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Interactive effects of polymethoxy flavones from *Citrus* on cell growth inhibition in human neuroblastoma SH-SY5Y cells

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Abstract—Much evidence indicates that typical phytochemicals such as resveratrol, epigallocatechin gallate, and curcumin have a growth inhibitory effect against cancer cells when each is tested separately. However, when fruits and vegetables including a mixture of phytochemicals are consumed, it is unclear whether this anti-proliferative activity is elicited in the body. Initially, we found that nobiletin, a typical polymethoxy flavone from *Citrus*, had a preventive effect on H_2O_2-induced apoptosis at 20–30 μM in human neuroblastoma SH-SY5Y cells. Nobiletin acted as a signal modulator to attenuate the activation of the intrinsic pathway of the apoptosis induced by H_2O_2 exposure. On the other hand, tangeretin and 5-demethyl nobiletin, which are also polymethoxy flavones from *Citrus*, were shown to have a growth inhibitory effect by us and others. These results led us to investigate the interactive effects of these polymethoxy flavones on cell growth. In the present study, we found that tangeretin, nobiletin, and 5-demethyl nobiletin exhibited a cancelling, synergistic, or additive effect when combinations of two of these three compounds were tested. As to the structure–activity relationship, the methyl group at C-5 in nobiletin was shown to contribute to the anti-proliferative effect. By the combined treatment with tangeretin and 5-demethyl nobiletin, the apoptotic cell population and the activity of caspase-3 were synergistically elevated. The finding that tangeretin and 5-demethyl nobiletin induced apoptosis by reducing the mitochondrial membrane potential suggested that an intrinsic pathway of apoptosis was synergistically activated by the combination treatment with tangeretin and 5-demethyl nobiletin. On the other hand, in the combined treatment including nobiletin, the growth inhibitory activity of tangeretin was reduced. These results indicate the relevance of the combination of phytochemicals for the enhancement of the anticancer effect.

1. Introduction

Chemoprevention is the use of a chemical substance of either natural or synthetic origin to prevent, hamper, arrest, or reverse a disease. Phytochemicals are bioactive non-nutrient components of various plant parts, such as seeds, leaves, and rhizomes. Recent epidemiological and preclinical testing have revealed the great potential of phytochemicals in combating cancer and other chronic diseases resulting from oxidative stress induced by free radicals.[1–6] In many dietary studies, the consumption of fruits and vegetables was found to have a significant protective effect.[2,3,5,6] The risk of cancer is reported to be two-fold higher in persons with a low intake of fruits and vegetables than in those with a high intake.[5] It has been established that cancer takes decades to develop in humans through the initiation, promotion, and progression stages for decades. Reactive oxygen species (ROS) play a crucial role in the pathophysiology associated with neoplasia.[1,4] Considerable attention has been focused on identifying naturally occurring anti-oxidative phenolic phytochemicals that are able to decrease ROS levels, but the efficacies of anti-oxidant therapies have been equivocal. By previous studies, we found that phytochemicals do not induce apoptosis directly by their anti-oxidant activity, but act as signal modulators on the apoptotic pathway that is inactivated in cancer cells.[7–12] In this present study, we initially found that nobiletin, a typical polymethoxy flavone from *Citrus*, prevented H_2O_2-induced apoptosis presumably by attenuating the activated intrinsic pathway of apoptosis. This finding prompted us to investigate the interactive effects between nobiletin and other polymethoxy flavones from *Citrus*.

Keywords: Growth inhibition; Synergistic effect; Polymethoxy flavones; Apoptosis; *Citrus*.
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Certainly, it may be possible to prevent the occurrence of cancer to some extent by phytochemicals; however, various phytochemicals in dietary vegetables and fruits could interact among them, resulting in a synergistic or additive, or cancelling effect on their anticancer action. In this study, we examined the interactive effects of three major polymethoxy flavones from *Citrus* on cell growth in vitro and found that various combinations of two of them resulted in a cancelling, synergistic, or additive effect on growth inhibition of human neuroblastoma SH-SY5Y cells, suggesting the relevance to take optimized phytochemicals for reducing the risk of cancer.

