Hypoxia-Selective Agents Derived from Quinoxaline 1,4-Di-*N*-oxides

Antonio Monge,*,† Juan A. Palop,† Adela López de Ceráin,‡ Virginia Senador,† Francisco J. Martínez-Crespo,† Yolanda Sainz,† Susana Narro,† Estrella García,‡ Carlos de Miguel,§ Mercedes González,† Elizabeth Hamilton,∥ Andy J. Barker,∥ Eric D. Clarke,⊥ and Daren T. Greenhow⊥

CIFA, Department of Medicinal Chemistry, Department of Toxicology, Department of Biochemistry, Universidad de Navarra, 31080, Pamplona, Spain, Zeneca Pharmaceuticals, Mereside, Alderley Park, Macclesfield, Cheshire SK10 4TG, U.K., Zeneca Agrochemicals, Jealott's Hill Research Station, Bracknell, Berks, RG12 6EY, U.K.

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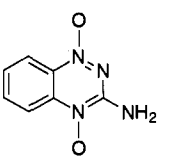
Hypoxic cells, which are a common feature of solid tumors, but not normal tissues, are resistant to both anticancer drugs and radiation therapy. Thus the identification of drugs with selective toxicity toward hypoxic cells is an important objective in anticancer chemotherapy. The benzotriazine di-*N*-oxide (SR 4233, Tirapazamine) has been shown to be an efficient and selective cytotoxin for hypoxic cells. Since the bioreductive activation of Tirapazamine is thought to be due to the presence of the 1,4-di-*N*-oxide moiety, a series of 3-aminoquinoxaline-2-carbonitrile 1,4-di-*N*-oxides with a range of electron-donating and -withdrawing substituents in the 6- and/or 7- positions has been synthesized and evaluated for toxicity to hypoxic cells. Electrochemical studies of the quinoxaline di-*N*-oxides and Tirapazamine showed that as the electron-withdrawing nature of the 6(7)-substituent increases, the reduction potential becomes more positive and the compound is more readily reduced. Apart from the unsubstituted **6a** and the 6,7-dimethyl derivative **6c**, the quinoxaline di-*N*-oxides have reduction potentials significantly more positive than Tirapazamine (E_{pc} −0.90 V). The most potent cytotoxins to cells in culture were the 6,7-dichloro and 6,7-difluoro derivatives **6i** and **6l**, which were 30-fold more potent than Tirapazamine. The 6(7)-fluoro and 6(7)-chloro compounds, **6e** and **6h**, showed the greatest hypoxia selectivity. Four of the compounds, **6e**, **6f**, **6h** and **6i**, killed the inner cells of multicellular tumor spheroids *in vitro*. *In vivo* Balb/c mice tolerated a dose of these four compounds twice the size of that of Tirapazamine. This study demonstrates that quinoxaline 1,4-di-*N*-oxides could provide useful hypoxia-selective therapeutic agents.

Introduction

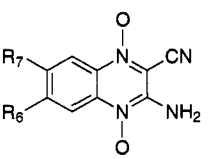
The oxygenation status of clonogenic cells in solid tumors has long been believed to be one of the major factors adversely affecting tumor response to radiotherapy.[1,2] There is now considerable evidence for the presence of hypoxia in human tumors, and this has been shown to influence the outcome of treatment for several types of malignancies.[2–5] Preclinical studies suggest that hypoxic cells may also be refractory to certain chemotherapeutic drugs.[6–8]

Differential drug toxicity toward hypoxic cells was first observed *in vitro* with metronidazole[8] and was confirmed with various nitro heterocycles in other cell lines.[9] The concept of bioreductive activation of drugs in hypoxic cells to produce a more toxic compound has been extensively reviewed, and three general classes of such agents are now known.[7,10,11] First are the quinone antibiotics of which mitomycin C is the prototype drug.[12] Second are the nitroimidazoles which were initially developed as radiation sensitizers, but also show preferential toxicity to hypoxic cells as dual function agents, e.g., RSU 1069.[13,14] The third class of bioreductive cytotoxins are the benzotriazine di-*N*-oxides of which Tirapazamine (3-amino-1,2,4-benzotriazine 1,4-di-*N*-oxide, WIN 59075, SR 4233) is the prototype compound.[15] Tirapazamine is the first drug to be introduced into the clinic purely as a bioreductive cytotoxic agent.[16]

Several groups have investigated the mechanism for the selective hypoxic toxicity of Tirapazamine and have found that the toxic species is an oxidizing radical which can be back-oxidized to the parent drug in the presence of oxygen.[17,18] The importance of the *N*-oxide group for the selective activity of Tirapazamine suggested to us the possibility of designing new heterocyclic *N*,*N*-dioxides and exploring their activity in hypoxic cells. We report here the preparation of compounds derived from the quinoxaline 1,4-di-*N*-oxides and their biological characterisation. It has also been suggested that the more negative the reduction potential the greater the hypoxia selectivity, to the point at which enzymes can no longer reduce the compound.[19] We have included electrochemical data to demonstrate whether the reduction potentials can be related to oxic/hypoxic selectivity.



