Structural Modifications of Thalidomide Produce Analogs with Enhanced Tumor Necrosis Factor Inhibitory Activity[1]

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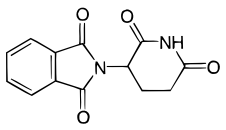
Introduction. Thalidomide (**1**) was developed initially as a "safe" sedative without the toxicity and addictive potential of barbiturates. The molecule, a synthetic derivative of glutamic acid, consists of a glutarimide ring and a phthaloyl ring. At physiologic pH, hydrolysis of thalidomide occurs at both the phthalimido and glutarimide rings.[2] It has therefore been postulated that thalidomide may act as a prodrug for one of its hydrolysis products or metabolites.



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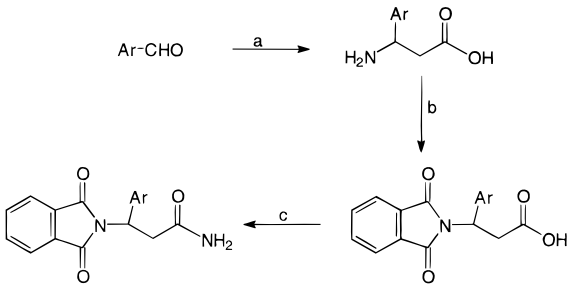
After a few years of use as a sedative, thalidomide was withdrawn from the market when its potent and tragic teratogenic properties became apparent.[3] However, during this time, it was noted that thalidomide was remarkably effective for the treatment of erythema nodusum leprosum (ENL), an acute inflammatory manifestation of lepromatous leprosy.[4,5] More recently, thalidomide was found to exert immunomodulatory and anti-inflammatory effects in a variety of disease states. These include graft-versus-host disease following bone marrow transplantation, rheumatoid arthritis, inflammatory bowel disease (IBD), cachexia in AIDS, and opportunic infections in AIDS.[6] In studies to define the physiological targets of thalidomide, the drug was found to have a wide variety of biological activities exclusive of its sedative effect including neurotoxicity,[7] teratogenicity,[8] suppression of tumor necrosis factor-α (TNFα) production by monocytes/macrophages[9] and the accompanying inflammatory toxicities associated with high levels of TNFα,[10] and inhibition of angiogenesis and neovascularization.[11] It is as yet unclear what the mechanism of action of the drug is and whether all the biological activities are mediated through the same pathway.

Because of the demonstrated effect of thalidomide on the production of TNFα, and the central role that TNFα plays in the immune response and the inflammatory cascade, we decided to focus on improving the TNFα-inhibiting properties of thalidomide by structure modification. We have designed and synthesized analogs of thalidomide optimized for their ability to control TNFα synthesis. Initial studies demonstrated the importance of an intact phthaloyl ring. Hydrolysis of the glutarimide ring affords either *N*-phthaloylglutamine or *N*-phthaloylisoglutamine. Analogs were therefore prepared based on the hydrolysis of the glutarimide ring of thalidomide.

Scheme 1[a]



[a] Reagents: (a) NH_4OAc, $CH_2(CO_2H)$, EtOH, reflux; (b) *N*-carbethoxyphthalimide, Na_2CO_3, CH_3CN/H_2O; (c) (1) CDI/THF, (2) concentrated NH_4OH.

Chemistry. Phthalimido β-amino amide derivatives were prepared as shown in Scheme 1. The β-amino β-aryl acids were prepared as previously described[12] by the treatment of aryl aldehydes with ammonium acetate and malonic acid in refluxing ethanol. The *N*-phthaloyl group was introduced by treating the β-amino acid with *N*-carbethoxyphthalimide in the presence of sodium carbonate.[13] Phthalimido amides were prepared by CDI activation of the carboxylic acid in THF at ambient temperature followed by treatment with excess ammonium hydroxide. The *N*-methyl amide derivative was prepared by a similar procedure using aqueous methylamine in place of ammonium hydroxide. Ester derivative **21** was prepared by direct condensation of methyl 3-amino-3-(3′,4′-dimethoxyphenyl)propionate hydrochloride with *N*-carbethoxyphthalimide in the presence of sodium carbonate. Substituted phthalimido groups were introduced by condensation of a substituted phthalic anhydride with methyl 3-amino-3-(3′,4′-dimethoxyphenyl)propionate hydrochloride in acetic acid or in acetic acid in the presence of sodium acetate. The aminophthaloyl-substituted compounds **23** and **25** were prepared by hydrogenation of the corresponding nitro compounds.

