

***N*-Phenyl-*N'*-(2-chloroethyl)urea analogues of combretastatin A-4: Is the *N*-phenyl-*N'*-(2-chloroethyl)urea pharmacophore mimicking the trimethoxy phenyl moiety?**

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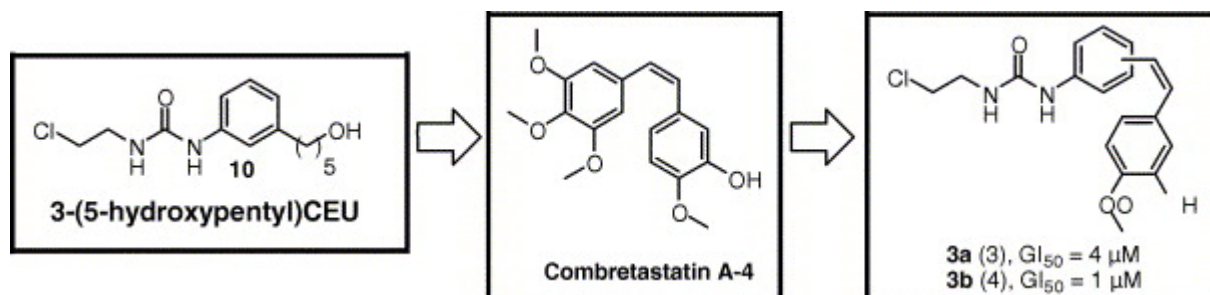
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Abstract

A series of novel *N*-phenyl-*N'*-(2-chloroethyl)urea derivatives potentially mimicking the structure of combretastatin A-4 were synthesized and tested for their cell growth inhibition and their binding to the colchicine-binding site of β -tubulin. Compounds **2a**, **3a**, and **3b** were found to inhibit cell growth at the micromolar level on four human tumor cell lines. Flow cytometric analysis indicates that the new compounds act as antimetotics and arrest the cell cycle in G₂/M phase. Covalent binding of **2a**, **3a**, and **3b** to the colchicine-binding site of β -tubulin was confirmed also using SDS-PAGE and competition assays.

Keywords. Phenyl chloroethylureas; Combretastatin analogues; Antimicrotubule agents; Anti- β -tubulin agents; Antimetotic agents; Soft alkylating agents; Anticancer drugs; Colchicine-binding site ligands.

Graphical abstract



1 Introduction

Microtubules are cytoskeletal components present in all eukaryotic cells and are recognized as playing key roles in intracellular transport, secretion, and maintenance of shape and scaffolding. They are also involved in cell division by forming the mitotic spindle during mitosis.¹ Drugs interfering with the microtubule dynamics such as *vinca* and *taxus* alkaloids are widely used in the management of several cancers.² Unfortunately, their effect is often hampered by chemoresistance and they exhibit biopharmaceutical properties suitable for the treatment of only a limited number of cancers.³ Combretastatins are a very interesting class of alternative antimitotic agents of natural origin which have received much attention due to their simple diaryl structure and their high potency as colchicine-binding agonists.⁴ Despite their very potent antitubulin activities, their therapeutic applications are limited by a high general toxicity.⁵ Therefore, several good candidates such as CA4-P,⁶ CA1-P,⁷ AVE8062,⁸ and phenstatin⁹ were in phase I/II clinical trials on advanced cancers and the results have been published.¹⁰ In this context, the promise to discover therapeutically useful colchicine site inhibitors (CSIs) has fueled innovative research programs of continued research.^{11, 12}

Over the years, we developed new antimicrotubule agents so called *N*-phenyl-*N'*-(2-chloroethyl)ureas (CEU). CEU covalently bind to the colchicine-binding site through a nucleophilic substitution involving the *N'*-(2-chloroethyl)urea pharmacophore, whereas combretastatins bind through electrostatic interactions.¹³ Recently, we showed using mass spectrometry that 4-*tert*-butyl-CEU covalently bind to β -tubulin isoform 5 by esterifying on the glutamic-198 residue (Glu198).¹⁴ Glu198 is part of a pocket that is slightly behind the two cysteine Cys239 and Cys354 residues, that are major keys for the binding of typical-CSIs involving the trimethoxyphenyl (TMP) moiety of these drugs.¹² Esterification of Glu198 by CEU may physically impede or alter the neighboring cysteine's pKa, similarly to what was described by Carvalho et al. for thioredoxin.¹⁵

