IB05204, a dichloropyridodithienotriazine, inhibits angiogenesis in vitro and in vivo

Beatriz Martinez-Poveda,1 Ramón Muñoz-Chápuli,2 Salvador Rodríguez-Nieto,3 José M. Quintela,4 Antonio Fernández,5 Miguel-Ángel Medina,1 and Ana R. Quesada1

1Departamento de Biología Molecular y Bioquímica and 2Departamento de Biología Animal, Facultad de Ciencias, Universidad de Málaga, Málaga, Spain; 3Institute of Environmental Medicine, Division of Toxicology, Karolinska Institutet, Stockholm, Sweden; 4Departamento de Química Fundamental, Facultad de Ciencias, Universidad de la Coruña, La Coruña, Spain; and 5Instituto Biomar S.A. Políg. Ind., edificio CEI, Onzonilla, León, Spain

Abstract
In the course of a blind screening program for inhibitors of angiogenesis, IB05204 (4,8-dichloro-12-phenylpyrido[5,6,4,5]dithieno[3,2-d:3,2-d]-1,2,3-ditriazine) was selected for its ability to inhibit endothelial tubule-like network formation on Matrigel. IB05204 inhibits the in vivo angiogenesis in the chorioallantoic membrane (CAM) and the mouse Matrigel plug assays. Antiangiogenic activity seems to be highly dependent on the chloro substituents because their removal results in a complete loss of the in vitro inhibitory activity of endothelial differentiation and in vivo antiangiogenic activity in CAM assay. Although IB05204 inhibits the growth of endothelial and tumor cells in culture, its antiangiogenic activity seems to be mainly dependent on the prevention of endothelial capillary-like tube formation and inhibition of endothelial migration because these effects are recorded at lower concentrations. IB05204 treatment inhibits matrix metalloproteinase-2 (MMP-2) production in endothelial and tumor cells, down-regulates endothelial cyclooxygenase-2 expression, and represses phosphorylation of endothelial Akt in response to serum stimulation, suggesting that IB05204 interferes with molecular mechanisms of cell migration and survival. IB05204 induces apoptosis in endothelial cells through cytochrome c release and caspase activation. Data here shown altogether indicate that IB05204 is a compound that interferes with several key steps of angiogenesis, making it a promising drug for further evaluation in the treatment of angiogenesis-related pathologies. [Mol Cancer Ther 2007;6(10):2675–85]

Introduction
Angiogenesis, a physiologic process involving the generation of new capillaries from preexisting vessels, is strictly controlled by a balance of stimulators and inhibitors, being restricted in adults to some processes related to the reproductive cycle and wound repair. However, angiogenesis is also a fundamental step in the transition of tumors from a dormant state to a malignant state, being considered one of the hallmarks of cancer, and playing an essential role in tumor growth, invasion, and metastasis (1). Furthermore, many other diseases are dependent on up-regulated angiogenesis. They include diabetic retinopathy, age-related macular degeneration, hemangioma, arthritis, and psoriasis, among others. Angiogenesis inhibitors are likely to change the face of medicine in the next decade, arising as an attractive therapeutic approach for the treatment of cancer and other angiogenesis-dependent diseases (2). There are different strategies for angiogenesis intervention based on the modulation of any of the different steps of the angiogenic process. Therefore, some angiogenesis inhibitors may act on the angiogenic signal or on one or several functions of activated endothelial cells (proliferation, adhesion to extracellular matrix, proteases production, migration, invasion, or differentiation). Recently, the first antiangiogenic agents have been approved for the treatment of cancer and blindness, encouraging expectations in their therapeutic potential (3).

In the course of a blind-screening program, the dichloro-substituted pentacyclic pyridodithienotriazine IB05204 (4,8-dichloro-12-phenylpyrido[5,6,4,5]dithieno[3,2-d:3,2-d]-1,2,3-ditriazine; Fig. 1) was selected by its ability to inhibit the endothelial cell differentiation in vitro. This compound had been initially synthesized as a reaction intermediate to afford the production of new antihistaminic pyridothienotriazines by nucleophilic displacement (4). IB05204 itself did not exhibit any significant antihistaminic activity.

Here, we show for the first time that IB05204 is a potent inhibitor of angiogenesis in vitro and in vivo, interfering several key steps of the angiogenic process. IB05204 inhibits proliferation of both endothelial and tumor cells and strongly reduces the migratory and proteolytic activities of endothelial cells and their ability to form a network of tubular-like structures on Matrigel in vitro at micromolar concentrations. IB05204 induces apoptosis in endothelial cells, as well as down-regulates cyclooxygenase-2 (COX-2)
expression. Finally, IB05204 also represses phosphorylation of endothelial Akt in response to serum stimulation, suggesting that IB05204 interferes with the molecular mechanisms of cell migration and survival.