The enantiomers of methyl 3-amino-3-(3′,4′-dimethoxyphenyl)propionate were prepared by the method of Davies.[14] The phthaloyl group was then introduced as described above. Chiral purity was determined by chiral HPLC analysis on a Daicel Crown-Pak R(+) column, and both isomers were found to be optically pure (>95% ee).

Results and Discussion. Inhibition of TNFα was measured in the supernatant of human PBMCs stimulated with LPS.[15] In this assay, thalidomide has an IC_{50} of 194 μM for inhibition of TNFα synthesis with a dose−response curve which maximizes at 60−70% inhibition of TNFα synthesis.[16] Simple phthalimidoalkyl amides which might mimic glutarimide hydrolysis products of thalidomide were evaluated for their ability to inhibit TNFα. Phthalimidoalkyl amides (**2**−**4**) derived from glycine, β-alanine, and γ-aminobutyric acid, respectively, were prepared and found to have minimal TNFα inhibition activity (0% inhibition at 980 μM, 22% inhibition at 458 μM, and 23% inhibition at 403 μM, respectively). In exploring the effects of substitution of simple phthalimidoalkyl amides, the phthalimido amide of 3-phenylpropionic acid, **5**, was prepared and found to be nearly equipotent to thalidomide (Table 1).

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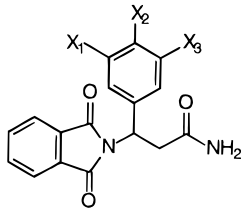
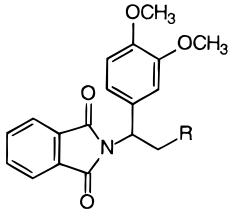
Table 1. TNFα Inhibition by *N*-Phthaloyl β-Amino β-Aryl Amides



compd	X_1	X_2	X_3	IC_{50} (μM) TNFα[a]
5	H	H	H	260
6	H	H	CN	89
7	H	CN	H	150
8	H	H	OCH_3	120
9	H	OCH_3	H	62
10	H	OCH_3	OCH_3	12
11	H	OEt	OEt	5.6
12	H	OPr	OPr	55
13	H	Cl	Cl	70
14	H	Ph		51
15	OCH_3	H	OCH_3	39

[a] IC_{50} for TNFα inhibition in LPS-stimulated human PBMCs.

Table 2. TNFα Inhibition by Amide Isosteres



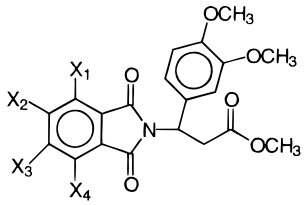
compd	R	IC_{50} (μM) TNFα[a]
16	$CONHCH_3$	12
17	$CONHCH_2CH_3$	53
18	CONHBn	84
19	CO_2H	60
20	CH_2OH	7.5
21	CO_2CH_3	2.9

[a] IC_{50} for TNFα inhibition in LPS-stimulated PBMCs.

The activity of **5** was optimized by exploring substitution patterns on the 3-phenyl ring. Substitution with an electron-withdrawing group or an electron-donating group in the meta or para position resulted in increased activity (Table 1). Substitution with a 3-cyano group (**6**) or a 4-cyano group (**7**) afforded compounds which are more active than thalidomide. Substitution with an electron-donating group such as 3-methoxy (**8**) or 4-methoxy (**9**) also afforded increases in activity. Thus, substitution effects appear to be mediated by steric effects. Disubstitution as illustrated by the 3,4-dimethoxy analog **10** afforded a synergistic effect with a compound 15-fold more potent than thalidomide. The 3,5-dimethoxyphenyl analog **15** was 3 times less active than **10**. Homologs of 3,4-dimethoxy substitution of **10** were prepared and evalutated. Substituents larger than diethoxy had decreased activity. The diethoxy analog **11** was found to be 2 times as active as **10**. Unlike thalidomide, these compounds can inhibit 100% of the TNFα formed by LPS stimulation.

