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A boronic acid chalcone analog of combretastatin A-4 as a potent anti-proliferation agent

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ABSTRACT

Chalcones represent a class of natural products that inhibits tubulin assembly. In this study we designed and synthesized boronic acid analogs of chalcones in an effort to compare biological activities with combretastatin A-4, a potent inhibitor of tubulin polymerization. Systematic evaluation of the positional effects of the carbonyl moiety towards inhibition of tubulin polymerization, cancer cell proliferation and angiogenesis revealed that placement of the carbonyl adjacent to the trimethoxybenzene A-ring resulted in more active compounds than when the carbonyl group was placed adjacent to the C-ring. Our study identified a boronic acid chalcone with inhibition towards 16 human cancer cell lines in the 10–200 nM range, and another three cell lines with GI_{50}-values below 10 nM. Furthermore, this drug has significant anti-angiogenesis effects demonstrated by HUVEC tube formation and aortic ring assay.

1. Introduction

Compounds with a chalcone-based structure have promise as agents for the treatment of human cancers. Moreover, they serve as precursors in the biosynthesis of flavonoids. The chalcone flavokawain A from kava extracts has strong anti-proliferative and apoptotic effects against human bladder cancer cells.[1] Moreover there are several reports demonstrating antiinflammatory,[2] antibacterial[3] and anticancer[4] activities of chalcones. Due to their abundance in plants and ease of synthesis, the chalcone class of compounds has provoked great interest for possible therapeutic uses.

The microtubule system of eukaryotic cells is an important target for the development of antitumor agents. Antimitotic agents cause mitotic arrest in eukaryotic cells by interfering with the normal microtubule polymerization/depolymerization process. Furthermore, these agents provide new perspectives on the inhibition of tumor cell growth and the suppression of cancer. Combretastatin A-4 (CA-4) is a structurally very simple natural product with potent cytotoxic activity (Fig. 1).[5] The mechanism of action of CA-4 involves reversible, high affinity binding in the colchicine site of tubulin.[6] The ease of synthesis of CA-4 analogs has led to the development of a number of diverse ligands designed to mimic CA-4. Ikeda et al.[7] and Edwards et al.[8] reported that chalcone analogs of CA-4 are potent inhibitory agents toward human cancer cells. Boronic acid-chalcone analogs have previously been reported in the literature as fluorescent probes[9] and antitumor agents.[10] Boronic acid derivatives are of considerable interest in drug development because of their moderate pH of 9–10 and air-stability. VELCADE®, a modified dipeptidyl boronic acid was approved by the FDA in 2003 for the treatment of multiple myeloma disease. Previously we designed boronic acid CA-4 analog **1** (Fig. 1), which was found to have potent anti-tubulin, anti-cancer activity, and have improved solubility compared to CA-4.[11] This led us to explore chalcone analogs incorporating the phenylboronic acid C-ring of analog **1**.

We designed chalcone analogs **4**, **6**,[4,7] **11**, and **14** as carbonyl-expanded analogs of CA-4 (Fig. 1). Analogs **11** and **14** are carbonyl-inverse analogs of chalcones **4** and **6**. Analogs **4** and **11** are analogs of our previously reported boronic acid analog **1**, whereas analogs **6** and **14** are directly derived from CA-4. We synthesized and evaluated these analogs for effects on tubulin polymerization, inhibition of [^{3}H]colchicine binding to tubulin, disruption of the microtubule network of A-10 cells, and inhibition of the growth of human MCF-7 breast cancer cells. Boronic acid analog **4** was also evaluated by the National Cancer Institute against a panel of 54 human cancer cell lines in an effort to survey activity against a broad range of human cancers.

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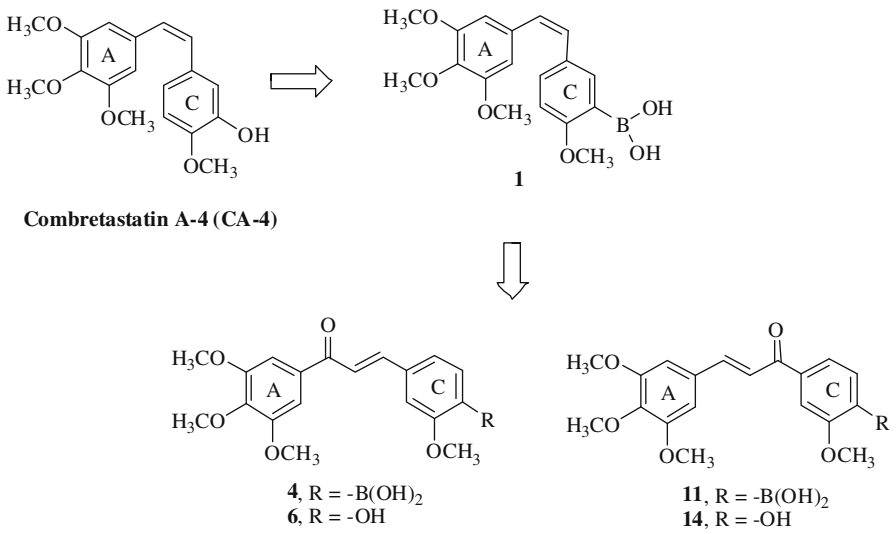


Figure 1. Chalcone analogs of CA-4.

