RESEARCH ARTICLE

In vitro and in vivo structure and activity relationship analysis of polymethoxylated flavonoids: Identifying sinensetin as a novel antiangiogenesis agent

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Scope: Polymethoxylated flavonoids are present in citrus fruit in a range of chemical structures and abundance. These compounds have potential for anticarcinogenesis, antitumor, and cardiovascular protective activity, but the effect on angiogenesis has not been well studied.
Methods and results: Human umbilical vein endothelial cells (HUVECs) in vitro and zebrafish (*Danio rerio*) in vivo models were used to screen and identify the antiangiogenesis activity of seven polymethoxylated flavonoids; namely, hesperetin, naringin, neohesperidin, nobiletin, scutellarein, scutellarein tetramethylether, and sinensetin. Five, excluding naringin and neohesperidin, showed different degrees of potency of antiangiogenesis activity. Sinensetin, which had the most potent antiangiogenesis activity and the lowest toxicity, inhibited angiogenesis by inducing cell cycle arrest in the G0/G1 phase in HUVEC culture and downregulating the mRNA expressions of angiogenesis genes *flt1, kdrl,* and *hras* in zebrafish.
Conclusion: The in vivo structure–activity relationship (SAR) analysis indicated that a flavonoid with a methoxylated group at the C3′ position offers a stronger antiangiogenesis activity, whereas the absence of a methoxylated group at the C8 position offers lower lethal toxicity in addition to enhancing the antiangiogenesis activity. This study provides new insight into how modification of the chemical structure of polymethoxylated flavonoids affects this newly identified antiangiogenesis activity.

Keywords:
Angiogenesis / Polymethoxylated flavonoid / Sinensetin / Structure–activity relationship / Zebrafish

1 Introduction

Angiogenesis is the establishment of the mature blood vessel network through expansion and remodeling of the preexisting vascular primordium. Blood vessel formation during angiogenesis involves the induction of new sprouts, coordinated and directed endothelial cell migration, proliferation, sprout fusion (anastomosis), and lumen formation [1]. Angiogenesis plays an important role in the development

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Abbreviations: DLAV, dorsal longitudinal anastomotic vessel; **EGFP**, enhanced green fluorescent protein; **FBS**, fetal bovine serum; **hpf**, hour-post fertilization; **hpt**, hour-post treatment; **HUVEC**, human umbilical vein endothelial cells; **ISV**, intersegmental vessel; **qPCR**, quantitative PCR; **SAR**, structure–activity relationship; **STE**, scutellarein tetramethylether; **VEGF**, vascular endothelial growth factor; **VRI**, VEGF receptor kinase inhibitor II; **XTT**, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide

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of cancer [2], psoriasis [3], asthma [4], etc. There is growing evidence that chronic inflammation and angiogenesis are codependent, involving increased cellular infiltration and proliferation as well as overlapping with the roles of regulatory growth factors and cytokines [5]. On the basis of ethnomedical knowledge, we are conducting a search for naturally occurring angiogenic substances that might lead to the discovery of novel angiogenic agents [6–8].

Epidemiological and experimental in vitro and in vivo evidence points to a possible protective effect of flavonoids against human cardiovascular diseases and cancer risks [9, 10]. Citrus fruits, such as orange, mandarin, and grapefruit, contain high levels of polymethoxylated flavones and flavanones, such as hesperidin, naringin, and neohesperidin, which are members of a flavonoid subclass with some hydroxyl groups capped by methylation [11, 12]. Polymethoxylated flavonoids have potential for chemoprevention of early changes associated with carcinogenesis and tumor growth in colon, skin, prostate, lung, and liver [13–19]. Compared to nonnmethoxylated flavones, methylation on the hydroxyl groups increases the selectivity of growth inhibitory activity toward cancer cells, with reduced toxicity to normal cells, particularly during early carcinogenesis, and the modification provides a unique mode of action on aromatase inhibition [12]. Although polymethoxylated flavones and flavanones have been reported to have promising anticancer and anticarcinogenesis activity, which are frequently associated with excessive angiogenesis, the effect of this subclass of flavonoids on angiogenesis remains unclear. Only two recent studies touched on the antiangiogenesis effect of polymethoxylated flavones and flavanones, focused mainly on nobiletin [20, 21]. It remain unclear that the relationship between chemical structural diversity of this subclass of flavonoids and mechanism of action underlying the angiogenesis activity.

The zebrafish (*Danio rerio*) has been regarded as an excellent vertebrate model for screening angiogenic compounds because antiangiogenesis clinical drugs elicit responses in zebrafish comparable with their effects in mammalian systems [22]. In addition, transgenic zebrafish expressing enhanced green fluorescent protein (EGFP) in the vasculature allows observation of the responses of live embryos to drugs in real time [23, 24]. The feasibility of screening pro/antiangiogenic compounds in a zebrafish model has been well demonstrated [6–8, 23]; however, the suitability of a zebrafish model for the structure–activity relationship (SAR) analysis of both the biological activity and toxicity has not been addressed.

In this study, five representative methoxylated flavonoids and two nonmethylated counterparts were selected for testing their SAR on antiangiogenesis and toxicity using both a live zebrafish embryo model and a human umbilical vein endothelial cell (HUVEC) model.

2 Materials and methods

2.1 Ethics statement

All animal experiments were done in accord with the ethical guidelines of the Institute of Chinese Medical Sciences, University of Macau and the protocols were approved by Institute of Chinese Medical Sciences, University of Macau.

2.2 Chemicals and reagents

Kaighn's modification of Ham's F12 medium, fetal bovine serum (FBS), PBS, penicillin–streptomycin, 0.25% (w/v) trypsin in 1 mM EDTA, and propidium iodide/RNase A were purchased from Invitrogen (Grand Island, NY). Endothelial cell growth supplement, heparin, gelatin, and dimethylsulfoxide (DMSO) were supplied by Sigma-Aldrich (St. Louis, MO). Hesperetin, neohesperidin, naringin, nobiletin, and sinensetin were purchased from Shanghai Tauto Biotech Co. (Shanghai, China). Scutellarein and scutellarein tetramethylether (STE) were purchased from INDOFINE Chemical Co. (Hillsborough, NJ). Vascular endothelial growth factor (VEGF) was obtained from R&D Systems (Minneapolis, MN). VEGF receptor kinase inhibitor II (VRI) obtained from Calbiochem (Billerica, MA) was dissolved in DMSO and used as a positive control. Hesperetin, neohesperidin, naringin, nobiletin, sinensetin, scutellarein, and STE were each dissolved in DMSO as 100 mM stock solutions. VEGF was dissolved in sterile PBS containing 0.1% (w/v) bovine serum albumin. Stock solutions were stored at −20°C.

