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A citrus polymethoxyflavonoid, nobiletin, is a novel MEK inhibitor that exhibits antitumor metastasis in human fibrosarcoma HT-1080 cells

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Abstract

The activation of mitogen-activated protein/extracellular signal-regulated kinase (MEK) is well known to be associated with tumor invasion and metastasis. We previously reported that a polymethoxyflavonoid, nobiletin (5,6,7,8,3′,4′-hexamethoxyflavone), derived from *Citrus depressa* (Hayata), inhibits the phosphorylation of MEK and thereby suppresses matrix metalloproteinase (MMP) expression in a tumor-metastasis stimulator, 12-*O*-tetradecanoyl phorbol 13-acetate (TPA)-stimulated human fibrosarcoma HT-1080 cells [Mol. Cancer Ther. 3 (2004) 839–847]. In the present study, we investigated whether or not nobiletin might directly influence MEK activity to exhibit the antitumor metastatic activity *in vitro*. MEK kinase assay using myelin basic protein (MBP) revealed that TPA-augmented MEK activity in HT-1080 cells and that the augmented MEK activity was diminished by nobiletin treatment. In addition, the decrease in MEK activity caused by nobiletin was found to inhibit the phosphorylation of extracellular regulated kinases (ERK), a downstream signaling factor for MEK. Furthermore, when an immunoprecipitated active MEK was incubated with nobiletin under cell-free conditions, nobiletin was found to inhibit the MEK-mediated MBP phosphorylation. In contrast, other citrus polymethoxyflavonoids such as 3-hydroxy-5,6,7,8,3′,4′-hexamethoxyflavone (natsudaidain) and 3,5,6,7,8,3′,4′-heptamethoxyflavone, did not directly inhibit MEK activity. Moreover, natsudaidain and 3,5,6,7,8,3′,4′-heptamethoxyflavone exhibited no or less inhibitory effect than nobiletin on the proMMP-9/progelatinase B production in HT-1080 cells. Therefore, these results provide novel evidence that nobiletin directly inhibits MEK activity and decreases the sequential phosphorylation of ERK, exhibiting the antitumor metastatic activity by suppressing MMP expression in HT-1080 cells.

Keywords: Flavonoid; Nobiletin; Natsudaidain; 3,5,6,7,8,3′,4′-Heptamethoxyflavone; MEK; ERK; Matrix metalloproteinase; Antitumor metastasis

Flavonoids derived from medicinal plants possess the pharmacological effect of preventing tumor progression by inhibiting tumor-cell proliferation and tumor invasion [1,2]. The prominent flavonoids, quercetin, and genistein, have been shown to exert an anti-tumorigenic effect on malignant tumors [3–7]. In addition, genistein has been reported to suppress the expression of membrane type 1 matrix metalloproteinase (MT1-MMP) and MMP-9/gelatinase B in human breast carcinoma cells [6,8]. Huang et al. [7] also found that quercetin suppresses the epidermal

Abbreviations: MEK, mitogen-activated protein/extracellular signal-regulated kinase; TPA, 12-*O*-tetradecanoyl phorbol 13-acetate; MMP, matrix metalloproteinase; ERK, extracellular regulated kinases; HEPT, 3,5,6,7,8,3′,4′-heptamethoxyflavone; MEM, Eagle's minimum essential medium; PBS(−), Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline; ECL, enhanced chemiluminescence; PKA, p21-activated kinase; PKC, protein kinase C.

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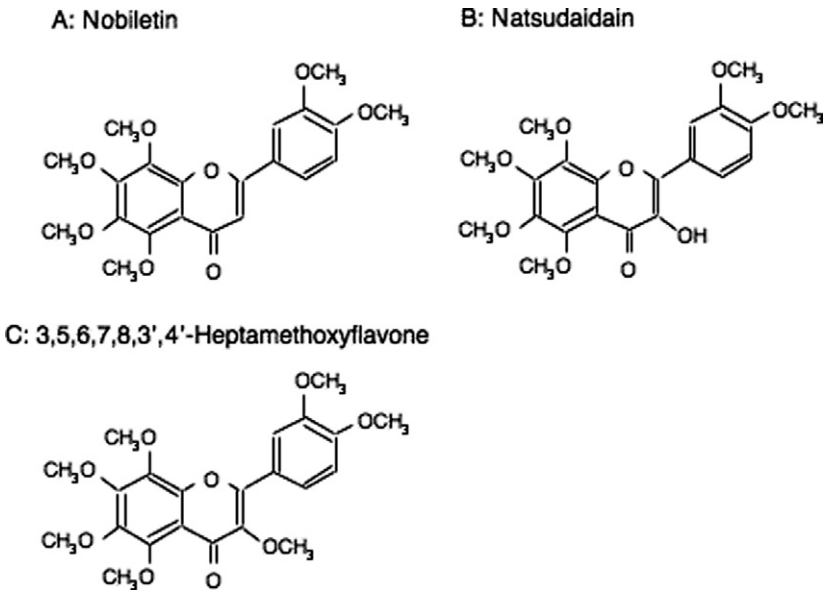

