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Synthesis of 7-amino-6-halogeno-3-phenylquinoxaline-2-carbonitrile 1,4-dioxides: a way forward for targeting hypoxia and drug resistance of cancer cells†

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To establish a new approach for the synthesis of quinoxaline 1,4-dioxides as hypoxia-selective cytotoxic agents, an original multi-step preparation of derivatives possessing the diamine moiety at position 7 was evaluated. Herein, we present the synthesis of a series of novel 7-amino-6-halogeno-3-phenylquinoxaline-2-carbonitrile 1,4-dioxides **13a–h**, **14a,b,g** based on the regioselective Beirut reaction. Comparison of antitumor properties of derivatives possessing the diamine moiety at position 7 with structurally close congeners possessing the corresponding amino groups at position 6 revealed key differences in the cytotoxicity profiles and HIF-1 α inhibition. All the synthesized 7-amino-6-halogeno derivatives **13a–h**, **14a,b,g** demonstrated significant cytotoxic activities against breast cancer cell lines (MCF7, MDA-MB-231) in normoxia and hypoxia with IC₅₀ values ranging from 0.1 to 7.6 μ M. Most of these novel derivatives can circumvent the multidrug resistance of tumor cells caused by P-glycoprotein over expression. The lead compounds **13a**, **14a** and **14b** can suppress the expression of HIF-1 α at low micromolar concentrations and induce apoptosis in breast cancer MCF7 cells. In addition, compound **14b** effectively inhibits BCL2 and ER α expression in MCF7 cells. The current research opens a new direction for targeting hypoxia and drug resistance of cancer cells.

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Introduction

The main challenge of rapidly proliferating solid tumors is hypoxia.¹ This phenomenon promotes chemo- and radio-resistance of cancer cells in hypoxic niches. An increase in hypoxic regions in tumor tissue correlates with the worst outcome of the disease and low effectiveness of treatment.^{2,3} Moreover, hypoxia in cancers changes cellular metabolism, leading to a more aggressive phenotype, and increases the expression of genes associated with angiogenesis and invasion tending toward metastasis in human cancers. Thus, this general hallmark of tumor cells, hypoxia, is one of the main targets for the development of new selective cytotoxic drugs for hypoxic cells. One way of targeting tumor hypoxia is to design hypoxic prodrugs that are activated in hypoxic tissue and thus selectively kill hypoxic tumor

cells.⁴ Cellular reductases can convert such prodrugs to agents with impressive antiproliferative potency against hypoxic tumor cells. Several classes of compounds display hypoxia selectivity in solid tumors, such as DNA alkylating agents, nitroaromatic compounds, and aromatic *N*-oxides.⁵ The most potent compound in this class is tirapazamine (TPZ) based on the benzotriazine 1,4-di-*N*-oxide

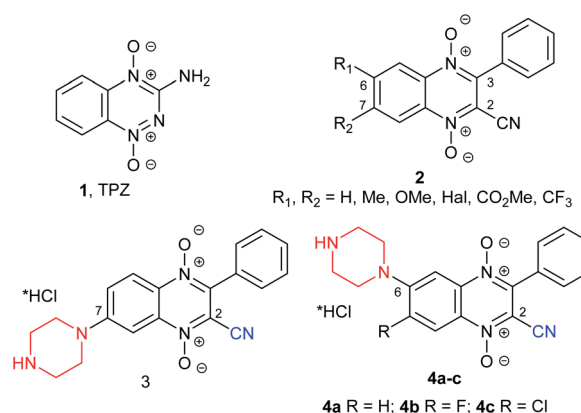


Fig. 1 Structures of the previously obtained hypoxia-selective cytotoxic agents.

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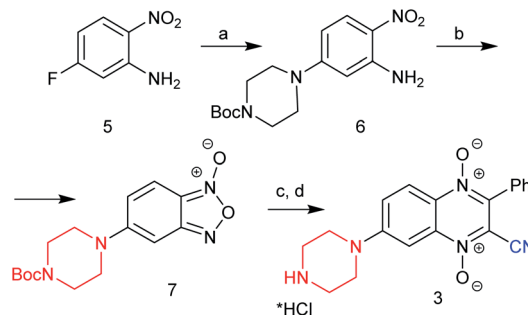
scaffold (**1**, Fig. 1). The hypoxic prodrug, tirapazamine, has been extensively tested in clinical trials, but results have been disappointing because of its poor extravascular transport. However, the unique one-electron reduction activation mechanism and encouraging antitumor profile have stimulated intense research efforts in the design and synthesis of a variety of its analogues. Based on the structural similarity between TPZ and quinoxaline 1,4-dioxide, a series of quinoxaline-2-carbonitrile 1,4-dioxides, **2–4** (ref. 6–9), have been synthesized and evaluated. Selected derivatives have shown a high hypoxia selectivity and antiproliferative potency against a panel of wild-type and drug-resistant tumor cell lines.⁹ Additionally, in an in-depth study of the Beirut reaction between monosubstituted benzofuroxans and benzoylacetone, we identified isomeric 6(7)-monosubstituted quinoxaline-2-carbonitrile 1,4-dioxide, **2**, which demonstrated significant differences in biological activity. Thus, in search for new knowledge regarding the possibility of chemical modification of the quinoxaline scaffold and for further exploration of the structure–activity relationships for quinoxaline-2-carbonitrile 1,4-dioxides, a novel water-soluble derivative with residues of cyclic diamines was obtained.¹⁰ Lead compound **4b** (for R = F, Fig. 1) suppressed HIF-1 α expression and demonstrated a strong anti-estrogenic effect.¹⁰ The position of the pharmacophore group in the heterocyclic core of 3-phenylquinoxaline-2-carbonitrile 1,4-dioxides (*e.g.*, compounds **3–4**, Fig. 1) and its structure are of fundamental importance for antiproliferative potency and hypoxic selectivity of these derivatives.^{10,11} Thus, derivative **3** with the piperazine residue in position 7 was noticeably more potent against MCF7 and MDA-MB-231 under normoxic (10 times) and hypoxic conditions (20 times) than its regioisomer **4a** (for R = H, Fig. 1). Similar differences in anticancer activity were observed for the series of isomeric derivatives, **2** (ref. 8).

Analysis of the antiproliferative properties of derivatives **2–4** (Fig. 1) showed that the position of the diamino residues in the quinoxaline ring may significantly effect interactions with targets and antiproliferative profiles. Thus, for in-depth analysis of the structural modifications of quinoxaline 1,4-dioxides, in this study, we aimed to estimate the role of the position of the cyclic diamino moiety in quinoxaline-2-carbonitrile 1,4-dioxides. For this purpose, a series of 6-halogeno-3-phenylquinoxaline-2-carbonitrile 1,4-dioxides bearing the basic fragment at position 7 in the quinoxaline ring were obtained and evaluated.

Results and discussion

Synthesis

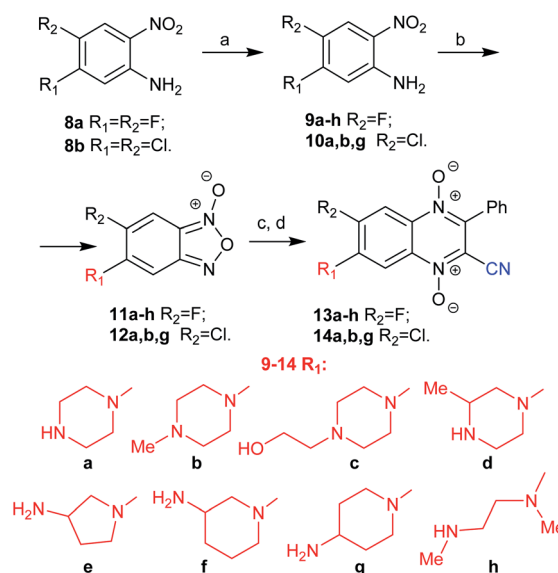
Earlier, it was shown that halogen atoms at position 6 of quinoxaline-2-carbonitrile 1,4-dioxides (due to activation with the electron-withdrawal effect of the cyano group) were more active than in position 7 in the reaction of nucleophilic substitution. Thus, the treatment of 6,7-dihalogeno-3-phenylquinoxaline-2-carbonitrile 1,4-dioxides **2** ($R_1 = R_2 = \text{Hal}$) with amines exclusively leads to 6-amino derivatives **4b** and **4c** (ref. 10). Meanwhile, the revision of the Beirut reaction showed that condensation of benzofuroxans with electron-donating substituents (for example, methyl and methoxy groups) and benzoylacetone leads only to the 7-isomers (derivatives **2**, $R_1 = \text{H}$, $R_2 =$



Scheme 1 New route for the synthesis of 7-(piperazin-1-yl)-quinoxaline-2-carbonitrile 1,4-dioxide (**3**). Reagents and conditions: (a) 1-Boc-piperazine, DMF, TEA, 50 °C, 4 h, 86%; (b) NaOCl, KOH(aq), DMF, 0–5 °C, 10–15 min, 81%; (c) BzCH₂CN, Et₃N, CHCl₃, r. t., 3 h, 71%; (d) HCl/MeOH, r. t., 68%.

Me, OMe; Fig. 1).⁸ Therefore, for the preparation of the 7-amino-derivatives of quinoxaline-2-carbonitrile 1,4-dioxides, we developed an original scheme based on similar heterocyclization of 5-amino-substituted benzofuroxans with benzoylacetone *via* the Beirut reaction (Schemes 1, 2).

First, for confirmation of this approach for the synthesis of the target 7-aminoquinoxaline-2-carbonitrile 1,4-dioxides, we examined the efficacy of this scheme for the preparation of 7-piperazinyl derivative **3**, which was obtained early from 7-fluoro derivative **2** ($R_1 = \text{H}$, $R_2 = \text{F}$; Fig. 1). Starting with 5-fluoro-2-nitroaniline (**5**) by nucleophilic substitution of an activated halogen atom at position 5 with Boc-piperazine, it converted into 2-nitro-5-(Boc-piperazinyl)aniline (**6**) in high yield (Scheme 1). The next step of this synthetic pathway was the preparation of amino-substituted benzofuroxans, which are key



Scheme 2 Synthesis of 7-amino-6-halogeno-3-phenyl-quinoxaline 1,4-dioxides **13a–h** and **14a,b,g**. Reagents and conditions: (a) *N*-Boc-protected diamine or diamine, DMF, TEA, r. t., 2–5 h, 61–90%; (b) NaOCl, KOH(aq), DMF, 0–5 °C, 10–15 min, 72–93%; (c) benzoylacetone, TEA, CHCl₃, r. t., 2 h; (d) HCl/MeOH or MsOH, r. t., 23–82%.



intermediates for the subsequent Beirut heterocyclization. Several methods can be successfully used for the synthesis of benzofuroxans.^{12–15} Among them, the most effective include thermolysis of 2-nitroarylazides and oxidative cyclization of 2-nitroanilines. However, the main limiting factors for the synthesis of benzofuroxans from 2-nitroarylazides are the two-stage method and the explosiveness of the arylazides used during isolation.¹⁵ Therefore, to obtain the key benzofuroxan, the one-stage method of oxidative cyclization of 2-nitroanilines was examined because it proceeds under mild conditions and does not require hazardous reagents. This method was found to be suitable for the preparation of aminobenzofuroxans. Thus, by treatment with sodium hypochlorite in the presence of a base, nitroaniline **6** can be cyclized into 5-Boc-aminobenzofuroxan **7** in high yield (Scheme 1). During the optimization of the conditions for the oxidation of 2-nitroaniline **6**, solvents, such as MeOH, EtOH, THF and DMF, and bases, such as NaOH and KOH, were tested. The highest yield of the target benzofuroxan **7** was achieved using an aqueous solution of potassium hydroxide as a base in DMF.

Due to the high efficiency of the previously described method for the synthesis of quinoxaline-2-carbonitrile 1,4-dioxides by the Beirut reaction,^{8,9,16,17} a similar procedure was used for the cyclization of the benzofuroxan **7** to quinoxaline-2-carbonitrile 1,4-dioxide **3**. Thus, the condensation of benzofuroxan **7** with benzoylacetonitrile in the presence of triethylamine afforded the corresponding Boc-derivative of **3** in a good yield (Scheme 1). The obtained Boc-intermediate of **3** was further purified by column chromatography and converted into the target quinoxaline-2-carbonitrile 1,4-dioxide **3** by treatment with a solution of HCl in methanol. Of note, the quinoxaline 1,4-dioxide **3** (Scheme 1), formed from benzofuroxan **7**, according to the physicochemical and spectral properties, was identical to derivative **3** (Fig. 1) previously obtained from quinoxaline 1,4-dioxide **2** ($R_1 = H$, $R_2 = F$) (ESI, Fig. S105†).^{8,9} Thus, the identical result of the counter synthesis additionally confirmed the position of the amino group in the quinoxaline core and supported the previous conclusion that the presence of electron-donating groups in the benzofuroxans contribute to the exceptional formation of 7-substituted quinoxaline-2-carbonitrile 1,4-dioxides in the Beirut reaction.⁸

Because the developed method was suitable for the synthesis of 7-aminoquinoxaline-2-carbonitrile 1,4-dioxide **3** (Scheme 1), it was used for the synthesis of an extended series of analogues with variation of the diamine residue at position 7 and the halogen atom at position 6 of the heterocyclic core. Thus, by substitution of the halogen atoms at the 5 position of nitroanilines **8a** and **8b** with different diamines **a–h**, 5-amino-nitroanilines **9a–h** and **10a,b,g** were obtained in good yields (Scheme 2). The mono Boc-protected diamines were used in the case of amines **a** and **d–h**. Additionally, to study the impact of the amino group on the biological properties of 3-phenylquinoxalin-2-carbonitrile 1,4-dioxide, linear diamine **h** was used. The procedure for the synthesis of benzofuroxan **7** (Scheme 1) was tested for the preparation of aminobenzofuroxans **11a–h** and **12a,b,g**. Thus, the oxidation of anilines **9a–h** and **10a,b,g** with sodium hypochlorite in the

presence of potassium hydroxide proceeds smoothly even in the case of derivatives **b** and **c** with ternary amino groups on the side chain and gave the key aminobenzofuroxans **11a–h** and **12a,b,g** in good yield (Scheme 2).

