UNIVERSITÉ DE LIÈGE

FACULTÉ DE MÉDECINE
Centre Interfacultaire de Recherche du Médicament
Laboratoire de Chimie Pharmaceutique
Professeur Bernard Pirotte

DESIGN, SYNTHESIS AND PHARMACOLOGICAL EVALUATION OF ORIGINAL NITROBENZENESULFONYLUREAS AND SULFONYLCYANOGUANIDINES AS THROMBOXANE A_2 RECEPTOR ANTAGONISTS

Insights into selectivity between thromboxane A_2 receptor isoforms (TPα and TPβ)

Julien Hanson

Pharmacien

Promoteurs :
Pr. J.-M. Dogné & Pr. B. Pirotte

Thèse présentée en vue de l'obtention du grade de Docteur en Sciences Pharmaceutiques

Année académique 2006-2007

Au terme de la rédaction de ce mémoire, je tiens à remercier les nombreuses personnes qui ont participé à ces travaux de recherches et sans qui la réalisation de ceux-ci n'aurait pas été possible. Ce projet est le fruit de nombreuses collaborations entre différents laboratoires de haut niveau.

J'aimerais tout d'abord exprimer à Monsieur le Professeur Bernard Pirotte ma plus profonde reconnaissance pour m'avoir donné les moyens d'entreprendre cette recherche au sein du Laboratoire de Chimie Pharmaceutique de l'Université de Liège. Son soutien, sa grande expérience et ses conseils judicieux m'ont beaucoup apporté.

Je voudrais également témoigner ici de mon plus profond respect pour Monsieur le Professeur Jaques Delarge qui m'a donné l'envie de poursuivre des études dans le domaine de la chimie pharmaceutique.

J'adresse également mes plus chaleureux remerciements et ma reconnaissance au Professeur Jean-Michel Dogné qui m'a octroyé sa confiance dès le premier jour et m'a permis de travailler dans les meilleures conditions. Ses qualités scientifiques, ses conseils, son soutien, ses encouragements et sa détermination m'ont aidé et apporté énormément.

During this Ph. D. work, I had the chance to join for four months the lab of Pr. Cecil Pace-Asciak, from the Hospital for Sick Children and University of Toronto (Canada). I wish to express my deepest gratitude to him for his support and for the impulsion to the present work. I also wish to address my warmest thanks for the people I worked with in this lab, particularly Denis and Na.

I also wish to address my gratitude to Pr. Therese B. Kinsella, from the University College Dublin (Ireland) for our active collaboration which allowed to generate many data presented in this dissertation.

Je souhaite également exprimer ma profonde reconnaissance à M. le Professeur Jean-Olivier Defraigne qui m'a gentiment permis de réaliser les cultures cellulaires dans son laboratoire (Centre de Chirurgie Expérimentale, CREDEC).

Je remercie vivement le Dr Jean-Louis David pour m'avoir fait bénéficier de son expérience et de sa compétence ainsi que des ressources de son laboratoire, sans lesquelles ce travail n'aurait pas été possible.

Je tiens particulièrement à exprimer ma profonde gratitude envers le Fonds pour la Formation à la Recherche dans l'Industrie et dans l'Agriculture (F.R.I.A) pour son soutien matériel qui m'a permis de mener à bien cette thèse de doctorat. J'exprime ma gratitude envers la Fondation Léon Fredericq, le Fonds National de la Recherche Scientifique (F.N.R.S) ainsi que l'Université de Liège pour m'avoir accordé les moyens financiers indispensables à la réalisation de ces travaux.

Ces années passées au sein du Laboratoire de Chimie Pharmaceutique de l'Université de Liège m'ont permis de rencontrer de nombreuses personnes que je salue ici. Je tiens plus particulièrement à témoigner ma reconnaissance à Philippe Neven pour sa patience, sa grande compétence, son amitié ainsi que pour les nombreuses conversations enrichissantes que nous avons partagées et qui m'ont aidé à mener à bien cette thèse de doctorat. Je n'oublie pas les personnes qui m'ont aidé tout au long de ce travail, avec qui j'ai collaboré et avec qui j'ai partagé de nombreux bons moments et pris beaucoup de plaisir à travailler, notamment : Annie Ooms, Anne-Lise Moray, Alexandre Ghuysen, Catherine Dujardin, Céline Cherdon, Didier Botty, Jean-François Renard, Jean-Paul Cheramy, Jérémie Ghiotto, Philippe Devel, Philippe Kolh, Pierre Drion, Stéphanie Rolin et Vincent Tchana-Sato. J'adresse également un tout grand merci à Yvette Abrassart pour les spectres infrarouge et Stéphane Counerotte pour les analyses élémentaires.

Je voudrais également remercier ma famille et mes proches pour leurs encouragements et leur soutien quotidiens.

Enfin, je tiens à remercier Domi pour sa présence, son écoute et son aide de tous les jours au long de la réalisation de ce travail.

Abstract

Design, synthesis and pharmacological evaluation of original nitrobenzenesulfonylureas and sulfonylcyanoguanidines as thromboxane A_2 receptor antagonists

Thromboxane A_2 (TXA_2) is an important mediator metabolized from arachidonic acid through the cyclooxygenase pathway, mainly in platelets and macrophages. It is a potent inducer of platelet aggregation and smooth muscle contraction. Its overproduction has been detected in pathologies such as stroke, asthma, myocardial infarction or atherosclerosis. The action of TXA_2 is mediated by a specific G-protein coupled receptor (TP) of which two alternative spliced isoforms, TPα and TPβ, have been described. The exact role of these two isoforms is not clearly understood. However, recent studies have described their implications in vascular physiology and pathology.

The inhibition of the action of TXA_2 on platelets and blood vessels would be interesting as original therapies against cardiovascular diseases. Consequently, the design of TP receptor antagonists remains of great interest in cardiovascular medicine. In the laboratory of medicinal chemistry (University of Liège, Belgium), several nitrobenzenesulfonylureas, derived from torasemide (a loop diuretic), have been previously described as TP receptor antagonists. Two compounds, BM573 and BM613 were among the most interesting molecules identified in that previous work.

The present project is divided in two parts. First, we have determined the pharmacological properties of BM573 and BM613 as thromboxane synthase inhibitors and TP receptor antagonists, *in vitro* and *in vivo*. In our assays, these two compounds were proved to have high affinity for both TPα and TPβ, to be potent antiplatelet agents, to inhibit thromboxane synthase and TP-mediated smooth muscle contraction. Additionally, they significantly reduced the size of the thrombus in a rat model of ferric chloride-induced arterial thrombosis. Consequently, we demonstrated that the TP receptor antagonists BM573 and BM613, belonging to the chemical family of nitrobenzenesulfonylureas, could be regarded as antiplatelet and

antithrombotic agents potentially useful in thromboxane-related diseases such as stroke or myocardial infarction.

Secondly, given the interesting pharmacological profile of BM573 and BM613, we have designed and synthesized several series of compounds derived from these two agents. We have evaluated the binding properties (affinity) of the first generation (+/- 35 original derivatives) of compounds on either TPα or TPβ, transiently expressed in COS-7 cell lines. Additionally, we have measured the ability of our drugs to inhibit the intracellular calcium ($[Ca^{2+}]_i$) mobilization upon TPα or TPβ stimulation. To confirm our results, we also assessed the antiplatelet properties of our drugs by means of determination of inhibition of human platelet aggregation. On the basis of the results obtained with these *in vitro* assays, we have synthesized and evaluated a second generation of derivatives (+/- 35 original compounds) and improved the selectivity of several original compounds for TP receptor isoforms.

The originality of this work was to evaluate a large library of synthetic compounds on both TP receptor isoforms, using specific pharmacological tests. By means of structure-activity relationship studies, we were able to identify chemical groups implicated in selectivity and to propose lead compounds for development of highly specific TPα or TPβ antagonists. Besides, we have identified an *in vivo* drug candidates for prevention of thrombosis and pathological platelet aggregation.

Résumé

Conception, synthèse et évaluation pharmacologique de nitrobenzènesulfonylurées et sulfonylcyanoguanidines en tant qu'antagonistes des récepteurs au thromboxane A_2

Le thromboxane A_2 (TXA_2) est un métabolite de la cascade de l'acide arachidonique (AA) par la voie des cyclooxygénases et de la thromboxane synthase, principalement formé dans les plaquettes et les macrophages. Le TXA_2 est un puissant inducteur de l'agrégation plaquettaire et de la contraction des muscles lisses vasculaires et bronchiques. Par ailleurs, une augmentation des taux en TXA_2 a été constatée dans différentes pathologies : l'infarctus du myocarde, l'atherosclérose, les accidents vasculaires cérébraux, ou encore l'asthme. L'action du TXA_2 sur les tissus résulte de la stimulation d'un récepteur appartenant à la famille des récepteurs couplés aux protéines G. Ce récepteur au TXA_2 (TP) présente deux isoformes générées par épissage alternatif, TPα et TPβ. Le rôle physiologique exact de ces deux isoformes n'est pas encore connu. Cependant, de récents travaux ont mis en évidence leur importance, notamment dans la physiologie vasculaire et dans certaines pathologies.

L'inhibition de l'action du TXA_2 au niveau des plaquettes et des vaisseaux sanguins pourrait donc être une stratégie thérapeutique innovante pour traiter et prévenir les maladies cardiovasculaires. En conséquence, le développement d'antagonistes des récepteurs TP reste d'un grand intérêt en médecine cardiovasculaire. Des études de pharmacomodulation avaient permis au Laboratoire de Chimie Pharmaceutique (Université de Liège, Belgique) d'identifier des nitrobenzènesulfonylurées, dérivées du torasémide (un diurétique de l'anse), présentant un puissant antagonisme des récepteurs TP. Parmi ceux-ci, deux composés, le BM573 et le BM613, faisaient parties des molécules les plus intéressantes identifiées au cours de ces précédentes recherches.

Ce projet est divisé en deux parties. Premièrement, nous avons déterminé les propriétés pharmacologiques du BM573 et du BM613 en tant qu'inhibiteurs de la thromboxane synthase et antagonistes des récepteurs TP, *in vitro* et *in vivo*. Au cours de nos expériences, ces deux composés se sont révélés posséder une grande affinité pour TPα et TPβ, être de puissants agents antiplaquettaires, des inhibiteurs de la thromboxane synthase et de la contraction des muscles lisses induite par le TXA_2. En outre, l'utilisation de ces produits dans un modèle de thrombose artérielle induite par le chlorure ferrique chez le rat a provoqué une réduction significative du thrombus formé. En conséquence, nous avons démontré que le BM573 et le BM613, appartenant à la famille chimique des nitrobenzenesulfonylurées, pouvaient être considérés comme des agents antiplaquettaires et antithrombotiques, potentiellement utiles en tant qu'agents thérapeutiques dans des pathologies associées au TXA_2 telles que l'infarctus du myocarde ou l'accident vasculaire cérébral.

Ensuite, nous nous sommes concentrés sur l'activité de cette famille de composés (les nitrobenzènesulfonylurées) vis-à-vis des deux isoformes du récepteur au thromboxane. Pour ce faire, nous avons conçu et synthétisé de nombreuses séries de composés dérivés du BM573 et du BM613. Nous avons tout d'abord évalué l'affinité de la première génération de composés (+/- 35 dérivés) sur des lignées cellulaires (COS-7) exprimant sélectivement soit TPα soit TPβ. De plus, nous avons mesuré la capacité de ces composés à inhiber la mobilisation de calcium intracellulaire ($[Ca^{2+}]_i$) induite par la stimulation des deux isoformes TPα et TPβ séparément. Nos résultats ont été confirmés sur agrégation plaquettaire humaine. Sur la base des résultats obtenus avec cette première génération de produits, nous avons synthétisé une seconde génération (+/- 35 dérivés) de composés, et avons réussi à augmenter la sélectivité en faveur de TPβ pour certains produits.

L'originalité de ce travail réside dans le fait que nous avons évalué un nombre de produits importants sur TPα et TPβ, au moyen de tests pharmacologiques spécifiques. Grâce à des études de relation structure-activité, nous avons identifié des groupements chimiques impliqués dans la sélectivité entre les deux isoformes. Nous pouvons donc proposer des structures "chef de file" pouvant être utiles pour le

développement de composés hautement sélectifs, soit pour TPα, soit pour TPβ. Par ailleurs, nous avons identifié *in vivo* des candidats pour le développement d'agents thérapeutiques pour la prévention des thromboses et des autres pathologies provoquées par une activation plaquettaires excessive.

Abbreviations list

AC	Adenylate cyclase	HUVEC	Human Umbilical Vein Endothelial Cells
ADP	Adenosine diphosphate	IP	Prostacyclin receptor
cAMP	cyclic adenosine monophosphate	IP_3	inositide triphosphate
ASA	acetylsalicylic acid, Aspirin	LDL	low-density lipoprotein
BAPTA	1,2-bis(2-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid	LOX	lipoxygenase
Ca^{2+}	Calcium ions	NO	nitric oxide
COPD	chronic obstructive pulmonary diseases	PUFA	polyunsaturated fatty acids
COS-7 cells	African green monkey kidney fibroblast-like cell line	PGD_2	Prostaglandin D_2
COX	cyclooxygenase	PGE_2	Prostaglandin E_2
CTA_2	carbocyclic TXA_2	$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
CYP	cytochrome P450	PGI_2	prostacyclin
DAG	diacylglycerol	PG	prostaglandin
DP	PGD_2 receptor	PGH_2	Prostaglandin H_2
ED	Extracellular domain	PIP_2	phosphatidylinositol 4, 5-bisphosphate

EGTA	Ethylene Glycol bis(2-aminoethyl) Tetra Acetate
EP	PGE_2 receptor
EPA	eicosapentaenoic acid
FP	$PGF_{2\alpha}$ receptors
GDP	guanosine diphosphate
GP	glycoprotein
GPCR	G protein-coupled receptors
GRK	G proteins receptor kinases
GTP	guanosine triphosphate
HEK293 cells	Human embryonic kidney cell line
HETE	hydroxyeicosatetraenoic acids
PKA	protein kinase A
PKC	protein kinase C
PLA_1	Phospholipase A_1
PLA_2	Phospholipase A_2
PLC	Phospholipase C
RGS	regulation of G protein signaling
TM	Transmembrane domain
TP	Thromboxane A_2 receptor
TXA_2	Thromboxane A_2
TXB_2	thromboxane B_2
TXRA	Thromboxane receptor antagonists
TXS	thromboxane synthase
TXSI	thromboxane synthase inhibitor
vWF	von Willebrand Factor

Table of contents

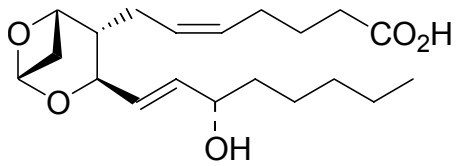
I. Introduction

I.1. Foreword

This introduction is intended to give a broad description of several aspects of the lipid mediator thromboxane A_2 (TXA_2). First, we will describe the complete human metabolism of TXA_2. This will be followed by a complete review of the physiology, pharmacology and biochemistry of its specific receptors. We will then emphasize the therapeutic interests of developing and studying agents able to counteract its actions. A short presentation on the previous works achieved in the TXA_2 modulator field will also be proposed.

I.2. Thromboxane A_2

TXA_2 (**1,** Figure I-1) belongs to the chemical family of eicosanoïd acids. Its structure is characterized by a 2,6-dioxabicyclo[3.1.1]heptane ring, a 2-heptenoic acid and a 3-hydroxy-1-octenyl chain, named α and ω (respectively), in a *trans* configuration (Figure I-1). TXA_2 is a product of the arachidonic acid metabolism. The aim of this section is to present metabolic pathway of this important lipid mediator.



1

Figure I-1. TXA_2 structure

I. 2. 1. Biosynthesis and metabolism

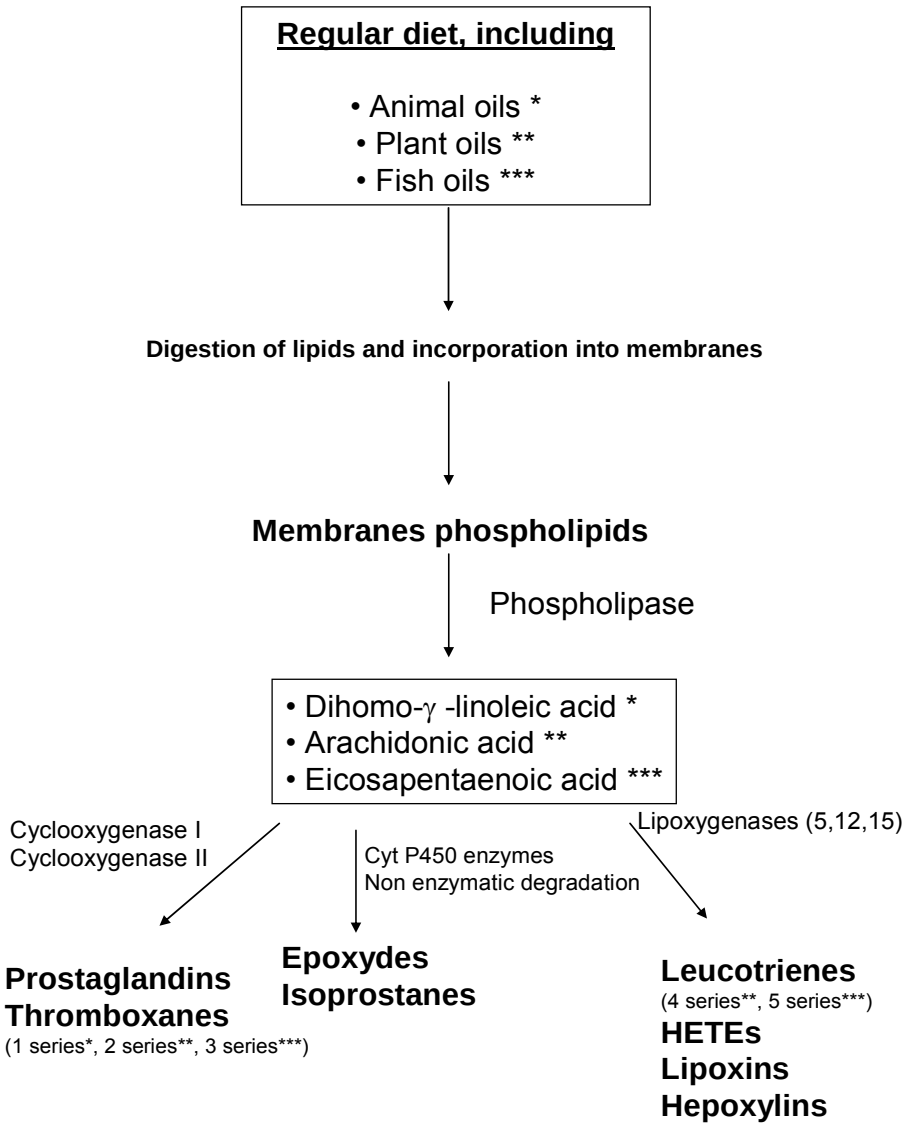
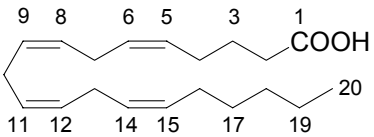


Figure I-2. General pathway for the biosynthesis of thromboxanes, prostaglandins, leucotrienes and other lipid mediators issued from polyinsaturated essential fatty acids

I.2.1.1. Essential fatty acids and eicosanoid family

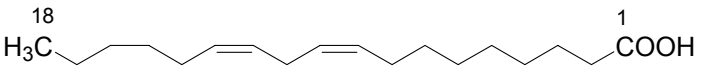
Eicosanoïds are members of a large family of mediators derived from 20 carbon atoms polyunsaturated fatty acids (PUFAs) released from cell membrane phospholipids, especially arachidonic acid or 5,8,11,14-eicosatetraenoic acid (**2**, Figure I-3). This family includes prostaglandins (PGs), thromboxanes (TXs), leucotrienes (LTs), and hydroxyeicosatetraenoic acids (HETEs).



2

Figure I-3. Arachidonic acid structure.

In mammals, arachidonic acid is the major substrate for eicosanoids of the 2-series synthesis. The arachidonic acid is directly taken from diet or is derived from linoleic acid, or 9, 12-octadecadienoic acid, an essential 18 carbon atoms PUFA (**3**, Vitamine F, Figure I-4).



3

Figure I-4. Chemical structure of linoleic acid.

Linoleic acid is also the precursor of another 20 carbon PUFA, dihomo-γ-linolenic acid or 8,11,14-eicosatrienoic acid (**4**, Figure I-5), which gives rise to the 1-series of eicosanoids. Eicosanoids of the 3-series are derived from a third type of PUFA, namely 5,8,11,14,17-

eicosapentaenoic acid or EPA (**5**, Figure I-3), which is mainly present in cold water fish oils.

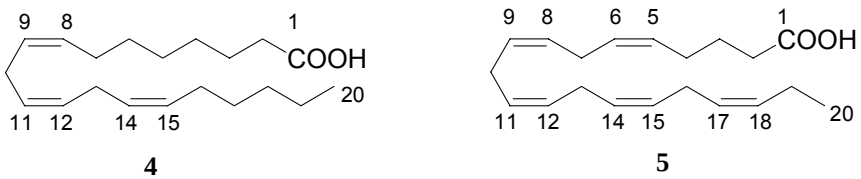


Figure I-3. Chemical structures of dihomo-γ-linolenic acid (4) and 5, 8, 11, 14, 17-eicosapentaenoic acid or EPA (5)

I.2.1.2. Membrane phospholipids

After food intake, essential fatty acids are stored in cellular membrane, mainly as phospholipids. These molecules are glycerophospholipids, characterized by the presence of both hydrophilic and hydrophobic domains. The 3-position of glycerol is hydrophilic and substituted by phosphatidylserine, phosphatidylethanolamine, phosphatidylcholine or phosphatidylinositol. The two other carbons (1 and 2) of the glycerol are substituted by fatty acids, linked by an ester bond. Usually, C_1 carries a saturated or monosaturated fatty acid whereas C_2 is mainly bound with an unsaturated fatty acid (i.e. arachidonic acid, dihomo-γ-linolenic acid and 5,8,11,14,17-eicosapentaenoic acid) (Figure I-4).

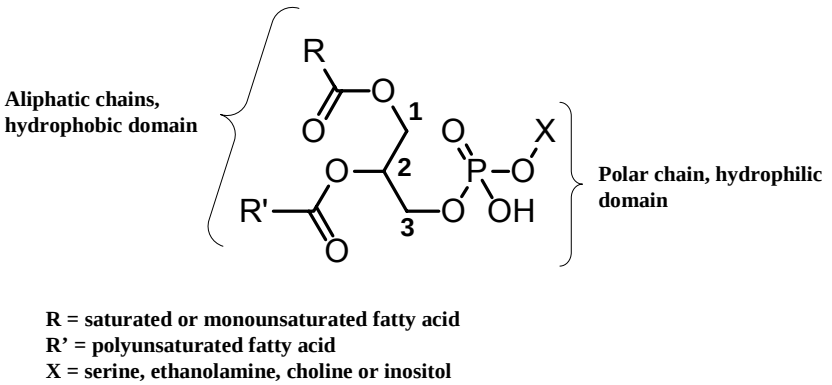


Figure I-4. General structure of membrane phospholipids

Release of fatty acid from glycerol by hydrolysis of the ester bond is achieved by the action of phospholipases, specific enzymes activated by several stimuli (i.e. chemical, physical or hormonal).

Several types of phospholipases have been identified :

- phospholipase A_1 (PLA_1) catalyses the release of saturated or monounsaturated fatty acids attached to C_1;
- phospholipase A_2 (PLA_2) is responsible for the release of C_2 polyunsaturated fatty acids;
- phospholipase C (PLC) hydrolyses a particular phospholipid (phosphatidylinositol or PIP_2) into two compounds : a diacylglycerol (DAG) and a phosphorylated base (inositide triphosphate or IP_3) (Ochocka and Pawelczyk 2003);

- phospholipase D (PLD) catalyses the release of C_3 substituents (choline, inositol, ethanolamine, serine).

Consequently, PLA_2 and C are the main enzymes of this family that play a central role in eicosanoid biosynthesis (Smith, Marnett *et al.* 1991). Once released, free arachidonic acid can enter into several metabolic pathways.

I.2.1.3. Arachidonic acid cascade

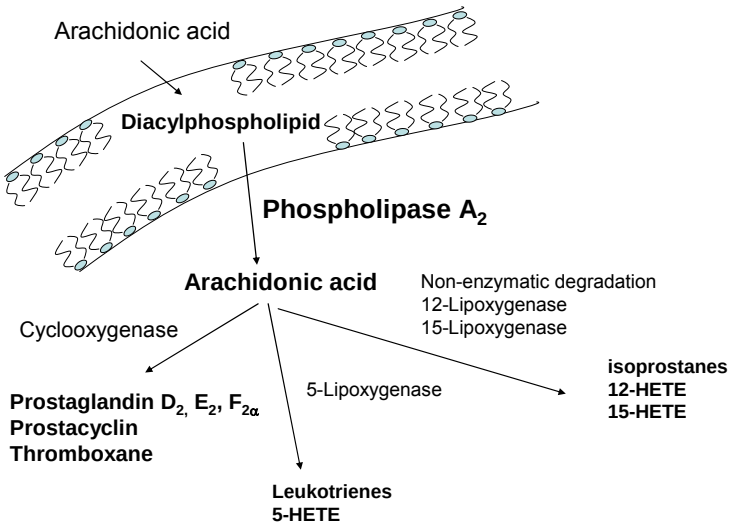


Figure I-5. Arachidonic acid metabolism

Two main enzymatic pathways for arachidonic acid metabolism have been highlighted : the cyclooxygenase (COX) pathway and the lipoxygenase (LOX) pathway. COX catalyses the production of prostaglandins D_2 (PGD_2), E_2 (PGE_2), $F_{2\alpha}$ ($PGF_{2\alpha}$), prostacyclin (PGI_2)

and TXA_2 (Bergstroem, Danielsson *et al.* 1964; Hamberg and Samuelsson 1967; Hamberg, Svensson *et al.* 1975). 5-, 12- and 15-LOX generate respectively leukotrienes (Murphy, Hammarstrom *et al.* 1979; Samuelsson 1983), hepoxylins (Pace-Asciak and Martin 1984) and lipoxins (Serhan, Hamberg *et al.* 1984) (Figure I-5). Arachidonic acid can also be oxygenated by cytochrome-dependent monoxygenases. The physiological roles of the metabolites generated by this pathway still has to be clarified.

Although all mediators issued from arachidonic acid metabolism are important, we will focus on COX pathway and TXA_2 biosynthesis.

Cyclooxygenase pathway

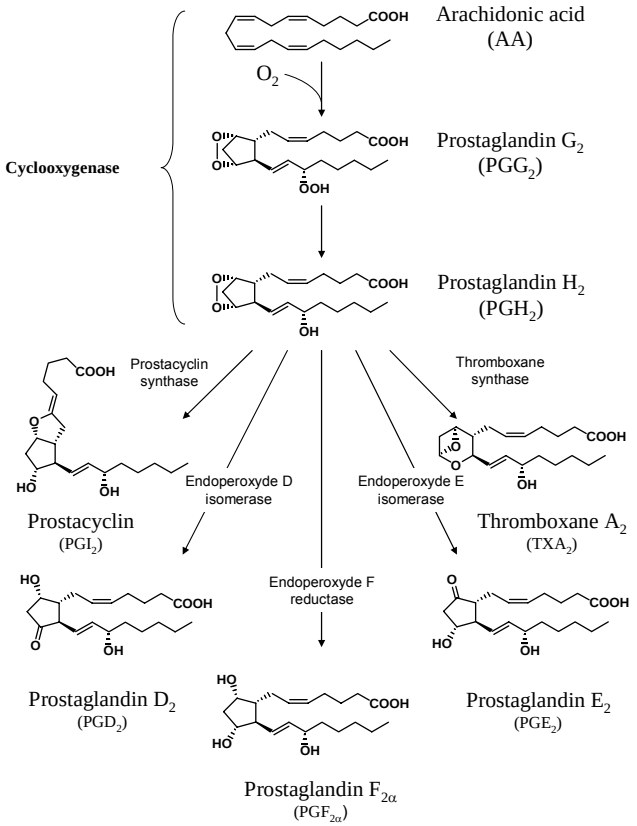


Figure I-6. Prostanoids biosynthesis by the arachidonic acid COX pathway.

Cyclooxygenases (or prostaglandin endoperoxide H synthases) are enzymes that catalyze the formation of PGs, TXA_2 and PGI_2. All non-steroidal anti-inflammatory drugs act via COX enzymes inhibition.

Initially, it was thought that there was only one COX, first described by Bergstroem and his collaborators (Bergstroem, Danielsson *et al.* 1964). In 1990, another type was discovered, leading to a distinction between COX-1 and COX-2 (Fu, Masferrer *et al.* 1990). COX-1, the first

to be discovered, is mainly characterized by a constitutive expression. It is expressed in many tissues and cell types, including platelets. Although initial studies concluded that COX-2 expression was inducible, it was recently demonstrated that the expression of COX-2 was also constitutive in some tissues such as kidney (Harris, McKanna *et al.* 1994), vascular endothelium (Cheng, Austin *et al.* 2002; McAdam, Catella-Lawson *et al.* 1999), or cortex (Yamagata, Andreasson *et al.* 1993)). COX-2 is also expressed in several pathological situations, including inflammation (Seibert, Zhang *et al.* 1994), and some cancers (colon (Taketo 1998),. liver (Shiota, Okubo *et al.* 1999), pancreas (Tucker, Dannenberg *et al.* 1999), lung (Wolff, Saukkonen *et al.* 1998), and breast (Hwang, Scollard *et al.* 1998)).

The arachidonic acid oxidation by COXs is a two-steps mechanism. First, COXs catalyse the oxydation and the ring closure of AA to form endoperoxide G_2 (Figure I-6). In the second step, the endoperoxidase activity of COX allows the formation of the pivotal endoperoxide prostaglandin H_2 (PGH_2). Indeed, PGH_2 is the common substrate for the synthesis of all subsequent prostanoids, as described in Figure I-6. Consequently, the specificity of prostanoids formation is achieved by their specific enzymes (Table I-1).

Substrate	Enzyme	Product
PGH_2	Prostacyclin synthase	PGI_2
PGH_2	Endoperoxide D isomerase	PGD_2
PGH_2	Endoperoxide F reductase	$PGF_{2\alpha}$
PGH_2	Endoperoxide E isomerase	PGE_2
PGH_2	Thromboxane synthase	TXA_2

Table I-1. Enzymes involved in prostanoid synthesis, using PGH_2 as substrate

Non-enzymatic pathways

More recently, it has been highlighted that arachidonic acid could undergo non-enzymatic degradation, giving rise to active compounds, namely isoprostanoïds (Fam and Morrow 2003). The physiological and pathological implications of these products are of particular importance and their formation is increased upon oxidative stress. Several recent reports have proposed that TXA_2 and some isoprostanes could indeed act on the same receptors (Cracowski, Devillier *et al.* 2001). This effect could be useful for the understanding and treatment of several diseases where TXA_2 receptor activation has been demonstrated.

I.2.1.4. Thromboxane synthase

In vivo, TXA_2 synthesis involves coordinated action of a series of enzymes, including phospholipases which release arachidonate from phospholipids, COX-1 or -2 which convert arachidonate into PGH_2, and, finally, thromboxane synthase (TXS).

TXS catalyzes the isomerization of PGH_2 into TXA_2. This activity was first described in platelets (Needleman, Moncada *et al.* 1976) and the enzyme was later purified and described as an hemoprotein belonging to the cytochrome P450 family (CYP) (Haurand and Ullrich 1985). This assumption was confirmed when the cDNA of the enzyme was cloned (Ohashi, Ruan *et al.* 1992; Yokoyama, Miyata *et al.* 1991). Human TXS was assigned as CYP 5A1. Unlike other microsomal CYPs that require the ubiquitous P450 reductase to shuttle electrons for the mono-oxygenation reaction, thromboxane synthase undergoes an isomerization reaction without reductase or molecular oxygen.

The TXS gene is transcribed as a 2.1-kb mRNA in hematopoietic cells, such as platelets, macrophages, monocytes and leukocytes, as well as in various tissues, particularly in lung, kidney, liver, spleen, prostate, placenta and thymus (Miyata, Yokoyama *et al.* 1994; Zhang, Xiao *et al.* 1997). The expression in these tissues is consistent with the biological activity of TXA_2, and the cell type specificity to produce TXA_2 is indeed dependent on the presence of TXS.

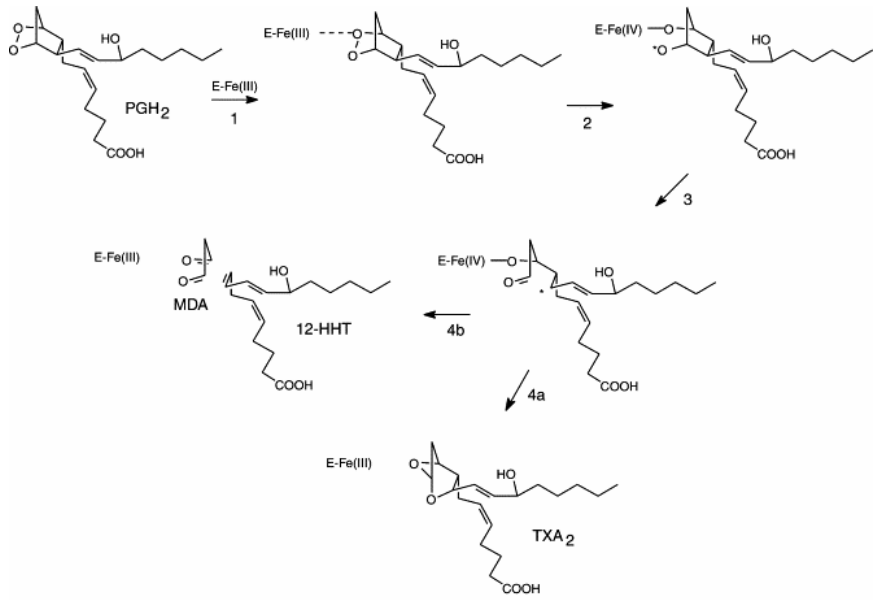


Figure I-7. Proposed mechanism for TXA_2 formation by TXS, according to Wang *et al.* (Wang and Kulmacz 2002)

The proposed mechanism for the isomerization of PGH_2 into TXA_2 postulates that in the first step, TXS heme iron interacts with the oxygen attached to C-9 of PGH_2 (Figure I-7, (Hecker and Ullrich 1989)). Then, The TXS heme is proposed to undergo a redox transition from resting Fe(III) to Fe(IV) upon opening of the endoperoxide in Step 2 of the mechanism in Figure I-7. Oxygen- and carbon-centered radicals are predicted to occur during TXS catalysis (Steps 2 and 3 in Figure I-7). Nevertheless, the exact mechanism of subsequent steps remains elusive (Wang and Kulmacz 2002).

I.2.1.5. Thromboxane A_2 degradation and Catabolism

TXA_2 is a chemical unstable mediator and is rapidly hydrolyzed into inactive thromboxane B_2 (TXB_2) (Figure I-8). *In vivo* TXA_2 half-life is 30 seconds.

Catabolism of TXA_2 leads to the formation of about 20 inactive compounds in different proportions in humans. The most important plasmatic products are TXB_2 and its dehydrogenation product, 11-dehydroTXB_2 (Catella, Healy *et al.* 1986). In urine, metabolites reflecting TXA_2 production are 11-dehydroTXB_2 and 2,3-dinorTXB_2 (Catella, Lawson *et al.* 1987). A third less important metabolite has also been identified in urines, 11-dehydro-2,3-dinorTXB_2 by Chiabrando *et al.* (Chiabrando, Rivoltella *et al.* 1993)(Figure I-8).

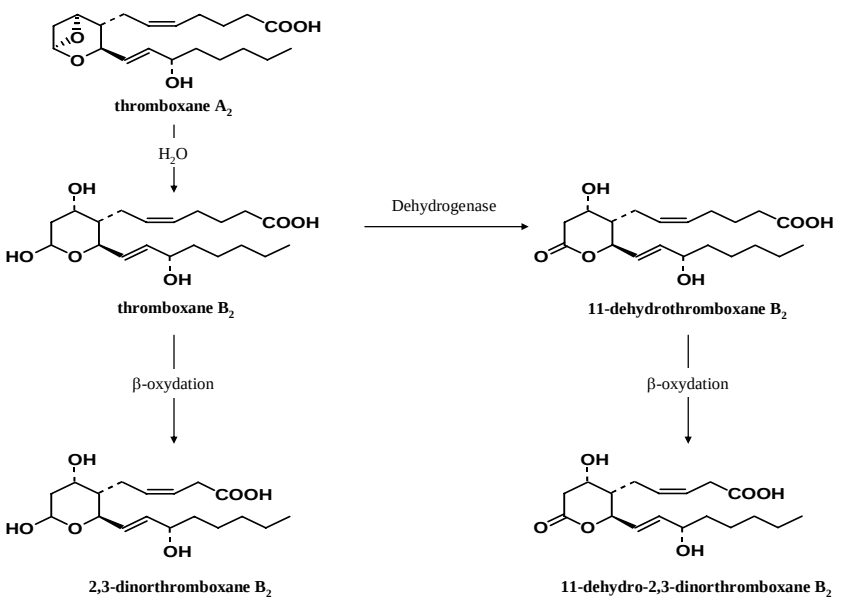


Figure I-8. TXA_2 catabolism.

I.3. Thromboxane A_2 receptor

The effects of TXA_2 are mediated through its specific receptor. The thromboxane receptor belongs to the most important superfamily of receptors : G protein-coupled receptors (GPCR), characterized by seven transmembrane domains receptors.

I. 3. 1. Pharmacology of G protein-coupled receptors

Before the complete description of TXA_2 receptor properties, we will briefly describe the general properties of GPCR function.

Signal transduction and regulation

These key families of proteins share a common molecular architecture consisting of seven transmembrane helices that are connected by three extracellular and three intracellular loops. GPCRs are characterized by the ability to couple to heterotrimeric G proteins which transduce a specific signal from a ligand-receptor interaction and act as effectors systems inside the cell. These proteins draw their name because they have a high affinity for GTP and are composed of three subunits : α, β and γ. The α subunit possesses a GTPase activity, since it is able to hydrolyze bound GTP.

When a receptor is stimulated by a cognate ligand, the Gα subunit of the G protein exchanges a molecule of bound GDP for GTP. The heterotrimeric protein becomes active until the GTPase activity hydrolyzes GTP for GDP thus turning off the system, which becomes available for a novel stimulation. Different classes of G protein exist and

are classified upon their properties as effectors but also on sequences homologies. To date, up to twenty Gα subunits have been described and are grouped into four major families, classified by their main functional properties (Nguyen Hwangpo and Iyengar 2005):

- $G\alpha_s$: this G protein subunit family stimulates the activity of adenylate cyclase, thus increasing cAMP levels ;

- $G\alpha_{i/o}$: $G\alpha_i$ family ($G\alpha_i$ 1, 2 and 3) inactivates adenylate cyclase and induces a decrease of cAMP levels. $G\alpha_o$ is mainly present in the nervous system and modulate the opening probability of voltage-gated Ca^{2+} channels ;

- $G\alpha_{q/11}$: this family of Gα subunit (including $G\alpha_{15}$ and $_{16}$) is coupled to the activation of PLC-β, which leads to the hydrolysis of phosphatidylinositol 4, 5-bisphosphate (PIP_2) and to the production of IP_3 and DAG (see also point I.2.1.2). The principal property of IP_3 is to release calcium ions (Ca^{2+}) from intracellular stores. DAG acts as an activator of protein kinase C (PKC) which regulates several processes inside the cell ;

- $G\alpha_{12/13}$: the specific role of these fourth family of G proteins subunit remains elusive. They are abundant in platelets and have been related to several downstream pathways (notably small GTPase RhoA).

On the other side, Gβγ complex forms one functional unit and can affect downstream effectors as well and as much as the Gα subunits.

Agonist activation of a GPCR not only results in the G protein-dependent activation of effector systems, but also sets in place a series of molecular interactions that allows for feedback regulation of G protein coupling, receptor endocytosis, and signaling through G protein-independent signal transduction pathways. For example, RGS (regulation of G protein signaling) proteins accelerate the hydrolysis of GTP by G proteins α subunits, and hence increase the rate of recovery of the effector from activation by $G\alpha_{GTP}$ or by $G\beta\gamma$ (Richman and Diversé-Pierluissi 2005). GRK (G proteins receptor kinases) and β-arrestins are proteins able to desensitize the receptor after agonist stimulation by endocytosis or by uncoupling of the receptor from its cognate G protein (Luttrell 2005; Ma, Gao *et al.* 2005). Additionally, activation of kinases by GPCRs (PKC for example) can also regulate the signaling of distinct GPCRs and thus lead to heterologous desensitization.

Another important feature in GPCR signal transduction regulation is their ability to form dimers, heterodimers or oligomers. In the past ten years, the vision of GPCR acting as monomeric entities has quickly evolved. First evidences for the concept that this family of receptor could be able to form dimers or other oligomeric species were obtained in experiments where inactive mutant receptors recovered their binding and functional activity when cotransfected (Maggio, Vogel *et al.* 1993a; Maggio, Vogel *et al.* 1993b). Subsequent key steps in this theory were achieved when it was demonstrated that the metabotropic $GABA_B$ was an obligatory heterodimer (Marshall, Jones *et al.* 1999) and opioids receptors could form heterodimers with new pharmacology (Jordan and

Devi 1999). The questions regarding this phenomenon remain controversial and are an active field of research.

Constitutive activity, inverse agonism and functional selectivity

Pharmacology of GPCR is complex and has quickly evolved during the past decade. We will briefly describe here the main basic principles of these receptors pharmacology.

The classical "receptor theory", evolved from the early days of experimental pharmacology to now and initially proposed that the interaction between a ligand and its receptor was able to elicit a functional cellular response (Rang 2006). Within this theory, a molecule able to "activate" the receptor protein was termed "agonist". It was soon observed that some chemical entities were able to counteract the agonist action, by displacing it from the active site of the receptor. These compounds, namely competitive antagonists, were though to be inert for the protein and to have a better steric affinity for the ligand binding pocket than the displaced agonist, thus preventing it to bind to and activate receptor.

Although this model permitted the fast development of modern pharmacology and was a powerful tool for elucidation of many questions regarding drugs action, it was rather imprecise. In several key papers from the Lefkowitz group in the early nineties it was evidenced that point mutation of a GCPR of the adrenergic receptor family were able to induce activity of the receptor, in the absence of bound agonist (Lefkowitz, Cotecchia *et al.* 1993). Beside, De Lean and co-workers proposed the concept of inverse agonism, whereas some antagonists may

be able to promote the dissociation of the receptor from G proteins (Wreggett and De Lean 1984). The later experimental confirmation of this concept, together with the discovery that non-mutated GPCR had basal activity, led to the finding of inverse agonists for a wide range of receptors. Indeed, many of the competitive antagonists in clinical practice were found to act as inverse agonists (Hill 2006). Consequently, recent model suggests that in the absence of any ligands, GPCR exists as a dynamic population of active and inactive form (Kenakin 2001). The receptor spontaneously crosses from inactive to active state and the nature of the ligand is indeed determined by its affinity for either active or inactive state. Thus, a compound with high affinity for active state will act as an agonist and induces this receptor species to redistribute in the system.

More interestingly, recent data suggested that ligands induce unique, ligand specific receptor conformations that can result in activation of distinct signal transduction pathways (Urban, Clarke *et al.* 2007). This phenomenon is generally reported to as "functional selectivity" and this change of perspective has been spurred by data emerging within the past decade in which ligands were shown to have quite diverse functional consequences mediated via a single receptor.

I. 3. 2. Thromboxane A_2 receptor gene and expression

The TXA_2 gene has been discovered in 1991 by Shuh Narumiya group (Hirata, Hayashi *et al.* 1991), using an oligonucleotide probe corresponding to its partial amino acid sequence. The cDNA was discovered in placenta and platelet-like MEG-01 cell lines and was found

to encode for a 343 amino acids protein, with seven putative transmembrane (TM) domains. Further work by the group identified that the gene was assigned to chromosome 19p13.3, and had three exons divided by 2 introns and 2 putative promoter regions (Nusing, Hirata *et al.* 1993). In 1994, Raychowdhury *et al.* isolated a TXA_2 receptor cDNA from a human endothelial library. The predicted amino acid sequence revealed a structure of 369 amino acids, in which a novel cytoplasmic tail replaced the carboxyl-terminal portion of the previously characterized TXA_2 receptor. The authors suggested that the mechanism for the generation of the alternative transcript expressed in placenta was a failure to utilize a potential splice site, thus resulting in the encoding of a cytoplasmic domain that, in the endothelial TXA_2 receptor, was an intron in the early discovered TXA_2 receptor (Raychowdhury, Yukawa *et al.* 1994)(Figure I-9).

Consequently, the two isoforms are identical with regard to their N-terminal 328 amino acid residues, but differ exclusively in their C-terminal cytoplasmic domains. The TXA_2 receptor was named TP (for Thromboxane Prostanoid), the two isoforms being subsequently termed TPα, for the initial placental peptide composed of 343 amino acid residues, and TPβ, for the 369 amino acid residues isoform initially found in endothelial cells (Coleman, Smith *et al.* 1994).

Further genetic analyses of the TP receptor gene confirmed the presence of two promoters, P1 and P2 and the functional regulation of TP expression by numerous transcription factors, P1 having the most important implication (D'Angelo, Davis *et al.* 1995; D'Angelo, Oliver *et al.* 1996; Kinsella, O'Mahony *et al.* 1994b). More recently, Coyle *et al.* have

demonstrated the existence of a third promoter, P3. This team suggested that expression of TPα and TPβ was subjected to differential regulation through the transcriptional activity of P1/P2 and P3, respectively, since their transcripts differed extensively in their 5' untranslated region (Jourdan, Aguejouf *et al.*) sequences (Coyle, Miggin *et al.* 2002) (Figure I-9). Indeed, there are a number of genetic examples where protein/receptor isoforms arise through differential splicing, being products of the same gene, but may be under the transcriptional control of separate or distinct promoters. Several agents acting on P3 and on TPβ expression have been later identified, including PPARγ, a nuclear receptor playing a prominent role in diabetes, resolution of inflammation or cardioprotective effect (Coyle and Kinsella 2005; Coyle and Kinsella 2006; Coyle, O'Keeffe *et al.* 2005).

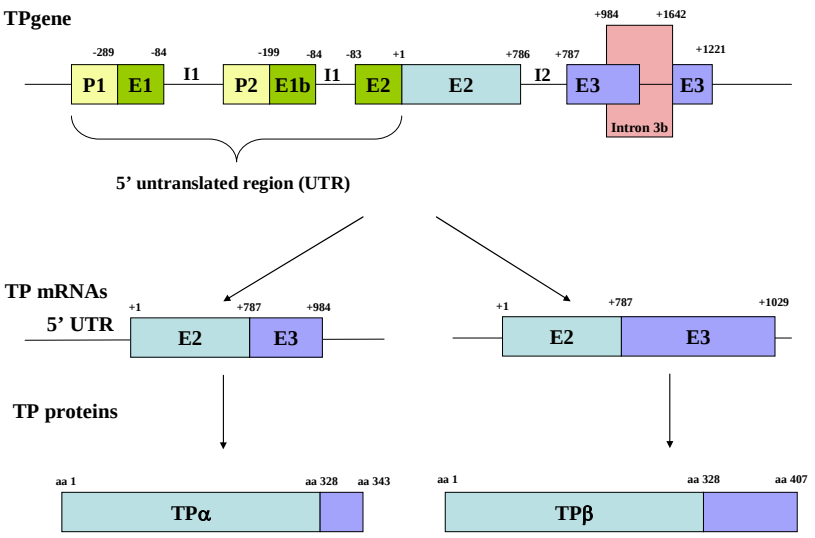


Figure I-9. Organization of thromboxane receptor gene. See text for details (adapted from (Coyle, Miggin *et al.* 2002)).

I. 3. 3. Distribution of TP receptors

Before the discovery of the isoforms, northern blot analyses confirmed the existence of TP mRNA in human MEG-01 cells (platelet-like cell lines), placental and lung tissue (Hirata, Hayashi *et al.* 1991; Swanson, Lei *et al.* 1992), in human erythroleukaemic (HEL) cells (Kinsella, O'Mahony *et al.* 1994a), and in mouse thymus, lung, spleen, ileum, brain and kidney (Namba, Sugimoto *et al.* 1992; Sugimoto, Narumiya *et al.* 2000).

Regarding the specific expression of the isoforms, early studies considered that TPα was the platelet/placental receptor (Hirata, Hayashi *et al.* 1991) whereas TPβ was the endothelial receptor, since it was initially discovered in endothelial cells (Raychowdhury, Yukawa *et al.* 1994).

More recent studies have addressed the specific expression pattern of TPα and TPβ using specific detection of mRNA. For example, Hirata *et al.* have demonstrated the presence of both mRNA in platelets (Hirata, Ushikubi *et al.* 1996). Further study conducted in 1998 by Miggin *et al.*, used PCR technology and specific probes to demonstrate that TP receptor was abundantly expressed at both the mRNA and protein level in tissues of relevance for TXA_2 biology. These tissues and cell types included erythroleukaemia cells, vascular and uterine smooth muscle, uterus and placental tissue, endothelium, epithelium, trophoblasts, thymus, liver and small intestine (all tissues studied within that study were from human). In that study, TPα was the most expressed isoform in nearly all tissues examined. Moreover, although TPα was expressed at approximately equal levels in all cell/tissue types analyzed, considerable

differences in TPβ mRNA expression were observed. Most strikingly, primary HUVECs (Human Umbilical Vein Endothelial Cells) were found to express low levels of TPβ and approximately 6-fold greater levels of TPα than TPβ (Miggin and Kinsella 1998).

More confusion was added in 1999 when a study conducted by Habib *et al* demonstrated the absence of TPβ in human platelets using specific antibody for both isoforms. The authors estimated that ≥ 50 fmol/mg of protein of TPβ could be detected with the specific antibodies, and suggested that TPβ was expressed at very low levels in platelets, if any (Habib, FitzGerald *et al.* 1999).

Collectively, these data point out that the relative expression of both isoforms proteins at the cell surface remains elusive, although one can consider that TPβ has probably a non-significant role in TXA_2 action on platelets.

I. 3. 4. Pharmacology of TP receptors

Abundant work has been reported on TP receptors pharmacology since the discovery of TXA_2. This section reviews the main discoveries and concepts related to TP receptors.

I.3.4.1. Pharmacological characterization

Early pharmacological studies and the two subtypes paradigm

Before the discovery of the human TXA_2 receptor gene, several teams have tried to characterize TP receptor pharmacologically. In terms

of binding studies, the TP receptor had been extensively characterized (Hall 1991), using both radiolabelled high affinity antagonists as well as stable TP agonists. Inhibition of specific binding correlated well with affinities predicted from biological activity, suggesting that the binding was meaningful. It is noteworthy that before the prostanoïd receptors nomenclature (TP, IP, DP, etc) the TXA_2 receptor was called the TXA_2/PGH_2 receptor since early studies had characterized PGH_2 as an agonist for TP receptors (Halushka 2000).

Following the first experiments, a controversy over the existence of pharmacologically distinct TP receptors on human platelets and vascular smooth muscle rapidly arose (Halushka 2000). Indeed, respectively in 1980 and 1981, possible existence of distinct platelet and vascular TP receptors was suggested by data produced by Lefer *et al.* (Lefer, Smith *et al.* 1980) and LeDuc *et al.* (LeDuc, Wyche *et al.* 1981). Additionally, Mais *et al.* (Mais, DeHoll *et al.* 1988; Mais, Dunlap *et al.* 1985), using a series of 13-azapinane TXA_2 analogs, provided strong evidence for the existence of receptors subtypes in vascular tissue and platelets, both issued from human species. Masuda *et al.* (Masuda, Mais *et al.* 1991), using the same compounds, conducted competition radioligand binding studies in rat and human platelets and cultured rat aortic smooth muscle cells. They found statistically significant differences in the rank order of potencies for these compounds in rat platelets compared to the smooth muscle cells, further supporting the notion that the platelet and vascular receptors represented unique subtypes.

It was also suggested at the time by Morinelli *et al.* (Morinelli, Niewiarowski *et al.* 1987) that platelets contained at least two TP receptor

subtypes. This was later supported by Takahara *et al.*, who postulated that platelets contained two TP receptor populations, one which mediated platelet shape change and the other aggregation (Takahara, Murray *et al.* 1990). Similarly, GR32191, a TXA_2 receptor antagonist (*vide infra*, point I.4.2.2.A., p. 61) was demonstrated to bind to two specific binding sites on human platelets (Armstrong, Humphrey *et al.* 1993a; Armstrong, Humphrey *et al.* 1993b), although further competition binding experiments suggested that these sites were identical (Armstrong and Wilson 1995).

Radioligand binding studies subsequently demonstrated different affinities in platelet and vascular TP receptors for the agonist ligand [^{125}I]-BOP (Dorn 1989; Morinelli, Okwu *et al.* 1989). Additionally, Morinelli *et al.* (Morinelli, Niewiarowski *et al.* 1987) demonstrated that the TXA_2 agonist, U46619, produced shape change and myosin light chain phosphorylation in platelets (for complete description of platelet aggregation, see point I.3.6.1.A, p. 36) with an EC_{50} value one-tenth of that for inducing aggregation, secretion and mobilization of calcium. Dorn subsequently demonstrated that [^{125}I]-BOP also bound to platelet TP receptors with a high and low affinity state (Dorn 1989). That group provided additional evidence to support the notion that shape change and aggregation were coupled to different receptors (Dorn and DeJesus 1991).

One receptor and two isoforms

Nevertheless, despite the evidence in support of distinct platelet and vascular TP receptors there were several reports refuting that notion. Swayne *et al.* (Swayne, Maguire *et al.* 1988) using a series of structurally

dissimilar TP receptor antagonists reported that they could find no evidence to support the notion that the platelet and vascular TP receptors represented unique subtypes. Mihara *et al.* (Mihara, Hara *et al.* 1989) failed to find any differences in the binding of receptor agonists and antagonists between platelets and vascular tissue in the pig. Hanasaki *et al.* (Hanasaki, Nakano *et al.* 1989) also failed to find any differences in rank order potency for a series of TP receptor agonists and antagonists in rat platelets and aortic smooth muscle cells.

The discovery of the TXA_2 receptor gene by Hirata *et al.* (Hirata, Hayashi *et al.* 1991) and the existence of two isoforms generated by alternative splicing (Raychowdhury, Yukawa *et al.* 1994) shed new light on the controversy. Indeed, genetic and molecular biology showed that there was only one gene coding for the receptor and that the two products only differed in the Carboxyl-terminal tail, leaving the putative ligand-binding site identical.

Moreover, most of the studies that characterized several subtypes for TXA_2 receptors used tissue from different species. In 1992, Ogletree *et al.* showed that there were significant differences between TP receptors among several species (Ogletree and Allen 1992), although in the same species, the receptors were quite similar. Moreover, the conclusions of those studies using human tissues have to be taken cautiously since they have been conducted on whole organs (Mais, DeHoll *et al.* 1988).

In 1998, Djellas and co-workers (Djellas, Antonakis *et al.* 1998) shed some light on the question of the importance of the interactions of TP receptors with G proteins in physiological conditions. They explored a possible molecular mechanism for platelet synergism that is commonly

seen between some aggregating agents in human platelets, for example thrombin and TXA_2. They demonstrated that thrombin caused an increase in the affinity for ligands by the TP receptors and that this increase seemed to be due to a raise in the amount of $G\alpha_q$ associated with the TP receptors. That $G\alpha_q$ can amplify the affinity of TP receptors for its ligands was further supported by the observations of Allan *et al.* who found that co-transfection of $G\alpha_q$ or $G\alpha_{13}$ with the α isoform of TP receptors into COS-7 cells resulted in a significant increase in I-BOP affinity (Allan, Higashiura *et al.* 1996). Similarly, Becker *et al.* demonstrated that co-transfection of $G\alpha_{13}$ with the β isoform increased the affinity for several agonists and also decreased the affinity of both isoforms for several antagonists (Becker, Garnovskaya *et al.* 1999). Consequently, consistent with recent development of GPCR pharmacological properties (see I. 3. 1., p. 15), Halushka proposed that differential coupling of TP receptor isoforms could change the affinity of the receptor for agonist or antagonist ligands, and could give a possible explanation for apparent TP receptor subtypes (Halushka 2000).

Besides, the presence of two binding sites coupled with two effectors systems has found some possible explanation recently, with the deep exploration of the TP receptor role in platelet aggregation. These mechanisms will be presented extensively at point I.3.6.1.A., p. 36.

I.3.4.2. Signal transduction by TP receptors

Signal transduction by TP receptors can lead to activation of many intracellular mechanisms. At least nine G proteins, G_q, G_{i2}, G_s, G_{11},

$G\alpha_{12}$, G_{13}, G_{15}, G_{16}, and G_h were demonstrated to some extent to couple to TP receptors. Coupling with these G proteins will eventually evoke an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$), activation of PLC, generation of IP_3 and DAG, activation of PKC, stimulation of myosin light chain kinase, exposure of GPIIb/IIIa binding sites, and intracellular alkalinization of platelets (Halushka 2000).

The most relevant signalling processes of TXA_2 are extensively described in section I.3.6.1 which details the physiology of TXA_2 with an emphasis of its roles in platelet aggregation.

I.3.4.3. TP receptor desensitization

Platelet TP receptors are known for a long time to undergo potent agonist-induced desensitization (Liel, Mais *et al.* 1988) and phosphorylation (Okwu, Ullian *et al.* 1992). It has subsequently been demonstrated that both TPα and TPβ receptors were subject to homologous, agonist-induced desensitization in transfected HEK293 cell lines (Habib, Vezza *et al.* 1997). This desensitization involves several mechanisms, including activation of PKC (Spurney 1998) and GRKs (Flannery and Spurney 2002).

Differences in the carboxy-terminal tail between the two isoforms and thus differences in sites for phosporylation imply that TPα and TPβ might undergo differential homologous/heterologous desensitization. For example, some studies indicated that TPβ, but not TPα, can undergo agonist-induced but also tonic internalization in a GRK- and arrestin-dependent manner (Parent, Labrecque *et al.* 2001; Parent, Labrecque *et al.*

1999). It is noteworthy that this is in contrast to what was reported for HEL and CHRF-288 (megakaryocytic) cells that express predominantly the α isoform and were found to undergo internalization (Dorn 1992; Parent, Labrecque *et al.* 1999).

Heterologous desensitization of TXA_2 receptors involves many other receptor/mediator systems, including subtype 1 of PGE_2 receptors (EP_1) and $PGF_{2\alpha}$ receptors (FP) (Kelley-Hickie and Kinsella 2004). Additionally, nitric oxide (NO) (Reid and Kinsella 2003) and stimulation of PGD_2 receptors (DP) (Foley, Kelley *et al.* 2001) have been shown to selectively desensitize TPα. Finally, PGI_2/IP mediated heterologous desensitization through cAMP has been widely documented (Manganello, Djellas *et al.* 1999; Murray, Shipp *et al.* 1990) and several data indicate that TPα, but not TPβ was subject to IP-receptor-induced desensitization in a protein kinase A (PKA)-dependent pathway (Walsh, Foley *et al.* 2000). The PGI_2/TXA_2 system will be described extensively at point I.3.6.2 (p.45).

I.3.4.4. TP receptor dimerization

Many GPCR have been demonstrated to function as homo-, hetero- or oligomers (see I. 3. 1. p.16). Different works performed on TP receptors have subsequently tried to evidence this phenomenon for TP receptors. In 2005, Laroche *et al.* demonstrated that TPα and TPβ could form homo- and heterooligomers in an agonist-independent process and suggested that dimers/oligomers constituted the functional unit for the receptor. Interestingly, in that study, TPα which does not undergo

constitutive or agonist-induced endocytosis on its own was subjected to both types of endocytosis when co-expressed with TPβ, indicating that TPα could display intracellular trafficking through heterodimer when complexed with TPβ (Laroche, Lepine *et al.* 2005). This homo/hetero oligo/dimerization of TP receptors was later confirmed by two studies which showed that TPα/TPβ dimerization enhanced isoprostane (Wilson, McGinley *et al.* 2007) but lowered U46619 (Sasaki, Miyosawa *et al.* 2006) mediated signal transduction. Thus, these data indicate that TP signaling could be dependent of the receptor isoforms heterodimerization.

Additionally, Wilson *et al.* postulated and demonstrated that TPα and IP formed functional heterodimers in an agonist-independent manner. Thus, IP/TPα dimerization was coincident with TP-cAMP generation, promoting a "PGI_2-like" cellular response to TP activation. Moreover, they demonstrated that IP/TPα interaction permitted reciprocal regulation of receptor endocytosis via the trafficking pathway determined by the activated dimeric partners. These results represented a possible mechanism by which PGI_2 and IP receptor may limit the cellular effects of TP receptors activation (Wilson, Dowling *et al.* 2007; Wilson, Roche *et al.* 2004).

I.3.4.5. Ligand-binding site

Although many ligands for TP receptors have been synthesized, the conformation of TP receptor remains elusive. Since the TP receptor belongs to the seven transmembrane-spanning class of receptors, it was

originally proposed that the binding domain of this receptor might also reside in TM7 (Hirata, Hayashi *et al.* 1991). Other investigators (Narumiya, Hirata *et al.* 1993) proposed that while TM7 interacted with the ligand carboxyl group, separate transmembrane regions also participated in ligand binding, *i.e.* TM3 coordinated with the prostanoid ring, and the TM4 and TM5 regions interacted with the alkyl chains.

More recently, site-directed mutagenesis studies have investigated the ligand coordination site for TP receptors. Specifically, Funk *et al.* (Funk, Furci *et al.* 1993) obtained four mutants with point mutations at TM7 of the TP receptor, *i.e.* between Leu^{291} and Trp^{299}. Three of these mutants completely lost binding activity to both antagonists and agonists. Although the fourth mutant, W299L, did not recognize the TP receptor antagonist SQ29548, it was able to bind with two different TP receptor agonists with the same affinities than those observed with the wild type receptor. In addition, Chiang *et al.* (Chiang, Kan *et al.* 1996) reported that mutations of S201A and S255A at TM5 and TM6, respectively, caused altered affinity to the agonist I-BOP but had no effect on the antagonist SQ29548 binding. Separate studies by Dorn *et al.* (Dorn, Davis *et al.* 1997) used receptor chimeras to evaluate ligand binding activity. They concluded that residues in TM1 constituted an important portion of the TP receptor binding site. Finally, reports from two different groups suggested that the putative disulfide bond between Cys^{105} and $Cys^{183/184}$ in extracellular domain (ED) 2 and ED3, respectively, played a critical role in receptor-ligand binding. In particular, mutants C105A and C183A from the human placenta TP receptor (Chiang, Kan *et al.* 1996) and mutants C105S and C184S from human K562 TP receptors (D'Angelo, Eubank *et*

al. 1996) did not show any binding activity for either agonists or antagonists. In addition, both groups reported that Cys^{102}, which is conserved in most seven transmembrane-spanning receptors including the TP receptor (but absent in other prostanoid receptors), also played an important, yet unspecified role in ligand binding.

In 1993, Yamamoto *et al.* (Yamamoto, Kamiya *et al.* 1993) proposed a receptor model where the ligand binding pocket of the TXA_2 receptor included a serine residue from segment V, an arginine residue from segment VII, and a large hydrophobic pocket between these two residues. More recently, Turek *et al.* (Turek, Halmos *et al.* 2002), using SQBAzide (a newly synthesized biotinylated photoaffinity probe (Halmos, Turek *et al.* 1999)) determined that extracellular loop II was critical for ligand binding. Subsequent work by this group led to the conclusion that five key amino acids within this region (Phe^{184}, Thr^{186}, Ser^{191}, Asp^{193}, and Ser^{201}) participated in TP receptor ligand binding and function (Khasawneh, Huang *et al.* 2006).

Using NMR (nuclear magnetic resonance) techniques, some studies determined that the domain within the second extracellular loop and the disulfide bond between the first extracellular loop and the extracellular loop 2 played a major role in forming the ligand recognition pocket (Ruan, Wu *et al.* 2004; So, Wu *et al.* 2003).

These collective data of several studies showed that transmembrane domains I, III, IV and VII as well as extracellular loop II and III were involved in ligand binding interaction (Chiang, Kan *et al.* 1996; D'Angelo, Eubank *et al.* 1996; Funk, Furci *et al.* 1993; Hirata, Hayashi *et al.* 1991; Narumiya, Hirata *et al.* 1993; Ruan, So *et al.* 2001;

Yamamoto, Kamiya *et al.* 1993). One could argue that all these models gave contradictory results. Nevertheless, there is no clue that one model excludes another one, given the important number of ligands (different chemical structures, agonists, antagonists, etc) and the methods used in these studies. Indeed, there is an obvious lack in 3D structure data for TP receptors, given the absence of absolute crystalline conformation.

I. 3. 5. Physiological significance of TPα and TPβ

The physiological significance for the presence of two isoforms of the TP receptor is still unclear. Nevertheless, several teams tried to decipher the exact role and function of the two isoforms.

Alternative splicing that generates receptor isoforms in the carboxyl-terminal tail is not only found for TP receptors. Among the prostanoid receptors family, mRNA splice variants have been identified for the EP_1, EP_3 and FP (Pierce and Regan 1998). Interestingly, except for the EP_1 receptors, the mechanisms giving rise to these receptor isoforms involves the use of splice sites located in the cytoplasmic carboxyl-termini of these receptors. Thus, the eight human EP_3 isoforms that have been identified are otherwise identical except for their carboxyl termini. Similarly, the optional use of a potential splice site encoding the carboxyl-terminus gives rise to each of the two FP isoforms. Because the carboxyl-termini of GPCR are generally implicated in interactions with G proteins, it is not surprising that these receptor isoforms differ mainly with respect to their activation of second messenger pathways and not in their pharmacological characteristics. Differences also exist with respect to their levels of constitutive activity and to their desensitization.

Besides its predominant role in platelet aggregation which will be described elsewhere (I.3.6.A. p. 36), TPα has also been proposed to be relevant for vascular homeostasis. Indeed, in 2000, Walsh *et al.* established that TPα, but not TPβ, was subject to cross-desensitization by IP mediated through direct PKA phosphorylation of the receptor at Serine 329 and the authors concluded that TPα might be the isoform physiologically relevant to TP:IP-mediated vascular homeostasis (Walsh, Foley *et al.* 2000).

More recently, Ashton and Ware discovered that TPβ was required for VEGF-induced endothelial cells migration and angiogenesis. Consequently, they proposed that selective inhibition of TPβ isoform could enhance myocardial revascularization after infarction (Ashton and Ware 2004).

Collectively, consecutive data resumed and presented in this section indicate that our knowledge of the TP receptor isoforms is still far from complete. The work required for the elucidation of isoform-specific physiological and pharmacological properties has still to be done.

I. 3. 6. Physiological and pathological implications of TXA_2

TXA_2 has an important but diversified role in organism. This part of the general introduction will describe the TXA_2 actions and their consequences on normal physiology and physiopathology.

I.3.6.1. Physiology of TXA_2

I.3.6.1.A. Hemostasis

In this section, we will briefly describe principal mechanisms of platelet aggregation and will focus on the interest of TXA_2 and its major role in hemostasis, platelet activation and aggregation.

Hemostasis literally means (Hemo = blood and stasis = the stop of flowing of blood). At site of vascular injury, thromboresistant endothelium is disrupted and prothrombotic subendothelial vessel wall constituents (eg, collagen) are exposed to blood. Three consecutive phenomena may then occur and their common action eventually conducts to hemostasis :

- vascular constriction ;
- platelet aggregation and plug formation (primary hemostasis) ;
- blood coagulation (secondary hemostasis) ;

Formation of fibrous tissues in the blood clot is sometimes considered as the fourth hemostasis step. It occurs at the cicatrisation and is responsible for definitive obturation of the lesion.

Vascular constriction

Vascular constriction allows sparing blood loss. An important hemorrhage can undeniably have dramatic consequences. Vascular spasm can persist from minutes to several hours and permits the coagulation system to be effective. The origin of this vascular reaction can be

multifactorial, but mainly a consequence of the liberation of several mediators from the wounded endothelium or the activated platelets.

In small vessels, platelets are directly responsible for the vasospams, after secretion of TXA_2 which has potent vasoconstrictor properties besides its proaggregant action. These physiological characteristics will be discussed further in this work.

Platelet aggregation and plug formation (primary hemostasis)

General considerations

Platelets are fundamentally involved in the physiological process of hemostasis. Platelets participation occurs in diversified ways including adhesion to the cut end of vessel or subendothelial components, activation, shape change and secretion, and formation of large platelet aggregates. Although there is no clear separation between these phenomena, we will describe them successively in this chapter.

Platelet adhesion and activation

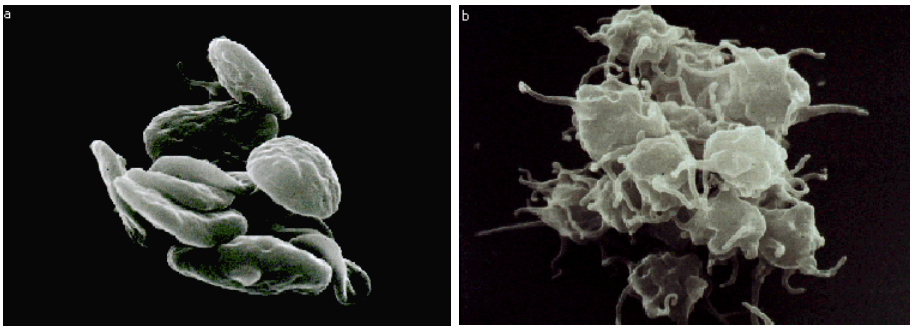
Under normal conditions, platelets circulate in the blood flow freely as small discoid bodies and do not adhere to normal vascular endothelial cells. Cut end or wound of a vessel provides numerous binding sites in the subendothelial matrix for resting platelets, including collagen and von Willebrand Factor (vWF), a multimeric adhesive protein associated with collagen in the vessel wall. The platelet adhesive receptors include some members of the integrin family, such as the fibrinogen/fibronectin receptor, integrin $\alpha_{IIb}\beta_3$, and the collagen receptor, integrin $\alpha_2\beta_1$, but also the glycoprotein Ib-V–IX complex (GPIb-V–IX), which is almost exclusively expressed in platelets and megakaryocytes.

Interactions between integrins and glycoproteins from immobilized platelets and subendothelial elements strengthen the activation of platelet and induce several signaling events inside platelets. Many processes involved in platelet activation are calcium-dependent, including liberation of arachidonic acid from membrane phospholipids by the enzyme PLA_2. This enzyme releases arachidonic acid from phosphatidylcholine and probably other phospholipids. Arachidonic acid is converted by COXs to prostaglandin endoperoxide and ultimately to TXA_2 as well as to other prostaglandins such as PGD_2. The latter inhibits platelet activation and acts as a negative feedback modulating platelet activities. DAG, like IP_3, both products of PLC, activates an ubiquitous enzyme, PKC (Nishizuka 1984).

Platelet secretion and shape change

The activation of platelet results in the reorganization of platelet cytoskeleton and secretion of both dense bodies and α-granules. Platelets contain several classes of granules in which intracellular constituents are sequestered, including dense bodies (containing serotonin, ATP, ADP, pyrophosphate and Ca^{2+}) and α-granules (containing fibrinogen, vWF, Factor V, high molecular weight kininogen, fibronectin, alpha1-antitryptsin, beta-thromboglobulin, platelet factor 4, and platelet-derived growth factor). Most of these constituents have procoagulant and proaggregant properties, and contribute to recruit circulating platelets. Secretion of the mediators contained in these granules results from activation of the platelet cytoskeletal apparatus. Indeed, polymerization of filamentous actin and phosphorylation of myosin are prominent events

that lead to fusion of the granule envelope with the membrane and external secretion of the granule content.



a **b**

Figure I-10. Electron microscopy of (a) resting and (b) activated platelets.

Platelet aggregation

Fibrinogen is a freely circulating plasma protein that appears to be the most important in the process of platelet aggregation. It is characterized by a divalent structure, thus allowing fibrinogen to form a bridge from platelets to platelets and thereby mediating aggregation.

It is well known that fibrinogen is unable to interact with resting platelets, but binds tightly to platelets activated by physiological agonists. Indeed, activation of platelets permits the transition of GPIIb/IIIa from a resting to an active state which is characterized by high affinity for circulating fibrinogen. Even if the final event in platelet aggregation is the transition of GPIIb/IIIa from an inactive to an active state, mediators implicated in platelet aggregation contributes in very different manners. Two of the critical changes in second messengers observed after proaggregant action are an increase in the intracellular calcium concentration and a decrease in cytosolic cAMP (Jin and Kunapuli 1998; Pulcinelli, Ciampa *et al.* 1999). Both of these changes are necessary for

inducing the conformational change and subsequent ability for fibrinogen to bind αIIbβ3 integrin (Brass, Manning *et al.* 1997; Shattil and Newman 2004). The change in the integrin that allows fibrinogen binding, termed "inside-out signaling" is generally accepted to be the central event in platelet aggregation, but the detailed mechanisms which lead from G protein activation to inside-out signaling are still not completely understood (Woulfe 2005). Besides TXA_2, three factors play a key role in platelet aggregation (Figure I-11) :

- Thrombin is a protease which is also a strong promoter of blood coagulation. It triggers platelet aggregation through activation of specific GPCRs (PAR-1 and PAR-4). These receptors promote platelet shape change, synthesis and release of TXA_2, release of granules content and activation of GPIIb/IIIa (Macfarlane, Seatter *et al.* 2001) ;

- ADP is regarded as a weak inducer of platelet aggregation, but induces complete aggregation by causing platelet secretion (of TXA_2 for example) and activation (Gachet 2006). Three receptors mediate ADP action at the platelet surface (ligand-gated cation channel $P2X_1$ and GPCRs $P2Y_1$ and $P2Y_{12}$). Collective activation of these three receptors is required for complete aggregation by ADP. $P2Y_{12}$ is the pharmacological target of antiplatelet agents ticlopidine and clopidogrel ;

- Collagen is the most thrombogenic component of the subendothelial layer. Besides its role in the adhesion of platelets, collagen is able to induce platelet aggregation. The

interaction between collagen and platelets is complex and mediated by several receptors, including integrin α2β1, and GPVI (Clemetson and Clemetson 2001).

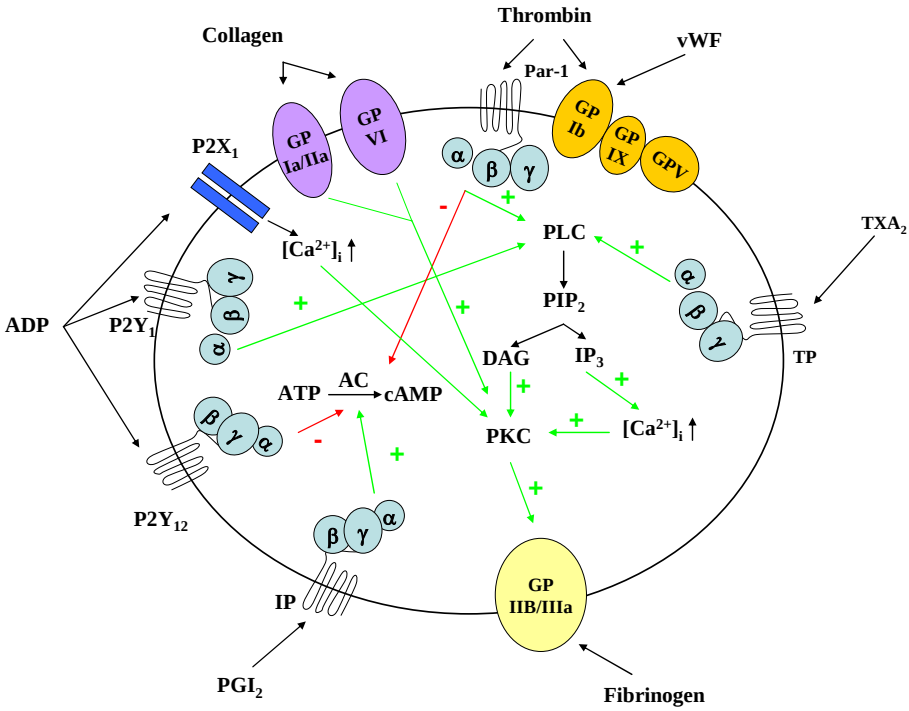


Figure I-11. Schematic representation of the role of different mediators in platelet activation and aggregation. See text for details.

Role of prostanoids and TXA_2

Early pharmacological studies indicated that platelets possess receptors for PGD_2, PGE_2 (EP_3 and EP_4), TXA_2 and PGI_2 (Armstrong 1996; Paul, Ashby *et al.* 1998). Although PGD_2 and PGE_2 might regulate platelet aggregation, TXA_2 and PGI_2 have antagonistic and intricate roles in the regulation of platelet aggregation and vascular homeostasis (see

I.3.6.2, p.44). Throughout the COX pathway, PGI_2 is the product of the isomerization of PGH_2 by prostacyclin synthase.

PGI_2 acts on a specific GPCR receptor termed IP which is, in platelets, mainly coupled to Gαs and to an increase in cAMP levels (Wise, Wong *et al.* 2002). Consequently, the physiological antagonism of platelet functions through activation of PKA (Siess 1989) and PGI_2 is a potent inhibitor of the platelet activation and aggregation, whatever the inducer (ADP, thrombin, collagen, TXA_2,…). For example, PKA includes myosin light chain phosphorylation, thus causing inhibition of Ca^{2+}/Calmodulin-dependent contractile processes such as secretion (Hathaway, Eaton *et al.* 1981). It is noteworthy that all inducers pathways are not equally sensitive to a rise in cAMP and that PGI_2 is characterized by a certain selectivity in inhibiting platelet aggregation; its inhibitory potency decreases with the aggregatory agent in the order TXA_2 >> thrombin > ADP (Manganello, Djellas *et al.* 1999).

TXA_2 is, just after thrombin, the dominant activator of platelet aggregation, due to the number of receptors expressed at the cell surface (around 1000 copies, compared to 2000 for thrombin receptors) (Brass, Manning *et al.* 1997). As we have seen before, TXA_2 mediates its action in platelets at least mainly through TPα receptors, since TPβ is expressed at very low levels, if any (see I. 3. 4., p. 24) (Habib, FitzGerald *et al.* 1999). TPα receptors are functionally coupled to $G\alpha_q$ and $G\alpha_{12/13}$ in platelets (Djellas, Manganello *et al.* 1999; Knezevic, Borg *et al.* 1993; Offermanns, Laugwitz *et al.* 1994; Offermanns, Toombs *et al.* 1997).

Although some studies also pointed out that TPα could be coupled to an increase of cAMP levels whereas TPβ would have the opposite effect on cAMP levels (Hirata, Ushikubi *et al.* 1996), it is unlikely that these coupling are relevant in platelets since several studies have shown that there were no changes in cAMP levels in platelets upon stimulation by U46619 (Brass, Woolkalis *et al.* 1988; Klages, Brandt *et al.* 1999; Paul, Jin *et al.* 1999).

In a recent paper by Li *et al.*, it was demonstrated that activation of platelet TP receptor by TXA_2 evoked two waves of platelet secretion which, respectively, preceded two waves of platelet aggregation (Li, Hu *et al.* 2003). The authors proposed that, consistent with previous reports, the first wave of platelet aggregation was a consequence of released ADP in the first wave of secretion. More importantly, the authors proposed that the second wave of secretion was dependent of concomitant action of Gβγ activated PI_3 kinase and integrin outside-in signaling, which finally induced irreversible second wave of aggregation.

Collectively, the depiction of the mechanism of action of several agonists involved in platelet aggregation shows that TXA_2 is involved in each pathway (thrombin, collagen, ADP,…) since production of AA is always implicated in platelet activation and aggregation.

Plug formation

The platelet aggregation mechanism provokes an overall auto-amplified accumulation of aggregated platelets at the injured site. The aggregate thus formed is called the platelet plug. It is a loose structure that is sufficient if the wound gap is not too large. Otherwise, the

bleeding will only stop after the formation of the clot, a consequence of *blood coagulation*.

Blood coagulation or secondary hemostasis

Blood coagulation is an extremely complex mechanism, and its description is outside the scope of this work.

I.3.6.1.B. Smooth muscles

We have seen at point I. 3. 3. (p.23) that TP receptors were expressed in smooth muscles cells. Thus, besides its implications in the process of platelet aggregation, TXA_2 is a potent inducer of smooth muscles contraction (Svenssen, Strandberg *et al.* 1977).

In the hemostasis process, the vasoconstrictor effects of TXA_2 are involved in prevention of blood loss. An overproduction of TXA_2 can have a deleterious effect through excessive vessel contraction, such as hypertension or preeclampsia (Cheever 2005; Schiffrin 2001; Walsh 2004). In vascular smooth muscles, both TPα and TPβ seem to contribute to the vasoconstrictor effect of TXA_2 (Miggin and Kinsella 1998).

TXA_2 is able to trigger a bronchospasm and plays a significant role in asthma. Several studies have pointed out that it could be produced by macrophages and platelets in lung in response to several stimuli (Godard, Chaintreuil *et al.* 1982).

I.3.6.2. The thromboxane-prostacyclin system

We have seen throughout the description of the production, regulation and action of TXA_2 that PGI_2 has a tight connection with that mediator.

As a general paradigm, it is though that COX-1 is mainly responsible for TXA_2 synthesis, because COX-1 and TXS are highly expressed in platelets. Besides, PGI_2 synthesis is principally dependent of COX-2 present in vascular vessel wall (McAdam, Catella-Lawson *et al.* 1999).

In the absence of any wound, normal endothelium releases the anti-aggregatory PGI_2 in the vascular bed. Upon platelet activation, PLA_2 releases AA from membrane phospholipids and TXA_2 is synthesized by sequential oxygenation of AA by COX and TXA_2 synthase. Indeed, TXA_2 is the major product of AA metabolism in platelets (Smith 1989). The proaggregant activity of TXA_2 acts as a positive feedback for the activation and recruitment of circulating platelets to the primary hemostatic plug. Additionally, in the endothelium, mediators like TXA_2 can trigger PGI_2 response by inducing Ca^{2+} increase and subsequent PLA_2 activation and PGI_2 synthesis (Ullrich, Zou *et al.* 2001). Consequently, the balance between the production and local concentration of PGI_2 and TXA_2, two antagonistic signals for the platelet aggregation, is a major parameter in this process.

In 2002, Cheng *et al.* have demonstrated that injury-induced vascular proliferation and platelet activation are enhanced in mice that are genetically deficient in the IP receptor but are depressed in mice

genetically deficient in the TP receptor or treated with a TP antagonist (Cheng, Austin *et al.* 2002). These results show that PGI_2 modulates platelet-vascular interactions *in vivo* and specifically limits the response to TXA_2. Indeed, the ratio between PGI_2 and TXA_2 seems to be more relevant than the levels of these mediators alone.

I.3.6.3. Correlation between TXA_2 and diseases

Numerous works have highlighted the relation of TXA_2 with several physiopathological processes. For example, a TXA_2 overproduction, detected by high levels of plasmatic TXB_2 or urinary 2,3-dinor-TXB_2, has been evidenced in many cardiovascular, pulmonary or renal diseases (Table I-2). In most cases, disequilibrium between TXA_2 and PGI_2 could contribute to the development of diseases, and we will try to describe when it is possible how this imbalance contributes to pathologies. Several aspects of TXA_2 physiopathology will be envisaged in this section, with an emphasis on cardiovascular diseases, where the contribution of TXA_2 has been well documented with recent data.

Pathological states	References
Unstable angina	(Hamm, Lorenz *et al.* 1987; Patrono and Renda 1997)
Myocardial infarction	(Bing, Yamamoto *et al.* 1999; Fitzgerald, Roy *et al.* 1986; Gurbel, Murugesan *et al.* 1999)
Atherosclerosis	(Mehta, Lawson *et al.* 1988; Titov 1999; Vanhoutte 1997)
Preeclampsia	(Friedman 1988; Walsh 1985)
Raynaud' disease	(Herrick, Illingworth *et al.* 1996; Reilly, Roy *et al.* 1986)
Thrombosis	(Fitzgerald, Wright *et al.* 1989; Saldeen, Nilsson *et al.* 1983; Saldeen, Saldeen *et al.* 1993)
Asthma	(Devillier and Bessard 1997; Iwamoto, Ra *et al.* 1988)
Cigarette Smoke-induced Bronchoconstriction	(Hong and Lee 1996; Matsumoto, Aizawa *et al.* 1996)

Table I-2. Main pathologies associated with TXA_2 overproduction.

I.3.6.3.A. Thromboxane A_2 and cardiovascular diseases

Atherosclerosis

Atherosclerosis, the major cause of ischemic coronary artery disease and cerebrovascular disease, is a chronic vascular disease in which inflammation and oxidative stress have important roles at every stage (Hansson 2005). The disease process develops and progresses in response to abnormal cholesterol deposits in the intima of large arteries. It is initiated by a phase of endothelial activation that is induced by inflammatory cytokines, oxidized low-density lipoproteins (ox-LDL) and/or changes in endothelial shear stress. This leads initially to the expression of endothelial adhesion molecules and chemokines, followed

by the recruitment and activation of circulating monocytes and lymphocytes (Dogné, Hanson *et al.* 2005). A variety of substances including cytokines, chemokines, and growth factors have been suggested to induce, amplify, and modify atherosclerosis, including PGI_2 and TXA_2 (FitzGerald, Smith *et al.* 1984). Atherosclerosis research efforts have recently focused on these two mediators, because of their deep implications in cardiovascular diseases, but also because their biosynthesis is increased in patients with atherosclerosis (FitzGerald, Smith *et al.* 1984).

There is growing evidence of a deep implication of COX-2 in pathogenesis of atherosclerosis, since this enzyme expression is induced at inflammatory sites (Linton and Fazio 2004). Moreover, it seems that PGI_2 increase in atherosclerosis is due to concomitant action of both COX-1 and COX-2, which is induced in atherosoclerotic plaque (Belton, Byrne *et al.* 2000). Besides, the activated platelets are though to be responsible for increased TXA_2 production in atheroslerosis (Pratico, Cyrus *et al.* 2000).

The atherosclerotic plaque is constituted of many cell types, including monocytes, macrophages, endothelial cells, T cells and platelets. Altogether, these cells exert a wide and complex array of interaction (Ross 1999). Ishizuka *et al.* showed that stimulation of endothelial cell TP receptors increased the expression of adhesion molecules such as ICAM-1 (Ishizuka, Suzuki *et al.* 1996). This molecule can promote monocyte adherence (Spagnuolo, Ellner *et al.* 1988) and is strongly expressed in atherosclerotic plaques of humans (Collins, Velji *et al.* 2000). The level of soluble ICAM-1 correlates with the severity of atherosclerosis (Springer 1990). Moreover, the infiltration of monocytes is a key "inflammatory"

event in early atherogenesis (Ross 1999). Thus, by this potential mechanism, TXA_2 might promote acceleration of plaque growth (Patrono and FitzGerald 1997).

These theoretical considerations on PGI_2 and TXA_2 implications in atherosclerosis have found strong rationale in several recent studies. Kobayashi *et al.* have recently studied atherosclerosis in genetically modified mice lacking either TP or IP receptors (Kobayashi, Tahara *et al.* 2004). They have discovered that mice lacking TP developed less atherosclerotic lesions. Besides, in the mice group lacking IP, the atherosclerotic plaque growth was accelerated. Additionally, the absence of IP appeared to affect the progression and nature of atherosclerotic plaques.

Thrombosis, stroke and myocardial infarction

The clinical manifestations of atherosclerosis include arterial thrombosis, and thus myocardial infarction and ischemic stroke. They are the consequences of either the rupture or erosion of an atherosclerotic plaque, which can trigger thrombus formation and lead to the occlusion of the vessel lumen (Hansson 2005). Since the platelet aggregation is one of the central mechanisms for arterial thrombus formation, TXA_2 has long been thought to have an important contribution in this phenomenon. Several data have now strengthened this hypothesis, first of all being the increased levels of TXA_2 in patient suffering from myocardial infarction or thrombosis (Table I-2).

Aspirin or acetylsalicylic acid (ASA) is a preferential inhibitor of COX (see point I.4.1.1, p. 54) and has been used for many years, at low

doses, in secondary prevention of myocardial infarction. The origin of its cardioprotective effect is well described. ASA irreversibly inactivates COX, preferentially COX-1. Since platelets have no nucleus, and are unable to synthesize proteins *de novo*, low doses ASA specifically inhibit TXA_2 biosynthesis by platelets and thus impair platelet activation and aggregation.

