

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

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Abstract

In the course of a blind screening program for inhibitors of angiogenesis, IB05204 (4,8-dichloro-12-phenylpyrido[5',6':4'',5'';3',2':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''3',2':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)

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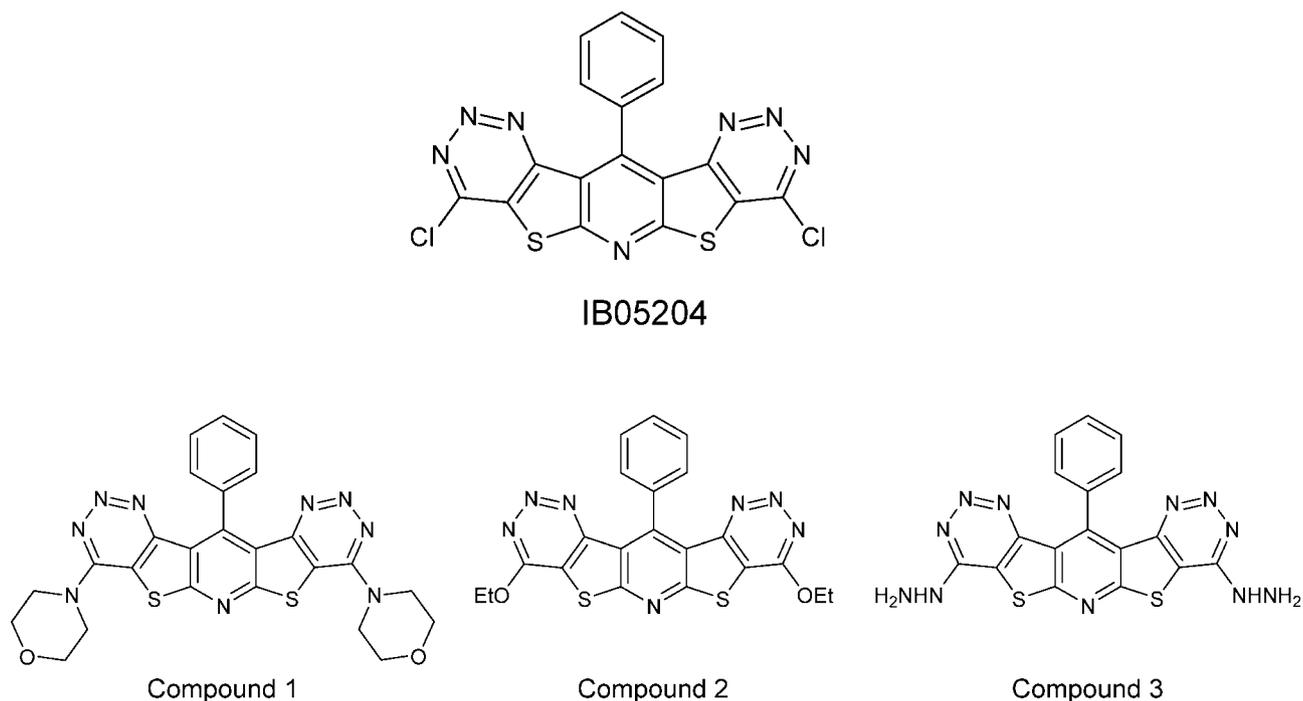


Figure 1. Chemical structure of IB05204 and derivatives.

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 -enamionitrile 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 $\mu\text{g}/\text{mL}$), and amphoterycin (1.25 $\mu\text{g}/\text{mL}$) 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 $\mu\text{g}/\text{mL}$), and amphoterycin (1.25 $\mu\text{g}/\text{mL}$) 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 $\mu\text{g}/\text{mL}$), and amphoterycin (1.25 $\mu\text{g}/\text{mL}$), supplemented with 3 mg/L endothelial cell growth supplement (Sigma) and 20% FBS in 5% CO_2 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 μ L 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 \times 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). 3×10^3 BAECs, 4×10^3 HUVECs, 2×10^3 HCT-116, and HT1080 in a total volume of 100 μ L 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 μ L 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 μ L 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 incubator, 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 μ L 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 μ L of Matrigel containing basic fibroblast growth factor (bFGF; 1 μ g/mL) and 4 μ mol/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 (13 μ m thick), and stained with H&E.

