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Design, synthesis and structure–activity relationship of 2-(3′,4′,5′-trimethoxybenzoyl)-benzo[*b*]furan derivatives as a novel class of inhibitors of tubulin polymerization

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ABSTRACT

The biological importance of microtubules in mitosis and cell division makes them an interesting target for the development of anticancer agents. Small molecules such as benzo[*b*]furans are attractive as inhibitors of tubulin polymerization. Thus, a new class of inhibitors of tubulin polymerization based on the 2-(3′,4′,5′-trimethoxybenzoyl)-benzo[*b*]furan molecular skeleton, with electron-donating (Me, OMe or OH) or electron-withdrawing (F, Cl and Br) substituents on the benzene ring, was synthesized and evaluated for antiproliferative activity, inhibition of tubulin polymerization and cell cycle effects. Adding a methyl group at the C-3 position resulted in increased activity. The most promising compound in this series was 2-(3′,4′,5′-trimethoxybenzoyl)-3-methyl-6-ethoxy-benzo[*b*]furan, which inhibits cancer cell growth at nanomolar concentrations and interacts strongly with tubulin by binding to the colchicine site.

1. Introduction

The microtubule system of eukaryotic cells plays important roles in regulating cell architecture, and it has an essential role in cell division, since microtubules are a key component of the mitotic spindle.[1] Microtubules are a dynamic cellular compartment in both neoplastic and normal cells. This dynamicity is characterized by the continuous turnover of αβ-tubulin heterodimers in the polymeric microtubules. They are involved in a variety of essential cellular processes, such as regulation of motility, cell signaling, formation and maintenance of cell shape, as well as transport of material within the cell.[2] Numerous chemically diverse antimitotic agents, many of which are derived from natural products, have been found to interact specifically with tubulin.[3]

Among the microtubule depolymerizing agents, combretastatin A-4 (CA4, **1**; Chart 1) is one of the more studied compounds. CA4, 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 structural simplicity, a wide number of CA4 analogues have been developed and evaluated in SAR studies.[6]

Among synthetic small molecule tubulin inhibitors, replacement of the olefinic bridge of **1** with a carbonyl group furnished a benzophenone-type CA4 analogue named phenstatin (**2a**). This compound demonstrated interesting efficacy in a variety of tumor models, while retaining the characteristics of **1**.[7] The 2-aminobenzophenone derivative **2b** also strongly inhibited cancer cell growth and tubulin polymerization and caused mitotic arrest, as did **2a**.[8]

In an earlier publication, we reported a series of methoxy-substituted 2-(3′,4′,5′-trimethoxybenzoyl)-benzo[*b*]furan derivatives with general structure **3**, with an amino or dimethylamino substituent at the 3-position of the benzo[*b*]furan skeleton (compounds **3a** and **3b**, respectively) as a new class of antimitotic agents.[9a] The concomitant presence of a methoxy group at the 6-position contributed to maximal activity. These compounds, along with the corresponding benzo[*b*]thiophene[9b,c] and indole[9d] derivatives, inhibited the growth of different cancer cell lines and tubulin polymerization by binding to the colchicine site of tubulin and

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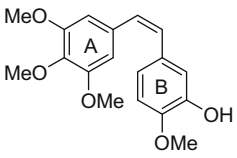
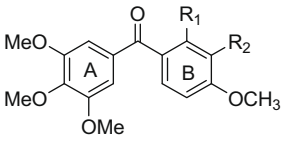
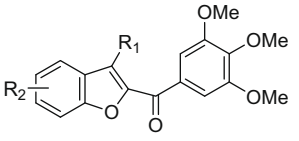


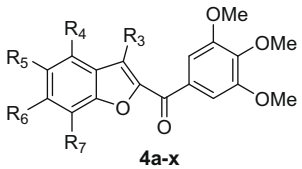
Combretastatin A-4 (CA-4), **1**



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

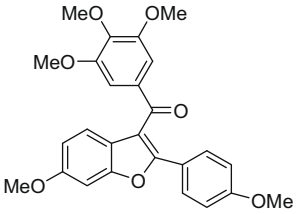


3
R_1=NH_2 or $N(Me)_2$
R_2=H, Me or OMe
R_1=NH_2, R_2=6-OCH_3, **3a**
R_1=$N(CH_3)_2$, R_2=6-OCH_3, **3b**

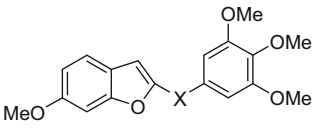


4a-x

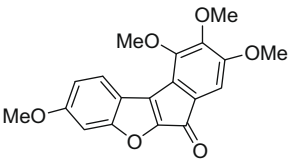
R_{3-7}=H, **4a**
R_4=OMe, $R_{3,\ 5-7}$=H, **4b**
R_5=OMe, $R_{3-4,\ 6-7}$=H, **4c**
R_6=OMe, $R_{3-5,\ 7}$=H, **4d**
R_7=OMe, R_{3-6}=H, **4e**
R_5=Me, $R_{3-4,\ 6-7}$=H, **4f**
R_5=Cl, $R_{3-4,\ 6-7}$=H, **4g**
R_5=Br, $R_{3-4,\ 6-7}$=H, **4h**
R_6=OMe, R_7=OH, R_{3-5}=H, **4i**
$R_{5,\ 6}$=OMe, $R_{3-4,\ 7}$=H, **4j**
R_{5-7}=OMe, R_{3-4}=H, **4k**
R_5=OH, $R_{3-4,\ 6-7}$=H, **4l**
R_6=OH, $R_{3-5,\ 7}$=H, **4m**
R_7=OH, R_{3-6}=H, **4n**
R_3=Me, R_{4-7}=H, **4o**
R_3=Me, R_4=OMe, R_{5-7}=H, **4p**
R_3=Me, R_5=OMe, $R_{4,\ 6-7}$=H, **4q**
R_3=Me, R_6=OMe, $R_{4-5,\ 7}$=H, **4r**
R_3=Me, R_7=OMe, R_{4-6}=H, **4s**
R_3=Me, R_6=OEt, $R_{4-5,\ 7}$=H, **4t**
R_3=Me, R_6=F, $R_{4-5,\ 7}$=H, **4u**
R_3=Me, R_4=OH, R_{5-7}=H, **4v**
R_3=Me, R_5=OH, $R_{4,\ 6-7}$=H, **4w**
R_3=Me, R_6=OH, $R_{4-5,\ 7}$=H, **4x**



3c



X=CS, **4y**
X=CHOH, **4z**
X=CHOMe, **4aa**
X=CH_2, **4ab**



4ac

Chart 1. Inhibitors of tubulin polymerization.

caused G_2–M phase arrest of the cell cycle. The higher potency of the 3-dimethylamino derivative **3b** allowed us to verify that an intramolecular hydrogen bond between the unsubstituted 3-amino group and the carbonyl oxygen of the 2-trimethoxybenzoyl moiety is not required for activity. These findings prompted us to study this class of compounds in more detail. We herein describe the synthesis and structure–activity relationship of 2-(3′,4′,5′-trimethoxybenzoyl)-benzo[*b*]furan derivatives in the continuation of our search for new potent antitubulin agents. 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. One of these compounds has structure **3c**, which incorporates the 3-(3,4,5-trimethoxybenzoyl)-6-methoxybenzo[*b*]furan ring system.

In the present investigation, the 3′,4′,5′-trimethoxyphenyl of the 2-benzoyl moiety was kept unchanged because it is the characteristic structural requirement for activity in a numerous inhibitors of tubulin polymerization, such as colchicine, CA4 and podophyllotoxin.[6] In the current studies we examined the importance of the 3-position of the benzo[*b*]furan skeleton by studying the effects of replacing the amino or dimethylamino substituents of compounds with general structure **3** with a hydrogen or a methyl group, to furnish derivatives with general structure **4**. Additional structure modifications were focused on the C-4/C-7 positions, as well as on the 2-carbonyl linker of the benzo[*b*]furan skeleton.

