

Design, Synthesis, and Biological Evaluations of 2,5-Diaryl-2,3-dihydro-1,3,4-oxadiazoline Analogs of Combretastatin-A4

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A total of 24 novel 2,5-diaryl-1,3,4-oxadiazoline analogs of combretastatin A-4 (CA-4, **1**) were designed, synthesized, and evaluated for biological activities. The compounds represent two structural classes; the Type I class has three methoxy groups on the A ring and the Type II class has a single methoxy group on the A ring. Biological evaluations demonstrate that multiple structural features control the biological potency. Four of the compounds, 2-(3'-bromophenyl)-5-(3'',4'',5''-trimethoxyphenyl)-2-acetyl-2,3-dihydro-1,3,4-oxadiazoline (**9i**), 2-(2',5'-dimethoxyphenyl)-5-(3''-methoxyphenyl)-2-acetyl-2,3-dihydro-1,3,4-oxadiazoline (**10h**), 2-(3',4',5'-trimethoxyphenyl)-5-(3''-methoxyphenyl)-2-acetyl-2,3-dihydro-1,3,4-oxadiazoline (**10i**), and 2-(3',5'-dimethoxyphenyl)-5-(3''-methoxyphenyl)-2-acetyl-2,3-dihydro-1,3,4-oxadiazoline (**10j**), have potent antiproliferative activities against multiple cancer cell lines. Mechanistic studies indicate that they retain the microtubule disrupting effects of compound **1**, including microtubule loss, the formation of aberrant mitotic spindles, and mitotic arrest. Compound **10i** inhibits purified tubulin polymerization and circumvents drug resistance mediated by P-glycoprotein and β III tubulin expression. The oxadiazoline analog **10i** is a promising lead candidate worthy of further investigation.

Introduction

A worldwide effort led by the U.S. National Cancer Institute to identify anticancer agents from plants led to the identification of the combretastatins from the African bush willow *Combretum caffrum*.^{1,2} Combretastatin A-4 (CA-4,^a **1**, Figure 1) is the most potent of the natural combretastatins and early work showed it inhibits tubulin polymerization and the proliferation of murine and human cancer cells.^{3,4} Early reports of the antitumor activity of the more water-soluble prodrug CA-4P (**2**) against murine P388 and B16 tumors were not encouraging.⁵ However, detailed mechanistic studies showed that compound **1** has specific effects on tumor vasculature, causing rapid vascular shutdown, leading to central tumor necrosis.^{6,7} The effects of prodrug **2** on tumor vasculature have been verified in human clinical trials.^{8,9} While compound **2** causes central hemorrhagic necrosis in animal tumor models, a rim of viable tumor cells remains, suggesting that the optimal use of **2** would be in combination with agents that target the tumor periphery. Compound **2** is currently being evaluated in phase II clinical trials for activity in anaplastic thyroid carcinoma, nonsmall cell lung cancer, and ovarian cancer in combination with conventional cytotoxic drugs including paclitaxel, carboplatin, and the antiangiogenic agent brevacizumab.¹⁰ Mechanistically, agent **1** rapidly alters HUVEC morphology, causing cell contraction, membrane blebbing, and disruption of endothelial monolayer permeability.¹¹ These effects are potentially mediated by a Rho/Rho kinase stimulated increase in actin stress fibers. Prodrug **2**

has also been shown to disrupt VE-cadherin signaling in HUVECs.¹² Most recently, it was shown to rapidly upregulate connective tissue growth factor (CTGF) in microvascular endothelial cells in a cell density dependent manner.¹³ Upregulation of CTGF, a pleiotropic endothelial cell growth factor, was observed only in subconfluent endothelial cell cultures or in endothelial cells growing as capillary-like tubules. Prodrug **2**-mediated activation of the CTGF might explain why thin, unstabilized tumor blood vessels are so susceptible to **1**, while other mature vessels are unaffected. It is not known which of these compound **1**-mediated signaling events are responsible for the specific antivascular effects leading to rapid catastrophic tumor vascular shutdown.

Significant efforts have been undertaken to develop analogs of compound **1** that retain the biological actions of the parent molecule but provide improved pharmacokinetic properties and better water solubility.¹⁴ In addition to prodrug **2**, notable analogs undergoing clinical evaluations include AVE 8062 (**3**), the amino acid derivative of AC-7739 (**4**) and the CA-1 (**5**) derivative CA-1P (**6**; OXI-4503).^{10,15} Our work in this area has yielded several new classes of analogs including pyrazoles¹⁶ (**7**) and pyrazolines (**8**, Figure 1) of **1**.¹⁷ These derivatives demonstrate a wide range of potencies. A significant drop in activity was observed in the pyrazole derivatives as compared to **1**. X-ray crystallography studies of a pyrazole analog of compound **7** showed that it adopted a planar conformation, as opposed to the nonplanar geometry of compound **1**.¹⁶ The structure of **1** contains two substituted benzene rings, referred to as the A and B rings, which fit into the A and B pockets within the colchicines binding site on tubulin. Modeling studies of **1** within the colchicines binding site of tubulin suggest that a geometrically planar analog of **1** would not

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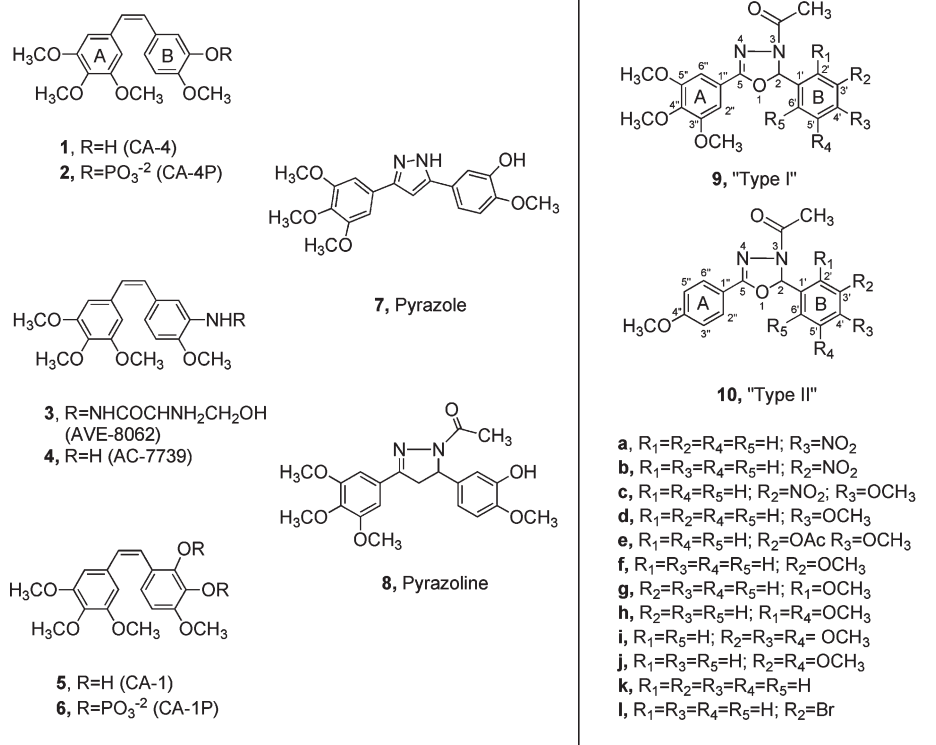
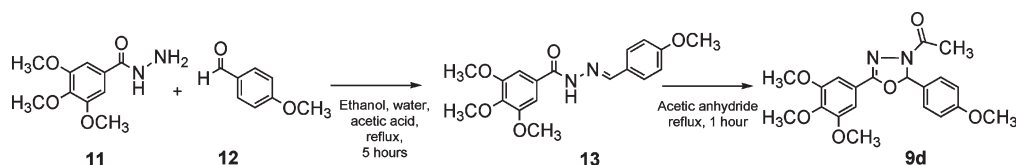


Figure 1. Structures of combretastatin-A4 (CA-4, **1**), its water-soluble derivate (CA-4P, **2**), its amino analogs (**3** and **4**), combretastatin-A1 (CA-1, **5**), combretastatin-A1 diphosphate (CA-1P, **6**), along with the pyrazole (**7**) and pyrazoline (**8**) analogs. Two general types of oxadiazoline analogs were generated, including the Type I compounds **9a–l** and the Type II compounds **10a–l**.

