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# 2-Arylamino-4-Amino-5-Aroylthiazoles. "One-Pot" Synthesis and Biological Evaluation of a New Class of Inhibitors of Tubulin Polymerization

**Romeo Romagnoli**[*,†], **Pier Giovanni Baraldi**[*,†], **Maria Dora Carrion**[†], **Olga Cruz-Lopez**[†], **Carlota Lopez Cara**[†], **Giuseppe Basso**[‡], **Giampietro Viola**[‡], **Mohammed Khedr**[||], **Jan Balzarini**[§], **Siavosh Mahboobi**[⊥], **Andreas Sellmer**[⊥], **Andrea Brancale**[||], and **Ernest Hamel**[#]

[†] Dipartimento di Scienze Farmaceutiche, Università di Ferrara, 44100 Ferrara, Italy

[‡] Dipartimento di Pediatria, Laboratorio di Oncoematologia, Università di Padova, 35131 Padova, Italy

[§] Rega Institute for Medical Research, Laboratory of Virology and Chemotherapy, Minderbroedersstraat 10, B-3000 Leuven, Belgium

[||] The Welsh School of Pharmacy, Cardiff University, King Edward VII Avenue, Cardiff CF10 3NB, U.K

[⊥] Faculty of Chemistry and Pharmacy, University of Regensburg, D-93040 Regensburg, Germany

[#] Toxicology and Pharmacology Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute at Frederick, National Institutes of Health, Frederick, Maryland 21702

## Abstract

The essential role of microtubules in mitosis makes them a major target of compounds useful for cancer therapy. In our search for potent antitumor agents, a novel series of 2-anilino-4-amino-5-aroylthiazoles was synthesized and evaluated for antiproliferative activity, inhibition of tubulin polymerization, and cell cycle effects. SAR was elucidated with various substitutions on the phenylamino and aroyl moiety at the 2- and 5-positions, respectively, of the 4-aminothiazole skeleton. Tumor cell exposure to several of these compounds led to the arrest of HeLa cells in the G2/M phase of the cell cycle and induction of apoptosis.

## Introduction

There has been in recent years an intense effort in the discovery and development of novel small molecules, many of which are natural products, able to inhibit tubulin polymerization and have potential for the treatment of cancer.[1,2] One of the most important antimitotic agents is combretastatin A-4[a] (CA-4, **1**; Chart 1). CA-4, isolated from the bark of the South African tree *Combretumcaffrum*,[3] is one of the well-known natural tubulin-binding molecules affecting microtubule dynamics by binding to the colchicine site.[4] Replacement of the double bond

*To whom correspondence should be addressed. For R.R.: phone, 39-(0)532-455303; fax, 39-(0)532-455953; rmr@unife.it. For P.G. B.: phone, 39-(0)532-455921; fax, 39-(0)532-455953; baraldi@unife.it.

**Supporting Information Available:** Detailed biological protocols, molecular modeling procedure, and spectroscopic data for compounds **5a**–**y**. This material is available free of charge via the Internet at http://pubs.acs.org.

[a]Abbreviations: CA-4, combretastatin A-4; EWG, electron-withdrawing group; ERG, electron-releasing group; SAR, structure–activity relationships; MeOH, methanol; MeONa, sodium methoxide.

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ofCA-4 with a carbonyl group furnished a synthetic benzophenone-typeCA-4 analogue named phenstatin (**2a**), which demonstrated interesting efficacy in a variety of tumor models.[5] The 2-aminobenzophenone derivative **2b** also strongly inhibited cancer cell growth and tubulin polymerization and caused mitotic arrest, as did **2a**.[6]

The classical bioisosteric equivalence between benzene and thiophene prompted us recently to synthesize a series of 2-(3′,4′,5′-trimethoxybenzoyl)-3-amino-5-phenyl thiophene derivatives with general formula **3**, in which the thiophene system replaced the benzene moiety in the 2-amino phenstatin analogue **2a**.[7] The analysis of structures of 2-aminobenzophenone and 2-aroyl-3-aminothiophenes (compounds **2b** and **3**, respectively) showed that the ortho relationship between the aroyl group and the 2-amino moiety plays an essential role in activity.

