Matrix Metalloproteinase Inhibitor, Marimastat, Decreases Peritoneal Spread of Gastric Carcinoma in Nude Mice

Masaru Kimata,1 Yoshihide Otani,1, 5 Tetsuro Kubota,1 Naoki Igarashi,1 Takeyoshi Yokoyama,1 Norihito Wada,1 Nobunari Yoshimizu,1 Masato Fujii,2 Kaori Kameyama,3 Yasunori Okada,3 Koichiro Kumai4 and Masaki Kitajima1

1Department of Surgery, 2Department of Otolaryngology, 3Department of Pathology and 4Center for Diagnostic and Therapeutic Endoscopy, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582

Marimastat, a matrix metalloprotease inhibitor, was examined for the ability to prevent peritoneal dissemination of a human gastric cancer xenograft, TMK-1. Even with novel approaches such as molecular targeting of cancer chemotherapy, peritoneal dissemination of gastric cancer has little sensitivity to anticancer drugs, and it is impossible to inhibit its growth completely. Intraperitoneal injection of TMK-1 into nude mice at 5×10^5 cells/body resulted in carcinomatous peritonitis that mimicked clinical cases. Continuous administration of marimastat (18 mg/kg/day) from 24 h after the tumor inoculation successfully inhibited the growth of peritoneal dissemination nodules. Combined administration of marimastat (18 mg/kg/day) and mitomycin C (MMC, 2 mg/kg) showed synergistic inhibition of growth of peritoneal dissemination, being superior to MMC alone (2 mg/kg). Although marimastat alone could not increase survival time with statistical significance, combined administration of marimastat and MMC had a survival benefit with statistical significance. The combination of marimastat and MMC increased the preventive effect on peritoneal dissemination. Marimastat seems to be a candidate for the prevention of peritoneal spread of gastric carcinoma.

Key words: MMP inhibitor — Gastric cancer — Peritoneal dissemination — Marimastat

An apparent increase in incidence of early gastric cancer in Japan has resulted from enhanced detection by a well-established screening program. However, outcomes remain unsatisfactory for patients with advanced gastric cancer. In our hospital, 3724 patients underwent surgery for gastric cancer between 1960 and 1997. At initial surgery 13.6% of these patients had macroscopically evident peritoneal dissemination of cancer. Peritoneal dissemination has represented the most common recurrence site for gastric cancer at our institution, comprising over 30% of all recurrences. The 5-year survival rate of the patients with this form of recurrence was only 4.8%, and survival beyond 3 years was uncommon. The usual treatment of gastric cancer is surgical operation and chemotherapy. However, it is impossible to resect all nodules of peritoneal dissemination by surgery. Furthermore, no sensitivity of peritoneal dissemination to anticancer drugs is apparent. Therefore, development of a novel and effective therapy for peritoneal dissemination is an urgent priority.

Generally, tumor invasion and metastasis from the primary site involve multiple steps including tumor cell attachment at a new site, matrix degradation, and cell locomotion. Similar events are likely to occur in peritoneal dissemination of gastric cancer. In this process the subperitoneal extracellular matrix would be degraded by matrix metalloproteinas (MMPs). Matrix degradation by MMPs is also important in angiogenesis, especially during the formation of peritoneal dissemination nodules. Pharmacologic inhibition of MMP in a gastric cancer patient should interfere with degradation of the extracellular matrix and subperitoneal angiogenesis, thus acting against peritoneal dissemination.

Several MMP inhibitors such as R94138, Batimastat, AG3340, BAY12-9566, and COL-3 have been synthesized as anticancer drugs and some of them are undergoing clinical trials. In particular, marimastat (BB-2516) has finished a phase III clinical trial after reportedly showing anticancer activity in phase II and III trials. However, these trials were designed for the treatment of primary tumor and few reports have considered the action of marimastat against peritoneal dissemination.

In the present study, we examined the preventive effects of marimastat, which inhibits MMP-1, -2, -3, -7, -9, -12, using human gastric cancer xenograft administered intraperitoneally to nude mice and investigated the possibility that marimastat could be a new preventive agent or treatment for peritoneal dissemination.

