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Synthesis and biological evaluation of 2-amino-3-(3′,4′,5′-trimethoxybenzoyl)-6-substituted-4,5,6,7-tetrahydrothieno[2,3-*c*]pyridine derivatives as antimitotic agents and inhibitors of tubulin polymerization

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ABSTRACT

Microtubules are among the most successful targets of compounds potentially useful for cancer therapy. A new series of inhibitors of tubulin polymerization based on the 2-amino-3-(3,4,5-trimethoxybenzoyl)-4,5,6,7-tetrahydrothieno[*b*]pyridine molecular skeleton was synthesized and evaluated for antiproliferative activity, inhibition of tubulin polymerization, and cell cycle effects. The most promising compound in this series was 2-amino-3-(3,4,5-trimethoxybenzoyl)-6-methoxycarbonyl-4,5,6,7-tetrahydrothieno[*b*]pyridine, which inhibits cancer cell growth with IC_{50}-values ranging from 25 to 90 nM against a panel of four cancer cell lines, and interacts strongly with tubulin by binding to the colchicine site. In this series of N^6-carbamate derivatives, any further increase in the length and in the size of the alkyl chain resulted in reduced activity.

The mitotic spindle is formed by microtubules that are generated by the polymerization of tubulin, and the spindle is an attractive target for the development of compounds useful in anticancer chemotherapy.[1] Besides being critical for cell division, the microtubule system of eukaryotic cells is involved in many fundamental cellular functions, including cell signaling, secretion, cell architecture in interphase, and intracellular transport.[2] A large number of antimitotic drugs displaying wide structural diversity, derived from natural sources or by screening compound libraries in combination with traditional medicinal chemistry, have been identified and shown to interfere with the tubulin system.[3]

One of the most important naturally occurring tubulin-binding agents is combretastatin A-4 (CA-4, **1**; Chart 1). CA-4, isolated from the bark of the South African tree *Combretum caffrum*,[4] strongly inhibits the polymerization of tubulin by binding to the colchicine site.[5] Because of its simple structure, a wide number of CA-4 analogues have been developed and evaluated in SAR studies.[6]

During our studies directed at the synthesis of new antitubulin agents, we previously reported the potent in vitro antitumor activity of a series of molecules with general structure **2**, characterized by the presence of a 2-amino-3-(3,4,5-trimethoxybenzoyl)-benzo[*b*]thiophene skeleton.[7] These compounds strongly inhibited tumor cell growth, as well as tubulin polymerization by binding to the colchicine site of tubulin, and caused arrest in the G2/M phase of the cell cycle. The trimethoxybenzoyl moiety is crucial for retaining potency in this and other series of molecules which occupy the colchicine site.[8]

Previously, investigators at Altana Pharma reported a series of 2-amido-3-cyano-4,5,6,7-tetrahydrothieno[2,3-*b*]pyridine analogues with general structure **3**, active at micromolar concentrations (IC_{50} = 0.2–5 μM) as antiproliferative agents against human colon adenocarcinoma (RKOp27) cells.[9]

As a part of our search for novel antimitotic agents, these findings prompted us to synthesize a new series of 2-amino-3-(3,4,5-trimethoxybenzoyl)-4,5,6,7-tetrahydrothieno[*b*]pyridine derivatives with general structure **4**, obtained by combining the 2-amino-3-(3′,4′,5′-trimethoxybenzoyl)thiophene portion of compound with general structure **2** with the N^6-substituted-4,5,6,7-tetrahydropyridine nucleus of general structure **3**.

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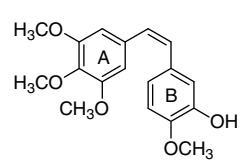
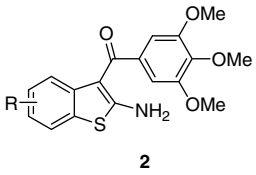
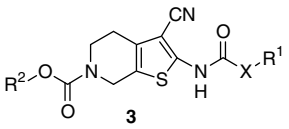


Combretastatin A-4 (CA-4), **1**



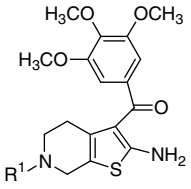
2

R=H, Me, OMe
2a, R=H



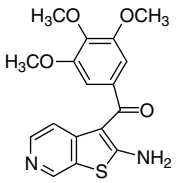
3

X=-CH=CH- or nothing
R^1=substituted or unsubstituted phenyl, 5 or 6-membered heterocyle
R^2=straight or branched alkyl chain