Fig. 1. Structure of nobiletin (A), natsudaidain (B), and 3,5,6,7,8,3′,4′-heptamethoxyflavone (C).

growth factor-induced production of MMPs-2 and -9 in human squamous carcinoma A431 cells. And we previously reported that nobiletin (5,6,7,8,3′,4′-hexamethoxyflavone) (Fig. 1A), a major component in the juice from *Citrus depressa* (Hayata), inhibits the invasive activity of human fibrosarcoma HT-1080 cells, not only by suppressing the expression of MMPs, but also by augmenting the production of tissue inhibitor of metalloproteinases-1 [9]. In addition, Minagawa et al. [10] reported that nobiletin prevents tumor-cell invasion due to a decrease of MMP-9 production in the peritoneal dissemination of human gastric carcinoma in severe combined immunodeficient mice. Furthermore, we reported the similar preventive effect of nobiletin for ECM breakdown due to the transcriptional suppression of MMPs-1, -3, and -9 in articular chondrocytes and synoviocytes from rabbits [11] and humans [12], respectively. Therefore, nobiletin is likely to be an effective candidate for antitumor metastatic therapy.

Various pathological events including tumor invasiveness are considered to result from abnormal regulation of the activation of intracellular signal molecules such as mitogen-activated protein kinase and tyrosine kinase [13,14]. Regarding the regulation of intracellular signaling by flavones, genistein, a well-known tyrosine kinase inhibitor, has been reported to block signal-transduction pathways mediated by mitogen-activated protein kinase in human neutrophils [15] and by 1-phosphatidylinositol 4-phosphate 5-kinase in human ovarian carcinoma OVCAR-5 cells [4]. Quercetin has been reported to inhibit protein kinase C and/or tyrosine kinase in human HL-60 leukemia cells [5], as does phosphatidylinositol kinase in human breast carcinoma MDA-MB-435 cells [3]. Furthermore, the overexpression of Ras and the augmented phosphorylation of mitogen-activated protein/extracellular signal-regulated kinase (MEK)1/2, a downstream kinase in the Ras signaling pathway, promote tumor invasion due to the augmentation of MMP expression [16,17]. We previously reported that nobiletin decreases the phosphorylation of MEK along with the inhibition of MMP expression, whereas there was no change in a conventional Ras/Raf-mediated signal in human fibrosarcoma HT-1080 cells [18]. In addition, the autophosphorylation mechanism of MEK1/2 using the recombinant protein has been reported [19,20]. Therefore, we speculate that nobiletin might directly regulate MEK activity, resulting in antitumor metastatic actions.

In the present study, we demonstrated that nobiletin directly inhibited MEK activity and decreases the sequential phosphorylation of ERK, thus exhibiting the antitumor metastatic activity by suppressing MMP expression in HT-1080 cells.

Materials and methods

Cell culture and treatment. Human fibrosarcoma HT-1080 cells (Health Science Research Resources Bank, Osaka, Japan) were cultured in Eagle's minimum essential medium (MEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (JRH Biosciences, Lenexa, KS) and nonessential amino acids (Invitrogen). After reaching confluence, the cells were treated with nobiletin, 3-hydroxy-5,6,7,8,3′,4′-hexamethoxyflavone (natsudaidain), and 3,5,6,7,8,3′,4′-heptamethoxyflavone (HEPT) (Fig. 1B and C, respectively) [21] in the presence or absence of 12-*O*-tetradecanoyl phorbol 13-acetate (TPA) (10 nM) in serum-free medium containing 0.2% lactalbumin hydrolysate (Sigma–Aldrich, St. Louis, MO) for up to 24 h. The harvested culture media were stored at −20 °C until use, and the cells were subjected to the preparation of the cytosol fraction.

