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Synthesis and biological evaluation of 2-(3′,4′,5′-trimethoxybenzoyl)-3-*N*,*N*-dimethylamino benzo[*b*]furan derivatives as inhibitors of tubulin polymerization

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Abstract

Molecules that target microtubules have an important role in the treatment of cancer. A new class of inhibitors of tubulin polymerization based on the 2-(3,4,5-trimethoxybenzoyl)-2-dimethylamino-benzo[*b*]furan 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-(3,4,5-trimethoxybenzoyl)-3-dimethylamino-6-methoxy-benzo[*b*]furan, which inhibits cancer cell growth at nanomolar concentrations and interacts strongly with tubulin by binding to the colchicine site.

Keywords

Microtubules; Tubulin polymerization; Colchicine binding site; Combretastatin A-4; Bioisosteric replacement

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1. Introduction

Compounds that are able to interfere with the microtubule-tubulin equilibrium in cells are useful in the treatment of human diseases.[1] The success of tubulin polymerization inhibitors as anticancer agents has stimulated significant interest in the identification of new compounds that may be more potent or more selective in targeted tissues or tumors.

Among the microtubule depolymerizing agents, combretastatin A-4 (CA-4, **1**; Chart 1) is one of more studied compounds. CA-4, isolated from the bark of the South African tree *Combretum caffrum*,[2] strongly inhibits the polymerization of tubulin by binding to the colchicine site.[3] Because of its simple structure, a wide number of CA-4 analogues have been developed and evaluated in SAR studies.[4]

We have earlier reported three different series of 2-(3′,4′,5′-trimethoxybenzoyl)-3-amino benzoheterocyclic derivatives with general structure **2**–**4**, characterized by the presence of the benzo[*b*]thiophene, 1*H*-indole, and 1-methylindole skeleton, respectively.[5,6] These compounds strongly inhibited tumor cell growth and tubulin polymerization by binding to the colchicine site of tubulin and caused G2/M phase arrest of the cell cycle. The C-6 methoxy group plays an essential role for the inhibition of tubulin polymerization within these series of molecules (compounds **2a**–**4a**).

As a part of our search for novel tubulin polymerization inhibitors, we synthesized and evaluated the biological properties of a new series of benzo[*b*]furan derivatives with general structure **5**. We based this synthesis on the bioisosteric replacement by furan of either the thiophene or pyrrole moieties that characterized the benzo[*b*]thiophene (**2**) and indole derivatives **3** and **4**. Since the 3,4,5-trimethoxybenzoyl substituent was demonstrated to be essential for the bioactivity of derivatives **2–4**, we maintained this substituent at the 2-position of the benzo[*b*]furan skeleton throughout the present investigation, and we examined the effects of various substituents at the 3-position. This furnished three different small series of compounds.

The first series (derivatives **5a–e**) was characterized by the presence of a 3-amino group in the benzo[*b*]furan skeleton and either no substituent (**5a**) or a methoxy group at each of the four possible positions on the benzene ring (compounds **5b–e**).

In the second series (compounds **5f–k**), the 3-amino moiety was replaced with a dimethylamino (**5f**) or acetamido group (**5k**), plus a methoxy group at each of the four positions of the benzene ring of the benzofuran moiety combined with the 3-dimethylamino group (**5g–j**) These compounds allowed us to determine whether the 3-amino substituent could restrict the conformation of the adjacent trimethoxybenzoyl moiety through an intramolecular hydrogen bond with the carbonyl oxygen.

In the third series (**5l–n**), we examined haloacetyl amides at position 3 of the benzo[*b*]furan moiety. We were stimulated to do this by previous studies in which the electrophilic nature of the haloacetyl moiety has been used in anticancer drug design.[7,8] The alkylation of sulfhydryl groups of tubulin by iodoacetamide[9] and the disruption of microtubules by *p*-bromophenacyl bromide[10] have been reported.

We should note that previous studies have yielded a limited series of tubulin inhibitors with the benzo[*b*]furan molecular skeleton as the core structure. These compounds have general structure **6**, which incorporates the 3-(3,4,5-trimethoxybenzoyl)-6-methoxybenzo[*b*]furan ring system.[11] The 6-methoxy substituent is important for maximal activity and appears to correspond to the 4-methoxy group in the B-ring of CA-4.[4]

2. Chemistry

Derivatives **5a–n** were synthesized as shown in Scheme 1. Refluxing 2-hydroxyaldehydes **7a–e** and nitroethane in glacial acetic acid in the presence of sodium acetate furnished the corresponding nitriles **8a–e** in good yields.[12] The subsequent reaction of **8a–e** with 2-bromo-1-(3,4,5-trimethoxyphenyl)ethanone[5] and potassium carbonate in refluxing acetone yielded the cyclized 3-amino benzo[*b*]furan derivatives **5a–e**, which were transformed into the corresponding 3-dimethylamino compounds **5f–j** by treatment with methyl iodide in DMF.

Acetylating or haloacetylating the 3-amino group of **5d** with acetyl chloride, chloroacetyl chloride, and bromoacetyl chloride in the presence of pyridine provided the acetamide derivatives **5k–m**, respectively. The iodoacetyl derivative **5n** was prepared from the bromoacetyl derivative **5m** by an exchange reaction using sodium iodide in *N*,*N*-dimethylacetamide.

3. Biological results and discussion

Table 1 summarizes the growth inhibitory effects of benzo[*b*]furan derivatives **5a–n** against murine leukemia (L1210), murine mammary carcinoma (FM3A), and human T-lymphoblastoid (Molt/4 and CEM) cells, with CA-4 (**1**) and **2a–4a** as reference compounds.

Compound **5i** was the most potent compound identified in this study, inhibiting the growth of L1210, FM3A, Molt/4, and CEM cancer cell lines with IC_{50}-values of 65, 59, 48, and 78 nM, respectively. CA-4 (**1**) and, to a lesser extent, **2a** were more potent as antiproliferative agents than **5i** with these four cell lines.

