

Development of Tubulin-Polymerization Inhibitors Based on the Thalidomide Skeleton

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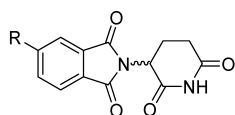
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We synthesized a series of compounds based on the potent tubulin-polymerization inhibitor 5-hydroxy-2-(2,6-diisopropylphenyl)-1*H*-isoindole-1,3-dione [5HPP-33 (3)], which is structurally derived from thalidomide (1), and investigated their inhibitory effects on tubulin polymerization. Direct interaction between 5HPP-33 (3) and α,β -tubulin heterodimer protein was demonstrated by means of a surface plasmon resonance study.

Key words thalidomide; phthalimide skeleton; tubulin polymerization inhibitor; surface plasmon resonance

Thalidomide (1) (Fig. 1) was developed and marketed in the late 1950's as a sedative/hypnotic drug, but subsequently had to be withdrawn from sale due to its teratogenicity.^{1–6} However, it was subsequently identified as an effective agent for the treatment of multiple myeloma (MM), AIDS, leprosy, and various cancers.^{1–5,7,8} The United States Food and Drug Administration (FDA) approved it for the treatment of erythema nodosum in leprosy (ENL) in 1998. In addition, official approval for the use of thalidomide (1) to treat MM has been applied for in Japan.

Thalidomide (1) has been discovered to have various biological activities, such as inhibition of tumor necrosis factor- α (TNF- α) production, and anti-inflammatory, anti-angiogenic, and cyclooxygenase (COX)-inhibitory activities.^{1–5,9,10} In the course of our studies of thalidomide metabolites, we found that 5-hydroxythalidomide (2) (Fig. 1) exhibited tubulin polymerization-inhibitory activity, though thalidomide (1) itself does not.^{11–15} Inhibition of tubulin function, including tubulin polymerization, is considered to be one of the molecular mechanisms of anti-tumor agents such as vinblastine and paclitaxel. Therefore, it was suspected that the efficacy of thalidomide (1) for the treatment of MM might be due to the tubulin polymerization-inhibitory activity of its metabolite, 5-hydroxythalidomide (2), at least in part. During our structural development studies of 5-hydroxythalidomide (2) based on tubulin polymerization-inhibitory activity, we obtained 5HPP-33 (3), which exhibits potent inhibitory activity, comparable with that of rhizoxin or colchicine.¹¹ However, Li *et al.* recently reported that 5HPP-33 (3) is not a tubulin polymerization inhibitor,¹⁶ but a tubulin polymerization enhancer, which prompted us to re-examine the effects elicited by 5HPP-33 (3) on tubulin polymerization/depolymerization, which led us to conclude that 5HPP-33 (3) acts a tubulin polymerization inhibitor, at least in our experimental conditions.



R = H, Thalidomide (1)
R = OH, 5-Hydroxythalidomide (2)

Fig. 1. Structures of Thalidomide (1) and Its Metabolite 5-Hydroxythalidomide (2)

We also reported that 5HPP-33 (3) exerts cell growth-inhibitory activity by causing cell cycle arrest and inducing apoptosis of human myelocytic cells IM9.¹¹ Additionally, we recently reported that fluorinated phthalimide analogs, FPP-33 (4), 4FPP-33 (5), and 4,7FPP-33 (6), exhibit potent tubulin polymerization-inhibitory activity (Fig. 2).¹⁷ However, whether these tubulin polymerization inhibitors, including 5HPP-33 (3), bind/interact with α,β -tubulin heterodimer protein remains unclear.

Therefore, we designed a hybrid compound of 5HPP-33 (3) and FPP-33 (4), *i.e.*, 5HFPP-33 (9), for which potent tubulin polymerization-inhibitory activity is expected. In this paper, we describe (1) structural development studies of 5HPP-33 (3) and its fluorinated derivatives based on tubulin polymerization-inhibitory activity, (2) analysis of the tubulin polymerization-inhibitory activity of 5HPP-33 (3), and (3) an experimental analysis of the interaction between 5HPP-33 (3) and α,β -tubulin heterodimer protein by means of a surface plasmon resonance (SPR) study.

Results and Discussion

Chemistry The tubulin-polymerization inhibitor, 5HPP-33 (3), was prepared as previously reported.¹¹ Compounds 7a–h and 9 were synthesized by usual organic synthetic methods or reported methods (Charts 1, 2). Briefly, condensation of 5-carboxyphthalic anhydride with neat 2,6-diisopropylaniline gave TCA-33 (7a), then condensation with ammonia, methylamine, and dimethylamine in the presence of *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide (EDCI), 1-hydroxybenzotriazole (HOBt), and *N,N*-diisopropylethylamine (DIPEA) afforded TAD-33 (7b), TDM-33 (7c), and TDMM-33 (7d), respectively. Reduction of 7a with $\text{BH}_3 \cdot \text{THF}$ gave the 5-hydroxymethyl analog TOL-33 (7e),