MATERIALS AND METHODS

Cell lines TMK-1, a human gastric carcinoma cell line
used for this investigation, was kindly supplied by Dr. S. Hirohashi of the Japanese National Cancer Center Research Institute. This cell line, established as a serially transplantable human tumor xenograft in nude mice by Ochiai et al., was obtained from cancer tissue from a 21-year-old man with gastric cancer. This cell line was also established as a cultured cell line by Ochiai et al. The cells were cultured in RPMI-1640 medium (GIBCO, Gaithersburg, MO) supplemented with 10% bovine serum (JRH, Lenexa, KS) and 100 IU/ml penicillin, 100 µg/ml streptomycin, and 250 µg/ml fungizone (GIBCO). In gelatin zymography, expression of MMP-2 and -9 was not recognized in culture medium obtained from TMK-1 solo-culture, but the expression of MMP-2 was recognized in culture medium obtained from the co-culture of TMK-1 and human fibroblast cells. BALB/3T3 clone A31 was established by Aaronson and Todaro in 1968 from disassociated 14- to 17-day-old BALB/c mouse embryos. The BALB/3T3 clone A31 possesses many properties similar to those of 3T3 fibroblasts derived from random-bred Swiss-mouse embryos (ATCC CCL-92). The cells were cultured in Dulbecco’s modified Eagle’s medium (Sigma, St. Louis, MO) supplemented with 10% calf serum and 100 IU/ml penicillin, 100 µg/ml streptomycin, and 250 µg/ml fungizone.

Agents Marimastat (Fig. 1), an inhibitor of MMPs, synthesized by British Biotech (London, UK), was kindly provided by Tanabe Co., Ltd. (Osaka). Marimastat has a low molecular weight of 331.42 and a collagen-mimicking hydroxamate structure that together facilitate chelation of the zinc ion in the active sites of MMPs. Mitomycin C (MMC) was purchased from Kyowa Hakko Kogyo Co., Ltd. (Tokyo) and dissolved in saline.

Nude mice BALB/c nu/nu male nude mice purchased from CLEA, Japan, Inc. (Tokyo) were maintained free of specified pathogens using an Isorack in the experimental animal center. Mice were given sterile food and water ad libitum. Four-to-six-week-old mice weighing 20 to 22 g were used for the experiment. In this experiment, we complied with the Guideline for the Care and Use of Laboratory Animals of Keio University School of Medicine.

MTT assay The assay method of Mosmann was used, including the modifications reported previously. After centrifugation, tumor cells were suspended in RPMI-1640 containing 10% fetal calf serum (FCS) and counted by the trypan blue dye exclusion method. Cell suspensions were then diluted to 2 × 10^7 to 10^8 cells/ml. Assays were performed using 96-well microplates; 100 µl of RPMI-1640 containing 10% FCS but without cells was placed in the front row of wells as a blank. In the remaining wells of the plates, 50 µl per well of cell suspension and 50 µl per well of drug diluted in RPMI-1640 were mixed to result in 10³ to 5 × 10^5 cells per well as determined in our previous study. Final concentrations were 10 µM and 50 µM for marimastat, and 1.0 µM, 3.0 µM, and 10.0 µM for MMC. After the plates had been incubated at 37°C in a humidified atmosphere containing 95% air and 5% CO₂ for 48 h, 10 µl of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma) dissolved in 5 mg/ml phosphate-buffered saline filtered through a 0.45-µm membrane filter (Millipore, Bedford, MA) was added to each well. Following an additional 4 h of incubation, 150 µl per well of 0.04 N HCl in DMSO was added to dissolve the MTT-formazan product. After thorough mixing with a mechanical plate mixer (model 250, Sonifer, Branson, Danbury, CT), supernatants were transferred to other plates at a volume of 150 µl per well following centrifugation for 5 min at 3000 rpm. The absorbance was read by a model 2550 enzyme immunoassay (EIA) reader (Bio-Rad, Hercules, CA). The absorbance at 570 nm was determined using a 630 nm reference wavelength.

