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BRIEF ARTICLE

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Synthesis and Biological Activities of (*R*)- and (*S*)-*N*-(4-Methoxyphenyl)-*N*,2,6-trimethyl-6,7-dihydro-5*H*-cyclopenta[*d*]pyrimidin-4-aminium Chloride as Potent Cytotoxic Antitubulin Agents

Aleem Gangjee,*[,†,∥] Ying Zhao,[†] Ernest Hamel,[‡] Cara Westbrook,[§] and Susan L. Mooberry*[,§,∥]

[†]Division of Medicinal Chemistry, Graduate School of Pharmaceutical Sciences, Duquesne University, 600 Forbes Avenue, Pittsburgh, Pennsylvania 15282, United States

[‡]Screening Technologies Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute at Frederick, National Institutes of Health, Frederick, Maryland 21702, United States

[§]Department of Pharmacology, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, Texas 78229, United States

Supporting Information

ABSTRACT: (*R*,*S*)-**1** is a potent antimitotic compound. (*R*)-**1**·HCl and (*S*)-**1**·HCl were synthesized from (*R*)- and (*S*)-3-methyladipic acid. Both enantiomers were potent inhibitors of cell proliferation and caused cellular microtubule loss and mitotic arrest. They inhibited purified tubulin assembly and the binding of [^{3}H]colchicine to tubulin, with (*S*)-**1** being about twice as potent. Cytotoxicity against 60 tumor cell lines, however, indicated that the (*S*)-isomer was 10- to 88-fold more potent than the (*R*)-isomer.

We[1,2] recently reported the discovery of a potent antitubulin (*R*,*S*)-*N*-(4-methoxyphenyl)-*N*,2,6-trimethyl-6,7-dihydro-5*H*-cyclopenta[*d*]pyrimidin-4-aminium chloride **1**. This compound is a water-soluble colchicine site binding, microtubule depolymerizing agent that inhibited the growth of cancer cells with GI_{50} in the nanomolar range. In addition, (*R*,*S*)-**1** overcomes the two most clinically relevant tumor resistance mechanisms that limit activity of microtubule targeting agents: overexpression of P-glycoprotein (Pgp)[3,4] and βIII-tubulin.[5−10] (*R*,*S*)-**1** was the most potent among a series of analogues evaluated and contains a chiral center at C6. It was therefore of interest to synthesize and determine the biological activities of the (*R*)-**1** and (*S*)-**1** enantiomers to ascertain the contribution of the individual isomers to the antitubulin and cytotoxic properties of the racemate and to determine if one enantiomer was more potent than the other. While there are numerous agents reported in the literature that target the colchicine binding site,[11,12] and some of these are currently in clinical trials as antitumor agents,[13] including combretastatin A-4 (CA4) (Figure 1) phosphate, there is little information regarding the activities of specific enantiomers of racemic colchicine site agents. Natural (−)-colchicine (Figure 1) has an aS,7S-absolute configuration and is much more active than its enantiomer, as is the case with other colchicinoids.[14,15] Chinigo et al.[16] synthesized 2-biphenyl-2,3-dihydroquinazoline analogue **2** (Figure 1). Racemic **2** and each enantiomer were evaluated. Racemic **2** was similar in potency to the (*S*)-isomer, while the (*R*)-isomer was 10-fold less potent as an inhibitor of tubulin assembly and [^{3}H]colchicine binding. The difference in activities of (*S*)-**2** and (*R*)-**2** was rationalized on the basis of molecular modeling.

We[1] had predicted, using molecular modeling, that the (*S*)- and (*R*)-isomers of **1** would be nearly equipotent in their effects on tubulin.

CHEMISTRY

(6*R*)-*N*-(4-Methoxyphenyl)-*N*,2,6-trimethyl-6,7-dihydro-5*H*-cyclopenta[*d*]pyrimidin-4-aminium chloride [(*R*)-**1**·HCl] was prepared from commercially available (*R*)-**3**-methyladipic acid (*R*)-**3** as shown in Scheme 1. (*R*)-**4** was synthesized via the reaction of (*R*)-**3** and concentrated sulfuric acid at reflux in ethanol followed by a Dieckmann condensation in the presence of elemental sodium in toluene and then with acetamidine hydrochloride. Chlorination of (*R*)-**4** with $POCl_3$ for 3 h afforded (*R*)-**5**. Reaction of (*R*)-**5** with 4-methoxy-*N*-methylaniline in the presence of 2−3 drops of concentrated HCl gave (*R*)-**1**. Anhydrous hydrochloric acid gas was bubbled through the anhydrous ether solution of (*R*)-**1** to give the HCl salt (*R*)-**1**·HCl as a white solid. Using the same methodology described for (*R*)-**1**·HCl in Scheme 1, (6*S*)-*N*-(4-methoxyphenyl)-*N*,2,6-trimethyl-6,7-dihydro-5*H*-cyclopenta[*d*]pyrimidin-4-aminium chloride [(*S*)-**1**·HCl] was synthesized from (*S*)-**3**.[17]

