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Thalidomide-based TNF-α inhibitors for neurodegenerative diseases

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Abstract. Inflammatory processes associated with the over-production of cytokines, particularly of TNF-α, accompany numerous neurodegenerative diseases, such as Alzheimer's disease, in addition to numerous systemic conditions, exemplified by rheumatoid arthritis and erythema nodosum leprosum (ENL). TNF-α has been validated as a drug target with Remicade and Enbrel available as prescription medications. Both, however, are large macromolecules, require injection and have limited brain access. The classical drug, thalidomide is being increasingly used in the clinical management of a wide spectrum of diseases. As its clinical value in treating ENL derives from its TNF-α inhibitory activity, thalidomide was chosen for structural modification for the discovery of novel and more potent isosteric analogues with appropriate lipophilicity to insure high brain penetration. TNF-α inhibitory activity was evaluated against lipopolysacharide (LPS) stimulated peripheral blood mononuclear cells (PBMC) in cell culture, whose viability was quantified to differentiate reductions in TNF-α secretion from that associated with cellular toxicity. Specific analogues potently inhibited TNF-α secretion, compared to thalidomide. This involved a post-transcriptional mechanism, as they decreased TNF-α mRNA stability *via* its 3'-untranslated region (UTR), as determined by luciferase activity in stably transfected cells with and without the 3'-UTR of human TNF-α.

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Key words: thalidomide, thalidomide analogues, TNF-α inhibitors, Alzheimer's disease, amyloid precursor protein, amyloid-β peptide, lipopolysacharide (LPS), peripheral blood mononuclear cells (PBMC), rheumatoid arthritis, erythema nodosum leprosum (ENL), amyotrophic lateral sclerosis (ALS), translational regulation

INTRODUCTION

Alzheimer's disease (AD) is the most prevalent dementia in the elderly and afflicts some 3 million people in the US and 5 million in Europe. It is considered to result from a series of biochemical steps in a pathogenic process that leads to amyloid formation and neurodegeneration in critical brain regions involved in memory and cognition (Lahiri et al. 2002, 2003a, Sambamurti et al. 2002, Selkoe 2001). Several genes have been identified that underpin a familial component of the disease process and provide insight into contributing pathological pathways. Amongst these are beta-amyloid precursor protein (APP) on 21q21, apolipoprotein E (ApoE) on 19q13, presenilin 1 (PS1) and 2 (PS2) on 14q24 and1q31-42, respectively, and alpha-antichymotrypsin (ACT). Each of these, independently and in a complex manner, affects APP and levels of the peptides, beta-amyloid ($A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$), generated from APP (Sambamurti et al. 2002).

In parallel with these, multiple other endogenous factors impact regulatory elements present on the APP gene as well as factors that regulate Aβ synthesis, deposition and clearance. Such elements include inflammatory cytokines and growth factors that are implicated in both inflammation and AD (Breitner 1997, Weninger and Yankner 2001). In this regard, several compounds have been identified in the AD brain that are known to promote and sustain inflammatory responses and alter APP levels. These include the inflammatory cytokines, interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α), as well as the prostaglandin generating cyclooxygenases, COX-1 and COX-2 (McGeer and McGeer 2001). The role of IL-1 in neuroinflammation and AD has recently been examined (Mrak and Griffin 2001) and, similarly, cytokine/ Aβ-induced glial activation, NF-kappaB, and apolipoprotein E play critical roles (Bales et al. 2000, Żekanowski et al. 2004). For example, IL-1 is the first proinflammatory cytokine secreted after the activation of macrophage/ microglial cells. IL-1 and TNF-α are expressed in microglia around developing amyloid plaques in brain cells (Das and Potter 1995, Griffin et al. 1989). The mechanisms underpinning their regulatory actions on APP processing are currently an area of significant interest (Lahiri et al. 2003b). These, like other environmental agents (Selkoe 2001), have been shown to stimulate APP turnover into its pathological Aβ form. In addition, astrocytes and microglia are the cellular surrounding of neurons and represent further and non-neuronal sources of APP (Akiyama et al. 2000, Funato et al. 1998). The deposition of amyloid plaques often are allied with enlarged microglia that, in turn, produce TNF-α and IL-1, potent mediators of astroglial proliferation and APP production (Nitsch et al. 1992).

