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Original article

# Cytotoxic 2′,5′-dihydroxychalcones with unexpected antiangiogenic activity

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**Abstract**

A series of 2′,5′-dihydroxychalcones were synthesized and evaluated for cytotoxicity against tumor cell lines and human umbilical venous endothelial cells (HUVEC). It was found that chalcones with electron-withdrawing substituents on the B ring exhibited potent cytotoxicity against a variety of tumor cell lines while compounds with electron-releasing groups were less potent in general. Those compounds with B ring replaced by extended or heteroaromatic rings exhibited significant bioactivity. Several compounds were shown to have marked cytotoxic selectivity towards HUVECs. Especially, among the synthesized compounds, 2-chloro-2′,5′-dihydroxychalcone (**2**-3) showed the highest selectivity index up to 66 in comparison to HCT116 cells. This compound also exhibited strong inhibitory effects on the HUVEC tube formation in an in vitro model. When administered into BDF1 mice bearing Lewis lung carcinoma cells at 50 mg $kg^{-1}$ $day^{-1}$, **2**-3 was found to inhibit the growth of tumor mass by 60.5%.


*Keywords:* 2′,5′-Dihydroxychalcone; Cytotoxicity; Antiangiogenic activity; Antitumor activity

## 1. Introduction

In a previous paper we have reported a series of 2,5-dihydroxychalcones (**1**) (Fig. 1) with potent cytotoxic activities in various tumor cell lines [1]. We also noted that some of them showed modest selectivity towards human umbilical venous endothelial cells (HUVEC). Those compounds with selective cytotoxicity against HUVECs may be potential for development as selective angiogenesis inhibitors. Indeed, antiangiogenesis has been widely accepted as a novel and interesting approach for anticancer drug development recently [2].

Structurally, **1** possesses two distinguished features; a hydroquinone and an α,β-unsaturated carbonyl. The hydroquinone has been known to be easily oxidised in vivo to quinone which elicits cytotoxicity per se or through a cascade of redox cycling [3]. The α,β-unsaturated carbonyl can be considered as a Michael acceptor, an active moiety often employed in the design of anticancer drugs [4]. A number of α,β-unsaturated ketones have demonstrated preferential activity toward thiol [5]. Alkylation with a cellular thiol such as glutathione (GSH) may also occur with chalcones like **1**, leading to adducts at β-position. Hence, α,β-unsaturated carbonyl-containing compounds may be free from problems of mutagenicity and carcinogenicity that are associated with many alkylating agents used in cancer chemotherapy [6].

Chemically, the alkylation of α,β-unsaturated carbonyl compounds like **1** with cellular thiols and other nucleophiles is greatly dependent on the electrophilicity of the β-carbon. In this view, we reason that the introduction of electron-withdrawing groups on B ring may increase the electrophilicity of the β-carbon, thus improve the bioactivity of the resulting compounds. However, our initial results showed that such compounds suffered unexpected chemical instability. In addition, it is difficult to synthesize such compounds with diverse substituted patterns in B ring. Therefore, we sought another solution by reallocation of the 2,5-

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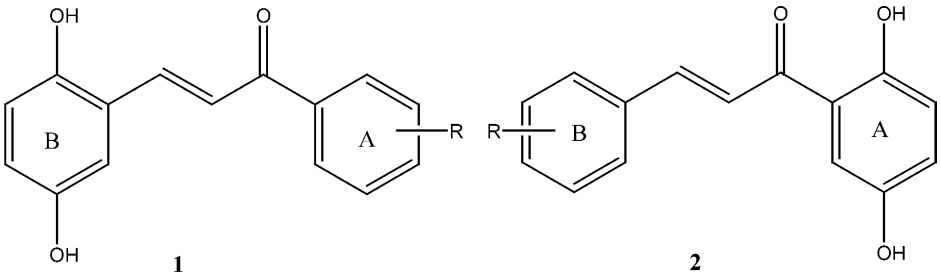


Fig. 1. Structures of 2,5-dihydroxychalcones (**1**) and 2′,5′-dihydroxychalcones (**2**).

dihydroxy functionality into ring A, and introduction of various electron-withdrawing groups into ring B. Thus, at the same time we could both conserve the important hydroquinone moiety and make the β-carbon more electron-deficient. The resulting compounds **2** are expected to be more bioactive than **1**. In this work we have synthesized and evaluated a series of compounds **2** for cytotoxicity in both tumor cell lines and HUVEC. And interestingly, we found compound **2**-3 (2-chloro-2,′-5′-dihydroxychalcone) with unexpectedly large cytotoxic selectivity against HUVECs. In this paper, the synthesis of the chalcones in series **2**, their cytotoxicity and detailed biological evaluation of compound **2**-3 as an antiangiogenic antitumor agent are presented and discussed.

## 2. Chemistry

Most of chalcones were synthesized by a Claisen–Schmidt condensation of appropriate benzaldehydes, protected as tetrahydropyranyl ether wherever a phenol group(s) was present, with 2′,5′-dihydroxyacetophenone, also protected as bistetrahydropyranyl ether (**3**) (Fig. 2), using barium hydroxide octahydrate as base. However, these conditions failed to give chalcone **2**-7 which possesses a nitro group on the A ring. Alternatively, this compound was obtained by condensation under acidic conditions, thus involving bubbling of hydrochloride gas into a solution of the two reactants in anhydrous ethanol. **2**-25 in particular was synthesized by condensation of **3** with cinnamaldehyde followed by deprotection as described for **2**-1. $^1$H-NMR spectra revealed that the olefinic bond in these chalcones adopted the *E* configuration (the coupling constants of the two olefinic protons were around 16 Hz).