2.3 Cell line and culture

HUVECs (Invitrogen) were cultured in medium with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 100 μg/mL heparin, 30 μg/mL endothelial cell growth supplement, 10% (v/v) heat-inactivated FBS, and 100 U/mL penicillin–streptomycin. Cells were incubated at 37°C in a humidified 5% CO_2 (v/v) atmosphere. Tissue culture flasks, 96-well plates, and 24 well plates were coated with 0.1% (w/v) gelatin before use. All assays were done using low passage cells (3–8 passages).

2.4 Maintenance of zebrafish and collection of embryos

Transgenic zebrafish *Tg(fli1a-EGFP)y1* were provided by the Zebrafish International Research Center (ZIRC, Oregon) and wild-type zebrafish were purchased from a local pet shop. Both stocks were maintained as described in the zebrafish handbook [25]. Stocks were maintained in a controlled environment (28.5°C with a 14 h light/10 h dark cycle) and fed with

brine shrimp in the morning and again in the afternoon (and occasionally with general tropical fish food). Embryos were collected in the morning and cultured in embryo medium at 28.5°C. At 24 h-post fertilization (hpf), the embryos were dechorionated with tweezers in a Petri dish coated with 1% (w/v) agarose, and then distributed into a six-well plate with 20–50 embryos/group before drug treatment, depending on the assay.

2.5 Morphological observations

Twenty-four hpf embryos were incubated in 3 mL of medium containing different concentrations of the test compounds. Embryos receiving DMSO (0.1–0.3%) were used as a vehicle control. Embryos receiving 150 nM VRI were used as a positive control. After drug treatment for 24 h, the embryos were anesthetized with freshly made 1% (w/v) tricaine (Sigma-Aldrich, St. Louis, MO) and inspected for viability and morphological changes using an Olympus Microscope System (IX81 Motorized Inverted Microscope [w/ZDC], IX2 universal control box, X-cite series 120, DP71 CCD camera). Photographs were captured at magnifications of 40× and 100×.

2.6 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, after incubation for 24 h, the complete medium was replaced with low serum (0.5% FBS) medium and incubated for a further 24 h. After this, the cells were exposed to various concentrations of drug medium (0.5% FBS medium) in the presence of VEGF (20 ng/mL). Cells receiving VEGF (20 ng/mL) were used as a control. The cells were incubated for an additional 48 h and cell proliferation was assessed with the Cell Proliferation Kit II (2,3-bis [2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide (XTT; Roche) in accordance with the manufacturer's instructions; 50 μL of XTT solution was placed into each well and the samples were incubated at 37°C for an additional 4 h. The absorbance of each well was measured with a multilabel-counter fluorescence 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 are expressed as the percentage of viable cells.

2.7 Cell cycle analysis of HUVECs

HUVCEs were seeded in 25 cm^2 flasks with complete medium. After the cells reached confluence, they were starved in low serum (0.5% FBS) medium for 24 h to render them quiescent. The samples were then treated with complete medium containing different concentrations of drugs for another 24 h. Cells receiving DMSO (0.1%) served as a vehicle control. After the drug treatment, the cells were trypsinized, washed with PBS, and then fixed in chilled 70% (v/v) ethanol at −20°C overnight. The 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 (BD FACSCanto™ BD Sciences, San Jose, CA). The fluorescent signal of stained cells was detected through the FL2-H channel. The DNA content in the G0/G1, S, and G2/M phases was analyzed using ModfitLT version 3.0 software (Verity Software House, Topsham, ME).

2.8 Total RNA extraction, reverse transcription, and real-time quantitative PCR (qPCR)

The 24 hpf zebrafish embryos were treated with different concentrations of sinensetin for 8 h. Each treatment group contained 40 embryos. Embryos receiving 0.1% DMSO served as a vehicle control. Treating with 150 nM VRI served as a positive control. The RNA extraction and real-time qPCR were done as described [26]. Briefly, after the drug treatment, total RNA of the embryos was extracted with an RNeasy Mini Kit QIAGEN (Valencia, CA). The quantity and quality of extracted RNA were estimated with a Quant-iT™ RNA BR Assay Kit (Invitrogen) and agarose gel electrophoresis, respectively. The extracted RNA was reverse transcribed to the single-stranded cDNA using the SuperScript® III First-Strand Synthesis System (Invitrogen) and the GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA). After that, real-time qPCR was done with the 7500 Real-Time PCR System (Applied Biosystems), the TaqMan® Universal PCR Master Mix (Applied Biosystems), and custom TaqMan® primers for zebrafish (Applied Biosystems). The primers were specific for *βactin* (GenBank ID AF057040), *flt1* (AY848694), *hras* (BC092826), *kdrl* (AY056466), and *vegfaa* (AF059661). Relative expression of the genes of interest was normalized to the amount of *βactin* by the relative quantification (ddCt) method described by the manufacturer.

2.9 Statistical analysis

All data are presented as mean ± SEM. from at least three independent experiments. Statistical significance was assessed by one-way analysis of variance (ANOVA) followed by the Tukey method and the level of statistically significant difference was set at $p < 0.05$.

