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Original article

Antimitotic and vascular disrupting agents: 2-Hydroxy-3,4,5-trimethoxybenzophenones

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ABSTRACT

2-Hydroxy-3,4,5-trimethoxybenzophenones (**8**–**16**) manifest pseudo-ring formation involving intramolecular hydrogen bonding of the 2-OH and the carbonyl group. Among the synthetic products described in this report, (3-hydroxy-4-methoxyphenyl)(2-hydroxy-3,4,5-trimethoxyphenyl)-methanone (**14**) and (3-amino-4-methoxyphenyl)(2-hydroxy-3,4,5-trimethoxy-phenyl)methanone (**16**) exhibit significant antiproliferative activity against KB cells with IC_{50} values of 11.1 and 11.3 nM, respectively. These two compounds also displayed tubulin affinity comparable to that of combretastatin A-4. In studies with human umbilical vein endothelial cells, compounds **14** and **16** revealed concentration-dependent vascular-disrupting properties. The results support the rationale of the pseudo-ring concept and suggest further investigation of A-ring modification in these benzophenones.

1. Introduction

Tumor vasculature associated with abnormal properties such as poor perfusion, hyperpermeability and disorganization is a basis for tumor proliferation and survival and targeting tumor vasculature has become an important approach in the development of anticancer therapy [1,2]. Among antivascular agents, vascular-disrupting agents (VDAs) with the capability of destroying established tumor vasculature causing widespread tumor necrosis and regression are particularly different from other anti-angiogenic strategies and have recently shown potential in clinical trials [3].

Tubulin-depolymerizing agents structurally related to combretastatin A-4 (CA-4, **1**) have exhibited promising results for cancer treatment. Recent reports have demonstrated that tubulin-binding agents not only inhibit microtubule polymerization at mitosis with consequent apoptosis in cancer cells but also overcome the problem of multi-drug resistance (MDR) in cancer chemotherapy. Moreover, they reveal the novel characteristic of selectively cytotoxic VDAs [4,5]. A variety of CA-4 derivatives such as combretastatin A-4 phosphate (CA-4P, fosbretabulin, **2**) and AVE-8062 (ombrabulin, **3**) (Fig. 1) are currently in clinical trials [6]. The clinical data however indicated unexpected systemic toxicity including cardiotoxicity, *neurotoxicity*, dose-limiting *toxicities*, *and other undesirable* pharmaceutical properties which impeded the development of microtubule-targeting agents for the treatment of cancer [7]. In the development of small molecules as antiproliferative agents based on the optimization of CA-4, benzophenone-contained analogs including phenstatin (**4**), hydroxyphenstatin (**5**), and compound **6** exhibit potent cytotoxicity. Furthermore, they displayed considerable pharmacological features and convenient synthetic access in which construction of Z-stilbene type of CA-4 derivatives was avoided [8–11].

Numerous studies have demonstrated that the trimethoxybenzene A ring of the combretastatins is pivotal for cytotoxic activity. Recently different trimethoxyindoles have been developed as surrogates of this trimethoxybenzene A-ring. For instance, in our previous studies [12–14], N-aryl-, N-benzyl-4,5,6-trimethoxyindoles, and N-aryl-5,6,7-trimethoxyindoles were found to be potent antiproliferative compounds and in addition to N1 substitution, C2 and C3 replacements of trimethoxyindoles were described [15,16]. Furthermore, 2-amino-3,4,5-trimethoxybenzophenones forming a pseudo-ring exhibited

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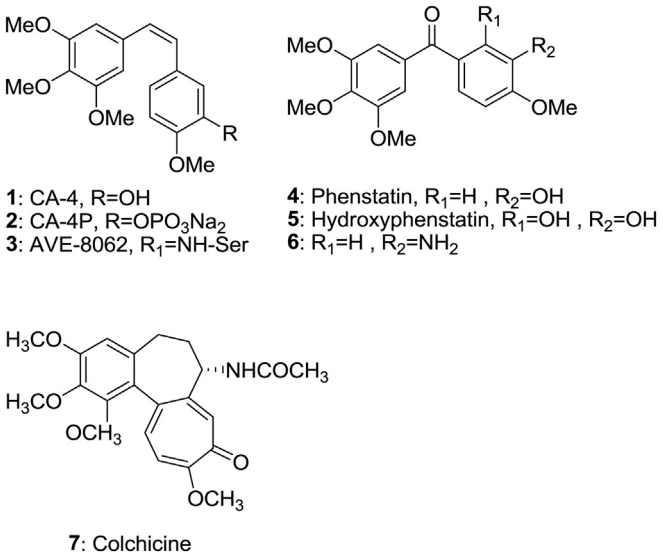


Fig. 1. Potential antitubulin agents.

marked antitubulin and antiproliferation activity [17]. These interesting results revealed that an extra ring fused with C1–C2 bond of 3,4,5-trimethoxybenzene is an acceptable form of A-ring modification. In an attempt to maintain both fused ring characteristics and the potency of the benzophenone structure, we have designed and synthesized a series of 2-hydroxy-3,4,5-trimethoxybenzophenones on the basis of pseudo-ring approach [18–20] and the relevant biological assays are reported in this paper (Fig. 2).

2. Results and discussion

2.1. Chemistry

The synthetic strategy for compounds **21** and **22** is shown in Scheme 1. Compound **18** was synthesized by a Baeyer–Villiger reaction in the presence of H_2O_2 [21]. Preparation of **19** and **20** was

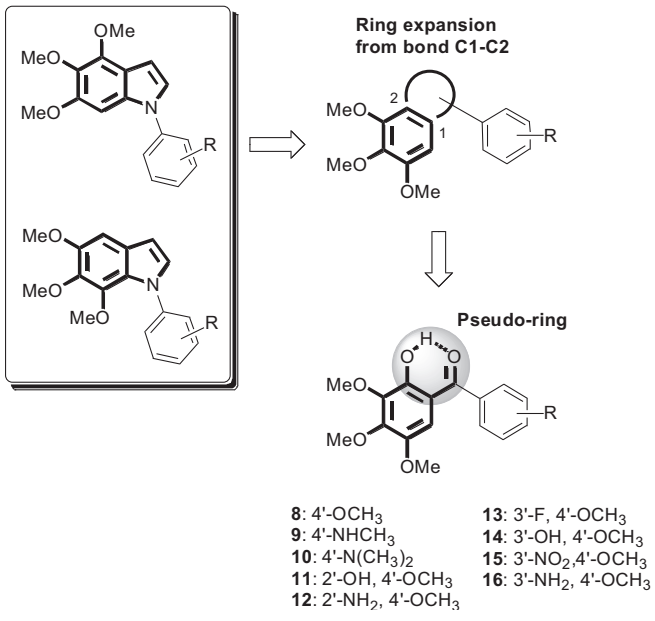


Fig. 2. Ring-opening strategy for novel anticancer agents.

achieved by treating compound **18** with hexamethylenetetramine and trifluoroacetic acid under Duff conditions [22] and then with tetrabutylammonium tribromide in CH_2Cl_2 [23]. The hydroxyl groups of **19** and **20** were protected by the reaction with *tert*-butyldimethylsilyl chloride (TBDMS-Cl) and methyl chloromethyl ether to afford compounds **21** and **22**, respectively.