Moreover, several authors now assume that the cardiovascular events associated with selective COX-2 inhibitors, claimed to be specific anti-inflammatory drugs with less gastrointestinal adverse effects, were due to selective depletion of PGI_2 levels compared to those of TXA_2 (Dogné, Hanson *et al.* 2006; Fitzgerald 2004; FitzGerald, Cheng *et al.* 2001; Frankish 2002; Konstam, Weir *et al.* 2001; Mukherjee, Nissen *et al.* 2001; Pratico and Dogné 2005; Schnitzer 2001). Supporting this assumption, rofecoxib was recently withdrawn from the market following first results of a 3-year randomized, placebo-controlled, double-blind clinical trial enrolling 2600 patients called APPROVe (Adenomatous Polyp Prevention on Vioxx). The study was halted prematurely, revealing a 3.9-fold increase in serious thromboembolic adverse events in the rofecoxib treated group (Bresalier, Sandler *et al.* 2005).

I.3.6.3.B. Thromboxane A_2 and asthma

In addition to its cardiovascular role, TXA_2 plays also a crucial role in the pathogenesis of bronchial asthma since it is a potent constrictor of bronchial smooth muscles and a stimulator of airway smooth muscle cells proliferation (Devillier and Bessard 1997; Tamaoki, Kondo *et al.* 2000). Several studies demonstrated increased concentrations

of this mediator and metabolites in bronchoalveolar lavage fluid, urine and plasma from asthmatic patients (Kumlin, Dahlen *et al.* 1992; Wenzel, Westcott *et al.* 1989). Activation of TP receptors present in bronchial smooth muscle cells by TXA_2 leads to intracellular calcium mobilization with bronchoconstriction as consequence (Billington, Hall *et al.* 1999; Capra, Habib *et al.* 2003). Prostanoid TP receptor activation also contributes to bronchial smooth muscle hyperplasia and airway remodelling which occur in response to chronic airway inflammation of asthma (Vignola, Mirabella *et al.* 2003). Using a model of allergen-induced cough in guinea pig, Xiang *et al.* demonstrated that airway mucous cells are an important source of TXA_2 and that this prostanoid facilitates cough (Xiang, Uchida *et al.* 2002).

TXA_2 is also implicated in chronic obstructive pulmonary diseases (COPD). Indeed, it was demonstrated in patients with COPD that urinary excretion of 11-dehydro-TXB_2, a TXA_2 metabolite, was significantly higher than in healthy subjects, suggesting an enhancement of platelet TXA_2 biosynthesis in such patients (Davi, Basili *et al.* 1997). Additionally, thromboembolic events such as pulmonary hypertension are complications of COPD and TXA_2 may play an important role in these complications (Cheever 2005).

I.3.6.3.C. Thromboxane A_2 and cancer

TXA_2 has also been implicated in cancer. TXA_2 would sustain tumor proliferation, angiogenesis, vascular wall adhesion and development of metastasis (Daniel, Liu *et al.* 1999; Nie, Lamberti *et al.* 2000).

Tumor proliferation

Studies performed with human cancer tissues such as lung cancer, squamous cell carcimonas of the larynx, glioblastomas or meningiomas revealed the presence of higher TXB_2 levels compared to normal tissues (Castelli, Chiabrando *et al.* 1989; McLemore, Hubbard *et al.* 1988; Pinto, Gori *et al.* 1993). Consistent with these data, Nigam *et al.* have demonstrated that several cancer cell lines were able to stimulate TXA_2 production (Nigam and Zakrzewicz 1991). Additionally, Nanji demonstrated that the organ preference for metastasis was positively correlated with the levels of TXS found in human organs, leading to the suggestion that presence of TXS was favorable for cancer development (Nanji 1993).

Tumor Angiogenesis

Angiogenesis is a necessary step in cancer development. Several data support the TXA_2 involvement in this process. First, TXA_2 is a potent stimulator of endothelial cells migration *in vitro* (Nie, Lamberti *et al.* 2000). Secondly, TP receptors antagonists such as SQ29548 have been demonstrated to possess inhibitory potency against the endothelial cells migration *in vitro* and anti-angiogenic profile in neo-vascularization models *in vivo* (Daniel, Liu *et al.* 1999).

Metastasis

Several cancer cell lines are able to induce platelet aggregation, which is an early step in hematogenous metastasis (Honn, Tang *et al.* 1992). Although tumor cells can use different mechanisms in order to induce platelet aggregation, such as the release of soluble mediators

(thrombin and ADP) or a direct interaction mediated by membrane glycoproteins, the final result of these interactions, is invariably a platelet aggregation accompanied by an important release of TXA_2 (Mehta, Lawson *et al.* 1986). Consequently, with regard to the roles of TXA_2 in cancer progression, it may be hypothesized that the TXA_2 release from the platelets could also contribute to tumor cell proliferation.

With regard to these hypothesis, it is noteworthy that several TP receptor antagonists related to the compounds presented in the present work were proved to reduce angiogenesis and sarcoma-cell-induced platelet aggregation (de Leval, Benoit *et al.* 2003; de Leval, Dassesse *et al.* 2006).

I.4. TXA_2 modulators

In this chapter, we will consider the different chemical compounds able to modulate TXA_2 actions. They are divided in three classes : compounds inhibiting TXA_2 production, compounds interacting with TP receptors or compounds acting on both targets.

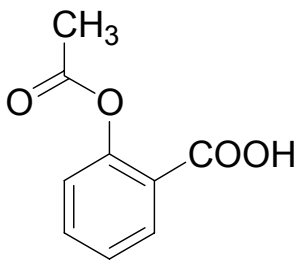
I. 4. 1. Chemical compounds inhibiting TXA_2 formation

Several ways to inhibit TXA_2 formation can be considered. COX inhibition will of course prevent the production of all prostanoïds, including TXA_2. TXS was a logical target for chemical compounds aiming to decrease TXA_2 levels.

I.4.1.1. Acetylsalicylic acid and other Cyclooxygenase inhibitors

I.4.1.1.A. Acetylsalicylic acid

Despite massive development efforts, acetylsalicylic acid (**6**, ASA or aspirin, Figure I-12) remains the first-choice drug for primary and especially secondary prevention of myocardial infarction. The effects of ASA are the result of the acetylation of a serine residue in the active site of COX, thus irreversibly inactivating the enzyme (Vane 1971). Consequently, ASA effects are limited to COX-1 when given at low dose (80 or 160 mg), since platelets cannot renew their proteins due to their lack of nucleus. ASA also inhibits endothelial cells COX-1 (Bennett 2001) and prevents a fraction of PGI_2 production by constitutive vascular endothelial COX-2 (Cheng, Austin *et al.* 2002). Physiological consequences are the selective inhibition of TXA_2, with higher PGI_2 levels, and thus a potent cardioprotective effect well evidenced by clinical data (Patrono, Garcia Rodriguez *et al.* 2005).



6

Figure I-12. Chemical structure of acetylsalicylic acid.

Despite all the acknowledge positive effects of ASA, and the increase understanding of its cardioprotective effects through TXA_2 production inhibition, some drawbacks of aspirin treatments for prevention of cardiovascular events have been observed. Indeed, many patients have a conserved platelet function despite aspirin treatment (Patrono 2003). This phenomenon is called "aspirin resistance" and its mechanisms are still not fully understood. Additionally, ASA triggers some serious allergic reaction such as asthma (Nettis, Colanardi *et al.* 2001). These negative elements have pushed to the research and development of other antiplatelet agents and compounds also able to counteract TXA_2 production and action.

I.4.1.1.B. Cyclooxygenase inhibitors

Since prostanoids are involved in inflammation process and pain, even before the elucidation of aspirin inhibition of COX, numerous compounds related or not to ASA have been synthesized as anti-inflammatory drugs, inhibiting COX enzymes. The complete chemical description of these compounds is out of the scope of this review.

Synthetically, all COX-1 preferential inhibitors have the same cardioprotective effect than aspirin, even if there are never used for this purpose, due to severe gastro-intestinal side effects.

On the other side, COX-2 mediated PGI_2 inhibition have received much attention due to the hypothesis that imbalance between TXA_2 and PGI_2 was responsible of cardiovascular events associated with rofecoxib (see, I.3.6.3.A. p. 47). Nevertheless, it is not clear whether or not this is related to a drug or a class effect (Dogné, Supuran *et al.* 2005).

I.4.1.2. Thromboxane synthase inhibitors

The selective inhibition of TXS prevents the conversion of PGH_2 to TXA_2 (Fiddler and Lumley 1990; Fitzgerald, Roy *et al.* 1986). This has the advantage that other arachidonic acid metabolites can still be produced. This is of particular interest in the prevention of platelet aggregation. Indeed, in the setting of platelet activation, as would occur locally at a vascular injury site, the platelet-produced endoperoxides may be taken by other cells such as smooth muscle cells and endothelial cells. In these cells, PGH_2 may be isomerised by the prostacyclin synthase to form PGI_2 which can then inhibit platelet aggregation and induce vasodilation. Therefore, with a TXS inhibitor (TXSI), the antithrombotic effect due to the presence of locally-produced PGI_2 could exceed that expected by blocking the COX as does aspirin. Additionally, the conversion of PGH_2 to E-type prostaglandins could help to reduce thrombus formation due to their vasodilatory action.

On the basis of these hypotheses, a series of TXSIs have been developed in the eighties. Several compounds such as dazoxiben (**7**, UK 37248), dazmagrel (**8**, UK 38485), pirmagrel (**10**, CGS 13080), ozagrel (**9**, OKY 046), CS-518 (**13**), OKY 1581 (**14**), isbogrel (**12**, CV 4151), and furegrelate (**15**, U63557A) (Figure I-13) have undergone clinical testing.

Unfortunately, these compounds were found less active than expected or sometimes ineffective although reducing the biosynthesis of TXA_2. Indeed, they failed to produce consistent effects in clinical conditions where an overproduction of TXA_2 has been detected (Dogné, de Leval *et al.* 2004). The disappointing clinical results with this class of

drugs can be explained by an incomplete blockage of TXS with the dosage used and mainly by the fact that TXSIs provoke an accumulation of the TXA_2 precursor, PGH_2, which is chemically more stable and exerts similar biological effects by acting at common receptors (Coleman, Humphrey *et al.* 1981; Hamberg, Svensson *et al.* 1974; Patrono 1990; Patscheke, Hornberger *et al.* 1990; Vermylen and Deckmyn 1992). As a consequence, few pharmaceutical companies have continued the clinical development of TXSIs as single drugs. Few original TXSI, such as camonagrel, have been described in the nineties and studied at the preclinical stage (Albet, Perez *et al.* 1998; Gryglewski, Szczeklik *et al.* 1995; Korbut and Gryglewski 1996; Villalobos, De La Cruz *et al.* 2003).

Nevertheless, most of the TXSIs cited above are mainly used as pharmacological tools in a series of recent *in vitro* and *in vivo* studies with the aim of evaluating the influence of TXA_2 in physiological or pathophysiological states. It is noteworthy that ozagrel hydrochloride (**9**, Figure I-13) was the first TXA_2 modulator released onto the market (in Japan, 1992) for the treatment of adult bronchial asthma.

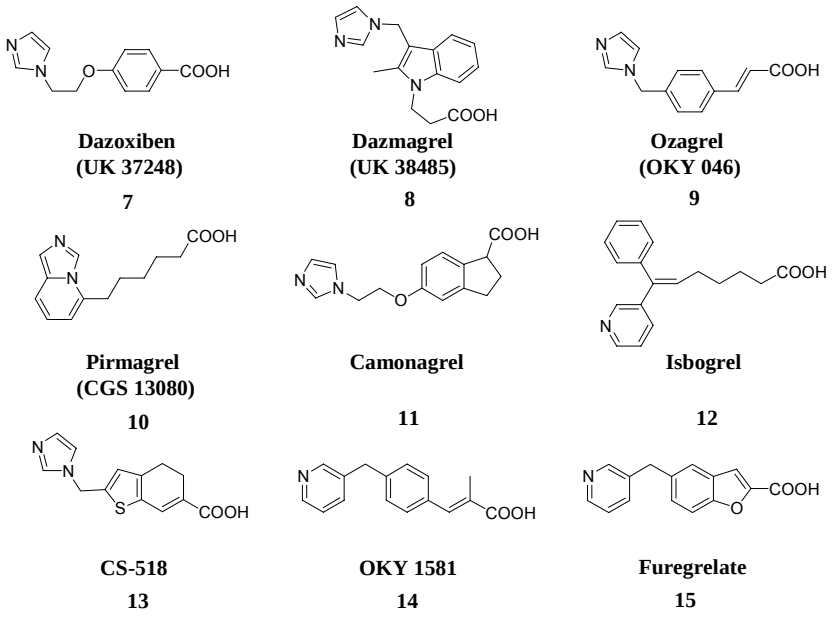


Figure I-13. Main thromboxane synthase inhibitors.

From a chemical point of view, all the TXSIs are characterized by an 1-imidazolyl or a 3-pyridinyl ring. Thereby, two structurally different classes of TXSIs have been described : TXSIs derived from imidazole and TXSIs derived from pyridine (Dogné, de Leval *et al.* 2000). The structural and energetic prerequisites for high binding affinities of TXSIs to TXS have been reported by Kato *et al.*. In his model, the pharmacophore for TXS inhibition comprises a 3-substituted pyridine or an *N*-substituted imidazole ring, and a carboxylic acid group separated by a distance of 0.85-1 nm (Figure I-14) (Kato, Ohkawa *et al.* 1985). This theory can be a way to explain the better activity of E isomer of isbogrel compared to Z isomer. Indeed the distance between pyridine nitrogen and carboxylic group of E isomer is 0.89 nm whereas this parameter is 0.42 nm for Z isomer. It was postulated by the same group that the

nitrogen atom of the heterocycle could form a complex with the iron atom of the heme group of the catalytic site of TXA_2 synthase (Hecker, Haurand *et al.* 1986). In addition, the side chain seemed to be oriented almost parallel to the plane of the heme. If this geometry is changed, a decrease in affinity is observed. From these data, it can be concluded that the inhibitors mimic the binding of PGH_2 with its carboxylic group at the carboxyl side chain and the endoperoxide oxygen atom at C9. The methyl side chain of PGH_2 does not seem to play a role in the formation of the enzyme-substrate complex.

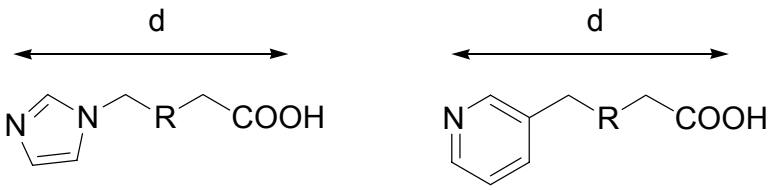


Figure I-14. Proposed pharmacophore for TXS inhibition. d has to be comprised between 0.85 an 1 nm.

I. 4. 2. Chemical compounds interacting with thromboxane receptors

I.4.2.1. TP receptor agonists

After the discovery of TXA_2, and due to its lack of stability, several TP receptor agonists have been developed as pharmacological tools (Figure I-15). The most used and well described are, with decreasing affinity : U46619 (**18**) > I-BOP (**16**)> STA_2 (**17**), (from the most to the less potent (Tsuboi, Sugimoto *et al.* 2002)). They have in common to present a stabilized TXA_2-like structure.

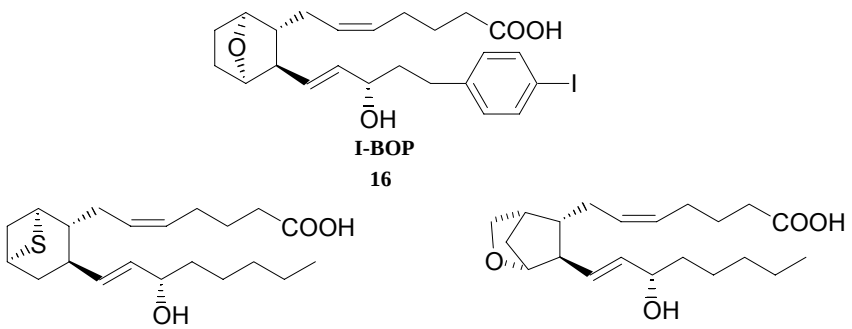


Figure I-15. TP receptor agonist.

I.4.2.2. TP receptor antagonists

Thromboxane receptor antagonists (TXRAs) block the action of TXA_2 and PGH_2 at the receptor level. Consequently, in contrast to TXSIs, TXRAs do not affect the synthesis of PGI_2 and other prostaglandins.

From a chemical point of view, TXRAs can be classified in two groups : the prostanoids TXRAs structurally related to the bicyclic physiological TP receptor ligands : PGH_2 and TXA_2 (Figure I-6 & Figure I-1) and the non-prostanoid TXRAs of which the majority is characterized by a phenylsulfonamido moiety (Figure I-17 & Figure I-18) (Dogné, de Leval *et al.* 2004).

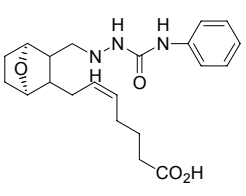
I.4.2.2.A. Prostanoid TXA_2 receptor antagonists

All these compounds bear a lipophilic hydrocarbon chain ended with a carboxylic function and a lipophilic side. The carboxylic and the ω-side chains of the PGH_2 ligands are fixed on a 5-atom ring (cyclopentane or tetrahydrofuran) in contrast to the TXA_2 related compounds characterized by a 3,3-dimethyltetrahydropyren ring (Figure I-16).

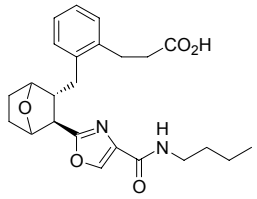
SQ29548 (**19**), ifetroban (**20**), and domitroban (**23**) (Figure I-16) are the most studied TXRAs derived from endoperoxide PGH_2. Semicarbazide SQ29548 is considered as one of the most potent ligand and antagonist of the TP receptors (Dogné, de Leval *et al.* 2004; Monshizadegan, Hedberg *et al.* 1992; Ogletree, Harris *et al.* 1985; Singh, Seth *et al.* 1997). Thereby, it remains the major TXRA used as pharmacological tool in *in vitro* studies and *in vivo* experiments performed

only in animals (Moore, Asboth *et al.* 2002; Okon, Golbabaie *et al.* 2002; Zou, Shi *et al.* 2002). Indeed, its development as potential clinical candidate has been stopped because of its mutagenic potential revealed in the Ames screen when incubated with a rat liver microsome fraction (Nakane, Reid *et al.* 1990). Its labelled derivative [5, 6-^{3}H]SQ29548 is frequently used as a tracer in TXA_2 receptors binding studies (Dogné, de Leval *et al.* 2004).

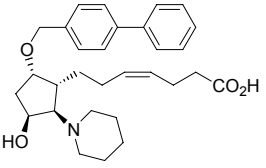
Another carboxamide derivative, ifetroban (**20**, BMS 180,291, Figure I-16), a proprietary Bristol-Myers Squibb, is a potent, long-acting, orally active TXA_2/PGH_2 receptor antagonist (Ogletree, Harris *et al.* 1993).



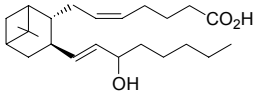
SQ29548
19



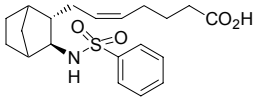
Ifetroban
20



Vapiprost
21



Pinane TXA_2 (PTA_2)
22



Domitroban (S1452)
23

Figure I-16. Prostanoid thromboxane receptor antagonists.

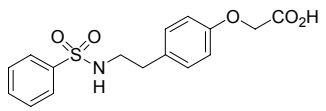
Narisada *et al.* opened the way to norbornane derivatives bearing a sulfonamide moiety (Narisada, Ohtani *et al.* 1988) and one compound of this family, S1452 (**23**, domitroban, Figure I-16), emerged as an original highly potent TXRA, characterized by a long-lasting antiplatelet activity (Fujimura, Shiga *et al.* 1996). Its antagonistic profile appeared to be non competitive, and in some isolated preparations, S1452 produced transient contractions indicative of a TXA_2/PGH_2 partial agonistic activity (Dube, Jakubowski *et al.* 1995). Although this compound exhibited anti-thrombotic effects, it has not been investigated for cardiovascular indications.

PTA_2 (**22**, pinane thromboxane A_2, Figure I-16) is a bicyclic TXRA derived from TXA_2, constructed on a 6,6-dimethylbicyclo[3.1.1]heptane or pinane ring and a carboxylic moiety. It was described as the first TP receptor antagonist (Nicolaou, Magolda *et al.* 1979). Nevertheless, a significant agonistic effect has been observed with this compound. It is frequently used as a pharmacological tool (Matz, Van Overloop *et al.* 2001; Sakai, Suzuki *et al.* 2002).

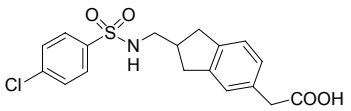
GR32191 (**21**, vapiprost, Figure I-16) is a two monocyclic prostanoid TXRAs characterized by a central ring similar to that of the $PGF_{2\alpha}$ (Figure I-6). It is a selective long-lasting TXRA after single and multiple oral administrations in healthy volunteers. Vapiprost is still being used as a pharmacological tool in a large number of studies (Kan, Jones *et al.* 2002; Vezza, Mezzasoma *et al.* 2002).

I.4.2.2.B. Non-prostanoid TXA_2 receptor antagonists of the benzenephenylsulfonamide type

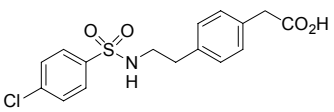
Sulotroban (**24**, Figure I-17) is the prototype of the "benzenesulfonamide" derivatives. Indeed, it was the first TXRA studied in clinical trials (Gresele, Deckmyn *et al.* 1984; Riess, Hiller *et al.* 1984; Stegmeier, Pill *et al.* 1984). Its development was discontinued because of its moderate effects in several clinical situations such as angina. This lack of activity could be explained by an agonistic activity revealed on vascular smooth muscle preparation (Karasawa, Kawakage *et al.* 1991; Miki, Kase *et al.* 1992). Moreover, sulotroban is a weak TXRA (Masereel, Damas *et al.* 1999). Consequently, a series of sulotroban derivatives have been developed with the aim of improving the antagonistic activity on TP receptors (Figure I-17 & Figure I-18). Thus, daltroban (**26**, BM 13,505), Z-335 (**25**), linotroban (**27**, HN-11500), ramatroban (**28**, BAY U 3405) and S-18886 (**29**) emerged as the most promising compounds of this class. From a chemical point of view, these compounds are characterized by a carboxylic acid function, a benzenesulfonamide moiety, and a "spacer" between these functional groups. The presence of an halogen atom on the benzene ring in the *para* position of the sulfonamide moiety also characterized the majority of these compounds.



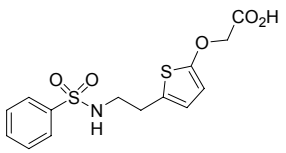
Sulotroban
24



Z335
25



Daltroban
26



Linotroban
27

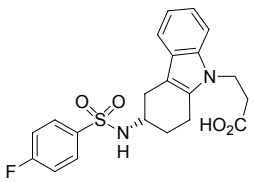
Figure I-17. benzenesulfonamide non-prostanoids TP receptor antagonists.

The structure of daltroban (**26**, Figure I-17), which has a phenylethyl group as a spacer, is the simplest of these molecules. It has been shown to be ten times more potent than sulotroban in *in vitro* experiments and in healthy volunteers. Nevertheless, as observed for sulotroban, evidence for partial agonistic properties at TP receptors on vascular smooth muscle have also been reported (Ogletree, Harris *et al.* 1993). Both sulotroban and daltroban have been used as pharmacological tools (Desjardins, Aubin *et al.* 2005; Grandel, Fink *et al.* 2000; Grandel, Hopf *et al.* 2005; Kudoh and Matsuki 2000).

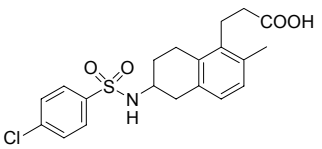
In 1999, Shinozaki and collaborators proposed the introduction of an indane ring as part of the structural rigid spacer in place of the phenylethyl group of daltroban. This modification led to Z335 (**25**, Figure I-17), which was found to be a potent TXRA in oral administration

(Shinozaki, Sato *et al.* 1999). It was studied as an antiplatelet and antithrombotic agent in rat and proved to be more active than cilostazol, a selective phosphodiesterase type III inhibitor (Tanaka, Ito *et al.* 1998; Tanaka, Sato *et al.* 2000). The pharmacokinetic and pharmacodynamic properties of Z-335 after single and multiple oral administrations to healthy volunteers were also reported. Z-335 was concluded to be safe and to provide long-lasting blockade of TP receptors on the basis of a once-daily regimen (Matsuno, Uematsu *et al.* 2002).

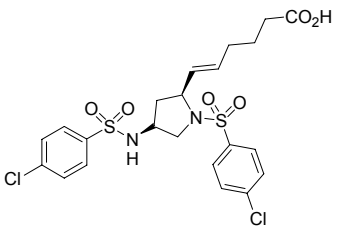
Linotroban (**27**, Figure I-17), developed by Nycomed company, is characterized by a thiophen ring as spacer in place of the benzene ring of sulotroban. It was early characterized as a potent antithrombotic agent (Roald, Barstad *et al.* 1994).



Ramatroban
28



S-18886
29



30

Figure I-18. Benzenesulfonylamide non-prostanoids TP receptor antagonists (2).

Ramatroban (**28**, Bay-U-3405) (Figure I-18) combines in its structure a 1,2,3,4-tetrahydrocarbazole ring as spacer and a 4-fluorobenzenesulfonamide group. It is a TXA_2/PGD_2 antagonist that has been launched in Japan for rhinitis mid-2000 by Bayer Yakuhin (Baynas®, tablets of 75 and 150 mg).

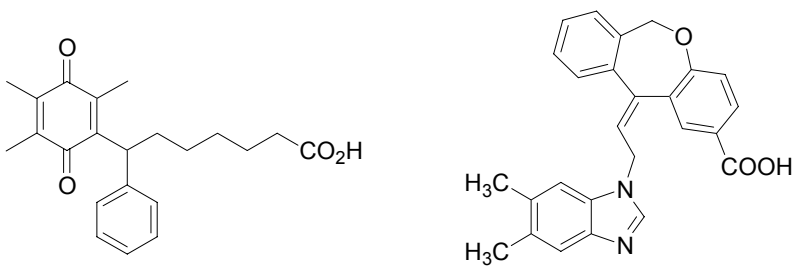
In a recent study, Marusawa and co-authors described the synthesis and the pharmacological evaluation of a new chemical skeleton with a non-prostanoid structure and focused on the conformational similarities of TXA_2 and daltroban. In an accurate 3D molecular model, the authors suggested that the sulfonamide moiety of daltroban might tentatively play a role as mimics of both the nuclear oxygen atoms and the allylic alcohol of TXA_2. On the basis of this prediction, they designed a new scaffold with two sulfonamide groups in the same molecules assuming that these two moieties might mimic both the nuclear oxygen atoms and the allylic alcohol of TXA_2. This original approach led to a series of 1-phenylsulfonyl-4-phenylsulfonylaminopyrrolidines as potent TXRA, of which compound **30** (Figure I-18) emerged as the most effective antiplatelet and antithrombotic agent (Marusawa, Setoi *et al.* 2002).

Finally, polysubstituted tetrahydronaphthalene derivatives were prepared as TP receptor antagonists. Within this series of compounds, S-18886 (**29**, Figure I-18) has been identified as an orally active, highly potent antagonist. Interestingly, this new compound have been claimed to reduce atherosclerotic plaque, in several animal models and conditions (Egan, Wang *et al.* 2005; Viles-Gonzalez, Fuster *et al.* 2005; Worth, Berry *et al.* 2005; Zuccollo, Shi *et al.* 2005). It has already been tried in human

and confirmed its antiplatelet activity (Gaussem, Reny *et al.* 2005). It is currently in clinical trials.

I.4.2.2.C. Miscellaneous compounds

The dibenzoxepin compound KW 3635 (**32**, Figure I-19) is a TP receptor antagonist which is characterized by an increased antiaggregant potency compared to sulotroban and daltroban (Karasawa, Kawakage *et al.* 1991). Nevertheless, it has never been evaluated on human to date.



Seratrodast
31

KW 3635
32

Figure I-19. Non-prostanoid TP receptor antagonists, miscellanous compounds.

Seratrodast (**31**, AA-2414, Figure I-19) is an orally active quinone derivative characterized to be a potent TP receptors antagonist (Fukumoto, Shiraishi *et al.* 1992; Kanao, Watanabe *et al.* 1989; Kurokawa, Matsumoto *et al.* 1994). It is the first TXRA that was developed as an

anti-asthmatic drug. It has also received marketing approval in Japan (1997). In human clinical studies, seratrodast showed a marked effect to improve clinical parameters in bronchial asthma and several relevant clinical studies have been described with it after 1999. Collectively, the results collected in humans suggested that blockade of TP receptors would be effective for asthma control and that addition of seratrodast to a conventional anti-asthmatic medication may be considered in the management of patients with mild to moderate asthma (Horiguchi, Tachikawa *et al.* 2002; Hoshino, Sim *et al.* 1999; Tamaoki, Kondo *et al.* 2000).

I. 4. 3. Thromboxane modulators expressing combined pharmacological properties

I.4.3.1. Combined thromboxane synthase inhibitors and thromboxane receptor antagonists

After the disappointing results given by either TXRA or TXSI in clinical trials, several authors postulated that improved molecules should present both pharmacological characteristics. As a result, TXSI action would result in an increase of the synthesis of the antiaggregatory and vasodilatory PGI_2 and TXRA effect could block the action of both TXA_2 and PGH_2 at a receptor level. Consistent with this key concept, a series of double-blind, placebo-controlled, crossover studies in healthy volunteers were carried out using a co-administration of the TXRA sulotroban and the TXSI dazoxiben. Results confirmed the greater interest for co-administration of both TXA_2 modulators compared to their independent

use (Gresele, Arnout *et al.* 1987). Therefore, a large variety of compounds associating both principles of action have been developed (Figure I-20). Most of these drugs retain the pyridinic ring of the TXSI isbogrel. Ridogrel (**33**), terbogrel (**34**), and picotamide (**35**) have been studied in clinical trials.

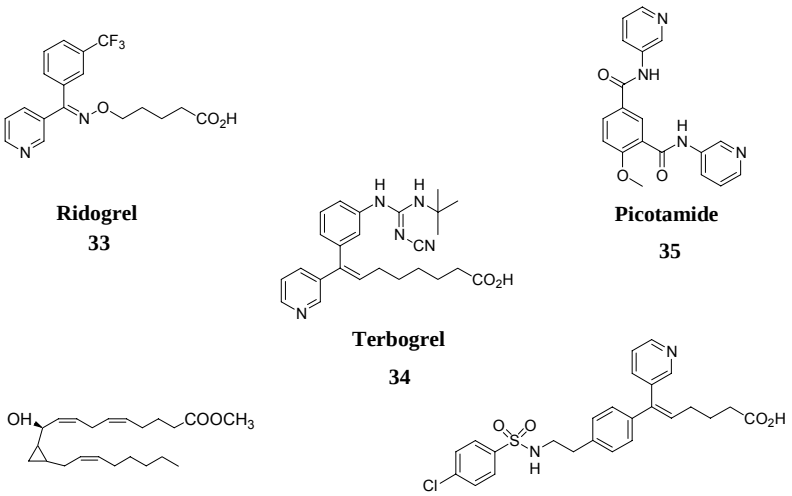


Figure I-20. Combined TXA_2 receptor antagonists and TXSI.

Ridogrel (**33**, Figure I-20), a pyridinic derivative, was the first dual inhibitor of TXA_2 to be studied in the clinic but it yielded disappointing results. Indeed, in the Ridogrel versus Aspirin Patency Trial (RAPT study), ridogrel did not significantly affect the primary endpoint of infarct-related artery patency at 2 weeks following streptokinase-induced lysis in acute myocardial infarction patients (1994; Hoet, Falcon *et al.* 1990). Evaluation of the pharmacological characteristics of ridogrel would suggest that the receptor affinity of the compound is indeed too

weak to be adequately qualified as a TXA_2 modulator with a dual mode of action (Heylen, De Clerck *et al.* 1991; Soyka, Heckel *et al.* 1994). This potent TXSI and weak TXRA was also evaluated in clinical studies in the treatment of inflammatory bowel diseases but gave disappointing or negative results (Carty, Rampton *et al.* 2001; Tytgat, Van Nueten *et al.* 2002).

Terbogrel (**34**, Figure I-20) is a close derivative of samixogrel (**37**, DTTX30). Both were designed and synthesized as combined TXA_2 receptor antagonists and TXSI. Samixogrel reflects structural elements of the TXSI isbogrel and of the TXRA daltroban. Nevertheless, it showed only moderate plasma levels combined with an unexpected high variability after oral administration in human volunteers presumably due to its very low solubility in aqueous solution under physiological conditions. Thereby, the modification of the benzenesulfonamide samixogrel led to the cyanoguanidine terbogrel. This drug showed a better pharmacokinetic profile (Soyka, Guth *et al.* 1999). It is currently under clinical evaluation as antithrombotic agent (Guth, Narjes *et al.* 2004).

Picotamide (**35**, Figure I-20) is another combined TXRA and TXSI. It is characterized by two pyridine rings each fixed to benzene by a carboxamide moiety. It was studied in man for the first time in 1978 (Schmutzler, Hartmann *et al.* 1978) and evaluated in a series of clinical conditions in patients suffering from chronic vascular pathology (Coto, Carrieri *et al.* 1986), peripheral arterial disease (Danese, Sergio *et al.* 1988), claudication (Coto, Cocozza *et al.* 1989; Girolami, Bernardi *et al.* 1999), diabetic vasculopathies (Pibiri, Petruzzo *et al.* 1990), myeloproliferative

disorders (Rafanelli, Grossi *et al.* 1990), deep venous thrombosis (Giberto, Canova *et al.* 1991), cerebral infarction (D'Andrea, Perini *et al.* 1995), thrombocytosis (Pogliani and Milani 1996), inflammatory bowel disease (Collins, Benson *et al.* 1996), primary Raynaud's phenomenon (Pancera, Sansone *et al.* 1997), myocardial infarction (Vetrano, Milani *et al.* 1999), and carotid atherosclerosis (Coto, Oliviero *et al.* 1998). It is currently marketed as antiplatelet and antithrombotic agent.

In 2002, Pace-Asciak *et al.* reported that PBT-3, an hepoxilin stable analog (**36**, Figure I-20) was a potent TP receptor antagonist with secondary TXS inhibition (Pace-Asciak, Reynaud *et al.* 2002). Interestingly, further studies by this group showed that PBT-3 had preferential affinity for TPα isoform in binding studies (Qiao, Reynaud *et al.* 2003).

I.4.3.2. Combined TXA_2 modulators and other pharmacological properties

Numerous TXA_2 modulators have been reported to possess other pharmacological properties. For example, some TXRAs were claimed to be IP receptor agonists (Yamada, Miyamoto *et al.* 2003), platelet activating factor (PAF) antagonists (Fujita and Seki 2002; Fujita, Seki *et al.* 2002a; Fujita, Seki *et al.* 2002b), leukotriene D_4 receptor antagonists (Arakida, Ohga *et al.* 2000; Arakida, Suwa *et al.* 1998), or angiotensin II receptor antagonists (Hwang, Yeh *et al.* 2000).

Several other teams have reported combined TXSI and aromatase inhibitors (Jacobs, Frotscher *et al.* 2000), 5-LOX inhibitors (Oketani,

Inoue *et al.* 2001; Oketani, Nagakura *et al.* 2001), and leukotriene D_4 receptor antagonists (Cardoso, de Brito *et al.* 2002).

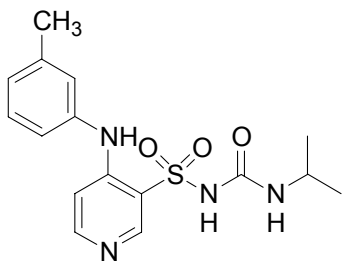
To date, all the compounds of these families had found limited applications as pharmacological tools or in the clinic.

II. Aims of the work

II.1. Previous work

The design of original molecules characterized by a specific biological activity can be achieved by several means. A typical approach is to modify the chemical structure of a known drug or active molecule in order to enhance a secondary activity and induce the loss of the primary activity. In the late nineties, this strategy was the one followed for the initial design of the compounds family presented in this work.

Torasemide (3-[4-[(3-methylphenyl)amino]pyridin-3-yl]sulfonyl-1-propan-2-yl-urea, **38**, Figure II-1) is a high-ceiling diuretic, acting on the $Na^+/H^+/2Cl^-$ exchanger of the Henle's loop and was first described in the seventies by Delarge *et al.* (Delarge and Lapiere 1973; Delarge and Lapiere 1978). It is marketed as a high ceiling diuretic in many countries (Demadex®, Torrem®)



38

Figure II-1. Chemical structure of torasemide or 3-[4-[(3-methylphenyl)amino]pyridin-3-yl]sulfonyl-1-propan-2-yl-urea.

In 1992, a study by Uchida *et al.* examined the effect of torasemide on the vasoconstriction induced by TXA_2 on the isolated canine coronary artery. The authors found that torasemide elicited a dose-dependent vasodilating action in the isolated canine coronary

arteries contracted by carbocyclic TXA_2 (CTA_2, a TXA_2 agonist), whereas indapamide or furosemide, two diuretics, had little effect on this preparation. These results suggested a TXA_2 modulator activity of torasemide, independent of its diuretic effects (Uchida, Kido *et al.* 1992). This theory was later confirmed since it was proved that torasemide was able to bind human platelet TP receptors (Masereel, Damas *et al.* 1999).

Consequently, in the Laboratory of Medicinal Chemistry of the University of Liège, a pharmacomodulation program started to investigate the effect of chemical modifications of torasemide on its TP receptor binding affinity. This program led to the synthesis of several compounds presenting structural modifications compared to torasemide (Dogné 2000). The modifications realized on the original molecules are presented in Figure II-2.

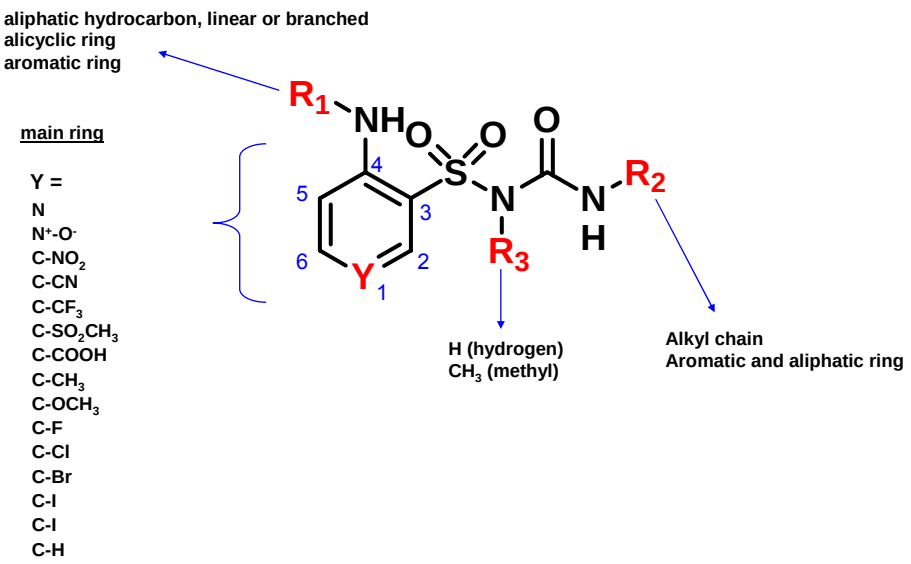


Figure II-2. Pharmacomodulation of torasemide achieved to improve TP receptor affinity.

This classical pharmacomodulation study identified the nitrobenzenic ring with a substituent in the *meta* and *para* positions to the nitro group to be the best configuration for high affinity on human platelet TP receptors. A pharmacophore for the TP receptors affinity was subsequently proposed. Beside the presence of the nitrobenzenic ring, it was postulated that a sulfonyl-urea or –cyanoguanidine group coupled with an aromatic or alicyclic ring as the R_1 (Figure II-2) substituent were the most interesting functional groups studied. The proposed pharmacophore identified in that previous study is presented in Figure II-3.

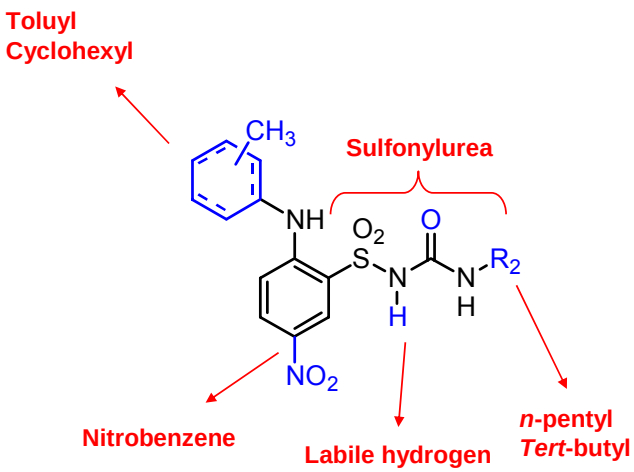


Figure II-3. Proposed pharmacophore for potent human platelet TP antagonistic activity.

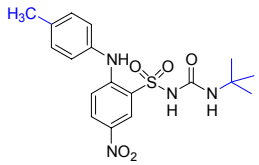
It is interesting to note that this family of compounds was not characterized by the presence of a carboxylic group. Nevertheless, one of the sulfonylurea protons is labile and the molecules behave as acids with a pKa close to the one of an alkyl carboxylic acid function. Indeed, if this labile hydrogen is substituted by a methyl group, there is a dramatic loss of affinity (Dogné 2000). Consequently, the sulfonylurea group is thought

to act as an isostere for the carboxylic acid function of the reference TP receptor antagonists (for the formulas of these kind of compounds, see introduction section I.4.2.2. p.61).

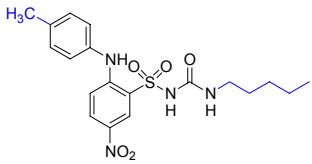
Within the framework of the pharmacomodulation, around one hundred active compounds were isolated and characterized (Dogné 2000). Most of these new families of molecules, namely nitrobenzenesulfonylureas, were subsequently demonstrated to act as antagonists, since they inhibited platelet aggregation.

II.2. Aims of the present work

From the initial research performed on sulfonylureas TP receptors antagonists, several compounds were selected for further pharmacological experiments. Indeed, several compounds were characterized by potent affinity for human platelet TP receptors in binding studies. The chemical structures of two of the most potent compounds in human platelet TP receptor binding studies are presented in Figure II-4. They are characterized by a methylated aromatic ring as R_1 and a *t*-butyl or *n*-pentyl alkyl chain as R_2.



BM573 (**39**)
1.3 ± 0.1 nM*



BM613 (**40**)
1.4 ± 0.2 nM*

* Binding affinity for human platelet TP receptors, IC_{50}

Figure II-4. Chemical structure and human platelet TP affinity of BM573 and BM613.

Two interconnected research programs were undertaken on the basis of these preliminary results.

Pharmacomodulation and structural exemplification

Although a pharmacophore was proposed, based on the initial experimental data, several chemical modifications were never envisaged in the early studies. Since the nitrobenzenic ring and the position of other groups on this ring had been identified as critical for the activity, we chose to conserve them and to focus on less-investigated structural components. Structures of BM573 and BM613 were chosen as a starting point because of their interesting pharmacological profile. The elements of the structure that were planned to be investigated are presented in Figure II-5, namely the nature and the position of the substituent of the second aromatic ring, the nature of the bond connecting the two rings, the urea group and R_2 side chain.

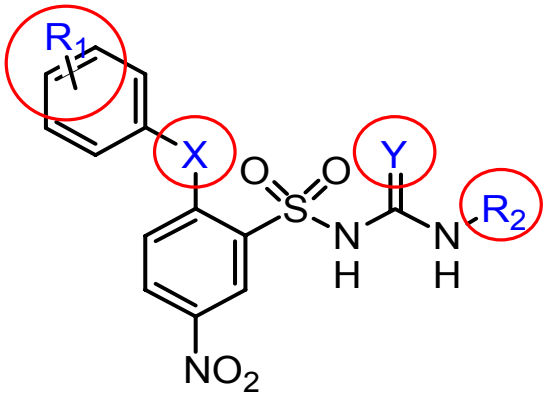


Figure II-5. Proposed chemical modifications of the general pharmacophore.

Pharmacological evaluation

Although the affinity for human platelet TP of most interesting compounds had been determined, little was known about their other pharmacological properties. Consequently, for the present project, we aimed to design specific pharmacological experiments for evaluation of available and newly synthesized compounds. Two principal aspects were planned to be explored :

- determination of BM573 and BM613 pharmacological properties related to TXA_2, both *in vitro* and *in vivo* ;

- pharmacological evaluation of all compounds (including BM573, BM613 and newly synthesized compounds) on both receptor isoforms.

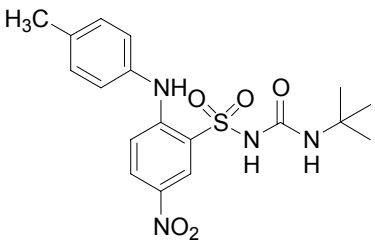
We intended to use informations collected with these experiments for synthesis of new compounds as well as for identification of influential groups for TP receptor antagonistic activity on both isoforms. This

orientation was particularly relevant since, in the literature, the systematic study of the influence of structure on TP isoforms specific activity (and thus selectivity) had never been investigated.

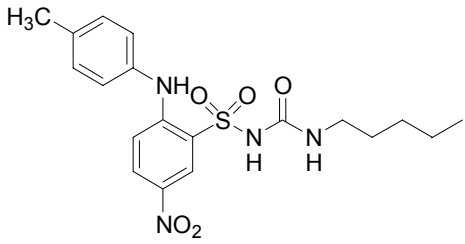
III. Results and discussion

III.1. BM573 and BM613

BM573 and BM613 (Figure III-1) pharmacological properties related to TXA_2 have been determined. The aim of these pharmacological studies was to confirm the potential therapeutic interest of these two compounds. This section (III.1) reports and discusses the results obtained during these experiments.



BM573



BM613

Figure III-1. Chemical structure of BM573 (39) and BM613 (40).

III. 1. 1. In vitro assays

The *in vitro* studies investigated the pharmacological interactions between BM613, BM573 and TXA_2. To do so, we considered the profile of these compounds in platelet aggregation, TXS inhibition, inhibition of aorta and trachea smooth muscle contractions, and binding on TP receptors.

III.1.1.1. Human ex vivo platelet aggregation

Principle of the test

To quantify the ability of platelets to aggregate, several tools have been developed. Diagnosis of particular blood diseases as well as

evaluation of new drugs need a rigorous evaluation of platelet function. The most frequently used protocol is the turbidimetric method developed by Born & Cross (Born and Cross 1963). The principle is based on the diffraction of the light by particles. When a light beam passes through a suspension of particles, it is diffracted, depending on the number and the size of the particles in suspension.

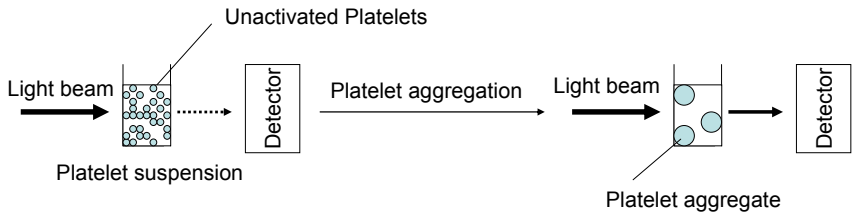
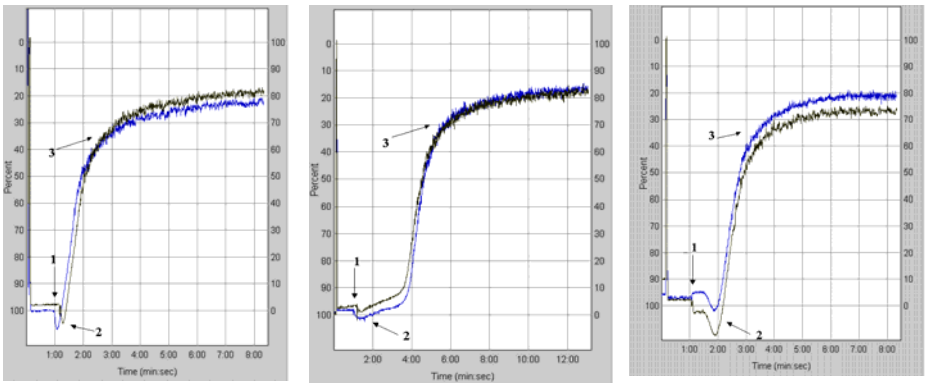


Figure III-2. Principle of Born & Cross method for measuring platelet aggregation.

In the Born & Cross method, a light beam passes through a platelet suspension and the quantity of light is measured by a detector placed after the sample. Upon platelet aggregation, the size of platelets aggregates will increase while the total number of free platelets will decrease. Consequently, less light will be diffracted and the detector will record an increase in light intensity (Figure III-2). The aggregometer has been developed based on these concepts. Variations in light transmission recorded by this device reflects the platelet physiology. When an agonist of platelet aggregation is added to a platelet suspension, platelets undergo activation and shape change. This step is characterized by an increase in platelets apparent volume and thus a decrease of transmitted light. Subsequent platelet aggregation gradually forms aggregates of increasing size. Transmitted light slightly increases until a plateau is reached (Figure III-3). The aggregation of platelet can be confirmed after the experiment by visual direct inspection of the test tube.



(a) (b) (c)

Figure III-3. Typical platelet aggregation course triggered by different inducers. (a) U46619, (b) ADP, (c) Collagen. In the graphs are indicated the different phases: 1) Induction, 2) shape change, 3) complete aggregation, plateau.

Preparation of platelet suspension is achieved by blood centrifugation. After the blood is withdrawn from healthy volunteers, it is centrifuged at 180 g for 10 minutes. The supernatant which is collected contains the platelets rich plasma (PRP). The remaining blood is subsequently centrifuged at 3000 g in order to retrieve plasma (platelets poor plasma, PPP). PRP is diluted with PPP to reach a final concentration of 300.10^3 platelets/µl. PRP is kept warmed at 37°C in the aggregometer and the adequate dilution of drug to test is introduced in the sample. Platelet aggregation is induced after 3 minutes incubation.

Light transmission (T) is measured throughout all the experiment, which is ended 8 minutes after induction of aggregation. Maximal light transmission (Tmax) is determined in the sample without drugs. Minimal light transmission (Tmin) is measured in PRP without inducer.

Percentage of platelet aggregation inhibition reflects the drug potency and is given by the following equation :

$$\% = 100 \text{ X } \left[1 - \frac{\text{T-Tmin}}{\text{Tmax-Tmin}}\right]$$

Results are expressed as IC_{50}, which is defined as the drug concentration required to inhibit 50% of platelet aggregation.

Results

The ability of BM613 and BM573 to prevent human platelet aggregation has been determined with three different agonists of platelet aggregation. Both BM613 and BM573 were able to inhibit (ED_{50} = 0.278 ± 0.186 μM and 0.240 ± 0.013 μM, respectively) platelet aggregation induced by the stable TXA_2 agonist U46619 (1 μM, final concentration). BM613 and BM573 also inhibited platelet aggregation induced by 600 μM arachidonic acid (ED_{50} = 0.375 ± 0.05 μM and 0.125 ± 0.015 μM, respectively). ADP is a weak inducer of platelet aggregation by itself, and it induces secretion of TXA_2 among other pro-aggregating substances. BM573, BM613 and SQ29548 (1 and 10 μM) inhibited the second wave of platelet aggregation induced by ADP, which is due to the secretion of TXA_2 (see Introduction, I.3.6.1.A. 36).

Drugs	U46619 (1 μM) ED_{50} (μM)	Arachidonic acid (600 μM) ED_{50} (μM)	ADP (2 μM)
SQ29548	0.034 ± 0.012	0.035 ± 0.003	Inhibition of second wave
BM573	0.240 ± 0.013	0.125 ± 0.015	Inhibition of second wave
BM613	0.278 ± 0.186	0.375 ± 0.05	Inhibition of second wave

Table III-1. Inhibition of human platelet aggregation induced by arachidonic acid, U46619 and ADP.

III.1.1.2. Thromboxane synthase inhibitory potency

Principle of the test

TXS is the enzyme converting PGH_2 into TXA_2 and is thus responsible for its production, particularly in platelets (see Introduction section I.2.1.4., p.12). Consequently, one can determine the total production of TXA_2 to evaluate the property of a given compound to inhibit TXS. Practically, it is TXB_2 that is quantified, due to TXA_2 instability. TXA_2 production is induced with arachidonic acid on PRP (prepared as described above). TXB_2 is measured by means of a competitive enzyme immunoassay.

Results

BM613 showed an inhibitory activity (IC_{50} = 0.15 ± 0.13 μM) comparable to the one of BM573, whose ability to inhibit TXS was evidenced in previous studies (IC_{50}= 0.053 ± 0.028 μM) (Rolin, Dogné *et al.* 2001). In this test, BM613 was about 100-fold more potent than the TXSI furegrelate (IC_{50} = 10.2 ± 6 μM, see introduction, section I.4.1.2, p. 56) chosen as reference compound.

III.1.1.3. Inhibition of rat aorta and guinea pig trachea contraction induced by U46619

Principle of the test

As previously described, TXA_2 plays a significant role in several physiopathological conditions (see introduction, I. 3. 6. p. 35). Beside the deleterious effects of pro-aggregatory TXA_2 effects, its vasoconstrictor potency can also contribute to the aggravation of the pathological conditions. It is consequently useful to observe the effect of our compounds on the vascular and bronchial smooth muscles. The myorelaxant effect of the compounds was measured on precontracted rat aorta and guinea-pig trachea. These evaluations have been performed in collaboration with Dr. Stephanie Rolin of the University of Namur-FUNDP (Pr B. Masereel).

In these experiments, the desired tissue, aorta or trachea, is dissected and removed from the euthanized animals and placed in a tissue bath. The tension is recorded, and after equilibration and stabilisation of the tension, Tmin (minimal tension observed in the absence of any added compound) is determined. A contraction is then triggered by addition of U46619. Tmax (maximal stabilized tension induced by TP receptor agonist) is measured and the compound to test is subsequently added at different concentrations. After relaxation, the resulting tension (T) is recorded.

Thus, for a given experimental drug concentration, the relaxation of the tissue is expressed as a percentage according to this equation :

$$\% = 100 \times \left[\frac{T - T\min}{T\max - T\min} \right]$$

Concentration curves were drawn and used to calculate ED_{50}, defined as the concentration of compound needed to reduce the U46619 induced tension by 50%.

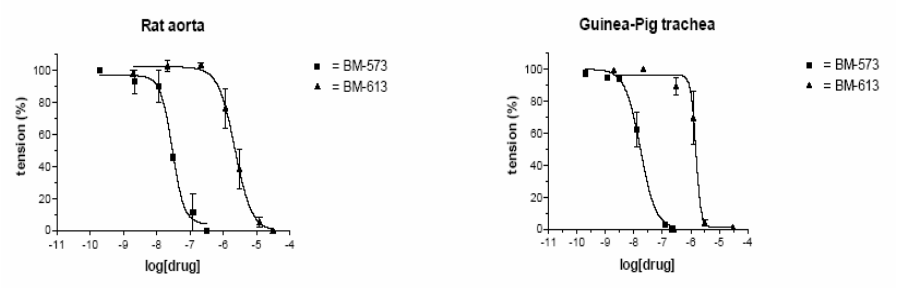


Figure III-4. Dose-dependent antagonistic effect of BM613 and BM573 on U46619 induced smooth muscle cell contraction. Experiments have been carried out on rat aorta and guinea-pig trachea. Data presented are representative of four independent experiments.

Results

On rat aorta, BM613 (ED_{50} = 2.4 ± 0.18 μM) was 100-fold less active than BM573 (ED_{50}: 28.4 ± 4.5 nM) and 1000-fold less active than SQ29548 (ED_{50}: 2.3 ± 0.07 nM) which was the most potent compound in these experiments (Table III-2). Additionally, the relaxing activity of BM613 and BM573 was measured on the guinea pig tracheal smooth muscles. Cumulative increasing concentrations of BM613 and BM573 caused a concentration-dependent relaxation of the contracted isolated trachea.

Compounds	Inhibition of contraction induced by U46619	
	Rat aorta ED50 (nM)	Guinea-pig trachea ED50 (nM)
SQ29548	2.3 ± 0.1	3.8 ± 0.6
BM573	28.4 ± 4.5	17.7 ± 3.9
BM613	2500 ± 180	1520 ± 250

Table III-2. Effect of BM613 on rat aorta and guinea pig trachea contraction induced by U46619. Results are expressed as mean ± SEM. ED_{50} represents the drug concentration reducing by 50% the rat aorta and the guinea-pig trachea tonus induced by 20 nM and 10 nM U46619, respectively.

Consistent with results obtained for aortic smooth muscles, BM613 (ED_{50} = 1.52 ± 0.25 μM) was much less active (100-fold) than BM573 (ED_{50} = 17.7 ± 3.9 nM) and than SQ29548 (1000-fold, ED_{50} = 3.8 ± 0.5 nM) which remains the most potent compound in this assay.

III.1.1.4. Radioligand binding assay

Principle

Chemical compounds have the ability to bind to proteins and macromolecules. Binding experiments exploit this property for the determination of drug-receptor interactions. To measure the quantity of drug that can bind to a given receptor, the most widely used tool are radiolabeled compounds, whose radioactivity can be easily quantified and reflects their concentrations. Two types of bindings can occur for chemicals in biological samples : the binding to the receptor is normally termed *specific binding*, whereas the binding to non-receptor tissue components is referred to as *non-specific binding*. Several kinds of ligand-binding studies can be realized (saturation, kinetic, competition and retardation), but we will focus on the two most relevant in our settings.

Saturation experiments examine the binding of the radioligand at equilibrium directly and can provide estimation of K_D (dissociation equilibrium constant for binding of a ligand, reflecting its affinity for a given receptor) and B_{max} (maximum binding capacity of radiolabeled ligand) (Figure III-5). For the simplest reaction :

$$R + L \rightleftharpoons RL$$

The binding equilibrium is given by the following equation :

$$B = B_{max} + \frac{[L]}{K_D + [L]}$$

where B is the amount of bound radioligand.

The determination of binding affinity in saturation experiments is only possible when a radiolabeled form of the ligand of interest is available. Consequently, the determination of affinity parameters have to be estimated indirectly, namely in competition binding experiments. In these experiments, one can determine the affinity of unknown compounds competing for the binding sites with a ligand that is available in a labeled form. It is a rapid mean for determining the affinity of novel compounds for a particular receptor for which a well-characterized radioligand is available. Practically, a fixed amount of radioligand (L) is equilibrated with the receptor preparation in the presence of a range of concentrations of the unlabeled competing compounds (I) (Figure III-5).

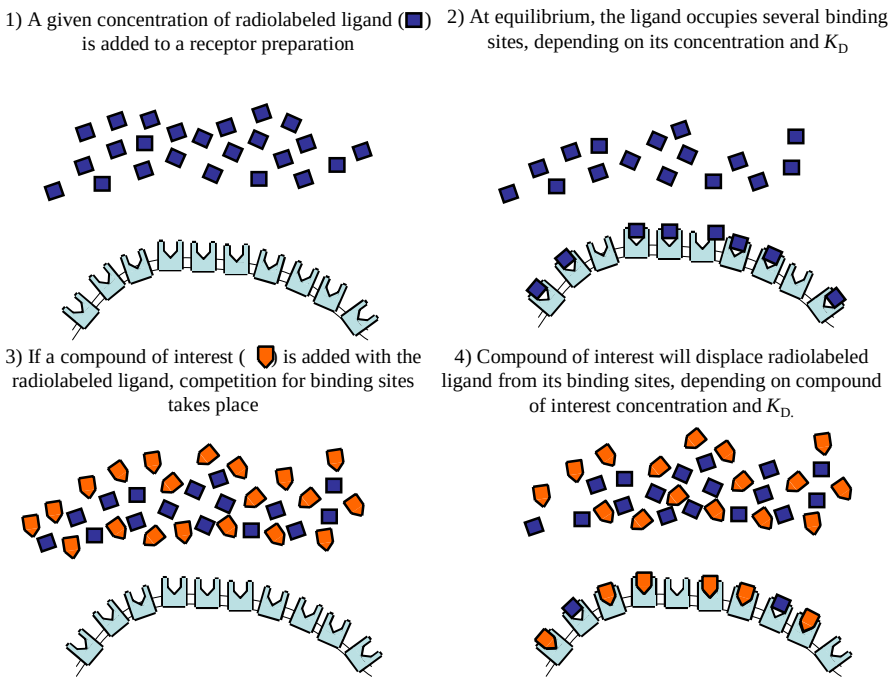


Figure III-5. Schematic representation of competitive binding experiments.

Displacement of radiolabeled drug increases with the concentration of added unlabeled compound, depending on the affinity of the drug for the binding sites. The amount of bound radioligand is usually plotted against log [I] (Figure III-6).

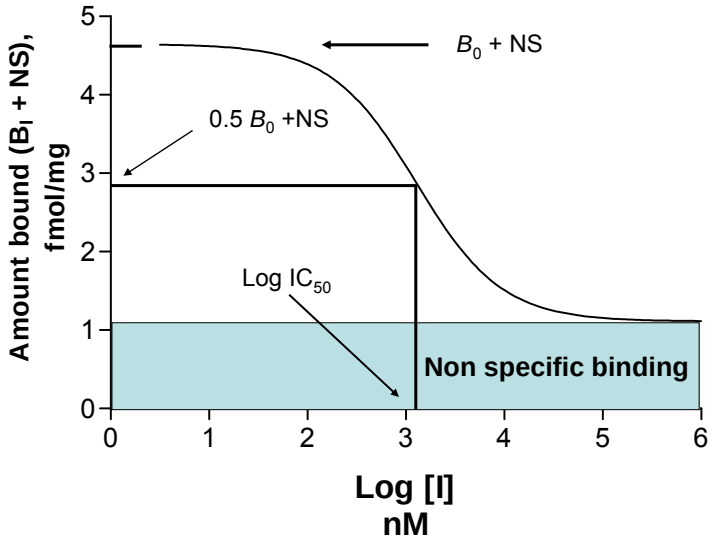


Figure III-6. Illustration of a competition experiment. B_0 is the specific binding of a fixed concentration of radioligand, in the absence of competitive ligand (I). Specific binding in the presence of a competitive ligand is denoted B_I. A constant amount of non-specific binding is assumed to be present. The concentration of Inhibitor that reduces specific binding by 50% is referred to as the IC_{50}. Adapted from Haylett (Haylett 2003).

The two main features of this curve are its position along the concentration axis and its slope. The position along the concentration axis indicates the IC_{50}, namely, the concentration of competing compound that reduces the specific binding by 50%. In the following experiments, results are expressed as IC_{50}.

IC_{50} of BM613 and BM573 was evaluated by measuring their ability to displace tritiated SQ29548, a strong TXRA used as the radiolabeled ligand. Beside binding on human washed platelets, affinity of our compounds have been determined on COS-7 cells line where TPα or TPβ was expressed, after transient transfection of appropriate plasmid carrying the sequence of either TPα or TPβ.

Results

BM613 and BM573 had already showed strong affinity (IC_{50} = 1.4 ± 0.2 nM) for human platelet TP receptor, ten-fold more potent than that of SQ29548 (IC_{50} = 21 ± 2 nM). Affinities of BM613 and BM573 for either TPα (IC_{50} = 2.1 ± 0.5 nM and 1.1 ± 0.2 nM, respectively) or TPβ (IC_{50} = 3.1 ± 0.7 nM and 0.8 ± 0.1 nM, respectively) expressed alone in COS-7 cells were not significantly different (p=0.28). BM573 was slightly more potent in this test.

Cpds	Affinity		
	Human Platelets IC_{50} (nM)	**TPα IC_{50} (nM)**	**TPβ IC_{50} (nM)**
SQ29548	21 ± 2	N.D.[e]	N.D.[e]
BM573	1.3 ± 0.1	1.1 ± 0.2	0.8 ± 0.1
BM613	1.4 ± 0.2	2.1 ± 0.5	3.1 ± 0.7

Table III-3. Binding affinity of BM613 and BM573 for human platelets TP, TPα and TPβ receptors.

III. 1. 2. In vivo antithrombotic activity of BM573 and BM613

In vivo experiments allow the confirmation of properties observed *in vitro*. In these experiments, compound is injected, metabolized, distributed among the tissues, etc. It was thus important to verify the antiplatelet potency of our compounds in an arterial thrombosis model.

III.1.2.1. Ferric chloride-induced rat arterial thrombosis

Principle of the test

To further test the hypothesis that BM573 and BM613 were potential antithrombotic agents acting on TP receptors and TXS, we evaluated their effects in a rat model of arterial thrombosis. Arterial thrombus formation is deeply implicated in thrombotic cardiovascular events. Indeed, the arterial thrombus formation results mainly from excessive platelet aggregation inside an arterial vessel. Consequently, potential antithrombotic drugs are evaluated *in vivo* for their ability to prevent thrombus formation in arteries.

Several models for the evaluation of antithrombotic potency have been described in animal studies. They have in common to induce a vessel lesion and differ mainly in the method of induction of thrombus formation. The most frequently referenced models are :

- mechanic induction (Hladovec 1986; Talbot, Ambler *et al.* 1991) ;
- electrostimulation (Salazar 1961) ;
- endothelium denudation (Tomaru, Uchida *et al.* 1987) ;
- injection of a photosensitization agent. After injection, the artery section to be studied is subjected to light stimulation, which induces thrombus formation. (Jourdan, Aguejouf *et al.* 1995; Kovacs, Tigyi-Sebes *et al.* 1975; Sato and Ohshima 1984; Watson, Dietrich *et al.* 1985) ;

- ferric chloride solution application (Dogné, Rolin *et al.* 2005; Kurz, Main *et al.* 1990).

For the evaluation of our compounds, we have chosen the ferric chloride model. Practically, a filter paper is soaked with a ferric chloride solution (50%) and placed on a portion of denuded rat abdominal aorta. The irritant solution rapidly induces a vascular injury, and subenthothelial components are exposed, including the highly proaggregant collagen. After completion of the experiment, the importance of platelet reaction is estimated with the thrombus volume. Compounds acting on platelet aggregation *in vivo* should reduce the size of the resulting thrombus. The different steps can be summarized as follow :

- anaesthesia (sodium pentobarbital) and compound injection (IV or IP) ;

- abdomen dissection ;

- denudation and exposition of abdominal aorta ;

- application of a filter paper disk saturated with the ferric chloride solution (50% m/v) on a portion of the aorta during 10 minutes ;

- 10 minutes after the removal of the filter paper from aorta, the aorta section is removed from animal, which is euthanized ;

- isolated aorta section is open longitudinally and the thrombus formed collected, dried and weighted.

BM613 was evaluated at the following doses : 5, 2, 1 and 0.5 mg/kg. BM573 at 5, 2, 0.5 and 0.1 mg/kg.

Results

In this experiment, BM613 significantly reduced the thrombus weight by 79%, 49% and 28%, respectively (Figure III-7). At 0.5 mg/kg, BM613 did not significantly reduce the thrombus weight.

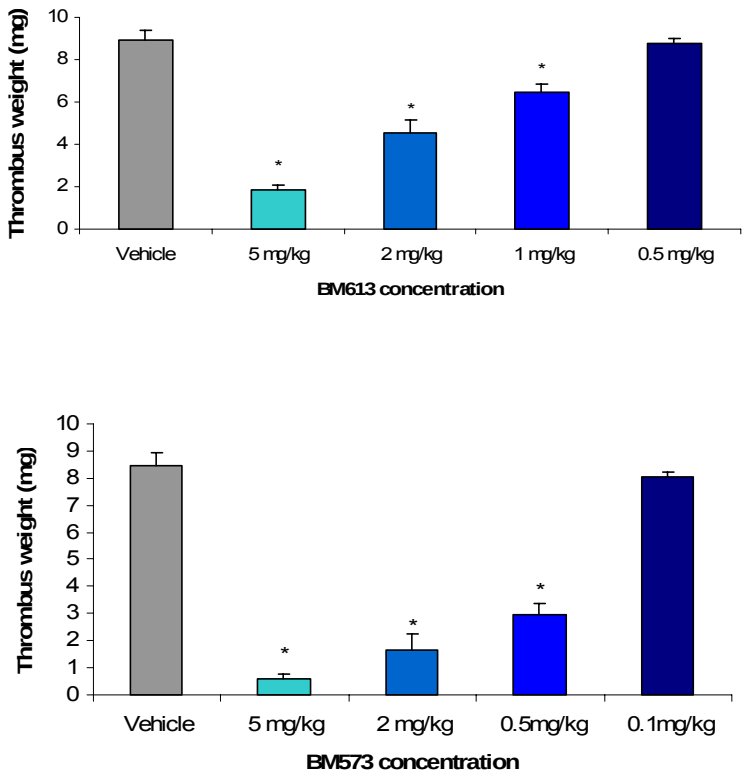


Figure III-7. Dose dependent inhibition arterial thrombus formation in rat by (A) BM613 and (B) BM573. *: p<0.05 compared to vehicle.

BM573, tested in the same model showed comparable activity. It significantly reduced the thrombus weight by 92%, 80% and 64%, at doses of 5, 2, and 0.5 mg/kg, respectively.

III. 1. 3. Discussion

BM613 and BM573 were selected from previous works for their potent binding affinities for human washed platelets TP receptors. However, little was known about their other pharmacological properties and therapeutic potential. In this series of experiments, we addressed their functional TXA_2 antagonism as well as extended affinity properties and *in vivo* efficacy.

Our first aim was to determine the *in vitro* TP receptor antagonistic potency of BM613 and BM573 in an inhibition of human platelet aggregation assay. BM613 was able to inhibit platelet aggregation induced by AA in the same concentration range than BM573 (Table III-1). Because COX inhibitors are also able to inhibit the AA-induced aggregation by blocking TXA_2 production, BM613 and BM573 activity was evaluated with U46619 which is a stable TP agonist. We have shown in this experiment that BM613 and BM573 completely inhibited platelet aggregation induced by U46619, thus demonstrating their action as TXRAs (Table III-1).

ADP provokes platelet aggregation by acting on specific purine receptors. Platelet aggregation induced by ADP is characterised by two waves. The first wave is due to a weak and reversible aggregation, which is the consequence of ADP action on its specific receptors. The second wave is due to TXA_2 synthesis and release, which provokes irreversible

and complete aggregation. Our compounds only inhibited the second wave (Table III-1) of ADP-induced platelet aggregation like other TXRAs (Reynaud, Hinek *et al.* 2002). This result highlights the lack of action of BM613 or BM573 on ADP receptors and confirms specific activity on TP receptors.

Compounds combining both actions on TP receptors and TXS have proved to be more therapeutically interesting and promising as antithrombotic agents (see introduction, section I.4.3.1, p.69). Blocking solely the production of TXA_2 provokes the accumulation of PGH_2, which is known to act on TP receptors (see introduction, section I.3.4.1, p.24). On the other side, with pure TP receptor antagonists, the benefit of redirecting production of other prostanoids (such as PGI_2) by accumulation of PGH_2 is lost. Thus, we also evaluated the potency of BM613 as TXSI by investigating the production of TXB_2 by human platelets activated by arachidonic acid. BM613 was 100-fold more potent that furegrelate, a clinically evaluated TXSI used as reference drug. These results demonstrate the efficacy of BM613 as a TXSI. Combined with the effect on platelet aggregation, BM613 can be considered as a well-balanced TXRA and TXSI. This is of great importance since the lack of efficacy of ridogrel (see Introduction, section I.4.3.1, P.69) was explained by its strong effect on TXS compared to TP receptors (Soyka *et al.*, 1999).

Further functional experiments were conducted on isolated rat aorta and guinea-pig trachea precontracted with U46619 (Figure III-4). In these experiments, BM573 had already shown good potency compared to SQ29548 (Rolin, Dogné *et al.* 2001). BM613 showed a 100-fold decreased activity in these experiments compared to BM573 (Table III-2). This

discrepancy between potency of BM613 to inhibit platelet aggregation and to inhibit smooth muscle cell contraction could find several explanations. First, the TP receptor may present conformational differences between species used in the test (human, rat and guinea-pig). Secondly, several authors initially stated in the eighties the existence of at least two pharmacological subtypes of TP receptor (see Introduction, I.3.4.1, p.24). A first subtype was thought to be present at the platelet surface and the second subtype on smooth muscle cells.

Thus, we postulated that BM613 could have a greater affinity for TPα than for TPβ and that this could be coupled with higher activity as an antiplatelet agent. In the frame of this hypothesis, BM573 would present no or weaker differences between TPα and TPβ in the same experiment. To test this second hypothesis, we have performed binding experiments on the two isolated receptors. BM613 and BM573 binding affinity was determined on separate isoforms using transiently transfected systems expressing selectively one or the other receptor. The results showed that there were no significant differences between the affinities of these compounds for the isoforms in this assay. Consequently, we concluded from these results that the discrepancy between BM573 and BM613 in relaxation of smooth muscles were rather the result of interspecies differences. Supporting this assertion, as stated in the introduction, some authors have already highlighted heterogeneity among TP receptors between different species (see introduction, I.3.4.1, p.24).

We further used a rat model of ferric chloride-induced thrombus formation to test the *in vivo* antithrombotic properties of BM613 and BM573. The drugs injected intravenously and intraperitoneally were able

to significantly reduce the weight of thrombus formed in the lumen of the aorta (Figure III-7). These results are in accordance with *in vitro* antiplatelet activity of BM613 and BM573. These experiments highlight strongly the potential of this chemical family of compounds as therapeutic agents in TXA_2-related diseases and more specifically where platelet activation is implicated. It is noteworthy that BM573, which was selected for other pharmacological experiments due to its slightly more important activity, was demonstrated to be active in several models of diseases related to TXA_2 (pulmonary embolism (Ghuysen, Lambermont *et al.* 2004), myocardial infarction (Rolin, Petein *et al.* 2003), endotoxic shock (Lambermont, Kolh *et al.* 2004) or atherosclerosis (Cyrus, Yao *et al.* 2006)).

III.2. Synthesis of original compounds

III. 2. 1. Introduction

Consistent with the initial project, a synthesis program was set up in order to study the structure-activity relationships of original series of compounds derived from BM573 and BM613 on TP receptors. The generation of the compounds could be split in two parts : one for the "first generation compounds", derived from BM573 and BM613 and the other for the "second generation compounds", which were designed from the results collected with the first generation compounds. Nevertheless, for the consistency of the present dissertation, we decided to report in this section the synthesis schemes for all the compounds investigated. Pharmacological evaluations and rational design are discussed in the next section (III.3. , p.126).

III. 2. 2. Background

In chapter II, we have detailed the results obtained by Dogné *et al.* (Dogné 2000) with nitrobenzenic series designed as TP receptor antagonists. The compounds evaluated in that preliminary study were synthesized according to the general scheme displayed in Figure III-8.

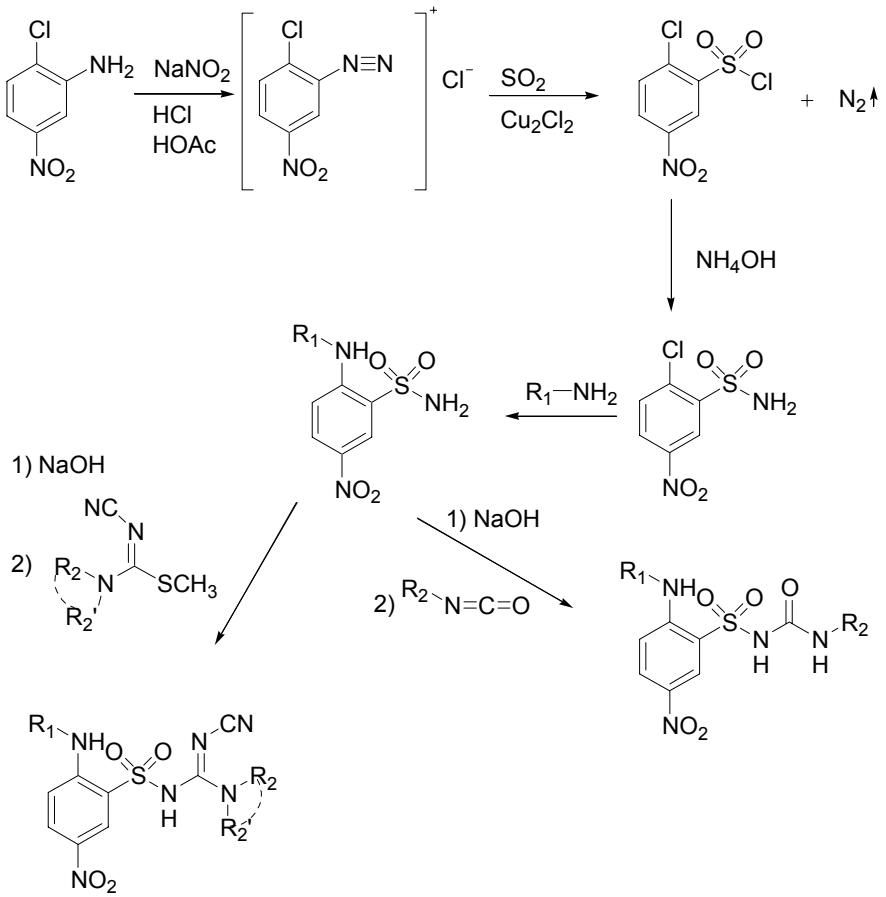


Figure III-8. General scheme for the synthesis of nitrobenzenesulfonylureas.

This general scheme was also followed for the synthesis of all compounds presented in this work. In the below sections, we describe the different steps of the synthetis pathway with their mechanisms and theoretical background.

III. 2. 3. Synthesis strategy

Consistent with previous work, structural modifications introduced to existing molecules followed the theoretical pharmacophore.

They mainly concerned the nature of R_1 and the bridge between the two rings (see Figure III-8 for general formula). Additionally, the length and nature of the side chain R_2 was also investigated, both in sulfonyurea and sulfonylcyanoguanidine series.

In order to achieve the synthesis of the modified compounds, we followed previously explored pathways and decided on a common intermediate for all final compounds synthesized. In this view, the common intermediate had to have a sufficiently activated nitrobenzenic ring, in order to modulate the nature of the bridge between the two cycles in the *para* position of the nitro group.

The chemical reaction that takes place in the addition of the second ring is a nucleophilic substitution in the *para* position of the nitro group and good knowledge of its mechanism is critical for the selection of the appropriate common intermediate.

Indeed, several types of nucleophilic substitutions may occur. Type 1 nucleophilic substitution or S_N1 (Figure III-9), is the less frequent case. It is a two-step mechanism where the initial compound undergoes an heterolytic dissociation (which determines the kinetic of the whole reaction), giving a tricoordinated carbocation (carbonium ion) and a leaving group (Y). This dissociation is immediately followed by the combination between the highly electrophilic carbonium and the nucleophilic species (Nu^-).

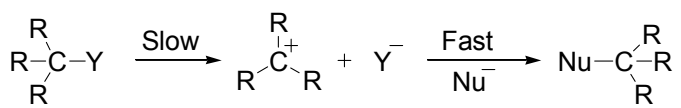


Figure III-9. Scheme for SN_1.

Type 2 nucleophilic substitution or S_N2 results from a concerted phenomenon, without intermediate species, but rather a kinetically determinant transition state (Figure III-10). Consequently the novel chemical bond is created concomitantly with the rupture of the initial chemical bond between the carbon atom and the leaving group.

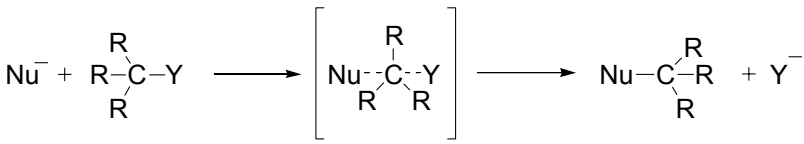


Figure III-10. Scheme for SN_2.

When the nucleophilic species reacts with a carbon atom from an aromatic ring, an addition-elimination mechanism arises. Although there is some formal resemblance with S_N2, the pathway depicted Figure III-11 must differ, because attack by Nu^- cannot take place from the back of the carbon carrying the leaving group, but must occur from the side, due to spatial configuration of the aromatic ring. It is often referred to as S_N2aro or S_NAr. The slow kinetic step is the addition of the nuceophile on the ring giving an transient intermediate, that can be isolated in some cases (Meisenheimer complex) (Figure III-11).

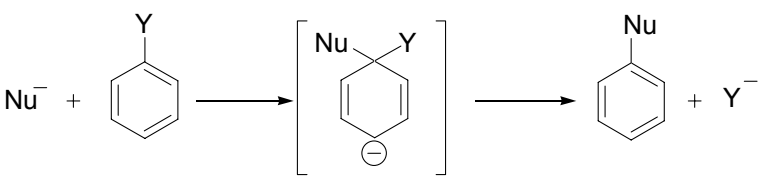


Figure III-11. Scheme for S_N2ar.

It is noteworthy that this particular type of substitution is less easy than aliphatic nucleophilic substitution. Consequently, the presence of an electron withdrawing group *para* and/or *ortho* to the substitution position

is often required. In our series of compounds, the nitro group, which is a very potent activator of nucleophilic addition with its –E effect (Figure III-12) is positioned *para* to the substitution location. Additionally, a sulfonamido group positioned *ortho* to the substitution site contributes to the activation as well.

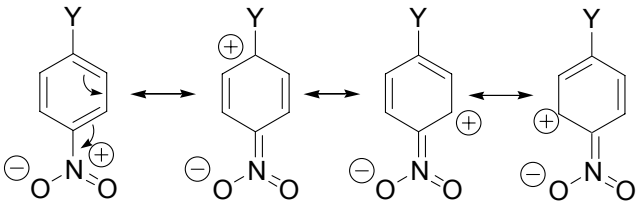


Figure III-12. Mesomeric form of *para*-nitrobenzene.

The kinetic of the substitution is dependent of several parameters, i.e. the nature of the leaving group and of the solvent. Regarding the nature of the leaving group, halogen atoms are the most widely used. The following reactivity trend is observed for S_NAr among halogens as leaving groups:

$$F > Cl > Br > I$$

This rank order of reactivity is the opposite compared to the classic S_N2, which is :

$$I > Br > Cl > F$$

In the case of S_NAr, the attack of the nucleophile limits the rate of the reaction. Consequently, the nucleophilic group will attack carbon with more ease when the bond is polarized, i.e. if the attached atom electronegativity is high.

Considering these parameters, the optimal intermediate for the synthesis of our compounds would be 2-fluoro-5-nitrobenzenesulfonamide. Nevertheless, we choose the 2-chloro-5-nitrobenzenesulfonamide (Figure III-13). Indeed, the nucleophilic potency of all chemical reagents used throughout this work was strong enough to adequately react with the chlorine-substituted intermediate, and thus did not require a fluorinated compound, which is far more expensive to prepare.

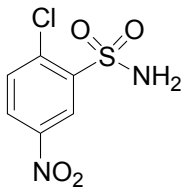


Figure III-13. Common intermediate, 2-chloro-5-nitrobenzenesulfonamide.

III. 2. 4. Compounds synthesis

III.2.4.1. From 2-chloro-5-nitroaniline (**41**) to 2-chloro-5-nitrobenzenesulfonamide (**43**)

2-Chloro-5-nitroaniline (**41**) was diazotized as depicted in Figure III-14. In an acid medium containing nitrite ions (NO_2^-), primary amines have the ability to be diazotized by nitronium ion, which is spontaneously formed in the acid medium. Practically, aliphatic primary amines give highly reactive and useless (except for very specific use) carbocations. In similar conditions, secondary aliphatic amines give nitrosamines and tertiary amines do not react.

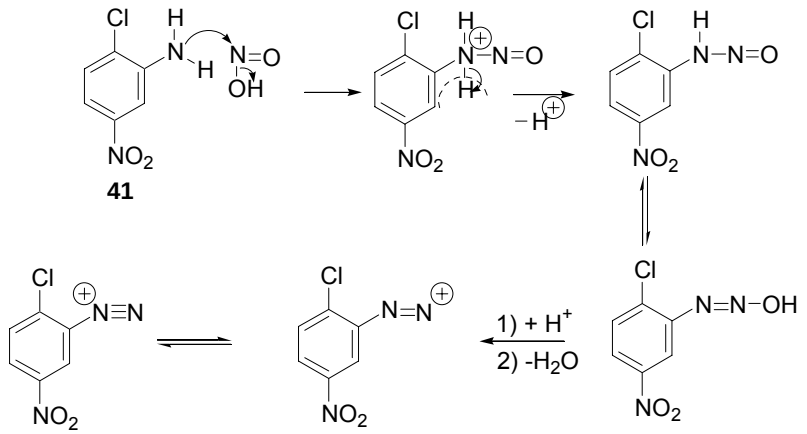


Figure III-14. Diazotization of 2-chloro-5-nitroaniline (41).

With regard to primary aromatic amines (anilines), the diazonium salt can be stabilized as a result of resonance phenomenon with π electrons of the aromatic ring (Figure III-15). These salts have to be formed and manipulated at a temperature below 5°C, because they violently react at warmer temperature to form phenols, with risk of explosion.

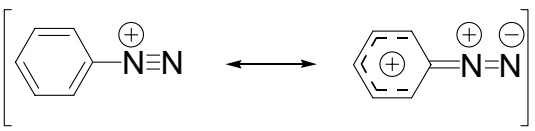


Figure III-15. Stabilisation of diazonium ion by aromatic π electrons.

Diazonium salts have the property to undergo several substitutions with release of molecular nitrogen. Indeed, nitrogen is an effective leaving group, due to the stability of the resulting molecule. The specific reaction used for our synthesis is a variant of the Sandmeyer reaction, first described by Meerwein *et al.* in 1957 (Figure III-16)(Meerwein, Dittmar *et al.* 1957).

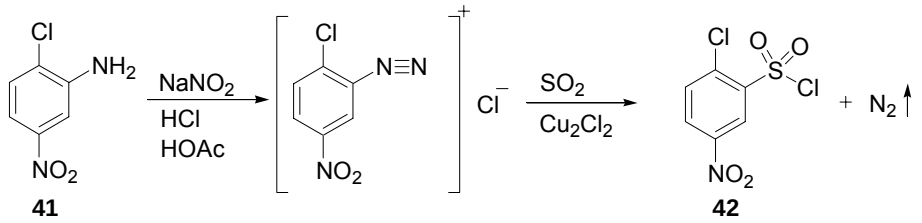


Figure III-16. Synthesis of sulfonyl chloride, proposed by Meerwein *et al.*(Meerwein, Dittmar *et al.* 1957).

In a typical Sandmeyer reaction, diazonium salt decomposes in the presence of copper (I) halides (such as copper (I) chloride) to form the desired aryl halide. The reaction involves a radical-mediated mechanism catalysed by copper (I). Meerwein, when he bubbled sulfurous anhydride in a diazonium salt/copper (I) chloride mixture, obtained a sulfonyl chloride instead of the expected aryl halide. This reaction leading to sulfonyl chloride is easily conducted, but is still poorly studied and its exact mechanism remains unknown.

Thus, we utilized the Meerwein *modus operandi* to synthesize the desired sulfonyl chloride **42** after diazotization. Resulting compound was subsequently poured into an ammoniacal solution, thus giving the desired sulfonamide **43**. This common intermediate was subsequently used as the starting reagent for the synthesis of all final compounds in each synthesis pathway (Figure III-17).

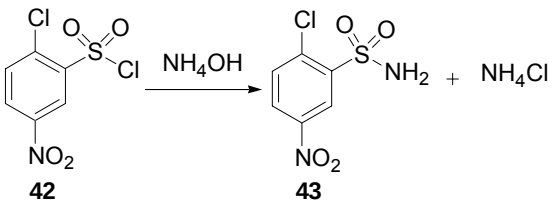


Figure III-17. Sulfonyl chloride reaction with ammonia gives the corresponding sulfonamide.

Practically, **41** was suspended in an hydrochloric-acetic acid mixture. The solution was maintained at a temperature below 5°C. Sodium nitrite was gradually added in order to obtain the diazonium salt. The mixture containing the diazonium salt was added to saturated acetic acid solution of sulphur dioxide in the presence of Cu_2Cl_2. The resulting sulphonyl chloride **42** precipitated in the medium after addition of ice. It was recovered on paper filter after filtration and then poured into a cooled ammonia solution. After 30 minutes agitation, the excess of ammonia was eliminated under reduced pressure. The pH was brought to 1 by addition of concentrated hydrochloric acid. The precipitate of 2-chloro-5-nitrobenzenesulfonamide **43** was collected by filtration, washed and dried.

