Effects of Immunomodulatory Derivatives of Thalidomide (IMiDs) and Their Analogs on Cell-Differentiation, Cyclooxygenase Activity and Angiogenesis

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Various analogs of known immunomodulatory derivatives of thalidomide (1) (IMiDs: 3, 5) were synthesized, focusing on cell-differentiation-inducing, cyclooxygenase-inhibitory and anti-angiogenesis activities. Among the prepared compounds, NIDO-33 (14) showed cell differentiation-inducing activity on HL-60 cells and anti-angiogenic activity on human umbilical vein endothelial cells (HUVEC). AIDO-00 (7) also showed anti-angiogenic activity. NIDO-11 (8) showed an enhancing effect on all-*trans* retinoic acid (ATRA)-induced HL-60 cell differentiation, and AIDO-30 (13) exhibited cyclooxygenase (COX)-inhibitory activity.

Key words thalidomide; immunomodulatory derivative of thalidomide (IMiD); differentiation; angiogenesis; cyclooxygenase

Thalidomide [α-(*N*-phthalimido)glutarimide, **1**] was originally used as a sedative/hypnotic drug, but was banned in the 1960s because of its serious teratogenicity.[1—4] Remarkably, thalidomide (**1**) was subsequently discovered to have various biological activities, including anti-inflammatory and anti-angiogenic properties, and was identified as an effective agent for the treatment of multiple myeloma (MM).[1,2] In 1998, the drug was approved in the U.S.A. for the treatment of Hansen's disease, under critical control known as S.T.E.P.S. (system for thalidomide education and prescribing safety), and has been used world-wide for the treatment of various cancers. Although the precise molecular basis of the drug's activities is not yet fully established, inhibition of tumor necrosis factor (TNF-α) production elicited by thalidomide (**1**) was initially considered to be one of the key action mechanisms.[1—4] Recently, a series of compounds has been created by chemical modification of thalidomide, focusing on the TNF-α production-inhibitory activity, to overcome the original devastating side effects.[1—4] Among them, 4-amino analogs of thalidomide (**1**), *i.e.*, CC-4047 (**3**) and CC-5013 (**5**), have been shown to possess potent anti-cancer and anti-inflammatory activities with little or none of the toxicity of thalidomide (**1**), and these compounds are known as IMiDs (immunomodulatory derivatives of thalidomide).[1,2] CC-5013 (**5**) appeared to be non-teratogenic when tested in the thalidomide-sensitive New Zealand rabbit preclinical model, in which thalidomide-associated teratogenicity can be detected.[1] Consequently, IMiDs (**3**, **5**) are under clinical development for the treatment of the myelodysplastic syndromes (MDS) and various cancers, including multiple myeloma (MM) and prostate cancer.[1,2]

In spite of the established activity of thalidomide (**1**) and IMiDs (**3**, **5**) as immunomodulatory agents, their mechanism(s) of action remain unclear.[1—4] Concerning thalidomide (**1**), studies of its TNF-α production-inhibitory activity and antiangiogenic activity have been reported.[1—4] We have engaged in extensive structural development studies of thalidomide (**1**), and have synthesized TNF-α production regulators,[3,4] inhibitors of various enzymes [cyclooxygenase (COX), nitrogen monoxide synthase (NOS), α-glucosidase, dipeptidylpeptidase type IV (DPP-IV), puromycin-sensitive aminopeptidase (PSA), histone deacetylase (HDAC), and μ-calpain],[3—6] anti-androgens,[3,4,7,8] cell differentiation inducers,[9] and other agents based on the thalidomide structure. During these studies, we found several previously unknown biological activities of thalidomide (**1**), including enhancing activity on all-*trans* retinoic acid (ATRA)-induced cell differentiation of human leukemia cell line HL-60[10] and COX-inhibitory activity.[11] Concerning the former activity, enhancement of HL-60 cell differentiation-induction is observed in the presence of the physiological concentration of ATRA, implying that thalidomide (**1**) may act as a cell differentiation inducer in the human body to elicit its anti-tumor activity.[10] Concerning the latter activity, the relationship(s) between COX inhibition and antiangiogenic activity has been well documented, *i.e.*, COX inhibition results in inhibition of the production of prostaglandin E_2, an inducer of vascular endothelial growth factor (VEGF), which plays a major role in angiogenesis.[12—14] The antiangiogenic activity of thalidomide (**1**) has been suggested to play a major role in the anti-tumor action of the drug,[1,2] and we also established that it (and its derivatives) inhibit tube formation of human umbilical vein endothelial cells (HUVEC).[15] These facts led us prepare various derivatives of IMiDs (**3**, **5**)/thalidomide (**1**), *i.e.*, compounds **2**, **4**, and **6**—**17**, and to investigate the effects of these compounds (**1**—**17**) on HL-60 cell differentiation, COX activity, and HUVEC tube formation.

