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Original article

Synthesis, antiproliferative activity, and *in silico* insights of new 3-benzoylamino-benzo[*b*]thiophene derivatives

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ABSTRACT

A new series of 3-benzoylamino-5-imidazol-5-yl-benzo[*b*]thiophenes and the parent amino derivatives were synthesized and screened as antitumor agents. All tested compounds showed concentration-dependent antiproliferative activity profile against HeLa cell line, exhibiting GI50 values in the low micromolar range. The most active compounds were tested in cell cycle perturbation experiments. A rapid accumulation of cells in the G2/M phase, with a concomitant reduction of cells in both the S and G0/G1 phases, was observed, suggesting that cell exposure to selected derivatives produces mitotic failure. To rationalize the biological results, the 3-benzoylamino-benzo[*b*]thiophenes were analyzed through the *in silico* VLAK protocol. Compounds presenting the 3,4,5-trimethoxy-benzoyl moiety were *in silico* classified as potential antimitotic agents or topoisomerase II inhibitors, in good agreement with the biological studies.

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1. Introduction

Heterocycles are heavily involved in life processes. DNA, RNA, proteins, and coenzymes are classical examples of fundamental biological macromolecules structured by heterocyclic moieties. The biomolecular standpoint of drug action confirms the broad biocompatibility and the versatile aptitude of heterocycles to mimic endogenous molecules and consequently to interact with biological entities [1]. These features make heterocyclic chemistry one of the major pivots for the development of new drugs, justifying the intense attention of the scientific community on this field.

Since long time, our research work has been focused on the design, the synthesis, and the biological evaluation of novel heterocyclic ring systems able to halt tumor cell proliferation [2–5]. Several of our tested molecules exhibited outstanding antiproliferative activities against a wide range of human cancer cell lines (Fig. 1).

In this regard, the indolo[3,2-*e*]-1,2,3-triazolo[1,5-*a*]pyrimidine derivatives of type **1**, designed as DNA-interacting drugs, showed a relevant antiproliferative effect on the renal and CNS human tumor cell lines [6]. In an analogous fashion, the isoindolo[2,1-*a*]quinoxalines **2** emerged as high promising antitumor scaffold, with GI50 values in the low micromolar to nanomolar concentrations. After treatment with **2**, the analysis of the cell cycle demonstrated an arrest in G2/M phase, in addition to cell apoptosis, displayed by mitochondrial depolarization, and activation of caspase-3, and caspase-9. Moreover, isoindole-quinoxaline derivatives inhibited tubulin polymerization similarly to colchicine and vinblastine in a concentration-dependent manner [7]. Recently, the *in silico* approach, performed through the Virtual Lock-and-Key (VLAK) protocol [8], allowed us to select new annelated thieno-triazolo and triazine series of type **3**, **4**, and **5** as new antitumor agents. These compounds, featuring by a sulfur atom in their scaffold and functionalized with proper side chains, demonstrated excellent *in vitro* and *in vivo* antiproliferative activity, exhibiting very low toxicity and high potency [9–11]. On the basis of these data, keeping our attention on the promising thieno heterocyclic ring systems, we

Abbreviations: TMB, 3,4,5-trimethoxy-benzoyl; VLAK, Virtual Lock-and-Key; DMSO, dimethyl sulfoxide; MA, mechanism of action; NCI, National Cancer Institute; ACAM, Anti-cancer Agent Mechanism.

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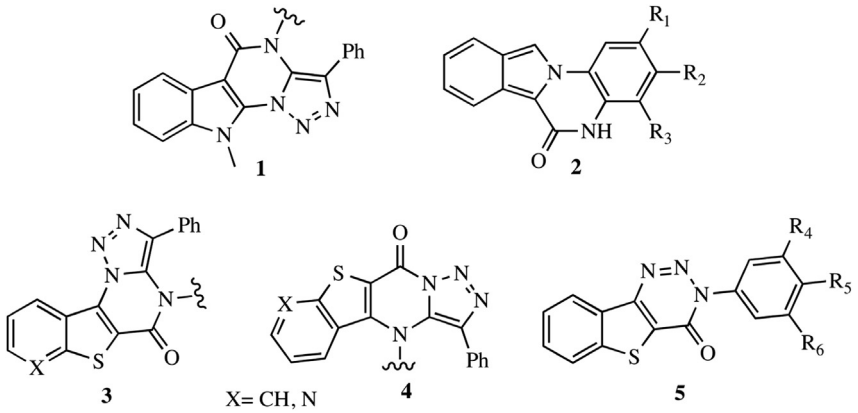


Fig. 1. Heterocyclic ring system series with outstanding antiproliferative activities.

decided to investigate a set of new benzo[*b*]thiophene derivatives of type **6** as anticancer compounds (Fig. 2). Several published works assigned to this scaffold a peculiar biological activity, suggesting the proposed molecules as potential bioisosteres of major microtubules assembly inhibitors. In fact, in the past few years, an ever increasing number of thieno derivatives as potent tubulin polymerization inhibitors have been proposed [12–14]. In this contest, Romagnoli and co-workers reported an interesting series of 2-(alkoxycarbonyl)-3-anilinobenzo[*b*]thiophenes of type **7**. These compounds, acting on the colchicine binding site of tubulin, showed an excellent antiproliferative activity, interfering with microtubules dynamics during cancer cell division processes [15,16].

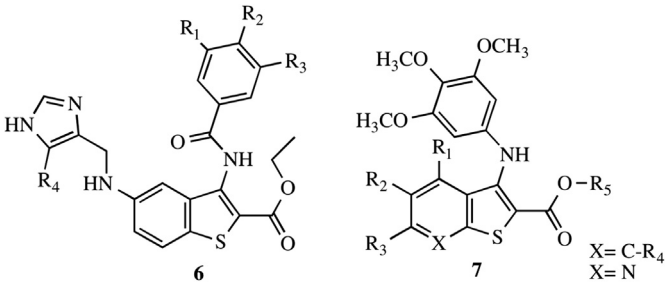


Fig. 2. Chemical structures of ethyl 3-benzoylamino-5-[(1H-imidazol-4-yl-methyl)-amino]-benzo[*b*]thiophene-2-carboxylates (**6**), 2-(alkoxycarbonyl)-3-anilino-benzo[*b*]thiophenes and thieno[2,3-*b*]pyridines (**7**).

2. Results and discussion

2.1. Chemistry

As shown in Scheme 1, the general synthetic route for the preparation of 3-benzoylamino-benzo[*b*]thiophene derivatives **6a**–**l** involved five steps, starting from the commercially available 2-fluorobenzonitrile (**8**). Pale yellow needles of 2-fluoro-5-nitrobenzonitrile (**9**) were obtained, in quantitative yield, by nitration of **8**, using a mixture of concentrated nitric and sulfuric acids, under inert atmosphere [17]. The presence of both cyano and nitro substituents on substrate **9** enhanced the reactivity of the *ortho*-fluorine atom, which readily underwent nucleophilic displacement by ethyl thioglycolate, in the presence of NaH (60% in oil dispersion) and dimethyl sulfoxide (DMSO). Subsequent intramolecular cyclization *in situ* led to the formation of the central core, and the isolation of the key intermediate 3-amino-benzo[*b*]thiophene **10**.

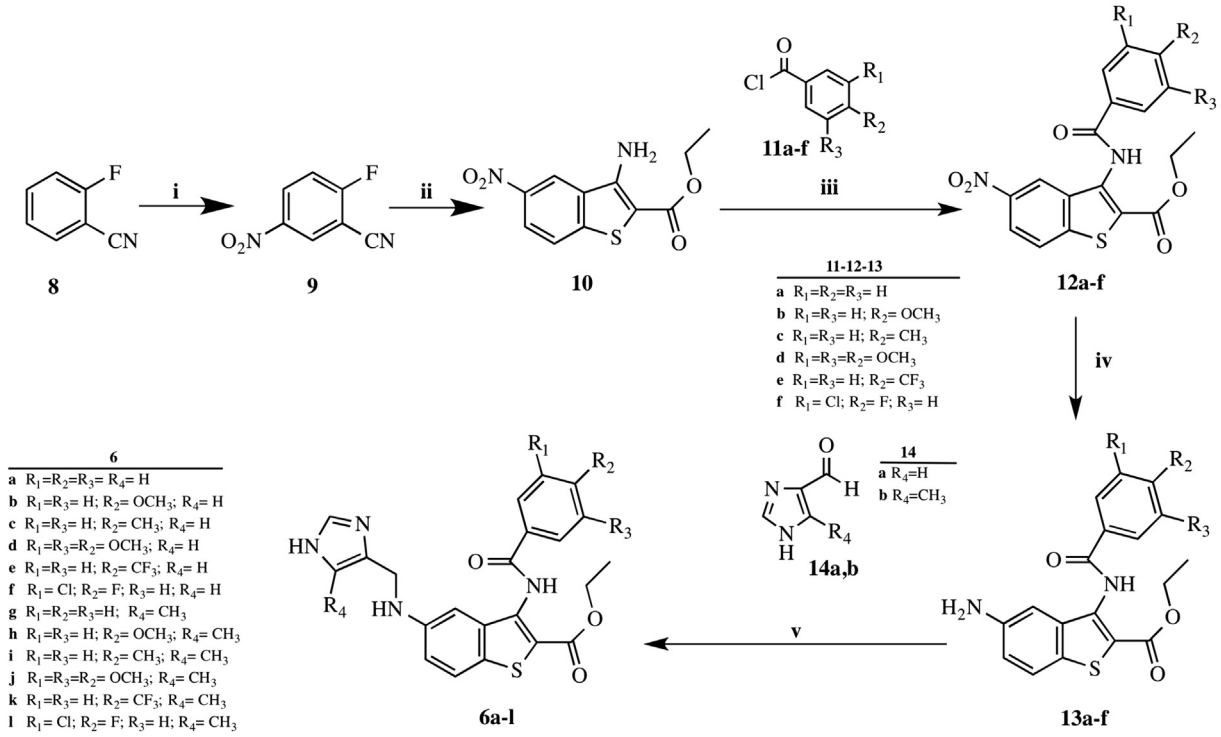
To introduce the benzoyl moiety, amino derivative **10** was treated with substituted benzoyl chlorides **11a**–**f**. The nucleophilic acyl substitution was carried out employing pyridine both as base and solvent. The use of other reaction media gave a significant decrease of the yields. Reduction of nitro group on compounds **12a**–**f** was realized through hydrogenation with 10% Pd/C in ethanol. After removal of the catalyst, amino derivatives **13a**–**f** were easily isolated as pure needles, in good yields. The last synthetic step provided the imidazole moiety insertion *via* a reductive amination in the presence of imidazole-4-carbaldehydes **14a**,**b**.