III.2.4.2. From 2-chloro-5-nitrobenzenesulfonamide (**43**) to 2-(cycloalkyl- or arylamino)-5-nitrobenzenesulfonamide (**44**)

Compounds of this series were obtained by aromatic nucleophilic substitution described at point III. 2. 3. (p. 107). The chlorine atom of **43** was substituted by appropriate anilines to give **44** series.

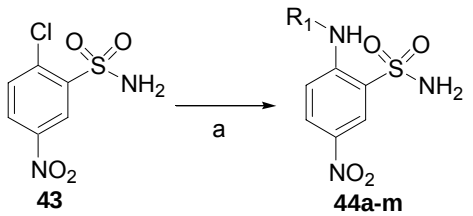
We envisaged several patterns for the second aromatic ring. Consistent with previous studies, where *o*-, *m*- and *p*-substituted anilines

were studied, we chose to investigate the specific role of the number and position of methyl groups. Several disubstituted and trisubstituted compounds were synthesized (Figure III-18). In addition, cyclohexylamine and 4-chloroaniline were also used as reagents and gave compounds **44a** and **44l**, respectively (Figure III-18). Although a general procedure has been followed, the reaction time and the product yield depended on the reactivity of each aniline.

The best conditions for these reactions were determined and a combination of methanol with a large excess of aniline appeared to be the adequate mixture for reaction with anilines in most cases.

Although methanol was not the most appropriate solvent for nucleophilic substitution, the mild conditions permitted to avoid the formation of degradation products, but the reaction time was increased. Moreover, the excess aniline allowed :

- to collect the proton from the weak acidic sulfonamido group ;
- to capture the proton released during reaction and thus to displace the equilibrium and to facilitate the reaction.



43 **44a-m**

44a R_1=cyclohexyl
44b R_1=2, 4, 6-trimethylphenyl
44c R_1=2-methylphenyl
44d R_1=3-methylphenyl
44e R_1=3-methyl-4-bromophenyl
44f R_1=2, 5-dimethylphenyl
44g R_1=2, 4-dimethylphenyl
44h R_1=2, 6-dimethylphenyl
44i R_1=4-methylphenyl
44j R_1=3, 4-dimethylphenyl
44k R_1=3, 5-dimethylphenyl
44l R_1=4-chlorophenyl
44m R_1=2, 3-dimethylphenyl

Figure III-18. Nucleophilic substitution of the chlorine atom from 2-chloro-5-nitrobenzenesulfonamide (43) by diversely substituted anilines. Reagent : a) appropriate aniline.

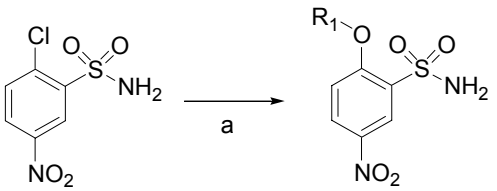
III.2.4.3. From 2-chloro-5-nitrobenzenesulfonamide (**43**) to 2-aryloxy-5-nitrobenzenesulfonamides (**45**)

For this series of compounds, where an oxygen bond replaces the NH bond, phenols were used as reagents instead of anilines. The mechanism of the substitution is identical, namely a S_NAr. The substitutions were first realized with appropriate methylphenol (*meta*, *para* or *ortho*) to give **45a-c** (Figure III-19). Second generation intermediate compounds (see below section III. 3. 2. 143) were synthesized following the same method and are depicted in Figure III-19 (compounds **45d-r**).

Phenols possess a greater reactivity in the setting of this nucleophilic substitution, and a large excess of reagent was not required for the reaction to be complete in a short time. All reactions were conducted in refluxing acetonitrile, which, as a solvent, combines a good activation of nucleophilic substitution and mild conditions. The rate of

the reaction is increased when a base is added, in order to neutralize release of an acidic proton during reaction. Potassium carbonate was used in these reactions for this purpose.

When the reaction was complete, the mixture was cooled and filtered in order to eliminate excess potassium carbonate. The filtrate was evaporated under reduced pressure and the residue was dissolved in methanol. The desired product was then isolated after addition of water, the insoluble material collected by filtration and further purified by crystallization or column chromatography.



43 **45a-r**

45a	R_1=4-methylphenyl	**45j**	R_1=4-iodophenyl
45b	R_1=3-methylphenyl	**45k**	R_1=3-iodophenyl
45c	R_1=2-methylphenyl	**45l**	R_1=4-methoxyphenyl
45d	R_1=4-chlorophenyl	**45m**	R_1=4-propoxyphenyl
45e	R_1=3-chlorophenyl	**45n**	R_1=4-ethoxyphenyl
45f	R_1=2-chlorophenyl	**45o**	R_1=3-methyl-4-Methoxyphenyl
45g	R_1=4-bromophenyl	**45p**	R_1=3-Methyl-4-chlorophenyl
45h	R_1=3-bromophenyl	**45q**	R_1=3-methoxyphenyl
45i	R_1=2-bromophenyl	**45r**	R_1=2-methoxy-4-methylphenyl

Figure III-19. Nucleophilic substitution of the chlorine atom from 2-chloro-5-nitrobenzenesulfonamide (43) by diversely substituted phenols. Reagent: a) appropriate phenol, K_2CO_3.

III.2.4.4. From 2-chloro-5-nitrobenzenesulfonamide (**43**) to 2-(arylthio)-5-nitrobenzenesulfonamides (**46**)

Conditions and methods employed for the synthesis of this series of compounds was similar to those used for the phenols. The nucleophile

was a thiophenol, in order to synthesize compounds with a sulphur bridge between the two cycles (compounds **46a-c**). The appropriate thiophenol was added to the mixture in acetonitrile in sufficient quantities (2 eq) together with potassium carbonate (Figure III-20).

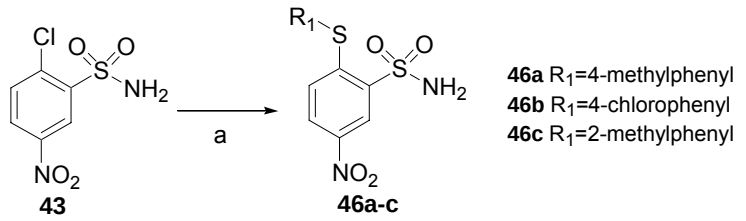


Figure III-20. Nucleophilic substitution of the chlorine atom from 2-chloro-5-nitrobenzenesulfonamide (43) by diversely substituted thiophenols. Reagent : a) appropriate thiophenol, K_2CO_3.

When the reaction was over, the mixture was cooled and subsequently filtered. The resulting solution was evaporated to dryness under reduced pressure. The desired product was further purified by crystallization or column chromatography.

III.2.4.5. From 2-(cycloalkylamino or arylamino, aryloxy or arylthio)-5-nitrobenzenesulfonamides (**44-46**) to 2-(cycloalkylamino or arylamino, aryloxy or arylthio)-5-nitrobenzenesulfonylureas (**47-49**)

Several strategies can be envisaged for the synthesis of the sulfonylurea group, starting from a sulfonamide. Indeed, when the distal nitrogen is solely monosubstituted (secondary sulfonamide), we can use an appropriate isocyanate as reagent (several of these agents are commercially available). When the distal nitrogen is substituted by two groups (tertiary sulfonamide), or included in a ring, the use of isocyanate

is impossible and alternative pathways have to be considered, like the synthesis of a carbamate.

Whatever the chosen pathway, the weak nucleophilicity of the sulfonamide group had to be enhanced by deprotonation. Consequently, all synthetic routes presented start with NaOH treatment (1 eq) of the starting sulfonamide intermediate, to give the corresponding sodium sulfonamidate salt (Figure III-21).

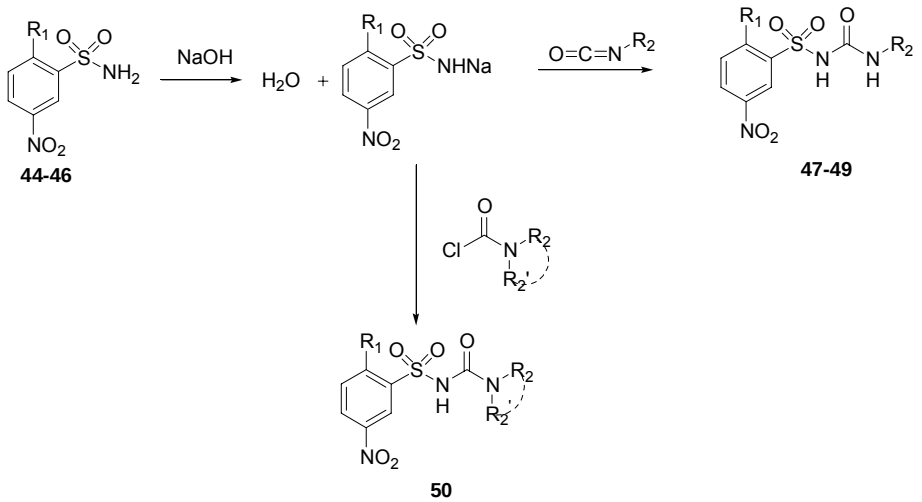
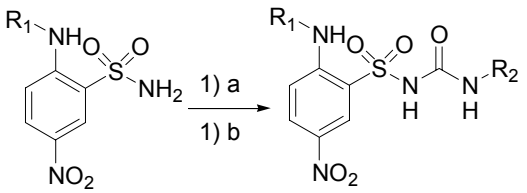


Figure III-21. General scheme for the synthesis of the desired nitrobenzenesulfonylureas.

The isocyanate route (47-49)

Most of the final compounds evaluated in this work have been synthesized according to this route. We have divided the compounds in several series in accordance to the nature of their intercyclic bridge.

Sulfonyureas of the **47** series, characterized by a NH bridge are displayed in Figure III-22. BM573 and BM613, belonging to this series, were resynthesized according to the abovementioned pathway for the purpose of pharmacological evaluations.



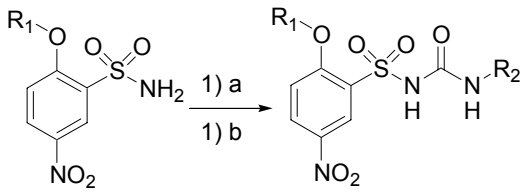
44 **47a-t**

47a R_1=cyclohexyl, R_2=hexyl
47b R_1=cyclohexyl, R_2=heptyl
47c R_1=cyclohexyl, R_2=octyl
47d R_1=2, 4, 6-trimethylphenyl, R_2=*n*-pentyl
47e R_1=2-methylphenyl, R_2=*n*-pentyl
47f R_1=3-methylphenyl, R_2=*n*-pentyl
47g R_1=3-methyl-4-bromophenyl, R_2=*t*-butyl
47h R_1=2, 5-dimethylphenyl, R_2=*n*-pentyl
47i R_1=2, 4-dimethylphenyl, R_2=*n*-pentyl
47j R_1=2, 6-dimethylphenyl, R_2=*n*-pentyl
47k R_1=4-methylphenyl, R_2=benzyl
47l R_1=4-methylphenyl, R_2=cyclohexyl
47m R_1=3, 4-dimethylphenyl, R_2=*n*-pentyl
47n R_1=3, 5-dimethylphenyl, R_2=*n*-pentyl
47o R_1=3-methyl-4-bromophenyl, R_2=*n*-pentyl
47p R_1=2, 6-dimethylphenyl, R_2=*sec*-butyl
47q R_1=2, 6-dimethylphenyl, R_2=*t*-butyl
47r R_1=4-methylphenyl, R_2=*sec*-pentyl
47s R_1=4-chlorophenyl, R_2=*n*-pentyl
47t R_1=2, 3-dimethylphenyl, R_2=*n*-pentyl

Figure III-22. Sulfonyureas of the 47 series, characterized by an "NH" bridge synthesized by the isocyanate route. Reagents : a) NaOH 10%, b) appropriate isocyanate.

Practically, sodium sulfonamidate was suspended in hot acetone and the appropriate alkyl isocyanate was added. The mixture was refluxed until completion of the reaction. When the reaction was finished, the mixture was evaporated under reduced pressure, and the crude product was dissolved in hot water and filtered. The filtrate was acidified with an hydrochloric acid solution. The precipitate was recovered by filtration, dried and purified by crystallization or on column chromatography.

Final compounds of the "oxygen bridge" family or **48** series have been obtained starting from compounds **45a-r**. All compounds synthesized are displayed in Figure III-23.

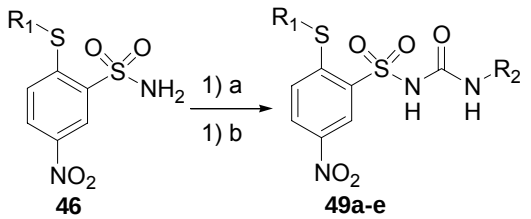


45 **48a-ao**

48a R_1=4-methylphenyl, R_2=*t*-butyl
48b R_1=4-methylphenyl, R_2=*n*-pentyl
48c R_1=4-methylphenyl, R_2=*n*-hexyl
48d R_1=4-methylphenyl, R_2=cyclohexyl
48e R_1=4-methylphenyl, R_2=*n*-butyl
48f R_1=3-methylphenyl, R_2=*n*-pentyl
48g R_1=2-methylphenyl, R_2=*n*-pentyl
48h R_1=2-methylphenyl, R_2=*i*-propyl
48i R_1=2-methylphenyl, R_2=*n*-butyl
48j R_1=2-methylphenyl, R_2=*t*-butyl
48k R_1=3-methylphenyl, R_2=*i*-propyl
48l R_1=3-methylphenyl, R_2=*n*-butyl
48m R_1=3-methylphenyl, R_2=*t*-butyl
48n R_1=4-methylphenyl, R_2=*i*-propyl
48o R_1=4-methylphenyl, R_2=*n*-propyl
48p R_1=2-bromophenyl, R_2=*t*-butyl
48q R_1=2-bromophenyl, R_2=*n*-pentyl
48r R_1=3-bromophenyl, R_2=*t*-butyl
48s R_1=3-bromophenyl, R_2=*n*-pentyl
48t R_1=4-bromophenyl, R_2=*t*-butyl
48u R_1=4-bromophenyl, R_2=*n*-pentyl
48v R_1=2-chlorophenyl, R_2=*t*-butyl
48w R_1=2-chlorophenyl, R_2=*n*-pentyl
48x R_1=3-chlorophenyl, R_2=*t*-butyl
48y R_1=3-chlorophenyl, R_2=*n*-pentyl
48z R_1=4-chlorophenyl, R_2=*t*-butyl
48aa R_1=4-chlorophenyl, R_2=*n*-pentyl
48ab R_1=3-iodophenyl, R_2=*t*-butyl
48ac R_1=3-iodophenyl, R_2=*n*-pentyl
48ad R_1=4-iodophenyl, R_2=*t*-butyl
48ae R_1=4-iodophenyl, R_2=*n*-pentyl
48af R_1=4-methoxyphenyl, R_2=*t*-butyl
48ag R_1=4-methoxyphenyl, R_2=*n*-pentyl
48ah R_1=4-propoxyphenyl, R_2=*t*-butyl
48ai R_1=4-propoxyphenyl, R_2=*n*-pentyl
48aj R_1=4-ethoxyphenyl, R_2=*t*-butyl
48ak R_1=4-ethoxyphenyl, R_2=*n*-pentyl
48al R_1=3-methoxyphenyl, R_2=*t*-butyl
48am R_1=3-methyl-4-chlorophenyl, R_2=*t*-butyl
48an R_1=3-methyl-4-chlorophenyl, R_2=*n*-pentyl
48ao R_1=2-methoxy-4-methylphenyl, R_2=*t*-butyl

Figure III-23. Sulfonyureas of the 48 series, characterized by a O bridge, synthesized by the isocyanate route. Reagents : a) NaOH 10%, b) appropriate isocyanate.

Finally, final compounds characterized by a sulphur bridge (**49** series) are depicted in Figure III-24.



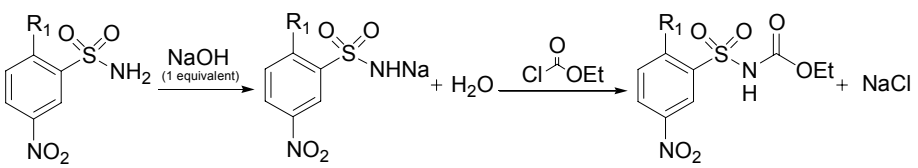
46 49a-e

49a R1=4-methylphenyl, R2=*t*-butyl
49b R1=4-methylphenyl, R2=*n*-pentyl
49c R1=2-methylphenyl, R2=*n*-pentyl
49d R1=4-chlorophenyl, R2=*t*-butyl
49e R1=4-chlorophenyl, R2=*n*-pentyl

Figure III-24. Sulfonyureas of the 49 series, characterized by a S bridge, synthesized by the isocyanate route. Reagents : a) NaOH 10%, b) appropriate isocyanate.

The carbamate route

The carbamate route consists in forming a sulfonylcarbamate intermediate after treatment of the appropriate sodium sulfonamidate with ethyl chloroformate (Figure III-25). Sodium sulfonamidate was dissolved in hot acetonitrile and 1.1 equivalent ethyl chloroformate was added to the mixture. At the end of the reaction, the solution was evaporated under reduced pressure and the residue was dissolved in methanol. After acidification of the solution by hydrochloric acid, the desired ethyl sulfonylcarbamate was precipitated by addition of cold water to methanolic solution. The precipitate was collected by filtration, washed, dried and further purified by crystallization.

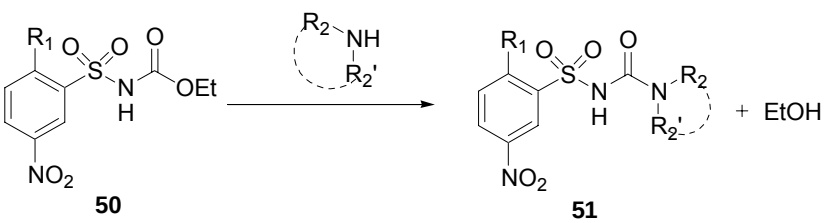


44 50

Figure III-25. Treatment of the sodium sulfonamidate intermediate with ethyl chloroformiate.

The reaction of secondary amines with the synthesized carbamate afforded the formation of the sulfonylurea reported in Figure III-26. The carbamate was dissolved in piperidine and reacted in a closed vessel at 150 °C during 5 H. After the reaction was complete, the formed product was precipitated in a water/methanol/chlorhydric acid mixture. The precipitate was recovered after filtration and purified by crystallization or by column chromatography.

This route is interesting because R_2' can be either hydrogen or another group and thus several products can be synthesized even if the isocyanate route is not possible (appropriate isocyanate not available for example).



51 R_1=4-methylphenylamino; R_2-R_2'=piperidino

Figure III-26. Reaction of carbamate with amine.

III.2.4.6. From 2-(arylamino or aryloxy)-5-nitrobenzenesulfonamides (**44-45**) to 2-(arylamino or aryloxy)-5-nitrobenzenesulfonycyanoguanidines (**54**)

The sulfonylcyanoguanidine function is usually formed by reaction of an appropriate reactive synthon with a sulfonamidate salt. Consistent with the pathway used for the synthesis of the sulfonyureas,

the nucleophilicity of the sulfonamide group had first to be increased by deprotonation.

Initially, Dogné *et al.* proposed a method using S, S'-dimethyl N-cyanodithioiminocarbonate as the starting reagent to prepare the reactive synthon (Dogné 2000). The resulting reagent was then used in the synthesis of the desired cyanoguanidines (Figure III-27).

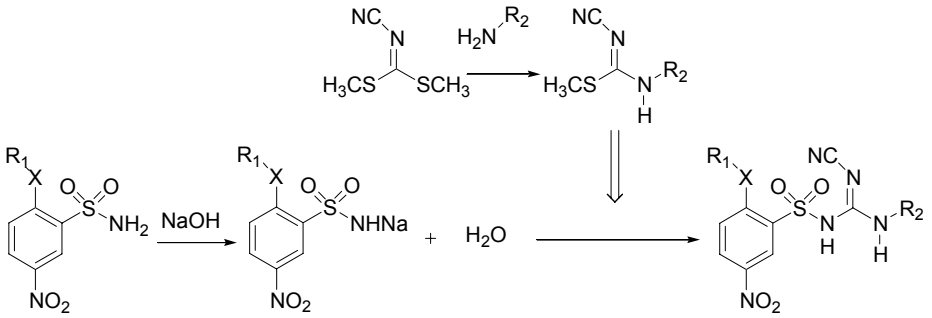
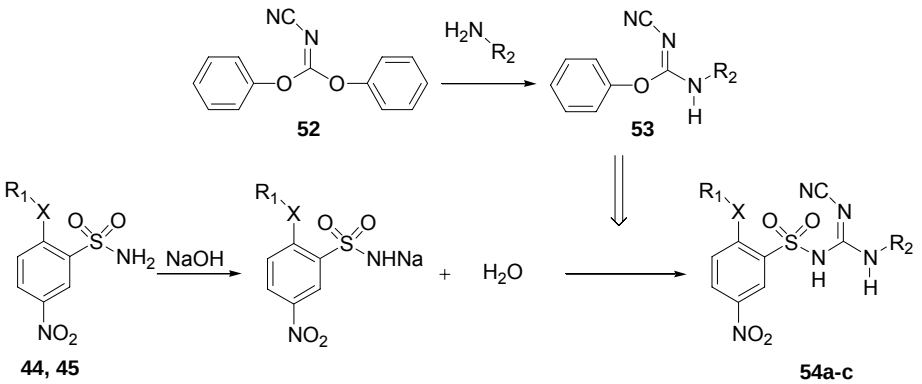


Figure III-27. Theoretical synthesis of sulfonycyanoguanidines with S, S'-dimethyl N-cyano-dithioiminocarbonate as reagent.

Unfortunately, this route did not give satisfactory results with the nitrobenzenic series. Indeed, the synthon and intermediate sulfonylurea showed poor reactivity or they were degraded by too drastic conditions. An alternative method was consequently proposed. Another reagent for the synthesis of cyanoguanidines function is available : diphenyl N-cyanocarbonimidate. This reagent was proven to be useful in milder conditions by Webb *et al.* (Webb and Labaw 1982; Webb, Eggleston *et al.* 1987). In that case, the methylthio moiety is replaced by a phenoxy moiety as the leaving group. Basic principle is identical and depicted in Figure III-28.



54a R_1=4-methylphenyl R_2=*n*-pentyl X=NH
54b R_1=3, 5-dimethylphenyl R_2=*n*-pentyl X=NH
54c R_1=4-methylphenyl R_2=*t*-butyl X=O

Figure III-28. Synthesis of sulfonycyanoguanidines 54a-c with diphenyl N-cyanocarbonimidate 52 as starting reagent.

The reactive synthon was prepared by dissolving diphenyl N-cyanocarbonimidate (**52**) in isopropanol. 1.1 equivalent of the appropriate amine was added to the medium. At the end of the reaction, water was added to the mixture and the resulting precipitate was recovered after filtration, and then purified by recrystallization.

Mechanism for the formation of sulfonylcyanoguanidines involves a nucleophilic substitution and thus the choice of the solvent was crucial. Because the second phenoxy group was less reactive after the first reaction, it was crucial to use the appropriate solvent for the next step of the reaction. After several experimentations, dimethyl formamide (DMF) was chosen as the solvent. It is noteworthy that it is one of the most adequate polar aprotic solvent recognized for nucleophilic substitution. These conditions allowed completion of the reactions at room temperature within 12 hours and markedly decreased by-products formation and degradation of compounds.

Sodium sulfonamidate was dissolved in DMF and 1.1 synthon equivalent was added to the mixture. When the reaction was completed, the solution was acidified at pH = 1 by hydrochloric acid and the desired nitrobenzenesulfonylcyanoguanidine (**54**) precipitated after addition of ice. The precipitate was collected by filtration and purified by crystallization or column chromatography.

III.3. Pharmacological evaluation of original compounds

In this section, we present the results of the pharmacological evaluations performed with the newly synthesized compounds. Two rounds of synthesis and pharmacological evaluations have been undertaken and are presented separately.

III. 3. 1. First pharmacomodulation

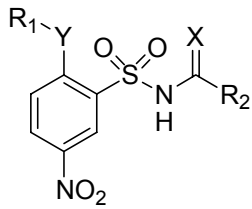
III.3.1.1. Background and design

Our first aim was to explore the influence of unknown structural modification of nitrobenzenesulfonylureas on TP receptor activity. Due to the lack of data about medicinal chemistry of TP receptor antagonists discriminating the two isoforms, we wished to establish structure-activity relationships for affinity and activity toward both TPα and TPβ separately. Therefore, we sought that this approach could highlight area(s) of the pharmacophore playing a critical role in selectivity, if we actually observed differences between the activities on the two isoforms.

The original derivatives were designed starting from the structures of BM573 and BM613, which displayed excellent pharmacological profiles (see section III.1., p.87). As stated in chapter II, the parts of the general structure of the compounds that we chose to modulate were the sulfonylurea function group, the nature of the intercyclic bridge as well as the nature of the second ring in the 2-position. Influence of the number and position of substituents on the second ring was also addressed. The nature and more specifically, the influence of the side chain's length attached to the distal nitrogen were concomitantly modulated.

The newly synthesized derivatives are presented below with respect to the structural variations operated on the structure of BM573 and BM613.

The following tables refer to the general formula :



Sulfonylurea function (X)

Compound	R1	R2	Y	X
54a	4-methylphenyl	*n*-pentylamino	NH	N-CN
54b	3, 5-dimethylphenyl	*n*-pentylamino	NH	N-CN

Table III-4. Compounds where sulfonylurea function (X=O) is substituted by a cyanoguanidine function (X=N-CN)

It is noteworthy that the cyanoguanidine group is an isostere of the urea group. Therefore, two nitrobenzenesulfonylcyanoguanidines

were synthesized, both bearing *n*-pentyl side chains. Compound **54a** is an isostere of BM573 and compound **54b** presents two methyl substituents on the second ring R_1, in the 3- and 5-positions.

Nature and substitution of the second ring (R_1) and the side chain (R_2)

Cpd	R_1	R_2
51	4-methylphenyl	piperidino
47a	Cyclohexyl	*n*-hexylamino
47b	Cyclohexyl	*n*-heptylamino
47c	Cyclohexyl	*n*-octylamino
47d	2, 4, 6-trimethylphenyl	*n*-pentylamino
47e	2-methylphenyl	*n*-pentylamino
47f	3-methylphenyl	*n*-pentylamino
47g	3-methyl-4-bromophenyl	*t*-butylamino
47h	2, 5-dimethylphenyl	*n*-pentylamino
47i	2, 4-dimethylphenyl	*n*-pentylamino
47j	2, 6-dimethylphenyl	*n*-pentylamino
47k	4-methylphenyl	benzylamino
47l	4-methylphenyl	cyclohexylamino
47m	3, 4-dimethylphenyl	*n*-pentylamino
47n	3, 5-dimethylphenyl	*n*-pentylamino
47o	3-methyl-4-bromophenyl	*n*-pentylamino
47p	2, 6-dimethylphenyl	*sec*-butylamino
47q	2, 6-dimethylphenyl	*t*-butylamino
47r	4-methylphenyl	*sec*-butylamino
47s	4-chlorophenyl	*n*-pentylamino
47t	2, 4-dimethylphenyl	*n*-hexyllamino

Table III-5. Compounds with X = O and Y = NH

These compounds are characterized by R_1 corresponding to a cycloalkyl group (**47a-c**), a mono-,di- or trimethyl-substituted benzene ring (**47d-r** and **47t**) or an halo-substituted benzene ring (chloro, **47s**). For compounds characterized by R_1 being a 4-methylphenyl moiety, the potential influence of the R_2 side chain was also explored (compounds **51**, **47k**, **47l**, **47r**). For other compounds, except **47a-c**, the R_2 side chain was either a *t*-butylamino or a *n*-pentylamino group, in order to permit direct comparison with BM573 and BM613.

Nature of the intercyclic bridge (Y)

Cpd	R_1	R_2	Y
48a	4-methylphenyl	*t*-butylamino	O
48b	4-methylphenyl	*n*-pentylamino	O
48c	4-methylphenyl	*n*-hexylamino	O
48d	4-methylphenyl	cyclohexylamino	O
48e	4-methylphenyl	*n*-butylamino	O
48f	3-methylphenyl	*n*-pentylamino	O
48g	2-methylphenyl	*n*-pentylamino	O
49a	4-methylphenyl	*t*-butylamino	S
49b	4-methylphenyl	*n*-pentylamino	S
49c	2-methylphenyl	*n*-pentylamino	S
49d	4-chlorophenyl	*t*-butylamino	S
49e	4-chlorophenyl	*n*-pentylamino	S

Table III-6. Compounds characterized by Y = O or S.

These sulfonylureas (X=O) are close analogues of BM573 and BM613, for which the intercyclic NH bridge is replaced by an oxygen atom (**48** series) or a sulfur atom (**49** series). Some other variations were

realized, such as the position of the methyl group on the second ring (**48f-g**). In the **48** series, **48c-e** explored the influence of the nature of the R_2 side chain.

III.3.1.2. Radioligand binding assay

Binding experiments directly reflect the affinity of a studied compound for a given receptor. The principle of the binding experiments remained the same than previously described (see point III.1.1.4, p.94). The system chosen for binding experiments was constituted of whole COS-7 cell lines overexpressing either TPα or TPβ. The cells were transiently transfected with plasmids containing the cDNA for TPα or TPβ proteins. This experiment was developed in collaboration with Pr Cecil Pace-Asciak at Hospital for Sick Children research institute at Toronto, Canada.

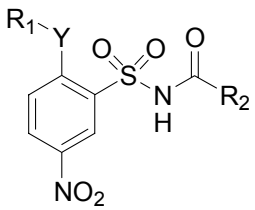
III.3.1.2.A. Screening

Principle and results

In order to select compounds for further investigation, the experiments were first performed as a "screening" assay. Consistent with experiments accomplished with BM573 and BM613, the labeled agent chosen was [^{3}H]SQ29548. Binding affinity (expressed as the IC_{50} value) of BM573 and BM613 in the settings of previous experiment was around 1 nM. Consequently, at a 1 nM concentration, these two compounds displaced about 50% of [^{3}H]SQ29548.

Results and discussion

The 35 compounds that were studied in this test are chemically close to the putative pharmacophore and we can expect their IC_{50} affinity to be found within the same concentration range. The first step in our study was to evaluate the displacement of [^{3}H]SQ29548 by the novel compounds at 1 nM. The aim of this rapid determination was to lead to a selection of the most interesting compounds for further investigations. Results for sulfonyureas are presented in Table III-7.



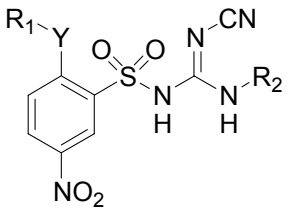
Cpd	R_1	R_2	Y	Binding affinity (%)[1]	
				TPα	TPβ
BM573	4-methylphenyl	*t*-butylamino	NH	49.5 ± 1.6	53.6 ± 5.5
BM613	4-methylphenyl	*n*-pentylamino	NH	39.6 ± 9.1	34.2 ± 2.9
47a	Cyclohexyl	*n*-hexylamino	NH	44.2 ± 16.5	42.5 ± 2.3
47b	Cyclohexyl	*n*-heptylamino	NH	29.5 ± 3.4	36.4 ± 11.1
47c	Cyclohexyl	*n*-octylamino	NH	17.1 ± 3.7	24.1 ± 4.4
47d	2, 4, 6-trimethylphenyl	*n*-pentylamino	NH	27.9 ± 3.0	27.5 ± 4.4
47e	2-methylphenyl	*n*-pentylamino	NH	45.3 ± 8.9	45.3 ± 3.0
47f	3-methylphenyl	*n*-pentylamino	NH	48.5 ± 3.4	35.8 ± 9.2
47g	3-methyl-4-bromophenyl	*t*-butylamino	NH	28.5 ± 5.4	25.0 ± 1.5
47h	2, 5-dimethylphenyl	*n*-pentylamino	NH	28.6 ± 3.4	33.1 ± 4.9
47i	2, 4-dimethylphenyl	*n*-pentylamino	NH	42.3 ± 4.0	39.9 ± 4.8
47j	2, 6-dimethylphenyl	*n*-pentylamino	NH	36.5 ± 4.7	54.0 ± 2.1
47k	4-methylphenyl	benzylamino	NH	32.9 ± 4.5	49.3 ± 3.5
47l	4-methylphenyl	Cyclohexylamino	NH	31.1 ± 2.9	29.0 ± 3.3

47m	3, 4-dimethylphenyl	*n*-pentylamino	NH	40.1 ± 8.8	48.7 ± 16.9
47n	3, 5-dimethylphenyl	*n*-pentylamino	NH	49.7 ± 5.1	49.7 ± 11.0
47o	3-methyl-4-bromophenyl	*n*-pentylamino	NH	32.1 ± 5.2	49.9 ± 13.0
47p	2, 6-dimethylphenyl	*sec*-butylamino	NH	48.4 ± 4.0	42.5 ± 6.0
47q	2, 6-dimethylphenyl	*t*-butylamino	NH	46.4 ± 2.2	46.3 ± 2.3
47r	4-methylphenyl	*sec*-butylamino	NH	40.7 ± 2.5	40.4 ± 6.5
47s	4-chlorophenyl	*n*-pentylamino	NH	48.9 ± 7.7	52.7 ± 2.2
47t	2, 3-dimethylphenyl	*n*-pentylamino	NH	44.6 ± 5.4	39.0 ± 7.8
48a	4-methylphenyl	*t*-butylamino	O	53.3 ± 5.7	44.7 ± 2.1
48b	4-methylphenyl	*n*-pentylamino	O	35.5 ± 2.8	48.5 ± 3.8
48c	4-methylphenyl	*n*-hexylamino	O	55.2 ± 2.1	46.1 ± 2.1
48d	4-methylphenyl	cyclohexylamino	O	49.8 ± 5.7	51.9 ± 3.8
48e	4-methylphenyl	*n*-butylamino	O	55.7 ± 1.7	56.0 ± 4.9
48f	3-methylphenyl	*n*-pentylamino	O	20.1 ± 10.3	22.8 ± 3.3
48g	2-methylphenyl	*n*-pentylamino	O	35.2 ± 1.6	36.8 ± 7.1
49a	4-methylphenyl	*t*-butylamino	S	47.0 ± 5.6	37.7 ± 6.3
49b	4-methylphenyl	*n*-pentylamino	S	38.5 ± 2.7	43.0 ± 1.1
49c	2-methylphenyl	*n*-pentylamino	S	33.4 ± 2.2	32.7 ± 7.7
49d	4-chlorophenyl	*t*-butylamino	S	32.5 ± 1.8	31.7 ± 3.4
49e	4-chlorophenyl	*n*-pentylamino	S	24.7 ± 7.6	21.3 ± 5.7
51	4-methylphenyl	piperidino	NH	46.2 ± 9.2	54.2 ± 7.8

[1] Expressed as the percentage of displaced [^{3}H]SQ29,548 by our compounds. Results are Mean ± standard deviation of at least three determinations (n≥3). Compounds were evaluated at a final concentration of 1 nM.

Table III-7. Displacement of radiolabeled [^{3}H]SQ29548 from TPα and TPβ by sulfonylureas at 1 nM.

The same experiment has been conducted on sulfonylcyanoguanidines. The results for compounds **54a** and **54b** are displayed in Table III-8 below.



Cpd	R_1	R_2	Y	Binding affinity (%)[1]	
				TPα	TPβ
54a	4-methylphenyl	*n*-pentyl	NH	25.5 ± 1.8	27.2 ± 5.2
54b	3, 5-dimethylphenyl	*n*-pentyl	NH	48.0 ± 6.4	53.4 ± 3.2

[1] Expressed as the percentage of displaced [^{3}H]SQ29,548 by our compounds. Results are Mean ± standard deviation of at least three determinations (n≥3). Compounds were evaluated at a final concentration of 1 nM.

Table III-8. Displacement of radiolabeled [^{3}H]SQ29548 from TPα and TPβ by sulfonylcyanoguanidines at 1 nM.

Discussion

All compounds evaluated displayed a high affinity for both TPα and TPβ receptor isoforms (Table III-7 & Table III-8). These affinities, expressed as the percentage of displaced [^{3}H]SQ29548, were all in the nanomolar range and were comparable to those of the reference compounds BM573 and BM613 used throughout this study. The affinities of BM573 and BM613 were in the same range than those found in previous experiments (see III.1.1.4, p.94). The chemical bond (O, S, or NH in the series **48a-g**, **49a-e** or **47a-t-54-51**, respectively) between the nitrobenzene and the second ring did not dramatically affect the affinity of the molecules for the TP receptors. Additionally, in the "NH" compounds family (**47a-t, 51,** and **54**), a critical loss of affinity was not observed as a result of the disubstitution of the distal nitrogen atom of

the urea group (**51**) or as a result of the replacement of the urea function with a cyanoguanidine moiety (**54**).

Interestingly, although all compounds showed comparable affinity between the two isoforms, some of them displayed higher differences in their affinities for TPα and TPβ. **47o** or **48b**, for example, were characterized by an highest affinity for TPβ, while **47f** or **48c** seemed to have a greater affinity for TPα.

III.3.1.2.B. Binding concentration-response curves

Principle and results

Compounds characterized by the highest affinity for the two isoforms as well as compounds characterized by the greatest differences in affinity for either TPα or TPβ were selected. Complete concentratione-response curves were determined with concentrations ranging from 10^{-6} to 10^{-11} M. The IC_{50} values were calculated from the curves obtained and a "selectivity ratio", defined as IC_{50} TPα /IC_{50} TPβ, was calculated (Table III-9).

Compounds	Binding affinity IC_{50} (nM)[1]		Ratio $IC_{50}TP\alpha/IC_{50}TP\beta$
	TPα	TPβ	
BM573	1.05 ± 0.43	0.78 ± 0.10	1.35
BM613	2.65 ± 1.59	3.53 ± 1.38	0.75
48a	1.26 ± 0.74	1.77 ± 0.69	0.71
48b	2.63 ± 0.94	0.65 ± 0.15	4.02
48c	0.72 ± 0.08	1.58 ± 0.21	0.45
48d	1.52 ± 0.70	0.79 ± 0.15	1.93
48e	0.74 ± 0.02	0.70 ± 0.24	1.05
49a	0.77 ± 0.33	1.74 ± 0.28	0.44
51	0.46 ± 0.11	0.68 ± 0.11	0.68
47a	0.71 ± 0.01	1.07 ± 0.26	0.67
47b	2.50 ± 1.21	2.68 ± 1.06	0.93
47f	1.01 ± 0.25	4.43 ± 0.47	0.23
47j	1.52 ± 0.31	1.48 ± 0.35	1.03
47k	1.90 ± 0.40	1.17 ± 0.12	1.62
47n	1.08 ± 0.33	0.70 ± 0.43	1.55
47o	1.82 ± 0.25	0.58 ± 0.27	3.11
47q	0.78 ± 0.15	0.56 ± 0.20	1.40

[1] Results are expressed as mean ± standard deviation of at least three determinations (n≥3).

Table III-9. Estimated IC_{50} values for displacement of [3H]SQ29,548 from TPα and TPβ

Structure-activity relationships and discussion

Regarding compounds **48a-e**, characterized by the presence of an oxygen atom as the bridge between the two aromatic rings, several concentration-response curves were determined and are summarized in Table III-9. All compounds of this family confirmed the screening results. Although the affinities of the compounds for both TPα and TPβ lie within the same range, several compounds exhibited a significant

selectivity ratio between the two receptor isoforms. For example, consistent with the results of the screening experiment, compound **48b** with R_2 = *n*-pentyl exhibited a significant ratio of 4.02 ($p<0.05$), which reflects a greater affinity for TPβ. Additionally, **48c** showed significant ($p<0.05$) preferential affinity for TPα confirmed in both tests.

In this family of compounds, it was apparent that the side chain (R_2) had an impact on the affinity. Compound **48e** with R_2 = *n*-butyl is the most potent compound in this assay and seems to bear the most interesting side chain among those evaluated in this test. In terms of the calculated selectivity ratio, the R_2 side chain was also found to be involved. On one hand, compounds for which R_2 are *n*-pentylamino or cyclohexylamino (**48b** and **48d** respectively) expressed a preferential affinity for TPβ (Table III-9). On the other hand, when R_2 corresponded to *t*-butyl or *n*-hexyl (**48a** and **48c** respectively), there was either a loss or no change in the selectivity ratio.

Additionally, the IC_{50} affinity of one compound of the **49** series, characterized by a sulfur atom as the intercyclic bridge, has been determined (compound **49a**, Table III-9). This compound showed a nanomolar affinity for the TP receptors. This observation confirms our previous statement that the nature of the intercyclic bridge (NH, S or O) does not critically influence the affinity of the compound for the receptors. The selectivity fully correlated with screening experiment and reinforced the hypothesis that a short alkyl chain (here with R_2 = *t*-butylamino) favours the preferential affinity for TPα isoform.

Compound **51** is characterized by a NH bridge between the two rings and the distal N urea atom incorporated in an alicyclic ring. Although the affinity is not dramatically influenced compared to the parent compounds BM573 and BM613, the incorporation of distal N urea atom into an alicyclic ring appears to be favourable for the affinity on both isoforms, with no influence on the selectivity ratio.

Finally, Table III-9 also presents IC_{50} values obtained with compounds of the **47** family, which are characterized by a NH bridge between the two rings. We observed that, when the compounds are characterized by a second ring being alicyclic, the length of the side chain influences the affinity. For example, compound **47a** with an R_2 = *n*-hexylamino, is characterized by a ~3 fold higher affinity for both isoforms than **47b** with R_2 = *n*-heptylamino. The results obtained with compound **47f** are of particular interest. Indeed, it is the most selective compound for TPα in this assay, characterized by R_2 = *n*-pentylamino and R_1 = *m*-methylphenyl. Consistent with results obtained with **48b**, compound **47o** displayed a greater affinity for TPβ than for TPα and is characterized by a bromo substituent in the 4-position of the second ring associated with R_2 being a *n*-pentylamino chain. Finally, it is important to notice that PBT-3 (see introduction, I. 4. 3., p. 69), an hepoxilin stable analog characterized as a selective TPα antagonist, exhibited a selectivity ratio of ~0.17 in the same assay (Qiao, Reynaud *et al.* 2003).

III.3.1.3. Functional assays

Binding assays provide very useful information on the affinity of a series of chemical compounds, and is the preferred method for directing

synthesis. Nevertheless, the efficacy of a compound at a given receptor can not be directly extrapolated from its affinity. Consequently, we aimed to study the activity of our compounds at both TPα and TPβ. In this section are described the results collected in an original, medium throughput functional test and in a platelet aggregation assay.

III.3.1.3.A. Measurement of intracellular calcium mobilization

Introduction

Both TPα and TPβ are coupled to G-protein Gαq. Therefore, stimulation of the receptor ultimately leads to a release of intracellular calcium ($[Ca^{2+}]_i$) from intracellular stores upon action of IP_3 (see introduction, I. 3. 1., p. 16). Consequently, the determination of Ca^{2+} flux represents an appropriate functional test for the evaluation of the agonistic/antagonistic potency of our newly synthesized compounds.

Ca^{2+} is an ubiquitous intracellular messenger controlling numerous activities within cells and its physiological importance was known for a long time. For the monitoring of Ca^{2+} flux and signalling, a crucial breakthrough was the development in the eighties by Tsien group of proper fluorescent dyes able to report variations in Ca^{2+} concentration (Grynkiewicz, Poenie *et al.* 1985).

Fluorescence

An oversimplification of fluorescence definition is that it is the immediate emission of light from a molecule or atom following the absorption of a radiation. In this short description of fluorescence phenomenon, we will restrict the basic description to molecular species.

Within molecules as well as in atoms, fluorescence results from specific electronic changes.

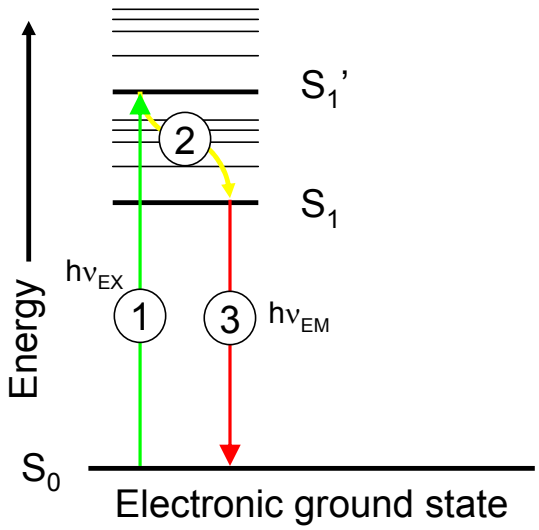


Figure III-29. Jablonski diagram illustrating the processes involved in the creation of an excited electronic singlet state by photon absorption and subsequent emission of fluorescence. See text for complete description of state 1, 2 and 3.

Fluorescence occurs in three stages :

- Excitation : a photon of energy $h\nu_{EX}$ that is absorbed by the fluorophore (the part of the molecule responsible for fluorescence) creates an excited state S_1' (Figure III-29) ;

- Excited-state lifetime : the excited state exist for a given period of time (typically 1-10 ns). During this state, fluorophore undergoes conformational changes or interaction with its environment, with a consequent dissipation of absorbed energy from S_1', yielding a relaxed singlet excited

state S_1 from which fluorescence emission originates (Figure III-29) ;

- Fluorescence emission : a photon of lower energy, $h\nu_{EM}$, is emitted, returning the fluorophore to its ground state S_0. Due to energy dissipation during the excited-state lifetime, the energy is lower and therefore the wavelength is higher (Figure III-29) ;

Detection and quantification of Calcium ions

To date, numerous Ca^{2+} indicators have been designed and described. Despite the improvement and diversification of their chemical and fluorescent properties, the principle of these dyes is based on a common characteristic. These molecules are characterized by the covalent combination of a Ca^{2+} chelating group and a fluorophore group. The Ca^{2+} binding properties of these indicators are formed by the presence of a tetracarboxylic acid core as found for example in EGTA (Ethylene Glycol bis(2-aminoethyl)Tetraacetic Acid). Binding of Ca^{2+} produces a wavelength shift in either the excitation or emission fluorescence spectra or a change in the emission intensity (Simpson 1999). Whereas the Ca^{2+} binding to EGTA is pH dependent, recent dyes are designed from an EGTA derivative, BAPTA (1, 2 - Bis(2-Aminophenoxy)ethane-*N*, *N*, *N*', *N*'-Tetraacetic Acid). Loading of these dyes inside cells commonly uses esterified forms (acetoxymethyl ester), which are able to cross the cell membranes and are subsequently hydrolyzed by esterases inside the cell.

The quantification of $[Ca^{2+}]_i$ can be calculated by several means depending on the nature of the dye. Two main families of indicators can be distinguished, based on the quantification method.

Non-ratiometric fluorescent dyes

With these indicators, there is little or no shift in the fluorescent spectra (neither excitation nor emission) ; however, a marked increase in fluorescence emission intensity can be observed and quantified upon Ca^{2+} binding. The parent dyes of this family, Quin-2 (Tsien's original Ca^{2+} dye (Tsien, Pozzan *et al.* 1982)), had poor quantum yield (efficiency of the fluorescence process). More recent indicators, like fluo-3 have overcome this issue and its fluorescence emission increases about 200 fold between Ca^{2+}-free and Ca^{2+}-saturated form.

Estimation of $[Ca^{2+}]i$ is calculated from the fluorescence signal (F). For calibration, the maximal fluorescence (F_{max}, Ca^{2+}-saturated form of the dye) as well as minimal fluorescence (dye Ca^{2+}-free fluorescence) must be determined. These parameters are determined usually *in situ* after the experiment, for example by subsequent addition to the mixture of a cell-disrupting agent that releases all Ca^{2+} and a potent Ca^{2+}-chelating agent such as EGTA. After subtraction of background fluorescence, $[Ca^{2+}]_i$ can be calculated for non-ratiometric indicators as follow :

$$[\mathrm{Ca}^{2+}]_\mathrm{i} = K_d.(F - F_{\min})/(F_{\max} - F)$$

Where K_d is the dissociation constant of the indicator for Ca^{2+}.

Ratiometric fluorescent dyes

Fura-2 is the archetypal dye of this family. In low [Ca^{2+}], fura-2 shows a broad excitation spectrum between 300 and 400 nm, with a peak at approx. 370 nm. The particularity of these dyes is that if fura-2, for example, is excited at 340 nm (emission monitored at 510 nm), Ca^{2+} binding will produce an increase in fluorescence, whereas a decrease in the fluorescent signal is observed when the dye is excited at 380 nm. When the dye is excited in quick succession at 340 and 380 nm, a ratio of the respective emission signals can be used to monitor [Ca^{2+}]. The advantage of this "ratiometric" measurement is that the ratio signal is not dependent on dye concentration, illumination intensity, or optical path length. Dye leakage and photobleaching frequently lead to a loss of indicator during an experiment. Under these conditions, a ratiometric dye leads to a more precise estimation of [Ca^{2+}].

For ratiometric indicators, the calibration is similar to that of single wavelength indicators. Hence, the maximum and minimum ratio values, R_{max} and R_{min} are required instead of F_{max} and F_{min}. [Ca^{2+}] is thus given by the following formula :

$$[Ca^{2+}]_i = \beta.K_d.(R - R_{min})/(R_{max} - R)$$

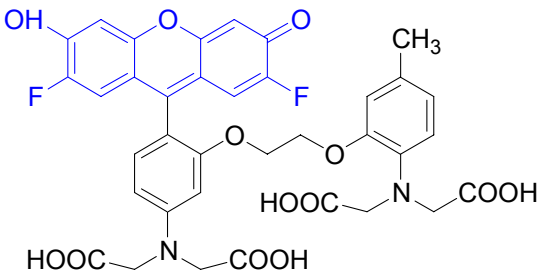
Where β is the ratio of the fluorescence intensities at the wavelength chosen for the denominator of R (e.g., 380 nm excitation for Fura-2) in zero and saturating [Ca^{2+}]. K_d is the dissociation constant of the indicator for Ca^{2+}.

Parameters of the experiment

Determination of a cell suspension Ca^{2+} flux upon response to an agonist is usually performed in a cuvette on a fluorescence spectrophotometer. Nevertheless, these protocols in our settings would have been laborious and time-consuming given the large amount of data to be collected. Consequently, we decided to increase our throughput capacity using a fluorescence plate reader.

We have based our protocol on a report by Lin *et al.* (Lin, Sadee *et al.* 1999) that proposed for the first time an effective and rapid assay to measure intracellular calcium using a fluorescence plate reader (Lin, Sadee *et al.* 1999). In this work, the authors proposed a general method for proper $[Ca^{2+}]_i$ determination in a multiwell plate format (96 wells). For the convenience of the experiment and to avoid time-consuming protocols, cell lines overexpressing either TPα or TPβ (HEK293) were set up in collaboration with Dr Kinsella from the University College Dublin, Ireland.

One crucial parameter to address is the kind of dye and we chose fluo-4 (**55**, Figure III-30). It is a recently developed dye designed from fluo-3, with a higher increase in fluorescence emission upon Ca^{2+} binding. Another advantage of it is that it has its excitation peak at 480 nm, in the visible spectra, which spare cells to be damaged by UV (340-360 nm) stimulation and reduces auto-fluorescence of cells. We chose Fluo-4 although it is not a ratiometric dye because of its high sensitivity, which is one of the most important feature in multiwell experiments. Additionally, we didn't need to determine the precise $[Ca^{2+}]_i$, but rather variations in concentrations.



Fluo-4 (**55**)

Figure III-30. Chemical structure of Ca^{2+} dye Fluo-4. In blue, the fluorophore. In black, the BAPTA moiety.

Our adaptations of Lin *et al.* method allowed the detection of $[Ca^{2+}]_i$ mobilization upon stimulation by U46619 in human TP platelets, as shown in Figure III-31.

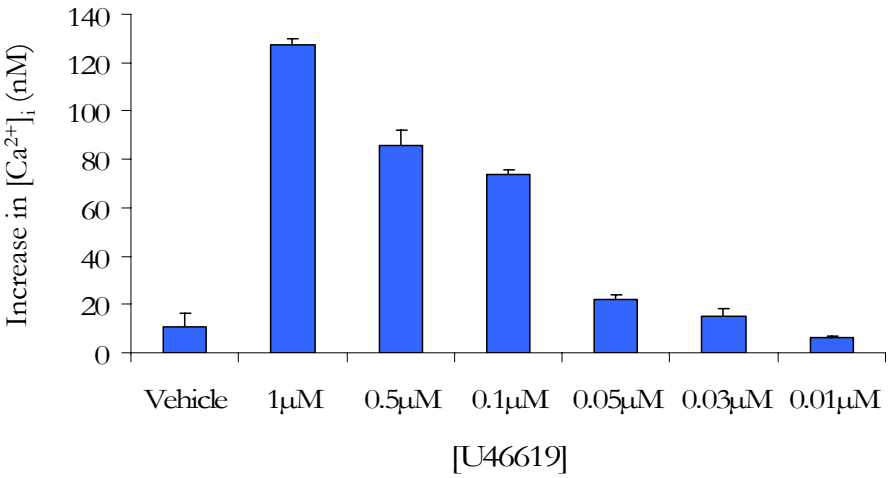


Figure III-31. Increase of $[Ca^{2+}]_i$ in fluo-4 loaded human platelets upon stimulation by increasing concentrations of U46619.

Similarly, we assessed the $[Ca^{2+}]_i$ mobilization in response to U46619 stimulation (1µM) in the HEK293 cell lines. Both TPα and TPβ

transfected cells responded in a comparable fashion, a pictured in Figure III-32.

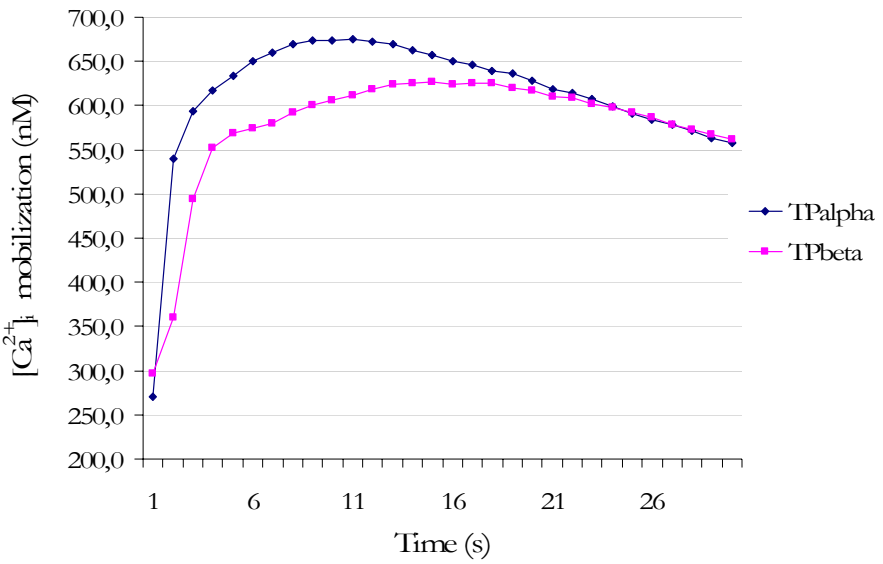


Figure III-32. Increase of $[Ca^{2+}]_i$ in fluo-4 loaded HEK293 cells line stably overexpressing TPα or TPβ after injection of U46619 (1µM, t=0 s).

Results

We first determined the effects of BM573 in this experiment and found that it inhibited $[Ca^{2+}]_i$ mobilization induced by U46619 (1µM) in a concentration-dependent manner. The calculated IC_{50}, defined as the concentration able to inhibit 50% of $[Ca^{2+}]_i$ mobilization, was 318 ± 202 nM for TPα and 53 ± 19 nM for TPβ (n = 6).

We then examined the effects of the selected compounds on agonist-induced intracellular signalling by the TPα and TPβ isoforms. We used concentrations ranging from 10^{-5} M to 10^{-9} M. As for the binding

experiment, a selectivity ratio was determined as the IC_{50} TPα /IC_{50} TPβ. Results collected in this evaluation are presented in Table III-10.

Compound	U46619-mediated $[Ca^{2+}]_i$ mobilization (nM)[1]		Ratio IC_{50}TPα/IC_{50}TPβ
	TPα	TPβ	
BM573	319 ± 202	53 ± 19	6.0
BM613	50 ± 1	14 ± 1	3.7
48a	58 ± 43	58 ± 4	1.0
48b	139 ± 88	55 ± 5	2.5
48c	526 ± 30	58 ± 1	9.0
48d	601 ± 11	70 ± 6	8. 6
48e	558 ± 34	53 ± 2	10.5
49a	293 ± 106	55 ± 3	5.3
51	65 ± 3	38 ± 3	1.7
47a	81 ± 3	46 ± 1	1.8
47b	93 ± 9	55 ± 2	1.7
47f	60 ± 16	45 ± 1	1.3
47j	81 ± 20	47 ± 0.4	1.7
47k	64 ± 2	45 ± 3	1.4
47n	538 ± 8	76 ± 6	7.0
47o	126 ± 26	43 ± 10	2.9
47q	57 ± 2	31 ± 10	1.8

[1] Results expressed as mean ± standard deviation of at least three determinations (n≥3).

Table III-10. Estimated IC_{50} values for the inhibition of $[Ca^{2+}]_i$ mobilization mediated by either TPα or TPβ upon stimulation by U46619 (1μM).

Structure-activity relationships and discussion

We aimed to extend the characterization of the binding-selected compounds through functional intracellular signalling studies. Hence, we

examined their properties on U46619-induced $[Ca^{2+}]_i$ mobilization in HEK293 cell lines stably over-expressing the individual TPα or TPβ isoforms.

The results obtained with the compounds in this assay (Table III-10) do not fully correlate with those obtained through competition binding studies (Table III-9). The discrepancy observed may be explained by the fact that the affinity is not the same property than activity. Thus, the mechanisms and intensities whereby a compound activates or deactivates a receptor might differ from affinity determined in binding studies. Hence, it is critical to evaluate active agents through different tests.

Firstly, it is noteworthy that all compounds assayed exhibited high activity as TPα and TPβ receptor antagonists. Compound **47q,** one of the most potent compound in this assay, was characterized by a 2,6-dimethylphenylamino group *ortho* to the sulfonamide group in the aromatic ring. As a result, we may suggest that a 2-6 substitution pattern by methyl groups may provide an interesting lead for further experimentations. Moreover, in this functional assay, each compound exhibited a better activity on TPβ compared with TPα, except with compound **48a** which possessed activities in the same concentration range. These data also confirmed the fact that the side chain of the molecules could play a role in selectivity. For example, activities on TPβ of compounds **48b-e** were within the same range than compound **48a** characterized by a *t*-butyl side chain. However, their activities on TPα were almost 10-fold less pronounced than that of **48a**. Additionally, it

should also be noted that the trend of potency of **48b-e** for TPα did not follow the pattern of the size or steric hindrance of the side chain. Indeed, TPα potency was ranked **48b** > **48c** ≅ **48e** > **48d**, indicating that *n*-pentyl > *n*-butyl ≅ *n*-hexyl > cyclohexyl.

We could not conclude from these results that the selectivity ratio was directly influenced by the length of the side chain. Nevertheless, it is notable that in this assay the *n*-pentyl side chain was responsible for the best activity in this series compared to other linear side chains.

III.3.1.3.B. Antiplatelet activity

Finally, since TP receptor directly mediates human platelet aggregation, we evaluated the effect of most interesting compounds in both previous tests in an *ex vivo* human platelet aggregation model. Results obtained with chosen compounds are presented in Table III-11. Principle of the test was identical to the one for platelet aggregation studies depicted at point III.1.1.1, p. 87.

Compound	Inhibition of platelet aggregation induced by 1 μM U46619 IC_{50} (μM)1
BM573	0.240 ± 0.013
BM613	0.278 ± 0.186
48a	0.300 ± 0.003
48b	0.670 ± 0.200
49a	0.800 ± 0.090
51	0.900 ± 0.006
47q	0.090 ± 0.007

[1] Results expressed as mean ± standard deviation of at least three determinations (n≥3).

Table III-11. Estimated IC_{50} values for the inhibition of platelet aggregation induced by U46619 (1μM).

Discussion

We measured the aggregation of platelets stimulated by U46619, a stable TXA_2 agonist/mimetic. Compounds **48a**, **48b**, **49a**, **51** and **47q** were selected because of their activities, affinities or apparent selectivity in different models. The results obtained are summarized in Table III-11. All compounds evaluated confirmed their activities as TP receptor antagonists. Indeed, each compound was able to inhibit platelet aggregation induced by U46619 at sub-micromolar concentrations. It should be highlighted that all the compounds evaluated in the three pharmacological models exhibited very good results for their affinities, activities as inhibitors of $[Ca^{2+}]_i$ and inhibition of platelet aggregation induced by U46619. Collective results highlighted compound **47q** as a potential lead of major interest since its IC_{50} for inhibition of platelet aggregation was 0.09 μM. Finally, it is noteworthy that compound **47q** is one of the most potent compounds on TPα and was also the most

potent inhibitor in platelet aggregation studies, consistent with previous findings that TPα is the dominant isoform expressed in human platelets, although it is equally active on TPβ (Table III-10).

Additionally, compounds of the **48** series and thus compounds with an oxygen atom as the intercyclic bridge, especially **48b**, proved to be of high interest in selectivity and could be regarded as a lead compound for further pharmacomodulation studies on differences of antagonistic activity between TPα and TPβ isoforms.

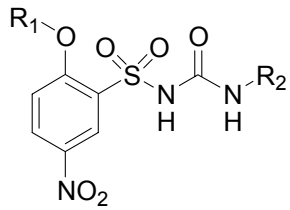
III. 3. 2. Exemplification of "O bridge" series

III.3.2.1. Design and background

For the second pharmacomodulation step, we wished to address the structure-activity relationships based on a functional assay. Indeed, it is more helpful to gather information on functional response to design useful selective compounds, so we focused on inhibition of $[Ca^{2+}]_i$ mobilization.

All the compounds tested were designed starting from **48** series, which combined interesting properties, both for selectivity and antagonist potency. Additionally, besides its interesting pharmacological properties, the presence of the oxygen bond was chemically interesting. Since phenols are more reactive in nucleophilic substitution (see III.2.4.3, p.116), we sought that several modification of R_1 would be obtained with more ease.

Several features of the structure were explored. First, we addressed the influence of the alkyl side chain R_2 with R_1 being substituted by methyl groups at 2- and 3-positions. We also completed the series of side chains with compounds characterized by a 4-methylphenyl moiety as R_1. Compounds prepared for these evaluations are presented in Table III-12.



Compounds	R_1	R_2
48h	2-methylphenyl	*i*-propyl
48i	2-methylphenyl	*n*-butyl
48j	2-methylphenyl	*t*-butyl
48k	3-methylphenyl	*i*-propyl
48l	3-methylphenyl	*n*-butyl
48m	3-methylphenyl	*t*-butyl
48n	4-methylphenyl	*i*-propyl
48o	4-methylphenyl	*n*-propyl

Table III-12. Compounds synthesized for evaluation of influence of side chain R_2.

Furthermore, we designed compounds to address the specific role of the methyl group, both in selectivity and activity. Former results showed that, in the **48** or **47** series, combination of a methyl or bromo substitutent in the 4-position of the second ring with a *n*-pentyl side chain favoured the TPβ affinity and activity (**48b** and **47o** for example) while

some compounds with no substituents in these positions showed a high affinity and activity for TPα (**47f, 47q**).

Consequently, in the **48** series, we designed various compounds in order to compare the methyl group with several other substituents. To this end, we designed and synthesized compounds **48p-48ae** (Table III-13), which are characterized by an halogen atom instead of a methyl group. Thus, monosubstituted chloro, bromo and iodo derivatives in 2- (only chloro and bromo), 3- and 4-positions derivatives were synthesized (Table III-13).

For each compound, two alkyl side chains R_2 were envisaged: *t*-butyl and *n*-pentyl, in order to have information on the influence of a long linear or short globular chain on these new compounds.

Cpd	R_1	R_2	Cpnd	R_1	R_2
48p	2-bromophenyl	*t*-butyl	**48x**	3-chlorophenyl	*t*-butyl
48q	2-bromophenyl	*n*-pentyl	**48y**	3-chlorophenyl	*n*-pentyl
48r	3-bromophenyl	*t*-butyl	**48z**	4-chlorophenyl	*t*-butyl
48s	3-bromophenyl	*n*-pentyl	**48aa**	4-chlorophenyl	*n*-pentyl
48t	4-bromophenyl	*t*-butyl	**48ab**	3-iodophenyl	*t*-butyl
48u	4-bromophenyl	*n*-pentyl	**48ac**	3-iodophenyl	*n*-pentyl
48v	2-chlorophenyl	*t*-butyl	**48ad**	4-iodophenyl	*t*-butyl
48w	2-chlorophenyl	*n*-pentyl	**48ae**	4-iodophenyl	*n*-pentyl

Table III-13. Compounds prepared for evaluation of the influence of the nature of the R_1 substituent. R_1 and R_2 refer to general formula presented Table III-12.

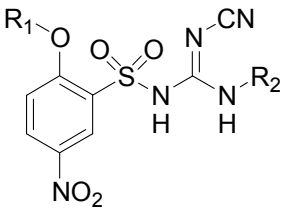
Additionally, we aimed to explore the influence of other substituents at the 4-position of the second aromatic ring R_1. Consequently, several 4 alkoxy derivatives were prepared (**48af-48ak**,

Table III-14). Additionally, some disubstituted compounds were synthesized. For example, two R_1 = 3-methyl-4-chlorophenyl compounds were synthesized since compound **47o** gave interesting results in the **47** series with a 3-methyl-4-bromo substitution pattern for R_1. As for previous series, the R_2 alkyl chain was either a *t*-butyl or *n*-pentyl moiety.

Compound	R_1	R_2
48af	4-methoxyphenyl	*t*-butyl
48ag	4-methoxyphenyl	*n*-pentyl
48ah	4-propoxyphenyl	*t*-butyl
48ai	4-propoxyphenyl	*n*-pentyl
48aj	4-ethoxyphenyl	*t*-butyl
48ak	4-ethoxyphenyl	*n*-pentyl
48al	3-methoxyphenyl	*t*-butyl
48am	3-methyl-4-chlorophenyl	*t*-butyl
48an	3-methyl-4-chlorophenyl	*n*-pentyl
48ao	2-methoxy-4-methylphenyl	*t*-butyl

Table III-14. Compounds prepared for evaluation of the influence of various substituents at the 4-position of the R_1 aromatic ring as well as disubstitution in the 48 series. R_1 and R_2 refer to general formula presented at Table III-12.

Finally, since cyanoguanidines proved to be quite active on both isoforms in the **47** family, we synthesized an aryloxy-substituted sulfonylcyanoguanidine, **54 c** (Table III-15).



Compound	R_1	R_2	X
54c	4-methylphenyl	*t*-butyl	N-CN

Table III-15. Compound prepared for evaluation of the influence of the sulfonyurea function.

III.3.2.2. Pharmacological evaluation

Two pharmacological tests were performed on these "second generation compounds" : inhibition of $[Ca^{2+}]_i$ mobilization and inhibition of platelet aggregation.

III.3.2.2.A. Measurement of intracellular calcium mobilization

Principle

Consistent with what we stated at point III.3.2.1, p. 150, we evaluated all compounds designed and synthesized in a functional inhibition of $[Ca^{2+}]_i$ mobilization assay. The procedure was identical to the one described in previous experiment (see III.3.1.3.A., p. 138).

Results, structure-activity relationships and discussion

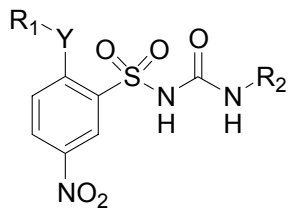
Results obtained are reported in Tables III-16 to III-19. For more readability and for an easier discussion of the structure-activity relationships, we have split the results in four tables, grouping the

compounds within their respective structural families. Results obtained with BM573 have been included to all tables as reference.

Influence of the position of the R_1 methyl group and of the R_2 side chain

We present in this section the first series of compounds that were prepared to address the influence of the R_2 alkyl chain and the position of the methyl substituent on the R_1 phenyl ring.

Tables III-16 to III-18 refer to the general formula :



cpds	R_1	R_2	Y	U46619-mediated $[Ca^{2+}]_i$ mobilization (nM)[1]		Ratio[2]
				TPα	TPβ	
BM573	4-methylphenyl	*t*-butyl	NH	319 ± 203	53 ± 19	6.0
48h	2-methylphenyl	*i*-propyl	O	8750 ± 446	501 ± 63	17.5
48i	2-methylphenyl	*n*-butyl	O	673 ± 339	42 ± 20	16.1
48j	2-methylphenyl	*t*-butyl	O	3760 ± 637	233 ± 88	16.1
48g	2-methylphenyl	*n*-pentyl	O	1630 ± 924	181 ± 101	9.0
48k	3-methylphenyl	*i*-propyl	O	2640 ± 492	205 ± 132	12.9
48l	3-methylphenyl	*n*-butyl	O	1910 ± 689	155 ± 30	12.3
48m	3-methylphenyl	*t*-butyl	O	398 ± 145	17 ± 8	23.2
48f	3-methylphenyl	*n*-pentyl	O	2530 ± 419	223 ± 68	11.3
48n	4-methylphenyl	*i*-propyl	O	8760 ± 3780	843 ± 124	10.4
48a	4-methylphenyl	*t*-butyl	O	58 ± 43	58 ± 4	1.0

48b	4-methylphenyl	*n*-pentyl	O	139 ± 88	55 ± 5	2.5
48c	4-methylphenyl	*n*-hexyl	O	526 ± 30	58 ± 1	9.0
48d	4-methylphenyl	cyclohexyl	O	601 ± 11	70 ± 6	8.6
48e	4-methylphenyl	*n*-butyl	O	558 ± 34	53 ± 2	10.5
48o	4-methylphenyl	*n*-propyl	O	4220 ± 2060	448 ± 328	9.4

[1] Results are expressed as mean ± standard deviation of at least three determinations (n≥3).

[2] $IC_{50}TP\alpha/IC_{50}TP\beta$.

Table III-16. Estimated IC_{50} values for the inhibition of $[Ca^{2+}]_i$ mobilization mediated by either TPα or TPβ upon stimulation by U46619 (1µM). First series : influence of the position of the methyl substituent and of the side chain.

All compounds tested within this assay displayed potent micro and submicromolar IC_{50} activity. Consistent with what we already observed in previous $[Ca^{2+}]_i$ mobilization experiments, all compounds tested were more potent on TPβ than on TPα isoform. BM573 has been included to Table III-16 as the reference compound. Results obtained with previously prepared compounds **48g** and **48f** (R_1 = 2- and 3-methyphenyl, respectively) have been included for direct comparison with newly synthesized compounds. All compounds bearing a 4-methylphenyl moiety as R_1 have also been included to Table III-16.

We first varied the position of the methyl group of the phenyl ring R_1. For each position, we synthesized and evaluated various derivatives with different alkyl chains R_2. In the 2-methylphenyl series (cpds **48h-g**), the *n*-butyl chain of analog **48i** was the best substituent and produced a 3 to10-fold increase in activity compared to other compounds of the series (**48h** with R_2 = *i*-propyl being the less active). Regarding the difference in activity between the two isoforms, all compounds were characterized by a ratio of ~16-17 in this "2" series except for analog **48g** with R_2 = *n*-pentyl which had a ratio of 9.

Meta substitution of the phenyl ring R_1 (3-methylphenyl series) seemed to produce little influence on activity, except for compound **48m** characterized by a ~2 (compared to **48i**) to 20-fold (**48h**) increase in activity on both isoforms. More interestingly, compound **48m** estimated selectivity ratio was the highest in this table (23.2) whereas other compounds of the series displayed a ~10 fold ratio. Finally, a methyl in 4-position of the phenyl ring generally produced the most potent compounds on TPα, although they were less selective. The two original R_2 alkyl chains evaluated in this series, *n*-propyl (**48o**) and *i*-propyl (**48n**) proved to induce an important (~10 fold) decrease in activity on both isoforms.

Taken as a whole, the results of Table III-16 highlight the following general trend for the most interesting R_2 substituents responsible for a marked activity on both isoforms :

n-butyl ≅ *t*-butyl > *n*-pentyl ≥ *n*-propyl ≅ *i*-propyl

For activity on both isoforms, the best substitution positions were found to be 4 > 3 ≥ 2.

Regarding the TPβ versus TPα selectivity, the best alkyl side chain was R_2 = *n*-butyl or *t*-butyl and the following trend was observed for these two side chains regarding the phenyl ring substitution : 3 ≥ 2 >> 4.

Influence of the nature and the position of halo-substituents on the R_1 phenyl ring

This series was designed to study the activity of halophenyl analogs and results obtained are presented in Table III-17.

cpds	R_1	R_2	Y	U46619-mediated $[Ca^{2+}]_i$ mobilization (nM)[1]		Ratio[2]
				TPα	TPβ	
BM573	4-methylphenyl	*t*-butyl	NH	319 ± 203	53 ± 19	6.0
48p	2-bromophenyl	*t*-butyl	O	2580 ± 1120	151 ± 73	17.0
48q	2-bromophenyl	*n*-pentyl	O	3130 ± 827	423 ± 164	7.4
48r	3-bromophenyl	*t*-butyl	O	683 ± 189	59 ± 30	11.6
48s	3-bromophenyl	*n*-pentyl	O	5860 ± 2420	413 ± 303	14.2
48t	4-bromophenyl	*t*-butyl	O	1660 ± 1290	523 ± 145	3.2
48u	4-bromophenyl	*n*-pentyl	O	1010 ± 257	120 ± 63	8.4
48v	2-chlorophenyl	*t*-butyl	O	2420 ± 1780	205 ± 156	11.8
48w	2-chlorophenyl	*n*-pentyl	O	3060 ± 775	387 ± 178	7.9
48x	3-chlorophenyl	*t*-butyl	O	698 ± 226	73 ± 9	9.6
48y	3-chlorophenyl	*n*-pentyl	O	2390 ± 526	201 ± 42	11.9
48z	4-chlorophenyl	*t*-butyl	O	3050 ± 1670	400 ± 277	7.6
48aa	4-chlorophenyl	*n*-pentyl	O	871 ± 362	98 ± 45	8.9
48ab	3-iodophenyl	*t*-butyl	O	470 ± 153	95 ± 35	4.9
48ac	3-iodophenyl	*n*-pentyl	O	4520 ± 1800	885 ± 389	5.1
48ad	4-iodophenyl	*t*-butyl	O	2150 ± 307	401 ± 212	5.4
48ae	4-iodophenyl	*n*-pentyl	O	2190 ± 1180	302 ± 153	7.2

[1] Results are expressed as mean ± standard deviation of at least three determinations (n≥3).

[2] $IC_{50}TP\alpha/IC_{50}TP\beta$.

Table III-17. Estimated IC_{50} values for the inhibition of $[Ca^{2+}]_i$ mobilization mediated by either TPα or TPβ upon stimulation by U46619 (1µM). Second series : influence of the nature and the position of halo-substituents on the R_1 phenyl ring.

Three halogens substituents were considered : the bromine, the chlorine and the iodine atoms. First, we varied the position of the bromine atom. Table III-17 shows that the replacement of the methyl group by a bromine atom had little or no positive effect, both in terms of potency and selectivity. It is interesting to note that compound 3-bromo-

substituted **48r** had a ~2 fold decrease in selectivity ratio compared with its potent 3-methyl-substituted analog **48m** (11.6 and 23.2, respectively). Consistent with our previous observation of the activity trend, **48r** was the most potent of its series, with a substituent in the 3-position and a *t*-butyl R_2 alkyl chain. Compounds of the 4-bromophenyl series were less potent than their 4-methylphenyl counterparts, but kept the same rank order for differences between the two isoforms.

In the "chlorophenyl" series, the same trends were observed, with little differences between this series and the "bromophenyl series. Again, substitution in the 3-position combined with a *t*-butyl R_2 side chain produced one of the most potent compound, **48x**, both on TPα and TPβ.

Interestingly, in the "iodophenyl" series, although compounds potencies were identical or somewhat decreased, selectivity ratio was diminished. To this extent, compound **48ab** was the most interesting since its 3-position substitution along with a *t*-butyl side chain R_2 increased its TPα potency while decreasing its TPβ activity. Thus, **48ab** was the most potent TPα halosubstituted derivative antagonist in this table with potency close to the one of **48m** (TPα IC_{50} : 470 ± 153 and 398 ± 145, respectively).

From this second table (Table III-17), we can deduce at this point that the best combination for our derivatives is a 3-position substituent on the R_1 phenyl ring and a *t*-butyl R_2 side chain. Following trends have been observed within these series :

TPα activity: $CH_3 \cong I > Cl \cong Br$

TPβ activity: $CH_3 > Br \cong Cl > I$

Selectivity ratio: $CH_3 > Br \cong Cl > I$

Finally, it interesting to point out that within the bromo and chloro derivatives, the combination of a 4-substituted phenyl moiety with a *n*-pentyl side chain provided the most active compounds whereas, surprisingly, in the 4-iodo series, the compounds displayed similar potency. In the 3-halo-substituted series (bromo, chloro and iodo), *t*-butyl side chain produced a ~10 fold increase in activity compared to compounds with a *n*-pentyl side chain (Table III-17).

Influence of other substituents on the R_1 phenyl ring

These compounds were prepared to address the influence of replacement of the methyl group by other chains, i.e. alkoxy moieties. Results are depicted in Table III-18.

cpds	R_1	R_2	Y	U46619-mediated $[Ca^{2+}]_i$ mobilization (nM)[1]		Ratio[2]
				TPα	TPβ	
BM 573	4-methylphenyl	*t*-butyl	NH	319 ± 203	53 ± 19	6.0
48af	4-methoxyphenyl	*t*-butyl	O	1940 ± 343	152 ± 76	12.7
48ag	4-methoxyphenyl	*n*-pentyl	O	2670 ± 1970	446 ± 416	6.0
48ah	4-propoxyphenyl	*t*-butyl	O	10760 ± 5150	875 ± 238	12.3
48ai	4-propoxyphenyl	*n*-pentyl	O	27230 ± 2650	4840 ± 2520	5.6
48aj	4-ethoxyphenyl	*t*-butyl	O	19190 ± 11410	1100 ± 401	17.5
48ak	4-ethoxyphenyl	*n*-pentyl	O	13560 ± 3440	749 ± 59	18.1
48al	3-methoxyphenyl	*t*-butyl	O	1970 ± 622	99 ± 61	19.9
48am	3-methyl-4-chlorophenyl	*t*-butyl	O	1060 ± 207	111 ± 54	9.6
48an	3-methyl-4-chlorophenyl	*n*-pentyl	O	3720 ± 769	565 ± 200	6.6
48ao	2-methoxy-4-methylphenyl	*t*-butyl	O	528 ± 133	52 ± 10	10.2

[1] Results are expressed as mean ± standard deviation of at least three determinations (n≥3).

[2] $IC_{50}TP\alpha/IC_{50}TP\beta$.

Table III-18. Estimated IC_{50} values for the inhibition of [Ca2+]i mobilization mediated by either TPα or TPβ upon stimulation by U46619 (1µM). Third series : influence of other substituents at position 4 of the R_1 aromatic ring and polysubstitution of R_1.

We first varied the 4-position of the phenyl ring and observed that there was systematically a loss in activity on both isoforms compared with their methyl group counterparts (**48a** and **48b**, see Table III-16). For both side chains (R_2 = *t*-butyl or *n*-pentyl), the loss of activity depended on the length of the alkoxy group. Interestingly, for thosee derivatives, the activity on TPβ decreased less markedly than activity on TPα, thus increasing the selectivity ratio (4-ethoxyphenyl, **48aj** and **48ak** > 4-methoxyphenyl, **48af** ≅ 4-propoxyphenyl, **48ah**, Table III-18).

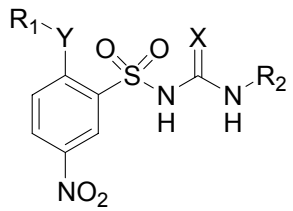
The most interesting compound of this Table III-18 (**48al**) was obtained with the combination of a methoxy group in the 3-position of the R_1 phenyl ring and a R_2 *t*-butyl side chain. Fully consistent with our previous observation, this compound displayed a potent antagonism of TPβ mediated $[Ca^{2+}]_i$ mobilization combined with a good selectivity ratio (19.9). The particular interest of this compound is that, although it is slightly less potent than its methylated or halogenated analogs (**48m**, **48r**, **48x** or **48ab**) on TPβ (IC_{50} = ~99 nM), it is characterized by a much lower activity on TPα (IC_{50} = ~2 μM).

Disubstituted compounds **48am** and **48an** proved to have the combined profile of monosubstituted 4-chlorophenyl and 3-methylphenyl analogs (**48z-48aa** and **48m**-**48f**, respectively). Thus, the presence of a methyl group at the 3-position of compound **48z** increased ~3-4 fold its activity, without reaching the potent activity of the 3-methylphenyl derivative **48m** (Table III-18).

The presence of a 2-methoxy group concomitantly with a 4 methyl group on the phenyl ring had little influence on TPβ activity but decreased ~10 fold the TPα activity compared to **48a**..

Influence of the sulfonylurea function in the "O bridge" series

The replacement of the sulfonylurea function in the oxygen bridge family of **54c** had a negative impact on the activity and generated a lower selectivity ratio (Table III-19).



cpds	R_1	R_2	Y	X	U46619-mediated $[Ca^{2+}]_i$ mobilization (nM)[1]		Ratio[2]
					TPα	TPβ	
BM573	4-methylphenyl	*t*-butyl	NH	O	319 ± 203	53 ± 19	6.0
54c	4-methylphenyl	*t*-butyl	O	N-CN	2080 ± 179	579 ± 53	3.6

[1] Results are expressed as mean ± standard deviation of at least three determinations (n≥3).

[2] $IC_{50}TP\alpha/IC_{50}TP\beta$.

Table III-19. Estimated IC_{50} values for the inhibition of $[Ca^{2+}]_i$ mobilization mediated by either TPα or TPβ upon stimulation by U46619 (1 µM). Fourth series : impact of the sulfonylurea function.

III.3.2.2.B. Antiplatelet activity

Finally, within this "oxygen bridge" family, we wished to confirm the antiaggregatory activity of the most interesting compounds. The procedure that we followed was similar to previous antiplatelet experiments (see III.1.1.1, p. 87). Compounds were selected with regard to their activity on TPα, TPβ or their selectivity ratio.

Results and discussion

48m was the compound with the optimal activity and selectivity and was chosen for evaluation on platelets (Figure III-33).

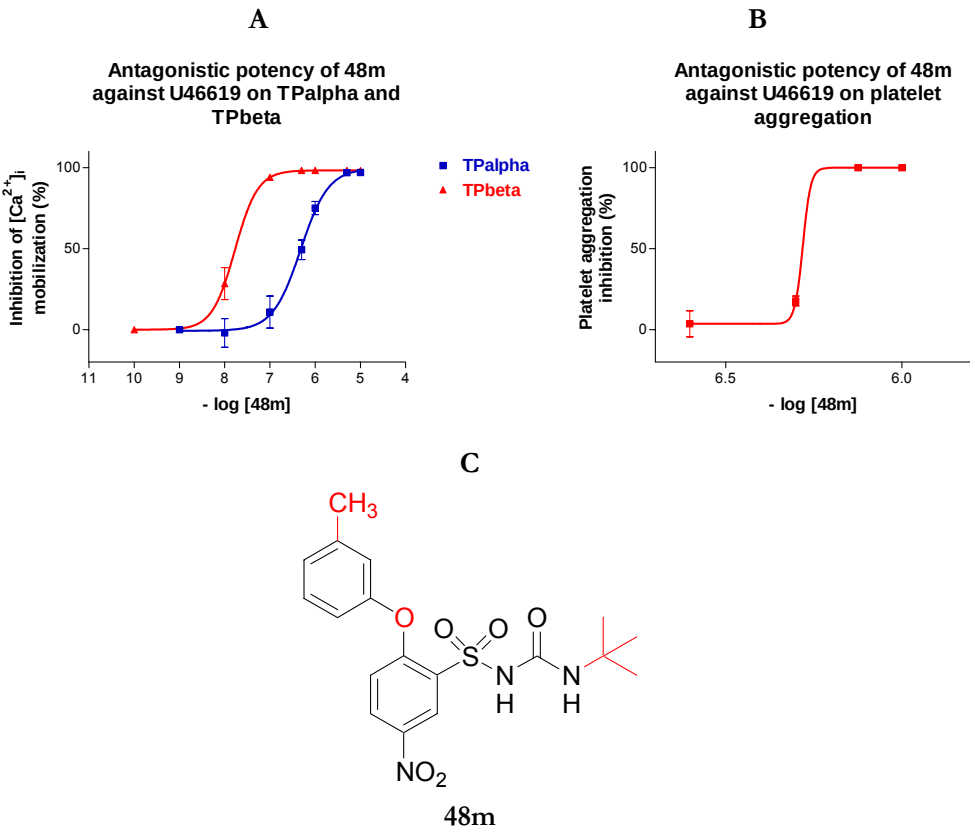


Figure III-33. A) concentration-response curves for inhibition of TP-induced $[Ca^{2+}]_i$ mobilization on separate isoforms. B) concentration-response curve for inhibition of platelet aggregation. C) structure of 48m.

Results on platelets with **48m** were consistent with previous observations. Inhibition of U46619 induced platelet aggregation (IC_{50}), was determined and found to be similar to the one for the inhibition of $[Ca^{2+}]_i$ mobilization triggered by TPα (513 ± 28 nM and 398 ± 145 nM, respectively). These results are conform to the hypothesis postulating that platelet aggregation is solely mediated by TPα isoform (see introduction

I.3.4.1, p. 24 and I.3.6.1.A., p. 36). Concentration-response curve obtained with this compound on platelets overlays with the TPα curve (Figure III-33, panel A and B). The interesting point is that **48m** is inactive or almost inactive on TPα (expressed in human platelets and in HEK293 cell lines) when used at 0.1μM, although is has complete activity on TPβ (expressed in HEK293 cell lines, Figure III-33, panel A). Thus, this compound is theoretically selective for TPβ at that concentration.

We have conducted similar experiments on platelets with other potentially selective compounds. Thus, **48ak**, displayed a similar profile with comparable IC_{50}s for inhibition of U46619 induced platelet aggregation and $[Ca^{2+}]_i$ mobilization. Nevertheless, the curves of this compound strongly shifted to the right (Figure III-34, panel A) suggesting it was less active on platelets (Figure III-34, panel B). Consequently, with a selectivity ratio within the same rank order, it could be more interesting since it is almost inactive on platelets below 10 μM while keeping a good activity on TPβ isoform expressed in HEK293 cell lines.

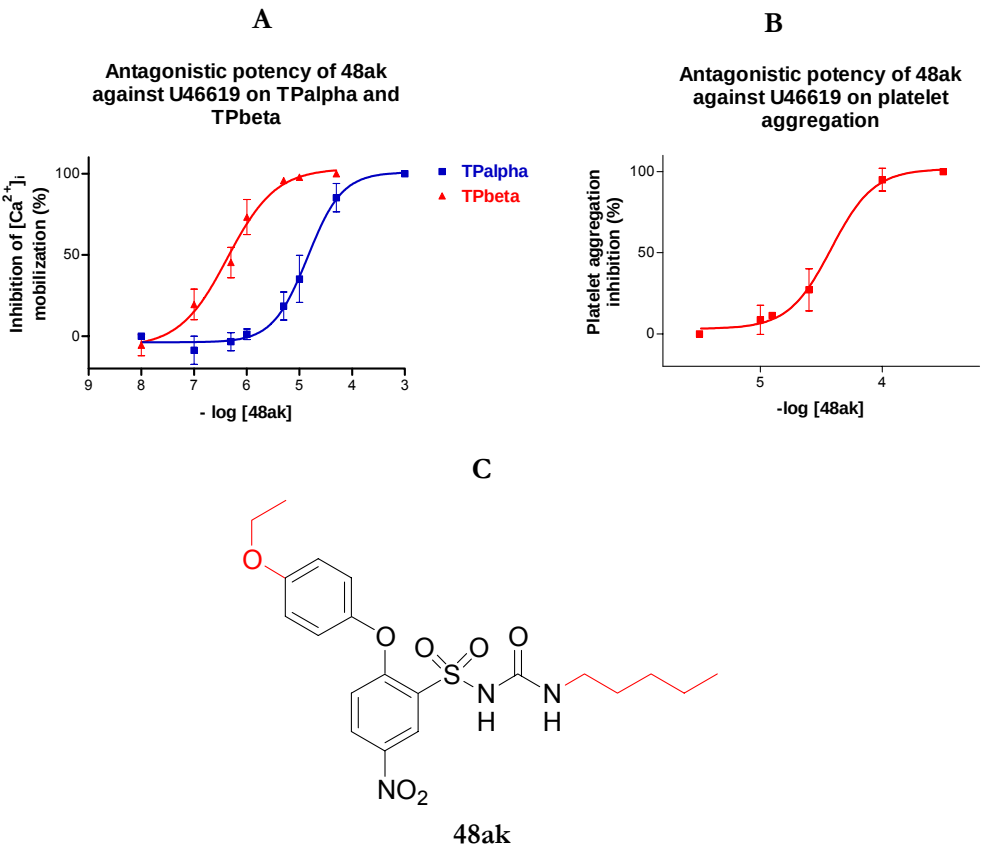


Figure III-34. A) concentration-response curves for inhibition of TP-induced $[Ca^{2+}]_i$ mobilization on separate isoforms. B) Antagonistic potency of 48ak on platelet aggregation at various concentrations. C) structure of 48ak.

