Effects of Putative Hydroxylated Thalidomide Metabolites on Blood Vessel Density in the Chorioallantoic Membrane (CAM) Assay and on Tumor and Endothelial Cell Proliferation

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Angiogenesis, in particular anti-angiogenesis, is an area of particular therapeutic interest in cancer treatment. Several anti-angiogenic agents are in the final stages of clinical trials. One of these agents, thalidomide, best known for its teratogenic potential, is showing promise against several tumor types. Thalidomide has been shown previously to require bio-activation to exert its anti-angiogenic effect in isolated blood vessels and endothelial cells. In this work, we confirmed these findings using the *in utero* chicken embryo chorioallantoic membrane (CAM) system. In particular, the anti-angiogenic effect of thalidomide is significantly enhanced by activation by human but not by rat liver microsomes. We also showed in the CAM assay that hydroxylation of thalidomide at either the 1'- or 5-position retained anti-angiogenic activity whereas its hydroxylation at the 4-position led to an inactive compound. We further demonstrated that thalidomide shows weak anti-proliferative activity against MDA-MB-231 human breast cancer cells in culture. Thalidomide showed slightly more anti-proliferative activity, however, against the SH-SY5Y human neuroblastoma and human umbilical vein endothelial cell (HUVEC) types. Furthermore, incubation of thalidomide with human liver microsomes added no additional anti-proliferative effect in these cell types *versus* thalidomide given alone. Finally, we report that none of the thalidomide metabolites tested had any anti-proliferative effect against the breast or neuroblastoma cells, but do possess appreciable anti-proliferative activity against the endothelial cells. In summary, this work suggests that hydroxylated thalidomide analogs based on putative metabolites of the drug possess significant anti-angiogenic activity and that exploring further derivatives of these as potential anti-angiogenic agents warrants further merit.

Key words thalidomide; angiogenesis; chorioallantoic membrane (CAM) assay; cytotoxicity

Angiogenesis, the process of blood vessel formation from pre-formed vessels, has recently become a primary target of anticancer therapy. This is due in part to the finding that removing the blood supply to a tumor can not only prevent further growth but can also starve the existing cells to death.²⁾ In the past several years there have been many potential anti-angiogenic agents developed acting by many mechanisms including interruption of growth factor receptor phosphorylation,^{3–5)} growth factor-receptor binding,^{6,7)} cell adhesion molecule expression and signaling^{8,9)} and alterations in endothelial cell apoptosis.^{10,11)} In addition, several agents such as thalidomide have been shown to inhibit angiogenesis by an as yet not fully understood mechanism.^{12–18)} Furthermore, thalidomide has been shown to reduce tumor growth both in model systems^{19–21)} and in patients.^{22–28)} Clinically, however, thalidomide has a fairly weak effect as a monotherapy in patients with solid tumors^{22–24)} but is showing tremendous promise against multiple myeloma.^{25–28)} It has been shown that activation of thalidomide by human or rabbit liver enzymes, but not by rat liver enzymes, significantly enhances the anti-angiogenic effect of the drug in rat aortic sections or isolated endothelial cells.²⁹⁾ This is very interesting because thalidomide is virtually non-toxic to rats when given orally (PO). Intraperitoneal (IP), administration however, commonly associated with “first-pass” hepatic metabolism,³⁰⁾ recapitulates the teratogenic effect of thalidomide in rats.³¹⁾ Kenyon *et al.*¹⁹⁾ also showed that thalidomide given IP was effective as an anti-angiogenic agent in the mouse corneal model, whereas thalidomide given PO was inactive. In addition, thalidomide teratogenesis was only found to occur after

activation of the drug by liver enzymes.^{13,32–35)} Furthermore, in a *Xenopus* oocyte assay of teratogenesis, activation of thalidomide by a single cytochrome P450 enzyme, CYP2E1, lead to a teratogenic compound whereas unactivated thalidomide was non-teratogenic.³⁵⁾ Thus metabolism of thalidomide is critical to its anti-angiogenic and teratogenic activity. However, the identity of the active anti-angiogenic and teratogenic moiety or moieties of thalidomide are still unknown.

The goal of this work was to explore the effect of putative hydroxylated thalidomide metabolites on blood vessel density and target cell proliferation. Since the anti-angiogenic activity of thalidomide was first reported,³⁶⁾ only a limited number of thalidomide metabolites have been tested for anti-angiogenic activity. Figure 1 shows known and hypothesized metabolites of thalidomide (structures **M1**–**M8**) based on extensive literature review.^{37–45)} Thalidomide can be metabolized through hydrolysis at the glutarimide and/or phthalimide ring to yield metabolites such as **M6**–**M8**. Its hydroxylation can occur on glutarimide or phthalimide ring to yield metabolites **M1**–**M5**. Interestingly, only metabolites **M5** (phthaloyl glutamic acid; PGA) and **M1** (4-hydroxy thalidomide) have been tested for their anti-angiogenic activities.^{19,36)}

Using an established angiogenesis assay, the chicken embryo chorioallantoic membrane (CAM) assay, we report that incubation of thalidomide with human (but not rat) liver microsomes increases anti-angiogenic activity of the drug. Thus it is likely that active anti-angiogenic metabolites of thalidomide exist. We became very interested as to whether any of

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the metabolites of thalidomide described from literature possess anti-angiogenic activity using the CAM assay. We were also interested whether thalidomide and these hydroxylated analogs showed selective anti-proliferative activity on endothelial cells or whether they were also cytotoxic to tumor cells derived from the breast or from neural tissue. These data presented here suggest that three of the putative metabolites of thalidomide are more active as anti-angiogenic agents than the parental drug in the CAM assay. We also found that thalidomide and thalidomide incubated with human microsomes have weak anti-proliferative activity on MDA-MB-231 human breast cancer, SH-SY5Y human neuroblastoma, and human umbilical vein endothelial cell (HUVEC) human umbilical vein endothelial cells. In addition we found no anti-proliferative activity of any of the thalidomide analogs tested on the MDA-MB-231 or SH-SY5Y cells but substantial anti-proliferative activity of these same metabolites against the HUVEC (endothelial) cells.