The reaction of the amino group with the aldehydes led to the formation of the corresponding imines, which, by mean of sodium cyanoborohydride addition, provided to a tightly selective reduction affording ethyl 3-benzoylamino-5-[(1H-imidazol-4-yl-methyl)-amino]-benzo[*b*]thiophene-2-carboxylates **6a**–**l**.

2.2. Biology

2.2.1. Antiproliferative activity

The antiproliferative activity of 3-benzoylamino-5-imidazol-4yl-benzo[*b*]thiophene derivatives **6a**–**l** and their corresponding precursors **13a**–**f** was evaluated on HeLa tumor cell line for 48 h using MTT based cell viability assay. All tested compounds showed concentration-dependent antiproliferative activity, but did not affect the cell membrane integrity in the concentration range 1–20 μM, as determined preliminary by the Trypan Blue exclusion method. For an easier analysis of the biological results, the tested molecules have been divided into three main subgroups: 5-amino-benzo[*b*]thiophenes **13a**–**f**, imidazole side-chain compounds **6a**–**f**, and methyl-imidazole side-chain derivatives **6g**–**l** (Table 1). The majority of our benzo[*b*]thiophene compounds proved a good growth inhibition effect, exhibiting GI50 values in the low micromolar range. As a general trend, 5-amino-benzo[*b*]thiophenes **13a**–**f** showed lower activity than the corresponding compounds with the imidazole side-chain **6**. Only when the benzoyl moiety was functionalized with 4-CF_3, the activity of the amino intermediate **13e** was more potent than imidazole derivatives **6e** and **6k**. The presence of the methyl moiety on the imidazole ring resulted in a significant increase of activity for the 3,4,5-trimethoxy (**6d** *vs* **6j**) and the 3-chloro-4-fluoro-benzoylamino-benzo[*b*]thiophene derivatives (**6f** *vs* **6l**), while it was irrelevant in the other cases. Relatively to the influence of the substituents on the benzoyl moiety there is no clear difference on activity between electron-withdrawing and electron-releasing substituents. Anyway, 3,4,5-trimethoxy benzoyl (TMB) derivatives **13d**, **6d**, and **6j** stand out as the most active of each subgroup.



Scheme 1. Synthesis of ethyl 3-benzoylamino-5-[(1H-imidazol-4-yl-methyl)-amino]-benzo[*b*]thiophene-2-carboxylates **6a**–**l**. Reagents and conditions: (i): HNO_3:H_2SO_4 (1:1), N_2, 0 °C, 2 h; (ii): ethyl thioglycolate/NaH 60%, anhydrous DMSO; (iii): pyridine, rt, 12 h; (iv): 10% Pd/C, H_2, ethanol, rt 2 h; (v): $NaCNBH_3$, ethanol/AcOH, 2 h.

2.2.2. Cell cycle analysis

The derivatives **13d**, **6j**, and **6l**, which showed the highest antiproliferative effect, were tested in cell cycle perturbation experiments to evaluate their potential influence on cell-cycle distribution. The flow cytometric analysis was performed after 24 and 48 h of incubation in order to detect the shifts in cell cycle distribution before a significant amount of cells underwent apoptosis. The working concentrations were fixed taking into account the GI50 values measured at 48 h. The histograms show the percentage of cells in the respective cell cycle phase (G1, S, and G2/M), along with the percentage of cells in the sub-G0/G1 (apoptotic cells) obtained by flow cytometry (Fig. 3). Untreated HeLa cells revealed a normal diploid distribution, presenting fast proliferation characteristics, with S + G2/M phase cells accounting for about 40% of the total cells (ctrl). After cell exposure to selected 3-benzoylamino-benzo[*b*]thiophene derivatives **13d**, **6j**, and **6l**, a rapid accumulation of cells in the G2/M phase, with a concomitant reduction of cells in both the S and G0/G1 phases, was observed. These changes occurred in a concentration-dependent manner. In fact, while in the experimental conditions a treatment with 1×GI50 produced only slight variations, a concentration of about 2×GI50 induced significant effects on cell cycle distribution. The time-dependent tendency of benzo[*b*]thiophenes effects was confirmed, after 48 h of treatment, by the deep alterations in the cell cycle distribution profile, with significant reduction of the cells number in all phases, and by the simultaneous increase of the cells population in subG0/G1, which is indicative of apoptotic cells (data not shown).

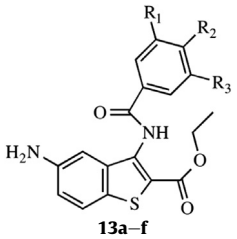
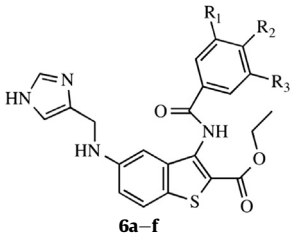
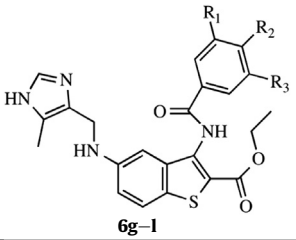
2.3. In silico insights

In attempt to rationalize biological results of selected hits on cell-cycle distribution and considering the structural features of the proposed derivatives of type **6** and **13**, similar to known antimitotic agents, the benzothiophenes were analyzed through the *in silico* VLAK protocol [18]. Mostly developed in the last years, as well as the recent protocol BIOTA [19], this protocol was employed in the design and optimization of lead compounds as anticancer drugs. The interesting results, concerning a wide set of heterocyclic derivatives, confirmed this *in silico* methodology as powerful approach in prediction capability of biological activities [9,11,20]. The VLAK protocol is focused on the search of molecular descriptors, which could be considered as the “pins of the lock”. The lock represents the biological target, or a specific mechanism of action (MA), which need a key (the molecule) to be tuned [20].

The first step of the VLAK protocol is the conversion of the biological targets, or the MAs, in “lock models” in which the keys (designed ligands) fit to modulate the biological activity [18]. In this study, the VLAK protocol was applied to predict the MA of 3-benzoylamino-benzo[*b*]thiophene derivatives by using the National Cancer Institute (NCI) Anti-cancer Agent Mechanism (ACAM) database as training set. Drug screening data are available for each structure as measurement of their growth inhibition ability over a panel of about 60 human tumor cell lines, and all tested molecules are explicitly designed as training set for neural network and multivariate analysis [21–23]. In particular, this database is constituted by 114 antitumor drugs ranked according to their MA (supporting information, Table 1) that can be considered as the locks in this protocol [24].

Thus, for each class of drugs (Alkylating Agents, Antimitotic Agents, Topoisomerase I Inhibitors, Topoisomerase II Inhibitors, RNA/DNA Antimetabolites, and DNA Antimetabolites), a “lock model” was generated starting from available known compounds. A set of 2D and 3D molecular descriptors was selected in order to best differentiate ACAM drugs database by MA. Once defined the lock model for each MA, compounds have been classified in terms of structure affinity to lock models (see computational details for protocol specifications). In Table 2 the percentages of affinity (A%) for the screened derivatives are reported.

Table 1
Cytotoxicity at 48 h of 3-benzoylamino-benzo[*b*]thiophene derivatives **13a**–**f**, **6a**–**f**, and **6g**–**l** against HeLa cell line expressed as GI50 values [GI50 ± SE (μM)].

	 **13a–f**	 **6a–f**	 **6g–l**
R_1=R_2=R_3=H	**a** >50	**a** 17.89 ± 2.03	**g** 23.32 ± 2.91
R_1=R_3=H; R_2=OCH_3	**b** >50	**b** 12.92 ± 0.94	**h** 8.22 ± 0.75
R_1=R_3=H; R_2=CH_3	**c** 24.44 ± 2.37	**c** 11.72 ± 1.17	**i** 11.42 ± 1.03
R_1=R_2=R_3=OCH_3	**d** 4.66 ± 0.31	**d** 6.79 ± 0.33	**j** 2.23 ± 0.04
R_1=R_3=H; R_2=CF_3	**e** 6.68 ± 0.57	**e** 16.84 ± 0.98	**k** 12.82 ± 1.09
R_1=Cl; R_2=F; R_3=H	**f** 12.29 ± 1.69	**f** 10.65 ± 1.32	**l** 2.91 ± 0.24

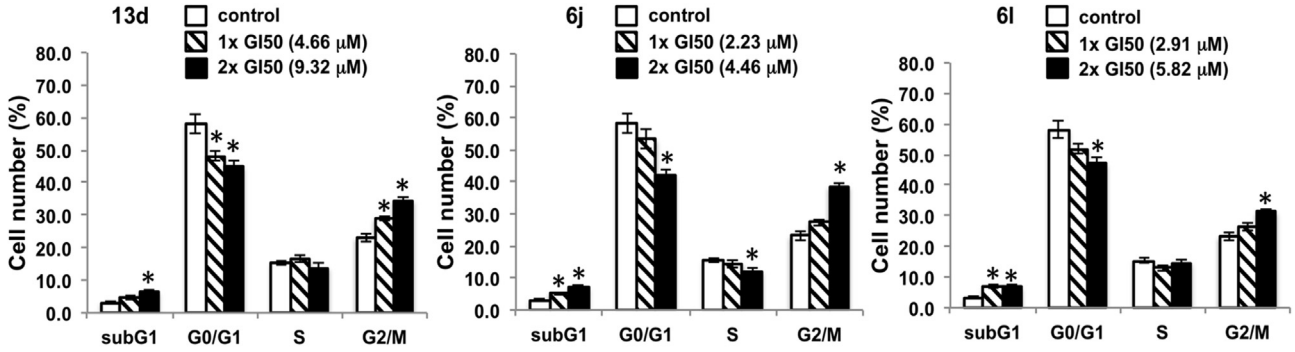


Fig. 3. Effects of compounds **13d**, **6j**, and **6l** on the cell cycle distribution of HeLa cells following 24 h treatment. The cells were cultured without any compound (ctr) or with compound used at the indicated concentrations. Cell cycle distribution was analyzed by the standard propidium iodide procedure as described in Methods. The histograms represent the percentages of cells in the respective cell cycle phase (G0/G1, S, and G2/M), along with the percentage of cells in the subG1 (dead cells) obtained by flow cytometry. Results are expressed as the mean of two independent experiments, performed in duplicate ±SE. Statistical analyses were performed using the Student's *t* test to determine the differences between the datasets. $^*p < 0.05$ denote significant differences from untreated control cells.