2. Results and discussion

2.1. Chemistry

The Claisen–Schmidt condensation was used as a general procedure to synthesize the chalcone analogs (Scheme 1). Base-promoted condensation was found to give the best results for chalcone synthesis.[12,13]

Chalcone analogs **4** and **6** were obtained under base-promoted condensation from ketone **2** and the appropriately substituted aldehyde (Scheme 2A and B). Chalcone **11** was prepared from 1-bromo-2-methoxybenzene (**7**) by Friedel–Crafts acylation,[14] followed by ketone protection, lithiation, treatment with trimethylborate, and acidic work-up to afford **10** in 77% yield. Standard Claisen–Schmidt condensation gave the substituted chalcone **11** in 57% yield (Scheme 2C). Analog **14** was synthesized from 3,4-dimethoxyphenyl ethanone **12**. Selective demethylation of **12** by acid-promoted hydrolysis afforded substituted phenol **13** as a white solid in 43% yield.[15] Standard Claisen–Schmidt condensation gave the substituted chalcone **14** in 68% yield (Scheme 2D).

2.2. Biological data

The biological activities of the chalcone analogs of CA-4 are summarized in Table 1. The data showed that the known chalcone **6** was the most potent inhibitor, with activity comparable with that of CA-4 in the tubulin assays. However, compound **6** was 3–4 fold less active than CA-4 as an inhibitor of MCF-7 cell growth and much less active in disrupting the microtubules of A-10 cells. Boronic acid chalcone analog **4** was weakly active as an inhibitor of colchicine binding and of tubulin polymerization, but nevertheless had significant cytotoxic activity against the MCF-7 cancer cells (IC_{50}, 0.9 μM). SAR analysis shows that placement of the carbonyl adjacent to the A-ring results in more active analogs than placement adjacent to the C-ring. In all assays, **4** was more active than **11** and **6** was more active than **14**.

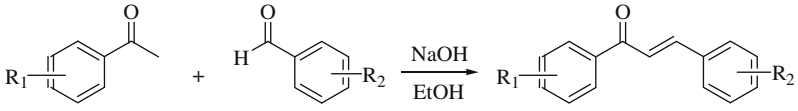
Figure 2 demonstrates the effects of compounds **4** and **6** on the microtubule networks of A-10 cells (Fig. 2). In the control (Fig. 2A), both the microtubule network and nuclei of the cells are clearly visible. Following the treatment with boronic acid **4**, extensive thinning of the network of microtubules occurred. In contrast, 20 μM of **6** caused complete disappearance of the microtubule network, with the disassembled tubulin, following its reaction with the antibodies used in the indirect immunofluorescence assay. This result is consistent with the relative activities of **4** and **6** as inhibitors of tubulin polymerization (Table 1).

To further survey the potential activity spectrum of boronic acid chalcone **4**, the compound was sent to the National Cancer Institute for evaluation in the human cancer cell screen.[16] Data was obtained for 54 human cancer cell lines (Table 2). Boronic acid chalcone **4** yielded GI_{50}'s between 10 and 200 nM in 16 of the 54 cell lines, and GI_{50}'s below 10 nM in three lines (colon cancer HCT-15, CNS cancer SNB-19, and ovarian cancer SK-OV-3.

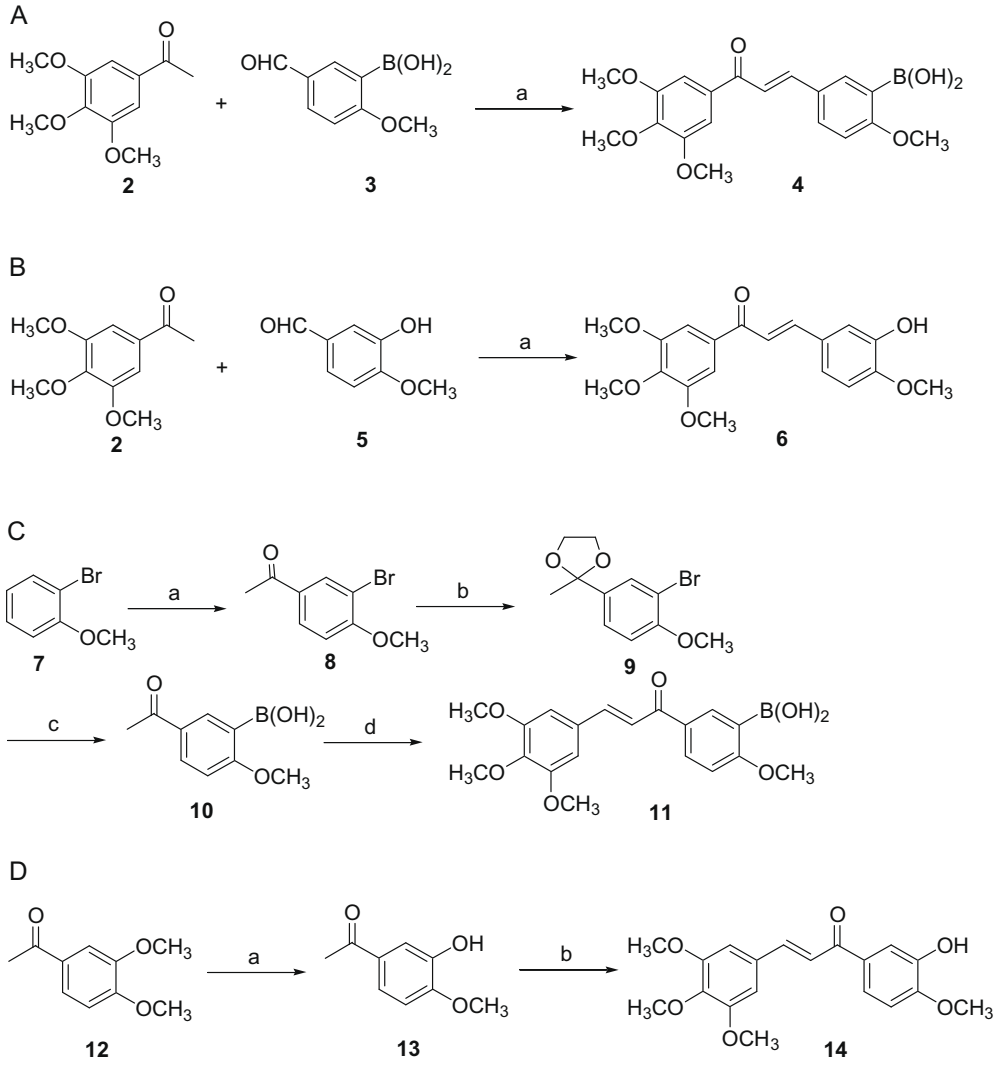
The data shown in Table 1 above demonstrates that boronic acid chalcone analog **4** is not a potent tubulin inhibitor. However, the highly cytotoxic activity toward the NCI panel of human cancer cells suggests it may have a different mechanism for cytotoxicity and further mechanistic studies are warranted.

