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# Characteristics of nobiletin-mediated alteration of gene expression in cultured cell lines

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ABSTRACT

Nobiletin, a polymethoxylated flavonoid that is highly contained in the peels of citrus fruits, exerts a wide variety of beneficial effects, including anti-proliferative effects in cancer cells, repressive effects in hyperlipidemia and hyperglycemia, and ameliorative effects in dementia at *in vitro* and *in vivo* levels. In the present study, to further understand the mechanisms of these actions of nobiletin, the nobiletin-mediated alterations of gene expression in three organ-derived cell lines – 3Y1 rat fibroblasts, HuH-7 human hepatocarcinoma cells, and SK-N-SH human neuroblastoma cells – were first examined with DNA microarrays. In all three cell lines, treatments with nobiletin (100 μM) for 24 h resulted in more than 200% increases in the expression levels of five genes, including the endoplasmic reticulum stress-responsive genes *Ddit3*, *Trib3*, and *Asns*, and in less than 50% decreases in the expression levels of seven genes, including the cell cycle-regulating genes *Ccna2*, *Ccne2*, and *E2f8* and the oxidative stress-promoting gene *Txnip*. It was also confirmed that in each nobiletin-treated cell line, the levels of the DDIT3 (DNA-damage-inducible transcript 3, also known as CHOP and GADD153) and ASNS (asparagine synthetase) proteins were increased, while the level of the TXNIP (thioredoxin-interacting protein, also known as VDUP1 and TBP-2) protein was decreased. All these findings suggest that nobiletin exerts a wide variety of biological effects, at least partly, through induction of endoplasmic reticulum stress and suppressions of oxidative stress and cell proliferation.



## 1. Introduction

Nobiletin (Fig. 1), a citrus polymethoxyflavonoid with six methoxy groups, is highly contained in the peels of citrus fruits. There is accumulating evidence that nobiletin exerts a wide variety of beneficial activities, including anti-dementia [1–8], anti-tumor [9–23], anti-metabolic syndrome, including anti-obesity, anti-hyperlipidemia, and anti-diabetes [18,21,24–30], and anti-inflammatory [31–35] activities at *in vivo* and *in vitro* levels. Nobiletin is thus expected to be clinically applicable for the treatment of these respective diseases. It has therefore become increasingly important to clarify the mechanisms of nobiletin-mediated biological effects, including adverse (toxic) effects.

The aim of the present study was to look closely into the nobiletin-mediated alterations of gene expression to further understand the mechanisms underlying nobiletin's effect on cellular events. We performed DNA microarray analyses by using total RNAs prepared from three different organ-derived cell lines: 3Y1 rat fibroblasts, HuH-7 human hepatocarcinoma cells, and SK-N-SH human neuroblastoma cells. Cells of each line were treated with 100 μM nobiletin, the concentration used in many previous studies, for 24 h. The results showed that five up-regulated and seven down-regulated genes were commonly identified in all three treated cell lines.

## 2. Materials and methods

### 2.1. Nobiletin

Nobiletin was extracted and isolated from *Citrus reticulata* peels as described previously [1,36]. This compound was dissolved at a concentration of 100 mM in dimethyl sulfoxide (DMSO) and then stored at −20 °C.

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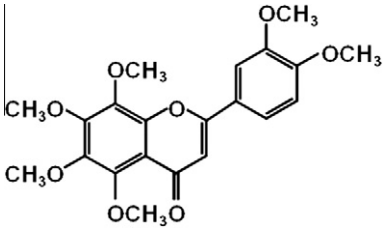

**Fig. 1.** Chemical structure of nobiletin.

### 2.2. Cell culture and treatments

The rat 3Y1-B clone 1–6 (3Y1) fibroblast and human HuH-7 hepatocellular carcinoma cell lines were obtained from the Japanese Cancer Research Resources Bank. The human SK-N-SH neuroblastoma cell line was obtained from RIKEN Cell Bank. The cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 4 mM L-glutamine, and 60 μg/ml kanamycin at 37 °C in a saturated humidity atmosphere of 95% air and 5% $CO_2$. Appropriate numbers of cells were incubated with 0.1% (v/v) DMSO as a vehicle (control) or 100 μM nobiletin for the appropriate time at 37 °C in the culture medium.

