

Antiangiogenic Activity of N-substituted and Tetrafluorinated Thalidomide Analogues

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ABSTRACT

Inhibition of angiogenesis is currently perceived as one of the promising strategies in the treatment of cancer. The antiangiogenic property of thalidomide has inspired a second wave of research on this teratogenic drug. Previous studies from our group and others demonstrated that metabolites of thalidomide are responsible for the drug's pharmacological actions. On the basis of the structures of these metabolites, we synthesized 118 thalidomide analogues. Preliminary screening selected 7 of these 118 analogues for more extensive testing in the current study. In the rat aortic ring assay, all 4 analogues in the N-substituted class and 2 of the 3 analogues in the tetrafluorinated class significantly inhibited microvessel outgrowth at 12.5–200 μM . Thalidomide failed to block angiogenesis at similar concentrations. Subsequently, the effects of these analogues on human umbilical vein endothelial cell proliferation and tube formation were determined. Those analogues showing antiangiogenicity in the rat aortic ring assay also demonstrated antiproliferative action in human umbilical vein endothelial cells. Cell proliferation was not affected by thalidomide. Interestingly, all 7 analogues as well as thalidomide suppressed tube formation. Two tetrafluorinated analogues consistently showed the highest potency and efficacy in all three assays. The *in vivo* toxicity of representative analogues from each class was also evaluated. Taken together, our results support the further development and evaluation of novel thalidomide analogues as antiangiogenic agents.

INTRODUCTION

Thalidomide [α -(N-phthalimido)-glutarimide] was first released in Europe and Canada as a rapid-acting, hangover-free sedative in 1956. Reports on phocomelia in infants of women who had taken thalidomide during pregnancy started to surface a few years later (1, 2), leading to its withdrawal from the market. Despite its teratogenicity, thalidomide was approved by the United States Food and Drug Administration in 1998 as a treatment for erythema nodosum leprosum. The clinical efficacy of thalidomide in inflammatory and autoimmune diseases was in part attributed to its ability to inhibit TNF- α production (3–5).

Over 30 years ago, Folkman (6) pioneered the notion that solid tumors cannot grow beyond 2–3 mm³ without the formation of new blood vessels. Inhibition of angiogenesis has been thereafter perceived as a promising strategy to treat cancer. D'Amato *et al.* (7) demonstrated that thalidomide inhibits basic fibroblast growth factor-induced angiogenesis in the rabbit cornea and tumor growth in rabbits. Subsequently, the antitumor activity of thalidomide was evaluated in numerous clinical trials. Partial response, stable disease, or tumor regression was reported in patients with multiple myeloma (8–10),

AIDS-related Kaposi's sarcoma (11), high-grade gliomas (12), hepatocellular carcinoma (13, 14), renal cell carcinoma (15, 16), and advanced melanoma (15). In androgen-independent prostate cancer, thalidomide caused a reduction in serum prostate-specific antigen and an improvement of clinical symptoms (17).

Our laboratory showed previously that one of the products of cytochrome P450 2C19 isozyme biotransformation of thalidomide, 5'-OH-thalidomide, is responsible for the drug's antiangiogenic activity (18–20). On the basis of the structure of the metabolite, we synthesized 118 thalidomide analogues and studied their structure–activity relationship in multiple angiogenesis assays. All analogues were preliminarily screened at 100 μM in the rat aortic ring assay. Seven of the 118 analogues were chosen because they demonstrated the highest efficacy. To our knowledge, thalidomide analogues synthesized by various groups were mostly tested for their ability to inhibit TNF- α production in monocytes (21–23). The current study investigated the effects of 7 of the 118 analogues from the N-substituted and tetrafluorinated classes on rat aortic microvessel outgrowth, HUVECs, and prostate cancer cells. Representative analogues from each class were also tested for *in vivo* toxicity.

MATERIALS AND METHODS

Thalidomide Analogues. Analogues were synthesized by Dr. Kurt Eger and his group at the University of Leipzig, Germany. Worldwide patents of these analogues have been filed. Their chemical structures are shown in Fig. 1.