Recently, investigators at Altana Pharma reported a series of [4-(imidazol-1-yl)thiazol-2-yl] phenylamine analogues with general structure **4**, active at submicromolar concentrations as antiproliferative agents against human colon adenocarcinoma (RKOp27) cells and that act as inhibitors of microtubule polymerization by interfering with the colchicine site of tubulin.[8]

As a part of our search for novel antimitotic agents, these findings prompted us to synthesize a new series of 2-arylamino- 4-amino-5-aroylthiazole derivatives with general structure **5**, obtained by incorporating the 3-amino and 2-aroyl moieties of compounds with general structure **3**, into the 4- and 5-positions, respectively, of the 2-arylaminothiazole nucleus of general formula **4**.[9]

While compounds **5a**–**f** were characterized by the unsubstituted 5-benzoylthiazole structure, we also prepared several derivatives in which the methoxy group was placed at the para (**5g**–**j**), meta (**5k**–**l**), or ortho (**5m**–**n**) position on the B-phenyl ring of the 5-benzoyl moiety. To analyze the effect of additional methoxy groups, we synthesized the 3′,4′-dimethoxy benzoyl (**5o**–**p**) and 3′,4′,5′-trimethoxybenzoyl (**5q**–**v**) analogues. Finally, to validate whether EWG's at the para-position of the B-ring can replace the methoxy group of compound **5j** with retention of activity, we prepared the chloro (**5w**), bromo (**5x**), and nitro (**5y**) analogues. Once one, two, or three methoxy substituents were placed on the 5-benzoyl moiety, we explored SAR by examining various substitutions with electron-withdrawing (F, Cl, Br, and $NO_2$) and electron-releasing (Me and MeO) groups on the A-phenyl ring of the 2-anilino moiety.

## Chemistry

The 2-anilino-4-amino-5-aroyl thiazoles **5a**–**y** were synthesized in acceptable yield (35–55%) by a one-pot, three-component reaction in MeOH of substituted phenyl isothiocyanates **6a**–**f** with α-bromoketones **7a**–**l** and cyanamide in a 1:1:1 ratio and in the presence of MeONa (in situ generated by methanol and sodium) as base (Scheme 1).[10]

## Results and Discussion

The novel series of thiazole derivatives **5a**–**y** were evaluated for their antiproliferative activity against a panel of five tumor cell lines and compared with the reference compounds CA-4 (**1**) and **4a**, as shown in Table 1. Of all the tested compounds, derivative **5q** possessed the highest overall cytostatic potency, with $IC_{50}$ values of 18, 23, 6, 9, and 14 nM against the L1210, FM3A, Molt4, CEM, and HeLa cell lines, respectively. It had comparable or slightly greater antiproliferative activity than CA-4 against three cell lines and was less potent in two lines. Comparing derivatives **5t** and **4a**, which shared a common 2-(*p*-methylanilino) thiazole moiety, **4a** was from 40- to 150-fold less active than **5t**.

In comparing the effect of ERG's or EWG's at the para-position of the A-phenyl ring, compounds with electron-donating methyl or methoxy groups were generally more cytostatic

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than those with the electron-withdrawing fluoro or chloro moieties. In six of the eight potent analogues (i.e., **5l**, **5n**, **5p–q**, and **5w–y**), there was a *para*-methoxy substitution on the A-phenyl ring, and the only inactive compound with this substituent was **5u**, while **5e** and **5j** had weak activity. The presence of the *para*-methoxy group in the A ring allowed a wide range of substituents in the B ring (*meta*- and *ortho*-methoxy groups in **5l** and **5n**, respectively; *para*-chloro, -bromo, and -nitro groups in **5w**, **5x**, and **5y**, respectively; and even a *meta*-, *para*-dimethoxy substitution in **5p**), but neither a bare B ring as in **5e**, a 4′-methoxy group as in **5j**, nor the 3′,4′, 5′-trimethoxy substitution pattern of **5u** were tolerated. However, the 3′,4′,5′-trimethoxy substitution pattern was tolerated with either no A ring substituent (the highly active **5q**) or the smaller A ring methyl substituent of **5t**.

The results also showed that, with the notable exception of **5q**, all compounds with no substituent in either ring A or ring B had minimal or reduced antiproliferative activity (**5a–g**, **5k**, **5m**, and **5o**). Again, with the exception of **5q**, the data showed that the ring B para-position is the least favorable for a methoxy group. However, para B ring EWG's are also associated with high antiproliferative activity when a *para*-methoxy-substituted A ring is also present (**5w–y**).