SR 4233



QUINOXALINE 1, 4-DI-*N*-OXIDE

Chemistry

The acetanilides **1** were prepared (Scheme 1) by acylation of the appropiate aromatic amines with acetic anhydride in acetic acid. Attempts to nitrate the acetanilides at 0–25 °C with 60% nitric acid and concentrated sulfuric acid afforded the 2-nitro derivatives **2**. Nitration of 4-phenylacetanilide yielded 4-(nitrophenyl)-2-nitroacetanilide **2q**. Hydrolysis of **2** in

† Department of Medicinal Chemistry.
‡ Department of Toxicology.
§ Department of Biochemistry.
∥ Zeneca Pharmaceuticals.
⊥ Zeneca Agrochemicals.
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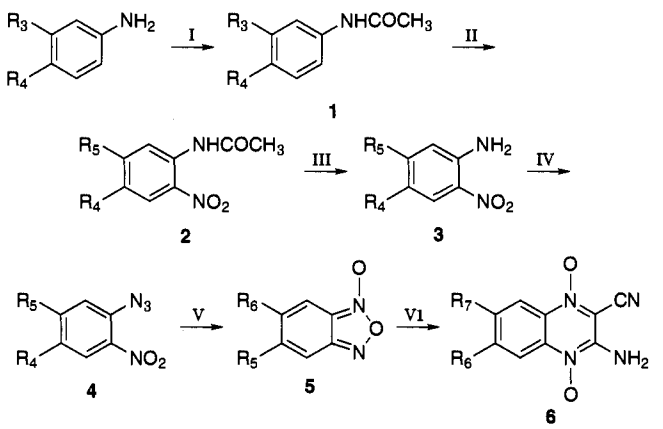
Scheme 1[a]



[a] (i) Ac_2O/AcOH/reflux/15 min; (ii) 60% HNO_3, H_2SO_4/0−20 °C/30 min; (iii) concentrated H_2SO_4/100 °C/15 min; (iv) concentrated HCl, H_2O/$NaNO_2$, H_2O; NaN_3/sodium acetate/H_2O; (v) toluene, reflux/2 h; (vi) $CH_2(CN)_2$/DMF/Et_3N/0−20 °C/24 h.

concentrated sulfuric acid gave the substituted 2-nitroanilines **3** in good yields. Diazotation of anilines **3** and subsequent treatment with sodium azide afforded the reactive 2-nitrophenyl azides **4**.[20] Cyclocondensation of the azides **4** in boiling toluene yielded the benzofuroxanes **5**. Beirut reaction[20,21] of substituted benzofuroxanes **5** with malononitrile in the presence of triethylamine as condensing base[22−24] at low temperatures (0−10 °C) gave the final 3-amino-2-quinoxalinecarbonitrile 1,4-di-*N*-oxides **6** in variable yields (8−75%). Theoretically two isomers are formed by Beirut reaction (6- and 7-substituted isomers), but mainly one of them is obtained. Technically, the workup and purification of the mixture afford only one isomer, probably the 7-isomer, while the minor isomer is discarded. However, compounds **6b**,**f**−**g** and **h** are obtained, after purification, as a mixture of both isomers (Table 1). In principle, product mixtures can be formed from unsymmetrically-substituted benzofuroxanes, although in practice considerable regiospecificity is often achieved. For example in the reaction of 5-substituted benzofuroxanes with benzoylacetonitrile, only 7-substituted quinoxaline 1,4-di-*N*-oxides are formed.[25] Separation of **6h** isomers was carried out by flash chromatography by eluting with ethyl acetate (7-chloro isomer) and TDA (6-chloro isomer). Both pure isomers were individually tested, demostrating equal activity. The 6(7)-amino derivative **6o** was prepared by hydrolysis of the acetamido group **6n**. Nitration of 6-methoxy compound **6d** with 60% nitric acid at room temperature for 10 h afforded the 7-methoxy-8-nitro compound **6p**. The (ethoxycarbonyl)quinoxaline **6r** was prepared by esterification of 5-(chloroformyl)benzofuroxane followed by Beirut reaction. Beirut reaction from carbonyl- or carboxybenzofuroxanes were unsuccessful or gave very low yields.

