Thiothalidomides: Novel Isosteric Analogues of Thalidomide with Enhanced TNF-α Inhibitory Activity†

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Thalidomide is being increasingly used in the clinical management of a wide spectrum of immunologically-mediated and infectious diseases, and cancers. However, the mechanisms underlying its pharmacological action are still under investigation. In this regard, oral thalidomide is clinically valuable in the treatment of erythema nodosum leprosum (ENL) and mutiple myeloma and effectively reduces tumor necrosis factor-α (TNF-α) levels and angiogenesis in vivo. This contrasts with its relatively weak effects on TNF-α and angiogenesis in in vitro studies and implies that active metabolites contribute to its in vivo pharmacologic action and that specific analogues would be endowed with potent activity. Our focus in the structural modification of thalidomide is toward the discovery of novel isosteric active analogues. In this regard, a series of thiothalidomides and analogues were synthesized and evaluated for their TNF-α inhibitory activity against lipopolysacharide (LPS)-stimulated peripheral blood mononuclear cells (PBMC), This was combined with a PBMC viability assay to differentiate reductions in TNF-α secretion from cellular toxicity. Two isosteric analogues of thalidomide, compounds **15** and **16**, that mostly reflect the parent compound, together with the simple structure, dithioglutarimide **19**, potently inhibited TNF-α secretion, compared to thalidomide, **1**. The mechanism underpinning this most likely is posttranscriptional, as each of these compounds decreased TNF-α mRNA stability via its 3′-UTR. The potency of **19** warrants further study and suggests that replacement of the amide carbonyl with a thiocarbonyl may be beneficial for increased TNF-α inhibitory action. In addition, an intact phthalimido moiety appeared to be requisite for TNF-α inhibitory activity.

Introduction

Thalidomide (*N*-α-phthalimidoglutarimide (**1**) (Figure 1)) is a glutamic acid derivative that was introduced onto the market as a sedative hypnotic in 1956, but was withdrawn in 1961 due to the development of severe congenital abnormalities in babies born to mothers using it for morning sickness.[1] Interest in the agent was reawakened after thalidomide was found clinically effective in the treatment of erythema nodosum leprosum (ENL)[2] and in the treatment of HIV wasting syndrome and various cancers.[3,4] Mechanistic studies of its ENL activity demonstrated an anti-TNF-α action.[5] Specifically, thalidomide enhances the degradation of tumor necrosis factor-α (TNF-α) RNA and, thereby, lowers its synthesis and secretion.[6,7] Further studies have defined it to be a costimulator of both CD8+ and CD4+ T cells,[8] an inhibitor of angiogenesis via its inhibitory actions on basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF),[9,10] and an inhibitor of the transcription factor, NFkB.[6,7] However, it is the action of thalidomide on TNF-α that provides the focus of the current study.

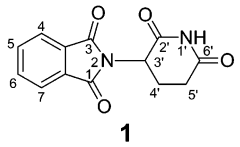

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Figure 1. The structure of thalidomide.

TNF-α and family members play pivotal roles in a variety of physiological and pathological processes, which include cell proliferation and differentiation, apoptosis, the modulation of immune responses, and induction of inflammation. TNF-α acts via two receptors, TNFR1 and 2. The former is expressed in all tissues and is the predominant signaling receptor for TNF-α. The latter is primarily expressed on immune cells and mediates more limited biological responses. The exposure of cells to TNF-α can result in activation

† Dedicated to our wonderful collaborator, mentor, and friend, Arnold Brossi, on his 80th birthday.

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of a caspase cascade leading to cell death via apoptosis. Indeed, major cell surface molecules capable of initiating apoptosis are members of the TNF family of ligands and receptors. For example, death-inducing members of the TNF receptor family each contain a cytoplasmic 'death domain' (DD), which is a protein−protein interaction motif critical for engaging downstream components of the signal transduction machinery.[11] Recently, TRAIL, the tumor necrosis factor-related apoptosis-inducing ligand, has been shown to selectively induce apoptosis of tumor cells, but not most normal cells.[12] It is suggested that TRAIL mediates thymocyte apoptosis and is important in the induction of autoimmune diseases. More often, however, TNF-α receptor binding induces the activation of transcription factors, AP-1 and NFkB, that thereafter induce genes involved in acute and chronic inflammatory responses.[13,14] Overproduction of TNF-α has thus been implicated in many inflammatory diseases, such as rheumatoid arthritis, graft-versus-host disease, and Crohn's disease, and it additionally exacerbates ENL, septic shock, AIDS, and dementia associated with Alzheimer's disease (AD). In this context, our present work, which examines novel analogues of thalidomide with enhanced TNF-α inhibitory activity, assumes significance.

The mechanisms underlying thalidomide's diverse actions, together with identification of the active species, remains an area of intense research. The compound is more active in vivo than would be predicted from its potency in in vitro studies.[15,16] This suggests that active metabolites largely account for its in vivo activity[17] and that specific analogues of thalidomide would be endowed with high potency. Indeed, seldom has such a simple molecule had such a controversial history and held so much therapeutic promise.[3,4,10,18] The focus of our research has been two pronged: (i) to elucidate the action of thalidomide on the regulation of TNF-α levels, and (ii) to develop analogues of clinical potential that potently inhibit TNF-α secretion.

A number of papers have been published on the design and synthesis of thalidomide analogues optimized to reduce TNF-α synthesis.[19−25] These have primarily focused on exploring structural modifications of the phthaloyl ring or glutarimide ring of thalidomide and have described *N*-phthaloyl 3-amino-3-arylpropionic acid derivatives, amino-phthaloyl-substituted and tetrafluorophthaloyl-substituted analogues of thalidomide, and N-substituted phthalimides with a simplified glutarimide moiety. In addition, following the demonstration that the antiangiogenic property of thalidomide is associated with its hydroxylated metabolites, the open ring metabolites,[15,26] syntheses of the hydroxylated and hydrolysis metabolites as inhibitors of angiogenesis or tumor metastasis have been reported.[23,24,27] In the current paper we focus on the synthesis and evaluation of novel sulfur analogues of thalidomide with enhanced biological activity.