In the series of 3′,4′,5′-trimethoxybenzoyl thiazole derivatives **5q–v**, the unsubstituted 2-phenylamino derivative **5q** was the most active inhibitor of cell growth. Introduction of a fluorine (**5r**) or chlorine (**5s**) into the ortho-position of A-phenyl ring caused on average a 27-fold reduction of potency relative to **5q**, while the *para*-methyl compound **5t** was almost as active as **5q**. The replacement of the *para*-methyl with a *para*-methoxy (**5u**), as noted above, was highly detrimental to activity, resulting, on average, in an 83-fold reduction of potency. The compound with a *meta*-methoxy group (**5v**) was similarly inactive.

As noted above, ERG's are not indispensable on the B-ring, as shown by the activity of derivatives **5w–y**. In fact, in the para-position EWG's resulted in compounds (**5w–y**) with substantially more cytostatic activity than was observed with the *para*-methoxy group of compound **5j**.

To investigate whether the antiproliferative activities of this novel series of 4-aminothiazole derivatives involved an interaction with tubulin, a selected series of compounds (**5a**, **5f**, **5l**, **5n**, **5p–q**, **5t**, and **5w**) were evaluated for their in vitro inhibition of tubulin polymerization and for their inhibitory effects on the binding of [$^3$H]colchicine to tubulin (Table 2). For comparison, CA-4 and **4a** were examined in contemporaneous experiments. In the assembly assay, compound **5t** was found to be the most active ($IC_{50}$, 0.72 $\mu$M) and it was 2- and 5-times more potent than CA-4 and **4a** ($IC_{50}$'s of 1.4 and 4.0 $\mu$M, respectively). Derivatives **5l**, **5n**, and **5w** had activity comparable to that of CA-4, while **5p** and **5q** were about half as potent as CA-4. Unexpectedly, derivatives **5a** and **5f** were 5- and 10-fold less active than **5t** as inhibitors of tubulin assembly although they were 2 orders of magnitude less potent in their effects on cell growth. The reduced antiproliferative activities of **5a** and **5f** may result from poor permeability into cells, poor solubility in the tissue culture medium, or any other mechanism limiting the accessibility of these molecules to cellular tubulin. Alternatively, these molecules may be exerting their effect by a different, as yet uncovered, mechanism of action.

For these eight compounds, **4a** and CA-4, the order of activity as inhibitors of tubulin assembly was **5t** > **5w** > CA-4 = **5l** = **5n** > **5q** > **5p** > **5f** > **4a** ≫ **5a**. The potent activity of derivatives **5l**, **5n**, and **5w** showed that the 3′,4′, 5′-trimethoxybenzoyl group was not necessary for inhibiting tubulin assembly.

In the colchicine binding studies, derivatives **5l**, **5t**, and **5w** were as potent as CA-4, which in these experiments inhibited colchicine binding by 87%. In general, inhibition of [$^3$H]colchicine

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binding to tubulin correlated more closely with antiproliferative activity than inhibition of tubulin assembly.

The effects of compounds **5l**, **5n**, **5p–q**, **5t**, and **5w** on the cell cycle were examined by flow cytometry after staining the cells with propidium iodide. HeLa cells were exposed for 24 h to different concentrations of the compounds. All tested compounds (see Figure 1 in the Supporting Information) caused an evident and rapid increase of cells in the G2-M phase, and this was already significant for all compounds at a concentration of 30 nM. Aconcomitant decrease of cells in the G1 and S phases was also observed. All the compounds also caused the appearance of a hypodiploid peak (sub-G1), indicative of apoptosis (see Figure 2 in the Supporting Information). Compared with the sub-G1 area (8.9%) in control cells, all compounds (except **5l**) showed a significant increase in apoptotic cells in a concentration-dependent manner (see Figure 3 in the Supporting Information).

We also performed a series of molecular docking simulations in the colchicine site of tubulin with the most active compound **5t**.[11] The proposed binding of this compound is shown in Figure 1, and it is possible to see how the trimethoxyphenyl moiety is placed in proximity of Cys241 (residue numbering based on the crystal structure used). Furthermore, binding is stabilized by the presence of three hydrogen bonds between the thiazole core and Thr179, Ser178, and Thr353. These residues (Cys241, Thr179, and Ser178) were also found to be involved in the binding of other tubulin binding agents.[12] Finally, the aromatic ring in position 2 of the thiazole is on the edge of the binding pocket.