4

R^1=CH_3, **4a**
R^1=C_2H_5, **4b**
R^1=n-C_3H_7, **4c**
R^1=$CH_2C_6H_5$, **4d**
R^1=$COCH_3$, **4e**
R^1=$COOCH_3$, **4f**
R^1=$COOCH_2CH_3$, **4g**
R^1=$COO(CH_2)_2CH_3$, **4h**
R^1=$COOCH_2CH(CH_3)_2$, **4i**
R^1=$COOC(CH_3)_3$, **4j**
R^1=$COOC_6H_5$, **4k**
R^1=$COOCH_2C_6H_5$, **4l**
R^1=$CONHCH(CH_3)_2$, **4m**
R^1=$CONH(CH_2)_3CH_3$, **4n**
R^1=$CONHC_6H_{11}$, **4o**
R^1=$CONHC_6H_5$, **4p**
R^1=$CONHCH_2C_6H_5$, **4q**
R^1=$CONHCH_2C_6H_4F$-p, **4r**
R^1=$CSNHCH_3$, **4s**
R^1=$CSNHCH_2CH_3$, **4t**
R^1=$CSNHCH(CH_3)_2$, **4u**
R^1=$CSNHC(CH_3)_3$, **4v**
R^1=$CSNHC_6H_{11}$, **4w**



5

Chart 1. Inhibitors of tubulin polymerization.

To the best of our knowledge, there have been no reports that molecules characterized by the presence of the 4,5,6,7-tetrahydrothieno[*b*]pyridine skeleton can inhibit tubulin polymerization. In order to explore the structure–activity relationships (SARs) at the N^6-position of the 2-amino-3-(3,4,5-trimethoxybenzoyl)-4,5,6,7-tetrahydrothieno[*b*]pyridine nucleus, we embarked upon the synthesis of different series of N^6-substituted derivatives, represented by alkyl compounds **4a–d**, amide **4e**, carbamates **4f–l**, ureas **4m–r**, and thioureas **4s–w**, characterized by the presence of alkyl chains of varying size. In addition, we explored the effect of bioisosteric replacement of the C-6 carbon atom of derivative **2a** with a nitrogen atom, to furnish the 2-amino-3-(3′,4′,5′-trimethoxybenzoyl)thieno[2,3-*c*]pyridine derivative **5**.

Synthesis of derivatives **4a–w** and **5** was carried out by the general methodology shown in Scheme 1. The Gewald reaction[10], applied to 3-(3,4,5-trimethoxyphenyl)-3-oxopropanenitrile,[11] sulfur, and N-substituted 4-piperidone **6a–l**[12] in the presence of triethylamine and ethanol at reflux, furnished the 2-amino-3-(3′,4′,5′-trimethoxybenzoyl)-6-substituted-4,5,6,7-tetrahydrobenzo[*b*]-thiophenes **4a–l**. [13] The N^6-*tert*-butoxycarbonyl (Boc) derivative **4j** was used as starting material for the synthesis of urea and thiourea derivatives **4m–r** and **4s–w**, respectively. Acetylation of the amino group of **4j**, using acetyl chloride and pyridine, yielded **7j**, which, followed by removal of the N^6-Boc protecting group with trifluoroacetic acid (TFA), afforded the derivative **8j**. The subsequent aromatization, by treatment with manganese dioxide (MnO_2) in refluxing toluene, furnished the corresponding N^2-acetyl-thieno[2,3-*c*]pyridine derivative, transformed by saponification into the final product **5**.