3 Results

3.1 Effects of citrus flavonoids on the morphology of zebrafish vasculature

Transgenic zebrafish embryos, *Tg(fli1a:EGFP)y1*, were used to evaluate the antiangiogenic activity of the seven flavonoids used in this study (hesperetin, naringin, neohesperidin, nobiletin, scutellarein, STE, and sinensetin, (Fig. 1b)). After the embryos were exposed to these seven flavonoids (each

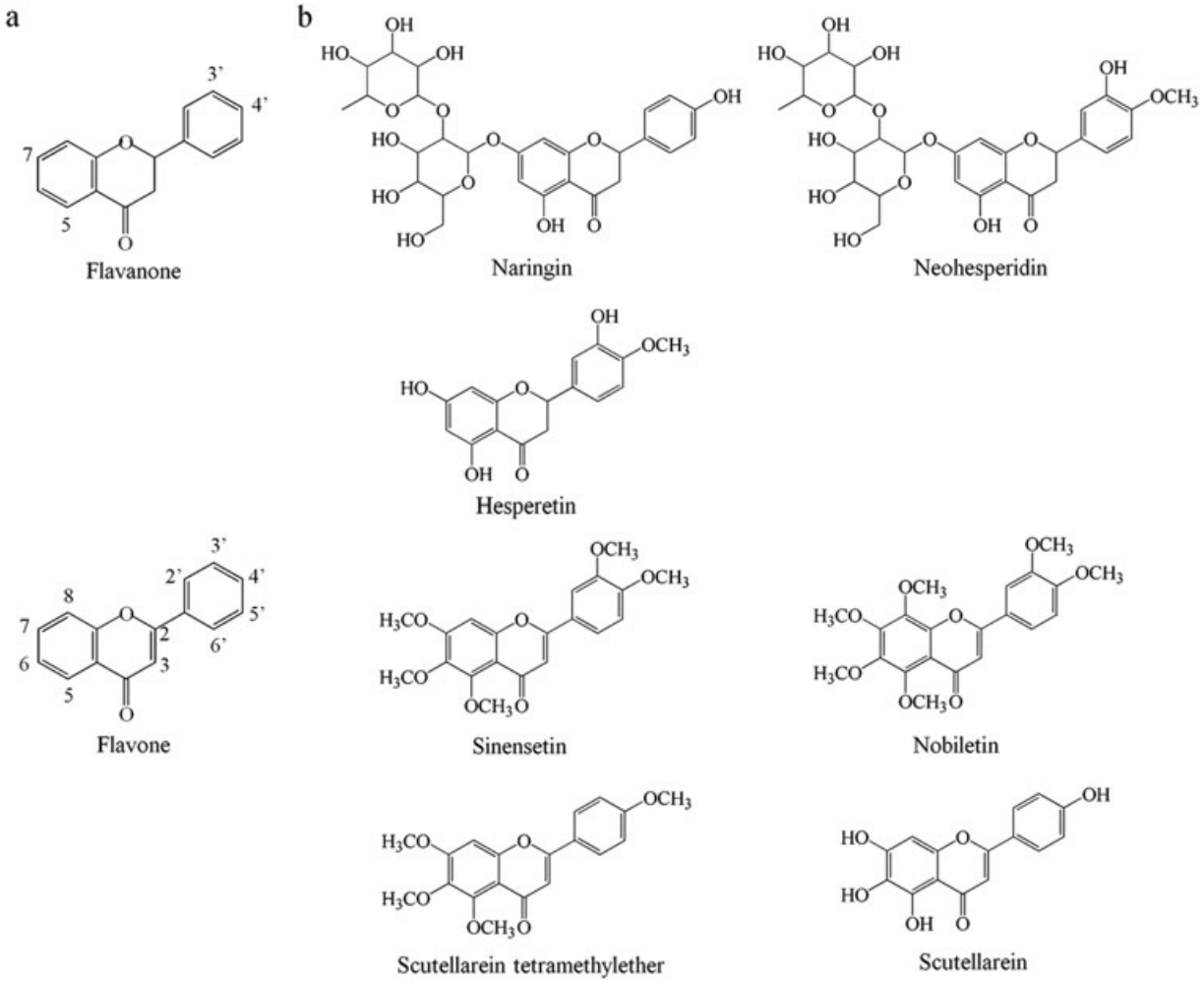

Figure 1. Chemical structures of (a) flavanone and flavone, (b) seven flavonoids.

at 30 μM) for 24 h, the formation of angiogenic vessels, intersegmental vessels (ISVs) and dorsal longitudinal anastomotic vessels (DLAVs), showed obvious growth inhibition in five of the seven flavonoid treatment groups (Fig. 2). Therefore, these five flavonoids (hesperetin, nobiletin, scutellarein, STE, and sinensetin) possess antiangiogenesis activity and sinensetin exhibited the strongest inhibitory effect on the formation of ISVs and DLAVs (Fig. 2h).

3.2 Effects of citrus flavonoids on HUVEC proliferation

Angiogenesis is a complex process that typically involves endothelial cell proliferation, migration, and alignment to form a tubular structure, so blockage of each step will disrupt the progress of angiogenesis. Inhibition of the seven flavonoids on cellular proliferation of VEGF-stimulated HUVEC was determined by the XTT assay. After treatment for 48 h, five of the seven flavonoids (except naringin and neohesperidin) exhibited an antiproliferative effect on HUVECs (Fig. 3). Consistent with the result of the zebrafish assay, sinensetin had the most potent inhibitory activity with an IC_{50} value at 24 μM, and the significant suppression of the proliferation of HUVECs induced by VEGF is concentration dependent (Fig. 3a). The inhibitory activity of these flavonoids on HUVEC proliferation was ranked as follows: sinensetin (IC_{50} 24 μM) > nobiletin (IC_{50} 62 μM) > hesperetin > STE > scutellarein. On the basis of the inhibition of HUVEC proliferation and ISVs formation in zebrafish embryos, sinensetin, nobiletin, and hesperetin were selected for further study and their antiangiogenesis activities were compared.

3.3 Antiangiogenesis activity of sinensetin, nobiletin, and hesperetin in zebrafish

The embryos after treatment with different doses of sinensetin for 24 h are shown in Fig. 4. Sinensetin suppression of the ISV development in zebrafish embryos compared to the control was concentration dependent. Even when embryos were treated with a lower concentration (3 μM),

