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New quinoxaline 1,4-di-*N*-oxides. Part 1: Hypoxia-selective cytotoxins and anticancer agents derived from quinoxaline 1,4-di-*N*-oxides

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Abstract—Hypoxic cells which are common feature of solid tumors are resistant to both anticancer drugs and radiation therapy. Thus, the identification of drugs with the selective toxicity toward hypoxic cells is an important target in anticancer chemotherapy. Tirapazamine has been shown to be an efficient and selective cytotoxin after bioreductive activation in hypoxic cells which is thought to be due to the presence of the 1,4-di-*N*-oxide. A new series of quinoxaline 1,4-di-*N*-oxides and fused quinoxaline di-*N*-oxides were synthesized and evaluated for hypoxic–cytotoxic activity on EAC cell line. Compound **10a** was the most potent cytotoxin IC_{50} 0.9 μg/mL, potency 75 μg/mL, and was approximately 15 times more selective cytotoxin (HCR > 111) than 3-aminoquinoxaline-2-carbonitrile which has been used as a standard (HCR > 7.5). Compounds **4** and **3a**,**b** were more selective than the standard. In addition, antitumor activity against Hepg2 (liver) and U251 (brain) human cell lines was evaluated, compounds **9c** and **8a** were the most active against Hepg2 with IC_{50} values 1.9 and 2.9 μg/mL, respectively, however, all the tested compounds were nontoxic against U251 cell line.

1. Introduction

Quinoxaline 1,4-di-*N*-oxide derivatives seem to have very interesting anticancer activity.[1] Hypoxic cells in solid tumors are an important target for cancer chemotherapy.[2] It has been proposed that these hypoxic cells play a negative role in the success of some antitumor therapies because of their resistance to radiotherapy and conventional chemotherapeutic agents.[3] Lin et al.[4] introduced the concept of bioreductive alkylation, that is the hypoxic cells in solid tumors could transform some drugs into cytotoxic species capable of alkylating DNA. Tirapazamine[5] is the first drug to be introduced into the clinic purely as bioreductive cytotoxic agent. In the last few years, extensive studies have been carried out on 3-aminoquinoxaline-2-carbonitrile 1,4-dioxide, its electrochemical measurements indicating that, it is more susceptible to reductive activation than tirapazamine[6] and causes redox-activated DNA damage analogous to it.[7] Herein, we report the preparation and cytotoxicities of several new quinoxaline 1,4-dioxides and fused quinoxalines, with the objective of determining the influence of different substituents in positions 2 and 3 of the quinoxaline ring on the biological activity.

2. Results and discussion

2.1. Chemistry

Scheme 1 shows the synthetic pathways to prepare the target compounds **3**–**7**. The key substrates 3-methylquinoxaline 1,4-dioxides **2a**–**c**[1] were obtained by the well-known Biuret reaction[8] between 5-chloro/methoxybenzo[*c*][1,2,5]oxadiazol 1-oxides (benzofuroxanes, **1a**,**b**)[9] and the appropriate β–diketone, acetyl acetone

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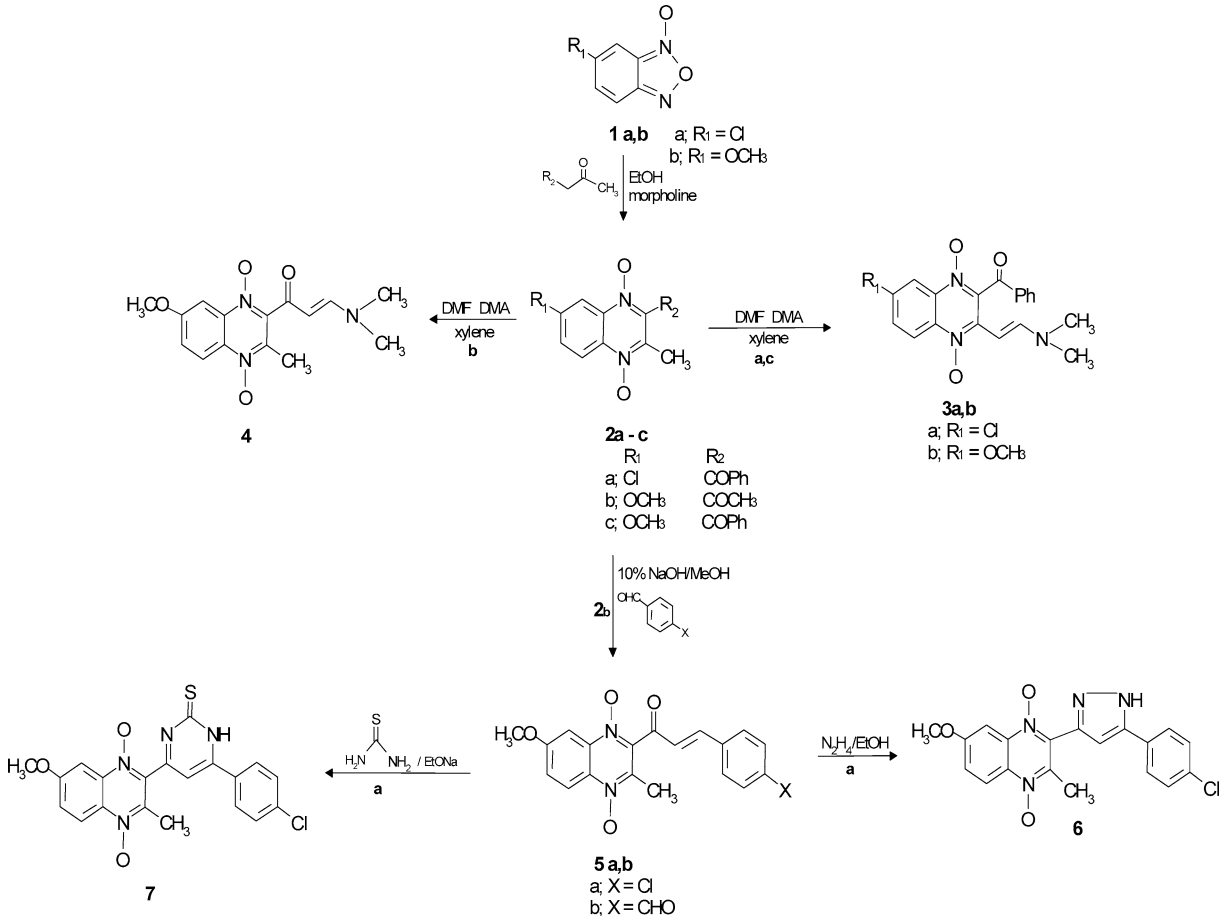


