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Research Article

New Biological Investigations on 3-Bromophenyl 6-Acetoxymethyl-2-oxo-2*H*-1-Benzopyran-3-Carboxylate as Anti-angiogenic Agent

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Strategy, Management and Health Policy				
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ABSTRACT The development of blood vessels inside tumors is required to provide the nutrients and oxygen needed for tumor growth and to allow the spread of cancer cells at a distance to form metastasis. Angiogenesis is also implicated in ocular diseases like age-related macular degeneration. The present work describes the potential anti-angiogenic properties of a coumarinic derivative, 3-bromophenyl 6-acetoxymethyl-2-oxo-2*H*-1-benzopyran-3-carboxylate (IK9), previously described as a potent inhibitor of HT 1080 fibrosarcoma cell invasion in vitro and tumor growth in vivo. In vivo, ex vivo, and in vitro models were used to delineate the anti-angiogenic properties of IK9. The anti-angiogenic effect of IK9 was demonstrated in vivo in a choroidal neovascularization mice model and additionally ex vivo in a rat aortic ring assay where it was more active than the known matrix metalloproteinase inhibitor Ro 28-2653. IK9 did not affect apoptosis, proliferation, or endothelial cell invasiveness in vitro. These findings suggest a complex mechanism of action of the compound via direct or indirect effects on endothelial cell properties. This study identifies IK9 as a new potent inhibitor of angiogenesis and suggests its potential use as a therapeutic agent. Drug Dev Res 71:209–218, 2010.

Key words: angiogenesis; cancer; age-related macular degeneration; anti-angiogenic agents; coumarin derivatives

INTRODUCTION

Angiogenesis is the sprouting of endothelial cells from preexisting vasculature to form new blood vessels. It is essential for organ growth and repair and occurs in a large number of physiological states (e.g., embryonic development or menstrual cycle). It is also implicated in pathological disorders, including wound healing, ocular disease, and cancer [Carmeliet, 2005; Folkman and Shing, 1992]. A finely regulated balance between

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pro- and anti-angiogenic factors is required in order to maintain blood vessel quiescence. Nevertheless, a deregulation can stimulate angiogenesis and induce tissue pathology. This can be observed in tumors in which the newly formed blood vessels not only provide nutrient and oxygen supply necessary for tumor growth, but are also the most important means of cancer cell dissemination [Folkman, 1971; Mazzocca and Carloni, 2009; Zetter, 1998]. The resulting metastasis represents one of the major challenges of effective cancer therapy and is responsible for more than 90% of cancer-related deaths [Gupta and Massague, 2006]. Targeting tumor angiogenesis is a key approach to anti-cancer therapy, with a large number of targets, including vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), mitogen-activated protein kinases (MAPK), and matrix metalloproteinases (MMPs) under investigation [Alfano et al., 2008; Zhong and Bowen, 2006].

Age-related macular degeneration (AMD) is a newer application for anti-angiogenic therapies [Noel et al., 2007]. This ocular disease is characterized by choroidal neovascularization (CNV) and is the leading cause of blindness in elderly patients. Several anti-vascular endothelial growth factor (VEGF) agents, including pegaptanib, bevacizumab, and ranibizumab, have been approved for human use [Noel et al., 2007].

3-Bromophenyl 6-acetoxymethyl-2-oxo-2*H*-1-benzopyran-3-carboxylate (IK9) was found to be potent in inhibiting cancer cell invasion in vitro and tumor growth in vivo, with rates comparable to those observed with inhibitors of matrix metalloproteinases (MMPs) such as GI 129471, Ro 28-2653 [Kempen et al., 2003, 2008], or BB94 [Maquoi et al., 2004]. The mechanism of action of IK9 remains unknown and could not be ascribed to the inhibition of MMPs (MMP-2 and MMP-9) or serine proteases (urokinase, α-chymotrypsin, human leukocyte elastase) [Kempen et al., 2003]. Some coumarinic derivatives, such as G8935, exert anti-cancer activity via receptor tyrosine kinase inhibition [Han et al., 2005]. Moreover, linomide, a quinolinone coumarin isostere immunomodulator, exhibits anti-angiogenic activity both in vitro and in vivo [Joseph et al., 1996; Shi et al., 2003; Vukanovic et al., 1993]. The present study explored the putative anti-angiogenic effect of IK9 using different models, including laser-induced model of choroidal neovascularization (CNV) in mice and a rat aortic ring assay [Devy et al., 2002; Lambert et al., 2001]. To better assess the mechanism of action of IK9, its effect on endothelial cell proliferation, apoptosis, migration and invasion was studied in HUVEC models. For each ex vivo and in vitro experiment, reference compounds were included, the MMP inhibitors Ro 28-2653 and BB94 and the pro-apoptotic and antiproliferative topoisomerase I inhibitor camptothecin.

