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Inhibition of Angiogenesis by Thalidomide Requires Metabolic Activation, Which Is Species-dependent

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ABSTRACT. Thalidomide has been shown to be an inhibitor of angiogenesis in a rabbit cornea micropocket model; however, it has failed to demonstrate this activity in other models. These results suggest that the anti-angiogenic effects of thalidomide may only be observed following metabolic activation of the compound. This activation process may be species specific, similar to the teratogenic properties associated with thalidomide. Using a rat aorta model and human aortic endothelial cells, we co-incubated thalidomide in the presence of either human, rabbit, or rat liver microsomes. These experiments demonstrated that thalidomide inhibited microvessel formation from rat aortas and slowed human aortic endothelial cell proliferation in the presence of human or rabbit microsomes, but not in the presence of rat microsomes. In the absence of microsomes, thalidomide had no effect on either microvessel formation or cell proliferation, thus demonstrating that a metabolite of thalidomide is responsible for its anti-angiogenic effects and that this metabolite can be formed in both humans and rabbits, but not in rodents. BIOCHEM PHARMACOL **55**;11:1827–1834, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. angiogenesis; thalidomide; aorta; metabolism

Angiogenesis is the formation of new blood vessels. In tumorigenesis, a network of blood vessels must be formed to sustain the nutrient and oxygen supply that a tumor requires for continued growth [1]. Several studies have shown that there is a direct correlation between tumor microvessel density and the incidence of metastases, and long-term survival [2–4]. It has been shown that tumor cells themselves can produce factors that stimulate the proliferation of endothelial cells and new capillary growth. These observations have led to the investigation of anti-angiogenic agents as possible therapeutic options for various cancers.

In the 1950s, thalidomide was marketed as a sedative in Europe, but eventually was withdrawn from the market when it was found to be a potent teratogen. Children of women who took thalidomide during pregnancy were born with dysmelia (stunted limb growth). It has been postulated that the limb defects seen with thalidomide were secondary to an inhibition of blood vessel growth in the developing limb bud [5]. *In vivo*, thalidomide inhibited basic fibroblast growth factor-induced angiogenesis, using a rabbit cornea micropocket assay, but failed to demonstrate any anti-angiogenic activity in the CAM† model of angiogenesis [5, 6]. We also failed to show in the present study that anti-angiogenic activity *in vitro* using both rat aorta and human aortic endothelial cell models of *in vitro* angiogenesis. Thalidomide has no teratogenic effect in rodents, and actually promoted the metastasis of prostate adenocarcinoma cells (PA-III) implanted in rats [7] and failed to show any anti-angiogenic properties in mice implanted with either B16-F10 melanoma or CT-26 colon carcinoma cells [8].

The activity of a thalidomide metabolite generated by human microsomes was demonstrated in the human leukemic cell line K562 [9]. K562 cells exposed to thalidomide metabolites exhibited a drastic morphologic change as well as an inhibition of cell division. Similar cultures exposed to only thalidomide were unaffected. Exposure of 3- and 4-day-old chick embryos to thalidomide and its hydrolytic products before and after metabolism with rat liver homogenate showed significant embryotoxic effects, primarily in those embryos treated with the metabolites formed following alkaline hydrolysis. These studies demonstrate that, in some cases, the effects of thalidomide are not directly associated with the parent molecule and, in fact, the pharmacologic and toxicologic effects can be traced to a metabolite. These data presented here suggest that thalidomide undergoes a metabolic activation to a compound with anti-angiogenic activity in humans and rabbits, but not in rodents.

MATERIALS AND METHODS

Materials

Thalidomide was obtained from EntreMed. Suramin was obtained from the Developmental Therapeutics Program,

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† *Abbreviations:* CAM, chicken chorioallantoic membrane; EBM, endothelial Eagle's Medium; ECOD, 7-ethoxycoumarin O-deethylation; and HAEC, human aortic endothelial cells.

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NCI. Doxorubicin and reagents for the NADPH-generating system were purchased from Sigma. TNP-470 was a gift from Takeda Chemical Industries, Ltd. All drugs were prepared as stock solutions in DMSO (Sigma). The concentrations of the stock solutions were prepared so that the final amount of DMSO that was present in the culture medium was 0.5% (v/v). Human liver microsomes were a gift from J. Collins, FDA. The human prostate carcinoma cell lines LNCaP and PC-3 were purchased from the American Type Culture Collection. HAEC and endothelial cell basal medium were obtained from Clonetics. All cell lines were grown as directed. The culture of HAEC was limited to 10 serial passages, and LNCaP cells were not used after passage 30. Matrigel® was purchased from Collaborative Biomedical. Cell-Titer nonradioactive cell proliferation kits were purchased from Promega.

Metabolism

Rat and rabbit liver microsomes were prepared by differential centrifugation [10]. The liver was removed from the animal and weighed, and a 20% (w/v) homogenate in 0.25 M sucrose was prepared. The homogenate was centrifuged at 12,500 *g* for 15 min; the supernatant was saved and then further centrifuged at 100,000 *g* for 45 min. The final microsomal pellet was resuspended in 5 mL of Tris buffer, pH 7.4. Microsomal cytochrome P450 activity was assessed by ECOD. ECOD is an overall assessment of microsomal activity; it is catalyzed by at least 11 different isozymes [11]. ECOD activity was measured by a previously described reversed-phase high performance liquid chromatographic method with fluorescence detection [12].

