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Antiproliferative potency of novel benzofuran-2-carboxamides on tumour cell lines: Cell death mechanisms and determination of crystal structure

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ABSTRACT

In this manuscript the synthesis and biological activity of novel heterocyclic derivatives of benzofuran-2-carboxamides **3a**–**j** and **6a**–**f** is presented. Biological evaluation *in vitro* revealed that only few compounds exerted concentration-depended antiproliferative effects on tumour cell lines at micromolar concentrations. In particular, 2-imidazolynyl substituted compound **6f** showed selectivity on SK-BR-3 cell line while 2-*N*-acetamidopyridyl substituted **3h** and 2-imidazolynyl substituted amide **3i** showed selective concentration-dependent antiproliferative effects on SW620 cell line. Compounds **3h** and **6f** induced apoptosis while compound **3i** acted through a cell death mechanism other than apoptosis.

1. Introduction

Small heteroaromatic molecules still represent the mainstay in classical chemotherapy for cancer treatment. However, the selectivity issue and better activity are still major requirements in development of novel anticancer drugs. The amide functionality is the common backbone of numerous organic molecules and natural products that bear diverse chemical and pharmacological features [1,2]. Consequently, amides play a key role in important life processes, whereas, for example, the amide bond is crucial for protein formation. Moreover, according to the Comprehensive Medicinal Chemistry database, amide bond is widely incorporated in the structure of 25% well known drugs. Amides are usually stable, neutral and have both hydrogen-bond acceptor/donator properties which are very important for the synthesis of versatile heteroaromatic molecules.

In addition, benzofurans and their derivatives possess a broad range of important biological activities including anticancer, antibacterial, antifungal and antiviral properties. They have already attracted considerable attention amongst organic and medicinal chemists in the last few years [3,4]. For example, benzofuran-2-carboxamides showed excellent oral uptake indexes and blood–brain barrier permeability, anti-dopamine action, potent methamphetamine potentiation, antiemetic action and low toxicity [5]. Another series of benzofuran-2-carboxamides proved to inhibit mammalian 5-lipoxygenase enzyme and their usefulness in the treatment of asthma, allergic disorders and certain cardiovascular disorders [6]. Heteroaromatic benzofuran-2,3-dicarboxamides were found to inhibit activated blood coagulation factor X which is a key enzyme located in the position of extrinsic and intrinsic coagulation cascade reactions [7]. Benzofuran-2,5-carboxamides, structurally related to netropsin with two pyrrole-amide units as well as amidine terminal units, have showed reduced cytotoxic activity [8]. A group of authors has recently published cytotoxic activity results of benzofuran-2-yl-pyridines towards human liver cancer cell line Hep-G2 [9]. Alkyl and cykloalkyl substituted benzofuran-2-carboxamides were found to be a selective and directly acting H3 receptor antagonists and can be used as active substances, particularly in the treatment or prevention of diseases

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which are associated with the modulation of H3 receptors [10]. Further on, 2-benzofuran derivatives containing heterocyclic nuclei have shown antibacterial activity towards *Staphylococcus aureus* and *Candida albicans* bacteria [11], and benzoxazole derivatives of benzofurane-2-carboxamides showed a broad spectrum of antibacterial activity as well. Some of tested compounds showed a significant activity towards *Pseudomonas aeruginosa* that was comparable to the potency of a commercial antibacterial drug Rifampicin [12]. 5-Sulfonyl substituted versatile benzofurane derivatives were found to act as a resistance-repellent and multidrug resistant retroviral HIV protease inhibitors [13]. At last, 2-(3′,4′,5′-trimethoxybenzoyl)-benzo[*b*]furan derivatives with electron-donating (Me, OMe or OH) or electron-withdrawing (F, Cl and Br) substituents on the benzene ring, have shown prominent inhibition properties on tubulin polymerization and the cell cycle [14]. Such a large amount of evidence proves a role of benzofuran scaffold in diverse biological activities and their therapeutic potential. It is therefore worth an effort to further investigate this class of compounds.

As a part of our commitment to search for novel potential anticancer agents related to heterocyclic amides, we already reported the synthesis of various cyano- or amidino-substituted heteroaromatic benzo[*b*]thiophene-2-carboxamides with strong inhibitory activities on several human cancer cell lines (Fig. 1) [15,16]. 2-Imidazolinyl-substituted derivatives of pyridyl-benzo[*b*]thiophene-2-carboxamides exhibited strong growth inhibitory effect in the low micromolar range. Only minor structural difference between compounds in position of heterocyclic nitrogen in respect to the amidine substituent had exceptionally strong impact on their biological action. We have shown that 2-pyridyl-benzo[*b*]thiophene-2-carboxamide derivative binds to DNA minor groove in a form of dimmer, while 5-pyridyl-benzo[*b*]thiophene-2-carboxamide derivative which had the most potent effect on the cell cycle, pointing towards tubulin as cellular target of its biological action.

Encouraged by the results from this study, we set out to explore and synthesize novel heteroaromatic benzofuran-2-carboxamide derivatives **3a**–**j** and **6a**–**f** (Fig. 2). All newly synthesized compounds were tested for their antiproliferative activity on the panel of several human tumour cell lines as well as normal human fibroblasts.

2. Results and discussion

2.1. Chemistry

Newly prepared carboxamides shown in Fig. 2 were synthesized according to two main procedures shown in Schemes 1 and 2, by conventional methods of organic synthesis used for preparation of similar heterocyclic amides.

Starting from benzofuran-2-carbonylchloride **1**, in the reaction with versatile substituted anilines and amino-pyridines **2a**–**j**, 2-aminobenzothiazoles **4a**, **4c** and **5a** and 2-aminobenzimidazoles **4b**, **4d** and **5b** corresponding benzofuran-2-carboxamides **3a**–**j** and **6a**–**f** were prepared. Compound **3c**, as hydrochloride salt, was prepared from compound **3b** with $HCl_{(g)}$. Cyano and nitro substituted 2-aminobenzazoles **4a**–**d** were prepared by earlier described methods [17]. 2-Imidazolynyl substituted 2-aminobenzazoles **5a**,**b** were prepared according to Scheme 3, in the Pinner reaction from corresponding cyano precursors **4a**,**b** [18,19].

The structure of all newly synthesized benzofuran-2-carboxanilides were determined by NMR analysis, based on the analysis of H–H coupling constants as well as chemical shifts. In ^{1}H NMR spectra, the presence of proton of CONH group was confirmed by one-proton singlet at 10.30 ppm to 13.48 ppm. The molecular and crystal structure for compound **3f** is given in Supplementary material.

2.2. Biological activity

The *in vitro* screening of benzo[*b*]furane amides derivatives (**3a**–**6f**) on human tumour cell lines and normal (diploid) human fibroblasts WI38 (control cell line) revealed a strong and non-specific cytotoxic effect of all tested compounds at higher tested concentrations (10–100 μM) (Table 1).

Majority of tested compounds were cytotoxic to normal human fibroblasts as well. Interestingly, SK-BR-3 cell line was less sensitive towards all tested compounds with the exception to 2-imidazolynyl substituted compound **6f**, that showed good selectivity on this cell line in the micromolar range. The antiproliferative activity of 2-imidazolynyl substituted benzimidazole amide **6f** is increased in comparison to the structurally related 2-imidazolynyl substituted benzothiazole amide **6e** due to substitution of a sulphur atom instead of nitrogen at position 1. The cell cycle analysis confirmed a strong antiproliferative effect of compound **6f** on SK-BR-3 cells witnessed by dramatic perturbations in the cell cycle induced at micromolar concentrations (Table 2). An increase of cells in the G1 phase ranging between 18 and 30% accompanied with an increase of cells in the sub G1 phase in comparison to control has been observed upon 24-h treatment. Similarly, 48-h treatment of cells with compound **6f** induced an increase of sub G1 cell population and increased S-phase cell population by 33% at lower compound concentration. Such strong perturbations of the cell cycle induced

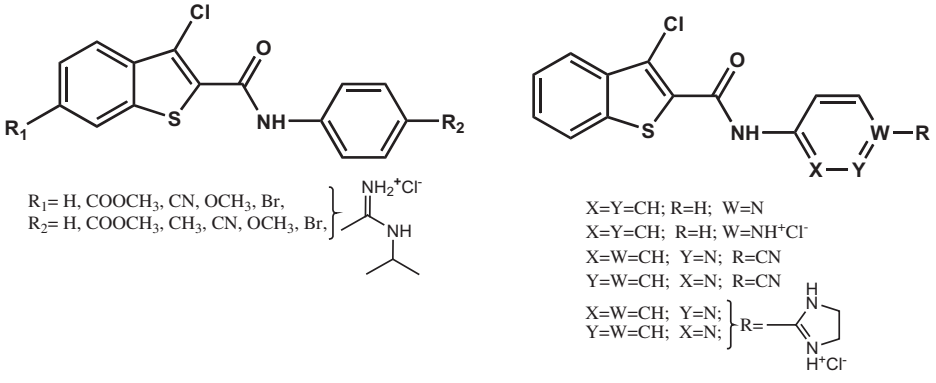


Fig. 1. Earlier prepared heteroaromatic benzo[*b*]thiophene-2-carboxamides.