In a first series (derivatives **4a–x**), the 3-amino group of the benzo[*b*]furan system was replaced by a hydrogen (**4a–n**) or a methyl (**4o–x**). These molecules possessed either no substituent (**4a** and **4o**) or a methoxy group at each of the four possible positions on the benzene ring (compounds **4b–e** and **4p–s**). In an effort to design compounds with improved polarity, we decided to introduce an additional hydroxy group at the C-7 position of compound **4d**, to obtain the C-6 methoxy, C-7 hydroxy derivative **4i**. An increase of the polarity in the 2-aroyl benzo[*b*]furan structure can be also obtained replacing the methoxy group of compounds **4b–e** and **4p–s** with a more hydrophilic hydroxyl moiety, to obtain the derivatives **4l–n** and **4v–x**. The hydroxyl moiety provides a site for the preparation of a phosphate prodrug, similar to what has been successfully done in the case of CA4 phosphate.[10] Keeping the C-6 methoxy group intact, compounds **4j** and **4k** were prepared with the aim of evaluating the effect on biological activity of one (**4j**) or two (**4k**) additional methoxy substituents at the C-5 and C-5, 7 positions, respectively. Besides the C-5 methoxy moiety, the substituents examined included electron-donating methyl (**4f**) and electron-withdrawing chlorine and bromine (**4g** and **4h**, respectively) groups. Finally, the 6-methoxy position (corresponding to the 4-methoxy group in the B-ring of CA4 and 2-aminobenzophenone **2b**) of **4r** was further studied by synthesizing the corresponding 6-ethoxy (**4t**) and 6-fluorine (**4u**) analogues.

Starting from compound **4d**, additional analogues were generated by modification of the 2-carbonyl function, which was converted into thiocarbonyl (**4y**), reduced to carbinol (**4z**) and to methylene (**4ab**) or transformed into a methyl ether (**4aa**).

Finally, by the synthesis of tetracyclic compound **4ac**, the phenyl of the 2-(3′,4′,5′-trimethoxy)benzoyl moiety and the 3-position of the benzo[*b*]furan skeleton were locked in a conformation in which these two ring were forced to be coplanar.

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]

2. Chemistry

The 2-(3′,4′,5′-trimethoxybenzoyl)benzo[*b*]furan derivatives **4a–l**, **4n–w**, **6m** and **6x** were synthesized by a 'one-step' condensation of the corresponding, variously substituted salicylaldehydes **5a–n** or 2-hydroxyacetophenones **5o–x** with 2-bromo-1-(3,4,5-trimethoxyphenyl)ethanone[9b] and anhydrous potassium carbonate in refluxing acetone (Scheme 1). For the *tert*-butyldimethylsilyl (TBDMS) analogues **5l**, **5n** and **5v–w**, the cyclization was accomplished by the concomitant removal of TBDMS-protecting group, to afford derivatives **4l**, **4n** and **4v–w**. The 6-hydroxy-2-aroyl benzo[*b*]furan derivatives **4m** and **4x** were obtained by cleavage of the 6-benzyloxy group of **6m** and **6x**, respectively, with a mixture of activated palladium on charcoal and ammonium formate.

The treatment of the 2-carbonyl moiety of **4d** with Lawesson′s reagent or its reduction with sodium borohydride furnished the thiocarbonyl or the carbinol derivatives **4y** and **4z**, respectively.[12] This latter compound was further converted to the methyl ether **4aa** by treatment with pyridinium *p*-toluensulfonate in a mixture of MeOH–THF or reduced to the methylene derivative **4ab** with triethylsilane in trifluoroacetic acid. The cyclic ketone **4ac** was synthesized by intramolecular cross-coupling of bromo derivative **7** catalyzed with palladium (0) tetrakistriphenylphosphine in *N*,*N*-dimethylacetamide at 130 °C. Derivative **7** was obtained by the chemoselective bromination of **4d** with NBS in acetonitrile.[13]

3. Biological results and discussion

Table 1 summarizes the growth inhibitory effects of 2-(3′,4′,5′-trimethoxy)benzo[*b*]furan derivatives **4a–ac** against murine leukemia (L1210), murine mammary carcinoma (FM3A), human T-lymphoblastoid (Molt/4 and CEM) and human cervix carcinoma (HeLa) cells, with CA4 (**1**), **3a** and **3b** as reference compounds. The 3-methyl-6-ethoxy derivative **4t** possessed the highest potency, inhibiting the growth of L1210, FM3A, Molt/4, CEM and HeLa cells with IC_{50} values of 2.0, 2.8, 1.2, 2.8 and 6.3 nM, respectively. These values are similar to those obtained with CA4 and from 20- to 100-fold higher activity than those obtained with the 3-amino derivatives **3a** and **3b**. However, this marked improvement in activity relative to **3a**–**b** may derive in part from the ethoxy substituent at C-6, since the analogue with a methoxyl at C-6 (**4r**) was approximately 10-fold less active than **4t** in four of the cell lines.

Comparing the 6-methoxy derivatives **3a**–**b**, **4d** and **4r**, the order of activity for the substituent at the 3-position of the benzo[*b*]furan moiety was methyl (**4r**) > hydrogen (**4d**) ≈ dimethylamino (**3b**) > amino (**3a**). With the four compounds IC_{50} values ranged from 3 to 27 nM for **4r**, from 59 to 110 nM for **4d**, from 48 to 78 for **3b**, and from 87 to 430 nM for **3a**. Comparing the activities of the carbonyl derivative **4d** with those of derivatives **4y–z** and **4aa–ab** indicated that the 2-carbonyl moiety could only be replaced with a thiocarbonyl group (**4y**) among the bridging moieties examined.

Comparing the 3-unsubstituted derivatives **4a–e** and **4l–m** with their 3-methyl congeners **4o–s** and **4w–x**, the introduction of a methyl at the C-3 position of the benzo[*b*]furan system generally increased antiproliferative activity against all five cell lines. The antiproliferative activities were also dependent on the substitution pattern on the benzene part of the benzo[*b*]furan moiety, with the most favorable position being C-6 for a methoxy group (**4r** was more active than **4p**, **4q** or **4s**, as was observed previously with the 3-aminobenzo[*b*]furan derivatives **3a**–**b**), while C-4 was the best position for an hydroxyl group (**4v** more active than **4w** or **4x**). In the absence of the C-3 methyl group, the derivative with the C-6 methoxyl moiety (**4d**) was more active than analogues with the methoxy at other positions (**4b**, **4c** and **4e**). Additional methoxy groups on the benzene ring (**4j** and **4k**) resulted in sharply reduced potency relative to **4d**. The introduction of an additional hydroxyl group at the C-7 position of **4d**, to furnish **4i**, caused a substantial loss of activity.

In the series of 3-unsubstituted derivatives, the only compound with a methoxy moiety with significant activity was **4d** and replacing the methoxy with the weaker electron-donating and more hydrophilic hydroxy group (compound **4m**) resulted in a drastic loss of activity. With the 3-methyl analogues **4p–r**, the substitution of the methoxy group at the C-4 or C-5 positions (**4v** and **4w**) with the hydroxy function caused an increase in the antiproliferative activity. A dramatic loss of activity was only observed when the C-6 methoxy (**4r**) was replaced with an hydroxy group (**4x**).

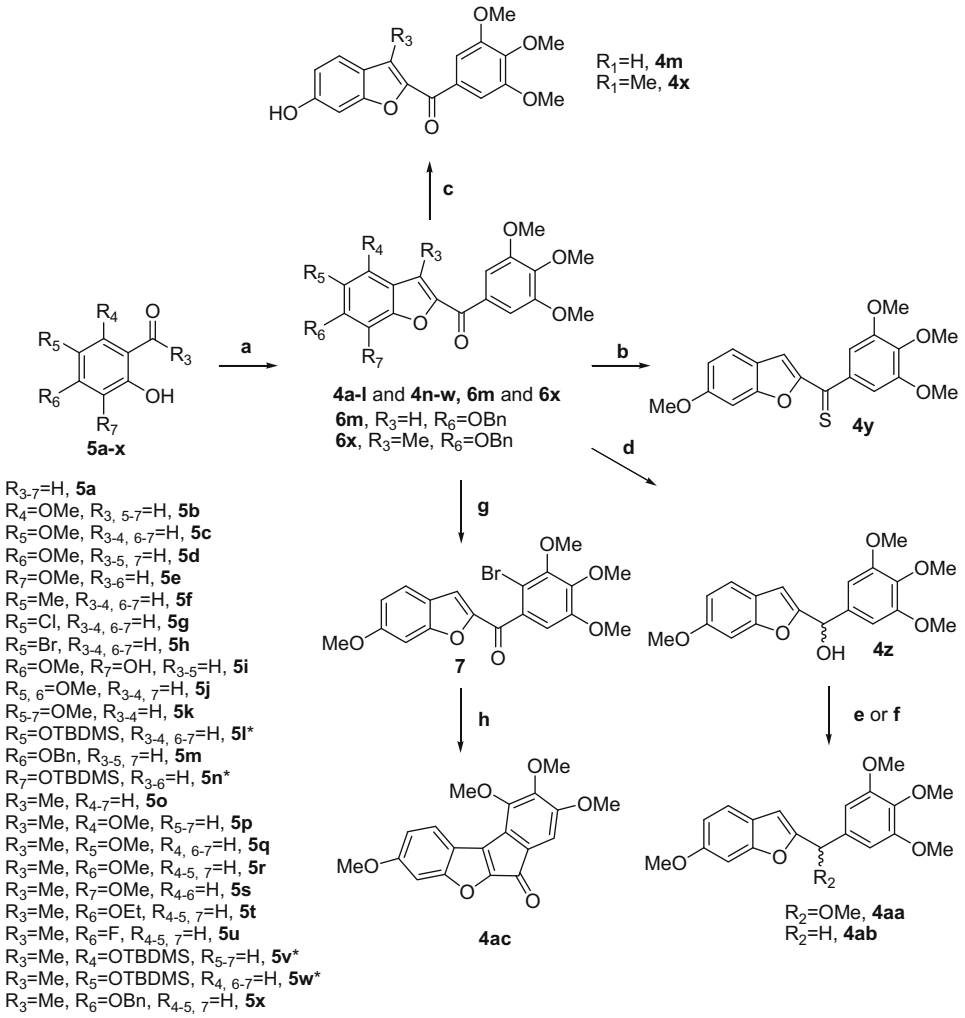
For the inactive C-5 substituted derivative **4c**, replacing the strong electron-donating methoxy function with the electron-withdrawing chlorine (**4g**) or bromine (**4h**) groups had no effect on activity, but replacement of the C-5 methoxy group with the moderately electron-donating methyl moiety (**4f**) slightly increased activity.