Scheme 1.

or benzoyl acetone. In principle, isomeric product mixtures can be formed from unsymmetrical 5(6)-substituted benzofuroxanes[10] although in practice, the workup and crystallization afford only one isomer probably the 7-isomer,[11] while the other one is discarded.

Reaction of 2-benzoyl-3-methylquinoxaline 1,4-dioxides **2a**,**c** with dimethylformamide–dimethylacetal (DMF–DMA) in xylene gave the enamine derivatives **3a**,**b**. On the other hand, when the 2-acetyl-3-methyl-7-methoxyquinoxaline 1,4-dioxide **2b** was reacted with the same reagent in xylene,[12] the enaminone derivative **4** was obtained in good yield (Table 1). Compound **2b** was employed as key intermediate in the synthesis of the two chalcone derivatives **5a**,**b**. Thus, when equimolar amounts of **2b** and 4-substituted benzaldehyde were reacted in the presence of 10% sodium hydroxide, the chalcones were obtained as yellow crystalline products. The chalcone **5a** was then used as a precursor for the preparation of pyrazole **6** and thiopyrimidine **7** derivatives through its cyclocondensation with hydrazine hydrate[13] and thiourea, respectively.

Scheme 2 shows the synthesis of compounds **8a**–**f** and **12a**,**b**. Treatment of benzofuroxanes **1a**,**b** with prepared intermediates[14] such as acetoacetanilides, benzoylacetanilides or cyanoacetanilides in ethanolic potassium carbonate produced 3-methyl/phenyl-2-carboxamide derivatives **8a**–**f** as well as 3-amino-2-carboxamide derivatives **9a**–**g**.

3-Arylquinoxaline 1,4-dioxides were obtained through reaction of benzofuroxanes with enamines derived from morpholine.[15] The instability of the prepared enamines, which in fact resulted in very low yields of the products, was a limiting factor to their use. In this work the bubbling of ammonia gas in a warm alcoholic solution of benzofuroxane **1a** and substituted acetophenones for 3 h followed by cooling, surprisingly, furnished yellow crystalline 6-chloro-2-substituted-phenylquinoxaline 1,4-dioxides **10a**,**b** in high yields (Table 1).

Moreover, reaction of **1a**,**b** with diketone, for example, indandione or dimidone in a warm ethanolic solution for 4 h in presence of ammonia gas, provided high yields of the indeno[1,2-*b*]quinoxalines **11a**,**b** and phenazines **12a**,**b**, respectively. All the prepared compounds were proved in light of their microanalysis and spectral data including IR, ^{1}H NMR and mass spectrum.