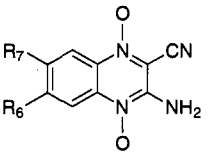
Electrochemical Studies

In DMF at a platinum working electrode the voltammetric response of 3-amino-2-quinoxalinecarbonitrile 1,4-di-*N*-oxides was complex. Tipically four to six reduction peaks were observed during forward cathodic scan, some of which may be linked to oxidation peaks on the reverse anodic scan. For example compound **6f** had cathodic peak potentials, E_{pc}, at −0.65, −0.73, −1.02, −1.44, and −1.58 V, with anodic peak potentials, E_{pa}, at −1.38, −1.11, −0.85, −0.70, and −0.58 V. Only the first reduction step was studied as a function of voltage sweep rate and switching potential.

Table 2 summarizes E_{pc} for the first reduction step of each quinoxaline di-*N*-oxide, plus Tirapazamine and anthraquinone-2-sulfonate. This potential was generally observed to be independent of voltage sweep rate and the potential for switching from cathodic to anodic scan. In most cases an anodic peak potential, E_{pa}, approximately 60 mV more positive than the first cathodic peak could be assigned. Data for **6f** are E_{pc} −0.65V, E_{pa} −0.58V, $E_{pa} - E_{pc}$= 0.07 V, at sweep rates of 0.02, 0.1, and 0.5 V/s. Given the complex nature of the voltammograms, anodic to cathodic peak current ratios (i_{pa}/i_{pc}) were not readily determined; however, the quinoxaline di-*N*-oxides appeared to have i_{pa}/i_{pc} ratios <0.5.

Tirapazamine gave E_{pc} −0.90 V, E_{pa} −0.83 V, $E_{pa} - E_{pc}$ = 0.07 V, and $i_{pa}/i_{pc} \approx 0.5$. Data for anthraquinone-2-sulfonate, AQS under these experimental conditions

Table 1. Preparation of 3-Amino-2-quinoxalinecarbonitrile 1,4-Di-*N*-oxides **6**



compd	R_6, R_7	yield, %	mp, °C	formula (C, H, N)
6a	H, H	75	190	$C_9H_6N_4O_2$
6b[a]	CH_3, H	52	245−247	$C_{10}H_8N_4O_2$
6c	CH_3, CH_3	28	>300	$C_{11}H_{10}N_4O_2$
6d	CH_3O, H	40	248−249	$C_{10}H_8N_4O_3$
6e	F, H	61	>300	$C_9H_5FN_4O_2$
6f[a]	CF_3, H	55	259−260	$C_{10}H_5F_3N_4O_2$
6g[a]	CF_3O, H	29	245−248	$C_{10}H_5F_3N_4O_3$
6h[a]	Cl, H	72	263−264	$C_9H_5ClN_4O_2$
6i	Cl, Cl	48	265	$C_9H_4Cl_2N_4O_2$
6j	CN, H	11	>300	$C_{10}H_5N_5O_2$[b]
6k	CH_3O, Cl	25	248 dec	$C_{10}H_7ClN_4O_3$
6l	F, F	63	>300	$C_9H_4F_2N_4O_2$
6m	Cl, F	11	>300	$C_9H_4ClFN_4O_2$
6n	CH_3CONH, H	10	269 dec	$C_{11}H_9N_5O_3$[c]
6o	NH_2, H	65	>300	$C_9H_7N_5O_2 \cdot HCl$
6p	CH_3O, 8-NO_2	18	270 dec	$C_{10}H_7N_5O_5$
6q	*p*-NO_2Ph, H	22	225 dec	$C_{15}H_9N_5O_4 \cdot 0.5H_2O$
6r	EtO_2C, H	8	178	$C_{12}H_{10}N_4O_4$

[a] Mixture of isomers. [b] N: calcd, 30.84; found, 30.36. [c] C: calcd, 50.96; found, 50.55.