BIOLOGICAL EVALUATIONS

All of the biological evaluations were carried out on the HCl salts of (*R*)-**1** and (*S*)-**1**. The microtubule disrupting effects of (*R*)-**1** and (*S*)-**1** were observed in a cell based phenotypic screen. Both compounds caused concentration dependent loss of the interphase microtubule network, similar to the effects of colchicine, CA4[20] and (*R*,*S*)-**1**.[1] The EC_{50} (concentration required to cause 50% loss of cellular microtubules) (Table 1) was calculated to be 56 nM for (*R*,*S*)-**1**, 23 nM for (*S*)-**1**, and 278 nM for (*R*)-**1**. Thus, in this assay the (*S*)-isomer was 12-fold more potent

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than the (*R*)-isomer. Consistent with effects on microtubules, (*R*)-**1** and (*S*)-**1** caused the formation of aberrant mitotic spindles (not shown) and mitotic accumulation when measured by flow cytometry. Besides the increase in the cells in G_2/M, with all three compounds, an increase in sub-G1 population representing apoptotic cells was observed with (*R*,*S*)-**1** and (*S*)-**1** but not with (*R*)-**1** at 18 h (see Supporting Information).

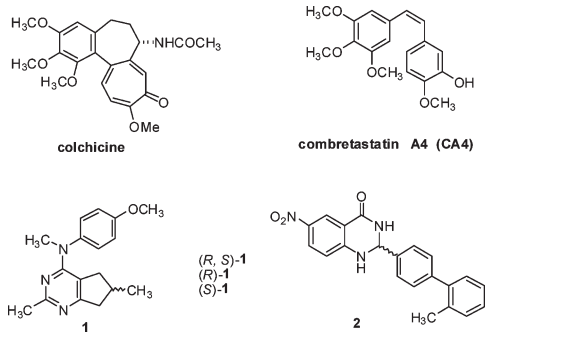
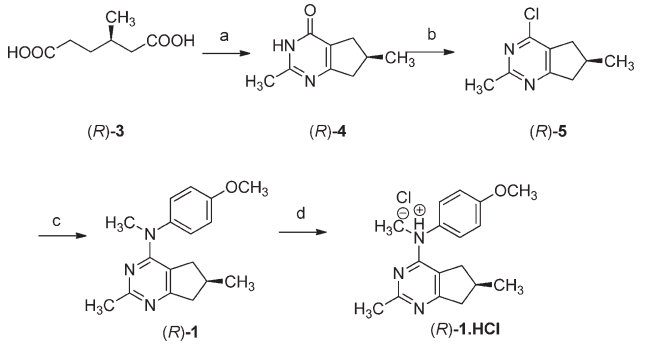


Figure 1

Scheme 1[a]



[a] Conditions: (a) (1) ethanol, conc sulfuric acid, reflux, 8 h; (2) Na, toluene, reflux, 3 h; (3) acetamidine hydrochloride, *t*-BuOH, *t*-BuOK, 25% over three steps; (b) $POCl_3$, reflux, 3 h; (c) 4-methoxy-*N*-methylaniline, *i*-PrOH, 2−3 drops HCl; (d) anhydrous HCl gas, anhydrous ether, 42% over three steps.

ANTIPROLIFERATIVE EFFECTS

(*R*)-**1** and (*S*)-**1** were tested for antiproliferative effects against the drug sensitive MDA-MB-435 cell line using the sulforhodamine B assay (SRB assay).[18,19] (*S*)-**1** and (*R*)-**1** had potent antiproliferative effects, with IC_{50} (concentration required to cause 50% inhibition of proliferation) of 12 ± 0.8 and 51 ± 4 nM. The (*S*)-enantiomer was over 4-fold more potent than the (*R*)-enantiomer and about 1.5 times more potent than the racemate (Table 1).

