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The Citrus Flavonoid, Nobiletin, Inhibits Peritoneal Dissemination of Human Gastric Carcinoma in SCID Mice

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The flavonoid nobiletin (5,6,7,8,3′,4′-hexamethoxyflavone), found in *Citrus depressa* Rutaceae, a popular citrus fruit in Okinawa, Japan, reportedly inhibits the production of pro-matrix metalloproteinase (proMMP)-1, 3, and 9 in rabbit synovial fibroblasts *in vitro*. In the present study, we demonstrated the inhibitory effects of nobiletin on the proliferation of the cancer cell line, TMK-1, and its production of MMPs. In the SCID mouse model, we found that nobiletin inhibited the formation of peritoneal dissemination nodules from TMK-1. The enzymatic activity of MMP-9 expressed in culture medium obtained from a co-culture of TMK-1 and mouse fibroblastic cells was inhibited by nobiletin in a concentration-dependent manner. In the SCID mouse model, total weight of dissemination nodules was significantly lower in the treated group compared with the vehicle control group (0.07 g vs. 0.78 g, *P*=0.0059). The total number of dissemination nodules was also significantly lower than in the vehicle control group (7.5 vs. 69.3/body, *P*=0.0001). These results suggest that nobiletin may be a candidate anti-metastatic drug for prevention of peritoneal dissemination of gastric cancer.

Key words: Flavonoid — Nobiletin — Gastric cancer — Peritoneal dissemination — Matrix metalloproteinase

In spite of the general decline in gastric cancer rates, this cancer remains one of the most important causes of death among Japanese people. In particular, peritoneal dissemination at the final stage of gastric cancer remains untreatable. Although several trials have attempted to control peritoneal dissemination of gastric cancer by chemotherapy and hyperthermia, no significant prolongation of survival was found. Peritoneal dissemination involves several steps, including tumor cell attachment, invasion and growth in the peritoneum. We investigated the role of matrix metalloproteinases (MMPs) in the invasion and metastasis of gastric cancer, and found a close relationship between MMPs expression and malignant potential of gastric cancer.[1,2] We postulated that an appropriate inhibitor of MMP could prevent peritoneal dissemination by controlling the initial steps, and demonstrated a preventive effect of the MMP inhibitor R-94138 in a peritoneal dissemination model using the human gastric cancer cell line TMK-1 in nude mice.[3]

The intake of citrus fruits is considered beneficial for health, and in a recent literature review of citrus flavonoids, a broad spectrum of biological activities including anti-carcinogenic and antitumor activities was discussed. It is commonly accepted that cancer formation can be prevented by the ingestion of certain foods,[4] and flavonoids in citrus fruits and juices are among the most prominent cancer-preventing agents. Epidemiological studies have indicated that flavonoid ingestion is associated with a reduced risk of certain forms of cancer.[5] Nobiletin (5,6,7,8,3′,4′-hexamethoxyflavone) is a polymethoxyflavonoid extracted from *Citrus depressa* Hayata (Rutaceae), a popular citrus fruit in Okinawa, Japan. Nobiletin is known to inhibit proliferation of human cancer cells[6] and production of MMPs[7,8] *in vitro*. Recently, nobiletin was shown to suppress PGE_2 production and COX-2 protein expression *in vitro*.[9] These observations encouraged us to examine the effects of nobiletin on peritoneal dissemination of gastric cancer *in vivo*. In the present study, we describe the inhibitory effects of nobiletin on the proliferation of the cancer cell line, TMK-1, and its production of MMPs. We also demonstrate a preventive effect of nobiletin on peritoneal dissemination of TMK-1 in the SCID mouse model.