2. Results

2.1. Preventive effect of nobiletin on H_2O_2-induced cell death in human neuroblastoma SH-SY5Y cells

Initially, we examined the anti-apoptotic activity of nobiletin against H_2O_2-induced cell death in human neuroblastoma SH-SY5Y cells. We have previously reported that the cell death elicited by H_2O_2 is mainly due to apoptosis[13,14] and that the apoptosis, which is exerted by the intrinsic pathway, is significantly reduced by the co-incubation with a lignophenol derivative, lig-8.[13] Therefore, we focused on the preventive effect of nobiletin on apoptosis. As shown in Figure 2, 60–70% of the apoptotic cell death induced by exposure of SH-SY5Y cells to 200 μM H_2O_2 for 12 h, determined by Hoechst33342 staining, was significantly reduced in a dose-dependent manner by nobiletin. At 30 μM nobiletin apoptotic cell death was reduced to approximately 30%. The DNA ladder formation was also decreased by the addition of 30 μM nobiletin (Fig. 2B), which was well correlated with the results shown in Figure 2A. Thus, we concluded that nobiletin exerted a preventive effect against H_2O_2-induced cell death of SH-SY5Y cells. In the time course, the apoptotic cell death examined by Hoechst33342 nuclear staining emerged 6 h after the treatment, concurrently with appearance of the active form of caspase-3 as detected by Western blot analysis (data not shown).[13] Since the anti-oxidant activity of nobiletin was not shown by the 1,1-diphenyl-2-picrylhydrazyl radical scavenging capacity test, nobiletin most likely affected the intrinsic pathway of apoptosis, which is known to be activated by H_2O_2 exposure.[13] Based on these observations, we compared the growth/survival signaling pathways, that is, the MAP kinases and the serine/threonine Akt kinase ones, between the treatments with H_2O_2 alone and that with H_2O_2 plus 30 μM nobiletin (Fig. 3). Interestingly, the level of phosphorylated Erk (p-Erk) obtained with H_2O_2 was drastically reduced at 1 h after the treatment and then was elevated at 3 h, but not back to the original level. By contrast, in the presence of nobiletin, the overall profile of the Erk phosphorylation pattern was similar to that for the H_2O_2 treatment, but the reduction in phosphorylation was much less. Furthermore, the level of phosphorylated Erk was restored to the control level. In the case of the Akt pathway, the level of phosphorylated Akt (p-Akt) was decreased after the treatment with H_2O_2, whereas its phosphorylation was rather increased from 3 h in the presence of nobiletin. These findings were expected from the expression profiles of p-Erk and p-Akt in NOB alone, that is, the p-Erk1/2 level was highly maintained compared with that in H_2O_2 or H_2O_2 plus NOB, and the expression level of p-Akt and Akt in 3 h-treatment were up-regulated, which reflected on that in H_2O_2 plus NOB. The levels of phos-

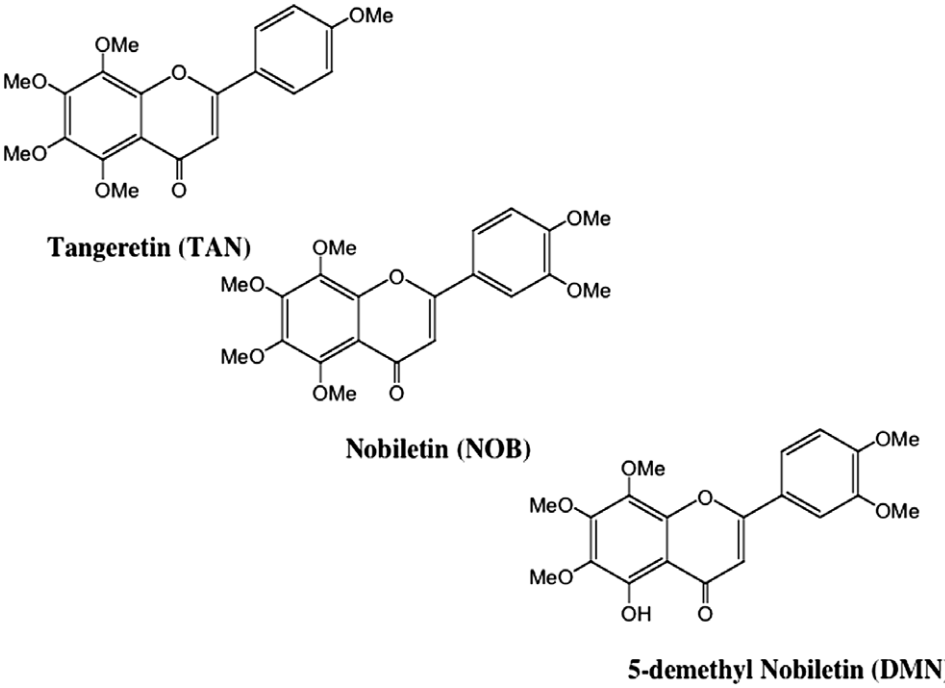


Figure 1. Chemical structure of polymethoxy flavones, tangeretin (TAN), nobiletin (NOB), and 5-demethyl nobiletin (DMN), which are intermediate products in the synthetic pathway of flavones in *Citrus*.

