Antiangiogenic Effect of TW37, a Small-Molecule Inhibitor of Bcl-2

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Abstract

Bcl-2 is an antiapoptotic protein that is up-regulated in several tumor types, and its expression levels have strong correlation to development of resistance to therapy and poor prognosis. We have shown recently that Bcl-2 also functions as a proangiogenic signaling molecule that activates a nuclear factor-κB−mediated pathway resulting in up-regulation of the angiogenic chemokines CXCL1 and CXCL8 by neovascular endothelial cells. Here, we evaluate the antiangiogenic effect of the novel small-molecule inhibitor of Bcl-2 (TW37) developed using a structure-based design strategy. We observed that TW37 has an IC_{50} of 1.8 μmol/L for endothelial cells but showed no cytotoxic effects for fibroblasts at concentrations up to 50 μmol/L. The mechanism of TW37-induced endothelial cell death was apoptosis, in a process mediated by mitochondrial depolarization and activation of caspase-9 and caspase-3. The effect of TW37 on endothelial cell apoptosis was not prevented by coexposure to the growth factor milieu secreted by tumor cells. Inhibition of the angiogenic potential of endothelial cells (i.e., migration and capillary sprouting assays) and expression of the angiogenic chemokines CXCL1 and CXCL8 were accomplished at subapoptotic TW37 concentrations (0.005-0.05 μmol/L). Notably, administration of TW37 i.v. resulted in a decrease in the density of functional human microvessels in the severe combined immunodeficient mouse model of human angiogenesis. In conclusion, we describe functionally separate proapoptotic and antiangiogenic mechanisms for a small-molecule inhibitor of Bcl-2 and show the potential for Bcl-2 inhibition as a target for antiangiogenic therapy. (Cancer Res 2006; 66(17): 8698-706)

Introduction

Tumor establishment and growth absolutely requires angiogenesis, development of new blood vessels from existing capillaries, to maintain a nutrient and oxygen source (1). Cancer, but also rheumatoid arthritis, retinopathy of prematurity, and diabetic retinopathy, displays uncontrolled blood vessel growth as a major factor in the progression of disease. Therefore, targeted inhibition of the pathologic angiogenesis may be an attractively specific and well-tolerated therapy in treatment of these conditions (2–5).

Most conventional anticancer therapies are intrinsically toxic and relatively nonspecific. Tumors may be inherently resistant, or

may develop selectively induced resistance, to conventional chemotherapeutics, such as cis-diamminodichloroplatinum (cisplatin; refs. 6, 7). Because the target of antiangiogenic drugs is nontransformed endothelial cells, the development of resistance to therapy is unlikely. Thus, development of antiangiogenic drugs has been an area of increasing interest over the last few years (8–10). The class of specific antiangiogenic drugs with largest clinical trial history is those blockading growth factor receptor pathways at ligand, receptor, or signaling levels (8, 10, 11). Within this group, the tyrosine kinase inhibitors have dominated in number and efficacy, with one exception being the humanized monoclonal anti–vascular endothelial growth factor (VEGF) antibody bevacizumab (Avastin; refs. 10, 12). Bevacizumab has been examined in phase I to phase III trials and has displayed tumor type–dependent results (13, 14). Encouraging results have been also observed for PTK787 (vatalanib), a VEGF receptor tyrosine kinase (RTK) inhibitor, and ZD6474, a VEGF/epidermal growth factor RTK inhibitor, in clinical trial (15, 16). Therefore, the concept of antiangiogenic therapy in cancer using small-molecule inhibitors to modulate the endothelial cell activation network has been well established. Most recently, the value of targeted combination antiangiogenic/antitumor therapy has been revealed elegantly in vivo using nanoparticle encapsulation with a slow release mechanism to deliver doxorubicin and combretastatin-A4 simultaneously to the tumor (17).

The expression of the prosurvival molecule Bcl-2 is up-regulated in a variety of tumor types (18–22). Studies have shown that modulation of Bcl-2 in tumor cells of varying lineage results in alteration of variables in tumor microvascular density (23–25). However, the direct involvement of endothelial cell Bcl-2 in the modulation of tumor-associated angiogenesis has only begun recently to be explored (26–28). Bcl-2 and other prosurvival or proapoptotic members of the Bcl-2 family maintain a balance within the cell that is biased toward survival through an intricate web of heterodimer and homodimer interactions. However, both external and internal stimuli may alter that balance toward apoptosis by inactivation of Bcl-2/Bcl-x_L, subsequently tipping the balance in favor of the proapoptotic family members. Binding of endogenous Bcl-2/Bcl-x_L ligands to the molecules allows release of Bcl-2 family members Bax/Bak, which insert into the mitochondrial membrane inducing membrane depolarization and subsequent activation of the caspase cascade (29, 30).

We have shown recently that Bcl-2 is directly proangiogenic through a pathway unrelated to its apoptotic function (28). We observed that Bcl-2 induces expression of the proangiogenic chemokines CXCL1 and CXCL8 in a nuclear factor-κB−dependent way. In the present study, we examine the activity of a small molecular inhibitor of Bcl-2 (TW37) on viability and angiogenic potential of human microvascular endothelial cells. We investigated whether inhibition of Bcl-2 function with TW37 alone is able to induce growth inhibition and apoptosis in endothelial cells

using cell cytotoxicity assays, fluorescence-activated cell sorting (FACS), and plate-based caspase assays. Using a collagen-based capillary sprouting assay, an *in vitro* migration assay, and ELISA, in addition to an *in vivo* model of human angiogenesis, we also investigated the antiangiogenic effect of blocking Bcl-2 function with TW37. We hypothesized that intervention of the Bcl-2 function by small-molecule inhibitors is sufficient for inhibition of the angiogenic potential of neovascular endothelial cells.

Materials and Methods

Cell culture. Primary human dermal microvascular endothelial cells (HDMEC) were purchased from Clonetics and cultured in endothelial cell growth medium (EGM2-MV; San Diego, CA). Oral squamous cell carcinoma-3 (OSCC3; gift from M. Lingen, University of Chicago); UM-SCC-17B, UM-SCC-74A, and UM-SCC-74B (gift from T. Carey, University of Michigan, Ann Arbor, MI); and LNCaP, MCF-7, human dermal fibroblasts (HDF), and Kaposi's sarcoma cells (SLK; gift from G. Nunez, University of Michigan, Ann Arbor, MI) were cultured in DMEM supplemented with 10% fetal bovine serum (Life Technologies/Invitrogen, Grand Island, NY). Tumor cell conditioned media were diluted 1:9 in EGM2-MV for testing of endothelial cell responses to treatment. Immunoassay for human VEGF (Quantikine, R&D Systems, Minneapolis, MN) was used to determine the concentration of this growth factor in tumor cell conditioned medium according to the manufacturer's protocol.