### 2.3. DNA microarray analysis

Total RNA was extracted from cells treated with vehicle and 100 μM nobiletin for 24 h using an RNeasy Mini Kit (Qiagen) in accordance with the manufacturer's instructions. One total RNA sample per nobiletin-treated cell line and two samples per vehicle (DMSO)-treated cell line were supplied for DNA microarray analysis. Total RNA (500 ng) was labeled with Cyanine-3 using a QuickAmp Labeling Kit (Agilent Technologies, Palo Alto, CA, USA). Fluorescently labeled targets were hybridized to a SurePrint G3 Rat GE 8 × 60 K DNA microarray for 3Y1 cells and a SurePrint G3 Human GE 8 × 60 K DNA microarray for HuH-7 and SK-N-SH cells (Agilent Technologies). Hybridization and wash processes were performed according to the manufacturer's instructions, and hybridized microarrays were scanned using an Agilent Microarray Scanner (Agilent Technologies). Feature Extraction software (Agilent Technologies) was employed for the image analysis and data extraction processes. Subio Platform (Subio, Tokyo, Japan) computer software was used to exclude the genes showing inappropriate expression values and to select genes whose expression levels in all nobiletin-treated cell lines were more than double or fewer than half the average expression levels in the corresponding vehicle controls. In other words, the genes whose raw signals were lower than 40 in both the nobiletin- and vehicle-treated cells of each cell line were excluded. When one of the two vehicle-treated samples was selected as a control, the genes whose expression levels in another vehicle-treated sample were more than double or fewer than half the expression levels in the control were also excluded.

### 2.4. Western blotting

Cells were lysed with lysis buffer (1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS in PBS) containing phenylmethylsulfonyl fluoride (PMSF), aprotinin, sodium orthovanadate and protease inhibitor cocktail, and the protein amounts in the obtained lysates were determined using BCA™ Protein Assay Reagent (Thermo Fisher Scientific Inc.). Fifteen micrograms per lane of total protein was subjected to SDS–PAGE (12.5% e-PAGEL; ATTO, Tokyo, Japan) and subsequently transferred onto a polyvinylidene fluoride (PVDF) membrane (GE Healthcare). The membranes were blocked with TBST buffer (150 mM NaCl, 0.1% Tween20, and 10 mM Tris–HCl, pH 7.6) containing 5% skim milk or BSA for 1 h at room temperature, followed by incubation overnight at 4 °C with monoclonal anti-DDIT3 (2000:1; Cell Signaling), monoclonal anti-ASNS (1000:1; Santa Cruz),monoclonal anti-TXNIP (1000:1; MBL), or polyclonal anti-β-ACTIN (5000:1; Cell Signaling) antibodies. Detection utilized anti-mouse IgG horseradish peroxidase (HRP)-linked antibody(Cell Signaling) for DDIT3 (5000:1), ASNS (10,000:1), and TXNIP (5000:1), and anti-rabbit IgG HRP-linked antibody (Cell Signaling) for β-ACTIN (10,000:1), and the SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific Inc.).

### 2.5. Alamar blue proliferation test

For evaluation of the effect of nobiletin on the proliferation, an Alamar blue assay kit (Invitrogen) was used. Cells were seeded at 5000 cells per well (0.5 ml) in 24-well microplates. Four wells were used for each time point. After 21 h, control cells (0 day) were applied to Alamar blue assay, while after 24 h the cells in the other wells were treated with DMSO (vehicle) or 100 μM nobiletin. After 21, 45, 69, and 93 h of the treated cells were applied to Alamar blue assay. The assays were basically carried out according to the manufacturer's instructions. Briefly, 50 μl of Alamar blue stock solution was added to the wells. The resazurin reduction in the cultures was determined after 3 h incubation with Alamar blue at 5% $CO_2$ and 37 °C by measuring the absorbance at an excitation wavelength of 550 nm and emission wavelength of 590 nm using a Wallac 1420 ARVOsx multilabel counter (Perkin Elmer). The fluorescence obtained for control cells (0 h) of each cell line was set to a value of 100, indicating 100% viability, and that for variously treated cells of the corresponding cell line was calculated accordingly.