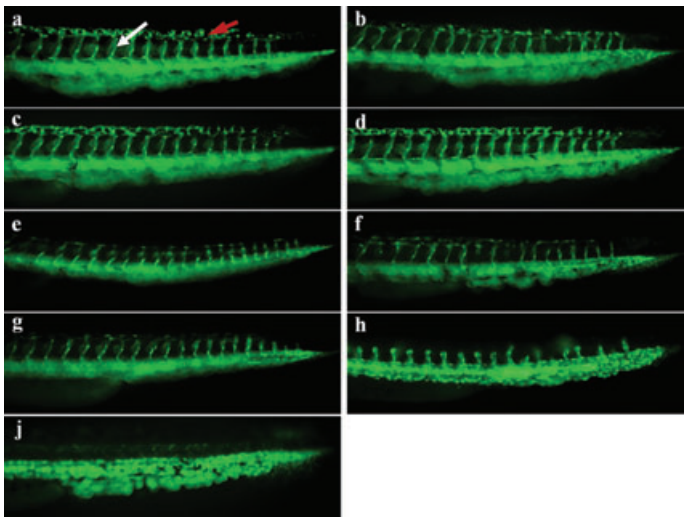

Figure 2. Inhibitory effects of seven flavonoids on angiogenic vessel (ISVs and DLAV) formation in *Tg(fli1a:EGFP)y1* zebrafish embryos at 48 hpf. Embryos were treated with (a) 0.1% DMSO; 30 μM of (b) hesperetin, (c) naringin, (d) neohesperidin, (e) nobiletin, (f) scutellarein, (g) scutellarein tetramethylether, (h) sinensetin, and (i) 150 nM of VRI for 24 h. Embryos receiving DMSO served as a vehicle control, while embryos receiving VRI served as a positive control. The white and grey arrows indicate ISVs and DLAV, respectively.

sinensetin affected the formation of ISVs with mild antiangiogenesis activity (Fig. 4). Concentrations of sinensetin up to 30 μM almost completely arrested the growth of ISVs (Fig. 4d). More than 80% of zebrafish embryos exhibited angiogenic defects after treatment with 10 μM sinensetin for 24 h compared to the control (Fig. 5a). Embryos with the antiangiogenesis phenotype were counted by observation of incomplete ISV formation. Treatment with sinensetin resulted in concentration-dependent decrease in number of complete ISVs (Fig. 5b). However, treatment with sinensetin did not disturb the well-established angiogenic vessels in the later developmental stage (120 hpf) of zebrafish (Supporting information Fig. S1).

Moreover, nobiletin presented a moderate-to-strong concentration-dependent, antiangiogenesis effect when the concentration was increased from 30 to 100 μM (Fig. 5b, supporting information Fig. S2).

Treatment of the embryos with 30 μM hesperetin for 24 h inhibited the development of ISVs only slightly (Supporting information Fig. S3), with ~30% of treated embryos affected (Fig. 5a), whereas a moderate inhibitory effect on ISV formation was observed for 100 μM hesperetin (Fig. 5b). In addition to the inhibition of ISV formation, all of the tested flavonoids showed concentration-dependent inhibition of the growth of dorsal longitudinal anastomotic vessel (DLAV) (Fig. 4, Supporting information Fig. S2, S3). The ranking of potency and effectiveness of the antiangiogenesis activity of these three flavonoids is the same as the result of the cell proliferation assay; i.e. sinensetin > nobiletin > hesperetin.

3.4 Lethal toxicity of sinensetin, nobiletin, and hesperetin on zebrafish

Survival rate or mortality is a common and direct parameter used to evaluate the toxicity of a compound. The beating heart of the zebrafish embryo is a parameter that indicates the living status of embryos. Thus, in order to determine the lethal toxicity of sinensetin, nobiletin, and hesperetin, the number of embryos with a heartbeat was determined every 12 h until 48 h posttreatment (hpt). The embryo survival rates after treatment with different concentrations of sinensetin, nobiletin, and hesperetin are shown in Fig. 6. Interestingly, although sinensetin had the highest level of potency of antiangiogenesis activity, its lethal toxicity was the lowest of the test compounds. At 48 hpt with 100 μM sinensetin, ~40% of embryos were alive (Fig. 6a). All embryos were dead after 48 h, even at the lowest dose of nobiletin (Fig. 6b); thus, nobiletin was shown to be the most toxic. Hesperetin showed intermediate lethal toxicity because >80% of embryos died at the highest concentration after incubation for 48 h (Fig. 6c). Moreover, three compounds also caused different degree of toxicity on embryonic development of zebrafish (e.g. lack of pigmentation, shorter trunk, etc.). The survival rate of zebrafish embryos and the antiangiogenesis activity of sinensetin, nobiletin, and hesperetin at 24 hpt are summarized in Table 1.

3.5 Sinensetin caused cell cycle arrest of HUVECs in the G0/G1 phase

The change in the cell cycle progression of HUVECs after the drug treatments was evaluated by flow cytometry. After 24 h of treatment, sinensetin induced a concentration-dependent accumulation of HUVECs in the G0/G1 phase of the cell cycle (Fig. 7). Treatment with 30 μM sinensetin significantly arrested the cell cycle in the G0/G1 phase accompanied by the reduction of S phase compared to the control, and the same phenomenon was found with 100 μM sinensetin. In conclusion, the inhibition of HUVEC proliferation by sinensetin may be contributed by the cell cycle arrest in the G0/G1 phase.