Although extensive studies exist regarding the structure−activity relationships between thalidomide and TNF-α, very little is known about the contribution of four amide carbonyl groups of thalidomide to its biological activity. The isosteric replacement of carbonyl group by a thiocarbonyl group provides isosteric analogues of thalidomide: thiothalidomides. A series of thiothalidomides and analogues were designed to explore their action on inhibition of TNF-α (Figure 2).

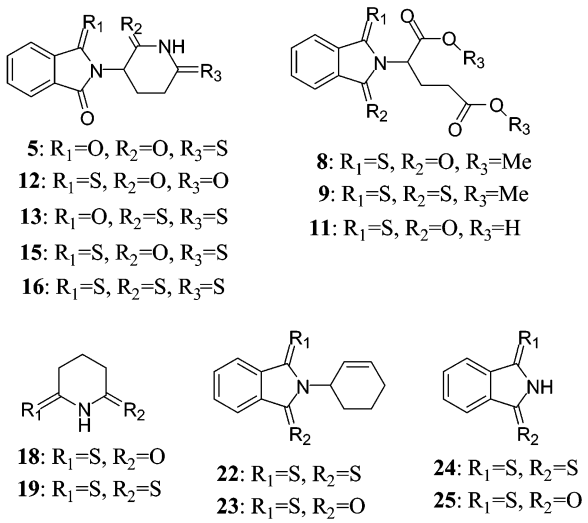


Figure 2. The structures of thiothalidomides and analogues.

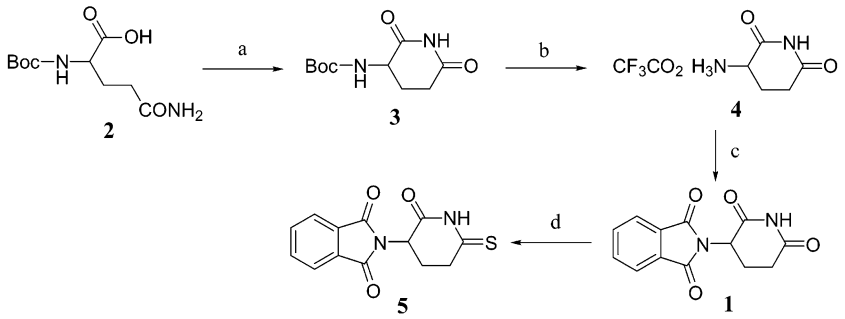
Chemistry

Monothiothalidomide **5** was prepared as shown in Scheme 1. *tert*-Butoxycarbonyl-L-glutamine **2** was refluxed with carbonyldiimidazole (CDI) in THF and cyclized to afford imide **3**.[21] Imide **3** then was treated with trifluoroacetic acid in CH_2Cl_2 to remove the protective group to generate aminoglutarimide trifluoroacetate **4**. Without further purification, compound **4** was reacted with phthalic anhydride in refluxing THF in the presence of triethylamine to produce thalidomide **1** in the total yield of 31% from compound **2**. Thalidomide **1** was thionated with Lawesson's reagent (LR)[28] to generate a single new product, whose structure was identified as 6′-thiothalidomide **5** by MS and 1D and 2D NMR spectra. The position of thiocarbonyl group was established from the HMBC cross-peak of H-5′/C-6′ (see Supporting Information: HMBC spectrum of compound **5**).

The synthesis of 3-thiothalidomide **12** is shown in Scheme 2. *N*-Phthaloyl-L-glutamic acid **6** was esterified to afford diester **7**. Compound **7** was thionated with LR at 110 °C to give compound **8** as a major product. Concurrently, compound **9** was separated as a minor product by chromatography. However, no thioesters were separated from the reaction mixture, as any ester reacts with LR at 130 °C.[27] Furthermore, we failed to obtain 3-thiothalidomide, **12**, through the cyclization of compound **8** with ammonia or amine, as ammonia reacts with the thioamide; reaction of compound **8** with benzylamine produced the unexpected compound **10**. In an alternative approach, compound **8** was hydrolyzed under acidic conditions to give diacid **11**. Compound **11** was then reacted with trifluoroacetamide to generate 1-thiothalidomide **12** in the presence of 1-hydroxybenzotriazole (HOBT) and 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (EDCI).[29]

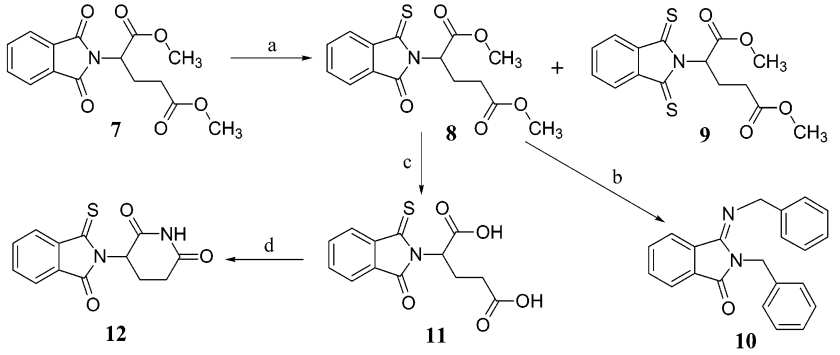
In the synthesis of dithiothalidomide, our initial attempt involved the reaction of monothiothalidomide with LR at reflux in toluene. Under such conditions, 2′,6′-dithiothalidomide was obtained in a yield of less than 2% (Scheme 3(a)). Such a yield was so low that

Scheme 1[a]