Results and Discussion

Chemistry Compounds **1**—**17** (Fig. 1) were prepared by usual organic synthetic methods (Chart 1) and gave analytical values close to those expected. Thalidomide (**1**) was prepared as previously reported.[16] The 4-amino derivative of thalidomide (**1**), *i.e.*, CC-4047 (**3**), and its decarbonylated analog, CC-5013 (**5**), were prepared as described by Muller *et al.*[17] The 4-nitro derivative of thalidomide (**1**), *i.e.*, 4NT (**2**), and its decarbonylated analog, H4NT (**4**), are intermediates in the synthesis of CC-4047 (**3**) and CC-5013 (**5**), respectively. Briefly, condensation of 3-aminopiperidine-2,6-dione with 3-nitrophthalic anhydride gave 4NT (**2**), and simi-

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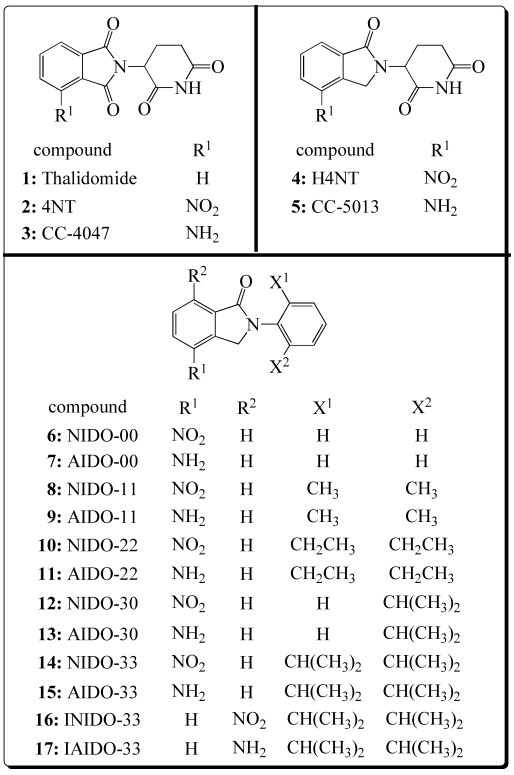

compound	R^1
1: Thalidomide	H
2: 4NT	NO_2
3: CC-4047	NH_2

compound	R^1
4: H4NT	NO_2
5: CC-5013	NH_2

compound	R^1	R^2	X^1	X^2
6: NIDO-00	NO_2	H	H	H
7: AIDO-00	NH_2	H	H	H
8: NIDO-11	NO_2	H	CH_3	CH_3
9: AIDO-11	NH_2	H	CH_3	CH_3
10: NIDO-22	NO_2	H	CH_2CH_3	CH_2CH_3
11: AIDO-22	NH_2	H	CH_2CH_3	CH_2CH_3
12: NIDO-30	NO_2	H	H	$CH(CH_3)_2$
13: AIDO-30	NH_2	H	H	$CH(CH_3)_2$
14: NIDO-33	NO_2	H	$CH(CH_3)_2$	$CH(CH_3)_2$
15: AIDO-33	NH_2	H	$CH(CH_3)_2$	$CH(CH_3)_2$
16: INIDO-33	H	NO_2	$CH(CH_3)_2$	$CH(CH_3)_2$
17: IAIDO-33	H	NH_2	$CH(CH_3)_2$	$CH(CH_3)_2$

Fig. 1. Structures of the Compounds Studied in This Paper

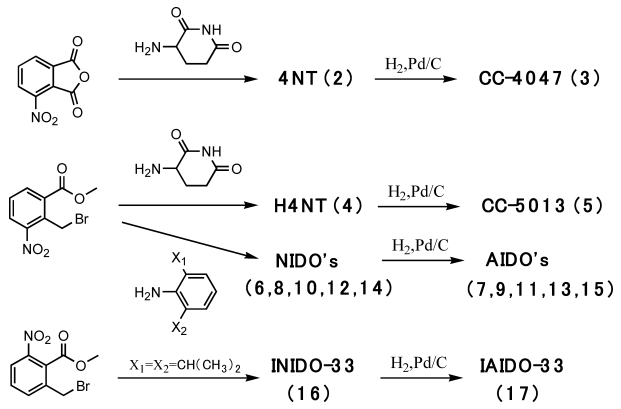

Chart 1

lar condensation with methyl (2-bromoethyl-3-nitro)benzoate gave H4NT (**4**).[17]

Our previous structural development studies of thalidomide (**1**) have indicated that replacement of the glutarimide moiety of thalidomide (**1**) with a non-substituted, a 2-alkyl-substituted or a 2,6-dialkyl-substituted phenyl ring affords compounds which partially retain various biological activities of thalidomide (**1**).[3,4] Therefore, amino- or nitrophthalimides with a *N*-phenyl, *N*-2-alkylphenyl or *N*-2,6-dialkylphenyl substituent (compounds **6**—**17**) were designed. Reactions similar to the condensation reaction used for the preparation of H4NT (**4**) with methyl (2-bromoethyl-3-nitro)benzoate using aniline, 2,6-dimethylaniline, 2,6-diethylaniline, 2-isopropylaniline and 2,6-diisopropylaniline, instead of 3-aminopiperidine-2,6-dione, gave NIDO-00 (**6**), NIDO-11 (**8**), NIDO-22 (**10**), NIDO-30 (**12**) and NIDO-33 (**14**), respectively. Reduction of these nitro compounds with hydrogen gas over Pd/C gave the corresponding amino derivatives [AIDO-00 (**7**), AIDO-11 (**9**), AIDO-22 (**11**), AIDO-30 (**13**) and AIDO-33 (**15**)]. Similar reactions using methyl (2-bromoethyl-6-nitro) benzoate instead of methyl (2-bromoethyl-3-nitro)benzoate gave INIDO-33 (**16**) and IAIDO (**17**), which are regioisomers of NIDO-33 (**14**) and AIDO-33 (**15**), respectively.