Thalidomide (*N*-α-phthalimidoglutarimide) is a glutamic acid derivative that was introduced as a sedative hypnotic in 1956, but was withdrawn in 1961 due to the development of grave congenital abnormalities in babies born to mothers using it for treating morning sickness (Eger et al. 1990). The compound was reintroduced as a therapeutic for leprosy and more recently has demonstrated potency in the treatment of a variety of cancers (Kumar et al. 2002, Richardson et al. 2002, Sheskin 1965). The initial mechanism underpinning the action of this compound was shown to be on inhibition of TNF-α protein expression and it was further demonstrated to act at the post-transcriptional level to facilitate turnover of the mRNA (Moreira et al. 1993, Sampaio et al. 1991). More recent work also has shown activity against COX2 protein expression, which may be mediated post-transcriptionally by similar AU-rich elements (AREs) found in the 3' UTRs of each mRNA (Chen and Shyu 1995, Kruys and Huez 1994, Kruys et al. 1989). The action of thalidomide to lower TNF-α levels is not particularly potent and it therefore represented an interesting lead compound for medicinal chemistry and is the focus of the present publication; particularly since the compound, unlike the macromolecules: Remicade (Centocor, Malvern, PA/Schering-Plough, Orange, NJ) and Enbril (Amgen, Thousand Oaks, CA/Wyeth, Princeton, NJ), is a small compound that can be taken orally rather than by direct injection.

METHODS

Thalidomide and analogues

Thalidomide together with a series of novel isosteric analogues were designed and synthesized. Specifically, in the synthesis of thalidomide, *tert*-butoxycarbonyl--L-glutamine was refluxed with carbonyl diimidazole (CDI) in THF, and cyclized to afford the imide. This was characterized and then was treated with trifluoroacetic acid in CH_2Cl_2 to remove the protective group to generate aminoglutarimide trifluoroacetate. Without further purification, this compound then was reacted with phthalic anhydride in refluxing THF in the

presence of triethylamine to produce thalidomide (1) (Fig. 1) in the total yield of 31%.

Thereafter, thalidomide (1) was thionated with Lawesson's reagent (LR) (Cava and Levinson 1985) to generate a single new product, whose structure was identified as 6′-thiothalidomide (2) by MS and 1D and 2D NMR spectra. The position of thiocarbonyl group was established from HMBC cross peak analysis (H-5′/C-6′).

For the synthesis of 3-thiothalidomide (3), *N*-phthaloyl--L-glutamic acid was esterified to afford its diester. This then was thionated with LR at 110°C to yield an intermediate that was hydrolyzed under acidic conditions and then was reacted with trifluoroacetamide to generate 3-thiothalidomide (3) in the presence of 1-hydroxy--benzotriazole (HOBt) and 1-[3-(dimethylamino)propyl] -3-ethyl carbodiimide hydrochloride (EDCI). 3-Thiothalidomide was separated and recrystallized to high purity.

In the synthesis of dithiothalidomide, monothiothalidomide was reacted with LR at reflux in toluene using pyridine as a catalyst for thionation. Specifically, monothiothalidomide (2) was thionated with LR to produce two dithiothalidomides, (4) and (5), in the yields of 45% and 31%, respectively. These were then separated