Phthalimido amides such as **10** were initially explored because of their relationship to thalidomide hydrolysis products. Isosteric replacement of the amide moiety revealed that the amide moiety was not optimal (Table 2). Compound **10** was chosen as the parent compound because of its high activity. Substituted amides larger than the *N*-methyl analog **16** resulted in decreased activity. The free carboxylic acid **19** was found to be 5-fold less active. The primary alcohol **20** afforded a slight increase in activity. Replacement of the amide moiety with a carboxymethyl group (compound **21**) produced a near 5-fold increase in activity.

Table 3. TNFα Inhibition by Ring-Substituted Phthaloyl Analogs



compd	X_1	X_2	X_3	X_4	IC_{50} (μM) TNFα[a]
22	H	H	NO_2	H	34
23	H	H	NH_2	H	0.38
24	H	H	H	NO_2	64
25	H	H	H	NH_2	0.45
26	H	H	H	OH	15
27	H	Ph		H	4.7
28	H	Cl	Cl	H	13
29	Cl	H	H	Cl	7.9
30	H	H	tBu	H	4.2

[a] IC_{50} for TNFα inhibition in LPS-stimulated human PBMCs.

To improve the activity of **21,** the effect of substitution of the aromatic ring of the phthaloyl ring was explored (Table 3). Dihalo substitution of the aromatic ring (**28** and **29**) decreased activity. Alkyl substitution had only a minor effect. Nitro group substitution in the 3- or 4-position decreased activity by more than 1 order of magnitude. However, 3- or 4-amino substitution (**23** and **25**, respectively) yielded compounds with submicromolar IC_{50}'s.

All the analogs described above were prepared and tested as racemic mixtures. Thalidomide has always been used clinically as a racemic mixture. The *S*-isomer is generally considered to be teratogenic with the *R*-isomer being a nonteratogenic sedative; however, this view is controversial.[17] Eriksson and co-workers recently reported that both isomers of thalidomide are rapidly racemized in plasma and *in vivo*.[18] This report suggests that even if only one isomer were teratogenic, there would be no difference in teratogenicity between the isomers *in vivo*. We prepared both isomers of thalidomide by a known procedure[19] and evaluated them for TNFα inhibition. Dose−response curves for the racemate and single isomers were similar (Figure 1) showing a very flat dose−response curve. The *R*-isomer **32** (IC_{50} = 3.1 μM) and *S*-isomer **33** (IC_{50} = 4.5 μM) of **21** were found to have TNFα synthesis inhibition activity similar to that of the racemate. This series of compounds, unlike thalidomide, do not have the acidic chiral hydrogen and would be expected to be chirally stable; thus differences in teratogenicity between the isomers could be of significance *in vivo*.

Conclusions. Using thalidomide as a lead structure, we have designed a new series of drugs which inhibit TNFα production to varying degrees in LPS-stimulated human PBMCs. Replacement of the amino glutarimide portion of thalidomide with β-amino β-aryl amino acid derivatives and substitution of the phthaloyl ring have resulted in analogs having TNFα inhibition potencies approaching 500 times that found for thalidomide.

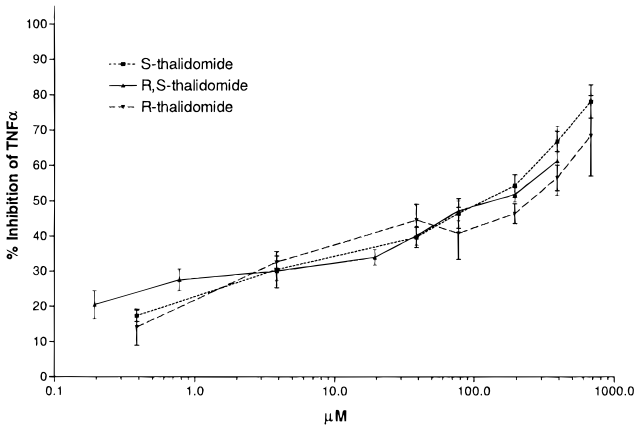


Figure 1. Inhibition of LPS-induced TNFα in LPS-stimulated human PBMC (error bars represent ±SEM).