Preparation of cytosol fraction. HT-1080 cells were washed once with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline [PBS(−)] and then homogenized in 10 mM Hepes–KOH (pH 7.8), 10 mM KCl, 0.1 mM EDTA, 0.1% Nonidet P-40, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 5 μM pepstatin, 10 μM leupeptin, and 1 mM sodium orthovanadate. After centrifugation at 5000*g* at 4 °C, the resultant supernatant was collected as the cytosol fraction and used for Western

blotting for phosphorylated proteins and in an *in vitro* kinase assay for MEK activity.

Western blotting. Aliquots (30 μg) of cytosol proteins were subjected to Western blotting using specific rabbit antibody against unphosphorylated or phosphorylated ERK (Thr202/Tyr204) (New England Biolaboratories, Beverly, MA) under non-reducing conditions [18]. The unphosphorylated or phosphorylated ERK was detected using enhanced chemiluminescence (ECL) Western blotting detection reagents (Amersham Bioscience, Tokyo, Japan) after being complexed with horseradish peroxidase-conjugated goat anti-rabbit IgG (New England Biolaboratories). Relative amounts of the immunoreactive proteins were quantified by densitometric scanning using Image Analyzer LAS-1000 plus (Fujifilm, Tokyo, Japan).

Immunoprecipitation and in vitro MEK assay. Aliquots (500 μg) of the cytosol proteins were incubated with rabbit anti-[phospho-MEK (Ser217/221)] antibody (New England Biolaboratories) for 18 h at 4 °C, and then with protein A-Sepharose (Amersham Bioscience) for another 2 h at 4 °C. After centrifugation at 10,000*g* at 4 °C, the resultant precipitate containing phosphorylated MEK was resuspended in Assay Dilution buffer I [20 mM MOPS (pH 7.2), 25 mM β-glycerol phosphate, 5 mM EGTA, 1 mM sodium orthovanadate, and 1 mM dithiothreitol] (Upstate Biotechnology, Lake Placid, NY). *In vitro* MEK assay was performed using a MEK substrate, myelin basic protein (MBP) (Upstate) [22] and [γ-^{32}P] ATP (5.5 kBq) (Dupont NEN, Boston, MA) for 15 min at 37 °C in Assay Dilution buffer I supplemented with or without nobiletin, natsudaidain or HEPT. After completing the reaction, the radioactivity of ^{32}P-labeled MBP spotted on p81 phosphocellulose paper (Upstate) was measured by a liquid scintillation counter (Aloka, Tokyo, Japan).

Statistical analysis. A one-way analysis of variance (ANOVA) was used for statistical analysis. The Fisher test was applied when multiple comparisons were performed.

Results

Decrease in MEK activity and ERK phosphorylation in nobiletin-treated HT-1080 cells

We previously reported that nobiletin exhibits antitumor metastatic action at 8–64 μM without cytotoxicity in HT-1080 cells [9]. Thus, to examine whether or not nobiletin might influence MEK activity in cultured HT-1080 cells, we treated the cells with the maximal concentration of nobiletin at 64 μM [9,18]. As shown in Fig. 2A, HT-1080 cells were found to constitutively express MEK activity, and the enzymic activity was enhanced by a tumor-metastasis stimulator, TPA [9,18]. When the cells were treated with nobiletin (64 μM) in the presence of TPA, nobiletin was found to decrease the TPA-augmented MEK activity. In addition, to confirm whether or not nobiletin might implicate a downstream signal of MEK, we examined the phosphorylation of ERK in the nobiletin-treated HT-1080 cells. As shown in Fig. 2B, the phosphorylated ERK was detected in untreated control cells and TPA augmented the ERK phosphorylation (*upper panel*). Nobiletin was found to interfere with the TPA-augmented ERK phosphorylation (40% inhibition) in HT-1080 cells (Fig. 2B, *lower panel*). However, there was no change in the level of ERK protein under treatment with nobiletin and/or TPA (Fig. 2B, *middle panel*). These results suggested that the decrease in MEK activity produced by nobiletin caused the inhibition of ERK phosphorylation without altering ERK production in HT-1080 cells.