Scheme 1. Representation of Hydrazone Intermediate and Oxadiazoline Synthesis



allow optimal interactions with this binding site.^{18,19} It is reasonable to assume that the pyrazole's loss of potency can be attributed to its incompatible conformation within the colchicines binding site of tubulin. Consistent with this relationship, pyrazolines have a less planar geometry than the pyrazoles and are more potent inhibitors of cell proliferation and antitubulin effects.

A new series of 2,5-diaryl-1,3,4-oxadiazoline analogs were designed to retain the geometric features of **1**. Compared to the pyrazolines (**8**), the oxadiazoline moiety should provide an optimal conformational geometry for interaction with the colchicine binding site while increasing the number of heteroatoms in the core structure. The net effect is an increase in the polarity of the molecule, which should enhance water solubility. Accordingly, 24 specific oxadiazoline analogs were synthesized and they are divided into two types (Figure 1). Type I compounds (oxadiazoline **9a–l**) contain a 3,4,5-trimethoxyphenyl group on the A ring and Type II compounds (oxadiazoline **10a–l**) contain a 4-methoxyphenyl moiety on the A ring. These analogs were synthesized and evaluated for biological activities. The most potent compounds were evaluated in mechanistic studies and attempts have been made to understand the structure activity relationships (SAR) among these compounds.

Chemistry

Oxadiazoline analogs were synthesized by adapting a procedure reported by Ali, Amer, and Abdel-Rahman.²⁰ As an example, the reaction of a hydrazide (**11**) with a benzaldehyde (**12**) in reflux with water and glacial acetic acid in ethanol for 5 h produced the hydrazone intermediate **13** (Scheme 1). The reaction mixture was poured into ice water and the precipitate was collected and dried. When needed, the hydrazones were purified by recrystallization in aqueous methanol. The yields of the hydrazones ranged between 60 and 98%. The chemical structures of the hydrazones were confirmed using 400 MHz ¹H NMR and IR. The oxadiazoline products were synthesized by refluxing the appropriate hydrazone intermediate in acetic anhydride for 1 h under dry conditions (Scheme 1). The reaction mixture was poured into ice–water, and the solid product was collected and washed with water. Most products were found to be homogeneous by TLC and 400 MHz ¹H NMR analyses, but when needed, heterogeneous products were readily purified by silica gel column chromatography using a methanol/chloroform eluent. Oxadiazoline product yields ranged from 25–70%. The structures of compounds **9a–l** and **10a–l** were confirmed using NMR, IR, mass spectrometry, and elemental analysis. The compounds that

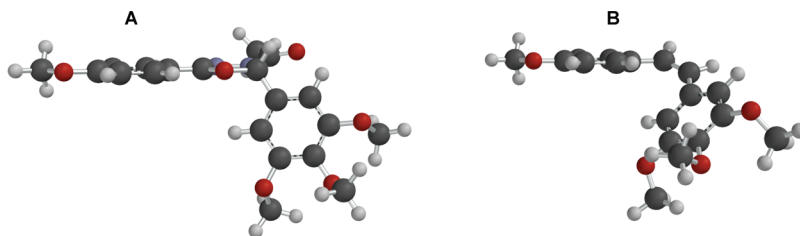


Figure 2. Molecular ball and stick model of oxadiazoline **10i** (A) and *cis*-4,3',4',5'-tetramethoxystilbene (B). Black represents carbon, blue nitrogen, gray hydrogen, and red oxygen.

showed a small amount (< 5%) of rotational isomers (about the amide bond) were analyzed by reversed phase HPLC (Nova Pak C₁₈ 3.6 × 150 mm column using methanol as the eluent). Each of the compounds showed a single peak with a retention time of 2–3 min, confirming the purity of the compounds.

To gain insight into the conformation of the oxadiazoline analogs, molecular modeling studies on compound **10i** were conducted using MacSpartan, version '04. The structure and conformation of **10i** was first optimized using molecular mechanics (MMFF) and molecular dynamics (equilibrium conformer search option and molecular mechanics). The optimal structure was further optimized using Hartree–Fock (equilibrium geometry optimization using the 3-21 G basis set) followed by density function theory (equilibrium geometry optimization using the B3LYP and 6-31 G* basis set) calculations. The resulting conformation of analog **10i** is shown in Figure 2A. It is evident that the oxadiazoline-containing compound adopts a nonplanar geometry similar to the parent compound **1**, unlike the previously reported pyrazole molecules. This result is consistent with earlier results from single X-ray diffraction studies indicating that the oxadiazoline structure adopts a puckered conformation.²¹ In addition, the conformation of *cis*-4,3',4',5'-tetramethoxystilbene, a dehydroxy analog of **1**, was also ascertained using the aforementioned modeling method, and a model is given in Figure 2B. It is worthy to note that both **10i** and *cis*-4,3',4',5'-tetramethoxystilbene possess a nonplanar or “twisted” geometry similar to the pyrazolines (**8**),¹⁷ and they show resemblance to the conformation of **1** itself.¹⁶

Biological Results and Discussion

Antiproliferative Effects of Oxadiazoline Compounds on Murine Cancer Cells. The antiproliferative effects of the 24 compounds were evaluated in murine L1210 leukemia cells and the IC₅₀ values determined (Table 1). Additionally, the compounds were evaluated in murine B16 melanoma cells (data not shown) with general concordance with L1210 data. The results reveal several interesting points including the broad range of potencies in this series. The novel oxadiazoline compounds are substantially less potent than compound **1** itself, which has reported IC₅₀ values ranging from 0.003 to 0.007 μM^{3–5} for L1210 and B16 cells. A correlation between the potencies of the Type I and Type II compounds could be expected, but this pattern was not observed. Not only is there no trend in L1210 potency with A ring alterations but a single adjustment to the B ring rarely affects a Type I compound in the same way that it affects a Type II compound. For example, a 3'-acetyl, 4'-methoxy B ring substitution in **9e** results in a compound with an IC₅₀ of 3.9 μM while this same substitution in the Type II compound (**10e**) results in a totally inactive compound (IC₅₀ > 100 μM). To explain these

Table 1. Sensitivity of L1210 Cells to Oxadiazoline Analogs^a

Type I	IC ₅₀ (μM)	Type II	IC ₅₀ (μM)
Ar =		Ar =	
	9a		10a
	4.2 ± 0.5		68.0 ± 4.9
	9b		10b
	39.0 ± 2.0		47.0 ± 2.9
	9c		10c
	58.0 ± 0.7		>100
	9d		10d
	57.0 ± 5.1		>100
	9e		10e
	3.9 ± 0.4		>100
	9f		10f
	45.0 ± 8.6		41.0 ± 6.4
	9g		10g
	48.0 ± 1.0		5.8 ± 0.6
	9h		10h
	55.0 ± 4.5		0.5 ± 0.05
	9i		10i
	>100		0.5 ± 0.06
	9j		10j
	5.9 ± 1.6		0.5 ± 0.1
	9k		10k
	6.4 ± 1.2		>100
	9l		10l
	0.6 ± 0.7		37.0 ± 6.0

^a The standard deviation for the IC₅₀ values is within 5–20% of the reported values.

inconsistencies, one possibility is to consider that compound **1** can bind in two orientations within the colchicine binding site of tubulin. For this reason the SAR of analogs of **1** gains complexity, as the B ring could orient within the standard A ring pocket of the binding site, if it results in a more energetically favorable conformation. Consequently, the effect of a single ring manipulation was less predictable and the methodical alterations of the A and B ring substituents were considered.

Compound **9i**, with 3'',4'',5''-trimethoxy A ring and 3',4',5'-trimethoxy B ring substitutions, was found to be entirely inactive (IC₅₀ > 100 μM), suggesting that this symmetric compound with two trimethoxy phenyl groups does

not interact well within the colchicine binding site. Compound **1**, with the 3'',4'',5''-trimethoxy A ring is very potent,⁴ implying the unfavorable nature of the 3',4',5'-trimethoxy B ring. At the other extreme, **10d**, a Type I compound with only a 4''-methoxy on the A ring and a 4'-methoxy substitution on the B ring is also inactive ($IC_{50} > 100 \mu M$). Interestingly, shifting the 4'-methoxy to the 3'-position (**10f**) on the Type II compound improves potency (IC_{50} 41.0 μM). The methoxy group in the 2'-position gives the highest potency of these three positions (**10g**, IC_{50} 5.8 μM). Together, these data suggest that a 4-methoxy substituted ring interacts poorly with one pocket of the binding site. Because of the potency of **10i** (IC_{50} 0.5 μM), which contains a trisubstituted methoxy ring that is predicted to only favorably interact with the A site, the single methoxy-substituted ring must favorably interact with the B site. This indicates that Type II compounds might position the 4''-methoxy A ring in the B ring pocket of the colchicine binding site on tubulin.

