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Quercetin inhibition of tumor invasion via suppressing PKCδ/ERK/AP-1-dependent matrix metalloproteinase-9 activation in breast carcinoma cells

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Quercetin (QUE; 3,5,7,3′,4′-tetrahydroxyflavone) has been shown to possess several beneficial biological activities including antitumor, anti-inflammation and antioxidant properties; however, the effects of QUE in preventing invasion by breast carcinoma cells are still undefined. Increases in the protein, messenger RNA and enzyme activity levels of matrix metalloproteinase (MMP)-9 were observed in 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-treated MCF-7 cells, and these were blocked by QUE, but not by quercitrin or rutin. A translocation of protein kinase C (PKC)δ from the cytosol to the membrane followed by activation of extracellular signal-regulated kinase (ERK) and c-Jun/activator protein-1 (AP-1) by TPA was demonstrated, and TPA-induced MMP-9 activation and migration were inhibited by the pan PKC inhibitor, GF109203X, the specific PKCδ inhibitor, rottlerin, an ERK inhibitor (PD98059) and an AP-1 inhibitor (curcumin). Application of QUE significantly suppressed TPA-induced activation of the PKCδ/ERK/AP-1-signaling cascade. To elucidate the importance of hydroxyl (OH) substitutions to QUE's inhibition of tumor migration, several structurally related flavones of QUE including 3′,4′-diOH, 3′,4′-diOCH$_3$, 3,5,7-triOH, 3,4′,4′-triOH, 3,3′,4′-triOCH$_3$, luteolin and fisetin were used. Results suggested that OH groups at both C3′ and C4′ play central roles in QUE's inhibition of TPA-induced MMP-9 activation and migration, and an additional OH at C3, C5 or C7 may increase the inhibitory potency of the 3′,4′-diOH flavone against TPA-induced MMP-9 activity and migration. The antitumor invasion and migration effects of breast carcinoma cells induced by QUE with the structure–activity relationship analysis were identified.

Introduction

Quercetin (QUE; 3,5,7,3′,4′-pentahydroxylflavone) is one of the active components of flavonoids that abundantly exist in onions, fruits and Chinese herbs. Several previous studies reported that QUE possesses various beneficial biological activities including antioxidant, anti-inflammation, anti-atherosclerosis and antitumor properties (1–3). The antitumor effects of QUE were identified through inhibiting the invasive potential of melanoma and prostate cancers (4,5), and suppression of tube formation in human umbilical vascular endothelial cells in response to antiangiogenic activity (6). Our previous studies reported that QUE has the ability to inhibit lipopolysaccharide- or 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced inflammation and matrix metalloproteinase (MMP)-9 expression in mouse macrophages and glioma cells, respectively (7,8). In addition, cellular protection by QUE via induction of heme oxygenase-1 was also reported (9). QUE in diet bioflavonoids often presents with its glycoside derivatives such as quercitrin (QUI; QUE-3-O-rhamnoside) and rutin (RUT; QUE-3-O-rutinoside), and studies demonstrated that QUE glycosides provide better solubility and absorption than that of QUE aglycone (10,11). However, although various bioactivity studies of QUE have been carried out, the anti-invasive capacity of QUE and its structure–activity relationship (SAR) analysis in breast cancer have not been yet analyzed.

Activation of MMPs in tumor invasion and metastasis has been reported via degrading the matrix surrounded the tumors (12–14). MMPs are a family of zinc-dependent proteases and divided into four subclasses based on the substrate including collagenase, gelatinase, stromelysin and membrane-associated MMPs (15). Tumor-secreted MMPs destroy extracellular matrix components in the surrounding tissues of the tumor, and subsequently tumor cells invade through the basement membrane of blood vessels and facilitate the spread to distant organs, resulting in organ failure and patient mortality. To date, 24 kinds of MMPs have been identified, and MMP-2 (gelatinase-A) and MMP-9 (gelatinase-B) are the most important mediators of tumor migration and invasion among these. The extents of MMP-2 and MMP-9 expressions in tumors are highly correlated with the metastatic potential (16–18).

Generally, MMP-2 is constitutive and overexpressed in highly metastatic tumors, whereas MMP-9 can be stimulated by the inflammatory cytokine, tumor necrosis factor-α, the growth factor, epidermal growth factor or phorbol ester, TPA, through activation of different intracellular-signaling pathways (19–21). Mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) are the predominant cascades participating in MMP-9 expression. In addition, transcriptional regulation by activating transcription factors including activator protein-1 (AP-1), nuclear factor-κ B (NF-κB) or Sp-1 was also reported to occur in the regulation of *MMP-9* gene expression. Therefore, agents possessing the ability to suppress the expression of MMP-2 or -9 deserve development for anti-breast cancer invasion and metastasis.

Several studies have indicated that inhibition of MMP expressions or enzyme activities can be used as early targets for treating cancer metastasis (22,23). Our previous studies demonstrated downregulation of MMP-9 and -2 by natural flavonoids, which suppress the invasiveness of gliomas and colorectal carcinoma *in vitro* and *in vivo* (24,25). However, studies regarding the molecular mechanisms by which QUE acts on the expression of MMP-9 and the invasiveness of breast cancer are still undefined. In the present study, QUE significantly suppressed *MMP-9* gene expression via blocking the protein kinase C (PKC)δ/extracellular signal-regulated kinase (ERK)/AP-1-signaling pathway, and consequently reductions in migration and invasion of human breast carcinoma cells were first identified.

Materials and methods

Chemicals

Antibodies against ERK, c-Jun N-terminal kinase (JNK), p38 and Akt were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against MMP-9, MMP-2 and α-tubulin were from Lab Vision (Fremont, CA). Antibodies against phospho-ERK, phospho-JNK, phospho-p38 and phospho-Akt were from Cell Signaling Technology (Beverly, MA). Antibodies against PKCα, PKCδ, c-Jun and phospho-c-Jun were obtained from Transduction Laboratories (Lexington, KY). Specific inhibitors of PI3K [LY294002 (LY)], MAPK family [PD98059 (PD), SP600125 (SP) and SB203580 (SB)] and gelatinase (SB3CT) were purchased from Calbiochem (La Jolla, CA). Specific inhibitors of PKCα (Go6979) and PKCδ [rottlerin (Rot)] were purchased from Tocris Cookson (Ellisville, MO). TPA was purchased from Sigma (St Louis, MO). QUE and its structural-related compounds were obtained from Indofine (Hillsborough, NJ).

Abbreviations: AP-1, activator protein-1; DPPH, 1,1-diphenyl-2-picrylhydrazyl; ERK, extracellular signal-regulated kinase; Fis, fisetin; GF, GF109203X; Go, Go6976; JNK, c-Jun N-terminal kinase; Lut, luteolin; LY, LY294002; MAPK, mitogen-activated protein kinase; MEM, modified Eagle's medium; MMP, matrix metalloproteinase; NF-κB, nuclear factor-κ B; OH, hydroxyl; PD, PD98059; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; QUE, quercetin; QUI, quercitrin; Rot, rottlerin; RUT, rutin; SAR, structure–activity relationship; SB, SB203580; SP, SP600125; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

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Cell culture

The human breast carcinoma cell lines, MCF-7 and MDA-MB-231, were obtained from American Type Culture Collection (Manassas, VA). Cells were maintained in modified Eagle's medium (MEM) supplemented with 5% heat-inactivated fetal bovine serum, 100 U penicillin–streptomycin, 1 mM sodium pyruvate and 0.1 mM non-essential amino acids at 37°C in a humidified incubator containing 5% CO_2. All culture reagents were purchased from Life Technologies (Gaithersburg, MD).