MATERIALS AND METHODS

Compounds

IK9 and Ro 28-2653 (Fig. 1) were obtained via previously described synthetic procedures [Daniewski et al., 2004; Pochet et al., 1996, 2000]. BB94 (Fig. 1) was obtained from British Biotech (British Pharmaceuticals, Oxford, UK). Camptothecin (Fig. 1) was obtained from Sigma (Bornem, Belgium).

Choroidal Neovascularization

Laser photocoagulation

CNV was induced in mice by laser burns as described previously [Lambert et al., 2001]. Mice were anesthetized with an ip injection of Avertin. Both pupils were dilated with 1% tropicamide; four burns were delivered (usually at the 3, 6, 9, and 12 o'clock positions around the optic disc) using a green argon laser (532 nm; 50-μm diameter spot size; 0.05-s duration; 400 mW). A suspension of IK9 (20 and 40 mg/kg) in propylene glycol/water (50/50) was given ip every second day for 2 weeks to treated mice. Control mice injected with propylene glycol/water (50/50) without IK9 were used as the reference. Animals were terminated after 14 days; eyes were enucleated and embedded in Tissue TeK (Miles Laboratories, Naperville, IL) and frozen in liquid nitrogen for cryostat sectioning.

Quantitation of choroidal neovascularization

A quantitative morphometric assessment of thickness of choroidal new vessels was carried out using a computer-assisted image analysis system (Olympus Micro Image version 3.0 for Windows 95/NT Olympus Optical Co. Europe GmbH). Microscopic images (working magnification of $\times 200$) of hematoxylin-stained eye sections were acquired via a video camera and were digitalized and analyzed. Neovascularization was estimated by the ratio B/C of the thickness from the bottom of the pigmented choroidal layer to the top of the neovascular membrane (B) to the thickness of the intact pigmented choroids adjacent to the lesion (C). Results are expressed as mean $\pm$ SEM ($n = 5$).

Flat mount of choroids

Before exsanguination, mice were injected (iv) with 200 μl fluorescein isothiocyanate (FITC)-conjugated dextran (2,000,000 Da, Sigma) in phosphate-buffered saline (PBS; pH 7.4). Immediately afterward, eyes were taken and fixed in paraformaldehyde 1%, pH 7.4 for 1 h at room temperature. Retinae were discarded, and the choroid was prepared in Vectashield

