New Hypoxia-selective Cytotoxines Derived from Quinoxaline 1,4-Dioxides

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A new series of quinoxaline 1,4-dioxides, structurally related to the benzotriazine tirapazamine **1** have been prepared starting from 5,6-dichlorobenzofuroxane **2**. The Beirut reaction between **2** and alkyl or aryl thiopropanones afforded the 2-methyl-3-alkyl(aryl)thioquinoxaline 1,4-dioxides **3a-3e**. Selective oxidation of **3** with *m*-chloroperbenzoic acid yielded the sulphinyl **4a-4c** and sulphonyl **5a-5b** derivatives. Replacement of the sulphonyl group of **5a** by chlorine or bromine gave **6** or **7**, while nucleophilic displacement by 3-(*N,N*-dimethylamino)propylamine afforded **8**. A dimer **9** was prepared from **5a** and hydrazine hydrate. The methylthio group of **3a** was replaced by using formamidine acetate giving the amino compound **10**. Bromination of the methyl group of **3b** afforded **11**, which reacted with 2-aminoethanol giving **12**. Compounds were tested as cytotoxic agents both in oxic and in hypoxic cells.

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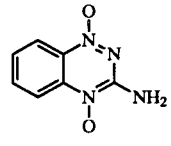
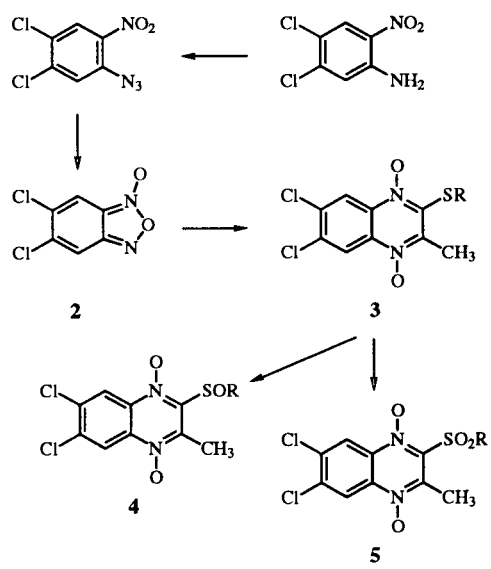
Hypoxic cells in solid tumors are an important target for cancer chemotherapy [1,2]. They are resistant to radiation and to some cytotoxic drugs, but may also be targeted specifically by drugs designed to be activated only in the absence of oxygen, providing selective bioactivation within tumor tissue. Lin and colleagues [3] 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 [4]. A compound of current interest in this regard is tirapazamine (3-amino-1,2,4-benzotriazine 1,4-dioxide) (**1**), which shows highly selective toxicity toward hypoxic cells both *in vitro* and in tumors *in vivo* [5,6]. Also, new quinoxaline 1,4-dioxides, structurally related to the benzotriazines, have demonstrated to be activated in hypoxia [7]. Herein, we report the preparation and the cytotoxicities of several new quinoxaline 1,4-dioxides, with the objective of determining the influence on the biological activity of different substituents in the quinoxaline ring.



1 Tirapazamine

As shown in Scheme 1, 5,6-dichlorobenzofuroxane **2** was prepared in two steps starting from commercial 4,5-dichloro-2-nitroaniline. Thus, the amine was diazotated and the diazonium salt formed replaced by sodium azide giving the corresponding 4,5-dichloro-2-nitrophenylazide [8]. The benzofuroxan was readily obtained by heating the azide in boiling toluene and was used without further purification. Beirut cyclocondensation between **2** and alkyl(aryl)thiopropanones in the presence of a base, such as bubbled ammonia [9] yielded 6,7-dichloro-2-methyl-3-alkyl(aryl)thioquinoxaline 1,4-dioxides **3** in variable yields (30-60%). Five new quinoxalines were prepared carrying out this method. The alkyl(aryl)thio derivatives **3** could be selectively oxidized [9] under controlled conditions to the sulphinyl analogues **4**. *m*-Chloroperbenzoic acid (MCPBA) was used as the oxidizing agent in an equimolecular concentration with respect to the thio-