Table 2. Peak Potential Data[a] for the First Reduction Step of Quinoxaline 1,4-Di-*N*-oxides, Tirapazamine, and Anthraquinone-2-sulfonate

compd	R_7	R_6	E_{pc}, V vs SCE
6a	H	H	−0.88
6c	CH_3	CH_3	−0.97
6e	F	H	−0.75
6f	CF_3	H	−0.65
6g	CF_3O	H	−0.73
6h	Cl	H	−0.74
6i	Cl	Cl	−0.62
6j	CN	H	−0.56
6l	F	F	−0.75
6m	Cl	F	−0.68
SR 4233	–	–	−0.90
AQS	–	–	−0.83

[a] Peak potentials (~±0.01 V) measured at a scan rate of 0.1 V/s.

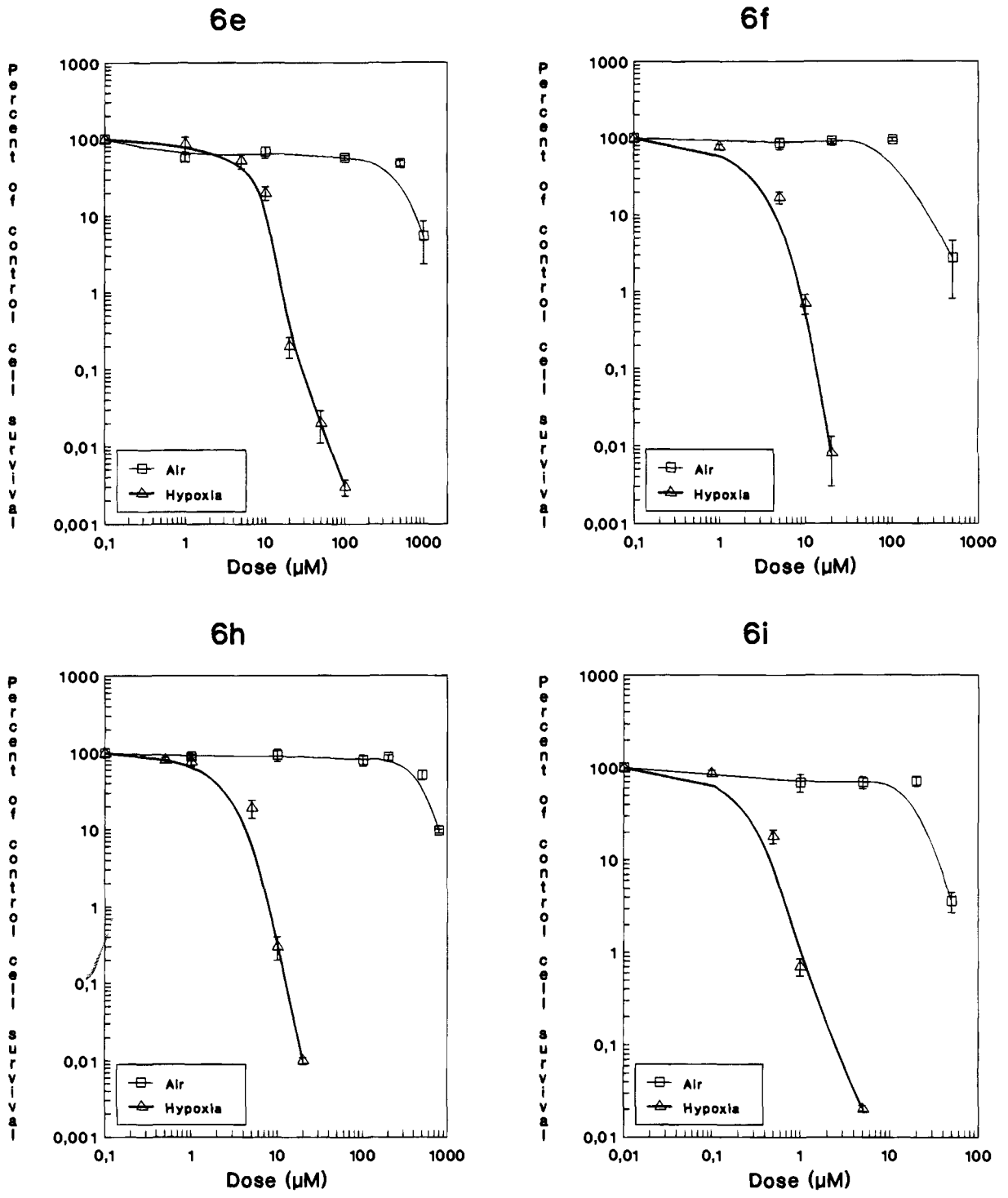


Figure 1. Dose–response in V-79 cells for 2 h treatment in air or hypoxia with **6e**, **6f**, **6h**, or **6i**. Each point represents the mean ± SD of at least three experiments.

were E_{pc} −0.83 V, E_{pa} −0.75 V, $E_{pa} - E_{pc}$ = 0.08 V and $i_{pa}/i_{pc} \approx 1$, consistent with reversible one electron reduction to its semiquinone.

