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Nobiletin, a Polymethoxylated Flavonoid from Citrus, Shows Anti-Angiogenic Activity in a Zebrafish In Vivo Model and HUVEC In Vitro Model

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ABSTRACT

Traditional Chinese medicinal herbs are a rich source of compounds with reported anti-inflammatory and anti-carcinogenic effects. Growing evidence shows the codependence of chronic inflammation and angiogenesis, and the potential benefits of targeting angiogenesis in the treatment of chronic inflammation and targeting inflammation in the treatment of diseases with impaired angiogenesis. We hypothesized that the anti-inflammatory activity of the natural compounds may owe at least some of its efficacy to their anti-angiogenic activity and hence we investigated the anti-angiogenic activity of these compounds in vivo in zebrafish embryos and in vitro in human umbilical vein endothelial cells (HUVECs). Nobiletin, a polymethoxylated flavonoid from citrus fruits, showed anti-angiogenic activity in both assays. Nobiletin inhibited the formation of intersegmental vessels (ISVs) in live transgenic zebrafish embryos expressing green fluorescent protein (GFP) in the vasculature. Cell cycle analysis of dissociated zebrafish embryo cells showed that nobiletin induced G0/G1 phase accumulation in a dose-dependent manner in GFP-positive endothelial cells. Nobiletin also dose-dependently induced *VEGF-A* mRNA expression. In HUVECs, nobiletin inhibited endothelial cell proliferation and, to a greater extent, tube formation in a dose-dependent manner. As in the in vivo study, nobiletin induced G0/G1 cell cycle arrest in HUVECs. However, this arrest was not accompanied by an increase in apoptosis, indicating a cytostatic effect of nobiletin. This study, for the first time, identifies nobiletin as having potent anti-angiogenic activity and suggests that nobiletin has a great potential for future research and development as a cytostatic anti-proliferative agent. J. Cell. Biochem. 112: 3313–3321, 2011.

KEY WORDS: NOBILETIN; FLAVONOID; ANTI-INFLAMMATORY; ANGIOGENESIS; ZEBRAFISH; CELL CYCLE

Angiogenesis plays an important role in the development of cancer and chronic inflammatory diseases including psoriasis, retinopathy, and rheumatoid arthritis [Folkman, 1995; Jackson et al., 1997]. There is growing evidence to suggest that chronic inflammation and angiogenesis are codependent, involving increased cellular infiltration and proliferation as well as over-lapping roles of regulatory growth factors and cytokines [Jackson et al., 1997]. Pharmacology of many anti-inflammatory drugs revealed at least some part of their efficacy was due to their anti-angiogenic effect [Jackson et al., 1997].

Traditional Chinese medicinal herbs have been used for thousands of years for treatment of chronic inflammation and

Kai Heng Lam and Deepa Alex contributed equally to the work.

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many compounds isolated from these herbs have been shown to possess anti-inflammatory or anti-carcinogenic activity. We hypothesized that these effects could be partly due to their anti-angiogenic activity. We used zebrafish embryos as a live in vivo model for an angiogenesis assay, in addition to a human umbilical vein endothelial cell (HUVEC) in vitro model. Zebrafish is an excellent animal model for the study of angiogenesis, with many anti-angiogenic drugs eliciting similar responses as in mammalian systems [Langheinrich, 2003], and especially due to the availability of transgenic zebrafish expressing GFP in the vasculature, allowing the rapid analysis of live embryos in response to drugs [Lawson and Weinstein, 2002; Norrby, 2006]. Recently, we demonstrated the feasibility of drug screening in zebrafish model and founded the anti-angiogenic effect of resveratrol derivative [Alex et al., 2010] and pro-angiogenic effects of *Angelica sinensis* and *Panax notoginseng* extracts [Lam et al., 2008; Hong et al., 2009].

In the course of our survey of natural compounds, with reported anti-inflammatory activity, for anti-angiogenic activity in the zebrafish angiogenesis model, we successfully identified nobiletin as showing potent anti-angiogenic activity. Nobiletin (5,6,7,8,3′,4′-hexamethoxyflavone), was first identified from the peel of citrus fruits, and along with its derivatives has been reported to have a broad range of biological effects. These effects include anti-inflammatory activity [Lin et al., 2003], especially for airway inflammation [Wu et al., 2006], and as an inhibitor of ROS production [Choi et al., 2007], indicating a similar effect compared to the traditional remedy where the citrus peel extract is used to relieve airway ailments. Nobiletin acts as an anti-carcinogenic compound through anti-proliferative activity, induction of apoptosis and cell cycle deregulation [Yoshimizu et al., 2004], and was shown to have anti-proliferative activity in human lung cancer [Luo et al., 2008] and colon cancer in mice [Miyamoto et al., 2008]. Furthermore, nobiletin was shown to cause cell cycle arrest at G1 phase [Morley et al., 2007], and also appears to inhibit the migration of gastric carcinoma in vivo [Minagawa et al., 2001]. In neurobiology, nobiletin has a demonstrated positive effect in rescuing memory deterioration in the Alzheimer's disease rat model [Matsuzaki et al., 2006] and olfactory-bulbectomized mice, as well as improved prevention of memory impairment in the Alzheimer's disease rat model [Onozuka et al., 2008].