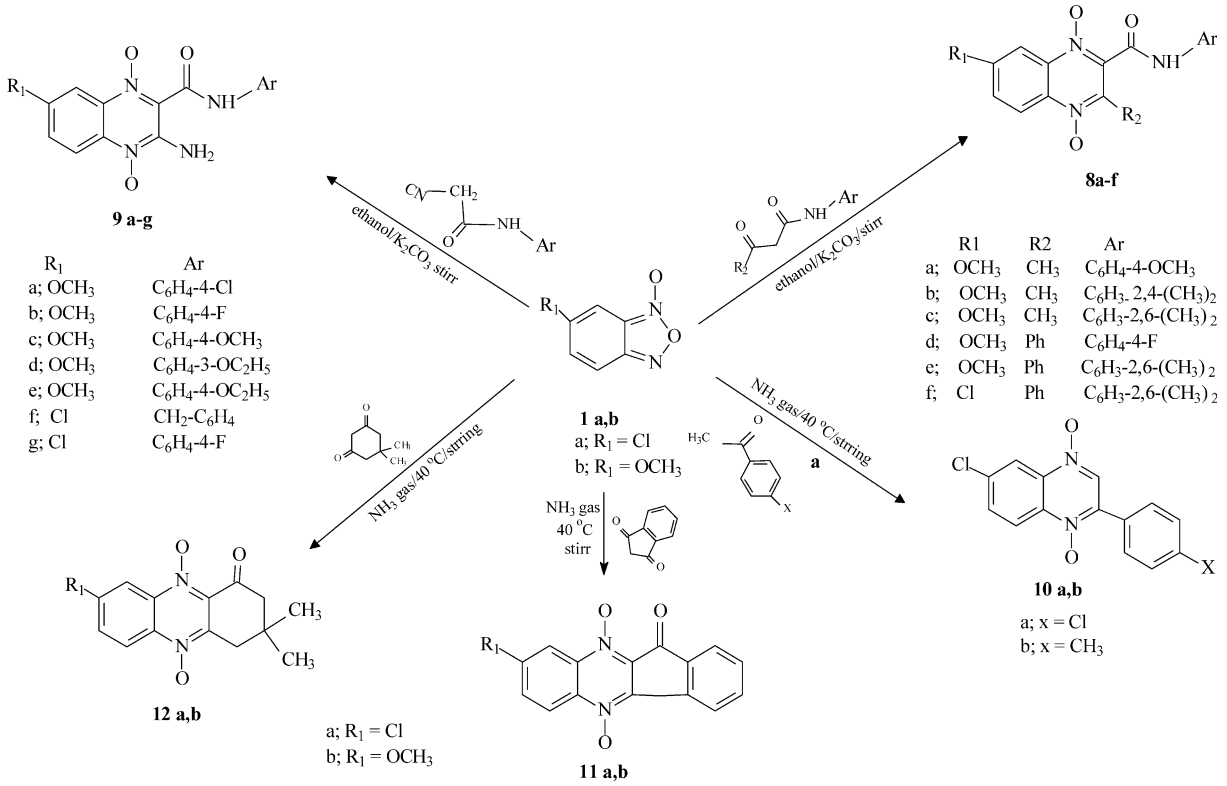
2.2. Antitumor screening

2.2.1. Activity against liver carcinoma (Hepg2) and brain tumor (U251) human cell lines. Antitumor screening was performed at the National Cancer Institute, Cancer Biology Department, Cairo, Egypt. Potential cytotoxic-

Table 1. Physical properties and molecular formula of the synthesized compounds

Compound	R_1	R_2	Ar	X	Mp (°C)	Yield (%)	Formula
3a	Cl	—	—	—	223–224	83	$C_{19}H_{16}ClN_3O_3$
3b	OCH_3	—	—	—	210–212	65	$C_{20}H_{19}N_3O_4$
4	—	—	—	—	239–240	89	$C_{15}H_{17}N_3O_4$
5a[b]	—	—	—	Cl	227–228	98	$C_{19}H_{15}ClN_2O_4$
5b[b]	—	—	—	CHO	215–216	97	$C_{20}H_{16}N_2O_5$
6[c]	—	—	—	—	169–170	56	$C_{19}H_{15}ClN_4O_3$
7	—	—	—	—	152–154	60	$C_{20}H_{15}ClN_4O_3S$
8a	OCH_3	CH_3	C_6H_4–4-OCH_3	—	248–250	85	$C_{18}H_{17}N_3O_5$
8b	OCH_3	CH_3	C_6H_3–2,4-$(CH_3)_2$	—	220–222	90	$C_{19}H_{19}N_3O_4$
8c	OCH_3	CH_3	C_6H_3–2,6-$(CH_3)_2$	—	232–234	87	$C_{19}H_{19}N_3O_4$
8d[a]	OCH_3	Ph	C_6H_4–4-F	—	240–241	67	$C_{22}H_{16}FN_3O_4$
8e[a]	OCH_3	Ph	C_6H_3–2,6-$(CH_3)_2$	—	258–260	80	$C_{24}H_{21}N_3O_4$
8f[a]	Cl	Ph	C_6H_3–2,6-$(CH_3)_2$	—	280–281	89	$C_{23}H_{18}ClN_3O_3$
9a	OCH_3	NH_2	C_6H_4–4-Cl	—	250–252	60	$C_{16}H_{13}ClN_4O_4$
9b	OCH_3	NH_2	C_6H_4–4-F	—	220–222	88	$C_{16}H_{13}FN_4O_4$
9c	OCH_3	NH_2	C_6H_4–4-OCH_3	—	243–245	80	$C_{17}H_{16}N_4O_5$
9d	OCH_3	NH_2	C_6H_4–3-OC_2H_5	—	217–219	77	$C_{18}H_{18}N_4O_5$
9e	OCH_3	NH_2	C_6H_4–4-OC_2H_5	—	187–190	83	$C_{18}H_{18}N_4O_5$
9f	Cl	NH_2	–CH_2–C_6H_5	—	217–218	79	$C_{16}H_{13}ClN_4O_3$
9g	Cl	NH_2	C_6H_4–4-F	—	210–212	77	$C_{15}H_{10}ClFN_4O_3$
10a	—	—	—	Cl	169–170	60	$C_{14}H_8Cl_2N_2O_2$
10b	—	—	—	CH_3	180–182	78	$C_{15}H_{11}ClN_2O_2$
11a	Cl	—	—	—	275–276	80	$C_{15}H_7ClN_2O_3$
11b	OCH_3	—	—	—	244–245	62	$C_{16}H_{10}N_2O_4$
12a	Cl	—	—	—	300–301	78	$C_{14}H_{13}ClN_2O_3$
12b	OCH_3	—	—	—	290–292	58	$C_{15}H_{16}N_2O_4$

All compounds are recrystallized from ethanol except.
[a] The solvent is DMF/EtOH.
[b] The solvent is MeOH.
[c] The solvent is dioxane.



