Synthesis and Enantiomeric Separation of 2-Phthalimidino-glutaric Acid Analogues: Potent Inhibitors of Tumor Metastasis

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Thalidomide is the common name of *N*-phthalimidoglutarimide, a molecule known to possess a wide variety of properties, including teratogenicity, reduction of TNF-α production, suppression of bFGF-induced angiogenesis, and inhibition of tumor metastasis.[1−5] Although extensive studies exist regarding the structure−activity relationship of the teratogenic and TNF-α suppressive abilities of thalidomide, very little is known about the structural elements of the drug required for inhibition of tumor metastasis.[5−7] In the present study, an attempt is made to identify the intricate components of the thalidomide structure responsible for its antimetastatic effects.

Thalidomide (**1**) is composed of two distinct moieties: the phthalimide and glutarimide rings. Previous reports have shown that hydrolysis of glutarimide group abolishes the ability of thalidomide to suppress TNF-α,[8,9] while its existence enhances teratogenicity.[6] To assess how important the presence of an intact glutarimide ring is for antitumor activity, 2-phthalimidino-glutaric acid (**5c**) was synthesized[6] and its biological activity was assessed in the B16BL6 melanoma experimental metastatic model.[10,11] In addition to a completely hydrolyzed glutarimide moiety, **5c** possesses a partially reduced phthalimide ring. Under physiological conditions, phthalimidino groups are less susceptible to hydrolysis than phthalimides and **5c** is expected to have a higher in vivo $t_{1/2}$ than **1**. If hydrolysis of the phthalimide moiety leads to inactivation of thalidomide, then **5c** is also expected to be more active than **1**. Several studies have suggested that thalidomide is a prodrug and hepatic hydroxylation is essential for activities such as teratogenicity or inhibition of angiogenesis.[14,15] In order to investigate the likelihood that metabolic hydroxylation is required for antitumor activity, derivatives of **5c** (**5a**,**b**) that underwent aromatic hydroxylation on the benzene ring of the phthalimidino group were prepared and assessed.

Another interesting characteristic of the thalidomide structure is the presence of a chiral carbon. Thus far, the majority of the reported structure−activity studies were performed with racemic mixtures of thalidomide, although some efforts were made to associate a particular enantiomer with a specific activity. These efforts produced conflicting data, partially due to the rapid racemization in the plasma of enantiomerically pure preparations of the drug.[7,10] To address this concern, (*R*)- and (*S*)-2-methyl-2-phthalimidino-glutaric acids (**10-*R***, **10-*S***), derivatives of 2-phthalimidino-glutaric acid that are unable to undergo in vivo racemization, were synthesized and tested for antimetastatic activity.

Chemistry. Analogues **5a**,**b**,**c** were prepared according to a synthetic method depicted in Scheme 1. Compounds **2a**,**b** were synthesized by treating 3′- and 4′-hydroxyphthalic acids with acetic anhydride. A coupling reaction between (DL) glutamic acid (**1**) and anhydride **2a**,**b** in refluxing pyridine, followed by treatment with acetic anhydride, gave, respectively, substituted *N*-phthaloyl-DL-glutamic anhydride **3a**,**b**. *N*-Phthaloyl-DL-glutamic anhydride (**3c**) was purchased from a commercial source. Acidic hydrolysis of **3a**−**c** followed by partial reduction of the *N*-phthaloyl ring by Zn dust in glacial acetic acid gave desired products **5a**−**c** in 40−60% yields.

Synthesis of **10-*R*** and **10-*S*** is shown in Schemes 2 and 3. A racemic mixture of 2-methyl-2-phthalimidinoglutaric acid (**7**) was prepared after reaction of phthalic anhydride with DL-2-methylglutamic acid in refluxing toluene over a Dean−Stark apparatus. Partial reduction of the *N*-phthaloyl ring of the resulting 2-methyl-2-phthalimidoglutaric acid (**6**) produced **7** in 60% yield.