Rat Aorta Cultures

Thoracic aortic cultures were prepared as previously described [13, 14]. The thoracic aorta was removed from Sprague–Dawley rats. Using a dissecting microscope, 1-mm long rings were cut. Each ring was rinsed eight times with culture medium, embedded in Matrigel®, and incubated in growth factor-free endothelial cell basal medium with 10 μg/mL of gentamicin, 100 units/mL of penicillin, 100 μg/mL of streptomycin, and 0.25 μg/mL of amphotericin at 37°, 5% CO_2. Every 24 hr, the supernatant was replaced with 8 μg/mL of thalidomide dissolved in culture medium. Parallel cultures were exposed to 8 μg/mL of thalidomide with 0.2 mg/mL of human, rabbit, or rat liver microsomes and an NADPH-generating system (0.015 M of $MgCl_2$, 4 nM of glucose-6-phosphate, and 20 U/mL of glucose-6-phosphate dehydrogenase in 0.1 M of Tris buffer, pH 7.4) to allow for metabolism. Control segments were exposed to culture medium with 0.5% DMSO with or without liver microsomes, 60 ng/mL of doxorubicin, 10 μg/mL of suramin, or 250 ng/mL of TNP-470. All drugs were tested at clinically achievable concentrations. The aortic preparations were cultured for 6 days, and microvessel growth was assessed daily. To confirm the presence of endothelial cells in the microvessel growth, they were stained with factor VIII and CD34 by a previously described method [2, 3, 15].

Cell Culture

HAEC were seeded into 96-well plates at a density of 1500 cells/well and allowed to attach overnight. Every 24 hr, the culture medium was aspirated, and the cells were exposed to 8 μg/mL of thalidomide dissolved in culture medium and 0.5% DMSO, or culture medium and 0.5% DMSO alone with or without growth factors. The effects of thalidomide were also assessed in the presence of human hepatic microsomes and an NADPH-generating system. On each day, cell proliferation was assessed using the Cell-Titer nonradioactive cell proliferation kit. LNCaP cells were plated in 12-well plates at 3×10^4 cells/well and allowed to attach for 48 hr. Every 24 hr, thalidomide was added to the cultures at 0.6, 6, or 60 μg/mL. Cell proliferation was assessed daily by trypsinizing and counting adherent cells using a Coulter Z1 counter.

LNCaP, PC-3, and HAEC were seeded into 96-well plates at a density of 1500 cells/well and allowed to attach for 48 hr. The culture medium was aspirated, and cells were exposed to 15, 30, or 60 ng/mL of doxorubicin. Every 24 hr, a single plate was removed, and cell proliferation was measured using the Cell-Titer kit. Cell viability was assessed for 6 days.

RESULTS

Metabolism

All microsomal preparations demonstrated metabolic cytochrome P450 activity. Upon incubation of 250 μg/mL of a 200 μg/mL solution of 7-ethoxycoumarin in the presence of 0.25 mg of rat microsomal preparation, 10.16 μg/mL of the metabolite umbelliferone was formed. Likewise, upon incubation of an 80 μg/mL solution of 7-ethoxycoumarin in the presence of 0.1 mg of human and rabbit microsomal preparation, 2.66 and 1.14 μg/mL of umbelliferone was formed. Umbelliferone was not present in any of the control chromatograms.

Rat Aorta Cultures

No growth inhibition of microvessels occurred in cultures containing thalidomide alone (Fig. 1C). In fact, these cultures actually appeared to have increased microvessel growth compared with the culture medium control (Fig. 1A). No inhibition was noted when microsomes were added to the control culture (Fig. 1B). Cultures incubated with thalidomide and human or rabbit hepatic microsomes (Fig. 2, B and C) showed a marked decrease in the formation of new microvessels compared with control cultures incubated with microsomes alone. Those cultures incubated with thalidomide and rat hepatic microsomes (Fig. 2A) demonstrated only a slight decrease in the formation of new microvessels. The cytotoxic agent doxo-

