Synthesis and Biological Evaluation of 2-Amino-3-(3′,4′,5′-trimethoxybenzoyl)-5-aryl Thiophenes as a New Class of Potent Antitubulin Agents

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A new series of compounds in which the 2-amino-5-chlorophenyl ring of phenstatin analogue **7** was replaced with a 2-amino-5-aryl thiophene was synthesized and evaluated for antiproliferative activity and for inhibition of tubulin polymerization and colchicine binding to tubulin. 2-Amino-3-(3′,4′,5′-trimethoxybenzoyl)-5-phenyl thiophene (**9f**) as well as the *p*-fluoro-, *p*-methyl-, and *p*-methoxyphenyl substituted analogues (**9i**, **j**, and **l**, respectively) displayed high antiproliferative activities with IC_{50} values from 2.5 to 6.5 nM against the L1210 and K562 cell lines. Compounds **9i** and **j** were more active than combretastatin A-4 as inhibitors of tubulin polymerization. Molecular docking simulations to the colchicine site of tubulin were performed to determine the possible binding mode of **9i**. The results obtained demonstrated that antiproliferative activity correlated well with the inhibition of tubulin polymerization and the lengthening of the G2/M phase of the cell cycle. Moreover, a good correlation was found between these inhibitory effects and the induction of apoptosis in cells treated with the compounds.

Introduction

Despite progress made in recent years in discovering new compounds with antiangiogenic activity, this research field is still considered one of the most promising for the development of new ways to treat diseases characterized by abnormal angiogenesis including tumors.[1] Research oriented toward the discovery of a new generation of agents useful in cancer chemotherapy has identified tubulin as a possible molecular target.[2]

The microtubule system of eukaryotic cells plays an important role in regulating cell architecture, and it has an essential role in cell division because microtubules are the key component of the mitotic spindle. Microtubules are a dynamic cellular compartment in both neoplastic and normal cells. This dynamicity is characterized by the continuous turnover of $\alpha\beta$-tubulin heterodimers in the polymeric microtubules. The discovery of natural and synthetic substances capable of interfering with the polymerization or depolymerization of microtubules has attracted much attention because microtubules are an attractive pharmacological target for anticancer drug discovery.[2] More recently, it was established that some tubulin binding agents also target the vascular system of tumors, inducing morphological changes in the endothelial cells of the tumor blood vessels so as to occlude flow.[3]

Combretastatin A-4 (CA-4, **1**), isolated from the bark of the South African tree *Combretum caffrum*,[4] is one of the well-known natural tubulin binding molecules affecting microtubule dynamics. CA-4 strongly inhibits the polymerization of tubulin by binding to the colchicine site.[5] CA-4 inhibits cell growth even at very low (nanomolar) concentrations, exhibiting inhibitory effects even on multidrug resistant (MDR) cancer cell lines.[4] The disodium phosphate prodrug of CA-4 (CA-4P, **2**) is water-soluble, and there have been promising results with **2** as a tumor vascular targeting agent in phase II clinical trials.[6] Its structural simplicity, along with its ability to selectively damage tumor neovasculature, makes CA-4 of great interest from the medicinal chemistry point of view. For these reasons, numerous CA-4 analogues have been synthesized and evaluated for their structure−activity relationships (SAR).[7] The replacement of the olefinic bridge of CA-4 with a carbonyl group furnished a benzophenone-type CA-4 analogue named phenstatin (**3**), which has been found to be a potent cytotoxic agent and inhibitor of tubulin polymerization, with activities differing little from those of CA-4.[8] Shifting the hydroxyl group from the C-3 to the C-2 position to furnish phenstatin isomer **4** dramatically decreased antiproliferative activity.[9] The replacement of the hydroxyl moiety with an amino group at the C-2 position of the benzophenone ring (compound **5**) increased cytotoxic activity by 100-fold compared with **4**, indicating that the amino and hydroxy groups are not bioequivalent at the C-2 position of these phenstatin analogues. 2-Aminobenzophenone derivative **5** showed significantly increased cytotoxicity against many human cancer cell lines with activity similar to the activities of phenstatin **3** and CA-4.[9] Changing the position of the methoxy group from C-4 to C-5, as in compound **6**, maintained antiproliferative activity. The replacement of the C-5 methoxy group with a chlorine atom, to give **7**, had little effect on activity, whereas its removal (**8**) reduced antiproliferative activity.[10]

The benzene−thiophene pair represents a prominent example of bioisosteres in drug research. Bioisosteres are groups of molecules that are structurally similar and show the same type

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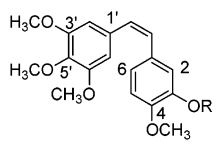
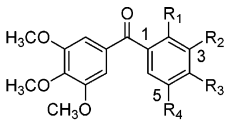
of biological activity. In the case of the 2-aminobenzene moiety, the C-3/C-4 ethylene group (-CH═CH-) can be replaced by a divalent sulfur (-S-) atom, producing a 2-aminothiophene. Then the C-4 and C-5 positions of the thiophene moiety (see general structure **9**) can be considered equivalent to the C-6 and C-5 positions, respectively, of the 2-aminobenzene moiety, numbered as in compounds **5**−**8**. The classical bioisosteric equivalence between benzene and thiophene prompted us to synthesize a series of 2-amino-3-(3′,4′,5′-trimethoxybenzoyl)-thiophene derivatives with general formula **9** in which a 2-aminothiophene system replaced the 2-aminobenzene moiety of compounds **5**−**8**.

The potent activity of C-5 methoxy and chloro derivatives **6** and **7** led us to synthesize a series of thiophene derivatives with various substituents at C-5 equivalent to the phenyl C-5 position of **5**−**8**. Our goal was to evaluate the steric and electronic effects of the substituents on antiproliferative activity. Besides hydrogen (compound **9a**), the substituents examined included halogens (bromine in **9b** and iodine in **9c**), alkyl groups (ethyl and *n*-pentyl, **9d** and **e**, respectively), and phenyl (**9f**) and substituted phenyl groups (**9g**−**o**). The latter compounds were the most active members of the series, and thus, we were able to analyze electron-withdrawing (F, Cl, and CF_3) and electron-donating (Me and MeO) substituents on the C-5 phenyl ring as well as positional effects with three methoxyl group isomers (**9l**−**n**). In addition, we synthesized and examined thiophene derivatives with C-4 substituents (methyl and phenyl, compounds **9p** and **q**, respectively) and three (**9r**−**t**) 4,5-disubstituted thienyl analogues.

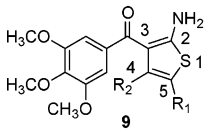
The removal of either the C′-3 or the C′-4 methoxy group of CA-4 causes a substantial loss of cytotoxicity,[11] and we demonstrated, through the syntheses of compounds **9u** and **v**, that a similar loss of antiproliferative activity occurs in our thiophene series relative to the 2-amino-5-phenylthiophene derivative **9f**.



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

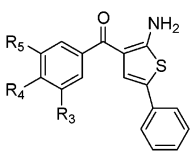


R_1=R_4=H, R_2=OH, R_3=OCH_3, Phenstatin, **3**
R_1=OH, R_2=R_4=H, R_3=OCH_3, **4**
R_1=NH_2, R_2=R_4=H, R_3=OCH_3, **5**
R_1=NH_2, R_2=R_3=H, R_4=OCH_3, **6**
R_1=NH_2, R_2, R_3=H, R_4=Cl, **7**
R_1=NH_2, R_2, R_3, R_4=H, **8**



9

9a, R_1=R_2=H
9b, R_1=Br, R_2=H
9c, R_1=I, R_2=H
9d, R_1=CH_3CH_2, R_2=H
9e, R_1=$CH_3(CH_2)_4$, R_2=H
9f, R_1=C_6H_5, R_2=H
9g, R_1=p-Cl-C_6H_4, R_2=H
9h, R_1=m,p-2Cl-C_6H_3, R_2=H
9i, R_1=p-F-C_6H_4, R_2=H
9j, R_1=p-CH_3-C_6H_4, R_2=H
9k, R_1=p-CF_3-C_6H_4, R_2=H
9l, R_1=p-MeO-C_6H_4, R_2=H
9m, R_1=m-MeO-C_6H_4, R_2=H
9n, R_1=o-MeO-C_6H_4, R_2=H
9o, R_1=o,m,p-3MeO-C_6H_2, R_2=H
9p, R_1=H, R_2=CH_3
9q, R_1=H, R_2=C_6H_5
9r, R_1=R_2=CH_3
9s, R_1=C_6H_5, R_2=CH_3
9t, R_1=R_2=C_6H_5