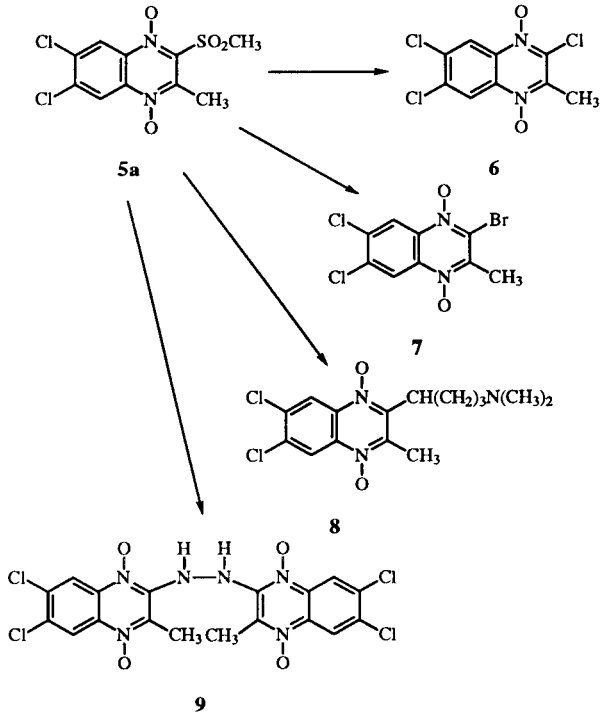
Scheme 1



quinoxaline. However, two equivalents of MCPBA were required for the synthesis of the sulphonyl derivatives **5**. This reaction was carried out in a two-phase system (aqueous phosphate buffer, *p*H = 7.5 and chloroform) and was monitored by tlc.

Nucleophilic displacement of the sulphonyl group (Scheme 2) in concentrated hydrochloric or hydrobromic acids afforded the 2-halo derivatives **6** and **7** in low yields (24 and 31%). Replacement of such a leaving group by 3-(*N,N*-dimethylamino)propylamine in dioxane/chloroform at 80° gave 6,7-dichloro-3-methyl-2-[3-(*N,N*-dimethylamino)propyl]aminoquinoxaline 1,4-dioxide **8** in 47% yield. The condensation of **5a** with hydrazine hydrate [11] in ethanol at room temperature afforded a deep red solid which was identified as the dimer **9**.

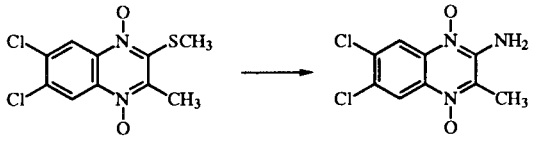
Scheme 2



5a **6** **7** **8** **9**

In Scheme 3 is shown the synthesis of 2-amino-6,7-dichloro-3-methylquinoxaline 1,4-dioxide **10** starting from the methylthio quinoxaline **3a** and formamidine

Scheme 3

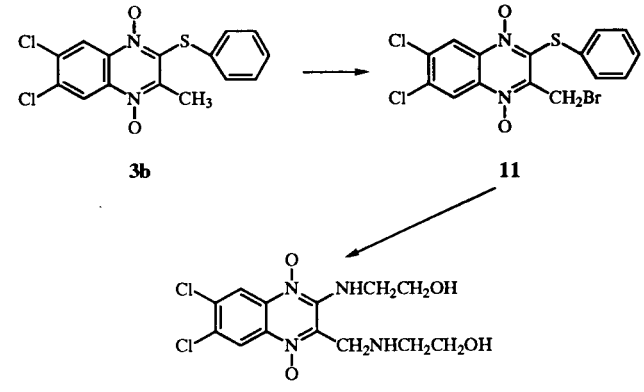


3a **10**

acetate [10] in the presence of hot ethoxyethanol (130°). Some amounts of monoreduced quinoxalines have been identified in this reaction.

6,7-Dichloro-2-methyl-6-phenylthioquinoxaline 1,4-dioxide **3b** was brominated in chloroform/carbon tetrachloride by using NBS (*N*-bromosuccinimide) and catalytic amounts of benzoyl peroxide (Scheme 4). The bromomethyl analogue **11** was treated with exccess 2-aminoethanol in chloroform. Replacement of both groups, bromine and phenylthio gave compound **12**.

