

## Effect of Combination Therapy with Matrix Metalloproteinase Inhibitor MMI-166 and Mitomycin C on the Growth and Liver Metastasis of Human Colon Cancer

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

Department of Surgery II, Hamamatsu University School of Medicine, 3600 Handa-cho, Hamamatsu 431-3192

Several synthetic inhibitors of matrix metalloproteinases (MMPs) show antitumor, antimetastasis and antiangiogenesis effects in various models. Synergistic effects of combinations with conventional cytotoxic agents were reported previously. In this study, we examined the effects of a new selective MMP inhibitor, MMI-166, on tumor growth, angiogenesis and metastasis in a liver metastatic model of human xenotransplanted colon cancer (TK-4). We also investigated the synergistic effects of MMI-166 and a conventional cytotoxic agent, mitomycin C (MMC), in this model. Mice transplanted orthotopically with TK-4 were divided into 4 groups; a control group (treated with vehicle solution), an MMI-166 group in which MMI-166 was orally administered (p.o.) at a dose of 200 mg/kg, 6 days/week for 5 weeks, an MMC group in which MMC was administered intraperitoneally (i.p.) at a dose of 2 mg/kg/week for 5 weeks, and a combination group (treated with MMI-166 and MMC). MMI-166 did not inhibit transplanted tumor growth, but significantly inhibited liver metastasis compared with the control group and MMC group ( $P < 0.01$ ). Significant antitumor and antimetastatic effects of the combination therapy were demonstrated. The microvessel density (MVD) detected by immunohistochemical staining with ER-MP12 antibody tended to be lower in the MMI-166 and the combination groups. These results suggest that MMI-166 has potential antimetastatic ability and a synergistic effect with MMC.

Key words: Matrix metalloproteinase inhibitor—Metastasis—Colon cancer—Mitomycin C

In tumor progression and metastasis, invasion and angiogenesis are essential. Among the various factors, matrix metalloproteinases (MMPs) play an important role in tumor invasion, as well as plasminogen activators, which function to degrade the extracellular matrix. Among the 18 MMPs identified to date, MMP-2 and MMP-9 have been reported to be associated with tumor invasion, metastasis and angiogenesis,<sup>1–5</sup> and these factors have also been identified to have a prognostic impact in various malignancies.<sup>6–9</sup> MMP-2 and MMP-9 have specific proteolytic activity against type IV collagen of the basement membrane.<sup>10</sup> The expression of MMP-2, -9 correlates closely with invasive and metastatic potentials of several tumors *in vivo*.<sup>4, 8, 11</sup> A correlation between angiogenesis and MMPs was also reported.<sup>6</sup>

Several MMP inhibitors including Batimastat,<sup>12–20</sup> Marimastat,<sup>21</sup> AG3340,<sup>22</sup> BPHA,<sup>1</sup> KB-R7785,<sup>23</sup> R94138,<sup>24</sup> CT1746<sup>25</sup> and BAY12-9566<sup>26</sup> have been synthesized as candidate drugs for cancer treatment. Most of them function to diminish the activity of MMP-2, -9, or -7. Antitumor effects of Batimastat on ovarian tumor,<sup>14, 20</sup> melanoma,<sup>19</sup> colon cancer,<sup>18</sup> breast cancer,<sup>16, 17</sup> hemangioma,<sup>9</sup> and lung cancer<sup>12</sup> have been recognized. The MMP

inhibitors Batimastat and Marimastat are presently undergoing Phase I, II and III clinical trials.<sup>3, 27–31</sup> BAY 12-9566 is in Phase I trial.<sup>26</sup>

Here, we examined a potent oral MMP inhibitor, MMI-166, which selectively inhibits MMP-2 and -9. Several MMP inhibitors reduce metastasis in various models by inhibiting angiogenesis.<sup>1, 9, 13, 23</sup> In this study, we examined the anti-tumor and anti-metastatic effects of MMI-166, and examined the microvessel density (MVD) to clarify the antiangiogenesis activity. Synergistic effects of MMP in combination with conventional cytotoxic agents were reported previously,<sup>3, 14, 24, 25</sup> and in this study we also examined the effect of MMI-166 and a conventional cytotoxic agent, mitomycin C (MMC), in a human colon cancer nude mouse model.

