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Synthesis and Biological Evaluation of 1,1-Dichloro-2,3-diarylcyclopropanes as Antitubulin and Anti-Breast Cancer Agents

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Abstract—*Z*-1,1-Dichloro-2,3-diphenylcyclopropane (**1**) is an effective anti-breast cancer agent in rodents and in cell culture. We recently determined that **1** inhibits tubulin assembly in vitro and causes microtubule loss in breast cancer cells, leading to accumulation in the G_2/M portion of the cell cycle. Aryl ring-halogenated, methoxylated and benzyloxylated derivatives of **1**, as well as its *E*-isomer and the dichlorocyclopropyl derivative of diethylstilbestrol (DES), were synthesized and tested for their ability to inhibit the assembly of tubulin into microtubules. Including **1**, 17 cyclopropyl compounds were tested. One (*Z*-1,1-dichloro-2-(4-methoxyphenyl)-3-phenylcyclopropane (**12**)) was found to be more active than **1**. In addition, *E*-1,1-dichlorocyclopropylDES (**17**) was more potent than DES. The *E*-isomer of **1** (**16**) was inactive. The cytostatic activities of the compounds against MCF-7 and MDA-MB231 human breast cancer cells, and their abilities to perturb microtubules in MCF-7 cells were also evaluated. *Z*-Dichloro-2-(4-fluorophenyl)-3-phenylcyclopropane (**5**), *Z*-1,1-dichloro-2-(4-fluorophenyl)-3-(4-methoxyphenyl)cyclopropane (**11**), and *Z*-1,1-dichloro-2-(4-methoxyphenyl)-3-phenylcyclopropane (**12**) were more potent than **1** against the breast cancer cells. © 1997 Elsevier Science Ltd.

Introduction

Z-1,1-Dichloro-2,3-diphenylcyclopropane (**1**) was first tested for biological activity as a part of a group of rigid, functionally minimized analogues of known olefinic estrogens, synthesized in an effort to reduce estrogenic activity.[1] In immature female mice, **1** displays no estrogenicity (i.e., is not uterotrophic) and possesses anti-uterotrophic (apparent anti-estrogenic) effects.[2,3] Compound **1** was also shown to have activity comparable to that of the clinically used anti-estrogen tamoxifen against the hormone-dependent 7,12-dimethylbenz[*a*]anthracene induced rat mammary tumor model and to be more effective than tamoxifen in reducing the incidence of new tumors in long-term dosing studies.[4] It is also active against the hormone-independent DMBA-4 transplantable metastatic rat mammary tumor model.[5]

It has been noted that **1** has almost no affinity for the classical high affinity–low capacity rat uterine estrogen type I receptor (ER), with a relative binding affinity less than 0.01% of that exhibited by estradiol.[2,3] Recently, we have undertaken a re-examination of the actions and mechanisms of **1**. We have shown that **1** is cytostatic against both ER-positive and ER-negative breast cancer cells in culture, suggesting its mode of action is independent of the estrogen receptor.[6] We also have noted that **1** inhibits the polymerization of isolated tubulin and perturbs the microtubule network of breast cancer cells in culture, leading to their accumulation in G_2/M, a strong indication of antimitotic activity.[7] To date, design, synthesis, and biological studies of **1** and its derivatives have concentrated on their potential anti-estrogenic activities.[1–3,8–12] Antitubulin effects represent a previously unstudied mechanism of action for dichlorodiphenylcyclopropanes, and our recent observations suggest that new structure–activity relationships might be developed for **1** and its analogues.

In the present study, we incorporated a variety of aryl ring moieties (methoxy and benzyloxy groups, halogens) into analogues of **1** in an effort to enhance antitubulin and anti-breast cancer activity. The compounds were synthesized and tested for their ability to inhibit tubulin polymerization and the binding of [^{3}H]colchicine to

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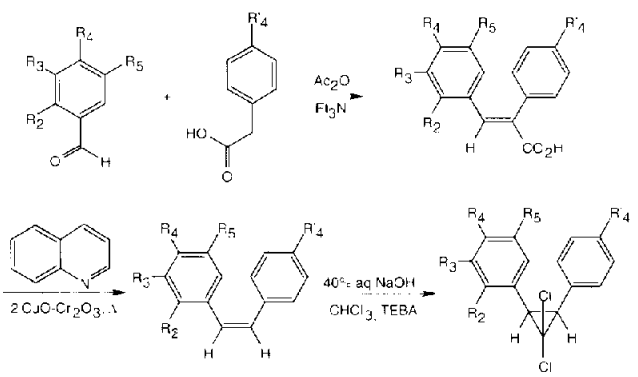
tubulin, cause cytostasis of MCF-7 and MDA-MB231 human breast cancer cell lines, and perturb microtubules in the MCF-7 cells.