Analyzing the results for compounds that showed the most significant biological effect (**13d**, **6j**, and **6l**) it is noteworthy that TMB derivatives **13d**, **6j** represent the most significant compounds in terms of affinity for the class of antimitotic agents (B), showing good values of A%. The structural features of these compounds, similar to the well-known antimitotic agents such as colchicine and combretastatin A-4, could justify these results (Fig. 4).

Molecule **6l** did not show high affinity percentage towards the antimitotic mechanism of action group (42.98), maybe because of the structural modifications involving the benzoyl moiety. Thus, in terms of structural analogies with known antimitotic compounds, these results highlight the importance of the TMB moiety and underline that the presence of the imidazole ring as substituent, essential for the biological activity, does not affect the structural analogy with known antimitotic agents. The difference in the affinity toward antimitotic agents for compound **6l** could be justified by the different functionalization of the benzoyl moiety rather than the presence of the methyl group on the imidazole ring. Another important aspect to be underlined is the good affinity percentage for topoisomerase II inhibitors showed by 3-benzoylamino-benzo[*b*]thiophene derivatives **6b**,**d**,**h**,**j**. It is worth noting that these findings are in agreement with the biological insights. In particular, the *in silico* studies make possible to hypothesize that observed arrest in the G2/M phase, after cell exposure to selected derivatives, involving mitotic failure can be associated, with both an antimitotic mechanism by interference with mitotic spindle formation and the DNA-Topoisomerase-II inhibition.

3. Conclusions

In this work, the synthesis and the biological evaluation of the new benzo[*b*]thiophene series of type **6** and the related amino precursors **13a**–**f** are reported. Generally, all tested compounds showed a good antiproliferative effect with GI50 values in a low micromolar range. Antiproliferative screening on HeLa tumor cell line underlined a significant growth inhibition effect of final imidazole-side chain compounds **6a**–**l**. Methyl insertion on the imidazole ring resulted in a notable increase of activity for the TMB (**6d** *vs* **6j**) and the 3-chloro-4-fluoro-benzoylamino-benzo[*b*]thiophene derivatives (**6e** *vs* **6k**), while it was irrelevant in the other cases.

No differences were observed in the biological activity by changing the substituents on the benzoyl moiety (both electron-withdrawing and electron-releasing substituents), and the TMB derivatives **13d**, **6d**, and **6j** resulted the most active of each subgroup. To evaluate their potential influence on cell-cycle distribution, derivatives **13d**, **6j**, and **6l** were also tested in cell cycle perturbation experiments. After cell exposure to benzo[*b*]thiophene derivatives, a rapid accumulation of cells in the G2/M phase, with a concomitant reduction of cells in both the S and G0/G1 phases, was observed.

To rationalize biological results of selected hits on cell-cycle distribution, considering the structural features of the proposed derivatives of type **6** and **13**, similar to known antimitotic agents, the benzothiophenes were analyzed through the *in silico* VLAK protocol. Compounds presenting the TMB moiety were classified as

Table 2
Percentage of affinity (A%) calculated for the tested compounds.

Compound	Alkylating agents	Antimitotic agents	DNA anti metabolites	Topoisomerase I inhibitors	Topoisomerase II inhibitors	RNA-DNA anti metabolites
13a	66.27	44.94	41.35	38.57	26.95	46.99
13b	64.59	53.90	38.99	53.55	36.60	50.29
13c	66.37	47.50	38.31	45.36	29.59	47.35
13d	46.27	**60.57**	23.35	56.25	56.32	45.25
13e	53.87	40.77	33.44	43.74	34.65	57.40
13f	60.85	40.22	43.58	45.92	30.79	54.95
6b	41.37	55.29	31.03	52.66	60.69	42.63
6c	43.16	48.52	29.74	48.83	52.80	40.57
6d	35.25	**56.39**	22.62	33.77	**62.67**	35.61
6e	40.22	38.92	31.11	38.89	45.41	49.06
6f	42.25	41.95	34.67	51.70	46.64	48.70
6g	44.48	45.58	30.19	46.60	47.89	40.24
6h	41.62	54.88	28.07	50.26	61.26	37.03
6i	43.40	49.57	26.70	47.40	54.34	38.56
6j	35.20	**56.74**	20.07	31.21	**61.32**	33.64
6k	41.46	42.30	28.77	41.64	44.37	44.63
6l	42.23	42.98	32.02	53.42	48.74	44.10

In bold are reported the highest A% values for the most active compounds (**13d**, **6d**, and **6j**) as antimitotic and topoisomerase II inhibitors classes.

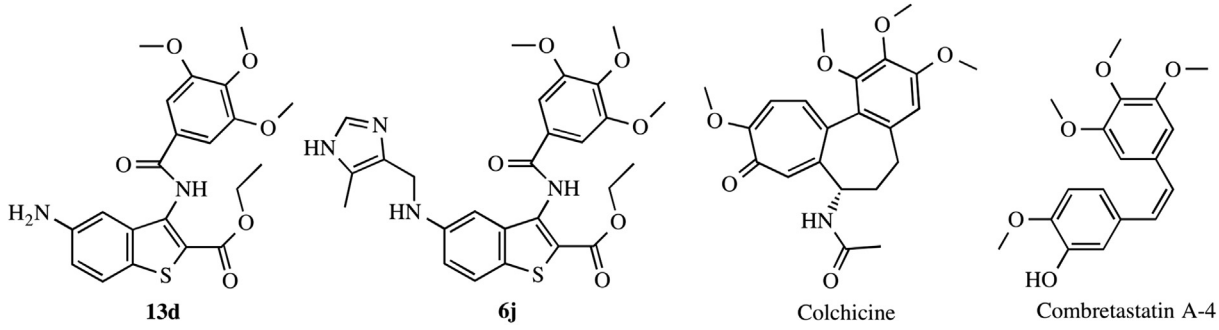


Fig. 4. TMB derivatives (**13d** and **6j**) with good percentage of affinity (A%) and well-known antimitotic agents (Conchicine and Combretastatin A-4).

potential antimitotic agents, showing high affinity percentage for this mechanism of action class. The presence of the imidazole ring does not seem to affect the mechanism of action as previously observed by biological assays. This is also underlined by the presence of several imidazole derivatives as the best ranked antimitotic agents in Table 2. Another important aspect to be underlined is the good percentage of affinity for topoisomerase II inhibitors showed by some other 3-benzoylamino-benzo[*b*]thiophene derivatives as **6b**,**d**,**h**,**j**. These findings could be in accordance with biological assays where a possible antimitotic mechanism or a potential inhibition of the topoisomerase II enzyme could justify the cell cycle arrest at G2/M checkpoint.

4. Experimental

4.1. Chemistry

Unless otherwise indicated, all reagents and solvents were purchased from commercial sources and used without further purification. All melting points (°C) were determined on a Büchi Tottoli capillary apparatus and are uncorrected; IR spectra were determined in bromoform with a Jasco FT/IR 5300 spectrophotometer.^{1}H NMR, ^{13}C NMR spectra were recorded, at 200 and 50.3 MHz respectively, in $CDCl_3$ or DMSO-d_6 solution, using a Bruker AC-E series 200 MHz spectrometer. Chemical shifts values are given in ppm and referred as the internal standard to tetramethylsilane (TMS). The following abbreviations are used: br s = broad signal, s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, rt = room temperature. The purity of all compounds screened in biological assays was determined to be >95% by HPLC/MS analysis. Mass spectra were performed using a GC and MS Shimadzu QP5050 with EI (75 ev). Microanalyses were in agreement with theoretical values ±0.4%. Thin layer chromatography was performed on precoated (0.25 mm) silica gel GF254 plates, compounds were detected with 254 nm UV lamp. Column chromatography was performed with Merck silica gel ASTM (230 and 400 mesh), or with a Biotage FLASH40i chromatography module (prepacked cartridge system). 2-fluorobenzonitrile (**8**) is commercially available. 2-fluoro-5-nitro-benzonitrile (**9**) was obtained following the procedure reported in literature [17].

