Matrix metalloproteinase inhibitor MMI-166 inhibits lymphogenous metastasis in an orthotopically implanted model of lung cancer

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Abstract
Matrix metalloproteinases (MMP) are considered to be critically involved in tumor invasion and the metastasis of various cancers. MMI-166 is a selective inhibitor of matrix metalloproteinase (MMP-2, MMP-9, and MMP-14). The purpose of this study was to evaluate the effects of MMI-166 on both the growth of the implanted tumor and the lymph node metastasis of the mediastinum and prolonging the life span, using an orthotopic implantation model of the Ma44-3 cancer cell line. We examined the anti-invasive effect of MMI-166 in lung cancer cell lines using an in vitro invasion assay. Next, we examined the anticancer effect of MMI-166 in vivo. MMI-166 (200 mg/kg body weight) or a vehicle was administered orally to the orthotopically implanted lung cancer model. MMI-166 dose-dependently inhibited the invasion of cancer cell lines with expressions of MMP-2 and/or MMP-9 in vitro. In vivo, MMI-166 significantly inhibited mediastinal lymph node metastasis in this orthotopic model (weight of the mediastinum: control, 0.089 ± 0.009 versus MMI-166, 0.069 ± 0.008 mg; P = 0.005; metastatic area: control, 93,495 ± 55,747 versus MMI-166, 22,747 ± 17,478 pixels; P = 0.045). MMI-166 prolonged the life span by 6 days in median survival time in the orthotopically implanted model (P = 0.039). These results showed that MMI-166 could possibly inhibit lymph node metastasis and prolong the life span in lung cancer patients. [Mol Cancer Ther 2005; 4(9):1409–16]

Introduction
Lung cancer is the most common cause of cancer death in Japan, North America, and Europe (1). The primary reason for the difficulty in treating lung cancer is that it is mostly identified at a very late stage. Lymphogenous or hematogenous metastasis occurs early in patients with lung cancer (2). Not only the status of distant metastasis but also the status of lymphatic metastasis is a major factor in the prognosis and assignment of treatment in patients with non–small cell lung cancer. Mountain (3) reported that the 5-year survival rates of N0, N1, and N2 disease were 38% to 67%, 25% to 55%, and 23%, respectively. There is an inverse correlation between the extent of lymph node metastasis and the survival rate of lung cancer patients.

Matrix metalloproteinases (MMP) are a family of structurally related zymogens capable of degrading the extracellular matrix, including the basement membrane. They are thought to be critically involved in tumor invasion and the metastasis of various cancers (4). In lung cancer, higher levels of MMP-2 have been shown in more invasive and metastatic tumors, giving an inverse prognostic effect (5, 6). MMPs also seem to be involved in angiogenesis by mediating tissue remodeling and penetration of the extracellular matrix by activated endothelial cells. Angiogenesis is an essential event for solid tumor growth at primary and secondary sites. The induction of angiogenesis also increases the risk of hematogenous metastasis (7). When angiogenesis is blocked, tumor growth and metastasis are suppressed (8). MMP-2 and MMP-9 null mice exhibited a reduction of experimental metastasis of mouse B16-BL6 melanoma cells and angiogenesis (9, 10). These observations have led to the development of a matrix metalloproteinase inhibitor (MMPI) to prevent tumor growth and metastasis.

Recently, many MMPIs were isolated and characterized, including batimastat, marimastat, prinomastat, and BAY12-9566 (11). MMI-166, a N-sulfonfyl amino acid derivative, is the third generation of MMPIs, and was designed to have a selective spectrum of MMP inhibition (MMP-2, MMP-9, and MMP-14; ref. 12). Many studies have shown that MMPIs inhibit hematogenous metastasis with regard to both the number and the size of tumor nodules (11). However, there have been few studies on the effect of MMPIs on lymphogenous metastasis (13, 14).
The purpose of this study was to evaluate the effects of MMI-166 on both the growth of the implanted tumor and the lymph node metastasis of the mediastinum using an orthotopically implanted severe combined immunodeficiency (SCID) mouse model of the Ma44-3 lung cancer cell line (15, 16).