Scheme 4



3b **11** **12**

Compounds were subjected to preliminary cytotoxic evaluation on V79 cells under hypoxic and aerobic conditions at 20, 5 and 1 μ*M* determining the survival fraction (SF). The most interesting compounds were: **3d** (tested at 1 μ*M*, $SF_{hypoxia}$: 2, SF_{air}: 17), **4c** (tested at 1 μ*M*, $SF_{hypoxia}$: 4, SF_{air}: 17). The halo derivatives **6** and **7** were potent (at 1 μ*M*) but not selective.

EXPERIMENTAL

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 mm Hg, 24 hours at about 80-100°). 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 Bruker AC-200E (200 MHz) instrument, with tetramethylsilane as the internal reference, at a concentration of about 0.1 g/ml and with dimethyl sulfoxide-d_6 and deuteriochloroform as solvents; 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 sol-

vents and the plates were scanned under ultraviolet light at 254 and 366 nm. Flash chromatography was carried out with silica gel 60 Merck (70-230 mesh ASTM).

4,5-Dichloro-2-nitroaniline was purchased from Aldrich, MCPBA from Merck. Tirapazamine, used as standard, was prepared as reported [12].

5,6-Dichlorobenzofuroxane (**2**).

A mixture of 4,5-dichloro-2-nitroaniline (17 mmoles), concentrated hydrochloric acid (20 ml) and water (60 ml) was stirred in a cooled system (0°) for 10 minutes. A solution of sodium nitrite (2 g) in water (10 ml) was added dropwise, and the resulting mixture was stirred at 0° for 15 minutes. The insoluble material was filtered off and the clear solution was added over a solution of sodium azide (2.50 g, 38 mmoles) and sodium acetate (50 g, 610 mmoles). The solid azide was collected and washed with water (73%). (**CAUTION:** all azides should not be heated in the solid state). This compound was used without further purification.

The azide was dissolved in toluene (170 ml) and the mixture was heated under reflux for 4 hours. After removal of the solvent a brown solid was obtained, identified as the benzofuroxan **2** (92%) and used without further purification.

Preparation of Alkyl(aryl)thiopropanones.

A cooled (0°) solution of sodium (45 mmoles) in ethanol (30 ml) and the corresponding thiol or thiophenol (45 mmoles) was vigorously stirred for 30 minutes. Chloropropanone (48 mmoles) was added and the final mixture was stirred and heated under reflux for 5 hours. After cooling the inorganic salts were filtered off. The solvent was removed and the yellow-brownish oil, identified as the thiopropanone, was used without further purification (yields from 75% to 95%).

6,7-Dichloro-2-methyl-3-methylthioquinoxaline 1,4-Dioxide (**3a**).

Dry ammonia was bubbled through a solution of 1-methylthiopropanone (1.10 g, 10.60 mmoles) and 5,6-dichlorobenzofuroxan **2** (2.30 g, 11.20 mmoles) in methanol (10 ml) at room temperature for 10 minutes. The mixture was allowed to stand at room temperature overnight. The resulting precipitate was collected and recrystallized from methanol/chloroform as yellow plates, (0.92 g, 30%), mp 184-185°; ir (potassium bromide): 3072 (CH), 1594 (C=N), 1374 (NO) cm^{-1}; ^1H nmr (deuteriochloroform): δ 2.65 (s, 3 H, CH_3), 2.82 (s, 3 H, SCH_3), 8.62 (s, 1 H, H_5), 8.66 (s, 1 H, H_8).

Anal. Calcd. for $C_{10}H_8Cl_2N_2O_2S$: C, 41.24; H, 2.75; N, 9.62. Found: C, 41.09; H, 2.77; N, 9.56.

6,7-Dichloro-2-methyl-3-phenylthioquinoxaline 1,4-Dioxide (**3b**).

The yield was 60%, mp 181-182°; ir (potassium bromide): 3095 (CH), 1600 (C=N), 1380 (NO) cm^{-1}; ^1H nmr (deuteriochloroform): δ 2.76 (s, 3 H, CH_3), 7.26 (m, 5 H, Ph), 8.55 (s, 1 H, H_5), 8.65 (s, 1 H, H_8).

Anal. Calcd. for $C_{15}H_{10}Cl_2N_2O_2S$: C, 50.99; H, 2.83; N, 7.93. Found: C, 51.00; H, 2.87; N, 7.90.

6,7-Dichloro-2-methyl-3-(4-chlorophenyl)thioquinoxaline 1,4-Dioxide (**3c**).