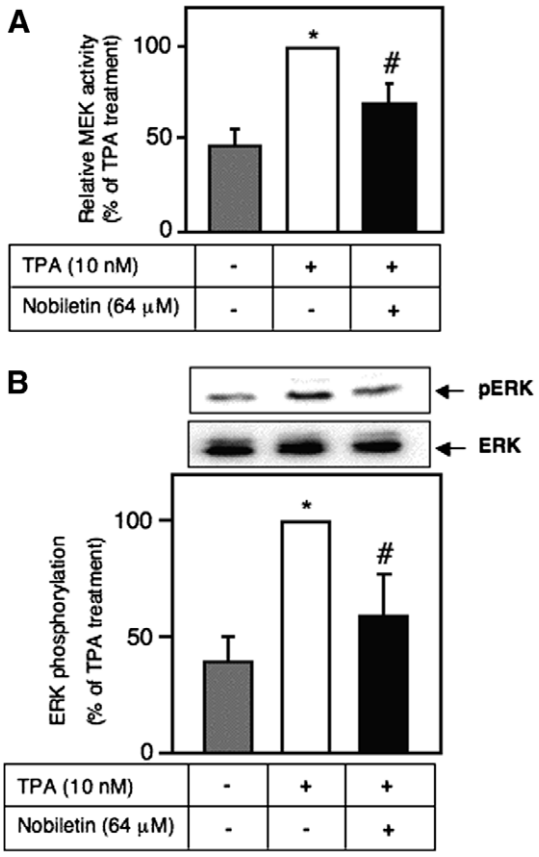

Fig. 2. Nobiletin inhibits the MEK activity and ERK phosphorylation in HT-1080 cells. Confluent HT-1080 cells were pretreated with nobiletin (64 μM) for 1 h, and then treated with TPA (10 nM) for another 3 h. (A) Immunoprecipitates from the cytosol fractions using phospho-MEK antibody were subjected to *in vitro* kinase assay for MEK as described in Materials and methods. The MEK activity was expressed by taking TPA-treated cells as 100%. (B) The harvested cytosol fractions were subjected to Western blot analysis for phosphorylated ERK (pERK) (*upper panel*) or ERK (*middle panel*), as described in Materials and methods. The relative amounts of pERK were quantified by densitometric scanning and expressed by taking TPA-treated cells as 100% (*lower panel*). Data are shown as means ± SD of three independent experiments. *Significantly different from untreated cells ($p < 0.05$). #Significantly different from TPA-treated cells ($p < 0.05$).

Nobiletin directly inhibits MEK activity and thereby causes antitumor metastatic action

Our previous report revealed that nobiletin does not influence the Ras activity and Raf phosphorylation in HT-1080 cells [18]. In addition, the autophosphorylation of MEK has been reported to stimulate the ERK signal [19,20]. Therefore, to address a hypothesis that nobiletin might directly inhibit MEK activity in HT-1080 cells, we carried out an *in vitro* kinase assay using the immunoprecipitated active MEK. As shown in Fig. 3, nobiletin was found to directly inhibit the enzymic activity of immunoprecipitated active MEK. In addition, to clarify whether or not the MEK inhibitory activity might be specific to nobiletin, we performed the same experiment using another polymethoxyflavonoids, natsudaidain and HEPT (Fig. 1B

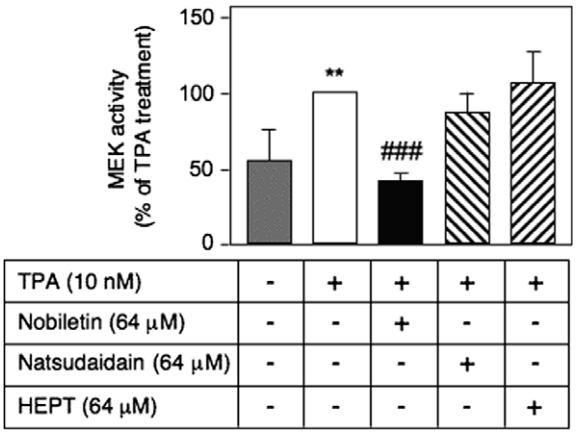

Fig. 3. Nobiletin, but not natsudaidain and HEPT, directly inhibits MEK activity *in vitro*. Confluent HT-1080 cells were treated with TPA (10 nM) for 1 h. After the treatment, the harvested cytosol fractions were subjected to immunoprecipitation using phospho-MEK antibody for the purification of active MEK. Nobiletin, natsudaidain, and HEPT (64 μM) were preincubated with the immunoprecipitated active MEK, and then the MEK activity was measured as described in Materials and methods. The MEK activity was expressed by taking TPA-treated cells as 100%. Data are shown as means ± SD of three independent experiments. **Significantly different from untreated cells ($p < 0.01$). ###Significantly different from TPA-treated cells ($p < 0.001$).