In this paper, we identified for the first time that nobiletin has potent anti-angiogenic activity in vivo in zebrafish embryos and in vitro in human endothelial cells through regulating cell cycle arrest and the VEGF pathway. Since angiogenesis plays an important pathological role in the progress of a wide range of diseases, our findings provide a rationale for future development of this compound as a potential drug or chemopreventive supplement to target diseases with excessive angiogenesis.

MATERIALS AND METHODS

CELL LINES AND CHEMICALS

HUVECs were obtained from ATCC. Nobiletin and naringin were purchased from Shanghai Tauto Biotech Co, China.

HUVECs were cultured in Kaighn's modification of Ham's F12 medium (F-12K) with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 100 μg/ml heparin, 30 μg/ml endothelial cell growth supplement (ECGS, Sigma–Aldrich, St. Louis), 10% heat-inactivated fetal bovine serum (FBS) and 100 U/ml penicillin-streptomycin (Gibco). Cells were incubated at 37°C in 5% CO_2 (v/v). Tissue culture flasks were pre-coated with 0.1% gelatin. All assays were conducted using low passage cells (4–6 passages). Nobiletin and naringin were dissolved in dimethylsulfoxide (DMSO, Sigma–Aldrich) as a 100 mM stock solution. The stock solution was diluted by sterilized Milli-Q (Millipore) water in different concentrations for use. Vascular endothelial growth factor receptor (VEGFR) inhibitors SU5416 (Calbiochem) and VEGFR-inhibitor II (Calbiochem, Catalogue No. 676481) were used as positive control for in vitro and in vivo experiment, respectively.

MAINTENANCE OF ZEBRAFISH AND COLLECTION OF EMBRYOS

Transgenic zebrafish-Tg(*fli1*:EGFP), which express green fluorescent protein(GFP) in endothelial cells, were kindly provided by ZFIN (Oregon) and wildtype zebrafish were purchased from a local pet shop, for use as the in vivo model. Both stocks were maintained as described in zebrafish handbook [Westerfield, 1993]. In brief, stocks were maintained in a controlled environment at a temperature of 28.5°C on a 14 h: 10 h light/dark cycle. Fish were fed twice daily with brine shrimp in the morning and afternoon, and also with general tropical fish food occasionally. Embryos were collected in the morning and cultured at 28.5°C in distilled water. After 1 day post-fertilization (dpf), embryos were dechorionated with tweezers in a Petri dish coated with 1% agarose, and distributed into a small Petri dish (35 mm × 10 mm) with 3 ml assay solution depending on the drug treatment. Embryos receiving DMSO (0.3%) served as a vehicle control and were equivalent to no treatment. All of the experiments were repeated at least three times, with 30 embryos per group.

CELL PROLIFERATION ASSAY

HUVECs were seeded onto 96-well gelatin coated plates at a density of 10^4 cells/well. In order to achieve a quiescent state, complete medium was replaced after 24 h incubation with low serum (0.5% FBS) medium and re-incubated for 24 h. After this, medium was replaced with various drug treatments diluted in low serum (0.5% FBS) medium containing 20 ng/ml of VEGF. Cells receiving DMSO (0.1%) served as vehicle control and 5 μM SU5416 served as positive control. Plates were incubated for an additional 48 h and cell proliferation assessed by the Cell Proliferation Kit II (XTT) (Roche) in accordance with the manufacturer's protocol. Fifty microliter of XTT test solution were added into each well and incubated for an additional 4 h at 37°C. The spectrophotometric absorbance of each well was measured using a Multilabel-counter fluorescent plate reader (PerkinElmer). The wavelength used to measure absorbance of the formazan product was 450 nm and the reference wavelength was 690 nm. The results were expressed as the percentage of proliferating cells.

MORPHOLOGICAL OBSERVATION OF ZEBRAFISH

After drug treatment, embryos were anesthetized using 1% tricaine (Sigma–Aldrich) and observed for morphological changes using an Olympus Spinning Disk Confocal Microscope System (IX81 Motorized Inverted Microscope (w/ZDC), IX2 universal control

box, X-cite series 120, Andor iXon EM CCD camera, DP71 CCD camera). Images were analyzed with SlideBook 4.2 and Adobe Photoshop 7.0.

ACRIDINE ORANGE (AO) STAINING OF ZEBRAFISH EMBRYOS

After 8 h drug treatment, embryos were washed in phosphate buffered saline (PBS) and stained with 1 μg/ml AO for 1 h at 28.5°C followed by three PBS washes. Photos were taken using the Confocal Microscope System described above.

CELL CYCLE ANALYSIS OF ZEBRAFISH ENDOTHELIAL CELLS AND HUVEC

Twenty Tg(*fli1*:EGFP) zebrafish embryos at 24 h post-fertilization (hpf) were exposed to various drug treatments in 3 ml medium in a Petri dish. After 8 h treatment, embryos were washed in PBS and digested in 500 μl trypsin lysis solution (0.5 g/L trypsin (1:250) in a solution of 0.14 M $CaCl_2$, 0.05 M KCl, 0.005 M glucose, 0.007 M $NaHCO_3$, and 0.7 mM EDTA), incubated for 30 min at 28.5°C, and triturated through a narrow-bore Pasteur pipette until they were completely dissociated. Trypsin digestion was terminated by adding 1 volume of stop solution (2 mM $CaCl_2$, 20% FBS). Cell suspensions were centrifuged at 1,000 g for 5 min at 4°C. Pellets were resuspended in 1 ml cold PBS with 1% FBS to wash away remaining trypsin, and re-centrifuged at 1,000 g for 5 min at 4°C. Resultant pellets were resuspended in 500 μl PBS. DRAQ5™ (Biostatus Ltd., UK) was added to the cells at a concentration of 20 μM and samples incubated at 37°C for 1 h. Clumps of cells and non-cellular debris were removed by passing cell suspensions through a nylon cell strainer with pore size of 40 μm (BD Falcon). Stained cell suspensions were analyzed immediately using a flow cytometer (BD FACS Canto™). During flow cytometric analysis, cell suspensions were kept constantly on ice. Measurements were recorded using a FITC filter (to detect the GFP positive cells from the transgenic zebrafish) and the PerCP-Cy5 channel to detect DNA content for cell cycle analysis.