Compounds **10h** and **10j** have a 2',5'-dimethoxy and a 3',5'-dimethoxy substituted B ring, respectively. These compounds are potent, with IC_{50} values of 500 nM. Compounds **9h** and **9j** have very different potencies (**9h**, IC_{50} 55.0 μM ; **9j**, IC_{50} 5.9 μM). The 2',5'-dimethoxy B ring on **9h** would be expected to interact poorly with the B ring pocket, whereas the 3',5'-dimethoxy B ring on **9j** favorably interacts with the binding site.

The pattern observed with **10d**, **10f**, and **10g** is not observed in the Type I compounds; **9d**, **9f**, and **9g** and all have low potencies with IC_{50} s of 57, 45, and 48 μM , respectively. The 4'-methoxy B ring substitution is the least potent in both Type I and Type II compounds. In an attempt to improve **9d** and **10d**, the 4'-methoxy substitution was replaced with a 4'-nitro group (**9a**, **10a**). This resulted in a modest improvement in potency of the Type II compound (**10a**, IC_{50} 68.0 μM). For the Type I compound, this change resulted in a dramatic, 13-fold increase in potency (**9a** IC_{50} 4.2 μM). The Type I compound (**9a**) demonstrates that the 4'-nitro group substitution is substantially more favorable than the 4'-methoxy in the B ring pocket. Shifting the nitro group to the 3'-position, **10a** to **10b**, results in a slight increase in potency (**10b**, IC_{50} 47.0 μM). In the Type I compound, however, this change in the position of the nitro group decreased potency 9-fold (**9b**, IC_{50} 39.0 μM), suggesting that in the B-ring pocket a *p*-nitro substitution is more favorable than an *m*-nitro substitution. The 3'-nitro substitution on compound **10b** may orient into the A-ring pocket, improving interaction and biological potency. This is consistent with the pattern observed with **10d**, **10f**, and **10g**, which suggests a preferable A-pocket interaction with an *ortho* or *meta* substitution, as compared to the *para* substitution.

Compounds **9c** and **10c**, with a 3'-nitro, 4'-methoxy B ring, are less potent than a single 3'-nitro substituted B ring. Replacing the 3'-nitro with a 3'-bromo group produces a more potent Type I compound (**9b**, IC_{50} 39.0 μM ; **9l**, IC_{50} 0.6 μM). In the Type II compounds, the 3'-nitro a 3'-bromo group (**10b**, IC_{50} 47.0 μM ; **10l**, IC_{50} 37 μM) have similar activities. Compounds **9k** and **10k** have no substitution on the B ring, and they have dramatically different potencies (**9k**, IC_{50} 6.4 μM ; **10k**, IC_{50} > 100 μM). Compound **9k** could favorably position the nonsubstituted ring in the B-ring pocket, however, the low potency of **10k** (> 100 μM) is consistent with the notion that the Type I and Type II compounds might orient differently within the colchicine binding site.

Table 2. Antiproliferative and Microtubule Depolymerizing Effects of Oxadiazoline Analogs^a

compound	IC_{50} (μM) \pm SD MDA-MB-435	EC_{50} (μM) microtubule depolymerization	ratio EC_{50}/IC_{50}
9e	3.3 \pm 0.2	10.2	3.1
9l	0.15 \pm 0.01	1.5	10.0
10h	0.20 \pm 0.02	1.3	6.5
10i	0.14 \pm 0.01	0.5	3.6
10j	0.11 \pm 0.01	1.0	9.1
1	0.003 \pm 0.0002	0.007	2.3

^aThe antiproliferative actions (IC_{50}) of the oxadiazoline analogs and compound **1** were measured using the SRB assay ($n = 3-5$) and EC_{50} values using indirect immunofluorescence techniques in at least three independent experiments.

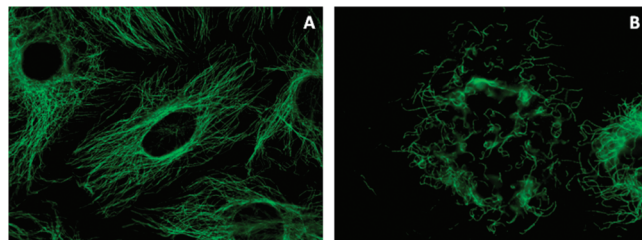


Figure 3. Effects of **10i** on cellular microtubules. A-10 cells were treated for 18 h with vehicle (A) or 1 μM **10i** (B) and cellular microtubule structures visualized with indirect immunofluorescence techniques. Normal interphase microtubules are visible in panel A. The **10i**-induced loss of interphase microtubules is shown in panel B.

Antiproliferative Effects against MDA-MB-435. In the development of chemical agents with potential application in human anticancer therapeutics, it was important to determine the activity of the most potent oxadiazolines in human cancer cells. The five most potent inhibitors of L1210 cell growth were evaluated in our reference human cell line, MDA-MB-435, a cell line originally designated as originating from breast but which has been shown definitively to be derived from the M14 melanoma cell line.²² The results, presented in Table 2, show that there is excellent correlation between the L1210 and MDA-MB-435 potencies, with 4 of the 5 compounds having submicromolar potency against human cancer cells. These compounds were evaluated further in mechanistic assays to determine if they retain the microtubule disrupting and antimitotic effects of the combretastatins.

Microtubule Depolymerizing Activity. With the goal of ascertaining the mechanism of action of the oxadiazoline compounds, the effects of **9e**, **9l**, **10h**, **10i**, and **10j** on interphase cellular microtubules were evaluated in A-10 smooth muscle cells. This cell line is valuable because the cells are relatively large and flat, and they arrest in interphase in response to tubulin binding antimitotics, consistent with the responses of other smooth muscle cells to these drugs.²³ All of the compounds caused a dose dependent loss of cellular microtubules, similar to the effects of **1**. Representative normal interphase microtubules and the microtubule depolymerizing effects of compound **10i** are shown in Figure 3. A range of concentrations was evaluated for each compound and the EC_{50} , the concentration required to cause 50% loss of cellular microtubules, as visualized by microscopy, was calculated (Table 2). The ratio of EC_{50} for microtubule depolymerization and the IC_{50} for inhibition of proliferation provides an indication of tight linkage of

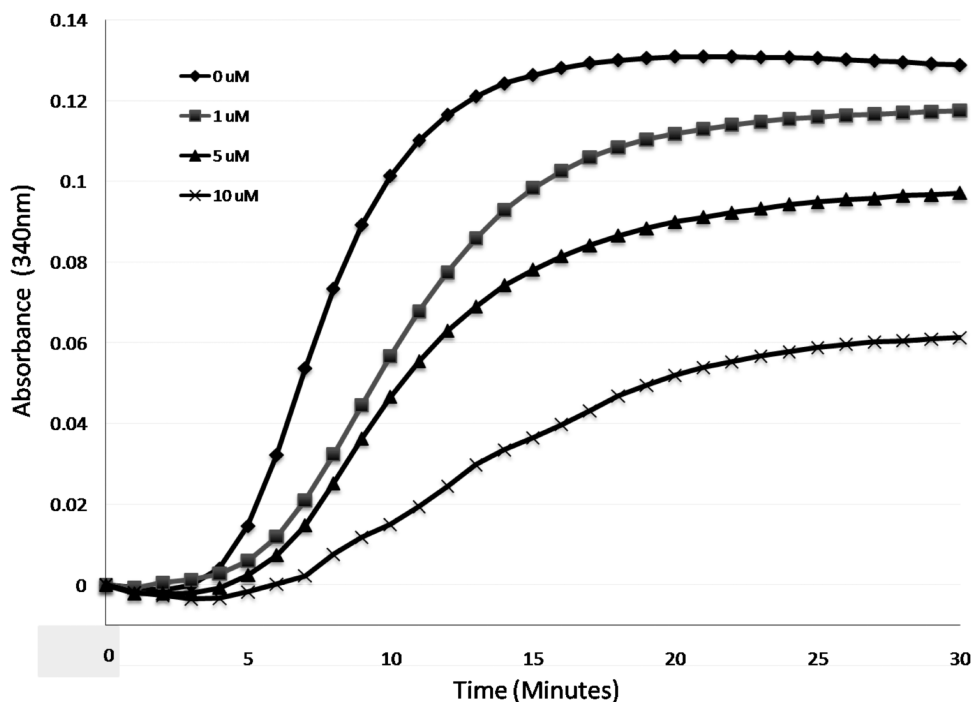


Figure 4. In vitro tubulin polymerization assays. The polymerization of bovine brain tubulin was monitored turbidimetrically by absorbance at 340 nm at 37 °C in the presence of a range of **10i** concentrations.