Materials and Methods

Chemicals

MMI-166, a N-sulfonyl amino acid derivative, was synthesized in Shionogi Research Laboratories (Osaka, Japan). MMI-166 selectively inhibits the activity of MMP-2, -9, and -14 (IC50s: 0.4, 90, and 100 nmol/L, respectively) but not the activity of MMP-1, -3 or -7 (IC50s: >1,000 nmol/L; ref. 12).

Animals

Male SCID mice (CB-17/ScicidJcl, CLEA Japan Inc., Tokyo, Japan) 6 to 8 weeks of age were used in this study, and were maintained in the Laboratory for Animal Experiments. The protocols of all animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Tokushima, School of Medicine, and were done according to their guidelines.

Cells and Cell Culture

Human non–small cell lung cancer cell lines Ma44, were kindly provided by Drs. Masuda and Takata (Osaka Prefecture Habikino Hospital, Osaka, Japan). A Ma44-3 cell line was cloned in our laboratory using the limiting dilution method. This cell line was cultured in RPMI 1640 (Sigma Chemical Co., St. Louis, MO) with 10% heat-inactivated fetal bovine serum (Bio Whittaker, Walkersville, MD) and maintained at 37°C in a humidified incubator with 5% CO2 in air. The human fibrosarcoma cell line, HT-1080, was purchased from the Health Science Research Bank, and was cultured in DMEM (Sigma Chemical) supplemented with 10% heat-inactivated fetal bovine serum and maintained at 37°C in a humidified incubator with 5% CO2 in air.

Gelatin Zymography

Lung cancer cell line Ma44-3 was seeded in RPMI 1640 containing 10% fetal bovine serum in 25 cm² tissue culture flasks. When the cell number reached subconfluent growth, the cells were washed and maintained in serum-free RPMI 1640 for 24 hours. After 24 hours of incubation, we harvested the conditioned media and counted the cell numbers. Supernatants were concentrated 10-fold by centrifugation using Centricon-30 concentrators (Millipore Co., Bedford, MA). The medium was stored at –20°C before testing. Gelatin zymography was carried out as described in a previous study (17). Electrophoresis was done at 20 mA per 1 mm thick gel and was terminated when the bromophenol blue marker reached the bottom of the gel. The lower gels were incubated with 2.5% Triton X-100 solution with gentle shaking at 20°C for 30 minutes and then rinsed for 30 minutes in 10 mmol/L Tris-HCl (pH 8.0). Metalloproteinase buffer [50 mmol/L Tris-HCl (pH 8.0), containing 0.5 mmol/L CaCl2 and 10⁻⁴ mol/L ZnCl2] was added, and the gels were incubated for 16 hours at 37°C. The gels were then stained for 10 minutes with 1% Coomassie brilliant blue R250 dissolved in 10% methanol-5% acetic acid, after which they were rapidly destained in the same solution without dye. Conditioned medium from HT-1080 fibrosarcoma cells was used as the collagenase standard. We quantified the band intensity (92 and 72 kDa) by gelatinoleticzymography using NIH image analysis (version 1.56; Wane Rasband; NIH, Bethesda, MD), and the gelatinolytic activity ratio was calculated. The intensity of the 92 kDa band in each sample was divided by the intensity of the 92 kDa band in HT-1080 cells, and the intensity of the 72 kDa band in each sample was divided by the intensity of the 72 kDa band in HT-1080 cells.

In vitro Invasion Assay

Tumor cell invasion through the reconstituted basement membrane (Matrigel; Collaborative Biomedical Products, Bedford, MA) was assayed using 24-well plates and a Transwell invasion chamber (Costar Corp., Cambridge, MA) equipped with 12-μm pore size polycarbonate membranes (Whatman Inc., Clifton, NJ) according to Saiki’s method (18). The upper surface of the membrane was coated with Matrigel 50 μg/chamber and the lower surface was coated with human fibronectin (Asahi Techno Glass Corp., Tokyo, Japan) 2 μg/chamber. Tumor cells (1 × 10⁵) were seeded on the reconstituted Matrigel in the upper compartment of the chamber, and the upper compartment of the chamber was filled with each concentration (0, 4, and 16 μmol/L) of MMI-166. After incubation for 6 hours (HT-1080) or 18 hours (Ma44-3) at 37°C, the cells on the upper surface of the membrane were completely removed by wiping with cotton swabs. The membranes were fixed in methanol and H&E-stained. The migrated cells on the lower surface were counted in five random fields/membranes at a magnification of ×100. Each assay was done in triplicate.