For HUVECs, cells were seeded onto 25 cm^2 flasks with complete medium. Cells were starved by using low serum (0.5% FBS) medium for 24 h to render them quiescent. Samples were then treated with complete medium containing different concentrations of nobiletin for another 24 h. Cells receiving DMSO (0.1%) served as vehicle control. After treatment, cells were trypsinized, washed with PBS and fixed in pre-chilled 70% ethanol at −20°C overnight. Fixed samples were washed with PBS, and then incubated with propidium iodide (5 μg/ml) and RNase (10 μg/ml) for 30 min. Stained cells were analyzed using a Flow Cytometer. DNA content analyses in G1, S, and G2/M phases were performed using ModfitLT Version 3.0 software (Verity Software House, Topsham).

TOTAL RNA EXTRACTION, REVERSE TRANSCRIPTION, AND REAL-TIME PCR

Total RNA was extracted from samples of 50 zebrafish embryos using RNeasy Mini Kit (QIAGEN) in accordance with the manufacturer's instructions. RNA was reverse transcribed to single-stand cDNA using SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen), followed by real-time PCR using TaqMan®Universal PCR Master Mix and custom Taqman primer for zebrafish *VEGF-A* (GenBank: AF059661, Applied Biosystems) in an ABI 7500 Real-Time PCR system (Applied Biosystems). Relative expression of VEGFa was normalized to the amount of β-actin in the same cDNA by using the relative quantification method described by the manufacturer.

TUNEL ASSAY

HUVECs were seeded onto 25 cm^2-flasks with complete medium. Once confluent, cells were starved by low serum (0.5% FBS) medium for 24 h to render them quiescent. Samples were then treated with complete medium containing different concentrations of nobiletin for another 24 h. Cells receiving DMSO (0.1%) served as vehicle control and 30 μM *t*-BHP served as positive control. After treatment, cells were harvested and then fixed in 1% (w/v) paraformaldehyde for 15 min. Following fixation, cells were further fixed in pre-chilled 70% ethanol at −20°C overnight. TUNEL assays (Terminal deoxynucleotidyl transferase dUTP nick end labeling) were performed using the APO-BrdU™ TUNEL Assay Kit (Invitrogen); cells were incubated for 1 h at 37°C with DNA-labeling solution containing TdT enzyme and BrdUTP, and then for 30 min with anti-BrdU antibody. Labeled cells were analyzed using the Flow Cytometer.

TUBE FORMATION ASSAY

The effect of nobiletin treatment on HUVEC differentiation was examined by in vitro tube formation on Matrigel (BD Sciences). 1×10^5 cells, in low serum medium containing various concentrations of nobiletin and 20 ng/ml of VEGF, were seeded onto Matrigel-coated 24-well plate at 37°C for 7 h. Cells receiving DMSO (0.1%) served as a vehicle control. The network-like structures were examined under an inverted microscope (Axiovert200, Carl Zeiss, Hong Kong) at 50× magnification.

STATISTICAL ANALYSIS

Each experiment was performed at least three times, and all values are presented as means ± SEM of triplicates. One-way ANOVA was used to analyze the statistical significance of the results. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Transgenic zebrafish embryos, Tg(*fli1*:EGFP) expressing EGFP under the control of the *fli-1* promoter, where the endothelial cells can be directly observed using fluorescence microscope, were used to study anti-angiogenic effect of nobiletin and its derivative naringin (Fig. 1). Figure 2A shows the inhibitory effect of different doses of nobiletin on intersegmental vessel (ISV) formation in zebrafish embryos. Zebrafish embryos at 24 hpf treated with nobiletin for 8 h showed a dose-dependent inhibition of ISV formation compared to vehicle control. For reference, embryos treated with 0.2 μM VEGFR inhibitor II served as positive control, which showed similar ISV regression. Naringin which is also a major flavonoid from citrus fruits, had no effect on ISV formation (Supplementary data; Fig. 1S). The apparent anti-angiogenic effect was most pronounced at a nobiletin concentration of between 30 and 100 μM, with higher

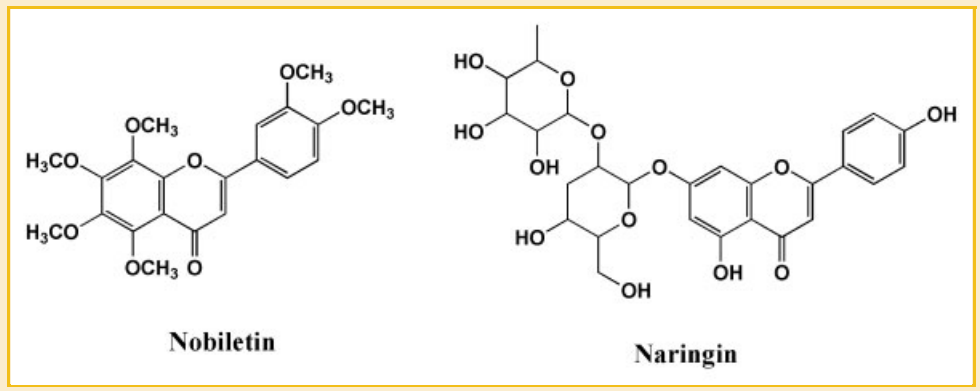

Fig. 1. Chemical structures of nobiletin and naringin.

