

Myricetin inhibits matrix metalloproteinase 2 protein expression and enzyme activity in colorectal carcinoma cells

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Abstract

Colorectal carcinoma is a leading cause of human mortality due to its high metastatic ability. Because the activation of matrix metalloproteinases (MMP) is a key factor in the metastatic process, agents with the ability to inhibit MMP activity have potential in the treatment of colorectal carcinoma. In the present study, among 36 flavonoids examined, myricetin was found to be the most potent inhibitor of MMP-2 enzyme activity in COLO 205 cells (IC₅₀ = 7.82 μmol/L). Myricetin inhibition of MMP-2 enzyme activity was also found in the human colorectal carcinoma cell lines COLO 320HSR, COLO 320DM, HT 29, and COLO 205-X (IC₅₀ = 11.18, 11.56, 13.25, and 23.51 μmol/L, respectively). In contrast, no inhibitory effect of MMP-2 protein expression or enzyme activity was observed in myricitrin (myricetin-3-rhamnoside)-treated cells. In 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-stimulated COLO 205 cells, an increase in MMP-2 protein expression and enzyme activity, as well as of protein kinase C (PKC) α protein translocation, extracellular signal-regulated kinase (ERK) 1/2 protein phosphorylation, and c-Jun protein expression was observed. ERK inhibitor (PD98059) and PKC inhibitors (GF-109203X and H-7), but not p38 inhibitor (SB203580) or c-*jun*-NH₂-kinase inhibitor (SP600125), significantly inhibited TPA-induced MMP-2 protein expres-

sion, with reduced ERK phosphorylation and c-Jun protein expression. Addition of myricetin but not myricitrin suppressed TPA-induced MMP-2 protein expression in COLO 205 cells by blocking the TPA-induced events, including translocation of PKCα from cytosol to membrane, phosphorylation of ERK1/2 protein, and induction of c-Jun protein expression. Addition of PD98059 or GF-109203X significantly enhanced the inhibitory effect of myricetin on MMP-2 enzyme activity induced by TPA. Furthermore, myricetin, but not myricitrin, suppressed TPA-induced invasion of COLO 205 cells in an *in vitro* invasion assay using Engelbreth-Holm-Swarm sarcoma tumor extract Matrigel-coated Transwells. Results of the present study indicate that myricetin significantly blocked both endogenous and TPA-induced MMP-2 enzyme activity by inhibiting its protein expression and enzyme activity. The blockade involved suppression of PKC translocation, ERK phosphorylation, and c-Jun protein expression. [Mol Cancer Ther 2005;4(2):281–90]

Introduction

The extracellular matrix is a complex structure supporting cells in mammalian tissues, and there are several biomolecules in the extracellular matrix including collagen and casein involved in maintaining the three-dimensional structure of the body (1). During carcinogenesis, degradation of the extracellular matrix occurs in the process of metastasis of malignant tumors and is involved in the migration and invasion of malignant tumor cells (2, 3). Matrix metalloproteinases (MMP) are proteins for extracellular matrix breakdown, and activation of these protein activities has been detected in malignant tumors including colon carcinoma, lung cancer, and hepatoma (4, 5). Both MMP-2 and MMP-9 have been shown to participate in the tumor metastatic process, and the more malignant the tumors the more potent MMP-2 and MMP-9 activity is in stimulating metastasis of tumors (6, 7). Therefore, an agent capable of inhibiting the activity of MMPs, especially MMP-2 and MMP-9, may potentially be an antitumor metastatic drug.

12-*O*-tetradecanoylphorbol-13-acetate (TPA), a potent tumor promoter, induces a variety of cellular responses including differentiation, proliferation, and apoptosis. Protein kinase C (PKC) activation by TPA via translocation of PKC proteins from cytoplasm to membrane has been shown to be an essential step in TPA-induced tumor promotion (8). At least five isoforms of PKC including α, β, γ, δ, and ε have been isolated from different human tissues, and activation of PKCα is involved in the formation of colorectal carcinoma. After PKC activation, a series of downstream genes such as mitogen-activated protein kinases (MAPK),

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c-Jun, and c-Fos is stimulated. Our previous study indicated that induction of extracellular signal-regulated kinase (ERK) 1/2 phosphorylation, ornithine decarboxylase, c-Jun, and cyclooxygenase 2 proteins is involved in TPA-induced transformation in NIH3T3 cells. Zeliadt et al. (9) indicated that TPA induced *MMP-13* gene expression via an ERK-dependent pathway in mouse keratinocytes. In human cervical cancers, TPA induced MMP-9 activity via activation of PKC δ and *c-jun*-NH₂-kinase (JNK). Arnott et al. (10) suggested that TPA was able to up-regulate MMP-9 expression that was closely correlated with tumor development. However, the exact mechanism of TPA regulation of MMPs in colorectal carcinoma is still unclear.

Colorectal cancer is the third leading cause of cancer mortality in the Western world and increases strikingly. Early diagnosis and surgery to remove primary tumor have increased the survival rate of patients with colorectal cancer. Several studies indicated that metastasis is a major factor that causes death in patients with colorectal cancer (11). Therefore, investigators keep continuing to search for effective agents to inhibit the metastasis of colorectal cancer.

Flavonoids are benzo- γ -pyrone derivatives with different numbers of hydroxyl substitutions in the structures. Beneficial effects of flavonoids include antioxidant, antitumor, and anti-inflammation effects (12, 13). Results of structure-activity relationship studies have indicated that OH substitutions may affect the biological functions of flavonoids, and increasing OH substitution seems to enhance the antioxidant activity of flavonoids. Our recent studies also showed that flavonoids such as quercetin and wogonin induced apoptosis of tumor cells, and addition of glycoside attenuated the apoptosis-inducing activity of flavonoids (14, 15). In addition, flavones with one or no OH substitution significantly inhibit epidermal growth factor-induced proliferation in A431 (16). These data suggest that chemical structure, especially OH substitution, plays important roles in the biological activities of flavonoids. However, the structure-activity relationship of flavonoids on inhibiting MMPs activity is still undelineated. In the present study, 36 structure-related flavonoids are used to investigate the inhibitory activities on MMP activity in colorectal carcinoma cells. The results suggest that myricetin is the most potent inhibitor on MMP-2 activity and TPA-induced cellular responses in COLO 205 cells. The inhibitory mechanism by myricetin via inhibiting MMP-2 gene expression and enzyme activity, and structure-activity relationship of flavonoids on MMP-2 inhibition were elucidated.

Materials and Methods

Cell Culture

The human colorectal carcinoma cells, including COLO 205, COLO 320HSR, COLO 320 DM, and HT 29 cells, were obtained from American Type Culture Collection (Rockville, MD). COLO 205-X cells were primary cultured from COLO 205-xenografted nude mice. These cells were grown

in RPMI 1640 containing 10% heat-inactivated fetal bovine serum and maintained at 37°C in a humidified incubator containing 5% CO₂. All culture reagents were purchased from Life Technologies, Inc. (Gaithersburg, MD).