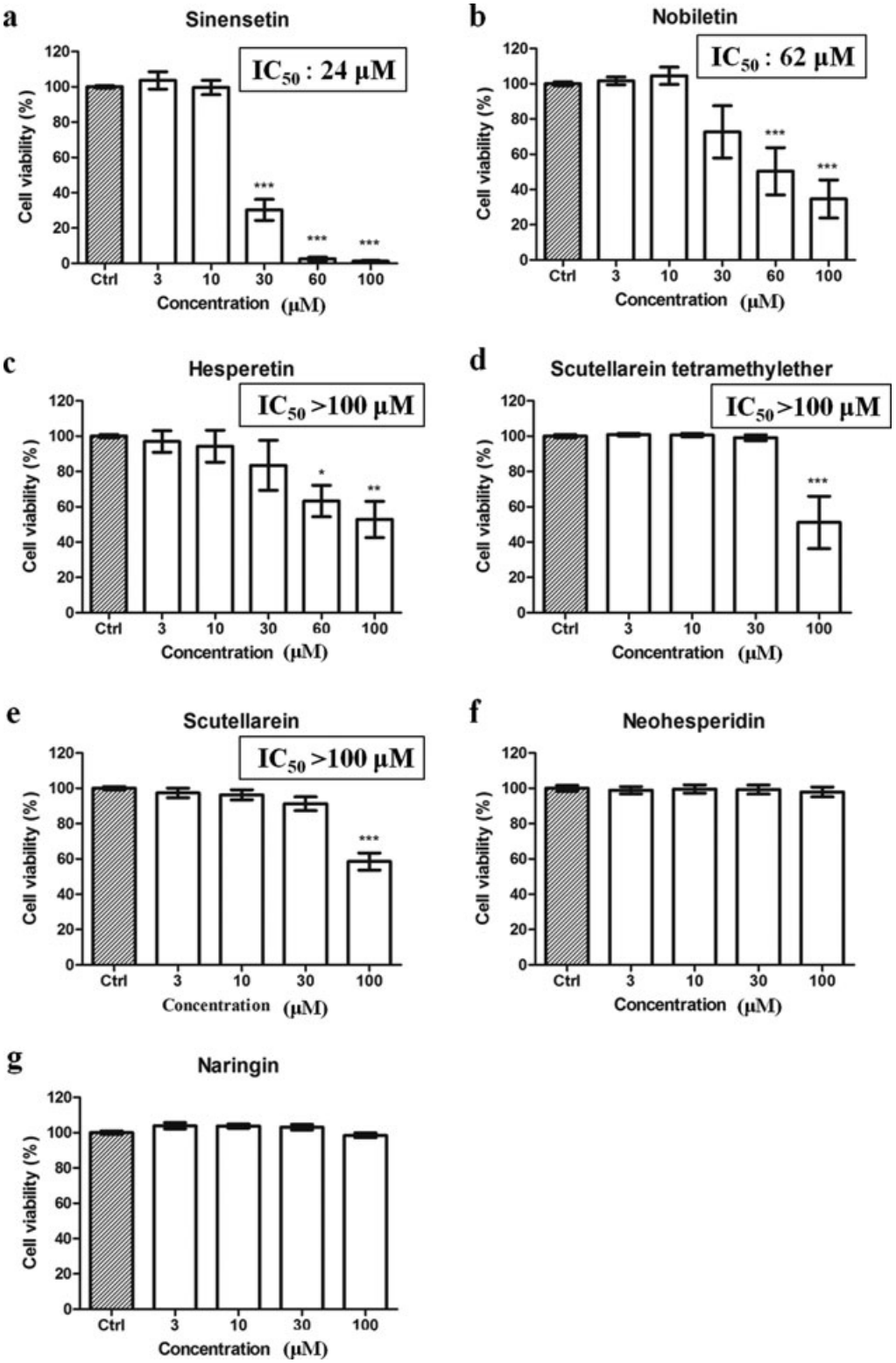

Figure 3. Inhibitory effects of (a) sinensetin, (b) nobiletin, (d) hesperetin, (e) scutellarein tetramethylether, (f) scutellarein, (g) neohesperidin, and (h) naringin on VEGF-induced proliferation of HUVECs. Cell viability was measured by XTT assay. Cells receiving VEGF (20 ng/mL) served as a control. Data are presented as the percentage of control in mean ± SEM, analyzed by one-way ANOVA, $n \geq 3$ independent experiments. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ versus control.

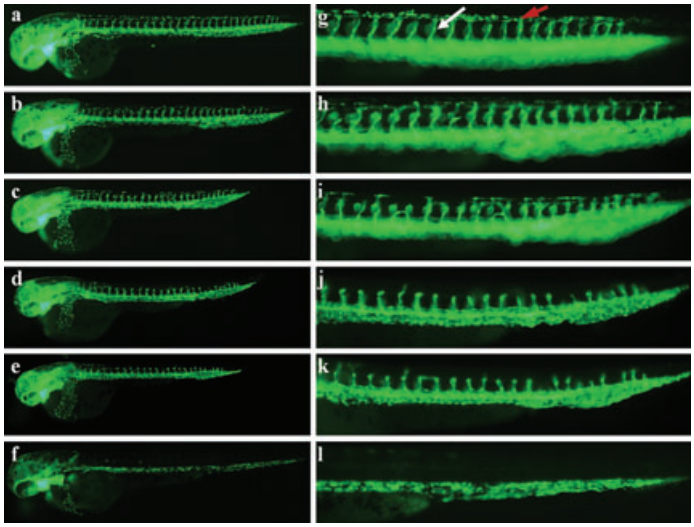

Figure 4. Lateral view of *Tg(fli1a:EGFP)y1* zebrafish embryos at 48 hpf showing angiogenic vessel (ISVs and DLAV) formation after treatment with sinensetin for 24 h. (a) 0.2% DMSO; (b–e) 3, 10, 30, and 100 μM of sinensetin; (f) 150 nM of VRI. Embryos receiving DMSO served as a vehicle control, while receiving VRI served as a positive control. The magnified views of (a–f) are shown in (g–l), respectively. The white and grey arrows indicate ISVs and DLAV, respectively.

3.6 Effect of sinensetin on zebrafish gene expression

Because sinensetin had the strongest antiangiogenesis activity in both the zebrafish and the HUVEC models, the effect of sinensetin on the change of mRNA expression of several genes (*flt1*, *hras*, *kdrl*, and *vegfaa*) that play important roles in angiogenesis was determined in zebrafish embryos. The expression of *flt1*, *hras*, and *kdrl* mRNA was concentration dependently downregulated in response to sinensetin (Fig. 8a, b, and c). After treatment with 30 μM sinensetin, the expression levels of *flt1*, *hras*, and *kdrl* mRNA were downregulated by 0.56-, 0.44-, and 0.52-fold respectively, compared to the control. VRI did not show an inhibitory effect as strong as sinensetin on suppressing the expression of *hras* and *kdrl* (0.41- and 0.36-fold, respectively, compared to the control). The level of expression of *vegfaa* mRNA was upregulated significantly by about 1.70-fold by treatment with 10 and 30 μM sinensetin. VRI increased the expression of *vegfaa* in the treated embryos but the difference was not statistically significant. Hence, these results suggest that the downregulation of expression of the *flt1*, *hras*, and *kdrl* genes induced by sinensetin could contribute to the antiangiogenesis effect of sinensetin observed in zebrafish.