The yield was 40%, mp 180-181°; ir (potassium bromide): 3095 (CH), 1594 (C=N), 1379 (NO) cm^{-1}; ^1H nmr (deuteriochloroform): δ 2.82 (s, 3 H, CH_3), 7.24 (s, 4 H, Ph), 8.53 (s, 1 H, H_5), 8.65 (s, 1 H, H_8).

Anal. Calcd. for $C_{15}H_9Cl_3N_2O_2S$: C, 46.45; H, 2.32; N, 7.23. Found: C, 46.71; H, 2.34; N, 7.31.

6,7-Dichloro-2-methyl-3-(4-nitrophenyl)thioquinoxaline 1,4-Dioxide (**3d**).

The yield was 32%, mp 192°; ir (potassium bromide): 3095 (CH), 1604 (C=N), 1338 (NO) cm^{-1}; ^1H nmr (deuteriochloroform): δ 2.88 (s, 3 H, CH_3), 7.30 (d, 2 H, H_2, H_6, J = 8 Hz, Ph), 8.12 (d, 2 H, H_3, H_5, J = 8 Hz, Ph), 8.55 (s, 1 H, H_5), 8.72 (s, 1 H, H_8).

Anal. Calcd. for $C_{15}H_9Cl_2N_3O_4S$: C, 45.23; H, 2.26; N, 10.55. Found: C, 45.34; H, 2.44; N, 10.16.

6,7-Dichloro-2-methyl-3-(2-diethylaminoethyl)thioquinoxaline 1,4-Dioxide (**3e**).

The yield was 30%, mp 107-109°; ir (potassium bromide): 3091 (CH), 1591 (C=N), 1317 (NO) cm^{-1}; ^1H nmr (deuteriochloroform): δ 0.76 (t, 6 H, 2 CH_3, J = 7 Hz), 2.30-2.43 (m, 4 H, 2 CH_2), 2.61 (t, 2 H, NCH_2, J = 6 Hz), 2.73 (s, 3 H, CH_3), 3.40 (t, 2 H, CH_2S, J = 6 Hz), 8.55 (s, 1 H, H_5 or H_8), 8.56 (s, 1 H, H_8 or H_5).

Anal. Calcd. for $C_{15}H_{19}Cl_2N_3O_2S$: C, 47.87; H, 5.05; N, 11.17. Found: C, 47.63; H, 5.18; N, 11.22.

6,7-Dichloro-2-methyl-3-methylsulphinylquinoxaline 1,4-Dioxide (**4a**).

A solution of *m*-chloroperbenzoic acid (MCPBA, 1.00 g, 5.80 mmoles) in chloroform (10 ml) was added dropwise over a cooled (0°) solution of **3a** (1.50 g, 5.11 mmoles) in chloroform (10 ml). The resulting mixture was stirred at room temperature for 12 hours. After washing with aqueous sodium bicarbonate solution, the organic layer was dried over sodium sulphate. After removal of the solvent a crude solid was obtained and recrystallized from methanol/chloroform as a yellow solid (1.10 g, 70%), mp 202-203°; ir (potassium bromide): 3096 (CH), 1606 (C=N), 1370 (NO), 1028 (SO) cm^{-1}; ^1H nmr (deuteriochloroform): δ 2.90 (s, 3 H, CH_3), 3.24 (s, 3 H, $SOCH_3$), 8.46 (s, 1 H, H_8), 8.63 (s, 1 H, H_5).

Anal. Calcd. for $C_{10}H_8Cl_2N_2O_3S$: C, 39.09; H, 2.61; N, 9.12. Found: C, 39.11; H, 2.70; N, 8.92.

6,7-Dichloro-2-methyl-3-phenylsulphinylquinoxaline 1,4-Dioxide (**4b**).

The yield was 78%, mp 161-162°; ir (potassium bromide): 3099 (CH), 1610 (C=N), 1329 (NO), 1053 (SO) cm^{-1}; ^1H nmr (deuteriochloroform): δ 2.86 (s, 3 H, CH_3), 7.44 (m, 3 H, H_3, H_4, H_5 Ph), 7.95 (m, 2 H, H_2, H_6 Ph), 8.56 (s, 1 H, H_5), 8.65 (s, 1 H, H_8).

Anal. Calcd. for $C_{15}H_{10}Cl_2N_2O_3S$: C, 48.78; H, 2.71; N, 7.59. Found: C, 48.50; H, 2.68; N, 7.70.

