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Benzo[*b*]thiophene-6-carboxamide 1,1-dioxides: Inhibitors of human cancer cell growth at nanomolar concentrations

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ABSTRACT

Benzo[*b*]thiophenesulfonamide 1,1-dioxide derivatives (BTS) were described as candidate antineoplastic drugs. In the hope of finding new compounds with improved antitumour activity and reduced toxicity, we have designed and synthesized a small series of benzo[*b*]thiophene-6-carboxamide 1,1-dioxide derivatives (BTC) structurally related with the best reported BTS. Growth inhibition of HTB-54, CCRF-CEM and HeLa tumour cells lines at nanomolar concentrations was exhibited by some of the BTC. Hydrophobic substituents on the carboxamide group increased cytotoxicity but substitution by a hydroxy group diminished it, thus pointing to the electronic density on benzo[*b*]thiophene nucleus as a determinant factor. The process of cell death induced by BTC derivatives was further analyzed in CCRF-CEM cells, where these compounds induced apoptosis in a time and dose-dependent manner and cell cycle arrest at S phase. BTC derivatives also induced a significant increase in intracellular ROS levels in this cell line. Previous treatment of the cells with the antioxidant *N*-acetyl-cysteine abrogated the induction of apoptosis by BTC indicating that ROS generation is a previous event required to trigger the BTC induced apoptotic process.

1. Introduction

Many efforts have been made to produce new arylsulfonamides[1–9] with improved antitumour activity and reduced toxicity. The last and the most promising *N*-acyl derivatives from Abbott Lab[8] as well as other aromatic sulfonamides (ASU, Fig. 1)[10] induce apoptosis through the bcl-2-dependent pathway. The core of ASU seems to be related with the arylsulfonylureas (BSU, Fig. 1) previously reported by us[11–13] and others.[14,15] Isozyme IX of human carbonic anhydrase (hCA IX) has emerged as another interesting target for the design of antitumour arylsulfonamides because it is highly over expressed in many cancer types while present in few normal tissues.[16,17] Within this group of compounds, we have reported the synthesis and in vitro antitumour activity of several benzo[*b*]thiophenesulfonamide 1,1-dioxide derivatives (BTS, Fig. 1).[2,11,18] These compounds, that showed a clear correlation between their lipophilicity (log *P*), their cytotoxic activities[2] and their ability to inhibit the tNOX activity of the plasma membrane,[19] induced in human leukaemia CCRF-CEM cells a typical process of apoptosis that included cell shrinkage, mitochondrial dysfunction, phosphatidylserine translocation to cell surface, caspase activation, chromatin condensation and internucleosomal DNA degradation.[12,13] BTS derivatives also induced in CCRF-CEM cells an accumulation of intracellular reactive oxygen species (ROS) and, since previous treatment of the cells with the antioxidant *N*-acetyl-cysteine abrogated the BTS induced cytochrome C release, caspase-3 activation and cell death, it has been proved that ROS are required for the BTS induced apoptotic effect.[12] Interestingly, N-substituted with short chains-BTS also showed some selectivity for the inhibition of the tumour-associated hCA IX over its cytosolyc counterpart hCA II.[16,18] Despite this fact, since some of the most potent inhibitors among the BTS are not cytotoxic (R = R_1 = H 2,3-dihydro derivative) and the contrary (R = benzyl, R_1 = H),[18] BTS cytotoxic activity seems to be unrelated with hCA inhibition.

Interestingly, benzo[*b*]thiophene-4-carboxamide 1,1-dioxide derivatives (hBTC, Fig. 2) have been described in the literature as preventive for various inflammatory and neoplastic diseases caused by an abnormal production of interleukin-6 or interleukin-12.[20] Moreover, 2-carboxamide analogues of BTS were patented as microbicides in phytosanitary practice.[21] Other carboxamide derivatives of heteroaryl nucleus including thiophene,[22,23] furane,[22] pyrrole,[22] pyrazole,[24] thiazole,[25,26] pyridine,[27] pyridazine,[28] pyridoindole[29] or thienopyridine[30] are suitable for the treatment of cancer because of their ability to inhibit different kinases. Remarkably, active heteroaryl carboxamides usually bring alternate aromatic systems, hydrogen bond donor/acceptors or lipophilic groups as in KI (Fig. 2).

Since substitution of the sulfonamide group of the BTS derivatives by a carboxamide group could improve their lipophilicity,

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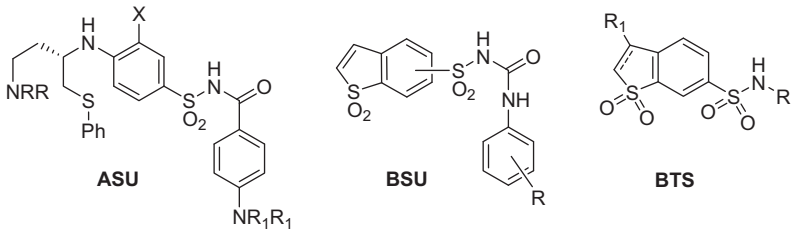


Figure 1.