Biological Studies

The compounds were tested for cytotoxicity to V-79 cells in a cloning assay, at a single dose, in air or hypoxia for 2 h. Results are presented in Table 3. At 20 μM all the compounds, except **6g**, were more toxic in hypoxia than in air. Compounds which gave a low surviving fraction in hypoxia were tested at different doses in air and hypoxia in at least three experiments to obtain dose–response curves. Graphs for **6e**,**f**,**h**,**i** are presented in Figure 1. Data for potency (the dose which gives 1% cell survival in hypoxia) and the hypoxic cytotoxicity ratio (HCR), the ratio between doses in air and in hypoxia giving the same cell survival, were calculated from these graphs and are shown in Table 3.

The hypoxic potency of the quinoxaline 1,4-di-*N*-oxides ranged between 1 and 30 μM and the HCR between 10 and 150. The reference compound Tirapazamine had a hypoxic potency of 30 μM and HCR of 75, under the same conditions. Although 11 quinoxaline

Table 3. Data of Potency and Hypoxic Selectivity of the Quinoxaline Derivatives

compd	potency[a]	HCR[b]
6a	30	80
6b	NT[c]	NT
6c	NT	NT
6d	30	>10
6e	15	100
6f	7	75
6g	NT	NT
6h	9	150
6i	1	80
6j	11	50
6k	10	<10
6l	1	<10
6m	2	10
6n	NT	NT
6o	NT	NT
6p	4	<10
6q	4	15
6r	NT	NT
SR-4233	30	75

[a] Potency: concentration in micromolar that gives 1% clonogenic cell survival in hypoxia. [b] HCR (hypoxic cytotoxicity ratio): dose in air/dose in hypoxia giving 99% of cell killing. [c] NT: not tested; 1% cell survival was not reached in the screening assay.

1,4-di-*N*-oxides were more potent than Tirapazamine, only five maintained or improved on its HCR selectivity: **6a,e,f,h,i** (Table 3).

In order to evaluate the ability of the quinoxaline 1,4-di-*N*-oxides to penetrate to the hypoxic cells in solid tumors, they were assayed in a multicellular tumor spheroid model *in vitro*. EMT-6 spheroids of 700–800 μm diameter were in air treated with the compounds for 2 h. They were trypsinized, to separate outer cell layers from the inner cells, and these two populations were cloned separately. The number of cells in each subpopulation was approximately the same (50% of inner cells and 50% of outer cells). Figure 2 shows the percentage of control plating efficiency for outer and inner cells of the treated spheroids. 100 μM Tirapazamine killed 80–90% of both outer and inner cells. At the same dose, compounds **6e,f,h** killed more inner than outer cells and gave 50–60% cell death. Compound **6i** was not toxic to either inner or outer cells and Doxorubicin, which was used as a positive control, killed more outer than inner cells, as was expected.[26]

In vivo toxicity of the selected quinoxaline 1,4-di-*N*-oxides was also evaluated after a single ip administration of the compounds. Results are presented in Table 4. Under our conditions, BALB/c mice tolerated a dose of quinoxaline 1,4-di-*N*-oxide at least twice that of Tirapazamine.

Results and Discussion

Many quinoxaline derivatives with different biological activities (bactericide, fungicide, herbicide, anti-HIV, etc.) have been described.[27-29] Some reduced quinoxalines have been patented as anticancer agents, e.g. 2,3,7-trichloro-6-[(methylamino)sulfonyl]quinoxaline[30] or chloroquinoxalinesulfonamide which is now in phase I clinical trial.[31] Others have been shown to increase the activity of antitumor agents, such as 1-(2-(diethylamino)ethyl)-3-(*p*-methoxybenzyl)-1,2-dihydroquinoxalin-2-one and their salts or derivatives of quinoxaline 1,4-di-*N*-oxides with nitrofurane.[32] Zeman et al.[15] refer to have examined the cytotoxicity of 40 substituted quinoxaline 1,4-di-*N*-oxides under aerobic and hypoxic