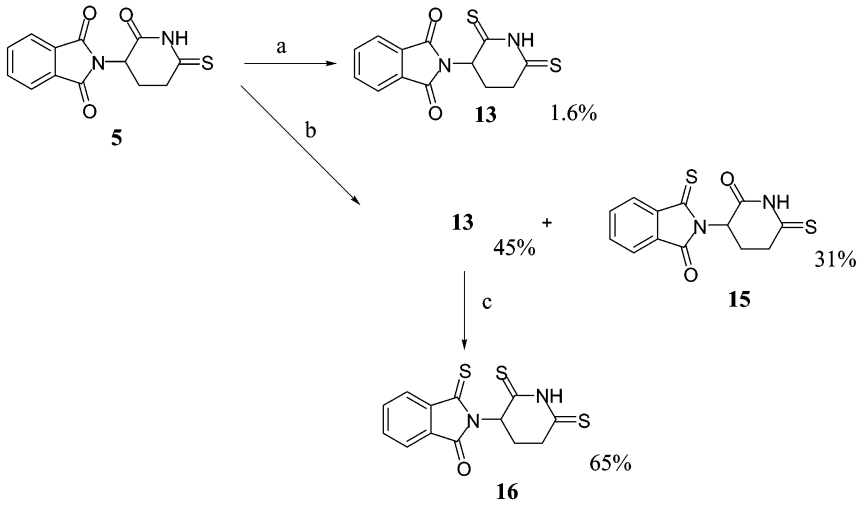
[a] Reagents: (a) CDI/THF; (b) CF_3COOH/CH_2Cl_2; (c) phthalic anhydride, Et_3N/THF; (d) Lawesson's reagent/toluene.

Scheme 2[a]



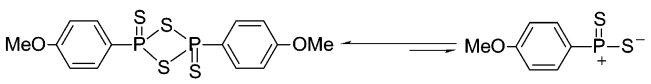
[a] Reagents: (a) Lawesson's reagent/toluene; (b) benzylamine; (c) HCl/HOAc; (d) F_3CCONH_2, HOBT, EDCI, Et_3N/CH_2Cl_2.

Scheme 3[a]



[a] Reagents: (a) Lawesson's reagent/toluene; (b) Lawesson's reagent, pyridine/toluene; (c) Lawesson's reagent, morpholine/toluene.

Scheme 4. Mechanism of Catalysis for Lawesson's Reagent



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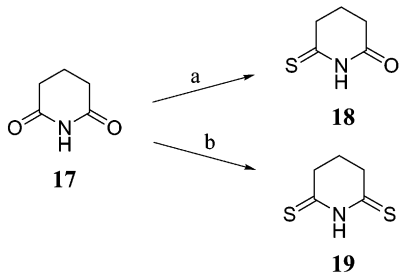
improvement was clearly essential and was undertaken by modifying the reaction conditions. It is believed that the mechanism underlying the reaction between LR and a carbonyl moiety is that a highly reactive dithiophosphine ylide **14**, rather than LR itself, likely is the active thionating agent (Scheme 4).[28] The Lewis base should be able to increase the reactivity of LR as the base may drive the unfavorable equilibrium and elevate the concentration of the ylide **14**. When pyridine was used as a catalyst for thionation, monothiothalidomide **5** was thionated with LR to produce two dithiothalidomides, **13** and **15**, in the yields of 45% and 31%, respectively (Scheme 3b,c). Dithiothalidomide **13** could be further thionated with LR in the presence of the stronger base, morpholine, to give trithiothalidomide **16** in the yield of 65%.

Glutarimide **17** was thionated with LR in THF at room temperature to afford compound **18** as a major product.[30] Glutarimide **17** also was refluxed with LR in toluene to produce dithioglutarimide **19** (Scheme 5).[31] Reaction of potassium phthalimide with 3-bromocyclohexene underwent the Gabriel reaction to give compound **21**. Thereafter, thionation of compound **21** with

Scheme 5[a]



[a] Reagents: (a) Lawesson's reagent/THF, rt; (b) Lawesson's reagent, reflux/toluene.

LR afforded compounds **22** and **23** (Scheme 6). Compounds **24** and **25** were prepared in a similar procedure to the preparation of compounds **22** and **23**.[32]

Results and Discussion

The action of the described thiothalidomide analogues to inhibit TNF-α secretion was assessed in human peripheral blood mononuclear cells (PBMC) and is shown in Table 1. Thalidomide, **1**, entirely lacked activity at 30 μM. A concentration of 100 μM was required for significant activity. The monothiothalidomides, 6′-thiothalidomide **5**, and 3-thiothalidomide **12** showed only marginal activity at 30 μM with 31% and 23% inhibition of TNF-α secretion, respectively. In contrast, the dithiothalidomides, including 2′,6′-dithiothalidomide **13** and 3,6′-dithiothalidomide **15**, exhibited more potent inhibitory activities with IC_{50} values of 20 μM and 11 μM, respectively. However, assessment of cell viability by MTS assay showed that **13** induced increasing cytotoxicity at higher concentrations. Trithiothalidomide **16** inhibited TNF-α production with an IC_{50} of 6 μM without accompanying toxicity. Compared with thalidomide, **1**, with an IC_{50} of ~200 μM for the inhibition of TNF-α synthesis, trithiothalidomide **16** is over 30-fold more active. Hence, successive replacement of a carbonyl with a thiocarbonyl group led to improved inhibitory activity compared to **1**, unassociated with toxicity. In this regard, the synthesized thiothalidomides possessed TNF-α lowering potency in the following decreasing order: trithiothalidomide **16** > dithiothalidomide **15** and **13** > monothiothalidomides **5** and **12** > thalidomide, **1**.

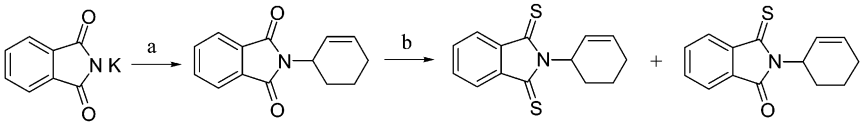
A comparison of the physical properties of thalidomide, **1**, and thiothalidomides shows that they have similar van der Waals radii and bond angles, although the C═S bond is slightly longer than the C═O bond. A possible explanation accounting for the elevated potency of the thiothalidomides is that their enhanced lipophilicity and loss of hydrogen bond acceptor capability potentially allows the attainment of higher intracellular drug levels. Interestingly, compounds **8**, **9**, and **11** are thio analogues of hydrolysis metabolites of thalidomide. Assessment of their TNF-α inhibitory action determined that the monothio analogue, **8**, has an IC_{50} of 20 μM without toxicity; demethylation (**11**) lowered potency. The dithio analogue, **9**, proved 2-fold more potent still than **8**, but induced cellular toxicity at lower concentrations. Intriguingly, thio analogues **22** and **23**, with a simplified glutarimide ring, were found to be active TNF-α inhibitors, albeit with some toxicity at 30 μM, with IC_{50} values (15 μM and 16 μM, respectively) that were greater than **12** (>30 μM) possessing a normal glutarimide ring.