## 3. Results

3Y1 rat fibroblast, HuH-7 human hepatocarcinoma, and SK-N-SH human neuroblastoma cells were treated with 100 μM nobiletin or with DMSO (vehicle control) for 24 h and the DNA microarray analyses were performed by use of total RNAs prepared from the treated cells. The genes whose expression levels in all nobiletin-treated cell lines were more than double or fewer than half the expression levels of the corresponding vehicle controls were selected and are listed in Table 1.

Among the listed genes, the expression levels of proteins encoded by the *Ddit3*, *Asns*, and *Txnip* genes were estimated by Western blotting. As shown in Fig. 2, the results demonstrated that the amounts of the DDIT3 and ASNS proteins were higher in all nobiletin-treated cell lines than in the corresponding vehicle controls, while the TXNIP protein level was lower in all the treated cell lines than in the controls, which was consistent with the results from DNA microarray analysis.

The effect of 100 μM nobiletin-treatment on the cell growth of the three cell lines was estimated by Alamar blue assay. The results showed that the growth of all cell lines was inhibited for 1–4 days by the treatment (Fig. 3).

## 4. Discussion

In the present study, DNA microarray analysis revealed that five genes were up-regulated (*Ddit3*, *Trib3*, *Asns*, *Slc6a9*, *Tmem116*) and seven genes were down-regulated (*Ccna2*, *Ccne2*, *Txnip*, *Depdc1*, *E2F8*, *Hist1h1b*, *Kif11*) in all three cell lines, 3Y1, HuH-7, and SK-N-SH cells, treated with 100 μM nobiletin for 24 h. Clarification of the significant roles played by these gene alterations may help

**Table 1**
List of genes whose expression was changed in all three cell lines, 3Y1, HuH-7, and SK-N-SH cells, treated with 100 μM nobiletin for 24 h.

| Gene symbol | Gene name | GenBank accession no. | | Probe ID | | Fold change (vs. control) | | |
|---|---|---|---|---|---|---|---|---|
| | | Rat | Human | Rat | Human | 3Y1 | HuH-7 | SK-N-SH |
| *Up-regulated* | | | | | | | | |
| Ddit3 | DNA-damage-inducible transcript 3 | NM_001109986 | NM_004083 | A_64_P059495 | A_23_P21134 | 2.71 | 4.95 | 19.05 |
| Trib3 | Tribbles homolog 3 (Drosophila) | NM_144755 | NM_021158 | A_64_P117901 | A_23_P210690 | 4.92 | 3.20 | 7.02 |
| Asns | Asparagine synthetase (glutamine-hydrolyzing) | NM_013079 | NM_001673 | A_44_P480267 | A_23_P145694 | 2.83 | 6.36 | 6.04 |
| Slc6a9 | Solute carrier family 6 (neurotransmitter transporter, glycine), member 9 | NM_053818 | NM_201649 | A_64_P034551<br>A_64_P059525 | A_33_P3402615<br>A_23_P11984 | 2.17<br>2.32 | 4.95 2.77 | 4.59 2.79 |
| Tmem116 | Transmembrane protein 116 | NM_001159625 | NM_138341 | A_44_P608167 | A_23_P105619 | 3.73 | 2.21 | 2.22 |
| *Down-regulated* | | | | | | | | |
| Txnip | Thioredoxin interacting protein | NM_001008767 | NM_006472 | A_44_P237621 | A_23_P97700 | 0.36 | 0.18 | 0.02 |
| Ccna2 | Cyclin A2 | NM_053702 | NM_001237 | A_64_P001740 | A_23_P58321 | 0.48 | 0.38 | 0.45 |
| Ccne2 | Cyclin E2 | NM_001108656 | NM_057749 | A_44_P272210 | A 33 P3217819<br>A_33_P3247022 | 0.43 | 0.38 0.46 | 0.19 0.32 |
| Depdc1 | DEP domain containing 1 | XM_001080406 | NM_001114120 | A_44_P213127 | A_33_P3387524 | 0.46 | 0.33 | 0.38 |
| E2f8 | E2F transcription factor 8 | XM_218601 | NM_024680 | A_64_P112034 | A_23_P35871 | 0.48 | 0.30 | 0.26 |
| Hist1h1b | Histone cluster 1, H1b | NM_001109417 | NM_005322 | A_44_P339798<br>A_64_P066979 | A_23_P250385 | 0.42<br>0.43 | 0.31 | 0.39 |
| Kif11 | Kinesin family member 11 | NM_001169112 | NM_004523 | A_42_P665319 | A_24_P227091 | 0.49 | 0.41 | 0.41 |