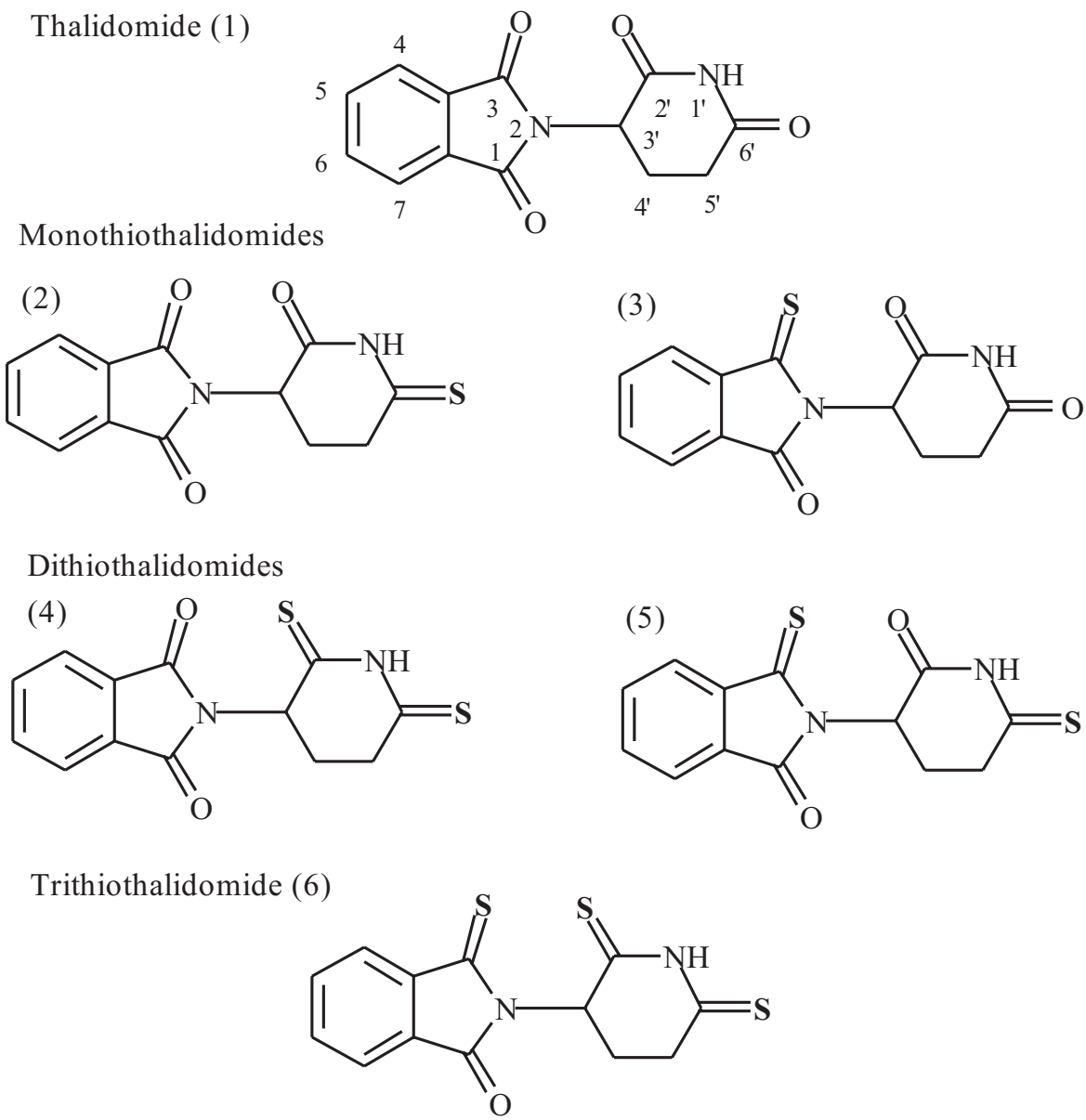

Fig. 1. Chemical structures of thalidomide and isosteric thiothalidomides analogues.

from each other, and recrystallized to high purity. Finally, dithiothalidomide (4) was thionated with LR in the presence of the stronger base, morpholine, to give trithiothalidomide (6) in the yield of 65%, which similarly was separated and recrystallized to high purity (Zhu et al. 2003).