Rat Aortic Ring Assay. Twelve-well tissue culture plates were coated with 250 μl of Matrigel (Becton-Dickinson, Bedford, MA) and allowed to gel for 30 min at 37°C and 5% CO₂. Thoracic aortas were excised from 8- to 10-week-old male Sprague Dawley rats. After careful removal of fibroadipose tissues, the aortas were cut into 1-mm-long cross-sections, placed on Matrigel-coated wells, and covered with an additional 250 μl of Matrigel. After the second layer of Matrigel had set, the rings were covered with EGM-II and incubated overnight at 37°C and 5% CO₂. EGM-II consists of endothelial cell basal medium (EBM-II; Clonetics, San Diego, CA) plus endothelial cell growth factors provided as the EGM-II Bulletkit (Clonetics). The culture medium was subsequently changed to EBM-II supplemented with 2% fetal bovine serum, 0.25 $\mu\text{g/ml}$ amphotericin B, and 10 $\mu\text{g/ml}$ gentamicin. Aortic rings were treated daily with either the vehicle (0.5% DMSO), CAI (12 $\mu\text{g/ml}$), thalidomide, CPS11, CPS16, CPS20, CPS45, CPS48, CPS49 (12.5–200 μM), or CPS13 (25–400 μM) for 4 days and photographed on the 5th day using a $\times 2.5$ objective. CAI, a known antiangiogenic agent, was used at higher than clinically achievable concentration as positive control. Experiments were repeated four times using aortas from four different rats. The area of angiogenic sprouting, reported in square pixels, was quantified using Adobe Photoshop.

Cell Lines. HUVECs (Clonetics) were maintained in EGM-II at 37°C and 5% CO₂. Human prostate cancer cell lines PC3 and LNCaP were obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained at 37°C and 5% CO₂ in RPMI 1640 supplemented with 10% fetal bovine serum and antibiotics (100 units/ml penicillin, 100 $\mu\text{g/ml}$ streptomycin, and 0.25 $\mu\text{g/ml}$ amphotericin B).

Cell Proliferation Assay. HUVECs, PC3, and LNCaP cells were seeded onto 12-well plates at a density of 30,000; 30,000; and 40,000 cells/well, respectively, and allowed to attach overnight at 37°C and 5% CO₂. The culture medium was then aspirated, and fresh culture medium containing either the

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²The abbreviations used are: TNF, tumor necrosis factor; CAI, carboxyamidotriazole; COX, cyclooxygenase; HUVEC, human umbilical vein endothelial cell.

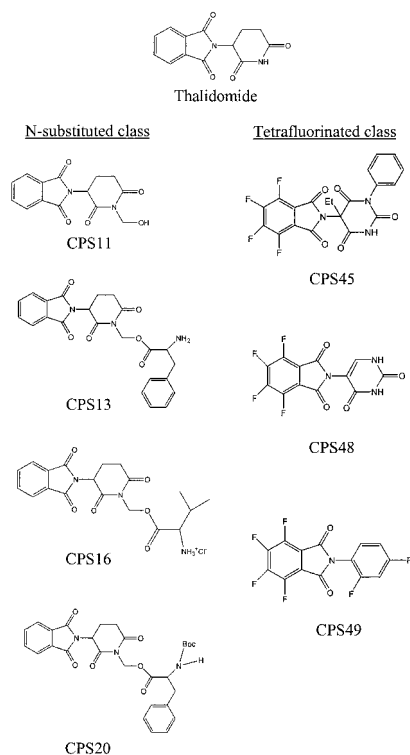


Fig. 1. Chemical structures of thalidomide and its analogues.

vehicle (0.5% DMSO), thalidomide, CPS11, CPS16, CPS20, CPS45, CPS48, CPS49 (12.5–200 μM), or CPS13 (25–400 μM) was added to each well every 24 h. After 48 h, cells were trypsinized and counted with a Coulter Z1 counter (Coulter Corp., Hialeah, FL). All experiments were done in duplicate and repeated three times.

Endothelial Cell Tube Formation Assay. Eight-well slide chambers were coated with 150 μl of Matrigel and allowed to gel at 37°C and 5% CO_2 for 30 min. HUVECs were then seeded at 30,000 cells/well in EGM-II containing either the vehicle (0.5% DMSO), thalidomide, CPS11, CPS16, CPS20, CPS45, CPS48, CPS49 (12.5–200 μM), or CPS13 (25–400 μM) and incubated at 37°C and 5% CO_2 for 16 h. After incubation, slides were washed in PBS, fixed in 100% methanol for 10 s, and stained with DiffQuick solution II (Dade Behring, Inc., Newark, DE) for 2 min. To analyze tube formation, each well was digitally photographed using a $\times 2.5$ objective. A threshold level was set to mask the stained tubes. The corresponding area was measured as the number of pixels using the MetaMorph software (Universal Imaging, Downingtown, PA). Experiments were repeated three times.