Fig. 2. Design of experiment I. TMK-1 human gastric carcinoma cells were injected intraperitoneally into 40 nude mice (5 × 10^6 cells/animal). Mice then were divided into treatment and control groups and underwent subcutaneous implantation of mini-osmotic pumps for continuous administration of marimastat in vehicle or of vehicle alone. Administration for 14 days began 7 days after tumor cell inoculation. At 21 days after tumor cell injection, the pumps were changed to continue administration for another 14 days. At 35 days after tumor cell injection, mice were killed and weighed. All peritoneal tumor nodules were collected and weighed. Doses of marimastat administered were 9, 18, 27, and 36 mg/kg/day (5 treated mice per dose-defined group; 5 untreated controls separately compared with each step; Table II).

Fig. 1. Three-dimensional structural formula of marimastat. [3R-2,2-dimethyl-13-methyl(carbamoyl)-propylcarbamoyl]-2S-hydroxy-5-methyl hexano-hydroxamic acid], which has a molecular weight of 331.42.
Richmond, CA) at 600 nm. The inhibition rate was calculated by using the formula, inhibition rate (\%) = (1 - mean absorbance of treated wells/mean absorbance of control wells) \times 100. The assay was regarded as evaluable when the mean absorbance of control wells was equal to or greater than 0.15. An inhibition rate of 50% or more was considered to indicate positive cytotoxicity.

Tumor inoculation and drug administration In experiment I, cultured TMK-1 cells (5×10^5 cells/mouse) were injected intraperitoneally into 40 nude mice (Fig. 2). Treatment was initiated 7 days later. Mice underwent subcutaneous implantation of mini-osmotic pumps (ALZET2002; ALZA, Palo Alto, CA) containing various doses of marimastat dissolved in 50% DMSO (Fig. 3), to continuously administer marimastat for 14 days. At 21 days after injection of TMK-1, pumps were changed to continue administration for another 14 days. At 35 days after tumor cell injection, mice were killed and weighed. All peritoneal tumor nodules were collected and weighed. Marimastat was administered at a dose of 18 mg/kg/day, while MMC was given as one dose of 2 mg/kg. The scheme for the control group followed that shown for the MMC group, without MMC injection.

In experiment II, TMK-1 human gastric carcinoma cells (5×10^6 cells/animal) were injected intraperitoneally into 20 nude mice four groups of 5 mice. All mice later underwent subcutaneous implantation of mini-osmotic pumps. Treatment was initiated 7 days after intraperitoneal injection of TMK-1 cells. In marimastat and combination therapy groups, pumps contained marimastat in vehicle; in control and MMC groups, pumps contained only vehicle. Pump contents were given continuously for 14 days beginning 7 days after cell injection. In MMC and combined therapy groups, MMC was injected intraperitoneally once on day 7. At 21 days after injection of TMK-1, pumps were changed to continue administration for another 14 days. At 35 days after tumor cell injection, mice were killed and weighed. All peritoneal tumor nodules were collected and weighed. Marimastat was administered at a dose of 18 mg/kg/day, while MMC was given as one dose of 2 mg/kg. The scheme for the control group followed that shown for the MMC group, without MMC injection.

Richmond, CA) at 600 nm. The inhibition rate was calculated by using the formula, inhibition rate (\%) = (1 - mean absorbance of treated wells/mean absorbance of control wells) \times 100. The assay was regarded as evaluable when the mean absorbance of control wells was equal to or greater than 0.15. An inhibition rate of 50% or more was considered to indicate positive cytotoxicity.