9u, R_3=R_4=MeO, R_5=H
9v, R_4=MeO, R_3=R_5=H

Chemistry. 2-Amino-3-(3′,4′,5′-trimethoxybenzoyl)thiophene derivatives with general structure **9** were synthesized as shown in the reaction sequence reported in Scheme 1. 2-Amino-3-aroyl thiophenes **9a**, **d**−**f**, **p**, **r**, and **u** and **v** were obtained by a one-step procedure (Gewald reaction[12]) applied to *β*-ketonitriles **10a**−**c**[13] and the appropriate aldehyde or ketone with sulfur and triethylamine (TEA) as base in refluxing ethanol. Compound **9q** was synthesized by a two-step procedure consisting of a Knoevenagel reaction of **10a** and acetophenone in benzene in the presence of *β*-alanine and acetic acid, followed after isolation and purification of the *E*- and *Z*-olefin isomers, by a cyclization in ethanol with sulfur in the presence of TEA.[14] The 5-unsubstituted thiophene derivatives **9a**, **p**, and **q** were transformed almost quantitatively into the corresponding *N*-phthalimido derivatives **11a**−**c** using phthalic anhydride in refluxing acetic acid.[15] The subsequent regioselective bromination of **11a**−**c** in a mixture of acetic acid and sodium acetate, using a stoichiometric amount of bromine,[16] furnished intermediate 5-bromothiophene derivatives **12a**−**c** in good yields. The formation of the 4-bromothiophene isomer of compound **11a** was not observed. The preparation of the thiophene 5-iodo substitution **12d** was carried out by stirring **11a** in acetic acid with *N*-iodosuccinimide (NIS) at 50 °C.[17]

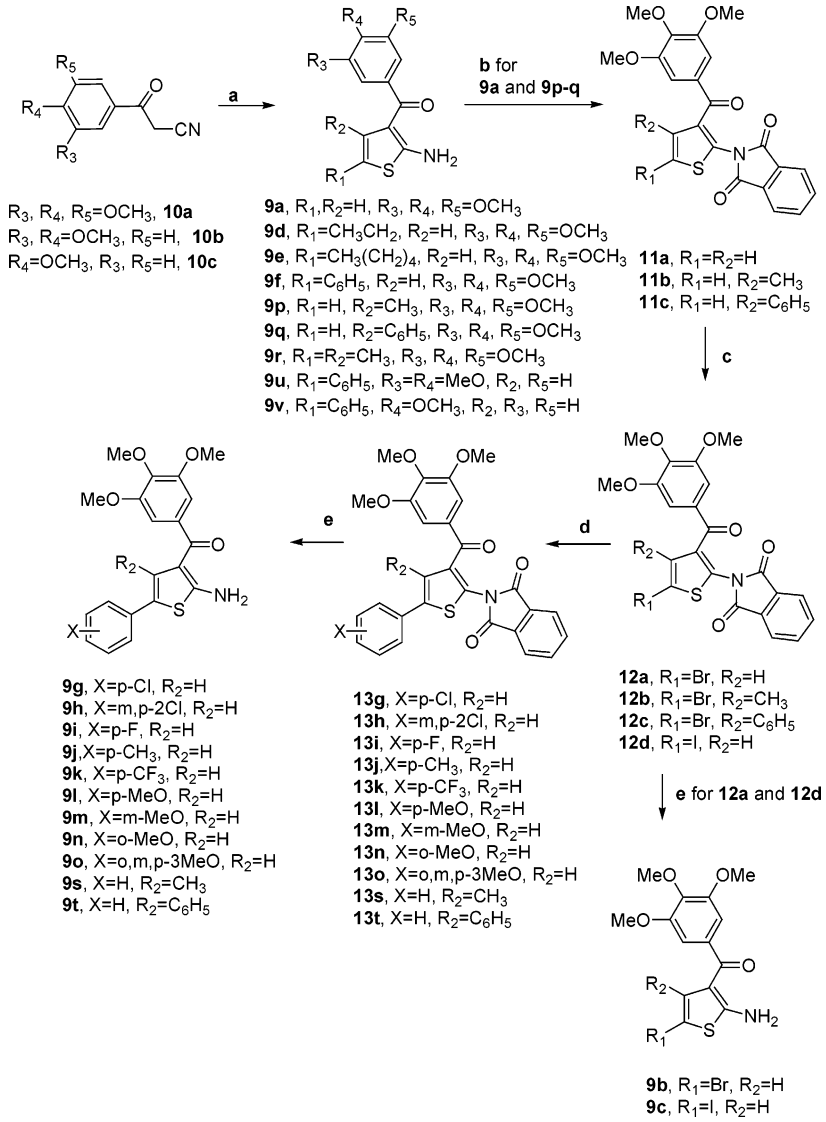
The eleven 5-aryl thiophene compounds **13g**−**o** and **s** and **t** were synthesized from key intermediates **12a**−**c** by a standard Suzuki cross-coupling reaction with appropriate aryl boronic acids under heterogeneous conditions ($Pd(PPh_3)_4$, K_2CO_3) in refluxing toluene.[18] The removal of the N-protected phthaloyl group was accomplished by the use of hydrazine in refluxing ethanol, to afford final compounds **9b** and **c**, **g**−**o**, and **s** and **t**.[19]

Results and Discussion

Effects on in Vitro Proliferation of the K562 and L1210 Tumor Cell Lines. Table 1 summarizes the effects of thiophene derivatives **9a**−**v** on the growth of murine L1210[20] and human K562[21a] leukemia cells with CA-4 (**1**) as the reference compound. L1210 and K562 cell lines are among the most used tumor cell lines for the screening of potential antitumor compounds.[21b,c] Seven compounds had activities in both cell lines comparable with the activities of CA-4, but most of the other compounds were minimally cytotoxic.

The 4,5-unsubstituted thiophene derivative **9a** displayed only modest antiproliferative activity, which was little affected with alkyl (ethyl, **9d** or *n*-pentyl, **9e**) or halogen (Br, **9b** or I, **9c**) substituents at C-5. In contrast, 9 out of 10 compounds with aryl substituents (**9f**−**o**, the exception being **9o**) at C-5 had significantly improved cytotoxicity relative to that of **9a**. In fact, 6 of the 7 most cytotoxic compounds were in this group. The contribution of the phenyl group to activity, as in **9f**, was position-dependent. Placing the phenyl at C-4 (**9q**) resulted in an inactive compound. The substitution on the phenyl ring had variable effects. A para fluoro group (**9i**) may have slightly improved antiproliferative activity, and all other para substituents prepared (Cl (**9g**), CH_3 (**9j**), OCH_3 (**9l**)), except *p*-CF_3 (**9k**), had minimal effects relative to those of **9f**. Only a slight loss of activity was observed when the methoxy substituent was moved from the para position to either the ortho (**9n**) or the meta (**9m**) position. However, either two (**9h**) or three (**9o**) substituents on the C-5 phenyl group led to a significant loss of antiproliferative activity, suggesting that steric factors account for the loss of activity observed with these two compounds. These data show no clear steric or electronic effects, except that multiple substituents on the phenyl ring or the strong

Scheme 1[a]



[a] Reagents: **a**: ketone or aldehyde, S_8, TEA, EtOH, 70 °C for 2 h; for **9q**: acetophenone, 10a, AcOH, β-alanine, benzene, rx, 16 h; **b**: phthalic anhydride, AcOH; c: Br_2, AcOH−AcONa for **11** a−**c**, r.t.; NIS, AcOH, 50 °C; **d**: $ArB(OH)_2$, $Pd(PPh_3)_4$, Na_2CO_3, PhMe, rx, 18 h; **e**: NH_2NH_2, EtOH.

electron-withdrawing group CF_3 were present in the least active C-5 aryl substituents.

Considering the substitution at the thiophene C-4 position, substituent size seemed to be a factor related to antiproliferative activity. The weak micromolar activity of 2-amino-3-(3′,4′,5′-trimethoxybenzoyl) thiophene (**9a**) was enhanced up to 12-fold (in the L1210 cell line) by the 4-methyl substitution (derivative **9p**) and eliminated by a 4-phenyl substituent (**9q**).

Starting from **9f**, with the C-5 phenyl substituent, the insertion of a second phenyl ring at C-4 yielded an inactive compound (**9t**). The steric hindrance of the C-4 phenyl group should result in conformational changes in the relative positions of the 3′,4′,5′-trimethoxybenzoyl and phenyl moieties at the C-3 and C-5 positions, respectively, and this could cause the inactivity of **9t**. In support of this idea, a methyl group at C-4 in the **9f** structure (i.e., compound **9s**) led to a small loss of cytotoxic activity, whereas the replacement of the C-5 phenyl ring in **9s** with a methyl group (compound **9r**) led to a significant loss of activity.

Synthesis of derivatives **9u** and **v**, both significantly less active than **9f**, demonstrated that the presence of the 3′,4′,5′-trimethoxybenzoyl moiety was essential for the optimal activity of **9f**. Its substitution with the 3′,4′-dimethoxybenzoyl and, especially, 4′-methoxybenzoyl moieties (compounds **9u** and **v**, respectively), led to the loss of antiproliferative activity.