As noted above, the activity of **4r** was increased 10-fold against L1210, FM3A, Molt/4 and CEM cells and 2-fold for HeLa cells when the C-6 methoxy group was replaced with an ethoxy moiety (**4t**). In contrast, an electron-withdrawing fluorine group at C-6 (**4u**) caused a dramatic reduction of antiproliferative activity.

Linking the *ortho*-position of the phenyl of the 2-(3′,4′,5′-trimethoxybenzoyl)moiety, to the 3-position of benzo[*b*]furan moiety, to yield the conformationally constrained derivative **4ac**, led to a completely inactive compound.

To confirm that the antiproliferative activities of these compounds were related to an interaction with the microtubule system, the most active compounds, **4b**, **4d**, **4p**–**r**, **4t**, **4v** and **4w**, along with the 6-fluoro derivative **4u**, were evaluated for their in vitro inhibition of tubulin polymerization and for their inhibitory effects on the binding of [^{3}H]colchicine to tubulin (in the latter assay, tubulin was examined at a concentration of 1 μM, while compounds and colchicine were at 5 μM).[14,15] For comparison, CA4 was examined in contemporaneous experiments as a reference compound (Table 2). The benzo[*b*]furan derivatives **4d**, **4r**, **4t** and **4v** with IC_{50} values of 0.70, 0.55, 0.43 and 0.57 μM, respectively, exhibited antitubulin activity about two times greater than that of CA4 (1.0 μM), while **4b**, **4q** and **4w** had IC_{50} values of 1.1–1.2 μM, essentially equivalent to that of CA4.

The order of inhibitory action on tubulin assembly was **4t** > **4r** > **4v** > **4d** > CA4 > **4b** > **4w** > **4q** > **4p** >> **4u**, which was consistent with the results of the antiproliferative assays, except that **4d** was more potent than **4v** and **4p** was more potent than **4q**. The most potent compound in this series was compound **4t**, with an IC_{50} value of 0.43 μM, which correlates well with its having the greatest antiproliferative activity. Compounds **4b**, **4q** and **4w** were as active as CA4 as inhibitors of tubulin assembly, although both compounds were less active in their effects on cell growth. Compound **4u** showed weak antitubulin polymerization activity, which is consistent with its low antiproliferative activity.



Scheme 1. Reagents and conditions: (a) (3,4,5-Trimethoxyphenyl)-2-bromo-ethanone, K_2CO_3, $(CH_3)2CO$, rt; (b) Lawesson's reagent, THF, rt from **4d**; (c) HCO_2NH_4, 10% Pd/C, MeOH, rt from **6m** and **6x**; (d) $NaBH_4$, MeOH, rt from **4d**; (e) PTSA, MeOH–THF, rt; (f) Et_3SiH, TFA, CH_2Cl_2, rt; (g) NBS, benzoyl peroxide, MeCN, rt from **4d**; (h) $Pd(Ph_3P)_4$, KOAc, DMA, 130 °C. For compounds **5l**, **5n** and **5v**–**w**, condition '**a**' led to a cyclization with concomitant removal of TBDMS group, to afford **4l**, **4n** and **4v**–**w**, respectively.

In the colchicine binding studies, compounds **4r** and **4t** strongly inhibited the binding of [^{3}H]colchicine to tubulin, since 97% and 96% inhibition, respectively, occurred with these agents and colchicine, both at 5 μM. These derivatives were as active as CA4, which in these experiments inhibited colchicine binding by 99%. These data indicate that **4r** and **4t** strongly bind to the colchicine site on tubulin.

Because molecules exhibiting effects on tubulin assembly should cause alteration of cell cycle parameters with preferential G_2–M blockade, flow cytometry analysis was performed to determine the effect of the compounds **4b**, **4d**, **4p–r**, **4t** and **4u–w** on K562 (human chronic myelogenous leukemia) cells. Cells were cultured for 24 h in the presence of each compound at the concentration able to inhibit 100% cell growth after 24 h (**4b** = 980 nM, **4d** and **4p** = 420 nM, **4q** = 230 nM, **4r** = 26 nM, **4t** = 16 nM, **4u** = 1.62 μM, **4v** = 530 nM, **4w** = 480 nM). Analysis of sub-G_0–G_1 (apoptotic peak, A), G_0–G_1, S, and G_2–M peaks revealed that the compounds caused somewhat different effects on cell cycle distribution (Fig. 1). All tested compounds caused an increase in the proportion of cells in the G_2–M peak relative to the untreated control, and compounds **4p** and **4u** also caused cells to accumulate in late S phase. For compounds **4b**, **4p**. **4u** and **4w**, the accumulation of cells in G_2–M phase was accompanied by the appearance of a significant sub-G_0–G_1 peak (*A* = 20–44%) due to apoptosis.

Molecular docking studies on this series of compounds in the colchicine site of tubulin were also performed. There was a comparable binding mode of these compounds to that of DAMA-colchicine co-crystallized in the tubulin structure used,[16] with the trimethoxyphenyl moiety occupying the same pocket as the corresponding ring A of the colchicinoid (Fig. 2).

The substituent in the position 6 of the benzofuran analogues, like the methoxy group on ring C of colchicinoids, falls into a hydrophobic region deep in the binding site (shown in green in Fig. 3). Furthermore, a polar area on the tubulin surface corresponding to the carbonyl group of Thr179 (residue numbering derived from the crystal structure used) with potential for accepting a hydrogen bond is close to the C-4 position of the benzo[*b*]furan group (shown in purple in Fig. 3). This feature could be exploited by the hydroxyl group of **4v** to establish a hydrogen bond with tubulin, which could compensate to the loss of the hydrophobic contact provided by the C-6 methoxy group, which is missing in **4v**, and justify the activity of this compound in the tubulin polymerization assay. It should be mentioned that the position observed for the hydroxyl group of **4v** is similar to the proposed binding location of the corresponding functional group of CA4, although in the case of **4v** is the carbonyl group of Thr179 that is closer to the inhibitor (2.54 Å), while for CA4 the hydroxyl group is placed close to Val181 (see Fig. 1, Supplementary data).[17]

Table 1
In vitro inhibitory effects of compounds **3a–b**, **4a–ac** and CA4 against the proliferation of murine leukemia (L1210), murine mammary carcinoma (FM3A), human T-lymphocyte (Molt/4 and CEM) and human cervix carcinoma (HeLa) cells