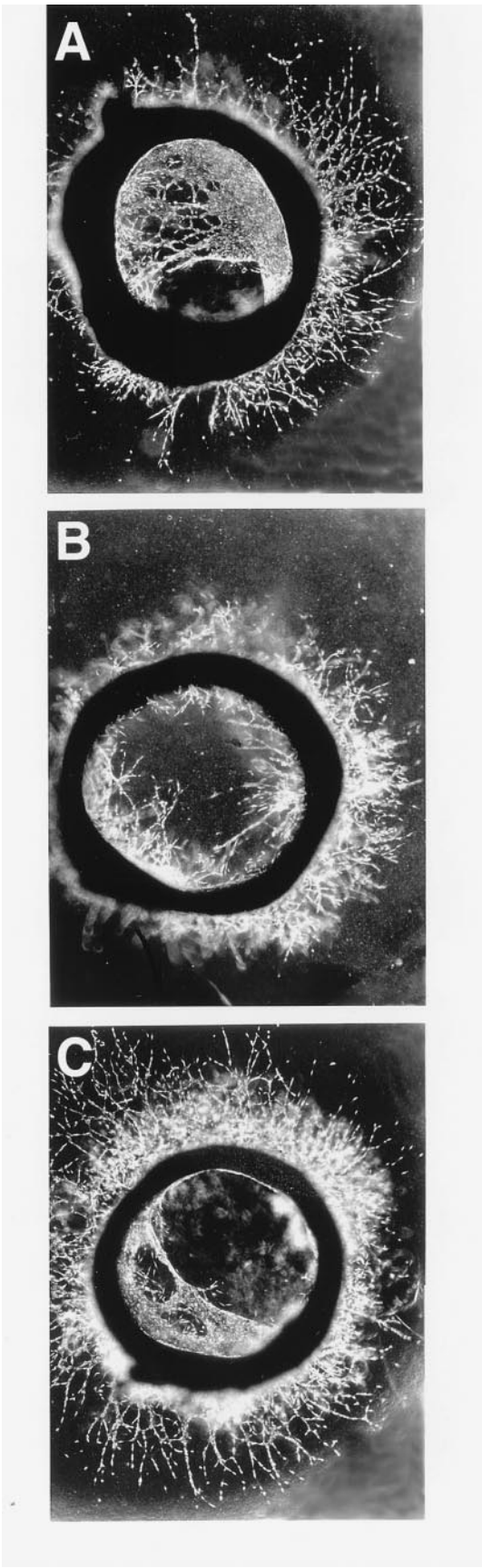

FIG. 1. Rat aortic sections treated with EBM and (A) DMSO vehicle, (B) human microsomes (0.2 mg/mL), and (C) thalidomide (8.0 μg/mL).

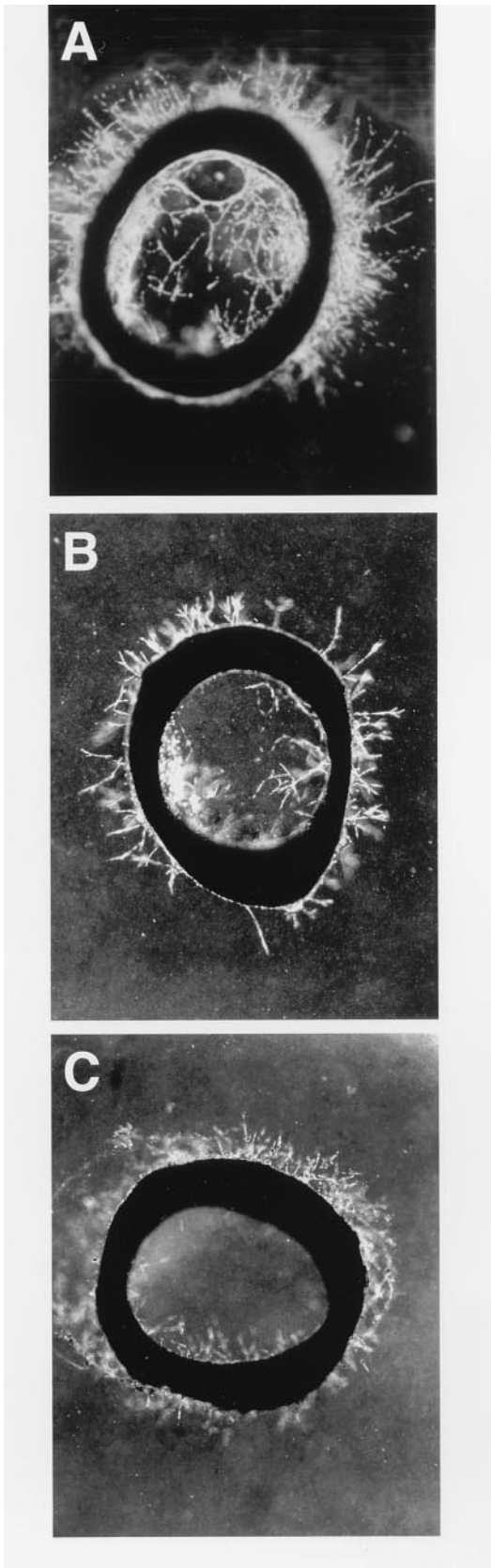

FIG. 2. Rat aortic sections treated with EBM and (A) thalidomide (8.0 μg/mL) and rat microsomes (0.2 mg/mL), (B) thalidomiode (8.0 μg/mL) and human microsomes (0.2 mg/mL), and (C) thalidomide (8.0 μg/mL) and rabbit microsomes (0.2 mg/mL).