Chemicals

Flavone, 3-OH flavone, 5-OH flavone, 6-OH flavone, 7-OH flavone, 2'-CH₃O flavone, 3-CH₃O flavone, 5-CH₃O flavone, 6-CH₃O flavone, flavanone, 2'-OH flavanone, 4'-OH flavanone, 6-OH flavanone, 7-OH flavanone, 4'-CH₃O flavanone, 5-CH₃O flavanone, 6-CH₃O flavanone, 7-CH₃O flavanone, kaempferol, quercetin, quercitrin, myricetin, myricitrin, baicalein, baicalin, morin, wogonin, fisetin, luteolin, rutin, taxifoin, naringenin, naringin, hesperidin, hesperetin, (\pm)catechin, (+)catechin, (-)catechin, and H-7 (isoquinoline-5-sulfonic 2-methyl-1-piperazide) were purchased from Sigma Chemical Co. (St. Louis, MO). GF-109203X was purchased from Calbiochem Co. (La Jolla, CA). Gelatin from porcine skin and MMP-2 proteins from human fibroblast for direct enzyme inhibition assay were purchased from Sigma. Antibodies for MMP-1, MMP-2, MMP-3, and MMP-9 protein detection in Western blotting were obtained from Oncogene Research Products (Cambridge, MA). EHS-Matrigel for *in vitro* invasion assay was purchased from BD Biosciences (Bedford, MA).

Cell Viability

Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) staining as described previously (15). Briefly, COLO 205 cells were plated at a density of 10⁵ cells per well into 24-well plates. After overnight growth, cells were treated with a different concentration of flavonoids described above for 12 hours. At the end of treatment, 30 μ L tetrazolium compound MTT and 270 μ L fresh RPMI medium were added. The supernatant was removed and formazon crystals were dissolved in DMSO. After incubation for 4 hours at 37°C, 200 μ L of 0.1 N HCl in 2-propanol was replaced per well to dissolve the tetrazolium crystals. At the end, absorbance at wavelength 600 nm was recorded using an ELISA plate reader.

Western Blots

Total cellular extracts (30 μ g) were prepared, separated on 8% SDS-polyacrylamide minigels for poly(ADP-ribose) polymerase detection and on 12% SDS-polyacrylamide minigels for MMP-1, MMP-2, MMP-3, and MMP-9 detection, and transferred to Immobilon polyvinylidenedifluoride membranes (Millipore, Bedford, MA). The membrane was incubated overnight at 4°C with 1% bovine serum albumin and at room temperature for 1 hour and then incubated with indicated antibodies for a further 3 hours at room temperature followed by incubation with alkaline phosphatase-conjugated anti-mouse IgG antibody for 1 hour. Protein was visualized by incubating with the colorimetric substrate nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate as described in our previous article (14).

Gelatin Zymography in Gel

COLO 205 cells at logarithmic growth phase were treated with 100 or 200 μ mol/L of myricetin and myricitrin in serum-free RPMI medium for 24 hours.

The conditioned media were collected and analyzed for MMPs using gelatin zymography (17). In brief, samples were mixed with SDS sample buffer without heating or reduction and applied to 10% polyacrylamide gels copolymerized with 0.1% gelatin (from porcine skin). The loading volume of each conditioned medium sample was normalized according to the cell number. After electrophoresis, gels were washed for 1 hour at room temperature in buffer containing 2.5% (v/v) Triton X-100 in 50 mmol/L Tris-Cl (pH 7.4) to remove SDS. Gels were then incubated at 37°C in reaction buffer (50 mmol/L Tris-Cl, pH 7.4, with 5 mmol/L CaCl₂, 1 μmol/L ZnCl₂, and 0.02% NaN₃) for 18 hours. Then the gels were stained with Coomassie brilliant blue R-250 (0.25%) in 10% acetic acid/20% ethanol and destained in the same solution without dye. The zones of gelatinolytic activity were detected as clear bands against a blue background and were quantified using a densitometer.

Direct MMP-2 Enzyme Activity Assay

MMP-2 (400 ng) from human fibroblasts were incubated with 0, 25, 50, 100, and 200 μmol/L of myricetin in 50 μL Tris buffer (50 mmol/L, pH 7.4) at 37°C for 30 minutes, respectively. Twenty microliters of incubated buffer were then analyzed for gelatinolytic activity as described above. The zones of gelatinolytic activity were detected as clear bands against a blue background and were quantified using a densitometer.

In vitro Invasion Assay

In vitro invasion assay was carried out by the method of Huang et al. (18). Briefly, 24-well Transwell units with 8 μmol/L porosity polycarbonate filters (Becton Dickinson, Franklin Lakes, NJ) were coated with 0.1 mL of 0.8 mg/mL Englebreth-Holm-Swarm sarcoma tumor extract (EHS Matrigel) at room temperature for 1 hour to form a genuine reconstituted basement membrane. COLO 205 cells (2 × 10⁵ cells per 0.4 mL RPMI) were placed in the upper compartment, and TPA (25 ng per 0.4 mL RPMI) was added in the lower compartment. The indicated Transwell plates were then incubated at 37°C for 48 hours in a humidified atmosphere with 5% CO₂. The cells migrated on the lower surface were quantified by MTT assay and observed under light microscope by Giemsa staining. Each treatment was assayed in triplicate, and two independent experiments were done.

Statistics

Values are expressed as the mean ± SE. The significance of the difference from the respective controls for each experimental test condition was assayed by using Student's *t* test for each paired experiment. A *P* value < 0.01 or < 0.05 was regarded as indicating a significant difference.

Results

Myricetin Suppresses MMP-2 Enzyme Activity in Human Colorectal Carcinoma Cell Line COLO 205 among 36 Flavonoids

Thirty-six structure-related flavonoids including flavones, flavanones, and catechins were used to investigate the inhibitory effect on MMP-2 enzyme activity in human colorectal carcinoma cells COLO 205. These compounds

possess a basic benzo-γ-pyrone in structure with different substitutions such as OH, OCH₃, and glycosides at different locations. Among them, flavones contain a double bond at C2-C3 of flavanones, and catechin possesses five OH substitutions at C3', C4', C3, C5, and C7 without an oxo group at C4 of flavanone. To investigate the inhibitory effect of tested compounds on MMP activity, release of MMPs in the medium was done by gelatin zymography. In COLO 205 cells, a detectable endogenous MMP-2, but not MMP-9, enzyme activity in the medium was examined in the absence of tested compounds. As elucidated in Table 1, myricetin at 200 μmol/L exhibited 90% inhibition of activity of MMP-2 enzyme released from COLO 205 cells. Kaempferol (200 μmol/L) also showed 47% inhibition of released MMP-2 enzyme activity in the medium. Other flavonoids examined in the present study exhibited <20% inhibition of released MMP-2 enzyme activity.

Results of MTT assay showed that flavone, 3-OH flavone, 2'-OCH₃ flavone, 2'-OH flavanone, 4'-OH flavanone, 6-OH flavanone, quercetin, wogonin, luteolin, and baicalein, at 200 μmol/L, showed significant cytotoxicity (>50%) in COLO 205 cells. Myricetin and kaempferol at 200 μmol/L exhibited 20% and 30% reduction in the viability of COLO 205 cells, respectively. Morphologic observations and DNA integrity assay showed that myricetin did not change the morphology of COLO 205 cells and no DNA laddering effect was detected in myricetin-treated COLO 205 cells (data not shown). These data suggest that myricetin is the most effective inhibitor among 36 flavonoids examined on the activity of MMP-2 released from COLO 205 cells.