Compound	IC_{50} (nM)[a]				
	L1210	FM3A/0	Molt4/C8	CEM/0	Hela
4a	>10,000	>10,000	8000 ± 100	>10,000	7900 ± 100
4b	410 ± 23	550 ± 42	360 ± 10	870 ± 50	410 ± 16
4c	>10,000	>10,000	9400 ± 500	>10,000	>10,000
4d	90 ± 2	100 ± 10	59 ± 12	70 ± 21	110 ± 20
4e	>10,000	>10,000	7800 ± 290	>10,000	>10,000
4f	7100 ± 580	8400 ± 300	2500 ± 160	7200 ± 210	>10
4g	>10,000	>10,000	>10,000	>10,000	>10,000
4h	>10,000	>10,000	>10,000	>10,000	>10,000
4i	>10,000	>10,000	>10,000	>10,000	>10,000
4j	1800 ± 400	1200 ± 400	770 ± 20	1900 ± 400	570 ± 110
4k	>10,000	>10,000	>10,000	>10,000	>10,000
4l	>10,000	>10,000	>10,000	>10,000	>10,000
4m	>10,000	>10,000	>10,000	>10,000	>10,000
4n	>10,000	>10,000	>10,000	>10,000	>10,000
4o	2100 ± 200	2100 ± 10	1300 ± 80	2000 ± 50	890 ± 130
4p	590 ± 40	570 ± 30	200 ± 70	490 ± 10	340 ± 30
4q	2300 ± 400	4400 ± 390	1900 ± 100	400 ± 150	1900 ± 200
4r	24 ± 0	27 ± 7	19 ± 2	20 ± 2	3.2 ± 0.6
4s	1700 ± 400	1700 ± 100	400 ± 30	2400 ± 150	530 ± 28
4t	2.0 ± 1.2	2.8 ± 1.0	1.2 ± 0.8	2.8 ± 0.6	6.3 ± 2.7
4u	3500 ± 200	3700 ± 100	2100 ± 0	2700 ± 200	2000 ± 0
4v	480 ± 32	330 ± 10	84 ± 21	100 ± 10	130 ± 24
4w	800 ± 31	430 ± 27	240 ± 30	430 ± 0	290 ± 10
4x	>10,000	>10,000	1300 ± 90	4300 ± 300	260 ± 16
4y	180 ± 0	320 ± 40	100 ± 10	220 ± 80	100 ± 25
4z	>10,000	7009 ± 530	3005 ± 120	>10,000	>10,000
4aa	>10,000	>10,000	>10,000	>10,000	4400 ± 290
4ab	2300 ± 400	2600 ± 600	770 ± 43	1500 ± 300	680 ± 10
4ac	>10,000	>10,000	>10,000	>10,000	>10,000
3a	430 ± 40	280 ± 160	140 ± 20	87 ± 22	n.d.
3b	65 ± 5	59 ± 5	48 ± 4	78 ± 3	n.d.
CA4	2.8 ± 1.1	42 ± 6.0	16 ± 1.4	1.9 ± 1.6	1.9 ± 1.6

n.d. = not determined.
[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 **4b**, **4d**, **4p–r**, **4t–w** and CA4

Compound	Tubulin assembly[a] IC_{50} ± SD (μM)	Colchicine binding[b] % ± SD
4b	1.1 ± 0.1	76 ± 2
4d	0.70 ± 0.08	79 ± 0
4p	1.8 ± 0.2	66 ± 0
4q	1.2 ± 0.1	65 ± 0
4r	0.55 ± 0.03	97 ± 1
4t	0.43 ± 0.06	96 ± 1
4u	6.9 ± 0.8	n.d.
4v	0.57 ± 0.03	85 ± 1
4w	1.1 ± 0.1	69 ± 3
CA4 (**1**)	1.0 ± 0.1	99 ± 2

n.d.: not determined.
[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 5 μM, respectively.

4. Conclusions

The SAR information indicated that the introduction of methyl at the C-3 position of the benzo[*b*]furan moiety resulted in increased activity compared with the corresponding 3-amino counterpart,[9a] thus revealing that this latter substituent is not essential for activity. Several compounds (**4d**, **4r**, **4t** and **4v**) showed excellent activity as inhibitors of tubulin polymerization, and were more potent than CA4 in this assay. The interaction with tubulin leads to cell cycle arrest in the G_2–M phase and to an apoptotic cell death. Our structure–activity relationship study indicates that a methoxy group located at the C-6 position of the benzo[*b*]-furan ring yields the most active compound. Changing its position from C-6 to C-4, C-5 or C-7 led to a reduction in potency. Replacement of methoxy by the more hydrophilic hydroxyl group turned out to be detrimental to potency in the case of the 2-unsubstituted benzo[*b*]furan series. The 2-(3′,4′,5′-trimethoxybenzoyl)-3-methyl-6-ethoxybenzo[*b*]furan derivative **4t** was the most potent analogue, with IC_{50} values ranging from 1.2 to 6.3 nM, in the same range as the values obtained with CA4. Compound **4t** also had excellent potency as an inhibitor of tubulin polymerization (IC_{50} = 0.43 μM). At the 2-position of the benzo[*b*]furan, the linker is much more effective as a carbonyl than as carbinol, methoxymethyl or simple methylene group. A thiocarbonyl linker, however, was compatible with good activity.

5. Experimental

5.1. Chemistry

5.1.1. Materials and methods

2-Hydroxybenzaldehyde (**5a**), 2-hydroxy-6-methoxybenzaldehyde (**5b**), 2-hydroxy-5-methoxybenzaldehyde (**5c**), 2-hydroxy-4-methoxybenzaldehyde (**5d**), 2-hydroxy-4-methoxybenzaldehyde (**5e**), 2-5-hydroxy-5-methylbenzaldehyde (**5f**), 2-hydroxy-5-chlorobenzaldehyde (**5g**), 2-hydroxy-5-bromobenzaldehyde (**5h**), 2-hydroxy-3-methoxybenzaldehyde (**5i**), 2-hydroxy-4-(benzyloxy) benzaldehyde (**5m**), 1-(2-hydroxyphenyl)ethanone (**5o**), 2-hydroxy-6-methoxyacetophenone (**5p**), 2-hydroxy-5-methoxyacetophenone (**5q**), 2-hydroxy-4-methoxyacetophenone (**5r**), 2-hydroxy-4-ethoxyacetophenone (**5t**), 2-hydroxy-4-fluoroacetophenone (**5u**) are commercially available and were used as received. For the preparation of **5j–l**, **5n**, **5s**, **5v–x** see Ref. 18.

^{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. Mass spectra were obtained by electrospray ionisation (ESI) in positive mode using a ESI Micromass ZMD 2000 mass spectrometer. 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 F_{254} 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).

5.2. General procedure for the synthesis of 2-(3′,4′,5′-trimethoxybenzoyl)-3-amino benzofuranes (4a–l, 4n–w, 6m and 6x)

To a solution of the appropriate substituted salicylaldehyde **5a–n** or 2-hydroxyacetophenone **5o–x** (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 reaction mixture was evaporated, and the

