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Design, synthesis, and biological evaluation of thiophene analogues of chalcones

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Abstract—Chalcones are characterized by possessing an enone moiety between two aromatic rings. A series of chalcone-like agents, in which the double bond of the enone system is embedded within a thiophene ring, were synthesized and evaluated for antiproliferative activity and inhibition of tubulin assembly and colchicine binding to tubulin. The replacement of the double bond with a thiophene maintains antiproliferative activity and therefore must not significantly alter the relative conformation of the two aryl rings. The synthesized compounds were found to inhibit the growth of several cancer cell lines at nanomolar to low micromolar concentrations. In general, all compounds having significant antiproliferative activity inhibited tubulin polymerization with an $IC_{50} < 2$ μM. Several of these compounds caused K562 cells to arrest in the G2/M phase of the cell cycle.

1. Introduction

The microtubule system of eukaryotic cells plays important roles in regulating cell architecture, and 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 α,β-tubulin heterodimers in the polymeric microtubules. They are involved in a variety of fundamental cellular processes, such as regulation of motility, cell signalling, 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] Chalcones with general structure **1** (Chart 1) are known to exhibit antimitotic properties caused by the inhibition of tubulin polymerization.[4] Chemically, they are open-chained molecules consisting of two aromatic rings linked by a three-carbon enone fragment. Several research groups have shown that the *s-cis* conformation of chalcones is important for their biological activity.[5] Their simple structure and ease of preparation make chalcones an attractive scaffold for structure–activity relationship (SAR) studies, and a wide number of substituted chalcones have been synthesized to evaluate the effects of various functional groups on biological activity.[4]

It has been reported that hydrogenation or bromination across the carbon–carbon double bond or its transformation into the corresponding epoxide dramatically

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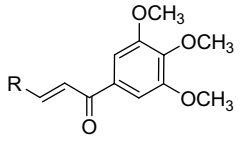
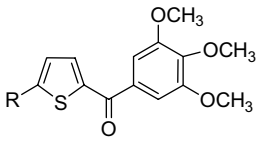


1a-g

1a, R=C_6H_5
1b, R=p-F-C_6H_4
1c, R=p-Cl-C_6H_4
1d, R=p-OCH_3-C_6H_4
1e, R=m-OCH_3-C_6H_4
1f, R=o-OCH_3-C_6H_4
1g, R=m,p-2(OCH_3)-C_6H_3



2a-p

2a, R=C_6H_5
2b, R=2-thienyl
2c, R=3-thienyl
2d, R=p-F-C_6H_4
2e, R=m-F-C_6H_4
2f, R=o-F-C_6H_4
2g, R=p-Cl-C_6H_4
2h, R=o-Cl-C_6H_4
2i, R=p-CH_3-C_6H_4
2j, R=o-CH_3-C_6H_4
2k, R=p-OCH_3-C_6H_4
2l, R=m-OCH_3-C_6H_4
2m, R=o-OCH_3-C_6H_4
2n, R=m,p-2(OCH_3)-C_6H_3
2o, R=p-NO_2-C_6H_4
2p, R=m-NO_2-C_6H_4

Chart 1. General structure of chalcones **1a–g** and thiophene derivatives **2a–p**.

reduced the chalcone activity.[6] In addition, heterocyclic derivatives of chalcones with the enone system replaced with a pyrazole[7] or pyrazoline[8] ring have been systematically investigated, and these analogues had a reduced activity when compared with the corresponding chalcone derivatives. These results demonstrated that the relative positions of the two aryl rings in the molecule play an essential role in the activity of chalcones and that the double bond seems to be a crucial moiety for biological activity. However, the clinical development of chalcones is limited by their chemical instability in vivo. The α,β-unsaturated carbonyl system in these compounds can undergo Michael reactions with biological nucleophiles, hence reducing their reliability as selective tubulin inhibitors.[9]

In this paper, we report a series of chalcone-like agents with general structure **2**, characterized by the presence of a 2-(3,4,5-trimethoxybenzoyl)-5-aryl/heteroaryl-thiophene nucleus. The trimethoxyphenyl ring was thought to be indispensable for retaining potency in the series of molecules which occupy the colchicine binding site.[7,8,10]

These new derivatives were developed with the aim to determine whether the replacement of the double bond of enone system of chalcones with a thiophene moiety would enable the molecules to adopt the correct geometry for activity. Furthermore, due to the absence of the α,β-unsaturated ketone moiety, an in vivo Michael reaction should not present an issue.

A series of molecular modelling studies were carried out on this class of compounds. Structure alignment between chalcone **1f** and thiophene analogue **2m** was performed with the Flexible Alignment tool in MOE and supports our hypothesis that the double bond could be replaced by the heterocyclic ring as a spacer between the two aromatic rings (Fig. 1).

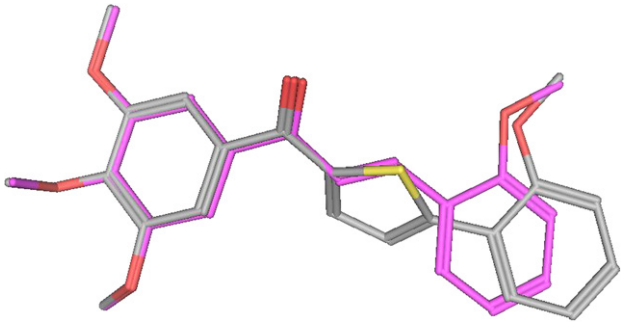


Figure 1. Structure alignment between **1f** (in magenta) and **2m** (in grey).