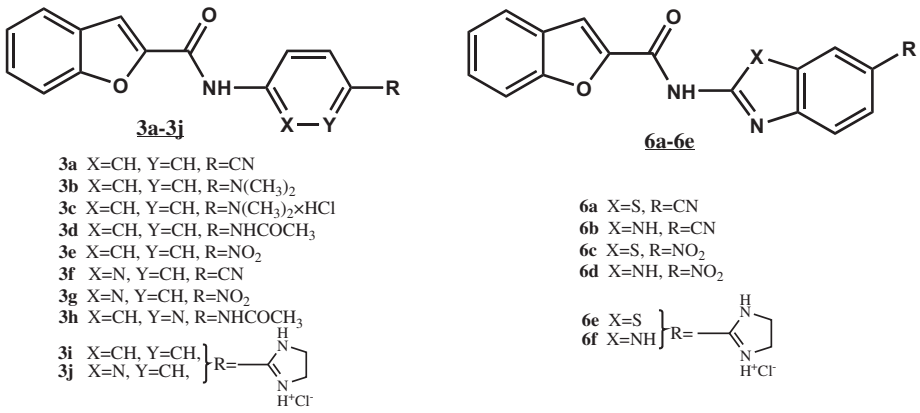


Fig. 2. Prepared heteroaromatic benzofuran-2-carboxamide derivatives **3a**–**j** and **6a**–**f**.

by very low compound concentrations confirm the strong antiproliferative effect previously observed in proliferation assays.

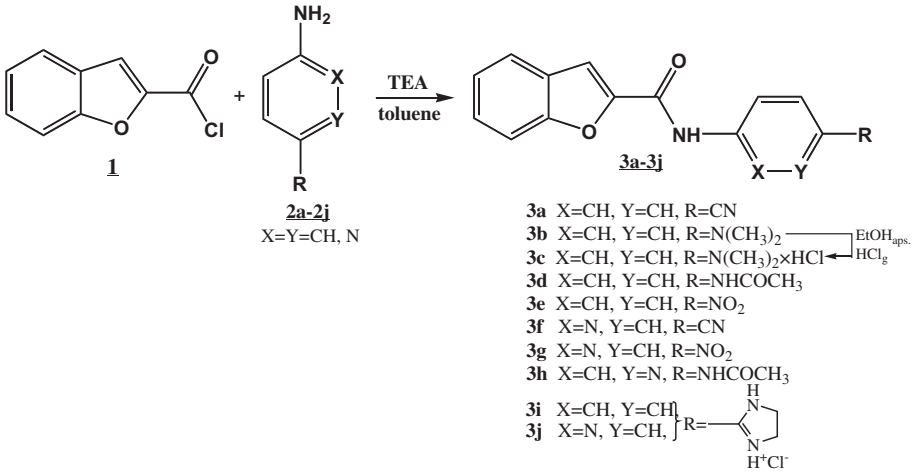
Cell death induction might be attributed to either the ability of benzofurane–carboxamide compounds to alkylate DNA and cause cytotoxic effects [20] or to DNA binding properties or changes of the cellular cytoskeleton that induce irreparable cell damage [15,16]. Current cancer chemotherapy primarily exerts the antitumour effect by triggering apoptosis in cancer cells where proteolytic enzymes, caspases, play a critical role in cell death execution [21]. Two major routes are known to involve different caspases in response to anticancer chemotherapy: the extrinsic (receptor) pathway and the mitochondria stimulated intrinsic pathway. Analysis of caspases 3, 8 and 9 activation upon treatment of SK-BR-3 cells with compound **6f** substantiate apoptosis activation in these cells (Fig. 3) which is consistent with antiproliferative results and cell cycle analysis. In some tumour cells, caspase-8 is known to be activated upon death receptor activation in quantities sufficient to directly activate downstream effector caspase-3 as well as a mitochondrial amplification loop required for full activation of caspases, inlcuding caspase 9 [22]. Our results speak in favour of this apoptotic mechanism in SK-BR-3 cells as well.

Similarly, 2-*N*-acetamidopyridyl substituted amide **3h** and 2-imidazolynyl substituted **3i** exerted a highly selective concentration-dependent antiproliferative effects in the micromolar range (0.1–1 μM) on SW620 cell line. In particular, pyridyl moiety of 2-*N*-acetamidopyridyl substituted amide **3h** correlated with increased antitumour activity and selectivity in comparison to the structurally related derivative 4-*N*-acetamidophenyl substituted amide **3d**. The cell cycle analysis of SW620 cells treated with compound **3h** showed increased S-phase cell population after 48-h. Similarly to compound **6f**, apoptosis induction in this cell line through death receptor activation and mitochondrial amplification [23] was corroborated by strong activation of tested caspases 3, 8 and 9 (Fig. 3).

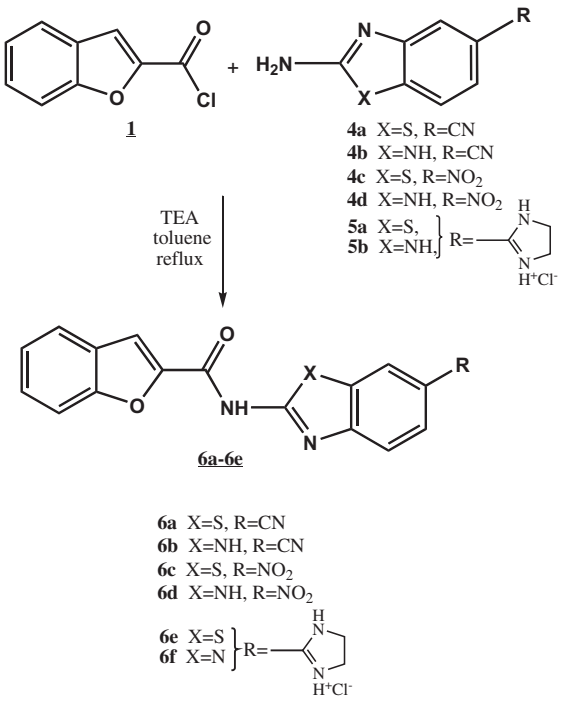
Further on, it seems that the imidazolynyl moiety of compound **3i** added to specificity and antiproliferative properties observed on SW620 cells. Contrary to compound **3h**, a small but statistically significant increase in G2/M cell population has been observed in SW620 cells treated with compound **3i** pointing to a cell death mechanism in SW620 other than apoptosis, *i.e.* mitotic catastrophe. Indeed, analysis of caspase activation showed no activation for caspases 3, 8 or 9 upon treatment of cells (Fig. 3). Our previous studies already showed that *N*-amidino-substituted benzimidazo[1,2-*a*]quinolones exerted a similar antiproliferative effect through mitotic catastrophe on SW620 cells as well [23]. This assumption however should be confirmed by additional tests (Table 3, Fig. 4).

3. Conclusions

We successfully prepared a series of novel heteroaromatic benzofuran-2-carboxamide derivatives **3a**–**j** and **6a**–**f**. In comparison to our previous results obtained by benzo[*b*]thiophene-2-



Scheme 1. Synthesis of benzofuran-2-carboxamide derivatives **3a**–**j**.