In conclusion, we have discovered a new class of structurally simple synthetic inhibitors of tubulin polymerization based on a 2-anilino-4-amino-5-aroylthiazole molecular skeleton. With the exception of 3′,4′,5′-trimethoxybenzoyl derivatives **5u–v**, we found that ERG's on the A-phenyl ring enhanced antiproliferative activity, while EWG's reduced antiproliferative activity. By comparing the effects of ERG's and EWG's on the B-phenyl ring, compounds (i.e., **5j**, **5w**, **5x**, and **5y**) with substituents with opposite electronic effects showed similar potency. We also showed by flow cytometry that selected compounds had cellular effects typical for microtubule- interacting agents, causing accumulation of apoptotic cells and cells in the G2/M phase of the cell cycle. The preparation of these compounds was carried out via an efficient procedure, and they constitute an interesting class of potent antitubulin agents which will be further evaluated as potential anticancer agents.

## Experimental Section

### General Procedure for the Synthesis of Compounds 5a–y

Sodium (188 mg, 8.2 mmol) was carefully dissolved in MeOH (10 mL) at ambient temperature. The resultant solution was added dropwise, over 10 min, to a mixture of cyanamide (345 mg, 8.2 mmol) and arylisothiocyanate (8.2 mmol, 1 equiv) dissolved in MeOH (5 mL), and cooled with an ice-bath. The appropriate phenacyl bromide (8.2 mmol, 1 equiv) was added in small portions, and the resulting mixture was stirred overnight at ambient temperature. The mixture was diluted with water (10 mL) and extracted with dichloromethane ($3 \times 15$ mL). The organic phase was washed with brine (10 mL), dried over $Na_2SO_4$, and evaporated. The residue was purified by silica gel column chromatography or crystallized from ethyl ether or petroleum ether.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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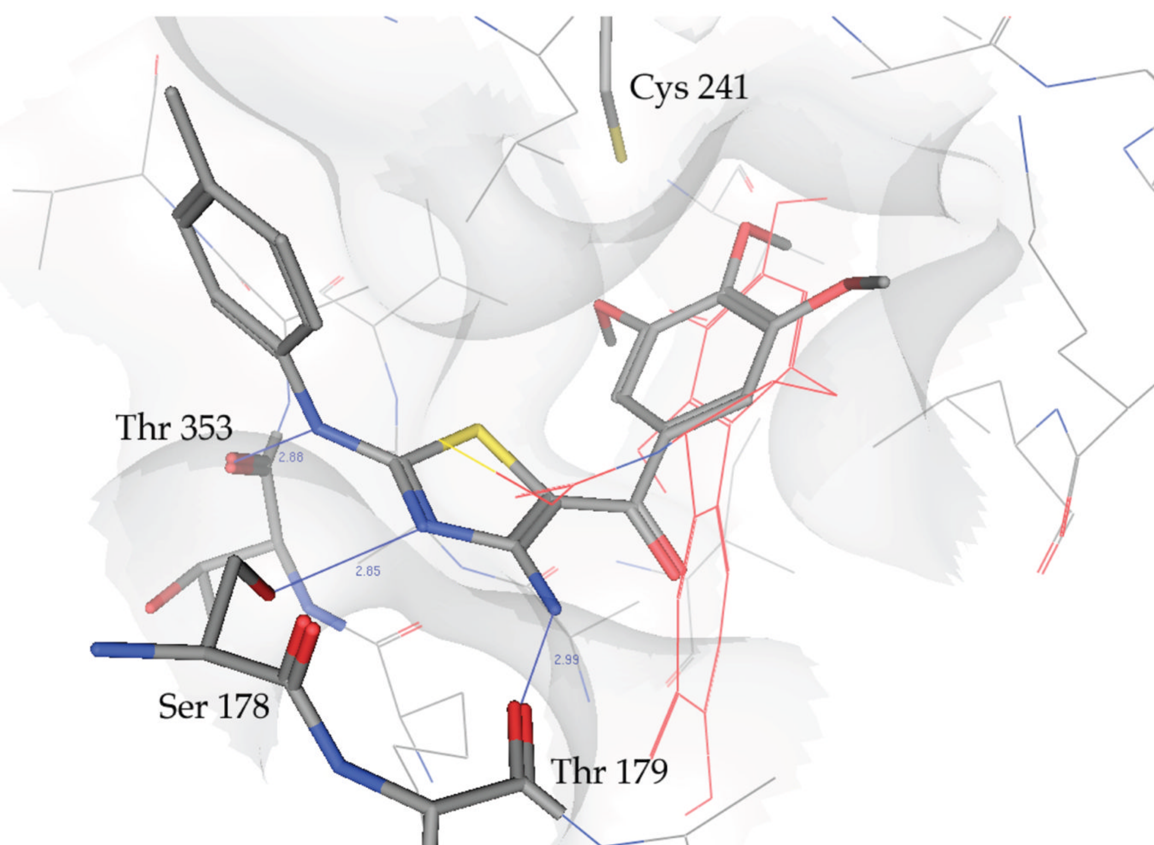