Cytotoxicity assays. The sulforhodamine B (SRB) cytotoxicity assay was used as described (31). Briefly, optimal cell density for cytotoxicity assay, 2×10^4 to 3×10^4 cells per well, was determined by growth curve analysis. HDMECs were seeded at 2.5×10^4 per well in a 96-well plate and allowed to adhere overnight. Drug or control was diluted in EGM2-MV and layered onto cells, which were allowed to incubate for times as indicated in the figures. Alternatively, HDMECs were coincubated with TW37 and 0 to 100 ng/mL recombinant human VEGF $(rhVEGF)_{165}$ or 0 to 100 ng/mL recombinant human CXCL8 (R&D Systems). Cells were fixed on the plates by addition of cold trichloroacetic acid (10% final concentration) and incubation for 1 hour at 4°C. Cellular protein was stained by addition of 0.4% SRB in 1% acetic acid and incubation at room temperature for 30 minutes. Unbound SRB was removed by washing with 1% acetic acid and the plates were air dried. Bound SRB was resolubilized in 10 mmol/L unbuffered Tris-base and absorbance was determined on a microplate reader at 560 nm (Genios Tecan, Tecan, Graz, Austria). Test results were normalized against initial plating density and drug-free controls. Data were obtained from triplicate wells per condition and are representative of at least three independent experiments.

Flow cytometry. Cells were seeded at either 3×10^5 to 5×10^5 per well in a six-well plate and allowed to adhere overnight. Medium was aspirated, and drug or controls, diluted in EGM2-MV medium, were added to the cells. Cells were incubated for times as indicated in the figures and assessed for apoptosis by hypotonic lysis and staining of DNA with propidium iodide (PI) as described (32). Apoptotic levels were determined by flow cytometry and cell cycle analysis of sub-G_1 fractions. Statistical significance for this assay and throughout this article was determined at the $P \leq 0.05$ level using one-way ANOVA and the Tukey post hoc test.

Fluorometric assay for caspase activity. The involvement of caspase-3 and caspase-9 on TW37-induced apoptosis was evaluated with a fluorometric assay. Cells were exposed to TW37 or vehicle control for times and concentrations as indicated in the figures. Both attached and floating cells were retrieved and lysed [cell lysis buffer (Biovision, Mountain View, CA). Cell extracts diluted in caspase assay 2× reaction buffer (Biovision) were assayed as described previously (32). Substrates Ac-DEVD-AMC and Ac-IETD-AMC, inhibitor Ac-DEVD-CHO and Ac-IETD-CHO, and recombinant proteins were obtained from Alexis Biochemicals (San Diego, CA). Data were obtained from triplicate wells per treatment from three independent experiments.

Confocal analysis of mitochondrial depolarization. HDMECs were cultured in LabTek eight-well chamber slides (Nalge Nunc International,

Naperville, IL) and exposed to varying concentrations of TW37 for 3 hours. Cells were washed in fresh medium to remove drug and then incubated with 100 nmol/L MitoTracker Red CMXRos (Molecular Probes/Invitrogen, Carlsbad, CA) for 30 minutes, washed finally in PBS, and fixed in 10% formalin at 4°C. Cells were finally exposed for 3 minutes to 300 nmol/L 4′,6-diamidino-2-phenylindole (DAPI) in PBS. Slides were mounted with Prolong Gold mounting medium (Molecular Probes) and visualized on an Olympus (Center Valley, CA) confocal microscope.

Chemokine immunoassay. HDMECs were treated with TW37 at concentrations as indicated in the figures. Supernatant media were collected after 24 hours and frozen at −80°C until required for assay. ELISAs (R&D Systems) were done for human CXCL1 and CXCL8 and the data were normalized against relevant treated cell density. ELISA protocols were followed as indicated in the manufacturer's instructions.

Migration assay. A fluorescent *in vitro* assay was used to determine the effect of TW37 on endothelial cell migration through a porous membrane (3 μm pores). Cells were stained with calcien AM (Molecular Probes) in complete medium for 15 to 20 minutes followed by incubation in fresh EGM2-MV for 20 minutes. Cells were trypsinized, counted, and seeded at 5×10^5 in the presence of 0 to 0.05 μmol/L TW37 in a volume of 300 μL medium per cell culture insert (BD/Falcon, Franklin Lakes, NJ). The inserts were placed in a 24-well companion culture plate (BD/Falcon), and the well was filled with 800 μL serum-free medium with or without rhVEGF$_{165}$ or TW37 and further incubated at 37°C for indicated time to allow migration. Cell-related fluorescence was determined at excitation and emission wavelengths of 485 and 535 nm, respectively, in a fluorometer (Genios). Data were obtained from triplicate wells per condition and are representative of three independent experiments.

Capillary sprouting assay. To investigate the effect of TW37 on the angiogenic potential of primary endothelial cells, a capillary sprouting assay was done as described previously (33). Briefly, six-well plates were precoated with 1.5 mL/well Vitrogen 100 collagen (Angiotech BioMaterials, Palo Alto, CA). HDMECs (5×10^5) were added to each well and allowed to adhere overnight. Cells were treated daily with 50 ng/mL rhVEGF$_{165}$ in fresh medium until day 5 when cells were treated concurrently with VEGF, drug, or control. Numbers of sprouts were assessed daily with a phase microscope at ×200. Six high-power fields were analyzed per well with triplicate wells per treatment.

Severe combined immunodeficient mouse model of human angiogenesis. Porous poly L-lactic acid scaffolds ($6 \times 6 \times 1$ mm) with an average pore diameter of 180 μm were fabricated as described previously (34). Just before implantation, scaffolds were seeded with 1×10^6 HDMECs in a 1:1 Matrigel/EGM2-MV mix. Male severe combined immunodeficient (SCID) mice (CB.17.SCID, Taconic, Hudson, NY) were anesthetized with ketamine and xylazine, and two scaffolds were implanted s.c. in the dorsal region of each mouse. At 10 days after transplantation, six mice per treatment were treated with 3 mg/kg or 30 mg/kg TW37 (in vehicle: PBS/Tween 80/ethanol) or vehicle alone i.v. for 5 consecutive days. At the end of the treatment period, mice were euthanized, and the scaffolds were retrieved, fixed overnight in 10% buffered formaldehyde at 4°C, and mounted on glass slides. Immunohistochemistry was done for Factor VIII (Lab Vision Corp., Freemont, CA) and microvessels were counted in 6 fields per scaffold and 12 scaffolds per treatment at ×200 magnification. Alternatively, sections were stained with H&E and occluded blood vessels were counted. The care and treatment of experimental animals was in accordance with University of Michigan institutional guidelines.