The inhibitory effect was calculated using the following formula: inhibitory effect (%) = [1 – (the average number of invading cells, 4 or 16 μmol/L MMI-166 / the average number of invading cells: 0 μmol/L MMI-166)] × 100.

Assessment of the Inhibitory Effect on Tumor Growth and the Toxicity of MMI-166 in the Subcutaneous Implantation Model

To determine the optimum dosage of MMI-166, we used a s.c. implantation model. Ma44-3 cells (2 × 10⁵ cells) suspended in PBS (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) were injected s.c. into the right groin of 8-week-old male SCID mice. Fifteen mice were divided into three groups (n = 5). The mice were given a dose of 0, 100, or 200 mg/kg body weight of MMI-166 five times a week by intragastric instillation from days 1 to 21 after implantation. MMI-166 was suspended in a vehicle (0.9% NaCl solution containing 0.5% carboxy-methyl-cellulose-Na, 0.9% benzyl alcohol, and 0.4% Tween 80; Sigma Chemical). The tumor volume was calculated on days 0, 1, 3, 7, 10, 14, 17 and 21 using the following formula: tumor volume (mm³) = [length (mm) × width (mm)]² / 2.
The body weight of these mice was measured on days 0, 1, 3, 7, 10, 14, 17, and 21 after implantation to monitor the toxicity of MMI-166.

Orthotopic Intrapulmonary Implantation Procedure
As in our previous studies (15, 16, 19), the mice were anesthetized by ether inhalation. A 1-cm transverse incision was made in the left lateral thorax in the right lateral decubitus position. The muscles were separated from the ribs by sharp dissection, and the intercostal muscles were exposed. We could see the left lung through the intercostal muscles. A 30-gauge needle was inserted 5 mm into the lung through the intercostal muscles, and Ma44-3 cells with 10 mg/mL Matrigel were inoculated into the left lung at a final volume of 10 μL medium (2.0 × 10⁴ cells). Finally, the skin incision was closed with 3-0 silk.

Assessment of the Inhibitory Effect of MMI-166 on Implanted Tumor Growth of the Lung or Mediastinal Lymph Node Metastasis
We investigated the inhibitory effect of MMI-166 on implanted lung tumor growth and mediastinal lymph node metastasis. Twelve mice with orthotopically implanted tumor cells were divided into two groups. The mice were treated orally with 0 or 200 mg/kg body weight of MMI-166 five times a week from days 1 to 13 after implantation. Our previous study showed that mediastinal lymph node metastasis was detected histologically on day 5 and that the median survival time of mice with no treatment was 16 days (19). Therefore, the mice were sacrificed on day 14 by ether inhalation and the left lung with the implanted tumor and mediastinal tissues was removed (Fig. 1A). The weight of the mediastinal tissues was measured immediately. The left lung and mediastinal tissues were fixated in 10% formalin and embedded in paraffin. To quantify the tumor volume at the implanted site in the left lung and the mediastinal metastatic area, tissues of the left lung and mediastinum were cut into 5-μm-thick paraffin sections at 300-μm intervals (Fig. 1B and C). All paraffin sections were stained with H&E and examined under a microscope. The slice with the largest section of tumor in the lung was selected, and the volume of the tumor was calculated by determining two perpendicular dimensions with callipers. The tumor volume was calculated using the following formula: tumor volume (mm³) = [length \( \times \) width \( \times \) (mm²)] / 2. In mediastinal metastasis, the metastatic areas in all sections of the mediastinal tissues were measured using NIH imaging, and calculated in each mouse.