MATERIALS AND METHODS

Drugs Nobiletin, a polymethoxyflavonoid, was isolated from the juice of *Citrus depressa* Hayata (Rutaceae) as described previously[7] (Fig. 1). We used a 128 m*M* dimeth-

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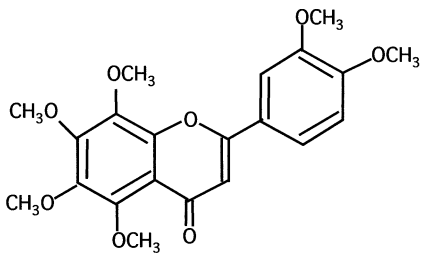

Fig. 1. Chemical structure of nobiletin.

yl sulfoxide (DMSO; Sigma Chemical Co., St. Louis, MO) solution of nobiletin for the experiments *in vitro* and 40 m*M* for those *in vivo*. The final DMSO concentration was less than 0.4% in the *in vitro* experiments.

Cell line TMK-1, a poorly differentiated human-stomach adenocarcinoma cell line, was established as a serially transplantable human tumor xenograft in nude mice by Tokuda *et al.*[10] from cancerous tissue of a 21-year-old male patient with gastric cancer, and was established as a cultured cell line by Ochiai *et al.*[11] This cell line was kindly supplied by Dr. S. Hirohashi, National Cancer Center Research Institute (Tokyo). MKN-45, a poorly differentiated human-stomach adenocarcinoma cell line, was derived from a gastric carcinoma of a 62-year-old female patient with liver metastasis by Dr. Hojo, Niigata University (Niigata), and was purchased from Dainippon Pharmaceuticals (Osaka). St-4, a poorly differentiated human-stomach adenocarcinoma cell line, was derived from a gastric carcinoma of a 45-year-old female patient at the National Cancer Center Research Institute (Tokyo) by Kubota *et al.*[12] This cell line is cultured in our laboratory.

Drug resistance assay The direct cytotoxicity of nobiletin was assessed by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay of human gastric cancer cell lines TMK-1, St-4 and MKN-45. Cell suspension (100 μl) containing 1×10^5 cells/ml was incubated with nobiletin (final concentration, 16–256 μM) in 100 μl of RPMI-1640 (Sigma Chemical Co.) in eight wells of a 96-well microtiter plate (SUMILON, Sumitomo Bakelite Co., Tokyo). Eight wells containing only cells in a drug-free medium, to which the vehicle was added, served as a control for cell survival. An additional eight wells, containing only culture medium were used to blank the spectrophotometer. Plates were then lidded and incubated for 48 h at 37°C in humidified air containing 5% CO_2. After 48 h culture, 40 μg (10 μl of a 4 mg/ml solution) of MTT (Sigma Chemical Co.) and 270 μg (10 μl of a 27 mg/ml solution) of sodium succinate (Wako Pure Chemical Industries, Osaka) were added to each well, and plates were incubated for a further 3 h at 37°C. During exposure, yellow MTT was reduced into purple formazan by viable, but not dead, cells. The formazan crystals were dissolved in 150 μl of DMSO (Nacalai Tesque, Inc., Kyoto) and the quantity of reduced product was measured by an enzyme-linked immunosorbent assay (ELISA) using an NJ-2300 microplate spectrophotometer at 540 and 630 nm (Immuno Reader, Nalge Nunc International, NY). The absorbance is linearly related to the number of viable cells. The cell survival was calculated by using the equation: (mean absorbance of drug wells/mean absorbance of control wells) $\times$ 100%. Mean absorbance of blank wells was used to adjust the absorbance of both control and tested wells. LC_{50}, the concentration of drug that was lethal to 50% of the cells, was used as a measure of *in vitro* drug cytotoxicity in each sample.

Conditioned culture media To examine the production of MMPs, TMK-1 cells and mouse fibroblast cells were co-cultured (1×10^6 cells each) for 24 h in RPMI-1640 supplemented with 10% fetal bovine serum (JRH Biosciences, Lenexa, KS) and antibiotic-antimycotic (GIBCO BRL, NY). After 24 h culture, the culture medium was changed to RPMI-1640 without the serum, and nobiletin was added to a final concentration of 16–64 μM. DMSO (0.05%) was added to the control culture medium. Cell cultures were incubated for 48 h at 37°C in a humidified atmosphere of 5% CO_2. After 48 h, the culture medium was harvested and centrifuged at 1000 rpm for 10 min, and the resulting supernatant was subjected to gelatin zymography.