Human Saphenous Vein Assay. Specimens of human saphenous vein were harvested during the course of surgery on an institution-approved protocol. Veins were cut into 2-mm-long cross-sections; cultured under similar conditions as described for the rat aortic rings; treated daily with CPS11, CPS13, CPS16, CPS20, CPS45, and CPS49 at 100 μM for 13 days; and photographed on the 14th day using a $\times 1.5$ objective. Images were analyzed as described above.

In Vivo Toxicity Study. Twelve-week-old male nude mice were given daily i.p. injections of either the vehicle (0.5% carboxymethylcellulose), CPS11, CPS45, or CPS49 at 12.5–200 mg/kg for 2 weeks. Five animals were included in each dose group. Maximum tolerated dose of each analogue was determined. Necropsy was performed on the survivors from each dose group by veterinary pathologists.

Statistics. All results were presented as mean \pm SE. Comparisons were made with one-way ANOVA followed by Dunnett's test with $P < 0.05$ as the criterion for statistical significance.

RESULTS

Effects of Thalidomide Analogues on Rat Aortic Angiogenesis. As shown in Fig. 2A, microvessel outgrowth was not signif-

icantly blocked by thalidomide at any concentrations. CAI at 12 $\mu\text{g/ml}$ (28.3 μM) markedly suppressed angiogenesis. In the N-substituted class, inhibition of angiogenesis was first evident with CPS11 and CPS13 at 25 μM . The analogues appeared to reduce microvessel outgrowth in a concentration-dependent manner. At 200 μM , all analogues demonstrated $\sim 90\%$ inhibition of angiogenesis, comparable with CAI. Fig. 2B showed that the tetrafluorinated analogue CPS45 was more potent than CPS49 and all of the N-substituted analogues, with 90% reduction of angiogenesis at 12.5 μM . Interestingly, CPS48 in the tetrafluorinated series failed to inhibit microvessel outgrowth. Representative images of aortic rings are illustrated in Fig. 3.

HUVEC Proliferation and Tube Formation in Response to Thalidomide Analogues. The N-substituted analogues inhibited HUVEC proliferation in a concentration-dependent fashion (Fig. 4A). However, only at higher concentrations (≥ 50 μM) did the inhibition reach statistical significance. HUVEC proliferation was not significantly decreased by any concentrations of thalidomide. As shown in Fig. 4B, CPS45 and CPS49 in the tetrafluorinated class potently reduced HUVEC proliferation by $>60\%$ at 12.5 μM . CPS48 did not affect HUVEC growth.

Tube formation appeared to be more sensitive to the effects of the analogues. Significant inhibition (50–60%) was observed with the N-substituted analogues CPS11, CPS16, and CPS20 at 12.5 μM (Fig. 5A). Surprisingly, thalidomide also blocked tube formation (Fig. 5A).