The subsequent Beirut condensation of benzofuroxans **11a–h** and **12a,b,g** with benzoylacetonitrile in the presence of triethylamine in chloroform selectively yielded the derivatives of 7-amino-6-halogenoquinoxaline-2-carbonitrile 1,4-dioxides **13a–h** and **14a,b,g** without their regioisomers (Scheme 2).

In the case of fluorine derivatives, the Boc intermediates were transformed into hydrochlorides of the target 7-amino-6-fluoroquinoxaline-2-carbonitrile 1,4-dioxides **13a–h** by treatment with HCl in methanol. Comparison of the solubility of derivative **4a** with hydrochlorides of **4b–c**, **13a** and **14a** showed that the introduction of a halogen atom into the benzene ring of quinoxaline 1,4-dioxides significantly decreased their solubility in water (ESI, Table S2†). Thus, the hydrochlorides of chlorine derivatives **14a,b,g** were poorly soluble in water, while their methanesulfonates obtained by treatment of the Boc intermediates with methanesulfonic acid had noticeably higher solubility (ESI, Table S2†). Therefore, the studies of the biological and physicochemical properties of compounds **14a,b,g** were carried out for their mesylates.

All final compounds of **13a–h** and **14a,b,g** were further purified by re-precipitation of their hydrochloride or mesylate salts. The purified compounds (purity >95%, HPLC) were used for analytical and biological studies. The structure of undescribed benzofuroxanes **7**, **11a–h**, and **14a,b,g** were studied by ¹H and ¹³C NMR analysis at 75 °C in DMSO-*d*₆.¹⁸ When ¹H and ¹³C NMR spectra of **11a** were recorded at room temperature, the aromatic region demonstrated very broad and poorly interpreted signals (ESI, Fig. S23 and S24†). Thus, at high temperature (75 °C), the signals of the aromatic region in ¹H and ¹³C NMR spectra were observed as precise narrow peaks, except for the signals regarding the quaternary carbon atoms of C-8 and C-9 (ESI, Fig. S25 and S26†).¹⁸ The structure of quinoxalin-2-carbonitrile 1,4-dioxide **3** (Fig. 1 and Scheme 1) was confirmed by HRMS ESI, HPLC, and NMR spectroscopies. Of note, in contrast to the earlier described 7(6)-monosubstituted quinoxaline-2-carbonitrile-1,4-dioxides **2** ($R_1 = H$), **3**, and **4a**, 7-amino-6-halogenderivatives **13a**, **14a**, and their previously obtained regioisomers **4b–c** showed differences in mobility on TLC and in retention times (*t*_R) in HPLC. As in the case of 6(7)-aminoderivatives **3** and **4a**, regioisomers **13a**, **14a**, **4b** and **4c** have identical ¹H NMR spectra and different ¹³C NMR spectra, which made it possible to distinguish the isomers from each other.

Comparison of the chemical shifts of the signals in the ¹³C NMR spectra of 3-phenylquinoxaline-2-carbonitrile 1,4-dioxide⁸ with the corresponding signals of 7- and 6-amino derivatives **13a**, **14a**, **4b**, and **4c** showed that changes in the chemical shifts of the signals correspond with increments (*f*^C) of the tertiary amino group in the ¹³C NMR spectra of arenes^{19,20} (ESI, Table S1†). Introduction of the amino group at position 7 (for **13a** and **14a**) led to a significant shift in signals in the *para*-position of the benzene ring (C-10) and pyrazine fragment (C-3). Thus, the analysis of the chemical shift increments in ¹³C NMR spectra confirmed the position of the amino group in the quinoxaline 1,4-dioxides obtained in the Beirut reaction.

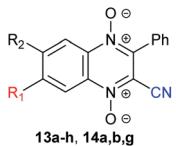


Antiproliferative activity

Novel 7-amino-6-halogeno-3-phenylquinoxaline-2-carbonitrile 1,4-dioxides **13a–h** and **14a,b,g** were tested against several mammalian cancer cell lines, including breast cancer cell lines

MCF7 and MDA-MB-231, under normoxic (21% O₂) and hypoxic (1% O₂) conditions and human myeloid leukemia K562 and its isogenic drug-resistant counterpart K562/4 (selected for resistance to doxorubicin (**Dox**); Pgp-positive).

Table 1 Structures and cytotoxicity (IC₅₀^b) of 7(6)-amino-3-phenylquinoxaline-2-carbonitrile 1,4-dioxides **13a–h** and **14a,b,g** under normoxic and hypoxic conditions

 13a-h, 14a,b,g								
			IC ₅₀ ^b (μM)					
Compound	R ₁	R ₂	MCF7			MDA-MB-231		
			N ^c	H ^d	HCR ^a	N ^c	H ^d	HCR ^a
13a		F	0.78 ± 0.08	0.65 ± 0.07	1.2	1.41 ± 0.02	0.61 ± 0.07	2.3
13b		F	1.1 ± 0.1	1.2 ± 0.1	0.9	1.72 ± 0.16	0.90 ± 0.08	1.9
13c		F	1.6 ± 0.2	0.8 ± 0.07	2.0	3.8 ± 0.2	1.7 ± 0.1	2.2
13d		F	0.93 ± 0.08	0.61 ± 0.07	1.5	0.89 ± 0.07	0.49 ± 0.06	1.8
13e		F	1.9 ± 0.2	2.1 ± 0.2	0.9	3.1 ± 0.2	1.6 ± 0.2	1.9
13f		F	1.6 ± 0.2	0.80 ± 0.08	2.0	2.0 ± 0.1	1.0 ± 0.1	2.0
13g		F	0.74 ± 0.08	0.62 ± 0.08	1.2	1.2 ± 0.1	0.57 ± 0.06	2.1
13h		F	21.8 ± 2.0	22.8 ± 2.1	1.0	18.0 ± 2.0	19.0 ± 2.2	0.9
14a		Cl	0.92 ± 0.08	0.22 ± 0.03	4.2	1.0 ± 0.1	0.40 ± 0.05	2.5
14b		Cl	5.8 ± 0.5	0.15 ± 0.1	39.8	7.0 ± 0.8	0.30 ± 0.04	23.3
14c		Cl	5.0 ± 0.5	3.8 ± 1.1	1.3	7.6 ± 0.7	2.2 ± 0.2	3.5
4b	F		2.0 ± 0.1	0.42 ± 0.03	4.7	1.5 ± 0.1	0.35 ± 0.02	4.3
4c	Cl		0.82 ± 0.05	0.23 ± 0.02	3.6	0.90 ± 0.05	0.29 ± 0.02	3.1
TPZ	—	—	24.2 ± 3.3	4.5 ± 1.1	5.4	39.5 ± 5.4	22.0 ± 2.1	1.8
Dox	—	—	0.31 ± 0.03	0.44 ± 0.03	0.7	0.32 ± 0.03	0.21 ± 0.01	1.5
CisPt	—	—	8.3 ± 0.8	11.4 ± 2.3	0.7	13.0 ± 1.2	16.2 ± 1.0	0.8

^a HCR, hypoxic cytotoxicity ratio: IC_{50(N)}/IC_{50(H)}. ^b IC₅₀, μM (mean ± S.D. of 3 experiments). ^c N = Normoxia: 21% of oxygen. ^d H = Hypoxia: 1% of oxygen.



Table 2 Circumventing MDR in leukemia cell lines by 7-amino-6-halogeno-3-phenylquinoxaline-2-carbonitrile 1,4-dioxides **13a–h** and **14a,b,g**

Compound	IC ₅₀ ^a (μM)		RI ^b
	K562	K562/4	
13a	1.0 ± 0.07	1.2 ± 0.07	1.2
13b	0.6 ± 0.02	1.1 ± 0.07	1.8
13c	2.5 ± 0.2	1.6 ± 0.09	0.6
13d	1.8 ± 0.09	1.2 ± 0.06	0.7
13e	1.0 ± 0.05	1.3 ± 0.05	1.3
13f	1.0 ± 0.03	0.4 ± 0.03	0.4
13g	1.1 ± 0.4	1.3 ± 0.08	1.2
13h	>50	>50	—
14a	3.0 ± 0.3	3.5 ± 0.5	1.1
14b	3.0 ± 0.5	4.0 ± 0.6	1.3
14g	5.5 ± 0.7	6.0 ± 0.8	1.1
4b	4.2 ± 0.5	4.0 ± 0.6	0.9
4c	2.0 ± 0.1	1.5 ± 0.2	0.8
Dox	0.3 ± 0.02	5.0 ± 0.7	11.7

^a IC₅₀, μM (mean ± S.D. of 3 experiments). ^b RI, resistance index: IC₅₀(K562/4)/IC₅₀(K562).

For the antiproliferative potencies of derivatives **13a–h** and **14a,b,g**, reference compounds **4b**, **4c**, **TPZ**, **Dox**, and **CisPt** are summarized in Tables 1 and 2. All new derivatives **13a–h** and **14a,b,g** inhibited tumor cell proliferation at submicromolar or low micromolar concentrations. The majority of synthesized compounds demonstrated high antiproliferative activities against adenocarcinoma cells, which were close to that of the piperazine derivatives **4b** and **4c** (Table 1). Moreover, the cytotoxic potencies of the synthesized quinoxalines **13a–h** and **14a,b,g** were 4.2–34.6 times and 1.2–45 times (under normoxic and hypoxic conditions, respectively) higher than for the reference **TPZ** agent against estrogen-dependent MCF7 breast cancer cells. Of note, the triple-negative breast cancer cell line MDA-MB-231 was a more sensitive cell line for the obtained compounds **13a–h** and **14a,b,g**, as observed in the IC₅₀ values, which were 5.2–49.4 times and 10–73 times, respectively, higher than those for **TPZ**. Additionally, as in the case of the previously obtained compounds (e.g., **3–4**), the inhibitory potency of these derivatives increased under hypoxic conditions. The most hypoxia-selective was the 6-chloro-7-methylpiperazine derivative **14b**, which showed 7.4- and 12.9-fold improvement compared with **TPZ** (HCR values of 5.4 and 1.8) with HCR values of 39.8 and 23.3, respectively. Of note, the structure of the diamine residue plays an important role in the biological activity of the derivatives of this scaffold. Thus, its analogues with different cyclic diamino groups in the side chain were less selective under hypoxic conditions but more potent for MCF7 and MDA-MB-231 with similar IC₅₀ values against these tumor cell lines. In striking contrast, compound **13h** with the linear *N,N'*-dimethylethylenediamine residue showed no activity against all tested cell lines. The relocation of the cyclic amino groups in the halogen-substituted 3-phenylquinoxaline-2-carbonitrile 1,4-dioxides **13a–g** and **14a,b,g** led to an increase

in the cytotoxic activity of these derivatives compared to the previously obtained 6-amino regioisomers¹⁰ but significantly reduced their solubility. Thus, in contrast to the previously obtained monosubstituted quinoxaline-2-carbonitrile 1,4-dioxides **3** and **4a**, fluoroderivatives **13a–g** demonstrated 3–6 times higher IC₅₀ values under normoxic conditions than the previously obtained regioisomers against the tested adenocarcinoma cell lines.¹⁰ Interestingly, replacement the fluorine atom at the 6 position with chlorine decreased the cytotoxicity of **14a,b,g** under normoxic conditions but improved the activity and selectivity in hypoxia.

Because decreased drug accumulation is one of the reasons for the MDR phenotype of tumor cells, the development of chemotherapeutic agents that can circumvent the P-glycoprotein (Pgp)-mediated efflux of numerous clinical drugs, including the “gold standard” doxorubicin (**Dox**), is an urgent goal in medicine. Importantly, most new derivatives are similarly potent for wild-type leukemia cells (K562) and sublines with an altered chemotherapeutic drug response (K562/4) with the expression of functional Pgp, which are critical for the cytotoxic properties of **Dox**.^{21–24} As shown in Table 2, **13a–g** (except for derivative **13h**) all effectively inhibited the growth of K562 cells as well as MDR-subline K562/4 at submicromolar or low micromolar concentrations. Overall, the introduction of a halogen atom at position 6 of the quinoxaline ring, as in the case of regioisomers **3** and **4b,c**, led to an increase in cytotoxicity of derivatives **13a** and **14a** in comparison with their isomer **4a**.¹⁰ Importantly, the majority of the obtained derivatives were more potent to Pgp expression of the K562/4 subline than for the parental cell line of K562. Thus, derivatives **13c**, **13d** and **13f** demonstrated a higher antiproliferative activity against Pgp-positive K562/4 cells than for the wild-type cell line K562, with a resistance index (RI) less than 1.

