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Tubulin-polymerization inhibitors derived from thalidomide

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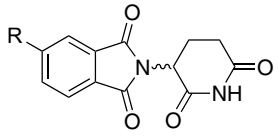
Abstract—2-(2,6-Diisopropylphenyl)-5-hydroxy-1*H*-isoindole-1,3-dione (5HPP-33), which was obtained during our previous structural development studies on thalidomide, was revealed to possess potent tubulin-polymerization-inhibiting activity, comparable to that of the known tubulin-polymerization inhibitors, rhizoxin and colchicine. A major metabolite of thalidomide, 5-hydroxythalidomide, which possesses a hydroxyl group at the position corresponding to that of 5HPP-33, also showed moderate inhibitory activity.

1. Introduction

Thalidomide (**1**: Fig. 1) is a sedative/hypnotic drug which was once withdrawn from the market in the 1960s because of its severe teratogenicity.[1,2] However, the drug has been established to be effective for the treatment of various diseases, including leprosy, myeloma, AIDS, and others.[3–5] It was approved in the United States for the treatment of leprosy in 1998, and clinical studies of its use for the treatment of various cancers, including multiple myeloma, colon cancer, prostate tumor, and breast cancer are on-going. The molecular mechanisms of the multiple pharmacological actions elicited by thalidomide are not clear, but its tumor necrosis factor (TNF)-α production-inhibitory activity has been well-documented.[4–6]



1: R=H, Thalidomide
2: R=OH, 5-Hydroxythalidomide

Figure 1. Structures of thalidomide (**1**) and one of its known metabolite, 5-hydroxythalidomide (**2**).

We have demonstrated that the TNF-α production-regulating activity of thalidomide is bidirectional, and that thalidomide is a multi-target drug.[2,4,5] In structural development studies of thalidomide, we have obtained TNF-α production regulators (including bidirectional ones, pure inhibitors, and pure enhancers),[7,8] androgen antagonists,[9–11] aminopeptidase inhibitors,[12–14] α-glucosidase inhibitors,[15,16] thymidine phosphorylase inhibitors,[17] cyclooxygenase (COX) inhibitors,[18,19] and nitric oxide synthase (NOS) inhibitors.[20,21] In the course of those studies, we noticed that some phenylphthalimide derivatives show potent cell proliferation-inhibiting activity toward human leukemia cell lines HL-60 (Table 1), THP-1 and IM9. Among the compounds listed in the table, 2-(2,6-diisopropylphenyl)-5-hydroxy-1*H*-isoindole-1,3-dione (5HPP-33: **11**), showed the most potent activity, with the IC_{50} value of approximately 5 μM. This cell growth-inhibitory activity seemed to be specific to 5HPP-33, though some features of the structure–activity relationship could be deduced. For example, introduction of a substituent (hydroxyl, amino or nitro group) at the 5-position seems to generate the activity [4HPP-00 (**6**), 4HPP-11 (**7**), 4APP-33 (**12**), and 4NPP-33 (**14**) are inactive, while the corresponding 5HPP-00 (**9**), 5HPP-11 (**10**), 5APP-33 (**13**), and 5NPP-33 (**15**) are active; the exception is 4HPP-33 (**8**), though the corresponding 5HPP-33 (**11**) is far more potent]. Detailed observation of cells treated with 5HPP-33 (**11**) indicated that the morphological change of the treated cells is similar with that of cells treated with tubulin-polymerization inhibitors, such as rhizoxin and colchicines, and we considered that 5HPP-33 might elicit its

Keywords: Thalidomide; Tubulin; Polymerization; Inhibitor.
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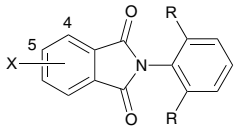
Table 1. Cell growth-inhibitory activity of phenylphthalimide derivatives (**3**–**15**) toward human leukemia cell line HL60