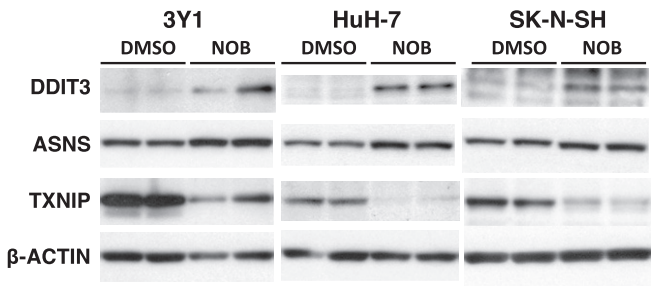


**Fig. 2.** Changes in expressions of DDIT3, ASNS, and TXNIP proteins in 3Y1, HuH-7, and SK-N-SH cells by treatment with 100 μM nobiletin (NOB) for 24 h. Total proteins prepared from two independently cultured plates per DMSO (vehicle)- or NOB-treated cells were assessed by Western blot analysis as described in Section 2.

to promote further investigation of the mechanisms of nobiletin-mediated biological effects, including adverse (toxic) effects.

The *Ddit3*, *Trib3*, and *Asns* genes encode DNA-damage-inducible transcript 3 protein (DDIT3, also known as CHOP and GADD153), tribbles homolog 3 protein (TRIB3, also known as TRB3), and asparagine synthetase (ASNS), respectively. The expression levels of these genes and proteins are well known to be increased in response to endoplasmic reticulum (ER) stress [37–39]. The present study demonstrated the increased expression of these three genes and DDIT3 and ASNS proteins in the nobiletin-treated cells by DNA microarray analysis and Western blotting. Therefore, it is likely that nobiletin has the capacity to induce ER stress. There have been many reports describing that nobiletin can cause apoptosis in various cancer cell lines [11,13,15,17], and these studies support the idea that nobiletin has chemopreventive potential in the development of cancer. Considering the facts that prolonged/excessive ER stress leads to cellular apoptosis [37,38], the nobiletin-induced apoptosis in cancer cell lines could be due to the ER stress. On the other hand, prolonged/excessive ER stress is thought to be a trigger for the cell loss and damage found in several common human diseases, including type 2 diabetes and neurodegeneration, and in chemical-induced toxicity [37,38]. Hence, it is necessary to carefully examine whether the induced expression of the *Ddit3*, *Trib3*, and *Asns* genes observed in the present study is involved in the beneficial effects or toxic potency of nobiletin.

