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P2 Receptors and Other, New Targets for Antithrombotic Drugs

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September 16-18, 2004, Castelvecchio Pascoli, Italy

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haematologica

The origin and power of a name

Ancient Greek

αιμα [aima] = blood;
αιματος [aimatos] = of blood,
λογος [logos]= reasoning

Scientific Latin

haematologicus (adjective) = related to blood

Scientific Latin

haematologica (adjective, plural and neuter, used as a noun) = hematological subjects

Modern English

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P2 RECEPTORS

P2 RECEPTORS IN THE CARDIOVASCULAR SYSTEM

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P2 receptors mediate the actions of extracellular nucleotides such as ATP, ADP, UTP, UDP, diadenosine polyphosphates (ApnA) or UDP-glucose. They regulate or participate to all the physiological functions of the cardiovascular system, including heart contractility and cardiac frequency, vascular tone, release of endothelial factors, angiogenesis, smooth muscle cells proliferation, haemostasis and immunity. Seven P2X ($P2X_{1-7}$) and eight P2Y ($P2Y_{1,2,4,6,11,12,13,14}$) subtypes have been identified so far. All of them have been found to be expressed in the cardiovascular system, i.e. in the heart, the vasculature, including smooth muscle cells and endothelial cells or in blood cells (*Kunapuli & Daniel, 1998; Boarder & Hourani, 1998; Di Virgilio et al., 2001; Wang et al., 2002*). However, although much is known about the effects of nucleotides in the various tissues and cells, the characterization of the P2 receptor subtypes involved is very difficult. This is due to several reasons: i) the field still lacks pharmacological tools such as selective agonists or antagonists although progress has been made; ii) most of the antibodies against the P2Y receptors used so far are not specific and poorly characterized; iii) RT-PCR has mostly been used to characterize the distribution pattern of the P2 receptors and it is well known that most often it is difficult to rely on this sole technique. Nevertheless, studies using combined techniques, selective antagonists and/or P2 receptor deficient mice allow now to draw a picture of the role of the P2 receptors in several aspects of the cardiovascular system. The earliest papers on the role of adenine compounds by Drury & Szent-Gyorgy in 1929 reported on their effect on the mammalian heart. P2 receptors are involved in cardiac function both indirectly via blood vessels and nerves and directly, via the cardiomyocytes. Here especially, much is to be done to clearly assign the effects of extracellular nucleotides to P2 receptor subtypes. These have been reported to be positive and negative inotropic effects as well as positive and negative chronotropic effects. The nucleotides also play a role in ischemic preconditioning. All known receptor subtypes have been found, by RT-PCR, in the cardiomyocytes (*Vassort, 2001*). The cardiac function and vascular tone were evaluated in $P2Y_1$ and in $P2X_1$ receptor deficient mice without detectable differences with the corresponding wild-type mice (*Gachet, unpublished work*). These data do not mean that these receptors are not involved in these functions but clearly either they only participate in the regulation of these functions or the deficient mice have compensated the receptor deficiency. Studies on isolated tissues from these mice should help to better characterize the individual role of these receptors. More is known concerning the vascular function and one has to subdivide it in endothelial function and smooth muscle cell function. In the endothelium, $P2Y_1$, $P2Y_2$ and possibly $P2Y_4$ and $P2Y_6$ are responsible for nucleotide-induced vasodilation through release of nitric oxide and prostacyclin (*Ralevic & Burnstock 1998, Kunapuli & Daniel 1998; Boarder & Hourani 1998; Kaiser & Buxton 2002*). In smooth muscle cells, $P2X_1$ and perhaps $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors are responsible for vasoconstriction. $P2Y_{12}$ receptors have been found to be expressed in rat capillary endothelial cells (Simon et al., 2002) while other found it on smooth muscle cells where it is also supposed to trigger vasoconstriction (*Wihlborg et al., 2004*). Vascular tone is also regulated by P2 receptors expressed at the perivascular nerve termini where ATP is coreleased with noradrenalin and neuropeptide Y (*Ralevic & Burnstock 1998*). In addition to their short term effects on vascular tone, nucleotides and P2 receptors are also involved in long term trophic effects on cell growth, proliferation and death which as great implications for diseases such as atherosclerosis and restenosis (*Burnstock 2002; Di Virgilio & Solini 2002*).

P2 receptors have also been shown to be expressed in all blood cells including platelets, monocytes, granulocytes, dendritic cells, red blood cells and platelets. ATP and ADP are major agonists for platelets where they act on $P2X_1$, $P2Y_1$ and $P2Y_{12}$ receptors in a coordinated fashion (*Gachet, 2001;*

Mahaut-Smith 2004). Several cell lines including macrophages and lymphocytes express the $P2X_7$ receptor responsible for the ATP-induced non-selective pore formation and release of cytokines (*Di Virgilio et al., 2001*). The $P2Y_{11}$ and $P2Y_{12}$ or $P2Y_{13}$ receptors have been shown to induce maturation and to regulate the function of monocyte-derived dendritic cells (*Wilkin et al., 2001; Marteau et al., 2004*). Finally, at least avian erythrocytes express the $P2Y_1$ receptor and probably other subtypes the role of which remain to be explored. Thus, the whole family of P2 receptors is present in the cardiovascular system where it regulates directly or in coordination with other major pathways all the physiological functions. As such, all these receptors could be pharmacological targets in diseases such as inflammation, atherothrombosis, undesired angiogenesis, hypertension, heart failure, and immune defects.

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P2 RECEPTORS IN OTHER SYSTEMS: $P2Y_{12}$

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The $P2Y_{12}$ receptor was originally cloned from rat and human cDNA libraries (Hollopeter et al., 2001; Zhang et al., 2001; Takasaki et al., 2001) and represents the elusive ADP-sensitive P2 receptor on platelets previously termed P2T, P2YADP and P2YT. This receptor is activated by adenine-di-phosphonucleotides and antagonized by the anti-platelet drugs ticlopidine and clopidrogrel, as well as by compounds of more recent synthesis such as ARL 67085 and AR-C 69931MX (*Ingall et al., 1999*). $P2Y_{12}$ has represented the first member of a P2Y receptor subgroup which now also includes the $P2Y_{13}$ and $P2Y_{14}$ receptors (*see Abbracchio et al., 2003 and references therein*). These receptors are structurally and phylogenetically correlated to each other and selectively couple to Gi/Go proteins and inhibition of adenylyl cyclase (ibidem). $P2Y_{12}$ is also expressed in non-platelet tissues, such as brain, brain-derived cells, and, to a lesser extent, spleen, suggesting physiopathological roles other than platelet aggregation. In rat brain, mRNA was found to be sparsely distributed throughout the neocortex, hippocampus, cerebellum and brainstem (*Sasaki et al., 2003*). Double-label in situ hybridization studies with markers for neuronal and glial cells suggested very low levels of expression (if any) in neuronal cells, but significant labeling of microglial cells (ibidem). In the facial nucleus, expression of $P2Y_{12}$ was greatly increased after facial nerve axotomy: $P2Y_{12}$ mRNA was found in microglial cells sorrounding axotomized motor neurons, suggesting a role for this receptor in microglial activation, proliferation and chemotaxis (ibidem). To confirm expression of this receptor in cells of the microglial lineage, $P2Y_{12}$ mRNA has been also found in N9 murine microglial cells (*Bianco et al., 2004*). In these cells, ADP and 2meSADP induced rapid and transient increases of intracellular calcium concentrations, which were partially antagonized by the $P2Y_1$ receptor antagonist MRS2179, and partially by the $P2Y_{12}/P2Y_{13}$ receptor antagonist ARC-69931MX, indicating that all these receptors contribute to modulation of intracellular calcium. These data also suggest that, besides inhibiting adenylyl cyclase, the $P2Y_{12}$ receptor can also couple to intracellular calcium increases, an effect which is likely mediated by the Gi/o-protein β-γ subunit. P2Y12 has been also detected in cells of the astroglial lineage (*primary astrocytic cultures from rat cortex, Fumagalli et al., 2003*) and proposed to be involved in generation of astrogliosis in the nucleus accumbens of rats (*Franke et al., 2001*). Differential levels of expression of $P2Y_{12}$ were found in rat C6 glioma cells, depending upon growth culture conditions (*Czajkowski et al., 2004*); in these cells, activation of P2Y12 reversed adrenergic receptor-induced differentiation into enhanced proliferation (*Van Kolen & Slegers, 2004*). Finally, despite lack of expression in brain neuronal cells (Sasaki et al., 2003), P2Y12 has been found in PC12 cells, a rat phaeochromocytoma cell line which is ontogenically related to sympathetic neurons and develops a neuronal phenotype upon treatment with nerve growth factor (Unterberger et al., 2002; Kubista et al., 2003). Activation of P2Y12 in these cells resulted in inhibition of voltage-activated Ca2+ currents, indicating a possible role in sympatho-effector transmission. Globally, these data confirm presence of the P2Y12 receptor in tissues other than platelets, and suggest roles for this receptor in microglial and astroglial response to brain injury as well as in the control of neuroendocrine cell function.

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P2 RECEPTORS IN OTHER SYSTEMS: $P2Y_1$

Léon C

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The $P2Y_1$ receptor was the first of the P2 receptor family to be cloned, in 1993. The human gene is located on chromosome 3 (3q24), close to the $P2Y_{12/13/14}$ receptors, suggesting a common ancestor.[1] This ADP receptor, which activates PLC≤ through G±q, has a widespread expression in adult tissues, $P2Y_1$ mRNA being expressed at highest levels in placenta, prostate and brain but also detected at varying levels in many other tissues.[2] Studies performed on chicks and rats during embryonic development have shown that it is also expressed early in a wide range of embryonic structures.[3] The dynamic expression of $P2Y_1$ is regulated in a developmental manner, pointing to its involvement in functions specific to embryonic life. These functions nevertheless do not seem to be crucial, since mice defective for expression of the $P2Y_1$ receptor develop and reproduce normally and have no gross abnormalities.[4] Apart from its role in hemostasis and thrombosis which is now firmly established, the characterization of a precise role of the $P2Y_1$ receptor in other tissues has long been hampered by a lack of selective agonists and antagonists distinguishing it from other nucleotide receptors. Many physiological functions have thus been attributed to the P2Y receptors without strong arguments. However, with the use of the selective antagonists now available and the development of $P2Y_1$ knockout mice, the involvement of the $P2Y_1$ receptor in some other physiological functions is now beginning to emerge. Its role in the regulation of insulin secretion/glucose homeostasis and bone remodeling will be developed here, as well as its contribution to the nervous system.

Glucose homeostasis. Pancreatic β-cells express a wide range of P2 receptors[5] and extracellular ATP has long been known to modulate insulin secretion.[6] Pancreatic islets are highly innervated and nucleotides, stored with acetylcholine or catecholamine in synaptic vesicles, may be liberated following sympathetic or parasympathetic stimulation. In addition, like in platelets or chromaffin cells, a pool of adenine nucleotides is present within insulin granules, with an ATP/ADP ratio of close to 1.[7] Hence these nucleotides could also play an important paracrine or autocrine feedback role. Several $P2Y_1$ receptor agonists (ADP, ADP≤S, 2-thioether ATP derivatives) have been found to be effective in stimulating insulin release in human and rat pancreas, although the subtype of receptor involved has not been precisely defined.[6] Conversely, nucleotides inhibit insulin release from mouse pancreatic islets, probably by direct inhibition of exocytosis.[8] Studies in $P2Y_1$-deficient mice have confirmed a role of this receptor in the maintenance of glucose homeostasis. $P2Y_{1-/-}$ mice exhibit a 10% increase in body weight, associated with 15% and 40% increases in glycemia and plasma insulin levels, respectively, which strongly favors the presence of a state of insulin resistance. Moreover, *in vitro* experiments showed enhanced insulin secretion in isolated pancreatic islets from $P2Y_{1-/-}$ mice, in the presence of high concentrations of glucose. Thus, at least in the mouse, the $P2Y_1$ receptor is involved in the negative regulation of insulin release, which would suggest an inhibitory function of $P2Y_1$ in glucose metabolism. This may act as an autocrine loop for the feedback control of insulin secretion through co-released ADP, thereby allowing the β-cells to rapidly adapt their response

to variations in plasma glucose.

Bone remodeling. The involvement of nucleotides in bone remodeling, while not totally understood, has been well documented. Nucleotides affect the cell activity of both bone-forming osteoblasts and bone-resorbing osteoclasts. Osteoblasts and osteoclasts express several types of P2 receptor and a role of the $P2Y_1$ receptor in osteoclastic bone resorption has been demonstrated using the $P2Y_1$ antagonist MRS2179.[9,10] However, it is not yet known whether this receptor acts directly on osteoclasts or indirectly through osteoblasts. $P2Y_{1-/-}$ mice have decreased plasma minerals level (magnesium, phosphorus and calcium), and preliminary studies with $P2Y_{1-/-}$ mice have revealed a slight defect in bone density. Since the difference did not reach statistical significance, further studies are needed to confirm a role of this receptor in mouse bone formation or remodeling.