We also tested the boronic acid compound **4** on the effect of MCF-7 cell cycle (Fig. 3). Based on this experiment, compound **4** did not cause significant G2/M phase arrest at the concentration as high as 5 μM, far above the IC_{50} (Table 1). The results further support the inhibitory effect on cancer cell proliferation was not realized through interfering tubulin assembly, which distinguished this compound from the regular anti-tubulin drugs.

We also tested the anti-angiogenesis property of compound **4** in HUVEC cells. Interestingly, this compound significantly inhibited the tube formation at the concentration of 1 μM (Fig. 4). Aortic ring assay also showed the compound **4** dramatically inhibited functional angiogenesis at a concentration of 5 μM (Fig. 5). The cancer vascular cells do not generate drug resistance as often as the cancer epithelium cells, and thus the discovery on drugs targeting angiogenesis is a very promising approach.



Scheme 1. General synthesis of chalcone analogs.



Scheme 2. Synthesis of chalcone analogs.

Table 1
Inhibition of tubulin polymerization, growth of MCF-7 human breast carcinoma cells, and the binding of [^{3}H]colchicine to tubulin, and EC_{50}'s for loss of microtubules in A-10 cells

Compound	Tubulin[a] IC_{50} (μM) ± SD	MCF-7[b] IC_{50} (μM) ± SD	[^{3}H]Colchicine binding inhibition[c] (% ± SD)		A-10 EC_{50}[d] (μM)
			5 μM	50 μM	
4	31 ± 3.4	0.9 ± 0.18	4.4 ± 8.6	56 ± 8.6	16.5
6	2.6 ± 0.2	0.11 ± 0.024	88 ± 6.3	101 ± 5.5	0.98
11	>40	>2.5	N/D	N/D	>15
14	>40	>2.5	N/D	N/D	18
CA-4	2.0 ± 0.1	0.032 ± 0.021	99 ± 2.0	N/D	<0.025

[a] Inhibition of tubulin polymerization. Tubulin was at 10 μM.
[b] Inhibition of growth of MCF-7 human breast carcinoma cells.
[c] Inhibition of colchicine binding. Compounds were tested at 5 and 50 μM. The tubulin concentration was 1 μM, and the [^{3}H]colchicine concentration was 5 μM. All experiments were performed in triplicate.
[d] The percentage of cellular microtubule loss was estimated visually over a range of concentrations. Dose response curves were generated and EC_{50} values calculated. The values represent the averages from two independent experiments.

3. Conclusion

We synthesized novel boronic acid analogs and the corresponding hydroxy chalcone analogs of CA-4. Boronic acid analog **4** was identified as a potent inhibitor of human cancer in cell proliferation and angiogenesis. Positional addition of boronic acid eliminated tubulin activity, but produced nanomolar cytotoxicity, warranting further studies on the mechanism of action and in vivo anti-tumor effects of this new boronic acid.

4. Experimental

4.1. Chemistry general methods

NMR spectra were recorded using a Varian-300 spectrometer for ^{1}H (300 MHz) and ^{13}C (75 MHz). Chemical shifts (δ) are given in ppm downfield from tetramethylsilane, as internal standard, and coupling constants (J-values) are in hertz (Hz). Mass spectra data were collected on a Finnigan MAT LC-Q mass spectrometer system using electrospray ionization (ESI). Elemental analyses were performed by Atlantic Microlabs. Melting points were obtained using an electrothermal digital melting point apparatus and are uncorrected. All purifications by flash chromatography were performed using Geduran silica gel 60, 35–75 μm (VWR). All solvents used were purified by pressure filtration through activated alumina. Reactions were run at ambient temperature and under a nitrogen atmosphere, unless otherwise noted, and were monitored by TLC using Merck 60 F_{254} silica gel aluminium sheets.

