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Original article

Synthesis and antiproliferative evaluation of some new amidino-substituted bis-benzothiazolyl-pyridines and pyrazine

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ABSTRACT

Novel diamidino substituted conformationally restricted derivatives of bis-benzothiazolyl-pyridines and pyrazine were synthesized and their antiproliferative activity against several human cancer cell lines were determinated. The synthetic approach used for preparation of isomeric amidinobenzotiazolyl disubstituted pyridines **3a**–**3k** and pyrazine **3l** was achieved by condenzation reaction of commercially available pyridine and pyrazine dicarboxylic acids with amidino- **2a** and 2-imidazolinyl-substituted 2-aminothiophenol **2b** in polyphosphoric acid in moderate to good yield. The condenzation reaction was greatly optimized. The targeted compounds were converted in the desired water soluble dihydrochloride salts by reaction of appropriate free base with concd HCl in ethanol or acetic acid. Antiproliferative assays revealed significant differences in antiproliferative activities of diamidino- and diimidazolinyl-derivatives, the latter exerting stronger concentration-dependent antiproliferative effects on tested tumor cell lines and thus being a prominent compound class for further chemical optimization and biological studies. Biological studies on SW620 cell line and BJ fibroblasts performed for the diimidazolinyl-derivative **3b** revealed oxidative stress as a possible mechanism of antiproliferative action and predicted antineoplastic properties for this class of compounds.

1. Introduction

Heterocyclic compounds from the series of benzothiazoles have been investigated due to their biological and pharmacological activity. These compounds have interesting biological properties including antiallergic [1], anti-inflammatory [1,2], antitumor [3–7] and analgesic [8,9] activities. Considering their mechanism of action as antitumor agents, it was shown that benzothiazole derivatives act as tyrosine kinase [10–13] and topoisomerase I and II inhibitors [14,15]. Therefore, various benzothiazole compounds are considerably interesting due to their potential for diverse pharmaceutical uses.

Although a variety of novel benzothiazole derivatives with antitumor activity were described so far, these are structurally different from compounds presented in this paper [16–18]. A group however, of similar compounds has been reported in the recent literature and describes the synthesis and cytotoxic evaluation of thiourea and *N*-bis-benzothiazole derivatives. This novel class of cytotoxic benzothiazolyl thiocarbamides has been achieved using a catalytic amount of 4-dimethylaminopyridine (DMAP) followed by its chemoselective oxidative cyclization with 1,3-di-*n*-butylimidazolium tribromide [bbim][Br_3] to afford the *N*-bis-benzothiazole derivatives. Synthesized compounds had significant antiproliferative activity on human monocytic cell lines U 937 and a mouse melanoma cell line B16-F10 cells [19].

Our previously published data showed accentuated antiproliferative effects for this class of compounds as well. We found that antiproliferative activity of amidino [20,21] and amino [22] substituted 2-phenylbenzothiazole derivatives strongly depends on the position of the substituent on 2-phenylbenzothiazole skeleton, as well as on the type of attached amidino substituent. We found that, in a series of unsubstituted, *N*-isopropyl substituted, as

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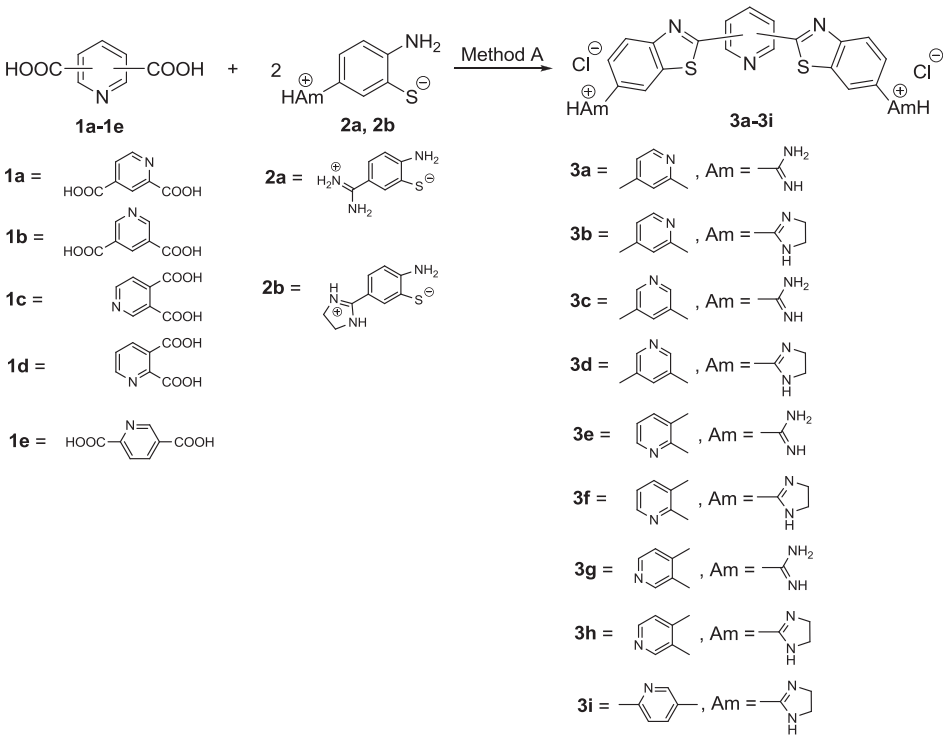


Method A 1.) PPA 120-140°C, 1h then 160-180°C, 2h; 2.) 2M NaOH; 3.) CH_3COOH/HCl or EtOH/HCl

Scheme 1.

well as 2-imidazolinyl mono- and bis-amidino derivatives of 2-phenylbenzothiazole, *N*-isopropyl substituted amidine possess less pronounced antiproliferative activity on tested tumor cell lines.

In relation with the above considerations, we designed and efficiently synthesized new diamidino-, diisopropylamidino- and diimidazolinyl-substituted derivatives of phenyl-benzothiazolyl- and bisbenzothiazolyl-furans and -thiophenes and evaluated their antiproliferative activity on tumor cell lines *in vitro*, DNA binding propensity and sequence-selectivity as well as cellular distribution. In addition, two compounds were chosen according to their differential effect for further biological studies including the cell cycle analysis and apoptosis induction in order to reveal a more detailed picture on the possible antiproliferative mechanisms and/or targets [23]. On the other hand, novel amidino substituted conformationally restricted derivatives of pentamidine were synthesized and their antiproliferative activity against several human cancer cell lines has been determined. It was found that introduction of furan-2,5-dicarboxamide core moiety increases antiproliferative activity as well as selectivity against certain tumor cell lines in comparison with amidino-substituted furan-mono-carboxamide. Unlike the furan series where isopropyl substituted amidine exhibits more potent overall antiproliferative activity and selectivity toward certain cell lines, the same was found for unsubstituted amidines in pyridine series [24].

