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Synthesis and preliminary biological evaluation of new anti-tubulin agents containing different benzoheterocycles

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Abstract—A new series of compounds, in which the 2-amino-4-methoxyphenyl ring of phenstatin analogue **5** was replaced with 2- or 3-amino-benzoheterocycles, was synthesized and evaluated for antiproliferative activity and inhibition of colchicine binding. The lack of activity of 3′,4′-dimethoxy- and 4′-methoxy-benzoyl derivatives (**8** and **9**, respectively) indicates that the 3′,4′,5′-trimethoxybenzoyl moiety is critical for the activity. Two compounds, **7** and **11**, displayed potent antiproliferative activity, with IC_{50} values ranging from 25 to 100 nM against a variety of cancer cell lines. Derivative **11** was more active than CA-4 as an inhibitor of tubulin polymerization. The results demonstrated that the antiproliferative activity was correlated with inhibition of tubulin polymerization.

Despite the progress made in recent years in the sector of new drugs endowed with antiangiogenic activity, this sector of research is still considered as one of the most promising fields for the discovery of new drugs for the treatment of diseases characterized by abnormal angiogenesis, and in particular for the treatment of tumors.[1] Research oriented toward the discovery of new generation agents useful in cancer chemotherapy has identified tubulin as a possible cellular target.[2a]

The microtubule cytoskeleton plays a very important role in regulating cell architecture. The microtubule systems of eukaryotic cells comprise a dynamic matrix in which heterodimers of tubulin polymerize to form microtubules both in neoplastic and normal cells. Consequently, natural and synthetic substances capable of altering the polymerization or depolymerization of microtubules have been effective as chemotherapeutic agents.[2b] Combretastatin A-4 (CA-4, **1**), isolated from an African willow, *Combretum caffrum* (*Combretaceae*),[3] shows interesting anticancer potential due to its antitubulin properties. CA-4 strongly binds to the colchicine site of tubulin.[4] This binding prevents tubulin polymerization and causes an antimitotic effect. CA-4 inhibits cell growth at low to mid-nanomolar concentrations.[3] The sodium phosphate prodrug of CA-4 (CA-4P, **2**) is water-soluble and has yielded promising results in current Phase I human cancer clinical trials.[5] Its structural simplicity, along with the ability to selectively damage tumor neovascularization, makes CA-4 of great interest from the medicinal chemistry viewpoint.

For these reasons, a large number of CA-4 analogues have been synthesized and evaluated in structure–activity relationship (SAR) studies.[6] Replacing the ethene

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bridge of CA-4 with a carbonyl group furnished a benzophenone derivative named Phenstatin (**3**), which was found to be a very strong cytotoxic agent with the same characteristics as CA-4.[7] Changing the position of the hydroxyl group from position C-3 to C-2, to furnish the Phenstatin isomer **4**, dramatically decreased bioactivity.[8] Replacing the hydroxyl moiety at the C-2 position of the benzophenone ring (compound **5**) with an amino group increased cytotoxicity 100-fold as compared with **4**, indicating that the amino and hydroxy groups were not bioequivalent at the C-2 position. The 2-amino benzophenone derivative **5** showed significantly increased cytotoxicity against many human cancer cell lines as compared with Phenstatin **3**, but **5** was nevertheless slightly less potent than CA-4.[8] Lack of the methoxy group (compound **6**) at the C-4 position resulted in significantly decreased growth inhibitory activity.

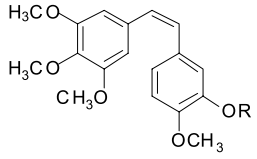
In this article, we report the synthesis and biological evaluation of new compounds **7**–**13**, that are structurally related to the 2-aminobenzophenones **5** and **6**, in which the 2-aminophenyl moieties of the latter derivatives were replaced with various 2- or 3-aminobenzoheterocycles such as benzo[*b*]thiophene, benzo[*b*]furan, and indole. We confirmed that a number of these new compounds are active cytotoxic agents with significant antitubulin activity. Since the removal of either the C′-4 or the C′-5 methoxy group causes a substantial loss of cytotoxicity in the CA-4 system,[9] through the synthesis of compounds **8** and **9**, we investigated the effect of the 3′,4′,5′-trimethoxybenzoyl group in the 2-amino benzo[*b*]thiophene derivative **7** on the antiproliferative activity. The 3-(3′,4′,5′-trimethoxybenzoyl)-indole, -benzofuran, and -benzothiophene molecular skeletons are the core structure of a series of antitubulin agents (compounds **14**–**16**, respectively), which showed activity comparable to that of CA-4.[10]

Compounds **7**–**9** were synthesized by a four-step synthesis described in Scheme 1.[11] 2-Amino-3-aroyl 4,5,6,7-tetrahydrobenzo[*b*]thiophenes **20**–**22** were obtained by the Gewald reaction[12] applied to β-ketonitriles **17**–**19**[13] and cyclohexanone. Acetylation of the amino group using a mixture of acetic anhydride and pyridine, and the subsequent dehydrogenation with Pd/C with heating afforded the benzo[*b*]thiophene derivatives **23**–**25**, which were transformed by saponification into the final products **7**–**9**.