Compound	R	X	Growth inhibition (%)		
			10 μM	6 μM	3 μM
PP-00 (**3**)	H	H	~0	~0	~0
PP-11 (**4**)	CH_3	H	~0	~0	~0
PP-33 (**5**)	$CH(CH_3)_2$	H	<5	~0	~0
4HPP-00 (**6**)	H	4-OH	~0	~0	~0
4HPP-11 (**7**)	CH_3	4-OH	~0	~0	~0
4HPP-33 (**8**)	$CH(CH_3)_2$	4-OH	8	~0	~0
5HPP-00 (**9**)	H	5-OH	34	<5	~0
5HPP-11 (**10**)	CH_3	5-OH	6	~0	~0
5HPP-33 (**11**)	$CH(CH_3)_2$	5-OH	~100	72	6
4APP-33 (**12**)	$CH(CH_3)_2$	4-NH_2	~0	~0	~0
5APP-33 (**13**)	$CH(CH_3)_2$	5-NH_2	23	<5	~0
4NPP-33 (**14**)	$CH(CH_3)_2$	4-NO_2	~0	~0	~0
5NPP-33 (**15**)	$CH(CH_3)_2$	5-NO_2	16	<5	~0

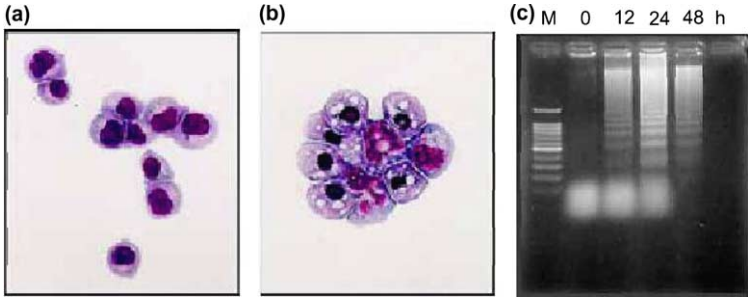


Figure 2. Morphological change of human myeloma cells, IM9, treated with 10 μM 5HPP-33 (**11**) and DNA ladder formation of the treated cells. Panel a: control IM9 cells. Panel b: IM9 cells treated with 5HPP-33 (**11**). Panel c: agarose gel electrophoresis of DNA extracted from the treated cells at the indicated times after the start of incubation with 10 μM 5HPP-33 (**11**). Line M: molecular weight markers.

cell growth-inhibitory activity by cell cycle arrest and inducing apoptosis of the cells (Fig. 2, detailed will be published elsewhere). The observations led us suspect tubulin as a target molecule of 5HPP-33. In fact, Hu et al. reported briefly that 5HPP-33 inhibits depolymerization of tubulin.[22] In this paper, we report (1) the effect of 5HPP-33 (**11**) and its derivatives on tubulin polymerization, and (2) the discovery of tubulin-polymerization-inhibiting activity of a known thalidomide metabolite, 5-hydroxythalidomide (**2**).

2. Results and discussion

First we investigated the effect of thalidomide (**1**), 5HPP-33 (**11**) and various derivatives on tubulin polymerization using the method previously described.[23,24] Briefly, microtubule protein was prepared from porcine brain.[25,26] Tubulin polymerization was followed by turbidity measurements at 37 °C in microtubule assemble buffer containing 100 mM 2-morpholinoethanesulfonic acid (MES), 1 mM EGTA, 0.5 mM $MgCl_2$, 1 mM 2-mercaptoethanol and 1 mM GTP (pH 6.5). Although the quantitative values differed from experiment to experiment, the results were basically reproducible. Typical sets of data are presented in Table 2 and Figure 3. Synthesis of all the compounds listed in Table 1 and their chemical/physical analytical data have already been reported.[27]

As shown in Table 2, 5HPP-33 (**11**) and its isoelectronic derivative, 5APP-33 (**13**), showed potent tubulin-polymerization-inhibiting activity with efficacy comparable to that of rhizoxin or colchicine. The structural requirement for the activity seems to be critical, because other analogs investigated, including regio-isomers of 5HPP-33 (**11**) and 5APP-33 (**13**) (4HPP-33: **12** and 4APP-33: **14**, respectively), and derivatives of 5HPP-33 (**11**) with less bulky alkyl groups (5HPP-00: **9** and 5HPP-11: **10**), showed no (**5**, **9**, **10**, **12**), or only slight (**8**) tubulin-polymerization-inhibiting activity. Nitro-substituted analogs (4NPP-33: **14** and 5NPP-33: **15**) were also inactive. The results suggest that the tubulin-polymerization-inhibiting activity of phenylphthalimide analogs is a spe-