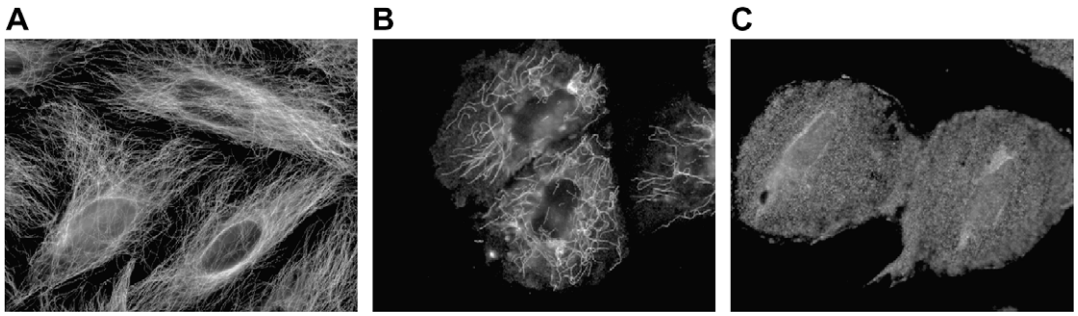


Figure 2. Effects of compounds **4** and **6** on interphase microtubules in A-10 cells. (A) Vehicle control; (B) 20 μM compound **4**; (C) 20 μM compound **6**.

4.2. General procedure a for the preparation of chalcones

To a stirred solution of an equivalent amount of the appropriately substituted acetophenone and benzaldehyde derivatives in ethanol was added aqueous NaOH solution (10% w/v, 4.0 equiv). The resulting solution was stirred at room temperature overnight, poured into water, and acidified to pH 4 with 1 N HCl. The resultant precipitate was filtered off, washed with water, and purified by recrystallization or flash chromatography.

4.2.1. 2-Methoxy-5-[(1*E*)-3-oxo-3-(3,4,5-trimethoxyphenyl)prop-1-en-1-yl]boronic acid (4)

General Procedure **A** was used for the reaction of 1-(3,4,5-trimethoxyphenyl)ethanone **2** (0.5 g, 2.38 mmol) and 5-formyl-2-methoxyboronic acid **3** (0.43 g, 2.38 mmol). The crude solid was recrystallized from EtOH–H_2O to afford product **4** (0.56 g, 63%) as yellow needles: mp 134–135 °C; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.18 (d, 1H, J = 2.1 Hz), 7.80 (d, 1H, J = 15.6 Hz), 7.70 (dd, 1H, J = 8.7, 2.1 Hz), 7.41 (d, 1H, J = 15.6 Hz). 7.24 (s, 2H), 6.96 (d, 1H, J = 8.7 Hz), 5.74 (s, 2H), 3.98 (s, 3H), 3.95 (s, 6H), 3.93 (s, 3H); ^{13}C NMR (75 MHz) δ 189.60, 166.32, 153.25, 144.60, 142.46, 137.16, 134.01, 133.87, 128.19, 120.24, 110.66, 106.24, 61.13, 56.58, 56.04; ESI m/z 373 $(M+H)^+$. Anal. Calcd for $C_{19}H_{21}BO_7 \cdot 0.9H_2O$: C, 58.71; H, 5.87. Found: C, 58.98, H, 5.97.

4.2.2. (2*E*)-3-(3-Hydroxy-4-methoxyphenyl)-1-(3,4,5-trimethoxyphenyl)prop-2-en-1-one (6)

General procedure A was used for the reaction of 1-(3,4,5-trimethoxyphenyl)ethanone **2** (0.5 g, 2.38 mmol) and 3-hydroxy-4-methoxybenzaldehyde **5** (0.36 g, 2.38 mmol). The crude solid was purified by flash chromatography using 1:3 EtOAc–hexanes to afford product **6** (0.37 g, 45%) as a bright yellow solid: mp 127–129 °C; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.75 (d, 1H, J = 15.6 Hz), 7.35 (d, 1H, J = 15.6 Hz), 7.31 (d, 1H, J = 1.8 Hz), 7.27 (s, 2H), 7.14 (dd, 1H, J = 8.4, 2.0 Hz), 6.88 (d, 1H, J = 9.0 Hz), 5.71 (s, 1H), 3.95 (s, 9H), 3.94 (s, 3H); ^{13}C NMR (75 MHz) δ 189.28, 153.24, 149.10, 146.07, 144.90, 142.39, 133.86, 128.59, 123.15, 119.87, 112.97, 110.74, 106.04, 61.11, 56.48, 56.15; ESI m/z 345 $(M+H)^+$. Anal. Calcd for $C_{19}H_{20}O_6$: C, 66.27; H, 5.85. Found: C, 66.47, H, 6.04.

4.2.3. 2-Methoxy-5-[(2*E*)-3-(3,4,5-trimethoxyphenyl)prop-2-enoyl]boronic acid (11)

To a stirred solution of 1-bromo-2-methoxybenzene **7** (4.68 g, 25.0 mmol) in CH_2Cl_2 (20 mL) was added $AlCl_3$ (8.03 g, 60.2 mmol). Acetic anhydride was added dropwise to the mixture. The resulting mixture was stirred at room temperature for 30 min, and heated to 40 °C for an additional 30 min. The solution was poured into concentrated HCl (20 mL) and ice (50 g), extracted with CH_2Cl_2 (3 × 30 mL), and the combined organic layers were washed with 1 N NaOH (30 mL), H_2O (30 mL), brine (30 mL), and dried over Na_2SO_4. The solution was concentrated, and the resulting crude solid was washed with petroleum ether to afford 1-(3-bromo-4-methoxyphenyl)ethanone **8** as a white solid (5.5 g, 96%): mp 86–88 °C (lit.[13] mp 86.5–87.5 °C). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.10 (d, 1H, J = 2.1 Hz), 7.85 (dd, 1H, J = 8.7, 2.1 Hz), 6.88 (d, 1H, J = 8.7 Hz), 3.91 (s, 3H), 2.50 (s, 3H); ^{13}C NMR (75 MHz) δ 195.68, 159.61, 133.82, 131.24, 129.61, 111.90, 111.16, 56.57, 26.41.