Soft agar assay

Cells were plated in 1 ml MEM containing 0.35% agarose and 5% fetal bovine serum overlaid with 1 ml of 0.7% agarose. Cultures were maintained for 3 weeks and refreshed with 1 ml MEM supplemented with 5% fetal bovine serum and subjected to the indicated treatment twice per week. Colonies were observed and photographed using a light microscope, and the number of colonies in each well was measured. Each value is derived form three independent experiments, and results are expressed as the mean ± SE.

Wound-healing assay

MCF-7 cells were seeded in a six-well plate and grown overnight to confluence. The monolayer cells were scratched with a 200 µl pipette tip to create a wound, and cells were washed twice with serum-free MEM to remove floating cells and then replaced with medium without serum. Cells were subjected to the indicated treatment for 24 h, and cells migrating from the leading edge were photographed at 0 and 24 h and counted at 24 h. Each value is derived from three randomly selected fields, and results are expressed as the mean of migrating cell numbers per field.

In vitro invasion assay

An *in vitro* invasion assay was carried out to examine tumor invasion as described previously (25). Briefly, 24-well transwell units with 8 µm polycarbonate Nucleopore filters (Corning, Corning, NY) were coated with 0.1 ml of 0.8 mg/ml Englebreth-Holm-Swarm sarcoma tumor extract (EHS Matrigel) at room temperature for 1 h to form a genuine reconstituted basement membrane. MCF-7 cells (2×10^5 cells) were placed in the upper compartment, and the conditioned medium derived from NIH3T3 fibroblasts was added to the lower compartment. The transwell plates were incubated at 37°C for 48 h in a humidified atmosphere with 5% CO_2. Cells migrating to the lower surface of the membrane were stained with Giemsa staining and observed with a light microscope.

Reverse transcription–polymerase chain reaction analysis

Total RNA was isolated with an RNA extraction kit (Amersham Pharmacia, Buckinghamshire, UK), and the concentration of total RNA was measured spectrophotometrically. RNA (2 µg) was converted to complementary DNA by a RT–PCR Bead kit (Amersham Pharmacia) according to the manufacturer's protocol. The amplification sequence protocol was 30 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 1 min. The polymerase chain reaction product of each sample was analyzed by electrophoresis in a 1.2% agarose gel and visualized by ethidium bromide staining. Oligonucleotide primer sequences used were as follows: MMP-9 5′-CACTGTCCACCCCTCAGAGC-3′ (sense) and 5′-GCCACTTGTCGGCGATAAGG-3′ (antisense); MMP-2 5′-GTGCTGAAGGACACACTAAAGAAGA-3′ (sense) and 5′-TTGCCATC-CTTCTCAAAGTTGTAGG-3′ (antisense) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) 5′-TGAAGGTCGGTGTGAACGGATTTGGC-3′ (sense) and 5′-CATGTAGGC CATGAGGTCC-ACCAC-3′ (antisense).

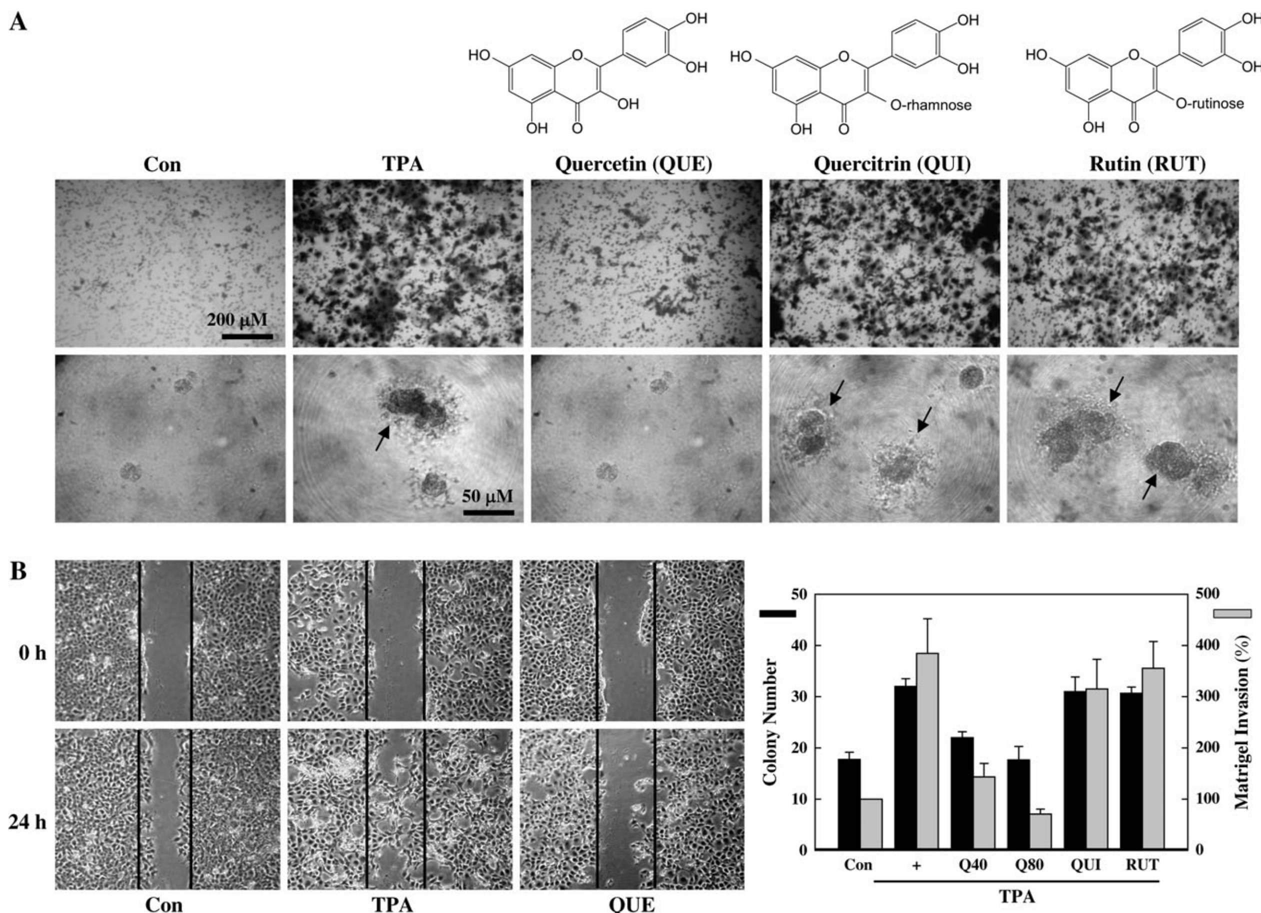

Fig. 1. QUE suppression of the invasion, migration and colony formation of human breast cancer MCF-7 cells. (**A**) MCF-7 cells were treated with QUE, QUI and RUT (80 µM) followed by TPA (50 ng/ml) treatment for 48 h. The invasive ability was evaluated by a Matrigel-coated *in vitro* invasion assay (upper panel), and colony formation of MCF-7 cells grown on soft agar was determined by a soft agar assay as described in Materials and Methods (lower panel). Quantification of the invasive colony formation of MCF-7 cells was evaluated (lower right), and data are expressed as the mean ± SE from three independent experiments. (**B**) Cells were scratched with a pipette tip and then pretreated with QUE, QUI and RUT (80 µM) followed by TPA (50 ng/ml) treatment for 24 h. Migrating cells were photographed under phase contrast microscopy.