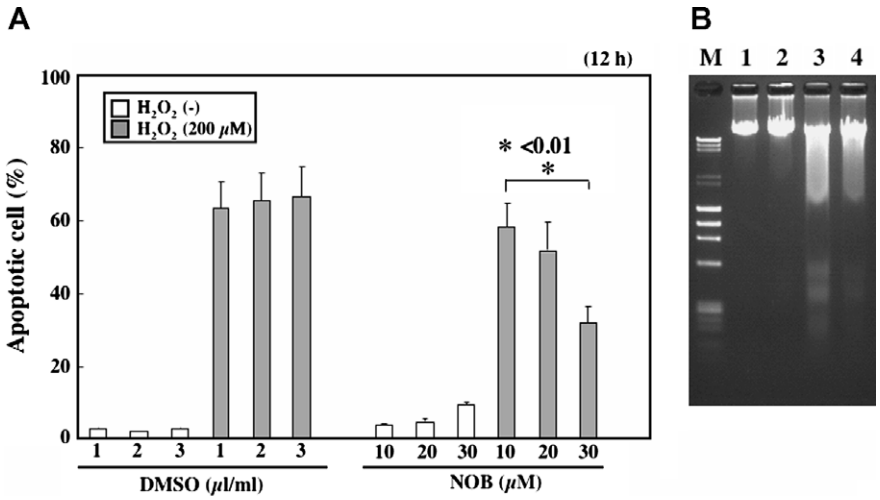


Figure 2. H_2O_2-induced apoptosis and its prevention by the treatment with nobiletin for 12 h in SH-SY5Y cells. (A) Apoptotic cell death evaluated by Hoechst33342 staining. Results are means ± SD of three-independent experiments. *Significant difference between the samples indicated by the bracket ($^*p < 0.01$). (B) Apoptotic cell death evaluated by DNA ladder formation. 1, control (untreated cells); 2, nobiletin 30 μM; 3, H_2O_2 200 μM; 4, H_2O_2 200 μM plus nobiletin 30 μM. M is a lane for DNA size markers.

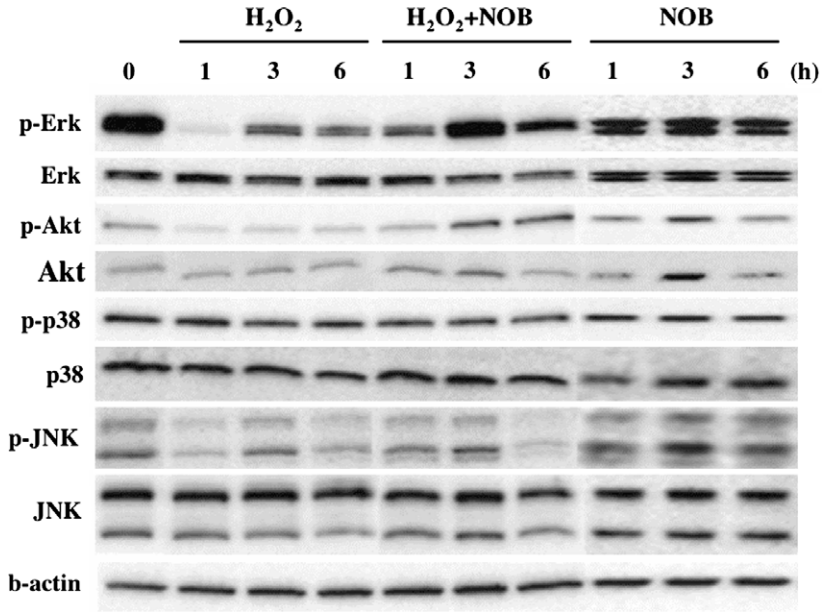


Figure 3. Activation of MAP kinases and serine/threonine kinase Akt in 30 μM nobiletin-treated SH-SY5Y cells over a 6-h period after the addition of 200 μM H_2O_2. Erk, p-Erk, p38, p-p38, JNK, p-JNK, Akt, and p-Akt were examined by Western blot analysis. Cells were inoculated 24 h before treatment at the concentration of 1×10^5. The cells were treated with 200 μM H_2O_2 and then nobiletin 30 μM was added or not at 1 h after the addition of the H_2O_2.

phorylated-p38 (p-p38) at 3 h and phosphorylated-JNK (p-JNK) at 1 h were higher after the treatment with nobiletin. Thus, the dampening of the growth/survival-related Erk1/2 and Akt by the H_2O_2 exposure was counteracted by the addition of nobiletin.