Scheme 2. Synthesis of benzofuran-2-carboxamide derivatives **6a**–**f**.

carboxamides, it seems that replacement of benzo[*b*]thiophene with benzo[*b*]furan seems to be detrimental for the antitumour activity. Indeed, all newly synthesized compounds were evaluated for the antiproliferative potency *in vitro* on human tumour cell lines and normal (diploid) human fibroblasts and showed non-selective effects on all tested cells with stronger antitumour activities detectable only in the highest tested concentrations. Obtained results however, revealed a potential for three compounds as selective antiproliferative compounds. In particular, 2-imidazolynyl substituted compound **6f** showed good selectivity on SK-BR-3 cell line while 2-*N*-acetamidopyridyl substituted amide **3h** and 2-imidazolynyl substituted compound **3i** showed selective concentration-dependent antiproliferative effects on MiaPaCa-2 and SW620 cell lines, respectively in the micromolar range. It seems that the antiproliferative activity of 2-imidazolynyl substituted benzimidazole amide **6f** is increased in comparison to the structurally related 2-imidazolynyl substituted benzothiazole amide **6e** due to substitution of a sulphur atom instead of nitrogen at position 1. In particular, pyridyl moiety of 2-*N*-acetamidopyridyl substituted amide **3h** correlated with increased antitumour activity and selectivity in comparison to the structurally related derivative 4-*N*-acetamidophenyl substituted amide **3d**. Further on, the imidazolynyl moiety of compound **3i** added to specificity and antiproliferative properties observed on SW620 cells. These compounds exerted different antiproliferative mechanisms, *i.e.* compounds **3h** and **6f** induced apoptosis through activation of caspases 3, 8 and 9 that might be indicative for activation of death receptors along with the intrinsic apoptotic pathway. Current cancer chemotherapy indeed, aims to activate apoptosis in cancer cells where the concomitant induction of the intrinsic apoptotic pathway plays a crucial role in chemotherapy potency towards tumour cells. Contrary, antiproliferative effect of compound **3i** relied on a cell death mechanism other than apoptosis, *i.e.* mitotic catastrophe that should be confirmed by additional studies.

Table 1
The inhibition effects of compounds **3a**–**6f** on the growth of selected tumour cells *in vitro*. The results are given as IC_{50} values in μM. The cell growth rate was evaluated by performing the MTT assay: experimentally determined absorbance values were transformed into a cell percentage growth (PG) using the formulae proposed by National Cancer Institute and described previously in Gazivoda et al. [20].

Compound	IC_{50}[a] (μM)					
	Cell lines					
	MCF-7	SK-BR-3	SW620	MiaPaCa-2	WI38	HeLa
3a	7.6	34.2	7.4	27.1	7.9	7.6
3b	35.6	>100	86	>100	>100	83.7
3c	8.5	>100	54.2	49.6	81.2	49.1
3d	45.1	>100	>100	>100	71.2	77.3
3e	4.9	>100	27.9	26.6	47.6	11.4
3i	26.2	40.5	6.4	31.5	23.3	27.5
3f	5.3	>100	69.2	44.7	52.4	23.9
3g	82.8	>100	76.5	73.1	37.8	42.4
3h	7.6	59.8	1.1	3.1	0.4	0.9
3j	>100	>100	67.1	>100	68.7	71.9
6a	21.5	>100	>100	>100	34.9	89.6
6c	63.9	>100	>100	>100	72.9	17.5
6e	50.9	>100	56.4	97.1	59.2	59.8
6b	>100	>100	9.2	>100	9.1	>100
6d	5.2	>100	6.9	9.3	8.6	9.2
6f	7	3.83	74.9	91.2	>100	68.5

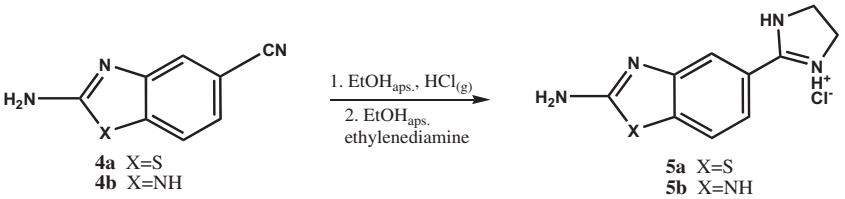
[a] IC_{50}; 50% inhibitory concentration, or compound concentration required to inhibit tumour cell proliferation by 50%. The IC_{50} values were calculated from dose–response curves using linear regression analysis by fitting the mean test concentrations that give PG values above and below the reference value. If, however, all of the tested concentrations produce PGs exceeding the respective reference level of effect (*e.g.* PG value of 50) for a given cell line, the highest tested concentration is assigned as the default value (>100). Each test point was performed in quadruplicate in three individual experiments.

4. Experimental

4.1. Chemistry

4.1.1. General methods

All chemicals and solvents were purchased from commercial suppliers including Aldrich, Fluka and Acros. Melting points were recorded on an Original Keller Mikroheitztisch apparatus (Reichert, Wien) and SMP11 Bibby apparatus. ^1H and ^{13}C NMR spectra were recorded on a Varian Gemini 300 or Varian Gemini 600 spectrophotometers at 300, 600, 150 and 75 MHz, respectively. All NMR spectra were measured in DMSO-d_6 solutions using TMS as an internal standard. Chemical shifts are reported in ppm (δ) relative to TMS. UV/vis spectra were recorded on Cary Eclipse 50



Scheme 3. Synthesis of 2-imidazolinyl substituted 2-aminobenzothiazole **5a** and 2-aminobenzimidazole **5b**.

Table 2
Flow cytometric analysis of the SK-BR-3 cell cycle upon 24- and 48-h treatments with compound **6f** at concentrations 5 μM and 10 μM. Statistically significant changes ($p < 0.05$) are denoted in bold and with an asterisk (*).

Compound **6f**		Cell percentage (% ± standard deviation)			
SK-BR-3					
Treatment		sub G1	G1	S	G2/M
24 h	Control/untreated	25.1 ± 0.4	40.1 ± 0.7	26.2 ± 2.3	33.7 ± 0.3
	5 μM	**33.1 ± 1.2***	**58.5 ± 0.4***	**31.5 ± 1.4***	**10 ± 1.1***
	10 μM	**31.5 ± 0.8 ***	**70 ± 0.2***	**23.6 ± 0.9***	**6.4 ± 0.6***
48 h	Control/untreated	27.5 ± 0.2	53.3 ± 1.5	35.2 ± 1.8	11.6 ± 0.3
	5 μM	**38.1 ± 0.6***	**21.8 ± 1.9***	**68.5 ± 1.4***	**9.7 ± 1.4***
	10 μM	**36.3 ± 0.9***	**52.8 ± 0.9***	**31.3 ± 1.1***	**15.9 ± 0.8***

(*) Statistically significant at $p < 0.05$.

Table 3
Flow cytometric analysis of the SW620 cell cycle upon 24- and 48-h treatments with compound **3i** at concentrations 5 μM and 10 μM. Statistically significant changes ($p < 0.05$) are denoted in bold and with an asterisk (*).

SW620		Cell percentage (% ± standard deviation)			
Treatment		Sub G1	G1	S	G2/M
Compound **3i**					
24 h	Control/untreated	6.9 ± 2.1	46.3 ± 1.8	41.1 ± 0.8	12.6 ± 1
	5 μM	7.1 ± 1.7	43.4 ± 2	43.9 ± 1.2	12.7 ± 0.9
	10 μM	10.4 ± 0.2	45.7 ± 1.9	40.9 ± 1.6	13.5 ± 3.5
48 h	Control/untreated	8.5 ± 1.5	51.1 ± 2.5	39.9 ± 0.5	8.9 ± 0.4
	5 μM	4.9 ± 0.3	46.8 ± 1.8	40.7 ± 3.3	8.3 ± 0.1
	10 μM	8.2 ± 1.7	**46.9 ± 0.4***	42.6 ± 0.5	**10.5 ± 0.9***
Compound **3h**					
24 h	Control/untreated	18.1 ± 3.8	52.6 ± 2.3	38.3 ± 0.4	9.3 ± 2.6
	5 μM	18.1 ± 2.4	46.8 ± 1.8	42.6 ± 3.2	10.7 ± 5
	10 μM	18.2 ± 5.5	43.5 ± 1.4	31.4 ± 11.1	25.3 ± 9.7
48 h	Control/untreated	5.8 ± 0.3	49.9 ± 1.4	41.6 ± 0.3	8.5 ± 1.6
	5 μM	4.7 ± 0.4	43.9 ± 0.5	44.9 ± 1.1	11.2 ± 0.6
	10 μM	4.3 ± 0.2	45.2 ± 0.3	**45.4 ± 0.2***	9.5 ± 0.5

spectrophotometer. Elemental analysis for carbon, hydrogen and nitrogen were performed on a Perkin–Elmer 2400 elemental analyzer and a Perkin–Elmer, Series II, CHNS Analyzer 2400. All compounds were routinely checked by TLC with Merck silica gel 60F-254 glass plates.