## 3. Biological results and discussion

### 3.1. Cytotoxicity

The synthesized chalcones were evaluated for cytotoxic activities in three cancer cell lines including B16 (murine melanoma), HCT116 (human colon cancer cells), and A431 (human epidermoid carcinoma). As already widely known, the formation of new blood vessels from endothelial cells, so-called angiogenesis, is prerequisite for the growth of solid tumors [7]. Inhibition of angiogenesis has been postulated to be an attractive approach for cancer treatment [2] and a compound showing a selective cytotoxicity against endothelial cells may be potential for further development as an angiogenesis inhibitor. For that reason,

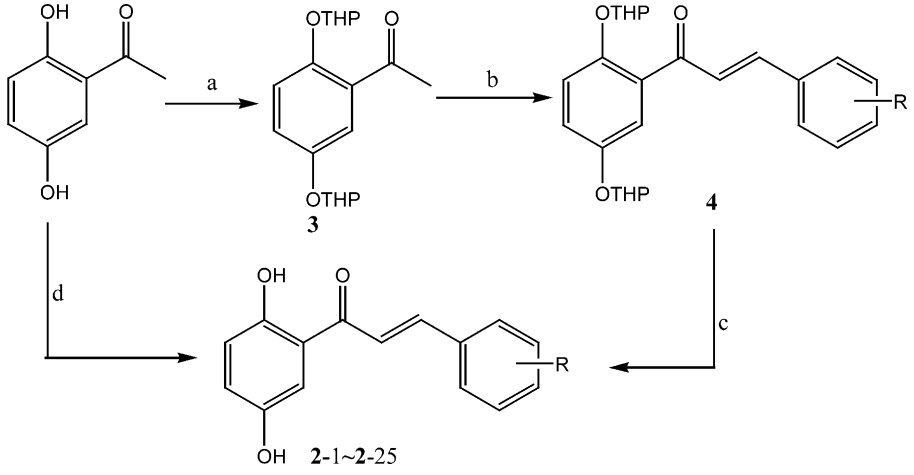


Fig. 2. (a) Pyridinium *p*-toluene sulfonate, 3,4-dihydro-2*H*-pyran, methylene chloride, r.t., 5 h. (b) ArCHO, $Ba(OH)_2 \cdot 8H_2O$, methanol, 40 °C, 12 h. (c) *p*-Toluene sulfonic acid, MeOH, 5 h. (d) HCl gas, ethanol, 4-$NO_2$–$C_6H_4CHO$.

HUVEC, a representative line of endothelial cells, was included in an assay panel in an attempt to unveil a selective cytotoxicity against this cell line of the compounds tested. The results expressed as $IC_{50}$ values are summarized in Table 1. For comparison of the potency between compounds within the series, the average potency (average $IC_{50}$ values) of each compound in four cell lines were calculated (see Section 4). The data are included in Table 1.

Furthermore, to compare the potency of compounds in series **2** with series **1**, the average potencies of the series **2** in each cell line and in all four cell lines are also calculated. The results are presented in Table 2. The data for series **1** shown in this table are adopted from Ref. [1].

To discern the electronic effects of the substituents on the bioactivity, we divided compounds in series **2** into four groups. Group **2**-EWG comprises of compounds (**2**-1 ~ 9) bearing electron-withdrawing substituents (EWG), meanwhile group **2**-ERG accommodates compounds (**2**-13 ~ 19) possessing electron-releasing substituents (ERG). Three compounds with one EWG and one ERG (**2**-10 ~ 12) were pooled in a **2**-MIX group. The last group (**2**-HET) houses compounds where the B rings were replaced with extended or heteroaromatic ones. The AP values of each group were calculated and included in Table 2.

As shown in Table 1, most of the synthesized compounds in series **2** exhibited significant cytotoxici-

Table 2
Average potency (AP) of series **1** and **2** and of each category [a] in series **2**

| Series | Category | Average Potency (AP) | | | | |
|---|---|---|---|---|---|---|
| | | B16 | HCT116 | A431 | HUVEC | AP |
| **2** | 2-EWG | 1.87 | 2.42 | 1.84 | 1.49 | 1.90 |
| | 2-MIX | 1.33 | 1.39 | 1.49 | 1.27 | 1.36 |
| | 2-ERG | 5.23 | 9.15 | 7.81 | 3.49 | 6.12 |
| | 2-HET | 1.68 | 3.00 | 2.13 | 1.68 | 2.12 |
| | AP | 2.52 | 3.99 | 3.31 | 1.98 | 2.87 |
| **1** [b] | AP | 4.47 | 5.62 | 5.13 | 4.52 | 4.90 |

[a] Category is classified as described in the text.
[b] Data adopted from Ref. [1].

Table 1
Cytotoxicity of synthesized chalcones in tumor cell lines [a] and HUVECs

| Cpd | R or A ring for | Cytotoxicity ($IC_{50}$ [b], μg $mL^{-1}$) | | | | |
|---|---|---|---|---|---|---|
| | | Cell line | | | | AP |
| | | B16 | HCT116 | A431 | HUVEC | |
| **2**-1 | H | 2.25 | 3.29 | 2.44 | 1.89 | 2.46 |
| **2**-2 | 2-F | 1.78 | 1.99 | 1.11 | 1.02 | 1.47 |
| **2**-3 | 2-Cl | 1.34 | 1.83 | 0.09 | 0.03 | 0.82 |
| **2**-4 | 3-Cl | 1.39 | 1.81 | 1.97 | 1.71 | 1.72 |
| **2**-5 | 4-Cl | 2.18 | 3.62 | 2.16 | 2.01 | 2.49 |
| **2**-6 | 4-Br | 1.84 | 2.36 | 2.54 | 1.52 | 2.06 |
| **2**-7 | 4-$NO_2$ | 1.99 | 2.38 | 2.56 | 2.01 | 2.23 |
| **2**-8 | 2,3,4,5,6-$(F)_5$ | 2.15 | 2.72 | 1.81 | 1.84 | 2.13 |
| **2**-9 | 2,5-$(F)_2$ | 1.99 | 1.84 | 1.84 | 1.42 | 1.77 |
| **2**-10 | 2-Br–5-$OCH_3$ | 1.10 | 1.18 | 1.29 | 1.24 | 1.20 |
| **2**-11 | 2-$OCH_3$–5-Br | 1.01 | 1.25 | 1.73 | 1.24 | 1.30 |
| **2**-12 | 2-Br–5-OH | 1.87 | 1.75 | 1.49 | 1.27 | 1.59 |
| **2**-13 | 4-$OCH_3$ | 3.59 | 9.09 | 3.75 | 5.48 | 5.47 |
| **2**-14 | 4-$N(CH_3)_2$ | 11.25 | > 30 | 13.33 | > 30 | > 21.14 |
| **2**-15 | 2,3-$(OH)_2$ | 1.00 | 5.26 | 2.58 | 1.48 | 2.58 |
| **2**-16 | 3,4-$(OH)_2$ | 2.90 | 13.73 | 6.27 | 3.00 | 6.47 |
| **2**-17 | 2,5-$(OH)_2$ | 10.16 | 14.46 | 26.65 | 4.78 | 14.01 |
| **2**-18 | 2,3,4-$(OCH_3)_3$ | 3.77 | 12.17 | 8.06 | 3.75 | 6.93 |
| **2**-19 | 3,4,5-$(OCH_3)_3$ | 3.84 | 9.35 | 7.81 | 3.49 | 6.12 |
| **2**-20 | 4-phenyl | 1.20 | 3.93 | 2.30 | 1.15 | 2.14 |
| **2**-21 | 2-naphtyl | 1.36 | 3.57 | 1.97 | 1.28 | 2.04 |
| **2**-22 | pyridin-3-yl | 2.21 | 2.69 | 1.71 | 2.04 | 2.16 |
| **2**-23 | indol-3-yl | 2.00 | 2.57 | 2.18 | 1.92 | 2.16 |
| **2**-24 | quinolin-2-yl | 1.97 | 2.49 | 2.37 | 2.49 | 2.33 |
| **2**-25 | CH=CHPh-(*t*) | 1.39 | 2.79 | 2.27 | 1.24 | 1.92 |
| ADR [c] | | 0.10 | 1.11 | 1.01 | 0.21 | 0.60 |