Effects on Tubulin Polymerization and on Colchicine Binding to Tubulin. To investigate whether the antiproliferative activities of these compounds were related to the interaction with the microtubule system, seven of the most active compounds (**9f** and **g**, **9i** and **j**, **9l** and **m**, and **9s**) were evaluated for the inhibition of the polymerization of purified tubulin and the binding of [^{3}H]colchicine to tubulin (Table 2).[22a,b] For comparison, the data of the potent antimitotic compounds CA-4 and podophyllotoxin are also presented. The results are consistent with the conclusion that tubulin is the intracellular target of these seven compounds because they were all potent inhibitors of both tubulin-dependent reactions with activities quantitatively similar to those of CA-4 and podophyllotoxin. Three compounds, **9g** and **9i** and **j**, were better inhibitors of assembly than either of the standard drugs, all seven compounds were better inhibitors of colchicine binding than podophyllotoxin, and the most cytotoxic of the thiophene derivatives, **9i**, was as potent as CA-4 as an inhibitor of colchicine binding, a property rarely observed in colchicine site drugs.

Table 1. In Vitro Activity of Compounds **9a**−**v** and CA-4 (**1**) against the Proliferation of Murine L1210 and Human K562 Leukemia Cell Lines

compd	IC_{50} (nM)[a] K562	L1210
9a	1267 ± 289	3150 ± 1900
9b	1677 ± 380	4250 ± 1060
9c	342 ± 43	1150 ± 490
9d	9000 ± 707	>10,000
9e	1490 ± 210	1325 ± 106
9f	3.75 ± 1.56	2.52 ± 0.70
9g	14.7 ± 1.52	6.96 ± 0.36
9h	362 ± 34	337 ± 18
9i	2.82 ± 1.82	2.15 ± 1.26
9j	4.72 ± 1.34	3.52 ± 0.36
9k	70.3 ± 14.1	73.7 ± 8.83
9l	4.47 ± 1.70	6.52 ± 0.35
9m	10.1 ± 3.36	20.8 ± 1.26
9n	7.75 ± 2.12	9.00 ± 2.82
9o	1100 ± 100	1650 ± 630
9p	219 ± 26	262 ± 3.52
9q	>10000	>10000
9r	790 ± 230	3900 ± 140
9s	6.42 ± 3.07	18.2 ± 2.20
9t	8500 ± 356	6950 ± 235
9u	85.5 ± 9.12	537 ± 18.8
9v	4670 ± 290	>10000
CA-4 **(1)**	3.6 ± 1.4	8.3 ± 2.1

[a] IC_{50} is the 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 **9f** and **g**, **i** and **j**, **l** and **m**, and **s**

compd	tubulin assembly[a] IC_{50} ± SD (μM)	colchicine binding[b] % ± SD
9f	1.9 ± 0.04	60 ± 5
9g	0.98 ± 0.10	68 ± 2
9i	1.1 ± 0.00	82 ± 3
9j	1.1 ± 0.04	71 ± 1
9l	1.7 ± 0.00	64 ± 1
9m	1.7 ± 0.00	58 ± 4
9s	2.2 ± 0.2	37 ± 2
Podophyllotoxin	1.7 ± 0.1	34 ± 10
CA-4 (**1**)	1.7 ± 0.2	82 ± 0.3

[a] Inhibition of tubulin polymerization. Tubulin was at 10 μM. [b] Inhibition of [^{3}H]colchicine binding. Tubulin, colchicine, and the test compound were at 1, 5, and 1 μM concentration, respectively.

We conclude that the antiproliferative activity of these compounds derives from an interaction with the colchicine site of tubulin and an interference with microtubule assembly. This indicates that the cellular actions of these agents would involve mitotic arrest due to the interference with the functions of the mitotic spindle and apoptotic cell death.[23−26]

Effects on Apoptosis. We determined whether compounds exhibiting high antitubulin activity induce apoptosis of K562 cells. The cells were treated with the most potent antiproliferative compounds and analyzed by light microscopy to determine the percentage of cells displaying an apoptotic phenotype. Figure 1 shows a representative image of K562 cells treated with **9f**, **i**, and **j**. These three compounds displayed the highest levels of apoptosis among the compounds examined. The average values of the percentage of apoptotic cells induced by these compounds were 87.5 ± 4.3% (**9f**), 20.5 ± 5.2% (**9j**), and 64.5 ± 6.2 (**9i**) in three independent experiments.

In agreement with the microscopic evaluation, the flow cytometric evalution of cells following treatment with compounds **9f**, **i**, and **j** showed the appearance of substantial numbers

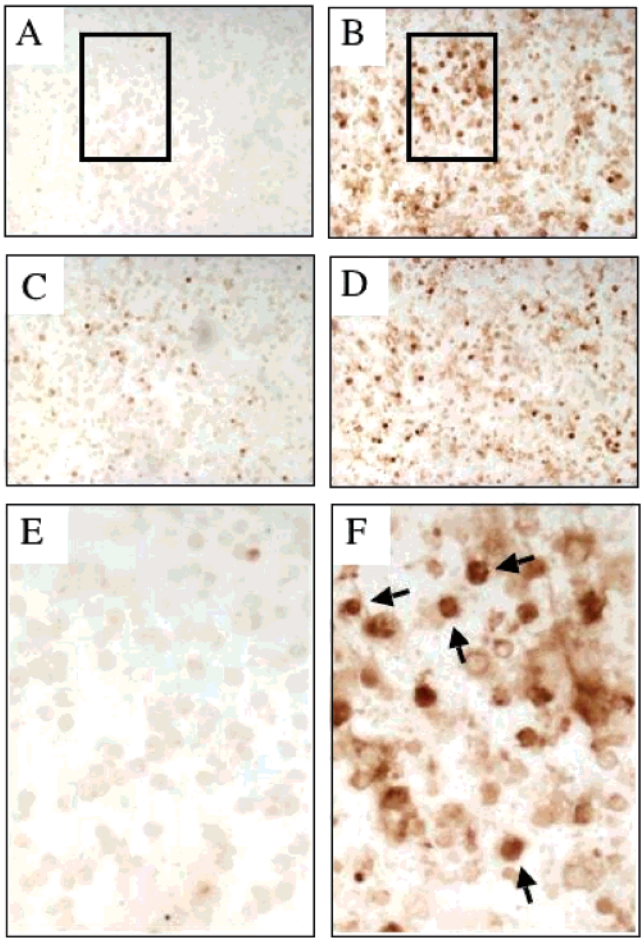


Figure 1. Effects of compounds **9f**, **i**, and **j** on apoptosis in K562 cells. The cells were cultured for 4 days without any compounds (A) or with compounds **9f** (B), **9i** (C), and **9j** (D) leading to 50% inhibition of cell growth. In panels E and F, enlargements of the areas represented by the boxes in panels A and B, respectively, are shown. Representative apoptotic cells are indicated by arrows in panel F.

of sub-G1 cells. In the first experiment (Figure 2, panels A−C) in which the cells were treated with 3 and 10 nM **9f**, there was a clear increase of the proportion of sub-G1 cells, indicative of extensive DNA breakage. Figure 2 (panels D−G) demonstrates that compounds **9i** and **j** as well as **f** induce a sharp increase in the proportion of sub-G1 cells. Thus, by two independent methods, we show that the most active thiophene derivatives cause apoptosis and, as expected from their antitubulin properties, are cytotoxic rather than cytostatic compounds.

Analysis of Cell Cycle. Because molecules exhibiting activity on tubulin binding should cause the alteration of cell cycle parameters leading to a preferential G2/M blockade,[5−7] the effects of the most active compound of the series (**9i**) on cell cycle distribution were analyzed in K562 cells cultured for 24 h in the presence of increasing amounts of the compound. The results of a representative experiment (Figure 3) show a dose-dependent increase in the proportion of G2-M cells occurring with a simultaneous decrease in S and G1 cells. As expected from the results shown in Figure 2, sub-G1 cells are found in K562 cells treated with the highest amount of **9i**. Similar effects on the cell cycle were obtained using the other active compounds (data not shown), suggesting that this class of molecules act, as expected from the data shown in Table 2, selectively on the G2/M phase of the cell cycle.