To this end, compound **48al** was the most interesting, since it displayed the best activity on TPβ, while being poorly active on platelets (IC_{50}>1 μM, Figure III-35).

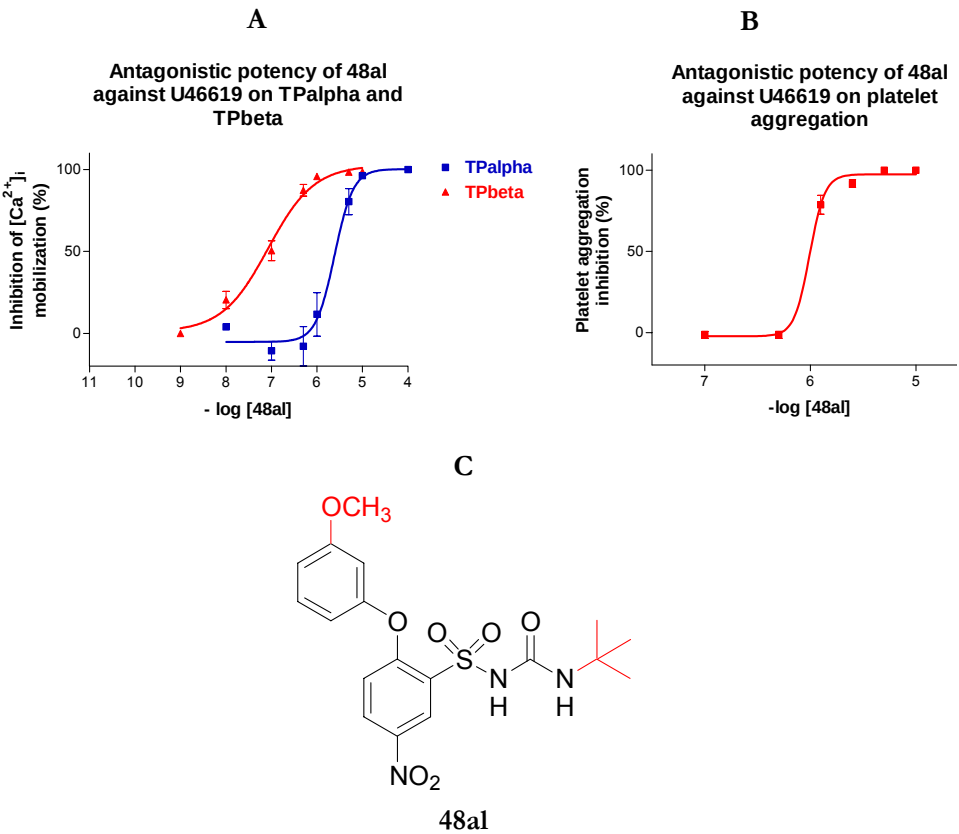


Figure III-35. A) concentration-response curves for inhibition of TP-induced $[Ca^{2+}]_i$ mobilization on separate isoforms. B) concentration response curve for inhibition of platelet aggregation. C) structure of 48al.

IV. Conclusions and perspectives

IV.1. Conclusions

IV. 1. 1. Pharmacological evaluation of BM573 and BM613

BM573 and BM613 are two nitrobenzenesulfonylureas that were identified as potential TP receptor antagonists in human platelet binding studies (Dogné 2000). In the present work, we have extended the pharmacological evaluation of these two compounds with several *in vitro* and *in vivo* experiments.

TXA_2 is considered as one of the most potent inducer of platelet aggregation (see introduction, I.3.6.1.A., p. 36). Thus, compounds antagonizing its action are known to behave like strong antiplatelet agents, whatever the inducer of platelet aggregation. Both BM573 and BM613 confirmed their antiplatelet potency in a platelet aggregation study. When they were incubated with a platelet suspension, these two compounds strongly inhibited the platelet aggregation phenomenon induced by arachidonic acid, the physiologic precursor of TXA_2, U46619, a synthetic TXA_2 analog and ADP, which triggers TXA_2 secretion by platelets. Consequently, these two compounds can be considered as potent *in vitro* antiplatelet agents.

We further demonstrated that BM613 was also an inhibitor of TXS in platelets. BM573 was proved to have the same property in previous study by Rolin *et al.* (Rolin, Dogné *et al.* 2001). Although BM613 was slightly less potent than BM573 in this test, we can conclude that BM613 is a combined TXA_2 antagonist and TXS inhibitor. This is of particular interest since it is thought that the combination of these

properties is optimal for counteracting the TXA_2 actions in a clinical setting.

Beside its pro-aggregating properties, TXA_2 is also a potent vaso- and broncho-constrictor. BM573 has already shown to be able to inhibit U46619-induced contraction of rat aorta and guinea-pig trachea smooth muscles (Rolin, Dogné *et al.* 2001). We performed the same experiment with BM613 and demonstrated its antagonistic potency on these tissues, although it was less potent than BM573.

In the last *in vitro* experiment of this section, we demonstrated that both BM613 and BM573 were characterized by potent affinities for both isolated TPα and TPβ, transiently expressed in COS-7 cells lines. Therefore, we can conclude from this experiment that, although these compounds have strong affinities for both TP receptors, they have no apparent binding selectivity toward the two isoforms.

Finally, both compounds were demonstrated to be antithrombotic agents *in vivo* since they prevented thrombus formation in a rat ferric chloride-induced thrombosis model.

Collectively, our results established that BM613 and BM573 could be considered as potent combined TXRA/TXSI. Moreover, they proved their preclinical interest as therapeutic agents in pathologic conditions such as thrombotic disorders (thrombosis, myocardial infarction, stroke,...), pulmonary embolism or asthma. Additionally, due to their properties against the TP receptors, their structure could serve as template for the design of original TP receptor antagonists and/or TXSI.

IV. 1. 2. Design, synthesis and pharmacological evaluation of original compounds

In this section, we aimed to study influence of structural modification of the parent compounds (BM573 & BM613) on TP receptors activity, with an emphasis on compounds activities toward TPα and TPβ isoforms. Within this project, 70 original compounds of two subsequent generations have been synthesized and evaluated as TPα and TPβ receptor antagonists and antiplatelet agents. Various synthetic routes were used and several parts of the original pharmacophore, proposed by Dogné *et al.* (Dogné 2000), were explored on a rational drug design basis. For the completion of this project, we have developed functional fluorimetric experiment to measure $[Ca^{2+}]_i$ mobilization. This experiment was designed on multiwells microplates to suit the evaluation of a large library of compounds.

First pharmacomodulation

Results collected on 35 "first generation" nitro-substituted benzenesulfonamide derivatives allowed to identify groups influencing antagonistic potency on separate TP receptor isoforms. Hence, we have studied the affinity and activity of these compounds on both TPα and TPβ. All compounds evaluated exhibited very high affinity for both TP receptors, mainly acting in the nanomolar range. Moreover, both receptor ligand binding and inhibition of $[Ca^{2+}]_i$ signalling concentration-response curves were determined with the most interesting compounds. In these

assays, all compounds confirmed their affinity and activity for the TP receptors.

According to the biological data we collected, we proposed some structural factors involved in affinity and activity on either TPα or TPβ in our series. This preliminary study identified templates which could lead to the development of selective TP receptor antagonists. The most promising compounds were evaluated on platelet aggregation and confirmed their TP receptor antagonism and their interest as anti-platelet agents.

In this study, one compound **47q** (JH48) was characterized by the most interesting profile displaying strong affinity coupled with potent antagonistic properties. So far, it is the most potent compound of the family of nitrobenzenesulfonylureas and sulfonylcyanoguanidines as antiplatelet agent. Consequently, the association of a 2, 6 dimethylphenyl substitution pattern for the second ring coupled with a NH bond between the rings and a t-butyl side chain gave the optimal structure for the antiplatelet activity so far. This structure should be retained as template for further generation of compounds designed as antiplatelet agents.

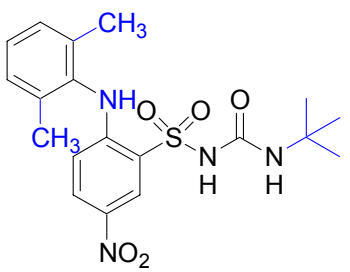


Figure IV-1. Chemical structure of compound 47q or JH48. The critical substituents for the activity on both isoforms are highlighted in blue.

Additionally, one particular family of compounds, namely the one characterized by an oxygen bridge between the two rings (family **48**), produced varied responses toward the two isoforms. The compounds of this family were selected for further exemplification.

Second pharmacomodulation

This second pharmacomodulation was devoted to the analysis of structure-activity relationships of compounds from the **48** family toward the two receptors isoforms. Thus, we presented the design, synthesis and SAR study of a series of 35 *N*-alkyl-*N*'-[2-(aryloxy)-5-nitrobenzenesulfonyl]-ureas and -cyanoguanidine. These compounds were designed based on results observed in the **48** family evaluated in the previous pharmacomodulation (i.e. **48a-48g**). The results collected in the functional inhibition of $[Ca^{2+}]_i$ mobilization test performed on both TP receptor isoforms allowed to define the optimal nature and position of several structural moieties for both activity and selectivity in this series. The best results in terms of selectivity were obtained with a *t*-butyl side chain coupled with a methyl or methoxy in the 3-position of the phenyl ring or with an alkoxy (methoxy, propoxy or ethoxy) in the 4-position of the phenyl ring (Figure IV-2). An exception was noted with compound **48ak**, characterized by an *n*-pentyl side chain and a 4-ethoxy substituent on the phenyl ring.

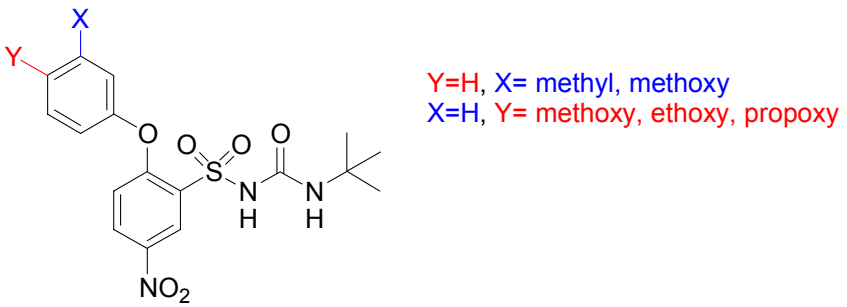


Figure IV-2. General structure of compounds characterized by the best selectivity ratios for TPβ activity in the 48 family.

From all the compounds evaluated, three were selected for their selectivity and activity and confirmed their TPα antagonistic potency in a human platelet aggregation test. We confirmed with this work the hypothesis that TPα is the predominant isoform expressed at the surface of platelets. The best compound for activity on TPβ in this series was compound **48m** (JH19) and its structure should serve as template for the design of compounds active on this isoform (Figure IV-3).

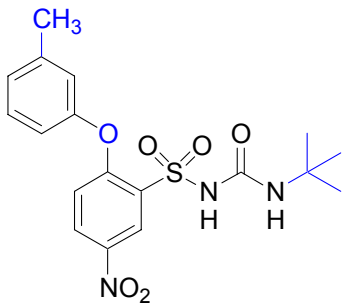


Figure IV-3. Chemical structure of compound 48m or JH19. The substitutents critical for the activity on TPβ are highlighted in blue.

In view of all results collected on all the compounds, it seems that the length of the molecule is a critical parameter, not only in terms of activity, but also for selectivity toward the two isoforms. In conclusion, it

is noteworthy that the present work represents the first extended SAR study addressing the selectivity among TP receptor isoforms.

IV.2. Perspectives

Several research perspectives can be envisaged following the results of this work. They can be subdivided in two parts: chemistry and pharmacology.

IV. 2. 1. Chemistry

The activity of the nitrobenzenesulfonylurea derivatives have been well defined by previous work as well as this one. Some compounds reached affinity and activity in the nanomolar range. Although chemical modifications could still be explored for the activity toward TP receptors, it seems now more interesting to focus on the influence on selectivity between the two isoforms.

Consequently, several modifications of the structure could be envisaged. Since slight variations of the pharmacophore have been extensively studied, further modifications should be more drastic.

Phenyl ring R_1 could be substituted by other aromatic or non-aromatic rings (heterocycle such as furane, thiophene, imidazole, pyridine, or a carbocycle such as cyclopentadiene). If we look more tightly at some selective compounds such as **48ak** or **48al**, a possible modification could be to replace the monocyclic benzene ring R_1 by a bicyclic ring such as indole, quinoline or naphatalene structure (Figure IV-4).

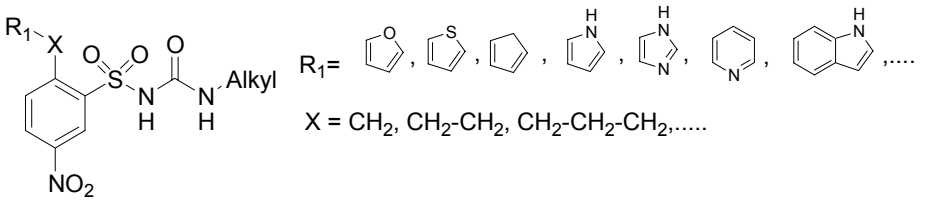


Figure IV-4. Possible modifications of the R_1 phenyl ring and the intercyclic bridge X.

Methylene, ethylene, propylene,… could replace the heteroatomic intercyclic bridge (Figure IV-4) or a direct coupling between the two rings could also be envisaged. A classic modification could be to decrease the flexibility of the structure, for example with tricyclic molecules (Figure IV-5).

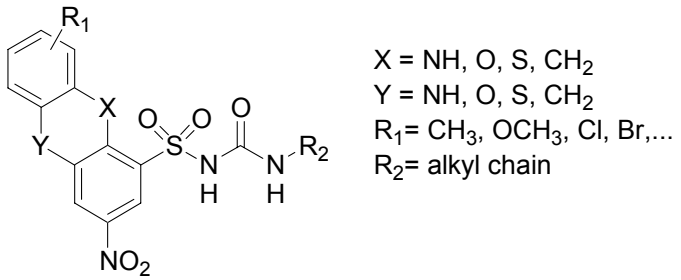


Figure IV-5. Possible tricyclic structure that would reduce flexibility of the molecule.

The modulation of the nitro group or the replacement of the central ring by an aromatic or non-aromatic central ring could be studied in terms of selectivity towards the two isoforms.

Finally, the sulfonylurea function could be changed for another acidic group, like the sulfonylcarbamate function, or could be included in an heterocycle.

Of course, since the compounds discussed in the present work were almost all more active on TPβ, optimal modifications would increase activity on TPα while decreasing activity on TPβ.

Beside the interest of our compounds for the study of the SARs, their properties could also be exploited as pharmacological tools. For example, direct binding parameters could be explored with tritiated compounds and functional parameters of the activation-deactivation of the receptors as well as localisation of receptors in tissues could be monitored with fluorescent derivatives.

IV. 2. 2. Pharmacology

Several pharmacological studies with the compounds presented in this work and their derivatives could be envisaged.

The exact definition of the mechanisms underlying the antagonistic potency of the compounds could be determined. For example, it is possible to demonstrate if our compounds act as pure antagonists or inverse agonists, with the [^{35}S]GTP-γ-S binding method. This property could be determined for the two TP receptor isoforms.

It would be interesting to develop an experiment addressing the role of TPβ in physiological conditions, on isolated cells or tissues. Thus, like for TPα and platelets, the compounds tested could confirm the selectivity of our compounds in physiological settings. Currently, it is difficult since the two isoforms are only expressed in humans and because no tissues where TPβ is expressed alone have been identified to date.

Conclusions and perspectives

We have proved that nitrobenzenesulfonylureas were potent TXA_2 receptor antagonists and some of them TXS inhibitors. Consequently, beside their evaluation as antiplatelet agents, these compounds could be evaluated in other disease models, such as atherosclerosis, hypertension, asthma, myocardial infarction, pulmonary embolism, cancer…. Consistent with this point, it is noteworthy that BM573 was proved to be active in some of these pathologies in animal models, notably atherosclerosis (Cyrus, Yao *et al.* 2006), angiogenesis (de Leval, Dassesse *et al.* 2003), pulmonary embolism (Ghuysen, Lambermont *et al.* 2004), myocardial infarction (Rolin, Petein *et al.* 2003; Kolh, Rolin *et al.* 2006), sceptic schock (Lambermont, Kolh *et al.* 2004). Besides their interest as therapeutic agents in such diseases, these TP receptor antagonists could be used as pharmacological tools to identify more precisely the role of TXA_2 in the development of different pathologies.

V. Materials and methods

V.1. Synthesis

V. 1. 1. Materials

All commercial chemicals (Sigma-Aldrich, Belgium) and solvents were reagent grade and were used without further purification unless otherwise stated. All reactions were followed by thin-layer chromatography (Silicagel $60F_{254}$ Merck®) and visualization was accomplished with UV light (254 nm). Elemental analyses (C, H, N, S) were determined on a Carbo Erba EA 1108 and were within ± 0.4% of the theoretical values. NMR spectra were recorded either on a Bruker Avance 500 or on a Bruker DRX-400 spectrometer using DMSO-*d*6 as solvent and tetramethylsilane as internal standard. For ^{1}H NMR spectra, chemical shifts are expressed in δ (ppm) downfield from tetramethylsilane. The abbreviations d, doublet; t, triplet; m, multiplet; br, broad were used throughout. Infrared spectra were recorded using a Perkin-Elmer® FT-IR 1750. All compounds described were re-crystallized from hot methanol (40°C)/H_2O mixture (60/40; 10 ml/100 mg of product) unless otherwise stated.

V. 1. 2. General procedure for the reaction of 43 with amines (47)

Compound **43** (0.01 mol) and the appropriate amine (0.05 mol) were dissolved in 3-chlorotoluene (30 ml) and refluxed for 12-96 hr. When the reaction was finished (monitored by tlc), the mixture was evaporated under reduced pressure. The residue was dissolved in an

aqueous NaOH solution (0.5 N, 10 ml/g of residue). This mixture was extracted with diethylether and the aqueous layer was separated and adjusted to pH=1 with 0.5 M hydrochloric acid. The precipitate which appeared was collected by filtration, washed with water and dried (Yield : 35-95 %).

2-(Cyclohexylamino)-5-nitrobenzenesulfonamide (44a)

Mp : 166-167°C. IR (KBr) νmax : 3375, 3262, 3098, 2938, 2923, 2855, 1830, 1603, 1584, 1534, 1498, 1451, 1429, 1337, 1316, 1294, 1269, 1239, 1156, 1140, 1127, 1093, 1041, 980, 925, 914, 822, 751, 650, 621, 589, 515, 496 cm^{-1}. ^1H NMR (DMSO) δ: 1.19-1.94 (m, 10H, cyclohexyl); 3.64 (d, 1H, Ph-NH-CH-); 6.75 (bs, 1H, Ph-NH-CH); 7.00 (d, 1H, J = 9 Hz, H-3); 7.77 (s, 2H, SO_2-NH_2); 8.16 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.50 (d, 1H, J = 2.5 Hz, H-6).

2-(2,4,6-Trimethylphenylamino)-5-nitrobenzenesulfonamide (44b)

Mp : 101-103°C. IR (KBr) νmax : 3353, 3272, 3094, 2920, 2853, 2623, 1598, 1582, 1521, 1496, 1450, 1325, 1297, 1215, 1153, 1124, 1049, 909, 856, 828, 746, 705, 652, 604, 592, 505 cm^{-1}. ^1H NMR (DMSO) δ: 2.09 (s, 6H, CH_3-2', CH_3-6'); 2.30 (s, 3H, CH_3-4'); 6.21 (d, 1H, J = 9 Hz, H-3); 7.05 (s, 2H, H_{aro}'); 7.85 (s, 2H, SO_2-NH_2); 8.01 (s, 1H, Ph-NH-Ph); 8.11 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.57 (d, 1H, J = 2.5 Hz, H-6).

2-(2-Methylphenylamino)-5-nitrobenzenesulfonamide (44c)

Mp : 163-164°C. IR (KBr) νmax : 3379, 3335, 3276, 3106, 1611, 1600, 1587, 1534, 1489, 1449, 1423, 1348, 1299, 1249, 1232, 1187, 1146, 1133, 1111, 1061, 961, 911, 820, 791, 756, 744, 709, 630, 587, 504, 477 cm^{-1}. ^1H NMR (DMSO) δ: 2.18 (s, 3H, CH_3-2'); 6.70 (d, 1H, J = 9 Hz, H-3); 7.27-7.41 (m, 4H, H_{aro}'); 7.88 (s, 2H, SO_2-NH_2); 8.16 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.27 (s, 1H, Ph-NH-Ph); 8.58 (d, 1H, J = 2.5 Hz, H-6).

2-(3-Methylphenylamino)-5-nitrobenzenesulfonamide (44d)

Mp : 144-146 °C. IR (KBr) νmax : 3379, 3335, 3276, 3106, 1611, 1600, 1587, 1534, 1489, 1449, 1423, 1348, 1299, 1249, 1232, 1187, 1146, 1133, 1111, 1061, 961, 911, 820, 791, 756, 744, 709, 630, 587, 504, 477 cm^{-1}. ^1H NMR (DMSO) δ: 2.34 (s, 3H, CH_3-3'); 7.10-7.20 (m, 4H, H_{aro}', H-3); 7.36

(t, 1H, J=8Hz, H-5'); 7.90 (s, 2H, SO_2-NH_2); 8.19 (dd, 1H, J=9Hz, J=2.5Hz, H-4); 8.38 (s, 1H, Ph-NH-Ph); 8.58 (d, 1H, J=2.5Hz, H-6).

2-(3-Methyl-4-Bromophenylamino)-5-nitrobenzenesulfonamide (44e)

Mp : 189-191°C. IR (KBr) νmax : 3352, 3317, 3253, 3070, 1597, 1573, 1531, 1487, 1429, 1352, 1236, 1148, 1132, 1056, 1028, 975, 952, 923, 901, 873, 836, 823, 815, 779, 745, 714, 700, 662, 593, 563, 508, 492, 474 cm^{-1}. ^1H NMR (DMSO) δ: 2.37 (s, 3H, CH_3-3'); 7.13 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-6'); 7.22 (d, 1H, J = 9 Hz, H-3); 7.35 (d, 1H, J = 2.5 Hz, H-2'); 7.65 (d, 1H, J = 9 Hz, H-5'); 7.90 (s, 2H, SO_2-NH_2); 8.19 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.36 (s, 1H, Ph-NH-Ph); 8.58 (d, 1H, J = 2.5 Hz, H-6).

2-(2, 5-Dimethylphenylamino)-5-nitrobenzenesulfonamide (44f)

Mp : 186-188°C. IR (KBr) νmax : 3384, 3291, 3097, 3055, 2922, 2628, 1605, 1582, 1547, 1522, 1500, 1449, 1418, 1338, 1234, 1156, 1127, 1053, 931, 917, 818, 810, 745, 703, 659, 640, 591, 561, 494 cm^{-1}. ^1H NMR (DMSO) δ: 2.12 (s, 3H, CH_3-2'); 2.31 (s, 3H, CH_3-5'); 6.68 (d, 1H, J = 9 Hz, H-3); 7.09-7.29 (m, 3H, H_{aro}'); 7.87 (s, 2H, SO_2-NH_2); 8.16 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.23 (s, 1H, Ph-NH-Ph); 8.57 (d, 1H, J = 2.5 Hz, H-6).

2-(2, 4-Dimethylphenylamino)-5-nitrobenzenesulfonamide (44g)

Mp : 179-181°C. IR (KBr) νmax : 3348, 3260, 3109, 3008, 2914, 1620, 1594, 1578, 1541, 1503, 1463, 1420, 1325, 1229, 1215, 1157, 1142, 1128, 1049, 908, 889, 876, 844, 821, 805, 756, 739, 710, 659, 602, 558, 535, 527, 498, 475, 461 cm^{-1}. ^1H NMR (DMSO) δ: 2.12 (s, 3H, CH_3-2'); 2.33 (s, 3H, CH_3-4'); 6.61 (d, 1H, J = 9 Hz, H-3); 7.13-7.22 (m, 3H, H_{aro}'); 7.88 (s, 2H, SO_2-NH_2); 8.14 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.20 (s, 1H, Ph-NH-Ph); 8.57 (d, 1H, J = 2.5 Hz, H-6).

2-(2,6-Dimethylphenylamino)-5-nitrobenzenesulfonamide (44h)

Mp : 197-198°C. IR (KBr) νmax : 3359, 3324, 3235, ,084, 2975, 2921, 2652, 1603, 1578, 1523, 1490, 1471, 1343, 1323, 1236, 1204, 1155, 1124, 1050, 913, 864, 829, 778, 766, 747, 732, 695, 654, 592, 555, 500, 478 cm^{-1}. ^1H NMR (DMSO) δ: 2.14 (s, 6H, CH_3-2', CH_3-6'); 6.22 (d, 1H, *J* = 9 Hz, H-3); 7.25 (s, 3H, H_{aro}'); 7.86 (s, 2H, SO_2-NH_2); 8.08 (s, 1H, Ph-N*H*-Ph); 8.14 (dd, 1H, *J* = 9 Hz, *J* = 2.5 Hz, H-4); 8.58 (d, 1H, *J* = 2.5 Hz, H-6).

2-(4-Methylphenylamino)-5-nitrobenzenesulfonamide (44i)

Mp : 161-163 °C. IR (KBr) νmax : 3352, 3330, 3254, 3087, 3070, 2917, 1598, 1579, 1531, 1511, 1487, 1459, 1430, 1357, 1330, 1229, 1148, 1131, 1053, 1017, 976, 938, 923, 903, 825, 806, 768, 746, 714, 703, 667, 650, 603, 573, 538, 503, 474 cm^{-1}. ^1H NMR (DMSO) δ: 2.34 (s, 3H, CH_3-4'); 7.08 (d, 1H, J = 9 Hz, H-3); 7.21-7.30 (m, 4H, H_{aro}'); 7.88 (s, 2H, SO_2-NH_2); 8.16 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.35 (s, 1H, Ph-NH-Ph); 8.57 (d, 1H, J = 2.5 Hz, H-6).

2-(3,4-Dimethylphenylamino)-5-nitrobenzenesulfonamide (44j)

Mp : 156-158°C. IR (KBr) νmax : 3380, 3343, 3248, 3103, 2966, 2917, 2623, 1595, 1528, 1493, 1461, 1421, 1318, 1228, 1211, 1154, 1126, 1052, 1023, 1009, 952, 905, 873, 842, 829, 816, 785, 759, 744, 701, 657, 619, 598, 564, 544, 501, 465 cm^{-1}. ^1H NMR (DMSO) δ: 2.25 (s, 6H, CH_3-3', CH_3-4'); 7.05-7.25 (m, 4H, H_{aro}', H-3); 7.87 (s, 2H, SO_2-NH_2); 8.16 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.32 (s, 1H, Ph-NH-Ph); 8.56 (d, 1H, J = 2.5 Hz, H-6).

2-(3, 5-Dimethylphenylamino)-5-nitrobenzenesulfonamide (44k)

Mp : 182-183°C. IR (KBr) νmax : 3365, 3338, 3253, 2916, 1607, 1578, 1532, 1489, 1344, 1318, 1296, 1236, 1151, 1126, 1054, 998, 967, 915, 901, 888, 856, 829, 757, 747, 723, 677, 647, 600, 555, 532, 507, 489 cm^{-1}. ^1H NMR (DMSO) δ: 2.30 (s, 6H, CH_3-3', CH_3-5'); 6.93 (d, 3H, H_{aro}'); 7.18 (d, 1H, J = 9 Hz, H-3); 8.022 (b, 2H, SO_2-NH_2); 8.18 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.57 (d, 1H, J = 2.5 Hz, H-6).

2-(4-Chlorophenylamino)-5-nitrobenzenesulfonamide (44l)

Mp : 191-194°C. IR (KBr) νmax : 3362, 3279, 3100, 3079, 2928, 2856, 1907, 1610, 1590 1528, 1504, 1473, 1411, 1352, 1268, 1246, 1211, 1192, 1159, 1127, 1055, 1015, 962, 913, 893, 862, 840, 761, 745, 737, 718, 696, 656, 601, 588, 533, 495 cm^{-1}. ^1H NMR (DMSO) δ: 7.20 (d, 1H, J = 9 Hz, H-3); 7.36-7.53 (m, 4H, H_{aro}'); 7.90 (s, 2H, SO_2-NH_2); 8.19 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.40 (s, 1H, Ph-NH-Ph); 8.58 (d, 1H, J = 2.5 Hz, H-6).

2-(2, 3-Dimethylphenylamino)-5-nitrobenzenesulfonamide (44m)

Mp : 162-165°C. IR (KBr) νmax : 3381, 3340, 3276, 3085, 2913, 1606, 1583, 1536, 1488, 1475, 1444, 1386, 1351, 1298, 1272, 1237, 1146, 1132, 1070, 910, 822, 786, 760, 745, 704, 654, 608, 588, 509, 473 cm^{-1}. ^1H NMR (DMSO) δ: 2.07 (s, 3H, CH_3-2'); 2.32 (s, 3H, CH_3-3'); 6.52 (d, 1H, J = 9

Hz, H-3); 7.10-7.25 (m, 3H, H'aro); 7.90 (s, 2H, SO_2-NH_2); 8.15 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.52 (s, 1H, Ph-NH-Ph); 8.55 (d, 1H, J = 2.5 Hz, H-6).

V. 1. 3. General procedure for the reaction of 5 with phenols (45)

Before starting the reaction, the phenolates were obtained from the corresponding phenols (0.05 mol) after their neutralization by 0.06 mol NaOH (in aqueous solution, 10 % w/v) in acetone. Evaporation under reduced pressure provided phenolate crystals. Compound **5** (0.01 mol) and the appropriate phenolate (0.05 mol) were dissolved in acetonitrile. The mixture was refluxed and potassium carbonate (0.007 mol) was added. After completion of the reaction monitored by TLC (12-36 H), the solution was acidified, filtered and the filtrate was evaporated under reduced pressure. The crude product was dissolved in methanol and ice was added. The resulting precipitate was collected by filtration. (Yield : 60-70%).

2-(4-Methylphenoxy)-5-nitrobenzenesulfonamide (45a)

Mp : 118-119°C. IR (KBr) νmax : 3363, 3277, 3103, 3082, 2924, 2855, 1904, 1606, 1586, 1524, 1505, 1469, 1413, 1349, 1266, 1245, 1210, 1192, 1160, 1127, 1055, 1017, 962, 912, 892, 862, 841, 802, 761, 747, 737, 717, 699, 655, 600, 587, 532, 494 cm^{-1}. ^1H NMR (DMSO) δ: 2.35 (s, 3H, CH_3-4'); 6.99 (d, 1H, J = 9 Hz, H-3); 7.13 (d, 2H, J = 9 Hz, H_{aro}'); 7.35 (d, 2H, J = 9 Hz, H_{aro}'); 7.80 (s, 2H, SO_2-NH_2); 8.37 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.62 (d, 1H, J = 2.5 Hz, H-6).

2-(-3-Methylphenoxy)-5-nitrobenzenesulfonamide (45b)

Mp : 118-120°C. IR (KBr) νmax : 3360, 3264, 3103, 3083, 2922, 2177, 1852, 1606, 1578, 1525, 1489, 1468, 1410, 1378, 1350, 1260, 1165, 1146, 1128, 1084, 1057, 1000, 936, 911, 887, 876, 832, 792, 749, 730, 711, 680, 655, 627, 593, 559, 534, 502 cm^{-1}. ^1H NMR (DMSO) δ: 2.36 (s, 3H, CH_3-

3'); 7.05 (m, 3H, H_{aro}'); 7.17 (d, 1H, J = 7.5 Hz, H_{aro}'); 7.43 (t, 1H, J = 7.8 Hz, H_{aro}'); 7.79 (s, 2H, SO_2-NH_2); 8.39 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); (d, 1H, J = 2.5 Hz, H-6).

2-(2-Methylphenoxy)-5-nitrobenzenesulfonamide (45c)

Mp : 160-163°C. IR (KBr) νmax : 3377, 3276, 3106, 2962, 2641, 2534, 1973, 1819, 1653, 1589, 1578, 1514, 1488, 1462, 1413, 1347, 1268, 1242, 1167, 1137, 1127, 1107, 1060, 909, 826, 810, 783, 759, 745, 734, 713, 702, 650, 631, 591, 557, 514, 475 cm^{-1}. ^1H NMR (DMSO) δ: 2.19 (s, 3H, CH_3-2'); 6.78 (d, 1H, J = 9 Hz, H-3); 7.1-7.5 (m, 4H, H_{aro}'); 7.8 (s, 2H, SO_2-NH_2); 8.37 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.62 (d, 1H, J = 2.5 Hz, H-6).

2-(4-Chlorophenoxy)-5-nitrobenzenesulfonamide (45d)

Mp : 180-181 °C. IR (KBr) νmax : 3351, 3270, 3102, 3083, 1923, 1895, 1607, 1595, 1581, 1523, 1485, 1470, 1413, 1403, 1348, 1295, 1264, 1242, 1193, 1160, 1143, 1127, 1102, 1087, 1057, 1012, 964, 913, 894, 860, 845, 833, 789, 751, 742, 719, 681, 651, 623, 592, 555, 515, 488 cm^{-1}. ^1H NMR (DMSO) δ: 7.01 (d, 1H, J = 9 Hz, H-3); 7.25 (d, 2H, J = 8.5 Hz, H_{aro}'); 7.58 (d, 2H, J = 8.5 Hz, H_{aro}'); 7.78 (s, 2H, SO_2-NH_2); 8.37 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.61 (d, 1H, J = 2.5 Hz, H-6). Anal ($C_{12}H_9ClN_2O_5S$) C, H, N, S.

2-(3-Chlorophenoxy)-5-nitrobenzenesulfonamide (45e)

Mp : 128-130 °C. IR (KBr) νmax : 3397, 3297, 3097, 3076, 2638, 2364, 1848, 1603, 1576, 1515, 1462, 1439, 1429, 1356, 1343, 1319, 1302, 1266, 1244, 1194, 1172, 1143, 1125, 1095, 1069, 1052, 1000, 965, 919, 886, 835, 801, 788, 745, 734, 707, 679, 653, 632, 596, 553, 532, 501, 468 cm^{-1}. ^1H NMR (DMSO) δ: 7.17 (d, 1H, J = 9 Hz, H-3); 7.2 (dd, 1H, J = 8 Hz, J =2 Hz H-6'); 7.35 (s, 1H, H-2'); 7.40 (d, 1H, J = 8 Hz, H-4'); 7.54 (t, 1H, J = 8 Hz, H-5'), 7.77 (s, 2H, SO_2-NH_2); 8.39 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.62 (d, 1H, J = 2.5 Hz, H-6). Anal ($C_{12}H_9ClN_2O_5S$) C, H, N, S.

2-(2-Chlorophenoxy)-5-nitrobenzenesulfonamide (45f)

Mp : 170-171 °C. IR (KBr) νmax : 3374, 3275, 3104, 1815, 1593, 1576, 1519, 1472, 1411, 1349, 1269, 1241, 1205, 1175, 1139, 1127, 1059, 948, 908, 827, 801, 770, 744, 736, 711, 678, 648, 627, 590, 558, 518, 492, 474 cm^{-1}. ^1H NMR (DMSO) δ: 6.83 (d, 1H, J = 9 Hz, H-3); 7.37 (d, 1H, J = 9 Hz, H-6'); 7.43 (t, 1H, J = 8 Hz, H-4'), 7.53 (t, 1H, J = 8 Hz, H-5'), 7.71

(d, 1H, J = 8 Hz, H-3'), 7.80 (s, 2H, SO_2-NH_2); 8.36 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.63 (d, 1H, J = 2.5 Hz, H-6). Anal ($C_{12}H_9ClN_2O_5S$) C, H, N, S.

2-(4-Bromophenoxy)-5-nitrobenzenesulfonamide (45g)

Mp : 170-171 °C. IR (KBr) νmax : 3366, 3280, 3098, 1898, 1606, 1594, 1576, 1523, 1480, 1470, 1414, 1399, 1348, 1294, 1263, 1243, 1192, 1160, 1126, 1065, 1009, 965, 927, 911, 892, 859, 845, 835, 787, 749, 717, 678, 645, 590, 550, 500, 484 cm^{-1}. ^1H NMR (DMSO) δ: 7.11 (d, 1H, J = 9 Hz, H-3); 7.19 (d, 2H, J = 8.5 Hz, H_{aro}'); 7.70 (d, 2H, J = 8.5 Hz, H_{aro}'); 7.78 (s, 2H, SO_2-NH_2); 8.37 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.61 (d, 1H, J = 2.5 Hz, H-6). Anal ($C_{12}H_9BrN_2O_5S$) C, H, N, S.

2-(3-Bromophenoxy)-5-nitrobenzenesulfonamide (45h)

Mp : 149-150 °C. IR (KBr) νmax : 3394, 3296, 3094, 3072, 1848, 1602, 1574, 1516, 1462, 1427, 1355, 1342, 1318, 1299, 1267, 1244, 1190, 1171, 1142, 1125, 1090, 1054, 998, 964, 926, 912, 886, 872, 834, 797, 785, 745, 734, 719, 696, 671, 648, 630, 594, 548, 528, 501, 466 cm^{-1}. ^1H NMR (DMSO) δ: 7.17 (d, 1H, J = 9 Hz, H-3); 7.25 (dd, 1H, J = 8 Hz, J =2 Hz H-6'); 7.46-7.49 (m, 2H, H_{aro}'); 7.54 (d, 1H, J = 8 Hz, H-4'), 7.77 (s, 2H, SO_2-NH_2); 8.39 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.62 (d, 1H, J = 2.5 Hz, H-6). Anal ($C_{12}H_9BrN_2O_5S$) C, H, N, S.

2-(2-Bromophenoxy)-5-nitrobenzenesulfonamide (45i)

Mp : 190-192 °C. IR (KBr) νmax : 3370, 3276, 3100, 1817, 1593, 1574, 1549, 1523, 1466, 1445, 1411, 1357, 1331, 1265, 1246, 1205, 1173, 1132, 1061, 1046, 1024, 933, 914, 901, 864, 830, 799, 769, 746, 729, 704, 658, 644, 625, 589, 528, 516, 460 cm^{-1}. ^1H NMR (DMSO) δ: 6.81 (d, 1H, J = 9 Hz, H-3); 7.33-7.36 (m, 2H, H_{aro}'); 7.56 (t, 1H, J = 8 Hz, H-5'), 7.80 (s, 2H, SO_2-NH_2); 7.85 (d, 1H, J = 8 Hz, H-3'); 8.36 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.63 (d, 1H, J = 2.5 Hz, H-6). Anal ($C_{12}H_9BrN_2O_5S$) C, H, N, S.

2-(4-Iodophenoxy)-5-nitrobenzenesulfonamide (45j)

Mp : 178-179 °C. IR (KBr) νmax : 3382, 3292, 3100, 1903, 1851, 1604, 1593, 1571, 1526, 1470, 1411, 1393, 1346, 1294, 1265, 1245, 1192, 1160, 1127, 1097, 1052, 1005, 965, 930, 910, 891, 841, 785, 745, 717, 704, 669, 641, 589, 546, 500, 483, 465 cm^{-1}. ^1H NMR (DMSO) δ: 7.04 (d, 2H, J = 8.5 Hz, H_{aro}'); 7.11 (d, 2H, J = 9 Hz, H-3); 7.77 (s, 2H, SO_2-NH_2); 7.85

(d, 2H, J = 8.5 Hz, H_{aro}'); 8.37 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.61 (d, 1H, J = 2.5 Hz, H-6). Anal ($C_{12}H_9IN_2O_5S$) C, H, N, S.

2-(3-Iodophenoxy)-5-nitrobenzenesulfonamide (45k)

Mp : 153-154 °C. IR (KBr) νmax : 3371, 3275, 3084, 1605, 1570, 1523, 1463, 1408, 1353, 1297, 1262, 1242, 1189, 1161, 1136, 1058, 996, 913, 872, 821, 804, 782, 748, 734, 717, 658, 625, 593, 557, 523, 503, 471 cm^{-1}. ^1H NMR (DMSO) δ: 7.13 (d, 1H, J = 9 Hz, H-3); 7.24 (dd, 1H, J = 8 Hz, J = 2 Hz H-6'); 7.31 (t, 1H, J = 8 Hz, H-5'); 7.61 (s, 1H, H-2'); 7.70 (d, 1H, J = 8 Hz, H-4');, 7.75 (s, 2H, SO_2-NH_2); 8.39 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.61 (d, 1H, J = 2.5 Hz, H-6). Anal ($C_{12}H_9IN_2O_5S$) C, H, N,S.

2-(4-Methoxyphenoxy)-5-nitrobenzenesulfonamide (45l)

Mp : 161-166 °C. IR (KBr) νmax : 3373, 3274, 3091, 2966, 2935, 2908, 2839, 2638, 2038, 1960, 1869, 1602, 1585, 1559, 1516, 1505, 1463, 1411, 1357, 1344, 1297, 1267, 1240, 1187, 1168, 1147, 1126, 1099, 1059, 1023, 1010, 926, 911, 891, 891, 844, 836, 803, 758, 748, 740, 693, 647, 603, 590, 552, 508 cm^{-1}. ^1H NMR (DMSO) δ: 3.80 (s, 3H, O-CH_3); 6.95 (d, 1H, J = 9 Hz, H-3); 7.07 (d, 2H, J = 9 Hz, H_{aro}'); 7.17 (d, 2H, J = 9 Hz, H_{aro}'); 7.73 (s, 2H, SO_2-NH_2); 8.36 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.60 (d, 1H, J = 2.5 Hz, H-6). Anal ($C_{13}H_{12}N_2O_6S$) C, H, N, S.

2-(4-Propoxyphenoxy)-5-nitrobenzenesulfonamide (45m)

Mp : 135-138 °C. IR (KBr) νmax : 3378, 3282, 3108, 3087, 2970, 2939, 2881, 2051, 1604, 1604, 1586, 1564, 1518, 1504, 1464, 1409, 1352, 1340, 1298, 1272, 1252, 1236, 1191, 1174, 1141, 1126, 1106, 1054, 1019, 971, 948, 908, 836, 818, 773, 747, 699, 655, 617, 589, 556, 517 cm^{-1}. ^1H NMR (DMSO) δ: 0.98 (t, 3H, J = 7 Hz, O-CH_2-CH_2-CH_3); 1.75 (m, 2H, O-CH_2-CH_2-CH_3); 3.96 (t, 2H, O-CH_2-CH_2-CH_3); 6.96 (d, 1H, J = 9 Hz, H-3); 7.06 (d, 2H, J = 9 Hz, H_{aro}'); 7.15 (d, 2H, J = 9 Hz, H_{aro}'); 7.73 (s, 2H, SO_2-NH_2); 8.36 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.60 (d, 1H, J = 2.5 Hz, H-6). Anal ($C_{15}H_{16}N_2O_6S$) C, H, N, S.

2-(4-Ethoxyphenoxy)-5-nitrobenzenesulfonamide (45n)

Mp : 158-162 °C. IR (KBr) νmax : 3394, 3286, 3094, 2981, 2923, 2879, 1949, 1602, 1586, 1504, 1466, 1393, 1344, 1298, 1272, 1250, 1234, 1188, 1172, 1142, 1115, 1046, 1008, 913, 892, 845, 826, 807, 757, 739, 698, 651, 615, 587, 545, 513, 485 cm^{-1}. ^1H NMR (DMSO) δ: 1.45 (t, 3H, J =7 Hz,

O-CH_2-CH_3); 4.06 (q, 2H, J =7 Hz, O-CH_2-CH_3); 6.90 (d, 1H, J = 9 Hz, H-3); 6.97 (d, 2H, J = 9 Hz, H_{aro}'); 7.08 (d, 2H, J = 9 Hz, H_{aro}'); 7.26 (s, 2H, SO_2-NH_2); 8.26 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.83 (d, 1H, J = 2.5 Hz, H-6). Anal ($C_{14}H_{14}N_2O_6S$) C, H, N, S.

2-(3-methyl-4-Methoxyphenoxy)-5-nitrobenzenesulfonamide (45o)

Mp : IR (KBr) νmax : 3351, 3254, 3108, 3009, 2984, 2948, 2846, 1824, 1607, 1587, 1517, 1508, 1468, 1410, 1354, 1292, 1275, 1258, 1241, 1196, 1163, 1138, 1124, 1052, 1030, 928, 907, 891, 860, 829, 818, 781, 756, 744, 718, 705, 683, 652, 608, 599, 581, 546, 499, 460 cm^{-1}. ^1H NMR (DMSO) δ: 2.37 (s, 3H, CH_3-4'), 6.79-6.90 (m, 2H, H-3, H-6'); 7.10-7.12 (m, 2H, H-2', H-5'); 7.67 (s, 2H, SO_2-NH_2); 8.32 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.59 (d, 1H, J = 2.5 Hz, H-6). Anal ($C_{12}H_9ClN_2O_5S$) C, H, N, S.

2-(3-Methyl-4-chlorophenoxy)-5-nitrobenzenesulfonamide (45p)

Mp : 182-184 °C. IR (KBr) νmax : 3350, 3261, 3088, 1918, 1609, 1586, 1572, 1521, 1469, 1414, 1351, 1260, 1243, 1164, 1153, 1049, 952, 914, 889, 875, 835, 824, 815, 750, 736, 713, 676, 651, 595, 561, 543, 516, 496 cm^{-1}. ^1H NMR (DMSO) δ: 2.36 (s, 3H, CH_3-4'), 7-7.11 (m, 2H, H-3, H-6'); 7.25 (d, 1H, J = 2.5 Hz, H-2'); 7.56 (d, 1H, J = 8.5 Hz, H-5'); 7.76 (s, 2H, SO_2-NH_2); 8.37 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.61 (d, 1H, J = 2.5 Hz, H-6). Anal ($C_{13}H_{11}ClN_2O_5S$) C, H, N, S.

2-(3-methoxyphenoxy)-5-nitrobenzenesulfonamide (45q)

Mp : 136-137 °C. IR (KBr) νmax : 3386, 3273, 3102, 2948, 2842, 2636, 1610, 1579, 1521, 1490, 1468, 1449, 1408, 1348, 1275, 1262, 1239, 1157, 1141, 1077, 1056, 1034, 996, 949, 916, 889, 848, 836, 817, 786, 746, 732, 713, 683, 632, 602, 575, 537, 507, 458 cm^{-1}. ^1H NMR (DMSO) δ: 3.785 (s, 1H, O-CH_3); 6.78 (d, 1H, J = 8 Hz, H-6'); 6.82 (s, 1H, H-2'); 6.91 (dd, 1H, J = 8 Hz, J =2 Hz H-4'); 7.07 (d, 1H, J = 9 Hz, H-3); 7.43 (t, 1H, J = 8 Hz, H-5'), 7.74 (s, 2H, SO_2-NH_2); 8.38 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.61 (d, 1H, J = 2.5 Hz, H-6). Anal ($C_{13}H_{12}N_2O_6S$) C, H, N, S.

2-(2-methoxy-4-methylphenoxy)-5-nitrobenzenesulfonamide (45r)

Mp : 148-152 °C. IR (KBr) νmax : 3373, 3274, 3091, 2966, 2935, 2908, 2839, 2638, 2038, 1960, 1869, 1602, 1585, 1559, 1516, 1505, 1463, 1411, 1357, 1344, 1297, 1267, 1240, 1187, 1168, 1147, 1126, 1099, 1059, 1023, 1010, 926, 911, 891, 891, 844, 836, 803, 758, 748, 740, 693, 647, 603, 590, 552, 508 cm^{-1}. ^1H NMR (DMSO) δ: 2.37 (s, 3H, CH_3-2'); 3.72 (s, 1H, O-

CH_3); 6.82 (d, 1H, J = 9 Hz, H-3); 6.90 (d, 1H, J = 8 Hz, H-6'); 7.00 (d, 1H, J = 8 Hz, H-5'); 7.13 (s, 1H, H-3'); 7.67 (s, 2H, SO_2-NH_2); 8.37 (d, 1H, J = 9 Hz, H-4); 8.60 (s, 1H, H-6);. Anal ($C_{14}H_{14}N_2O_6S$) C, H, N, S.

V. 1. 4. General procedure for the reaction of 43 with thiophenols (46)

Compound **43** (0.01 mol) and the appropriate thiophenol (0.05 mol) were dissolved in acetonitrile (30 ml). The mixture was refluxed and potassium carbonate (0.01 mol) was added. When the reaction was finished (0.1-1 hr), the solution was acidified, filtered and the filtrate was evaporated under reduced pressure. The resulting crude oil was dissolved in methanol and ice was added. The precipitate was recovered by filtration. (Yield : 50-75%).

2-(4-Methylphenylthio)-5-nitrobenzenesulfonamide (46a)

Mp : 161-162°C. IR (KBr) νmax : 3367, 3266, 3099, 3045, 2919, 2592, 1922, 1829, 1590, 1572, 1512, 1444, 1385, 1354, 1301, 1244, 1211, 1168, 1141, 1123, 1105, 1088, 1036, 1015, 917, 899, 873, 841, 817, 798, 773, 740, 710, 687, 652, 593, 572, 553, 542, 512, 486 cm^{-1}. ^1H NMR (DMSO) δ: 2.41 (s, 3H, CH_3-4'); 7 (d, 1H, J = 9 Hz, H-3); 7.48 (m, 4H, H_{aro}'); 7.94 (s, 2H, SO_2-NH_2); 8.22 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.63 (d, 1H, J = 2.5 Hz, H-6).

2-(4-Chlorophenylthio)-5-nitrobenzenesulfonamide (46b)

Mp : 195-197°C. IR (KBr) νmax : 3368, 3266, 3097, 2360, 2343, 1923, 1591, 1573, 1517, 1476, 1444, 1390, 1354, 1292, 1267, 1244, 1167, 1141, 1124, 1090, 1037, 1012, 917, 900, 874, 834, 773, 748, 739, 710, 686, 654, 594, 579, 553, 538, 507, 486 cm^{-1}. ^1H NMR (DMSO) δ: 7.09 (d, 1H, J = 9 Hz, H-3); 7.65 (s, 4H, H_{aro}'); 7.92 (s, 2H, SO_2-NH_2); 8.22 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.63 (d, 1H, J = 2.5 Hz, H-6).

2-(2-Methylphenylthio)-5-nitrobenzenesulfonamide (46c)

Mp : 113-115 °C. IR (KBr) νmax : 3366, 3266, 3102, 1588, 1573, 1517, 1471, 1439, 1385, 1338, 1243, 1164, 1124, 1095, 1058, 1038, 911, 895,

835, 762, 739, 715, 687, 595, 579, 554, 519, 487, 470 cm^{-1}. ^1H NMR (DMSO) δ: 2.28 (s, 3H, CH_3-2'); 6.83 (d, 1H, J = 9 Hz, H-3); 7.4-7.7 (m, 4H, H_{aro}'); 7.96 (s, 2H, SO_2-NH_2); 8.20 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.63 (d, 1H, J = 2.5 Hz, H-6).

V. 1. 5. General procedure for the preparation of sulfonylureas with isocyanates (39, 40, 47, 48, and 49)

The appropriate sulfonamide (0.01 mol) was dissolved in acetone (30 ml). 0.01 mol NaOH (10% aqueous sol. w/v) was added. The mixture was gently mixed during 10 minutes and then was evaporated under reduced pressure. The resulting solid was resuspended in acetone (30 ml) and gently refluxed. The appropriate isocyanate (0.02 mol) was added to the mixture. At the end of the reaction (0.1-1 hr), the mixture was evaporated under reduced pressure and the crude product was washed with AcOEt. The solid was collected by filtration and dissolved in an aqueous NaOH solution (0.5 N; 20 ml). The resulting solution was adjusted to pH=1 with hydrochloric acid (12N) and the solid which precipitated was isolated by filtration (Yield : 40-60%).

Compounds with an amino bridge (47)

N-*t*-Butyl-*N*'-[2-(4-methylphenylamino)-5-nitrobenzenesulfonyl]urea (BM573)

Mp : 126-127 °C. ^1H NMR (DMSO) δ: 1.19 (s, 9H, NH-C(CH_3)$_3$); 2.34 (s, 3H, CH_3-4'); 6.58 (s, 1H, CO-N*H*-C-); 7.07 (d, 1H, J = 9 Hz, H-3); 8.18 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.58 (d, 1H, J = 2.5 Hz, H-6); 8.79 (s, 1H, Ph-N*H*-Ph); 10.59 (s, 1H, SO_2-N*H*-CO-). Anal ($C_{18}H_{22}N_4O_5S$) C, H, N, S.

N-*n*-Pentyl-*N*'-[2-(4-methylphenylamino)-5-nitrobenzenesulfonyl]urea (BM613)

Mp : 145-147 °C. ^{1}H NMR (DMSO) δ: 0.8 (t, 3H, J =7 Hz, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 1.1-1.35 (m, 6H, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 2.34 (s, 3H, CH_3-4'); 2.96 (q, 2H, J =7 Hz, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 6.87 (bs, 1H, CO-N*H*-CH_2); 7.06-7.31 (m, 4H, H_{aro}'); 8.18 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.60 (d, 1H, J = 2.5 Hz, H-6); 8.72 (s, 1H, Ph-N*H*-Ph); 10.99 (s, 2H, SO_2-NH_2). Anal ($C_{19}H_{24}N_4O_5S$) C, H, N, S.

***N-n*-Hexyl-*N'*-[2-(cyclohexylamino)-5-nitrobenzenesulfonyl]urea (47a)**

Mp : 115-116 °C. IR (KBr) νmax : 3385, 2932, 2857, 1683, 1602, 1583, 1543, 1497, 1463, 1329, 1296, 1270, 1237, 1189, 1150, 1125, 1088, 1046, 982, 901, 827, 749, 651, 621, 599, 571, 508 cm^{-1}. ^{1}H NMR (DMSO) δ: 0.8 (t, 2H, *J* =7 Hz, -CH_2-CH_2-CH_3); 1.28-1.93 (m, 19H, cyclohexyl, hexyl); 2.96 (q, 2H, *J* =7 Hz, NH-CH_2-CH_2-); 3.65 (bs, 1H, Ph-N*H*-cyclohexyl); 6.57 (bs, 1H, CO-N*H*-CH_2); 7.01 (m, 2H, H-3, Ph-NH-C*H*-); 8.17 (dd, 1H, *J* = 9 Hz, *J* = 2.5 Hz, H-4); 8.52 (d, 1H, *J* = 2.5 Hz, H-6); 10.96 (s, 1H, SO_2-N*H*-CO-). Anal ($C_{19}H_{30}N_4O_5S$) C, H, N, S.

***N-n*-Heptyl-*N'*-[2-(cyclohexylamino)-5-nitrobenzenesulfonyl]urea (47b)**

Mp : 117-118 °C. IR (KBr) νmax : 3385, 3363, 3091, 2930, 2858, 1682, 1602, 1583, 1540, 1496, 1468, 1449, 1433, 1350, 1331, 1306, 1297, 1270, 1239, 1188, 1149, 1126, 1094, 1045, 989, 933, 900, 825, 795, 749, 653, 622, 600, 570, 512 cm^{-1}. ^{1}H NMR (DMSO) δ: 0.83 (t, 2H, *J* =7 Hz, -CH_2-CH_3); 1.27-1.93 (m, 21H, cyclohexyl, heptyl); 2.96 (q, 2H, *J* =7 Hz, NH-CH_2-CH_2-); 3.65 (bs, 1H, Ph-N*H*-cyclohexyl); 6.56 (bs, 1H, CO-N*H*-CH_2); 7.01 (m, 2H, *J* = 9 Hz, H-3, Ph-NH-C*H*-); 8.17 (dd, 1H, *J* = 9 Hz, *J* = 2.5 Hz, H-4); 8.52 (d, 1H, *J* = 2.5 Hz, H-6); 10.96 (s, 1H, SO_2-N*H*-CO-). Anal ($C_{20}H_{32}N_4O_5S$) C, H, N, S.

***N-n*-Octyl-*N'*-[2-(cyclohexylamino)-5-nitrobenzenesulfonyl]urea (47c)**

Mp : 93-94 °C. IR (KBr) νmax : 3385, 390, 2931, 2856, 1686, 1603, 1584, 1543, 1497, 1468, 1453, 1351, 1329, 1296, 1269, 1238, 1188, 1149, 1125, 1095, 1045, 983, 941, 900, 823, 797, 767, 749, 719, 651, 624, 600, 570, 551, 497 cm^{-1}. ^{1}H NMR (DMSO) δ: 0.84 (t, 2H, *J* =7 Hz, -CH_2-CH_3); 1.13-1.93 (m, 23H, 2.96 (q, 2H, *J* =7 Hz, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 3.65 (bs, 1H, Ph-N*H*-cyclohexyl); 6.56 (bs, 1H, CO-N*H*-CH_2); 7.01 (m, 2H, *J* = 9 Hz, H-3, Ph-NH-C*H*-); 8.17 (dd, 1H, *J* = 9 Hz, *J* = 2.5 Hz, H-4); 8.52 (d, 1H, *J* = 2.5 Hz, H-6); 10.96 (s, 1H, SO_2-N*H*-CO-). Anal ($C_{21}H_{34}N_4O_5S$) C, H, N, S.

_N_-_n_-Pentyl-_N'_-[2-(2,4,6-trimethylphenylamino)-5-nitrobenzenesulfonyl]urea (47d)

Mp : 143-145 °C. IR (KBr) νmax : 3379, 2956, 2929, 2859, 1682, 1599, 1581, 1548, 1500, 1466, 1325, 1242, 1217, 1151, 1123, 1049, 974, 901, 857, 837, 759, 748, 705, 652, 600, 499 cm^{-1}. ^1H NMR (DMSO) δ: 0.8 (t, 2H, *J* =7 Hz, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 1.16-1.33 (m, 6H, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 2.05 (s, 6H, CH_3-2' and CH_3-6'); 2.29 (s, 3H, CH_3-4'); 2.95 (q, 2H, *J* =7 Hz, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 6.21 (d, 1H, *J* = 9 Hz, H-3); 6.94 (sb, 1H, CO-N*H*-CH_2); 7.06 (s, 2H, H-3', H-5'); 8.13 (dd, 1H, J_1=2.5 Hz, J_2=9 Hz, H-4), 8.2 (s, 1H, Ph-N*H*-Ph); 8.59 (d, 1H, *J* = 2.5 Hz, H-6); 10.79 (s, 1H, SO_2-N*H*-CO-NH). Anal ($C_{21}H_{28}N_4O_5S$) C, H, N, S.

_N_-_n_-Pentyl-_N'_-[2-(2-methylphenylamino)-5-nitrobenzenesulfonyl]urea (47e)

Mp : 127-128 °C. IR (KBr) νmax : 3360, 3198, 2953, 2930, 2869, 1701, 1668, 1609, 1582, 1547, 1501, 1467, 1372, 1330, 1241, 1227, 1188, 1153, 1125, 1052, 956, 903, 876, 828, 746, 704, 662, 635, 609, 583, 539, 514, 498 cm^{-1}. ^1H NMR (DMSO) δ: 0.8 (t, 2H, *J* =7 Hz, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 1.11-1.35 (m, 6H, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 2.14 (s, 3H, CH_3-2'); 2.96 (q, 2H, *J* =7 Hz, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 6.61 (d, 1H, *J* = 9 Hz, H-3); 6.9 (sb, 1H, CO-N*H*-CH_2); 7.27-7.42 (m, 4H, H_{aro}'); 8.17 (dd, 1H, *J* = 9 Hz, *J* = 2.5 Hz, H-4); 8.48 (s, 1H, Ph-N*H*-Ph); 8.61 (d, 1H, *J* = 2.5 Hz, H-6); 10.91 (s, 1H, SO_2-N*H*-CO-NH). Anal ($C_{19}H_{24}N_4O_5S$) C, H, N, S.

_N_-_n_-Pentyl-_N'_-[2-(3-methylphenylamino)-5-nitrobenzenesulfonyl]urea (47f)

Mp : 128-130 °C. IR (KBr) νmax : 3335, 3108, 2956, 2930, 2871, 1670, 1602, 1578, 1501, 1333, 1234, 1156, 1124, 1051, 1028, 954, 895, 865, 833, 770, 744, 709, 661, 637, 600, 503 cm^{-1}. ^1H NMR (DMSO) δ: 0.8 (t, 2H, *J* =7 Hz, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 1.12-1.37 (m, 6H, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 2.34 (s, 3H, 3'-CH_3); 2.97 (q, 2H, *J* =7 Hz, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 6.87 (sb, 1H, CO-N*H*-CH_2); 7.11-7.19 (m, 4H, H_{aro}'); 7.37 (t, 1H, *J* = *8*, H-5'); 8.2 (dd, 1H, J_1=2.5 Hz, J_2=9 Hz, H-4); 8.61 (d, 1H, *J* = 2.5 Hz, H-6); 8.75 (s, 1H, Ph-N*H*-Ph); 11 (s, 1H, SO_2-N*H*-CO-NH). Anal ($C_{19}H_{24}N_4O_5S$) C, H, N, S.

_N_-_t_-Butyl-_N'_-[2-(3-methyl-4-bromophenylamino)-5-nitrobenzenesulfonyl]urea (47g)

Mp : 141-143 °C. IR (KBr) νmax : 3344, 3101, 2968, 2929, 1712, 1686, 1597, 1534, 1502, 1476, 1395, 1365, 1328, 1230, 1156, 1126, 1052, 1029, 954, 922, 897, 871, 819, 778, 759, 745, 699, 647, 590, 510, 474 cm^{-1}. ^1H NMR (DMSO) δ: 1.2 (s, 9H, NH-C(CH_3)$_3$); 2.37 (s, 3H, CH_3-3'); 6.59 (s, 1H, CO-N*H*-C-(CH_3)$_3$); 7.08-7.32 (m, 3H, H_{aro}'); 7.66 (d, 1H, *J* = 9 Hz, H-6'); 8.2 (dd, 1H, J_1=2.5 Hz, J_2=9 Hz, H-4); 8.59 (d, 1H, *J* = 2.5 Hz, H-6); 8.82 (s, 1H, Ph-N*H*-Ph); 10.6 (s, 1H, SO_2-N*H*-CO-NH). Anal ($C_{18}H_{21}BrN_4O_5S$) C, H, N, S.

_N_-_n_-Pentyl-_N'_-[2-(2, 5-dimethylphenylamino)-5-nitrobenzenesulfonyl]urea (47h)

Mp : 126-128 °C. IR (KBr) νmax : 3362, 3098, 2956, 2929, 2860, 1764, 1670, 1603, 1584, 1543, 1504, 1466, 1378, 1328, 1236, 1155, 1123, 1050, 965, 899, 871, 828, 794, 746, 703, 662, 639, 597, 501 cm^{-1}. ^1H NMR (DMSO) δ: 0.8 (t, 2H, *J* =7 Hz, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 1.12-1.35 (m, 6H, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 2.1 (s, 3H, CH_3-2'); 2.3 (s, 3H, CH_3-5'); 2.99 (q, 2H, *J* =7 Hz, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 6.61 (d, 1H, *J* = 9 Hz, H-6'); 6.96 (bs, 1H, CO-N*H*-CH_2); 7.1-7.3 (m, 3H, H_{aro}', H-3); 8.19 (dd, 1H, *J* = 9 Hz, *J* = 2.5 Hz, H-4); 8.43 (s, 1H, Ph-N*H*-Ph); 8.6 (d, 1H, *J* = 2.5 Hz, H-6); 11 (s, 1H, SO_2-N*H*-CO-). Anal ($C_{20}H_{24}N_6O_4S$) C, H, N, S.

_N_-_n_-Pentyl-_N'_-[2-(2, 4-dimethylphenylamino)-5-nitrobenzenesulfonyl]urea (47i)

Mp : 127-129 °C. IR (KBr) νmax : 3372, 3098, 2955, 2930, 2870, 1668, 1600, 1585, 1544, 1501, 1467, 1335, 1217, 1156, 1123, 1051, 1033, 962, 901, 875, 828, 790, 755, 744, 700, 652, 601, 547, 504 cm^{-1}. ^1H NMR (DMSO) δ: 0.8 (t, 2H, *J* =7 Hz, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 1.1-1.35 (m, 6H, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 2.09 (s, 3H, CH_3-2'); 2.32 (s, 3H, CH_3-4'); 2.96 (q, 2H, *J* =7 Hz, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 6.54 (d, 1H, *J* = 9 Hz, H-3); 6.89 (bs, 1H, CO-N*H*-CH_2); 7.14 (s, 2H, H_{aro}'); 7.22 (s, 1H, H-3'); 8.14 (dd, 1H, *J* = 9 Hz, *J* = 2.5 Hz, H-4); 8.40 (s, 1H, Ph-N*H*-Ph); 8.59 (d, 1H, *J* = 2.5 Hz, H-6); 10.89 (s, 1H, SO_2-N*H*-CO-). Anal ($C_{20}H_{26}N_4O_5S$) C, H, N, S.

_N_-_n_-Pentyl-_N'_-[2-(2, 6-dimethylphenylamino)-5-nitrobenzenesulfonyl]urea (47j)

Mp : 129-131 °C. IR (KBr) νmax : 3397, 3351, 3096, 2955, 2929, 2859, 1678, 1603, 1582, 1526, 1499, 1379, 1324, 1264, 1240, 1220, 1205, 1155, 1125, 1109, 1051, 906, 884, 824, 768, 748, 729, 691, 652, 597, 507 cm^{-1}.

^{1}H NMR (DMSO) δ: 0.8 (t, 2H, *J* =7 Hz, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 1.1-1.35 (m, 6H, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 2.1 (s, 6H, CH_3-2', CH_3-6'); 2.96 (q, 2H, *J* =7 Hz, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 6.1 (d, 1H, *J* = 9 Hz, H-3); 6.56 (bs, 1H, CO-N*H*-CH_2); 7.22 (s, 3H, H_{aro}'); 8.04 (dd, 1H, *J* = 9 Hz, *J* = 2.5 Hz, H-4); 8.54 (d, 1H, *J* = 2.5 Hz, H-6); 8.74 (s, 1H, Ph-N*H*-Ph); 11 (s, 1H, SO_2-N*H*-CO-). Anal ($C_{20}H_{26}N_4O_5S$) C, H, N, S.

_N_-Benzyl-_N'_-[2-(4-methylphenylamino)-5-nitrobenzenesulfonyl]urea (47k)

Mp : 148-150 °C. IR (KBr) νmax : 3391, 3269, 3101, 3041, 2924, 1683, 1625, 1597, 1578, 1549, 1513, 1501, 1476, 1454, 1328, 1252, 1222, 1157, 1125, 1037, 904, 877, 841, 824, 809, 744, 698, 658, 598, 581, 540, 511, 503 cm^{-1}. ^{1}H NMR (DMSO) δ: 2.34 (s, 3H, CH_3-4'); 4.20 (d, 1H, *J* = 2.75 Hz); 7.05-7.3 (m, 10H, H_{aro}', H-5); 7.45 (bs, 1H, CO-N*H*-CH_2); 8.18 (dd, 1H, *J* = 9 Hz, *J* = 2.5 Hz, H-4); 8.61 (d, 1H, *J* = 2.5 Hz, H-6); 8.66 (s, 1H, Ph-N*H*-Ph); 11.2 (s, 1H, SO_2-N*H*-CO-). Anal ($C_{21}H_{20}N_4O_5S$) C, H, N, S.

_N_-Cyclohexyl-_N'_-[2-(4-methylphenylamino)-5-nitrobenzenesulfonyl]urea (47l)

Mp : 166-168 °C. IR (KBr) νmax : 3348, 3066, 2932, 2857, 1686, 1658, 1597, 1539, 1513, 1500, 1471, 1335, 1253, 1222, 1158, 1125, 1036, 970, 906, 884, 868, 841, 824, 806, 768, 744, 699, 657, 611, 579, 549, 537, 497 cm^{-1}. ^{1}H NMR (DMSO) δ: 1-1.8 (m, 10H, cyclohexyl); 2.34 (s, 3H, CH_3-4'); 3.34 (b, 1H , NH-C*H*-); 6.80 (bs, 1H, CO-N*H*-CH_2); 7.07 (d, 1H, *J* = 9 Hz, H-3); 7.19-7.3 (m, 4H, H_{aro}'); 8.18 (dd, 1H, *J* = 9 Hz, *J* = 2.5 Hz, H-4); 8.60 (d, 1H, *J* = 2.5 Hz, H-6); 8.73 (s, 1H, Ph-N*H*-Ph); 10.79 (s, 1H, SO_2-N*H*-CO-). Anal ($C_{20}H_{24}N_4O_5S$) C, H, N, S.

_N_-_n_-Pentyl-_N'_-[2-(3, 4-dimethylphenylamino)-5-nitrobenzenesulfonyl]urea (47m)

Mp : 88-90 °C. IR (KBr) νmax : 3362, 3100, 2956, 2931, 2860, 1763, 1670, 1599, 1542, 1504, 1468, 1327, 1235, 1210, 1156, 1124, 1051, 1023, 957, 899, 871, 826, 745, 700, 656, 597, 503 cm^{-1}. ^{1}H NMR (DMSO) δ: 0.8 (t, 2H, *J* =7 Hz, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 1.1-1.35 (m, 6H, NH-CH2-CH_2-CH_2-CH_2-CH_3); 2.25 (s, 6H, CH_3-3', CH_3-4'); 2.96 (q, 2H, *J* =7 Hz, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 6.94 (bs, 1H, CO-N*H*-CH_2); 7.02-7.27 (m, 3H, H_{aro}', H-3); 8.19 (dd, 1H, *J* = 9 Hz, *J* = 2.5 Hz, H-4); 8.61 (d, 1H, *J* = 2.5 Hz, H-6); 8.68 (s, 1H, Ph-N*H*-Ph); 11.07 (s, 1H, SO_2-N*H*-CO-). Anal ($C_{20}H_{26}N_4O_5S$) C, H, N, S.

_N-n_-Pentyl-_N'_-[2-(3, 5-dimethylphenylamino)-5-nitrobenzenesulfonyl]urea (47n)

Mp : 122-125 °C. IR (KBr) νmax : 3571, 3420, 3366, 3323, 3239, 3106, 2957, 2931, 2960, 1705, 1680, 1609, 1580, 1526, 1501, 1474, 1421, 1349, 1330, 1302, 1254, 1235, 1156, 1122, 1051, 1029, 956, 921, 904, 868, 826, 755, 745, 675, 647, 605, 506 cm^{-1}. ^1H NMR (DMSO) δ: 0.8 (t, 2H, *J* =7 Hz, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 1.1-1.35 (m, 6H, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 2.30 (s, 6H, CH_3-3', CH_3-5'); 6.87 (bs, 1H, CO-N*H*-CH_2); 6.92-6.94 (m, 3H, H_{aro}'); 7.18 (d, 1H, *J* = 9 Hz, H-3); 8.20 (dd, 1H, *J* = 9 Hz, *J* = 2.5 Hz, H-4); 8.60 (d, 1H, *J* = 2.5 Hz, H-6); 8.67 (s, 1H, Ph-N*H*-Ph); 11.00 (s, 1H, SO_2-N*H*-CO-). Anal ($C_{20}H_{26}N_4O_5S$) C, H, N, S.

_N-n_-Pentyl-_N'_-[2-(3-methyl-4-bromophenylamino)-5-nitrobenzenesulfonyl]urea (47o)

Mp : 130-132 °C. IR (KBr) νmax : 3344, 3100, 2956, 2930, 2870, 2194, 1668, 1584, 1540, 1499, 1477, 1332, 1231, 1157, 1124, 1052, 1029, 956, 899, 873, 826, 816, 780, 744, 699, 646, 601, 560, 502 cm^{-1}. ^1H NMR (DMSO) δ: 0.8 (t, 2H, *J* =7 Hz, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 1.1-1.35 (m, 6H, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 2.37 (s, 3H, CH_3-3'); 2.96 (q, 2H, *J* =7 Hz, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 6.87 (bs, 1H, CO-N*H*-CH_2); 7.08 (dd, 1H, *J* = 9 Hz, *J* = 2.5 Hz, H-6'); 7.22 (d, 1H, *J* = 9 Hz, H-3); 7.32 (d, 1H, *J* = 2.5 Hz, H-2'); 7.65 (d, 1H, *J* = 9 Hz, H-5'); 8.20 (dd, 1H, *J* = 9 Hz, *J* = 2.5 Hz, H-4); 8.61 (d, 1H, *J* = 2.5 Hz, H-6); 8.75 (s, 1H, Ph-N*H*-Ph); 11.02 (s, 1H, SO_2-N*H*-CO-). Anal ($C_{19}H_{23}BrN_4O_5S$) C, H, N, S.

N-sec-Butyl-_N'_-[2-(2,6-dimethylphenylamino)-5-nitrobenzenesulfonyl]urea (47p)

Mp : 97-99 °C. IR (KBr) νmax : 3364, 3099, 2969, 2931, 2877, 1680, 1605, 1582, 1532, 1500, 1471, 1380, 1334, 1307, 1239, 1205, 1154, 1124, 1094, 1050, 1022, 997, 933, 902, 879, 830, 771, 747, 691, 651, 591, 506 cm^{-1}. ^1H NMR (DMSO) δ: 0.76 (t, 2H, *J* =7 Hz, NH-CH_2-CH-$(CH_3)_2$); 0.98 (d, 6H, *J* =7 Hz, CH_2-CH-$(CH_3)_2$); 1.34 (m, 1H, CH_2-CH-$(CH_3)_2$); 2.10 (s, 6H, CH_3-2', CH_3-6'); 6.22 (d, 1H, *J* = 9 Hz, H-3); 6.79 (bs, 1H, CO-N*H*-CH_2); 7.26 (s, 3H, H_{aro}'); 8.14 (dd, 1H, *J* = 9 Hz, *J* = 2.5 Hz, H-4); 8.29 (s, 1H, Ph-N*H*-Ph); 8.59 (d, 1H, *J* = 2.5 Hz, H-6); 10.59 (s, 1H, SO_2-N*H*-CO-). Anal ($C_{19}H_{24}N_4O_5S$) C, H, N, S.

_N-t_-Butyl-_N'_-[2-(2,6-dimethylphenylamino)-5-nitrobenzenesulfonyl]urea (47q)

Mp : 95-97 °C. IR (KBr) νmax : 3370, 3098, 2970, 2928, 1692, 1639, 1605, 1582, 1545, 1501, 1472, 1394, 1365, 1327, 1307, 1264, 1205, 1153, 1124, 1094, 1050, 1031, 950, 922, 902, 874, 829, 769, 747, 691, 650, 587, 508, 469 cm^{-1}. ^1H NMR (DMSO) δ: 1.18 (s, 9H, NH-C(CH_3)$_3$); 2.12 (s, 6H, CH_3-2', CH_3-6'); 6.22 (d, 1H, *J* = 9 Hz, H-3); 6.66 (s, 1H, CO-N*H*-CH_2); 7.26 (s, 3H, H_{aro}'); 8.15 (dd, 1H, *J* = 9 Hz, *J* = 2.5 Hz, H-4); 8.28 (s, 1H, Ph-N*H*-Ph); 8.57 (d, 1H, *J* = 2.5 Hz, H-6); 10.42 (s, 1H, SO_2-N*H*-CO-). Anal ($C_{19}H_{24}N_4O_5S$) C, H, N, S.

N-sec-Butyl-_N'_-[2-(4-methylphenylamino)-5-nitrobenzenesulfonyl]urea (47r)

Mp : 104-106 °C. IR (KBr) νmax : 3349, 3097, 2969, 2931, 2876, 1684, 1597, 1532, 1513, 1501, 1461, 1380, 1328, 1221, 1155, 1123, 1052, 1020, 998, 935, 903, 881, 824, 804, 770, 745, 699, 656, 604, 490 cm^{-1}. ^1H NMR (DMSO) δ: 0.76-1.39 (m, 9H, alkyl); 2.34 (s, 3H, CH_3-4'); 3.47 (m, 2H, CO-NH-CH_2-); 6.72 (bs, 1H, CO-N*H*-CH_2); 7.07 (d, 1H, *J* = 9 Hz, H-3); 7.18-7.31 (m, 4H, H_{aro}'); 8.18 (dd, 1H, *J* = 9 Hz, *J* = 2.5 Hz, H-4); 8.60 (d, 1H, *J* = 2.5 Hz, H-6); 8.74 (s, 1H, Ph-N*H*-Ph); 10.81 (s, 1H, SO_2-N*H*-CO-). Anal ($C_{19}H_{24}N_4O_5S$) C, H, N, S.

_N-n_-Pentyl-_N'_-[2-(4-Chlorophenylamino)-5-nitrobenzenesulfonyl]urea (47s)

Mp : 124-126 °C. IR (KBr) νmax : 3390, 3264, 3105, 2958, 2931, 2859, 1685, 1628, 1605, 1590, 1578, 1539, 1506, 1477, 1397, 1329, 1223, 1158, 1125, 1094, 1051, 1027, 1015, 963, 903, 869, 832, 810, 744, 718, 653, 589, 507 cm^{-1}. ^1H NMR (DMSO) δ: 0.8 (t, 2H, *J* =7 Hz, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 1.1-1.35 (m, 6H, NH-CH2-CH_2-CH_2-CH_2-CH_3); 2.96 (q, 2H, *J* =7 Hz, NH-C*H*2-CH_2-CH_2-CH_2-CH_3); 6.89 (bs, 1H, CO-N*H*-CH_2); 7.21 (d, 1H, *J* = 9 Hz, H-3); 7.33-7.54 (m, 4H, H_{aro}'); 8.21 (dd, 1H, *J* = 9 Hz, *J* = 2.5 Hz, H-4); 8.62 (d, 1H, *J* = 2.5 Hz, H-6); 8.81 (s, 1H, Ph-N*H*-Ph); 11.02 (s, 1H, SO_2-N*H*-CO-). Anal ($C_{18}H_{21}ClN_4O_5S$) C, H, N, S.

_N-n_-Pentyl-_N'_-[2-(2, 3-dimethylphenylamino)-5-nitrobenzenesulfonyl]urea (47t)

Mp : 139-142 °C. IR (KBr) νmax : 3374, 3342, 3312, 3290, 3081, 2973, 2934, 1703, 1687, 1666, 1603, 1578, 1529, 1499, 1470, 1388, 1330, 1231, 1159, 1124, 1093, 1052, 944, 901, 829, 790, 758, 746, 651, 590, 508 cm^{-1}. ^1H NMR (DMSO) δ: 0.8 (t, 2H, J =7 Hz, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 1.1-1.35 (m, 6H, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 2.07 (s, 3H, CH_3-2'); 2.32

(s, 3H, CH_3-3'); 2.96 (q, 2H, J =7 Hz, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 6.52 (d, 1H, J = 9 Hz, H-3); 6.89 (bs, 1H, CO-NH-CH_2); 7.10-7.25 (m, 3H, H'aro); 8.15 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.52 (s, 1H, Ph-N*H*-Ph); 8.55 (d, 1H, J = 2.5 Hz, H-6); 10.75 (s, 1H, SO_2-N*H*-CO-). Anal ($C_{20}H_{26}N_4O_5S_2$) C, H, N, S.

Compounds with an oxygen bridge (48)

N-t-Butyl-*N'*-[2-(4-methylphenoxy)-5-nitrobenzenesulfonyl]urea (48a)

Mp : 162-165 °C. IR (KBr) νmax : 3335, 3222, 3108, 2977, 2932, 2872, 1688, 1660, 1607, 1587, 1557, 1527, 1505, 1472, 1410, 1396, 1349, 1265, 1243, 1192, 1160, 1138, 1126, 1103, 1057, 1037, 1018, 921, 904, 879, 838, 802, 748, 693, 648, 601, 582, 537, 493, 475 cm^{-1}. ^1H NMR (DMSO) δ: 1.20 (s, 9H, NH-C(CH_3)$_3$); 2.39 (s, 3H, CH_3-4'); 6.3 (s, 1H, CO-N*H*-C-); 6.99-7.45 (m, 5H, H_{aro}', H-3); 8.43(dd, 1H, *J* = 9 Hz, *J* = 2.5 Hz, H-4); 8.62 (d, 1H, *J* = 2.5 Hz, H-6); 10.5 7.8 (s, 1H, SO_2-N*H*-CO-NH). Anal ($C_{18}H_{21}N_3O_6S$) C, H, N, S.

N-n-Pentyl-*N'*-[2-(4-methylphenoxy)-5-nitrobenzenesulfonyl]urea (48b)

Mp : 148-150 °C. IR (KBr) νmax : 3344, 3105, 2929, 2859, 1702, 1666, 1604, 1585, 1550, 1523, 1505, 1470, 1410, 1348, 1267, 1191, 1162, 1137, 1125, 1106, 1056, 1019, 964, 904, 872, 836, 802, 759, 747, 733, 711, 650, 600, 589, 568, 540, 493 cm^{-1}. ^1H NMR (DMSO) δ: 0.81 (t, 2H, *J* =7 Hz, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 1.12-1.35 (m, 6H, NH-CH_2-CH_2-CH_2-CH_2-CH_3); (s, 3H, CH_3-4'); 3.00 (q, 2H, *J* =7 Hz, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 6.47 (bs, 1H, CO-N*H*-CH_2); 6.99-7.45 (m, 5H, H_{aro}', H-3); 8.43 (dd, 1H, *J* = 9 Hz, *J* = 2.5 Hz, H-4); 8.65 (d, 1H, *J* = 2.5 Hz, H-6); 11 (s, 1H, SO_2-N*H*-CO-NH). Anal ($C_{19}H_{23}N_3O_6S$) C, H, N, S.

N-n-Hexyl-*N'*-[2-(4-methylphenoxy)-5-nitrobenzenesulfonyl]urea (48c)

Mp : 128-130 °C. IR (KBr) νmax : 3335, 3102, 2954, 2928, 2857, 1664, 1605, 1587, 1550, 1525, 1505, 1471, 1409, 1345, 1265, 1242, 1193, 1162, 1137, 1125, 1102, 1055, 1017, 987, 905, 889, 836, 802, 746, 647, 588, 566, 493 cm^{-1}. ^1H NMR (DMSO) δ: 0.8-1.3 (m, 11H, H-hexyl); 2.36 (s, 3H, CH_3-4'); 2.97 (q, 2H, *J* =7 Hz, NH-CH_2-); 6.47 (bs, 1H, CO-N*H*-CH_2); 6.99-7.45 (m, 5H, H_{aro}', H-3); 8.43 (dd, 1H, *J* = 9 Hz, *J* = 2.5 Hz, H-4), 8.65 (d, 1H, *J* = 2.5 Hz, H-6); 11 (s, 1H, SO_2-N*H*-CO-). Anal ($C_{20}H_{25}N_3O_6S$) C, H, N, S.

***N*-Cyclohexyl-*N'*-[2-(4-methylphenoxy)-5-nitrobenzenesulfonyl]urea (48d)**

Mp : 170-173 °C. IR (KBr) νmax : 3356, 3106, 2928, 2858, 1691, 1662, 1606, 1585, 1543, 1526, 1505, 1473, 1350, 1260, 1191, 1161, 1134, 1124, 1055, 1040, 1019, 969, 913, 898, 861, 843, 826, 804, 760, 748, 731, 693, 652, 608, 597, 571, 547, 531, 511, 489 cm^{-1}. ^1H NMR (DMSO) δ: 0.9-1.8 (m, 11H, cyclohexyl); 2.36 (s, 3H, CH_3-4'); 6.37 (d, 1H, CO-N*H*-cyclohexyl); 6.99-7.45 (m, 5H, H_{aro}', H-3); 8.43 (dd, 1H, *J* = 9 Hz, *J* = 2.5 Hz, H-4), 8.65 (d, 1H, *J* = 2.5 Hz, H-6); 11 (s, 1H, SO_2-N*H*-CO-). Anal ($C_{20}H_{23}N_3O_6S$) C, H, N, S.

***N*-*n*-Butyl-*N'*-[2-(4-methylphenoxy)-5-nitrobenzenesulfonyl]urea (48e)**

Mp : 207-210 °C. IR (KBr) νmax : 3343, 3184, 3110, 2955, 2919, 2868, 1886, 1702, 1665, 1605, 1585, 1548, 1523, 1504, 1470, 1411, 1350, 1301, 1265, 1245, 1211, 1190, 1162, 1138, 1126, 1106, 1057, 1043, 1018, 1003, 984, 930, 909, 895, 863, 838, 802, 759, 747, 734, 538, 509, 492 cm^{-1}. ^1H NMR (DMSO) δ: 0.8 (t, 2H, *J* =7 Hz, NH-CH_2-CH_2-CH_2-CH_3); 1.1-1.4 (m, 4H, NH-CH_2- CH_2-CH_2-CH_3); 2.36 (s, 3H, CH_3-4'); 2.96 (q, 2H, *J* =7 Hz, NH-CH_2-CH_2-CH_2-CH_3); 6.46 (bs, 1H, CO-N*H*-CH_2); 6.99-7.45 (m, 5H, H_{aro}', H-3); 8.43 (dd, 1H, *J* = 9 Hz, *J* = 2.5 Hz, H-4); 8.65 (d, 1H, *J* = 2.5 Hz, H-6); 11 (s, 1H, SO_2-N*H*-CO-). Anal ($C_{18}H_{21}N_3O_6S$) C, H, N, S.

***N*-*n*-Pentyl-*N'*-[2-(3-methylphenoxy)-5-nitrobenzenesulfonyl]urea (48f)**

Mp : 157-158 °C. IR (KBr) νmax : 3343, 3189, 3109, 2955, 2931, 2871, 1703, 1664, 1603, 1578, 1551, 1528, 1468, 1344, 1262, 1166, 1143, 1128, 1103, 1056, 1036, 1002, 978, 937, 927, 903, 895, 869, 837, 817, 792, 747, 731, 714, 689, 656, 633, 593, 570, 536, 504, 477, 457 cm^{-1}. ^1H NMR (DMSO) δ: 0.8 (t, 2H, *J* =7 Hz, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 1-1.6 (m, 6H, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 2.35 (s, 3H, CH_3-3'); 2.98 (q, 2H, *J* =7 Hz, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 6.48 (bs, 1H, CO-N*H*-CH_2), 6.96-7.10 (m, 3H, H_{aro}'); 7.21 (d, 1H, *J* = 9 Hz, H-3); 7.43 (t, 1H, *J* = 8 Hz, H-5'); 8.43 (dd, 1H, *J* = 9 Hz, *J* = 2.5 Hz, H-4); (d, 1H, *J* = 2.5 Hz, H-6); 11 (s, 1H, SO_2-N*H*-CO-). Anal ($C_{19}H_{23}N_3O_6S$) C, H, N, S.

***N*-*n*-Pentyl -*N'*-[2-(2-methylphenoxy)-5-nitrobenzenesulfonyl]urea (48g)**

Mp : 150-153 °C. IR (KBr) νmax : 3332, 3103, 2952, 2930, 2858, 1685, 1658, 1602, 1578, 1550, 1525, 1471, 1348, 1269, 1243, 1214, 1166, 1141, 1127, 1109, 1056, 1036, 975, 904, 875, 832, 808, 783, 758, 745, 731, 649, 629, 588, 519, 505 cm^{-1}. ^1H NMR (DMSO) δ: 0.8 (t, 2H, *J* =7 Hz, NH-

CH_2-CH_2-CH_2-CH_2-CH_3); 1.1-1.35 (m, 6H, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 2.1 (s, 3H, CH_3-2'); 2.96 (q, 2H, *J* =7 Hz, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 6.45 (bs, 1H, CO-N*H*-CH_2); 6.78 (d, 1H, *J* = 9 Hz, H-6'); 7.08 (d, 1H, *J* = 9 Hz, H-6); 7.3-7.46 (m, 3H, H_{aro}'); 8.41 (dd, 1H, *J* = 9 Hz, *J* = 2.5 Hz, H-4); 8.65 (d, 1H, *J* = 2.5 Hz, H-6); 10.98 (s, 1H, SO_2-N*H*-CO-). Anal ($C_{19}H_{23}N_3O_6S$) C, H, N, S.

