JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

Cite This: J. Agric. Food Chem. 2018, 66, 13423−13434

pubs.acs.org/JAFC

Nobiletin Down-Regulates c-KIT Gene Expression and Exerts Antileukemic Effects on Human Acute Myeloid Leukemia Cells

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Supporting Information

ABSTRACT: Nobiletin, a dietary citrus flavonoid, has been reported to possess several biological activities such as antioxidant, anti-inflammatory, and anticancer properties. The aim of this study was to investigate the antileukemic effects of nobiletin and its underlying mechanisms on human acute myeloid leukemia (AML) cells. We demonstrated that nobiletin (0−100 μM) significantly reduced cell viability from 100.0 ± 9.6% to 31.1 ± 2.8% in human AML THP-1 cell line. Nobiletin arrested cell cycle progression in G1 phase and induced myeloid cell differentiation in human AML cells. Microarray analysis showed that mRNA expression of the c-*KIT* gene, a critical proto-oncogene associated with leukemia progression, was dramatically reduced in nobiletin-treated AML cells. Furthermore, we verified that AML cells treated with nobiletin (40 and 80 μM) for 48 h markedly suppressed c-KIT mRNA expression (from 1.00 ± 0.07-fold to 0.62 ± 0.08- and 0.30 ± 0.05-fold) and reduced the level of c-KIT protein expression (from 1.00 ± 0.11-fold to 0.60 ± 0.15- and 0.34 ± 0.05-fold) by inhibition of *KIT* promoter activity. The knockdown of c-KIT expression by shRNA attenuated cancer cell growth and induced cell differentiation. Moreover, we found that the overexpression of c-KIT abolished nobiletin-mediated cell growth inhibition in leukemia cells. These results indicate that nobiletin exerts antileukemic effects through the down-regulation of c-KIT gene expression in AML cells. Finally, we demonstrated that the combination of a conventional AML chemotherapeutic agent, cytarabine, with nobiletin resulted in more reduction of cell viability in AML cells. Our current findings suggest that nobiletin is a novel c-KIT inhibitor and may serve as a chemo-preventive or -therapeutic agent against human AML.

KEYWORDS: *nobiletin, flavonoid, AML, c-KIT, cytarabine*

INTRODUCTION

Acute myeloid leukemia (AML) is a highly heterogeneous malignancy characterized by the accumulation of immature myeloid cells in the bone marrow and by aberrant regulation in hematopoietic proliferation, differentiation, and apoptosis. AML is the most common myeloid leukemia in adults. AML patients can be classified into different risk categories according to their genetic and molecular abnormalities.[1] With traditional intensive chemotherapy regimens, only 40% of patients younger than 60 years old survive more than 5 years, and even patients with favorable-risk core binding factor leukemia have high mortality rate of 56% at 10 years.[2] Indeed, over half of AML patients are older than 60 years, and the treatment outcomes in this group remain poor, with a median overall survival of less than 1 year.[3] To improve outcomes in AML, a number of recurrently mutated genes involved in cell proliferation, cell survival, or cell differentiation, such as *KIT*[4] and *FLT3*,[5] have been identified. These discoveries are now being translated into better predictive markers or targeted therapies. Recent studies have tended to develop new agents, alone or in combination with chemotherapy, to target dysregulated molecular mechanisms for AML treatment.[6]

The c-KIT protein (also designated CD117) is encoded by the *KIT* gene and is highly expressed in hematopoietic stem cells, so it is commonly used as a phenotypic marker for these stem cells. The c-Kit protein is a member of type III receptor tyrosine kinases (RTK), and it dimerizes and autophosphorylates after the binding of its specific ligand, the stem cell factor (SCF), and then activates several downstream signaling pathways to regulate cell growth, proliferation, and differentiation.[7] The *KIT* gene is a proto-oncogene, and deregulation of this gene in different ways, such as overexpression or gain-of function mutations, can result in several types of tumors, including AML.[8,9] Overexpression or activation of c-KIT protein has been shown to be associated with malignant cell proliferation in human AML cells.[10] Recently, global gene expression studies also found the *KIT* gene to be highly expressed in AML, independent of its mutation status.[11,12] AML cells highly express c-KIT protein, which also affects the malignant phenotype of this cancer. The survival rate of c-KIT(+) AML patients is significantly shorter than c-KIT(−) AML patients. The higher c-KIT-expressing AML patients have shown lower complete remission rate.[13] Therefore, in AML, there is a rationale to use tyrosine kinase inhibitors (TKIs) to target both overexpressed and mutated c-KIT. In fact, clinical trials combining conventional chemotherapy with c-KIT inhibitors are ongoing in patients with AML.[14] However, because of the expanding spectrum of TK

Received: October 15, 2018
Accepted: December 3, 2018
Published: December 3, 2018

DOI: 10.1021/acs.jafc.8b05680
J. Agric. Food Chem. 2018, 66, 13423−13434

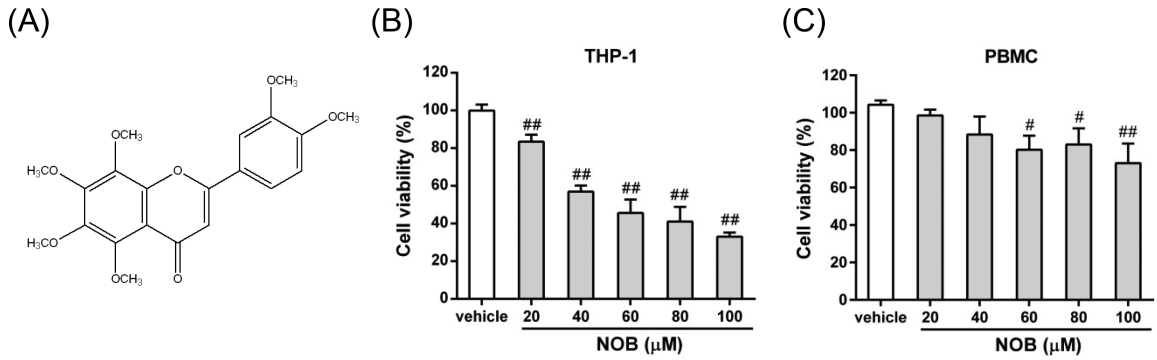

Figure 1. Effects of NOB on cell growth in THP-1 cells. (A) Chemical structure of nobiletin (5,6,7,8,3′,4′-hexamethoxyflavone, NOB). (B) THP-1 cells or (C) PBMCs were treated with vehicle (0.1% DMSO) or NOB (20, 40, 60, 80, and 100 μM) for 48 h. The viability of NOB-treated cells was measured using an MTT assay. The data represent the mean ± SD of three independent experiments. #p < 0.05 and ##p < 0.01 represent significant differences compared to the vehicle-treated group.

mutations, TKI-therapy resistance has led to the search for second or third generation TKIs, monoclonal antibodies for blocking c-KIT activation, or novel small molecules for the down-regulation of the c-KIT gene expression in AML cells to improve target potency and to overcome resistant clones.

Nobiletin (5,6,7,8,3′,4′-hexamethoxyflavone, NOB) (Figure 1A), a dietary polymethoxyflavone (PMF) flavonoid that is mainly isolated from citrus fruits, has been reported to exhibit several biological activities including anti-inflammatory, antiatherogenic, neuroprotective, neurotrophic, and antitumor properties.[15−18] Several studies have demonstrated that NOB suppresses cell proliferation, induces cell cycle arrest and cell apoptosis, and inhibits cell invasion in various type of solid tumors including breast cancer, lung cancer, gastric cancer, colon cancer, and glioma.[19−23] In human nonsolid tumor studies, NOB has been reported to possess antileukemic effects such as suppressing leukemia cell proliferation and inducing apoptosis in various types of AML cell lines. NOB inhibited the cell proliferation and induced apoptosis involving MAPK- and caspase-dependent signals in human AML HL-60 cells.[24] NOB also induced cell differentiation in HL-60 cells.[25] NOB was reported to strongly inhibit P-glycoprotein function and induce cell cycle arrest in human leukemia MOLT-4 cells.[26] NOB also potentiates the cytolytic activity of an NK cell line against cancer cells via enhancing granzyme B expression.[27]

Even though NOB is considered a nontoxic constituent of dietary phytochemicals, its antitumor action mainly involves the induction of cell death. There is little evidence to date that NOB can significantly target key regulators related to leukemogenic proliferation or improve differentiation of hematopoietic progenitors, which may provide a potentially strategy to specifically inhibit leukemia cell growth but avoid complications of toxicity in normal tissue. In this study, we aim to investigate the antileukemia effects of NOB and their underlying mechanisms in human AML cells. We focused on the investigation of NOB modulation of the expression of the proto-oncogene *KIT*, which was associated with the regulation of cell proliferation and differentiation in human AML cells. We also demonstrated the effect of combining a conventional AML chemotherapy agent, cytarabine (Ara-C), with NOB to suppress cell growth in human AML cells.