Tumor inoculation and drug administration In experiment I, cultured TMK-1 cells (5×10^5 cells/mouse) were injected intraperitoneally into 40 nude mice (Fig. 2). Treatment was initiated 7 days later. Mice underwent subcutaneous implantation of mini-osmotic pumps (ALZET2002; ALZA, Palo Alto, CA) containing various doses of marimastat dissolved in 50% DMSO (Fig. 3), to continuously administer marimastat for 14 days. At 21 days after injection of TMK-1, pumps were retrieved and new pumps were implanted. Thus, marimastat was administered for 28 consecutive days. A control group for each dose was given the vehicle (50% DMSO) alone in the same manner. At 35 days after injection of TMK-1, mice were killed and weighed, and all peritoneal tumor nodules were collected and weighed. Doses of marimastat administered were 9, 18, 27 and 36 mg/kg/day. Each treated and control group included 5 mice. In experiment II, TMK-1 was injected i.p. into 20 nude mice by the same method (Fig. 3). Five mice each were assigned to a control group, a marimastat group, a MMC group, or a combination therapy group. In the control group, only vehicle (50% DMSO) was administered by the subcutaneously implanted pump. Marimastat (18 mg/kg/day) was administered by pump for 28 days in marimastat group. In the MMC group, MMC (2 mg/kg) was injected i.p. at 7 days after injection of TMK-1 cells, and these animals also underwent implantation of mini-osmotic pumps containing vehicle. The
combination therapy group received MMC i.p. as above and also received marimastat via implanted pump. At 35 days after injection of TMK-1 cells, mice were killed and weighed. All peritoneal tumor nodules then were collected and weighed.

**Survival experiment** TMK-1 cells were injected into 40 nude mice by the same method as above (Fig. 4), and 10 mice each were assigned to control, marimastat, MMC, and combination therapy groups. All mice underwent subcutaneous implantation of mini-osmotic pumps 7 days after cell injection. In MMC and combination therapy groups, MMC (2 mg/kg i.p.) was injected once 7 days after injection of TMK-1 cells. In control and MMC groups the pumps contained 50% DMSO. In the marimastat and combination therapy groups, marimastat (18 mg/kg/day) was administered continuously via the pumps. Pumps were reimplanted every 2 weeks until the mice

| Table I. Cytotoxic Effect of Marimastat and MMC on TMK-1 |
|-------------------|-------------|-------------|
| Agent             | Concentration (µM) | Inhibition rate (%) |
| Marimastat        | 10          | 2.64        |
| Marimastat        | 50          | 1.10        |
| MMC               | 1.0         | 0.00        |
| MMC               | 3.0         | 43.38       |
| MMC               | 10.0        | 50.84       |

Fig. 5. Macroscopic appearance of peritoneally disseminated TMK-1 cell nodules at 5 weeks after intra-peritoneal tumor cell injection (5×10^5 cells/mouse). All nodules appeared glossy and whitish. Many peritoneal dissemination nodules were observed throughout the peritoneal cavity in control group. Some peritoneal nodules were mainly observed on the mesentery in the marimastat group. The largest nodule, 17.0 mm in diameter and 0.53 g in weight, was seen in the control group, representing confluence of many smaller nodules.

Fig. 6. Dose-dependent antitumor effect of marimastat on TMK-1 cells. Inhibition increased in a dose-dependent manner up to 26 mg/kg/day of marimastat.
died of peritoneal dissemination. The nodules of disseminated tumor were examined postmortem.

Statistical analysis Statistical analysis was performed by Mann-Whitney U test (Stat View, SAS Institute, Cary, NC). \( P < 0.05 \) was chosen as the criterion for statistical significance.

RESULTS

In the MTT assay, marimastat did not show cytotoxicity to TMK-1 cells (Table I). MMC showed cytotoxic activity in the same systems, with growth inhibition rates of 43.38\% and 50.84\% at 3.0 and 10.0 \( \mu M \), respectively (Table I).

Figs. 5, 6 and Table II show the results of experiment I. The weight of peritoneal tumor nodules in the marimastat group receiving 36 mg/kg/day was 1.68±0.49 g (mean±SD), while in the control group, nodules weighed 11.42±0.92 g. Formation of peritoneal nodules as judged from nodule weight was significantly suppressed in all treated groups (\( P < 0.05 \)), except at the dose of 9 mg/kg/day. Inhibition rates were 39.7\% for 9 mg/kg/day, 63.3\% for 18 mg/kg/day, 89.9\% for 27 mg/kg/day, and 85.3\% for 36 mg/kg/day, representing dose-dependent inhibition (Fig. 6). No severe side effect was evident in terms of body weight loss (Table II). No deformity or inflammation of knee joints was evident macroscopically after the mice were killed.