Statistical Analysis

All data are expressed as means \pm 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 gelatinograms 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 μ mol/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 μ L/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 2×10^6 cells were resuspended in 100 μ L 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 μ g digitonin (5 min, room temperature). Samples were centrifuged (13,000 rpm, 5 min), and supernatants (soluble cytosolic fraction) were collected and mixed with 6 \times sample buffer; pellets (mitochondria and nuclei) were resuspended in the same volume of 2 \times sample buffer. In Akt phosphorylation studies, BAECs were serum-deprived overnight; after treatment with 5 or 10 μ mol/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 \times sample buffer. For poly(ADP-ribose)polymerase (PARP) or Lamin A cleavage studies, BAECs were grown 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 \times sample buffer.

Cell lysates in 2 \times sample buffer were denaturalized for 5 min at 95°C and separated by SDS-PAGE. After proteins

electro-transference, nitrocellulose membranes (PRO-TRAN, 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 Biosite); 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% β -mercaptoethanol] for 30 min at 50°C with shaking.

Analysis of Oligonucleosomal DNA Cleavage

Sub-confluent BAECs were treated with 10 μ mol/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 μ mol/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 μ g/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 μ g/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 μ mol/L, 14 h), and then, attached and detached cells were harvested. A total of 5×10^5 cells were resuspended in 25 μ L PBS and snap-frozen in wells of a 96-well opaque microtiter plate on liquid nitrogen. After thawing on ice, 50 μ L 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 μ mol/L of selective fluorogenic substrates (caspase-2: Ac-VDVAD-AMC; caspase-3: Ac-DEVD-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 ($\lambda_{excitation}$ 355/ $\lambda_{emission}$ 460 nm) 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

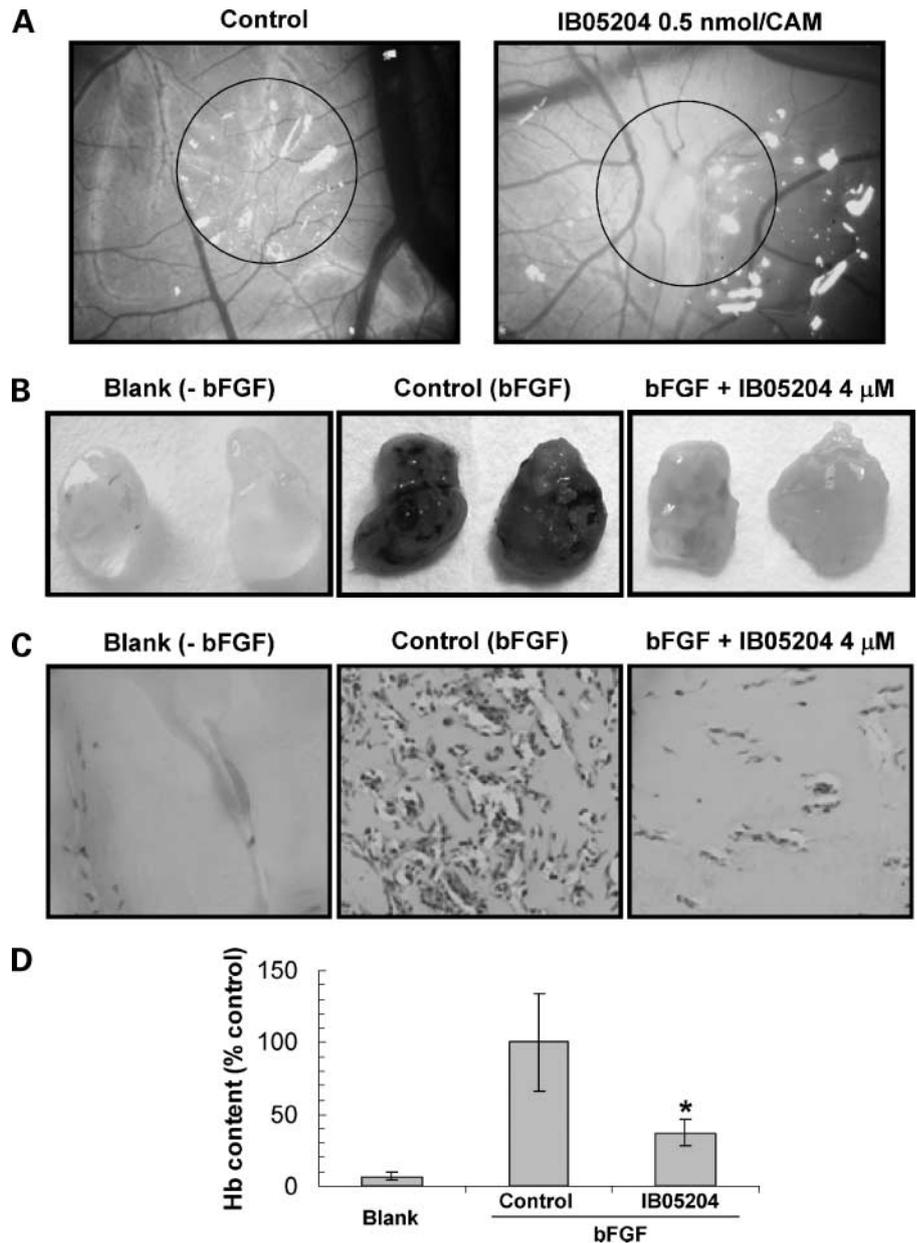
Table 1 summarizes the evaluation of the *in vivo* inhibition of angiogenesis in the CAM assay by IB05204 and related compounds, showing that the antiangiogenic activity of IB05204 is maintained as low as 0.1 nmol per CAM, where 75% of the eggs scored positive. No significant antiangiogenic activity 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