2. Chemistry

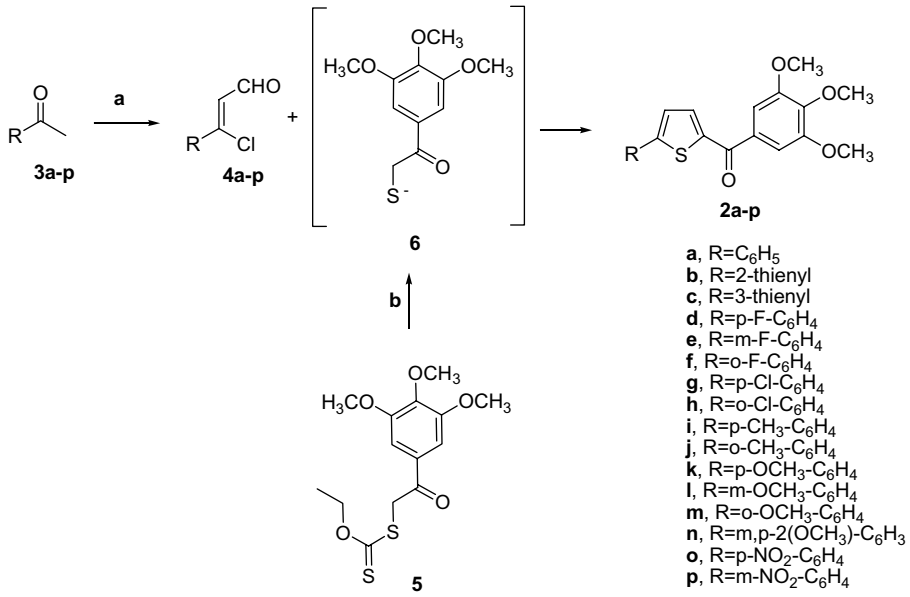
2-(3′,4′,5′-Trimethoxybenzoyl)-5-arylthiophene derivatives with general structure **2** were synthesized by a two-step procedure, as shown in Scheme 1. The first step consisted of the synthesis of β-chlorocinnamaldehydes **4a–p** , which in turn were obtained in good yields (40–80%) by Vilsmeier–Haack chloroformylation applied to commercially available acetophenones **3a–p** , treated with *N*,*N*-dimethylformamide and phosphorus oxychloride.[11] The 'one-pot' cyclization in refluxing ethanol of **4a–p** with the α-mercapto ketone anion **7**, generated in situ by treating *O*-ethyl-*S*-[2-oxo-2-(3,4,5-trimethoxyphenyl)-ethyl]dithiocarbonate **6**[12] with piperidine, produced the 2-(3′,4′,5′-trimethoxybenzoyl)-5-arylthiophene derivatives **2a–p** in moderate to good yields (40–60%).

3. Results and discussion

Table 1 summarizes the antiproliferative effects of thiophene derivatives **2a–p** against a panel of tumour cell lines using chalcones **1a–g**[13] as reference compounds. Several derivatives demonstrated substantial growth inhibitory effects against murine leukaemia (L1210), murine mammary carcinoma (FM3A), human T-lymphoblastoid (Molt/4 and CEM) and human cervix carcinoma (HeLa) cells. In general, the antiproliferative activities of the tested compounds were more pronounced against Molt/4 and HeLa cells. Of the tested compounds, derivatives **2abdfimn** possessed the highest overall potency, with IC_{50} values of 0.2–1.4 μM against the five cell lines. In general, the thiophene derivatives **2adglmn** are more active than their chalcone analogues with general structure **1**. In particular, the methoxy group at the 2′-position of the phenyl ring enhanced antiproliferative activity.

Replacement of the phenyl ring of compound **2a** by the bioisosteric 2-thienyl group (compound **2b**) had minor effects on antiproliferative activity against the five cell lines. The isomeric 3-thienyl derivative **2c** was less active than **2b**, although the difference between the two compounds was minimal in the Molt-4 cell line.

Substitution on the phenyl ring had variable effects. Introduction of a weak electron-withdrawing group (EWG), a *para*-fluorine atom (**2d**), had little overall ef-



Scheme 1. Reagents: (a) $POCl_3$, DMF, 65 °C, 5 h; (b) piperidine, EtOH, reflux, 2 h.

Table 1. In vitro inhibitory effects of compounds **1a–g** and **2a–p** against the proliferation of murine leukaemia (L1210), murine mammary carcinoma (FM3A), human T-lymphocyte (Molt/4 and CEM) and human cervix carcinoma (HeLa) cells

Compound	IC_{50} (μM)[a]				
	L1210	FM3A/0	Molt4/C8	CEM/0	HeLa
2a	0.56 ± 0.05	0.46 ± 0.18	0.40 ± 0.02	0.48 ± 0.06	0.31 ± 0.07
2b	0.53 ± 0.11	0.46 ± 0.01	0.30 ± 0.07	0.43 ± 0.08	0.47 ± 0.02
2c	1.3 ± 0.81	1.8 ± 0.30	0.37 ± 0.09	1.3 ± 1.02	1.1 ± 0.31
2d	0.59 ± 0.06	1.1 ± 0.03	0.40 ± 0.09	0.56 ± 0.27	0.30 ± 0.01
2e	2.4 ± 0.21	5.6 ± 4.0	1.2 ± 0.11	2.1 ± 0.20	1.7 ± 0.01
2f	0.52 ± 0.03	0.87 ± 0.46	0.33 ± 0.04	0.48 ± 0.05	1.4 ± 0.01
2g	2.2 ± 0.41	4.6 ± 0.21	1.3 ± 0.06	2.3 ± 0.51	1.5 ± 0.21
2h	0.75 ± 0.12	1.7 ± 0.31	1.0 ± 0.90	0.49 ± 0.07	1.6 ± 0.10
2i	0.49 ± 0.01	0.47 ± 0.04	0.38 ± 0.04	0.36 ± 0.01	0.41 ± 0.03
2j	2.2 ± 0.30	2.1 ± 0.01	0.60 ± 0.05	1.2 ± 0.51	1.6 ± 0.02
2k	2.3 ± 0.20	2.4 ± 0.40	0.46 ± 0.36	2.2 ± 0.51	1.0 ± 0.60
2l	2.5 ± 0.10	2.4 ± 0.42	1.8 ± 0.51	2.0 ± 0.01	1.4 ± 0.11
2m	0.51 ± 0.03	0.44 ± 0.04	0.18 ± 0.09	0.37 ± 0.03	0.16 ± 0.09
2n	0.52 ± 0.01	0.96 ± 0.10	0.38 ± 0.01	0.54 ± 0.10	0.50 ± 0.01
2o	>10	>10	>10	>10	>10
2p	2.1 ± 0.12	2.4 ± 0.11	1.7 ± 0.11	1.8 ± 0.21	1.4 ± 0.12
1a	>10	>10	>10	>10	>10
1b	>10	>10	>10	>10	>10
1c	>10	>10	>10	>10	>10
1d	2.8 ± 0.42	8.4 ± 1.0	1.4 ± 0.31	1.5 ± 0.11	1.3 ± 0.40
1e	4.3 ± 1.9	11 ± 1.0	3.1 ± 0.11	2.2 ± 0.42	3.7 ± 0.72
1f	2.1 ± 0.01	7.9 ± 0.01	1.9 ± 0.32	1.8 ± 0.21	1.4 ± 0.22
1g	8.1 ± 2.3	10 ± 2.2	3.1 ± 1.8	1.9 ± 0.21	2 ± 0.22

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

fect on antiproliferative activity against L1210, Molt4, CEM and HeLa cells, but increasing the size of the halogen from fluorine to chlorine (**2g**) caused loss of antiproliferative activity. Moving the fluorine from the *para* (**2d**) to the *ortho* position (**2f**) slightly enhanced antiproliferative activity in four of the five cell lines, the exception being the HeLa cells. In contrast, the *meta*-fluoro derivative **2e** was substantially less potent than **2d** and **2f**. The replacement of the *para*-fluoro group with a nitro moiety, to give **2o**, eliminated antiproliferative activity (IC_{50}'s > 10 μM). Shifting the nitro group to the *meta* position (compound **2p**) enhanced activity with respect to **2o**. Compound **2p** had antiproliferative activities comparable to those of the *meta*-fluoro derivative **2e** against L1210, Molt-4, CEM and HeLa cell lines. Thus, selected, single EWG's can be placed at all three positions in the phenyl ring with only minor effects on antiproliferative activity,

but no modification improved activity relative to the unsubstituted **2a**.

