Inhibition of Spontaneous Rat Osteosarcoma Lung Metastasis by 3S-[4-(N-hydroxyamino)-2R-isobutylsuccinyl]amino-1-methoxy-3,4-dihydrocarbostyril, a Novel Matrix Metalloproteinase Inhibitor

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In the present experiment, we examined the effects of OPB-3206, 3S-[4-(N-hydroxyamino)-2R-isobutylsuccinyl]amino-1-methoxy-3,4-dihydrocarbostyril, a novel metalloproteinase inhibitor, on the growth and metastasis of transplantable osteosarcomas (spontaneous osteosarcoma, selected lung metastatic lesions; S-SLM), which were previously established in rats. OPB-3206 inhibited the activities of interstitial collagenase, gelatinases A and B, and stromelysin in vitro. After oral administration to rats, its serum concentration peaked at 40 min and the drug was no longer detectable at 8 h. When OPB-3206 was orally administered at 0%, 0.1% and 0.4% in the diet for 4 weeks, starting 7 days after subcutaneous transplantation of osteosarcomas to male Fischer 344 rats, numbers of lung metastatic nodules were significantly reduced by the highest dose, while the growth of subcutaneous tumors was not affected. Zymographic analysis showed the presence of pro matrix metalloproteinase (proMMP)-2, proMMP-9 and MMP-9 activities in S-SLM. In animals fed 0.4% OPB-3206, the activity of proMMP-9 was increased, but that for MMP-9 had become undetectable. The results thus suggest that OPB-3206 selectively inhibits lung metastasis of rat transplantable osteosarcomas by inhibiting MMP-9 activation.

Key words: Rat transplantable osteosarcomas — Metalloproteinase inhibitor — Spontaneous metastasis — OPB-3206

Osteosarcomas readily spread via the bloodstream to the lungs, resulting in metastases that make the prognosis poor after noncurative or even curative treatments.1) To prevent the development of secondaries, it is therefore important to give optimal chemotherapeutic treatment. Previously, we established transplantable osteosarcomas with rapid growth and high lung metastatic potential after serial transplantation into the subcutaneous back space of syngeneic rats.2–7) They represent useful osteosarcoma models for testing novel anti-metastatic therapies.

Expression and activation of matrix metalloproteinases (MMPs) appear to play important roles in metastatic processes,8–13) such as local tumor expansion through adjacent normal tissues, invasion of blood vessels and lymphatics, and extravasation at distant sites. MMPs also appear to be involved in angiogenesis, mediating tissue remodeling and penetration of the extracellular matrix by new capillaries.13) An MMP inhibitor should, therefore, have the potential to inhibit both tumor growth and spread.

OPB-3206, 3S-[4-(N-hydroxyamino)-2R-isobutylsuccinyl]amino-1-methoxy-3,4-dihydrocarbostyril, is a novel agent which was designed to be administered orally, and to inhibit MMP activity by binding reversibly to the zinc-binding region of MMPs. In this study, we investigated the effects of orally administered OPB-3206 on tumor growth and spontaneous lung metastasis in our rat transplantable osteosarcoma model. We also explored the effects of OPB-3206 on mRNA levels of MMP-2, MMP-9, TIMP-1 and TIMP-2, and on the gelatinolytic activities of MMP-2 and MMP-9.

MATERIALS AND METHODS

Chemical OPB-3206 (Fig. 1) was provided by Otsuka Pharmaceutical Co., Ltd., Shiga. In vitro effect of OPB-3206 on interstitial collagenase, gelatinases and stromelysin Human fibroblast Detroit 551 cells were cultured in Eagle’s minimum essential medium (MEM) with 10% fetal bovine serum, 1% non-essential amino acids (NEAA), 1 mM sodium pyruvate, 20 mM N-(2-hydroxyethyl)piperazine-N’-2-ethanesulfonic acid (HEPES), 0.1% non-essential amino acids, and 0.4 mg/mL bovine insulin. Cells were seeded at a density of 2.5 x 10⁵ cells/well in 2 mL MEM with 10% fetal bovine serum. After 12 h, cells were incubated for 16 h with Sadler gelatinase assay system (unlabeled), 3S-[4-(N-hydroxyamino)-2R-isobutylsuccinyl]amino-1-methoxy-3,4-dihydrocarbostyril, or 3S-[4-(N-hydroxyamino)-2R-isobutylsuccinyl]amino-1-methoxy-3,4-dihydrocarbostyril, and incubated for an additional 24 h. Assay supernatants were assayed for gelatinase activities. Gelatinolytic activities of the supernatant were determined using a zymography gel containing type IV collagen as described above. Neutrophil elastase activity was determined using Trypsin substrate and Gelatinase substrate, respectively.