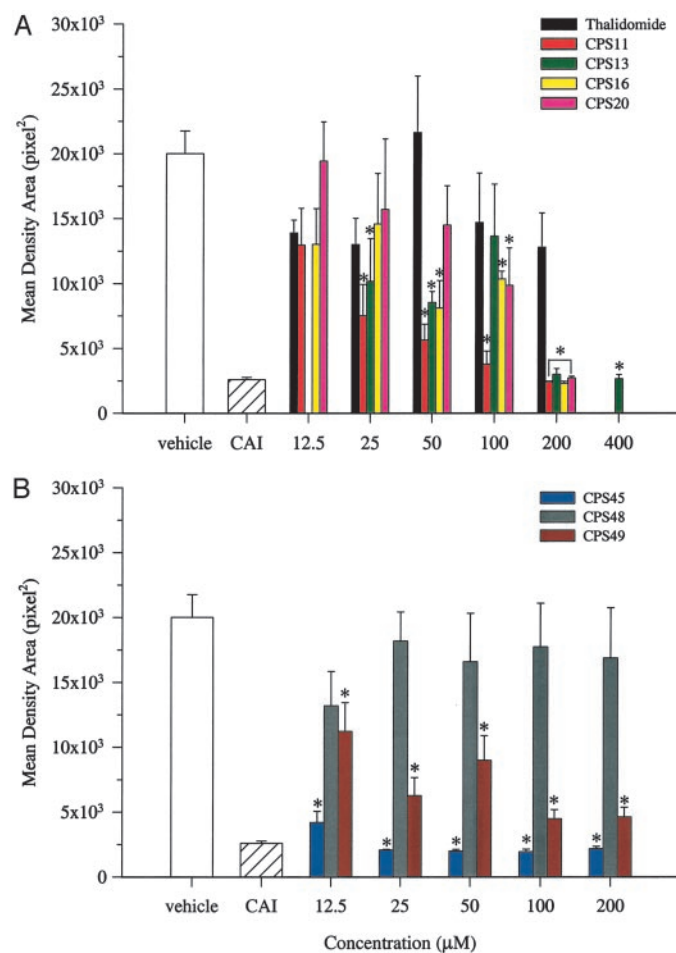


Fig. 2. Bar graphs of the total area of microvessel outgrowth measured as square pixel in response to the vehicle control (0.5% DMSO), CAI (12 $\mu\text{g/ml}$), or various thalidomide analogues (12.5–400 μM) in the N-substituted class (A) and tetrafluorinated class (B). Bars, mean \pm SE. *, significantly ($P < 0.05$) different from vehicle control.

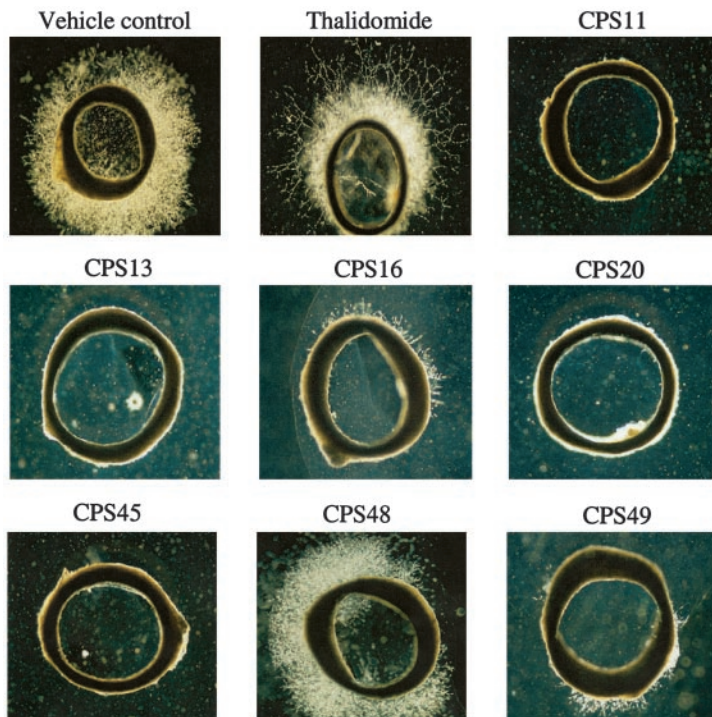


Fig. 3. Representative images of rat aortic rings treated with the vehicle control (0.5% DMSO), thalidomide, or the indicated thalidomide analogues at 200 μM .

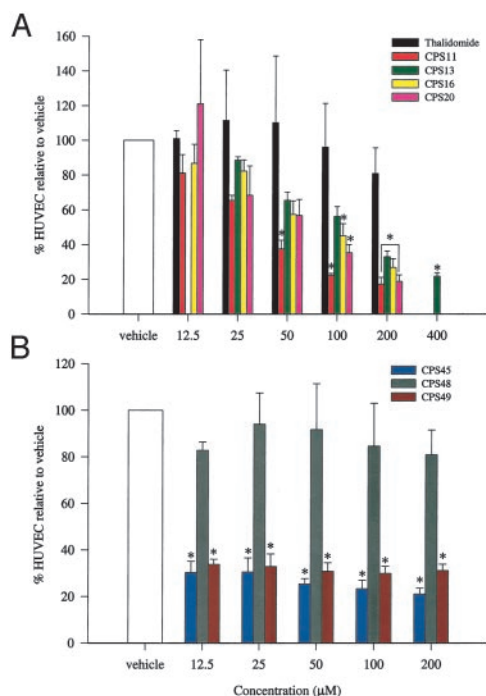


Fig. 4. Bar graphs of the percentage of HUVECs relative to the vehicle control (0.5% DMSO) versus treatment with various thalidomide analogues (12.5–400 μM) in the N-substituted class (A) and tetrafluorinated class (B) for 48 h. Bars, mean \pm SE. *, significantly ($P < 0.05$) different from vehicle control.