In experiment II, peritoneal tumor nodules were significantly suppressed in all treatment groups (\( P < 0.01 \)). The peritoneal tumor nodules weighed 9.37±0.92 g in the con-

| Treatment            | \( n^a \) | Body weight (g)\(^b\) | T/C\(^c\) |
|----------------------|----------|------------------------|-----------|
| Control\(^d\)        | 5        | 26.05±3.60             | 100       |
| Marimastat (9 mg/kg/day)\(^e\) | 5        | 29.47±3.60             | 113.1     |
| Control              | 5        | 33.06±4.66             | 100       |
| Marimastat (18 mg/kg/day) | 5        | 29.97±2.62             | 90.7      |
| Control              | 5        | 32.70±4.16             | 100       |
| Marimastat (27 mg/kg/day) | 5        | 28.30±3.30             | 104.8     |
| Control              | 5        | 32.60±4.85             | 100       |
| Marimastat (36 mg/kg/day) | 5        | 29.59±2.72             | 90.8      |

\( a \) Number of mice.
\( b \) Body weight of nude mouse in grams as mean±SD at 5 weeks after tumor injection.
\( c \) Treated group/control group ratio of body weights of mice (%).
\( d \) Vehicle (50% DMSO) was administered as a control.
\( e \) Marimastat was administered subcutaneously for 4 weeks using an osmotic pump, starting on day 7 after tumor cell injection.

| Table III. Antitumor Effect of Marimastat and MMC on Intraperitoneally Disseminated TMK-1 |
|-----------------------------------------------|
| Treatment                        | \( n^a \) | Tumor weight (g)\(^b\) | T/C\(^c\) | Body weight (g)\(^d\) | T/C\(^e\) |
| Control\(^f\)                     | 5        | 9.37±0.92              | 100       | 32.60±4.84             | 100       |
| Marimastat\(^g\)                  | 5        | 3.43±1.29\(^f\)       | 26.6      | 29.58±2.72             | 83.9      |
| MMC                              | 5        | 0.68±0.42\(^f\)       | 7.2       | 27.35±2.06             | 83.9      |
| Marimastat+MMC                    | 5        | 0.12±0.16\(^g\)       | 1.3       | 29.02±1.01             | 89.1      |

\( a \) Number of mice.
\( b \) Total disseminated tumor weight in grams as mean±standard deviation at 5 weeks after tumor injection.
\( c \) Treated group/control group ratio of tumor weights (%).
\( d \) Body weight of nude mouse in grams as mean±SD at 5 weeks after tumor injection.
\( e \) Treated group/control group ratio of body weights of mice (%).
\( f \) Vehicle (50% DMSO) was administered as a control.
\( g \) Marimastat was administered subcutaneously at a dose of 18 mg/kg/day for 4 weeks using an osmotic pump, starting on day 7.

\( * P < 0.01 \).
trol group (mean±SD): 3.43±1.29 g (T/C 26.6%) in the marimastat group (18 mg/kg/day), 0.68±0.42 g (T/C 7.2%) in MMC group (2 mg/kg i.p.); and 0.12±0.16 g (T/C 1.3%) in the combination therapy group (marimastat 18 mg/kg/day + MMC 2 mg/kg i.p.) (Table III).

The results of the survival experiment are shown in Fig. 7. Mice treated with marimastat alone survived longer than the control group, but the difference fell short of statistical significance. Mice treated with MMC alone and also those receiving a combination of MMC and marimastat survived significantly longer than control mice (P<0.01). One mouse in the MMC group and two mice in the combination group survived until 6 months after tumor inoculation, when they were killed. Absence of metastatic nodules was confirmed at autopsy. At the time of their deaths, other mice had peritoneal nodules constituting more than 40% of their body weight. No distant metastasis to liver was observed in any mouse investigated.