In this regard, thalidomide is composed of two distinct moieties: the glutarimide and phthalimide rings. Thioglutarimides and thiophthalimides were thus synthesized and evaluated to assess the effect of thio-analogues of these two moieties on TNF-α levels. Monothioglutarimide **18** minimally inhibited TNF-α secretion at a concentration of 30 μM; however, dithioglutarimide **19** exerted a potent inhibitory effect with an IC_{50} of 8 μM and a lack of toxicity. An intact phthaloyl ring is believed to be required for the inhibition of TNF-α secretion.[19] Surprisingly, such a simple structure, dithioglutarimide **19**, proved to be 25-fold more active than **1**. In contrast, 2′,6′-dithiothalidomide **13**, a phthalimido-substituted dithioglutarimide, is less active than dithioglutarimide **19** and induces toxicity at high concentration. Monothiophthalimide **25** showed a marginal TNF-α activity at a concentration of 30 μM without toxicity. Interestingly, however, dithiophthalimide **24** was found to possess potent activity with an IC_{50} of 3 μM. Although it was associated with toxicity at 30 μM, its inhibition of TNF-α occurred at a log lower concentration that was well tolerated.

As described, compounds **15**, **16**, and **19** potently inhibited TNF-α secretion without toxicity. As a consequence, additional studies were undertaken to elucidate the mechanism underpinning this action. Gene and protein expressions are controlled at the level of transcription, posttranscription, RNA stability, and translation under different physiological stimuli. Recently, posttranscriptional pathways have been recognized to provide a major means of regulating eukaryotic gene expression. In this regard, TNF-α and other cytokines and protooncogenes are known to be regulated at the posttranscriptional level.[6,7,33,34] Multiple proteins, including the four cloned proteins AUF1, HuR, TTP, and HuD have been shown to bind to a region of the mRNA that contains adenylate/uridylate (AU)-rich elements (AREs) in the 3′-untranslated region (UTR). These proteins mediate RNA turnover and decay and hence translational efficiency.[35,36] The stability of TNF-α mRNA is largely regulated at its 3′-UTR, which contains a well characterized ARE. Although AREs are found in a number of different cytokine and protooncogene RNAs, the pathways by which they induce degradation are highly specific for a given ARE, suggesting some cellular specificity. When the AREs from different cytokines are complexed with AUF1, different binding affinities are observed. Notably, however, the highest affinity for AUF1 is to human and then mouse TNF-α.

To determine the involvement of the 3′-UTR in the action of our thalidomide analogues, their ability was assessed to inhibit reporter gene activity in cells containing the TNF-α 3′-UTR versus a control vector (Figure 3). Specifically, this cell-based assay comprised of two stably transfected cell lines derived from the mouse macrophage line, RAW264.7. One line, designated "luciferase only", expressed a luciferase reporter construct without any UTR sequences, whereas the other, designated "luciferase + TNF UTRs", expressed a luciferase reporter construct with the entire 3′- UTR of human TNF-α inserted directly downstream of the luciferase coding region. Compounds **15**, **16**, and **19**

Scheme 6[a]



[a] Reagents: (a) 3-bromocyclohexene/DMF; (b) Lawesson's reagent/toluene.

Table 1. Inhibition of LPS-Induced TNF-α Production in PBMC and Cell Viability[a]

compd	% inhibition at 30 μM	IC_{50} (μM)	cell viability at 30 μM	at 3 μM	at 0.3 μM
5	31	>30	>100	90	96
8	56	20	93	99	96
9	85	10	57	86	89
11	20	>30	86	93	93
12	23	>30	94	100	94
13	52	20	69	87	94
15	61	11	>100	87	94
16	79	6	94	86	90
18	15	>30	>100	84	86
19	75	8	>100	98	99
22	86	15	50	94	96
23	85	16	57	89	99
24	95	3	54	83	83
25	34	>30	>100	94	94

[a] Thalidomide (**1**) totally lacked activity at 30 μM (IC_{50} ~ 200 μM).

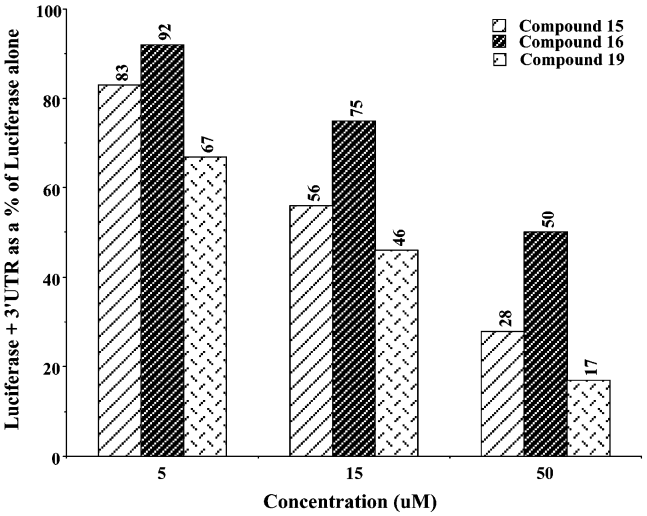


Figure 3. The action of compounds **15**, **16**, and **19** in cells (mouse macrophage cell line, RAW264.7.) possessing a luciferase reporter element plus the 3′-UTR of human TNF-α compared to cells lacking the 3′-UTR. All agents lowered luciferase reporter activity in cells stably expressing the 3′-UTR. Thalidomide **1** lacked activity at 50 μM.

exerted differential effect on the two cell lines in a dose-dependent manner, consistent with their ability to inhibit TNF-α production via the 3′-UTR.