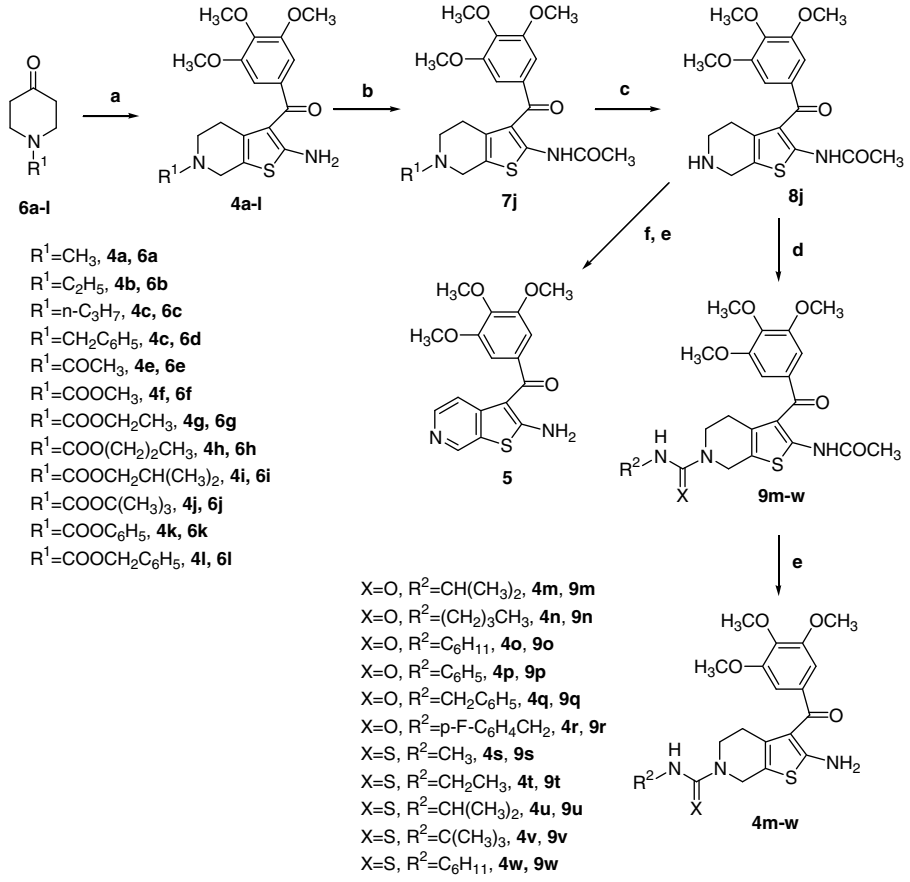
Compound **8j** was further condensed with different isocyanates or isothiocyanates, in the presence of triethylamine, to afford the corresponding ureas **9m–** and thioureas **9s–w**, which were transformed by hydrolysis with NaOH into the final products **4m–r** and **4s–w**, respectively, in good yields.

Table 1 summarizes the antiproliferative activity of N^6-substituted-4,5,6,7-tetrahydrothieno[*b*]pyridine derivatives **4a–w** and the thieno[2,3-*c*]pyridine **5** against the growth of murine leukemia (L1210), murine mammary carcinoma (FM3A), and human T-lymphoblastoid (Molt/4 and CEM) cells, using CA-4 (**1**) and the benzo[b]thiophene derivative **2a** as reference compounds. The results indicated that *N*^{6}-methyl and ethyl carbamates **4f** and **4g**,[14] respectively, showed the most potent antiproliferative activities, while N^6-branched alkyl or aryl carbamates as well as alkyl, acetyl, urea, and thiourea functionalities decreased activity drastically.

Of all the tested compounds, the N^6-methyl carbamate derivative **4f** possessed the highest potency, inhibiting the growth of L1210, FM3A, Molt/4, and CEM cancer cell lines with IC_{50}s of 25, 46, 45, and 90 nM, respectively.

The results indicated that a non-basic nitrogen atom at the N^6-position of 4,5,6,7-tetrahydrothieno[*b*]pyridine nucleus was important for inhibition of cell growth. In fact, in the series of *N*^{6}-alkyl derivatives **4a–d**, only the ethyl derivative **4b** showed moderate potency (IC_{50} = 1.7–2.1 μM), whereas for the methyl, propyl, and benzyl analogues (compounds **4a**, 4c, and **4d**, respectively), the IC_{50} was greater than 10 μM in all four cell lines. The *N*^{6}-acetyl derivative **4e** also showed moderate antiproliferative activity, with IC_{50}-values of 1.1–3.9 μM:

In the series of carbamates **4f–l**, a small substituent size was important for good activity. Only the methyl and ethyl derivatives **4f** and **4g**, respectively, showed potent antiproliferative activity, with **4f** more active than **4g**. Specifically, **4f** and **4g** had similar activity against FM3A cells, but **4f** was 4-, 5-, and 6-fold more po-



Scheme 1.