Results

TW37 cytotoxicity in endothelial cells is dose dependent and is unaffected by mediators of endothelial cell survival. TW37 is a novel nonpeptide small-molecule inhibitor designed using a structure-based design strategy (Fig. 1*A*). TW37 targets the BH3-binding groove in Bcl-2 where proapoptotic Bcl-2 proteins, such as

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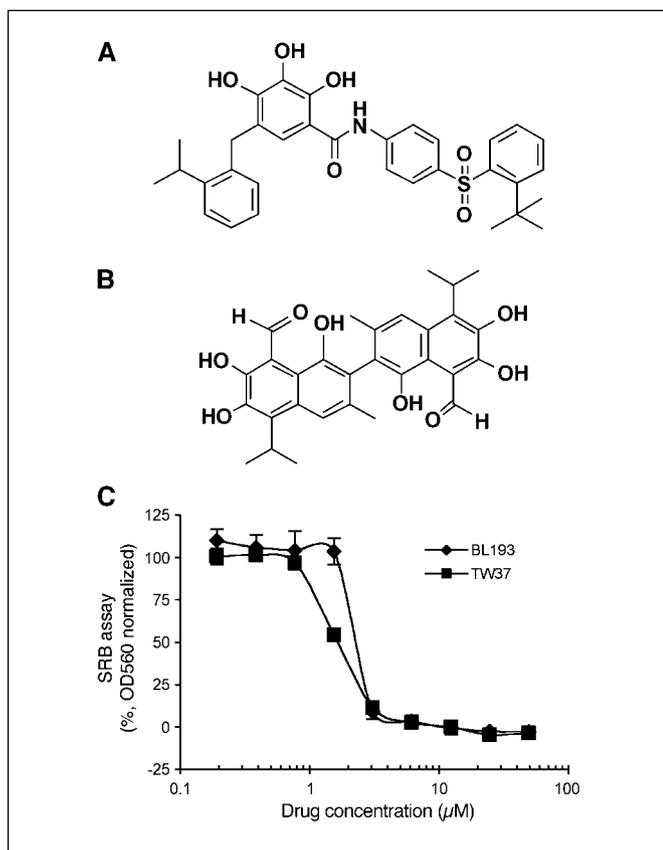
Figure 1. Structure and activity of small-molecule inhibitors of Bcl-2. Structure diagram for the small-molecule inhibitors of Bcl-2 studied here: TW37 (*A*) and BL193 (*B*). *C*, effects of TW37 or BL193 on HDMEC density as determined by SRB assay. Results are normalized against vehicle control and initial plating density. Experiments were done in triplicate wells per condition. Representative of three independent experiments.

Bak, Bax, and Bid bind.[5] We selected BL193 [(−)-gossypol; Fig. 1*B*] for use as control for TW37 because BL193 also has an inhibitory effect on Bcl-2. In fluorescence polarization–based binding assays using recombinant Bcl-2 and Bcl-x_L proteins, TW37 binds to Bcl-2 and Bcl-x_L with Ki values of 290 and 1110 nmol/L, respectively. In comparison, BL193 binds to Bcl-2 and Bcl-x_L proteins with Ki values of 320 and 480 nmol/L, respectively, in the same binding assays. Hence, both TW37 and BL193 are potent inhibitors of Bcl-2. However, TW37 has higher affinity for Bcl-2 and is also more selective for Bcl-2 over Bcl-x_L than is BL193.

Initial screening for effect of BL193 and TW37 on endothelial cells was carried out using a cytotoxicity assay that allowed for the determination of effect of the drugs on both cell growth and cell death. A 72-hour time point was determined to be optimal for full effect of TW37 dose-response curve on HDMEC, with no further change occurring at 96 hours (data not shown) and was used throughout. The IC_{50}s were approximately 1.8 and 2.2 μmol/L for TW37 and BL193, respectively (Fig. 1*C*).

CXCL8 and VEGF are proangiogenic factors secreted by many tumor cells. Additionally, CXCL8 is secreted by endothelial cells and can act in an autocrine manner (28, 35, 36). As the tumor milieu is rich in angiogenic and growth stimuli, we next investigated the effect of these two major endothelial mitogenic and prosurvival agents on the effect of TW37 on endothelial cell growth. We observed that the cytotoxic activity of TW37 was unaffected by the presence of mitogenic and angiogenic factors, CXCL8 and VEGF, respectively (Fig. 2*A* and *B*). To more closely simulate tumor-related angiogenic conditions, HDMECs were exposed to TW37 in the presence of conditioned medium from several head and neck carcinoma tumor lines (Fig. 2*C* and *D*) and from the sarcoma cell line SLK (Fig. 2*E*). We observed that the response of endothelial cells to TW37 was not affected by any of the tumor cell conditioned media tested here. We also studied the specificity of effects of TW37 by doing SRB experiments with primary HDF. We observed that TW37 had no effect on the fibroblasts exposed to the same concentration range as the endothelial cells (Fig. 2*F*). However, TW37 is able to inhibit growth of MCF-7, LNCaP, and SLK tumor cell lines in ranges equal to or lower than those required to inhibit endothelial cell growth (Fig. 2*F*). These data show that proliferating endothelial cells are susceptible to Bcl-2 inhibition and suggest that the cytotoxic effect of TW37 is cell type specific.

Inhibition of Bcl-2 by TW37 or BL193 induces apoptosis in endothelial cells. The cytotoxicity assays allowed measurement of growth inhibition and, to a limited extent, cytotoxicity but did not identify the mechanism responsible for these responses. Bcl-2 is a key survival checkpoint molecule in the apoptosis signaling pathway (37), and small-molecule inhibitors of Bcl-2 have been found to induce apoptosis in tumor cells (38). Therefore, in endothelial cells, overall growth inhibition induced by an inhibitor of Bcl-2 may be expected to involve apoptosis. We observed that increasing concentrations of BL193 (Fig. 3*A*) and TW37 (Fig. 3*B*) were correlated with significantly increased apoptosis of endothelial cells compared with vehicle control. At concentrations of 0.5 μmol/L and below, no significant apoptosis was observed in HDMEC compared with untreated controls. The higher levels of apoptosis displayed by BL193 at 5 μmol/L compared with TW37 may result from nonspecific interactions and their resultant toxicities. The broader active range in both assays and greater molecular specificity of TW37 confirmed it as our main test compound and indicated that it may have greater potential as a drug than BL193.

