Prevention of liver metastasis of human colon cancer by selective matrix metalloproteinase inhibitor MMI-166

Kouji Oba, Hiroyuki Konno *, Tatsuo Tanaka, Megumi Baba, Kinji Kamiya, Manabu Ohta, Takeshi Kaneko, Tsuyoshi Shouji, Akira Igarashi, Satoshi Nakamura

Department of Surgery II, Hamamatsu University School of Medicine, 1-20-1 Handayama, Hamamatsu 431-3192, Japan

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Abstract

MMI-166 is a selective inhibitor of matrix metalloproteinase (MMP)-2 and MMP-9. Mice implanted a human colon cancer orthotopically received 200 mg/kg of MMI-166 orally for 5 weeks. Gelatin zymography demonstrated that the administration of MMI-166 remarkably decreased the active MMP-2 expression. Histological examination revealed that MMI-166 showed prominent effect on reduction of the invasive feature of the cancer cells and showed inhibitory effect on tumor vasculature, resulting in the significant decrease of microvessel density of the implanted tumor and liver metastasis compared with the control group. Conclusively, MMI-166 is a potent antiangiogenic oral agent for a human colon cancer.

Keywords: MMI-166; Colon cancer; Liver metastasis; Zymography; Microvessel density

1. Introduction

Various potent matrix metalloproteinase (MMP) inhibitors (MMIs) including batimastat [1,2], marimastat [3], BAY12-9566 [4], AG3340 [5], COL-3 [6], CT1746 [7], KB-R7785 [8], R-94138 [9] and BPHA [10] were synthesized and several clinical trials have already started and an antitumor effect of some of these inhibitors has been demonstrated [11–18]. Several MMP inhibitors have been reported to reduce metastasis in various models by inhibiting angiogenesis [8,10]. However, the fact that some of them show the inhibitory effect against a broad spectrum of MMPs suggests that they may cause various adverse effects [11,13,16,17].

In colon cancer, MMP-2 and MMP-9 have been shown to degrade mainly the extracellular matrix [19], and these MMPs have a specific proteolytic activity for type IV collagen in the basement membrane [20]. In addition, the expression of MMP-2 and MMP-9 is closely related to the invasive and metastatic potential of several tumors in vivo [21–23], and has also been identified as having a prognostic impact in various malignancies [24,25]. Furthermore, both MMPs have been reported to contribute to tumor angiogenesis [26,27], and a correlation between their expression and microvessel density (MVD) has been reported [28].

MMI-166 was synthesized as a specific inhibitor of MMP-2 and -9 and was demonstrated to have an excellent antitumor effect on experimental lung cancer, colon cancer and pancreatic cancer [29,30]. In the present study, we demonstrated experimentally that MMI-166 showed a prominent effect on reduction...
of the invasive feature and the tumor vasculature, which resulted in the significant decrease of MVD and liver metastasis of a human colon cancer.

2. Materials and methods

2.1. Animals

Male BALB/c nu/nu mice were purchased from Clea Japan, Inc. (Tokyo, Japan) and were used at 5 weeks of age.

2.2. Materials

MMI-166, an N-sulfonylamino acid derivative, was a kind gift from the Discovery Research Laboratories of Shionogi Co. Ltd. (Osaka, Japan). Its enzyme inhibition profile was as follows: IC50 value > 10 μM for MMP-1 (collagenase), 0.002 μM for M, 72 000 gelatinase (MMP-2), 1.801 μM for MMP-3 (stromelysin), > 10 μM for MMP-7 (matrilysin), and 0.053 μM for M, 92 000 gelatinase (MMP-9). The compound was obtained as a white powder and was suspended in a vehicle (saline containing 0.5% carboxymethylcellulose-Na, 0.9% benzyl alcohol, and 0.4% Tween 80) for oral administration.

2.3. Human colon cancer xenograft

TK-4, a human colon cancer xenograft of well differentiated adenocarcinoma, was used in this study. TK-4 was established from a surgical specimen at our department in 1993 and has been maintained by subcutaneous passage [31].

2.4. Experimental design

The method of orthotopic implantation has been reported elsewhere. Briefly, small pieces of TK-4 tumor tissue (200 mg) were resected aseptically during the exponential growth phase from tumors subcutaneously implanted in nude mice. The cecum of each recipient was carefully exteriorized, and a tumor piece was sutured to the cecal surface with a 6-0 Vicryl transmural suture (Ethicon Inc., Somerville, NJ, USA). Then the intestine was returned to the abdominal cavity, and the abdominal wall and skin were closed with 6-0 Vicryl sutures.

To evaluate the effect of MMI-166 on tumor growth and liver metastasis, 25 mice were randomly divided into two groups. Ten mice were given MMI-166 orally at a daily dose of 200 mg/kg on 6 days per week for 5 weeks starting on the fifth day after orthotopic implantation (the MMI-166 group). In addition, the same volume of vehicle was given to another 15 mice (the control group). All of the mice were killed at 6 weeks after tumor implantation. Autopsy was performed immediately and the tumors growing on the cecum were removed and weighed. The liver of each animal was processed for routine histological examination to detect metastases after careful macroscopic examination.