Gelatin zymography The culture media were electrophoretically separated on an 11% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel impregnated with gelatin (1 mg/ml). After incubation, the gels were rinsed twice in 2.5% Triton X-100, and twice in the incubation buffer [200 m*M* NaCl, 5 m*M* $CaCl_2$, 1 μM $ZnCl_2$ and 50 m*M* Tris-HCl (pH 7.6)], then incubated overnight at 37°C in the incubation buffer. The gels were stained with 0.25% Coomassie Brilliant Blue R-250 and destained in 10% acetic acid and 5% isopropanol in H_2O. Gelatinolytic enzymes were detected as transparent bands on the background of the Coomassie Blue-stained gel. Relative enzyme activity was quantified by computer-assisted image analysis of the negatively stained bands according to the method described by Davies *et al.*[13] ProMMP-2 (M_r 72 000), activeMMP-2 (M_r 62 000), proMMP-9 (M_r 92 000) and activeMMP-9 (M_r 86 000) were identified by comparing them with known gelatinolytic activities from conditioned media of HT-1080 cells.[14]

Animals Severe combined immuno-deficient (SCID) mice were kindly supplied by the Central Institute for Experimental Animals (Kanagawa). The animals were maintained under specific pathogen-free conditions using an isorack at our experimental animal center and given sterile food and water *ad libitum*. Five-week-old male mice weighing 19–21 g were used for the experiment.

Peritoneal dissemination model In this experiment, cultured TMK-1 cells (1×10^6 cells/body) were injected intraperitoneally (i.p.) into 10 SCID mice, and the mice were randomized into control and treated groups, one week after tumor inoculation. The treated group was administered nobiletin at 21 mg/kg/day for two weeks using subcutaneously implanted mini-osmotic pumps (ALZA Co., Palo Alto, CA). The control group was administered an equivalent amount of vehicle, 50% DMSO, using the same technique. These mice were sacrificed three weeks after tumor inoculation. Total weight and total number of peritoneal dissemination nodules were measured (Fig. 2). This experiment was conducted according to the Guideline for the Care and Use of Laboratory Animals of Keio University School of Medicine.

Statistical analysis Statistical analysis was performed using Student's *t* test and $P<0.05$ was taken as the criterion of statistical significance.

RESULTS

Inhibition of cell proliferation The direct cytotoxicity of nobiletin was assessed by MTT assay of human gastric cancer cell lines, TMK-1, St-4 and MKN-45. The results are shown in Fig. 3. Nobiletin showed direct cytotoxicity on TMK-1 in a concentration-dependent manner, with an LC_{50} of 156 μM. In contrast, nobiletin had no significant direct cytotoxicity on MKN-45 and St-4, showing LC_{50} values of 494 and 655 μM, respectively.

Inhibition of MMP activity A gelatin zymogram illustrating the inhibition of proMMP-9 production by nobiletin is shown in Fig. 4. The positive control showed strong proMMP-9 (92 kDa), activeMMP-9 (86 kDa), proMMP-2 (72 kDa), and activeMMP-2 (62 kDa) activity. ProMMP-9, proMMP-2, and activeMMP-2 activities were also observed in the culture medium obtained from the co-culture of TMK-1 and BALB/cA mouse fibroblastic cells, although no activeMMP-9 was expressed in that medium. ProMMP-9 activity was completely inhibited by the addition of nobiletin, to 57% of the control activity at 16 μM nobiletin, 51% at 32 μM, and 32% at 64 μM (Fig. 4, A and B). No significant reduction was observed in proMMP-2 or activeMMP-2 after nobiletin treatment.