Molecular modeling experiments based on the assumption that CEU are esterifying Glu198 suggested that the phenyl group of CEU might be positioned in the vicinity of Cys239 and Cys354 in a similar manner as reported with colchicinoids and combretastatin;

where the 2-and the 3-methoxyl groups are anchored nearby Cys239 and Cys354, respectively^{11, 12, 16} (Figure 1). It has been long propounded that the presence of the TMP ring moiety is crucial to obtain relevant cytotoxic and antitubulin responses¹² but different biological results have been obtained by other groups over the aromatic cycle.^{16, 19} Moreover, structural similarities between 3-(5-hydroxypentyl)CEU^{17, 18} (**10**, Figure 2), CA-4, and several colchicinoids¹¹ raised the hypothesis that the *N*-phenyl-*N'*-(2-chloroethyl)urea moiety of CEU may replace the TMP ring of CA-4. We therefore carried out the synthesis of the designed hybrids of CA-4 and CEU with the aim to induce both cell growth inhibition and irreversible colchicine-binding site inactivation (Figure 2).

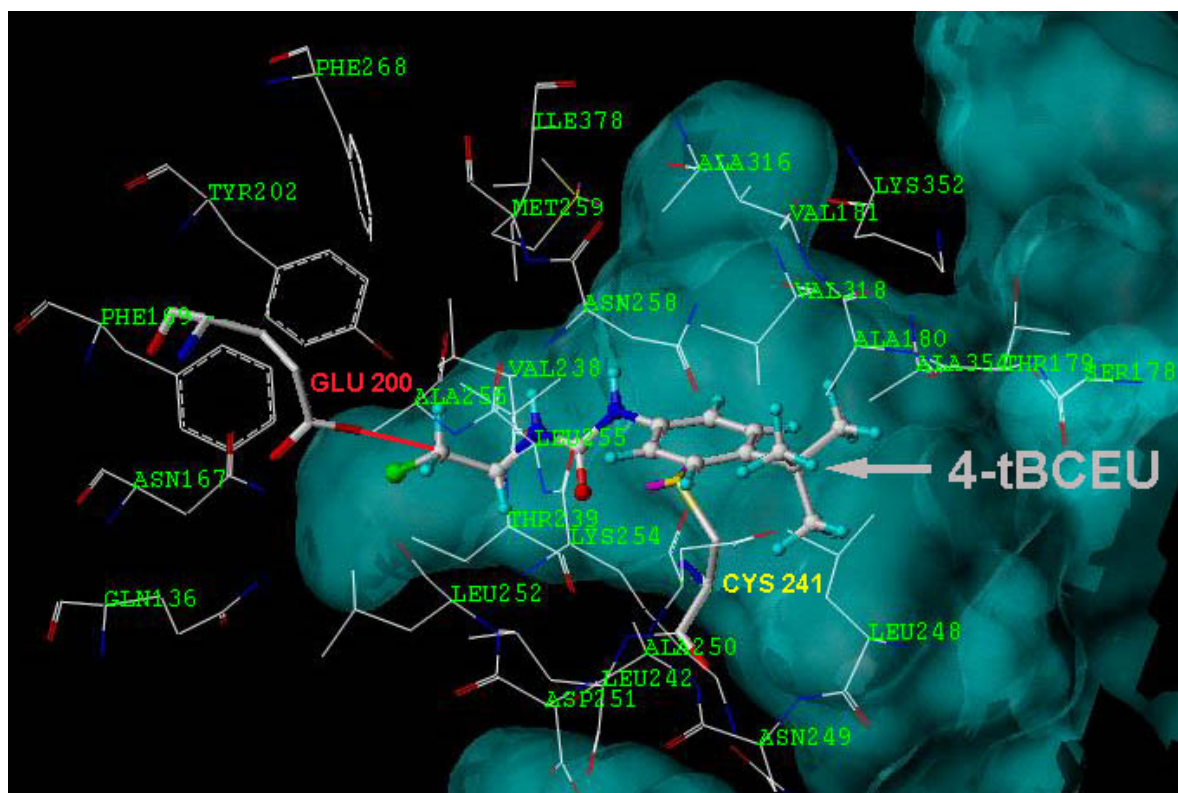


Figure 1 A molecular model of the docking of 4-*t*BCEU into the colchicine-binding site on β -tubulin that were generated with Sybyl 7.0 using an Octane 2 linked to a Onyx3800 supercomputer. The protein structure was obtained from protein data bank number 1SA0.²⁰

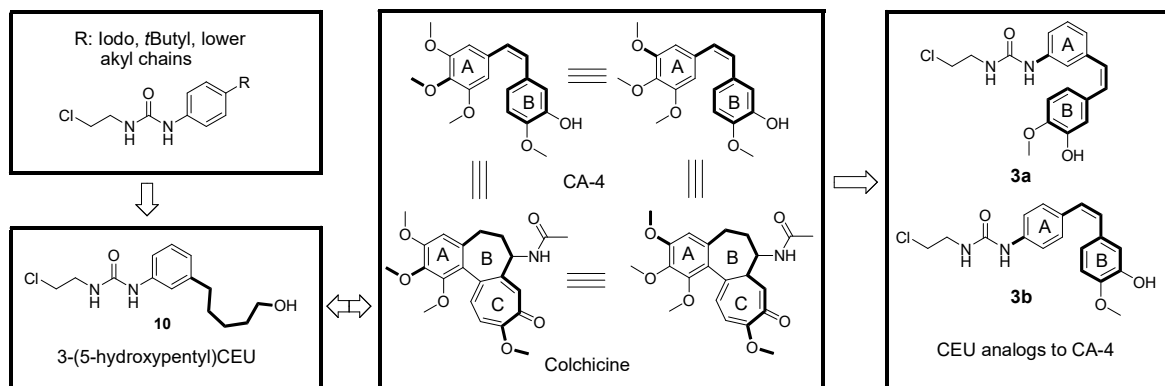
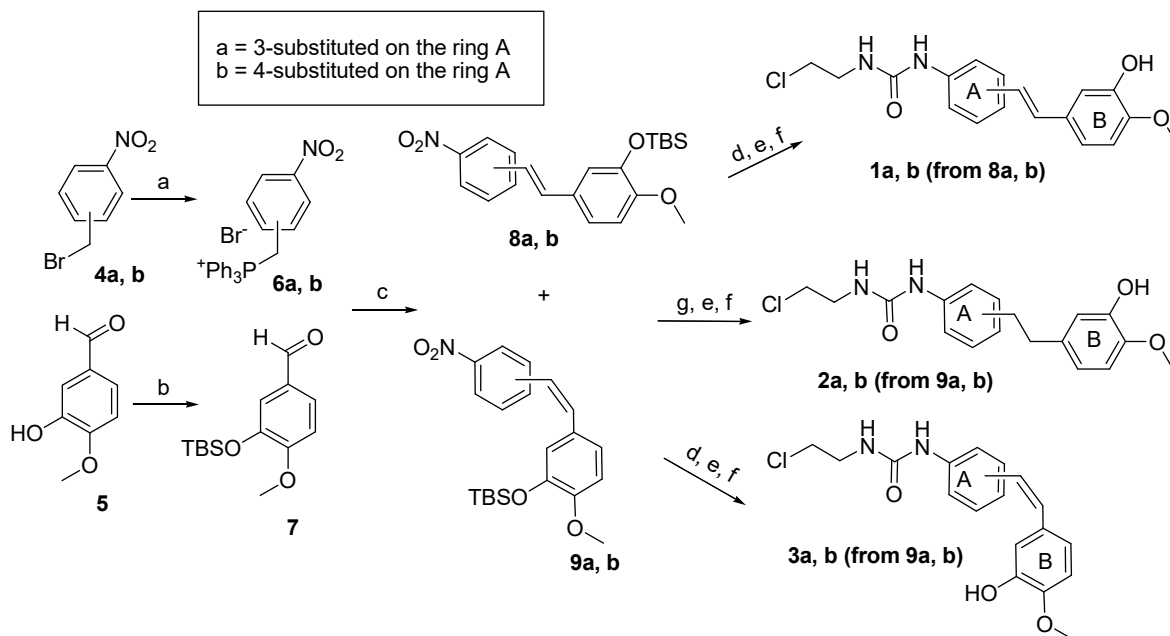