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acid (HEPES), and 50 µg/ml kanamycin sulfate under 95% air and 5% CO2. Human fibrosarcoma HT-1080 cells were similarly cultured without the sodium pyruvate. Mouse colon carcinoma 26 cells were also cultured in RPMI-1640 medium under the same conditions.

Prointerstitial collagenase was partially purified by diethylaminoethyl (DEAE)-cellulose and heparin-agarose column chromatography from the conditioned medium of Detroit 551 cells, cultured as above with 50 ng/ml PMA. Mouse colon carcinoma 26 cells, cultured in serum-free RPMI-1640 medium with 20 µM NaCl, 5 mM CaCl2, 0.01% (w/v) Briji 35 and 0.1% (v/v) DMS containing the test compound (dissolved in DMS), with 0.1% (w/v) of fluorescence-labeled gelatin as the substrate. For the assay of gelatinase A, the reaction was started by adding the partially purified proenzyme to a solution of 1 mM mercury (II) p-aminophenylacetate, 50 mM Tris-HCl pH 7.5, 10 mM CaCl2, 0.01% (w/v) Briji 35 and 0.1% (v/v) DMS containing the test compound, with 0.1% (w/v) fluorescence-labeled gelatin as the substrate. The reaction was conducted for 0.5 h at 37°C, and stopped by adding a 1/2 amount of 30% (w/v) trichloroacetic acid solution. The mixture was allowed to stand at 4°C for 30 min, then the undigested substrate was precipitated by centrifugal separation for 10 min at 10,000 rpm, 4°C. To one part of the obtained supernatant, 50 parts by volume of 0.3 M phosphate buffer (pH 8.5) was added, and the fluorescence intensity was measured at 520 nm (E520)/495 nm (E495).

Inhibition of stromelysin was measured using fluorescence-labeled casein, according to the method of Miyazaki et al. [16] and Sally. [17] The partially purified proenzyme was activated by incubation for 2 h at 37°C in 1 mM mercury (II) p-aminophenylacetate. The enzyme reaction was started by adding the activated enzyme to a solution of 50 mM Tris-HCl pH 7.5, 1 mM CaCl2, 0.01% (w/v) Briji 35 and 0.1% (v/v) DMS containing the test compound, with 0.25% (w/v) of fluorescence-labeled casein as the substrate. The reaction was conducted for 3 h at 37°C, and stopped by adding an equivalent amount of 5% (w/v) trichloroacetic acid solution. Afterwards, the undigested substrate was precipitated by centrifugation for 15 min at 3,000 rpm, 4°C. To one part of the obtained supernatant, 4 parts by volume of 0.3 M phosphate buffer (pH 8.5) was added. Fluorescence intensity was then measured at 520 nm (E520)/495 nm (E495).

Serum concentration of orally administered OPB-3206 in rats Male-specific pathogen-free rats of SD strain, 4 weeks of age, were purchased from CLEA Co., Ltd., Tokyo. The animals were acclimatized and housed in groups of 10 rats each in Ekon PC facilities. Solid food (CL-2, CLEA, sterilized by γ-ray irradiation) and water (autoclaved, sterilized water prepared at Fuji Memorial Research Institute, Otsuka Pharmaceutical Co., Ltd., Shiga) were provided ad libitum. The animals were 5 weeks old when used in the experiments. OPB-3206 was
were handled in accordance with our institution guidelines for animal welfare.