Cell Culture Studies: Freshly prepared PBMCs were used in all studies. Specifically, a blood sample, 40 ml, was drawn from a volunteer, immediately mixed with sodium heparin (50 U/ml) and then was diluted to a total volume of 50 ml with sterile PBS. Samples (20 ml) of this preparation were then layered on an equal volume of Ficoll-Paque and were centrifuged (800 g, 20 min). The Ficoll/plasma interface, that contained PBMCs, was then collected, diluted to 200 ml with PBS, and was centrifuged (800 g, 15 min) to pellet the cells. Subsequently, the recovered pellet was re-suspended in 37°C tissue culture medium (RPMI/1 mM sodium pyruvate/10% heat inactivated FBS/2 mM Glutamax) and was placed on ice. Finally, recovered cells were counted, pipetted (1×10^5 cells in 200 μl) into 96 well plates, and incubated for 60 minutes (37°C, 5% CO_2). Thereafter, appropriate concentrations of thalidomide, novel analogues or vehicle (10 μl DMSO) were added to duplicate wells. Following an additional 60 minutes of incubation, a 10 μl sample of lipopolysacharide (LPS)(100 ng/ml in supplemented medium) or vehicle was added to induce stimulated and unstimulated cells, respectively, which were then incubated for 16 hours. Supernatants, thereafter, were collected for TNF-α measurement by ELISA assay (Pierce-Endogen human TNF-α mini kit, Rockford, IL) using the monoclonal antibodies, M303E and M302B (Pierce-Endogen), for capture and detection, respectively. Finally, ELISA plates were read at 450 nm λ and TNF-α levels were determined from a calibration curve of six-points that was run concurrently with the experimental samples. The action of thalidomide and analogues on PBMC viability was determined by MTS assay (Promega, Madison, WI) utilizing the cells from which TNF-α levels in supernatant samples were measured, as described.

TNF-α 3'-UTR luciferase activity: two stably transfected cell lines derived from the mouse macrophage line, RAW264.7, were utilized that expressed either: (i) a luciferase reporter construct with the entire 3'-UTR of human TNF-α inserted directly downstream of the luciferase coding region (termed: "luciferase + TNF-α UTR"); or (ii) a luciferase reporter construct without any UTR sequences (termed: "luciferase alone"). Thalidomide and analogue (6) were added, as described above, in a concentration-dependent manner and after 16 hours incubation (37°C, 5% CO_2) the media was removed, the cells were lysed and luciferase activity was assayed using Steady-glo luciferase assay reagent (Promega). Background activity was subtracted and assay results were expressed as a ratio of the +3'-UTR to –3'-UTR (control) values, and then expressed as a percent of controls.

RESULTS

The action of the described thiothalidomide analogs to inhibit TNF-α secretion was assessed in human peripheral blood mononuclear cells (PBMCs) and is shown in Table I. Thalidomide, itself, entirely lacked activity at 30 μM. A concentration of 100 μM was required for significant inhibition of TNF-α secretion. In contrast, monothiothalidomides, 6'-thiothalidomide (2) and 3-thiothalidomide (3) showed marginal activity at 30 μM with 31% and 23% inhibition of TNF-α activity, respectively. Notably, the dithiothalidomides, 2', 6'-dithiothalidomide (4) and 3, 6'-dithiothalidomide (5), exhibited greater inhibitory activities with IC_{50} values of 20 μM and 11 μM, respectively. However, assessment of cell viability by MTS assay showed that (4) induced some cytotoxicity at higher concentrations. Of greatest interest, trithiothalidomide (6) inhibited TNF-α production with an IC_{50} of 6 μM without any accompanying toxicity. Compared with thalidomide (1) with an IC_{50} of some 200 μM for the inhibition of TNF-α synthesis, trithiothalidomide (6) proved to be over 30-fold more active. Hence, replacement of a carbonyl with a thiocarbonyl group led to an increased inhibitory activity compared to thalidomide, unassociated with toxicity, that additionally provided an elevation in liophilicity, as determined by cLog P values (Table I). The synthesized thiothalidomides hence possessed potent TNF-α lowering potency in the following decreasing order trithiothalidomide (6) > dithiothalidomide (5) and (4) > monothiothalidomides (2) and (3) > thalidomide (1).