Interestingly, both the halogen atom and the amino group position had no significant effect on the ability to block the growth of leukemia cells, while the structure of the diamine in the heterocyclic nucleus was found to be of decisive importance for the overall level of activity of the compounds and, more importantly, showed a strong effect on overcoming the resistance of cells with the overexpression of transmembrane transporter Pgp. Compound **13f** with the 3-aminopiperidine residue in the side chain notably stands out with its antiproliferative activity against Pgp-positive cells, whereas doxorubicin is 8.8-fold less active against the MDR subline. The resistance index (RI) for the **13f** derivative is 30-fold higher compared to that of **Dox** (RI value of 11.7). Of note, the introduction of the linear diamine into the quinoxaline 1,4-dioxide moiety led to a dramatic decrease in activity against all tested tumor cell lines. Additionally, the 7-aminoderivatives of 6-halogenoquinoxaline-2-carbonitrile 1,4-dioxide showed higher apoptotic potency for both leukemia cell lines (K562; K562/4) than the previously obtained series of their regioisomeric analogues (**3**, **4**).¹⁰ In striking contrast to the previously obtained regioisomers **3** and **4a** (Fig. 1), compound **14a** had similar IC₅₀ values against all tested cell lines compared to its 6-substituted analogue **4b** (Fig. 1 and Tables 1 and 2).¹⁰ Nevertheless, the



location of the substituents also plays an important role in the cytotoxic properties.

Overall, comparison of the cytotoxicity of the obtained derivatives **13a–g** with the previously obtained regioisomeric analogues¹⁰ showed that compounds **13a–g** containing an amino group at position 7 of the quinoxaline core were 2–5 times more cytotoxic than their isomers.

HIF-1 α targeting

Three compounds, **13a**, **14a** and **14b** were selected for further in-depth study. The antiproliferative effects of the chosen compounds on MCF7 cells varied in IC₅₀ values from 0.7 to 5.8 μ M in normoxia and from 0.1 to 0.6 μ M in hypoxia. To analyze the signaling pathways in the MCF7 line, cells were cultivated in hypoxia (1% O₂), and then, protein expression was assessed by immunoblotting. Control MCF7 cells were incubated in normoxia (21% O₂). As shown in Fig. 2, hypoxia induces a significant accumulation of HIF-1 α in MCF7 cells.

At low concentration, **13a** slightly decreased the hypoxia-induced expression of HIF-1 α , and the effects of **14a** and **14b** were more pronounced. At high doses, all three selected compounds completely blocked HIF-1 α expression, suggesting their potency as anti-HIF1 agents. More effective inhibition of HIF-1 α was revealed for **14b**, which was characterized by a high HCR (39.8, Table 1).

Research on HIF inhibition by quinoxaline derivative treatment is under way and is actively being developed by several research groups. Mona Diab-Assef and colleagues have shown that quinoxaline 1,4-dioxides are hypoxia-selective active compounds, and HIF-1 α is considered one of their targets. The most cytotoxic 2-benzoyl-3-phenyl-6,7-dichloroquinoxaline 1,4-dioxide (DCQ) was potent at 1 μ M with an HCR of 100 and significantly reduced the levels of HIF-1 α transcript and protein.²⁵ HIF-1 α was inhibited in cell lysates of MCF7 and MDA-MB-231 after 6 h of exposure to 5 μ M DCQ under normoxia or hypoxia in one study.²⁶ Thus, DCQ is active at micromolar concentrations, while the new quinoxaline derivatives obtained in our study block the expression of HIF-1 α at a concentration of 300 nM.

Hypoxia stimulates the active growth of blood vessels in tumors. The new vessels supply a tumor with oxygen and

nutrients, supporting its growth. Blocking angiogenesis (vascular growth) is one of the promising approaches for the treatment of solid neoplasms. Quinoxaline 1,4-dioxides, due to their ability to inhibit HIF-1 α , are considered potential agents targeting angiogenesis.²⁷ DCQ showed cytotoxic effects against Lewis lung carcinoma and EMT-6 mammary adenocarcinoma cells under hypoxic conditions as described previously.²⁷ Treatments with DCQ and 2-benzoyl-3-phenylquinoxaline 1,4-dioxide (BPQ) inhibited HIF-1 α and VEGF secretion, which are key proangiogenic factors, with DCQ being more potent than BPQ. Another derivative, 3-(4-bromophenyl)-2-(ethylsulfonyl)-6-methylquinoxaline 1,4-dioxide (Q39), has shown great anti-proliferative activity in extensive cell lines in normoxia and hypoxia.²⁸ Weng Q. and colleagues showed that pretreatment of SMMC-7721 cells with Q39 at a micromolar concentration significantly inhibited hypoxia-induced HIF-1 α protein accumulation in a dose-dependent manner.²⁸ Suppression of HIF-1 α accumulation by Q39 correlated with dephosphorylation of mTOR and 4E-BP1.

Assessment of apoptosis

Quinoxaline derivatives are active inducers of tumor cell death, as discussed in several papers.^{29–32} For instance, quinoxaline dioxide DCQ causes the death of various types of breast cancer. DCQ causes a significant accumulation of ROS in cells, which leads to their death. In the work of Khaled Ghattass and colleagues, it was revealed that 3–5 μ M DCQ can induce the death of MCF7 and MDA-MB-231 cells.³³ Apoptosis in cells is assessed by the presence of specific markers, and one of these markers is cleaved PARP.³⁴ MCF7 cells were treated with compounds **13a**, **14a** and **14b** for 3 days, and then, the level of

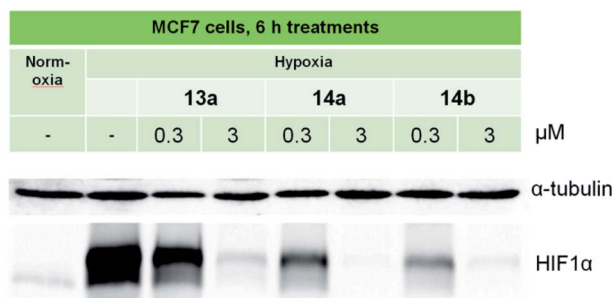


Fig. 2 Immunoblotting of the HIF-1 α protein in MCF7 cells treated with lead compounds. Antibodies against α -tubulin were used to control loading.

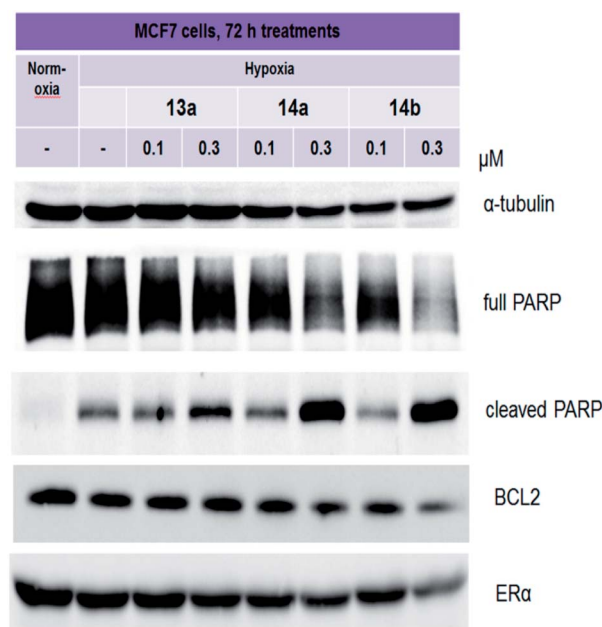


Fig. 3 Immunoblotting of PARP, BCL2, and ER α in MCF7 cells treated with lead compounds **13a**, **14a** and **14b**. Antibodies against α -tubulin were used to control loading.



full and cleaved PARP was determined in the samples. As shown in Fig. 3, all three selected compounds increased the accumulation of cleaved PARP. Compound **13a** causes moderate accumulation of cleaved PARP, whereas **14a** and **14b** induce its significant accumulation. BCL2 belongs to a large family of proteins that are involved in the regulation of cell death. By blocking the mitochondrial pathway, BCL2 prevents apoptosis, and this protein is considered a target for future therapies.^{35,36} Quinoxaline drug candidates XK469 and CQS exhibit anti-proliferative and proapoptotic effects against cancers, including through the mitochondria.^{37,38} In a recent work by Yukari Ono and colleagues,³⁸ quinoxaline-1,3,4-oxadiazole hybrid derivatives were considered as potential inhibitors of the anti-apoptotic BCL2 protein. RT-PCR analysis demonstrated that novel derivatives inhibit BCL2 expression.

In our work, the expression of BCL2 was analyzed by immunoblotting. Derivative **14a** caused a slight decrease in BCL2 expression, whereas the effects of compound **14b** were more pronounced. Considering that ER α is the main driver of the growth of hormone-dependent cancers, we analyzed its expression in MCF7 cells. As shown in Fig. 3, at submicromolar concentrations, **14b** decreased ER α expression.

Thus, the selected compounds applied at submicromolar concentrations have a complex effect on the signaling pathways in cancer cells: they block HIF1 α and induce apoptosis. Moreover, the lead compound **14b** inhibits BCL2 and ER α expression.

Conclusions

We developed a convenient method for preparing halogen-substituted quinoxaline-2-carbonitrile 1,4-dioxides bearing a diamino side chain at position 7 of the heterocyclic ring starting from amino-substituted 2-nitroanilines **9a–h** and **10a,b,g**. The high regioselectivity of the Beirut reaction was shown in the case of benzofuroxans with electron-donating substituents, which made it possible to obtain 7-amino-6-halogenoquinoxaline-2-carbonitrile 1,4-dioxides. The presence of an amino group in the quinoxaline core allowed us to improve the biological properties and the aqueous solubility of these compounds. All the obtained compounds (except **13h** with the linear amine residue) were highly efficient against adenocarcinoma cell lines (MCF7, MDA-MB-231). The most hypoxia-selective 6-chlorosubstituted quinoxaline-2-carbonitrile 1,4-dioxide, **14b**, bearing the *N*-methylpiperazine moiety, demonstrated superior HCR values for MCF7 and MDA-MB-231 cell lines at 39.8 and 23.3, respectively, and an IC₅₀ < 0.5 μ M under hypoxic conditions. This compound was also 7.4- and 12.9-fold more potent than the reference hypoxic cytotoxin, **TPZ** (HCR values of 5.4 and 1.8, respectively) (Table 1). Furthermore, compounds **13a–g** demonstrated similar toxicity for the MDR cell line K562/4 and its K562 wild-type cells (RI values between 0.4 and 1.8; Table 2) as opposed to being otherwise resistant to Pgp-transported drugs, including doxorubicin (**Dox**) (RI = 11.7; Table 2). Of note, the introduction of a halogen atom independent of the position in the quinoxaline ring, as in the case of regioisomers **4a**, **4b** and **4c**, leads to an increase in the

cytotoxicity of derivatives **13a** and **14a** compared with that of compound **3**.¹⁰ It was revealed that the structure of the diamine in the quinoxaline core is of critical importance for the biological activity of such compounds. Thus, the screening of cytotoxicity properties of the obtained compounds showed that derivative **13h** with the linear diamine moiety (Tables 1 and 2) in the quinoxaline scaffold exhibited no activity against all tested cancer cells. The selected compounds **13a**, **14a** and **14b** acted *via* HIF-1 α inhibition and induced apoptosis under hypoxia. Moreover, ER α and BCL2 expression was inhibited in hormone-dependent breast cancer cells after treatment with lead compound **14b** at submicromolar concentration. Therefore, these derivatives could be potential leaders for further structural modifications and biological studies, especially *in vivo* evaluations. Novel 7-amino-6-halogeno-3-phenylquinoxaline-2-carbonitrile 1,4-dioxides are of great interest for the development of new approaches for the targeting of tumors with large hypoxic regions.