To determine if the tumor cell–secreted mediators protect endothelial cells against apoptosis induced by inhibition of Bcl-2 function, we exposed primary endothelial cells to TW37 in the presence of conditioned medium from carcinoma or sarcoma cell lines. As VEGF is believed to be a primary mediator of endothelial cell survival (33), we measured the levels of that cytokine in the recovered conditioned medium by immunoassay (Fig. 3*C*). High pg/mL levels (equivalent to 45-400 nmol/L) of VEGF were found in all conditioned media. The proportion of apoptotic HDMEC exposed to TW37 in the presence or absence of tumor cell conditioned medium is indicated (Fig. 3*D-H*). Although overall patterns were similar to those of TW37 in the presence of normal culture medium, exposure in the presence of tumor cell conditioned medium showed a significant trend for potentiation of the proapoptotic effect of TW37 at the highest concentration (50 μmol/L). There was no protection seen among the tumor cell conditioned medium about the effects of TW37 on the apoptotic profile of endothelial cells.

Fluorometric assays were done to investigate the activation of caspase-9 and caspase-3 in TW37-induced apoptosis of endothelial cells. As caspase activation is a relatively early signal in the apoptotic process, it may be expected to occur earlier than DNA fragmentation. Indeed, the caspase-9 activity was observed between 0 to 10 hours with a peak between 2 to 4 hours of
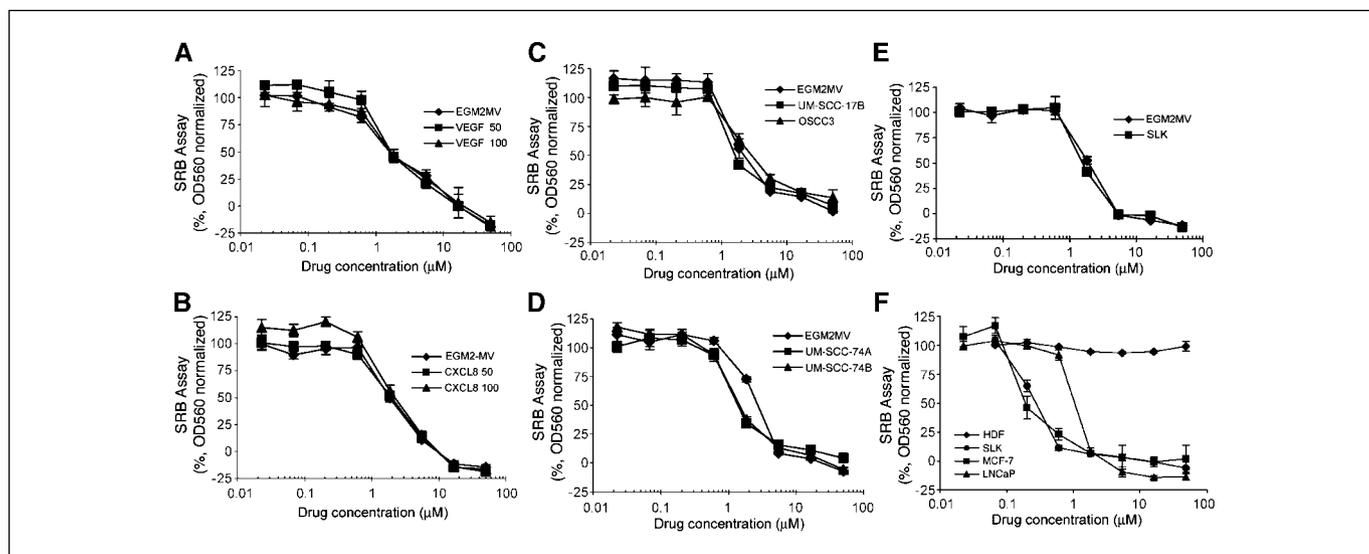
Figure 2. TW37 cytotoxicity on primary endothelial cells is unaffected by VEGF, CXCL8, or tumor-derived proangiogenic soluble mediators. Effect of coincubation of TW37 with 0 to 100 ng/mL VEGF (*A*) or CXCL8 (*B*) on HDMEC cells. *C* and *D*, effect of coincubation of HDMEC with TW37 and conditioned medium from four head and neck carcinoma lines OSCC3 and UM-SCC-17B or UM-SCC-74A and UM-SCC-74B head and neck tumor cell lines. *E*, alternatively, HDMECs were coincubated with TW37 and conditioned medium from Kaposi's sarcoma cell line (SLK). *F*, effect of TW37 on primary HDF and tumor cell lines SLK and human breast cancer line MCF-7 or human prostate cancer line LNCaP. In all cases, results are normalized against control and initial plating density. Representative of at least three independent experiments done in triplicate wells per condition.

exposure (Fig. 4*A*). Interestingly, caspase-3 activation induced by TW37 was almost exactly coincidental with the caspase-9 activity (Fig. 4*A*). Caspase-3 activity was induced at 5 μmol/L but not 0.5 μmol/L or lower (Fig. 4*B*), which correlates well with equivalent dose effects on mitochondrial depolarization (Fig. 4*D*). In all cases, competitive inhibitors of caspase-3 and caspase-9 were able to attenuate or entirely abolish detectable enzymatic activity, thus verifying assay specificity. Attenuation of caspase-9 activity in endothelial cells stably expressing a dominant-negative mutant caspase-9 caused a reduction in the ability of TW37 to induce cell death (Fig. 4*C*). These data showed a role for caspase-9 activity in the apoptotic pathway triggered by TW37. A precursor to caspase-9

activation is the release of cytochrome *c* as the mitochondria become leakier after activation of Bax/Bak (30). In Fig. 4*D*, we show the effect of TW37 on retention of MitoTracker by endothelial cell mitochondria. Individual mitochondria are readily apparent in control cells and also those exposed to subapoptotic concentrations of TW37 (0.5 μmol/L) for 3 hours. However, exposure to 5 μmol/L TW37 for the same period resulted in almost complete depolarization of the mitochondria as indicated by the highly diffuse and virtually undetectable MitoTracker staining.