Turning to the effects of an electron-releasing group (ERG) on the phenyl moiety, we found that a *p*-methyl (**2i**) group caused only minor changes in antiproliferative activity relative to the unsubstituted **2a**. Reduced activity occurred when the methyl substituent was moved from the *para* to *ortho* position (compound **2j**).

The antiproliferative activity of the thiophene derivatives can be further characterized in terms of the substitution pattern and the number of methoxy groups on the phenyl ring (compounds **2k–n**). With the exception of the Molt-4 cells, *p*-methoxy derivative (**2k**) was 3–5-fold less active than **2a**. Moreover, changing the methoxy group from the *para* to the *meta* position (**2l**) maintained antiproliferative activity, while potency was considerably increased with the *ortho* -methoxy derivative (**2m**), which was about 3–10-fold more active than **2k** and **2l**. With L1210 cells, potency was little changed comparing **2m** with the *m*,*p*-dimethoxy derivative (**2n**), but about a 2-fold loss of activity occurred with the other four cell lines. Moreover, **2n** had a greater potency than the mono-substituted *para* (**2k**) and *meta* (**2l**) methoxy derivatives.

By comparing the effects of ERG's and EWG's on the phenyl at the C5-thiophene position, no clear influence on antiproliferative activity was observed. In fact, several compounds characterized by the presence of substituents with opposite electronic effects showed the same potency. For example, compound **2d** containing the electron-withdrawing fluoro group showed the same antiproliferative potency as compound **2i** containing the electron-donating methyl group.

The more active compounds (**2abdfijmn**) 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, the compounds and tubulin were examined at a concentration of 1 μM with the colchicine at 5 μM) (Table 2).[14,15] For comparison, the antitubulin agent CA-4 was examined in contemporaneous experiments as a reference compound.[16] With the exception of compounds **2f** and **2n**, all new compounds were comparable or superior to CA-4 as inhibitors of tubulin assembly, with the order of activity being **2b = 2i>2d>2m**>CA-4> **2a>2j>2f>2n**. Compounds **2b** and **2i** were the most active (IC_{50}, 0.8 μM), having twice the potency of CA-4 (IC_{50}, 1.4 μM), while the others had IC_{50}'s ranging from 1 to 3 μM.

In the colchicine binding studies, compounds **2abdfim** potently inhibited the binding of [^{3}H]colchicine to tubulin, since 66–82% inhibition occurred with these agents at 1 μM and the colchicine at 5 μM. None, however, was quite as potent as CA-4, which in these experiments inhibited colchicine binding by 87%. While this group of compounds were all potent in the biological assays (inhibition of cell growth, tubulin assembly and colchicine binding), correlations between the three assay types were imperfect. Thus, while compounds **2b** and **2i** were the best inhibitors of tubulin assembly, their effects on colchicine binding were matched by compounds **2a** and **2m**, which were about half as active as assembly inhibitors.

The effects on the cell cycle of a selected group of compounds (**2abdfijmn**) were examined by flow cytometry after staining of the cells with propidium iodide. K562 (human leukaemia) cells were exposed for 24 h in the presence of each compound used at a cytostatic concentration (1.3 μM **2a**, 1.7 μM **2 b**, 2.2 μM **2d**, 1.0 μM **2f**, 1.4 μM **2i**, 4.0 μM **2j**, 1.5 μM **2m** and 2.1 μM **2n**). Table 3 lists the percentage of cells arrested in the G2-M phase. All compounds caused an increase in the G2-M peak. As shown in Figure 2, compounds **2a** and **2d** induced a modest increase of cells in the S and G2-M phases of the cell cycle relative to the control. Compounds **2bfijmn** were the most active in inducing a G2-M block with a marked decrease of cells in S and G0–G1. These data suggest that this class of molecules act selectively on the G2-M phase of the cell cycle. Moreover, derivatives **2j** and **2m** induced apoptosis to the greatest extent, as shown by the appearance of a relatively large sub-Go-Gi peak.

Molecular docking studies of **2m** in the colchicine binding site of tubulin were performed,[12] and the results showed good overlap of the trimethoxyphenyl moiety of the thiophene analogue with the corresponding group

Table 2. Inhibition of tubulin polymerization and colchicine binding by compounds **2abdfijmn** and CA-4

Compound	Tubulin assembly[a] IC_{50} ± SD (μM)	Colchicine binding[b] % ±SD
2a	1.5 ± 0.1	74 ± 3
2b	0.8 ± 0.1	82 ± 2
2d	1.1 ± 0.1	66 ± 2
2f	2.0 ± 0.1	66 ± 0.7
2i	0.8 ± 0.1	74 ± 0.5
2j	1.7 ± 0.3	49 ± 5
2m	1.3 ± 0.1	74 ± 0.5
2n	3.0 ± 0.2	28 ± 4
CA-4	1.4 ± 0.1	87 ± 3

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

Table 3. Percentage of K562 cells in G2-M phase of cell cycle after 24 h treatment with compounds **2abdfijmn** used at a cytostatic concentration

Compound	Cytostatic concentration (μM)	Percentage cells in G2-M phase (%)
Control	0.0	13
2a	1.3	18
2b	1.7	18
2d	2.2	75
2f	1.0	21
2i	1.4	81
2j	4.0	85
2m	1.5	62
2n	2.1	57