Table 1
In vitro inhibitory effects of compounds **2a**, **4a-w**, **5**, and CA-4 (**1**) against the proliferation of murine leukemia (L1210), murine mammary carcinoma (FM3A), and human T-lymphocyte (Molt/4 and CEM) cells

Compound	IC_{50}[a] (nM)			
	L1210	FM3A	Molt4/C8	CEM
4a	>10,000	>10,000	>10,000	>10,000
4b	2100 ± 100	1900 ± 0.0	1800 ± 0.0	1700 ± 0.0
4c	>10,000	>10,000	>10,000	>10,000
4d	>10,000	>10,000	>10,000	>10,000
4e	3900 ± 290	1200 ± 100	1100 ± 60	1400 ± 20
4f	25 ± 1	46 ± 1.3	45 ± 3.1	90 ± 1.7
4g	95 ± 3.3	57 ± 3.8	290 ± 25	440 ± 30
4h	1100 ± 80	1400 ± 100	470 ± 0.00	1200 ± 30
4i	>10,000	>10,000	>10,000	>10,000
4j	>10,000	>10,000	>10,000	>10,000
4k	>10,000	>10,000	>10,000	>10,000
4l	>10,000	>10,000	7500 ± 140	8300 ± 500
4m	>10,000	>10,000	>10,000	>10,000
4n	>10,000	>10,000	>10,000	>10,000
4o	>10,000	>10,000	>10,000	>10,000
4p	>10,000	>10,000	>10,000	>10,000
4q	>10,000	>10,000	>10,000	>10,000
4r	>10,000	>10,000	>10,000	>10,000
4s	4900 ± 470	2800 ± 110	1600 ± 60	1800 ± 70
4t	6800 ± 430	6900 ± 290	4800 ± 370	6700 ± 550
4u	>10,000	>10,000	>10,000	>10,000
4v	>10,000	>10,000	>10,000	>10,000
4w	1100 ± 60	1400 ± 50	750 ± 58	730 ± 71
5	370 ± 160	400 ± 170	340 ± 40	1000 ± 900
2a	90 ± 3	100 ± 0	73 ± 9	74 ± 15
CA-4 (1)	2.8 ± 1.1	42 ± 6	1.6 ± 1.4	1.9 ± 1.6

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

tent then **4g** against L1210, CEM and Molt4 cells, respectively. A further increase in the length of the straight alkyl chain, to furnish the propyl derivative **4h**, caused 10-, 25-, 1.5-, and 3-fold reductions in activity with the L1210, FM3A, Molt-4, and CEM cells, respectively. With still bulkier carbamate moieties, there was essentially total loss of activity. All urea derivatives **4m–r** were also inactive (IC_{50} > 10 μM).

It is noteworthy that for the active compounds **4f** and **4g**, the replacement of the carbamate group with a thiourea, to furnish the corresponding derivatives **4s** and **4t**, produced a dramatic drop in potency (IC_{50} = 1.6–4.9 μM and 4.8–6.9 μM for **4s** and **4t**, respectively, versus IC_{50} = 25–90 nM and 57–440 nM for **4f** and **4g**, respectively).

In the series of thiourea derivatives **4s–w**, the cyclohexyl thiocarbamoyl derivative **4w** resulted in the most active compound,

Table 2
Inhibition of tubulin polymerization and colchicine binding by compounds **2a**, **4f-g**, **5**, and CA-4

Compound	Tubulin assembly[a] IC_{50} ± SD (μM)	Colchicine binding[b] ±SD (%)	
		1 μM inhibitor	5 μM inhibitor
2a	1.9 ± 0.1	25 ± 2	71 ± 1
4f	0.6 ± 0.02	71 ± 0.6	84 ± 3
4g	0.6 ± 0.03	67 ± 2	82 ± 2
5	5.4 ± 0.4	n.d.	27 ± 0.1
CA-4 (1)	1.2 ± 0.1	90 ± 1	99 ± 0.7

n.d., not done.
[a] Inhibition of tubulin polymerization. Tubulin was at 10 μM.
[b] Inhibition of [^{3}H]colchicine binding. Tubulin and colchicine were at 1 and 5 μM, respectively, and the tested compound was at the indicated concentration.