Results

Chemistry

Ten new *Z*-1,1-dichloro-2,3-diphenylcyclopropanes were synthesized beginning with a mixed Perkin condensation of substituted phenylacetic acids with appropriately substituted benzaldehydes in triethylamine/acetic anhydride (Scheme 1).[11,12] The resulting *E*-cinnamic acids were decarboxylated with copper chromite in quinoline at 180–190 °C. The products of decarboxylation were largely the corresponding *Z*-stilbenes, which were separated from their *E*-isomers by crystallization or silica gel column chromatography. The *Z*-1,1-dichloro-2,3-diphenylcyclopropanes were obtained by benzyltriethylammonium chloride-catalyzed phase transfer dichlorocyclopropanation of the *Z*-stilbenes. The target compounds were purified either by crystallization or by column chromatography over basic alumina or flash SiO_2. Physical and spectral data are shown in Table 1. Compound **1**, its *E*-isomer (**16**), *Z*-1,1-dichloro-2-(4-methoxyphenyl)-3-phenylcyclopropane (**12**), *Z*-1,1-dichloro-2-(4-benzyloxyphenyl)-3-phenylcyclopropane (**13**), *Z*-1,1-dichloro-2,3-bis(4-methoxyphenyl)cyclopropane (**14**), and *Z*-1,1-dichloro-2,3-bis(4-benzyloxyphenyl)cyclopropane (**15**) were prepared previously or as described.[1,11,12] *E*-1,1-dichloro-2,3-diethyl-2,3-bis(4-hydroxyphenyl)cyclopropane (**17**) was prepared by dichlorocarbene addition to *E*-1,2-bis(4-methoxyphenyl)-3-hexene, followed by didemethylation with $AlCl_3$/*n*-butanethiol. All compounds



2. $R_2 = OCH_3$, $R_3 = H$, $R_4 = OCH_3$, $R_5 = H$, $R'_4 = H$
3. $R_2 = OCH_3$, $R_3 = H$, $R_4 = H$, $R_5 = OCH_3$, $R'_4 = H$
4. $R_2 = H$, $R_3 = OCH_3$, $R_4 = OCH_3$, $R_5 = OCH_3$, $R'_4 = H$
5. $R_2 = H$, $R_3 = H$, $R_4 = F$, $R_5 = H$, $R'_4 = H$
6. $R_2 = F$, $R_3 = H$, $R_4 = F$, $R_5 = H$, $R'_4 = H$
7. $R_2 = F$, $R_3 = H$, $R_4 = H$, $R_5 = F$, $R'_4 = H$
8. $R_2 = H$, $R_3 = H$, $R_4 = Cl$, $R_5 = H$, $R'_4 = H$
9. $R_2 = Cl$, $R_3 = H$, $R_4 = Cl$, $R_5 = H$, $R'_4 = H$
10. $R_2 = H$, $R_3 = H$, $R_4 = Br$, $R_5 = H$, $R'_4 = H$
11. $R_2 = H$, $R_3 = H$, $R_4 = F$, $R_5 = H$, $R'_4 = OCH_3$

Scheme 1.

had proton NMR and mass spectra consistent with the proposed structures or as previously reported, and all new compounds yielded acceptable combustion elemental analyses.

One curious observation was made from the proton NMR spectra of some of the new cyclopropanes. In our previous experience with *Z*-dichlorodiphenylcyclopropanes, the signal for the benzylic cyclopropyl hydrogens has invariably appeared as a singlet, regardless of spectrometer field strength, near 3 ppm. This signal for several of the new compounds (**2**, **3**, **4**, **7**, and **8**) appeared as an AB quartet, with coupling constants ranging from 11 to 13 Hz. The non-equivalence of the cyclopropyl protons in only these five compounds cannot be easily explained solely on asymmetry due to substitution pattern nor on bulk or electronic effects at a given position. It is perhaps more curious that the signals for these chemically non-equivalent protons are magnetically equivalent in all of the remaining derivatives.