Myricetin Concentration-Dependently Inhibits MMP-2 Enzyme Activity in Colorectal Carcinoma Cell Lines COLO 205, COLO 320HSR, COLO 320DM, HT-29, and COLO 205-X

Previous data suggested that myricetin exhibited MMP-2 inhibitory effect without severe cytotoxicity in COLO 205 cells. Therefore, several colorectal carcinoma cells including COLO 320HSR, COLO 320DM, HT-29, and COLO 205-X cells were used to investigate the MMP-2 inhibition by myricetin. Figure 1A shows the structures of myricetin and its related glycosylated compound, myricitrin. Myricitrin contains a rhamnose at C3 of myricetin. Myricetin, but not myricitrin, dose-dependently inhibits MMP-2 enzyme activity released from COLO 205, COLO 320HSR, COLO 320DM, HT-29, and COLO 205-X cells (Fig. 1B). The IC₅₀ values of myricetin on MMP-2 activity in COLO 205, COLO 320HSR, COLO 320DM, HT-29, and COLO 205-X cells are 7.82, 11.18, 11.56, 13.25, and 23.51 μmol/L, respectively. MTT assay showed that myricetin only at 200 μmol/L exhibited slight but significant reduction on the viability of COLO 205 cells. These data suggest that myricetin inhibition of MMP-2 enzyme activity was not due to its cytotoxic effect on cells.

Myricetin Inhibits MMP-2 Released from COLO 205 by Decreasing MMP-2 Protein Expression and Inhibiting MMP-2 Enzyme Activity

Two possibilities might be involved in myricetin inhibition of activity of MMP-2 enzyme released from

Table 1. MMP-2 inhibitory effects of flavonoids with their cytotoxicity in human colorectal COLO 205 cells

Compound	Cytotoxicity (%) [*]	MMP-2 inhibition (%) [†]
Flavone	71 ± 0.23	—
3-OH flavone	47 ± 0.1	—
5-OH flavone	23 ± 0.11	—
6-OH flavone	38 ± 0.12	—
7-OH flavone	31 ± 0.09	—
2'-CH ₃ O flavone	47 ± 0.06	—
3-CH ₃ O flavone	33 ± 0.001	11
5-CH ₃ O flavone	30 ± 0.012	—
6-CH ₃ O flavone	25 ± 0.12	—
Kaempferol	35 ± 0.13	47
Quercetin	59 ± 0.12	—
Quercitrin	9 ± 0.18	—
Myricetin	23 ± 0.11	90
Myricitrin	9 ± 0.08	—
Baicalin	47 ± 0.12	—
Baicalin	20 ± 0.15	—
Morin	16 ± 0.1	—
Wogonin	71 ± 0.1	—
Luteolin	46 ± 0.11	13
Rutin	13 ± 0.11	—
Flavanone	24 ± 0.11	20
2'-OH flavanone	69 ± 0.13	—
4'-OH flavanone	54 ± 0.09	—
6-OH flavanone	55 ± 0.14	11
7-OH flavanone	21 ± 0.13	—
4'-CH ₃ O flavanone	27 ± 0.12	10
5-CH ₃ O flavanone	25 ± 0.11	15
6-CH ₃ O flavanone	23 ± 0.16	11
7-CH ₃ O flavanone	18 ± 0.24	12
Taxifoin	19 ± 0.21	—
Naringenin	21 ± 0.17	—
Naringin	20 ± 0.13	—
Hesperidin	23 ± 0.07	—
Hesperetin	21 ± 0.01	—
(±)Catechin	15 ± 0.1	—
(+)Catechin	17 ± 0.11	11
(-)Catechin	45 ± 0.07	—

^{*}Human colorectal COLO 205 cells were treated with indicated compounds (200 μmol/L) for 24 hours and the cell viability was analyzed by MTT assay. The cytotoxicity was measured by the equation [(absorbance of control group) - (absorbance of compound-treated group)]/(absorbance of control group) × 100%.

[†]Human colorectal COLO 205 cells were treated with indicated compounds (200 μmol/L) for 24 hours. The conditioned media were collected and normalized by cell numbers before gelatin zymography analysis. The density of each band was quantified by densitometry analysis. The inhibition percentage was measured by the equation [(density of control group) - (density of compound-treated group)]/(density of control group) × 100%.

COLO 205 cells: one is to block MMP-2 protein expression and the other is to directly inhibit MMP-2 enzyme. Results of Western blotting indicated that myricetin inhibited endogenous MMP-2 protein expression in COLO 205 cells without affecting that of MMP-1, MMP-3, and MMP-9 proteins. In contrast, myricitrin did not affect any MMP protein expression in COLO 205 cells (Fig. 2A). Further-

more, whether myricetin or myricitrin might directly affect MMP-2 enzyme was examined by direct incubation of different concentrations of myricetin or myricitrin with the medium taken from that culturing COLO 205 at 37°C for 30 minutes, and MMP-2 activity was examined by gelatin zymography. It indicated that myricetin, but not myricitrin, directly inhibited MMP-2 enzyme activity *in vitro* (Fig. 2B). These data suggested myricetin inhibition of activity of MMP-2 enzyme released from COLO 205 cells might be through both decreasing MMP-2 protein expression and/or suppressing MMP-2 enzyme activity.

Myricetin Directly Inhibited MMP-2 Activity Using Purified MMP-2 Protein

To elucidate if myricetin directly blocked MMP-2 activity, a purified MMP-2 protein isolated from human fibroblasts was incubated with or without indicated doses (25, 50, 100, 200 μmol/L) of myricetin or myricitrin at 37°C for 30 minutes, and MMP-2 enzyme activity was analyzed by gelatin zymography. Results shown in Fig. 2C and D indicated that purified MMP-2 protein was able to digest gelatin at three clear zones, and myricetin, but not myricitrin, concentration-dependently inhibited MMP-2 enzyme activity, as indicated by a reduction in gelatin digestion in the gel. Results from Western blot analysis using a specific MMP-2 antibody indicated that neither myricetin nor myricitrin caused a decrease in MMP-2 protein level. These data provide direct evidence suggesting that myricetin directly inhibits MMP-2 activity.

TPA Induction of MMP-2 Activity in COLO 205 Cells through PKC-α Translocation, ERK Phosphorylation, and c-Jun Expression

We further investigated if myricetin blocked TPA-induced MMP-2 enzyme activity. TPA is a potent tumor promoter in carcinogenesis. As shown in Fig. 3A, TPA time-dependently induced both MMP-2 protein expression and enzyme activity released from COLO 205 cells by Western blotting and gelatin zymography. An increase in c-Jun, but not MMP-1, MMP-3, and MMP-9, protein was observed in a time-dependent manner under TPA treatment. Furthermore, PKCα translocation from cytosol to membrane and activation of ERK1/2, but not p38 and JNKs, were detected in TPA-treated COLO 205 cells by Western blotting. These data revealed events including induction of MMP-2 protein expression and enzyme activity and increases in phosphorylated ERK1/2 protein and c-Jun protein expression with translocation of PKCα from cytosol to membrane in TPA-treated COLO 205 cells.

