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Tubulin polymerization inhibitors with a fluorinated phthalimide skeleton derived from thalidomide

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Abstract—4,7-Difluoro-2-(2,6-diisopropylphenyl)-1*H*-isoindole-1,3-dione [4,7FPP-33 (**14**)] has a potent tubulin-polymerization-inhibiting activity comparable with those of the known tubulin-polymerization inhibitors rhizoxin and colchicine. The structure–activity relationship for fluorine substitution was elucidated.

Thalidomide (**1**) (Fig. 1) was introduced as a sedative–hypnotic agent, but withdrawn from the market in the 1960s because of serious teratogenicity.[1,2] However, it has since been established to be effective for the treatment of various diseases, including leprosy, cancers, and AIDS.[3–5] The United States Food and Drug Administration (FDA) approved thalidomide (**1**) for the treatment of erythema nodosum leprosum (ENL) in 1998.[6] Clinical studies of its use for the treatment of various cancers, including multiple myeloma (MM), colon cancer, prostate cancer, and breast cancer, are ongoing.

Although thalidomide (**1**) was known to inhibit the synthesis of tumor necrosis factor (TNF)-α and to show anti-angiogenic activity, its mechanism of action is complex and might include a range of molecular targets.[2,4,7,8] We have demonstrated that the TNF-α production-regulating activity of thalidomide (**1**) is bidirectional, and that thalidomide (**1**) is a multi-target drug.[2,9] In structural development studies of thalidomide (**1**), we have obtained TNF-α production regulators (including bidirectional ones, pure inhibitors, and pure enhancers),[9,10] androgen antagonists,[11–13] aminopeptidase inhibitors,[14–16] α-glucosidase inhibitors,[17,18] thymidine phosphorylase inhibitors,[19] cyclooxygenase (COX) inhibitors,[20,21] and nitric oxide synthase (NOS) inhibitors.[22] In the course of those studies, we discovered that the thalidomide metabolite 5-hydroxythalidomide (**2**) (Fig. 1) possesses tubulin polymerization-inhibiting activity, whereas thalidomide does not.[23] Inhibition of tubulin function, including tubulin polymerization inhibition, is considered to be one of the molecular mechanisms of anti-tumor agents, including vinblastine and taxol. Therefore, effectiveness of thalidomide (**1**) for the treatment of MM might be attributed to the tubulin polymerization-inhibiting activity elicited by its metabolite, 5-hydroxythalidomide (**2**), at least in part. During our structural development studies on 5-hydroxythalidomide (**2**) based on its tubulin polymerization-inhibiting activity, we obtained 5HPP-33 (**4**) (Fig. 1), which exhibits potent tubulin polymerization-inhibiting activity, comparable with that of the tubulin-polymerization inhibitors rhizoxin and colchicine.[23] Moreover, 5HPP-33 (**4**) exerts cell growth-inhibitory activity by means of cell cycle arrest and inducing apoptosis of IM9.[23] In this paper, we present further structural development studies of phthalimide-type tubulin polymerization inhibitors based on thalidomide structure.

First, we investigated the effect of thalidomide (**1**) and various derivatives on tubulin polymerization using the method previously described.[24,25] Briefly, microtubule protein was prepared from porcine brain.[26,27] Tubulin polymerization was followed by means of turbidity measurements at 37 °C in microtubule assemble buffer containing 100 mM 2-morpholinoethanesulfonic acid

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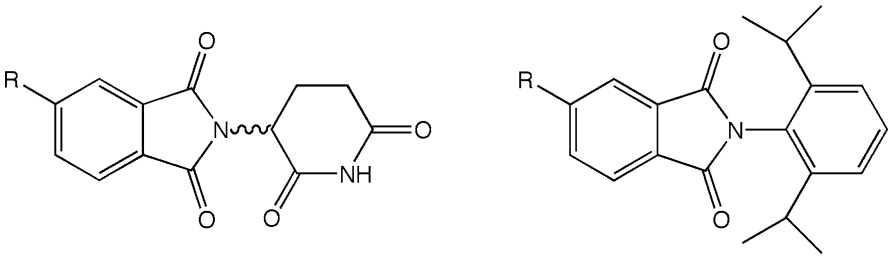