MATERIALS AND METHODS

Chemicals Thalidomide (racemic mixture) was purchased from Calbiochem (La Jolla, CA, U.S.A.). VEGF was purchased from Landing Biotech (Cambridge, MA, U.S.A.); bFGF was purchased from Peprotech (Rocky Hill, NJ, U.S.A.). Human liver microsomes were from Gentest (Woburn, MA, U.S.A.) and rat liver microsomes were from In Vitro Technologies (Baltimore, MD, U.S.A.). All other chemicals and starting materials were purchased from Sigma Chemical (St. Louis, MO, U.S.A.) or Aldrich Chemical (Milwaukee, WI, U.S.A.).

Synthesis of Thalidomide Metabolites The synthesis of thalidomide metabolites was accomplished using reported synthetic procedures^{46,47} and confirmed by NMR and MS. (This part has already mentioned in chemical section.)

Microsomal Incubation of Thalidomide A racemic mixture of thalidomide (Calbiochem) was used and incubation of the drug completed as previously described by Bauer *et al.*²⁹ Briefly, 200 μ g human or rat liver microsomes were permeabilized using 0.1 M potassium phosphate buffer with 0.5% Triton X-100 on ice for 10 min. A 10 μ l aliquot containing 100 μ g of thalidomide is then added and pre-incubated for 2 min at 37 °C in a water bath with shaking. One microliter of 100 mM NADPH is added and the mixture incubated for 10–30 min at 37 °C.

Microsomal cytochrome P450 activity was assessed by ethoxyresorufin *O*-deethylase (ECOD). ECOD is an overall assessment of P450 activity because it is converted by at least 11 CYP isozymes.⁴⁸ An essentially non-fluorescent substrate 6,8-difluoro-7-ethoxy-4-methylcoumarin (DiFMUEt) was chosen (Molecular Probes, Eugene, OR, U.S.A.) that is converted by ECOD to a blue fluorescent product, 6,8-difluoro-7-OH-4-methylcoumarin (Molecular Probes manual), that was measured with an imaging system (Kodak-NEN ImageStation). A standard curve of the purified product (Molecular Probes) was also generated to determine the amount of substrate converted to product. In duplicate experiments with a sample size of 3 per experiment, a 10 min incubation of DiFMUEt led to a $9.2 \pm 2.6\%$ conversion to product with human microsomes and a $10.1 \pm 3.8\%$ conversion to product with rat microsomes (data not shown in graphical form).

Thus the human and rat microsomes used in these studies possessed similar metabolic potential.

CAM Assay The CAM assay used was a modified procedure based on Sheu⁴⁹ and Brooks.⁵⁰ Briefly, fertile leghorn chicken eggs (Truslow Farms, MD, U.S.A.) were allowed to incubate in a humidified environment at 37.5 °C until 10 d of incubation. The pro-angiogenic factors human vascular endothelial growth factor (VEGF)-165 and basic fibroblast growth factor (bFGF) (200 ng each) were then added to saturation to a microbial testing disk and placed onto the CAM by breaking a small hole in the superior surface of the egg. Anti-angiogenic compounds were then added 8 h after the VEGF/bFGF at saturation to the same microbial testing disk and embryos allowed to incubate for an additional 40 h. After 48 h, CAMs were removed, quickly fixed with 4% paraformaldehyde in PBS, placed onto Petri dishes, and digitized images taken at 7.5 $\times$ using a Nikon dissecting microscope and Scion Imaging system. A 1 $\times$ 1-cm grid was then added to the digital CAM images and the average number of vessels within 5–7 grids counted as a measure of vascularity. AGM-1470 (a kind gift of the NCI Developmental Therapeutics testing program), a very active anti-angiogenic agent currently undergoing clinical trials for cancer,^{51,52} was used as a positive control. Ten ng/embryo AGM-1470 caused 60–70% suppression in angiogenesis in all experiments (data not shown).

Cell Culture All cells were from American Type Culture Collection (ATCC) and were maintained at 37 °C in a humidified environment containing 5% CO₂. The MDA-MB-231 and SH-SY5Y cells were maintained in Eagle's Dulbecco's modified medium containing 10% (fetal bovine serum) FBS. The HUVEC cells were grown in EBM-MV2 medium from Clonetics (San Diego, CA, U.S.A.).

Prior to treatment, the tumor cell lines were seeded to 50% confluence, a point of logarithmic growth. HUVECs were seeded either to confluence, a point mimicking the environment of endothelial cells lining a blood vessel, or to 50% confluence, a point of cell growth mimicking an endothelial cell undergoing angiogenic proliferation. HUVECs were pre-stimulated with 50 ng/ml VEGF for 12 h, a dose and time found to be optimal for angiogenic stimulation (data not shown). Cells were then treated with thalidomide metabolite doses (10, 33.3, 100, 333.3 μ M), ranging from non-toxic to lethal (Figs. 4, 5). Specifically, thalidomide metabolites were placed upon cells in culture for 8 h, a time approximating the elimination half-life of the parental drug in blood^{53–55} and the cell proliferation ELISA run as described below.