Phosphorylation of ERK1/2 Proteins Located Downstream of PKCα Translocation in TPA-Induced MMP-2 Activity

To elucidate if phosphorylation of ERK1/2, translocation of PKCα from cytosol to membrane, and induction of c-Jun protein are essential events in TPA-induced MMP-2 enzyme activity, pharmacologic studies using specific inhibitors including PD98059 (mitogen-activated protein/ERK kinase

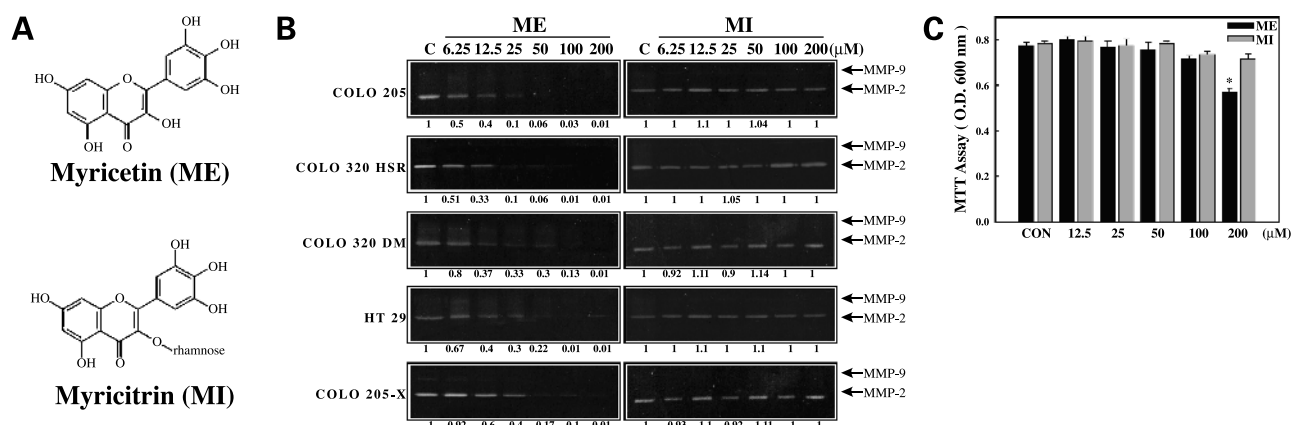


Figure 1. Differential inhibitory effect of myricetin (*ME*) and its glycoside myricitrin (*MI*) on MMP-2 enzyme activity in human colorectal carcinoma cells. **A**, structures of myricetin and myricitrin. The latter contains a rhamnose at C3 of myricetin. **B**, myricetin but not myricitrin inhibits activity of MMP-2 enzyme released from indicated colorectal carcinoma cells. Cells were treated with different concentrations (6.25, 12.5, 25, 50, 100, and 200 $\mu\text{mol/L}$) of myricetin or myricitrin for 24 h, and 20 μL of medium were applied for gelatin zymography. **C**, cytotoxicity of myricetin and myricitrin in COLO 205 cells was evaluated by MTT assay as described in Materials and Methods. Columns, mean from three independent experiments; bars, SE. *, $P < 0.05$, significantly different from control group as analyzed by Student's t test. The intensity of each band was quantitated by densitometry analysis and expressed as folds of control. Gelatin zymography was done at least thrice, and the results are representative of all of the data. CON, control; O.D., optical density (absorbance).

inhibitor), SB203580 (p38 inhibitor), SP600120 (JNK inhibitor), H-7 (PKA and PKC inhibitor), and GF-109203X (specific PKC inhibitor) were done. The results indicated that TPA treatment induced significant morphologic changes in COLO 205 cells by microscopic observation with (Fig. 4, bottom) or without (Fig. 4, top) Giemsa staining. Addition of PD98059, H-7, and GF-109203X, but not SB203580 and SP600120, showed a significant inhibition of TPA-induced morphologic changes in COLO 205 cells (Fig. 4A). In addition, PD98059, GF-109203X, and H 7, but not SB203580 or SP600120, inhibit TPA-induced MMP-2 protein expression with a decrease in activity of MMP-2 enzyme released from COLO 205 cells (Fig. 4B). Moreover, PD98059, GF-109203X, and H 7 significantly blocked TPA-induced ERK1/2 protein phosphorylation and c-Jun protein expression (Fig. 4C). These data suggest that activation of ERK1/2 located downstream of PKC activation was followed by inducing c-Jun protein expression, and these events occurred upstream of MMP-2 activation induced by TPA.

Myricetin Suppression of TPA-Induced MMP-2 Activity Is Accompanied by Inhibition of PKC α Translocation, ERK1/2 Protein Phosphorylation, and c-Jun Protein Expression in COLO 205 Cells

The possibility that myricetin inhibited TPA-induced MMP-2 activity was further examined. Results shown in Fig. 5A indicated that myricetin significantly inhibited TPA-induced activity of MMP-2 enzyme released from COLO 205 cells. In addition, TPA-induced PKC α translocation, ERK1/2 protein phosphorylation, and c-Jun and MMP-2 protein expression were significantly blocked by myricetin, but not by myricitrin (Fig. 5B). Addition of low concentrations of PD98059 (1, 2, and 4 $\mu\text{mol/L}$) or GF-109203X (0.5, 1, and 2 $\mu\text{mol/L}$) enhanced the MMP-

2 inhibition of myricetin (5 $\mu\text{mol/L}$) under TPA treatment (Fig. 5C). These data provided additional evidence that myricetin inhibited TPA-induced MMP-2 activity via blocking intracellular signaling transduction.

Anti-invasive Effect of Myricetin in COLO 205 Cells

We further investigated if myricetin blocked TPA-induced invasion in COLO 205 cells. *In vitro* invasion assay using a Transwell chamber coated with a reconstituted basement membrane (EHS Matrigel) was done to examine the anti-invasive effect of myricetin. In this assay system, COLO 205 cells were cultured on the upper chamber in the presence or absence of myricetin or myricitrin (100 and 200 $\mu\text{mol/L}$), and TPA (100 ng/mL) was added to the lower chamber. After 24 hours incubation, COLO 205 cells in the lower chamber were observed microscopically with (Fig. 6A, bottom) or without (Fig. 6A, top) Giemsa staining, and the number of viable cells in the lower chamber was quantified by MTT assay (Fig. 6B). Results shown in Fig. 6A indicated that COLO 205 cells did not penetrate the EHS-coated filter in the absence of TPA in the lower chamber (control group). Addition of TPA induced invasion of COLO 205 cells from the upper to the lower chambers in the absence of myricetin or myricitrin. Addition of myricetin, but not myricitrin, significantly reduced TPA-induced invasion in COLO 205 cells. Results of quantification of cells in the lower chambers showed that myricetin but not myricitrin inhibited TPA-induced invasion in COLO 205 cells (Fig. 6B).

Discussion

Results of the present study show that myricetin significantly inhibits MMP-2 activity and tumor invasion in colorectal carcinoma cells. Myricetin inhibition of MMP-2

