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Cell differentiation inducers derived from thalidomide

Tomomi Noguchi, Hiroyuki Miyachi, Ryohei Katayama, Mikihiko Naito and Yuichi Hashimoto*

Institute of Molecular and Cellular Biosciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan

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Abstract—5-Hydroxy- and 4-amino-2-(2,6-diisopropylphenyl)-1*H*-isoindole-1,3-dione (5HPP-33 and 4APP-33, respectively) have been shown to possess cell differentiation-inducing activity toward human leukemia cell line HL-60.

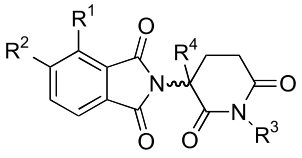
1. Introduction

Thalidomide (Thal: **1**, Fig. 1), which was once marketed as a sedative agent, but subsequently withdrawn because of its severe teratogenicity, is now known to have immunomodulatory activity, and is effective for the treatment of various diseases, including leprosy, multiple myeloma (MM), AIDS, and various cancers.[1–5] The anti-MM activity of Thal (**1**) is of particular interest, because it overcomes the resistance of human MM cells to conventional drug therapy.[6] Although various pharmacological effects elicited by Thal (**1**), including tumor necrosis factor (TNF)-α production-regulating activity, anti-angiogenic activity, cyclooxygenase (COX)-inhibiting activity, and nitric oxide synthase (NOS)-inhibiting activity,[1–5,7–9] have been reported, the mechanism of its anti-MM activity is unclear. One possibility would be differentiation-inducing activity.

We recently reported that Thal (**1**) and its two major metabolites, 5-hydroxythalidomide (5HT: **2**) and *N*-hydroxythalidomide (NHT: **3**) (Fig. 1), possess potent cell differentiation-enhancing activity toward human leukemia cell line HL-60.[10] However, although they enhanced the HL-60 cell differentiation induced by all-*trans*-retinoic acid (ATRA), none of them showed intrinsic cell differentiation-inducing activity. Nevertheless, their enhancing effect is apparent at the physiological concentration of ATRA, suggesting that thalidomide may act as if it were a differentiation inducer under physiological conditions.

Now, a screening study of our thalidomide-related compounds has shown that 5-hydroxy- and 4-amino-2-(2,6-diisopropylphenyl)-1*H*-isoindole-1,3-dione [5HPP-33 (**8**) and 4APP-33 (**9**), respectively (Table 1)] each possess direct HL-60 cell differentiation-inducing activity.

In this letter, we describe the HL-60 cell differentiation-inducing activity of 5HPP-33 (**8**) and 4APP-33 (**9**), assessed by means of nitroblue tetrazolium (NBT) assay, and we present the results of fluorescence-activated cell sorter (FACS) analysis of the treated cells.



	R^1	R^2	R^3	R^4	
1:	H	H	H	H	Thalidomide (Thal)
2:	H	OH	H	H	5-Hydroxythalidomide (5HT)
3:	H	H	OH	H	*N*-Hydroxythalidomide (NHT)
4:	H	NH_2	H	CH_3	5-AMT
5:	NH_2	H	H	CH_3	4-AMT

Figure 1. Structures of thalidomide (**1**) and its two major metabolites, 5-hydroxythalidomide (**2**) and *N*-hydroxythalidomide (**3**).

Keywords: Thalidomide; HL-60; Cell differentiation; Phenylphthalimide.
* Corresponding author. Tel.: +3 5841 7848; fax: +3 5841 8495; e-mail: bmcyfh@iam.u-tokyo.ac.jp

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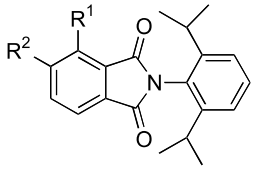
Table 1. HL-60 cell differentiation-inducing activity of compounds **1**–**10**



Compound	R^1	R^2	Concentration (M)	Cell proliferation (%)	NBT positivity (%)
None	—	—	—	100	2
ATRA	—	—	2×10^{-9}	45	52
1,25-$(OH)_2$-VD_3	—	—	1×10^{-8}	55	39
1: Thal	—	—	1×10^{-5}	101	1
2: 5HT	—	—	1×10^{-5}	93	2
3: NHT	—	—	1×10^{-5}	100	1
4: 5AMT	—	—	1×10^{-5}	103	1
5: 4AMT	—	—	1×10^{-5}	92	2
6: PP-33	H	H	1×10^{-5}	24	9
7: 4HPP-33	OH	H	1×10^{-5}	76	3
8: 5HPP-33	H	OH	1×10^{-6}	94	3
8: 5HPP-33	H	OH	3×10^{-6}	43	8
8: 5HPP-33	H	OH	5×10^{-6}	10	55
9: 4APP-33	NH_2	H	3×10^{-6}	73	2
9: 4APP-33	NH_2	H	1×10^{-5}	25	16
10: 5APP-33	H	NH_2	1×10^{-5}	53	5

2. Results and discussion

Compounds **1**–**10** were prepared by usual organic synthetic methods, as described previously.[10–13] The structures of the synthesized compounds were confirmed by NMR and mass spectroscopy and elemental analysis.