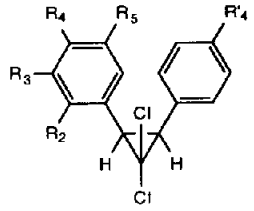
Biological results and discussion

Table 2 summarizes the data we obtained with compounds **1–17** in five biological assays. The agents were evaluated for effects on the growth of two breast cancer cell lines, the estrogen-dependent MCF-7 line and the estrogen-independent MDA-MB231 line. The MCF-7 cells were also evaluated by indirect immunofluorescence for drug effects on their microtubule cytoskeleton. All agents were evaluated for effects on the polymerization of purified tubulin in comparative studies with the weakly active DES (**18**) and with the potent *cis*-stilbene antimitotic agent combretastatin A-4 (**19**). The compounds that inhibited tubulin polymerization were also examined for inhibitory effects on the binding of [^{3}H]colchicine to tubulin.

There were no striking differences between the two cell lines in the inhibitory effects observed with the different agents. The most substantial difference was the ca. fivefold greater sensitivity of MCF-7 cells as compared with MDA-MB231 cells with compounds **12** and **17**. Four agents were more active than compound **1** with the MCF-7 cells. These were compounds **5**, **11**, **12**, and **17**, and these agents except **17** were also more active against the MDA-MB231 cells. Several additional agents had activities comparable to **1** (up to half as active) in both cell lines (compounds **4**, **6**, **7**, **8**, and **10**).

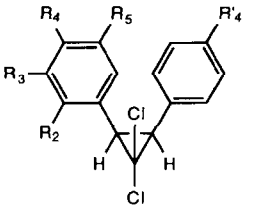
Perturbation of cellular microtubule arrays was observed when the MCF-7 cells were exposed to 100 μM **1**, **17**, and DES (**18**). More potent perturbation was caused by compounds **5**, **11**, and **12**. The effects of **12** on cellular microtubule networks became apparent at a concentration of 50 μM, while effects of **5** and **11** were evident at 10 μM. No other compound exhibited microtubule perturbing effects on MCF-7 cells at 100 μM under the conditions of the assay.

Table 1. Physical data for new *Z*-1,1-dichloro-2,3-diphenylcyclopropanes



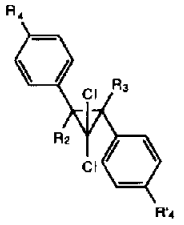
Compd	R_2	R_3	R_4	R_5	R'_4	^{1}H NMR (δ, $CDCl_3$, 200 MHz)	mp (°C)	Elemental analysis (C, H, Cl)	Yield (overall, %)
2	OCH_3	H	OCH_3	H	H	7.3–6.3 (m, 8H, ArH), 3.79 (s, 3H, OCH_3), 3.60 (s, 3H, OCH_3), 3.29 (q, 2H, benzylic H, J = 13 Hz)	60–61	$C_{17}H_{16}Cl_2O_2$ (C,H,Cl)	45
3	OCH_3	H	H	OCH_3	H	7.3–7.0, (m, 5H, ArH), 6.70 (s, 2H ArH), 6.50 (1H, ArH), 3.60 (s, 3H, OCH_3), 3.55 (s, 3H, OCH_3), 3.32 (q, 2H, benzylic H, J=13 Hz)	79–81	$C_{17}H_{16}Cl_2O_2$ (C,H,Cl)	34
4	H	OCH_3	OCH_3	OCH_3	H	7.5–7.1 (m, 5H, ArH), 6.20 (s, 2H, ArH), 3.85 (s, 3H, OCH_3), 3.62 (s, 6H, OCH_3), 3.26, (2H, q, benzylic H, J=12 Hz)	oil	$C_{18}H_{18}Cl_2O_3$ (C,H,Cl)	9
5	H	H	F	H	H	7.4 6.8 (m, 9H, ArH), 3.25 (s, 2H, benzylic H)	oil	$C_{15}H_{11}Cl_2F$ (C,H,Cl)	5
6	F	H	F	H	H	7.6–6.7 (m, 8H, ArH), 3.20 (s, 2H, benzylic H)	oil	$C_{15}H_{10}Cl_2F_2$ (C,H,Cl)	5
7	F	H	H	F	H	7.6–6.8 (m, 8H, ArH), 3.20 (q, 2H, benzylic H, J=11 Hz)	oil	$C_{15}H_{10}Cl_2F_2$ (C,H,Cl)	5
8	H	H	Cl	H	H	7.6–6.8 (m, 9H, ArH), 3.30 (q, 2H, benzylic H, J=12 Hz)	oil	$C_{15}H_{11}Cl_3$ (C,H,Cl)	20
9	Cl	H	Cl	H	H	7.6–6.6 (m, 8H, ArH), 3.20 (s, 2H, benzylic H)	oil	$C_{15}H_{10}Cl_4$ (C,H,Cl)	5
10	H	H	Br	H	H	7.5–7.0 (m, 9H, ArH), 3.20 (s, 2H, benzylic H)	oil	$C_{15}H_{11}BrCl_2$ (C,H,Cl)	5
11	H	H	F	H	OCH_3	7.1–6.1 (m, 8H, ArH), 3.77 (s, 3H, OCH_3), 3.24 (s, 2H, benzylic H)	oil	$C_{16}H_{13}Cl_2FO$ (C,H,Cl)	24

