Correlation of Antiangiogenic and Antitumor Efficacy of N-biphenyl Sulfonyl-phenylalanine Hydroxyacid (BPHA), an Orally-active, Selective Matrix Metalloproteinase Inhibitor

Ryuji Maekawa, Hideo Makii, Hiroshi Yoshida, Kanji Hojo, Hidekazu Tanaka, Tohru Wada, Naomi Uchida, Yukihito Takeda, Hisanori Kasai, Hiroyuki Okamoto, Hiroshiye Tsuzuki, Yoshikazu Kambayashi, Fumihiko Watanabe, Kenji Kawada, Ken-ichi Toda, Mitsuki Ohtani, Kenji Sugita, and Takayuki Yoshioka\(^1\)

Discovery Research Laboratories, Shionogi and Company, Ltd., 12-4 Sogisu, 5-Chome Fukushima-ku, Osaka 553-0002, Japan \(\text{[R. M., H. M., H. Y., K. H., H. Ta., T. W., N. U., Y. T., H. K., H. O., H. Ts., Y. K., F. W., K. K., M. O., K. S., T. Y.], and Department of Dermatology, Faculty of Medicine, Kyoto University, Sakyo-ku, Kyoto 606, Japan \[K. S.\]}

ABSTRACT

The antiangiogenic activity and antitumor efficacy of a newly developed matrix metalloproteinase (MMP) inhibitor were examined. N-biphenyl sulfonyl-phenylalanine hydroxyacid (BPHA) potently inhibits MMP-2, -9, and -14, but not MMP-1, -3, or -7. In contrast, (-)BPHA, an enantiomer of BPHA, was inactive against all MMPs tested. Daily oral administration of 200 mg/kg BPHA, but not (-)BPHA in mice resulted in potent inhibition of tumor-induced angiogenesis, primary tumor growth, and liver metastasis. The growth inhibition activity of BPHA was 48% and 45% in a B16-BL6 melanoma and F2 hemangio-endothelioma model, respectively. BPHA also showed 42% inhibition of the liver metastasis of C-1H human colon carcinoma cells. These results indicate that selective MMP inhibition is correlated with antiangiogenic and antitumor efficacy and that the selective MMP inhibitor BPHA has therapeutic potential.

INTRODUCTION

MMPs\(^2\) are a class of structurally related enzymes that function in the degradation of extracellular matrix proteins that constitute connective tissue (1). MMPs are essential for tumor cells to penetrate the basement membrane, gain access to blood vessels, exit blood vessels, and colonize at distant sites (metastasis; Refs. 2–4). Angiogenesis, a neovascularization process crucial to sustain progressive tumor growth by supplying oxygen and nutrients, also involves proteolytic degradation via extracellular matrix by activated endothelial cells (5–8). The accumulated evidence has demonstrated that angiogenesis or resultant tumor vascularity could be related to metastasis and patient survival (9–13). In many preclinical and clinical studies, increased MMP activity has been detected in a wide range of cancers including lung (14), prostate (15), breast (16), head and neck (17), ovarian (18), and pancreas (19) and correlated to their invasive and metastatic potential (20–23). Therefore, MMPs may be useful targets as a new class of inhibitory drugs.

Several novel MMP inhibitors have been identified and are presently being investigated in clinical trials (24–29). A broad-type MMP inhibitor, BB-2516 (British Biotech) and AG3340 (Agouron) are presently in Phase III trials. The selective inhibitor BAY 12-9566 (Bayer) is in Phase II/III trials. Chiroscience has selective MMP inhibitors D-1927 and D-2163 in Phase I trials.

However, few studies have been focused on relationships between enzyme inhibitory activity of MMP inhibitors and their antitumor activity. Recently, we have developed p.o.-active MMP inhibitors as antitumor agents (30). We have found that BPHA strongly inhibits MMP-2, -9, and -14, but not MMP-1, -3, or -7, and that (-)BPHA, an enantiomer of BPHA, had no inhibitory activity. Using these compounds, we demonstrate in this study that the MMP inhibitory activity correlates directly with in vivo antiangiogenic and antitumor activity.

MATERIALS AND METHODS

Animals. BDF1 and C57BL/6 mice (female, 7–9 weeks of age) were purchased from Japan SLC, Inc. (Shizuoka, Japan). Athymic BALB/c nude mice (female, 7–9 weeks of age) were purchased from CLEA Japan, Inc. (Tokyo, Japan).