to expand and further optimise our series of BTS derivatives we decided to test the replacement of the 6-sulfonamide group within our BTS series[2] by a set of alternative carboxamides. Thus, here we describe the synthesis and biological evaluation of new benzo[*b*]-thiophene-6-carboxamide 1,1-dioxide derivatives (BTC, Fig. 2). Series of BTC was built from the residues of the more active BTS's and some other residues as reference. Substituents at BTC position 3 were not considered because of R_1 electron releasing groups in BTS drove to inactive compounds. Neither the 2,3-dihydro derivatives from BTC were studied because their BTS analogues were not very cytotoxic. The studied BTC series starts from an unsubstituted 6-carboxamide (**4a**) as the reference and continues with derivatives including 4-methoxyphenyl (**4b**) and butyl (**4d**) groups, the best cytotoxic *N*-aryl and *N*-alkyl residues found within the BTS series. Phenylethylamines are much less toxic than arylamines[31] and so, since we expect a better metabolic profile *N*-2-(4-methoxyphenyl)ethyl BTC (**4c**) was also included in the series.

2. Results and discussion

BTC's **4a**–**4d** were obtained in 35–75% yield from the 6-aminobenzo[*b*]thiophene 1,1-dioxide **1** following typical methods as described in Scheme 1. The 6-ciano derivative **2** was prepared by diazotization and subsequent treatment of the mixture with cuprous cyanide at 40 °C. Acid **3** was obtained by refluxing **2** in 37% hydrochloric acid. The reaction of **3** with thionyl chloride yielded its acyl chloride, which was then treated with amines and triethylamine at room temperature to give the corresponding amides **4**. To compare, new *N*-2-(4-methoxyphenyl)ethyl (**5c**) and *N*-benzyl-*N*-methyl (**6e**) BTS derivatives and 6-hydroxybenzo[*b*]thiophene 1,1-dioxide (**7**) were also prepared from **1** through known methods.[2,32]

The growth inhibitory activity of these compounds was tested in vitro by examining their cytotoxic effects against HeLa (cervix epitheloid carcinoma), HTB-54 (lung carcinoma), HT-29 (colon carcinoma), MEL-AC (melanoma), K-562 (myelocytic leukaemia) and CCRF-CEM (lymphocytic leukaemia) cells through an MTT-based colorimetric assay.[33] The response parameter GI_{50}, concentration that reduces by 50% the growth of treated cells, is shown in Table 1. As can be observed, under the described conditions BTC derivatives **4a**–**4d** strongly inhibited growth of every cell line tested, with HeLa, HTB-54 and CCRF-CEM as the most sensitive ones. As expected, N-substitution modulates BTC cytotoxicity: compounds **4b**, **4c** and **4d** that carry hydrophobic groups attached to the carboxamide one displayed a stronger toxicity against every tested cell line than the lead compound **4a**. The *N*-aryl BTC derivative **4b** came up as the most active compound with GI_{50} values ranging from 2 to 4 nM against HTB-54, CCRF-CEM and HeLa cells, and from 47 to 760 nM against HT-29, K-562 and MEL-AC cells. Compound **4b** was also active at the same level of commercial Doxorubicin (GI_{50} values of 33 nM in CCRF-CEM, 6 nM in MEL-AC and 20 nM in K-562 and HeLa cells).[2,19] However, cytotoxicity of the *N*-aralkyl or *N*-alkyl BTC derivatives **4c** and **4d** was similar against every tested cell line apart from HTB-54. On the other hand, when cytotoxic activities of BTC and BTS were compared we found again that the N-substitution modulates activity of the compounds: the *N*-aryl derivative was the most active among the tested compounds in both series (GI_{50} values of **5b** ranging 1–200 nM).[2] However, N-substituents from BTC or BTS produce slightly different effects depending on the tested cell line. Thus, while 2-(4-methoxyphenyl)ethyl BTC **4c** and BTS **5c** derivatives showed similar GI_{50} values at HTB-54 and MEL-AC cell lines, **5c** was much more cytotoxic against K-562 and CCRF-CEM cells. On the contrary, cytotoxic activities of N-unsubstituted **4a** and **5a** derivatives were within the same order of magnitude at every cell line tested, except for HTB-54 cells that were more sensitive to **4a**. Moreover, since GI_{50} of *N*-benzyl BTS **5e** and its N-methylated derivative **6e** compare in most of the tested cell lines, free NH group seems to play a residual role in the studied activity. Last, electronic density on the benzo[*b*]thiophene 1,1-dioxide nucleus also seems to be important for the cytotoxic activity: as GI_{50} of **4a**, **5a** and **7** reflects, carboxamide and sulfonamide, two electron withdrawing groups, are both more active than hydroxy, an electron releasing group. These results, that point to the electrophilic nature of the thiophene 1,1-dioxide moiety as the main cause of the cytotoxic properties of benzo[*b*]thiophene 1,1-dioxide derivatives, are well in agreement with the lost of cytotoxicity of the BTS derivatives when the double bond of the thiophene was hydrogenated.[11,13] Interestingly, the benzo[*b*]thiophene nucleus of BTC allows more simple cytotoxic compounds than other reported heteroaryl carboxamides. Thus, BTC **4b** displays a slightly lower cytotoxic activity against K562 cells than the specifically designed pyridocarboxamide KI (GI_{50} 1.6 nM[27]). However, compound **4b** is more toxic against HeLa cervix carcinoma cells than reported *N*-(4-aryl-2-thiazoly)benzamides or their equivalent pyridocarboxamides[34] and, it is also worth noting that **4b** inhibits the growth of both, HTB-54 lung carcinoma and CCRF-CEM lymphocytic