### MATERIALS AND METHODS

**Animals** Male BALB/c *nu/nu* mice were obtained from Clea, Japan, Inc. (Tokyo). Animals were used for this study at 5 weeks of age.

**Materials** Matrix metalloproteinase inhibitor MMI-166 was kindly provided by Shionogi Co., Ltd. (Osaka). Its *in vitro* IC<sub>50</sub> values are 2 nM for gelatinase A (MMP-2) and 53 nM for gelatinase B (MMP-9). An aqueous suspension

E-mail: otam@hama-med.ac.jp

of MMI-166 (a white powder) was prepared by homogenizing the drug in 0.5% carboxymethyl cellulose Na, 0.9% benzyl alcohol and 0.4% Tween 80 in saline. A control solution of the vehicle without MMI-166 was also prepared. MCC was purchased from Kyowa Hakko (Tokyo) and dissolved in saline.

**Preparation of human colon cancer xenografts** Human colon cancer xenograft, TK-4 (a well-differentiated carcinoma line) was used in this study (Fig. 1). This xenograft was established from surgical specimens in our department and was maintained by serial transplantation in nude mice. In gelatin zymography, TK-4 tumors expressed MMP-2, but the expression of MMP-9 was not recognized (data not shown).

**Experimental design** For the xenograft orthotopic transplantation, tumor tissue specimens weighing 200 mg were sutured onto the surface of the cecum. Mice were randomly divided into 4 groups and the experiment was started on day 5 after transplantation. The groups were the control group treated with the same volume of vehicle, the MMC group in which animals were given MMC i.p. at a dose of 2 mg/kg/week for 5 weeks, the MMI-166 group in which animals were given MMI-166 p.o. at a dose of 200 mg/kg, 6 days/week for 5 weeks, and the combination group in which animals were given MMC i.p. at a dose of 2 mg/kg/week and MMI-166 p.o. at a dose of 200 mg/kg, 6 days/week for 5 weeks. Mice were sacrificed on day 40 after transplantation. The mice were weighed and

autopsy was performed immediately. The tumors growing on the cecal wall were removed and weighed. One part of the tumor was placed in 10% formalin, embedded in paraffin, sectioned, and stained with H&E. The other part was placed in Tissue-Tek O.C.T. Compound (Sakura Finetechnical Co., Ltd., Tokyo), and then snap-frozen and stored at  $-80^{\circ}\text{C}$  for immunohistochemistry and gelatin zymography. The liver metastases were counted macroscopically and subjected to routine histological examinations (Fig. 2).

**Immunohistochemical analysis for MVD** Intratumoral microvessels were determined by immunostaining using a rat monoclonal antibody (ER-MP12) with the avidin-biotin-peroxidase complex technique as previously described.<sup>32)</sup> For determination of the MVD in transplanted tumors, the three most vascular areas within one section were chosen and the stained vessels were counted under a light microscope at 200-fold magnification. The average count was recorded as the MVD for each animal.

**Statistical analysis** The statistical analysis was performed with Student's *t* test and the  $\chi^2$  test, and  $P < 0.05$  was taken as the criterion of statistical significance.

## RESULTS

**Therapeutic effect on tumor growth and liver metastasis** All tumor pieces transplanted in the cecum grew, as previously reported.<sup>33)</sup> Actual tumor weights in each group were as follows: control group,  $1.102 \pm 0.96$  g; MMC