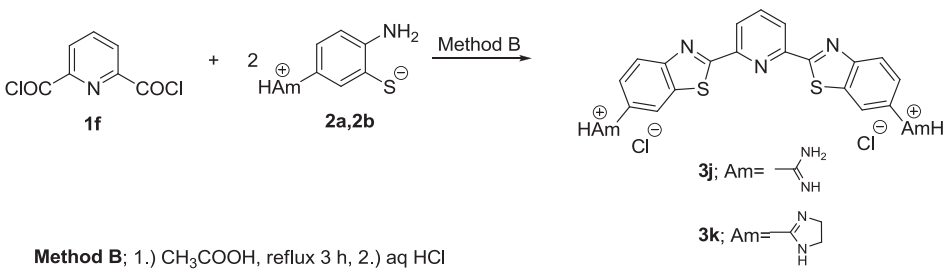
As a continuation of these previous studies, we present the synthesis and biological evaluation of newly synthesized amidino substituted conformationally restricted derivatives of bisbenzothiazolyl-pyridines and pyrimidine.

2. Results and discussion

2.1. Chemistry

The synthetic approach used to prepare isomeric amidinobenzotiazolyl disubstituted pyridines **3a**–**3k** is outlined in Schemes 1 and 2.

Recently, we found a convenient and efficient method for the synthesis of amidino substituted benzothiazolyl compounds by condensation reaction of carboxylic acids or acyl chlorides and



Method B; 1.) CH_3COOH, reflux 3 h, 2.) aq HCl

Scheme 2.

amidino substituted *o*-aminothiophenoles [25]. Commercially available isomeric pyridine dicarboxylic acids **1a**–**1e** with amidino-**2a** and 2-imidazolinyl-substituted 2-aminothiophenol **2b** were condensed in polyphosphoric acid (method A) and isolated as corresponding bis-amidinosubstituted free bases. The desired water soluble dihydrochloride salts **3a**–**3i** were prepared by reaction of appropriate crude free base with concd HCl in ethanol or acetic acid. We optimized reaction conditions of condensation reaction in PPA and found that gradual heating of reaction mixture significantly affected the yield of product. It showed that the usual condensation reaction condition of *o*-aminothiophenol and carboxylic acid in PPA at 180 °C, through 2–3 h gives product in low to moderate yield, with some unidentified byproducts. Heating for the first 1 h at 120–140 °C, than at 160–180 °C in PPA drastically reduced the quantity of byproducts and after conversion of the corresponding free base into dihydrochloride salts improved overall yield of pure product. The diamidino bisbenzothiazolyl compounds **3a**–**3i** were isolated in a moderate to good yield of about 50–75%. Our research shows that the condensation of acyl chlorides and zwitterions **2a** and **2b** in acetic acid is also an efficient method to prepare bis-amidino substituted bisbenzothiazolyl derivatives [23]. Consequently, condensation reaction of commercially available pyridine-2,6-dicarbonyl dichloride (**1f**) with corresponding amidino-substituted 2-amithiophenole **2a** and **2b** in acetic acid gave target dihydrochloride salts **3j** and **3k** in a good yield of 78% and 75%, respectively. Besides isomeric disubstituted pyridines using method A, we have also prepared pyrazine derivative 2,5-bis[6-(4,5-dihydro-1*H*-imidazol-2-yl)1,3-benzothiazol-2-yl]pyrazine dihydrochloride (**3l**) in a moderate yield of 46% (Scheme 3).

Unexpectedly, using method A from pyridine-2,5-dicarboxylic (**1e**) and pyrazine-2,5-dicarboxylic acid (**1g**) and 5-amidinium-2-aminobenzothiolate (**2a**) we did not succeed to prepare corresponding bisamidino dibenzothiazolyl compounds. The structure of compounds was determined by ^{1}H and ^{13}C NMR spectroscopy, as well as elemental analysis.

2.2. Antitumor activity

The antiproliferative *in vitro* screening of novel diamidino-bisbenzothiazolyl pyridines and pyrazine derivatives (**3a**–**3l**) revealed different patterns of activity for diimidazolinyl- derivatives **3b**, **3d**, **3f**, **3h**, **3i**, **3k** and **3l** that were highly active on tested tumor cell lines in a concentration-dependent manner at micromolar concentrations between 0.1 and 10 μM (Table 1). Contrary, diamidino-compounds **3a**, **3c**, **3e** and **3g** showed antiproliferative effects only at highest tested concentrations (10 or 100 μM) while compound **3j** was inactive. In particular, the presence of imidazolo-moiety increased the selectivity towards SK-BR-3, HeLa and SW620 cell lines, while both imidazolo- and amidino-moieties contributed to antiproliferative effects on the MCF-7 and MiaPaCa-2 cell lines. Cytotoxic effect was observed for all tested compounds except for derivative **3j** on normal human fibroblasts (Table 1).

The observed difference in experimentally obtained antiproliferative data for diamidino- and diimidazolinyl-derivatives may arise due to several reasons. One of them is a difference in compounds' lipophilicity and their ability to form H-bond formations. These molecular features are known to considerably influence membrane permeability as well as to modulate interactions with potential biological molecular targets such as membrane transporters [27]. According to fragment-based algorithm for calculating lipophilicity coefficients clog *P* and clog *D*, diimidazolinyl-derivatives are generally more lipophilic than diamidino-analogs. This is exemplified in Table 2 by comparison of clog *P* and clog *D* values of 2-phenyl imidazoline and benzamidine fragments. In addition, ability to form H-bonds is lower for diimidazolinyl – compounds than for diamidino-analogs, regardless their ionization state. Such physicochemical profile of diimidazolinyl-derivatives, may account for their better membrane permeability by passive diffusion.

Considering the observed prominent antiproliferative effect of tested diimidazolinyl-derivatives, we performed *in silico* analyses of biological activity spectra to elicit possible targets and biological mechanisms of action.

Table 1
The inhibition effects of compounds **3a**–**3l** on the growth of selected tumor cells and normal human fibroblasts *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 formulas proposed by National Cancer Institute and described previously in Gazivoda et al. [26].

Substance No.	IC_{50}[a] (μM)					
	Cell lines					
	MCF-7	SK-BR-3	SW620	MiaPaCa-2	BJ	HeLa
3a	78.9	>100	>100	61.1	2.92	>100
3b	1.4	2.3	0.2	2.9	0.02	0.3
3c	>100	>100	>100	86.7	15	>100
3d	0.9	0.5	0.42	6.6	0.16	0.3
3e	23.2	>100	>100	71.7	34.67	39.2
3f	6.9	21.5	31.1	27.3	0.77	17.2
3g	17.6	>100	>100	88.6	0.08	>100
3h	16.3	63.6	47.3	57.2	2.87	40.6
3i	4.4	3.4	2.8	4.4	0.34	3.4
3j	>100	>100	>100	>100	>100	>100
3k	0.3	3.2	0.8	4.6	0.04	1.3
3l	1.2	0.9	1.8	5.1	0.07	1.9

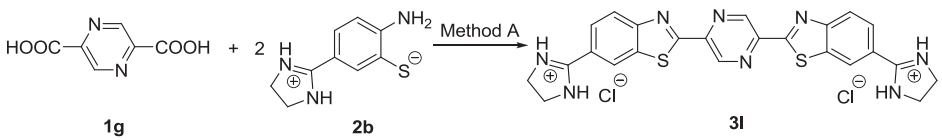
[a] IC_{50}; 50% inhibitory concentration, or compound concentration required to inhibit tumor cell proliferation by 50%.