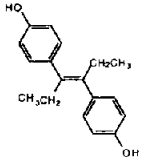
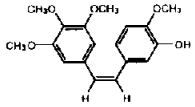
Table 2. Inhibition of tubulin polymerization and binding of [^{3}H]colchicine using purified protein, perturbation of microtubules in MCF-7 cells, and growth inhibitory activity against MCF-7 and MDA-MB231 cells



Compd	R_2	R_3	R_4	R_5	R'_4	Tubulin polym. inhibition, IC_{50} (μM) ±SD	Colchicine binding, % inhibition ±SD	Cellular microtubule perturbation (μM)	IC_{50} (μM) MCF-7 (24 h) ±SD	IC_{50} (μM) MDA-MB231 (24 h) ± SD
1	H	H	H	H	H	10±2[a]	17±4	100	14±4	20±2
2	OCH_3	H	OCH_3	H	H	>40	–	–	40±6	70±9
3	OCH_3	H	H	OCH_3	H	>40	–	–	38±4	>100
4	H	OCH_3	OCH_3	OCH_3	H	>40	–	–	25±3	32±2
5	H	H	F	H	H	[b]	[b]	–	2±0.7	7±1
6	F	H	F	H	H	>40	–	10	21±2	32±3
7	F	H	H	F	H	>40	–	–	19±3	25±5
8	H	H	Cl	H	H	[b]	[b]	–	15±2	38±4
9	Cl	H	Cl	H	H	>40	–	–	60±6	>100
10	H	H	Br	H	H	>40	–	–	27±3	19±2
11	H	H	F	H	OCH_3	>40	–	10	0.9±0.2	3±1
12	H	H	OCH_3	H	H	8.2±0.7	48±2	50	0.8±0.3	4±1
13	H	H	OCH_3	H	OCH_3	>40	–	–	35±6	>100
14	H	H	OCH_2Ph	H	H	>40	–	–	>100	>100
15	H	H	OCH_2Ph	H	OCH_2Ph	>40	–	–	>100	>100

Table 2. Continued.