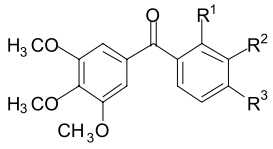
The two ligands based on the 3-amino-benzo[*b*]thiophene molecular skeleton (**10** and **11**), along with the 3-amino-benzo[*b*]furan **12**, were prepared in excellent yield by a 'one-pot' cyclization reaction between 2-cyanothiophenol **26**, 4-methyl-2-cyanothiophenol **27**, and 2-cyanophenol **28**, respectively, with 2-bromo-3′,4′,5′-trimethoxyacetophenone **29**[14] and K_2CO_3 in acetone (Scheme 2). Compounds **26** and **27** were synthesized by the condensation of commercially available 2-nitrobenzonitrile **30** and 2-nitro-4-methylbenzonitrile **31**, respectively, with benzylmercaptan anion and subsequent S-debenzylation with aluminium chloride.[15]

Compound **13** was prepared following the procedure reported in Scheme 3. Derivative **32**[16] was reacted with bromoacetophenone **29** to give the *N*-ethoxycarbonyl indole intermediate, which was converted into the corresponding *N*-unsubstituted indole **13** after alkaline hydrolysis.

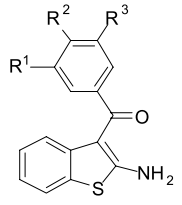
In Table 1, we report the in vitro antiproliferative activity of compounds **7**–**13** and **20** against a panel of tumor cell lines, using CA-4 as reference compound. It is noteworthy that the antiproliferative effects of **7**–**8**, **10**–**11**, and **20** were more pronounced against Molt/4 and CEM as compared with L1210 and FM3A. Compounds



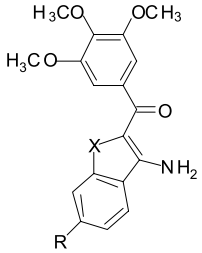
R=H, Combretastatin A-4 (CA-4), **1**
R=PO_3Na_2, CA-4P, **2**



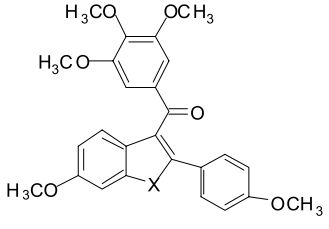
R^1=H, R^2=OH, R^3=OCH_3, Phenstatin, **3**
R^1=OH, R^2=H, R^3=OCH_3, **4**
R^1=NH_2, R^2=H, R^3=OCH_3, **5**
R^1=NH_2, R^2=H, R^3=H, **6**



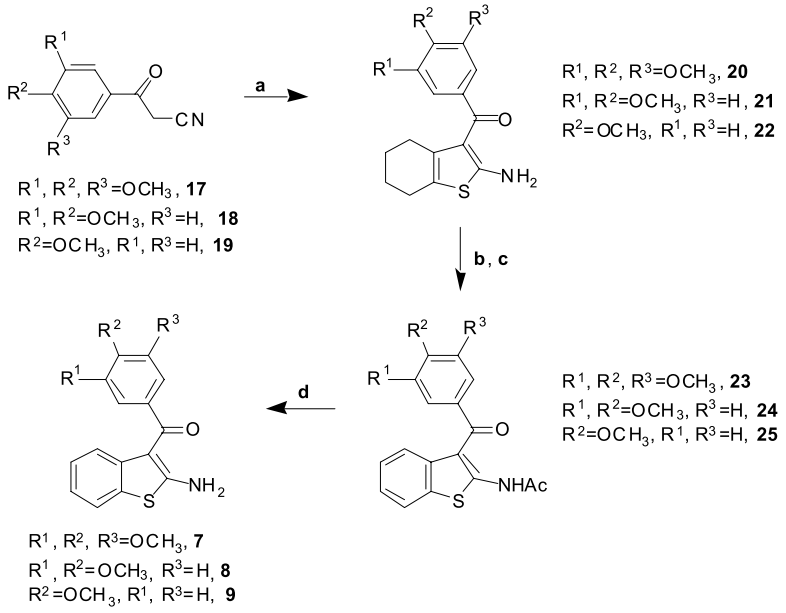
R^1, R^2, R^3=OCH_3, **7**
R^1, R^2=OCH_3, R^3=H, **8**
R^2=OCH_3, R^1, R^3=H, **9**