Central nervous system. Concerning the central nervous system (CNS), numerous studies have demonstrated an effect of nucleotides at the level of neural or glial cells. Astrocytes express several P2Y receptors, and the $P2Y_1$ was suggested to play a role in the induction and/or maintenance of reactive gliosis 11 and in astrocytic Ca^{2+} oscillations potentially involved in gliotransmission or astrocyte-neuron communication.[12] The $P2Y_1$ receptor also appears to be highly expressed in neurons of the CNS[2] where it may be involved in pre- or post-synaptic neuronal functions,[13] and in proliferation and migration during early CNS development.[14] At the behavioral level, the $P2Y_1$ has been shown to be involved in the modulation of anxiety in the rat.[15]

Conclusions. Despite the multiple effects of nucleotides in physiological processes, our understanding of the role of the $P2Y_1$ receptor outside the cardiovascular system is still in its infancy. Studies have been complicated by the complex interactions between P2 and P1 receptors, which act in a coordinated manner in most tissues. Just as it may be a potential target for antithrombotic therapy, the widespread expression of the $P2Y_1$ receptor and its participation in at least some aspects of insulin secretion and bone remodeling nevertheless suggest it could be used to develop novel therapeutic approaches in other pathologies.

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Oral Communications I

P2Y1 AND P2Y12 - INVOLVEMENT IN CLOTTING TIME SHORTENING CAUSED BY ACTIVATION OF THROMBIN RECEPTORS PAR-1 AND PAR-4

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Background. Human platelets express two protease-activated receptors (PAR) activated by thrombin, PAR1 and PAR4. PAR1 responds to thrombin levels of about 1 nmol/L, while PAR4 needs a tenfold higher concentration to respond. The activation of the two PAR receptors might also be accomplished using small peptides mimicking the terminal part of the extracellular tail that will be exposed as thrombin cleaves the receptor. *Aim.* The aim of this study

was to examine if ADP released from activated platelets would affect the clotting time of whole blood samples where clotting had been accelerated by the addition of PAR1- or PAR4-activating peptides (SFLLRN or AYPGKF, respectively), using the P2Y1 antagonist MRS2179 (0.2 mmol/L) and the P2Y12 antagonist AR-C69931MX (0.1 mmol/L). *Methods.* Free oscillation rheometry (FOR) was used to measure clotting times in whole blood samples collected without any anticoagulants. The blood samples were preincubated with the antagonists for 3 minutes before the addition of the PAR activating peptides. Results The clotting times for samples with the PAR1 activating peptide (0.03 mmol/L) were significantly increased by the addition of AR-C69931MX and also when both antagonists were added (n=5). With the PAR4 activating peptide (0.3 mmol/L), the clotting times were only significantly increased when both antagonists were added together (n=5). When both PAR activating peptides were used together, significant prolongations were seen with both antagonists (n=5). *Conclusions*. Although the number of samples analysed so far is quite small, making it hard to draw any conclusions, it seems that endogenous ADP is less important for platelets activated via the PAR4 receptor.

BLOOD LEUKOCYTES HAVE A MAJOR INFLUENCE ON THE RESPONSE OF PLATELETS TO ADP AND ATP

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It is well known that ADP induces platelet aggregation. Recently we found that ATP can also do so, but only in whole blood, not in PRP. We demonstrated an involvement of leukocytes (*Stafford et al. ATVB 2003;23:1928-33*). Here we have studied further the role of leukocytes in platelet aggregation induced by ATP and also by ADP. The investigations involved 1) studies in blood from patients with pathologically raised leukocyte counts as well as healthy volunteers, and 2) determination by HPLC of the rate of conversion of ATP and ADP into other nucleotides and nucleosides. Blood was collected into hirudin. In some experiments leukocytes were separated using MonoPoly Resolving Medium and added to whole blood or PRP. Platelet aggregation was measured using a platelet counting technique. ATP, ADP, AMP, adenosine and inosine were measured using a novel reversed phase chromatographic HPLC procedure using a gradient elution method. Platelet aggregation in blood from patients was markedly altered compared with blood from healthy volunteers. There was a remarkable decrease in the aggregation induced by ADP and the response to ATP was more rapid and more reversible. Conversely platelet aggregation to ADP in PRP prepared from the patients was not reduced. Adding normal leukocytes to blood from healthy volunteers reproduced these findings. Adding normal leukocytes to PRP resulted in marked inhibition of aggregation induced by ADP, while at the same time enabling the platelets to respond to ATP. Adding ATP or ADP to blood plasma resulted in very little conversion of either of these nucleotides to AMP or adenosine. AMP added to plasma was converted to adenosine and then inosine. Similar results were obtained in PRP. When ATP or ADP was added to plasma that contained leukocytes, conversion to AMP was very rapid. High concentrations of ATP or ADP (100 μM) were removed within 8 min. With ATP there was formation of ADP before conversion to AMP. These results are consistent with leukocytes providing the means of rapidly converting ATP to ADP and ADP to AMP. Platelets do not possess this activity. AMP is then converted by plasma enzymes to adenosine and inosine. These interconversions result in ATP causing platelet aggregation in whole blood. Responses to both ATP and ADP are radically different in patients with raised leukocyte counts. The latter is likely to contribute to abnormal haemostasis in such patients.

CLONING AND CHARACTERIZATION OF RAT $P2Y_{13}$ RECEPTOR CONFIRM HIGH SIMILARITY WITH $P2Y_{12}$ RECEPTOR

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The human $P2Y_{13}$ receptor has been recently identified as a new P2Y receptor sharing a high sequence homology with the $P2Y_{12}$ receptor as well as similar functional properties: coupling to Gi, responsiveness to adenine di-phospho-nucleotides and blockade by AR-C69931MX. Here we report the cloning of rat $P2Y_{13}$ receptor, its tissue distribution and pharmacological characterization. Rat $P2Y_{13}$ is 79% and 87% identical to human and mouse $P2Y_{13}$, respectively, and is branched together with $P2Y_{12}$ and $P2Y_{14}$ in the dendrogram of P2Y receptors. In a similar way to its human hortologue, $rP2Y_{13}$ showed highest expression levels in spleen, followed by liver and brain, suggesting important roles in the nervous and immune systems. Moderate, albeit significant, levels were observed in heart and aorta. Expression of r

$P2Y_{13}$ receptor in 1321N1 cells induced the appearance of responses to the typical $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptor agonists ADP and 2MeSADP, as detected by stimulation of $[^{35}S]GTP\gamma S$ binding. Agonist activities were higher in cells transfected with $rP2Y_{13}$ receptor in the presence of the $G\alpha_{16}$ subunit; in all cases agonist effects were abolished by pertussis toxin pretreatment, confirming coupling to G_i. At variance from both *human* and *mouse* receptors, on the rat receptor ADP was more potent than 2MeSADP. Other nucleotides and sugar-nucleotides were ineffective. Activation of $rP2Y_{13}$ receptor by ADP and 2MeSADP was completely inhibited by nM concentrations of the P2Y12 receptor antagonist AR-C69931MX (IC_{50}= 26±1.9). In contrast, no inhibition of $rP2Y_{13}$ receptor was induced by the selective $P2Y_1$ receptor antagonist MRS2179. These results confirm that $P2Y_{13}$ receptor is more similar to the $P2Y_{12}$ receptor than initially believed, as also suggest by the short distance between these two genes in both human and rat genome and by the high homology (48%) between the two receptor proteins.

STUDY OF THE ASSOCIATION OF PFA-100® CLOSURE TIME AND THE SKIN BLEEDING TIME WITH THE SEVERITY OF BLEEDING SYMPTOMS IN PATIENTS SCREENED FOR BLEEDING DIATHESIS

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We compared the association of PFA-100® closure time (CT) and the Bleeding Time (BT) with the severity of bleeding history in 128 consecutive patients referred to our Center from June 2002 through June 2003 to be screened for bleeding disorders, due to the presence of bleeding symptoms or the casual finding of abnormal screening tests of hemostasis. All patients underwent a careful medical interview and were assigned a *bleeding score*, based on the number, type, frequency and severity (need for blood transfusion and/or surgical or medical intervention) of bleeding symptoms. In addition, all patients underwent a first-line screening, which included PT, APTT, BT and PFA-100® CT (with both the collagen-ADP and the collagen-epinephrine cartridges). The search for Von Willebrand disease (VWD), platelet function disorders (PFD), clotting factor defects and abnormalities of fibrinolysis was performed according to the results of the first line screening tests and the severity and type of bleeding history. Seven (6%) patients had type-1 VWD, 7 (6%) PFD, 29 (23%) defects of coagulation, 18 (14%) defects of the contact system or lupus anticoagulant, while in 67 (52%) all tests gave normal results. The sensitivity of PFA-100® for VWD was 86% (both cartridges), for PFD 71% (collagen-epinephrine) and 14% (collagen-ADP). The sensitivity of BT for VWD was 29%, for PFD 57%. After dividing the patient population in four quartiles of distribution, according to the severity of the bleeding score (null, low, intermediate and severe), only the CT values of collagen-epinephrine showed a progressive and significant prolongation from the first to the fourth quartile (p=0.04). No association of BT and collagen-ADP CT with the severity of bleeding history was found. In conclusion, PFA-100 showed a better sensitivity than BT for VWD and PFD. CT with the collagen-epinephrine cartridge was significantly associated with the severity of the bleeding history.

PLATELET AGGREGATION STUDIES: AUTOLOGOUS PLATELET-POOR PLASMA INHIBITS PLATELET AGGREGATION WHEN ADDED TO PLATELET-RICH PLASMA TO NORMALIZE PLATELET COUNT

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Platelet aggregation (PA) studies are important for diagnosing patients with defects of platelet function. Due to the high variability of the test, results obtained with platelet-rich plasma (PRP) of the patient should be compared to those of control PRP, run in parallel. Platelet counts in the two PRPs should be adjusted to the same value, using autologous platelet-poor plasma (PPP) for proper dilution. We investigated whether or not dilution of PRP with autologous PPP affects the results of PA studies. Study 1. We re-evaluated the results obtained with 83 control PRPs in PA studies run with the same instrument from 1999 through 2001. Twenty-six PRPs had been diluted with PPP, while 57 were used undiluted. The mean (range) platelet count was 301×10^9/L (120-436) in diluted-PRPs and 376 (177-588) in undiluted-PRPs ($p<0.001$). The mean maximal percent of PA induced by PAF 0.2 μM, ADP 2 μM or collagen 2 μg/mL was significantly lower in diluted-PRPs than in undiluted-PRPs (24.4 vs 50.2, 39.2 vs 54.7, 72 vs 76). PA correlated with platelet count in diluted-PRPs (PAF, ρ=0.41; ADP, ρ=0.37; collagen, rho=0.33), but not in undiluted-PRPs (PAF, rho=0.09; ADP, ρ=-0.10; collagen, ρ=0.02). Study 2. We tested the hypothesis that adenine nucleotides

(AN) released from cells into PPP during its preparation inhibit PA. Platelet counts of normal PRPs (n=7) were diluted to 300, 220 and 150 with PPP plus saline (S-PRP) or 0.5 U/mL apyrase (APY-PRP), which degrades AN. PA decreased with the extent of PRP dilution both in S-PRP and APY-PRP, but was significantly higher in diluted APY-PRP than in diluted S-PRP. Therefore, platelet count (range, ≈150-500) in PRP is not a determinant of in vitro PA. AN (and, possibly, other factors) in PPP inhibit PA. Platelet count should not be adjusted with PPP to the lowest value when comparing PA in different samples.

PROBLEMS WITH DRUGS OF PROVEN ANTITHROMBOTIC ACTIVITY

RESISTANCE TO ANTIPLATELET AGENTS: REVIEW OF THE LITERATURE AND SOME METHOLOGICAL CONSIDERATIONS

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Aspirin and clopidogrel are inhibitors of platelet aggregation that display good antithrombotic activity. In the last few years, the problem of "aspirin resistance" has been largely emphasized in the medical literature, although its definition and probably even its real existence are still uncertain. More recently, clopidogrel resistance has also been investigated.

ASPIRIN RESISTANCE

Definition. The term "aspirin resistance" has been given different definitions by different researchers and I think that an effort should be made to propose a universally acceptable definition of this phenomenon. A list of definitions that have been given to "aspirin resistance" follows, with my personal considerations relative to each of them.

1) Failure of aspirin to prevent clinical events associated to vascular occlusion. This phenomenon has been called "clinical aspirin resistance",[1] but it should be termed "treatment failure":[2] it can be observed with any kind of treatment and is expected to be particularly frequent for drugs, like aspirin and all other antithrombotic agents, that are used to prevent multifactorial diseases, such as those associated with vascular occlusions. Therefore, the definition of "aspirin resistance" that is based on clinical outcomes is certainly unacceptable.