MATERIALS AND METHODS

Chemicals. Nobiletin (NOB) was purchased from Tokyo Chemical Industry (Tokyo, Japan). Dimethyl sulfoxide (DMSO), cytarabine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), RPMI-1640 medium, nonessential amino acids (NEAA), and other chemicals, unless otherwise indicated, were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

Cell Culture. The THP-1, U-937, and HL-60 cell lines were obtained from Bioresource Collection and Research Center (Hsinchu, Taiwan). Peripheral blood mononuclear cells (PBMCs) were from one healthy volunteer donor and prepared using Ficoll-Paque Plus Reagent (GE Healthcare, Buckinghamshire, UK). These cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) (ThermoFisher Scientific, Inc., Rockford, IL, USA) and 1% NEAA in a 5% CO_2 incubator at 37 °C.

Analysis of Cell Viability by MTT Assay. Cells were treated with the vehicle (0.1% DMSO) or NOB for indicated periods, followed by incubation with 1 mg/mL MTT solution for an additional 3 h at 37 °C. The cell pellets were collected by centrifugation and dissolved in DMSO. The extent of the reduction of MTT formazan crystals produced by mitochondria was determined as the absorbance at 550 nm.

Flow Cytometric Analysis. For cell cycle analysis, the cells were seeded on six-well plates and treated with vehicle or NOB (40 and 80 μM) for 24−72 h. For analysis of cell cycle distribution, the cells were harvested and fixed with 70% ethanol in PBS and stored at −20 °C for a minimum of 24 h. The cells were washed with ice-cold PBS and incubated in propidium iodide (PI) staining buffer (20 μg/mL PI, 200 μg/mL RNaseA and 0.1% Triton X-100 in PBS) in the dark at room temperature for 30 min. Cell cycle analysis was carried out on the FACSCalibur, and cell distribution was analyzed by Cell Quest Pro software (BD Biosciences, San Jose, CA) to determine the fractions of cells in the sub-G1, G1, S, and G2/M phases. For detection of cell-surface c-KIT (CD117), the cells were incubated with an antihuman CD117 monoclonal antibody (Cell Signaling Technology, Danvers, MA, USA) at 37 °C for 1 h. The cells were washed with PBS and incubated with Alexa Fluor 488 dye (Thermo Fisher Scientific) at 37 °C for 30 min. After washing with PBS twice, the cells were suspended in PBS and measured using flow cytometric analysis. The level of c-KIT on the cell surface was expressed as the relative percentage of the geometric mean fluorescence intensity.

Quantitative Reverse Transcription-PCR (Q-RT-PCR). The total RNA was extracted using the RNA mini kit (Geneaid, New Taipei City, Taiwan). Reverse transcription was performed using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystem). Quantitative real-time PCR was performed in a reaction mixture that contained cDNA and specific primers [CD11b,[28] 5′-ATCCGCAGTGAGAACACGTATG-3′ (forward) and 5′- AGAGCCATCAATCAAGAAGGC-3′ (reverse); c-KIT (Genebank sequence NM̄000222.2), 5′-TGACTTACGACAGGCTCGTG-3′ (forward) and 5′-CCACTGGCAGTACAGAAGCA-3′ (reverse); GAPDH,[29] 5′-CATGAGAAGTATGACAACAGCCT-3′ (forward) and 5′-AGTCCTTCCACGATACCAAAGT-3′ (reverse)] and Maxima

DOI: 10.1021/acs.jafc.8b05680
J. Agric. Food Chem. 2018, 66, 13423−13434

SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific). Amplification was carried out in a Roche LightCycler 480 Real-Time PCR System (Roche Diagnostics). PCR conditions were performed according to manufacturer's instruction. The $\Delta\Delta C_t$ method was used for data analysis, and gene expression was estimated in triplicate samples and was normalized to GAPDH expression levels.

RNA Preparation and cDNA Microarray Analysis. RNA preparation and microarray analyses were performed as previously described.[29] Briefly, the cells were treated with NOB (80 μM) for 0, 24, or 48 h, and cellular RNA was extracted using TRIzol reagent according to the manufacturer's instruction (Thermo Fisher Scientific). RNA concentration was measured, with $OD_{260}/OD_{280} \geq 1.8$ and $OD_{260}/OD_{230} \geq 1.5$ for acceptable purity. The integrity of RNA samples was determined using the Agilent RNA 6000 Nano assay (Agilent Technology, Inc., Santa Clara, California, USA), and an RNA integrity number (RIN) value >6 was required. The RNA samples for the generation of amino allyl antisense RNA (aa-aRNA) were obtained using Eberwine-based amplification with Amino Allyl MessageAmp II aRNA Amplification Kit (Ambion, CA, USA). The Cy5-labeled aRNAs were prepared for microarray hybridization to the Human Whole Genome One Array Version 6.1 (HOA 6.1, Phalanx Biotech Group, Hsinchu, Taiwan). The fluorescence intensities of each spot were analyzed by GenePix 4.1 (Molecular Devices, Sunnyvale, CA, USA). The normalized spot intensities were transformed to $\log_2$ ratios of gene expression, and the differentially expressed genes were established at $\log_2(\text{ratio}) \geq 1.0$ or ≤ -1.0 and p-value < 0.05 for selection criteria and further analysis.

Western Blot Analysis. The cells were treated with vehicle or NOB (40 and 80 μM) for 24 or 48 h. Total cellular lysates were prepared using RIPA buffer (ThermoFisher Scientific). Nuclear extracts were prepared using the NE-PER nuclear and cytoplasmic extraction reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. The protein lysates were separated by 10% or 12% SDS-PAGE, then transferred onto a PVDF membrane (PerkinElmer, Boston, MA, USA). The membranes were incubated with specific antibodies for human proteins: c-KIT, p21 Waf1/Cip1, cyclin E1, CDK6, AP-2α, cleaved-caspase 3, and PARP1 (Cell Signaling Technology, Danvers, MA, USA); p27 Kip1 and p16 INK4A (ABclonal, Woburn, MA, USA); SP1 (Santa Cruz Biotechnology); cyclin D1, caspase 3, and HDAC2 (GeneTex, Irvine, CA, USA); β-actin (Sigma-Aldrich); and actin (Thermo Fisher Scientific). The blots were incubated with HRP-conjugated secondary antibodies (Santa Cruz Biotechnology), and proteins were detected using the Amersham ECL Prime Western Blotting Detection Reagent. The signal was visualized on Amersham Hyperfilm ECL (GE Healthcare, Buckinghamshire, UK).