**Preparation of cDNA probes** MMP-2, MMP-9, TIMP-1 and TIMP-2 cDNA fragments were obtained by reverse transcriptase-polymerase chain reaction (RT-PCR) amplification. Total RNAs were prepared from normal rat skin and bone tissues and cDNAs were synthesized using Moloney-murine leukemia virus reverse transcriptase (GIBCO BRL, Rockville, MD) and 20-mer oligodeoxythymidyl acid primers. Appropriate oligonucleotide-pairs were synthesized for PCR amplification. The sequences of the primers were: MMP-2, 5'-TGATCTCGAGTGGCCTC-3' and 5'-TGCGGACACGACGATTC-3'; MMP-9, 5'-AGCGAGACACTAAAGGCGATC-3' and 5'-TGCGAGGAAATATAGGACC-3'; TIMP-1, 5'-CCAAGATCATCGAGACCACCT-3' and 5'-GCCAGGGCAGTGGATCGCCT-3'; TIMP-2, 5'-TGACAGAGGAGCAGGAAAAGT-3' and 5'-TATTCTCCTTCTTCCACAC-3'. Thirty microliters of mineral oil was layered over 200 µl of reaction mixture to prevent evaporation. PCR conditions were: MMP-2, 40 cycles at 94°C for 45 s, 60°C for 1 min, and 72°C for 1 min; MMP-9, 40 cycles at 94°C for 45 s, 63°C for 1 min, and 72°C for 1 min; TIMP-1, 30 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min; TIMP-2, 35 cycles at 94°C for 45 s, 58°C for 1 min, and 72°C for 1 min. DNA amplified by PCR was electrophoresed in 1% agarose gels and the DNA fragments were extracted using Wizard PCR Prep (Promega Co., WI). Their sequences were determined with an ABI PRISM Genetic Analyzer (Perkin-Elmer Co., NJ). The lengths of the probes were: MMP-2, 0.41 kb; MMP-9, 0.47 kb; TIMP-1, 0.42 kb; TIMP-2, 0.44 kb. cDNA probes for MMP-3 and MMP-7 were generous gifts from Dr. Hiroshi Sato (Department of Molecular Oncology, Cancer Research Institute, Kanazawa University, Kanazawa).

**Northern blot analysis** Total RNA was isolated from tissues using the lithium chloride-urea method. Twenty microgram aliquots were applied and electrophoresed in 1% agarose/formaldehyde gels. Gels were capillary-blotted in 20× standard saline citrate (SSC) onto Biozyme nylon membranes (Biolabs Tag Support Co., New York, NY), which were baked at 80°C. Radiolabeled DNA probes were prepared with [α-32P]dCTP using the DNA labeling system (Amersham Japan, Tokyo). Nylon membranes were pretreated with a mixture of 50% formamide, 5× SSC, 0.1 M phosphate buffer, pH 7.4, 5× Denhardt's solution, 0.1% sodium dodecyl sulfate (SDS), 5 mM EDTA, and 100 µg of salmon testis DNA for 1 h at 37°C. Then hybridization was performed for 2 h in the same buffer with the labeled probes at 42°C. Nylon membranes were washed in 2× SSC and 0.1% SDS at room temperature and twice in 2× SSC and 0.1% SDS at 55°C for 20 min, then densitometric quantification was performed with
a BAS1000 image analyzer (Fujix, Tokyo). Northern blots were reprobed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to confirm loading and transfer of similar amounts of RNA for each sample. GAPDH levels were also used to normalize the densitometric quantification.

**Zymography** Gelatin zymography to reveal gelatinase activity was performed according to the method of Nakajima et al.\textsuperscript{19} We used 8 SOS, 8 S-SLM, 8 0.1% OPB-3206 and 8 0.4% OPB-3206 treated S-SLM from the same numbers of tumor-bearing rats, respectively, for zymography and densitometric analysis. Standardized aliquots of tissue homogenate were subjected to electrophoresis in 10% SDS polyacrylamide gels polymerized with 0.1% gelatin. Gels were washed three times each for 30 min in Triton X-100 to remove SDS, then incubated for 16 h at 37°C in 50 mM Tris-HCl buffer, pH 7.4, containing 200 mM ZnCl\textsubscript{2} and 10 mM CaCl\textsubscript{2}. Another set of gels was incubated under identical conditions in the presence of 20 mM EDTA to confirm that the proteins exerting gelatinase activity were MMPs. Gels were fixed and stained with 0.5% Coomassie blue R-250 (Nacalai Tesque, Kyoto) in a mixture of 30% methanol and 10% acetic acid for 15 min at room temperature, and destained with the same solution without Coomassie blue. They were dried for 2 days at room temperature and quantitated with image analyzing software Mac BAS version 2.52 (Fujix).