The results presented in Table 1 show that the location of the methoxy group on the benzene portion of the benzo[*b*]furan moiety plays a critical role in inhibition of cell growth, and the most favorable position for the substituent was at C-6, as was observed previously with the thiophene and indole series.[5,6] In the series of 3-amino benzo[*b*]furan derivatives **5b–e**, the 6-methoxy derivative **5d** had the greatest antiproliferative activity, with IC_{50}-values ranging from 87 to 430 nM against the four cell lines. The 4-methoxy analogue **5b** was also over 10-fold less active than **5d** in the four cell lines. From the point of view of SAR, the oxygen biostere (**5d**) did not enhance activity relative to sulfur atom (**2a**), but did relative to the nitrogen atom (**3a**).

With the exception of the C-4 methoxy derivative **5g**, the methoxy substituent at C-5, C-6, or C-7 (**5h–j**, respectively) in the 3-dimethylamino series increased antiproliferative activity relative to both the unsubstituted **5f** and their parent 3-amino counterparts **5c–e**. This suggests that it would be worthwhile introducing dimethylamino modification at the 3-amino group into the thiophene, indole, and methylindole series described previously.[5,6]

The antiproliferative activity of **5i** was superior to that of 1*H*-indole derivative **3a**, comparable to that of 1-methylindole **4a**, and slightly less than that of benzo[*b*]thiophene **2a**. Therefore, even though the good activity of **5i** required a further modification of the 3-amino group, we conclude that there is validity to the idea of bioisosteric equivalence between benzo[*b*]furan, 1-methylindole, and benzo[*b*]thiophene in this class of compounds. Moreover, comparing the structures of **5i** with those of **6ab**, we conclude that the 3-dimethylamino group of **5i** is analogous to the right-hand substituted phenyl ring of **6ab** (see Chart 1).

Changing the substituent at C-3 from the dimethylamino group (**5i**) to an acetamido moiety (**5k**) caused only a minimal reduction in antiproliferative activities. Consequently, **5k**, like **5i**, was superior in its antiproliferative properties to the amine, **5d**.

Compound **5k** was also more potent than 3-haloacetamido derivatives **5l–n** against all four cell lines. Of note in haloacetamido series, increasing the size of the halogen atom resulted in increased antiproliferative activity, with the order being I (**5n**) > Br (**5m**)>Cl (**5k**). This order is also inversely related to the electron-withdrawing properties of the halide atoms.

To confirm that the antiproliferative activities of these compounds, like those of the benzothiophene and indole series,[5,6] were related to an interaction with the microtubule system, **5d**, **5i**, and **5k–n** were evaluated for their inhibitory effects on tubulin polymerization and on the binding of [^{3} H]colchicine to tubulin (Table 2).[13,14] Comparing benzo-fused heterocyclic compounds **2a–4a** and **5i**, those containing either a sulfur (**2a**) or a nitrogen (**3a** and **4a**) in the benzoheterocyclic ring are less effective than oxygen analogue **5i** as an inhibitor of tubulin polymerization and [^{3} H] colchicine binding.

The order of inhibitory action on tubulin assembly was **5i > 5d > 5k > 5n > 5l > 5m**, which was consistent with the results of the antiproliferative assays, except that **5d** was more potent than **5k**. The most potent compound in this series was compound **5i**, with an IC_{50} value of 0.9 μM. This is in agreement with **5i** being the compound with the greatest antiproliferative activity. Compounds **5d** and **5i** were as active as CA-4 as inhibitors of tubulin assembly, although both compounds were less active in their effects on cell growth.

In the colchicine binding studies, compounds **5d** and **5i** potently inhibited the binding of [^{3}H] colchicine to tubulin, since 73% and 76% inhibition, respectively, occurred with these agents at 1 μM with colchicine at 5 μM. These derivatives were slightly less potent than CA-4, which in these experiments inhibited colchicine binding by 86%, but **5i** was twofold more potent than its 1-methylindole counterpart **4a**.

Because molecules exhibiting effects on tubulin assembly should cause the 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. Cells were cultured for 24 h in the presence of each compound at the IC_{50} value determined for 24 h of growth (**5d** = 120 nM, **5i** = 68 nM, **5k** = 85 nM, **5l** = 400 nM, **5m** = 212 nM, **5n** = 115 nM). Analysis of sub-G_0-G_1 (apoptotic peak), G_0-G_1, S, and G_2-M peaks revealed that the studied compounds caused somewhat different effects on cell cycle distribution (Fig. 1). While all six compounds caused an increase in the proportion of cells in the G2-M peak, compounds **5d** and **5l** also caused cells to accumulate in late S phase. In contrast, compound **5n** caused the greatest percentage increase in the percentage of cells in apoptotic cells (subG0-G1 peak), as well as substantial proportion of cells in S phase.

We performed a series of molecular modeling studies on the series of compounds reported here. In particular, we investigated the use of three different docking software packages, Plants,[15] the Surflex module implemented in Sybyl[16] and MOE,[17] in identifying possible binding conformations for this family of compounds. We also wanted to determine whether any combination of algorithm/scoring function would generate a good correlation between a calculated biological activity and the corresponding experimental value for future use as a predictive model. The results obtained with the different software packages were also rescored using the scoring functions implemented in the CScore module of Sybyl.[16]

As noted previously, no correlation between the output of the scoring functions and the experimental data was observed.[18] However, a correlation was observed between the tubulin polymerization assay results and the calculated RMSD value of the trimethoxyphenyl group of the compounds reported here and the corresponding ring in the DAMA-colchicine co-crystallized with tubulin.[19] The best correlation was observed with the MOE docking program (r = 0.8), and the pose with the lowest RMSD value for compound **5i** is shown in Figure 2.

