Hypoxia-Selective Agents Derived from 2-Quinoxalinecarbonitrile 1,4-Di-*N*-oxides. 2

Antonio Monge,*,† Francisco J. Martínez-Crespo,† Adela López de Ceráin,‡ Juan A. Palop,† Susana Narro,† Virginia Senador,† Ana Marín,‡ Yolanda Sainz,† Mercedes González,† Elizabeth Hamilton,§ and Andy J. Barker§

Departments of Medicinal Chemistry and Toxicology, CIFA, Universidad de Navarra, 31080 Pamplona, Spain, and Zeneca Pharmaceuticals, Mereside, Alderley Park, Macclesfield, Cheshire SK10 4TG, U.K.

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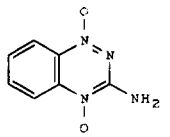
Hypoxic cells are an important target for antitumor therapy because tumors are typically characterized by such cells. Virtually all tumors which are present as solid masses contain hypoxic cells, while normal cells generally have an adequate supply of oxygen. Accordingly, antitumor agents can be made selective for tumors by virtue of high activity under hypoxic conditions. The initial purpose of this work was to determine the influence of different groups in position 3. Thus, the synthesis of some 3-NH-substituted derivatives (**2a**, **3a**, **4a**) starting from 3-amino-2-quinoxalinecarbonitrile 1,4-di-*N*-oxide (**1a**) is described. Reductive deamination of compounds **1a**–**k** provides the 2-quinoxalinecarbonitriles **5a**–**k**, which are more potent, while selectivity is maintained or increased in some derivatives. The compound 7-(4-nitrophenyl)-2-quinoxalinecarbonitrile 1,4-di-*N*-oxide (**5k**) is 150-fold more potent than tirapazamine (3-amino-1,2,4-benzotriazine 1,4-di-*N*-oxide), which has been used as a standard. Three derivatives (**5g**,**i**,**k**) show a hypoxic cytotoxicity ratio (HCR) ≥ 200, better than that of tirapazamine (HCR = 75) in V79 cells. Replacement of the 3-amino group by chlorine affords the potent but nonselective 3-chloro derivatives **6a**–**k** showing similar toxicities under both aerobic and hypoxic conditions. These compounds were used as intermediates for the synthesis of a new series of water-soluble compounds derived from 3-[[(*N*,*N*-dialkylamino)alkyl]amino]-2-quinoxalinecarbonitrile 1,4-di-*N*-oxides **10a**–**i** and **11a**–**i**. The 7-chloro and the 7-trifluoromethyl derivatives **10b**,**f** have demonstrated high potency (0.4 and 0.3 μM) and excellent selectivity (HCR = 250 and 340). Several 7-chloro analogues, **12b**, **13b.1**,**b.2**, and **14b**, and the dimer **16b** have been prepared and evaluated in order to determine the optimum lateral chain in position 3, which appears to be the [(*N*,*N*-dimethylamino)propyl]amino moiety.

Introduction

There is evidence that hypoxic cells exist in both animal and human tumors and that oxygen-deficient malignant cells are significantly more resistant to ionizing radiation than their aerobic counterparts. In addition, they may be more resistant to most chemotherapeutic agents, since hypoxic cells may be blocked or slowly moving through the cell cycle, and are distal to tumor vasculature, making them more difficult to reach with adequate drug concentrations.[1,2]

The oxygen deficit of solid tumors can, however, be considered to be a form of vulnerability, susceptible to selective therapeutic attack by chemotherapeutic agents that have chemical and physical properties which permit a preferential exploitation of the hypoxic state. Lin and colleagues were the first to point out that the greater reductive environment in tumors might be exploitable by developing drugs that are preferentially reduced to cytotoxic species in tumors.[3,4] Such bioreductive cytotoxic drugs have the feature of being preferentially toxic to hypoxic compared to aerobic cells.[5]

At least three distinct structural classes of compounds have been identified which exhibit selectivity for the hypoxic cells in solid tumors: quinone antibiotics, nitroaromatic compounds, and benzotriazine di-*N*-oxides.[6–8] The lead compound in the third of these classes (tirapazamine) shows high selective toxicity toward hypoxic cells both *in vitro* and in tumors *in vivo*.[9,10] Tirapazamine has been used as a standard in the biological screenings (Table 1).



TIRAPAZAMINE

Recently, Naylor and colleagues have reported the preparation of a series of imidazo[1,2-*a*]quinoxaline mono-*N*-oxides as bioreductively activated cytotoxins.[11] To date, few quinoxaline 1,4-di-*N*-oxides have been described as potential selective cytotoxic agents, but various related quinoxaline 1,4-di-*N*-oxides possess antibacterial activity, and this activity is increased by 2 orders of magnitude under anaerobic conditions.[12,13] We have demonstrated that quinoxaline 1,4-di-*N*-oxides are selective cytotoxic agents in hypoxia.[14–16] Preliminary studies of structure–activity relationships suggested the importance of the cyano group in the 2 position; also, mild electron-withdrawing substituents in the 6(7) position, e.g., Cl, F, and CF_3, increased potency under hypoxic conditions.