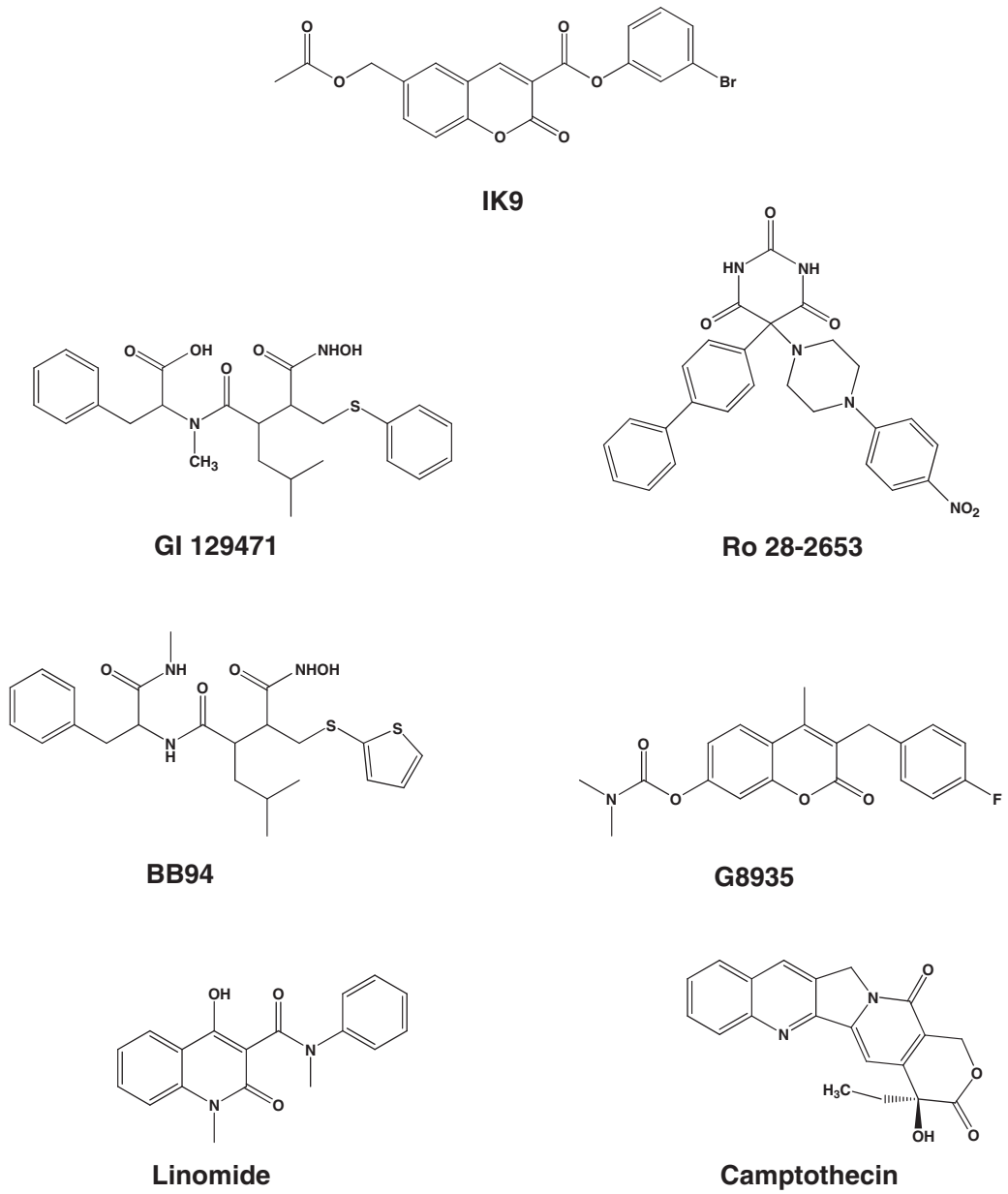

Fig. 1. Compounds mentioned in this work (IK9: the coumarinic derivative investigated as anti-angiogenic agent; GI 129471, Ro 28-2653 and BB94: MMP inhibitors used as references; G8935: a coumarinic derivative known for exerting anti-cancer activity; linomide: a quinolinone coumarin isostere anti-angiogenic agent; camptothecin: a pro-apoptotic and antiproliferative topoisomerase I inhibitor used as reference).

medium (Vector Laboratories, Burlingame, CA) for confocal microscopy analysis. Spatial distribution of FITC was examined by a confocal inverse Leica TCS SP2 microscope (Leica Microsystems, Germany), equipped with an argon laser and an AOTF laser (acousto-optical tunable filter). The digitalized pictures were obtained via a Plan-Apo water-immersion $\times 10$ (NA 0.4) or $\times 63$ (NA 1.2) objective with 1024×1024 pixel resolution. FITC was visualized at 488-nm excitation wavelength with a dichroic mirror RSP500. Emitted light was scattered and recorded at 500–550 nm. For each lesion, optic sections series were recorded with a 1.67-µm gap. After successive scanning of each interval, three-dimensional (3D) fluorescence pictures were reconstructed using Leica software. Pictures were exported in TIFF format and treated by Adobe Photoshop. Quantitation was realized by measuring of total vessel fluorescence surface (Scion Image for Windows beta 4.0.2), and statistical analysis was done using Student's *t*-test (GraphPad Prism 4.0). Results were expressed as mean $\pm$ SEM ($n = 5$).

Aortic Ring Assay

Ex vivo angiogenesis was studied by culturing rings of rat aorta in 3D collagen gels as described [Blacher et al., 2001; Devy et al., 2002; Nicosia and Ottinetti, 1990a,b]; 6 ml of MCDB 131 (Gibco), supplemented with 25 mM $NaHCO_3$, 1% glutamine, 100 UI/ml penicillin, and 100 µg/ml streptomycin, containing IK9 (2, 5, and 10 µM) or Ro 28-2653 (5 µM), were added to Petri dishes with aortic rings in a 3D collagen gel. These were kept at 37°C in a humid atmosphere (5% CO_2). The rings were observed after 6 and 9 days by phase-contrast microscopy with an Olympus microscope and pictures were taken. Image analysis was performed on a WorkStation Sun SPARC30, using the software Visilog 5.0 (Noesis). Results were expressed as a percentage (±SEM) of control ($n = 3$).

Proliferation and Apoptosis Assays

Cell culture

Human umbilical vein endothelial cells (HUVEC; ATCC CC-2519) were maintained in MCDB 131 (Gibco) supplemented with 2 mM L-glutamine, 100 U/ml penicillin/streptomycin, 50 µg/ml heparin, 5 µg/ml ECGS, 25 mM HEPES, and 10% (v/v) fetal calf serum (FCS) at 37°C in a humid atmosphere (5% CO_2 and 95% O_2).