Molecular Modeling Studies. Molecular docking simulations to the colchicine site of tubulin were performed to determine a

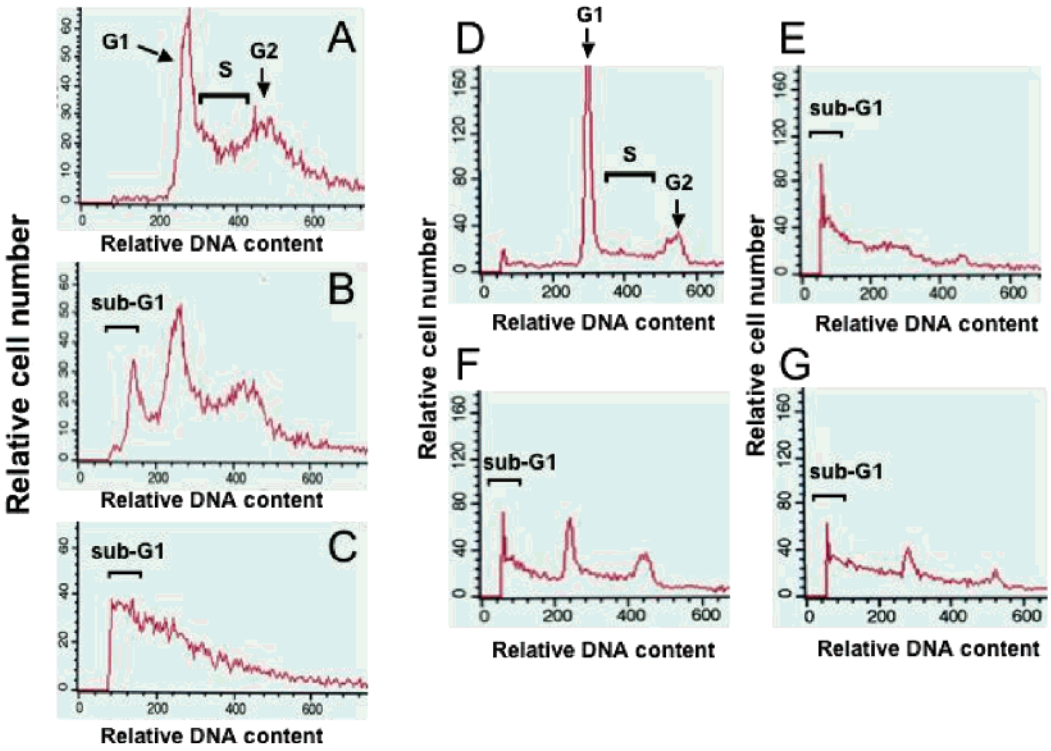


Figure 2. Effects of compounds **9f** (B, C, E), **9i** (F), and **9j** (G) on DNA content/cell following the treatment of K562 cells for 4 days. The cells were cultured without any compounds (A, D) or with compounds **9f**, **i**, or **j** leading to 50% (B) and 75% (C, E−G) inhibition of cell growth. Cell cycle distribution was analyzed by the standard propidium iodide procedure as described in the Experimental Section. Sub-G1, G1, S, and G2 cells are indicated.

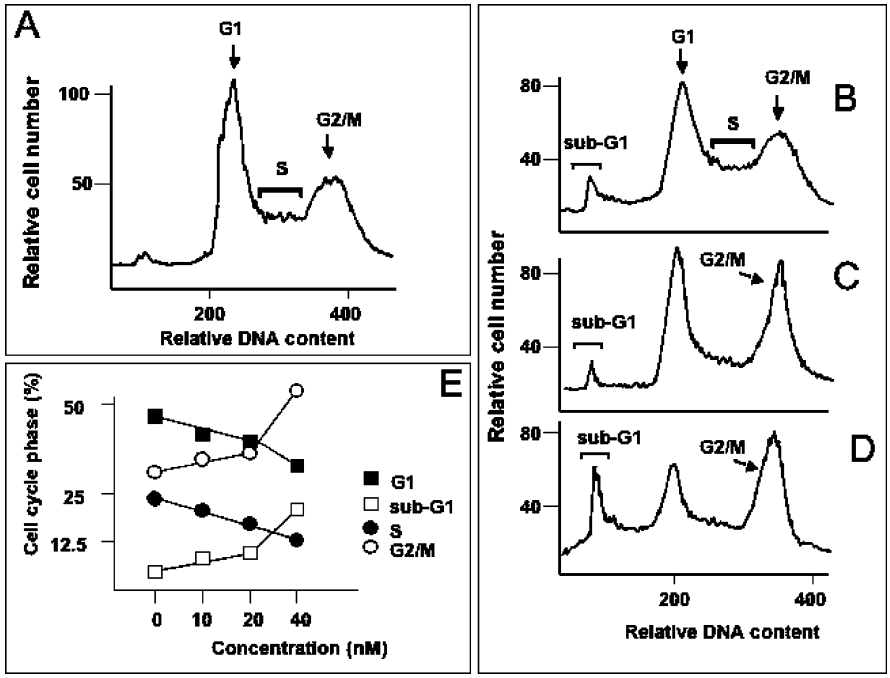


Figure 3. Effects of compound **9i** on the cell cycle distribution of K562 cells**.** The cells were cultured for 24 h without the compound (A) or withcompound **9i** leading to 25% (B), 50% (C), and 75% (D) inhibition of cell growth. Cell cycle distribution was analyzed by the standard propidium iodide procedure as described in the Experimental Section. Sub-G1, G1, S and G2 cells are indicated. Quantitative analysis of the data shown in panels A−D (E).

possible binding mode of **9i**. The recently reported crystallographic 3D structure of tubulin was retrieved from the Protein Data Bank.[27] All docking runs were performed to the colchicine site of the macromolecule, applying the Lamarckian genetic algorithm (LGA) of AutoDock 3.0.[28]

To set a consistent flexible docking protocol for compound **9i**, the cocrystallized ligand *N*-deacetyl-*N*-(2-mercaptoacetyl)-colchicine (DAMA-colchicine) was redocked into the binding site of the protein using the above algorithm. The pose of the original X-ray crystallographic reference structure was successfully reproduced (rmsd: 0.79 Å, $\Delta G_{bind} = -12.07$ kcal/mol).

Concerning compound **9i**, the calculated free energies of binding were used as the parameter for the selection of the cluster of docking poses to be evaluated. The docked ligand

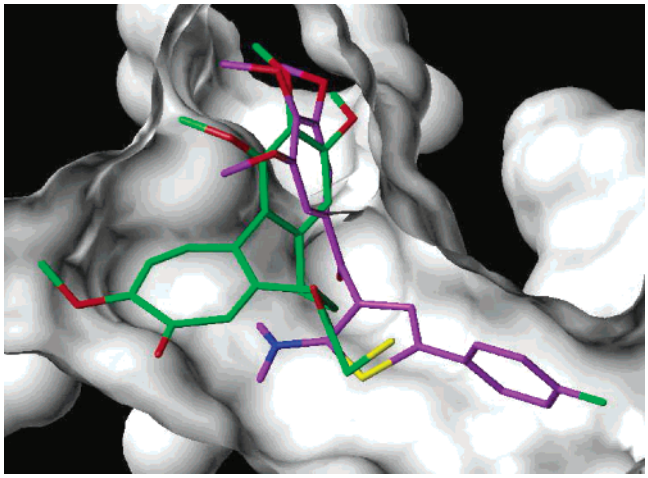


Figure 4. DAMA-colchicine and **9i** docked into the binding site of tubulin. (The Connoly surface is shown in gray.) The carbon atoms of DAMA-colchicine and **9i** are green and violet, respectively. The Connoly surface of the region along the *z*-axis is, on purpose, not highlighted.

Figure 5. Compound **9i** (carbon atom are violet) docked into the tubulin binding pocket. Both side chains of important active site amino acids and hydrogen bonds are shown: Leu-252 (angle N−H...O = 132.74°, distance = 2.675 Å), Asn-258 (angle O...H−N = 162.16°, distance = 2.716 Å), and Gln-11 (angle C═O...F = 136.08°, distance = 2.545 Å). Amino acid residue numbers are as in the crystal structure.[27]

showed a similar orientation to that of DAMA-colchicine in the tubulin binding pocket. Subsequently, the selection of the putative bioactive conformation was performed mainly on a geometrical basis, namely, the conformation with the best fit to the common trimethoxybenzoyl moiety in terms of rmsd. The selected pose of **9i** had an estimated free energy of binding of −10.11 kcal/mol, and the trimethoxybenzoyl ring of the ligand superimposed well on the same moiety of DAMA-colchicine (Figure 4). This finding is in agreement with data reported in the literature concerning the possible binding mode of other potent colchicine site inhibitors of tubulin polymerization.[29,30]

Moreover, the binding of compound **9i** to tubulin was stabilized by hydrogen bonding interactions with Leu-252, Asn-258, and Gln-11 of β-tubulin (Figure 5). The results of our docking studies are consistent with the high inhibitory activity observed for compound **9i**. The residue numbering system used here is from the crystal structure.[27]

