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Bioorganic & Medicinal Chemistry Letters
Bioorganic & Medicinal Chemistry Letters 16 (2006) 3068–3072

# Anti-angiogenic activity of basic-type, selective cyclooxygenase (COX)-1 inhibitors

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Received 23 December 2005; revised 26 January 2006; accepted 7 February 2006
Available online 2 March 2006

**Abstract**—Indole- and indoline-type basic COX-1-selective competitive inhibitors, 5-amino-1-(3,5-dimethylbenzoyl)-1*H*-indole (**1**) and 5-amino-1-(3,5-dimethylphenyl)-2,3-dihydro-1*H*-indole (**2**), were found to possess anti-angiogenic activity estimated as a tube formation-inhibition using human umbilical vein endothelial cells (HUVECs).


Thalidomide is a sedative and/or hypnotic drug, which was used from the late 1950s to the early 1960s, but was withdrawn from the market because of severe teratogenicity.[1] In spite of this, thalidomide research was continued, due to the serendipitous finding of the drug's effectiveness against various intractable diseases, such as leprosy, AIDS, and certain kinds of cancers.[2] Finally, the United States Food and Drug Administration (FDA) gave marketing approval to thalidomide for the treatment of Hansen's disease in 1998, with special precautions for usage. At present, several clinical studies of thalidomide for the treatment of multiple myeloma, colon cancer, prostate cancer, and other conditions are ongoing in the US.[3]

The effectiveness of thalidomide for the treatment of certain kinds of cancers was suggested to be mediated through inhibition of tumor necrosis factor (TNF)-α production- and anti-angiogenic activity.[4] However, we suspected that cyclooxygenase (COX) might be another anticancer-related molecular target of thalidomide.

Prostaglandin and thromboxane biosynthesis involves the conversion of arachidonic acid to prostaglandin $H_2$ ($PGH_2$), a reaction catalyzed by the sequential actions of COX and prostaglandin endoperoxidase synthase (PGHS).[5] Three isozymes of COX (COX-1, COX-2, and COX-3) are known to date, of which COX-1 and COX-2 have been well defined. COX-1 is reported to be constitutively expressed in many organs or tissues, while COX-2 is inducible with various stimuli. However, recent molecular-biological studies have indicated that this simple paradigm has many exceptions. For example, COX-1 can be regulated during development,[6] while COX-2 is constitutively expressed in the brain[7] and in reproductive tissues.[8] Often, both isozymes are involved in physiological and pathophysiological conditions, while in some cases, each isozyme plays a distinct role.

COX-2 has been detected in various tumors and its roles in carcinogenesis and angiogenesis have been well documented.[9] Therefore, COX-2 is thought to be a promising therapeutic target for cancer.[10] Attempts have been made to apply COX-2 inhibitors, such as celecoxib, rofecoxib, and sulindac, for chemoprevention of various cancers, including colon and prostate cancers.[11] However, current clinical studies of a COX-2-selective inhibitor, rofecoxib (Vioxx), for preventing recurrence of colorectal polyps in patients with a history of colorectal adenomas were discontinued and the drug was withdrawn from the market because its use was associated with an increased incidence of cardiovascular events, such as heart attack and stroke.[12]

Very recently, experimental results have also indicated a possible involvement of the other isoform of COX, COX-1, in angiogenesis, thereby providing the rationale for the development of selective COX-1 inhibitors.[13] However, the effectiveness of COX-1 inhibitors as angiogenesis inhibitors has not yet been well established.

*Keywords*: Angiogenesis; COX-1; COX-1 inhibitor; HUVEC.
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doi:10.1016/j.bmcl.2006.02.021

We have recently demonstrated that thalidomide directly inhibits COX-1/COX-2 with efficacy comparable to that of the representative drug, aspirin.[14] Our earlier work on the COX-inhibiting activity of thalidomide afforded a new structural type of COX-1-selective inhibitors, such as compounds **1** and **2**; the $IC_{50}$s of **1** for COX-1 and COX-2 are 1.9 μM and undetermined (13.8 % inhibition at 30 μM), respectively, and those of **2** are 1.8 and 55 μM, respectively.[15] These compounds are structurally unique, because most of the previously reported COX-1-selective and non-selective COX inhibitors are acidic or neutral compounds.