[a] Cancer cell lines: B16, murine melanoma; HCT116, colon cancer; A431, human epidermoid carcinoma.
[b] A sample's concentration produces a 50% reduction in cell growth. The values shown were the averages from a triplicate experiment with the individual value varied less than 10%.
[c] Adriamycin, used as a positive control.

ties. Noteworthy, a review of the AP values of the two series **1** and **2** shows that compounds in series **2** are in average twofold more potent than those in **1** (the AP values of **2** and **1** are 2.87 and 4.90 μg $mL^{-1}$, respectively. For example, **2**-3 showed average $IC_{50}$ value of 0.82 versus 1.32 μg $mL^{-1}$ of the corresponding compound **1**-3 which bears a 2-Cl substituent on the A ring [1]. Thus, though we have not yet confirmed by calculation of the atomic charges of the β-carbons of the two series, these results strongly support our earlier hypothesis that by reallocation of the 2,5-dihydroxy into A ring and introduction of the EWGs into B ring, the series **2** would be expected to be more active than **1**.

Perusal of the data summarized in Table 2 indicates that compounds with EWGs (**2**-1 ~ 9, group **2**-EWG) showed most potent cytotoxicities while compounds with ERGs (**2**-13, -19) were much less active (the AP values of **2**-EWG and **2**-ERG are 1.90 and 6.12 μg $mL^{-1}$, respectively). These results clearly prove the favorable effects of the EWGs for the bioactivity. However, it is unclear that compounds possess mixed substituents (**2**-10 ~ 12, **2**-MIX group) and compounds where the B ring has been replaced by extended aromatic or heteroaromatic rings (**2**-20 ~ 25, **2**-HET group) also exhibited good activities. For the case of **2**-HET group, it is possible that the extended aromatic rings or the heteroatoms may increase the binding of those compounds to appropriate receptors through a van der Waals force or hydrogen bonding.

### 3.2. Antiangiogenic activity of 2-3

Interestingly, the results of the screening of chalcones synthesized in this work in HUVECs revealed several compounds with significant preferential cytotoxicity toward HUVEC, in reference to tumor cells. In order to get a quick view of the compound's cytotoxic selectivity, a selectivity index (SI), which refers to the cytotoxic selectivity of the compound towards HUVEC in comparison to three tumor cell lines, was calculated (see Section 4). As a result we found nine out of 25 compounds synthesized, including **2**-2, -3, -15, -16, -17, -18, -19, -20, -25 displayed SI values larger than 2 (data not shown). Strikingly, compound **2**-3 in this series was revealed to have distinguished SI value of 36, respectively. Noteworthy, **2**-3 also showed significant selectivity in human epidermoid carcinoma cells (A431) with the $IC_{50}$ value in this cell line being 15- and 20-fold lower than that in B16 and HCT116, respectively. Biologically, the two cell lines A431 and HUVEC share a common property. The growth of A431 could be stimulated by three to fourfold in the presence of epidermal growth factors (EGF) meanwhile HUVEC's growth is completely dependent on growth factors like EGF, PDGF (platelet-derived growth factors), and FGF (fibroblast growth factors). Thus, it is likely that **2**-3 might inhibit A431 and HUVEC growth by antagonizing EGFs. Whatever an exact mechanism would be, as stressed earlier, compounds displaying selective cytotoxicity towards HUVEC, like **2**-3, may be potential for further development as angiogenesis inhibitor. Therefore, **2**-3 was chosen as a candidate for evaluation in an in vitro angiogenesis assay using HUVEC. Because a 24 h incubation was used in this assay model, an $IC_{50}$ value for a 24 h incubation of **2**-3 was determined (0.45 μg $mL^{-1}$) using SRB assay. Accordingly, **2**-3 was assayed at four concentrations of 0.3, 0.1, 0.03 and 0.01 μg $mL^{-1}$. As a positive inhibitory control, suramin, an established angiogenesis inhibitor, was also added at non-growth inhibitory concentrations of 100, 30, 10 μM. For suramin, an $IC_{50}$ value for a 24 h incubation using SRB assays was $>100$ μM. The results from these assays are summarized in Table 3.

As shown in Table 3, the addition of **2**-3 at the concentrations of 0.1 and 0.3 μg $mL^{-1}$ resulted in the complete inhibition of the HUVEC tube formation. Though signs of cytotoxicity were observed at 0.3 μg $mL^{-1}$, the lower concentration of 0.1 μg $mL^{-1}$ manifested no cytotoxicity while the complete inhibition was still observed. This concentration was 4.5-fold lower than the $IC_{50}$ value of the compound against HUVECs measured under the same conditions, e.g. cell concentration ($8 \times 10^4$ cells/mL), and time course (24 h). Compared to the $IC_{50}$ values of the compound in tumor cell lines, it was revealed that this concentration was more than tenfold lower than the average cytotoxicity (AVR-C) in three tumor cell lines (B16, HCT116, A431). Especially, at 0.03 μg $mL^{-1}$, the concentration 36-fold lower than the AVR-C value, **2**-3 considerably inhibited the tube formation induced by HUVEC. The inhibition of HUVEC tube formation at this concentration was comparable to that of 10 μM, or 15 μg $mL^{-1}$ of suramin, one established angiogenesis inhibitor currently in some clinical uses. Collectively, these results suggest that **2**-3 is a cytotoxic agent ($IC_{50}$ values in tumor cell lines in the range of 1 μg $mL^{-1}$) as well as a

Table 3
Summary of the inhibitory effects of **2**-3 on in vitro HUVEC assay

| **2**-3 | | Suramin | |
|---|---|---|---|
| Concentration (μg $mL^{-1}$) | Activity [a] | Concentration (μM) | Activity [a] |
| 0.3 (1.10) [b] | +++ | 100 | +++ |
| 0.1 (0.37) | +++ | 30 | +++ |
| 0.03 (0.11) | ++ | 10 | ++ |
| 0.01 (0.04) | + | 3 | – |

[a] The activity was evaluated in a double-blind manner and designed as follows: –, inactive; +, slight; ++, moderate; +++, strong to complete inhibition.