First, we investigated the HL-60 cell differentiation-inducing activity of the compounds. Measurement of HL-60 cell differentiation was performed, as described previously.[14] Briefly, HL-60 cells were incubated in RPMI1640 medium in the presence or absence of a test compound for 3 days. Treated HL-60 cells were mixed with phosphate-buffered saline (PBS) containing 0.2% NBT and 20 nM 12-*O*-tetradecanoylphorbol 13-acetate (TPA) in a 1:1 (v/v) ratio and incubated at 37 °C for 20 min. NBT positivity was measured by counting 200–300 cells and the results were expressed as the percentage of NBT-positive cells. The cell differentiation was also confirmed morphologically by microscopy after Wright–Giemsa staining, using ATRA and 1α, 25-dihydroxyvitamin D_3 [1,25-$(OH)_2$-VD_3] as positive control compounds, which have been established to induce differentiation of HL-60 cells to mature granulocytes and monocytes, respectively. Of course, the percentage values differed from experiment to experiment, but the results were basically reproducible and a typical set of data is presented in Table 1.

As shown in Table 1, Thal (**1**) and its two metabolites, 5HT (**2**) and NHT (**3**), did not show HL-60 cell differentiation-inducing activity, in agreement with a previous report.[10] Recently, a group at Celgene Co. reported that introduction of an amino group into the 4-position of Thal (**1**) (corresponding to R^1 in Fig. 1), that is, CC-4047 (Actimid) results in enhanced anti-MM activity.[1] In addition, we reported that introduction of a methyl group at the α-nitrogen atom of Thal (**1**) enhances various biological activities, including TNF-α production-regulating activity and COX-inhibiting activity.[7,13,15] Therefore, we examined the effects of both an amino group and a methyl group, that is, 5AMT (**4**) and 4AMT (**5**). Briefly, 3-amino-3-methylpiperidine-2-one was treated with *o*- or *meta*-nitrophthalic anhydride. Oxidation of the piperidine ring with 3-chloroperoxybenzoic acid, followed by reduction of the nitro group, gave 5AMT (**4**) and 4AMT (**5**). But these compounds did not show any differentiation-inducing activity.

On the other hand, during our structural development studies of Thal (**1**),[2–5] we had noticed that 2-(2,6-diisopropylphenyl)-1*H*-isoindole-1,3-dione (PP-33: **6**, Table 1) showed moderate cell growth-inhibitory activity, which led us to investigate its cell differentiation-inducing activity. PP-33 (**6**) was judged to possess weak HL-60 cell differentiation-inducing activity (9% NBT positivity at 10 μM, Table 1), so we next investigated the activity of various derivatives of PP-33 (**6**).

Introduction of an electron-withdrawing group(s), including a nitro group and halogens, resulted in compounds with rather high cytotoxicity (data not shown). However, we found active compounds among the derivatives bearing an electron-donating group, that is, 5HPP-33 (**8**) and 4APP-33 (**9**). 5HPP-33 (**8**) showed potent and dose-dependent HL-60 cell differentiation-inducing activity, as shown in Table 1. The NBT positivity value of the cells treated with 5 μM 5HPP-33 (**8**) reached 55%, though the relative ratio of NBT positivity and cell growth inhibition of 5HPP-33 (**8**) was worse than that of ATRA or 1,25-$(OH)_2$-VD_3. The positional isomer of 5HPP-33 (**8**), that is, 4HPP-33 (**7**), did not

show apparent cell differentiation-inducing activity even at 10 μM concentration. Interestingly, 5APP-33 (**10**), which is isoelectronic to active 5HPP-33 (**8**), was inactive, but its regioisomer, 4APP-33 (**9**), which is isoelectronic to inactive 4HPP-33 (**7**), was active, though the activity was lower than that of 5HPP-33 (**8**). We cannot interpret the structure–activity relationship at this stage.

Next, we analyzed the treated HL-60 cells by means of FACS to characterize the differentiated cell type. It has been well established that various cell surface clusters of differentiation antigens (CDs) are induced at particular stages/types of differentiation. Among such antigens, CD11b and CD14 are well characterized as granulocyte/monocyte-specific and monocyte-specific antigens, respectively.[16] So, we analyzed the expression of these antigens by indirect immunofluorescence staining and flow cytometry.[16] Briefly, HL-60 cells, treated or not treated with test compounds (1×10^6 cells), were washed with phosphate-buffered saline (PBS) and incubated with fluorescent agent-conjugated antibody [monoclonal anti-human CD11b FITC (fluorescein isothiocyanate) conjugate, mouse IgG1 isotype, Sigma (FITC-CD11b) or monoclonal anti-human CD14 clone UCHM-1 PE (*R*-phycoerythrin) conjugate, mouse immunoglobulin, Sigma (PE-CD14)] in PBS containing 0.5% bovine serum albumin (BSA) and 0.1% NaN_3 (staining buffer) at 4 °C for 30 min. After the incubation, the cells were washed with staining buffer, treated with paraformaldehyde (1% in PBS), and then analyzed with a flow cytometer (Cytomics FC500, Beckman Coulter). The results of FACS analysis are presented in Figure 2.