Figure 2 Structure homologies between **10**, CA-4, and colchicinoids.

2 Chemistry

Starting from commercially available nitrobenzyl bromides **4a** and **4b**, the triphenylphosphonium bromide salts were prepared by addition of triphenylphosphine in *m*-xylene to yield compound **6a**²⁰ and **6b**²¹. The phenolic group of **5** was protected with the *tert*-butyldimethylsilyl group (TBDMS) using *tert*-butyldimethylsilyl chloride, imidazoles as a base in DMF to yield compound **7**.²² The conjugation of **4a**, **b**, and **7** to **8a**, **b**, and **9a**, **b** using the Wittig-coupling was performed as described by Cushman et al.²³ Following the careful separation of the isomers by flash chromatography, the nitro group of **8a**, **8b** and **9a**, **9b** was reduced with zinc dust in acetic acid⁸ into the corresponding silylated E- and Z-amino stilbenes. The nitro and the alkenyl groups of **9a**, **b** were reduced simultaneously by catalytic hydrogenation using H₂ and Pd/C.¹⁷ The 2-chloroethylurea moiety was added by the nucleophilic addition of 2-chloroethylisocyanate onto the corresponding anilines. Removal of TBDMS group was carried out using TBAF²³ to yield compounds **1a**, **b**,²⁴ **2a**, **b**,²⁵ and **3a**, **b**²⁶ (see Scheme 1).

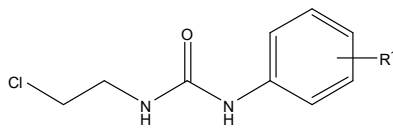


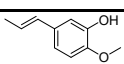

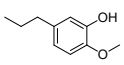
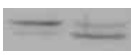
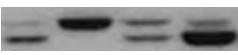
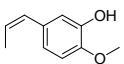
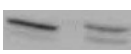

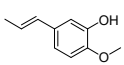

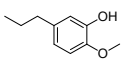
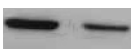
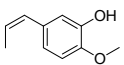
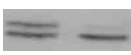
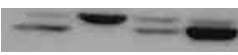
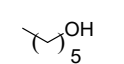
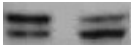

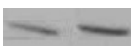


Scheme 1 Reagents and conditions: (a) PPh_3 , *m*-xylene, reflux, 16 h; (b) TBDMSiCl , imidazole, DMF, rt, 16 h; (c) NaH , DCM, rt, 16 h; (d) Zn , AcOH, rt, 2 h; (e) 2-chloroethylisocyanate, DCM, rt, 16 h; (f) TBAF 1 M, THF, rt, 16 h; (g) Pd/C 10%, H_2 (38 PSI), EtOH, rt, 8 h.

3 Biology

The cell growth inhibitory activity of CA-4-CEU hybrids **1a, b**, **2a, b**, **3a, b**, CA-4, and **10** was assessed on human colon carcinoma (HT-29), skin melanoma (M21), breast carcinoma (MCF-7), and breast hormone-independent adenocarcinoma (MDA-MB-231). As shown in Table 1, the 1,2-diarylethenyl *Z*-isomers **3a** and **3b** are significantly more active than the *E*-isomers **1a** and **1b**, and the saturated 1,2-diarylethanyl derivatives **2a** and **2b**. A similar effect of the special conformation of the biaryl bridge on the biological activity is already well documented.^{12, 19, 27} Unexpectedly CEU substituted in position 4 showed a better cell growth inhibition than CEU substituted in position 3. It could be that the covalent binding of the drugs with β -tubulin introduced spatial constraints that are partly compensated by shifting the (3-hydroxy-4-methoxyphenyl)ethenyl moiety from position 3 to position 4 of the phenyl moiety of CEU (see Scheme 1).