2.3. In silico prediction of biological activity spectra

The most probable molecular targets at the cellular level that might be responsible for the observed antiproliferative activity of newly synthesized amidinobenzotiazolyl disubstituted pyridines

Table 2
ACD/Lab clog *P* and clog *D* (at pH 7.4) values as well as numbers of H-bond donating (hbd) and accepting (hba) atoms of 2-phenyl imidazoline and benzamidine fragments, calculated at http://www.chemspider.com/.

Calculated molecular properties	clog *P*	clog *D*	hbd	hba
Benzamidine	0.887	−1.111	3	2
2-Phenyl imidazoline	2.226	0.332	1	2



Scheme 3.

Table 3
PASS prediction of human activity spectra for diamidino compound **3e** (at Pa ≥ 0.5).[a] Similar activities have been predicted for all synthesized diamidino-derivatives.

Imidamide **3e**		
Pa	Pi	*Biological targets*
0.693	0.01	Granzyme B inhibitor
0.745	0.079	Fibrinogen receptor antagonist
0.636	0.007	Factor VIIa inhibitor
0.64	0.016	Tissue kallikrein inhibitor
0.58	0.003	Urokinase-type plasminogen activator receptor antagonist
0.552	0.053	HERG channel antagonist
0.531	0.044	Carboxypeptidase U inhibitor
0.527	0.053	Calmodulin antagonist
Pa	Pi	*Pharmacological effects*
0.827	0.005	Platelet aggregation inhibitor
0.745	0.005	Anticoagulant
0.725	0.014	Antithrombotic
0.656	0.005	Antiprotozoal (Trypanosoma)
0.578	0.004	Plasminogen activator inhibitor
0.582	0.045	Nucleotide metabolism regulator
0.562	0.046	Antidiarrheal
0.504	0.003	Antiprotozoal (Babesia)
0.597	0.099	Antinephritic
0.499	0.005	Antithrombocytopenic
0.502	0.041	Antiasthmatic

[a] The predicted activity spectrum is presented in PASS by the list of activities with the probabilities "to be active" (Pa) and "to be inactive" (Pi) calculated for each activity [28]. The list is arranged in descending order of Pa–Pi; therefore, more probable activities are at the top of the list. The probability Pa reflects the similarity of a molecule under prediction with the structures of molecules, which are the most typical in a sub-set of "actives" in the structure–activity relationship (SAR) of PASS.

were assessed by use of the software PASS (Prediction of Activity Spectra of Substances). PASS is also suitable for estimation of additional activities including side-effects risks. PASS is based on structure–activity relationships (SAR) analyses of the training sets by use of probabilistic ligand-based methodology, 2D structural representation of molecules and regularly updated experimental data of high-quality [28].

Molecular targets and pharmacological activities of tested compounds were predicted with probability Pa ≥ 0.5. Possible activities have been determined for all diamidino- and diimidazolinyl-derivatives. Highly similar activities were assessed per each group and are presented in Tables 3 and 4 for compounds **3e** and **3b**, respectively. Differences in predicted activities for these two classes of derivatives may be ascribed to interactions of their amidino- and imidazolynil-moieties, respectively. Majority of diamidino-compounds (Table 3) have been predicted to target proteases or inhibit/act as antagonists of proteins involved in blood coagulation. According to PASS predictions, diamidino-compounds may also inhibit granzyme B, an enzyme that cleaves and activates caspases −3, −7, −9 and 10. It is thus possible that diamidino-derivatives exerted poor antiproliferative activities on tumor cell lines due to inhibitory effect on this enzyme. Diamidino-compounds are moreover predicted to act as HERG voltage-gated potassium channel antagonists. This is a serious safety issue and their systemic administration may cause severe side effects by prolonging the QT interval and in some cases leading to potentially life-threatening ventricular arrhythmia.

Similarly, but with smaller probability Pa < 0.5, diimidazolinyl-derivatives may also act as HERG antagonists. Additionally, diimidazolinyl-derivatives are agonists of imidazoline I1 receptor and may thus have unwanted effects on blood pressure as well (Table 4). Inhibition of imidazoline I1 receptor however, may play a role in inhibition of cell proliferation substantiated by obtained experimental results. Moreover, diimidazolinyl-derivatives may act as inhibitors of diamine oxidase, an enzyme that catalyzes the degradation of substances involved in cell proliferation, tumor formation, and possibly apoptosis. In conclusion this points to diimidazolinyl-substituted benzothiazolyl pyridines as more potent antineoplastic drugs than their diamidino-analogs. Interestingly, all predicted targets of diimidazolinyl-derivatives participate in modulation of inflammatory response (Table 4).

PASS is based on SAR models for 'target families' such as GPCRs, kinases, ion channels, nuclear receptors and proteases but also for DNA and proteins included in its modulating. Nevertheless, majority of predicted targets in this study are cytosolic and/or membrane proteins. This result might be attributed to the fact that tested bisbenzothiazole derivatives represent a novel chemical class and the structures may be different from known compounds included in the PASS training set. Therefore, the action of tested compounds on other possible targets (e.g. DNA) may not be excluded.

Table 4
PASS prediction of human activity spectra for diimidazolinyl compound **3b** (at Pa ≥ 0.5).[a] Similar activities have been predicted for all other synthesized diimidazolinyl-derivatives.

Imidazole **3b**		
Pa	Pi	*Biological targets*
0.8	0.003	Imidazoline I1 receptor agonist
0.798	0.023	Diamine oxidase inhibitor
0.632	0.046	GABA A receptor antagonist
0.558	0.01	5 − Hydroxytryptamine 3 agonist
0.551	0.042	Calmodulin antagonist
0.521	0.038	Interleukin 1 antagonist
Pa	Pi	*Pharmacological effects*
0.785	0.01	Nucleotide metabolism regulator
0.699	0.008	Insulin promoter
0.567	0.015	Angiogenesis inhibitor
0.51	0.054	Antiarthritic
0.518	0.086	Antineoplastic (brain cancer)

[a] Meanings of parameters Pa and Pi are given under Table 3.