Apoptosis assay

HUVEC cell suspension (62,500 cells/ml; 2 ml/well) was seeded in 6-well plates and incubated for 24 h. Culture medium was then discarded and replaced by 2 ml of medium containing IK9 (10 µM), camptothecin (0.1 µM), or vehicle (0.1% DMSO). After 48-h incubation, harvested cells were centrifuged at 300 g for 5 min and kept at 4°C. After removal of supernatant and washing with 1 ml binding buffer (10 mM HEPES/NaOH pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$), cells were marked by adding 100 µl binding buffer, 10 µl Annexin V-FITC (Becton-Dickinson, Erembodegem, Belgium) and 10 µl propidium iodide (50 µg/ml), to measure the respective percentages of apoptotic and necrotic cells, followed by incubation at room temperature protected from light for 15 min; 400 µl of binding buffer was then added to each tube and flow cytometry analysis performed within 1 h, using a Coulter Epics XL cytometer (Beckman Coulter, Brea, CA). For each assay, three replicates ($n = 3$) were performed, each in triplicate. Results were expressed as a percentage (±SEM) of cells positive for Annexin V-FITC or PI.

Proliferation assay

HUVEC cell suspension (30,000 cells/ml; 150 µl/well) was seeded in 96-well plates and incubated for 24 h. Culture medium was then discarded and replaced with 150 µl of medium containing IK9 (10 µM), camptothecin (0.1 µM), or vehicle (0.1% DMSO). These were changed every 24 h. After 24, 48, or 72 h of incubation, media were discarded and plates stored at −80°C until samples were to be assayed. Analysis of proliferation was done using CyQuant® Cell Proliferation Kit (Molecular Probes, Invitrogen, Leiden, Netherlands). When ready for quantification, plates were thawed and 150 µl of a solution containing 20-fold diluted lysis buffer and 400-fold diluted fluorescent marker in sterile water was added to each well. After 2–5 min at room temperature protected from light, sample fluorescence was measured (480-nm excitation; 520-nm emission maxima). The cell numbers per well were determined using a standard curve previously created for converting sample fluorescence values into cell numbers. For each assay, three replicates ($n = 3$) were performed, each in triplicate. Results were expressed as number of cells (±SEM) at 0, 24, 48, and 72 h.

Invasion assay

The inhibitory potency of IK9 (10 µM) on endothelial cell invasion was assayed using a Boyden chamber test, with transwell cell culture inserts (6.5-mm diameter; 8-µm pore size). Membranes were coated with type IV collagen and lower wells of the chambers filled with 600 µl MCDB 131 supplemented with 10% FBS (acting as chemoattractant), 1% BSA and 0.6 µl of an inhibitor solution (10^{-2} M in DMSO). The filters were recovered with 100 µl of cell suspension (10^5 cells), 200 µl MCDB 131 supplemented with 0.1% BSA and 0.3 µl of inhibitor solution. Chambers were subsequently incubated for 24 h in a humid atmosphere at 37°C. After incubation, cells at the bottom of the filters were fixed for 30 min in absolute methanol at −20°C and stained with Giemsa (4% in sterile water) for 20 min. Excess dye was removed by washing with water and cells on the upper surface of filters were removed by scrapping with a cotton swab. Cells reaching the lower surface of the filters were visualized using a light microscope (Vanox AH3, Olympus, Hamburg, Germany) and counted using ImageJ software. For each assay, three replicates ($n = 3$) were performed, each in triplicate. Results were expressed as a percentage (±SEM) of the migration of control cells.

RESULTS

In Vivo Effects of IK9 on Choroidal Neovascularization in Mice

The anti-angiogenic effect of IK9 was tested on CNV in vivo. Choroidal lesioning was assessed by determining the B/C ratio (B: thickness from the

bottom of the pigmented choroidal layer to the top of the neovascular lesion to C: the thickness of the intact adjacent choroid) (Fig. 2A). This ratio was reduced in mice treated with 20 and 40 mg/kg IK9 (Fig. 2B). To determine the total surface of new blood vessels, choroidal flat mounts from FITC-labeled dextran-perfused mice were analyzed using confocal microscopy (Fig. 2C). Again, the fluorescence surface