4.1.1. Synthesis of ethyl 3-amino-5-nitro-benzo[b]thiophene-2-carboxylate (*10*)

According to the literature procedure [9], ethyl thioglycolate (1.2 mL, 11 mmol) was added to a stirred mixture of NaH 60% dispersion in mineral oil (0.36 g, 15 mmol) in dry DMSO (10 mL). After stirring at room temperature for 20 min, a solution of 2-fluoro-5-nitrobenzonitrile (**9**) (1.66 g, 10 mmol) in dry DMSO (20 mL) was added dropwise. The reaction was stirred at room temperature for further 3 h and then was poured onto stirred water/ice. The precipitate was collected by filtration and dried to give orange-red needles of **10**, 2.48 g. Yield 93%. Mp 207–208 °C. IR ν_{max}: 3491, 3367 (NH_2), 1657 (CO) cm^{-1}. ^{1}H NMR ($CDCl_3$) δ: 1.41 (t, 3H, J = 8.0 Hz, CH_3), 4.42 (q, 2H, J = 8.0 Hz, CH_2), 6.03 (br s, 2H, exchange with D_2O, NH_2), 7.84 (d, 1H, J = 8.8 Hz, H-7), 8.29 (dd, 1H, J = 2.0, 8.8 Hz, H-6), 8.59 (d, 1H, J = 2.0 Hz, H-4); ^{13}C NMR (DMSO-d_6) δ: 14.3 (q), 60.2 (t), 96.9 (s), 119.4 (d), 122.0 (d), 124.3 (d), 131.4 (s), 144.5 (s), 144.9 (s), 149.7 (s), 163.9 (s). Anal. Calcd. (%) for

$C_{11}H_{10}N_2O_4S$: C, 49.62; H, 3.79; N, 10.52; found: C, 49.64; H, 3.81; N, 10.48.

*4.1.2. General procedure for the synthesis of ethyl 3-benzoylamino-5-nitro-benzo[b]thiophene-2-carboxylates **12a–f***

To a suspension of amine **10** (0.37 mmol) and pyridine (0.56 mmol) was added the appropriate benzoyl chloride **11** (0.56 mmol). The reaction mixture was stirred at room temperature for about 12 h, and then poured onto stirred water/ice. The precipitate was collected by filtration, dried over a night. The crude was crystallized from ethyl acetate.

*4.1.2.1. Ethyl 3-benzoylamino-5-nitro-benzo[b]thiophene-2-carboxylate (**12a**).* Yield 95%. Mp 200–201 °C. IR ν_{max}: 3606 (NH), 1687, 1679 (CO) cm^{-1}. ^1H NMR ($CDCl_3$) δ: 1.43 (t, 3H, $J = 8.0$ Hz, CH_3), 4.44 (q, 2H, $J = 8.0$ Hz, CH_2), 7.48–7.69 (m, 3H, H-3′, H-4′, H-5′), 7.89 (d, 1H, $J = 8.8$ Hz, H-7), 8.11 (dd, 2H, $J = 2.0$, 8.0 Hz, H-2′, H-6′), 8.32 (dd, 1H, $J = 2.0$, 8.8 Hz, H-6), 9.24 (d, 1H, $J = 2.0$ Hz, H-4), 10.69 (s, 1H, NH). ^{13}C NMR ($CDCl_3$) δ: 14.2 (q), 62.3 (t), 117.3 (s), 121.7 (d), 123.3 (d), 124.2 (d), 127.9 (d), 128.6 (s), 129.0 (d), 132.8 (d), 132.9 (s), 141.0 (s), 144.5 (s), 145.1 (s), 164.1 (s), 165.3 (s). Anal. Calcd. (%) for $C_{18}H_{14}N_2O_5S$: C, 58.37; H, 3.81; N, 7.56; found: C, 58.34; H, 3.84; N, 7.53.

*4.1.2.2. Ethyl 3-(4-methoxy-benzoylamino)-5-nitro-benzo[b]thiophene-2-carboxylate (**12b**).* Yield 82%. Mp 191–192 °C. IR ν_{max}: 3601 (NH), 1696, 1672 (CO) cm^{-1}. ^1H NMR ($CDCl_3$) δ: 1.43 (t, 3H, $J = 8.0$ Hz, CH_3), 3.91 (s, 3H, OCH_3), 4.44 (q, 2H, $J = 8.0$ Hz, CH_2), 7.05 (d, 2H, $J = 8.0$ Hz, H-3′,H-5′), 7.89 (d, 1H, $J = 9.0$ Hz, H-7), 8.07 (d, 2H, $J = 8.0$ Hz, H-2′, H-6′), 8.32 (dd, 1H, $J = 2.0$, 9.0 Hz, H-6), 9.25 (d, 1H, $J = 2.0$ Hz, H-4), 10.64 (s, 1H, NH). ^{13}C NMR ($CDCl_3$) δ: 14.2 (q), 55.6 (q), 62.3 (t), 114.1 (d), 114.2 (d), 121.2 (d), 121.6 (s), 121.8 (s), 126.8 (s), 132.3 (d), 132.8 (d), 141.3 (s), 141.8 (s), 144.5 (s), 145.0 (s), 164.2 (s), 164.8 (s). Anal. Calcd. (%) for $C_{19}H_{16}N_2O_6S$: C, 56.99; H, 4.03; N, 7.00; found: C, 56.95; H, 4.07; N, 7.03.

*4.1.2.3. Ethyl 3-(4-methyl-benzoylamino)-5-nitro-benzo[b]thiophene-2-carboxylate (**12c**).* Yield 98%. Mp 231–232 °C. IR ν_{max}: 3607 (NH), 1700, 1677 (CO) cm^{-1}. ^1H NMR ($CDCl_3$) δ: 1.43 (t, 3H, $J = 8.0$ Hz, CH_3), 2.47 (s, 3H, CH_3), 4.44 (q, 2H, $J = 8.0$ Hz, CH_2), 7.37 (d, 2H, $J = 8.8$ Hz, H-3′,H-5′), 7.90 (d, 1H, $J = 8.0$ Hz, H-7), 8.00 (d, 2H, $J = 8.8$ Hz, H-2′, H-6′), 8.33 (dd, 1H, $J = 2.0$, 8.0 Hz, H-6), 9.25 (d, 1H, $J = 2.0$ Hz, H-4), 10.66 (s, 1H, NH). ^{13}C NMR ($CDCl_3$) δ: 14.2 (q), 21.6 (q), 62.3 (t), 118.5 (s), 121.7 (d), 123.3 (d), 124.2 (d), 128.0 (d), 129.7 (d), 130.3 (s), 132.9 (s), 141.2 (s), 143.6 (s), 144.5 (s), 145.1 (s), 164.1 (s), 165.3 (s). Anal. Calcd. (%) for $C_{19}H_{16}N_2O_5S$: C, 59.37; H, 4.20; N, 7.29; found: C, 59.40; H, 4.17; N, 7.32.

*4.1.2.4. Ethyl 3-(3,4,5-trimethoxy-benzoylamino)-5-nitro-benzo[b]thiophene-2-carboxylate (**12d**).* Yield 99%. Mp 211–213 °C. IR ν_{max}: 3607 (NH), 1680, 1668 (CO) cm^{-1}. ^1H NMR ($CDCl_3$) δ: 1.46 (t, 3H, $J = 8.0$ Hz, CH_3), 3.98 (s, 3H, OCH_3), 4.02 (s, 6H, $2\times OCH_3$), 4.45 (q, 2H, $J = 8.0$ Hz, CH_2), 7.38 (s, 2H, H-2′,H-6′), 7.93 (d, 1H, $J = 8.0$ Hz, H-7), 8.36 (dd, 1H, $J = 2.0$, 8.0 Hz, H-6), 9.32 (d, 1H, $J = 2.0$ Hz, H-4), 10.72 (s, 1H, NH). ^{13}C NMR ($CDCl_3$) δ: 14.2 (q), 56.4 (q), 61.0 (t), 62.3 (q), 105.3 (d), 117.0 (s), 117.5 (d), 121.7 (d), 121.9 (d), 128.4 (s), 131.3 (s), 132.8 (s), 141.1 (s), 145.1 (s), 145.6 (s), 153.4 (s), 164.2 (s), 165.0 (s). Anal. Calcd. (%) for $C_{21}H_{20}N_2O_8S$: C, 54.78; H, 4.38; N, 6.08; found: C, 54.76; H, 4.40; N, 6.10.

*4.1.2.5. Ethyl 3-(4-trifluoromethyl-benzoylamino)-5-nitro-benzo[b]thiophene-2-carboxylate (**12e**).* Yield 74%. Mp 236–237 °C. IR ν_{max}: 3616 (NH), 1695, 1684 (CO) cm^{-1}. ^1H NMR ($CDCl_3$) δ: 1.44 (t, 3H, $J = 8.0$ Hz, CH_3), 4.46 (q, 2H, $J = 8.0$ Hz, CH_2), 7.85 (d, 2H, $J = 8.0$ Hz, H-3′, H-5′), 7.92 (d, 1H, $J = 8.0$ Hz, H-7), 8.23 (d, 2H, $J = 8.0$ Hz, H-2′, H-6′), 8.36 (dd, 1H, $J = 2.0$, 8.0 Hz, H-6), 9.27 (d, 1H, $J = 2.0$ Hz, H-4), 10.83 (s, 1H, NH). ^{13}C NMR ($CDCl_3$) δ: 14.2 (q), 62.5 (t), 117.7 (s), 120.9 (s), 121.9 (d), 123.5 (s), 124.1 (d), 125.0 (s), 126.1 (d), 128.4 (d), 130.6 (d), 132.7 (s), 140.5 (s), 144.4 (s), 151.5 (s), 164.0 (s), 164.2 (s). Anal. Calcd. (%) for $C_{19}H_{13}F_3N_2O_5S$: C, 52.06; H, 2.99; N, 6.39; found: C, 52.10; H, 2.93; N, 6.42.