concentrations becoming toxic to the embryos over time. Figure 2B shows the overall percentage of zebrafish embryos with visible inhibition of ISVs when observed under fluorescent microscope (anti-angiogenic phenotype) upon treatment with different concentrations of nobiletin, and shows that higher concentrations of nobiletin (30–100 μM) caused obvious inhibition of ISV formation. The anti-angiogenic effect of nobiletin on ISVs was the most pronounced for 24–48 hpf embryos when rapid angiogenesis at ISVs occurred. At later stages of embryos when ISVs were already established, nobiletin treatment did not cause visible changes in blood vessel morphology (Supplementary data; Fig. 2S). This indicates that nobiletin causes anti-angiogenesis by blocking new blood vessel formation and not by destruction of existing vasculature.

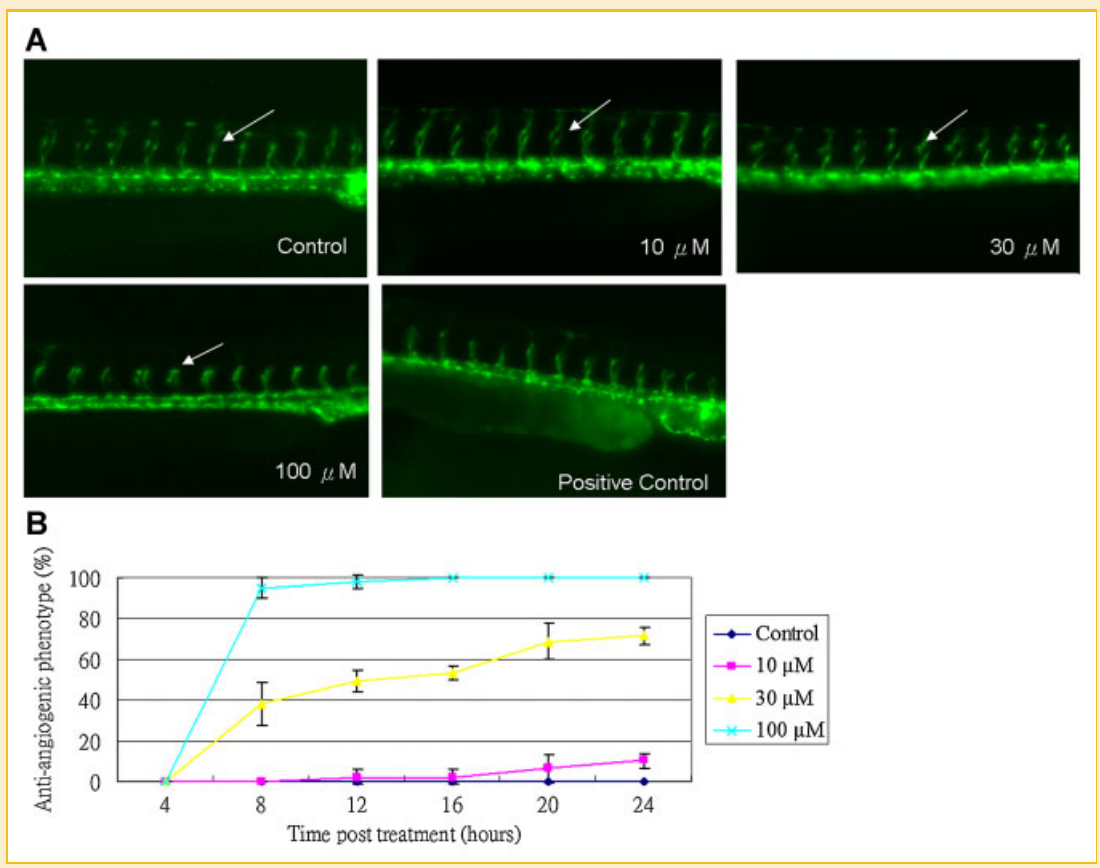

Fig. 2. In vivo anti-angiogenic effect of nobiletin in zebrafish embryos. A: The effect of nobiletin on blood vessel formation in ISVs captured after treatment of 24-hpf Tg(*fli1*:EGFP) zebrafish embryos for 8 h. The ISVs were observed under a confocal fluorescent microscope. The white arrows indicate the ISVs. B: Quantitative analysis of the percentage of anti-angiogenic phenotype after the treatment with nobiletin. 0.1% DMSO and VEGFR kinase inhibitor served as control and positive control, respectively. All data are presented as mean ± SEM of three individual experiments. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

Angiogenesis is a complex process, typically involving endothelial cell proliferation and alignment to form tubular structures [Risau, 1997). We further explored the anti-angiogenic activity of nobiletin in human endothelial cell proliferation using XTT assay, a colorimetric assay to measure the activity of enzymes that reduce water-soluble XTT dye (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) to orange formazan thereby assessing viability of cells. We also measured the ability of endothelial cells to form tubular structures using Matrigel model. As shown in Figure 3A, nobiletin dose-dependently inhibited HUVEC proliferation with significant inhibition at 60 and 100 μM ($P < 0.001$). Also, nobiletin dose-dependently inhibited tube formation (Fig. 3B–D), with significant inhibition ($P < 0.05$) observed at 100 μM concentration (Fig. 3E), indicating nobiletin also inhibits endothelial cell differentiation on Matrigel. Tube formation was quantified by measuring branching points upon treatment with nobiletin (Fig. 3E).