Preparation of Reporter Constructs, Plasmid Transfection, and Luciferase Reporter Gene Assay. The 5′ regulatory sequence of the human c-KIT promoter from nucleotide −887 to +50 was PCR-amplified using primers [KIT−P1-F: 5′-gcggtaccctagccctaa-aactgtcg-3′ and KIT-Pro-R: 5′-gcaagcttagtaggagcagaacgcagag-3′] with human genomic DNA (Novagen, San Diego, CA, USA) as a template and was inserted into the pGL3-basic vector (Promega) to construct a KIT promoter/luciferase reporter construct (pGL3-KIT-P1). The constructs contained the 5′-deletion of c-KIT promoter were generated using specific forward primers [KIT−P2-F: 5′-gcggtacctcccctccccatccccatg-3′, KIT−P3-F: 5′-gcggtaccagcttcacaa-agcgagcgg-3′, KIT−P4-F: 5′-gcggtaccggagtcaagagcggggag-3′ and KIT−P5-F: 5′-gcggtacctgggaggaggggctgctgct-3′], reverse primer KIT-Pro-R and pGL3-KIT-P1 as a template for PCR amplification. The PCR amplified DNA fragments were inserted into the pGL3-basic vector and designated as pGL3-KIT-P2 to pGL3-KIT-P5. For reporter construct transfection, THP1 cells (8×10^5/well) were seeded in six-well plates and cotransfected with reporter plasmids and *Renilla* luciferase vector using TransIT −2020 Transfection Reagent (Mirus Bio, Madison, WI, USA). After transfection for 24 h, the cells were treated with vehicle or NOB (40 and 80 μM) for an additional 48 h. The cells were harvested using Passive Lysis Buffer (Promega), and luciferase activities were determined using the Dual-Luciferase Reporter Assay System Kit (Promega). The intensities of the luciferase reactions measured in the lysates of the transfected cells were normalized to the activity of the *Renilla* luciferase internal control. For overexpression of c-KIT protein, THP-1 cells were transfected with pCMV-AC control vector or pCMV-AC-KIT expression plasmids (OriGene Technologies, Rockville, MD, USA) using TransIT −2020 Transfection Reagent for 24 h. The plasmid-transfected cells were treated with vehicle or NOB (80 μM) for an additional 48 h, and cell viability was determined.

Generation of c-KIT-Knockdown Stable Cell Clones. The control LacZ- and c-KIT-knockdown THP-1 cells were established by lentiviruses encoding specific shRNAs (LacZ-shRNA clone: TRCN0000072233; KIT-shRNA clone: TRCN0000195226) obtained from the National RNAi Core Facility (Academia Sinica, Taipei, Taiwan). Knockdown clones (shLacZ or shKIT cell clones) were selected with puromycin (2 μg/mL) and isolated for a single and pure stable cell clone. The mRNA and protein levels of c-KIT in the knockdown clones were characterized by Q-RT-PCR and Western blot analysis.

Combination Analysis. To evaluate the pharmacological interactions of the combination of cytarabine and nobiletin, the combination index (CI) was calculated for quantitative determination of different drug interactions, where CI < 1, = 1, and > 1 indicate synergism, additive effect (summation), and antagonism, respectively.[30] The CI was calculated on $CI = (C_A)/(C_x)_A + (C_B)/(C_x)_B$, where $(C_x)_A$ and $(C_x)_B$ are the doses of drug A and drug B, alone, inhibiting x%, whereas (C_A) and (C_B) are the doses of drug A and drug B in combination that gives the experimentally observed x% inhibition.

Statistical Analysis. All experiments were performed at least three times, and the data were expressed as the mean ± SD. The results were analyzed using one-way ANOVA with Dunnett's post hoc test, and a p value < 0.05 was considered statistically significant.

RESULTS

Effects of Nobiletin on the Cell Growth in Human Acute Myeloid Leukemia Cell Lines. Polymethoxyflavones have been reported to inhibit cancer cell proliferation in several kinds of tumors including acute myeloid leukemia (AML).[24] To investigate the effect of polymethoxyflavone nobiletin (NOB) on the viability of AML cells, human myeloid leukemia THP-1 cells were treated with vehicle (0.1% DMSO) and NOB (20−100 μM) for 48 h, and the viability was examined using the MTT assay. As shown in Figure 1B, THP-1 cells treated with NOB for 48 h showed significantly decreased cell viability in a concentration-dependent manner. The viability of cells treated with NOB (20, 40, 60, 80, and 100 μM) decreased from 100.0 ± 9.6% to 86.7 ± 9.8%, 59.0 ± 2.4%, 43.4 ± 5.0%, 37.3 ± 5.8%, and 31.1 ± 2.8%, respectively, compared to the vehicle-treated group ($p < 0.01$). We also examined the effect of NOB on peripheral blood mononuclear cells (PBMCs). As shown in Figure 1C, low cytotoxicity was detected in NOB-treated PBMC cells. The viability of PBMCs treated with NOB (20−100 μM) was significantly higher than that of NOB-treated THP-1 leukemia cells. The inhibitory concentration of 50% cell growth (IC_{50}) was 54.8 μM for NOB in THP-1 cells. Similar inhibitory effects of cell growth were also detected in the human AML cell lines U-937 and HL-60 cells (Figure S1A,B). The IC_{50} value was 45.2 μM and 45.8 μM in U-937 and HL-60 cells, respectively. These data supported that nobiletin significantly inhibited cell growth in different human AML cells.

Nobiletin Altered Cell Cycle Distribution and Promoted Cell Differentiation in Human Acute Myeloid Leukemia Cells. To assess whether the inhibition of cell growth in THP-1 cells observed with NOB treatment was the result of cell cycle arrest or induction of apoptosis, the cells