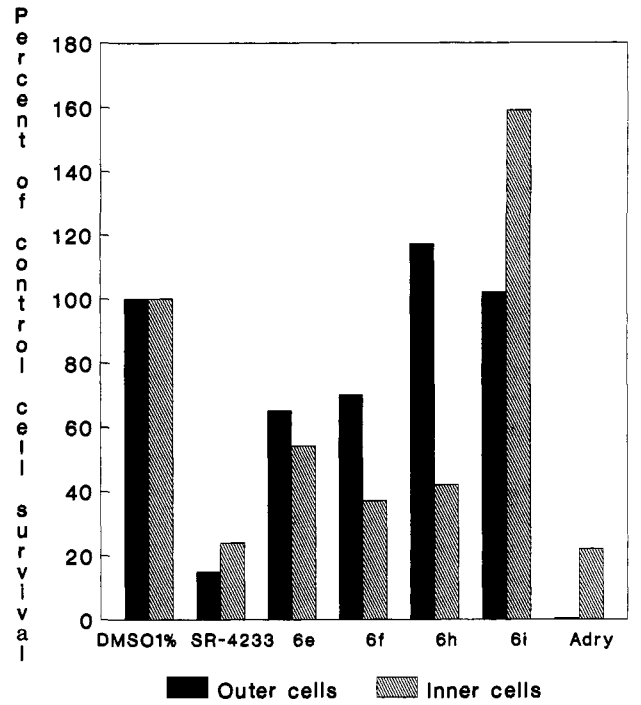


Figure 2. Percent control survival for outer and inner cells of EMT-6 spheroids treated for 2 h with 100 μM **6e**, **6f**, **6h**, or **6i**, Tirapazamine (100 μM), or adriamycin (1.5 μg/mL). Control spheroids were dosed with solvent (1% DMSO). Results of one representative experiment.

Table 4. *In Vivo* Toxicity of Quinoxaline 1,4-Di-*N*-oxides

compd	MTD,[a] mmol/kg	MTD, mg/kg
6e	0.6	176
6f	0.5	135
6h	0.6	142
6i	0.8	217
SR-4233	0.3	71

[a] MTD (maximum tolerated dose): dose giving 20% weight loss within 3 days of a single ip administration.

conditions. Although they found preferential cytotoxicity to hypoxic CHO cells in three instances (unpublished data), they did not go on with these compounds. More recently, Naylor et al.[33] have found differential hypoxic:oxic toxicities of some 8-(alkylamino)-substituted phenylimidazo[1,2-*a*]quinoxalines.

All the quinoxaline 1,4-di-*N*-oxides shown above, with the exception of **6g**, are more toxic in hypoxia, but their potency and hypoxia selectivity depends on the substituents at positions 6 and 7. In general, the compounds of the series with an electron-withdrawing substituent in position 6 are the most cytotoxic in hypoxia (**6e,f,h,j**). When two electron-withdrawing substituents in positions 6 and 7 are present, the HCR is generally lower. Compare for example compound **6e** with the corresponding **6l**. Derivatives with electrodonating substituents in positions 6 and 7 are less potent (e.g., **6b–d,o**). This is in agreement with the structure–activity relationships found for benzotriazines, possibly suggesting a similar mechanism of toxicity.[34]

The electrochemical studies show that the potential for the first reduction step of the quinoxaline 1,4-di-*N*-oxide without benzo substituents, **6a**, is similar to the benzotriazene Tirapazamine. Substituent effects in the 6 and/or 7 positions for quinoxaline and benzotriazine 1,4-di-*N*-oxides are expected to be similar. As the electron-withdrawing nature of the substituents in-

creases, the potential for the first reduction step becomes more positive and the compound more readily reduced. The E_{pc} data more closely reflects the substituent parameter σ_{meta}. The effect of introducing 6,7-disubstitution is less readily predicted. The 6,7-difluoro compound **6l** has a similar E_{pc} to its monofluoro analogue **6e** and the monochloro compound **6g**, whereas the 6,7-dichloro analogue **6i** is reduced, as expected, at a more positive potential. Introducing 6,7-disubstitution may affect radical stability; more detailed electrochemical or pulse radiolysis studies would be required to explore this issue.