Materials and Methods

Materials

Cell culture media were purchased from Life Technologies and Cambrex. Fetal bovine serum (FBS) was a product of Harlan-Seralab. Matrigel was purchased from Becton Dickinson. Supplements and other chemicals not listed in this section were obtained from Sigma Chemicals Co. Plastics for cell culture were supplied by NUNC. IB05204 was prepared as shown in ref. 4 starting from the readily available 2-amino-6-chloro-3,5-dicyano-4-phenylpyridine by means of a route based on the preparation of a β-enaminonitrile intermediate and its diazotation. Compounds 1, 2, and 3 were prepared from IB05204 by nucleophilic displacement, as described (4). All the pyridodithienotriazines were dissolved in DMSO at a concentration of 2 mg/mL and stored at −20°C until use. Fertilized chick eggs were obtained from Granja Santa Isabel.

Screening of Antiangiogenic Compounds

A chemical compound library containing some 1,000 compounds was obtained from Instituto Biomar (León, Spain). Compounds were dissolved in DMSO and were tested for their ability to inhibit the endothelial tube formation on Matrigel with the microplate assay described below at nontoxic concentrations. As a secondary screening, the antiangiogenic activity of those compounds selected in the primary screening was assayed in vivo [chorioallantoic membrane (CAM) assay]. From this screening, IB05204 was chosen to be further characterized for its antiangiogenic activity.

Cell Culture

Human colon carcinoma HCT-116 and human fibrosarcoma HT1080 cells were maintained in DMEM containing glucose (4.5 g/L), glutamine (2 mmol/L), penicillin (50 IU/mL), streptomycin (50 μg/mL), and amphoterycin (1.25 μg/mL) supplemented with 10% FBS. Bovine aortic endothelial cells (BAEC) were maintained in DMEM containing glucose (1 g/L), glutamine (2 mmol/L), penicillin (50 IU/mL), streptomycin (50 μg/mL), and amphoterycin (1.25 μg/mL) supplemented with 10% FBS (5). Human umbilical vascular endothelial cells (HUVEC) were isolated from human umbilical cords by collagenase digestion (6) and maintained in medium 199 containing HEPES (10 mmol/L), L-glutamine (2 mmol/L), heparin (10 mg/mL), penicillin (50 IU/mL), streptomycin (50 μg/mL), and amphoterycin (1.25 μg/mL), supplemented with 3 mg/L endothelial cell growth supplement (Sigma) and 20% FBS in 5% CO2 and 37°C.

Endothelial Cell Differentiation Assay: Tube Formation on Matrigel

Wells of a 96-well plate were coated with 50 μL of Matrigel (10.5 mg/mL) at 4°C and allowed to polymerize at 37°C for a minimum of 30 min (7). Some 5 × 10^4 BAECs were added in 200 μL of DMEM. For HUVECs, 2.5 × 10^4
cells were added in 200 μL of Medium 199 supplemented with 5% FBS. Finally, different amounts of IB05204 were added and incubated at 37°C in a humidified chamber with 5% CO₂. After incubation for 7 h, cultures were observed (200× magnification), photographed with a NIKON inverted microscope DIAPHOT-TMD (NIKON Corp.), and evaluated by two different observers. Those assays where no tubular structures could be observed were considered as positive.

**Cell Growth Assay**

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma Chemical Co.) dye reduction assay in 96-well microplates was used as previously described (8). 3 × 10³ BAECs, 4 × 10³ HUVECs, 2 × 10³ HCT-116, and HT1080 in a total volume of 100 μL of their respective growth media were incubated with serial dilutions of IB05204. After 3 days of incubation (37°C, 5% CO₂ in a humid atmosphere), 10 μL of MTT (5 mg/mL in PBS) were added to each well, and the plate was incubated for a further 4 h (37°C). The resulting formazan was dissolved in 150 μL of 0.04 N HCl–2 propanol and read at 550 nm. All determinations were carried out in triplicate. IC₅₀ value was calculated as the concentration of IB05204 yielding 50% of cell survival.

**CAM Assay**

CAM assay was done as described (7). Fertilized chick eggs were incubated horizontally at 38°C in a humidified chamber, windowed by day 3 of incubation, and processed by day 8. The compounds to be tested were added to a 0.7% solution of methylcellulose in water, and 10 μL drops of this solution were allowed to dry on a Teflon-coated surface in a laminar flow hood and implanted on the CAM. After 48 h reincubation, the CAM was examined under a stereomicroscope by two different observers. The assay was scored as positive when both of them reported a significant reduction of vessels in the treated area.