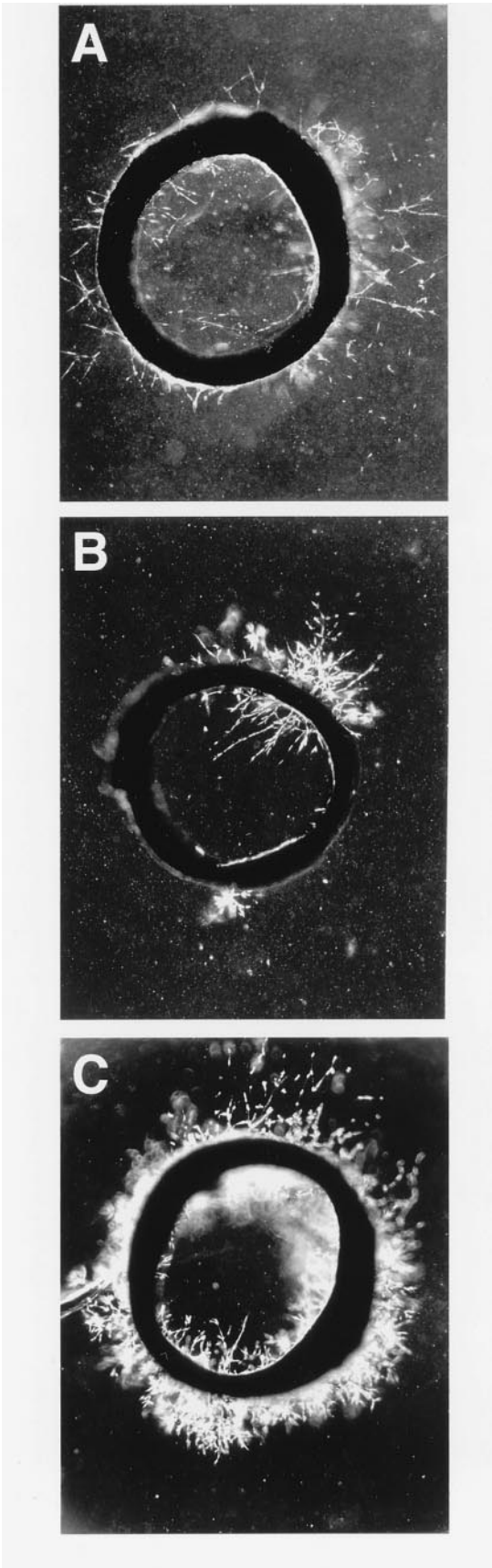

FIG. 3. Rat aortic sections treated with EBM and (A) TNP-470 (250 ng/mL), (B) suramin (10 μg/mL), and (C) doxorubicin (60 ng/mL).

rubicin (Fig. 3C) showed no effect on microvessel growth compared with the culture medium control. Several other pharmacological agents with anti-angiogenic activity were assayed. Both suramin (Fig. 3B) and TNP-470 (Fig. 3A) showed strong inhibition of microvessel growth in this model system. Microvessel formations stained positive for both factor VIII and CD34 (data not shown).

Cell Culture

In both the presence and the absence of growth factors, thalidomide alone seemed to stimulate proliferation of HAEC compared with controls (Fig. 4, A and B). When the cells were incubated with human microsomes and thalidomide in the presence of growth factors, there was little, if any, effect on cell proliferation. However, when the growth factors were deleted from the culture medium, there was a significant decrease in cell proliferation compared with controls as well as cells treated with thalidomide alone. This decrease in cell proliferation appeared to be dependent on the amount of microsomes added. In LNCaP cells, thalidomide alone seemed to have a slight cytotoxic effect at all three concentrations tested. After a 120-hr exposure, treated cells were approximately 90% of controls (Fig. 4C).

All three cell lines showed a decreased proliferation in response to treatment with the cytotoxic agent doxorubicin (Fig. 5). However, there were marked differences in the sensitivities of the cell lines to doxorubicin. Both of the prostate carcinoma cell lines, LNCaP and PC-3, were more sensitive to higher concentrations of doxorubicin. In addition, LNCaP cells showed a slightly higher cytotoxicity than PC-3 cells at the same concentration of doxorubicin. In contrast, HAEC demonstrated no differences in cell proliferation at any of the doxorubicin concentrations used.

DISCUSSION

Folkman and colleagues [16, 17] have demonstrated that angiogenesis is a prerequisite for expansion of solid tumors beyond 1–3 mm^3 and have suggested that angiogenesis is often activated during the early, preneoplastic stages in the development of a tumor. Thus, inhibition of angiogenesis should be effective in inhibiting the growth of a malignancy. D'Amato *et al.* [5] reported that oral thalidomide, a potent teratogen, is a highly effective inhibitor of angiogenesis in the rabbit cornea micropocket assay. They did not observe any anti-angiogenic activity in other model systems (endothelial cell culture and the CAM assay) and speculated that a metabolite of thalidomide was responsible for the activity. Subsequently, several investigators attempted to reproduce those data in rodent models, without success [7, 8]. We elected to initially utilize the rat aorta model of angiogenesis and compare the activity of thalidomide with or without liver microsomes. Our results confirm the potent anti-angiogenic properties of thalidomide if co-incubated with human microsomes or rabbit micro-