***N*-*i*-Propyl-*N'*-[2-(2-methylphenoxy)-5-nitrobenzenesulfonyl]urea (48h)**

Mp : 196-198°C. IR (KBr) νmax : 3349, 3107, 2978, 2933, 1659, 1603, 1579, 1528, 1470, 1389, 1347, 1266, 1244, 1163, 1138, 1127, 1109, 1056, 953, 912, 865, 834, 808, 783, 757, 745, 732, 703, 650, 630, 604, 588, 518 cm^{-1}. ^1H NMR (DMSO) δ: 1 (d, 6H, -CH-(CH_3)$_2$); 2.10 (s, 3H, CH_3-2'); 3.60 (m, 1H, -C_H-(CH_3)$_2$); 6.31(d, 1H, CO-N*H*-CH); 6.78 (d, 1H, J = 9 Hz, H-3); 7.1-7.5 (m, 4H, H_{aro}'); 8.42 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.62 (d, 1H, J = 2.5 Hz, H-6); 10.85 (s, 1H, N*H*-CO-NH). Anal ($C_{17}H_{19}N_3O_6S$) C, H, N, S.

***N*-*n*-Butyl-*N'*-[2-(2-methylphenoxy)-5-nitrobenzenesulfonyl]urea (48i)**

Mp : 179-181 °C. IR (KBr) νmax : 3344, 3099, 2958, 2932, 2873, 1701, 1691, 1663, 1592, 1577, 1543, 1523, 1469, 1409, 1348, 1272, 1243, 1163, 1142, 1127, 1108, 1056, 1044, 1005, 982, 909, 894, 864, 846, 833, 809, 781, 757, 745, 733, 715, 704, 689, 649, 630, 595, 509, 475 cm^{-1}. ^1H NMR (DMSO) δ: 0.79 (t, 3H, NH-CH_2-CH_2-CH_2-CH_3); 1.14-1.34 (m, 4H, NH-CH_2-CH_2-CH_2-CH_3); 2.19 (s, 3H, CH_3-2'); 2.99 (q, 2H, NH-CH_2-CH_2-CH_2-CH_3); 6.50 (t, 1H, NH-CO-N*H*); 6.78 (d, 1H, J = 9 Hz, H-3); 7.1-7.5 (m, 4H, H_{aro}'); 8.37 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.62 (d, 1H, J = 2.5 Hz, H-6); 11.07 (s, 1H, N*H*-CO-NH). Anal ($C_{18}H_{21}N_3O_6S$) C, H, N, S.

***N*-*t*-Butyl-*N'*-[2-(2-methylphenoxy)-5-nitrobenzenesulfonyl]urea (48j)**

Mp : 150-153 °C. IR (KBr) νmax : 3370, 3104, 2967, 2930, 1672, 1603, 1593, 1579, 1524, 1471, 1395, 1344, 1268, 1219, 1174, 1161, 1139, 1126, 1110, 1062, 1036, 946, 904, 879, 835, 809, 782, 748, 733, 703, 649, 630, 605, 586, 520 cm^{-1}. ^1H NMR (DMSO) δ: 1.17 (s, 9H, NH-C(CH_3)$_3$); 2.12 (s, 3H, CH_3-2'); 6.24 (s, 1H, N*H*-C(CH_3)$_3$); 6.79 (d, 1H, J = 9 Hz, H-3); 7.1-7.5 (m, 4H, H_{aro}'); 8.40 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.64 (d, 1H, J = 2.5 Hz, H-6); 10.52 (s, 1H, N*H*-CO-NH). Anal ($C_{12}H_9BrN_2O_5S$) C, H, N, S.

***N*-*i*-Propyl-*N'*-[2-(3-methylphenoxy)-5-nitrobenzenesulfonyl]urea (48k)**

Mp : 173-174 °C. IR (KBr) νmax : 3349, 3106, 2982, 2894, 2521, 1669, 1604, 1578, 1526, 1469, 1388, 1350, 1264, 1247, 1168, 1141, 1056, 1002, 935, 908, 889, 865, 833, 790, 755, 743, 728, 706, 681, 654, 631, 593, 572, 560, 532, 487 cm^{-1}. ^1H NMR (DMSO) δ: 1.01 (d, 6H, CH-(CH_3)$_2$); 2.36 (s, 3H, CH_3-3'); 3.61 (m, 1H, C*H*-(CH_3)$_2$); 6.29 (d, 1H, CO-N*H*-CH); 7.01 (m, 3H, H_{aro}'); 7.18 (d, 1H, J = 7.5 Hz, H_{aro}'); 7.42 (t, 1H, J = 7.8 Hz, H_{aro}'); 8.41 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.63 (d, 1H, J = 2.5 Hz, H-6); 10.69 (s, 1H, N*H*-CO-NH). Anal ($C_{17}H_{19}BrN_3O_6S$) C, H, N, S.

***N*-*n*-Butyl-*N'*-[2-(3-methylphenoxy)-5-nitrobenzenesulfonyl]urea (48l)**

Mp : 183-184 °C. IR (KBr) νmax : 3334, 3106, 2956, 2929, 2870, 1702, 1665, 1605, 1578, 1547, 1529, 1468, 1350, 1263, 1247, 1167, 1141, 1105, 1057, 1044, 1004, 934, 906, 887, 869, 843, 789, 746, 686, 655, 632, 593, 572, 532, 509, 478 cm^{-1}. ^1H NMR (DMSO) δ: 1.35 (t, 3H, CH_2-CH_2-CH_2-CH_3); 1.10-1.34 (m, 4H, CH_2-CH_2-CH_2-CH_3); 2.36 (s, 3H, CH_3-3'); 2.98 (q, 2H, NH-CH_2-CH_2-CH_2-CH_3); 6.48 (bs, 1H, CO-N*H*-butyl); 7.05 (m, 3H, H_{aro}'); 7.17 (d, 1H, J = 7.5 Hz, H_{aro}'); 7.43 (t, 1H, J = 7.8 Hz, H_{aro}'); 8.43 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.65 (d, 1H, J = 2.5 Hz, H-6); 10.99 (s, 1H, N*H*-CO-NH). Anal ($C_{18}H_{21}N_3O_6S$) C, H, N, S.

***N*-*t*-Butyl-*N'*-[2-(3-methylphenoxy)-5-nitrobenzenesulfonyl]urea (48m)**

Mp : 150-152 °C. IR (KBr) νmax : 3375, 3207, 3109, 2970, 2932, 2872, 1698, 1681, 1604, 1579, 1551, 1528, 1471, 1428, 1395, 1365, 1348, 1264, 1250, 1229, 1163, 1144, 1128, 1058, 1033, 1001, 937, 897, 876, 831, 792, 746, 728, 708, 688, 656, 631, 607, 586, 533, 475 cm^{-1}. ^1H NMR (DMSO) δ: 1.17 (s, 9H, NH-C(CH_3)$_3$); 2.36 (s, 3H, CH_3-3'); 6.28 (s, 1H, N*H*-C(CH_3)$_3$); 7.05 (m, 3H, H_{aro}'); 7.20 (d, 1H, J = 7.5 Hz, H_{aro}'); 7.45 (t, 1H, J = 7.8 Hz, H_{aro}'); 8.42 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.62(d, 1H, J = 2.5 Hz, H-6); 10.99 (s, 1H, N*H*-CO-NH). Anal ($C_{18}H_{21}N_3O_6S$) C, H, N, S.

***N*-*i*-Propyl-*N'*-[2-(4-methylphenoxy)-5-nitrobenzenesulfonyl]urea (48n)**

Mp : 183-184 °C. IR (KBr) νmax : 3346, 3103, 2969, 2925, 2893, 2522, 1887, 1689, 1670, 1603, 1584, 1539, 1527, 1505, 1469, 1408, 1352, 1264, 1235, 1212, 1189, 1161, 1135, 1105, 1055, 1020, 952, 909, 896, 863, 850, 833, 804, 760, 743, 733, 696, 678, 648, 602, 586, 542, 489 cm^{-1}. ^1H NMR (DMSO) δ: 1.01 (d, 6H, CH-(CH_3)$_2$), 2.35 (s, 3H, CH_3-4'); 3.61 (m, 1H, C*H*-(CH_3)$_2$), 6.31 (d, 1H, CO-N*H*-CH); 6.99 (d, 1H, J = 9 Hz, H-3); 7.06 (d, 2H, J = 9 Hz, H_{aro}'); 7.36 (d, 2H, J = 9 Hz, H_{aro}'); 8.42 (dd, 1H, J = 9

Hz, J = 2.5 Hz, H-4); 8.62 (d, 1H, J = 2.5 Hz, H-6); 10.80 (s, 1H, N*H*-CO-NH). Anal ($C_{17}H_{19}N_3O_6S$) C, H, N, S.

***N-n*-Propyl-*N'*-[2-(4-methylphenoxy)-5-nitrobenzenesulfonyl]urea (48o)**

Mp : 206-208 °C. IR (KBr) νmax : 3343, 3107, 2952, 2928, 2874, 1702, 1666, 1605, 1586, 1547, 1524, 1505, 1470, 1411, 1348, 1265, 1237, 1190, 1162, 1127, 1056, 1037, 1019, 976, 913, 862, 840, 802, 758, 747, 735, 716, 651, 600, 589, 567, 539, 493 cm^{-1}. ^1H NMR (DMSO) δ: 0.77 (t, 3H, J = 8 Hz; CH_2-CH_2-CH_3); 1.33 (m, 2H, CH_2-CH_2-CH_3); 2.35 (s, 3H, CH_3-4'); 2.94 (m, 2H, CH_2-CH_2-CH_3); 6.48 (bs, 1H, CO-N*H*-CH_2-CH_2-CH_3); 6.99 (d, 1H, J = 9 Hz, H-3); 7.13 (d, 2H, J = 9 Hz, H_{aro}'); 7.35 (d, 2H, J = 9 Hz, H_{aro}'); 8.42 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.64 (d, 1H, J = 2.5 Hz, H-6); 11.02 (s, 1H, N*H*-CO-NH). Anal ($C_{17}H_{19}N_3O_6S$) C, H, N, S.

***N-t*-Butyl-*N'*-[2-(2-bromophenoxy)-5-nitrobenzenesulfonyl]urea (48p)**

Mp : 169-173 °C. IR (KBr) νmax : 3388, 3105, 2971, 2933, 2872, 1721, 1692, 1595, 1575, 1530, 1467, 1444, 1395, 1347, 1266, 1243, 1205, 1163, 1139, 1127, 1057, 1028, 943, 905, 880, 832, 799, 969, 745, 709, 659, 647, 625, 605, 585, 520, 461 cm^{-1}. ^1H NMR ($CDCl_3$) δ: 1.19 (s, 9H, NH-C(CH_3)$_3$); 6.39 (s, 1H, N*H*-C(CH_3)$_3$); 6.76 (d, 1H, J = 9 Hz, H-3); 7.28-7.48 (m, 4H, H_{aro}'); 7.74 (d, 1H, J = 8 Hz, H-3'); 8.35 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.89 (d, 1H, J = 2.5 Hz, H-6). Anal ($C_{17}H_{18}BrN_3O_6S$) C, H, N, S.

***N-n*-Pentyl-*N'*-[2-(2-bromophenoxy)-5-nitrobenzenesulfonyl]urea (48q)**

Mp : 130-135 °C. IR (KBr) νmax : 3343, 3104, 2955, 2929, 2859, 1691, 1663, 1597, 1575, 1536, 1467, 1350, 1265, 1241, 1206, 1172, 1130, 1103, 1056, 1046, 1027, 954, 909, 828, 799, 770, 744, 735, 709, 687, 659, 646, 625, 593, 522 cm^{-1}. ^1H NMR ($CDCl_3$) δ: 0.83 (t, 3H, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 1.10-1.38 (m, 6H, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 2.36 (s, 3H, CH_3-4'); 3.08 (q, 2H, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 6.46 (bs, 1H, NH-CO-N*H*-CH_2-CH_2-CH_2-CH_2-CH_3); 6.77 (d, 1H, J = 9 Hz, H-3); 7.26-7.29 (m, 2H, H_{aro}'); 7.45 (t, 1H, J = 8 Hz, H-5'), 7.73 (d, 1H, J = 8 Hz, H-3'); 7.89 (bs, 1H, N*H*-CO-NH); 8.35 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.90 (d, 1H, J = 2.5 Hz, H-6). Anal ($C_{18}H_{20}BrN_3O_6S$) C, H, N, S.

***N-t*-Butyl-*N'*-[2-(3-bromophenoxy)-5-nitrobenzenesulfonyl]urea (48r)**

Mp : 148-149 °C. IR (KBr) νmax : 3396, 3104, 2979, 2869, 1697, 1604, 1576, 1533, 1466, 1429, 1395, 1350, 1267, 1243, 1219, 1188, 1162, 1127,

1086, 1057, 1032, 999, 954, 908, 869, 847, 840, 789, 766, 750, 732, 695, 668, 654, 627, 598, 582, 545, 475 cm^{-1}. ^1H NMR ($CDCl_3$) δ: 1.17 (s, 9H, NH-C(CH_3)$_3$); 6.32 (s, 1H, N*H*-C(CH_3)$_3$); 6.94 (d, 1H, J = 9 Hz, H-3); 7.16 (dd, 1H, J = 8 Hz, J =2 Hz H-6'); 7.36-7.40 (m, 2H, H_{aro}'); 7.51 (d, 1H, J = 8 Hz, H-4'); 7.79 (bs, 1H, N*H*-CO-NH); 8.37 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.88 (d, 1H, J = 2.5 Hz, H-6). Anal ($C_{17}H_{18}BrN_3O_6S$) C, H, N, S.

***N*-*n*-Pentyl-*N'*-[2-(3-bromophenoxy)-5-nitrobenzenesulfonyl]urea (48s)**

Mp : 136-138 °C. IR (KBr) νmax : 3343, 3105, 2931, 2869, 1704, 1668, 1635, 1605, 1576, 1548, 1527, 1465, 1349, 1260, 1237, 1188, 1167, 1127, 1104, 1060, 1039, 1000, 976, 909, 882, 833, 803, 780, 747, 734, 705, 685, 669, 650, 629, 592, 573, 525, 499, 473 cm^{-1}. ^1H NMR ($CDCl_3$) δ: 0.83 (t, 3H, NH-CH_2-CH_2-CH_2-C*H*$_3$); 1.08-1.39 (m, 6H, NH-CH_2-C*H*$_2$-C*H*$_2$-C*H*$_2$-CH_3); 3.10 (q, 2H, NH-C*H*$_2$-CH_2-CH_2-CH_2-CH_3); 6.45 (bs, 1H, NH-CO-N*H*-CH_2-CH_2-CH_2-CH_2-CH_3), 6.95 (d, 1H, J = 9 Hz, H-3); 7.14 (dd, 1H, J = 8 Hz, J =2 Hz H-6'); 7.35-7.39 (m, 2H, H_{aro}'); 7.50 (d, 1H, J = 8 Hz, H-4'), 7.99 (bs, 1H, N*H*-CO-NH); 8.37 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.88 (d, 1H, J = 2.5 Hz, H-6). Anal ($C_{18}H_{20}BrN_3O_6S$) C, H, N, S.

***N*-*t*-Butyl-*N'*-[2-(4-bromophenoxy)-5-nitrobenzenesulfonyl]urea (48t)**

Mp : 173-174 °C. IR (KBr) νmax : 3410, 3355, 3251, 3103, 2969, 2932, 2871, 1721, 1698, 1601, 1578, 1524, 1472, 1441, 1396, 1347, 1265, 1244, 1196, 1159, 1140, 1124, 1097, 1067, 1056, 1027, 1011, 904, 881, 847, 831, 789, 752, 645, 587, 508, 472 cm^{-1}.^1H NMR ($CDCl_3$) δ: 1.17 (s, 9H, NH-C(CH_3)$_3$); 6.34 (s, 1H, N*H*-C(CH_3)$_3$); 6.91 (d, 1H, J = 9 Hz, H-3); 7.11 (d, 2H, J = 8.5 Hz, H_{aro}'); 7.62 (d, 2H, J = 8.5 Hz, H_{aro}'); 8.35 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.86 (d, 1H, J = 2.5 Hz, H-6). Anal ($C_{17}H_{18}BrN_3O_6S$) C, H, N, S.

***N*-*n*-Pentyl-*N'*-[2-(4-bromophenoxy)-5-nitrobenzenesulfonyl]urea (48u)**

Mp : 166-168 °C. IR (KBr) νmax : 3336, 3102, 2958, 2932, 2859, 1702, 1661, 1597, 1575, 1551, 1527, 1481, 1469, 1408, 1347, 1262, 1244, 1191, 1161, 1125, 1106, 1068, 1056, 1028, 1010, 956, 908, 871, 833, 787, 747, 645, 590, 571, 508, 489 cm^{-1}. ^1H NMR (DMSO) δ: 0.81 (t, 3H, NH-CH_2-CH_2-CH_2-C*H*$_3$); 1.08-1.33 (m, 6H, NH-CH_2-C*H*$_2$-C*H*$_2$-C*H*$_2$-CH_3); 2.94 (q, 2H, NH-C*H*$_2$-CH_2-CH_2-CH_2-CH_3); 6.44 (bs, 1H, NH-CO-N*H*-CH_2-CH_2-CH_2-CH_2-CH_3), 7.13 (d, 1H, J = 9 Hz, H-3); 7.19 (d, 2H, J =

8.5 Hz, H_{aro}'); 7.71 (d, 2H, J = 8.5 Hz, H_{aro}'); 8.43 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.64 (d, 1H, J = 2.5 Hz, H-6); 10.98 (s, 1H, N*H*-CO-NH). Anal ($C_{18}H_{20}BrN_3O_6S$) C, H, N, S.

***N-t*-Butyl-*N'*-[2-(2-chlorophenoxy)-5-nitrobenzenesulfonyl]urea (48v)**

Mp : 186-187 °C. IR (KBr) νmax : 3365, 3186, 3099, 2968, 2906, 1669, 1595, 1578, 1537, 1522, 1471, 1395, 1344, 1267, 1249, 1162, 1141, 1126, 1063, 1036, 948, 917, 905, 877, 837, 801, 769, 752, 745, 713, 681, 647, 629, 585, 570, 524, 467, 454 cm^{-1}. ^1H NMR (DMSO) δ: 1.16 (s, 9H, NH-C(C*H*$_3$)$_3$); 6.21 (s, 1H, N*H*-C(CH$_3$)$_3$); 6.86 (d, 1H, J = 9 Hz, H-3); 7.35 (d, 1H, J = 9 Hz, H-6'); 7.46 (t, 1H, J = 8 Hz, H-4'), 7.56 (t, 1H, J = 8 Hz, H-5'), 7.75 (d, 1H, J = 8 Hz, H-3'), 8.41 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.65 (d, 1H, J = 2.5 Hz, H-6); 10.94 (s, 1H, N*H*-CO-NH). Anal ($C_{17}H_{18}ClN_3O_6S$) C, H, N, S.

***N-n*-Pentyl-*N'*-[2-(2-chlorophenoxy)-5-nitrobenzenesulfonyl]urea (48w)**

Mp : 132-133 °C. IR (KBr) νmax : 3343, 3106, 2956, 2931, 2870, 1664, 1597, 1577, 1529, 1470, 1448, 1347, 1268, 1241, 1207, 1168, 1127, 1104, 1062, 1029, 956, 907, 830, 799, 768, 745, 713, 680, 647, 626, 591, 525, 492 cm^{-1}. ^1H NMR (DMSO) δ: 0.83 (t, 3H, NH-CH$_2$-CH$_2$-CH$_2$-C*H*$_3$); 1.10-1.34 (m, 6H, NH-CH$_2$-C*H*$_2$-C*H*$_2$-C*H*$_2$-CH$_3$); 2.94 (q, 2H, NH-C*H*$_2$-CH$_2$-CH$_2$-CH$_2$-CH$_3$); 6.40 (bs, 1H, NH-CO-N*H*-CH$_2$-CH$_2$-CH$_2$-CH$_2$-CH$_3$); 6.85 (d, 1H, J = 9 Hz, H-3); 7.34 (d, 1H, J = 9 Hz, H-6'); 7.44 (t, 1H, J = 8 Hz, H-4'), 7.53 (t, 1H, J = 8 Hz, H-5'), 7.73 (d, 1H, J = 8 Hz, H-3'), 8.40 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.66 (d, 1H, J = 2.5 Hz, H-6) 10.50 (s, 1H, N*H*-CO-NH). Anal ($C_{18}H_{20}ClN_3O_6S$) C, H, N, S.

***N-t*-Butyl-*N'*-[2-(3-chlorophenoxy)-5-nitrobenzenesulfonyl]urea (48x)**

Mp : 154-156 °C. IR (KBr) νmax : 3397, 3106, 2979, 2843, 2768, 2362, 1697, 1605, 1578, 1534, 1467, 1433, 1395, 1352, 1268, 1243, 1219, 1193, 1162, 1127, 1087, 1056, 1032, 1001, 979, 956, 915, 896, 869, 848, 840, 802, 791, 768, 750, 732, 703, 674, 657, 628, 598, 583, 546, 523, 475 cm^{-1}. ^1H NMR ($CDCl_3$) δ: 1.18 (s, 9H, NH-C(C*H*$_3$)$_3$); 6.27 (s, 1H, N*H*-C(CH$_3$)$_3$); 6.94 (d, 1H, J = 9 Hz, H-3); 7.11 (dd, 1H, J = 8 Hz, J =2 Hz H-6'); 7.25 (s, 1H, H-2'); 7.36 (d, 1H, J = 8 Hz, H-4'); 7.44 (t, 1H, J = 8 Hz, H-5'), 7.58 (s, 1H, N*H*-CO-NH); 8.37 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.87 (d, 1H, J = 2.5 Hz, H-6). Anal ($C_{17}H_{18}ClN_3O_6S$) C, H, N, S.

***N-n*-Pentyl-*N'*-[2-(3-chlorophenoxy)-5-nitrobenzenesulfonyl]urea (48y)**

Mp : 158-159 °C. IR (KBr) νmax : 3343, 3106, 2953, 2933, 2868, 1704, 1670, 1606, 1578, 1547, 1529, 1467, 1351, 1260, 1236, 1193, 1167, 1147, 1140, 1121, 1104, 1087, 1059, 1039, 1002, 977, 915, 888, 868, 848, 835, 806, 777, 748, 737, 725, 706, 675, 653, 630, 593, 571, 532, 500, 472 cm^{-1}. ^1H NMR ($CDCl_3$) δ: 0.83 (t, 3H, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 1.10-1.38 (m, 6H, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 2.36 (s, 3H, CH_3-4'); 3.08 (q, 2H, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 6.46 (bs, 1H, NH-CO-N*H*-CH_2-CH_2-CH_2-CH_2-CH_3); 6.94 (d, 1H, J = 9 Hz, H-3); 7.10 (dd, 1H, J = 8 Hz, J =2 Hz H-6'); 7.26 (s, 1H, H-2'); 7.35 (d, 1H, J = 8 Hz, H-4'); 7.43 (t, 1H, J = 8 Hz, H-5'), 8.09 (bs, 1H, N*H*-CO-NH); 8.37 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.87 (d, 1H, J = 2.5 Hz, H-6). Anal ($C_{18}H_{20}ClN_3O_6S$) C, H, N, S.

_N_-_t_-Butyl-_N'_-[2-(4-chloroxyphenoxy)-5-nitrobenzenesulfonyl]urea (48z)

Mp : 161-163 °C. IR (KBr) νmax : 3409, 3339, 3234, 3104, 2975, 2933, 2872, 1721, 1699, 1603, 1582, 1524, 1486, 1472, 1394, 1348, 1266, 1196, 1158, 1141, 1124, 1089, 1057, 1028, 1013, 904, 880, 853, 834, 791, 752, 717, 665, 650, 586, 518, 486 cm^{-1}. ^1H NMR (DMSO) δ: 1.16 (s, 9H, NH-C(CH_3)$_3$); 6.24 (s, 1H, N*H*-C(CH_3)$_3$); 7.13 (d, 1H, J = 9 Hz, H-3); 7.19 (d, 2H, J = 8.5 Hz, H_{aro}'); 7.61 (d, 2H, J = 8.5 Hz, H_{aro}'); 8.43 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.62 (d, 1H, J = 2.5 Hz, H-6); 10.51 (s, 1H, N*H*-CO-NH). Anal ($C_{17}H_{18}ClN_3O_6S$) C, H, N, S.

_N_-_n_-Pentyl-_N'_-[2-(4-chloroxyphenoxy)-5-nitrobenzenesulfonyl]urea (48aa)

Mp : 162-164 °C. IR (KBr) νmax : 3335, 3102, 2959, 2933, 2860, 1702, 1661, 1602, 1580, 1551, 1523, 1485, 1470, 1404, 1347, 1263, 1244, 1192, 1161, 1126, 1090, 1056, 1029, 1013, 956, 903, 872, 842, 790, 747, 651, 591, 578, 518, 502, 483 cm^{-1}. ^1H NMR (DMSO) δ: 0.82 (t, 3H, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 1.10-1.32 (m, 6H, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 2.95 (q, 2H, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 6.44 (bs, 1H, NH-CO-N*H*-CH_2-CH_2-CH_2-CH_2-CH_3); 7.13 (d, 1H, J = 9 Hz, H-3); 7.19 (d, 2H, J = 8.5 Hz, H_{aro}'); 7.58 (d, 2H, J = 8.5 Hz, H_{aro}'); 8.43 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.64 (d, 1H, J = 2.5 Hz, H-6); 10.97 (s, 1H, N*H*-CO-NH). Anal ($C_{18}H_{20}ClN_3O_6S$) C, H, N, S.

_N_-_t_-Butyl-_N'_-[2-(3-iodophenoxy)-5-nitrobenzenesulfonyl]urea (48ab)

Mp : 154-157 °C. IR (KBr) νmax : 3395, 2976, 2868, 1694, 1603, 1573, 1530, 1463, 1424, 1395, 1349, 1266, 1242, 1217, 1188, 1161, 1127, 1056,

1031, 997, 950, 906, 870, 845, 788, 766, 750, 714, 691, 657, 625, 598, 582, 544, 475 cm^{-1}. ^1H NMR (DMSO) δ: 1.18 (s, 9H, NH-C(CH_3)$_3$); 6.29 (s, 1H, N*H*-C(CH_3)$_3$); 7.13 (d, 1H, J = 9 Hz, H-3); 7.24 (dd, 1H, J = 8 Hz, J =2 Hz H-6'); 7.35 (t, 1H, J = 8 Hz, H-5'); 7.49 (s, 1H, H-2'); 7.75 (d, 1H, J = 8 Hz, H-4'); 8.44 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.61 (d, 1H, J = 2.5 Hz, H-6); 10.97 (s, 1H, N*H*-CO-NH). Anal ($C_{17}H_{18}IN_3O_6S$) C, H, N,S.

***N-n*-Pentyl-*N'*-[2-(3-iodophenoxy)-5-nitrobenzenesulfonyl]urea (48ac)**

Mp : 123-126 °C. IR (KBr) νmax : 3324, 3104, 2958, 2933, 2871, 1698, 1659, 1604, 1572, 1547, 1530, 1463, 1417, 1346, 1291, 1263, 1237, 1214, 1185, 1169, 1136, 1127, 1104, 1055, 997, 951, 910, 871, 835, 802, 776, 746, 734, 714, 703, 680, 655, 626, 597, 575, 522, 503, 470 cm^{-1}. ^1H NMR (DMSO) δ: 0.80 (t, 3H, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 1.10-1.34 (m, 6H, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 2.95 (q, 2H, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 6.47 (bs, 1H, N*H*-CO-NH-CH_2-CH_2-CH_2-CH_2-CH_3); 7.13 (d, 1H, J = 9 Hz, H-3); 7.22 (dd, 1H, J = 8 Hz, J =2 Hz H-6'); 7.32 (t, 1H, J = 8 Hz, H-5'); 7.48 (s, 1H, H-2'); 7.73 (d, 1H, J = 8 Hz, H-4'); 8.43 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.63 (d, 1H, J = 2.5 Hz, H-6); 10.97 (s, 1H, N*H*-CO-NH). Anal ($C_{18}H_{20}IN_3O_6S$) C, H, N,S.

***N-t*-Butyl-*N'*-[2-(4-iodophenoxy)-5-nitrobenzenesulfonyl]urea (48ad)**

Mp : 176-179 °C. IR (KBr) νmax : 3410, 3349, 3246, 3103, 2966, 2930, 2868, 1720, 1698, 1600, 1574, 1527, 1471, 1439, 1394, 1345, 1263, 1242, 1197, 1160, 1139, 1123, 1096, 1054, 1027, 1007, 903, 878, 847, 828, 787, 753, 711, 639, 586, 506, 471 cm^{-1}. ^1H NMR (DMSO) δ: 1.16 (s, 9H, NH-C(CH_3)$_3$); 6.23 (s, 1H, N*H*-C(CH_3)$_3$); 6.98 (d, 2H, J = 8.5 Hz, H_{aro}'); 7.14 (d, 2H, J = 9 Hz, H-3); 7.88 (d, 2H, J = 8.5 Hz, H_{aro}'); 8.42 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.62 (d, 1H, J = 2.5 Hz, H-6); 10.50 (s, 1H, N*H*-CO-NH). Anal ($C_{17}H_{18}IN_3O_6S$) C, H, N, S.

***N-n*-Pentyl-*N'*-[2-(4-iodophenoxy)-5-nitrobenzenesulfonyl]urea (48ae)**

Mp : 151-154 °C. IR (KBr) νmax : 3344, 3101, 2956, 2928, 2858, 1703, 1665, 1596, 1572, 1550, 1525, 1470, 1395, 1345, 1263, 1244, 1193, 1162, 1126, 1056, 1024, 1008, 965, 903, 877, 835, 786, 748, 717, 641, 590, 573, 505, 483 cm^{-1}. ^1H NMR (DMSO) δ: 0.80 (t, 3H, NH-CH_2-CH_2-CH_2-CH_3); 1.08-1.33 (m, 6H, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 2.93 (q, 2H, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 6.43 (bs, 1H, NH-CO-N*H*-CH_2-CH_2-CH_2-CH_2-CH_3); 6.98 (d, 2H, J = 8.5 Hz, H_{aro}'); 7.13 (d, 2H, J = 9 Hz, H-3);

7.86 (d, 2H, J = 8.5 Hz, H_{aro}'); 8.42 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.63 (d, 1H, J = 2.5 Hz, H-6); 10.97 (s, 1H, N*H*-CO-NH). Anal ($C_{18}H_{20}IN_3O_6S$) C, H, N, S.

***N*-*t*-Butyl-*N'*-[2-(4-methoxyphenoxy)-5-nitrobenzenesulfonyl]urea (48af)**

Mp : 176-177 °C. IR (KBr) νmax : 3338, 3211, 3110, 2971, 2934, 2841, 2054, 1689, 1661, 1606, 1588, 1556, 1527, 1505, 1470, 1442, 1409, 1395, 1352, 1266, 1249, 1240, 1191, 1163, 1136, 1128, 1099, 1068, 1056, 1032, 1009, 952, 905, 879, 844, 804, 761, 747, 694, 646, 602, 580, 570, 548, 525, 492, 475 cm^{-1}. ^1H NMR (DMSO) δ: 1.17 (s, 9H, NH-C(CH_3)$_3$); 3.80 (s, 3H, O-CH_3); 6.28 (s, 1H, N*H*-C(CH_3)$_3$); 6.98 (d, 1H, J = 9 Hz, H-3); 7.11 (s, 4H, H_{aro}'); 8.42 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.61 (d, 1H, J = 2.5 Hz, H-6); 10.48 (s, 1H, N*H*-CO-NH). Anal ($C_{18}H_{21}N_3O_7S$) C, H, N, S.

***N*-*n*-Pentyl-*N'*-[2-(4-methoxyphenoxy)-5-nitrobenzenesulfonyl]urea (48ag)**

Mp : 170-172 °C. IR (KBr) νmax : 3310, 3209, 3112, 3007, 2952, 2932, 2869, 2050, 1695, 1655, 1605, 1587, 1564, 1524, 1504, 1472, 1443, 1409, 1345, 1299, 1270, 1241, 1190, 1165, 1141, 1125, 1104, 1055, 1030, 1007, 958, 908, 881, 835, 803, 758, 745, 695, 675, 651, 593, 569, 514, 462 cm^{-1}. ^1H NMR (DMSO) δ: 0.80 (t, 3H, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 1.10-1.35 (m, 6H, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 2.96 (q, 2H, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 3.80 (s, 3H, O-CH_3); 6.46 (bs, 1H, NH-CO-N*H*-CH_2-CH_2-CH_2-CH_2-CH_3); 6.98 (d, 1H, J = 9 Hz, H-3); 7.07-7.12 (m, 4H, H_{aro}'); 8.42 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.63 (d, 1H, J = 2.5 Hz, H-6); 10.94 (s, 1H, N*H*-CO-NH). Anal ($C_{19}H_{23}N_3O_7S$) C, H, N, S.

***N*-*t*-Butyl-*N'*-[2-(4-propoxyphenoxy)-5-nitrobenzenesulfonyl]urea (48ah)**

Mp : 145-149 °C. IR (KBr) νmax : 3333, 3221, 3110, 2977, 2936, 2874, 1691, 1659, 1606, 1588, 1559, 1528, 1504, 1470, 1440, 1396, 1349, 1264, 1251, 1235, 1188, 1162, 1136, 1125, 1103, 1056, 1035, 1020, 977, 917, 904, 878, 840, 785, 773, 756, 695, 646, 616, 580, 520, 474 cm^{-1}. ^1H NMR (DMSO) δ: 0.98 (t, 3H, J =7 Hz, O-CH_2-CH_2-CH_3); 1.17 (s, 9H, NH-C(CH_3)$_3$); 1.75 (m, 2H, O-CH_2-CH_2-CH_2-CH_3); 3.96 (t, 2H, O-CH_2-CH_2-CH_2-CH_3); 6.28 (s, 1H, N*H*-C(CH_3)$_3$); 6.98 (d, 1H, J = 9 Hz, H-3); 7.09 (s, 4H, H_{aro}'); 8.41 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.60 (d, 1H, J = 2.5 Hz, H-6); 10.97 (s, 1H, N*H*-CO-NH). Anal ($C_{20}H_{25}N_3O_7S$) C, H, N, S.

***N*-*n*-Pentyl-*N'*-[2-(4-propoxyphenoxy)-5-nitrobenzenesulfonyl]urea (48ai)**

Mp : 148-150°C. IR (KBr) νmax : 3339, 3186, 3108, 2933, 2871, 1660, 1605, 1588, 1555, 1527, 1504, 1470, 1407, 1346, 1297, 1268, 1252, 1237, 1189, 1164, 1138, 1126, 1106, 1056, 1008, 978, 911, 838, 784, 746, 698, 651, 616, 593, 572, 520 cm^{-1}. ^1H NMR (DMSO) δ: 0.81 (t, 3H, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 0.98 (t, 3H, J =7 Hz, O-CH_2-CH_2-CH_3); 1.10-1.34 (m, 6H, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 1.75 (m, 2H, O-CH_2-CH_2-CH_3); 2.95 (q, 2H, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 3.96 (t, 2H, O-CH_2-CH_2-CH_3); 6.46 (bs, 1H, NH-CO-N*H*-CH_2-CH_2-CH_2-CH_2-CH_3); 6.99 (d, 1H, J = 9 Hz, H-3); 7.05-7.10 (m, 4H, H_{aro}'); 8.41 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.62 (d, 1H, J = 2.5 Hz, H-6); 10.93 (s, 1H, N*H*-CO-NH). Anal ($C_{21}H_{27}N_3O_7S$) C, H, N, S.

***N-t*-Butyl-*N'*-[2-(4-ethoxyphenoxy)-5-nitrobenzenesulfonyl]urea (48aj)**

Mp : 178-179 °C. IR (KBr) νmax : 3333, 3207, 3113, 2980, 2934, 2877, 1688, 1658, 1607, 1587, 1560, 1530, 1505, 1472, 1443, 1396, 1368, 1349, 1267, 1249, 1236, 1188, 1162, 1136, 1124, 1101, 1071, 1055, 1007, 952, 917, 905, 880, 863, 842, 829, 804, 756, 734, 695, 646, 615, 580, 522, 476 cm^{-1}. ^1H NMR (DMSO) δ: 1.17 (s, 9H, NH-C(CH_3)$_3$); 1.45 (t, 3H, J =7 Hz, O-CH_2-CH_3); 4.06 (q, 2H, J =7 Hz, O-CH_2-CH_3); 6.28 (s, 1H, N*H*-C(CH_3)$_3$); 6.98 (d, 1H, J = 9 Hz, H-3); 7.0 (s, 4H, H_{aro}'); 8.42 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.61 (d, 1H, J = 2.5 Hz, H-6); 10.47 (s, 1H, N*H*-CO-NH). Anal ($C_{19}H_{23}N_3O_7S$) C, H, N, S.

***N-n*-Pentyl-*N'*-[2-(4-ethoxyphenoxy)-5-nitrobenzenesulfonyl]urea (48ak)**

Mp : 182-185 °C. IR (KBr) νmax : 3322, 3202, 3109, 2950, 2931, 2868, 2365, 1696, 1657, 1604, 1587, 1563, 1524, 1504, 1472, 1407, 1344, 1298, 1272, 1239, 1189, 1165, 1140, 1125, 1115, 1053, 1006, 958, 910, 884, 838, 798, 767, 745, 698, 675, 652, 616, 596, 571, 516, 460 cm^{-1}. ^1H NMR (DMSO) δ: 0.81 (t, 3H, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 1.10-1.36 (m, 9H, NH-CH_2-CH_2-CH_2-CH_2-CH_3; O-CH_2-CH_3); 2.95 (q, 2H, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 4.05 (t, 2H, O-CH_2-CH_3); 6.46 (bs, 1H, NH-CO-N*H*-CH_2-CH_2-CH_2-CH_2-CH_3); 6.98 (d, 1H, J = 9 Hz, H-3); 7.05-7.10 (m, 4H, H_{aro}'); 8.42 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.62 (d, 1H, J = 2.5 Hz, H-6); 10.47 (s, 1H, N*H*-CO-NH). Anal ($C_{20}H_{25}N_3O_7S$) C, H, N, S.

***N-t*-Butyl-*N'*-[2-(3-methoxyphenoxy)-5-nitrobenzenesulfonyl]urea (48al)**

Mp : 128-131 °C. IR (KBr) νmax : 3394, 3105, 3068, 3034, 2980, 2869, 2838, 1698, 1604, 1579, 1541, 1526, 1491, 1471, 1449, 1421, 1394, 1366, 1351, 1278, 1264, 1242, 1217, 1164, 1142, 1073, 1054, 1028, 996, 949,

918, 899, 882, 856, 800, 779, 748, 705, 681, 665, 648, 628, 602, 569 cm^{-1}. ^1H NMR ($CDCl_3$) δ: 1.18 (s, 9H, NH-C(CH_3)$_3$); 3.82 (s, 1H, O-CH_3); 6.36 (s, 1H, N*H*-C(CH_3)$_3$); 6.76-6.78 (m, 2H, H-6', H-2'); 6.90 (dd, 1H, J = 8 Hz, J =2 Hz H-4'); 6.96 (d, 1H, J = 9 Hz, H-3); 7.38 (t, 1H, J = 8 Hz, H-5'), 7.46 (bs, 1H, N*H*-CO-NH); 8.34 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.85 (d, 1H, J = 2.5 Hz, H-6). Anal ($C_{18}H_{21}N_3O_7S$) C, H, N, S.

***N*-*t*-Butyl-*N'*-[2-(3-methyl-4-chlorophenoxy)-5-nitrobenzenesulfonyl]urea (48am)**

Mp : 143-147 °C. IR (KBr) νmax : 3345, 3246, 3106, 2977, 2933, 1689, 1662, 1606, 1576, 1530, 1471, 1396, 1350, 1268, 1243, 1163, 1152, 1126, 1054, 1034, 951, 922, 900, 876, 833, 814, 746, 702, 648, 606, 585, 467 cm^{-1}.^1H NMR ($CDCl_3$) δ: 1.16 (s, 9H, NH-C(CH_3)$_3$); 2.41 (s, 3H, CH_3-4'); 6.34 (s, 1H, N*H*-C(CH_3)$_3$); 6.92 (d, 1H, J = 9 Hz, H-3); 6.99 (dd, 1H, J = 8 Hz, J =2 Hz H-6'); 7.10 (s, 1H, H-2'); 7.45 (d, 1H, J = 8.5 Hz, H-5'); 7.74 (bs, 1H, N*H*-CO-NH); 8.35 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.85 (d, 1H, J = 2.5 Hz, H-6). Anal ($C_{18}H_{20}ClN_3O_6S$) C, H, N, S.

***N*-*n*-Pentyl-*N'*-[2-(3-methyl-4-chlorophenoxy)-5-nitrobenzenesulfonyl]urea (48an)**

Mp : 174-176 °C. IR (KBr) νmax : 3336, 3103, 2956, 2930, 2860, 1663, 1604, 1575, 1550, 1528, 1470, 1403, 1347, 1268, 1243, 1167, 1152, 1125, 1101, 1053, 1004, 953, 921, 896, 873, 831, 815, 745, 647, 591, 506 cm^{-1}. ^1H NMR ($CDCl_3$) δ: 0.82 (t, 3H, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 1.12-1.39 (m, 6H, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 2.36 (s, 3H, CH_3-4'); 3.10 (q, 2H, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 6.48 (bs, 1H, NH-CO-N*H*-CH_2-CH_2-CH_2-CH_2-CH_3), 6.92 (d, 1H, J = 9 Hz, H-3); 6.99 (dd, 1H, J = 8 Hz, J =2 Hz H-6'); 7.10 (d, 1H, J =5 Hz, H-2'); 7.45 (d, 1H, J = 8.5 Hz, H-5'); 7.73 (bs, 1H, N*H*-CO-NH); 8.35 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.86 (d, 1H, J = 2.5 Hz, H-6). Anal ($C_{19}H_{22}ClN_3O_6S$) C, H, N, S.

***N*-*t*-Butyl-*N'*-[2-(2-methoxy-4-methylphenoxy)-5-nitrobenzenesulfonyl]urea (48ao)**

Mp : 160-162 °C. IR (KBr) νmax : 3382, 3095, 3074, 2967, 2938, 2865, 2776, 1699, 1638, 1607, 1584, 1547, 1520, 1506, 1470, 1410, 1391, 1351, 1291, 1271, 1246, 1231, 1199, 1162, 1123, 1057, 1033, 955, 927, 901, 887, 841, 830, 782, 748, 722, 697, 646, 611, 534, 516, 459 cm^{-1}. ^1H NMR (DMSO) δ: 1.17 (s, 9H, NH-C(CH_3)$_3$); 2.37 (s, 3H, CH_3-2'); 3.72 (s, 1H, O-CH_3); 6.24 (s, 1H, N*H*-C(CH_3)$_3$); 6.82 (d, 1H, J = 9 Hz, H-3); 6.90 (d, 1H, J = 8 Hz, H-6'); 7.00 (d, 1H, J = 8 Hz, H-5'); 7.127 (s, 1H, H-3');

8.37 (d, 1H, J = 9 Hz, H-4); 8.60 (s, 1H, H-6); 10.42 (s, 1H, N*H*-CO-NH). Anal ($C_{19}H_{23}N_3O_7S$) C, H, N, S.

Compounds with a sulphur bridge (49)

N-t-Butyl-*N'*-[2-(4-methylphenylthio)-5-nitrobenzenesulfonyl]urea (49a)

Mp : 181-183 °C. IR (KBr) νmax : 3329, 3217, 3100, 2968, 2930, 2870, 1692, 1661, 1592, 1576, 1560, 1519, 1492, 1447, 1395, 1367, 1344, 1270, 1242, 1220, 1161, 1146, 1124, 1100, 1087, 1064, 1043, 1017, 950, 917, 894, 870, 835, 816, 797, 773, 740, 709, 679, 638, 583, 544, 510, 465 cm^{-1}. ^1H NMR (DMSO) δ: 1.16 (s, 9H, NH-C(CH_3)$_3$); 2.4 (s, 3H, CH_3-4'); 6.36 (s, 1H, CO-N*H*-CH_2); 7.07 (d, 1H, *J* = 9 Hz, H-3); 7.4-7.48 (m, 4H, H_{aro}'); 8.27 (dd, 1H, *J* = 9 Hz, *J* = 2.5 Hz, H-4); 8.63 (d, 1H, *J* = 2.5 Hz, H-6); 10.63 (s, 1H, SO_2-N*H*-CO-). Anal ($C_{18}H_{21}N_3O_5S_2$) C, H, N, S.

N-n-Pentyl-*N'*-[2-(4-methylphenylthio)-5-nitrobenzenesulfonyl]urea (49b)

Mp : 149-151 °C. IR (KBr) νmax : 3345, 3102, 2953, 2928, 2868, 1664, 1592, 1574, 1547, 1520, 1492, 1446, 1343, 1244, 1211, 1167, 1123, 1100, 1088, 1062, 1041, 1017, 963, 906, 888, 870, 835, 812, 770, 739, 710, 680, 637, 592, 509, 494 cm^{-1}. ^1H NMR (DMSO) δ: 0.8 (t, 2H, *J* =7 Hz, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 1.1-1.35 (m, 6H, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 2.4 (s, 3H, CH_3-4'); 2.96 (q, 2H, *J* =7 Hz, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 6.5 (bs, 1H, CO-N*H*-CH_2); 7.03 (d, 1H, *J* = 9 Hz, H-3); 7.39- 7.48 (m, 4H, H_{aro}'); 8.26 (dd, 1H, *J* = 9 Hz, *J* = 2.5 Hz, H-4); 8.65 (d, 1H, *J* = 2.5 Hz, H-6); 11.12 (s, 1H, SO_2-N*H*-CO-). Anal ($C_{19}H_{23}N_3O_5S_2$) C, H, N, S.

N-n-Pentyl-*N'*-[2-(2-methylphenylthio)-5-nitrobenzenesulfonyl]urea (49c)

Mp : 156-158 °C. IR (KBr) νmax : 3337, 3100, 2955, 2932, 2861, 1666, 1622, 1576, 1520, 1458, 1382, 1345, 1262, 1230, 1164, 1142, 1122, 1073, 1040, 958, 912, 879, 835, 762, 739, 714, 676, 659, 584, 465 cm^{-1}. ^1H NMR (DMSO) δ: 0.8 (t, 2H, *J* =7 Hz, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 1.1-1.35 (m, 6H, NH-CH2-CH_2-CH_2-CH_2-CH_3); 2.21 (s, 3H, CH_3-2'); 2.96 (q, 2H, *J* =7 Hz, NH-C*H*2-CH_2-CH_2-CH_2-CH_3); 6.50 (bs, 1H, CO-N*H*-CH_2); 6.86 (d, 1H, *J* = 9 Hz, H-3); 7.40-7.65 (m, 4H, H_{aro}'); 8.25 (dd, 1H, *J* = 9 Hz, *J* = 2.5 Hz, H-4); 8.67 (d, 1H, *J* = 2.5 Hz, H-6); 11.18 (s, 1H, SO_2-N*H*-CO-). Anal ($C_{19}H_{23}N_3O_5S_2$) C, H, N, S.

***N*-*t*-Butyl-*N'*-[2-(4-chlorophenylthio)-5-nitrobenzenesulfonyl]urea (49d)**

Mp : 139-142 °C. IR (KBr) νmax : 3388, 3101, 2971, 2932, 1690, 1592, 1575, 1524, 1475, 1448, 1393, 1344, 1264, 1245, 1220, 1163, 1125, 1091, 1058, 1041, 1013, 892, 873, 826, 772, 748, 739, 708, 679, 588, 501, 465 cm^{-1}. ^1H NMR (DMSO) δ: 1.18 (s, 9H, NH-C(CH_3)$_3$); 6.36 (s, 1H, CO-N*H*-CH_2); 7.17 (d, 1H, *J* = 9 Hz, H-3); 7.57-7.67 (m, 4H, H_{aro}'); 8.29 (dd, 1H, *J* = 9 Hz, *J* = 2.5 Hz, H-4); 8.65 (d, 1H, *J* = 2.5 Hz, H-6); 10.68 (s, 1H, SO_2-N*H*-CO-). Anal ($C_{17}H_{18}ClN_3O_5S_2$) C, H, N, S.

***N*-*n*-Pentyl -*N'*-[2-(4-chlorophenylthio)-5-nitrobenzenesulfonyl]urea (49e)**

Mp : 131-135 °C. IR (KBr) νmax : 3354, 3102, 2956, 2930, 2859, 1666, 1592, 1575, 1548, 1523, 1475, 1448, 1390, 1344, 1244, 1165, 1124, 1091, 1040, 1013, 957, 908, 889, 825, 770, 739, 679, 589, 498 cm^{-1}. ^1H NMR (DMSO) δ: 0.8 (t, 3H, J =7 Hz, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 1.1-1.35 (m, 6H, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 2.96 (q, 2H, J =7 Hz, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 6.52 (bs, 1H, CO-N*H*-CH_2); 7.15 (d, 1H, J = 9 Hz, H-3); 7.58-7.66 (m, 4H, H_{aro}'); 8.28 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.67 (d, 1H, J = 2.5 Hz, H-6); 11.15 (s, 1H, SO_2-N*H*-CO). Anal ($C_{18}H_{20}ClN_3O_5S_2$) C, H, N, S.

Procedure for the synthesis of *N*-[2-(4-methylphenylamino)-5-nitrobenzenesulfonyl]-piperidine-1-carboxamide (51)

Compound **44i** (0.01 mol) was dissolved in acetone. 0.01 mol NaOH (10% sol. w/v) was added. The mixture was gently mixed during 10 minutes and was evaporated under reduced pressure. The solid was resuspended in acetone (10 ml). A large excess of ethyl chloroformate (0.03 mol) was added dropwise under vigorous stirring. The mixture was stirred for 15 minutes and the solution was evaporated under reduced pressure. The ethyl carbamate obtained was purified by crystallization in methanol. Ethyl 2-(4-methylphenylamino)-5-nitrobenzenesulfonyl carbamate (**14**, 0.01 mol) was dissolved in a mixture of anhydrous toluene (40 ml) and piperidine (0.02 mol). The resulting solution was gently

refluxed overnight. At the end of the reaction, the solution was evaporated under reduced pressure and the residue was dissolved into an aqueous NaOH solution (0.5N, 20ml). This mixture was extracted with diethylether. The aqueous layer was recovered and adjusted to pH=1 with hydrochloric acid (0.5N). The precipitate which appeared was recovered by filtration (yield : 65%).

Mp : 139-141 °C. ^{1}H NMR ($CDCl_3$) δ: 1.60 (b, 6H, piperidine); 2.38 (s, 3H, C*H*$_3$-4'); 3.39 (s, 4H, piperidine); 7.03 (d, 1H, *J* = 9 Hz, H-3); 7.17-7.29 (m, 4H, H_{aro}'); 8.07 (dd, 1H, *J* = 9 Hz, *J* = 2.5 Hz, H-4); 8.75 (d, 1H, *J* = 2.5 Hz, H-6); 8.85 (s, 1H, Ph-N*H*-Ph); 11.14 (s, 1H, $S0_2$-N*H*-CO-). Anal ($C_{19}H_{22}N_4O_5S$) C, H, N, S.

Procedure for the preparation of *N-n*-pentyl-*N'*-cyano-O-phenylisourea (53)

A mixture of **52** (0.01 mol) and *n*-pentylamine (0.015 mol) in isopropanol (30 ml) was stirred at room temperature for 10-15 minutes. The solution was evaporated and the crude oil was crystallized in cold methanol.

Mp : 134-136°C. ^{1}H NMR (DMSO) δ: 0.86 (t, 2H, *J* =7 Hz, NH-CH_2-CH_2-CH_2-CH_2-C*H*$_3$); 1.1-1.35 (m, 6H, NH-CH_2-C*H*$_2$-C*H*$_2$-C*H*$_2$-CH_3); 3.26 (q, 2H, *J* =7 Hz, NH-C*H*$_2$-CH_2-CH_2-CH_2-CH_3); 7.06-7.41 (m, 5H, H_{aro}).

General procedure for the preparation of nitrobenzenesulfonylcyanoguanidines (54)

The appropriate sulfonamide (0.01 mol) was dissolved in acetone (30 ml). 0.01 mol NaOH (10% aqueous sol. w/v) was added. The mixture was gently mixed during 10 minutes and was evaporated under reduced pressure. The solid was resuspended in dimethylformamide (20ml) at room temperature and the appropriate N-alkyl-*N'*-cyano-O-

phenylisourea was added. The mixture was stirred at RT for 20-24H. At the end of the reaction, the mixture was evaporated under reduced pressure and the crude oil was suspended in a mixture of methanol and hydrochloric acid (5N aqueous solution) from which crystals of the desired product appeared.

_N-n_-Pentyl-_N'_-[2-(4-methylphenylamino)-5-nitrobenzenesulfonyl]-_N''_-cyanoguanidine (54a)

Mp : 162-166 °C. IR (KBr) νmax : 3382, 3358, 3091, 2963, 2934, 2862, 2719, 2191, 1634, 1597, 1579, 1556, 1513, 1467, 1427, 1416, 1358, 1330, 1315, 1220, 1154, 1125, 1080, 1049, 1018, 926, 902, 870, 838, 822, 804, 754, 745, 691, 661, 634, 598, 563, 513, 494, 455 cm^{-1}. ^1H NMR (DMSO) δ: 0.77 (t, 2H, *J*=7Hz, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 1.06-1.32 (m, 6H, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 2.32 (s, 3H, CH_3-4'); 3.09 (b, 2H, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 6.35 (b, 1H, S02-N*H*-C=N-CN-); 6.91 (bs, 1H, C=N-CN-N*H*-CH_2); 7.21-7.39 (m, 4H, H_{aro}', H-3); 8.12 (dd, 1H, *J*=9Hz, *J*=2.5Hz, H-4); 8.51 (d, 1H, *J*=2.5Hz, H-6); 9.06 (s, 1H, Ph-N*H*-Ph). Anal ($C_{20}H_{24}N_6O_4S$) C, H, N, S.

_N-n_-Pentyl-_N'_-[2-(3,5-dimethylphenylamino)-5-nitrobenzenesulfonyl]-_N''_-cyanoguanidine (54b)

Mp : 149-151 °C. IR (KBr) νmax : 3384, 3336, 3101, 2957, 2927, 2858, 2205, 1620, 1581, 1533, 1501, 1470, 1430, 1334, 1240, 1153, 1137, 1125, 1051, 998, 933, 893, 857, 825, 804, 746, 714, 699, 663, 596, 568, 495 cm^{-1}. ^1H NMR (DMSO) δ: 0.77 (t, 2H, *J*=7Hz, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 1.06-1.32 (m, 6H, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 2.26 (s, 6H, CH_3-3', CH_3-5'); 3.08 (b, 2H, NH-CH_2-CH_2-CH_2-CH_2-CH_3); 6.81 (s, 2H, H-2', H-6'); 7.17 (s, 1H, H-4'); 7.32 (bs, 1H, H-3); 8.14 (d, 1H, *J*=9Hz, H-4); 8.51 (s, 1H, H-6); 9.11 (s, 1H, Ph-N*H*-Ph). Anal ($C_{21}H_{26}N_6O_4S$) C, H, N, S.

_N-t_-Butyl-_N'_-[2-(4-methylphenoxy)-5-nitrobenzenesulfonyl]-_N''_-cyanoguanidine (54c)

Mp : 161-163 °C. IR (KBr) νmax : 3368, 3084, 2984, 2935, 2869, 2769, 2194, 1622, 1585, 1524, 1506, 1472, 1393, 1367, 1350, 1277, 1242, 1216, 1193, 1181, 1149, 1136, 1124, 1054, 1040, 1019, 1008, 930, 902, 865, 836, 803, 773, 759, 744, 732, 694, 668, 625, 597, 579, 562, 536, 518, 491, 467 cm^{-1}. ^1H NMR (DMSO) δ: 1.09 (s, 9H, N*H*-C(CH_3)$_3$); 2.34 (s, 3H, CH_3-

4'); 5.65 (bs, 1H, N*H*-CNCN-NH-C(CH_3)$_3$); 6.85 (d, 1H, J=9Hz, H-3); 7.09 (d, 2H, J=9Hz, H_{aro}'); 7.31 (d, 2H, J=9Hz, H_{aro}'); 8.28 (dd, 1H, J=9Hz, J=2.5Hz, H-4); 8.60 (d, 1H, J=2.5Hz, H-6). Anal ($C_{19}H_{21}N_5O_5S$) C, H, N, S.

Elemental analysis of final compounds

	Formula		Elemental analysis
47a	$C_{19}H_{30}N_4O_5S$	Calcd(found)	53.50 (53.66)%C; 7.09 (6.9)%H; 13.14 (13.35)%N; 7.52 (7.78)%S
47b	$C_{20}H_{32}N_4O_5S$	Calcd(found)	54.52 (54.25)%C; 7.32 (7.26)%H; 12.72 (12.74)%N; 7.28 (7.69)%S
47c	$C_{21}H_{34}N_4O_5S$	Calcd(found)	55.48 (55.31)%C; 7.54 (7.42)%H; 12.32 (12.01)%N; 7.05 (7.03)%S
47d	$C_{21}H_{28}N_4O_5S$	Calcd(found)	56.23 (55.95)%C; 6.29 (6.08)%H; 12.49 (12.61)%N; 7.15 (7.24)%S
47e	$C_{19}H_{24}N_4O_5S$	Calcd(found)	54.27 (54.13)%C; 5.75 (5.64)%H; 13.32 (13.41)%N; 7.63 (7.26)%S
47f	$C_{19}H_{24}N_4O_5S$	Calcd(found)	54.27 (54.50)%C; 5.75 (5.98)%H; 13.32 (13.54)%N; 7.63 (7.38)%S
47g	$C_{18}H_{21}BrN_4O_5S$	Calcd(found)	44.54 (44.16)%C; 4.36 (4.72)%H; 11.54 (11.21)%N; 6.61 (6.22) %S
47h	$C_{20}H_{24}N_6O_4S$	Calcd(found)	55.28 (55.36) ()%C; 6.03 (5.71)%H; 12.89 (12.89)%N; 7.38 (7.78)%S
47i	$C_{20}H_{26}N_4O_5S$	Calcd(found)	55.28 (55.35)%C; 6.03 (6.42)%H; 12.89 (13.25)%N; 7.38 (7.75)%S
47j	$C_{20}H_{26}N_4O_5S$	Calcd(found)	55.28 (54.97)%C; 6.03 (5.71)%H; 12.89 (12.70)%N; 7.38 (6.91)%S
47k	$C_{21}H_{20}N_4O_5S$	Calcd(found)	57.26 (57.55)%C; 4.58 (4.95)%H; 12.72 (13.11)%N; 7.28 (6.99)%S
47l	$C_{20}H_{24}N_4O_5S$	Calcd(found)	55.54 (55.93)%C; 5.59 (6.05)%H; 12.95 (13.27)%N; 7.41 (7.12)%S
47m	$C_{20}H_{26}N_4O_5S$	Calcd(found)	55.28 (54.96)%C; 6.03 (5.65)%H; 12.89 (12.88)%N; 7.38 (7.69)%S
47n	$C_{20}H_{26}N_4O_5S$	Calcd(found)	55.28 (55.10)%C; 6.03 (6.34)%H;

			12.89 (12.85)%N; 7.38 (6.92)%S
47o	$C_{19}H_{23}BrN_4O_5S$	Calcd(found)	45.70 (45.83)%C; 4.64 (4.94)%H; 11.22 (11.33)%N; 6.42 (6.14)%S
47p	$C_{19}H_{24}N_4O_5S$	Calcd(found)	54.27 (54.09)%C; 5.75 (6.17)%H; 13.32 (13.08)%N; 7.62 (7.25)%S
47q	$C_{19}H_{24}N_4O_5S$	Calcd(found)	54.27 (54.63)%C; 5.75 (6.03)%H; 13.32 (13.07)%N; 7.62 (7.84)%S
47r	$C_{19}H_{24}N_4O_5S$	Calcd(found)	54.27 (53.91)%C; 5.75 (6.05)%H; 13.32 (13.69)%N; 7.62 (7.55)%S
47s	$C_{18}H_{21}ClN_4O_5S$	Calcd(found)	50.88 (50.58)%C; 4.98 (5.17)%H; 13.19 (12.81)%N; 7.55 (7.11)%S
47t	$C_{20}H_{26}N_4O_5S_2$	Calcd(found)	55.60 (55.60)%C; 6.03 (6.38)%H; 12.89 (12.87)%N; 7.38 (7.00)%S
48a	$C_{18}H_{21}N_3O_6S$	Calcd(found)	53.06 (52.99)%C; 5.20 (5.55)%H; 10.31 (10.43)%N; 7.87 (7.35)%S
48b	$C_{19}H_{23}N_3O_6S$	Calcd(found)	54.14 (53.75)%C; 5.50 (5.77)%H; 9.97 (9.87)%N; 7.61 (7.26)%S
48c	$C_{20}H_{25}N_3O_6S$	Calcd(found)	55.39 (55.02)%C; 5.57 (5.68)%H; 9.67 (9.61)%N; 7.38 (7.70)%S
48d	$C_{20}H_{23}N_3O_6S$	Calcd(found)	55.42 (55.12)%C; 5.35 (5.06)%H; 9.69 (9.65)%N; 7.40 (7.80)%S
48e	$C_{18}H_{21}N_3O_6S$	Calcd(found)	53.06 (52.81)%C; 5.20 (5.52)%H; 10.31 (10.58)%N; 7.87 (7.92)%S
48f	$C_{19}H_{23}N_3O_6S$	Calcd(found)	54.14 (54.21)%C; 5.50 (5.71)%H; 9.97 (10.09)%N; 7.61 (7.23)%S
48g	$C_{19}H_{23}N_3O_6S$	Calcd(found)	54.14 (54.24)%C; 5.50 (5.41)%H; 9.97 (10.13)%N; 7.61 (7.89)%S
48h	$C_{17}H_{19}N_3O_6S$	Calcd(found)	51.90 (51.72)%C; 4.87 (5.03)%H; 10.68 (10.75)%N; 8.15 (8.02)%S
48i	$C_{18}H_{21}N_3O_6S$	Calcd(found)	53.06 (53.23)%C; 5.20 (5.39)%H; 10.31 (10.39)%N; 7.87 (7.69)%S
48j	$C_{18}H_{21}N_3O_6S$	Calcd(found)	53.06 (53.03)%C; 5.20 (5.09)%H; 10.31 (10.35)%N; 7.87 (8.18)%S
48k	$C_{17}H_{19}N_3O_6S$	Calcd(found)	51.90 (52.26)%C; 4.87 (4.95)%H; 10.68 (10.79)%N; 8.15 (8.14)%S

48l	$C_{18}H_{21}N_3O_6S$	Calcd(found)	53.06 (53.29)%C; 5.20 (5.41)%H; 10.31 (10.47)%N; 7.87 (7.50)%S
48m	$C_{18}H_{21}N_3O_6S$	Calcd(found)	53.06 (52.80)%C; 5.20 (5.51)%H; 10.31 (10.40)%N; 7.87 (7.66)%S
48n	$C_{17}H_{19}N_3O_6S$	Calcd(found)	51.90 (51.94)%C; 4.87 (5.23)%H; 10.68 (10.78)%N; 8.15 (7.99)%S
48o	$C_{17}H_{19}N_3O_6S$	Calcd(found)	51.90 (52.11)%C; 4.87 (4.93)%H; 10.68 (10.70)%N; 8.15 (8.40)%S
48p	$C_{17}H_{18}BrN_3O_6S$	Calcd(found)	43.23 (42.94)%C; 3.84 (4.16)%H; 8.90 (8.85)%N; 6.79 (6.92)%S
48q	$C_{18}H_{20}BrN_3O_6S$	Calcd(found)	44.45 (44.17)%C; 4.15 (4.52)%H; 8.64 (8.79)%N; 6.59 (6.82)%S
48r	$C_{17}H_{18}BrN_3O_6S$	Calcd(found)	43.23 (43.15)%C; 3.84 (4.24)%H; 8.90 (9.03)%N; 6.79 (7.09)%S
48s	$C_{18}H_{20}BrN_3O_6S$	Calcd(found)	44.45 (44.77)%C; 4.15 (4.35)%H; 8.64 (8.89)%N; 6.59 (6.49)%S
48t	$C_{17}H_{18}BrN_3O_6S$	Calcd(found)	43.23 (43.46)%C; 3.84 (4.09)%H; 8.90 (9.05)%N; 6.79 (7.16)%S
48u	$C_{18}H_{20}BrN_3O_6S$	Calcd(found)	44.45 (44.32)%C; 4.15 (3.80)%H; 8.64 (8.59)%N; 6.59 (6.41)%S
48v	$C_{17}H_{18}ClN_3O_6S$	Calcd(found)	47.72 (47.52)%C; 4.24 (4.31)%H; 9.82 (9.91)%N; 7.49 (7.77)%S
48w	$C_{18}H_{20}ClN_3O_6S$	Calcd(found)	48.93 (48.67)%C; 4.56 (4.23)%H; 9.51 (9.46)%N; 7.26 (6.91)%S
48x	$C_{17}H_{18}ClN_3O_6S$	Calcd(found)	47.72 (47.78)%C; 4.24 (3.86)%H; 9.82 (9.79)%N; 7.49 (7.24)%S
48y	$C_{18}H_{20}ClN_3O_6S$	Calcd(found)	48.93 (48.56)%C; 4.56 (4.88)%H; 9.51 (9.64)%N; 7.26 (7.35)%S
48z	$C_{17}H_{18}ClN_3O_6S$	Calcd(found)	47.72 (47.55)%C; 4.24 (4.29)%H; 9.82 (9.91)%N; 7.49 (7.79)%S
48aa	$C_{18}H_{20}ClN_3O_6S$	Calcd(found)	48.92 (49.05)%C; 4.56 (4.38)%H; 9.51 (9.69)%N; 7.26 (7.53)%S
48ab	$C_{17}H_{18}IN_3O_6S$	Calcd(found)	39.32 (39.41)%C; 3.49 (3.10)%H; 8.09 (8.40)%N; 6.17 (6.17)%S
48ac	$C_{18}H_{20}IN_3O_6S$	Calcd(found)	40.54 (40.72)%C; 3.78 (3.43)%H;

			7.88 (8.28)%N; 6.01 (6.18)%S
48ad	$C_{17}H_{18}IN_3O_6S$	Calcd(found)	39.32 (39.51)%C; 3.49 (3.18)%H; 8.09 (8.10)%N; 6.17 (5.98)%S
48ae	$C_{18}H_{20}IN_3O_6S$	Calcd(found)	40.54 (40.93)%C; 3.78 (3.41)%H; 7.88 (8.20)%N; 6.01 (5.95)%S
48af	$C_{18}H_{21}N_3O_7S$	Calcd(found)	51.06 (50.91)%C; 5.00 (4.90)%H; 9.92 (10.22)%N; 7.57 (7.24)%S
48ag	$C_{19}H_{23}N_3O_7S$	Calcd(found)	52.16 (52.23)%C; 5.30 (5.54)%H; 9.61 (9.91)%N; 7.33 (7.35)%S
48ah	$C_{20}H_{25}N_3O_7S$	Calcd(found)	53.20 (53.24)%C; 5.58 (5.74)%H; 9.31 (9.48)%N; 7.10 (6.91)%S
48ai	$C_{21}H_{27}N_3O_7S$	Calcd(found)	54.18 (54.25)%C; 5.85 (6.20)%H; 9.03 (9.32)%N; 6.89 (6.64)%S
48aj	$C_{19}H_{23}N_3O_7S$	Calcd(found)	52.16 (52.31)%C; 5.30 (5.25)%H; 9.61 (9.85)%N; 7.33 (7.20)%S
48ak	$C_{20}H_{25}N_3O_7S$	Calcd(found)	53.20 (53.29)%C; 5.58 (5.76)%H; 9.31 (9.71)%N; 7.10 (6.95)%S
48al	$C_{18}H_{21}N_3O_7S$	Calcd(found)	51.06 (50.94)%C; 5.00 (5.28)%H; 9.92 (9.92)%N; 7.57 (7.47)%S
48am	$C_{18}H_{20}ClN_3O_6S$	Calcd(found)	48.92 (48.68)%C; 4.56 (4.95)%H; 9.51 (9.43)%N; 7.26 (7.32)%S
48an	$C_{19}H_{22}ClN_3O_6S$	Calcd(found)	50.05 (50.27)%C; 4.86 (5.26)%H; 9.22 (9.38)%N; 7.03 (7.10)%S
48ao	$C_{19}H_{23}N_3O_7S$	Calcd(found)	52.16 (52.35)%C; 5.30 (5.42)%H; 9.61 (9.52)%N; 7.33 (7.28)%S
49a	$C_{18}H_{21}N_3O_5S_2$	Calcd(found)	51.05 (50.66)%C; 5.00 (4.93)%H; 9.92 (9.90)%N; 15.14 (14.98)%S
49b	$C_{19}H_{23}N_3O_5S_2$	Calcd(found)	52.16 (52.27)%C; 5.08 (5.39)%H; 9.60 (9.75)%N; 14.66 (14.27)%S
49c	$C_{19}H_{23}N_3O_5S_2$	Calcd(found)	52.16 (52.52)%C; 5.30 (5.65)%H; 9.60 (9.26)%N; 14.65 (14.30)%S
49d	$C_{17}H_{18}ClN_3O_5S_2$	Calcd(found)	45.99 (46.20)%C; 4.09 (4.31)%H; 9.47 (9.43)%N; 14.44 (14.43)%S
49e	$C_{18}H_{20}ClN_3O_5S_2$	Calcd(found)	47.21 (47.35)%C; 4.40 (4.59)%H; 9.18 (9.06)%N; 14.00 (13.72)%S

54a	$C_{20}H_{24}N_6O_4S$	Calcd(found)	54.04 (54.00)%C; 5.44 (5.58)%H; 18.91 (18.81)%N; 7.21 (7.52)%S
54b	$C_{21}H_{26}N_6O_4S$	Calcd(found)	53.95 (53.93)%C; 5.82 (5.98)%H; 17.97 (17.85)%N; 6.86 (6.69)%S
54c	$C_{19}H_{21}N_5O_5S$	Calcd(found)	50.77 (50.97)%C; 5.16 (5.10)%H; 15.58 (15.57)%N; 7.13 (7.00)%S
51	$C_{19}H_{22}N_4O_5S$	Calcd(found)	54.53 (54.15)%C; 5.30 (5.41)%H; 13.39 (13.17)%N; 7.66 (7.30)%S

V.2. Pharmacological evaluations

V. 2. 1. Materials

Furegrelate sodium salt (5-(3-pyridinylmethyl)-2-benzofurancarboxylate), SQ29548 [[1S-[1, 2(Z), 3, 4]]-7-[3-[[2-[(phenylamino)carbonyl]hydrazino]methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid], [^{3}H]SQ29548 and U46619 (9.11-dideoxy-9.11-methanoepoxy-prostaglandin F2) were purchased from Cayman Chemical (MI, USA). TPα and TPβ cDNA were as described previously (Allan *et al.*, 1996; Becker *et al.*, 1998). Human coronary artery smooth muscle cells (HCASMc) were obtained from CellWorks (Buckingham, U.K.). Dulbecco's modified Eagle's medium (DMEM) supplemented with glucose (4.5 g/l), foetal bovine serum (FBS) and antibiotic/antimycotic solution were purchased from Invitrogen life technologies (Carlsbad, CA). Male Sprague–Dawley rats, weighing 250–300 g were housed in a temperature-controlled room before being used in the present experiments. All experimental procedures and protocols used in this investigation have been carried out in accordance with the

Declaration of Helsinki (Publication No. 85-23. revised 1985) and were reviewed and approved by the Ethics Committee of the Medical Faculty of the University of Liège.

V. 2. 2. Methods

V.2.2.1. Radioligand Binding Assays

V.2.2.1.A. TPα and TPβ

COS-7 cells were maintained in Dulbecco's modified Eagle's medium (Hyclone Laboratories, Logan, UT) supplemented with 10% fetal bovine serum and 1% of solution containing 10,000 units/ml penicillin G, 10,000 μg/ml streptomycin, and 25 μg/ml amphotericin (Cellgro; Mediatech, Herndon, VA). Cells were grown at 37°C in a humidified atmosphere of 95% O_2 and 5% CO_2. cDNAs (the cDNA was generously provided by Prof. Perry Halushka, Medical University of South Carolina, USA) for the TPα and TPβ were subcloned into pcDNA3, the resultant plasmids, pcDNA3:TPα and pcDNA3:TPβ were introduced into COS-7 cells by the DEAE-dextran/chloroquine method. 48 h post-transfection, cells were harvested by centrifugation at 500*g* for 5 min and washed three times in ice-cold phosphate-buffered saline. Cells were resuspended in buffer containing 25 mM HEPES/125 mM NaCl/10 μM indomethacin, pH 7.4, and kept on ice for the binding study. Binding reactions were carried out on 5 x 10^5 cells in a total volume of 0.2 ml in the above buffer with 10 nM [^{3}H]SQ29,548 (Perkin Elmer Life and analytical services, Boston, MA) added to all tubes in

triplicate, containing various concentrations of studied compounds (10^{-9} M for screening assay or 10^{-6} to 10^{-11} M for competition binding curves) in 1 µl of ethanol. Additional tubes containing excess unlabeled SQ29,548 (10 µM) (Cayman Chemical Co, Ann Arbor, MI) were included to assess the extent of non-specific binding. Binding was allowed to take place for 30 min at 37°C; free radioligand was removed by rapid vacuum filtration through Whatman (Maidstone, UK) GF/B glass fiber filters pre-washed with the cell suspension buffer. The tubes and the filters were rapidly washed with ice-cold 10 mM Tris buffer, pH 7.4 (three times with 3 ml). The radioactivity on the filters containing the ligand-receptor complexes was counted in 10 ml of Ecolite scintillation fluid (ICN, St. Laurent, QC, Canada) in a Beckman (model LS 3800) liquid scintillation counter. The binding experiments were performed on whole cells.

V.2.2.1.B. Human coronary artery smooth muscle cells

HCASM cells were maintained in HCASMc basal medium supplemented with HCASMc growth supplement (CellWorks) at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. HCAM cells were re-suspended in the binding buffer (25 mM Tris-HCl, pH 7.4, 5 mM $CaCl_2$,10 µM indomethacin, 50 µg/ml PMSF). For the binding assay, 50 µg of protein was incubated with [3H]SQ29548 (30 Ci/mol, 1 µM, PerkinElmer Life Sciences) in the presence of the drug at the indicated concentrations (0-100 µM) in a 0.1 ml reaction volume with vigorous shaking at room temperature for 60 min. The reaction was then terminated by adding 1 ml of ice-cold washing buffer (25 mM Tris-HCl, pH 7.4). The unbound ligand was filtered under vacuum through a

Whatman GF/C glass filter (Whatman, Clifton, NJ) presoaked with the ice-cold washing buffer. The radioactivity of the TP receptor-bound [^{3}H]SQ29548 remaining on the glass filter was counted in 8 ml of scintillation mixture (NEN) using a Beckman counter (Fullerton, CA).

V.2.2.2. Calcium measurements

HEK.TPα and HEK.TPβ cell lines, stably over-expressing HA-tagged forms of TPα and TPβ in human embryonic kidney (HEK) 293 cells have been previously described (Walsh, Foley *et al.* 2000). HEK 293 cells or their stable cell line equivalents were routinely grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). Measurement of intracellular calcium concentration ($[Ca^{2+}]_i$) mobilization either in HEK.TPα or HEK.TPβ cells was carried out using fluorescent microplate reader Fluoroskan Ascent FL equipped with two dispenser (thermo electron corporation, Finland) according to modified method of Lin *et al.* (Lin, Sadee *et al.* 1999). Briefly, cells were trypsinized and washed twice with Krebs-HEPES buffer (118 mM, NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 11.7 mM D-Glucose, 1.3 mM $CaCl_2$, 10 mM HEPES, pH 7.4), and incubated for one hour with fluorescent dye Fluo-4/AM (5 μg/ml; Molecular Probes, Invitrogen, Merelbeke Belgium). Cells were then rinsed three times with Krebs-HEPES buffer and 150 μl of a suspension of cells in that buffer was loaded into each well of a 96-well plate at a density of 150,000 cells/well. Cells were incubated 10 min with various concentrations of the test compound (10^{-5} to 10^{-8} M final; 10 μl) prior to stimulation with U46619 (1 μM final, 50 μl). In all cases, compound (1

mM) was diluted in dimethylsulfoxide (DMSO)/PBS (30/70) prior to further dilution in PBS. Fluorescence emission was read at 538 nM. At the end of each experiment, fluorescence intensities were calibrated for determination of $[Ca^{2+}]_i$ values by permeating cells with 1% Triton X-100 to release all the dye (Fmax) and subsequently chelating with 10 mM EGTA (Fmin). Calcium concentrations were calculated using equation $[Ca^{2+}]_i = Kd(F - F_{min})/(Fmax - F)$, assuming a Kd of 345 nM for Fluo-4. The results (IC_{50}) presented are the concentration required to inhibit 50% of the normal rise of $[Ca^{2+}]_i$ upon stimulation with 1 μM U46619, determined in the absence of any compounds. The IC_{50} were calculated by non-linear regression analysis (GraphPad Prism software) from at least three concentration-response curves.

V.2.2.3. Platelet aggregation studies

The anti-aggregant efficacy has been determined according to the turbidimetric Born's method (Born and Cross 1963). The blood was drawn from healthy donors of both genders, aged 20-30. The subjects were free from medication for at least 14 days. No significant differences in the results were observed between the donors in our experiments. Blood was centrifuged (10 min 180g) and supernatant was recolted (platelet rich plasma, PRP). The tubes were subsequently centrifuged at 3000g and the supernatant was recolted (platelet poor plasma or PPP). Platelet concentration of PRP was adjusted to 3×10^8 cells/ml by dilution with PPP. Platelet aggregation of PRP was studied using a double channel aggregometer (Chronolog Corporation, Chicago, IL) connected to a linear recorder as previously described. PRP (294 μl) was added in a

silanised cuvette and stirred (1000 rpm). Each compound was diluted (1 mM) in dimethylsulfoxide (DMSO)/PBS (30/70) and preincubated in PRP for three minutes at 37°C before the aggregating agent was added. Platelet aggregation was initiated by addition of a fresh solution of U46619 (1 μM final). To evaluate platelet aggregation, the maximum increase in light transmission was determined from the aggregation curve 6 minutes after addition of the inducer. The substance concentration preventing 50% of platelet aggregation (IC_{50}) induced by U46619 was calculated by non-linear regression analysis (GraphPad Prism software) from at least three dose-response curves.

V.2.2.4. Statistical analysis

Radioligand binding assays

Results are expressed as the mean ± standard deviation from at least three determinations ($n \geq 3$). Statistical differences between TP isoforms have been determined using unpaired t-test between IC_{50}s values. p values of less than 0.05 were considered to be significant.

Ferric chloride-induced rat arterial thrombosis

Results are expressed as the mean ± standard error of the mean (Gaussem, Reny *et al.*) and statisticalsignificance was determined by a Mann-Whitney test. Probability values of less than 0.05 were considered to be significant.

V.2.2.5. Rat aorta relaxation

Endothelium-denuded thoracic aorta rings, obtained from rats (Wistar, 250–300 g) anaesthetised with sodium pentobarbital (80 mg/kg, i.p.), were suspended under a tension ofone gram in a Krebs solution (mM: NaCl 118, KCl 5.4, $CaCl_2$ 2.5, $MgCl_2$.6 H_2O 1.5, $NaHCO_3$ 25,NaH_2PO_4 1.2 and glucose 10; pH 7.4) which was bubbled with O_2–CO_2 (95–5%) at 37°Cin a 20 ml tissue bath (EMKA Technologies, Paris, France). The muscle tension of the aortic rings was isometrically recorded with a force-displacement transducer IT1 (EMKA Technologies, Paris, France). The buffer was renewed every 15 minutes during the equilibration period (1 h) before exposing the rings to U46619 (20 nM). When a stable tension was obtained (15 min), cumulative increasing concentrations of potential antagonist were added to the bath until tension returned to the base-line value. The ED50 value of each drugs was assessed for at least 4 concentration-response curves obtained from separate preparations and corresponded to the concentration which reduced to 50% the tension induced by U46619 (20 nM). The ED_{50} were calculated by non-linear regression analysis (GraphPad Prism software).

V.2.2.6. Guinea-pig trachea relaxation

Trachea was removed from guinea pigs (Hartley, 250–300 g) anesthetized with sodium pentobarbital (80 mg/kg, i.p.), and carefully cleaned of connective tissue. Trachea strips were suspended in the organ bath (20 ml) and the experiment progressed in the same conditions as

those described above for the rat aorta except for the concentration of U46619 (10 nM), the contraction inducer.

V.2.2.7. Thromboxane synthase activity

PRP preparation is identical to that described for the platelet aggregation experiments. Each drug was dissolved in dimethylsulfoxide (DMSO) and diluted with a Tyrode-Hepes buffer (mM: NaCl 137, KCl 2.7, NaH_2PO_4.- H_2O 0.4, D-glucose 5, $NaHCO_3$ 12, Hepes; pH 7.4). To 900 µl of PRP, 50 µl NaCl 0.9% and 10 µl of drug solution were added. After 6 minutes incubation at 37°C under stirring (600 rpm), aggregation was induced by 40 µl sodium arachidonate (0.6 mM final). After 4 minutes, the reaction was stopped by addition of indomethacin (50 µl, 0.02 M in ethanol). The sample was immediately centrifuged (17500 g, for 10s) and the supernatant was removed and frozen (-80 °C) until assayed for TXB_2. Basal and maximal production of TXB_2 was estimated in the absence and in the presence of arachidonic acid, respectively. Evaluation was done in triplicate on concentrations ranging from 10 to 0.1 µM. TXS activity was expressed as the TXB_2 production which was measured by using a competitive enzyme immunoassay (TXB_2 EIA Kit, Cayman ChemicalCompany, MI).

V.2.2.8. Ferric chloride-induced rat arterial thrombosis

The experiments were carried out according to the modification of the method described by Kurz *et al.* (Kurz *et al.*, 1990). Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). After an abdominal midline incision, the abdominal aorta was exposed carefully. A

filter paper disk (diameter 8 mm) saturated with 50% (w/v) ferric chloride solution was placed on the surface of the artery for 10 min. The artery was isolated 10 min after removing the disk and then opened lengthwise. The thrombus was scraped out and placed on a filter paper to remove any water and its wet weight was measured immediately. Results are expressed in mg of thrombus weight by kg of rat weight. BM613, BM573 and placebo were injected intravenously 5 minutes prior to application of ferric chloride solution. The results are expressed as the mean of results from four separate experiments.

VI. Bibliography

[The Ridogrel Versus Aspirin Patency Trial (RAPT)] "Randomized trial of ridogrel, a combined thromboxane A_2 synthase inhibitor and thromboxane A_2/prostaglandin endoperoxide receptor antagonist, versus aspirin as adjunct to thrombolysis in patients with acute myocardial infarction." *Circulation*, **1994**, 89, (2), 588-95.

Albet, C., Perez, A., Rozman, E., Sacristan, A. and Ortiz, J. A. "Pharmacokinetics of Camonagrel in experimental animal: rat, rabbit and dog." *Eur J Drug Metab Pharmacokinet*, **1998**, 23, (2), 251-4.

Allan, C. J., Higashiura, K., Martin, M., Morinelli, T. A., Kurtz, D. T., Geoffroy, O., Meier, G. P., Gettys, T. W. and Halushka, P. V. "Characterization of the cloned HEL cell thromboxane A_2 receptor: evidence that the affinity state can be altered by G alpha 13 and G alpha q." *J Pharmacol Exp Ther*, **1996**, 277, (2), 1132-9.

Arakida, Y., Ohga, K., Suwa, K., Okada, Y., Morio, H., Yokota, M., Miyata, K., Yamada, T. and Honda, K. "Effect of YM158, a dual lipid mediator antagonist, on immediate and late asthmatic responses, and on airway hyper-responsiveness in guinea pigs." *Jpn J Pharmacol*, **2000**, 82, (4), 287-94.

Arakida, Y., Suwa, K., Ohga, K., Yokota, M., Miyata, K., Yamada, T. and Honda, K. "In vitro pharmacologic profile of YM158, a new dual antagonist for LTD_4 and TXA_2 receptors." *J Pharmacol Exp Ther*, **1998**, 287, (2), 633-9.

Armstrong, R. A. "Platelet prostanoid receptors." *Pharmacol Ther*, **1996**, 72, (3), 171-91.

Armstrong, R. A., Humphrey, P. P. and Lumley, P. "Characteristics of the binding of [3H]-GR32191 to the thromboxane (TP-) receptor of human platelets." *Br J Pharmacol*, **1993a**, 110, (2), 539-47.

Armstrong, R. A., Humphrey, P. P. and Lumley, P. "Reduction in the number of thromboxane receptors on human platelets after exposure to GR32191." *Br J Pharmacol*, **1993b**, 110, (2), 548-52.

Armstrong, R. A. and Wilson, N. H. "Aspects of the thromboxane receptor system." *Gen Pharmacol*, **1995**, 26, (3), 463-72.

Ashton, A. W. and Ware, J. A. "Thromboxane A_2 receptor signaling inhibits vascular endothelial growth factor-induced endothelial cell differentiation and migration." *Circ Res*, **2004**, 95, (4), 372-9.

Becker, K. P., Garnovskaya, M., Gettys, T. and Halushka, P. V. "Coupling of thromboxane A_2 receptor isoforms to Galpha13: effects on ligand binding and signalling." *Biochim Biophys Acta*, **1999**, 1450, (3), 288-96.

Belton, O., Byrne, D., Kearney, D., Leahy, A. and Fitzgerald, D. J. "Cyclooxygenase-1 and -2-dependent prostacyclin formation in patients with atherosclerosis." *Circulation*, **2000**, 102, (8), 840-5.

Bennett, J. S. "Novel platelet inhibitors." *Annu Rev Med*, **2001**, 52, 161-84.

Bergstroem, S., Danielsson, H. and Samuelsson, B. "The Enzymatic Formation of Prostaglandin E_2 from Arachidonic Acid Prostaglandins and Related Factors." *Biochim Biophys Acta*, **1964**, 90, 207-10.

Billington, C. K., Hall, I. P., Mundell, S. J., Parent, J. L., Panettieri, R. A., Jr., Benovic, J. L. and Penn, R. B. "Inflammatory and contractile agents sensitize specific adenylyl cyclase isoforms in human airway smooth muscle." *Am J Respir Cell Mol Biol*, **1999**, 21, (5), 597-606.

Bing, R. J., Yamamoto, T., Yamamoto, M., Kakar, R. and Cohen, A. "New look at myocardial infarction: toward a better aspirin." *Cardiovasc Res*, **1999**, 43, (1), 25-31.

Born, G. V. and Cross, M. J. "The Aggregation of Blood Platelets." *J Physiol*, **1963**, 168, 178-95.

Brass, L. F., Manning, D. R., Cichowski, K. and Abrams, C. S. "Signaling through G proteins in platelets: to the integrins and beyond." *Thromb Haemost*, **1997**, 78, (1), 581-9.

Brass, L. F., Woolkalis, M. J. and Manning, D. R. "Interactions in platelets between G proteins and the agonists that stimulate phospholipase C and inhibit adenylyl cyclase." *J Biol Chem*, **1988**, 263, (11), 5348-55.

Bresalier, R. S., Sandler, R. S., Quan, H., Bolognese, J. A., Oxenius, B., Horgan, K., Lines, C., Riddell, R., Morton, D., Lanas, A., Konstam, M. A. and Baron, J. A. "Cardiovascular events associated with rofecoxib in a colorectal adenoma chemoprevention trial." *N Engl J Med*, **2005**, 352, (11), 1092-102.

Capra, V., Habib, A., Accomazzo, M. R., Ravasi, S., Citro, S., Levy-Toledano, S., Nicosia, S. and Rovati, G. E. "Thromboxane prostanoid receptor in human airway smooth muscle cells: a relevant role in proliferation." *Eur J Pharmacol*, **2003**, 474, (2-3), 149-59.

Cardoso, C. R., de Brito, F. C., da Silva, K. C., de Miranda, A. L., Fraga, C. A. and Barreiro, E. J. "Design, synthesis and pharmacological evaluation of novel pyrazolo[3,4-b]thieno[2,3-d] pyridine acid derivatives: a new class of anti-inflammatory and anti-platelet agents." *Bioorg Med Chem Lett*, **2002**, 12, (1), 9-12.