The tetrafluorinated analogues CPS45 and CPS49 were found to be the most potent and efficacious, inhibiting tube formation at 12.5 μM by 93 and 80%, respectively (Fig. 5B). CPS48 blocked tube formation as well. Representative tube images are shown in Fig. 6.

Effects of Thalidomide Analogues on Human Prostate Cancer Cells. The androgen-independent PC3 and androgen-dependent LNCaP cells displayed higher resistance to the antiproliferative ef-

fects of the N-substituted analogues than HUVECs (Fig. 7, A and C). Significant reduction in cell number was only evident at 200 μM for CPS11 and CPS20 and at 400 μM for CPS13 in both cell lines (Fig. 7, A and C). CPS16 significantly suppressed proliferation at 200 μM only in LNCaP cells (Fig. 7C). As shown in Fig. 7D, the number of LNCaP cells was significantly decreased by the tetrafluorinated analogues CPS45 (12.5–200 μM) and CPS49 (25–200 μM). However, CPS45 and CPS49 inhibited PC3 cell proliferation only at ≥ 50 μM and 200 μM , respectively (Fig. 7B). Thalidomide and CPS48 did not

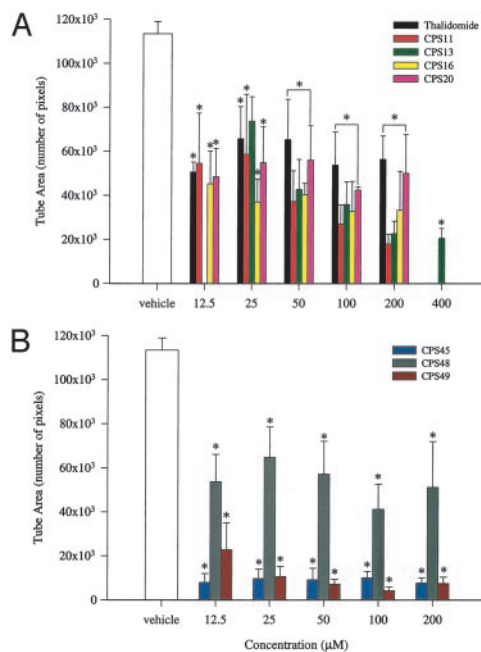


Fig. 5. Bar graphs of the total tube area in the microscopic field measured as number of pixels in response to the vehicle control (0.5% DMSO) or various thalidomide analogues (12.5–400 μM) in the N-substituted class (A) and tetrafluorinated class (B). Bars, mean \pm SE. *, significantly ($P < 0.05$) different from vehicle control.

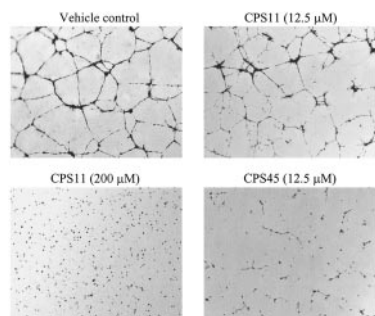


Fig. 6. Representative images of the tube formation assay showing the higher potency of the tetrafluorinated analogue CPS45 compared with the N-substituted analogue CPS11. Vehicle control is 0.5% DMSO.

affect proliferation of LNCaP and PC3 cells at any concentrations (Fig. 7A–D).

Human Venous Angiogenesis in Response to Thalidomide Analogues. The N-substituted analogues CPS11, CPS13, and CPS16 at 100 μM inhibited angiogenesis by 90, 50, and 70%, respectively (data not shown). Microvessel outgrowth was suppressed by the tetrafluorinated analogues CPS45 and CPS49 at similar concentrations by 87 and 40%, respectively (data not shown). In comparison with the rat aorta, human saphenous vein appeared to be less sensitive to the antiangiogenic effect of CPS49.