[b] Numbers in parenthesis show the corresponding concentration in μM.

selective angiogenesis inhibitor with large selectivity index ( > 36). In the in vitro model, **2**-3 was shown to be more potent than suramin.

### 3.3. Antitumor activity of 2-3

The chalcones prepared in this work showed considerable cytotoxicity in tumor cell lines, and compound **2**-3 was found to selectively inhibit angiogenesis in the in vitro model. To examine whether these chalcones exert in vivo antitumor activity, one representative compound, **2**-3, was chosen for in vivo experiments in BDF1 mice bearing Lewis lung carcinoma cells. The compound was evaluated at the maximum tolerated dose of 50 mg $kg^{-1}$ $day^{-1}$. The administration of the compound was scheduled to include 6 injections, from day 1 after tumor cell transplantation and continued every two days until day 11. The body weights were recorded and body weight changes were calculated. The results are included in Table 4. Tumor volumes were tracked from day 11 and the inhibition rates, calculated as described in the Section 4, are shown in Table 4. Etoposide, one clinical anticancer agent, was dosed to one group of mice at 36 mg $kg^{-1}$ $day^{-1}$ on days 1, 5 and 9, and used as a positive control. The results shown in Table 4 demonstrate that **2**-3 showed efficacy in this model. On day 15, the tumor masses in the **2**-3 treated group were inhibited by 60.5% compared to the vehicle treated control. However, this inhibition rate was still lower than that of etoposide (73.6% on day 15). To ascertain whether the antiangiogenic activity of this compound contributed to its in vivo antitumor activity, an indirect approach was used. Compound **2**-4, structurally similar to **2**-3, thus their pharmacokinetics would be expected to be similar, was experimented in parallel at 50 mg $kg^{-1}$ $day^{-1}$ with the same injection schedule. It was interesting to observe that, on day 15, **2**-3 was found to be less potent than **2**-3; the IR value of **2**-4 was 47.3%, lower than that (60.5%) of **2**-3. In vitro, the two compounds were almost equipotent towards tumor cell lines ($IC_{50}$ values of 1.34 and 1.39 μg $mL^{-1}$ in B16 cells, for instance), however, **2**-4 did not show any selective angiogenesis inhibitory activity meanwhile **2**-3 was shown to be a strong angiogenesis inhibitor. Thus, the antiangiogenic activity of **2**-3 was likely one mechanism of its in vivo antitumor activity. It was noted that the inhibition rate of **2**-3 on the growth of Lewis lung carcinoma cells in BDF1 mice was highest on day 11 (72.6%), the last day of the compound injection. After the interruption of the compound injection, the tumors continued to growth. On day 16, the IR value decreased to just 45.9%. Similar phenomena have been documented for other angiogenesis inhibitors like TNP-470, endostatin, and thalidomide [8]. And for angiogenesis inhibitors, continual administration of drugs has been reported to be required to effect regression of tumors. From these perspectives, the tumor masses in mice treated with **2**-3 were challenged with a second round of treatment with three additional injections on days 16, 17, and 18. On day 19, a significant regression of tumors in this group was achieved with the inhibition rate recorded as 57.4%. Meanwhile, a similar supplement of **2**-4 did not cause any shrinkage of tumor masses in the group treated with **2**-4 (Fig. 3), indicating that tumor cells in this group had become refractory to this agent.

In summary, we have synthesized a series of 2′,5′-dihydroxychalcones (**2**) and found that most of compounds in this series showed potent cytotoxicity against tumor cell lines. In overall, the series **2** was found to be twofold more potent than series **1**. The electron-with-

Table 4
Antitumor activity of **2**-3 and **2**-4

| Cpd | R | Cytotoxicity ($IC_{50}$ [a], μg $mL^{-1}$) | | | Dose (mg $kg^{-1}$ $day^{-1}$) | Antitumor activity | | |
|---|---|---|---|---|---|---|---|---|
| | | B16 | AVR-C [b] | HUVEC | | BWC (%) [c] | IR [d] | |
| **2**-3 | **2**-Cl | 1.34 | 1.09 | 0.03 | 50 | | 72.6 | (day 11) |
| | | | | | | +10.8 | 60.5 | (day 15) |
| | | | | | | | 45.9 | (day 16) |
| | | | | | | | 57.4 | (day 19) |
| **2**-4 | **3**-Cl | 1.39 | 1.72 | 1.71 | 50 | +5.9 | 51.2 | (day 11) |
| | | | | | | | 47.3 | (day 15) |
| | | | | | | | 39.7 | (day 16) |
| | | | | | | | 37.5 | (day 19) |
| ETP [e] | | 0.10 | 1.11 | 0.21 | 36 | 0 | 73.6 | (day 15) |

[a] A sample's concentration produces a 50% reduction in cell growth. The values shown were the averages from a triplicate experiment.
[b] Average $IC_{50}$ values in three cell lines B16, A431 and HCT116.
[c] Body weight change.
[d] Inhibition rate of tumor growth in BDF1 mice bearing Lewis lung carcinoma cells (see Section 4 for calculation method); **2**-3 and **2**-4 were injected every 2 days from day 1 after tumor cell transplantation until day 11; a second round of three doses were supplemented every day from day 16.
[e] Etoposide, a positive control, was dosed on days 1, 5 and 9.