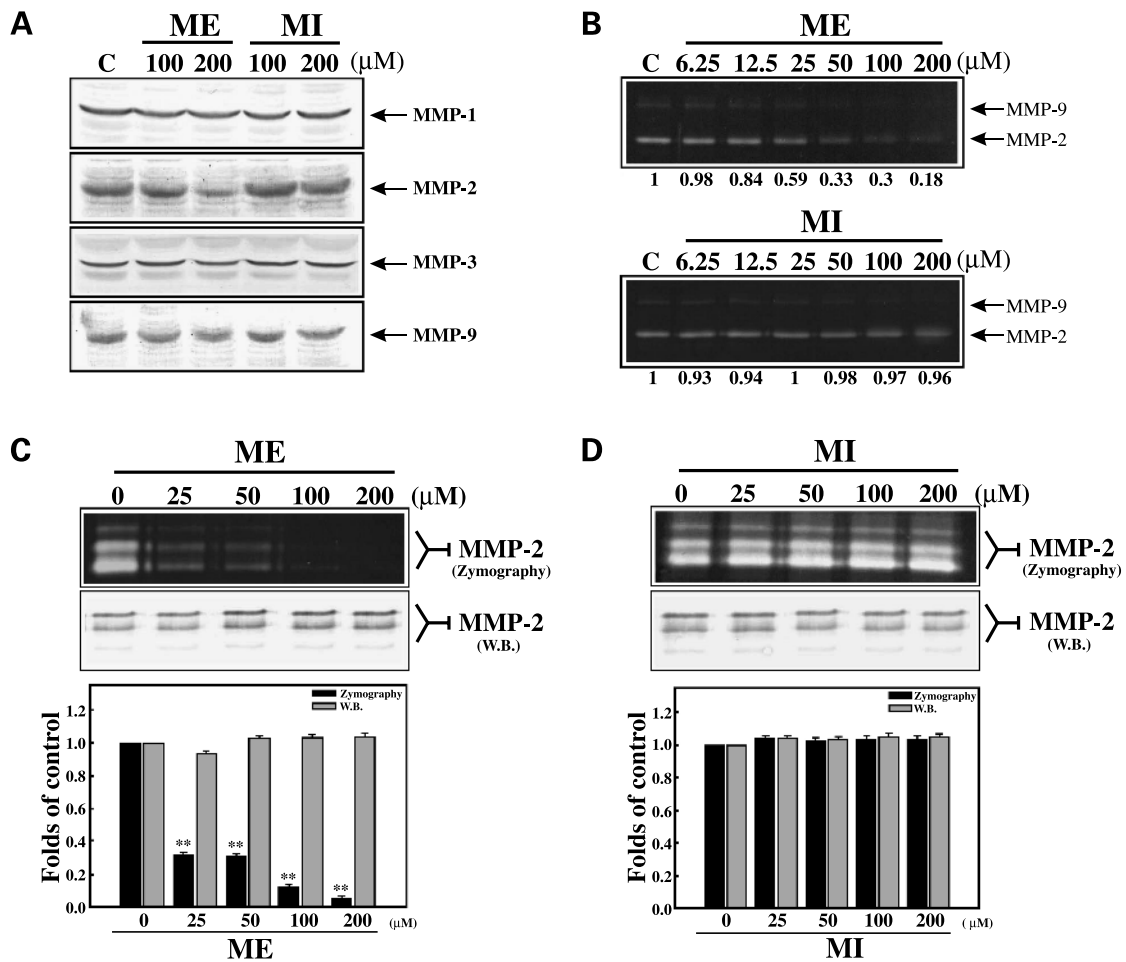


Figure 2. Myricetin but not myricitrin inhibits both MMP-2 protein expression and enzyme activity. **A**, myricetin inhibits MMP-2 protein expression in COLO 205 cells. Cells were treated with myricetin or myricitrin (100 or 200 $\mu\text{mol/L}$) for 24 h, and expression of MMP-1, MMP-2, MMP-3, and MMP-9 protein in cells was detected by Western blotting using specific antibodies. **B**, myricetin inhibits MMP-2 enzyme activity in the medium. The conditioned medium after 24 h incubation of COLO 205 cells was incubated with different concentrations (6.25, 12.5, 25, 50, 100, and 200 $\mu\text{mol/L}$) of myricetin or myricitrin at 37°C for 30 min. Activity of MMP-2 enzyme in each sample was analyzed by gelatin zymography. **C** and **D**, myricetin inhibited MMP-2 enzyme activity using purified MMP-2 protein as a substrate. Purified MMP-2 protein (500 ng) from human fibroblast was incubated with different concentrations (25, 50, 100, and 200 $\mu\text{mol/L}$) of myricetin (**C**) or myricitrin (**D**) at 37°C for 30 min. Activity of MMP-2 enzyme in each sample was analyzed by gelatin zymography (*top*). The amount of MMP-2 protein in each sample was detected by Western blotting (*W.B.*) using a specific anti-MMP-2 antibody (*middle*). Quantification of MMP-2 enzyme activity under different treatments was done by densitometry analysis and expressed as a fold of control (*bottom*). *Columns*, mean from three independent experiments; *bars*, SE. **, $P < 0.01$, significantly different from control group as analyzed by Student's t test. Gelatin zymography and Western blotting were done at least thrice, and the results are representative of all of the data.

activity was via a decrease in MMP-2 protein expression and a direct inhibition of MMP-2 enzyme activity. In the presence of TPA, myricetin suppressed PKC activation, ERK1/2 protein phosphorylation, and c-Jun protein expression together with a reduction in MMP-2 protein expression and enzyme activity. Inhibition of TPA-induced tumor invasion by myricetin was shown by *in vitro* invasion assay in the present study. These results suggest that myricetin, a compound capable of blocking both MMP-2 protein expression and enzyme activity, is a potentially effective antitumor agent.

Flavonoids are known to possess several biological functions including antitumor, antioxidant, and anti-inflammation activities. Structure-activity analysis has

shown that the number and location of OH substitutions are essential for the biological effects of flavonoids (19, 20). Results of the present study indicated that myricetin significantly inhibited MMP-2 activity. However, quercetin, which is a potent antioxidant and an apoptotic inducer, did not suppress MMP-2 activity. In contrast to quercetin, myricetin contains an additional OH substitution at C4' of the quercetin structure. In addition, an OH group at C4' is also found in kaempferol and luteolin, and both showed about 50% and 13% inhibition of MMP-2 enzyme activity, respectively. These results suggest that OH at C4' of flavone is important for MMP-2 inhibition.

In addition, glycoside has been shown to affect the biological functions of flavonoids via increasing their

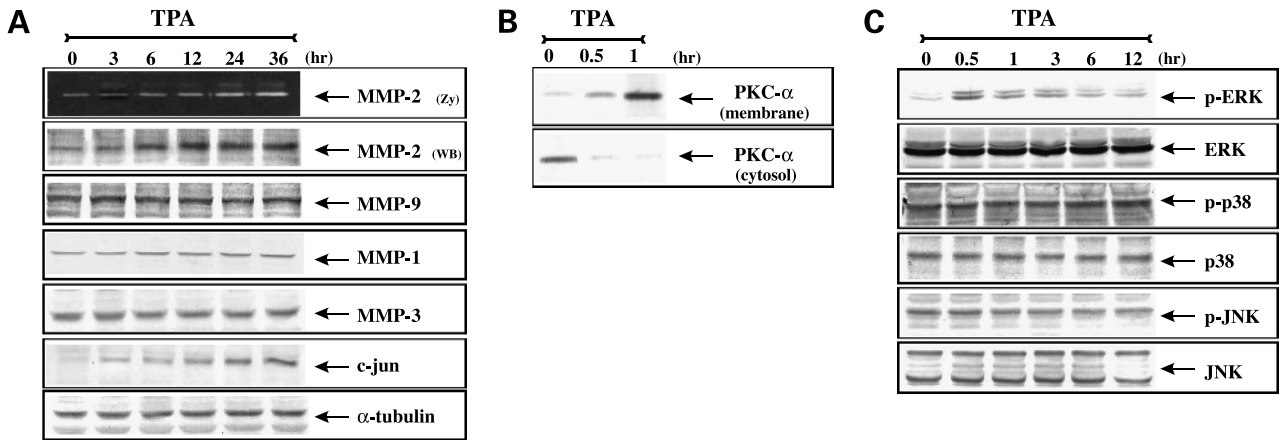


Figure 3. TPA time-dependently induced MMP-2 protein and enzyme activity, *c-jun* protein, ERK1/2 protein phosphorylation, and PKC α translocation in COLO 205 cells. **A**, COLO 205 cells were treated with TPA (50 ng/mL) for indicated time points, and MMP-2 activity in the medium and expression of indicated proteins including MMP-1, MMP-2, MMP-3, and MMP-9 proteins were analyzed by gelatin zymography and Western blotting, respectively. **B**, TPA induced PKC α translocation from cytosol to membrane. Cells were treated with TPA for 30 min, and the fractions of membrane and cytosol were collected as described in Materials and Methods. The expression of PKC α protein was detected by Western blotting. **C**, TPA induced ERK1/2 but not p38 and JNK protein phosphorylation in COLO 205 cells. Cells were treated with TPA (50 ng/mL) for different times, and expression of indicated proteins was detected by Western blotting using specific antibodies. *ERK*, *p38*, and *JNK* indicate the total amount of indicated proteins in cells, and *p-ERK*, *p-p38*, and *p-JNK* represent the respective phosphorylated proteins. Western blotting was done at least thrice, and the results are representative of all of the data.