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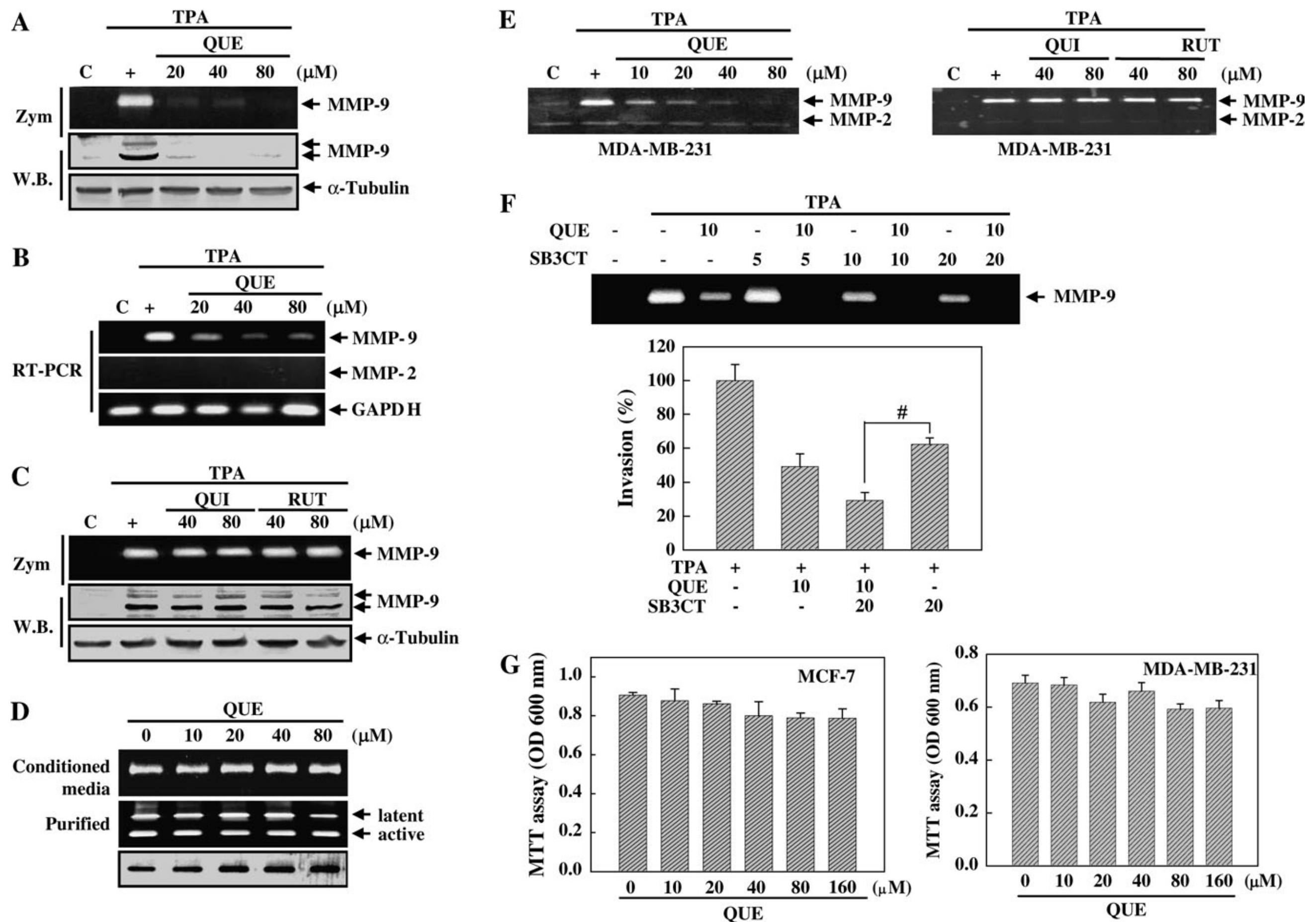

Fig. 2. QUE downregulated TPA-induced *MMP-9* gene expression in MCF-7 and MDA-MB-231 cells. (**A**) MCF-7 cells were treated with QUE (20, 40 and 80 μM) followed by TPA treatment for 24 h. MMP-9 enzyme activity was analyzed by gelatin zymography (Zym) and protein expression by western blotting (W.B.). Bands were representative as latent- and active-MMP-9 protein. (**B**) Cells were treated with QUE and TPA for 4 h, and *MMP-2*, *MMP-9* and *GAPDH* messenger RNA levels were measured by a reverse transcription–polymerase chain reaction analysis. (**C**) MCF-7 cells were treated with QUI and RUT (40 and 80 μM) in the presence or absence of TPA for 24 h, and the MMP-9 level was evaluated by western blotting and gelatin zymography. (**D**) MMP-9 derived from TPA-treated conditioned medium (upper panel) or from a purified mouse fibrosarcoma (middle panel) were incubated with QUE (10, 20, 40 and 80 μM) for 30 min as described in Materials and Methods and then subjected to gelatin zymography. Western blotting analysis of the MMP-9 protein level was used as the loading control (lower panel). (**E**) MDA-MB-231 cells were treated with the same conditions as described in (D), and the conditioned medium was subjected to gelatin zymography. (**F**) MCF-7 cells were treated with QUE (10 μM) and SB3CT (5, 10 and 20 μM) in the presence or absence of TPA, and the MMP-9 level was evaluated by gelatin zymography (upper). Cells were treated with QUE (10 μM), SB3CT (20 μM) or cotreated with QUE and SB3CT in the presence of TPA for 48 h, and the invasive ability was evaluated by a transwell invasion assay (lower). Data are expressed as a percentage of invaded cells from three independent experiments. #Denotes *P* value <0.05 was regarded as a significant difference. (**G**) MCF-7 and MDA-MB-231 cells were treated with the same conditions as described in (A), the viability of cells was evaluated by an 3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium bromide (MTT) assay and data are expressed as the mean ± SE from three independent experiments.

Western blotting

Cell lysates were prepared by suspending cells in lysis buffer [50 mM Tris–HCl (pH 7.4), 1% Nonidet P-40, 150 mM NaCl, 1 mM ethylene glycol tetra-acetic acid, 0.025% sodium deoxycholate, 1 mM sodium fluoride, 1 mM sodium orthovanadate and 1 mM phenylmethylsulfonyl fluoride], and equal amounts of protein were prepared and separated on 8% sodium dodecyl sulfate–polyacrylamide gels and then transferred to Immobilon polyvinylidene difluoride membranes (Millipore, Bedford, MA). The membrane was blocked with 1% bovine serum albumin at room temperature for 1 h and then incubated with specific indicated primary antibodies for a further 3 h followed by incubating with respective alkaline phosphatase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h. Expression of protein was visualized by incubating with the colorimetric substrates, nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate.

Gelatin zymography

The enzymatic activities of MMP-2 and MMP-9 were determined by gelatin zymography. Briefly, cells were seeded and allowed to grow to confluence for 24 h and then maintained in serum-free medium. The conditioned media were collected 24 h after stimulation, mixed with non-reducing sample buffer and subjected to electrophoresis in a 10% polyacrylamide gel containing 0.1% (wt/vol) gelatin. The gel was washed with washing buffer containing 2.5% Triton X-100 and 50 mM Tris–HCl (pH 7.5) and incubated at 37°C for 24 h in 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 5 mM $CaCl_2$, 1 mM $ZnCl_2$ and 40 mmol/l NaN_3. The gel was stained with 0.25% (wt/vol) Coomassie brilliant blue in 45% (vol/vol) methanol and 1% (vol/vol) acetic acid.