4.1.2. General method for the preparation of compounds *3a*, *3b*, *3d–j* and *6a–f*

To a solution of benzofurane-2-carbonyl chloride **1** in dry toluene, a solution of corresponding anilines and amino-pyridines **2a–j**, 2-aminobenzothiazoles **4a**, **4c** and **5a** and 2-aminobenzimidazoles **4b**, **4d** and **5b** in dry toluene was added dropwise, followed by the addition of Et_3N. The mixture was refluxed for several hours. After cooling, the resulting products were filtered off and recrystallized from methanol to obtain benzofurane-2-carboxamides **3a**, **3b**, **3d–j** and **6a–f**.

*4.1.2.1. N-(4-Cyanophenyl)benzofuran-2-carboxamide **3a**.* Compound **3a** was prepared using above described method, from benzofurane-2-carbonyl chloride **1** (1.36 g, 7.53 mmol) and 4-cyanoaniline **2a** (0.89 g, 7.53 mmol) in dry toluene (35 mL) followed by the addition of Et_3N (1.60 mL, 11.34 mmol). The mixture was refluxed for 24 h and worked up as it is described to give 0.73 g (33%) of white crystals; mp 196–197 °C; ^{1}H NMR (600 MHz, DMSO-d_6): $\delta = 10.54$ (s, 1H, NH_{amide}), 8.09 (d, 1H, $J = 7.86$ Hz, $H_{arom.}$), 7.95 (d, 1H, $J = 8.88$ Hz, $H_{arom.}$), 7.84 (s, 1H, $H_{arom.}$), 7.78 (d, 2H, $J = 8.96$ Hz, $H_{arom.}$), 7.70 (t, 1H, $J = 7.88$ Hz, $H_{arom.}$), 7.56 (t, 1H, $J = 7.78$ Hz, $H_{arom.}$), 6.99 (d, 2H, $J = 8.78$ Hz, $H_{arom.}$); ^{13}C NMR (75 MHz, DMSO-d_6): $\delta = 157.7$ (s), 155.3 (s), 148.6 (s), 136.0 (s), 135.3 (s), 131.9 (d, 2C), 131.8 (d), 128.4 (d), 127.8 (s), 124.8 (d, 2C), 123.9 (d), 118.4 (s), 112.8 (d), 112.4 (d); UV (EtOH) λ_{max}/nm = 318; elemental analysis calcd. (%) for $C_{16}H_{10}N_2O_2$: C 73.27, H 3.84, N 10.68; found C 73.12, H 3.59, N 10.47.

*4.1.2.2. N-(4-N,N-Dimethylaminophenyl)benzofuran-2-carboxamide **3b**.* Compound **3b** was prepared using above described method, from

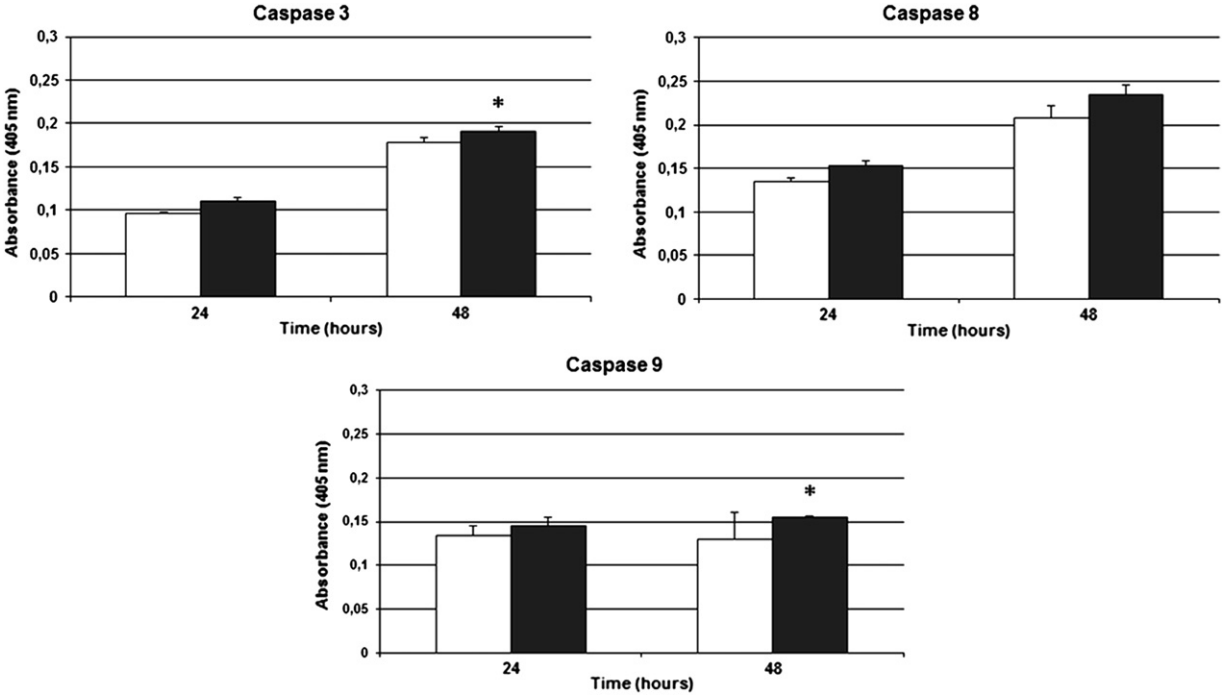


Fig. 3. Activation of caspases 3, 8 and 9 in SK-BR-3 cell line after 24 and 48 h treatments with compound **6f** at a concentration of 5 μM. Control; white columns. Compound **6f**; dark grey columns.

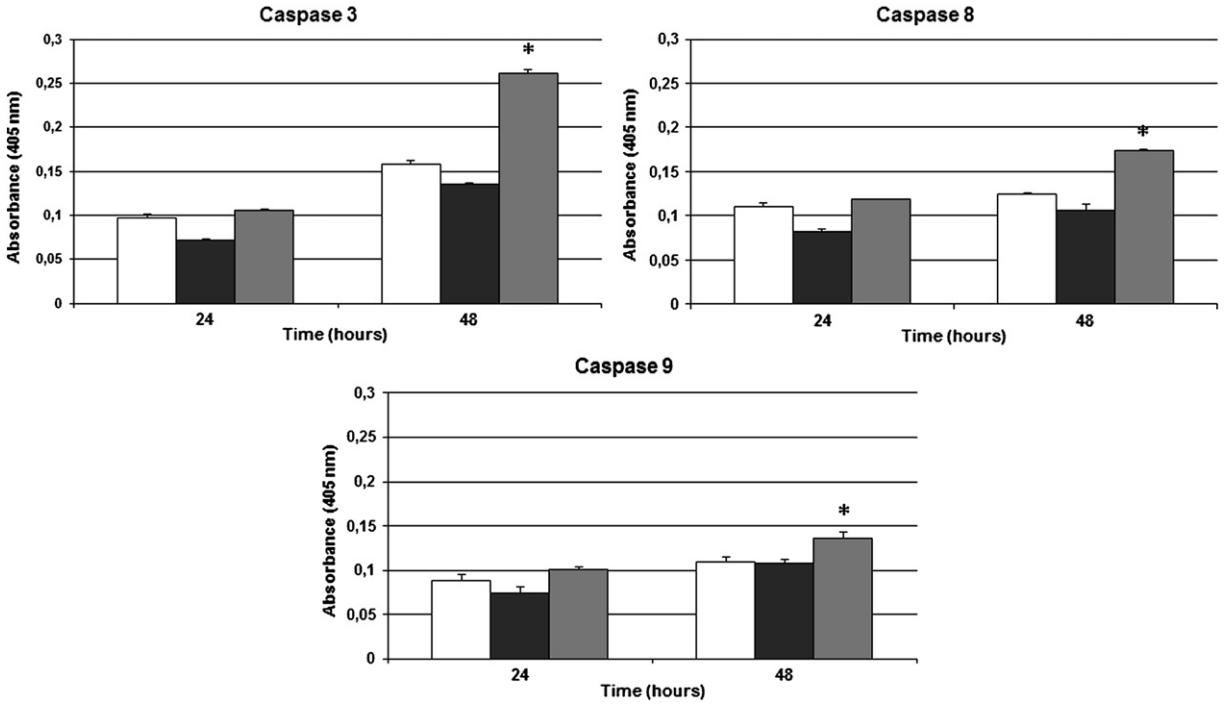


Fig. 4. Activation of caspases 3, 8 and 9 in SW620 cell line after 24 and 48 h treatments with compounds **3i** and **3h** at a concentration 10 μM. Control; white columns. Compound **3i**; dark grey columns. Compound **3h**; light grey columns.