2) Failure of aspirin to inhibit platelet function "in vivo" or "in vitro". Platelet function *in vivo* has been measured by the bleeding time, while platelet function in vitro has, in most instances, been measured by light transmission aggregometry or by global techniques that evaluate primary hemostasis, such as the PFA-100® system, or, more rarely, the Ultegra Rapid Platelet Function Assay-ASA. All these techniques, albeit to different degrees, are sensitive to several variables. Among these, platelet TxA2 production, which is the pharmacological target of aspirin, is usually of marginal importance, and one should not expect aspirin to inhibit completely platelet functions that are not regulated by TxA2 only. In addition to using inadequate techniques to measure the response to aspirin, most published studies had an inadequate experimental design, because they failed to compare the results obtained before aspirin ingestion with those after aspirin. Given the high inter-individual variability of platelet function tests, this could lead to inaccurate and somewhat paradoxical conclusions. For instance, studies with PFA-100® usually defined aspirin resistant those subjects whose collagen-epinephrine CT was lower than the upper limit of the normal range (the normal range is between approximately 80 sec and 180 sec). Let us consider two hypothetical subjects: subject 1 has a baseline CT of 80 sec which increases to 179 sec during aspirin treatment; subject 2 has a baseline CT of 180 sec which remains unmodified during aspirin treatment. Paradoxically, a study that measures CT during aspirin treatment only, would classify subject 1 as "aspirin resistant" and subject 2 as "aspirin responder". To avoid this kind of paradoxical results, studies of platelet function should be performed both before and after aspirin intake.

3) Failure of aspirin to inhibit TxA2 production. Lacking a reproducible and highly sensitive and specific method to study TxA2-dependent platelet function, the pharmacological response to aspirin treatment should be assessed by measuring the degree of inhibition of TxA2 production. This could be done by measuring either serum TxB2 or the urinary excretion of TxB2 metabolites. Therefore, based on the available techniques, the only acceptable definition of aspirin resistance should rely on the demonstration of an insufficient inhibition of TxA2 production.

For the sake of clarity, in the remaining part of this review, I will refer to failure of aspirin to inhibit TxA2 production, with the term "true" aspirin resistance, and to failure of aspirin to inhibit platelet function "in vivo" or "in vitro" (without demonstration of inadequate inihibition of TxA2 production), with the term "unproven" aspirin resistance.

Mechanisms. "True" aspirin resistance. The following potential mechanisms could be considered responsible for "true" aspirin resistance: 1) decreased bioavalaibility of aspirin; 2) competition of aspirin with other NSAIDs (such a ibuprofen) preventing aspirin access at Ser530 of COX-1;[3] 3) accelerated platelet

turnover, introducing newly formed, non-aspirinated platelets into blood stream;[3] 4) transcellular formation of TxA2 by aspirinated platelets from prostaglandin H2 released by other blood cells or vascular cells;[3] 5) TxA2 production by the aspirin-insensitive COX-2 in newly-formed platelets or other cells;[4] 6) (theoretical) presence of variant COX-1 that is less responsive to aspirin inhibition. Another mechanism of aspirin resistance that should never be laid aside is lack of compliance, which, in a recent study, accounted for the majority of poor aspirin response and was the only significant mediator of poor clinical outcome.[5]

Mechanisms. "Unproven" aspirin resistance. The mechanisms responsible for insufficient platelet function inhibition during aspirin therapy should be looked for among the several variables that affect the platelet function tests that have been used: increased sensitivity to ADP-induced GPIIb/IIIa activation, increased responsiveness to collagen, high plasma levels of VWF, GPIIb/IIIa polymorphisms, etc. Factors related to the subject, such as hyperlipidemia, cigarette smoking and physical or mental stress, could also play a role.

Clinical consequences. "True" aspirin resistance. Eikelboom et al showed that suboptimal reduction of urinary 11-dehydro TxB2 levels during aspirin treatment is associated with heightened risk for future myocardial infarction and cardiovascular death, indeed suggesting that "true" aspirin resistance may be a clinically relevant phenomenon.[6] Inadequate inhibition by aspirin of TxA2 biosynthesis can be observed in patients on treatment with ibuprofen, due to competition of the two drugs at the COX-1 level; observational studies and post-hoc analysis suggested that ibuprofen blunts the cardioprotective effect of aspirin, although the question is still controversial.

Clinical consequences. "Unproven" aspirin resistance. An association between suboptimal platelet function inhibition during aspirin treatment and heightened incidence of cardiovascular or cerebrovascular events has been described. These interesting findings, if confirmed in larger studies, could bear important clinical implications, because they suggest that monitoring platelet function during antiplatelet therapy can be useful to predict the risk of treatment failures. However, the phenomenon that they describe should not be termed "aspirin resistance", because it is determined to a large extent by variables that cannot be inhibited by aspirin (see above).

CLOPIDOGREL RESISTANCE

Less well known than aspirin resistance, but certainly better characterized is "clopidogrel resistance".

Definition. Correctly, the term has never been used to refer to treatment failures, despite the fact the clopidogrel is only marginally superior to aspirin in preventing cardiovascular events.[7] The extent of the platelet aggregation response *in vitro* to ADP has been used to define "clopidogrel resistance" in the large majority of studies that have been published so far. Needless to say, the aforementioned general pitfalls of in vitro platelet aggregation apply not only to studies of "aspirin resistance" but also to those of "clopidogrel resistance". In addition, although ADP is the most appropriate aggregating agent in this context, because clopidogrel antagonizes the ADP receptor P2Y12, it must be noted that platelets express also a second ADP receptor, P2Y1, which causes the initial wave of ADP-induced platelet aggregation.8 Since the extent of residual, P2Y1-dependent platelet aggregation induced by ADP varies widely among patients with congenital P2Y12-deficiency or normal subjects in whom P2Y12 function had been completely blocked in vitro by saturating concentrations of specific antagonists, ADP-induced platelet aggregation may not be the most suitable test to measure the individual response to clopidogrel. A better and more specific test would be measurement of the extent of ADP-induced inhibition of adenylyl cyclase, which is uniquely mediated by P2Y12. This could be accomplished by measuring the inhibition by ADP of prostaglandin-induced platelet cyclic AMP increase or phosphorilation of vasodilator-stimulated phosphoprotein (VASP).

Mechanisms. Clopidogrel is a pro-drug, which needs to be metabolized by the liver to an active metabolite with anti-aggregating activity.[9] Therefore, its pharmacological effect can be detected only some time after its first administration and, more importantly, the plasma levels of the active metabolite, and, consequently, the degree of inhibition of platelet aggregation induced by ADP, vary widely among subjects. In published studies, about 50% of the patients were either clopidogrel non responders or low responders. Inter-individual variability in platelet inhibition by clopidogrel correlated well with the metabolic activity of the hepatic cytochrome P450, which activates the pro-drug to its active metabolite.[10] Interference with clopidogrel metabolism by other drugs that are frequently given to patients with atherosclerosis, such as atorvastatin, can increase the number of patients who are resistant to clopidogrel, although this is still a controversial issue.

Clinical consequences. A recent study of 60 patients undergoing coronary angioplasty confirmed the high inter-individual variability of platelet inhibition by clopidogrel and showed that patients with clopidogrel resistance (mean ADP-induced platelet aggregation on day 6 of treatment = 103±8% of baseline) are at increased risk for recurrent cardiovascular events.[11]

CONCLUSIONS. Aspirin and the thienopyridines ticlopidine and clopidogrel are antiplatelet agents that display good antithrombotic activity. For investigational purposes, aspirin and clopidogrel resistance

should be evaluated in compliant patients by studying the specific target of each drug; measurements should be done both before and after drug administration (Table 1). At present, aspirin and clopidogrel resistance should not be looked for in the clinical setting, because there is no definite demonstration of an association with clinical events conditioning cost-effective changes in patient management.

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Table 1. Recommendations for studying aspirin and clopidogrel resistance.

1. Rule out patient non compliance
2. Measure the function of specific targets of the antiplatelet drugs:
   a. COX-1 for aspirin (laboratory measurement: levels of serum TxB2 or urinary TxB2 metabolites)
   b. P2Y12 for clopidogrel (laboratory measurement: inhibition by ADP of prostaglandin-induced increase in platelet cyclic AMP or phosphorylation of VASP)
3. Measure the "response" to the antiplatelet drugs: baseline vs after treatment

At present, aspirin and clopidogrel resistance should be studied for investigational purposes only; they should not be looked for in the clinical setting, because no definite demonstration of an association with clinical events conditioning cost-effective changes in patient management is available yet.

CLOPIDOGREL RESPONSE VARIABILITY AND DRUG RESISTANCE

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Clopidogrel, a thienopyridine, is a prodrug that requires metabolic activation by hepatic cytochrome P 450 3A4 in order to exert its antiplatelet effect.[1] The active metabolite has been identified and irreversibly binds via a disulfide bond to the adenosine diphosphate (ADP) G-coupled $P2Y_{12}$ receptor.[2] $P2Y_{12}$ has been reported as a central mediator of the hemostatic response. Inhibition of $P2Y_{12}$ attenuates both the amplification of platelet aggregation and the stability of the thrombus.[3] In combination with aspirin, clopidogrel is currently the drug of choice to prevent stent thrombosis.[4] Stent thrombosis remains an important and potentially lethal clinical problem (incidence 0.9%, mortality 8.9%).[5] The standard clopidogrel regimen is a 300 mg loading dose followed by 75 mg daily.[6] The dosing regimen of clopidogrel used for coronary stenting was based primarily on studies in normal volunteers and stable patients with coronary artery disease not undergoing stenting. Clopidogrel is administered to the vast majority of patients without any assessment of platelet inhibition. Moreover, there is no uniform consensus about the preferred methodology to measure clopidogrel-induced platelet inhibition. A theoretical concern for the development of stent thrombosis exists in those patients who have high platelet reactivity despite ongoing therapy with antiplatelet agents. In our early investigations we had observed modest inhibition of 5 μM ADP-induced platelet aggregation following chronic clopidogrel and aspirin therapy (37±14% inhibition of baseline aggregation).[7] We have long recognized the phenomena of clopidogrel resistance and response variability in our investigations of platelet reactivity following coronary stent implantation and have recently reported our findings.[6] Our studies were conducted in patients treated with chronic aspirin therapy who were undergoing elective stenting. Patients were administered a 300 mg clopidogrel loading dose in the catheterization laboratory immediately after successful stenting followed by a 75 mg daily maintenance dose. Heparin was administered as the anticoagulant and GP IIb/IIIa inhibitors were withheld per protocol. We serially assessed ADP-induced platelet aggregation and surface receptor expression at baseline and for the following 30 days. Using light transmittance aggregometry we observed high rates of non-responsiveness to clopidogrel. At both 24 hours and 5 days post-stenting the incidence of

clopidogrel resistance was ~30%. Similar high resistance rates were also measured by ADP-stimulated p-selectin expression.6 Since this initial report others have followed supporting our conclusion that clopidogrel resistance is indeed a real phenomenon.[8–12] In 2003, the European Society of Cardiology Congress dedicated a session on this subject. We have also examined the durability of platelet inhibition induced by chronic clopidogrel therapy.[13] In general, patients who are early responders at 24 hours post-loading will remain responsive over 30 days. Roughly one-half of early non-responders will become responders by 30 days. The mechanism(s) underlying these findings remain unclear but may involve upregulation of hepatic CYP 3A4 by clopidogrel[14] and the early enhancement of platelet reactivity by stenting that subsequently wanes as time accrues following the procedure.[4,15] Recent interest has focused on the detection of downstream intracellular signaling events that follow the engagement of ADP with the $P2Y_{12}$ receptor. The inhibition of adenylate cyclase by the G-coupled $P2Y_{12}$ receptor decreases cyclic AMP production leading in turn to a reduction in the activation of specific protein kinases.[16] Vasodilator stimulated phosphoprotein (VASP) is phosphorylated by these kinases and the degree of phosphorylation is a marker of clopidogrel-induced P2Y12 receptor inhibition. The level of VASP phosphorylation has recently been proposed as a predictive marker of stent thrombosis. A fall in VASP phosphorylation following stimulation by ADP indicates incomplete inhibition of the $P2Y_{12}$ receptor by clopidogrel.[10] Recent data from Lau *et al.* support the important role of CYP 3A4 activity in relation to the antiplatelet effect induced by clopidogrel. These investigators have demonstrated that inducers of CYP 3A4 activity (e.g. rifampin©) enhance clopidogrel's antiplatelet effect whereas agents that compete with clopidogrel for the active site (e.g. erythromycin) attenuate the effect.[1,9] These data and other preliminary studies that have reported measurements of active metabolite levels suggest that response variability is partially explained by insufficient active metabolite generation. Controversy exists regarding the optimal clopidogrel loading and maintenance dose regimens. We have recently presented data suggesting that a 300 mg load produces inferior inhibition as compared to 600 mg.[17] These results support those of an earlier study also conducted in patients undergoing elective stenting.[18] A recent large clinical trial, also conducted in elective patients, compared a 600 mg clopidogrel loading dose to the same loading dose combined with abciximab therapy.19 The finding of equivalent primary endpoint rates in both arms lends further support to use of the 600 mg clopidogrel loading dose in elective stenting. We are currently completing a 2x2 factorial investigation to evaluate the antiplatelet effects of *standard* versus *high* dose clopidogrel treatment strategies in the presence and absense of eptifibatide. In conclusion, response variability and resistance definitely occur to clopidogrel.Preliminary data appear to confirm the hypothesis that patients with reactive or nonresponsive platelets are at risk for thrombotic events. However, the magnitude of the clinical effect remains unknown and overall has been underinvestigated. Important questions that must be answered are: A) What is the relation of clopidogrel resistance and high platelet reactivity to the occurrence of stent thrombosis, recurrent myocardial infarction, stroke and death?; B) Is there a threshold of platelet reactivity that correlates with the onset of thrombotic risk?; and C) What is the cost of administering clopidogrel to non-responsive patients? Finally, our understanding of the clinical relevance of drug resistance and high platelet reactivity should be facilitated by the use of validated point of service devices. The mechanisms of the response variability to clopidogrel remain incompletely defined. The contribution of intra- and extracellular pathways are under investigation.