Direct MMP-9 enzyme activity assay

One hundred nanograms of purified MMP-9 from human fibroblasts was incubated with 0, 10, 20, 40 and 80 μM QUE in 50 μl Tris buffer (50 mM, pH 7.4) at 37°C for 30 min, respectively. Twenty microliters of incubated buffer was analyzed for gelatinolytic activity as described as above.

Measuring MMP-9 activity

MMP-9 activity was quantified by using the fluorimetric SensoLyte^{TM} 490 MMP-9 assay kit (Anaspec, San Jose, CA) according to the manufacturer's instructions. Briefly, cells were treated with TPA and indicated flavonoids in serum-free medium for 24 h, the supernatant of conditioned medium was collected and centrifuged for 5 min at 10 000 r.p.m. Samples containing MMP-9 were then incubated with 4-aminophenylmercuric acetate for activating pro-MMP-9 followed by initiating the enzyme reaction. The activity of

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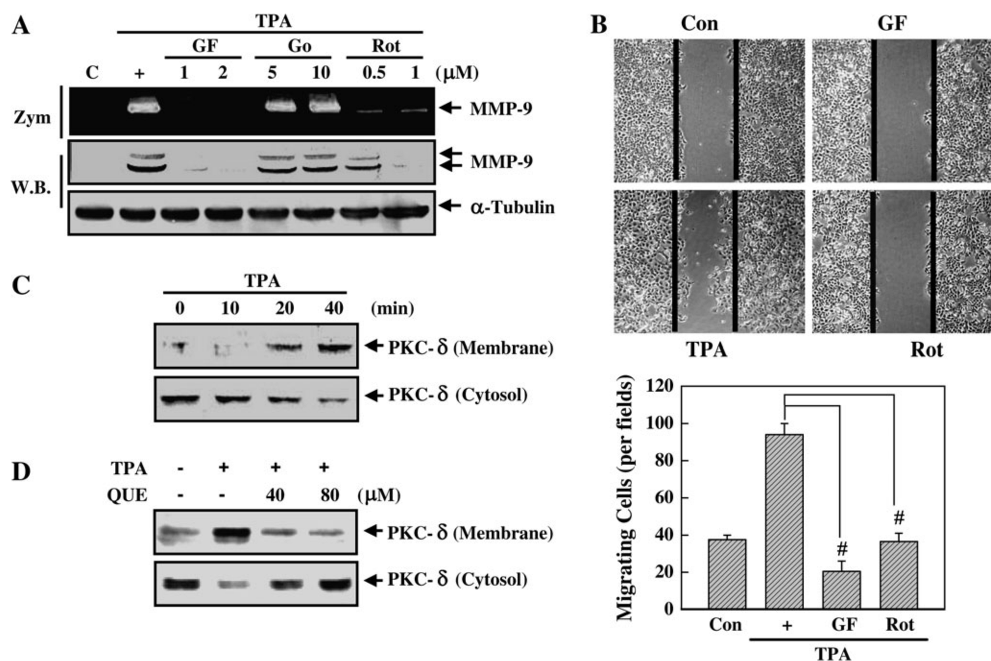

Fig. 3. Effects of QUE on TPA-induced PKCδ activation. (**A**) MCF-7 cells were treated with GF (1 and 2 μM), Go (5 and 10 μM) or Rot (0.5 and 1 μM) followed by the addition of TPA, and the MMP-9 level was evaluated by gelatin zymography and western blotting. (**B**) Cells were pretreated with GF (2 μM) and Rot (0.5 μM) followed by TPA treatment, and the ability of cells to migrate was evaluated by a wound-healing assay as described in Materials and Methods. Data are expressed as the mean ± SE from three independent experiments. #Denotes P value of <0.05 was regarded as a significant difference compared with the TPA-treated group. (**C**) Cells were treated with TPA for different time intervals, the subcellular level of cells was extracted as described in Materials and Methods, and the level of PKCδ in the cytosolic and membrane fraction was analyzed by western blotting. (**D**) Cells were treated with QUE (40 and 80 μM) followed by TPA treatment for 20 min, and the PKCδ level was evaluated as described above.

MMP-9 was detected by fluorescence microplate reader (Bio-Tek Instruments, Burlington, VT) at excitation/emission wavelengths = 360 nm/460 nm.

Luciferase assay

MCF-7 cells were grown in a 10 cm culture dish, subconfluent cells were replaced with serum-free Opti-MEM (Gibco-BRL, Grand Island, NY) and cells were transfected with the pAP-1-Luciferase reporter construct using LipofectAMINE 2000. After transfection, the medium was replaced, and cells were cultured for 24 h. Cells were plated in 24-well plates and treated with 50 ng/ml TPA for 6 h. Luciferase activities were measured by a luminometer (Berthold Detection Systems, Oak Ridge, TN) using the Dual-Luciferase Reporter Assay (Promega, Madison, WI) according to the manufacturer's instructions. The transfected efficiency was normalized by cotransfection with phRL-TK as an internal control, and all transient transfections were repeated in three independent experiments.

1,1-Diphenyl-2-picrylhydrazyl-free radical-scavenging activity

1,1-Diphenyl-2-picrylhydrazyl (DPPH) assay has been used extensively to examine the free radical-scavenging activity of chemicals. Briefly, DPPH is a stable-free radical with an absorbance at 517 nm, and a reduction in optical density 517 value is described as a representative of free radical-scavenging activity after chemicals stimulation. In the present study, QUE and its structurally related compounds were added to 0.5 ml methanol to a concentration of 40 μM and then mixed with 50 μl of 500 mM DPPH in methanol for 20 min while being protected from light. The scavenging activities of compounds against the DPPH radical were calculated by the equation: [1 − (absorbance by compounds)/(absorbance with dimethyl sulfoxide treatment)] × 100 at 517 nm.

Statistical analyses

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

Results

QUE, but not QUI or RUT inhibited TPA-induced invasion and migration in human breast cancer cells

In vitro invasion and migration assays including transwell, soft agar and wound-healing assays were used to investigate the differential inhibitory effects of QUE and its glycoside, QUI (QUE-3-O-rhamnoside) and RUT (QUE-3-O-rutinoside) on the invasive potency of breast carcinoma MCF-7 cells. QUI and RUT, respectively, contain a rhamnose and rutinose at C3 of QUE. As illustrated in Figure 1A, TPA induction of invasion and colony formation was, respectively, detected by transwell and soft agar assays, and the addition of QUE, but not QUI or RUT, significantly prevented its induction. In the same part of the experiment, data of the wound-healing assay indicated that migration of MCF-7 cells was increased by TPA incubation and inhibited by adding QUE (Figure 1B), but not by QUI and RUT (data not shown). Quantitative data derived from three independent experiments supported QUE effectively preventing the migration and invasion of breast carcinoma MCF-7 cells elicited by TPA.