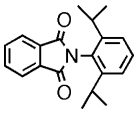
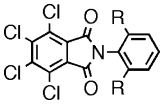
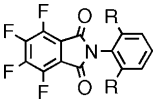
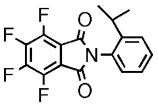
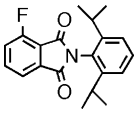
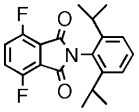
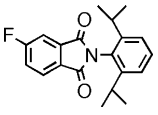
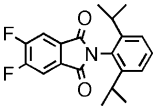
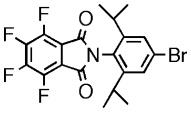
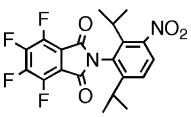
Figure 1. Structures of thalidomide (**1**), one of its metabolites, 5-hydroxythalidomide (**2**), PP-33 (**3**), and 5HPP-33 (**4**).

(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 1. The compounds listed in Table 1 were synthesized by usual organic synthetic methods; for chemical/physical analytical data, see Refs. 28–36. The concentration of all test compounds was 20 μM.

As shown in Table 1, non-substituted diisopropylphenylphthalimide [PP-33 (**3**)] and its chlorinated derivatives, including CPP-00 (**5**), CPP-11 (**6**), and CPP-33 (**7**), showed no or only weak tubulin polymerization-inhibiting activity. On the other hand, the fluorinated derivatives of PP-33 (**3**), that is, FPP-33 (**11**), 4FPP-33 (**13**), and 4,7FPP-33 (**14**), showed very potent tubulin polymerization-inhibiting activity. The structural requirement for the activity seems to be critical, because derivatives of FPP-33 (**11**) with less bulky alkyl groups (FPP-00: **8**, FPP-11: **9**, FPP-22: **10**, and FPP-03: **12**) showed only slight tubulin polymerization-inhibiting activity. The longer the alkyl group, the higher the tubulin polymerization-inhibiting activity; FPP-33 (**11**) > FPP-22 (**10**) > FPP-11 (**9**) > FPP-00 (**8**). The IC_{50} of FPP-33 (**11**) was 16 μM under the experimental conditions used. The results suggest that the tubulin polymerization-inhibiting activity of phenylphthalimide analogs is a specific feature of the 2,6-diisopropylphenylphthalimide structure substituted with fluorine.

We therefore investigated the effect of fluorine-substitution in detail. Among mono-fluorinated derivatives (Table 1), 4FPP-33 (**13**), which has an *ortho*-fluorine substituent, showed more potent tubulin polymerization-inhibiting activity than 5FPP-33 (**15**), which contains fluorine at the *meta* position. Among the difluorinated derivatives, 4,7FPP-33 (**14**) was more potent than 5,6FPP-33 (**16**). The IC_{50} values of 4FPP-33 (**13**) and 4,7FPP-33 (**14**) were 17 and 7.8 μM, respectively, under these experimental conditions. 4,7FPP-33 (**14**) showed more potent tubulin polymerization-inhibiting activity than FPP-33 (**11**) and was as potent as 5HPP-33 (**4**, IC_{50} = 6.9 μM). The order of tubulin polymerization-inhibiting activity of the fluorinated derivatives was 4,7FPP-33 (**14**) > FPP-33 (**11**) > 4FPP-

Table 1. Tubulin polymerization-inhibiting activity of test compounds (20 μM)

		Tubulin polymerization inhibition (%) at 20 μM
	Colchicine	78
	Rhizoxin	81
	PP-33 (**3**)	3
	R = H: CPP-00 (**5**) R = Me: CPP-11 (**6**) R = ^{i}Pr: CPP-33 (**7**)	6 0 0
	R = H: FPP-00 (**8**) R = Me: FPP-11 (**9**) R = Et: FPP-22 (**10**) R = ^{i}Pr: FPP-33 (**11**)	7 10 18 71 (IC_{50} = 16 μM)
	FPP-03 (**12**)	10
	4FPP-33 (**13**)	73 (IC_{50} = 17 μM)
	4,7FPP-33 (**14**)	78 (IC_{50} = 7.8 μM)
	5FPP-33 (**15**)	9
	5,6FPP-33 (**16**)	8
	FPP-33-*p*-Br (**17**)	11
	FPP-33-*m*-NO_2 (**18**)	10