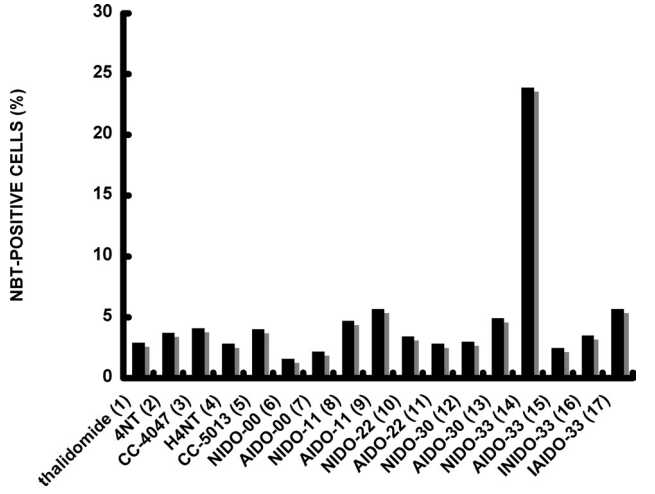

Fig. 2. HL-60 Cell Differentiation-Inducing Activity of Compounds **1**—**17** (10 μM)

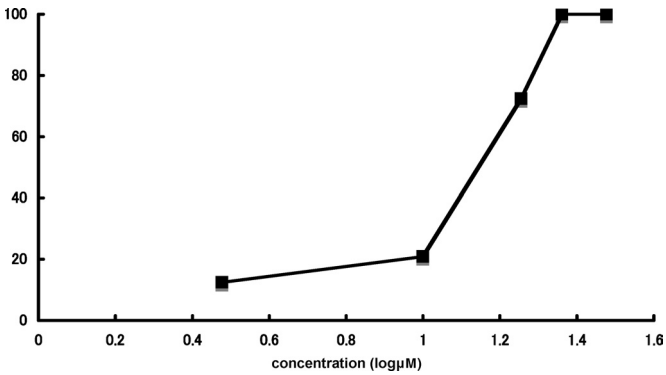

Fig. 3. HL-60 Cell Differentiation-Inducing Activity of NIDO-33 (**14**)

Effects on HL-60 Cell Differentiation First we examined the HL-60 cell differentiation-inducing activity of the prepared compounds (**1**—**17**); this was estimated in terms of the nitroblue tetrazolium (NBT)-reducing activity of the cells as previously reported.[18] Of course, the measured NBT-positive percentage values differed from experiment to experiment, but the results were basically reproducible. A typical set of data is presented in Fig. 2. As shown in the figure, almost all the compounds, except NIDO-33 (**14**), were inactive or had very weak HL-60 cell differentiation-inducing activity (2—6% NBT-positive cells) at 10 μM under the experimental conditions used (generally, non-treated HL-60 cells contain 2—5% NBT-positive cells). Only NIDO-33 (**14**) showed apparent HL-60 cell differentiation-inducing activity (*ca.* 25% NBT-positive cells at 10 μM). This activity was confirmed to be dose-dependent, as shown in Fig. 3. The HL-60 cell differentiation-inducing activity of NIDO-33 (**14**) could be observed at a concentration as low as 1 μM, and almost all the cells were differentiated at the concentration of 23 μM (Fig. 3). To examine the features of the cells differentiated with NIDO-33, Wright-Giemsa staining and fluorescence-acti-

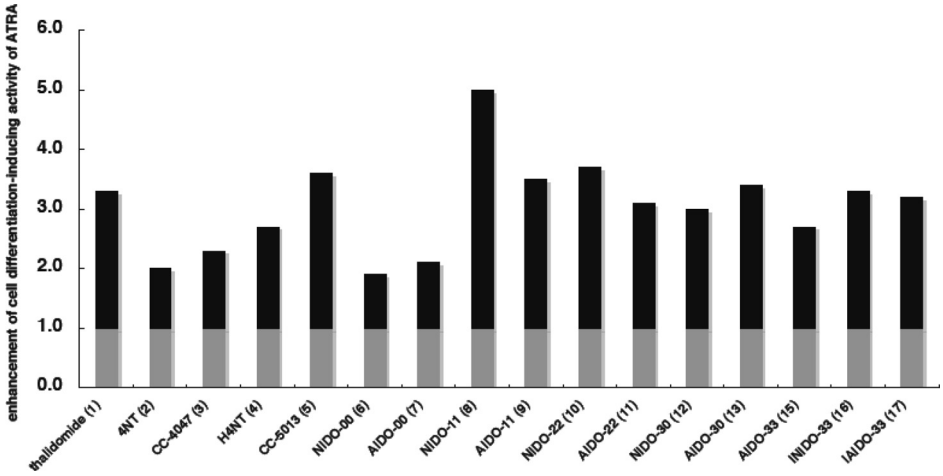

Fig. 4. Effects of Compounds **1**—**13** and **15**—**17** on ATRA-Induced HL-60 Cell Differentiation