We have also reported the anti-angiogenic activity of thalidomide and its metabolites, using a human umbilical vein endothelial cell (HUVEC) assay.[16] The study clearly indicated that thalidomide, as well as its main metabolites 5-hydroxythalidomide and *N*-hydroxythalidomide, exhibits moderate anti-angiogenic activity. Although the exact mechanism(s) of anti-angiogenic activity elicited by thalidomide and its metabolites is unknown, we hypothesized that a COX-1-mediated pathway might be involved in angiogenesis, and these compounds might act by inhibiting this pathway. Therefore, we expected that compounds, **1** and **2**, possess more potent anti-angiogenic activity than thalidomide. In this paper, we would like to report the anti-angiogenic activity of compounds **1** and **2**.

The preparation of **1**, **2**, **10**, **14**, **18** (2,3-dihydro-1-(3,5-dimethylphenyl)-1*H*-indole), and **19** (2,3-dihydro-5-methoxy-1-(3,5-dimethylphenyl)-1*H*-indole) was described previously.[15] The N-benzoyl-substituted indoles and the N-benzoyl-substituted indolines were prepared according to the preparation of **1**. Briefly, 5-nitroindole sodium (or 5-nitroindoline sodium) was prepared from 5-nitroindole (or 5-nitroindoline) and NaH, with 3,5-disubstituted-benzoyl chloride in *N*,*N*-dimethylformamide to afford **4**–**6** and **11**–**13**. These nitro-derivatives were reduced with 10% Pd–C to afford amino derivatives **7**–**9** and **15**–**17**. The structures of the synthesized compounds were confirmed by $^1$H NMR, mass spectroscopy, and elemental analysis. SC-560 (SC), DUP-697 (DUP), and Ibuprofen (IBU) were purchased from Funakoshi. Co. Ltd (Japan).

A kinetic study of the inhibition of COX-1-mediated oxidation of arachidonic acid was performed with an enzyme immunoassay-based COX inhibitor screening assay kit purchased from Cayman Chemical (Ann Arbor, MI, Catalog No. 560101) according to the supplier's protocol.

HUVEC tube formation assay[16] was performed as follows: human umbilical vein endothelial cells (HUVECs) were plated on Matrigel and treated with the test compounds for 6 h, then tube formation was measured as previously reported.[16] Briefly, 6-well plates were coated with 1.5 mL of the Matrigel basement membrane matrix (Becton Dickinson) and allowed to gel at 37 °C under an atmosphere of 5% $CO_2$ in air for 30 min. Then, HUVECs were plated at $5 \times 10^5$ cells/well in DMEM containing the vehicle (0.5% DMSO) and growth factors (hEGF, VEGF, hFGF-β, and $R_3$-IGF-1, as well as FBS) in the presence or absence of various concentrations of the test compounds and incubated at 37 °C under 5% $CO_2$ 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 Meta Morph software (Universal Imaging, Downingtown, PA). Experiments were repeated at least three times.

To assess the cytotoxicity of the test compounds, HUVECs were treated with various concentrations of these compounds at 37 °C for 6 h. After the incubation, the viability of the treated cells was measured by direct counting under a microscope. The $LD_{50}$ values of these compounds were more than 100 μM.