DOI: 10.1021/acs.jafc.8b05680
J. Agric. Food Chem. 2018, 66, 13423−13434

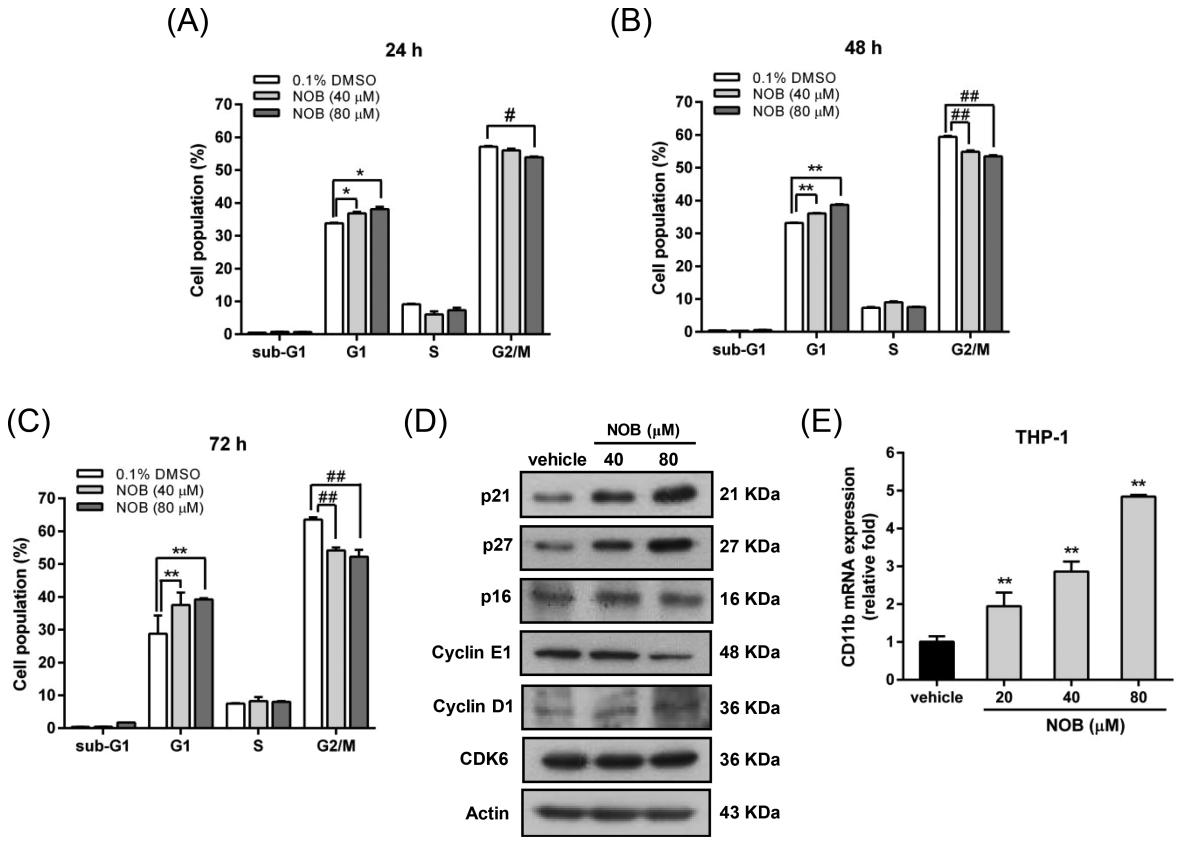

Figure 2. Effects of NOB on cell cycle distribution and cell differentiation. THP-1 cells were treated with vehicle or NOB (40 and 80 μM) for (A) 24 h, (B) 48 h, (C) 72 h, and the cell populations in sub-G1, G1, S, and G2/M phases were detected by flow cytometric analysis. The experiments were replicated three times. The data represent the mean ± SD of three independent experiments. $*p < 0.05$, # $p < 0.05$, $**p < 0.01$, and ##$p <$ 0.01 represent significant differences compared to the vehicle-treated group. (D) THP-1 cells were treated with vehicle or NOB (40 and 80 μM) for 48 h. The expression of p21, p27, p16, cyclin E1, cyclin D1, CDK6, and actin proteins was detected by Western blot analysis. The immunoblots were performed at three independent experiments. A represent blot is shown. (E) THP-1 cells were treated with vehicle or NOB (20, 40, and 80 μM) for 48 h. The expression of CD11b mRNA was measured by Q-RT-PCR analysis. The data represent the mean ± SD of three independent experiments. $**p < 0.01$ represents significant differences compared to the vehicle-treated group.

were treated with NOB (40 and 80 μM) for 24−72 h, and the cell cycle distribution was examined using flow cytometric analysis. As shown in Figure 2A−C, treatment of THP-1 cells with NOB (40 and 80 μM) for 24−72 h resulted in a significant accumulation of cell populations in the G1 phase in a time-dependent manner compared to the vehicle (0.1% DMSO) group. Moreover, we also found that NOB significantly reduced the cell population in the G2/M phase. However, there was no significant increase in the cell population in the sub-G1 phase (the cell death population) in NOB-treated cells. To investigate the mechanism of G1 phase arrest, the levels of cell cycle regulators such as p21 Waf1/Cip1 (p21), p27 Kip1 (p27), p16 INK4A (p16), cyclin E1, cyclin D1, and cyclin-dependent kinase 6 (CDK6) controlling the G1 phase arrest and G1/S phase transition[31] were analyzed by Western blot analysis. As shown in Figure 2D, the levels of p21 and p27 proteins were significantly increased, while cyclin E1 was reduced in NOB-treated THP-1 cells. The levels of p16, cyclin D1, and CDK6 were not markedly altered by NOB. These results verify nobiletin induced cell cycle arrest in the G1 phase and suppressed cell proliferation but did not induce cell death in THP-1 leukemia cells.

We further investigated whether NOB induces cell differentiation in human AML cell lines. The mRNA expression of a monocytic differentiation marker gene, CD11b, was examined using Q-RT-PCR in NOB-treated AML cell lines. As shown in Figure 2E, NOB significantly increased CD11b expression in THP-1 cells. After 48 h treatment, NOB (20, 40, and 80 μM) increased mRNA expression of CD11b to approximately 1.9-, 2.9-, and 4.8-fold compared to the vehicle-treated group, respectively ($p < 0.01$). Similar effects for promoting cell differentiation were also demonstrated in NOB-treated U-937 and HL-60 cells compared to vehicle-treated cell groups (Figure S2A,B). These results indicated that nobiletin also promoted cell differentiation in human AML cells.

Differential Gene Expression in Nobiletin-Treated THP-1 Cells. To investigate the potential genes associated with the NOB-mediated inhibition of cell growth, we examined the differential expression of mRNAs by a genome-wide microarray analysis in NOB-treated leukemia cells. THP-1 cells were treated with NOB (80 μM) for 0 h, 24 h, and 48 h; then the RNA expression profiles were analyzed using Human OneArray expression microarrays. On the basis of microarray data analysis, as shown in Table 1, a total of 396 and 231 genes were significantly up-regulated ($\log_2$ ratio ≥1.0), and 299 and 397 genes were down-regulated ($\log_2$ ratio ≤ −1.0), in cells treated with NOB for 24 and 48 h, respectively, compared to that of the 0 h-treated group. Furthermore, Gene Ontology enrichment analysis showed that 10 and 16 gene transcripts

DOI: 10.1021/acs.jafc.8b05680
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Table 1. Gene Expression Analysis of NOB-Treated THP-1 Cells by Human cDNA Microarray

	24 h versus 0 h	48 h versus 0 h
genes in up-regulated expression	396	231
genes in down-regulated expression	299	397
genes in down-regulation of cell proliferation-related genes	10	16

Table 2. Down-Regulation of Cell Proliferation-Related Genes in NOB-Treated THP-1 Cells

	fold changes $\log_2$ (ratio)[a]	
genes	$\log_2$ (24 h/0 h)	$\log_2$ (48 h/0 h)
KIT	−1.38	−1.77
TP63	−2.03	−1.85
LGR4	−1.95	−1.75
PA2G4	−1.1	−1.33

[a]Fold changes represents the fold change of mean normalized density of two replicates for each group. Only mRNAs ($p < 0.05$) with $\log_2$(ratio) ≤ −1.0 were presented.

related to cell proliferation were significantly down-regulated in the cells treated with NOB for 24 and 48 h, respectively (Figure 3A). Among these down-regulated genes, the expression of four genes, namely, *KIT*, *TP63*, *LGR4*, and *PA2G2*, was significantly reduced in the cells treated with NOB for 24 h as well as 48 h (Table 2). It has been reported that the *KIT* gene, a critical proto-oncogene receptor tyrosine kinase, is highly expressed in AML cells and activation of c-KIT has been shown to be associated with human AML progression. Therefore, the c-KIT was chosen for further investigation of NOB-inhibited cell growth. The microarray data showed that the relative fold change of mRNA expression in the *KIT* (c-*KIT*) gene was pronounced and dramatically reduced to approximately to 0.4- and 0.3-fold in a time-dependent manner compared with the 0 h group (Figure 3B). We further validated the microarray data using Q-RT-PCR analysis. THP1 cells were treated with NOB (40 and 80 μM) for 24 and 48 h, and the level of c-KIT mRNA was measured. As shown in