2.4. Effect on selected biological targets predicted by PASS

Due to possible severe side effect of tested compounds predicted by PASS related to systemic administration, we assessed their potential for local administration on chosen biological targets, namely inflammatory modulators and apoptosis effectors caspases 3/7. Diimidazolinyl-derivative **3b** was tested for assessment of effects on cytokine and chemokine expression and caspase 3/7 activation in SW620 tumor cell line and normal human fibroblasts BJ.

Among a panel of tested cytokines and chemokines, compound **3b** induced an increased secretion of IL8 both in fibroblasts BJ and SW620 cells (Fig. 1) at tested submicromolar concentration. Increased IL-8 secretion may point to oxidative stress as a possible mechanism of antiproliferative action and predicted antineoplastic properties for compound **3b** [29]

Interestingly, we managed to detect activation of caspases 3/7 by compound **3b**. Caspases activation was however detectable in SW620 only at concentrations between 0.1 and 10 μM (Fig. 2) while the highest tested concentration (100 μM) expectedly failed to activate the tested caspases. Activation of caspases 3/7 was six levels lower in SW620 cells in comparison with BJ cells at 10 μM as witnessed by measured luminescence values (Fig. 2). Moreover, compound **3b** failed to activate caspases 3/7 in normal fibroblasts at other tested concentrations. This might be attributed to inhibition of caspase activation or other mechanisms of cell death induction that might involve oxidative stress previously witnessed in cytokine/chemokine excretion analyses.

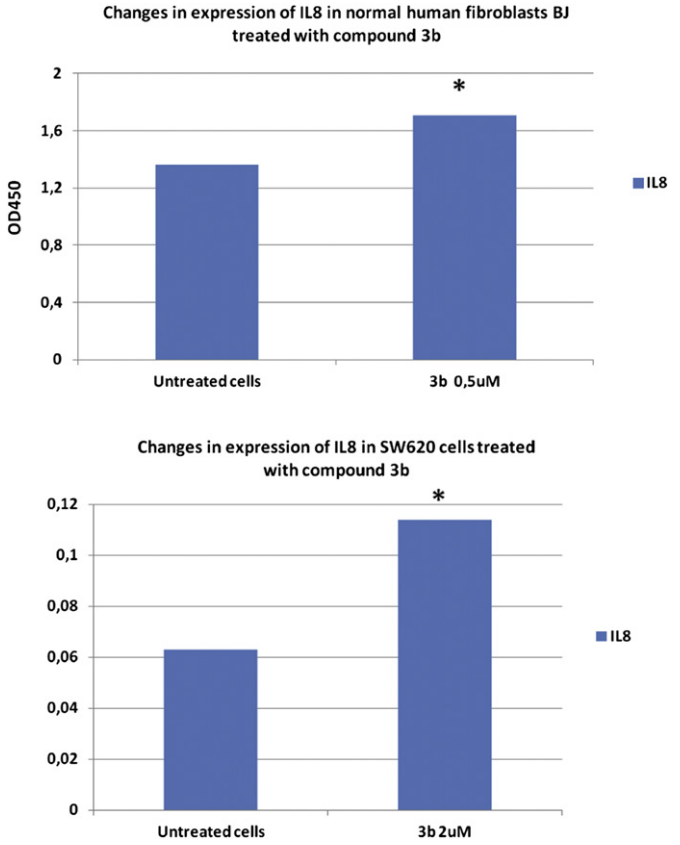


Fig. 1. Induction of IL-8 secretion into the growth medium of normal human fibroblasts BJ and SW620 treated with compound **3b** at concentrations above IC50 (0.5 μM and 2 μM respectively).

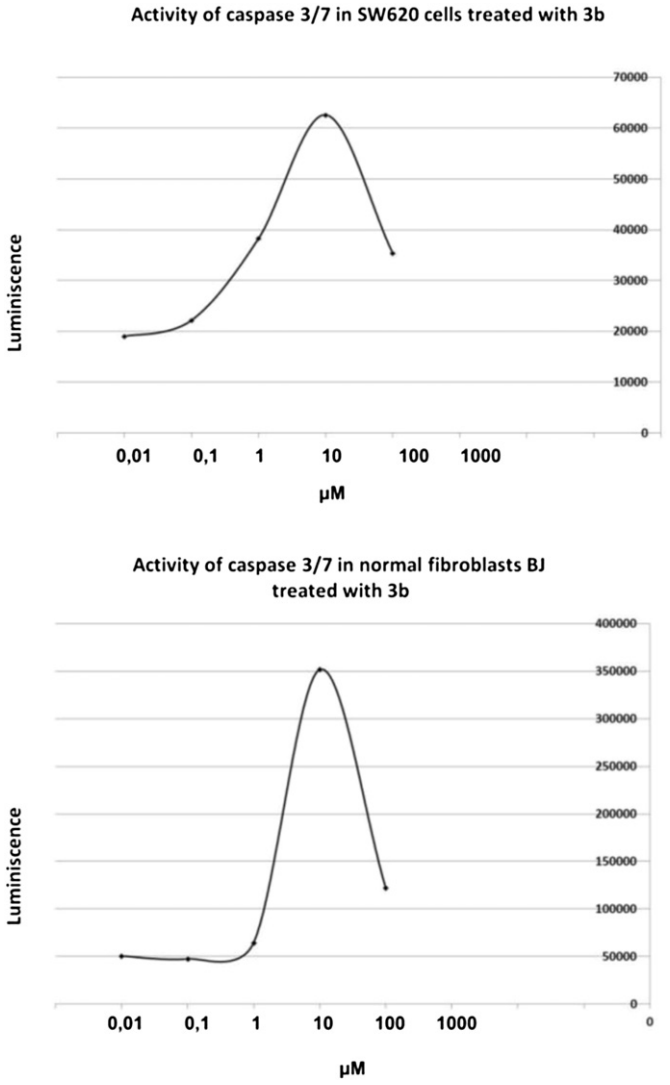


Fig. 2. The activity of caspase 3/7 in SW620 cells and normal fibroblasts BJ treated with compound **3b** at concentrations 100, 10, 1, 0.1 and 0.01 μM represented as log [*M*] (1×10^{-8}–1×10^{-4}) upon 24-h treatment.

3. Experimental

3.1. Chemistry

Melting points were determined on a Koffler hot stage microscope. ^{1}H and ^{13}C NMR spectra were recorded on Bruker Avance DPX 300 or Brucker AV-600 spectrometers using TMS, as an internal standard and the deuterated solvents were used, as mentioned. Elemental analysis for carbon, hydrogen and nitrogen were performed on a Perkin–Elmer 2400 elemental analyzer. Analyses are indicated only as symbols of elements, and analytical results obtained are within 0.4% of the theoretical value. Mass spectra were recorded with the Agilent 1100 Series LC/MSD Trap SL spectrometer. Synthesis of 5-amidinium-2-aminobenzothiolate **2a** and 5-(imidazolinium-2-yl)-2-aminobenzothiolate hydrate **2b** were prepared according to the literature [25].