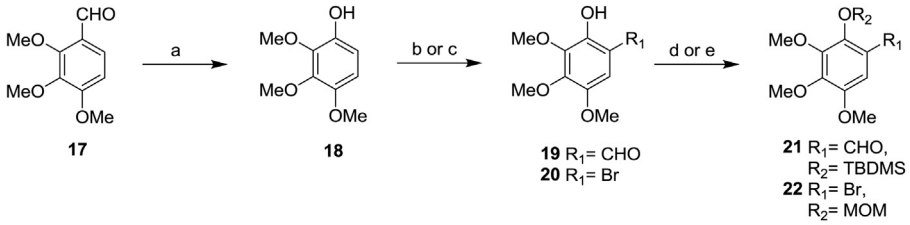
The synthesis of 2-hydroxy-3,4,5-trimethoxybenzoyl derivatives is described in Schemes 2 and 3. Compound **23a**–**b** was synthesized by reacting compound **21** with the appropriate substituted Grignard reagent followed by oxidation with pyridinium dichromate (PDC). Removal of the TBMDS group was accomplished with tetrabutylammonium fluoride (TBAF) affording compounds **8** and **14**. Compound **22** was sequentially treated with *n*-BuLi, various benzaldehydes, and PDC to afford the corresponding TBDMS-protected benzophenones **24a**, **24b**, **24e**, and **24f**. Surprisingly, compound **10** was obtained simultaneously during the PDC oxidation. Reduction of the nitro group of compounds **24c** and **24f** by iron powder yielded compounds **24d** and **24g**. Finally, the deprotection of MOM group under acidic conditions provided the desired compounds **9**–**16**. In compounds such as **8**–**16**, NMR signals corresponding to C2–OH are observed at downfield regions (δ 11–12 ppm). This is attributed to the formation of the intramolecular hydrogen bond shown in Fig. 2.

2.2. Biological evaluation

2.2.1. In vitro cell growth inhibitory activity

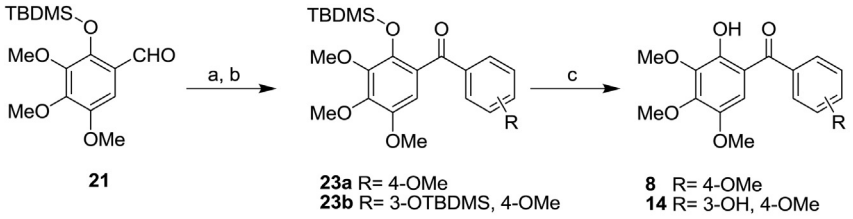
Reference compounds, all synthesized compounds (**8**–**16**), and CA4 and colchicine were evaluated for cytotoxic activity against three human cancer cell lines, cervical carcinoma KB cells, stomach carcinoma MKN45 cells and colorectal carcinoma HT29 cells (Table 1).

HT29 cells are not sensitive to the compounds tested, with the exception of compound **16**. Comparison of compounds **8**, **9** and **10** revealed a substitution effect at C4 of the B-ring. Compound **9** with an N-methylamino group, showed the most potent activity against KB and MKN45 cells with IC_{50} values of 30.9 and 43.5 nM, respectively. Conversion of the 4-NHMe to 4-OMe (**8**) resulted in a 5-fold decrease of potency. Addition of an extra methyl group, as in compound **10**, led to a 10-fold decrease in cytotoxicity. In the B-ring modification, additional –F, $-NH_2$, and –OH substituents accompanied by a 4-OMe were found to improve biological activity and the effect of –F, $-NH_2$, and –OH at the C2 or C3 position was therefore evaluated. Compounds with additional substitution at C2 or C3, with the exception of compound **16**, showed considerable loss of inhibitory activity with MKN45 cells. The comparison of **8**, **11**, and **12** demonstrated that a 2-NH_2 group was associated with a 2-fold increase of inhibitory activity against KB cells but a 2-OH substituent has no influence on the potency. Compound **15** bearing a nitro group displayed a dramatic loss of cytotoxicity indicating the electron-withdrawing NO_2 group is disfavored. A similar phenomenon is observed in compound **13** with a fluorine atom at C3 position. Notably, the addition of a 3-OH (**14**) or a 3-NH_2 (**16**) led to a 13-fold improvement of activity, compared with **8**, against KB cells with IC_{50} values of 11.1 and 11.3 nM, respectively. Comparison of compounds **11** and **12** with compounds **14** and **16** revealed that the 3-OH and 3-NH_2 substituents respectively provided a 13- and 6-fold enhancement of antiproliferative activity against KB cells. Since 3′-hydroxy-4′-methoxyphenyl (**14**) and 3′-amino-4′-methoxyphenyl substitution (**16**) produces the same B-ring as in CA-4 and AVE-8062, this result indicates that such pseudo-ring methodology, which maintains the conformation of A-ring/Z-double bond part of CA-4 is feasible. In general, this group of compounds displays no cytotoxicity against HT29 cells and the introduction of additional groups at C2 or C3 is detrimental to



[a]Reagents and conditions: (a) 30% H_2O_2, H_2SO_4, MeOH, rt; (b) hexamethylenetetramine, TFA, reflux; (c) tetrabutylammonium tribromide, DCM, rt; (d) TBDMSCl, DIPEA, DCM, 0 °C to r.t; (e) MOMCl, DIPEA, DCM, 0 °C to r.t.

Scheme 1. Synthetic approaches to compounds **21** and **22**. Reagents and conditions: (a) 30% H_2O_2, H_2SO_4, MeOH, rt; (b) hexamethylenetetramine, TFA, reflux; (c) tetrabutylammonium tribromide, DCM, rt; (d) TBDMSCl, DIPEA, DCM, 0 °C to r.t; (e) MOMCl, DIPEA, DCM, 0 °C to r.t.



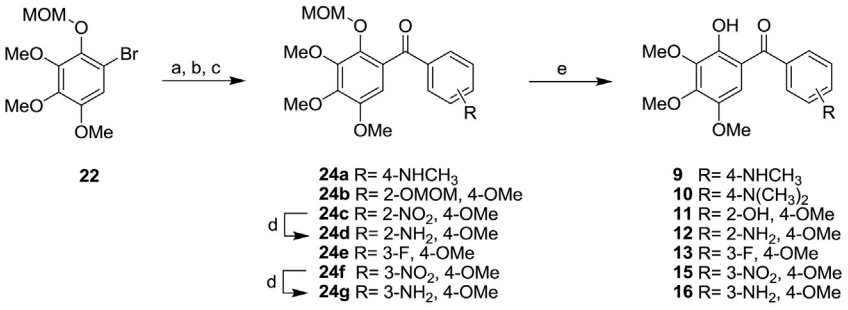
[a]Reagents and conditions: (a) substituted phenyl magnesium bromide, THF, 0 °C to rt; (b) PDC, DCM, r.t; (c) TBAF, THF, 0 °C to rt.