Scheme 2.

ity of the selected compounds **4**, **6**, **8a**,**d**, **9b**,**c**,**f**, **10a**, **11a**, and **12b** was tested using the method of Skehan and Storeng.[16] Two human cell lines: liver carcinoma (Hepg2) and brain tumor (U251) were incubated with five concentrations 0–10 μg/mL[16,17] for each compound. The growth inhibitory action of the selected compounds is summarized in Table 2. The results showed that compounds **9c** and **8a** were the most cytotoxic of all the tested compounds with IC_{50} 1.91 and 2.91 μg/mL, respectively. Compound **9b** and the fused quinoxaline **11a** showed moderate activity with IC_{50} 6.97 and 4.55 μg/mL, respectively. The rest of the tested compounds were nontoxic against the liver carcinoma (Hepg2), all the tested compounds were also nontoxic against the brain tumor. In open chain structures as the carboxamide derivatives substituted with a methoxyl group in the 7-position and different substituents in the 3-position as **9c** and **8a**,**d**, it was observed that as the lipophilicity of the substituent in position 3 increased the toxicity decreased in the order **9c** > **8a** > **8d** with IC_{50} values 1.91, 2.91, and >10 μg/mL, respectively. Activity of **9c** over **9b** (IC_{50} = 1.91 and 6.97 μg/mL) suggests that an electron-donating group in position 4 of the 2-aryl carboxamide moiety imparts higher cytotoxicity than an electron-withdrawing group, **9c** showed cytotoxicity approximately threefold that of **9b**. The combination of a lipophilic group in the 3-position and an electron-withdrawing group in the 4-phenylcarboxamide seems to abolish the activity completely and this is demonstrated in the 2-(4-fluoro-phenyl)-carboxamide-3-phenylquinoxaline 1,4-dioxide **8d**. In order to assess the activity of quinoxaline 1,4-dioxide bearing a heterocyclic ring at position 2, 2-pyrazolyl quinoxaline **6** was tested and the result revealed that it was inactive. On the other hand, the tricyclic derivative **12b** was also inactive and this shows that rigidification is not favorable for the antitumor activity. However, the tetracyclic **11a** was active with an IC_{50} 4.55 μg/mL.

2.2.2. Activities against Ehrlich ascites carcinoma (EAC) cell line: Hypoxia-selective cytotoxicity screening. These biological studies were performed at the National Center for Radiation Research and Technology (NCRRT), Cairo, Egypt. A preliminary screening on compounds **3a**,**b**, **4**, **6**, **7**, **8a**,**d**–**f**, **9b**,**c**,**f**,**g**, **10a**,**b**, **11a**, and **12b** was performed against EAC cells.[18] The tumor cell suspensions were incubated with different concentrations in micrograms per mililiter[16,17] for each compound in air (oxia).

Table 2. Growth inhibitory action (IC_{50}) of the tested compounds against Hepg2 and U251 human cell lines

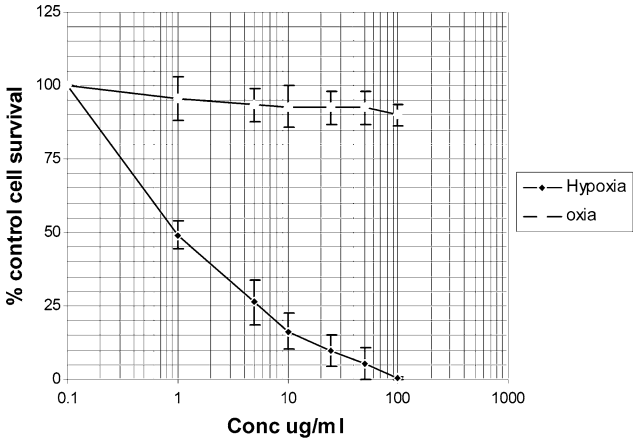
Compound	Hepg2	U251
4	>10	>10
6	>10	>10
8a	2.91	>10
8d	>10	>10
9b	6.97	>10
9c	1.91	>10
9f	>10	>10
10a	>10	>10
11a	4..55	>10
12b	>10	>10

Table 3. Data of IC_{50}, potency, and hypoxic selectivity of the tested quinoxaline derivatives

Compound	IC_{50} hypoxia[a] (μg/mL)	Potency[b] (μg/mL)	HCR[c]
Reference (**B**)	13.5	>100	>7.5
3a	11.5	100	>8.7
3b	8.5	90	9.41
4	10	100	>10
6	>100	>100	≃1
7	90	>100[d]	>1.1
8e	50	>100	>2
8f	25	100	4.34
9b	20	>100	3.25
9g	60	>100	>1.6
10a	0.9	75	>111
10b	20	>100	>5
11a	30	>100	>3.3

* Other compounds could not be accurately determined because at dose higher than 25 μg/mL, they precipitated in the culture medium.
[a] Concentration in (μg/mL) causing 50% mortality in cell numbers.
[b] Concentration that gives 1% cell survival in hypoxia.
[c] Hypoxic cytotoxicity ratio (dose in air/dose in hypoxia giving the same cell survival, IC_{50}).
[d] Potency of tested compound was more than 100 μg/mL.