Table 1. Inhibition of *in vivo* angiogenesis by IB05204 and related compounds

Dose (nmol/CAM)	IB05204	Compound 1	Compound 2	Compound 3
0	0 (0/20)	0 (0/20)	0 (0/20)	0 (0/20)
0.05	20 (1/5)	ND	ND	ND
0.1	75 (3/4)	ND	ND	ND
0.2	100 (4/4)	ND	ND	ND
0.5	100 (3/3)	ND	ND	ND
1	100 (3/3)	ND	0 (0/3)	ND
2	100 (4/4)	0 (0/3)	0 (0/3)	ND
4	100 (4/4)	0 (0/3)	0 (0/4)	0 (0/4)
10	100 (3/3)	0 (0/4)	0 (0/3)	25 (1/4)

NOTE: CAM assay was carried out with different doses of the compounds as described in Materials and Methods. Data are given as percentage of inhibition. Between brackets, the number of eggs with inhibited angiogenesis in their CAMs per total number of treated egg CAMs is indicated. Abbreviation: ND, not determined.

Figure 2. Effect of IB05204 on *in vivo* angiogenesis. **A**, CAM assay of IB05204. *Left*, methylcellulose disc containing the substance vehicle alone. *Right*, methylcellulose disc containing 0.5 nmol of IB05204. *Circles*, locations of the methylcellulose discs. **B–D**, bFGF-induced Matrigel-plug assay containing or lacking 4 $\mu\text{mol/L}$ IB05204, as described in Materials and Methods. **B**, representative Matrigel plugs that contained no bFGF (*blank*), bFGF alone, or bFGF plus IB05204 were photographed. **C**, representative sections of the plugs stained by H&E. **D**, the total hemoglobin (*Hb*) content in the Matrigel plugs was quantified as an indicator of blood vessel formation. Data are expressed as means \pm SD of at least five animals. *, $P < 0.005$ versus bFGF alone.



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 $\mu\text{mol/L}$ 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 indicator of neovascularization. As shown in Fig. 2D, 4 $\mu\text{mol/L}$ 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. IC_{50} values of this antiproliferative effect were 14.0 ± 1.7 and 17.0 ± 5.6 $\mu\text{mol/L}$ (means of three different experiments \pm SD),

for BAEC and HUVEC, respectively. Data obtained with HCT116 colon carcinoma and HT1080 fibrosarcoma cell lines ($IC_{50} = 4.2 \pm 1.0$ and $9.1 \pm 2.4 \mu\text{mol/L}$, respectively; means of three different experiments \pm 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 $2 \mu\text{mol/L}$ for BAE and $5 \mu\text{mol/L}$ 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 $10 \mu\text{mol/L}$ (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, $2 \mu\text{mol/L}$ 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 $10 \mu\text{mol/L}$ 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 $10 \mu\text{mol/L}$ 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 $10 \mu\text{mol/L}$ IB05204.