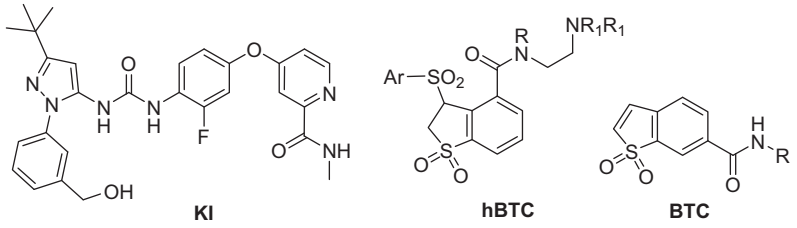
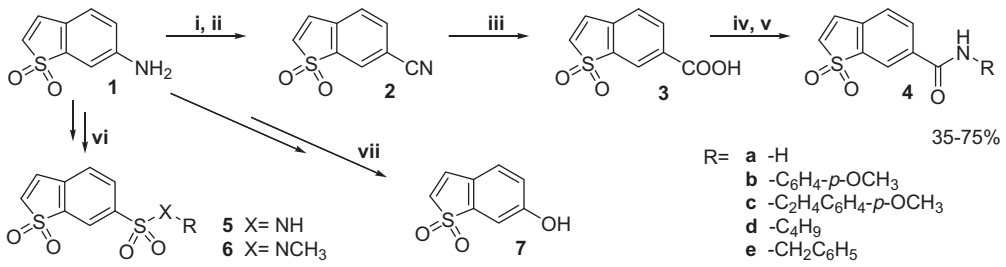
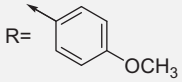
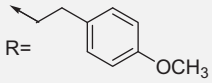
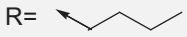
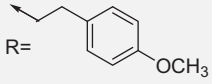
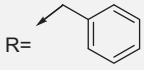
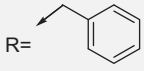


Figure 2.



Scheme 1. Reagents and conditions: (i) $NaNO_2$, HCl (aq); (ii) CuCN (aq), ethyl acetate; (iii) HCl 37%, reflux; (iv) $SOCl_2$ toluene, reflux; (v) RNH_2, toluene, (vi) as previously described,[2] (vii) following the general procedure of Ref. 32.

Table 1
Cytotoxic activities (GI_{50}, μM) of BTC **4a–4d**, BTS **5a**, **5c**, **5e**, **6e** and hydroxy derivative **7** against tumour cell lines

Compound	HTB-54	K-562	MEL-AC	HT-29	CCRF-CEM	HeLa
4a R = –H	0.22	7.85	3.52	5.19	3.55	0.65
4b R= 	0.002	0.047	0.76	0.35	0.004	0.003
4c R= 	0.64	2.66	1.32	2.08	0.73	0.040
4d R= 	0.09	2.73	1.42	2.32	0.28	0.08
5a R = –H	5.89[a]	29.4[a]	7.14[a]	8.55[a]	2.86[a]	2.21
5c R= 	0.62	0.006	1.93	0.22	0.005	0.12
5e R= 	0.70[a]	0.72[a]	0.40[a]	0.84[a]	0.33[a]	0.010
6e R= 	0.40	38.1	2.30	35.0	0.63	0.010
7	7.53	51.1	24.0	2.04	19.2	N.D.

[a] From Ref. 2 N.D.: not determined.

leukaemia cells at levels that compare to those reported for the better patented benzo[*b*][1,6]naphthyridine-4-carboxamides against the related LLTC and P388 cell lines[35] and to those reported for the best ASU against H146 small lung cancer cells (GI_{50} 30 nM,[8] Fig. 1).

To further analyze the process of cell death induced by BTC derivatives, the apoptotic status and cell cycle phase distribution was determined in leukaemia CCRF-CEM cells. Apoptosis was evaluated by measuring the exposure of phosphatidylserine on the cell membranes after 12–48 h of treatment with 0–6 μM of the corresponding BTC. Obtained results are shown in Figure 3. As can be observed, each one of the tested compounds was able to induce apoptosis in a time and dose-dependent fashion. Moreover, as aforementioned for the cytotoxic activity, induction of apoptosis was stronger with compound **4b** than with any other tested compound. Thus, after 12 h of treatment with 6 μM **4b** the incidence of apoptotic cells at the culture was 30.4% (vs 2.6% in untreated control cells), a value that raised to 83% after 48 h of treatment (vs 2.9% in untreated control cells). This result resembles the previously reported for BTS,[12] where induction of a typical apoptotic process was observed. Cell cycle distribution was determined by measurement of the DNA content of cells exposed to 2.5 μM of the corresponding BTC for 24 h using propidium iodide (PI) staining and flow cytometry. Obtained results are shown in Figure 4. A significant increase of the hypodipliod subG1 population was detected for each one of the tested compounds, a result that confirms their role as inducers of apoptosis. At the same time a significant increase of the cell percent in the S phase, as well as a reduction of the G_0/G_1 cell population was detected. These data are consistent with cell cycle arrest at the S phase. Additional work to unravel the specific regulatory proteins responsible for the cell cycle arrest is under progress.