BrdU Cell Proliferation ELISA A commercially available (Amersham-Pharmacia) BrdU ELISA kit was used as a measure of cellular proliferation. After drug treatment, cells were allowed to grow for 24 h then BrdU labeling solution added overnight at a concentration found non-toxic to all cell lines (10 μ M). The cells were then fixed to the plate with methanol and heat, blocked using 5% milk, probed with an anti-BrdU antibody-horseradish peroxidase (HRP) conjugate, colorimetric HRP substrate added and the color change corresponding to cell proliferation measured using a colorimetric plate reader. AGM-1470, a kind gift of the NCI testing program and previously shown to target its anti-angiogenic activity to endothelial cells,⁵⁶ was used as a positive control for cell kill in the studies with the HUVEC cells (data not

Fig. 1. Hydroxylated Metabolites of Thalidomide from Literature Review

Thalidomide is a phthalimide ring joined at the nitrogen to a glutarimide ring. Hydroxylated metabolites of the drug include 4-hydroxy thalidomide (M1), 5-hydroxy thalidomide (M2), 1'-hydroxy thalidomide (M3), 4,1'-dihydroxy thalidomide (M4), 5,1'-dihydroxy thalidomide (M5), phthaloyl glutamic acid (PGA) (M6), 4-hydroxy phthaloyl glutamic acid (M7), and 5-hydroxy phthaloyl glutamic acid (M8).

shown). Ten nM AGM-1470 resulted in a 45–60% inhibition in proliferation in all experiments (data not shown). Cisplatin, shown to be an active anti-proliferative agent against many tumor cell types, was used as a positive control for cell kill in the tumor cell studies. 1 μ M cisplatin resulted in a 50–60% reduction in proliferation in both cell lines in all experiments (data not shown).

Statistical Evaluation of Data Graphs, averages, standard deviations, one-way analysis of variance (ANOVA) and unpaired Student's *t*-test at the 95% confidence interval were performed using Prism 2.0 software (GraphPad Software).

RESULTS

Effect of Microsomal Incubation of Thalidomide on Blood Vessel Density in the CAM Assay In the first article describing thalidomide as an anti-angiogenic agent, D'Amato *et al.*³⁶⁾ found that thalidomide exhibited anti-angiogenic activity in the rabbit corneal micropocket assay while it was not effective in the chicken embryo CAM assay. In addition, Bauer *et al.*²⁹⁾ found that thalidomide required metabolic activation by human and rabbit but not rat liver microsomes to possess anti-angiogenic activity in isolated endothelial cells or rat aortic sections. We hypothesized that in the CAM assay, in which angiogenic factors and anti-angiogenic agents are placed locally on a vascularized membrane, thalidomide was not being adequately metabolically activated. We first attempted to reproduce the effect of thalidomide alone and thalidomide activated by liver microsomes using the chicken embryo CAM assay. In the CAM assay, growth factors (bFGF and VEGF) were placed onto a bacterial testing disk and this placed on top of the CAM, a highly vascularized membrane in a growing chicken egg. Eight hours after growth factor administration the thalidomide analogs were added to the same disk and 40 h later the change in blood vessel density assessed in the local area of disk placement. Figure 2A represents digitized images of representative CAMs treated for 48 h with vehicle, VEGF/bFGF alone or VEGF/bFGF plus 100 μ g thalidomide pre-incubated with human liver microsomes. The amount of vessels, particularly smaller vessels, was significantly increased with growth factor treatment, and such increase was

prevented when treating with thalidomide pre-incubated with human liver microsomes.

Figure 2B graphically represents vessel counts of CAMs treated with 100 μ g thalidomide alone, microsomes alone, or 100 μ g thalidomide pre-activated with various concentrations of rat or human microsomes. Thalidomide pre-treated with rat liver microsomes (dark bars) showed little effect on CAM vasculature (Fig. 2B). In contrast, thalidomide pre-treated with 0.2 (but not with 0.1 or 1.0) mg/ml human microsomes (light bars) showed a significant suppression of blood vessel density (48% mean inhibition of blood vessel density, Student's *t*-test $p < 0.05$). Figure 2C shows a dose-response relation of thalidomide alone (open circles), thalidomide pre-incubated with 0.2 mg/ml rat liver microsomes for 10 min (open triangles), or thalidomide pre-incubated for 10 or 30 min with 0.2 mg/ml human microsomes (closed triangles and closed squares, respectively) in the CAM assay. In this study, thalidomide alone showed a significant suppression in blood vessel density only at the highest dose of 100 μ g thalidomide (20% mean suppression at 100 μ g, Student's *t*-test $p < 0.05$). In addition, both a 10- and 30-min pre-incubation of thalidomide with human microsomes showed significant suppression of blood vessels at both 10 and 100 μ g *versus* thalidomide given alone (Student's *t*-test, $p < 0.05$). Finally, thalidomide pre-incubated by rat liver microsomes, however, showed no suppression of blood vessel density at any dose tested.

Effect of Putative Thalidomide Metabolites on Blood Vessel Density in the CAM Assay Hydroxylated "closed ring" and "open ring" putative thalidomide metabolites (Fig. 1) were then synthesized.⁴⁷⁾ The purity of all putative metabolites was determined by NMR and by HPLC^{57,58)} and found to be over 95% in all cases. Figure 3 represents data from the eight putative thalidomide metabolites tested in the CAM assay. Hydroxylation of thalidomide on the C4-position of the phthalimide ring (M1) led to a weakly anti-angiogenic compound (Fig. 3A; 14% mean inhibition of blood vessel density at 100 μ g). In contrast, hydroxylation of thalidomide on the C5-position of the aromatic ring (M2) or on the nitrogen (C1'-position) of the glutarimide ring (M3) led to active anti-angiogenic agents (mean inhibitions of 47% and 50% at 100 μ g, respectively). Hydroxylation at both po-