6,7-Dichloro-2-methyl-3-(4-chlorophenyl)sulphinylquinoxaline 1,4-Dioxide (**4c**).

The yield was 52%, mp 198-199°; ir (potassium bromide): 3096 (CH), 1603 (C=N), 1312 (NO), 1011 (SO) cm^{-1}; ^1H nmr (deuteriochloroform): δ 2.84 (s, 3 H, CH_3), 7.42 (d, 2 H, H_3, H_5, J = 8 Hz, Ph), 7.93 (d, 2 H, H_2, H_6, J = 8 Hz, Ph), 8.48 (s, 1 H, H_5), 8.60 (s, 1 H, H_8).

Anal. Calcd. for $C_{15}H_9Cl_3N_2O_3S$: C, 44.61; H, 2.23; N, 6.93. Found: C, 44.75; H, 2.22; N, 6.91.

6,7-Dichloro-2-methyl-3-methylsulphonylquinoxaline 1,4-Dioxide (**5a**).

A solution of MCPBA (1.86 g, 10.80 mmoles) in chloroform (18 ml) was added dropwise over a cooled solution of **3a** (0.80 g, 2.70 mmoles) in chloroform (10 ml). The resulting mixture was stirred for 12 hours. The organic layer was washed with sodium bicarbonate. The crude solid obtained after removal of the solvent was chromatographed by eluting with ethyl acetate giving a yellow solid (0.81 g, 92%), mp 195-196°; ir (potassium bromide): 3094 (CH), 1595 (C=N), 1371 (NO), 1149 (SO_2) cm^{-1}; ^1H nmr (deuteriochloroform): δ 3.03 (s, 3 H, CH_3), 3.67 (s, 3 H, SO_2CH_3), 8.63 (s, 1 H, H_8), 8.70 (s, 1 H, H_5).

Anal. Calcd. for $C_{10}H_8Cl_2N_2O_4S$: C, 37.15; H, 2.48; N, 8.67. Found: C, 37.03; H, 2.54; N, 8.37.

6,7-Dichloro-2-methyl-3-phenylsulphonylquinoxaline 1,4-Dioxide (**5b**).

The yield was 85%, mp 172-173°; ir (potassium bromide): 3090 (CH), 1595 (C=N), 1356 (NO), 1128 (SO_2) cm^{-1}; ^1H nmr (deuteriochloroform): δ 2.54 (s, 3 H, CH_3), 7.50 (t, 2 H, H_3, H_5, J = 8 Hz, Ph), 7.69 (t, 1 H, H_4, J = 8 Hz, Ph), 8.08 (d, 2 H, H_2, H_6, J = 8 Hz, Ph), 8.17 (s, 1 H, H_5), 8.49 (s, 1 H, H_8).

Anal. Calcd. for $C_{15}H_{10}Cl_2N_2O_4S$: C, 46.75; H, 2.60; N, 7.27. Found: C, 46.61; H, 2.20; N, 6.95.

2,6,7-Trichloro-3-methylquinoxaline 1,4-Dioxide (**6**).

A mixture of **5a** (0.50 g, 1.51 mmoles) and concentrated hydrochloric acid (4 ml) was heated at 80° for 30 minutes. After stirring at room temperature overnight the crude solid was collected, washed with methanol and recrystallized from methanol/chloroform as a yellow solid (0.11 g, 24%), mp 175-176°; ir (potassium bromide): 3075 (CH), 1595 (C=N), 1380 (NO) cm^{-1}; ^1H nmr (deuteriochloroform): δ 2.82 (s, 3 H, CH_3), 8.73 (s, 2 H, H_5, H_8).

Anal. Calcd. for $C_9H_5Cl_3N_2O_2$: C, 38.64; H, 1.79; N, 10.02. Found: C, 38.85; H, 1.78; N, 10.07.

2-Bromo-6,7-dichloro-3-methylquinoxaline 1,4-Dioxide (**7**).

A solution of **5a** (0.32 g, 0.91 mmoles) in 48% hydrobromic acid (3 ml) was heated at 80° for 30 minutes. The mixture was stirred at room temperature for 12 hours. Water (10 ml) was added and the resulting precipitate was collected, washed with methanol and recrystallized from methanol/chloroform as an orange solid (0.10 g, 31%), mp 157-159°; ir (potassium bromide): 3073 (CH), 1592 (C=N), 1313 (NO) cm^{-1}; ^1H nmr (deuteriochloroform): δ 2.87 (s, 3 H, CH_3), 8.70 (s, 2 H, H_5, H_8).