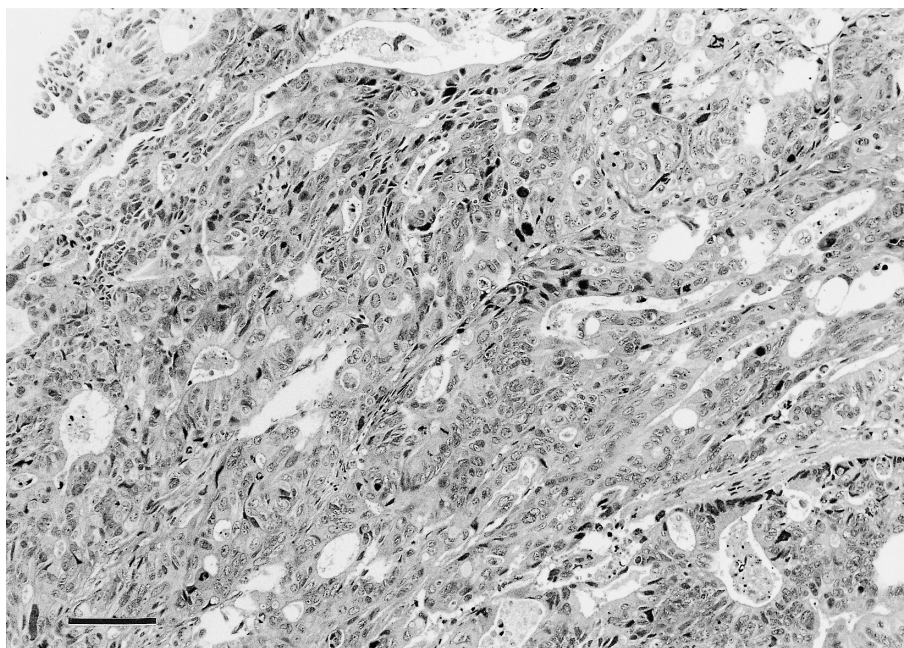


Fig. 1. TK-4 (a well-differentiated adenocarcinoma), which was established in Department of Surgery II, Hamamatsu University School of Medicine. Bar 50  $\mu\text{m}$ . Liver metastatic rate of orthotopic implantation is 60–84%.

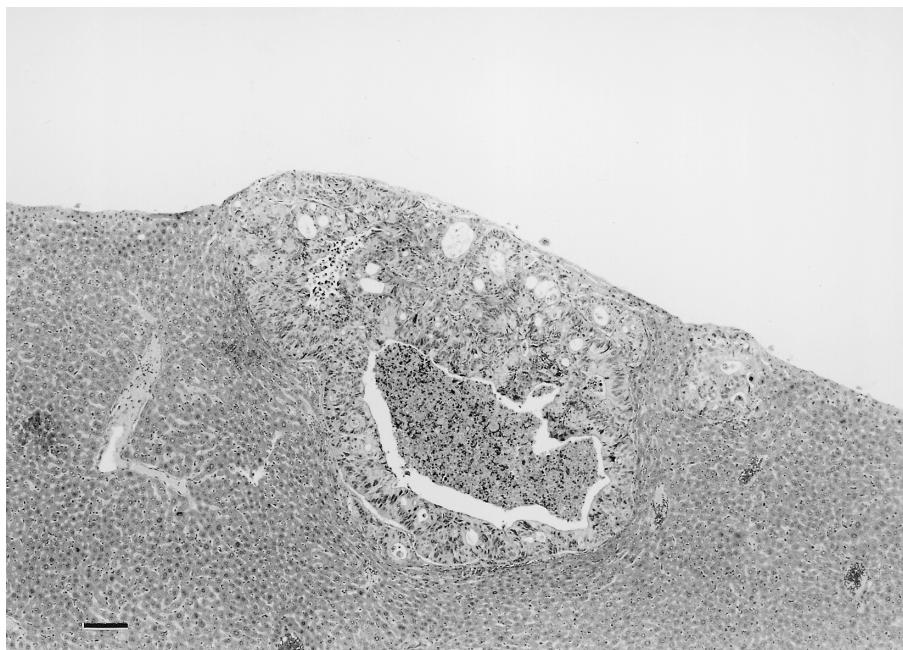


Fig. 2. Microscopic finding of liver metastasis. The metastatic lesions formed nodules in normal liver tissues. Bar 50  $\mu$ m.