**Mouse Matrigel Plug Assay**

The Matrigel plug assay was done as described previously (7). C57BL/6 female mice were injected s.c. near the abdominal midline, via a 21-gauge needle with about 300 μL of Matrigel containing basic fibroblast growth factor (bFGF; 1 μg/mL) and 4 μmol/L IB05204. Control mice received the same volume of Matrigel with bFGF mixed with a corresponding amount of DMSO without IB05204. Blank mice were injected with Matrigel containing a corresponding dose of PBS and DMSO. After injection, the Matrigel rapidly formed a single, solid gel plug. Mice were sacrificed 4 days after injection. Plugs were then removed and photographed, and the extent of neovascularization was assessed by measuring the hemoglobin content using the Drabkin’s Reagent Kit (Sigma). Some of the excised Matrigel plugs were fixed with 10% formalin, embedded in paraffin, sectioned (15 μm thick), and stained with H&E.

**Statistical Analysis**

All data are expressed as means ± SD. One-tailed Student’s t-test was used for evaluations of pair of means to establish which groups differed from the control group.

**Conditioned Media and Gelatinograms**

BAECs and HT1080 cells were grown at 75% confluency in six-well plates. After two washes with PBS, each well received the indicated concentration of IB05204 in 1.5 mL of DMEM/0.1% bovine serum albumin (BSA) containing 200 Komberg international units of aprotinin/mL. After 24 h of incubation, conditioned media were collected, and matrix metalloproteinase-2 (MMP-2) and MMP-9 activities were detected in gelatingrams as described (9).

**Endothelial Cell Migration Assay**

The migratory activity of HUVEC was assessed using a wound migration assay (10). Confluent monolayers in six-well plates were wounded with pipette tips, giving rise to one acellular 1-mm-wide lane per well. After washing, cells were supplied with 1.5 mL complete medium in the absence (controls) or presence of 2 μmol/L IB05204. Wounded areas were photographed at zero time, and after 2, 4, and 6 h of incubation, plates were observed under a microscope, and photos were taken from the same areas as those recorded at zero time.

**Western Blot Analyses**

For COX-2 expression analysis, sub-confluent HUVECs were stimulated with 50 ng/mL phorbol 12-myristate 13-acetate (PMA) during 4 h and 30 min in the absence (controls) or presence of different concentrations of IB05204. Cell culture dishes were washed twice with ice-cold PBS before adding on ice lysis buffer (50 mmol/L Tris (pH, 7.4), 150 mmol/L NaCl, 1% Triton X-100, 0.25% sodium deoxycholate, 1 mmol/L EDTA, 1 mmol/L sodium orthovanadate, 30 mmol/L β-glycerophosphate, 30 mmol/L sodium fluoride, 5 mmol/L disodium Ppi, 5 μL/mL protease inhibitor cocktail]. After scraping, lysates were kept on ice for 15 min and centrifuged (13,000 rpm, 15 min, 4°C), and supernatants were kept at –80°C until Western blot analysis. For cytochrome c release from the mitochondria study, attached and unattached 14-h IB05204-treated BAECs were harvested, centrifuged, and washed with PBS. About 2 × 10⁶ cells were resuspended in 100 μL of assay buffer (MSH buffer, 50 mmol/L KCl, 1 mmol/L EGTA, 5 mmol/L succinate, 5 mmol/L MgCl₂) and permeabilized with 10 μg digitonin (5 min, room temperature). Samples were centrifuged (13,000 rpm, 5 min), and supernatants (soluble cytosolic fraction) were collected and mixed with 6 × sample buffer; pellets (mitochondria and nuclei) were resuspended in the same volume of 2 × sample buffer. In Akt phosphorylation studies, BAECs were serum-deprived overnight; after treatment with 5 or 10 μmol/L IB05204 for 2 h in serum absence, cells were stimulated for 10 min with 10% FCS supplemented culture medium. Cells were washed with ice-cold PBS and lysed with 2 × sample buffer. For poly(ADP-ribose)polymerase (PARP) or Lamin A cleavage studies, BAECs were growth until sub-confluence and treated for 14 h either with the indicated concentration of IB05204 or with the vehicle (DMSO, control). Attached and unattached cells were harvested and lysed in 2 × sample buffer.

Cell lysates in 2 × sample buffer were denaturalized for 5 min at 95°C and separated by SDS-PAGE. After proteins
electro-transfer, nitrocellulose membranes (PROTRAN, Schleider & Schuell) were blocked in blocking buffer (5% w/v dry nonfat milk in TBS-T) and probed (overnight, 4°C) with primary antibodies diluted in TBS-T plus 1% BSA and 0.02% sodium azide: anti-PARP monoclonal antibody (mAb; BD Biosciences), recognizing the 116-kDa intact form of PARP and the 85-kDa cleaved fragment; anti-cleaved lamin-A mAb (Cell Signaling Technology), recognizing the small fragment of cleaved lamin-A (28 kDa); anti-Akt, anti–phospho-Akt (Ser473) rabbit polyclonal antibodies (pAb; Cell Signaling Technology); anti–COX-2 mAb (Santa Cruz Biotechnology); anti–β-actin mAb (Sigma); anti-glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH) rabbit pAb (Nordic Biotise); anti-cytochrome c mAb (BD Biosciences). After three washes with TBS-T, membranes were probed with secondary horseradish peroxidase-linked antibodies (diluted 1:10,000 in blocking buffer) for 1 h at room temperature and developed using the enhanced chemiluminescence system (Amersham). For antibody reprobing, membranes were incubated in stripping solution [62.5 mmol/L Tris-HCl (pH, 6.8), 2% SDS, and 0.77% 3-mercaptoethanol] for 30 min at 50°C with shaking.