From the docking results, it is possible to observe how the methoxy group of the benzofuran ring of **5i** can establish two hydrogen bonds with Lys352 and Val181 of β-tubulin. This could explain the difference in activity between **5i** and the structurally similar compounds **5f**, **5g**, **5h**, and **5j**, which were not able to establish the same interactions, given the different position of the methoxy group on the benzofuran ring. Furthermore, compounds **5g**, **5h**, and **5j** were not docked successfully in the colchicine site by any of the three software packages used.

4. Conclusions

In conclusion, the synthesis and biological evaluation of a new class of synthetic antitubulin compounds based on the 2-(3′,4′,5′-trimethoxybenzoyl) benzo[*b*]furan skeleton is described. The results showed that compounds **5d** and **5i**, with a methoxy substitutent at the C-6 position of the benzofuran ring, exhibited the best antiproliferative activity in the 3-amino and 3-dimethylamino benzo[*b*]furan series, respectively, while shifting the methoxy group in both series to the C-3, C-5, or C-7 position resulted in major losses in activity.

The dimethyl substitution on the amino group at the 3-position of the benzo[*b*]furan core usually enhanced antiproliferative activity, with the dimethylamino derivatives **5f** and **5h–j** being more potent than their amino counterparts **5a** and **5c–e**. In the series of 3-haloacetamido derivatives **5l–n**, the order of antiproliferative activity was I > Br > Cl, but the greatest activity was observed with the unsubstituted acetamido derivative **5k**. Compound **5k** was almost as active as **5i**, the most potent antiproliferative agent in the series.

The IC_{50}-values for **5i** in the cell lines examined ranged from 48 to 75 nM. The antiproliferative activity of **5i** was equivalent to that of the previously reported 1-methylindole derivative **4a** and slightly less than that of benzo[*b*]thiophene **2a**. Compound **5i** strongly inhibited both the polymerization of tubulin and the binding of [^{3}H]colchicine to tubulin, suggesting that **5i** bound to tubulin at a site overlapping the colchicine site. The antimitotic activity of **5i** was demonstrated by flow cytometric analysis that showed that **5i** had cellular effects typical of agents that bind to tubulin, causing accumulation of cells in G2-M. We also were readily able to model **5i** into the colchicine site, with excellent overlap with the colchicinoid bound in X-ray crystal structure.

5. Experimental

5.1. Chemistry

5.1.1. Materials and methods—2-Hydroxybenzaldehyde (**7a**), 2-hydroxy-6-methoxybenzaldehyde (**7b**), 2-hydroxy-5-methoxybenzaldehyde (**7c**), 2-hydroxy-4-methoxybenzaldehyde (**7d**), 2-hydroxy-3-methoxybenzaldehyde (**7e**), and 2-hydroxybenzonitrile (**8a**) are commercially available and were used as received.

^{1}H NMR spectra were recorded on a Bruker AC 200 spectrometer. Chemical shifts (δ) are given in ppm upfield from tetramethylsilane as internal standard, and the spectra were recorded in appropriate deuterated solvents, as indicated. Melting points (mp) were determined on a Buchi–Tottoli apparatus and are uncorrected. All products reported showed ^{1}H NMR spectra in agreement with the assigned structures. Elemental analyses were conducted by the Microanalytical Laboratory of the Chemistry Department of the University of Ferrara. All reactions were carried out under an inert atmosphere of dry nitrogen, unless otherwise described. Standard syringe techniques were applied for transferring dry solvents. Reaction courses and product mixtures were routinely monitored by TLC on silica gel (precoated F254 Merck plates) and visualized with aqueous $KMnO_4$. Flash chromatography was performed using 230–400 mesh silica gel and the indicated solvent system. Organic solutions were dried

over anhydrous Na_2SO_4. Calcium chloride was used in the distillation of DMF, and the distilled solvent was stored over molecular sieves (3 Å).

5.1.2. General procedure A for the synthesis of 2-(3′,4′,5′-trimethoxybenzoyl)-3-amino benzo[*b*]furan (5a–e)—To a solution of **7a–e** (1 mmol) in dry acetone (15 mL) was added 2-bromo-1-(3,4,5-trimethoxyphenyl)-ethanone (289 mg, 1 mmol) and anhydrous potassium carbonate (276 mg, 2 mmol) while stirring, and the reaction mixture was refluxed for 18 h. After cooling, the solvent was evaporated, and the residue was dissolved in a mixture of dichloromethane (15 mL) and water (5 mL). The organic layer was washed with brine, dried and evaporated to obtain a residue, which was purified by flash column chromatography. Then the final solid product was recrystallized from petroleum ether.

5.1.2.1. (3-Aminobenzofuran-2-yl)(3,4,5-trimethoxyphenyl)-methanone (5a): Following general procedure A, the crude residue purified by flash chromatography using ethyl acetate/petroleum ether 4:6 (v:v) as eluent furnished **5a** as a yellow solid (89% yield); mp 134–136 °C. ^{1}H NMR ($CDCl_3$) δ: 3.95 (s, 3H), 3.97 (s, 6H), 6.04 (br s, 2H), 7.28 (t, J = 8.4 Hz, 1H), 7.43 (d, J = 8.4 Hz, 1H), 7.53 (t, J = 8.4 Hz, 1H), 7.60 (s, 2H), 7.64 (d, J = 8.4 Hz, 1H). Anal. Calcd for $C_{18}H_{17}NO_5$: C, 66.05; H, 5.23; N, 4.28. Found: C, 65.95; H, 5.11; N, 4.17.