Further in vitro study was carried out both in oxic and hypoxic conditions, for the active compounds, following the reported method.[6,11,19,20] Three response parameters were calculated, IC_{50}, potency, and hypoxic cytotoxicity ratio (HCR) (Table 3). Where IC_{50} value corresponds to the compound's concentration causing 50% mortality in net cell numbers, the potency is the dose which gives 1% cell survival in hypoxia, while HCR is the ratio between doses in air and in hypoxia giving the same cell survival (calculated at IC_{50}).[21,22] Compound **10a** was the most potent cytotoxin, IC_{50} 0.9 μg/mL, potency 75 μg/mL, and was approximately 15 times more hypoxia-selective cytotoxin (HCR > 111) than 3-aminoquinoxaline-2-carbonitrile (**B**) which has been used as a standard (HCR > 7.5). Moreover, it is approximately 22 times more selective than its counterpart **10b** (HCR > 5) which may be due to the electron-withdrawing effect of 4-Cl group on the 3-phenyl moiety of **10a**. Compounds



Dose-respnse curve in oxia and hypoxia of compound 10a

Figure 1.

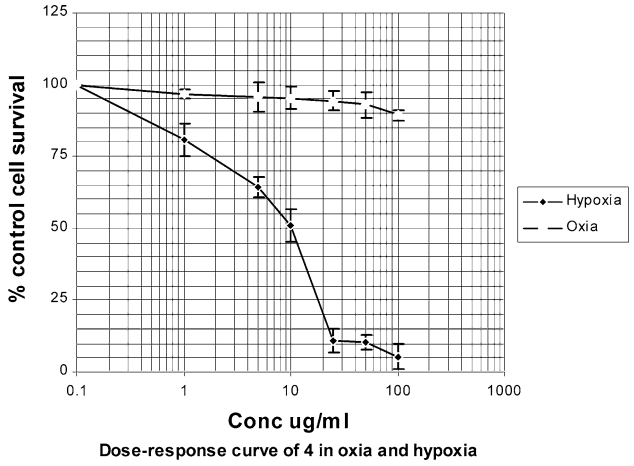


Figure 2.

3a,**b** and **4** were more selective (HCR > 8.7, 9.4, and >10, respectively) than the standard (**B**). Poor selectivity observed by compounds **6** and **7** is thought to be due to the heterocyclic moiety (pyrazole/pyrimidine) at 2-position. Dose–response curves for **10a** and **4** are presented in Figures 1 and 2.

3. Experimental

3.1. Synthesis

Melting points were determined on electrothermal 9100 digital melting point apparatus and are uncorrected. ^{1}H NMR spectra were recorded in DMSO-d_6 on Varian Gemini 200 (200 MHz) using tetramethylsilane (TMS) as an internal standard (chemical shift in δ parts per million). The IR spectra were performed on a Perkin-Elmer 1600 FTIR in KBr pellets. Elemental microanalysis (C, H, and N) was performed on a Perkin-Elmer 2400 analyzer from vacuum-dried samples. All compounds were within ±0.4% of the theoretical values. The mass spectra were recorded on a Hewlett-Packard 5988-A instrument at 70 eV. Chemicals were purchased from E. Merck (Darmstadt, Germany), Sigma–Aldrich (Germany); solvents used were of the highest grade. Compound **B** was prepared according to the reported methods.[6] MF: $C_9H_6N_4O_2$, mp 240–242 °C; yield: 93%.

3.1.1. 2-Benzoyl-3-(2-dimethylamino-vinyl)-7-substituted-quinoxaline 1,4-di-*N*-oxides (3a,b). A mixture of **2a** or **2c** (0.01 mol) and DMF–DMA (0.015 mol) in xylene (30 mL) was refluxed for 6 h. The solvent was removed and the red precipitate was crystallized (Table 1). IR (KBr, cm^{-1}) **3a**,**b** 1674–1678 (CO), 1630–1590 (C=N), 1320–1350 (NO). ^{1}H NMR **3a**: δ 2.49 (s, 6H, N $(CH_3)_2$), 4.3 (d, 1H, NC*H*=CH); 7.5–7.9 (m, 5H, Ph-H); 8.0 (d, 1H, H_6); 8.2 (s, 1H, H_8); 8.3 (d, 1H, H_5); 9.4 (d, 1H, C*H*=CH).

3.1.2. 7-Methoxy-3-methyl-2-(3-dimethylamino-2-propenoyl)-quinoxaline 1,4-dioxide (4). A mixture of **2b** (2.48 g, 0.01 mol) and DMF–DMA (1.78 g, 0.015 mol) in xylene (30 mL) was refluxed for 8 h. After removal of the solvent, the product was crystallized (Table 1). IR (KBr, cm^{-1}): 1654 (CO) 1638–1595 (C=N), 1320–1350 (NO). ^{1}H NMR: δ 2.3 (s, 3H, CH_3-quinox.); 2.8, 3.0 (2s, 6H, N $(CH_3)_2$); 3.9 (s, 3H, OCH_3); 5.2 (d, 1H, NC*H*=CH); 7.4 (d, 2H, COC*H*=CH + H_6); 7.7 (s, 1H, H_8); 8.3 (d, 1H, H_5).