Analysis of Oligonucleosomal DNA Cleavage

Sub-confluent BAECs were treated with 10 μmol/L IB05204 for 14 h. After treatment, attached and unattached cells were pelleted, and DNA fragmentation was analyzed as described (11). As an internal control, 2-methoxyestradiol–treated BAECs (10 μmol/L, 24 h) were used.

4,6-Diamidino-2-phenylindole Staining

Cells were seeded in six-well plates on coverslips and grown to sub-confluence. After treatments with the indicated concentrations of IB05204 for 14 h, cells were washed (PBS) and fixed (formalin solution, Sigma). Coverslips were stained [1 μg/mL 4,6-diamidino-2-phenylindole (DAPI)], mounted on slides (DAKO Cytomation Fluorescent Mounting Medium, DAKO), and observed under a fluorescence microscope (Leica, TCS-NT).

Flow Cytometry Analysis

Attached and unattached treated and control BAECs were harvested, washed (PBS), and fixed (70% ethanol, 1 h on ice). Pelleted cells were incubated (1 h protected from light) with RNase-A (0.1 mg/mL) and propidium iodide (40 μg/mL) during 1 h shaking and protected from light. Percentage of sub-G1 population was determined using a FACScan cytometer (BD).

Caspases Activity Assay

Sub-confluent BAECs were treated with IB05204 (5 μmol/L, 14 h), and then, attached and detached cells were harvested. A total of 5 × 10^5 cells were resuspended in 25 μL PBS and snap-frozen in wells of a 96-well opaque microtiter plate on liquid nitrogen. After thawing on ice, 50 μL of assay buffer [caspases-3 and -8; 0.1 mol/L HEPES (pH 7.2), 10% sucrose, 0.1% CHAPS; caspases-2 and -9; 0.1% MES (pH, 6.5), 10% polyethylene glycol, 0.1% CHAPS] containing 50 μmol/L of selective fluorogenic substrates (caspase-2: Ac-VDVAD-AMC; caspase-3: Ac-DEVAD-AMC; caspase-8: Ac-IETD-AMC; caspase-9: Ac-LEHD-AMC, Peptide Institute, Osaka, Japan) were added per well. Cleavage of the fluorogenic substrates was monitored at 37°C (λ excitation 355/λ emission 460) using a Fluoroscan II microplate reader (Thermo Electron Co.). Slopes of the fluorescence curves were used as activity values and were represented as percentage of control values. Data from duplicate samples were used in each experiment.

Results

IB05204 Inhibits In vivo Angiogenesis in the Chick CAM and the Mouse Matrigel Plug Assays

Table 1 summarizes the evaluation of the in vivo inhibition of angiogenesis in the CAM assay by IB05204 and related compounds, showing that the antiangiogenic activity of IB05204 is maintained as low as 0.1 nmol per CAM, where 75% of the eggs scored positive. No significant antiangiogenic activity in the CAM assay was exhibited at 10 nmol/CAM by compounds 1, 2, and 3. IB05204 antiangiogenic effect was observed as an inhibition of the ingrowth of new vessels in the area covered by the methylcellulose discs. The peripheral vessels (relative to the position of the disc) grew centrifugally, avoiding the treated area, where a decrease in the vascular density.
could be observed (Fig. 2A). Signs of irritation or inflammation, such as a whitening of the CAM, were not observed.

To determine whether IB05204 could inhibit the bFGF-induced angiogenesis in the Matrigel plug assay, Matrigels containing bFGF with or without 4 μmol/L IB05204 were injected s.c. into mice, and the Matrigel plugs were excised after 4 days. The Matrigel plugs containing bFGF alone were apparently red (Fig. 2B, middle) due to neovascularization. We confirmed the formation of numerous blood vessels by histologic analysis of the plugs (Fig. 2C, middle). On the other hand, the Matrigel plugs treated with both bFGF and IB05204 were only partially red, indicating decreased blood vessel formation also confirmed by histologic analysis (Fig. 2B and C, right). The Matrigel plugs without bFGF were colorless and showed the absence of vasculature (Fig. 2B and C, left). Furthermore, we measured the hemoglobin content in the plugs as an indicator of neovascularization. As shown in Fig. 2D, 4 μmol/L IB05204 significantly inhibited the bFGF-induced angiogenesis in the Matrigel plug in vivo assay.