**Statistical analyses** Statistical analyses were performed using a personal computer as described elsewhere.\textsuperscript{20} To assess the statistical significance of inter-group differences in quantitative data, the Dunnett multiple comparison test was performed after one-way analysis of variance.
OPB-3206 Inhibits Lung Metastasis of Rat Osteosarcomas

RESULTS

In vitro effect of OPB-3206 on interstitial collagenase, gelatinases and stromelysin OPB-3206 inhibited the activities of interstitial collagenase, gelatinase A, gelatinase B and stromelysin. The IC_{50} values were calculated to be 7 \times 10^{-7} M (interstitial collagenase), 5 \times 10^{-6} M (gelatinase A), 5 \times 10^{-7} M (gelatinase B), and 2 \times 10^{-6} M (stromelysin) (Fig. 2).

Serum concentration of orally administered OPB-3206 in rats The serum concentration of orally administered OPB-3206 reached a maximum 40 min after the administration after 4 h it had been almost completely excreted (Fig. 3).

Inhibition of lung metastasis but not of growth of osteosarcomas transplanted into subcutaneous space by OPB-3206 Growth curves of transplanted subcutaneous tumors are shown in Fig. 4; no effect was observed in rats given OPB-3206 at either 0.1% or 0.4% in the diet. Representative macroscopic findings for lungs are shown in Fig. 5, and the numbers of lung nodules and the lung weights are given in Table I. Lung weights of the rats given 0% or 0.1% OPB-3206 were significantly greater than in the case of 0.4% OPB-3206 administration (P<0.001). The numbers of lung nodules in rats given 0.4% OPB-3206 were also markedly decreased (P<0.001). However, 0.1% OPB-3206 did not have any effect on the numbers of lung nodules.

Northern blot analysis of MMP-2, MMP-3, MMP-7, MMP-9, TIMP-1 and TIMP-2 Representative blots are shown in Fig. 6. A single mRNA transcript was evident for each enzyme in all of the examined samples with the exceptions of two transcripts noted for TIMP-2. The results of densitometric analysis of MMP-2, MMP-3, MMP-7, MMP-9, TIMP-1 and TIMP-2 are summarized in Table II. Northern analysis revealed that OPB-3206 did not affect the levels of MMP or TIMP mRNAs.

Gelatinolytic activity Representative gelatinolytic pat-
terns of proMMP-9, MMP-9 and proMMP-2 are shown in Fig. 7. Three gelatin-degrading activities, at positions corresponding to molecular weights of 92 kDa, 88 kDa and 72 kDa, were observed in the SOS case (lanes 1 and 2). However, the 92-kDa activity was not detected in S-SLM without OPB-3206 (lanes 3 and 4) and the 88-kDa activity was not detected in S-SLM with 0.4% OPB-3206 (lanes 5 and 6). All bands were abolished by addition of EDTA to the reaction medium (data not shown), confirming that the activity was due to metalloproteinase. Gelatinolytic activities relative to that of proMMP-2 in SOS are shown in Table III. The active form of MMP-2, 68kDa, was not detected in any of the cases examined. ProMMP-9 activity was increased and MMP-9 was decreased by 0.4% OPB-3206 in the S-SLM case.