We have previously demonstrated that the N-substituted phenylindoline and N-substituted phenylindole skeletons are useful scaffolds for the development of COX inhibitors other than the clinically used diaryl heterocyclic COX inhibitors, such as celecoxib.[15] We have also demonstrated that strongly electron-donating groups, such as an unsubstituted amino group at the 5- or 4-position of the indoline and/or indole skeleton, favor potent and COX-1-selective inhibitory activity.[15] In the previous study, the substituent on the *N*-phenyl, and/or *N*-benzoyl group, was fixed to 3,5-dimethyl group, considering the early SAR of the series of compounds, but the substituent was not optimized. Therefore, we synthesized 3,5-disubstituted-benzoyl derivatives and assayed their COX-inhibiting activity. Results are summarized in Table 1. Roughly speaking, in the case of the 5-nitroindole and 5-nitroindoline series, all compounds which have $CF_3$, $C(CH_3)_3$, and Cl as a 3,5-substituent did not show apparent COX-1-inhibiting activity (**4**, **5**, **6**, **11**, and **12**), or weak inhibitory activity (**13**). As for COX-2, these compounds also show no activity at the concentration of 100 μM (**11**–**13**), or showed very weak inhibitory activity (**4**–**6**). In the case of 5-aminoindole and 5-aminoindoline series, all the compounds show moderate to high COX-1 inhibitory activity, except **8**. As for COX-2, all these compounds show weak inhibitory activity at the concentration of 100 μM. As a whole, none of the compounds show superior activity and selectivity than 3,5-dimethyl derivative (**1**). Therefore, we selected compounds **1** and **2** for further study (Fig. 1).

First, we analyzed the inhibitory mode of COX-1 inhibitors **1** and **2**, because the screening method that we previously used[17] could not completely exclude false-positive results from simple anti-oxidant compounds. The Lineweaver–Burk plot depicted in Figure 2 clearly indicated that compounds **1** and **2** are true competitive inhibitors of COX-1. Thus, we confirmed that these compounds inhibit COX-1 by competing directly with the natural substrate, arachidonic acid, at the ligand binding site.

We then investigated the anti-angiogenic activity of representative COX inhibitors ((SC (COX-1-selective inhibitor), DUP (COX-2-selective inhibitor), IBU (non-selective COX inhibitor)), and our COX-1-selective

**Table 1.** COX-inhibitory activity

| Compound | R | Inhibition[a] (%) | |
|---|---|---|---|
| | | COX-1 | COX-2 |
| **3** | $CH_3$ | 14.2 | 6.33 |
| **4** | $CF_3$ | ia[b] | 17.6 |
| **5** | $C(CH_3)_3$ | ia[b] | 28.2 |
| **6** | Cl | ia[b] | 24.1 |
| Aspirin | | 21.9 | 15.7 |

| Compound | R | Inhibition[a] (%) | |
|---|---|---|---|
| | | COX-1 | COX-2 |
| **1** | $CH_3$ | 88.1 | 13.8 |
| **7** | $CF_3$ | 68.6 | 24.1 |
| **8** | $C(CH_3)_3$, | 18.3 | 20.8 |
| **9** | Cl | 76.0 | 34.1 |
| DUP | | ia[b] | 88.2 |

| Compound | R | Inhibition[a] (%) | |
|---|---|---|---|
| | | COX-1 | COX-2 |
| **10** | $CH_3$ | 68.6 | 53.5 |
| **11** | $CF_3$ | ia[b] | ia[b] |
| **12** | $C(CH_3)_3$ | ia[b] | ia[b] |
| **13** | Cl | 29.7 | 2.99 |
| SC | | 95.1 | 28.5 |

| Compound | R | Inhibition[a] (%) | |
|---|---|---|---|
| | | COX-1 | COX-2 |
| **14** | $CH_3$ | 47.3 | 4.48 |
| **15** | $CF_3$ | 37.4 | 20.1 |
| **16** | $C(CH_3)_3$ | 36.5 | 35 |
| **17** | Cl | 55.3 | 22.5 |

[a] Compounds were screened for inhibitory activity on COX at the concentration of 30 μM.
[b] ia, inactive at the concentration of 30 μM.