We performed a detailed toxicity assay of nobiletin on zebrafish embryos. As shown in Figure 4A, nobiletin was progressively toxic to zebrafish embryos at 24–48 hpf. Moreover, nobiletin seemed to cause delayed development of zebrafish embryos, as can be observed from the lower level of pigmentation in body and eyes, larger yolk sacs, and shorter trunk upon drug treatment. However, AO staining of zebrafish embryos to observe apoptosis did not show any obvious difference between control and nobiletin treated embryos (Fig. 4B). This indicates that nobiletin causes growth arrest

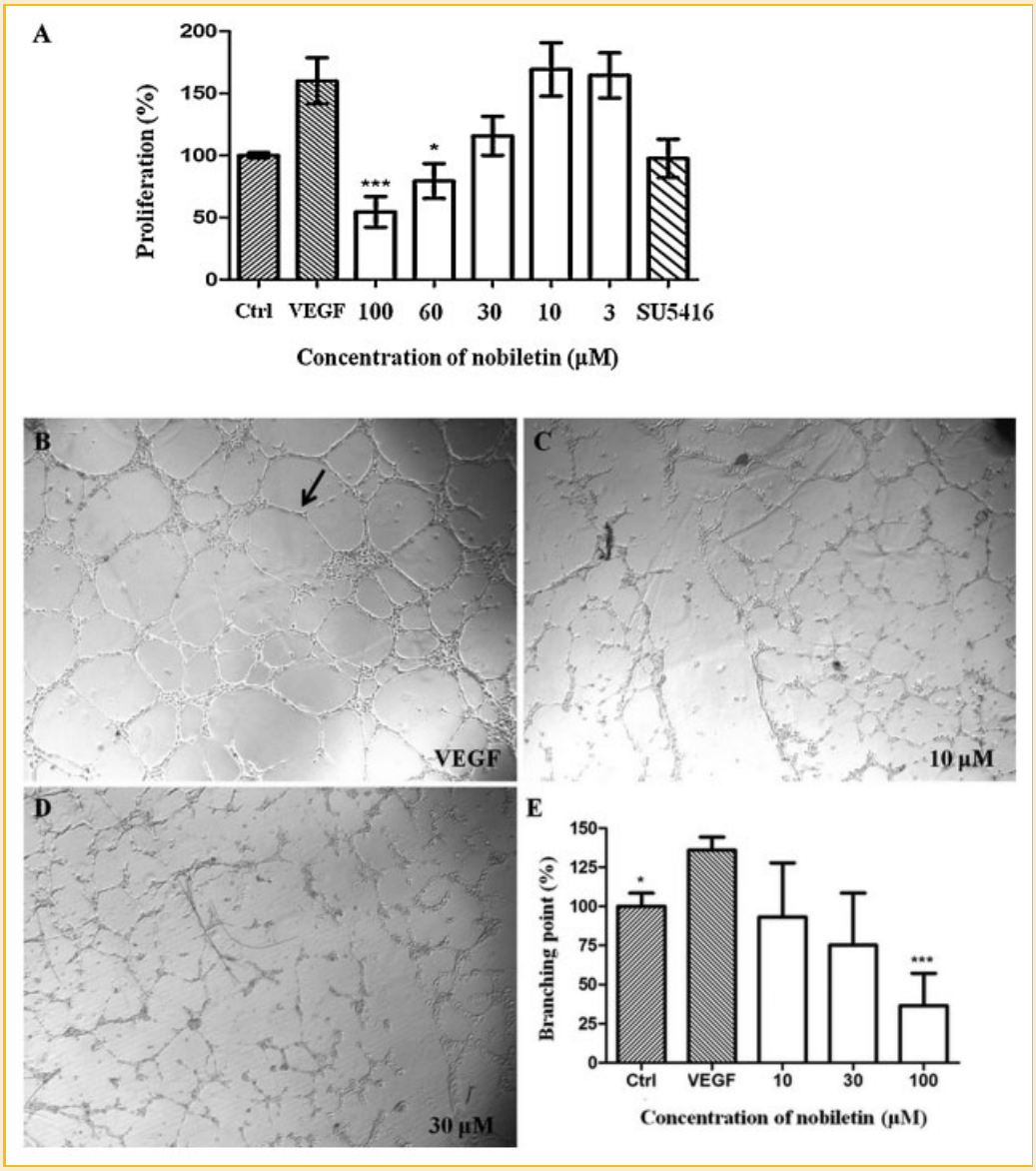

Fig. 3. In vitro anti-angiogenic effect of nobiletin in HUVECs. Inhibitory effect of nobiletin on VEGF-induced HUVEC proliferation as measured by XTT assay (A). Result represented as 100% of vehicle-control. Tube formation assay (B–D), showing morphological features of nobiletin-treated HUVECs on Matrigel. The branching points (black arrows) decrease after the treatment with nobiletin (E). Quantitative data are presented as mean ± SEM of three individual experiments. * $P < 0.05$, *** $P < 0.001$, versus VEGF-control.