Tumors. The Lewis murine lung carcinoma was obtained from the National Cancer Institute (Bethesda, MD) and are maintained by serial s.c. transplantation as tumor fragments in C57BL/6 mice. B16-BL6 murine melanoma, Colon 26 murine colon cancer, Ma44 human lung squamous cell carcinoma, and C-1 human colon cancer were provided by Dr. I. J. Fidler (D. Anderson Cancer Center, Houston, TX), Dr. T. Tsuruo (Tokyo University, Tokyo, Japan), Dr. T. Komiya (Habikino Hospital, Osaka, Japan), and Dr. T. Kubota (Keio University, Tokyo, Japan), respectively. HT-1080 human fibrosarcoma was purchased from American Type Culture Collection (Manassas, VA). These cell lines were maintained by in vitro passage using Eagle’s MEM (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% FCS (Life Technologies, Inc., Rockville, MD). The C-1H human colon cancer was established in our laboratory by repeat passage of C-1 liver metastases via intrasplenic injection. F2 murine hemangio-endothelioma was established by Dr. K. Toda (31).

Chemicals. BPHA, (-)BPHA, and BB-2516 (32) were used in this study.

Enzyme Assays. For MMP-1 assay, a commercially available assay kit with natural substrate was used (Yagai, Yamagata, Japan), as described previously (30). MMP-2 and -9 were purified from the culture supernatant of HT-1080 cells (33) with enzyme activities assayed using natural substrate. A cDNA clone of MMP-14 was provided by Dr. Seiki (Tokyo University). Recombinant MMP-14 was expressed in Escherichia coli, and a 28-kDa protein, including a catalytic core domain starting with Arg\(^{109}\) was purified by DEAE-Sepharose column chromatography. MMP-3 and -7 were obtained from Yagai. The enzyme activities of MMP-3, -7, and -14 were assayed in a reaction buffer [300 mM NaCl, 10 mM CaCl\(_2\), 0.005% Brij35, 0.01% Na\(_2\)AsO\(_4\), and 50 mM Tris-HCl (pH 7.5)] using 20 mM MOAC-Pro-Leu-Gly-Leu-Ap(Ala)Arg-NH\(_2\), as substrate. After a 90-min incubation at 25°C, fluorescence was measured with excitation and emission at 320 nm and 405 nm, respectively, with a Fluoroscan Ascent (Labsystems, Helsinki, Finland). For enzyme inhibition assays, the enzymes were preincubated with inhibitor for 60 min.

Gelatin Zymography. Gelatin zymography was carried out as described elsewhere (34). Briefly, the supernatant was prepared by incubating 1 × 10\(^6\) tumor cells in a 6-cm culture dish in serum-free DMEM (Nissui Pharmaceutical Co.) for 24 h. The culture supernatant (10 μl) was applied to nonreduced SDS-PAGE using a 7.5% gel containing 0.1% gelatin. After electrophoresis, the gel was soaked in 2.5% Triton X-100 solution at room temperature with gently shaking for 1 h. The gels were then incubated overnight in reaction buffer [50 mM Tris-HCl (pH 7.5), 10 mM CaCl\(_2\), and 0.01% Brij35] at 37°C and stained with Coomassie Brilliant Blue.

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1 To whom requests for reprints should be addressed, at Discovery Research Laboratories, Shionogi and Company, Ltd., 12-4 Sogisu, 5-Chome Fukushima-ku, Osaka 553-0002 Japan. Phone: 81-6-6458-5861; Fax: 81-6-6458-0987; E-mail: takayuki.yoshioka@shionogi.co.jp.

2 The abbreviations used are: MMP, matrix metalloproteinase; BPHA, N-biphenylsulfonfyl-phenylalanine hydroxyacid.
concentration of $5 \times 10^6$ cells/mL. A Millipore chamber (Millipore Co., Bedford, MA) was filled with 0.2 ml of either a cell suspension or HBSS and implanted s.c. into the dorsal side of mice (day 0). The compounds were administrated p.o. twice a day from days 0–3. At 4 days after implantation, a wide rectangular incision was made in the skin on the dorsal side of anesthetized mice, and the skin was carefully ablated. Histological analyses and computer image analyses were performed, as described previously (35).