3.1.1. General synthetic procedures for preparation of target compounds *3a*–*3l*

Method A: To a stirred solution of corresponding pyridine dicarboxylic acid **1a**–**1e** (84 mg, 0.5 mmol) or pyrazine-2,5-dicarboxylic acid **1g** (102 mg, 0.5 mmol) in polyphosphoric acid (15 g) dissolved at 120 °C under nitrogen 5-amidinium-2-aminobenzothiolate **2a** (175 mg, 1.1 mmol) or 5-(imidazolinium-2-yl)-2-aminobenzothiolate hydrate **2b** (232 mg, 1.1 mmol) was added. The mixture was heated at 120–140 °C under nitrogen, until it becomes homogenous (1 h) and then for additional 2 h at 160–180 °C. The reaction mixture was cooled and water (100 mL) was added. The resulting precipitate was filtered off, washed with water and dried. The crude product was suspended in water (50 mL) and made alkaline with 2.5 M NaOH (10 mL). The corresponding free base was filtered off, washed with water and dried under vacuum. The free base was converted into dihydrochloride salt and purified by crystallization from appropriate solvents as described below.

Method B: To a stirred solution of 5-amidinium-2-aminobenzothiolate **2a** (175 mg, 1.1 mmol) or 5-(imidazolinium-2-yl)-2-aminobenzothiolate hydrate **2b** (232 mg, 1.1 mmol) in acetic acid (10 mL), pyridine-2,6-dicarbonyl dichloride **1f** (102 mg, 0.5 mmol) in acetic acid (5 mL) was added under nitrogen and refluxed for 3 h. The reaction mixture was cooled and resulting precipitate filtered off, washed with acetone and dried under vacuum over KOH. The crude product was dissolved in water (20 mL) and to the stirred solution concd hydrochloride acid (2 mL) was added. After cooling overnight the resulting solid was filtered off, washed with acetone and dried. The pure dihydrochloride salt was obtained by crystallization as described below.

*3.1.1.1. 2,2′-(Pyridine-2,4-diyl)bis(1,3-benzothiazole-6-carboximidamid) dihydrochloride (**3a**).* To a solution of the free base prepared by *method A* in acetic acid (20 mL) concd HCl (2 mL) was added and

stirred for 1 h. The resulting precipitate was filtered off, washed with acetone and dried. Crystallization from water/1 M HCl/acetone mixture gave pure compound **3a** 176 mg (64.3%) as colorless solid: mp > 300 °C. ^{1}H NMR (300 MHz, DMSO-d_6) (δ ppm): 9.56 (br s, 4H, H-Amd), 9.28 (br s, 4H, H-Amd), 9.04–9.01 (m, 2H), 8.83 (s, 1H), 8.76 (s, 1H), 8.47–8.41 (m, 2H, H-Bt), 8.35 (d, 1H, J = 4.8 Hz, H-Py), 8.04–7.98 (m, 2H, H-Bt). ^{13}C NMR (75 MHz, D_2O/DMSO-d_6) (δ ppm): 172.1, 168.6, 164.8, 164.7, 156.1, 155.7, 151.4, 149.8, 139.7, 135.7, 135.3, 125.9, 125.6, 124.6, 124.2, 123.4, 122.6, 122.4, 117.1. LC–MS (ESI) m/z: 430.3 [(M + H$^+$) calcd for free base $C_{21}H_{15}N_7S_2$, 429.08]. Analysis calcd for $C_{21}H_{17}Cl_2N_7S_2 \times 2.5H_2O$ (547.48): C, 46.07; H, 4.05; N, 17.91, Cl, 12.93. Found C, 45.97; H, 4.03; N, 17.55; Cl, 13.14%.

*3.1.1.2. 2,2′-(Pyridine-2,4-diyl)bis[6-(4,5-dihydro-1H-imidazol-2-yl)-1,3-benzothiazole] dihydrochloride (**3b**).* To a solution of the free base prepared by *method A* in acetic acid (30 mL) concd HCl (2.5 mL) was added and stirred for 1 h. The resulting precipitate was filtered off, washed with acetone and dried. Crystallization from 1 M HCl gave pure compound **3b** 223 mg (74.4%) as colorless solid: mp > 300 °C. ^{1}H NMR (600 MHz, DMSO-d_6) (δ ppm): 10.79 (s, 2H, H-Amd), 10.74 (s, 2H, H-Amd), 9.03 (d, 1H, J = 4.1 Hz, H-Py), 9.00 (s, 1H), 8.95 (s, 1H), 8.89 (s, 1H), 8.49 (d, 1H, J = 8.3 Hz, H-Bt), 8.46 (d, 1H, J = 8.6 Hz, H-Bt), 8.34 (d, 1H, J = 4.2 Hz, H-Py), 8.18–8.13 (m, 2H, H-Bt), 4.08 (s, 8H, H–CH_2). ^{13}C NMR (75 MHz, D_2O/DMSO-d_6) (δ ppm): 172.3, 168.7, 164.1, 164.0, 156.2, 155.7, 151.5, 149.4, 139.2, 135.7, 135.2, 126.0, 125.8, 124.3 (2C), 123.3, 123.2, 123.0, 118.8, 118.3, 116.8, 45.1. LC–MS (ESI) m/z: 482.4 [(M + H$^+$) calcd for free base $C_{25}H_{19}N_7S_2$, 481.11]. Analysis calcd for $C_{25}H_{21}Cl_2N_7S_2 \times 2.5H_2O$ (599.56): C, 50.08; H, 4.37; N, 16.34, Cl, 11.82. Found C, 50.24; H, 4.33; N, 16.45; Cl, 11.80%.

*3.1.1.3. 2,2′-(Pyridine-3,5-diyl)bis(1,3-benzothiazole-6-carboximidamide) dihydrochloride (**3c**).* To a solution of the free base prepared by *method A* in acetic acid (40 mL) concd HCl (2.0 mL) was added and stirred for 2 h. The resulting precipitate was filtered off, washed with acetone and dried. Crystallization from water/1 M HCl/acetone mixture gave pure compound **3c** 135 mg (48.5%) as colorless solid: mp > 300 °C. ^{1}H NMR (300 MHz, DMSO-d_6) (δ ppm): 9.61 (br s, 4H, H-Amd), 9.52 (d, 2H, J = 2.0 Hz, H-Py), 9.36 (br s, 4H, H-Amd), 9.12 (t, 1H, J = 2.0 Hz, H-Py), 8.81 (d, 2H, J = 1.5 Hz, H-Bt), 8.40 (d, 2H, J = 8.6 Hz, H-Bt), 8.02 (dd, 2H, J = 1.7 Hz, J = 8.6 Hz, H-Bt). LC–MS (ESI) m/z: 430.3 [(M + H$^+$) calcd for free base $C_{21}H_{15}N_7S_2$, 429.08]. Analysis calcd for $C_{21}H_{17}Cl_2N_7S_2 \times 3H_2O$ (556.49): C, 45.32; H, 4.17; N, 17.62, Cl, 12.74. Found C, 45.46; H, 4.08; N, 17.85; Cl, 13.04%.