2.2. Interactive effects between polymethoxy flavones of *Citrus*, tangeretin, nobiletin, and 5-demethyl nobiletin on cell growth inhibition

5-Demethyl nobiletin, which was demethylated at the 5-methoxy group, exhibited a considerable growth inhibitory effect on SH-SY5Y cells (Fig. 4), indicating the critical role of the 5-methoxy group in the anti-apoptotic effect of nobiletin. As another major polymethoxy flavone of *Citrus*, we selected tangeretin which has a growth inhibitory effect,[15,16] and investigated the interactive effects on growth inhibition of 3 two-compound combinations (tangeretin × nobiletin, tangeretin × 5-demethyl nobiletin, and nobiletin × 5-demethyl nobiletin) in SH-SY5Y cells. The IC_{50} values of tangeretin and 5-demethyl nobiletin were estimated to be 41.2 μM and 32.5 μM, respectively. As shown in Figure 4, the combined treatment with tangeretin and 5-demethyl nobiletin exhibited a greater anti-proliferative activity than was obtained with either tangeretin or 5-demethyl

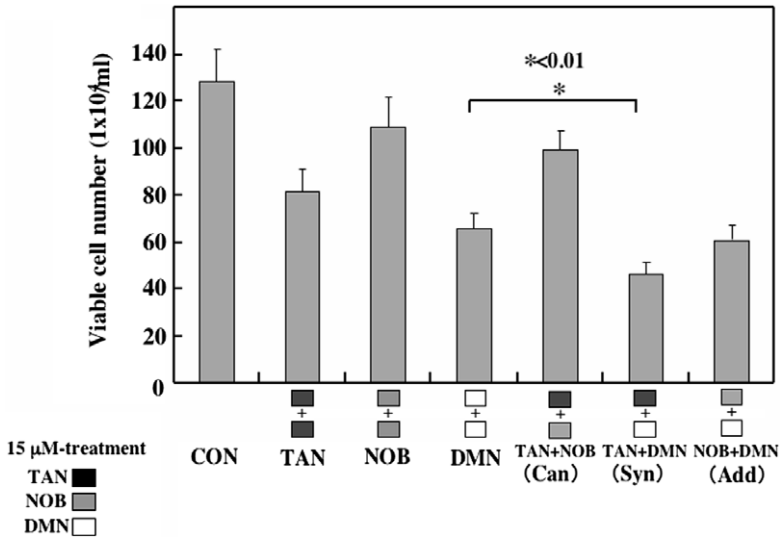


Figure 4. Growth inhibition of SH-SY5Y cells by treatment with single flavonoid and by combined treatments as indicated. Viable cell number was determined by the trypan-blue dye-exclusion test at 48 h after the treatment with the flavone(s). Results are means ± SD of three-independent experiments, each performed in duplicate. Each treatment was performed at 30 μM. Each box deserves 15 μM-treatment. Black box, tangeretin; gray box, nobiletin; open box, 5-demethyl nobiletin. Can, cancelling effect; Add, additive effect; Syn, synergistic effect. *Significant difference between two samples ($^*p < 0.01$).