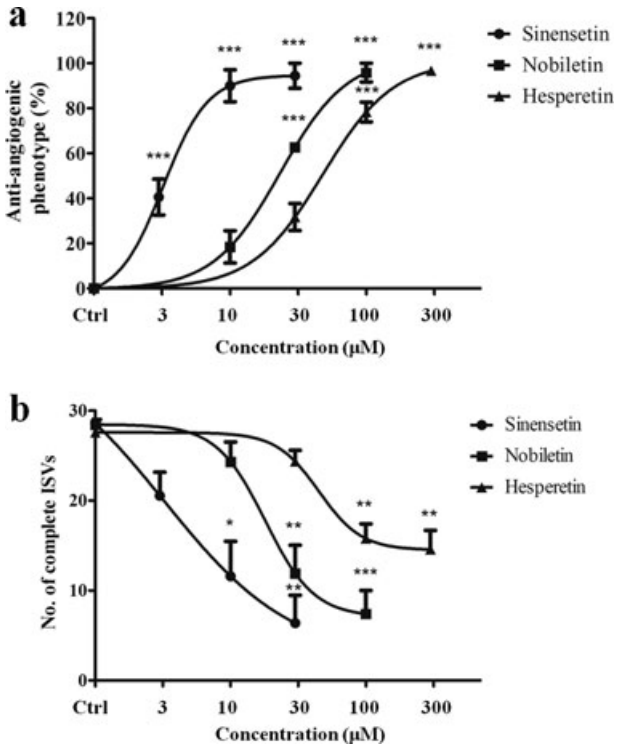

Figure 5. (a) The percentage of zebrafish embryos with anti-angiogenic phenotype; and (b) number of complete ISVs in zebrafish embryos after sinensetin, nobiletin, and hesperetin treatments for 24 h. Embryos with any incomplete ISVs were defined as displaying antiangiogenic phenotype. Embryos receiving 0.2% DMSO in sinensetin and nobiletin treatments, and 0.3% DMSO in hesperetin treatments served as a vehicle control. Data are presented as mean ± SEM, and were analyzed by one-way ANOVA, $n \geq 3$ independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ versus control.

4 Discussion

Although polymethoxylated flavones and flavanones have potential for reducing cancer risk, their antiangiogenesis activity was unclear. Zebrafish has been validated as a versatile model for rapid in vivo drug screening [23] and we used this model for SAR analysis of seven polymethoxylated flavonoids for the antiangiogenesis activity and toxicity. In brief, five of the seven flavonoids, sinensetin, nobiletin, scutellarein, STE,

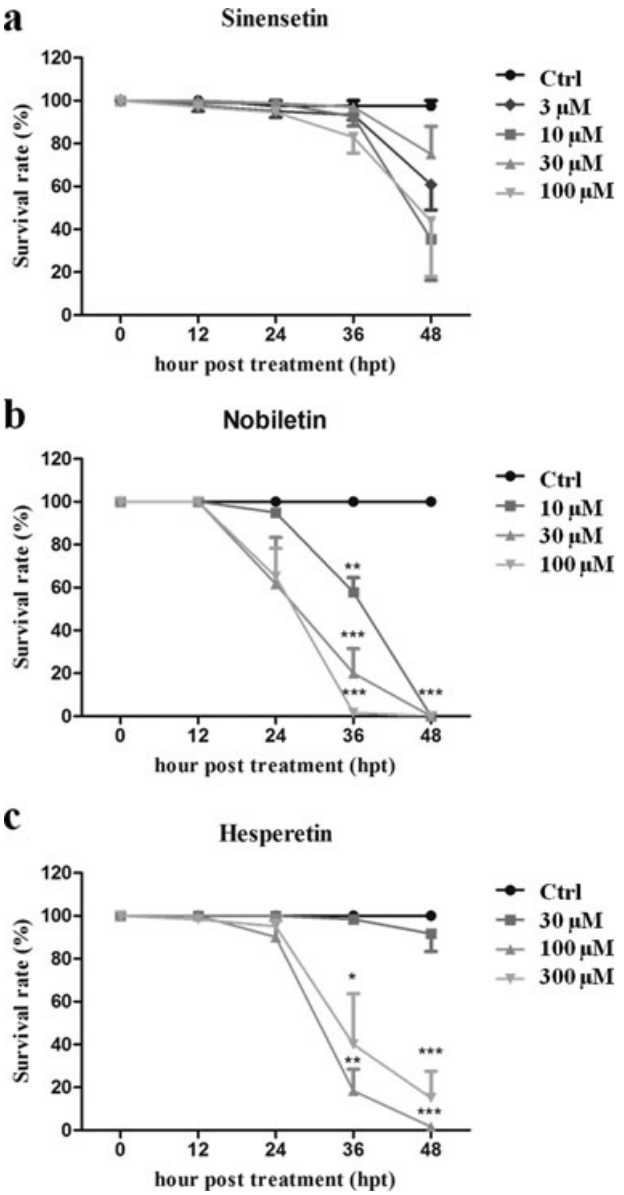

Figure 6. Survival rate of zebrafish embryos after treatment with various concentrations of (a) sinensetin; (b) nobiletin; and (c) hesperetin for 48 h. Embryos receiving 0.2% DMSO in sinensetin and nobiletin treatments, and 0.3% DMSO in hesperetin treatments served as a vehicle control. Data are presented as mean ± SEM, and were analyzed by one-way ANOVA, $n \geq 3$ independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ versus control.

and hesperetin, showed inhibitory effects on the proliferation of endothelial cells in vitro and in vivo, and we have identified the major chemical structure modification that makes sinensetin the most potent antiangiogenesis compound with the lowest lethal toxicity among these compounds. The antiangiogenesis activity of sinensetin in zebrafish involves the induction of cell cycle arrest in the G0/G1 phase in HUVECs and differential expression of target mRNAs in the VEGF signaling pathway.

In this study, seven flavonoids showed different levels of potency of antiangiogenesis activity in both the in vitro HUVEC and the in vivo zebrafish angiogenic assays (Figs. 2 and 3). The trend of antiangiogenesis activity of these seven flavonoids is similar in vitro and in vivo, which suggests the antiangiogenesis activity observed in zebrafish embryos may probably attributable to direct action on endothelial cells after absorption into the blood circulation in vivo. The relationship between antiangiogenesis activity and chemical structure was further examined. Naringin, neohesperidin, and hesperetin are dihydroflavones. Neohesperidin is the product of glycosylation of hesperetin at the C7 position in the A-ring. Compared to hesperetin, the antiangiogenesis effect of neohesperidin was reduced significantly. Naringin, which also has a glycosylation group at the C7 position, displayed no observable antiangiogenesis activity in vitro or in vivo. These findings suggest that glycosylation at the C7 position is not favorable to the antiangiogenesis effect.