As TNF-α protein levels changed without significant alterations in mRNA levels (data not shown), protein expression is presumably regulated via translational control (at the posttranscriptional level). There is precedence for translational (protein) control through either the 3′- or 5′-UTR regions of a number of critical proteins that are current drug targets. For example, levels of the β-amyloid precursor protein (APP) that is central to the development of AD can be regulated by either UTR. Turnover and translation of APP mRNA is regulated by a 29-nucleotide instability element within the 3′-UTR, located 200 nucleotides downstream from the stop codon.[37] This 3′-UTR element acts as an mRNA destabilizer whose function can be inhibited by the presence of growth factors. In contrast, different cytokines,[38] including TNF-α[39] and iron,[40] can up regulate APP protein synthesis at the level of its 5′-UTR, whereas, interestingly, the anticholinesterase, phenserine, that is currently in clinical trials for AD lowers APP protein levels with concurrent maintenance of mRNA steady-state levels through translational modification within the same 5′-UTR element.[41] A further example is that of the human immunodeficiency virus 1 (HIV-1) Trans-activating transduction (tat) protein, which binds trans-activation-responsive region (TAR) RNA.[42] Tat is brought into contact with the transcription machinery after binding the TAR element, which is a 59-residue stem-loop RNA found at the 5′ end of all HIV-1 transcripts.[42] Finally, thalidomide (**1**) has been reported to lower cyclooxygenase-2 (Cox-2) biosynthesis via its 3′-UTR[43] that appears to likewise contain an ARE that can regulate Cox-2 mRNA stability.[44] Our studies of analogues **15**, **16**, and **19** confirm the regulation of TNF-α protein levels by thalidomide (**1**) via its 3′-UTR, whether the 5′-UTR contains a similar element that is accessible to pharmacological manipulation remains to be determined, as does action against Cox-2.

Our future studies are aimed at assessing whether the in vitro activity of the described thalidomide analogues translates into pertinent in vivo models, such as in mice challenged with LPS.[33] In terms of the mechanism of TNF-α signaling, there is recent in vivo evidence showing that the AU-rich 3′-UTRs of TNF-α and IL-6 are downstream to MAPKAP kinase 2 (MK2) signaling. MK2 is an essential component of mechanisms that regulate biosynthesis of IL-6 at the level of mRNA stability, and of TNF-α mainly through TNF-ARE-dependent translational control.[45] In addition, future in vitro studies involving the induction of other cytokines and deletion of the ARE will define the specificity of the most potent compounds described herein, as well as ARE-independent actions. Knockout mice for the TNF ARE or the destabilizing protein TTP both have elevated levels of TNF-α and increased inflammation. Hence, the interaction between the TNF-α ARE and the cognate RNA binding proteins appears to represent an attractive target for regulation at the posttranscriptional level.

In conclusion, we have synthesized a novel series of thiothalidomide analogues that are more potent inhibitors of TNF-α production in LPS-induced human PBMCs than thalidomide, **1**. The isosteric replacement of successive carbonyl groups by a thiocarbonyl leads to an increasing inhibition with the number moieties replaced (trithiothalidomide **16** > dithiothalidomide **15** and **13** > monothiothalidomides **5** and **12** > thalidomide **1**). Preliminary studies indicate that the induced TNF-α inhibition involved posttranscriptional mechanisms and

occurred via its 3′-UTR. Like thalidomide **1**, **15**, and **16** effectively inhibited angiogenesis, whereas the potent TNF-α inhibitor, dithioglutarimide **19**, was inactive in angiogenesis assays (data not shown); suggesting that different structure/activity relations are responsible for these pharmacological actions. TNF-α has been validated as a drug target with Remicade (Cetocor, Malvern, PA; Schering-Plough, Orange, NJ) and Enbrel (Amgen, Thousand Oaks, CA; Wyeth-Ayerst, Princeton, NJ) on the market; however, both are large macromolecules and hence require injection. The development of small, druglike molecules to potently and safely inhibit TNF-α likely will be of significant clinical value.

Experimental Section

General. Melting points were determined with a Fisher-Johns apparatus and are uncorrected. ^{1}H NMR, ^{13}C NMR, and 2D NMR were recorded on a Bruker AC-300 spectrometer. Mass spectra and high-resolution mass spectra (HRMS) were recorded on a VG 7070 mass spectrometer and a Agilent Technologies 5973N GC-MS (CI). All exact mass measurements show an error of less than 5 ppm. Elemental analyses were performed by Atlantic Microlab, Inc., Norcross, GA.

3-(*tert*-Butoxycarbonylamino)-2,6-piperidinedione (3). A mixture of *N*-(*tert*-butoxycarbonyl)-L-glutamine (4.92 g, 20 mmol) and carbonyl diimidazole (3.24 g, 20 mmol) in THF (100 mL) was refluxed for 16 h. Thereafter, solvent was removed, and the crude product was recrystallized from hot EtOAc to give compound **3** (2.04 g, 45%) as white crystals: mp 214−215 °C; ^{1}H NMR (DMSO-d_6) δ 4.22 (dd, J = 6.2 Hz, J = 11.0 Hz, 1H), 2.77−2.65 (m, 1H), 2.45 (m, 1 H), 1.96−1.87 (m, 2H), 1.40 (s, 9H); MS (CI/CH_4) m/z 227 [M − 1]$^+$.