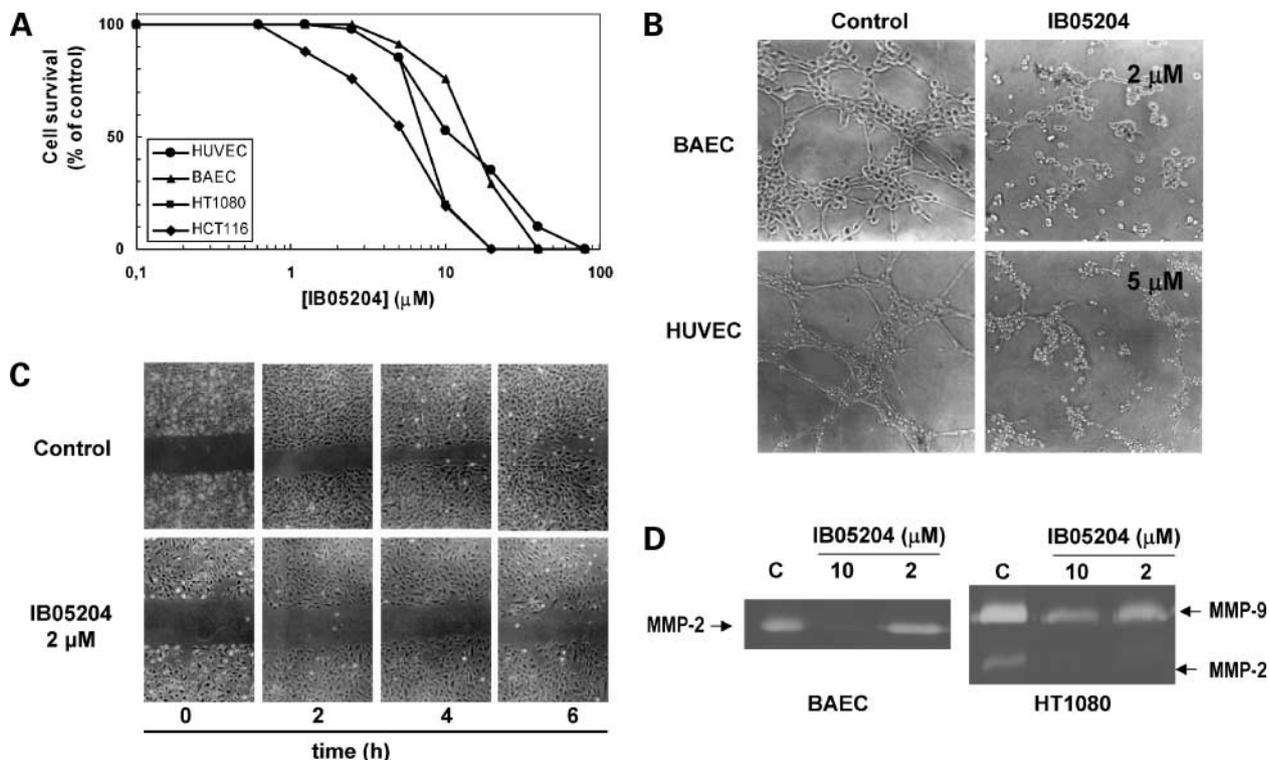


Figure 3. **A**, dose-dependent effect of IB05204 on the *in vitro* growth of HUVEC (●), BAEC (▲), HT1080 (■), and HCT116 (◆) cells. Cell proliferation is represented as a percentage of control cell growth in cultures containing no drug. *Point*, mean of triplicates; SD values were always lower than 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 $5 \mu\text{mol/L}$ IB05204 inhibited BAEC and HUVEC tubulogenesis, respectively (*right*). Cells were photographed 7 h after seeding under an inverted microscope ($\times 200$). **C**, effect of IB05204 on endothelial cell migration. Confluent HUVEC monolayers were wounded, and fresh culture medium was added either in the absence or presence of $2 \mu\text{mol/L}$ 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 $10 \mu\text{mol/L}$ IB05204 and nontreated cells (control) were normalized for equal cellular density and used for gelatin zymography as indicated in Materials and Methods.