**DISCUSSION**

Peritoneal dissemination of gastric cancer has little sensitivity to anticancer drugs, and it is impossible to control its growth completely. In the present study we have demonstrated that marimastat successfully inhibited the growth of peritoneal dissemination nodules in nude mice model. Furthermore, combined administration of marimastat and a conventional anticancer drug, MMC, afforded stronger inhibition of the growth of peritoneal dissemination than marimastat alone or MMC alone. Although marimastat had inhibitory activity on the enzymatic activities of MMP-1, -2, -7, -9 and -14 (MT1-MMP), inhibition of nodule formation in the combination group was smaller than T/C in the MMC group multiplied by T/C in the combination therapy group (marimastat 18 mg/kg/day, 0.42 g (T/C 26.6%) in the combination therapy group (marimastat 18 mg/kg/day + MMC 2 mg/kg i.p.) (Table III).

In conclusion, marimastat inhibited peritoneal dissemination of human gastric cancer in nude mice when given by controlled subcutaneous administration, and this MMP inhibition showed a synergistic effect with MMC, which significantly prolonged the survival of mice. Marimastat is believed to be suitable for long-term administration without producing severe adverse effects. A clinical study of marimastat and MMC in combination appears warranted for patients with peritoneal dissemination of gastric cancer.

(Received February 19, 2002/Revised April 25, 2002/Accepted May 2, 2002)
REFERENCES

1) Liotta, L. A., Stee, P. S. and Sterler-Stevenson, W. Cancer metastasis and angiogenesis; an imbalance of positive and negative regulation. *Cell*, 64, 327–336 (1991).

2) Steward, W. P. Marimastat (BB2516): current status of development. *Cancer Chemother. Pharmacol.*, 43 (Suppl.), S56–S60 (1999).

3) Igarashi, N., Kubota, T., Otani, Y., Matsuaki, S. W., Watanabe, M., Teramoto, T., Kumai, K., Tamaki, K., Tazawa, K., Kobayashi, T. and Kitajima, M. Preventive effect of matrix metalloproteinase inhibitor, R-94338, in combination with mitomycin C or cisplatin on peritoneal dissemination of human gastric cancer cell line TMK-1 in nude mice. *Jpn. J. Cancer Res.*, 90, 116–121 (1999).

4) Prontera, C., Mariani, B., Rossi, C., Poggi, A. and Rotilio, D. Inhibition of gelatinase A (MMP-2) by batimastat and captopril reduces tumor growth and lung metastasis in mice bearing Lewis lung carcinoma. *Int. J. Cancer*, 81, 761–766 (1999).

5) Giavazzi, R., Giarofalo, A., Ferri, C., Lucchini, V., Bone, E. A., Chiar, S., Brown, P. D., Nicoletti, M. I. and Taraboletti, G. Batimastat, a synthetic inhibitor of matrix metalloproteinases, potentiates the antitumour activity of cisplatin in ovarian carcinoma xenografts. *Clin. Cancer Res.*, 4, 985–992 (1998).

6) Eccles, S. A., Box, G. M., Court, W. J., Bone, E. A., Thomas, W. and Brown, P. D. Control of lymphatic and hemogenous metastasis of a rat mammary carcinoma by matrix metalloproteinase inhibitor batimastat. *Cancer Res.*, 56, 2815–2822 (1996).

7) Price, A., Shi, Q., Morris, D., Wilcox, M. E., Brasher, P. M., Newcastel, N. B., Shalinsky, D., Zou, O., Appelt, K., Johnson, R. N., Yong, V. W., Edwards, D. and Forsyth, P. Marked inhibition of tumor growth in a malignant glioma tumor model by a novel synthetic matrix metalloproteinase inhibitor AG3340. *Clin. Cancer Res.*, 5, 845–854 (1999).

8) Rowsinsky, E. K., Humphrey, R., Hammond, L. A., Aylesworth, C., Smetzer, L., Hidalgo, M., Morrow, M., Smith, L., Garner, A., Sorensen, J. M., Van Hoff, D. D. and Eckhardt, S. G. Phase I and pharmacologic study of the specific matrix metalloproteinase inhibitor BAY 12-19566 on a protracted oral daily dosing schedule in patients with solid malignancies. *J. Clin. Oncol.*, 18, 178–186 (2000).