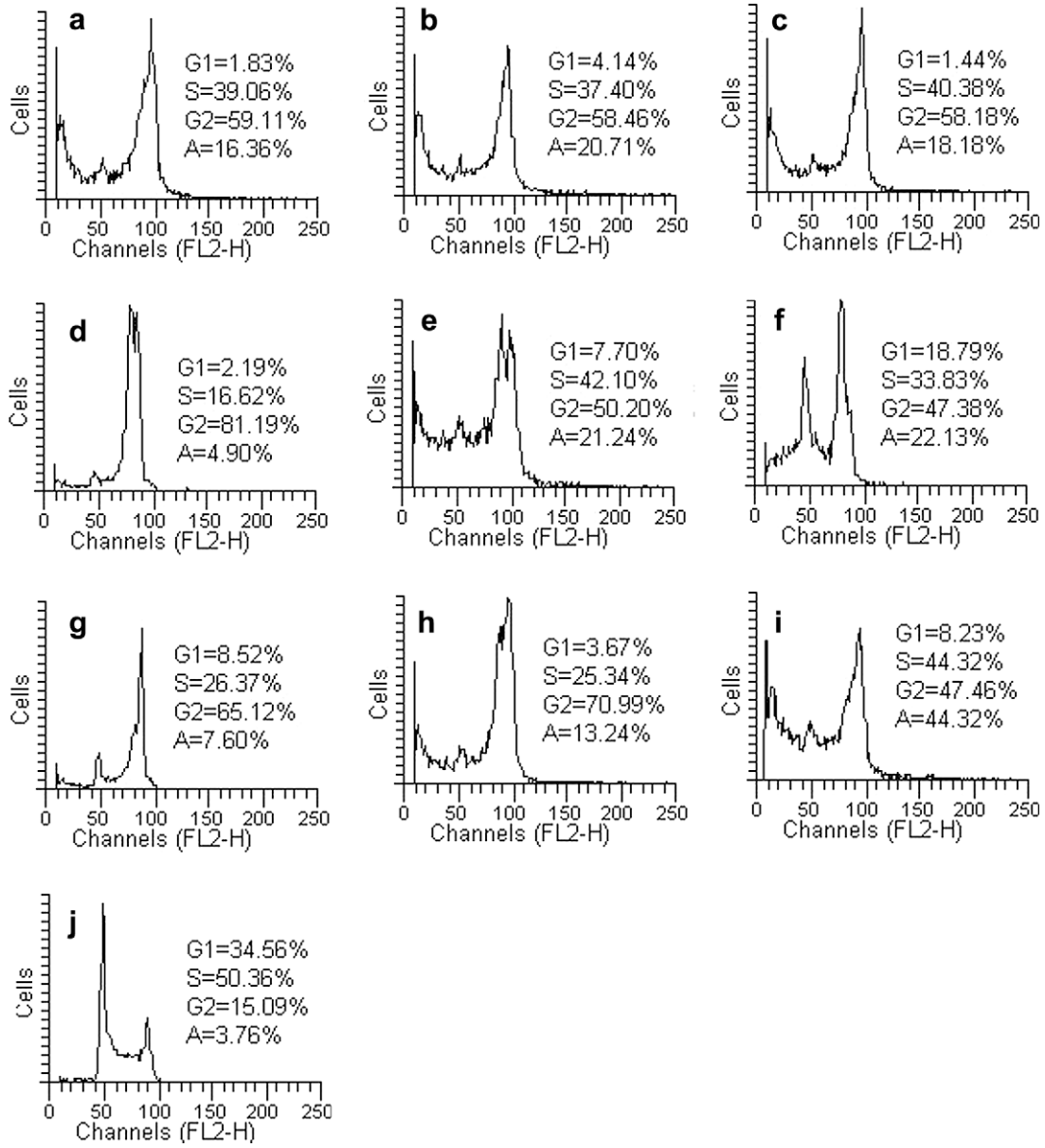


Figure 1. Effects of compounds **4d** (a), **4b** (b), **4q** (c), **4r** (d), **4p** (e), **4w** (f), **4v** (g), **4t** (h) and **4u** (i) on DNA content/cell following the treatment of K562 cells for 24 h. Cell cycle distribution was analyzed by the standard propidium iodide procedure as described in Section 5. Control is reported in Figure 1 as panel j.

residue was dissolved in a mixture of dichloromethane (15 mL) and water (5 mL). The organic layer was washed with brine, dried and concentrated under reduced pressure to obtain a residue, which was purified by flash column chromatography. The final product was recrystallized from petroleum ether.

5.2.1. (Benzofuran-2-yl)(3,4,5-trimethoxyphenyl)methanone (4a)

The residue was chromatographed with EtOAc/petroleum ether 3:7 as eluent to give **4a** as a white solid, yield: 93%, mp 94–96 °C. ^{1}H NMR ($CDCl_3$) δ: 3.95 (s, 6H), 3.96 (s, 3H), 7.36 (m, 3H), 7.50 (t, J = 8.0 Hz, 1H), 7.53 (s, 1H), 7.64 (d, J = 8.0 Hz, 1H), 7.76 (d, J = 8.0 Hz, 1H). Anal. Calcd for $C_{18}H_{16}O_5$: C, 69.22; H, 5.16. Found: C, 69.01; H, 5.02.

5.2.2. (4-Methoxybenzofuran-2-yl)(3,4,5-trimethoxyphenyl)methanone (4b)

The residue was chromatographed with EtOAc/petroleum ether 3:7 as eluent to give **4b** as a white solid, yield: 85%, mp 118–120 °C. ^{1}H NMR ($CDCl_3$) δ: 3.95 (s, 3H), 3.96 (s, 6H), 3.98 (s, 3H), 7.12 (m, 2H), 7.34 (s, 2H), 7.49 (s, 1H), 7.52 (d, J = 9.2 Hz, 1H). Anal. Calcd for $C_{19}H_{18}O_6$: C, 66.66; H, 5.30. Found: C, 66.47; H, 5.12.

5.2.3. (5-Methoxybenzofuran-2-yl)(3,4,5-trimethoxyphenyl)methanone (4c)

The residue was chromatographed with EtOAc/petroleum ether 2:8 as eluent to give **4c** as a white solid, yield: 83%, mp 154–156 °C. ^{1}H NMR ($CDCl_3$) δ: 3.88 (s, 3H), 3.96 (s, 6H), 3.97 (s, 3H), 6.70 (d, J = 7.6 Hz, 1H), 7.25 (m, 1H), 7.32 (s, 2H), 7.43 (d, J = 8.4 Hz, 1H), 7.62 (d, J = 1.0 Hz, 1H). Anal. Calcd for $C_{19}H_{18}O_6$: C, 66.66; H, 5.30. Found: C, 66.38; H, 5.03.

5.2.4. (6-Methoxybenzofuran-2-yl)(3,4,5-trimethoxyphenyl)methanone (4d)

The residue was chromatographed with EtOAc/petroleum ether 3:7 as eluent to give **4d** as a white solid, yield: 89%, mp 115–117 °C. ^{1}H NMR ($CDCl_3$) δ: 3.99 (s, 3H), 3.94 (s, 6H), 3.95 (s, 3H), 6.97 (dd, J = 8.8 and 2.2 Hz, 1H), 7.01 (d, J = 2.4 Hz, 1H), 7.29 (s,

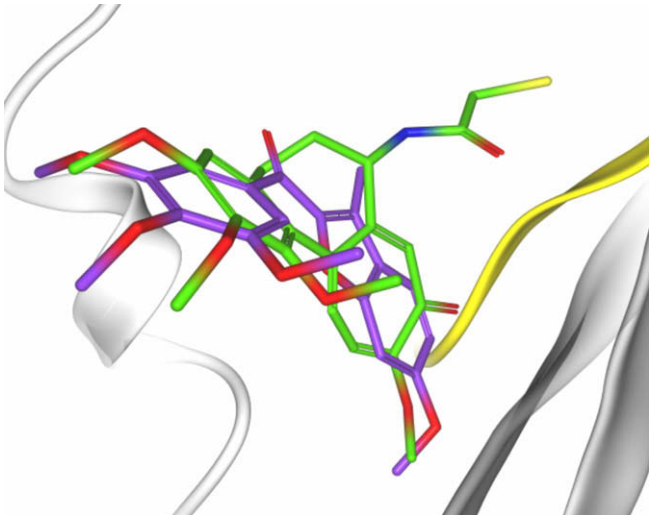


Figure 2. Putative binding mode of compound **4r** (colored in purple); DAMA-colchicine colored in green.

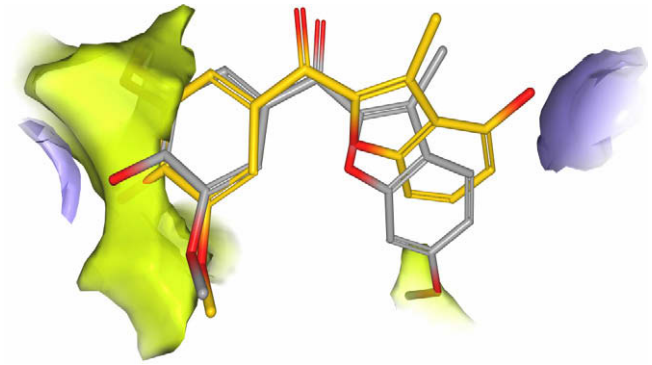


Figure 3. Putative binding mode of compound **4r** (colored in gray) and **4v** (colored in yellow). Lipophilic potential map represented in green; H-bond donor potential map represented in purple.

2H), 7.48 (s, 1H), 7.60 (d, J = 8.8 Hz, 1H). Anal. Calcd for $C_{19}H_{18}O_6$: C, 66.66; H, 5.30. Found: C, 66.42; H, 5.14.

5.2.5. (7-Methoxybenzofuran-2-yl)(3,4,5-trimethoxyphenyl)methanone (4e)

The residue was chromatographed with EtOAc/petroleum ether 3:7 as eluent to give **4e** as a yellow solid, yield: 95%, mp 116–118 °C. ^{1}H NMR ($CDCl_3$) δ: 3.95 (s, 3H), 3.96 (s, 6H), 4.03 (s, 3H), 6.96 (d, J = 7.6 and 2.0 Hz, 1H), 7.27 (t, J = 7.6 Hz, 1H), 7.33 (d, J = 7.6 Hz, 1H), 7.44 (s, 2H), 7.57 (s, 1H). Anal. Calcd for $C_{19}H_{18}O_6$: C, 66.66; H, 5.30. Found: C, 66.39; H, 5.09.