Conclusions

In conclusion, we have discovered a new class of simple synthetic inhibitors of tubulin polymerization based on the molecular skeleton of 2-amino-3-(3′,4′,5′-trimethoxybenzoyl)-thiophene. The compounds were evaluated for their antiproliferative activities against both L1210 and K562 cells. Seven of the 22 new compounds displayed potent activity, differing little from that of CA-4. SAR studies showed that the most active analogues had a phenyl or a substituted phenyl group in the 5-position of the thiophene ring along with a hydrogen or a small substituent (methyl, compound **9s**) in the 4-position. Indeed, the absence of a 5-aryl moiety (**9a**), including its replacement by a halogen (**9b** and **c**) or an alkyl substituent (**9d** and **e**), led to sharply reduced activity. The investigations reported here were aimed at the elucidation of the mechanism of action by which these compounds exert their antiproliferative activity. As the molecular target of the compounds, we identified tubulin, the major constituent of the cellular microtubule system. Six of the seven most cytotoxic members of the series were all evaluated for effects on tubulin assembly and inhibition of colchicine binding. The IC_{50} values in a relatively narrow range (1−2.2 μM) were obtained for **9f** and **g**, **i** and **j**, **l** and **m**, and **9s** as inhibitors of tubulin assembly. The three most potent inhibitors of tubulin assembly (**9g** and **i** and **j**) also exhibited high levels of induction of apoptosis and DNA breakage. Compound **9i**, with the highest antiproliferative activity, was the strongest inhibitor both of tubulin assembly and [^{3}H]-colchicine binding to tubulin. The results of docking studies are consistent with the observed biological data for compound **9i**. The binding of this derivative to tubulin was stabilized by hydrogen-bonding interactions with Leu-252, Asn-258, and Gln-11 of β-tubulin. Cell-cycle distribution analysis shows that **9i**, as expected from its inhibition of tubulin assembly, acts on the G2/M phase of the cell cycle. These derivatives constitute an interesting new class of antitubulin agents with the potential to be clinically developed for cancer treatment. Among their attractive properties are the ease of synthesis and chemical stability.

Experimental Section

Chemistry. Materials and Methods. 1-Phenylethanone, 2,5-dihydroxy-1,4-dithiane, 2,5-dihydroxy-2,5-dimethoxy-1,4-dithiane, butanal, heptanal, phenylacetaldehyde, and 2-butanone are commercially available and 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 the 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 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 hydride and calcium chloride were used in the distillation of dioxane and DMF, respectively, and the distilled solvents were stored over molecular sieves (3 Å). In high-pressure hydrogenation experiments, a Parr shaker on a high-pressure autoclave was used.

Synthesis of (2-Amino-4-phenylthiophen-3-yl)-(3,4,5-trimethoxyphenyl)-methanone (9q). A mixture of 3-oxo-3-(3,4,5-trimethoxyphenyl)-propionitrile (**10a**) (588 mg, 2.5 mmol), 1-phenylethanone (2.5 mmol), β-alanine (0.5 mmol), acetic acid (0.6 mL), and benzene (15 mL) was refluxed under Dean−Stark conditions for 16 h. The mixture was cooled, washed with water (5 mL) and brine (5 mL),

dried, evaporated, and purified by column chromatography (EtOAc−petroleum ether 3:7) to furnish the E/Z mixture of Knoevenagel's adduct as a yellow oil (48% yield). The oil was dissolved in EtOH (5 mL), and sulfur (64 mg, 2 mmol) and TEA (250 μL, 2 mmol) were added. The mixture was stirred for 2 h at 50 °C. The mixture was cooled to r.t. and evaporated, and the residue was dissolved in dichloromethane (DCM, 10 mL), washed with water (2 mL) and brine (2 mL), dried, evaporated, purified by column chromatography (EtOAc−petroleum ether 3:7), and crystallized from petroleum ether. Yellow solid (55% yield); mp 154−156 °C. ^{1}H NMR ($CDCl_3$): δ 3.69 (s, 3H), 3.71 (s, 6H), 6.22 (bs, 2H), 6.59 (s, 2H), 7.02 (m, 6H). Anal. ($C_{20}H_{19}NO_4S$): C, H, N.

General Procedure A for the Synthesis of Compounds 9a, d−f, p−r, u and v. To a suspension of aroylacetonitrile derivative **10a**, **b**, or **c** (5 mmol), TEA (0.44 mL, 5 mmol), and sulfur (164 mg, 5 mmol) in EtOH (10 mL) was added the appropriate ketone or aldehyde (5 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.

(2-Aminothiophen-3-yl)-(3,4,5-trimethoxyphenyl)-methanone (9a). 2,5-Dihydroxy-1,4-dithiane (thioacetaldehyde dimer), 3-oxo-3-(3,4,5-trimethoxyphenyl)-propionitrile (**10a**), TEA, and sulfur were reacted according to general procedure A. Purification by silica gel column chromatography (petroleum ether−EtOAc 3:7) afforded **9a** as a yellow powder (74% yield); mp 140−142 °C. ^{1}H NMR ($CDCl_3$): δ 3.89 (s, 3H), 3.91 (s, 6H), 6.16 (d, J = 6.2 Hz, 1H), 6.28 (bs, 2H), 6.94 (s, 2H), 7.00 (d, J = 6.2 Hz, 1H). Anal. ($C_{14}H_{15}NO_4S$): C, H, N.

(2-Amino-5-ethylthiophen-3-yl)-(3,4,5-trimethoxyphenyl)-methanone (9d). Butanal, 3-oxo-3-(3,4,5-trimethoxyphenyl)-propionitrile (**10a**), TEA, and sulfur were reacted according to general procedure A. Purification by silica gel column chromatography (petroleum ether−EtOAc 2:8) afforded **9d** as a yellow oil (65% yield). ^{1}H NMR ($CDCl_3$): δ 1.21 (t, J = 7.6 Hz, 3H), 2.62 (q, J = 7.6 Hz, 2H), 3.89 (s, 3H), 3.90 (s, 6H), 6.33 (bs, 2H), 6.59 (s, 2H), 6.94 (s, 1H). Anal. ($C_{16}H_{19}NO_4S$): C, H, N.

(2-Amino-5-pentylthiophen-3-yl)-(3,4,5-trimethoxyphenyl)-methanone (9e). Heptanal, 3-oxo-3-(3,4,5-trimethoxyphenyl)-propionitrile (**10a**), TEA, and sulfur were reacted according to general procedure A. Purification by silica gel column chromatography (petroleum ether−EtOAc 2:8) afforded **9e** as a yellow oil (61% yield). ^{1}H NMR ($CDCl_3$): δ 1.31 (m, 9H), 2.63 (t, J = 7.6 Hz, 2H), 3.87 (s, 3H), 3.88 (s, 6H), 6.34 (bs, 2H), 6.61 (s, 2H), 6.90 (s, 1H). Anal. ($C_{19}H_{25}NO_4S$): C, H, N.

(2-Amino-5-phenylthiophen-3-yl)-(3,4,5-trimethoxyphenyl)-methanone (9f). Phenylacetaldehyde (50% solution in water), 3-oxo-3-(3,4,5-trimethoxyphenyl)-propionitrile (**10a**), triethylamine, and sulfur were reacted according to general procedure A. Purification by silica gel column chromatography (toluene−ethyl ether 7:3) afforded **9f** as a yellow solid (78% yield); mp 63−64 °C. ^{1}H NMR ($CDCl_3$): δ 3.89 (s, 3H), 3.92 (s, 6H), 6.26 (bs, 2H), 6.97 (s, 2H), 7.18 (s, 1H), 7.36 (m, 5H). Anal. ($C_{20}H_{19}NO_4S$): C, H, N.

(2-Amino-4-methylthiophen-3-yl)-(3,4,5-trimethoxyphenyl)-methanone (9p). 2,5-Dimethyl-2,5-dihydroxy-1,4-dithiane, 3-oxo-3-(3,4,5-trimethoxyphenyl)-propionitrile (**10a**), TEA, and sulfur were reacted according to general procedure A. Purification by silica gel column chromatography (petroleum ether−EtOAc 2:8) afforded **9p** as a yellow powder (50% yield); mp 148−150 °C. ^{1}H NMR ($CDCl_3$): δ 1.82 (s, 3H), 3.89 (m, 9H), 5.86 (s, 1H), 6.43 (bs, 2H), 6.77 (s, 2H). Anal. ($C_{15}H_{17}NO_4S$): C, H, N

(2-Amino-4,5-dimethylthiophen-3-yl)-(3,4,5-trimethoxyphenyl)-methanone (9r). 2-Butanone, 3-oxo-3-(3,4,5-trimethoxyphenyl)-propionitrile (**10a**), TEA, and sulfur were reacted according to general procedure A. Purification by silica gel column chromatography (petroleum ether−EtOAc 3:7) afforded **9r** as a yellow powder (68% yield); mp 78−80 °C. ^{1}H NMR ($CDCl_3$): δ 1.65 (s, 3H), 2.14 (s, 3H), 3.86 (s, 6H), 3.89 (s, 3H), 6.28 (bs, 2H), 6.78 (s, 2H). Anal. ($C_{16}H_{19}NO_4S$): C, H, N.