*4.1.2.6. Ethyl 3-(3-chloro-4-fluoro-benzoylamino)-5-nitro-benzo[b]thiophene-2-carboxylate (**12f**).* Yield 57.4%. Mp 218–219 °C. IR ν_{max}: 3620 (NH), 1693, 1684 (CO) cm^{-1}. ^1H NMR ($CDCl_3$) δ: 1.47 (t, 3H, $J = 8.0$ Hz, CH_3), 4.49 (q, 2H, $J = 8.0$ Hz, CH_2), 7.36 (t, 1H, $J = 10.0$ Hz, H-5′), 7.94 (d, 1H, $J = 8.0$ Hz, H-7), 8.03 (dq, 1H, $J = 2.0$, 10.0 Hz, H-6′), 8.23 (dd, 1H, $J = 2.0$, 8.0 Hz, H-6), 8.37 (dd, 1H, $J = 2.0$, 10.0 Hz, H-2′), 9.24 (d, 1H, $J = 2.0$ Hz, H-4), 10.83 (s, 1H, NH). ^{13}C NMR ($CDCl_3$) δ: 14.2 (q), 62.5 (t), 117.3 (d, $J = 5$ Hz), 117.4 (s), 121.8 (d), 122.5 (s), 123.4 (s), 123.9 (d), 128.1 (d), 130.3 (s), 131.4 (s), 132.6 (d), 140.5 (s), 144.4 (s), 145.2 (s), 161.0 (d, $J = 30$ Hz), 163.2 (s), 164.2 (s). Anal. Calcd. (%) for $C_{18}H_{12}ClFN_2O_5S$: C, 51.13; H, 2.86; N, 6.63; found: C, 51.16; H, 2.90; N, 6.65.

*4.1.3. General procedure for the synthesis of ethyl 5-amino-3-benzoylamino-benzo[b]thiophene-2-carboxylates **13a–f***

A suspension of 5-nitro compound **12a–f** (1 mmol), 10% Pd/C (0.05 g) in ethanol was subjected to hydrogenation using a *Parr* Hydrogenation apparatus at 500 psi for 2 h. After then, the catalyst was removed by filtration and the filtrate was concentrated under reduced pressure. The crude was recrystallized from ethanol.

*4.1.3.1. Ethyl 5-amino-3-benzoylamino-benzo[b]thiophene-2-carboxylate (**13a**).* Yield 91%. Mp 184–185 °C. IR ν_{max}: 3612, 3356 (NH_2), 3293 (NH), 1688, 1664 (CO) cm^{-1}. ^1H NMR ($CDCl_3$) δ: 1.42 (t, 3H, $J = 8.0$ Hz, CH_3), 4.41 (q, 2H, $J = 8.0$ Hz, CH_2), 4.83 (br s, 2H, NH_2) 6.96 (dd, 1H, $J = 2.0$, 8.0 Hz, H-6), 7.49 (d, 1H, $J = 2.0$ Hz, H-4), 7.52–7.63 (m, 5H, C_6H_5), 8.10 (d, 1H, $J = 8.0$ Hz, H-7), 10.48 (s, 1H, NH). ^{13}C NMR ($CDCl_3$) δ: 14.3 (q), 61.6 (t), 111.0 (d), 119.0 (d), 123.2 (d), 127.8 (d), 128.9 (d), 130.4 (s), 131.1 (s), 132.4 (d), 133.9 (s), 139.1 (s), 143.6 (s), 146.6 (s), 161.2 (s), 165.9 (s). Anal. Calcd. (%) for $C_{18}H_{16}N_2O_3S$: C, 63.51; H, 4.74; N, 8.23; found: C, 63.53; H, 4.72; N, 8.21.

*4.1.3.2. Ethyl 5-amino-3-(4-methoxy-benzoylamino)-benzo[b]thiophene-2-carboxylate (**13b**).* Yield 98%. Mp 160–161 °C. IR ν_{max}: 3613, 3360 (NH_2), 3296 (NH), 1683, 1660 (CO) cm^{-1}. ^1H NMR (DMSO-d_6) δ: 1.19 (t, 3H, $J = 8.0$ Hz, CH_3), 3.91 (s, 3H, OCH_3), 4.24 (q, 2H, $J = 8.0$ Hz, CH_2), 5.08 (br s, 2H, NH_2) 6.94 (d, 1H, $J = 2.0$ Hz, H-4), 7.09–7.17 (m, 3H, H-6, H-3′, H-5′), 7.50 (d, 1H, $J = 8.0$ Hz, H-7), 8.07 (d, 2H, $J = 8.8$ Hz, H-2′, H-6′), 10.04 (s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ: 13.9 (q), 55.6 (q), 60.8 (t), 113.5 (d), 113.7 (d), 118.8 (d), 119.2 (d), 126.3 (s), 127.2 (s), 129.6 (d), 130.2 (s), 134.2 (s), 138.8 (s), 139.7 (s), 161.7 (s), 161.9 (s), 164.9 (s). Anal. Calcd. (%) for $C_{19}H_{18}N_2O_4S$: C, 61.61; H, 4.90; N, 7.56; found: C, 61.59; H, 4.93; N, 7.52.

*4.1.3.3. Ethyl 5-amino-3-(4-methyl-benzoylamino)-benzo[b]thiophene-2-carboxylate (**13c**).* Yield 70%. Mp 208–209 °C. IR ν_{max}: 3613, 3360 (NH_2), 3297 (NH), 1682, 1665 (CO) cm^{-1}. ^1H NMR (DMSO-d_6): δ 1.18 (t, 3H, $J = 8.0$ Hz, CH_3), 2.42 (s, 3H, CH_3), 4.23 (q, 2H, $J = 8.0$ Hz, CH_2), 5.32 (br s, 2H, NH_2) 6.90–6.95 (m, 2H, H-4, H-6), 7.38 (d, 2H, $J = 8.0$ Hz, H-3′, H-5′), 7.64 (d, 1H, $J = 8.0$ Hz, H-7), 7.97 (d, 2H, $J = 8.8$ Hz, H-2′, H-6′), 10.20 (s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ: 14.5 (q), 21.5 (q), 61.3 (t), 105.8 (d), 118.3 (d), 123.0 (d), 125.9 (s), 127.8 (d), 129.0 (d), 131.0 (s), 135.5 (s), 137.1 (s), 141.9 (s), 146.5 (s), 161.9 (s), 163.0 (s), 165.2 (s). Anal. Calcd. (%) for $C_{19}H_{18}N_2O_3S$: C, 64.39; H, 5.12; N, 7,90; found: C, 64.42; H, 5.15; N, 7.93.

*4.1.3.4. Ethyl 5-amino-3-(3,4,5-trimethoxy-benzoylamino)-benzo[b] thiophene-2-carboxylate (**13d**).* Yield 93%. Mp 182–183 °C. IR ν_{max}: 3404, 3316 (NH_2), 3213 (NH), 1696, 1655 (CO) cm^{-1}. ^1H NMR (DMSO-d_6) δ: 1.20 (t, 3H, $J = 8.0$ Hz, CH_3), 3.76 (s, 3H, OCH_3), 3.88 (s, 6H, 2×OCH_3), 4.22 (q, 2H, $J = 8.0$ Hz, CH_2), 5.34 (br s, 2H, NH_2) 6.87 (d, 1H, $J = 2.0$ Hz, H-4), 6.93 (dd, 1H, $J = 2.0$, 8.0 Hz, H-6), 7.41 (s, 2H, H-2′, H-6′), 7.65 (d, 1H, $J = 8.0$ Hz, H-7), 10.22 (s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ: 14.0 (q), 56.1 (q), 60.1 (q), 60.8 (t), 99.5 (s), 105.3 (d), 105.7 (d), 118.3 (s), 123.0 (d), 125.8 (d), 128.9 (s), 135.2 (s), 137.1 (s), 140.5 (s), 146.5 (s), 152.7 (s), 161.8 (s), 164.6 (s). Anal. Calcd. (%) for $C_{21}H_{22}N_2O_6S$: C, 58.59; H, 5.15; N, 6.51; found: C, 58.63; H, 5.17; N, 6.55.

*4.1.3.5. Ethyl 5-amino-3-(4-trifluoromethyl-benzoylamino)-benzo[b] thiophene-2-carboxylate (**13e**).* Yield 80%. Mp 198–199 °C. IR ν_{max}: 3612, 3356 (NH_2), 3293 (NH), 1688, 1664 (CO) cm^{-1}. ^1H NMR (DMSO-d_6) δ: 1.18 (t, 3H, $J = 8.0$ Hz, CH_3), 4.22 (q, 2H, $J = 8.0$ Hz, CH_2), 5.34 (br s, 2H, NH_2) 6.89 (d, 1H, $J = 2.0$ Hz, H-4), 6.93 (dd, 1H, $J = 2.0$, 8.0 Hz, H-6), 7.66 (d, 1H, $J = 8.0$ Hz, H-7), 7.98 (d, 2H, $J = 8.8$ Hz, H-3′, H-5′), 8.25 (d, 2H, $J = 8.8$ Hz, H-2′, H-6′), 10.54 (s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ: 14.0 (q), 60.9 (t), 119.1 (s), 118.3 (d), 120.8 (s), 123.1 (d), 123.7 (s), 125.5 (d), 126.9 (s), 128.6 (d), 129.1 (d), 134.4 (s), 137.1 (s), 146.6 (s), 147.9 (s), 161.7 (s), 164.3 (s). Anal. Calcd. (%) for $C_{19}H_{15}F_3N_2O_3S$: C, 55.88; H, 3.70; N, 6.86; found: C, 55.84; H, 3.73; N, 6.82.

*4.1.3.6. Ethyl 5-amino-3-(3-chloro-4-fluoro-benzoylamino)-benzo[b] thiophene-2-carboxylate (**13f**).* Yield 52%. Mp 226–227 °C. IR ν_{max}: 3615, 3360 (NH_2), 3290 (NH), 1689, 1667 (CO) cm^{-1}. ^1H NMR ($CDCl_3$) δ: 1.18 (t, 3H, $J = 8.0$ Hz, CH_3), 4.23 (q, 2H, $J = 8.0$ Hz, CH_2), 5.35 (br s, 2H, NH_2), 6.87 (d, 1H, $J = 2.0$ Hz, H-4), 6.93 (dd, 1H, $J = 2.0$, 10.0 Hz, H-2′), 7.65 (d, 1H, $J = 8.0$ Hz, H-7), 7.66 (t, 1H, $J = 10.0$ Hz, H-5′), 8.08 (dq, 1H, $J = 2.0$, 10.0 Hz, H-6′), 8.29 (dd, 1H, $J = 2.0$, 8.0 Hz, H-6), 10.34 (s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ: 14.0 (q), 61.1 (t), 99.5 (s), 117.3 (d), 118.5 (s), 120.0 (d), 122.6 (s), 123.2 (s), 125.8 (d), 128.9 (d), 131.5 (s), 134.3 (d), 137.0 (s), 141.1 (s), 146.6 (s), 161.6 (d), 163.2 (s), 164.3 (s). Anal. Calcd. (%) for $C_{18}H_{14}ClFN_2O_3S$: C, 55.03; H, 3.59; N, 7.13; found: C, 55.04; H, 3.60; N, 7.22.