2-(2-Oxo-6-thioxo-3-piperidinyl)-1*H*-isoindole-1,3(2*H*)-dione (5). Compound **3** (1.14 g, 5 mmol) was suspended in CH_2Cl_2 (100 mL). To the mixture was added CF_3COOH (10 mL), and this then was stirred at room temperature for 4 h. The solvent was evaporated to give crude **4** (1.25 g): ^{1}H NMR (DMSO-d_6) δ 11.42 (s, 1H), 8.70 (br, 2H), 4.31 (dd, J = 5.4 Hz, J = 13 Hz), 2.88−2.72 (m, 2H), 2.25−2.09 (m, 2H). A mixture of crude **4** (1.25 g), phthalic anhydride (0.89 g, 6 mmol), and Et_3N (1.39 mL, 10 mmol) in THF (150 mL) was refluxed for 2 days. The reaction mixture was concentrated, and the residue was crystallized from ethyl acetate to give thalidomide (**1**) (0.89 g, 69%) as white crystals; mp 276 °C (lit. 276−279 °C). The mixture of thalidomide **1** (258 mg, 1 mmol) and Lawesson's reagent (222 mg, 0.55 mmol) in toluene (50 mL) was stirred at reflux for 12 h; thereafter, solvent was removed under vacuum. The resulting residue was purified by column chromatography using CH_2Cl_2 as the eluent to afford compound **5** (200 mg, 73%) as a yellow solid: mp 225−226 °C; ^{1}H NMR (DMSO-d_6) δ 12.83 (s, 1H, NH), 8.00−7.92 (m, 4H, Ph), 5.32 (dd, J = 5.6 Hz, J = 12.9 Hz, 1H, H-3′), 3.28−3.25 (m, 2H, H-5′), 2.60−2.54 (m, 1H, H-4′), 2.17−2.10 (m, 1H, H-4′); ^{13}C NMR (DMSO-d_6) δ 208.7(C-6′), 165.3(C-2′), 165.2(C-1 and C-3), 133.1(C-5 and C-6), 129.3 (C-3a, C-7a), 121.7 (C-4 and C-7), 46.9 (C-3′), 38.9 (C-5′), 21.79 (C-4′); MS (CI/CH_4) m/z 274 (M$^+$); Anal. ($C_{13}H_{10}N_2O_3S$) C, H, N.

Dimethyl 2-(1,3-dihydro-1,3-dioxo-2*H*-isoindol-2-yl)-pentanedioate (7). To a solution of *N*-phthaloyl-L-glutamic acid (200 mg, 0.72 mmol) in methanol (10 mL) was added, dropwise, thionyl chloride (1 mL). The reaction mixture was refluxed for 6 h. The solvent was removed under reduced pressure, dissolved in ethyl acetate (100 mL), and then washed with saturated aqueous Na_2CO_3 solution (2 × 30 mL) and water (2 × 30 mL). The ethyl acetate layer was dried over Na_2SO_4 and then evaporated, leaving an oil, which upon purification by silica gel chromatography, using CH_2Cl_2:EtOAc (1:1) as the eluent, gave compound **7** (161 mg, 73%) as an oil; ^{1}H NMR ($CDCl_3$) δ 7.87−7.84 (m, 2H), 7.75−7.72 (m 2H), 4.91 (dd, J = 5 Hz, J = 9 Hz, 1H), 3.73 (s, 3H), 3.62 (s, 3H), 2.67−2.56 (m, 1H), 2.51−2.44 (m, 1H), 2.41−2.35 (m, 2H).

Dimethyl 2-(1,3-Dihydro-1-oxo-3-thioxo-2*H*-isoindol-2-yl)pentanedioate (8) and Dimethyl 2-(1,3-Dihydro-1,3-dithioxo-2*H*-isoindol-2-yl)pentanedioate (9). A mixture of compound **7** (144 mg, 0.47 mmol) and LR (191 mg, 0.47 mmol) in toluene was stirred in a 110 °C oil bath for 10 h. The solvent was then evaporated, and the residue was purified by column chromatography (silica gel) using CH_2Cl_2 as the eluent, to obtain compound **9** (17 mg, 11%) as a dark red oil. Thereafter, using CH_2Cl_2:EtOAc (10:1) as the eluent the more polar component **8** (105 mg, 70%) was obtained as a red oil.

Compound **8**: ^{1}H NMR ($CDCl_3$) δ 7.98−7.96 (m, 1H), 7.81−7.70 (m, 3H), 5.53 (dd, J = 5.1 Hz, J = 10 Hz, 1H), 3.70 (s, 3H), 3.59 (s, 3H), 2.76−2.56 (m, 2H), 2.40−2.33 (m, 2H); MS (CI/CH_4) m/z 321 (M$^+$).

Compound **9**: ^{1}H NMR ($CDCl_3$) δ 7.87−7.84 (m, 2H), 7.73−7.68 (m. 2H), 6.09 (dd, J = 5 Hz, J = 10 Hz, 1H), 3.70 (s, 3H), 3.58 (s, 3H), 2.81−2.63 (m, 2H), 2.40−2.24 (m, 2H); MS (DEI) m/z 337 (M$^+$); HRMS (DEI) calcd for $C_{15}H_{15}NO_4S_2$ 337.0442 (M$^+$), found 337.0449.

2-(1,3-Dihydro-1-oxo-3-thioxo-2*H*-isoindol-2-yl)pentanedioic Acid (11). Compound **8** (350 mg, 1.09 mmol) was stirred with a 1:1 mixture of acetic acid glacial and concentrated HCl in a 100 °C oil bath for 2.5 h. Ethyl acetate (100 mL) and ice−water (30 mL) was added. The ethyl acetate layer was separated, washed with ice−water, dried over Na_2SO_4, and concentrated. The resulting syrup was crystallized with ether to afford compound **11** as red crystals (253 mg, 79%); mp 157 °C; ^{1}H NMR (DMSO-d_6) δ 8.04−7.96 (m, 1H), 7.91−7.74 (m, 3H), 5.43 (dd, J = 5.1 Hz, J = 9.6 Hz, 1H), 2.42−2.33 (m, 2H), 2.30−2.26 (m, 2H); MS (DEI) m/z 293 (M$^+$); HRMS (DEI) calcd for $C_{13}H_{11}NO_5S$ 293.0358 (M$^+$), found 293.0363; Anal. ($C_{13}H_{11}NO_5S$) H, N; C: calcd, 53.24; found, 53.88.