Carty, E., Rampton, D. S., Schneider, H., Rutgeerts, P. and Wright, J. P. "Lack of efficacy of ridogrel, a thromboxane synthase inhibitor, in a placebo-controlled, double-blind, multi-centre clinical trial in active Crohn's disease." *Aliment Pharmacol Ther*, **2001**, 15, (9), 1323-9.

Castelli, M. G., Chiabrando, C., Fanelli, R., Martelli, L., Butti, G., Gaetani, P. and Paoletti, P. "Prostaglandin and thromboxane synthesis by human intracranial tumors." *Cancer Res*, **1989**, 49, (6), 1505-8.

Catella, F., Healy, D., Lawson, J. A. and FitzGerald, G. A. "11-Dehydrothromboxane B_2: a quantitative index of thromboxane A_2 formation in the human circulation." *Proc Natl Acad Sci U S A*, **1986**, 83, (16), 5861-5.

Catella, F., Lawson, J. A., Fitzgerald, D. J. and FitzGerald, G. A. "Analysis of multiple thromboxane metabolites in plasma and urine." *Adv Prostaglandin Thromboxane Leukot Res*, **1987**, 17B, 611-4.

Cheever, K. H. "An overview of pulmonary arterial hypertension: risks, pathogenesis, clinical manifestations, and management." *J Cardiovasc Nurs*, **2005**, 20, (2), 108-16; quiz 117-8.

Cheng, Y., Austin, S. C., Rocca, B., Koller, B. H., Coffman, T. M., Grosser, T., Lawson, J. A. and FitzGerald, G. A. "Role of prostacyclin in the cardiovascular response to thromboxane A_2." *Science*, **2002**, 296, (5567), 539-41.

Chiabrando, C., Rivoltella, L., Alberti, E., Bonollo, M., Djurup, R. and Fanelli, R. "Urinary excretion and origin of 11-dehydro-2,3-dinor-thromboxane B_2 in man." *Prostaglandins*, **1993**, 45, (5), 401-11.

Chiang, N., Kan, W. M. and Tai, H. H. "Site-directed mutagenesis of cysteinyl and serine residues of human thromboxane A_2 receptor in insect cells." *Arch Biochem Biophys*, **1996**, 334, (1), 9-17.

Cipollone, F., Iezzi, A., Fazia, M., Zucchelli, M., Pini, B., Cuccurullo, C., De Cesare, D., De Blasis, G., Muraro, R., Bei, R., Chiarelli, F., Schmidt, A. M., Cuccurullo, F. and Mezzetti, A. "The receptor RAGE as a progression factor amplifying arachidonate-dependent inflammatory and proteolytic response in human atherosclerotic plaques: role of glycemic control." *Circulation*, **2003**, 108, (9), 1070-7.

Cipollone, F., Prontera, C., Pini, B., Marini, M., Fazia, M., De Cesare, D., Iezzi, A., Ucchino, S., Boccoli, G., Saba, V., Chiarelli, F., Cuccurullo, F. and Mezzetti, A. "Overexpression of functionally coupled cyclooxygenase-2 and prostaglandin E synthase in symptomatic atherosclerotic plaques as a basis of prostaglandin E_2-dependent plaque instability." *Circulation*, **2001**, 104, (8), 921-7.

Clemetson, K. J. and Clemetson, J. M. "Platelet collagen receptors." *Thromb Haemost*, **2001**, 86, (1), 189-97.

Coleman, R. A., Humphrey, P. P., Kennedy, I., Levy, G. P. and Lumley, P. "Comparison of the actions of U46619, a prostaglandin H_2-analogue, with those of prostaglandin H_2 and thromboxane A_2 on some isolated smooth muscle preparations." *Br J Pharmacol*, **1981**, 73, (3), 773-8.

Coleman, R. A., Smith, W. L. and Narumiya, S. "International Union of Pharmacology classification of prostanoid receptors: properties, distribution, and structure of the receptors and their subtypes." *Pharmacol Rev*, **1994**, 46, (2), 205-29.

Collins, C. E., Benson, M. J., Burnham, W. R. and Rampton, D. S. "Picotamide inhibition of excess in vitro thromboxane B_2 release by colorectal mucosa in inflammatory bowel disease." *Aliment Pharmacol Ther*, **1996**, 10, (3), 315-20.

Collins, R. G., Velji, R., Guevara, N. V., Hicks, M. J., Chan, L. and Beaudet, A. L. "P-Selectin or intercellular adhesion molecule (ICAM)-1 deficiency substantially protects against atherosclerosis in apolipoprotein E-deficient mice." *J Exp Med*, **2000**, 191, (1), 189-94.

Coto, V., Carrieri, P., Cocozza, M., Oliviero, U., Picano, T., Spinoli, G. and Cacciatore, L. "[Antiplatelet activity of picotamide in patients with chronic vascular pathology]." *Minerva Cardioangiol*, **1986**, 34, (9), 601-4.

Coto, V., Cocozza, M., Oliviero, U., Lucariello, A., Picano, T., Coto, F. and Cacciatore, L. "Clinical efficacy of picotamide in long-term treatment of intermittent claudication." *Angiology*, **1989**, 40, (10), 880-5.

Coto, V., Oliviero, U., Cocozza, M. and Milani, M. "Long-term safety and efficacy of picotamide, a dual-action antithromboxane agent, in diabetic patients with carotid atherosclerosis: a 6-year follow-up study." *J Int Med Res*, **1998**, 26, (4), 200-5.

Coyle, A. T. and Kinsella, B. T. "Characterization of promoter 3 of the human thromboxane A receptor gene. A functional AP-1 and octamer motif are required for basal promoter activity." *Febs J*, **2005**, 272, (4), 1036-53.

Coyle, A. T. and Kinsella, B. T. "Synthetic peroxisome proliferator-activated receptor gamma agonists rosiglitazone and troglitazone suppress transcription by promoter 3 of the human thromboxane A_2 receptor gene in human erythroleukemia cells." *Biochem Pharmacol*, **2006**, 71, (9), 1308-23.

Coyle, A. T., Miggin, S. M. and Kinsella, B. T. "Characterization of the 5' untranslated region of alpha and beta isoforms of the human thromboxane A_2 receptor (TP). Differential promoter utilization by the TP isoforms." *Eur J Biochem*, **2002**, 269, (16), 4058-73.

Coyle, A. T., O'Keeffe, M. B. and Kinsella, B. T. "15-deoxy Delta12,14-prostaglandin J_2 suppresses transcription by promoter 3 of the human thromboxane A_2 receptor gene through peroxisome proliferator-activated receptor gamma in human erythroleukemia cells." *Febs J*, **2005**, 272, (18), 4754-73.

Cracowski, J. L., Devillier, P., Durand, T., Stanke-Labesque, F. and Bessard, G. "Vascular biology of the isoprostanes." *J Vasc Res*, **2001**, 38, (2), 93-103.

Cyrus, T., Yao, Y., Ding, T., Dogné, J. M. and Pratico, D. "Thromboxane receptor blockade improves the anti-atherogenic effect of thromboxane A_2 suppression in LDLR KO mice." *Blood*, **2006**,

D'Andrea, G., Perini, F., Hasselmark, L., Alecci, M. and Cananzi, A. R. "Effect of picotamide and aspirin, combined or alone, on platelet aggregation in patients with cerebral infarction." *Funct Neurol*, **1995**, 10, (2), 91-8.

D'Angelo, D. D., Davis, M. G., Houser, W. A., Eubank, J. J., Ritchie, M. E. and Dorn, G. W., 2nd. "Characterization of 5' end of human thromboxane receptor gene. Organizational analysis and mapping of protein kinase C--responsive elements regulating expression in platelets." *Circ Res*, **1995**, 77, (3), 466-74.

D'Angelo, D. D., Eubank, J. J., Davis, M. G. and Dorn, G. W., 2nd. "Mutagenic analysis of platelet thromboxane receptor cysteines. Roles in ligand binding and receptor-effector coupling." *J Biol Chem*, **1996**, 271, (11), 6233-40.

D'Angelo, D. D., Oliver, B. G., Davis, M. G., McCluskey, T. S. and Dorn, G. W. "Novel role for Sp1 in phorbol ester enhancement of human platelet thromboxane receptor gene expression." *J Biol Chem*, **1996**, 271, (33), 19696-704.

Danese, C., Sergio, G., Giunta, G., Landi, M. T., Renzulli, C., Panciocco, M. G., De Rossi, M. G. and Perego, M. A. "Effects of prolonged picotamide therapy on platelet activity in patients with peripheral arterial disease." *Curr Med Res Opin*, **1988**, 11, (4), 221-6.

Daniel, T. O., Liu, H., Morrow, J. D., Crews, B. C. and Marnett, L. J. "Thromboxane A_2 is a mediator of cyclooxygenase-2-dependent endothelial migration and angiogenesis." *Cancer Res*, **1999**, 59, (18), 4574-7.

Davi, G., Basili, S., Vieri, M., Cipollone, F., Santarone, S., Alessandri, C., Gazzaniga, P., Cordova, C. and Violi, F. "Enhanced thromboxane biosynthesis in patients with chronic obstructive pulmonary disease. The Chronic Obstructive Bronchitis and Haemostasis Study Group." *Am J Respir Crit Care Med*, **1997**, 156, (6), 1794-9.

de Leval, X., Benoit, V., Delarge, J., Julemont, F., Masereel, B., Pirotte, B., Merville, M. P., David, J. L. and Dogné, J. M. "Pharmacological evaluation of the novel thromboxane modulator BM567 (II/II). Effects of BM567 on osteogenic sarcoma-cell-induced platelet aggregation." *Prostaglandins Leukot Essent Fatty Acids*, **2003**, 68, (1), 55-9.

de Leval, X., Dassesse, T., Dogné, J. M., Waltregny, D., Bellahcene, A., Benoit, V., Pirotte, B. and Castronovo, V. "Evaluation of original dual thromboxane A_2 modulators as antiangiogenic agents." *J Pharmacol Exp Ther*, **2006**, 318, (3), 1057-67.

de Leval, X. J., Dassesse, T., Benoit, V., Pirotte, B., Merville, M. P., David, J. L., Castronovo, V. and Dogné, J. M. "Pharmacological evaluation of BM-573, a dual thromboxane A_2 receptor antagonist and thromboxane synthase inhibitor, as potential anti-metastatic agent." *Blood*, **2003**, 102, (11), 72b-72b.

Delarge, J. and Lapiere, C. L. "[Synthesis and properties of some substituted anilinopyridines]." *J Pharm Belg*, **1973**, 28, (3), 283-90.

Delarge, J. and Lapiere, C. L. "[A new class of high ceiling diuretics : derivatives of 1-alkyl-3-(4-phenylaminopyridin-3-yl)sulfonylurea (author's transl)]." *Ann Pharm Fr*, **1978**, 36, (7-8), 369-79.

Desjardins, F., Aubin, M. C., Carrier, M. and Perrault, L. P. "Decrease of endothelin receptor subtype ETB and release of COX-derived products contribute to endothelial dysfunction of porcine epicardial coronary arteries in left ventricular hypertrophy." *J Cardiovasc Pharmacol*, **2005**, 45, (6), 499-508.

Devillier, P. and Bessard, G. "Thromboxane A_2 and related prostaglandins in airways." *Fundam Clin Pharmacol*, **1997**, 11, (1), 2-18.

Djellas, Y., Antonakis, K. and Le Breton, G. C. "A molecular mechanism for signaling between seven-transmembrane receptors: evidence for a redistribution of G proteins." *Proc Natl Acad Sci U S A*, **1998**, 95, (18), 10944-8.

Djellas, Y., Manganello, J. M., Antonakis, K. and Le Breton, G. C. "Identification of Gα13 as one of the G-proteins that couple to human platelet thromboxane A2 receptors." *J Biol Chem*, **1999**, 274, (20), 14325-30.

Dogné, J. M. **2000**. "Conception, synthèse et évaluation pharmacologique de dérivés originaux du torasémide à propriétés antagonistes du thromboxane A_2 et inhibitrices de la thromboxane synthase". Department of Pharmacy, Laboratory of Medicinal Chemistry. Liège, University of Liège**:** 239.

Dogné, J. M., de Leval, X., Delarge, J., David, J. L. and Masereel, B. "New trends in thromboxane and prostacyclin modulators." *Curr Med Chem*, **2000**, 7, (6), 609-28.

Dogné, J. M., de Leval, X., Hanson, J., Frederich, M., Lambermont, B., Ghuysen, A., Casini, A., Masereel, B., Ruan, K. H., Pirotte, B. and Kolh, P. "New developments on thromboxane and prostacyclin modulators part I: thromboxane modulators." *Curr Med Chem*, **2004**, 11, (10), 1223-41.

Dogné, J. M., Hanson, J. and Pratico, D. "Thromboxane, prostacyclin and isoprostanes: therapeutic targets in atherogenesis." *Trends Pharmacol Sci*, **2005**, 26, (12), 639-44.

Dogné, J. M., Hanson, J., Supuran, C. and Pratico, D. "Coxibs and cardiovascular side-effects: from light to shadow." *Curr Pharm Des*, **2006**, 12, (8), 971-5.

Dogné, J. M., Rolin, S., Petein, M., Tchana-Sato, V., Ghuysen, A., Lambermont, B., Hanson, J., Magis, D., Segers, P., Pirotte, B., Masereel, B., Drion, P., D'Orio, V. and Kolh, P. "Characterization of an original

model of myocardial infarction provoked by coronary artery thrombosis induced by ferric chloride in pig." *Thromb Res*, **2005**, 116, (5), 431-42.

Dogné, J. M., Supuran, C. T. and Pratico, D. "Adverse cardiovascular effects of the coxibs." *J Med Chem*, **2005**, 48, (7), 2251-7.

Dorn, G. W., 2nd. "Distinct platelet thromboxane A_2/prostaglandin H_2 receptor subtypes. A radioligand binding study of human platelets." *J Clin Invest*, **1989**, 84, (6), 1883-91.

Dorn, G. W., 2nd. "Regulation of response to thromboxane A_2 in CHRF-288 megakaryocytic cells." *Am J Physiol*, **1992**, 262, (4 Pt 1), C991-9.

Dorn, G. W., 2nd, Davis, M. G. and D'Angelo, D. D. "Structural determinants for agonist binding affinity to thromboxane/prostaglandin endoperoxide (TP) receptors. Analysis of chimeric rat/human TP receptors." *J Biol Chem*, **1997**, 272, (19), 12399-405.

Dorn, G. W., 2nd and DeJesus, A. "Human platelet aggregation and shape change are coupled to separate thromboxane A_2-prostaglandin H_2 receptors." *Am J Physiol*, **1991**, 260, (2 Pt 2), H327-34.

Dube, G. P., Jakubowski, J. A., Brune, K. A., Bemis, K. G. and Kurtz, W. L. "In vivo effects of a novel thromboxane A_2/prostaglandin H_2 (TXA_2/PGH_2) partial agonist, (+)5(Z)-7-[3-endo-phenylsulfonylamino[2.2.1]- bicyclohept-2-exo-yl]-heptenoic acid [(+)-S-145], on vascular, platelet and cardiac function." *J Pharmacol Exp Ther*, **1995**, 272, (2), 799-807.

Egan, K. M., Wang, M., Fries, S., Lucitt, M. B., Zukas, A. M., Pure, E., Lawson, J. A. and FitzGerald, G. A. "Cyclooxygenases, thromboxane, and atherosclerosis: plaque destabilization by cyclooxygenase-2 inhibition combined with thromboxane receptor antagonism." *Circulation*, **2005**, 111, (3), 334-42.

Fam, S. S. and Morrow, J. D. "The isoprostanes: unique products of arachidonic acid oxidation-a review." *Curr Med Chem*, **2003**, 10, (17), 1723-40.

Fiddler, G. I. and Lumley, P. "Preliminary clinical studies with thromboxane synthase inhibitors and thromboxane receptor blockers. A review." *Circulation*, **1990**, 81, (1 Suppl), I69-78; discussion I79-80.

Fitzgerald, D. J., Roy, L., Catella, F. and FitzGerald, G. A. "Platelet activation in unstable coronary disease." *N Engl J Med*, **1986**, 315, (16), 983-9.

Fitzgerald, D. J., Wright, F. and FitzGerald, G. A. "Increased thromboxane biosynthesis during coronary thrombolysis. Evidence that platelet activation and thromboxane A_2 modulate the response to tissue-type plasminogen activator in vivo." *Circ Res*, **1989**, 65, (1), 83-94.

Fitzgerald, G. A. "Coxibs and cardiovascular disease." *N Engl J Med*, **2004**, 351, (17), 1709-11.

FitzGerald, G. A., Cheng, Y. and Austin, S. "COX-2 inhibitors and the cardiovascular system." *Clin Exp Rheumatol*, **2001**, 19, (6 Suppl 25), S31-6.

FitzGerald, G. A., Smith, B., Pedersen, A. K. and Brash, A. R. "Increased prostacyclin biosynthesis in patients with severe atherosclerosis and platelet activation." *N Engl J Med*, **1984**, 310, (17), 1065-8.

Flannery, P. J. and Spurney, R. F. "Desensitization of the mouse thromboxane A_2 receptor (TP) by G protein-coupled receptor kinases (Grks)." *Prostaglandins Other Lipid Mediat*, **2002**, 70, (1-2), 79-90.

Foley, J. F., Kelley, L. P. and Kinsella, B. T. "Prostaglandin D_2 receptor-mediated desensitization of the alpha isoform of the human thromboxane A_2 receptor." *Biochem Pharmacol*, **2001**, 62, (2), 229-39.

Frankish, H. "Why do COX-2 inhibitors increase risk of cardiovascular events?" *Lancet*, **2002**, 359, (9315), 1410.

Friedman, S. A. "Preeclampsia: a review of the role of prostaglandins." *Obstet Gynecol*, **1988**, 71, (1), 122-37.

Fu, J. Y., Masferrer, J. L., Seibert, K., Raz, A. and Needleman, P. "The induction and suppression of prostaglandin H_2 synthase (cyclooxygenase) in human monocytes." *J Biol Chem*, **1990**, 265, (28), 16737-40.

Fujimura, A., Shiga, T., Kumagai, Y., Ohashi, K., Ebihara, A. and Kotegawa, T. "Pharmacokinetics of a new thromboxane A_2 receptor antagonist, S-1452, and its effect on platelet aggregation in healthy volunteers." *J Clin Pharmacol*, **1996**, 36, (5), 409-13.

Fujita, M. and Seki, T. "Syntheses and bioactivities of novel carbamates combining platelet activating factor (PAF) receptor antagonist with thromboxane synthase inhibitor (TxSI)." *Bioorg Med Chem Lett*, **2002**, 12, (10), 1383-6.

Fujita, M., Seki, T., Inada, H., Shimizu, K., Takahama, A. and Sano, T. "Approach to dual-acting platelet activating factor (PAF) receptor antagonist/thromboxane synthase inhibitor (TxSI) based on the link of PAF antagonists and TxSIs." *Bioorg Med Chem Lett*, **2002a**, 12, (3), 341-4.

Fujita, M., Seki, T., Inada, H., Shimizu, K., Takahama, A. and Sano, T. "Novel agents combining platelet activating factor (PAF) receptor antagonist with thromboxane synthase inhibitor (TxSI)." *Bioorg Med Chem Lett*, **2002b**, 12, (5), 771-4.

Fukumoto, S., Shiraishi, M., Terashita, Z., Ashida, Y. and Inada, Y. "Synthesis and thromboxane A_2/prostaglandin H_2 receptor antagonistic activity of phenol derivatives." *J Med Chem*, **1992**, 35, (12), 2202-9.

Funk, C. D., Furci, L., Moran, N. and Fitzgerald, G. A. "Point mutation in the seventh hydrophobic domain of the human thromboxane A_2 receptor allows discrimination between agonist and antagonist binding sites." *Mol Pharmacol*, **1993**, 44, (5), 934-9.

Gachet, C. "Regulation of platelet functions by P2 receptors." *Annu Rev Pharmacol Toxicol*, **2006**, 46, 277-300.

Gaussem, P., Reny, J. L., Thalamas, C., Chatelain, N., Kroumova, M., Jude, B., Boneu, B. and Fiessinger, J. N. "The specific thromboxane receptor antagonist S18886: pharmacokinetic and pharmacodynamic studies." *J Thromb Haemost*, **2005**, 3, (7), 1437-45.

Ghuysen, A., Lambermont, B., Dogné, J. M., Kolh, P., Tchana-Sato, V., Morimont, P., Magis, D., Hanson, J., Segers, P. and D'Orio, V. "Effect of BM-573 [N-terbutyl-N'-[2-(4'-methylphenylamino)-5-nitro-

benzenesulfonyl]urea], a dual thromboxane synthase inhibitor and thromboxane receptor antagonist, in a porcine model of acute pulmonary embolism." *J Pharmacol Exp Ther*, **2004**, 310, (3), 964-72.

Giberto, M., Canova, G., Masini, R., Damerio, M. A. and Filippi, M. "[Picotamide monohydrate prophylaxis of deep venous thrombosis in patients undergoing general surgery. Clinical experience]." *Clin Ter*, **1991**, 137, (5), 339-42.

Girolami, B., Bernardi, E., Prins, M. H., ten Cate, J. W., Prandoni, P., Hettiarachchi, R., Marras, E., Stefani, P. M., Girolami, A. and Buller, H. R. "Antithrombotic drugs in the primary medical management of intermittent claudication: a meta-analysis." *Thromb Haemost*, **1999**, 81, (5), 715-22.

Godard, P., Chaintreuil, J., Damon, M., Coupe, M., Flandre, O., Crastes de Paulet, A. and Michel, F. B. "Functional assessment of alveolar macrophages: comparison of cells from asthmatics and normal subjects." *J Allergy Clin Immunol*, **1982**, 70, (2), 88-93.

Grandel, U., Fink, L., Blum, A., Heep, M., Buerke, M., Kraemer, H. J., Mayer, K., Bohle, R. M., Seeger, W., Grimminger, F. and Sibelius, U. "Endotoxin-induced myocardial tumor necrosis factor-alpha synthesis depresses contractility of isolated rat hearts: evidence for a role of sphingosine and cyclooxygenase-2-derived thromboxane production." *Circulation*, **2000**, 102, (22), 2758-64.

Grandel, U., Hopf, M., Buerke, M., Hattar, K., Heep, M., Fink, L., Bohle, R. M., Morath, S., Hartung, T., Pullamsetti, S., Schermuly, R. T., Seeger, W., Grimminger, F. and Sibelius, U. "Mechanisms of cardiac depression caused by lipoteichoic acids from Staphylococcus aureus in isolated rat hearts." *Circulation*, **2005**, 112, (5), 691-8.

Gresele, P., Arnout, J., Deckmyn, H., Huybrechts, E., Pieters, G. and Vermylen, J. "Role of proaggregatory and antiaggregatory prostaglandins in hemostasis. Studies with combined thromboxane synthase inhibition and thromboxane receptor antagonism." *J Clin Invest*, **1987**, 80, (5), 1435-45.

Gresele, P., Deckmyn, H., Arnout, J., Lemmens, J., Janssens, W. and Vermylen, J. "BM 13.177, a selective blocker of platelet and vessel wall thromboxane receptors, is active in man." *Lancet*, **1984**, 1, (8384), 991-4.

Gryglewski, R. J., Szczeklik, A., Korbut, R., Swies, J., Musial, J., Krzanowski, M. and Maga, P. "The mechanism of anti-thrombotic, thrombolytic and fibrinolytic actions of camonagrel--a new thromboxane synthase inhibitor." *Wien Klin Wochenschr*, **1995**, 107, (9), 283-9.

Grynkiewicz, G., Poenie, M. and Tsien, R. Y. "A new generation of Ca^{2+} indicators with greatly improved fluorescence properties." *J Biol Chem*, **1985**, 260, (6), 3440-50.

Gurbel, P. A., Murugesan, S. R., Lowry, D. R. and Serebruany, V. L. "Plasma thromboxane and prostacyclin are linear related and increased in patients presenting with acute myocardial infarction." *Prostaglandins Leukot Essent Fatty Acids*, **1999**, 61, (1), 7-11.

Guth, B. D., Narjes, H., Schubert, H. D., Tanswell, P., Riedel, A. and Nehmiz, G. "Pharmacokinetics and pharmacodynamics of terbogrel, a combined thromboxane A_2 receptor and synthase inhibitor, in healthy subjects." *Br J Clin Pharmacol*, **2004**, 58, (1), 40-51.

Habib, A., FitzGerald, G. A. and Maclouf, J. "Phosphorylation of the thromboxane receptor alpha, the predominant isoform expressed in human platelets." *J Biol Chem*, **1999**, 274, (5), 2645-51.

Habib, A., Vezza, R., Creminon, C., Maclouf, J. and FitzGerald, G. A. "Rapid, agonist-dependent phosphorylation in vivo of human thromboxane receptor isoforms. Minimal involvement of protein kinase C." *J Biol Chem*, **1997**, 272, (11), 7191-200.

Hall, S. E. "Thromboxane A_2 receptor antagonists." *Med Res Rev*, **1991**, 11, (5), 503-79.

Halmos, T., Turek, J. W., Le Breton, G. C. and Antonakis, K. "Synthesis and biological characterization of sqbazide, a novel biotinylated photoaffinity probe for the study of the human platelet thromboxane A_2 receptor." *Bioorg Med Chem Lett*, **1999**, 9, (20), 2963-8.

Halushka, P. V. "Thromboxane A_2 receptors: where have you gone?" *Prostaglandins Other Lipid Mediat*, **2000**, 60, (4-6), 175-89.

Hamberg, M. and Samuelsson, B. "On the mechanism of the biosynthesis of prostaglandins E-1 and F-1-alpha." *J Biol Chem*, **1967**, 242, (22), 5336-43.

Hamberg, M., Svensson, J. and Samuelsson, B. "Thromboxanes: a new group of biologically active compounds derived from prostaglandin endoperoxides." *Proc Natl Acad Sci U S A*, **1975**, 72, (8), 2994-8.

Hamberg, M., Svensson, J., Wakabayashi, T. and Samuelsson, B. "Isolation and structure of two prostaglandin endoperoxides that cause platelet aggregation." *Proc Natl Acad Sci U S A*, **1974**, 71, (2), 345-9.

Hamm, C. W., Lorenz, R. L., Bleifeld, W., Kupper, W., Wober, W. and Weber, P. C. "Biochemical evidence of platelet activation in patients with persistent unstable angina." *J Am Coll Cardiol*, **1987**, 10, (5), 998-1006.

Hanasaki, K., Nakano, K., Kasai, H. and Arita, H. "Biochemical characterization and comparison of rat thromboxane A_2/prostaglandin H_2 receptors in platelets and cultured aortic smooth muscle cells." *Biochem Pharmacol*, **1989**, 38, (18), 2967-76.

Hansson, G. K. "Inflammation, atherosclerosis, and coronary artery disease." *N Engl J Med*, **2005**, 352, (16), 1685-95.

Harris, R. C., McKanna, J. A., Akai, Y., Jacobson, H. R., Dubois, R. N. and Breyer, M. D. "Cyclooxygenase-2 is associated with the macula densa of rat kidney and increases with salt restriction." *J Clin Invest*, **1994**, 94, (6), 2504-10.

Hathaway, D. R., Eaton, C. R. and Adelstein, R. S. "Regulation of human platelet myosin light chain kinase by the catalytic subunit of cyclic AMP-dependent protein kinase." *Nature*, **1981**, 291, (5812), 252-6.

Haurand, M. and Ullrich, V. "Isolation and characterization of thromboxane synthase from human platelets as a cytochrome P-450 enzyme." *J Biol Chem*, **1985**, 260, (28), 15059-67.

Haylett, D. G. "Direct Measurement of Drugs Binding to Receptor." *Textbook of Receptor Pharmacology*, **2003**, J. C. Foreman and T. Johansen, CRC Press**:** 153-180.

Hecker, M., Haurand, M., Ullrich, V. and Terao, S. "Spectral studies on structure-activity relationships of thromboxane synthase inhibitors." *Eur J Biochem*, **1986**, 157, (1), 217-23.

Hecker, M. and Ullrich, V. "On the mechanism of prostacyclin and thromboxane A_2 biosynthesis." *J Biol Chem*, **1989**, 264, (1), 141-50.

Herrick, A. L., Illingworth, K., Blann, A., Hay, C. R., Hollis, S. and Jayson, M. I. "Von Willebrand factor, thrombomodulin, thromboxane, beta-thromboglobulin and markers of fibrinolysis in primary Raynaud's phenomenon and systemic sclerosis." *Ann Rheum Dis*, **1996**, 55, (2), 122-7.

Heylen, L., De Clerck, F., Somers, Y. and Leysen, J. E. "Thromboxane A_2/prostaglandin endoperoxide (TXA_2/PG-END) receptor binding properties in human platelets of ridogrel, a combined TXA_2 synthase inhibitor--TXA_2/PG-END receptor antagonist." *Blood Coagul Fibrinolysis*, **1991**, 2, (5), 617-21.

Hill, S. J. "G-protein-coupled receptors: past, present and future." *Br J Pharmacol*, **2006**, 147 Suppl 1, S27-37.

Hirata, M., Hayashi, Y., Ushikubi, F., Yokota, Y., Kageyama, R., Nakanishi, S. and Narumiya, S. "Cloning and expression of cDNA for a human thromboxane A_2 receptor." *Nature*, **1991**, 349, (6310), 617-20.

Hirata, T., Ushikubi, F., Kakizuka, A., Okuma, M. and Narumiya, S. "Two thromboxane A_2 receptor isoforms in human platelets. Opposite coupling to adenylyl cyclase with different sensitivity to Arg60 to Leu mutation." *J Clin Invest*, **1996**, 97, (4), 949-56.

Hladovec, J. "A new model of arterial thrombosis." *Thromb Res*, **1986**, 41, (5), 659-64.

Hoet, B., Falcon, C., De Reys, S., Arnout, J., Deckmyn, H. and Vermylen, J. "R68070, a combined thromboxane/endoperoxide receptor antagonist and thromboxane synthase inhibitor, inhibits human platelet activation in vitro and in vivo: a comparison with aspirin." *Blood*, **1990**, 75, (3), 646-53.

Hong, J. L. and Lee, L. Y. "Cigarette smoke-induced bronchoconstriction: causative agents and role of thromboxane receptors." *J Appl Physiol*, **1996**, 81, (5), 2053-9.

Honn, K. V., Tang, D. G. and Chen, Y. Q. "Platelets and cancer metastasis: more than an epiphenomenon." *Semin Thromb Hemost*, **1992**, 18, (4), 392-415.

Horiguchi, T., Tachikawa, S., Kondo, R., Shiga, M., Hirose, M. and Fukumoto, K. "Study on the usefulness of seratrodast in the treatment of chronic pulmonary emphysema." *Arzneimittelforschung*, **2002**, 52, (10), 764-8.

Hoshino, M., Sim, J., Shimizu, K., Nakayama, H. and Koya, A. "Effect of AA-2414, a thromboxane A_2 receptor antagonist, on airway inflammation in subjects with asthma." *J Allergy Clin Immunol*, **1999**, 103, (6), 1054-61.

Hwang, D., Scollard, D., Byrne, J. and Levine, E. "Expression of cyclooxygenase-1 and cyclooxygenase-2 in human breast cancer." *J Natl Cancer Inst*, **1998**, 90, (6), 455-60.

Hwang, T. L., Yeh, Y. A., Chern, J. W. and Teng, C. M. "Pharmacological characterization of EK112, a new combined angiotensin II and thromboxane A_2 receptor antagonist." *Gen Pharmacol*, **2000**, 34, (1), 25-31.

Ishizuka, T., Suzuki, K., Kawakami, M., Hidaka, T., Matsuki, Y. and Nakamura, H. "Thromboxane A_2 receptor blockade suppresses intercellular adhesion molecule-1 expression by stimulated vascular endothelial cells." *Eur J Pharmacol*, **1996**, 312, (3), 367-77.

Iwamoto, I., Ra, C., Sato, T., Tomioka, H. and Yoshida, S. "Thromboxane A_2 production in allergen-induced immediate and late asthmatic responses. Its possible role in inducing the late response." *J Asthma*, **1988**, 25, (3), 117-24.

Jacobs, C., Frotscher, M., Dannhardt, G. and Hartmann, R. W. "1-imidazolyl(alkyl)-substituted di- and tetrahydroquinolines and analogues: syntheses and evaluation of dual inhibitors of thromboxane A_2 synthase and aromatase." *J Med Chem*, **2000**, 43, (9), 1841-51.

Jin, J. and Kunapuli, S. P. "Coactivation of two different G protein-coupled receptors is essential for ADP-induced platelet aggregation." *Proc Natl Acad Sci U S A*, **1998**, 95, (14), 8070-4.

Jordan, B. A. and Devi, L. A. "G-protein-coupled receptor heterodimerization modulates receptor function." *Nature*, **1999**, 399, (6737), 697-700.

Jourdan, A., Aguejouf, O., Imbault, P., Doutremepuich, F., Inamo, J. and Doutremepuich, C. "Experimental thrombosis model induced by free radicals. Application to aspirin and other different substances." *Thromb Res*, **1995**, 79, (1), 109-23.

Kan, K. K., Jones, R. L., Ngan, M. P. and Rudd, J. A. "Actions of prostanoids to induce emesis and defecation in the ferret." *Eur J Pharmacol*, **2002**, 453, (2-3), 299-308.

Kanao, M., Watanabe, Y., Kimura, Y., Saegusa, J., Yamamoto, K., Kanno, H., Kanaya, N., Kubo, H., Ashida, S. and Ishikawa, F. "Thromboxane A_2 synthetase inhibitors. 2. Syntheses and activities of tetrahydronaphthalene and indan derivatives." *J Med Chem*, **1989**, 32, (6), 1326-34.

Karasawa, A., Kawakage, M., Shirakura, S., Higo, K., Kubo, K., Ohshima, E. and Obase, H. "Antiplatelet effects of the novel thromboxane A_2 receptor antagonist sodium (E)-11-[2-(5,6-dimethyl-1-benzimidazolyl)-ethylidene]-6,11- dihydrodibenz[b,e] oxepine-2-carboxylate monohydrate." *Arzneimittelforschung*, **1991**, 41, (12), 1230-6.

Kato, K., Ohkawa, S., Terao, S., Terashita, Z. and Nishikawa, K. "Thromboxane synthetase inhibitors (TXSI). Design, synthesis, and evaluation of a novel series of omega-pyridylalkenoic acids." *J Med Chem*, **1985**, 28, (3), 287-94.

Kelley-Hickie, L. P. and Kinsella, B. T. "EP1- and FP-mediated cross-desensitization of the alpha (alpha) and beta (beta) isoforms of the human thromboxane A_2 receptor." *Br J Pharmacol*, **2004**, 142, (1), 203-21.

Kenakin, T. "Inverse, protean, and ligand-selective agonism: matters of receptor conformation." *Faseb J*, **2001**, 15, (3), 598-611.

Khasawneh, F. T., Huang, J. S., Turek, J. W. and Le Breton, G. C. "Differential mapping of the amino acids mediating agonist and antagonist coordination with the human thromboxane A_2 receptor protein." *J Biol Chem*, **2006**, 281, (37), 26951-65.

Kinsella, B. T., O'Mahony, D., Lawson, J. A., Pratico, D. and Fitzgerald, G. A. "Cellular activation of thromboxane receptors." *Ann N Y Acad Sci*, **1994a**, 714, 270-8.

Kinsella, B. T., O'Mahony, D. J. and FitzGerald, G. A. "Phosphorylation and regulated expression of the human thromboxane A_2 receptor." *J Biol Chem*, **1994b**, 269, (47), 29914-9.

Kitahara, M., Ichikawa, M., Kinoshita, T., Shiozawa, S., Shigematsu, H. and Komiyama, A. "Prostacyclin inhibits the production of MMP-9 induced by phorbol ester through protein kinase A activation, but does not affect the production of MMP-2 in Human cultured mesangial cells." *Kidney Blood Press Res*, **2001**, 24, (1), 18-26.

Klages, B., Brandt, U., Simon, M. I., Schultz, G. and Offermanns, S. "Activation of G_{12}/G_{13} results in shape change and Rho/Rho-kinase-mediated myosin light chain phosphorylation in mouse platelets." *J Cell Biol*, **1999**, 144, (4), 745-54.

Knezevic, I., Borg, C. and Le Breton, G. C. "Identification of G_q as one of the G-proteins which copurify with human platelet thromboxane A_2/prostaglandin H_2 receptors." *J Biol Chem*, **1993**, 268, (34), 26011-7.

Kobayashi, T., Tahara, Y., Matsumoto, M., Iguchi, M., Sano, H., Murayama, T., Arai, H., Oida, H., Yurugi-Kobayashi, T., Yamashita, J. K., Katagiri, H., Majima, M., Yokode, M., Kita, T. and Narumiya, S. "Roles of thromboxane A_2 and prostacyclin in the development of atherosclerosis in apoE-deficient mice." *J Clin Invest*, **2004**, 114, (6), 784-94.

Kolh, P., Rolin, S., Tchana-Sato, V., Petein, M., Ghuysen, A., Lambermont, B., Hanson, J., Magis, D., Segers, P., Masereel, B., D'Orio, V. and Dogné, J. M. "Evaluation of BM573, a novel TXA_2 synthase inhibitor and receptor antagonist, in a porcine model of myocardial

ischemia-reperfusion." *Prostaglandins Other Lipid Mediat*, **2006**, 79, (1-2), 53-73.

Konstam, M. A., Weir, M. R., Reicin, A., Shapiro, D., Sperling, R. S., Barr, E. and Gertz, B. J. "Cardiovascular thrombotic events in controlled, clinical trials of rofecoxib." *Circulation*, **2001**, 104, (19), 2280-8.

Korbut, R. and Gryglewski, R. J. "A paradox of thrombogenesis by streptokinase and its prevention by iloprost and camonagrel." *Methods Find Exp Clin Pharmacol*, **1996**, 18, (3), 167-74.

Kovacs, I. B., Tigyi-Sebes, A., Trombitas, K. and Gorog, P. "Evans blue: an ideal energy-absorbing material to produce intravascular microinjury by HE-NE gas laser." *Microvasc Res*, **1975**, 10, (1), 107-24.

Kudoh, A. and Matsuki, A. "Bradykinin-induced inositol 1,4,5-triphosphate in neonatal rat cardiomyocytes is activated by endotoxin." *Shock*, **2000**, 14, (6), 635-9.

Kumlin, M., Dahlen, B., Bjorck, T., Zetterstrom, O., Granstrom, E. and Dahlen, S. E. "Urinary excretion of leukotriene E4 and 11-dehydro-thromboxane B_2 in response to bronchial provocations with allergen, aspirin, leukotriene D_4, and histamine in asthmatics." *Am Rev Respir Dis*, **1992**, 146, (1), 96-103.

Kurokawa, T., Matsumoto, T., Ashida, Y., Sasada, R. and Iwasa, S. "Antagonism of the human thromboxane A_2 receptor by an anti-asthmatic agent AA-2414." *Biol Pharm Bull*, **1994**, 17, (3), 383-5.

Kurz, K. D., Main, B. W. and Sandusky, G. E. "Rat model of arterial thrombosis induced by ferric chloride." *Thromb Res*, **1990**, 60, (4), 269-80.

Lambermont, B., Kolh, P., Ghuysen, A., Segers, P., Dogné, J. M., Tchana-Sato, V., Morimont, P., Benoit, P., Gerard, P., Masereel, B. and D'Orio, V. "Effect of a novel thromboxane A_2 inhibitor on right ventricular-arterial coupling in endotoxic shock." *Shock*, **2004**, 21, (1), 45-51.

Laroche, G., Lepine, M. C., Theriault, C., Giguere, P., Giguere, V., Gallant, M. A., de Brum-Fernandes, A. and Parent, J. L. "Oligomerization of the alpha and beta isoforms of the thromboxane A_2 receptor:

relevance to receptor signaling and endocytosis." *Cell Signal*, **2005**, 17, (11), 1373-83.

LeDuc, L. E., Wyche, A. A., Sprecher, H., Sankarappe, S. K. and Needleman, P. "Analogues of arachidonic acid used to evaluate structural determinants of prostaglandin receptor and enzyme specificities." *Mol Pharmacol*, **1981**, 19, (2), 242-7.

Lefer, A. M., Smith, E. F., 3rd, Araki, H., Smith, J. B., Aharony, D., Claremon, D. A., Magolda, R. L. and Nicolaou, K. C. "Dissociation of vasoconstrictor and platelet aggregatory activities of thromboxane by carbocyclic thromboxane A_2, a stable analog of thromboxane A_2." *Proc Natl Acad Sci U S A*, **1980**, 77, (3), 1706-10.

Lefkowitz, R. J., Cotecchia, S., Samama, P. and Costa, T. "Constitutive activity of receptors coupled to guanine nucleotide regulatory proteins." *Trends Pharmacol Sci*, **1993**, 14, (8), 303-7.

Li, N., Hu, H. and Hjemdahl, P. "Aspirin treatment does not attenuate platelet or leukocyte activation as monitored by whole blood flow cytometry." *Thromb Res*, **2003**, 111, (3), 165-70.

Liel, N., Mais, D. E. and Halushka, P. V. "Desensitization of platelet thromboxane A_2/prostaglandin H_2 receptors by the mimetic U46619." *J Pharmacol Exp Ther*, **1988**, 247, (3), 1133-8.

Lin, K., Sadee, W. and Quillan, J. M. "Rapid measurements of intracellular calcium using a fluorescence plate reader." *Biotechniques*, **1999**, 26, (2), 318-22, 324-6.

Linton, M. F. and Fazio, S. "Cyclooxygenase-2 and inflammation in atherosclerosis." *Curr Opin Pharmacol*, **2004**, 4, (2), 116-23.

Luttrell, L. M. "Regulators of GPCR activity." *The G Protein-Coupled Receptor Handbook*, **2005**, L. A. devi, Humana Press**:** 159-198.

Ma, L., Gao, J. and Chen, X. "G Protein-Coupled Receptor Kinases." *The G Protein-Coupled Receptor Handbook*, **2005**, L. A. devi, Humana Press**:** 149-158.

Macfarlane, S. R., Seatter, M. J., Kanke, T., Hunter, G. D. and Plevin, R. "Proteinase-activated receptors." *Pharmacol Rev*, **2001**, 53, (2), 245-82.

Maggio, R., Vogel, Z. and Wess, J. "Coexpression studies with mutant muscarinic/adrenergic receptors provide evidence for intermolecular "cross-talk" between G-protein-linked receptors." *Proc Natl Acad Sci U S A*, **1993a**, 90, (7), 3103-7.

Maggio, R., Vogel, Z. and Wess, J. "Reconstitution of functional muscarinic receptors by co-expression of amino- and carboxyl-terminal receptor fragments." *FEBS Lett*, **1993b**, 319, (1-2), 195-200.

Mais, D. E., DeHoll, D., Sightler, H. and Halushka, P. V. "Different pharmacologic activities for 13-azapinane thromboxane A_2 analogs in platelets and blood vessels." *Eur J Pharmacol*, **1988**, 148, (3), 309-15.

Mais, D. E., Dunlap, C., Hamanaka, N. and Halushka, P. V. "Further studies on the effects of epimers of thromboxane A_2 antagonists on platelets and veins." *Eur J Pharmacol*, **1985**, 111, (1), 125-8.

Manganello, J. M., Djellas, Y., Borg, C., Antonakis, K. and Le Breton, G. C. "Cyclic AMP-dependent phosphorylation of thromboxane A_2 receptor-associated $G\alpha_{13}$." *J Biol Chem*, **1999**, 274, (39), 28003-10.

Marshall, F. H., Jones, K. A., Kaupmann, K. and Bettler, B. "GABAB receptors - the first 7TM heterodimers." *Trends Pharmacol Sci*, **1999**, 20, (10), 396-9.

Marusawa, H., Setoi, H., Sawada, A., Kuroda, A., Seki, J., Motoyama, Y. and Tanaka, H. "Synthesis and biological activity of 1-phenylsulfonyl-4-phenylsulfonylaminopyrrolidine derivatives as thromboxane A_2 receptor antagonists." *Bioorg Med Chem*, **2002**, 10, (5), 1399-415.

Masereel, B., Damas, J., Fontaine, J., Lembege, M., Lacan, F., Nuhrich, A., Delarge, J., Pochet, L. and Dogné, J. M. "Thromboxane A_2 receptor antagonism in man and rat by a sulphonylcyanoguanidine (BM-144) and a sulphonylurea (BM-500)." *J Pharm Pharmacol*, **1999**, 51, (6), 695-701.

Masuda, A., Mais, D. E., Oatis, J. E., Jr. and Halushka, P. V. "Platelet and vascular thromboxane A_2/prostaglandin H_2 receptors. Evidence for different subclasses in the rat." *Biochem Pharmacol*, **1991**, 42, (3), 537-44.

Matsumoto, K., Aizawa, H., Inoue, H., Takata, S., Shigyo, M. and Hara, N. "Role of thromboxane A_2 and cholinergic mechanisms in bronchoconstriction induced by cigarette smoke in guinea-pigs." *Eur Respir J*, **1996**, 9, (12), 2468-73.

Matsuno, H., Uematsu, T., Niwa, M., Kozawa, O., Nagashima, S., Kohno, K., Kato, H., Kawabata, Y., Yoshida, Y. and Kanamaru, M. "Pharmacokinetic and pharmacodynamic properties of a new thromboxane receptor antagonist (Z-335) after single and multiple oral administrations to healthy volunteers." *J Clin Pharmacol*, **2002**, 42, (7), 782-90.

Matz, R. L., Van Overloop, B., Gaubert, E. and Gairard, A. "Maturation reveals a decrease in endothelium-dependent contraction induced by depolarization in the aorta of spontaneously hypertensive rats." *Life Sci*, **2001**, 69, (15), 1791-804.

McAdam, B. F., Catella-Lawson, F., Mardini, I. A., Kapoor, S., Lawson, J. A. and FitzGerald, G. A. "Systemic biosynthesis of prostacyclin by cyclooxygenase COX-2: the human pharmacology of a selective inhibitor of COX-2." *Proc Natl Acad Sci U S A*, **1999**, 96, (1), 272-7.

McLemore, T. L., Hubbard, W. C., Litterst, C. L., Liu, M. C., Miller, S., McMahon, N. A., Eggleston, J. C. and Boyd, M. R. "Profiles of prostaglandin biosynthesis in normal lung and tumor tissue from lung cancer patients." *Cancer Res*, **1988**, 48, (11), 3140-7.

Meerwein, H., Dittmar, G., Göllner, R., Hafner, K., Mensch, F. and Steinfort, O. "Verfahren zur herstellung aromatischer sulfonsäurechloride, eine neue modifikation der Sandmeyer reaktion." *Chemische Berichte*, **1957**, 90, (6), 841-852.

Mehta, J. L., Lawson, D., Mehta, P. and Saldeen, T. "Increased prostacyclin and thromboxane A_2 biosynthesis in atherosclerosis." *Proc Natl Acad Sci U S A*, **1988**, 85, (12), 4511-5.

Mehta, P., Lawson, D., Ward, M. B., Lee-Ambrose, L. and Kimura, A. "Effects of thromboxane A_2 inhibition on osteogenic sarcoma cell-induced platelet aggregation." *Cancer Res*, **1986**, 46, (10), 5061-3.

Miggin, S. M. and Kinsella, B. T. "Expression and tissue distribution of the mRNAs encoding the human thromboxane A_2 receptor (TP) alpha and beta isoforms." *Biochim Biophys Acta*, **1998**, 1425, (3), 543-59.

Mihara, S., Hara, S., Ueda, M., Ide, M. and Fujimoto, M. "Comparison of vascular and platelet thromboxane A_2/prostaglandin H_2 receptors in the pig." *Eur J Pharmacol*, **1989**, 160, (3), 313-21.

Miki, I., Kase, H. and Ishii, A. "Differences in activities of thromboxane A_2 receptor antagonists in smooth muscle cells." *Eur J Pharmacol*, **1992**, 227, (2), 199-204.

Miyata, A., Yokoyama, C., Ihara, H., Bandoh, S., Takeda, O., Takahashi, E. and Tanabe, T. "Characterization of the human gene (TBXAS1) encoding thromboxane synthase." *Eur J Biochem*, **1994**, 224, (2), 273-9.

Monshizadegan, H., Hedberg, A. and Webb, M. L. "Characterization of binding of a specific antagonist, [3H]-SQ29,548, to soluble thromboxane A_2/prostaglandin H_2 (TP) receptors in human platelet membranes." *Life Sci*, **1992**, 51, (6), 431-7.

Moore, F., Asboth, G. and Lopez, B. A. "Thromboxane receptor signalling in human myometrial cells." *Prostaglandins Other Lipid Mediat*, **2002**, 67, (1), 31-47.

Morinelli, T. A., Niewiarowski, S., Daniel, J. L. and Smith, J. B. "Receptor-mediated effects of a PGH_2 analogue (U46619) on human platelets." *Am J Physiol*, **1987**, 253, (5 Pt 2), H1035-43.

Morinelli, T. A., Okwu, A. K., Mais, D. E., Halushka, P. V., John, V., Chen, C. K. and Fried, J. "Difluorothromboxane A_2 and stereoisomers: stable derivatives of thromboxane A_2 with differential effects on platelets and blood vessels." *Proc Natl Acad Sci U S A*, **1989**, 86, (14), 5600-4.

Mukherjee, D., Nissen, S. E. and Topol, E. J. "Risk of cardiovascular events associated with selective COX-2 inhibitors." *Jama*, **2001**, 286, (8), 954-9.

Murphy, R. C., Hammarstrom, S. and Samuelsson, B. "Leukotriene C: a slow-reacting substance from murine mastocytoma cells." *Proc Natl Acad Sci U S A*, **1979**, 76, (9), 4275-9.

Murray, R., Shipp, E. and FitzGerald, G. A. "Prostaglandin endoperoxide/thromboxane A_2 receptor desensitization. Cross-talk with adenylate cyclase in human platelets." *J Biol Chem*, **1990**, 265, (35), 21670-5.

Nakane, M., Reid, J. A., Han, W. C., Das, J., Truc, V. C., Haslanger, M. F., Garber, D., Harris, D. N., Hedberg, A., Ogletree, M. L. and et al. "7-Oxabicyclo[2.2.1]heptyl carboxylic acids as thromboxane A_2 antagonists: aza omega-chain analogues." *J Med Chem*, **1990**, 33, (9), 2465-76.

Namba, T., Sugimoto, Y., Hirata, M., Hayashi, Y., Honda, A., Watabe, A., Negishi, M., Ichikawa, A. and Narumiya, S. "Mouse thromboxane A_2 receptor: cDNA cloning, expression and northern blot analysis." *Biochem Biophys Res Commun*, **1992**, 184, (3), 1197-203.

Nanji, A. A. "Thromboxane synthase and organ preference for metastases." *N Engl J Med*, **1993**, 329, (2), 138-9.

Narisada, M., Ohtani, M., Watanabe, F., Uchida, K., Arita, H., Doteuchi, M., Hanasaki, K., Kakushi, H., Otani, K. and Hara, S. "Synthesis and in vitro activity of various derivatives of a novel thromboxane receptor antagonist, (+/-)-(5Z)-7-[3-endo-[(phenylsulfonyl)amino]bicyclo[2.2.1] hept-2-exo-yl]heptenoic acid." *J Med Chem*, **1988**, 31, (9), 1847-54.

Narumiya, S., Hirata, N., Namba, T., Hayashi, Y., Ushikubi, F., Sugimoto, Y., Negishi, M. and Ichikawa, A. "Structure and function of prostanoid receptors." *J Lipid Mediat*, **1993**, 6, (1-3), 155-61.

Needleman, P., Moncada, S., Bunting, S., Vane, J. R., Hamberg, M. and Samuelsson, B. "Identification of an enzyme in platelet microsomes which generates thromboxane A_2 from prostaglandin endoperoxides." *Nature*, **1976**, 261, (5561), 558-60.

Nettis, E., Colanardi, M. C., Ferrannini, A. and Tursi, A. "Update on sensitivity to nonsteroidal antiinflammatory drugs." *Curr Drug Targets Immune Endocr Metabol Disord*, **2001**, 1, (3), 233-40.

Nguyen Hwangpo, T. and Iyengar, R. "Heterotrimeric G Proteins and Their Effector Pathways." *The G Protein-Coupled Receptors Handbook* **2005**, L. A. devi, Humana Press**:** 109-134.

Nicolaou, K. C., Magolda, R. L., Smith, J. B., Aharony, D., Smith, E. F. and Lefer, A. M. "Synthesis and biological properties of pinane-thromboxane A_2, a selective inhibitor of coronary artery constriction, platelet aggregation, and thromboxane formation." *Proc Natl Acad Sci U S A*, **1979**, 76, (6), 2566-70.

Nie, D., Lamberti, M., Zacharek, A., Li, L., Szekeres, K., Tang, K., Chen, Y. and Honn, K. V. "Thromboxane A_2 regulation of endothelial cell migration, angiogenesis, and tumor metastasis." *Biochem Biophys Res Commun*, **2000**, 267, (1), 245-51.

Nigam, S. and Zakrzewicz, A. "Tumour cell proliferation by thromboxane A_2: a receptor-mediated event." *Adv Prostaglandin Thromboxane Leukot Res*, **1991**, 21B, 925-8.

Nishizuka, Y. "The role of protein kinase C in cell surface signal transduction and tumour promotion." *Nature*, **1984**, 308, (5961), 693-8.

Nusing, R. M., Hirata, M., Kakizuka, A., Eki, T., Ozawa, K. and Narumiya, S. "Characterization and chromosomal mapping of the human thromboxane A_2 receptor gene." *J Biol Chem*, **1993**, 268, (33), 25253-9.

Ochocka, A. M. and Pawelczyk, T. "Isozymes delta of phosphoinositide-specific phospholipase C and their role in signal transduction in the cell." *Acta Biochim Pol*, **2003**, 50, (4), 1097-110.

Offermanns, S., Laugwitz, K. L., Spicher, K. and Schultz, G. "G proteins of the G_{12} family are activated via thromboxane A_2 and thrombin receptors in human platelets." *Proc Natl Acad Sci U S A*, **1994**, 91, (2), 504-8.

Offermanns, S., Toombs, C. F., Hu, Y. H. and Simon, M. I. "Defective platelet activation in G alpha(q)-deficient mice." *Nature*, **1997**, 389, (6647), 183-6.

Ogletree, M. L. and Allen, G. T. "Interspecies differences in thromboxane receptors: studies with thromboxane receptor antagonists

in rat and guinea pig smooth muscles." *J Pharmacol Exp Ther*, **1992**, 260, (2), 789-94.

Ogletree, M. L., Harris, D. N., Greenberg, R., Haslanger, M. F. and Nakane, M. "Pharmacological actions of SQ29548, a novel selective thromboxane antagonist." *J Pharmacol Exp Ther*, **1985**, 234, (2), 435-41.

Ogletree, M. L., Harris, D. N., Schumacher, W. A., Webb, M. L. and Misra, R. N. "Pharmacological profile of BMS180291: a potent, long-acting, orally active thromboxane A_2/prostaglandin endoperoxide receptor antagonist." *J Pharmacol Exp Ther*, **1993**, 264, (2), 570-8.

Ohashi, K., Ruan, K. H., Kulmacz, R. J., Wu, K. K. and Wang, L. H. "Primary structure of human thromboxane synthase determined from the cDNA sequence." *J Biol Chem*, **1992**, 267, (2), 789-93.

Oketani, K., Inoue, T. and Murakami, M. "Effect of E3040, an inhibitor of 5-lipoxygenase and thromboxane synthase, on rat bowel damage induced by lipopolysaccharide." *Eur J Pharmacol*, **2001**, 427, (2), 159-66.

Oketani, K., Nagakura, N., Harada, K. and Inoue, T. "In vitro effects of E3040, a dual inhibitor of 5-lipoxygenase and thromboxane A_2 synthetase, on eicosanoid production." *Eur J Pharmacol*, **2001**, 422, (1-3), 209-16.

Okon, E. B., Golbabaie, A. and van Breemen, C. "In the presence of L-NAME SERCA blockade induces endothelium-dependent contraction of mouse aorta through activation of smooth muscle prostaglandin H_2/thromboxane A_2 receptors." *Br J Pharmacol*, **2002**, 137, (4), 545-53.

Okwu, A. K., Ullian, M. E. and Halushka, P. V. "Homologous desensitization of human platelet thromboxane A_2/prostaglandin H_2 receptors." *J Pharmacol Exp Ther*, **1992**, 262, (1), 238-45.

Pace-Asciak, C. R. and Martin, J. M. "Hepoxilin, a new family of insulin secretagogues formed by intact rat pancreatic islets." *Prostaglandins Leukot Med*, **1984**, 16, (2), 173-80.

Pace-Asciak, C. R., Reynaud, D., Demin, P., Aslam, R. and Sun, A. "A new family of thromboxane receptor antagonists with secondary

thromboxane synthase inhibition." *J Pharmacol Exp Ther*, **2002**, 301, (2), 618-24.

Pancera, P., Sansone, S., Secchi, S., Covi, G. and Lechi, A. "The effects of thromboxane A_2 inhibition (picotamide) and angiotensin II receptor blockade (losartan) in primary Raynaud's phenomenon." *J Intern Med*, **1997**, 242, (5), 373-6.

Parent, J. L., Labrecque, P., Driss Rochdi, M. and Benovic, J. L. "Role of the differentially spliced carboxyl terminus in thromboxane A_2 receptor trafficking: identification of a distinct motif for tonic internalization." *J Biol Chem*, **2001**, 276, (10), 7079-85.

Parent, J. L., Labrecque, P., Orsini, M. J. and Benovic, J. L. "Internalization of the TXA_2 receptor alpha and beta isoforms. Role of the differentially spliced cooh terminus in agonist-promoted receptor internalization." *J Biol Chem*, **1999**, 274, (13), 8941-8.

Patrono, C. "Biosynthesis and pharmacological modulation of thromboxane in humans." *Circulation*, **1990**, 81, (1 Suppl), I12-5; discussion I22-3.

Patrono, C. "Aspirin resistance: definition, mechanisms and clinical read-outs." *J Thromb Haemost*, **2003**, 1, (8), 1710-3.

Patrono, C. and FitzGerald, G. A. "Isoprostanes: potential markers of oxidant stress in atherothrombotic disease." *Arterioscler Thromb Vasc Biol*, **1997**, 17, (11), 2309-15.

Patrono, C., Garcia Rodriguez, L. A., Landolfi, R. and Baigent, C. "Low-dose aspirin for the prevention of atherothrombosis." *N Engl J Med*, **2005**, 353, (22), 2373-83.

Patrono, C. and Renda, G. "Platelet activation and inhibition in unstable coronary syndromes." *Am J Cardiol*, **1997**, 80, (5A), 17E-20E.

Patscheke, H., Hornberger, W. and Zehender, H. "Pathophysiological role of thromboxane A_2 and pharmacological approaches to its inhibition." *Z Kardiol*, **1990**, 79 Suppl 3, 151-4.

Paul, B. Z., Ashby, B. and Sheth, S. B. "Distribution of prostaglandin IP and EP receptor subtypes and isoforms in platelets and human umbilical artery smooth muscle cells." *Br J Haematol*, **1998**, 102, (5), 1204-11.

Paul, B. Z., Jin, J. and Kunapuli, S. P. "Molecular mechanism of thromboxane A_2-induced platelet aggregation. Essential role for p2t(ac) and alpha(2a) receptors." *J Biol Chem*, **1999**, 274, (41), 29108-14.

Pibiri, L., Petruzzo, P., De Giudici, A. and Brotzu, G. "[Picotamide: prevention and therapy of diabetic vasculopathies. A double-blind clinical study]." *Clin Ter*, **1990**, 133, (4), 233-7.

Pierce, K. L. and Regan, J. W. "Prostanoid receptor heterogeneity through alternative mRNA splicing." *Life Sci*, **1998**, 62, (17-18), 1479-83.

Pinto, S., Gori, L., Gallo, O., Boccuzzi, S., Paniccia, R. and Abbate, R. "Increased thromboxane A_2 production at primary tumor site in metastasizing squamous cell carcinoma of the larynx." *Prostaglandins Leukot Essent Fatty Acids*, **1993**, 49, (1), 527-30.

Pogliani, E. and Milani, M. "Safety and efficacy of picotamide, a dual anti-thromboxane agent, in patients with thrombocytosis and a previous thromboembolic event: a 1-year observational study." *J Int Med Res*, **1996**, 24, (3), 311-5.

Pratico, D., Cyrus, T., Li, H. and FitzGerald, G. A. "Endogenous biosynthesis of thromboxane and prostacyclin in 2 distinct murine models of atherosclerosis." *Blood*, **2000**, 96, (12), 3823-6.

Pratico, D. and Dogné, J. M. "Selective cyclooxygenase-2 inhibitors development in cardiovascular medicine." *Circulation*, **2005**, 112, (7), 1073-9.

Pulcinelli, F. M., Ciampa, M. T., Favilla, M., Pignatelli, P., Riondino, S. and Gazzaniga, P. P. "Concomitant activation of Gi protein-coupled receptor and protein kinase C or phospholipase C is required for platelet aggregation." *FEBS Lett*, **1999**, 460, (1), 37-40.

Qiao, N., Reynaud, D., Demin, P., Halushka, P. V. and Pace-Asciak, C. R. "The thromboxane receptor antagonist PBT-3, a hepoxilin stable

analog, selectively antagonizes the TPalpha isoform in transfected COS-7 cells." *J Pharmacol Exp Ther*, **2003**, 307, (3), 1142-7.

Rafanelli, D., Grossi, A., Vannucchi, A. M., Cinotti, S., Morfini, M. and Ferrini, P. R. "The effect of picotamide on platelet function in patients with myeloproliferative disorders." *Thromb Haemost*, **1990**, 63, (3), 525-6.

Rang, H. P. "The receptor concept: pharmacology's big idea." *Br J Pharmacol*, **2006**, 147 Suppl 1, S9-16.

Raychowdhury, M. K., Yukawa, M., Collins, L. J., McGrail, S. H., Kent, K. C. and Ware, J. A. "Alternative splicing produces a divergent cytoplasmic tail in the human endothelial thromboxane A_2 receptor." *J Biol Chem*, **1994**, 269, (30), 19256-61.

Reid, H. M. and Kinsella, B. T. "The alpha, but not the beta, isoform of the human thromboxane A_2 receptor is a target for nitric oxide-mediated desensitization. Independent modulation of Tp alpha signaling by nitric oxide and prostacyclin." *J Biol Chem*, **2003**, 278, (51), 51190-202.

Reilly, I. A., Roy, L. and Fitzgerald, G. A. "Biosynthesis of thromboxane in patients with systemic sclerosis and Raynaud's phenomenon." *Br Med J (Clin Res Ed)*, **1986**, 292, (6527), 1037-9.

Reynaud, D., Hinek, A. and Pace-Asciak, C. R. "The hepoxilin analog PBT-3 inhibits heparin-activated platelet aggregation evoked by ADP." *FEBS Lett*, **2002**, 515, (1-3), 58-60.

Richman, R. W. and Diversé-Pierluissi, M. A. "RGS Proteins." *The G Protein-Coupled Receptors Handbook*, **2005**, L. A. devi, Humana Press**:** 135-148.

Riess, H., Hiller, E., Reinhardt, B. and Brauning, C. "Effects of BM 13.177, a new antiplatelet drug in patients with atherosclerotic disease." *Thromb Res*, **1984**, 35, (4), 371-8.

Roald, H. E., Barstad, R. M., Engen, A., Kierulf, P., Skjorten, F. and Sakariassen, K. S. "HN-11500--a novel thromboxane A_2 receptor antagonist with antithrombotic activity in humans at arterial blood flow conditions." *Thromb Haemost*, **1994**, 71, (1), 103-9.

Rolin, S., Dogné, J. M., Michaux, C., Delarge, J. and Masereel, B. "Activity of a novel dual thromboxane A_2 receptor antagonist and thromboxane synthase inhibitor (BM573) on platelet function and isolated smooth muscles." *Prostaglandins Leukot Essent Fatty Acids*, **2001**, 65, (2), 67-72.

Rolin, S., Petein, M., Tchana-Sato, V., Dogné, J. M., Benoit, P., Lambermont, B., Ghuysen, A., Kolh, P. and Masereel, B. "BM573, a dual thromboxane synthase inhibitor and thromboxane receptor antagonist, prevents pig myocardial infarction induced by coronary thrombosis." *J Pharmacol Exp Ther*, **2003**, 306, (1), 59-65.

Ross, R. "Atherosclerosis--an inflammatory disease." *N Engl J Med*, **1999**, 340, (2), 115-26.

Ruan, K. H., So, S. P., Wu, J., Li, D., Huang, A. and Kung, J. "Solution structure of the second extracellular loop of human thromboxane A_2 receptor." *Biochemistry*, **2001**, 40, (1), 275-80.

Ruan, K. H., Wu, J., So, S. P., Jenkins, L. A. and Ruan, C. H. "NMR structure of the thromboxane A_2 receptor ligand recognition pocket." *Eur J Biochem*, **2004**, 271, (14), 3006-16.

Sakai, H., Suzuki, T., Murota, M., Takahashi, Y. and Takeguchi, N. "Nitric oxide-induced Cl- secretion in isolated rat colon is mediated by the release of thromboxane A_2." *J Physiol*, **2002**, 543, (Pt 1), 261-71.

Salazar, A. E. "Experimental myocardial infarction. Induction of coronary thrombosis in the intact closed-chest dog." *Circ Res*, **1961**, 9, 1351-6.

Saldeen, P., Nilsson, I. M. and Saldeen, T. "Increased synthesis of thromboxane B_2 and 6-keto-PGF1 alpha in hand veins from patients with deep venous thrombosis." *Thromb Res*, **1983**, 32, (5), 461-7.

Saldeen, T. G., Saldeen, P., Nichols, W. W., Lawson, D. L., Nicolini, F. A. and Mehta, J. L. "Increased production of thromboxane A_2 by coronary arteries after thrombolysis." *Am Heart J*, **1993**, 125, (2 Pt 1), 277-84.

Samuelsson, B. "Leukotrienes: mediators of immediate hypersensitivity reactions and inflammation." *Science*, **1983**, 220, (4597), 568-75.

Sasaki, M., Miyosawa, K., Ohkubo, S. and Nakahata, N. "Physiological significance of thromboxane A_2 receptor dimerization." *J Pharmacol Sci*, **2006**, 100, (4), 263-70.

Sato, M. and Ohshima, N. "Platelet thrombus induced in vivo by filtered light and fluorescent dye in mesenteric microvessels of the rat." *Thromb Res*, **1984**, 35, (3), 319-34.

Schermuly, R. T., Kreisselmeier, K. P., Ghofrani, H. A., Samidurai, A., Pullamsetti, S., Weissmann, N., Schudt, C., Ermert, L., Seeger, W. and Grimminger, F. "Antiremodeling effects of iloprost and the dual-selective phosphodiesterase 3/4 inhibitor tolafentrine in chronic experimental pulmonary hypertension." *Circ Res*, **2004**, 94, (8), 1101-8.

Schiffrin, E. L. "A critical review of the role of endothelial factors in the pathogenesis of hypertension." *J Cardiovasc Pharmacol*, **2001**, 38 Suppl 2, S3-6.

Schmutzler, R., Hartmann, H., Casale, G. and Magliano, A. "Picotamide: effects on coagulation, fibrinolysis and platelet aggregation during prolonged administration in the aged." *Age Ageing*, **1978**, 7, (4), 246-50.

Schnitzer, T. J. "Is concern about cardiovascular events with the new coxib class of drugs justified?" *Medscape Womens Health*, **2001**, 6, (5), 8.

Seibert, K., Zhang, Y., Leahy, K., Hauser, S., Masferrer, J., Perkins, W., Lee, L. and Isakson, P. "Pharmacological and biochemical demonstration of the role of cyclooxygenase 2 in inflammation and pain." *Proc Natl Acad Sci U S A*, **1994**, 91, (25), 12013-7.

Serhan, C. N., Hamberg, M. and Samuelsson, B. "Lipoxins: novel series of biologically active compounds formed from arachidonic acid in human leukocytes." *Proc Natl Acad Sci U S A*, **1984**, 81, (17), 5335-9.

Shattil, S. J. and Newman, P. J. "Integrins: dynamic scaffolds for adhesion and signaling in platelets." *Blood*, **2004**, 104, (6), 1606-15.

Shinozaki, K., Sato, H., Iwakuma, T., Sato, R., Kurimoto, T. and Yoshida, K. "Synthesis and thromboxane A_2 antagonistic activity activity of indane derivatives." *Bioorg Med Chem Lett*, **1999**, 9, (3), 401-6.

Shiota, G., Okubo, M., Noumi, T., Noguchi, N., Oyama, K., Takano, Y., Yashima, K., Kishimoto, Y. and Kawasaki, H. "Cyclooxygenase-2 expression in hepatocellular carcinoma." *Hepatogastroenterology*, **1999**, 46, (25), 407-12.

Siess, W. "Molecular mechanisms of platelet activation." *Physiol Rev*, **1989**, 69, (1), 58-178.

Simpson, A. W. "Fluorescent measurement of $[Ca^{2+}]c$. Basic practical considerations." *Methods Mol Biol*, **1999**, 114, 3-30.

Singh, J., Seth, S. D., Manchanda, S. C. and Seth, S. "Protective actions of a thromboxane receptor antagonist, SQ29548 on the ischemic myocardium: morphologic and hemodynamic effects." *Prostaglandins Leukot Essent Fatty Acids*, **1997**, 56, (2), 105-10.

Smith, W. L. "The eicosanoids and their biochemical mechanisms of action." *Biochem J*, **1989**, 259, (2), 315-24.

Smith, W. L., Marnett, L. J. and DeWitt, D. L. "Prostaglandin and thromboxane biosynthesis." *Pharmacol Ther*, **1991**, 49, (3), 153-79.

So, S. P., Wu, J., Huang, G., Huang, A., Li, D. and Ruan, K. H. "Identification of residues important for ligand binding of thromboxane A_2 receptor in the second extracellular loop using the NMR experiment-guided mutagenesis approach." *J Biol Chem*, **2003**, 278, (13), 10922-7.

Soyka, R., Guth, B. D., Weisenberger, H. M., Luger, P. and Muller, T. H. "Guanidine derivatives as combined thromboxane A_2 receptor antagonists and synthase inhibitors." *J Med Chem*, **1999**, 42, (7), 1235-49.

Soyka, R., Heckel, A., Nickl, J., Eisert, W., Muller, T. H. and Weisenberger, H. "6,6-Disubstituted Hex-5-enoic acid derivatives as combined thromboxane A_2 receptor antagonists and synthetase inhibitors." *J Med Chem*, **1994**, 37, (1), 26-39.

Spagnuolo, P. J., Ellner, J. J., Hassid, A. and Dunn, M. J. "Mediation of augmented monocyte adhesiveness by thromboxane." *Inflammation*, **1988**, 12, (1), 1-9.

Springer, T. A. "Adhesion receptors of the immune system." *Nature*, **1990**, 346, (6283), 425-34.

Spurney, R. F. "Role of C-terminal serines in desensitization and phosphorylation of the mouse thromboxane receptor." *J Biol Chem*, **1998**, 273, (43), 28496-503.

Stegmeier, K., Pill, J., Muller-Beckmann, B., Schmidt, F. H., Witte, E. C., Wolff, H. P. and Patscheke, H. "The pharmacological profile of the thromboxane A_2 antagonist BM 13.177. A new anti-platelet and anti-thrombotic drug." *Thromb Res*, **1984**, 35, (4), 379-95.

Sugimoto, Y., Narumiya, S. and Ichikawa, A. "Distribution and function of prostanoid receptors: studies from knockout mice." *Prog Lipid Res*, **2000**, 39, (4), 289-314.

Svenssen, J., Strandberg, K., Tuvemo, T. and Hamberg, M. "Thromboxane A_2: effects on airway and vascular smooth muscle." *Prostaglandins*, **1977**, 14, (3), 425-36.

Swanson, M. L., Lei, Z. M., Swanson, P. H., Rao, C. V., Narumiya, S. and Hirata, M. "The expression of thromboxane A_2 synthase and thromboxane A_2 receptor gene in human uterus." *Biol Reprod*, **1992**, 47, (1), 105-17.

Swayne, G. T., Maguire, J., Dolan, J., Raval, P., Dane, G., Greener, M. and Owen, D. A. "Evidence for homogeneity of thromboxane A_2 receptor using structurally different antagonists." *Eur J Pharmacol*, **1988**, 152, (3), 311-9.

Takahara, K., Murray, R., FitzGerald, G. A. and Fitzgerald, D. J. "The response to thromboxane A_2 analogues in human platelets. Discrimination of two binding sites linked to distinct effector systems." *J Biol Chem*, **1990**, 265, (12), 6836-44.

Taketo, M. M. "COX-2 and colon cancer." *Inflamm Res*, **1998**, 47 Suppl 2, S112-6.

Talbot, M. D., Ambler, J., Butler, K. D., Lees, C. M., Mitchell, K. A., Peters, R. F., Tweed, M. F. and Wallis, R. B. "The effects of recombinant desulphatohirudin on arterial thrombosis in rats." *Haemostasis*, **1991**, 21 Suppl 1, 73-9.

Tamaoki, J., Kondo, M., Nakata, J., Nagano, Y., Isono, K. and Nagai, A. "Effect of a thromboxane A_2 antagonist on sputum production and its physicochemical properties in patients with mild to moderate asthma." *Chest*, **2000**, 118, (1), 73-9.

Tanaka, T., Ito, S., Higashino, R., Fukuta, Y., Fukuda, Y., Takei, M., Kurimoto, T. and Tamaki, H. "A new thromboxane receptor antagonist, Z-335, ameliorates experimental thrombosis without prolonging the rat tail bleeding time." *Thromb Res*, **1998**, 91, (5), 229-35.

Tanaka, T., Sato, R. and Kurimoto, T. "Z-335, a new thromboxane A_2 receptor antagonist, prevents arterial thrombosis induced by ferric chloride in rats." *Eur J Pharmacol*, **2000**, 401, (3), 413-8.

Titov, V. N. "[Atherosclerosis as a problem of general biology: cell adaptation to deficiency of essential fatty acids]." *Vestn Ross Akad Med Nauk*, **1999**, (10), 53-7.

Tomaru, T., Uchida, Y., Masuo, M., Kato, A. and Sugimoto, T. "Experimental canine arterial thrombus formation and thrombolysis: a fiberoptic study." *Am Heart J*, **1987**, 114, (1 Pt 1), 63-9.

Tsien, R. Y., Pozzan, T. and Rink, T. J. "Calcium homeostasis in intact lymphocytes: cytoplasmic free calcium monitored with a new, intracellularly trapped fluorescent indicator." *J Cell Biol*, **1982**, 94, (2), 325-34.

Tsuboi, K., Sugimoto, Y. and Ichikawa, A. "Prostanoid receptor subtypes." *Prostaglandins Other Lipid Mediat*, **2002**, 68-69, 535-56.

Tucker, O. N., Dannenberg, A. J., Yang, E. K., Zhang, F., Teng, L., Daly, J. M., Soslow, R. A., Masferrer, J. L., Woerner, B. M., Koki, A. T. and Fahey, T. J., 3rd. "Cyclooxygenase-2 expression is up-regulated in human pancreatic cancer." *Cancer Res*, **1999**, 59, (5), 987-90.

Turek, J. W., Halmos, T., Sullivan, N. L., Antonakis, K. and Le Breton, G. C. "Mapping of a ligand-binding site for the human thromboxane A_2 receptor protein." *J Biol Chem*, **2002**, 277, (19), 16791-7.

Tytgat, G. N., Van Nueten, L., Van De Velde, I., Joslyn, A. and Hanauer, S. B. "Efficacy and safety of oral ridogrel in the treatment of ulcerative colitis: two multicentre, randomized, double-blind studies." *Aliment Pharmacol Ther*, **2002**, 16, (1), 87-99.

Uchida, T., Kido, H., Yamanaga, K., Okita, M. and Watanabe, M. "A novel loop diuretic, torasemide, inhibits thromboxane A_2-induced contraction in the isolated canine coronary artery." *Prostaglandins Leukot Essent Fatty Acids*, **1992**, 45, (2), 121-4.

Ullrich, V., Zou, M. H. and Bachschmid, M. "New physiological and pathophysiological aspects on the thromboxane A_2-prostacyclin regulatory system." *Biochim Biophys Acta*, **2001**, 1532, (1-2), 1-14.

Urban, J. D., Clarke, W. P., von Zastrow, M., Nichols, D. E., Kobilka, B., Weinstein, H., Javitch, J. A., Roth, B. L., Christopoulos, A., Sexton, P. M., Miller, K. J., Spedding, M. and Mailman, R. B. "Functional selectivity and classical concepts of quantitative pharmacology." *J Pharmacol Exp Ther*, **2007**, 320, (1), 1-13.

Vane, J. R. "Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs." *Nat New Biol*, **1971**, 231, (25), 232-5.

Vanhoutte, P. M. "Endothelial dysfunction and atherosclerosis." *Eur Heart J*, **1997**, 18 Suppl E, E19-29.

Vermylen, J. and Deckmyn, H. "Thromboxane synthase inhibitors and receptor antagonists." *Cardiovasc Drugs Ther*, **1992**, 6, (1), 29-33.

Vetrano, A., Milani, M. and Corsini, G. "Effects of aspirin or picotamide, an antithromboxane agent, in combination with low-intensity oral anticoagulation in patients with acute myocardial infarction: a controlled randomized pilot trial." *G Ital Cardiol*, **1999**, 29, (5), 524-8.

Vezza, R., Mezzasoma, A. M., Venditti, G. and Gresele, P. "Prostaglandin endoperoxides and thromboxane A_2 activate the same receptor isoforms in human platelets." *Thromb Haemost*, **2002**, 87, (1), 114-21.

Vignola, A. M., Mirabella, F., Costanzo, G., Di Giorgi, R., Gjomarkaj, M., Bellia, V. and Bonsignore, G. "Airway remodeling in asthma." *Chest*, **2003**, 123, (3), 417S-422S.

Viles-Gonzalez, J. F., Fuster, V., Corti, R., Valdiviezo, C., Hutter, R., Corda, S., Anand, S. X. and Badimon, J. J. "Atherosclerosis regression and TP receptor inhibition: effect of S18886 on plaque size and composition--a magnetic resonance imaging study." *Eur Heart J*, **2005**, 26, (15), 1557-61.

Villalobos, M. A., De La Cruz, J. P., Escalante, R., Arrebola, M. M., Guerrero, A. and Sanchez de la Cuesta, F. "Effects of camonagrel, a selective inhibitor of platelet thromboxane synthase, on the platelet-subendothelium interaction." *Pharmacology*, **2003**, 69, (1), 44-50.

Walsh, M. T., Foley, J. F. and Kinsella, B. T. "The alpha, but not the beta, isoform of the human thromboxane A_2 receptor is a target for prostacyclin-mediated desensitization." *J Biol Chem*, **2000**, 275, (27), 20412-23.

Walsh, S. W. "Preeclampsia: an imbalance in placental prostacyclin and thromboxane production." *Am J Obstet Gynecol*, **1985**, 152, (3), 335-40.

Walsh, S. W. "Eicosanoids in preeclampsia." *Prostaglandins Leukot Essent Fatty Acids*, **2004**, 70, (2), 223-32.

Wang, L. H. and Kulmacz, R. J. "Thromboxane synthase: structure and function of protein and gene." *Prostaglandins Other Lipid Mediat*, **2002**, 68-69, 409-22.

Watson, B. D., Dietrich, W. D., Busto, R., Wachtel, M. S. and Ginsberg, M. D. "Induction of reproducible brain infarction by photochemically initiated thrombosis." *Ann Neurol*, **1985**, 17, (5), 497-504.

Webb, L. R. and Labaw, C. S. "Diphenyl Cyanocarbonimidate. A versatile synthon for the construction of heterocyclic systems." *Journal of heterocyclic chemistry*, **1982**, 19, (5), 1205-1206.

Webb, R. L., Eggleston, D. S., Labaw, C. S., Lewis, J. J. and Wert, K. "Diphenyl Cyancarbonimidate and Dichlorodiphenoxymethane as

Synthons for the Construction of Heterocyclic-Systems of Medicinal Interest." *Journal of Heterocyclic Chemistry*, **1987**, 24, (1), 275-278.

Wenzel, S. E., Westcott, J. Y., Smith, H. R. and Larsen, G. L. "Spectrum of prostanoid release after bronchoalveolar allergen challenge in atopic asthmatics and in control groups. An alteration in the ratio of bronchoconstrictive to bronchoprotective mediators." *Am Rev Respir Dis*, **1989**, 139, (2), 450-7.

White, J. G. "Platelets and atherosclerosis." *Eur J Clin Invest*, **1994**, 24 Suppl 1, 25-9.

Wilson, S. J., Dowling, J. K., Zhao, L., Carnish, E. and Smyth, E. M. "Regulation of thromboxane receptor trafficking through the prostacyclin receptor in vascular smooth muscle cells: role of receptor heterodimerization." *Arterioscler Thromb Vasc Biol*, **2007**, 27, (2), 290-6.

Wilson, S. J., McGinley, K., Huang, A. J. and Smyth, E. M. "Heterodimerization of the alpha and beta isoforms of the human thromboxane receptor enhances isoprostane signaling." *Biochem Biophys Res Commun*, **2007**, 352, (2), 397-403.

Wilson, S. J., Roche, A. M., Kostetskaia, E. and Smyth, E. M. "Dimerization of the human receptors for prostacyclin and thromboxane facilitates thromboxane receptor-mediated cAMP generation." *J Biol Chem*, **2004**, 279, (51), 53036-47.

Wise, H., Wong, Y. H. and Jones, R. L. "Prostanoid signal integration and cross talk." *Neurosignals*, **2002**, 11, (1), 20-8.

Wolff, H., Saukkonen, K., Anttila, S., Karjalainen, A., Vainio, H. and Ristimaki, A. "Expression of cyclooxygenase-2 in human lung carcinoma." *Cancer Res*, **1998**, 58, (22), 4997-5001.

Worth, N. F., Berry, C. L., Thomas, A. C. and Campbell, J. H. "S18886, a selective TP receptor antagonist, inhibits development of atherosclerosis in rabbits." *Atherosclerosis*, **2005**, 183, (1), 65-73.

Woulfe, D. S. "Platelet G protein-coupled receptors in hemostasis and thrombosis." *J Thromb Haemost*, **2005**, 3, (10), 2193-200.

Wreggett, K. A. and De Lean, A. "The ternary complex model. Its properties and application to ligand interactions with the D_2-dopamine receptor of the anterior pituitary gland." *Mol Pharmacol*, **1984**, 26, (2), 214-27.

Xiang, A., Uchida, Y., Nomura, A., Iijima, H., Sakamoto, T., Ishii, Y., Morishima, Y., Masuyama, K., Zhang, M., Hirano, K. and Sekizawa, K. "Involvement of thromboxane A_2 in airway mucous cells in asthma-related cough." *J Appl Physiol*, **2002**, 92, (2), 763-70.

Yamada, N., Miyamoto, M., Isogaya, M., Suzuki, M., Ikezawa, S., Ohno, M., Otake, A. and Umemura, K. "TRA-418, a novel compound having both thromboxane A_2 receptor antagonistic and prostaglandin I_2 receptor agonistic activities: its antiplatelet effects in human and animal platelets." *J Thromb Haemost*, **2003**, 1, (8), 1813-9.

Yamagata, K., Andreasson, K. I., Kaufmann, W. E., Barnes, C. A. and Worley, P. F. "Expression of a mitogen-inducible cyclooxygenase in brain neurons: regulation by synaptic activity and glucocorticoids." *Neuron*, **1993**, 11, (2), 371-86.

Yamamoto, Y., Kamiya, K. and Terao, S. "Modeling of human thromboxane A_2 receptor and analysis of the receptor-ligand interaction." *J Med Chem*, **1993**, 36, (7), 820-5.

Yokoyama, C., Miyata, A., Ihara, H., Ullrich, V. and Tanabe, T. "Molecular cloning of human platelet thromboxane A synthase." *Biochem Biophys Res Commun*, **1991**, 178, (3), 1479-84.

Zhang, L., Xiao, H., Schultz, R. A. and Shen, R. F. "Genomic organization, chromosomal localization, and expression of the murine thromboxane synthase gene." *Genomics*, **1997**, 45, (3), 519-28.

Zou, M. H., Shi, C. and Cohen, R. A. "High glucose via peroxynitrite causes tyrosine nitration and inactivation of prostacyclin synthase that is associated with thromboxane/prostaglandin H_2 receptor-mediated apoptosis and adhesion molecule expression in cultured human aortic endothelial cells." *Diabetes*, **2002**, 51, (1), 198-203.

Zuccollo, A., Shi, C., Mastroianni, R., Maitland-Toolan, K. A., Weisbrod, R. M., Zang, M., Xu, S., Jiang, B., Oliver-Krasinski, J. M., Cayatte, A. J., Corda, S., Lavielle, G., Verbeuren, T. J. and Cohen, R. A. "The thromboxane A_2 receptor antagonist S18886 prevents enhanced atherogenesis caused by diabetes mellitus." *Circulation*, **2005**, 112, (19), 3001-8.

VII. Appendix

Publications related to the dissertation

0022-3565/05/3131-293–301$20.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
Vol. 313, No. 1
79301/1197533
Printed in U.S.A.

JPET 313:293–301, 2005

In Vitro and in Vivo Pharmacological Characterization of BM-613 [*N*-*n*-Pentyl-*N*′-[2-(4′-methylphenylamino)-5-nitrobenzenesulfonyl]urea], a Novel Dual Thromboxane Synthase Inhibitor and Thromboxane Receptor Antagonist

Julien Hanson,[1] Stephanie Rolin,[1] Denis Reynaud, Na Qiao, Leanne P. Kelley, Helen M. Reid, François Valentin, John Tippins, B. Therese Kinsella, Bernard Masereel, Cecil Pace-Asciak, Bernard Pirotte, and Jean-Michel Dogné

Natural and Synthetic Drugs Research Centre, Department of Pharmacy, Laboratory of Medicinal Chemistry, University of Liège, Liège, Belgium (J.H., J.-M.D., B.P.); Department of Pharmacy, University of Namur, Namur, Belgium (S.R., B.M.); Programme in Integrative Biology, Research Institute, The Hospital for Sick Children, Toronto, Ontario, Canada (D.R., N.Q., C.P.-A.); Department of Biochemistry, Conway Institute for Biomolecular and Biomedical Research, University College Dublin, Ireland (L.P.K., H.M.R., B.T.K.); and Department of Biological Sciences, Imperial College, London, United Kingdom (F.V., J.T.)

Received October 15, 2004; accepted November 18, 2004

ABSTRACT

Thromboxane A_2 (TXA_2) is a key mediator of platelet aggregation and smooth muscle contraction. Its action is mediated by its G protein-coupled receptor of which two isoforms, termed TPα and TPβ, occur in humans. TXA_2 has been implicated in pathologies such as cardiovascular diseases, pulmonary embolism, atherosclerosis, and asthma. This study describes the pharmacological characterization of BM-613 [*N*-*n*-pentyl-*N*′-[2-(4′-methylphenylamino)-5-nitrobenzenesulfonyl]urea], a new combined TXA_2 receptor antagonist and TXA_2 synthase inhibitor. It exhibits a strong affinity for human platelet TP receptors (IC_{50} = 1.4 nM), TPα and TPβ expressed in COS-7 cells (IC_{50} = 2.1 and 3.1 nM, respectively), and TPs expressed in human coronary artery smooth muscle cells (IC_{50} = 29 μM). BM-613 shows a weak ability to prevent contraction of isolated rat aorta (ED_{50} = 1.52 μM) and guinea pig trachea (ED_{50} = 2.5 μM) induced by TXA_2 agonist U-46619 (9.11-dideoxy-9.11-methanoepoxy-prostaglandin F_2). Besides, BM-613 antagonizes TPα (IC_{50} = 0.11 μM) and TPβ (IC_{50} = 0.17 μM) calcium mobilization induced by U-46619 and inhibits human platelet aggregation induced by U-46619 (ED_{50} = 0.278 μM), arachidonic acid (ED_{50} = 0.375 μM), and the second wave of ADP. BM-613 also dose dependently prevents TXA_2 production by human platelets (IC_{50} = 0.15 μM). In a rat model of ferric chloride-induced thrombosis, BM-613 significantly reduces weight of formed thrombus by 79, 49, and 28% at 5, 2, and 1 mg/kg i.v., respectively. In conclusion, BM-613 is a dual and potent TP receptor antagonist and TXA_2 synthase inhibitor characterized by a strong antiplatelet and antithrombotic potency. These results suggest that BM-613 could be a potential therapeutic drug for thrombotic disorders.