Inhibition of tumor nodule formation Three weeks after tumor inoculation of SCID mice, multiple peritoneal dis-

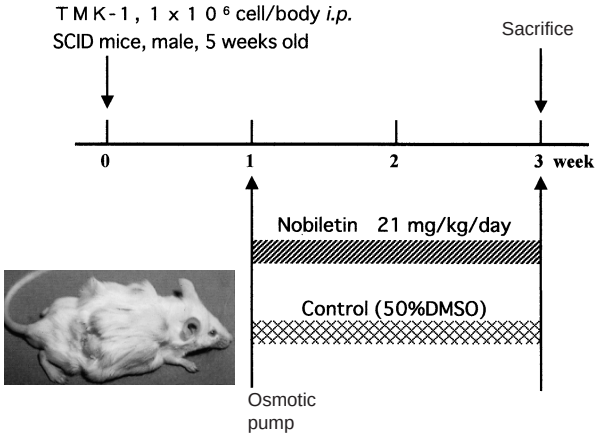

Fig. 2. Preparation of the peritoneal dissemination model and administration of nobiletin for two weeks. One week after tumor inoculation, the treated group was administered nobiletin 21 mg/kg/day for two weeks using subcutaneously implanted mini-osmotic pumps. The control group was given an equivalent amount of vehicle, 50% DMSO, using the same technique. These mice were sacrificed three weeks after tumor inoculation.

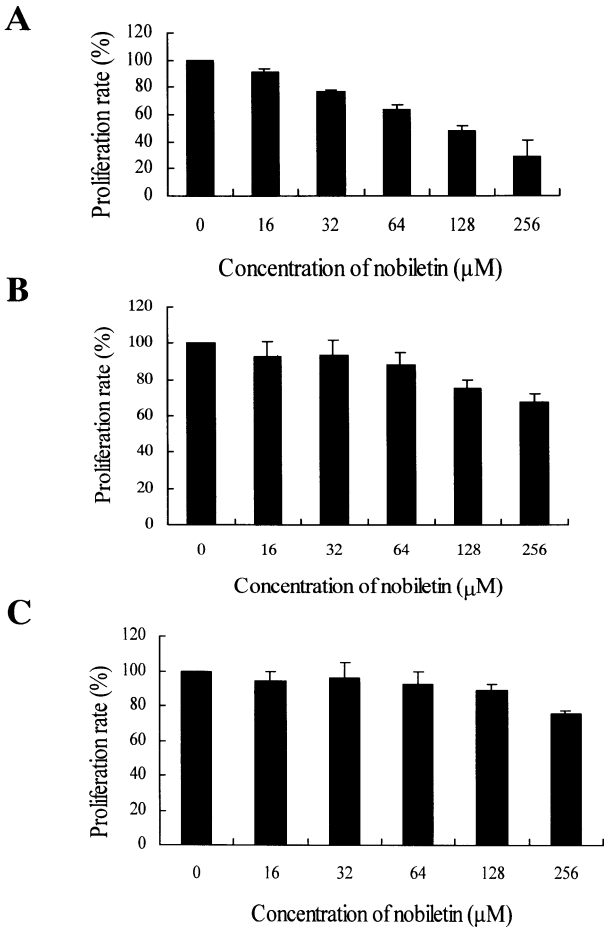

Fig. 3. The effect of nobiletin on cell proliferation. The direct cytotoxicity of nobiletin was assessed by MTT assay on human gastric cancer cell lines, TMK-1 (A), MKN-45 (B) and St-4 (C). Nobiletin showed direct cytotoxicity on TMK-1 in a concentration-dependent manner. On the other hand, nobiletin had no significant cytotoxic effect on MKN-45 and St-4. The results are the mean±SD of three experiments.