The two most desirable properties of any pharmaceutical preparation must surely be selectivity and potency. Any agent that has such properties should have a high therapeutic index, that is, the effective dose should be orders of magnitude below the toxic one.[17] Several of the quinoxalines previously prepared meet this criteria, but their low water solubilities constitute a disadvan-

† Department of Medicinal Chemistry, Universidad de Navarra.
‡ Department of Toxicology, Universidad de Navarro.
§ Zeneca Pharmaceuticals.
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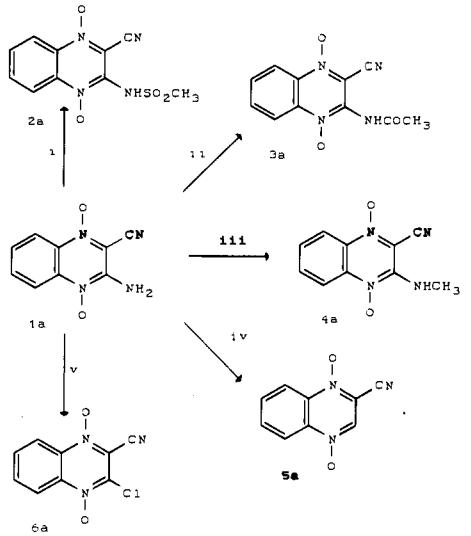
Scheme 1[a]



[a] Conditions: (i) CH_3SO_2Cl, $NaHCO_3$, dioxane, 100 °C; (ii) $(CH_3CO)_2O$, heat; (iii) $(CH_3)_2SO_4$, $NaHCO_3$, dioxane, 90 °C; (iv) *tert*-butyl nitrite, DMF, nitrogen, 70 °C; (v) *tert*-butyl nitrite, acetonitrile, $CuCl_2$, nitrogen, 70 °C.

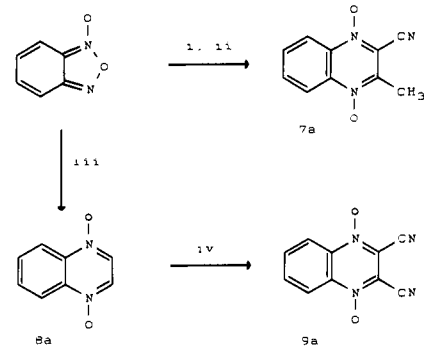
tage for use *in vivo*. Thus, we have concentrated our efforts on development of more potent and selective hypoxic cell cytotoxins and preferably water-soluble. We describe now the synthesis and the cytotoxicities of deaminated and 3-(alkylamino)quinoxaline 1,4-di-*N*-oxide derivatives. The deaminated compounds **5a–k** were not water-soluble, but their interesting *in vitro* features constitute a new approach in the design of bioreductive quinoxalines.

Results and Discussion

Chemistry. In order to determine the optimum substituent for hypoxic cytotoxicity at position 3, a series of quinoxaline 1,4-di-*N*-oxides was prepared starting from the 6(7)-unsubstituted quinoxaline **1a** as shown in Scheme 1. Reaction of 3-aminoquinoxaline **1a** with methanesulfonyl chloride in dry dioxane as solvent afforded the corresponding sulfonamide **2a**. Treatment of **1a** with acetic anhydride in dry dioxane at room temperature gave the amide **3a**. Methylation of **1a** afforded the 3-*N*-methylamino derivative **4a**. Compound **5a** was prepared from **1a** under reductive deamination conditions, which involved the diazotization of the 3-amino group by use of *tert*-butyl nitrite at 65–70 °C and subsequent displacement of the diazonium salt thus formed by a hydrogen donor, such as DMF. Attempts to diazotize the 3-amino group by using the conventional Sandmeyer reaction were unsuccessful, probably due to its low basicity and the low solubility of compounds. However, reduced aminoquinoxalines were readily converted to the chloro derivatives.[15] The amino group of **1a** could be replaced by chlorine using *tert*-butyl nitrite in dry acetonitrile and in the presence of copper(II) chloride as chlorine donor. The low basicity and solubility of **1a** gave, in poor yield (2%), a bright yellow solid which was identified as the 3-halo compound **6a**.

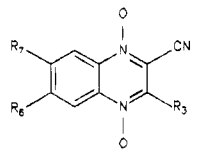
Scheme 2 describes the synthesis of the 3-methyl-2-quinoxalinecarbonitrile 1,4-di-*N*-oxide (**7a**) from benzofuroxan and 5-methylisoxazole under basic conditions. Beirut reaction between benzofuroxan and vinyl acetate in the presence of diethylamine as base gave the

Scheme 2[a]



[a] Conditions: (i) NH_4Cl, NH_3, methanol; (ii) 5-methylisoxazole, KOH, methanol; (iii) vinyl acetate, Et_2NH, ethyl acetate, 0 °C; (iv) KCN, $K_3Fe(CN)_6$, ethanol/water, 0 °C.