vated cell sorter (FACS) analysis using granulocyte/monocyte-specific CD11b FITC (fluorescein isothiocyanate) conjugate and monocyte-specific CD14 phycoerythrin conjugate were performed as described previously.[9,19] The results indicated that NIDO-33 (**14**) induces HL-60 cell differentiation to mature monocytes, as does 1α,25-dihydroxyvitamin D_3 [1,25-$(OH)_2$-VD_3]. Typical HL-60 monocytic differentiation inducers are rather complex molecules, including 1,25-$(OH)_2$-VD_3 and 12-*O*-tetradecanoylphorbol 13-acetate (TPA), while HL-60 granulocytic differentiation inducers include quite simple compounds, such as dimethylsulfoxide (DMSO).[20] NIDO-33 (**14**) can be classified as a rather simple/small molecule, so it can be considered as unusual for a HL-60 monocytic differentiation inducer. The fact that only NIDO-33 (**14**) was found to be active among the various structurally related derivatives suggests that the structure of the compound is critically recognized in the cell differentiation-inducing assay system, though the mechanism involved is not known.

Next we investigated the enhancing effect of the prepared compounds (**1**—**17**) on ATRA-induced HL-60 cell differentiation (Fig. 4). Although compounds **1**—**13** and **15**—**17** themselves do not possess apparent HL-60 cell differentiation-inducing activity at 10 μM (Fig. 2), they showed potent HL-60 cell differentiation-inducing activity at the same concentration in the presence of a physiological concentration (0.6 nM) of ATRA (Fig. 4). In the figure, the level of NBT-positive cells (%) that appeared in the presence of 0.6 nM ATRA alone (*ca.* 20%) was defined as unity (1.0), and the relative values are presented on the vertical scale. Thalidomide (**1**) enhanced 0.6 nM ATRA-induced HL-60 cell differentiation *ca.* 3.2-fold, which is in accordance of our previous report.[10] As for the IMiDs, CC-5013 (**5**) showed more potent activity than thalidomide (**1**), but CC-4047 (**3**) was less potent than thalidomide (**1**), suggesting that this cell differentiation-enhancing activity is not the basis for the superiority of IMiDs as anti-tumor agents compared with thalidomide (**1**), though this activity is likely contribute at least in part to the effectiveness of IMiDs.

Exchange of one carbonyl group of the phthalimide moiety of 4NT (**2**) and CC-4047 (**3**) to a methylene group [H4NT (**4**) and CC-5013 (**5**), respectively] resulted in an increase of the activity, *i.e.*, the activity decreased in the order of **4**>**2**, and **5**>**3**. In this series of compounds (**2**—**5**), the 4-amino analogs (**3**, **5**) are more potent than the corresponding 4-nitro analogs (**2**, **4**, respectively). On the other hand, in the *N*-arylphthalimide analog series (**6**—**13**, **16**, **17**), there is no clear tendency for 4-amino analogs to be more potent than the corresponding 4-nitro analogs. Remarkably, the most potent compound among those prepared was the 4-nitro analog, NIDO-11 (**8**), which enhanced the 0.6 nM ATRA-induced HL-60 cell differentiation *ca.* 5-fold (almost 100% NBT-positive cells) at 10 μM (Fig. 4). The cell differentiation induction-enhancing activity of NIDO-11 (**8**) was shown to be dose-dependent. Wright-Giemsa staining and FACS data indicated that NIDO-11 (**8**) enhances ATRA-inducing HL-60 cell differentiation to mature granulocytes, though the mechanism of the enhancing activity is not known. Similar enhancement of ATRA-induced HL-60 cell differentiation has been found with TPA and tubulin disruptors at low concentrations.[10,21] However, none of the compounds (**1**—**13**, **15**—**17**) possessed TPA-like activity (*i.e.*, monocytic differentiation-inducing activity toward HL-60 cells), nor did they affect tubulin polymerization.

Although we found a HL-60 cell differentiation inducer, NIDO-33 (**14**), and a potent cell differentiation-induction enhancer, NIDO-11 (**8**), the structure–activity relationship(s) remain to be established. The HL-60 cell differentiation-inducing activity and the enhancement of this activity described here appear to be independent phenomena, because the most active compound in the two assay systems was different, *i.e.*, NIDO-33 (**14**) and NIDO-11 (**8**), though their structures are very similar. NIDO-33 (**14**) and NIDO-11 (**8**) appear to be unique lead compounds for the development of novel types of agents which might be useful for the differentiation-inducing therapy of cancers.

COX-Inhibitory Activity COX is an enzyme which catalyzes the synthesis of prostaglandins from arachidonic acid, and is well-known as a target molecule of non-steroidal anti-inflammatory drugs (NSAIDs), including aspirin.[22—24] There are two isoforms of COX. COX-1 is constitutively expressed in most tissues, whereas COX-2 is inducible. Overexpression of COX has been detected in various tumors and its role in carcinogenesis and angiogenesis has been well-documented.[12—14,24—26] In the past, we suspected that COX is another target molecule of thalidomide (**1**), because the drug is