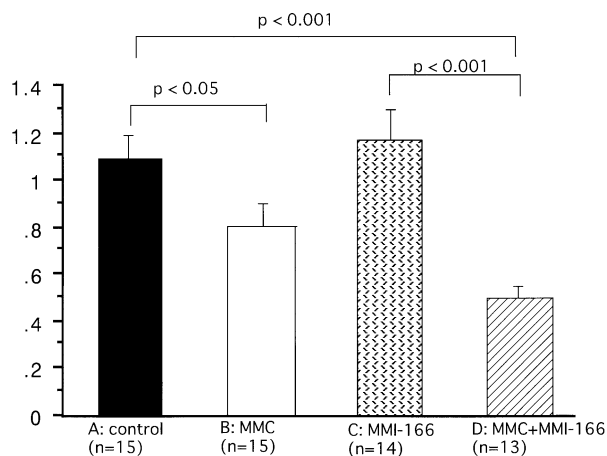


Fig. 3. Inhibition of transplanted tumor by MMC and MMI-166. MMI-166 did not inhibit the tumor growth, but the combination therapy showed a significant synergistic effect of inhibition of tumor growth. Error bars, SD.

group,  $0.807 \pm 0.96$  g; MMI group,  $1.175 \pm 0.130$  g; and combination group,  $0.501 \pm 0.052$  g. Administration of MMC significantly inhibited the primary tumor growth ( $P < 0.03$ ), but no such antitumor effect of MMI-166 administration was observed. Combination therapy amplified the inhibitory effect on tumor growth compared with MMC therapy ( $P < 0.013$ ) (Fig. 3).

Histological difference between the tumors treated with and without MMI-166 was clearly apparent (Fig. 4). The tumor margin adjacent to normal tissue in the cecal wall could be readily identified in the MMI and the combination groups, whereas cancer nests invaded the cecal submucosa in the control group.

Table I shows the numbers of mice with liver metastasis and metastatic foci in the four groups. No liver metastasis developed in the MMI-166 and combination groups ( $P < 0.01$ ), although it was observed in mice of the control groups and in mice of the MMC group. MMC failed to inhibit liver metastasis.

**Immunohistochemical analysis for MVD** In all three treated groups, MVD was significantly reduced compared with the control group. Development of microvessels was inhibited in the three treated groups. MVD in the combination therapy was lowest among the groups, although the difference was not significant ( $P = 0.155$  vs. MMC,  $= 0.13$  vs. MMI-166) (Fig. 5).

**Body weight and spleen weight** No significant differences in body weight and spleen weight were found in the 4 groups, and there were no severe side effects in any group (Fig. 6).

## DISCUSSION

We demonstrated here that the orally active MMP inhibitor, MMI-166, has antimetastatic efficacy. This is the first report to describe the antimetastatic efficacy of an