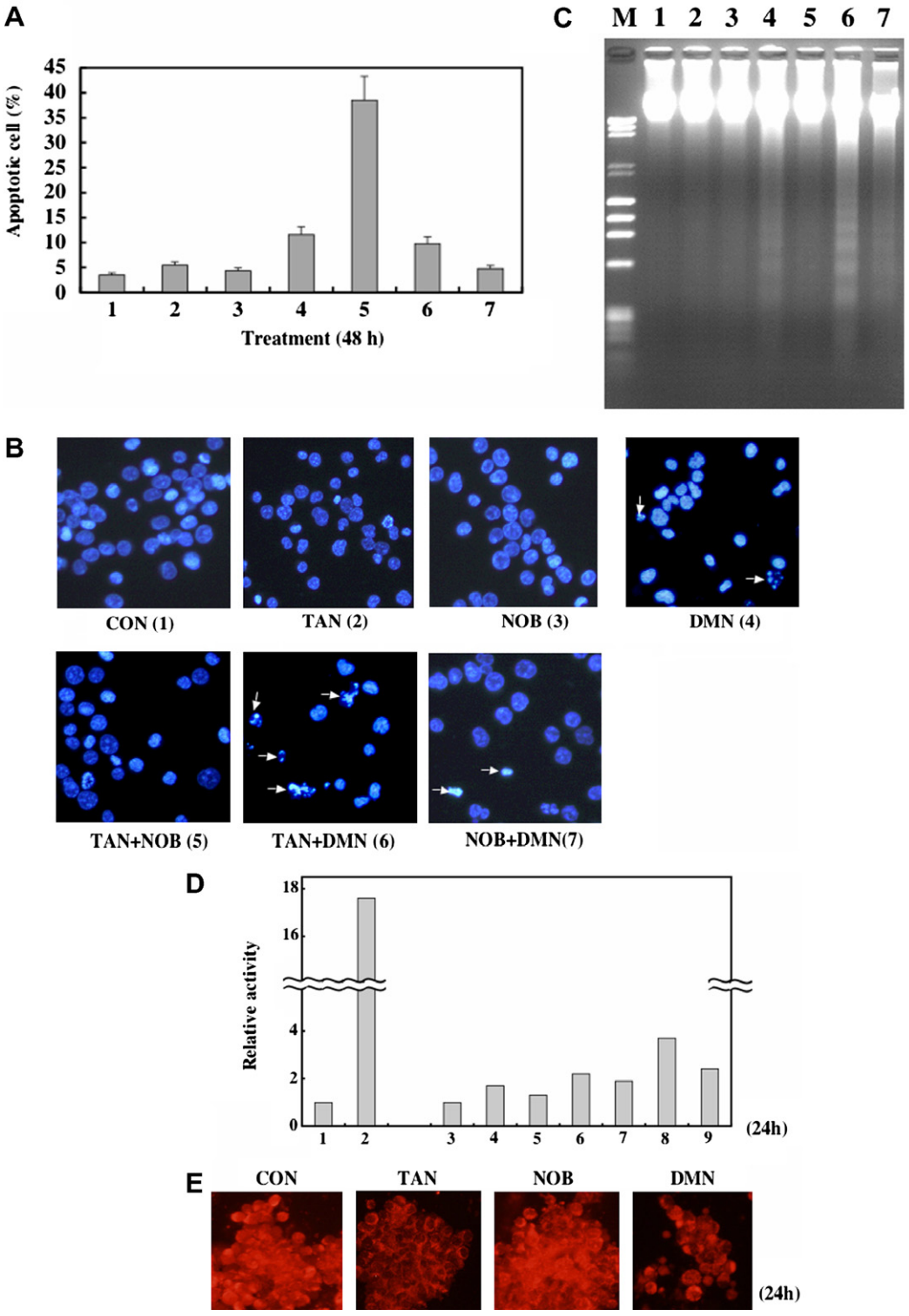
nobiletin alone (synergistic effect). The combination of nobiletin and 5-demethyl nobiletin exhibited an additive growth inhibitory effect; that is, the effect was almost the same as that in the single treatment with 30 μM 5-demethyl nobiletin alone, which has a higher anti-proliferative activity than nobiletin. On the other hand, the combination of tangeretin with nobiletin had a cancelling effect on the tangeretin-induced growth inhibition; that is, the effect of tangeretin was lowered by the co-incubation with nobiletin. Thus, the combined treatment with these polymethoxyl flavones showed various combination-dependent interactive effects (cancelling, synergic, and additive) on cell growth inhibition.

The cell death caused by these compounds was mainly due to apoptosis which was evaluated in terms of Hoechst33342 nuclear staining, the DNA ladder formation, and caspase-3 activity (Fig. 5A–D). As to the apoptosis, the activation of caspase-8 and changes in the expression levels of bcl-2 and bax were not observed by Western blot analysis (data not shown). Since the caspase-3 activation monitored by the appearance of its active form by Western blot analysis did not show quantitative differences between the treatments, we examined its activation by the colorimetric protease assay (Fig. 5D). Hoechst33342 nuclear staining indicated that the rate of apoptotic cell death shown in Figure 5A reflected in the growth inhibition (Fig. 4). The levels of caspase-3 protease activity for the various treatments appear to be compatible with those of cell death (Fig. 5A and D). Thus, the results on growth inhibition, population of apoptotic cells, and caspase-3 activity were almost consistent among the various treatments. Since both tangeretin and 5-demethyl nobiletin reduced the mitochondrial membrane potential in SH-SY5Y cells (Fig. 5E), the activation of the intrinsic pathway in the combination treatments appears to have almost paralleled the results of the apoptosis assays. In the synergistic effect between tangeretin and 5-demethyl nobiletin on growth inhibition, we surveyed the profile of signaling pathways including the Erk and Akt, which were significant in the preventive effect of nobiletin on the H_2O_2-induced apoptosis in SH-SY5Y cells (Fig. 3). The levels of p-Erk and p-Akt were reduced at 24 h after the treatment by the combination of 15 μM tangeretin with 15 μM 5-demethyl nobiletin, compared with those detected following treatment with either alone at 30 μM (Fig. 6).