(2-Amino-5-phenylthiophen-3-yl)-(3,4-dimethoxyphenyl)-methanone (9u). Phenylacetaldehyde (50% solution in water), 3-oxo-3-(3,4-dimethoxyphenyl)-propionitrile (**10b**), TEA, and sulfur were reacted according to general procedure A. Purification by silica gel column chromatography (petroleum ether−EtOAc 3:7) afforded afforded **9u** as a yellow solid (66% yield); mp 112−114 °C. ^{1}H NMR ($CDCl_3$): δ 3.93 (s, 3H), 3.95 (s, 3H), 6.28 (bs, 2H), 6.91 (s, 1H), 6.95 (d, J = 7.6 Hz, 1H), 7.20 (s, 1H), 7.33 (m, 6H). Anal. ($C_{19}H_{17}NO_3S$): C, H, N.

(2-Amino-5-phenylthiophen-3-yl)-(4-methoxyphenyl)-methanone (9v). Phenylacetaldehyde, 3-oxo-3-(4-methoxyphenyl)-propionitrile (**10c**), TEA, and sulfur were reacted according to general procedure A. Purification by silica gel column chromatography (petroleum ether−EtOAc 3:7) afforded **9v** as an orange gum (56% yield). ^{1}H NMR ($CDCl_3$): δ 3.88 (s, 3H), 6.94 (bs, 2H), 6.99 (s, 1H), 7.32 (m, 7H), 7.73 (d, J = 8.8 Hz, 2H). Anal. ($C_{18}H_{15}NO_2S$): C, H, N.

General Procedure B for the Preparation of Compounds 11a−c. To a suspension of compound **9a**, **p**, or **q** (6 mmol) in acetic acid (20 mL) was added phthalic anhydride (7.2 mmol, 1.07 g). After stirring for 18 h at reflux, the solvent was evaporated and the residue dissolved in EtOAc (30 mL). The organic solution was washed with a saturated solution of $NaHCO_3$ (10 mL), water (10 mL), and brine (10 mL), dried, and concentrated. The crude product was purified by crystallization from petroleum ether.

2-[3-(3,4,5-Trimethoxybenzoyl)-thiophen-2-yl]-isoindole-1,3-dione (11a). Following general procedure B, starting from compound **9a**, derivative **11a** was obtained as a yellow solid (75% yield); mp 153−155 °C. ^{1}H NMR ($CDCl_3$): δ 3.72 (s, 3H), 3.83 (s, 6H), 6.98 (s, 2H), 7.41 (m, 2H), 7.77 (m, 2H), 7.84 (m, 2H).

2-[4-Methyl-3-(3,4,5-trimethoxybenzoyl)-thiophen-2-yl]-isoindole-1,3-dione (11b). Following general procedure B, starting from compound **9p**, derivative **11b** was obtained as a yellow solid (60% yield); mp 108−110 °C. ^{1}H NMR ($CDCl_3$): δ 2.34 (s, 3H), 3.61 (s, 3H), 3.79 (s, 6H), 6.91 (s, 2H), 7.08 (s, 1H), 7.70 (m, 4H).

2-[4-Phenyl-3-(3,4,5-trimethoxybenzoyl)-thiophen-2-yl]-isoindole-1,3-dione (11c). Following general procedure B, starting from compound **9q**, derivative **11c** was obtained as a yellow solid (68% yield); mp 152−154 °C. ^{1}H NMR ($CDCl_3$): δ 3.62 (s, 3H), 3.82 (s, 6H), 6.92 (s, 2H), 7.12 (m, 6H), 7.62 (m, 2H), 7.72 (m, 2H).

General Procedure C for the Preparation of Compounds 12a−c. To an acetic acid solution (12 mL) of derivatives **11a**, **b**, or **c** (5 mmol) containing sodium acetate (5.5 mmol), bromine (5 mmol) was added dropwise with stirring at r.t over 5 min. After 1 h, the acetic acid was removed in vacuo, and the crude residue diluted with EtOAc (25 mL), washed with an aqueous 10% sodium thiosulfate solution (10 mL), a saturated solution of $NaHCO_3$ (10 mL), water (5 mL), and brine (5 mL), and it was dried and concentrated. The resulting residue was recrystallized from petroleum ether.

2-[5-Bromo-3-(3,4,5-trimethoxybenzoyl)-thiophen-2-yl]-isoindole-1,3-dione (12a). Yellow solid (82% yield); mp 175−177 °C. ^{1}H NMR ($CDCl_3$): δ 3.69 (s, 3H), 3.83 (s, 6H), 6.94 (s, 2H), 7.37 (s, 1H), 7.73 (m, 2H), 7.84 (m, 2H).

2-[5-Bromo-4-methyl-3-(3,4,5-trimethoxybenzoyl)-thiophen-2-yl]-isoindole-1,3-dione (12b). Yellow solid (86% yield); mp 183−184 °C. ^{1}H NMR ($CDCl_3$): δ 2.33 (s, 3H), 3.62 (s, 3H), 3.80 (s, 6H), 6.96 (bs, 2H), 7.67 (m, 2H); 7.74 (m, 2H).

2-[5-Bromo-4-phenyl-3-(3,4,5-trimethoxybenzoyl)-thiophen-2-yl]-isoindole-1,3-dione (12c). Yellow solid (71% yield); mp 163−165 °C. ^{1}H NMR ($CDCl_3$): δ 3.61 (s, 3H), 3.78 (s, 6H), 6.90 (s, 2H), 7.08 (m, 5H), 7.60 (m, 2H), 7.68 (m, 2H).

2-[5-Iodo-3-(3,4,5-trimethoxybenzoyl)-thiophen-2-yl]-isoindole-1,3-dione (12d). A mixture of derivative **11a** (1 mmol) and NIS (4.4 mmol, 1.1 equiv) in acetic acid was heated at 50 °C for 6 h. Acetic acid was removed under reduced pressure, the crude residue was diluted with EtOAc (25 mL), washed with a saturated solution of $NaHCO_3$ (10 mL), water (5 mL), and brine (5 mL), and dried and concentrated. The resulting residue was recrystallized from petroleum ether. Compound **12d** was obtained as a brown solid

(38% yield); mp 179−181 °C. ^{1}H NMR ($CDCl_3$): δ 3.38 (s, 3H), 3.68 (s, 6H), 6.81 (s, 2H), 7.65 (s, 1H), 7.84 (m, 4H).

General Procedure D (Suzuki Coupling) for the Synthesis of Compounds 13g−o, s, and t. A mixture of thiophene derivatives **12a**, **b**, or **c** (0.5 mmol), potassium carbonate (104 mg, 0.75 mmol, 1.5 equiv), the appropriate aryl boronic acid (1 mmol, 2 equiv), and tetrakis(triphenylphosphine)palladium (13.5 mg, 0.012 mmol) in dry toluene (10 mL) was stirred at 100 °C under nitrogen for 18 h, cooled to ambient temperature, and evaporated in vacuo. The residue was dissolved with EtOAc (30 mL), and the resultant solution was washed sequentially with 5% $NaHCO_3$ (10 mL), water (10 mL), and brine (10 mL). The organic layer was dried, filtered, and evaporated, and the residue was purified by flash chromatography on silica gel.

2-[5-(4-Chlorophenyl)-3-(3,4,5-trimethoxybenzoyl)-thiophen-2-yl]-isoindole-1,3-dione (13g). The title compound was prepared by general procedure D from **12a** and *p*-chlorophenylboronic acid. EtOAc−petroleum ether (2:8) was used for flash chromatography. Yellow solid (90% yield); mp 216−218 °C. ^{1}H NMR ($CDCl_3$): δ 3.62 (s, 3H), 3. 76 (s, 6H), 6.92 (s, 2H), 7.19 (s, 1H), 7.35 (d, J = 8.8 Hz, 2H), 7. 50 (d, J = 8.8 Hz, 2H), 7. 51 (s, 1H), 7.74 (m, 2H), 7.77 (m, 2H).

2-[5-(3,4-Dichlorophenyl)-3-(3,4,5-trimethoxybenzoyl)-thiophen-2-yl]-isoindole-1,3-dione (13h). The title compound was prepared by general procedure D from **12a** and *m*,*p*-dichlorophenylboronic acid. EtOAc−petroleum ether (2:8) was used for flash chromatography. Yellow solid (70% yield); mp 225−226 °C. ^{1}H NMR ($CDCl_3$): δ 3.72 (s, 3H), 3.84 (s, 6H), 6.97 (s, 2H), 7.45 (m, 3H), 7. 51 (s, 1H), 7.74 (m, 2H), 7.79 (m, 2H).