Because accumulation of ROS has been proposed to be an essential event in apoptotic responses induced by some antitumour drugs,[36–41] we next decided to test the effect of BTC on intracellular ROS level. *N*-(4-Hydroxyphenyl)retinamide (4-HPR), which has been reported to induce ROS overproduction on cervical carcinoma

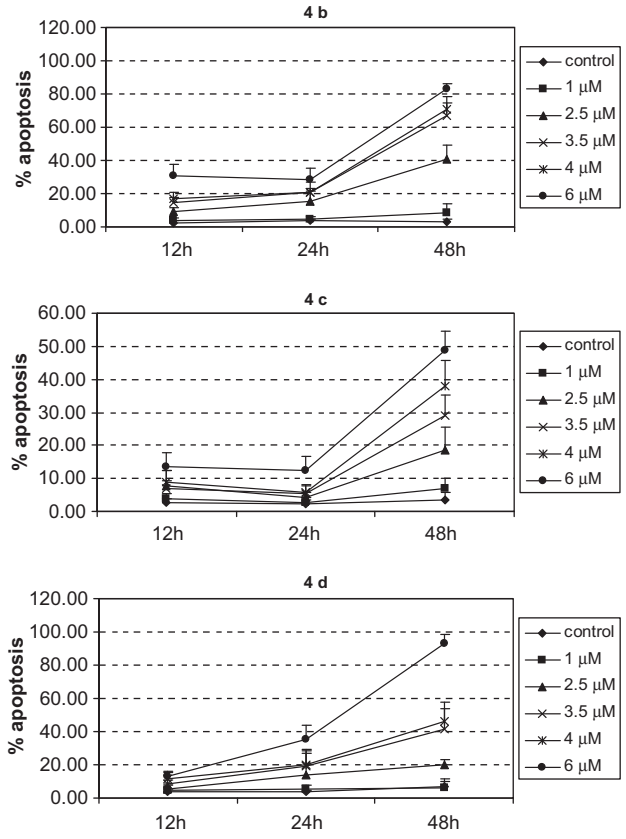


Figure 3. Apoptotic effect of BTC **4b**–**4d** at 12, 24 and 48 h. CCRF-CEM cells were incubated in the presence of BTC **4b**, **4c** and **4d** at the indicated concentration. The percentage of apoptotic cells was determined by flow cytometry. Values represent means ± SD of five independent experiments (duplicate wells).

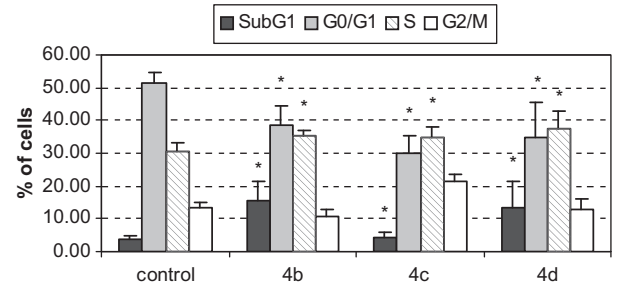


Figure 4. BTC effect on cell cycle phase distribution. CCRF-CEM cells were treated with 2.5 μM **4b**, **4c** or **4d** for 24 h. After treatment, cells were processed for propidium iodide staining and cellular DNA content determined by flow cytometry. Data were obtained from 10,000 events and the percentage of cells in the Sub G_1 G_0/G_1, S and G_2/M phase was determined by EXPO 32 ADC Analysis System. Results are expressed as mean ± SD of six independent experiments (duplicate wells). *p <0.05 respect to control cells.

cells,[42] was used as a positive control. As shown in Figure 5, BTC derivatives induced a significant increase in intracellular ROS level. To investigate if the observed accumulation of ROS was necessary for the apoptotic effect of BTC, induction of apoptosis by **4b** was analyzed in the presence or absence of the antioxidant *N*-acetylcysteine (NAC), which was added to the culture medium 1 h earlier than **4b**. As shown in Figure 6, the effect of **4b** was inhibited by the previous addition of NAC, indicating that BTC-induced ROS generation is a previous event required to trigger the apoptotic process. In this sense, BTC are also similar to previously reported BTS[12] and to other antitumour drugs that have been shown to induce apoptosis through a mechanism mediated by ROS.[36–41] Additional work is required to find out the origin of BTC-induced ROS. In this sense, we have previously shown that BTS are able to inhibit the tNOX activity of the plasma membrane of CCRF-CEM and other tumour cells.[13,19] The putative implication of this enzyme and/or other ROS-regulating systems of the cell in BTC-induced ROS generation is an interesting issue to be analyzed in future experiments.