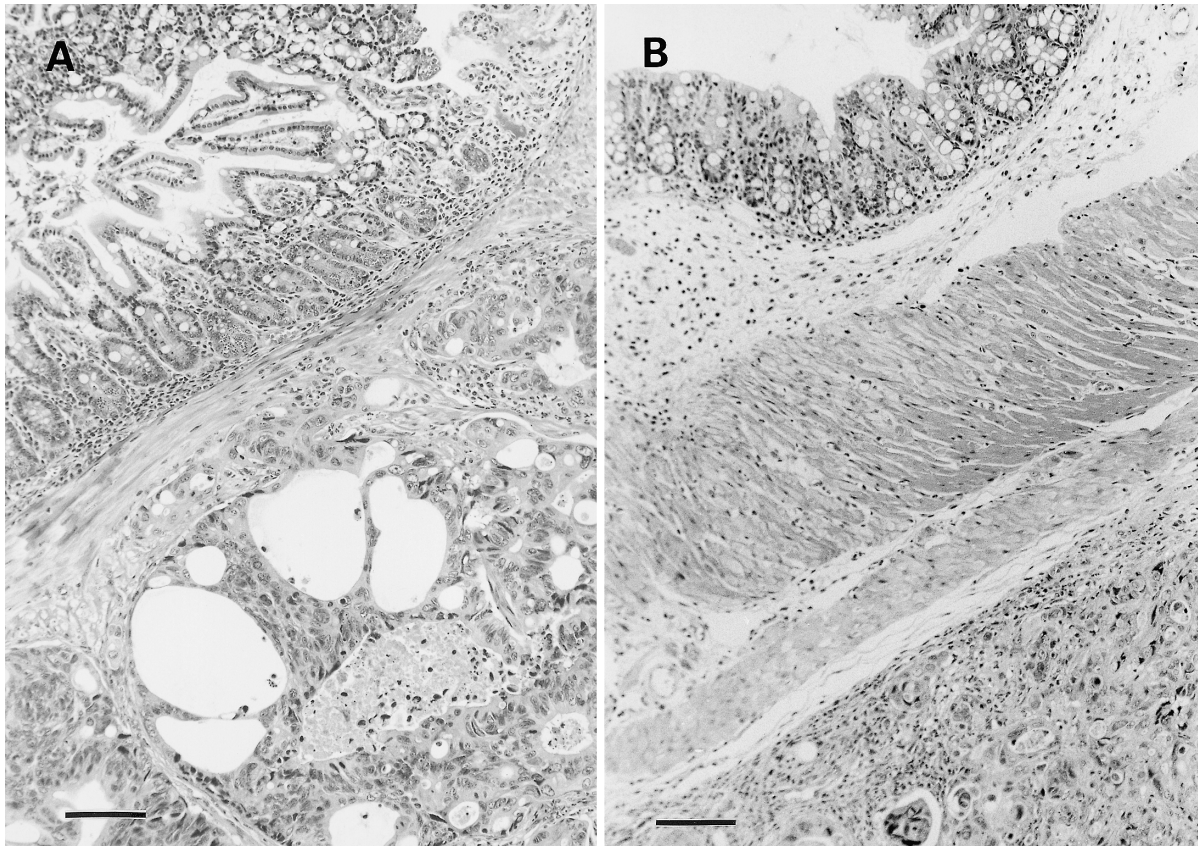


Fig. 4. Histological appearance from the invaded lesion from the implanted tumor. A: Massive invasion of cancer cells is observed in the control group. B: The layered construction of the cecum in the MMI-166 group is maintained. Bar 50  $\mu$ m.

Table I. Inhibitory Effect of MMI-166 and MMC on Liver Metastasis

Group	Number of mice with liver metastasis (%)	Mean number of metastatic liver foci
A (Control)	9/15 (60) <sup>d</sup>	1.40±0.35 <sup>d</sup>
B (MMC)	7/15 (46.7) <sup>a)</sup>	1.68±0.49 <sup>a)</sup>
C (MMI-166)	0/14 (0) <sup>b, e)</sup>	0.00 <sup>c, e)</sup>
D (MMC+MMI-166)	0/13 (0) <sup>b, e)</sup>	0.00 <sup>c, e)</sup>

a) NS, b)  $P < 0.01$ , c)  $P < 0.001$ , relative to control by Student's  $t$  test.

d) NS, e)  $P < 0.01$ , relative to group B (MMC) by Student's  $t$  test.

orally active MMP inhibitor in a nude mouse liver-metastatic model. Furthermore, the synergy of antimetastasis and antiangiogenesis actions of this inhibitor and MMC was clearly apparent in our model. Previously, we investigated and reported the antimetastatic effects of various angiogenesis inhibitors, and the inhibitory effect of MMI-166 was as potent as that of TNP-470 or VEGF-neutralizing antibody.<sup>34, 35)</sup>