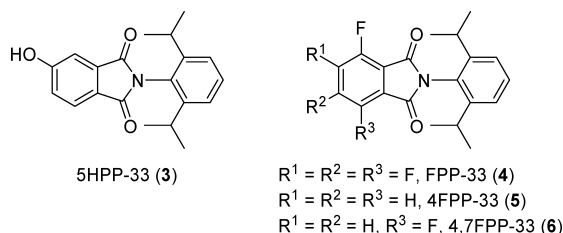
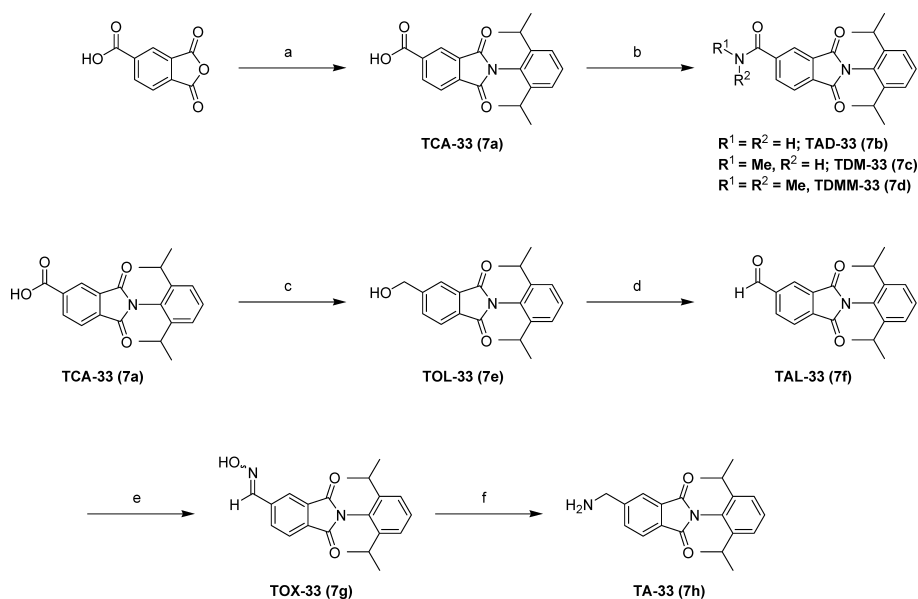


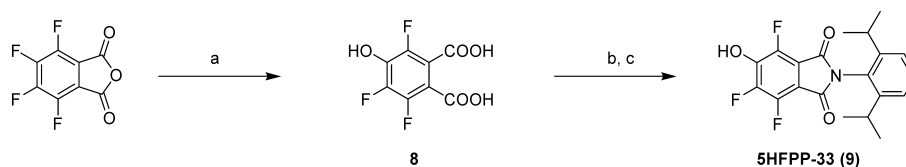
Fig. 2. Potent Tubulin Polymerization Inhibitors with a Phthalimide Skeleton Derived from Thalidomide (1)

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Reagents and conditions: (a) 2,6-diisopropylaniline, 200 °C, 2 h (32%); (b) NHR^1R^2 , EDCI, HOBT, DIPEA, RT, 10 h (8–18%); (c) BH_3 , THF, THF, RT, overnight (62%); (d) MnO_2 , CH_2Cl_2 , RT, 24 h (60%); (e) $HONH_2 \cdot HCl$, pyridine, EtOH, RT, 4 h (99%); (f) 10% Pd/C, H_2 (0.3 MPa), 2 N HCl, EtOH, RT, 1.5 h, (9%).

Chart 1



Reagents and conditions: (a) KOH, H_2O , 90 °C, 9 h (85%); (b) Ac_2O , 100 °C, 10 min; (c) 2,6-diisopropylaniline, pyridine, 120 °C, 4 h (19%).

Chart 2

which was oxidized with MnO_2 to afford the 5-formyl analog TAL-33 (7f). Condensation of 7f with hydroxylamine hydrochloride in the presence of pyridine gave the 5-hydroxyimino analog TOX-33 (7g), whose hydroxylamino group was reduced with hydrogen gas over Pd/C to afford the corresponding 5-aminomethyl analog TA-33 (7h) (Chart 1). The fluorinated compound 5HFPP-33 (9) was synthesized from 4,5,6,7-tetrafluorophthalic anhydride. Replacement of fluorine with a hydroxyl group at the 5-position of 4,5,6,7-tetrafluorophthalic anhydride was performed by treatment with KOH aqueous solution to give 5-hydroxy-4,6,7-trifluorophthalic acid (8).^{18,19} Compound 8 was dehydrated with Ac_2O , followed by condensation with 2,6-diisopropylaniline in pyridine to give 5HFPP-33 (9) (Chart 2).

Inhibitory Effects of the Prepared Compounds on Tubulin Polymerization We examined the tubulin polymerization-inhibitory activity of the prepared compounds (7a–h, 9) using the method previously described.^{20,21} Microtubule protein was prepared from porcine brain.^{22,23} Tubulin polymerization was followed by means of 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 measured turbidity (Abs_{400}) values of polymerized tubulin differed from experiment to experiment, the results (calculated IC_{50} values of the compounds) were basically reproducible. A typical set of data is presented in Figs. 3 and 4. In these figures, the con-

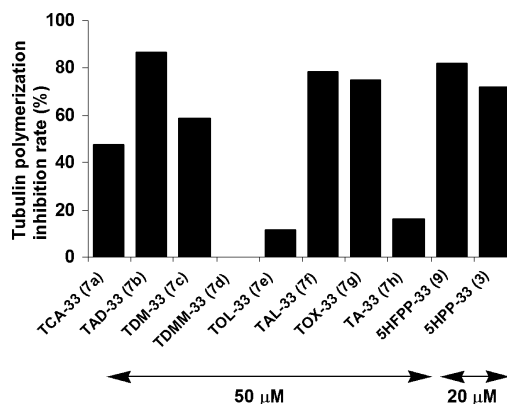


Fig. 3. Tubulin Polymerization-Inhibitory Activity of Compounds 7a–h, 9, and 3

centration of the test compounds was 50 μM , except for 5HFPP-33 (9, 20 μM). As shown in Fig. 3, although TDMM-33 (7d), TOL-33 (7e), and TA-33 (7h) exhibited little or no tubulin polymerization-inhibitory activity, other compounds showed moderate to high activity. The inhibitory activity of the amide compounds 7b–d was influenced by the number of methyl groups on the nitrogen atom of amide group. The inhibitory activity of the compounds decreased in the order of non-substituted compound TAD-33 (7b; *ca.* 85%) > mono-substituted compound TDM-33 (7c; *ca.* 60%) > di-substituted compound TDMM-33 (7d; 0%). The compounds bearing hydroxymethyl and aminomethyl moieties, TOL-33 (7e) and