Compd	R_2	R_3	R_4	R'_4	Tubulin polym. inhibition, IC_{50} (μM)	Colchicine binding, % inhibition	Cellular microtubule perturbation (μM)	IC_{50} (μM) MCF-7	IC_{50} (μM) MDA-MB231
16	H	H	H	H	>40	–	–	>100	>100
17	CH_2CH_3	CH_2CH_3	OH	OH	7.0±0.9	46±0.3	100	7±2	36±7
Diethylstilbestrol (**18**)					12±1	17±3	100	47±4	50±8
Combretastatin A-4 (**19**)					1.1±0.4	97±2	nd[c]	nd	nd

[a]The IC_{50} value reported for **1** in this study differs slightly from that which we report in reference 7 (6.7±0.9 μM). The reason for this difference is the time that elapsed between our original experimental discovery reported in reference 7 of compound **1**'s antitubulin effects and the experiments given in this study. The tubulin preparation was the same in each case, but several months elapsed between the biochemical evaluations. Tubulin preparations can give different absolute results after extended storage, but relative potencies generally remain constant. We believe such an effect occurred in this instance. The tubulin polymerization IC_{50} values for all compounds in this study were determined within one week of each other, and thus a fair comparison of their relative potencies may be made. [b]An initial survey of compounds **5** and **8** shortly after their synthesis yielded preliminary IC_{50} values of 4 μM and inhibition of [^{3}H]colchicine binding. When definitive experiments were performed several months later, however, no activity was detected. Since many of the dichlorocyclopropanes are unstable in polar media,[6] definitive evaluation of **5** and **8** will require their resynthesis. [c]Not determined.

Significant inhibitory effects on the polymerization of purified tubulin were observed with compounds **1**, **12**, **17**, and perhaps **5** and **8** (see note in Table 2). With the specific tubulin preparation used, compound **1** (IC_{50}, 10 μM) was slightly more inhibitory than DES (**18**) (IC_{50}, 12 μM). The *para*-methoxy analogue **12** (IC_{50}, 8.2 μM) was somewhat more inhibitory than **1**. Interestingly, the most potent of the cyclopropyl analogues as a tubulin polymerization inhibitor with this particular tubulin preparation was the dichlorocyclopropyl derivative of DES, compound **17**. The dichlorocyclopropanes were all, however, much less potent than the *cis*-stilbene combretastatin A-4 (**19**) (IC_{50}, 1.1 μM).

In terms of antiproliferative activity, the phenyl rings of compound **1** tolerate a variety of substituents. Activity was enhanced with a single *para*-fluoro group (**5**), a single *para*-methoxy group (**12**), and asymmetric *para*-fluoro and *para*-methoxy groups (**11**). In addition, more potent antiproliferative activity was achieved by introduction of the dichlorocyclopropyl group into DES to give compound **17**. DES was stimulatory to MCF-7 cells up to 1 μM under the assay conditions used, and antiproliferative at higher concentrations. The growth IC_{50} of DES in both MCF-7 and MDA-MB231 cells was ca. 50 μM. Its cyclopropyl congener, on the other hand, was not stimulatory at any concentration, and yielded much lower IC_{50} values in the two cell lines (7 and 36 μM, respectively). The *E*-analogue of **1** (compound **16**) was inactive as an antiproliferative agent. Antiproliferative activity was little affected by trimethoxy (**4**) substitution, two fluoro groups (**6** and **7**), a *para*-chloro group (**8**), or a *para*-bromo group (**10**) on one phenyl ring. Significant loss of activity occurred with two methoxy groups (**2** and **3**) or two chloro groups (**9**) on one phenyl ring, symmetric *para*-methoxy groups (**13**), or bulky, hydrophobic *para*-benzyloxy substituents on one (**14**) or both (**15**) phenyl rings.

Despite the significant number of agents in this series with antiproliferative activity, only compounds **5**, **11**, **12**, and **17** mimicked compound **1** in its perturbation of cellular microtubules. DES was equipotent with **17** and **1** in this effect. The *para*-fluoro analogue **5** and the *para*-fluoro *para*-methoxy analogue **11** were the most potent microtubule perturbing agents, while the *para*-methoxy analogue **12** gave results intermediate to those of **1** and **5**/**11**.

The inhibitory activities of these agents with purified tubulin were not in complete agreement with the cellular assays. Although **12** and **17** were more potent than **1** and DES in the tubulin assays, the potent antiproliferative and microtubule-perturbing agent **11** had no detectable activity with purified tubulin. Similarly, we could find no evidence for an interaction with purified tubulin of compounds **4**, **5**, **6**, **7**, and **10**. With the exception of **5**, these negative findings were supported by the observations made in immunofluorescence analysis of MCF-7 cells (i.e., no perturbation of cellular microtubules).