5.1.2.2. (3-Amino-4-methoxybenzofuran-2-yl)(3,4,5-trimethoxyphenyl)methanone(5b): Following general procedure A, the crude residue purified by flash chromatography using ethyl acetate/petroleum ether 4:6 (v:v) as eluent furnished **5b** as a yellow solid (78% yield); mp 177–179 °C. ^{1}H NMR ($CDCl_3$) δ: 3.91 (s, 3H), 3.94 (s, 3H), 3.97 (s, 6H), 6.52 (br s, 2H), 6.55 (d, J = 7.8 Hz, 1H), 6.98 (d, J = 7.8 Hz, 1H), 7.42 (t, J = 7.8, 1H), 7.57 (s, 2H). Anal. Calcd for $C_{19}H_{19}NO_6$: C, 63.86; H, 5.35; N, 3.92. Found: C, 63.67; H, 5.21; N, 3.78.

5.1.2.3. (3-Amino-5-methoxybenzofuran-2-yl)(3,4,5-trimethoxyphenyl)methanone (5c): Following general procedure A, the crude residue purified by flash chromatography using ethyl acetate/petroleum ether 4:6 (v:v) as eluent furnished **5c** as a yellow solid (76% yield); mp 125–127 °C. ^{1}HNMR ($CDCl_3$) δ: 3.88 (s, 3H), 3.93 (s, 3H), 3.94 (s, 6H), 6.99 (s, 1H), 7.13 (d, J = 8.8 Hz, 1H), 7.26 (br s, 2H), 7.32 (d, J = 8.8 Hz, 1H), 7.58 (s, 2H). Anal. Calcd for $C_{19}H_{19}NO_6$: C, 63.86; H, 5.35; N, 3.92. Found: C, 63.72; H, 5.14; N, 3.80.

5.1.2.4. (3-Amino-6-methoxybenzofuran-2-yl)(3,4,5-trimethoxyphenyl)methanone(5d): Following general procedure A, the crude residue purified by flash chromatography using ethyl acetate/petroleum ether 4:6 (v:v) as eluent furnished **5d** as a yellow solid (77% yield); mp 167–169 °C. ^{1}H NMR ($CDCl_3$) δ: 3.89 (s, 3H), 3.94 (s, 3H), 3.97 (s, 6H), 6.87 (br s, 2H), 6.91 (d, J = 8.2 Hz, 1H), 7.48 (d, J = 8.2 Hz, 1H), 7.52 (s, 1H), 7.54 (s, 2H). Anal. Calcd for $C_{19}H_{19}NO_6$: C,63.86;H, 5.35; N, 3.92. Found: C, 63.77; H, 5.22; N, 3.74.

5.1.2.5. (3-Amino-7-methoxybenzofuran-2-yl)(3,4,5-trimethoxyphenyl)methanone(5e): Following general procedure A, the crude residue purified by flash chromatography using ethyl acetate/petroleum ether 4:6 (v:v) as eluent furnished **2e** as a yellow solid (87% yield); mp 171–173 °C. ^{1}H NMR ($CDCl_3$) δ: 3.93 (s, 3H), 3.97 (s, 6H), 3.99 (s, 3H), 6.84 (br s, 2H), 6.97 (d, J = 6.8 Hz, 1H), 7.21 (m, 2H), 7.66 (s, 2H). Anal. Calcd for $C_{19}H_{19}NO_6$: C, 63.86; H, 5.35; N, 3.92. Found: C, 63.59; H, 5.18; N, 3.80.

5.1.3. General procedure B for the synthesis of 2-(3,4,5-trimethoxybenzoyl)-3-dimethylamino-benzo[*b*]furans (5f–j)—Sodium hydride (60% oil dispersion, 48 mg, 1 mmol) was carefully added to an ice-cooled solution of CH_3I (93 μL, 1.5 mmol) and **5a–e** (0.56 mmol) in 2 mL of anhydrous DMF. The reaction vessel was sealed and the mixture was stirred at 40 °C for 48 h. After this period, CH_3I (124 μL, 2 mmol) was added, and after 72 h the

reaction mixture was diluted with cold water (1 mL) and extracted with CH_2Cl_2 (3 × 5 mL). The combined organic extracts were washed with water (2 mL) and brine, dried and concentrated in vacuo. The resulting residue was purified by flash chromatography using a mixture of ethyl acetate/petroleum ether as eluent. The final solid product was recrystallized from petroleum ether.

5.1.3.1. (3-(Dimethylamino)benzofuran-2-yl)(3,4,5-trimethoxyphenyl)methanone (5f): Following general procedure B, the crude residue purified by flash chromatography using ethyl acetate/petroleum ether 3:7 (v:v) as eluent furnished **5f** as a yellow oil (46% yield). ^{1}H NMR ($CDCl_3$) δ: 3.32 (s, 6H), 3.93 (s, 3H), 3.96 (s, 6H), 7.30 (t, J = 8.2 Hz, 1H), 7.44 (d, J = 8.2 Hz, 1H), 7.56 (t, J = 8.2 Hz, 1H), 7.64 (s, 2H), 7.68 (d, J = 8.2 Hz, 1H). Anal. ($C_{20}H_{21}NO_5$): C, H, N. Anal. ($C_{19}H_{19}NO_6$): C, H, N. Anal. Calcd for $C_{20}H_{21}NO_5$: C, 67.45; H, 5.96; N, 3.94. Found: C, 67.45; H, 5.82; N, 3.83.

5.1.3.2. (3-(Dimethylamino)-4-methoxybenzofuran-2-yl) (3,4,5-trimethoxyphenyl) methanone (5g): Following general procedure B, the crude residue purified by flash chromatography using ethyl acetate/petroleum ether 3:7 (v:v) as eluent furnished **5g** as a yellow solid (43% yield); mp 148–150 °C. ^{1}H NMR ($CDCl_3$) δ: 3.52 (s, 6H), 3.92 (s, 3H), 3.96 (s, 6H), 3.98 (s, 3H), 6.52 (d, J = 7.8 Hz, 1H), 7.00 (d, J = 7.8 Hz, 1H), 7.40 (t, J = 7.8, 1H), 7.62 (s, 2H). Anal. Calcd for $C_{21}H_{23}NO_6$: C, 65.44; H, 6.02; N, 3.63. Found: C, 65.29; H, 5.88; N, 3.51.