semination nodules of TMK-1 were observed in the visceral peritoneum of the mice, although no metastasis was observed in the parietal peritoneum (Fig. 5). From each control and treatment group, we excluded two mice that showed no peritoneal dissemination nodules. We determined the total weight and total number of peritoneal dissemination nodules macroscopically. The vehicle control group had 0.78±0.21 g peritoneal dissemination nodules per body (n=4). The weight of the peritoneal dissemination nodules was significantly lower (0.07±0.05 g per body) in the group treated with nobiletin (n=4, P=0.0059, Figs. 5, 6). The total number of dissemination nodules in the vehicle control group was 69.3±6.1 nodules per body, whereas in the group treated with nobiletin the total was significantly lower (7.5±9.0 nodules per body; P=0.0001, data not shown). We examined total body weight and the weights of the principal organs (liver, spleen, kidney, and stomach) in control and treated mice (Table I). Total body and liver weight differed significantly between the control and the treatment groups, but we observed no ascites, lymph nodes metastasis or liver metastasis. No significant differences were observed in other organs between the control and the treatment groups.

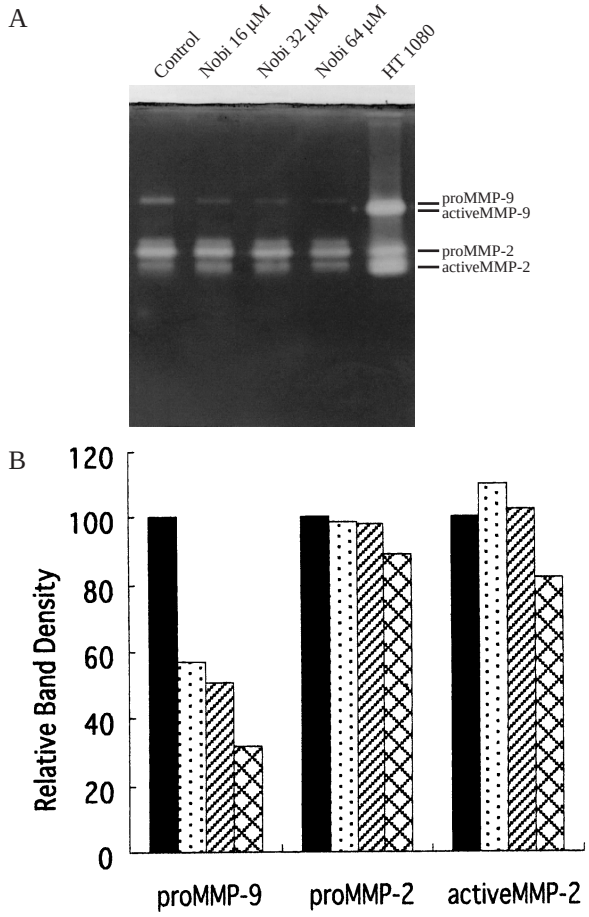

Fig. 4. Gelatin zymography. A, gelatin zymogram illustrating the inhibition of proMMP-9 by nobiletin in culture medium obtained from the co-culture of TMK-1 and BALB/cA mouse fibroblastic cells. The positive control showed strong activity of active and proforms of MMP-2 (72 kDa and 62 kDa) and MMP-9 (92 kDa and 86 kDa), which was identified by comparison with the known gelatinolytic activities of HT1080 cells. B, the gelatinolytic activities of the culture medium were expressed as relative band density. We observed a significant reduction in proMMP-9 levels compared with the control band. ■ control, ▯ Nobi 16 μM, ▨ Nobi 32 μM, ⊠ Nobi 64 μM.