Scheme 2. Synthetic approaches to compounds **8** and **14**. Reagents and conditions: (a) substituted phenyl magnesium bromide, THF, 0 °C to r.t; (b) PDC, DCM, r.t; (c) TBAF, THF, 0 °C to rt.

MKN45 inhibitory activity, but these changes, together with retention of the B-rings in CA-4 and AVE-8062 leads to remarkable improvement of antiproliferative activity. Compounds **14** and **16** were further evaluated for antiproliferative activity against a variety of resistant cell lines and the results are shown in Table 2. Despite the high level of expression of drug-resistant efflux protein (MDR/P-gp or MRP) in KB-Vin10, KB-S15, and KB-7D cells, compounds **14** and **16** show similar efficacy in parental cells and in these resistant cell lines.

2.2.2. Inhibition of tubulin polymerization and colchicine binding activity

To investigate whether the pseudo-ring approach affects the original interaction mode with the microtubule system, compounds **14** and **16** and the reference compounds colchicine and CA-4 were evaluated in the tubulin inhibition assay and for colchicine binding activity (Table 3). Compounds **14** and **16** exhibited comparable antitubulin activity to that of colchicine but with IC_{50} values of 4.1 and 3.7 μM, respectively, are slightly less potent than CA-4. In the [^{3}H]colchicine binding assay, compound **16** has a similar affinity as CA-4 for the colchicine binding site. The interaction between compound **14** and tubulin is slightly weaker than is shown by compound **16**, and this is consistent with the results of the tubulin inhibition assay. Compounds **14** and **16** inhibited polymerization of pure MAP-rich tubulins in a concentration-dependent manner (Fig. 3.) and were identified as competitive inhibitors targeting the colchicine binding site in tubulin (Fig. 4.). Taken together, the marked cytotoxicity of compounds **14** and **16** is associated with the inhibition of microtubules, and supports the pharmacological mechanism of pseudo-ring products.



[a]Reagents and conditions: (a) *n*-BuLi, THF, -78 °C to 0 °C; (b) substituted benzaldehyde, THF, 0 °C; (c) PDC, DCM, r.t; (d) Fe, NH_4Cl, IPA-H_2O, reflux; (e) 1 N HCl, MeOH, rt.

Scheme 3. Synthetic approaches to compounds **9**–**16**. Reagents and conditions: (a) *n*-BuLi, THF, −78 °C to 0 °C; (b) substituted benzaldehyde, THF, 0 °C; (c) PDC, DCM, r.t; (d) Fe, NH_4Cl, IPA-H_2O, reflux; (e) 1 N HCl, MeOH, rt.

Table 1
Antiproliferative activity of compounds **8**–**16** and reference compounds.

Compd	Cell type (IC_{50} nM ± SD[a])		
	KB	MKN45	HT29
8	145.6 ± 48	235 ± 0.8	> 5000
9	30.9 ± 3.8	43.5 ± 1.2	206 ± 30.7
10	517.3 ± 59.3	412 ± 76.6	1093 ± 109
11	146.3 ± 5.9	484 ± 128	> 5000
12	70.3 ± 10.2	447 ± 11.3	3301 ± 116
13	183 ± 40.6	1130 ± 107	> 5000
14	11.1 ± 0.8	493 ± 35.1	2500
15	3667 ± 190	> 5000	1312 ± 560
16	11.3 ± 1.1	13.1 ± 2.3	51.8 ± 15.6
CA4	2.5 ± 0.8	3.2 ± 1.5	87.2 ± 27.8
colchicine	12.4 ± 2.5	14.9 ± 2.5	12.8 ± 1.3

[a] SD: standard deviation. All experiments were independently performed at least three times.

2.2.3. *In vitro vascular disrupting effect*

Recent reports indicate that antitubulin agents also exhibit a vascular disrupting effect, causing destruction of tumor vasculature. Compounds **14** and **16** were explored for the VDA property using a human umbilical vein endothelial cells (HUVECs) culture assay. HUVECs on Matrigel were allowed to form capillary tubes in the presence of VEGF (20 ng/mL) and then were exposed to different concentrations of selected compounds. The results shown in Fig. 5A indicated that compounds **14** and **16** both reveal a concentration-dependent inhibition of the formation of capillary tubes. In addition, compounds **14** and **16** with the concentration value of 500 nM entirely destroyed established tubes without altering cell viability (Fig. 5B.).

3. Conclusion

This paper studies the introduction of C2−OH of the A-ring to form intramolecular H-bond-containing benzophenone derivatives. The existence of a pseudo-ring structure is supported by a distinct downfield signal in the NMR spectra of the compounds derived from trimethoxyindole. A series of 2-hydroxy-3,4,5-trimethoxybenzophenones were synthesized (**8**–**16**) and were found generally to exhibit superior activity against KB cells than against MKN45 and HT29 cells. In addition, C3−NH_2 and C3−OH substituents on the B-ring contributed to an improvement of cytotoxicity. This result is similar to those obtained with CA-4 and AVE-8062, and supports the pseudo-ring rationale. Among the synthetic compounds, compounds **14** and **16** showed marked activity against KB cells with IC_{50} values of 11.1 and 11.3 nM, respectively. These two compounds were identified as inhibitors of tubulin polymerization which bind at the colchicine binding site. In studies of HUVECs tube formation assay, compounds **14** and **16** have been recognized as vascular disrupting agents with concentration-dependent suppression activity.

In summary, this paper not only widens the exploration of A-ring modification but also identifies a series of 2-hydroxy-3,4,5-trimethoxybenzophenones as novel promising anti-mitotic and anti-vascular agents.

Table 2
Growth inhibition of compounds **14**, **16** and reference compounds against drug-resistant cell lines.

Compd	(IC_{50} nM ± SD[a])			
	Parental cells	Multidrug resistant cells		
	KB	KB-VIN10 (P-gp170/MDR↑)	KB-S15 (P-gp170/MDR↑)	KB-7D (MRP↑)
14	11.1 ± 0.8	13.8 ± 3.3	10 ± 1.0	11.7 ± 2.3
16	11.3 ± 1.1	8.0 ± 4.1	7.4 ± 3.3	7.5 ± 4.3
Vincristine	0.9 ± 0.4	96.4 ± 8.5	2.1 ± 0.6	1.5 ± 0.6
Paclitaxel	5.1 ± 2.3	14,800 ± 890	135 ± 7.8	8.4 ± 0.3
VP-16	1500 ± 500	26,400 ± 2800	3500 ± 400	46,100 ± 5400

[a] SD: standard deviation. All experiments were independently performed at least three times.

Table 3
Inhibition of tubulin polymerization and colchicine binding inhibition by compounds **14**, **16**, and reference compounds.