hydrophilic properties. Our previous studies showed that rutinoides and rhamnoglucoside attenuated apoptosis-inducing activity and nitric oxide synthesis inhibition of flavonoids (14, 15, 21). In the present study, all the tested glycosylated compounds including quercitrin, myricitrin,

baicalin, rutin, naringin, and hesperedin did not inhibit activity of MMP-2 enzyme released from COLO 205 cells. A significant difference was found in myricetin and its glycoside myricitrin. Myricitrin, which contains a rhamnoside at C3 of myricetin, did not inhibit endogenous and

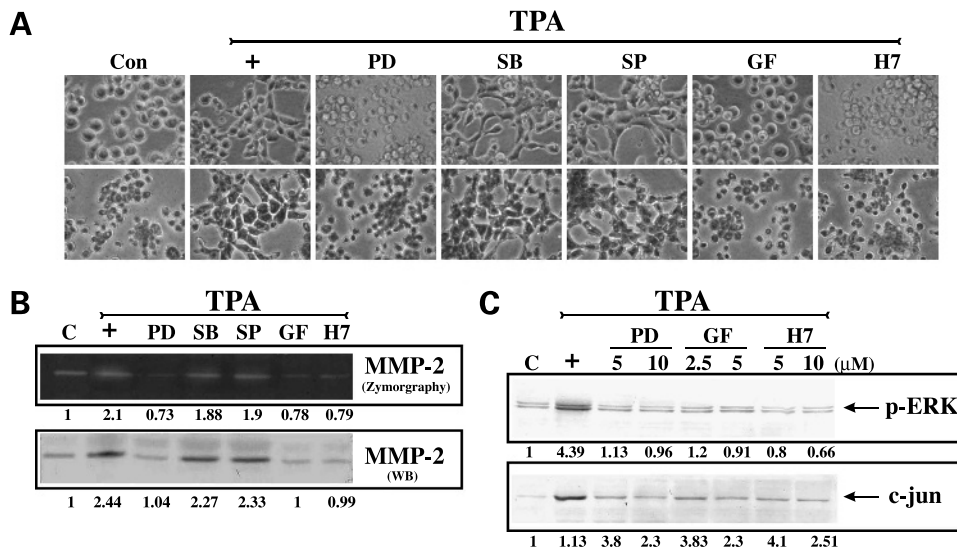


Figure 4. Induction of PKC α translocation, ERK1/2 protein phosphorylation, and *c-Jun* protein expression involved in TPA-induced transformation and MMP-2 enzyme activity. **A**, activation of PKC α and ERK1/2 protein participated in TPA-induced transformation in COLO 205 cells. Cells were treated with 10 μ mol/L PD98059 (*PD*), 10 μ mol/L SB203580 (*SB*), 10 μ mol/L SP600125 (*SP*), 10 μ mol/L GF-109203X (*GF*), or 10 μ mol/L H 7 for 30 min followed by incubation with TPA (50 ng/mL) for an additional 24 h. Cell morphology was detected microscopically with (*bottom*) or without (*top*) Giemsa staining. **B**, PD98059, GF-109203X, and H 7 inhibited TPA-induced MMP-2 protein expression and activity of enzyme released from COLO 205 cells. As described in **A**, MMP-2 protein (*bottom*) and enzyme activity (*top*) in cells and medium were analyzed by Western blotting and gelatin zymography, respectively. **C**, PD98059, GF-109203X, H 7 inhibited TPA-induced ERK1/2 protein phosphorylation and *c-Jun* protein expression in COLO 205 cells as described in **A**. Cells were pretreated with PD98059 (5, 10 μ mol/L), GF-109203X (2.5, 5 μ mol/L), or H 7 (5, 10 μ mol/L) for 30 min followed by addition of TPA and incubation for an additional 30 min for *p-ERK* detection or 6 h for *c-jun* protein detection by Western blotting using specific antibodies. Western blotting was done at least thrice, and the results are representative of all of the data.

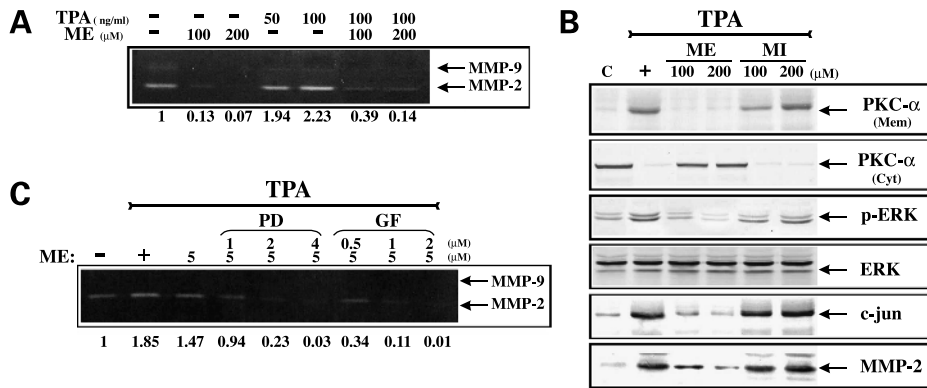


Figure 5. Myricetin inhibits TPA-induced MMP-2 protein expression and enzyme activity by blocking PKC α translocation, ERK1/2 protein phosphorylation, and c-Jun protein expression in COLO 205 cells. **A**, myricetin inhibited TPA-induced MMP-2 protein expression and enzyme activity in COLO 205 cells. Cells were treated with TPA (50 or 100 ng/mL) for 24 h in the presence or absence of myricetin (100 or 200 μ mol/L) pretreatment for 1 h. The MMP-2 enzyme activity in the medium was analyzed by gelatin zymography. **B**, myricetin inhibited TPA-induced PKC α translocation, ERK1/2 protein phosphorylation, and c-Jun protein expression in COLO 205 cells. Cells were pretreated with myricetin or myricitrin (100 or 200 μ mol/L) for 1 h followed by addition of TPA (50 ng/mL). The expression of indicated proteins was detected by Western blotting using specific antibodies as described in Fig. 4. **C**, addition of PD98059 or GF-109203X enhances the MMP-2 inhibition of myricetin. Cells were treated with or without PD98059 (1, 2, 4 μ mol/L) or GF-109203X (0.5, 1, 2 μ mol/L) for 30 min followed by addition of myricetin (5 μ mol/L) and TPA for a further 24 h. MMP-2 enzyme activity in medium was examined by gelatin zymography. Gelatin zymography and Western blotting were done at least thrice, and the results are representative of all of the data.

TPA-induced MMP-2 enzyme activity and invasion. These data support the notion that glycosides are negative moieties on MMP-2 inhibition and invasion in colorectal carcinoma cells.