Subapoptotic concentrations of TW37 inhibit the angiogenic potential of endothelial cells. We used an assay for endothelial capillary sprouting on a three-dimensional collagen
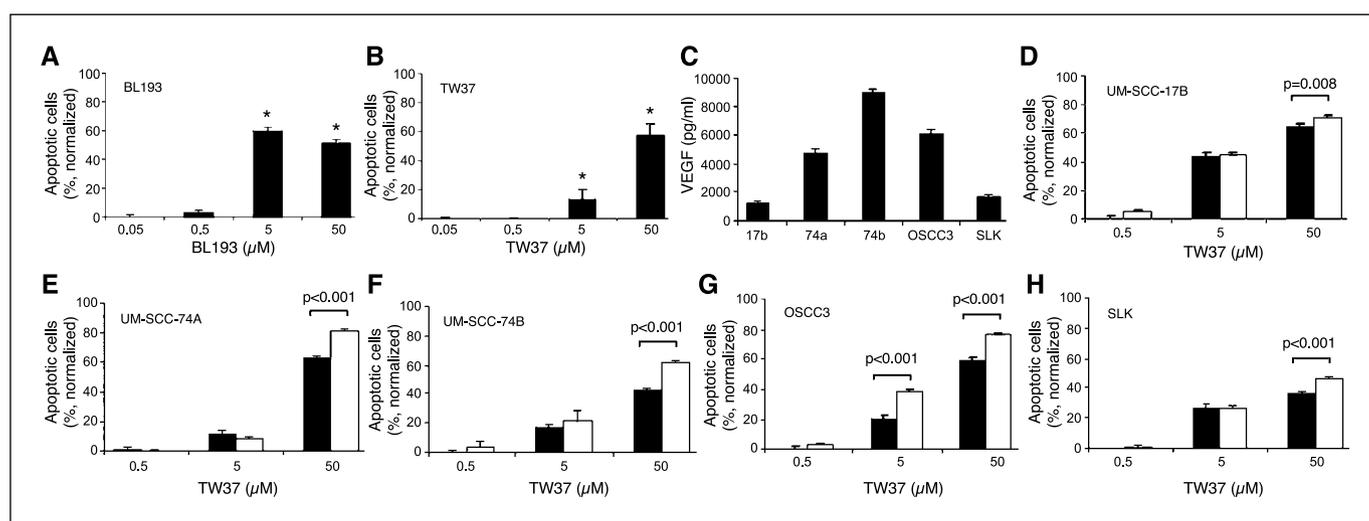
Figure 3. Small-molecule inhibitors of Bcl-2 induce apoptosis of primary endothelial cells. HDMEC exposed to 0 to 50 μmol/L BL193 (*A*) or TW37 (*B*) for 72 hours were harvested, stained with PI, and subjected to flow cytometry for identification of the proportion of apoptotic cells (sub-G_1). *, $P \leq 0.05$. *C*, VEGF concentrations in the conditioned medium of the tumor cell lines used here. *D*, HDMECs were also exposed to TW37 in the presence (*clear columns*) or absence (*filled columns*) of tumor cell conditioned medium for 72 hours, then harvested, and analysed by flow cytometry. Proportion of sub-G_1 levels in TW37-treated cells in comparison with vehicle-treated cells. Representative of at least three independent experiments done in triplicate wells per condition.
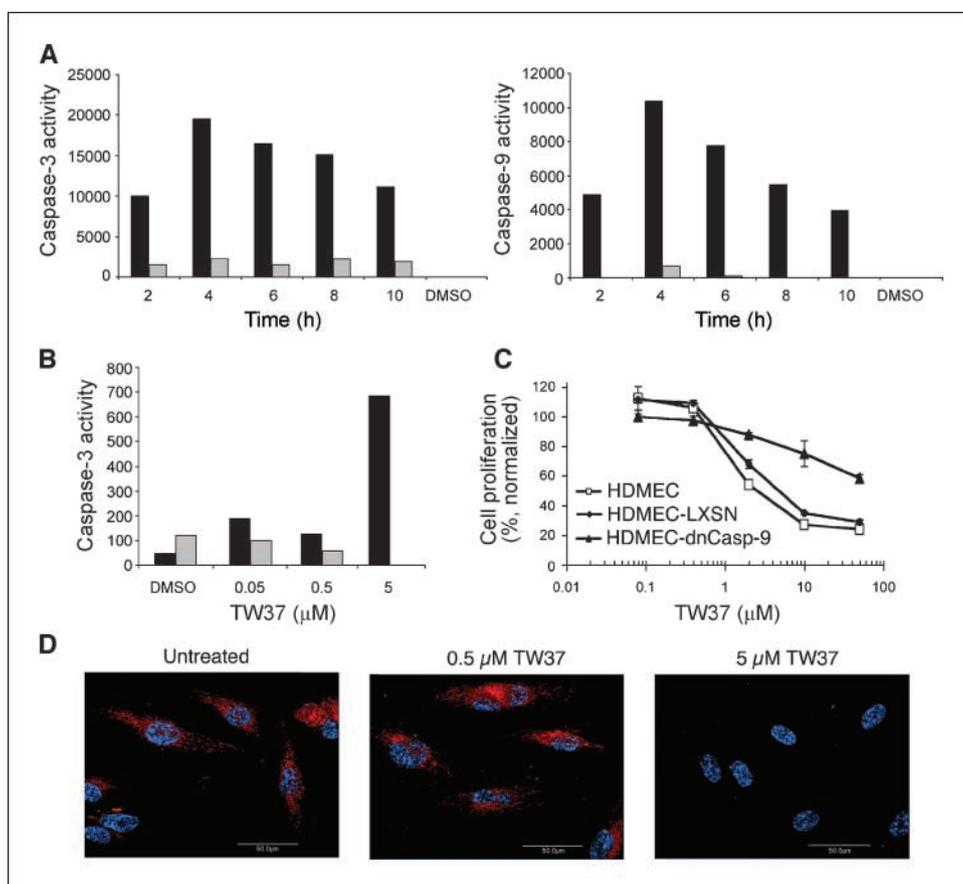
Figure 4. TW37 induces caspase-9 and caspase-3 activity and acts on the mitochondria. HDMECs were exposed to TW37 for times indicated and then harvested, and cell lysates were analyzed for caspase activity. *A*, caspase-9 and caspase-3 activity normalized against untreated controls after exposure to 50 μmol/L TW37 (*black columns*) for the times indicated. In both panels, inhibitors of the relevant caspases were used to evaluate specificity of TW37-induced activity (*gray columns*). Alternatively, cells were incubated with vehicle (DMSO) or with EGM2-MV alone. *B,* caspase-3 activity at 3 hours was assayed after treatment with TW37 over a concentration range, including both apoptotic and nonapoptotic doses of the drug. *C,* HDMEC expressing a dominant-negative caspase-9 (*HDMEC-dnCaspase-9*), the empty vector control (*HDMEC-LXSN*), or untransduced HDMEC were exposed to TW37 for 72 hours and evaluated with the SRB assay. *D,* HDMECs were exposed to 0 to 5 μmol/L TW37 for 3 hours and then stained with MitoTracker Red CMXRos (mitochondria; *red*) and DAPI (nucleus; *blue*). Images were captured on an Olympus confocal microscope. Representative of at least three independent experiments.