Thromboxane A_2 (TXA_2) is a key lipid mediator characterized by several implications in physiological homeostasis, including platelet aggregation and vascular and bronchial smooth muscle constriction (Hamberg et al., 1975; Moncada and Vane, 1978). An overproduction of TXA_2 has been associated with many pathological states such as myocardial infarction, thrombosis and thrombotic disorders, unstable angina, pulmonary embolism, shock, atherosclerosis, preeclampsia, and asthma (Dogné et al., 2004a).

TXA_2 is a metabolite of arachidonic acid (AA), a 20-carbon

This work was supported by the Fonds National de la Recherche Scientifique and Wellcome Trust. J.H. is funded by the Fonds pour la Formation à la Recherche dans l'Industrie et dans l'Agriculture.
[1] These authors contributed equally to this work.
Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.
doi:10.1124/jpet.104.079301.

ABBREVIATIONS: TXA_2, thromboxane A_2; AA, amino acid(s); COX, cyclooxygenase; PGH_2, prostaglandin endoperoxide H_2; TXS, thromboxane synthase; TXRA, thromboxane receptor antagonist; TXSI, thromboxane synthase inhibitor; ASA, acetylsalicylic acid; BM-613, *N*-*n*-pentyl-*N*′-[2-(4′-methylphenylamino)-5-nitrobenzenesulfonyl]urea; BM-573, *N*-*tert*-butyl-*N*′-[2-(4′-methylphenylamino)-5-nitrobenzenesulfonyl]urea; SQ-29548, [1*S*-[1,2(*Z*),3,4]]-7-[3-[[2-[(phenylamino)carbonyl]hydrazino]methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-5-heptenoic acid; U-46619, 9.11-dideoxy-9.11-methanoepoxy-prostaglandin F_2; PBS, phosphate-buffered saline; HCASMC, human coronary artery smooth muscle cell; DMSO, dimethyl sulfoxide; PRP, platelet-rich plasma; PPP, platelet-poor plasma; HEK, human embryonic kidney; TXB_2, thromboxane B_2; S18886, 3-((6*R*)-6-{[(4-chlorophenyl)sulfonyl]amino}-2-methyl-5,6,7,8 tetrahydro-1-naphthalenyl) propanoic acid, sodium salt.

essential fatty acid stored in membrane phospholipids. Free AA can be released mainly by phospholipase A_2 upon stimulation. TXA_2 is synthesized in two steps: first the action of cyclooxygenase (COX) on free AA leads to the formation of prostaglandin H_2 (PGH_2) which can be subsequently metabolized by thromboxane synthase (TXS) into TXA_2.

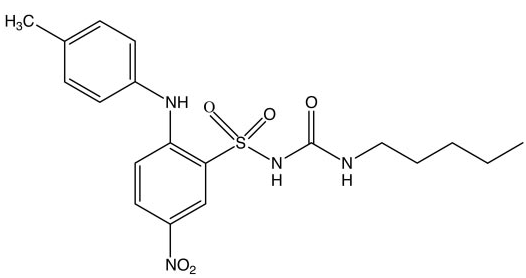
COX exists in two main isoforms, COX-1 and COX-2, which are encoded by two separate genes. COX-1 is expressed constitutively in most tissues mediating "housekeeping" functions, whereas COX-2 expression is mainly induced at sites of inflammation by various stimuli (Dogné et al., 2004a). The second is also expressed in a constitutive manner in endothelium (Cheng et al., 2002). TXA_2 synthesis mainly occurs in platelets where COX-1 and thromboxane synthase are highly expressed.

TXA_2 actions are mediated by its G protein-coupled receptor, referred to as the TP receptor (Coleman et al., 1994). In 1991, Hirata et al. reported that human TP receptor was coded by one gene (Hirata et al., 1991), and in 1994, Raychowdhury et al. highlighted the existence of an isoform generated by alternative splicing (Raychowdhury et al., 1994). The two isoforms, named TPα and TPβ, share the same first 328 amino acids but differ by the length of their carboxy-terminal tails (15 amino acids of the α isoform being replaced by 79 amino acids in the β isoform).

Given the wide implications of TXA_2 in several pathologies, studies have been undertaken to find therapeutic agents able to counteract the negative effects of TXA_2. Indeed, therapeutic strategies include blocking the synthesis of TXA_2 by the inhibition of TXS, antagonizing its action at the receptor level, or both. Thus, several TP receptor antagonists (TXRAs), such as sulotroban (Stegmeier et al., 1984), thromboxane synthase inhibitors (TXSIs), such as furegrelate (Gorman et al., 1983), and drugs combining both properties, such as ridogrel and terbogrel, have been developed. Dual compounds proved to be more interesting in therapeutics and promising antithrombotic agents (Dogné et al., 2004a).

In addition, acetylsalicylic acid (ASA, aspirin) is a nonsteroidal, anti-inflammatory drug exerting its effects by irreversibly acetylating the active site of COX. In platelets, the inhibition is effective throughout the platelets' life time because they lack a nucleus. Since TXA_2 is mainly produced by platelets, low-dose ASA has become the most common antiplatelet therapy used for secondary prevention of cardiovascular disease. Nevertheless, ASA is far from being a panacea since it has been associated with Reye's syndrome and allergic reaction (asthma), and almost one-third of patients receiving low-dose ASA do not respond, demonstrating a link between platelet function and the persistence of TXA_2 production (Patrono, 2003). These latter developments have strengthened the interest for development of other antithrombotic drugs such as TXA_2 modulators.

Herein, we report the in vitro and in vivo pharmacological profile of BM-613 (Fig. 1), a new TXA_2 modulator synthesized in our laboratory. BM-613 is a chemical derivative of BM-573, another thromboxane modulator combining TXRA and TXSI activities that has been widely described in literature (Rolin et al., 2001; Dogné et al., 2004b; Ghuysen et al., 2004; Lambermont et al., 2004). BM-613 has been developed as a dual TXRA and TXSI characterized by high activity on platelets compared with smooth muscle. In this study, we present several in vitro tests performed to characterize BM-613 effects on TXS, platelets, and smooth muscle. We have also carried out specific binding and antagonism of intracellular calcium concentration ($[Ca^{2+}]_i$) mobilization tests to compare the affinity and activity of our two compounds on the distinct TP receptors isoforms. Finally, an in vivo test is presented to evaluate the potential of BM-613 as an antithrombotic drug.



BM-613

Fig. 1. Chemical structure of BM-613.

Materials and Methods

Materials

BM-613 and BM-573 were synthesized in our laboratory. The sodium salts were dissolved in propyleneglycol and diluted with physiological saline. Furegrelate sodium salt [5-(3-pyridinylmethyl)-2-benzofurancarboxylate], SQ-29548, [^{3}H]SQ-29548, and U-46619 were purchased from Cayman Chemical (Ann Arbor, MI). They were dissolved in ethanol and diluted with physiological saline. Phosphate-buffered saline (PBS) was purchased from Cambrex Bio Science (Petit-Rechain, Belgium). dRhodamine terminator cycle sequencing kit was from Applied Biosystems (Foster City, CA). TPα and TPβ cDNA were as described previously (Allan et al., 1996; Becker et al., 1998). Human coronary artery smooth muscle cells (HCASMCs) were obtained from CellWorks (Buckingham, UK). Dulbecco's modified Eagle's medium supplemented with 4.5 g/l glucose, fetal bovine serum, and antibiotic/antimycotic solution were purchased from Invitrogen (Carlsbad, CA).

Animals

Male Sprague-Dawley rats, weighing 250 to 300 g, were housed in a temperature-controlled room before being used in the present experiments. All experimental procedures and protocols used in this investigation have been carried out in accordance with the Declaration of Helsinki (Publication 85-23. revised 1985) and were reviewed and approved by the Ethics Committee of the Medical Faculty of the University of Liège.

Cell Culture and Transfection

COS-7 cells were maintained in Dulbecco's modified Eagle's medium (Hyclone Laboratories, Logan, UT) supplemented with 10% fetal bovine serum and 1% of solution containing 10,000 units/ml penicillin G, 10,000 μg/ml streptomycin, and 25 μg/ml amphotericin (Cellgro; Mediatech, Herndon, VA). Cells were grown at 37°C in a humidified atmosphere of 95% O_2 and 5% CO_2. cDNAs for the TPα and TPβ were subcloned into pcDNA3; the resultant plasmids, pcDNA3:TPα and pcDNA3:TPβ, were introduced into COS-7 cells by the DEAE-dextran/chloroquine method. After 48 h post-transfection, cells were harvested by centrifugation at 500*g* for 5 min and washed three times in phosphate-buffered saline. Protein concentrations were determined using the Bradford assay (Bradford, 1976). Cells were resuspended in buffer containing 25 mM HEPES, 125 mM

NaCl, and 10 μM indomethacin, pH 7.4, and kept on ice for the binding study. TP expression was determined according to Miggin and Kinsella (1998) using 10 nM [^{3}H]SQ-29548 and a single saturating amount of SQ-29548 (10 μM).

Sequence Analysis

TPα, TPβ, and TPtailless sequences were confirmed on both strands using the ABI Big Dye Terminator Cycle Sequencing Ready Reaction kit, and the products were resolved on an ABI Prism 310 genetic analyzer (Applied Biosystems). The sequences were assembled and analyzed using the ClustalW Sequence analysis.

Radioligand Binding Assay

The binding experiments were performed on whole cells.

Human Platelet. The TXA_2 receptor binding study was carried out on human washed platelets suspended in a calcium- and magnesium-free Tyrode-HEPES buffer (137 mM NaCl, 2.7 mM KCl, 0.4 mM NaH_2PO_4, 12 mM $NaHCO_3$, 5 mM D-glucose, and HEPES; pH 7.4) to a concentration 2×10^8 cells/ml (Dogné et al., 2000, 2001). Freshly prepared samples of this suspension (0.5 ml) were incubated with [^{3}H]SQ-29548 (5 nM final concentration, 0.1 ml) for 60 min at 25°C. The displacement was initiated by addition of the studied ligand dissolved in the same buffer (0.4 ml). After incubation (30 min, 25°C), ice-cold Tris-HCl buffer (10 mM, pH 7.4; 4 ml) was added, the sample was rapidly filtered through a glass fiber filter (Whatman GF/C; Whatman, Maidstone, UK), and the tube rinsed twice with ice-cold buffer (4 ml). The filters were then placed in plastic scintillation vials containing an emulsion-type scintillation mixture (4 ml), and the radioactivity was counted. The amount of [^{3}H]SQ-29548 specifically bound to human platelet TXA_2 receptors (Bs, %) was calculated from the following equation: $Bs = 100 \times (B - NSB)/Bt$, where total binding (Bt) and nonspecific binding (NSB) are the radioactivity of 5 nM [^{3}H]SQ-29548 bound to the platelets incubated in the absence of any competing ligand and in the presence of unlabeled 50 μM SQ-29548, respectively. B is the radioactivity of the filtered platelets incubated with 5 nM [^{3}H]SQ-29548 and the studied compound at a concentration ranging from 10^{-5} to 10^{-10} M. In each experiment, NSB varied between 5 and 7% of Bt. For each drug, three concentration-response curves were performed in triplicate. The drug concentration which reduced the amount of specifically bound [^{3}H]SQ-29548 by 50% (IC_{50}) was determined by nonlinear regression analysis (GraphPad Prism software; GraphPad Software, Inc., San Diego, CA).

TPα and TPβ. Competition binding curves were carried out on COS-7 cells expressing TP receptor isoforms. Binding reactions were carried out on 5×10^5 cells in a total volume of 0.2 ml in the above-mentioned buffer with 10 nM [^{3}H]SQ-29548 added to all tubes in triplicate, containing various concentration of BM-613 and BM-573 (10^{-6}–10^{-11} M) in 1 μl of ethanol. Additional tubes containing excess unlabeled 10 μM SQ-29548 were included to assess the extent of nonspecific binding. Binding was allowed to take place for 30 min at 37°C; free radioligand was removed by rapid vacuum filtration through Whatman GF/B glass fiber filters prewashed with the cell suspension buffer. The tubes and the filters were rapidly washed with ice-cold 10 mM Tris buffer, pH 7.4 (three times with 3 ml). The radioactivity on the filters containing the ligand-receptor complexes was counted in 10 ml of Ecolite scintillation fluid (MP Biomedicals, St. Laurent, QC, Canada) in a Beckman (model LS 3800) liquid scintillation counter.

Human Coronary Artery Smooth Muscle Cells. HCASMCs were maintained in HCASMC basal medium supplemented with HCASMC growth supplement (CellWorks) at 37°C in a humidified atmosphere of 95% air and 5% CO_2. HCASMCs were resuspended in the binding buffer (25 mM Tris-HCl, pH 7.4, 5 mM $CaCl_2$, 10 μM indomethacin, 50 μg/ml phenylmethylsulfonyl fluoride). For the binding assay, 50 μg of protein was incubated with [^{3}H]SQ-29548 (30 Ci/mol, 1 μM; PerkinElmer Life and Analytical Sciences, Boston, MA) in the presence of the drug at the indicated concentrations (0–100 μM) in a 0.1-ml reaction volume with vigorous shaking at room temperature for 60 min. The reaction was then terminated by adding 1 ml of ice-cold washing buffer (25 mM Tris-HCl, pH 7.4). The unbound ligand was filtered under vacuum through a Whatman GF/C glass filter (Whatman, Clifton, NJ) presoaked with the ice-cold washing buffer. The radioactivity of the TP receptor-bound [^{3}H]SQ-29548 remaining on the glass filter was counted in 8 ml of scintillation mixture (PerkinElmer Life and Analytical Sciences) using a Beckman counter (Beckman Coulter, Fullerton, CA).

Human ex Vivo Platelet Aggregation

The antiaggregant potency has been determined according to the turbidimetric Born's method (Born and Cross, 1963). The blood was drawn from 10 healthy donors of both genders, aged 20 to 30 years. The subjects were free from medication for at least 14 days. No significant differences in the results were observed between the donors in our experiments. Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were prepared as described previously (Dogné et al., 2000, 2001). Platelet concentration of PRP was adjusted to 3×10^8 cells/ml by dilution with PPP. Platelet aggregation of PRP was studied using a double channel aggregometer (Chronolog Corporation, Chicago, IL) connected to a linear recorder as described previously (Harris et al., 1979). PRP (294 μl) was added in a silanized cuvette and stirred (1000 rpm). Each drug was diluted (1 mM) in dimethyl sulfoxide (DMSO)/PBS (30:70) and preincubated in PRP for 3 min at 37°C before the aggregating agent was added. Platelet aggregation was initiated by addition of a fresh solution of arachidonic acid (600 μM final) or U-46619 (1 μM final). To evaluate platelet aggregation, the maximum increase in light transmission was determined from the aggregation curve 6 min after addition of the inducer. The drug concentration preventing 50% of platelet aggregation (ED_{50}) induced by arachidonic acid and U-46619 was calculated by nonlinear regression analysis (GraphPad Prism software) from at least three dose-response curves.

Rat Aorta Relaxation

Endothelium-denuded thoracic aorta rings, obtained from rats (Wistar, 250–300 g) anesthetized with sodium pentobarbital (80 mg/kg i.p.), were suspended under a tension of 1 g in a Krebs' solution (118 mM NaCl, 5.4 mM KCl, 2.5 mM $CaCl_2$, 1.5 mM $MgCl_2 \cdot 6H_2O$, 25 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, and 10 mM glucose, pH 7.4), which was bubbled with O_2, CO_2 (95:5%) at 37°C in a 20-ml tissue bath (EMKA Technologies, Paris, France). The muscle tension of the aortic rings was isometrically recorded with a force displacement transducer IT1 (EMKA Technologies). The buffer was renewed every 15 min during the equilibration period (1 h) before exposing the rings to 20 nM U-46619. When a stable tension was obtained (15 min), cumulative increasing concentrations of potential antagonist were added to the bath until tension returned to the baseline value. The ED_{50} value of each drug was assessed for at least four concentration-response curves obtained from separate preparations and corresponded to the concentration which reduced to 50% the tension induced by 20 nM U-46619. The ED_{50} values were calculated by nonlinear regression analysis (GraphPad Prism software).

Guinea Pig Trachea Relaxation

Tracheae were removed from guinea pigs (Hartley, 250–300 g) anesthetized with sodium pentobarbital (80 mg/kg i.p.) and carefully cleaned of connective tissue. Tracheal strips were suspended in the organ bath (20 ml), and the experiment progressed in the same conditions as those described above for the rat aorta, except for the concentration of 10 nM U-46619, the contraction inducer.

Calcium Measurements

HEK.TPα and HEK.TPβ cell lines, stably overexpressing hemagglutinin-tagged forms of TPα and TPβ in human embryonic kidney

(HEK) 293 cells have been described previously (Walsh et al., 2000). HEK 293 cells or their stable cell line equivalents were routinely grown in minimal Eagle's medium containing 10% fetal bovine serum. For measurements of $[Ca^{2+}]_i$ mobilization, approximately 48 h before transfection, HEK.TPα and HEK.TPβ stable cell lines were plated in 10-cm culture dishes at a density of 2×10^6 cells/dish in 8 ml of media; thereafter, cells were transiently transfected with 10 μg of pADVA and 25 μg of pCMV:Gαq, coding for Gαq, using the calcium phosphate/DNA coprecipitation procedure essentially as described previously (Kinsella et al., 1997; Hayes et al., 1999). Measurement of $[Ca^{2+}]_i$ mobilization either in Gαq-transfected HEK.TPα and HEK.TPβ cells was carried out in Fura-2/acetoxymethyl ester-preloaded cell lines cells, essentially as described previously (Kinsella et al., 1997). Briefly, cells were stimulated at 50 s with either 1 μM U-46619, or for competition studies, BM-573 or BM-613; cells were prestimulated with 10^{-9} to 10^{-3} M BM-573 or BM-613 for 5 min before stimulation with 1 μM U-46619. In all cases, the stock solution (100 mM BM-573 or BM-613 in DMSO) was diluted with PBS to achieve the desired working solution and 20 μl of the vehicle or drug in vehicle was added to 2 ml of cells; the vehicle had no effect on $[Ca^{2+}]_i$ mobilization by either TP isoform and had no effect on experimental data. The ratio of the fluorescence at 340 nm to that at 380 nm is a measure of $[Ca^{2+}]_i$ (Grynkiewicz et al., 1985), assuming a K_d value of 225 nM Ca^{2+} for Fura-2/acetoxymethyl ester. The results presented in the Figs. 3 and 4 are either representative or mean data from three or four independent experiments. Alternatively, mean percentage reductions in U-46619-mediated $[Ca^{2+}]_i$ mobilization in the presence of BM-573 (10^{-9}–10^{-3} M) are expressed as a percentage of U-46619-mediated $[Ca^{2+}]_i$ mobilization in the absence of BM-573.

Thromboxane Synthase Activity

PRP preparation is identical to that described for the platelet aggregation experiments. Each drug was dissolved in DMSO and diluted with a Tyrode-HEPES buffer (137 mM NaCl, 2.7 mM KCl, 0.4 mM $NaH_2PO_4 \cdot H_2O$, 5 mM D-glucose, 12 mM $NaHCO_3$, and HEPES, pH 7.4). To 900 μl of PRP, 50 μl of 0.9% NaCl and 10 μl of drug solution were added. After 6-min incubation at 37°C under stirring (600 rpm), aggregation was induced by 40 μl of sodium arachidonate (0.6 mM final). After 4 min, the reaction was stopped by adding 50 μl of indomethacin (0.02 M in ethanol). The sample was immediately centrifuged (17,500*g* for 10 s), and the supernatant was removed and frozen (−80°C) until assayed for TXB_2. Basal and maximal production of TXB_2 was estimated in the absence and in the presence of arachidonic acid, respectively. Evaluation was done in triplicate on concentrations ranging from 10 to 0.1 μM. Thromboxane synthase activity was expressed as the TXB_2 production, which was measured by using a competitive enzyme immunoassay (TXB_2 enzyme immunoassay kit; Cayman Chemical).

Ferric Chloride-Induced Rat Arterial Thrombosis

The experiments were carried out according to the modification of the method described by Kurz et al. (1990). Rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.). After an abdominal midline incision, the abdominal aorta was exposed carefully. A filter paper disk (8 mm in diameter) saturated with 50% (w/v) ferric chloride solution was placed on the surface of the artery for 10 min. The artery was isolated 10 min after removing the disk and then opened lengthwise. The thrombus was scraped out and placed on a filter paper to remove any water, and its wet weight was measured immediately. Results are expressed in milligrams of thrombus weight per kilogram of rat weight. BM-613 (5, 2, 1, and 0.5 mg/kg) and placebo were injected intravenously 5 min before application of ferric chloride solution. The results are expressed as the mean of results from four separate experiments.

Statistical Analysis

Results are expressed as the mean ± S.E.M., and statistical significance was determined by a Mann-Whitney *U* test. Probability values of less than 0.05 were considered to be significant.

Results

In Vitro Functional Assays

Inhibition of Human Platelet Aggregation. The ability of BM-613 to prevent human platelet aggregation has been evaluated in vitro with three different agonists of platelet aggregation. BM-613 was able to inhibit (ED_{50} = 0.278 ± 0.186 μM) platelet aggregation induced by the stable TXA_2 agonist U-46619 (1 μM). This effect is in the same order of potency as results obtained with BM-573 (ED_{50} = 0.240 ± 0.013 μM). In these experiments, BM-613 produced a shift to the right of the concentration-response curve of U-46619 (data not shown). BM-613 also inhibited platelet aggregation induced by 600 μM arachidonic acid (ED_{50} = 0.375 ± 0.050 μM) in the same concentration range as BM-573 (ED_{50} = 0.125 ± 0.015 μM). ADP is a weak inducer of platelet aggregation by itself, and it induces secretion of TXA_2 among other proaggregating substances. BM-613 (1 and 10 μM) inhibited the second wave of platelet aggregation induced by ADP, which is due to the secretion of TXA_2 (Table 1).

Thromboxane Synthase Inhibitory Potency. Thromboxane synthase is the enzyme converting PGH_2 into TXA_2. BM-613 was evaluated in a thromboxane activity test and showed an inhibitory activity (IC_{50} = 0.15 ± 0.13 μM) comparable with that obtained with BM-573 (IC_{50} = 0.053 ± 0.028 μM). It was 100-fold more potent than the thromboxane synthase inhibitor furegrelate (IC_{50} = 10.2 ± 6 μM), chosen as reference drug.

Inhibition of Rat Aorta and Guinea Pig Trachea Contraction Induced by U-46619. The TXA_2 receptor antagonism properties of BM-613 were studied on isolated rat thoracic aorta precontracted by the TXA_2 agonist U-46619 (20 nM) (Table 2). The concentration that caused 50% of relaxation (ED_{50}) was calculated from concentration-response curves (Table 2). On rat aorta, BM-613 (ED_{50} = 2.5 ± 0.18 μM) was 100-fold less active than BM-573 (ED_{50} = 28.4 ± 4.5 nM) and 1000-fold less active than SQ-29548 (ED_{50} = 2.3 ± 0.07 nM), which was the most potent compound in these experiments. To evaluate the potential therapeutic interest

TABLE 1

Inhibition of human platelet aggregation induced by arachidonic acid, U-46619, and ADP

Compounds were evaluated at a concentration of 1 and 10 μ M in experiments using ADP as an inducer. Results are expressed as mean ± S.E.M. ED_{50} represents the drug concentration inhibiting 50% of human platelet aggregation.

Drug	1 μM U-46619 ED_{50}	600 μM Arachidonic Acid ED_{50}	2 μM ADP
		μM	
SQ-29548	0.034 ± 0.012	0.035 ± 0.003	Inhibition of second wave
BM-573	0.240 ± 0.013	0.125 ± 0.015	Inhibition of second wave
BM-613	0.278 ± 0.186	0.375 ± 0.050	Inhibition of second wave

TABLE 2
Effect of BM-613 on rat aorta and guinea pig trachea contraction induced by U-46619
Results are expressed as mean ± S.E.M. ED_{50} represents the drug concentration reducing by 50% the rat aorta and guinea pig trachea tonus induced by 20 and 10 nM U-46619, respectively.

Drug	Inhibition of Contraction Induced by U-46619	
	Rat Aorta ED_{50}	Guinea Pig Trachea ED_{50}
	nM	
SQ-29548	2.3 ± 0.1	3.8 ± 0.6
BM-573	28.4 ± 4.5	17.7 ± 3.9
BM-613	2500 ± 180	1520 ± 250

of BM-613 in the treatment of asthma, the relaxing activity of BM-613 was measured on the guinea pig trachea contracted by 10 nM U-46619 (Table 2), which is considered as a potent constrictor of the bronchopulmonary tract (Coleman et al., 1981). Cumulative increasing concentrations of BM-613 caused a concentration-dependent relaxation of the contracted isolated trachea. The drug concentration required to decrease by 50% the muscular tonus (ED_{50}) induced by U-46619 was calculated from these curves (Table 2). BM-613 (ED_{50} = 1.52 ± 0.25 μM) was much less active than BM-573 (ED_{50} = 17.7 ± 3.9 nM) and than SQ-29548 (ED_{50} = 3.8 ± 0.5 nM), which remains the most potent compound.

Radioligand Binding Assay. Affinity of BM-613 for TP receptors has been evaluated by measuring its ability to displace radiolabeled SQ-29548, a strong TXRA, using different cells types (Table 3). Binding experiments have been conducted on human washed platelets, human coronary artery smooth muscle cells, and COS-7 cells where TPα and TPβ were expressed. BM-613 showed strong affinity (IC_{50} = 1.4 ± 0.2 nM) for human platelet TP receptor. It was as active as BM-573 (IC_{50} = 1.3 ± 0.1 nM) and 10-fold more potent than SQ-29548 (IC_{50} = 21 ± 2 nM). Affinities of BM-613 for either TPα (IC_{50} = 2.1 ± 0.5 nM) or TPβ (IC_{50} = 3.1 ± 0.7 nM) expressed alone in COS-7 cells are not significantly different (p = 0.28). BM-573 was slightly more potent in this test, but again, there was little difference in affinity for the two isoforms. In binding studies performed on HCASMCs, BM-613 (IC_{50} = 29 ± 7 μM) showed an affinity comparable with the reference drug, BM-573 (IC_{50} = 37 ± 1 μM).

Intracellular Signaling by Assessment of Intracellular Calcium Mobilization. The effect of BM-613 on intracellular signaling by the TP isoforms was investigated by comparing its effect to BM-573 in intracellular calcium mobilization mediated by TPα and TPβ stably overexpressed in HEK 293 cells, in response to the selective TXA_2 mimetic U-46619. It has been reported that for efficient TPα and TPβ coupling to phospholipase Cβ activation in HEK 293 cells, it is necessary to cotransfect cells with a member of the G_q family of heterotrimeric G proteins (Kinsella et al., 1997; Walsh et al., 1998). Hence, throughout these studies, HEK.TPα and HEK.TPβ cells were routinely cotransfected with pCMV:Gα_q, encoding Gα_q. In HEK.TPα cells, both BM-573 (IC_{50} = 0.17 μM) and BM-613 (IC_{50} = 0.11 μM) exhibited concentration-dependent antagonism of U-46619-mediated $[Ca^{2+}]_i$ mobilization (Figs. 3 and 4). Similarly, in HEK.TPβ cells, both BM-573 (IC_{50} = 0.38 μM) and BM-613 (IC_{50} = 0.30 μM) antagonized U-46619-mediated $[Ca^{2+}]_i$ mobilization to levels that were not significantly different from those observed in HEK.TPα cell lines (BM-573, p = 0.6; BM-613, p = 0.8; Table 4).

TABLE 3
Binding affinity of BM-613 for TP receptor on different cell types
Tests were performed in triplicate, and results are expressed as mean ± S.E.M.

Drug	Affinity			
	Human Platelets IC_{50}[a]	TPα IC_{50}[b]	TPβ IC_{50}[c]	HCASMCs IC_{50}
	nM			
SQ-29548	21 ± 2	N.D.	N.D.	N.D.
BM-573	1.3 ± 0.1	1.1 ± 0.2	0.8 ± 0.1	37,000 ± 1000
BM-613	1.4 ± 0.2	2.1 ± 0.5	3.1 ± 0.7	29,000 ± 7000

N.D., not determined.
[a] Human washed platelets; [b] COS-7 cells expressing TPα; [c] COS-7 cells expressing TPβ.

In Vivo Antithrombotic Activity of BM-613

To further test the hypothesis that BM-613 was a potential antithrombotic agent acting on TP receptors and TXS, we evaluated its effect in a rat model of arterial thrombosis. It has been described previously that application of ferric chloride solution on vascular vessels induced thrombus formation (Kurz et al., 1990). In this model, topical application of ferric chloride [50% (w/v)] for 10 min to the abdominal aorta induced marked thrombi in vehicle-treated rats (8.95 ± 0.92 mg/kg). Intravenously injected at 5, 2, and 1 mg/kg, BM-613 significantly reduced the thrombus weight by 79, 49, and 28%, respectively (Fig. 5). At 0.5 mg/kg, BM-613 did not significantly reduce the thrombus weight. Intraperitoneally injected, BM-573 tested in the same model showed similar activity (Dogné et al., 2004b).

Discussion

TXA_2 is a key mediator involved in platelet aggregation and smooth muscle contraction. It is synthesized by the action of TXS on PGH_2, an unstable endoperoxide formed by action of COX on free AA. TXA_2 mediates its action by a specific G protein-coupled receptor named TP receptor. In humans but not in nonprimates, this receptor is expressed as two isoforms, termed TPα and TPβ, that arise by alternative splicing. Since TXA_2 has been implicated in several pathologies such as myocardial infarction, thrombosis, and thrombotic disorders, unstable angina, pulmonary embolism, shock, atherosclerosis, preeclampsia, and asthma (Dogné et al., 2004a), efforts have been made to develop agents able to antagonize TXA_2 actions.

Aspirin is the antiplatelet drug commonly used for secondary prevention of cardiovascular disease. Nevertheless, ASA has been associated with Reye's syndrome and allergic reaction (asthma), and almost one-third of patients receiving low-dose ASA do not respond, demonstrating a link between platelet function and the persistence of TXA_2 production (Patrono, 2003). Although the etiology of this "aspirin resis-

TABLE 4
Summary of IC_{50} determinations of effects of BM-573 and BM-613 on U-46619-mediated $[Ca^{2+}]_i$ mobilization by TPα and TPβ

Drug	TPα IC_{50}	TPβ IC_{50}	p Value[a]
	μM		
BM-573	0.17	0.38	0.6
BM-613	0.11	0.30	0.8

[a] Mann-Whitney U test to compare differences between TPα and TPβ.

tance" is still not fully explained, some authors have pointed out the possible role of oxidative products such as the non-enzymatic isoprostane derivatives of AA acting on TP receptor (Cipollone et al., 2000) or COX-2-dependent TXA_2 formation (Patrono, 2003). Besides the aspirin resistance, there is growing evidence that TP receptor is deeply involved in growing of atherosclerotic plaque. Recently, Cayatte et al. (2000) have shown that the TP receptor antagonist S18886 reduces atherogenesis, whereas ASA has no effect, thus demonstrating the superiority of TP receptor antagonist in these tests (Cayatte et al., 2000). These results were explained by the TP receptor activation by other mediators that can still promote growth of the atherosclerotic plaque.

These recent developments in our knowledge on TXA_2 biology have highlighted the limitations of ASA and the growing interest of TXA_2 modulators in several pathologies. Here, we describe in vitro and in vivo pharmacological characterization of BM-613, a novel dual TXRA and TXSI developed in our laboratory. It is a close derivative of BM-573, another TXRA and TXSI described in the literature (Rolin et al., 2001; Dogné et al., 2004b; Ghuysen et al., 2004; Lambermont et al., 2004).

Our first aim was to determine the in vitro TP receptor antagonistic potency of BM-613 in a test of inhibition of human platelet aggregation. BM-613 was able to inhibit platelet aggregation induced by AA in the same concentration range as BM-573 (Table 1). Because COX inhibitors are also able to inhibit the AA-induced aggregation by blocking TXA_2 production, BM-613 activity was evaluated with U-46619, which is a stable TP agonist. We have shown in this experiment that BM-613 completely inhibited platelet aggregation induced by U-46619, thus demonstrating its action as TXRA (Table 1). Moreover, BM-613 produced a shift to the right of the concentration-response curve of U-46619, suggesting a competitive type antagonism of platelet TP receptors (data not shown). ADP provokes platelet aggregation by acting on specific purine receptors. Platelet aggregation induced by ADP is characterized by two waves. The first wave is due to a weak and reversible aggregation, which is the consequence of ADP action on its specific receptors. The second wave is due to TXA_2 synthesis and release, which provokes irreversible and complete aggregation. Our compound only inhibited the second wave (Table 1) of ADP-induced platelet aggregation, like other TXRAs (Reynaud et al., 2002). This result highlights the lack of action of BM-613 on ADP receptors and confirms specific activity on TP receptors.

Compounds combining both actions on TP receptors and TXS have proved to be more therapeutically interesting and promising as antithrombotic agents (Gresele et al., 1987). Indeed, stopping exclusively the production of TXA_2 provokes the accumulation of PGH_2, another TP receptor agonist (Coleman et al., 1981). In contrast, with pure TP receptor antagonists, the benefit of redirecting production of other prostanoids (such as prostacyclin) by accumulation of PGH_2 is lost. Thus, we also evaluated the potency of BM-613 as TXSI by investigating the production of the stable TXA_2 metabolite, thromboxane B_2 (TXB_2), by human platelets activated by arachidonic acid. The IC_{50} value determined for BM-613 was 100-fold lower than that of furegrelate, a clinically evaluated TXSI used as reference drug. These results demonstrated the efficacy of BM-613 as a TXSI (Mohrland et al., 1990). Combined with the effect on platelet aggregation, BM-613 can be considered as a well balanced TXRA and TXSI. This is of great importance since the lack of efficacy of ridogrel was explained by its strong effect on TXS compared with TP receptors (Soyka et al., 1999).

Further functional experiments were conducted on isolated rat aorta and guinea pig trachea precontracted by U-46619 (Fig. 2). In these experiments, BM-573 has already shown good potency compared with SQ-29548 (Rolin et al., 2001). BM-613 showed a 100-fold decreased activity in these experiments compared with BM-573 (Table 2). This discrepancy between potency of BM-613 to inhibit platelet aggregation and to inhibit smooth muscle cell contraction could find several explanations. First, the TP receptor may present conformational differences between species used in the test (human, rat, and guinea pig). Second, several authors initially stated in the 1980s that at least two pharmacological subtypes of TP receptor existed (Mais et al., 1985; Saussy et al., 1985). The first subtype was thought to be present at the platelet surface (Takahara et al., 1990), responsible for platelet aggregation. The second subtype, detected in smooth muscle cells was thought to be responsible for smooth muscle contraction (Mais et al., 1988). However, it is still controversial whether the two isoforms are responsible for pharmacological differences described in some studies because none of the initial tissue-selective antagonists of TP receptors were able to discriminate between the two isoforms. Moreover, the exact physiological significance of the existence of two iso-

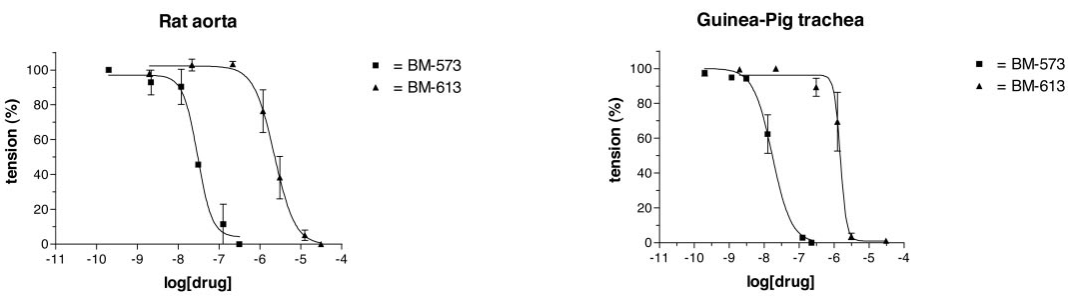


Fig. 2. Dose-dependent antagonistic effect of BM-613 and BM-573 on U-46619 induced smooth muscle cell contraction. Experiments have been carried out on rat aorta and guinea pig trachea. Data presented are representative of four independent experiments.

forms is still unclear, although it has been shown that TPα was the only isoform expressed in platelets (Habib et al., 1999) and that both TPα and TPβ were expressed in smooth muscle cells (Miggin and Kinsella, 1998). Recently, Qiao et al. (2003) showed for the first time that the hepoxilin-stable analog PBT-3 could selectively bind the TPα isoform in transfected COS-7 cells. Thus, we postulated that BM-613 could have a greater affinity for TPα than TPβ and that this could be coupled with higher activity as an antiplatelet agent.

To test this second hypothesis, we have performed binding experiments on several cell types. BM-613 and BM-573 affinity was evaluated on washed platelets, COS-7 cells expressing either TPα or TPβ, and HCASMCs. In these experiments, BM-613 showed high affinity for human platelet TP receptor. BM-613 was also characterized by great affinity for both TPα and TPβ expressed in COS-7 cells. This result confirmed the potent affinity of BM-613 for human platelet TP receptor of which TPα is predominant (Habib et al., 1999). Since TPα and TPβ are both expressed in smooth muscle cells (Miggin and Kinsella, 1998), lower affinity for TPβ should occur when performing tests on smooth muscle cells. BM-613 and BM-573 exhibited very similar affinities for HCASMC TP receptors, which is in accordance with their TPβ binding properties.

Since the binding affinity of a compound for different receptors cannot reflect its antagonistic or agonistic properties, we performed studies on intracellular signaling by TPα and TPβ. BM-613 dose dependently inhibits $[Ca^{2+}]_i$ mobilization upon stimulation by U-46619 in HEK 293 cells cotransfected with Gαq and either TPα or TPβ (Figs. 3 and 4). Nevertheless, BM-613 exhibited no significant differences in antagonistic potency against the two isoforms in these experiments (Table 4). We conclude from these results that the weak activity of BM-613 in inhibiting smooth muscle cell contraction was due to interspecies differences in TP receptor configuration.

We further used a rat model of ferric chloride-induced thrombus formation to test the antithrombotic properties of BM-613. The drug, injected intravenously, was able to significantly reduce the weight of thrombus formed in the lumen of the aorta (Fig. 5). These results are in accordance with in vitro antiplatelet activity of BM-613 and with results obtained with BM-573 in similar conditions (Dogné et al., 2004b).

In conclusion, we have presented herein a new compound, BM-613, that is characterized by high affinity for human platelets and HCASMC TP receptors and TPα and TPβ receptors. These binding affinities are comparable with those of BM-573, our compound used as reference. Moreover, our compound is an antagonist of human platelet TP receptors TPα and TPβ and an inhibitor of TXS. Moreover, it is characterized by antagonist properties for platelet aggregation

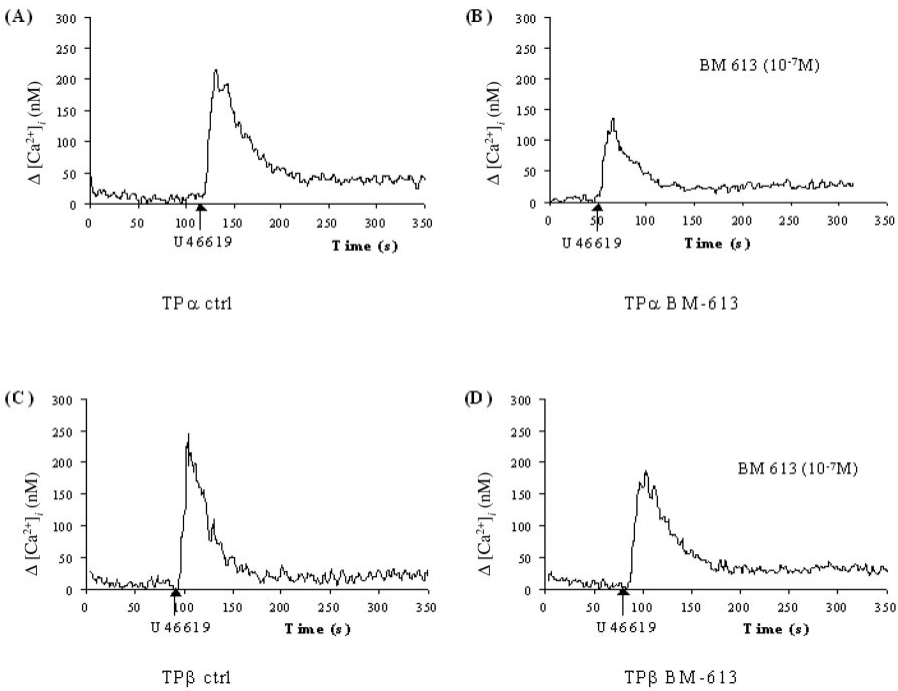


Fig. 3. A and B, HEK.TPα cells, transiently cotransfected with pCMV:Gαq, were either stimulated with 1 μM U-46619 alone (A) or preincubated with BM-613 (10^{-7}) for 5 min before stimulation with 1 μM U-46619 (B). Data presented in A and B are representative profiles from three independent experiments. C and D, HEK.TPβ cells, transiently cotransfected with pCMV:Gαq, were either stimulated with 1 μM U-46619 alone (C) or preincubated with BM-613 (10^{-7}) for 5 min before stimulation with 1 μM U-46619 (D). Data presented in C and D are representative profiles from three independent experiments.

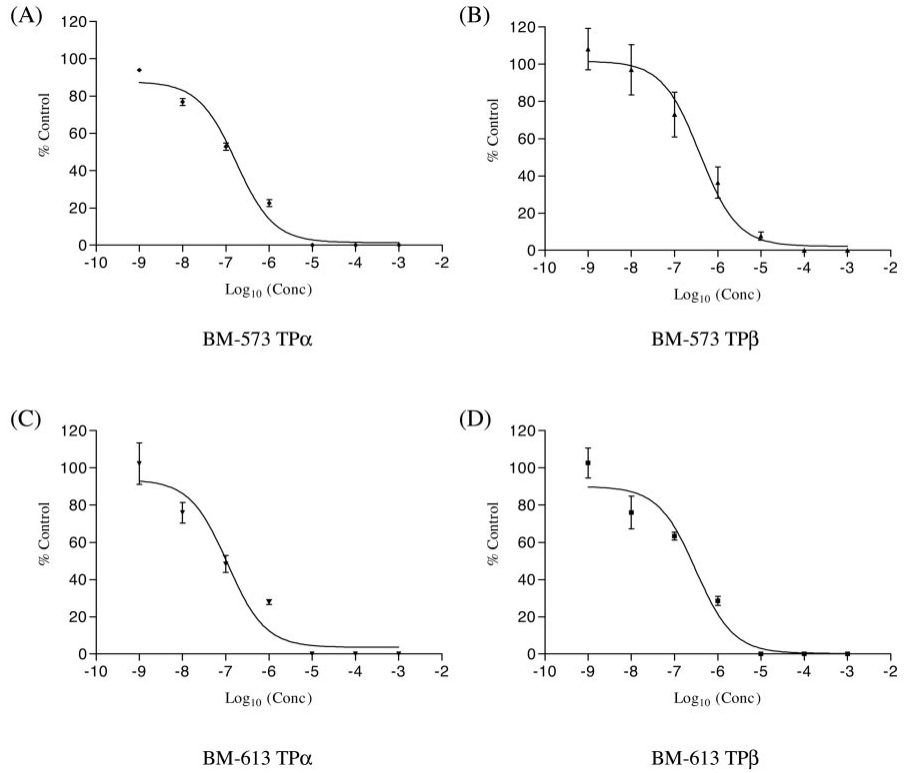


Fig. 4. A and C, HEK.TPα cells, transiently cotransfected with pCMV:Gαq, were incubated with either BM-573 (A, 10^{-9}–10^{-3} M) or BM-613 (C, 10^{-9}–10^{-3} M) for 5 min followed by stimulation with 1 μM U-46619. B and D, HEK.TPβ cells, transiently cotransfected with pCMV:Gαq, were incubated with either BM-573 (B, 10^{-9}–10^{-3} M) or BM-613 (D, 10^{-9}–10^{-3} M) for 5 min followed by stimulation with 1 μM U-46619. Mean changes in U-46619-mediated $[Ca^{2+}]_i$ mobilization in the presence of the drug were expressed as a percentage of U-46619-mediated $[Ca^{2+}]_i$ mobilization in the absence of the drug.

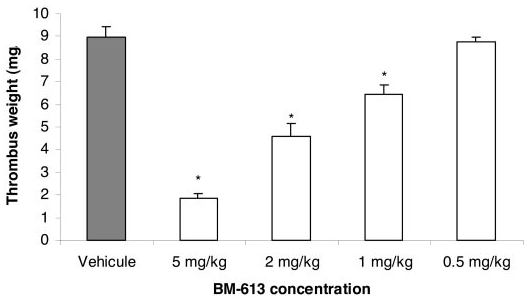


Fig. 5. Dose-dependent effects of BM-613 on thrombus weight (milligrams per kilogram) induced by application of ferric chloride solution [50% (w/v)] on rat abdominal aorta. Results are expressed as the mean ± S.E.M. *, $p < 0.05$. Data presented are representative of four independent experiments.

and calcium mobilization in the same range of concentration than the potent BM-573. Nevertheless, BM-613 is less powerful than reference BM-573 in counteracting rat and guinea pig smooth muscle contraction. It seems that this difference is due to interspecies variations. However, given the therapeutic interest of tissue-selective compounds, these results need to be confirmed in further experiments. BM-613 is also an in vivo antithrombotic agent, suggesting that it could be a potential therapeutic agent for cardiovascular diseases and thrombotic disorders.

Acknowledgments

We are grateful to Dr. Perry V. Halushka (Medical University of South Carolina, Charleston, SC) for providing cDNA for the TPα and TPβ receptor isoforms. We also thank Philippe Neven for excellent technical assistance.

References

Allan CJ, Higashiura K, Martin M, Morinelli TA, Kurtz DT, Geoffroy O, Meier GP, Gettys TW, and Halushka PV (1996) Characterization of the cloned HEL cell thromboxane A2 receptor: evidence that the affinity state can be altered by G alpha 13 and G alpha q. *J Pharmacol Exp Ther* **277:**1132–1139.

Becker KP, Ullian M, and Halushka PV (1998) Cloning and characterization of an endogenous COS-7 cell thromboxane A2 receptor. *Biochim Biophys Acta* **1403:** 109–114.

Born GV and Cross MJ (1963) The aggregation of blood platelets. *J Physiol (Lond)* **168:**178–195.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:**248–254.

Cayatte AJ, Du Y, Oliver-Krasinski J, Lavielle G, Verbeuren TJ, and Cohen RA (2000) The thromboxane receptor antagonist S18886 but not aspirin inhibits

atherogenesis in apo E-deficient mice: evidence that eicosanoids other than thromboxane contribute to atherosclerosis. *Arterioscler Thromb Vasc Biol* **20:**1724–1728.
Cheng Y, Austin SC, Rocca B, Koller BH, Coffman TM, Grosser T, Lawson JA, and FitzGerald GA (2002) Role of prostacyclin in the cardiovascular response to thromboxane A2. *Science (Wash DC)* **296:**539–541.
Cipollone F, Ciabattoni G, Patrignani P, Pasquale M, Di Gregorio D, Bucciarelli T, Davi G, Cuccurullo F, and Patrono C (2000) Oxidant stress and aspirin-insensitive thromboxane biosynthesis in severe unstable angina. *Circulation* **102:**1007–1013.
Coleman RA, Humphrey PP, Kennedy I, Levy GP, and Lumley P (1981) Comparison of the actions of U-46619, a prostaglandin H2-analogue, with those of prostaglandin H2 and thromboxane A2 on some isolated smooth muscle preparations. *Br J Pharmacol* **73:**773–778.
Coleman RA, Smith WL, and Narumiya S (1994) International Union of Pharmacology classification of prostanoid receptors: properties, distribution and structure of the receptors and their subtypes. *Pharmacol Rev* **46:**205–229.
Dogné JM, de Leval X, Hanson J, Frederich M, Lambermont B, Ghuysen A, Casini A, Masereel B, Ruan KH, Pirotte B, et al. (2004a) New developments on thromboxane and prostacyclin modulators part I: thromboxane modulators. *Curr Med Chem* **11:**1223–1241.
Dogné JM, de Leval X, Neven P, Rolin S, Wauters J, David JL, Delarge J, and Massereel B (2000) Effects of a novel non-carboxylic thromboxane A2 receptor antagonist (BM-531) derived from torasemide on platelet function. *Prostaglandins Leukotrienes Essent Fatty Acids* **62:**311–317.
Dogné JM, Hanson J, de Leval X, Kolh P, Tchana-Sato V, de Leval L, Rolin S, Ghuysen A, Segers P, Lambermont B, et al. (2004b) Pharmacological characterization of *N*-tert-butyl-*N*′-[2-(4′-methylphenylamino)-5-nitrobenzenesulfonyl]urea (BM-573), a novel thromboxane A_2 receptor antagonist and thromboxane synthase inhibitor in a rat model of arterial thrombosis and its effects on bleeding time. *J Pharmacol Exp Ther* **309:**498–505.
Dogné JM, Wouters J, Rolin S, Michaux C, Pochet L, Durant F, Delarge J, and Masereel B (2001) Design, synthesis and biological evaluation of a sulfonylcyanoguanidine as thromboxane A2 receptor antagonist and thromboxane synthase inhibitor. *J Pharm Pharmacol* **53:**669–680.
Ghuysen A, Lambermont B, Dogné JM, Kolh P, Tchana-Sato V, Morimont P, Magis D, Hanson J, Segers P, and D'Orio V (2004) Effect of BM-573 [*N*-terbutyl-*N*′-[2-(4′-methylphenylamino)-5-nitro-benzenesulfonyl]urea], a dual thromboxane synthase inhibitor and thromboxane receptor antagonist, in a porcine model of acute pulmonary embolism. *J Pharmacol Exp Ther* **310:**964–972.
Gorman RR, Johnson RA, Spilman CH, and Aiken JW (1983) Inhibition of platelet thromboxane A2 synthase activity by sodium 5-(3′-pyridinylmethyl)benzofuran-2-carboxylate. *Prostaglandins* **26:**325–342.
Gresele P, Arnout J, Deckmyn H, Huybrechts E, Pieters G, and Vermylen J (1987) Role of proaggregatory and antiaggregatory prostaglandins in hemostasis. Studies with combined thromboxane synthase inhibition and thromboxane receptor antagonism. *J Clin Investig* **80:**1435–1445.
Grynkiewicz G, Poenie M, and Tsien RY (1985) A new generation of Ca2+ indicators with greatly improved fluorescence properties. *J Biol Chem* **260:**3440–3450.
Habib A, FitzGerald GA, and Maclouf J (1999) Phosphorylation of the thromboxane receptor alpha, the predominant isoform expressed in human platelets. *J Biol Chem* **274:**2645–2651.
Hamberg M, Svensson J, and Samuelsson B (1975) Thromboxanes: a new group of biologically active compounds derived from prostaglandin endoperoxides. *Proc Natl Acad Sci USA* **72:**2994–2998.
Harris DN, Asaad MM, Phillips MB, Goldenberg HJ, and Antonaccio MJ (1979) Inhibition of adenylate cyclase in human blood platelets by 9-substituted adenine derivatives. *J Cyclic Nucleotide Res* **5:**125–134.
Hayes JS, Lawler OA, Walsh MT, and Kinsella BT (1999) The prostacyclin receptor is isoprenylated. Isoprenylation is required for efficient receptor-effector coupling. *J Biol Chem* **274:**23707–23718.
Hirata M, Hayashi Y, Ushikubi F, Yokota Y, Kageyama R, Nakanishi S, and Narumiya S (1991) Cloning and expression of cDNA for a human thromboxane A2 receptor. *Nature (Lond)* **349:**617–620.
Kinsella BT, O'Mahony DJ, and Fitzgerald GA (1997) The human thromboxane A2 receptor alpha isoform (TP alpha) functionally couples to the G proteins Gq and G11 in vivo and is activated by the isoprostane 8-epi prostaglandin F2 alpha. *J Pharmacol Exp Ther* **281:**957–964.
Kurz KD, Main BW, and Sandusky GE (1990) Rat model of arterial thrombosis induced by ferric chloride. *Thromb Res* **60:**269–280.
Lambermont B, Kolh P, Ghuysen A, Segers P, Dogné JM, Tchana-Sato V, Morimont P, Benoit P, Gerard P, Masereel B, et al. (2004) Effect of a novel thromboxane A2 inhibitor on right ventricular-arterial coupling in endotoxic shock. *Shock* **21:**45–51.
Mais DE, DeHoll D, Sightler H, and Halushka PV (1988) Different pharmacologic activities for 13-azapinane thromboxane A2 analogs in platelets and blood vessels. *Eur J Pharmacol* **148:**309–315.
Mais DE, Saussy DL Jr, Chaikhouni A, Kochel PJ, Knapp DR, Hamanaka N, and Halushka PV (1985) Pharmacologic characterization of human and canine thromboxane A2/prostaglandin H2 receptors in platelets and blood vessels: evidence for different receptors. *J Pharmacol Exp Ther* **233:**418–424.
Miggin SM and Kinsella BT (1998) Expression and tissue distribution of the mRNAs encoding the human thromboxane A2 receptor (TP) alpha and beta isoforms. *Biochim Biophys Acta* **1425:**543–559.
Mohrland JS, Vander Lugt JT, and Lakings DB (1990) Multiple dose trial of the thromboxane synthase inhibitor furegrelate in normal subjects. *Eur J Clin Pharmacol* **38:**485–488.
Moncada S and Vane JR (1978) Pharmacology and endogenous roles of prostaglandin endoperoxides, thromboxane A2 and prostacyclin. *Pharmacol Rev* **30:**293–331.
Patrono C (2003) Aspirin resistance: definition, mechanisms and clinical read-outs. *J Thromb Haemost* **1:**1710–1713.
Qiao N, Reynaud D, Demin P, Halushka PV, and Pace-Asciak CR (2003) The thromboxane receptor antagonist PBT-3, a hepoxilin stable analog, selectively antagonizes the TPalpha isoform in transfected COS-7 cells. *J Pharmacol Exp Ther* **307:**1142–1147.
Raychowdhury MK, Yukawa M, Collins LJ, McGrail SH, Kent KC, and Ware JA (1994) Alternative splicing produces a divergent cytoplasmic tail in the human endothelial thromboxane A2 receptor. *J Biol Chem* **269:**19256–19261.
Reynaud D, Hinek A, and Pace-Asciak CR (2002) The hepoxilin analog PBT-3 inhibits heparin-activated platelet aggregation evoked by ADP. *FEBS Lett* **515:**58–60.
Rolin S, Dogné JM, Michaux C, Delarge J, and Masereel B (2001) Activity of a novel dual thromboxane A(2)receptor antagonist and thromboxane synthase inhibitor (BM-573) on platelet function and isolated smooth muscles. *Prostaglandins Leukotrienes Essent Fatty Acids* **65:**67–72.
Saussy DL Jr, Mais DE, Knapp DR, and Halushka PV (1985) Thromboxane A2 and prostaglandin endoperoxide receptors in platelets and vascular smooth muscle. *Circulation* **72:**1202–1207.
Soyka R, Guth BD, Weisenberger HM, Luger P, and Muller TH (1999) Guanidine derivatives as combined thromboxane A2 receptor antagonists and synthase inhibitors. *J Med Chem* **42:**1235–1249.
Stegmeier K, Pill J, Muller-Beckmann B, Schmidt FH, Witte EC, Wolff HP, and Patscheke H (1984) The pharmacological profile of the thromboxane A2 antagonist BM 13.177. A new anti-platelet and anti-thrombotic drug. *Thromb Res* **35:**379–395.
Takahara K, Murray R, FitzGerald GA, and Fitzgerald DJ (1990) The response to thromboxane A2 analogues in human platelets. Discrimination of two binding sites linked to distinct effector systems. *J Biol Chem* **265:**6836–6844.
Walsh MT, Foley JF, and Kinsella BT (1998) Characterization of the role of N-linked glycosylation on the cell signaling and expression of the human thromboxane A2 receptor alpha and beta isoforms. *J Pharmacol Exp Ther* **286:**1026–1036.
Walsh MT, Foley JF, and Kinsella BT (2000) The alpha, but not the beta, isoform of the human thromboxane A2 receptor is a target for prostacyclin-mediated desensitization. *J Biol Chem* **275:**20412–20423.

Address correspondence to: Julien Hanson, Natural and Synthetic Drugs Research Centre, Department of Pharmacy, Laboratory of Medicinal Chemistry, University of Liège, 1, Av de L'hôpital, 4000 Liège, Belgium. E-mail: j.hanson@ulg.ac.be

Synthesis and Pharmacological Evaluation of Novel Nitrobenzenic Thromboxane Modulators as Antiplatelet Agents Acting on Both the Alpha and Beta Isoforms of the Human Thromboxane Receptor

Julien Hanson,[†,*] Denis Reynaud,[‡] Na Qiao,[‡] Philippe Devel,[†] Anne-Lise Moray,[†] Jean-François Renard,[†] Leanne P. Kelley,[§] Jean-Yves Winum,[⊥] Jean-Louis Montero,[⊥] B. Therese Kinsella,[§] Bernard Pirotte,[†] Cecil R. Pace-Asciak,[‡] and Jean-Michel Dogné[†,||]

Natural and Synthetic Drugs Research Centre, Department of Pharmacy, Laboratory of Medicinal Chemistry, University of Liège, 1, Av. de l'Hôpital, B-4000 Liège, Belgium, Program in Integrative Biology, Research Institute, The Hospital for Sick Children, 555 University Avenue, Toronto, Ontario, Canada M5G 1X8, School of Biomolecular & Biomedical Science, Conway Institute for Biomolecular and Biomedical Research, University College Dublin, Ireland, Department of Pharmacy, University of Namur, FUNDP, 61 rue de Bruxelles, B-5000 Namur, Belgium, and Université Montpellier II, Laboratoire de Chimie Biomoleculaire, UMR 5032, Ecole Nationale Superieure de Chimie de Montpellier, 8 rue de l'Ecole Normale, 34296 Montpellier Cedex, France

Received February 1, 2006

Thromboxane A_2 (TXA_2) is an arachidonic acid metabolite involved in pathologies such as stroke, myocardial infarction, and atherosclerosis. Consequently, the design of TXA_2 receptor (TP) antagonists remains of great interest in cardiovascular medicine. The actions of TXA_2 are mediated by its specific G-protein coupled receptor of which two alternative spliced isoforms, TPα and TPβ, have been described in humans. In this study, we report the synthesis of a series of original *N*-alkyl-*N*′-[2-(cycloalkyl, alkylaryl)-5-nitrobenzenesulfonyl]urea and *N*-alkyl-*N*′-[2-(alkylaryl)-5-nitrobenzenesulfonyl]-*N*″-cyanoguanidines and outline their pharmacological evaluation using the individual TPα and TPβ isoforms. Among compounds analyzed, several of them exhibited greater affinity and/or functional activity for either TPα or TPβ. The most promising molecules were also found to be antiplatelet agents. From the present results, structural features involved in isoform selectivity can be proposed, and thereby several lead compounds have been identified for the further development of selective TP isoform antagonists.

Introduction

The lipid mediator thromboxane A_2 (TXA_2)[a] plays a key role in several physiologic processes including platelet aggregation as well as vascular and bronchial smooth muscle constriction.[1,2] An overproduction of TXA_2 has been associated with many pathological states such as myocardial infarction, thrombosis, unstable angina, pulmonary embolism, septic shock, atherosclerosis, preeclampsia, and asthma.[3] TXA_2, a metabolite of arachidonic acid (AA) released mainly by phospholipase (PL)-A_2 from membrane phospholipids, is primarily synthesized through the sequential actions of cyclooxygenase (COX) and thromboxane synthase (TXS). COX exists as two main isoforms, COX-1 and COX-2, which are encoded by two separate genes. COX-1 is expressed constitutively in most tissues mediating "housekeeping" functions, whereas COX-2 expression is mainly induced at sites of inflammation by various stimuli.[3] COX-2 is also expressed in a constitutive manner in the brain, the vascular endothelium, and the kidney.[4] TXA_2 synthesis mainly occurs in platelets where both COX-1 and TXS are highly expressed.

The actions of TXA_2 are mediated by its specific G-protein coupled receptor (GPCR), referred to as the TXA_2 receptor or TP.[5] In 1991, Hirata and colleagues reported that the human TP was encoded by a single gene,[6] and in 1994 Raychowdhury et al. identified a second isoform, generated by alternative splicing.[7] The two human TP isoforms, consequently named TPα and TPβ, share the first 328 amino acids but differ exclusively within their carboxy-terminal tails (15 amino acids of the α isoform being replaced by 79 amino acids in the β isoform).

In the 1980s, several compounds with differential activities for TPs expressed in platelets or various types of smooth muscle were characterized[8] and none of them selectively bind to TPα or TPβ.

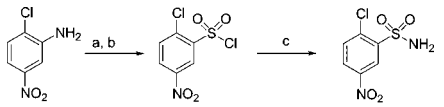
Currently only one compound, 5,8-decadienoic acid, 10-hydroxy-10-[2-(2*Z*)-2-octenylcyclopropyl]-, methyl ester, (5*Z*,8*Z*,-10*S*)- (9CI) (PBT-3), a hepoxilin antagonist, has been described as a preferential TPα antagonist.[9] While the exact role of the TPα and TPβ isoforms has not been fully elucidated to date, a number of independent investigations highlight potentially important physiological differences between them. For example, Coyle et al. have recently demonstrated that the expression of TPα and TPβ is under the control of two distinct promoters.[10,11] Ashton et al. have also demonstrated that a specific inhibitor of TPβ would be useful to enhance postmyocardial infarction revascularization since the stimulation of TPβ seems to be responsible for vascular endothelial growth factor-induced endothelial cell differentiation and migration.[12] On the other hand, since TPα is the predominant TP isoform expressed in platelets,[13] specific inhibitor(s) of TPα may be beneficial as antiplatelet agent(s). Moreover, it has been established that TPα, but not TPβ, is subject to cross-desensitization of signaling by prostacyclin leading to the proposal that TPα is the predominant TP isoform involved in vascular hemeostasis.[14] The potential benefits of TP receptor/isoform selective antagonists in atherosclerosis have been recently highlighted where it was demonstrated that compounds antagonizing TP receptors reduced

* Corresponding author. Phone: +3243664382, Fax: +3243664362, E-mail: J.hanson@ulg.ac.be.
† University of Liège.
‡ The Hospital for Sick Children.
§ University College Dublin.
|| University of Namur.
⊥ Ecole Nationale Superieure de Chimie de Montpellier.

[a] Abbreviations: TXA_2, thromboxane A_2; TXS, thromboxane synthase; PLA_2, phospholipase A_2; AA, arachidonic acid; GPCR, G-protein coupled receptor; COX, cyclooxygenase; TP, thromboxane receptor; PLC, phospholipase C; IP_3, inositol 1,4,5-triphosphate.

10.1021/jm060108a CCC: $33.50
Published on Web 05/24/2006 PAGE EST: 8.6

Scheme 1[a]



3 4 5

[a] Reagents: (a) $NaNO_2$, HCl; (b) SO_2, HOAc, Cu_2Cl_2; (c) NH_4OH, Δ.

atherosclerotic plaque growth.[15] Several groups have also proposed that the adverse cardiovascular effects of the selective COX-2 inhibitor rofecoxib were due to its reduction of prostacylin synthesis without affecting TXA_2 levels, thereby altering the balance between the anti- and prothrombotic actions of prostacyclin and TXA_2 within the vasculature.[16]

In the current study, we describe the synthesis of a series of novel nitrobenzenesulfonylureas and nitrobenzenesulfonylcyanoguanidines and outline their pharmacological evaluation as TP receptor antagonists. For the first time, the structure−activity relationships of this large series of compounds for both TPα and/or TPβ isoforms are discussed. The antiplatelet activity of the most pharmacologically interesting compounds is also described.

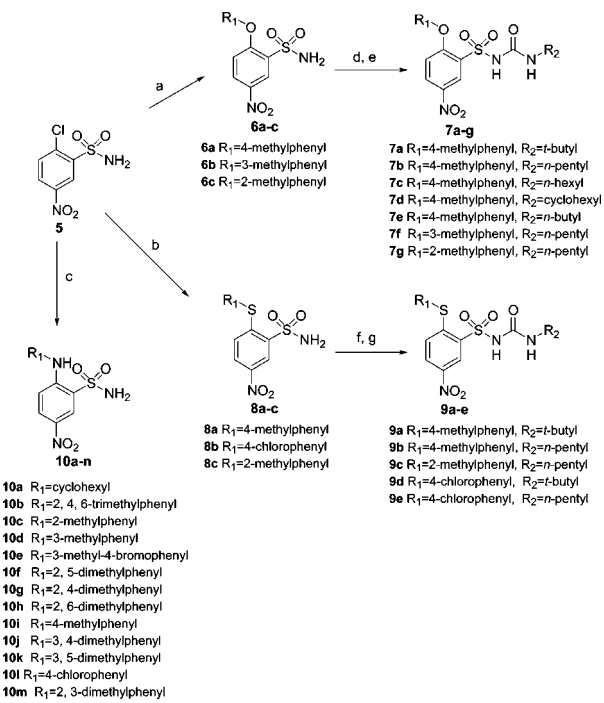
Chemistry. The compounds evaluated in this work were synthesized according to the synthesis pathway described in Schemes 1−3. In the first step, which is common to all compounds described, commercially available 2-chloro-5-nitroaniline (**3**) reacted with sodium nitrite in acidic medium, and the resulting solution of diazonium salt was mixed with a solution of sulfur dioxide in acetic acid in the presence of Cu(I) to generate 2-chloro-5-nitrobenzenesulfonyl chloride (**4**) according to the Meerwein variation of the Sandmeyer reaction.[17] Compound **4** was poured into a solution of ammonium hydroxide and gently heated, resulting in the synthesis of 2-chloro-5-nitrobenzenesulfonamide (**5**) which is the common intermediate for the compounds studied (Scheme 1).

The nucleophilic substitution of the chlorine atom in the position para to the nitro group by either amines, phenols, or thiols led to the synthesis of the intermediates **6a**−**c**, **8a**−**c**, and **10a**−**n**, respectively. Deprotonation of the sulfonamide group of compounds **6a**−**c** followed by reaction with the appropriate isocyanate led to the synthesis of compounds **7a**−**g** (Scheme 2).

Intermediates **8a**−**c** are characterized by a sulfur atom as a bridge between the two aromatic rings. These compounds were synthesized by reacting thiophenols, instead of phenols, with compound **5** in the presence of K_2CO_3. As with the compounds **6a**−**c**, intermediates **8a**−**c** were deprotonated and reacted with the appropriate isocyanates to afford the formation of compounds **9a**−**e** (Scheme 2).

Compounds **10a**−**m** were obtained by reacting the appropriate amines with compound **5**. The sulfonamide group of compounds **10a**−**m** was deprotonated and then submitted to different reaction conditions. For the synthesis of sulfonylcyanoguanidines **13a**−**b**, a reactive synthon was first prepared by direct reaction of diphenyl *N*-cyanoiminocarbonate (**11**) with *n*-pentylamine. This synthon (**12**) directly reacted with the sulfonamidate salts of **10i** and **10m** to generate compounds **13a**−**b** (Scheme 3).

Scheme 2[a]



6a R_1=4-methylphenyl
6b R_1=3-methylphenyl
6c R_1=2-methylphenyl

7a R_1=4-methylphenyl, R_2=*t*-butyl
7b R_1=4-methylphenyl, R_2=*n*-pentyl
7c R_1=4-methylphenyl, R_2=*n*-hexyl
7d R_1=4-methylphenyl, R_2=cyclohexyl
7e R_1=4-methylphenyl, R_2=*n*-butyl
7f R_1=3-methylphenyl, R_2=*n*-pentyl
7g R_1=2-methylphenyl, R_2=*n*-pentyl

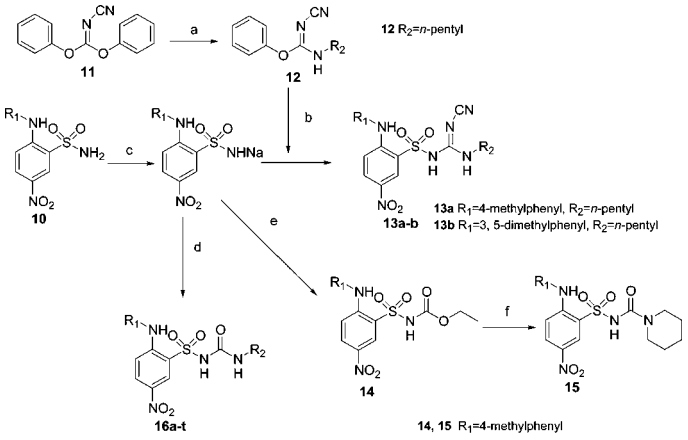
8a R_1=4-methylphenyl
8b R_1=4-chlorophenyl
8c R_1=2-methylphenyl

9a R_1=4-methylphenyl, R_2=*t*-butyl
9b R_1=4-methylphenyl, R_2=*n*-pentyl
9c R_1=2-methylphenyl, R_2=*n*-pentyl
9d R_1=4-chlorophenyl, R_2=*t*-butyl
9e R_1=4-chlorophenyl, R_2=*n*-pentyl

10a R_1=cyclohexyl
10b R_1=2, 4, 6-trimethylphenyl
10c R_1=2-methylphenyl
10d R_1=3-methylphenyl
10e R_1=3-methyl-4-bromophenyl
10f R_1=2, 5-dimethylphenyl
10g R_1=2, 4-dimethylphenyl
10h R_1=2, 6-dimethylphenyl
10i R_1=4-methylphenyl
10j R_1=3, 4-dimethylphenyl
10k R_1=3, 5-dimethylphenyl
10l R_1=4-chlorophenyl
10m R_1=2, 3-dimethylphenyl

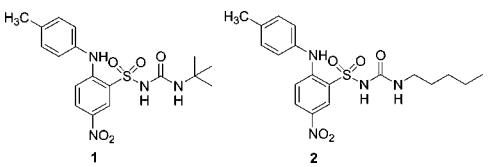
[a] Reagents: (a) R_1-ONa, K_2CO_3; (b) R_1-SH, K_2CO_3; (c) R_1-NH_2, K_2CO_3; (d) NaOH; (e) $R_2-N{=}C{=}O$; (f) NaOH; (g) $R_2-N{=}C{=}O$.

Scheme 3[a]



16a R_1=cyclohexyl, R_2=hexyl
16b R_1=cyclohexyl, R_2=heptyl
16c R_1=cyclohexyl, R_2=octyl
16d R_1=2, 4, 6-trimethylphenyl, R_2=*n*-pentyl
16e R_1=2-methylphenyl, R_2=*n*-pentyl
16f R_1=3-methylphenyl, R_2=*n*-pentyl
16g R_1=3-methyl-4-bromophenyl, R_2=*t*-butyl
16h R_1=2, 5-dimethylphenyl, R_2=*n*-pentyl
16i R_1=2, 4-dimethylphenyl, R_2=*n*-pentyl
16j R_1=2, 6-dimethylphenyl, R_2=*n*-pentyl
16k R_1=4-methylphenyl, R_2=benzyl
16l R_1=4-methylphenyl, R_2=cyclohexyl
16m R_1=3, 4-dimethylphenyl, R_2=*n*-pentyl
16n R_1=3, 5-dimethylphenyl, R_2=*n*-pentyl
16o R_1=3-methyl-4-bromophenyl, R_2=*n*-pentyl
16p R_1=2, 6-dimethylphenyl, R_2=*sec*-butyl
16q R_1=2, 6-dimethylphenyl, R_2=*t*-butyl
16r R_1=4-methylphenyl, R_2=*sec*-pentyl
16s R_1=4-chlorophenyl, R_2=*n*-pentyl
16t R_1=2, 3-dimethylphenyl, R_2=*n*-pentyl

[a] Reagents: (a) R_3-NH_2, 2-propanol; (b) DMF; (c) NaOH; (d) $R_2-N=C=O$; (e) ethyl chloroformate; (f) piperidine.



1 **2**

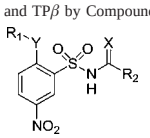
Figure 1. Chemical structures of **1** and **2**, two TP receptor antagonists used as references in pharmacological tests.

Compounds **10a**−**m** also reacted, after deprotonation, with appropriate isocyanates to generate the sulfonylureas (**16a**−**t**). Finally, one compound (**15**), bearing a sulfonyl group for which the distal nitrogen atom was fully substituted, was obtained in two steps: (i) reaction of **10i** as the sodium salt with ethyl chloroformate followed by (ii) reaction with piperidine that led to formation of **15** (Scheme 3).

Pharmacological Evaluation. The overall aim of this study was to evaluate a series of novel nitro-substituted benzene sulfonamide derivatives as pharmacological antagonists of the TPα and TPβ isoforms of the human TXA_2 receptor. Consequently, we synthesized several analogues of **1** (BM-573) and **2** (BM-613, both structures are presented in Figure 1), two potent TP antagonists characterized by a nitrobenzenic ring bearing a sulfonylurea moiety.[18−22] The binding affinities of each TP isoform for each test compound was assessed through competition binding studies in COS-7 cell lines transiently transfected with respective plasmids encoding TPα or TPβ. Experiments were initially performed at a single concentration (1 nM) of the 35 novel test compounds or reference compounds, **1** and **2**. Competition binding studies were performed with [^{3}H]SQ29,548 {5-heptenoic acid, 7-[(1*S*,2*R*,3*R*,4*R*)-3-[[2-[(phenylamino)carbonyl]hydrazino]methyl]-7-oxabicyclo[2.2.1]hept-2-yl]-, (5Z)-(9CI)}, a selective TP antagonist, as the radioligand. It has been previously confirmed that SQ29,548 had no apparent selectivity for either the TPα or TPβ isoform.[23] Thereafter, those test compounds that displayed the highest affinities for one or both TP isoforms as well as compounds characterized by the greatest differences in affinity for either TPα or TPβ were selected for a complete concentration−response curve determination with concentrations ranging from 10^{-6} to 10^{-11} M. Values for the inhibitory concentration $(IC)_{50}$ were obtained from the concentration−response curves and a "selectivity index", defined as the IC_{50} TPα/IC_{50} TPβ ratio, was calculated.

Because binding experiments do not reflect the specific activity and pharmacological profile of a molecule/ligand at its receptor, we also examined the effects of the compounds under study on agonist-induced intracellular signaling by the individual TPα and TPβ isoforms. To this end, the ability of the compounds to antagonize TPα and TPβ-mediated intracellular calcium mobilization ($[Ca^{2+}]_i$) in response to the TXA_2 mimetic

Table 1. Displacement of Radiolabeled [^{3}H]SQ29,548 from TPα and TPβ by Compounds at 1 nM



compound	R_1	R_2	Y	X	binding affinity (%)[a] TPα	TPβ
1	4-methylphenyl	*tert*-butylamino	NH	O	49.5 ± 1.6	53.6 ± 5.5
2	4-methylphenyl	*n*-pentylamino	NH	O	39.6 ± 9.1	34.2 ± 2.9
16a	cyclohexyl	*n*-hexylamino	NH	O	44.2 ± 16.5	42.5 ± 2.3
16b	cyclohexyl	*n*-heptylamino	NH	O	29.5 ± 3.4	36.4 ± 11.1
16c	cyclohexyl	*n*-octylamino	NH	O	17.1 ± 3.7	24.1 ± 4.4
16d	2,4,6-trimethylphenyl	*n*-pentylamino	NH	O	27.9 ± 3.0	27.5 ± 4.4
16e	2-methylphenyl	*n*-pentylamino	NH	O	45.3 ± 8.9	45.3 ± 3.0
16f	3-methylphenyl	*n*-pentylamino	NH	O	48.5 ± 3.4	35.8 ± 9.2
16g	3-methyl-4-bromophenyl	*tert*-butylamino	NH	O	28.5 ± 5.4	25.0 ± 1.5
16h	2,5-dimethylphenyl	*n*-pentylamino	NH	O	28.6 ± 3.4	33.1 ± 4.9
16i	2,4-dimethylphenyl	*n*-pentylamino	NH	O	42.3 ± 4.0	39.9 ± 4.8
16j	2,6-dimethylphenyl	*n*-pentylamino	NH	O	36.5 ± 4.7	54.0 ± 2.1
16k	4-methylphenyl	benzylamino	NH	O	32.9 ± 4.5	49.3 ± 3.5
16l	4-methylphenyl	cyclohexylamino	NH	O	31.1 ± 2.9	29.0 ± 3.3
16m	3,4-dimethylphenyl	*n*-pentylamino	NH	O	40.1 ± 8.8	48.7 ± 16.9
16n	3,5-dimethylphenyl	*n*-pentylamino	NH	O	49.7 ± 5.1	49.7 ± 11.0
16o	3-methyl-4-bromophenyl	*n*-pentylamino	NH	O	32.1 ± 5.2	49.9 ± 13.0
16p	2,6-dimethylphenyl	*sec*-butylamino	NH	O	48.4 ± 4.0	42.5 ± 6.0
16q	2,6-dimethylphenyl	*tert*-butylamino	NH	O	46.4 ± 2.2	46.3 ± 2.3
16r	4-methylphenyl	*sec*-butylamino	NH	O	40.7 ± 2.5	40.4 ± 6.5
16s	4-chlorophenyl	*n*-pentylamino	NH	O	48.9 ± 7.7	52.7 ± 2.2
16t	2,3-dimethylphenyl	*n*-pentylamino	NH	O	44.6 ± 5.4	39.0 ± 7.8
7a	4-methylphenyl	*tert*-butylamino	O	O	53.3 ± 5.7	44.7 ± 2.1
7b	4-methylphenyl	*n*-pentylamino	O	O	35.5 ± 2.8	48.5 ± 3.8
7c	4-methylphenyl	*n*-hexylamino	O	O	55.2 ± 2.1	46.1 ± 2.1
7d	4-methylphenyl	cyclohexylamino	O	O	49.8 ± 5.7	51.9 ± 3.8
7e	4-methylphenyl	*n*-butylamino	O	O	55.7 ± 1.7	56.0 ± 4.9
7f	3-methylphenyl	*n*-pentylamino	O	O	20.1 ± 10.3	22.8 ± 3.3
7g	2-methylphenyl	*n*-pentylamino	O	O	35.2 ± 1.6	36.8 ± 7.1
9a	4-methylphenyl	*tert*-butylamino	S	O	47.0 ± 5.6	37.7 ± 6.3
9b	4-methylphenyl	*n*-pentylamino	S	O	38.5 ± 2.7	43.0 ± 1.1
9c	2-methylphenyl	*n*-pentylamino	S	O	33.4 ± 2.2	32.7 ± 7.7
9d	4-chlorophenyl	*tert*-butylamino	S	O	32.5 ± 1.8	31.7 ± 3.4
9e	4-chlorophenyl	*n*-pentylamino	S	O	24.7 ± 7.6	21.3 ± 5.7
13a	4-methylphenyl	*n*-pentylamino	NH	N−CN	25.5 ± 1.8	27.2 ± 5.2
13b	3,5-dimethylphenyl	*n*-pentylamino	NH	N−CN	48.0 ± 6.4	53.4 ± 3.2
15	4-methylphenyl	piperidino	NH	O	46.2 ± 9.2	54.2 ± 7.8

[a] Expressed as the percentage of displaced [^{3}H]SQ29,548 by our compounds. Results are mean ± standard deviation of at least three determinations ($n \geq 3$). Compounds were evaluated at a final concentration of 1 nM.

U46619 {5-Heptenoic acid, 7-[(1*R*,4*S*,5*S*,6*R*)-6-[(1*E*,3*S*)-3-hydroxy-1-octenyl]-2-oxabicyclo[2.2.1]hept-5-yl]-, (5*Z*)- (9Cl)} was evaluated in HEK 293 cell lines stably overexpressing either TPα (HEK.TPα cells) or TPβ (HEK.TPβ cells). HEK.TPα and/or HEK.TPβ cells were preloaded with fluorescent dye Fluo-4 and inhibition of the TXA_2 mimetic U46619 (1 μM) stimulation of $[Ca^{2+}]_i$ mobilization was determined in the absence or presence of increasing concentrations of the compounds, using concentrations ranging from 10^{-5} M to 10^{-8} M. As for the competition binding studies, a selectivity index was determined. Finally, since the TP receptor directly mediates human platelet aggregation, the efficacy of test compounds in an ex vivo human platelet aggregation model was evaluated.

Results and Discussion

The results obtained with the initial competition-binding studies are presented in Table 1. All compounds evaluated displayed a high affinity for both TPα and TPβ receptor isoforms. These affinities, expressed as the percentage displacement of [^{3}H]SQ29,548, were all in the nanomolar range and were comparable to that of the reference compounds **1** and **2** (Figure 1) used throughout this study. The affinities of **1** and **2** were in the same range of order of previously published data.[22] The bridging atom (O, S, or NH in the series **7a**−**g**, **9a**−**e**, or **13**, **15**, **16a**−**t**, respectively) between the nitrobenzene and the second ring did not affect the affinity of the molecules for the TP receptors. Additionally, in the "NH" compounds family (**13**, **15**, and **16a**−**t**), a critical loss of affinity was not observed as a result of the disubstitution of the distal nitrogen atom of the urea group or as a result of the replacement of the urea function with a cyanoguanidine isoster moiety. Thereafter, further experiments focused on those compounds that either displayed the highest affinity for the TP receptors or on those that showed significant differences in their TPα/TPβ isoform selectivity.

For compounds **7a**−**e**, characterized by the presence of an oxygen atom as the bridge between two aromatic rings, several concentration−response curves were determined and are summarized in Table 2. Although the affinities of the compounds for both TPα and TPβ lie within the same range, certain compounds exhibited a significant ratio of selectivity between the two receptor isoforms. For example, compound **7b** exhibited a significant ratio of 4.02 ($p < 0.05$), which was a result of a greater affinity for TPβ. In this family of compounds, it was apparent that the side chain (R_2) had an impact on the affinity.

Table 2. Estimated IC_{50} Values for Displacement of [^{3}H]SQ29,548 from TPα and TPβ

compound	binding affinity IC_{50} (nM)[a] TPα	TPβ	ratio[a] IC_{50} TPα/IC_{50} TPβ
1	1.05 ± 0.43	0.78 ± 0.10	1.35
2	2.65 ± 1.59	3.53 ± 1.38	0.75
7a	1.26 ± 0.74	1.77 ± 0.69	0.71
7b	2.63 ± 0.94	0.65 ± 0.15	4.02
7c	0.72 ± 0.08	1.58 ± 0.21	0.45
7d	1.52 ± 0.70	0.79 ± 0.15	1.93
7e	0.74 ± 0.02	0.70 ± 0.24	1.05
9a	0.77 ± 0.33	1.74 ± 0.28	0.44
15	0.46 ± 0.11	0.68 ± 0.11	0.68
16a	0.71 ± 0.01	1.07 ± 0.26	0.67
16b	2.50 ± 1.21	2.68 ± 1.06	0.93
16f	1.01 ± 0.25	4.43 ± 0.47	0.23
16j	1.52 ± 0.31	1.48 ± 0.35	1.03
16k	1.90 ± 0.40	1.17 ± 0.12	1.62
16n	1.08 ± 0.33	0.70 ± 0.43	1.55
16o	1.82 ± 0.25	0.58 ± 0.27	3.11
16q	0.78 ± 0.15	0.56 ± 0.20	1.40

[a] Results expressed as mean ± standard deviation of at least three determinations ($n \geq 3$).

For example in terms of TP isoform affinity, compound **7e** with R_2 = *n*-butyl was the most potent compound in this assay and seems to bear the most interesting side chain, among those tested, for these compounds. In terms of the calculated selectivity ratio, the side chain was also found to be involved. On one hand, compounds for which R_2 corresponds to *n*-pentyl or cyclohexyl (**7b** and **7d**, respectively) expressed a preferential affinity for TPβ (Table 2). On the other hand, when R_2 corresponds to *tert*-butyl or *n*-hexyl (**7a** and **7c**, respectively), there was either a loss or no change in the selectivity ratio.

Additionally, Table 2 reports the results of the determination of the IC_{50} value of compound **9a**, characterized by a sulfur atom as the intercycle bridge. This compound expressed a nanomalor affinity for the TP receptors in our model. This observation is consistent with our previous statement that the nature of the intercycle bridge (NH, S, or O) did not markedly influence the affinity of the compound for TPα/TPβ.

Compound **15** is characterized by having a NH as the bridge between the two rings and having the distal N urea atom incorporated into a ring. Although the affinity of compound **15** for TPα or TPβ was not markedly improved compared to the reference compounds **1** and **2** (Table 2), the incorporation of the distal N urea atom into an alicyclic ring appeared to be favorable for the affinity of both isoforms, with no influences on the selectivity.

Finally, with compounds of the **16** family (**16a**−**t**) bearing a NH bridge between the two rings, data on Table 2 indicates that, when the compounds were characterized by a second ring (R_1), namely alicyclic, the length of the side chain influenced the affinity. For example, compound **16a** with R_2 = *n*-hexyl was characterized by a ∼3-fold higher affinity than **16b** with R_2 = *n*-heptyl The results obtained with compound **16f** for which R_2 = *n*-pentyl and R_1 = *m*-methylphenyl were of particular interest, since it is the most selective compound for TPα isoform. Finally, it is noteworthy that PBT-3, a stable analogue of hepoxilin which is known to be a selective TPα antagonist, was reported to exhibit a selectivity ratio of ∼0.17 in the same assay.[9]

Thereafter, we sought to extend the characterization of the selected high affinity test compounds through functional intracellular signaling studies. Hence, we further examined the properties of the various test compounds through assessment of U46619-induced intracellular calcium ($[Ca^{2+}]_i$ mobilization

Table 3. Estimated IC_{50} Values for the Inhibition of $[Ca^{2+}]_i$ Mobilization Mediated by Either TPα or TPβ upon Stimulation by U46619 (1 μM)

compound	U46619-mediated $[Ca^{2+}]_i$ mobilization (nM)[a] TPα	TPβ	ratio[a] IC_{50} TPα/IC_{50} TPβ
1	318.89 ± 202.54	53.10 ± 19.38	6.01
2	50.04 ± 0.73	13.56 ± 1.08	3.69
7a	58.34 ± 43.01	57.63 ± 3.97	1.01
7b	139.32 ± 87.93	55.38 ± 4.85	2.52
7c	526.42 ± 29.61	58.48 ± 0.58	9.00
7d	600.71 ± 11.16	70.15 ± 5.90	8.56
7e	558.04 ± 33.91	52.89 ± 2.54	10.55
9a	293.54 ± 106.53	55.17 ± 3.45	5.32
15	64.81 ± 3.54	38.52 ± 2.84	1.68
16a	81.22 ± 3.50	46.17 ± 1.32	1.76
16b	92.90 ± 9.03	55.55 ± 2.26	1.67
16f	60.39 ± 16.07	45.53 ± 1.50	1.33
16j	80.72 ± 20.39	47.10 ± 0.39	1.71
16k	64.47 ± 1.84	45.01 ± 2.59	1.43
16n	537.86 ± 8.18	76.50 ± 6.39	7.03
16o	126.28 ± 26.07	43.19 ± 9.98	2.92
16q	56.89 ± 2.35	30.78 ± 9.81	1.85

[a] Results expressed as mean ± standard deviation of at least three determinations ($n \geq 3$).

in HEK293 cell lines stably overexpressing the individual TPα or TPβ isoforms). Calcium was the obvious second messenger to evaluate since TPα or TPβ each couple to Gq/phospholipase (PL)C activation, leading to inositol 1,4,5-trisphosphate (IP_3)-mediated $[Ca^{2+}]_i$ mobilization from intracellular stores.[24,25] The $[Ca^{2+}]_i$ mobilization was measured with cells loaded with fluorescent dye Fluo-4, whose fluorescence increases upon binding with calcium ion released in response to the TXA_2 mimetic U46619. Consequently, the ability of the compounds to antagonize U46619-mediated $[Ca^{2+}]_i$ mobilization was evaluated.

The results obtained with the test compounds in this assay (Table 3) do not fully correlate with those obtained through competition binding studies (Table 2). This can be explained by the fact that the affinity is not the same property as activity, i.e., that affinity does not necessarily reflect the agonistic or antagonistic properties of a compound at its receptor. Moreover, the mechanism and intensity whereby a compound activates or deactivates a receptor cannot be measured through competition binding studies. Hence, when evaluating a receptor ligand (agonist/antagonist), it is critical to evaluate that agent through a number of independent mechanisms, such as through binding studies and functional studies as outlined herein.