IB05204 Inhibits the Growth of Endothelial and Tumor Cells

Angiogenesis involves local proliferation of endothelial cells. We investigated the ability of IB05204 to inhibit the growth of endothelial and tumor cells. IC₅₀ values of this antiproliferative effect were 14.0 ± 1.7 and 17.0 ± 5.6 μmol/L (means of three different experiments ± SD),
for BAEC and HUVEC, respectively. Data obtained with HCT116 colon carcinoma and HT1080 fibrosarcoma cell lines (IC50 = 4.2 ± 1.0 and 9.1 ± 2.4 μmol/L, respectively; means of three different experiments ± SD) show that IB05204 is not a specific inhibitor of endothelial cell growth (Fig. 3A).

### IB05204 Inhibits the Capillary Tube Formation by Endothelial Cells

The final event during angiogenesis is the organization of endothelial cells in a three-dimensional network of tubes. In vitro, endothelial cells plated on Matrigel align themselves forming cords, already evident a few hours after plating (Fig. 3B, left). Fig. 3B (right) shows that IB05204 was able to completely inhibit the BAE and HUVEC alignment and cord formation. The minimal concentration of compound yielding a complete inhibition of endothelial morphogenesis on Matrigel was 2 μmol/L for BAEC and 5 μmol/L for HUVECs. The concentrations required to inhibit the differentiation of BAEcs and HUVECs, did not affect their viability after 7 h (results not shown). Compounds 1, 2, and 3 did not inhibit the BAEC tube formation on Matrigel when added at 10 μmol/L (results not shown).

### IB05204 Inhibits the Migratory and Proteolytic Capabilities of Endothelial Cell

Angiogenesis involves the acquisition by endothelial cells of the capability to migrate through the extracellular matrix, degrade the basement membrane, and in general, remodel the extracellular matrix. As shown in Fig. 3C, 2 μmol/L IB05204 produced a significant inhibition of the migratory capability of HUVECs. Gelatin zymography of conditioned media of IB05204-treated BAECs (Fig. 3D, left) shows that 10 μmol/L IB05204 produced a complete inhibition of MMP-2 secretion by endothelial cells. This effect does not seem to be endothelial specific because a complete inhibition of MMP-2 secretion by HT1080 tumor cells was observed after treatment with 2 or 10 μmol/L IB05204. A lower effect on MMP-9 levels was observed after HT1080 treatment with the mentioned concentrations of IB05204 (Fig. 3D, right).

### IB05204 Down-regulates the COX-2 Expression by Endothelial Cell

COX-2 seems to play a significant role as a positive regulator of tumor angiogenesis. As shown in Fig. 4A, IB05204 inhibited in a dose-dependent manner the COX-2 expression in HUVECs, with complete inhibition at 10 μmol/L IB05204.

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**Figure 3.** A, dose-dependent effect of IB05204 on the in vitro growth of HUVEC (●), BAEC (▲), HT1080 (■), and HCT116 (●) cells. Cell proliferation is represented as a percentage of control cell growth in cultures containing no drug. Point, mean of triplicates; SD values were always lower than 10% of the mean values and are omitted for clarity. B, effect of IB05204 on endothelial cell tubulogenesis in vitro. BAECs and HUVECs seeded on Matrigel formed tubes (left). Addition of 2 or 5 μmol/L IB05204 inhibited BAEC and HUVEC tubulogenesis, respectively (right). Cells were photographed 7 h after seeding under an inverted microscope (×200). C, effect of IB05204 on endothelial cell migration. Confluent HUVEC monolayers were wounded, and fresh culture medium was added in the absence or presence of 2 μmol/L IB05204. Photographs were taken at the beginning of the assay and after 2, 4, and 6 h of incubation. D, effect of IB05204 on endothelial and tumor cell gelatinases secretion. Conditioned media of BAECs and HT1080 cells treated with 2 or 10 μmol/L IB05204 and nontreated cells (control) were normalized for equal cellular density and used for gelatin zymography as indicated in Materials and Methods.
significant increases in sub-G1 population were observed by 1.5- and 4.5-fold compared with control. However, no A
dependent manner. Thus, after treatment with 2 and
containing 10% FCS and lysed for Western blot analysis of phospho-Akt.
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overnight by serum deprivation and treated with IB05204 for 2 h in the
Discussion
The formation of new blood vessels is a complex multistep process. Endothelial cells resting in the parent vessels are
activated by an angiogenic signal and stimulated to
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may be a potential target for pharmacologic intervention (3).
IB05204 antiangiogenic activity was firstly detected using the in vitro differentiation assay for endothelial cells. Our
results show that IB05204 completely inhibits capillary-like tube formation by BAEC or HUVEC at concentrations
(2 and 5 μmol/L, respectively) that are lower than those
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IB05204 activates caspases-2, -3, -8, and -9 and PARP and lamin A cleavage in BAEC
Because caspase activation plays a central role in the induction of apoptosis, fluorometric assays were done to
investigate the activation of caspases-2, -3, -8, and -9 in IB05204-treated endothelial cells. As shown in Fig. 5C, the initiator caspases-2, -8, and -9 and the effector caspase-3 were significantly activated in BAEC after treatment with
IB05204 (5 μmol/L, 14 h). Activated caspases cleave PARP and lamin A, among other cellular substrates, which leads to all phenomena of the apoptotic morphology. As shown in Fig. 5D, PARP was cleaved from the 116-kDa intact form into a 85-kDa fragment after BAEC treatment with 2 or 5 μmol/L of IB05204. Treatment of BAEC with 5 μmol/L IB05204 caused lamin A cleavage, generating a fragment of 28 kDa, and that, by the addition of 25 μmol/L of the pan-caspase inhibitor N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (ZVAD), inhibited lamin A cleavage, which indicates that IB05204 induces apoptosis in BAEC through a caspase-dependent pathway (Fig. 5E).
IB05204 Induces Cytochrome c Release from Mitochondria
To determine whether IB05204 targets mitochondrial permeability in endothelial cells, the distribution of cytochrome c protein between cell cytosol and mitochondria was measured by Western blotting. As shown in Fig. 5F, cytochrome c was found to be effectively released from the mitochondria into the cytosol after treating BAEC with 5 μmol/L IB05204.