*4.1.4. General procedure for the synthesis of ethyl 3-benzoylamino-5-[(1H-imidazol-4-yl-methyl)-amino]-benzo[b]thiophene-2-carboxylates **6a-l***

To a suspension of the **13a–f** (1.25 mmol) and the appropriate aldehyde (1.6 mmol) in dry ethanol (10 mL) was added acetic acid up to pH 4. The mixture was stirred for 30 min, and then $NaCNBH_3$ (1.6 mmol) was added continuing the stirring at room temperature for further 6–24 h. The reaction solvent was removed under reduced pressure and the crude product was purified by chromatography column using dichloromethane/methanol 98:2 as eluant. Recrystallized from ethanol.

*4.1.4.1. Ethyl 3-benzoylamino-5-[(1H-imidazol-4-yl-methyl)-amino]-benzo[b]thiophene-2-carboxylate (**6a**).* Yield 76%. Mp 184–185 °C. IR ν_{max}: 3301, 3245 (NH), 1684, 1668 (CO) cm^{-1}. ^1H NMR (DMSO-d_6) δ: 1.19 (t, 3H, $J = 8.0$ Hz, CH_3), 4.24 (q, 2H, $J = 8.0$ Hz, CH_2), 4.52–4.54 (m, 3H, NH, NCH_2) 6.89 (s, 1H, H-5″), 7.19 (s, 1H, H-2″), 7.29 (dd, 1H, $J = 2.0$, 8.0 Hz, H-6), 7.56–7.65 (m, 5H, C_6H_5), 7.73 (d, 2H, $J = 8.0$ Hz, H-7), 8.04 (d, 1H, $J = 2.0$ Hz, H-4) 10.39 (s, 1H, NH), 12.10 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ: 13.5 (q), 46.1 (t), 60.4 (t), 100.3 (d), 119.7 (s), 122.8 (s), 122.9 (d), 125.9 (s), 128.4 (d), 129.5 (d), 131.1 (s), 132.5 (s), 134.4 (d), 135.1 (d), 135.7 (d), 137.0 (d), 146.2 (s), 149.5 (s), 161.6 (s), 165.9 (s). Anal. Calcd. (%) for $C_{22}H_{20}N_4O_3S$: C, 62.84; H, 4.79; N, 13.32; found: C, 62.86; H, 4.80; N, 13.35.

*4.1.4.2. Ethyl 5-[(1H-imidazol-4-yl-methyl)-amino]-3-(4-methoxy-benzoylamino)-benzo[b]thiophene-2-carboxylate (**6b**).* Yield 62%. Mp 124–125 °C. IR ν_{max}: 3306, 3243 (NH), 1682, 1667 (CO) cm^{-1}. ^1H NMR (DMSO-d_6) δ: 1.15 (t, 3H, $J = 8.0$ Hz, CH_3), 3.86 (s, 3H, OCH_3), 4.20 (q, 2H, $J = 8.0$ Hz, CH_2), 4.28 (d, 2H, $J = 2.0$ Hz, NCH_2) 5.34 (t, 1H, $J = 2.0$ Hz, NH), 6.91 (s, 1H, H-5″), 7.04–7.16 (m, 4H, H-3′, H-5′, H-2″, H-6), 7.54 (d, 1H, $J = 8.0$ Hz, H-7), 7.58 (d, 1H, $J = 2.0$ Hz, H-4), 8.06 (d, 2H, $J = 8.0$ Hz, H-2′, H-6′) 10.06 (s, 1H, NH), 11.96 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ: 14.0 (q), 40.1 (t), 55.4 (q), 60.8 (t), 99.5 (s), 103.5 (d), 113.6 (d), 113.7 (s), 117.7 (d), 122.0 (s), 122.5 (s), 122.9 (d), 126.2 (d), 129.7 (d), 134.8 (d), 135.6 (s), 137.0 (s), 146.6 (s), 161.9 (s), 162.0 (s), 164.9 (s). Anal. Calcd. (%) for $C_{23}H_{22}N_4O_4S$: C, 61.32; H, 4.92; N, 12.44; found: C, 61.31; H, 4.94; N, 12.48.

*4.1.4.3. Ethyl 5-[(1H-imidazol-4-yl-methyl)-amino]-3-(4-methyl-benzoylamino)-benzo[b]thiophene-2-carboxylate (**6c**).* Yield 58%. Mp 138–139 °C. IR ν_{max}: 3307, 3253 (NH), 1680, 1669 (CO) cm^{-1}. ^1H NMR (DMSO-d_6) δ: 1.18 (t, 3H, $J = 8.0$ Hz, CH_3), 2.42 (s, 3H, CH_3), 4.14 (d, 2H, $J = 2.0$ Hz, NCH_2), 4.23 (q, 2H, $J = 8.0$ Hz, CH_2), 6.11 (t, 1H, $J = 2.0$ Hz, NH), 6.91–6.93 (m, 2H, H-4, H-5″), 7.06 (dd, 1H, $J = 2.0$, 8.0 Hz, H-6), 7.38 (d, 2H, $J = 8.0$ Hz, H-3′, H-5′), 7.56 (s, 1H, H-2″), 7.68 (d, 1H, $J = 8.0$ Hz, H-7), 7.97 (d, 2H, $J = 8.0$ Hz, H-2′, H-6′), 10.23 (s, 1H, NH), 11.93 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ: 14.0 (q), 21.0 (q), 40.4 (t), 60.8 (t), 103.4 (d), 117.4 (d), 117.7 (d), 122.7 (s), 122.9 (d), 126.1 (s), 127.8 (d), 129.0 (d), 131.3 (s), 134.8 (d), 135.3 (s), 137.0 (s), 141.8 (s), 146.6 (s), 161.9 (s), 165.3 (s), 172.0 (s). Anal. Calcd. (%) for $C_{23}H_{22}N_4O_3S$: C, 63.58; H, 5.10; N, 12.89; found: C, 63.60; H, 5.12; N, 12.85.

*4.1.4.4. Ethyl 5-[(1H-imidazol-4-yl-methyl)-amino]-3-(3,4,5-trimethoxy-benzoylamino)-benzo[b]thiophene-2-carboxylate (**6d**).* Yield 94%. Mp 165–166 °C. IR: 3301, 3245 (NH), 1684, 1668 (CO) cm^{-1}. ^1H NMR (DMSO-d_6) δ: 1.20 (t, 3H, $J = 7.0$ Hz, CH_3), 3.76 (s, 3H, OCH_3), 3.89 (s, 6H, 2×OCH_3), 4.34 (d, 2H, $J = 1.8$ Hz, NCH_2), 4.24 (q, 2H, $J = 7.0$ Hz, CH_2), 6.14 (t, 1H, $J = 1.8$ Hz, NH), 6.89 (d, 1H, $J = 1.8$ Hz, H-4) 6.95 (s, 1H, H-5″), 7.07 (dd, 1H, $J = 1.8$, 8.8 Hz, H-6), 7.40 (s, 2H, H-2′, H-6′), 7.58 (s, 1H, H-2″), 7.70 (d, 1H, $J = 8.8$ Hz, H-7), 10.25 (s, 1H, NH), 12.22 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ: 14.0 (q), 40.2 (t), 56.0 (q), 60.1 (q), 60.2 (t), 99.5 (s), 103.3 (s), 105.3 (d), 117.7 (d), 122.9 (d), 126.1 (d), 129.1 (s), 129.5(s), 134.8 (s), 135.2 (d), 137.0 (s), 140.4 (s), 146.6 (s), 152.6 (d), 156.9 (s), 161.8 (s), 164.9 (s). Anal. Calcd. (%) for $C_{25}H_{26}N_4O_6S$: C, 58.81; H, 5.13; N, 10.97; found: C, 58.83; H, 5.14; N, 10.95.

*4.1.4.5. Ethyl 5-[(1H-imidazol-4-yl-methyl)-amino]-3-(4-trifluoromethyl-benzoylamino)-benzo[b]thiophene-2-carboxylate (**6e**).* Yield 84%. Mp 192–193 °C. IR ν_{max}: 3298, 3233 (NH), 1679, 1665 (CO) cm^{-1}. ^1H NMR (DMSO-d_6) δ: 1.21 (t, 3H, $J = 6.0$ Hz, CH_3), 4.25 (q, 2H, $J = 6.0$ Hz, CH_2), 4.53–4.56 (m, 3H, NCH_2, NH), 6.90 (1, 1H, H-5″), 7.19 (d, 1H, $J = 2.0$ Hz, H-4), 7.57 (s, 1H, H-2″), 7.74 (d, 1H, $J = 8.0$ Hz, H-7), 7.99 (d, 2H, $J = 10.0$ Hz, H-3′, H-5′), 8.22 (d, 2H, $J = 10.0$ Hz, H-2′, H-6′), 10.56 (s, 1H, NH), 11.93 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ: 14.0 (q), 40.0 (t), 60.9 (t), 99.5 (s), 113.0 (d), 117.9 (d), 121.1 (d), 125.4 (s), 125.5 (s), 126.2 (d), 128.6 (d), 129.0 (s), 130.6 (d), 134.4 (d), 134.8 (s), 136.9 (s), 137.8 (s), 146.7 (s), 161.7 (s), 164.5 (s), 166.3 (s). Anal. Calcd. (%) for $C_{23}H_{19}F_3N_4O_3S$: C, 56.55; H, 3.92; N, 11.47; found: C, 56.53; H, 3.91; N, 11.49.

