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ENHANCED POTENCY OF PERFLUORINATED THALIDOMIDE DERIVATIVES FOR INHIBITION OF LPS-INDUCED TUMOR NECROSIS FACTOR-α PRODUCTION IS ASSOCIATED WITH A CHANGE OF MECHANISM OF ACTION

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Abstract: Perfluorination of phthalimides leads to dramatically increased potency as inhibitors of TNF-α production. We examined the enantiodependence for several tetrafluorophthalimides and α-methylthalidomide, **3**. Only **3** exhibited strikingly enantiodependent activity. The key structural determinant for the enhanced activity is the tetrafluorophthaloyl group, which confers enhanced potency and a change in the mechanism of inhibition.

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Despite its teratogenicity, thalidomide (**1**) is a uniquely effective drug for the treatment of erythema nodosum leprosum (ENL) and other inflammatory diseases owing to its immunomodulatory and anti-inflammatory activity.[1] There has been considerable interest in the molecular mechanisms of action of thalidomide in the immune system. Kaplan and coworkers reported that thalidomide selectively inhibits TNF-α production from human monocytes stimulated with lipopolysaccharide (LPS), offering a possible explanation for the immunomodulatory effects of the drug.[2]

As thalidomide selectively inhibits LPS-stimulated TNF-α production, we have been using thalidomide as a molecular probe to gain insight into the LPS-mediated signaling pathway in monocytes. Using a photoaffinity label derived from thalidomide, we isolated and identified a major thalidomide binding protein from monocytes as α_1-acid-glycoprotein (AGP).[3a] Indirect evidence exists supporting a role of AGP in LPS-stimulated TNF-α production.[3] Further study, however, has been hampered by the low potency of thalidomide which has an IC_{50} of over 200 μM for TNF-α inhibition.

In an effort to increase the potency of thalidomide and eliminate its teratogenic activity, we synthesized a series of thalidomide analogs by replacing the four aromatic hydrogens with halogens.[4] We found that tetrafluorothalidimide, **2**, and the series of tetrafluorophthalimides exhibited an over 500-fold increase in potency for the inhibition of TNF-α over the corresponding non-fluorinated analogs, with tetrafluorinated methyl [3-phthalimido-3-(3,4-dimethoxyphenyl)] propionate (F_4CC-1104), **6**, being the most potent.[4a] A key question has been whether this new class of tetrafluorophthalimides shares the same mechanism of action as thalidomide. We report herein that the mechanism of action of tetrafluorothalidomide and other tetrafluorophthalimides is distinct from that of thalidomide as demonstrated, in part, by the dependence of activity on the chirality of alkyl groups attached to tetrafluorophthaloyl moiety.

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Thalidomide has been used as a classic example of the importance of chirality in drug action; it has been suggested that only one of the two enantiomers of thalidomide has teratogenic or immunomodulatory activity.[5] This notion has now been invalidated by the demonstration that thalidomide rapidly racemizes under physiological conditions.[5b,6] However, use of α-methyl thalidomide, **3**, in which the labile hydrogen is replaced with a methyl group, indicated significant selectivity between the enantiomers. (*S*)-α-methylthalidomide was shown to be a more active than (*R*)-α-methylthalidomide for inhibition of production of TNF-α from LPS-stimulated human monocytes.[5b] Consistent with this observation is the finding that only (*S*)-α-methylthalidomide has enhancing effects on the production of TNF-α from TPA-treated HL-60 cells.[5c] We became interested in studying the enantiodependence of (*R*)- and (*S*)-α-methyl tetrafluorothalidomide, **4**. For comparison, we also synthesized and tested pairs of the enantiomers for F_4CC-1104, **6**, and FPTP, **7**.[7]

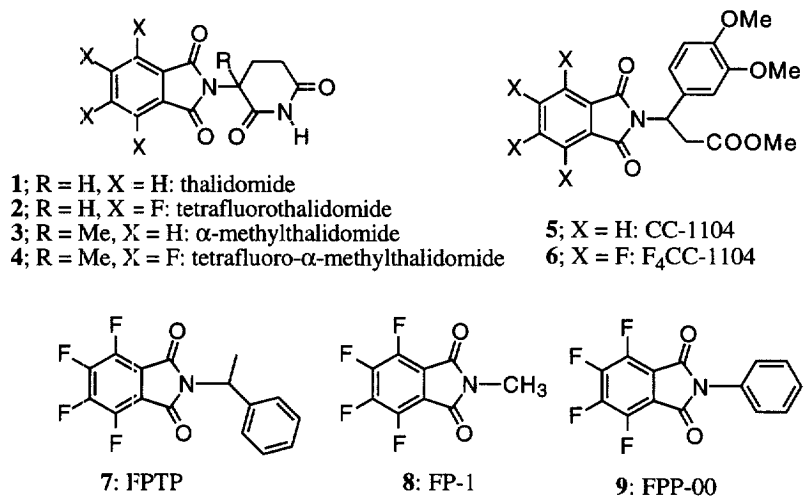

Figure 1. TNF-α Inhibitors Evaluated in this Study.