Table 1 Cell growth inhibition of **1a, b**, **2a, b**, **3a, b**, **10**, and CA-4.



#	R ¹	Isomer/ Position	Tumor cell lines IC ₅₀ (μM)*				SDS-Page β-Tubulin		Competition assay (48 h)			
			HT-29	M21	MCF-7	MDA-MB-231	24 h	48 h	Ctl	Col	CA-4	Vbl
1a		E/m	68.2	22.7	28.2	16.5						
2a		m	9.6	4.7	11.6	4.1						
3a		Z/m	9.2	4.0	4.5	4.9						
1b		E/p	1.18	8.38	14.0	17.4						
2b		p	15.3	16.3	15.9	11.0						
3b		Z/p	2.4	0.97	1.65	0.86						
10		m	0.16	0.27	0.48	0.23						
CA-4			0.089	0.002	0.003	0.002						
DMSO												

*IC₅₀= Drug concentration inhibiting 50 % of cell growth; Ctl= Control (treated only with the CEU).

CA-4 is known to block cell cycle in G₂/M phase due to microtubule depolymerization and cytoskeleton disruption.¹⁷ The cell growth inhibitory potency of CA-4-CEU hybrid derivatives prompted us to evaluate their effect on the cell cycle.²⁸ As shown in Table 2, the treatments of M21 cells with CEU derivatives showed that compounds **2a**, **3a**, **2b**, and **3b** caused a significant accumulation of cells in G₂/M, suggesting that these CEU, like combretastatin, might inhibit the tubulin polymerization and consequently initiate apoptosis.

Table 2 Cell cycle evaluation of M21 cells treated with **1a**, **b**, **2a**, **b**, **3a**, **b**, **10**, and CA-4.

Compd	Conc (μM)	Apoptotic cells and cell cycle phase (% of population)			
		Apoptosis	G ₁	S	G ₂ /M
1a	24	8.3	37.2	16.6	22.5
	80	5.3	38.1	18.7	21.3
	200	4.5	40.3	19.0	19.6
2a	24	12.5	23.8	20.7	27.5
	80	18.7	15.2	16.4	34.9
	200	6.9	13.5	15.7	42.6
3a	24	44.8	22.1	12.3	12.5
	80	15.0	18.4	16.4	35.5
	200	21.7	14.7	10.5	41.3
1b	24	9.9	38.1	12.9	19.5
	80	5.5	38.8	15.6	21.0
	200	11.9	37.4	21.9	12.4
2b	24	2.8	37.8	9.9	30.2
	80	2.3	15.5	13.4	45.1
	200	2.6	7.2	16.3	47.9
3b	6	10.7	11.4	15.8	43.3
	21	17.6	6.8	9.7	47.2
	53	9.7	8.1	12.5	48.0
CA-4	0.015	10.7	9.4	15.0	46.4
	0.050	15.8	10.1	14.6	40.6
	0.125	11.,6	8.2	14.7	45.4
DMSO		2.6	45.8	19.4	17.6

To confirm our hypothesis that the *N*-(2-chloroethyl)phenylurea moiety may mimic the TMP ring of CA-4, we have assessed the potential of **1a**, **b**, **2a**, **b**, **3a**, **b**, combretastatin, and **10** to covalently bind to β -tubulin using SDS-PAGE.²⁹ As shown in Table 1, cells treated with compounds **2a**, **3a**, and **3b** exhibited a second immunoreactive band of β -tubulin as reported previously with other antimicrotubule CEU,¹³ strongly suggesting the nucleophilic addition of compounds **2a**, **3a**, **3b**, and **10** to β -tubulin. No covalent binding to tubulin was observed for compounds **1a**, **b**, and **2b**. The competition assay between colchicine and the CEU shows the disappearance of the band corresponding to the CEU-tubulin by-product, suggesting that compounds **2a**, **3a**, and **3b** are covalently binding to the colchicine-binding site. Combretastatin A4 did not compete efficiently with CEU for the colchicine-binding site. This might be related to the fact that the rate constant for dissociation of combretastatin ($4.8 \times 10^{-3} \text{ s}^{-1}$)⁸ is significantly lower than for colchicine,

which is essentially irreversible.³⁰ As expected, vinblastine did not compete either with compounds **2a**, **3a** and **3b**; vinblastine binding to tubulin using a different binding site.