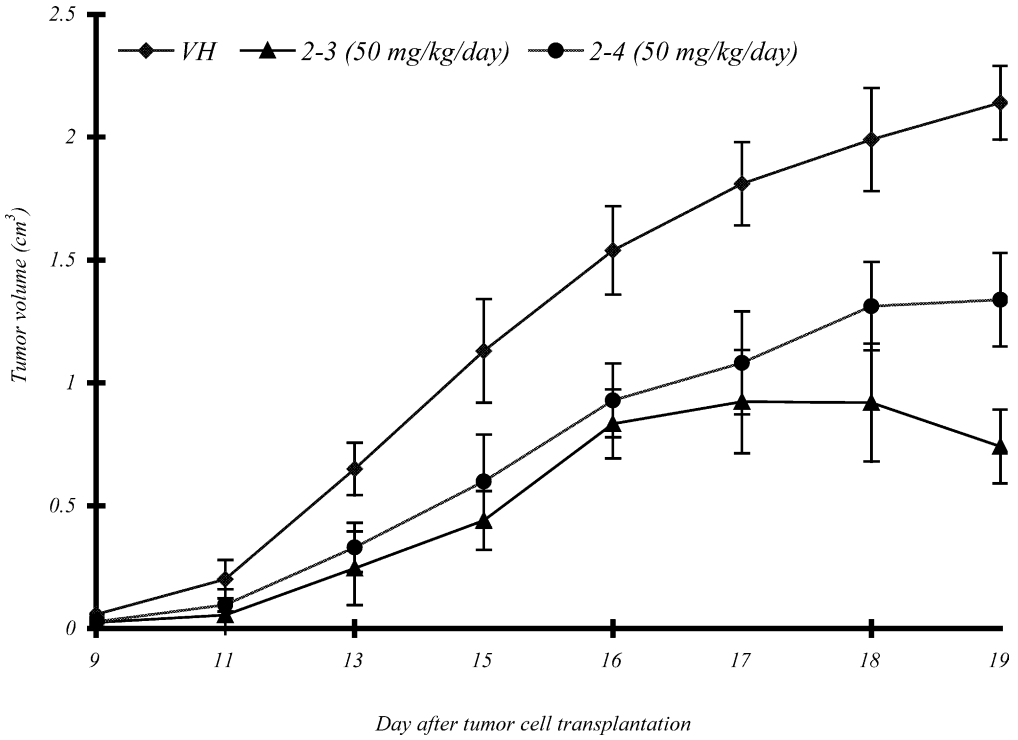


Fig. 3. Tumor volumes in **2**-3- and **2**-4-treated mice. The data plotted as averages ± S.D. The tumor volumes of the treated groups were significantly ($p < 0.05$) lower than the negative control (vehicle) group. ◆, vehicle group; ●, **2**-4 treated group; ▲, **2**-3 treated group. Each group of ten mice was used. Samples were injected at 50 mg $kg^{-1}$ $day^{-1}$ every other day from day 1 after tumor cell transplantation until day 11; a second round of three doses were supplemented every day from day 16.

drawing groups proved to be favorable for bioactivity. A number of compounds showed significant cytotoxic selectivity towards HUVE cells. Especially, compound **2**-3 (2-chloro-2′,5′-dihydroxychalcone) was revealed to be a strong angiogenesis inhibitor with marked antitumor activity. Through the experimental results, the antiangiogenic property of **2**-3 was suggested to play a role in its antitumor activity.

## 4. Experimental protocols

### *4.1. Chemistry*

Chemicals and solvents were reagent grade and used without further purification. Tumor cell lines were obtained from a cancer cell bank at the Korea Research Institute of Bioscience and Biotechnology (KRIBB). Media, Sera and other reagents used for cell culture were purchased from GIBCO Co. Ltd. Melting points were determined on an electrothermal melting point apparatus and are uncorrected. IR spectra were recorded on a Jasco Report-100 IR spectrometer. $^1$H NMR spectra were recorded on Varian-Gemini 300 or 90 MHz spectrometers using tetramethylsilane as internal standard. Mass spectra were determined with a JEOL JMS-D-100 mass spectrometer. Elemental analyses were within ±0.4% of the theoretical values, unless otherwise noted. Analytical thin layer chromatography was performed on a plastic sheet (0.2 mm) precoated with silica gel 60 $F_{254}$ (E. Merck). Silica gel 60 (70-230 mesh, E. Merck) was used for column chromatography.

#### *4.1.1. (E)-1-(2,5-Dihydroxyphenyl)-3-phenyl-2-propene-1-one (2-1)*

2′,5′-Dihydroxyacetophenone (3.8 g, 25 mmol) and pyridinium *p*-toluene sulfonate (0.15 g, 0.6 mmol) were stirred at room temperature (r.t.) for 0.5 h in methylene chloride (100 mL), and then 3,4-dihydro-2*H*-pyran (13 mL) in methylene chloride (20 mL) was added dropwise. The reaction mixture was stirred at r.t) for 4 h, then washed twice with water, dried and evaporated in vacuo. The residue yielded crude 2′,5′-bis(tetrahydropyran-2-yloxy)acetophenone (**3**).

Crude **3**, benzaldehyde (2.6 g, 25 mmol) and barium hydroxide octahydrate (8.15 g, 25 mmol) were dissolved in methanol (100 mL). The reaction mixture was stirred for 12 h at 40 °C and then evaporated in vacuo. Water (100 mL) was added and the mixture was neutralized with HCl (1M, 35 mL) and extracted with methylene chloride. The organic layer was separated, washed with water, dried and evaporated in vacuo. The residue yielded crude (*E*)-1-[2,5-bis(tetrahydropyran-2-yloxy)-phenyl]-3-phenyl-2-propene-1-one (**4**).

Crude **4** and *p*-toluenesulfonic acid (0.2 g, 1.05 mmol) were dissolved in methanol (100 mL). The reaction

mixture was stirred for 4 h at r.t., and then evaporated in vacuo. Water (100 mL) was added and the resulting mixture was neutralized with 5% $NaHCO_3$ (50 mL), and extracted with ethyl acetate. The organic layer was separated, washed with water, dried and concentrated in vacuo. The residue was purified on a silica gel column eluted with hexane–ethyl acetate (4:1) to give **2**-1 (4.2 g, 65%) as pale yellow solids; IR (KBr) 3400, 1670 $cm^{-1}$; $^1$H-NMR (DMSO-$d_6$, 90 MHz) $\delta$ 11.89 (1H, s), 9.58 (1H, s), 7.98 (1H, d, $J = 15.12$ Hz), 7.71–7.32 (5H, m), 7.15 (1H, d, $J = 2.12$ Hz), 7.07–6.91 (2H, m), 6.79 (1H, d, $J = 15.10$ Hz).

The following chalcones were synthesized by the same procedure described above unless otherwise noted.

*4.1.2. (E)-1-(2,5-Dihydroxyphenyl)-3-(2-fluorophenyl)-2-propene-1-one (**2**-2)*

Red crystals; yield 81%; $^1$H-NMR (DMSO-$d_6$, 90 MHz) $\delta$ 12.00 (1H, s), 10.18 (1H, s), 8.11 (1H, s, $J =$ 15.21 Hz), 7.42 (1H, s, $J = 15.21$ Hz), 7.32–6.91 (4H, m), 6.88–6.73 (3H, m).