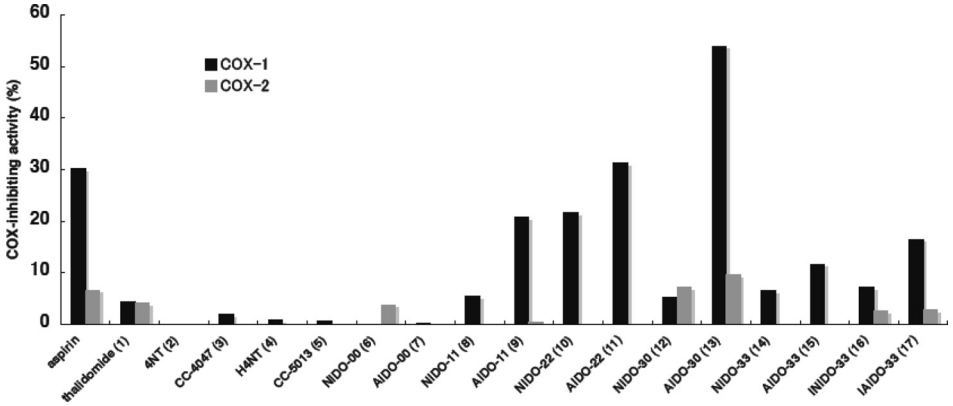

Fig. 5. COX-Inhibitory Activities of Compounds **1**—**17** (100 μM)

effective against colon and prostate cancers and possesses anti-angiogenic activity,[27,28] in which COX plays an important role. Indeed, we found that thalidomide (**1**) itself possesses COX-inhibiting activity.[11] We therefore examined the COX-inhibitory activity of IMiDs (**3**, **5**) and their derivatives (Fig. 5). Thalidomide (**1**) showed weak inhibitory activity toward both COX-1 and COX-2, as already reported.[11] IMiDs (**3**, **5**), however, showed only very weak or no COX-inhibitory activity, suggesting that COX inhibition might not be related to the superior immunomodulatory activity of IMiDs (**3**, **5**).

Among the compounds prepared here, compounds **8**—**17** showed rather potent COX-1-inhibiting activity, and compounds **6**, **12**, and **13** showed rather potent COX-2-inhibiting activity (Fig. 5). As regards COX-1-inhibitory activity, AIDO-30 (**13**) was the most potent, having a higher potency than that of aspirin (Fig. 5). The amino analogs (**9**, **11**, **13**, **15**, **17**) were more potent than the corresponding nitro analogs (**8**, **10**, **12**, **14**, **16**, respectively). Among the 4-amino analogs, a mono-isopropyl substituent at the *ortho* position of the *N*-phenyl ring seemed to be the best, *i.e.*, the activity decreased in the order of mono-isopropyl [AIDO-30 (**13**)]>2,6-diethyl [AIDO-22 (**11**)]>2,6-dimethyl [AIDO-11 (**9**)]>2,6-diisopropyl [AIDO-33 (**15**)]. On the other hand, among the 4-nitro analogs, the mono-isopropyl-substituted analog [NIDO-30 (**12**)] is almost the least potent, and 2,6-diethyl substitution seemed to be the best, *i.e.*, the activity decreased in the order of 2,6-diethyl [NIDO-22 (**10**)]>2,6-diisopropyl [NIDO-33 (**16**)]>2,6-dimethyl [NIDO-11 (**8**)]= mono-isopropyl [NIDO-30 (**12**)].

As for COX-2-inhibitory activity, AIDO-30 (**13**) was also the most potent among the prepared compounds. Its COX-2-inhibitory activity was higher than that of aspirin (Fig. 5), though it would still be classified as a COX-1-selective inhibitor. AIDO-30 (**13**) should be a useful lead compound for the development of a unique class of COX inhibitors, because the structure of the compound is rather simple, and it possesses basic nature, which is rare among reported COX inhibitors.

Anti-angiogenic Activity As mentioned above, the relationship between COX-inhibitory activity and antiangiogenic activity has been well-documented.[12—14] So, we examined the antiangiogenic activity of the prepared compounds by means of assay of tube formation-inhibitory activity toward HUVEC cells. The HUVEC cells were treated with 100 μM test compounds according to the reported method,[15] and a typical set of data is shown in Table 1. Thalidomide (**1**) showed moderate activity, which is in consistent with our previous report.[15] One of the IMiDs, CC-5013 (**5**), showed more potent activity than thalidomide (**1**), while the activity of the other IMiD, CC-4047 (**3**), was comparable to that of thalidomide (**1**). The nitro analog, 4NT (**2**), showed potent activity, comparable with that of CC-5013 (**5**).

Table 1. HUVEC Tube Formation-Inhibiting Activity of Compounds **1**—**17** (100 μM)

Compounds	Inhibition of tube formation (%)
Thalidomide (**1**)	26
4NT (**2**)	32
CC-4047 (**3**)	21
H4NT (**4**)	25
CC-5013 (**5**)	32
NIDO-00 (**6**)	8
AIDO-00 (**7**)	33
NIDO-11 (**8**)	28
AIDO-11 (**9**)	19
NIDO-22 (**10**)	0
AIDO-22 (**11**)	8
NIDO-30 (**12**)	0
AIDO-30 (**13**)	0
NIDO-33 (**14**)	33
AIDO-33 (**15**)	22
INIDO-33 (**16**)	21
IAIDO-33 (**17**)	16

Among the *N*-aryl derivatives prepared (**6**—**17**), NIDO-22 (**10**), (**12**) and AIDO-30 (**13**) were completely inactive, but the others showed moderate to potent tube formation-inhibitory activity on HUVEC (Table 1). AIDO-00 (**7**) and NIDO-33 (**14**) showed potent activity, comparable with that of CC-5013 (**5**). No clear structure–activity relationship could be extracted from our data, but, at least, the activity (Table 1) does not seem to correlate with the COX-inhibitory activity of the compounds (Fig. 5). The molecular basis of the HUVEC tube formation inhibition observed in our experiments should therefore be different from that of the activity exhibited by NSAIDs. Further investigation of the mechanism of HUVEC tube formation inhibition and structural development studies to elucidate the structure–activity relationships are in progress.