*3.1.1.4. 2,2′-(Pyridine-3,5-diyl)bis[6-(4,5-dihydro-1H-imidazol-2-yl)-1,3-benzothiazole] dihydrochloride (**3d**).* To a suspension of the free base prepared by *method A* in ethanol (35 mL) concd HCl (0.5 mL) was added, stirred for 2 h, and cooled overnight. The resulting precipitate was filtered off, washed with diethyl-ether and dried. Crystallization from water/acetone mixture gave pure compound **3d** 153 mg (50.3%) as colorless solid: mp = 288–290 °C. ^{1}H NMR (300 MHz, DMSO-d_6) (δ ppm): 10.70 (br s, 4H, H-Amd), 9.54 (s, 2H, H-Py), 9.13 (s, 1H, H-Py), 8.91 (s, 2H, H-Bt), 8.44 (d, 2H, J = 8.3 Hz, H-Bt), 8.15 (d, 2H, J = 8.4 Hz, H-Bt), 4.04 (s, 8H, H–CH_2). ^{13}C NMR (75 MHz, D_2O, 70 °C) (δ ppm): 167.9, 164.3, 155.7, 149.5, 135.0, 131.8, 127.8, 126.0, 123.7, 123.0, 118.7, 44.7. LC–MS (ESI) m/z: 482.4 [(M + H$^+$) calcd for free base $C_{25}H_{19}N_7S_2$, 481.11]. Analysis calcd for $C_{25}H_{21}Cl_2N_7S_2 \times 3H_2O$ (608.56): C, 49.34; H, 4.47; N, 16.11, Cl, 11.65. Found C, 49.51; H, 4.54; N, 16.01; Cl, 11.83%.

*3.1.1.5. 2,2′-(Pyridine-2,3-diyl)bis(1,3-benzothiazole-6-carboximidamide) dihydrochloride (**3e**).* To a solution of the free base prepared by *method A* in acetic acid (40 mL) concd HCl (2.0 mL) was added and stirred for 2 h. To the reaction mixture acetone (150 mL) was added, cooled overnight, and the resulting precipitate was filtered off, washed with acetone and dried. Crystallization from water/1 M HCl/acetone mixture gave pure compound **3e** 160 mg (57.5%) as pale yellow solid: mp > 300 °C. ^{1}H NMR (300 MHz, DMSO-d_6) (δ ppm): 9.57 (br s, 2H, H-Amd), 9.52 (br s, 2H, H-Amd), 9.29 (br s, 2H, H-Amd), 9.25 (br s, 2H, H-Amd), 9.01 (dd, 1H, J = 1.5 Hz, J = 4.7 Hz, H-Py), 8.74 (d, 1H, J = 1.7 Hz, H-Bt), 8.71 (d, 1H, J = 1.7 Hz, H-Bt), 8.38 (dd, 1H, J = 1.5 Hz, J = 7.8 Hz, H-Py), 8.28 (d, 1H, J = 8.6 Hz, H-Bt), 7.98 (dd, 1H, J = 1.8 Hz, J = 8.6 Hz, H-Bt), 7.88 (dd, 1H, J = 4.8 Hz, J = 7.8 Hz, H-Py), 7.80 (dd, 1H, J = 1.8 Hz, J = 8.6 Hz, H-Bt), 7.65 (d, 1H, J = 8.6 Hz, H-Bt). ^{13}C NMR (75 MHz, D_2O/DMSO-d_6, 80 °C) (δ ppm): 169.8, 168.8, 164.7, 164.6, 154.7, 154.2, 150.1, 146.3, 138.8, 135.2, 134.8, 127.0, 124.6, 124.5, 124.4, 124.0, 123.7, 122.6, 122.2, 121.5, 121.4. LC–MS (ESI) m/z: 430.3 [(M + H$^+$) calcd for free base $C_{21}H_{15}N_7S_2$, 429.08]. Analysis calcd for $C_{21}H_{17}Cl_2N_7S_2 \times 3H_2O$ (556.49): C, 45.32; H, 4.17; N, 17.62, Cl, 12.74. Found C, 45.25; H, 4.18; N, 17.81; Cl, 12.63%.

*3.1.1.6. 2,2′-(Pyridine-2,3-diyl)bis[6-(4,5-dihydro-1H-imidazol-2-yl)-1,3-benzothiazole] dihydrochloride (**3f**).* To a solution of the free base prepared by *method A* in ethanol (25 mL) concd HCl (0.5 mL) was added, stirred for 2 h, and cooled overnight. The resulting precipitate was filtered off, washed with diethyl-ether and dried. Crystallization from water/acetone mixture gave pure compound **3f** 192 mg (63.1%) as pale yellow solid: mp = 295–298 °C. ^{1}H NMR (300 MHz, DMSO-d_6) (δ ppm): 10.89 (br s, 4H, H-Amd), 9.02 (d, 1H, J = 4.3 Hz, H-Py), 8.96 (s, 1H, H-Bt), 8.87 (s, 1H, H-Bt), 8.39 (d, 1H, J = 7.2 Hz, H-Py), 8.33 (d, 1H, J = 8.4 Hz, H-Bt), 8.18 (d, 1H, J = 8.6 Hz, H-Bt), 8.00 (d, 1H, J = 8.2 Hz, H-Bt), 7.88 (dd, 1H, J = 4.6 Hz, J = 7.5 Hz, H-Py), 7.65 (d, 1H, J = 8.2 Hz, H-Bt), 4.07 (s, 4H, H–CH_2), 4.02 (s, 4H, H–CH_2). LC–MS (ESI) m/z: 482.4 [(M + H$^+$) calcd for free base $C_{25}H_{19}N_7S_2$, 481.11]. Analysis calcd for $C_{25}H_{21}Cl_2N_7S_2 \times 3H_2O$ (608.56): C, 49.34; H, 4.47; N, 16.11, Cl, 11.65. Found C, 49.66; H, 4.35; N, 16.27; Cl, 11.53%.