Table 1. Results of the Dose–Response Assays of 2-Quinoxalinecarbonitrile 1,4-Di-*N*-oxides



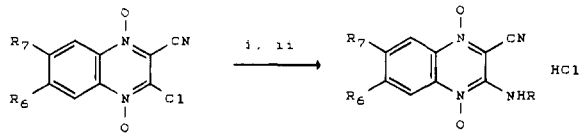
compd	R_3	R_6	R_7	formula	*P*[a]	HCR[b]
tirapazamine					30	75
1a	NH_2	H	H	$C_9H_6N_4O_2$	30	80
1b[c]	NH_2	H	Cl	$C_9H_5ClN_4O_2$	9	150
1c	NH_2	Cl	Cl	$C_9H_4Cl_2N_4O_2$	1	80
1d[c]	NH_2	H	F	$C_9H_5FN_4O_2$	15	100
1e	NH_2	F	F	$C_9H_4F_2N_4O_2$	1	<10
1f[c]	NH_2	H	CF_3	$C_{10}H_5F_3N_4O_2$	7	75
1g[c]	NH_2	H	CH_3	$C_{10}H_8N_4O_2$	NT[d]	NT
1h	NH_2	CH_3	CH_3	$C_{11}H_{10}N_4O_2$	NT	NT
1i	NH_2	H	OCH_3	$C_{10}H_8N_4O_3$	30	>10
1j	NH_2	Cl	OCH_3	$C_{10}H_7ClN_4O_3$	10	<10
1k	NH_2	H	*p*-O_2N-Ph	$C_{15}H_9N_5O_4$	4	15
2a	$NHSO_2CH_3$	H	H	$C_{10}H_8N_4O_4S$	NT	NT
3a	$NHCOCH_3$	H	H	$C_{11}H_8N_4O_3$	NT	NT
4a	$NHCH_3$	H	H	$C_{10}H_8N_4O_2$	NT	NT
5a	H	H	H	$C_9H_5N_3O_2$	5	>100
5b	H	H	Cl	$C_9H_4ClN_3O_2$	3	30
5c	H	Cl	Cl	$C_9H_3Cl_2N_3O_2$[e]	0.7	10
5d	H	H	F	$C_9H_4FN_3O_2$	2	>50
5e	H	F	F	$C_9H_3F_2N_3O_2$	0.3	5
5f	H	H	CF_3	$C_{10}H_4F_3N_3O_2$	6	80
5g	H	H	CH_3	$C_{10}H_7N_3O_2$	6	200
5h	H	CH_3	CH_3	$C_{11}H_9N_3O_2$	14	>7
5i	H	H	OCH_3	$C_{10}H_7N_3O_3$	5	>200
5j	H	Cl	OCH_3	$C_{10}H_6ClN_3O_3$[f]	0.4	>50
5k	H	H	*p*-O_2N-Ph	$C_{15}H_8N_4O_4$	0.2	200
7a	CH_3	H	H	$C_{10}H_7N_3O_2$	10	15

[a] Potency= dose in micromolar which gives 1% of control cell survival in hypoxia. [b] Hypoxic cytotoxicity ratio = the dose in air divided by the dose in hypoxia giving 1% of control cell survival. [c] Mixture of isomers. [d] NT = not tested; 1% cell survival was not reached in the screening assay (at 20 μM). [e] C: calcd, 42.19; found, 42.66. [f] C: calcd, 47.72; found, 48.16.

unsubstituted quinoxaline 1,4-di-*N*-oxide (**8a**). Oxidative cyanation of **8a** using potassium cyanide and potassium ferricyanide provided the 2,3-quinoxalinedicarbonitrile 1,4-di-*N*-oxide (**9a**).[18]

The compounds described in Schemes 1 and 2 were tested in a cloning assay using V79 cells. The deaminated analogue **5a** showed the best profile as potential bioreductive agent. Consequently, the next purpose of this work was to prepare a series of 6(7)-substituted 3-deaminated compounds (**5a–k**, Table 1) in order to establish structure–activity relationships for increased toxicity under hypoxic conditions. Reaction of *tert*-butyl

Scheme 3[a]



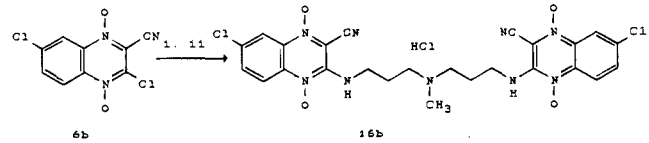
[a] Conditions: (i) H_2N-R, CH_2Cl_2, 20 °C, 3 days; (ii) concentrated HCl, acetone.

nitrite with 6(7)-substituted 3-amino quinoxalines **1a–k** in DMF at 65–70 °C resulted in the formation of 2-quinoxalinecarbonitrile 1,4-di-*N*-oxides **5a–k** in low yields (8–26%). Reaction time for complete evolution of nitrogen was nearly 30 min.[19] In principle, product mixtures can be formed from unsymmetrically substituted quinoxalines, but technically, the workup and purification of the mixture afford only one isomer, the 7-isomer, while the minor isomer is discarded. Compounds **5a–k** were isolated by flash chromatography eluting with a gradient of toluene/ethyl acetate and recrystallized from ethyl acetate as bright yellow crystals. Their low water solubility represented a serious problem for the *in vivo* tests. Our next objective was the preparation of 3-(alkylamino)quinoxalines having basic nitrogens on the lateral chain and that would permit the synthesis of the corresponding hydrochlorides.

The 3-chloro compounds **6a–k** were used as intermediates for the preparation of final derivatives **10–16**. **6a–k** were prepared by treating the arylamines **1a–k** with *tert*-butyl nitrite and anhydrous cupric chloride in acetonitrile at 65 °C.[20,21] The major process competing with aryl halide formation when the molar ratio of $CuCl_2$ to amine was ≤0.5 was reduction of the arylamine to the corresponding arene. Reduction to **5a–k** was effectively minimized by the use of sufficient copper(II) chloride so that the $CuCl_2$:$ArNH_2$ molar ratio was >0.5. Derivatives **6a–k** were obtained in low yield (2–21%). For example, 3,6,7-trichloro-2-quinoxalinecarbonitrile 1,4-di-*N*-oxide (**6c**) was isolated in a very low 2% yield; the presence of two electron-withdrawing fluoro groups at the 6 and 7 positions inhibited this reaction entirely. Also, the remarkably low solubilities shown by **1c,e** may have a decisive influence in this reaction.