Experimental part

General methods

NMR spectra of all synthesized compounds were recorded on a Varian VXR-400 instrument at 400 MHz (¹H NMR) and 100 MHz (¹³C NMR). Chemical shifts were measured in DMSO-*d*₆ using TMS as an internal standard. NMR spectra of benzofuroxans **7**, **11a–h**, **10a,b,g** were recorded at 80 °C. Analytical TLC was performed on silica gel F254 plates (Merck) and column chromatography on Silica Gel Merck 60. Melting points were determined on a Buchi SMP-20 apparatus and are uncorrected. High-resolution mass spectra were recorded by electron spray ionization on a Bruker Daltonics micro OTOF-QII instrument. UV spectra were recorded on a Hitachi-U2000 spectrophotometer. IR spectra were recorded on Nicolet iS10 Fourier transform IR spectrometer (Nicolet iS10 FT-IR, Madison, WI, USA). HPLC was performed using Shimadzu Class-VP V6-12SP1 system (GraseSmart RP-18, 6 $\times$ 250 mm). Eluents: A, H₃PO₄ (0.01 M); B, MeCN. All solutions were evaporated at reduced pressure on a Buchi-R200 rotary evaporator at a temperature below 50 °C. All solvents, chemicals, and reagents were obtained from Sigma-Aldrich (unless specified otherwise) and used without purification. The purity of compounds **13a–h** and **14a,b,g** was >95% as determined by HPLC analysis. All products were dried under vacuum at room temperature. The synthesis of compounds **9b**, **10b** based on the nucleophilic substitution of halogen atom in the nitroanilines **8a–b** and subsequent oxidation of **9b**, **10b** into benzofuroxans **9b** and **10b** respectively, have been described previously.^{39–45}

5-(tert-Butyl-piperazine-1-yl-carboxylate)-2-nitroaniline (6). To a stirring mixture of 5-fluoro-2-nitroaniline (**5**, 1.0 g, 6.4 mmol) in *N,N*-dimethylformamide (10 mL) the 1-Boc-piperazine (3.0 g, 16.0 mmol) and Et₃N (1 mL) were added at 50 °C. The mixture was stirred for 4 h at 50 °C. The mixture was poured in water (20 mL) and stirred for 1 h when cooling. The precipitate was filtered, washed with water (30 mL) and dried. Yield 1.78 g (5.5 mmol, 86%), yellow powder, mp 125–130 °C. ¹H NMR (400 MHz; DMSO-*d*₆; Me₄Si) δ 8.13 (1 H, s, H_{Ar}), 7.93 (1H, s, H_{Ar}), 7.34 (2H, s, NH₂), 6.60 (1H, s, H_{Ar}), 3.51–3.49 (4H, br m, CH₂), 3.05–



3.03 (4H, br m, CH₂), 1.44 (9H, s, CH₃); ¹³C NMR (100 MHz; DMSO-*d*₆) 154.2 (CO), 153.5 (C–N), 145.9 (C–NH₂), 127.1 (CH), 126.3 (CH), 112.4 (C–NO₂), 107.4 (CH), 78.7 (C(CH₃)₃), 49.6 (2 × CH₂), 42.8 (2 × CH₂), 27.6 (3 × CH₃). HRMS (ESI) calculated for C₁₅H₂₃N₄O₄⁺ (M + H)⁺ 323.1714, found 323.1687.

6-(tert-Butyl-piperazine-1-yl-carboxylate)benzofuroxan (7). The nitroaniline **6** (1.0 g, 3.1 mmol) was dissolved in DMF (30 mL^{−1}) and the 0.05 mL^{−1} 50% aqueous solution of potassium hydroxide was added. The mixture was cooled to 0–5 °C and a solution of sodium hypochlorite (5 mL, 13%) was added dropwise with vigorous stirring. After the addition was complete, the reaction mixture was stirred for 10 min. The resulting solution was poured into cold water (50 mL). The precipitate was filtered, washed with water (30 mL) and dried. The product was crystallized from toluene to give the compound **7** (0.8 g, 81%), yellow solid, mp 150–152 °C. ¹H NMR (400 MHz; DMSO-*d*₆; Me₄Si) δ 7.52–7.45 (2H, m, H-4, H-5), 6.41 (1H, s, H-7), 3.50–3.47 (4H, m, CH₂), 3.37–3.35 (4H, m, CH₂), 1.44 (9H, s, CH₃); ¹³C NMR (100 MHz; DMSO-*d*₆) δ 154.4 (CO), 131.7–131.4 (m, 5-CH), 127.9–127.7 (m, 8-C), 121.8–120.3 (m, 4-CH), 115.9–115.5 (m, 9-C), 89.8–89.2 (m, 7-CH), 79.6 (C(CH₃)₃), 43.2 (2 × CH₂), 47.1 (2 × CH₂), 28.6 (CH₃). HRMS (ESI) calculated for C₁₅H₂₁N₄O₄⁺ (M + H)⁺ 321.1557, found 321.1547.

7-(Piperazine-1-yl)-3-phenylquinoxaline-2-carbonitrile-1,4-dioxide hydrochloride (3). To a stirring mixture of benzofuroxan **7** (100 mg, 0.31 mmol) and benzoylacetone nitrile (68 mg, 0.47 mmol) in chloroform (10 mL) triethylamine (200 μL) was added at room temperature. The mixture was stirred for 3 h. The formed precipitate was filtered and washed with cold chloroform (3 mL). The crude product was crystallized from ethanol–toluene mixture gave pure appropriate *N*-Boc-intermediate (0.98 g, 71%). A red powder of Boc-intermediate was dissolved in DCM (5 mL), then the 3 M solution of HCl in MeOH (2 mL) was added and the reaction mixture was stirred at room temperature for 2 h. The solvent was evaporated and residue was dissolved in boiling water (5 mL), filtered and evaporated under vacuum. The product was precipitated with water–acetone–ether mixture (1 : 3 : 1) and dried. Yield 0.73 g (2.1 mmol, 68%), red solid, mp 223–234 °C. HPLC (LW = 300 nm, gradient B 20/60% (30 min)) *t*_R = 10.3 min, purity 95.2%. λ_{max} (EtOH)/nm 222, 299, 361, 479. IR ν_{max}, (film)/cm 3393, 2980, 2911, 2718, 2493, 1710, 1600, 1519, 1505, 1456, 1389, 1324, 1316, 1268, 1192, 1151, 1030, 996, 953. ¹H NMR (400 MHz; DMSO-*d*₆; Me₄Si) δ 9.65 (2H, br s, NH₂⁺), 8.32 (1H, d, *J* = 9.5 Hz, H-5), 7.88 (1H, d, *J* = 9.5 Hz, H-6), 7.70–7.44 (6H, m, C₆H₅, H-8), 3.82–3.75 (4H, m, CH₂), 3.48–3.39 (4H, m, CH₂); ¹³C NMR (100 MHz; DMSO-*d*₆) δ 151.9 (7-C), 139.9 (3-C), 138.0 (10-C), 132.3 (9-C), 130.7 (4'-CH), 130.4 (2 × 2'-CH), 128.4 (2 × 3'-CH), 127.9 (1'-C), 123.6 (5-CH), 121.5 (6-CH), 120.1 (2-C), 111.4 (CN), 98.6 (8-CH), 43.8 (2 × CH₂), 42.1 (2 × CH₂). HRMS (ESI) calculated for C₁₉H₁₈N₅O₂⁺ (M + H)⁺ 348.1455, found 348.1441.

5-(tert-Butyl-piperazine-1-yl-carboxylate)-4-fluoro-2-nitroaniline (9a). This compound was prepared from **8a** and 1-Boc-piperazine as described for **6**. Yield 1.4 g (4.1 mmol, 72%), yellow powder, mp 228–230 °C. ¹H NMR (400 MHz; DMSO-*d*₆; Me₄Si) δ 7.64 (1H, d, *J* = 14.5 Hz, H_{Ar}), 7.38 (2H, s, NH₂), 6.42 (1H, d, *J* = 8.2 Hz, H_{Ar}), 3.46–3.14 (4H, br m, CH₂), 3.14–3.12

(4H, m, CH₂), 1.42 (9H, s, CH₃); ¹³C NMR (100 MHz; DMSO-*d*₆) δ 153.8 (d, *J* = 10.7 Hz, CO), 146.9 (d, *J* = 9.9 Hz, C–N), 145.1 (C–NH₂), 145.0 (d, *J* = 238.5, C–F), 122.0 (d, *J* = 8.4 Hz, C–NO₂), 111.2 (d, *J* = 26.1 Hz, CH), 104.5 (CH), 79.2 (C(CH₃)₃), 48.9 (2 × CH₂), 43.4 (2 × CH₂), 28.0 (3 × CH₃). HRMS (ESI) calculated for C₁₅H₂₂FN₄O₄⁺ (M + H)⁺ 341.1620, found 341.1614.

4-Fluoro-5-(methylpiperazine-1-yl)-2-nitroaniline (9b). This compound was prepared from **8a** and *N*-methylpiperazine as described in.⁴¹ Yield 1.3 g (5.1 mmol, 89%), yellow powder, mp 158–160 °C (lit.,⁴¹ 154–155 °C). ¹H NMR (400 MHz; DMSO-*d*₆; Me₄Si) δ 7.60 (1H, d, *J* = 14.5 Hz, H_{Ar}), 7.31 (2H, s, NH₂), 6.41 (1H, d, *J* = 14.5 Hz, H_{Ar}), 3.20–14 (4H, br m, CH₂), 2.47–2.42 (4H, br m, CH₂), 2.21 (3H, s, CH₃); ¹³C NMR (100 MHz; DMSO-*d*₆) δ 146.9 (d, *J* = 9.9 Hz, C–N), 144.9 (d, *J* = 239.2, C–F), 144.8 (C–NH₂), 121.8 (d, *J* = 7.7 Hz, C–NO₂), 110.9 (d, *J* = 26.1 Hz, CH), 104.0 (CH), 53.9 (2 × CH₂), 48.8 (2 × CH₂), 45.3 (CH₃).

4-Fluoro-5-(hydroxyethylpiperazine-1-yl)-2-nitroaniline (9c). To a stirring mixture of 4,5-difluoro-2-nitroaniline **8a** (1 g, 5.7 mmol) in *N,N*-dimethylformamide (7 mL^{−1}) the 1-hydroxyethylpiperazine (3.7 g, 28.7 mmol) was added at 50 °C. The mixture was stirred for 4 h at 50 °C. The mixture was poured in water (20 mL) and stirred for 1 h when cooling. The precipitate was filtered, washed with water (30 mL) and dried. Yield 1.2 g (4.4 mmol, 76%), yellow powder, mp 155–156 °C. ¹H NMR (400 MHz; DMSO-*d*₆; Me₄Si) δ 7.60 (1H, d, *J* = 14.5 Hz, H_{Ar}), 7.34 (2H, s, NH₂), 6.39 (1H, d, *J* = 8.2 Hz, H_{Ar}), 4.45 (1H, t, *J* = 4.5 Hz, OH), 3.53 (2H, q, *J* = 6.3 Hz, CH₂CH₂OH), 3.16–3.14 (4H, br m, CH₂), 2.55–2.53 (4H, br m, CH₂), 2.43 (2 H, t, *J* = 6.3 Hz, CH₂–CH₂OH); ¹³C NMR (100 MHz; DMSO-*d*₆) *d* 147.2 (d, *J* = 10.7 Hz, C–N), 145.2 (C–NH₂), 145.1 (d, *J* = 239.2 Hz, CF), 121.8 (d, *J* = 8.4, C–NO₂), 111.1 (d, *J* = 26.1 Hz, CH), 104.1 (CH), 60.1 (CH₂), 58.5 (CH₂), 52.7 (CH₂), 49.1 (CH₂). HRMS (ESI) calculated for C₁₂H₁₈FN₄O₃⁺ (M + H)⁺ 285.1357, found 285.1374.

5-(tert-Butyl-3-methylpiperazine-1-yl-carboxylate)-4-fluoro-2-nitroaniline (9d). This compound was prepared from **8a** and *N*-Boc-2-methylpiperazine as described for **6**. Yield 1.6 g (4.7 mmol, 81%), yellow powder, mp 213–215 °C. ¹H NMR (400 MHz; DMSO-*d*₆; Me₄Si) *d* 7.64 (1H, d, *J* = 14.5 Hz, H_{Ar}), 7.38 (2H, s, NH₂), 6.40 (1H, d, *J* = 8.2 Hz, H_{Ar}), 4.20–4.18 (1H, m, CH), 3.80 (1H, d, *J* = 13.3 Hz, CH), 3.43 (2H, t, *J* = 11.3 Hz, CH₂), 3.16 (1H, t, *J* = 11.3 Hz, CH), 2.95 (1H, d, *J* = 13.3, 3.1 Hz, CH), 2.80 (1H, td, *J* = 13.3, 3.1 Hz, CH), 1.41 (9H, s, CH₃), 1.18 (3H, d, *J* = 6.6 Hz, CH₃); ¹³C NMR (100 MHz; DMSO-*d*₆) δ 153.6 (CO), 147.7 (d, *J* = 10.7 Hz, C–N), 145.2 (C–NH₂), 144.9 (d, *J* = 238.5 Hz, CF), 121.9 (d, *J* = 8.4 Hz, C–NO₂), 111.2 (d, *J* = 26.1 Hz, CH), 104.1 (CH), 79.1 (C(CH₃)₃), 53.6 (CH₂), 48.5 (CH₂), 46.7 (CH₂), 38.2 (CH), 28.1 (C(CH₃)₃), 15.3 (CH₃). HRMS (ESI) calculated for C₁₆H₂₄FN₄O₄⁺ (M + H)⁺ 355.1776, found 355.1764.