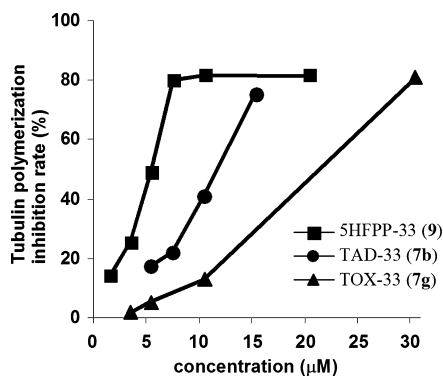


Fig. 4. Dose-Dependence of Inhibition of Tubulin Polymerization by TAD-33 (7b), TOX-33 (7g), and 5HFPP-33 (9)

TA-33 (7h), exhibited low inhibitory effects on tubulin polymerization, amounting to about 10% and 15%, respectively. Compounds substituted with a carbamoyl [TAD-33 (7b)], a formyl [TAL-33 (7f)], or a hydroxyimino group [TOX-33 (7g)], showed more potent inhibitory activity, amounting to about 85%, 80%, and 75%, respectively.

As expected, 5HFPP-33 (9), which is a tri-fluorinated derivative of 5HPP-33 (3), was found to be the most potent inhibitor of tubulin polymerization among our compounds. As shown in Fig. 4, 5HFPP-33 (9) was also a potent inhibitor, with the inhibition amounting to 82% at 7.5 μM. Its IC_{50} value was calculated to be about 5 μM, whereas those of TAD-33 (7b) and TOX-33 (7g) were about 16 μM and 11 μM, respectively. These results indicate that compounds substituted with a protic or a carbon-heteroatom double bond (C=O or C=N)-containing functional group at the 5-position showed moderate to high tubulin polymerization-inhibitory activity. In addition, introduction of fluorine atoms on the phthalimide skeleton enhanced the inhibitory potency.

Next, we investigated the tubulin depolymerization-inhibitory activity of TOX-33 (7g) and 5HFPP-33 (9). Depolymerization of tubulin was induced by cooling of the tubulin fraction polymerized at 37 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.^{20–22} As shown in Fig. 5, once-polymerized tubulin was depolymerized by cooling at 0 °C (Fig. 5, black circles). The tubulin depolymerized by cooling could be re-polymerized again by warming at 37 °C. The addition of 15 μM TOX-33 (7g) or 10 μM 5HFPP-33 (9) to polymerized tubulin at 37 °C did not affect the cooling-induced depolymerization step (Fig. 5, white squares and white circles, respectively). As expected, the tubulin depolymerized by cooling did not re-polymerize so effectively upon being warmed in the presence of TOX-33 (7g) or 5HFPP-33 (9) (at least, they did not enhance the re-polymerization) (Fig. 5, white squares and white circles, respectively). Thus, TOX-33 (7g) and 5HFPP-33 (9) showed no inhibitory effect on tubulin depolymerization, and they themselves do not induce tubulin depolymerization, at least in our system. Our findings suggest that TOX-33 (7g) and 5HFPP-33 (9) bind only to α,β -tubulin heterodimer protein to inhibit tubulin polymerization, but not to polymerized tubulin. These profiles of the effects of the compounds on tubulin polymerization/depolymerization are quite similar with those that we previously observed for 5HPP-33 (3).¹⁷ To examine the binding site(s) on

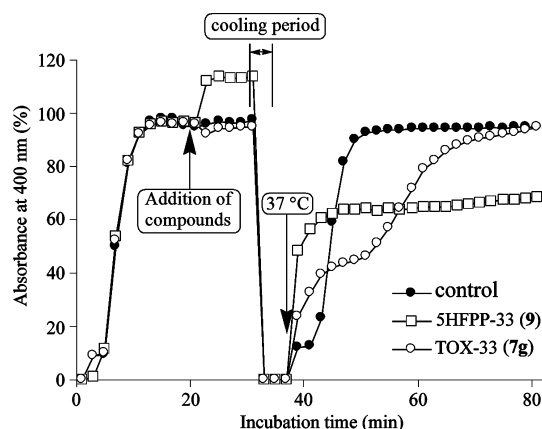


Fig. 5. Effects of Potent Tubulin Polymerization Inhibitors with a Phthalimide Skeleton on Tubulin Depolymerization

tubulin of our novel polymerization inhibitors, we performed binding competition studies using commercially available radio-labeled colchicine and vinblastine, but no binding competition was observed (data not shown). The results suggested that these molecules do not bind to the site at which colchicine or vinblastine binds.

Recently, Li *et al.* reported the effect of 5HPP-33 (3) on tubulin polymerization/depolymerization.¹⁶ They found that 5HPP-33 (3) does not inhibit tubulin polymerization, but enhances the polymerization and inhibits cold-induced tubulin depolymerization. The reason for this conflict between the results of Li *et al.* and our findings is not yet clear. However, there were a number of differences between the experimental conditions in the two studies: (1) Li *et al.* used purified α,β -tubulin heterodimer protein, while we used microtubule protein, *i.e.*, microtubule-associated proteins (MAPs)-containing tubulin fraction, (2) the incubation buffer of Li *et al.* contained a high concentration of DMSO (12% v/v), while ours contained less than 2% v/v DMSO, and (3) the incubation buffer of Li *et al.* contained a high concentration of glutamate (0.4 M), while ours did not contain glutamate.