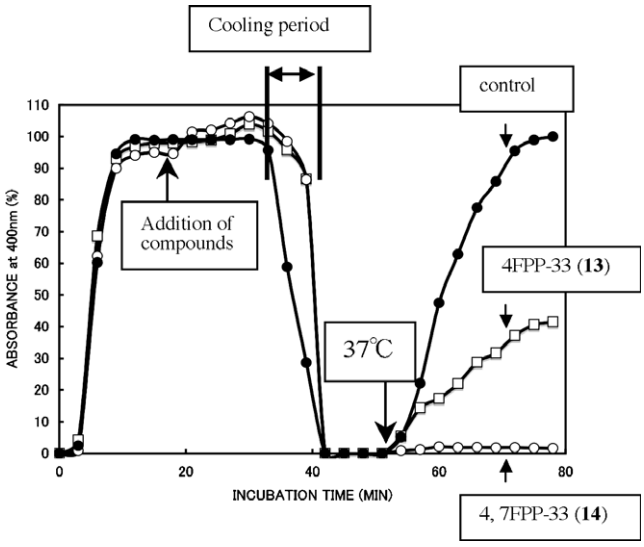


Figure 2. Effects of representative compounds on tubulin depolymerization.

33 (**13**) > 5FPP-33 (**15**) and 5,6FPP-33 (**16**). The results suggest that fluorine-substitution at the *ortho* position is mandatory for potent activity, while *meta* substitution is not so effective. Substitution on the diisopropylphenyl ring, FPP-33-*p*-Br (**17**) and FPP-33-*m*-NO_2 (**18**), diminished the activity.

Next we investigated the tubulin depolymerization-inhibiting activity of 4FPP-33 (**13**) and 4,7FPP-33 (**14**). Depolymerization of tubulin was induced by cooling the tubulin fraction polymerized at 37–0 °C, or by addition of 4 mM $CaCl_2$ at 37 °C, and was followed by means of turbidity measurements as described previously.[24–26] As shown in Figure 2, once-polymerized tubulin was depolymerized by cooling (Fig. 2, black circles). The tubulin depolymerized by cooling could be re-polymerized again by warming at 37 °C. The addition of 10 μM 4FPP-33 (**13**) or 4,7FPP-33 (**14**) to polymerized tubulin at 37 °C did not affect the cooling-induced depolymerization step (Fig. 2, white square and white circle, respectively). As expected, the tubulin depolymerized by cooling did not repolymerize upon warming in the presence of 4FPP-33 (**13**) or 4,7FPP-33 (**14**) (Fig. 2, open square and open circle, respectively). Thus, 4FPP-33 (**13**) and 4,7FPP-33 (**14**) showed no inhibiting effect on tubulin depolymerization, at least in our system. Our finding indicates that 4FPP-33 (**13**) and 4,7FPP-33 (**14**) bind only to heterodimeric α/β-tubulin protein, but not to polymerized tubulin. Concerning the binding site(s) on tubulin of our novel polymerization inhibitors, our preliminary studies indicated that they do not compete with colchicines nor vinblastine on tubulin-binding. Determination of the binding site will be planned.

In conclusion, we have developed a potent tubulin polymerization inhibitor, 4,7FPP-33 (**14**), by structural elaboration of thalidomide.