No preferential toxicity to inner cells of EMT-6 spheroids in air was obtained with Tirapazamine at 100 μM. Similar data were obtained with V-79 multicellular spheroids. The 100 μM Tirapazamine was highly toxic to V-79 single cell suspensions in hypoxia but did not kill V-79 cell suspensions in air (Table 3). These data suggest that the outer cells of spheroids cultured in air are more hypoxic than single cells in aerobic suspension cultures, while the inner cells of spheroids in air are less hypoxic than single cell suspensions under nitrogen. Previous workers have found that only when spheroids are grown under nitrogen can the inner cells reduce the quinone mitomycin C.[36]

The most selective quinoxaline 1,4-di-*N*-oxide in spheroids is **6h**, which killed no outer cells and 60% of inner cells. This is consistent with the HCR data (Table 3), which shows that **6h** has the highest HCR in this series. All the quinoxaline 1,4-di-*N*-oxides were less toxic to spheroids than Tirapazamine. The reason for this reduced toxicity, which may be related to the higher MTD of the quinoxaline 1,4-di-*N*-oxides than of Tirapazamine in mice (Table 4), is under investigation.

The above results demonstrate that quinoxaline 1,4-di-*N*-oxides could provide useful hypoxia-selective therapeutic agents.

Experimental Section

Chemistry. Melting points were determined using a Mettler FP82+FP80 apparatus and are uncorrected. Elemental analyses were obtained from vacuum-dried samples (over phosphorous pentoxide at 3–4 mmHg, 24 h at about 80–100 °C). Elemental analyses were performed on a Carlo-Erba Strumentazione 1106 Analyzer. 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 ^{1}H-NMR spectra were obtained on a Brucker AC-200E (200 MHz) instrument, with tetramethylsilane as the internal reference, at a concentration of about 0.1 g/mL and with dimethyl-d_6 sulfoxide (DMSO-d_6) as the solvent; the chemical shifts are reported in ppm of tetramethylsilane in δ units. 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 = 254 and 366 nm. Column chromatography was carried out with silica gel 60 Merck (70–230 mesh ASTM).

3-Aminobenzo-1,2,4-triazine 1,4-di-*N*-oxide[22] (Tirapazamine) and 3-amino-2-quinoxalinecarbonitrile 1,4-di-*N*-oxide (**6a**) were prepared as reported.[22,24]

General Procedure for the Preparation of Acetanilides 1. Substituted aniline (55.00 mmol) was added to a stirred solution of acetic anhydride (8 mL) and acetic acid (8 mL), and the mixture was heated under reflux for 15 min. The cooled reaction was poured into ice–water. The crude precipitate was filtered off and washed with water (yields varying between 72 and 87%).

General Procedure for the Preparation of 2-Nitroacetanilides 2. Substituted acetanilide 1 (59.00 mmol) was added in portions to a cooled (−4 °C) mixture of 60% nitric acid (20 mL) and concentrated sulfuric acid (20 mL). The mixture was stirred at 0 °C for 10 min and then at room temperature for 30 min. The acidic solution was poured into ice–water, giving a precipitate which was washed with water. Sometimes recrystallization from ethanol was required (yields between 64 and 91%).

General Procedure for the Preparation of 2-Nitroanilines 3. 2-Nitroacetanilide **2** (35.00 mmol) was dissolved in concentrated sulfuric acid (30 mL), and the solution was heated at 100 °C for 15 min. The reaction mixture was cooled and poured into crushed ice, affording a crude solid which was filtered off and washed with water (yields between 71 and 93%).

General Procedure for the Preparation of 2-Nitrophenyl Azides (4). Powdered 2-nitroaniline **3** (17.00 mmol) was added to a solution of concentrated HCl (20 mL) in water (60 mL). The suspension was stirred at room temperature for 5 min. The mixture was cooled in an ice bath, and a solution of $NaNO_2$ (2.00 g, 29.00 mmol) in water (10 mL) was added dropwise. Stirring was continued at 0 °C for 15 min. After filtering, the clear solution was added over a solution of NaN_3 (2.50 g, 38 mmol) and sodium acetate (50.00 g, 0.36 mol) in water (150 mL). The crude solid was filtered off and washed with water (yields between 14 and 68%). Solid azides could not be heated at high temperatures.

General Procedure for the Preparation of Benzofuroxanes (5). A solution of the azide **4** (10 mmol) in toluene (25 mL) was added dropwise over boiling toluene (75 mL). The mixture was refluxed for 2 h. After removal of the solvent a brown-yellowish solid was obtained and identified as benzofuroxane **5** (yields between 47 and 88%).