Therefore, we first compared the polymerization profile of purified tubulin and MAPs-containing tubulin (Fig. 6A). We found that purified tubulin without MAPs hardly polymerizes in the absence of a high concentration of DMSO (Fig. 6A, line 4). The observed increase of turbidity was *ca.* 0.025 $OD_{400}/1-2$ mg protein/ml (Li *et al.* measured the turbidity at OD_{351} , and the increase was 0.04–0.09 judging from their data¹⁶). The turbidity increase at the same concentration of MAPs-containing tubulin fraction generally reached 0.24 OD_{400} (this value is defined as 100% in Figs. 6A–C). The inability of purified tubulin protein to polymerize/assemble has been already reported.²⁴ Addition of DMSO at the final concentration of 12% v/v to purified tubulin resulted in rapid and efficient polymerization, as indicated by the turbidity increase (in Fig. 6A, line 2). This phenomenon has also been reported by Himes *et al.*^{24,25} Interestingly, addition of 12% v/v DMSO moderately inhibited the polymerization of MAPs-containing tubulin (Fig. 6A, line 3 vs. line 1). Our tentative interpretation/speculation is that MAPs are mandatory for tubulin polymerization, as reported,¹⁷ but that DMSO can partially and functionally substitute for MAPs as regards both interaction with tubulin and function as a tubulin polymerization helper.^{24,25} This effect of 12% v/v DMSO on pu-

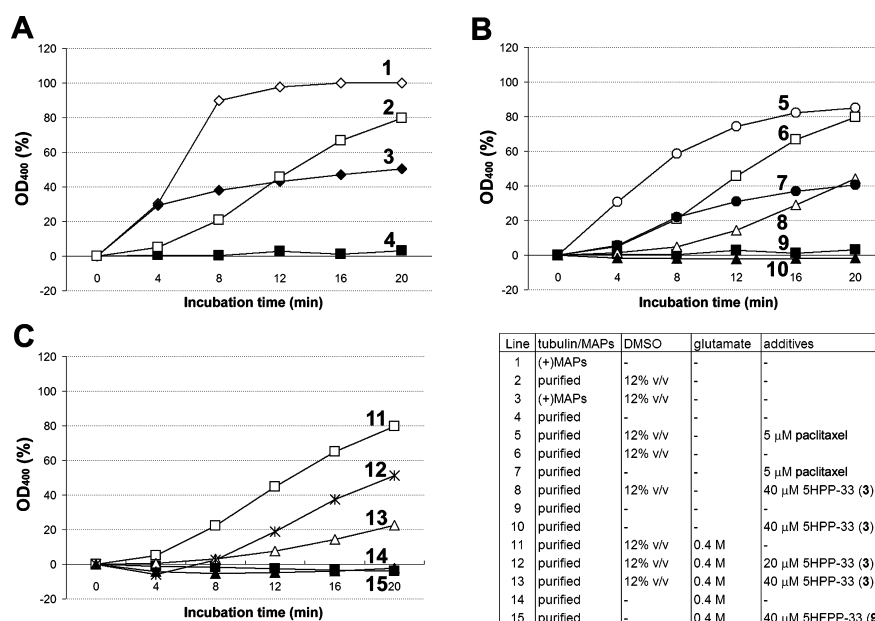


Fig. 6. Effects of 5HPP-33 (3) and Additives on Tubulin Polymerization

(A) Comparison of purified tubulin and MAPs-containing tubulin, and effect of DMSO. Line 1: MAPs-containing tubulin. Line 2: purified tubulin in the presence of 12% v/v DMSO. Line 3: MAPs-containing tubulin in the presence of 12% v/v DMSO. Line 4: purified tubulin. (B) Effects of 5HPP-33 (3) and paclitaxel on polymerization of purified tubulin in the presence or absence of DMSO. Line 5: 5 μ M paclitaxel and 12% v/v DMSO. Line 6: 12% v/v DMSO. Line 7: 5 μ M paclitaxel. Line 8: 40 μ M 5HPP-33 (3) and 12% v/v DMSO. Line 9: no additive. Line 10: 40 μ M 5HPP-33 (3). (C) Effects of 5HPP-33 (3) and 5HPP-33 (9) on polymerization of purified tubulin in the presence of 0.4 M glutamate and in the presence or absence of DMSO. Line 11: 12% v/v DMSO. Line 12: 20 μ M 5HPP-33 (3) and 12% v/v DMSO. Line 13: 40 μ M 5HPP-33 (3) and 12% v/v DMSO. Line 14: no additive. Line 15: 40 μ M 5HPP-33 (9).

rified tubulin polymerization was also observed in the presence of 0.4 M glutamate (Fig. 6C, line 11). Addition of 0.4 M glutamate alone did not enhance the polymerization of purified tubulin (Fig. 6C, line 14).

Paclitaxel (5 μ M) showed potent polymerization-enhancing effects on purified tubulin (Fig. 6B, line 7), which is in good accordance with the results reported by Li *et al.*,¹⁶ though its effect is less potent than that of 12% v/v DMSO (Fig. 6B, line 6). The effects of paclitaxel and DMSO seemed to be additive (Fig. 6B, line 5). However, 5HPP-33 (3) seemed to inhibit polymerization of purified tubulin in the presence of 12% v/v DMSO (Fig. 6, line 8), and dose-dependency for the inhibition of polymerization of purified tubulin was observed in the presence of 12% v/v DMSO and 0.4 M glutamate (Fig. 6C, lines 12, 13). No depolymerization-inhibitory activity of 5HPP-33 (3) was observed under any of the conditions described above (data not shown). Although precise analysis is still required, the effect of DMSO and/or MAPs cannot be excluded.