and C). The *in vitro* kinase assay revealed that neither natsudaidain nor HEPT inhibited MEK activity (Fig. 3). Furthermore, to address the hypothesis that the inhibition of MEK activity might implicate antitumor metastasis, we compared the effects of nobiletin, natsudaidain, and HEPT on the production of proMMP-9 in HT-1080 cells. As shown in Fig. 4, in contrast to nobiletin, natsudaidain, and HEPT were found to show no or less inhibitory action in proMMP-9 production. In addition, we confirmed that nobiletin, natsudaidain, nor HEPT directly inhibits the gelatinolytic activity of MMP-9 *in vitro* (data not shown). Thus, these results suggested that nobiletin is a dominant polymethoxyflavonoid with direct inhibitory activity against MEK, exhibiting antitumor metastatic action.

Discussion

Since mutational activation of Ras protein promotes tumor proliferation and metastasis [23,24], the inhibition of Ras signaling including Raf and MEK is likely to be an effective strategy for anti-tumorigenesis and antitumor metastasis [24,25]. For instance, a specific MEK inhibitor, PD98059, has been reported to exhibit the antitumor cell proliferation and the antitumor invasion and metastasis activities against various tumor cell species *in vivo* and *in vitro* [9,25]. In the present study, by applying the *in vitro* kinase assay to the immunoprecipitated active MEK and MBP, we demonstrated that nobiletin directly inhibited the MEK activity. Furthermore, the inhibition of MEK activity by nobiletin caused the sequential suppression of ERK phosphorylation and the production of proMMP-9 in HT-1080 cells. Similar inhibition of MEK activity and proMMP-9 production by nobiletin was also observed in human squamous carcinoma SAS cells (data not shown). As with PD98059, a synthetic flavonoid, these results suggest that nobiletin is yet another natural flavonoid with inhibitory activity against MEK. Taken together with our previous reports that nobiletin exhibits the antitumor metastasis *in vivo* and *in vitro* [9,10], the antitumor metastatic actions of nobiletin are likely to result from the direct inhibition of MEK activity in tumor cells.

