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THALIDOMIDE ANALOGS AND PDE4 INHIBITION

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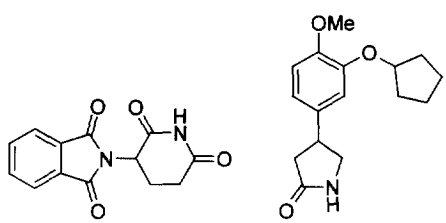
Abstract. N-Phthaloyl 3-amino-3-arylpropionic acid analogs of thalidomide that are potent inhibitors of tumor necrosis factor-α are reported. These compounds were found to be potent inhibitors of phosphodiesterase 4.

Introduction

Tumor necrosis factor-α (TNF-α) is a key cytokine in the inflammatory cascade. Excessive TNF-α levels have been found to be associated with a number of inflammatory and autoimmune conditions including rheumatoid arthritis, Crohn's disease, aphthous ulcers, erythema nodosum leprosum in leprosy, septic shock, cachexia, graft versus host disease, asthma, ARDS, and AIDS.[1] Thus, control of TNF-α levels could be a key to the treatment of a wide range of diseases. The validity of this approach has recently been demonstrated by the clinical benefit observed in the treatment of rheumatoid arthritis and Crohn's disease by TNF-α antibodies and TNF-α soluble receptors.[2] In 1991, thalidomide (**1**) was reported to be a selective inhibitor of TNF-α production in activated monocytes.[3] Although thalidomide has a tragic history because of its teratogenic properties, it has never totally disappeared from pharmaceutical use because of its effective immunomodulatory properties.[4] In a program to increase the TNF-α inhibitory potency of thalidomide and eliminate/decrease its teratogenic potency we have prepared numerous analogs of thalidomide.



1 **5**

We recently reported on a series of thalidomide analogs (**2** and **3**) derived from 3-amino-3-arylpropionic acids, which are potent inhibitors of TNF-α.[5] This series of thalidomide analogs are much more potent inhibitors of TNF-α than thalidomide (TNF-α IC_{50} = ~200 μM)[5]. The mechanism of thalidomide's inhibition of TNF-α levels is unknown, although it was reported by the Kaplan group that it decreases TNF-α mRNA stability.[6] We have continued to explore the mechanism of action of thalidomide and these analogs. It is well documented that elevated levels of cAMP inhibit TNF-α production in activated monocytes and peripheral blood mononuclear

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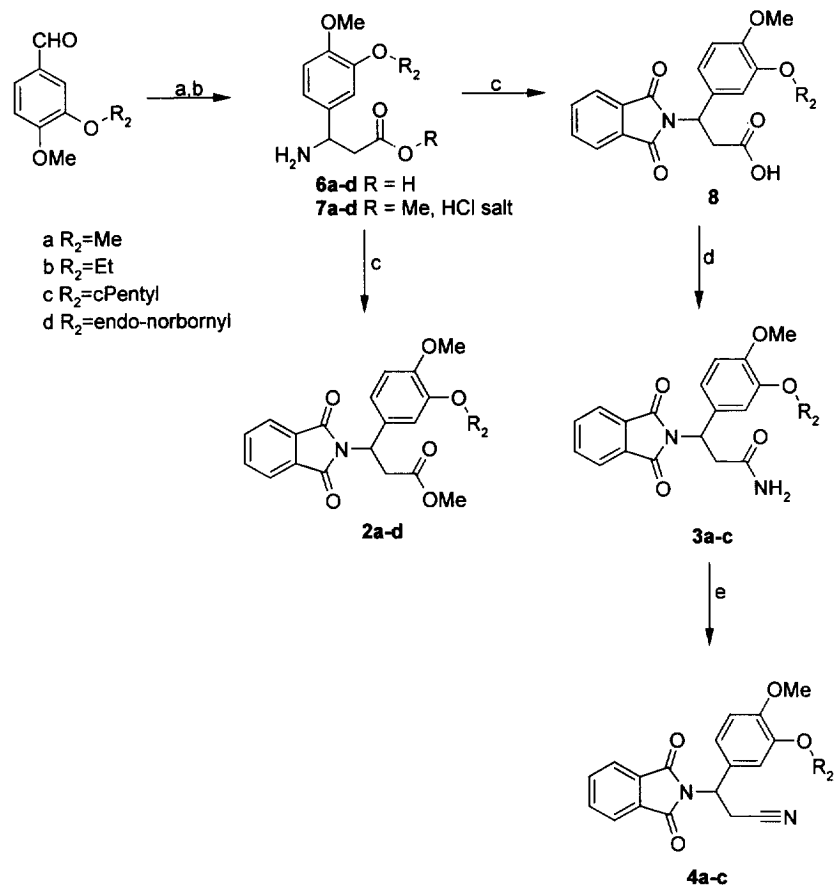
cells (PBMC).[7] Cellular levels of cAMP are controlled by adenylate cyclase and the cAMP phosphodiesterases (PDEs).[8] PDE4 is the major enzyme found in monocytes, the major producers of TNF-α in the inflammatory cascade. Inhibition of PDE4 has been shown to be an effective method for inhibition of TNF-α production in activated monocytes and PBMC. We wish to report the discovery that these thalidomide analogs (**2** and **3**) and the related nitriles (**4**) are potent inhibitors of PDE4.