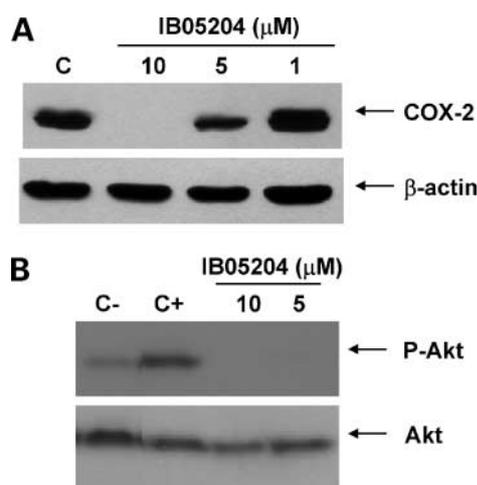


Figure 4. Effect of IB05204 on endothelial COX-2 expression and Akt phosphorylation. **A**, HUVECs were grown to sub-confluence, treated with the indicated concentrations of IB05204 in the presence of 50 ng/mL PMA and lysed, and COX-2 was detected by Western blot analysis. Detection of β -actin was used as loading control. **B**, sub-confluent BAECs were starved overnight by serum deprivation and treated with IB05204 for 2 h in the absence of serum. Cells were stimulated for 10 min with culture medium containing 10% FCS and lysed for Western blot analysis of phospho-Akt. Membranes were stripped off and reprobed for detection of total Akt. C+, BAEC stimulated with serum; C-, nonstimulated BAECs.

IB05204 Inhibits the Serum-Induced Akt Phosphorylation

Akt, also known as protein kinase B, regulates essential cellular functions, such as migration, proliferation, differentiation, 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 μ mol/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 μ mol/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 μ mol/L IB05204. These results suggested that IB05204 could selectively induce apoptosis on 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 μ mol/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 μ mol/L IB05204 for 14 h induced DNA laddering, a hallmark of apoptosis.

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 μ mol/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 μ mol/L of IB05204. Treatment of BAEC with 5 μ mol/L IB05204 caused lamin A cleavage, generating a fragment of 28 kDa, and that, by the addition of 25 μ mol/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 μ mol/L IB05204.

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 synthesize and release degradative enzymes, allowing endothelial cells to migrate, proliferate, and finally, differentiate to give rise to capillary tubules. Any of these steps 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 μ mol/L, respectively) that are lower than those required for endothelial proliferation inhibition (IC_{50} = 14 and 17 μ mol/L for BAEC and HUVEC, respectively). Furthermore, IB05204 antiproliferative effect does not seem to be endothelial cell specific because these IC_{50} values are similar or even higher to those obtained with tumor cells (IC_{50} = 4.2, and 9.1 μ mol/L for HCT-116 and HT1080 cell