QUE inhibited MMP-9 enzyme activity via reducing its gene expression at both the protein and messenger RNA levels

MMP-9 activation has been shown in the metastasis of breast carcinoma cells; therefore, the effects of QUE on TPA-induced MMP-9 enzyme activity and gene expression were examined. As shown in Figure 2A, treatment of MCF-7 cells with QUE significantly suppressed TPA-induced MMP-9 protein expression and enzyme activity. Data of the reverse transcription–polymerase chain reaction analysis showed that the level of MMP-9, but not MMP-2, messenger RNA increased with TPA treatment and was blocked by adding QUE (Figure 2B). However, the addition of QUI or RUT produced no effect on TPA-induced MMP-9 protein expression or enzyme activity (Figure 2C). In order to examine if QUE reduces MMP-9 enzyme activity through the direct inhibition of the MMP-9 enzyme, incubation of QUE with conditioned medium derived from TPA-treated MCF-7 cells or purified MMP-9 protein derived from human fibroblasts followed by gelatin zymography analysis was performed. As illustrated in Figure 2D, there was no significant difference between the groups with or without QUE treatment. Suppression of MMP-9 enzyme activity by QUE, but not QUI or RUT, was also identified in MDA-MB-231 cells (Figure 2E). SB3CT is a specific MMP-9

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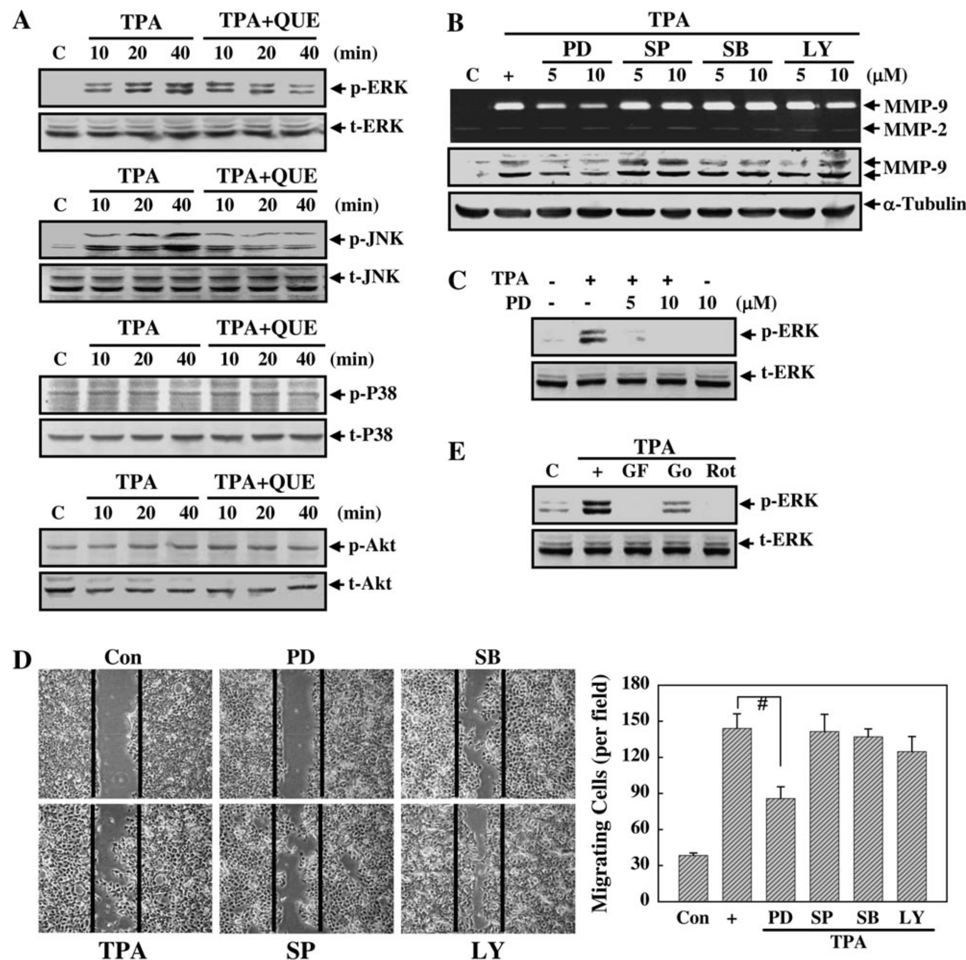

Fig. 4. Effects of QUE on TPA-stimulated phosphorylation of MAPK and PI3K. (**A**) MCF-7 cells were treated with QUE (40 μM) in the presence or absence of TPA for different time intervals, the cellular extracts were blotted using specific antibodies. (**B**) Cells were treated with PD, SP, SB or LY followed by the addition of TPA, the MMP-9 level was evaluated by western blotting and gelatin zymography. (**C**) Cells were treated with PD (PD; 10, 20 and 40 μM) followed by the addition of TPA for 20 min, and the cellular extracts were blotted using specific antibodies. (**D**) Cells were pretreated with PD, SP, SB and LY (10 μM) followed by TPA treatment, and the ability of cells to migrate was evaluated by a wound-healing assay as described in Materials and Methods. Data are expressed as the mean ± SE from three independent experiments. #Denotes P value of <0.05 was regarded as a significant difference compared with the TPA-treated group. (**E**) Cells were pretreated with GF (10 μM), Go (10 μM) and Rot (1 μM) followed by TPA addition, expression of phosphorylated ERK was done by western blotting.

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inhibitor, and SB3CT treatment enhanced the inhibitory effect of QUE on TPA-induced MMP-9 enzyme activity and invasion of MCF-7 cells (Figure 2F). No significant cytotoxic effects of QUE on the viability of MCF-7 and MDA-MB-231 were identified by the 3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium bromide assay (Figure 2G).

QUE suppresses PKCδ activation elicited by TPA

Activation of PKCs has been shown to be correlated with the potential of tumor metastasis (26–28). TPA has been reported as a PKC activator, and activation of the PKC isoforms including α, β, δ and ε by TPA has been identified (29–31). However, contributions of PKC isoforms in TPA-induced invasion and migration in breast cancer are still unclear. Pharmacological studies using the three PKC inhibitors including GF109203X (GF; a broad inhibitor of PKC isoforms), Rot (a specific PKCδ inhibitor) and Go6976 (Go; a specific PKCα inhibitor) were performed in the present study. As shown in Figure 3A, incubation of MCF-7 cells with GF and Rot, but not Go, inhibited TPA-induced MMP-9 protein expression and enzyme activity. Data from the wound-healing assay revealed that the addition of GF and Rot significantly inhibited the TPA-induced migration of MCF-7 cells (Figure 3B). Translocation of PKCδ protein from the cytosol to the membrane was detected in TPA-treated cells (Figure 3C), but was blocked by the addition of QUE (Figure 3D). No significant cytotoxic effect elicited by GF (2 μM) and Rot (0.5 μM) in MCF-7 cells was detected (data not shown). This suggests that PKCδ activation is involved in TPA-induced MMP-9 activation and migration, which was inhibited by the addition of QUE.

QUE suppresses ERK activation located downstream of PKCδ

Induction of MAPK- or PI3K-signaling pathway is involved in the expression of MMP-9 (19,20); however, studies regarding the mechanism of QUE's downregulation of MMP-9 through suppression of MAPK or PI3K have not been carried out. As shown in Figure 4A, induction of ERK and JNK phosphorylation, but not that of p38 MAPK or Akt, elicited by TPA was detected, and these were blocked by pretreatment with QUE. Pharmacological inhibitors of MAPK and PI3K including PD (an ERK inhibitor), SP (a JNK inhibitor), SB (a p38 MAPK inhibitor) and LY (an Akt inhibitor) were used to identify the molecular mechanisms by which TPA induces MMP-9 expression. As shown in Figure 4B, only treatment of cells with PD abrogated TPA-induced MMP-9 expression as well as ERK activation (Figure 4C) and migration of MCF-7 cells (Figure 4D), whereas treatment with SP, SB or LY did not.