Scheme 1



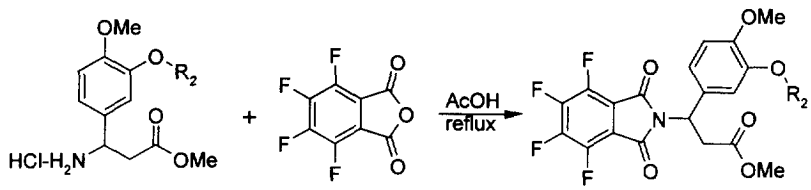
Regents: (a) NH_4OAc, $CH_2(CO_2H)_2$, EtOH, reflux; (b) $SOCl_2$/MeOH; (c) N-carbethoxyphthalimide, Na_2CO_3, CH_3CN/H_2O; (d) (1) CDI/THF, (2) concentrated NH_4OH; (e) $SOCl_2$ or $(COCl)_2$/DMF/pyridine.

Chemistry

The ester and amide analogs were prepared as previously described (Scheme 1).[5] The nitrile analogs were prepared from the corresponding amides. 3-Amino-3-arylpropionic acids were prepared as previously described by treatment of a substituted benzaldehyde with malonic acid and ammonium acetate in refluxing EtOH. Substituted 3,4-dialkoxybenzaldehydes were commercially available or prepared as previously described.[9] The N-phthaloyl carboxylic acids (**8a-c**) were prepared using a standard Nef reaction. The N-

phthaloyl carboxylic methyl esters **2a-d** were prepared by conversion of the 3-amino-3-arylpropionic acid (**6a-d**) to the methyl esters (**7a-d**) by treatment with $SOCl_2$ in MeOH, followed by treatment with Nef's reagent in the presence of Na_2CO_3 (Scheme 1). Conversion of phthaloyl carboxylic acids (**8a-c**) to the corresponding phthaloyl amides (**3a-c**) was accomplished by activation of the carboxylic acid with carbonyldiimidazole (CDI) followed by treatment with conc. NH_4OH. The amides **3a-c** were dehydrated to the nitriles with $SOCl_2$.or $(COCl)_2$.[10] The tetrafluorophthaloyl analogs **9a** and **9b** were prepared by condensation of **7a** and **7b**, respectively with tetrafluorophthalic anhydride in refluxing acetic acid (Scheme 2).

Scheme 2



9a R_2=Me
9b R_2=Et

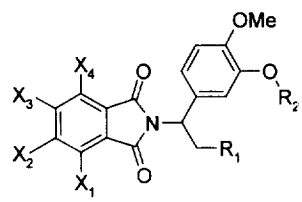
Biological Assays

TNF-α inhibitory activity was measured in lipopolysacharide (LPS) stimulated PBMC as previously reported.[5] Crude PDE4 extract was obtained from U937 cells using the method of Hill and Mitchell as described below.[11] Cells (1 x 10^9) were washed in PBS and lysed in cold homogenization buffer (20 mM Tris-HCl, pH 7.1, 3 mM 2-mercaptoethanol, 1 mM $MgCl_2$, 0.1 mM EGTA, 1 μM PMSF, 1μg/mL leupeptin). Following homogenization in a Dounce homogenizer the supernatant was collected by centrifugation and loaded onto a Sephacryl S-200 column equilibrated in homogenization buffer. PDE was eluted in homogenization buffer and the rolipram sensitive fractions pooled and stored in aliquots. PDE activity was assayed using the protocol adapted from Hill and Mitchell[11] and based on assay described by Thompson et al.[12] Enzyme activity was assayed in 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$ and 1 μM cAMP (of which 1% was ^3H cAMP) in the presence of varying concentrations of inhibitors. The amount of extract used was pre-determined to ensure that reactions were within the linear range and consumed less than 15% of the total substrate. Reactions were performed at 30 °C for 30 min and terminated by boiling for 2 min. The samples were then chilled and treated with snake venom (1 mg/mL) at 30 °C for 15 min. Unused substrate was removed by incubation with 200 μL AG1-X8 resin (BioRad) for 15 min. Samples were then spun at 3000 rpm for 5 min and 50 μL of the aqueous phase taken for counting. Each data point was carried out in duplicate with activity expressed as percentage of control. IC_{50}s was determined from dose response curves derived from three independent experiments. TNF-α and PDE4 IC_{50}s were calculated by non-linear regression analysis (variable slope) using Prism by GraphPad Software, Inc.