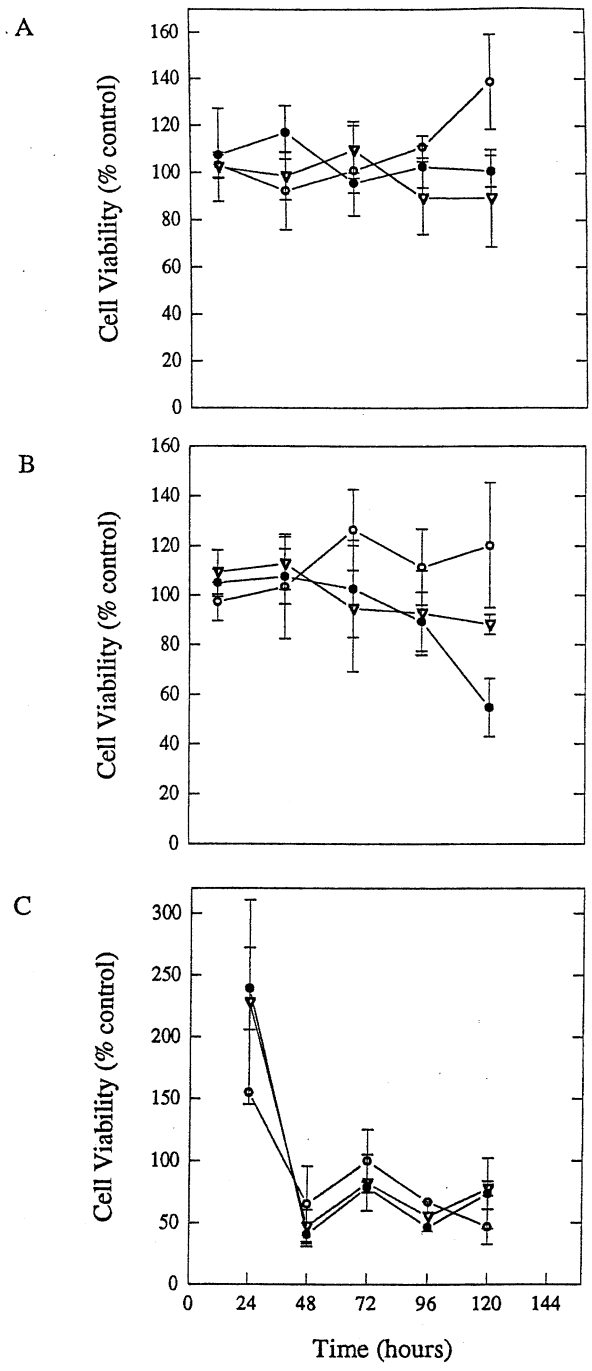

FIG. 4. Effect on HAEC of thalidomide in the presence or absence of growth factors. HAEC, plated at 1500 cells/well, were incubated in the presence of (○) thalidomide (8.0 μg/mL), (●) thalidomide (8.0 μg/mL) and human microsomes (0.1 mg/mL), and (▽) thalidomide (8.0 μg/mL) and human microsomes (0.03 mg/mL). (A) With the addition of supplemental growth factors. (B) without the addition of supplemental growth factors. (C) LNCaP cells treated with thalidomide at: (○) 0.6 μg/mL, (●) 6.0 μg/mL, and (▽) 60.0 μg/mL. Each value is the mean ± SD of 5 experiments.

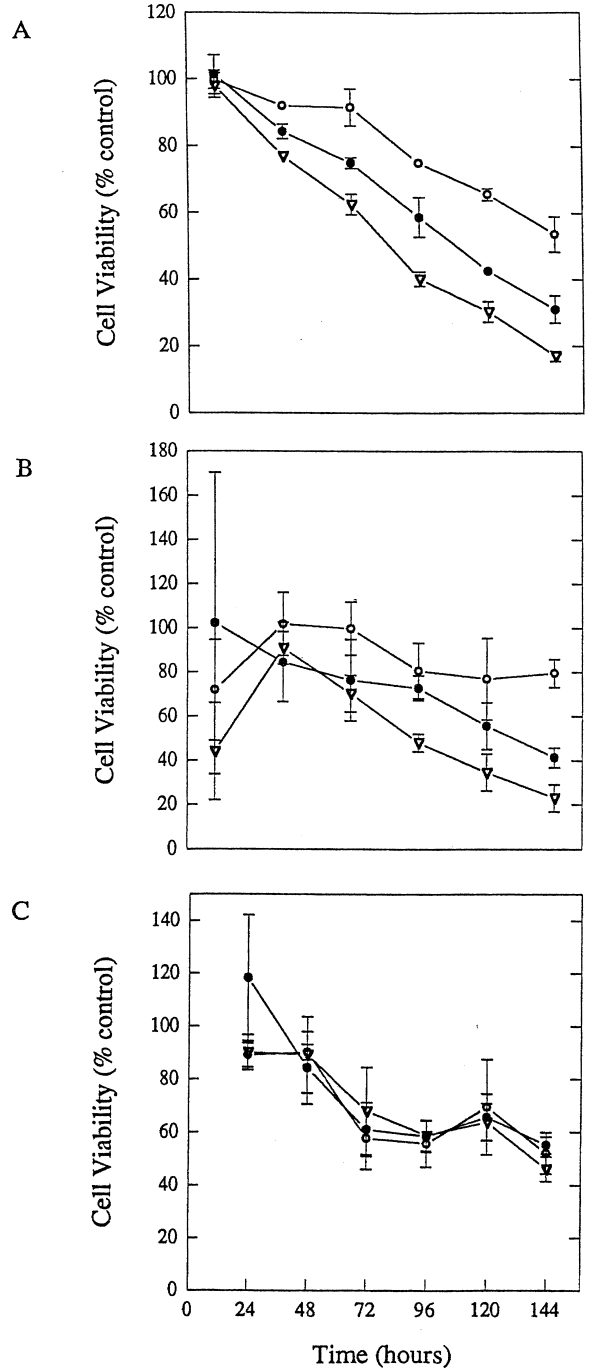

FIG. 5. Effect of doxorubicin on the growth of three cell lines. (A) LNCaP cells, (B) PC-3 cells, and (C) HAEC, each plated at 1500 cells/well, were incubated in the presence of doxorubicin at: (○) 15 ng/mL, (●) 30 ng/mL, and (▽) 60.0 ng/mL. Each value is the mean ± SD of 5 experiments.