Ketone **8** (0.5 g, 2.18 mmol) was treated with ethylene glycol (0.73 mL, 13.1 mmol) and p-TsOH·H_2O (0.07 g, 0.37 mmol) in benzene (50 mL) under reflux. A Dean–Stark trap was used to remove the water generated during reaction. The resulting mixture was cooled to room temperature, washed with saturated $NaHCO_3$ (20 mL), H_2O (20 mL), brine (20 mL), and dried over Na_2SO_4. The solution was concentrated and purified by flash chromatography using 1:19 EtOAc–hexanes to afford 2-(3-bromo-4-methoxyphenyl)-2-methyl-1,3-dioxolane **9** as a yellow oil (0.57 g, 96%): ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.65 (d, 1H, J = 2.4 Hz), 7.37 (dd, 1H, J = 8.4, 2.1 Hz), 6.85 (d, 1H, J = 8.7 Hz), 4.02 (m, 2H), 3.88 (s, 3H), 3.76 (m, 2H), 1.62 (s, 3H); ^{13}C NMR (75 MHz) δ 155.23, 137.06, 130.24, 125.43, 111.37, 111.16, 107.87, 64.32, 56.06, 27.48.

Compound **9** (0.44 g, 1.61 mmol) was dissolved in dry THF (25 mL), cooled to −78 °C, and BuLi (0.97 mL, 2.43 mmol, 2.5 M in hexane) was added dropwise. The resulting mixture was stirred at −78 °C for an additional 1.5 h, followed by the dropwise addition of trimethyl borate (0.25 g, 2.43 mmol). The mixture was warmed to room temperature and stirred overnight. The reaction was quenched with 3 N HCl (10 mL) and stirred at room temperature for 30 min, followed by extraction with ether (3 × 30 mL). The combined ether layers were extracted with 1 N NaOH (2 × 10 mL), and the combined aqueous phase was re-acidified with 1 N HCl to pH 4. After re-acidification, the mixture was extracted with ether (2 × 30 mL). The ether layers were combined and dried over Na_2SO_4. The solution was concentrated and purified by flash chromatography using 1:1 EtOAc–hexanes to afford 5-acetyl-2-methoxyboronic acid **10** as a white solid (0.24 g, 77%): ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.46 (d, 1H, J = 2.4 Hz), 8.10 (dd, 1H, J = 8.7, 2.2 Hz), 6.97 (d, 1H, J = 8.7 Hz), 6.45 (broad, 2H), 3.98 (s, 3H), 2.59 (s, 3H); ^{13}C NMR (75 MHz) δ 197.54, 168.10, 138.32, 133.61, 130.76, 110.27, 110.11, 56.13, 26.66.

General procedure **A** was used for the reaction of 3,4,5-trimethoxybenzaldehyde (0.24 g, 1.23 mmol) and 5-acetyl-2-methoxyboronic acid **10** (0.24 g, 1.23 mmol). The crude solid was recrystallized from EtOAc–hexanes to afford product **11** (0.26 g, 57%) as a pale yellow solid: mp 148–150 °C; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.55 (d, 1H, J = 2.4 Hz), 8.20 (dd, 1H, J = 8.7, 2.4 Hz), 7.73 (d, 1H, J = 15.3 Hz), 7.46 (d, 1H, J = 15.6 Hz). 7.03 (d, 1H, J = 9.0 Hz), 6.86 (s, 2H), 6.23 (broad, 2H), 4.00 (s, 3H), 3.91 (s, 6H), 3.89 (s, 3H); ^{13}C NMR (75 MHz) δ 189.66, 168.20, 153.61, 144.77, 137.96, 134.29, 131.72, 130.72, 121.30, 110.50, 105,87, 61.19, 56.42, 56.18; ESI m/z 373 $(M+H)^+$. Anal. Calcd for $C_{19}H_{21}BO_7$: C, 61.32; H, 5.69. Found: C, 61.29, H, 5.95.