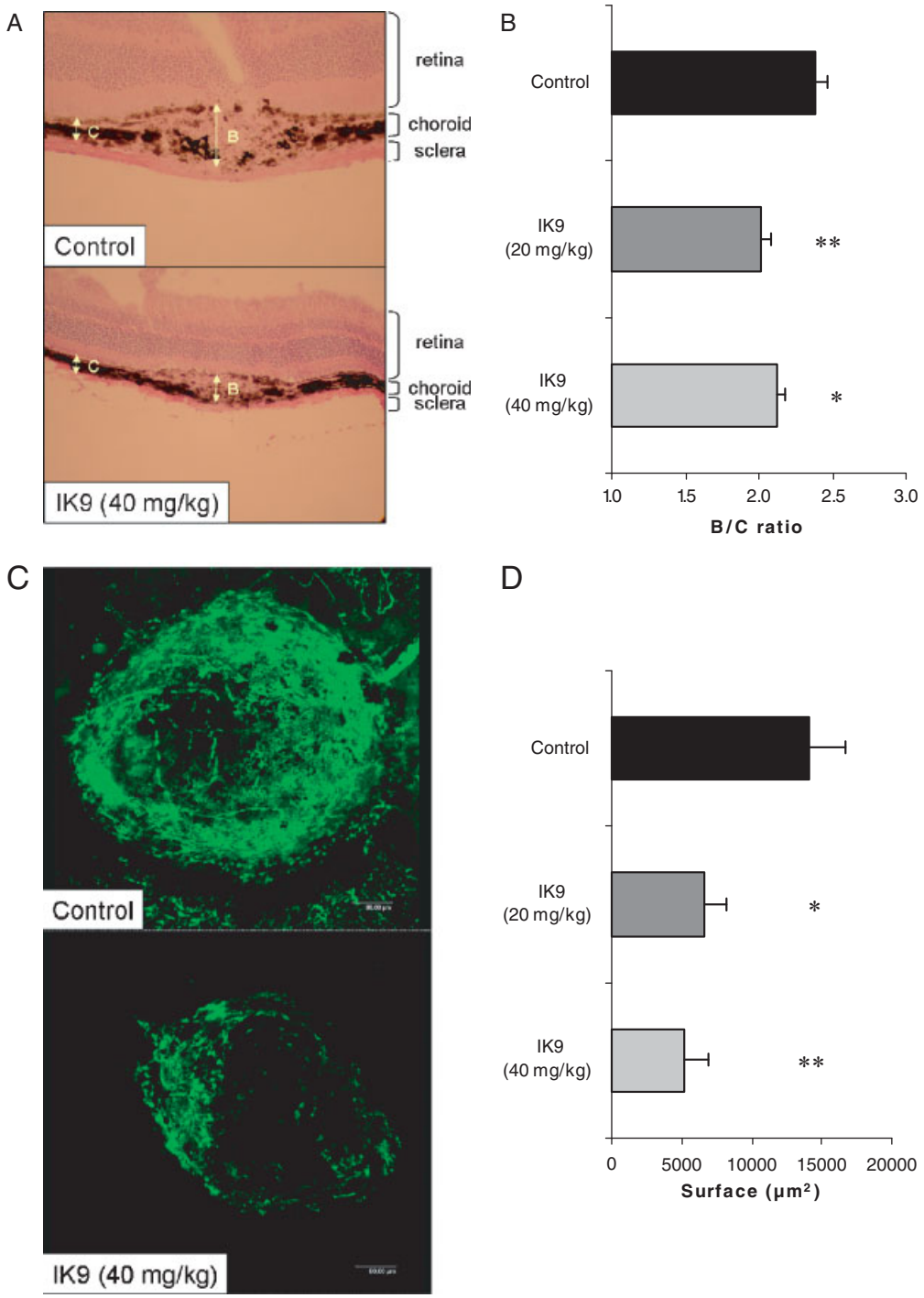

Fig. 2. Choroidal neovascularization, induced by laser burns in mice, was estimated by measuring (**A**) the B/C ratio of the thickness from the bottom of the pigmented choroidal layer to the top of the neovascular membrane to the thickness of the intact pigmented choroids adjacent to the lesion. Corresponding results (**B**) are expressed as mean ± SEM ($n = 5$) (*, $P < 0.05$; **, $P < 0.01$). The total area of neovascularization was measured on flat mount (see examples in **C**) and corresponding results (**D**) are expressed as mean ± SEM ($n = 5$) (*, $P < 0.05$; **, $P < 0.01$). [Color figure can be viewed in the online issue which is available at www.interscience.wiley.com]

corresponding to the angiogenic response was decreased twofold with 20 and 40 mg/kg IK9 (Fig. 2D).