Compound	Tubulin[a] IC_{50} ± SD (μM)	Colchicine binding[b] (%)	
		1 μM	5 μM
14	4.1	79.3 ± 1.2	88.2 ± 1.4
16	3.7	81.1 ± 2.1	91.7 ± 1.5
colchicine	4.9	32.2 ± 3.3	72.4 ± 0.6
CA4	1.9	86.2 ± 0.6	93.5 ± 0.3

[a] Inhibition of tubulin polymerization.
[b] Inhibition of [^{3}H]colchicine binding. Tubulin was at 1 μM; [^{3}H]colchicine was at 5 μM.

4. Experimental section

4.1. Chemistry

Nuclear magnetic resonance (^{1}H NMR and ^{13}C NMR) spectra were obtained with Bruker Fourier 300 and DRX-500 spectrometers, with chemical shift reported in parts per million (ppm, δ) downfield from TMS as an internal standard. High-resolution mass spectra (HRMS) were recorded with a FINNIGAN MAT 95S Mass Spectrometer. Purity of the final compounds was determined using a Hitachi 2000 series HPLC system using C-18 column (Agilent ZORBAX Eclipse XDB-C18 5 μm, 4.6 mm × 150 mm) with the solvent system (elution conditions: mobile phase A consisting of acetonitrile; mobile phase B consisting of water containing 0.1% formic acid + 10 mmol NH_4OAc) and was found to be ≥95%. Flash column chromatography was done using silica gel (Merck Kieselgel 60, no. 9385, 230–400 mesh ASTM). All reactions were carried out under an atmosphere of dry nitrogen.

4.1.1. *2,3,4-Trimethoxyphenol (**18**)*

A solution of 2,3,4-trimethoxybenzaldehyde (20 g, 100.9 mmol) and sulfuric acid (2 mL) in MeOH (200 mL) was stirred at 0 °C. To the previous solution, 30% H_2O_2 (13.6 mL, 131.2 mmol) was added dropwise at 0 °C and then was stirred at room temperature for 30 min. The solution was evaporated and extracted with EtOAc. The crude product was purified through chromatography to provide a transparent oil (14.8 g, 79%; EtOAc/hexane, R_f = 0.25). ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.79 (s, 3H), 3.88 (s, 3H), 3.94 (s, 3H), 5.54 (s, 1H), 6.54 (d, 1H, J = 9.0 Hz), 6.62 (d, 1H, J = 9.0 Hz).

4.1.2. *2-Hydroxy-3,4,5-trimethoxybenzaldehyde (**19**)*

A solution of 2,3,4-trimethoxyphenol (14.7 g, 80 mmol) and hexamethylenetetramine (11.4 g, 80 mmol) in trifluoroacetic acid (80 mL) was heated under reflux for 4 h. The stirred solution was cooled to room temperature and ice was added to the cooled mixture. Subsequently, the mixture was extracted with EtOAc and the organic layer was evaporated. The product was purified through chromatography to provide a solid (6.9 g, 41%; EtOAc/hexane, R_f = 0.2). ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.81 (s, 3H), 3.89 (s, 3H), 3.99 (s, 3H), 6.73 (s, 1H), 9.72 (s, 1H), 10.93 (s, 1H).

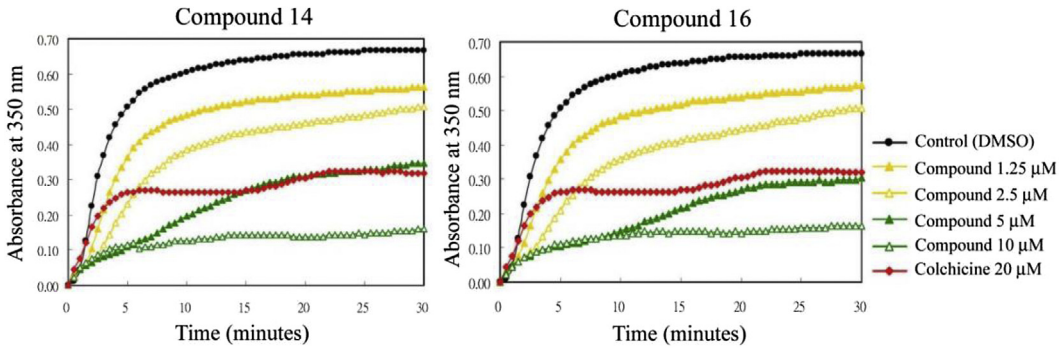


Fig. 3. Compounds **14** and **16** depolymerize microtubule *in vitro* by binding to the colchicine-binding site. Effect of compounds **14** and **16** on *in vitro* tubulin polymerization. MAP-rich tubulins were incubated at 37 °C in the absence [dimethyl sulfoxide (DMSO) control] or presence of drugs (colchicine or serial concentrations of compounds **14** and **16**). Absorbance at 350 nm was measured every 30 s for 30 min and is presented as the polymerized microtubule increases.

*4.1.3. 6-Bromo-2,3,4-trimethoxyphenol (**20**)*

To a solution of 2,3,4-trimethoxyphenol (2 g, 10.9 mmol) in CH_2Cl_2 (60 mL)-MeOH (40 mL) was added tetrabutylammonium tribromide (5.6 g, 11.4 mmol) in portions at room temperature. Subsequently, the resulting mixture was stirred at room temperature for 2 h. The solvent was evaporated and the crude product was purified through chromatography to afford the product (2.3 g, 81%; EtOAc/hexane = 1:6, $R_f = 0.3$). ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.80 (s, 3H), 3.87 (s, 3H), 3.96 (s, 3H), 5.65 (brs, 1H), 6.77 (s, 1H).

*4.1.4. 2-((Tert-Butyldimethylsilyl)oxy)-3,4,5-trimethoxybenzaldehyde (**21**)*

To a solution of 2-hydroxy-3,4,5-trimethoxybenzaldehyde (6.1 g, 28.8 mmol) and *N*,*N*-diisopropylethylamine (15.4 mL, 86.3 mmol) in CH_2Cl_2 (56 mL) was added dropwise *tert*-butyldimethylsilyl chloride (5.3 g, 34.5 mmol) at 0 °C. Subsequently, the resulting mixture was stirred at room temperature for 6 h. The solvent was evaporated and the crude product was purified through chromatography to afford the product (7.59 g, 81%; EtOAc/hexane = 95:5, $R_f = 0.2$). ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.20 (s, 6H), 1.00 (s, 9H), 3.81 (s, 3H), 3.85 (s, 3H), 3.97 (s, 3H), 7.07 (s, 1H), 10.33 (s, 1H).

*4.1.5. 1-Bromo-3,4,5-trimethoxy-2-(methoxymethoxy)benzene (**22**)*

To a solution of 6-bromo-2,3,4-trimethoxyphenol (1 g, 3.8 mmol) and *N*,*N*-diisopropylethylamine (2 mL, 10.2 mmol) in CH_2Cl_2 (8 mL) was added dropwise methyl chloromethyl ether (0.4 mL, 4.6 mmol) at 0 °C. Subsequently, the resulting mixture was stirred at room temperature for 4 h. The solvent was evaporated and the crude product was purified through chromatography to afford the product (0.93 g, 80%; EtOAc/hexane = 1:6, $R_f = 0.6$). ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.63 (s, 3H), 3.80 (s, 3H), 3.85 (s, 3H), 3.88 (s, 3H), 5.09 (s, 2H), 6.82 (s, 1H).