Table 2. Tubulin-polymerization-inhibiting activity of phthalimide analogs

Compound	Polymerization inhibition (%)[a]	Compound	Polymerization inhibition (%)[a]
PP-33 (**5**)	<10	Thalidomide (**1**)	<10
4HPP-33 (**8**)	7	5-Hydroxy-thalidomide (**2**)[b]	32
5HPP-00 (**9**)	<10	Rhizoxin	88
5HPP-11 (**10**)	<10	Colchicine	78
5HPP-33 (**11**)	83		
4APP-33 (**12**)	<10		
5APP-33 (**13**)	81		
4NPP-33 (**14**)	<10		
5NPP-33 (**15**)	<10		

[a] Calculated from the change of the turbidity at 400 nm after incubation in the presence of test compound (20 μM) for 30 min at 37 °C compared with that after incubation in the absence of the test compound.

[b] The concentration of the test compound was changed to 50 μM.

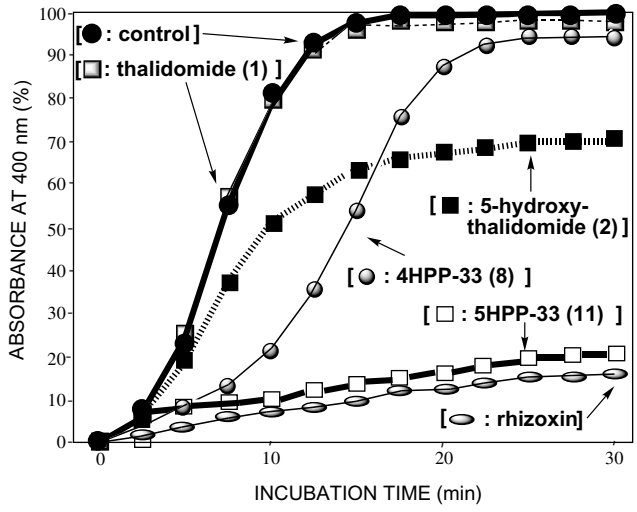


Figure 3. Time course curves of inhibition of tubulin polymerization monitored in terms of turbidity (absorbance at 400 nm). A tubulin preparation was incubated at 37 °C in the presence or absence (control) of test compound. The test concentration was 10 μM for 4HPP-33 (**8**), 5HPP-33 (**11**) and rhizoxin, and 50 μM for thalidomide (**1**) and 5-hydroxythalidomide (**2**).

cific feature of the 2,6-di-isopropylphenylphthalimide structure substituted with an electron-donating group at the 5-position.

Although the tubulin-polymerization-inhibiting activity of 5HPP-33 (**11**) is consistent with the cell growth-inhibitory activity of the compound, that of 5APP-33 (**13**) is not (Table 1). This might be explained by a difference in the cell membrane permeability of the two compounds (details will be discussed elsewhere).

Time course curves of the inhibition of tubulin polymerization by selected compounds are shown in Figure 3. 4-HPP-33 (**8**), a regio-isomer of 5HPP-33 (**11**), showed an unusually shaped curve. Though we cannot yet interpret this phenomenon, the result was reproducible in repeated. 4HPP-33 (**8**) appears to retard the initiation of tubulin polymerization, while having little effect on the final degree of tubulin polymerization.