Both the (*R*)- and (*S*)-enantiomers of **4, 6** and **7** were synthesized by condensation of the corresponding optically pure amines with tetrafluorophthalic anhydride. The two enantiomers of α-methyl-α-aminoglutarimide used for the synthesis of tetrafluoro-α-methylthalidomide, **4**, were prepared as described earlier.[5c] Both enantiomers of the amines used for the synthesis of F_4CC-1104, **6**, were prepared by the method of Davies.[8] The optical purity of the final products were determined by chiral HPLC analysis on a DAICEL OJ column. All isomers were obtained with high optical purity (>99% e.e.).[9]

We measured the inhibitory activity of each enantiomer of **4**, **6** and **7** on LPS-stimulated TNF-α production from THP-1 cells.[3,4,10] The two enantiomers of α-methylthalidomide, **3**, were used as a control in this assay.

As shown in Figure 2A, α-methylthalidomide, **3**, exhibited a significant selectivity between the two enantiomers: only the (*S*)-isomer inhibited TNF-α production from THP-1 cells to an appreciable degree at 200 μM. This result is in agreement with previous reports using primary human monocytes.[5b,11] In contrast, both enantiomers of tetrafluoro-α-methylthalidomide, **4**, exhibited almost the same inhibitory activity on TNF-α production (Figure 2B).

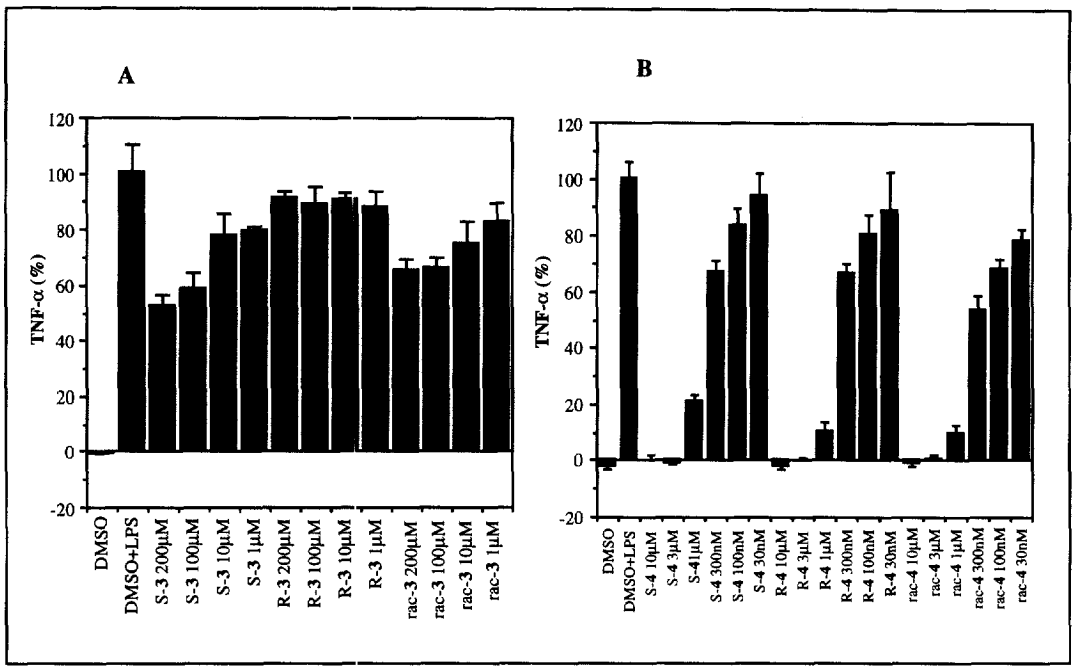

Figure 2. Enantiodependence of Effects of **3** and **4** on TNF-α Production.

Table 1. Inhibition of TNF-α by Tetrafluorophthalimides.