*4.1.6. General procedure A for synthesis of 2-(tert-butyldimethylsilyloxy)-3,4,5-trimethoxy- benzophenone (**23a** and **23b**)*

A solution of the substituted benzene magnesium bromide was prepared by achieving the reaction of substituted bromobenzene (1.2 equiv), magnesium (1.3 equiv) and a catalytic amount of iodine in THF (0.5 M) under reflux condition for 4 h. The solution was cooled to room temperature and then was added to a solution of 2-((tert-butyldimethylsilyl)oxy)-3,4,5-trimethoxybenzaldehyde (1 equiv) in THF (0.5 M) at 0 °C. The solution was stirred at 0 °C for 1 h and then was quenched with water. The mixture was extracted with EtOAc and the organic layer was collected. The crude product was purified through chromatography (EtOAc/hexane) to afford the substituted benzhydrol. To a solution of the substituted benzhydrol in CH_2Cl_2 (0.2 M), pyridinium dichromate (1.5 equiv) and 4Å molecular sieves (0.3 g) was added at room temperature. The resulting mixture was stirred at room temperature for 12 h. The mixture was diluted with EtOAc and filtered through a pad of Celite. The organic layer was collected and the crude product was purified by flash

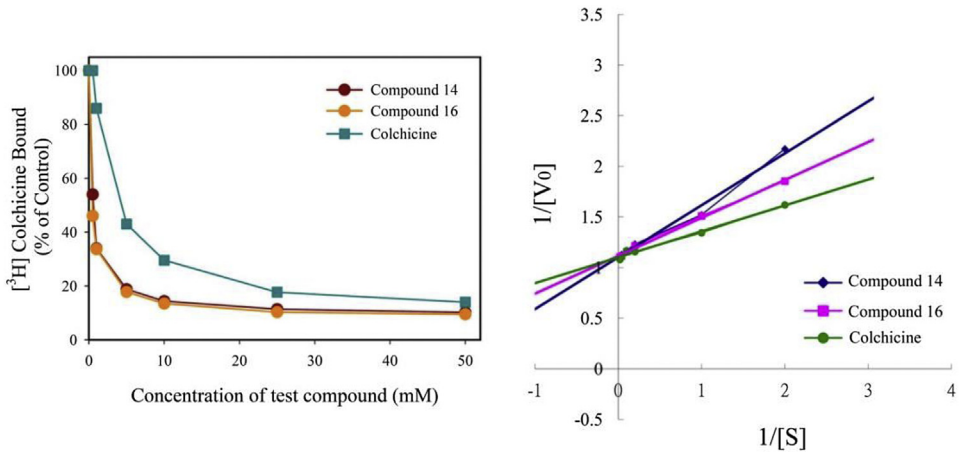


Fig. 4. The binding site of compounds **14** and **16** on tubulin was examined by using a competition-binding scintillation proximity assay, as described under *Materials and Methods*. The 100% binding represents ^{3}H-labeled ligand binding of the control group without tested compounds. The double-reciprocal plot indicates that compounds **14** and **16** are competitive inhibitors for the colchicine-binding site on tubulin. Each data point represents the mean ± S.D.

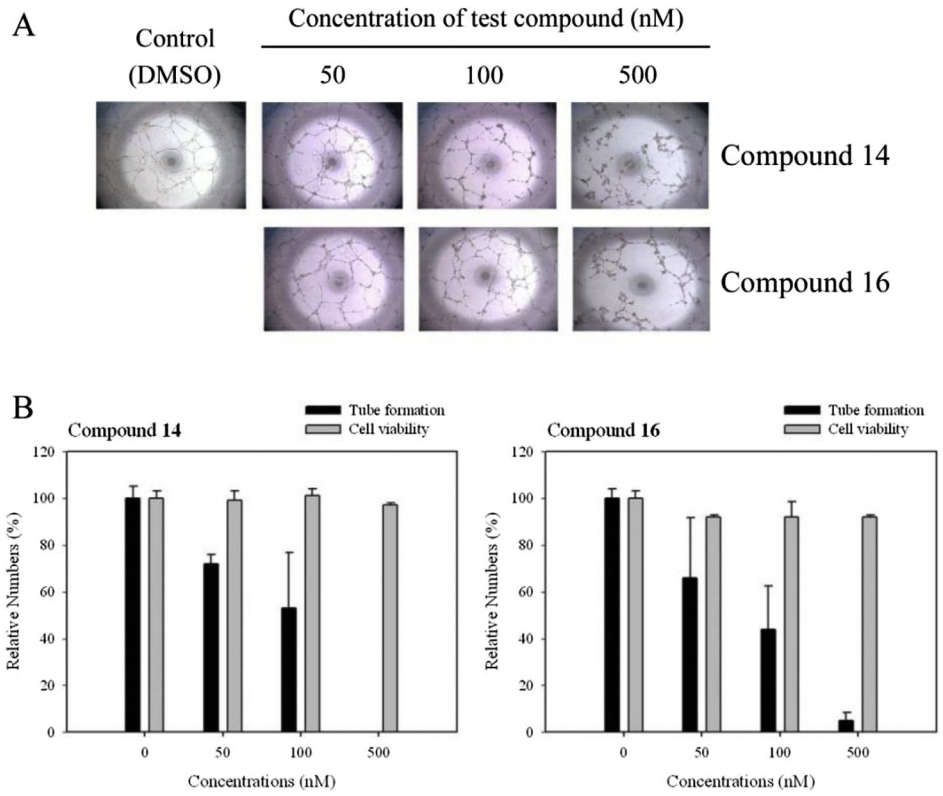


Fig. 5. Investigation of the vascular disrupting activity of compounds **14** and **16**. HUVECs were plated on Matrigel and allowed to form capillary tubes in the presence of VEGF (20 ng/mL) followed by exposure to different concentrations of test compound. Cultures were photographed, and the number of capillary tube networks was determined by counting under a microscope (original magnification of 100×). Data reflect the mean number of capillary tube networks to vehicle control group (DMSO) ± the standard deviation (SD) from three separate experiments. In the meantime, cell viability was also determined in parallel.

chromatography (EtOAc/hexane) to yield the substituted benzophenone.

*4.1.6.1. (2-((Tert-Butyldimethylsilyl)oxy)-3,4,5-trimethoxyphenyl)(4-methoxyphenyl)methanone (**23a**).* The title compound was obtained from 2-((*tert*-butyldimethylsilyl)oxy)-3,4,5-trimethoxybenzaldehyde (1.7 g, 5.20 mmol) according to general procedure A (1.17 g, 52%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.03 (s, 6H), 0.64 (s, 9H), 3.81 (s, 3H), 3.85 (s, 3H), 3.87 (s, 3H), 3.93 (s, 3H), 6.66 (s, 1H), 6.88–6.92 (m, 2H), 7.79–7.83 (m, 2H).