Next we investigated the tubulin-depolymerization-inhibiting activity of 5HPP-33 (**11**). Hu et al. briefly reported that 5HPP-33 shows inhibitory activity toward depolymerization of tubulin.[22] Depolymerization of tubulin was induced by cooling the tubulin fraction polymerized at 37 °C to 0 °C, or by addition of 4 mM $CaCl_2$ at 37 °C, and was followed by means of turbidity measurements as described previously.[23–25] As shown in Figure 4 (circles), once-polymerized tubulin was depolymerized by cooling (Fig. 4, black circles) or by addition of $CaCl_2$ (Fig. 4, gray circles). The tubulin depolymerized by cooling could be re-polymerized again by warming at 37 °C. The addition of 10 μM 5HPP-33 (**11**) to polymerized tubulin at 37 °C did not affect the cooling- or $CaCl_2$-induced depolymerization step (Fig. 4, gray squares). As expected, the tubulin depolymerized by cooling did not re-polymerize by warming on the presence of 5HPP-33 (**11**) (Fig. 4, gray squares). Thus, 5HPP-33 (**11**) showed no inhibiting effect on tubulin depolymerization, at least in our system, which is contrary to the result reported by Hu et al.[22] Our finding indicates that 5HPP-33 (**11**) binds only to heterodimeric α/β-tubulin protein, but not to polymerized tubulin.

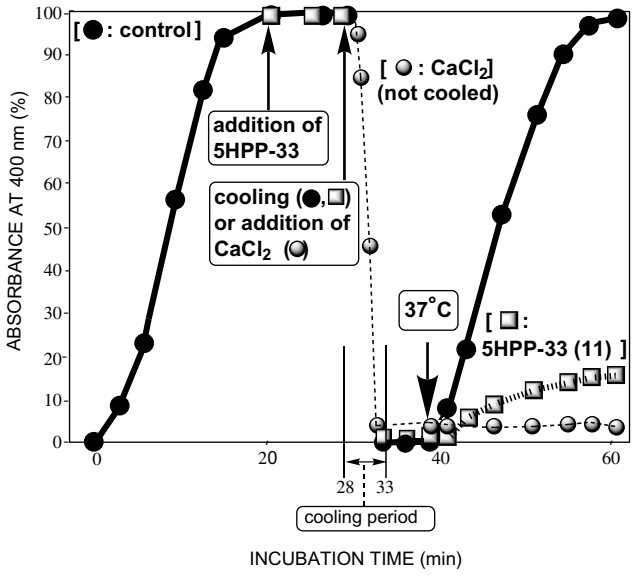


Figure 4. Time course curves of tubulin polymerization/depolymerization monitored in terms of turbidity (absorbance at 400 nm). A tubulin preparation was incubated at 37 °C (starting at time 0 min) to polymerize it in the absence of 5HPP-33 (**11**), then cooled to 0 °C (black circles, the cooling was started at time 28 min) in the absence (black circles) or presence of 10 μM 5HPP-33 (**11**) (gray squares: 5HPP-33 was added at time 20 min). The time course of cooling-induced depolymerization (cooling period: time 28–33 min) was not monitored (black circles and gray squares). The treated tubulin fractions were heated again to 37 °C at the time 38 min. Gray circles: the polymerized tubulin was treated with 4 mM $CaCl_2$ (added at time 28 min) without cooling.

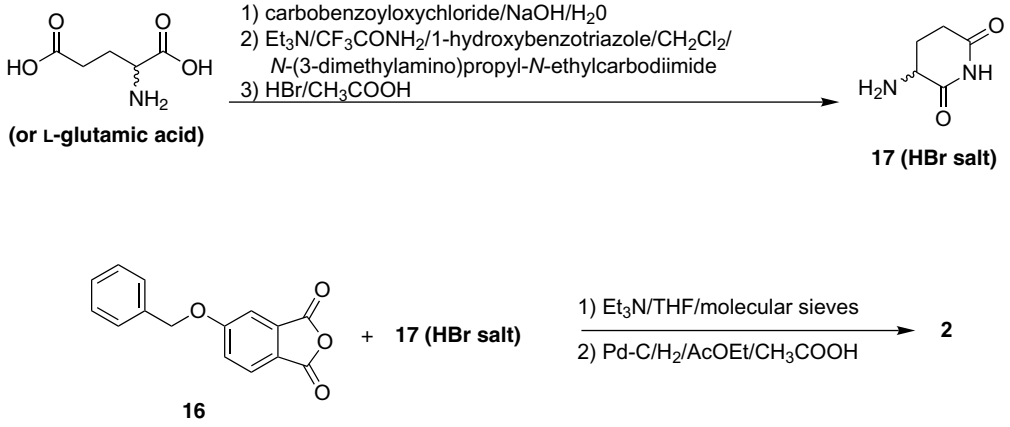


Figure 5. Preparation of 5-hydroxythalidomide (**2**).