Table 2
NCI Cancer Cell line GI_{50} values

Cell line	GI_{50} (μM) Boronic acid chalcone 4
Leukemia	
CCRF-CEM	2.39
K-562	3.46
MOLT-4	3.60
RPMI-8226	1.12
SR	2.18
Non-small cell lung cancer	
A549/ATCC	0.45
EKVX	1.31
HOP-62	0.098
HOP-92	0.38
NCI-H226	4.14
NCI-H23	0.14
NCI-H322M	8.44
NCI-H460	0.028
NCI-H522	0.20
Colon cancer	
COLO 205	2.20
HCC-2998	0.41
HCT-116	0.37
HCT-15	<0.01
KM12	15.4
SW-620	0.037
CNS Cancer	
SF-268	0.80
SF-295	2.73
SF-539	0.035
SNB-19	<0.01
U251	0.025
Melanoma	
LOX IMVI	0.060
MALME-3M	5.49
M14	1.19
SK-MEL-2	3.33
SK-MEL-5	0.31
UACC-257	4.41
UACC-62	0.034
Ovarian cancer	
IGROV1	0.088
OVCAR-3	2.08
OVCAR-4	2.06
OVCAR-5	1.81
OVCAR-8	0.30
SK-OV-3	<0.01
Renal cancer	
786-0	0.39
A498	4.76
ACHN	0.52
CAKI-1	6.39
RXF 393	0.39
SN12C	0.46
TK-10	20.90
UO-31	0.32
Prostate cancer	
PC-3	0.25
DU-145	0.050
Breast cancer	
MCF7	0.034
NCI/ADR-RES	0.050
MDA-MB-231/ATCC	0.11
HS 578T	0.046
MDA-MB-435	1.95
BT-549	0.021

4.2.4. (2*E*)-1-(3-Hydroxy-4-methoxyphenyl)-3-(3,4,5-trimethoxyphenyl)prop-2-en-1-one (14)

1-(3,4-Dimethoxyphenyl)ethanone **12** (2.0 g, 11.1 mmol) was dissolved in concentrated sulfuric acid (10 mL) at room temperature. The resulting solution was heated to 65 °C and stirred overnight. The mixture was cooled to room temperature, poured into ice (65 g), and extracted with CH_2Cl_2 (4 × 25 mL). The combined CH_2Cl_2 phase was extracted into 1 N NaOH (2 × 20 mL). The aqueous phase was acidified with concentrated HCl to pH 3, extracted with CH_2Cl_2 (3 × 30 mL), and the combined CH_2Cl_2 extracts were dried over Na_2SO_4. The solution was concentrated and purified by flash column chromatography using 3:7 EtOAc–hexanes to afford **13** as a white solid (0.8 g, 43%): mp 86–88 °C (lit.[14] mp 92–93 °C); ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.55 (m, 2H), 6.89 (d, 1H, J = 9.0 Hz), 3.96 (s, 3H), 2.54 (s, 3H); ^{13}C NMR (75 MHz) δ 197.50, 151.08, 145.58, 130.80, 122.03, 114.57, 110.02, 56.09, 26.39. ESI m/z 167 $(M+H)^+$.

General procedure **A** was used for the reaction of 3,4,5-trimethoxybenzaldehyde (0.35 g, 1.81 mmol) and 1-(3-hydroxy-4-methoxyphenyl)ethanone **13** (0.30 g, 1.81 mmol). The crude solid was purified by flash column chromatography using 1:4 EtOAc–hexanes to afford product **14** (0.43 g, 68%) as a pale yellow solid: mp 130–132 °C; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.72 (d, 1H, J = 15.6 Hz), 7.65 (m, 2H), 7.40 (d, 1H, J = 15.6 Hz), 7.82 (d, 1H, J = 9.0 Hz), 6.86 (s, 2H), 5.73 (broad, 1H), 3.99 (s, 3H), 3.93 (s, 6H), 3.90 (s, 3H); ^{13}C NMR (75 MHz) δ 188.89, 153.56, 150.86, 145.69, 144.41, 140.33, 131.89, 130.65, 122.20, 121.14, 114.90, 110.22, 105.67, 61.11, 56.33, 56.20; ESI m/z 345 $(M+H)^+$. Anal. Calcd for $C_{19}H_{20}O_6$: C, 66.27; H, 5.85. Found: C, 66.43, H, 5.99.

5. Biological testing

5.1. Tubulin polymerization

Tubulin polymerization was followed turbidimetrically at 350 nm in Beckman model DU-7400 and DU-7500 spectrophotometers equipped with electronic temperature controllers, as previously described in detail.[17] The tubulin concentration was 10 μM (1.0 mg/mL).

5.2. MCF-7 Cell proliferation assay

IC_{50} values for inhibition of cell growth were obtained by measuring the amount of total cell protein with the sulforhodamine B (SRB) assay.[18] The MCF-7 cells were grown for 24 h in the presence and absence of the drug. IC_{50} values were determined after an additional 48 h.

5.3. [^{3}H]Colchicine binding assay

The binding of [^{3}H]colchicine to tubulin was measured by the DEAE-cellulose filter method, as previously described in detail.[19] The tubulin concentration was 1.0 μM (0.1 mg/mL), and the [^{3}H]colchicine concentration was 5.0 μM.

5.4. NCI Human cancer cell proliferation assay[20]

Compound **4** was tested against human tumor cell lines at five concentrations in a 10-fold serial dilution. After a 48 h incubation time period, an SRB protein assay was used to determine inhibition of cell growth. GI_{50} values were calculated from log dose response curves.