2.5. Gelatin zymography

Specimens of the primary tumor were stored frozen at −80°C until homogenization in special buffer. Five specimens in the control group and five specimens in the MMI-166 group, randomly selected, were analyzed with gelatin zymography. The protein concentration of the tissue lysate was determined from Yagai Inc. (Yamagata, Japan). Aliquots of lysate (20 μl) were diluted in 20 μl of sample buffer, and were applied to prepared gels. After electrophoresis (10 mA/29 mV/20 min + 20 mA/80 mV/80 min), each gel was washed and incubated for 25 h in enzyme reaction buffer at 37°C. Then the gel was stained with Coomassie brilliant blue and destained. Gelatinolytic activity was visualized as clear bands against a blue background. The levels of activities were compared between the control group and the MMI-166 group by means of densitometry.

2.6. Immunohistochemical staining and determination of the MVD

Before immunohistochemical staining, tissue specimens from the resected tumors were embedded in Tissue-Tek O.C.T. compound (Sakura Fine Technical Co. Ltd., Tokyo, Japan) and were frozen in 2-methylbutane cooled with liquid nitrogen. Then sections with a thickness of 6 μm were cut on a cryostat. Microvessels were highlighted by the immunostaining of endothelial cells with a rat monoclonal antibody (ER-MP12) using the avidin–biotin–peroxidase complex technique. The three areas within each
section containing the most capillaries and small venules were selected, and the stained vessels were carefully counted under a light microscope at a magnification of 200×. The average count for the three areas was calculated as the MVD for each animal.

2.7. Statistical analysis

Data on the tumor weight, body weight, spleen weight, number of metastatic foci, and MVD are reported as the mean ± standard deviation and analysis of significance was done by Student’s t-test. The chi-square test was used to compare the number of mice in each group with liver metastasis. Quantitative analysis with densitometry was compared with Mann–Whitney U-test. For all analyses, \( P < 0.05 \) was considered significant.

3. Results

3.1. Gelatin zymography

Detection of gelatinase activity in the experimental tumors by zymography is shown in Fig. 1. MMP-2 expression was revealed in the tumors by zymography. The level of MMP-2 activity in the MMI-166 group was significantly reduced compared with that in control group (\( P = 0.0472 \)). Gelatinolytic bands corresponding to 92 or 82 kDa were not recognized, so MMP-9 activity was not detected.

3.2. Inhibitory effect of MMI-166 on liver metastasis

A suppressive effect of MMI-166 on liver metastasis was significantly demonstrated when compared with the control group (Table 1; \( P = 0.0031 \)). Liver metastasis was observed in 12 mice (12/15, 80%) from the control group, whereas only two mice (2/10, 20%) showed liver metastasis in the MMI-166 group. The administration of MMI-166 also clearly decreased the number of metastatic foci in the liver (Table 1).

3.3. Inhibitory effect of MMI-166 on tumor growth

The implanted tumors grew on the wall of the cecum, as reported previously [31]. The actual tumor weight at the end of the experiment was \( 0.793 ± 0.355 \) g in the control group (\( n = 15 \)) and \( 0.743 ± 0.352 \) g in the MMI-166 group (\( n = 10 \)), with no significant difference in tumor growth between the groups.

3.4. Histological features treated by MMI-166

Fig. 2 shows photomicrographs (H&E staining) of metastatic foci in the livers (Fig. 2A,B) and implanted tumors on cecal walls (Fig. 2C,D). Tumors of mice from the control group showed invasion at the metastatic sites (Fig. 2A), whereas the tumor margin was clearly separated from the adjacent liver tissue and there was internal necrosis of tumors in the MMI-166 group (Fig. 2B). At implanted sites, tumors showed invasion from the serosa to the submucosa in the control group (Fig. 2C), whereas tumors did not invade beyond muscular layer in the MMI-166 group (Fig. 2D). Thus, an inhibitory effect of MMI-

Table 1

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<th>Group</th>
<th>No. of mice with liver metastasis</th>
<th>No. of foci of liver metastasis (^a)</th>
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<tr>
<td>Control group</td>
<td>12/15 (80.0%)</td>
<td>3.75 ± 2.26</td>
</tr>
<tr>
<td>MMI-166 group</td>
<td>2/10 (20.0%)(^b)</td>
<td>1.00 ± 0.00(^c)</td>
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\(^a\) Mean ± SD.  
\(^b\) \( P < 0.01 \) versus control group.  
\(^c\) Not significant.
166 on tumor cell invasion at both metastatic sites and implanted sites was demonstrated.

3.5. Immunohistochemical assessment of the tumor vasculature

Immunohistochemical staining with ER-MP12 revealed positive cells in the microvessels of the implanted tumors. Numerous brown-stained endothelial cells showed elongation and tube-formation in the control group, whereas endothelial cells formed clusters and there was less tube-formation in the MMI-166 group (Fig. 3). The MVD of the MMI-166 group (35.8 ± 6.9) was significantly smaller than that of the control group (46.3 ± 12.7) (P = 0.0332).