5-(3-tert-Butyl-aminopyrrolidine-1-yl-carboxylate)-4-fluoro-2-nitroaniline (9e). This compound was prepared from **8a** and 3-*N*-Boc-aminopyrrolidine as described for **6**. Yield 1.3 g (3.7 mmol, 64%), yellow powder, mp 182–184 °C. ¹H NMR (400 MHz; DMSO-*d*₆; Me₄Si) δ 7.57 (1H, d, *J* = 15.2 Hz, H_{Ar}), 7.26 (2H, s, NH₂), 7.21 (1H, d, *J* = 5.1 Hz, NH), 5.95 (1H, d, *J* = 8.2 Hz, H_{Ar}), 4.08–4.07 (1H, m, CH), 3.65–3.54 (2H, m, CH₂), 3.45–3.44 (1H, m, CH), 3.30–3.27 (1H, m, CH), 2.10–2.05 (1H, m, CH), 1.89–1.85 (1H, m, CH), 1.39 (9H, s, CH₃); ¹³C NMR (100 MHz;



DMSO- d_6) δ 155.3 (CO), 145.7 (C-NH₂), 143.4 (d, J = 11.5 Hz, C-N), 142.9 (d, J = 236.9 Hz, CF), 119.3 (d, J = 7.7 Hz, C-NO₂), 111.2 (d, J = 26.1 Hz, CH), 98.0 (CH), 77.9 (C(CH₃)₃), 55.2 (CH), 49.6 (CH₂), 47.8 (CH₂), 30.1 (CH), 28.2 (CH₃). HRMS (ESI) calculated for C₁₅H₂₂FN₄O₄⁺ (M + H)⁺ 341.1620, found 341.1602.

5-(3-*tert*-Butyl-aminopiperidine-1-yl-carboxylate)-4-fluoro-2-nitroaniline (9f). This compound was prepared from **8a** and 3-Boc-aminopiperidine as described for **6**. Yield 1.4 g (3.9 mmol, 68%), yellow powder, mp 176–179 °C. ¹H NMR (400 MHz, DMSO- d_6 ; Me₄Si) δ 7.61 (1H, d, J = 14.5 Hz, H_{Ar}), 7.35 (2H, s, NH₂), 6.91 (1H, d, J = 7.0 Hz, H_{Ar}), 6.41 (1H, d, J = 8.2 Hz, NH), 3.49–3.32 (4H, m, CH₂), 2.77–2.72 (1H, m, CH), 2.67–2.61 (1H, m, CH), 1.84–1.71 (2H, m, CH₂), 1.60–1.54 (1H, m, CH), 1.38 (9H, s, CH₃); ¹³C NMR (100 MHz, DMSO- d_6) δ 154.9 (CO), 147.3 (d, J = 9.2 Hz, C-N), 145.2 (C-NH₂), 145.0 (d, J = 238.5 Hz, CF), 121.7 (d, J = 7.7 Hz, C-NO₂), 111.1 (d, J = 26.1 Hz, CH), 104.2 (CH), 77.8 (C(CH₃)₃), 54.4 (CH), 49.4 (CH₂), 46.8 (CH₂), 29.5 (CH₂), 28.3 (CH₃), 23.2 (CH₂). HRMS (ESI) calculated for C₁₆H₂₄FN₄O₄⁺ (M + H)⁺ 355.1776, found 355.1752.

5-(4-*tert*-Butyl-aminopiperidine-1-yl-carboxylate)-4-fluoro-2-nitroaniline (9g). This compound was prepared from **8a** and 4-*N*-Boc-aminopiperidine as described for **6**. Yield 1.3 g (3.7 mmol, 65%), yellow powder, mp 217–220 °C. ¹H NMR (400 MHz, DMSO- d_6 ; Me₄Si) δ 7.60 (1H, d, J = 14.5 Hz, H_{Ar}), 7.35 (2H, s, NH₂), 6.91 (1H, d, J = 7.8 Hz, H_{Ar}), 6.41 (1H, d, J = 8.2 Hz, NH), 3.53–3.45 (3H, m, CH, CH₂), 2.85 (2H, t, J = 10.9 Hz, CH₂), 1.83–1.80 (2H, m, CH₂), 1.48 (2H, q, J = 10.9 Hz, CH₂), 1.38 (9H, s, CH₃); ¹³C NMR (100 MHz, DMSO- d_6) δ 154.9 (CO), 147.3 (d, J = 9.9 Hz, C-N), 145.3 (C-NH₂), 145.1 (d, J = 238.5 Hz, CF), 121.6 (d, J = 8.4 Hz, C-NO₂), 111.1 (d, J = 26.8 Hz, CH), 104.2 (CH), 77.6 (C(CH₃)₃), 48.3 (CH₂), 46.9 (CH₂), 31.4 (CH), 28.3 (CH₃). HRMS (ESI) calculated for C₁₆H₂₄FN₄O₄⁺ (M + H)⁺ 355.1776, found 355.1742.

***tert*-Butyl (2-((5-amino-2-fluoro-4-nitrophenyl)(methylamino)ethyl)(methyl)carbamate (9h).** This compound was prepared from **8a** and *tert*-butyl methyl(2-(methylamino)ethyl)carbamate as described for **6**. Yield 1.5 g (4.4 mmol, 77%), yellow powder, mp 96–100 °C. ¹H NMR (400 MHz, DMSO- d_6 ; Me₄Si) δ 7.58 (1H, d, J = 15.6 Hz, H_{Ar}), 7.13 (2H, s, NH₂), 6.21 (1H, d, J = 8.6 Hz, H_{Ar}), 3.54 (2H, t, J = 6.0 Hz, CH₂), 3.38 (2H, t, J = 6.0 Hz, CH₂), 2.97 (3H, s, CH₃), 2.77 (3H, s, CH₃), 1.35 (9H, s, CH₃); ¹³C NMR (100 MHz, DMSO- d_6) δ 154.3 (CO), 145.3 (d, J = 9.2 Hz, C-N), 144.9 (C-NH₂), 143.5 (d, J = 237.7 Hz, CF), 120.3 (d, J = 8.4 Hz, C-NO₂), 111.0 (d, J = 27.6 Hz, CH), 100.8 (CH), 78.3 (C(CH₃)₃), 51.2 (CH₃), 46.2 (CH₃), 39.6 (CH₂), 33.9 (CH₂), 27.7 (CH₃). HRMS (ESI) calculated for C₁₀H₁₆N₄O₂⁺ (M + H)⁺ 343.1776, found 343.1791.

5-(*tert*-Butyl-piperazine-1-yl-carboxylate)-4-chloro-2-nitroaniline (10a). This compound was prepared from **8b** and 1-Boc-piperazine as described for **6**. Yield 1.6 g (4.4 mmol, 90%), yellow powder, mp 143–145 °C. ¹H NMR (400 MHz, DMSO- d_6 ; Me₄Si) δ 7.93 (1H, s, H_{Ar}), 7.34 (2H, s, NH₂), 6.60 (1H, s, H_{Ar}), 3.50–3.48 (4H, m, CH₂), 3.06–3.03 (4H, br m, CH₂), 1.44 (9H, s, CH₃); ¹³C NMR (100 MHz, DMSO- d_6) δ 155.1 (CO), 154.4 (C-N), 146.9 (C-NH₂), 127.3 (CH), 126.1 (C-Cl), 114.4 (C-NO₂), 108.4 (CH), 79.6 (C(CH₃)₃), 50.6 (CH₂), 43.8 (CH₂), 28.5 (CH₃). HRMS (ESI) calculated for C₁₅H₂₂ClN₄O₄⁺ (M + H)⁺ 357.1324, found 357.1337.

4-Chloro-5-(methylpiperazine-1-yl)-2-nitroaniline (10b). This compound was prepared from **8b** and *N*-methylpiperazine as described in.⁴³ Yield 0.9 g (3.3 mmol, 83%), yellow powder, mp 204–205 °C (lit.,⁴³ 202 °C). ¹H NMR (400 MHz; DMSO- d_6 ; Me₄Si) δ 7.95 (1H, s, H_{Ar}), 7.53 (2H, s, NH₂), 7.52 (1H, s, H_{Ar}), 3.13–3.05 (4H, br m, CH₂), 2.55–2.52 (4H, br m, CH₂), 2.26 (3H, s, CH₃); ¹³C NMR (100 MHz; DMSO- d_6) δ 154.9 (C-N), 146.6 (C-NH₂), 128.2 (C-NO₂), 125.0 (CH), 113.8 (C-Cl), 107.4 (CH), 54.3 (CH₂), 50.2 (CH₂), 45.6 (CH₃).

5-(4-*tert*-Butyl-aminopiperidine-1-yl-carboxylate)-4-chloro-2-nitroaniline (10g). This compound was prepared from **8b** and 4-*N*-Boc-aminopiperidine as described for **6**. Yield 0.9 g (2.4 mmol, 61%), yellow powder, mp 156–159 °C. ¹H NMR (400 MHz; DMSO- d_6 ; Me₄Si) δ 7.90 (1H, s, H_{Ar}), 7.49 (2H, s, NH₂), 6.92 (1H, d, J = 7.4 Hz, NH), 6.57 (1H, s, H_{Ar}), 3.41–3.38 (3H, m, CH₂, CH), 2.70 (2H, d, J = 11.3 Hz, CH₂), 1.82 (2H, br d, J = 11.3 Hz, CH₂), 1.53 (2H, q, J = 10.5 Hz, CH₂), 1.39 (9H, s, CH₃); ¹³C NMR (100 MHz; DMSO- d_6) δ 155.3 (CO), 154.9 (C-N), 146.6 (C-NH₂), 126.7 (CH), 124.9 (C-NO₂), 113.9 (C-Cl), 107.4 (CH), 77.6 (C(CH₃)₃), 49.6 (CH₂), 46.9 (CH₂), 31.6 (CH), 28.3 (CH₃). HRMS (ESI) calculated for C₁₆H₂₄ClN₄O₄⁺ (M + H)⁺ 371.1481, found 371.1470.

6-(*tert*-Butyl-piperazine-1-yl-carboxylate)-5-fluorobenzofuroxan (11a). This compound was prepared from fluoronitroaniline **9a** as described for **7**. Yield 0.7 g (2.1 mmol, 72%), yellow powder, mp 125–126 °C. ¹H NMR (400 MHz; DMSO- d_6 ; Me₄Si) δ 7.55 (1H, d, J = 11.7 Hz, H-4), 6.78 (1H, d, J = 5.9 Hz, H-7), 3.51–3.48 (4H, br m, CH₂), 3.14–3.12 (4H, br m, CH₂), 1.44 (9H, s, CH₃); ¹³C NMR (100 MHz; DMSO- d_6) δ 159.6 (d, J = 268.4 Hz, 6-CF), 154.3 (CO), 145.5 (d, J = 14.6 Hz, 5-CH), 100.8–100.1 (m, 8-C), 98.0–97.8 (m, 9-C), 79.6 (C(CH₃)₃), 55.3 (2 × CH₂), 43.4 (2 × CH₂), 28.5 (CH₃). HRMS (ESI) calculated for C₁₅H₂₀FN₄O₄⁺ (M + H)⁺ 339.1463, found 339.1469.

5-Fluoro-6-(methylpiperazine-1-yl)benzofuroxan (11b). This compound was prepared from **9b** as described in ref. 45. Yield 0.9 g (3.4 mmol, 87%), yellow powder, mp 86–87 °C (lit.,⁴⁵ 84–86 °C). ¹H NMR (400 MHz; DMSO- d_6 ; Me₄Si) δ 7.50 (1H, d, J = 12.1 Hz, H-7), 6.69 (1H, d, J = 7.4 Hz, H-4), 3.17–3.14 (4H, br m, CH₂), 2.55–2.53 (4H, br m, CH₂), 2.25 (3H, s, CH₃); ¹³C NMR (100 MHz; DMSO- d_6) δ 158.9 (d, J = 262.2 Hz, 6-CF), 144.9 (d, J = 13.0 Hz, 5-CH), 99.5 (br m, 8-C), 96.4 (br m, 4-CH), 96.4 (br m, 9-C), 53.7 (2 × CH₂), 49.6 (2 × CH₂), 45.1 (CH₃). HRMS (ESI) calculated for C₁₁H₁₄FN₄O₂⁺ (M + H)⁺ 253.1095, found 253.1126.

5-Fluoro-6-(hydroxyethylpiperazine-1-yl)benzofuroxan (11c). This compound was prepared from **9c** as described for **7**. Yield 0.9 g (3.2 mmol, 93%), yellow powder, mp 120–123 °C. ¹H NMR (400 MHz; DMSO- d_6 ; Me₄Si) δ 7.48 (1H, d, J = 12.1 Hz, H-7), 6.70 (1H, d, J = 7.4 Hz, H-4), 4.57 (1H, br s, OH), 3.60 (2H, t, J = 5.9 Hz, CH₂CH₂OH), 3.20 (4H, br m, CH₂), 2.76–2.72 (4H, m, CH₂), 2.60 (2H, t, J = 5.9 Hz, CH₂CH₂OH); ¹³C NMR (100 MHz; DMSO- d_6) δ 158.9 (d, J = 262.2 Hz, 6-CF), 144.8 (5-CH), 99.3 (br m, 7-CH), 99.0 (br m, 8-C), 98.0 (br m, 9-C), 96.6 (br m, 4-CH), 59.4 (CH₂OH), 57.8 (CH₂CH₂OH), 52.0 (2 × CH₂), 49.3 (2 × CH₂). HRMS (ESI) calculated for C₁₂H₁₆FN₄O₃⁺ (M + H)⁺ 283.1201, found 283.1211.



