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Angiogenesis inhibitors derived from thalidomide

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Abstract—5-Hydroxy-2-(2,6-diisopropylphenyl)-1*H*-isoindole-1,3-dione (5HPP-33: **10**), which was obtained during our previous structural development studies on thalidomide, was revealed to possess potent anti-angiogenic activity in a human umbilical vein endothelial cell (HUVEC) assay. Thalidomide (**1**) and its metabolite, 5-hydroxythalidomide (5-HT: **2**), which possesses a hydroxyl group at the position corresponding to that of 5HPP-33, as well as IMiDs (immunomodulatory derivatives of thalidomide: **3** and **5**), also showed weak or moderate activity in the same assay.

Thalidomide (**1**) is a sedative/hypnotic drug, which was banned in the 1960s because of its teratogenicity.[1,2] It has since been established to be effective for the treatment of various diseases, including graft-versus-host disease, cancers, AIDS, and other angiogenesis-dependent disorders.[3–5] The United States Food and Drug Administration (FDA) gave approval to thalidomide for the treatment of erythema nodosum leprosum (ENL) in 1998.[6] Furthermore, immunomodulatory derivatives of thalidomide (IMiDs), in particular the 4-amino analogues (CC-4047: **3** and CC-5013: **5**), are under clinical development for the treatment of various cancers, including multiple myeloma (MM), solid tumors, and prostate cancer.[4,5] Although the precise mechanisms of action are unknown, the anti-angiogenic effects of thalidomide and IMiDs are believed to be associated with its antimyeloma activity.[7,8] Moreover, the anti-angiogenic effects of IMiDs are mediated through the inhibition of endothelial cell growth, rather than through cytotoxic mechanisms.[8] In the present study, we measured the anti-angiogenic activity of thalidomide derivatives (**1**–**6**) using a human umbilical vein endothelial cell (HUVEC) assay. Based on the results, we suspected that phenylphthalimides derived from thalidomide might also possess anti-angiogenic activity and we performed some structural development studies.

All the compounds **1**–**17** (Fig. 1) were prepared by usual organic synthetic methods and gave analysis values close to those expected. Thalidomide (**1**), 5-HT (**2**), CC-4047 (**3**), and CC-5013 (**5**) were prepared as described previously, and 4-NT (**4**) and 4-NHT (**6**) are intermediates in the preparation of CC-4047 (**3**) and CC-5013 (**5**), respectively.[9–12] Similar reactions using 2,6-diisopropylaniline, instead of 3-aminopiperidine-2,6-dione, gave 4AHPP-33 (**16**) and 4NHPP-33 (**17**).[13,14] Preparation of compounds **7**–**15** has already been reported.[9–15]

First, we investigated the effect of thalidomide (**1**), its metabolites (5-hydroxythalidomide: 5-HT, **2**), IMiDs (CC-4047: **3**, CC-5013: **5**), and its nitro-substituted analogues, 4-NT (**4**) and 4-NHT (**6**), using HUVEC tube formation assay.[16] HUVECs were plated on Matrigel and treated with test compounds for 6 h, and tube formation was measured as previously reported.[16] Briefly, six-well plates were coated with 1.5 mL of the Matrigel basement membrane matrix (Becton Dickinson) and allowed to gel at 37 °C under a 5% CO_2 atmosphere for 30 min. Then, HUVECs were plated at 5.0×10^5 cells/well in DMEM containing the vehicle (0.5% DMSO) and growth factors (hEGF, VEGF, hFGF-B, and R^3-IGF-1, as well as FBS) in the presence or absence of test compounds (100 μM) and incubated at 37 °C under a 5% CO_2 atmosphere for 6 h. After incubation, each well was photographed using a ×5 objective to analyze tube formation. The corresponding area was measured as the number of pixels using MetaMorph software (Universal Imaging, Downingtown, PA). Experiments were

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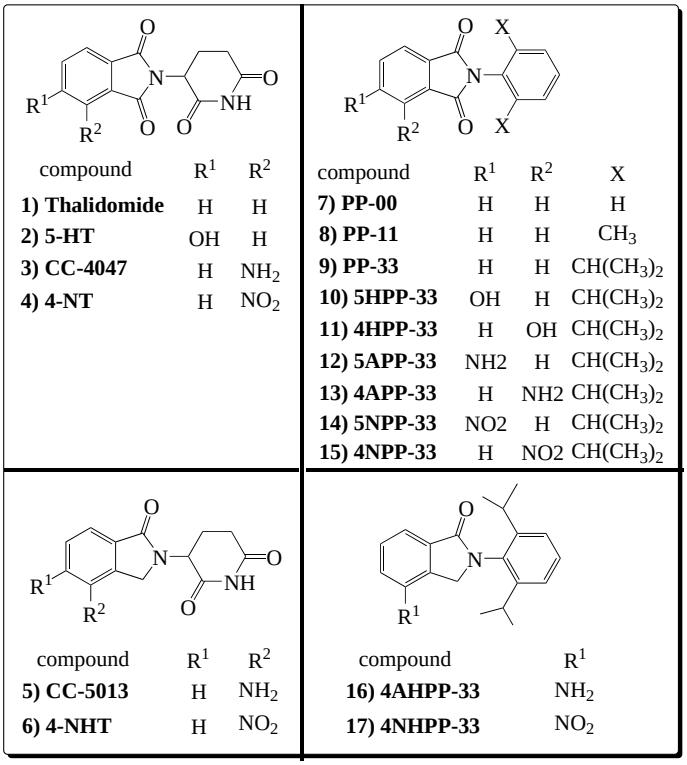