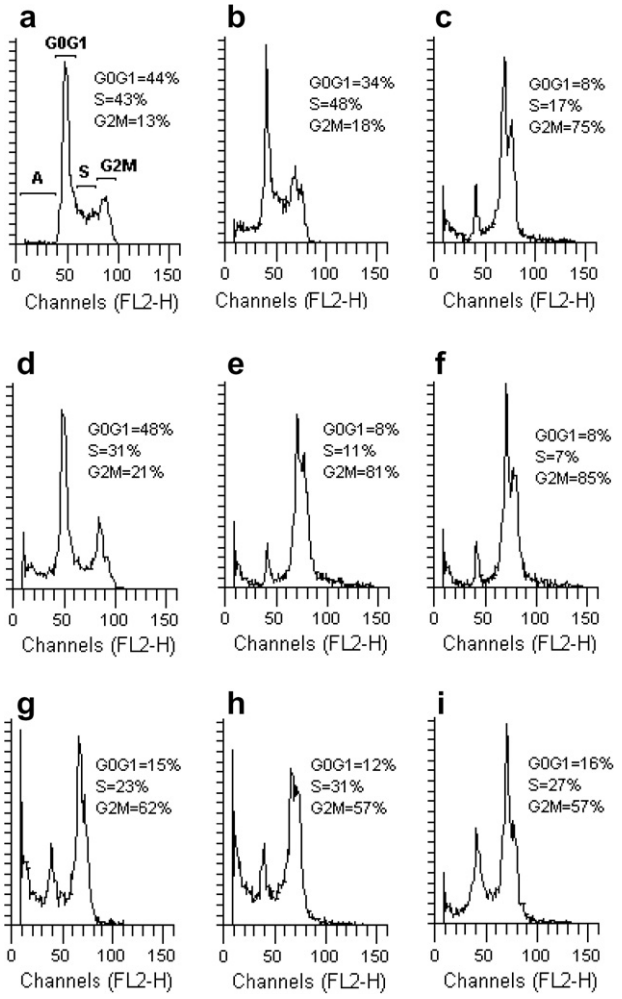


Figure 2. Effects of compounds **2a** (panel b), **2b** (**c**), **2d** (**d**), **2f** (**e**), **2i** (**f**), **2j** (**g**), **2m** (**h**) and **2n** (**i**), on DNA content/cell following treatment of K562 cells for 24 h. The cells were cultured without compound (control, panel a) or with each compound used at a cytostatic concentration. Cell cycle distribution was analyzed by the standard propidium iodide procedure. Control cells at the sub-G0–G1 (labelled A), G0–G1, S, and G2-M phases of the cell cycle are indicated in panel (a).

of the DAMA–colchicine co-crystallized with the tubulin. A hydrogen bond was also formed between the *ortho*-methoxy group of **2m** and βAsn101 (residue numbering as in the crystal structure used), suggesting a possible structural explanation for the reduced activity of **2k** and **2l** where the methoxy group was present in the *para*- and *meta*-positions, respectively, on the aromatic ring (Fig. 3).

4. Conclusions

In conclusion, we have discovered a new class of simple synthetic inhibitors of tubulin polymerization, based on a 2-(3′,4′,5′-trimethoxybenzoyl)-5-aryl/heteroaryl-thiophene molecular skeleton. SAR studies revealed that the replacement of the double bond of the enone system with a thiophene nucleus between the aryl and 3′,4′,5′-trimethoxybenzoyl moieties of chalcones with general structure **1**, enhanced activity of this class of compounds as antiproliferative agents. All the synthesized compounds inhibited the growth of cancer cell lines at nanomolar to low micromolar concentrations. Compound **2m**, with a *ortho*-methoxy phenyl group at the 5-position of thiophene ring, exhibited the greatest antiproliferative activity among the compounds tested in this study. The best results for the inhibition of antiproliferative activity were obtained with the *p*-F-phenyl (**2d**), the *o*-*F*-phenyl (**2f**), the *p*-CH_3-phenyl (**2i**), the *o*-OCH_3-phenyl (**2m**) and the *p*, *m*-$(OCH_3)_2$-phenyl (**2n**) derivatives at the 5-position of the thiophene ring. There was also no clear difference in effect on activity between an EWG versus an ERG, once substituent size was taken into account. We identified tubulin as the molecular target of the molecules, since the compounds with the greatest inhibitory effects on cell growth strongly inhibited tubulin assembly and binding of colchicine to tubulin. These activities differed little from those of CA-4, and all the most active compounds had quantitatively similar effects in the tubulin assays, varying within a narrow range (IC_{50}'s for assembly, 0.8–2 μM with 10 μM tubulin; 49–82% inhibition of the binding of 5 μM colchicine, with the inhibitor and tubulin both at 1 μM). We also showed by flow cytometry that a set of the active compounds had cellular effects typical of agents that bind to tubulin, causing the accumulation of cells in the G2/M phase of the cell cycle. Molecular modelling studies were also performed, and the proposed binding mode for compound **2m** in the colchicine site of tubulin was consistent with the experimental data.

5. Experimental

5.1. Chemistry

5.1.1. Materials and methods. All acetophenones are commercially available and were used as received. ^1H and ^{13}C NMR spectra were recorded on a Bruker AC 200 and Varian 400 Mercury Plus spectrometer, respectively. Chemical shifts (δ) are given in ppm upfield from tetramethylsilane as internal standard, and the spectra were recorded in appropriate deuterated solvents, as indicated. Positive-ion electrospray ionization (ESI) mass spectra were recorded on a double-focusing Finnigan MAT 95 instrumental with BE geometry. Melting points (mp) were determined on a Buchi–Tottoli apparatus and are uncorrected. All products reported showed ^1H 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.

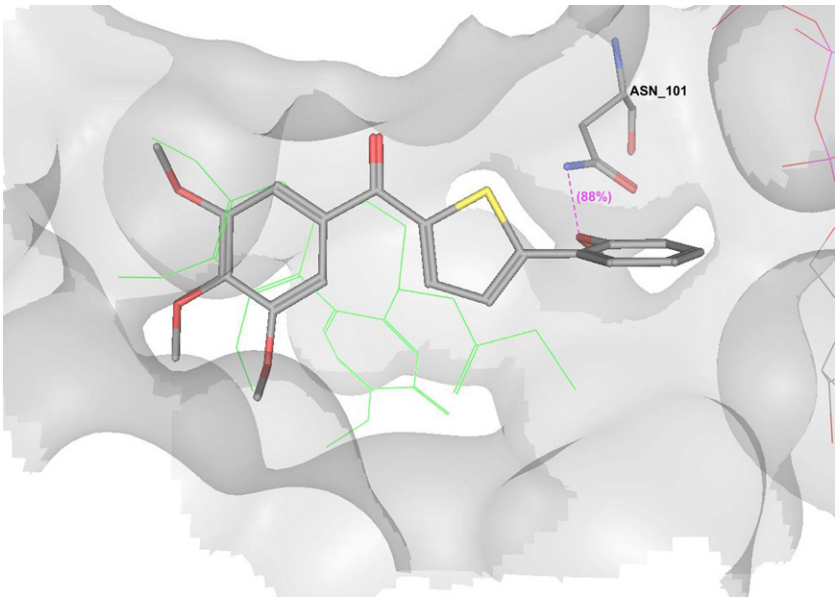


Figure 3. Proposed binding mode of **2m**. DAMA–colchicine is represented in green.

5.1.2. General procedure A for the synthesis of compounds 4a–p. $POCl_3$ (3.1 g, 20 mmol) was added dropwise over 20 min, maintaining the temperature below 25 °C, to an ice-cooled stirred solution of dry DMF (10 mL). After 10 min, the appropriate acetophenone derivative (5 mmol) dissolved in DMF (5 mL) was added dropwise to the DMF-$POCl_3$ complex. The reaction mixture was stirred for 1 h at 5 °C, heated at 70 °C for 5 h, cooled at room temperature and finally poured in a ice-cold saturated NaOAc water solution (20 mL). The resulting mixture was extracted with ethyl acetate (3 × 15 mL). The organic phase was washed with water (3 × 10 mL), brine (10 mL), dried over Na_2SO_4 and concentrated in vacuo. The resulting residue was purified by silica gel column chromatography to yield β-chlorocinnamaldehydes **4a–p**.