*4.1.3. (E)-3-(2-Chlorophenyl)-1-(2,5-dihydroxyphenyl)-2-propene-1-one (**2**-3)*

Red crystals; yield 76%; $^1$H-NMR (DMSO-$d_6$, 90 MHz) $\delta$ 12.27 (1H, s), 9.98 (1H, s), 8.08 (1H, s, $J =$ 14.39 Hz), 7.45 (1H, s, $J = 14.42$ Hz), 7.27–7.11 (4H, m), 6.85–6.73 (2H, m), 6.70 (1H, d, $J = 8.21$ Hz).

*4.1.4. (E)-3-(3-Chlorophenyl)-1-(2,5-dihydroxyphenyl)-2-propene-1-one (2-4)*

Red crystals; yield 59%; $^1$H-NMR (DMSO-$d_6$, 90 MHz) $\delta$ 11.79 (1H, s), 9.37 (1H, s), 8.01 (1H, s, $J =$ 15.87 Hz), 7.41 (1H, s, $J = 15.87$ Hz), 7.31 (1H, d, $J =$ 7.89 Hz), 7.29–7.02 (2H, m), 6.98 (1H, d, $J = 8.12$ Hz), 6.86–6.71 (2H, m).

*4.1.5. (E)-3-(4-Chlorophenyl)-1-(2,5-dihydroxyphenyl)-2-propene-1-one (**2**-5)*

Red crystals; yield 65%; IR (KBr) 3360, 1670 $cm^{-1}$; $^1$H-NMR (DMSO-$d_6$, 300 MHz) $\delta$ 11.98 (1H, s), 9.89 (1H, s), 7.85 (1H-β, d, $J = 16.3$ Hz), 7.78 (1H-α, d, $J =$ 15.3 Hz), 7.75–7.71 (H-2, H-6, m), 7.48–7.42 (H-6′, H-3, H-5, m), 7.05 (H-4′, dd, $J = 8.12$ Hz), 6.84 (H-3′, d, $J = 8.12$ Hz).

*4.1.6. (E)-3-(4-Bromophenyl)-1-(2,5-dihydroxyphenyl)-2-propene-1-one (**2**-6)*

Red crystals; yield 43%; IR (KBr) 3400, 1680 $cm^{-1}$; $^1$H-NMR (DMSO-$d_6$, 300 MHz) $\delta$ 12.41 (1H, s), 10.27 (1H, s), 8.10 (1H-β, d, $J = 16.10$ Hz), 7.93 (1H-α, d, $J =$ 16.10 Hz), 7.91–7.73 (H-2, H-6, m), 7.42–7.31 (H-6′, H-3, H-5, m), 7.12 (H-4′, dd, $J = 2.50$, 8.41 Hz), 6.83 (H-3′, d, $J = 8.41$ Hz).

*4.1.7. (E)-1-(2,5-dihydroxyphenyl)-3-(4-nitrophenyl)-2-propene-1-one (**2**-7)*

A 100 mL two-neck flask was charged with 1.52 g of 2′,5′-dihydroxyacetophenone (10 mmol), 1.51 g of 4-nitrobenzaldehyde (10 mmol) dissolved in 30 mL of ethanol. Dry HCl gas was bubbled into the reaction mixture for 3 h. The mixture was kept stirring for a further 9 h and ethanol was removed. The residue was dissolved in water and extracted with methylene chloride twice (50 mL each). The methylene chlororide layers were combined and concentrated in vacuo. The crude product was purified on a silica gel column eluting with gradient ethyl acetate in hexane to give a reddish solid; yield 70%; IR (KBr) 3350, 1650 $cm^{-1}$; $^1$H-NMR (DMSO-$d_6$, 300 MHz) $\delta$ 12.10 (1H, s), 10.12 (1H, s), 8.02 (H-β, d, $J = 16.00$ Hz), 7.91 (H-α, d, $J = 16.00$ Hz), 7.65–7.84 (H-2, H-6, m), 7.27–7.45 (H-6′, H-3, H-5, m), 7.07 (H-4′, dd, $J = 2.50$, 8.41 Hz), 6.85 (H-3′, d, $J = 7.89$ Hz).

*4.1.8. (E)-1-(2,5-Dihydroxyphenyl)-3-(2,3,4,5,6-pentafluorophenyl)-2-propene-1-one (**2**-8)*

Red crystals; yield 78%; IR (KBr) 3350, 1670 $cm^{-1}$; $^1$H-NMR (DMSO-$d_6$, 90 MHz) $\delta$ 11.03 (1H, s), 9.22 (1H, s), 7.88 (1H, d, $J = 15.93$ Hz), 7.50 (1H, d, $J =$ 15.93 Hz), 7.18 (1H, d, $J = 2.70$ Hz), 7.02 (1H, dd, $J =$ 2.70, 8.82 Hz), 6.82 (1H, d, $J = 8.82$ Hz).

*4.1.9. (E)-3-(2,5-Difluorophenyl)-1-(2,5-dihydroxyphenyl)-2-propene-1-one (**2**-9)*

Red crystals; yield 57%; IR (KBr) 3520, 1680 $cm^{-1}$; $^1$H-NMR (DMSO-$d_6$, 90 MHz) $\delta$ 12.17 (1H, s), 10.27 (1H, s), 8.12 (H, d, $J = 15.38$ Hz), 7.96 (H, d, $J = 2.24$ Hz), 7.87 (H, d, $J = 15.38$ Hz), 7.55–7.41 (2H, m), 7.46–6.99 (3H, m).

*4.1.10. (E)-3-[(**2**-Bromo-5-methoxy)phenyl]-1-(2,5-dihydroxyphenyl)-2-propene-1-one (**2**-10)*

Red crystals; yield 81%; IR (KBr) 3420, 1670 $cm^{-1}$; $^1$H-NMR (DMSO-$d_6$, 90 MHz) $\delta$ 12.41 (1H, s), 10.06 (1H, s), 8.01 (H, d, $J = 16.15$ Hz), 7.75–7.56 (3H, m), 7.27–6.85 (5H, m), 3.91 (3H, s).

*4.1.11. (E)-3-[(5-Bromo-2-methoxy)phenyl]-1-(2,5-dihydroxyphenyl)-2-propene-1-one (**2**-11)*

Red crystals; yield 69%; IR (KBr) 3400, 1680 $cm^{-1}$; $^1$H-NMR (DMSO-$d_6$, 90 MHz) $\delta$ 12.07 (1H, s), 10.06 (1H, s), 8.02 (H, d, $J = 15.75$ Hz), 7.91 (H, d, $J = 2.43$ Hz), 7.83 (H, d, $J = 15.75$ Hz), 7.71–7.59 (1H, m), 7.35–6.85 (4H, m), 3.98 (3H, s).