In Vivo Toxicity of Thalidomide Analogues. As shown in Fig. 8A, all mice injected with CPS11 at 12.5–100 mg/kg survived the 2-week daily treatment schedule. Mortality was 20% after four doses of the compound at 200 mg/kg. Fig. 8B demonstrated the much higher toxicity of CPS49 relative to CPS11. All mice given CPS49 at 50–200 mg/kg died within 4 days of treatment. CPS49 at 25 mg/kg caused 20% mortality. When given at 12.5 mg/kg, all mice survived the treatment regimen. The maximum tolerated doses of CPS11 and CPS49 were determined to be 100 and 12.5 mg/kg, respectively. For CPS49 at 25 and 12.5 mg/kg, the major histopathology included chronic inflammation of the pancreas, the epididymis, and some

accessory male sex glands, increased extramedullary hematopoiesis in the spleen, as well as granulocytic hyperplasia in the bone marrow. CPS11 at 200 and 100 mg/kg was associated with granulocytic hyperplasia in the bone marrow and increased incidence of minimal to mild nephropathy. Surprisingly, all mice injected with the other tetrafluorinated analogue CPS45, which also showed high potency *in vitro*, at 12.5–200 mg/kg survived the 2-week treatment regimen (data not shown). The administration of CPS45 was associated with histiocytosis in lymph nodes.

DISCUSSION

Our results demonstrated the efficacy of thalidomide analogues to inhibit angiogenesis in multiple assays. The tetrafluorinated analogues CPS45 and CPS49 were the most potent and efficacious, blocking microvessel outgrowth in the rat aorta as well as HUVEC proliferation and tube formation at 12.5 μM . In contrast, CPS48 did not show activity in the rat aortic ring and HUVEC proliferation assays. Gütschow *et al.* (24) previously tested these three analogues for their ability to block lipopolysaccharide-induced TNF- α production in human monocytes. Parallel to our findings, CPS45 and CPS49 at 25 μM were shown to completely suppress TNF- α production, whereas CPS48 failed to do so. This suggests that tetrafluoro substitution at the phthalimido ring is crucial but not sufficient for potent angiogenesis and TNF- α inhibitory actions. Being a polar isostere of CPS49, the lack of biological activity of CPS48 might be attributed to its decreased lipophilicity. Niwayama *et al.* (21) reported that the increase in potency of the tetrafluorothalidomides with regard to TNF- α inhibition is unique to fluorine substitution as other halogen atoms fail to produce similar effects. Replacement of the imido hydrogen in the glutarimide ring (N-substitution) with the current moieties also generated analogues with reasonable, albeit less potent, antiangiogenic activity. In general, fluorine-containing molecules are much more hydrophobic and, therefore, more bioactive compared with their hydrocarbon counterparts. It has also been suggested that fluorine substitution confers stereoelectronic changes to drug molecules and, in

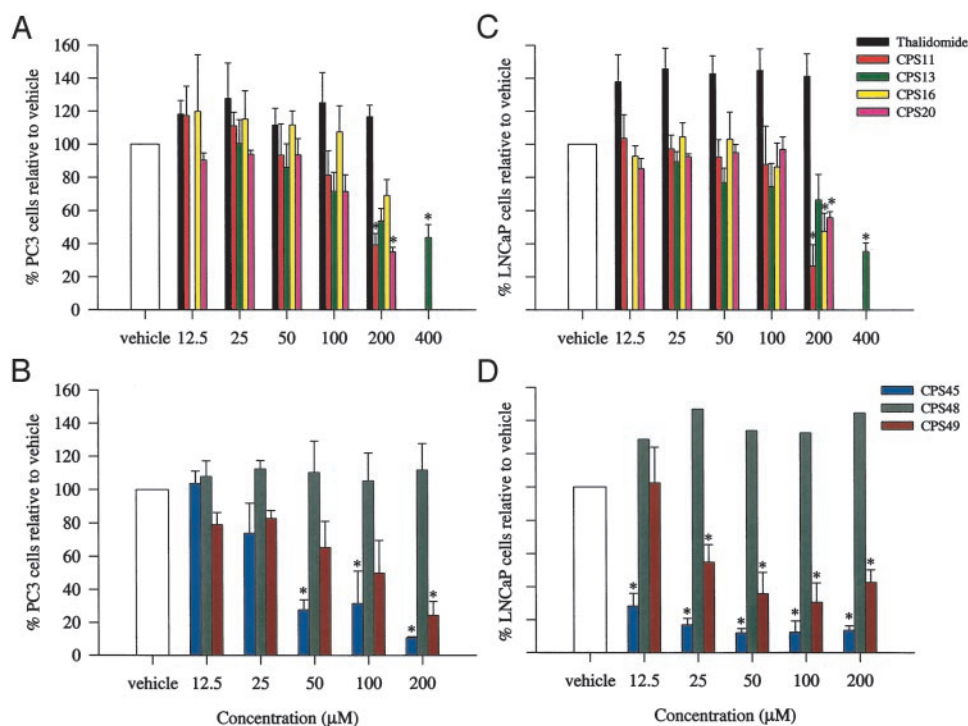


Fig. 7. Bar graphs of the percentage of androgen-independent PC3 cells (A and B) and androgen-dependent LNCaP cells (C and D) relative to the vehicle control (0.5% DMSO) versus treatment with various thalidomide analogues (12.5–400 μM) for 48 h. Bars, mean \pm SE. *, significantly ($P < 0.05$) different from vehicle control.