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5.1.3. (*Z*)-3-Chloro-3-(thiophen-2-yl)acrylaldehyde (4b). Following general procedure **A**, compound **4b** was purified by chromatography eluting with petroleum ether–ethyl acetate (9:1). Yellow solid, yield: 42%, mp 38–40 °C. ^{1}H NMR ($CDCl_3$) δ: 6.59 (d, J = 6.8 Hz, 1H), 7.13 (dd, J = 4.8 and 3.6 Hz, 1H), 7.55 (dd, J = 4.8 and 1.2 Hz, 1H), 7.67 (dd, J = 4.8 and 1.2 Hz, 1H), 10.1 (d, J = 6.8 Hz, 1H).

5.1.4. (*Z*)-3-Chloro-3-(thiophen-3-yl)acrylaldehyde (4c). Following general procedure **A**, compound **4c** was purified by chromatography eluting with petroleum ether–ethyl acetate (9:1). Yellow solid, yield: 68%, mp 77–79 °C. ^{1}H NMR ($CDCl_3$) δ: 6.60 (d, J = 6.8 Hz, 1H), 7.34 (dd, J = 5.0 and 1.4 Hz, 1H), 7.55 (dd, J = 5.2 and 3.0 Hz, 1H), 7.94 (dd, J = 3.0 and 1.6 Hz, 1H), 10.1 (d, J = 6.8 Hz, 1H).

5.1.5. (*Z*)-3-Chloro-3-(4-fluorophenyl)acrylaldehyde (4d). Following general procedure **A**, compound **4d** was purified by chromatography eluting with petroleum ether–ethyl acetate (8:2). Brown oil, yield: 52%. ^{1}H NMR ($CDCl_3$) δ: 6.60 (d, J = 7.0 Hz, 1H), 7.15 (t, J = 8.2 Hz, 2H), 7.76 (dd, J = 8.6 and 5.2 Hz, 2H), 10.1 (d, J = 7.0 Hz, 1H).

5.1.6. (*Z*)-3-Chloro-3-(3-fluorophenyl)acrylaldehyde (4e). Following general procedure **A**, compound **4e** was purified by chromatography eluting with petroleum ether–ethyl acetate (9:1). Yellow solid, yield: 60%, mp 40–42

°C. ^{1}H NMR ($CDCl_3$) δ: 6.65 (d, J = 6.6 Hz, 1H), 7.18 (m, 1H), 7.43 (m, 3H), 10.1 (d, J = 6.6 Hz, 1H).

5.1.7. (*Z*)-3-Chloro-3-(2-fluorophenyl)acrylaldehyde (4f). Following general procedure **A**, compound **4f** was purified by chromatography eluting with petroleum ether–ethyl acetate (8:2). Brown oil, yield: 51%. ^{1}H NMR ($CDCl_3$) δ: 6.68 (d, J = 6.8 Hz, 1H), 7.17 (m, 2H), 7.42 (m, 1H), 7.73 (t, J = 7.8 Hz, 1H), 10.3 (d, J = 6.8 Hz, 1H).

5.1.8. (*Z*)-3-Chloro-3-(4-chlorophenyl)acrylaldehyde (4g). Following general procedure **A**, compound **4g** was purified by chromatography eluting with petroleum ether–ethyl acetate (9:1). Yellow solid, yield: 58%, mp 90–92 °C. ^{1}H NMR ($CDCl_3$) δ: 6.65 (d, J = 6.8 Hz, 1H), 7.43 (dd, J = 6.8 and 1.8 Hz, 1H), 7.72 (d, J = 6.8 and 1.8, 2H), 10.2 (d, J = 6.8 Hz, 1H).

5.1.9. (*Z*)-3-Chloro-3-(3-chlorophenyl)acrylaldehyde (4h). Following general procedure **A**, compound **4h** was purified by chromatography eluting with petroleum ether–ethyl acetate (9:1). Yellow oil, yield: 68%. ^{1}H NMR ($CDCl_3$) δ: 6.38 (d, J = 7.0 Hz, 1H), 7.44 (m, 4H), 10.2 (d, J = 7.0 Hz, 1H).

5.1.10. (*Z*)-3-Chloro-3-(4-methoxyphenyl)acrylaldehyde (4k). Following general procedure **A**, compound **4k** was purified by chromatography eluting with petroleum ether–ethyl acetate (9:1). Yellow solid, yield: 54%, mp 58–60 °C. ^{1}H NMR ($CDCl_3$) δ: 3.87 (s, 3H), 6.61 (d, J = 6.8 Hz, 1H), 6.94 (dd, J = 6.8 and 2.0 Hz, 1H), 7.72 (d, J = 6.8 and 2.0, 2H), 10.2 (d, J = 6.8 Hz, 1H).

5.1.11. (*Z*)-3-Chloro-3-(3-nitrophenyl)acrylaldehyde (4p). Following general procedure **A**, compound **4p** was purified by chromatography eluting with petroleum ether–ethyl acetate (2:8). Yellow solid, yield: 80%, mp 81–83 °C. ^{1}H NMR ($CDCl_3$) δ: 6.76 (d, J = 6.8 Hz, 1H), 7.73 (t, J = 8.0 Hz, 1H), 8.07 (d, J = 8.0 Hz, 1H), 8.38 (dd, J = 8.0 and 1.4 Hz, 1H), 8.64 (d, J = .4 Hz, 1H), 10.2 (d, J = 6.8 Hz, 1H).

5.2. General procedure B for the synthesis of 5-aryl/heteroaryl-thiophen-2-yl-(3,4,5-trimethoxyphenyl)-methanones 2a–p

Piperidine (0.2 mL, 2 mmol) was added to a stirred solution of dithiocarbonic acid *O*-ethyl ester *S*-[2-oxo-2-(3,4,5-trimethoxyphenyl)-ethyl] ester **5** (330 mg, 1 mmol) dissolved in ethanol (20 mL). The reaction mixture was stirred for 30 min at room temperature. The corresponding β-chlorocinnamaldehyde (1 mmol) was added, and the solution was stirred at reflux for 3 h, after which ethanol was removed under reduced pressure and the residue dissolved in dichloromethane (15 mL). The organic phase was washed with a 5% v/v solution of HCl (2 × 5 mL), a saturated solution of sodium carbonate (2 × 5 mL), brine (5 mL) and dried over sodium sulfate. After concentration under reduced pressure, the residue was purified by silica gel column chromatography (petroleum ether–EtOAc, 7:3) to furnish the corresponding final compounds **2a**–**p**.