4 Conclusion

In summary, we have shown that new CA-4-CEU hybrid derivatives are cytotoxic on tumor cells through their nucleophilic covalent binding to β -tubulin in the colchicine-binding site. These CA-4-CEU antimitotic agents that arrest the cell cycle in G₂/M phase might be an alternative to the TMP ring such as an atypic-CSI. We therefore conclude that other structural bridges between the two aromatic rings might be investigated such as sulfonamides, sulfonates, amine or amide derivatives, cyclic or heterocyclic moiety. These results of further optimized biological activities of CA-4-CEU hybrid derivatives will be reported in due course.

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6 References and notes

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24. (E)-1-(2-Chloroethyl)-3-(3-(3-hydroxy-4-methoxystyryl)-phenyl)urea (**1a**): mp 191-193 °C; ¹H NMR (200 MHz, DMSO-d₆) δ 9.02 (s, 1H), 8.67 (s, 1H), 7.59 (s, 1H), 7.06 (m, 7H), 6.42 (s, 1H), 3.80 (s, 3H), 3.68 (t, 2H, J = 6.0 Hz), 3.45 (m, 2H); ¹³C NMR (50 MHz, DMSO-d₆) δ 155.1, 146.7, 140.6, 137.8, 130.1, 129.0, 128.3, 126.3, 119.0, 119.5, 118.5, 116.9, 115.5, 113.0, 112.3, 55.7, 44.4, 41.3. ESIMS (m/z) 371.2 [M+2+Na]⁺, 369.2 [M+Na]⁺, 349.2 [M+3]⁺, 347.2 [M+1]⁺, 346.1 [M]⁺.³¹ (E)-1-(2-Chloroethyl)-3-(4-(3-hydroxy-4-methoxystyryl)phenyl)urea (**1b**): mp 208-210 °C; ¹H NMR (200 MHz, DMSO-d₆) δ 8.14 (s, 1H), 7.46 (m, 4H), 7.10 (s, 1H), 6.96 (m, 4H), 6.13 (s, 1H), 3.85 (s, 3H), 3.69 (t, 2H, J = 6.0 Hz), 3.67 (m, 2H); ¹³C NMR (50 MHz, DMSO-d₆) δ 155.7, 148.0, 147.5, 140.5, 132.2, 127.4,