antiproliferative effects and the compound's tubulin-dependent mechanisms of action.^{24,25} Compound **10i** had a ratio of 3.5, **9e** a ratio of 3.1, **10h** a ratio of 6.5, and **1** a ratio of 2.3 (Table 2). The ratios are within the ranges we have seen with 2-methoxyestradiol analogs²⁴ and a large subset of polysubstituted pyrrole compounds.²⁵ Interestingly, the other potent antiproliferative compounds, and **10j** and **9l** did not demonstrate correspondingly potent microtubule depolymerization effects, and their ratios of EC₅₀/IC₅₀ ratios were 8.8 and 10, respectively (Table 2). This suggests that these compounds might exert cytotoxicity through mechanisms in addition to tubulin inhibition as has been shown with similar molecules that can bind directly to DNA.²⁶

Interaction of Compound 10i with Purified Bovine Brain Tubulin. The potent antiproliferative and microtubule depolymerizing effects prompted further mechanistic studies on **10i**. The effects of **10i** on purified tubulin polymerization were evaluated in a biochemical assay. In this assay, **10i** caused a concentration-dependent inhibition of tubulin polymerization (Figure 4). The concentration required to cause 50% inhibition of tubulin polymerization was 9.6 μ M. In similar assays, 2–3 μ M of **1** is needed to cause this effect.⁴ The tubulin data are consistent with the lower potency of **10i** for cellular microtubule and antiproliferative effects and strongly implicate a direct interaction of **10i** with tubulin.

Antimitotic Effects of Compound 10i. In addition to causing the loss of cellular microtubules, colchicine, compound **1** and their analogs initiate the formation of highly abnormal mitotic spindles.^{27–29} The effects of **10i** on mitotic spindles were evaluated in HeLa cells. HeLa cells, unlike the smooth muscle cells, arrest in the G₂/M phase of the cell cycle in response to tubulin binding drugs.^{28,29} Microtubules were visualized 18 h after treatment with vehicle or 0.5 μ M **10i**. Normal bipolar mitotic spindles emanating from γ -tubulin-containing centrosomes were observed and they aligned the chromosomes in the metaphase plate (Figure 5A). In

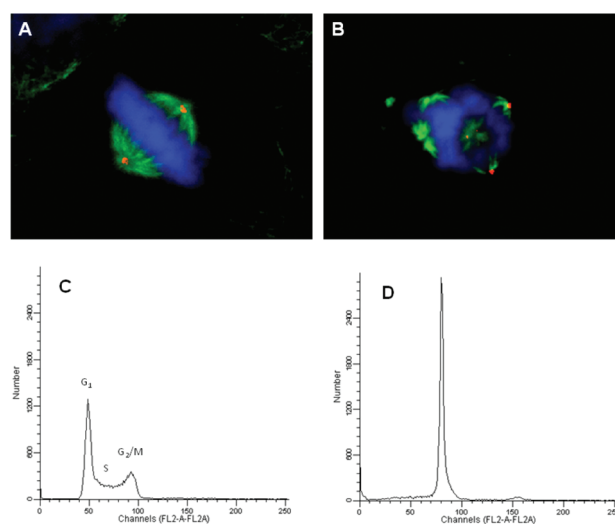


Figure 5. Effects of **10i** on mitotic spindles and cell cycle distribution. HeLa cells were treated with vehicle (A) or 0.50 μ M **10i** (B) for 18 h, fixed and cellular structures visualized by indirect immunofluorescence. Cellular microtubules were visualized with a β -tubulin antibody (green), centrosomes using a γ -tubulin antibody (red), and DNA using DAPI (blue). For the cell cycle distribution studies, MDA-MB-435 cells were treated for 18 h with vehicle (A) or 0.50 μ M **10i** then stained with Kirshan's reagent and evaluation by flow cytometry.

contrast, compound **10i** initiated the formation of highly aberrant, multipolar mitotic spindles (Figure 5B). At least seven mitotic spindles, three of which contained γ -tubulin, were visible in a single representative focal plane (Figure 5B). The DNA was not well organized into a metaphase plate, suggesting these cells did not have the potential to successfully divide into two equivalent daughter cells with normal complements of DNA.

Table 3. Sensitivity of Cell Lines Expressing Pgp and β III Tubulin

cell line	IC ₅₀ (μ M) \pm SD 1	IC ₅₀ (μ M) \pm SD 10i
SK-OV-3	0.0033 \pm 0.0002	0.34 \pm 0.01
SK-OV-3-MDR 1–6/6 ^a	0.0035 \pm 0.0005	0.45 \pm 0.01
Rr ^c	1.8	1.3
HeLa	0.0029 \pm 0.0003	0.23 \pm 0.03
WT β III ^b	0.0033 \pm 0.0008	0.27 \pm 0.02
Rr ^c	1.1	1.2

^aSK-OV-3-MDR 1–6/6 cells were generated from SK-OV-3 by adenoviral expression of MDR-1. ^bHeLa cells were transfected with the β III tubulin isotype to generate the WT β III cells. ^cThe Rr values were calculated by dividing the IC₅₀ of the genetically engineered line by the IC₅₀ of the parental cell line.

The cell cycle distribution of cells treated with **10i** was further evaluated by flow cytometry to determine whether the formation of abnormal mitotic spindles caused cells to accumulate in the G₂/M phase of the cell cycle. MDA-MB-435 cells were treated for 18 h with vehicle or 0.5 μ M **10i**. A normal cell cycle distribution for MDA-MB-435 cells was seen in the vehicle treated cells (Figure 5C). In contrast, in the **10i**-treated cells complete G₂/M accumulation occurred, indicative of mitotic blockade. Paclitaxel caused an identical effect (data not shown). These data, combined with the cellular microtubule depolymerizing effects and the ability of **10i** to directly inhibit purified tubulin assembly, provide strong evidence that this oxadiazoline retains the cellular antitubulin mechanisms of action of **1**.

Compound 10i can Circumvent Clinically Relevant Mechanisms of Drug Resistance. One major limitation of anticancer drugs is intrinsic or acquired multidrug resistance. Tumor expression of the ATP-dependent drug efflux pump, P-glycoprotein (Pgp), is associated with anticancer treatment failure.³⁰ A second clinically relevant mechanism of drug resistance to tubulin-targeted agents, including the taxanes and vinorelbine, is the expression of the β III isotype of tubulin.³¹ Drugs that can overcome these mechanisms of resistance might have advantages in the treatment of drug resistant tumors. The ability of the oxadiazoline **10i** to overcome these mechanisms of resistance was tested in isogenic cell line pairs. The sensitivity of the Pgp expressing SK-OV-3-MDR-6-1-6/6 cell line was compared to the sensitivity of the parental cell line, SK-OV-3.³² IC₅₀s were measured for each cell line and relative resistance (Rr) values were calculated by dividing the IC₅₀ of the genetically engineered cell line by the IC₅₀ of the parental cell line. In this cell line pair, the Pgp substrate paclitaxel had an Rr of 860 and the non-Pgp substrate 2-methoxyestradiol had an Rr value of 2.6.³² As shown in Table 3, **1** has an Rr of 1.8 and **10i** has an Rr of 1.3, suggesting that **10i**, like **1**, is a poor substrate for transport by Pgp. The effect of β III tubulin expression was evaluated using the WT β III and HeLa cell line pair.³² In this cell line pair, paclitaxel, which is susceptible to β III-mediated resistance, has an Rr of 4.7, while 2-methoxyestradiol, which is unaffected by β III expression, has a Rr of 1.0.³² The Rr value for compound **10i** was 1.2 and a Rr of 1.1 was measured for compound **1** in these cell lines (Table 3). These data suggest that the oxadiazoline **10i** retains the ability of **1** to overcome drug resistance mediated by the expression of Pgp and the β III isotype of tubulin. Therefore, these drugs would be expected to have advantages in the treatment of multidrug resistant tumors.