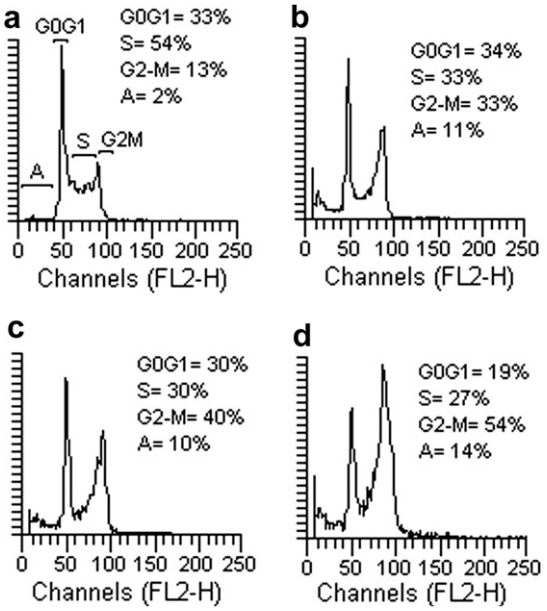


Figure 1. Effects of compounds **4f** (panel b), **4g** (panel c), and **5** (panel d) on DNA content/cell following treatment of K562 cells for 24 h. The cells were cultured without compound (panel a) or with compound used at the concentration leading to 50% cell growth inhibition after 24 h of treatment. Cell cycle distribution was analyzed by the standard propidium iodide procedure. Sub-G0-G1 (apoptotic peak, A), G0-G1, S, and G2-M cells are indicated in the panel (a).

with IC_{50}s of 0.73–1.1 μM. Comparing urea and thiourea derivatives with the same substitution at the N^6-position (**4m** vs 4u and **4o vs 4w**), while the isopropyl derivatives **4m** and **4u** were both inactive, the cyclohexyl thiourea derivative 4w was more active than the urea derivative **4o**.

Finally, comparing the benzo[*b*]thiophene **2a** with the thieno[2,3-*c*]pyridine **5**, replacement of the benzene with the bioisosteric pyridine ring led to a significant loss of activity. Compound **5** was 4- to 6-fold less active than **2a** against L1210, FM3A, and Molt-4 cells. Reduction in potency was even more pronounced with the CEM cells, with **5** being 13-fold less potent than **2a**. Moreover, the corresponding tetrahydrothieno[*b*]pyridine analogue of **5** resulted inactive (IC_{50} > 10 μM), indicating that the aromaticity of the pyridine ring fused with the thiophene was critical for activity.

To investigate whether the antiproliferative activities of these compounds were related to an interaction with the microtubule system, compounds **4f–g** and **5** and reference derivatives **2a** and CA-4 were evaluated for inhibitory effects on tubulin polymerization, and on the binding, of [^{3}H]colchicine to tubulin (Table 2).[15,16] For compounds **2a** and **5**, there was a positive correlation between inhibition of both tubulin polymerization and colchicine binding, and antiproliferative activity. However, relative to **2a**, both **4f** and **4g** were disproportionately more active as assembly inhibitors, and these compounds also had greater activity as inhibitors of colchicine binding. The IC_{50}s of 0.6 μM obtained with **4f** and **4g** are among the lowest ever observed in this assembly assay, and half that obtained in simultaneous experiments for CA-4 (IC_{50}, 1.2 μM). Nonetheless, CA-4 had greater antiproliferative activity and a greater inhibitory effect on the four cell lines than both **4f** and **4g**. In addition, we should note that the similar effects of **4f** and **4g** in the tubulin-based assays differed from the greater activity observed with **4f** in the antiproliferative studies. This could derive from preferential cellular uptake of **4f** relative to **4g**. Alternatively, it is possible that cellular tubulin differs from the neural tubulin used in the biochemical assays in its affinities for the two compounds.

Because molecules exhibiting effects on tubulin assembly should cause alteration of cell cycle parameters, with preferential G2-M blockade, flow cytometry analysis was performed to determine the effect of the most active compounds on K562 (human chronic myelogenous leukemia) cells.[17] Cells were cultured for 24 h in the presence of each compound at the IC_{50} determined for 24 h of growth (**4f** = 70 nM, **4g** = 80 nM, **5** = 500 nM). Figure 1 shows that these molecules caused a marked increase in the percentage of cells blocked in the G2-M phase of the cell cycle, with a simultaneous decrease of cells in S and G0-G1. These data confirm that this class of derivatives acts selectively on the G2-M phase of the cell cycle, as expected for inhibitors of tubulin assembly.