5.1.3.3. (3-(Dimethylamino)-5-methoxybenzofuran-2-yl) (3,4,5-trimethoxyphenyl) methanone (5h): Following general procedure B, the crude residue purified by flash chromatography using ethyl acetate/petroleum ether 3:7 (v:v) as eluent furnished **5h** as a yellow oil (44% yield). ^{1}H NMR ($CDCl_3$) δ: 3.42 (s, 6H), 3.86 (s, 3H), 3.90 (s, 3H), 3.93 (s, 6H), 7.00 (s, 1H), 7.12 (d, J = 8.4 Hz, 1H), 7.34 (d, J = 8.4 Hz, 1H), 7.60 (s, 2H). Anal. Calcd for $C_{21}H_{23}NO_6$: C, 65.44; H, 6.02; N, 3.63. Found: C, 65.33; H, 5.91; N, 3.53.

5.1.3.4. (3-(Dimethylamino)-6-methoxybenzofuran-2-yl) (3,4,5-trimethoxyphenyl) methanone (5i): Following general procedure B, the crude residue purified by flash chromatography using ethyl acetate/petroleum ether 3:7 (v:v) as eluent furnished **5i** as a yellow oil (42% yield). ^{1}H NMR ($CDCl_3$) δ: 3.38 (s, 6H), 3.88 (s, 3H), 3.94 (s, 3H), 3.96 (s, 6H), 6.92 (d, J = 8.0 Hz, 1H), 7.18 (d, J = 8.0 Hz, 1H), 7.54 (s, 1H), 7.68 (s, 2H). Anal. Calcd for $C_{21}H_{23}NO_6$: C, 65.44; H, 6.02; N, 3.63. Found: C, 65.31; H, 5.88; N, 3.48.

5.1.3.5. (3-(Dimethylamino)-7-methoxybenzofuran-2-yl) (3,4,5-trimethoxyphenyl) methanone (5j): Following general procedure B, the crude residue purified by flash chromatography using ethyl acetate/petroleum ether 3:7 (v:v) as eluent furnished **5j** as a yellow solid (93% yield); mp 148–150 °C. ^{1}H NMR ($CDCl_3$) δ: 3.50 (s, 6H), 3.90 (s, 3H), 3.94 (s, 6H), 3.98 (s, 3H), 6.94 (d, J = 6.8 Hz, 1H), 7.18 (m, 2H), 7.62 (s, 2H). Anal. Calcd for $C_{21}H_{23}NO_6$: C, 65.44; H, 6.02; N, 3.63. Found: C, 65.34; H, 5.92; N, 3.52.

5.1.4. Synthesis of (3-acetylamino-6-methoxybenzofuran-2-yl)-(3,4,5-trimethoxyphenyl)methanone (5k)—To a solution of **5d** (1 mmol, 358 mg) and pyridine (3 mmol, 242 μL) in dry dichloromethane (5 mL), acetyl chloride (3 mmol, 212 μL) was added at 0 °C. The reaction mixture was stirred for 2 h at room temperature, diluted with dichloromethane (5 mL), washed with water (4 mL), dried over Na_2SO_4, and concentrated in vacuo. The crude residue purified by flash chromatography using ethyl acetate/petroleum ether 4:6 (v:v) as eluent furnished **5k** as a yellow solid (90% yield) after recrystallization from petroleum ether; mp 172–173 °C. ^{1}H NMR ($CDCl_3$) δ: 2.17 (s, 3H), 3.91 (s, 3H), 3.94 (s, 3H), 3.97 (s, 6H), 6.88 (s, 1H), 6.90 (d, J = 9.2 Hz, 1H), 7.53 (s, 2H), 8.47 (d, J = 9.2 Hz, 1H), 10.9

(s, 1H). Anal. ($C_{21}H_{21}NO_7$): C, H, N. Anal. Calcd for $C_{21}H_{21}NO_7$: C, 63.15; H, 5.30; N, 3.51. Found: C, 63.02; H, 5.18; N, 3.38.

5.1.5. Synthesis of (3-chloroacetylamino-6-methoxybenzofuran-2-yl)(3,4,5-trimethoxyphenyl)methanone (5l)—To a solution of **5d** (1 mmol, 358 mg) and pyridine (3 mmol, 242 μL) in dry dichloromethane (5 mL), chloroacetyl chloride (3 mmol, 239 μL) was added at 0 °C. The reaction mixture was stirred for 1 h at room temperature, diluted with dichloromethane (10 mL), washed with water (5 mL), dried over Na_2SO_4, and concentrated in vacuo. The crude residue purified by flash chromatography using ethyl acetate/petroleum ether 3:7 (v:v) as eluent furnished **5l** as a green solid (>95% yield) after recrystallization from petroleum ether; mp 123–124 °C. ^{1}H NMR ($CDCl_3$) δ: 3.91 (s, 3H), 3.97 (s, 9H), 4.31 (s, 2H), 6.91 (s, 1H), 6.95 (d, J = 9.2 Hz, 1H), 7.55 (s, 2H), 8.47 (d, J = 9.2 Hz, 1H), 11.7 (s, 1H). Anal. Calcd for $C_{21}H_{20}ClNO_7$: C, 58.14; H, 4.65; N, 3.23. Found: C, 58.01; H, 4.48; N, 3.11.