Figure 5. Apoptotic cell death of SH-SY5Y cells caused by the treatment with flavone(s) for 48 h. (A and B) Graphic results (A) and photomicrographs (B) for apoptotic cell death evaluated by Hoechst33342 staining. The results in (A) are means of three-independent experiments. 1, control (untreated cells); 2, tangeretin 30 μM; 3, nobiletin 30 μM; 4, 5-demethyl nobiletin 30 μM; 5, tangeretin 15 μM plus nobiletin 15 μM; 6, tangeretin 15 μM plus 5-demethyl nobiletin 15 μM; 7, nobiletin 15 μM plus 5-demethyl nobiletin 15 μM. (C) Apoptotic cell death evaluated by DNA ladder formation. 1, control (untreated cells); 2, tangeretin 30 μM; 3, nobiletin 30 μM; 4, 5-demethyl nobiletin 30 μM; 5, tangeretin 15 μM plus nobiletin 15 μM: 6, tangeretin 15 μM plus 5-demethyl nobiletin 15 μM; 7, nobiletin 15 μM plus 5-demethyl nobiletin 15 μM. (D) Activity of caspase-3 was measured colorimetrically by using an assay kit. Column designations: 1 and 2, Jurkat cells were treated (2) or not (1) with anti-Fas antibody (CH-11) as a reference of caspase-3-dependent apoptosis; 3, control (untreated SH-SY5Y cells); 4, tangeretin 30 μM; 5, nobiletin 30 μM; 6,5-demethyl nobiletin 30 μM; 7, tangeretin 15 μM plus nobiletin 15 μM: 8, tangeretin 15 μM plus 5-demethyl nobiletin 15 μM; 9, nobiletin 15 μM plus 5-demethyl nobiletin 15 μM. The levels for control cells (lanes 1 and 3) were taken as 1.0. The results are means of three-independent experiments, each performed in duplicate. (E) Mitochondrial membrane potential in SHSY-5Y cells treated with the indicated compound at 30 μM for 24 h. The cells were stained with Mito-Tracker Red (magnification 400×).

3. Discussion

We found that nobiletin had a protective effect against H_2O_2-induced apoptosis. As tangeretin and 5-demethyl nobiletin, other polymethoxy flavones, have anti-proliferative activity, we decided to examine the possible interactive effects of various combinations of these three flavones in this present study. We demonstrated that the combination treatments using two compounds among tangeretin, nobiletin, and 5-demethyl nobiletin, exhibited the interactive effects on cell growth (cancelling, synergistic, or additive) of human neuroblastoma SH-SY5Y cells. As to the anti-apoptotic effect of nobiletin, we considered that nobiletin prevented the H_2O_2-induced activation of the intrinsic pathway leading to apoptosis, as in the case of lig-8,[13] and that the growth-related signaling of the MAP kinases including Erk and the serine/threonine kinase Akt may be associ-



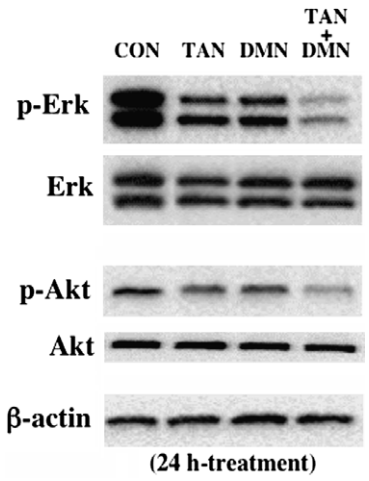


Figure 6. Down-regulation of the phosphorylation of MAP kinase Erk and Akt kinase in the synergic effect of the combined treatment with 15 μM tangeretin and 15 μM 5-demethyl nobiletin for 24 h in SH-SY5Y cells. Erk, p-Erk, Akt, and p-Akt were examined by Western blot analysis.

ated with this preventive effect. On the other hand, we also demonstrated that tangeretin and 5-demethyl nobiletin had a significant apoptosis-inducing activity (tangeretin < 5-demethyl nobiletin) in SH-SY5Y cells (Fig. 5A). Interestingly, the combination treatments (tangeretin × nobiletin, tangeretin × 5-demethyl nobiletin, nobiletin × 5-demethyl nobiletin) exhibited different effects, that is, cancelling, synergistic, and additive, respectively.

We previously reported that the several phytochemicals included in stilbenoids, terpenoids, and xanthones exhibited a cell growth inhibitory effect by inducing apoptosis through the intrinsic pathway via mitochondria where the phytochemicals could associate with the molecules involved in permeability transition pore proteins (PTPP).[7–12] Such signaling was probably related to the down-regulation of p-Erk and/or p-Akt level(s). Perhaps phytochemicals primarily associate with PTPP, leading to the activation of the intrinsic pathway, which is enhanced through the modulating the growth-related signaling(s) including those by MAP kinases and/or Akt. Therefore, we consider that the synergistic effect found in the combination treatment with tangeretin and 5-demethyl nobiletin would be due to enhanced activation of the intrinsic pathway. As expected, the ratio of apoptotic cells examined by Hoechst33342 staining, DNA ladder formation, and the caspase-3 activity was synergistically increased in such a combination compared with the results for treatment with each compound alone. Furthermore, the effect on the phosphorylated levels of Erk and Akt by the combination treatment could enhance the apoptotic cell death. On the contrary, our data suggest that the combination treatments including nobiletin may result in attenuating the intrinsic pathway activated by tangeretin or 5-demethyl nobiletin alone.