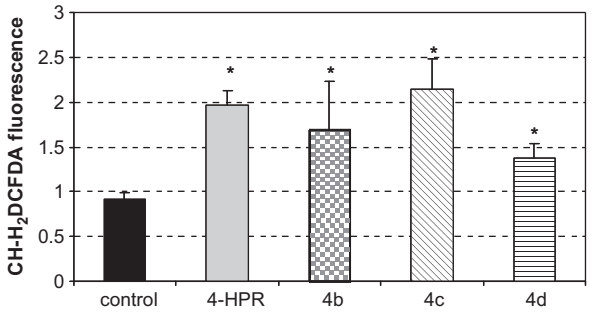


Figure 5. Incubation of CCRF-CEM cells with BTC induces an increase in ROS production. Cells were incubated in the presence or absence (control) of **4b**, **4c** and **4d** (2.5 μM) for 24 h. *N*-(4-Hydroxyphenyl)retinamide (4-HPR) was used as a positive control. Reactive oxygen species were measured using CM-H_2DFDA. Results are presented as the fold increased with respect to untreated control cells. Each bar represents mean ± SD of two independent experiments (triplicate wells). *p <0.05 respect to control cells.

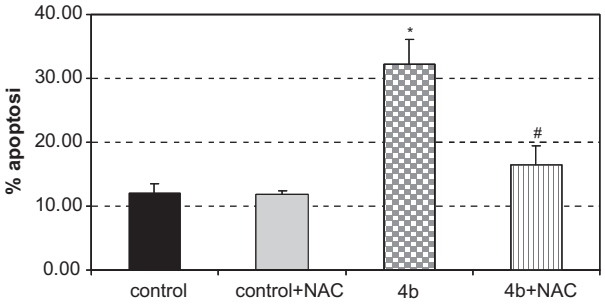


Figure 6. NAC prevents the apoptotic process induced by compound **4b**. The percentage of apoptotic cells in the culture was determined by flow cytometry after 24 h in the absence (control) or presence of 2.5 μM **4b**, 1 mM *N*-acetyl-cysteine (NAC) or both (**4b** + NAC). Cells were preincubated with NAC for 1 h before **4b** addition. Results are presented as the mean ± SD of three independent experiments (triplicate wells). *p <0.001with respect to the control, #p <0.001with respect to **4b**.

In conclusion, BTC is a novel family of potent cytotoxic compounds that confirms the thiophene 1,1-dioxide nucleus as the true lead to obtain improved antitumour agents. BTC induce cell cycle arrest, ROS over generation and apoptosis in leukaemia CCRF-CEM cells. Additional experiments are now in progress to help clarify their mechanism of action and specificity towards tumour cells.

3. Experimental

Melting points were determined in a Mettler FP82HT-FP80 system and are uncorrected. Routine monitoring of reactions was performed using Allegra SilG/UV_{254} (0.20 mm). All chromatographic separations were performed using silica gel (Merck 60 230–400 mesh). ^{1}H NMR spectra were recorded on a Varian 200 MHz spectrometer with TMS as internal standard. Chemical shifts are reported in ppm and coupling constants in hertz. IR spectra were recorded on a Nicolet-Avatar 360 FT-IR spectrophotometer. Elemental analyses were carried out on a Carlo Erba EA1108 elemental analyser from vacuum-dried samples (over phosphorus pentoxide at 3–4 mmHg, 6–12 h at about 30–70 °C).

3.1. Synthesis of precursors

3.1.1. Benzo[*b*]thiophene-6-carbonitrile 1,1-dioxide (2)

6-Aminobenzo[*b*]thiophene 1,1-dioxide **1**[2] (10.0 g, 55 mmol) was dissolved in a mixture of water (100 mL) and HCl (37%, 10 mL, 121 mmol) by heating if necessary. This solution was cooled at −10 °C and a solution of $NaNO_2$ (4.0 g, 58 mmol) in water (15 mL) was added drop wise. The resulting mixture was stirred at −5 °C for 30 min to form the diazonium salt. The mixture was cautiously neutralized by adding dry sodium carbonate with constant stirring, using litmus paper to determine the end-point. A solution of NaCN (12.4 g, 253 mmol) in water (60 mL) was added to a solution of CuCl (9.8 g, 99 mmol) in water (60 mL) at *T* <20 °C. This mixture was stirred at room temperature for 30 min and then ethyl acetate (200 mL) was added. To this mixture, the solution of the neutralized diazonium salt was slowly added with stirring at 5 °C. Stirring was continued for 1 h at room temperature and at 40 °C for 30 min. Cold water (500 mL) was added and the mixture was extracted with ethyl acetate (2 × 200 mL). Extracts were washed with $NaHCO_3$ (aq, 3 × 200 mL, water), dried ($MgSO_4$) and evaporated at reduced pressure. The solid material was purified by column chromatography (dichloromethane) to give 6-cyanobenzo[*b*]thiophene 1,1-dioxide (4.79 g, 45.3%): IR (KBr, cm^{-1}) 1151, 1309 (SO_2); 2228 (CN); ^1H NMR ($CDCl_3$ δ): 7.95 (s, 1H, H-7); 7.86 (d, J_{4-5} = 7.7 Hz, 1H, H-4); 7.50 (d, 1H, H-5); 7.27 (d, J_{3-2} = 7.0 Hz, 1H, H-3); 6.91 (d, 1H, H-2); Anal. Calcd ($C_9H_5NO_2S$): C, 56.53; H, 2.64; N, 7.33; S, 16.77. Found: C, 55.99; H, 2.73; N 7.07; S, 16.73.