Amines **10–16** were prepared from compounds with mild electron-withdrawing groups in position 6(7), such as chlorine, fluorine, or trifluoromethyl, and electron-donating substituents (methyl, methoxy) as shown in Scheme 3. Attempts to alkylate the 3-amino group of **1a–k** with *N,N*-dimethyl-3-chloropropylamine were unsuccessful. Reaction between **6** and 3-(*N,N*-dimethylamino)propylamine afforded the most interesting derivatives **10**: **10b**, with a 7-chloro group, and **10f**, with a 7-trifluoromethyl group, showed the best *in vitro* profile. More **10b** analogues have been identified in order to determine the influence of the lateral chain in hypoxia selectivity. Reaction of **6b** with 2-(*N,N*-diethylamino)ethylamine gave **11b** and with 2-(*N,N*-dimethylamino)ethylamine afforded **12b**. Treating the mixture of isomers of **6b** with 3-morpholinylpropylamine gave a mixture of **13b.1,b.2**. Both isomers were conveniently

Scheme 4[a]



[a] Conditions: (i) $[H_2N(CH_2)_3]_2NCH_3$, CH_2Cl_2, 20 °C, 3 days; (ii) concentrated HCl, acetone.

separated by flash chromatography eluting with a gradient of dichloromethane/ethyl acetate/methanol. When **6b** was reacted with 2-aminoethanol, **14b** was isolated and identified as the free base. Condensation of **6b** and 3-(1-imidazolyl)propylamine yielded **15b**. Also, the synthesis of the dimer **16b** was described by reaction between **6b** and the primary diamine (Scheme 4). All amines, except **14b**, were prepared as hydrochloride salts by dissolving the free base in acetone and adding 3–5 drops of concentrated hydrochloric acid. The red solids obtained were recrystallized from acetone/methanol.

Biological Studies. The quinoxaline derivatives were tested in a cloning assay using V79 cells. Suspension cultures were established from exponentially growing cells and gassed with pure air or nitrogen for 30 min before dosing with the compounds. Treatment lasted 2 h and gassing was continuous during this time. All the compounds were tested at 20 μM in duplicate flasks in both air and nitrogen. The compounds that were toxic in hypoxia and inactive in air were tested in three more assays at different doses to obtain a dose–response curve in air and hypoxia. The potency was defined as the dose in micromolar which gives 1% of control cell survival in hypoxia. The hypoxic cytotoxicity ratio (HCR) was calculated by dividing the dose in air by the dose in hypoxia giving 1% of control cell survival.

The previous related quinoxalines **1a–k**, which were tested as hypoxic cytotoxins, demonstrated potencies between 1 and 30 μM and HCRs between 10 and 150 (Table 1).[14] Under the same conditions, tirapazamine showed a potency of 30 μM and HCR = 75. We found that 6(7)-substituents such as halogens, trifluoromethyl, or cyano increased the potency with respect to the unsubstituted quinoxaline. It also might be that the presence of the amino group at the 3 position played some role in determining cytotoxicity. Sulfonation, acetylation, and methylation of **1a** gave the inactive compounds **2a**, **3a** and **4a**. Compounds **5** were surprisingly more toxic in hypoxia than their aminated counterparts (Table 1), demonstrating that the amino group is not absolutely necessary for cytotoxicity. Similarly, the deaminated benzotriazine SR4482 has been reported to be 1.75-fold more toxic but one-half as selective as tirapazamine.[22,23] Also, deaminated quinoxalines **5a–k** showed a different pattern of activity with respect to the aminated **1a–k**, suggesting a different mechanism of action.[16] Thus, deaminated compounds **5a–k** with electron-donating groups at position 7, e.g., methyl or methoxy, exhibited better activity than compounds **5** with electron-withdrawing groups, e.g., chlorine or fluorine. For example, the 7-methyl analogue **5g** showed an interesting HCR = 200 and the 7-methoxy analogue an HCR > 200. However, in the 7-halo compounds **5b–d**, we found a substantial lack of hypoxia selectivity. The 7-(4-nitrophenyl) derivative **5k** reached the highest toxicity (potency = 0.2 μM), being 20-fold more potent