As trithiothalidomide potently inhibited TNF-α secretion without toxicity, additional studies were undertaken with this compound, as a representative of the class, to elucidate the mechanism underpinning this action. TNF-α together with other cytokines and protooncogenes are known to be regulated at the post-transcriptional level. As thalidomide has been reported to act *via* the 3'-UTR of TNF-α,

Table I

Inhibition of LPS-induced TNF-α production in PBMCs and cell viability

Compounds	Percent inhibition at 30 μM	Percent cell viability at 30 μM	Percent cell viability at 0.3 μM	IC_{50} (μM)	cLog P value (lipophilicity)
1	Zero	100	100	~200	-0.83
2	31	100	96	>30	-0.57
3	23	94	94	>30	-0.57
4	52	69	94	20	-0.56
5	61	100	94	11	-0.56
6	79	94	90	6	-0.30

specific studies were undertaken to assess whether or not such a mechanism could be exploited by trithiothalidomide. As illustrated in Fig. 2., trithiothalidomide lowered the luciferase reporter element activity in mouse macrophage cell line, RAW264.7, that stably expressed the 3'-UTR of human TNF-α, when expressed as a percent of cells without the 3'-UTR. Without alterations in mRNA levels (not shown), the action of the compounds can be considered to be *via* translational regulation.

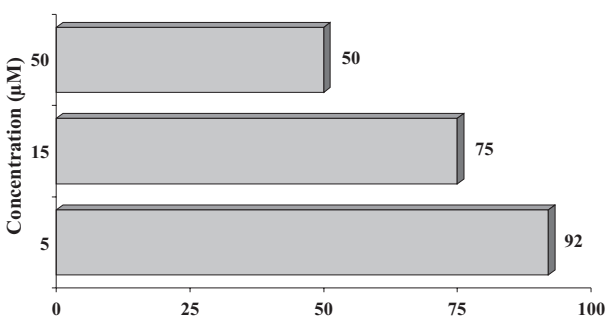

Fig. 2. The action of trithiothalidomide (6) in cells (mouse macrophage cells, RAW264.7) possessing a luciferase reporter element plus the 3'-UTR of human TNF-α compared to cells lacking the 3'-UTR.

DISCUSSION

There is a reported inverse relationship between AD pathology and the use of anti-inflammatory drugs, although this observation remains controversial (Veld et al. 2001, Zandi and Breitner 2001). Several recent studies have reported that the use of non-steroidal anti-inflammatory drugs is associated with a lowered risk of AD (Veld et al. 2001, Weninger and Yankner 2001) with particular agents, such as Celastrol that is both an anti-inflammatory and anti-oxidant, showing potential as an AD treatment (Allison et al. 2001). Unfortunately, recent prospective placebo-controlled clinical trials, such as with Celebrex, Viox and prednisone, have not supported the beneficial action of this drug class in AD subjects and hence have questioned the anti-inflammatory approach *via* COX inhibitors. An alternative strategy is to target specific cytokines *via* COX-independent mechanisms.

In this regard, the targeting of TNF-α synthesis represents a fresh tactic, as TNF-α and other cytokines and protooncogenes are known to be regulated at the post-transcriptional level. Indeed, a number of proteins, AUF1 (Wilson et al. 2001), HuR (Dean et al. 2001), tristetraprolin (TTP) (Carballo et al. 1998) and the related proteins, TIA-1 and TIAR, have been shown to bind to a region of the RNA. These proteins mediate RNA turnover and translational efficiency, with AUF1 and HuR (Brennan and Steitz 2001, Fan and Steitz 1998) reported to stabilize the RNA and TTP acting to destabilize the RNA (Lai et al. 1999), although in some cell types AUF1 may destabilize RNAs (Xu et al. 2001). Each of these proteins is known to interact with AREs present in the 3' UTRs of the mRNAs. Both *in vivo* and *in vitro* studies have shown that the TNF-α ARE is intimately involved with the post-transcriptional regulation of TNF-α mRNA. As an example, transgenic mice with a 69-nucleotide deletion spanning the ARE develop chronic inflammatory arthritis and inflammatory bowel disease (Kontoyiannis et al. 1999). Furthermore, the absence of the ARE profoundly increased TNF-α levels determined in the serum, macrophage explants and synovial fibroblasts of these mice. The molecular basis for the increased TNF-α levels was recognized to be *via*