*4.1.4.6. Ethyl 5-[(1H-imidazol-4-yl-methyl)-amino]-3-(3-chloro-4-fluoro-benzoylamino)-benzo[b]thiophene-2-carboxylate (**6f**).* Yield 43%. Mp 162–163 °C. IR ν_{max}: 3306, 3253 (NH), 1690, 1669 (CO) cm^{-1}. ^1H NMR ($CDCl_3$) δ: 1.34 (t, 3H, $J = 8.0$ Hz, CH_3), 4.26–4.30 (m, 3H, NH, NCH_2), 4.32 (q, 2H, $J = 8.0$ Hz, CH_2), 6.82 (dd, 1H, $J = 2.0$, 8.0 Hz, H-6), 6.96 (s, 1H, H-5″), 7.24 (t, 1H, $J = 8.0$ Hz, H-5′), 7.43 (d, 1H, $J = 8.0$ Hz, H-7), 7.47 (s, 1H, H-2″), 7.73 (d, 1H, $J = 2.0$ Hz, H-4),

7.89 (dq, 1H, $J = 2.0$, 8.0 Hz, H-6′), 8.07 (dd, 1H, $J = 2.0$, 8.0 Hz, H-2′), 9.73 (s, 1H, NH), 10.45 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ: 14.0 (q), 40.6 (t), 60.9 (t), 103.0 (d), 117.0 (d), 117.9 (d), 119.6 (s), 120.0 (s), 123.0 (d), 123.4 (s), 126.1 (s), 129.0 (d), 129.1 (d), 130.3 (d), 131.7 (s), 134.4 (s), 134.8 (d), 136.9 (s), 146.8 (s), 161.7 (s), 163.3 (s), 172.2 (s). Anal. Calcd. (%) for $C_{22}H_{18}ClFN_4O_3S$: C, 55.87; H, 3.84; N, 11.85; found: C, 55.91; H, 3.81; N, 11.88.

*4.1.4.7. Ethyl 3-benzoylamino-5-[(5-methyl-1H-imidazol-4-yl-methyl)-amino]-benzo[b]thiophene-2-carboxylate (**6g**).* Yield 49%. Mp 169–171 °C. IR ν_{max}: 3607, 3336 (NH), 1671, 1543 (CO) cm^{-1}. ^{1}H NMR (DMSO-d_6) δ: 1.22 (t, 3H, $J = 6.0$ Hz, CH_3), 2.24 (s, 3H, CH_3), 4.22–4.33 (m, 4H, OCH_2, NCH_2), 5.27 (t, 1H, $J = 4.0$ Hz, NH), 7.27 (d, 1H, $J = 8.0$ Hz, H-7), 7.52 (s, 1H, H-2″), 7.60–7.66 (m, 5H, C_6H_5), 8.12 (d, 1H, $J = 2.0$ Hz, H-4), 8.15 (dd, 1H, $J = 2.0$, 8.0 Hz, H-6), 10.25 (s, 1H, NH), 11.96 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ: 10.0 (q), 14.0 (q), 39.0 (t), 60.8 (t), 102.8 (d), 117.9 (d), 122.9 (s), 123.5 (d), 125.0 (s), 125.9 (s), 127.8 (d), 128.4 (d), 129.2 (d), 131.0 (s), 132.3 (s), 132.7 (d), 146.5 (s), 147.5 (s), 160.8 (s), 165.4 (s), 171.9 (s). Anal. Calcd. (%) for $C_{23}H_{22}N_4O_3S$: C, 63.58; H, 5.10; N, 12.89; found: C, 63.54; H, 5.06; N, 12,86.

*4.1.4.8. Ethyl 5-[(5-methyl-1H-imidazol-4-yl-methyl)-amino]-3-(4-methoxy-benzoylamino)-benzo[b]thiophene-2-carboxylate (**6h**).* Yield 34%. Mp 165–1667 °C. IR ν_{max}: 3603, 3328 (NH), 1670, 1611 (CO) cm^{-1}. ^{1}H NMR (DMSO-d_6) δ: 1.18 (t, 3H, $J = 6.2$ Hz, CH_3), 2.09 (s, 3H, CH_3), 3.87 (s, 3H, OCH_3), 4.06 (br s, 2H, CH_2), 4.22 (q, 2H, $J = 6.2$ Hz, CH_2), 6.07 (br s, 1H, NH), 6.87 (s, 1H, H-4), 7.00–7.18 (m, 3H, H-6, H-3′, H-5′), 7.51 (s, 1H, H-2″), 7.67 (d, 1H, $J = 8.2$ Hz, H-7), 8.06 (d, 2H, $J = 8.0$ Hz, H-2′, H-6′), 10.17 (s, 1H, NH), 11.36 (s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ: 10.0 (q), 14.0 (q), 40.1 (t), 55.4 (q), 60.7 (t), 99.5 (s), 102.9 (d), 110.5 (s), 113.6 (s), 117.9 (d), 121.3 (s), 122.5 (d), 122.8 (s), 126.1 (d), 129.5 (s), 129.7 (d), 133.1 (d), 135.6 (s), 137.0 (s), 146.5 (s), 162.0 (s), 164.8 (s). Anal. Calcd. (%) for $C_{24}H_{24}N_4O_4S$: C, 62.05; H, 5.21; N, 12.06; found: C, 62.09; H, 5.24; N, 12.10.

*4.1.4.9. Ethyl 5-[(5-methyl-1H-imidazol-4-yl-methyl)-amino]-3-(4-methyl-benzoylamino)-benzo[b]thiophene-2-carboxylate (**6i**).* Yield 32%. Mp 164–166 °C. IR ν_{max}: 3615, 3360 (NH), 1689, 1667 (CO) cm^{-1}. ^{1}H NMR (DMSO-d_6) δ: 1.18 (t, 3H, $J = 6.2$ Hz, CH_3), 2.09 (s; 3H, CH_3), 2.42 (s, 3H, CH_3), 4.06 (br, s, 2H, CH_2), 4.22 (q, 2H, $J = 6.2$ Hz, CH_2), 6.05 (br s, 1H, NH), 6.88 (d, 1H, $J = 2.0$ Hz, H-4), 7.06 (dd, 1H, $J = 2.0$, 8.6 Hz, H-6), 7.38 (d, 2H, $J = 8.0$ Hz, H-3′, H-5′), 7.44 (s, 1H, H-2″), 7.67 (d, 1H, $J = 8.6$ Hz, H-7), 8.98 (d, 2H, $J = 8.0$ Hz, H-2′, H-6′), 10.23 (s, 1H, NH), 11.84 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ: 10.1 (q), 14.0 (q), 21.0 (q), 39.0 (t), 60.8 (t), 102.8 (d), 117.9 (d), 122.8 (d), 126.1 (s), 127.8 (d), 128.9 (d), 129.6 (s), 131.2 (s), 133.1 (d), 135.4 (s), 137.0 (s), 141.8 (s), 146.6 (s), 161.9 (s), 165.3 (s), 171.1 (s), 172.1 (s). Anal. Calcd. (%) for $C_{24}H_{24}N_4O_3S$: C, 64.27; H, 5.39; N, 12.49; found: C, 64.31; H, 5.36; N, 12.51.

*4.1.4.10. Ethyl 5-[(5-methyl-1H-imidazol-4-yl-methyl)-amino]-3-(3,4,5-trimethoxy-benzoylamino)-benzo[b]thiophene-2-carboxylate (**6j**).* Yield 56%. Mp 167–168 °C. IR ν_{max}: 3617, 3363 (NH), 1690, 1665 (CO) cm^{-1}. ^{1}H NMR (DMSO-d_6) δ: 1.25 (t, 3H, $J = 6.2$ Hz, CH_3), 2.16 (s; 3H, CH_3), 3.81 (s, 3H, OCH_3), 3.94 (s, 6H, OCH_3x2), 4.35 (d, 2H, $J = 1.6$ Hz, CH_2), 4.29 (q, 2H, $J = 7.0$ Hz, CH_2), 6.23 (t, 1H, $J = 1.6$ Hz, NH), 6.85 (d, 1H, $J = 1.6$ Hz, H-4), 7.11 (dd, 1H, $J = 2.0$, 8.8 Hz, H-6), 7.46 (s, 2H, H-2′, H-6′), 7.62 (s, 1H, H-2″), 7.74 (d. 1H, $J = 8.8$ Hz, H-7), 10.27 (s, 1H, NH), 12.09 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ: 14.0 (q), 21.0 (q), 40.1 (t), 56.0 (q), 60.1 (q), 60.8 (t), 99.5 (s), 102.7 (s), 105.3 (d), 117.9 (s), 123.0 (d), 126.1 (s), 129.1 (s), 133.2 (d), 135.2 (s), 137.0 (s), 140.3 (s), 140.4 (s), 146.6 (s), 152.6 (d), 161.8 (s), 164.8 (s), 172.0 (s). Anal. Calcd. (%) for $C_{26}H_{28}N_4O_6S$: C, 59.53; H, 5.38; N, 10.68; found: 59.56; H, 5.40; N, 10.66.