benzofurane-2-carbonyl chloride **1** (0.20 g, 1.10 mmol) and 4-*N*,*N*-dimethylaminoaniline **2b** (0.17 g, 1.10 mmol) in dry toluene (15 mL) followed by the addition of Et_3N (0.22 mL, 1.54 mmol). The mixture was refluxed for 18 h and worked up as it is described to give 0.22 g (77%) of green powder; mp 154–155 °C; ^{1}H NMR (600 MHz, DMSO-d_6): δ = 10.30 (s, 1H, NH_{amide}), 7.79 (d, 1H, J = 7.74 Hz, $H_{arom.}$), 7.69 (d, 1H, J = 8.64 Hz, $H_{arom.}$), 7.68 (s, 1H, $H_{arom.}$), 7.60 (d, 2H, J = 8.88 Hz, $H_{arom.}$), 7.47 (t, 1H, J = 7.68 Hz, $H_{arom.}$), 7.34 (t, 1H, J = 7.56 Hz, $H_{arom.}$), 6.72 (d, 2H, J = 8.64 Hz, $H_{arom.}$), 2.86 (s, 6H, CH_3); ^{13}C NMR (75 MHz, DMSO-d_6): δ = 156.5 (s), 154.8 (s), 149.8 (s), 148.0 (s), 128.3 (s), 127.8 (s), 127.7 (d), 124.2 (d), 123.2 (d), 122.4 (d, 2C), 112.9 (d, 2C), 112.3 (d), 110.3 (d), 40.8 (q, 2C); UV (EtOH) λ_{max}/nm = 329, 273; elemental analysis calcd. (%) for $C_{17}H_{16}N_2O_2$: C 72.84, H 5.75, N 9.99; found C 73.04, H 5.58, N 10.18.

4.1.2.3. N-(4-Acetamidophenyl)benzofuran-2-carboxamide ***3d***. Compound **3d** was prepared using above described method, from benzofurane-2-carbonyl chloride **1** (0.30 g, 1.67 mmol) and 4-acetamidoaniline **2d** (0.25 g, 1.67 mmol) in dry toluene (20 mL) followed by the addition of Et_3N (0.32 mL, 2.32 mmol). The mixture was refluxed for 24 h and worked up as it is described to give 0.30 g (61%) of light pink crystals; mp 252–254 °C; ^{1}H NMR (600 MHz, DMSO-d_6): δ = 10.43 (s, 1H, NH_{amide}), 9.90 (s, 1H, NH), 7.80 (d, 1H, J = 7.74 Hz, $H_{arom.}$), 7.72 (d, 1H, J = 7.92 Hz, $H_{arom.}$), 7.71 (d, 2H, J = 8.98 Hz, $H_{arom.}$), 7.69 (s, 1H, $H_{arom.}$), 7.54 (d, 2H, J = 8.88 Hz, $H_{arom.}$), 7.48 (dt, 1H, J_1 = 8.34 Hz, J_2 = 1.24 Hz, $H_{arom.}$), 7.35 (dt, 1H, J_1 = 8.28 Hz, J_2 = 1.22 Hz, $H_{arom.}$), 2.03 (s, 3H, $COCH_3$); ^{13}C NMR (75 MHz, DMSO-d_6): δ = 168.3 (s), 157.2 (s), 154.9 (s), 149.0 (s), 147.9 (s), 130.6 (s), 127.8 (d), 127.6 (s), 124.4 (d), 123.5 (d), 122.0 (d), 121.9 (d, 2C), 112.4 (d, 2C), 111.5 (d), 23.8 (q); UV (EtOH) λ_{max}/nm = 322; elemental analysis calcd. (%) for $C_{17}H_{14}N_2O_3$: C 69.38, H 4.79, N 9.52; found C 69.12, H 4.96, N 9.76.

4.1.2.4. N-(4-Nitrophenyl)benzofuran-2-carboxamide ***3e***. Compound **3e** was prepared using above described method, from benzofurane-2-carbonyl chloride **1** (0.20 g, 1.10 mmol) and 4-nitroaniline **2e** (0.15 g, 1.10 mmol) in dry toluene (20 mL) followed by the addition of Et_3N (0.21 mL, 1.54 mmol). The mixture was refluxed for 16 h and worked up as it is described to give 0.17 g (55%) of white crystals; mp 212–213 °C; ^{1}H NMR (600 MHz, DMSO-d_6): δ = 11.09 (s, 1H, NH_{amide}), 8.28 (d, 2H, J = 7.20 Hz, $H_{arom.}$), 8.09 (d, 2H, J = 7.26 Hz, $H_{arom.}$), 7.88 (d, 1H, J = 0.72 Hz, $H_{arom.}$), 7.85 (d, 1H, J = 7.74 Hz, $H_{arom.}$), 7.74 (dd, 1H, J_1 = 8.34 Hz, J_2 = 1.20 Hz, $H_{arom.}$), 7.37 (dt, 1H, J_1 = 8.38 Hz, J_2 = 1.12 Hz, $H_{arom.}$); ^{13}C NMR (75 MHz, DMSO-d_6): δ = 157.6 (s), 155.1 (s), 148.5 (s), 145.2 (s), 143.2 (s), 128.1 (d), 127.5 (s), 125.3 (d, 2C), 124.5 (d), 123.6 (d), 120.6 (d, 2C), 112.5 (d), 112.4 (d); UV (EtOH) λ_{max}/nm = 319; elemental analysis calcd. (%) for $C_{15}H_{10}N_2O_4$: C 63.83, H 3.57, N 9.92; found C 63.95, H 3.74, N 10.13.

4.1.2.5. N-(5-Cyanopyridin-2-yl)benzofuran-2-carboxamide ***3f***. Compound **3f** was prepared using above described method, from benzofurane-2-carbonyl chloride **1** (1.00 g, 5.54 mmol) and 2-amino-5-cyanopyridine **2f** (0.66 g, 5.54 mmol) in dry toluene (30 mL) followed by the addition of Et_3N (1.16 mL, 8.34 mmol). The mixture was refluxed for 30 h and worked up as it is described to give 0.41 g (28%) of light yellow crystals; mp 171–172 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ = 11.42 (brs, 1H, NH_{amide}), 8.89 (t, 1H, J = 1.43 Hz, $H_{arom.}$), 8.32 (t, 1H, J = 1.43 Hz, $H_{arom.}$), 8.10 (s, 2H, $H_{arom.}$), 7.84 (d, 1H, J = 7.80 Hz, $H_{arom.}$), 7.72 (d, 1H, J = 8.37 Hz), 7.53 (dt, 1H, J_1 = 7.79 Hz, J_2 = 1.10 Hz, $H_{arom.}$); ^{13}C NMR (75 MHz, DMSO-d_6): δ = 157.4 (s), 154.7 (s), 154.2 (s), 152.0 (d), 147.3 (s), 142.0 (d), 127.7 (d), 126.8 (s), 123.9 (d), 123.2 (d), 117.1 (s), 114.0 (d), 112.2 (d), 112.0 (d), 104.1 (s); UV (EtOH) λ_{max}/nm = 321; elemental analysis calcd. (%) for $C_{15}H_9N_3O_2$: C 68.44, H 3.45, N 15.96; found C 68.69, H 3.56, N 15.72.