Ex Vivo Effects of IK9 on Angiogenesis

The ex vivo anti-angiogenic effect of IK9 was assessed using an aortic ring assay and was compared with that of the MMP inhibitor, Ro 28-2653 (5 μM). In control conditions, neovasculaturization was visible around the initial portion of the aortic fragment. Sprouting of endothelial cells was partially reduced by Ro 28-2653 and almost completely abolished with increasing concentrations of IK9 (Fig. 3). With Ro 28-2653 treatment, the number of new vessels was reduced, while branching and maximal length of the new vessels remained unchanged. A reduction of all three parameters was observed with IK9, further confirming its ability to block angiogenesis (Fig. 4).

In Vitro Effects of IK9 on Apoptosis, Proliferation, and Mobility of Endothelial Cells (HUVEC)

To investigate the mechanism of action of IK9, its effect on HUVEC apoptosis, proliferation, and invasiveness was assessed. A basal apoptosis rate of around 30% was observed under control conditions. Camptothecin (CPT; 100 nM) produced approximately 60% of apoptosis, while IK9 (10 μM) had an apoptosis rate similar to that observed under basal conditions (Fig. 5A). In the proliferation assay CPT (100 nM) decreased HUVEC growth but a growth profile similar to that of control was obtained with IK9 (10 μM; Fig. 5B). The anti-invasive potency of IK9 was assessed by measuring the number of HUVECs passing through type IV collagen-coated transwell culture inserts. The broad-spectrum MMP inhibitor, BB94 (10 μM) produced a slight reduction (~10%) in HUVEC invasion. IK9 (10 μM) had no effect on HUVEC invasion (data not shown).

DISCUSSION

Certain coumarinic derivatives exert anti-cancer activities [Han et al., 2005; Pan et al., 2009]. Among these, IK9 impaired in vivo tumorigenesis and in vitro invasiveness of HT 1080 fibrosarcoma cells [Kempen et al., 2003]. In the present study, IK9 displayed anti-angiogenic effects in two models, the in vivo CNV model and the ex vivo aortic ring assay, extending the therapeutic potential of IK9 to ocular disease and providing the first evidence of an angio-inhibitory effect of this compound. The anti-angiogenic properties of IK9 were demonstrated in the CNV via two different methods of quantitation: (1) on histological eye sections, and (2) on flat mounted choroids. It was further confirmed in the rat aortic ring assay that three measured parameters—number of new vessels, branching, and the maximal length of new vessels—were reduced. These findings suggest that the previously observed inhibitory effect on tumor development in vivo was not exclusively due to an inhibition of HT 1080 cell invasion [Kempen et al., 2003], but also to a reduction of tumor angiogenesis. Angiogenesis is a multi-faceted process occurring as a result of a switch to pro-angiogenic signaling under physiological or pathological conditions that involves multiple cell types [Gerhardt and Betsholtz, 2003; Hanahan and Folkman, 1996]. Among them, endothelial cells play a key role: they proliferate and sprout from the preexistent vessels.

Afterward, the endothelial cell masses invade the extracellular matrix and undergo a reorganization to form vascular tubes [Rundhaug, 2005], a process referred to as "sprouting angiogenesis." The neoformation of blood vessels can also rely on the recruitment of progenitor endothelial cells issued from the bone marrow, a process called vasculogenesis [Asahara et al., 1999; Carmeliet, 2005]. Both sprouting angiogenesis and vasculogenesis

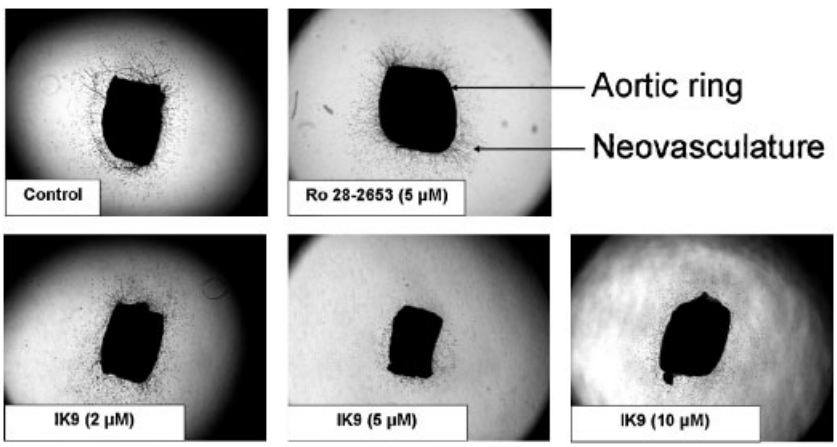

Fig. 3. Aortic ring assay. Rat aorta rings were cultured in a three-dimensional collagen gel, treated as indicated in the pictures and photographed after 9 days in culture.