9) Rudek, M. A., Figg, W. D., Dyer, V., Dahut, W., Turner, M. L., Steinberg, S. M., Liewehr, D. J., Kohler, D. R., Pluda, J. M. and Reed, E. Phase I clinical trial of oral COL-3, a matrix metalloproteinase inhibitor, in patients with refractory metastatic cancer. *J. Clin. Oncol.*, 19, 584–592 (2001).

10) Cianfrocca, M., Cooley, T. P., Lee, J. Y., Rudek, M. A., Scadden, D. T., Ratner, L., Pluda, J. M., William, D. F., Brown, S. E. and Dezube, B. J. Matrix metalloproteinase inhibitor COL-3 in the treatment of AIDS-related Kaposi’s sarcoma: a phase I AIDS Malignancy Consortium study. *J. Clin. Oncol.*, 20, 153–159 (2002).

11) Rasmussen, H. S. and McCann, P. P. Matrix metalloproteinase inhibition as a novel anticancer strategy: a review with special focus on Batimastat and Marimastat. *Pharmacol. Ther.*, 75, 69–75 (1997).

12) Nemunaitis, J., Poole, C., Primrose, J. and Rosemurgy, A. Combined analysis of studies of the effects of the matrix metalloproteinase inhibitor marimastat on serum tumor markers in advanced cancer: selection of a biologically active and tolerable dose for longer-term studies. *Clin. Cancer Res.*, 4, 1101–1109 (1998).

13) Miller, A. W., Brown, P. D., Moor, J., Galloway, W. A., Cornish, A. G. and Lenehan, T. J. Results of single and repeat dose studies of the oral matrix metalloproteinase inhibitor marimastat in healthy male volunteers. *Br. J. Clin. Pharmacol.*, 45, 21–26 (1998).

14) Bramhall, S. R., Rosemurgy, A., Brown, P. D., Bowry, C. and Buckels, J. A. C. for the Marimastat Pancreatic Cancer Study Group. Marimastat as first-line therapy for patients with unresectable pancreatic cancer: a randomized trial. *J. Clin. Oncol.*, 19, 3447–3455 (2001).

15) Tokuda, Y., Nagura, H., Maruo, K., Uemura, Y., Yoshimura, S., Tamaoki, K., Kondo, Y., Ogoshi, Y. and Mitomi, T. An immunohistochemical study of human gastric carcinoma in nude mice and athymic rats with special reference to secretory component production. *Jpn. J. Cancer Clin.*, 27, 1605–1612 (1981) (with Japanese with English abstract).

16) Ochiai, A., Yasui, W. and Tahara, E. Growth-promoting effect of gastrin on human gastric carcinoma cell line TMK-1. *Jpn. J. Cancer Res. (Gann)*, 76, 1064–1071 (1985).

17) Aaronson, S. A. and Todaro, G. J. Development of 3T3-like lines from Balb-c mouse embryo cultures: transformation susceptibility to SV40. *J. Cell. Physiol.*, 72, 141–148 (1968).

18) Wojtowicz-Praga, S. M., Dickson, R. B. and Hawkins, M. J. Matrix metalloproteinase inhibitors. *Invest. New Drugs*, 15, 61–75 (1997).

19) Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods*, 65, 55–63 (1983).

20) Suto, A., Kubota, T., Shimoyama, Y., Ishibiki, K. and Abe, O. MTT assay with reference to clinical effect of chemotherapy. *J. Surg. Oncol.*, 42, 28–32 (1989).

21) Furukawa, T., Kubota, T., Suto, A., Takahara, T., Yamaguchi, H., Takeuchi, T., Kase, S., Kodaira, S., Ishibiki, K. and Kitajima, M. Clinical usefulness of chemotherapy testing using the MTT assay. *J. Surg. Oncol.*, 48, 188–193 (1991).