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29. FPP-22 (**10**). MS (FAB): $[M+H]^+ = 352$. ^1H NMR (500 MHz/$CDCl_3$/δ): 7.40 (t, $J = 7.7$ Hz, 1H), 7.23 (d, $J = 7.7$ Hz, 2H), 2.41(q, $J = 7.7$ Hz, 4H), 1.13 (t, $J = 7.7$ Hz, 6H). Anal. Calcd for $C_{18}H_{13}F_4NO_2$: C, 61.54; H, 3.73; N, 3.99. Found: C, 61.25; H, 3.86; N, 3.95. Mp: 113–115 °C.
30. FPP-03 (**12**). MS (FAB): $[M+H]^+ = 338$. ^1H NMR (500 MHz/$CDCl_3$/δ): 7.49 (m, 2H), 7.31 (m, 1H), 7.10 (d, $J = 7.7$ Hz, 1H), 2.73 (sept, $J = 6.84$ Hz, 1H), 1.20 (d, $J = 6.8$ Hz, 6H). Anal. Calcd for $C_{17}H_{11}F_4NO_2$: C, 60.54; H, 3.29; N, 4.15. Found: C, 60.47; H, 3.49; N, 4.07. Mp: 151–153 °C.
31. 4FPP-33 (**13**). MS (FAB): $[M+H]^+ = 326$. ^1H NMR (500 MHz/$CDCl_3$/δ): 7.82 (m, 2H), 7.47 (q, $J = 7.9$ Hz, 2H), 7.29 (d, $J = 7.9$ Hz, 2H), 2.70 (sept, $J = 6.7$ Hz, 2H) 1.17 (t, $J = 6.7$ Hz, 12H). Mp: 164–165 °C.
32. 4,7FPP-33 (**14**). MS (FAB): $[M+H]^+ = 344$. ^1H NMR (500 MHz/$CDCl_3$/δ): 7.47 (m, 3H), 7.29 (d, $J = 7.9$ Hz, 2H), 2.69 (sept, $J = 6.7$ Hz, 2H), 1.54 (d, $J = 6.7$ Hz, 12H). Anal. Calcd for $C_{20}H_{19}F_2NO_2$: C, 69.96; H, 5.58; N, 4.08. Found: C, 69.69; H, 5.74; N, 4.00. Mp: 167–168 °C.
33. 5FPP-33 (**15**). MS (FAB): $[M+H]^+ = 326$. ^1H NMR (500 MHz/$CDCl_3$/δ): 7.96 (d, $J = 4.6$ Hz, 1H), 7.63 (d, $J = 4.6$ Hz, 1H), 7.47 (m, 2H), 7.28 (d, $J = 7.6$ Hz, 2H), 2.67 (sept, $J = 6.7$ Hz, 2H) 1.15 (d, $J = 6.7$ Hz, 12H). Anal. Calcd for $C_{20}H_{20}FNO_2$: C, 73.83; H, 6.20; N, 4.30. Found: C, 73.72; H, 6.16; N, 4.28. Mp: 167–168 °C.
34. 5,6FPP-33 (**16**). MS (FAB): $[M+H]^+ = 343$. ^1H NMR (500 MHz/$CDCl_3$/δ): 7.72 (t, $J = 7.1$ Hz, 2H), 7.41 (t, $J = 7.7$ Hz, 1H), 7.23 (d, $J = 7.7$ Hz, 2H), 2.60 (sept, $J = 6.8$ Hz, 2H), 1.10 (d, $J = 6.8$ Hz, 12H). Anal. Calcd for $C_{20}H_{19}F_2NO_2$: C, 69.96; H, 5.58; N, 4.08. Found: C, 69.69; H, 5.74; N, 4.00. Mp: 162–163 °C.
35. FPP-33-*p*-Br (**17**). MS (FAB): $[M+H]^+ = 458, 460$. ^1H NMR (500 MHz/$CDCl_3$/δ): 7.38 (s, 2H), 2.58 (sept, $J = 6.8$ Hz, 2H), 1.14 (d, $J = 6.8$ Hz, 12H). Anal. Calcd for $C_{20}H_{16}F_2BrNO_2$: C, 52.42; H, 3.52; N, 3.06. Found: C, 52.26; H, 3.53; N, 2.83. Mp: 174–175 °C.
36. FPP-33-*m*-NO_2 (**18**). MS (FAB): $[M+H]^+ = 421$. ^1H NMR (500 MHz/$CDCl_3$/δ): 7.64 (d, $J = 8.6$ Hz, 1H), 7.40 (d, $J = 8.6$ Hz, 1H), 2.95 (sept, $J = 6.8$ Hz, 1H), 2.56 (sept, $J = 6.7$ Hz, 1H), 1.24 (d, $J = 6.8$ Hz, 6H), 1.16 (d, $J = 6.7$ Hz, 6H). Anal. Calcd for $C_{20}H_{16}F_4N_2O_4$: C, 56.61; H, 3.80; N, 6.60. Found: C, 56.32; H, 3.87; N, 6.55. Mp: 182–183 °C.