Unlike thalidomide, analogs in this series inhibit 100% of the TNFα synthesis in LPS-stimulated PBMCs. The difference in dose−response curves is suggestive of a possible change in mechanism of action which is being investigated. Other studies are in progress to (a) examine the effect of these analogs on leukocyte cytokine production and their associated toxicities, (b) further the understanding of the mechanism of action of these analogs,[20] and (c) expand the structure−activity relationship. The toxicology of compounds in this series is currently being evaluated.

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References

(1) Muller, G.; Corral, L.; Shire, M.; Kaplan, G.; Stirling, D. Novel inhibitors of tumor necrosis factor-α. Presented in part at the 210th American Chemical Society National Meeting, Chicago, IL, Fall 1995; Med. Chem., Abstract 84.

(2) (a) Schumacher, H.; Smith, R. L.; Williams, R. T. The metabolism of thalidomide: the spontaneous hydrolysis of thalidomide in solution. *Brit. J. Pharmacol.* **1965**, *25*, 324. (b) Georgopoulos, A.; Koch, H. P.; Czejka, M. J. In-vitro kinetics and metabolism of α-phthalimido-glutarimide. *Int. J. Exp. Clin. Chemother.* **1990**, *3* (3), 135−141.

(3) D'Arcy, P. F.; Griffin, J. P. Thalidomide Revisited. *Adverse Drug React. Toxicol. Rev.* **1994**, *13* (2), 65−76.

(4) Sheskin, J. Thalidomide in the treatment of lepra reactions. *Clin. Pharmacol. Ther.* **1965**, *6*, 303.

(5) (a) Hendler, S. S.; McCarthy, M. F. Thalidomide for Autoimmune Disease. *Med. Hypotheses* **1983**, *10*, 437−443. (b) Crawford, C. L. Use of Thalidomide in Leprosy. *Adverse Drug React. Toxicol. Rev.* **1994**, *13* (4), 177−192.

(6) (a) Gutierrez-Rodriguez, O. Thalidomide, A Promising New Treatment for Rheumatoid Arthritis. *Arthritis Rheum.* **1984**, *27* (10), 1118−1121. (b) Peterson, D. L.; Georghiou, P. R.; Allworth, A. M.; Kemp, R. J. Thalidomide as Treatment of Refactory Apthous Ulceration Related to Human Immunodeficiency Virus. *Infection Clin. Infect. Dis.* **1995**, *20*, 250−254. (c) Schuler, U.; Ehninger, G. Thalidomide: Rationale for Renewed Use in Immunological Disorders. *Drug Safety* **1995**, *12* (6), 364−369. (d) Vogelsang, G. B.; Hess, A. D.; Gordon, G.; Brundrette, R.; Santos, G. W. Thalidomide Induction of Bone Marrow Transplantation Tolerance. *Trans. Proc.* **1987**, *XIX* (1), 2658−2661. (e) Klausner, J.; Makonkawkeyoon, S.; Akarasewi, P.; Nakata, K.; Kasinrerk, W.; Corral, L.; Dewar, R.; Lane, C.; Freedman, V.; Kaplan, G. The Effect of Thalidomide on the Pathogenesis of Human Immunodeficiency Virus Type 1 and M. tuberculosis Infection. *J. Acquired Immune Defic. Syndr.* **1996**, *11*, 247−257.

(7) Crawford, C. L. Thalidomide Neuropathy. *Lepr. Rev.* **1969**, *40*, 126−128.

(8) (a) Smithells, R. W.; Newman, C. G. H. Recognition of thalidomide defects. *J. Med. Genet.* **1992**, *29*, 716−723.