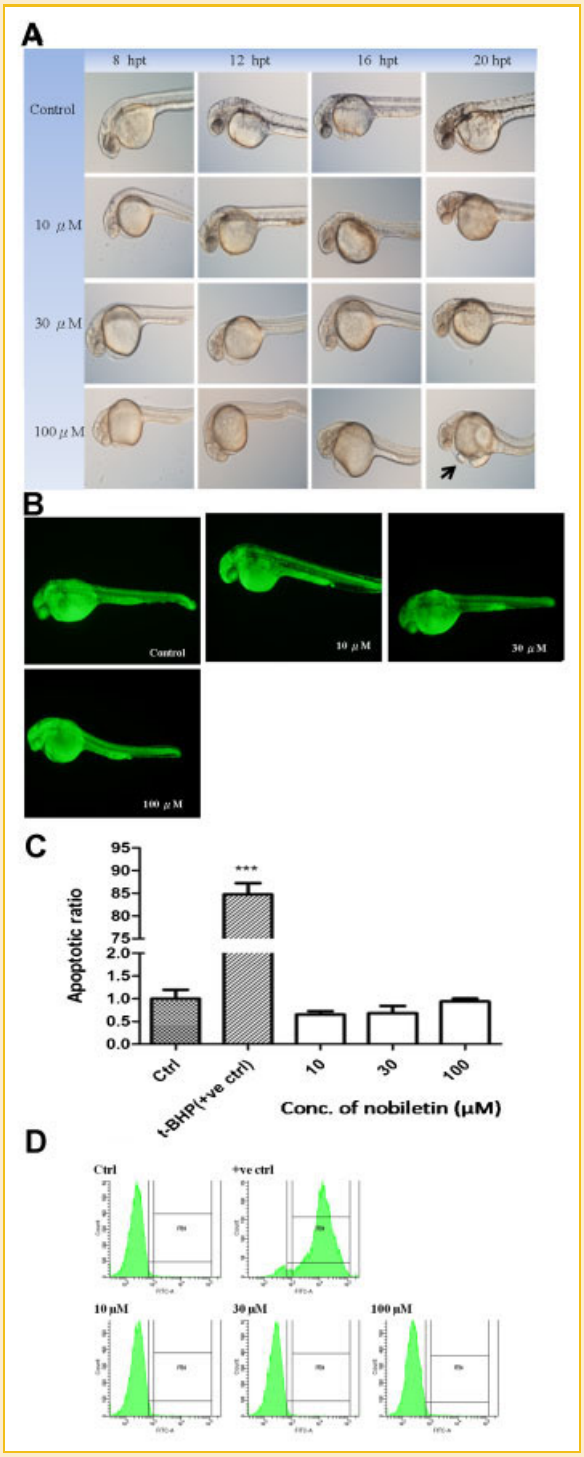

Fig. 4. Toxicity and apoptotic effects of nobiletin. A: The morphological features of zebrafish embryos observed and captured after treating 24-hpf embryos with nobiletin for 8, 12, 16, and 20 h. The black arrow indicates heart edema. B: The apoptotic morphology investigated by AO staining in zebrafish embryos at 32 hpf after treatment with nobiletin for 8 h. C: The apoptotic ratio caused by nobiletin on HUVECs after treatment for 24 h measured by TUNEL staining. All data are presented as mean ± SEM of three individual experiments. D: Flow cytometric analysis of a representative TUNEL assay. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

and eventual toxicity to the zebrafish cells without activating overall apoptosis. Indeed, in HUVECs, even the highest concentration of nobiletin was unable to induce appreciable apoptosis compared to controls (Fig. 4C, D), although at that concentration it did inhibit cell proliferation and tube formation.

Nobiletin has been previously reported to exert its activity by modulation of cell cycle progression in different cell types [Yoshimizu et al., 2004; Morley et al., 2007]. To further analyze the mechanism of action of nobiletin in vitro and in vivo, we performed cell cycle analyses of zebrafish embryo cells and HUVECs using flow cytometry (Fig. 5). In the case of zebrafish embryo cells, the endothelial GFP-expressing cells (measured in the FITC channel, Y-axis) and the DNA content of the cells (measured by PerCP-Cy5, X-axis) were plotted to differentiate the GFP-positive and GFP-negative cells, represented in green and blue dots respectively in Figure 5A–E. For reference, the dot plot of wild-type zebrafish embryos is shown in Figure 5A, where there are no GFP positive cells. Figure 5F shows the cell-cycle distribution of the endothelial (GFP-positive) cells from five independent experiments after treatment of zebrafish embryos at 24 hpf with different concentrations of nobiletin for 8 h. Nobiletin significantly ($P < 0.05$) induced dose-dependent accumulation of cells in G0/G1 phase in endothelial cells (Fig. 5F). Nobiletin also caused G0/G1 phase accumulation in non-endothelial cells (Fig. 5G); however, to a slightly lesser extent than that in endothelial cells, indicating that it may be more selective toward inhibition of endothelial cell proliferation.

In HUVECs, nobiletin was able to dose-dependently cause cell cycle arrest at G0/G1 phase (Fig. 5H), with 30 and 100 μM nobiletin causing highly significant ($P < 0.01$) accumulation of cells in G0/G1 phase with a concomitant decrease of cells in S and G2/M phase. These results show that nobiletin causes G0/G1 cell cycle arrest in both zebrafish embryo cells and HUVECs.