The ability of (*R*)-**1** and (*S*)-**1** to circumvent Pgp-mediated drug resistance was evaluated by using an SK-OV-3 isogenic cell line pair (Table 2). In this cell line pair, the relative resistance (Rr) to paclitaxel, a well-known Pgp substrate, was over 1600 while Rr values of 1.8, 3.8, 1.4, and 3.6 were obtained for (*R*,*S*)-**1**, (*S*)-**1**, (*R*)-**1**, and CA4, respectively. Thus, all four compounds are poor substrates for transport by Pgp and hence have advantages over some clinically used tubulin-targeting drugs, including paclitaxel.

A second mechanism of drug resistance that can lead to chemotherapy failure with tubulin targeting agents is the expression of the βIII isotype of tubulin.[5−10] An isogenic HeLa cell line pair[21] was used to evaluate the effects of βIII tubulin expression on the activities of (*R*)-**1** and (*S*)-**1** in comparison with paclitaxel and CA4. (*S*)-**1**, (*R*)-**1**, and CA4 had Rr of 0.7, 0.7, and 0.98, respectively (Table 2), in this cell line pair, suggesting that, like (*R*,*S*)-**1**, they overcome drug resistance mediated by βIII tubulin compared with paclitaxel, which had a Rr of 6.6. These results suggest that (*R*)- **1** and (*S*)-**1** would circumvent tumor resistance because of the overexpression of βIII tubulin.

Confirming the microtubule studies in the A-10 cells, we found that (*R*)-**1** and (*S*)-**1**, like the racemic mixture,[1] strongly inhibited the polymerization of purified bovine brain tubulin (Table 1). The (*S*)-enantiomer was slightly more active than the racemic mixture, while the (*R*)-enantiomer was slightly less active. The compounds were also examined as inhibitors of [^{3}H]colchicine binding, and the differences between the two enanatiomers and the racemic mixture were more convincing in this assay. As in the assembly assay, the order of activity was (*S*)-**1** > (*R*,*S*)-**1** > (*R*)-**1**. The compounds were compared with CA4 in both assays, and the *cis*-stilbene was somewhat more active than (*S*)-**1**. These results with tubulin corroborate our molecular modeling prediction[1] that the (*R*)-**1** and (*S*)-**1** enantiomers would have similar activity against tubulin.

Table 1. Effects of (*R*,*S*)-1·HCl, (*S*)-1·HCl, and (*R*)-1·HCl in cellular assays and on purified tubulin[a]

	cellular effects in MDA-MB-435 cells					% inhibition ± SD of colchicine binding	
compd	IC_{50} ± SD (nM)	EC_{50} (nM)	EC_{50}/IC_{50}	% G_2/M ($3 \times IC_{50}$)	inhibition of tubulin assembly, IC_{50} ± SD (μM)	5 μM inhibitor	1 μM inhibitor
H_2O control				6.1			
(*R*,*S*)-1·HCl	17 ± 0.7	56	3	65.9	1.5 ± 0.04	87 ± 2	59 ± 2
(*S*)-**1**·HCl	12 ± 0.8	23	2	49.2	1.3 ± 0.2	93 ± 0.9	70 ± 0.04
(*R*)- 1·HCl	51 ± 4	278	5	91.4	2.1 ± 0.1	77 ± 2	37 ± 3
CA4	3.4 ± 0.6	13	4	88.4	1.2 ± 0.2	98 ± 1	85 ± 0.01

[a] The IC_{50} for inhibition of proliferation was determined in MDA-MB-435 cells using the SRB assay,[18,19] $n = 3$. The EC_{50}, the concentration that causes 50% loss of interphase microtubules, was determined in A-10 cells using immunofluorescence, $n = 3$. The ratio EC_{50}/IC_{50} provides an indication of the linkage between the microtubule depolymerizing effects and inhibition of proliferation. MDA-MB-435 cells were treated with each compound at 3 × IC_{50} for inhibition of proliferation and cell cycle distribution studied after 18 h by flow cytometry. The percentage of cells in each phase of the cell cycle was determined using the Modfit program (Verity software). Inhibition of tubulin assembly and colchicine binding (Table 1) were carried out as previously reported.[1]