Conclusion

We examined the effects of thalidomide (**1**), IMiDs (**3**, **5**) and their analogs (**2**, **4**, **6**—**17**) on HL-60 cell differentiation, COX activity and angiogesis. Although we could not elucidate the structural basis for the superiority of IMiDs (**3**, **5**) over thalidomide, we identified analogs with HL-60 cell differentiation-inducing activity [NIDO-33 (**14**)], enhancing activity on ATRA-induced HL-60 cell differentiation [NIDO-11 (**8**)], COX-inhibitory activity [AIDO-30 (**13**)], and HUVEC tube formation-inhibitory activity [AIDO-00 (**7**) and NIDO-33 (**14**)]. Thalidomide (**1**) possesses HL-60 cell differentiation-enhancing, COX-inhibitory, and anti-angiogenic effects, and IMiDs (**3**, **5**) possess HL-60 cell differentiation-enhancing and anti-angiogenic effects. In this sense, it can be said that separation of these activities by structural development based on IMiDs (**3**, **5**) has been partially successful. In addition, NIDO-33 (**14**) showed HL-60 cell differentiation-inducing activity, which is not elicited by thalidomide (**1**) or IMiDs (**3**, **5**). Our results imply that further structural development studies of thalidomide (**1**) and IMiDs (**3**, **5**) might allow us to fully separate these biological activities, leading to a range of unique biologically active compounds. Further structural development studies and investigation of the molecular mechanisms of action are in progress.

Experimental

Chemicals NIDO-00 (**6**): MS (FAB): M+1=255. ^{1}H-NMR (500 MHz/$CDCl_3$/δ): 5.35 (s, 2H), 7.26 (t, J=7.7 Hz, 1H), 7.48 (t, J=7.7 Hz, 2H), 7.76 (t, J=7.7 Hz, 1H), 7.91 (d, J=7.7 Hz, 2H), 8.28 (d, J=7.7 Hz, 1H), 8.47 (d, J=7.7 Hz, 1H). *Anal.* Calcd for $C_{14}H_{10}N_2O_3$: C, 66.14; H, 3.96; N, 11.02. Found: C, 65.91; H, 4.18; N, 10.91. mp: 131—133.5.

AIDO-00 (**7**): MS (FAB): M+1=225. ^{1}H-NMR (500 MHz/$CDCl_3$/δ): 4.47 (s, 2H), 5.51 (s, 2H), 6.82 (d, J=7.3 Hz, 1H), 6.95 (d, J=7.3 Hz, 1H), 7.16 (t, J=7.3 Hz, 1H), 7.20 (t, J=7.9 Hz, 1H), 7.43 (t, J=7.9 Hz, 2H), 7.86 (d, J=7.9 Hz, 2H). *Anal.* Calcd for $C_{14}H_{12}N_2O$: C, 74.98; H, 5.39; N, 12.49. Found: C, 74.93; H, 5.57; N, 12.45. mp: 181—182.

NIDO-11 (**8**): MS (FAB): M+1=283. ^{1}H-NMR (500 MHz/$CDCl_3$/δ): 2.21 (s, 6H), 5.07 (s, 2H), 7.19 (d, J=7.2 Hz, 2H), 7.25 (t, J=7.2 Hz, 1H), 7.78 (t, J=7.7 Hz, 1H), 8.32 (d, J=7.7 Hz, 1H), 8.48 (d, J=7.7 Hz, 1H). *Anal.* Calcd for $C_{16}H_{14}N_2O_3$: C, 68.07; H, 5.00; N, 9.92. Found: C, 67.81; H, 5.14; N, 9.82. mp: 138.5—139.5.

AIDO-11 (**9**): MS (FAB): M+1=253. ^{1}H-NMR (500 MHz/$CDCl_3$/δ): 2.20 (s, 6H), 3.75 (s, 2H), 4.44 (s, 2H), 6.90 (d, J=7.5 Hz, 1H), 7.15 (d, J=7.5 Hz, 2H), 7.21 (t, J=7.5 Hz, 1H), 7.35 (t, J=7.5 Hz, 1H), 7.42 (d, J=7.9 Hz, 1H). *Anal.* Calcd for $C_{16}H_{16}N_2O$: C, 76.16; H, 6.39; N, 11.10. Found: C, 76.12; H, 6.50; N, 11.16. mp: 211—212.5.