5.2.1. (3,4,5-Trimethoxyphenyl)-(5-phenylthiophen-2-yl)-methanone (2a). Following procedure B, compound **2a** was isolated as a yellow oil. Yield: 57%. ^{1}H NMR ($CDCl_3$) δ: 3.93 (s, 3H), 3.95 (s, 6H), 7.16 (s, 2H), 7.37 (d, J = 4.0 Hz, 1H), 7.43 (m, 3H), 7.67 (d, J = 4.0 Hz, 1H), 7.69 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 56.4 (2×), 61.1, 106.8 (2×), 123.9 (2×), 126.4 (2×), 129.2 (2×), 133.4 (2×), 135.6 (2×), 141.9 (2×), 153.1 (2×), 187.0. MS-ESI (*m*/*z*): 355.3 $[M+1]^+$. Anal. Calcd for $C_{20}H_{18}O_4S$: C, 67.78; H, 5.12. Found: C, 67.56; H, 5.01.

5.2.2. (3,4,5-Trimethoxyphenyl)(5-(thiophen-2-yl)thiophen-2-yl)methanone (2b). Following general procedure B, compound **2b** was isolated as a yellow solid, yield: 38%; mp 94–96 °C. ^{1}H NMR ($CDCl_3$) δ: 3.92 (s, 3H), 3.94 (s, 6H), 7.02 (m, 1H), 7.13 (s, 2H), 7.23 (d, J = 4.0 Hz, 1H), 7.35 (m, 2H), 7.60 (d, J = 4.0 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 56.4 (2×), 61.1, 106.7 (2×), 124.2 (2×), 125.9, 126.7 (2×), 128.4 (2×), 133.2, 135.5, 136.3, 146.2, 153.1, 186.8. MS-ESI (*m*/*z*): 361.4 $[M+1]^+$. Anal. Calcd for $C_{18}H_{16}O_4S_2$: C, 59.98; H, 4.47. Found: C, 59.78; H, 4.35.

5.2.3. (3,4,5-Trimethoxyphenyl)(5-(thiophen-3-yl)thiophen-2-yl)methanone (2c). Following general procedure B, compound **2c** was isolated as a pink solid. Yield: 54%; mp 122–124 °C. ^{1}H NMR ($CDCl_3$) δ: 3.92 (s, 3H), 3.94 (s, 6H), 7.12 (s, 2H), 7.24 (d, J = 3.8 Hz, 1H), 7.38 (m, 2H), 7.59 (m, 1H), 7.62 (d, J = 3.8 Hz, 1H).^{13}C NMR (100 MHz, $CDCl_3$) δ: 56.4 (2×), 61.1, 106.7 (2×), 122.3, 123.9 (2×), 126.0, 127.2, 133.3, 134.8, 135.6 (2×), 141.3, 147.8, 153.1, 187.0. MS-ESI (*m*/*z*): 361.3 $[M+1]^+$. Anal. Calcd for $C_{18}H_{16}O_4S_2$: C, 59.98; H, 4.47. Found: C, 59.68; H, 4.32.

5.2.4. (5-(4-Fluorophenyl)thiophen-2-yl)(3,4,5-trimethoxyphenyl)methanone (2d). Following general procedure B, compound **2d** was isolated as a white solid. Yield: 48%, mp 127–128 °C. ^{1}H NMR ($CDCl_3$) δ: 3.93 (s, 6H), 3.95 (s, 3H), 7.15 (s, 2H), 7.18 (t, J = 7.8 Hz, 2H), 7.29 (d, J = 3.8 Hz, 1H), 7.63 (d, J = 3.8 Hz, 1H), 7.68 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 56.4 (2×), 61.1, 106.8 (2×), 116.2, 116.4, 123.9, 128.2, 128.3, 129.7, 133.2, 135.6 (2×), 141.9, 142.2, 151.9, 153.1 (2×), 187.0. MS-ESI (*m*/*z*): 373.5 $[M+1]^+$. Anal. Calcd for $C_{20}H_{17}FO_4S$: C, 64.50; H, 4.60; F, 5.10. Found: C, 64.33; H, 4.51; F, 4.89.

5.2.5. (5-(3-Fluorophenyl)thiophen-2-yl)(3,4,5-trimethoxyphenyl)methanone (2e). Following general procedure B, compound **2e** was isolated as a white solid. Yield: 46%, mp 129–131 °C. ^{1}H NMR ($CDCl_3$) δ: 3.93 (s, 6H), 3.95 (s, 3H), 7.16 (s, 2H), 7.36 (d, J = 4.0 Hz, 1H), 7.46 (m, 4H), 7.68 (d, J = 4.0 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 56.4 (2×), 61.0, 106.8 (2×), 113.1, 113.4, 115.9, 116.1, 122.2, 124.5, 130.8, 130.9, 133.1, 135.4, 141.9, 142.8, 151.3, 153.1, 186.9. MS-ESI (*m*/*z*): 373.4 $[M+1]^+$. Anal. Calcd for $C_{20}H_{17}FO_4S$: C, 64.50; H, 4.60; F, 5.10. Found: C, 64.41; H, 4.48; F, 4.93.

5.2.6. (5-(2-Fluorophenyl)thiophen-2-yl)(3,4,5-trimethoxyphenyl)methanone (2f). Following general procedure B,

compound **2e** was isolated as a white solid. Yield: 48%, mp 123–125 °C. ^{1}H NMR ($CDCl_3$) δ: 3.93 (s, 3H), 3.94 (s, 6H), 7.10 (m, 1H), 7.18 (s, 2H), 7.32 (m, 1H), 7.42 (d, J = 3.8 Hz, 1H), 7.56 (m, 1H), 7.64 (m, 1H), 7.68 (d, J = 3.8 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 56.4 (2×), 61.1, 106.8 (2×), 116.6, 116.8, 124.8, 127.1, 127.2, 129.1, 130.4, 130.5, 133.2, 135.0, 141.9, 142.6, 145.8, 153.1, 187.1. MS-ESI (*m*/*z*): 373.5 $[M+1]^+$. Anal. Calcd for $C_{20}H_{17}FO_4S$: C, 64.50; H, 4.60; F, 5.10. Found: C, 64.41; H, 4.48; F, 4.90.

5.2.7. (5-(4-Chlorophenyl)thiophen-2-yl)(3,4,5-trimethoxyphenyl)methanone (2g). Following general procedure B, compound **2g** was isolated as a white solid. Yield: 62%, mp 137–139 °C. ^{1}H NMR ($CDCl_3$) δ: 3.93 (s, 3H), 3.96 (s, 6H), 7.15 (s, 2H), 7.34 (d, 4.0 Hz, 1H), 7.43 (d, J = 8.6 Hz, 2H), 7.61 (d, J = 8.6 Hz, 2H), 7.66 (d, 4.0 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ : 56.4 (2×), 61.1, 106.8 (2×), 124.2, 127.6 (2×), 129.5 (2×), 131.9, 133.1, 135.1, 135.5, 141.9, 142.5, 151.6, 153.1 (2×), 186.9. MS-ESI (*m*/*z*): 389.9 $[M+1]^+$. Anal. Calcd for $C_{20}H_{17}ClO_4S$: C, 61.77; H, 4.41; Cl, 9.12. Found: C, 61.63; H, 4.33; Cl, 8.96.