Thus, it appears likely that this class of structurally simple agents probably inhibits cell proliferation by more than one mechanism of action. Classical antiestrogenicity (i.e., competition for the type I estrogen receptor) seems unlikely as the compounds displayed antiproliferative actions against the estrogen-receptor negative MDA-MB231 cells. An interaction with tubulin may play an important role with such agents as **1**, **12**, and **17**, and probably **5** and **11** (perhaps by metabolic activation), but additional modes of action likely account for the antiproliferative properties of the other active agents.

Experimental

Chemistry

General. ^1H NMR spectra were measured at 200.057 MHz in $CDCl_3$ on a Varian XL-200 spectrometer at ambient temperature. Spectra are reported in parts per million downfield of internal $(CH_3)_4Si$. Gas chromatography–mass spectra were determined on a Hewlett–Packard 5791 mass selective detector interfaced to a Hewlett–Packard 5890 Series II GC equipped with a 12 m HP-1 (methylsilicone, 0.2 mm i.d., 0.33 μm film thickness) fused silica capillary column (Hewlett–Packard). The carrier gas was high purity He (8 psi column pressure), and the temperature zones used were as follows: injector 150 °C; GC oven 0–2 min isothermal at 100 °C, 20 °C/min linear ramp to 310 °C, 1 min isothermal at 310 °C; detector interface 220 °C. The spectra were determined in electron ionization (70 V, GC–EI–MS) or positive chemical ionization (CH_4, 200 V, GC–PCI–MS) modes. Pneumatically-assisted electrospray mass spectra (API-MS) were determined with a Perkin–Elmer/Sciex API *I* mass spectrometer with an atmospheric pressure ionization source and an articulated IonSpray interface linked with glass capillary tubing to a Hewlett–Packard 1090 Series II LC equipped with a Hewlett–Packard 1040 UV-vis diode array detector (LC–MS). Analytes were dissolved in CH_3OH and injected into a flowing stream (40 μL/min) of 2:1:1 H_2O:CH_3OH:CH_3CN containing 1 mM $NH_4O_2CCH_3$ and 0.5% CH_3CO_2H through the LC without a column and introduced into the ion source of the mass spectrometer directly. High purity air was used as the nebulizing gas and was maintained at an operating pressure of 40 psi. High purity N_2 was used as the curtain gas flowing at 0.6 L/min. The IonSpray interface was maintained at 5 kV and the orifice voltage at 70–90 V. The quadrupole was scanned from *m/z* 150 to 450 in 9–11 s per scan at a resolution of *m/z* 0.1. All target compounds were judged >99% pure by capillary GC and gave M^+, $[M+H]^+$/$[M+NH_4]^+$ ion clusters indicative of their calculated masses and isotopic ratios.

Synthetic starting materials, reagents and solvents were purchased from Aldrich Chemical Co. (Milwaukee, WI) or Fisher Scientific (Pittsburgh, PA) and were of the highest available purity. Column chromatography was performed over silica gel (Merck flash grade 9385, 230–

400 mesh, Aldrich Chemical Co.) or basic alumina (Brockman activity I, 60–325 mesh, Fisher Scientific) using petroleum ether (bp 30–60 °C) neat or mixed with CH_2Cl_2 or EtOAc as the eluent. Evaporations were carried out in vacuo on a rotary evaporator or under a stream of dry N_2. Melting points were taken on a Fisher–Johns stage apparatus and are uncorrected. Reaction progress and product purity were monitored by analytical TLC on strips of Eastman–Kodak plastic-backed SiO_2 60 F_{254}. Combustion elemental analyses were done by Atlantic Microlab, Inc., Norcross, GA. Analytical results were within ±0.4% of theoretical values, and are denoted in Table 1 by the formula, followed by the elements analyzed. Melting points and highest overall yields of the new compounds are also listed in Table 1.

Compounds **1** and **12–17** were prepared in previous investigations or by modifications of those methods as described in the text.[1,11,12] A representative example of the synthetic method follows. No attempts were made to optimize yields.