DISCUSSION

It is evident that MMPs, a family of zinc-dependent endoproteinases whose enzymatic activities are directed against components of the extracellular matrix, are critically involved in metastasis by cancer cells.8–13 In humans, 16 members of this family have been identified by cloning and sequencing.21, 22 As a therapeutic strategy to prevent cancer metastasis after operations, or in inoperable cases, the use of inhibitors of MMPs has been highlighted. OPB-3206 was recently developed as one such inhibitor and in the present experiment, it was proven to inhibit MMPs activities. In addition, OPB-3206 at a dose of 0.4% significantly inhibited lung metastasis from sub-

| Treatment     | No. of rats | Relative signal intensity |
|---------------|-------------|--------------------------|
|               | MMP-2       | MMP-3       | MMP-7       | MMP-9       | TIMP-1       | TIMP-2       |
| Control       | 8           | 0.65±0.03   | 0.42±0.08   | 0.31±0.04   | 0.73±0.28   | 0.26±0.08   | 0.96±0.09   |
| 0.4% OPB-3206 | 8           | 0.61±0.05   | 0.41±0.01   | 0.29±0.11   | 0.75±0.19   | 0.28±0.14   | 1.02±0.21   |

a) Densitometric quantification was carried out on northern blots with a BAS 1000 image analyzer. The values were normalized to the GAPDH level.

b) Mean±SD.

Fig. 6. Representative results of northern blot analysis for mRNA expression of MMP-2, MMP-3, MMP-7, MMP-9, TIMP-1 and TIMP-2 in S-SLM rat transplantable osteosarcomas. Control (lanes 1 and 2), S-SLM without OPB-3206 treatments; OPB-3206 (lanes 3 and 4), S-SLM with 0.4% OPB-3206 treatment.

Table II. Densitometric Quantification of MMP-2, MMP-7, MMP-9, TIMP-1 and TIMP-2 mRNA Expression in S-SLM Rat Transplantable Osteosarcomas

Fig. 7. Zymography of transplantable osteosarcomas. Equal amounts (5 µg) of total protein from tumors were loaded. Lanes 1 and 2, SOS; lanes 3 and 4, S-SLM in animals without OPB-3206; lanes 5 and 6, S-SLM in animals given 0.4% OPB-3206 in diet.
OPB-3206 Inhibits Lung Metastasis of Rat Osteosarcomas

For this in vivo study, the OPB-3206 doses were decided on the basis of the IC50 and serum concentrations in rats. No animals died during the experimental period and no clinical toxic signs were observed. Fig. 8 shows that there was no significant difference of body weight between the control and OPB-3206-treated groups. Other MMP inhibitors such as batimastat and AG3340 have been found to inhibit both growth and metastasis of transplanted tumors.23, 24) The possible reason for the differences from the present case is that agents such as batimastat and marimastat also influence angiogenesis. Previously, we reported that AGM-1470 inhibited both growth and metastasis of transplantable osteosarcomas in rats.25–27) Therefore, the present results indicate that OPB-3206 does not act by inhibiting angiogenesis, although further studies are required to confirm this.

Our northern blot analysis revealed no effects of OPB-3206 on mRNA levels of MMP-2, MMP-3, MMP-7, MMP-9, TIMP-1 and TIMP-2, but on zymography, increased proMMP-9 was detected along with a decrease of MMP-9 at the catalytic activity level in S-SLM. Thus, OPB-3206 may inhibit activation from proMMP-9 to MMP-9 without affecting TIMP-1 or TIMP-2 expression. OPB-3206 is capable of inhibiting the activation of all MMPs, which have similar structures, by its binding to the active site of proMMPs. Therefore, OPB-3206 might inhibit all MMPs which contribute to activate MMP-9. MMPs facilitate tumor cell invasion and metastasis by at least three distinct mechanisms.28, 29) The first is that their proteinase action removes physical barriers to invasion through the degradation of extracellular matrix macromolecules such as collagens, laminins and proteoglycans. The second is that MMPs have the ability to modulate cell adhesion. In order for cells to move through extracellular matrix, they must be able to form new cell-matrix and cell-cell attachments and break existing ones. The third is that MMPs may act on other extracellular matrix components to uncover hidden biological activities, as exemplified by the production of the angiogenesis inhibitor angiostatin in plasma by MMP action.28, 29) The present findings of inhibition of osteosarcoma lung metastasis by OPB-3206 may reflect the proteinase action of MMP-9. The results are clearly of interest in relation to chemotherapy for lung metastasis in osteosarcoma clinics.

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