Dimethyl ester of DL-2-methyl-2-phthalimidinoglutaric acid (**8**) is prepared with subsequent methylation of **7**. The ChiroCLEC-BL catalyst specifically hydrolyzed one methyl ester of the *S*-enantiomer of **8** to give acid/ester **9-*S*(−)** in 95% ee, leaving the *R*-enantiomer intact as diester **9-*R*(+)**. Acid/ester **9-*S*(−)** was separated from diester **9-*R*(+)** by silica gel flash column chromatography. $CHCl_3$/MeOH 95:5 mixture was used as elution solvent. Subsequently, the two products were hydrolyzed by an AcOH/HCl mixture to give **10-*S*** ($[\alpha]^{25}_D$ (c = 1, methanol) = −46.0°) and **10-*R*** ($[\alpha]^{25}_D$ (c = 1, methanol) = +46.0°) pure enantiomers in 95% ee as evaluated by chiral HPLC (Figure 1). Absolute configuration was determined by comparison of specific rotation $[\alpha]^{25}_D$ of compound **10-*R*** and **10-*S*** to the analogous compounds *R*(+)- and *S*(−)-*N*(2′,6′-dioxopiperiden-3-yl)-phthalimidine, which has been previously determined on the resolved enantiomers.[16]

The two enantiomers of DL-2-methyl-2-phthalimidinoglutaric acid (**7**) were resolved by chiral HPLC column Welk-01 (10 mm × 750 mm) and eluted with CH_3CN/MeOH/H_2O/HOAC 1:1:5:0.1 mixture. The retention time for the *S*(−)-isomer was 30.46 min and for the *R*(+)-isomer, 31.76 min, respectively, at a flow rate of 2 mL/min at 230 nm (Figure 1).

Biology. Thalidomide, 2-phthalimidinoglutaric acid (PGA) analogues **5a**,**b**,**c**, racemic mixture **7**, and enantiomers **10-*R*** and **10-*S*** were tested for their ability to inhibit B16BL6 pulmonary experimental metastasis according to the following protocol.[12,13] B16BL6 melanoma cells (5×10^4) were injected intravenously into the tail veins of C57B1/6 mice. Treatment was initiated 3 days later. Groups of five mice received daily intraperitoneal injections of various concentrations of thali-

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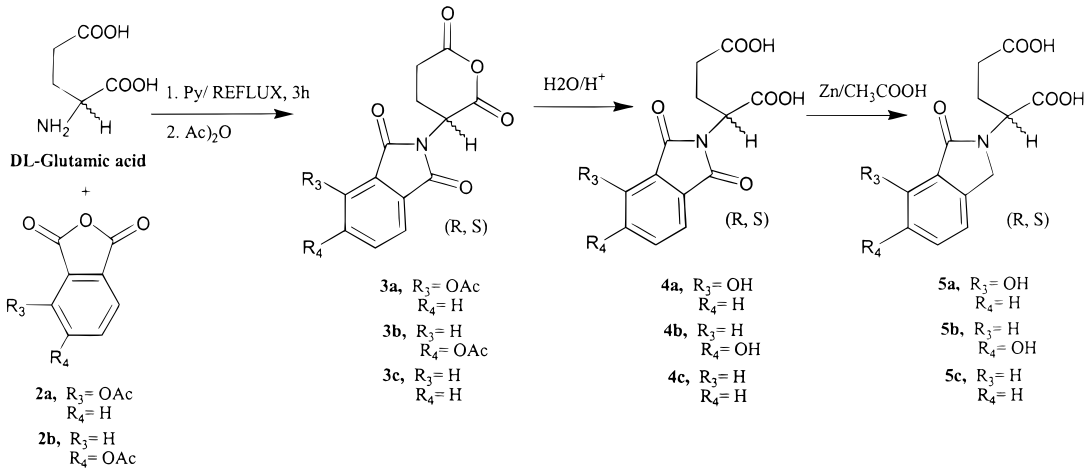
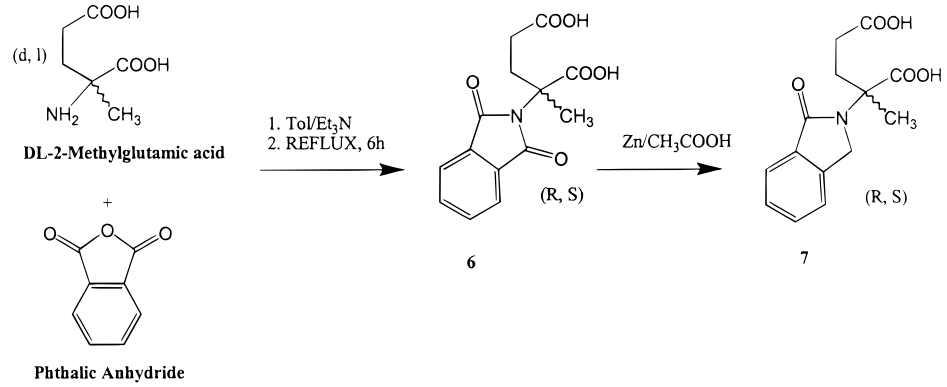
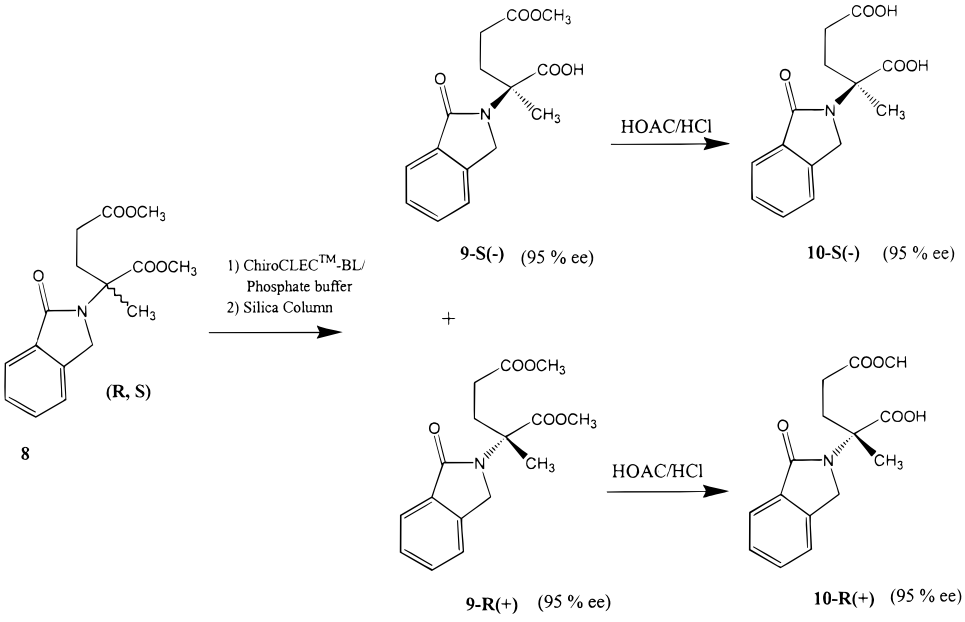
Scheme 1