**Figure 1.**
Proposed binding mode of **5t** in the colchicine site. DAMA-colchicine in red.

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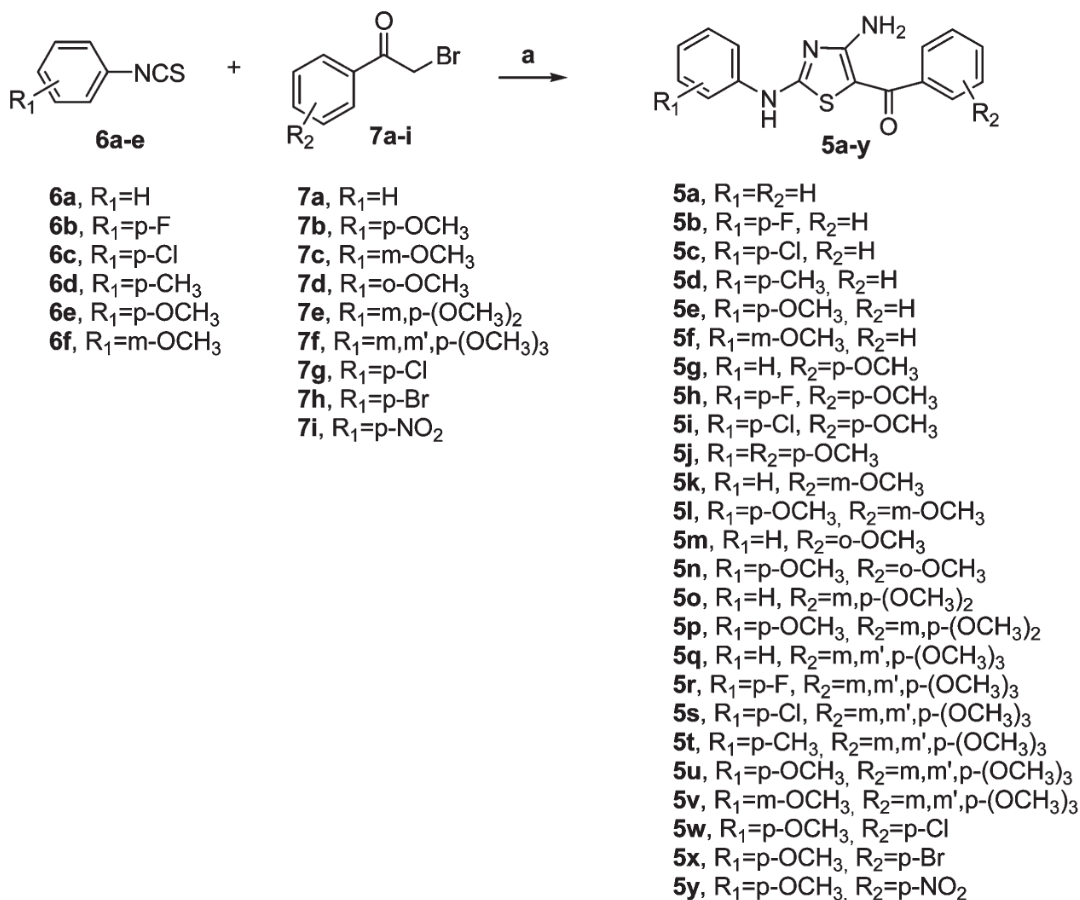