DISCUSSION

The present study demonstrated for the first time that the citrus flavonoid, nobiletin, extracted from *C. depressa* Rutaceae, effectively prevented peritoneal dissemination

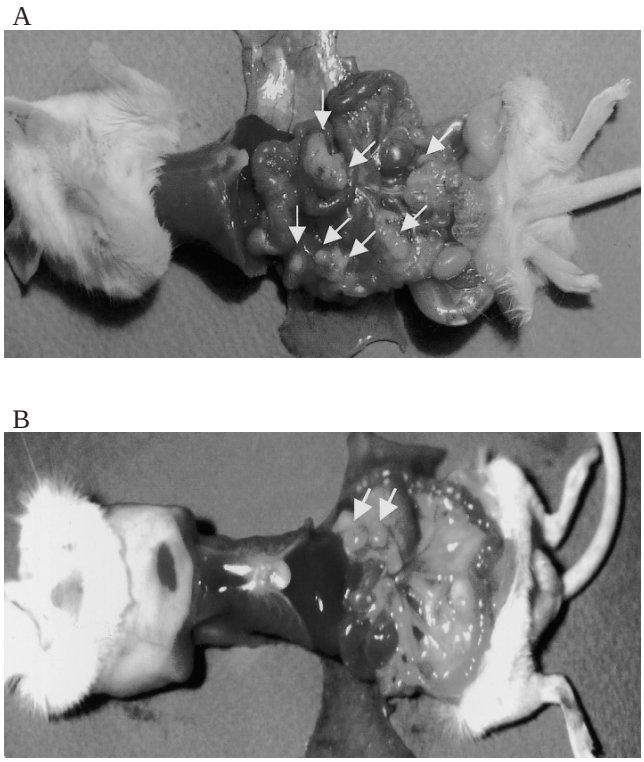

Fig. 5. Peritoneal dissemination of TMK-1. We observed multiple peritoneal dissemination nodules from TMK-1 three weeks after tumor inoculation in SCID mice. Nodules were obvious in the visceral peritoneum of mice (white arrow). We determined the total weight and total number of peritoneal dissemination nodules, macroscopically. Nobiletin significantly suppressed the formation of peritoneal dissemination nodules in the treatment group (B) compared with the control group (A).

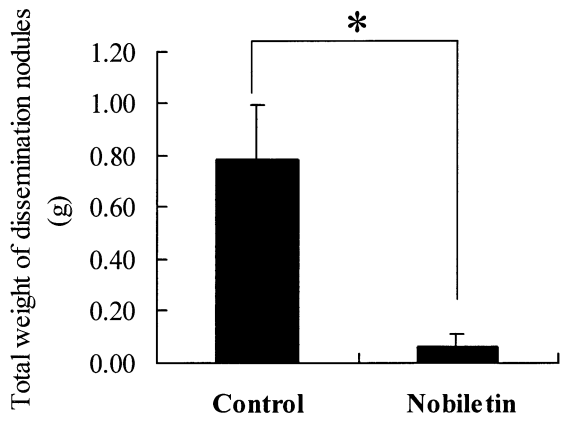

Fig. 6. Total weight of peritoneal dissemination nodules per mouse. The total weight of peritoneal dissemination nodules was significantly suppressed in the nobiletin-treated group compared with mice in the control group. Results are expressed as mean±SD (control, n=4; nobiletin, n=4). * P=0.0059.

Table I. Total Body and Principal Organ Weights of SCID Mice

	Control (n=4)	Nobiletin (n=4)	P value
Total body (g)	26.5±1.24	29.9±1.78	0.0234
Liver (g)	1.42±0.08	1.30±0.04	0.0419
Spleen (g)	0.060±0.005	0.065±0.010	0.2446
Kidney (right) (g)	0.20±0.022	0.18±0.010	0.1272
(left) (g)	0.20±0.027	0.19±0.010	0.5232
Stomach (g)	0.21±0.052	0.23±0.060	0.7005

of human gastric cancer cells in SCID mice. Recently, it was reported that citrus flavonoids exert an anti-inflammatory effect[9] and cytotoxic effects on some types of cancer cells.[6, 15] Accordingly, *C. depressa* Rutaceae has attracted attention in Japan. Although *C. depressa* Rutaceae contains six polymethoxyflavonoids, nobiletin was selected for the present study because of its effective suppression of proMMP-9 production.[7] In addition, nobiletin was previously shown to have antiproliferative effects on a human squamous cell carcinoma *in vitro*.[6] In the present study, although nobiletin inhibited the proliferation of TMK-1 (human gastric cancer cells), no significant effect was observed on MKN-45 and St-4, suggesting that nobiletin shows preferential inhibitory effects on proliferation of human gastric cancer cells. We speculate that the difference of inhibitory effect may be due to the difference of permeability of the cell membrane in each gastric cancer cell line.