4.1.2.6. N-(5-Nitropyridin-2-yl)benzofuran-2-carboxamide ***3g***. Compound **3g** was prepared using above described method, from benzofurane-2-carbonyl chloride **1** (0.20 g, 1.10 mmol) and 2-amino-5-nitropyridine **2g** (0.15 g, 1.10 mmol) in dry toluene (20 mL) followed by the addition of Et_3N (0.21 mL, 1.54 mmol). The

mixture was refluxed for 20 h and worked up as it is described to give 0.16 g (52%) of light grey crystals; mp 203–204 °C; ^{1}H NMR (600 MHz, DMSO-d_6): δ = 11.72 (s, 1H, NH_{amide}), 9.24 (d, 1H, J = 2.58 Hz, $H_{arom.}$), 8.68 (dd, 1H, J_1 = 9.08 Hz, J_2 = 2.82 Hz, $H_{arom.}$), 8.41 (d, 1H, J = 0.72 Hz, $H_{arom.}$), 8.16 (d, 1H, J = 0.98 Hz, $H_{arom.}$), 7.84 (d, 1H, J = 7.68 Hz, $H_{arom.}$), 7.74 (dd, 1H, J_1 = 8.40 Hz, J_2 = 0.86 Hz, $H_{arom.}$), 7.52 (dt, 1H, J_1 = 7.44 Hz, J_2 = 1.20 Hz, $H_{arom.}$), 7.38 (dd, 1H, J_1 = 7.56 Hz, J_2 = 1.12 Hz, $H_{arom.}$); ^{13}C NMR (75 MHz, DMSO-d_6): δ = 157.8 (s), 154.6 (s), 149.1 (d), 146.7 (s), 143.1 (d), 128.6 (d), 127.1 (s), 123.5 (d), 123.0 (d), 116.3 (s), 115.2 (d), 113.4 (d), 111.7 (d), 103.7 (s); UV (EtOH) λ_{max}/nm = 320; elemental analysis calcd. (%) for $C_{14}H_9N_3O_4$: C 59.37, H 3.20, N 14.84; found C 59.60, H 3.03, N 14.70.

4.1.2.7. N-(2-Acetamidopyridin-3-yl)benzofuran-2-carboxamide ***3h***. Compound **3h** was prepared using above described method, from benzofurane-2-carbonyl chloride **1** (0.20 g, 1.10 mmol) and 2-acetamido-5-aminopyridine **2h** (0.17 g, 1.10 mmol) in dry toluene (20 mL) followed by the addition of Et_3N (0.21 mL, 1.54 mmol). The mixture was refluxed for 24 h and worked up as it is described to give 0.22 g (68%) of white crystals; mp 222–224 °C; ^{1}H NMR (600 MHz, DMSO-d_6): δ = 10.64 (s, 1H, NH_{amide}), 10.43 (s, 1H, NH), 8.71 (d, 1H, J = 2.58 Hz, $H_{arom.}$), 8.11 (dd, 1H, J_1 = 8.94 Hz, J_2 = 2.58 Hz, $H_{arom.}$), 8.05 (d, 1H, J = 8.94 Hz, $H_{arom.}$), 7.81 (d, 1H, J = 7.86 Hz, $H_{arom.}$), 7.74 (s, 1H, $H_{arom.}$), 7.69 (d, 1H, J = 8.64 Hz, $H_{arom.}$), 7.49 (dt, 1H, J_1 = 8.66 Hz, J_2 = 1.44 Hz, $H_{arom.}$), 7.35 (t, 1H, J = 8.26 Hz, $H_{arom.}$), 2.07 (s, 1H, $COCH_3$); ^{13}C NMR (75 MHz, DMSO-d_6): δ = 169.4 (s), 157.2 (s), 154.9 (s), 148.9 (s), 148.6 (s), 140.5 (d), 131.3 (s), 130.8 (d), 127.7 (d), 127.5 (s), 124.3 (d), 123.5 (d), 113.6 (d), 112.4 (d), 111.4 (d), 24.2 (q); UV (EtOH) λ_{max}/nm = 316; elemental analysis calcd. (%) for $C_{16}H_{13}N_3O_3$: C 65.08, H 4.44, N 14.23; found C 65.24, H 4.59, N 14.54.

4.1.2.8. N-[4-(2-Imidazolynyl)phenyl]benzofuran-2-carboxamide hydrochloride ***3i***. Compound **3i** was prepared using above described method, from benzofurane-2-carbonyl chloride **1** (0.08 g, 0.41 mmol) and 4-(2-imidazolynyl)aniline hydrochloride **2i** (0.08 g, 0.41 mmol) in dry toluene (10 mL) followed by the addition of Et_3N (0.08 mL, 0.57 mmol). The mixture was refluxed for 18 h and worked up as it is described to give 0.11 g (78%) of white powder; mp >290 °C; ^{1}H NMR (600 MHz, DMSO-d_6): δ = 10.60 (s, 1H, NH_{amide}), 9.94 (s, 2H, $NH_{imidazolynyl}$), 8.11 (d, 2H, J = 8.94 Hz, $H_{arom.}$), 8.02 (d, 2H, J = 8.88 Hz, $H_{arom.}$), 7.95 (s, 1H, $H_{arom.}$), 7.74 (d, 1H, J = 8.28 Hz, $H_{arom.}$), 7.53 (d, 1H, J = 8.18 Hz, $H_{arom.}$), 7.37 (t, 2H, J = 8.16 Hz, $H_{arom.}$), 7.23 (t, 2H, J = 8.18 Hz, $H_{arom.}$), 3.98 (s, 4H, CH_2); ^{13}C NMR (75 MHz, DMSO-d_6): δ = 164.2 (s), 157.2 (s), 154.8 (s), 148.0 (s), 130.7 (d, 2C), 129.8 (d), 127.5 (d), 127.0 (s), 123.1 (d), 119.9 (d), 116.8 (s), 112.8 (d, 2C), 111.8 (d), 107.0 (s), 45.2 (t, 2C); UV (EtOH) λ_{max}/nm = 320; elemental analysis calcd. (%) for $C_{18}H_{16}N_3O_2Cl$: C 63.25, H 4.72, N 12.29; found C 63.47, H 4.50, N 12.49.

4.1.2.9. N-[5-(2-Imidazolynyl)pyridin-2-yl]benzofuran-2-carboxamide hydrochloride ***3j***. Compound **3j** was prepared using above described method, from benzofurane-2-carbonyl chloride **1** (0.09 g, 0.50 mmol) and 2-amino-5-(2-imidazolynyl)pyridine hydrochloride **2j** (0.10 g, 0.50 mmol) in dry toluene (15 mL) followed by the addition of Et_3N (0.10 mL, 0.70 mmol). The mixture was refluxed for 22 h and worked up as it is described to give 0.12 g (70%) of white powder; mp >290 °C; ^{1}H NMR (600 MHz, DMSO-d_6): δ = 9.94 (brs, 2H, $NH_{imidazolynyl}$), 8.84 (s, 1H, NH_{amide}), 8.61 (d, 1H, J = 2.46 Hz, $H_{arom.}$), 7.94 (dd, 2H, J_1 = 8.88 Hz, J_2 = 2.58 Hz, $H_{arom.}$), 7.74 (d, 1H, J = 7.74 Hz, $H_{arom.}$), 7.62 (dd, 1H, J_1 = 8.34 Hz, J_2 = 0.98 Hz, $H_{arom.}$), 7.53 (d, 1H, J = 1.12 Hz, $H_{arom.}$), 7.43 (dt, 1H, J_1 = 8.46 Hz, J_2 = 1.32 Hz, $H_{arom.}$), 7.31 (dt, 1H, J_1 = 7.92 Hz, J_2 = 0.98 Hz, $H_{arom.}$), 3.87 (s, 4H, CH_2); ^{13}C NMR (75 MHz, DMSO-d_6): δ = 163.7 (s), 163.5 (s), 158.8 (s), 154.6 (s), 151.5 (d), 149.6 (s), 137.0 (d), 127.6 (s), 127.2 (d), 124.1 (d), 123.2 (d), 112.2 (d), 109.8 (d), 107.8 (d), 106.0 (s), 45.6 (t, 2C); UV (EtOH) λ_{max}/nm = 322; elemental analysis calcd. (%) for $C_{17}H_{15}N_4O_2Cl$: C 59.57, H 4.41, N 16.34; found C 59.68, H 4.60, N 16.62.