*4.1.12. (E)-3-[(5-Bromo-2-hydroxy)phenyl]-1-(2,5-dihydroxyphenyl)-2-propene-1-one (**2**-12)*

Red crystals; yield 48%; IR (KBr) 3340, 1640 $cm^{-1}$; $^1$H-NMR (DMSO-$d_6$, 90 MHz) $\delta$ 12.31 (1H, s), 10.12

(1H, s), 9.21 (1H, s), 8.07 (1H, d, $J = 14.57$ Hz), 7.63–7.25 (3H, m), 7.05–6.75 (4H, m).

*4.1.13. (E)-1-(2,5-Dihydroxyphenyl)-3-(4-methoxyphenyl)-2-propene-1-one (**2**-13)*

Yellow crystals; yield 83%; IR (KBr) 3380, 1670 $cm^{-1}$; $^1$H-NMR (DMSO-$d_6$, 90 MHz) $\delta$ 12.06 (1H, s), 9.28 (1H, s), 8.02–7.90 (4H, m), 7.63 (1H, d, $J = 2.70$ Hz), 7.18–7.09 (3H, m), 6.93 (1H, d, $J = 8.82$ Hz), 3.88 (3H, s).

*4.1.14. (E)-1-(2,5-Dihydroxyphenyl)-3-(4-dimethylaminophenyl)-2-propene-1-one (**2**-14)*

Yellow crystals; yield 61%; m.p. 147–149 °C; IR (KBr) 3350, 1670 $cm^{-1}$; $^1$H-NMR (DMSO-$d_6$, 90 MHz) $\delta$ 12.35 (1H, s), 9.16 (1H, s), 7.82 (1H, d, $J =$ 14.49 Hz), 7.72 (2H, d, $J = 8.45$ Hz), 7.53 (1H, d, $J =$ 2.75 Hz), 7.00 (1H, dd, $J = 2.75$, 8.81 Hz), 6.86 (1H, dd, $J = 15.00$ Hz), 6.78 (2H, dd, $J = 8.45$ Hz), 6.69 (1H, dd, $J = 8.81$ Hz), 3.00 (6H, s).

*4.1.15. (E)-1-(2,5-Dihydroxyphenyl)-3-(2,3-dihydroxyphenyl)-2-propene-1-one (**2**-15)*

Yellow crystals; yield 55%; m.p. 145–148 °C; IR (KBr) 3380, 1670 $cm^{-1}$; $^1$H-NMR (DMSO-$d_6$, 90 MHz) $\delta$ 12.21 (1H, s), 9.32 (1H, s), 8.87 (2H, br), 7.89 (1H, d, $J = 14.51$ Hz), 7.32–7.05 (3H, m), 7.00–6.81 (3H, m), 6.56 (1H, d, $J = 14.51$ Hz).

*4.1.16. (E)-1-(2,5-Dihydroxyphenyl)-3-(2,5-dihydroxyphenyl)-2-propene-1-one (**2**-16)*

Yellowish crystals; yield 35%; m.p. 144–145 °C; IR (KBr) 3345, 1680, 1610, 1590 $cm^{-1}$; $^1$H-NMR (DMSO-$d_6$, 90 MHz) $\delta$ 7.99 (1H, d, $J = 16.11$ Hz), 7.41 (1H, d, $J = 16.11$ Hz), 6.88–7.02 (3H, m), 6.44–6.75 (3H, m).

*4.1.17. (E)-1-(2,5-Dihydroxyphenyl)-3-(3,4-dihydroxyphenyl)-2-propene-1-one (**2**-17)*

Yellowish crystals; yield 28%; IR (KBr) 3350, 1680, 1615, 1585 $cm^{-1}$; $^1$H-NMR (DMSO-$d_6$, 90 MHz) $\delta$ 8.10 (1H, d, $J = 16.23$ Hz), 7.45 (1H, d, $J = 16.22$ Hz), 6.88–7.11 (3H, m), 6.51–6.87 (3H, m).

*4.1.18. (E)-1-(2,5-Dihydroxyphenyl)-3-(2,3,4-trimethoxyphenyl)-2-propene-1-one (**2**-18)*

Yellowish crystals; yield 59%; m.p. 128–130 °C; IR (KBr) 3340, 2910, 1670, 1620, 1580 $cm^{-1}$; $^1$H-NMR (DMSO-$d_6$, 300 MHz) $\delta$ 7.95 (1H, d, $J = 15.59$ Hz), 7.58 (1H, d, $J = 15.59$ Hz), 7.29 (1H, d, $J = 8.6$ Hz), 6.66 (1H, dd, $J = 7.82$, 2.40 Hz), 6.51 (1H, d, $J = 8.61$ Hz), 6.44 (1H, d, $J = 7.82$ Hz), 6.41 (1H, d, $J = 2.40$ Hz), 3.95 (3H, s), 3.89 (3H, s), 3.88 (3H, s).

*4.1.19. (E)-1-(2,5-Dihydroxyphenyl)-3-(3,4,5-trimethoxyphenyl)-2-propene-1-one (**2**-19)*

Yellow crystals; yield 65%; m.p. 188–191 °C; IR (KBr) 3360, 1680 $cm^{-1}$; $^1$H-NMR (DMSO-$d_6$, 90 MHz) $\delta$ 11.93 (1H, s), 9.23 (1H, s), 8.00–7.54 (3H, m), 7.33 (2H, s), 7.04 (1H, d, $J = 2.75$ Hz), 6.88 (1H, d, $J = 8.84$ Hz), 3.86 (6H, s), 3.81 (3H, s).

*4.1.20. (E)-1-(2,5-Dihydroxyphenyl)-3-(4-phenylphenyl)-2-propene-1-one (**2**-20)*

Yellow crystals; yield 68%; m.p. 147–150 °C; IR (KBr) 3400, 1680 $cm^{-1}$; $^1$H-NMR (DMSO-$d_6$, 90 MHz) $\delta$ 11.97 (1H, s), 10.01 (1H, s), 7.91 (1H, d, $J =$ 15.24 Hz), 7.51–22 (10H, m), 7.13–6.84 (3H, m).

*4.1.21. (E)-1-(2,5-Dihydroxyphenyl)-3-(2-naphthyl)-2-propene-1-one (**2**-21)*

Yellow crystals; yield 45%; m.p. 235–237 °C; IR (KBr) 3380, 1660 $cm^{-1}$; $^1$H-NMR (DMSO-$d_6$, 90 MHz) $\delta$ 8.26 (1H, s), 8.11 (1H, d, $J = 15.51$ Hz), 7.65–8.01 (4H, m), 7.57 (1H, d, $J = 16.00$ Hz), 7.52–7.13 (2H, m), 6.77–6.89 (3H, m).