matrix that allowed culture and growth of capillary sprouts over a period of days upon stimulation with VEGF (33). The effect of TW37 on angiogenic sprouting was examined over a dose range that included proapoptotic and nonapoptotic concentrations, previously determined by flow cytometry. Figure 5A shows that 5 μmol/L TW37 completely abolished endothelial cell sprouting, indeed this concentration completely killed the cells cultured in collagen over 4 to 5 days. Sprouting was not significantly reduced in a consistent manner in the presence of 0.05 or 0.5 μmol/L TW37 when compared with initial day of treatment. However, these lower concentrations of TW37 were sufficient to prevent inductive effect of VEGF on sprouting, suggesting an angiostatic effect for the small-molecule inhibitor at this subapoptotic concentration range. Notably, the 0.05 μmol/L dose of TW37 was a concentration that we observed to have a possible angiostatic action in the sprouting assay (Fig. 5A) and yet displayed no significant apoptotic activity as indicated by FACS analyses (Fig. 3).

Chemotaxis is an integral part of angiogenesis. Therefore, we decided to determine the effects of subapoptotic concentrations of TW37 on endothelial cell migration. TW37 at 0.005 and 0.05 μmol/L significantly inhibited migration of primary endothelial cells ($P < 0.05$) in response to 50 ng/mL VEGF (Fig. 5B). Vehicle at concentrations equivalent to those found in the TW37 preparations had no inhibitory effect on migration (data not shown). As determined by ELISA, TW37 was also able to inhibit secretion of the chemotactic and proliferative chemokines CXCL1 and CXCL8 in a manner and range similar to that displayed by BL193 in our previous study (28). Notably, this effect was observed at concentrations far below those inducing apoptosis, 0.0005 to 0.5 μmol/L

(Fig. 5C and D). A reproducible trend was observed for increasing inhibition of CXCL1 and CXCL8 with increasing drug concentration (Fig. 5C and D). We observed that CXCL1 and CXCL8 expression levels were significantly lower for each TW37 concentration tested here than for the highest vehicle concentration. Therefore, the inhibitory effect on CXCL1 and CXCL8 expression is drug specific. Taken together, these data showed that the observed inhibition of the angiogenic potential of endothelial cells mediated by TW37 is not due solely to its proapoptotic effect. Indeed, subapoptotic concentrations of TW37 have an angiostatic effect *in vitro*.

SCID mouse model of human angiogenesis. We have developed a murine model of humanized vasculature (34) that has allowed us to investigate the biological effect of TW37 on human microvascular endothelial cell *in vivo*. Using this model, we observed a significant decrease in total blood vessel number ($P < 0.05$) comparing both 3 and 30 mg/kg TW37 against vehicle control (Fig. 6A and B). In addition to reduction in total number of blood vessels, we observed that an unusual number of occluded vessels were occurring in the treated groups. We assessed the levels of vessel occlusion by counting completely blocked vessels and determining their number as a percentage of total vessel number. Both drug concentrations mediated a significant increase in the number of occluded vessels when compared with control (Fig. 6C and D).

Discussion

We have shown recently that Bcl-2 is a proangiogenic signaling molecule in addition to its well-known effect on cell survival (28).