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Oral Communications II

CLOPIDOGREL THERAPY DOES NOT OFFSET THE PLATELET HYPEREACTIVITY ASSOCIATED WITH COMMON PLATELET RECEPTOR POLYMORPHISMS

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Background. Sequence variations within the $P2Y_{12}$, protease-activated receptor-1 (PAR-1) and glycoprotein IIIa (GPIIIa) genes have been linked to platelet aggregation phenotypes. A wide inter-patient variability exists both in platelet reactivity prior to medication and in the efficacy of clopidogrel. *Aim.* To determine whether these genotypes influence the response to clopidogrel. *Methods.* 54 patients listed for elective percutaneous coronary intervention were studied using optical aggregometry (OA), whole-blood single platelet counting aggregometry, annexin V binding, P-selectin expression, and platelet-monocyte conjugate formation, with ADP and TRAP as agonists. Platelet reactivity was measured at baseline, 4 hours post clopidogrel 300 mg (prior to PCI), and 10 and 28 days following clopidogrel 75 mg daily. Each patient was genotyped for P2Y12 haplotype, the PAR-1 intervening sequence A>T 14 dimorphism, and the GPIIIa PlA1/A2 genotype. Results: Increased platelet responses to ADP were associated both with the presence of the P2Y12 H2 haplotype (% OA to ADP 5 μmol/L, H1/H1 (n=32) v H1/H2 or H2/H2 (n=22), 33.9 ± 3.6 v 41.5 ± 3.7, $p<0.01$) and with PlA1 homozygotes (% P-selectin expression to ADP 10 μmol/L, PlA1/A1 (n=41) v PlA1/A2 or PlA2/A2 (n=13), 23.7 ± 1.4 v 19.7 ± 1.8, $p<0.01$). Increased platelet responses to TRAP were associated with the PAR-1 A allele homozygotes (annexin V % positive events to TRAP 20 μmol/L, A/A (n=44) v A/T or T/T (n=10), 18.0 ± 1.6 v 12.1 ± 2.4, $p<0.01$). These patterns of platelet reactivity were observed both before and during clopidogrel therapy. None of these genotypes influenced the inhibitory activity of clopidogrel. *Conclusions.* Common sequence variations within the $P2Y_{12}$ and GPIIIa genes influence ADP-induced platelet responses, and within the PAR-1 gene influence TRAP-induced platelet responses. The higher platelet reactivity associated with the risk alleles persists during clopidogrel therapy. These patients potentially have a higher risk of thromboembolic events and may warrant more effective anti-platelet therapy.

EVALUATION OF CLOPIDOGREL EFFICACY BY FLOW CYTOMETRIC ASSESSMENT OF VASP PHOSPHORYLATION IN PATIENTS WITH ATHEROTHROMBOTIC DISEASES

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Background: Clopidogrel, a selective and irreversible inhibitor of P2Y12 platelet receptors, is an antiplatelet agent widely used in the prevention of atherothrombotic complications. It is a prodrug which must be metabolised in the liver to acquire its antiaggregatory properties. However, since clinical thrombosis still occurs in 5 to 10% of patients under clopidogrel treatment while platelet aggregation remains unchanged in up to 30% of such patients. The concept of clopidogrel resistance has been put forward. In platelets, the phosphorylation state of the vasodilator-stimulated phosphoprotein (VASP-P), an intracellular actin regulatory protein, is correlated with the degre of inhibition of the $P2Y_{12}$ receptor by clopidogrel or competitive $P2Y_{12}$ antagonists and inhibition of ADP-induced platelet aggregation. *Aim.* To use a

VASP-P assay to evaluate the efficacy of clopidogrel therapy for the prevention of platelet activation in patients presenting atherothrombotic diseases. *Methods.* VASP-P was measured by quantitative flow cytometry (Platelet VASP®, Diagnostica Stago/Biocytex). The platelet reactivity index (PRI) was calculated from the difference in VASP-P fluorescence intensity (FI) between resting (+PGE1) and activated platelets (+ADP) (IRP% = [FIPGE1-FIADP]/FIPGE1). The PRI was determined in healthy donors and in patients with cardiovascular diseases treated or not with clopidogrel (75 mg/day). Results: The PRI was (mean±SD) 78.3±4.6% in healthy donors (n=47), 79.0±4.1% in patients without clopidogrel (n=34) and decreased to 61.1±17.0% in patients receiving clopidogrel (n=33) (p<0.0001). In the clopidogrel group, the PRI was widely dispersed (from 6.6 to 85.8%) and 33% of these patients had a PRI equivalent to values in healthy donors or patients not receiving clopidogrel. *Conclusions.* One third of our patients under clopidogrel therapy displayed no inhibition of platelet activation, possibly due to interindividual differences in liver metabolism and to drug dosage. Whether these *unprotecte* patients are more susceptible to recurrent ischemic complications will now be investigated in follow-up studies.

GLYCOPROTEIN IIB/IIIA AND $P2Y_{12}$ ANTAGONISTS YIELD ADDITIVE INHIBITION OF PLATELET AGGREGATION, GRANULE SECRETION AND PROCOAGULANT RESPONSES

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Glycoprotein IIb/IIIa (GPIIb/IIIa) antagonists, including abciximab and tirofiban, are administered concurrently with clopidogrel, a $P2Y_{12}$ antagonist, and aspirin in some patients undergoing percutaneous coronary intervention. We studied the effects of, and interactions between, abciximab, tirofiban, aspirin and the $P2Y_{12}$ antagonist cangrelor (AR-C69931MX) on platelet aggregation, dense granule release and procoagulant responses *in vitro*. Blood was obtained from healthy volunteers and anticoagulated with hirudin. Aspirin (100 μM), tirofiban (50 nM), abciximab (4 μg/mL) and cangrelor (100 nM) were studied alone and in combination. Platelets were stimulated, in whole blood, with either collagen (4 μg/mL) or TRAP (20 μM). Platelet aggregation, dense granule secretion and platelet procoagulant responses were assessed by single-platelet counting, release of 14C-5-HT from preloaded platelets and flow cytometry (FITC-annexin V binding and microparticle formation) respectively.

Agonist	*Test*			*Saline*			*Abciximab*			
	n=7	*Saline*	*ASA*	*Can*	*ASA+ Can*	*Saline*	*ASA*	*Can*	*ASA+ Can*
Coll	DGS	27	14	19	9	23	7	7	3
Coll	AV	80	35	56	9	39	6	8	4
Coll	MP	23	8	15	2	11	2	2	1
TRAP	DGS	23	22	11	12	21	21	14	15
TRAP	AV	71	65	37	27	30	22	11	9
TRAP	MP	16	15	6	5	6	5	3	2

% dense granule secretion (DGS), annexin V binding (AV) and % microparticle formation (MP) in response to collagen (Coll) or TRAP in the presence of aspirin (ASA), cangrelor (Can) and abciximab.

All the antagonists inhibited collagen-induced aggregation, dense granule secretion and procoagulant responses (p<0.05, all tests) and combinations of abciximab or tirofiban with aspirin and/or cangrelor gave additive inhibition (p<0.01 aggregation, p<0.05 annexin V, p<0.02 microparticle formation) with greatest inhibition seen when abciximab or tirofiban was combined with both aspirin and cangrelor (p<0.001 aggregation, p<0.05 annexin V). Abciximab and tirofiban but not aspirin or cangrelor inhibited TRAP-induced aggregation (p<0.05) and procoagulant responses (p<0.01) and, again, there was additive inhibition of these parameters when abciximab or tirofiban was combined with cangrelor (p<0.05). The GPIIb/IIIa receptor plays an important role in amplification of platelet activation (via outside-in signalling) such that there are important interactions between GPIIb/IIIa antagonists and inhibitors of both $P2Y_{12}$ receptor activation (cangrelor) and, to a lesser extent, thromboxane A2 generation (aspirin). These interactions are likely to have important influences on the safety and efficacy of combination antiplatelet therapies.

N0204-003 INHIBITS ADP-INDUCED PLATELET FUNCTION VIA ANTAGONSIM AT $P2Y_1$ AND $P2Y_{12}$ RECEPTORS

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The main ADP receptors on platelets are $P2Y_1$ and $P2Y_{12}$ and both need to be occupied for a full platelet aggregation response. $P2Y_1$ initiates the response and $P2Y_{12}$ sustains the response. Antagonists that act at either of these receptors are actual or potential antithrombotic agents. Currently the anti-thrombotic focus has mainly been on development of $P2Y_{12}$ antagonists such as clopidogrel and cangrelor. Antagonists that act at the $P2Y_1$ receptor could also be effective antithrombotic agents. Also, a combi-

nation of antagonists that act at both of these receptors could be beneficial. Recently we studied a range of compounds for their ability to inhibit ADP-induced platelet responses. Studies were performed in hirudinized whole blood or platelet-rich plasma (PRP) from normal healthy volunteers. Measurements of platelet aggregation, platelet shape change, intracellular cAMP, VASP phosphorylation and intracellular Ca^{2+} were performed. The results were compared with those obtained using the $P2Y_1$ antagonist MRS2179 and the $P2Y_{12}$ antagonist AR-C69931MX (cangrelor). Three agents (N0204-001, N0204-002 and N0204-003) inhibited ADP-induced platelet aggregation in a competitive fashion. N0204-003 was the most potent. N0204-003 proved to be a selective inhibitor of ADP-induced aggregation in that its effects on aggregation induced by 5-HT and by PAF were identical to those of MRS2179 and AR-C69931MX; it only reduced the ADP component of the aggregation response. An effect at platelet P2Y1 receptors was demonstrated: like MRS2179, N0204-003 inhibited ADP-induced shape change and also ADP-induced increases in intracellular Ca^{2+}. An effect at platelet $P2Y_{12}$ receptors was also demonstrated: N0204-003 inhibited the ADP-induced reduction in cAMP in forskolin-stimulated platelets that occurs via this receptor. It also reduced inhibition of VASP phosphorylation by ADP. The intracellular Ca^{2+} response to the combination of ADP and thrombin receptor activating peptide (TRAP) occurs in two phases mediated by $P2Y_1$ followed by $P2Y_{12}$. The combination of MRS2179 and AR-C69931MX reduces the Ca^{2+} response to that seen with TRAP alone. N0204-003 behaved like the combination of MRS2179 and AR-C69931MX. It also behaved like a combined $P2Y_1$ and $P2Y_{12}$ antagonist in its effects on collagen-induced platelet aggregation. N0204-003 had no effect on the platelet shape change induced by α,β-methylene-ATP indicating that it does not act at $P2X_1$ receptors. We conclude that N0204-003 inhibits platelet function through antagonism at both $P2Y_1$ and $P2Y_{12}$ receptors on platelets.

DEVELOPMENT OF REVERSIBLE $P2Y_{12}$ RECEPTOR ANTAGONISTS

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ADP-induced platelet aggregation requires the simultaneous activation of $P2Y_1$ and $P2Y_{12}$ receptors. Antagonism of either receptor results in inhibition of platelet aggregation and prevention of thrombosis. We have developed novel reversible $P2Y_{12}$ antagonists for potential use as antithrombotic agents. In this study we have characterized the antiplatelet activity of some of the most attractive compounds *in vitro* and *in vivo*. INS50589 and related compounds produced a dose-dependent and reversible inhibition of ADP-induced platelet aggregation. Administration of $P2Y_{12}$ antagonists in mice resulted in inhibition of *ex vivo* ADP-induced platelet aggregation and prevention of mortality induced by systemic thromboembolism triggered by the administration of collagen and epinephrine. Intravenous administration of $P2Y_{12}$ receptor antagonists in rats, cynomolgus monkeys and dogs produced dose-dependent inhibition of ADP-induced platelet aggregation. Steady-state effect on platelet aggregation occurred within 30 minutes of initiating continuous IV infusion, was maintained during the infusion at all doses administered, and the effect was rapidly reversed upon termination of the infusion. No adverse effects on clinical signs or histological observations were observed in preliminary non-clinical toxicological studies. In conclusion, we have developed well tolerated, potent and reversible $P2Y_{12}$ antagonists for potential use in acute episodes of vascular thrombosis where rapid, and readily reversible pharmacokinetics are necessary for maintaining a tight control of platelet function.