**Scheme 1a.**
[a] Reagents: (a) $NH_2CN$, Na, MeOH, rt.

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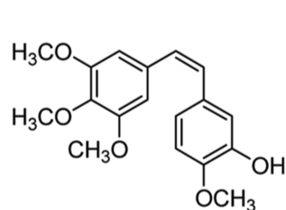
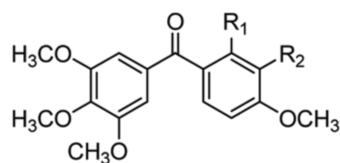
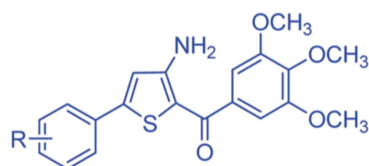



Combretastatin A-4 (CA-4), **1**

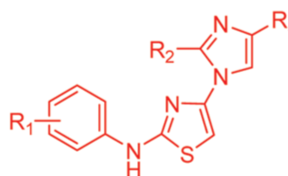



$R_1$=H, $R_2$=OH, Phenstatin, **2a**
$R_1$=$NH_2$, $R_2$=H, **2b**



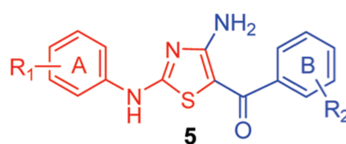

**3**

R=H, halogen, $CH_3$, $CF_3$, MeO




**4**

$R_1$=H, Br, Me or OMe
$R_2$ and $R_3$=H or Me
**4a**, $R_1$=p-Me, $R_2$=Me, $R_3$=H




**5a**, $R_1$=$R_2$=H
**5b**, $R_1$=p-F, $R_2$=H
**5c**, $R_1$=p-Cl, $R_2$=H
**5d**, $R_1$=p-$CH_3$, $R_2$=H
**5e**, $R_1$=p-$OCH_3$, $R_2$=H
**5f**, $R_1$=m-$OCH_3$, $R_2$=H
**5g**, $R_1$=H, $R_2$=p-$OCH_3$
**5h**, $R_1$=p-F, $R_2$=p-$OCH_3$
**5i**, $R_1$=p-Cl, $R_2$=p-$OCH_3$
**5j**, $R_1$=$R_2$=p-$OCH_3$
**5k**, $R_1$=H, $R_2$=m-$OCH_3$
**5l**, $R_1$=p-$OCH_3$, $R_2$=m-$OCH_3$
**5m**, $R_1$=H, $R_2$=o-$OCH_3$
**5n**, $R_1$=p-$OCH_3$, $R_2$=o-$OCH_3$
**5o**, $R_1$=H, $R_2$=m,p-$(OCH_3)_2$
**5p**, $R_1$=p-$OCH_3$, $R_2$=m,p-$(OCH_3)_2$
**5q**, $R_1$=H, $R_2$=m,m',p-$(OCH_3)_3$
**5r**, $R_1$=p-F, $R_2$=m,m',p-$(OCH_3)_3$
**5s**, $R_1$=p-Cl, $R_2$=m,m',p-$(OCH_3)_3$
**5t**, $R_1$=p-$CH_3$, $R_2$=m,m',p-$(OCH_3)_3$
**5u**, $R_1$=p-$OCH_3$, $R_2$=m,m',p-$(OCH_3)_3$
**5v**, $R_1$=m-$OCH_3$, $R_2$=m,m',p-$(OCH_3)_3$
**5w**, $R_1$=p-$OCH_3$, $R_2$=p-Cl
**5x**, $R_1$=p-$OCH_3$, $R_2$=p-Br
**5y**, $R_1$=p-$OCH_3$, $R_2$=p-$NO_2$

**Chart 1.**
Inhibitors of Tubulin Polymerization

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**Table 1**

In Vitro Inhibitory Effects of Compounds **4a**, **5a–y** and CA-4 (**1**)