Fig. 2. Effect of Microsomal Incubation of Thalidomide on Blood Vessel Density in the CAM Assay

(A) Digitized images of (left to right) untreated CAM, CAM treated with 48 h VEGF/bFGF (200 ng each), and 48 h VEGF/bFGF (200 ng each) followed by 40 h 100 µg thalidomide pre-incubated for 10 min with human liver microsomes. (B) Effect of 100 µg thalidomide given alone or pre-incubated for 10 min with various concentrations of either human (light bars) or rat (dark bars) microsomes on blood vessel density in the CAM assay. VEGF/bFGF (200 ng each) were administered on a disk to the CAMs for 48 h and drug was added to the same disk 8 h later (total 40 h with drug) as in Materials and Methods. Data are represented as the mean $\pm$ standard deviation of 5–12 replicates from 2–3 separate experiments. Data are expressed as a percent of blood vessels per unit volume of VEGF/bFGF treated CAMs, as described in Materials and Methods. * $p < 0.05$ (versus thalidomide alone) by Student's *t*-test. (C) Effect of 10 or 30 min pre-incubation of 1, 10, and 100 µg thalidomide with human microsomes on blood vessel density in the CAM assay. Untreated (no VEGF/bFGF) blood vessel density is added at the bottom left of the graph (small open square; dotted line) for reference. Data are represented as the mean $\pm$ standard deviation of 4–16 replicates from 2–4 separate experiments. Data are expressed as a percent of blood vessels per unit volume of VEGF/bFGF treated CAMs, as described in Materials and Methods.

sitions (M5) also led to a very active compound (64% mean inhibition at 100 µg), whereas hydroxylation at both the 4- and the 1'-position of thalidomide (M4) led to a weakly active compound (14% mean inhibition at 100 µg). Thus the position of hydroxylation seems critical for anti-angiogenic activity of thalidomide.

Next, three putative thalidomide metabolites with the glutarimide ring hydrolyzed were tested in the CAM assay. The

Fig. 3. Effect of 1, 10, and 100 µg/Embryo Hydroxylated Putative Thalidomide Metabolites on Blood Vessel Density in the CAM Assay

VEGF/bFGF (200 ng each) were administered on a disk to the CAMs for 48 h and drug was added to the same disk 8 h later (total 40 h with drug) as in Materials and Methods. Untreated (no VEGF/bFGF) blood vessel density is added at the bottom left of each graph (small open square; dotted line) for reference. Data are represented as the mean $\pm$ standard deviation of 5–12 replicates from 2–3 separate experiments. Data are expressed as a percent of blood vessels per unit volume of VEGF/bFGF treated CAMs, as described in Materials and Methods.

first, phthaloyl glutamic acid (PGA), was previously shown to be active as an anti-angiogenic agent in the mouse corneal model of angiogenesis.¹⁹ We found that PGA (M6) was almost as active as Thalidomide pre-incubated with human liver microsomes in the CAM assay (Fig. 3B; 39% mean inhibition at 100 µg). In contrast, hydroxylation at either the 4- or the 5-position of PGA led to slightly less active compounds (22 and 30% mean inhibition at 100 µg, respectively).

Effect of Thalidomide and Its Putative Metabolites on Cancer Cell Proliferation The effect of the thalidomide and its hydroxylated analogs on tumor cell proliferation was next examined. Doses ranging from 10 to 333 µM were chosen based on previous findings in humans that a single oral dose of 200 mg gave a peak blood level of *ca.* 1.5 µg/ml (6 µM).^{53,54} Naively assuming that a patient taking a large dose of 1200 mg thalidomide will have a peak blood level around 9 µg/ml (36 µM), doses approximately one log higher and lower than this dose were chosen. The MDA-MB-231

Fig. 4. Effect of Microsomal Incubation of Various Concentrations of Thalidomide on MDA-MB-231 Human Breast Cancer (A), SH-SY5Y Human Neuroblastoma (B) or Human Umbilical Vein Endothelial Cell (HUVEC) Cell Proliferation

Cells were treated with drug for 8 h, allowed to grow for 2 d, exposed to BrdU overnight, and a BrdU ELISA used to measure cell proliferation as described in Materials and Methods. Data are represented as the mean $\pm$ standard deviation of 8–17 replicates from 2–3 separate experiments. Data are expressed as a percent of BrdU uptake (cell proliferation) of untreated cells as described in Materials and Methods.

breast tumor cell line was chosen because it is very metastatic when implanted into mice⁵⁹) and can induce angiogenesis in cellular systems.⁶⁰) Because of the potential of thalidomide to cause neuropathy, a neuroblastoma cell line, SH-SY5Y, was also chosen. This cell line retains many neuronal properties including nerve growth factor-induced neurite outgrowth^{61,62}) and neuronal-like action potentials^{63,64}) and has been used as a model for drug-induced neurotoxicity.^{65,66})

As shown in Fig. 4A, thalidomide alone and thalidomide pre-incubated with human microsomes weakly inhibited cell proliferation in the MDA-MB-231 breast cells (54 and 62% mean inhibition of proliferation at 333 μ M, respectively). Furthermore, incubation of thalidomide by human microsomes did not enhance anti-proliferative activity versus thalidomide given alone. Thalidomide showed slightly more anti-proliferative activity against the SH-SY5Y neuroblastoma cells (Fig. 4B) than the MDA-MB-231 cells, and caused almost complete inhibition of cell proliferation at 333 μ M. In the SH-SY5Y cells (Fig. 4A), like the breast cells, incubation of

thalidomide by liver microsomes did not enhance anti-proliferative activity *versus* thalidomide given alone. Furthermore, none of the putative hydroxylated metabolites (M1–M8) tested showed any anti-proliferative effect on either MDA-MB-231 or SH-SY5Y cells even at the highest doses (Figs. 5B, 5C, 5E, 5F, 5H, 5I).