3.1.2. Benzo[*b*]thiophene-6-carboxylic acid 1,1-dioxide (3)

A suspension of benzo[*b*]thiophene-6-carbonitrile 1,1-dioxide **2** (4.36 g, 23 mmol) in HCl (37%, 25 mL, 0.30 mol) was refluxed for 8 h. The resulting yellow solid was filtered, washed with water and dried to give the carboxylic acid **3** (3.02 g, 63.0%). IR (KBr, cm^{-1}): 1159, 1301 (SO2); 1704 (CO); 3468 (OH).

3.2. General procedure for the synthesis of the benzo[*b*]thiophene-6-carboxamide 1,1-dioxide derivatives (4)

A solution of the benzo[*b*]thiophene-6-carboxylic acid 1,1-dioxide **3** (0.34 g, 1.6 mmol) was treated with thionyl chloride (0.6 mL, 8.3 mmol) in boiling dry toluene (25 mL) for 30 min. The mixture was evaporated to afford a crude solid of the corresponding acid chloride which was identified by IR spectroscopy (1751, C=O). It was sufficiently pure for the next step. A solution of the crude acid chloride in dry toluene (20 mL) was added to a mixture of the appropriate amine (1.60 mmol) and triethylamine (1.60 mmol) in toluene (5 mL) (or CH_2Cl_2 for the butyl derivative) at 0 °C with stirring. Stirring was continued for 30–60 min at room temperature. Solvents were removed under vacuum. The residual material was dissolved in ethyl acetate (100 mL), washed successively with 5% HCl (2 × 50 mL), $NaHCO_3$ (aq 2 × 50 mL) and water (50 mL), dried over $MgSO_4$ and evaporated. The solid was purified by crystallization. The following products were obtained using the general procedure.

3.2.1. Benzo[*b*]thiophene-6-carboxamide 1,1-dioxide (4a)

Trough a stirred solution of the acid chloride from **3** in dry toluene (20 mL), ammonia gas was passed for 30 min. The solvent was removed and the yellow solid was recrystallized from ethanol to give compound **4a** (0.25 g, 74.0%): Mp 216–217 °C; IR (KBr, cm^{-1}): 1129.36, 1160, 1290 (SO_2); 1676 (CO); 3340, 3299 (NH_2). ^1H NMR ($CDCl_3$ δ): 7.95 (s, 1H, H-7); 7.86 (d, J_{4-5} = 7.7 Hz, 1H, H-4); 7.50 (d, 1H, H-5); 7.27 (s, J_{3-2} = 7.0 Hz, 1H, H-3); 6.91 (s, 1H, H-2). Anal. Calcd ($C_9H_7NO_3S$): C, 51.67; H, 3.37; N, 6.69; S, 15.33. Found: C, 51.59; H, 3.55; N, 6.46; S, 14.89.

3.2.2. *N*-(4-Methoxy)phenylbenzo[*b*]thiophene-6-carboxamide 1,1-dioxide (4b)

Starting from the acid **3** and *p*-anisidine, compound **4b** was obtained and recrystallized from ethanol (0.25 g, 49.1%): Mp 215–216 °C; IR (KBr, cm^{-1}): 1252, 1288 (SO_2); 1669 (CO); 3381 (NH). ^1H NMR (DMSO-d_6 δ): 10.37 (s, 1H, NH); 8.39 (s, 1H, H-7); 8.24 (d, J_{4-5} = 7.7 Hz; 1H, H-5); 7.76–7.72 (m, 2H, H-4, H-3); 7.68 (d, $J_{2'-3'}$ = 9.2 Hz, 2H, H-2′, H-6′); 7.54 (d, J_{2-3} = 6.6 Hz, 1H, H-2); 6.95 (d, 2H, H-3′, H-5′); 3.75 (s, 3H, O–CH_3). Anal. Calcd ($C_{16}H_{13}NO_4S$): C, 60.94; H, 4.16; N, 4.44; S, 10.17. Found: C, 60.49; H, 4,41; N, 4.39; S, 10.28.