2-[5-(4-Fluorophenyl)-3-(3,4,5-trimethoxybenzoyl)-thiophen-2-yl]-isoindole-1,3-dione (13i). The title compound was prepared by general procedure D from **12a** and *p*-fluorophenylboronic acid. EtOAc−petroleum ether (2:8) was used for flash chromatography. Yellow solid (85% yield); mp 198−200 °C. ^{1}H NMR ($CDCl_3$): δ 3.72 (s, 3H), 3.83 (s, 6H), 7.00 (s, 2H), 7.65 (s, 1H), 7.70 (m, 4H), 7.84 (m, 2H), 7.86 (m, 2H).

2-[5-(4-Methylphenyl)-3-(3,4,5-trimethoxybenzoyl)-thiophen-2-yl]-isoindole-1,3-dione (13j). The title compound was prepared by general procedure D from **12a** and *p*-methylphenylboronic acid. EtOAc−petroleum ether (3:7) was used for flash chromatography. Yellow solid (86% yield); mp 202−204 °C. ^{1}H NMR ($CDCl_3$): δ 2.38 (s, 3H), 3.69 (s, 3H), 3.82 (s, 6H), 7.00 (s, 2H), 7.24 (d, J = 9.2 Hz, 2H), 7.50 (d, J = 9.2 Hz, 2H), 7.52 (s, 1H), 7.76 (m, 2H), 7.82 (m, 2H).

2-[5-(4-Trifluoromethylphenyl)-3-(3,4,5-trimethoxybenzoyl)-thiophen-2-yl]-isoindole-1,3-dione (13k). The title compound was prepared by general procedure D from **12a** and *p*-trifluoromethylphenylboronic acid. EtOAc−petroleum ether (3:7) was used for flash chromatography. Yellow solid (72% yield); mp 206−207 °C. ^{1}H NMR ($CDCl_3$): δ 3.70 (s, 3H), 3.83 (s, 6H), 7.01 (s, 2H), 7.65 (s, 1H), 7.70 (d, J = 4.2 Hz, 2H), 7.75 (d, J = 4.2 Hz, 2H), 7.83 (m, 2H), 7.85 (m, 2H).

2-[5-(4-Methoxyphenyl)-3-(3,4,5-trimethoxybenzoyl)-thiophen-2-yl]-isoindole-1,3-dione (13l). The title compound was prepared by procedure D from **12a** and *p*-methoxyphenylboronic acid. EtOAc−petroleum ether (3:7) was used for flash chromatography. Yellow solid (82% yield); mp 181−182 °C. ^{1}H NMR ($CDCl_3$): δ 3.69 (s, 3H), 3.85 (s, 9H), 6.93 (s, 2H), 6.98 (d, J = 9.2 Hz, 2H), 7.45 (s, 1H), 7.57 (d, J = 9.2 Hz, 2H), 7.37 (m, 2H), 7.84 (m, 2H).

2-[5-(3-Methoxyphenyl)-3-(3,4,5-trimethoxybenzoyl)-thiophen-2-yl]-isoindole-1,3-dione (13m). The title compound was prepared by general procedure D from **12a** and *m*-methoxyphenylboronic acid. EtOAc−petroleum ether (3:7) was used for flash chromatography. Yellow solid (75% yield); mp 202−204 °C. ^{1}H NMR ($CDCl_3$): δ 3.69 (s, 3H), 3.86 (s, 9H), 6.98 (m, 1H), 7.00 (s, 2H), 7.05 (m, 1H), 7.12 (m, 1H), 7.34 (m, 1H), 7.54 (s, 1H), 7.76 (m, 2H), 7.83 (m, 2H).

2-[5-(2-Methoxyphenyl)-3-(3,4,5-trimethoxybenzoyl)-thiophen-2-yl]-isoindole-1,3-dione (13n). The title compound was prepared by general procedure D from **12a** and *o*-methoxyphenylboronic acid. EtOAc−petroleum ether (3:7) was used for flash chromatography. Yellow solid (60% yield); mp 198−200 °C. ^{1}H NMR ($CDCl_3$): δ 3.73 (s, 3H), 3.86 (s, 9H), 6.98 (m, 2H), 7.00 (s, 2H), 7.15 (m, 1H), 7.37 (m, 1H), 7.56 (s, 1H), 7.78 (m, 2H), 7.85 (m, 2H).

2-[3-(3,4,5-Trimethoxybenzoyl)-5-(3,4,5-trimethoxyphenyl)-thiophen-2-yl]-isoindole-1,3-dione (13o). The title compound was prepared by general procedure D from **12a** and *m*,*m*′,*p*-trimethoxyphenylboronic acid. EtOAc−petroleum ether (4:6) was used for flash chromatography. Yellow solid (60% yield); mp 181−182 °C. ^{1}H NMR ($CDCl_3$): δ 3.66 (s, 3H), 3.82 (s, 6H), 3.89 (s, 3H), 3.92 (s, 6H), 6.82 (s, 1H), 6.98 (s, 2H), 7.50 (s, 2H), 7.74 (m, 2H), 7.85 (m, 2H).

2-[4-Methyl-5-phenyl-3-(3,4,5-trimethoxybenzoyl)-thiophen-2-yl]-isoindole-1,3-dione (13s). The title compound was prepared by general procedure D from **12b** and phenylboronic acid. EtOAc−petroleum ether (2:8) was used for flash chromatography. Yellow cream solid (62% yield); mp 143−144 °C. ^{1}H NMR ($CDCl_3$): δ 2.33 (s, 3H), 3.62 (s, 3H), 3.82 (s, 6H), 6.98 (s, 2H), 7.48 (m, 5H), 7.75 (m, 4H).

2-[4,5-Diphenyl-3-(3,4,5-trimethoxybenzoyl)-thiophen-2-yl]-isoindole-1,3-dione (13t). The title compound was prepared by general procedure D from **12c** and phenylboronic acid. EtOAc−petroleum ether (2:8) was used for flash chromatography. Yellow solid (71% yield); mp 167−169 °C. ^{1}H NMR ($CDCl_3$): δ 3.64 (s, 3H), 3.84 (s, 6H), 7.00 (s, 2H), 7.48 (m, 10H), 7.75 (m, 4H).

General Procedure E for the Synthesis of Compounds 9b, c, g−o, s, and t. A stirred suspension of a thiophene derivative (one of **13b and c**, **g−o**, and **s** and **t**; 0.5 mmol) and hydrazine monohydrate (29 μL, 0.6 mmol, 1.2 equiv) in abs EtOH (10 mL) was refluxed for 3 h. The reaction mixture was then left at r.t. for 1 h, by which time the starting material was completely solubilized. The solvent was evaporated, and the residue was partitioned between EtOAc (10 mL) and water (5 mL). The separated organic phase, which was washed with brine (2 mL) and dried, was concentrated in vacuo to obtain a residue that was purified by column chromatography using petroleum ether−EtOAc (7:3) as the eluent to give the desired products **9b** and **c**, **g−o**, or **s** and **t**.

(2-Amino-5-bromothiophen-3-yl)-(3,4,5-trimethoxyphenyl)-methanone (9b). Yellow powder (67% yield); mp 119−120 °C; ^{1}H NMR ($CDCl_3$): δ 3.90 (m, 9H), 6.88 (s, 2H), 7.01 (s, 1H), 7.27 (bs, 2H). Anal. ($C_{14}H_{14}BrNO_4S$): C, H, N.

(2-Amino-5-iodothiophen-3-yl)-(3,4,5-trimethoxyphenyl)-methanone (9c). Yellow powder (70% yield); mp 134−135 °C; ^{1}H NMR ($CDCl_3$): δ 3.78 (s, 3H), 3.82 (s, 6H), 6.81 (s, 2H), 7.10 (s, 1H), 8.44 (bs, 2H). Anal. ($C_{14}H_{14}INO_4S$): C, H, N.

[2-Amino-5-(4-chlorophenyl)-thiophen-3-yl]-(3,4,5-trimethoxyphenyl)-methanone (9g). Yellow powder (65% yield); mp 184−186 °C; ^{1}H NMR ($CDCl_3$): δ 3.83 (s, 6H), 3.86 (s, 3H), 6.89 (s, 2H), 6.97 (bs, 2H), 7.09 (s, 1H), 7.20 (m, 4H). Anal. ($C_{20}H_{18}ClNO_4S$): C, H, N.

[2-Amino-5-(3,4-dichlorophenyl)-thiophen-3-yl]-(3,4,5-trimethoxyphenyl)-methanone (9h). Yellow powder (55% yield); mp 159−161 °C; ^{1}H NMR ($CDCl_3$): δ 3.83 (s, 6H), 3.86 (s, 3H), 6.87 (s, 2H), 6.92 (bs, 2H), 7.11 (s, 1H), 7.18 (s, 1H), 7.36 (m, 2H). Anal. ($C_{20}H_{17}Cl_2NO_4S$): C, H, N.