Furthermore, phosphorylation of ERK was blocked by treatment with GF and Rot, but not with Go (Figure 4E), suggesting that QUE suppression of ERKs is located downstream of PKCδ, and consequently inhibits MMP-9 expression. Addition of PD, SB, SP and LY showing no cytotoxicity against the viability of MCF-7 cells is identified by 3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium bromide assay (data not shown).

QUE inhibits AP-1 activities of MMP-9

The MMP-9 promoter contains two important transcriptional elements, which are the binding sites of AP-1 and NF-κB. To further determine the involvement of the activation of AP-1 or NF-κB in stimulating MMP-9 expression induced by TPA, cells were pretreated with curcumin (an AP-1 inhibitor) and pyrrolidin dithiocarbamate (PDTC) (a NF-κB inhibitor) followed by the addition of TPA. As shown in Figure 5A, treating cells with curcumin, but not PDTC, effectively suppressed TPA-induced MMP-9 protein expression and enzyme activity. Figure 5B shows that time-dependent inductions of c-Jun protein expression and phosphorylation were identified in TPA-treated cells, whereas c-fos did not change after exposure to TPA, and the expression of c-Jun protein was inhibited by curcumin (Figure 5C). Moreover, the inductions of c-Jun protein expression and AP-1 promoter activities by TPA were significantly suppressed by treatment with GF, Rot and PD (Figure 5D and E). Pretreatment with QUE showing the significant inhibition against TPA-induced c-Jun protein expression and AP-1 promoter activity were also identified (Figure 5F and G).

The 3′,4′-diOH group plays a major role in QUE suppression of MMP-9 expression

To investigate the SARs of QUE against MMP-9 expression, the related structural analogs of QUE were employed and are depicted in Figure 6A, as represented in (a) QUE, (b) 3′,4′-diOH flavone, (c) 3′,4′-diOCH$_3$ flavone and (d) 3,5,7-triOH flavone. Results showed that QUE and 3′,4′-diOH flavone at the doses of 10 and 20 μM possessed inhibitory activity against MMP-9 protein expression and enzyme activity (Figure 6B) with suppressing ERK phosphorylation and c-Jun expression induced by TPA (Figure 6C). The 3,5,7-triOH flavone showing less potent inhibition against TPA-induced MMP-9 and c-Jun protein than those elicited by QUE and 3′,4′-diOH flavone was observed. However, no effect of 3′,4′-diOCH$_3$ flavone on TPA-induced events was detected (Figure 6B and C). Furthermore, QUE has been reported to be a potent antioxidant, and the expression of MMP-9 is often associated with induction of intracellular reactive oxygen species production. To further examine whether QUE suppression of MMP-9 expression is due to its antioxidant activity, a DPPH-free radical-scavenging assay was carried out. As shown in Figure 6D, QUE and 3′,4′-diOH flavone exhibited efficient antioxidant activities against DPPH-free radicals; however, neither 3′,4′-diOCH$_3$ flavone nor 3,5,7-triOH flavone had an effect on the DPPH-scavenging activity, confirming that the antioxidant property of QUE is correlated with inhibition of MMP-9 expression. These data indicate that the diOH groups at C3′ and C4′ in QUE play a central role against MMP-9 expression in association with TPA-induced cascades.

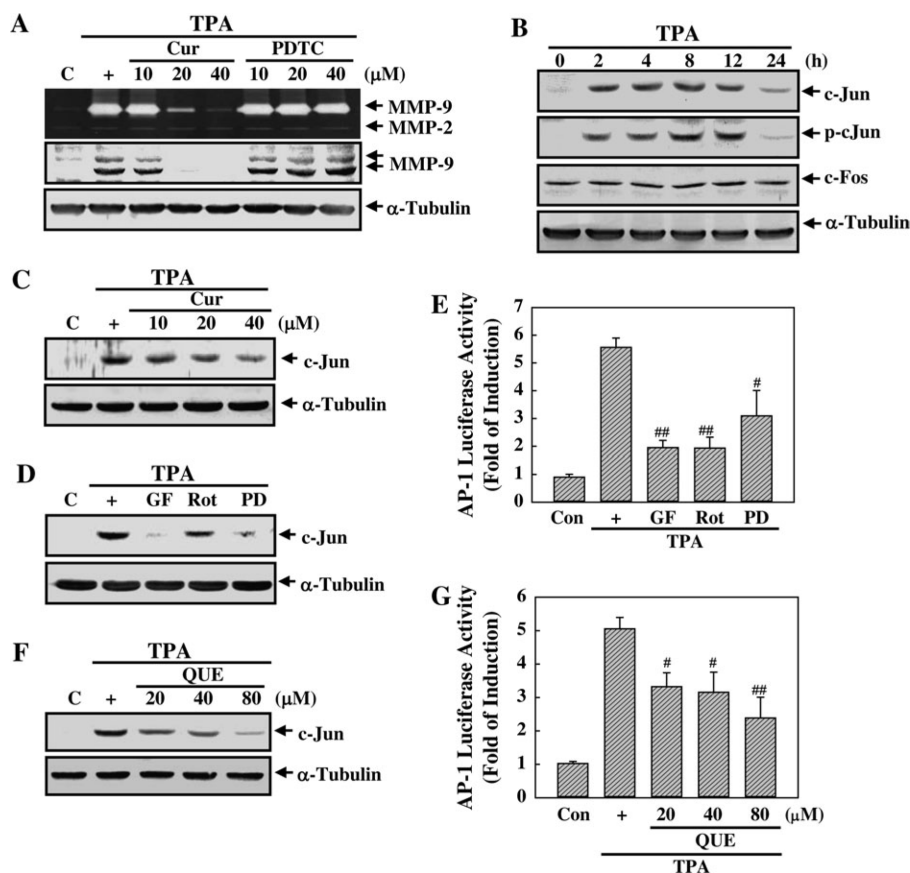

Fig. 5. Effects of QUE on the transcriptional regulation of MMP-9. (**A**) MCF-7 cells were treated with curcumin or PDTC (10, 20 and 40 μM) followed by TPA addition, the MMP-9 level was analyzed by western blotting and gelatin zymography. (**B**) Cells were treated with TPA for different time intervals, and the cellular extract was subjected to western blotting. Cells were pretreated with curcumin (**C**), GF, Rot, PD (**D**) or QUE (**F**) followed by the addition of TPA, and the expression of c-Jun was analyzed by western blotting. (**E**) Cells were transiently transfected with pAP-1-luc and then treated with GF, Rot, PD or QUE (**G**) in the presence of TPA for 6 h followed by measurement of reporter activity. The representative data are shown by luciferase activity relative to the control. Data are expressed as the mean ± SE of three independent experiments. #Denotes P value of <0.05 and ##P value of <0.01 were regarded as a significant difference compared with the TPA-treated group.