NEW DRUGS FOR OLD TARGETS

CS-747 (LY640315): A NEW THIENOPYRIDINE-TYPE $P2Y_{12}$ ANTAGONIST

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Clopidogrel, and to a lesser extent ticlopidine, are widely used antiplatelet agents of the thienopyridine class of oral platelet aggregation inhibitors. CS-747 (LY640315) is a novel thienopyridine providing more potent antiplatelet activity *in vivo*. Like ticlopidine and clopidogrel, CS 747 is inactive *in vitro*. Following oral dosing, an active metabolite is formed that binds to and irreversibly blocks the $P2Y_{12}$ platelet adenosine diphosphate (ADP) receptor, thus inhibiting platelet aggregation mediated by this receptor. The anti-aggregatory effects of CS-747 in rats are evident 0.5 hour after a single oral administration and remain for

up to 72 hours.[1] Furthermore, high-grade platelet inhibition in rats is achieved more rapidly following single doses of CS-747 than with clopidogrel. The platelet inhibitory effects of CS-747 following repeat dosing were studied in rats, dogs, and monkeys, and in each case, cumulative platelet inhibition was observed. Maximal inhibition of ADP-induced platelet aggregation by CS-747 was reached after 3 to 5 days and was maintained throughout drug administration. On a dose comparison basis, CS-747 was approximately 10- and 100-fold more potent than clopidogrel and ticlopidine, respectively. After drug discontinuation, platelet function gradually returned over subsequent days. While most effective against ADP-induced aggregation, CS-747 also inhibited aggregation induced by other platelet activators. Oral administration of CS-747 to rats antagonized the binding of [^{3}H]-2-methylthio-ADP to platelets and ADP-mediated attenuation of PGE1-induced adenylyl cyclase activation, but did not inhibit platelet shape change. These results are consistent with the irreversible blockade of platelet $P2Y_{12}$ ADP receptors following oral dosing with CS-747. The antithrombotic effects of CS-747 were investigated and compared with those of clopidogrel and ticlopidine in several experimental rodent models of thrombosis. In these models, CS-747 (on a mg/kg basis) demonstrated approximately 10 and 100 times more antithrombotic activity compared to clopidogrel and ticlopidine, respectively. CS-747 prolonged the bleeding time in rats, similar to the effects of clopidogrel and ticlopidine. The degree of bleeding time prolongation was related to the dose and inherent antiplatelet potency. Combined administration of CS-747 with aspirin to rats and dogs produced markedly potent inhibition of collagen-induced platelet aggregation compared to each agent alone. The combination of CS 747 with aspirin resulted in a more pronounced antithrombotic effect in a rat arteriovenous shunt thrombosis model, but did not show any significant prolongation of the bleeding time compared to each agent alone. These results suggest that the combination of CS-747 with aspirin will provide greater antithrombotic efficacy than either agent alone. A hepatic metabolite of CS-747 (R-138727) has been identified and studied *in vitro*,[2,3] demonstrating direct and irreversible inhibitory effects on human platelets that is most effective against ADP-induced aggregation. The active metabolite also antagonized [^{3}H]-2-methylthio-ADP binding to platelets, ADP-mediated neutralization of PGE1-induced adenylyl cyclase activation, and ADP-induced [^{125}I]-fibrinogen binding. Initial clinical studies examined the effects of CS-747 on *ex vivo* ADP-induced platelet aggregation following both single- and multiple-dose administration in healthy volunteers. Single oral dosing resulted in dose-dependent inhibition of platelet aggregation in the 2.5 mg to 75 mg range, achieving approximately 70% inhibition at the higher doses of CS-747. Repeat dosing at 24-hour intervals with either 2.5 mg or 10 mg CS-747 resulted in inhibition of ADP-induced platelet aggregation which accumulated over the days of dosing, reaching a steady state by Days 2-4. As was observed with the single dose studies, recovery following the last dose on Day 9 was gradual over the subsequent wash out period. Previous studies in which the platelet-inhibitory activity of clopidogrel were followed under identical conditions confirmed that 10 mg CS-747 would be expected to achieve equal or greater inhibition of aggregation as 75 mg clopidogrel, the currently approved maintenance dose. This clinical experience is consistent with the preclinical profile of CS-747 and further supports the conclusion that following oral administration, CS-747 is converted to an active metabolite that binds to and irreversibly inhibits platelet P2Y12 ADP receptors. *Conclusion.* Thienopyridine P2Y12 inhibitory prodrugs are valuable agents for the treatment of occlusive vascular diseases. CS-747 is a novel and more recently developed member of this class. The attractive preclinical profile of CS-747 shows it to be more potent and faster acting than ticlopidine and clopidogrel. Successful Phase I clinical studies have guided the selection of doses currently being evaluated in patients with atherothrombotic disease. CS-747 (LY640315) is expected to provide improved benefit over existing therapies.

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SINGLE DOSE PHARMACOKINETICS AND PHARMACODYNAMICS OF AZD6140- AN ORAL REVERSIBLE ADP RECEPTOR ANTAGONIST

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Background. AZD6140 is an oral, direct acting, reversible ADP receptor antagonist acting at the $P2Y_{12}$ receptor. AZD6140 potently inhibits the binding of the $P2Y_{12}$ specific ligand AR-C98937 to human washed platelets (Ki 2 nM). It is currently under development for the prevention of arterial throm-

boembolic events. Aim: This study evaluated the tolerability, pharmacokinetics and pharmacodynamics of single doses of AZD6140 in healthy volunteers. *Methods*. Ascending single doses of AZD6140 oral suspension from 30 to 400 mg were studied in 13 healthy volunteers (12 male, 1 female). Blood samples for determination of AZD6140 and AR-C126910 (active metabolite) plasma levels were obtained at multiple timepoints after dosing through 36 hours. ADP stimulated platelet aggregation in platelet rich plasma using optical aggregometry was measured at 0 (predose), 2, 4, 12 and 24 hours after dosing. Safety assessments (adverse events, vital signs, ECG, laboratory) were also done. *Results*. AZD6140 was well tolerated in this study with no dose related adverse events and no effects on vital signs, ECG or laboratory tests. The pharmacokinetic parameters of AZD6140 are shown below. AZD6140 was rapidly absorbed and showed linear, dose proportional pharmacokinetics best fit by a two compartment open model. Platelet aggregation inhibition was dose and time dependent with the 300 and 400 mg doses achieving and maintaining high levels of inhibition over 24 hours. *Conclusion*. AZD6140 is a promising new oral antiplatelet agent with a favorable pharmacokinetic and pharmacodynamic profile.

Table 1. AZD6140 pharmacokinetic parameters following administration of single oral doses: Study SC 532 5171.

Dose (mg)	*N*	C_{max} *(ng/mL)*	t_{max} *(h)*	*AUC (ng.h/mL)*	*CL/F (mL/min/kg)*	$t_{1/2}$ *(h)*
30	7	158 (20.5)	1.50 (1.00-2.00)	995 (14.3)	6.63 (17.7)	7.71 (13.1)
100	9	565 (28.9)	1.50 (1.00-4.10)	3612 (20.4)	6.37 (22.4)	7.19 (18.9)
200	8	1237 (32.2)	1.49 (1.00-3.00)	7960 (25.7)	5.56 (24.1)	8.02 (14.1)
300	8	1721 (18.1)	1.50 (1.00-3.05)	12849 (22.6)	5.17 (23.5)	7.51 (14.0)
400	7	2653 (21.0)	1.50 (1.00-2.00)	18062 (23.7)	4.88 (25.8)	7.82 (13.2)

Table shows geometric mean (CV), except for tmax where median (range) is shown.

NITRIC-OXIDE RELEASING ASPIRIN

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Despite more than a century of use, the benefits of aspirin treatment have not ceased to be exploited in different therapeutic areas, from inflammation to cardiovascular disease, through cancer chemoprevention. Aspirin is approved for the treatment of acute coronary heart disease, and is also recommended by the American Heart Association for secondary cardiovascular prevention, in patients at risk. However, aspirin presents some limitations too, such as gastro-intestinal ulceration with consequent bleeding, even at low doses for long term treatment; an antiplatelet activity limited to the thromboxane (Tx)A2-dependent pathways of platelet activation; the lack of any proven anti-atherosclerotic activity. Nitric Oxide (NO) is a small gaseous free radical molecule playing a very important role as mediator of the vasoactive and antithrombotic properties of the endothelium and of other blood cell types, showing also anti-inflammatory effects. In addition, it has been proven to be a protective agent for the gastrointestinal mucosa. An elegant approach for exploiting the properties of both aspirin and NO, and possibly to increase overall activity and tolerability, has been to create a new molecule in which aspirin and a NO-donating group are covalently linked. NCX 4016 (nitroaspirin) is able to release enzymatically, thus in the presence of cells or plasma, both components. Nitroaspirin has been extensively studied in vitro and shown to inhibit several aspect of platelet function, imparing platelet aggregation induced by different stimuli, platelet adhesion to collagen under flow conditions, high-shear stress-induced platelet activation and to inhibit the expression of adhesion molecules (GPIIb/IIIa and P-selectin) in response to thrombin, actions that aspirin does not exert.[1,2] At the molecular level, NCX4016 acetylates COX-1 with a similar potency as aspirin, but in addition it acts as a weak inhibitor of COX-2 in human whole blood[3] and it also interferes with lipoxygenase activity. Moreover, NCX4016 reduces the expression of cytokines (TNFα, IL-6) by LPS-stimulated monocytes.[4] Furthermore, it inhibits rat aortic smooth muscle cell proliferation.[5] Nitroaspirin has been tested in several animal models of thrombosis, tissue ischemia and/or atherogenesis, with interesting results. Namely, studies in the rat have shown antithrombotic activity in an extracorporeal circuit upon chronic administration of the drug.[6] Moreover, nitroaspirin was effective in preventing platelet pulmonary thromboembolism *in vivo*, in mice and rabbits.[7] Interestingly, the antithrombotic effects of nitroaspirin seem to be accompanied by only a minor pro-hemorrhagic activity.[8] In addition, the combination of nitroaspirin with the ADP-receptor antagonist, clopidogrel, leads to an enhancement of the antithrombotic activity which appears to be larger than that observed with the combination aspirin plus clopidogrel, but with a lower prohemorrhagic potential.[8] Nitroaspirin also exerts tissue protective effects in ischemia/reperfusion damage models, such as focal cerebral ischemia in the spontaneous hypertensive rats[9] or myocardial ischemia in the rabbit or rat[10] or even in the anesthetized pig.[11] Moreover, chronic treatment with NCX4016 was able to restore the impaired endothelium-dependent relaxation to acetylcholine in diabetic rats.[12] Finally, and very interestingly, nitro-

aspirin prevented restenosis and atherogenesis in hypercholesterolemic mice or aged rats,[13,14] in ApoE knockout mice[15] or even in normal mice after localized, oxygen radicals-induced arterial damage.[8] Another interesting observation shows that NCX 4016, but not aspirin, was able to blunt significantly the early rise of matrix metalloproteinase 9 (MMP9) and the late rise of MMP2 in plasma of mice injected with sub-lethal doses of the inflammatory stimulus lipopolysaccaride.[16] Given that MMPs are thought to play an important role in vascular inflammation and remodelling, blunting effects of NCX 4016 on MMPs rise further suggest potential benefit in clinical conditions associated with chronic inflammation, such as atherosclerosis, diabetes, etc. All the above pharmacological effects in animals were associated with no gastrointestinal damage, differently from aspirin, even in presence of a complete inhibition of gastric prostaglandin synthesis.[17] NItroaspirin has been tested in humans in phase I and phase II studies and is now under phase III clinical testing. A phase I study in healthy volunteers has shown antiinflammatory effects, not shared by aspirin, by reducing the rise of a number of cytokines and other inflammatory markers elicited by the i.v. administration of LPS.[18] A study of gastric tolerability in healthy volunteers, comparing 800 mg b.i.d. NCX 4016 with 420 mg b.i.d. aspirin for seven days, has been carried out showing significantly better gastric tolerability.[19] In this study NCX 4016 inhibited ex vivo platelet aggregation induced by arachidonic acid (AA) and suppressed platelet thromboxane production to an extent similar to aspirin, at least when a low dose of the platelet stimulus was used.[19] Endothelial dysfunction is an early marker of atherosclerosis and an independent predictor of ischemic events in patients with intermittent claudication (IC). Ischemia-reperfusion injury, associated with walking under ischemia, worsens systemic endothelial dysfunction, reducing nitric oxide (NO) biosynthesis or activity. A phase II study was thus set up to evaluate whether nitroaspirin in comparison with aspirin prevents ischemia reperfusion injury-induced endothelial dysfunction in IC.[20] A study on the effects of NCX4016 vs aspirin on the in vivo platelet activation induced acutely in diabetic patients by a short-term, controlled hyperglycemia has also been performed and recently complete.[21] Finally, a large, international, multicenter, randomised, double-blind study in 440 patients with intermittent claudication is now underway to assess whether the addition of NCX 4016 for six months to conventional treatment, including low-dose aspirin, improves the walking capacity of patients with peripheral arterial disease, stage II. On the basis of the above summarized pharmacologic characteristics, NCX-4016 appears to possess a unique therapeutic profile. Theoretical limitations to the anti-atherothrombotic activities of this molecule in the clinical could be: side-effects due to the vasoactive properties of NO; incomplete protection from in vivo platelet activation due to the relatively short duration of the NO-effects; development of vascular or platelet tolerance to NO. Only large clinical studies comparing NCX 4016 with established treatments will say whether all the above-described properties translate into short and long-term clinical advantage in cardiovascular diseases.