VEGF and its receptors are the most important signaling molecules involved in neovascularization. We used real-time quantitative PCR to measure the relative levels of mRNA expression for *VEGF-A* and its receptors, *flk1*, *flt1*, and *flk4* after nobiletin treatment. Only *VEGF-A* showed a dose-dependent increase, with 10, 30, and 100 μM nobiletin resulting in a significant increase ($P < 0.05$ for 30 and 100 μM), in zebrafish embryos after treatment with nobiletin (Fig. 6). An increase in *VEGF-A* expression was unexpected, owing to the fact that nobiletin showed an anti-angiogenic effect in zebrafish embryos. However, this could be because of feedback regulation of growth factors, like VEGF, due to a nobiletin-induced growth delay in zebrafish embryos.

DISCUSSION

In this study, using both in vitro and in vivo assays, we demonstrate for the first time the anti-angiogenic activity of nobiletin through modulation of cell cycle and the VEGF-A pathway, indicating to a new mode of pharmacological activity for nobiletin.

Nobiletin inhibited angiogenesis in rapidly developing blood vessels in the ISVs of zebrafish embryos (at around 24–48 hpf) with no significant anti-angiogenesis observed in ISVs at later stages of development. This indicates that nobiletin did not exert any vascular

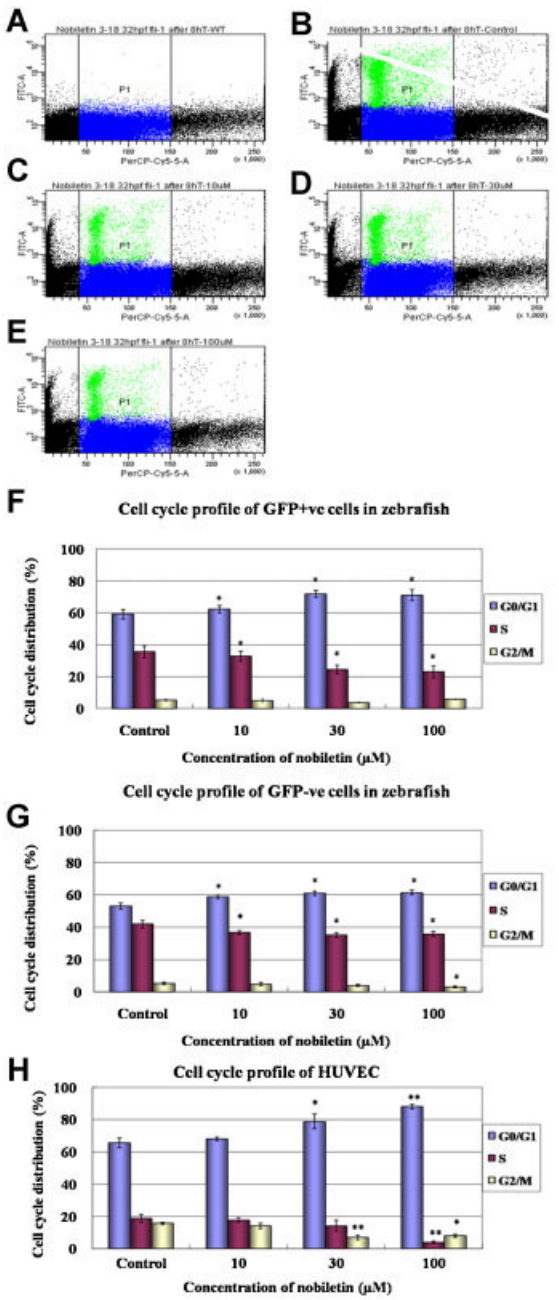

Fig. 5. Cell cycle modulation by nobiletin in zebrafish embryos and HUVECs. The effect of nobiletin on the cell cycle in endothelial cells of Tg(*fli1*:EGFP) zebrafish embryos. Wild-type zebrafish embryos (A) and Tg(*fli1*:EGFP) zebrafish embryos (B–E) were treated with different concentrations of nobiletin for 8 h, dissociated cells were then stained with DRAQ5™ and analyzed by flow cytometry for changes in intensity of FITC and PerCP-Cy5-5 signals. The green color indicates the endothelial cells. Quantitative analyses of the percentage of cell cycle distribution of endothelial cells (GFP + ve cells) in zebrafish embryos (F) and non-endothelial cells (G) were done using ModfitLT Version 3.0 software. 0.1% DMSO served as control. H: The effect of different concentrations of nobiletin on cell cycle distribution in HUVEC samples treated for 24 h. All data are presented as mean ± SEM of three individual experiments. * $P < 0.05$, ** $P < 0.01$ versus control. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

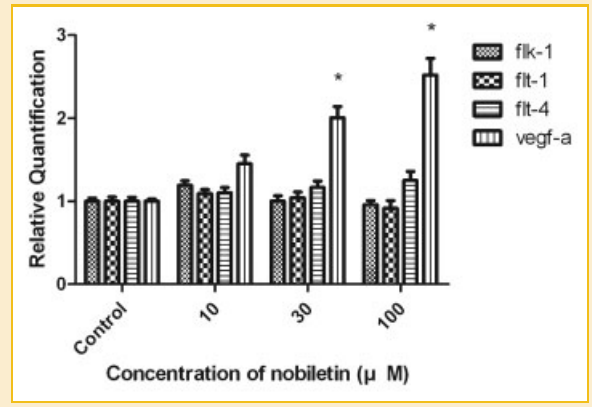

Fig. 6. The effect of nobiletin on the mRNA expression in zebrafish embryos. The expression levels of *flk-1*, *flt-1*, *flt-4*, and *VEGFa* mRNA after nobiletin treatment. 0.1% DMSO served as control. All data are presented as mean ± SEM of three individual experiments. * $P < 0.05$ versus control.