Synthetic method

***Z*-1,1-Dichloro-2-(3,4,5-trimethoxyphenyl)-3-phenylcyclopropane (4).** A round-bottom flask equipped with a magnetic stir bar and reflux condenser with a $CaCl_2$ drying tube was charged with a suspension of 3,4,5-trimethoxybenzaldehyde (10.98 g, 56 mmol) and phenylacetic acid (7.6 g, 56 mmol) in triethylamine (10 mL) and acetic anhydride (20 mL). The mixture was stirred and heated to reflux overnight. The reaction mixture was cooled to room temperature and poured into a separatory funnel. Et_2O (100 mL) was added and the solution was extracted with 10% aq NaOH (3 × 200 mL). The aqueous layer was acidified with conc HCl, and the resulting solid containing 2-phenyl-3-(3,4,5-trimethoxyphenyl)acrylic acid (6.2 g, 19.72 mmol, 35% crude yield; LC–MS *m/z* 315.2 ($[M+H]^+$)) was collected by vacuum filtration.

A portion of the crude acrylic acid (6 g, ca. 19.08 mmol) was dissolved in quinoline (30 mL) in a 3-neck round-bottom flask equipped with a magnetic stir bar, thermometer, and reflux condenser with a $CaCl_2$ drying tube. The mixture was charged with 2CuO Cr_2O_3 (0.5 g, 6.42 mmol) and heated to 180–190 °C with stirring for 2 h. The resulting mixture was cooled to room temperature, diluted with conc HCl (25 mL) and filtered through Celite. The filtrate was extracted with EtOAc (3 × 50 mL). The combined organic layers were washed with brine (50 mL), dried over Na_2SO_4, filtered, and evaporated to give a tan semi-solid. The crude product was separated by flash SiO_2 chromatography (petroleum ether to 9:1 petroleum ether:EtOAc) to give the desired *Z*-1-(3,4,5-trimethoxyphenyl)-2-phenylethene as an oil (0.5 g, 1.85 mmol, 10% yield from the acrylic acid; ^1H NMR ($CDCl_3$) δ 2.7 (s, 9 H, 3 × OCH_3), 5.5 (s, 2 H, 1-phenyl ArH), 5.65 (dd, 2 H, J = 16 Hz, olefinic H), 6.6 (m, 5 H, 2-phenyl ArH); GC–PCI–MS *m/z* 271.2 ($[M+H]^+$)) and *E*-1-(3,4,5-trimethoxyphenyl)-2-phenylethene as a white solid (1 g, 20% yield from the crude acrylic acid; mp 45–47 °C; ^1H NMR ($CDCl_3$) δ 2.9 (s, 9 H, 3 × OCH_3), 5.6 (s, 2 H, 1-phenyl ArH), 5.7 (dd, 2 H, J = 6 Hz, olefinic H); GC–PCI–MS *m/z* 271.3 ($[M+H]^+$)).

Z-1-(3,4,5-trimethoxyphenyl)-2-phenylethene (0.2 g, 740 μmol) was dissolved in $CHCl_3$ (2 mL) and placed in a round-bottom flask equipped with a stir bar. Benzyltriethylammonium chloride (20 mg, 88 μmol) was added, and the solution was treated dropwise with chilled 40% (w/v) aq NaOH with rapid stirring. The flask was subsequently equipped with a reflux condenser with a $CaCl_2$ drying tube and stirring was continued for 48 h. The resulting brown mixture was placed in a separatory funnel, and the organic layer was collected. The aqueous layer was extracted with $CHCl_3$ (2 × 10 mL). The organic layers were combined, washed with brine (10 mL), H_2O (10 mL), dried over Na_2SO_4, filtered, and evaporated to give a straw oil. Three passages through short (ca. 0.5 g) flash SiO_2 columns (petroleum ether) gave 90 mg (36% yield from olefin) of **4** as a clear oil (GC-PCI-MS *m/z* 353.1 ($[(1,1\text{-di-}^{35}Cl)M+H]^+$)).