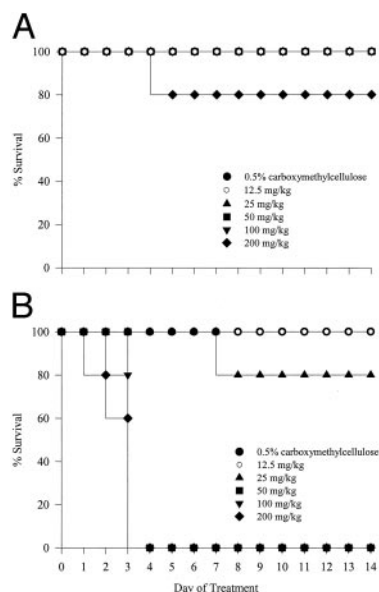


Fig. 8. Survival graphs of male nude mice given daily i.p. injections of the vehicle (0.5% carboxymethylcellulose), CPS11 (A), or CPS49 (B) at 12.5–200 mg/kg for 2 weeks.

turn, stronger binding to biological targets (25). These *in vitro* data were reverberated in the much lower maximum tolerated dose of CPS49 (12.5 mg/kg) compared with CPS11 (100 mg/kg). The relative lack of *in vivo* lethal toxicity of CPS45 (200 mg/kg), which demonstrated similar potency to CPS49 *in vitro*, was unexpected and remains to be understood. Contrary to the analogues, thalidomide itself failed to suppress rat aortic angiogenesis and HUVEC proliferation, consistent with previous reports which showed that thalidomide metabolites are responsible for the pharmacological actions of the drug (18, 19). It is unclear, however, why thalidomide exhibited activity in the tube formation assay. The differential sensitivity of rat aorta and human vein to the antiangiogenic effects of the thalidomide analogues demonstrates the importance of using multiple systems in the preclinical screening of new compounds.

The precise mechanisms of action of thalidomide and its analogues are not completely understood. Thalidomide has been shown to alleviate inflammatory processes by enhancing TNF- α mRNA degradation (4). Kenyon *et al.* (26) reported that thalidomide, but not strong TNF- α inhibitors, blocks basic fibroblast growth factor-induced angiogenesis, suggesting that the antiangiogenic action of thalidomide may be mediated by TNF- α -independent pathways in the mouse cornea model. However, the tumor microenvironment, with infiltrating macrophages as the source of TNF- α , is much more complex. It remains to be determined whether TNF- α is involved in thalidomide-induced antiangiogenicity in solid malignancies.

COX-2, an inducible enzyme with a role in carcinogenesis and angiogenesis (27–29), has been recently described as another target of thalidomide and its analogues (30). Fujita *et al.* (31) showed that thalidomide and its analogues reduce prostaglandin E₂ production in the murine macrophage-like cell line RAW 264.7 by decreasing the stability of COX-2 mRNA. Subsequently, it has been demonstrated that introduction of different substituents at different positions of the phthalimido ring differentially changes the selectivity of the analogues for COX-1 and COX-2 (30). Taken together, future design of antiangiogenic thalidomide analogues should aim at generating or optimizing structures which possess both TNF- α and COX-2 inhibitory actions. It is important to note that thalidomide and its analogues may not necessarily share similar mechanisms of action. Indeed, Niwayama *et al.* provided evidence that nonfluorinated and fluori-

nated phthalimides inhibit cytokine production through interaction with distinct target proteins (25).

In summary, we have shown that thalidomide analogues inhibit rat aortic and human venous microvessel outgrowth as well as HUVEC proliferation and tube formation, further supporting the preclinical and clinical development of these compounds as angiogenesis inhibitors. The *in vivo* antiangiogenic and antitumor activities of our thalidomide analogues in prostate cancer are currently being investigated.

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