Regarding body weight and spleen weight at the time of sacrifice, there was no significant difference between the two groups (Table 2).
4. Discussion

During angiogenesis, degradation of ECM is an essential part of the process of endothelial cell proliferation. In this early part of the process of angiogenesis, MMPs play an important role as well as plasminogen activators. Therefore, MMP inhibitors have attracted attention as antiangiogenic agents and have been shown to have antiangiogenic activity [28]. Several studies have also demonstrated that synthetic MMPIs inhibited tumor angiogenesis and growth [8,10,32,33]. However, broad-spectrum MMP inhibitors have been demonstrated to have adverse effects. It was reported in a clinical study that marimastat, the inhibitor of MMP-1, -2, -3, -7, and -9, caused adverse events such as inflammation of tendons and ligaments [13]. On the other hand, BAY12-9566, the selective inhibitor of MMP-2, -3 and -9, has not been reported to cause such events in a phase I study and other early clinical studies [14,15]. In the present study, MMI-166 did not cause body weight loss, and no physical or behavioral changes were noticed, probably because MMI-166 was a narrow-spectrum MMP inhibitor.

MMI-166 is a novel narrow-spectrum MMP inhibitor, which selectively inhibits MMP-2 and MMP-9. There have been several reports regarding the role of both MMP-2 and MMP-9 in angiogenesis [34]. In the present study, gelatin zymography of implanted tumors revealed that MMP-2 activity was expressed in TK-4 and was decreased in mice treated with MMI-166, whereas MMP-9 activity was not detected in implanted tumors. Considering that MMI-166 inhibits both MMP-2 and MMP-9, but only MMP-2 was expressed in TK-4 tumors, inhibition of MMP-2 activity seemed to be vital for preventing liver metastasis in the present experiment. MMP-2 expression has been detected in several types of cancer [27,35], and is correlated with tumor angiogenesis [26,27], especially VEGF expression in breast cancer [26] and lung cancer [35]. In MMP-2-deficient mice, tumor angiogenesis was reduced in the dorsal air sac assay, and tumor growth was suppressed for B16-BL16 melanoma as well as Lewis lung carcinoma [36].

In the present study, MMI-166 reduced tumor invasion, since it was demonstrated histologically that the tumor edge was microscopically defined from normal tissue in the MMI-166 group, not only in the cecal wall but also in the liver. This inhibition of tumor invasion may also play an important role in the inhibition of liver metastasis. However, it was reported that batimastat did not influence the extravasation of tumor cells from the hepatic circulation, as shown

![Image](A)

Fig. 3. Immunohistological analysis of inhibitory effect of MMI-166 on tumor angiogenesis. Microvessels were highlighted by staining endothelial cells immunohistochemically with ER-MP12. Numerous brown-stained endothelial cells showed elongation and tube formation in the control group (A). Although positively stained endothelial cells were identified, they formed clusters and there was less tube formation in the MMI-166 group (B). Magnification: ×250.

Table 2

<table>
<thead>
<tr>
<th>Group</th>
<th>Body weight</th>
<th>Spleen weight</th>
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<tr>
<td>Control group</td>
<td>21.667 ± 1.665</td>
<td>0.153 ± 0.048</td>
</tr>
<tr>
<td>MMI-166 group</td>
<td>22.472 ± 0.874b</td>
<td>0.170 ± 0.057b</td>
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*Values are mean ± SD.

*Not significant.
by intravital videomicroscopy, whereas it reduced vessel density of liver metastasis in a mouse B16F1 melanoma model [33]. Therefore, MMI-166 may show an antimetastatic effect by decreasing the number of circulating tumor cells and by prevention of angiogenesis in the early period after tumor implantation at metastatic sites. Moreover, MMI-166 reduced tumor invasion at a metastatic site in liver, so that it may also inhibit the secondary metastasis subsequent to the liver metastasis including intrahepatic metastasis.

Histologically, MMI-166 was demonstrated to inhibit the development of tumor vasculature. An MMP inhibitor, KB-R7785, was reported to affect endothelial cell invasion and capillary sprouting, whereas TNP-470, a fumagilline derivative, may interfere with maturation of newly formed vessels [8]. In the present study, we observed immunohistochemically that endothelial cells in the implanted tumor could not form mature vessels involving sprouting, elongation and tube formation in the MMI-166 group. This suggests that MMP inhibitors may primarily affect the early steps of angiogenesis. It was demonstrated in a recent study that MMI-166 inhibited the liver metastasis of experimental pancreatic cancer and that MMI-166 significantly reduced MVD whereas it did not reduce tumor cell proliferation [30]. These findings are compatible with our results, and it is suggested that MMI-166 showed an inhibitory effect on liver metastasis by means of the reduction of invasion and angiogenesis.

MMI-166 has another advantage as an antiangiogenic agent, which is an orally active agent. Oral administration for the long term is one of the optimal strategies in antiangiogenic therapy, since antiangiogenic therapy is expected to have an impact on patient survival through long-term treatment.

Collectively, MMI-166 is a potent inhibitor of liver metastasis in a human colon cancer, reducing invasion of tumor cells and tumor angiogenesis.

References