3.1.3. 7-Methoxy-3-methyl-2-[3(4-substituted-phenyl)-propenoyl]-quinoxaline 1,4-di-*N*-oxides (5a,b). A mixture of **2b** (2.48 g, 0.01 mol) and 4-chlorobenzaldehyde or terphthaldehyde (0.01 mol) in methanol (60 mL) was stirred, while a few drops of sodium hydroxide (10% in methanol) was added. Bright yellow crystals separated within 15 min. The mixture was stirred for an additional 30 min, filtered, washed with water, and crystallized (Table 1). IR (KBr, cm^{-1}) **5a**: weak band at 1678 (CO–CH=CH–), 1620 (C=N), 1320–1350 (NO). ^{1}H NMR **5a**: δ 2.3 (s, 3H, CH_3-quinox.); 4.0, (s, 3H, OCH_3); 5.4 (d, 1H, COCH=C*H*); 7.3 (d, 1H, CO–C*H*=CH); 7.4, 7.7 (2d, 4H, Ar-H, AB system J = 8.4); 7.6 (d, 1H, H_6); 7.8 (s, 1H, H_8); 8.4 (d, 1H, H_5). IR (KBr, cm^{-1}) **5b**: 1770 (HCO); 1681 (CO). MS (*m*/*z*, %) **5b**: 364 (M^+, 4.0); 332 [M^+-32 (2O), 23.5]; 173 (100).

3.1.4. 3-[5-(4-Chloro-phenyl)-1*H*-pyrazol-3-yl]-6-methoxy-2-methyl-quinoxaline-1,4- di-*N*-oxide (6). A solution of hydrazine hydrate 98% (0.01 mol) in ethanol (10 mL) was added to a boiling solution of **5a** (3.79 g, 0.01 mol) in ethanol (30 mL), the mixture was refluxed for 7 h. After the solvent removal, the product was crystallized (Table 1). IR (KBr, cm^{-1}): 3321 (NH), 1616 (C=N), 1320–1350 (NO). MS (*m*/*z*, %): 382 (M^+, 9.2); 359 (6.1), 357 (18.5), 148 (100).

3.1.5. 2-[6-(4-Chloro-phenyl)-2-thioxo-1*H*-pyrimidin-2yl]-7-methoxy-3-methyl-quinoxaline 1,4-di-*N*-oxide (7). A mixture of (3.79 g, 0.01 mol), thiourea (0.67 g, 0.01 mol), and sodium ethoxide in ethanol (50 mL) was refluxed for 10 h. The product was washed with water and crystallized (Table 1). IR (KBr, cm^{-1}): 3445 (NH), 1598 (C=N), 1320–1350 (NO). MS (*m*/*z*, %): 426 (M^+, 29.1); 428 (M+2, 14.2); 355 (100).

3.1.6. 7-Substituted-3-methyl/phenyl-2-[N-(substituted-phenyl)-amino-carbonyl]-quinoxaline 1,4-di-*N*-oxides (8a–f). A warm solution of 5(6)-substituted benzofuroxane and acetoacetanilides or benzoylacetanilides (0.01 mol) in ethanol (50 mL) was stirred at room temperature in the presence of catalytic amount of potassium carbonate. The yellow products precipitated in a period of 2 h and were crystallized (Table 1). IR (KBr, cm^{-1}) **8a**–**f**: 3242–3261 (NH), 1656–1665 (CO), 1630–1590 (C=N), 1320–1350 (NO). ^{1}H NMR **8a**: δ 2.4 (s, 3H, CH_3); 3.8 (s, 3H, OCH_3); 4.0 (s, 3H, OCH_3); 6.9, 7.5 (2d, 4H, Ar-H, AB system J = 9 Hz); 7.6 (d, 1H, H_6); 7.8 (s, 1H, H_8); 8.4 (d, 1H, H_5); 11.0 (s, 1H, NH–D_2O exchangeable). MS (*m*/*z*, %) **8a**: 355 (M^+, 65.4); 338 [M^+−17(OH), 100], 322 (55.1). ^{1}H NMR **8b**: δ 2.2, 2.3 (2s, 6H, 2CH_3); 2.4, (s, 3H, CH_3-quinox.); 4.0 (s, 3H, OCH_3); 6.9–7.1, 7.5 (2m, 3H, Ar-H); 7.5 (d, 1H, H_6); 7.8 (s, 1H, H_8); 8.4 (d, 1H, H_5); 10.2 (s, 1H, NH–D_2O exchangeable). ^{1}H NMR **8c**: δ 2.34 (s, 6H,

$2CH_3$); 2.4 (s, 3H, CH_3-quinox.); 3.9 (s, 3H, OCH_3); 7.0–7.4 (m, 3H, Ar-H); 7.45 (d, 1H, H_6); 7.74 (s, 1H, H_8); 8.3 (d, 1H, H_5); 10.3 (s, 1H, NH–D_2O exchangeable). MS (*m*/*z*, %) **8c**: 353 (M^+, 6.2), 337 [M^+-16 (O), 12.5], 320 [M^+-33 (2O+H), 100].^{1}H NMR **8d**: δ 3.8 (s, 3H, OCH_3); 7.05 (d, 2H, AB system, $J = 9$ Hz); 7.55 (d, 1H, H_6); 7.7 (d, 2H, AB system, $J = 9$ Hz); 7.83 (s, 1H, H_8); 8.3 (d, 1H, H_5); 10.2 (s, 1H, NH). MS (*m*/*z*, %) **8d**: 405 (M^+, 19.3); 373 [M^+-32 (2O), 28.4]; 109 (100). ^{1}H NMR **8e**: δ 1.8 (s, 6H, $2CH_3$); 4.0 (s, 3H, OCH_3); 7.0, 7.5 (2m, 8H, Ar-H); 7.6 (d, 1H, H_6); 7.9 (s, 1H, H_8); 8.4 (d, 1H, H_5); 10.1 (s, 1H, NH). MS (*m*/*z*, %) **8f**: 419 (M^+, 9.57); 403 [M^+-16 (O), 23.2]; 386 [M^+-33 (2O+H), 100].