Figure 1. Structures of compounds studied in this letter.

repeated at least three times. Of course, the values differed from experiment to experiment, but the results were basically reproducible and a typical set of data is presented.

As shown in Figures 2 and 3, thalidomide (**1**) exhibited moderate anti-angiogenic activity (ca. 26% inhibition at 100 μM). One of its major metabolites, 5-HT (**2**), also showed moderate activity, comparable to that of thalidomide (**1**). One of the IMiDs, CC-4047 (**3**), the 4-amino analogue of thalidomide (**1**), is less active than thalidomide (**1**) (ca. 21% inhibition at 100 μM). However, introduction of an electron-withdrawing nitro group instead of an electron-donating amino group at the same 4-position, that is, 4-NT (**4**), resulted in more potent anti-angiogenic activity than that of thalidomide (**1**) (ca. 32% inhibition at 100 μM). The decarbonyl derivative of CC-4047 (**3**), that is, CC-5013 (**5**), is also more potent than thalidomide (**1**) (ca. 32% inhibition at 100 μM). Replacement of the electron-donating amino group of CC-5013 (**5**) with an electron-withdrawing nitro group, that is, 4-NHT (**6**), showed just the opposite effect to that in the case of CC-4047 (**3**). CC-5013 (**5**) showed more potent anti-angiogenic activity than 4-NHT (**6**).

In our previous structural development studies of thalidomide, we have obtained TNF-α production regulators (including bidirectional ones, pure inhibitors, and pure enhancers),[9,15] androgen antagonists,[17–19] aminopeptidase inhibitors,[20–22] α-glucosidase inhibitors,[23,24] thymidine phosphorylase inhibitors,[25] cyclooxygenase (COX) inhibitors,[26,27] and nitric oxide synthase (NOS) inhibitors.[28,29] In the course of those studies, we noticed that the phenylphthalimide analogues also possess some interesting activities. Therefore, we investigated the anti-angiogenic activity of phenylphthalimide analogues of thalidomide using a HUVEC assay system.

As shown in Figures 2 and 4, PP-33 (**9**) possessed rather potent anti-angiogenic activity. The structural requirement for the activity seems to be clear, because derivatives of PP-33 (**9**) with less bulky alkyl groups (PP-00: **7** and PP-11: **8**) showed no or only slight anti-angiogenic activity. These results suggest that the anti-angiogenic activity of phenylphthalimide analogues is a specific feature of the 2,6-diisopropylphenylphthalimide structure.

Next, we investigated the effect of substituents introduced at the 4- or 5-position of the phthalimide moiety of PP-33 (**9**), that is, compounds **10**–**15**. The results are shown in Figures 2 and 5. 5HPP-33 (**10**), which possesses a hydroxyl group at the position corresponding to that of 5-HT (2), showed quite potent anti-angiogenic activity dose dependently (ca. 59% inhibition at 100 μM, ca. 41% inhibition at 30 μM, and ca. 8% inhibition at 10 μM). Its isoelectronic amino derivative, 5APP-33 (**12**), also showed potent anti-angiogenic activity, though it was less potent than 5HPP-33 (**10**). The regio-isomers of 5HPP-33 (**10**) and 5APP-33 (**12**), that is, 4HPP-33 (**11**) and 4APP-33 (**13**), respectively, are less active than the corresponding 5-substituted analogues. Interestingly, analogues substituted with an electron-withdrawing nitro group (5NPP-33: **14** and 4NPP-33: **15**) showed position dependency, in contrast to the analogues bearing an electron-donating