*4.1.4.11. Ethyl 5-[(5-methyl-1H-imidazol-4-yl-methyl)-amino]-3-(4-trifluoromethyl-benzoylamino)-benzo[b]thiophene-2-carboxylate (**6k**).* Yield 34%. Mp 171–173 °C. IR ν_{max}: 3612, 3294 (NH), 1691, 1644 (CO) cm^{-1}. ^{1}H NMR (DMSO-d_6) δ: 1.18 (t, 3H, $J = 7.0$ Hz, CH_3), 2.08 (s, 3H, CH_3), 4.05 (br s, 2H, CH_2), 4.22 (q, 2H, $J = 7.0$ Hz, CH_2), 6.03 (br s, 1H, NH), 6.88 (d, 1H, $J = 2.0$ Hz, H-4), 7.06 (dd, 1H, $J = 2.0$, 8.6 Hz, H-6), 7.44 (s, 1H, H-2″), 7.68 (d, 1H, $J = 8.6$ Hz, H-7), 7.97 (d, 2H, $J = 8.2$ Hz, H-3′, H-5′), 8.20 (d, 2H, $J = 8.2$ Hz, H-2′, H-6′), 10.53 (s, 1H, NH), 11.67 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 10.3 (q), 14.0 (q), 39.1 (t), 60.9 (t), 99.5 (s), 102.4 (d), 118.1 (d), 122.9 (d), 123.6 (s), 125.4 (s), 125.5 (d), 126.1 (s), 128.7 (d), 131.9 (s), 133.1 (d), 134.5 (s), 137.0 (s), 137.9 (s), 146.8 (s), 161.7 (s), 164.5 (s), 172.0 (s). Anal. Calcd. (%) for $C_{24}H_{21}F_3N_4O_3S$: C, 57.36; H, 4.21; N, 12.15; found: C, 57.46; H, 4.31; N, 12.25.

*4.1.4.12. Ethyl 5-[(5-methyl-1H-imidazol-4-yl-methyl)-amino]-3-(3-chloro-4-fluoro-benzoylamino)-benzo[b]thiophene-2-carboxylate (**6l**).* Yield 35%. Mp 184.7–186 °C. IR ν_{max}: 3607, 3332 (NH), 1701, 1655 (CO) cm^{-1}. ^{1}H NMR (DMSO-d_6) δ: 1.24 (t, 3H, $J = 7.1$ Hz, CH_3), 2.15 (s, 3H, CH_3), 4.11 (br s, 2H, CH_2), 4.28 (q, 2H, $J = 7.1$ Hz, CH_2), 6.10 (br s, 1H, NH), 6.92 (d, 1H, $J = 1.7$ Hz, H-4), 7.12 (dd, 1H, $J = 8.8$ Hz, H-6), 7.47 (s, 1H, H-2″), 7.66–7.75 (m, 2H, H-7, H-2′), 8.13 (dd, 1H, $J = 2.1$, 13.3 Hz, H-6′), 8.34 (dd, 1H, $J = 2.0$, 7.1 Hz, H-5′), 10.50 (s, 1H, NH) 11.96 (br s, 1H, NH). ^{13}C NMR (DMSO-d_6) δ: 10.5 (q), 14.0 (q), 39.1 (t), 60.9 (t), 99.5 (s), 102.4 (d), 116.9 (d), 117.4 (d), 118.1 (d), 119.6 (s), 119.9 (s), 123.0 (d), 123.5 (s), 126.1 (s), 129.2 (d), 130.3 (d), 131.7 (s), 134. 5 (s), 137.0 (s), 146.8 (s), 161.7 (s), 163.3 (s), 172.2 (s). Anal. Calcd. (%) for $C_{23}H_{20}ClFN_4O_3S$: C, 56.73; H, 4.14; N, 11.50; found: C, 56.77; H, 4.10; N, 11.54.

4.2. Biology

4.2.1. Chemicals and reagents

Propidium iodide, ribonuclease A (RNAse A), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), and DMSO were obtained from Sigma Aldrich (St. Louis, MO, USA). RPMI, fetal bovine serum (FBS), phosphate buffered saline (PBS), L-glutamine solution (200 mM), trypsin-EDTA solution (170.000 U/l trypsin and 0.2 g/l EDTA) and penicillin-streptomycin solution (10.000 U/ml penicillin and 10 mg/ml streptomycin) were purchased from Lonza (Verviers, Belgium).

4.2.2. Cell culture

The cancer cell line HeLa (human epithelial cervical cancer) was obtained from American Type Culture Collection (ATCC) (Rockville, MD, USA). The cells were cultured in RPMI supplemented with 5% FBS, 2 mM L-glutamine, 50 IU/ml penicillin, and 50 μg/ml streptomycin and maintained in a humidified atmosphere with 5% CO_2 at 37 °C. The cells were routinely cultured in 75 cm^2 culture flasks and were trypsinized using trypsin-EDTA when the cells reached approximately 80% confluence. Exponentially growing cells were used for experiments.

4.2.3. Antiproliferative evaluation assay

3-benzoylamino-benzo[*b*]thiophene derivatives **6a**–**l** and **13a**–**f** were submitted to the MTT assay to assess the growth inhibition activity against HeLa cells. The MTT assay is a measurement of cell metabolic activity, quite effective in estimating cell proliferation, which is based on the protocol first described by Mossmann [25]. The assay was performed as previously described [26]. The cells were seeded into a series of standard 96-well plates in 100 μl of complete culture medium at 1.0×10^4 cells/cm^2. Cells were incubated for 24 h under 5% CO_2 at 37 °C and the medium was then replaced with 100 μl of fresh medium supplemented by 5% (v/v) FBS containing the treatments. Benzothiophene derivatives were

previously dissolved in DMSO to obtain 20 mM stock solutions. Working solutions were freshly prepared on the day of testing by dilutions of the stock solutions in the complete culture medium. For the experiments a concentration range from 50 to 0.05 μM was used. 24 h after seeding aliquots of 100 μl of different solutions at the appropriate concentrations were added to the appropriate wells and the cells were incubated for 48 h without renewal of the medium. In each experiment, DMSO concentration never exceeded 0.25% and culture medium with 0.25% DMSO was used as control. After the incubation time, cells were washed and 100 μL FBS-free medium containing 0.5 mg/mL of MTT was added. The medium was discarded after a 4 h incubation at 37 °C and formazan blue formed in the cells was dissolved in DMSO. The absorbance (optical density, OD) at 570 nm of MTT-formazan was measured in a microplate reader. As the absorbance is directly proportional to the number of living, metabolically active cells, the percentage of growth (PG) to with respect to untreated cell control for each drug concentrations was calculated according to one of the following two expressions:

$$\text{if } (\text{ODtest} - \text{ODtzero}) \geq 0, \text{then}$$
$$\text{PG} = 100 \times (\text{ODtest} - \text{ODtzero})/(\text{ODctr} - \text{ODtzero})$$
$$\text{if } (\text{ODtest} - \text{ODtzero}) < 0, \text{then}$$
$$\text{PG} = 100 \times (\text{ODtest} - \text{ODtzero})/\text{ODtzero}$$

where ODtzero is the average of optical density measurements before exposure of cells to the test compound, ODtest is the average of optical density measurements after the desired period of time, and ODctr is the average of optical density measurements after the desired period of time with no exposure of cells to the test compound. The concentration necessary for 50% of growth inhibition (GI50) for each derivative was calculated from concentration–response curves using linear regression analysis by fitting the test concentrations that give PG values above and below the reference value (50%). Each result was the mean value of three separate experiments performed in quadruplicate.

4.2.4. Cell-cycle analysis

Effects of 3-benzoylamino-benzo[b]thiophene derivatives **13d**, **6j**, and **6l** exposure on cell-cycle were performed by DNA staining with propidium iodide (PI) and flow cytometry analysis. HeLa cells were seeded on 12 well plates at a density of 2.0×10^4 cells/cm^2, and treated 24 h after seeding without or with indicated concentrations of the test compound for 24 h. Following the treatments, cells were collected, washed in PBS and stained with staining solution (20 μg/ml propidium iodide, 200 μg/ml RNAse A and 0.1% Triton X-100 in PBS), for 30 min at 37 °C. The DNA contents of more than 10,000 cells were subjected to fluorescence-activated cell sorting (FACS) analysis (Coulter® Epics® XLTM, Beckman) and the percentage of cells belonging to the different compartments of the cell cycle was determined. All experiments were performed in duplicate and reproduced two times.

4.2.5. Statistical analysis

All data are expressed as mean ± SD. Statistical difference was calculated using unpaired Student's *t*-test. Bonferroni least-significance difference test was used to examine difference between group means. Values of p lower than 0.05 were considered significant.

4.3. Computational details

The compounds of the Anti-Cancer Agents Mechanism Database (NCI ACAM Database) [21–23], were drawn and optimized *in vacuo* by Ligprep software of MAESTRO SUITE [27]. The NCI ACAM Database entries containing cations or consisting of a mix of two structures were excluded. Thus the starting database was constituted from 114 compounds (Table 1 in supporting information) classified in six mechanism of action: 30, Alkylating Agents; 13, Antimitotic Agents; 24, Topoisomerase I Inhibitors; 15, Topoisomerase II Inhibitors; 16, RNA/DNA Antimetabolites; 16, DNA Antimetabolites (Table 2). 2D and 3D molecular descriptors were calculated for all structures to achieve all the structural information useful for building a lock model database. Each descriptor value range is delimited by μDj(MA) ± σDj(MA), where (MA) is a specific mechanism of action, μDj(MA) is the molecular descriptor j average value and σDj(MA) is the standard deviation of descriptor j. When the molecular descriptor value Dj of a tested structure X falls within the defined range (μDj ± σDj) $\alpha = 1$ (i.e. D1, D3, and Dj), otherwise $\alpha = 0$. Therefore, after processing descriptor values as mentioned above, every training set database structure is converted into a binary sequence. Then all of the binary values are summed, so the higher is the number of fitted pins (descriptors with $\alpha = 1$), the higher will be the affinity of the investigated compound to a specific class with potential anticancer mechanism. Thus, the percentage of affinity A% (Eq. (1)) for each compound belonging to training set database has been defined for each class (MA) as:

$$A\% = \sum \alpha i, j(MA) \Big/ D_{tot} * 100 \quad (1)$$

where $\Sigma\alpha i, j$ (MA) is the sum of all fitted molecular descriptors for the MA class and D_{tot} is the total of the molecular descriptors used in the VLAK protocol.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2014.12.002.

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