somes. However, no activity was noted when rat microsomes were added to the culture with thalidomide. Furthermore, we confirmed this observation using human aortic endothelial cell cultures by adding thalidomide with and without microsomes.

There are five primary metabolites of thalidomide [4-OH-thalidomide, 3-OH-thalidomide, 3′-OH-thalidomide, 4′-OH-thalidomide, and 5′-OH-thalidomide] (see Fig. 6), and the antiangiogenic property could be the result of either of these compounds, or of an intermediate [18–23]. Also, thalidomide undergoes rapid spontaneous hydro-

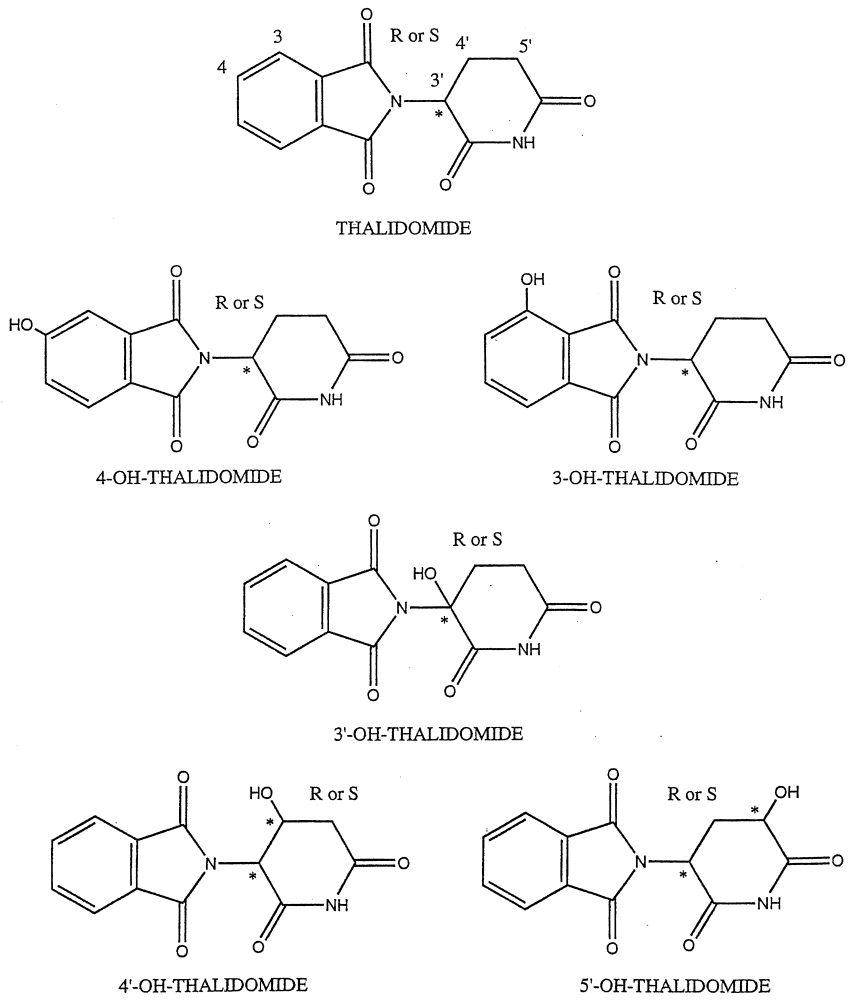

FIG. 6. Structures of thalidomide and the five known metabolites.

lysis in aqueous solutions at a pH of 6.0 or greater to form three primary products [4-phthalimidoglutaramic acid, 2-phthalimidoglutaramic acid, and α-(*o*-carboxybenzamido)glutarimide] and eight minor products (see Fig. 7) [18–23]. Furthermore, each of the five metabolites of the parent compound undergoes similar hydrolysis.

Less than 15% of thalidomide is present 24 hr following an oral dose (terminal half-life ≈8 hr) [24]. Likewise, the parent compound is degraded rapidly in an *in vitro* system by spontaneous hydrolysis (assuming a pH greater than 6) [25]. In our angiogenesis model systems, thalidomide was added only at the beginning of the experiment, and growth was monitored over 5 days. Thus, the hydrolysis products listed above were present in the cultured wells, but anti-angiogenic activity was not noted without the addition of microsomes. Therefore, none of the breakdown products generated from the hydrolysis process are responsible for the activity, although an actively metabolized product of one these hydrolysis compounds could be responsible.