Binding Analysis of 5HPP-33 (3) with α,β -Tubulin Heterodimer Although 5HPP-33 (3) strongly inhibited tubulin polymerization under our experimental conditions (especially for MAPs-containing tubulin), the presence or absence of interaction between 5HPP-33 (3) and α,β -tubulin heterodimer protein remained unclear (*vide supra*). Thus, to examine this issue, we conducted surface plasmon resonance (SPR) measurements using a SPR670 instrument (Nippon Laser & Electronics Lab.). For the analysis, the sensor chip was treated with 4,4-dithiodibutyric acid (DDA) to introduce a linker segment for connection to tubulin protein. Immobilization of α,β -tubulin heterodimer protein onto the sensor chip with the sulfanylbutyric acid-modified surface was carried out by treatment of the modified sensor chip with *N*-hydroxysuccinimide and EDCI in 100 mM MES buffer solution, followed by

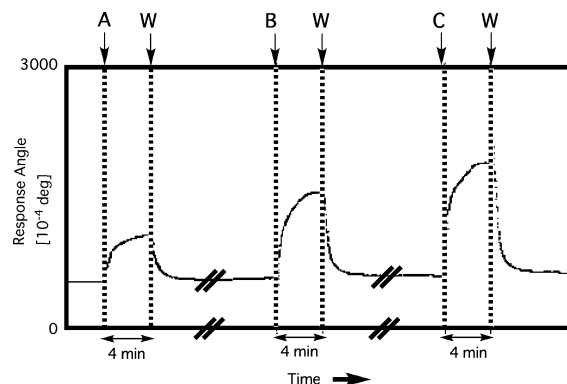


Fig. 7. SPR Measurement of Tubulin-5HPP-33 (3) Interaction

5HPP-33 (3) was injected at the time indicated by arrows "A" (100 μ M), "B" (200 μ M) and "C" (400 μ M). Washing procedures were started at the times indicated by arrows "W".

incubation with α,β -tubulin heterodimer protein. After immobilization of the protein, unreacted succinimide terminals on the sensor chip were blocked with ethanolamine. When 5HPP-33 (3) was injected onto the α,β -tubulin heterodimer protein-bearing sensor chip, a distinct, dose-dependent increase of the degree of difference line was detected (Fig. 7, arrows A, B, C). Washing the sensor chip with running buffer caused disappearance of the increase (Fig. 7, arrow W), suggesting dissociation of 5HPP-33 (3) from the tubulin-bearing chip. These results indicate that 5HPP-33 (3) binds/interacts directly and reversibly with α,β -tubulin heterodimer protein. From the SPR analysis, the binding constant of 5HPP-33 (3) with α,β -tubulin heterodimer protein was calculated to be $4.5 \times 10^6 \text{ M}^{-1}$ under our experimental conditions. The calculated value is in good agreement with the IC_{50} value of 5HPP-33 (3) for inhibition ($6.9 - 7.9 \times 10^6 \text{ M}^{-1}$)^{11,17} of tubulin polymerization.

Conclusion

We examined the tubulin polymerization-inhibitory activity of a series of phthalimide analogs (**7a–h**, **9**). Analogs bearing a substituent with protic character (TAD-33; **7b**) or having a carbon–heteroatom double bond-containing functional group (TAL-33; **7f**, TOX-33; **7g**) at the 5-position showed potent inhibitory activity. 5HFPP-33 (**9**), which is a fluorinated derivative of 5HPP-33 (**3**), also exhibited potent activity. In SPR experiments, we observed direct binding/interaction of 5HPP-33 (**3**) with α,β -tubulin heterodimer protein. Further structural development studies, investigation of the molecular mechanisms involved, and studies to identify the binding site(s) are in progress.

Experimental

General ^1H -NMR (500 MHz) and ^{13}C -NMR (125 MHz) spectra were recorded on a JEOL JNM- α 500 spectrometer. Mass spectra were obtained on JEOL JMA-HX 110 spectrometer with *m*-nitrobenzyl alcohol. Melting points determined on Yanaco MP-J3 micro melting point apparatus are uncorrected. Flash column chromatography was performed on silica gel 60 Kanto Kagaku (40–100 μm).

5-Carboxy-2-(2,6-diisopropylphenyl)-1H-isoindole-1,3-dione (TCA-33, **7a)** A mixture of trimellitic anhydride (192 mg, 1.00 mmol) and 2,6-diisopropylaniline (192 mg, 1.00 mmol) was stirred at 200 °C for 2 h, then cooled to room temperature. The crude product was purified by silica-gel column chromatography (hexane/ethyl acetate=1/8) and recrystallized from hexane and ethyl acetate to give TCA-33 (**7a**) (113 mg, 0.320 mmol, 32%) as a white powder; mp 234 °C (sublimation). ^1H -NMR (CDCl_3) δ : 1.17 (12H, d, $J=6.8$ Hz), 2.69 (2H, hept, $J=6.8$ Hz), 7.31 (2H, d, $J=7.7$ Hz), 7.49 (1H, t, $J=7.7$ Hz), 8.10 (1H, d, $J=7.9$ Hz), 8.59 (1H, d, $J=7.9$ Hz), 8.71 (1H, s). Anal. Calcd for $\text{C}_{21}\text{H}_{21}\text{NO}_4$: C, 71.78; H, 6.02; N, 3.99. Found: C, 71.52; H, 6.15; N, 3.89.