The proposed mechanism of action is also supported by docking studies of compound **4g** in the colchicine site of tubulin[18] (methodology reported previously).[7] Figure 2 shows how the trimethoxyphenyl moiety of **4g** is situated in the same pocket on β-tubulin as the structurally analogous ring A of the co-crystallized DAMA-colchicine. In this model, the carbonyl group of the carbamate of **4g** overlaps the carbonyl group of ring C of DAMA-colchicine. Furthermore, the alkyl substituent of the carbamate lies in a small

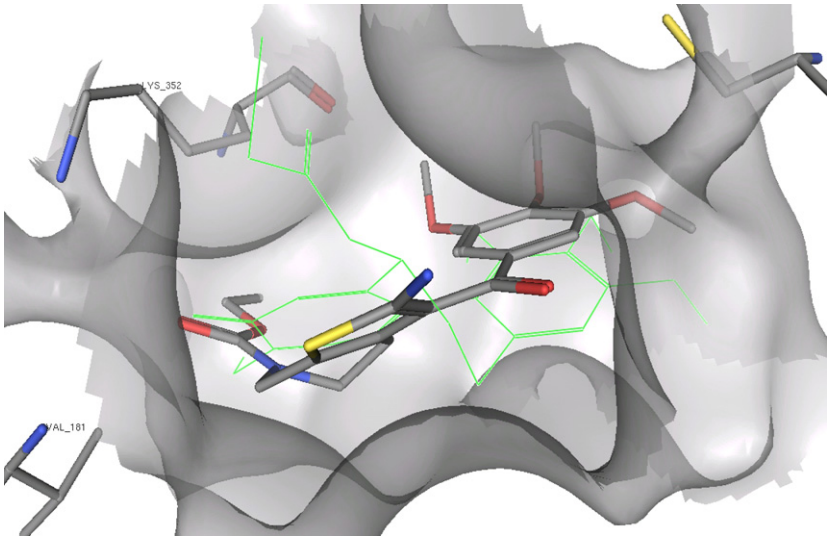


Figure 2. Docking pose of compound **4g** in the colchicine site. DAMA-colchicine in green.

hydrophobic pocket deep in the binding cavity, which can only accommodate a small group like methyl or ethyl. This model thus is consistent with the SARs observed in the antiproliferative studies, and is in accord with previously reported results.[19]

In conclusion, the synthesis and the SAR of a series of 2-amino-3-(3,4,5-trimethoxybenzoyl)-6-substituted-4,5,6,7-tetrahydrothieno[*b*]pyridines, which incorporated partial structures of both 2-amino-3-(3,4,5-trimethoxybenzoyl)-benzo[*b*]thiophene and 2-acetamido-3-cyano-6-alkoxycarbonyl-4,5,6,7-tetrahydrothieno [2,3-*b*]pyridine with general structures **2** and **3**, respectively, are described. In particular, compounds **4f** and **4g** are the best amalgamation of structures 2 and 3. Derivatives **4f** and **4g** were highly active as inhibitors of tubulin assembly, with IC_{50}s half that of CA-4. They also were strong inhibitors of the binding of colchicine to tubulin, although somewhat less active than CA-4. Consistent with their antitubulin activity, both **4f** and **4g** caused cells to arrest in the G2/M phase of the cell cycle.

Molecular docking studies with **4g** into the colchicine site[18] provided a rationale for our observations. The trimethoxybenzene ring and the carbamate carbonyl of **4g** could bind in the same manner as the trimethoxybenzene ring A and the ring C carbonyl, respectively, of DAMA-colchicine in the crystal structure.[18] An adjacent pocket in β-tubulin could readily accommodate only a methyl or ethyl group, consistent with the SAR observations.

Finally, we should note that the synthesis of **4f** was efficient and produced the compound in high yield. Thus, **4f** represents the lead compound of an interesting new class of antitubulin agents with potential to be developed clinically for anticancer chemotherapy.