[2-Amino-5-(4-fluorophenyl)-thiophen-3-yl]-(3,4,5-trimethoxyphenyl)-methanone (9i). Yellow powder (78% yield); mp 174−175 °C; ^{1}H NMR ($CDCl_3$): δ 3.90 (s, 6H), 3.92 (s, 3H), 6.95 (s, 2H), 7.02 (bs, 2H), 7.05 (m, 2H), 7.09 (s, 1H), 7.34 (m, 2H). Anal. ($C_{20}H_{18}FNO_4S$): C, H, N.

[2-Amino-5-(4-methylphenyl)-thiophen-3-yl]-(3,4,5-trimethoxyphenyl)-methanone (9j). Yellow powder (80% yield); mp 184−185 °C; ^{1}H NMR ($CDCl_3$): δ 2.33 (s, 3H), 3.89 (s, 3H), 3.92 (s, 6H), 6.97 (s, 2H), 6.99 (bs, 2H), 7.12 (d, J = 8.4 Hz, 2H), 7.13 (s, 1H), 7.28 (d, J = 8.4 Hz, 2H). Anal. ($C_{21}H_{21}NO_4S$): C, H, N.

[2-Amino-5-(4-trifluoromethylphenyl)-thiophen-3-yl]-(3,4,5-trimethoxyphenyl)-methanone (9k). Yellow powder (70% yield); mp 199−200 °C; ^{1}H NMR ($CDCl_3$): δ 3.90 (s, 6H), 3.93 (s, 3H), 6.96 (s, 2H), 7.09 (bs, 2H), 7.29 (s, 1H), 7.45 (d, J = 8.6 Hz, 2H), 7,56 (d, J = 8.6 Hz, 2H). Anal. ($C_{21}H_{18}F_3NO_4S$): C, H, N.

[2-Amino-5-(4-methoxyphenyl)-thiophen-3-yl]-(3,4,5-tri-methoxyphenyl)-methanone (9l). Yellow powder (84% yield); mp 115−

116 °C; ^{1}H NMR ($CDCl_3$): δ 3.81 (s, 3H), 3.90 (s, 6H), 3.92 (s, 3H), 6.87 (d, J = 8.8 Hz, 2H), 6.97 (s, 2H), 6.98 (bs, 2H), 7.04 (s, 1H), 7.32 (d, J = 8.8 Hz, 2H). Anal. ($C_{21}H_{21}NO_5S$): C, H, N.

[2-Amino-5-(3-methoxyphenyl)-thiophen-3-yl]-(3,4,5-tri-methoxyphenyl)-methanone (9m). Yellow powder (76% yield); mp 160−161 °C; ^{1}H NMR ($CDCl_3$): δ 3.81 (s, 3H), 3.90 (s, 6H), 3.92 (s, 3H), 6.75 (m, 1H), 6.91 (m, 1H), 6.96 (s, 2H), 7.01 (bs, 2H), 7.18 (s, 1H), 7.20 (m, 2H). Anal. ($C_{21}H_{21}NO_5S$): C, H, N.

[2-Amino-5-(2-methoxyphenyl)-thiophen-3-yl]-(3,4,5-trimeth-oxyphenyl)-methanone (9n). Yellow powder (72% yield); mp 64−66 °C; ^{1}H NMR ($CDCl_3$): δ 3.82 (s, 3H), 3.90 (s, 6H), 3.93 (s, 3H), 6.98 (s, 2H), 6.99 (bs, 2H), 7.08 (s, 1H), 7.13 (d, J = 8.4 Hz, 1H), 7.32 (m, 2H), 7.34 (d, J = 8.6 Hz, 1H). Anal. ($C_{21}H_{21}NO_5S$): C, H, N.

[2-Amino-5-(3,4,5-trimethoxyphenyl)-thiophen-3-yl]-(3,4,5-tri-methoxyphenyl)-methanone (9o). Yellow powder (80% yield); mp 153−154 °C; ^{1}H NMR ($CDCl_3$): δ 3.84 (s, 3H), 3.87 (s, 6H), 3.90 (s, 6H), 3.93 (s, 3H), 6.59 (s, 2H), 6.98 (m, 2H), 6.99 (s, 2H), 7.06 (s, 1H). Anal. ($C_{23}H_{25}NO_7S$): C, H, N.

(2-Amino-4-methyl-5-phenylthiophen-3-yl)-(3,4,5-trimeth-oxyphenyl)-methanone (9s). Yellow powder (75% yield); mp 130−131 °C; ^{1}H NMR ($CDCl_3$): δ 1.83 (s, 3H), 3.89 (s, 9H), 6.40 (bs, 2H), 6.87 (s, 2H), 7.36 (m, 5H). Anal. ($C_{21}H_{21}NO_4S$): C, H, N.

(2-Amino-4,5-diphenylthiophen-3-yl)-(3,4,5-trimethoxyphenyl)-methanone (9t). Yellow powder (61% yield); mp 153−155 °C; ^{1}H NMR ($CDCl_3$): δ 3.70 (s, 9H), 6.60 (s, 2H), 6.86 (bs, 2H), 7.02 (m, 5H), 7.18 (m, 5H). Anal. ($C_{26}H_{23}NO_4S$): C, H, N.

Inhibition of the Growth of Murine L1210 and Human K562 Cells. The murine lymphocytic L1210 and the human chronic myelogenous K562 leukemia cell lines were obtained from the American Type Culture Collection. All test compounds were dissolved in dimethyl sulfoxide (DMSO) at 1 mg/mL immediately before use and diluted in the medium before addition to the cells. Both cell lines were cultured in an RPMI 1640 medium (GIBCO) supplemented with 10% bovine fetal calf serum (Flow, Irvine, U.K.), 2 mM L-glutamine (GIBCO), 10 mM β-mercaptoethanol, 100 U/mL of penicillin, and 100 μg/mL of streptomycin. To determine the effects of the studied compounds on cell growth, exponentially growing L1210 and K562 cells were exposed to increasing concentrations of drugs, and the cell number/mL value was determined after 48 h using a model ZBI Coulter Counter (Coulter Electronics, Hialeah, FL). The results were expressed as IC_{50} values (concentration causing 50% inhibition relative to untreated controls). All experiments were repeated at least three times. For each drug concentration, duplicate cultures were used. Considering the possible antiproliferative effects of DMSO, control cultures were always performed using the maximum levels of DMSO employed for the administration of tested compounds. The concentration of DMSO in the array was never higher than 0.01% and did not affect the cell growth of the employed cell lines.

Effects on Tubulin Polymerization and Colchicine Binding to Tubulin. Bovine brain tubulin was purified as previously described.[31] To evaluate the effect of the compounds on tubulin assembly in vitro,[22a] the compounds were preincubated across a concentration range with 10 μM tubulin in a glutamate buffer at 30 °C and then cooled to 0 °C. After the addition of GTP, the mixtures were 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%. The capacity of the test compounds to inhibit colchicine binding to tubulin was measured as described,[22b] except that the reaction mixtures contained 1 μM tubulin, 5 μM [^{3}H]-colchicine, and 1 μM test compound.

Analysis of the Cell Cycle and the Detection of Apoptosis. After treatment, the cells were rinsed twice with a PBS solution. For cell cycle analysis, the cells (1×10^6) were treated with propidium iodide for 30 min and injected into a flow cytometer, as described in detail elsewhere.[32,33] For the measurement of apoptosis, the cells were fixed for 25 min in 4% paraformaldehyde at r.t. The apoptotic cells were detected by the DeadEnd Colorimetric Apoptosis Detection System (Promega) as described elsewhere[34] and their number calculated as a percentage of the total number of cells evaluated in three independent experiments. We previously demonstrated that the employed concentrations of DMSO (used as vehicle) did not alter the cell cycle parameters of treated cells.

Molecular Modeling. The crystal structure of DAMA-colchicine, as deposited in the Protein Data Bank (pdb ID: ISA0),[27] was used as the reference structure. The 3D model of **9i** molecule was built by assembling the fragments from the SYBYL 7.0 software package standard library (Tripos, St. Louis, MO). The resulting geometry was optimized by semiempirical molecular orbital calculations using the AM1 Hamiltonian[35] (module MOPAC implemented in SYBYL). The docking studies were carried out by means of the AutoDock suite of software[28] exploiting the 3D X-ray crystallography structure of the DAMA-colchicine−tubulin complex. The flexible docking simulations were performed using LGA. The 3D grids describing the interactions of the atom types of the molecules under investigation (DAMA-colchicine and **9i**) were precomputed using the autogrid facility. In all the calculations, the energy scoring grids ($91 \times 125 \times 105$ points, spacing 0.286 Å) were centered on the geometric center of the inhibitor's coordinates. The compounds were subjected to 100 runs of the AutoDock search in which the default values of the other parameters were used.

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Supporting Information Available: Elemental analyses for compounds **9a**−**v**. This material is available free of charge via the Internet at http://pubs.acs.org.

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