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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

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### 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 [<sup>3</sup>H]colchicine to tubulin, with (*S*)-**1**, being about twice as potent. Cytotoxicity against a panel of 60 tumor cell lines, however, indicated that the (*S*)-isomer was 10- to 88-fold more potent than the (*R*)-isomer.

### Keywords

Antimitotic agents; Cytotoxicity; Enantiomers

We<sup>1,2</sup> 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<sub>50</sub> values 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)<sup>3,4</sup> and  $\beta$ III-tubulin.<sup>5-10</sup> (*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. Whilst there are numerous agents reported in the literature that target the colchicine binding site,<sup>11,12</sup> and some of these are currently in clinical trials as antitumor agents,<sup>13</sup> including combretastatin A-4 (CA4) (Fig. 1) phosphate,

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**Supplemental Information Available:** Results from flow cytometry data, NCI 60 cancer cell line screen, Table 3 and elemental analysis are available free of charge via the internet at <http://pubs.acs.org>.

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.<sup>14,15</sup> Chinigo et al.<sup>16</sup> synthesized 2-biphenyl-2,3-dihydroquinazoline analog **2** (Fig. 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 [<sup>3</sup>H]colchicine binding. The difference in activities of (*S*)-**2** and (*R*)-**2** were rationalized on the basis of molecular modeling.

We<sup>1</sup> 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. Compound (*R*)-**4** was synthesized via the reaction of (*R*)-**3** and conc. 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<sub>3</sub> for 3 h afforded (*R*)-**5**. Reaction of (*R*)-**5** with 4-methoxy-*N*-methylaniline in the presence of 2-3 drops of conc. 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**.<sup>17</sup>

## 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<sup>20</sup> and (*R,S*)-**1**.<sup>1</sup> The EC<sub>50</sub> (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 than the (*R*)-isomer. Consistent with effects on microtubules, both (*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<sub>2</sub>/M, with all three compounds, an increase in sub-G1 population, representing apoptotic cells was observed with both (*R,S*)-**1** and (*S*)-**1**, but not with (*R*)-**1** at 18 h. (see Supplemental Information).

## Antiproliferative effects

Compounds (*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).<sup>18, 19</sup> (*S*)-**1** and (*R*)-**1** had potent antiproliferative effects, with IC<sub>50</sub> values (concentration required to cause 50% inhibition of proliferation) of 12.0 ± 0.8 nM and 51.3 ± 3.7 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 1,600, 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  $\beta$ III isotype of tubulin.<sup>5-10</sup> An isogenic HeLa cell line pair<sup>21</sup> was used to evaluate the effects of  $\beta$ III tubulin expression on the activities of (*R*)-**1** and (*S*)-**1**, in comparison with paclitaxel and CA4. Compounds (*S*)-**1**, (*R*)-**1** and CA4 had *R*<sub>r</sub> values 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  $\beta$ III tubulin as compared with paclitaxel, which had a *R*<sub>r</sub> of 6.6. These results suggest that both (*R*)-**1** and (*S*)-**1** would circumvent tumor resistance due to the overexpression of  $\beta$ III tubulin.

Confirming the microtubule studies in the MDA-MB-435 cells, we found that (*R*)-**1** and (*S*)-**1**, like the racemic mixture,<sup>1</sup> both 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 [<sup>3</sup>H]colchicine binding, and the differences between the two enantiomers 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<sup>1</sup> that the (*R*)-**1** and (*S*)-**1** enantiomers would have similar activity against tubulin.