NIDO-22 (**10**): MS (FAB): M+1=311. ^{1}H-NMR (500 MHz/$CDCl_3$/δ): 1.22 (t, J=7.7 Hz, 6H), 2.57—2.43 (m, 4H), 7.25 (d, J=7.7 Hz, 2H), 7.38 (t, J=7.7 Hz, 1H), 7.79 (t, J=7.7 Hz, 1H), 8.32 (d, J=7.7 Hz, 1H), 8.49 (d, J=7.7 Hz, 1H). *Anal.* Calcd for $C_{18}H_{18}N_2O_3$: C, 69.66; H, 5.85; N, 9.03. Found: C, 69.69; H, 5.99; N, 8.86. mp: 160.5—162.

AIDO-22 (**11**): MS (FAB): M+1=281. ^{1}H-NMR (500 MHz/$CDCl_3$/δ): 1.18 (t, J=7.7 Hz, 6H), 2.56—2.40 (m, 4H), 3.72 (s, 2H), 4.43 (s, 2H), 6.89 (d, J=7.7 Hz, 1H), 7.20 (d, J=7.7 Hz, 2H), 7.31 (t, J=7.7 Hz, 1H), 7.34 (t, J=7.7 Hz, 1H), 7.40 (d, J=7.7 Hz, 1H). *Anal.* Calcd for $C_{18}H_{18}N_2O\cdot 1/8H_2O$: C, 76.50; H, 7.22; N, 9.91. Found: C, 76.75; H, 7.25; N, 9.72. mp: 226—227.

NIDO-30 (**12**): MS (FAB): M+1=297. ^{1}H-NMR (500 MHz/$CDCl_3$/δ): 1.25 (d, J=6.9 Hz, 6H), 2.94 (sept, J=6.8 Hz, 1H), 5.18 (s, 2H), 7.22 (d, J=7.5 Hz, 1H), 7.30 (t, J=7.5 Hz, 1H), 7.42 (d, J=7.5 Hz, 1H), 7.46 (t, J=7.5 Hz, 1H), 7.78 (t, J=7.7 Hz, 1H), 8.30 (d, J=7.7 Hz, 1H), 8.48 (d, J=7.7 Hz, 1H),. *Anal.* Calcd for $C_{17}H_{16}N_2O_3$: C, 68.91; H, 5.44; N, 9.45. Found: C, 68.75; H, 5.54; N, 9.42. mp: 157—158.

AIDO-30 (**13**): MS (FAB): M+1=267. ^{1}H-NMR (500 MHz/$CDCl_3$/δ): 1.23 (d, J=6.6 Hz, 6H), 2.99 (sept, J=6.6 Hz, 1H), 3.75 (s, 2H), 4.54 (s, 2H), 6.90 (d, J=7.7 Hz, 1H), 7.20 (d, J=7.7 Hz, 1H), 7.27 (t, J=7.7 Hz, 1H), 7.44—7.34 (m, 4H). *Anal.* Calcd for $C_{17}H_{18}N_2O$: C, 76.66; H, 6.81; N, 10.52. Found: C, 76.37; H, 6.90; N, 10.50. mp: 194—195.

INIDO-33 (**16**): MS (FAB): M+1=339. ^{1}H-NMR (500 MHz/$CDCl_3$/δ): 1.23 (dd, J=6.7, 4.9 Hz, 12H), 2.74 (sept, J=6.7 Hz, 2H), 4.62 (s, 2H), 7.27 (d, J=8.4 Hz, 2H), 7.42 (t, J=8.4 Hz, 2H), 7.84—7.74 (m, 3H). *Anal.* Calcd for $C_{20}H_{22}N_2O_3$: C, 70.99; H, 6.55; N, 8.28. Found: C, 70.97; H, 6.56; N, 8.21. mp: 238—240.

IAIDO-33 (**17**): MS (FAB): M+1=309. ^{1}H-NMR (500 MHz/$CDCl_3$/δ): 1.21 (d, J=6.6 Hz, 12H), 2.82 (sept, J=6.6 Hz, 2H), 4.48 (s, 2H), 6.65 (d, J=8.1 Hz, 1H), 6.75 (d, J=7.3 Hz, 1H), 7.39—7.23 (m, 4H). *Anal.* Calcd for $C_{20}H_{24}N_2O$: C, 77.89; H, 7.84; N, 9.08. Found: C, 77.96; H, 7.86; N, 9.00. mp: 190—191.5.

Cell Culture HL-60 cells were cultured in RPMI1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco BRL) at 37 °C under a 5% CO_2 atmosphere. HUVECs were cultured in EBM-2 medium supplemented with growth factors (hEGF, VEGF, hFGF-B, and R3-IGF-1, as well as FBS) at 37 °C under a 5% CO_2 atmosphere.

Assay of Cell Differentiation-Inducing Activity Measurement of HL-60 cell differentiation was performed as described previously.[9,18] Briefly, HL-60 cells were incubated in RPMI 1640 medium in the presence or absence of a test compound (10 μM) with or without 0.6 nM ATRA for 3 d. Treated HL-60 cells were mixed with phosphate-buffered saline (PBS) containing 0.2% nitroblue tetrazolium (NBT) and 20 μM 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 all-*trans* retinoic acid (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. The treated HL-60 cells were analyzed by means of FACS to characterize the differentiated cell type.[19] Briefly, HL-60 cells (1×10^6 cells), treated or not treated with a test compound (10 μM), 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).[9,19]

Assay of Anti-angiogenesis Activity HUVECs were plated on Matrigel and treated with test compounds (100 μM) for 6 h, and tube formation was measured as previously reported.[15,29] 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 under 5% CO_2 in air for 30 min. Then, HUVECs were plated at 5.0×10^5 cells/well in DMEM containing the vehicle (0.5% DMSO) with 10% FBS in the presence or absence of a test compound and incubated at 37 °C under 5% CO_2 in air 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, U.S.A.).