5.1.6. Synthesis of (3-bromoacetylamino-6-methoxybenzofuran-2-yl)(3,4,5-trimethoxyphenyl)methanone (5m)—To a solution of **5d** (1 mmol, 358 mg) and pyridine (3 mmol, 242 μL) in dry dichloromethane (5 mL), bromoacetyl chloride (3 mmol, 250 μL) was added at 0 °C. After 3 h at the same temperature, the reaction mixture was diluted with dichloromethane (5 mL), washed with water (5 mL), dried over Na_2SO_4, and concentrated in vacuo. The crude residue purified by flash chromatography using ethyl acetate/petroleum ether 3:7 (v:v) as eluent furnished 5 m as a green solid (78% yield) after recrystallization from petroleum ether; mp 99–100 °C. ^{1}H NMR ($CDCl_3$) δ: 3.89 (s, 3H), 3.92 (s, 6H), 3.97 (s, 3H), 4.13 (s, 2H), 6.91 (s, 1H), 6.94 (d, J = 9.2 Hz, 1H), 7.54 (s, 2H), 8.45 (d, J = 9.2 Hz, 1H), 11.6 (s, 1H). Anal. Calcd for $C_{21}H_{20}BrNO_7$: C, 52.73; H, 4.21; N, 2.93. Found: C, 52.62; H, 4.04; N, 2.78.

5.1.7. Synthesis of (3-iodoacetylamino-6-methoxybenzofuran-2-yl)(3,4,5-trimethoxyphenyl)methanone (5n)—A mixture of *5m* (1 mmol, 478 mg) and NaI (10 mmol, 1.5 g) in *N*,*N*-dimethylacetamide (5 mL) was stirred at room temperature for 18 h. *N*,*N*-dimethylacetamide was evaporated under reduced pressure, followed by addition of dichloromethane (15 mL) and a solution of $Na_2S_2O_3$ (10%, 5 mL). The organic layer was washed with water (5 mL), brine (5 mL), and dried over Na_2SO_4. After removal of the solvent under reduced pressure, the crude residue purified by flash chromatography using ethyl acetate/petroleum ether 4:6 (v:v) as eluent furnished **5n** as a yellow solid (58% yield) after recrystallization from petroleum ether; mp 140–141 °C. ^{1}H NMR ($CDCl_3$) δ: 3.72 (s, 2H), 3.90 (s, 3H), 3.97 (s, 6H), 3. 98 (s, 3H), 6.89 (s, 1H), 6.92 (d, J = 8.8 Hz, 1H), 7.54 (s, 2H), 8.45 (d, J = 8.8 Hz, 1H), 11.5 (s, 1H). Anal. Calcd for $C_{21}H_{20}INO_7$: C, 48.02; H, 3.84; N, 2.67. Found: C, 47.88; H, 3.74; N, 2.56.

5.2. Cell growth inhibitory activity

Murine leukemia L1210, murine mammary carcinoma FM3A, and human T-lymphocyte Molt 4 and CEM cells were suspended at 300,000–500,000 cells/mL of culture medium, and 100 μL of a cell suspension was added to 100 μL of an appropriate dilution of the test compounds in wells of 96-well microtiter plates. After incubation at 37 °C for two (L1210 and FM3A) or three (Molt 4 and CEM) days, cell number was determined using a Coulter counter. The IC_{50} value was defined as the compound concentration required to inhibit cell proliferation by 50%.

5.3. Effects on tubulin polymerization and on colchicine binding to tubulin

Bovine brain tubulin was purified as described previously.[20] To evaluate the effect of the compounds on tubulin assembly *in vitro*,[13] varying concentrations were preincubated with 10 μM tubulin in glutamate buffer at 30 °C and then cooled to 0 °C. After addition of GTP, the

mixtures were transferred to 0 °C cuvettes in a recording spectrophotometer and warmed to 30 °C, and the assembly of tubulin was observed turbidimetrically. The IC_{50} value was defined as the compound concentration that inhibited the extent of assembly by 50% after a 20 min incubation. The ability of the test compounds to inhibit colchicine binding to tubulin was measured as described,[14] except that the reaction mixtures contained 1 μM tubulin, 5 μM [^{3}H]colchicine, and 1 μM test compound.

5.4. 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 (myeloblastic leukemia) by flow cytometric analysis after staining with propidium iodide. Cells were exposed for 24 h to each compound used at a concentration corresponding to the IC_{50} determined after a 24 h incubation. After treatment, the cells were washed once in ice-cold PBS and resuspended at 1 × 106 per mL in a hypotonic fluoro-chrome 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 histogram, using a FAC-Scan 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.).

5.5. Molecular modeling

All molecular modeling studies were performed on a MacPro dual 2.66 GHz Xeon running Ubuntu 7. The tubulin structure was downloaded from the PDB (http://www.rcsb.org/–PDB code: 1SA0).[19] Hydrogen atoms were added to the protein using Molecular Operating Environment (MOE) 2006.08[17] and minimized using the MMFF94x forcefield until a RMSD gradient of 0.05 kcal mol^{-1} $Å^{-1}$ was reached. The docking simulations with MOE were performed using the Alpha Triangle placement method and the London dG scoring method. Plants[15] was used from the graphical interface included in ZODIAC[21] with the default settings. The Surflex module included in Sybyl 7.3[16] was used with the default settings with a ligand-based (DAMA-colchicine) protomodel. The results obtained with each software package were then rescored using the CScore module in Sybyl 7.3. The RMSD of the trimethoxyphenyl moiety for each of the results obtained was calculated in comparison with ring A of the colchicine analogue.[22]

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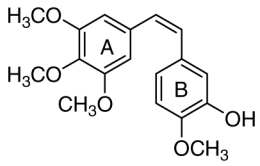
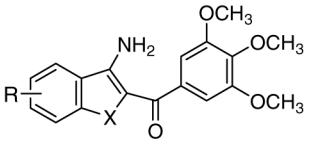
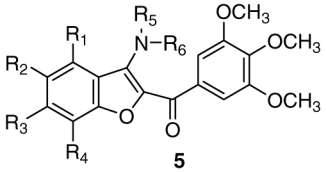