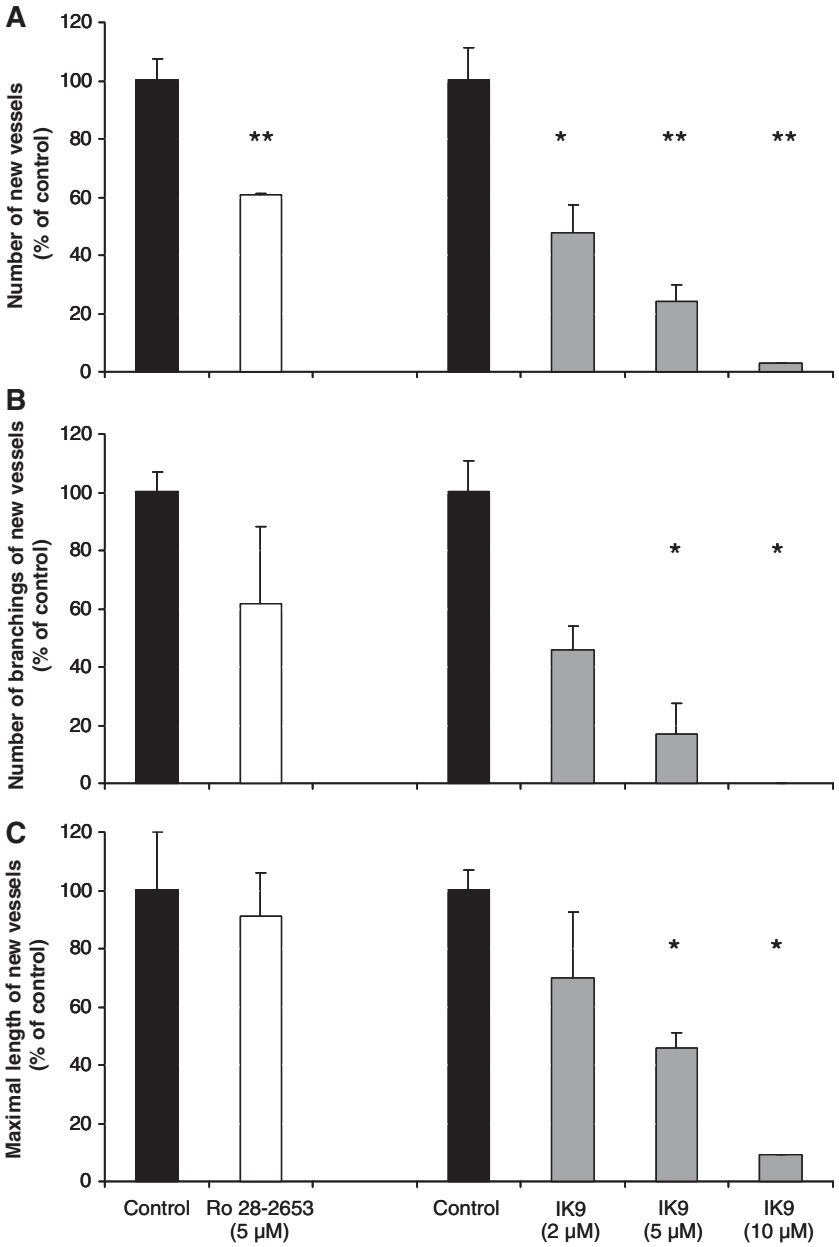

Fig. 4. Aortic ring assay: quantitation of the neovascularization. Neovascularization was assessed by measuring (**A**) the number of new vessels, (**B**) the number of branchings and (**C**) the maximal length of the new vessels. Results are expressed as percentages versus respective individual controls (mean ± SEM; $n = 3$). (*, $P < 0.05$; **, $P < 0.01$).

contribute to choroidal angiogenesis [Jost et al., 2007; Sengupta et al., 2003; Tomita et al., 2004]. The control of sprouting angiogenesis by IK9 is clearly supported by its inhibitory effect on endothelial cell spreading from the aortic fragments. This 3D model using a collagen matrix recapitulated different steps of angiogenesis (e.g., endothelial cell activation, migration, proliferation, and differentiation into tube-like structures) [Berndt et al., 2008]. In an attempt to provide mechanistic information, the IK9 effect was evaluated in less complex 2D culture models in which endothelial cell (HUVEC) properties were investigated separately [Berndt et al., 2008; Bruyère and Noel, 2009].