2,3-Dihydro-3-thioxo-2-(2,6-dioxo-3-piperidinyl)-1*H*-isoindol-1-one (12). A mixture of compound **8** (81 mg, 0.276 mmol), trifluoroacetamide (57 mg, 0.50 mmol), 1-hydroxybenzotriazole (145 mg, 1.07 mmol), 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (200 mg, 1.04 mmol), and triethylamine (0.21 mL, 1.51 mmol) in CH_2Cl_2 (1.5 mL) was stirred at ambient temperature for 3 days. Water (10 mL) and CH_2Cl_2 (10 mL) were added. The dichloromethane layer was separated, washed with water, dried over Na_2SO_4, and evaporated under reduced pressure. Purification by chromatography, with EtOAc:CH_2Cl_2 (1:10) as the eluent, gave compound **12** (48 mg, 63%) as a red solid: mp 255 °C; ^{1}H NMR ($CDCl_3$) δ 8.00−7.98 (m, 1H), 7.80−7.71 (m, 3H), 5.63 (br, 1H), 2.98−2.70 (m, 3H), 2.18−2.15 (m, 1H); MS (CI/CH_4) m/z 274 (M$^+$); Anal. ($C_{13}H_{10}N_2O_3S$) C, H, N.

2-(2, 6-Dithioxo-3-piperidinyl)-1*H*-isoindole-1,3(2*H*)-dione (13) and 2,3-Dihydro-3-thioxo-2-(2-oxo-6-thioxo-3-piperidinyl)-1*H*-isoindol-1-one (15). The mixture of **5** (146 mg, 0.533 mmol), LR (108 mg, 0.267 mmol), and pyridine (21 μL) in toluene was stirred at 110 °C under an atmosphere of N_2 for 12 h. Thereafter, additional LR (108 mg, 0.267 mmol) and pyridine (21 μL) were added, and the reaction mixture was stirred for a further 12 h. The solvent was removed under vacuum, and the residue was purified by column chromatography with CH_2Cl_2:petroleum ether (2:1, 10:1) and then CH_2Cl_2:EtOAc (10:1) as eluents to afford **13** (30 mg, 45%), **15** (21 mg, 31.5%), and starting material **5** (83 mg).

Compound **13** (yellow solid): mp 263−265 °C; ^{1}H NMR ($CDCl_3$) δ 7.78−7.74 (m, 2 H), 7.66−7.63 (m, 2 H), 5.00 (dd, J = 4.9 Hz, 11.9 Hz, 1 H), 3.43−3.35 (m, 1 H), 2.95−2.84 (m, 2 H), 2.08−2.06 (m, 1 H); MS (DEI) m/z 290 (M$^+$); HRMS (DEI) calcd for $C_{13}H_{10}N_2O_2S_2$ 290.0184 (M$^+$), found 290.0185; Anal. ($C_{13}H_{10}N_2O_2S_2$) C, H, N.

Compound **15** (red solid): mp 240−242 °C; ^{1}H NMR ($CDCl_3$) δ 9.44 (s, 1 H), 8.05−8.02 (m, 1 H), 7.86−7.76 (m, 3 H), 5.75−5.64 (m, 1 H), 3.57−3.52 (m, 1 H), 3.09−2.99 (m, 2 H), 2.19−2.12 (m, 1 H). ^{13}C NMR (DMSO): 208.16, 207.98, 166.10, 165.39, 134.32, 133.11, 132.42, 124.30, 122.15, 121.11, 49.64, 21.29; MS (DEI) m/z 291 (MH$^+$); HRMS (DEI) calcd for $C_{13}H_{11}N_2O_2S_2$ 291.0262 (M$^+$), found 291.0264; Anal. ($C_{13}H_{10}N_2O_2S_2 \cdot 0.5H_2O$) C, H, N.

2,3-Dihydro-3-thioxo-2-(2,6-dithioxo-3-piperidinyl)-1*H*-isoindol-1-one (16). The mixture of compound **13** (29 mg, 0.1 mmol), LR (22 mg, 0.054 mmol), and morpholine (9 μL, 0.1

mmol) in toluene (10 mL) was stirred at reflux under an atmosphere of N_2 for 16 h. The solvent was removed under vacuum, and the residue was purified by column chromatography using CH_2Cl_2:petroleum ether (1:1) as the eluent to afford compound **16** (20 mg, 65%) as a red solid: mp 244 °C; ^{1}H NMR ($CDCl_3$) δ 10.81 (s, 1H), 8.05−8.01 (m, 1H), 7.91−7.75 (m, 3H), 5.92 (m, 1H), 3.57−3.52 (m, 1H), 3.13−2.97 (m, 2H), 2.18−2.15 (m, 1H); MS(DEI) *m/z* 306 (M^+); HRMS (DEI) calcd for $C_{13}H_{10}N_2OS_3$ 305.9955 (M^+), found 305.9951; Anal. ($C_{13}H_{10}N_2OS_3 \cdot 0.5H_2O$) C, H, N.

6-Thioxo-2-piperidinone (18). The mixture of glutarimide (0.45 g, 4 mmol) and LR (0.809 g, 2 mmol) in THF (30 mL) was stirred at room temperature for 2 days. The solvent was evaporated under vacuum, and the residue was purified by column chromatography using petroleum ether:EtOAc (1:1) as the eluent to give compound **18** as a yellow solid (0.361 g, 70%): mp 135 °C; ^{1}H NMR ($CDCl_3$) δ 2.96 (t, J = 5.7 Hz, 2 H), 2.58 (t, J = 5.8 Hz, 2 H), 1.96 (m, 2 H); MS (CI/CH_4) *m/z* 129 (M^+); Anal. (C_5H_7NOS) C, H, N.