*4.1.22. (E)-1-(2,5-Dihydroxyphenyl)-3-(pyridin-3-yl)-2-propene-1-one (**2**-22)*

Yellow crystals; yield 35%; m.p. 119–122 °C; IR (KBr) 3400, 1680 $cm^{-1}$; $^1$H-NMR (DMSO-$d_6$, 90 MHz) $\delta$ 12.35 (1H, s), 10.12 (1H, s), 7.91 (1H, d, $J =$ 14.57 Hz), 7.75 (1H, s), 7.71–7.31 (4H, m), 7.15–6.85 (3H, m).

*4.1.23. (E)-1-(2,5-Dihydroxyphenyl)-3-(indol-3-yl)-2-propene-1-one (**2**-23)*

Yellow crystals; yield 38%; m.p. 117–121 °C; IR (KBr) 3380, 1680 $cm^{-1}$; $^1$H-NMR (DMSO-$d_6$, 90 MHz) $\delta$ 11.99 (1H, s), 9.48 (1H, s), 8.21 (1H, s), 7.97 (1H, d, $J = 15.21$ Hz), 7.48 (1H, d, $J = 15.21$ Hz), 7.32–6.99 (5H, m), 6.75–6.88 (2H, m).

*4.1.24. (E)-1-(2,5-Dihydroxyphenyl)-3-(quinolin-2-yl)-2-propene-1-one (**2**-24)*

Yellow crystals; yield 45%; m.p. 141–145 °C; IR (KBr) 3350, 1690 $cm^{-1}$; $^1$H-NMR (DMSO-$d_6$, 90 MHz) $\delta$ 12.00 (1H, s), 9.78 (1H, s), 8.21 (1H, d, $J =$ 14.38 Hz), 8.07 (1H, s), 7.71–7.56 (5H, m), 7.48 (1H, d, $J = 14.40$ Hz), 7.15–6.88 (3H, m).

*4.1.25. all-trans-1-(2,5-Dihydroxyphenyl)-5-phenyl-2,4-dienepentane-1-one (**2**-25)*

Yellow crystals; yield 87%; m.p. 142–144 °C; IR (KBr) 3360, 1680 $cm^{-1}$; $^1$H-NMR (DMSO-$d_6$, 90 MHz) $\delta$ 11.75 (1H, s), 9.25 (1H, s), 7.88–7.26 (8H, m), 7.13 (1H, dd, $J = 8.81$, 2.22 Hz), 6.91 (1H, s), 6.85 (1H, d, $J = 8.81$ Hz).

### 4.2. Biological assays

#### 4.2.1. Cells and cell cultures

Tumor cell lines were obtained from a cancer cell bank at Korea Research Institute of Bioscience and Biotechnology (KRIBB) and cultured in DMEM supplemented with 10% FBS. Human umbilical venous endothelial cells (HUVEC) were purchased from Sanko Junyaku Co., Ltd., Tokyo, Japan, and were grown in M-199 supplemented with heparin (5 U $mL^{-1}$), endothelial cell growth supplement (ECGS, 200 μg $mL^{-1}$) and 20% FBS. HUVECs were preserved frozen between passages 3 and 4. Biological experiments were carried out with cells between passages 5–10.

#### 4.2.2. Cytotoxicity assays

On day 0, 180 μL of a cell suspension (in culture medium, $3 \times 10^4$ cells $mL^{-1}$ for tumor cells, $5 \times 10^4$ cells $mL^{-1}$ for HUVECs) were seeded in each well of 96 well plates. The plates were incubated in a 5% $CO_2$ incubator at 37 °C for 24 h, then samples in 20 μL culture medium were added at various concentrations. The plates were incubated for another 48 h for tumor cell lines and 72 h for HUVECs. Cytotoxicity was measured by SRB's method as described in literature [9,10]. Compounds were examined in three independent assays, and the values, expressed $IC_{50}$ (a concentration produces 50% reduction in cell growth), were the averages of three determinations (S.D. $\leq$ 10%). The average potency of each compound in all cell lines was the calculated as the average of $IC_{50}$ values (average cytotoxicity). When the $IC_{50}$ values were greater than 30 μg $mL^{-1}$, this figure was used. The SI was calculated by an equation: SI = [average of $IC_{50}$ value of the compound in tumor cell lines (B16, HCT116, A431)]/$IC_{50}$ value of the compound in HUVEC.

#### 4.2.3. In vitro angiogenesis assay

In vitro angiogenesis assays were performed using a method reported previously [11] with slight modifications. Briefly, matrigel was thawed on ice to prevent premature polymerization. An aliquot of 200 μL matrigel was seeded into each well of a 24-well tissue culture plate and allowed to polymerize at 37 °C for 30 min. Into each well of the matrigel-seeded plate were added 500 μL of M-199, with or without a test sample. Finally, 500 μL of a cell suspension ($1.6 \times 10^5$ cells $mL^{-1}$ in a complete HUVEC culture medium) were added. After incubation for 18 h at 37 °C in a 5% $CO_2$ humidified atmosphere, the formation of HUVEC induced network was observed under microscope and four different phase-contrast microscopic fields ( × 100) per well were photographed. The inhibition was estimated in a double-blind manner and designed as +++ (strong, complete), ++ (moderate), + (slight), and − (inactive).

#### 4.2.4. Antitumor experiments

Antitumor experiments were carried as described in our previous reports [10,12]. Briefly, Lewis lung carcinoma cells were inoculated s.c. into BDF1 mice on day 0 ($1 \times 10^7$ cells/mouse/0.2 mL PBS). Test compounds were dissolved in a medium comprising of 5% DMSO and 20% Cremophor®. Each compound was dosed with six injections from day 1 and every 2 days after. The second round of three injections was administered everyday from day 16 until day 18. Body weights were tracked every day and tumor sizes were measured with calipers from day 11. Tumor volumes were calculated by the following equation: tumor volume ($mm^3$) = [length (mm) × width $(mm)^2$]/2. The inhibition rate was evaluated as (1 − T/C) × 100% (where T is the mean tumor volume of the treated group, C is the mean tumor volume of the control group). Each group consisted of ten mice. Etoposide was administered at days 1, 5 and 9 at 36 mg $kg^{-1}$ $day^{-1}$. No death was recorded within the experimented period. Student's *t*-test was used for all statistical analyses.

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