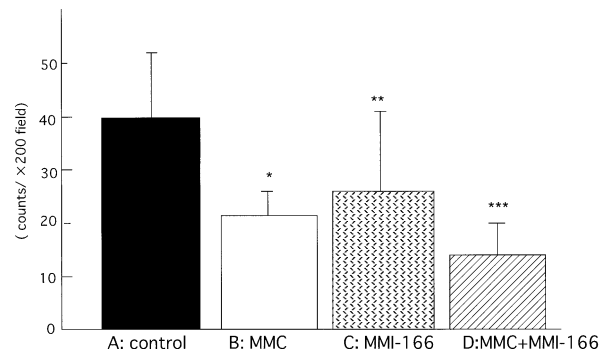


Fig. 5. The MVD visualized by immunohistological staining with ER-MP12 antibody. \*  $P < 0.027$ , \*\*  $P < 0.0017$ , \*\*\*  $P < 0.001$ , from control. Error bars, SD.

Several MMP inhibitors have been demonstrated to have antiangiogenic activity,<sup>1, 9, 13, 23)</sup> mainly because the degradation of ECM is essential to the process of angiogenesis. It has been shown in several studies with different

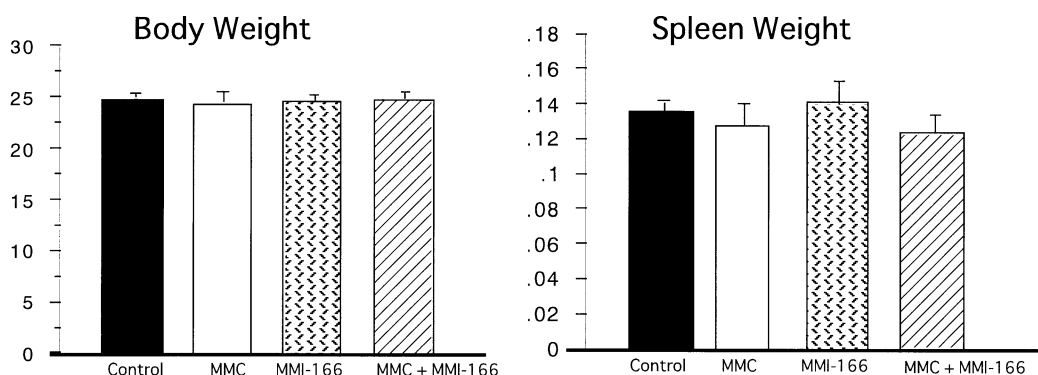


Fig. 6. Body weight and spleen weight were not significantly different among the 4 groups.

approaches that synthetic MMP inhibitors can inhibit angiogenesis in malignant tumors. Schnapper *et al.* demonstrated the importance of type IV collagenases in new blood vessel formation in an *in vitro* model of angiogenesis.<sup>36</sup> Furthermore, vascular endothelial growth factor (VEGF) expression was significantly related to expression of MMP-2 in breast cancer<sup>6</sup> and lung cancer.<sup>37</sup> Thymidine phosphorylase expression was also correlated to pro MMP-2 and activated MMP-9 expression.<sup>6</sup> In MMP-2-deficient mice, tumor-induced angiogenesis was suppressed according to dorsal air sac assay, and the implanted tumor volume decreased for B16-BL16 melanoma and Lewis lung carcinoma.<sup>2</sup> MMP-2 is expressed in several tumors<sup>4, 7, 11, 37</sup> and is related to prognosis<sup>19</sup> and angiogenesis.<sup>6</sup>

Considering that MMI-166 selectively inhibited both MMP-2 and MMP-9, and only MMP-2 is expressed in TK-4, inhibition of MMP-2 activity of TK-4 is crucial in preventing liver metastasis of this strain (data not shown). MMP-2 has been reported to enhance tumor angiogenesis. Histological findings revealed that MMI-166 was effective in reducing not only tumor angiogenesis, but also tumor invasion. The tumor margin adjacent to normal tissue in the cecal wall could be identified clearly in the groups treated with MMI-166. This suggested that the tumor invasion was inhibited by MMI-166, and this might have contributed to the antimetastatic effect. In the metastatic sites, however, MMP inhibitors may not inhibit the extravasation of tumor cells. Wylie *et al.* showed by intravital videomicroscopy that Batimastat did not affect the extravasation of cells from the liver circulation, whereas it reduced the mean diameter of liver metastasis after injection of B16F1 melanoma cells in a mouse model.<sup>13</sup>