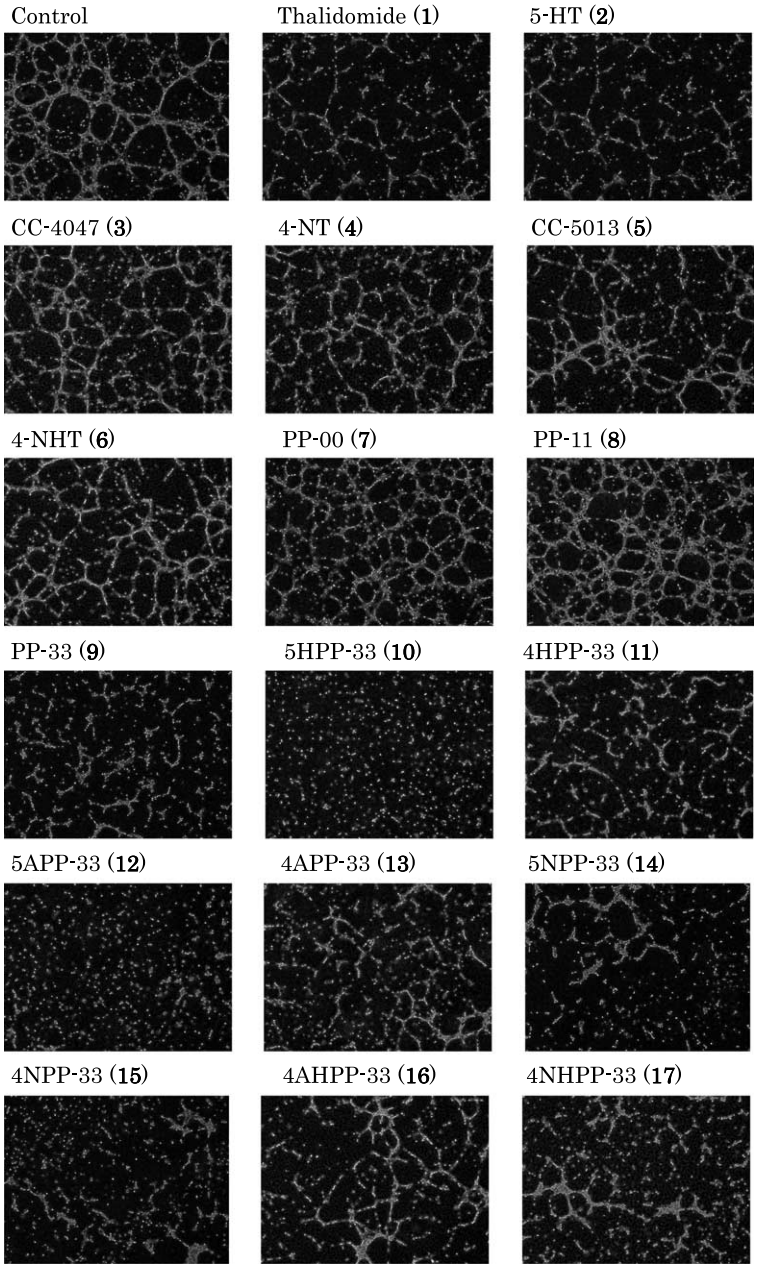

Figure 2. HUVEC tube formation assay.

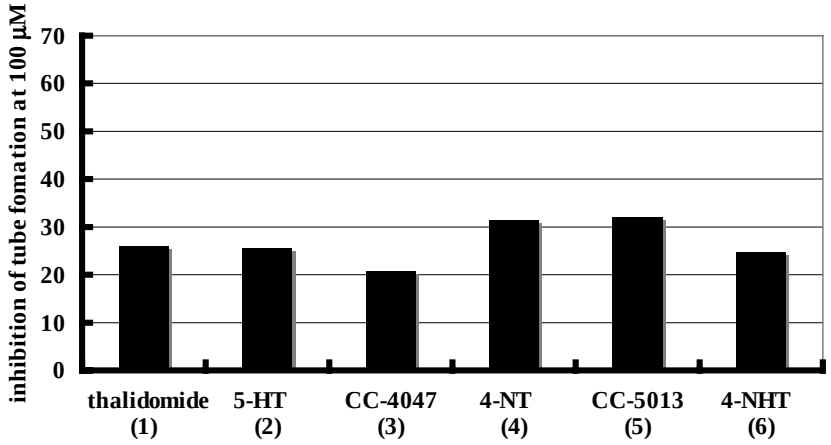

Figure 3. HUVEC tube formation-inhibiting activity of thalidomide (**1**) and derivatives (**2**–**6**).