The potent cell growth-inhibitory activity and tubulin-polymerization-inhibiting activity of 5HPP-33 (**11**) led us suspect a relation between inhibition of tubulin polymerization and the usefulness of thalidomide (**1**) as an anti-tumor agent, especially for multiple myeloma. Recently, so-called immunomodulatory drugs based on thalidomide (IMiDs), especially amino-substituted thalidomide analogs, have been reported to be superior candidates for anti-tumor agents.[6] IMiDs, as well as thalidomide itself, were found to possess the ability to directly induce growth arrest and caspase-dependent apoptosis of tumor cells, though the activity of thalidomide (**1**) is less potent than those of IMiDs.[6,28] These activities elicited by thalidomide (**1**) and IMiDs are similar to those elicited by 5HPP-33 (**11**). Though thalidomide itself did not show any tubulin-polymerization-inhibiting activity (Table 2 and Fig. 3), one or more of its metabolites (or chemical decomposition products) might possess tubulin-polymerization-inhibiting activity. Thalidomide (**1**) is known to be both chemically and metabolically unstable, and the structures of various metabolites have been reported.[29] Because the 5-hydroxyl group of 5HPP-33 (**11**) seemed to be critical for the tubulin-polymerization-inhibiting activity, we focused on the thalidomide metabolite with a hydroxyl group at the corresponding position, that is, 5-hydroxythalidomide (**2**), which is a major microsomal metabolite of thalidomide.[29]

5-Hydroxythalidomide (**2**) was prepared by condensation of 4-benzyloxyphthalic anhydride (**16**)[30] with 3-aminopiperidine-2,6-dione (**17**),[31,32] which was derived from racemic glutamic acid, followed by deprotection (Fig. 5).[33] Optically pure *S*-form of 5-hydroxythalidomide could be obtained by using L-glutamic acid in place of racemic glutamic acid, but it readily undergoes racemization under physiological conditions (pH 7, 37 °C, less than 12 h).[34] Therefore, we used racemic 5-hydroxythalidomide (**2**) in the following experiments.

As shown in Table 2 and Figure 3, 5-hydroxythalidomide (**2**) showed moderate tubulin-polymerization-inhibiting activity. Though the activity is less potent than that of 5HPP-33 (**11**), the activity might explain the usefulness of thalidomide (**1**) as an anti-tumor agent, at least in part. In this sense, 5-hydroxythalidomde (**2**) might be an active form of thalidomide (**1**), although the cell growth inhibitory activity of 5-hydroxythalidomide (**2**) is weaker than that of 5HPP-33 (**11**) [5-hydroxythalidomide (**2**) inhibited the cell growth of HL-60 cells with a potency of 20–25% at 10 μM, while 5-HPP-33 (**2**) inhibited HL-60 cell growth almost completely at the same concentration]. Possible biological activities of other thalidomide metabolites remain to be investigated.

In conclusion, we have developed a potent tubulin-polymerization inhibitor, 5HPP-33 (**11**), by structural elaboration of thalidomide. Based on the structural relationship between 5HPP-33 (**11**) and one of the major metabolites of thalidomide, 5-hydroxythalidomide (**2**), we found that the metabolite also possesses tubulin-polymerization-inhibiting activity.

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References and notes

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33. Hydroxythalidomide (**2**). Mp >300 °C. MS(FAB): M+1 = 275. ^{1}H NMR (500 MHz/d_6-DMSO/δ): 2.00 (m, 2H), 2.87 (m, 2H), 3.00 (br s, 1H), 5.07 (dd, J = 12.8, 5.5 Hz, 1H), 7.15 (m, 2H), 7.72 (d, J = 7.7 Hz, 1H), 11.07 (s, 1H). Anal. Calcd for $C_{13}H_{10}N_2O_3$: C, 56.94; H, 3.68; N, 10.22. Found: C, 56.78; H, 3.87; N, 10.06.
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