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Table 2. (*R*,*S*)-**1**·HCl, (*S*)-**1**·HCl, and (*R*)-**1**·HCl Circumvent Pgp and βIII-Tubulin Mediated Resistance[a]

	$IC_{50} \pm SD$ (nM)			$IC_{50} \pm SD$ (nM)		
drug	SK-OV-3	SK-OV-3 MDR-1-6/6	Rr[a]	HeLa	WTβIII	Rr[a]
(*R*,*S*)-**1**·HCl	34.5 ± 1.4	60.9 ± 4.4	1.8	37.3 ± 4.1	23.9 ± 1.7	0.6
(*S*)-**1**·HCl	16.4 ± 1.6	62.6 ± 6.7	3.8	19.0 ± 0.9	13.3 ± 0.5	0.7
(*R*)-**1**·HCl	85.9 ± 4.5	119.8 ± 11.3	1.4	92.9 ± 5.6	67.7 ± 1.3	0.7
paclitaxel	2.95 ± 0.07	4,875 ± 153	1622	1.38 ± 0.13	9.05 ± 51.1	6.6
CA4	6.05 ± 0.61	22.0 ± 6.9	3.6	4.09 ± 0.05	4.02 ± 0.26	0.98

[a] Rr: relative resistance.

(*R*,*S*)-**1**·HCl, (*S*)-**1**·HCl, and (*R*)-**1**·HCl were selected by the National Cancer Institute for evaluation in their preclinical 60 cancer cell line screen.[22] The GI_{50} values in nM are listed in Table 3 (Supporting Information). All three compounds were potent inhibitors of almost all of the cancer cell lines. However, more so than the previous evaluations, the (*S*)-**1** enantiomer was clearly superior to the (*R*,*S*)-**1** racemate, which in turn was somewhat more potent than the (*R*)-**1** enantiomer. (*S*)-**1** showed single digit nanomolar GI_{50} against 51 of the tumors evaluated, and (*R*,*S*)-**1** and (*R*)-**1** were at least 10-fold less potent than the (*S*)-isomer in most of the tumors evaluated. Thus, chirality at the 6-position of **1** does not play a major role in inhibition or binding to tubulin as demonstrated by the results in Table 1, and this validates our earlier prediction from molecular modeling. However, this is not consistent with the 10- to 88-fold potency increase of the (*S*)-isomer over the (*R*)-isomer toward cancer cells in the NCI panel. Hence, an unknown mechanism(s), perhaps facilitated transport and/or lack of metabolism, is responsible for the much greater potency of (*S*)-**1** over (*R*)-**1** in most of the NCI cancer cell lines.

SUMMARY

The (*R*)- and (*S*)-enantiomers of the potent cytotoxic antitubulin racemate (*R*,*S*)-**1** were synthesized from (*R*)- and (*S*)-3-methyladipic acid. (*R*)-**1** and (*S*)-**1** were potent inhibitors of cancer cells in culture and bind to the colchicine site on tubulin. The (*S*)-enantiomer is more active than the (*R*)-enantiomer against cellular microtubule loss and in the inhibition of tubulin assembly. Both enantiomers cause a G_2/M cell cycle arrest. (*R*)-**1** and (*S*)-**1** circumvent tumor resistance because of overexpression of Pgp and βIII tubulin, thus overcoming two important mechanisms that hamper the clinical use of the most important antitubulin agents. Against the NCI tumor cell line panel (*S*)-**1** is a single digit nanomolar inhibitor of 51 cancer cell lines and is 10- to 88-fold more potent against most of the cell lines than (*R*,*S*)-**1** or (*R*)-**1**. These results suggest that the (*S*)-isomer has significant potential and should be further examined in murine preclinical studies to evaluate its antitumor efficacy.