Figure 5. Capillary sprouting and migration assays for effect of TW37 on angiogenic potential of VEGF-stimulated endothelial cells. *A,* HDMECs were cultured on collagen and stimulated with 50 ng/mL VEGF for 5 days. Starting on day 5, HDMECs were cultured in EGM2-MV supplemented with 50 ng/mL VEGF in the absence (□) or presence of TW37. The concentrations of TW37 used here were 0.05 (●), 0.5 (♦), and 5 (▲) μmol/L. Sprouts were counted daily from six microscopic fields randomly selected from each of three wells per condition. *B,* HDMECs were stained with calcien AM, exposed to 0.005 (♦) or 0.05 (■) μmol/L TW37 in the presence of 50 ng/mL VEGF, and allowed to migrate for the indicated times. Controls were serum-free medium supplemented with 50 ng/mL VEGF (▲) or without VEGF (○). The fluorescence of migrated cells was measured at 485 nm emission. For analysis of chemokine secretion, HDMECs were incubated with TW37 or vehicle at indicated concentrations and the 24-hour supernatant was analysed for CXCL1 (*C*) and CXCL8 (*D*) levels by ELISA. Experiments were done in triplicate wells per condition.
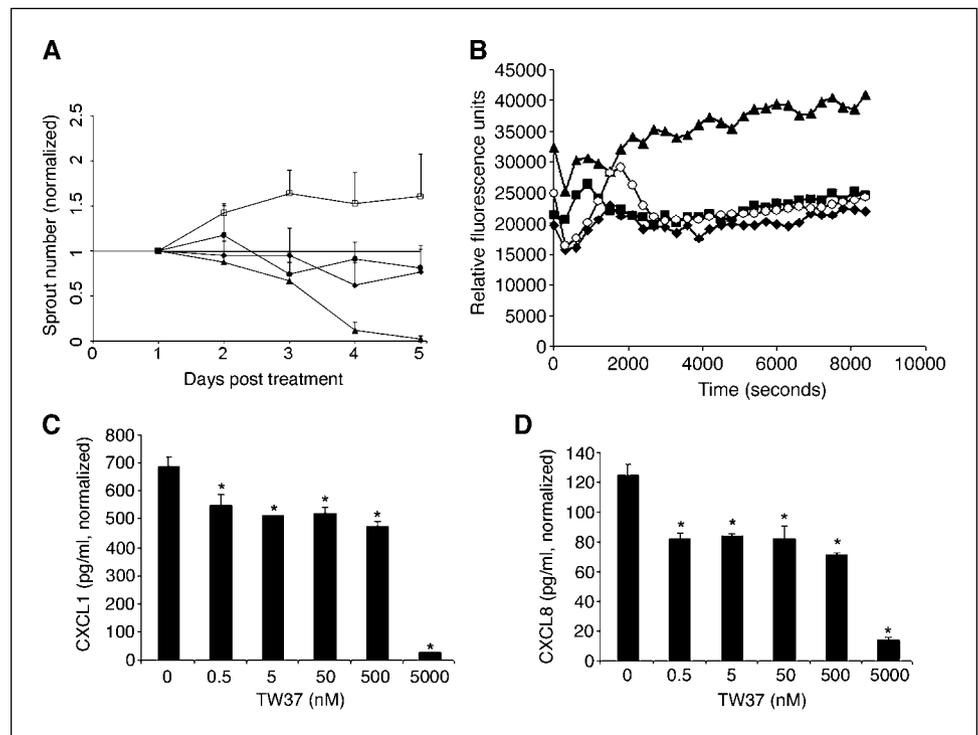
The Bcl-2 proangiogenic pathway can be triggered by VEGF or by the growth factor milieu secreted by tumor cells and results in the up-regulation of the proangiogenic chemokines CXCL1 and CXCL8 (28). These data suggest that small-molecule inhibitors of Bcl-2 may have an antiangiogenic effect that is mediated by the inhibition of Bcl-2-mediated expression of proangiogenic chemokines. Our laboratory has also shown that Bcl-2 up-regulation in the endothelial cells lining the vessels of a carcinoma or a sarcoma is sufficient to accelerate tumor progression (27). Here, we showed that the novel small-molecule inhibitor of Bcl-2 (TW37) inhibits the angiogenic potential of endothelial cells when used in nanomolar concentrations and induces apoptosis of primary endothelial cells, but not primary fibroblasts, in concentrations up to 50 μmol/L.
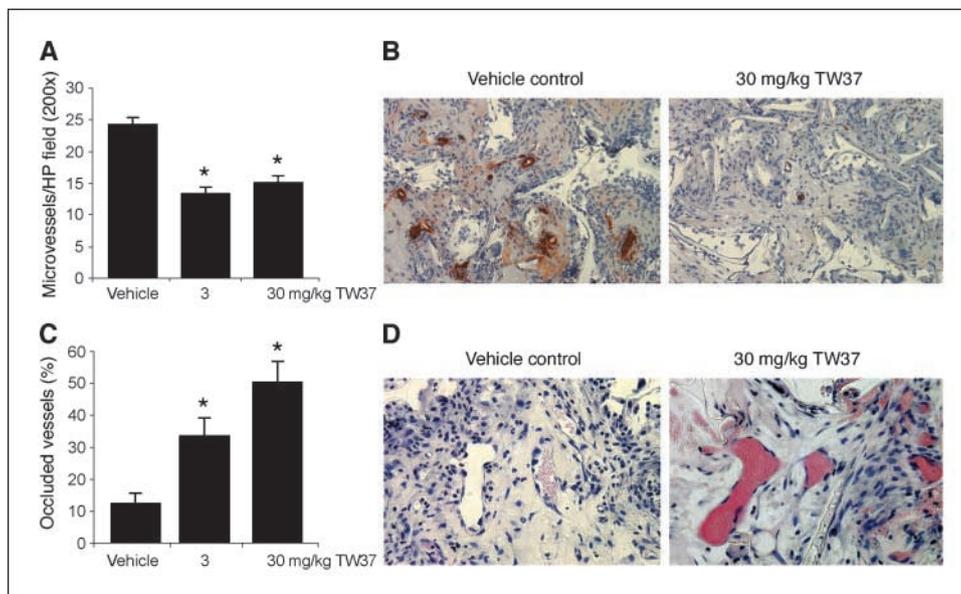
Figure 6. TW37 inhibits angiogenesis *in vivo*. HDMECs were seeded in biodegradable scaffolds and implanted in the s.c. of SCID mice. Mice were treated with TW37 (3 or 30 mg/kg) or vehicle (PBS/Tween 80/ethanol) for 5 days via i.v. injections. At the end of the experimental period, scaffolds were retrieved, tissue sections were prepared and stained, and the vessels were counted under high-power (×200) magnification. *A* and *B,* factor VIII staining was used for identification of blood vessels. A significant (*P* < 0.05) reduction in the microvessel density was observed with both concentrations of TW37. *C* and *D,* blood vessel occlusions were counted in H&E-stained sections under high power (×200), and counts were normalized as percentage of total blood vessel numbers. TW37 at both concentrations induced significantly (*P* < 0.05) higher percentages of occluded blood vessels relative to total number of blood vessels compared with controls. Photographs of occluded blood vessels are at ×400 magnification. Data are derived from 6 high-power fields per scaffold, and 12 scaffolds from independent mice were analyzed per experimental group.

Bcl-2 expression correlates with poor prognosis in many cancer types, lymphoma, prostate carcinoma, and colorectal neoplasia, and is also associated with resistance to both chemotherapy and radiotherapy (39–42). Recently, a breast cancer cell line was generated, with resistance to YC137, a small-molecule inhibitor of Bcl-2, which displayed a reduced expression of Bcl-2, Stat3, and epidermal growth factor receptor HER-2 (43). However, the authors further showed that resistance to the Bcl-2 inhibitor resulting from Bcl-2 down-regulation corresponded with an increased sensitivity of the cells to traditional chemotherapeutic agents, such as paclitaxel or Adriamycin. These data suggest that, in tumors with Bcl-2 inhibitor-driven down-regulation of Bcl-2 function, combination therapy would prevent this avenue of escape. In general, studies involving small-molecule inhibitors of Bcl-2 or Bcl-x_L have indeed shown increasing efficacy in tumor types that present up-regulated Bcl-2 expression (38, 43–47). However, in the present study, we investigate the therapeutic potential of targeting Bcl-2-related angiogenic functions in endothelial cells. Importantly, differentiated endothelial cells have a low rate of turnover and are unlikely to cause subclones with resistance to the Bcl-2 small-molecule inhibitors.

In the current investigation, we tested the small-molecule inhibitors BL193 and TW37 that belong to two different chemical classes. We reason that the use of two structurally distinctively different small-molecule inhibitors of Bcl-2 can provide a cross-validation of our results. We initially examined these two compounds for their ability to inhibit endothelial cell growth. BL193 was used as comparison for TW37 as its proapoptotic antitumor activities have been well described (38, 45). Both compounds showed an equivalent dose-dependent inhibition of endothelial cell growth over the low micromolar range. This was similar to the activity of BL193 in various tumor cell lines (45). IC_{50}s for BL193 and TW37 were lower than those of most other published Bcl-2 small-molecule inhibitors, Z24, >10 μmol/L (46), and HA14-1, 8.7 to 26.6 μmol/L (48). Given our previous work showing that VEGF enhances endothelial cell survival and maintains angiogenesis by inducing expression of Bcl-2 (33) and that up-regulation of Bcl-2 enhances angiogenesis (28), it is noteworthy that TW37 endothelial cell growth-inhibitory activity is unaffected by the presence or absence of VEGF and other prosurvival and proangiogenic stimuli. This suggested that therapeutic blockade of Bcl-2 function with low micromolar concentrations of TW37 may inhibit angiogenesis despite the presence of a strong protective signal for endothelial cells.