*4.1.6.2. (2-(Tert-butyldimethylsilyloxy)-3,4,5-trimethoxyphenyl)(3-(tert-butyldimethylsilyloxy)-4-methoxyphenyl)methanone (**23b**).* The title compound was obtained from 2-((*tert*-butyldimethylsilyl) oxy)-3,4,5-trimethoxy- benzaldehyde (2 g, 6.13 mmol) according to general procedure A (2.22 g, 64%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.64 (s, 9H), 0.96 (s, 9H), 3.80 (s, 3H), 3.84 (s, 6H), 3.91 (s, 3H), 6.63 (s, 1H), 6.83 (d, 1H, J = 8.4 Hz), 7.34 (d, 1H, J = 2.1 Hz), 7.46 (dd, 1H, J = 2.1, 8.4 Hz).

*4.1.7. General procedure B for cleavage of tert-butyldimethylsilyl group to provide **8** and **14***

TBAF solution (2 equiv) was added to a stirred solution of TBDMS-protected 2-hydroxybenzophenone (1 equiv) in THF (0.2 M) at 0 °C and the resulting mixture was stirred at room temperature for 1 h. The reaction solution was quenched with water. The crude product was extracted with EtOAc and purified by flash chromatography (EtOAc/hexane).

*4.1.7.1. (2-Hydroxy-3,4,5-trimethoxyphenyl)(4-methoxyphenyl) methanone (**8**).* The title compound was obtained from compound **23a** (200 mg, 0.47 mmol) according to general procedure B (147 mg, 98%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.68 (s, 3H), 3.84 (s, 3H), 3.92 (s, 3H), 4.00 (s, 3H), 6.85 (s, 1H), 6.95 (d, 2H, J = 8.5 Hz), 7.65 (d, 2H, J = 9.0 Hz), 12.09 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 55.2, 56.3, 60.8, 61.0, 110.2, 113.4, 113.6, 130.2, 131.2, 141.3, 144.5, 149.1, 153.1, 162.6, 198.7. HRMS-ESI calcd. for $C_{17}H_{19}O_6$ $[M + H]^+$ 319.1182, found 319.1181.

*4.1.7.2. (2-Hydroxy-3,4,5-trimethoxyphenyl)(3-hydroxy-4-methoxyphenyl)methanone (**14**).* The title compound was obtained from compound **23b** (2.12 g, 3.77 mmol) according to general procedure B (1.06 g, 84%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.71 (s, 3H), 3.95 (s, 6H), 3.97 (s, 3H), 4.04 (s, 3H), 5.71 (brs, 1H), 6.90 (s, 1H), 6.93 (d, 1H, J = 8.4 Hz), 7.23–7.30 (m, 2H), 12.10 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 56.1, 56.7, 61.1, 61.3, 109.9, 110.6, 113.8, 115.6, 122.5, 131.3, 141.5, 144.7, 145.5, 149.5, 150.0, 153.4, 199.0. HRMS-ESI calcd. for $C_{17}H_{19}O_7$ $[M + H]^+$ 335.1131, found 335.1138.

*4.1.8. General procedure C for synthesis of 2-(methoxymethoxy)-3,4,5-trimethoxy benzophenone (**24a–c**, **24e–f** and **10**)*

A stirred solution of 1-bromo-3,4,5-trimethoxy-2-(methoxymethoxy)benzene (1.2 equiv) in THF (0.13 M) was cooled to −78 °C and *n*-BuLi (1.2 equiv, 1.6 M in hexane) was added dropwise. The resulting solution was stirred at −78 °C for 1 h. The previous solution was added slowly to a solution of the substituted benzaldehyde (1 equiv) in THF (0.3 M) at 0 °C. The solution was stirred at 0 °C for 1 h and then was quenched with water. The mixture was extracted with EtOAc and the organic layer was collected. The crude product was purified through chromatography to afford (EtOAc/hexane) the substituted benzhydrol. To a solution of the substituted benzhydrol (0.1 M) in CH_2Cl_2, pyridinium dichromate (2 equiv) and

4Å molecular sieves (0.3 g) was added at room temperature. The resulting mixture was stirred at room temperature for 6 h. The mixture was diluted with EtOAc and filtered through a pad of Celite. The organic layer was collected and the crude product was purified by flash chromatography (EtOAc/hexane) to yield the substituted benzophenone.

*4.1.8.1. (4-(Methylamino)phenyl)(3,4,5-trimethoxy-2-(methoxymethoxy)phenyl)methanone (**24a**).* The title compound was obtained from 4-(methylamino)benzaldehyde (600 mg, 4.44 mmol) according to general procedure C (330 mg, 21%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.09 (s, 3H), 3.27 (s, 3H), 3.74 (s, 3H), 3.87 (s, 3H), 4.81 (s, 2H). 6.60 (s, 1H), 7.19 (d, 2H, J = 8.5 Hz), 7.83 (d, 2H, J = 8.5 Hz), 8.60 (s, 1H).

*4.1.8.2. (4-Methoxy-2-(methoxymethoxy)phenyl)(3,4,5-trimethoxy-2-(methoxymethoxy)phenyl)methanone (**24b**).* The title compound was obtained from 4-methoxy-2-(methoxymethoxy)benzaldehyde (500 mg, 2.55 mmol) according to general procedure C (510 mg, 47%). ^{1}H NMR (300 MHz, $CDCl_3$): δ3.26 (s, 3H) 3.30 (s, 3H) 3.79 (s, 3H) 3.82 (s, 3H), 3.88 (s, 3H), 3.91 (s, 3H), 4.83 (s, 2H), 4.98 (s, 2H), 6.56 (dd, 1H, J = 2.4, 8.7 Hz), 6.66 (d, 1H, J = 2.4 Hz), 6.73 (s, 1H), 7.59 (d, 1H, J = 8.7 Hz).

*4.1.8.3. (4-Methoxy-2-nitrophenyl)(3,4,5-trimethoxy-2-(methoxymethoxy)phenyl)methanone (**24c**).* The title compound was obtained from 4-methoxy-2-nitrobenzaldehyde (800 mg, 4.42 mmol) according to general procedure C (760 mg, 42%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.23 (s, 3H), 3.82 (s, 3H), 3.83 (s, 3H), 3.90 (s, 3H), 3.94 (s, 3H), 4.74 (s, 1H), 7.03 (s, 1H), 7.13 (dd, 1H, J = 2.4, 8.4 Hz), 7.41–7.47 (m, 2H).

*4.1.8.4. (3-Fluoro-4-methoxyphenyl)(3,4,5-trimethoxy-2-(methoxymethoxy)phenyl)methanone (**24e**).* The title compound was obtained from 3-fluoro-4-methoxybenzaldehyde (200 mg, 1.3 mmol) according to general procedure C (130 mg, 22%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.19 (s, 3H), 3.82 (s, 3H), 3.94–3.95 (m, 9H), 4.90 (s, 2H), 6.63 (s, 1H), 6.94–7.00 (m, 1H), 7.59–7.66 (m, 2H).