(9) Sampaio, E. P.; Sarno, E. N.; Galilly, 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.

(10) (a) Koch, H. P. Thalidomide and Congeners as Anti-inflammatory Agents. *Prog. Med. Chem.* **1985**, *22*, 166−242. (b) Gunzler, V. Thalidomide- A Therapy for the Immunological Consequences of HIV Infection? *Med. Hypotheses* **1989**, *30*, 105−109.

(11) 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.

(12) (a) Kalvin, D.; Woodard, R. Synthesis of (4R)-D,L-[4-^{2}H]- and (4S)-D,L-[4-^{2}H]Homoserine Lactones. *J. Org. Chem.* **1985**, *50*, 2259−2263. (b) Madigan, D.; Muller, G.; Walters, D.; Culberson, J.; DuBois, G.; Carter, J.; Nagarajan, S.; Klix, R.; Ager, D.; Klade, C. Eur. Patent Appl. 344819 A1, Aug. 23, 1989.

(13) Shealy, Y. F.; Opliger, C. E.; Montgomery, J. A. Synthesis of D- and L- Thalidomide and Related Studies. *J. Pharm. Sci.* **1968**, *57*, 757−764.

(14) Davies, S. G.; Ichihar, O. Asymmetric Synthesis of R-β-Amino butanoic acid and S-β-Tyrosine: Homochiral Lithium Amide Equivalent for Michael Additions to α,β-Unsaturated Esters. *Tetrahedron Asymmetry* **1991**, *2*, 183.

(15) Assay for inhibition of TNF synthesis by human PBMCs**:** Human PBMCs from normal donors were obtained by Ficoll−Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) density centrifugation. Cells (10^6 cells/mL) were cultured in RPMI (Gibco Laboratories, Grand Island, NY) supplemented with 10 AB+ serum (Biocell, Rancho Dominguez, CA), 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco). Test compounds were dissolved in DMSO (Sigma Chemical, St. Louis, MO) at 20 mg/mL; further dilution was done with culture medium. The final DMSO concentration in all assays including the controls was 0.25%. Test compounds were added to cells 1 h prior to the addition of LPS. PBMCs (10^6 cells/mL) were stimulated with 1 μg/mL of LPS from Salmonella minnesota R595 (List Biological Labs, Campbell, CA). Cells, in triplicate, were incubated with LPS for 18−20 h at 37 °C in 5% CO_2. Supernatants were then harvested and assayed for cytokine levels. In some experiments, supernatants were kept frozen at −70 °C until use. Cell viability was assayed by Trypan blue exclusion dye method. The concentration of TNFα in the culture supernatants was determined by ELISA (ENDOGEN, Boston, MA) according to the manufacturer's directions. All compounds were assayed in a minimum of three separate experiments. Percent inhibition was determined as 100 × [1 − (cytokine(experimental)/cytokine(control))].

(16) Thalidomide was routinely tested at 194 μM as a positive control and found to inhibit TNF production by 50%. All other IC_{50}'s were calculated by nonlinear regression analysis (variable slope) using Prism by GraphPad Software, Inc.

(17) Wintersk, W.; Frankus, E. Thalidomide enantiomers. *Lancet* **1992**, *339*, 365.

(18) Eriksson, T.; Bjorkman, S.; Roth, B.; Fyge, A.; Hoglund, P. Stereospecific Determination, Chiral Inversion In Vitro and Pharmacokinetics in Human of the Enantiomers of Thalidomide. *Chirality* **1995**, *7*, 44−52.

(19) Reepmeyer, J. Separation of R- and S-Thalidomide by Reversed-Phase HPLC with β-Cyclodextrin in the Mobile Phase. *Chirality* **1996**, *8*, 11−17.

(20) Corral, L.; Muller, G.; Moreira, A.; Chen, Y.; Wu, M.; Stirling, D.; Kaplan, G. Selection of novel analogues of thalidomide with enhanced TNF-α inhibitory activity. *Mol. Med.*, in press.

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