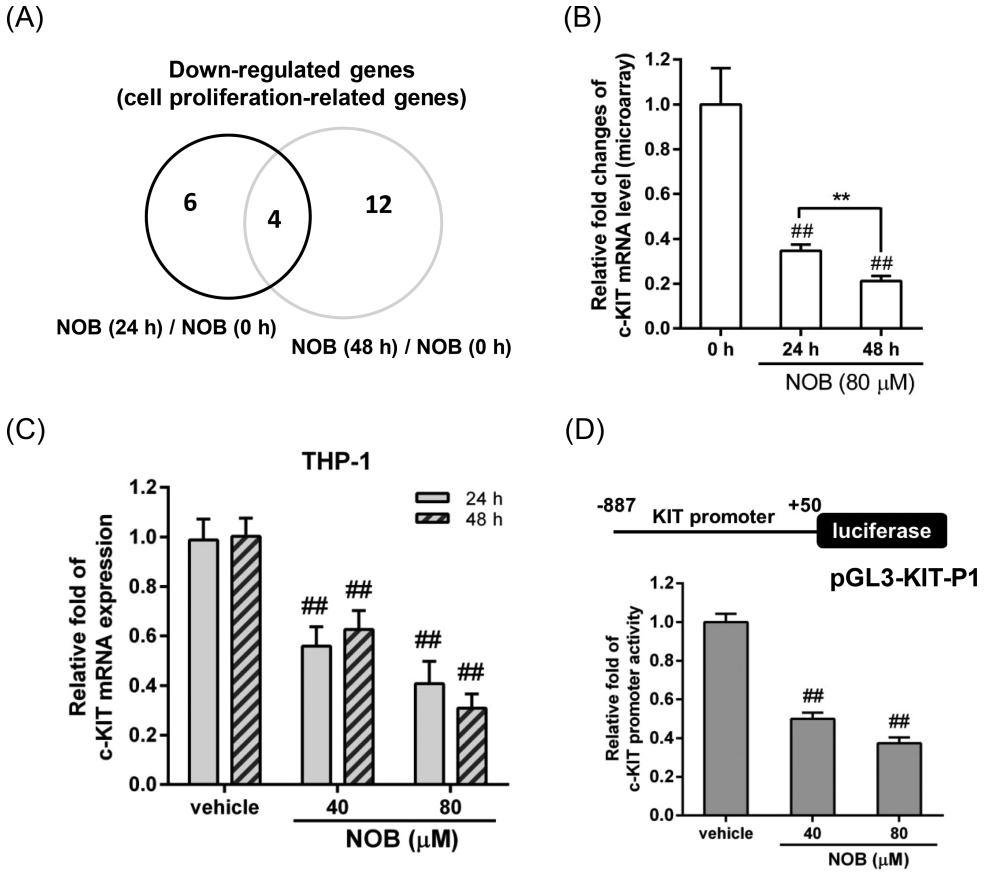

Figure 3. Gene expression alternations in NOB-treated AML cells. (A) THP-1 cells were treated with NOB (80 μM) for 0, 24, or 48 h, and the mRNA expression profiles were determined using the Human OneArray expression microarray. Genes associated with the cell proliferation process categories based on Gene Ontology terms were down-regulated in cells treated with NOB for 24 or 48 h as compared to those treated for 0 h. (B) On the basis of microarray analysis, relative fold changes of c-Kit mRNA expression in NOB (80 μM)-treated cells for 24 or 48 h were analyzed. The microarray experiment was duplicated. The data represent the mean ± SD of three independent experiments. ##$p < 0.01$ represents significant differences compared to the 0 h group. **$p < 0.01$ represents significant differences compared to the 24 h group. (C)THP-1 cells were treated with vehicle or NOB (40 and 80 μM) for 24−48 h, and c-Kit mRNA levels were determined by Q-RT-PCR analysis. The data represent the mean ± SD of three independent experiments. ##$p < 0.01$ represents significant differences compared to the vehicle-treated cells. (D) THP-1 cells were transfected with the *KIT* promoter-luciferase reporter construct containing the DNA region of the human KIT gene promoter from −887 bp to +50 bp (pGL3-KIT-P1) and the *Renilla* luciferase control plasmid for 24 h, and these cells were then treated with vehicle or NOB (40 and 80 μM) for 24 h. The luciferase activities were measured and normalized to the *Renilla* luciferase control. The data represent the mean ± SD from three independent experiments. ##$p < 0.01$ represents significant differences compared to the vehicle-treated cells.

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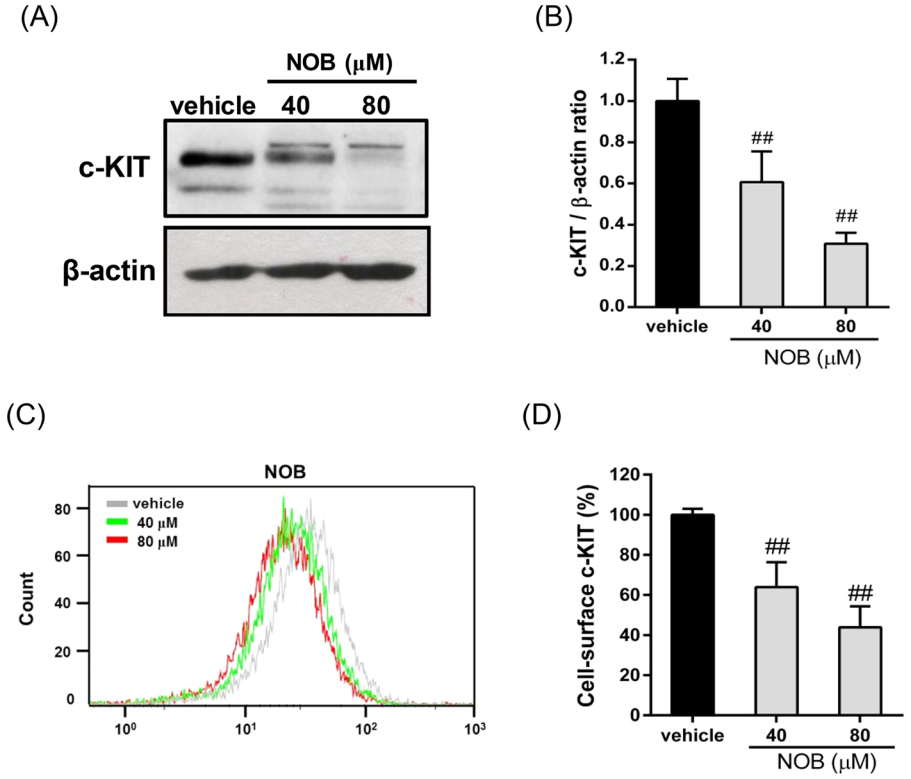

Figure 4. Effects of NOB on c-KIT protein expression in THP-1 cells. (A) THP-1 cells were treated with vehicle or NOB (40 and 80 μM) for 48 h. The c-Kit protein in total cell lysates was detected by Western blot analysis. A representative blot is shown. (B) Normalized intensity of c-Kit protein versus β-actin represents the mean ± SD of three independent experiments. ##p < 0.01 represents significant differences compared to the vehicle-treated cells. (C) THP-1 cells were treated with vehicle or NOB (40 and 80 μM) for 48 h. The amount of cell-surface c-Kit protein was measured by flow cytometric analysis. A representative histogram is shown. (D) Summary of cell-surface c-KIT protein levels. The data represent the mean ± SD of three independent experiments. ##p < 0.01 represents significant differences compared to the vehicle-treated cells.

Figure 3C, NOB (40 and 80 μM) markedly decreased c-KIT mRNA expression in THP-1 cells by approximately 43% and 60% (from 1.00 ± 0.08-fold to 0.57 ± 0.08- and 0.41 ± 0.09-fold) for 24 h treatment, and 40% and 70% for 48 h (from 1.00 ± 0.07-fold to 0.62 ± 0.08- and 0.30 ± 0.05-fold) treatment compared to vehicle groups, respectively (p < 0.01). Additionally, we also examined the level of c-KIT mRNA expression in NOB-treated U-937 or HL-60 cells. Similar suppressive effects of c-KIT mRNA expression by NOB were also detected in U-937 or HL-60 cells (Figure S3A,B). Furthermore, we examined the effect of NOB on *KIT* promoter activity in AML cells. The THP-1 cells were transfected with a luciferase reporter plasmid containing the 5′-flanking sequence of the human *KIT* gene promoter from −887 bp to +50 bp (pGL3-KIT-P1). After plasmid transfection for 24 h, the cells were treated with vehicle or NOB for 24 h. As shown in Figure 3D, NOB (40 and 80 μM) significantly suppressed *KIT* promoter activity by approximately 50% and 63% compared to that of the vehicle-treated cells. These above results suggested that nobiletin down-regulated c-KIT mRNA expression by suppression of promoter activity in human AML cells.

Nobiletin Suppressed c-KIT Protein Expression in Human AML Cells. To further verify the effect of NOB on c-KIT, protein levels were examined using Western blot analysis in human AML cells. As shown in Figure 4A and B, the level of total cellular c-KIT protein in NOB (40 and 80 μM)-treated THP-1 cells for 48 h displayed a dramatic decrease of approximately 40% and 75% (from 1.00 ± 0.11-fold to 0.60 ± 0.15- and 0.34 ± 0.05-fold) in a dose-dependent manner compared to that of vehicle-treated cells. Moreover, we examined the level of cell-surface c-KIT protein by flow cytometric analysis. Our data showed that NOB (40 and 80 μM) significantly attenuated cell-surface c-KIT protein in THP-1 cells compared to vehicle-treated cells (p < 0.01) (Figure 4C,D). This result demonstrated that NOB reduced c-KIT protein expression in human AML cells.