The *Txnip* gene encodes thioredoxin-interacting protein (TXNIP, also known as VDUP1 and TBP-2). TXNIP binds to thioredoxin, which functions as one of the major antioxidant defense systems of the cells, and blocks its function, thereby inhibiting the antioxidant defense system [40,41]. The present study demonstrated the decreased expression of the *Txnip* gene and TXNIP protein in the nobiletin-treated cells, suggesting that nobiletin-treatment can promote the facilitation of the antioxidant defense system in the treated cells. There is accumulating evidence that up-regulation of the *Txnip* gene and its protein is a possible causative factor of several common human diseases, including type 2 diabetes and Alzheimer's disease (AD), which the beneficial effects of nobiletin could target. For example, enhancement of TXNIP expression suppresses insulin sensitivity in the muscles and glucose-stimulated insulin secretion in the pancreas and leads to pancreatic β-cell apoptosis [42]. It has also been reported that TXNIP-null mice are resistant to diabetes, showing that TXNIP is necessary for the induction of insulin resistance [42,43]. A close relationship between TXNIP expression and AD has been pointed out. TXNIP is overexpressed in the hippocampus and in the entorhinal cortex of 5XFAD mice [44], a model of AD, which expresses neuronal human amyloid precursor protein (APP) carrying three AD familiar mutations (Swedish, Florida, London) and presenilin 1 (PS1) containing 2 mutations (M146L and L286V) [45]. The β-amyloid peptide (Aβ) induces TXNIP expression in brain-derived endothelial cells (RBE4) and the induced expression is dependent on the receptor of advanced glycation end products (RAGE) [44]. In other words, it is most likely that suppression of the induced TXNIP expression can improve the aberrant responses, as mentioned above. Judging from these facts, the down-regulation of TXNIP expression observed in the present study would seem to play an important role in the mechanisms underlying the beneficial effects of nobiletin. However, in carcinogenesis, the *Txnip* gene is regarded as a tumor suppressor gene [41,46], inconsistent with the anti-tumor effect of nobiletin. Some of the reasons for this are that down-regulation or loss of TXNIP expression is observed in a wide range of primary human tumor tissues and human cancer cell lines [41,46] and that TXNIP-KO mice are more susceptible to chemical-induced cancers, including hepatocellular carcinoma [47,48] and bladder cancer [49]. Therefore, it will be necessary to examine the interaction between the nobiletin-induced down-regulation of

