AUGMENTATION BY PHTHALIMIDES OF PHORBOL ESTER-INDUCED EXPRESSION OF TUMOR NECROSIS FACTOR ALPHA MESSAGE

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N(α)-Phthalimidoglutarimide (thalidomide), 2-(2,6-diisopropylphenyl)-1*H*-isoindole-1,3-dione (PP-33) and its 4,5,6,7-tetrafluoro derivative (FPP-33) augmented 12-*O*-tetradecanoylphorbol 13-acetate-induced production by human leukemia HL-60 cells of both tumor necrosis factor alpha (TNF- α) mRNA and secreted TNF- α protein. Intracellular TNF- α protein production was increased to a lesser extent.

KEY WORDS tumor necrosis factor; phenylphthalimide; thalidomide; phorbol ester; HL-60

Tumor necrosis factor alpha (TNF- α) is a cytokine with pleiotropic biological effects which plays a critical role in certain physiological defensive responses, but causes damage to the host when produced in excess.¹⁾ TNF- α is produced by activated monocytes/macrophages, and is synthesized as a precursor protein of M_r 26K (pre-TNF- α) which is buried in the cell membrane and is processed into a secreted 17K mature form through cleavage of an Ala-Val bond between residues 76-77 by metalloproteinases.²⁾

Because TNF- α possesses both favorable effects (*e.g.*, direct tumor-killing activity and stimulation of the host immune system) and unfavorable effects (*e.g.*, induction of endotoxic shock and tissue inflammation), regulators of TNF- α production would be useful as biological response modifiers (BRMs).³⁾ Among such BRMs that regulate TNF- α production, N(α)-phthalimidoglutarimide (thalidomide, **1**) has been well documented. Thalidomide was previously used as a hypnotic/sedative agent, but was withdrawn because of its teratogenicity.⁴⁾ Despite its disappearance from the market, thalidomide is potentially useful in the treatment of graft-*versus*-host disease (GVHD), leprosy, Behcet's disease, malaria, acquired immunodeficiency syndrome (AIDS), and other related diseases.⁵⁻⁷⁾ The effects elicited by thalidomide have been attributed to its inhibitory effect on TNF- α production, although the molecular mechanism (target molecules) has not been elucidated.⁶⁻⁸⁾

Recently, we reported that thalidomide (**1**) enhanced TNF- α production by human leukemia cell line HL-60 stimulated with 12-*O*-tetradecanoylphorbol 13-acetate (TPA),⁹⁾ and we have developed potent enhancers of TNF- α production by TPA-stimulated HL-60 cells, including 2-(2,6-diisopropylphenyl)-1*H*-isoindole-1,3-dione (PP-33, **2**) and its 4,5,6,7-tetrafluoro derivative (FPP-33, **3**).^{10,11)} In this communication, we show that these three compounds affect the amounts of TNF- α mRNA and intracellular TNF- α protein (*in*-TNF- α), as well as that of secreted TNF- α protein (*ex*-TNF- α), in TPA-stimulated HL-60 cells.

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The effects of thalidomide, PP-33, and FPP-33 on TPA-induced TNF- α expression by HL-60 cells were examined at the mRNA, intracellular TNF- α protein (*in*-TNF- α), and secreted/extracellular TNF- α protein (*ex*-TNF- α) levels. Cells (5×10^5 cells/ml) were treated with 3 nM TPA or not treated, in the presence or absence of drugs (concentrations indicated in the figure and table) in RPMI 1640 medium containing 10% v/v FBS.^{10,11} Secreted *ex*-TNF- α in the medium was measured by ELISA (Amersham) as described previously.^{10,11} For assay of the *in*-TNF- α , the cells were collected, washed, and then solubilized in 1% Triton X-100. *In*-TNF- α in the lysate was measured by ELISA.^{10,11} For assay of TNF- α mRNA, total RNA was extracted from the cells by the acid guanidium-phenol-chloroform method¹² and subjected to Northern blot analysis after separation by agarose/formaldehyde gel electrophoresis and transfer to a Hybond-N⁺ nylon membrane (Amersham). The cDNA of human TNF- α (British Biotechnology) was amplified and labeled with digoxigenin by the use of a DIG DNA Labeling Kit (Boehringer Mannheim). Hybridization and detection were carried out using a DIG Luminescent Detection Kit for Nucleic Acids (Boehringer Mannheim).

As previously reported,⁹⁻¹¹ thalidomide enhanced TPA-induced TNF- α production, and PP-

Table 1. Effect of Thalidomide, PP-33 and FPP-33 on TPA-induced *ex*-TNF- α /*in*-TNF- α Production by HL-60 Cells

	Controls		Thalidomide ^a		PP-33 ^a		FPP-33 ^a	
	Negative (-TPA)	Positive ^a (+TPA)	10 μ M	100 μ M	1 μ M	10 μ M	10 nM	100 nM
<i>ex</i> -TNF- α (pg/ 10^5 cells)	<2	36	48	59	79	234	53	126
<i>ex</i> -TNF- α (%)	0	100	123	145	220	650	148	351
<i>in</i> -TNF- α (pg/ 10^5 cells)	<2	13	12	17	15	31	14	22
<i>in</i> -TNF- α (%)	0	100	92	130	115	238	107	169

a. In the presence of 3 nM TPA. Cells were incubated for 16 h.

33 and FPP-33 also showed potent TNF- α production-enhancing activity at low concentrations (Table 1). These three compounds also increased the amount of *in*-TNF- α (Table 1), but the extent of the increase was smaller than that of *ex*-TNF- α . This result suggests that the effect elicited by these compounds cannot be attributed to stimulation of the secreting/processing step of *in*-TNF- α .

Fig. 1. Northern Blot Analysis of TNF- α mRNA

Cells were treated as described in the text for the indicated period. Lane *a*: nontreated; *b*: treated with 3 nM TPA alone; *c*: treated with TPA and 100 μ M thalidomide; *d*: treated with TPA and 10 μ M PP-33; *e*: treated with TPA and 0.1 μ M FPP-33.

Northern blot analysis (Fig. 1) showed that PP-33 (10 μ M) and FPP-33 (0.1 μ M) increased the amount of TNF- α mRNA which is expressed TPA dependently. The effect of thalidomide (100 μ M) was not clear, and this is consistent with the relatively weak TNF- α production-enhancing activity (less than 150% at the above concentration) compared to those of PP-33 (650%) and FPP-33 (351%) (Table 1). The results suggest that the TNF- α production-enhancing activity of these compounds could be attributed predominantly to the increase in the TNF- α mRNA level.

It should be noted that FPP-33, which enhances *ex*-TNF- α production less efficiently than PP-33 (Table 1), seems to enhance TNF- α mRNA more efficiently than PP-33. Although we cannot interpret this discrepancy at this stage, a possible explanation would be the difference in the incubation time between assay for TNF- α mRNA (1 – 6 h) and that for *ex*-TNF- α (16 h).

Because thalidomide, PP-33 and FPP-33 are considered to be cell type- and/or inducer-specific TNF- α production regulators,^{7,9-11)} further investigation using these compounds should contribute to the understanding of the molecular mechanism of regulation of TNF- α expression.

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