Characterization of Nobiletin-Responsive Element within KIT Promoter. These above data revealed that nobiletin down-regulated the mRNA and protein expression of c-KIT in human AML cells. To investigate the possible mechanism by which NOB down-regulated c-KIT gene expression, the NOB-responsive DNA region within human *KIT* promoter was characterized. To elucidate the regulatory DNA elements within *KIT* promoter that are response to NOB, plasmids contained deleted DNA segments of *KIT* promoter (pGL3-KIT-P1−P5) (Figure S4A) were individually transferred into THP-1 cells followed by treatment of vehicle or NOB (80 μM) for 24 h. As shown in Figure S4B, NOB obviously suppressed transcriptional activity in those cells transfected with plasmids from pGL3-KIT-P1 (−887/+50) to pGL3-KIT-P4 (−300/+50). In contrast, the transcriptional activity was strongly eliminated in cells transfected with construct that bears the promoter sequence −120 to +50 (pGL3-KIT-P5) and the luciferase activity was not significantly changed by treatment of NOB. These results indicate that the NOB-responsive elements within the *KIT* gene promoter reside between the −300 and −119 positions. Transcription

DOI: 10.1021/acs.jafc.8b05680
J. Agric. Food Chem. 2018, 66, 13423−13434

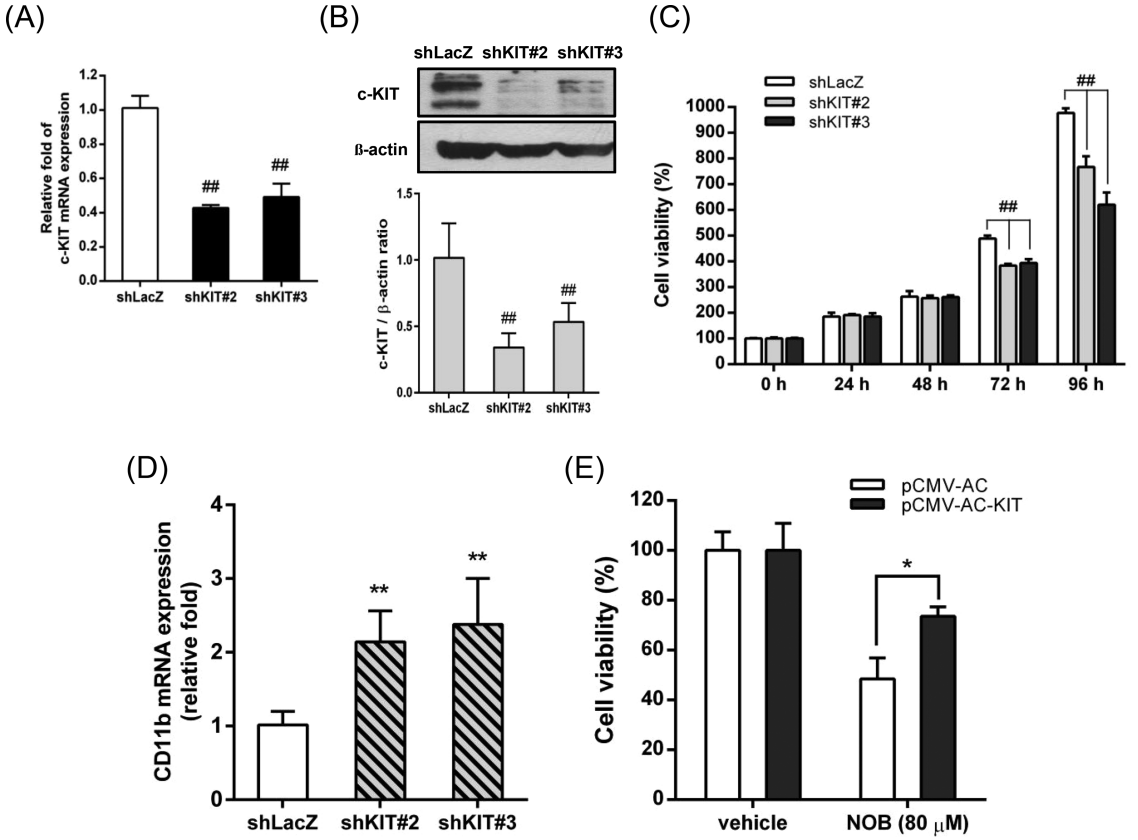

Figure 5. Effects of c-KIT overexpression and knockdown on cell growth and differentiation in THP-1 cells. (A) Negative control LacZ-knockdown (shLacZ) and KIT-knockdown THP-1 cells (shKIT#2 and shKIT#3) were established as described in the Materials and Methods. The c-Kit mRNA levels were determined by Q-RT-PCR analysis. (B) c-KIT and β-actin protein levels were measured by Western blot analysis. The experiments were triplicated, and a representative blot is shown. The intensity of c-KIT versus β-actin protein is normalized and presented as the mean ± SD from triplicated experiments. (C) Cell viability of shLacZ, shKIT#2, and shKIT#3 THP-1 cells were analyzed by MTT assay at 0-, 24-, 48-, 72- and 96-h time points. The data represent the mean ± SD of three independent experiments. ##$p < 0.01$ represents significant differences compared to the shLacZ control group. (D) The expression of CD11b mRNA was measured by Q-RT-PCR analysis. The data represent the mean ± SD of three independent experiments. **$p < 0.01$ represents significant differences compared to the shLacZ control group. (E) pCMV-AC control vector or pCMV-AC-KIT expression plasmid was transfected to THP-1 cells for 24 h, which was followed by treatment with vehicle or NOB (80 μM) for 24 h. Cell viability was determined by MTT assay. The viability of vehicle-treated group (pCMV-AC- and pCMV-AC-KIT-transfected cells) was expressed as 100%, respectively. *$p < 0.05$ represent a significant difference compared to the NOB-treated pCMV-AC-transfected group.

factors including SP1[32] and AP2[33] have been predicted to interact with this DNA region (−300 to −119) for transcriptional activation of *KIT* gene promoter (Figure S4C). We further to investigate whether SP1 and AP2 transcription factors were involved in the NOB-mediated down-regulation of c-KIT transcriptional activity. THP-1 cells were treated with NOB (40 and 80 μM), and nuclear extracts were prepared for Western blot analysis. The level of nuclear SP1 proteins were not significantly changed in NOB-treated cells. Nobiletin slightly increased AP2 protein level in THP-1 cells (Figure S4D,E). These data showed that transcription factors SP1 and AP2 were not involved in the nobiletin-mediated reduction of c-KIT promoter activity in human AML cells.

Effect of c-KIT Expression on Cell Growth and Differentiation in Human AML Cells. We further examined the cell growth of THP-1 cells when c-KIT expression was reduced by shRNA knockdown. Two stable clones with c-KIT knockdown, shKIT#2 and shKIT#3, were established, and their c-KIT expression was significantly reduced compared to parental THP-1 cells, as confirmed by Q-RT-PCR (Figure 5A) and Western blot analysis (Figure 5B). Our results showed that the knockdown of c-KIT expression significantly attenuated cell growth with 72−96 h culture compared to that of the shLacZ-control cells (Figure 5C) ($p < 0.01$). Moreover, the monocytic differentiation marker CD11b in the c-KIT-knockdown clones was significantly increased by two-fold compared to the shLacZ-control cells (Figure 5D). These above results suggested that c-KIT is involved in the response to NOB-mediated cell growth inhibition and differentiation in human AML cells. Moreover, to confirm that the suppression of cell growth by NOB is associated with down-regulation of c-KIT expression, the effect of c-KIT overexpression on NOB-mediated reduction of cell viability was investigated. The THP-1 cells were transfected with the control vector (pCMV-AC) or plasmid for c-KIT overexpression, which was followed by treatment with vehicle or NOB (80 μM) for 48 h, and cell viability was measured using the MTT assay. The viability of vehicle-treated group (pCMV-AC- and pCMV-AC-KIT-transfected cells) was expressed as 100%, respectively, and the percentage of viability in NOB-treated cells was calculated by respective plasmid-transfected cells with vehicle treatment. As shown in Figure 5E, in NOB-treated cells, overexpression of the c-KIT protein (pCMV-AC-KIT group) significantly