Sinensetin, nobiletin, and hesperetin were subjected to concentration range analysis of both the antiangiogenesis activity and lethal toxicity in zebrafish (Figs. 4–6, supporting information Figs. S2 and S3). Interestingly, we found that sinensetin was the most potent as well as the least toxic among these three flavonoids. Nobiletin differs from sinensetin by having methylation at the C8 position. Compared with sinensetin, the 8-OCH_3 group in nobiletin reduced the antiangiogenesis activity and increased the lethal toxicity to zebrafish. Furthermore, result of sinensetin-treated 120 hpf zebrafish suggests that sinensetin affects mainly the growing angiogenic vessels and not the preexisting vasculature. In addition, the comparison of sinensetin and STE showed that 3′-OCH_3 is favorable to the antiangiogenesis effect. Comparison of the activity and structure of STE and scutellarein indicates that methylation of the OH groups at the C4′, C5, C6, and/or C7 positions might increase the activity.

Table 1. The survival rate (%) and antiangiogenic effect (+) of three flavonoids on zebrafish embryos

Flavonoid	Concentration (μM)				
	3	10	30	100	300
Sinensetin	95.0 ± 2.9 (+)	98.8 ± 1.3 (++)	98.8 ± 1.3 (+++)	94.6 ± 2.1 (+++)	N/A
Nobiletin	N/A	94.9 ± 0.1 (+)	61.7 ± 21.7 (++)	65.0 ± 13.2 (+++)	N/A
Hesperetin	N/A	N/A	100 (+)	90.0 ± 10.0 (++)	95.0 ± 2.9 (++)

The data are presented as mean ± SEM of at least three individual experiments. + is the estimated arbitrary unit of antiangiogenesis activity of the flavonoid. +, mild; ++, moderate; +++, strong. N/A, not applicable.

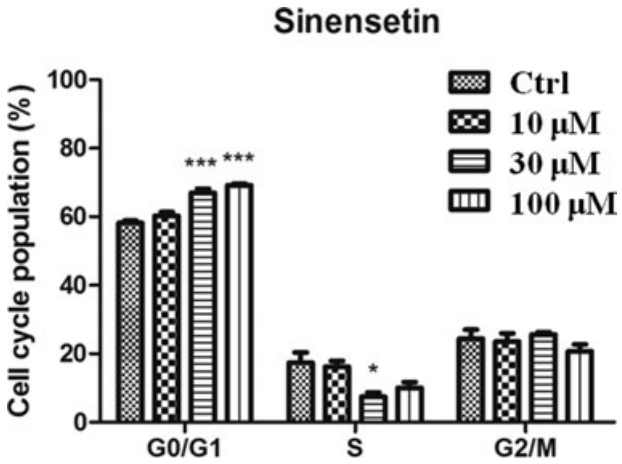

Figure 7. Cell cycle analysis of HUVECs treated with sinensetin for 24 h. Sinensetin caused G0/G1 arrest of cell cycle at higher concentration (30 and 100 μM). Cells treated with 0.1% DMSO served as a vehicle control. Data are presented as mean ± SEM, and were analyzed by one-way ANOVA, $n \geq 3$ independent experiments. $^{*}p < 0.05$ and $^{***}p < 0.001$ versus control.

Our further investigation of the mechanism underlying the antiangiogenesis activity of sinensetin showed that the inhibition of endothelial cell proliferation involved cell cycle arrest in G0/G1 phase (Fig. 7). Gene expression analysis supports the finding that sinensetin is antiangiogenic in zebrafish (Fig. 8). Sinensetin caused significant concentration-dependent downregulation of *flt1, kdrl,* and *hras* mRNA expression in zebrafish embryos. VEGF receptors and RAS are immensely important in triggering angiogenesis and transmitting an angiogenic signal, respectively, and downregulation of the expression of these genes could be a molecular clue to how sinensetin blocks angiogenesis. Surprisingly, *vegfaa* (the zebrafish ortholog of human VEGF), which encodes an angiogenesis-inducing factor, was upregulated by sinensetin and VRI. The increment in *vegfaa* expression could be a feedback regulation that compensates for the antiangiogenesis activity of sinensetin and VRI.

Citrus fruits, especially their peels, contain high abundance of polymethoxylated flavonoids. Previous study showed that ~4.4 g of sinensetin and 8.6 g of nobiletin could be extracted from each kilogram of the peels of *Citrus reticulata* Tangerine [27]. In some Asian countries, there is a