5-Carbamoyl-2-(2,6-diisopropylphenyl)-1H-isoindole-1,3-dione (TAD-33, **7b)** To a solution of TCA-33 (**7a**) (100 mg, 0.285 mmol) in DMF (20 ml) were added 1-ethyl-3-(3'-*N,N*-dimethylaminopropyl)carbodiimide, hydrochloride (EDCI) (110 mg, 0.574 mmol), 1-hydroxybenzotriazole (HOBt) (81.0 mg, 0.599 mmol), diisopropylethylamine (151 mg, 1.17 mmol), and ammonium chloride (30.0 mg, 0.561 mmol). The mixture was stirred at ambient temperature overnight, diluted by adding ethyl acetate, washed successively with dil. HCl, saturated aqueous NaHCO_3 , and brine, dried over MgSO_4 , and evaporated under reduced pressure. The residue was purified by silica-gel column chromatography (hexane/ethyl acetate=1/1) to give TAD-33 (**7b**) (8.0 mg, 0.023 mmol, 8%) as a white powder; mp 181 °C (sublimation). ^1H -NMR (CDCl_3) δ : 1.10 (12H, d, $J=6.8$ Hz), 2.61 (2H, hept, $J=6.8$ Hz), 5.69 (1H, bs), 6.13 (1H, bs), 7.23 (2H, d, $J=7.8$ Hz), 7.41 (1H, t, $J=7.8$ Hz), 8.00 (1H, d, $J=7.7$ Hz), 8.27 (1H, s), 8.28 (1H, d, $J=7.7$ Hz). Anal. Calcd for $\text{C}_{21}\text{H}_{23}\text{N}_2\text{O}_3$: C, 71.98; H, 6.33; N, 7.99. Found: C, 71.86; H, 6.48; N, 7.76.

5-(*N*-Methylamino)carbonyl-2-(2,6-diisopropylphenyl)-1H-isoindole-1,3-dione (TDM-33, **7c)** To a solution of TCA-33 (**7a**) (100 mg, 0.285 mmol) in DMF (20 ml) were added 1-ethyl-3-(3'-*N,N*-dimethylaminopropyl)carbodiimide, hydrochloride (EDCI) (110 mg, 0.574 mmol), 1-hydroxybenzotriazole (HOBt) (81 mg, 0.599 mmol), diisopropylethylamine (151 mg, 1.17 mmol), and methylamine (40 wt% in water, 50.0 mg, 0.644 mmol). The mixture was stirred at ambient temperature overnight, diluted by adding ethyl acetate, washed successively with dil. HCl, saturated aqueous NaHCO_3 , and brine, dried over MgSO_4 , and evaporated under reduced pressure. The residue was purified by silica-gel column chromatography (hexane/ethyl acetate=1/1) to give TDM-33 (**7c**) (8.0 mg, 0.022 mmol, 8%) as a white powder; mp 163 °C. ^1H -NMR (CDCl_3) δ : 1.16 (6H, d, $J=6.8$ Hz), 1.17 (6H, d, $J=6.8$ Hz), 2.68 (2H, hept, $J=6.8$ Hz), 3.00 (3H, d, $J=5.1$ Hz), 6.43 (1H, bs), 7.31 (2H, d, $J=7.7$ Hz), 7.47 (1H, t, $J=7.7$ Hz), 8.04 (1H, d, $J=7.7$ Hz), 8.32 (1H, d, $J=7.7$ Hz), 8.33 (1H, s). FAB-MS m/z : 365.1842 (Calcd for $\text{C}_{22}\text{H}_{25}\text{N}_2\text{O}_3$: 365.1865).

5-(*N,N*-Dimethylamino)carbonyl-2-(2,6-diisopropylphenyl)-1H-isoindole-1,3-dione (TDDM-33, **7d)** To a solution of TCA-33 (**7a**) (100 mg, 0.285 mmol) in DMF (20 ml) were added 1-ethyl-3-(3'-*N,N*-dimethylaminopropyl)carbodiimide, hydrochloride (EDCI) (110 mg, 0.574 mmol), 1-hydroxybenzotriazole (HOBt) (81 mg, 0.599 mmol), diisopropylethylamine (151 mg, 1.17 mmol), and dimethylamine (40 wt% in water, 62.5 mg, 0.555 mmol). The mixture was stirred at ambient temperature overnight, di-

luted by adding ethyl acetate, washed successively with dil. HCl, saturated aqueous NaHCO_3 , and brine, dried over MgSO_4 , and evaporated under reduced pressure. The residue was purified by silica-gel column chromatography (hexane/ethyl acetate=1/1) to give TDDM-33 (**7d**) (20.0 mg, 0.053 mmol, 19%) as a white powder; mp 134 °C. ^1H -NMR (CDCl_3) δ : 1.15 (12H, d, $J=6.8$ Hz), 2.68 (2H, hept, $J=6.8$ Hz), 3.05 (3H, s), 3.17 (3H, s), 7.29 (2H, d, $J=8.1$ Hz), 7.46 (1H, t, $J=8.1$ Hz), 7.85 (1H, dd, $J=1.3$, 7.7 Hz), 7.98 (1H, d, $J=1.3$ Hz), 8.00 (1H, d, $J=7.7$ Hz). FAB-MS m/z : 379.2017 (Calcd for $\text{C}_{23}\text{H}_{27}\text{N}_2\text{O}_3$: 379.2022).

5-Hydroxymethyl-2-(2,6-diisopropylphenyl)-1H-isoindole-1,3-dione (TOL-33, **7e)** To a solution of TCA-33 (**7a**) (877 mg, 2.50 mmol) in THF (10 ml) was added $\text{BH}_3\cdot\text{THF}$ (1.0 M THF solution, 3.0 ml, 3.00 mmol) at 0 °C, and the mixture was stirred at ambient temperature overnight. The reaction was quenched by adding diluted HCl. The mixture was extracted with ethyl acetate, washed with brine, dried over MgSO_4 , and evaporated under reduced pressure. The residue was purified by silica-gel column chromatography (hexane/ethyl acetate=1/1) to give TOL-33 (**7e**) (357 mg, 1.06 mmol, 42%) as a white powder; mp 136 °C. ^1H -NMR (CDCl_3) δ : 1.15 (6H, d, $J=6.8$ Hz), 1.16 (6H, d, $J=6.8$ Hz), 2.15 (1H, t, $J=4.7$ Hz), 2.71 (2H, hept, $J=6.8$ Hz), 4.89 (2H, d, $J=4.7$ Hz), 7.29 (2H, d, $J=7.7$ Hz), 7.46 (1H, t, $J=7.7$ Hz), 7.81 (1H, d, $J=7.5$ Hz), 7.95 (1H, d, $J=7.5$ Hz), 7.99 (1H, s). FAB-MS m/z : 338.1711 (Calcd for $\text{C}_{21}\text{H}_{24}\text{NO}_3$: 338.1756).