Using doxorubicin as a control for cytotoxicity, we compared thalidomide in cultured tumor cells (PC-3 and LNCaP), the rat aorta angiogenesis model, and cultured endothelial cells. When co-incubated with microsomes (human or rabbit), thalidomide significantly inhibited the formation of microvessels, whereas doxorubicin had no effect. Similarly, thalidomide plus microsomes inhibited the proliferation of aortic endothelial cells (when supplemental growth factors were not added). In tumor cells, doxorubicin significantly reduced the cell number, whereas thalidomide had a minimal effect (either alone or in the presence of microsomes). This suggests that the anti-angiogenic property of thalidomide is through the inhibition of proliferation of endothelial cell growth rather than a cytotoxic effect.

Based on the preclinical data demonstrating anti-angiogenic activity, thalidomide entered Phase II clinical trials in solid tumors (prostate cancer, brain cancer, breast cancer, and Kaposi's sarcoma). Preliminary data suggest some antitumor activity with minimal side-effects. The doses being utilized (200–1200 mg/day) in those trials are based on prior experience with the agent in other disease settings (i.e. graft-versus-host disease [26], lepromatous leprosy, aphthous stomatitis, discoid lupus erythematosus, and HIV wasting). With the high dose regimen (1200 mg/day), peak steady-state plasma concentrations of the parent compound are approximately 10 μg/mL.

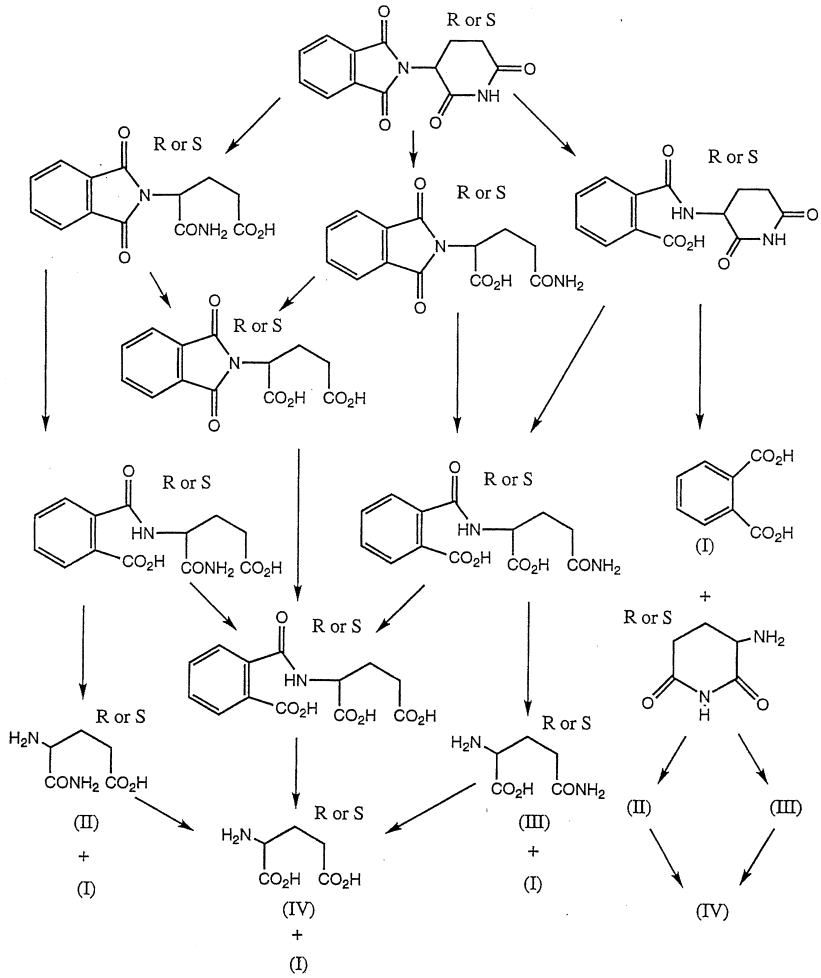

FIG. 7. Structure of thalidomide and the hydrolysis pathway for degradation.

In conclusion, a metabolite of thalidomide is responsible for its anti-angiogenic property. This metabolite appears to be formed in species higher than rodents. Current efforts are ongoing to synthesize and isolate potential candidate compounds, but preliminary data suggest an epoxide intermediate. Furthermore, these data are important in supporting the clinical efforts ongoing with this agent [27]. If the anti-angiogenic activity can be attributed to a single metabolite, then efforts can be made to identify that product in patient samples and to correlate pharmacodynamic results with antitumor effects.