Compound	IC_{50} (nM)
1	>2 x 10^5
2	490 (± 138)
(*S*)-**4**	443 (± 68)
(*R*)-**4**	494 (± 53)
(±)-**4**	378 (± 47)
(*S*)-**6**	313 (± 26)
(*R*)-**6**	250 (± 36)
(±)-**6**	249 (± 13)
(*S*)-**7**	367 (± 25)
(*R*)-**7**	434 (± 7)
(±)-**7**	415 (± 99)
8	413 (± 57)
9	425 (± 125)

A lack of enantioselectivity was also observed for the two enantiomers of F_4CC-1104, **6**.[12] Another tetrafluorophthalimide, FPTP, **7**, showed only negligible enantiodependence, although it has been previously shown that the two enantiomers have differential inhibition of okadaic acid-induced TNF-α production in HL-60 cells.[7] These observations suggest that tetrafluorothalidomide and other related tetrafluorophthalimides differ from thalidomide in the LPS/THP-1 system in their mechanism of inhibition of TNF-α production. In addtion, where tested, these compounds exhibit comparable potency and also lack enantioselectivity against LPS-stimulated human peripheral blood monocytes.[10] We note that when different stimuli were used in nondifferentiated THP-1 cells and HL-60 cells, enantioselectivity was observed for several tetrafluorophthalimides.[13] The precise underlying mechanism is not known.

In addition to the lack of enantioselectivity of these analogs, we noticed that all three tetrafluorophthalimides exhibited more or less equal potency with IC_{50}s of around 300-400 nM (Table 1). We therefore synthesized and examined the inhibitory effects of N-phenyl tetrafluorophthalimide (FPP-00, **8**) and N-methyl tetrafluorophthalimide (FP-1, **9**). We found that both **8** and **9** are as potent as the chiral tetrafluorophthalimides with IC_{50}s of ~400 nM. These results demonstrate that the tetrafluorophthaloyl group is both necessary and sufficient for inhibition of TNF-α production in monocytes. As nonfluorinated phthalimides show enantioselectivity, these results suggest that there is a mechanistic change upon replacement of the aromatic hydrogens of thalidomide by fluorines.[12]

Three additional pieces of evidence support our contention that there is a change of mechanism upon fluorination of thalidomide. First, thalidomide, **1**, has been shown to bind AGP.[3] We examined whether tetrafluorothalidomide, **2**, or F_4CC-1104, **6**, could bind to AGP in a competitive assay using a photoaffinity label derived from thalidomide. Preliminary experiments indicate that neither **2** nor **6** compete for the thalidomide binding site on AGP (data not shown). Second, thalidomide, **1**, was found to be selective for inhibition of TNF-α secretion in human monocytes without affecting the secretion of other cytokines such as IL-6 or IL-1β.[2] However, unlike thalidomide, **1**, both (±)-tetrafluorothalidomide, **2**, and (±)-F_4CC-1104, **6**, were found to inhibit the production of IL-6 (IC_{50}: ca. 700 nM for **2** and ca. 300 nM for **6**) and IL-1β (IC_{50}: ca. 400 nM for **2** and **6**), in addition to TNF-α. Third, although thalidomide, **1**, is reported to block TNF-α production by accelerating TNF-α mRNA degradation, we did not observe this effect for tetrafluorothalidomide, **2**, or F_4CC-1104, **6** (data not shown).

To further assess the specificity of these compounds, we have also examined the effect of both **2** and **6** on PMA/ionomycin-stimulated TNF-α production in T cells. Neither **2** nor **6** affected TNF-α production in Jurkat T cells, suggesting that they are specific for the LPS-mediated signaling pathway in monocytes and ruling out the possibility that these compounds possess a pleotropic inhibitory effect on cytokine production (data not shown). Together, these results further highlight the difference between thalidomide and its closest fluorinated analog, tetrafluorothalidomide.

Fluorine is a close steric mimic of hydrogen, and incorporation of fluorine into drugs often improves their biological activity.[14] Such improvements have been attributed to either the enhanced lipophilicity of fluorinated compounds or to stereoelectronic changes imparted to compounds by fluorine substitution resulting in more avid binding to their respective target molecules. The evidence presented in this paper indicates that fluorinated thalidomide derivatives are an exceptional case in which their mechanism of action bears apparently no relation to that of the parent compound, and suggests that nonfluorinated and fluorinated phthalimides mediate inhibition of cytokine production through interaction with distinct target proteins. The dramatically increased potency of the

tetrafluorothalidomide and tetrafluorophthalimides therefore offers a unique opportunity to use these fluorinated analogs as probes of the LPS-stimulated signaling pathway in monocytes.