DOI: 10.1021/acs.jafc.8b05680
J. Agric. Food Chem. 2018, 66, 13423−13434

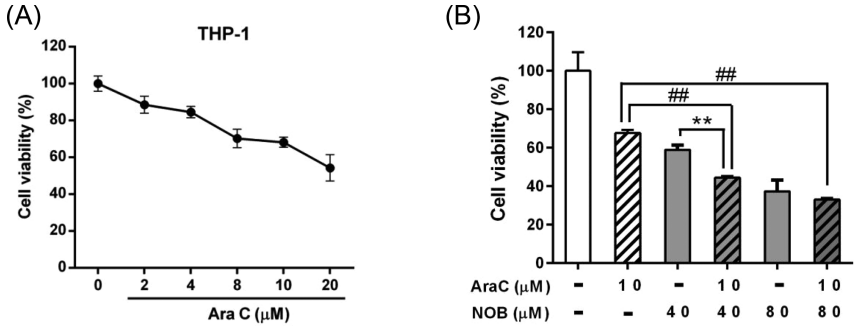

Figure 6. Effects of combination treatment with cytarabine (Ara C) and NOB in THP1 cells. (A) THP-1 cells were treated with cytarabine (Ara C) (0−20 μM) for 48 h. Cell viability was measured by MTT assay. (B) THP-1 cells were pretreated with cytarabine (Ara C) (10 μM) for 12 h, followed by treatment of cells with vehicle or NOB (40 or 80 μM) for a further 36 h. Cell viability was measured by MTT assay. The data represent the mean ± SD of three independent experiments. ##$p < 0.01$ represents significant differences compared to the NOB-untreated group. **$p < 0.01$ represents significant differences compared to the Ara C-untreated group.

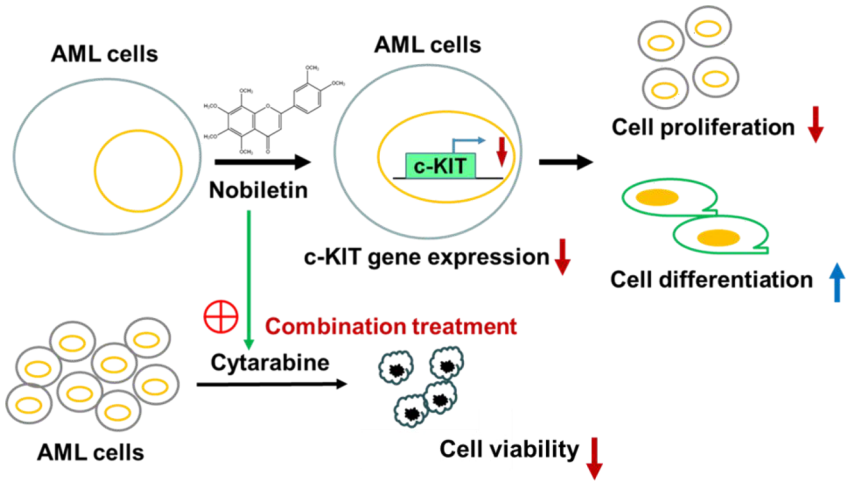

Figure 7. Hypothetic model for the antileukemic effects of NOB on human AML cells. Nobiletin suppresses cancer cell proliferation and promotes cell differentiation via down-regulation of proto-oncogene c-KIT expression in human AML cells. The combination of a conventional AML chemotherapeutic agent, cytarabine, with nobiletin resulted in enhancing cell viability reduction.

elevated the cell viability as compared with control vector-transfected cells (pCMV-AC group) ($p < 0.05$). These data indicated that c-KIT overexpression rescued viability of NOB-treated cancer cells and verified that NOB-reduced c-KIT expression was associated with inhibition of cell growth in THP-1 cells.

Effect of Combination Treatment of Cytarabine and Nobiletin on Human AML Cells. The nucleoside analog cytarabine (Ara-C) has been the first-line of chemotherapeutic drug in AML treatment for several decades. However, some patients are generally intolerant to high doses of Ara-C due to high toxicity. To examine whether NOB can sensitize AML cells to Ara-C, we investigated the effects of combined treatment of Ara-C with NOB in AML cells. First, the viability of Ara-C (2−20 μM)-treated THP-1 cells was examined, and the data showed that Ara-C reduced cell viability in a dose-dependent manner in THP-1 cells (Figure 6A). Next, THP-1 cells were treated with Ara-C (10 μM) alone, NOB alone (40 or 80 μM), or both compounds, and the cell viability was analyzed using MTT assay. As shown in Figure 6B, the cells treated with a combination of Ara-C and NOB demonstrated an enhanced cytotoxic effect for the attenuation of the cell viability compared with Ara-C- or NOB-only treated cells ($p < 0.01$). The values of combination index (CI) were calculated in combination of Ara-C (10 μM) with NOB (40 and 80 μM) were 0.98 (CI = 1) and 1.24 (CI > 1), respectively. These data suggested that summation was found at lower concentration of NOB; however, antagonism was found at higher concentration of NOB. The significant antileukemic effects of combined Ara-C and NOB treatment were also demonstrated in U-937 and HL-60 cells (Figure S5A,B). Furthermore, to elucidate the mechanism of the combination of Ara-C and NOB on the suppression of cell viability, the apoptotic effects were evaluated in these compounds-treated THP-1 cells. The cells treated with Ara-C (10 μM) alone significantly increased the levels of apoptotic markers cleaved-caspase 3 and PARP1. However, the cells treated with a combination of Ara-C (10 μM) and NOB (40 or 80 μM) reduced the levels of these activated apoptotic proteins as compared with Ara-C alone-treated group (Figure S6). These results suggested that the combination of cytarabine and NOB suppress the cell viability by cell growth inhibition but not elevation of cell apoptosis.

DISCUSSION

Targeting protein tyrosine kinases is a compelling strategy to treat human cancer with aberrant kinase activities. A large variety of kinase mutations have been reported in AML, and several have been clinically explored for targeted inhibition.[34] Studies have shown that the c-KIT receptor was expressed in

DOI: 10.1021/acs.jafc.8b05680
J. Agric. Food Chem. 2018, 66, 13423−13434

blast cells in most cases of AML and in some case of chronic myelogenous leukemia (CML) in blastic crisis but was expressed at low levels or not at all acute lymphoblastic leukemia (ALL).[35] Activating mutations in c-KIT have also been described, predominantly in patients with CBF-AML, and are associated with worse prognosis.[4] The c-KIT protein has been targeted by tyrosine kinase inhibitors, such as imatinib or dasatinib, in AML.[36] In this study, we demonstrated that NOB dramatically suppressed cell proliferation and induced cell differentiation of AML cells through down-regulation of c-KIT gene expression by transcriptional repression of promoter activity. Additionally, we demonstrated that NOB combined with cytarabine is a more effective treatment to reduce viability of cancer cells (Figure 7). Our current findings suggest that NOB represents a novel c-KIT inhibitor for the treatment of AML. In contrast to other c-KIT inhibitors, which usually interfere with c-KIT protein activity via targeting its receptor, this transcriptional inhibition of c-KIT expression appears to be a new mechanism and is thus considered as a novel therapeutic approach. Moreover, NOB in combination with cytarabine (Ara-C) markedly enhances the cytotoxic effect on AML cells at considerably reduced doses of cytarabine, which could be used for therapeutic applications for patients with AML.