EXPERIMENTAL SECTION

Analytical samples were dried in vacuo (0.2 mmHg) in a CHEM-DRY drying apparatus over P_2O_5 at 50 °C. Melting points were determined on a digital MEL-TEMP II melting point apparatus with Fluke 51 K/J electronic thermometer and are uncorrected. Nuclear magnetic resonance spectra for protons (^{1}H NMR) were recorded on Bruker Avance II 400 (400 MHz) and 500 (500 MHz) NMR systems. The chemical shift values are expressed in ppm (parts per million) relative to tetramethylsilane as an internal standard: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad singlet. Thin-layer chromatography (TLC) was performed on Whatman Sil G/UV254 silica gel plates with a fluorescent indicator, and the spots were visualized under 254 and 366 nm illumination. Proportions of solvents used for TLC are by volume. Column chromatography was performed on a 230−400 mesh silica gel (Fisher Scientific) column. Elemental analyses were performed by Atlantic Microlab, Inc., Norcross, GA. Elemental compositions are within ±0.4% of the calculated values and indicate >95% purity of the compounds. Fractional moles of water or organic solvents found in some analytical samples could not be prevented despite 24−48 h of drying in vacuo and were confirmed where possible by their presence in the ^{1}H NMR spectra. All solvents and chemicals were purchased from Sigma-Aldrich Co. or Fisher Scientific Inc. and were used as received. All biological assays were performed as described previously.[1]

General Procedure for (*R*)-4 and (*S*)-4. (*R*)-3-Methyladipic acid [or (*S*)-3-methyladipic acid] (1.60 g, 10 mmol) was heated at reflux in ethanol/concentrated sulfuric acid solution (35 mL, v/v = 2.5/1) for 8 h. The solution was neutralized with ammonium hydroxide to pH 7, then diluted with ethyl acetate (100 mL) and washed with water. The organic phase was dried with anhydrous sodium sulfate and evaporated to afford a light yellow liquid that was used in the next step without further purification. The yellow liquid was dissolved in anhydrous toluene (100 mL), and sodium (0.23 g) was added to the solution. The mixture was heated at reflux for 3 h and cooled, neutralized with 1 N hydrochloric acid solution, and washed with water. After drying with anhydrous sodium sulfate, the organic phase was separated and evaporated to afford a light brown liquid. The liquid was used in the next step without further purification. The light brown liquid was diluted with *t*-BuOH. Acetamidine hydrochloride (1.13 g, 12 mmol) and potassium *tert*-butoxide (1.34 g, 12 mmol) were added, and the mixture was heated at reflux overnight. The mixture was cooled and the precipitate collected by filtration. The residue was washed with warm methanol twice (30 mL × 1, 15 mL × 1). The filtrate and washings were combined, and then 3 g of silica gel was added and the solvent removed in vacuo to afford a dry plug. This plug was placed on the top of a silica gel column and eluted with 1% methanol in chloroform. Fractions containing the product were pooled and evaporated to afford (*R*)-**4** [or (*S*)-**4**] as a white solid.

(*R*)-2,6-Dimethyl-6,7-dihydro-3*H*-cyclopenta[*d*]pyrimidin-4(5*H*)-one [(*R*)-4]. (*R*)-**4** (0.41 g, 25%) was synthesized from (*R*)-**3** (1.6 g, 10 mmol) using the general procedure described above: TLC R_f = 0.30 ($CHCl_3$/CH_3OH, 10:1); mp 173.8−175.4 °C; ^{1}H NMR (DMSO-d_6) δ 1.06(d, 3 H, *J* = 6.8 Hz, 3H), 2.16 (m, 1H), 2.24 (s, 3H), 2.31 (m, 1H), 2.44 (m, 1H), 2.77 (m, 1H), 2.86 (m, 1H), 12.16 (br, 1 H, OH, exch).

(*S*)-2,6-Dimethyl-6,7-dihydro-3*H*-cyclopenta[*d*]pyrimidin-4(5*H*)-one [(*S*)-4]. (*S*)-**4** (0.17 g, 18%) was synthesized from (*S*)-**3** (1.0 g, 6.24 mmol) using the general procedure described above: TLC R_f = 0.30 ($CHCl_3$/CH_3OH, 10:1); mp 173.9−175.4 °C; ^{1}H NMR (DMSO-d_6) δ 1.06 (d, *J* = 6.8 Hz, 3H), 2.16 (m, 1H), 2.24 (s, 3H), 2.30 (m, 1H), 2.44 (m, 1H), 2.77 (m, 1H), 2.87 (m, 1H), 12.15 (br, 1H, exch).