*3.1.1.7. 2,2′-(Pyridine-3,4-diyl)bis(1,3-benzothiazole-6-carboximidamide) dihydrochloride (**3g**).* To a solution of the free base prepared by *method A* in acetic acid (50 mL) concd HCl (2.5 mL) was added and stirred for 2 h. To the reaction mixture acetone (150 mL) was added and the resulting precipitate was filtered off washed with acetone and dried. Crystallization from water/1 M HCl/acetone mixture gave pure compound **3g** 186 mg (66.8%) as pale yellow solid: mp = 238–242 °C. ^{1}H NMR (300 MHz, DMSO-d_6) (δ ppm): 9.54 (br s, 4H, H-Amd), 9.30 (br s, 4H, H-Amd), 9.21 (s, 1H, H-Py), 9.17 (d, 1H, J = 5.1 Hz, H-Py), 8.70–8.68 (m, 2H, H-Bt), 8.23–8.19 (m, 2H, H-Bt), 8.07 (d, 1H, J = 5.1 Hz, H-Py), 7.94–7.91 (m, 2H, H-Bt). ^{13}C NMR (75 MHz, D_2O/DMSO-d_6, 80 °C) (δ ppm): 166.4, 166.1, 164.6, 164.5, 154.1, 154.0, 149.5, 148.2, 139.4, 135.1, 135.0, 126.5, 124.8, 124.7, 124.3, 124.0, 123.8, 122.7, 122.4, 121.5, 121.4. LC–MS (ESI) m/z: 430.3 [(M + H$^+$) calcd for free base $C_{21}H_{15}N_7S_2$, 429.08]. Analysis calcd for $C_{21}H_{17}Cl_2N_7S_2 \times 3H_2O$ (556.49): C, 45.32; H, 4.17; N, 17.62, Cl, 12.74. Found C, 45.21; H, 4.23; N, 17.90; Cl, 12.78%.

*3.1.1.8. 2,2′-(Pyridine-3,4-diyl)bis[6-(4,5-dihydro-1H-imidazol-2-yl)-1,3-benzothiazole] dihydrochloride (**3h**).* To a solution of the free base prepared by *method A* in ethanol (25 mL) concd HCl (0.5 mL) was added and stirred for 2 h. The reaction mixture was concentrated to 5 mL and the resulting precipitate filtered off, washed with diethyl-ether and dried. Crystallization from water/acetone mixture gave pure compound **3h** 187 mg (61.5%) as pale yellow solid: mp = 255–258 °C. ^{1}H NMR (600 MHz, DMSO-d_6) (δ ppm): 11.01 (s, 2H, H-Amd), 11.0 (s, 2H, H-Amd), 9.24 (d, 1H, J = 0.7 Hz, H-Py), 9.05 (d, 1H, J = 5.0 Hz, H-Py), 8.94 (d, 1H, J = 1.6 Hz, H-Bt),

8.93 (d, 1H, $J = 1.7$ Hz, H-Bt), 8.25–8.23 (m, 2H, H-Bt), 8.17–8.16 (m, 2H, H-Bt), 8.11 (dd, 1H, $J = 5.2$ Hz, $J = 0.6$ Hz, H-Py), 4.03 (s, 8H, H–CH_2). ^{13}C NMR (75 MHz, D_2O/DMSO-d_6) (δ ppm): 167.8, 167.5, 166.1, 166.0, 156.1, 156.0, 149.6, 148.6, 143.0, 137.2, 129.1, 127.0, 126.9, 126.7, 124.9, 124.6, 124.0, 123.9, 120.7, 120.4, 45.3. LC–MS (ESI) m/z: 482.4 [($M + H^+$) calcd for free base $C_{25}H_{19}N_7S_2$, 481.11]. Analysis calcd for $C_{25}H_{21}Cl_2N_7S_2 \times 3H_2O$ (608.56): C, 49.34; H, 4.47; N, 16.11, Cl, 11.65. Found C, 49.18; H, 4.55; N, 16.31; Cl, 11.80%.

*3.1.1.9. 2,2′-(Pyridine-2,5-diyl)bis[6-(4,5-dihydro-1H-imidazol-2-yl)-1,3-benzothiazole] dihydrochloride (**3i**).* To a solution of the free base prepared by *method A* in acetic acid (25 mL) concd HCl (2.5 mL) was added and stirred for 1 h. The resulting precipitate was filtered off, washed with acetone and dried. Crystallization from water/1 M HCl/acetone mixture gave pure compound **3i** 158 mg (51.2%) as pale yellow solid: mp > 300 °C. ^{1}H NMR (600 MHz, DMSO-d_6) (δ ppm): 10.59 (br s, 4H, H-Amd), 9.49 (s, 1H, H-Py), 8.82–8.77 (m, 3H, 2H-Bt, H-Py), 8.57 (d, 1H, $J = 8.2$ Hz, H-Py), 8.40 (d, 2H, $J = 8.6$ Hz, H-Bt), 8.07–8.06 (m, 2H, H-Bt), 4.03 (s, 8H, H–CH_2). ^{13}C NMR (75 MHz, D_2O, 80 °C) (δ ppm): 172.5, 168.9, 164.6, 164.4, 156.6, 156.2, 150.6, 147.8, 136.4, 136.3, 135.4, 129.3, 126.3, 126.1, 124.6, 124.3, 123.6, 123.4, 121.6, 118.8, 118.7, 45.3. LC–MS (ESI) m/z: 482.4 [($M + H^+$) calcd for free base $C_{25}H_{19}N_7S_2$, 481.11]. Analysis calcd for $C_{25}H_{21}Cl_2N_7S_2 \times 3.5H_2O$ (617.57): C, 48.62; H, 4.57; N, 15.88, Cl, 11.48. Found C, 48.57; H, 4.66; N, 15.65; Cl, 11.73%.

*3.1.1.10. 2,2′-(Pyridine-2,6-diyl)bis(1,3-benzothiazole-6-carboximidamide) dihydrochloride (**3j**).* Using general *method B* and crystallization from water gave pure compound **3j** 211 mg (78.4%) as colorless crystals: mp > 300 °C. ^{1}H NMR (600 MHz, DMSO-d_6) (δ ppm): 9.35 (br s, 8H, H-Amd), 8.75 (d, 2H, $J = 1.5$ Hz, H-Bt), 8.55 (d, 2H, $J = 7.8$ Hz, H-Py), 8.35–8.33 (m, 3H, 2H-Py, H-Bt), 7.95 (dd, 2H, $J = 1.7$ Hz, $J = 8.6$ Hz, H-Bt). ^{13}C NMR (75 MHz, D_2O, 60 °C) (δ ppm): 173.0, 164.8, 156.1, 148.4, 139.9, 136.0, 125.3, 123.8, 123.6, 122.7, 122.4. LC–MS (ESI) m/z: 430.3 [($M + H^+$) calcd for free base $C_{21}H_{15}N_7S_2$, 429.08]. Analysis calcd for $C_{21}H_{17}Cl_2N_7S_2 \times 2H_2O$ (538.47): C, 46.84; H, 3.93; N, 18.21, Cl, 13.17. Found C, 46.67; H, 3.65; N, 18.19; Cl, 13.28%.