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9. The physical data for the new compounds are as follows:

(*S*)-**4**: mp 199-200 °C; $[\alpha]_D^{20}$ +30.7° (*C* 0.081, $CHCl_3$); ^1H NMR (δ, $CDCl_3$, 500 MHz): 2.05 (3H, m), 2.09-2.16 (1H, m), 2.71-2.76 (3H, m), 7.95 (1H, br.s); HRMS *m/z* calcd for $C_{14}H_8O_4N_2F_4$ 344.0420, found 344.0433 (M^+); Anal. calcd for $C_{14}H_8O_4N_2F_4$: C, 48.85; H, 2.34; N, 8.14. Found: C, 49.05; H, 2.47; N, 7.92.

(*R*)-**4**: mp 200-201°C; $[\alpha]_D^{20}$ -31.5° (*C* 0.121, $CHCl_3$); ^1H-NMR (δ, $CDCl_3$, 500 MHz): 2.05 (3H, m), 2.11-2.15 (1H, m), 2.71-2.76 (3H, m), 7.97 (1H, br.s); HRMS *m/z* calcd for $C_{14}H_8O_4N_2F_4$ 344.0420, found 344.0401(M^+); Anal. Calcd for $C_{14}H_8O_4N_2F_4$: C, 48.85; H, 2.34; N, 8.14. Found: C, 48.95; H, 2.47; N, 8.30.

(*S*)-**6**: $[\alpha]_D^{20}$ -3.4° (*C* 0.10, $CHCl_3$); ^1H NMR (δ, $CDCl_3$, 250 MHz): 7.1 (2H, m), 6.8 (1H, br.d), 5.70 (1H, dd, *J* = 7.1, 10.0), 3.90 (1H, s), 3.88 (3H, s), 3.79 (1H, dd, *J* = 17.5, 10.0), 3.65 (3H, s), 3.12 (1H, *J* = 17.5, 7.1); HRMS *m/z* calcd. for $C_{20}H_{15}O_6NF_4$ 441.08355, found 441.08351(M^+).

(*R*)-**6**: $[\alpha]_D^{20}$ +3.0°(*C* 0.10, $CHCl_3$); ^1H-NMR (δ, $CDCl_3$, 250 MHz): 7.1 (2H, m), 6.8 (1H, br.d), 5.70 (1H, dd, *J* = 7.1, 10.0), 3.90 (1H, s), 3.88 (3H, s), 3.79 (1H, dd, *J* = 17.5, 10.0), 3.65 (3H, s), 3.12 (1H, *J* = 17.5, 7.1); HRMS *m/z* calcd for $C_{20}H_{15}O_6NF_4$ 441.08355, found 441.08351(M^+).

8: ^1H NMR (δ, $CDCl_3$, 250 MHz): 3.12(3H, s), HRMS *m/z* calcd. for $C_9H_3O_2NF_4$ 233.00999, found 223.00992(M^+)

10. Comparable potency was observed for selected compounds [(±)-**2**, (±)-**6**] when tested using human peripheral blood monocytes. The chiral compounds, **4**, **6**, and **7** did not exhibit enantiodependence when assayed with primary human monocytes (IC_{50} values: (*S*)-**4**; 432 (±30) nM, (*R*)-**4**; 345 (±55) nM, (±)-**4**; 365 (±30) nM, (*S*)-**6**; 101 (±1) nM, (*R*)-**6**; 108 (±10) nM, (±)-**6**; 101 (±1) nM, (*S*)-**7**; 177 (±1) nM, (*R*)-**7**; 180 (±24) nM, (±)-**7**; 166 (±23) nM. All the assays were conducted at least in duplicate. None of the compounds used in this assay showed significant toxicity up to a dose of 1 μM for tetrafluorophthalimides or 200 μM for α–methylthalidomide, as judged by Trypan Blue exclusion.

11. In this system, racemic thalidomide (**1**) and (*S*)-α-methylthalidomide have roughly the same potency.

12. The IC_{50} values for the nonfluorinated CC-1104, **5**[12a] are 3.1 μM for the (*R*)-isomer and 4.5 μM for the (*S*)-isomer.[12b] (a) Corral, L. G.; Muller, G. W.; Moreita, A. L.; Chen, Y.; Wu, M.; Stirling, D.; Kaplan, G. *Mol. Medicine* **1996**, *2*, 506. (b) Muller, G.; Corral, L. G.; Shire, M. G.; Wang, H.; Moreira, A.; Kaplan, G.; Stirling, D. I. *J. Med. Chem.* **1996**, *39*, 3238.

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