5.2.6. (3,4,5-Trimethoxyphenyl)(5-methylbenzofuran-2-yl)methanone (4f)

The residue was chromatographed with EtOAc/petroleum ether 3:7 as eluent to give **4f** as a white solid, yield: 88%, mp 106–107 °C. ^{1}H NMR ($CDCl_3$) δ: 2.47 (s, 3H), 3.94 (s, 6H), 3.96 (s, 3H), 7.30 (d, J = 9.2 Hz, 1H), 7.33 (s, 2H), 7.47 (s, 1H), 7.52 (m, 2H). Anal. Calcd for $C_{19}H_{18}O_5$: C, 69.93; H, 5.56. Found: C, 69.77; H, 5.33.

5.2.7. (5-Chlorobenzofuran-2-yl)(3,4,5-trimethoxyphenyl)methanone (4g)

The residue was chromatographed with EtOAc/petroleum ether 2:8 as eluent to give **4g** as a white solid, yield: 72%, mp 136–138 °C. ^{1}H NMR ($CDCl_3$) δ: 3.95 (s, 6H), 3.97 (s, 3H), 7.33 (s, 2H), 7.44 (m, 2H), 7.56 (d, J = 8.8 Hz, 1H), 7.72 (d, J = 2.0 Hz, 1H). Anal. Calcd for $C_{18}H_{15}ClO_5$: 62.35; H, 4.36; Cl, 10.22. Found: 62.06; H, 4.14; Cl, 10.01.

5.2.8. (5-Bromobenzofuran-2-yl)(3,4,5-trimethoxyphenyl)methanone (4h)

The residue was chromatographed with EtOAc/petroleum ether 2:8 as eluent to give **4h** as a white solid, yield: 83%, mp 144–146 °C. ^{1}H NMR ($CDCl_3$) δ: 3.94 (s, 6H), 3.96 (s, 3H), 7.33 (s, 2H), 7.47 (s, 1H), 7.50 (d, J = 8.8 Hz, 1H), 7.56 (dd, J = 8.8 and 2.0 Hz, 1H), 7.88 (d, J = 2.0 Hz, 1H). Anal. Calcd for $C_{18}H_{15}BrO_5$: C, 55.26; H, 3.86; Br, 20.42. Found: C, 55.02; H, 3.68; Br, 20.17.

5.2.9. (7-Hydroxy-6-methoxybenzofuran-2-yl)(3,4,5-trimethoxyphenyl)methanone (4i)

The residue was chromatographed with EtOAc/petroleum ether 4:6 as eluent to give **4i** as a white solid, yield: 83%, mp 128–130 °C. ^{1}H NMR ($CDCl_3$) δ: 3.91 (s, 3H), 3.93 (s, 6H), 3.95 (s, 3H), 6.97 (d, J = 8.6 Hz, 1H), 7.28 (s, 2H), 7.32 (d, J = 8.6 Hz, 1H), 7.50 (s, 1H), 10.2 (s, 1H). Anal. Calcd for $C_{19}H_{18}O_7$: C, 63.68; H, 5.06. Found: C, 63.50; H, 4.88.

5.2.10. (5,6-Dimethoxybenzofuran-2-yl)(3,4,5-trimethoxyphenyl)methanone (4j)

The residue was chromatographed with EtOAc/petroleum ether 4:6 as eluent to give **4j** as a yellow solid, yield: 87%, mp 145–147 °C. ^{1}H NMR ($CDCl_3$) δ: 3.94 (s, 6H), 3.95 (s, 6H), 3.98 (s, 3H), 7.08 (s, 1H), 7.11 (s, 1H), 7.29 (s, 2H), 7.47 (s, 1H). Anal. Calcd for $C_{20}H_{20}O_7$: C, 64.51; H, 5.41. Found: C, 64.40; H, 5.20.

5.2.11. (5,6,7-Trimethoxybenzofuran-2-yl)(3,4,5-trimethoxyphenyl)methanone (4k)

The residue was chromatographed with EtOAc/petroleum ether 4:6 as eluent to give **4k** as a yellow solid, yield: 73%, mp 100–102 °C. ^{1}H NMR ($CDCl_3$) δ: 3.92 (s, 3H), 3.93 (s, 3H), 3.95 (s, 3H), 3.96 (s, 3H), 3.97 (s, 3H), 4.24 (s, 3H), 6.84 (s, 1H), 7.40 (s, 2H), 7.51 (s, 1H). Anal. Calcd for $C_{21}H_{22}O_8$: C, 62.68; H, 5.51. Found: C, 62.44; H, 5.38.

5.2.12. (5-Hydroxybenzofuran-2-yl)(3,4,5-trimethoxyphenyl)-methanone (4l)

The residue was chromatographed with EtOAc/petroleum ether 4:6 as eluent to give **4l** as a yellow solid, yield: 80%, mp 155–157 °C. ^{1}H NMR ($CDCl_3$) δ: 3.94 (s, 3H), 3.96 (s, 6H), 7.06 (dd, J = 8.8 and 2.4 Hz, 1H), 7.11 (d, J = 2.4 Hz, 1H), 7.33 (s, 2H), 7.44 (s, 1H), 7.48 (d, J = 8.8 Hz, 1H), 7.52 (s, 1H). Anal. Calcd for $C_{18}H_{16}O_6$: C, 65.85; H, 4.91. Found: C, 65.68; H, 4.67.

5.2.13. (7-Hydroxybenzofuran-2-yl)(3,4,5-trimethoxyphenyl)-methanone (4n)

The residue was chromatographed with EtOAc/petroleum ether 3:7 as eluent to give **4n** as a yellow solid, yield: 74%, mp 166–168 °C. ^{1}H NMR ($CDCl_3$) δ: 3.94 (s, 3H), 3.96 (s, 6H), 7.07 (d, J = 7.6 and 2.0 Hz, 1H), 7.22 (t, J = 7.6 Hz, 1H), 7.28 (m, 3H), 7.53 (s, 2H). Anal. Calcd for $C_{18}H_{16}O_6$: C, 65.85; H, 4.91. Found: C, 65.59; H, 4.39.

5.2.14. (3,4,5-Trimethoxyphenyl)(3-methylbenzofuran-2-yl)-methanone (4o)

The residue was chromatographed with EtOAc/petroleum ether 1:9 as eluent to give **40** as a white solid, yield: 85%, mp 108–110 °C. ^{1}H NMR ($CDCl_3$) δ: 2.65 (s, 3H), 3.94 (s, 6H), 3.96 (s, 3H), 7.36 (t, J = 7.2 Hz, 1H), 7.50 (s, 2H), 7.52 (m, 2H), 7.70 (dd, J = 8.0 and 1.0 Hz, 1H). Anal. Calcd for $C_{19}H_{18}O_5$: C, 69.93; H, 5.56. Found: C, 69.68; H, 5.38.

5.2.15. (4-Methoxy-3-methylbenzofuran-2-yl)(3,4,5-trimethoxyphenyl)methanone (4p)

The residue was chromatographed with EtOAc/petroleum ether 2:8 as eluent to give **4p** as a white solid, yield: 90%, mp

107–109 °C. ^{1}H NMR ($CDCl_3$) δ: 2.78 (s, 3H), 3.9 (s, 3H), 3.95 (s, 6H), 3.96 (s, 3H), 6.65 (d, J = 7.6 Hz, 1H), 7.13 (d, J = 7.6 Hz, 1H), 7.37 (m, 3H). Anal. Calcd for $C_{20}H_{20}O_6$: C, 67.41; H, 5.66. Found: C, 67.20; H, 5.38.

5.2.16. (5-Methoxy-3-methylbenzofuran-2-yl)(3,4,5-trimethoxyphenyl)methanone (4q)

The residue was chromatographed with EtOAc/petroleum ether 2:8 as eluent to give **4q** as a white solid, yield: 81%, mp 122–124 °C. ^{1}H NMR ($CDCl_3$) δ: 2.62 (s, 3H), 3.90 (s, 3H), 3.93 (s, 6H), 3.96 (s, 3H), 7.07 (m, 1H), 7.12 (d, J = 8.6 Hz, 1H), 7.42 (m, 3H). Anal. Calcd for $C_{20}H_{20}O_6$: C, 67.41; H, 5.66. Found: C, 67.12; H, 5.24.