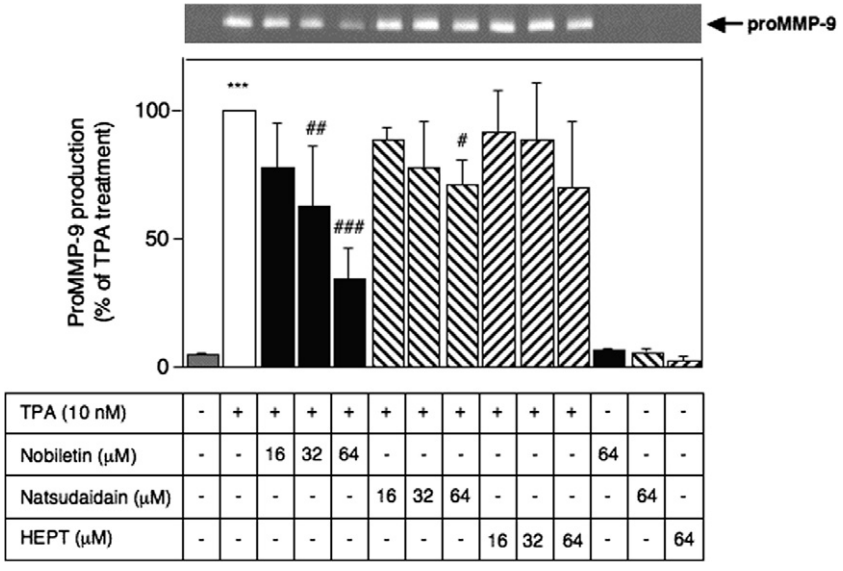

Fig. 4. No or less inhibitory effect of natsudaidain and HEPT on the production of proMMP-9 in HT-1080 cells compared to nobiletin. Confluent HT-1080 cells were treated with nobiletin (16–64 μM), natsudaidain (16–64 μM), or HEPT (16–64 μM) in the presence or absence of TPA (10 nM). The harvested culture media were subjected to gelatin zymography. The relative amounts of proMMP-9 were quantified by densitometric scanning and expressed by taking TPA-treated cells as 100%. Data are shown as means ± SD of three independent experiments.***Significantly different from untreated cells ($p < 0.001$). #,##,###Significantly different from TPA-treated cells ($p < 0.05$, 0.01, and 0.001, respectively).

The activation of MEK has been reported to be regulated not only by a conventional Ras/Raf signal pathway [16,17,24,25] but also by the autophosphorylation of MEK [19,20] and another novel mechanism involving p21-activated kinase (PAK) [26]. We previously reported that nobiletin inhibited the phosphorylation of MEK without affecting Ras activity and Raf phosphorylation in the TPA-stimulated HT-1080 cells [18]. Regarding the inhibition of MEK phosphorylation by nobiletin, our findings suggest a possible mechanism whereby the nobiletin-mediated direct inhibition of MEK activity causes a decrease in MEK autophosphorylation. On the other hand, Wen-Sheng [27] reported protein kinase C (PKC)-mediated MEK activation via the Ras/Raf independent pathway in TPA-treated hepatoma cell HepG2. In addition, Menard and Mattingly [28] reported that TPA-mediated PKC activation stimulates PAK1 in *myc*-PAK1 plasmid transfected COS7 cells. Therefore, another possible mechanism to explain the inhibition of MEK phosphorylation is that nobiletin may interfere with novel signaling toward MEK, such as a PKC/PAK pathway.

Regarding the structure activity of flavonoids in terms of their biological activities, Iwase et al. [29] reported that HEPT, which has another 3-methoxy residue in the C ring, exhibits more potent anticarcinogenesis activity than nobiletin, suggesting that the increase in methoxy residue in flavonoids is associated with the enhancement of biological activity. In the present study, we revealed that HEPT failed to inhibit MEK activity and proMMP-9 production, indicating that the 3-methoxy addition in nobiletin diminishes the inhibitory activity against MEK and the antitumor metastatic action. However, we earlier demonstrated that HEPT sustained the inhibition of cell proliferation in HT-1080 cells, equally as well as nobiletin (data not shown). Thus, the effect of methoxy substitution in flavonoids may be dependent on cell species and/or types of cell reactions. In particular, the 3-methoxy addition in nobiletin is likely to contribute to the negative regulation against its antitumor metastasis.

Ferial et al. [30] reported that in rat brain, a coplanar flavonoid structure is requisite for the inhibition of PKC activity. Sartor et al. [31] also reported the relationship between the planar structure of a flavonoid and the inhibitory activity against leukocyte lactase and gelatinase from human neuroblastoma and HT-1080 cells by investigating 27 flavonoids. We preliminarily performed a computer analysis to estimate the planarity of the three rings in nobiletin, natsudaidain, and HEPT, and demonstrated that nobiletin possesses the coplanar structure, but a twist exists between the B and A/C rings in natsudaidain and HEPT (data not shown). In addition, as in the case of HEPT, the structural change in natsudaidain resulted in no inhibitory activities against MEK and less inhibitory activity than nobiletin against proMMP-9 production in HT-1080 cells. Therefore, these results allow us to speculate that the 3-substitution to methoxy or hydroxy residue changes the coplanar nobiletin structure, which results in diminishing the MEK inhibitory activity of nobiletin. This hypothesis will be clarified by evaluating the planar structure of these flavonoids in detail, and by investigating whether nobiletin, but not natsudaidain and HEPT, may directly interact with MEK protein to inhibit the enzymic activity.

In conclusion, we demonstrated that nobiletin directly inhibited MEK activity and the sequential ERK activation, which resulted in the suppression of proMMP-9 production in HT-1080 cells. Taken together with the fact that there was no or less inhibition of MEK activity and pro MMP-9 production, respectively, using other nobiletin derivatives, namely, natsudaidain and HEPT, these results provide novel evidence that nobiletin is a natural MEK inhibitor that exhibits the antitumor metastatic effect.