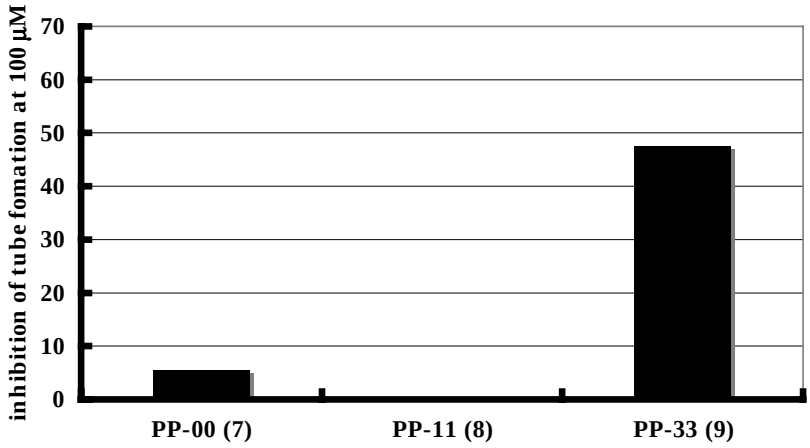

Figure 4. HUVEC tube formation-inhibiting activity of phenylphthalimide (PP-00: **7**) and its alkylated analogues (PP-11: **8** and PP-33: **9**).

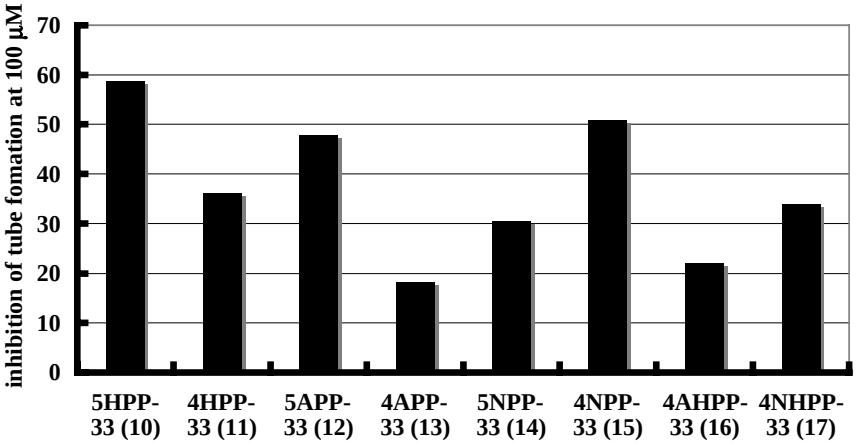

Figure 5. HUVEC tube formation-inhibiting activity of 2,6-diisopropylphthalimide analogues.

group, that is, 4NPP-33 (**15**) was more potent than 5NPP-33 (**14**).

A similar reversal of effect between derivatives with an electron-donating amino and an electron-withdrawing nitro substituent was found in the case of decarbonylation. While decarbonylation of 4APP-33 (**13**) to 4AHPP-33 (**16**) resulted in enhancement of the activity, the same derivatization of 4NPP-33 (**15**) to afford 4NHPP-33 (**17**) produced a decrease of the activity.

The results suggest that for potent anti-angiogenic activity of phenylphthalimide analogues, a 2,6-diisopropylphenyl structure and an electron-donating substituent at the 5-position or an electron-withdrawing substituent at the 4-position of the phenylphthalimide moiety are required.

In conclusion, we have discovered potent phenylphthalimide-type inhibitors of angiogenesis in a HUVEC tube formation assay. Among the prepared compounds, 5HPP-33 (**10**) showed the most potent activity. Angiogenesis has recently become a primary target of anticancer therapy. Further structural development studies based on the present compounds may yield potent and non-teratogenic derivatives.

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13. 4-AHPP-33 (**16**). MS (FAB): M+1 = 309. ^{1}H NMR (500 MHz/CDCl $_3$/δ): 1.20 (dd, J = 5.1, 6.9 Hz, 12H), 2.78 (sept, J = 6.9 Hz, 2H), 3.76 (s, 2H), 4.40 (s, 2H), 6.92 (d, J = 7.7 Hz, 1H), 7.25 (d, J = 7.7 Hz, 1H), 7.44–7.35 (m, 4H). Anal. Calcd for $C_{20}H_{24}N_2O{\cdot}1/3H_2O$: C, 76.40; H, 7.91; N, 8.91. Found: C, 76.12; H, 7.78; N, 8.60.
14. 4-NHPP-33 (**17**). MS (FAB): M+1 = 339. ^{1}H NMR (500 MHz/CDCl $_3$/δ): 1.20 (dd, J = 6.8, 15.3 Hz, 12H), 2.72 (sept, J = 6.8 Hz, 2H), 5.02 (s, 2H), 7.26 (d, J = 7.7 Hz, 2H), 7.41 (t, J = 7.7 Hz, 1H), 7.77 (t, J = 7.7 Hz, 1H), 8.30 (d, J = 7.7 Hz, 4H), 8.47 (d, J = 8.1 Hz, 1H). Anal. Calcd for $C_{20}H_{22}N_2O_3$: C, 70.99; H, 6.55; N, 8.28. Found: C, 71.02; H, 6.58; N, 8.17.
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