Assay of Cyclooxygenase-Inhibitory Activity Inhibitory activity of test compounds (100 μM) on COX-1 and COX-2 was determined with the use of a Colorimetric COX (ovine) Inhibitor Screening Assay Kit (Cayman, No. 760111), according to the protocol recommended by the supplier.[11]

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References

1) Bartlett J. B., Dredge K., Dalglish A. G., *Nat. Rev. Cancer*, **4**, 314—322 (2004).
2) Bartlett J. B., Tozer A., Stirling D., Zeldis J. B., *British J. Cancer*, **93**, 613—619 (2005).
3) Hashimoto Y., *Bioorg. Med. Chem.*, **10**, 461—479 (2002).
4) Hashimoto Y., *Curr. Med. Chem.*, **5**, 163—178 (1998).
5) Kakuta H., Takahashi H., Sou S., Kita T., Nagasawa K., Hashimoto Y., *Recent Res. Develop. Med. Chem.*, **1**, 189—211 (2001).
6) Hashimoto Y., Tanatani A., Nagasawa K., Miyachi H., *Drugs Future*, **29**, 383—391 (2004).
7) Hashimoto Y., Miyachi H., *Bioorg. Med. Chem.*, **13**, 5080—5093

(2005).
8) Ishioka T., Kubo A., Koiso Y., Nagasawa K., Itai A., Hashimoto Y., *Bioorg. Med. Chem.*, **10**, 1555—1566 (2002).
9) Noguchi T., Miyachi H., Katayama R., Naito M., Hashimoto Y., *Bioorg. Med. Chem. Lett.*, **15**, 3212—3215 (2005).
10) Noguchi T., Shinji C., Kobayashi H., Makishima M., Miyachi H., Hashimoto Y., *Biol. Pharm. Bull.*, **28**, 563—564 (2005).
11) Noguchi T., Shimazawa R., Nagasawa K., Hashimoto Y., *Bioorg. Med. Chem. Lett.*, **12**, 1043—1046 (2002).
12) Tsujii M., Kawano S., Tsuji S., Sawaoko H., Hori M., DuBois R. N., *Cell*, **93**, 705—716 (1998).
13) Sales K. J., Katz A. A., Howard B., Soeters R. P., Millar R. P., Jabbou H. N., *Cancer Res.*, **62**, 424—432 (2002).
14) Jones M. K., Wang H., Peskar B. M., Levin E., Itani R. M., Sarfeh I. J., Tarnawski A. S., *Nature Medicine*, **5**, 1418—1423 (1999).
15) Noguchi T., Fujimoto H., Sano N., Miyajima A., Miyachi H., Hashimoto Y., *Bioorg. Med. Chem. Lett.*, **15**, 5509—5513 (2005).
16) Nishimura K., Hashimoto Y., Iwasaki S., *Chem. Pharm. Bull.*, **42**, 1157—1159 (1994).
17) Muller G. W., Chen R., Huang S.-Y., Corral L. G., Wong L. M., Patterson R. T., Chen Y., Kaplan G., Stirling D. I., *Bioorg. Med. Chem. Lett.*, **9**, 1625—1630 (1999).
18) Kagechika H., Kawachi E., Hashimoto Y., Himi T., Shudo K., *J. Med. Chem.*, **31**, 2182—2192 (1988).
19) Okabe-Kado J., Kasukabe T., Honma Y., *Leuk. Res.*, **26**, 569—576 (2002).
20) Koeffler H. P., *Blood*, **62**, 709—721 (1983).
21) Nakajima O., Sugishita Y., Hashimoto Y., Iwasaki S., *Biol. Pharm. Bull.*, **17**, 742—744 (1994).
22) Smith W. L., Garavito R. M., DeWitt D. L., *J. Biol. Chem.*, **271**, 33157—33160 (1996).
23) Taketo M. M., *J. Natl. Cancer Inst.*, **90**, 1529—1536 (1998).
24) Vane J. R., *Nature* (London), **367**, 215—216 (1994).
25) Prescott S. M., Fitzpatrick F. A., *Biochim. Biophys. Acta*, **1470**, M69—M78 (2000).
26) Reddy B. S., Hirose Y., Lubet R., Steele V., Kelloff G., Paulson S., Seibert K., Rao C. V., *Cancer Res.*, **60**, 293—297 (2000).
27) D'Amato R. J., Loughnan M. S., Flynn E., Folkman J., *Proc. Natl. Acad. Sci. U.S.A.*, **91**, 4082—4085 (1994).
28) Shimazawa R., Miyachi H., Takayama H., Kuroda K., Kato F., Kato M., Hashimoto Y., *Biol. Pharm. Bull.*, **22**, 224—226 (1999).
29) Ng S. S. W., Gutschow M., Weiss M., Hauschildt S., Teubert U., Hecker T. K., Luzzio F. A., Kruger E. A., Eger K., Figg W. D., *Cancer Res.*, **63**, 3189—3194 (2003).