(S)-FORM OF α-METHYL-N(α)-PHTHALIMIDOGLUTARIMIDE, BUT NOT ITS (R)-FORM, ENHANCED PHORBOL ESTER-INDUCED TUMOR NECROSIS FACTOR-α PRODUCTION BY HUMAN LEUKEMIA CELL HL-60: IMPLICATION OF OPTICAL RESOLUTION OF THALIDOMIDAL EFFECTS

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The rate of racemization of N(α)-phthalimidoglutarimide (thalidomide) was determined as its half life to be 566 min at pH 7.4/37℃. This fast racemization of thalidomide resulted in no apparent difference between (S)- and (R)-forms of the compound on enhancing activity of phorbol ester-induced tumor necrosis factor (TNF)-α production by human leukemia HL-60 cells. Optically pure forms of structurally related analog of thalidomide, (S)- and (R)-α-methyl-N(α)-phthalimidoglutarimides (methylthalidomides), which do not racemize under the physiological condition, were prepared. Only (S)-form of methylthalidomide, but not its (R)-form, elicited TNF-α production-enhancing effect, suggesting that the (S)-isomer of thalidomide would be the active form in terms of thalidomidal biological response modifying effects.

KEYWORDS thalidomide; racemization; tumor necrosis factor; α-methyl-N(α)-phthalimidoglutarimide

N(α)-Phthalimidoglutarimide (thalidomide) is a hypnotic/sedative agent which has been withdrawn from the market mostly because of its teratogenicity.[1] In spite of this, the drug has been drawing attention as an orphan drug which would be useful in treatment of graft-versus-host disease (GVHD), leprosy, Behcet's disease, acquired immunodeficiency sydrome (AIDS), and other related diseases.[2,3] The usefulness of thalidomide in the treament of these diseases has partly been ascribed to its activity as a biological response modifier (BRM) which regulates tumor necrosis factor (TNF)-α production by stimulated macrophages.[3,4]

Recently, we reported that thalidomide enhanced 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced production of TNF-α by human leukemia cell lines including HL-60, K562 and U937.[5] Though the widely prevailing hypothesis is that only one optical isomer of thalidomide is biologically active, we did not observe any difference between (S)- and (R)-forms of the compound on the TNF-α production-enhancing activity, probably because of its fast racemization.[5]

For the further development of superior thalidomidal BRM's as well as the investigation of molecular mechanisms of thalidomidal action, it would be mandatory to know which optical isomer is the active form. In this paper, we report the rate of racemization of thalidomide and TNF-α production enhancing activity of a non-racemizable thalidomide derivative, i.e., α-methyl-N(α)-phthalimidoglutarimide (methylthalidomide).

First we investigated the rate of racemization of thalidomide. Optically pure isomers of thalidomide were prepared as described previously.[5] Each isomer was incubated under various conditions, and the racemization was monitored by high performance liquid chromatography (HPLC) using the chiral separation column CHIRALCEL OJ (Daicel Chemical Industry) eluted with ethanol [the separation factor (α) of (R)- and (S)-forms was 1.211]. As an example, the result of one set of typical experiments using (R)-thalidomide incubated under various buffer conditions is shown in Fig. 1. The half completion time ($t_{0.5}$) of

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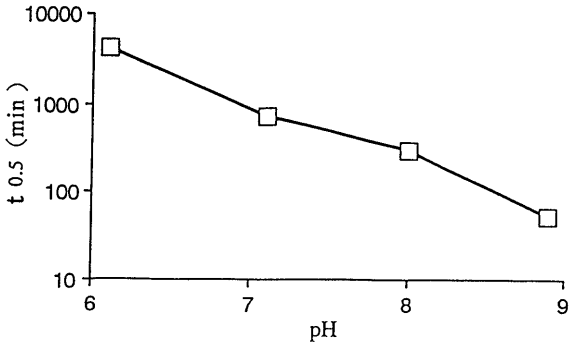

Fig. 1. Rate of Racemization of (R)-Thalidomide

(R)-Thalidomide was incubated in citrate (10mM), phosphate (50mM), or Tris-HCl (10 mM) buffers of indicated pH at 37℃. The contents of (R)- and (S)-forms were measured by HPLC, and the rate [$t_{0.5}$ (min): half completion time of the racemization was defined as the incubation period necessary to reach the ratio of the starting isomer versus its enantiomer was defined to be 2:1] was plotted against pH.

racemization of thalidomide at pH 7.4/37℃ was determined to be 566 min under the experimental conditions. The racemization was not observed during incubation for 48 h at pH lower than 6. Our results shown in Fig. 1 suggest that optically pure forms of thalidomide racemize fast under the physiological conditions, even though the racemization rate would be different in the cells. This fast racemization would make it difficult to analyze biological activity differences between enantiomers of thalidomide.

To overcome this problem, we prepared a non-racemizable thalidomide analog, methylthalidomide. As the fast racemization of thalidomide is attributed to its acidic proton at the α-position, its conversion to methyl group is expected to inhibit the racemization. Methylthalidomide was prepared as racemate by the method described by Knabe & Omlor.[6] Optical resolution of the correctly analyzed (±)-methylthalidomide was performed by HPLC equipped with CHIRALCEL OD column eluted with n-hexane:ethanol (80:20) at 40℃ (Daicel Chemical Industry) with the separation factor (α) of 1.372. The optical purities of (S)- and (R)-methylthalidomides thus obtained were more than 99 %ee each by HPLC analysis, and showed $[\alpha]_D^{22}$ = + 31.0 °, c= 1.00, dioxan (lit. + 38.5 °)[6] and $[\alpha]_D^{22}$ = - 36.9 °, c=1.00, dioxan (lit. - 38.2 °)[6], respectively.