**In Vivo Tumor Growth Assay.** The experimental procedures have been described previously (36). All experiments consisted of 5–10 mice/group. On day 0, $2 \times 10^3$ B16-BL6 cells, or $2 \times 10^5$ F2 cells were implanted i.d. into the back of BDF1 and BALB/c nude mice, respectively. The experimental compounds were suspended in vehicle (saline including 0.4% Tween 80, 0.5% carboxy-methylcellulose, and 0.9% benzylalcohol) and were p.o. administered daily from day 1. Tumor size and body weight were scored throughout each experiment. Growth inhibitory activity was estimated from the treated:control ratio (36).

**In Vivo Liver Metastasis Model.** C-1H human colon carcinoma cells ($5 \times 10^3$) were injected into the spleen of BALB/c nude mice, and the spleen was removed after tumor inoculation. The compounds were p.o. administered daily from day 1. On day 25, the mice were sacrificed and the liver with tumor nodules were excised and weighed. All in vivo studies were performed with the approval of the Shionogi Animal Care and Use Committee.

### Statistics

The statistical significance in the present experiments was evaluated using Dunnett’s test (37).

## RESULTS

### MMP Inhibitory Activity of BPHAs

The inhibitory activity of BPHA and (-)BPHA against various human MMPs was examined (Table 1). BPHA inhibited activities of MMP-2, -9, and -14 (MT1-MMP), with the $IC_{50}$ of 10–20 nM, but did not inhibit MMP-1, -3, and -7 (the $IC_{50}$ were 974, >1000, and 795 nM, respectively). In contrast, (-)BPHA, an enantiomer of BPHA, had no inhibitory activity against all enzymes tested. Neither BPHA nor (-)BPHA inhibited typical serine proteinases (neutrophil elastase, plasmin, trypsin, and chymotrypsin), cysteine proteinases (cathepsins B and L), aspartic proteinase (HIV-1 protease), or metalloproteinase (aminopeptidase M; data not shown).

We next examined the MMP inhibitory activity of BPHAs against murine MMPs using gelatin zymography. As shown in Fig. 1, the gelatinase activity of MMP-2 and -9, derived from murine as well as from human tumor cells, was completely inhibited by BPHA at 20 $\mu$M (Fig. 1, center), but not by (-)BPHA even at 100 $\mu$M (Fig. 1, right).

**Pharmacokinetics of BPHAs.** The plasma concentration of BPHAs after oral administration (200 mg/kg) in mice was determined by high-performance liquid chromatography. The maximum plasma concentrations of BPHA and (-)BPHA were 910 nM and 840 nM, respectively. The area under the plasma concentration curves of BPHA and (-)BPHA was 0.98 $\mu$g hr/ml and 1.07 $\mu$g hr/ml, respectively. The pharmacokinetic profiles for BPHA and (-)BPHA were, thus, similar. The plasma level of BPHA 24 h after oral administration was ~30 nM, which was higher than the $IC_{50}$ against MMP-2, -9, and -14 (data not shown).

**Correlation of Antiangiogenic Activity of BPHAs with Their MMP Inhibitory Activity.** To evaluate the antiangiogenic activity of BPHAs, we used the dorsal air sac-chamber assay with transplantation in the dorsal side of mice of a Millipore chamber filled with human HT-1080 fibrosarcoma, which is an angiogenesis inducer. In the control experiments in which the chamber was filled with HBSS, few newly developed blood vessels were detected macroscopically (Fig. 2A), as well as in the skin section (Fig. 3A), indicating that the nonspecific blood vessel formation induced by the dorsal air-sac procedure was minimal. In contrast, after implantation of the chamber with HT-1080 tumor cells, the number of blood vessels had markedly increased with newly formed blood vessels branching out from large blood vessels (Figs. 2B and 3, B and E). Severe hemorrhage was also observed.

In mice, p.o. administration of 200 mg/kg BPHA clearly suppressed tumor-induced angiogenesis (Figs. 2C and 3, C and F). In (-)BPHA-treated mice, however, induction of angiogenesis was not affected (Figs. 2D and 3, D and G).

We then measured angiogenesis by analyzing vertical sections of the skin under light microscopy (Fig. 3, E-G). The total vascular area and number of vessels beneath the musculus cutaneus/1-mm width of skin section were estimated using an image analyzer. The total vascular area and number of vessels/unit width were reduced by 79% and 75% in mice treated with BPHA, respectively (Table 2; $P < 0.01$ compared with vehicle control). In mice treated with (-)BPHA, the MMP inhibitory activity-deficient enantiomer, no significant inhibition was found compared with the control. These results indicate that the antiangiogenic activity of BPHAs is closely correlated with MMP inhibitory activity.