General Procedure for (*R*)-5 and (*S*)-5. A mixture of (*R*)-**4** [or (*S*)-**4**] and $POCl_3$ (10 mL) was heated at reflux for 3 h. The mixture

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was cooled and evaporated at reduced pressure. The residue was diluted with chloroform (50 mL), cooled in an ice bath, and neutralized carefully with ammonium hydroxide. The organic portion was washed with water (3 × 30 mL) and dried with anhydrous sodium sulfate. To this was added 1 g of silica gel, and concentration of the organic solvent afforded a dry plug. This plug was placed on the top of a silica gel column, and the column was eluted with 20% hexane in chloroform. Fractions containing the product (TLC) were pooled and evaporated to afford (*R*)-**5** [or (*S*)-**5**] as a light yellow liquid. (*R*)-**5** and (*S*)-**5** were unstable and were used for the next step without further characterization.

General Procedure for (*R*)-1·HCl and (*S*)-1·HCl. (*R*)-**5** [or (*S*)-**5**] and *N*-methyl-4-methoxyaniline were dissolved in isopropanol (5 mL). To this solution was added 37% hydrochloric acid (2−3 drops). The mixture was heated at reflux for 3−6 h. Then the mixture was cooled and the solvent evaporated at reduced pressure. The residue was diluted with chloroform, neutralized with ammonium hydroxide, and then washed with water (2 × 30 mL). After the mixture was dried with anhydrous sodium sulfate, 1 g of silica gel was added and the solvent evaporated under reduced pressure to give a dry plug. This plug was placed on the top of a silica gel column, and the column was eluted with chloroform. Fractions containing the product (TLC) were pooled and evaporated to afford pure (*R*)-**1** [or (*S*)-**1**] as a liquid. (*R*)-**1** [or (*S*)-**1**] was dissolved in anhydrous ether (10 mL), and anhydrous hydrochloric acid gas was bubbled into the solution until no further solid precipitated. The white solid was collected by filtration and dried over P_2O_5 to afford (*R*)-**1**·HCl [or (*S*)-**1**·HCl].

(6*R*)-*N*-(4-Methoxyphenyl)-*N*,2,6-trimethyl-6,7-dihydro-5*H*-cyclopenta[*d*]pyrimidin-4-aminium [(*R*)-1·HCl]. (*R*)-**1**·HCl (0.22 g, 42%) was synthesized from (*R*)-**4** (0.3 g, 1.16 mmol) using the general procedure described above: mp 196.6−197.4 °C; ^{1}H NMR (DMSO-d_6): δ 0.87 (d, J = 6.8 Hz, 3H), 1.43 (m, 1H), 1.95 (m, 1H), 2.30 (m, 1H), 2.46 (m, 1H), 2.62 (s, 3H), 3.04 (m, 1H), 3.52 (s, 3H), 3.81 (s, 3H), 7.04 (d, J = 8.9 Hz, 2H), 7.35 (d, J = 8.8 Hz, 2H), 15.12 (br, 1H, exch). Anal. ($C_{17}H_{22}N_3OCl·0.2H_2O$) C, H, N, Cl.

(6*S*)-*N*-(4-Methoxyphenyl)-*N*,2,6-trimethyl-6,7-dihydro-5*H*-cyclopenta[*d*]pyrimidin-4-aminium [(*S*)-1·HCl]. (*S*)-**1**·HCl (0.12 g, 36%) was synthesized from (*S*)-**4** (0.2 g, 6.24 mmol) using the general procedure described above: mp 196.7−197.6 °C; ^{1}H NMR (DMSO-d_6): δ 0.87 (d, J = 6.8 Hz, 3H), 1.43 (m, 1H), 1.95 (m, 1H), 2.31 (m, 1H), 2.46 (m, 1H), 2.62 (s, 3H), 3.04 (m, 1H), 3.52 (s, 3H), 3.81 (s, 3H), 7.04 (d, J = 8.9 Hz, 2H), 7.35 (d, J = 8.8 Hz, 2H), 15.10 (br, 1H, exch). Anal. ($C_{17}H_{22}N_3OCl$) C, H, N, Cl.

■ ASSOCIATED CONTENT

Supporting Information. Flow cytometry data, NCI 60 cancer cell line screen, Table 3, and elemental analysis results. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*For A.G.: phone, 412-396-6070; fax, 412-396-5593,; e-mail, gangjee@duq.edu. For S.L.M.: phone, 210-567-4788; fax, 210-567-4300; e-mail, mooberry@uthscsa.edu.

Author Contributions

‖These authors contributed equally to this work.

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■ ABBREVIATIONS USED

CA4, combretastatin A-4; MDR, multidrug resistance; Pgp, P-glycoprotein; Rr, relative resistance

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