5.2.8. (5-(2-Chlorophenyl)thiophen-2-yl)(3,4,5-trimethoxyphenyl)methanone (2h). Following general procedure B, compound **2h** was isolated as a yellow solid, yield: 37%; mp 210–212 °C. ^{1}H NMR ($CDCl_3$) δ: 3.94 (s, 3H), 3.95 (s, 6H), 7.12 (m, 1H), 7.18 (s, 2H), 7.31 (m, 1H), 7.42 (d, J = 3.8 Hz, 1H), 7.56 (m, 1H), 7.64 (d, J = 9.0 Hz, 1H), 7.69 (d, J = 3.8 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 56.2, 56.4, 61.1, 106.9 (2×), 126.5, 127.3, 128.6, 129.3, 129.7, 130.0, 130.3, 130.9, 131.4, 132.0, 133.2, 134.4, 152.5, 153.1, 187.1. MS-ESI (*m*/*z*): 389.3 $[M+1]^+$. Anal. Calcd for $C_{20}H_{17}ClO_4S$: C, 61.77; H, 4.41; Cl, 9.12. Found: C, 61.71; H, 4.193; Cl, 9.01.

5.2.9. (3,4,5-Trimethoxyphenyl)(5-*p*-tolylthiophen-2-yl)methanone (2i). Following general procedure B, compound **2i** was isolated as a white solid. Yield: 47%; mp 135–137 °C. ^{1}H NMR ($CDCl_3$) δ: 2.40 (s, 3H), 3.93 (s, 3H), 3.94 (s, 6H), 7.15 (s, 2H), 7.22 (d, J = 8.4 Hz, 2H), 7.32 (d, J = 4.0 Hz, 1H), 7.57 (d, J = 8.4 Hz, 2H), 7.65 (d, J = 4.0 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 21.4, 56.4 (2×), 61.1, 106.8 (2×), 123.4 (2×), 126.3 (2×), 129.9 (2×), 130.6, 133.4, 135.7 (2×), 139.5, 141.6, 153.1, 153.5, 187.0. MS-ESI (*m*/*z*): 369.4 $[M+1]^+$. Anal. Calcd for $C_{21}H_{20}O_4S$: C, 68.46; H, 5.47. Found: C, 68.34; H, 5.36.

5.2.10. (3,4,5-Trimethoxyphenyl)(5-*o*-tolylthiophen-2-yl)methanone (2j). Following general procedure B, compound **2j** was isolated as a white solid. Yield: 38%; mp 99–101 °C. ^{1}H NMR ($CDCl_3$) δ: 2.48 (s, 3H), 3.94 (s, 3H), 3.95 (s, 6H), 7.09 (m, 1H), 7.13 (d, J = 4.0 Hz, 1H), 7.18 (s, 2H), 7.32 (m, 2H), 7.44 (m, 1H), 7.70 (d, J = 4.0 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 21.3, 56.4 (2×), 61.1, 106.8 (2×), 125.6, 126.3, 127.5, 128.0, 129.0, 130.1, 130.2, 130.3, 131.2, 133.2, 134.8, 141.8, 152.5, 153.1, 187.1. MS-ESI (*m*/*z*): 369.3 $[M+1]^+$. Anal. Calcd for $C_{21}H_{20}O_4S$: C, 68.46; H, 5.47. Found: C, 68.28; H, 5.31.

5.2.11. (3,4,5-Trimethoxyphenyl)(5-(4-methoxyphenyl)-thiophen-2-yl)methanone (2k). Following general procedure B, compound **2k** was isolated as a white solid. Yield: 34%; mp 93–96 °C. ^{1}H NMR ($CDCl_3$) δ: 3.86 (s, 3H), 3.93 (s, 6H), 3.94 (s, 3H), 6.91 (d, J = 8.8 Hz, 2H), 7.14 (s, 2H), 7.22 (d, J = 4.0 Hz, 1H), 7.62 (d, J = 8.8 Hz, 2H), 7.66 (d, J = 4.0 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ:55.5, 56.4 (2×), 61.0, 105.8, 106.7 (2×), 114.6 (2×), 122.8, 126.1, 127.8 (2×), 132.5, 133.4, 135.9, 141.1, 153.1, 153.4, 160.6, 187.1. MS-ESI (*m*/*z*): 385.3 $[M+1]^+$. Anal. Calcd for $C_{21}H_{20}O_5S$: C, 65.61; H, 5.24. Found: C, 65.44; H, 5.03.

5.2.12. (3,4,5-Trimethoxyphenyl)(5-(3-methoxyphenyl)thiophen-2-yl)methanone (2l). Following general procedure B, compound **2l** was isolated as a white solid, yield: 53%; mp 138–140 °C. ^{1}H NMR ($CDCl_3$) δ: 3.87 (s, 3H), 3.92 (s, 3H), 3.94 (s, 6H), 6.92 (s, 1H), 7.16 (s, 2H), 7.21 (s, 1H), 7.30 (m, 2H), 7.37 (d, J = 3.8 Hz, 1H), 7.65 (d, J = 3.8 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 55.5, 56.4 (2×), 61.1, 106.8 (2×), 112.0 (2×), 114.7 (2×), 118.9, 124.1, 130.3, 133.2, 134.6, 135.5, 142.1, 153.1 (2×), 160.1, 187.0. MS-ESI (*m*/*z*): 385.4 $[M+1]^+$. Anal. Calcd for $C_{21}H_{20}O_5S$: C, 65.61; H, 5.24. Found: C, 65.50; H, 5.11.

5.2.13. (3,4,5-Trimethoxyphenyl)(5-(2-methoxyphenyl)thiophen-2-yl)methanone (2m). Following general procedure B, compound **2m** was isolated as a brown solid, yield: 69%; mp 135–137 °C. ^{1}H NMR ($CDCl_3$) δ: 3.93 (s, 3H), 3.94 (s, 3H), 3.98 (s, 6H), 7.05 (t, J = 7.6 Hz, 1H), 7.18 (s, 2H), 7.22 (d, J = 8.4 Hz, 1H), 7.37 (t, J = 7.6 Hz, 1H), 7.54 (d, J = 4.2 Hz, 1H), 7.66 (d, J = 4.2 Hz, 1H), 7.74 (d, J = 8.4 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 55.7, 56.4 (2×), 61.1, 106.8 (2×), 111.8, 121.1 (2×), 125.8, 128.7, 130.2, 130.5, 133.7, 134.5, 141.6, 142.0, 148.5, 153.0, 156.2, 187.6. MS-ESI (*m*/*z*): 385.6 $[M+1]^+$. Anal. Calcd for $C_{21}H_{20}O_5S$: C, 65.61; H, 5.24. Found: C, 65.44; H, 5.12.