Scheme 2



Scheme 3



domide, PGA analogues, or vehicle. Fourteen days after tumor cell inoculation, the animals were sacrificed, the lungs were removed, and the surface pulmonary metastases were counted. Activity is expressed as T/C (the ratio of number of lung surface metastases of treated (T) vs vehicle-treated control (C) animals). Generally the vehicle-treated control animals have 100−200 metastases visible. Dose−response curves for thalidomide and individual analogues are presented in Figure 2. Table 1 summarizes activity for each compound at a dose of 0.4 mmol/kg of thalidomide or 2-phthalimidinoglutaric acid analogues.

At 0.4 mmol/kg dose, administration of **5c** acid led to 80% reduction of pulmonary metastases, while thalidomide exhibited minimal inhibition. Significant inhibition of metastasis (>30%) by thalidomide was seen only at

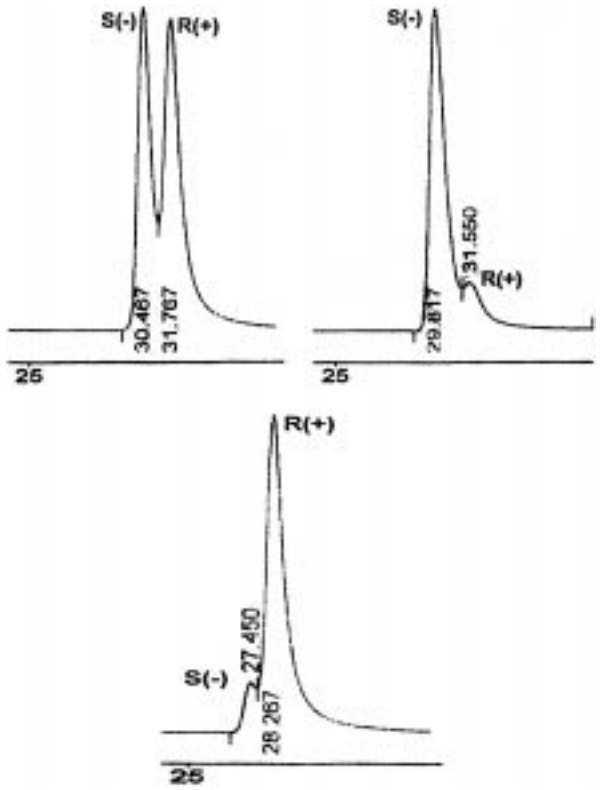


Figure 1. . Chiral resolution of 2-methyl-2-phthalimidino-glutaric acid (**8**) by chiral HPLC column Welk-01 (10 mm × 750 mm) eluted with a $CH_3CN/MeOH/H_2O/HOAC$ 1:1:5:0.1 mixture at a flow rate of 2 mL/min at 230 nm.