5.5. Indirect immunofluorescence

Drug effects on the microtubule network of A-10 cells were evaluated by indirect immunofluorescent techniques as previously described.[21] Cells were plated onto glass cover slips and treated with compounds at varying concentrations for 18 h. The cells were fixed and microtubules visualized using a β-tubulin antibody, and the nuclei was stained with 4,6-diamidino-2-phenylindole. Cells were examined with a Nikon ES800 fluorescence microscope,

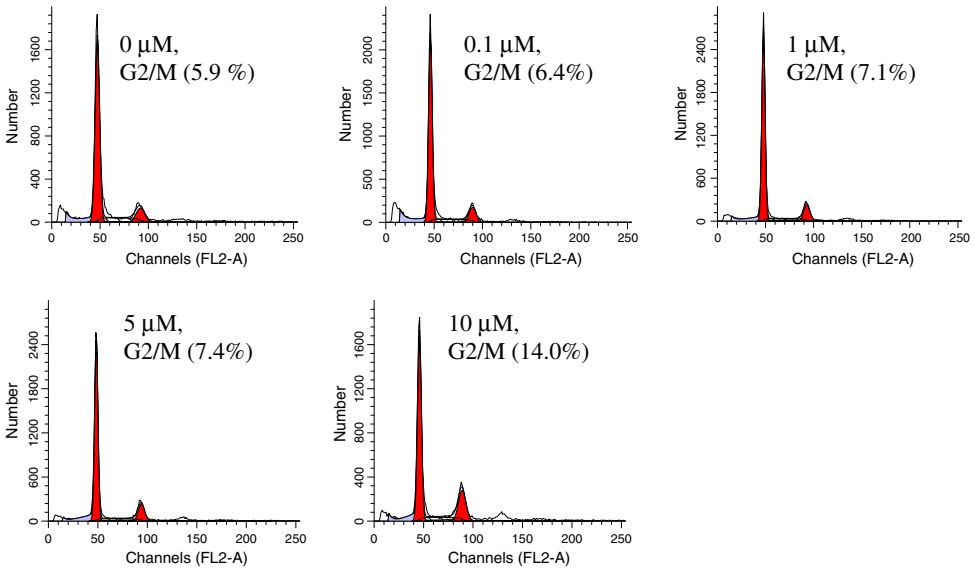


Figure 3. The effect of compound **4** on the cell cycle of MCF-7 cells after 48 h treatment.

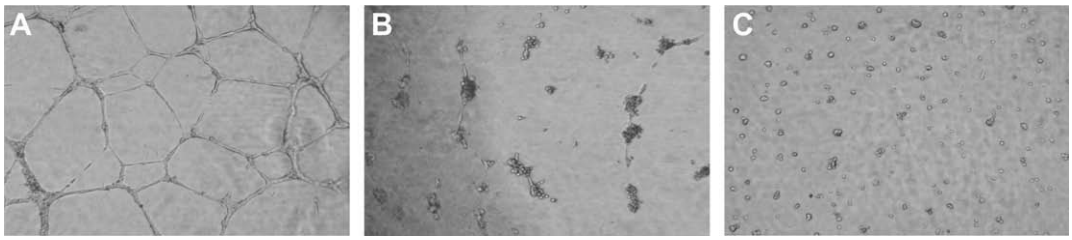


Figure 4. Anti-angiogenesis affects of compound **4** in MCF-7 cell line. (A) Control; (B) 1 μM of compound **4**; (C) 10 μM of compound **4**.

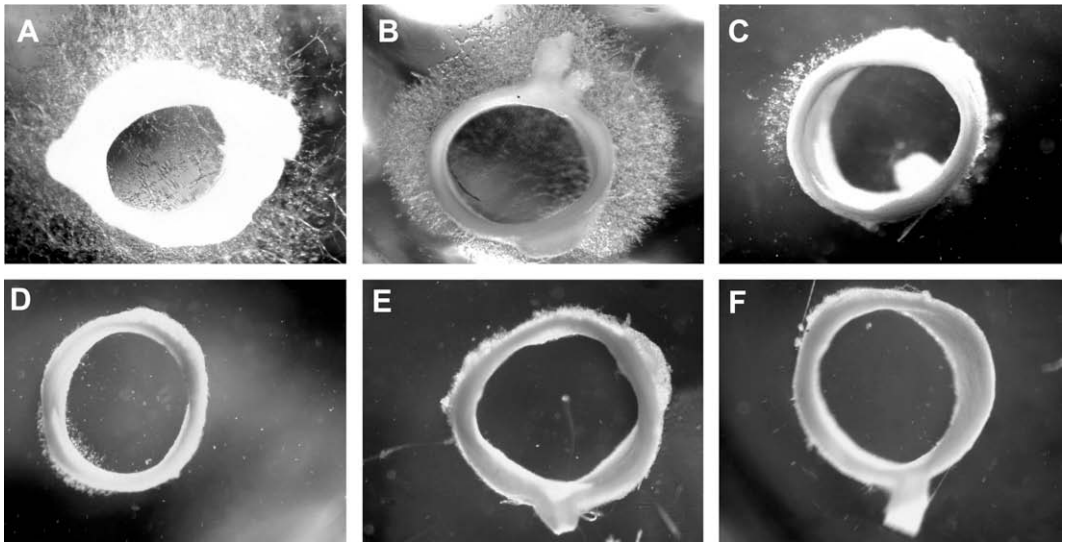


Figure 5. The aortic ring assay on the inhibitory effects of compound **4**. (A) Control; (B) 1 μM of compound **4**; (C) 5 μM of compound **4**; (D) 10 μM of compound **4**; (E) 5 0 μM of compound **4**; (F) 100 μM of compound **4**.

images were captured with a Photometrics Cool Snap FX3 camera, and the data was compiled using Metamorph® software.