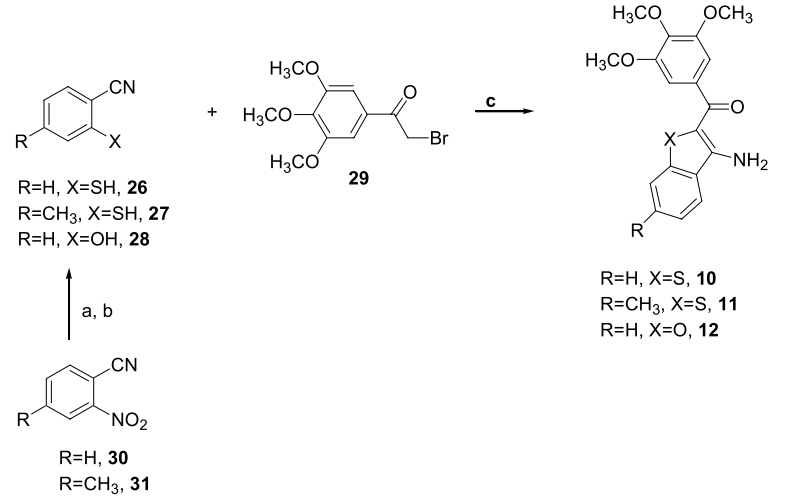
X=S, R=H, **10**
X=S, R=CH_3, **11**
X=O, R=H, **12**
X=NH, R=H,**13**



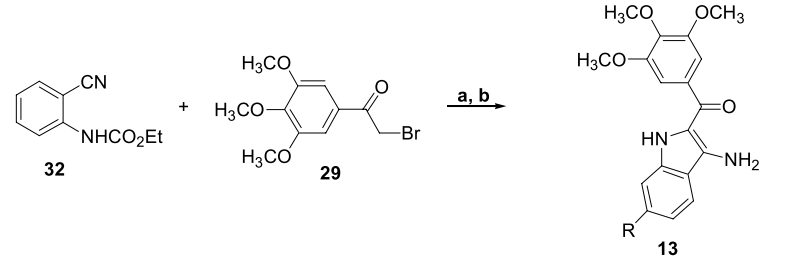
X=NH, **14**
X=O, **15**
X=S, **16**



Scheme 1. Reagents and conditions: (a) cyclohexanone, S_8, morpholine, EtOH, 70 °C for 1 h then 18 h at rt; (b) Ac_2O, pyridine, reflux; (c) 10% Pd/C moistened with a 50% water, 130 °C, 18 h; (d) KOH, EtOH, reflux, 2 h.



Scheme 2. Reagents and conditions: (a) $C_6H_5CH_2SH$, KOH, DMF; (b) $AlCl_3$, C_6H_6; (c) K_2CO_3, acetone, reflux, 12 h.



Scheme 3. Reagents and conditions: (a) NaH, DMF, rt, 24 h; (b) NaOH, EtOH/H_2O, reflux, 1 h.

7 and **11** demonstrated substantial growth-inhibitory effects on the proliferation of murine leukemia (L1210), murine mammary carcinoma (FM3A), and human T-lymphoblastoid (Molt/4 and CEM) cells. However, CA-4 was more potent than any of the new compounds in all cell lines examined.

Comparing the molecules which possess the trimethoxy benzoyl moiety, the benzofused heterocyclic compounds containing a nitrogen (**13**) or an oxygen (**12**) atom in the heterocyclic ring are much less effective than their sulfur analogues **7** and **10**. Comparing the two benzo[*b*]thiophene isomers **7** and **10**, the latter was about 4-fold less

Table 1. In vitro growth inhibition activity of compounds **7**–**13**, **20**, and CA-4 against murine leukemia (L1210), murine mammary carcinoma (FM3A), and human T-lymphoblast (Molt/4 and CEM) cells