Nobiletin, a flavonoid rich in citrus fruits, has been demonstrated to play a pivotal role in chemoprevention.[37] In the present study, we found that NOB (20−100 μM) strongly inhibited cell growth in acute myeloid leukemia cell lines including THP-1, U-937, and HL-60 cells. Nobiletin has been reported to inhibit cell proliferation through cell cycle arrest in leukemia cell lines.[24,26] In the present study, we found that NOB induced G1 phase arrest in THP-1 cells. Similarly, NOB has been demonstrated to inhibit cell growth by inducing G0/G1 arrest in other tumors.[38,39] The cell cycle arrest in late G1 phase or G1/S transition was known to be associated with increased cell cycle regulators and cyclin-dependent kinase (CDK) inhibitors such as p21 Waf1/Cip1, and p27 Kip1, and reduced the level of cyclin E.[31] In the present study, we found that NOB increased the levels of p21 and p27 as well as attenuated cyclin E1 protein level. Our data suggested that NOB may alter cell cycle distribution and arrest cell cycle in late G1 phase to impede S phase progression. In this study, we investigated the antileukemic effects of NOB on AML cell lines in vitro. NOB is a polymethoxyflavone phytochemical, similar to several flavonoids, and has a poor water solubility and low oral bioavailability in vivo. Singh et al.[40] reported on the pharmacokinetic studies of NOB that oral administration of compound (50 mg/kg) in male Sprague−Dawley rats, that the maximal concentrations (C_{max}) of NOB in plasma was 1.78 μg/mL (equivalent to 4.42 μM), time to maximum concentration (t_{max}) was around 1 h, and the half-life ($t_{1/2}$) was 1.8 h. Manthey et al.[41] reported that oral administration of NOB (50 mg/kg) by gavage in rats, at 8 h time point, NOB and its metabolites such as glucuronides and aglycones also could be detected in plasma and the level of NOB in blood at this time point was 2.7 μg/mL (equivalent to 6.71 μM). NOB possessed the long half-lives and remained for prolonged periods up to 24 h after administration. We demonstrated suppression of leukemia cell growth by NOB at concentrations 20−100 μM, which are used in several anticancer studies, though it is higher than what can be reached in vivo. To enhance the oral bioavailability and bioactivity of NOB in vivo, novel formulation or modification such as nanoemulsion delivery system or chemical modification may improve its solubility and achieve the concentration employed in this study.[42,43]

AML is characterized by aberrant regulation in hematopoietic proliferation, apoptosis, and differentiation. Therefore, differentiation therapy, which means induction of leukemia cancer cells toward differentiation, appears to be an alternative approach in the application of natural products.[44] Differentiation therapy with all-trans retinoic acid (ATRA) has been successfully developed to treat acute promyelocytic leukemia (APL). Numerous natural or synthetic compounds alone or in combination with ATRA effectively induced leukemia cells toward differentiation in vitro and in vivo.[45] Herein, we showed that NOB significantly induced the expression of the differentiation marker CD11b in a dose-dependent manner in THP-1, U937, and HL-60 cells. Our results indicated that NOB exhibited regulatory activities to induce myeloid cell differentiation in AML cells. In the present study, we proposed that c-KIT inhibition is possibly involved in the effects of NOB-mediated cell differentiation because knockdown of c-KIT expression alone promotes cell differentiation. However, hematopoietic differentiation is a multifaceted process that depends on complex regulatory networks involving transcriptional, post-transcriptional, and epigenetic regulation of various genes' expression. Thus, the detailed molecular mechanisms associated with NOB-mediated cell differentiation via c-KIT down-regulation need to be further investigated.

Overexpression or mutations of the *KIT* gene has been demonstrated to drive tumorigenesis in several types of cancer including leukemia.[46] Targeting c-KIT or its downstream signal pathways is useful in treating cancers associated with increased c-KIT activity. Different approaches have been developed for this strategy. Imatinib mesylate is one example of successful solid tumor treatment with a tyrosine kinase-targeted drug, which was originally designed for the treatment of CML. Imatinib then was applied to treat GIST, with good outcomes.[47] Nevertheless, it has been reported that different classes of activating c-KIT mutations respond differently to different kinase inhibitors.[48] This highlights the need to identify specific variants of mutant KIT expressed by individual patients when one considers rational therapy. Unfortunately, most patients will gradually develop resistance to kinase inhibitor such as imatinib. The resistance mechanism may be ascribed to the occurrence of secondary mutations in the *KIT* gene. Therefore, it is very important to discover more effective second-line or third-line drugs that can be made available for personalized treatment to combat resistance.[34] Inhibitors for down-regulation of *KIT* transcriptional levels, rather than targeting kinase activity, offer a novel strategy for cancer therapy. In the present study, analysis from microarray data, real-time PCR, and Western blot analysis revealed that NOB functions as a potential c-KIT inhibitor to attenuate both RNA and protein expression. We further demonstrated the reduction effects of NOB on c-KIT gene expression through transcriptional suppression of its promoter activity. The NOB-responsive elements within the *KIT* gene promoter were also identified to locate at the DNA sequence between the −300 and −119 positions, which possessed the binding sites for the transcription factor SP1 and AP2 to activate transcription. However, we further demonstrated that SP1 and AP2 proteins may be not the critical regulators for NOB-mediated c-KIT down-regulation. Recently, it has been reported that small molecules that directly interact with the *KIT* promoter to suppress c-KIT mRNA transcription are associated with potent

DOI: 10.1021/acs.jafc.8b05680
J. Agric. Food Chem. 2018, 66, 13423−13434

inhibition of cell growth in GIST cells.[49,50] Whether NOB can interact with *KIT* promoter and the detail mechanism by which NOB modulates the promoter activity remains unclear and needs to be further investigated.

In the present study, we demonstrated that knockdown c-KIT alone impairs cell proliferation. Moreover, overexpression of the c-KIT protein in THP-1 cells could increase cell viability in NOB-treated cells. These data supported that the c-KIT protein is a critical target for the effects of NOB-mediated antileukemic activities. In the present study, we found that the attenuated cell growth in silencing of c-KIT cells was observed at 72−96 h; however, NOB inhibits the protein expression of c-KIT and the cell proliferation at 48 h. These data implied that not only c-KIT protein is involved in the antileukemic effect of NOB. In addition to c-KIT down-regulation by NOB, the microarray data showed that NOB dramatically down-regulated the mRNA expression of proliferation-related genes such as *TP63*, *LGR4*, and *PA2G2*. These genes may be involved in the NOB-mediated suppression of leukemia cell growth and need to be further investigated. Our current results suggest a novel mechanism of antileukemic activity for NOB, which could reduce the expression level of c-KIT mRNA and protein, unlike other small molecule inhibitors that target c-KIT kinase activity. These findings suggest that NOB has potential utilities in the treatment of AML with aberrant c-KIT expression.

NOB and other natural products represent safe and low-toxic agents, the application of which is under investigation in clinical practice. An increasing understanding of the pathogenesis of AML, facilitated by next-generation sequencing, has spurred the development of new drugs in the treatment of AML, particularly small molecules that target the disease via molecular modulation. Various new drugs, such as tyrosine kinase inhibitors, epigenetic inhibitors, and monoclonal antibodies, are currently being investigated in clinical trials. It is likely that the highest response rates will be achieved when new molecularly targeted therapies are combined with traditional chemotherapy. In the present study, we found that NOB (20−100 μM) induced low cytotoxicity on normal PBMCs. The drug combination analysis data suggested that concentration-dependent drug interactions were found within Ara-C and NOB combination, additive effect was found at lower concentration of NOB (40 μM); however, antagonistic effect was found at higher concentration of NOB (80 μM). We further demonstrated that the combination of Ara-C and NOB reduce the cell viability through inhibition of cell proliferation, not by promotion of cell apoptosis. These results suggested that low concentration of NOB combined with cytarabine treatment resulted in more suppressive effects on leukemia cell growth. Our current findings reveal that NOB represents a nontoxic and effective agent to combine with traditionally chemotherapeutic agents to target AML cell proliferation by interference with c-KIT overexpression.

In conclusion, the current study demonstrated that NOB is a c-KIT inhibitor and exerts antileukemic effects via the suppression of the gene expression of c-KIT in human acute myeloid leukemia cells. NOB sensitized myeloid leukemia cells to the chemotherapeutic cytarabine. Our current findings support that the polymethoxyflavone NOB may serve as a chemo-preventive or chemotherapeutic agent against human hematological malignancies.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.8b05680.

Effects of NOB on cell growth in U-937 and HL-60 cells; effects of NOB on myeloid cell differentiation in U-937 and HL-60 cells; effects of NOB on c-KIT mRNA expression in U-937 and HL-60 cells; characterization of nobiletin-responsive element within human c-KIT promoter; effects of combination treatment of cytarabine (Ara C) and NOB in U-937 and HL-60 cells; effects of combination treatment of cytarabine (Ara-C) and NOB on cell apoptosis in THP-1 cells (PDF)

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Funding

This research was supported by Grant No. TCRD 103−017 (to P.-Y.C.) from Buddhist Tzu Chi General Hospital and Grant No. MOST-105−2320-B-320−004-MY3 (to J.-H.Y.) from the Ministry of Science and Technology, Taiwan.

Notes

The authors declare no competing financial interest.

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