NF-κB is also a molecule that contributes to growth. It was reported that curcumin enhanced the apoptosis-inducing activity of Taxol by perturbing of signalings including those by NF-κB and Akt.[17]

Thus, we demonstrated three different effects, that is, cancelling, synergy, and addition, on cell growth inhibition by the use of three polymethoxy flavones from *Citrus*. These in vitro results also indicated that in order to strengthen the growth inhibitory effect, a better combination of phytochemicals could exist.[18,19] This is particularly important, because these findings indicate the necessity of supplement of the extractions including the phytochemicals exhibiting a synergistic effect on chemoprevention and excluding those having a cancelling effect. For better chemopreventive effects, we must find better combinations among phytochemicals included in *Citrus*.

A similar in vivo study was reported by Wallig[19] who showed that two compounds, indol-3-carbinol (I3C) and 1-cyano-3-butane (Crambene) synergistically enhanced detoxication enzyme activity in rat. High dose-combination dietary doses of I3C and Crambene demonstrated enhanced protection from aflatoxin.

We previously reported that all of the phytochemicals tested induced apoptosis through the intrinsic pathway via mitochondria.[7–12] Similarly, tangeretin and 5-demethyl nobiletin exhibited growth suppression through the induction of apoptosis mediated by this pathway, by which they may target different molecules on mitochondria. These findings suggest that the synergistic effect is due to the enhanced activation of this pathway, with or without modulating the signaling of MAP kinase Erk1/2 and/or the serine/threonine Akt kinase. However, another signaling pathway(s) may be associated with the synergistic effect.

The results in this study are preliminary and represent only an event of truly demonstrating the occurrence of synergy, addition and cancelling in in vitro growth inhibition. The unraveling of the mechanisms of the synergistic, additive, and cancelling effects will be necessary to provide firm and definite answers to how these phytochemicals act in vivo.

4. Conclusions

Nobiletin, a typical polymethoxy flavone from *Citrus*, had a preventive effect on H_2O_2-induced apoptosis at 20–30 μM in human neuroblastoma SH-SY5Y cells. Nobiletin acted as a signal modulator to attenuate the activation of intrinsic pathway in the apoptosis induced by H_2O_2 exposure. Tangeretin and 5-demethyl nobiletin, which are also polymethoxy flavones from *Citrus*, were shown to have a growth inhibitory effect. These results led us to investigate the interactive effects of these polymethoxy flavones on cell growth. We found that tangeretin, nobiletin, and 5-demethyl nobiletin exhibited a canceling, synergistic, or addition effect in various combinations of two compounds. Regarding the structure–activity relationship, the 5-methoxy group of 5-demethyl nobiletin was shown to contribute to the anti-apoptotic

effect, as its removal resulted in an anti-proliferative effect. By the combined treatment with tangeretin and 5-demethyl nobiletin, the apoptotic cell population and the activity of caspase-3 were synergistically elevated. The finding that tangeretin and 5-demethyl nobiletin-induced apoptosis through the reduction of the mitochondrial membrane potential suggested that the intrinsic pathway of apoptosis was synergistically activated by the combined treatment with them. On the other hand, in the combined treatment including nobiletin, the growth inhibitory activity by tangeretin alone was reduced. These results indicate the relevance of the combination of phytochemicals for the enhancement of anti-cancer effect.

5. Experimental

5.1. Agents

Three polymethoxy flavones, tangeretin, nobiletin, and 5-demethyl nobiletin (Fig. 1), were purified from *Citrus reticulata* to greater than 98% as described in our previous studies.[20] They were dissolved in DMSO at the concentration of 10 mM, and further diluted to the desired working concentration before use.

5.2. Cell culture, morphological study, and cell viability

Human neuroblastoma SH-SY5Y cells were grown in RPMI-1640 medium supplemented with 5% (v/v) heat-inactivated fetal bovine serum (Sigma, Tokyo) and 2 mM L-glutamine under an atmosphere of 95% air and 5% CO_2 at 37 °C. Cell viability was determined by the trypan blue dye-exclusion test. Co-treatment or single treatment of the cells with the compound(s) was started at 24 h after the cells had been plated at the concentration of $1–1.5 \times 10^5$/ml cells. In H_2O_2-induced cell death, nobiletin was added into the medium 1 h after the addition of H_2O_2. In all the experiments tested, the control cells were co-incubated with DMSO alone.