Analytical Measurement To Estimate Aqueous Solubility. One major limitation of the combretastatins compounds **1** and **6** (CA-1) is low aqueous solubility; this led to the

Table 4. Relative Aqueous Solubility of Oxadiazoline Analogs^a

compound	aqueous solubility (μ M)
9e	112
9l	66
10h	259
10i	257
10j	94
1	350

^aThe relative aqueous solubilities were measured using a protocol based on Millipore's MultiScreen aqueous solubility filter plate assay. Maximal solubility in this assay is 500 μ M.

synthesis and use of the more water-soluble prodrugs **2** and **6**. Excellent water solubility would provide a significant advantage for new analogs of **1**. There was a good expectation that the increased number of heteroatoms in the core structure of the oxadiazoline analogs would facilitate aqueous solubility. An analytical assay derived from the shake-flask solubility method was used to evaluate the relative aqueous solubility of the most potent oxadiazoline analogs. This assay is rapid and highly reproducible, requires only small quantities of material, and provides good, but not perfect, correlation with the shake-flask solubility method. The results (Table 4) show that there was a wide range of aqueous solubilities in this subset of compounds. Compounds **10h** and **10i** have aqueous solubilities in the same range as compound **1**, while compounds **9e**, **9l**, and **10j** have lower aqueous solubility.

In summary, the synthesis of novel 2,5-diaryl-1,3,4-oxadiazolines as antitubulin, antimetabolic cytotoxins is described. Compound **10i** exhibits promising antiproliferative activities and a mechanism of action identical to prodrug **2** in vitro. The promising activities of this compound will be investigated further with in vivo murine antitumor experiments, which will be reported in due course.

Experimental Section

Solvents and organic reagents were purchased from Aldrich or Fisher and were used without further purification. Melting points (mp) were performed using a Mel-temp instrument, and the results were uncorrected. Infrared (IR) spectra were recorded with a Midac M1700 FT-IR instrument as films on KBr discs, unless stated otherwise. Proton NMR spectra were recorded on a Varian INOVA 400 MHz Fourier transform spectrometer using an internal deuterium lock. Chemical shifts are quoted in parts per million downfield from tetramethylsilane. High-resolution mass spectra (HRMS) and low-resolution mass spectra (LRMS) were provided by the Mass Spectrometry Laboratory, University of South Carolina, Columbia, SC. Elemental analyses were performed by Midwest Microlab, LLC, Indianapolis, IN. Reactions were monitored using thin-layer chromatography (TLC) using commercially available precoated plates (Merck Kieselgel 60 F254 silica). Visualization was achieved with UV light at 254 nm or I₂ vapor staining. In addition to NMR and elemental analysis, HPLC analysis was used to determine the purity (> 95%) of the compounds. Compounds were dissolved in methanol (1.5 mL). A reversed phase Nova Pak C₁₈ 3.9 \times 150 mm column attached to a Perkin-Elmer Series 200 pump coupled to a Hitachi UV-vis detector was used. Each sample was injected at a volume of 5 μ L, the eluent was methanol and the flow rate was 0.5 mL/min.

Representative Experimental Procedure for the Synthesis of Oxadiazoline 9d via Hydrazone Intermediate 13. To a stirred solution of hydrazide **11** (226 mg, 1 mmol) and *p*-anisaldehyde (0.12 mL, 1 mmol) in ethanol (25 mL), water (5 mL) was added followed by dropwise addition of glacial acetic acid (0.2 mL). The resulting mixture was refluxed for 5 h, after which the

solution was poured into ice water. The mixture was stirred until a precipitate formed, which was collected using suction filtration and dried, followed by recrystallization in aqueous methanol, giving hydrazone **13** as a pale yellow solid (0.19 g, 54%); R_f [methanol/chloroform (2:98)] 0.47; mp 74–76 °C; δ_H (400 MHz, $CDCl_3$) 8.66 (1H, s br), 8.26 (1H, s), 7.53 (2H, d, $J = 8.4$), 7.24 (1H, s), 7.06 (1H, s), 6.94 (2H, d, $J = 8.4$), 3.93 (9H, s), and 3.86 (3H, s); ν_{max} (CH_2Cl_2)/ cm^{-1} 3484, 1645, 1501, 1332, 1126, and 735; LRMS (EI^+) m/z (rel intensity) 344 (20%, M^+), 211 (35%), and 195 (100%); HRMS calcd for $C_{18}H_{20}N_2O_5^+$, 344.1372 (M^+); found, 344.1373 (M^+). 2-(4'-Methoxyphenyl)-5-(3'',4'',5''-trimethoxyphenyl)-2-acetyl-2,3-dihydro-1,3,4-oxadiazoline **9d** was synthesized by addition of acetic acid (2 mL) to hydrazone **13** (100 mg, 0.29 mmol), and the resulting solution was heated to reflux for 1 h. The reaction mixture was poured into ice water, and the resulting solid product was filtered and washed with copious amounts of water, followed by drying, to give **9d** as a white solid (74 mg, 66%); R_f [methanol/chloroform (2:98)] 0.31; mp 117–119 °C; δ_H (400 MHz, $CDCl_3$) 7.41 (2H, dt, $J = 8.8, 3.2$), 7.11 (2H, s), 7.02 (1H, s), 6.92 (2H, dt, $J = 8.8, 3.2$), 3.91 (9H, s), 3.81 (3H, s), and 2.35 (3H, s); ν_{max} (KBr disk)/ cm^{-1} 2939, 1667, 1583, 1462, 1416, 1233, 1127, 1001, 843, and 694; LRMS (EI^+) m/z (rel intensity) 386 (30%, M^+) and 195 (100%); HRMS calcd for $C_{20}H_{22}N_2O_5^+$, 386.1478 (M^+); found, 386.1484 (M^+).

2-(4'-Nitrophenyl)-5-(3'',4'',5''-trimethoxyphenyl)-2-acetyl-2,3-dihydro-1,3,4-oxadiazoline (9a). Pale yellow solid (47 mg, 73%); R_f [methanol/chloroform (2:98)] 0.27; mp 141–142 °C; δ_H (400 MHz, $CDCl_3$) 8.28 (2H, d, $J = 8.8$), 7.69 (2H, d, $J = 8.8$), 7.14 (1H, s), 7.11 (2H, s), 3.95 (3H, s), 3.94 (6H, s), and 2.38 (3H, s); ν_{max} (KBr disk)/ cm^{-1} 2940, 2847, 1666, 1584, 1525, 1505, 1416, 1175, 1233, 1129 1020, 910, and 731; LRMS (EI^+) m/z (rel intensity) 401 (25%, M^+), 195 (100%).

2-(3'-Nitrophenyl)-5-(3'',4'',5''-trimethoxyphenyl)-2-acetyl-2,3-dihydro-1,3,4-oxadiazoline (9b). White powder (73 mg, 65%); R_f [methanol/chloroform (2:98)] 0.44; mp 64–66 °C; δ_H (400 MHz, $CDCl_3$) 8.35 (1H, s), 8.28 (1H, d, $J = 8.4$), 7.87 (1H, d, $J = 8.4$), 7.65 (1H, t, $J = 8.4$), 7.14 (1H, s), 7.12 (2H, s), 3.92 (6H, s), 3.91 (3H, s), and 2.39 (3H, s); ν_{max} (KBr disk)/ cm^{-1} 2916, 2848, 1662, 1584, 1536, 1504, 1462, 1416, 1351, 1130, 1001, 733, and 633; LRMS (EI^+) m/z 401 (25%, M^+), 359 (20%), and 195 (100%).

2-(3'-Nitro-4-methoxyphenyl)-5-(3'',4'',5''-trimethoxyphenyl)-2-acetyl-2,3-dihydro-1,3,4-oxadiazoline (9c). White solid (85 mg, 75%); R_f [methanol/chloroform (2:98)] 0.35; mp 176–178 °C; δ_H (400 MHz, $CDCl_3$) 7.95 (1H, d, $J = 2.4$), 7.71 (1H, dd, $J = 8.4, 2.4$), 7.14 (1H, d, $J = 8.4$), 7.10 (2H, s), 7.04 (1H, s), 3.98 (3H, s), 3.92 (6H, s), 3.91 (3H, s), and 2.37 (3H, s); ν_{max} (KBr disk)/ cm^{-1} 2917, 2846, 1666, 1622, 1578, 1540, 1500, 1468, 1420, 1359, 1250, 1233, 1135, 1001, and 733; LRMS (ES^+) m/z 441 (25%, M^+), 389 (15%), and 195 (100%).