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References

[1] S.N. Nichenametla, T.G. Taruscio, D.L. Barney, J.H. Exon, A review of the effects and mechanisms of polyphenolics in cancer, Crit. Rev. Food Sci. Nutr. 46 (2006) 161–183.
[2] Y. Li, H. Fang, W. Xu, Recent advance in the research of flavonoids as anticancer agents, Mini Rev. Med. Chem. 7 (2007) 663–678.
[3] R.L. Singhal, Y.A. Yeh, N. Praja, E. Olah, G.W. Sledge Jr., G. Weber, Quercetin down-regulates signal transduction in human breast carcinoma cells, Biochem. Biophys. Res. Commun. 208 (1995) 425–431.
[4] F. Shen, G. Weber, Synergistic action of quercetin and genistein in human ovarian carcinoma cells, Oncol. Res. 9 (1997) 597–602.
[5] T.B. Kang, N.C. Liang, Studies on the inhibitory effects of quercetin on the growth of HL-60 leukemia cells, Biochem. Pharmacol. 54 (1997) 1013–1018.
[6] Z.M. Shao, J. Wu, Z.Z. Shen, S.H. Barsky, Genistein exerts multiple suppressive effects on human breast carcinoma cells, Cancer Res. 58 (1998) 4851–4857.
[7] Y.T. Huang, J.J. Hwang, P.P. Lee, F.C. Ke, J.H. Huang, C.J. Huang, C. Kandaswami, E. Middleton Jr., M.T. Lee, Effects of luteolin and quercetin, inhibitors of tyrosine kinase, on cell growth and metastasis-associated properties in A431 cells overexpressing epidermal growth factor receptor, , Br. J. Pharmacol. 128 (1999) 999–1010.
[8] M. Yu, E.T. Bowden, J. Sitlani, H. Sato, M. Seiki, S.C. Mueller, E.W. Thompson, Tyrosine phosphorylation mediates Con A-induced membrane type 1-matrix metalloproteinase expression and matrix metalloproteinase-2 activation in MDA-MB-231 human breast carcinoma cells, Cancer Res. 57 (1997) 5028–5032.
[9] T. Sato, L. Koike, Y. Miyata, M. Hirata, Y. Mimaki, Y. Sashida, M. Yano, A. Ito, Inhibition of activated protein-1 binding activity and phosphatidylinositol 3-kinase pathway by nobiletin, a polymethoxy flavonoid, results in augmentation of tissue inhibitor of metalloproteinases-1 production and suppression of production of matrix metalloproteinase 1 and 9 in human fibrosarcoma HT-1080 cells, Cancer Res. 62 (2002) 1025–1029.
[10] A. Minagawa, Y. Otani, T. Kubota, N. Wada, T. Furukawa, K. Kumai, K. Kameyama, Y. Okada, M. Fujii, M. Yano, T. Sato, A. Ito, M. Kitajima, The citrus flavonoid, nobiletin, inhibits peritoneal dissemination of human gastric carcinoma in SCID mice, Jpn. J. Cancer Res. 92 (2001) 1322–1328.