Nobiletin inhibited the formation of peritoneal dissemination nodules in TMK-1 at the dose of 21 mg/kg/day, the maximum concentration of nobiletin that could be obtained in 50% DMSO solution. We observed no side effects such as body weight loss or death in either treated or control mice. In addition, in treated mice, weight loss was only observed in the liver, but not in any other organs, suggesting that administration of nobiletin has no severe adverse effects. Although we did not examine the inhibition of MMP-9 activity by nobiletin in the dissemination nodules of TMK-1 cells, the activity of MMP-9 in the culture medium obtained from the co-culture of TMK-1 and mouse fibroblast cells was inhibited by the addition of nobiletin. In addition, we confirmed the increased activity of MMP-9 in the dissemination nodules of TMK-1 cells. A previous *in vitro* study indicated that nobiletin down-regulated the production of proMMP-1, 3, 9 in both synovial cells and chondrocytes.[7] Peritoneal dissemination of gastric carcinoma is considered a complex process involving tumor cell attachment to extracellular matrix, its degradation and locomotion,[16] and we have previously revealed an important role of MMP in the progression of gastric cancer.[1] In addition, we previously demonstrated the preventive effect of a synthetic MMP inhibitor, R-94138, in a peritoneal dissemination model using TMK-1 in nude mice.[3] These results suggested that in the present study, the inhibition of dissemination nodule formation from TMK-1 by nobiletin occurs at least partially through the inhibition of MMP-9 and other MMPs.

Our previous study suggested that nobiletin inhibits MMPs transcription or transcription factors because it down-regulates proMMP-9 production, as shown by mRNA measurements.[7] AP-1, NF-κB, Sp-1, and Ets transcription factors are known to regulate the MMP-9 promoter.[17] Recently, *KiSS-1* was reported to repress MMP-9 expression by down-regulating NF-κB binding to the MMP-9 promoter because of an IκBα-induced block of NF-κB nuclear translocation.[18] On the other hand, hypothemycin, which inhibits Ras-mediated cellular signaling, was found to reduce the transcription of Ras-inducible genes, *MMP-1*, *MMP-3*, *MMP-9*, *TGF-β*, and *VEGF*.[19] Further molecular analysis of transcription factors is required for a better understanding of the inhibitory effect of nobiletin on MMPs.

Nobiletin has been shown to exert an anti-ulcer effect,[20] an anti-invasive effect[8, 21] and an anti-inflammatory effect,[9] and to inhibit cAMP-phosphodiesterase *in vitro*.[22, 23] In addition, nobiletin suppresses the production of PGE_2 and the expression of COX-2 protein.[9] Recently, COX-2 was reported to be frequently overexpressed in colorectal neoplasms.[24, 25] This protein also plays a role in colorectal tumorigenesis and tumor progression,[26–29] and modulates the production of angiogenic factors by colon cancer cells.[30] Furthermore, COX-2 expression has been reported to be up-regulated not only in colorectal neoplasms, but also in gastric, breast, esophageal, pancreatic,

and lung carcinoma.[31–35] COX-2 overexpression enhances lymphatic invasion and metastasis of human gastric carcinoma.[36] Thus, nobiletin may exhibit its antitumor activity through both MMP and COX-2 inhibition.

The present study suggests that nobiletin may be a candidate anti-metastatic drug for the prevention of peritoneal dissemination of gastric cancer and it may increase the survival of gastric cancer patients as compared with patients treated with conventional cytotoxic therapy alone.

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