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THROMBOXANE RECEPTOR ANTAGONISTS

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Renewed interest in thromboxane receptor (TP) antagonists is related to the role of aspirin-insensitive TP agonists in atherothrombosis. These include isoprostanes and isothromboxanes, products of non-enzymatic lipid peroxidation, as well as aspirin-resistant thromboxane production that can derive from inflammatory cells and/or newly formed platelets expressing cyclooxygenase-2.

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Oral Communications III

PLATELET EXPRESSION OF NON-FUNCTIONAL P2X1DELL ION CHANNELS IN MICE REDUCES ARTERIAL THROMBOSIS

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ATP is the physiological agonist at the $P2X_1$ ion channel. *In vitro*, $P2X_1$ mediates a transient intracellular Ca^{2+}-influx responsible for platelet shape change, and contributes to platelet aggregation induced by low doses of collagen and by shear forces. More importantly, over-expression of platelet $P2X_1$ ion channels in transgenic mice, as well as $P2X_1$ gene-disruption revealed a role for this ion channel in the formation of platelet thrombi, particularly in conditions in which shear forces are high.

Presently, we have further investigated the role of platelet $P2X_1$ in a murine model of shear stress-controlled thrombosis. To this aim, we have generated transgenic mice expressing non-functional human $P2X_1$delL subunits in the megakaryocytic cell lineage, i.e. a naturally occurring mutant identified in a patient with a severe bleeding disorder. Platelets of these mice display defective of $P2X_1$ function during in vitro collagen-induced platelet aggregation, resembling that due to treatment of WT murine platelets with the selective $P2X_1$ antagonist NF279. Surprisingly, in both cases, platelet shape change produced by the $P2X_1$ agonist α,β-meATP was normal, suggesting a dual platelet $P2X_1$ functionality. Photochemically induced thrombosis in murine intestinal arterioles and venules, was then analysed via real-time videomicroscopy.

We compared vessel occlusion times in WT mice and in transgenic mice overexpressing human $P2X_1$ or non functional human $P2X_1$delL, as well as during the pharmacological inhibition of this ion channel with NF279. Overexpression of human $P2X_1$ strongly shortened arterial occlusion times, whereas occlusion was delayed in arterioles of mice expressing P2X1delL, and this to a similar extent as in WT mice treated with NF279. NF279 did not further affect arterial thrombosis in P2X1delL-expressing mice. In

contrast, thrombosis in venules did not significantly differ. Thus, these data demonstrate that, in addition to ADP, co-released ATP, by activating the platelet P2X1 ion channel controls shear- and platelet-dependent thrombosis, a process that can efficiently be inhibited with NF279 *in vivo*.

P2X$_1$ SYNERGISES WITH P2Y$_1$ AND GI COUPLED-RECEPTORS TO ENHANCE CA^{2+} MOBILIZATION AND AGGREGATION

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Activation of the P2X$_1$ receptor by its specific agonist, α,β-methylene ATP (α,β-M-ATP) results in a transient Ca^{2+}influx which has been shown to amplify platelet activation by other agonists. Using flow cytometric determination of calcium mobilisation and whole blood single-platelet counting assessment of microaggregation, we studied the interactions between P2X$_1$, P2Y and β2A-adrenergic receptors. Blood samples from healthy volunteers were anticoagulated with hirudin in the presence of apyrase (1U/ml). PRP was labelled with fluo-3 AM for studies of calcium mobilisation and diluted 1:100 at the time of agonist stimulation. Platelet aggregation was measured by whole blood single-platelet counting. Epinephrine (EPI, 0-10 μM), ADP (0-1 μM), α,β-M-ATP (0-3 μM) were added either singularly or in combination. The following antagonists were used: MRS2159 10 μM (P2X$_1$), MRS2179 100 μM (P2Y1), AR-C69931MX 100nM (P2Y$_{12}$), yohimbine 10 μM (β2A) and, to prevent Ca^{2+} influx, EGTA. Co-stimulation with both P2X$_1$ and P2Y agonists lead to an increase in the peak and duration of Ca^{2+} levels. This effect was evident at low concentrations of both agonists and was strongly inhibited by either P2X$_1$ or P2Y$_1$ antagonists. α,β-M-ATP amplified ADP-induced (P2Y-mediated) platelet aggregation. Co-stimulation of Gi-coupled receptor (using EPI) and P2X$_1$ increased the duration of the Ca^{2+} response which was prevented by the addition of the P2X$_1$ antagonist or EGTA confirming that it is mediated by Ca^{2+} influx. This combination of agonists was also associated with an increase in the extent of platelet aggregation, which was dependent on concomitant P2Y receptor activation by released ADP. These studies have utilised two techniques that can sensitively detect subtle interactions between receptors. The P2X$_1$ receptor amplifies calcium mobilisation and platelet aggregation mediated via P2Y receptors. Activation of Gi-coupled receptors amplifies P2X$_1$-mediated Ca^{2+} mobilization. These techniques may further understanding of the complex interactions that occur in the presence of multiple agonists.

Receptors	*Time point*	*0.1μMαβ M-ATP*	*0.03μM ADP*	*αβM-ATP + ADP*
P2X$_1$ + P2Y	Peak	0.13	0.12	0.36*
	30sec	0.11	0.11	0.19*
		0.1μMαβ M-ATP	*10μM EPI*	*αβ M-ATP + EPI*
P2X$_1$ + Gi	Peak	0.18	0.08	0.23
	30sec	0.13	0.09	0.17**

*Ca^{2+} levels (μM) in response to co-stimulation of P2X$_1$ + P2Y and P2X$_1$ + Gi *p<0.02, **p<0.001.*

EFFECT OF NF449, A NOVEL SELECTIVE P2X$_1$ RECEPTOR ANTAGONIST, ON PLATELET ACTIVATION AND THROMBOSIS

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The platelet P2X$_1$ receptor has been shown to play a role in platelet activation and the thrombosis of small arteries. Synthesis of a potent antagonist of this receptor, NF449 (4,4',4'',4'''-(carbonylbis(imino-5,1,3-benzenetriylbis(carbonylimino))) tetrakis-benzene-1,3-disulfonic acid), which displays good selectivity for P2X$_1$ among the P2X$_3$, P2X$_7$, P2Y$_1$, P2Y$_2$ and P2Y11 receptor subtypes, was recently reported (*Kassack* 2004). Our aim was to determine whether NF449 selectively antagonizes the platelet P2X1 receptor and how it affects platelet functions in vitro and *in vivo*. The selective and stable P2X$_1$ agonist αβMeATP induced an influx of calcium (EC_{50}=200±30 nM) and transient shape change (EC_{50}=880±310 nM) of washed human platelets, provided the ADP-removing enzyme apyrase (0.9U/mL) was added to prevent desensitization of the P2X1 receptor. Both events were dose-dependently inhibited by NF449 (IC_{50}=104±4nM and 83±13nM, respectively). However, under conditions where the P2X$_1$ was desensitized, i.e. in the presence of a lower concentration of apyrase (0.02 U/mL), NF449 inhibited ADP-induced platelet aggregation (IC_{50}=34±8 μM) and the P2Y$_1$ receptor-mediated $[Ca^{2+}]_i$ rise (IC_{50}=6±2 μM), indicating that it could also antagonize P2Y1. In contrast, NF449 did not reverse the P2Y$_{12}$ receptor-mediated inhibition of adenylyl cyclase. Thus, P2X$_1$ activation could be totally inhibited at 0.3 μM NF449, leaving the P2Y$_1$ and P2Y$_{12}$ receptors fully functional. At this concentration, NF449 inhibited collagen-induced platelet aggregation, confirming a role of the P2X$_1$ receptor

in platelet activation to this agent. In an *in vivo* model of systemic thromboembolism induced by infusion of a mixture of collagen and adrenaline, mice injected with NF449 (50 mg/kg, iv) displayed reduced platelet consumption, reflecting decreased intravascular platelet aggregation, as compared to mice injected with saline (33±2 versus 71±4%). Hence this molecule would appear to be effective *in vivo*. Overall our results indicate that NF449 constitutes a new tool to investigate the functions of the $P2X_1$ receptor and a starting compound to design more selective and potent $P2X_1$ antagonists.

STUDIES OF THE SPECIFICITY OF THE SURAMIN ANALOGUE (NF449) AS $P2X_1$ RECEPTOR ANTAGONIST

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It has been shown that $P2X_1$ plays an important role in platelet aggregation and thrombus formation under high shear conditions, making it an attractive molecular target for antithrombotic intervention. In this study, we further characterized the suramin analogue, 4,4',4'',4'''-(carbonylbis(imino-5,1,3-benzenetriyl-bis(carbonylimino)))tetrakis-benzene-1,3-disulfonic acid (NF449), a compound of proven antagonistic activity (IC_{50}=0.05 nM) on $P2X_1$ recombinantly expressed in Xenopus oocytes. *Study 1.* NF 449 was examined at $P2Y_1$, $P2Y_2$, and $P2Y_{11}$ receptors recombinantly expressed in 1321N1 astrocytoma cells using as agonists 2-methylthio-ADP for $P2Y_1$, UTP for $P2Y_2$, and ATP≥S for $P2Y_{11}$, respectively. The effect of NF449 on these receptors was estimated by measuring the inhibition of agonist-induced increase in intracellular Ca^{2+}. NF449 had no effect at $P2Y_1$ and $P2Y_2$ up to a concentration of 3.2 mM. $P2Y_{11}$ receptors were inhibited by NF449 with an IC_{50} of ~180 μM. Study 2. The effect of NF449 on platelet function was studied in normal human platelet-rich plasma (PRP) anticoagulated with PPACK, in the presence of apyrase (0.5 U/mL), to prevent $P2X_1$ desensitization. Inhibition of the $P2X_1$-dependent platelet shape change induced by 1 and 10 μM α,β,methylene-ATP was complete at NF449 concentrations of 10^{-5M} and 10^{-4M}, respectively. Only at the highest concentration tested (10^{-4M}), NF449 partially inhibited ADP (1-10 μM)-induced platelet aggregation, but had no effects on ADP-induced shape change or adenylyl cyclase inhibition. $P2X_1$ desensitization with α,β,methylene-ATP had no inhibitory effects on ADP-induced platelet responses, indicating that the inhibitory effects of NF449 on these platelet responses were not mediated by $P2X_1$ inhibition. NF449 (10^{-5} and 10^{-4}) partially inhibited collagen (2 μg/mL)-induced platelet aggregation; similar effects were observed after $P2X_1$ desensitization with α,β,methylene-ATP. In conclusion, NF449 proved a rather specific antagonist of $P2X_1$, receptor recombinanlty expressed on astrocytoma cells. Its antagonist activity was less specific in platelets.