Biological evaluations

General. MCF-7 human breast carcinoma cells (28th passage) were a gift from Dr Marc Lippman, Lombardi Cancer Center, Georgetown University. MDA-MB231 human breast carcinoma cells were from the American Type Culture Collection. MEM culture medium, glutamine, fetal calf serum (FCS), and HBSS were from Gibco-BRL. IMEM culture medium was purchased from Biofluids. Cells were maintained in phenol red-containing MEM supplemented with 10% FCS and glutamine (final concentration 0.58 mg/ml) at 37 °C (95% humidity, 5% CO_2). One week prior to the start of experiments MEM was replaced with IMEM without phenol red. Permanox slides were purchased from Nunc. PERMFLUOR mounting fluid was obtained from Fisher Scientific. DES, antibodies, GTP (repurified by triethylammonium bicarbonate gradient anion exchange chromatography), and monosodium glutamate were obtained from Sigma Chemical Co. [^{3}H]Colchicine was from DuPont. Combretastatin A-4 was a generous gift from Professor George Pettit, Arizona State University.

Tubulin polymerization inhibition assay. Electrophoretically homogenous bovine brain tubulin was prepared as described previously.[13] Inhibition of glutamate-induced assembly of purified tubulin was performed as described previously.[14] Reaction mixtures (0.25 mL) contained tubulin and varying drug concentrations and were preincubated for 15 min at 30 °C. The reaction mixtures were then placed on ice and GTP was added, and they were transferred to cuvettes held at 0 °C. The temperature was increased to 30 °C, and the reaction was followed turbidimetrically at 350 nm for 20 min in

Gilford recording spectrophotometers equipped with electronic temperature controllers. Two control reaction mixtures without drug were present in each experiment. The IC_{50} value was defined as the drug concentration necessary to inhibit the extent of the reaction by 50%. Final concentrations of the reaction components were: 10 μM tubulin (1.0 mg/mL) 0.8 M monosodium glutamate (pH 6.6 with HCl in 2 M stock solution), 10 μM tubulin (1 mg/mL), 0.4 mM GTP, and 4% (v/v) DMSO.

Inhibition of [^{3}H]colchicine binding to isolated tubulin. This was performed as described previously.[14] Reaction mixtures contained 1.0 μM tubulin, 5.0 μM [^{3}H]colchicine, and inhibitor at 50 μM, except that 5.0 μM combretastatin A-4 was used. Incubation was for 10 min at 37 °C.

Cytostasis assays.[6] MCF-7 or MDA-MB231 cells (3×10^5) were seeded in 25 cm^2 flasks in 6 mL of IMEM (non-phenol red-containing, Biofluids) with 5% fetal calf serum (Gibco) and were allowed to attach for 24 h. The medium was discarded and cells were gently washed with Hank's balanced salt solution. IMEM with 5% FCS containing drugs (predissolved in DMSO; final DMSO concentration, 0.4%) at different concentrations (0, 10^{-7}, 10^{-6}, 10^{-5}, and 10^{-4} M) was added and incubated at 37 °C for 24 h. Each concentration was tested in triplicate flasks. After drug exposure, medium was transferred to a sterile centrifuge tube. Attached cells were gently trypsinized, collected, and added to the tube containing the medium (and detached cells). The cells were pelleted by centrifugation, the number of viable cells was determined microscopically on a hemacytometer by trypan blue exclusion in blinded triplicate, and IC_{50} values (drug concentration necessary for 50% growth inhibition relative to control cultures) were determined graphically ($n = 9$).

Indirect immunofluorescence detection of microtubule perturbation.[15-17] MCF-7 cells (800 cells/40 μL) were plated on sterile eight-well microscope slides and allowed to attach and grow at 37 °C for 24 h, then treated with drugs at various concentrations (10^{-5} to 10^{-7} M and vehicle controls) for 24 h. The cells were fixed with 3% formaldehyde in PBS, permeabilized in CH_3OH at −20 °C for 5 min, treated with 1% Triton X-100 for 20 min at room temperature, preincubated with 2% skim milk in PBS for 30 min at 37 °C, and incubated with mouse monoclonal anti-β-tubulin antibody overnight at 4 °C. The wells were rinsed with PBS, incubated for 6–8 h with fluorescein-5-isothiocyanate-conjugated goat-antimouse antibody, rinsed with 2% skim milk in PBS, rinsed again with PBS, mounted with PERMFLUOR, and allowed to dry at room temperature for two days. Slides were scored by fluorescence microscopy, where a concentration was considered to give a positive result when microtubule perturbation was observed in 50% of 1000 cells.

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