3.2.3. *N*-(4-Methoxyphenyl)ethylbenzo[*b*]thiophene-6-carboxamide 1,1-dioxide (4c)

Starting from acid **3** and 2-(4-methoxyphenyl)ethylamine, compound **4c** was obtained and recrystallized from ethanol (0.18 g, 32.4%): Mp 149–150 °C; IR (KBr, cm^{-1}): 1252, 1288 (SO_2); 1669 (CO); 3381 (NH); ^1H NMR (DMSO-d_6 δ): 8.83 (t, J_{NH-CH2} = 5.5 Hz, 1H, NH); 8.21 (s, 1H, H-7); 8.12 (d, J_{5-4} = 7.9 Hz, 1H, H-5); 7.71–7.67 (m, 2H, H-4, H-3); 7.51 (d, J_{2-3} = 7.0 Hz, 1H, H-2); 7.15 (d, $J_{2'-3',6'-5'}$ = 8.4 Hz, 2H, H-2′, H-6′); 6.85 (d, 2H, H-3′, H-5′); 3.71 (s, 3H, –OCH_3); 3.51–3.41 (m, 2H, NH–CH_2–CH_2); 2.79 (t, $J_{CH2-CH2}$ = 7.0 Hz, 2H, –CH_2CH_2–C_6H_4). Anal. Calcd ($C_{18}H_{17}NO_4S$): C, 62.96; H, 4.99; N, 4.08; S, 9.34. Found: C, 62.54; H, 4.82; N, 3.95; S, 9.32.

3.2.4. *N*-Butylbenzo[*b*]thiophene-6-carboxamide 1,1-dioxide (4d)

Starting from the acid **3** and *n*-butylamine, compound **4d** was obtained and recrystallized from hexane/ethyl acetate (0.23 g, 53.6%): Mp 121–122 °C; IR (KBr, cm^{-1}): 1148, 1304 (SO_2); 1637 (CO); 3286 (NH_2). ^1H NMR (DMSO-d_6 δ): 8.73 (t, J_{NH-CH2} = 5.5 Hz, 1H, NH); 8.15 (s, 1H, H-7); 8.15 (d, J_{5-4} = 7.9 Hz, 1H, H-5); 7.70 (m, 2H, H-4, H3); 7.52 (d, J_{3-2} = 7.0 Hz, 1H, H-2); 3.27 (m 2H, NH–CH_2); 1.56–1.23 (m, 4H, CH_2–CH_2–CH_3); 0.91 (t, $J_{CH3-CH2}$ = 7.3 Hz, 3H, –CH_3). Anal. Calcd ($C_{13}H_{15}NO_3S$): C, 58.85; H, 5.70; N, 5.28; S, 12.09. Found: C, 58,57; H, 5.89; N, 5.33; S, 12.02.

3.3. General procedure for the synthesis of the benzo[*b*]thiophene-6-sulfonamide 1,1-dioxide derivatives (5 and 6)

Compounds **5** and **6** were obtained from 6-aminobenzo[*b*]thiophene 1,1-dioxide **1** and the corresponding amine by the method previously reported by us.[2]

3.3.1. *N*-(4-Methoxyphenyl)ethylbenzo[*b*]thiophene-6-sulfonamide 1,1-dioxide (5c)

Starting from **1** (0.33 g, 1.8 mmol) and 2-(4-methoxyphenyl)ethylamine (0.27 g, 1.8 mmol), compound **5c** was obtained and recrystallized from 2-propanol (0.25 g, 36.0%): Mp 123–124 °C; IR (KBr) cm^{-1}: 1141, 1305 (SO_2); 1247 (C_{ar}–O–C); 3290 (NH). ^1H NMR ($CDCl_3$ δ): 8.07 (s, 1H, H-7); 7.5 (d, J_{54} = 8.1 Hz, 1H, H-5); 7.44 (d, 1H, H-4); 7.25 (d, J_{32} = 7.0 Hz, 1H, H-3); 6.99 (d, $J_{2'3',6'5'}$ = 8.8 Hz, 2H, H-2′, H-6′); 6.88 (d, 1H, H-2); 6.79 (d, 2H, H-3′, H-5′); 4.44 (t, 1H, NH); 3.76 (s, 3H, –OCH_3); 3.24 (c, 2H, –NHCH_2–); 2.73 (t, *J* = 7.0 Hz, 2H, CH_2–CH_2–C_6H_4). Anal. Calcd ($C_{17}H_{17}NO_5S_2$): C, 53.81; H, 4.52; N, 3.69; S, 16.90. Found: C, 54.20; H, 4.36; N, 3.60; S, 16.71.

3.3.2. *N*-Benzyl-*N*-methyl-benzo[*b*]thiophene-6-sulfonamide 1,1-dioxide (6e)

Starting from **1** (0.33 g, 1.8 mmol) and *N*-benzylmethylamine (0.22 g, 1.8 mmol), compound **6e** was obtained and recrystallized from 2-propanol (0.13 g, 20%): Mp 105–106 °C; IR (KBr) cm^{-1}: 1146, 1302 (SO_2). ^1H NMR ($CDCl_3$ δ): 8.10 (s, 1H, H-7); 8.02 (d, J_{54} = 8.1 Hz, 1H, H-5); 7.54 (d, 1H, H-4); 7.32–7.24 (m, 6H, H-3, C_6H_5); 6.90 (d, J_{23} = 7.0 Hz, 1H, H-2); 4.20 (s, 2H, –CH_2–) 2.66 (s,

3H, $-CH_3$). Anal. Calcd ($C_{16}H_{15}NO_4S_2$): C, 55.00; H, 4.33; N, 4.01; S, 18.35. Found: C, 54.99; H, 4.08; N, 3.87; S, 18.22.