Effect of Thalidomide and Its Putative Hydroxylated Metabolites on Endothelial Cell Proliferation The effects of thalidomide and its hydroxylated analogs on HUVEC proliferation were next examined. As shown in Fig. 4C, thalidomide alone and thalidomide pre-incubated with liver microsomes showed substantial anti-proliferative activity against the HUVECs (100% and 67% mean inhibition of proliferation at 333 μ M, respectively). Further, thalidomide pre-incubated with microsomes actually showed less anti-proliferative activity *versus* thalidomide alone. Figure 5 showed the results of putative thalidomide metabolites on HUVEC cell proliferation. All of the hydroxylated analogs showed increased cell kill *versus* thalidomide alone or thalidomide pre-incubated by human liver microsomes (Fig. 5 *vs.* Fig. 4C). The most active hydroxylated thalidomide analogs against HUVEC proliferation were 4-hydroxy thalidomide (M1; 100% mean inhibition at 333 μ M), 1'-hydroxy thalidomide (M3; 87% mean inhibition at 333 μ M), 5,1'-dihydroxy thalidomide (M5; 88% mean inhibition at 333 μ M), PGA (M6; 100% inhibition at 333 μ M) and 5-hydroxy PGA (M8; 91% mean inhibition at 333 μ M).

DISCUSSION

Angiogenesis inhibition remains a very active, and based on early clinical data, an extremely relevant area of cancer research. There are a number of novel agents in development for anti-angiogenic therapy as well as some older agents being used toward this end. In the case of thalidomide, an old drug best known for its teratogenic potential is finding new life as both an anti-inflammatory and anti-angiogenic agent. It has been shown with aortic sections and endothelial cells in culture that thalidomide must first be activated to exert its anti-angiogenic activity.²⁹) We now confirm these findings using the *in utero* chicken embryo CAM system; that the anti-angiogenic activity of thalidomide is significantly enhanced by incubation by human but not by rat liver microsomes. We also found that thalidomide alone possessed weak anti-angiogenic activity, in contrast to the study by D'Amato.³⁶) This could be due to the fact that in the D'Amato report, angiogenesis was stimulated by bFGF alone,³⁶) whereas in our studies angiogenesis was stimulated using both bFGF and VEGF. We believe that this combination of growth factors more closely mimics that seen in the extracellular milieu during tumor-related angiogenesis. In addition, in the D'Amato, paper,³⁶) pellets of bFGF were implanted into the CAM whereas in our studies growth factors were applied locally to the surface of the CAM. Thus we are assessing a localized anti-angiogenic effect, versus more of a systemic effect with the D'Amato work.³⁶)

Because increased activity of thalidomide was observed when pre-incubated with human (but not rat) liver microsomes, it is likely that active metabolites of this drug exist. Through extensive literature review,^{37–45}) a number of hydroxylated metabolites of thalidomide have been character-

Fig. 5. Effect of Various Concentrations of Hydroxylated Putative Thalidomide Metabolites on Human Umbilical Vein Endothelial Cell (A, D, G), MDA-MB-231 Human Breast Cancer (B, E, H) or SH-SY5Y Human Neuroblastoma (C, F, I) Cell Proliferation

Cells were treated with drug for 8 h, allowed to grow for 2 d, exposed to BrdU overnight, and a BrdU ELISA used to measure cell proliferation as described in Materials and Methods. Data are represented as the mean $\pm$ standard deviation of 6–16 replicates from 2–3 separate experiments. Data are expressed as a percent of BrdU uptake (cell proliferation) of untreated cells as described in section Materials and Methods.

ized. Thus the goal of the remainder of this work was to explore the effect of putative hydroxylated thalidomide metabolites on blood vessel density and target cell proliferation. We now demonstrate that hydroxylation of thalidomide at either the 1'- or 5-position of thalidomide is activating with respect to anti-angiogenic activity in the CAM assay whereas hydroxylation at the 4-position leads to an inactive compound.

Because we found putative metabolites of thalidomide that were active in a functional angiogenesis assay, we next sought to define the cellular target for the drug and these metabolites. We further show that thalidomide alone has weak anti-proliferative activity against MDA-MB-231 human breast cancer cells in culture. Thalidomide also shows slightly more anti-proliferative activity against the SH-SY5Y human neuroblastoma and human umbilical vein endothelial cell (HUVEC) types. Furthermore, incubation of thalidomide with human liver microsomes added no additional anti-proliferative activity in these cell types *versus* thalidomide given alone. Finally, we report that none of the putative thalidomide metabolites tested had any anti-proliferative activity against the breast or neuroblastoma cells, but did possess appreciable anti-proliferative activity against the endothelial cells. This implies that endothelial cells are a cellular target for hydroxylated putative metabolites of thalidomide.

There has been a great deal of speculation over the years that thalidomide acts through a reactive epoxide intermediate to exert its teratogenic (and potentially its anti-angiogenic) activity.^{29,33,67} This intermediate is then hydrolyzed to yield

hydroxylation of the phthalimide ring at the 4- and 5-position. These two hydroxylation products were found in the plasma of rabbits but not rats orally fed thalidomide.³⁹ Further, both 5- and 5'-hydroxy analogs of thalidomide were isolated after activated with S9 fraction of human liver cells.⁵⁷ Thus the differential response of rats versus rabbits and humans to thalidomide could be explained by this difference in hydroxylation. Our finding that 5-hydroxy thalidomide, a product of the epoxide intermediate, has an active anti-angiogenic effect in the CAM assay implies that the reactive arene intermediate is not the only active thalidomide metabolite. In addition, hydroxylation of thalidomide at the 4-position led to a weakly active anti-angiogenic compound. Since the mechanism of anti-angiogenic action of thalidomide is unknown, it is hard to ascertain why a simple change of one position on the aromatic ring could cause such extreme changes in activity. This finding does, however, imply a discrete anti-angiogenic target for the drug. The glutarimide ring of thalidomide has been found to be non-enzymatically hydrolyzed at basic pH.^{39,68,69} This hydrolysis abrogates the anti-TNF α anti-inflammatory activity of the drug.⁷⁰ We have found in the CAM assay, as has been previously shown in a corneal angiogenesis model,¹⁹ that the cleavage of the glutarimide ring to form PGA retains anti-angiogenic activity.