Film In situ Zymography
Two orthotopically implanted mice treated with 0 or 200 mg/kg MMI-166 were sacrificed 8 hours after MMI-166 administration on day 14. Fresh lung tumor and mediastinal metastasis tissues of the mice were embedded in cryomold OCT 4583 Compound (Miles, Inc., Elkhart, IN) and immediately frozen on dry ice. These frozen blocks were then sliced sequentially using a TISSUTEK II cryostat microtome (Miles) to prepare serial frozen thin sections (8 μm thick). These thin sections were placed on FIZ-GN films, a polyethylene terephthalate-base film coated with cross-linked gelatin, 7 μm thick (Fuji, Tokyo Japan). As a negative control, FIZ-GI films, treated with 1,10-phenanthroline, a chelating agent functioning as MMPI, were also used to fix one of the serial sections. These films were incubated in a moist chamber (Cosmo Bio Co., Ltd., Tokyo, Japan) at 37°C for 8 hours. After incubation, the specimens were stained in a Coplin jar for 4 minutes with Biebrich scarlet (Aldrich, Milwaukee, WI), which was diluted to a 1.0% solution with distilled water. These films were then decolored for 10 minutes with distilled water, followed by counterstaining with Mayer’s hematoxylin solution (Muto, Tokyo, Japan) for 2 minutes and rinsed with water for 10 minutes. Degraded gelatin was not stained with Biebrich scarlet, and areas of gelatinolytic activity were visualized as white to weak red on a red background. In serial sections, if
there were more weakly red stained areas on the FIZ-GN film than on the FIZ-GI film, it suggested that there was gelatinolytic activity from MMPs but not from other proteases. One of the serial sections was fixed with 4% paraformaldehyde fixative and rinsed thoroughly with PBS, followed by staining with H&E solution (Wako Pure Chemical Industries, Ltd., Osaka, Japan). For quantitative analysis of the gelatinolytic activity, the observed photographic image under the light microscope was scanned with a Photograph-300Z scanner (Fuji).

Survival of Orthotopically Implanted Mice Treated with MMI-166
To examine the life-prolonging activity of MMI-166 in an orthotopically implanted SCID mouse model, 20 mice orthotopically implanted with tumor cells were divided into two groups (n = 10). These mice were treated orally with 0 or 200 mg/kg body weight of MMI-166 five times a week from day 1 until death.

Statistical Analysis
Statistical analysis was done using the unpaired $t$ test (SPSS for Windows Version 11.0.1). The Kaplan-Meier method was used to construct curves for overall survival, and the log-rank test was used to determine survival rates (SPSS for Windows Version 11.0.1). Significance was defined as a $P$ value $<0.05$.

Results
Gelatinolytic Activity of Lung Cancer Cell Line Ma44-3
The gelatinolytic activity was assessed in human lung cancer cell line Ma44-3 by quantitative gelatinolytic zymography. Figure 2A shows a zymogram of the culture supernatants from the standard (human fibrosarcoma cell line HT-1080) and human lung cancer cell line Ma44-3. MMP-2 and MMP-9 migrated at position 72 and 92 kDa, respectively.

In vitro Invasion Assay
The inhibitory effect of invasion to the basement membrane (Matrigel) by MMI-166 was evaluated using the in vitro invasion assay (Fig. 2B). The inhibitory effects of 4 and 16 μmol/L of MMI-166 in Ma44-3 were 42.9% ($P = 0.001$) and 54.9% ($P < 0.001$), respectively. MMI-166 inhibited the invasion to Matrigel in a dose-dependent manner.

Effect of MMI-166 on the Tumor Growth of Subcutaneous Implantation of Ma44-3 Cells
To examine the therapeutic effect of MMI-166, SCID mice were inoculated s.c. with Ma44-3 cells. The changes in the mean volume of s.c. tumors are shown in Fig. 3. There was no significant difference in body weight on day 21 among three groups. No toxicity was observed at doses of 100 or 200 mg/kg body weight of MMI-166. The volumes of s.c. tumors treated with 0, 100, and 200 mg/kg body weight of MMI-166 on day 21 were 1,272 ± 273, 742 ± 64, and 474 ± 118 mm$^3$, respectively. The growth of s.c. tumors in mice treated with 200 mg/kg of MMI-166 was significantly suppressed compared with that of tumors in mice not treated with MMI-166 on days 7, 10, 14, 17, and 21 ($P = 0.002, 0.024, 0.001, 0.0001$, and $0.001$), and the growth of s.c. tumors in the mice treated with 100 mg/kg MMI-166 was also significantly suppressed on days 14, 17, and 21 ($P = 0.004, 0.026$, and $0.011$). The inhibitory effect of MMI-166 was dose-dependent in the two treatment groups.
Inhibitory Effect of MMI-166 on Implanted Tumor Growth or Mediastinal Lymph Node Metastasis in an Orthotopically Implanted SCID Mouse Model