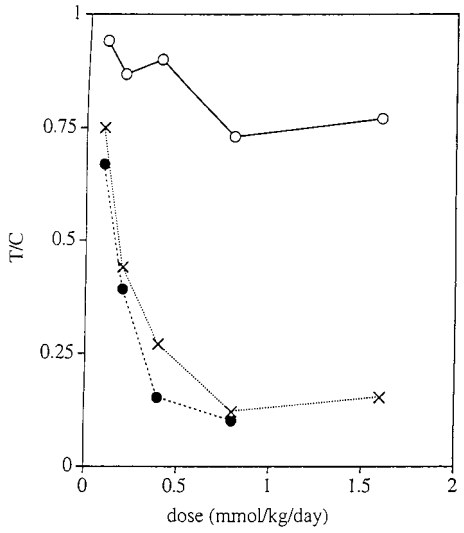
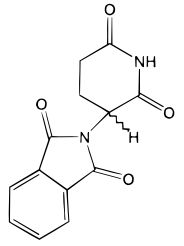


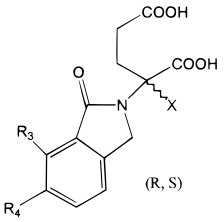
Figure 2. Dose−response of thalidomide, PGA, and *S*-(−)-α-methyl-PGA on inhibition of melanoma metastases. Mice were treated i.p. with vehicle or with the indicated doses of thalidomide (open circles), PGA (**5c**, crosses), or *S*-(−)-α-methyl-PGA (**10a**, filled circles) for 11 days, and the T/C ratio was determined as described in the Biology section. Values shown are the mean of five animals per group, and the standard deviation for each group was less than 20%.

concentrations equal to or higher than 3 mmol/kg (data not shown). This suggests that **5c** is at least 7 times more potent than thalidomide. In contrast, no appreciable antimetastatic effect was observed after administration of either 0.4 or 3 mmol/kg of 2-phthalimidino-glutaric acid analogues **5a**,**b**. Compound **7** exhibited the same inhibitory ability as **5c**. Enantiomeric separation of **7** gave rise to a relatively inactive (**10-*R***) and a potent (**10-*S***) antimetastatic.

Table 1. Structure−Activity Relationship of Thalidomide and 2-Phthalimidino-glutaric Acid Analogues[a]



Thalidomide (**1**)



2-Phthalimidinoglutaric Acid Analogs

compd	X	R_3	R_4	T/C at 0.4 mmol/kg
1				0.90
5a	H	OH	H	0.95
5b	H	H	OH	0.90
5c	H	H	H	0.20
7	CH_3	H	H	0.25
10-*R*(+)	CH_3	H	H	0.70
10-*S*(−)	CH_3	H	H	0.15

[a] Substitution for 2-phthalimidino-glutaric acid analogues is indicated in the table. The number of lung metastases of treated (T) and nontreated or control (C) is expressed as a ratio, T/C. Details of the ratio are described in the Experimental Methods (Supporting Information).

Results and Discussion. Thalidomide (Thalomid) has recently been approved by the U.S. FDA for use in treatment of Hansen's disease (leprosy) and is currently under clinical trial in several centers as an anticancer agent. Here we have identified the structural components of thalidomide which are responsible for its anticancer effects and designed analogues, which have increased potency as antitumor agents.