Anal. Calcd. for $C_9H_5BrCl_2N_2O_2$: C, 33.33; H, 1.54; N, 8.64. Found: C, 33.70; H, 1.61; N, 8.65.

6,7-Dichloro-2-methyl-3-[(3-dimethylamino)propylamino]quinoxaline, 4-Dioxide (**8**).

3-(*N,N*-Dimethylamino)propylamine (0.13 g, 1.30 mmoles) was carefully added over a solution of **5a** (0.50 g, 1.50 mmoles) in dioxane (10 ml) and chloroform (3 ml). The resulting mixture was heated at 80° for 10 hours. After removal of the solvent an oil was obtained. Flash chromatography eluting with dichloromethane/methanol (90/10) afforded a red-brownish solid (0.21 g, 47%), mp 186°; ir (potassium bromide): 3440 (NH), 2949-2863 (CH), 1629 (C=N), 1370 (NO) cm^{-1}; ^1H nmr (deuteriochloroform): δ 1.88 (q, 2 H, CH_2, J = 6 Hz), 2.25 (s, 6 H, $N(CH_3)_2$), 2.44-2.52 (m, 2 H, NCH_2), 2.73 (s, 3 H, CH_3), 3.66 (t, 2 H, NCH_2, J = 6 Hz), 7.69 (s, 1 H, NH), 8.50 (s, 1 H, H_8), 8.54 (s, 1 H, H_5).

Anal. Calcd. for $C_{14}H_{18}Cl_2N_4O_2$: C, 48.70; H, 5.22; N, 16.23. Found: C, 48.61; H, 5.34; N, 16.03.

N,N'-Bis(6,7-dichloro-1,4-dioxo-3-methyl-2-quinoxalinyl)-hydrazine (**9**).

Hydrazine hydrate (0.15 g, 3.10 mmoles) was added over a solution of **5a** (1.00 g, 3.10 mmoles) in ethanol (20 ml). The mixture was stirred at room temperature for 10 hours. After removal of the solvent an oil was obtained and recrystallized from acetone-light petroleum ether as a deep red solid (1.06 g, 66%), mp 236°; ir (potassium bromide): 3410 (NH), 3091 (CH), 1568 (C=N), 1328 (NO) cm^{-1}; ^1H nmr (dimethyl sulfoxide-d_6): δ 2.55 (s, 6 H, 2 CH_3), 3.37 (s, 2 H, 2 NH), 8.35 (s, 4 H, 2 H_5, 2 H_8).

Anal. Calcd. for $C_{18}H_{12}Cl_4N_6O_4$: C, 41.70; H, 2.32; N, 16.22. Found: C, 41.31; H, 2.70; N, 16.41.

2-Amino-6,7-dichloro-3-methylquinoxaline 1,4-Dioxide (**10**).

A complete mixture of **3a** (0.50 g, 1.70 mmoles) and formamidine acetate (0.60 g, 5.81 mmoles) in ethoxyethanol (6 ml) was heated under reflux for 30 minutes. After removal of the solvent a gum was obtained. Flash chromatography eluting with methanol/dichloromethane (9/1) gave a yellow solid (0.13 g, 30%), mp 235-236°; ir (potassium bromide): 3413 (NH), 3327 (NH), 1598 (C=N), 1344 (NO) cm^{-1}; ^1H nmr (dimethyl sulfoxide-d_6): δ 2.54 (s, 3 H, CH_3), 7.84 (s, 2 H, NH_2), 8.41 (s, 1 H, H_8), 8.48 (s, 1 H, H_5).

Anal. Calcd. for $C_9H_7Cl_2N_3O_2$: C, 41.54; H, 2.69; N, 16.15. Found: C, 41.62; H, 2.68; N, 16.24.

2-Bromomethyl-6,7-dichloro-3-phenylthioquinoxaline 1,4-Dioxide (**11**).