5-Formyl-2-(2,6-diisopropylphenyl)-1H-isoindole-1,3-dione (TAL-33, **7f)** To a solution of TOL-33 (**7e**) (357 mg, 1.06 mmol) in CH_2Cl_2 (10 ml) was added MnO_2 (1.00 g, 11.5 mmol), and the mixture was stirred at ambient temperature overnight. The reaction mixture was filtered through a pad of Celite, which was then washed with CH_2Cl_2 , and the combined filtrate and washing was concentrated under reduced pressure. The residue was purified by silica-gel column chromatography (hexane/ethyl acetate=3/1) to give TAL-33 (**7f**) (310 mg, 0.924 mmol, 87%) as a white powder; mp 153 °C. ^1H -NMR (CDCl_3) δ : 1.17 (12H, d, $J=6.8$ Hz), 2.68 (2H, hept, $J=6.8$ Hz), 7.31 (2H, d, $J=7.7$ Hz), 7.48 (1H, t, $J=7.7$ Hz), 8.15 (1H, d, $J=7.7$ Hz), 8.35 (1H, d, $J=7.7$ Hz), 8.47 (1H, s), 10.22 (1H, s). FAB-MS m/z : 336.1602 (Calcd for $\text{C}_{21}\text{H}_{22}\text{NO}_3$: 336.1600).

5-(Hydroxyimino)methyl-2-(2,6-diisopropylphenyl)-1H-isoindole-1,3-dione (TOX-33, **7g)** To a solution of TAL-33 (**7f**) (40.0 mg, 0.119 mmol) in a mixture of EtOH (15 ml) and pyridine (8 ml) was added hydroxylamine hydrochloride (8.1 mg, 0.117 mmol). The mixture was stirred at 90 °C for 4 h, then cooled to room temperature, diluted with ethyl acetate, and washed successively with 2 N HCl, saturated aqueous NaHCO_3 , H_2O , and brine. The organic layer was dried over MgSO_4 and concentrated under reduced pressure. The residue was purified by silica-gel column chromatography (hexane/ethyl acetate=5/1) to give TOX-33 (**7g**) (35.0 mg, 0.100 mmol, 84%) as a white powder; mp 215 °C (sublimation). ^1H -NMR (CDCl_3) δ : 1.16 (12H, d, $J=6.8$ Hz), 2.70 (2H, hept, $J=6.8$ Hz), 7.29 (2H, d, $J=7.7$ Hz), 7.46 (1H, t, $J=7.7$ Hz), 7.56 (1H, s), 7.99 (1H, d, $J=7.9$ Hz), 8.00 (1H, d, $J=7.9$ Hz), 8.21 (1H, s), 8.27 (1H, s). FAB-MS m/z : 351.1731 (Calcd for $\text{C}_{21}\text{H}_{23}\text{N}_2\text{O}_3$: 351.1709).

5-Aminomethyl-2-(2,6-diisopropylphenyl)-1H-isoindole-1,3-dione (TA-33, **7h)** To a solution of TOX-33 (**7g**) (25.0 mg, 0.071 mmol) in a mixture of EtOH (20 ml) and 2 N HCl (1 ml) was added 10% Pd-C (10 mg), and the mixture was stirred at ambient temperature for 1.5 h under an H_2 atmosphere (0.3 MPa). The reaction mixture was filtered through a pad of Celite and the filtrate was concentrated under reduced pressure. The residue was taken up in Et_2O and filtered. The filtrate was concentrated under reduced pressure, and the residue was purified by recrystallization (hexane and ethyl acetate) to give TA-33 (**7h**) (8.0 mg, 0.024 mmol, 33%) as a white powder; mp 170 °C. ^1H -NMR (D_2O) δ : 1.01 (12H, d, $J=6.8$ Hz), 2.64 (2H, hept, $J=6.8$ Hz), 4.32 (2H, s), 7.35 (2H, d, $J=8.1$ Hz), 7.50 (1H, t, $J=8.1$ Hz), 7.89 (1H, d, $J=7.9$ Hz), 7.95 (1H, s), 7.96 (1H, d, $J=7.9$ Hz). FAB-MS m/z : 337.1951 (Calcd for $\text{C}_{21}\text{H}_{25}\text{N}_2\text{O}_3$: 337.1916).

4-Hydroxy-3,5,6-trifluorophthalic Acid (8**)**^{18,19)} To a solution of 85% KOH (2.0 g, 30.3 mmol) in H_2O (10 ml) was added 3,4,5,6-tetrafluorophthalic anhydride (1.0 g, 4.54 mmol) at 90 °C. The reaction mixture was stirred for 9 h at the same temperature, then cooled to room temperature. The reaction was quenched by adding conc. HCl to *ca.* pH 2. The resulting acidic solution was extracted with ethyl acetate, and the organic solution was washed with brine, dried over MgSO_4 , and concentrated under reduced pressure to give **8** (0.912 g, 85%) as a white powder. ^1H -NMR ($\text{DMSO}-d_6$) δ : 11.89 (2H, bs). ^{13}C -NMR (CD_3OD) δ : 111.5 (d, $^2J_{\text{C-F}}=13.0$ Hz), 120.1 (d, $^2J_{\text{C-F}}=16.6$ Hz), 140.2 (dd, $^2J_{\text{C-F}}=14.8$, 14.8 Hz), 143.2 (dd, $^1J_{\text{C-F}}=247.8$ Hz, $^2J_{\text{C-F}}=11.1$ Hz), 146.9 (d, $^1J_{\text{C-F}}=246.0$ Hz), 147.8 (dd, $^1J_{\text{C-F}}=253.4$ Hz, $^2J_{\text{C-F}}=11.1$ Hz), 165.4 (s), 166.4 (s). FAB-MS m/z : 332 ($\text{M}+\text{H}^+$).