The mixture (*R,S*)-**1**•HCl and both (*S*)-**1**•HCl and (*R*)-**1**•HCl were selected by the National Cancer Institute for evaluation in their preclinical 60 cancer cell line screen.<sup>22</sup> The GI<sub>50</sub> 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. Compound (*S*)-**1** showed single digit nanomolar GI<sub>50</sub> values 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 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. Both (*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 as well as in the inhibition of tubulin assembly. Both enantiomers cause a G<sub>2</sub>/M cell cycle arrest. (*R*)-**1** and (*S*)-**1** circumvent tumor resistance due to overexpression of Pgp and  $\beta$ III tubulin, thus overcoming two important mechanisms that hamper the clinical use of 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 either (*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

Analytical samples were dried in vacuo (0.2 mm(add space)Hg) in a CHEM-DRY drying apparatus over P<sub>2</sub>O<sub>5</sub> 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 (<sup>1</sup>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 <sup>1</sup>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.<sup>1</sup>

### 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/conc. 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 reaction mixture was cooled and the precipitate collected by filtration. The residue was washed with warm methanol twice (30 mL x1, 15 mL x1). 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]

Compound (*R*)-4 (0.41 g, 25%) was synthesized from (*R*)-3 (1.6 g, 10 mmol) using the general procedure described above: TLC R<sub>f</sub> 0.30 (CHCl<sub>3</sub>/CH<sub>3</sub>OH, 10:1); mp 173.8-175.4 °C; <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>) 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]

Compound (*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<sub>f</sub> 0.30 (CHCl<sub>3</sub>/CH<sub>3</sub>OH, 10:1); mp 173.9-175.4 °C; <sup>1</sup>H NMR (DMSO-*d*<sub>6</sub>) 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  $\text{POCl}_3$  (10 mL) was heated at reflux for 3 h. The reaction mixture 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 ( $3 \times 30$  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. Both compounds (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

Compound (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 reaction was cooled and the solvent evaporated at reduced pressure. The residue was diluted with chloroform, neutralized with ammonium hydroxide and then washed with water ( $2 \times 30$  mL). After drying 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 compound (R)-1 [or (S)-1] as a liquid. Compound (R)-1 [or (S)-1] was dissolved in anhydrous ether (10 mL), and anhydrous hydrochloric acid gas was bubbled into the solution till no further solid precipitated. The white solid was collected by filtration and dried over  $\text{P}_2\text{O}_5$  to afford (R)-1•HCl [or (S)-1•HCl].

### (6R)-N-(4-Methoxyphenyl)-N,2,6-trimethyl-6,7-dihydro-5H-cyclopenta[d]pyrimidin-4-aminium [(R)-1•HCl]

Compound (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;  $^1\text{H}$  NMR ( $\text{DMSO}-d_6$ ):  $\delta$  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. ( $\text{C}_{17}\text{H}_{22}\text{N}_3\text{OCl} \cdot 0.2\text{H}_2\text{O}$ ) C, H, N, Cl.

### (6S)-N-(4-Methoxyphenyl)-N,2,6-trimethyl-6,7-dihydro-5H-cyclopenta[d]pyrimidin-4-aminium [(S)-1•HCl]

Compound (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;  $^1\text{H}$  NMR ( $\text{DMSO}-d_6$ ):  $\delta$  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. ( $\text{C}_{17}\text{H}_{22}\text{N}_3\text{OCl}$ ) C, H, N, Cl.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations

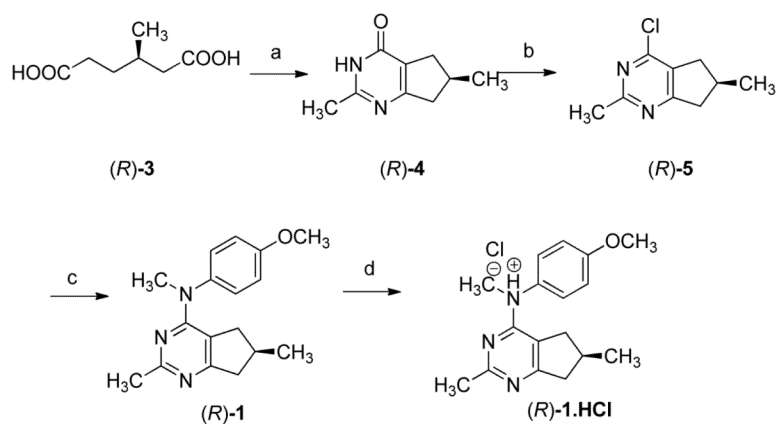
<b>CA4</b>	Combretastatin A-4
<b>MDR</b>	multidrug resistance
<b>Pgp</b>	P-glycoprotein
<b>Rr</b>	relative resistance