N-Bromosuccinimide (NBS, 1.33 g, 7.50 mmoles) and catalytic amounts of benzoyl peroxide were added over a solution of **3b** (2.65 g, 7.50 mmoles) in carbon tetrachloride (35 ml). The complete mixture was heated under reflux for 77 hours. After cooling a precipitate was obtained. Recrystallization from methanol/chloroform afforded a yellow solid (1.77 g, 55%), mp 187°; ir (potassium bromide): 3091 (CH), 1598 (C=N), 1368 (NO) cm^{-1}; ^1H nmr (deuteriochloroform): δ 5.14 (s, 2 H, CH_2), 7.32-7.35 (m, 3 H, H_2, H_4, H_6 Ph), 7.48-7.52 (m, 2 H, H_3, H_5 Ph), 8.58 (s, 1 H, H_5), 8.73 (s, 1 H, H_8).

Anal. Calcd. for $C_{15}H_9BrCl_2N_2O_2S$: C, 41.67; H, 2.08; N, 6.48. Found: C, 41.67; H, 2.09; N, 6.09.

6,7-Dichloro-2-(2-hydroxyethylamino)-3-(2-hydroxyethylamino)methylquinoxaline 1,4-Dioxide (**12**).

2-Aminoethanol (2.00 g, 32.80 mmoles) was added over a solution of **11** (0.30 g, 6.90 mmoles) in chloroform (5 ml). The resulting mixture was stirred at room temperature for 4 days. The organic layer was washed with water and dried over sodium sulphate. After removal of the solvent an oil was obtained and crystallized from acetone-light petroleum ether (1.55 g, 62%), mp 159-160°; ir (potassium bromide): 3391 (OH), 2929 (CH), 1578 (C=N), 1364 (NO) cm^{-1}; ^1H nmr (dimethyl sulfoxide-d_6): δ 3.56-3.61 (m, 4 H, 2 CH_2O), 3.75-3.80 (m, 4 H, 2 NCH_2), 4.96 (s, 2 H, CH_2N), 4.78 (s, 2 H, 2 OH), 4.94 (s, 2 H, 2 NH), 8.30 (s, 1 H, H_8), 8.73 (s, 1 H, H_5).

Anal. Calcd. for $C_{13}H_{16}Cl_2N_4O_4$: C, 42.98; H, 4.41; N, 15.43. Found: C, 42.59; H, 4.15; N, 15.75.

Biological Methods.

In vitro selective cytotoxicity in hypoxia was evaluated by a clonogenic assay after 2 hours of treatment of V79 suspension cultures gassed with air or nitrogen.

Cells.

V79 cells (Chinese hamster lung fibroblasts) [13,14] were obtained from ECACC (European Collection of Animal Cell Cultures), and maintained in logarithmic-phase 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 foetal bovine serum (FBS) and Penicillin/Streptomycin 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 Erlenmeyer flasks: 2•10^4 cells/ml in 30 ml of EMEM containing 10% v/v FBS and HEPES 10 m*M*. The Erlenmeyer flasks were tightly closed with rubber caps which were perforated with two needles of 19G•40 mm to provide gas inlet and outlet. The Erlenmeyer flasks were submerged and stirred in a water bath at 37°, where they were gassed with humidified air or nitrogen.

Treatment.

Drug solutions were prepared just before the assay was carried out. Stock solutions, 150-fold more concentrated, were prepared in pure dimethyl sulfoxide (DMSO). Thirty minutes after starting to gas the suspension cultures, 0.2 ml of the stock solution was added to the 30 ml of total medium. In every assay there was an Erlenmeyer flask with 0.2 ml of dimethyl sulfoxide (Negative control). For screening, treatment lasted two hours during which gassing was continuous.

Cloning.

After treatment cells were centrifuged and resuspended in plating medium (EMEM supplemented with 15% v/v FBS and Penicillin/Streptomycin 100 U/100 μg/ml. The cell density was determined with a Hemocytometer and 10^2-10^5 cells were plated in 30 mm 6-well plates to give a final volume of 2 ml/well. Plates were incubated at 37° in 5% carbon dioxide for 7 days and were stained with aqueous crystal violet. Colonies with more than 64 cells were counted. The plating efficiency (PE) was calculated by dividing the number of clones by the number of cells seeded. The survival fraction (SF) is the percentage of PE of treated cultures with respect to the control.

Screening Assays.

Compounds were tested at 20, 5 and 1 μ*M* in duplicate flasks both in aerobic and hypoxic conditions.

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