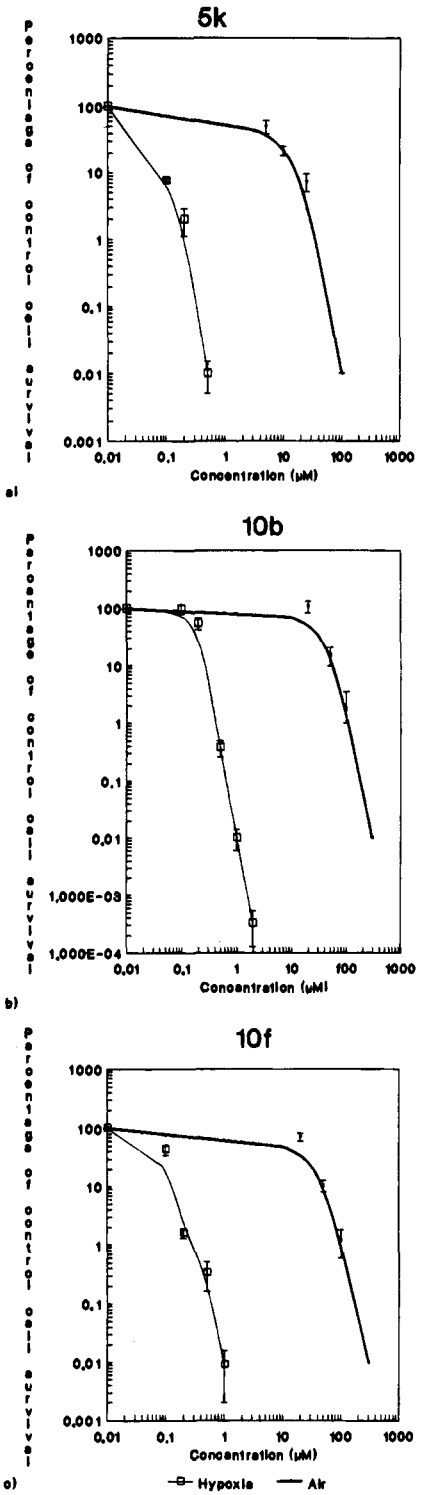


Figure 1. Dose–response curves in air and hypoxia of compounds **5k** (a), **10b** (b), and **10f** (c).

than **1k** or 150-fold more potent than the benzotriazine. The promising HCR = 200 shown by **5k** makes it a potential candidate for further *in vivo* studies (Figure 1a).

We also assayed the 3-chloro-2-quinoxalinecarbonitrile 1,4-di-*N*-oxides **6a–k**. They were potent toxins in both oxic and hypoxic cells (potency between 0.2 and 1 μM), but the differential air/nitrogen toxicity was not significant. For this reason, dose–response assays have not been carried out. These results confirm that minor changes in the basic structure of the quinoxaline may affect the electrochemical properties and, subsequently, the activity.

Compound **7a**, bearing an electron-donating methyl group in position 3, exhibited a potency of 10 μM but a decreased selectivity (HCR = 15) (Table 1). The unsubstituted quinoxaline 1,4-di-*N*-oxide (**8a**) was not active at 20 μM; this suggests the presence of the electron-withdrawing cyano group at position 2 is important. However, the presence of a second cyano group (compound **9a**) decreased the selectivity, as in the case of compounds **6a–k**.

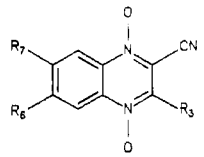
Nucleophilic displacement of the chlorine gave the most interesting compounds. Results of potency and selectivity are presented in Table 2. We selected basic lateral chains in order to prepare the corresponding water-soluble salts. Compounds **10a–i**, bearing an (*N,N*-dimethylamino)propylamino group, demonstrated high selective cytotoxicity. In particular **10b,f** showed excellent HCR values: 250 and 340, respectively, and potency below 1 μM (Figure 1b,c). Although less potent, the presence of a 7-methyl or 7-methoxy substituent also gave interesting hypoxia selectivity, in the range of the 7-chloro- or 7-(trifluoromethyl)quinoxalines. We focused our attention on the development of a series of 6(7)-chloro-3-(alkylamino)quinoxalines for the relevant activity shown by **10b**; also we considered the difficulties (mainly low yields) of preparing 6(7)-trifluoromethyl analogues (see the supporting information). Potency was maintained when an (*N,N*-diethylamino)ethylamino group was present at the 3 position, while an important decreasing of selectivity was achieved for compounds **11a–f**. The (*N,N*-dimethylamino)ethylamino analogue **12b** was as potent as **10b**; however, its HCR was considerably lower (HCR = 100). The 3-(morpholinylpropyl)amino isomers **13b.1,b.2** were individually tested showing high selectivity (HCR for the major isomer = 300) but less potency than **10b**. Replacement of the chloro atom of **6b** by 2-aminoethanol afforded the poorly selective **14b**. The 3-(imidazolylpropyl)amino compound **15b** showed similar features as **14b**. Finally we prepared the dimer **16b**, which demonstrated a good potency but virtually complete lack of selectivity.

Conclusion

In an attempt to develop new hypoxia-selective cytotoxins, the structure–activity relationships for quinoxaline 1,4-di-*N*-oxides have been studied. We found that one new compound, **5k**, which contains no substituent at the 3 position of the aryl ring, was 150-fold more toxic to hypoxic cells *in vitro* than tirapazamine.[16] Also, **5k** showed a better profile than its 3-amino analogue **1k**, demonstrating that the amino group is not necessary for activity. Two promising compounds, bearing a [(dimethylamino)propyl]amino basic lateral chain, **10b,10f**, were developed. They are potent, selective, and water-soluble toxins. Some attempts to modulate the **10b** structure were tried, but minor changes to the lateral chain affected the activity, sometimes detrimentally, e.g., two methylene groups between the nitrogens or dimerization.

In summary, we conclude that several quinoxalines have a good *in vitro* profile, such as **5k** or **10b,f**, and they might have useful *in vivo* properties, which will be reported elsewhere.