5.6. Cell cycle assay

Breast cancer cell line MCF-7 (2×10^5 cells/ml) were seeded into 6-well plates in 2 mL DMEM culture media (GIBCO BRL Company; Grand Island, USA) with 5% fetal calf serum for each well. Incubation at 37 °C overnight, allow the cells to attach to the bottom of the wells. Different concentration of compound **4** was added to each well. Cell cycle was measured by flow cytometry after 48 h treatment.

5.7. Angiogenesis assay in vitro

HUVEC (human umbilical vein endothelial cells, 1×10^5 cells) were seeded on the surface of Matrigel in 96 well plates in EBM-

2 medium. Several dose ranges of compound **4** were added to each well. The tube formation was observed after 16 h treatment.

5.8. Aortic ring assay

Normal 1–2 month old rats were sacrificed and the thoracic aortas were excised. The aorta was immediately transferred into a 100 mm petri dish in ice cooled PBS buffer. The fibroadipose tissue was removed under dissection microscope. The arteries were cut into 1–1.5 mm long cross sections and washed five times with PBS. To the cover slip in 6-well plates was added 80 μL of Matrigel (Chemicon International), and the aortic sections submerged into the gel and incubate for 15 min (37 °C, 5% CO_2). EBM2 (1.5 mL) with different concentration of compound **4** was added to each well. The formation of sprouts on the rings were examined on day 6, and compared with the control group.

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References and notes

1. Zi, X.; Simoneau, A. R. *Cancer. Res.* **2005**, *65*(8), 3479.
2. Won, S. J.; Liu, C. T.; Tsao, L. T.; Weng, J. R.; Ko, H. H.; Wang, J. P.; Lin, C. N. *Eur. J. Med. Chem.* **2005**, *40*, 103.
3. Nielsen, S. F.; Boesen, T.; Larsen, M.; Schønning, K.; Kromann, H. *Bioorg. Med. Chem.* **2004**, *12*, 3047.
4. Ducki, S.; Forrest, R.; Hadfield, J. A.; Kendall, A.; Lawrence, N. J.; McGown, A. T.; Rennish, D. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 1051.
5. Pettit, G. R.; Cragg, G. M.; Herald, D. L.; Schmidt, J. M.; Lohavanijaya, P. *Can. J. Chem.* **1982**, *60*, 1374.
6. Lin, C. M.; Ho, H. H.; Pettit, G. R.; Hamel, E. *Biochemistry* **1989**, *28*, 6984.
7. Ikeda, S.; Kimure, U.; Ashizawa, T.; Gomi, K.; Saito, H. Jpn, Kokai Tokkyo Koho JP 08, 188,546; *Chem. Abs.* **1996**, *125*, 221359a.
8. Edwards, M. L.; Stemerick, D. M.; Sunkara, P. S. *J. Med. Chem.* **1990**, *33*, 1948.
9. Dicesare, N.; Lakowicz, J. R. *Tetrahedron Lett.* **2002**, *43*, 2615.
10. Autom, R. J.; Sukumaran, K.; Kuttan, G.; Rao, M. N. A.; Subbaraju, V.; Kuttan, R. *Cancer Lett.* **1995**, *97*, 33.
11. Kong, Y.; Grembecka, J.; Edler, M. C.; Hamel, E.; Mooberry, S. L.; Sabat, M.; Rieger, J.; Brown, M. L. *Chem. Biol.* **2005**, *12*, 1007.
12. Kumar, S. K.; Hager, E.; Pettit, C.; Gurulingappa, H.; Davidson, N. E.; Khan, S. R. *J. Med. Chem.* **2003**, *46*, 2813.
13. Dhar, D. N.; Lal, J. B. *J. Org. Chem.* **1958**, *23*, 1159.
14. Laali, K. K.; Koser, G. K.; Subramanyam, S.; Forsyth, D. A. *J. Org. Chem.* **1993**, *58*, 1385.
15. Brossi, A.; Gurien, H.; Rachlin, A. I.; Teitel, S. *J. Org. Chem.* **1967**, *32*, 1269.
16. Monks, A.; Scudiero, A.; Skehan, P.; Shoemaker, R.; Paull, K.; Vistica, D.; Hose, C.; Langley, J.; Cronise, P.; Vaigro-Wolff, A.; Gray-Goodrich, M.; Campbell, H.; Mayo, J.; Boyd, M. *J. Natl. Cancer Inst.* **1991**, *83*, 757.
17. Hamel, E. *Cell Biochem. Biophys.* **2003**, *38*, 1.
18. Skehan, P.; Storeng, R.; Scudiero, D.; Monks, A.; McMahon, J.; Vistica, D.; Warren, J. T.; Bokesch, H.; Kenney, S.; Boyd, M. R. *J. Natl. Cancer Inst.* **1990**, *82*, 1107.
19. Verdier-Pinard, P.; Lai, J. Y.; Yoo, H. D.; Yu, J. R.; Marquez, B.; Nagle, D. G.; Nambu, M.; White, J. D.; Falck, J. R.; Gerwick, W. H.; Day, B. W.; Hamel, E. *Mol. Pharmacol.* **1998**, *53*, 62.
20. For additional details concerning this screen and the NCI's drug discovery and development program, visit http://dtp.nci.nih.gov/branches/btb/ivclsp.html.
21. Tinley, T. L.; Randall-Hlubek, D. A.; Leal, R. M.; Jackson, E. M.; Cessac, J. W.; Quada, J. C., Jr.; Hemscheidt, T. K.; Mooberry, S. L. *Cancer Res.* **2003**, *63*, 3211.