*4.1.8.5. (4-Methoxy-3-nitrophenyl)(3,4,5-trimethoxy-2-(methoxymethoxy)phenyl)methanone (**24f**).* The title compound was obtained from 4-methoxy-3-nitrobenzaldehyde (603 mg, 1.3 mmol) according to general procedure C (1 g, 75%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.17 (s, 3H), 3.84 (s, 3H), 3.95 (s, 3H), 3.97 (s, 3H), 4.04 (s, 3H), 4.89 (s, 2H), 6.68 (s, 1H), 7.14 (d, 1H, J = 8.7 Hz), 8.08 (d, 1H, J = 2.1, 8.7 Hz), 8.32 (d, 1H, J = 2.1 Hz).

*4.1.8.6. (4-(Dimethylamino)phenyl)(2-hydroxy-3,4,5-trimethoxyphenyl)methanone (**10**).* The title compound was obtained from 4-(dimethylamino)benzaldehyde (370 mg, 2.5 mmol) according to general procedure C (380 mg, 46%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.03 (s, 6H), 3.79 (s, 3H), 3.86 (s, 3H), 3.91 (s, 3H), 6.61 (d, 2H, J = 11.0 Hz), 6.63 (s, 1H), 7.28 (d, 2H, J = 11.5 Hz). ^{13}C NMR (75 MHz, $CDCl_3$): δ 39.9, 56.0, 60.7, 61.0, 106.9, 110.4, 125.3, 127.6, 132.6, 140.5, 144.4, 145.0, 147.6, 153.5, 194.0. HRMS-ESI calcd. for $C_{18}H_{22}NO_5$ $[M + H]^+$ 332.1498, found 332.1499.

*4.1.9. General procedure D for reduction of nitro group to provide **24d** and **24g***

To a solution of nitro compound (1 equiv) in isopropanol and water (5:1, 0.1 M) iron powder (2 equiv) and ammonium chloride (2 equiv) was added. The mixture was heated to reflux for 2 h and then diluted with EtOAc. The mixture was filtered off with celite and the organic layer was collected. The crude product was purified by flash chromatography (EtOAc/hexane) to afford the desired compound.

*4.1.9.1. (2-Amino-4-methoxyphenyl)(3,4,5-trimethoxy-2-(methoxymethoxy)phenyl)methanone (**24d**).* The title compound was obtained from compound **24c** (600 mg, 1.5 mmol) according to general procedure D (400 mg, 72%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.08 (s, 3H), 3.88 (s, 3H), 3.90 (s, 3H), 3.97 (s, 3H), 3.99 (s, 3H), 4.93 (s, 2H), 6.66–6.71 (m, 2H), 7.04 (s, 1H), 7.69–7.74 (m, 1H).

*4.1.9.2. (3-Amino-4-methoxyphenyl)(3,4,5-trimethoxy-2-(methoxymethoxy)phenyl)methanone (**24g**).* The title compound was obtained from compound **24f** (710 mg, 1.8 mmol) according to general procedure D (516 mg, 78%). ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.23 (s, 3H), 3.80 (s, 3H), 3.90–3.95 (m, 12H), 4.93 (s, 2H), 6.61 (s, 1H), 6.78 (d, 1H, J = 8.4 Hz), 7.22 (dd, 1H, J = 2.1, 8.4 Hz), 7.31 (d, 1H, J = 1.8 Hz).

*4.1.10. General procedure E for cleavage of methoxymethoxy group to provide **9**–**15***

2 N HCl (5 mL) was added at room temperature to a stirred solution of MOM-protected 2-hydroxybenzophenone (1 equiv) in MeOH (5 mL) and the resulting mixture was stirred for 4 h. The solution was evaporated *in vacuo* and diluted with water. The diluted solution was extracted with EtOAc. The organic layer was collected and the crude product was purified by flash chromatography (EtOAc/hexane) to afford the substituted 2-hydroxybenzophenone.

*4.1.10.1. (2-Hydroxy-3,4,5-trimethoxyphenyl)(4-(methylamino)phenyl)methanone (**9**).* The title compound was obtained from compound **24a** (365 mg, 1.0 mmol) according to general procedure E (301 mg, 83%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 2.93 (d, 3H, J = 4.5 Hz, NCH_3), 3.76 (s, 3H), 3.97 (s, 3H), 4.05 (s, 3H), 4.33 (brs, 1H), 6.63 (d, 2H, J = 9.0 Hz), 6.96 (s, 1H), 7.63 (d, 2H, J = 8.5 Hz), 12.13 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 30.0, 56.6, 61.1, 61.3, 110.6, 111.1, 114.4, 126.0, 132.1, 141.6, 144.5, 148.7, 152.7, 152.8, 198.2. HRMS-ESI calcd. for $C_{17}H_{20}NO_5$ $[M + H]^+$ 318.1341, found 318.1340.

*4.1.10.2. (2-Hydroxy-3,4,5-trimethoxyphenyl)(2-hydroxy-4-methoxyphenyl)methanone (**11**).* The title compound was obtained from compound **24b** (423 mg, 1.0 mmol) according to general procedure E (272 mg, 81%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.77 (s, 3H), 3.84 (s, 3H), 3.94 (s, 3H), 4.01 (s, 3H), 6.44 (dd, 1H, J = 2.0, 9.0 Hz), 6.50 (d, 1H, J = 2.0 Hz), 6.79 (s, 1H), 7.54 (d, 1H, J = 9.0 Hz), 9.50 (s, 1H), 11.64 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 55.0, 56.0, 60.6, 100.9, 106.7, 108.9, 113.0, 115.6, 134.3, 141.0, 145.0, 147.2, 148.1, 164.7, 165.5, 198.7. HRMS-ESI calcd. for $C_{17}H_{19}O_7$ $[M + H]^+$ 335.1131, found 335.1143.

*4.1.10.3. (2-Amino-4-methoxyphenyl)(2-hydroxy-3,4,5-trimethoxyphenyl)methanone (**12**).* The title compound was obtained from compound **24d** (320 mg, 0.79 mmol) according to general procedure E (221 mg, 84%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.88 (s, 3H), 3.90 (s, 3H), 3.98 (s, 3H), 4.03 (s, 3H), 6.29 (s, 1H), 6.65 (dd, 1H, J = 2.0, 9.5 Hz), 6.67–6.68 (m, 1H), 7.14 (s, 1H), 7.89 (d, 1H, J = 9.5 Hz). ^{13}C NMR (75 MHz, $CDCl_3$): δ 55.1, 56.2, 60.8, 61.1, 88.8, 106.5, 109.1, 111.9, 119.5, 123.9, 140.9, 141.7, 144.1, 146.7, 159.1, 161.4, 161.8. HRMS-ESI calcd. for $C_{17}H_{20}NO_6$ $[M + H]^+$ 334.1291, found 334.1190.