Table 2. Results of Dose–Response Assays of 3-[[(*N*,*N*-Dialkylamino)alkyl]amino]-2-quinoxalinecarbonitrile 1,4-Di-*N*-oxides **10–16**



compd	R_3	R_6	R_7	formula	P[a]	HCR[b]
10a	$NH(CH_2)_3NMe_2$	H	H	$C_{14}H_{17}N_5O_2 \cdot HCl$	1	300
10b	$NH(CH_2)_3NMe_2$	H	Cl	$C_{14}H_{16}ClN_5O_2 \cdot HCl$	0.4	250
10d	$NH(CH_2)_3NMe_2$	H	F	$C_{14}H_{16}FN_5O_2 \cdot HCl \cdot H_2O$[c]	0.6	170
10f	$NH(CH_2)_3NMe_2$	H	CF_3	$C_{15}H_{16}F_3N_5O_2 \cdot HCl \cdot H_2O$[d]	0.3	340
10g	$NH(CH_2)_3NMe_2$	H	CH_3	$C_{15}H_{19}N_5O_2 \cdot HCl$	1	300
10i	$NH(CH_2)_3NMe_2$	H	OCH_3	$C_{15}H_{19}N_5O_3 \cdot 2HCl \cdot 1.5H_2O$	2	200
11a	$NH(CH_2)_2NEt_2$	H	H	$C_{15}H_{19}N_5O_2 \cdot HCl \cdot 0.25H_2O$	0.9	120
11b	$NH(CH_2)_2NEt_2$	H	Cl	$C_{15}H_{18}ClN_5O_2 \cdot HCl \cdot 0.5H_2O$	0.5	40
11d	$NH(CH_2)_2NEt_2$	H	F	$C_{15}H_{18}FN_5O_2 \cdot HCl \cdot 0.6H_2O$	0.4	75
11f	$NH(CH_2)_2NEt_2$	H	CF_3	$C_{16}H_{18}F_3N_5O_2 \cdot HCl$	0.6	50
11g	$NH(CH_2)_2NEt_2$	H	CH_3	$C_{16}H_{21}N_5O_2 \cdot HCl$	3	100
11i	$NH(CH_2)_2NEt_2$	H	OCH_3	$C_{16}H_{21}N_5O_3 \cdot HCl \cdot 0.5H_2O$	1	100
12b	$NH(CH_2)_2NMe_2$	H	Cl	$C_{13}H_{14}ClN_5O_2 \cdot HCl \cdot 0.25H_2O$	0.3	100
13b.1	$NH(CH_2)_3$-morph	Cl	H	$C_{16}H_{18}ClN_5O_3 \cdot HCl$	0.8	>125
13b.2	$NH(CH_2)_3$-morph	H	Cl	$C_{16}H_{18}ClN_5O_3 \cdot HCl$	2	300
14b	$NHCH_2CH_2OH$	H	Cl	$C_{11}H_9N_4O_3$	4	75
15b	$NH(CH_2)_3$-imid	H	Cl	$C_{15}H_{13}ClN_6O_2 \cdot HCl \cdot 0.5H_2O$	2	>50
16b	$[NH(CH_2)_3]_2NCH_3$	H	Cl	$C_{25}H_{23}Cl_2N_9O_4 \cdot HCl$	0.7	10

[a] Potency = dose in micromolar which gives 1% of control cell survival in hypoxia. [b] Hypoxic cytotoxicity ratio = the dose in air divided by the dose in hypoxia giving 1% of control cell survival. [c] N: calcd, 19.47; found, 19.05. [d] N: calcd, 17.48; found, 17.02.

Experimental Section

General. Melting points were determined using a Mettler FP82+FP80 apparatus and are uncorrected. Elemental analyses were obtained from vacuum-dried samples (over phosphorus pentoxide at 3–4 mmHg, 24 h, at ca. 80–100 °C). Infrared spectra were recorded on a Perkin-Elmer 681 apparatus, using potassium bromide tablets for solid products and sodium chloride plates for liquid products; the frequencies are expressed in cm^{-1}. The ^1H NMR spectra were obtained on a Brucker AC-200E (200 MHz) instrument, with tetramethylsilane as the internal reference, at a concentration of ca. 0.1 g/mL and with dimethyl sulfoxide-d_6 (DMSO-d_6) as the solvent; the chemical shifts are reported in parts per million (ppm) of tetramethylsilane in δ units, and the J values are given in hertz (Hz). The mass spectra were recorded on a Hewlett-Packard 5988-A instrument at 70 eV.

Thin-layer chromatography (TLC) was carried out on silica gel (DSF-5, Cammaga 0.3 mm thickness) with the indicated solvents, and the plates were scanned under ultraviolet light at 254 and 366 nm. Column chromatography was carried out with Merck silica gel 60 (70–230 mesh ASTM). Elemental analyses were performed on a Carlo-Erba 1106 Instrumentazione and are within ±0.4% of the calculated values except where otherwise stated. 3-Aminobenzo-1,2,4-triazine 1,4-dioxide (tirapazamine) and 6- and/or 7-substituted 3-amino-2-quinoxalinecarbonitrile 1,4-di-*N*-oxides **1a–j** were prepared as reported.[14,24]

3-[(Methylsulfonyl)amino]-2-quinoxalinecarbonitrile 1,4-Di-*N*-oxide (2a). A mixture of **1a** (1.00 g, 4.90 mmol), methanesulfonyl chloride (0.59 g, 5.15 mmol), sodium bicarbonate (1.00 g, 12.00 mmol), and dry dioxane (20 mL) was stirred at 20 °C for 48 h. The crude solid was collected, washed with diethyl ether, and recrystallized from dioxane: 39% yield; mp 256 °C; IR (KBr) 3413–3325, 2228, 1360 cm^{-1}; ^1H NMR (DMSO-d_6) δ 3.49 (s, 3 H, CH_3), 7.38–7.43 (m, 1 H, H_6), 7.70–7.74 (m, 3 H, NH, H_5, H_7), 8.10–8.14 (d, 1 H, H_8). Anal. ($C_{10}H_8N_4O_4S$) C, H, N.