Whereas BL193, Z24, and YC137 are all more active in tumor cells engineered to express, or constitutively overexpressing, Bcl-2, or Bcl-2 and Bcl-x_L (43, 45, 46), unstimulated endothelial cells express relatively low levels of Bcl-2 under normal growth conditions (33). Therefore, it is reasonable to deduce from our data that Bcl-2 expression levels in endothelial cells do not dictate sensitivity to Bcl-2 inhibitors. We suggest instead that the level of reliance on Bcl-2 prosurvival function determines sensitivity to inhibitors of Bcl-2 antiapoptotic family members. This observation agrees with Real et al. (43) who reached a similar conclusion from observation of the effect of the Bcl-2 inhibitor YC137 on hematopoietic cells overexpressing and reliant on Bcl-2. It would seem reasonable to suggest then that cancers need not necessarily overexpress Bcl-2 in order for Bcl-2 inhibitors to be effective.

Unexpectedly, the tumor conditioned medium showed a significant trend for potentiation of TW37-induced apoptosis, which was reflected in results from both tumor types (i.e.,

carcinoma and sarcoma). Possible explanations may include either a synergistic interaction of the drug and tumor secreted inhibitors of angiogenesis, increased rate of drug uptake due to secreted carrier interactions, or an increased dependency on Bcl-2 function for endothelial cells exposed to the cytokine milieu secreted by tumor cells. Further studies will be done to understand the reasons for this trend. Using primary cells, we expected and indeed saw some variation in sensitivity to the compounds both over time and between different primary cell batches. For this reason, we ran individual vehicle controls for every single FACS assay run to act as internal comparisons for each conditioned medium sample tested.

Induction of apoptosis results in release of cytochrome *c* from the mitochondria, which together with Apaf-1 and caspase-9 in presence of dATP forms the apoptosome (49). The apoptosome subsequently activates caspase-9, which in turn activates caspase-3. The exact mechanism by which the Bcl-2 family members interact to cause cytochrome *c* release is still unclear, but it seems likely that both suppression of Bcl-2 activity and activation of Bax/Bak to induce mitochondrial membrane permeability are required (30). In the present study, the mitochondrial pathway for apoptosis was activated after inhibition of the antiapoptotic Bcl-2 checkpoint with TW37 as evidenced by mitochondrial depolarization and induction of caspase-9 followed by caspase-3. Interestingly, in a chemoresistant lymphoma cell line, 2 μmol/L BL193 maximally activated caspase-3 and caspase-9 at 8 and 24 hours, respectively (38). Here, TW37 induced significant caspase activity with maximum induction of caspase-9 and caspase-3 almost coincidental over 2 to 4 hours. We observed that TW37 concentrations unable to induce mitochondrial depolarization were also unable to induce caspase-3 induction above control levels. In contrast, caspase-3-inducing concentrations caused complete mitochondrial depolarization. These data showed that, in primary endothelial cells, the blockade of Bcl-2 function induces assembly of a functional apoptosome with rapid activation of caspase-9 and caspase-3 most probably via a mitochondrial pathway.

Of note, we observed a disparity between the effectiveness of TW37 in inhibition of growth in the SRB assay and levels of apoptosis induced by similar concentrations in the cytometry assay. For example, 5 μmol/L TW37 induced between approximately 80% to 100% growth inhibition as shown by SRB but between approximately 15% to 40% apoptosis depending on conditions in the flow cytometry studies (Figs. 2 and 3). Therefore, we looked at the effect of the drugs on endothelial angiogenic variables to see if Bcl-2 inhibition by TW37 was indeed solely due to apoptosis or whether it also had a specifically antiangiogenic component. Angiogenesis involves cellular activation, migration, orientation, and vessel tube formation. The capillary sprout assay is a well-recognized *in vitro* assay for investigation of the differentiation properties of endothelial cells in the presence or absence of angiogenic stimuli. Notably, subapoptotic concentrations of TW37 inhibited VEGF-induced sprouting of endothelial cells in collagen. In addition, migration assays were done to determine whether TW37 disrupted the chemotactic component of angiogenesis. We observed that doses of TW37 lower than the ones required for apoptosis significantly and consistently inhibited migration. Interestingly, the subapoptotic concentrations of TW37 that inhibited migration corresponded to equivalent concentrations of TW37 in the present study and BL193 in our previous study (28), both of which inhibited CXCL8 and CXCL1 levels. Although our observations on capillary sprouting and migration may not be related by cause and effect, they all describe specific

angiogenic functions that are inhibited by small-molecule inhibitors of Bcl-2. This work is to our knowledge the first description of an endothelial cell-specific antiangiogenic effect of Bcl-2 inhibition and one in which a mechanism other than apoptosis or direct cell cycle inhibition may be involved.

To evaluate the effect of TW37 on angiogenesis *in vivo*, we used the SCID mouse model of human angiogenesis. TW37 was given i.v. for 5 days and resulted in a significant reduction in neovasculature, compared with controls, presumably by one or more of the mechanisms described by our *in vitro* studies. Surprisingly, both concentrations of TW37 induced vascular occlusion in the angiogenic vessels within the scaffolds. We did not observe any loss of animals or signs of major thrombotic events during the course of TW37 treatment, suggesting that the occluding effects were specific to the neovessels within the scaffolds. Antitumor therapies using the occlusion of tumor neovasculature have been reported (50). We speculate that TW37 may have potential antitumor effects via a similar mechanism.

The value of a cancer therapy that targets both the tumor body and its associated neovasculature is apparent in ongoing clinical studies using combined antiangiogenic and traditional chemotherapeutics. Tumor-specific drugs, such as the small-molecule inhibitors of Bcl-2, which also display antiangiogenic activity, have, at the very least, the potential to allow effective administration of lower doses of the more toxic traditional chemotherapeutics. In conclusion, our study showed that TW37 is a novel small-molecule inhibitor of Bcl-2 that induces significant levels of apoptosis in endothelial cells at a low micromolar concentration range. We further show that TW37 has significant antiangiogenic properties at nanomolar concentrations that are unrelated to induction of endothelial cell apoptosis. The evidence we present here suggests that the Bcl-2 signaling pathway is a novel target for antiangiogenic therapy.

Acknowledgments

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Antiangiogenic Effect of TW37, a Small-Molecule Inhibitor of Bcl-2

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