5-Hydroxy-4,6,7-trifluoro-2-(2,6-diisopropylphenyl)-1H-isoindole-1,3-

dione (5HFPP-33, 9) A solution of 4-hydroxy-3,5,6-trifluorophthalic acid (**8**) (1.00 g, 4.24 mmol) in Ac₂O (1.0 ml) was stirred at 100 °C for 10 min and the solvent was removed under reduced pressure. The residue was dissolved in pyridine (25 ml), and to this solution was added 2,6-diisopropylaniline (752 mg, 4.24 mmol). The mixture was stirred at 120 °C for 4 h, then cooled to room temperature, diluted with ethyl acetate, and washed with 1 N HCl. The organic layer was dried over MgSO₄ and concentrated under reduced pressure. The residue was purified by silica-gel column chromatography (ethyl acetate) and then recrystallized (hexane and ethyl acetate) to give 5HFPP-33 (**9**) (303 mg, 0.803 mmol, 19%) as a white powder; mp 199 °C. ¹H-NMR (CDCl₃) δ: 1.16 (12H, d, *J*=6.8 Hz), 2.66 (2H, hept, *J*=6.8 Hz), 7.27 (2H, d, *J*=7.7 Hz), 7.41 (1H, t, *J*=7.7 Hz). *Anal.* Calcd for C₂₀H₁₀F₃NO₃: C, 63.66; H, 4.81; N, 3.71. Found: C, 63.31; H, 4.74; N, 3.60.

Tubulin Polymerization Measurement Microtubule protein was prepared from porcine brain as described.^{22,23} A typical run was as follows: fresh brains were cooled on ice, washed with aqueous solution containing 100 mM 2-morpholinoethanesulfonic acid (MES), 1 mM EGTA, 0.5 mM MgCl₂, pH 6.5, and homogenized in 0.5 ml/g of MES buffer (100 mM MES, 1 mM EGTA, 0.5 mM MgCl₂, 1 mM 2-mercaptoethanol, and 1 mM GTP, pH 6.5) under ice-cooling. After centrifugation (100000 *g*, 30 min, 4 °C), the supernatant was mixed with an equal volume of glycerol buffer (MES buffer and 8 M glycerol, pH 6.5), and the mixture was warmed at 37 °C for 30 min to polymerize tubulin. The polymerized microtubule protein was collected by centrifugation (100000 *g*, 45 min, 25 °C) and resuspended in MES buffer, and the suspension was chilled (0 °C, 30 min) to allow depolymerization. After centrifugation of the suspension (100000 *g*, 60 min, 4 °C), the supernatant was collected. The same polymerization/depolymerization process was repeated, then an equal volume of glycerol buffer was added to the supernatant solution, and the mixture was stored at -80 °C. The microtubule protein thus obtained was used as MAPs-containing tubulin for polymerization/depolymerization assay after the polymerization/depolymerization process described above was once more repeated. This tubulin fraction was further purified by phosphocellulose column chromatography²² and its purity was evaluated by polyacrylamide gel electrophoresis. It was then used as purified tubulin for polymerization/depolymerization assay. Tubulin polymerization was followed by means of turbidity measurements at 37 °C in microtubule assemble buffer containing 100 mM MES, 1 mM EGTA, 0.5 mM MgCl₂, 1 mM 2-mercaptoethanol, and 1 mM GTP (pH 6.5), in the presence or absence of 12% v/v DMSO and/or 0.4 M glutamate. Although the measured turbidity (Abs.₄₀₀) values of polymerized tubulin differed from experiment to experiment, the results (calculated IC₅₀ values of the compounds) were basically reproducible.

Surface Plasmon Resonance Interaction of 5HPP-33 (**3**) and α,β -tubulin heterodimer protein was monitored by surface plasmon resonance analysis using a SPR670 instrument (Nippon Laser & Electronics Lab.). Preparation of the sensor chip on which α,β -tubulin heterodimer protein was immobilized was carried out as follows. (1) Immobilization of sulfanylbutyric acid on the sensor chip by soaking the chip in 1 mM dithiodibutyric acid (DDA) ethanol solution at 4 °C overnight. (2) Activation of carboxyl groups with *N*-hydroxysuccinimide (NHS). This operation was carried out using EDCI (25 mg) in Milli Q water (1 ml) and NHS (15 mg) in 1,4-dioxane (10 ml) for 30 min at room temperature. (3) Immobilization of α,β -tubulin heterodimer protein was performed by injection of the protein (50 μ g/ml in 100 mM MES buffer solution, 60 μ M) on the sensor chip. (4) Blocking of unreacted surface was performed by treatment of the tip with 1 M ethanolamine solution in Milli Q water (60 μ l). 5HPP-33 (**3**) was dissolved at the concentration of 100, 200 or 400 μ M in 100 mM MES buffer (containing 1–2% v/v DMSO). This solution was injected at 25 °C at a flow rate of 15 μ l/min onto the surface of the sensor chip upon which α,β -tubulin heterodimer protein was immobilized. Using the sensor chip which was directly blocked with

ethanolamine (*i.e.*, sensor chip without tubulin protein) did not afford increase of the degree of difference line, eliminating the possibility that 5HPP-33 itself gives false positives.

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