It is very interesting that none of the putative metabolites of thalidomide tested showed any anti-proliferative activity against MDA-MB-231 breast or SH-SY5Y neuroblastoma cells. Thalidomide alone did, however, show some weak anti-

proliferative activity in these two tumor cell types. In addition, incubation of thalidomide by human microsomes did not increase anti-proliferative activity against either cell type *versus* thalidomide given alone. Thus it appears that thalidomide itself represents the cytotoxic compound in these cancer cell types. Thalidomide also showed increased anti-proliferative activity against the neuronal-like SH-SY5Y neuroblastoma cells *versus* MDA-MB-231 breast cells. This selectivity may reflect the ability of thalidomide to target neuronal cells to cause peripheral neuropathy, an area we are currently being explored further.

In contrast to the findings in the tumor cells, hydroxylated thalidomide analogs were very active against HUVEC proliferation. This suggests that the target cell type for these putative metabolites are the endothelial cells and not the tumor cells. A general lack of correlation, however, was found between the activity in the CAM assay (Figs. 2, 3) and the HUVEC proliferation data (Figs. 4, 5) of specific hydroxylated analogs. For instance, 4-hydroxy thalidomide was not active in the CAM assay whereas it was one of the most active compounds against HUVEC proliferation (Figs. 3A, 5A, respectively). There are a couple possible explanations for this observation. The first is that the concentrations in the discs placed onto the CAMs ($1\text{--}100\text{ }\mu\text{g}$ per $25\text{ }\mu\text{l}$ = $0.15\text{--}15\text{ mM}$) were much higher than those used in the HUVEC proliferation assays ($10\text{--}333\text{ }\mu\text{M}$). The high concentration in the disk, however, is most likely slowly released onto the CAM and distributed in gradients over the surface of the membrane. This could explain why putative metabolites show more potent activity in the CAM assay *versus* on HUVEC proliferation assay but does not explain why some putative metabolites are more active against the endothelial cells *versus* in the CAM assay. A second explanation of the discrepancy between the CAM and HUVEC data is that the HUVECs grown in monolayers do not represent an adequate system for assessing the anti-angiogenic activity of thalidomide analogs. This disparity could be due to the lack of three-dimensional interactions between the endothelial cells themselves and the endothelial cells and the basement membrane. Cell adhesion molecules are critical to both angiogenesis and tumor growth (reviewed in^{71,72}) and it is clear that the signals produced in cells grown in monolayer culture are quite different from those in a three-dimensional growth environment. A current area of research by our laboratory is exploring the effects of three-dimensional growth conditions, basement membrane composition, stimulation of angiogenesis by tumor cells *versus* purified growth factors, and endothelial cell source on the process of tumor-related angiogenesis.

Our goal was to explore the effect of putative hydroxylated thalidomide metabolites on blood vessel density and target cell proliferation. We have demonstrated that the position of the hydroxyl group on the glutarimide or phthalimide ring of thalidomide has a substantial effect on blood vessel inhibition in the CAM assay. In contrast, putative hydroxylated thalidomide metabolites do not possess anti-proliferative activity against human tumor cells but do cause substantial cytotoxicity against isolated endothelial cells.

Summary This work suggests that active anti-angiogenic metabolites of thalidomide may exist. Targeting the effects of the metabolites at the level of the endothelial cells,

and that exploring analogs of these metabolites as potential anti-angiogenic agents warrant further merit.