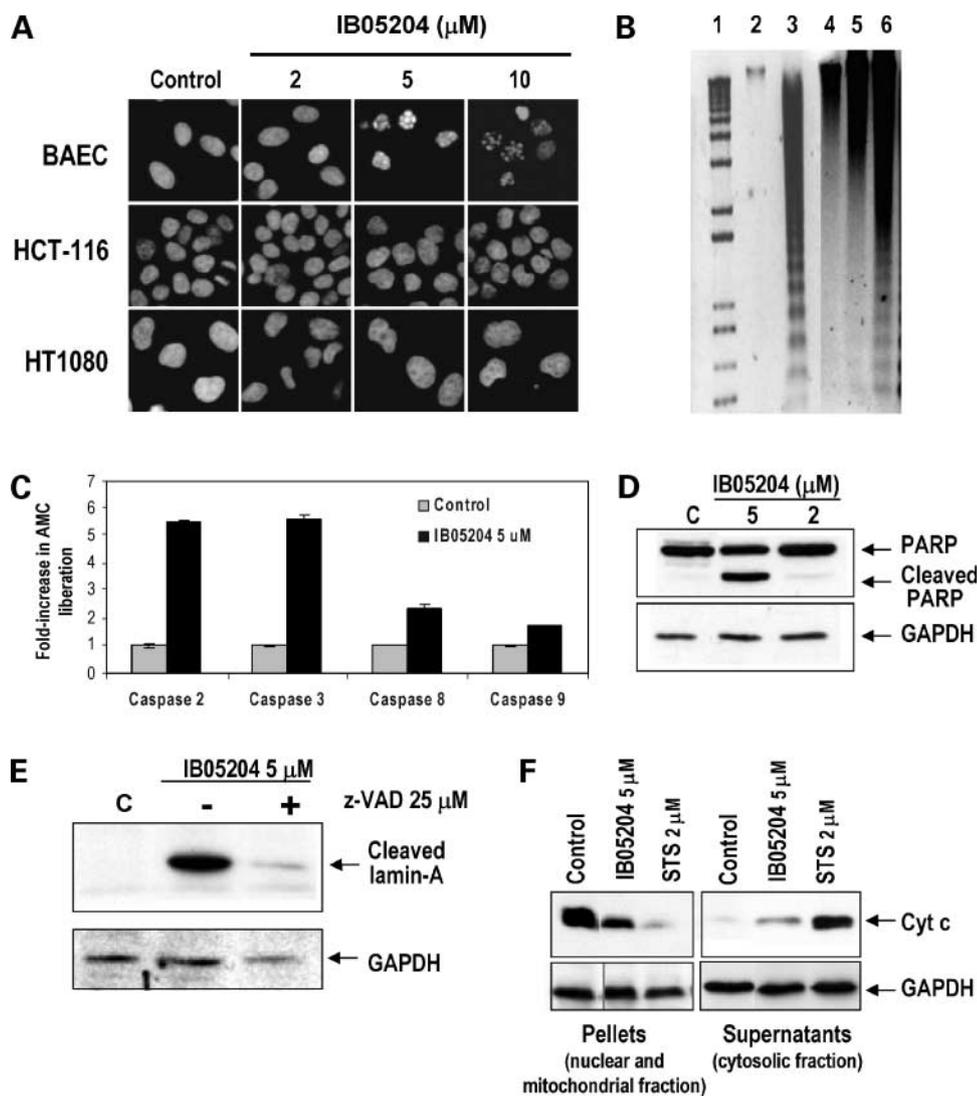


Figure 5. **A**, effect of IB05204 in DNA condensation. Cells were grown on covers, treated with the indicated concentrations of IB05204 for 14 h, fixed with formalin, and stained with 1 $\mu\text{g}/\text{mL}$ DAPI. Covers were mounted on slides, and nuclei were observed under fluorescence microscope. **B**, effect of IB05204 in BAEC DNA fragmentation. *Lane 1*, DNA molecular size ladder; *lane 2*, nontreated cells; *lane 3*, cells treated with 10 μM /L 2-methoxyestradiol; *lane 4*, cells treated with 2 μM /L IB05204; *lane 5*, cells treated with 5 μM /L IB05204; *lane 6*, cells treated with 10 μM /L IB05204. **C**, effect of IB05204 in BAEC caspase activation. Attached and unattached BAECs treated for 14 h with the indicated concentrations of IB05204 were harvested, and caspase activity was measured by fluorescence liberation using a fluorescence microplate reader. Slopes of the obtained curves were taken as activity values and represented as percentage of control values (mean \pm SD). **D**, Western blot detection of PARP cleavage in IB05204-treated BAECs. GAPDH was used as loading control. **E**, Western blot detection of cleaved lamin-A in IB05204-treated BAECs with or without the preincubation of cells with the pan-caspase inhibitor 25 μM /L z-VAD. **F**, effect of IB05204 in cytochrome *c* release from mitochondria. Cells were permeabilized with 10 μg digitonin, and cytosolic fraction was separated from nuclear and mitochondrial fraction. Cytochrome *c* (Cyt *c*) in both fractions was detected by Western blot, and GAPDH was used as loading control. Treatment of BAEC with 2 μM /L staurosporine (STS) for 6 h was used as internal control of mitochondrial cytochrome *c* release.

lines, respectively). The 2 μM /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 quinazolinamino-benzoquinones and pyrazine-pyridine biheteroaryls 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 aeropylsinin-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 $\mu\text{mol/L}$ IB05204 inhibited and 10 $\mu\text{mol/L}$ 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 $\mu\text{mol/L}$ 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 IC_{50} 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 sorafenib) are multitargeted 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 multitargeted 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 $\mu\text{mol/L}$ 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 $\mu\text{mol/L}$ 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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IB05204, a dichloropyridodithienotriazine, inhibits angiogenesis *in vitro* and *in vivo*

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