| compd | $IC_{50}$[a] (nM) | | | | |
|---|---|---|---|---|---|
| | **L1210** | **FM3A** | **Molt4** | **CEM** | **HeLa** |
| **5a** | 8100 ± 200 | 8200 ± 100 | 7300 ± 1100 | 3900 ± 800 | 6100 ± 1600 |
| **5b** | >10000 | >10000 | >10000 | >10000 | >10000 |
| **5c** | >10000 | >10000 | >10000 | >10000 | >10000 |
| **5d** | 2800 ± 400 | 1500 ± 900 | 210 ± 130 | 370 ± 120 | 900 ± 260 |
| **5e** | 850 ± 170 | 730 ± 390 | 230 ± 140 | 300 ± 10 | 230 ± 50 |
| **5f** | 8200 ± 600 | 5000 ± 800 | 1500 ± 200 | 2900 ± 300 | 3200 ± 1100 |
| **5g** | >10000 | >10000 | 6900 ± 1900 | 9100 ± 5000 | >10000 |
| **5h** | >10000 | >10000 | >10000 | >10000 | >10000 |
| **5i** | >10000 | >10000 | >10000 | >10000 | >10000 |
| **5j** | 470 ± 58 | 630 ± 110 | 320 ± 70 | 1200 ± 110 | 330 ± 10 |
| **5k** | 7600 ± 2200 | 6200 ± 1900 | 1300 ± 100 | 2300 ± 200 | 1700 ± 0.0 |
| **5l** | 35 ± 14 | 50 ± 26 | 4.0 ± 2.3 | 9.9 ± 1.8 | 37 ± 12 |
| **5m** | 1100 ± 600 | 1100 ± 200 | 1300 ± 0.0 | 730 ± 490 | 1200 ± 100 |
| **5n** | 31 ± 11 | 52 ± 15 | 18 ± 2 | 17 ± 3 | 17 ± 0.0 |
| **5o** | >10000 | >10000 | 5500 ± 3700 | 6900 ± 1600 | 6000 ± 900 |
| **5p** | 38 ± 28 | 71 ± 26 | 37 ± 31 | 10 ± 2 | 52 ± 12 |
| **5q** | 18 ± 0.0 | 23 ± 10 | 6 ± 1.1 | 9.4 ± 3.6 | 14 ± 1 |
| **5r** | 900 ± 480 | 58 ± 100 | 280 ± 170 | 400 ± 20 | 440 ± 40 |
| **5s** | 420 ± 170 | 420 ± 10 | 110 ± 10 | 190 ± 70 | 180 ± 40 |
| **5t** | 31 ± 6.0 | 15 ± 3.0 | 21 ± 1.1 | 41 ± 3.5 | 86 ± 10 |
| **5u** | 1500 ± 1000 | 2200 ± 900 | 480 ± 150 | 1100 ± 0.0 | 560 ± 40 |
| **5v** | 2100 ± 300 | 2000 ± 300 | 530 ± 0.00 | 1300 ± 0.0 | 1000 ± 200 |
| **5w** | 84 ± 30 | 59 ± 37 | 13 ± 7 | 19 ± 6.9 | 83 ± 6 |
| **5x** | 110 ± 0.00 | 100 ± 0.00 | 16 ± 6 | 39 ± 11 | 79 ± 4 |
| **5y** | 280 ± 110 | 310 ± 160 | 29 ± 11 | 31 ± 12 | 350 ± 60 |
| **4a** | 1800 ± 71 | 1200 ± 0 | 510 ± 11 | 1500 ± 71 | 530 ± 64 |
| CA-4 | 2.8 ± 1.1 | 42 ± 6.0 | 16 ± 1.4 | 12 ± 2.5 | 1.9 ± 1.6 |

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[a] $IC_{50}$ = compound concentration required to inhibit tumor cell proliferation by 50%.

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**Table 2**

Inhibition of Tubulin Polymerization and Colchicine Binding by Compounds **4a**, **5l**, **5n**, **5p–q**, **5t**, **5w**, and CA-4

| compd | tubulin assembly[a] $IC_{50}$ ± SD (μM) | colchicine binding[b] % ± SD |
|---|---|---|
| **5a** | 8.0 ± 0.7 | 35 ± 2 |
| **5f** | 3.6 ± 0.4 | 43 ± 2 |
| **5l** | 1.4 ± 0.2 | 84 ± 2 |
| **5n** | 1.4 ± 0.2 | 77 ± 4 |
| **5p** | 2.8 ± 0.3 | 63 ± 0.2 |
| **5q** | 2.5 ± 0.3 | 75 ± 2 |
| **5t** | 0.72 ± 0.01 | 87 ± 1 |
| **5w** | 1.1 ± 0.1 | 89 ± 2 |
| **4a** | 4.0 ± 0.4 | 58 ± 3 |
| CA-4 (1) | 1.4 ± 0.1 | 87 ± 3 |

[a]Inhibition of tubulin polymerization. Tubulin was at 10 μM.

[b]Inhibition of [$^3$H]colchicine binding. Tubulin, colchicine, and tested compound were at 1, 5, and 1 μM, respectively.