4.1.2.10. N-(6-Cyanobenzothiazol-2-yl)benzofuran-2-carboxamide ***6a***. Compound **6a** was prepared using above described method, from benzofurane-2-carbonyl chloride **1** (0.30 g, 1.67 mmol) and 2-amino-6-cyanobenzothiazole **4a** (0.29 g, 1.67 mmol) in dry toluene (20 mL) followed by the addition of Et_3N (0.32 mL, 2.32 mmol). The mixture was refluxed for 18 h and worked up as it is described to give 0.40 g (75%) of light white powder; mp >290 °C; ^{1}H NMR (600 MHz, DMSO-d_6): δ = 13.48 (s, 1H, NH_{amide}), 8.59 (s, 1H, $H_{arom.}$), 8.16 (s, 1H, $H_{arom.}$), 7.90 (d, 1H, J = 7.56 Hz, $H_{arom.}$), 7.87–7.83 (m, 2H, $H_{arom.}$), 7.74 (d, 1H, J = 8.40 Hz, $H_{arom.}$), 7.54 (t, 1H, J = 7.98 Hz, $H_{arom.}$), 7.38 (t, 1H, J = 7.78 Hz, $H_{arom.}$); ^{13}C NMR (75 MHz, DMSO-d_6): δ = 164.7 (s), 157.2 (s), 155.0 (s), 148.7 (s), 135.5 (s), 129.7 (d), 129.0 (s), 128.0 (d), 127.0 (d), 126.7 (s), 124.1 (d), 123.5 (d, 2C), 119.1 (s), 113.2 (d), 112.1 (d), 105.5 (s); UV (EtOH) λ_{max}/nm = 327; elemental analysis calcd. (%) for $C_{17}H_9N_3O_2S$: C 63.94, H 2.84, N 13.16; found C 63.82, H 3.03, N 13.30.

4.1.2.11. N-(6-Cyanobenzimidazol-2-yl)benzofuran-2-carboxamide ***6b***. Compound **6b** was prepared using above described method, from benzofurane-2-carbonyl chloride **1** (0.30 g, 1.67 mmol) and 2-amino-5(6)-cyanobenzimidazole **4b** (0.26 g, 1.67 mmol) in dry toluene (15 mL) followed by the addition of Et_3N (0.32 mL, 2.32 mmol). The mixture was refluxed for 16 h and worked up as it is described to give 0.37 g (76%) of yellow powder; mp 286–287 °C; ^{1}H NMR (600 MHz, DMSO-d_6): δ = 12.72 (brs, 2H, NH_{amide}, $NH_{benzimidazole}$), 8.02 (s, 1H, $H_{arom.}$), 7.92 (s, 1H, $H_{arom.}$), 7.84 (d, 1H, J = 7.86 Hz, $H_{arom.}$), 7.74 (d, 1H, J = 8.34 Hz, $H_{arom.}$), 7.62 (d, 1H, J = 8.10 Hz, $H_{arom.}$), 7.55 (dd, 1H, J_1 = 8.28 Hz, J_2 = 1.02 Hz, $H_{arom.}$), 7.51 (t, 1H, J = 7.92 Hz, $H_{arom.}$), 7.36 (t, 1H, J = 7.56 Hz, $H_{arom.}$); ^{13}C NMR (75 MHz, DMSO-d_6): δ = 164.4 (s), 155.3 (s), 150.4 (s), 148.6 (s), 134.7 (s), 132.3 (s), 128.0 (d), 127.4 (s), 126.0 (d), 124.4 (d), 123.7 (d, 2C), 120.5 (s), 112.9 (d), 112.6 (d, 2C), 103.8 (s); UV (EtOH) λ_{max}/nm = 329; elemental analysis calcd. (%) for $C_{17}H_{10}N_4O_2$: C 67.55, H 3.33, N 18.53; found C 67.71, H 3.14, N 18.69.

4.1.2.12. N-(6-Nitrobenzothiazol-2-yl)benzofuran-2-carboxamide ***6c***. Compound **6c** was prepared using above described method, from benzofurane-2-carbonyl chloride **1** (0.30 g, 1.67 mmol) and 2-amino-6-nitrobenzothiazole **4c** (0.32 g, 1.67 mmol) in dry toluene (20 mL) followed by the addition of Et_3N (0.32 mL, 2.32 mmol). The mixture was refluxed for 12 h and worked up as it is described to give 0.34 g (61%) of beige powder; mp >290 °C; ^{1}H NMR (600 MHz, DMSO-d_6): δ = 13.63 (s, 1H, NH_{amide}), 9.11 (s, 1H, $H_{arom.}$), 8.31 (dd, 1H, J_1 = 8.88 Hz, J_2 = 1.20 Hz, $H_{arom.}$), 8.24 (s, 1H, $H_{arom.}$), 7.97 (d, 1H, J = 8.76 Hz, $H_{arom.}$), 7.89 (d, 1H, J = 7.88 Hz, $H_{arom.}$), 7.76 (d, 1H, J = 8.46 Hz, $H_{arom.}$), 7.55 (t, 1H, J = 7.76 Hz, $H_{arom.}$), 7.39 (t, 1H, J = 7.56 Hz, $H_{arom.}$); ^{13}C NMR (150 MHz, DMSO-d_6): δ = 164.2 (s), 155.6 (s), 154.7 (s), 153.8 (s), 138.4 (s), 135.8 (s), 132.9 (s), 128.6 (d), 128.1 (d), 127.2 (s), 124.6 (d), 124.0 (d), 122.4 (d), 119.6 (d), 113.8 (d), 112.62 (d); UV (EtOH) λ_{max}/nm = 337; elemental analysis calcd. (%) for $C_{16}H_9N_3O_4S$: C 56.63, H 2.67, N 12.38; found C 56.81, H 2.85, N 12.53.

4.1.2.13. N-(6-Nitrobenzimidazol-2-yl)benzofuran-2-carboxamide ***6d***. Compound **6d** was prepared using above described method, from benzofurane-2-carbonyl chloride **1** (0.30 g, 1.67 mmol) and 2-amino-5(6)-nitrobenzimidazole **4d** (0.30 g, 1.67 mmol) in dry toluene (20 mL) followed by the addition of Et_3N (0.32 mL, 2.32 mmol). The mixture was refluxed for 18 h and worked up as it is described to give 0.36 g (68%) of light yellow powder; mp

>290 °C; ^{1}H NMR (600 MHz, DMSO-d_6): δ = 12.72 (brs, 2H, NH_{amide}, $NH_{benzimidazole}$), 8.02 (s, 1H, $H_{arom.}$), 7.92 (s, 1H, $H_{arom.}$), 7.84 (d, 1H, J = 7.86 Hz, $H_{arom.}$), 7.74 (d, 1H, J = 8.34 Hz, $H_{arom.}$), 7.62 (d, 1H, J = 8.10 Hz, $H_{arom.}$), 7.55 (dd, 1H, J_1 = 8.28 Hz, J_2 = 1.02 Hz, $H_{arom.}$), 7.51 (t, 1H, J = 7.92 Hz, $H_{arom.}$), 7.36 (t, 1H, J = 7.56 Hz, $H_{arom.}$); ^{13}C NMR (75 MHz, DMSO-d_6): δ = 163.4 (s), 154.9 (s), 154.7 (s), 142.1 (s), 133.4 (s), 130.7 (s), 127.7 (d), 126.9 (s), 123.9 (d), 123.2 (d, 2C), 117.7 (d), 112.3 (d), 112.0 (d, 2C), 104.7 (s); UV (EtOH) λ_{max}/nm = 335; elemental analysis calcd. (%) for $C_{16}H_{10}N_4O_4$: C 59.63, H 3.13, N 17.38; found C 59.79, H 3.28, N 17.60.

*4.1.2.14. N-[6-(2-Imidazolynyl)benzothiazol-2-yl]benzofuran-2-carboxamide hydrochloride **6e**.* Compound **6e** was prepared using above described method, from benzofurane-2-carbonyl chloride **1** (0.06 g, 0.33 mmol) and 2-amino-6-(2-imidazolynyl)benzothiazole hydrochloride **5a** (0.09 g, 0.33 mmol) in dry toluene (15 mL) followed by the addition of Et_3N (0.07 mL, 0.46 mmol). The mixture was refluxed for 18 h and worked up as it is described to give 0.09 g (69%) of white powder; mp >290 °C; ^{1}H NMR (600 MHz, DMSO-d_6): δ = 12.50 (s, 1H, NH_{amide}), 10.23 (s, 2H, $NH_{imidazolynyl}$), 8.37 (d, 1H, J = 1.56 Hz, $H_{arom.}$), 7.85 (dd, 1H, J_1 = 8.52 Hz, J_2 = 1.74 Hz, $H_{arom.}$), 7.75 (d, 1H, J = 7.86 Hz, $H_{arom.}$), 7.63 (d, 1H, J = 8.16 Hz, $H_{arom.}$), 7.55 (s, 1H, $H_{arom.}$), 7.48 (d, 1H, J = 8.52 Hz, $H_{arom.}$), 7.44 (dt, 1H, J_1 = 8.34 Hz, J_2 = 1.44 Hz, $H_{arom.}$), 7.32 (t, 1H, J = 7.56 Hz, $H_{arom.}$), 4.05 (s, 4H, CH_2); ^{13}C NMR (150 MHz, DMSO-d_6): δ = 170.7 (s), 164.9 (s), 158.8 (s), 158.2 (s), 154.6 (s), 149.6 (s), 132.0 (s), 127.6 (s), 127.3 (d), 127.1 (d), 124.1 (d), 123.2 (d), 122.4 (d), 117.8 (d), 114.1 (s), 112.2 (d), 109.8 (d), 46.6 (t, 2C); UV (EtOH) λ_{max}/nm = 324; elemental analysis calcd. (%) for $C_{19}H_{15}N_4O_2ClS$: C 57.21, H 3.79, N 14.05; found C 57.47, H 3.95, N 14.30.