3.1.7. 3-Amino-7-substituted-2-[N-(substituted-phenyl)-amino-carbonyl]-quinoxaline 1,4-di-*N*-oxides (9a–e,g) and 3-amino-7-chloro-2-benzyl-amino-carbonyl quinoxaline 1,4-di-*N*-oxides (9f). A warm solution of 5(6)-substituted benzofuroxane **1a**,**b** (0.01 mol) and the appropriate cyanoacetanilide (0.01 mol) in ethanol (50 mL) was stirred at room temperature in the presence of catalytic amount of potassium carbonate. The yellow products precipitated in a period of 2 h and were crystallized (Table 1). IR (KBr, cm^{-1}) **9a**–**g**: 3400–3251 (NH_2, NH), 1661–1655 (CO), 1617–1593 (C=N), 1320–1350 (NO). MS (*m*/*z*, %) **9a**: 360 (M^+, 8.48); 328 [M^+-32 (2O), 1.67], 127 (100). ^{1}H NMR **9b**: δ 3.9 (s, 3H, OCH_3); 7.1–8.7 (m, 7H, Ar-H), 10.1, 12.4 (2s, 3H, NH_2, NH–D_2O exchangeable). MS (*m*/*z*, %) **9b**: 344 (M^+, 6.6), 312 [M^+-32 (2O), 100]. ^{1}H NMR **9c**: δ 3.70 (s, 3H, OCH_3); 3.77 (s, 3H, OCH_3); 6.8, 7.3 (2d, 4H, AB system, $J = 9$ Hz); 7.4 (d, 1H, H_6); 7.8 (s, 1H, H_8); 8.4 (d, 1H, H_5); 8.8 (br s, 3H, NH_2, NH–D_2O exchangeable). MS (*m*/*z*, %) **9c**: 356 (M^+, 7.5), 340 [M^+-16 (O), 11.5], 124 (100). ^{1}H NMR **9d**: δ 1.2 (t, 3H, CH_2CH_3); 3.9 (s, 3H, OCH_3), 4.0 (q, 2H, CH_2CH_3), 6.6–7.2 (m, 4H, Ar-H), 6.7 (d, 1H, H_6), 7.2 (s, 1H, H_8), 8.00 (d, 1H, H_5), 10.2, 12.1 (2s, 3H, NH_2, NH–D_2O exchangeable). ^{1}H NMR **9e**: δ 1.2 (t, 3H, CH_2CH_3); 3.8 (s, 1H, OCH_3); 3.9 (q, 2H, CH_2CH_3); 6.8, 7.2 (2d, 4H, Ar-H, AB system $J = 9$ Hz); 7.8 (d, 1H, H_6); 8.2 (d, 1H, H_5); 8.4 (s, 1H, H_8) 10.2, 12.4 (br s, 3H, NH_2, NH–D_2O exchangeable); ^{1}H NMR **9f**: δ 4.6, 5.4 (2d, 2H, N–CH_2); 7.2–7.4 (m, 6H, 5Ph-H + H_6); 7.7 (s, 1H, H_8); 7.8 (d, 1H, H_5); 8.6 (t, 1H, NH–D_2O exchangeable), 9.3 (br s, 2H, NH_2–D_2O exchangeable). MS (*m*/*z*, %) **9f**: 344 (M^+, 30.3), 312 [M^+-32 (2O), 5.4], 92 (100). MS (*m*/*z*, %) **9g**: 348 (M^+, 34.2); 178 (65.7), 111 (100).

3.1.8. 6-Chloro-2-(4-chloro-phenyl)-quinoxaline 1,4-di-*N*-oxide (10a) and 6-chloro-2-*p*-tolyl-quinoxaline-1,4-di-*N*-oxide (10b). A mixture of 5(6)-chloro-benzofuroxane **1a** (1.79 g, 0.01 mol) and 4-methyl or chloro acetophenone (0.01 mol) in ethanol (50 mL) was stirred at 60 °C, while ammonia gas was bubbled into the solution for 3 h stirring continued for another 4 h, after which the reaction mixture was cooled overnight, after solvent removal the products were crystallized (Table 1). IR (KBr, cm^{-1}) **10a**,**b**: 3079 (CH-aromatic) 2854 (CH-aliphatic); 1608 (C=N), 1320–1350 (NO). ^{1}H NMR **10a**: 7.55–8.6 (m, 6H, 4 Ar-H + H_7 + H_8), 8.94 (s, 1H, H_5), 9.34 (s, 1H, H_3). MS (*m*/*z*, %) **10a**: 308 (M^+ 2, 51.5); 306 (M^+, 78.6); 290 (M−16 (O), 100). ^{1}H NMR **10b**: 2.4 (s, 3H, CH_3); 7.3, 7.9–8.5 (2m, 6H, 4Ar-H + H_7 + H_8); 8.9 (s, 1H, H_5); 9.2 (s, 1H, H_3).