### Correlation of Antitumor Efficacy of BPHAs with Their MMP Inhibitory Activity

The antitumor efficacy of BPHAs was evaluated against murine B16-BL6 melanoma and F2 hemangio-endothelioma implanted i.d. As shown in Table 3, daily p.o. administration of BPHA, but not (-)BPHA, exerted significant ($P < 0.01$) growth inhibition in both tumor models. The growth inhibition activity of BPHA was as potent as BB-2516 in the B16-BL6 melanoma model (Table 3).

We next examined the therapeutic efficacy of BPHA on experimental hepatic metastasis formed in a human colon cancer model. The increase in liver weight due to C-1H tumor-cell growth was inhibited by 42% ($P < 0.05$) in BPHA-treated mice (Table 4). (-)BPHA, however, had no inhibitory activity (Table 4). These results indicated that tumor growth inhibitory activity and the antimetastatic activity of BPHAs were closely correlated with MMP inhibitory activity. During daily treatment at the effective dose for antitumor activity (200 mg/kg), BPHA did not exhibit any toxic effect on body weight loss or on hematopoietic cells (data not shown).

## DISCUSSION

We have found in this study that one compound and its enantiomer exhibit different MMP inhibitory activity. Recent elucidation

**Table 1 Profile of inhibitory activity of BPHAs against human MMPs**

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>BPHA ($IC_{50}$) (nM)</th>
<th>(-)BPHA ($IC_{50}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-1</td>
<td>974</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>MMP-2</td>
<td>12</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>MMP-3</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>MMP-7</td>
<td>795</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>MMP-9</td>
<td>16</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>MMP-14</td>
<td>17</td>
<td>&gt;1000</td>
</tr>
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of the crystallographic structures of MMP-inhibitor complexes have demonstrated the model for enzyme-inhibitor interaction (38). In the present model, unfavorable steric repulsion between an α-substituent of hydroxamate of (-)BPHA and S1 subsite of the enzyme may explain the instability of binding between (-)BPHA and MMPs (38).

Because both tumor-derived and host stroma-derived MMPs may play a pivotal role in tumor growth (3), the effect of MMP inhibitory activity on murine MMPs is important for a therapeutic experimental model. We have confirmed the cross-inhibitory effect of BPHA on murine MMP-2 and -9 by gelatin zymography.

In this study, we demonstrated that BPHA, but not (-)BPHA, showed significant antiangiogenic activity, antitumor growth, and antimetastatic efficacy in various tumor models. As both compounds showed similar pharmacokinetic profiles, the difference found in the biological activities of these compounds is mainly due to the inhibitory activity against MMP-2, -9, and -14. The present study is consistent with our previous study showing reduced angiogenesis and tumor progression in MMP-2-deficient mice (35). These studies support the role of MMPs such as MMP-2 in angiogenesis, tumor growth, and metastasis.

BPHA selectively inhibits MMP activity, in contrast to broad-spectrum MMP inhibitors such as BB-94 and BB-2516 (26, 28). However, BPHA, which is active against MMP-2, -9, and -14, demonstrates similar antiangiogenic and antitumor activity as broad-spectrum MMP inhibitors.

These findings have important implications for the therapeutic potential of BPHA that may reduce the incidence of adverse events such as pain and tenderness in joints, pain affecting shoulders and

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Fig. 2. Inhibition of tumor-induced angiogenesis by BPHAs. Representative views from the inner side of the dorsal skin segment. Dorsal skin of the mice 4 days after implantation of a chamber filled with HBSS only (A) or HT-1080 cells (B-D). The implanted mice were treated p.o. daily with vehicle control (B), 200 mg/kg BPHA (C), or 200 mg/kg (-)-BPHA (D). Magnitude, ×6.4.
Fig. 3. Histological analysis of vertical skin sections. H&E staining of skin vertical sections 4 days after implantation of a chamber filled with HBSS only (A) or a chamber filled with HT-1080 cells (B-G). The implanted mice were treated p.o. daily with vehicle control (B and E), 200 mg/kg BPHA (C and F), and 200 mg/kg (-)BPHA (D and G). A-D, magnification: ×87.5. E-G, magnification: ×350. Arrowhead, blood vessels beneath the musculi cutaneus.


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