Supplementary data

Detailed synthesis and spectroscopic data for compounds **4a–w**, **5**, **6h–i**, **7–8j**, and **9m–w** can be found in the online version. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.08.006.

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12. Phenyl isocyanate, benzyl isocyanate, 4-fluorobenzyl isocyanate, isopropyl isocynate, butyl isocyanate, cyclohexyl isocyanate, methyl isothiocyanate, ethyl isothiocyanate, isopropyl isothiocyanate, cyclohexyl isothiocyanate, *tert*-butyl isothiocyanate, 1-methyl-4-piperidone (**6a**), 1-ethyl-4-piperidone (**6b**), 1-*n*-propyl-4-piperidone (**6c**), 1-benzyl-4-piperidone (**6d**), 1-acetyl-4-piperidone (**6e**), 1-ethoxycarbonyl-4-piperidone (**6g**), n-propyl 4-oxopiperidine-1-carboxylate (**6h**), isobutyl 4-oxopiperidine-1-carboxylate (**6i**), 1-*tert*-butyloxycarbonyl-4-piperidone (**6j**), and 1-benzyloxycarbonyl-4-piperidone (**6l**) are commercially available and were used as received. For the synthesis of 4-oxo-piperidine-1-carboxylic acid methyl ester (**6f**) see: (a) Xu, G.; Kannan, A.; Hartman, T. L.; Wargo, H.; Watson, K.; Turpin, J. A.; Buckheit, R. W.; Johnson, A. A.; Pommier, Y.; Cushman, M. *Bioorg. Med. Chem.* **2002**, *10*, 2807; For the synthesis of 4-oxo-piperidine-1-carboxylic acid phenyl ester (**6k**) see: (b) Mauleon, D.; Antunez, S.; Rosell, G. *Farmaco* **1989**, *44*, 1109.
13. General procedure for the synthesis of compounds (**4a–l**). To a suspension of 3-oxo-3-(3,4,5-trimethoxyphenyl)-propionitrile (2.55 g., 10 mmol), TEA (1.54 mL, 11 mmol), and sulfur (352 mg, 11 mmol) in EtOH (50 mL) was added the appropriate 1-substituted-4-piperidone **6a–l** (10 mmol). After stirring for 2 h at 70 °C, the solvent was evaporated and the residue diluted with DCM (15 mL). After washing with water (2 × 5 mL) and brine (5 mL), the organic layer was dried and evaporated. The crude product was purified by column chromatography and crystallized from petroleum ether.
14. Characterization of compound **4f**. Yellow solid, mp 85–87 °C. ^{1}H NMR ($CDCl_3$) δ: 2.07 (t, J = 5.0 Hz, 2H), 3.44 (t, J = 5.0 Hz, 2H), 3.74 (s, 3H), 3.86 (s, 6H), 3.90 (s, 3H), 3.95 (bs, 2H), 4.43 (s, 2H), 6.72 (s, 2H). Characterization of compound **4g**. Yellow solid, mp 65–67 °C. ^{1}H NMR ($CDCl_3$) δ: 1.26 (t, J = 7.0 Hz, 3H), 2.14 (t, J = 5.4 Hz, 2H), 3.44 (t, J = 5.4 Hz, 2H), 3.86 (s, 6H), 3.89 (s, 3H), 3.90 (bs, 2H), 4.18 (q, J = 7.0 Hz, 2H), 4.42 (s, 2H), 6.72 (s, 2H).
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17. Flow cytometric analysis of cell cycle distribution. The effects of the most active compounds of the series on cell cycle distribution were studied on K562 cells by flow cytometric analysis after staining with propidium iodide. Cells were exposed 24 h to each compound used at a concentration corresponding to the IC_{50} evaluated after a 24 h incubation. After treatment, the cells were washed once in ice-cold PBS and resuspended at 1×10^6 per mL in a hypotonic fluorochrome solution containing propidium iodide (Sigma) at 50 μg/mL in 0.1% sodium citrate plus 0.03% (v/v) nonidet P-40 (Sigma). After a 30-min incubation, the fluorescence of each sample was analyzed as single-parameter frequency histograms by using a FACScan flow cytometer (Becton Dickinson, San Jose, CA). The distribution of cells in the cell cycle was analyzed with the ModFit LT3 program (Verity Software House, Inc.).
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