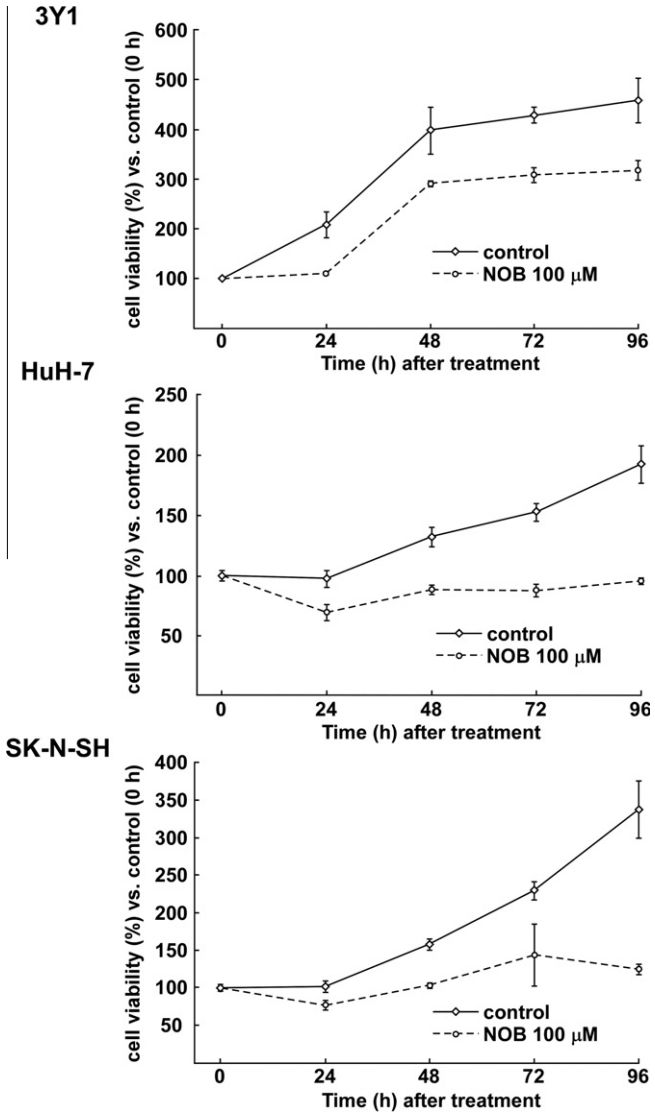


**Fig. 3.** Inhibitory effect of 100 μM nobiletin (NOB)-treatment on proliferation of 3Y1, HuH-7, and SK-N-SH cells. The fluorescence obtained for control cells (0 h) of each cell line by Alamar blue proliferation test was set to a value of 100, indicating 100% viability, and that for variously treated cells of the corresponding cell line was calculated accordingly. Results are represented as the means ± SD of $n = 4$.

TXNIP expression and carcinogenesis in order to allow nobiletin to be applied to clinical use.

The *Depdc1* gene encodes DEP (Dishevelled, Egl-10, and Pleckstrin) domain-containing protein 1A and was previously determined to be up-regulated in bladder cancer, breast cancer, and lung adenocarcinomas [50–52]. DEPDC1 expression was hardly detectable in any normal tissues except the testis, so DEPDC1 has been considered to be a cancer/testis antigen [50,51]. The protein encoded by the *E2f8* gene belongs to the E2F family member of transcription factors. It was reported that E2F8 was strongly up-regulated in human hepatocellular carcinoma and that the ectopic overexpression of E2F8 promoted cell proliferation, colony formation, and tumorigenicity, whereas E2F8 knockdown in human hepatocellular carcinoma cell lines, including HuH-7, inhibited these phenotypes [53]. The DNA microarray analysis in the present study showed the decreased expression of *Depdc1* and *E2f8* genes in all three nobiletin-treated-cell lines. Therefore, these actions of nobiletin may play some important roles in the anti-cancer effect of nobiletin.

Our results showed the inhibitory effect of 100 μM nobiletin-treatment on the cell growth of three cell lines, in good agreement with previous reports using other cell lines [11,13–15,17,20,22]. Both cyclin A2 (CCNA2) and E2 (CCNE2) proteins serve as positive regulators in the process of the cell cycle. Therefore, the decreased expression of both genes shown in the present study might indicate a delay in the process of the cell cycle. DDIT3 and E2F8, whose gene expressions were increased and decreased, respectively, after nobiletin treatment in the present study have also been known to lead to cell cycle arrest [54] and to positively regulate the cell cycle [53], respectively.

The proteins encoded by the *Slc6a9*, *Hist1h1b*, *Kif11*, and *Tmem116* genes are glycine transporter GLYT1 (SLC6A9), which is a member of the sodium- and chloride-dependent neurotransmitter transporter family SLC6, histone 1 H1b, kinesin family member 11, which is a motor protein, and transmembrane protein 116, whose function remains unclear, respectively. To make sense of the changes in expression of these genes will also be important for understanding the beneficial and adverse (toxic) effects exerted by nobiletin.

In conclusion, nobiletin-treatment caused increased expression of *Ddit3*, *Trib3*, *Asns*, *Slc6a9*, and *Tmem116* genes and decreased expression of *Ccna2*, *Ccne2*, *Txnip*, *Depdc1*, *E2F8*, *Hist1h1b*, and *Kif11* genes in all 3Y1 rat fibroblasts, HuH-7 human hepatocarcinoma cells, and SK-N-SH human neuroblastoma cells. These findings may help to promote further investigation of the mechanisms of nobiletin-mediated biological effects, including adverse (toxic) effects.

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