NEW POTENTIAL TARGETS

THE $P2Y_1$ RECEPTOR AS A NEW POTENTIAL TARGET FOR ANTIPLATELET DRUGS

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Blood platelets express three separate P2 receptors, the ionotropic $P2X_1$ channel activated by ATP and the metabotropic $P2Y_1$ and $P2Y_{12}$ receptors coupled to Gq and Gi2 respectively, both activated by ADP. The $P2X_1$ receptor is involved in platelet shape change, activation by collagen and thrombus formation under high shear (*Rolf et al., 2001; Oury et al., 2001; Hechler et al., 2003*). The $P2Y_1$ receptor initiates platelet aggregation through mobilisation of intracellular calcium stores while the $P2Y_{12}$ receptor amplifies the responses to all agonists and stabilizes the aggregates (*Gachet, 2001*). Among the platelet P2 receptors, the $P2Y_{12}$ receptor is the molecular target of the efficient antiplatelet drug clopidogrel (*Herbert & Savi, 2003*) and several competitive antagonists which are under clinical evaluation (*Humphries, 2000*). Its expression seems to be limited to the megakaryocytic lineage although several other cell types have been shown to express $P2Y_{12}$ transcripts. It is thus a well established target for antiplatelet drugs (*Herbert & Savi, 2003; Conley & Delaney, 2003*). Less is known concerning the $P2X_1$ and the $P2Y_1$ receptors which are broadly expressed in many tissues. However, studies in knock-out mice and experimental thrombosis models using selective antagonists have shown that both the $P2X_1$ and the $P2Y_1$ receptors are potential targets for new antithrombotic drugs. This paper will focus on the $P2Y_1$ receptor only. When platelets are activated by ADP, the $P2Y_1$ receptor promotes the shape change and weak and transient aggregation. When the $P2Y_1$

receptor is inhibited platelet aggregation and shape change do not occur in response to ADP indicating that this receptor is necessary for platelet aggregation to ADP (*Hechler et al., 1998*). It has also been demonstrated to be involved in collagen-induced platelet shape change (*Mangin et al., 2004*). Finally, P2Y1-deficient mice display mildly prolonged bleeding time as compared to the wild type (*Fabre et al., 1999; Léon et al., 1999*). Obviously, such a key role in platelet activation and haemostasis, suggested this receptor to play a role in arterial thrombosis. Indeed, P2Y1-deficient mice display increased resistance to thromboembolism induced either by a mixture of collagen and adrenaline or by tissue factor (*Léon et al., 1999; Léon et al., 2001*). In the latter case, in vivo thrombin generation was reduced in the knock-out animals suggesting the $P2Y_1$ receptor to be involved somehow in thrombin generation. This was confirmed later, showing that the $P2Y_1$ receptor is involved in P-selectin exposure and subsequent formation of platelet-leucocyte aggregates as well as exposure of tissue factor on leucocytes (*Léon et al., 2003*). In both cases of experimental thromboembolism, mortality as well as platelet consumption were reduced in the deficient mice. Moreover, IV administration to mice of the $P2Y_1$ antagonist MRS2179 similarly resulted in prolongation of the bleeding time, inhibition of *ex vivo* platelet aggregation to ADP and resistance to thromboembolism induced by collagen and adrenalin or by tissue factor. These results clearly demonstrated that the P2Y1 receptor represents a potential target for antithrombotic therapy (*Baurand & Gachet, 2003*). Models of localized arterial thrombosis have also been used to further evaluate the $P2Y_1$ receptor as a target for antiplatelet therapy. In a model of superfusion of ferric chloride to induce injury of mesenteric arterioles and intravital microscopy examination it could be shown that $P2Y_1$ inhibition was as efficient as $P2Y_{12}$ inhibition. Indeed, either $P2Y_1$ knock-out mice or MRS2179 treated mice (50 mg/kg, IV, bolus) displayed similar reduced thrombosis scores as clopidogrel treated mice (50 mg/kg, PO). Interestingly, the treatment of $P2Y_1$ deficient mice by clopidogrel resulted in additive inhibition of thrombosis, suggesting that a combination of drugs targeting both $P2Y_1$ and $P2Y_{12}$ could be of benefit (*Lenain et al., 2003*). A second model of localized arterial thrombosis consisted in laser injury of mesenteric arterioles. In this case, $P2Y_1$-deficient mice or MRS2179 treated mice again displayed reduced thrombosis as compared to the wild type but to a lesser extend as compared to mice treated with maximal doses of clopidogrel. This model revealed differences in the behaviour of $P2Y_1$ and $P2Y_{12}$ treated animals, with the suggestion that in $P2Y_1$-deficient mice the thrombus could be less stable and embolize more easily, while the $P2Y_{12}$ inhibition resulted in the absence of any thrombus formation (*Lenain et al., unpublished data*). Whether combination of $P2Y_1$ and $P2Y_{12}$ antagonists could have additive or synergistic effects remains to be determined in such a model. One problem is the requirement of potent and selective antagonists of the $P2Y_1$ receptor. Such compounds only begin to exist as for example MRS2500 which has an EC50 around 1 nM as antagonist of platelet aggregation to ADP, which should help in further assessing the relevance of the $P2Y_1$ receptor as a target for antithrombotic drugs (*Jacobson 2003*). In addition, numerous models of experimental thrombosis, where the $P2Y_{12}$ targeting drugs have been demonstrated to be efficient, have not yet been used to evaluate $P2Y_1$ antagonists. These include stenosed and injured coronary arteries in animals such as dogs or non human primates, the Folts's model of cyclic flow variations and other well known models. All these models would require high amounts of compounds which are not yet available. Thus, one has to hope for new potent, in vivo stable, $P2Y_1$ antagonists to further evaluate this target for itself and /or in combination with other P2 receptor antagonists. The idea here is that one could combine the tissue selectivity of $P2Y_{12}$ with the lesser impact of $P2Y_1$ on the bleeding time in order to build compounds with high efficacy and also better safety.

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NEW POTENTIAL TARGETS FOR ANTIPLATELET DRUGS: P2X1

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Despite the fact that the currently available drugs inhibiting platelet function have a good risk-to-benefit ratio, they suffer some drawbacks, which justifies the unceasing search for agents that can further improve the clinical outcome of patients with ather-

osclerosis through greater efficacy and/or safety. New platelet targets for potential antithrombotic drugs include several receptors and effectors that are important for platelet function. They have recently been reviewed.[1] The ideal antithrombotic drug should selectively target the "bad" fibrin and platelet aggregates of pathologic thrombi, without affecting the "good" fibrin and platelet aggregates of the hemostatic plug. The search for such a drug, with an ideal benefit-to-risk ratio, has been unsuccessful so far and chances are high that it will be elusive also in the near future, because, based on our current knowledge, it is difficult to identify a pathogenic mechanism operating selectively during pathologic thrombosis that is not involved also in normal hemostasis. Perhaps the only exception is represented by P2X1, an ATP receptor that is expressed on the platelet surface, which seems to be important for pathological platelet thrombus formation at high shear.

The P2X1 receptor is a fast ATP-gated cation channel that is present in blood platelets and other cells of the body. Its role in platelet activation has been difficult to study because it undergoes rapid desensitization by ATP released during the preparation of platelet-rich plasma (PRP). Rolf and Mahuat-Smith showed that the non hydrolysable P2X1 agonist, α–β-methylene-ATP induces a transient platelet shape change in platelet preparations to which the ATP degrading enzyme apyrase was added to prevent receptor desensitization.[2] Further studies performed using the traditional light-transmission aggregometer have indicated that P2X1 may have a marginal role in platelet aggregation induced by threshold concentrations of collagen.[3] However, these studies may not reflect accurately the situation at sites of vascular injury, where platelets interact with thrombogenic subendothelial components under flow conditions that generate variable levels of shear stress, which are highest in small arterioles and, in pathological conditions, at the top of atherosclerotic plaques partially occluding the arterial lumen. Using a cone-and-plate viscometer that allows monitoring platelet aggregation in real-time, we studied the role of P2X1 in shear-induced platelet aggregation (SIPA) using PPACK-anticoagulated human PRP containing apyrase (1.5 U/mL), which prevented P2X1 desensitization. The results were compared to those obtained 30 sec after the addition of α–β–methylene-ATP (10 µmol/L) to apyrase-PRP, which caused P2X1 desensitization. Using this experimental approach, we showed that platelet aggregation in apyrase-PRP started after a lag-phase of 15.5±7.7 s (n=8) from the application of high shear stress (108 dynes/cm^2) and the signal measuring light transmission reached a maximal amplitude of 3.8±2.5 nW. In contrast, after P2X1 desensitization with α–β–methylene-ATP, platelet aggregation was delayed (lag phase, 36.3±15.2, $p<0.001$) and its amplitude was decreased (1.9±1.5, $p<0.01$). The mean concentrations of platelet factor 4 (PF4) 30 s after the application of shear stress were 388±92 ng/mL in platelet-poor plasma (PPP) from apyrase-PRP and 274±69 in PPP from apyrase-PRP after P2X1 desensitization with α–β–methylene-ATP ($p=0.02$), indicating that P2X1 plays a role in platelet secretion induced by high shear stress. These results indicate that P2X1 stimulation by released ATP may play an important role in accelerating and amplifying platelet secretion and aggregation under flow conditions that are relevant particularly for the development of thrombi at sites of severe arterial stenosis, where very high shear forces are generated.[4] The important role of P2X1 in SIPA has recently been confirmed by other investigators.[5] Hechler *et al*., in studies of P2X1-/- mice, showed that, *in vitro*, the P2X1 receptor contributes to the response of platelets exposed to collagen under flow conditions characterized by elevated shear stress, and enhances thrombosis in injured small arteries *in vivo*.[6] The bleeding time was normal in most of the P2X1-/- mice, indicating that lack of the P2X1 receptor has a modest impact if any on primary hemostasis in the microcirculation, opening the intriguing possibility that such target may be more relevant for pathological thrombus formation and less essential for normal hemostasis.

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GPVI: A NEW TARGET FOR ANTITHROMBOTIC DRUGS

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Platelet-collagen interactions play a fundamental role in the process of arterial thrombosis. Therefore, collagen receptors are attracting targets for the development of new antithrombotic drugs. Platelets contain two well-characterized, functionally impor-

tant receptors interacting directly with collagen, the integrin $\alpha 2\beta 1$ that has a wide tissue expression and GPVI which expression is restricted to the platelet lineage. GPVI, a 62 kDa glycoprotein, is non-covalently complexed with the Fc receptor (FcR) γ chain and present at a moderate number of copies on the platelet surface (~3000). The GPVI/FcRγ complex serves as the major activating receptor for collagen.[1] Interaction of collagen with GPVI acts in concert with soluble agonists such as ADP and thromboxane A2 to initiate signals that lead to activation of platelet integrins in turn leading to firm adhesion at sites of vascular injury and to platelet aggregation.[2,3] Collagen interaction with GPVI also stimulates platelet procoagulant activity that results from the exposure of phosphatidylserine and the shedding of microparticles. Variations in GPVI content lead to a corresponding change in GPVI-induced prothrombinase activity. Antagonising collagen interaction with GPVI thus appears as a promising mean to limit thrombus initiation and growth. This is supported by the observation that mice deficient in the GPVI-FcRγ complex are protected from thrombosis in different models.[4,5] Furthermore, deficiency in the FcRγ chain protects arteries from neointimal hyperplasia after injury[6]. A very interesting point is that GPVI deficiency does not produce an unacceptable bleeding tendency suggesting that GPVI antagonists could constitute safe antithrombotic drugs. Collagen interaction with GPVI can be antagonised at two levels, GPVI and collagen. GPVI recognizes a Gly-Pro-hydroxyPro motif apparently present at a low density on collagen fibres. It is thus conceivable that a molecule able to bind to this motif would prevent its interaction with platelet GPVI. Such an approach has been made using a soluble recombinant protein composed of the extracellular domain of GPVI fused with the human IgG Fc domain. This protein binds to collagen but hardly inhibits the aggregation of human platelets.[7] It has been reported to inhibit thrombus formation in an arterial model of thrombosis in mice[8] but this was not confirmed in an other study. However, the use of such a large, Fc-bearing molecule as a drug in human does not appear realistic. The production of small molecules mimicking the collagen binding site on GPVI is a prerequisite to go on this approach. The only antagonists of GPVI that have so far been produced are antibodies. Monoclonal antibodies (MoAb) and scFv antibodies have been reported to inhibit GPVI interaction with collagen. The Fab fragments of the MoAb 9O12.2 and the scFv antibody 10B12 have similar effects:[2,3,9] inhibition of collagen-induced platelet secretion and integrins activation, of platelet procoagulant activity and of thrombus growth in flow conditions. Efforts have now to be made to prove the efficacy of these antibodies in animal models of thrombosis. It is expected that the *in vivo* effect will be prolonged in relation with a depletion in platelet GPVI as already observed in mice treated with MoAbs and in patients with anti-GPVI auto-antibodies.[4,10,11] The production of small inhibitors is a longer term objective. GPVI has not yet been crystallized but a three-dimensional model of its extracellular domain can be constructed using the x-ray structure of KIRs with which it presents large homologies. By comparing the sequences of human and mouse GPVI to which 10B12 binds differently on one hand, and by a phage display approach using 9O12.2 on an other hand, residues more or less involved in collagen binding have been identified. Mapping precisely the collagen binding site on GPVI will open the possibility to screen small molecule libraries.