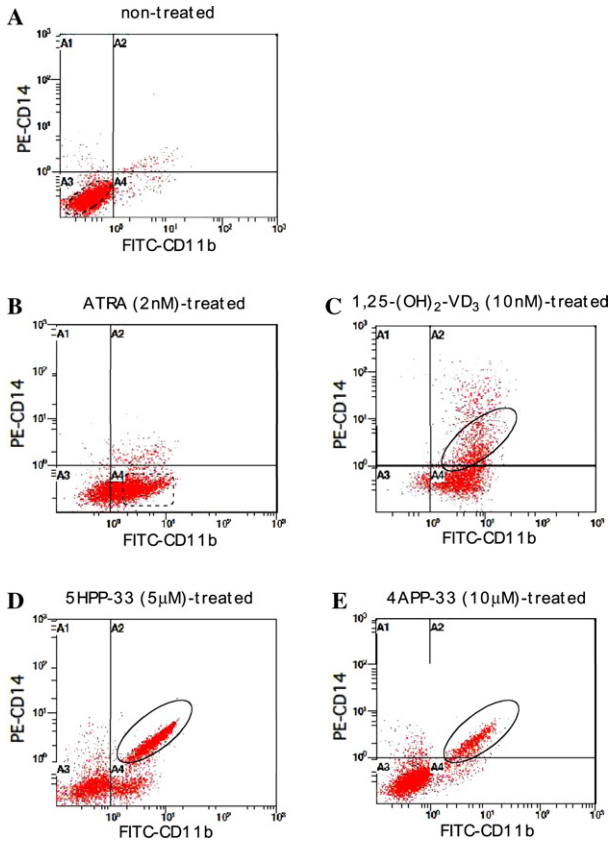


Figure 2. FACS analysis of HL-60 cells treated with ATRA, 1,25-$(OH)_2$-VD_3, 5HPP-33 (**8**), and 4APP-33 (**9**). Vertical and horizontal scales: relative fluorescence of PE-CD14 and FITC-CD11b, respectively.

As shown in Figure 2, the majority of non-treated HL-60 cells apparently did not express CD11b or CD14, that is, almost all the cells are distributed in the left bottom A3 region (panel A). ATRA treatment results in a shift of the distribution to the right bottom A4 region (panel B), which indicates induction of granulocyte/monocyte-specific CD11b antigen expression, but not monocyte-specific CD14 antigen. This result is in accordance with the established ATRA-induced HL-60 cell differentiation of mature granulocytes. Treatment with 1,25-$(OH)_2$-VD_3 resulted in a shift of the distribution to the right upper A2 region (panel C), indicating induction of both CD11b and CD14 antigen expression, and therefore the treated cells were suggested to be differentiated to mature monocytes, because CD14 is monocyte-specific. This result is also in accordance with the established 1,25-$(OH)_2$-VD_3-induced HL-60 differentiation to monocytes. The patterns of FACS analysis of 5HPP-33 (**8**)- and 4APP-33 (**9**)-treated cells are very similar to that of 1,25-$(OH)_2$-VD_3-treated cells, though the shift of distribution to the A4 region is less apparent than with the latter (panels D and E). The results suggest that 5HPP-33 (**8**) and 4APP-33 (**9**) induce HL-60 cell differentiation to mature monocytes, as 1,25-$(OH)_2$-VD_3 does. However, monocytic differentiation induction by 5HPP-33 (**8**) and 4APP-33 (**9**) was not inhibited by *N*-benzyl-1α,25-dihydroxyvitamin D_3-26,23-lactam [(23*S*,25*S*)-DLAM-1P], which is a competitive vitamin D_3 antagonist.[17] The results suggest that nuclear vitamin D_3 receptor (VDR) is not associated with the 5HPP-33 (**8**)- and/or 4APP-33 (**9**)-induced monocytic cell differentiation. VDR is established to act as a heterodimer with another nuclear receptor, retinoid X receptor (RXR). 5HPP-33 (**8**) and 4APP-33 (**9**) did not bind to or activate RXR (data not shown). The molecular target and mechanism of 5HPP-33 (**8**)- and 4APP-33 (**9**)-induced cell differentiation remain to be investigated.

In conclusion, we have found two small-molecular, thalidomide-related HL-60 cell monocytic differentiation inducers, 5HPP-33 (**8**) and 4APP-33 (**9**). Typical HL-60 monocytic differentiation inducers so far known are rather large and complex molecules, including 1,25-$(OH)_2$-VD_3 and TPA. Though the activity of 5HPP-33 (**8**) and 4APP-33 (**9**) is of micromolar order, these compounds appear to be unique as differentiation inducers because of their small size and simple structures. Molecular-level investigation of the cell differentiation-induction mechanism of these compounds and further structural development studies are continuing.

Acknowledgments

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