Compound	IC_{50}[a] (nM)			
	L1210	FM3A	Molt4/C8	CEM
7	90 ± 3.2	100 ± 0	73 ± 9	74 ± 15
20	2240 ± 122	5680 ± 315	1580 ± 135	2150 ± 234
8	3700 ± 60	4700 ± 2500	1700 ± 200	1700 ± 500
9	>10,000	>10,000	9200 ± 200	>10,000
10	350 ± 320	1800 ± 300	290 ± 10	310 ± 20
11	58 ± 50	71 ± 5	34 ± 3	24 ± 2
12	>10,000	>10,000	>10,000	>10,000
13	>10,000	>10,000	>10,000	>10,000
CA-4 (**1**)	2.8 ± 1.1	42 ± 6.0	16 ± 1.4	1.9 ± 1.6

Data are expressed as means ± SE from the dose–response curves of at least three independent experiments.
[a] IC_{50}, compound concentration required to inhibit tumor cell proliferation by 50%.

active than **7** with L1210, Molt/4, and CEM cells, and almost 20-fold less active with FM3A cells. The reduced antiproliferative activity of the 4,5,6,7-tetrahydrobenzo[*b*]thiophene derivative **20** as compared with **7** demonstrates that the aromaticity of the benzene ring fused with the thiophene is critical for activity.

The series of derivatives **7**–**9** demonstrates that all three methoxy groups are essential for activity. Substitution of the trimethoxybenzene group with 3′,4′-dimethoxybenzoyl and 4′-methoxybenzoyl moieties (compounds **8** and **9**, respectively), almost eliminated growth inhibition activity.

Compound **10**, with a 3-(3′,4′,5′-trimethoxybenzoyl)-2-amino-benzo[*b*]thiophene nucleus, had moderate antiproliferative effects on the growth of L1210, Molt4, and CEM cells (IC_{50} = 290–350 nM) and about 5-fold lower activity against the FM3A cell line. The introduction of a lipophilic and electron-releasing methyl group at the C-6 position of **10**, to afford the derivative **11**, resulted in an increase in the antitumor activity by one order of magnitude. This compound was especially effective against CEM and Molt4/C8 cells, with IC_{50}'s of 24 and 34 nM, respectively.

To investigate whether the antiproliferative activities of these compounds were related to an interaction with tubulin, compounds **7**, **10**, **11**, and **20** were evaluated for inhibition of the polymerization of purified tubulin.[17] The same compounds were also examined for inhibitory effects on the binding of [^{3}H]colchicine to tubulin[18] (Table 2).

Compound **20** did not greatly alter tubulin assembly at concentrations as high as 40 μM nor did it inhibit colchicine binding to tubulin. Compounds **7**, **10**, and **11** all strongly inhibited tubulin assembly, and compound **11** seemed to be even more active than the reference compound CA-4. Compound **7** was half as active (IC_{50} = 3.1 μM) and **10** about one-third as active (IC_{50} = 4.2 μM) as **11**. Thus, the order of inhibitory effects on tubulin polymerization was **11** > CA-4 > **7** > **10** >> **20**. This order of activity as inhibitors of tubulin assembly correlates well with their order of activity as cytotoxic agents, except that CA-4 was more cytotoxic than compound **11**.

Table 2. Inhibition of tubulin polymerization and colchicine binding by compounds **7**, **10**, **11**, and **20**

Compound	Tubulin assembly[a] IC_{50} ± SD (μM)	Colchicine binding[b] % ± SD	
		5 μM drug	2 μM drug
7	3.1 ± 0.3	57 ± 1	ND
20	>40	3 ± 0.3	ND
10	4.2 ± 0.7	39 ± 9	ND
11	1.3 ± 0.8	88 ± 9	77 ± 4
CA-4 (**1**)	2.0 ± 0.3	97 ± 6	95 ± 3

ND, not determined.
[a] Inhibition of tubulin polymerization. Tubulin was at 10 μM.
[b] Inhibition of [^{3}H]colchicine binding. Tubulin was at 1 μM, [^{3}H]colchicine at 5 μM.

When inhibitory effects on colchicine binding were evaluated, however, CA-4 proved to be somewhat more potent than compound **11**, which was the most potent of the new agents, but otherwise order of activity in the two tubulin-based assays was identical.

In conclusion, we have discovered a new type of inhibitor of tubulin polymerization based on the 2-(3′,4′,5′-trimethoxybenzoyl)-3-amino-benzo[*b*]thiophene molecular skeleton. Thus far the promising compound in this series is the 2-(3′,4′,5′-trimethoxybenzoyl)-3-amino-6-methylbenzo[*b*]thiophene derivative **11**. Compound **11** is a potent antiproliferative agent and inhibitor of tubulin polymerization through binding to the colchicine-binding site of tubulin. A noteworthy point was that the preparation of **11** was carried out via an efficient synthesis and it represents the lead compound of an interesting new class of antitubulin agents with potential utility for the treatment of human cancer.

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