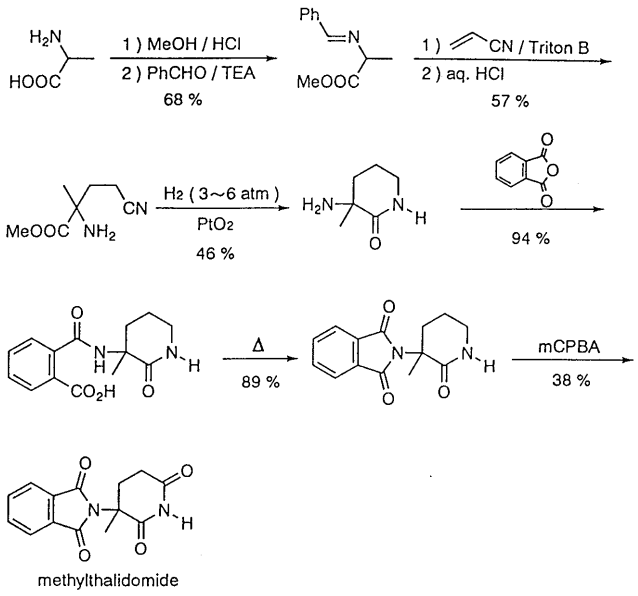

Chart 1. Preparation of Methylthalidomide

Methylthalidomide was prepared from alanine with the overall yield of 5%. m.p. 248-249.5 ℃, Mass (M+H) 273, ^{1}H-NMR(d_6-DMSO): 1.91 (s, 3H), 2.16 (m, 1H), 2.57 (m, 2H), 2.70 (m, 1H), 7.85 (m, 4H), 11.01 (s, 1H). Anal., Calcd for $C_{14}H_{12}N_2O_4$, C, 61.75; H, 4.45; N, 10.29. Found, C, 61.64; H, 4.44; N, 10.31.

Effects on TPA-induced TNF-α production by HL-60 cells of prepared compounds were measured according to the previously described method.[5] Briefly, exponentially growing HL-60 cells (2 x 10^5 cells/ml) were treated with 1 nM TPA in the presence or absence of the compounds (concentration indicated) at 37 ℃ for

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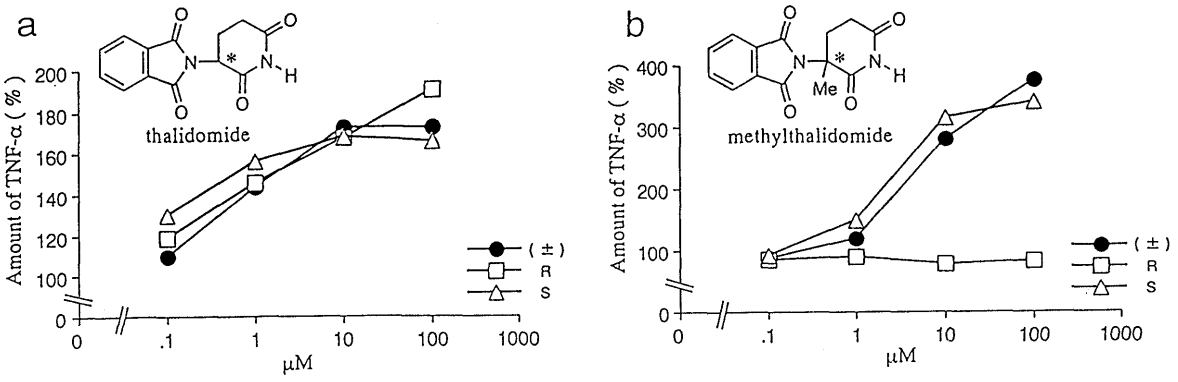

Fig. 2. Enhancement of TPA-Induced TNF-α Production by HL-60 Cells
(a) Thalidomide. (b) Methylthalidomide.

24 h. Then the concentration of TNF-α in the cell culture supernatant was measured by the use of human TNF-α ELISA system (Amersham Co.)(Fig. 2). TNF-α production by HL-60 was TPA-dependent,[5] and the concentration of TNF-α produced in the presence of 1 nM TPA alone was defined as 100%. As expected, both (S)- and (R)-thalidomides, as well as its racemate, similarly enhanced the TNF-α production dose-dependently (Fig. 2a). On the other hand, methylthalidomide showed differences between (S)- and (R)-enantiomers in their TNF-α production-enhancing activity: only (S)-enantiomer was active and no effects was observed by addition of (R)-form (Fig. 2b). Moreover, response of HL-60 cells in TNF-α production enhancement was higher for (S)-methylthalidomide (approximately 350% at 100μM) compared with thalidomide (approximately 170% at 100 μM). This might be attributed to higher hydrophobicity of methylthalidomide than of thalidomide.[7]

In conclusion, [1] we determined the rate of racemization of thalidomide, which suggests difficulty in distinguishing activity elicited by (S)- and (R)-thalidomides in general biological assay, [2] we found that (S)-form, but not (R)-form, of methylthalidomide is active in TPA-induced TNF-α production enhancement, which suggests that, in thalidomide, (S)-form would also be the active form. Methylthalidomide might be a useful probe to distinguish thalidomidal activity elicited by (S)- and/or (R)-thalidomide(s). Though the teratogenic activity of methylthalidomide has not been reported as far as we know, it was reported that the sedative effect of methylthalidomide is far weaker than that of thalidomide.[7] In our TPA-induced TNF-α production enhancement assay system, (S)-methylthalidomide seemed to be more active than thalidomide. Further structural modification for the development of thalidomidal BRM's seems promising.

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