First, it is noteworthy that all compounds assayed exhibited high activity as TPα and TPβ receptor antagonists. Compound **16q**, one of the most potent compound in this assay, was characterized by a 2,6-dimethylphenylamino group ortho to the sulfonamide group in the aromatic ring. As a result, it is suggestive that a pattern of substitution by methyl groups in the 2- and 6- positions of the second ring may provide an interesting lead for further experimentations. Moreover, in this functional assay, each compound exhibited a better activity on TPα compared with TPβ, except with compound **7a** which possessed activities in the same concentration range. These data also confirmed the fact that the side chain of the molecules could play a role in selectivity. For example, activities on TPβ of compounds **7b**−**e** were within the same range as compound **7a** characterized by a *tert*-butyl side chain. Nevertheless, their activities on TPα were almost 10-fold less pronounced than that of **7a**. Additionally, it should also be noted that the rank order of potency of **7b**−**e** for TPα did not follow the pattern of the size or steric hindrance of the side chain. Indeed, TPα potency

Table 4. Estimated IC_{50} Values for the Inhibition of Platelet Aggregation Induced by U46619 (1 μM)

compound	inhibition of platelet aggregation induced by 1 μM U46619 IC_{50} (μM)[a]
1	0.240 ± 0.013
2	0.278 ± 0.186
7a	0.300 ± 0.003
7b	0.670 ± 0.200
9a	0.800 ± 0.090
15	0.900 ± 0.006
16q	0.090 ± 0.007

[a] Results expressed as mean ± standard deviation of at least three determinations (n≥3).

was ranked 4b > 4c ≅ 4e > 4d, indicating that *n*-pentyl > *n*-butyl ≅ *n*-hexyl > cyclohexyl. We could not conclude from these results that the selectivity ratio is directly influenced by the length of the side chain. Nevertheless, it is notable that in this assay the *n*-pentyl side chain was responsible for the best activity in this series compared to other linear side chains.

Finally, we sought to confirm the activity of our most interesting molecules on human platelets by assessment of their ability to inhibit platelet aggregation in response to the TXA_2 mimetic U46619. Compounds **7a**, **7b**, **9a**, **15**, and **16q** were selected because of their ability to inhibit U46619-mediated $[Ca^{2+}]_i$ release, affinities, or apparent selectivity in different models. The results obtained are summarized in Table 4. First, compounds evaluated were indeed confirmed to act as TP receptor antagonists inhibiting platelet aggregation in response to 1 μM U46619 at sub-micromolar concentrations ranging from 0.09 to 0.9 μM.

It should be highlighted that all the compounds evaluated in the three pharmacological assessment models exhibited promising results in terms of their affinities, as assessed through competition of radioligand binding, measurements of inhibition of $[Ca^{2+}]_i$ mobilization, and inhibition of U46619-induced platelet aggregation. Collective results highlighted compound **16q** as a potential lead of major interest since its IC_{50} for inhibition of platelet aggregation was 0.09 μM. Finally, it is noteworthy that compound **16q** is one of the most potent compounds on TPα and is also the most potent inhibitor in platelet aggregation studies. These data are consistent with previous findings that TPα is the dominant isoform expressed in human platelets,[13] although this compound was equally active on TPβ (Table 2).

Conclusions

We have synthesized and evaluated a series of some 40 novel nitro-substituted benzenesulfonamide derivatives designed as antagonists of the TP/TXA_2 receptors. Since there are two TP isoforms in humans, the development of selective compounds for TPα and/or TPβ is clearly of great clinical interest for human diseases. Hence, we have studied the affinity and activity of our compounds on both TPα and TPβ. All compounds evaluated exhibited very high affinity for both TP receptors, mainly acting in the nanomolar range. Moreover, both receptor ligand binding and inhibition of $[Ca^{2+}]_i$ signaling concentration−response curves have been determined with the most interesting compounds. In these assays, all compounds confirmed their affinity and activity for the TP receptors.

According to the biological data presented herein, we can propose in our series of nitrobenzene-sulfonylureas and -sulfonylcyanoguanidines some structural factors involved in affinity and activity on either TPα or TPβ, which could lead to the development of selective TP receptor antagonists. Finally, the most promising compounds were evaluated on platelet aggregation and confirmed their potent TP receptor antagonism and their interest as antiplatelet agents.

Experimental Section

Chemistry. All commercial chemicals (Sigma-Aldrich, Belgium) and solvents are reagent grade and were used without further purification unless otherwise stated. Compounds **5**, **10d**, **8a**, and **8c** were already described.[26,27] **6c** was commercially available (AmbinterStock Screening Collection). Nevertheless, these compounds were synthesized in our lab according to the method of preparation described below. All reactions were followed by thin-layer chromatography (silica gel $60F_{254}$ Merck) and visualization was accomplished with UV light (254 nm). Elemental analyses (C, H, N, S) were determined on a Carbo Erba EA 1108 and were within ±0.4% of the theoretical values. NMR spectra were recorded either on a Bruker Avance 500 or on a Bruker DRX-400 spectrometer using DMSO-d_6 as solvent and tetramethylsilane as internal standard. For ^{1}H NMR spectra, chemical shifts are expressed in δ (ppm) downfield from tetramethylsilane. The abbreviations d, doublet; t, triplet; m, multiplet; br, broad were used throughout. Infrared spectra were recorded using a Perkin-Elmer FT-IR 1750. All compounds described were recrystallized from hot methanol (40 °C)/H_2O mixture (60/40; 10 mL/100 mg of product) unless otherwise stated.

General Procedure for the Reaction of 5 with Amines (10). Compound **5** (0.01 mol) and the appropriate amine (0.05 mol) were dissolved in 3-chlorotoluene (30 mL) and refluxed for 12−96 h. When the reaction was finished (monitored by tcl), the mixture was evaporated under reduced pressure. The residue was dissolved in an aqueous NaOH solution (0.5 N, 10 mL/g of residue). This mixture was extracted with diethyl ether and the aqueous layer was separated and adjusted to pH 1 with 0.5 M hydrochloric acid. The precipitate which appeared was collected by filtration, washed with water and dried (yield: 35−95%).

General Procedure for the Reaction of 5 with Cresols (6). Before starting the reaction, the cresolates were obtained from the corresponding cresols (0.05 mol) after their neutralization by 0.06 mol NaOH (in aqueous solution, 10% w/v) in acetone. Evaporation under reduced pressure provided crystals of the cresolates.

Compound **5** (0.01 mol) and the appropriate cresolate (0.05 mol) were dissolved in acetonitrile. The mixture was refluxed and potassium carbonate (0.007 mol) was added. After completion of the reaction monitored by TLC (12−36 h), the solution was acidified and filtered and the filtrate was evaporated under reduced pressure. The crude product was dissolved in methanol and ice was added. The resulting precipitate was collected by filtration (yield: 60−70%).

General Procedure for the Reaction of 5 with Thiophenols (8). Compound **5** (0.01 mol) and the appropriate thiophenol (0.05 mol) were dissolved in acetonitrile (30 mL). The mixture was refluxed and potassium carbonate (0.007 mol) was added. When the reaction was finished (0.1−1 h), the solution was acidified and filtered and the filtrate was evaporated under reduced pressure. The resulting crude oil was dissolved in methanol and ice was added. The precipitate was recovered by filtration (yield: 50−75%).

General Procedure for the Preparation of Sulfonylureas with Isocyanates (1, 2, 7, 9, and 16). The appropriate sulfonamide (0.01 mol) was dissolved in acetone (30 mL). NaOH (0.01 mol) (10% aqueous sol. w/v) was added. The mixture was gently mixed during 10 min and then was evaporated under reduced pressure. The resulting solid was resuspended in acetone (30 mL) and gently refluxed. The appropriate isocyanate (0.02 mol) was added to the mixture. At the end of the reaction (0.1−1 h), the mixture was evaporated under reduced pressure and the crude product was washed with AcOEt. The solid was collected by filtration and dissolved in an aqueous NaOH solution (0.5 N; 20 mL). The resulting solution was adjusted to pH 1 with hydrochloric acid (12 N), and the solid which precipitated was isolated by filtration (yield 40−60%).

***N-tert*-Butyl-*N′*-[2-(4-methylphenylamino)-5-nitrobenzenesulfonyl]urea (1).** Mp: 126−127 °C. Anal. ($C_{18}H_{22}N_4O_5S$) C, H, N, S.

***N-n*-Pentyl-*N′*-[2-(4-methylphenylamino)-5-nitrobenzenesulfonyl]urea (2).** Mp: 145−147 °C. Anal. ($C_{19}H_{24}N_4O_5S$) C, H, N, S.

***N-tert*-Butyl-*N′*-[2-(4-methylphenoxy)-5-nitrobenzenesulfonyl]-urea (7a).** Mp: 162−165 °C. Anal. ($C_{18}H_{21}N_3O_6S$) C, H, N, S.

***N-n*-Pentyl-*N′*-[2-(4-methylphenoxy)-5-nitrobenzenesulfonyl]-urea (7b).** Mp: 148−150 °C. Anal. ($C_{19}H_{23}N_3O_6S$) C, H, N, S.

***N-n*-Hexyl-*N′*-[2-(4-methylphenoxy)-5-nitrobenzenesulfonyl]-urea (7c).** Mp: 128−130 °C. Anal. ($C_{20}H_{25}N_3O_6S$) C, H, N, S.

***N*-Cyclohexyl-*N′*-[2-(4-methylphenoxy)-5-nitrobenzenesulfonyl]urea (7d).** Mp: 170−173 °C. Anal. ($C_{20}H_{23}N_3O_6S$) C, H, N, S.

***N-n*-Butyl-*N′*-[2-(4-methylphenoxy)-5-nitrobenzenesulfonyl]-urea (7e).** Mp: 207−210 °C. Anal. ($C_{18}H_{21}N_3O_6S$) C, H, N, S.

***N-n*-Pentyl-*N′*-[2-(3-methylphenoxy)-5-nitrobenzenesulfonyl]-urea (7f).** Mp: 157−158 °C. Anal. ($C_{19}H_{23}N_3O_6S$) C, H, N, S.

***N-n*-Pentyl −*N′*-[2-(2-methylphenoxy)-5-nitrobenzenesulfonyl]urea (7g).** Mp: 150−153 °C. Anal. ($C_{19}H_{23}N_3O_6S$) C, H, N, S.

***N-tert*-Butyl-*N′*-[2-(4-methylphenylthio)-5-nitrobenzenesulfonyl]urea (9a).** Mp: 181−183 °C. Anal. ($C_{18}H_{21}N_3O_5S_2$) C, H, N, S.

***N-n*-Pentyl-*N′*-[2-(4-methylphenylthio)-5-nitrobenzenesulfonyl]urea (9b).** Mp: 149−151 °C. Anal. ($C_{19}H_{23}N_3O_5S_2$) C, H, N, S.

***N-n*-Pentyl-*N′*-[2-(2-methylphenylthio)-5-nitrobenzenesulfonyl]urea (9c).** Mp: 156−158 °C. Anal. ($C_{19}H_{23}N_3O_5S_2$) C, H, N, S.

***N-tert*-Butyl-*N′*-[2-(4-chlorophenylthio)-5-nitrobenzenesulfonyl]urea (9d).** Mp: 139−142 °C. Anal. ($C_{17}H_{18}ClN_3O_5S_2$) C, H, N, S.

***N-n*-Pentyl −*N′*-[2-(4-chlorophenylthio)-5-nitrobenzenesulfonyl]urea (9e).** Mp: 131−135 °C. Anal. ($C_{18}H_{20}ClN_3O_5S_2$) C, H, N, S.

***N-n*-Hexyl-*N′*-[2-(cyclohexylamino)-5-nitrobenzenesulfonyl]-urea (16a).** Mp: 115−116 °C. Anal. ($C_{19}H_{30}N_4O_5S$) C, H, N, S.

***N-n*-Heptyl-*N′*-[2-(cyclohexylamino)-5-nitrobenzenesulfonyl]-urea (16b).** Mp: 117−118 °C. Anal. ($C_{20}H_{32}N_4O_5S$) C, H, N, S.

***N-n*-Octyl-*N′*-[2-(cyclohexylamino)-5-nitrobenzenesulfonyl]-urea (16c).** Mp: 93−94 °C. Anal. ($C_{21}H_{34}N_4O_5S$) C, H, N, S.

***N-n*-Pentyl-*N′*-[2-(2,4,6-trimethylphenylamino)-5-nitrobenzenesulfonyl]urea (16d).** Mp: 143−145 °C. Anal. ($C_{21}H_{28}N_4O_5S$) C, H, N, S.

***N-n*-Pentyl-*N′*-[2-(2-methylphenylamino)-5-nitrobenzenesulfonyl]urea (16e).** Mp: 127−128 °C. Anal. ($C_{19}H_{24}N_4O_5S$) C, H, N, S.

***N-n*-Pentyl-*N′*-[2-(3-methylphenylamino)-5-nitrobenzenesulfonyl]urea (16f).** Mp: 128−130 °C. Anal. ($C_{19}H_{24}N_4O_5S$) C, H, N, S.

***N-tert*-Butyl-*N′*-[2-(3-methyl-4-bromophenylamino)-5-nitrobenzenesulfonyl]urea (16g).** Mp: 141−143 °C. Anal. ($C_{18}H_{21}BrN_4O_5S$) C, H, N, S.

***N-n*-Pentyl-*N′*-[2-(2, 5-dimethylphenylamino)-5-nitrobenzenesulfonyl]urea (16h).** Mp: 126−128 °C. Anal. ($C_{20}H_{24}N_6O_4S$) C, H, N, S.

***N-n*-Pentyl-*N′*-[2-(2, 4-dimethylphenylamino)-5-nitrobenzenesulfonyl]urea (16i).** Mp: 127−129 °C. Anal. ($C_{20}H_{26}N_4O_5S$) C, H, N, S.

***N-n*-Pentyl-*N′*-[2-(2, 6-dimethylphenylamino)-5-nitrobenzenesulfonyl]urea (16j).** Mp: 129−131 °C. Anal. ($C_{20}H_{26}N_4O_5S$) C, H, N, S.

***N*-Benzyl-*N′*-[2-(4-methylphenylamino)-5-nitrobenzenesulfonyl]urea (16k).** Mp: 148−150 °C. Anal. **($C_{21}H_{20}N_4O_5S$) C, H, N, S.**. ***N*-Cyclohexyl-*N′*-[2-(4-methylphenylamino)-5-nitrobenzenesulfonyl]urea (16l).** Mp: 166−168 °C. Anal. ($C_{20}H_{24}N_4O_5S$) C, H, N, S.

***N-n*-Pentyl-*N′*-[2-(3, 4-dimethylphenylamino)-5-nitrobenzenesulfonyl]urea (16m).** Mp: 88−90 °C. Anal. ($C_{20}H_{26}N_4O_5S$) C, H, N, S.

***N-n*-Pentyl-*N′*-[2-(3, 5-dimethylphenylamino)-5-nitrobenzenesulfonyl]urea (16n).** Mp: 122−125 °C. Anal. ($C_{20}H_{26}N_4O_5S$) C, H, N, S.

***N-n*-Pentyl-*N′*-[2-(3-methyl-4-bromophenylamino)-5-nitrobenzenesulfonyl]urea (16o).** Mp: 130−132 °C. Anal. ($C_{19}H_{23}BrN_4O_5S$) C, H, N, S.

***N-sec*-Butyl-*N′*-[2-(2,6-dimethylphenylamino)-5-nitrobenzenesulfonyl]urea (16p).** Mp: 97−99 °C. Anal. ($C_{19}H_{24}N_4O_5S$) C, H, N, S.

***N-tert*-Butyl-*N′*-[2-(2,6-dimethylphenylamino)-5-nitrobenzenesulfonyl]urea (16q).** Mp: 95−97 °C. Anal. ($C_{19}H_{24}N_4O_5S$) C, H, N, S.

***N-sec*-Butyl-*N′*-[2-(4-methylphenylamino)-5-nitrobenzenesulfonyl]urea (16r).** Mp: 104−106 °C. Anal. ($C_{19}H_{24}N_4O_5S$) C, H, N, S.

***N-n*-Pentyl-*N′*-[2-(4-Chlorophenylamino)-5-nitrobenzenesulfonyl]urea (16s).** Mp: 124−126 °C. Anal. ($C_{18}H_{21}ClN_4O_5S$) C, H, N, S.

***N-n*-Pentyl-*N′*-[2-(2, 3-dimethylphenylamino)-5-nitrobenzenesulfonyl]urea (16t).** Mp: 139−142 °C. Anal. ($C_{20}H_{26}N_4O_5S_2$) C, H, N, S.

Procedure for the preparation of *N*-*n*-Pentyl-*N′*-cyano-*O*-phenylisourea (12). A mixture of **11** (0.01 mol) and *n*-pentylamine (0.015 mol) in 2-propanol (30 mL) was stirred at room temperature for 10−15 min. The solution was evaporated, and the crude oil was crystallized in cold methanol. Mp: 134−136 °C. ^{1}H NMR (DMSO) δ: 0.86 (t, 2H, J = 7 Hz, NH−CH_2−CH_2−CH_2−CH_2−CH_3); 1.1−1.35 (m, 6H, NH−CH2−CH_2−CH_2−CH_2−CH_3); 3.26 (q, 2H, J = 7 Hz, NH−CH_2-CH_2−CH_2−CH_2−CH_3); 7.06−7.41 (m, 5H, H_{aro}).

General Procedure for the Preparation of Nitrobenzenesulfonylcyanoguanidines (13). The appropriate sulfonamide (0.01 mol) was dissolved in acetone (30 mL). NaOH (0.01 mol) (10% aqueous sol. w/v) was added. The mixture was gently mixed during 10 min and was evaporated under reduced pressure. The solid was resuspended in dimethylformamide (20 mL) at room temperature and the appropriate *N*-alkyl-*N′*-cyano-*O*-phenylisourea was added. The mixture was stirred at RT for 20−24 h. At the end of the reaction, the mixture was evaporated under reduced pressure and the crude oil was suspended in a mixture of methanol and hydrochloric acid (5 N aqueous solution) from which crystals of the desired product appeared.

***N-n*-Pentyl-*N′*-[2-(4-methylphenylamino)-5-nitrobenzenesulfonyl]-*N″*-cyanoguanidine (13a).** Mp: 162−166 °C. Anal. ($C_{20}H_{24}N_6O_4S$) C, H, N, S.

***N-n*-Pentyl-*N′*-[2-(3,5-dimethylphenylamino)-5-nitrobenzenesulfonyl]-*N″*-cyanoguanidine (13b).** Mp: 149−151 °C. Anal. ($C_{21}H_{26}N_6O_4S$) C, H, N, S.

Procedure for the Synthesis of *N*-[2-(4-Methylphenylamino)-5-nitrobenzenesulfonyl]-piperidine-1-carboxamide (15). Compound **10i** (0.01 mol) was dissolved in acetone. NaOH (0.01 mol) (10% sol. w/v) was added. The mixture was gently mixed during 10 min and was evaporated under reduced pressure. The solid was resuspended in acetone (10 mL). A large excess of ethyl chloroformate (0.03 mol) was added dropwise under vigorous stirring. The mixture was stirred for 15 min, and the solution was evaporated under reduced pressure. The ethyl carbamate obtained was purified by crystallization in methanol. Ethyl 2-(4-methylphenylamino)-5-nitrobenzenesulfonylcarbamate (**14**, 0.01 mol) was dissolved in a mixture of anhydrous toluene (40 mL) and piperidine (0.02 mol). The resulting solution was gently refluxed overnight. At the end of the reaction, the solution was evaporated under reduced pressure, and the residue was dissolved into an aqueous NaOH solution (0.5 N, 20 mL). This mixture was extracted with diethyl ether. The aqueous layer was recovered and adjusted to pH 1 with hydrochloric acid (0.5 N). The precipitate which appeared was recovered by filtration (yield: 65%). Mp: 139−141 °C. ^{1}H NMR ($CDCl_3$) δ: 1.60 (b, 6H, piperidine); 2.38 (s, 3H, CH_3-4′); 3.39 (s, 4H, piperidine); 7.03 (d, 1H, J = 9 Hz, H-3); 7.17−7.29 (m, 4H, H_{aro}′); 8.07 (dd, 1H, J = 9 Hz, J = 2.5 Hz, H-4); 8.75 (d, 1H, J = 2.5

Hz, H-6); 8.85 (s, 1H, Ph-N*H*-Ph); 11.14 (s, 1H, SO_2−N*H*-CO-). Anal. ($C_{19}H_{22}N_4O_5S$) C, H, N, S.

Radioligand Binding Assay. COS-7 cells were maintained in Dulbecco's modified Eagle's medium (Hyclone Laboratories, Logan, UT) supplemented with 10% fetal bovine serum and 1% of solution containing 10 000 units/mL penicillin G, 10 000 μg/mL streptomycin, and 25 μg/mL amphotericin (Cellgro; Mediatech, Herndon, VA). Cells were grown at 37 °C in a humidified atmosphere of 95% O_2 and 5% CO_2. cDNAs (the cDNA was generously provided by Prof. Perry Halushka, Medical University of South Carolina) for the TPα and TPβ were subcloned into pcDNA3, the resultant plasmid, pcDNA3:TPα and pcDNA3:TPβ were introduced into COS-7 cells by the DEAE-dextran/chloroquine method. Forty eight hours posttransfection, cells were harvested by centrifugation at 500*g* for 5 min and washed three times in ice-cold phosphate-buffered saline. Cells were resuspended in buffer containing 25 mM HEPES/125 mM NaCl/10 μM indomethacin, pH 7.4, and kept on ice for the binding study. Binding reactions were carried out on 5×10^5 cells in a total volume of 0.2 mL in the above buffer with 10 nM [^{3}H]SQ29,548 (Perkin-Elmer Life and analytical services, Boston, MA) added to all tubes in triplicate, containing various concentrations of studied compounds (10^{-9} for screening assay or 10^{-6} to 10^{-11} M for competition binding curves) in 1 μL of ethanol. Additional tubes containing excess unlabeled SQ29,548 (10 μM) (Cayman Chemical Co, Ann Arbor, MI) were included to assess the extent of nonspecific binding. Binding was allowed to take place for 30 min at 37 °C; free radioligand was removed by rapid vacuum filtration through Whatman (Maidstone, UK) GF/B glass fiber filters prewashed with the cell suspension buffer. The tubes and the filters were rapidly washed with ice-cold 10 mM Tris buffer, pH 7.4 (three times with 3 mL). The radioactivity on the filters containing the ligand−receptor complexes was counted in 10 mL of Ecolite scintillation fluid (ICN, St. Laurent, QC, Canada) in a Beckman (model LS 3800) liquid scintillation counter. The binding experiments were performed on whole cells.

Calcium Measurements. HEK.TPα and HEK.TPβ cell lines, stably overexpressing HA-tagged forms of TPα and TPβ in human embryonic kidney (HEK) 293 cells have been previously described.[28] HEK 293 cells or their stable cell line equivalents were routinely grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). Measurement of $[Ca^{2+}]_i$ mobilization either in HEK.TPα or HEK.TPβ cells was carried out using fluorescent microplate reader Fluoroskan Ascent FL equipped with two dispenser (thermo electron corporation, Finland) according to modified method of Lin et al.[29] Briefly, cells were trypsinized, washed twice with Krebs-HEPES buffer (118 mM, NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 11.7 mM D-glucose, 1.3 mM $CaCl_2$, 10 mM HEPES, pH 7.4), and incubated for 1 h with fluorescent dye Fluo-4/AM (5 μg/mL; Molecular Probes, Invitrogen, Merelbeke Belgium). Cells were then rinsed three times with Krebs-HEPES buffer and 150 μL of a suspension of cells in that buffer was loaded into each well of a 96-well plate at a density of 150 000 cells/well. Cells were incubated 10 min with various concentrations of the test compound (10^{-5} to 10^{-8} M final; 10 μL) prior to stimulation with U46619 (1 μM final, 50 μl). In all cases, compound (1 mM) was diluted in dimethyl sulfoxide (DMSO)/PBS (30/70) prior to further dilution in PBS. Fluorescence emission was read at 538 nM. At the end of each experiment, fluorescence intensities were calibrated for determination of intracellular calcium concentration ($[Ca^{2+}]_i$) values by permeabilizing cells with 1% Triton X-100 to release all the dye (Fmax) and subsequently chelating with 10 mM EGTA (F_{min}). Calcium concentrations were calculated using equation $[Ca^{2+}]_i = K_d(F - F_{min})/(F_{max} - F)$, assuming a *K*d of 385 nM for Fluo-4. The results (IC_{50}) presented are the concentration required to inhibit 50% of the normal rise of $[Ca^{2+}]_i$ upon stimulation with 1 μM U46619, determined in the absence of any compounds. The IC_{50}s were calculated by nonlinear regression analysis (GraphPad Prism software) from at least three concentration−response curves.

Human in Vitro Platelet Aggregation. The antiaggregant potency has been determined according to the turbidimetric Born's method.[30] The blood was drawn from 10 healthy donors of both genders, aged 20−30. The subjects were free from medication for at least 14 days. No significant differences in the results were observed between the donors in our experiments. Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were prepared as previously described.[26,31] Platelet concentration of PRP was adjusted to 3×10^8 cells/mL by dilution with PPP. Platelet aggregation of PRP was studied using a double channel aggregometer (Chronolog Corporation, Chicago, IL) connected to a linear recorder as previously described.[32] PRP (294 μL) was added in a silanised cuvette and stirred (1000 rpm). Each compound was diluted (1 mM) in dimethyl sulfoxide (DMSO)/PBS (30/70) and preincubated in PRP for three minutes at 37 °C before the aggregating agent was added. Platelet aggregation was initiated by addition of a fresh solution of U46619 (1 μM final). To evaluate platelet aggregation, the maximum increase in light transmission was determined from the aggregation curve 6 min after addition of the inducer. The substance concentration preventing 50% of platelet aggregation (IC_{50}) induced by U46619 was calculated by nonlinear regression analysis (GraphPad Prism software) from at least three dose−response curves.

Statistical Analysis. Results are expressed as the mean ± standard deviation from at least three determinations ($n \geq 3$). Statistical differences between TP isoforms have been determined using unpaired t-test between IC_{50}s values. *p* values of less than 0.05 were considered to be significant.

Acknowledgment. Julien Hanson is funded by the "Fonds pour la Formation à la Recherche dans l'Industrie et dans l'Agriculture" (FRIA., from Belgium). This work was supported by a grant from the Fonds National de la Recherche Scientifique (FNRS) from Belgium. The authors are grateful to Pierre Close for his scientific advice and wish to thank MM. Philippe Neven and Didier Botty for excellent technical assistance. B.T.K. acknowledges the support of the Wellcome Trust.

Supporting Information Available: NMR, elemental analysis, melting points, and IR peaks for all compounds presented. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Hamberg, M.; Svensson, J.; Samuelsson, B. Thromboxanes: a new group of biologically active compounds derived from prostaglandin endoperoxides. *Proc. Natl. Acad. Sci. U.S.A.* **1975**, *72*, 2994−2998.
(2) Moncada, S.; Ferreira, S. H.; Vane, J. R. Bioassay of prostaglandins and biologically active substances derived from arachidonic acid. *Adv. Prostaglandin. Thromboxane. Res.* **1978**, *5*, 211−236.
(3) Dogne, J. M.; de Leval, X.; Hanson, J.; Frederich, M.; Lambermont, B. et al. New developments on thromboxane and prostacyclin modulators part I: thromboxane modulators. *Curr. Med. Chem.* **2004**, *11*, 1223−1241.
(4) Cheng, Y.; Austin, S. C.; Rocca, B.; Koller, B. H.; Coffman, T. M. et al. Role of prostacyclin in the cardiovascular response to thromboxane A2. *Science* **2002**, *296*, 539−541.
(5) Coleman, R. A.; Smith, W. L.; Narumiya, S. International Union of Pharmacology classification of prostanoid receptors: properties, distribution, and structure of the receptors and their subtypes. *Pharmacol. Rev.* **1994**, *46*, 205−229.
(6) Hirata, M.; Hayashi, Y.; Ushikubi, F.; Yokota, Y.; Kageyama, R. et al. Cloning and expression of cDNA for a human thromboxane A2 receptor. *Nature* **1991**, *349*, 617−620.
(7) Raychowdhury, M. K.; Yukawa, M.; Collins, L. J.; McGrail, S. H.; Kent, K. C. et al. Alternative splicing produces a divergent cytoplasmic tail in the human endothelial thromboxane A2 receptor. *J. Biol. Chem.* **1994**, *269*, 19256−19261.
(8) Mais, D. E.; Saussy, D. L., Jr.; Chaikhouni, A.; Kochel, P. J.; Knapp, D. R. et al. Pharmacologic characterization of human and canine thromboxane A2/prostaglandin H2 receptors in platelets and blood vessels: evidence for different receptors. *J. Pharmacol. Exp. Ther.* **1985**, *233*, 418−424.

(9) Qiao, N.; Reynaud, D.; Demin, P.; Halushka, P. V.; Pace-Asciak, C. R. The thromboxane receptor antagonist PBT-3, a hepoxilin stable analogue, selectively antagonizes the TPalpha isoform in transfected COS-7 cells. *J. Pharmacol. Exp. Ther.* **2003**, *307*, 1142−1147.
(10) Coyle, A. T.; Kinsella, B. T. Characterization of promoter 3 of the human thromboxane A receptor gene. A functional AP-1 and octamer motif are required for basal promoter activity. *FEBS J.* **2005**, *272*, 1036−1053.
(11) Coyle, A. T.; O'Keeffe, M. B.; Kinsella, B. T. 15-deoxy Delta12, 14-prostaglandin J2 suppresses transcription by promoter 3 of the human thromboxane A2 receptor gene through peroxisome proliferator-activated receptor gamma in human erythroleukemia cells. *FEBS J.* **2005**, *272*, 4754−4773.
(12) Ashton, A. W.; Ware, J. A. Thromboxane A2 receptor signaling inhibits vascular endothelial growth factor-induced endothelial cell differentiation and migration. *Circ. Res.* **2004**, *95*, 372−379.
(13) Habib, A.; FitzGerald, G. A.; Maclouf, J. Phosphorylation of the thromboxane receptor alpha, the predominant isoform expressed in human platelets. *J. Biol. Chem.* **1999**, *274*, 2645−2651.
(14) Reid, H. M.; Kinsella, B. T. The alpha, but not the beta, isoform of the human thromboxane A2 receptor is a target for nitric oxide-mediated desensitization. Independent modulation of Tp alpha signaling by nitric oxide and prostacyclin. *J. Biol. Chem.* **2003**, *278*, 51190−51202.
(15) Egan, K. M.; Wang, M.; Fries, S.; Lucitt, M. B.; Zukas, A. M. et al. Cyclooxygenases, thromboxane, and atherosclerosis: plaque destabilization by cyclooxygenase-2 inhibition combined with thromboxane receptor antagonism. *Circulation* **2005**, *111*, 334−342.
(16) Dogne, J. M.; Supuran, C. T.; Pratico, D. Adverse cardiovascular effects of the coxibs. *J. Med. Chem.* **2005**, *48*, 2251−2257.
(17) Meerwein, H.; Dittmar, G.; Gollner, R.; Hafner, K.; Mensch, F. et al. Aromatic diazo compounds. II. Preparation of aromatic sulfonyl chlorides, a new modification of the Sandmeyer reaction. *Chem. Ber.* **1957**, *90*, 841−852.
(18) Dogne, J. M.; Hanson, J.; de Leval, X.; Kolh, P.; Tchana-Sato, V. et al. Pharmacological characterization of N-*tert*-butyl-N′-[2-(4′-methylphenylamino)-5-nitrobenzenesulfonyl]urea (BM-573), a novel thromboxane A2 receptor antagonist and thromboxane synthase inhibitor in a rat model of arterial thrombosis and its effects on bleeding time. *J. Pharmacol. Exp. Ther.* **2004**, *309*, 498−505.
(19) Ghuysen, A.; Lambermont, B.; Dogne, J. M.; Kolh, P.; Tchana-Sato, V. et al. Effect of BM-573 [N-terbutyl-N′-[2-(4′-methylphenylamino)-5-nitro-benzenesulfonyl]urea], a dual thromboxane synthase inhibitor and thromboxane receptor antagonist, in a porcine model of acute pulmonary embolism. *J. Pharmacol. Exp. Ther.* **2004**, *310*, 964−972.
(20) Rolin, S.; Petein, M.; Tchana-Sato, V.; Dogne, J. M.; Benoit, P. et al. BM-573, a dual thromboxane synthase inhibitor and thromboxane receptor antagonist, prevents pig myocardial infarction induced by coronary thrombosis. *J. Pharmacol. Exp. Ther.* **2003**, *306*, 59−65.
(21) Tchana-Sato, V.; Dogne, J. M.; Lambermont, B.; Ghuysen, A.; Magis, D. et al. Effects of BM-573, a thromboxane A2 modulator on systemic hemodynamics perturbations induced by U-46619 in the pig. *Prostaglandins Other Lipid Mediat.* **2005**, *78*, 82−95.
(22) Hanson, J.; Rolin, S.; Reynaud, D.; Qiao, N.; Kelley, L. P. et al. In vitro and in vivo pharmacological characterization of BM-613 [N-*n*-pentyl-N′-[2-(4′-methylphenylamino)-5-nitrobenzenesulfonyl]urea], a novel dual thromboxane synthase inhibitor and thromboxane receptor antagonist. *J. Pharmacol. Exp. Ther.* **2005**, *313*, 293−301.
(23) Parent, J. L.; Labrecque, P.; Orsini, M. J.; Benovic, J. L. Internalization of the TXA2 receptor alpha and beta isoforms. Role of the differentially spliced cooh terminus in agonist-promoted receptor internalization. *J. Biol. Chem.* **1999**, *274*, 8941−8948.
(24) Kinsella, B. T.; O'Mahony, D. J.; Fitzgerald, G. A. The human thromboxane A2 receptor alpha isoform (TP alpha) functionally couples to the G proteins Gq and G11 in vivo and is activated by the isoprostane 8-epi prostaglandin F2 alpha. *J. Pharmacol. Exp. Ther.* **1997**, *281*, 957−964.
(25) Walsh, M. T.; Foley, J. F.; Kinsella, B. T. Characterization of the role of N-linked glycosylation on the cell signaling and expression of the human thromboxane A2 receptor alpha and beta isoforms. *J. Pharmacol. Exp. Ther.* **1998**, *286*, 1026−1036.
(26) Dogne, J. M.; Wouters, J.; Rolin, S.; Michaux, C.; Pochet, L. et al. Design, synthesis and biological evaluation of a sulfonylcyanoguanidine as thromboxane A2 receptor antagonist and thromboxane synthase inhibitor. *J. Pharm. Pharmacol.* **2001**, *53*, 669−680.
(27) Wagner, A. W.; Banholzer, R. Heterocycles with endocyclic sulfur−nitrogen double bond. I. 1-Substituted 5-nitro-1H-1,3,2-benzodithiazole 3,3-dioxides. *Berichte* **1963**, *96*, 1177−1186.
(28) Walsh, M. T.; Foley, J. F.; Kinsella, B. T. The alpha, but not the beta, isoform of the human thromboxane A2 receptor is a target for prostacyclin-mediated desensitization. *J. Biol. Chem.* **2000**, *275*, 20412−20423.
(29) Lin, K.; Sadee, W.; Quillan, J. M. Rapid measurements of intracellular calcium using a fluorescence plate reader. *Biotechniques* **1999**, *26*, 318−322, 324−316.
(30) Born, G. V.; Cross, M. J. The Aggregation of Blood Platelets. *J. Physiol.* **1963**, *168*, 178−195.
(31) Dogne, J. M.; de Leval, X.; Neven, P.; Rolin, S.; Wauters, J. et al. Effects of a novel non-carboxylic thromboxane A2 receptor antagonist (BM-531) derived from torasemide on platelet function. *Prostaglandins, Leukotrienes Essent. Fatty Acids* **2000**, *62*, 311−317.
(32) Harris, D. N.; Asaad, M. M.; Phillips, M. B.; Goldenberg, H. J.; Antonaccio, M. J. Inhibition of adenylate cyclase in human blood platelets by 9-substituted adenine derivatives. *J. Cyclic Nucleotide Res.* **1979**, *5*, 125−134.

JM060108A

Design, synthesis and SAR study of a series of *N*-alkyl-*N*'-[2-(aryloxy)-5-nitrobenzenesulfonyl]-ureas and –cyanoguanidine as selective antagonists of the TPα and TPβ isoforms of the human thromboxane A_2 receptor.

Julien Hanson[1,*], Jean-Michel Dogné[1,3], Jérémie Ghiotto[1], Anne-Lise Moray[1], B. Therese Kinsella[2], Bernard Pirotte[1]

[1] Drug Research Center, Laboratory of Medicinal Chemistry, University of Liège, 1, Av. de l'Hôpital, B-4000 Liège, Belgium.

[2] School of Biomolecular & Biomedical Science, Conway Institute for Biomolecular and Biomedical Research, University College Dublin, Ireland.

[3] Department of Pharmacy, University of Namur, FUNDP, 61 rue de Bruxelles, B-5000 Namur, Belgium.

[*] To whom correspondence should be addressed

Julien Hanson

Drug Research Center, Laboratory of Medicinal Chemistry, University of Liège, 1, Av de L'hôpital, 4000 Liège, Belgium.

Phone : +3243664382

Fax : +3243664362

E-mail : J.hanson@ulg.ac.be

Submitted for publication in

Journal of Medicinal Chemistry

Abstract

The prostanoid thromboxane (TX)A_2 exerts its pro-aggregant and constrictive actions upon binding to its specific TXA_2 receptor, or TP, a member of the G protein coupled receptor (GPCR) superfamily. In human, TXA_2 signals through two distinct TP isoforms, known as TPα and TPβ. Herein, we describe the design, synthesis and SAR study of a series of original *N*-alkyl-*N*'-[2-(aryloxy)-5-nitrobenzenesulfonyl]-ureas and –cyanoguanidine compounds. SAR study was based on the results of a functional assay, TP-mediated intracellular calcium ($[Ca^{2+}]_i$) mobilization performed on the two separate isoforms. Thus, optimal nature and position of several structural moieties was defined for both activity and selectivity toward the individual TPα and TPβ isoforms. Three compounds (**9h**, **9af** and **9ag**), showing increased selectivity for TPβ relative to TPα (23.2:1, 18.1:1, 19.9:1, respectively), were selected for further experiment and confirmed their activity in a platelet aggregation assay. This study represents the first extended SAR study dealing with the identification of isoform selective antagonists for the human TXA_2 receptor.

Introduction

The lipid mediator thromboxane (TX)A_2 plays a key role in several physiologic processes including platelet aggregation and vascular and bronchial smooth muscle constriction [1,2]. An overproduction of TXA_2 has been associated with many pathological states such as myocardial infarction, thrombosis, unstable angina, pulmonary embolism, septic shock, atherosclerosis, preeclampsia and asthma [3]. TXA_2, a metabolite of arachidonic acid (AA) released mainly by phospholipase (PL)A_2 from membrane phospholipids, is primarily synthesized through the sequential actions of cyclooxygenase (COX) and TX synthase in platelets [3].

The actions of TXA_2 are mediated by its specific G-protein coupled receptor (GPCR), referred to as the TXA_2 receptor or TP [4]. In 1991, Hirata *et al.* reported that the human TP is encoded by one gene [5] and, in 1994, Raychowdhury *et al.* identified the existence of a second isoform generated by alternative splicing [6]. The two human TP isoforms, namely TPα and TPβ, share the first 328 amino acids but differ within their carboxyl-terminal tails (15 last amino acids of the α isoform being replaced by 79 amino acids in the β isoform). To date, the exact role of the TPα and TPβ isoforms is not fully understood although several studies have established that they may operate distinct physiological functions [7-9]. It has been proposed, for example, that specific antagonism of TPβ would be useful to enhance revascularization post-myocardial infarction since the stimulation of TPβ seemed to be responsible for vascular endothelial growth factor-induced endothelial cell differentiation and migration [10]. On the other hand, because TPα appears to be the predominant TP isoform expressed in platelets [11], specific inhibitor(s) of TPα may be beneficial as more specific anti-platelet agent(s).

Hence, the design and synthesis of selective isoform specific TP receptor antagonists would be of great interest not only in therapeutics but also as tools in pharmacological sciences to decipher TP isoform specific roles. Despite their great interest, the identification of such TPα and/or TPβ isoform specific agonists/antagonists has been poorly studied. Although early pharmacological reports have described selective TXA_2 receptor (TP) antagonists for separate tissues, the initial observations have never been confirmed on isolated TP isoforms [12]. Through previous investigations, our group have addressed the influence of the chemical structure of a series of nitrobenzenesulfonyl-ureas and –cyanoguanidines on selective antagonistic potency for TPα or TPβ [13]. In that study, we reported the synthesis and functional characterization of a series of derivatives of the potent TP receptor antagonist BM573 (**1**, figure 1)[14] and, from such investigations, a family of original *N*-alkyl-*N*'-[2-(4-methylphenoxy)-5-nitrobenzenesulfonyl]ureas compounds was selected for further detailed characterization through both chemical optimization and functional biologic/pharmacologic readouts. Three of the most interesting previously reported compounds **2**, **3** and **4** are illustrated in figure 1. In the present study, we describe

the design, synthesis and SAR study of a series of original nitrobenzenesulfonylureas derived from the latter **2**, **3** and **4** chemical leads. We have characterized the antagonistic potency and TP-isoform selectivity of the new compounds using a functional pharmacological test, namely inhibition of intracellular calcium ($[Ca^{2+}]_i$) mobilization in model mammalian cell lines that specifically over-express the individual TPα or TPβ isoforms. The *anti*-platelet activity of some of the most interesting compounds was also determined in aggregation assays.

Chemistry

The compounds evaluated in this work were synthesized according to the synthesis pathway described in schemes 1-3. In the first step, which is common to all compounds described, commercially available 2-chloro-5-nitroaniline (**5**) reacted with sodium nitrite in acidic medium and the resulting diazonium salt solution was mixed with a solution of sulfur dioxide in acetic acid in the presence of Cu(I) to generate 2-chloro-5-nitrobenzenesulfonyl chloride (**6**) according to the Meerwein variation of the Sandmeyer reaction [15]. Compound **6** was poured into a solution of ammonium hydroxide and gently heated, resulting in the synthesis of 2-chloro-5-nitrobenzenesulfonamide (**7**) which is the common intermediate giving access to the compounds studied (scheme 1).

The nucleophilic substitution of the chlorine atom in the *para* position to the nitro group by various phenols led to the synthesis of the intermediates **8a-p**. Deprotonation of the sulfonamide group of compounds **8a-p** followed by reaction with the appropriate isocyanate led to the synthesis of compounds **9a-aj** (scheme 2).

For the synthesis of the sulfonylcyanoguanidine **12**, a reactive synthon was first prepared by direct reaction of diphenyl N-cyanoiminocarbonate (**10**) with *t*-butylamine. This synthon (**11**) directly reacted with the sulfonamidate salt of **8a** (**9**) to generate compound **12** (scheme 3).

Pharmacological Evaluation

Thereafter, we wished to address the structure-activity relationships (SAR) of the latter compounds based on a functional pharmacological evaluation. To this end, the ability of the compounds to antagonize TPα and TPβ-mediated intracellular calcium ($[Ca^{2+}]$i) mobilization in response to the TXA_2 mimetic U46619 was evaluated in human embryonic kidney (HEK) 293 cell lines stably over-expressing either TPα (HEK.TPα cells) or TPβ (HEK.TPβ cells). Indeed, both TPα and TPβ are coupled to the Gq/phospholipase C effector system [8]and, hence, can readily induce a rise in $[Ca^{2+}]_i$ upon agonist stimulation of the TP receptor. HEK.TPα and/or HEK.TPβ cells were preloaded with Ca^{2+} fluorescent dye Fluo-4 and inhibition of $[Ca^{2+}]_i$ mobilization in response to the well characterized TXA_2 mimetic U46619 (1 μM) stimulation was determined in the absence or presence of increasing concentrations of the test compounds, using concentrations ranging from 10^{-9} M to 10^{-5} M.

Concentration-response curves were determined and the IC_{50}, defined as the concentration (M) of compound required to inhibit 50% of U46619-induced $[Ca^{2+}]_i$ mobilization, value (M) were calculated for each compound tested. A selectivity index, defined as ratio of the IC_{50} (M) of TPα relative to the IC_{50} (M) of TPβ (i.e IC_{50} TPα/ IC_{50} TPβ) was subsequently determined.

Furthermore, since TXA_2 is a potent inducer of platelet aggregation, the ability of the most interesting test compounds to prevent aggregation of human platelets in response to the TXA_2 mimetic U46619 was also evaluated. In this test, the compounds were incubated at concentrations ranging from 10^{-9} to 10^{-5} M with isolated human platelets and aggregation was triggered upon stimulation by U46619 (1μM).

Results and Discussion

In a previous study, we demonstrated the activity of a family of nitrobenzenesulfonylureas presenting an oxygen bond between the two aromatic rings as selective antagonists of the TPα and TPβ isoforms of the human TXA_2 receptor (TP)[13]. The three most interesting leads identified in those previous investigations [13], namely compounds **2**, **3** and **4**, are presented in figure 1 and their relative potency toward the TP receptor isoforms in table 1. One single parameter was modulated in that preliminary study, namely the side chain on the sulfonylurea group. Consequently, herein, we designed and synthesized several series of *N*-alkyl-*N*'-[2-(aryloxy)-5-nitrobenzenesulfonyl]ureas, derived form **2**, **3** and **4** (figure 1) in order to more precisely estimate the structure-activity relationships (SAR) for activity and selectivity within this family of compounds toward the individual TP receptor isoforms.

Several features of the structure were explored. Previous work on nitrobenzene sulfonylurea suggested that the position and the nature of the nitro group as well as the position of the sulfonylurea moiety and the second aromatic ring were optimal, so these parameters were kept constant (Dogné *et al.*, unpublished data). As shown in table 1, we first varied the position of the methyl group on the aromatic ring R_1 (2 and 3-position) and we addressed the influence of various alkyl side chains R_2 for each position. We also completed the series of side chains in the R_1=4-methylphenyl moiety series. All compounds examined in the U46619-mediated $[Ca^{2+}]_i$ mobilization assay displayed potent micro and submicromolar IC_{50} activities. Consistent with previous observations, all compounds tested were more potent on TPβ than on TPα [13] in this assay. Throughout the studies presented in tables 1, 2, 3 and 4, **1** has been included as the reference compound. Results obtained with previously described compounds **9b** and **9a** (R_1 = 2- and 3-methyphenyl, respectively) have been included for comparison with newly synthesized compounds. Parent compounds **2**, **3** and **4**, bearing a 4-methylphenyl moiety as R_1 have also been included into table 1.

In the 2-methylphenyl series (compounds **9b-e**), the R_2 *n*-butyl chain of analog **9d** was found to be the best substituent and produced a 3- to10-fold increase in activity compared to other compounds of the series (**9c** with an *i*-propyl chain as R_2 being the less active). Regarding the difference of activity between the two isoforms, all compounds were characterized by an isoform selectivity ratio (IC_{50} TPα/IC_{50} TPβ) of ~16-17 in this 2-substituted series except for analog **9b** with R_2=*n*-pentyl which had a lower ratio of 9. The higher ratio clearly suggested increased selectivity for TPβ relative to TPα. Meta substitution of the phenyl ring R_1 (3-methylphenyl series) seemed to induce little influence on activity, except for compound **9h** characterized by a ~2 (compared to **9d**) to 20-fold (compared to **9c**) increase in activity on both isoforms. More interestingly, compound **9h** estimated selectivity ratio was the highest in this table (23.2, table 1) whereas other compounds of this series displayed a ~10 fold ratio. In previous work, the compounds having a 4-methylphenyl moiety as the aromatic ring R_1 proved generally to be the most potent on TPα [13]. The two original R_2 alkyl chains evaluated in this aryloxy series, *n*-propyl (**9j**) and *i*-propyl (**9i**) produced an important (~10 fold) decrease of activity on both isoforms. Collectively, the results of Table 1 highlighted the following general trend for most active R_2 substituents on both isoforms: *t*-butyl ≅ *n*-butyl > *n*-pentyl ≥ *n*-propyl ≅ *i*-propyl. With compounds with *t*-butyl and *n*-pentyl chains, the best substitution positions for activity on both isoforms were 4 > 3 ≥ 2. Regarding the selectivity, the best alkyl side chain was R_2 = *n*-butyl or *t*-butyl and the following trend was observed for these side chains regarding the phenyl ring substitution: 3 ≥ 2 >> 4.

Furthermore, we designed compounds to address the specific role in selectivity and activity of the nature of the substituent on the phenyl ring R_1. Former results showed that, in other series, combination of a methyl or bromo substituent in the 4-position of the second ring with a *n*-pentyl side chain favoured the TPβ activity while some compounds with no substituents in these positions showed a high affinity and activity on TPα [13]. Consequently, we wished to compare methyl group with several other substituents. To this end, we designed and prepared compounds which were characterized by an halogen atom instead of the methyl group. Thus, monosubstituted derivatives with a chlorine, bromine and iodine atom in the 2-(except for iodine series), 3- and 4-position were synthesized (**9k-9z**, scheme 2). For each compound, two alkyl side chains R_2 were envisaged: *t*-butyl and *n*-pentyl, in order to collect information on the influence of a long linear or short globular chain in this series. Table 2 shows that the replacement of a methyl group by a bromine atom had little or no positive effect, both in terms of potency and selectivity. It is interesting to note that compound **9m** (3-bromophenyl) had a ~2 fold decrease of selectivity ratio compared with its potent methylated analog **9h** (11.6 and 23.2, respectively). Consistent with our previous observation on the activity trend, **9m**, with a substituent in

3-position and a *t*-butyl R_2 alkyl chain, was the most potent compound of its series. Compounds of the 4-bromophenyl series were less potent than their methyl counterparts, but kept the same rank order of selectivity ratio. In the chlorophenyl series, the same trends were observed, with little differences compared to the bromophenyl compounds series. Once again, the 3-position substitution of phenyl ring combined with a *t*-butyl side chain produced the most potent compound, **9s**, both on TPα and TPβ (table 2). Interestingly, in the iodophenyl series, although compounds potencies were in the same range as the other halo-substituted compounds, selectivity ratio was diminished. To this extent, compound **9w** was the most representative since its 3-position substitution along with a *t*-butyl side chain R_2 increased its TPα potency while decreasing its TPβ activity (table 2). Thus, **9w** was the most potent TPα halo-substituted antagonist in this table with a potency close to the one of **9h** (TPα IC_{50} 470 ± 153 nM and 398 ± 145 nM, respectively). From this table (Table 2), we can presume that the best combination for our derivatives is a substituent at the 3-position of the phenyl ring R_1 and a *t*-butyl side chain R_2. Following trends have been observed within these series: TPα activity: $CH_3 \cong I > Cl \cong Br$; TPβ activity: $CH_3 > Br \cong Cl > I$; selectivity toward TPβ isoform: $CH_3 > Br \cong Cl > I$. Additionally, it is interesting to point out that within bromo and chloro derivatives, the combination of a 4-substituted phenyl moiety with a *n*-pentyl side chain produced the most selective compounds whereas, surprisingly, in the 4-iodo series, compounds were less selective. In the series with a 3-substituted phenyl moiety (bromo, chloro and iodo), a *t*-butyl side chain produced a ~10 fold increase in activity compared to compounds with *n*-pentyl side chain (table 2).

We subsequently aimed to explore the influence of longer substituents at the 4-position of the second aromatic ring R_1. Consequently, several derivatives with a 4-alkoxyphenyl moiety as R_1 were prepared (**9aa-9af**, scheme 2). Moreover, some disubstituted compounds were synthesized (**9ag-9aj**). The R_2 alkyl chain in this series was either a *t*-butyl or a *n*-pentyl moiety. We observed a systematically important loss of activity on both isoforms for compounds with alkoxy groups at the 4-position of the phenyl ring R_1 compared with the parent methylated compounds (**2** and **3**). For both side chains (R_2 = *t*-butyl or *n*-pentyl), the loss of activity depended on the length of the alkoxy group. Interestingly, the activity on TPβ decreased less markedly than activity on TPα, thus increasing the selectivity ratio toward the TPβ isoform (4-ethoxyphenyl, **9ae** and **9af** > 4-methoxyphenyl, **9aa** ≅ 4-propoxyphenyl, **9ac**). The most interesting compound of this table (**9ag**) was obtained with the combination of a methoxy group in the 3-position of the phenyl ring R_1 and the R_2 side chain being a *t*-butyl moiety. Fully consistent with observations in other series, this compound displayed a potent antagonism of TPβ mediated $[Ca^{2+}]_i$ mobilization combined with a good selectivity ratio (19.9). The particular interest of this

compound is that, although slightly less potent than its methylated or halogenated analogs (**9h**, **9m**, **9s** or **9w**) on TPβ (IC_{50} = ~99 nM), it is characterized by a much lower activity on TPα (IC_{50} = ~2 μM). Disubsituted compounds **9ah** and **9ai** proved to have a combined profile of monosubstituted 4-chlorophenyl and 3-methylphenyl analogs (**9u-9v** and **9h-9a**, respectively). Thus, the presence of a methyl group at the 3-position of compound **9u** increased ~3-4 fold its activity, without reaching the potent activity of the solely 3-methyl-substituted **9h** (table 3 and 1, respectively). The presence of a 2-methoxy group concomitant with a 4-methyl group on the phenyl ring had little influence on TPβ activity but decreased ~10 fold the TPα activity compared to **2** (Table 3 and 1, respectively). Since cyanoguanidines proved to be quite active at both isoforms in other series [13], we synthesized an oxygen bonded cyanoguanidine, **10** (scheme 3). This replacement of sulfonylurea had a negative impact on the activity and generated a minor selectivity ratio (Table 4).

Finally, within this "oxygen bridged" family of chemical compounds, we wished to confirm the antiplatelet activity of the most potent compounds. Selection relied on their activity on TPα, TPβ or the importance of their selectivity ratio. **9h** was the first to be evaluated because it displayed the optimal structure for both activity and selectivity among the compounds presented herein. The IC_{50} of compound **9h** for inhibition of U46619-induced platelet aggregation was found to be similar to the one for the inhibition of $[Ca^{2+}]_i$ mobilization triggered by TPα isoform (513 ± 28 nM and 398 ± 145 nM, respectively). Concentration-response curve obtained with this compound on platelets can overlay with the TPα curve (Figure 2, panels A and B). These results confirmed the theory postulating that platelet aggregation is solely mediated by TPα isoform. It is noteworthy that **9h** is inactive or almost inactive on TPα (expressed in platelets and in HEK.TPα cells) when tested at 0.1μM, although is has complete antagonistic activity on TPβ (expressed in HEK.TPβ cells) at this concentration (Figure 2, panel A). Thus, this compound is theoretically selective for TPβ at this concentration.

We have conducted similar experiments on platelets with other potentially selective compounds. Thus, **9af** displayed a similar profile with comparable IC_{50}s for inhibition of U46619-induced platelet aggregation and $[Ca^{2+}]_i$ mobilization. This compound had nevertheless its curves strongly shifted to the right in inhibition of $[Ca^{2+}]_i$ mobilization (Figure 3, panel A) and was thus less active on platelets (IC_{50} : 40 ± 9 μM, figure 3, panel B). Consequently, with a selectivity ratio within the same rank order, it could be more interesting since it is almost inactive on platelets at concentrations below 10μM but keeps a good activity on TPβ isoform expressed in HEK293 cell lines. Similarly, compound **9ag** was one of the most interesting compounds, since it displayed one of the best activity on TPβ, while being poorly active on platelets (IC_{50} : 985 ± 49 nM).

Conclusions

TXA_2 exerts its pro-aggregatory and constrictive actions upon binding to specific GPCR named TP. In humans, two isoforms (TPα and TPβ) of this receptor have been described that differ exclusively in their C-carboxyl terminal tail domains. Herein, we present the design, synthesis and SAR study of a series of 35 original N-alkyl-N'-[2-(aryloxy)-5-nitrobenzenesulfonyl]-ureas and -cyanoguanidine. These compounds were designed based on a previous report by our group that highlighted this family as potentially interesting in terms of antagonistic activity as well as selectivity toward the individual TP isoforms. Results obtained in an inhibition of $[Ca^{2+}]_i$ mobilization test performed on both TPα and TPβ isoforms allowed us to define the optimal nature and position of several structural moieties for both activity and selectivity. The study represents the first extended SAR study dealing with selectivity among TP receptor isoforms. Three compounds were selected for their selectivity and activity and confirmed their TPα antagonistic potency in a human platelet aggregation test.

Acknowledgments

Julien Hanson is funded by the "Fonds pour la Formation à la Recherche dans l'Industrie et dans l'Agriculture" (F.R.I.A., from Belgium). This work was supported by a grant from the « Fonds National de la Recherche Scientifique » (FNRS) from Belgium. The authors wish to thank M. Philippe Neven for excellent technical assistance. B.T. Kinsella acknowledges the financial support of the Wellcome Trust and Science Foundation Ireland.

Supporting information available : Elemental analysis

Experimental section

Chemistry. All commercial chemicals (Sigma-Aldrich, Belgium) and solvents are reagent grade and were used without further purification unless otherwise stated. Compounds **1**, **7**[16], **2**, **3**, **4**, **8a-b and 9a-b** [13] were synthesized as previously described. 2-(2-Methylphenoxy)-5-nitrobenzenesulfonamide (**8c**) was commercially available (*AmbinterStock Screening Collection*). Nevertheless, this compound was synthesized in our lab according to the method of preparation described below. All reactions were followed by thin-layer chromatography (Silicagel $60F_{254}$ Merck®) and visualization was accomplished with UV light (254 nm). Elemental analyses (C, H, N, S) were determined on a Carbo Erba EA 1108 and were within ± 0.4% of the theoretical values. NMR spectra were recorded on a Bruker Avance 500 spectrometer using DMSO-*d*6 or $CDCl_3$ as solvent and tetramethylsilane as internal standard. For ^{1}H NMR spectra, chemical shifts are expressed in δ (ppm) downfield from tetramethylsilane. The abbreviations s, singulet; d, doublet; t, triplet; m, multiplet; br, broad were used throughout. Infrared spectra were recorded using a Perkin-Elmer® FT-IR 1750. All compounds described were re-crystallized from hot methanol (40°C)/H_2O mixture (60/40; 10ml/100mg of product) unless otherwise stated.

General procedure for the reaction of 5 with phenols

Before starting the reaction, the phenolates were obtained from the corresponding cresols (0.05 mol) after their neutralization by 0.06 mol NaOH (in aqueous solution, 10 % w/v) in acetone. Evaporation under reduced pressure provided crystals of the phenolates.

Compound **5** (0.01 mol) and the appropriate phenolate (0.05 mol) were dissolved in acetonitrile. The mixture was refluxed and potassium carbonate (0.007 mol) was added. After completion of the reaction monitored by TLC (12-36 h), the solution was acidified by means of hydrochloric acid solution (10M), filtered and the filtrate was evaporated under reduced pressure. The crude product was dissolved in methanol and ice was added. The resulting precipitate was collected by filtration. (Yield : 60-70%).

General procedure for the preparation of sulfonylureas with isocyanates

The appropriate sulfonamide (0.01 mol) was dissolved in acetone (30 ml) and 0.01 mol NaOH (10% aqueous sol. w/v) was added. The mixture was gently mixed during 10 minutes and then was evaporated under reduced pressure. The resulting solid was resuspended in acetone (30 ml) and gently refluxed. The appropriate isocyanate (0.02 mol) was added to the mixture. At the end of the reaction (0.1-1 hr), the mixture was evaporated under reduced pressure and the crude product was washed with AcOEt. The solid was collected by filtration and

dissolved in an aqueous NaOH solution (0.5 N; 20 ml). The resulting solution was adjusted to pH=1 with hydrochloric acid (12N) and the solid which precipitated was isolated by filtration (Yield : 40-60%).

Procedure for the preparation of N-*tert*-butyl-N'-cyano-O-phenylisourea

A mixture of **10** (0.01 mol) and *tert*-butylamine (0.015 mol) in isopropanol (30ml) was stirred at room temperature for 10-15 minutes. The solution was evaporated and the crude oil was crystallized in cold methanol.

General procedure for the preparation of nitrobenzenesulfonylcyanoguanidines

The appropriate sulfonamide (0.01 mol) was dissolved in acetone (30ml). 0.01 mol NaOH (10% aqueous sol. w/v) was added. The mixture was gently mixed during 10 minutes and was evaporated under reduced pressure. The solid was resuspended in dimethylformamide (20ml) at room temperature and the appropriate N-alkyl-N'-cyano-O-phenylisourea was added. The mixture was stirred at RT for 20-24H. At the end of the reaction, the mixture was evaporated under reduced pressure and the crude oil was suspended in a mixture of methanol and hydrochloric acid (5N aqueous solution) from which crystals of the desired product appeared.

Calcium measurements

HEK.TPα and HEK.TPβ cell lines, stably over-expressing HA-tagged forms of TPα and TPβ in human embryonic kidney (HEK) 293 cells have been previously described [8]. HEK 293 cells or their stable cell line equivalents were routinely grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). Measurement of $[Ca^{2+}]i$ mobilization either in HEK.TPα or HEK.TPβ cells was carried out using fluorescent microplate reader Fluoroskan Ascent FL equipped with two dispenser (thermo electron corporation, Finland) according to modified method of Lin *et al.* [17]. Briefly, cells were trypsinized and washed twice with Krebs-HEPES buffer (118 mM, NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 11.7 mM D-Glucose, 1.3 mM $CaCl_2$, 10 mM HEPES, pH 7.4), and incubated for one hour with fluorescent dye Fluo-4/AM (5 µg/ml; Molecular Probes, Invitrogen, Merelbeke Belgium). Cells were then rinsed three times with Krebs-HEPES buffer and 150 µl of a suspension of cells in that buffer was loaded into each well of a 96-well plate at a density of 150,000 cells/well. Cells were incubated 10 min with various concentrations of the test compound (10^{-5} to 10^{-8} M final; 10 µl) prior to stimulation with U46619 (1 µM final, 50 µl). In all cases, compound (1 mM) was diluted in dimethylsulfoxide (DMSO)/PBS (30/70) prior to further dilution in PBS. Fluorescence emission was read at 538 nM. At the end of each experiment, fluorescence intensities were calibrated for determination of intracellular calcium concentration ($[Ca^{2+}]i$) values by permeabilizing cells with 1% Triton X-100 to release all the dye (Fmax) and subsequently chelating with 10 mM EGTA (Fmin). Calcium concentrations were calculated using equation $[Ca^{2+}]_i = Kd(F - F_{min})/(Fmax - F)$, assuming a Kd of 385 nM for

Fluo-4. The results (IC_{50}) presented are the concentration required to inhibit 50% of the normal rise of $[Ca^{2+}]_i$ upon stimulation with 1 µM U46619, determined in the absence of any compounds. The IC_{50} were calculated by non-linear regression analysis (GraphPad Prism software) from at least three concentration-response curves.

Human *ex vivo* platelet aggregation

The anti-aggregatory potency has been determined according to the turbidimetric Born's method [18]. The blood was drawn from ten healthy donors of both genders, aged 20-30. The subjects were free from medication for at least 14 days. No significant differences in the results were observed between the donors in our experiments. Platelet-rich plasma (PRP) and platelet-poor plasma (PPP) were prepared as previously described [13]. Platelet concentration of PRP was adjusted to 3.10^8 cells/ml by dilution with PPP. Platelet aggregation of PRP was studied using a double channel aggregometer (Chronolog Corporation, Chicago, IL). PRP (294 µl) was added in a silanised cuvette and stirred (1000 rpm). Each drug was diluted (1 mM) in dimethylsulfoxide (DMSO)/PBS (30/70) and preincubated in PRP for three minutes at 37°C before the aggregating agent was added. Platelet aggregation was initiated by addition of a fresh solution of U46619 (1 µM final). To evaluate platelet aggregation, the maximum increase in light transmission was determined from the aggregation curve 6 minutes after addition of the inducer. The drug concentration preventing 50% of platelet aggregation (IC_{50}) induced by arachidonic acid and U46619 was calculated by non-linear regression analysis (GraphPad Prism software) from at least three dose-response curves.

References

(1) Hamberg, M.; Svensson, J.; Samuelsson, B. Thromboxanes: a new group of biologically active compounds derived from prostaglandin endoperoxides. *Proc Natl Acad Sci U S A* **1975**, *72*, 2994-2998.

(2) Moncada, S.; Ferreira, S. H.; Vane, J. R. Bioassay of prostaglandins and biologically active substances derived from arachidonic acid. *Adv Prostaglandin Thromboxane Res* **1978**, *5*, 211-236.

(3) Dogne, J. M.; de Leval, X.; Hanson, J.; Frederich, M.; Lambermont, B. et al. New developments on thromboxane and prostacyclin modulators part I: thromboxane modulators. *Curr Med Chem* **2004**, *11*, 1223-1241.

(4) Coleman, R. A.; Smith, W. L.; Narumiya, S. International Union of Pharmacology classification of prostanoid receptors: properties, distribution, and structure of the receptors and their subtypes. *Pharmacol Rev* **1994**, *46*, 205-229.

(5) Hirata, M.; Hayashi, Y.; Ushikubi, F.; Yokota, Y.; Kageyama, R. et al. Cloning and expression of cDNA for a human thromboxane A2 receptor. *Nature* **1991**, *349*, 617-620.

(6) Raychowdhury, M. K.; Yukawa, M.; Collins, L. J.; McGrail, S. H.; Kent, K. C. et al. Alternative splicing produces a divergent cytoplasmic tail in the human endothelial thromboxane A2 receptor. *J Biol Chem* **1994**, *269*, 19256-19261.

(7) Reid, H. M.; Kinsella, B. T. The alpha, but not the beta, isoform of the human thromboxane A2 receptor is a target for nitric oxide-mediated desensitization. Independent modulation of Tp alpha signaling by nitric oxide and prostacyclin. *J Biol Chem* **2003**, *278*, 51190-51202.

(8) Walsh, M. T.; Foley, J. F.; Kinsella, B. T. The alpha, but not the beta, isoform of the human thromboxane A2 receptor is a target for prostacyclin-mediated desensitization. *J Biol Chem* **2000**, *275*, 20412-20423.

(9) Kinsella, B. T. Thromboxane A2 signalling in humans: a 'Tail' of two receptors. *Biochem Soc Trans* **2001**, *29*, 641-654.

(10) Ashton, A. W.; Ware, J. A. Thromboxane A2 receptor signaling inhibits vascular endothelial growth factor-induced endothelial cell differentiation and migration. *Circ Res* **2004**, *95*, 372-379.

(11) Habib, A.; FitzGerald, G. A.; Maclouf, J. Phosphorylation of the thromboxane receptor alpha, the predominant isoform expressed in human platelets. *J Biol Chem* **1999**, *274*, 2645-2651.

(12) Halushka, P. V. Thromboxane A(2) receptors: where have you gone? *Prostaglandins Other Lipid Mediat* **2000**, *60*, 175-189.

(13) Hanson, J.; Reynaud, D.; Qiao, N.; Devel, P.; Moray, A. L. et al. Synthesis and pharmacological evaluation of novel nitrobenzenic thromboxane modulators as antiplatelet agents acting on both the alpha and beta isoforms of the human thromboxane receptor. *J Med Chem* **2006**, *49*, 3701-3709.

(14) Dogne, J. M.; Hanson, J.; de Leval, X.; Kolh, P.; Tchana-Sato, V. et al. Pharmacological characterization of N-tert-butyl-N'-[2-(4'-methylphenylamino)-5-nitrobenzenesulfonyl]urea (BM-573), a novel thromboxane A2 receptor antagonist and thromboxane synthase inhibitor in a rat model of arterial thrombosis and its effects on bleeding time. *J Pharmacol Exp Ther* **2004**, *309*, 498-505.

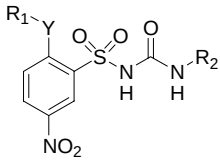
(15) Meerwein, H.; Dittmar, G.; Gollner, R.; Hafner, K.; Mensch, F. et al. Aromatic diazo compounds. II. Preparation of aromatic sulfonyl chlorides, a new modification of the Sandmeyer reaction. . *Chemische Berichte* **1957**, *90*, 841-852.

(16) Dogne, J. M.; Wouters, J.; Rolin, S.; Michaux, C.; Pochet, L. et al. Design, synthesis and biological evaluation of a sulfonylcyanoguanidine as thromboxane A2 receptor antagonist and thromboxane synthase inhibitor. *J Pharm Pharmacol* **2001**, *53*, 669-680.

(17) Lin, K.; Sadee, W.; Quillan, J. M. Rapid measurements of intracellular calcium using a fluorescence plate reader. *Biotechniques* **1999**, *26*, 318-322, 324-316.

(18) Born, G. V.; Cross, M. J. The Aggregation of Blood Platelets. *J Physiol* **1963**, *168*, 178-195.

Table 1. Estimated IC_{50} values for the inhibition of $[Ca^{2+}]_i$ mobilization mediated by either TPα or TPβ upon stimulation by U46619 (1μM). First series : influence of the position of R_1 methyl substituent and of the side chain.



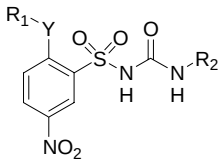
cpds	R_1	R_2	Y	Inhibition of U46619-mediated $[Ca^{2+}]_i$ mobilization (IC_{50}, nM)[1]		Ratio[2]
				TPα	TPβ	
1	4-methylphenyl	*t*-butyl	NH	319 ± 203	53 ± 19	6.01
2[3]	4-methylphenyl	*t*-butyl	O	58 ± 43	58 ± 4	1.0
3[3]	4-methylphenyl	*n*-pentyl	O	139 ± 88	55 ± 5	2.5
4[3]	4-methylphenyl	*n*-butyl	O	558 ± 34	53 ± 2	10.5
9a	3-methylphenyl	*n*-pentyl	O	2530 ± 419	223 ± 68	11.3
9b	2-methylphenyl	*n*-pentyl	O	1630 ± 924	181 ± 101	9.0
9c	2-methylphenyl	*i*-propyl	O	8750 ± 446	501 ± 63	17.5
9d	2-methylphenyl	*n*-butyl	O	673 ± 339	42 ± 20	16.1
9e	2-methylphenyl	*t*-butyl	O	3760 ± 637	233 ± 88	16.1
9f	3-methylphenyl	*i*-propyl	O	2640 ± 492	205 ± 132	12.9
9g	3-methylphenyl	*n*-butyl	O	1910 ± 689	155 ± 30	12.3
9h	3-methylphenyl	*t*-butyl	O	398 ± 145	17 ± 8	23.2
9i	4-methylphenyl	*i*-propyl	O	8760 ± 3780	843 ± 124	10.4
9j	4-methylphenyl	*n*-propyl	O	4220 ± 2060	448 ± 328	9.4

[1] Results are expressed as mean ± SD of three separate experiments

[2] IC_{50}TPα/IC_{50}TPβ

[3] Data already published [13]

Table 2. Estimated IC_{50} values for the inhibition of $[Ca^{2+}]_i$ mobilization mediated by either TPα or TPβ upon stimulation by U46619 (1μM). Second series : influence of nature and position of R_1 phenyl ring substituents.

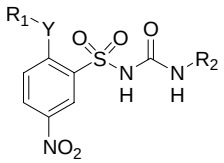


cpds	R_1	R_2	Y	Inhibition of U46619-mediated $[Ca^{2+}]_i$ mobilization (IC_{50}, nM)[1]		Ratio[2]
				TPα	TPβ	
1	4-methylphenyl	*t*-butyl	NH	319 ± 203	53 ± 19	6.01
9k	2-bromophenyl	*t*-butyl	O	2580 ± 1120	151 ± 73	17.0
9l	2-bromophenyl	*n*-pentyl	O	3130 ± 827	423 ± 164	7.4
9m	3-bromophenyl	*t*-butyl	O	683 ± 189	59 ± 30	11.6
9n	3-bromophenyl	*n*-pentyl	O	5860 ± 2420	413 ± 303	14.2
9o	4-bromophenyl	*t*-butyl	O	1660 ± 1290	523 ± 145	3.2
9p	4-bromophenyl	*n*-pentyl	O	1010 ± 257	120 ± 63	8.4
9q	2-chlorophenyl	*t*-butyl	O	2420 ± 1780	205 ± 156	11.8
9r	2-chlorophenyl	*n*-pentyl	O	3060 ± 775	387 ± 178	7.9
9s	3-chlorophenyl	*t*-butyl	O	698 ± 226	73 ± 9	9.6
9t	3-chlorophenyl	*n*-pentyl	O	2390 ± 526	201 ± 42	11.9
9u	4-chlorophenyl	*t*-butyl	O	3050 ± 1670	400 ± 277	7.6
9v	4-chlorophenyl	*n*-pentyl	O	871 ± 362	98 ± 45	8.9
9w	3-iodophenyl	*t*-butyl	O	470 ± 153	95 ± 35	4.9
9x	3-iodophenyl	*n*-pentyl	O	4520 ± 1800	885 ± 389	5.1
9y	4-iodophenyl	*t*-butyl	O	2150 ± 307	401 ± 212	5.4
9z	4-iodophenyl	*n*-pentyl	O	2190 ± 1180	302 ± 153	7.2

[1] Results are expressed as mean ± SD of three separate experiments

[2] $IC_{50}TP\alpha/IC_{50}TP\beta$

Table 3. Estimated IC_{50} values for the inhibition of [Ca2+]i mobilization mediated by either TPα or TPβ upon stimulation by U46619 (1µM). Third series : influence of large substituent at position 4 of R_1 aromatic ring and polysubstitution of R_1.

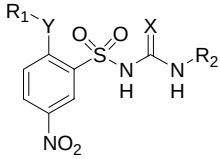


cpds	R_1	R_2	Y	Inhibition of U46619-mediated $[Ca^{2+}]_i$ mobilization (IC_{50}, nM)[1]		Ratio[2]
				TPα	TPβ	
1	4-methylphenyl	*t*-butyl	NH	319 ± 203	53 ± 19	6.01
9aa	4-methoxyphenyl	*t*-butyl	O	1940 ± 343	152 ± 76	12.7
9ab	4-methoxyphenyl	*n*-pentyl	O	2670 ± 1970	446 ± 416	6.0
9ac	4-propoxyphenyl	*t*-butyl	O	10760 ± 5150	875 ± 238	12.3
9ad	4-propoxyphenyl	*n*-pentyl	O	27230 ± 2650	4840 ± 2520	5.6
9ae	4-ethoxyphenyl	*t*-butyl	O	19190 ± 11410	1100 ± 401	17.5
9af	4-ethoxyphenyl	*n*-pentyl	O	13560 ± 3440	749 ± 59	18.1
9ag	3-methoxyphenyl	*t*-butyl	O	1970 ± 622	99 ± 61	19.9
9ah	3-methyl-4-chlorophenyl	*t*-butyl	O	1060 ± 207	111 ± 54	9.6
9ai	3-methyl-4-chlorophenyl	*n*-pentyl	O	3720 ± 769	565 ± 200	6.6
9aj	2-methoxy-4-methylphenyl	*t*-butyl	O	528 ± 133	52 ± 10	10.2

[1] Results are expressed as mean ± SD of three separate experiments

[2] IC_{50}TPα/IC_{50}TPβ

Table 4. Estimated IC_{50} values for the inhibition of $[Ca^{2+}]_i$ mobilization mediated by either TPα or TPβ upon stimulation by U46619 (1µM). Fourth series : impact of the sulfonylurea function.



cpds	R_1	R_2	Y	X	Inhibition of U46619-mediated $[Ca^{2+}]_i$ mobilization (IC_{50}, nM)[1]		Ratio[2]
					TPα	TPβ	
1	4-methylphenyl	*t*-butyl	NH	O	319 ± 203	53 ± 19	6.01
13c	4-methylphenyl	*t*-butyl	O	N-CN	2080 ± 179	579 ± 53	3.6

[1] Results are expressed as mean ± SD of three separate experiments

[2] $IC_{50}TP\alpha/IC_{50}TP\beta$

Figure Legends :

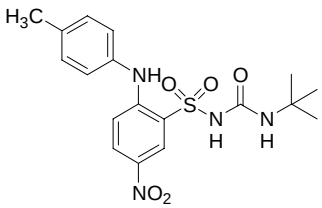
Figure 1. Chemical structures of 1 (BM573), 2, 3 and 4.

Figure 2. A) Concentration-response curves for inhibition of $[Ca^{2+}]_i$ mobilization by 9h on separate isoforms. B) Concentration response curve for inhibition of platelet aggregation by 9h

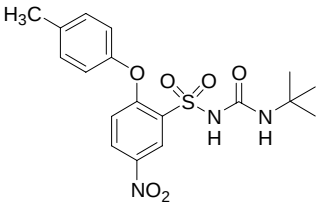
Figure 3. A) Concentration-response curves for inhibition of $[Ca^{2+}]_i$ mobilization by 9af on separate isoforms. B) Concentration response curve for inhibition of platelet aggregation by 9af

Figure 4. A) Concentration-response curves for inhibition of $[Ca^{2+}]_i$ mobilization by 9ag on separate isoforms. B) Concentration response curve for inhibition of platelet aggregation by 9ag

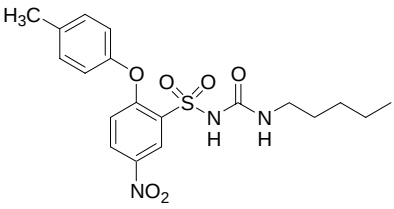
Figure 1



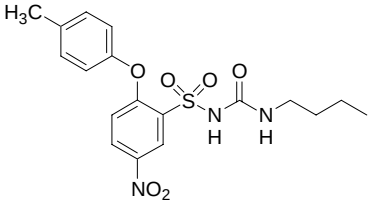
1 (BM573)



2



3



4

Figure 2

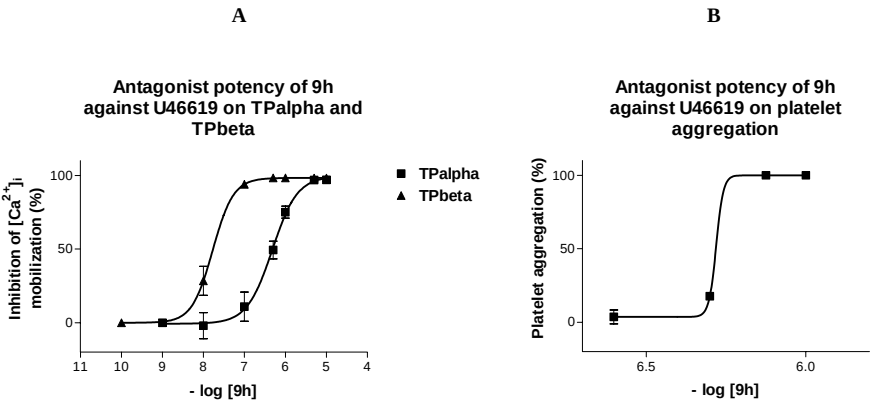

A
B
Antagonist potency of 9h against U46619 on TPalpha and TPbeta
Inhibition of [Ca2+]i mobilization (%)
100
50
0
11
10
9
8
7
6
5
4
- log [9h]
TPalpha
TPbeta
Antagonist potency of 9h against U46619 on platelet aggregation
Platelet aggregation (%)
100
50
0
6.5
6.0
- log [9h]

Figure 3

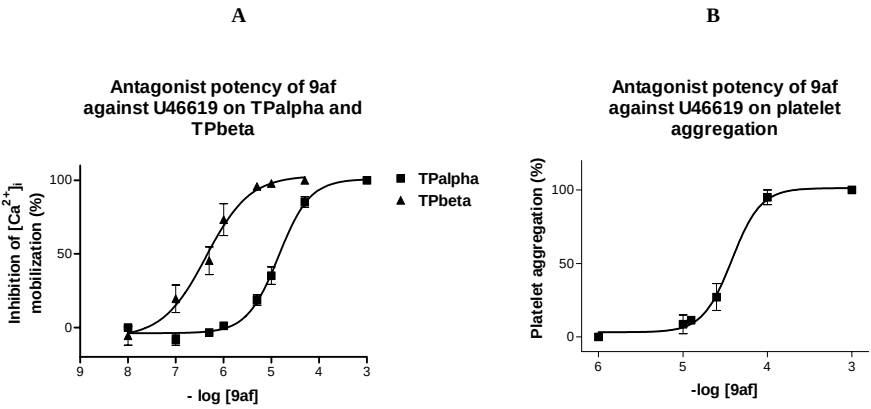
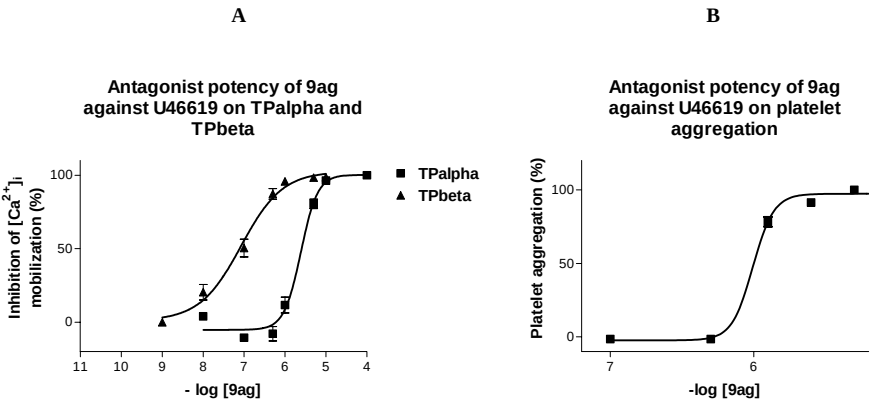
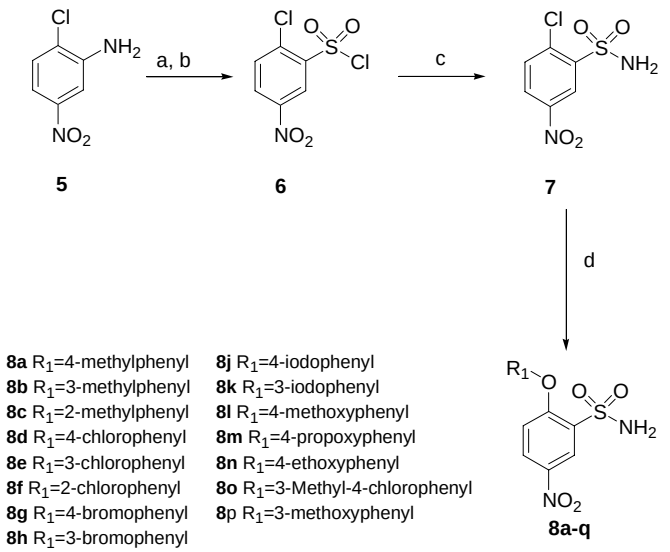

A
B
Antagonist potency of 9af against U46619 on TPalpha and TPbeta
Inhibition of [Ca2+]i mobilization (%)
100
50
0
9
8
7
6
5
4
3
- log [9af]
TPalpha
TPbeta
Antagonist potency of 9af against U46619 on platelet aggregation
Platelet aggregation (%)
100
50
0
6
5
4
3
-log [9af]

Figure 4


A
B
Antagonist potency of 9ag against U46619 on TPalpha and TPbeta
Inhibition of [Ca2+]i mobilization (%)
100
50
0
11
10
9
8
7
6
5
4
- log [9ag]
TPalpha
TPbeta
Antagonist potency of 9ag against U46619 on platelet aggregation
Platelet aggregation (%)
100
50
0
7
6
-log [9ag]

Scheme 1

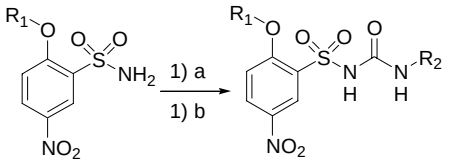


5 **6** **7** **8a-q**

8a R_1=4-methylphenyl
8b R_1=3-methylphenyl
8c R_1=2-methylphenyl
8d R_1=4-chlorophenyl
8e R_1=3-chlorophenyl
8f R_1=2-chlorophenyl
8g R_1=4-bromophenyl
8h R_1=3-bromophenyl
8i R_1=2-bromophenyl
8j R_1=4-iodophenyl
8k R_1=3-iodophenyl
8l R_1=4-methoxyphenyl
8m R_1=4-propoxyphenyl
8n R_1=4-ethoxyphenyl
8o R_1=3-Methyl-4-chlorophenyl
8p R_1=3-methoxyphenyl

Reagents: (a) $NaNO_2$, HCl; (b) SO_2, HOAc, Cu_2Cl_2 ; (c) NH_4OH, Δ ; (d) appropriate phenol

Scheme 2

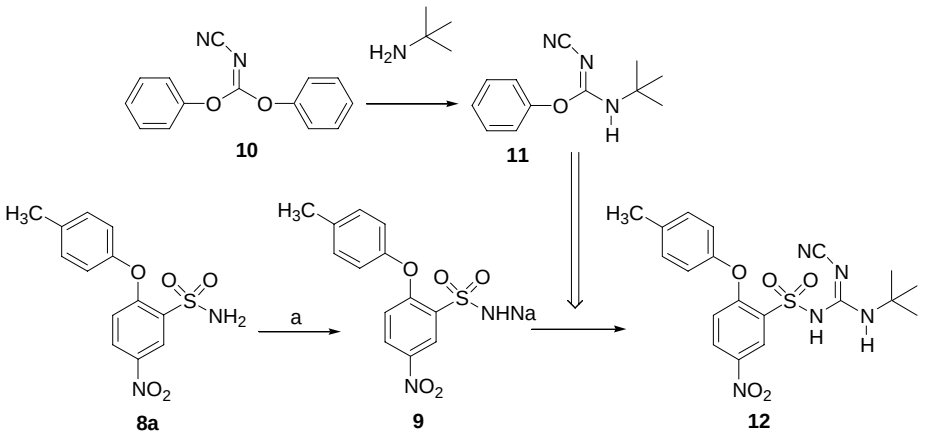


8 **9a-ao**

9a R1=3-methylphenyl, R2=*n*-pentyl
9b R1=2-methylphenyl, R2=*n*-pentyl
9c R1=2-methylphenyl, R2=*i*-propyl
9d R1=2-methylphenyl, R2=*n*-butyl
9e R1=2-methylphenyl, R2=*t*-butyl
9f R1=3-methylphenyl, R2=*i*-propyl
9g R1=3-methylphenyl, R2=*n*-butyl
9h R1=3-methylphenyl, R2=*t*-butyl
9i R1=4-methylphenyl, R2=*i*-propyl
9j R1=4-methylphenyl, R2=*n*-propyl
9k R1=2-bromophenyl, R2=*t*-butyl
9l R1=2-bromophenyl, R2=*n*-pentyl
9m R1=3-bromophenyl, R2=*t*-butyl
9n R1=3-bromophenyl, R2=*n*-pentyl
9o R1=4-bromophenyl, R2=*t*-butyl
9p R1=4-bromophenyl, R2=*n*-pentyl
9q R1=2-chlorophenyl, R2=*t*-butylamino
9r R1=2-chlorophenyl, R2=*n*-pentyl
9s R1=3-chlorophenyl, R2=*t*-butyl
9t R1=3-chlorophenyl, R2=*n*-pentyl
9u R1=4-chlorophenyl, R2=*t*-butyl
9v R1=4-chlorophenyl, R2=*n*-pentyl
9w R1=3-iodophenyl, R2=*t*-butyl
9x R1=3-iodophenyl, R2=*n*-pentyl
9y R1=4-iodophenyl, R2=*t*-butyl
9z R1=4-iodophenyl, R2=*n*-pentyl
9aa R1=4-methoxyphenyl, R2=*t*-butyl
9ab R1=4-methoxyphenyl, R2=*n*-pentyl
9ac R1=4-propoxyphenyl, R2=*t*-butyl
9ad R1=4-propoxyphenyl, R2=*n*-pentyl
9ae R1=4-ethoxyphenyl, R2=*t*-butyl
9af R1=4-ethoxyphenyl, R2=*n*-pentyl
9ag R1=3-methoxyphenyl, R2=*t*-butyl
9ah R1=3-methyl-4-chlorophenyl, R2=*t*-butyl
9ai R1=3-methyl-4-chlorophenyl, R2=*n*-pentyl
9aj R1=2-methoxy-4-methylphenyl, R2=*n*-pentyl

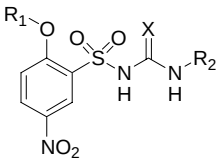
Reagents: (a) NaOH 10% ; (b) R_2-N=C=O.

Scheme 3



Reagents : (a) NaOH 10%.

Table of Contents Graphic



R_1=methylphenyl, halophenyl, alkoxyphenyl
R_2=*t*-butyl, *n*-pentyl, *i*-propyl, *n*-butyl
X=O, N-CN