- 127.1, 127.0, 119.2, 119.0, 118.9, 113.0, 112.4, 56.2, 44.8, 42.5. ESIMS (m/z) 371.1 $[M+2+Na]^+$, 369.1 $[M+Na]^+$, 349.1 $[M+3]^+$, 347.1 $[M+1]^+$, 346.1 $[M]^+$.³¹
25. 1-(2-Chloroethyl)-3-(3-(3-hydroxy-4-methoxyphenethyl)-phenyl)urea (**2a**): mp 134-136 °C; ¹H NMR (200 MHz, CDCl₃ and CD₃OD) δ 7.09 (m, 3H), 6.75 (m, 1H), 6.65 (m, 2H), 6.55 (m, 1H), 3.76 (s, 3H), 3.53 (m, 4H), 2.73 (m, 4H); ¹³C NMR (50 MHz, CDCl₃ and CD₃OD) δ 156.3, 145.5, 145.3, 142.8, 138.8, 135.0, 128.8, 123.2, 119.7, 119.6, 117.2, 115.0, 111.1, 55.9, 44.4, 41.7, 37.9, 36.9. ESIMS (m/z) 373.1 $[M+2+Na]^+$, 371.1 $[M+Na]^+$, 351.2 $[M+3]^+$, 349.2 $[M+1]^+$, 348.1 $[M]^+$.³¹ 1-(2-Chloroethyl)-3-(4-(3-hydroxy-4-methoxyphenethyl)phenyl)urea (**2b**): mp 139-140 °C; ¹H NMR (200 MHz, DMSO-d₆) δ 7.99 (s, 1H), 7.39 (m, 2H), 7.09 (m, 2H), 6.81 (m, 1H), 6.72 (m, 1H), 6.62 (m, 1H), 6.08 (s, 1H), 3.71 (s, 3H), 3.69 (m, 2H), 3.52 (m, 2H), 2.76 (m, 4H); ¹³C NMR (50 MHz, DMSO-d₆) δ: 155.9, 147.2, 146.5, 139.0, 136.0, 135.7, 129.3, 120.0, 119.0, 116.0, 112.3, 56.2, 44.8, 38.0, 37.9, 30.6. ESIMS (m/z) 373.1 $[M+2+Na]^+$, 371.1 $[M+Na]^+$, 351.2 $[M+3]^+$, 349.2 $[M+1]^+$, 348.1 $[M]^+$.³¹
26. (Z)-1-(2-Chloroethyl)-3-(3-(3-hydroxy-4-methoxystyryl)-phenyl)urea (**3a**): mp 97-98 °C; ¹H NMR (200 MHz, CDCl₃ and CD₃OD) δ 7.12 (m, 1H), 6.98 (m, 2H), 6.77 (m, 1H), 6.60 (m, 3H), 6.25 (m, 2H), 3.67 (s, 3H), 3.35 (m, 4H); ¹³C NMR (50 MHz, CDCl₃ and CD₃OD) δ 156.4, 146.6, 145.2, 138.8, 138.2, 130.1, 129.9, 128.6, 123.4, 121.0, 119.8, 118.2, 115.3, 110.9, 55.5, 44.0, 41.5. ESIMS (m/z) 371.1 $[M+2+Na]^+$, 369.1 $[M+Na]^+$, 349.3 $[M+3]^+$, 347.3 $[M+1]^+$, 346.2 $[M]^+$.³¹ (Z)-1-(2-chloroethyl)-3-(4-(3-hydroxy-4-methoxystyryl)phenyl)urea (**3b**): mp 119-121 °C; ¹H NMR (200 MHz, DMSO-d₆) δ 8.15 (s, 1H), 7.42 (m, 2H), 7.17 (m, 2H), 6.74 (m, 3H), 6.16 (m, 2H), 3.82 (s, 3H), 3.69 (t, 2H, J = 6.0 Hz), 3.53 (m, 2H); ¹³C NMR (50 MHz, DMSO-d₆) δ 155.8, 147.5, 147.0, 140.1, 131.7, 131.6, 130.0, 129.4, 129.1, 121.2, 118.5, 116.1, 112.1, 56.1, 44.8, 42.5. ESIMS (m/z) 371.2 $[M+2+Na]^+$, 369.2 $[M+Na]^+$, 349.2 $[M+3]^+$, 347.3 $[M+1]^+$, 346.1 $[M]^+$.³¹
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28. Exponentially growing M21 cells were treated with 3-, 10-, and 25-fold the IC₅₀ of CEU and CA-4 for 24 h followed by evaluation of cell cycle distribution by flow cytometry using propidium iodide.¹³
29. M21 cells were incubated for 24 and 48 h, respectively, with 10-fold the IC₅₀ concentrations of the studied compounds. The competition assays were performed with drugs having shown significant binding to β-tubulin. M21 cells were treated with the drug (10-fold the IC₅₀) and colchicine (5 μM), CA-4 (50 nM), and vinblastine (5 μM). The cells were harvested and the proteins were quantified and analyzed using SDS-PAGE.¹³
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31. ESIMS spectra were carried out in the Mass Spectroscopy Laboratory of Molecular Medicine Research Centre, Medical Sciences Bldg, University of Toronto (http://www.medresearch.utoronto.ca/pmsc_home.html).