an elevated TNF-α mRNA stability and translational activation of the TNF-α mRNA. Similar effects of the ARE on TNF-α mRNA stability and translation have been observed in reporter gene assay systems (Han et al. 1990), transcriptional pulsing assays (Xu et al. 1997) and *in vitro* decay systems (Ford et al. 1999). While these AREs are found in a number of different cytokine and protooncogene mRNAs (Bakheet et al. 2001, Bhattacharya et al. 1999), the pathways by which they induce degradation are recognized to be highly specific for a given ARE suggesting some cellular specificity (Chen and Shyu 1995).

The TNF-α family members play critical functions in a array of physiological and pathological processes, which include the induction of inflammation, apoptosis, modulation of the immune status as well as cellular proliferation and differentiation. TNF-α acts *via* two receptors, TNFR1 and 2; with the former being the predominant signaling receptor that is expressed in all tissues. Whereas the latter is principally expressed on immune cells, to mediate more restricted biological responses. Exposure of cells to TNF-α can induce activation of a caspase cascade and cause cell death *via* apoptosis. In reality, many cell surface molecules that are proficient at instigating apoptosis are members of the TNF family of ligands and receptors. In inflammation, TNF-α receptor binding induces the activation of transcription factors, AP-1 and NFκB, that thereafter induce genes involved in acute and chronic inflammatory responses (Begg and Baltimore 1996, Thanos and Maniatis 1995), with TNF-α overproduction being implicated in numerous inflammatory diseases, such as rheumatoid arthritis, septic shock, AIDS, graft-versus-host and Crohn's diseases in addition to AD. In this context, potent, bioavailable and well-tolerated TNF-α inhibitors could prove of wide clinical utility.

The former anxiolytic non-barbiturate hypnotic drug, thalidomide, has been demonstrated to enhance the degradation of TNF-α RNA and, thereby, lower its synthesis and secretion (Kruys et al. 1989, Moreiera et al. 1993) Further studies have defined it to additionally be a co-stimulator of both CD8+ and CD4+ T cells (Haslett et al. 1998), an inhibitor of angiogenesis, likely *via* its inhibitory actions on basic fibroblast and vascular endothelial growth factors (D'Amato et al. 1994), and an inhibitor of the transcription factor, NFκB (Begg and Baltimore 1996) It thus represents an interesting lead compound as a focus for medicinal chemistry, particularly since the mechanisms underlying its diverse actions, together with identification of the active species responsible for each of the described actions remain to be fully elucidated. Interestingly, the agent is more potent *in vivo* than would be predicted from its *in vitro* activity (Bauer et al. 1998, Meierhofer and Wiedermann 2003) suggesting that active metabolites largely account for its *in vivo* action (Price et al. 2002), and that specific analogues with high potency may, indeed, be synthesized. This proved to be the case, whereby successive replacement of carbonyl groups by thiocarbonyls provided incremental increases in TNF-α inhibitory activity of up to 30-fold, in the case of trithiothalidomide, without toxicity.

An evaluation of the comparative physical properties of thalidomide and thiothalidomides is currently the focus of x-ray crystallographic analysis, with preliminary studies suggesting alike Van der Waals radii and bond angles, although the C=O bond is not as long as the C=S one. A plausible justification of the increased potency of the thiothalidomides versus thalidomide is their greater lipophilicity and loss of hydrogen bond acceptors to potentially allow higher intracellular drug levels and blood-brain barrier penetrability. However, calculations of cLog P values indicated relatively modest increased lipophilicity (Table I), supporting the involvement of additional factors.