*4.1.2.15. N-[6-(2-Imidazolynyl)benimidazol-2-yl]benzofuran-2-carboxamide hydrochloride **6f**.* Compound **6f** was prepared using above described method, from benzofurane-2-carbonyl chloride **1** (0.10 g, 0.55 mmol) and 2-amino-5(6)-(2-imidazolynyl)benzimidazole hydrochloride **5b** (0.13 g, 0.55 mmol) in dry toluene (20 mL) followed by the addition of Et_3N (0.11 mL, 0.77 mmol). The mixture was refluxed for 18 h and worked up as it is described to give 0.18 g (86%) of white powder; mp >290 °C; ^{1}H NMR (600 MHz, DMSO-d_6): δ = 13.37 (s, 1H, $NH_{benzimidazole}$), 11.48 (s, 1H, NH_{amide}), 10.38 (brs, 2H, $NH_{imidazolynyl}$), 8.93 (s, 1H, $H_{arom.}$), 8.06 (d, 1H, J = 1.32 Hz, $H_{arom.}$), 7.89 (dd, 1H, J_1 = 8.46 Hz, J_2 = 1.76 Hz, $H_{arom.}$), 7.75 (d, 1H, J = 7.86 Hz, $H_{arom.}$), 7.63 (d, 1H, J = 8.68 Hz, $H_{arom.}$), 7.56 (d, 1H, J = 8.46 Hz, $H_{arom.}$), 7.44 (t, 1H, J = 8.48 Hz, $H_{arom.}$), 7.32 (t, 1H, J = 7.74 Hz, $H_{arom.}$); 4.12 (s, 4H, CH_2); ^{13}C NMR (150 MHz, DMSO-d_6): δ = 164.6 (s), 158.4 (s), 154.1 (s), 152.0 (s), 149.1 (s), 134.4 (s), 130.0 (s), 127.1 (s), 126.7 (d), 124.2 (d), 123.6 (d), 122.7 (d), 116.4 (s), 111.8 (d), 111.6 (d, 2C), 109.3 (d), 45.2 (t, 2C); UV (EtOH) λ_{max}/nm = 323; elemental analysis calcd. (%) for $C_{19}H_{16}N_5O_2Cl$: C 59.77, H 4.22, N 18.34; found C 59.50, H 4.17, N 18.60.

*4.1.3. Preparation of N-(4-N,N-dimethylaminophenyl)benzofuran-2-carboxamide hydrochloride **3c***

A stirred suspension of compound **3b** (0.10 g, 0.36 mmol) in absolute ethanol (10 mL) was saturated with $HCl_{(g)}$. After 24 h of stirring small amount of diethylether was added, resulting product was filtered off and washed with diethylether to give 0.09 g (79%) of red powder; mp >290 °C; ^{1}H NMR (600 MHz, DMSO-d_6): δ = 10.72 (s, 1H, NH_{amide}), 7.88 (br s, 1H, NH^+), 7.82 (d, 2H, J = 7.74 Hz, $H_{arom.}$), 7.80 (s, 1H, $H_{arom.}$), 7.71 (d, 2H, J = 8.28 Hz, $H_{arom.}$), 7.50 (t, 2H, J = 7.98 Hz, $H_{arom.}$), 7.36 (t, 2H, J = 7.84 Hz, $H_{arom.}$), 3.06 (s, 6H, CH_3); ^{13}C NMR (150 MHz, DMSO-d_6): δ = 157.1 (s), 155.3 (s), 150.2 (s), 14,910 (s), 129.0 (s), 128.3 (d), 128.1 (s), 124.7 (d), 123.6 (d), 123.0 (d, 2C), 113.4 (d, 2C), 112.8 (d), 110.7 (d), 41.5 (q, 2C); UV (EtOH) λ_{max}/nm = 330, 273; elemental analysis calcd. (%) for $C_{17}H_{17}N_2O_2Cl$: C 64.46, H 5.41, N 8.84; found C 64.59, H 5.27, N 8.54.

4.2. Cell culturing and cell morphology

The cell lines HeLa (cervical carcinoma), SW620 (colorectal adenocarcinoma, metastatic), MiaPaCa-2 (pancreatic carcinoma), MCF-7 (breast epithelial adenocarcinoma, metastatic), SK-BR-3 (breast adenocarcinoma, metastatic) and WI38 (normal diploid human fibroblasts) were cultured as monolayers and maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/mL streptomycin in a humidified atmosphere with 5% CO_2 at 37 °C. The evaluation of cell morphology was performed under the light invert microscope Olympus (magnification 200×).

4.3. Antitumour activity assays

The panel of monolayer tumour cell lines was inoculated into standard 96-well microtiter plates on day 0, at 3000–6000 cells per well depending on the doubling time of the specific cell line. Test compounds were then added in five 10-fold dilutions (0.01–100 μM) followed by 72-h incubation. Cell viability was quantitatively determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. Experimentally obtained absorbances were transformed into cell percentage growth (PG) using the formulae proposed by NIH and described previously. The IC_{50} values for each compound were calculated from concentration–response curves using linear regression analysis by fitting the test concentrations that give PG values above and below the reference value. If, however, all of the tested concentrations produce PGs exceeding the respective reference level of effect (*e.g.* PG value of 50) for a given cell line, the highest tested concentration is assigned as the default value (in the screening data report, that default value is preceded by a ">" sign). Each test point was performed in quadruplicate in three individual experiments. The results were statistically analyzed (ANOVA, Tukey post-hoc test at $p < 0.05$). Finally, the effects of the tested substances were evaluated by plotting the mean percentage growth for each cell type in comparison to control on dose–response graphs.

4.4. Cell cycle analysis

A total of 5×10^5 cells were seeded per petridish. After 24 h SK-BR-3 cells were treated with the compound **6f** while SW620 cells were treated with compound **3i** and **3h**. The concentration of tested compounds was 5 μM and 10 μM. After 24 h and 48 h the attached cells were trypsinized, combined with floating cells, washed with PBS and fixed with 70% ethanol. Immediately before the analysis, the cells were washed again with PBS and stained with 1 μg/mL of propidium iodide (PI) with the addition of 0.2 μg/mL of RNAse A. The stained cells were then analyzed with Becton Dickinson FACScalibur flow cytometer (10,000 counts were measured). Each test point was performed in duplicate. The percentage of the cells in each cell cycle phase was based on the obtained DNA histograms and determined using the WinMDI 2.9 and Cylchred software. Statistical analysis was performed in Microsoft Excel by using the ANOVA at $p < 0.05$.

4.5. Caspase colorimetric protease assay

To determent activation status of caspase 3, 8 and 9 the Apo-Target caspase colorimetric protease assay was used. In compliances with the protocol, 6×10^6 cells were seated in petridish.

SW620 were treated with compound **3i** and **3h** at a concentration of 10 μM while SK-BR-3 cells were treated with compound 6f at a concentration of 5 μM. After 24 and 48 h cells were collected, washed once in PBS, and 50 μL of lysis buffer was added to the pellet. After 10 min of incubation on ice, samples were centrifuged at 13,000 rpm for 1 min and protein lysates were stored at −80 °C. Protein concentration was measured by DC protein assay (Bio-Rad). Each protein extracts was diluted to a concentration of 3 mg/mL. In a 96-well plate 50 μL of diluted samples, 50 μL of reaction buffer, containing DTT, and 5 μL of caspase substrate was added. The plate was incubated for 90 min, protected from light at a 37 °C. All samples were done in duplicate and absorbance was measured at 405 nm.

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Appendix A. Supplementary material

Supplementary material related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2012.11.009.

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