There were no surgical deaths caused by the implantation of Ma44-3 cells. No metastasis was observed in the contralateral lung, liver, kidneys, or adrenal glands. The effective dose of MMI-166 was determined to be 200 mg/kg body weight from the s.c. implantation experiment. Ma44-3 cells were injected into a lung of 12 mice that were treated orally with 0 or 200 mg/kg body weight of MMI-166 during days 1 to 13 after implantation. In all mice of the control group (n = 6) and treated group (n = 6), tumors formed in the inoculated site of the left lung. One mouse in the control group died from tumor on day 12. It developed a large tumor on the inoculated site, along with mediastinal metastases, and bloody effusion in the thorax. As the tumor tissue of the mouse had decayed, we could not measure the tumor volume on the inoculated site or the areas of mediastinal metastasis. The tumor volumes in the control (n = 5) and treated groups (n = 6) were 10.033 ± 11.196 and 7.060 ± 5.781 mm³ (Fig. 4A). There was no significant difference in tumor volume between the groups (P = 0.611).

To compare the degree of mediastinal lymph node metastasis in both groups, we examined the weight of the mediastinal tissues and metastatic area of the mediastinum. The weight of the mediastinal tissue in the control (n = 5) and treated (n = 6) groups was 0.089 ± 0.009 and 0.069 ± 0.008 mg, respectively (Fig. 4B). The weight of the mediastinum in the treated group was significantly less than that in the control group (P = 0.005). The metastatic area of the mediastinum in the control (n = 5) and treated (n = 6) groups was 93,495 ± 55,747 and 22,747 ± 17,478 pixels, respectively (Fig. 4C). There was a significant difference in metastatic area between the groups (P = 0.045). Mice treated with MMI-166 showed inhibited mediastinal lymph node metastasis, and this effect was statistically significant.

Film in situ Zymography

MMP activity in the in vivo conditions of the implanted tumors and mediastinal metastatic lesions was examined by film in situ zymography (Fig. 5). Lung tumors in the control group showed gelatinolytic activity on GN film comparing with those on GI film, and lung tumors in the MMP-166 group showed weak activity on GN film (Fig. 5A). Mediastinal metastatic lesions in the control group showed gelatinolytic activity on GN film, whereas, mediastinal metastasis in the MMP-166 group showed no activity on GN film (Fig. 5B). It suggested that MMP-166 successfully inhibited the MMP activity of mediastinal metastatic lesions in vivo, but moderately inhibited that of the implanted tumor in the lung.

Life-Prolonging Activity of MMI-166 in an Orthotopically Implanted SCID Mouse Model

We compared the life-prolonging activity in mice treated with the vehicle control (n = 10) and in those treated with MMI-166 (n = 10) in an orthotopically implanted SCID mouse model (Fig. 6). In the vehicle control group, the first death occurred on day 12, and the median survival time was 17 days. In the MMI-166 group, however, the first death occurred on day 15, and the mean survival time was 23 days. The mice treated with MMI-166 showed a marked prolongation of survival as compared with that in the mice not treated with MMI-166 (P = 0.039).