Combretastatin A-4 (CA-4), **1**



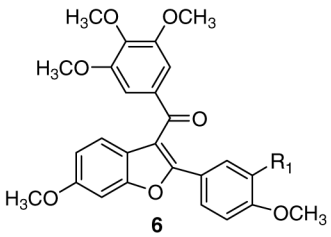
R=H, Me or OMe
X=S, benzo[b]thiophene derivatives **2**
X=NH, 1-*H*-indole derivatives **3**
X=NMe, 1-methylindole derivatives **4**

X=S, R=6-OMe, **2a**
X=NH, R=6-OMe, **3a**
X=NMe, R=6-OMe, **4a**



5

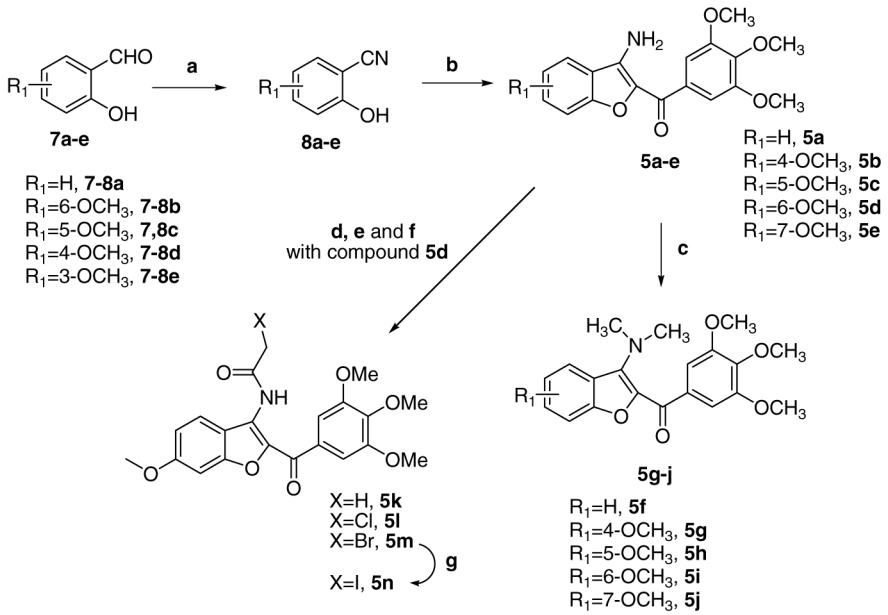
$R_{1\text{-}6}$=H; **5a**
R_1=OCH_3, $R_{2\text{-}6}$=H; **5b**
R_2=OCH_3, $R_{1,\ 3\text{-}6}$=H, **5c**
R_3=OCH_3, $R_{1\text{-}2,\ 4\text{-}6}$=H; **5d**
R_4=OCH_3, $R_{1\text{-}3,\ 5,\ 6}$=H; **5e**
$R_{1\text{-}4}$=H, $R_{5,\ 6}$=CH_3; **5f**
R_1=OCH_3, $R_{2\text{-}4}$=H, $R_{5,6}$=CH_3; **5g**
R_2=OCH_3, $R_{1,\ 3\text{-}4}$=H, $R_{5,6}$=CH_3; **5h**
R_3=OCH_3, $R_{1\text{-}2,\ 4\text{-}}$=H, $R_{5,6}$=CH_3; **5i**
R_4=OCH_3, $R_{1\text{-}3}$=H, $R_{5,\ 6}$=CH_3; **5j**
R_3=OCH_3, $R_{1\text{-}2,\ 4\text{--}5}$=H, R_6=$COCH_3$; **5k**
R_3=OCH_3, $R_{1\text{-}2,\ 4\text{--}5}$=H, R_6=$COCH_2Cl$; **5l**
R_3=OCH_3, $R_{1\text{-}2,\ 4\text{--}5}$=H, R_6=$COCH_2Br$; **5m**
R_3=OCH_3, $R_{1\text{-}2,\ 4\text{--}5}$=H, R_6=$COCH_2I$; **5n**



6

R_1=H, **6a**
R_1=OH, **6b**

Chart 1.
Inhibitors of tubulin polymerization.



Scheme 1.

Reagents and conditions: (a) $C_2H_5NO_2$, NaOAc; AcOH; (b) 2-bromo-1-(3,4,5-trimethoxyphenyl)ethanone, K_2CO_3, $(CH_3)_2CO$, reflux, 18 h, (c) Me1, NaH, DMF; (d) CH_3COCl, pyridine, CH_2Cl_2, rt; (e) $ClCH_2COCl$, pyridine, CH_2Cl_2, rt; (f) $BrCH_2COBr$, pyridine, CH_2Cl_2, rt; (g) Nal, $CH_3CON(CH_3)_2$, rt.