Results and Discussion

These thalidomide analogs were screened for their ability to inhibit TNF-α in LPS stimulated human PBMC.[5] PDE4 inhibitory activity was assayed with PDE4 enzyme isolated from the U937 cells, a promonocytic cell line. A good correlation between TNF-α inhibition and PDE4 inhibition was observed for the majority of compounds (Table 1). Thalidomide was inactive in the PDE4 assay (IC_{50} > 500 μM). A separation in mechanism of action between these analogs and thalidomide was found. These compounds appear to inhibit TNF-α by elevation of cellular cAMP levels.

Table 1: TNF-α and PDE4 Inhibition by Thalidomide



compd	R_2	R_1	X_1	X_2	X_3	X_4	TNF-α IC_{50} [μM]	PDE4 IC_{50} [μM]
2a	Me	CO_2Me	H	H	H	H	2.9	2.5
2b	Et	CO_2Me	H	H	H	H	0.70	0.23
2c	cPentyl	CO_2Me	H	H	H	H	1.6	1.7
2d	norbornyl	CO_2Me	H	H	H	H	2.4	0.74
3a	Me	$CONH_2$	H	H	H	H	13	9.4
3b	Et	$CONH_2$	H	H	H	H	2.7	2.0
3c	cPentyl	$CONH_2$	H	H	H	H	2.5	1.1
4a	Me	CN	H	H	H	H	1.7	1.3
4b	Et	CN	H	H	H	H	0.12	0.13
4c	cPentyl	CN	H	H	H	H	1.6	0.35
9a	Me	CO_2Me	F	F	F	F	0.26	4.7
9b	Et	CO_2Me	F	F	F	F	0.38	2.2

The prototypical PDE4 inhibitor, rolipram (PDE4 IC_{50}= 0.40 μM and TNF-α IC_{50}= 0.15 μM),[13] **5** contains a 3-cyclopentoxy-4-methoxyphenyl moiety, which correlated with the 3,4-dimethoxypheny moiety found in these thalidomide analogs. The SAR of the 3,4-dialkoxyphenyl moiety in rolipram type PDE4 inhibitors is well developed. A 3-cyclopentoxy-4-methoxyphenyl moiety along with other large hydrophobic 3-

alkoxy substituents such as endo-norbornyloxy is preferred.[14] The SAR of the 3-alkoxy group was explored in this series (Table 1). Interestingly, these PDE4 inhibitors do not directly follow the SAR of known rolipram type analogs. The 3-cyclopentoxy-4-methoxyphenyl analog **2c** was only 2-fold more active as a PDE4 inhibitor than the previously reported 3,4-dimethoxy analog **2a**. The 3-ethoxy-4-methoxy analog **2b** was over 7-fold more active as a PDE4 inhibitor than **2c**. The differences in TNF-α inhibitory activities were smaller but followed the same trend. The smaller differences in the TNF-α inhibitory potencies are probably due to cell based effects of the TNF-α inhibition in which the compound must enter the cell to be active. In the amide series, the 3-cyclopentoxyl analog **3c** was 2-fold more active than the 3-ethoxy analog **3b** and a magnititude more active as a PDE4 inhibitor. However, **3b** and **3c** were equipotent as TNF-α inhibitors. Isosteric replacement of the amide/ester moiety with a nitrile was investigated. The nitriles **4a-c** were significantly more potent as PDE4 inhibitors than the amide analogs but afforded only slight improvements over the ester analogs. The 3-ethoxy-4-methoxy nitrile is the most potent TNF-α inhibitor of the reported compounds with an IC_{50} of 120 nM. In this series of compounds the smaller 3-ethoxy substituent appears to be preferred over larger 3-alkoxy substituent.

Recently other researchers reported the tetrafluorophthaloyl analog of **3a** as a potent TNF-α inhibitor in LPS stimulated THP-1 cells.[15] We also prepared this compound, **9a** and the 3-ethoxy-4-methoxy analog **9b** and found them to be potent inhibitors of TNF-α in LPS stimulated PBMC (Table 1). However, both compounds were found to be cytotoxic at 10 and 100 μM in this assay which put the TNF-α inhibition results in question.[16] The non-fluorinated analogs described were not cytotoxic at the highest concentration (100 μM) tested in this assay. Both **9a** and **9b** were found to be approximately 10- to 15-fold less active as PDE4 inhibitors compared to their TNF-α inhibitory activity. Whether the differences in the TNF-α and PDE4 inhibitory activity are due to the cytotoxicity of the compounds in the TNF-α inhibition is unknown.

In conclusion, we have determined that these thalidomide analogs are potent inhibitors of PDE4. It is proposed that these thalidomide analogs control TNF-α levels by inhibition of PDE4. Thalidomide was found to be inactive against PDE4 (IC_{50} > 500 μM). Although thalidomide was inactive against PDE4 the possibility that one or more of its metabolites or degradation products inhibits PDE4 has not been eliminated. Using thalidomide as a lead structure we have discovered a novel series of potent PDE4 inhibitors. The most active compound reported here is >1,500 times more potent (IC_{50}) as a TNF-α inhibitor than thalidomide. Future publications from our laboratories will further describe the SAR and report the potential therapeutic value of these PDE4 inhibitors.

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16. Cytotoxicity was determined as described in reference 5.