disruption, but rather inhibited formation of new blood vessels, perhaps through inhibition of endothelial cell proliferation, migration, loop formation, or some other process involved in angiogenesis. In fact, results from cell cycle analysis of zebrafish embryo cells showed that G1 arrest was slightly more pronounced in endothelial cells with a corresponding decrease in S phase, although not statistically significant, compared to non-endothelial cells possibly indicating a certain selectivity of nobiletin to endothelial cells in inhibiting cell proliferation. Although nobiletin caused slight toxicity and overall growth arrest in these zebrafish embryos, this did not significantly alter the apoptosis pattern in whole zebrafish embryos. A resveratrol derivative that showed significant anti-angiogenesis in zebrafish embryos through vascular targeting and inhibition of endothelial cell proliferation was also found to be toxic when used on zebrafish embryos at around 24–36 hpf [Belleri et al., 2005].

The citrus flavonoids nobiletin and tangeretin have been shown to be anti-proliferative in a variety of cancer cell lines and in vivo rodent models [Kandaswami et al., 1992; Kawaii et al., 1999; Murakami et al., 2000; Suzuki et al., 2004; Morley et al., 2007]. Although several studies about nobiletin-induced anti-proliferation were shown to proceed through modulation of molecular pathways regulating proliferation and cell survival [Ohnishi et al., 2004; Morley et al., 2007; Xiao et al., 2008], these studies were entirely done using in vitro models. Here, we show that nobiletin inhibits angiogenesis by regulating cell cycle progression through G0/G1 arrest in vivo, corroborating previously reported in vitro data. Also, in HUVECs, nobiletin caused inhibition of proliferation and tube formation, as well as significantly induced G1 arrest without significant induction of apoptosis. All these results indicate the cytostatic effect of nobiletin on endothelial cells, which is similar to that reported by Morley et al. in breast and colon cancer cell lines [Morley et al., 2007]. In their study, physiologically relevant concentrations of nobiletin and tangeretin treatment (54 μM and 60–100 μM, respectively) resulted in significant growth inhibition of breast and colon cancer cells with accumulation of cells in G1 phase without any effect on apoptosis. They proposed that these

citrus flavonoids were clearly cytostatic and not cytotoxic in their anti-proliferation activity.

Agents that are anti-angiogenic and cytostatic, and not cytotoxic, may be desirable in a cancer chemopreventative regimen where they do not cause cytotoxicity to normal cells while offering anti-proliferative effects on developing tumor cells as well as discouraging any new blood vessel formation through angiogenesis to feed these tumors. Walle proposed that methoxylated flavones, like nobiletin and tangeretin, were a superior cancer chemopreventive flavonoid subclass owing to their dramatically increased metabolic stability and membrane transport in the intestine/liver, thus improving oral bioavailability [Walle, 2007]. This is consistent with our earlier work where a trimethoxylated derivative of resveratrol proved to be a more potent anti-angiogenic and anti-proliferative agent than resveratrol [Alex et al., 2010]. Interestingly, nobiletin came up as one of the most promising natural cancer-preventive compounds in an extensive screening of extracts from Asian vegetables and fruits [Murakami and Ohigashi, 2006].

Nobiletin was reported to be anti-proliferative more selectively to tumor cell lines compared to normal cells including endothelial cells [Wu et al., 2006]. However, in our study using the zebrafish embryo angiogenesis model and HUVECs, nobiletin showed anti-proliferative effects on endothelial cells. Our results showing the anti-angiogenic activity of nobiletin in zebrafish embryos, where new blood vessels are formed through multiple steps, are more physiologically relevant and reflect the effect of this compound in vivo. However, the fact that non-endothelial cells in zebrafish embryos also showed G1 cell cycle arrest points to the anti-proliferation effect on other cell types as well. Perhaps nobiletin causes an overall cytostatic effect on proliferating cells, with certain selectively toward endothelial cells, by a common mechanism involving G1 arrest.

Gene expression data showed that nobiletin induced *VEGF-A* expression without any effect on the expression of its receptors. This was in contrast to our previous study, where the anti-angiogenic activity of *trans*-3,5,4′-trimethoxystilbene was accompanied by downregulation of *VEGF-receptor 2* (*VEGFR2* or *flk-1*) [Alex et al., 2010], indicating that nobiletin induced anti-angiogenesis through a different mechanism. The increase in *VEGF-A* expression could be because of a feedback regulation resulting from the anti-angiogenic effect and/or an overall embryonic growth delay induced by nobiletin.

Our findings provide a rationale for future development of nobiletin for treatment of diseases with impaired angiogenesis including various inflammatory diseases. Also, the anti-proliferative effect of nobiletin on endothelial cells without causing apoptosis makes it an excellent candidate for development as a chemopreventive agent against development of cancer or other chronic inflammatory diseases.

SUPPORTING INFORMATION

Effect of naringin on zebrafish embryos ISV formation and effect of nobiletin on 72 h postfertilized embryos is provided as supporting information.

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