22) Otani, Y., Okazaki, I., Arai, M., Kameyama, K., Wada, N., Maruyama, K., Yoshino, K., Kitajima, M., Hosoda, Y. and Tsuchiya, M. Gene expression of interstitial collagenase (matrix metalloproteinase 1) in gastrointestinal tract cancers. *J. Gastroenterol.*, 29, 391–397 (1994).

23) Sakurai, Y., Otani, Y., Kameyama, K., Hosoda, Y.,
Okazaki, I., Kubota, T., Kumai, K. and Kitajima, M. Expression of interstitial collagenase (matrix metalloproteinase-1) in gastric cancers. *Jpn. J. Cancer Res.*, 88, 401–406 (1997).

24) Okada, Y., Nagase, H. and Harris, E. D., Jr. A metalloproteinase from human rheumatoid synovial fibroblasts that digests connective tissue matrix components. *J. Biol. Chem.*, 261, 14245–14255 (1986).

25) Tolnay, E., Wiethege, T., Kuhnen, C., Wulf, M., Voss, B. and Muller, K. M. Expression of type IV collagenase correlates with the expression of vascular endothelial growth factor in primary non-small cell lung cancer. *J. Cancer Res. Clin. Oncol.*, 123, 652–658 (1997).

26) Kurizaki, T., Toi, M. and Tominaga, T. Relationship between matrix metalloproteinase expression and tumor angiogenesis in human breast carcinoma. *Oncol. Rep.*, 5, 673–677 (1998).

27) Ito, T., Tanioka, M., Yoshida, H., Yoshioka, T., Nishimoto, H. and Itohara, S. Reduced angiogenesis and tumor progression in gelatinase A-deficient mice. *Cancer Res.*, 58, 1048–1051 (1998).

28) Charvat, S., Le Griel, C., Chignol, M. C., Schmitt, D. and Serres, M. Ras-transfection up-regulated HaCaT cell migration inhibition by Marimastat. *Clin. Exp. Metastasis*, 17, 677–685 (1999).

29) Peterson, M., Porter, K. E., Loftus, I. M., Thompson, M. M. and London, N. J. Marimastat inhibits neointimal thickening in a model of human arterial intimal hyperplasia. *Eur. J. Vasc. Endovasc. Surg.*, 19, 461–467 (2000).

30) Zhu, W. H., Guo, X., Villaschi, S. and Francesco, N. R. Regulation of vascular growth and regression by matrix metalloproteinases in the rat aorta model of angiogenesis. *Lab. Invest.*, 80, 545–555 (2000).

31) Lozonschi, L., Sunamura, M., Kobori, M., Egawa, S., Ding, L. and Matsuno, S. Controlling tumor angiogenesis and metastasis of C26 murine colon adenocarcinoma by a new matrix metalloproteinase inhibitor, KB-R7785, in two tumor models. *Cancer Res.*, 59, 1252–1258 (1999).

32) Taraboletti, G., Garofalo, A., Beloti, D., Drudis, T., Borsotti, P., Scanziani, E., Brown, P. D. and Giavazzi, R. Inhibition of angiogenesis and murine hemangioma growth by batimastat, a synthetic inhibitor of matrix metalloproteinases. *J. Natl. Cancer Inst.*, 87, 293–298 (1995).

33) Minagawa, A., Otani, Y., Kubota, T., Wada, N., Furukawa, T., Kumai, K., Kameyama, K., Okada, Y., Fujii, M., Yano, M., Sato, T., Ito, A. and Kitajima, A. The citrus flavonoid, nobiletin, inhibits peritoneal dissemination of human gastric carcinoma in SCID mice. *Jpn. J. Cancer Res.*, 92, 1322–1328 (2001).

34) Murakami, A., Nakamura, Y., Torikai, K., Tanaka, T., Koshiba, T., Koshimizu, K., Kuwahara, S., Takahashi, Y., Ogawa, K., Yano, M., Tokuda, H., Nishino, H., Mimaki, Y., Sashida, Y., Kitanaka, S. and Ohigashi, H. Inhibitory effect of citrus nobiletin on phorbol ester-induced skin inflammation, oxidative stress, and tumor promotion in mice. *Cancer Res.*, 60, 5059–5066 (2000).