*3.1.1.11. 2,2′-(Pyridine-2,6-diyl)bis[6-(4,5-dihydro-1H-imidazol-2-yl)-1,3-benzothiazole] dihydrochloride (**3k**).* Using general *method B* and crystallization from water/1 M HCl mixture gave pure compound **3k** 222 mg (75.2%) as colorless solid: mp > 300 °C. ^{1}H NMR (600 MHz, DMSO-d_6) (δ ppm): 8.89 (d, 2H, $J = 1.3$ Hz, H-Bt), 8.57 (d, 2H, $J = 7.8$ Hz, H-Py), 8.39 (d, 2H, $J = 8.6$ Hz, H-Bt), 8.36 (d, 1H, $J = 7.8$ Hz, H-Py), 8.11 (dd, 2H, $J = 1.6$ Hz, $J = 8.6$ Hz, H-Bt), 4.04 (s, 8H, H–CH_2). ^{13}C NMR (75 MHz, D_2O/DMSO-d_6, 80 °C) (δ ppm): 170.9, 162.2, 154.3, 146.3, 137.9, 134.1, 123.7, 122.0, 121.3, 121.1, 116.2, 43.0. LC–MS (ESI) m/z: 482.4 [($M + H^+$) calcd for free base $C_{25}H_{19}N_7S_2$, 481.11]. Analysis calcd for $C_{25}H_{21}Cl_2N_7S_2 \times 2H_2O$ (590.55): C, 50.85; H, 4.27; N, 16.60, Cl, 12.01. Found C, 51.12; H, 4.23; N, 16.81; Cl, 11.92%.

*3.1.1.12. 2,5-Bis[6-(4,5-dihydro-1H-imidazol-2-yl)1,3-benzothiazol-2-yl]pyrazine dihydrochloride (**3l**).* To a solution of the free base prepared by *method A* in acetic acid (40 mL) concd HCl (2.0 mL) was added and stirred for 2 h. The resulting precipitate was filtered off, washed with acetone and dried. Crystallization from water/1 M HCl/acetone mixture gave pure compound **3l** 136 mg (46.0%) as yellow solid: mp > 300 °C. ^{1}H NMR (300 MHz, DMSO-d_6) (δ ppm): 10.65 (br s, 4H, H-Amd), 9.70 (s, 2H, H-Pyr), 8.86 (d, 2H, $J = 1.4$ Hz, H-Bt), 8.48 (d, 2H, $J = 8.7$ Hz, H-Bt), 8.10 (dd, 2H, $J = 1.5$ Hz, $J = 8.6$ Hz, H-Bt), 4.06 (s, 8H, H–CH_2). LC–MS (ESI) m/z: 483.3 [($M + H^+$) calcd for free base $C_{24}H_{18}N_8S_2$, 482.11]. Analysis calcd for $C_{24}H_{20}Cl_2N_8S_2 \times 2H_2O$ (591.54): C, 48.73; H, 4.09; N, 18.94, Cl, 11.99. Found C, 48.91; H, 4.05; N, 18.94; Cl, 11.72%.

3.2. Cell culturing

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) and BJ (normal diploid human fibroblasts), were cultured as monolayers and maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal 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.

3.3. Proliferation assays

The panel cell lines were inoculated onto a series of standard 96-well microtiter plates on day 0, at 3000 cells to 6000 cells per well according to the doubling times of specific cell line. Test agents were then added in five, 10-fold dilutions (0.01–100 μM) and incubated for further 72 h. Working dilutions were freshly prepared on the day of testing in the growth medium. The solvent (DMSO) was also tested for eventual inhibitory activity by adjusting its concentration to be the same as in the working concentrations (DMSO concentration never exceeded 0.1%). After 72 h of incubation, 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 formulas proposed by NIH and described previously [26]. This method directly relies on control cells behaving normally at the day of assay because it compares the growth of treated cells with the growth of untreated cells in control wells on the same plate – the results are therefore a percentile difference from the calculated expected value.

The IC_{50} and LC_{50} values for each compound 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 (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.

3.4. Assessment of cytokine and chemokine secretion

For detection of 12 cytokines or chemokines expression (IL1A, IL1B, IL2, IL4, IL6, IL8, IL10, IL12, IL17A, IFNg, TNFa, and GM-CSF) a multi-analyte ELISArray (Qiagen) was used. 1,00,000 cells/well were seeded in 12-well plates. BJ cell line was treated with compounds **3b** and **3l** at concentration of 0.5 μM while SW620 cells were treated with compound **3b** at concentration of 2 μM and **3l** at concentration of 50 μM for 24 h. The culturing medium was used for the detection of cytokine levels according to the producer protocol. Absorbance was measured at 450 nm.

3.5. Activity of caspases **3** and **7**

Detection of apoptosis has been measured by assessment of caspase 3 and 7 activity with ApoTox-Glo™ triplex assay (Promega).

10,000 cells per well were seeded in 96 well plates and treated with compound **3b** for 24 h at concentrations 0.01 μM, 0.1 μM, 1 μM, 10 μM and 100 μM. Caspase activity was detected by addition of 100 μL of Caspase-Glo 3/7 reagent on treated cells. Plate was briefly mixed with orbital shaker and incubated at room temperature for 30 min prior to luminescence measurement.

3.6. Chemoinformatics – in silico analysis

Simple structural characteristics and o/w lipophilicity coefficients clog *P* and clog *D* were calculated using free Internet server ChemSpider (ACD/Lab values at http://www.chemspider.com/).

Tentative predictions of biological targets and pharmacological activities were made by web-service PASS (www.pharmaexpert.ru/PASSOnline/services.php) which is based on the identification of substructure features typical for active molecules [28].

4. Conclusion

Novel diamidino substituted conformationally restricted derivatives of bis-benzothiazolyl-pyridines and pyrazine were synthesized. Commercially available isomeric pyridine and pyrazine dicarboxylic acids were condensed with amidino- and/or 2-imidazolinyl-substituted 2-aminothiophenole in polyphosphoric acid and isolated as the corresponding diamidino-substituted free bases. The desired water soluble dihydrochloride salts were prepared by reaction of appropriate crude free base with concd HCl in ethanol or acetic acid. Antiproliferative assays revealed significant differences in antiproliferative activities of diamidino- and diimidazolinyl-derivatives, the latter exerting stronger concentration-dependent antiproliferative effects and thus being a prominent compound class for further chemical optimization and biological studies. Observed experimental differences between diamidino- and diimidazolinyl-derivatives may be due to difference in molecular features like lipophilicity and H-bond formation ability, which influence membrane permeability as well as interactions with molecular targets. Interestingly, the presence of imidazolo-moiety increased the selectivity towards some tumor cell lines (SK-BR-3, HeLa and SW620) while both imidazolo- and amidino-moieties contributed to increased antiproliferative effect on the growth of MCF-7 and MiaPaCa-2 cell lines. Possible severe HERG channel blockade side effect of tested compounds predicted by PASS is related to systemic administration. Their potential thus, lies in local administration directed towards chosen biological targets. Analysis of caspases 3/7 activation and excretion of a panel of cytokines and chemokines for diimidazolinyl-derivative **3b** pointed to oxidative stress as one of possible mechanisms of antiproliferative action and predicted antineoplastic properties for tested diimidazolinyl-derivatives.

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