IB05204 Inhibits the Serum-Induced Akt Phosphorylation
Akt, also known as protein kinase B, regulates essential cellular functions, such as migration, proliferation, differentation, and apoptosis. We examined whether IB05204 was able to inhibit the phosphorylation of Akt induced by serum in BAEC. Treatment of the BAEC with serum resulted in a significant enhancement of Akt phosphorylation that was completely blocked by the presence of 5 or 10 μmol/L IB05204 (Fig. 4B).

IB05204 Induces DNA Condensation and Fragmentation in Endothelial Cells in vitro
As a first approach to determine whether IB05204 could induce apoptosis in endothelial and tumor cells, nuclear morphology was investigated in BAECs, HCT-116, and HT1080 cells after 14 h treatment with different concentrations of IB05204. Figure 5A shows that 5 μmol/L IB05204 induced chromatin condensation in endothelial cells, whereas no significant changes on the nuclear morphology were observed in colon carcinoma or fibrosarcoma cells treated with 10 μmol/L IB05204. These results suggested that IB05204 could selectively induce apoptosis in endothelial cells.

To confirm the putative apoptotic activity of IB05204 on endothelial cells, cell cycle analysis was done in IB05204-treated BAEC after propidium iodide staining. Flow-cytometric analysis showed that IB05204 significantly increased apoptotic sub-G1 cells in a concentration-dependent manner. Thus, after treatment with 2 and 5 μmol/L IB05204 for 14 h, apoptotic cells were increased by 1.5- and 4.5-fold compared with control. However, no significant increases in sub-G1 population were observed when colon cancer HCT-116 cells were treated with IB05204 in the same experimental conditions (results not shown). Apoptosis induction in BAEC by IB05204 was also confirmed by the DNA fragmentation assay. Figure 5B shows that the treatment of BAEC with 10 μmol/L IB05204 for 14 h induced DNA laddering, a hallmark of apoptosis.

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(IC50 = 4.2, and 9.1 μmol/L for HCT-116 and HT1080 cell