A major problem for research on thalidomide is that its mechanism of action in cancer is not known. Thalidomide is known to reduce secretion of TNF-α from activated monocytes, and this effect is the basis for its use in Hansen's disease and treatment of skin lesions associated with Kaposi's sarcoma. However, several lines of evidence indicate that this mechanism is not related to the anticancer effect of thalidomide.[21] Previous studies have clearly shown that the oral dose of thalidomide required for antiangiogenic activity is significantly higher than that known to reduce TNF-α levels.[3,4] Furthermore, the TNF-α-lowering ability of thalidomide requires the glutarimide group or another lipophilic group in place of the glutarimide ring,[17,7] whereas data presented here and by others demonstrate that the antiangiogenic effects remain after this moiety is disrupted.[20,21,7] Recent studies also indicated a poor correlation between TNF-α activity and antitumor activity.[21] Although there are many mechanisms described for thalidomide including inhibition of integrin expression, inhibition of IL-12 production, stimulation of IL-2 synthesis, inhibition of IgM synthesis, and increase in gap junction intracellular communication,[22] none of these mechanisms have been shown to be relevant to the antitumor activity of thalidomide. Due to the lack of a valid in vitro assay to assess thalidomide's activity, we chose an in vivo tumor assay for analysis of the analogues presented here. The B16BL6 metastasis model covers several aspects of tumor growth (adhesion, invasion, seeding, proliferation, and angio-

genesis) and thus is an ideal assay for identifying antimetastatic agents.[19]

These structure−activity relationship studies on thalidomide's ability to inhibit B16BL6 metastasis revealed that the glutarimide moiety of thalidomide is not essential for antimetastatic activity, since a completely hydrolyzed glutarimide group in **5c**, **7**, **10a**, and **10b** does not render the molecule inactive. Conversely, data in this paper suggest that the antimetastatic action of thalidomide resides in the phthalimide group. Partial reduction of the phthalimide moiety results in a thalidomide analogue with enhanced activity compared to thalidomide, presumably by increasing the in vivo $t_{1/2}$ of the drug by removing one of the known sites of in vivo hydrolysis. Also, aromatic substitution (hydroxylation) on phthalimide gives rise to inactive derivatives. Whether this is due to changing the spatial or electronic requirements for binding to the cellular target or whether this is due to increased secretion following sulfate or glucuronidation is not known. This will be assessed in future studies by synthesis of ethers of **5a** and **5b**. The data indicate that hepatic hydroxylation of thalidomide is not required for inhibition of metastasis, as was at one time proposed.[18]

Previous reports on enantioselectivity of thalidomide and analogues such as *N*-(2′,6′-dioxopiperiden-3′-yl)-phthalimidine are questionable, since preparations of unstable enantiomers, e.g., nonmethylated chiral carbon, racemized in vitro and in vivo.[14] Heger et al. suggested that preparations of derivatives locked in each optical configuration would be required to confirm selective effects of optical isomers of thalidomide analogues.[16] We report here the preparation and purification of **10a** and **10b**, which are the stabilized enantiomers of **5c**. As the activity of racemic **5c** is indistinguishable from that of racemic **7**, the presence of the methyl group does not affect the antitumor activity, enabling us to ignore the alkyl group's contributions to lipophilicity, volume, and access to and binding to the cellular target. The clear difference between the activities of **10a** and **10b** indicates that the cellular target of this series of molecules is enantioselective and prefers the *S*(−)-enantiomer.

Collectively, this is the first study on the structural requirements for antimetastatic activity of thalidomide and thalidomide analogues. Further, this report clearly shows that a specific property of these analogues is enantioselective. This property, as well as the increased stability of the PGA series of analogues compared to the parent, thalidomide, may also be helpful in identifying the cellular target of these molecules, and so defining the mechanism of the anticancer action of thalidomide.