General Procedure for the Preparation of the 3-Amino-2-quinoxalinecarbonitrile 1,4-Di-*N*-oxides (6). A mixture of benzofuroxane **5** (10 mmol) and malononitrile (10.6 mmol) was stirred for 10 min at 0 °C (ice bath). Over the cooled suspension was added a solution of triethylamine (5 drops) in *N*,*N*-DMF (3 mL). The mixture was allowed to stand at room temperature over 24 h and filtered off. The solid product was washed with diethyl ether and recrystallized from dioxane (yields between 8 and 75%).

Electrochemical Method. Voltammetric responses for quinoxaline di-*N*-oxides were obtained by cyclic voltammetry. Experiments were carried out in *N*,*N*-dimethylformamide (Aldrich, HPLC grade) with 0.1 M tetrabutylammonium bromide (Aldrich, 99%) as the supporting electrolyte and purged with nitrogen at room temperature. Typically 1–2 mg of compound was used in a cell volume of ~4 mL. A three-electrode cell configuration was used, with platinum wire working and secondary electrodes, with a saturated calomel reference electrode. Voltammograms were obtained using a HI-TEK DT2101 potentiostat, HI-TEK PPRI waveform generator, and Philips PM 8271 XY recorder. Voltage scan rates ranged from 0.02 to 0.5 V/s. Anthraquinone-2-sulfonate sodium salt, AQS (Hopkins and Williams), was used as standard.

Biology. Cells. V-79 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, containing 10% v/v fetal bovine serum (FBS) and penicillin/streptomycin 100 units/100 μg/mL.

Aerobic and Hypoxic Cytotoxicity. Suspension Cultures. Monolayers of V-79 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 19G 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 nitrogen.

Treatment. Compound solutions were prepared just before dosing. Stock solutions, 150-fold more concentrated, were prepared in pure dimethyl sulfoxide (DMSO). 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 haemocytometer and 10^2-10^3 cells were plated in six-well plates to give a final volume of 2 mL per 30 mm 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 $PE_{treated}/PE_{control} \times 100$.

Cytotoxicity in Multicellular Tumor Spheroids. Spheroids Formation. Multicellular tumor spheroids were obtained according to the methods of Sutherland and Durand[37] and Sutherland et al.[38] EMT-6 cells in exponential growth were trypsinized, counted, and seeded into a 250 mL stirrer vessel at a density of 10^5 cells/mL in 70 mL of EMEM (10% FBS). The vessel was placed on a magnetic base with a stirring speed of 30 rpm and incubated at 37 °C. Two days later more medium was added, and then the medium was replaced every third day. Spheroids were measured with a calibrated ocular micrometer on an inverted microscope. They reached the treatment size of 700–800 μm in 10–12 days.

Treatment. The spheroids were plated out in an agar-based 24-well plate (1.5% agar; 250 μL/well) with 1 mL of EMEM (10%FBS)/well; 24 h later they were dosed with compounds, diluted just before the assay was carried out. They were dissolved in pure DMSO and diluted in such a way that, upon adding 250 μL/well to the 1.25 mL of final volume, we obtained the desired concentration of compound and 1% of DMSO.

Spheroids were dosed with the test compounds for 2 h. One plate was dosed with DMSO 1% and another one with Adriamycin (1 μg/mL) (positive control).

Spheroid Trypsinization. The method was based on that of Freyer and Sutherland.[39] Spheroids were transferred to a centrifuge tube with 5 mL of PBS and allowed to settle, and the PBS was removed. Two milliliters of 0.125% trypsin solution was added, and the spheroids were transferred to the outer track of an organ culture dish and rotated at 35 rpm for 4–5 min. Trypsin action was stopped by adding 3 mL of EMEM (15% FBS), and the spheroids and single outer cells were transferred to a centrifuge tube. Once the spheroids had settled, the suspension containing single outer cells was pipetted into another tube. The remainder of the spheroids were washed with 5 mL of PBS and then trypsinized with 3 mL of Trypsin solution until single cells were obtained. The suspensions of outer and inner cell layers were then spun down, resuspended in 0.5 mL of medium, and counted. Plating, cell incubation, staining, and fixing were performed as described above.

***In Vivo* Toxicity.** The maximum tolerated dose (MTD) was determined in BALB/c female mice, 3–4 months of age. Four animals per dose group were injected intraperitoneally with a single dose of compound suspended in 5% Tween 80 in saline. The maximum tolerated dose was that which produced 20% body weight loss on the third day.

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Supplementary Material Available: IR (KBr), ^1H NMR (DMSO-d_6), and MS (EI, 70 eV) data for **6** (4 pages). Ordering information is given on any current masthead page.

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