6-(*tert*-Butyl-3-methylpiperazine-1-yl-carboxylate)-5-fluorobenzofuroxan (11d). This compound was prepared from **9d** as described for **7**. Yield 0.8 g (2.4 mmol, 86%), yellow powder, mp 81–82 °C. ^1H NMR (400 MHz; DMSO- d_6 ; Me $_4$ Si) δ 7.54 (1H, d, J = 11.7 Hz, H-7), 6.74 (1H, d, J = 12.2 Hz, H-4), 4.27–4.21 (1H, br m, CH), 3.84 (1H, br d, J = 13.3 Hz, CH), 3.44–3.35 (2H, m, CH $_2$), 3.23–3.17 (1H, m, CH $_2$), 2.93 (1H, d, J = 13.3 Hz, CH), 2.84 (1H, t, J = 11.7 Hz, CH), 1.43 (9H, s, C(CH $_3$) $_3$), 1.23 (3H, s, CH $_3$); ^{13}C NMR (100 MHz; DMSO- d_6) δ 159.6 (d, J = 262.4 Hz, 6-CF), 154.2 (CO), 146.2 (5-C), 100.3 (br m, 8-C), 97.7 (br m, 9-C), 79.5 (C(CH $_3$) $_3$), 55.0 (CH $_2$), 54.9 (CH), 50.1 (CH $_2$), 47.1 (CH $_2$), 28.52 (C(CH $_3$) $_3$), 15.7 (CH $_3$). HRMS (ESI) calculated for C $_{16}$ H $_{22}$ FN $_4$ O $_4$ $^+$ (M + H) $^+$ 353.1620, found 353.1591.

6-(3-*tert*-Butyl-aminopyrrolidin-1-yl-carboxylate)-5-fluorobenzofuroxan (11e). This compound was prepared from **9e** as described for **7**. Yield 0.9 g (2.5 mmol, 90%), yellow powder, mp 190–193 °C. ^1H NMR (400 MHz; DMSO- d_6 ; Me $_4$ Si) δ 7.70 (1H, d, J = 15.6 Hz, H-7), 7.31 (1H, s, NH), 7.11 (1H, br s, H-4), 4.11–4.03 (1H, m, CH), 3.83–3.75 (1H, m, CH), 3.68–3.66 (1H, m, CH), 3.57–3.54 (1H, m, CH), 3.46–3.44 (1H, m, CH), 2.12–2.02 (1H, m, CH), 1.92–1.80 (1H, m, CH), 1.39 (9H, s, C(CH $_3$) $_3$); ^{13}C NMR (100 MHz; DMSO- d_6) δ 154.9 (CO), 145.3 (d, J = 240.8 Hz, 6-CF), 141.9 (d, J = 12.3 Hz, 5-C), 121.6 (d, J = 8.4 Hz, 4-CH), 110.4 (d, J = 28.4 Hz, 7-CH), 101.3–100.1 (m, 8-C), 96.9–95.4 (m, 9-C), 77.6 (C(CH $_3$) $_3$), 55.6 (CH $_2$), 49.8 (CH), 48.9 (CH $_2$), 30.3 (CH $_2$), 27.9 (C(CH $_3$) $_3$). HRMS (ESI) calculated for C $_{15}$ H $_{20}$ FN $_4$ O $_4$ $^+$ (M + H) $^+$ 339.1463, found 339.1482.

6-(3-*tert*-Butyl-aminopiperidine-1-yl-carboxylate)-5-fluorobenzofuroxan (11f). This compound was prepared from **9f** as described for **7**. Yield 0.8 g (2.2 mmol, 81%), yellow powder, mp 164–168 °C. ^1H NMR (400 MHz; DMSO- d_6 ; Me $_4$ Si) δ 7.60–7.59 (1H, m, H-7), 6.88 (1H, d, J = 6.6 Hz, H-4), 3.52–3.31 (4H, m, CH $_2$), 2.79 (1H, t, J = 10.5 Hz, CH), 2.67 (1H, t, J = 10.5 Hz, CH), 1.82–1.79 (2H, m, CH $_2$), 1.64–1.51 (1H, m, CH), 1.38 (9H, s, CH $_3$); ^{13}C NMR (100 MHz; DMSO- d_6) δ 154.9 (CO), 143.6 (d, J = 275.5 Hz, 6-CF), 141.0 (C-5), 101.3–100.6 (m, 8-C), 95.8–94.8 (m, 9-C), 77.7 (C(CH $_3$) $_3$), 55.2 (CH $_2$), 50.2 (CH), 46.5 (CH $_2$), 29.2 (CH $_2$), 28.2 (C(CH $_3$) $_3$), 23.1 (CH $_2$). HRMS (ESI) calculated for C $_{16}$ H $_{22}$ FN $_4$ O $_4$ $^+$ (M + H) $^+$ 353.1620, found 353.1625.

6-(4-*tert*-Butyl-aminopiperidine-1-yl-carboxylate)-5-fluorobenzofuroxan (11g). This compound was prepared from **9g** as described for **7**. Yield 0.9 g (2.4 mmol, 88% d_6 ; Me $_4$ Si), yellow powder, mp 160–162 °C. ^1H NMR (400 MHz; DMSO- d_6 ; Me $_4$ Si) δ 7.47 (1H, d, J = 11.7 Hz, NH), 6.67 (1H, d, J = 5.5 Hz, H-7), 6.61 (1H, br s, H-4), 3.49–3.46 (1H, br m, CH), 2.88–2.83 (2H; m, CH $_2$), 2.73–2.70 (1H, m, CH), 2.52–2.48 (1H, m, CH), 1.87–1.85 (2H, m, CH $_2$), 1.57 (2H, q, J = 10.9 Hz, CH $_2$), 1.38 (9H, s, CH $_3$); ^{13}C NMR (100 MHz; DMSO- d_6) δ 159.1 (d, J = 267.6 Hz, 6-CF), 154.6 (CO), 145.3 (5-CH), 99.6 (br m, 8-C), 96.4 (br m, 9-C), 77.4 (C(CH $_3$) $_3$), 48.9 (CH $_2$), 46.7 (CH $_2$), 31.1 (CH), 27.9 (C(CH $_3$) $_3$). HRMS (ESI) calculated for C $_{16}$ H $_{22}$ FN $_4$ O $_4$ $^+$ (M + H) $^+$ 353.1620, found 353.1611.

6-((2-((*tert*-Butoxycarbonyl)(methyl)amino)ethyl)(methyl)amino)-5-fluoro benzofuroxan (11h). This compound was prepared from **9h** as described for **7**. Yield 0.8 g (2.4 mmol, 82%), yellow oil. ^1H NMR (400 MHz; DMSO- d_6 ; Me $_4$ Si) δ 7.51

(1H, br s, H-7), 6.34 (1H, br s, H-4), 3.38–3.36 (2H, m, CH $_2$), 2.95 (3H, s, CH $_3$), 2.73 (3H, s, CH $_3$), 1.33 (9H, s, C(CH $_3$) $_3$), 1.28–1.22 (2H, br m, CH $_2$); ^{13}C NMR (100 MHz; DMSO- d_6) δ 159.3 (d, J = 272.2 Hz, 6-CF), 154.2 (CO), 143.1 (5-C), 111.6 (d, J = 26.2 Hz, 7-CH), 100.9–100.3 (m, 8-C), 95.5 (d, J = 8.7 Hz, 4-CH), 90.7–90.3 (m, 9-C), 78.2 (C(CH $_3$) $_3$), 51.5 (CH $_3$), 45.6 (CH $_3$), 39.7 (CH $_2$), 33.8 (CH $_2$), 27.6 (C(CH $_3$) $_3$). HRMS (ESI) calculated for C $_{15}$ H $_{22}$ FN $_4$ O $_4$ $^+$ (M + H) $^+$ 341.1620, found 341.1612.

6-(*tert*-Butyl-piperazine-1-yl-carboxylate)-5-chlorobenzofuroxan (12a). This compound was prepared from **10a** as described for **7**. Yield 0.9 g (2.4 mmol, 85%), yellow powder, mp 154–155 °C. ^1H NMR (400 MHz; DMSO- d_6 ; Me $_4$ Si) δ 7.90 (1H, s, H-7), 7.01 (1H, s, H-4), 3.51 (4 H, t, J = 4.7 Hz, CH $_2$), 3.05 (4H, t, J = 4.7 Hz, CH $_2$), 1.44 (9H, s, CH $_3$); ^{13}C NMR (100 MHz; DMSO- d_6) δ 154.4 (CO), 151.7 (5-C), 136.5 (6-CCl), 116.7 (m, 8-C), 101.9 (m, 9-C), 79.6 (C(CH $_3$) $_3$), 51.6 (2 $\times$ CH $_2$), 43.7 (2 $\times$ CH $_2$), 28.6 (C(CH $_3$) $_3$). HRMS (ESI) calculated for C $_{15}$ H $_{20}$ ClN $_4$ O $_4$ $^+$ (M + H) $^+$ 355.1168, found 355.1155.

5-Chloro-6-(methylpiperazine-1-yl)benzofuroxan (12b). This compound was prepared from **10b** as described in.⁴³ Yield 0.9 g (3.3 mmol, 89%), yellow powder, mp 110–113 °C (lit.,⁴³ 115–116 °C). ^1H NMR (400 MHz; DMSO- d_6 ; Me $_4$ Si) δ 7.97 (1H, s, H-7), 6.98 (1H, s, H-4), 3.09–2.99 (4H, br m, CH $_2$), 2.53–2.40 (4H, br m, CH $_2$), 2.22 (3H, s, CH $_3$); ^{13}C NMR (100 MHz; DMSO- d_6) δ 151.2 (5-C), 135.7 (6-CCl), 115.9 (br s, 8-C), 100.3 (br s, 9-C), 53.9 (2 $\times$ CH $_2$), 50.8 (2 $\times$ CH $_2$), 45.1 (CH $_3$). HRMS (ESI) calculated for C $_{11}$ H $_{14}$ ClN $_4$ O $_4$ $^+$ (M + H) $^+$ 269.0800, found 269.0817.

6-(4-*tert*-Butyl-aminopiperidine-1-yl-carboxylate)-5-chlorobenzofuroxan (12g). This compound was prepared from **10g** as described for **7**. Yield 0.8 g (2.3 mmol, 84%), yellow powder, mp 156–159 °C. ^1H NMR (400 MHz; DMSO- d_6 ; Me $_4$ Si) δ 7.89 (1H, s, H-7), 6.94 (1H, s, H-4), 6.91 (1H, d, J = 6.3 Hz, NH), 3.47 (1H, d, J = 10.9 Hz, CH), 3.37 (2H, d, J = 12.1 Hz, CH $_2$), 2.79 (2H, t, J = 10.9 Hz, CH $_2$), 1.88 (2H, d, J = 12.1 Hz, CH $_2$), 1.62 (2H, q, J = 10.9 Hz, CH $_2$), 1.41 (9H, s, CH $_3$); ^{13}C NMR (100 MHz; DMSO- d_6) δ 154.6 (CO), 151.9 (5-C), 136.0 (6-CCl), 115.8 (m, 8-C), 100.3 (m, 9-C), 89.6 (7-CH), 83.1 (4-CH), 77.4 (C(CH $_3$) $_3$), 50.2 (2 $\times$ CH $_2$), 46.7 (CH), 31.3 (2 $\times$ CH $_2$), 28.0 (C(CH $_3$) $_3$). HRMS (ESI) calculated for C $_{16}$ H $_{22}$ ClN $_4$ O $_4$ $^+$ (M + H) $^+$ 321.1324, found 321.1356.

6-Fluoro-7-(piperazine-1-yl)-3-phenylquinoxaline-2-carbonitrile-1,4-dioxide hydrochloride (13a). This compound was prepared from **11a** as described for **3**. Yield 0.1 g (0.3 mmol, 56%), yellow powder, mp 233–235 °C. λ_{max} (EtOH)/nm 226, 236, 298, 341, 417. IR ν_{max} (film)/cm $^{-1}$ 3434, 2971, 2848, 2731, 2507, 2238, 1616, 1511, 1492, 1455, 1388, 1330, 1275, 1215, 1134, 918. HPLC (LW = 294 nm, gradient B 15/70% (30 min)) t_{R} = 15.6 min, purity 99.6%. ^1H NMR (400 MHz; DMSO- d_6 ; Me $_4$ Si) δ 9.41 (2H, br s, NH $_2^+$), 8.28 (1H, d, J = 13.0 Hz, H-5), 7.82 (1H, d, J = 8.3 Hz, H-8), 7.75–7.70 (2H, m, C $_6$ H $_5$), 7.64–7.59 (3H, m, C $_6$ H $_5$), 3.88–3.74 (2H, br m, CH $_2$), 3.61–3.57 (2H, br m, CH $_2$), 3.33–3.30 (4H, br m, CH $_2$); ^{13}C NMR (100 MHz; DMSO- d_6) δ 158.8 (d, J = 260.7 Hz, 6-CF), 144.1 (d, J = 10.7 Hz, 7-C), 142.3 (3-C), 135.1 (9-C), 130.8 (d, J = 13.8 Hz, 10-C), 131.4 (4'-CH), 130.7 (2 $\times$ 2'-CH), 128.9 (2 $\times$ 3'-CH), 128.0 (1'-C), 120.5 (2-C), 111.5 (CN), 107.6 (d, J = 28.4 Hz, 5-CH), 107.1 (8-CH), 46.9 (2 $\times$



CH₂), 42.8 (2 × CH₂). HRMS (ESI) calculated for C₁₉H₁₇FN₅O₂⁺ (M + H)⁺ 366.1361, found. 366.1341.