5.2.17. (6-Methoxy-3-methylbenzofuran-2-yl)(3,4,5-trimethoxyphenyl)methanone (4r)

The residue was chromatographed with EtOAc/petroleum ether 2:8 as eluent to give **4r** as a white solid, yield: 77%, mp 101–103 °C. ^{1}H NMR ($CDCl_3$) δ: 2.61 (s, 3H), 3.89 (s, 3H), 3.94 (s, 6H), 3.96 (s, 3H), 6.99 (m, 2H), 7.39 (s, 2H), 7.59 (d, J = 9.6 Hz, 1H). Anal. Calcd for $C_{20}H_{20}O_6$: C, 67.41; H, 5.66. Found: C, 67.19; H, 5.44.

5.2.18. (7-Methoxy-3-methylbenzofuran-2-yl)(3,4,5-trimethoxyphenyl)methanone (4s)

The residue was chromatographed with EtOAc/petroleum ether 2:8 as eluent to give **4s** as a white solid, yield: 71%, mp 143–145 °C. ^{1}H NMR ($CDCl_3$) δ: 2.66 (s, 3H), 3.95 (s, 3H), 3.97 (s, 6H), 4.00 (s, 3H), 6.97 (dd, J = 6.0 and 2.8 Hz, 1H), 7.27 (m, 2H), 7.53 (s, 2H). Anal. Calcd for $C_{20}H_{20}O_6$: C, 67.41; H, 5.66. Found: C, 67.27; H, 5.49.

5.2.19. (6-Ethoxy-3-methylbenzofuran-2-yl)(3,4,5-trimethoxyphenyl)methanone (4t)

The residue was chromatographed with EtOAc/petroleum ether 1.5:8.5 as eluent to give **4t** as a yellow solid, yield: 81%, mp 98–100 °C. ^{1}H NMR ($CDCl_3$) δ: 1.41 (t, J = 7.2 Hz, 3H), 2.60 (s, 3H), 3.93 (s, 6H), 3.95 (s, 3H), 4.12 (q, J = 7.2 Hz, 2H), 6.97 (m, 2H), 7.38 (s, 2H), 7.55 (d, J = 9.2 Hz, 1H). Anal. Calcd for $C_{21}H_{22}O_6$: C, 68.10; H, 5.99. Found: C, 67.89; H, 5.79.

5.2.20. (6-Fluoro-3-methylbenzofuran-2-yl)(3,4,5-trimethoxyphenyl)methanone (4u)

The residue was chromatographed with EtOAc/petroleum ether 1:9 as eluent to give **4u** as a brown solid, yield: 79%, mp 130–132 °C. ^{1}H NMR ($CDCl_3$) δ: 2.62 (s, 3H), 3.93 (s, 6H), 3.96 (s, 3H), 7.08 (dd, J = 9.0 and 2.6 Hz, 1H),), 7.16 (dd, J = 9.4 and 2.2 Hz, 1H), 7.38 (s, 2H), 7.66 (dd, J = 8.8 and 5.2 Hz, 1H). Anal. Calcd for $C_{19}H_{17}FO_5$: C, 66.27; H, 4.98. Found: C, 66.03; H, 4.76.

5.2.21. (4-Hydroxy-3-methylbenzofuran-2-yl)(3,4,5-trimethoxyphenyl)methanone (4v)

The residue was chromatographed with EtOAc/petroleum ether 3:7 as eluent to give **4v** as a yellow solid, yield: 65%, mp 203–205 °C. ^{1}H NMR ($CDCl_3$) δ: 2.82 (s, 3H), 3.93 (s, 3H), 3.96 (s, 6H), 5.83 (bs, 1H), 6.59 (d, J = 8.2 Hz, 1H), 7.11 (d, J = 8.2 Hz, 1H), 7.29 (m, 1H), 7.39 (s, 2H). Anal. Calcd for $C_{19}H_{18}O_6$: C, 66.66; H, 5.30. Found: C, 66.48; H, 5.11.

5.2.22. (5-Hydroxy-3-methylbenzofuran-2-yl)(3,4,5-trimethoxyphenyl)methanone (4w)

The residue was chromatographed with EtOAc/petroleum ether 4:6 as eluent to give **4w** as a yellow solid, yield: 53%, mp 181–183 °C. ^{1}H NMR ($CDCl_3$) δ: 2.59 (s, 3H), 3.93 (s, 3H), 3.96 (s, 6H), 7.01 (m, 1H), 7.06 (d, J = 6.4 Hz, 1H), 7.38 (d, J = 6.4 Hz, 1H), 7.41 (s, 1H), 7.42 (s, 2H). Anal. Calcd for $C_{19}H_{18}O_6$: C, 66.66; H, 5.30. Found: C, 66.50; H, 5.16.

5.2.23. (6-(Benzyloxy)benzofuran-2-yl)(3,4,5-trimethoxyphenyl)methanone (6m)

The residue was chromatographed with EtOAc/petroleum ether 4:6 as eluent to give **6m** as a pink solid, yield: 61%, mp 182–184 °C. ^{1}H NMR ($CDCl_3$) δ: 3.94 (s, 3H), 3.96 (s, 6H), 4.35 (s, 2H), 6.51 (d, J = 2.4 Hz, 1H), 7.10 (dd, J = 8.6 and 2.4 Hz, 1H), 7.28 (m, 5H), 7.29 (s, 2H), 7.47 (s, 1H), 7.61 (d, J = 8.6 Hz, 1H). ESI-MS m/z 419.1 $[M+1]^+$.

5.2.24. (6-(Benzyloxy)-3-methylbenzofuran-2-yl)(3,4,5-trimethoxyphenyl)methanone (6x)

The residue was chromatographed with EtOAc/petroleum ether 2:8 as eluent to give **6x** as a white solid, yield: 68%, mp 110–112 °C. ^{1}H NMR ($CDCl_3$) δ: 2.61 (s, 3H), 3.93 (s, 3H), 3.95 (s, 6H), 5.15 (s, 2H), 7.07 (m, 2H), 7.37 (s, 2H), 7.44 (m, 5H), 7.56 (d, J = 8.6 Hz, 1H). ESI-MS m/z 433.2 $[M+1]^+$.

5.3. General procedure for the synthesis of 4m and 4x

A suspension of benzyloxy derivative **6m** or **6x** (1 mmol) ammonium formate (630 mg, 10 mmol) and activated Pd on charcoal (10%, 150 mg) in a mixture of THF/MeOH (1:1, 20 mL) was heated to reflux for 1 h. The catalyst was filtered over Celite, the solvent removed under reduced pressure and the product purified by column chromatography (silica gel, EtOAc/petroleum ether 1:1).

5.3.1. (6-Hydroxybenzofuran-2-yl)(3,4,5-trimethoxyphenyl)-methanone (4m)

The residue was chromatographed with EtOAc/petroleum ether 1:1 as eluent to give **4m** as a yellow solid, yield: 46%, mp 141–143 °C. ^{1}H NMR ($CDCl_3$) δ: 3.84 (s, 3H), 3.88 (s, 6H), 6.87 (dd, J = 8.6 and 2.0 Hz, 1H), 6.92 (s, 1H), 7.23 (s, 2H), 7.66 (d, J = 8.4 Hz, 1H), 7.77 (s, 1H), 10.1 (s, 1H). Anal. Calcd for $C_{18}H_{16}O_6$: C, 65.85; H, 4.91. Found: C, 65.58; H, 4.72.

5.3.2. (6-Hydroxy-3-methylbenzofuran-2-yl)(3,4,5-trimethoxyphenyl)methanone (4x)

The residue was chromatographed with EtOAc/petroleum ether 1:1 as eluent to give **4x** as a yellow solid, yield: 46%, mp 211–213 °C. ^{1}H NMR ($CDCl_3$) δ: 2.08 (s, 3H), 3.77 (s, 3H), 3.85 (s, 6H), 6.86 (dd, J = 8.6 and 2.0 Hz, 1H), 6.99 (d, J = 2.0 Hz, 1H), 7.29 (s, 2H), 7.62 (d, J = 8.6 Hz, 1H), 10.1 (bs, 1H). Anal. Calcd for $C_{19}H_{18}O_6$: C, 66.66; H, 5.30. Found: C, 66.38; H, 5.08.