![Figure 4. Effect of IB05204 on endothelial COX-2 expression and Akt phosphorylation.](image-url)
The 2 μmol/L IB05204 causes a significant inhibition of BAECs migratory abilities, suggesting that the antiangiogenic activity of IB05204 is dependent on the prevention of capillary-like tube formation and endothelial migration rather than proliferation. Some criteria by which a chemotherapeutic agent might be considered to have meaningful antiangiogenic activity have been recently suggested (12). Considering that IB05204 interferes with endothelial cell function at nontoxic concentrations, this compound could be considered an antiangiogenic compound. This assessment is reinforced by the angiogenesis-inhibitory activity of IB05204 in the CAM and the mouse Matrigel plug assays, two of the most widely used in vivo angiogenesis assays (13). Our results show that IB05204 inhibits the neovascularization of the CAM and the Matrigel plug at concentrations that are orders of magnitude lower than those required for other inhibitors of angiogenesis (7, 14–18). IB05204 antiangiogenic activity seems to be highly dependent on the chloro substituents because their removal results in a complete loss of the in vitro inhibitory activity of endothelial differentiation and in vivo antiangiogenic activity. Halogen substituents in aromatic rings are found in other previously described angiogenesis inhibitors, including SU11248 (sunitinib) and BAY 43-9006 (sorafenib), the only two small size inhibitors of angiogenesis currently approved for the treatment of cancer patients (19). The fluoro-dihydroindol derivatives SU11248 and SU14813 inhibit vascular endothelial growth factor receptor-2 (VEGFR-2) and platelet-derived growth factor receptor-β (PDGFR-β), among other receptor tyrosine kinases (20, 21). The chloro-diaryl urea BAY 43-9006 exhibits antitumoral activity, based on its inhibition of the Raf/ mitogen-activated protein (MAP)/ERK kinase (MEK)/Erk signaling pathway, and inhibits tumor neovascularization by targeting the VEGFR and PDGFR pathways (22). Other halogen-containing compounds are the inhibitors of VEGFR-2 ZD4190, ZD6474, PTK787 and KRN951, and the bromotyrosine aeroplysinin-1 isolated from several marine lines, respectively). The 2 μmol/L IB05204 causes a significant inhibition of BAECs migratory abilities, suggesting that the antiangiogenic activity of IB05204 is dependent on the prevention of capillary-like tube formation and endothelial migration rather than proliferation. Some criteria by which a chemotherapeutic agent might be considered to have meaningful antiangiogenic activity have been recently suggested (12). Considering that IB05204 interferes with endothelial cell function at nontoxic concentrations, this compound could be considered an antiangiogenic compound. This assessment is reinforced by the angiogenesis-inhibitory activity of IB05204 in the CAM and the mouse Matrigel plug assays, two of the most widely used in vivo angiogenesis assays (13). Our results show that IB05204 inhibits the neovascularization of the CAM and the Matrigel plug at concentrations that are orders of magnitude lower than those required for other inhibitors of angiogenesis (7, 14–18). IB05204 antiangiogenic activity seems to be highly dependent on the chloro substituents because their removal results in a complete loss of the in vitro inhibitory activity of endothelial differentiation and in vivo antiangiogenic activity. Halogen substituents in aromatic rings are found in other previously described angiogenesis inhibitors, including SU11248 (sunitinib) and BAY 43-9006 (sorafenib), the only two small size inhibitors of angiogenesis currently approved for the treatment of cancer patients (19). The fluoro-dihydroindol derivatives SU11248 and SU14813 inhibit vascular endothelial growth factor receptor-2 (VEGFR-2) and platelet-derived growth factor receptor-β (PDGFR-β), among other receptor tyrosine kinases (20, 21). The chloro-diaryl urea BAY 43-9006 exhibits antitumoral activity, based on its inhibition of the Raf/ mitogen-activated protein (MAP)/ERK kinase (MEK)/Erk signaling pathway, and inhibits tumor neovascularization by targeting the VEGFR and PDGFR pathways (22). Other halogen-containing compounds are the inhibitors of VEGFR-2 ZD4190, ZD6474, PTK787 and KRN951, and the bromotyrosine aeroplysinin-1 isolated from several marine
sponges (3, 23). Structure-function studies with quinazolinonamino-benzoquinones and pyrazine-pyridine biheteroaryl yls reveal that halogen substitution increases the activity of the compounds as inhibitors of the kinase domain of VEGFR-2 (24, 25). Although evidences point out to the relevance of electron-withdrawing halogen substituents in the antiangiogenic inhibitor molecules, their role in the mechanism of the reaction involved in the inhibition of the receptor tyrosine kinase activity remains obscure and deserves further studies.

A positive proteolytic balance is required for capillary sprouting and lumen formation during angiogenesis. MMPs play a central role during angiogenesis (26). Endothelial cells constitutively secrete MMP-2, which is required for the tumor to trigger the angiogenic response (27). When endothelial cells are cultured on Matrigel, the formation of tubular networks is increased by the addition of recombinant MMP-2 and decreased when neutralizing antibody is added (28). Our data show that incubation with IB05204 inhibits MMP-2 secretion by BAEC. Similar decreases in MMP-2 activity in the conditioned media of endothelial cells have been described for curcumin (29), halofuginone (30), and aeropylsin-1 (7), and they have been suggested to lead to the inhibition of the endothelial cell tube formation in vitro. The inhibition of MMP-2 production by IB05204 does not seem to be endothelial specific because a similar effect was observed in HT1080 tumor cells.

COX-2, a key enzyme in the synthesis of prostanoids, is highly up-regulated in tumor cells, stromal cells, and angiogenic endothelial cells during tumor progression. Recent experiments have shown that COX-2 promotes tumor angiogenesis, and COX-2 inhibition inhibits angiogenesis in a number of tumor models. Therefore, the targeting of COX-2, either by inhibiting its enzymatic activity or by blocking its transcription, might be useful in combating angiogenesis-dependent diseases (31). Our results show that 5 μmol/L IB05204 inhibited and 10 μmol/L IB05204 completely abrogated the expression of COX-2 in HUVEC, suggesting the possibility that the antiangiogenic activity of IB05204 could be also due to a direct modulation of the endothelial COX-2 expression.