5.2.14. (3,4,5-Trimethoxyphenyl)(5-(3,4-dimethoxyphenyl)thiophen-2-yl)methanone (2n). Following general procedure B, compound **2n** was isolated as a yellow solid, yield: 53%; mp 133–135 °C. ^{1}H NMR ($CDCl_3$) δ: 3.92 (s, 6H), 3.94 (s, 3H), 3.96 (s, 6H), 6.92 (d, J = 8.4 Hz, 1H), 7.15 (s, 2H), 7.17 (s, 1H), 7.28 (d, J = 3.8 Hz, 1H), 7.30 (d, J = 8.4 Hz, 1H), 7.64 (d, J = 3.8 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 45.6, 56.1, 56.4 (2×), 61.1, 106.3, 106.8, 109.5, 110.3, 111.6, 119.3, 123.1, 126.4, 130.6, 133.4, 135.8, 141.4, 141.8, 149.4, 153.1, 153.2, 187.0. MS-ESI (*m*/*z*): 415.4 $[M+1]^+$. Anal. Calcd for ($C_{22}H_{22}O_6S$): C, 63.75; H, 5.35. Found: C, 63.61; H, 5.17.

5.2.15. (3,4,5-Trimethoxyphenyl)(5-(4-nitrophenyl)thiophen-2-yl)methanone (2o). Following general procedure B, compound **2o** was isolated as a yellow solid. Yield: 25%, mp 162–165°C. ^{1}H NMR ($CDCl_3$) δ: 3.87 (s, 3H), 3.94 (s, 6H), 7.16 (s, 2H), 7.52 (d, J = 4.0 Hz, 1H), 7.76 (d, J = 8.8 Hz, 2H), 8.28 (d, J = 8.8 Hz, 2H), 8.34 (d, J = 4.0 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 56.5 (2×), 61.1, 106.9 (2×), 124.7 (2×), 126.0, 126.9 (2×), 132.7, 135.3, 139.4, 142.3, 144.6, 147.8, 149.2, 153.2 (2×), 186.8. MS-ESI (*m*/*z*): 400.5 $[M+1]^+$.

Anal. Calcd for $C_{20}H_{17}NO_6S$: C, 60.14; H, 4.29; N, 3.51. Found: C, 60.02; H, 4.10; N, 3.38.

5.2.16. (3,4,5-Trimethoxyphenyl)(5-(3-nitrophenyl)thiophen-2-yl)methanone (2p). Following general procedure B, compound **2p** was isolated as a white solid. Yield: 43%, mp 141–143 °C. ^{1}H NMR ($CDCl_3$) δ: 3.94 (s, 6H), 3.96 (s, 3H), 7.17 (s, 2H), 7.48 (d, $J = 4.0$ Hz, 1H), 7.64 (s, 1H), 7.72 (d, $J = 4.0$ Hz, 1H), 7.98 (dd, $J = 7.8$ and 1.8 Hz, 1H), 8.22 (dd, $J = 7.8$ and 1.8 Hz, 1H), 8.54 (t, $J = 1.8$ Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 56.5 (2×), 61.1, 106.9 (2×), 121.0 (2×), 123.5 (2×), 125.5 (2×), 130.4 (2×), 132.0, 132.8, 135.3, 143.8, 149.4, 153.2, 186.8. MS-ESI (*m*/*z*): 400.5 $[M+1]^+$. Anal. Calcd for $C_{20}H_{17}NO_6S$: C, 60.14; H, 4.29; N, 3.51. Found: C, 60.00; H, 4.12; N, 3.18.

5.3. Growth inhibitory activity

Murine leukaemia L1210, murine mammary carcinoma FM3A, 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 microtiter plates. After incubation at 37 °C for two days, cell number was determined using a Coulter counter.

To determine the growth inhibitory activity of the tested compounds on human myeloid leukaemia K562 cell line, 2×10^5 cells were plated into 25 mm wells (Costar, Cambridge, UK) in 1 mL of complete medium and treated with different concentrations of each drug. After 24 h of incubation, the number of viable cells was determined using a Coulter counter.

The IC_{50} was defined as the compound concentration required to inhibit cell proliferation by 50%.

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

Bovine brain tubulin was purified as described previously.[14] To evaluate the effect of the compounds on tubulin assembly in vitro, varying concentrations were preincubated with 10 μM tubulin in glutamate buffer at 30 °C and then cooled to 0 °C. After the 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} was defined as the compound concentration that inhibited the extent of assembly by 50% after a 20 min incubation. The capacity 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 1 μM test compound.

5.5. Flow cytometric analysis of cell cycle distribution and apoptosis

The effects of the tested compounds of the series on cell cycle distribution were studied on K562 cells (myeloblastic leukaemia) by flow cytometric analysis after staining with propidium iodide. Cells were exposed 24 h to each compound used at a concentration corresponding to the cytostatic concentration. After treatment cells were washed once in ice-cold PBS and resuspended at 1×10^6 mL in a hypotonic fluorochrome solution containing propidium iodide (Sigma) 50 μg/mL in 0.1% sodium citrate plus 0.03% (v/v) nonidet P-40 (Sigma). After 30 min of incubation, the fluorescence of each sample was analyzed as single-parameter frequency histograms by using a FACScan flow cytometer (Becton–Dickinson, San Jose, CA). The distribution of cells in the cell cycle was analyzed with the ModFit LT3 program (Verity Software House, Inc.).

5.6. Molecular modelling

All molecular modelling studies were performed on a MacPro dual 2.66 GHz Xeon running Ubuntu 7, using Molecular Operating Environment (MOE) 2006.08.[17] The tubulin structure was downloaded from the PDB data bank (http://www.rcsb.org/-PDBcode:1SA0).[18] Hydrogen atoms were added to the protein with MOE and minimized keeping all the heavy atoms fixed until a RMSD gradient of 0.05 kcal mol^{-1} Å^{-1} was reached. Ligand structures were built with MOE and minimized using the MMFF94x forcefield until a RMSD gradient of 0.05 kcal mol^{-1} Å^{-1} was reached. The docking simulations were performed using the Alpha Triangle placement method and the London dG scoring method. 300 results for each ligand were generated, and the position of the trimethoxyphenyl moiety was compared with ring A of the colchicine analogue co-crystallized with the protein, discarding the results with a RMSD value >3 Å.[19] The best scored result of the remaining conformations for each ligand was further analyzed. The protein/ligand complexes were minimized using the MMFF94x force field, until a RMSD gradient of 0.1 kcal mol^{-1} Å^{-1} was reached.

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