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REFERENCES AND NOTES

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- 2) Benjamin L. E., Keshet E., *Proc. Natl. Acad. Sci. U.S.A.*, **94**, 8761—8766 (1997).
- 3) Laird A. D., Vajkoczy P., Shawver L. K., Thurnher A., Liang C., Mohammadi M., Schlessinger J., Ullrich A., Hubbard S. R., Blake R. A., Fong T. A., Strawn L. M., Sun L., Tang C., Hawtin R., Tang F., Shenoy N., Hirth K. P., McMahon G., Cherrington J. M., *Cancer Res.*, **60**, 4152—4160 (2000).
- 4) Fong T. A., Shawver L. K., Sun L., Tang C., App H., Powell T. J., Kim Y. H., Schreck R., Wang X., Risau W., Ullrich A., Hirth K. P., McMahon G., *Cancer Res.*, **59**, 99—106 (1999).
- 5) Panek R. L., Lu G. H., Dahrting T. K., Batley B. L., Connolly C., Hamby J. M., Brown K. J., *J. Pharmacol. Exp. Ther.*, **286**, 569—577 (1998).
- 6) DeVore R. F., Fehrenbacher L., Herbst R. S., Langer C. J., Kelly K. J., Gaudreault Holmgren E., Novotny W. F., *Amer. Assoc. Clin. Oncol.*, Vol. 1896, San Francisco (2000).
- 7) Sledge G., Miller K., Novotny W., Gaudreault J., Ash M., Cobleigh M., *Amer. Assoc. Clin. Oncol.*, pp. #5C-2000, San Francisco (2000).
- 8) Klotz O., Park J. K., Pleyer U., Hartmann C., Baatz H., *Graefes Arch. Clin. Exp. Ophthalmol.*, **238**, 88—93 (2000).
- 9) Mitjans F., Meyer T., Fittschen C., Goodman S., Jonczyk A., Marshall J. F., Reyes G., Piulats J., *Int. J. Cancer*, **87**, 716—723 (2000).
- 10) O'Reilly M. S., Holmgren L., Shing Y., Chen C., Rosenthal R. A., Cao Y., Moses M., Lane W. S., Sage E. H., Folkman J., *Cold Spring Harb. Symp. Quant. Biol.*, **59**, 471—842 (1994).
- 11) Ahmed M. H., Arai T., Konno H., Nahar L., Tanaka T., Izumiyama N., Takubo K., Nakamura S., Baba S., *Jpn. J. Cancer Res.*, **88**, 977—981 (1997).
- 12) Stephens T. D., Bunde C. J., Fillmore B. J., *Biochem. Pharmacol.*, **59**, 1489—1499 (2000).
- 13) Stephens T. D., Fillmore B. J., *Teratology*, **61**, 189—195 (2000).
- 14) Sauer H., Gunther J., Hescheler J., Wartenberg M., *Am. J. Pathol.*, **156**, 151—158 (2000).
- 15) Dixon S. C., Kruger E. A., Bauer K. S., Figg W. D., *Cancer Chemother. Pharmacol.*, **43**, S78—S84 (1999).
- 16) Liu J., Razani B., Tang S., Terman B. I., Ware J. A., Lisanti M. P., *J. Biol. Chem.*, **274**, 15781—15785 (1999).
- 17) Minchinton A. I., Fryer K. H., Wendt K. R., Clow K. A., Hayes M. M., *Anticancer Drugs*, **7**, 339—343 (1996).
- 18) Battagay E. J., *J. Mol. Med.*, **73**, 333—346 (1995).
- 19) Kenyon B. M., Browne F., D'Amato R. J., *Exp. Eye Res.*, **64**, 971—978 (1997).
- 20) Moreira A. L., Friedlander D. R., Shif B., Kaplan G., Zagzag D., *J. Neurooncol.*, **43**, 109—114 (1999).
- 21) Kruse F. E., Joussen A. M., Rohrschneider K., Becker M. D., Volcker H. E., *Graefes Arch. Clin. Exp. Ophthalmol.*, **236**, 461—466 (1998).
- 22) Baidas S. M., Winer E. P., Fleming G. F., Harris L., Pluda J. M., Crawford J. G., Yamauchi H., Isaacs C., Hanfelt J., Tefft M., Flockhart D., Johnson M. D., Hawkins M. J., Lippman M. E., Hayes D. F., *J. Clin. Oncol.*, **18**, 2710—2717 (2000).
- 23) Fine H. A., Figg W. D., Jaecle K., Wen P. Y., Kyritsis A. P., Loeffler J. S., Levin V. A., Black P. M., Kaplan R., Pluda J. M., Yung W. K., *J. Clin. Oncol.*, **18**, 708—715 (2000).
- 24) Eisen T., Boshoff C., Mak I., Sapunar F., Vaughan M. M., Pyle L., Johnston S. R., Ahern R., Smith I. E., Gore M. E., *Br. J. Cancer*, **82**, 812—817 (2000).
- 25) Singhal S., Mehta J., Desikan R., Ayers D., Roberson P., Eddlemon P., Munshi N., Anaissie E., Wilson C., Dhodapkar M., Zeddis J., Barlogie B., *N. Engl. J. Med.*, **341**, 1565—1571 (1999).
- 26) Juliusson G., Celsing F., Turesson I., Lenhoff S., Adriansson M., Malm