It has been shown that flavonoids containing more hydroxyl substitutions exhibit stronger antioxidant activity (22, 23). Wang and Joseph (24) indicated that OH groups at C3' and C4' of the B ring and a 2,3 double bond in conjugation with a 4-oxo group in the C ring, along with the polyphenolic structures, were crucial for the protection from H₂O₂-induced oxidative stress. Cos et al. (25) indicated the OH groups at C5 and C7 and a double bond between C2 and C3 were important for the inhibitory activity of flavonoids on xanthine oxidase activity. In contrast to OH substitutions, methoxy (OCH₃) addition inactivated both antioxidant and prooxidant activities of the flavonoids, and hydroxylation but not methoxylation at C2 of flavone showed high affinity on benzodi-

azepine receptors (26, 27). To elucidate the relationship between the antioxidant activity and MMP-2 inhibition of flavonoids, anti-1,1-diphenyl-2-picrylhydrazyl radical activity of indicated flavonoids was examined. It indicated that flavonoids with more than two OH substitutions, such as quercetin, kaempferol, baicalein, myricetin, myricitrin, and toxifolin, exhibited potent anti-1,1-diphenyl-2-picrylhydrazyl radical activity (data not shown). However, results of the present study indicated that myricetin but not others exhibited significant inhibition on MMP-2 enzyme activity. This suggests that myricetin inhibition of invasion and MMP-2 enzyme activity is not only due to its antioxidant activity but also to its suppression of MMP-2 protein expression and enzyme activity via blocking intracellular signaling transduction processes.

TPA, a tumor promoter, has been used extensively in induction of tumor progression *in vitro* and *in vivo*.

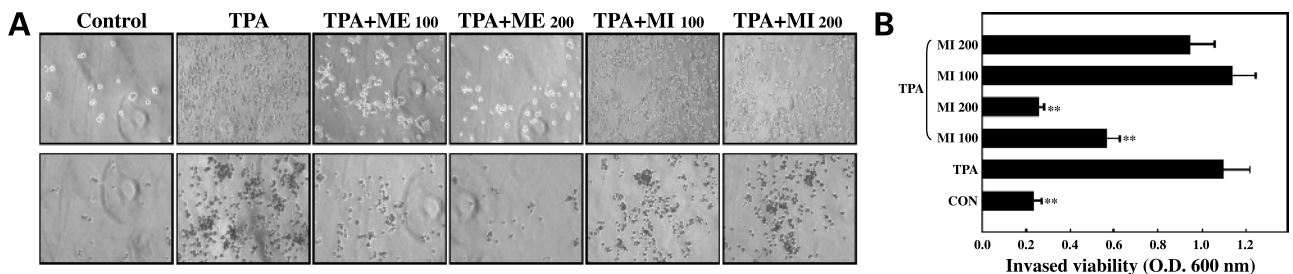


Figure 6. Anti-invasion effect of myricetin *in vitro*. **A**, myricetin but not myricitrin inhibited invasion of COLO 205 cells. *In vitro* invasion assay using Transwells coated with EHS Matrigel was done. Cells in upper chambers were treated with or without myricetin or myricitrin (100 or 200 μ mol/L), and TPA (100 ng/mL) was added into the lower chambers for 24 h. In the control group, no TPA added in the lower well. **A**, cells in the lower chambers were observed microscopically (*top*) and cells in the lower chambers were detected by Giemsa staining and observed microscopically (*bottom*). **B**, quantification of cells in the lower chambers, which was done by MTT assay. *Columns*, mean from three independent experiments; *bars*, SE. **, *P* < 0.01, significantly different from TPA-treated group as analyzed by Student's *t* test.

Activation of PKCs via translocation of PKCs from cytosol to membrane has been identified under TPA treatment (28). In addition to PKCs, phosphorylation of MAPKs, including ERK1/2, p38, and JNK, and induction of c-Jun gene expression were detected in TPA-induced proliferation (8, 29). Hah and Lee (30) reported that TPA induced the invasion of the hepatocellular carcinoma cells through MMP-9 but not MMP-2 secretion. However, the effect of TPA on MMP-2 enzyme activity and invasion in colorectal carcinoma cells is still undefined. Results of the present study indicated that TPA induced invasion and MMP-2 protein expression in COLO 205 cells. Sequential induction of PKC α translocations, ERK1/2 protein phosphorylation, and c-Jun protein expression were involved. Myricetin inhibited TPA-induced MMP-2 enzyme activity and invasion via blocking activated signaling cascades. These data suggested that addition of TPA was able to induce invasion via activation of MMP-2 enzyme activity in colorectal carcinoma cells, which was blocked by myricetin (Fig. 7).

Several natural products such as curcumin, epigallocatechin, and resveratrol have been found to inhibit MMP activity and invasion in different assay systems (31–33). The mechanism of inhibition was attributed to blockade of MMP gene expression but not enzyme activity. In the present study, myricetin inhibited both endogenous and TPA-induced MMP-2 enzyme activity and invasion in colorectal carcinoma cells. The most important event was that myricetin inhibition of MMP-2 released from cells was through blocking both MMP-2 protein expression and enzyme activity. The inhibition by myricetin of MMP-2 protein expression may be due to interruption of signaling transduction cascades such as PKC translocations and ERK1/2 proteins phosphorylation. However, the reason for myricetin-inhibited MMP-2 enzyme activity is still unclear. Several previous studies showed the metal-chelating activity of flavonoids (34, 35). Therefore, it is suggested that a direct inhibitory effect of myricetin on MMP-2 enzyme activity may be through chelating metals such as zinc and calcium, which are essential for the activity of MMP-2.

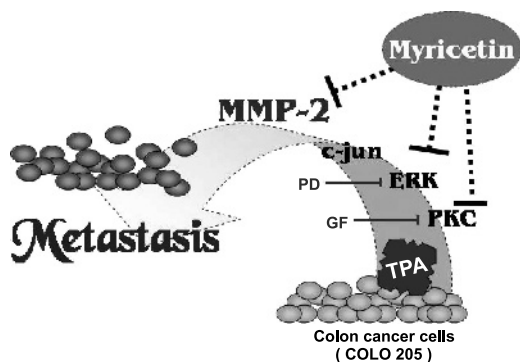


Figure 7. A proposed mechanism for myricetin in suppressing MMP-2 enzyme activity and that induced by TPA in colorectal carcinoma cells.

In conclusion, we have provided evidence demonstrating that myricetin inhibits invasion and both MMP-2 protein expression and enzyme activity in colorectal carcinoma cells in both unstimulated and TPA-stimulated conditions. Therefore, myricetin is potentially a useful anti-invasive agent in the treatment of colorectal carcinoma.