2,6-Piperidinedithione (19). The mixture of glutarimide (0.34 g, 3 mmol) and LR (1.22 g, 3 mmol) in toluene (30 mL) was stirred at reflux for 3 h. The solvent was evaporated under vacuum, and the residue was purified by column chromatography using petroleum ether:EtOAc (20:1) as the eluent to give compound **19** as a yellow solid (0.286 g, 66%): mp 103 °C; ^{1}H NMR ($CDCl_3$) δ 3.02 (t, J = 6.3 Hz, 4H), 1.98 (t, J = 6.3 Hz, 2H); MS (CI/CH_4) *m/z* 145 (M^+); Anal. ($C_5H_7NS_2$) C, H, N.

2-(3-Cyclohexenyl)-1*H*-isoindole-1,3(2*H*)-dione (21). A mixture of potassium phthalimide (1.85 g, 3 mmol) and 3-bromocyclohexene (1.79 g, 3 mmol) in DMF (15 mL) was stirred in a 100 °C oil bath for 12 h. The cooled reaction mixture was poured into ice−water. The solid was collected by filtration and purified by flash chromatography with CH_2Cl_2 as the eluent to afford compound **21** (1.6 g, 72%) as pink crystals; mp 114 °C; ^{1}H NMR ($CDCl_3$) δ 7.73−7.69 (m, 2H), 7.62−7.58 (m, 2H), 5.85−5.82 (m, 1H), 5.47−5.44 (m, 1H), 4.80−4.78 (m, 1H), 2.14−2.00 (m, 3H), 1.86−1.78 (m, 2H), 1.64−1.58 (m, 1H).

2-(3-Cyclohexenyl)-1*H*-isoindol-1,3(2*H*)-dithione (22) and 2,3-Dihydro-3-thioxo-2-(3-cyclohexenyl)-1*H*-isoindol-1-one (23). The mixture of compound **21** (68 mg, 0.3 mmol) and LR (121 mg, 0.3 mmol) in toluene was refluxed under N_2 for 10 h. The solvent was removed under vacuum, and the residue was purified by column chromatography using petroleum ether as the eluent to obtain compound **22** (37 mg, 48%) as a dark green solid. Then, using CH_2Cl_2:petroleum ether (1:1) as the eluent, the more polar component **23** (23 mg, 32%) was obtained as a red solid.

Compound **22**: mp 93 °C; ^{1}H NMR ($CDCl_3$) δ 7.65−7.60 (m, 2H), 7.49−7.42 (m, 2H), 5.92−5.88 (m, 1H), 5.66−5.63 (m, 1H), 5.47−5.43 (m, 1H), 2.40−2.35 (m, 1H), 1.99−1.95 (m, 2H), 1.75−1.59 (m, 3H); MS (CI/CH_4) *m/z* 259 (M^+); Anal. ($C_{14}H_{13}NS_2$) C, H, N.

Compound **23**: mp 67−68 °C; ^{1}H NMR ($CDCl_3$) δ 7.94−7.91 (m, 1H), 7.73−7.64 (m, 3H), 5.92−5.88 (m, 1H), 5.60−5.51 (m, 2H), 2.27−2.10 (m, 3H), 1.96−1.76 (m, 2H), 1.81−1.70 (m, 1H); MS (CI/CH_4) *m/z* 243 (M^+); Anal. ($C_{14}H_{13}NOS$) C, H, N.

Assessment of Compounds on the Secretion of TNF-α by PBMCs. Freshly prepared PBMCs were utilized in all studies. Blood, 40 mL, was drawn from a volunteer, immediately mixed with 50 U/mL Na heparin and was diluted to 50 mL total volume with sterile PBS. Samples, 20 mL, of this preparation then was layered on 20 mL of Ficoll-Paque and was centrifuged (800*g*, 20 min). The Ficoll/serum interface, containing PBMCs, was collected, diluted to 200 mL with PBS, and then was centrifuged (800*g*, 15 min) to pellet the cells. Thereafter, the recovered pellet was resuspended in 37 °C tissue culture medium (RPMI/1 mM Sodium pyruvate/10% heat inactivated FBS/2 mM Glutamax) and placed on ice. Recovered cells were counted, pipetted (200 μL of 5 × 105/mL) into 96-well plates, and incubated for 1 h (37 °C, 5% CO_2). Thereafter, appropriate concentrations of test compounds or vehicle (10 μL of DMSO) were added to duplicate wells. Following a further hour of incubation, a 10 μL sample of lipopolysaccharide (LPS)(100 ng/mL in supplemented medium) or vehicle was added to induce stimulated and unstimulated cells, respectively, and the cells were incubated overnight. Sixteen hours later, supernatants were collected for quantification of TNF-α levels by ELISA assay (Pierce-Endogen human TNF-α minikit, Rockford, IL) and the use of specific capture and detection monoclonal antibodies, M303E and M302B (Pierce-Endogen), respectively. ELISA plates were read at 450 nm λ, and TNF-α levels were determined from a six-point calibration curve that was run concurrently with the test samples. The effect of test drug concentrations on the cellular viability of PBMCs was assessed by MTS assay (Promega, Madison, WI) of the cells that provided the supernatant samples assayed for TNF-α levels, described above.

Assessment of Compounds on TNF-α/Luciferase Activity. This cell-based assay utilized two stably transfected cell lines derived from the mouse macrophage line, RAW264.7. One line, designated "luciferase only" expressed a luciferase reporter construct without any UTR sequences. The other line, designated "luciferase + TNF-α UTR" expressed a luciferase reporter construct with the entire 3′-UTR of human TNF-α inserted directly downstream of the luciferase coding region. Compounds were added in a concentration-dependent manner, as above, and at the end of the incubation period (16 h, 37 °C, 5% CO_2) the media was removed, cells were lysed, and luciferase activity was assayed with Steady-glo luciferase assay reagent (Promega) according to the supplier's directions. Background was subtracted, and data from this assay was expressed as a ratio of the +3′-UTR to -3′-UTR (control) values and was expressed as a percent as shown in Figure 3. In this manner, compounds that show a differential effect on the two cell lines, with and without a 3′-UTR, are highlighted.

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Supporting Information Available: HMBC spectrum of **5**. This material is available free of charge via the Internet at http://pubs.acs.org.

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