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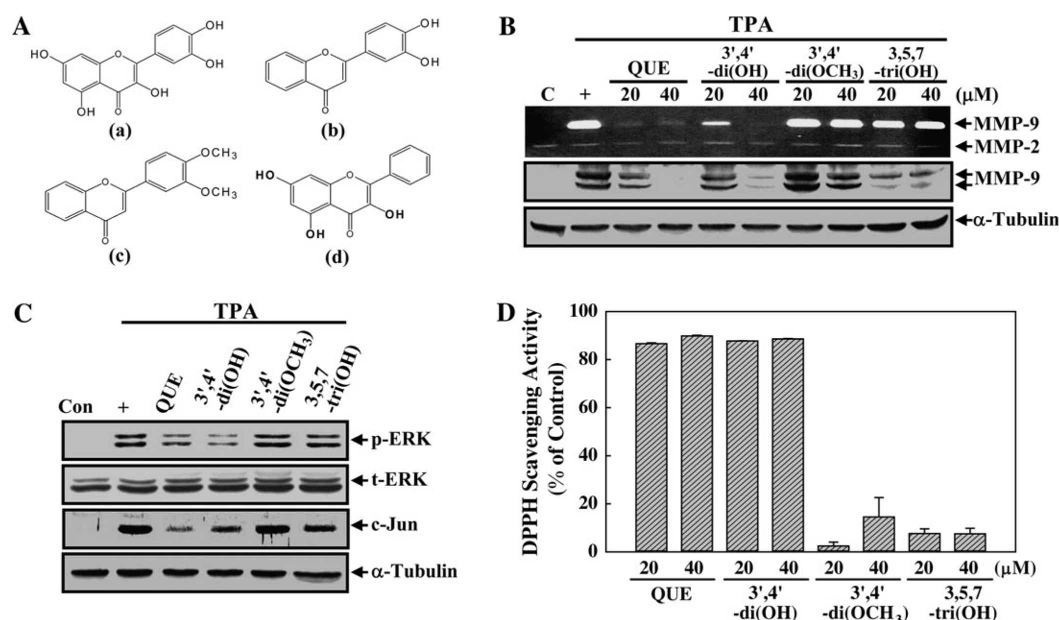

Fig. 6. The 3′,4′-diOH groups in QUE mediate the central inhibitory activity against MMP-9 expression. (**A**) Four structural analogs of QUE are depicted and represented as (**a**) QUE, (**b**) 3′,4′-diOH flavone, (**c**) 3′,4′-diOCH$_3$ flavone and (**d**) 3,5,7-triOH flavone. (**B**) Cells were treated with the indicated flavonoids (20 and 40 μM) and TPA for 24 h and the MMP-9 level was evaluated by gelatin zymography and western blotting. (**C**) Cells were treated with the indicated flavonoid (40 μM) and TPA for 30 min (for ERK detection) and 2 h (for c-Jun detection), and cell extracts were subjected to western blotting. (**D**) The antioxidant ability of the indicated flavonoid (20 and 40 μM) was evaluated by measuring DPPH scavenging as described in Materials and Methods. Data are represented as the percentage of the vehicle treatment relative to the indicated flavonoids and are expressed as the mean ± SE.

The hydroxyl group at C3, C5 or C7 increases the inhibitory potency of the 3′,4′-diOH group against MMP-9 expression

In order to provide more insight into the SAR analysis of QUE, six structurally related analogs of QUE including 3′,4′-diOH flavone, 3,5,7-triOH flavone, 3,3′,4′-triOH flavone, 3,3′,4′-triOCH$_3$ flavone, 3,7,3′,4′-tetraOH flavone [fisetin (Fis)] and 5,7,3′,4′-tetraOH flavone [luteolin (Lut)] were employed. As shown in Figure 7A, the gelatin zymographic analysis showed that the inhibitory capacities against MMP-9 of QUE, 3,3′,4′-triOH flavone, Lut and Fis were better than that of 3′,4′-diOH flavone; however, treatment with 3,5,7-triOH flavone and 3,3′,4′-triOH$_3$ flavone had less inhibitory activity against MMP-9 (Figure 7A, upper). Utilizing the MMP-9 enzyme activity assay also revealed that QUE, 3,3′,4′-triOH flavone and Lut exhibited >75% inhibitory capacity, and 3′,4′-diOH flavone and Fis exhibited ∼60% inhibitory activities; however, <30% inhibition was detected with 3,5,7-triOH flavone and 3,3′,4′-triOH$_3$ flavone treatment (Figure 7A, middle). Similar inhibitory potencies were also determined by the DPPH-free radical-scavenging assay (Figure 7A, lower) and *in vitro* invasion assay (Figure 7B). Together, the above results from Figures 6 and 7 demonstrate that the 3′,4′-diOH group plays a major active role in QUE suppression of MMP-9 expression, whereas the hydroxyl (OH) group at C3, C5 or C7 in QUE may increase the suppressive potency of the 3′,4′-diOH group in QUE (Figure 7C).

Discussion

QUE is the active component of dietary flavonoids and possesses several biological activities, especially useful in cancer treatment. QUE has been shown to suppress cytokine and growth factor-induced invasiveness (32,33) and was reported to possess antimetastatic potential against melanoma and prostate cancers (34,35). However, the role of QUE against TPA-induced MMP-9 expression and invasiveness of breast cancer is still unclear. The present study showed that QUE effectively suppressed TPA-induced *MMP-9* gene expression via suppressing the PKCδ/ERK/AP-1 cascades with consequence suppression of colony formation, tumor migration and invasion by human breast carcinoma cells.

Upregulation and activation of PKCs are highly correlated with an increased invasiveness in glioma, breast and colorectal carcinomas (26–28,36). PKC isozymes are grouped into three subclasses including conventional (PKCα, β and γ), novel (PKCδ, ε, η and θ) and atypical (PKCζ and λ) forms. It has been reported that TPA can activate PKCα, β, δ and ε in different cell types (29–31); however, studies indicated that stimulation of PKCδ activity by TPA is critical for the induction of MMP-9 expression and invasiveness of tumor cells. Woo *et al.* (37) reported that suppression of TPA-mediated PKCδ activation represses MMP-9 expression and invasion in cervical cancer. Liu *et al.* (30) delineated that activation of PKCδ rather than PKCα is critical for MMP-9 induction in MCF-7 cells. In addition, blocking of PKCδ activation by QUE was correlated with the ability to suppress hypoxia-induced malignant progression of fibrosarcomas (38). In agreement with the previous study, stimulation of both PKCα (data not shown) and PKCδ activations by TPA was observed in the present work; however, using the specific inhibitors of PKC isozymes demonstrated that blocking PKCδ (by treatment with Rot and GF), but not PKCα (by treatment with Go), completely attenuated TPA-induced MMP-9 expression and cell migration, and QUE's ability to suppress activation of PKCδ may therefore reduce the metastatic potential.

Inductions of MAPK and PI3K are involved in MMP-9 expression in different cell types; however, the signaling pathway related to MMP-9 expression evoked by TPA in MCF-7 cells is still unclear. Our present study found that TPA can activate both ERK and JNK, but not p38 or Akt in MCF-7 cells, and these can be diminished by QUE treatment. However, only the blocking of ERK by PD suppressed TPA-induced MMP-9 expression and cell migration. These findings suggest that suppression of ERK activation is critical for QUE-mediated MMP-9 inhibition. Activation of ERK subsequently stimulates two cis-acting regulatory elements including the binding sites of AP-1 and NF-κB, both of which play an important role in controlling *MMP-9* gene expression. Hong *et al.* (39) reported that TPA activates AP-1 with an increase in MMP-9 expression in Caki-1 cells. Shin *et al.* (40) indicated that TPA-induced upregulation of MMP-9 is mediated by the NF-κB cascade in human lung epithelial cells. However, QUE's involvement in the transcriptional regulation of MMP-9 is still unclear. The present