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References

1. Folkman J, Tumor angiogenesis: Therapeutic implications. *N Engl J Med* **181:** 182–186, 1971.
2. Weidner N, Semple JP, Welch WR and Folkman J, Tumor angiogenesis and metastasis–correlation in invasive breast carcinoma. *N Engl J Med* **324:** 1–8, 1991.
3. Weidner N, Carroll PR, Flax J, Blumenfeld W and Folkman J, Tumor angiogenesis correlates with metastasis in invasive prostate carcinoma. *Am J Pathol* **143:** 401–409, 1993.
4. Heimann R, Ferguson D, Powers C, Recant WM, Weichselbaum RR and Hellman S, Angiogenesis as a predictor of long-term survival for patients with node-negative breast cancer. *J Natl Cancer Inst* **88:** 1764–1769, 1996.
5. D'Amato RJ, Loughnan MS, Flynn E and Folkman J, Thalidomide is an inhibitor of angiogenesis. *Proc Natl Acad Sci USA* **91:** 4082–4085, 1994.
6. Vesela D, Vesely D and Jelinek R, Embryotoxicity in chick embryo of thalidomide hydrolysis products following metabolic activation by rat liver homogenate. *Funct Dev Morphol* **4:** 313–316, 1994.
7. Pollard M, Thalidomide promotes metastasis of prostate adenocarcinoma cells (PA-III) in L-W rats. *Cancer Lett* **101:** 21–24, 1996.
8. Gutman M, Szold A, Ravid A, Lazzauskas T, Merimsky O and Klausner JM, Failure of thalidomide to inhibit tumor growth and angiogenesis *in vivo*. *Anticancer Res* **16:** 3673–3678, 1996.
9. Hatfill SJ, Fester ED, DeBeer DP and Bohm L, Induction of morphological differentiation in the human leukemic cell line K562 by exposure to thalidomide metabolites. *Leukemia Res* **15:** 129–136, 1991.
10. Gibson GG and Skett P, *Introduction to Drug Metabolism*, 2nd Edn. Chapman & Hall, New York, 1994.
11. Ryan DE and Levin W, Purification and characterization of hepatic microsomal cytochrome P-450. *Pharmacol Ther* **45:** 153–239, 1990.
12. Evans RR and Relling MV, Automated high-performance liquid chromatographic assay for the determination of 7-ethoxycoumarin and umbelliferone. *J Chromatogr* **578:** 141–145, 1992.

13. Nicosia RF and Ottinetti O, Growth of microvessels in serum-free matrix culture of rat aorta: A quantitative assay of angiogenesis *in vitro*. *Lab Invest* **63:** 115–122, 1990.
14. Carlini RG, Reyes AA and Rothstein M, Recombinant human erythropoietin stimulates angiogenesis *in vitro*. *Kidney Int* **47:** 740–745, 1995.
15. Pinkus GS, Etheridge CL and O'Connor EM, Are keratin proteins a better tumor marker than epithelial membrane antigen? A comparative immunohistochemical study of various paraffin-embedded neoplasms using monoclonal and polyclonal antibodies. *Am J Clin Pathol* **85:** 269–277, 1986.
16. Folkman J, Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat Med* **1:** 27–31, 1995.
17. Hanahan D and Folkman J, Patterns and emerging mechanisms of angiogenic switch during tumorigenesis. *Cell* **86:** 353–364, 1996.
18. Schmahl HJ, Nau H and Neubert D, The enantiomers of the teratogenic thalidomide analogue EM 12: 1. Chiral inversion and plasma phamacokinetics in the marmoset monkey. *Arch Toxicol* **62:** 200–204, 1988.
19. Winckler K, Klinkmüller KD and Schmahl HJ, Determination of the thalidomide analogues 2-(2,6-dioxopiperidine-3-yl)phthalimidine (EM 12), 2-(2,6-dioxopiperidine-4-yl)phthalimidine (EM 16) and their metabolites in biological samples. *J Chromatogr* **488:** 417–425, 1989.
20. Czejka MJ and Koch HP, Determination of thalidomide and its major metabolites by high-performance liquid chromatography. *J Chromatogr* **413:** 181–187, 1987.
21. Fabro S, Schumacher H, Smith RL, Stagg RBL and Williams RT, The metabolism of thalidomide: Some biological effects of thalidomide and its metabolites. *Br J Pharmacol* **25:** 352–362, 1965.
22. Günzler V, Hanauske-Abel HM, Tschank G and Schulte-Wissermann H, Immunological effects of thalidomide: Inactivity of the drug and several of its hydrolysis products in mononucleocyte proliferation tests. *Arzneimittelforschung* **36:** 1138–1141, 1986.
23. Jönsson NA, Chemical structure and teratogenic properties: III. A review of available data on structure–activity relationships and mechanism of action of thalidomide analogues. *Acta Pharm Suec* **9:** 521–542, 1972.
24. Chen TL, Vogelsang GB, Petty BG, Brundrett RB, Noe DA, Santos GW and Colvin OM, Plasma pharmacokinetics and urinary excretion of thalidomide after oral dosing in healthy male volunteers. *Drug Metab Dispos* **17:** 402–405, 1989.
25. Simmons BR, Lush RM and Figg WD, A reversed-phase high performance liquid chromatography method using solid phase extraction to quantitate thalidomide in human serum. *Anal Chim Acta* **339:** 91–97, 1997.
26. Vogelsang GB, Farmer ER, Hess AD, Altamonte V, Beschorner WE, Jabs DA, Corio RL, Levin LS, Colvin OM, Wingard JR and Santos GW, Thalidomide for the treatment of chronic graft-versus-host disease. *N Engl J Med* **326:** 1055–1058, 1992.
27. Gordon GB, Spielberg SP, Blake DA and Balasubramanian V, Thalidomide teratogenesis: Evidence for a toxic arene oxide metabolite. *Proc Natl Acad Sci USA* **78:** 2545–2548, 1981.