5.3. Assessment of apoptosis

For assessment of the morphological characteristics of apoptosis, the cells were stained with Hoechst33342 (5 μg/ml) at 37 °C for 30 min, washed once with PBS, resuspended, pipetted dropwise onto a glass slide, and examined by fluorescence microscopy using an Olympus microscope (Tokyo, Japan) equipped with an epi-illuminator and appropriate filters. The cells with condensed and fragmented nuclei stained with Hoechst33342 were assessed to be apoptotic. In all conditions examined, necrotic cell death by H_2O_2 was negligible (less than 2% of the total cells). Approximately 200 cells were counted in four different fields and three-independent experiments were performed. To examine nucleosomal DNA fragmentation by agarose gel electrophoresis, cellular DNA was extracted from whole cells by ethanol precipitation after phenol/chloroform preparation. RNase was added to the DNA solution at the final concentration of 20 μg/ml, and the mixture was incubated at 37 °C for 30 min. After electrophoresis on a 2.5% agarose gel, DNA was visualized by ethidium bromide staining.

5.4. Western blot analysis

Before and after treatment with the compounds, SH-SY5Y cells were washed twice with PBS, lysed in lysis buffer, and then homogenized. The lysis buffer consisted of 2 × PBS, 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 25 × Complete®, a mixture of protease inhibitors (Roche, Penzberg Germany)®, and Phosphatase Inhibitor Cocktail® 1 and 2 (Sigma–Aldrich Co.). A suitable amount of protein of each cell lysate was separated by SDS–PAGE by using a 7.5%, 10%, or 12.5% polyacrylamide gel and the separated proteins were then electroblotted onto a PVDF membrane (Du Pont, Boston, MA). After blockage of non-specific binding sites for 1 h by 5% non-fat milk in TPBS (PBS and 0.1% Tween 20), the membrane was incubated overnight at 4 °C with various antibodies. They included anti-human caspase-3 (Santa Cruz Biotechnology, CA), anti-human caspase-8 (MBL, Nagoya, Japan), anti-human bcl-2 (Santa Cruz Biotechnology), anti-human bax (Santa Cruz Biotechnology), anti-human p44/42 MAP Kinase (Erk1/2; Cell Signaling Technology Inc., MA), anti-human phospho-p44/42 MAPK (Thr202/Tyr204, p-Erk1/2; Cell Signaling Technology Inc.), anti-human p38 MAP kinase (p38, Cell Signaling Technology Inc.), anti-human phospho-p38 MAP kinase (Thr180/Tyr182, p-p38; Cell Signaling Technology Inc.), anti-human SAPK/JNK (JNK, Cell Signaling Technology Inc.), anti-human phospho-SAPK/JNK (Thr183/Tyr185, p-JNK; Cell Signaling Technology Inc.), anti-human Akt (Cell Signaling Technology Inc.), anti-human phospho-Akt (Ser473, p-Akt; Cell Signaling Technology Inc.), and anti-human β-actin (Sigma–Aldrich Co.). Each electroblotted membrane was then washed three times with TPBS, incubated further with alkaline phosphatase-conjugated goat anti-mouse antibody (Promega, Madison, WI) or anti-rabbit antibody (New England Biolabs, Beverly, MA) at room temperature, and then washed three times with TPBS. The immunoblot was visualized by use of an enhanced chemiluminescence detection kit (New England Biolabs).

5.5. Caspase activity assay

Caspase-3 activity was measured colorimetrically with a commercial assay kit (Alexis Biochemicals, Lausen, Switzerland). Briefly, the cells untreated or treated with the compound(s) were washed twice in ice-cold PBS; and cell lysates were then prepared according to the manufacturer's instructions. Cell lysates containing 50 μg of protein were incubated with pNA-conjugated substrate, and the release of pNA was measured at 405 nm by using a microplate reader NJ-2300 (NUNC, Rockilde, Denmark).

5.6. Measurement of mitochondrial membrane potential by using Mito-Traker probe

The mitochondrial membrane potential was measured by use of a fluorescent dye, Mito-Tracker Orange

(Molecular Probes, #M-7511, Eugene, OR) which accumulates selectively in active mitochondria and becomes to be fluorescent when oxidized. After the cells had been treated with Mito-Tracker Orange solution and washed twice with PBS, the cells were resuspended in PBS. The fluorescence of Mito-Tracker Orange in the cells was observed under a fluorescence microscope (Olympus).

5.7. Statistics

Differences were statistically evaluated by one-way ANOVA followed by Fisher's PLSD. A p-value less than 0.05 was considered to be statistically significant.

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