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GAS6

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The coagulation pathway is composed of a series of protease reactions leading to the formation of a blood clot. Vitamin K-dependent proteins present in plasma are essential in the serine protease cascade. More recently, a role of several of these proteins on the inflammatory response is being deciphered. This effect is mediated through regulation of *classical* coagulation and fibronolytic reactions and also through direct interaction with cells. Gas6 and protein S are special members of the vitamin K-dependent proteins, as they do not contain a serine protease domain.[1,2] Instead, they have LG (laminin globular-like) modules capable of interacting with the three receptor tyrosine kinases of the Axl family, named Axl, Sky and Mer.[3-6] In contrast to protein S, Gas6 does not seem to have a direct effect on the coagulation cascade at physiological concentrations.[7] Instead, it has a potential effect in regulating cellular homeostasis through its antiapoptotic function and its capacity to induce cellular migration, aggregation and mitosis.[8,9] The gas6 knockout mouse has been an important model for suggesting possible roles of this molecule *in vivo*. Surprisingly, the mice showed a marked effect of the mutation on the thrombotic response.[10,11] Gas6 deficient mice form fewer thrombi when challenged with several stimuli, including bacterial endotoxin, nephrotoxic serum, and collagen-epinephrine injection, mechanical and chemical vascular wall damage, or blood stasis.[11] Gas6 also affects the cellular content of organs under stress situations, as observed in the progress of glomerulonephritis and atherosclerotic lesions. Recent studies centered in the effect of Gas6 in the vessel wall have shown that this molecule could be involved in processes of response to vascular injury, in models of restenosis, transplant arteriosclerosis and endotoxemia. In the vessel wall, Gas6 and its receptors are expressed in fibroblasts, pericytes and smooth muscle cells. The expression of Gas6 and its receptors is upregulated and activation of Gas6 receptors is increased in conditions of vascular injury, inflammation and repair.[12,13] The mechanism of action of Gas6 is in part mediated by its effect on the vascular endothelium. Endothelial cells deficient in Gas6 have a blunted response to mediators of inflammation, including a decreased induction of tissue factor expression. Carotid arteries and the aorta of mice deficient in Gas6 are less able to regulate vasodilation, especially after bacterial LPS-induced inflammation. This latter effect seems to be due to a reduced capacity of the Gas6-deficient endothelium to produce nitric oxide. Therefore, Gas6 appears to be a novel, important component in the regulation of responses of the vasculature both in acute inflammatory situations and in more chronic pathologies. Additionally, a clear thrombotic effect of Gas6 is mediated by its role on platelets.[11] Platelets of mice deficient in Gas6 have an aggregation deficiency when stimulated by ADP and collagen. Secretion of dense granule stores is also significantly impaired in Gas6-/- platelets, as shown by a decreased ATP release. Compared to WT platelets, release of ATP from Gas6-/- platelets was significantly decreased in response to ADP, collagen or U46619, when these agonists were used at low concentrations that only caused platelet shape changes or reversible platelet aggregation. ATP release from Gas6-/- platelets was also reduced in response to high concentrations of ADP or thrombin, consistent with the incomplete degranulation of thrombin-stimulated Gas6-/- platelets seen on ultrastructural preparations. However, release of ATP was normal or only slightly reduced when Gas6-/- platelets were stimulated with high concentrations of collagen (10 μg/mL) or U46619, which cause irreversible platelet aggregation. An autocrine role for Gas6 in platelets is suggested by the finding that Gas6 is present in alpha-granules and, following platelet activation, becomes secreted and bound to the platelet surface, most likely via Gas6 receptors. Since Gas6 deficient platelets have normal expression of the Gas6 receptors Mer, Axl or Tyro3, the platelet defects were not related to downregulation of these receptors. The platelet dysfunction of the Gas6-/- mice is translated *in vivo* into a protection of these mice towards pulmonary embolism induced by jugular injection of aggregating agents as collagen-epinephrine. Interestingly, this protective effect could be elicited by treating wild type mice with specific antibodies against Gas6. Collectively, the data on the literature are consistent with a model whereby Gas6 is released from the α-granules upon initial stimulation of platelets by several agonists. Subsequently, Gas6 amplifies, via signaling through one or more of its receptors, the intracellular signals generated from the ADP-, collagen-, TXA2- and thrombin-receptors. Similar phenotypes have been described for other mutant mice, as for instance that of the AKT1, AKT2 and PI3K deficient mice 14-16. Gas6 stimulation of the receptor tyrosine kinase family leads to Akt activation, therefore, a likely pathway for Gas6 function on platelets is the ligand-RTK-PI3K-Akt activation pathway. Among the Axl family of receptors, the MerTK receptor is the most likely candidate for being the target of Gas6 action on platelets. MerTK receptor was the predominantly expressed receptor of the family in human and murine platelets, whereas Axl and Tyro3

are not expressed or to a less extent than MerTK. MerTK-deficient mice generated by targeted disruption of the mer receptor gene showed impaired platelet aggregation induced by collagen, U46619, and PAR4 thrombin receptor agonist peptide at low concentrations *in vitro*. Again, this effect is reflected in an inhibition of acute arterial thrombosis in vivo in the MerTK-deficient mice. The available data, therefore, provides the first evidence that the Gas6-MerTK interaction participates in regulation of platelet function *in vitro* and platelet-dependent thrombosis *in vivo*.[17] From these studies, Gas6 arises as a component of the thrombo-inflammatory response. These results tend to suggest an implication of Gas6 in human pathology. This hypothesis would be further supported by genetic studies in the context cardiovascular disease.[18]

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PHOSPHOINOSITIDE 3-KINASE INHIBITORS AS POTENTIAL ANTITHROMBOTIC DRUGS

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Phosphoinositide 3-kinases (PI3K) are a family of enzymes able to phosphorylate phosphoinositides, a class of membrane phospholipids, at the D3 position of their inositol ring. These lipid kinases are activated by growth factors, cytokines, hormones or adhesive molecules and produce D3-phosphoinositides which are potent lipid second messengers (*Rameh and Cantley* 1999). D3-phosphoinositide binding domains such as pleckstrin homogy (PH), FYVE or Phox (PX) domains, allow specific interactions between PI3K products and several proteins involved in signal transduction or in intracellular trafficking (*Toker 2002, Payrastre 2001*). These domains play a critical role for signal-dependent membrane localization and, in some cases, in conformation changes of these proteins. PI3K products are now considered as key players in the spatio-temporal organization of several signaling pathways but also in vesicular trafficking and in cytoskeleton dynamics. D3-phosphoinositides are present in low amounts and their level is tightly controlled through the activation of the various PI3K isoforms and several specific phosphoinositide phosphatases. Three classes of PI3K (class I A and B, class II and class III) have been identified, based on their structure, mechanism of regulation and substrate specificity (Vanhaesebroeck and Waterfield 1999). Class I (A and B) PI3K has been investigated extensively these last years whereas classes II and III are still poorly characterized. Class IA PI3K is composed of a p110 catalytic subunit (either p110α, p110β or p110δ, encoded by 3 distinct genes) and a regulatory subunit (p85α, p55α, p50α, p85β and p55γ, derived from 3 distinct genes). These PI3Ks are classically activated via specific interac-

tions of their regulatory subunit with phosphotyrosine-containing proteins. Class IB PI3K is composed of a p110γ catalytic subunit associated with a p101 regulatory subunit and is activated by G protein-coupled receptors via G-protein β/γ subunits. The three classes of PI3K are present in human platelets where they produce the different D3-phosphoinositides (phosphatidylinositol 3-monophosphate; phosphatidylinositol 3,4-bisphosphate; phosphatidylinositol 3,5-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate). Initially, the use of two unrelated PI3K inhibitors, wortmannin and LY294002, which do not distinguish between the different forms of PI3K, suggested a role of these lipid kinases in the control of platelet activation induced by some agonists. These studies indicated that PI3Ks are important in regulating a broad range of functional platelet responses. They are critical for platelet activation via the collagen receptor GPVI and via FcγRIIa in cooperation with the ADP receptor P2Y12. In these cases, PI3K and its product phosphatidylinositol 3,4,5-trisphosphate are essential for PLCγ2 activation and Ca^{2+} signaling (*Gratacap et al. 1998, Pasquet et al. 1999*). There is also abundant evidence that PI3K play an important role in promoting and maintaining integrin GpIIbIIIa activation (*Trumel et al. 2001, Yap et al. 2002*). Recently, an implication of PI3K in platelet activation and cytoskeleton organization downstream of GpIb-IX-V has also been suggested (*Resendiz et al. 2003, Yap et al. 2002*). The development of mouse deficient in PI3K isoforms and the synthesis of PI3K-isoform-specific inhibitors provide now more precise informations on the role of these enzymes in different aspects of platelet activation. Both p110γ and p110δ deficient mice are viable and no specific bleeding problems have been described, suggesting that these PI3K isoforms are not essential in the haemostatic process. However, using p110γ deficient mice platelets, it has been shown that this isoform was implicated downstream of the P2Y12 ADP receptor (*Hirsh et al 2001*). Since deletion of p110α and p110β isoforms is embryonic lethal, the development of megakaryocyte-targeted deletion of these proteins has yet to be performed to obtain direct evidence for their implication in platelet activation. A role of these PI3K isoforms is however suggested by the observation that platelets from p85α deficient mice have an impaired response to GPVI stimulation (*Watanabe et al. 2003*). Recently, using a p110β selective inhibitor, S.P. Jackson's group suggested an important role of this class IA PI3K isoform in the regulation of platelet functions. In this presentation, we will give an overview of the different PI3Ks, of their mechanism of regulation and of their implication in platelet functions. Moreover, data showing the role of phosphatases able to specifically regulate the level of PI3K products, particularly the 5-phosphatases SHIP1 and SHIP2 (*Giuriato 2003*), will be presented. Since PI3Ks can function independently of their lipid kinase activity, as docking proteins or as protein kinases, these aspects of the PI3K signaling will be evoked. Finally, the use of isoform-specific inhibitors of PI3K as potential antithrombotic drugs will be discussed.

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SECONDARY MEDIATORS OF PLATELET AGGREGATION: CD40L, P-SELECTIN, SLAM

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Thrombus formation (platelet aggregates) provides the structural framework for the occlusive events causing acute myocardial infarction and thrombotic

stroke. Current antithrombotic strategies are aimed at inhibiting platelet aggregation by targeting the primary platelet adhesion receptor GP IIb-IIIa, via direct blockage or inhibition of signaling events that activate the "inside-out" function of the receptor. Theses successful therapies are however, often accompanied by undesirable bleeding complications. Thus, understanding the molecular mechanisms regulating platelet-platelet interactions (both platelet aggregation and thrombus cohesion) may provide insights into the development of new therapeutic strategies. One such possibility are secondary reactions following platelet activation and/or GP IIb-IIIa engagement that may be responsible for thrombus stability but not haemostasis. P-selectin, CD40L (CD154), and SLAM are three platelet-derived secondary agonists which affect thrombus stability via different mechanisms. The generation of microparticles triggered by increased levels of membrane or soluble P-selectin is proposed to stabilize platelet aggregates in vivo. CD40L expressed on the surface of activated platelets and present as a soluble form in plasma is a ligand for both CD40 and GP IIb-IIIa and has been shown to directly activate platelets. Finally, signaling of the homophilic adhesion molecule SLAM activated by proximity during platelet aggregation stabilizes platelet-platelet interactions. Targeting one of these three molecules and or more of these secondary platelet agonists may constitute a safer approach for chronic antithrombotic indications.

TARGETING PROTEASE-ACTIVATED RECEPTORS ON THE INSIDE WITH PEPDUCINS

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Identification of new pathways involved in platelet activation and the development of novel therapeutics to prevent platelet-mediated vessel occlusion are a major focus of cardiovascular pharmaceutical research. The G protein-coupled transmembrane receptors (GPCRs) are important targets in human platelets and blood vessels. Interaction of GPCRs with their corresponding ligands triggers rapid activation of platelets and endothelial cells, leading to thrombus formation. Protease-activated receptors (PARs) are key regulators of platelet function. Activation of PAR1 and PAR4 with the major procoagulant protease, thrombin, leads to platelet shape change, degranulation and aggregation. Recently, we described a new class of compounds called pepducins,[1] which specifically modulate GPCR function by blocking receptor-G protein signaling on the inside surface of the plasma membrane. The PAR4 pepducin antagonist (P4pal-10, PZ-410) was previously shown to protect against systemic platelet activation in mice.[2] PZ-410 also cross-inhibits PAR1 in human platelets suggesting that this pepducin may be useful as an anti-PAR compound. In this study, we describe the *in vivo* pharmacological properties of PZ-410. Single bolus intravenous injection of fluorescently-labeled PZ-410 into mice gave sustained drug levels in platelets and plasma for 5 h, followed by elimination with a half-life of 3.5 h. The pharmacokinetic profile correlates well with pharmacodynamic data obtained from tail bleeding measurements. After a single bolus intravenous injection of PZ-410, tail bleeding time increased to 210±30 s, one hour post injection, as compared to vehicle-treated animals (60±15 s) and this effect lasted over 4 h. Subcutaneous administration of PZ-410 also significantly prolonged tail bleeding time for 6 h. The efficacy of PZ-410 was then tested in an established model of ferric chloride-induced thrombosis of the mouse carotid artery. PZ-410 prevented full occlusion of the carotid artery in 70% of treated animals (n=15) compared to 15% of vehicle treated mice (n=16). Thus, PZ-410 represents a potent PAR antagonist with a favorable pharmacological profile as an antithrombotic agent.

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