5.3.3. Synthesis of (6-methoxybenzofuran-2-yl)(3,4,5-trimethoxyphenyl)methanethione (4y)

To a magnetically stirred solution of **4d** (1 mmol) in anhydrous THF at room temperature was added Lawesson's reagent (1.1 mmol), and the mixture was stirred for 1 h. The solvent was evaporated in vacuo, and the residue was dissolved in dichloromethane (15 mL). The organic extract was washed with water (5 mL), dried over Na_2SO_4 and concentrated in vacuo. The residue purified by flash chromatography (ethyl acetate/petroleum ether 3:7) furnished **4y** as a white powder, yield: 96%, mp 143–145 °C. ^{1}H NMR ($CDCl_3$) δ: 3.90 (s, 3H), 3.92 (s, 6H), 3.94 (s, 3H), 6.94 (dd, J = 8.8 and 2.4 Hz, 1H), 7.10 (s, 2H), 7.12 (d, J = 2.4 Hz, 1H), 7.37 (s, 1H), 7.62 (d, J = 8.8 Hz, 1H). Anal. Calcd for $C_{19}H_{18}O_5S$: C, 63.67; H, 5.06; S, 8.95. Found: C, 63.48; H, 4.88; S, 8.74.

5.3.4. Synthesis of (6-methoxybenzofuran-2-yl)(3,4,5-trimethoxyphenyl)methanol (4z)

To a cooled solution of **4d** (1 mmol) in dioxane (5 mL) was added $NaBH_4$ (1 mmol), and then the mixture was allowed to stir at room temperature under nitrogen for 2 h. The solvent was concentrated under reduced pressure, and aqueous hydrochloric acid (1 M, 2 mL) was added to the residue. The mixture was extracted

with dichloromethane (2 × 10 mL) and the organic layer washed with water (2 × 5 mL) and brine (5 mL), dried with Na_2SO_4 and concentrated under reduced pressure to give a light yellow oil residue. Purification by flash column chromatography (petroleum ether/ethyl acetate 1:1) gave **4z** as a white solid, which was recrystallized from petroleum ether. Yield: 84%, mp 148–150 °C. ^{1}H NMR ($CDCl_3$) δ: 3.84 (s, 6H), 3.86 (s, 6H), 5.87 (bs, 1H), 6.47 (s, 1H), 6.57 (bs, 1H), 6.73 (s, 2H), 6.84 (dd, J = 8.6 and 2.4 Hz, 1H), 7.00 (d, J = 2.4 Hz, 1H), 7.37 (d, J = 8.6 Hz, 1H). Anal. Calcd for $C_{19}H_{20}O_6$: C, 66.27; H, 5.85. Found: C, 66.04; H, 5.67.

5.3.5. Preparation of 6-methoxy-2-(methoxy(3,4,5-trimethoxyphenyl)methyl)benzofuran (4aa)

A mixture of **4z** (86 mg, 0.26 mmol) and pyridinium *p*-toluensulfonate (125 mg, 0.5 mmol) in a mixture of THF-MeOH (10 mL, 0.5:9.5) was stirred for 15 h in a sealed tube. The reaction mixture was evaporated under vacuum, the crude mixture was dissolved with dichloromethane (5 mL) and the organic phase was washed with water (1 mL) and brine (1 mL), dried over Na_2SO_4 and concentrated under reduced pressure. The residue was chromatographed with EtOAc/petroleum ether 3:7 as eluent to give **4aa** as a white solid, yield: 96%, mp 148–150 °C. ^{1}H NMR ($CDCl_3$) δ: 3.46 (s, 3H), 3.84 (s, 3H), 3.85 (s, 3H), 3.87 (s, 6H), 5.29 (bs, 1H), 6.46 (d, J = 1.0 Hz, 1H), 6.71 (s, 2H), 6.84 (dd, J = 8.4 and 2.2 Hz, 1H), 7.01 (d, J = 2.2 Hz, 1H), 7.35 (d, J = 8.6 Hz, 1H). Anal. Calcd for $C_{20}H_{22}O_6$: C, 67.03; H, 6.19. Found: C, 66.88; H, 6.07.

5.3.6. Preparation of 2-(3,4,5-trimethoxybenzyl)-6-methoxybenzofuran (4ab)

To a magnetically stirred suspension of **4z** (0.5 mmol) in dichloromethane (3 mL) at room temperature was added trifluoroacetic acid (410 μL, 5.5 mmol) and then triethylsilane (890 μL, 5.5 mmol). After 15 min, the solvent was evaporated in vacuo, and the residue was dissolved in dichloromethane (20 mL). The organic layer was washed with an aqueous saturated solution of $NaHCO_3$ (5 mL) and brine (5 mL), dried (Na_2SO_4) and concentrated in vacuo. Flash chromatography using ethyl acetate–petroleum ether 1:1, furnished **4ab** as a brown oil, yield: 94%. ^{1}H NMR ($CDCl_3$) δ: 3.83 (s, 6H), 3.84 (s, 6H), 4.01 (s, 2H), 6.34 (d, J = 1.0 Hz, 1H), 6.51 (s, 2H), 6.82 (dd, J = 8.4 and 2.4 Hz, 1H), 6.98 (d, J = 2.4 Hz, 1H), 7.37 (d, J = 8.5 Hz, 1H). Anal. Calcd for $C_{19}H_{20}O_5$: C, 69.50; H, 6.14. Found: C, 69.31; H, 5.95.

5.3.7. Synthesis of (2-bromo-3,4,5-trimethoxyphenyl)(6-methoxybenzofuran-2-yl)methanone (7)

To a solution of **4d** (342 mg, 1 mmol.) in acetonitrile (10 mL) was added a mixture of benzoyl peroxide (48 mg, 0.2 mmol) and NBS (214 mg, 1.2 mmol). The mixture was heated under reflux for 2 h. The solvent was removed under reduced pressure, and the residue dissolved in EtOAc (15 mL), which was washed successively with a saturated solution of $NaHCO_3$ (5 mL), water (5 mL) and brine (5 mL), dried (Na_2SO_4) and concentrated in vacuo. Purification by flash column chromatography (petroleum ether/ethyl acetate 7:3) gave **7** as a white solid. Yield: 65%, mp 144–145 °C. ^{1}H NMR (DMSO-d_6) δ: 3.83 (s, 6H), 3.85 (s, 3H), 3.86 (s, 3H), 6.99 (dd, J = 8.8 and 2.2 Hz, 1H), 7.15 (s, 1H), 7.36 (d, J = 2.4 Hz, 1H), 7.54 (s, 1H), 7.68 (d, J = 8.8 Hz, 1H). ESI-MS m/z 421.0/423.0 $[M+1]^+/[M+3]^+$.

5.3.8. Synthesis of 3,8,9,10-tetramethoxy-6*H*-benzo[*b*]indeno[1,2-*d*]furan-6-one (4ac)

A mixture of bromo derivative **7** (160 mg, 0.38 mmol), Pd$(Ph_3P)_4$ (12 mg) and potassium acetate (40 mg, 0.42 mmol) in 2 mL of *N*,*N*-dimethylacetamide was heated at 160 °C for 5 h. The solvent was then removed under reduced pressure and the residue purified by flash chromatography on silica gel using EtOAc/petroleum ether 2:8 as eluent. Yield: 46%, brown solid, mp 124–125 °C. ^{1}H NMR (DMSO-d_6) δ: 3.88 (s, 6H), 3.93 (s, 3H), 4.04 (s, 3H), 6.92 (s, 1H), 7.00 (m, 2H), 7.73 (d, J = 9.2 Hz, 1H). Anal. Calcd for $C_{19}H_{16}O_6$: C, 67.05; H, 4.74. Found: C, 66.89; H, 4.59.

5.4. Cell growth inhibitory activity

Murine leukemia L1210, murine mammary carcinoma FM3A and human T-lymphocyte Molt 4 and CEM and human cervix carcinoma (HeLa) 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 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%.

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

Bovine brain tubulin was purified as described previously.[19] To evaluate the effect of the compounds on tubulin assembly in vitro,[14] 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,[15] except that the reaction mixtures contained 1 μM tubulin, 5 μM [^{3}H]colchicine and 5 μM test compound.

5.6. Flow cytometric analysis of cell cycle distribution

For details, see Ref. 9a.

5.7. Molecular modeling

All molecular modeling studies were performed on a MacPro dual 2.66 GHz Xeon running Ubuntu 8. The tubulin structure was downloaded from the PDB data bank (http://www.rcsb.org/ - PDB code: 1SA0). Docking simulations were performed using PLANTS[20] and results examined using ZODIAC.[21] H-Bond and lipophilicity maps were created using ZODIAC.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.08.027.

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