Supporting Information Available: Experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Cahen, R. L. Evaluation of the teratogenicity of drugs. *Clin. Pharmacol. Ther.* **1964**, *5*, 480−514.
(2) Bazzoli, A. S.; Manson, J.; Scott, W. J.; Wilson, J. G. The effects of thalidomide and two analogues on the regenerating forelimb of the newt. *J. Embryol. Exp. Morph.* **1977**, *41*, 125−135.
(3) Sampaio, E. P.; Sarno, E. N.; Gallily, R.; Cohn, Z. A.; Kaplan, G. Thalidomide selectively inhibits tumor necrosis factor α production by stimulated human monocytes. *J. Exp. Med.* **1991**, *173*, 699−703.
(4) D'Amato, R. J.; Loughnan, M. S.; Flynn, E.; Folkman, J. Thalidomide is an inhibitor of angiogenesis. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 4082−4085.
(5) Minchinton, A. I.; Fryer, K. H.; Wendt, K. R.; Clow, K. A.; Hayes, M. M. The effect of thalidomide on experimental tumors and metastases. *Anti-Cancer Drugs* **1996**, *7*, 339−343.
(6) Helm, F.-Ch.; Frankus, E.; Graudums, I.; Flohe, L. Comparative teratological investigation of compounds structurally and pharmacologically related to thalidomide. *Drugs Res.* **1981**, *31*, 941−949.
(7) Miyachi, H.; Azuma, A.; Ogasawara, A.; Uchimura, E.; Watanabe, N.; Kobayashi, Y.; Kato, F.; Kato, M.; Hashimoto, Y. Novel biological response modifiers: Phthalimides with tumor necrosis factor-α production-regulating activity. *J. Med. Chem.* **1997**, *40* (18), 2858−2865.
(8) Corral, L. G.; Kaplan, G.; Stirling, D.; Wu, M.; Chen, Y.; Moriera, A. L.; Muller, G. W. Selection of novel analogs of thalidomide with enhanced tumor necrosis factor-α inhibitory activity. *Mol. Med.* **1996**, *2* (4), 506−515.
(9) Shannon, E. J.; Sandoval, F.; Krahenbuhl, J. L. Hydrolysis of thalidomide abrogates its ability to enhance mononuclear cell synthesis of IL-2 as well as its ability to suppress the synthesis of TNF-α. *Immunopharmacology* **1996**, 990.
(10) Nicolson, G. L.; Brunson, K. W.; Fidler, I. J. Specificity of arrest, survival, and growth of selected metastatic variant cell lines. *Cancer Res.* **1978**, *38*, 4105−4111.
(11) Hart, I. R. The selection and characterization of an invasive variant of the B16 melanoma. *Am. J. Pathol.* **1979**, *97*, 587−600.
(12) Braun, A. G.; Weinreb, S. L. Teratogen metabolism: spontaneous decay products of thalidomide and thalidomide analogues are not bioactivated by liver microsomes. *Teratog. Carcinog. Mutagen.* **1985**, *5*, 149−158.
(13) Bauer, K. S.; Dixon, S. C.; Figg, W. D. Inhibition of angiogenesis by thalidomide requires metabolic activation, which is species-dependent. *Biochem. Pharmacol.* **1998**, *55* (11), 1827−1834.
(14) Schmahl, H.-J.; Heger, W.; Nau, H. The enantiomers of the teratogenic analog EM12. *Toxicol. Lett.* **1989**, *45*, 23−33.
(15) Kenyon, B. M.; Browne, F.; D'Amato, R. J. Effects of thalidomide and related metabolites in a mouse cornea model of neovascularization. *Exp. Eye Res.* **1997**, *64* (6), 971−978.
(16) Heger, W.; Schmahl, H. J.; Klug, S.; Felies, A.; Nau, H.; Merker, H. J.; Neubert, D. Embryotoxic effects of thalidomide derivatives in the non-human primate callithrix jacchus. IV. Teratogenicity of micrograms/kg doses of the EM12 enantiomers. *Teratog. Carcinog. Mutagen.* **1994**, *14*, 115−122.
(17) Muller, G. W.; Corral, L. G.; Shire, M. G.; Wang, H.; Moreira, A.; Kaplan, G.; Stirling, D. I. Structural modifications of thalidomide produce analogs with enhanced tumor necrosis factor inhibitory activity. *J. Med. Chem.* **1996**, *39*, 3238−3240.
(18) Gordon, G. B.; Spielberg, S. P.; Blake, D. A.; Balassubramanian, V. Thalidomide teratogenesis: evidence for a toxic arene oxide metabolite. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 2545−2548.
(19) Post, G.; Doll, J.; Hart, I. R.; Fidler, I. J. In vitro selection of murine B16 melanoma varients with enhanced tissue-invasive properties. *Cancer Res.* **1980**, *40*, 1636−44.
(20) Kenyon, B. M.; Browne, F.; D'Amato, R. J. Effects of thalidomide and related metabolites in a mouse corneal model of neovascularization. *Exp. Eye Res.* **1997**, *64*, 971−978.
(21) Shimazawa, R.; Miyachi, H.; Takayama, H.; Kuroda, K.; Kato, F.; Kato, M.; Hashimoto, Y. Antiangiogenic activity of tumor necrosis factor-a production regulators derived from thalidomide. *Biol. Pharm. Bull.* **1999**, *22*, 224−226.
(22) Stirling, D.; Sherman, M.; Strauss, S. Thlidomide. A surprising recovery. *J. Am. Pharm. Assoc.* **1997**, *37*, 306−13.

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