6-Fluoro-7-(methylpiperazine-1-yl)-3-phenylquinoxaline-2-carbonitrile-1,4-dioxide hydrochloride (13b). To a stirring mixture of corresponding benzofuroxan **11b** (0.1 g, 0.4 mmol) and benzoylacetone nitrile (0.86 g, 0.6 mmol) in chloroform (10 mL) triethylamine (200 μL) was added at room temperature. The mixture was stirred for 2–4 h. The formed precipitate was filtered and washed with cold chloroform (3 mL). The crude product was crystallized from ethanol–toluene mixture gave pure compound **13b** (0.11 g, 70%). The yellow solid obtained after crystallization was dissolved in the mixture of water (2 mL) and hydrochloric acid (0.3 mL^{−1}, 4.6 mmol). Then the resulting solution residue was filtered and evaporated under vacuum. The product was precipitated with water–acetone–ether mixture (1 : 3 : 1) and dried. The yellow solid was collected by filtration, successively washed with acetone, Et₂O, *n*-hexane and dried. Yield 0.1 g (0.3 mmol, 66%), yellow solid, mp 218–220 °C. λ_{max} (EtOH)/nm 220, 242, 291, 359. HPLC (LW = 290 nm, gradient B 15/70% (35 min)) t_R = 15.7 min, purity 97.7%. ¹H NMR (400 MHz; DMSO-*d*₆; Me₄Si) δ 11.48 (1H, br s, NH⁺), 8.28 (1H, d, *J* = 13.0 Hz; H-5), 7.82 (1H, d, *J* = 8.3 Hz, H-8), 7.75–7.70 (2H, m, C₆H₅), 7.64–7.59 (3H, m, C₆H₅), 3.91–3.81 (2H, m, CH₂), 3.60–3.43 (4H, br m, CH₂), 3.32–3.23 (2H, br m, CH₂); ¹³C NMR (100 MHz; DMSO-*d*₆) δ 158.3 (d, *J* = 260.1 Hz, 6-CF), 143.3 (d, *J* = 10.7 Hz, 7-C), 141.9 (3-C), 134.8 (d, *J* = 11.4 Hz, 10-C), 134.6 (9-C), 130.9 (4'-CH), 130.2 (2 × 2'-CH), 128.5 (2 × 3'-CH), 127.6 (1'-C), 120.1 (2-C), 111.1 (CN), 107.2 (d, *J* = 28.9 Hz, 5-CH), 106.9 (8-CH), 51.8 (2 × CH₂), 46.5 (2 × CH₂), 41.9 (CH₃). HRMS (ESI) calculated for C₂₀H₁₉FN₅O₂⁺ (M + H)⁺ 380.1517, found. 380.1491.

6-Fluoro-7-(1-hydroxyethylpiperazine-1-yl)-3-phenylquinoxaline-2-carbonitrile-1,4-dioxide hydrochloride (13c). This compound was prepared from **11c** as described for **13b**. Yield 0.08 g (0.2 mmol, 57%), orange powder, mp 217–218 °C. λ_{max} (EtOH)/nm 220, 242, 293, 359. HPLC (LW = 290 nm, gradient B 15/70% (35 min)) t_R = 15.2 min, purity 96.8%. ¹H NMR (400 MHz; DMSO-*d*₆; Me₄Si) δ 11.14 (1H, br s, NH⁺), 8.27 (1H, d, *J* = 12.9 Hz, H-5), 7.82 (1H, d, *J* = 8.1 Hz, H-8), 7.74–7.72 (2H, m, C₆H₅), 7.62–7.61 (3H, m, C₆H₅), 5.50–5.36 (1H, br m, OH), 3.94–3.79 (4H, m, CH₂), 3.74–3.64 (2H, m, CH₂CH₂OH), 3.59–3.47 (2H, m, CH₂CH₂OH), 3.35–3.23 (4H, m, CH₂); ¹³C NMR (100 MHz; DMSO-*d*₆) δ 158.3 (d, *J* = 259.4 Hz, 6-CF), 143.3 (d, *J* = 11.4 Hz, 7-C), 141.9 (3-C), 134.8 (d, *J* = 9.2 Hz, 10-C), 134.7 (9-C), 131.0 (4'-CH), 130.3 (2 × 2'-CH), 128.5 (2 × 3'-CH), 127.6 (1'-C), 120.1 (2-C), 111.1 (CN), 107.2 (d, *J* = 28.9 Hz, 5-CH), 106.8 (8-CH), 57.1 (CH₂), 55.2 (CH₂), 50.9 (2 × CH₂), 46.4 (2 × CH₂). HRMS (ESI) calculated for C₂₁H₂₁FN₅O₂⁺ (M + H)⁺ 410.1623, found. 410.1618.

6-Fluoro-7-(2-methylpiperazine-1-yl)-3-phenylquinoxaline-2-carbonitrile-1,4-dioxide hydrochloride (13d). This compound was prepared from **11d** as described for **3**. Yield 0.07 g (0.18 mmol, 63%), yellow powder, mp 243–245 °C (dec). λ_{max} (EtOH)/nm 220, 242, 294, 361. HPLC (LW = 290 nm, gradient B 15/70% (35 min)) t_R = 16.4 min, purity 98.0%. ¹H NMR (400 MHz; DMSO-*d*₆; Me₄Si) δ 9.61 (2H, br s, NH₃⁺), 8.26 (1H, d, *J* = 12.9 Hz, H-5), 7.82 (1H, d, *J* = 8.1 Hz, H-8), 7.73–7.71 (2H, m,

C₆H₅), 7.61–7.60 (3H, m, C₆H₅), 3.78 (2H, d, *J* = 12.0 Hz, CH₂), 3.50–3.39 (3H, br m, CH, CH₂), 3.24–3.13 (2H, m, CH₂), 1.35 (3H, d, *J* = 6.4 Hz, CH₃); ¹³C NMR (100 MHz; DMSO-*d*₆) δ 158.3 (d, *J* = 260.2 Hz, 6-CF), 143.6 (d, *J* = 11.5 Hz, 7-C), 141.9 (3-C), 134.7 (9-C), 134.6 (d, *J* = 10.7 Hz, 10-C), 130.9 (4'-CH), 130.2 (2 × 2'-CH), 128.5 (2 × 3'-CH), 127.6 (1'-C), 120.1 (2-C), 111.1 (CN), 107.2 (d, *J* = 28.2 Hz, 5-CH), 106.8 (8-CH), 52.4 (CH₂), 50.0 (CH₂), 46.1 (CH₂), 41.9 (CH), 15.5 (CH₃). HRMS (ESI) calculated for C₂₀H₁₉FN₅O₂⁺ (M + H)⁺ 380.1517, found 380.1505.

7-(3-Aminopyrrolidine-1-yl)-6-fluoro-3-phenylquinoxaline-2-carbonitrile-1,4-dioxide hydrochloride (13e). This compound was prepared from **11e** as described for **13a**. Yield 0.02 g (0.07 mmol, 23%), yellow powder, mp 205–207 °C. λ_{max} (EtOH)/nm 223, 310, 364, 490. HPLC (LW = 300 nm, gradient B 20/60% (30 min)) t_R = 11.2 min, purity 95.2%. ¹H NMR (400 MHz; DMSO-*d*₆; Me₄Si) δ 8.51 (3H, br s, NH₃⁺), 8.16 (1H, d, *J* = 13.9 Hz, H-5), 7.75–7.70 (2H, m, C₆H₅), 7.61–7.57 (3H, m, C₆H₅), 7.24 (1H, d, *J* = 8.1 Hz, H-8), 3.96 (2H, d, *J* = 10.2 Hz, CH₂), 3.88–3.85 (1H, m, CH₂), 3.72–3.63 (1H, br m, CH), 3.42–3.37 (1H, br m, CH₂), 2.40–2.14 (2H, br m, CH₂); ¹³C NMR (100 MHz; DMSO-*d*₆) δ 155.5 (d, *J* = 257.9 Hz, 6-CF), 140.4 (d, *J* = 12.9 Hz, 7-C), 140.1 (3-C), 131.4 (d, *J* = 9.9 Hz, 10-C), 130.8 (9-C), 130.8 (4'-CH), 130.4 (2 × 2'-CH), 128.4 (2 × 3'-CH), 127.8 (1'-C), 119.8 (2-C), 111.3 (CN), 106.7 (d, *J* = 28.9 Hz, 5-CH), 99.9 (d, *J* = 6.9 Hz, 8-CH), 53.4 (CH), 49.4 (CH₂), 47.9 (CH₂), 28.8 (CH₂). HRMS (ESI) calculated for C₁₉H₁₇FN₅O₂⁺ (M + H)⁺ 366.1361, found 366.1357.

7-(3-Aminopiperidine-1-yl)-6-fluoro-3-phenylquinoxaline-2-carbonitrile-1,4-dioxide hydrochloride (13f). This compound was prepared from **11f** as described for **13a**. Yield 0.05 g (0.14 mmol, 51%), yellow powder, mp 233–235 °C. λ_{max} (EtOH)/nm 222, 243, 296, 368, 418. HPLC (LW = 290 nm, gradient B 15/70% (35 min)) t_R = 16.7 min, purity 98.0%. ¹H NMR (400 MHz; DMSO-*d*₆; Me₄Si) δ 8.50 (3H, br s, NH₃⁺), 8.23 (1H, d, *J* = 12.6 Hz, H-5), 7.77–7.72 (3H, m, H-8, C₆H₅), 7.61–7.60 (3H, m, C₆H₅), 3.78 (1H, d, *J* = 10.3 Hz, CH₂), 3.48 (2H, d, *J* = 10.3 Hz, CH₂), 3.17 (1H, t, *J* = 10.3 Hz, CH₂), 3.09–3.04 (1H, br m, CH), 2.08–2.05 (1H, br m, CH₂), 1.96–1.90 (1H, br m, CH₂), 1.72–1.68 (2H, br m, CH₂); ¹³C NMR (100 MHz; DMSO-*d*₆) δ 158.4 (d, *J* = 260.9 Hz, 6-CF), 144.5 (d, *J* = 10.7 Hz, 7-C), 141.7 (3-C), 134.7 (9-C), 134.4 (d, *J* = 11.5 Hz, 10-C), 130.9 (4'-CH), 130.3 (2 × 2'-CH), 128.5 (2 × 3'-CH), 127.6 (1'-C), 119.9 (2-C), 111.1 (CN), 107.0 (d, *J* = 28.9 Hz, 5-CH), 106.7 (8-CH), 52.5 (CH), 49.8 (CH₂), 46.4 (CH₂), 27.3 (CH₂), 22.2 (CH₂). HRMS (ESI) calculated for C₂₀H₁₉FN₅O₂⁺ (M + H)⁺ 380.1517, found 380.1503.

7-(4-Aminopiperidine-1-yl)-6-fluoro-3-phenylquinoxaline-2-carbonitrile-1,4-dioxide hydrochloride (13g). This compound was prepared from **11g** as described for **13a**. Yield 0.06 g (0.15 mmol, 53%), yellow powder, mp > 250 °C. λ_{max} (EtOH)/nm 298, 368, 450. HPLC (LW = 298 nm, gradient B 15/70% (35 min)) t_R = 16.6 min, purity 95.6%. ¹H NMR (400 MHz; DMSO-*d*₆; Me₄Si) δ 8.35 (3H, br s, NH₃⁺), 8.21 (1H, d, *J* = 13.1 Hz, H-5), 7.76–7.72 (3H, m, H-8, C₆H₅), 7.61 (3H, br m, C₆H₅), 3.74 (2H, d, *J* = 11.6 Hz, CH₂), 3.30–3.26 (1H, br m, CH), 3.06 (2H, t, *J* = 11.6 Hz, CH₂), 2.11 (2H, d, *J* = 10.9 Hz, CH₂), 1.77 (2H, q, *J* = 10.9 Hz, CH₂); ¹³C NMR (100 MHz; DMSO-*d*₆) δ 158.5 (d, *J* = 259.4 Hz, 6-CF), 144.5 (d, *J* = 10.7 Hz, 7-C), 141.6 (3-C), 134.2 (d, *J* = 10.7 Hz, 10-C), 130.9 (4'-CH), 130.3 (2 × 2'-CH), 128.4 (2 × 3'-CH), 127.6 (1'-C), 119.9 (2-



C), 111.2 (CN), 106.9 (d, $J = 28.9$ Hz, 10-CH), 106.2 (9'-CH), 47.9 ($2 \times \text{CH}_2$), 46.9 ($2 \times \text{CH}_2$), 29.3 (CH). HRMS (ESI) calculated for $\text{C}_{20}\text{H}_{19}\text{FN}_5\text{O}_2^+ (\text{M} + \text{H})^+$ 380.1517, found 380.1552.