2-(3'-Acetoxy-4'-methoxyphenyl)-5-(3'',4'',5''-trimethoxyphenyl)-2-acetyl-2,3-dihydro-1,3,4-oxadiazoline (9e). White powder (73 mg, 65%); R_f [methanol/chloroform (2:98)] 0.35; mp 117–119 °C; δ_H (400 MHz, $CDCl_3$) 7.38 (1H, d, $J = 8.4$), 7.14 (1H, s), 7.11 (2H, s), 7.01 (1H, s), 6.98 (1H, d, $J = 8.4$), 3.92 (6H, s), 3.91 (3H, s), 3.83 (3H, s), 2.35 (3H, s), and 2.30 (3H, s); ν_{max} (KBr disk)/ cm^{-1} 3100, 2940, 2841, 1769, 1666, 1583, 1513, 1463, 1416; 1271, 1200, 1126, 1002, and 733; LRMS (ES^+) m/z (rel intensity) 444 (30%, M^+), 402 (15%), and 195 (100%). Anal. ($C_{22}H_{24}N_2O_8 \cdot H_2O$) C, H.

2-(3'-Trimethoxyphenyl)-5-(3'',4'',5''-trimethoxyphenyl)-2-acetyl-2,3-dihydro-1,3,4-oxadiazoline (9f). Yellow solid (16 mg, 14%); R_f [methanol/chloroform (2:98)] 0.45; mp 135–137 °C; δ_H (400 MHz, $CDCl_3$) 7.36 (1H, s), 7.33 (1H, t, $J = 8.0$), 7.11 (2H, s), 7.09 (1H, d, $J = 8.0$), 7.03 (1H, d, $J = 8.0$), 6.94 (1H, d, $J = 8.0$), 3.91 (6H, s), 3.90 (3H, s), 3.82 (3H, s), and 2.39 (3H, s); ν_{max} (KBr disk)/ cm^{-1} 2917, 2848, 1662, 1613, 1583, 1515, 1463, 1416, 1130, 1033, 732, and 696; LRMS (EI^+) m/z (rel intensity) 386 (25%, M^+), 344 (15%), and 195 (100%).

2-(2'-Methoxyphenyl)-5-(3'',4'',5''-trimethoxyphenyl)-2-acetyl-2,3-dihydro-1,3,4-oxadiazoline (9g). Yellow powder (63 mg, 56%); R_f [methanol/chloroform (2:98)] 0.35; mp 151–153 °C; δ_H (400 MHz, $CDCl_3$) 7.38 (1H, t, $J = 8.0$), 7.37 (1H, s), 7.32 (1H, d, 8.0), 7.10 (2H, s), 6.96 (1H, d, $J = 8.0$), 6.98 (1H, t, $J = 8.8$), 3.91 (6H, s), 3.90 (3H, s), 3.88 (3H, s), and 2.38 (3H, s); ν_{max} (KBr disk)/ cm^{-1} 2916, 2848, 1666, 1583, 1463, 1416, 1248, 1129, 911, 732, and 668; LRMS (EI^+) m/z (rel intensity) 386 (M^+ , 35%) and 195 (100%).

2-(2',5'-Dimethoxyphenyl)-5-(3'',4'',5''-trimethoxyphenyl)-2-acetyl-2,3-dihydro-1,3,4-oxadiazoline (9h). White powder (40 mg, 36%); R_f [methanol/chloroform (2:98)] 0.37; mp 187–189 °C; δ_H (400 MHz, $CDCl_3$) 7.33 (1H, s), 7.09 (2H, s), 6.90 (2H, s), 6.87 (1H, s), 3.94 (3H, s), 3.91 (6H, s), 3.89 (3H, s), 3.75 (3H, s), and 2.38 (3H, s); ν_{max} (KBr disk)/ cm^{-1} 2917, 2848, 1665, 1613, 1583, 1515, 1462, 1416, 1130, 1033, and 732; LRMS (EI^+) m/z (rel intensity) 416 (M^+ , 20%) and 195 (100%). Anal. ($C_{21}H_{24}N_2O_7$) C, H.

2-(3',4',5'-Trimethoxyphenyl)-5-(3'',4'',5''-trimethoxyphenyl)-2-acetyl-2,3-dihydro-1,3,4-oxadiazoline (9i). White solid (79 mg, 68%); R_f [methanol/chloroform (2:98)] 0.25; mp 162–163 °C; δ_H (400 MHz, $CDCl_3$) 7.12 (2H, s), 6.87 (1H, s), 6.70 (2H, s), 3.93 (3H, s), 3.91 (6H, s), 3.87 (6H, s), 3.85 (3H, s), and 2.39 (3H, s); ν_{max} (KBr disk)/ cm^{-1} 2916, 2848, 1666, 1582, 1503, 1331, 1234, 1127, 911, and 730; LRMS EI m/z (rel intensity) 466 (M^+ , 25%) and 195 (100%).

2-(3',5'-Dimethoxyphenyl)-5-(3'',4'',5''-trimethoxyphenyl)-2-acetyl-2,3-dihydro-1,3,4-oxadiazoline (9j). White powder (22 mg, 20%); R_f [methanol/chloroform (2:98)] 0.46; mp 80–82 °C; δ_H (400 MHz, $CDCl_3$) 7.11 (2H, s), 6.99 (1H, s), 6.64 (2H, d, $J = 2.0$), 6.48 (1H, t, $J = 2.0$), 3.93 (6H, s), 3.91 (3H, s), 3.79 (6H, s), and 2.37 (3H, s, Ac); ν_{max} (KBr disk)/ cm^{-1} 2917, 2848, 2359, 2342, 1662, 1583, 1416, 1250, 1158, 1173, 909, and 732; LRMS (ES^+) m/z (rel intensity) 416 (M^+ , 29%) and 195 (100%).

2-Phenyl-5-(3'',4'',5''-trimethoxyphenyl)-2-acetyl-2,3-dihydro-1,3,4-oxadiazoline (9k). White solid (77 mg, 77%); R_f [methanol/chloroform (2:98)] 0.75; mp 175–177 °C; δ_H (400 MHz, $CDCl_3$) 7.49 (2H, dd, $J = 7.6, 2.4$), 7.42 (3H, m), 7.13 (2H, s), 7.07 (1H, s), 3.94 (6H, s), 3.91 (3H, s), and 2.37 (3H, s); ν_{max} (KBr disk)/ cm^{-1} 2916, 2848, 1667, 1582, 1462, 1416, 1250, 1127, and 762; LRMS (EI^+) m/z 356 (rel intensity) (M^+ , 35%), 314 (15%), and 195 (100%).

2-(3'-Bromophenyl)-5-(3'',4'',5''-trimethoxyphenyl)-2-acetyl-2,3-dihydro-1,3,4-oxadiazoline (9l). Off-white solid (60 mg, 54%); R_f [methanol/chloroform (2:98)] 0.77; mp 134–136 °C; δ_H (400 MHz, $CDCl_3$) 7.61 (1H, s), 7.54 (1H, d, $J = 8.0$), 7.44 (1H, d, $J = 8.0$), 7.29 (1H, t, $J = 8.0$), 7.11 (2H, s), 7.01 (1H, s), 3.92 (6H, s), 3.91 (3H, s), and 2.38 (3H, s); ν_{max} (KBr disk)/ cm^{-1} 2916, 2850, 1585, 1557, 1504, 1416, 1331, 1233, 1128, and 733; LRMS (EI^+) m/z 434 (M^+ , 17%), 392 (10%), and 195 (100%); HRMS calcd for $C_{19}H_{19}BrN_2O_5^+$, 434.0478 (M^+); found, 434.0470 (M^+). Anal. ($C_{19}H_{19}BrN_2O_5$) C, H.

2-(4'-Nitrophenyl)-5-(3'-methoxyphenyl)-2-acetyl-2,3-dihydro-1,3,4-oxadiazoline (10a). Yellow powder (50 mg, 44%); R_f [methanol/chloroform (2:98)] 0.69; mp 99–101 °C; δ_H (400 MHz, $CDCl_3$) 8.26 (2H, d, $J = 8.9$), 7.84 (2H, d, $J = 8.9$), 7.69 (2H, d, $J = 8.8$), 7.13 (1H, s), 6.97 (2H, d, $J = 8.8$), 3.89 (3H, s), and 2.36 (3H, s); ν_{max} (KBr disk)/ cm^{-1} 2916, 2848, 1666, 1608, 1520, 1536, 1414, 1367, 1257, 1174, 1066, and 1027; LRMS (EI^+) m/z 341 (M^+ , 12%), 299 (10%), and 135 (100%).