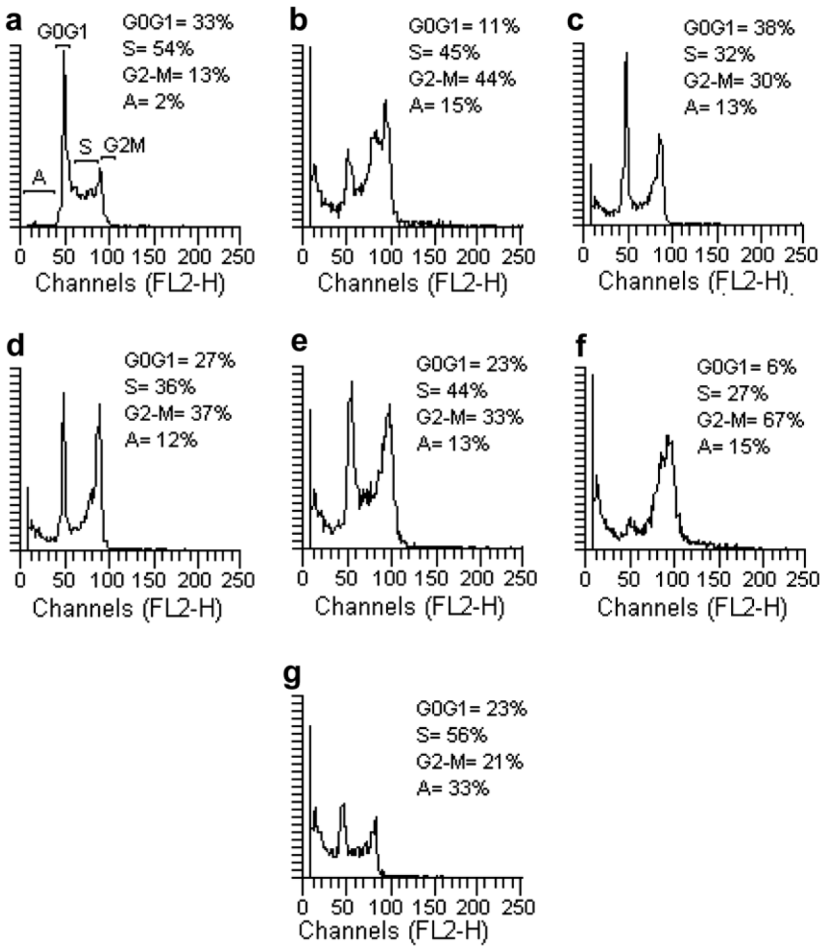


Fig. 1.

Effects of compounds **5d** (b), **5i** (c), **5k** (d), **5l** (e), **5m** (f), and **5n** (g) on DNA content/cell following treatment of K562 cells for 24 h. The cells were cultured without compound (Control, 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 as described in Section 5. Sub-G0-G1 (A), G0-G1, S, and G2-M cells are indicated in the control panel.

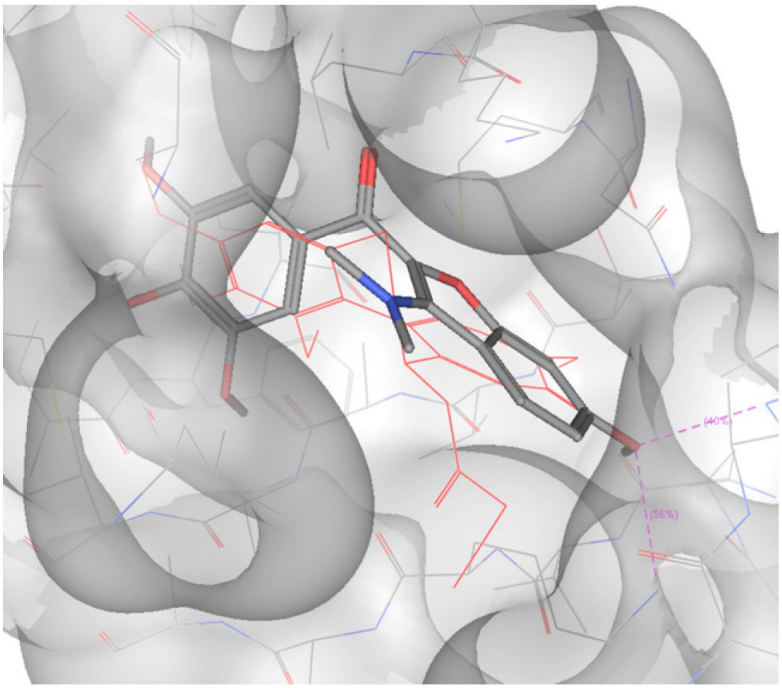


Fig. 2.
Docking pose of **5i**. DAMA-colchicine is represented in red.

Table 1

In vitro inhibitory effects of compounds **2–4a**, **5a–n**, and CA-4 (**1**) on 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**
5a	>10,000	>10,000	>10,000	>10,000
5b	7500 ± 900	7900 ± 2100	1800 ± 100	6700 ± 100
5c	>10,000	>10,000	>10,000	>10,000
5d	430 ± 40	280 ± 160	140 ± 20	87 ± 22
5e	>10,000	>10,000	4400 ± 1400	7700 ± 400
5f	>10,000	8800 ± 1300	2700 ± 500	>10,000
5g	>10,000	>10,000	>10,000	>10,000
5h	1400 ± 110	1200 ± 40	470 ± 22	1700 ± 100
5i	65 ± 5	59 ± 5	48 ± 4	78 ± 3
5j	1100 ± 100	1200 ± 900	670 ± 380	1400 ± 80
5k	94 ± 7	100 ± 0.00	62 ± 4.2	78 ± 3
5l	1100 ± 90	1100 ± 90	310 ± 28	460 ± 33
5m	480 ± 15	560 ± 12	240 ± 19	210 ± 11
5n	170 ± 30	260 ± 60	86 ± 5	120 ± 0.0
2a	39 ± 16	46 ± 12	10 ± 7	7.7 ± 2.9
3a	970 ± 24	1600 ± 30	630 ± 10	320 ± 90
4a	69 ± 37	97 ± 3	57 ± 7	71 ± 5
CA-4 (**1**)	2.8 ± 1.1	42 ± 6	16 ± 1.4	1.9 ± 1.6

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

Table 2

Inhibition of tubulin polymerization and colchicine binding by compounds **2–4a**, **5d**, **5i**, **5k–n**, and CA-4

Compound	Tubulin assembly[a] IC_{50} ± SD (μM)	Colchicine binding[b] % ± SD
5d	1.1 ± 0.1	73 ± 5
5i	0.90 ± 0.0	76 ± 5
5k	1.6 ± 0.0	51 ± 7
5l	2.0 ± 0.1	47 ± 5
5m	2.3 ± 0.5	49 ± 2
5n	1.8 ± 0.1	54 ± 1
2a	1.3 ± 0.1	60 ± 0
3a	>40	nd
4a	4.6 ± 0.1	38 ± 5
CA-4 (**1**)	1.2 ± 0.1	86 ± 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. nd, not determined.