No evidence was observed for an effect of IK9 on HUVEC growth, insofar as proliferation and apoptosis of these cells were unchanged. Considering the marked inhibitory effect of IK9 on HT 1080 cell invasion [Kempen et al., 2003], a putative similar effect on endothelial cells was examined. However, no inhibition on HUVEC invasion was observed even at high

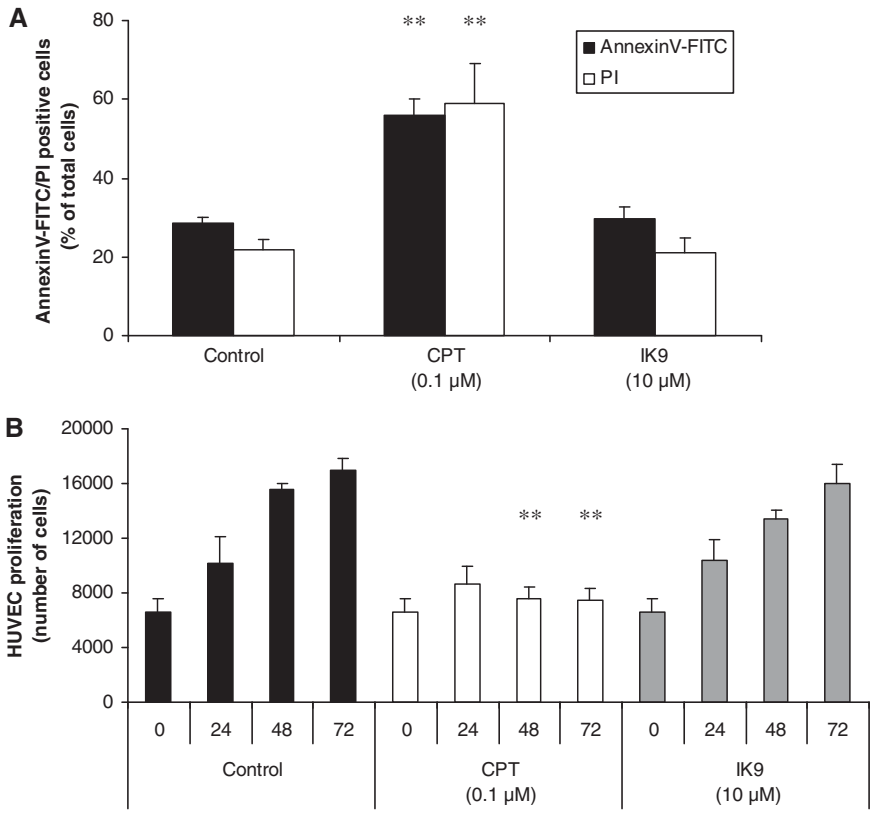

Fig. 5. Apoptosis and proliferation assays. **A**: Effect of CPT and IK9 on HUVEC apoptosis: measured using Annexin V-FITC as apoptosis marker and propidium iodide (PI) as necrosis marker. Results are expressed as percentage of annexin V-FITC/PI-positive cells (mean ± SEM; *, $P<0.05$; **, $P<0.01$). **B**: Effect of CPT and IK9 on HUVEC proliferation: measured using CyQuant® Cell Proliferation Kit. Results are expressed as the number of cells at 0, 24, 48, and 72 h of treatment (mean ± SEM; $n=3$; *, $P<0.05$; **, $P<0.01$).

concentrations. The apparent discrepancy between the lack of IK9 effect on endothelial cell properties in 2D systems and the dramatic effect observed in vivo in the CNV model and ex vivo in the aortic ring assay may have several explanations.

The present findings might pinpoint the importance of the matrix microenvironment of endothelial cells to achieve IK9 inhibitory effects with the compound controlling the release/generation of an angiogenic inhibitor from the collagen matrix or interfere with endothelial cell to matrix interaction [Kalluri, 2003], Alternatively, the possibility of an indirect effect of the compound on endothelial cells by interfering with perivascular cells present in the in vivo and ex vivo models, but lacking in the 2D monocultures of endothelial cells cannot be excluded. Additionally, IK9 may affect signal transduction via receptor tyrosine kinases like VEGFR and PDGFR, which are activated by the corresponding growth factors VEGF and PDGF [Andrae et al., 2008; Ostman, 2004; Shibuya, 2001]. While modulation of tyrosine kinase activity has been observed with coumarinic derivatives, IK9 was unable to inhibit VEGF or PDGF-mediated signal transduction through the mitogen-activated kinase pathway (Dr. C. Haan, University of Luxembourg, 2008 personal communication), when investigated in two established cell systems (HUVEC for VEGF and 3T3 fibroblasts for PDGF). Further studies are thus required to unravel the mechanism of action of IK9. In conclusion, the present study provides new evidence for the anti-angiogenic potency of a coumarinic derivative and extends the potential therapeutic applications of IK9 to ocular diseases.

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