2-(3'-Nitrophenyl)-5-(3'-methoxyphenyl)-2-acetyl-2,3-dihydro-1,3,4-oxadiazoline (10b). Yellow solid (82 mg, 73%); R_f [methanol/chloroform (2:98)] 0.51; mp 144–146 °C; δ_H (400 MHz, $CDCl_3$) 8.34 (1H, s), 8.26 (1H, d, $J = 8.0$), 7.87 (1H, d, $J = 8.0$), 7.85 (2H, d, $J = 8.8$), 7.61 (1H, t, $J = 8.0$), 7.13 (1H, s), 6.97 (2H, d, $J = 8.8$), 3.88 (3H, s, OCH_3 ; Ar^B), and 2.37 (3H, s, CH_3 ; Ac); ν_{max} (KBr disk)/ cm^{-1} 2916, 2848, 2360, 1665, 1620, 1585, 1540, 1420, 1367, 1275, 1250, 1240, 1130, 1011, and 735; LRMS m/z 341 (M^+ , 12%), 299 (10%), and 135 (100%).

2-(3'-Nitro-4'-methoxyphenyl)-5-(3''-methoxyphenyl)-2-acetyl-2,3-dihydro-1,3,4-oxadiazoline (10c). White powder (27 mg, 24%); R_f [methanol/chloroform (2:98)] 0.47; mp 146–148 °C; δ_H (400 MHz, $CDCl_3$) 7.96 (1H, s), 7.83 (2H, d, $J = 8.8$), 7.72 (1H, d, $J = 8.6$), 7.13 (1H, d, $J = 8.6$), 6.98 (1H, s), 6.97 (2H, d, $J = 8.8$), 3.98 (3H, s), 3.88 (3H, s), and 2.36 (3H, s, CH_3); ν_{max} (KBr disk)/ cm^{-1} 2920, 2850, 1667, 1622, 1583, 1536, 1462, 1416, 1279, 1249, 1230, 1087, 1011, and 735; LRMS (ES^+) m/z (rel intensity) 371 (M^+ , 12%), 329 (8%), and 135 (100%).

2-(4'-Methoxyphenyl)-5-(3''-methoxyphenyl)-2-acetyl-2,3-dihydro-1,3,4-oxadiazoline (10d). Yellow oil (108 mg, 95%); R_f [methanol/chloroform (2:98)] 0.37; δ_H (400 MHz, $CDCl_3$) 7.83 (2H, d, $J = 8.8$), 7.41 (2H, d, $J = 8.8$), 7.02 (1H, s), 6.95 (2H, d, $J = 8.8$), 6.91 (2H, d, $J = 8.8$), 3.89 (3H, s), 3.80 (3H, s), and 2.34 (3H, s); ν_{max} (KBr disk)/ cm^{-1} 2919, 2849, 1630, 1461, 1255, 908, and 733; LRMS (EI^+) m/z (rel intensity) 326 (M^+ , 18%), 282 (13%), and 135 (100%).

12-(3'-Acetoxy-4'-methoxyphenyl)-5-(3''-methoxyphenyl)-2-acetyl-2,3-dihydro-1,3,4-oxadiazoline (10e). Colorless oil (68 mg, 59%); R_f [methanol/chloroform (2:98)] 0.40; δ_H (400 MHz, $CDCl_3$) 7.82 (2H, d, $J = 8.9$), 7.38 (1H, d, $J = 8.4$), 7.16 (1H, s), 7.02 (1H, s), 6.96 (2H, d, $J = 8.9$), 6.86 (1H, d, $J = 8.4$), 3.87 (3H, s), 3.83 (3H, s), 2.33 (3H), and 2.29 (3H, s); ν_{max} (KBr disk)/ cm^{-1} 3250, 2942, 2840, 1770, 1670, 1580, 1513, 1463, 1420, 1271, 1200, 1130, 1001, and 733; LRMS (EI^+) m/z (rel intensity) 384 (M^+ , 15%), 342 (15%), 151 (10%), and 135 (100%). Anal. ($C_{20}H_{20}N_2O_6 \cdot 2H_2O$) C, H.

2-(3'-Methoxyphenyl)-5-(3''-methoxyphenyl)-2-acetyl-2,3-dihydro-1,3,4-oxadiazoline (10f). Colorless oil (70 mg, 61%); R_f [methanol/chloroform (2:98)] 0.44; δ_H (400 MHz, $CDCl_3$) 7.84 (2H, d, $J = 8.8$), 7.32 (1H, t, $J = 8.0$), 7.07 (1H, d, $J = 8.0$), 7.02 (1H, s), 7.01 (1H, s), 6.94 (2H, d, $J = 8.8$), 6.92 (1H, d, $J = 8.0$), 3.86 (3H, s, OCH_3 ; $Ar^{A/B}$), 3.83 (3H, s, OCH_3 ; $Ar^{A/B}$), and 2.33 (3H, s, CH_3 ; Ac); ν_{max} (KBr disk)/ cm^{-1} 2916, 2848, 1666, 1463, 1416, 1248, 1129, 1027, 911, and 735; LRMS (EI^+) m/z (rel intensity) 326 (M^+ , 20%), and 135 (100%).

2-(2'-Methoxyphenyl)-5-(3''-methoxyphenyl)-2-acetyl-2,3-dihydro-1,3,4-oxadiazoline (10g). White solid (290 mg, 93%); R_f [methanol/chloroform (2:98)] 0.49; mp 187–189 °C; δ_H (400 MHz, $CDCl_3$) 7.80 (2H, d, $J = 8.8$), 7.37–7.28 (2H, m), 6.97 (1H, s), 6.96–6.89 (2H, m), 6.91 (2H, d, $J = 8.8$), 3.86 (3H, s), 3.84 (3H, s), and 2.37 (3H, s); ν_{max} (KBr disk)/ cm^{-1} 2916, 2848, 1666, 1583, 1463, 1416, 1249, 1129, 910, 732, and 669; LRMS (EI^+) m/z (rel intensity) 326 (M^+ , 20%) and 135 (100%).

2-(2',5'-Dimethoxyphenyl)-5-(3''-methoxyphenyl)-2-acetyl-2,3-dihydro-1,3,4-oxadiazoline (10h). Colorless oil (65 mg, 57%); R_f [methanol/chloroform (2:98)] 0.56; δ_H (400 MHz, $CDCl_3$) 7.80 (2H, d, $J = 8.8$), 7.31 (1H, s), 6.92 (2H, d, $J = 8.8$), 6.88 (2H, s), 6.85 (1H, s), 3.86 (3H, s), 3.83 (3H, s), 3.73 (3H, s), and 2.33 (3H, s); ν_{max} (KBr disk)/ cm^{-1} 2920, 2850, 1660, 1614, 1580, 1515, 1461, 1250, 1235, 1173, 1129, 909, and 732; LRMS (EI^+) m/z (rel intensity) 356 (M^+ , 20%) and 135 (100%); HRMS calcd for $C_{19}H_{20}N_2O_5^+$, 356.1372 (M^+); found, 356.1365 (M^+). Anal. ($C_{19}H_{20}N_2O_5$) C, H.

2-(3',4',5'-Trimethoxyphenyl)-5-(3''-methoxyphenyl)-2-acetyl-2,3-dihydro-1,3,4-oxadiazoline (10i). White powder (60 mg, 54%); R_f [methanol/chloroform (2:98)] 0.31; mp 148–150 °C; δ_H (400 MHz, $CDCl_3$) 7.84 (2H, d, $J = 8.8$), 6.97 (1H, s), 6.95 (1H, s), 6.92 (2H, d, $J = 8.8$), 6.71 (1H, s), 3.91 (3H, s), 3.89 (3H, s), 3.87 (3H, s), 3.84 (3H, s), and 2.38 (3H, s); ν_{max} (KBr disk)/ cm^{-1} 2918, 2849, 1610, 1503, 1463, 1327, 1257, 1174, 1128, 908, and 733; LRMS (EI^+) m/z (rel intensity) 386 (M^+ , 25%) and 135 (100%); HRMS calcd for $C_{20}H_{22}N_2O_6^+$, 386.1478 (M^+); found, 386.1484 (M^+). Anal. ($C_{20}H_{22}N_2O_6$) C, H.