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**Scheme 1.a.**

<sup>a</sup> 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 3 steps; (b) POCl<sub>3</sub>, reflux, 3 h; (c) 4-methoxy-*N*-methylaniline, *i*-PrOH, 2-3 drops HCl; (d) anhydrous HCl gas, anhydrous ether, 42% over 3 steps.



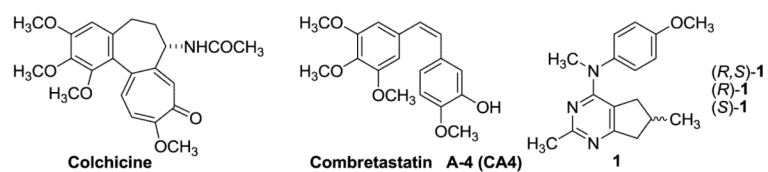
**Figure 1.**

Table 1

Effects of (R,S)-1•HCl, (S)-1•HCl, and (R)-1•HCl in cellular assays and on purified tubulin.

Cmpd	MDA-MB-435 cells				Inhibition of	
	Cellular effects				Tubulin Assembly	Colchicine Binding
	IC <sub>50</sub> (nM) ± SD	EC <sub>50</sub> (nM)	EC <sub>50</sub> /IC <sub>50</sub>	% G <sub>2</sub> /M (3×IC <sub>50</sub> )	IC <sub>50</sub> (μM) ± SD	% inhibition ± SD
					5 μM inhibitor	1 μM inhibitor
H <sub>2</sub> O control				6.1		
(R,S)-1•HCl	17 ± 0.7	56	3	65.9	1.5 ± 0.04	87 ± 2
(S)-1•HCl	12 ± 0.8	23	2	49.2	1.3 ± 0.2	93 ± 0.9
(R)-1•HCl	51 ± 4	278	5	91.4	2.1 ± 0.1	77 ± 2
CA4	3.4 ± 0.6	13	4	88.5	1.2 ± 0.2	98 ± 1
						85 ± 0.01

The IC<sub>50</sub> for inhibition of proliferation was determined in MDA-MB-435 cells using the SRB assay,<sup>18, 19</sup> n=3. The EC<sub>50</sub>, the concentration that causes 50% loss of interphase microtubules was determined in A-10 cells using immunofluorescence n=3. The ratio of EC<sub>50</sub>/IC<sub>50</sub> 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<sub>50</sub> 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 Modfit Program (Verity Software). Inhibition of tubulin assembly and colchicine binding (Table 1) were carried out as previously reported.<sup>1</sup>

**Table 2**

(*R,S*)-**1•HCl**, (*S*)-**1•HCl** and (*R*)-**1•HCl** Circumvent Pgp and  $\beta$ III-tubulin mediated resistance.<sup>a</sup>

Drug	IC <sub>50</sub> ± SD (nM)			IC <sub>50</sub> ± SD (nM)		
	SK-OV-3	SK-OV-3 MDR-1/6/6	R <sub>r</sub> <sup>a</sup>	HeLa	WTβIII	R <sub>r</sub> <sup>a</sup>
( <i>R,S</i> )- <b>1•HCl</b>	34.5 ± 1.4	60.9 ± 4.4	1.8	37.3 ± 4.1	23.9 ± 1.7	0.6
( <i>S</i> )- <b>1•HCl</b>	16.4 ± 1.6	62.6 ± 6.7	3.8	19.0 ± 0.9	13.3 ± 0.5	0.7
( <i>R</i> )- <b>1•HCl</b>	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	1,622	13.8 ± 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

<sup>a</sup>R<sub>r</sub> – Relative resistance.