3-Acetamido-2-quinoxalinecarbonitrile 1,4-Di-*N*-oxide (3a). A mixture of **1a** (1.10 g, 5.44 mmol) and acetic anhydride (30 mL) was heated until complete dissolution. The reaction mixture was allowed to cool, and the resulting yellow crystals were collected and washed with methanol (80% yield). A sample was recrystallized from dioxane: mp 208 °C; IR (KBr) 3257, 1713, 1332 cm^{-1}; ^1H NMR (DMSO-d_6) δ 2.28 (s, 3 H, CH_3), 7.97–8.50 (m, 4 H, H_5, H_6, H_7, H_8), 11.28 (s, 1 H, NH). Anal. ($C_{11}H_8N_4O_3$) C, H, N.

3-(Methylamino)-2-quinoxalinecarbonitrile 1,4-Di-*N*-oxide (4a). A mixture of **1a** (2.03 g, 10.05 mmol), dimethyl sulfate (1.40 g, 11.11 mmol), $NaHCO_3$ (1.00 g, 11.90 mmol), and dry dioxane (25 mL) was stirred and heated at 90 °C for 4 h. After cooling a brown solid was obtained. Recrystallization from dioxane afforded orange crystals (34% yield): mp 208 °C; IR (KBr) 3350, 1352 cm^{-1}; ^1H NMR (DMSO-d_6) δ 3.36 (s, 3 H, CH_3), 7.83 (t, J = 7.6 Hz, 1 H, H_6), 8.07–8.23 (m, 2 H, H_5, H_7), 8.39 (d, J = 8.4 Hz, 1 H, H_8). Anal. ($C_{10}H_8N_4O_2$) C, H, N.

General Procedure for the Preparation of the 7-Substituted 2-Quinoxalinecarbonitrile 1,4-Di-*N*-oxides 5a–k. A mixture of the 6- and/or 7- substituted 3-amino-2-quinoxalinecarbonitrile 1,4-di-*N*-oxide **1** (11.00 mmol) and DMF (60 mL) was stirred and heated at 65 °C under nitrogen. *tert*-Butyl nitrite (4 mL) was added, and effervescence was observed. The complete mixture was stirred and heated at 65 °C for 10 min, and additional *tert*-butyl nitrite (3 mL) was added. The reaction mixture was heated at 70 °C for 2 h and then allowed to cool. After removal of the solvent under reduced pressure, the impure oil was purified by flash chromatography eluting with toluene/EtOAc. Final recrystallization from EtOAc afforded the corresponding **5a–k** (yields between 8% and 26%) as bright yellow crystals. ^1H NMR studies indicated the presence of a single isomer, the major 7-isomer, while the minor 6-isomer was discarded.[14]

General Procedure for Preparation of 7-Substituted 3-Chloro-2-quinoxalinecarbonitrile 1,4-Di-*N*-oxides 6a–k. A suspension of $CuCl_2$ (50.0 mmol) in acetonitrile (100 mL) was stirred and heated under a nitrogen atmosphere at 70 °C for 30 min. The appropriate 3-amino-2-quinoxalinecarbonitrile 1,4-di-N-oxide **1a–k** (25.0 mmol) and *tert*-butyl nitrite (2 mL) were added, and the reaction mixture was stirred and heated at 70 °C for 15 min. Further addition of *tert*-butyl nitrite (5 × 2 mL) was necessary to complete the reaction. After stirring and heating for 2 h, the mixture was allowed to cool. Inorganic salts were filtered off and, after removal of the solvent and the excess of *tert*-butyl nitrite, a dark oil was obtained. Flash chromatography was carried out eluting with a gradient of toluene/EtOAc. A yellow solid was obtained, and final recrystallization from EtOAc afforded bright yellow crystals (yields between 2% and 21%). **6d** was obtained as a mixture of

isomers and both of them were conveniently separated and identified (see the supporting information).

3-Methyl-2-quinoxalinecarbonitrile 1,4-Di-*N*-oxide (7a). A solution of 5-methylisoxazole (0.64 g, 7.70 mmol), potassium hydroxide (0.27 g, 4.80 mmol), and methanol (3 mL) was added to a mixture of benzofuroxan (1.20 g, 8.80 mmol), ammonium chloride (0.47 g, 8.80 mmol) and methanol (2 mL). Dry ammonia gas was bubbled into the mixture for 15 min. After stirring at room temperature for 15 h, a precipitate was obtained. Recrystallization from methanol/chloroform afforded a yellow solid (85% yield): mp 176 °C; IR (KBr) 3093, 2240, 1378 cm^{-1}; ^1H NMR (DMSO-d_6) δ 2.62 (s, 3 H, CH_3), 8.00–8.11 (m, 2 H, H_6, H_7), 8.42–8.51 (m, 2 H, H_5, H_8). Anal. ($C_{10}H_7N_3O_2$) C, H, N.