2-(3',5'-Dimethoxyphenyl)-5-(3''-methoxyphenyl)-2-acetyl-2,3-dihydro-1,3,4-oxadiazoline (10j). White solid (76 mg, 73%); R_f [methanol/chloroform (2:98)] 0.48; mp 98–100 °C; δ_H (400 MHz, $CDCl_3$) 7.82 (2H, d, $J = 8.8$), 6.98 (1H, s), 6.94 (2H, d, $J = 8.8$), 6.63 (2H, d, $J = 2.4$), 6.47 (1H, t, $J = 2.4$, CH), 3.86 (3H, s), 3.78 (6H, s), and 2.36 (3H, s); ν_{max} (KBr disk)/ cm^{-1}

2917, 2849, 1731, 1609, 1456, 1257, 1158, 908, and 732; LRMS (EI^+) m/z (rel intensity) 356 (30%, M^+), 314 (6%), 179 (8%), and 135 (100%); HRMS calcd for $C_{19}H_{20}N_2O_5^+$, 356.1372 (M^+); found, 356.1376 (M^+).

2-Phenyl-5-(3''-methoxyphenyl)-2-acetyl-2,3-dihydro-1,3,4-oxadiazoline (10k). White powder (51 mg, 44%); R_f [methanol:chloroform (2:98)] 0.65; mp 149–151 °C; δ_H (400 MHz, $CDCl_3$) 7.85 (2H, d, $J = 8.8$), 7.48 (2H, dd, $J = 4.8$, 8.0), 7.41–7.28 (3H, m), 7.07 (1H, s), 6.95 (2H, d, $J = 8.8$), 3.87 (3H, s), and 2.36 (3H, s); ν_{max} (KBr disk)/ cm^{-1} 2917, 2849, 1630, 1606, 1510, 1461, 1312, 1257, 1174, 908, and 733; LRMS (EI^+ ; rel intensity) m/z 296 (M^+ , 10%), 254 (8%), 151 (18%), and 135 (100%). Anal. ($C_{17}H_{16}N_2O_3$) C, H.

2-(3'-Bromophenyl)-5-(3''-methoxyphenyl)-2-acetyl-2,3-dihydro-1,3,4-oxadiazoline (10l). White powder (56 mg, 63%); R_f [methanol/chloroform (2:98)] 0.39; mp 115–117 °C; δ_H (400 MHz, $CDCl_3$) 7.84 (2H, d, $J = 8.8$), 7.61 (1H, s), 7.53 (1H, d, $J = 8.0$), 7.45 (1H, d, $J = 8.0$), 7.28 (1H, t, $J = 8.0$), 7.01 (1H, s), 6.96 (2H, d, $J = 8.8$), 3.88 (3H, s), and 2.36 (3H, s); ν_{max} (KBr disk)/ cm^{-1} 2917, 2849, 1644, 1557, 1509, 1462, 1425, 1364, 1316, 1256, 1174, 908, and 733; LRMS (EI^+) m/z (rel intensity) 296 (M^+ , 10%), 254 (8%), 151 (18%), and 135 (100%).

Inhibition of L1210 and B16 Cell Growth. Murine leukemia L1210 cells were obtained from American Type Tissue Culture Collection (ATCC). The murine melanoma B16-F₀ cell line was obtained from the Cancer Center of the Greenville Hospital System. The cell lines were grown in Dulbecco's Modified Eagle Medium (DMEM, Atlanta Biochemicals) supplemented with 10% fetal bovine serum, HEPES buffer (2 mM, Mediatech Cellgro), L-glutamine (2 mM, Mediatech Cellgro), and penicillin/streptomycin (50000 units penicillin, 50000 mg streptomycin, Atlanta Biologicals). Cells were maintained at 37 °C in a 5% humidified CO₂ atmosphere. Cultured cells (8000 cells/well) were plated into 96-well flat-bottom tissue culture plates. The drug solutions were diluted in DMEM and added to each well (5 μ L/well). Quadruplicate wells were prepared for each drug concentration. The plates were incubated for 72 h at 37 °C in a 5% CO₂ atmosphere. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) was dissolved in PBS (5 mg/mL). After the indicated cell incubation period, 10 μ L stock MTT solution was added to each well, and the plates were further incubated for 4 h at 37 °C in a 5% CO₂ atmosphere. After this final incubation, 100 μ L acidic isopropanol solution (16 μ L 12.1 N HCl in 5 mL isopropanol) was added to each well. The contents of the wells were mixed and the plates developed at room temperature for 15 min. The plates were then read on a Dynatech Plate Reader, utilizing Dynex Revelation 3.2 software, at a test wavelength of 570 nm and a reference wavelength of 630 nm. Dose response curves were generated and the IC₅₀ values were extrapolated from curves.

Sulforhodamine B (SRB) Assay. The antiproliferative effects of the oxadiazoline compounds against human cancer cells were evaluated using the SRB assay³³ as previously described.³⁴ The sources of the MDA-MB-435, SK-OV-3, SK-OV-3 MDR-1-6/6, HeLa, and WT β III cell lines and the procedures used to generate the drug resistant clones have been described.³² Cells were incubated with compounds for 48 h. Each IC₅₀ value represents the mean of 3–5 independent experiments, each performed in triplicate.

Indirect Immunofluorescence and Calculation of EC₅₀ Values. Cellular structures were visualized in A-10 smooth muscle cells using indirect immunofluorescence techniques, as previously described.³⁴ Cells were treated for 18–24 h with vehicle, compound **1**, or a wide range of concentrations of the oxadiazoline analogs. Microtubules were visualized with a β -tubulin antibody, centrosomes with a γ -tubulin antibody, and DNA with DAPI. Images were acquired with a digital camera and compiled and colorized using NIS Elements AR 3.0 software. The EC₅₀ values for cellular microtubule depolymerization were determined in A-10 cells by evaluating the microtubule depolymeriz-

ing effects of a range of concentrations of each compound by indirect immunofluorescence. The percent microtubule loss for each concentration was estimated visually in at least three independent experiments. A dose–response plot was generated from the averages of the experiments and the EC_{50} values were calculated by linear regression.

In Vitro Tubulin Polymerization. The kinetics of microtubule assembly in vitro were measured using the tubulin polymerization assay kit (Cytoskeleton Inc.) according to manufacturer's directions. Briefly, 300 μ g of purified bovine brain tubulin was added to tubulin assembly buffer (80 mM Na-Pipes, pH 6.9, 1 mM EGTA, 1 mM $MgCl_2$, 1 mM GTP, and 5% glycerol) and the indicated compound in a final volume of 100 μ L. Microtubule polymerization was followed by absorbance at 340 nm at 37 °C in a Spectromax Plus 96 well plate spectrophotometer (Molecular Devices).

Cell Cycle Analysis. MDA-MB-435 cells were incubated for 24 h with 0.05 μ M **10i**, vehicle, or 0.0125 μ M paclitaxel as a positive control. The cells were harvested, stained with Krishan's reagent, and analyzed using a FacsCalibur flow cytometer. Data were plotted as propidium iodide intensity versus the number of events.

Aqueous Solubility Assay. Our aqueous solubility assay is a slightly modified version of the MultiScreen Solubility Filter Plate protocol developed by Millipore. The assay is based on the shake-flask solubility method that utilizes UV–vis spectroscopy for quantification. Maximum aqueous solubility for this assay is 500 μ M. The assay is conducted at room temperature at pH 7.4. Tamoxifen, ketoconazole, and furosemide span the entire range of aqueous solubilities in this assay (30–500 μ M) and were used as reference standards. Standard curves, covering the range of 3.13–500 μ M, were generated for each compound using a 4:1 mixture of aqueous buffer (pH 7.4) to acetonitrile. Each compound was dissolved in DMSO, and the final DMSO concentration was 2.5% (v/v). Each sample was analyzed by UV–vis spectroscopy at 10 nm increments, from 260–800 nm, to determine the optimal wavelength and standard curves constructed. To measure the aqueous solubility of the reference standards and the oxadiazoline compounds, a 500 μ M solution of each compound was prepared in aqueous buffer, mixed for 6 h, and briefly centrifuged to pellet any undissolved compound. A (4:1) mixture of supernatant to acetonitrile was added to a 96-well acrylic plate and analyzed by UV–vis spectroscopy. The drug concentration in the supernatant was determined from the standard curve.

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Supporting Information Available: 1H NMR (400 MHz) spectra of the final compounds **9a–I** and **10a–I** and elemental analysis data for **9e**, **9h**, **9l**, **10e**, **10h**, and **10i** are provided. HPLC tracings of compounds **9b**, **9c**, **9f**, **10c**, **10d**, and **10f** are also included. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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