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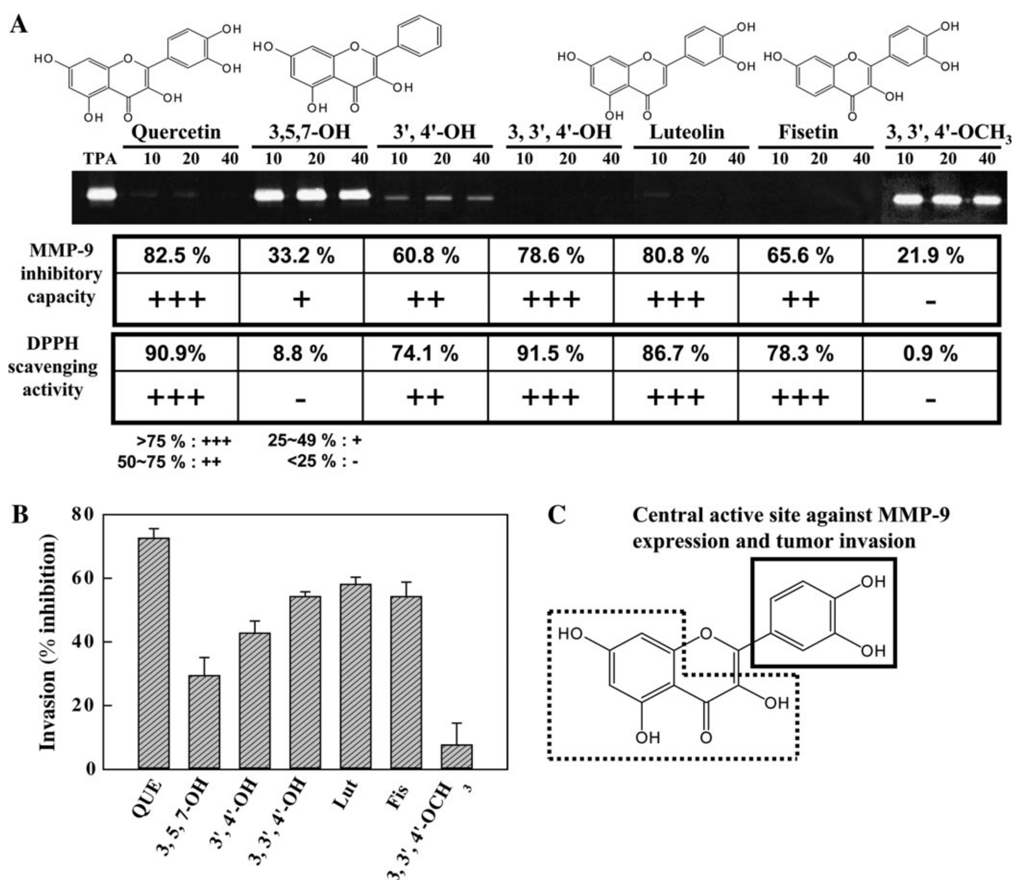

Fig. 7. The OH group at C3, C5 or C7 increases the inhibitory potency of 3′,4′-diOH groups in QUE against MMP-9 expression. (**A**) QUE and its structural-related analogs (10, 20 and 40 μM) were coincubated with TPA for 24 h, the conditioned media were collected and subjected to gelatin zymography (upper), and the inhibitory capacity of the different flavonoids at a dose of 40 μM against MMP-9 was examined using a fluorometric MMP-9 activity assay kit as described in Materials and Methods. Data are represented by the equation: [1 − (compound-treated group/TPA-treated group)] × 100%, and each value was computed from at least three independent experiments in triplicate (middle). The antioxidant properties of differential flavonoids (40 μM) were examined by a DPPH-free radical analysis. Data were represented by the equation: [1 − (compound-treated group/dimethyl sulfoxide-treated group)] × 100%, and each value was computed from at least three independent experiments in triplicate (lower). (**B**) The anti-invasive ability of QUE and its structural analogs were assessed by cotreatment with the indicated flavonoid (40 μM) and TPA for 48 h via an *in vitro* transwell invasion assay. Data are expressed as the percentage of inhibition by the equation: [1 − (the invaded cell number of compound-treated group/TPA-treated group)] × 100%. Each value was computed from at least three independent experiments in triplicate. (**C**) The proposed SAR of QUE in suppressing TPA-induced MMP-9 expression and the invasiveness of breast cancer cells. The 3′,4′-diOH group plays a central role in QUE inhibition of TPA-induced MMP-9 activation and migration (solid line), and the OH group at C3, C5 or C7 increases the inhibitory potency of 3′,4′-diOH flavone against TPA-induced MMP-9 activity and migration (dot line).

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study showed that activation of AP-1, but not NF-κB, was involved in TPA-induced MMP-9 expression in MCF-7 cells. Stimulation of the AP-1 component, c-Jun expression and its promoter activity was observed in TPA-treated cells, and these were blocked by pretreatment with Rot and PD. This indicates that TPA induction of AP-1 activation is located downstream of the PKCδ and ERK cascades that critically participate in MMP-9 expression. Moreover, QUE's significant suppression of c-Jun expression accompanied by a reduction in AP-1 transcriptional activity therefore inhibited *MMP-9* gene expression.

Flavonoid glycosides are derivatives of flavonoids produced during biosynthetic processing and often coexist with their aglycones in plants. Flavonoid glycosides are reported to enhance their hydrophilic ability and prevent lipid peroxidation *in vitro* (41); however, studies have supported aglycone flavonoids being the main active moiety for bioactivities. Our previous studies showed the apoptosis-inducing effect and neuroprotective activity of QUE, but not its glycosides, QUI or RUT (9,42). Data from our recent (43) and current work also support QUE, but not QUI or RUT, having the ability to inhibit the proliferation and invasion of breast tumor cells. Suppression of the migratory and invasive ability by QUE, but not QUI or RUT, while reducing MMP-9 expression in MCF-7 and MDA-MB-231 cells was identified in the present study. Several studies have indicated that numerous bioactivities exhibited by flavonoids are attributed to their antioxidant properties, and these are correlated to hydroxylation patterns in the flavone backbone. Sim *et al.* (44) reported that higher numbers of OH groups are present in flavones possessing greater antioxidant and anti-MMPs activities, whereas Zhang *et al.* (45) argued that the hydroxylated position is more important in breast cancer-resistant protein inhibition. However, the SAR analysis regarding the relationship between the antioxidant potential and MMP-9 suppression by QUE is still unclear. Data from the present study showed that the anti-MMP-9 effect of QUE is associated with its antioxidant activity and that was attributed to the 3′,4′-diOH group in the B-ring, but not the 3,5,7-triOH group in the A- or C-ring of QUE. We further determined the inhibitory extent of QUE and its structural analogs against MMP-9 activity, and the respective invasiveness potentials of MCF-7 cells were QUE (3,5,7,3′,4′-pentaOH flavone) > Lut (5,7,3′,4′-tetraOH flavone) > 3,3′,4′-triOH flavone > Fis (3,7,3′,4′-tetraOH flavone) > 3′,4′-diOH flavone > 3,5,7-triOH flavone > 3,3′,4′-triOH_3 flavone. It is therefore speculated that (i) dihydroxyl groups at C3′ and C4′ of QUE are crucial for MMP-9 inhibition and (ii) the position rather than the number of -OH groups in QUE is more important for the inhibition of MMP-9. Together, our present study provides the molecular mechanism and the probable active structure for QUE

against MMP-9 expression. Suppression of the PKCδ/ERK/AP-1-signaling pathway by QUE downregulates MMP-9 expression, and the inhibitory activity of QUE was predominantly mediated by 3′,4′-diOH groups, whereas the 3, 5 or 7-OH group in QUE potentiated the inhibitory effect of the 3′,4′-diOH groups. QUE therefore has the potential to be a potent chemopreventive drug in therapeutic strategies for the metastasis of breast cancer in the future.

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