Interestingly, Lozonchi *et al.* found differences of inhibitory pattern on tumor angiogenesis between the MMP inhibitor KB-R7785, and TNP-470, a fumagillin derivative.<sup>23</sup> Administration of MMP inhibitor reduced the

expansion of vascular areas, generating centrally avascular tumors with only a rim of peripheral neovascularization in a dorsal skinchamber model. Avascular areas, however, were not seen after administration of TNP-470, as opposed to KB-R7785. TNP-470 did not affect the vascular density, but affected the vessel diameter. These findings suggested that KB-R7785 may primarily affect the early steps of angiogenesis, while TNP-470 may interfere more with later steps, possibly including maturation of newly formed vessels. Therefore, there may be a synergistic inhibitory effect of combined therapy with MMP inhibitor and TNP-470 on tumor angiogenesis.

Because MMC also inhibited the MVD in the primary tumors (Fig. 5), MMC may have a cytotoxic effect on both tumor cells and endothelial cells. However, the number of metastatic foci in the liver was not decreased by MMC treatment. It is possible that MMC may have a cytotoxic effect on endothelial cells after the tumor reaches by a certain volume.

An enhanced effect of MMI-166 and MMC was also demonstrated in the present study. Several reports have revealed synergistic effects of MMP inhibitors and cytotoxic agents *in vivo*. The combination of either Batimastat or Marimastat and either cisplatin or cyclophosphamide has synergistic antitumor and survival-prolongation effects in lung and ovarian cancer mouse models.<sup>3, 14</sup> The combination of MMC and R94138 showed an increased preventive effect on peritoneal dissemination in a nude mouse model.<sup>24</sup> The mechanisms of antitumor action of these combinations are not yet known. In a study of induction of Fas-mediated apoptosis in Ewing's sarcoma cell lines, a combination effect of MMP inhibitors and doxorubicin was observed.<sup>38</sup> In another report, the combination of Batimastat and interferon  $\gamma$  showed a strong synergistic effect in an ovarian cancer model *in vivo*.<sup>15</sup> In addition, our previous study revealed an enhanced therapeutic effect of VEGF neutralizing antibody and MMC.<sup>39</sup> The combi-

nation of antiangiogenic therapy and chemotherapy thus appears to be a useful strategy for colorectal cancer. Though the mechanisms of these combination therapies remain unclear, the combination of MMP inhibitors with chemotherapeutic agents or other antiangiogenic agents may be an option for cancer treatment.

Pan-MMP inhibitors have been reported to have adverse effects. Marimastat, which inhibits MMP-1, 2, 3, 7, 9, 12, commonly induces musculoskeletal events such as inflammation of the tendons and ligaments.<sup>12, 29)</sup> In patients treated with BAY12-9566, which inhibits MMP-2, 3, 9, such musculoskeletal events were not observed in a Phase I study and other early clinical studies.<sup>26, 40, 41)</sup> In the present study, MMI-166 did not induce body weight loss or other serious adverse effects, probably because of its narrow inhibitory spectrum.

In conclusion, our results indicate that MMI-166 has potential antimetastatic ability and a synergistic therapeutic

effect with MMC. The advantages of MMI-166 in clinical use are as follows. 1) MMI-166 has a small molecular weight and can be orally administered, which may improve patient compliance. 2) The adverse effects are less than those of broad-type MMP inhibitors and cytotoxic agents. 3) The synergistic effect with cytotoxic agents may afford amplified antitumor and antimetastatic potential.

#### ACKNOWLEDGMENTS

We thank Ryuji Maekawa PhD. and Takayuki Yoshioka PhD. (Discovery Research Laboratories, Shionogi Co., Ltd.) for providing the MMP inhibitor and for helpful suggestions during this study.

(Received January 22, 2001/Revised March 30, 2001/Accepted April 6, 2001)

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