As trithiothalidomide potently inhibited TNF-α secretion without toxicity, additional studies were undertaken to clarify the mechanism behind this action. To determine whether or not this inhibition was due to the involvement of the 3'-UTR in the action of our thalidomide analogues, the ability of the most potent one was assessed to inhibit reporter gene activity in cells containing the human TNF-α 3'-UTR *versus* a control vector. In this regard, trithiothalidomide exerted differential effects on the two cell lines in a dose-dependent manner, consistent with its ability to inhibit TNF-α production *via* the 3'-UTR (Fig. 2). Whether this inhibition is due to a decrease in the stability or translational efficiency remains to be determined. Clearly, however, a post-transcriptional pathway is involved, and such pathways have now been recognized to provide a major means of regulating eukaryotic gene expression. In this regard, thalidomide has been reported to lower COX-2 biosynthesis *via* its 3'-UTR (Fujita et al. 2001), which appears to likewise contain an ARE that can regulate COX-2 mRNA stability (Sheng et al. 2000). Whether or not the thiothalidomides possess similar elevated potency as COX-2 inhibitors remains to be determined.

As described, changes within the brain milieu can alter APP metabolism and presumably the course of AD development (Breitner 1997, Buxbaum et al. 1992). Alterations in APP processing by cytokines are supported by the observation that activated microglial cells are consistently associated with developing lesions and these cells are major sources of TNF-α and IL-1as well as other potent stimulators of APP in surrounding astrocytes. Specifically, cytokines, such as IL-1, have the ability to activate non-neuronal cells to produce APP and hence kindle the progression of AD (Buxbaum et al. 1992, Goldgaber et al. 1989). In this regard, specific cytokines mediate their action on *APP* gene expression *via* regulatory elements present in the APP promoter region and 5'-UTR mRNA, where their actions, together with those of different growth factors and interleukins on *APP* gene expression, like thalidomides action on the 3'-UTR of TNF-α mRNA, are only beginning to be understood (Lahiri and Nall, 1995, Rogers et al. 1999). The 5'-UTR of APP mRNA that confers translational control of APP protein synthesis in U373 MG cells (Rogers et al. 1999). The 5'-UTR of APP mRNA shows structural and functional homology to the iron regulatory element (IRE) sequence in the 5'-UTR of ferritin mRNA (Rogers et al. 1999, 2002a), and hence can be upregulated by iron whose concentration is reported elevated in AD brain (Rogers et al. 2002b). In addition, the 5'-UTR likewise contains at least one *TGF-β1* responsive element, specifically a CAGA box that is able to interact with Smads complexes (Lahiri and Ge 2000, Lahiri et al. 2003b). Signaling events leading to transcriptional activation initiated by members of the TGF-β superfamily are known to be mediated by Smad proteins (Burton et al. 2002, Derynck et al. 1998). A further example is the AD experimental drug, phenserine: an acetylcholinesterase inhibitor that is currently in phase 3 clinical trials, that likewise regulates APP protein levels through the action of a translational enhancer in the APP-mRNA 5'-UTR (Lahiri et al. 2003b, Shaw et al. 2001); thereby lowering APP and hence Aß levels. The action of TNF-α on APP regulation likely is at the transcriptional level, but these former examples provide support to the contention that, as demonstrated by the actions of thalidomide and analogue on the 3'-UTR of TNF-α mRNA, pharmacological regulation at the post-transcriptional level is clearly achievable with small drug-like compounds. Furthermore, reductions in TNF-α synthesis, a valuable entity alone, could lead to secondary beneficial actions at the level of APP by breaking the self-propagating cycle that drives AD pathogenesis.

CONCLUSION

In summation, we synthesized a novel series of thiothalidomide analogues that are dramatically more potent inhibitors of TNF-α production in LPS-induced human PBMCs than thalidomide. The isosteric replacement of successive carbonyl groups by a thiocarbonyl leads to an increasing inhibition with the number moieties replaced (trithiothalidomide > dithiothalidomide > monothiothalidomides > thalidomide) coupled with an elevated lipophilicity associated with blood-brain barrier penetrability. Preliminary studies indicate that the induced TNF-α inhibition involved post-transcriptional mechanisms and occurred *via* its 3'-UTR.

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