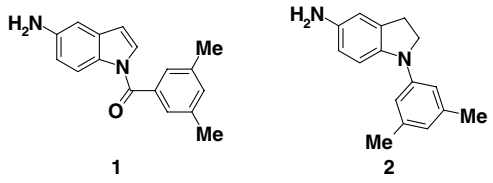


**Figure 1.** Chemical structures of the basic-type selective COX-1 inhibitors **1** and **2**.

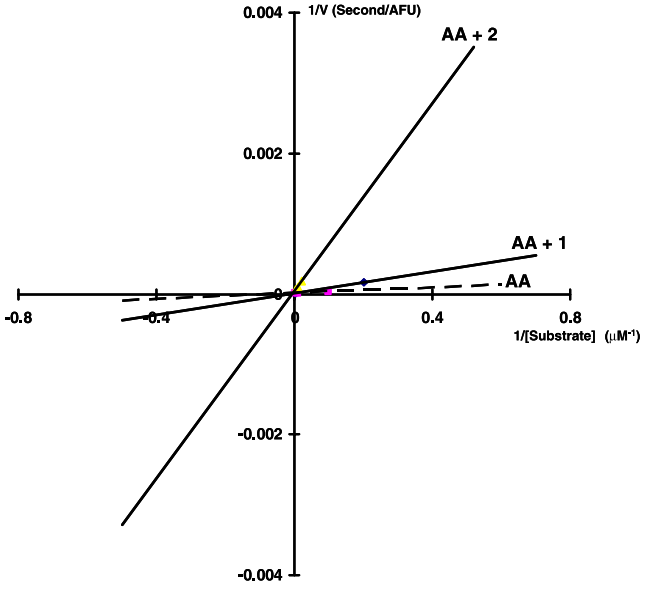


**Figure 2.** Lineweaver–Burk plots for compounds **1** and **2**. AA means arachidonic acid.

inhibitors **1** and **2** at the concentration of 100 μM (Fig. 3). Compounds **18** and **19**, both of which structurally resemble **1** and **2**, but which lack COX-inhibitory activity, were also assayed.

As can be seen from Figure 3, the reference compounds SC, DUP, and IBU exhibited anti-angiogenic activity. SC, a COX-1-selective inhibitor, exhibited moderate anti-angiogenic activity (ca. 50% inhibition at 100 μM). IBU, which is a well-known non-selective COX inhibitor, also exhibited moderate anti-angiogenic activity, comparable to that of SC (ca. 35% inhibition at

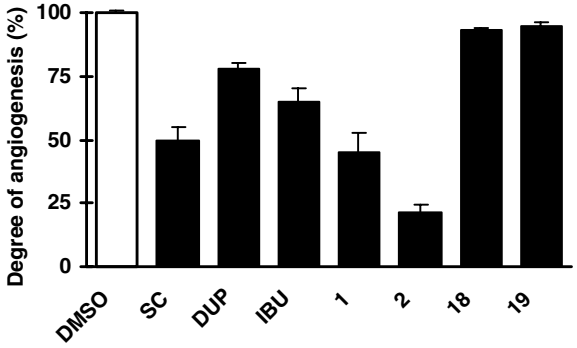


**Figure 3.** Anti-angiogenic effects of representative COX inhibitors (**SC**, **DUP**, and **IBU**) and our derivatives.

100 μM). DUP, a COX-2-selective inhibitor, also showed anti-angiogenic activity. However, the potency of DUP (ca. 25% inhibition at 100 μM) is weaker than that of SC. It is reported that HUVEC apparently does not express COX-2 in the absence of endogenous stimuli, such as IL-1β,[18] so DUP might not show potent activity in these cells. In the case of our compounds, both **1** and **2** exhibited moderate anti-angiogenic activity, comparable or superior to that of SC (ca. 50% and 75% inhibition at 100 μM, respectively), whereas **18** and **19**, which have similar structures to **1** and **2**, but do not show apparent COX-inhibitory activity, lacked anti-angiogenic activity. These results indicate that the anti-angiogenic activity of **1** and **2** is mediated through the COX-1 pathway, at least in part, but not via an unknown indole and/or indoline structure-dependent pathway.

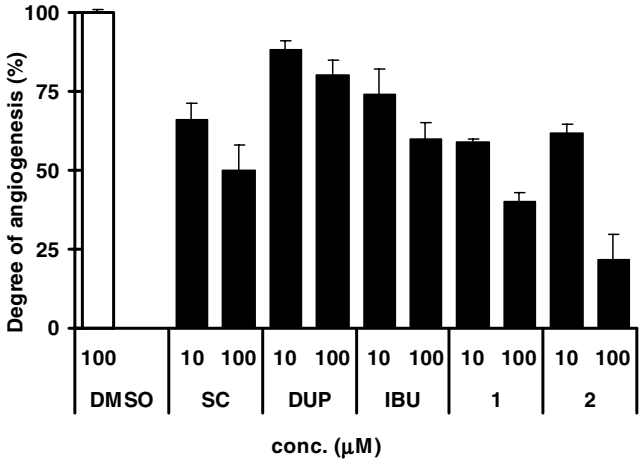


**Figure 4.** Dose–response relationship of representative COX inhibitors (**SC**, **DUP**, and **IBU**) and our derivatives (**1**, **2**).