3.3.3. 6-Hydroxybenzo[*b*]thiophene 1,1-dioxide (7)

Compound **7** was obtained following the method for preparation of phenols from diazonium ions via generation and oxidation of aryl radicals by copper salts, previously reported.[32] 6-Aminobenzo[*b*]thiophene 1,1-dióxido **1** (0.31 g, 1.7 mmol) was dissolved at −5 °C in 3 mL of H_2SO_4 35%. Then a solution of $NaNO_2$ (0.12 g, 1.7 mmol in water 1 mL) was dropwise added with stirring at −5 °C. Subsequently a few crystals of urea were added to decompose any excess sodium nitrite and then a solution of Copper(II) nitrate hemi(pentahydrate) (0.48 g, 2.1 mmol in water 90 mL) was dropwise added with stirring at −5 °C. To the stirred mixture solid Copper(I) oxide (0.24 g 1.7 mmol) was added and the stirring was continued for 1 h at −5 °C. Cold water (50 mL) was added and the mixture was extracted with ethyl acetate. The extracts were dried, evaporated and the solid material was purified by column chromatography (toluene/dioxane 4:3) to give **7** (0.05 g, 16.2%). IR (HATR) cm^{-1}: 1146, 1288 (SO_2); 3394 (OH). ^1H NMR (DMSO-d_6 δ): 10.61 (s, 1H, OH); 7.50 (d, J_{32} = 7.0 Hz, 1H, H-3); 7.38 (d, J_{45} = 8.1 Hz, 1H, H-4); 7.11 (d, 1H, H-2); 7.1 (s, 1H, H-7); 7.0 (d, 1H, H-5). Ms: 182 [M^+], 153, 125, 97, 89.

3.4. Biological evaluation

3.4.1. Cells and cell culture

American Type Culture Collection (ATCC, Manassas, VA, USA) or European Collection of Cell Cultures (ECACC, Porton Down, Salisbury, UK) provided human tumour cell lines. Six cell lines were used: two human leukaemia (K-562 and CCRF-CEM) and four human solid tumours, one colon carcinoma (HT-29), one lung carcinoma (HTB54), one cervix epitheloid carcinoma (HeLa) and one melanoma (MEL-AC). MEL-AC cells were kindly provided by Dr. Natalia López-Moratalla (Universidad de Navarra, Pamplona, Spain). Cells were grown in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% faetal calf serum, 2 mM L-glutamine, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 10 mM HEPES buffer (pH 7.4).

3.4.2. Cytotoxicity analysis

The cytotoxic effect of each substance was tested at five different doses between 0.01 and 100 μM. In the case of compound **4b** additional doses between 10^{-5} and 10^{-2} μM were tested. Each substance was initially dissolved in DMSO at a concentration of 0.1 M, and serial dilutions were prepared using culture medium. The plates with cells from the different lines, to which media containing the substance under test were added, were incubated for 72 h at 37 °C in a humidified atmosphere containing 5% CO_2. Cell viability was then determined by assaying for the reduction of MTT to formazan.[33]

3.4.3. Assessment of apoptosis

The apoptotic status of the cells was evaluated by measuring the exposure of phosphatidylserine on the cell membranes using *Annexin V-FITC Kit* (BD Pharmingen, San Jose, CA, USA)[43] under the conditions described by the manufacturer. Briefly, after incubation with and without BTC, cells (5×10^5) were pelleted and washed in PBS. Cells were then stained with annexin V-FITC and propidium iodide for 15 min at 4 °C in the dark and analyzed on a Coulter Epics XL flow cytometer (Beckman Coulter, Miami, Florida, USA).

3.4.4. Cell cycle analysis

CCRF-CEM cells were cultured in complete medium in six-well plates (10^6 cells/ml; 3 ml) for 24 h with o without BTC at 37 °C. Harvested cells (700 g, 6 min, 4 °C) were washed with PBS and fixed in 70% ethanol at 4 °C 20 min. After fixation cells were recovered by centrifugation, suspended in PBS containing IP (50 μg/ml) and RNAse (200 μg/ml) and incubated at 37 °C for 30 min. Cell cycle analysis was performed using a COULTER Epics XL flow cytometer (Beckman Coulter, Miami, FL, USA). Cells were excited with an argon laser emitting at 488 nm and propidium iodide was detected using 620 nm band pass filter. Cell cycle distribution was determined by EXPO 32 ADC cell cycle analysis software. A doublet discriminatory gate in a FL3/Aux FL3 plot was established to ensure only authentic targeted events were permitted for analysis, and G_0/G_1, S and G_2/M regions were determined manually in a FL3-n events histogram. Data from 10,000 cells were collected for each data file.

3.4.5. Measurement of ROS

Cells were grown at a density of 1×10^6 cells/ml in a six well plate and cultured in the presence or in the absence of compound BTC (2.5 μM) for 24 h. Generation or intracellular ROS was examined using the oxidation-sensitive fluorescent probe CM-H_2DCFDA (Molecular Probes, Eugene, OR, USA). Briefly, the cells (2×10^6) were incubated with 1 μM CM-H_2DCFDA for 60 min at 37 °C. Subsequently, the cellular fluorescence was analyzed using a Coulter Epics XL flow cytometer (Beckman Coulter, Miami, Florida, USA).

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