References

- Mercapide J, Lopez De Cicco R, Castresana JS, Klein-Szanto AJ. Stromelysin-1/matrix metalloproteinase-3 (MMP-3) expression accounts for invasive properties of human astrocytoma cell lines. *Int J Cancer* 2003; 106:676–82.
- Bernardo MM, Fridman R. TIMP-2 (tissue inhibitor of metalloproteinase-2) regulates MMP-2 (matrix metalloproteinase-2) activity in the extracellular environment after pro-MMP-2 activation by MT1 (membrane type 1)-MMP. *Biochem J* 2003;374:739–45.
- Hotary KB, Allen ED, Brooks PC, Datta NS, Long MW, Weiss SJ. Membrane type I matrix metalloproteinase usurps tumor growth control imposed by the three-dimensional extracellular matrix. *Cell* 2003;114: 33–45.
- Maekawa R, Maki H, Yoshida H, et al. Correlation of antiangiogenic and antitumor efficacy of *N*-biphenyl sulfonyl-phenylalanine hydroxamic acid (BPHA), an orally-active, selective matrix metalloproteinase inhibitor. *Cancer Res* 1999;59:1231–5.
- Rosenblum G, Meroueh SO, Kleinfeld O, et al. Structural basis for potent slow binding inhibition of human matrix metalloproteinase-2. *J Biol Chem* 2003;278:27009–15.
- Shamamian P, Schwartz JD, Pocock BJ, et al. Activation of progelatinase A by neutrophil elastase, cathepsin G, and proteinase-3: a role for inflammatory cells in tumor invasion and angiogenesis. *J Cell Physiol* 2001;189:197–206.
- Bourguignon LY, Gunja-Smith Z, Iida N, et al. CD44v (3, 8-10) is involved in cytoskeleton-mediated tumor cell migration and matrix metalloproteinase (MMP-9) association in metastatic breast cancer cells. *J Cell Physiol* 1998;176:206–15.
- Ko CH, Shen S-C, Lin H-Y, et al. Flavanones structure related inhibition on TPA-induced tumor promotion through suppression of extracellular signal-regulated protein kinases: involvement of prostaglandin E2 in anti-promotive process. *J Cell Physiol* 2002;193:93–102.
- Zeliadt NA, Warmka JK, Winston SE, et al. Tumor promoter-induced MMP-13 gene expression in a model of initiated epidermis. *Biochem Biophys Res Commun* 2004;317:570–7.
- Arnott CH, Scott KA, Moore RJ, et al. Tumor necrosis factor- α mediates tumor promotion via a PKC α - and AP-1-dependent pathway. *Oncogene* 2002;21:4728–38.
- Hinoda Y, Okayama N, Takano N, et al. Association of functional polymorphisms of matrix metalloproteinase (MMP)-1 and MMP-3 genes with colorectal cancer. *Int J Cancer* 2002;102: 526–9.
- Chen YC, Shen SC, Lee WR, et al. Wogonin and fisetin induction of apoptosis through activation of caspase 3 cascade and alternative expression of p21 protein in hepatocellular carcinoma cells SK-HEP-1. *Arch Toxicol* 2002;76:351–9.
- Lee WR, Shen S-C, Lin H-Y, Hou WC, Yang LL, Chen Y-C. Wogonin and fisetin induce apoptosis in human promyeloleukemic cells, accompanied by a decrease of reactive oxygen species, and activation of caspase-3 and Ca²⁺-dependent endonuclease. *Biochem Pharmacol* 2002; 63:225–36.
- Chen YC, Shen SC, Lin HY. Rutinoside at C7 attenuates the apoptosis-inducing activity of flavonoids. *Biochem Pharmacol* 2003;66: 1139–50.
- Shen SC, Chen YC, Hsu FL, Lee WR. Differential apoptosis-inducing effect of quercetin and its glycosides in human promyeloleukemic HL-60 cells by alternative activation of the caspase 3 cascade. *J Cell Biochem* 2003;89:1044–55.
- Shen SC, Ko CH, Hsu KC, Chen YC. 3-OH flavone inhibition of epidermal growth factor-induced proliferation through blocking prostaglandin E2 production. *Int J Cancer* 2004;108:502–10.
- Fishman DA, Liu Y, Ellerbroek SM, Stack MS. Lysophosphatidic acid promotes matrix metalloproteinase (MMP) activation and

MMP-dependent invasion in ovarian cancer cells. *Cancer Res* 2001; 61:3194–9.

18. Huang YT, Hwang JJ, Lee LT, et al. Inhibitory effects of a luteinizing hormone-releasing hormone agonist on basal and epidermal growth factor-induced cell proliferation and metastasis-associated properties in human epidermoid carcinoma A431 cells. *Int J Cancer* 2002;99: 505–13.

19. Shen SC, Ko CH, Tseng SW, Tsai SH, Chen YC. Structurally-related anti-tumor effects of flavanones *in vitro* and *in vivo*: involvement of caspase 3 activation, p21 gene expression and reactive oxygen species production. *Toxicol Appl Pharmacol* 2004;197:84–95.

20. Ko CH, Shen SC, Chen YC. Hydroxylation at C4' or C6 is essential for apoptosis-inducing activity of flavanone through activation of the caspase 3 cascade and production of reactive oxygen species. *Free Radic Biol Med* 2004;36:897–910.

21. Lin HY, Uan SH, Shen SC, Hsu FL, Chen YC. Inhibition of lipopolysaccharide-induced nitric oxide production by flavonoids in RAW264.7 macrophages involves heme oxygenase-1. *Biochem Pharmacol* 2003;66:1821–32.

22. Arora A, Nair MG, Strasburg GM. Structure-activity relationships for antioxidant activities of a series of flavonoids in a liposomal system. *Free Radic Biol Med* 1998;24:1355–63.

23. Fiorani M, De Sanctis R, De Bellis R, Dachà M. Intracellular flavonoids as electron donors for extracellular ferricyanide reduction in human erythrocytes. *Free Radic Biol Med* 2002;32:64–72.

24. Wang H, Joseph JA. Structure-activity relationships of quercetin in antagonizing hydrogen peroxide-induced calcium dysregulation in PC12 cells. *Free Radic Biol Med* 1999;27:683–94.

25. Cos P, Ying L, Calomme M, et al. Structure-activity relationship and classification of flavonoids as inhibitors of xanthine oxidase and superoxide scavengers. *J Nat Prod* 1998;61:71–6.

26. Cao G, Sofic E, Prior RL. Antioxidant and prooxidant behavior of

flavonoids: structure-activity relationships. *Free Radic Biol Med* 1997; 22:749–60.

27. Huen Michael-SY, Hui KM, Leung Justin-WC, et al. Naturally occurring 20-hydroxyl-substituted flavonoids as high-affinity benzodiazepine site ligands. *Biochem Pharmacol* 2003;66:2397–407.

28. Fahrman M, Kaufhold M, Rieg T, Seidler U. Different actions of protein kinase C isoforms α and epsilon on gastric acid secretion. *Br J Pharmacol* 2002;136:938–46.

29. Hess A, Wijayanti N, Neuschäfer-Rube AP, Katz N, Kietzmann T, Immenschuh S. Phorbol ester-dependent activation of peroxiredoxin I gene expression via a protein kinase C, Ras, p38 mitogen-activated protein kinase signaling pathway. *J Biol Chem* 2003;278:45419–34.

30. Hah N, Lee ST. An absolute role of the PKC-dependent NF- κ B activation for induction of MMP-9 in hepatocellular carcinoma cells. *Biochem Biophys Res Commun* 2003;305:428–33.

31. Garbisa S, Sartor L, Biggin S, Salvato B, Benelli R, Albini A. Tumor gelatinases and invasion inhibited by the green tea flavanol epigallocatechin-3-gallate. *Cancer* 2001;91:822–32.

32. Woo JH, Lim JH, Kim YH, et al. Resveratrol inhibits phorbol myristate acetate-induced matrix metalloproteinase-9 expression by inhibiting JNK and PKC δ signal transduction. *Oncogene* 2004;23:1845–53.

33. Shishodia S, Potdar P, Gairola CG, Aggarwal BB. Curcumin (diferuloylmethane) down-regulates cigarette smoke-induced NF- κ B activation through inhibition of I κ B α kinase in human lung epithelial cells: correlation with suppression of COX-2, MMP-9 and cyclin D1. *Carcinogenesis* 2003; 24:1269–79.

34. Mira L, Fernandez MT, Santos M, Rocha R, Florencio MH, Jennings KR. Interactions of flavonoids with iron and copper ions: a mechanism for their antioxidant activity. *Free Radic Res* 2002;36:1199–208.

35. Fernandez MT, Mira ML, Florencio MH, Jennings KR. Iron and copper chelation by flavonoids: an electrospray mass spectrometry study. *J Inorg Biochem* 2002;92:105–11.