3.1.9. 8-Substituted-11-oxo-indeno [1,2-*b*] quinoxaline-5,10-dioxides (11a,b). A mixture of 5(6)-substituted benzofuroxane **1a**,**b** (0.01 mol) and indanedione (0.01 mol) in ethanol (70 mL) was stirred and heated at 60 °C, while ammonia gas was bubbled into the solution for 4 h after cooling and evaporating the solvent in a rotary evaporator the products were crystallized (Table 1). IR (KBr, cm^{-1}) **11a**,**b**: 1712–1715 (CO), 1638–1590 (C=N), 1320–1350 (NO). ^{1}H NMR **11a**: 7.25–8.3 (m, 6H, Ar-H), 8.85 (s, 1H, H_9). MS (*m*/*z*, %) **11a**: 298 (M^+, 75.3); 300 (M+2, 30.4); 282 (M^+-16 (O), 100). ^{1}H NMR **11b**: 3.75 (s, 3H, OCH_3), 7.16–8.2 (m, 6H, Ar-H), 8.6 (s, 1H, H_9).

3.1.10. 3,3-Dimethyl-1-oxo-8-substituted-3,4-dihydro-2*H*-phenazine-5,10-di-*N*-oxides (12a,b). The same procedure as **11a**,**b**. IR (KBr, cm^{-1}) **12a**,**b**: 1650 (CO), 1611 (C=N), 1320–1350 (NO). ^{1}H NMR **12b**: δ 1.5 (s, 6H, $2CH_3$); 1.7 (s, 2H, CH_2), 3.6 (s, 3H, OCH_3); 5.4 (s, 2H, CH_2–CO); 7.8 (d, 1H, H_6); 8.4 (s, 1H, H_8); 8.6 (d, 1H, H_5).

3.2. Antitumor screening

3.2.1. Activity against Hepg2 and U251. Cells were plated in 96-multiwell plate (10^4 cells/well) for 24 h before treatment with the compounds to allow attachment of cell to the wall of the plate. Different concentrations of the compounds under test (0.1, 2.5, 5, and 10 μg/mL) were solubilized in dimethylsulfoxide (DMSO) and were added to the cell monolayer of the two human cell lines. Monolayer cells were incubated with the compounds for 48 h at 37 °C and in atmosphere of 5% CO_2. After 48 h, cells were fixed, washed, and stained with sulforhodamine B stain. The color intensity was measured in an ELISA reader.[16]

3.2.2. Activity against EAC experimental cell line. Animals, chemicals, and facilities: Female Swiss albino mice weighing 25–30 g obtained from (the holding company of biological products and vaccines, VACSERA, Cairo, Egypt) were housed at a constant temperature (24 ± 2 °C) with alternating 12 h light and dark cycles and fed standard laboratory food (Milad Co., Cairo, Egypt) and water ad libitum. All chemicals and reagents were from Sigma–Aldrich, Germany and Merck, Germany.

3.2.3. Aerobic and hypoxic cytotoxicity

3.2.3.1. Cells. Ehrlich Ascites carcinoma cells EAC were obtained by needle aspiration of ascitic fluid from pre-inoculated mice, under aseptic conditions. Tumor cell suspension (2.5×10^6/mL) was prepared.

3.2.3.2. Suspension cultures. Cell suspensions were prepared in sterile growth medium RPMI-164 (Sigma–Aldrich, Germany), supplemented with 10% v/v fetal bovine serum (FBS) (Sigma–Aldrich, Germany) and penicillin–streptomycin 100 U/100 μg/mL. To 0.9 mL of the prepared suspension culture different concentrations (1–100 μg/mL) of the prepared compounds were added to the media (0.1 mL). Suspension cultures were

introduced into two sets of sterile tubes for aerobic and anaerobic assays. For the hypoxic condition evacuated tubes were used. These vacated tubes were tightly closed with rubber caps which were perforated with two needles of (19 G), to provide gas (N_2 gas) inlet and outlet. The tubes were incubated at 37 °C under oxic (O_2 air) or hypoxic (N_2 gas) with pressure of (5 barometer). Supplementation of N_2 gas continued for 2 h.

3.2.3.3. Preparation of tested compounds. Drug solutions, with various dilutions, were freshly prepared in pure (DMSO). Drug solutions (0.1 mL) were added to each tube in the two sets of aerobic and anaerobic tubes corresponding to (100, 50, 25, 10, 5, and 1 μg/mL). This condition was applied for at least three times for each tested compound. Control group without any treatment (positive control) and group treated with 0.1 mL DMSO (negative control) were performed parallel to compounds tested in each experiment.

3.2.3.4. Determination of total cell counts and viable cell numbers. After 2 h exposure to the compound, the cells were centrifuged. Cell numbers were determined with a hemocytometer (Sigma catalogue) after staining with Trypan blue (Trypan blue exclusion test).[23] Trypan blue is one of several stains recommended for use in dye exclusion procedures for viable cell counting. This method is based on the principle that live cells do not take up certain dyes, whereas dead cells do. The percent of control cell survival for the treated suspension cultures was calculated.

$$\%\,\text{cell viability} = \text{total viable cells (unstained)}/\text{total cells} \times 100.$$

4. Conclusion

In our attempt to synthesize and evaluate the anticancer activity and hypoxia-selectivity of quinoxaline 1,4-dioxide derivatives, we were overwhelmed by the results of **10a**. It was 15 times more selective than the standard suggesting that it may act by the same mechanism. Compounds **4** and **3a**,**b** elicited more selectivity than the standard. Moreover, compounds **9c** and **8a** were the most potent antitumor agents against Hepg2 (liver) human cell line. Our future studies would be concerned with the in vivo examination of these compounds.

Supplementary data

Supplementary data associated with this article including calculation details and predicted activities can be found, in the online version, at doi:10.1016/j.bmc.2006.06.038.

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