- C., *Br. J. Haematol.*, **109**, 89—96 (2000).
- 27) Hideshima T., Chauhan D., Shima Y., Raju N., Davies F. E., Tai Y. T., Treon S. P., Lin B., Schlossman R. L., Richardson P., Muller G., Stirling D. I., Anderson K. C., *Blood*, **96**, 2943—2950 (2000).
 - 28) Pini M., Baraldi A., Pietrasanta D., Allione B., Depaoli L., Salvi F., Levis A., *Haematologica*, **85**, 1111—1112 (2000).
 - 29) Bauer K. S., Dixon S. C., Figg W. D., *Biochem. Pharmacol.*, **55**, 1827—1834 (1998).
 - 30) Gut I., Nerudova J., Kopecky J., Holeccek V., *Arch. Toxicol.*, **33**, 151—161 (1975).
 - 31) Kotoh T., Dhar D. K., Masunaga R., Tabara H., Tachibana M., Kubota H., Kohno H., Nagasue N., *Surgery*, **125**, 536—544 (1999).
 - 32) Nau H., *Environ. Health Perspect.*, **70**, 113—129 (1986).
 - 33) Gordon G. B., Spielberg S. P., Blake D. A., Balasubramanian V., *Proc. Natl. Acad. Sci. U.S.A.*, **78**, 2545—2548 (1981).
 - 34) Lash J. W., Saxen L., *Dev. Biol.*, **28**, 61—70 (1972).
 - 35) Fort D. J., Stover E. L., Bantle J. A., Finch R. A., *Teratog. Carcinog. Mutagen*, **20**, 35—47 (2000).
 - 36) D'Amato R. J., Loughnan M. S., Flynn E., Folkman J., *Proc. Natl. Acad. Sci. U.S.A.*, **91**, 4082—4085 (1994).
 - 37) Smith R. L., Williams R. A., Williams R. T., *Life Sci.*, **7**, 333—336 (1962).
 - 38) Schumacher H. J., Wilson J. G., Terapane J. F., Rosedale S. L., *J. Pharmacol. Exp. Ther.*, **173**, 265—269 (1970).
 - 39) Schumacher H., Smith R. L., Williams R. T., *Br. J. Pharmacol.*, **25**, 338—351 (1968).
 - 40) Schumacher H., Smith R. L., Williams R. T., *Br. J. Pharmacol.*, **25**, 324—337 (1965).
 - 41) Schumacher H., Blake D. A., Gillette J. R., *J. Pharmacol. Exp. Ther.*, **160**, 201—211 (1968).
 - 42) Nicholls P. J., *J. Pharm. Pharmacol.*, **18**, 46—48 (1966).
 - 43) Hanauske-Abel H., Gunzler V., *Z. Naturforsch. [C]*, **32**, 241—248 (1977).
 - 44) Eriksson T., Bjorkman S., Fyge A., Ekberg H., *J. Chromatogr.*, **582**, 211—216 (1992).
 - 45) Meyring M., Chankvetadze B., Blaschke G., *J. Chromatogr. A*, **876**, 157—167 (2000).
 - 46) Shah J. H., Swartz G. M., Papathanassiou A. E., Treston A. M., Fogler W. E., Madsen J. W., Green J. G., *J. Med. Chem.*, **42**, 3014—3017 (1999).
 - 47) Robin S., Zhu J., Galons H., Pham-Huy C., Claude J. R., Tomas A., Viossat B., *Tetrahedron: Asymmetry*, **6**, 1249—1252 (1995).
 - 48) Ryan G., Ades I. Z., *Int. J. Biochem.*, **21**, 1025—1031 (1989).
 - 49) Sheu J. R., Fu C. C., Tsai M. L., Chung W. J., *Anticancer Res.*, **18**, 4435—4441 (1998).
 - 50) Brooks P. C., Montgomery A. M., Cheres D. A., *Methods Mol. Biol.*, **129**, 257—269 (1999).
 - 51) Dezube B. J., Von Roenn J. H., Holden-Wiltse J., Cheung T. W., Remick S. C., Cooley T. P., Moore J., Sommadossi J. P., Shriver S. L., Suckow C. W., Gill P. S., *J. Clin. Oncol.*, **16**, 1444—1449 (1998).
 - 52) Stadler W. M., Kuzel T., Shapiro C., Sosman J., Clark J., Vogelzang N. J., *J. Clin. Oncol.*, **17**, 2541—2545 (1999).
 - 53) Chen T. L., Vogelsang G. B., Petty B. G., Brundrett R. B., Noe D. A., Santos G. W., Colvin O. M., *Drug Metab. Dispos.*, **17**, 402—405 (1989).
 - 54) Noormohamed F. H., Youle M. S., Higgs C. J., Kook K. A., Hawkins D. A., Lant A. F., Thomas S. D., *AIDS Res. Hum. Retroviruses*, **15**, 1047—1052 (1999).
 - 55) Figg W. D., Raju S., Bauer K. S., Tompkins A., Venzon D., Bergan R., Chen A., Hamilton M., Pluda J., Reed E., *J. Pharm. Sci.*, **88**, 121—159 (1999).
 - 56) Zhang Y., Griffith E. C., Sage J., Jacks T., Liu J. O., *Proc. Natl. Acad. Sci. U.S.A.*, **97**, 6427—6432 (2000).
 - 57) Eriksson T., Bjorkman S., Roth B., Bjork H., Hoglund P., *J. Pharm. Pharmacol.*, **50**, 1409—1416 (1998).
 - 58) Teo S. K., Sabourin P. J., O'Brien K., Kook K. A., Thomas S. D., *J. Biochem. Mol. Toxicol.*, **14**, 140—147 (2000).
 - 59) Price J. E., Polyzos A., Zhang R. D., Daniels L. M., *Cancer Res.*, **50**, 717—721 (1990).
 - 60) Garrido T., Riese H., Aracil M., Perez-Aranda A., *Br. J. Cancer*, **71**, 770—775 (1995).
 - 61) Spinelli W., Sonnenfeld K. H., Ishii D. N., *Cancer Res.*, **42**, 5067—5073 (1982).
 - 62) Recio-Pinto E., Lang F. F., Ishii D. N., *Proc. Natl. Acad. Sci. U.S.A.*, **81**, 2562—2566 (1984).
 - 63) Johansson S., *Acta Physiol. Scand.*, **151**, 331—341 (1994).
 - 64) Reuveny E., Narahashi T., *Brain Res.*, **603**, 64—73 (1993).
 - 65) Ehrlich M., *Clin. Exp. Pharmacol. Physiol.*, **22**, 291—292 (1995).
 - 66) Kolsch H., Ludwig M., Lutjohann D., Prange W., Rao M. L., *Neurochem. Int.*, **36**, 507—512 (2000).
 - 67) Braun A. G., Weinreb S. L., *Biochem. Pharmacol.*, **33**, 1471—1477 (1984).
 - 68) Tanaka A., Hasegawa A., Urakubo G., *Chem. Pharm. Bull.*, **13**, 1263—1265 (1965).
 - 69) Fabro S., Schumacher H., Smith R. L., Stagg R. B., Williams R. T., *Br. J. Pharmacol.*, **25**, 352—362 (1965).
 - 70) Shannon E. J., Sandoval F., Krahenbuhl J. L., *Immunopharmacology*, **36**, 9—15 (1997).
 - 71) Mizejewski G. J., *Proc. Soc. Exp. Biol. Med.*, **222**, 124—138 (1999).
 - 72) Hynes R. O., Bader B. L., Hodivala-Dilke K., *Braz. J. Med. Biol. Res.*, **32**, 501—510 (1999).