Discussion

Angiogenesis is initiated by the release of proteases that allow degradation of the basement membrane and migration of endothelial cells into the interstitial space. Endothelial cell proliferation then occurs with eventual differentiation into mature blood vessels (20). There are several classes of agents which target the different steps involved in angiogenesis (21). These include (a) drugs inhibiting MMP, (b) drugs that block endothelial cell signaling by vascular endothelial growth factor and its receptor, (c) drugs that are similar to

Figure 4. Inhibitory effect of MMI-166 on implanted lung tumor and mediastinal lymph node metastasis in an orthotopic implantation model. Mice were treated orally with 0 mg/kg body weight (vehicle control) or 200 mg/kg body weight (MMI-166) of MMI-166 five times a week from day 1 to 14. Mice were sacrificed on day 14. A, volume of the implanted lung tumor; B, mediastinal weight; C, total metastatic area of the mediastinum.
endogenous inhibitors of angiogenesis, and (d) miscellaneous agents. MMI-166 belongs to the third generation of MMP inhibitors. It is a novel synthetic N-sulfonyl amino acid derivative designed to be given orally, and has a selective spectrum of MMP inhibition (MMP-2, MMP-9, and MMP-14; ref. 12). In this study, we examined the anti-invasive effect of MMI-166, a selective inhibitor of MMP-2 and MMP-9, in lung cancer cell line Ma44-3 in vitro. MMI-166 dose-dependently inhibited the invasion of cancer cell lines with the expressions of MMP-2 and/or MMP-9 through a basement membrane-like barrier.

Several studies have reported that MMI-166 (100 or 200 mg/kg body weight) suppressed the tumor growth of pancreatic cancer implanted orthotopically and head and neck cancer implanted s.c. (22, 23). Katori et al. and Matsushita et al. showed that MMI-166 suppressed tumor growth through the inhibition of angiogenesis and the induction of apoptosis accompanied by the reduction of MMP production and activity (22, 23). This study also showed that the Ma44-3 lung cancer cell line moderately expressed both MMP-9 and MMP-2, and that 200 mg/kg body weight of MMI-166 significantly suppressed the
growth of the Ma44-3 tumor implanted s.c. However, MMI-166 did not significantly suppress the growth of the Ma44-3 tumor implanted orthotopically. Our film in situ zymography results also showed that the inhibitory effect of MMI-166 in orthotopically implanted tumors is inadequate. We suspect that this discrepancy results from using a model orthotopically implanted with a cell suspension and Matrigel, whereas the s.c. model did not involve Matrigel. Matrigel enhances the growth of various human tumor xenografts in immunodeficient mice (24–26), and stimulates angiogenesis in tumors (27). It is necessary to examine the close relationship between Matrigel and the inhibitory effect of tumor growth of MMI-166.

Many studies have also reported that the MMP inhibitor inhibits hematogenous metastasis in animal models (11). However, there have been few studies on the effects of MMP inhibitors on lymphogenous metastasis. Yamashita et al. reported that a MMP inhibitor, ONO-4817, inhibited lymph node metastasis of gastric cancer in an orthotopically implanted model (14). Matsuoka et al. showed that a MMP inhibitor, R-94138, inhibited lymph node metastasis of gastric cancer in an orthotopically implanted model (13). MMI-166 has also been reported to inhibit the hematogenous metastasis of various cancers in animal models (12, 22, 27), there has been no report evaluating whether or not MMI-166 inhibits lymphogenous metastasis until now. This study showed that MMI-166 significantly inhibited mediastinal lymph node metastasis in an orthotopically implanted lung cancer model of the Ma44-3 cell line with MMP-2 and MMP-9 activities, and it strongly inhibited gelatinolytic activity localized in mediastinal metastatic lesions. As a result, MMI-166 prolonged the life span in our orthotopic implanted model. Some studies have reported that lymph node metastasis in lung cancer closely correlated with the activation of MMP-2 and MT1-MMP, which are specific activators of MMP-2 on the cell surface (5, 6). We speculated that MMI-166 with the inhibitory potential of MMP-2 activity contributes to the suppression of mediastinal lymph node metastasis in lung cancer, whereas previous reports show that MMI-166 inhibits tumor angiogenesis (22, 23). The mechanism inhibiting lymph node metastasis in this study may include both the suppression of extracellular matrix degradation and the inhibition of angiogenesis at metastatic sites.

In conclusion, our data indicate that MMI-166 significantly inhibited mediastinal lymph node metastasis, and prolonged the life span in the orthotopically implanted model. MMI-166 could possibly inhibit lymph node metastasis and prolong the life span in lung cancer patients.

References
Molecular Cancer Therapeutics

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