*4.1.10.4. (3-Fluoro-4-methoxyphenyl)(2-hydroxy-3,4,5-trimethoxyphenyl)methanone (**13**).* The title compound was obtained from compound **24e** (140 mg, 0.37 mmol) according to general procedure E (110 mg, 88%). ^{1}H NMR (300 MHz, $CDCl_3$):

δ 3.74 (s, 3H), 3.96 (s, 3H), 3.98 (s, 3H), 4.06 (s, 3H), 6.85 (s, 1H), 7.02–7.09 (m, 1H), 7.47–7.49 (m, 1H), 7.50–7.52 (m, 1H), 11.96 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 56.3, 56.6, 61.1, 61.3, 110.1, 112.4, 112.5, 113.5, 117.0, 117.3, 126.4, 126.5, 130.6, 130.7, 141.6, 144.8, 153.5, 197.6. HRMS-ESI calcd. for $C_{17}H_{18}FO_6$ $[M + H]^+$ 337.1087, found 337.1100.

*4.1.10.5. (2-Hydroxy-3,4,5-trimethoxyphenyl)(4-methoxy-3-nitrophenyl)methanone (**15**).* The title compound was obtained from compound **24f** (150 mg, 0.37 mmol) according to general procedure E (116 mg, 86%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.74 (s, 3H), 3.96 (s, 3H), 4.07 (s, 3H), 4.08 (s, 3H), 6.77 (s, 1H), 7.23 (d, 1H, $J = 9.0$ Hz), 7.96 (d, 1H, $J = 2.0$, 9.0 Hz), 8.25 (d, 1H, $J = 3.0$ Hz). ^{13}C NMR (75 MHz, $CDCl_3$): δ 56.6, 56.9, 61.1, 61.3, 109.4, 113.0, 113.5, 127.1, 130.0, 135.0, 139.0, 141.6, 145.1, 150.0, 153.6, 155.5, 196.3. HRMS-ESI calcd. for $C_{17}H_{18}NO_8$ $[M + H]^+$ 364.1032, found 364.1056.

*4.1.10.6. (3-Amino-4-methoxyphenyl)(2-hydroxy-3,4,5-trimethoxyphenyl)methanone (**16**).* The title compound was obtained from compound **24g** (300 mg, 0.8 mmol) according to general procedure E (226 mg, 85%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 3.72 (s, 3H), 3.92 (s, 3H), 3.95 (s, 3H), 4.04 (s, 3H), 6.83 (d, 1H, $J = 8.5$ Hz), 6.95 (s, 1H), 7.08–7.09 (m, 2H), 12.16 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 55.5, 56.5, 60.9, 61.1, 109.0, 110.7, 113.9, 114.9, 120.7, 130.7, 136.4, 141.3, 144.4, 149.1, 150.3, 153.1, 199.4. HRMS-ESI calcd. for $C_{17}H_{20}NO_6$ $[M + H]^+$ 334.1291, found 364.1298.

4.2. Biology

Reagents for cell culture were obtained from Gibco-BRL Life Technologies (Gaithersburg, MD). Microtubule-associated protein (MAP)-rich tubulin was purchased from Cytoskeleton, Inc. (Denver, CO). [^{3}H]Colchicine (specific activity, 60–87 Ci/mmol) was purchased from PerkinElmer Life Sciences (Boston, MA).

4.2.1. Cell growth inhibitory assay

Human cancer cell lines (KB, MKN45, and HT29) used in this study were procured from American Type Culture Collection (Rockville, MD) and grown in Dulbecco's modified Eagle's medium, minimal essential medium, or RPMI 1640 medium. Resistant cell lines KB-Vin10, KB-7D, and KB-S15 were maintained in medium containing additional 10 nM vincristine, 7 μM VP16, and 50 nM paclitaxel, respectively. All cell cultures were supplemented with 10% fetal bovine serum, 2 μM glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin and incubated in a humidified atmosphere (95% air and 5% CO_2) at 37 °C. KB-Vin10 and KB-S15 cell lines were resistant to vincristine and paclitaxel, respectively, and both overexpressed the MDR drug efflux protein. KB-7D cells were resistant to VP16 and overexpressed MRP. All resistant cell lines were incubated in drug-free medium for 3 days before being harvested for the growth inhibition assay. *In vitro* growth inhibition was assessed with the methylene blue assay [24]. Exponentially growing cells were seeded into 24-well culture plates at a density of 8000–20,000 cells per mL per well (depending on the doubling time of the cell line) and allowed to adhere overnight. Cells were incubated with various concentrations of drugs for 72 h. Then the A595 of the resulting solution from 1% N-lauroylsarcosine extraction was measured. Fifty percent growth inhibition (IC_{50}) was calculated on the basis of the A595 of untreated cells (taken as 100%). The values shown are the means and standard errors of at least three independent experiments performed in duplicate.

4.2.2. Tubulin polymerization in vitro assay [11,25]

Turbidimetric assays of microtubules were performed as described by Bollag et al. [24]. In brief, microtubule-associated protein (MAP)-rich tubulin (from bovine brain, Cytoskeleton, Inc.) was dissolved in reaction buffer [100 mM PIPES (pH 6.9), 2 mM $MgCl_2$, and 1 mM GTP] in preparing the 4 mg/mL tubulin solution. The tubulin solution (240 μg of MAP-rich tubulin per well) was placed in a 96-well microtiter plate in the presence of test compounds or 2% (v/v) DMSO as a vehicle control. The increase in absorbance at 350 nm was measured in a PowerWave X Microplate Reader (BIO-TEK Instruments, Winooski, VT) at 37 °C and recorded every 30 s for 30 min. The area under the curve (AUC) was used to determine the concentration that inhibited tubulin polymerization by 50% (IC_{50}). The AUCs of the untreated control and 10 μM colchicine were set to 100 and 0% polymerization, respectively, and the IC_{50} was calculated by nonlinear regression in at least three experiments.

4.2.3. Tubulin competition binding scintillation proximity assay [26–28]

This assay was performed in a 96-well plate. In brief, 0.08 μM [^{3}H]colchicine was mixed with the test compound and 0.5 μg of special long-chain biotin-labeled tubulin (0.5 μg) then incubated in 100 μL of reaction buffer [80 mM PIPES (pH 6.8), 1 mM EGTA, 10% glycerol, 1 mM $MgCl_2$, and 1 mM GTP] for 2 h at 37 °C. Then 80 μg of streptavidin-labeled SPA beads was added to each reaction mixture. The radioactive counts were then directly measured with a scintillation counter and the inhibition constant (Ki) was calculated using the Cheng–Prusoff equation [29].

4.2.4. Capillary disruption assays [30]

Capillary disruption assays were conducted in a 96-well plate format using human umbilical vein endothelial cells (HUVECs) plated at a density of 2×10^4 cells/well in 20% FBS M199 medium containing 20 ng/mL VEGF on a Matrigel layer (BD Biosciences). Capillaries were allowed to form over a 4 h period before the addition of test compound or vehicle control. Images were acquired immediately following addition of the compound and 4 h after exposure to the test compound. Tube formation was quantified by measuring the network number of capillary structures manually by counting under a microscope (original magnification of 100×).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2014.02.061.

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