Figures 4 and 5 show the dose-dependency of the anti-angiogenic effects of COX inhibitors. All the compounds tested showed dose-dependent anti-angiogenic activity, and the rank order of inhibitory activity is as follows; **2** > SC > **1** > IBU > DUP (none of these compounds showed cytotoxicity at the concentration of 300 μM). Compound **2** was found to possess highly potent anti-angiogenic activity, comparable with that of the well-known COX-1-selective antagonist, SC.

In conclusion, we have discovered that the novel basic-type selective COX-1 inhibitors, **1** and **2**, exhibit apparent anti-angiogenic activity in a HUVEC tube formation assay. Compound **2** was the most potent among the prepared compounds. Angiogenesis has recently become a primary target of anticancer therapy. A recent report has indicated that COX-1 is over-expressed in a significant number of ovarian cancers.[19] Further, ovarian cancer is known to be highly vascular and is a primary cancer in which current anti-angiogenic therapies are being tested.[19] Therefore, our present results may yield new candidate drugs for the treatment of ovarian cancer.

## Acknowledgments

We thank Dr. Hideki Nohara (University of Tokyo) for helpful discussions and technical assistance. The work described here was partly supported by Grants-in-Aid for Scientific Research from The Ministry of Education, Culture, Sports, Science and Technology, Japan, and the Japan Society for the Promotion of Science.

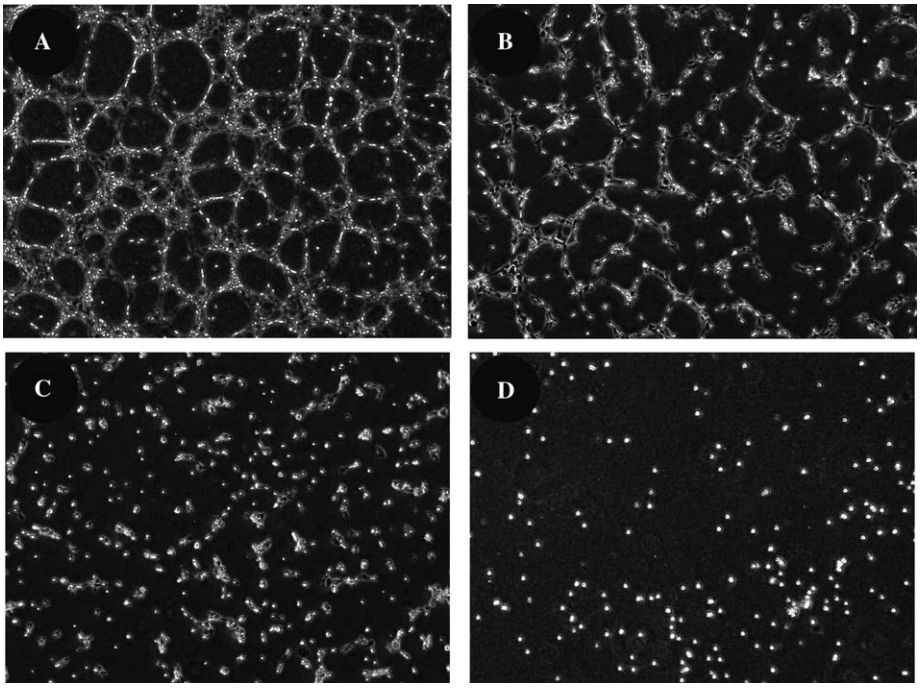


**Figure 5.** Photographic representation of the dose-dependent inhibition of **2** on the HUVEC tube-formation. (A) Control; (B) 10 μM **2**; (C) 100 μM **2**; (D) 300 μM **2**.

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