Quinoxaline 1,4-Di-*N*-oxide (8a). Benzofuroxan (4.08 g, 30.00 mmol) and diethylamine (2.19 g, 30.00 mmol) were dissolved in ethyl acetate (15 mL). The mixture was stirred at 0 °C while a solution of vinyl acetate (5.16 g, 60.00 mmol) in ethyl acetate (5 mL) was added dropwise. The mixture was stirred at 0 °C for 3 h. After stirring at room temperature for an additional 72 h and removal of the solvent, a crude solid was obtained. Recrystallization from 2-propanol afforded a yellow solid (79% yield): mp 241 °C; IR (KBr) 3096, 1372 cm^{-1}; ^1H NMR (DMSO-d_6) δ 7.80–7.95 (m, 2 H, H_6, H_7), 8.42–8.47 (m, 2 H, H_5, H_8), 8.53 (s, 2 H, H_2, H_3). Anal. ($C_8H_6N_2O_2$) C, H, N.

2,3-Quinoxalinedicarbonitrile 1,4-Di-*N*-oxide (9a). A mixture of quinoxaline 1,4-di-*N*-oxide (**8a**; 1.00 g, 6.20 mmol), potassium ferricyanide (2.44 g, 7.40 mmol), potassium cyanide (2.44 g, 30.10 mmol), ethanol (70 mL), and water (30 mL) was stirred at 0 °C for 3 h. The precipitated crude solid was purified by flash chromatography eluting with ethyl acetate. A yellow solid was obtained (18% yield): mp 224 °C; IR (KBr) 2236, 1365 cm^{-1}; ^1H NMR (DMSO-d_6) δ 8.24–8.29 (m, 2 H, H_6, H_7), 8.59–8.63 (m, 2 H, H_5, H_8). Anal. ($C_{10}H_4N_4O_2$) C, H, N.

General Procedure for Preparation of 6- and/or 7-Substituted 3-[[(*N*,*N*-Dialkylamino)alkyl]amino]-2-quinoxalinecarbonitrile 1,4-Di-*N*-oxide Hydrochlorides 10–16. A mixture of the corresponding 3-chloro-2-quinoxalinecarbonitrile 1,4-di-*N*-oxide **6** (0.68 mmol), potassium carbonate (0.68 mmol), and dichloromethane (200 mL) was stirred while the amine (0.70 mmol) was added dropwise. The resulting mixture was stirred for 3 days at room temperature and monitored by TLC. Inorganic salts were filtered off and, after removal of the solvent, the residue was chromatographed by eluting with a gradient of dichloromethane/ethyl acetate/methanol. The red solid thus obtained was dissolved in dry acetone. Addition of 3–5 drops of concentrated HCl gave a red precipitate which was recrystallized from methanol/acetone. **13b** was obtained as a mixture of isomers (**13b.1**,**b.2**), which were conveniently separated by flash chromatography and individually tested (Table 2).

Biology: Cells. V79 cells (Chinese hamster lung fibroblasts) were obtained from ECACC (European Collection of Animal Cell Cultures) and maintained in logarithmic growth as subconfluent monolayers by trypsinization and subculture to (1–2) × 10^4 cells/cm^2 twice weekly. The growth medium was EMEM (Eagle's Minimal Essential Medium), containing 10% (v/v) fetal bovine serum (FBS) and penicillin/streptomycin at 100 U/100 μg/mL.

Aerobic and Hypoxic Cytotoxicity: Suspension Cultures. Monolayers of V79 cells in exponential growth were trypsinized, and suspension cultures were set up in 50 mL glass flasks: 2 × 10^4 cells/mL in 30 mL of EMEM containing 10% (v/v) FBS and HEPES (10 mM). The glass flasks were stoppered with rubber caps perforated with two 19 G needles to provide gas inlet and outlet. The flasks were submerged and stirred in a water bath at 37 °C, where they were gassed with humidified air or pure nitrogen.

Treatment. Compound solutions were prepared just before dosing. Stock solutions, 150-fold more concentrated, were prepared in pure dimethyl sulfoxide (DMSO) or sterilized distilled water. Thirty minutes after the start of gassing, 0.2 mL of the stock compound solution was added to each flask, two flasks per dose. In every assay there was one flask with 0.2 mL of DMSO (negative control) and another with 0.06 μM nitracrine (positive control).

Cloning. After 2 h exposure to the compound, the cells were centrifuged and resuspended in plating medium (EMEM plus 15% (v/v) FBS and penicillin/streptomycin). Cell numbers were determined with a hemocytometer and 10^2–10^3 cells were plated in 6-well plates to give a final volume of 2 mL/30 mm of well. Plates were incubated at 37 °C in 5% CO_2 for 7 days and then stained with aqueous crystal violet. Colonies with more than 64 cells were counted. The plating efficiency (PE) was calculated by dividing the number of colonies by the number of cells seeded. The percent of control cell survival for the compound-treated cultures was calculated as $PE_{treated}/PE_{control} \times 100$.

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Supporting Information Available: Yields, melting points, and IR (KBr), ^1H NMR (DMSO-d_6), and MS (EI, 70 eV) data (11 pages). Ordering information is given on any current masthead page.

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