6-Fluoro-7-(1-methyl(2-(methylamino)ethyl)amino)-3-phenylquinoxaline-2-carbonitrile-1,4-dioxide hydrochloride (13h). This compound was prepared from **11h** as described for **13a**. Yield 0.05 g (0.14 mmol, 48%), orange powder, mp 219–220 °C. λ_{max} (EtOH)/nm 300, 365, 466. HPLC (LW = 300 nm, gradient B 15/70% (35 min)) $t_{\text{R}} = 15.09$ min, purity 95.0%. ^1H NMR (400 MHz; DMSO- d_6 ; Me $_4$ Si) δ 9.33 (2H, br s, NH_2^+), 8.16 (1H, d, $J = 13.9$ Hz, H-5), 7.76–7.71 (2H, m, C $_6$ H $_5$), 7.62–7.57 (3H, m, C $_6$ H $_5$), 7.55 (1H, d, $J = 8.5$ Hz, H-8), 3.98–3.73 (4H, br m, CH $_2$), 3.20 (3H, s, CH $_3$), 2.57 (3H, s, CH $_3$); ^{13}C NMR (100 MHz; DMSO- d_6) δ 157.0 (d, $J = 259.4$ Hz, 6-CF), 143.3 (d, $J = 10.7$ Hz, 7-C), 140.8 (3-C), 134.69 (9-C), 132.6 (d, $J = 10.7$ Hz, 10-C), 130.8 (4'-CH), 130.3 ($2 \times 2'$ CH), 128.4 ($2 \times 3'$ CH), 127.7 (1'-C), 119.8 (2-C), 111.2 (CN), 106.9 (d, $J = 29.8$ Hz, 5-CH), 103.2 (8-CH), 50.0 (CH $_3$), 45.9 (CH $_3$), 38.9 (CH $_2$), 32.6 (CH $_2$). HRMS (ESI) calculated for $\text{C}_{19}\text{H}_{19}\text{FN}_5\text{O}_2^+ (\text{M} + \text{H})^+$ 368.1498, found 368.1510.

6-Chloro-7-(piperazine-1-yl)-3-phenylquinoxaline-2-carbonitrile-1,4-dioxide hydrochloride (14a). This compound was prepared from **12a** as described for **13a**. Yield 0.08 g (0.21 mmol, 75%), orange powder, mp 252–255 °C. λ_{max} (EtOH)/nm 224, 296, 363. IR ν_{max} (film)/cm $^{-1}$ 3370, 2979, 2910, 2853, 2238, 1725, 1604, 1515, 1449, 1366, 1300, 1230, 1168, 1088, 987. HPLC (LW = 290 nm, gradient B 20/60% (33 min)) $t_{\text{R}} = 12.6$ min, purity 98.5%. ^1H NMR (400 MHz; DMSO- d_6 ; Me $_4$ Si) δ 8.92 (2H, br s, NH_2^+), 8.54 (1H, s, H-8), 7.93 (1H, s, H-5), 7.74–7.71 (2H, m, C $_6$ H $_5$), 7.63–7.61 (3H, m, C $_6$ H $_5$), 3.46–3.44 (4 H, br m, CH $_2$), 3.37–3.34 (4H, br m, CH $_2$); ^{13}C NMR (100 MHz; DMSO- d_6) δ 151.5 (7-C), 142.4 (3-C), 136.4 (9-C), 135.3 (10-C), 134.9 (6-CCl), 131.1 (4'-CH), 130.2 ($2 \times 2'$ CH), 128.5 ($2 \times 3'$ CH), 127.5 (1'-C), 122.1 (5-CH), 120.6 (2-C), 111.0 (CN), 109.0 (8-CH), 47.7 (CH $_2$), 42.9 (CH $_2$). HRMS (ESI) calculated for $\text{C}_{19}\text{H}_{17}\text{ClN}_5\text{O}_2^+ (\text{M} + \text{H})^+$ 382.1065, found 382.1088.

6-Chloro-7-(methylpiperazine-1-yl)-3-phenylquinoxaline-2-carbonitrile-1,4-dioxide methanesulfonate (14b). To a stirring mixture of corresponding benzofuroxan **12b** (0.1 g, 0.37 mmol) and benzoylacetone nitrile (0.81 g, 0.56 mmol) in chloroform (15 mL) triethylamine (200 μL) was added at room temperature. The mixture was stirred for 2–4 h. The formed precipitate was filtered and washed with cold chloroform (3 mL). The crude product was crystallized from ethanol-toluene mixture gave compound **14b** (0.124 g, 85%). The orange solid obtained after crystallization was dissolved in the mixture of chloroform (2 mL) and methanesulphonic acid (50 μL , 0.5 mmol). Then the resulting solution residue was filtered and evaporated under vacuum. The product was precipitated with methanol-ether mixture (1 : 3) and dried. The orange solid was collected by filtration, successively washed with acetone, Et $_2$ O, *n*-hexane and dried. Yield 0.98 g (2.4 mmol, 82%), yellow solid, mp 215–217 °C. λ_{max} (EtOH)/nm 224, 296, 359. HPLC (LW = 3290 nm, gradient B 20/60% (33 min)) $t_{\text{R}} = 12.7$ min, purity 98.5%. ^1H NMR (400 MHz; DMSO- d_6 ; Me $_4$ Si) δ 9.96 (1H, br s, NH^+), 8.54 (1H, s, H-5), 7.95 (1H, s, H-8), 7.73 (2H, br m, C $_6$ H $_5$), 7.62 (3H, br m, C $_6$ H $_5$), 4.03–3.98 (4H, br m, CH $_2$), 3.76 (2H, br d, $J = 10.9$ Hz, CH $_2$), 3.63 (2H, br d, $J = 10.9$ Hz, CH $_2$), 2.94 (3H, s, CH $_3$), 2.40

(3H, s, CH $_3\text{SO}_3^-$); ^{13}C NMR (100 MHz; DMSO- d_6) δ 151.1 (7-C), 142.5 (3-C), 136.5 (6-CCl), 135.3 (9-C), 135.0 (10-C), 131.2 (4'-CH), 130.3 ($2 \times 2'$ CH), 128.6 ($2 \times 3'$ CH), 127.5 (1'-C), 122.1 (8-CH), 120.7 (2-C), 111.1 (CN), 109.2 (5-CH), 52.4 (CH $_2$), 47.7 (CH $_2$), 42.4 (CH $_3$), 39.8 (CH $_3\text{SO}_3^-$). HRMS (ESI) calculated for $\text{C}_{20}\text{H}_{19}\text{ClN}_5\text{O}_2^+ (\text{M} + \text{H})^+$ 396.1222, found 396.1259.

7-(4-Aminopiperidine-1-yl)-6-chloro-3-phenylquinoxaline-2-carbonitrile-1,4-dioxide methanesulfonate (14g). This compound was prepared from **12g** as described for **14b**. Yield 0.06 g (0.16 mmol, 60%), orange powder, mp 248–250 °C. λ_{max} (EtOH)/nm 231, 265, 299, 368. HPLC (LW = 290 nm, gradient B 20/60% (33 min)) $t_{\text{R}} = 13.7$ min, purity 97.7%. ^1H NMR (400 MHz; DMSO- d_6 ; Me $_4$ Si) δ 8.49 (1H, s, H-5), 8.07 (3H, br s, NH_3^+), 7.87 (1H, s, H-8), 7.71 (2H, br m, C $_6$ H $_5$), 7.62–7.61 (3H, br m, C $_6$ H $_5$), 3.62–3.58 (2H, br m, CH $_2$), 3.33–3.23 (1H, br m, CH), 2.97 (2H, t, $J = 10.5$ Hz, CH $_2$), 2.38 (3H, s, CH $_3\text{SO}_3^-$), 2.10 (2H, br d, $J = 10.5$ Hz, CH $_2$), 1.78 (2H, q, $J = 10.5$ Hz, CH $_2$); ^{13}C NMR (100 MHz; DMSO- d_6) δ 153.0 (7-C), 142.5 (3-C), 136.9 (9-C), 136.0 (6-CCl), 134.9 (10-C), 131.5 (4'-CH), 130.7 ($2 \times 2'$ CH), 128.9 ($2 \times 3'$ CH), 127.9 (1'-C), 122.3 (5-CH), 120.9 (2-C), 111.6 (CN), 109.0 (8-CH), 49.5 (CH $_2$), 47.4 (CH $_2$), 40.2 (CH $_3\text{SO}_3^-$), 29.9 (CH). HRMS (ESI) calculated for $\text{C}_{20}\text{H}_{19}\text{ClN}_5\text{O}_2^+ (\text{M} + \text{H})^+$ 396.1222, found 396.1143.

Cell lines and antiproliferative assay

The MCF7 (ATCC HTB-22) and MDA-MB-231 (ATCC HTB-26) human breast cancer cells and K562 (ATCC CCL-243) chronic myelogenous leukaemia cells were obtained from the ATCC collection. The multidrug-resistant (MDR) subline K562/4 (kind gift of Dr Alexander Shtil, Blokhin National Medical Research Center of Oncology, Russia) was obtained by stepwise selection of K562 cells for survival under the continuous exposure to **Dox**. This subline expresses the MDR1 gene and functional P-glycoprotein and is characterized by a high resistance index for **Dox**.⁴⁶ The MCF7 and MDA-MB-231 cells were cultured in standard 4.5 g L $^{-1}$ glucose DMEM medium (Gibco) supplemented with 10% FCS (HyClone), 2 mM L-glutamine, 50 U mL $^{-1}$ penicillin, 50 μg mL $^{-1}$ streptomycin (PanEco), and 100 μg mL $^{-1}$ sodium pyruvate (Santa Cruz) at 37 °C, 5% CO $_2$, and 80–85% humidity in NuAir incubator. Suspension myelogenous leukaemia cells (K562, K562/4) were propagated in RPMI-1640 (PanEco) with 5% FCS (HyClone), 2 mM L-glutamine, 100 U mL $^{-1}$ penicillin, and 100 μg mL $^{-1}$ streptomycin at 37 °C, 5% CO $_2$, and 80–85% humidity in Binder incubator. Cells in the logarithmic phase of growth were used in the experiments. The growth inhibitory activity of compounds was assessed by the MTT test based on the metabolism of the MTT reagent (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (Appllichem) in living cells, with modifications as described previously in ref. 9.

Briefly, the cells were seeded in 24-well plates (Corning) in 900 μL of the standard medium. Compounds at different concentrations in 100 μL of the appropriate medium were added, and the cells were grown for 72 h. For assessment of hypoxic cytotoxicity ratio, hypoxia (1% O $_2$) conditions were simulated in Binder multigas incubator, as described in ref. 9. After incubation with compounds, the medium was removed, the MTT reagent that was dissolved in the medium was added to

the final concentration of 0.2 mg mL⁻¹ to each well, and the incubation was performed for 2 h. Then the cell supernatants were removed and purple formazan crystals were dissolved in DMSO (350 µL per well). Culture plates were gently shaken, and the absorbance was measured at 571 nm with a reference wavelength of 630 nm on a MultiScan reader (ThermoFisher). The viability of the cells was expressed as a percentage of control. Dose-response curves were analyzed by regression analysis using sigmoid curves (log(concentration) vs. normalized absorbance). In this study, the half-maximal inhibitory concentration (IC₅₀) values were determined using GraphPad Prism (Version 7.0).

Immunoblotting

MCF7 cells were washed twice, and incubated for 10 min on ice in the total lysis buffer containing 50 mM Tris-HCl, pH 7.4, 1% SDS, 1% Igepal CA-630, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF; 1 µg mL⁻¹ each aprotinin, leupeptin, pepstatin; 1 mM Na-orthovanadate and 1 mM NaF. Samples were sonicated 5 times for 5 s each at 30% output, centrifuged for 5 min at 15 000g, and supernatants were then used as total cell extracts. Total protein content was determined by Bradford method.

MCF7 cell lysates were separated in 10% SDS-PAGE under reducing conditions, transferred to a nitrocellulose membrane (GE HealthCare), and processed according to a standard protocol. HIF-1α, full and cleaved PARP, ERα and BCL2 antibodies were obtained from Cell Signaling Technology; the antibodies against α-tubulin (Cell Signaling Technology) were added to standardize loading. Goat anti-rabbit IgGs (Jackson ImmunoResearch) conjugated to horseradish peroxidase were used as secondary antibodies. Signals were detected using the ECL reagent as described in Mruk and Cheng's protocol⁴⁷ and an ImageQuant LAS4000 system (GE HealthCare).

Conflicts of interest

There are no conflicts to declare.

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