[11] J. Ishiwa, T. Sato, Y. Mimaki, Y. Sashida, M. Yano, A. Ito, Citrus flavonoid, nobiletin, suppresses the production and gene expression of matrix metalloproteinase 9/gelatinase B in rabbit synovial fibroblasts, J. Rheumatol. 271 (2000) 20–25.
[12] N. Lin, T. Sato, Y. Takayama, Y. Mimaki, Y. Sashida, M. Yano, A. Ito, Novel anti-inflammatory actions of nobiletin, a citrus polymethoxy flavonoid, on human synovial fibroblasts and mouse macrophages, Biochem. Pharmacol. 65 (2003) 2065–2071.
[13] P.M. Campbell, C.J. Der, Oncogenic Ras and its role in tumor cell invasion and metastasis, Semin. Cancer Biol. 14 (2004) 105–114.
[14] E. Viala, J. Pouyssegur, Regulation of tumor cell motility by ERK mitogen-activated protein kinases, Ann. NY Acad. Sci. 1030 (2004) 208–218.
[15] A. Mocsai, Z. Jakus, T. Vantus, G. Berton, C.A. Lowell, E. Ligeti, Kinase pathways in chemoattractant-induced degranulation of neutrophils: the role of p38 mitogen-activated protein kinase activated by Src family kinases, J. Immunol. 164 (2000) 4321–4331.
[16] E.J. Bernhard, S.B. Gruber, R.J. Muschel, Direct evidence linking expression of matrix metalloproteinase 9 (92-kDa gelatinase/collagenase) to the metastatic phenotype in transformed rat embryo cells, Proc. Natl. Acad. Sci. USA 91 (1994) 4293–4297.
[17] B.P. Himelstein, E.J. Lee, H. Sato, M. Seiki, R.J. Muschel, Transcriptional activation of the matrix metalloproteinase-9 gene in an H-ras and v-myc transformed rat embryo cell line, Oncogene 14 (1997) 1995–1998.
[18] Y. Miyata, T. Sato, M. Yano, A. Ito, Activation of protein kinase C βII/ε-c-Jun NH_2-terminal kinase pathway and inhibition of mitogen-activated protein/extracellular signal-regulated kinase 1/2 phosphorylation in antitumor invasive activity induced by the polymethoxy flavonoid, nobiletin, Mol. Cancer Ther. 3 (2004) 839–847.
[19] C.F. Zheng, K.L. Guan, Properties of MEKs, the kinases that phosphorylate and activate the extracellular signal-regulated kinases, J. Biol. Chem. 268 (1993) 23933–23939.
[20] A.M. Gardner, R.R. Vaillancourt, C.A. Lange-Carter, G.L. Johnson, MEK-1 phosphorylation by MEK kinase, Raf, and mitogen-activated protein kinase: analysis of phosphopeptides and regulation of activity, Mol. Biol. Cell 5 (1994) 193–201.
[21] M. Itoigawa, K. Takeya, H. Furukawa, Cardiotonic flavonoids from Citrus plants (Rutaceae), Biol. Pharm. Bull. 17 (1994) 1519–1521.
[22] S. Matsuda, H. Kosako, K. Takenaka, K. Moriyama, H. Sakai, T. Akiyama, Y. Gotoh, E. Nishida, Xenopus MAP kinase activator: identification and function as a key intermediate in the phosphorylation cascade, EMBO J. 11 (1992) 973–982.
[23] K. Giehl, Oncogenic Ras in tumour progression and metastasis, Biol. Chem. 386 (2005) 193–205.
[24] J.M. Shields, K. Pruitt, A. McFall, A. Shaub, C.J. Der, Understanding Ras: 'it ain't over 'til it's over', Trends Cell Biol. 10 (2000) 147–154.
[25] D.T. Dudley, L. Pang, S.J. Decker, A.J. Bridges, A.R. Saltiel, A synthetic inhibitor of the mitogen-activated protein kinase cascade, Proc. Natl. Acad. Sci. USA 92 (1995) 7686–7689.
[26] E.R. Park, S.T. Eblen, A.D. Catling, MEK1 activation by PAK: a novel mechanism, Cell. Signal. 19 (2007) 1488–1496.
[27] W. Wen-Sheng, Protein kinase C α trigger Ras and Raf-independent MEK/ERK activation for TPA-induced growth inhibition of human hepatoma cell HepG2, Cancer Lett. 239 (2006) 27–35.
[28] R.E. Menard, R.R. Mattingly, Cell surface receptors activate p21-activated kinase 1 via multiple Ras and PI3-kinase-dependent pathways, Cell Signal. 15 (2003) 1099–1109.
[29] Y. Iwase, Y. Takemura, M. Ju-ichi, M. Yano, C. Ito, H. Furukawa, T. Mukainaka, M. Kuchide, H. Tokuda, H. Nishino, Cancer chemopreventive activity of 3,5,6,7,8,3′,4′-heptamethoxyflavone from the peel of citrus plants, Cancer Lett. 163 (2001) 7–9.
[30] P.C. Ferriola, V. Cody, E. Middleton Jr., Protein kinase C inhibition by plant flavonoids. Kinetic mechanisms and structure–activity relationships, Biochem. Pharmacol. 38 (1989) 1617–1624.
[31] L. Sartor, E. Pezzato, I. Dell'Aica, R. Caniato, S. Biggin, S. Garbisa, Inhibition of matrix-proteases by polyphenols: chemical insights for anti-inflammatory and anti-invasion drug design, Biochem. Pharmacol. 64 (2002) 229–237.