One proposed mechanism for COX-2 inhibitor antiangiogenic activity is the induction of apoptosis in endothelial cells (32). COX-2 inhibitors have been reported to block phosphorylation of Akt in endothelial, mural, and endothelial progenitor cells (33–36). On the other hand, the observation that IB05204 inhibited endothelial cell migration and morphogenesis at concentrations that had little or no effect on cell growth suggested that IB05204 could preferentially target cellular motility. The serine/threonine protein kinase Akt signaling axis is activated by a variety of stimuli in endothelial cells and regulates multiple critical steps in angiogenesis, including endothelial cell survival, migration, and differentiation (37). Here, we show that 5 μmol/L IB05204 completely inhibited BAEC serum-induced Akt phosphorylation and suggested a possible proapoptotic activity of this compound. It has been previously reported that a number of endogenous and exogenous angiogenesis inhibitors do induce endothelial cell apoptosis, thereby preventing the growth of primary tumors and their metastases (7, 38–43).

Apoptosis is associated with characteristic morphologic changes, including chromatin condensation, nuclear fragmentation, cell shrinkage, plasma membrane blebbing, and the formation of apoptotic bodies (44). Our studies on the nuclear morphology of BAEC revealed that IB05204 induces nuclear changes, characterized by chromatin condensation and nuclear fragmentation. This result was also confirmed by the monitoring of the cell cycle distribution, showing an increase of the percentage of cells with sub-diploid DNA content, a hallmark of an apoptotic cell population (45). In contrast, a similar dose of IB05204 did not cause any observable effect in the cancer cell lines HCT-116 and HT1080. The fact that IB05204 induces apoptosis in endothelial cells at doses that do not affect the morphology of tumor cells, whereas the IC50 for the tumor cells are lower than that for the endothelial cells, suggests that different mechanisms are responsible for the growth inhibition activity of this compound in different cell types, so that IB05204 might be modulating more than one molecular target. This is not surprising because, as mentioned before, other halogenated compounds (e.g., sunitinib and sorafemib) are multtargeted small-molecule tyrosine kinase inhibitors, simultaneously inhibiting multiple pathways and resulting in an antiangiogenic effect and a direct antitumoral effect by inhibition of oncogene-activated pathways. The clinical success of multitargeted compounds indicates that chances of success in eradicating cancer will increase when catching tumors in the crossfire of more than one pathway-inhibiting agent (46). The different effects of IB05204 on different cell types suggest that this compound could also be a multtargeted compound. Moreover, the pleiotropic nature of the activity of IB05204 on endothelial cells in vitro resembles that of some receptor tyrosine kinase inhibitors. A further characterization of the putative modulation of different signaling pathways by IB05204 will be of our interest in the near future.

Apoptosis can be induced by two pathways: the extrinsic death receptor cascade or the intrinsic mitochondria cascade. In both pathways, caspases (cysteine aspartases) are critical components of the apoptotic machinery. All caspases are produced in cells as catalytically inactive zymogens and must undergo activation during apoptosis (47). Here, we show that 5 μmol/L IB05204 induces the activation of the upstream initiator caspases-2, -8, and -9, and the downstream effector caspase-3. When caspases are activated, they are capable of cleaving many important cellular substrates, including the DNA repair enzyme PARP and lamin A, a nuclear protein that maintains the normal nuclear structure. Incubation with 2 or 5 μmol/L of IB05204 induced PARP cleavage in BAEC. IB05204-induced lamin A cleavage was inhibited by the addition of ZVAD, a widely used caspase inhibitor, indicating that IB05204 induces apoptosis in BAEC through a caspase-dependent...
pathway. A precursor to caspase activation is the release of cytochrome c, as the mitochondria become leakier after the activation of the mitochondrial pathway of induction of apoptosis. In this study, IB05204 effectively induced cytochrome c release from the mitochondria into the cytosol. Taken together, these results suggest that IB05204 induces apoptosis through cytochrome c release and caspase activation in endothelial cells and imply that it may be a good antiangiogenic agent. However, further studies of the biological mechanisms and the *in vivo* effects of IB05204 are required.

In conclusion, we have shown for the first time that IB05204 is able to inhibit certain functions of endothelial cells, namely, differentiation, proliferation, and migration, and it exhibits a potent inhibition of *in vivo* angiogenesis. IB05204 antiangiogenic activity could be related to its ability to inhibit the endothelial MMP-2 secretion, to down-regulate endothelial COX-2 expression, and to inhibit Akt phosphorylation. IB05204 induces endothelial cell apoptosis through cytochrome c release and caspase activation. Although additional studies will be needed to elucidate the molecular mechanisms underlying the antiangiogenic activity of IB05204, data presented here suggest its potential in therapeutic applications for the treatment of angiogenesis-related malignancies.

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