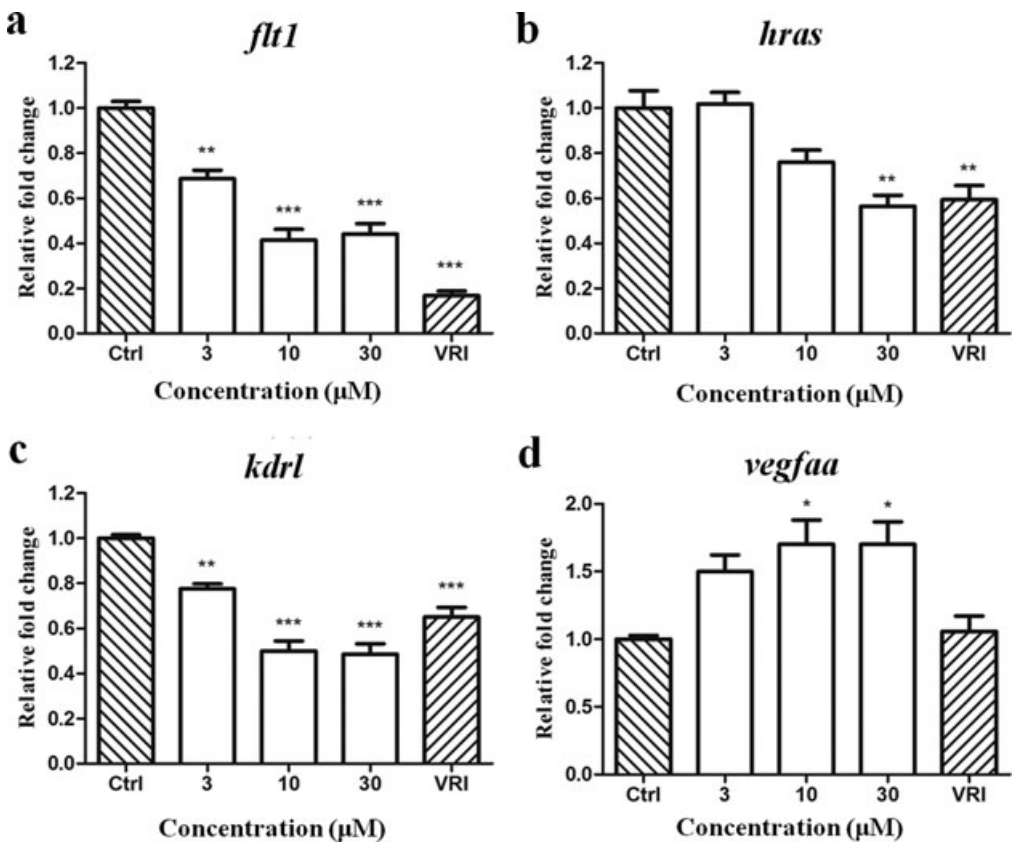

Figure 8. The change in the gene expression of zebrafish treated with sinensetin for 8 h. (a) *flt1*, (b) *hras,* and (c) *kdrl* were downregulated, while (d) *vegfaa* was upregulated after the treatment. *β actin* was used as internal standard. Embryos receiving 0.1% DMSO served as a vehicle control, while receiving 150 nM of VRI served as a positive control. Data are presented as mean ± SEM, and were analyzed by one-way ANOVA, $n \geq 3$ independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ versus control.

high consumption of citrus fruits and their peels as food and natural remedies. For example, the peel of shaddock (*Citrus maxima*) and the peel of mature citrus (*Pericarpium Citri Reticulatae*) are commonly used as food and tea in China. It has been shown that the maximum concentrations of sinensetin and nobiletin in rat plasma after oral administration of 10 g/kg of *Orthosiphon stamineus* extract (equivalent to 15 mg/kg of sinensetin) and 50 mg/kg of nobiletin were about 0.1 μg/mL and 1.78 μg/mL, respectively [28, 29]. The bioavailabilities of sinensetin and nobiletin, 9.4 and 10%, respectively, are relatively high when compared with that of (—)-epigallocatechin-3-gallate (EGCG) (0.1% in rat), which is the principal active polyphenol in green [28, 30, 31]. In several epidemiological studies, it was shown that the consumption of five cups of green tea per day could lower the risks of breast, liver, and/or lung cancers [32–34]. When several cups of green tea were taken at once, the active concentrations of EGCG could reach 10–100 μM in plasma. Lee et al. (2002) showed that the mean peak plasma EGCG level in human was 78 ng/mL (0.17 μM) after drinking two cups of tea (equivalent to 195 mg of EGCG) [35]. In our present study, the active concentrations of selected polymethoxylated flavonoids ranged from 3 to 30 μM on antiangiogenesis assays. However, there is no published data on the plasma concentrations of polymethoxylated flavonoids in human after oral consumption of shaddock peel or citrus peel. More recently, long-term dietary hydroxylated polymethoxyflavones treatment has been shown to inhibit colonic tumor formation in mice through antiangiogenesis by suppressing the expression of proangiogenic factors such as VEGF and COX-2 [36]. Therefore, further study on the beneficial effects of the long-term consumption of dietary hydroxylated polymethoxyflavones shall be warranted.

Zebrafish is an excellent in vivo model for physiologically relevant whole organism and behavior-based screening [37], which cannot be achieved with conventional in vitro systems. Because the pharmaceutical industry frequently encounters a high risk of failure in the development of a new drug, particularly at later stages, there is a trend in drug discovery strategy to exclude potentially toxic compounds at an early stage. However, one limitation of the current drug screening in vitro assays is that these assays can usually only attribute potential therapeutic action to a specific molecular target and/or cell type. These assays provide no information about hidden toxicity or any side effect due to interaction with other molecular targets and cell types and, more importantly, leading to overall toxicity to the whole organism. Recently, in vivo SAR analysis of active compounds that takes bioavailability and ADME (absorption, distribution, metabolism, and excretion) properties of the screened compound into account, has been realized in a zebrafish model [38, 39]. However, there is no reported study on content screen for both activity and toxicity in zebrafish. In this study, we found that compared to sinensetin, the 8-OCH_3 group in nobiletin has the opposite effects; the antiangiogenesis activity is reduced and the toxicity to zebrafish is increased. This study proved the concept of the feasibility of using in vivo SAR analysis in zebrafish to identify major chemical determinants of both the efficacy of antiangiogenesis activity and toxicity.

Many nonclinical studies have shown that polymethoxylated flavones and flavanones have both anticarcinogenesis and antitumor growth activity. The present study, for the first time, systematically evaluated the SAR of selected representative flavonoid on angiogenesis in vivo and in vitro, leading to identification of sinensetin as the most potent antiangiogenic compound. Most studies of polymethoxylated flavonoids have been focused mainly on nobiletin. Recently, other workers have reported that nobiletin exhibited antiangiogenesis activity in a chick embryo chorioallantoic membrane assay in vivo, supporting the presence of an antiangiogenesis potential of this polymethoxylated flavonoid subclass [21]. Our SAR analysis further demonstrates that flavonoids with the presence and/or the absence of methoxylated groups at the C3′ and C8 positions elicit stronger antiangiogenesis activity and the absence of a methoxylated group at the C8 position reduces the systemic toxicity to zebrafish.

In conclusion, the present study made new findings that correlate the chemical structure of flavonoids to their antiangiogenesis effects and lethal toxicity as well as facilitating future work on the design and chemical modification of flavonoids for antiangiogenesis therapy. The promising results reported here provide insight into the action of citrus flavonoids on angiogenesis and could lead to new therapy for the treatment of diseases associated with excessive angiogenesis. This study provides a new methodological model for the exploitation and utilization of zebrafish in high content of biological activity and SAR analysis.

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