3-Amino-2-quinoxalinecarbonitrile. New Fused Quinoxalines with Potential Cytotoxic Activity

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Starting with 3-amino-2-quinoxalinecarbonitrile 1,4-dioxide **1**, a new series of quinoxaline derivatives was prepared through chemical modifications of the 2-cyano and 3-amino groups. Nitration of 3-amino-2-quinoxalinecarbonitrile **3** afforded the 7-nitro derivative **6**. Diazotation of **3** gave the 3-chloro compound **9**. 2,3-Quinoxalinedicarbonitrile **14** was obtained from **9**. Pyridazino[4,5-*b*]quinoxalines **15** and **16** were prepared by condensing **14** with hydrazine hydrate. A triazolo[4,5-*b*]quinoxaline **18**, a isothiazolo[4,5-*b*]quinoxaline **20** and two pyrazolo[3,4-*b*]quinoxalines **21** and **22** were identified. Compounds were tested as cytotoxic agents both in oxic and in hypoxic cells.

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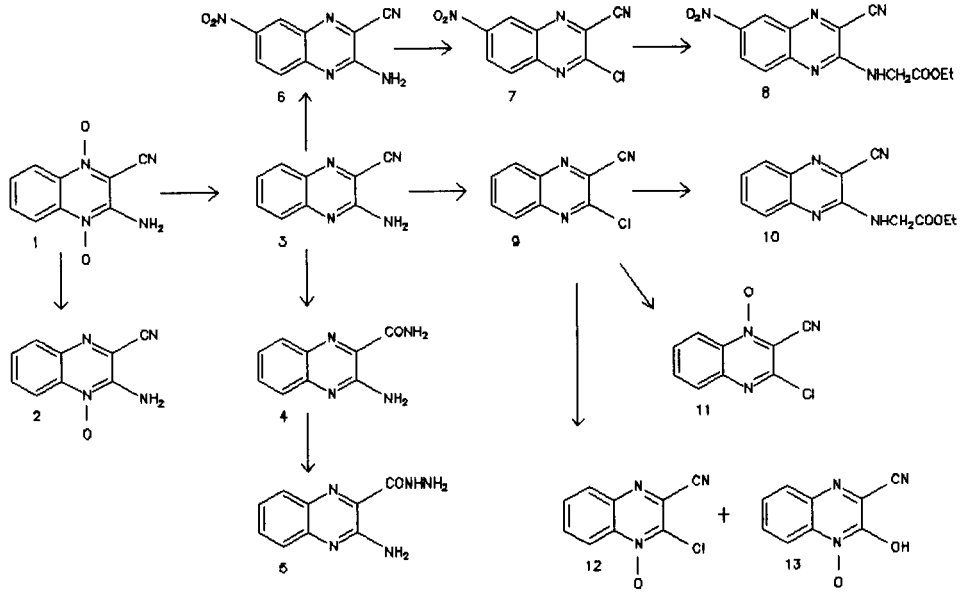
Solid tumors are refractory to cytotoxic agents because they do not reach the poorly vascularized regions of the tumors. Also, the cellular populations in solid tumors are physiologically more heterogeneous with respect to oxygenation and proliferation than are the cellular components of well-vascularized tumors [1]. It has been established that hypoxic cells exist in solid tumors and that these cells are relative resistant to the cytotoxic effects of ionizing radiation [2-6].

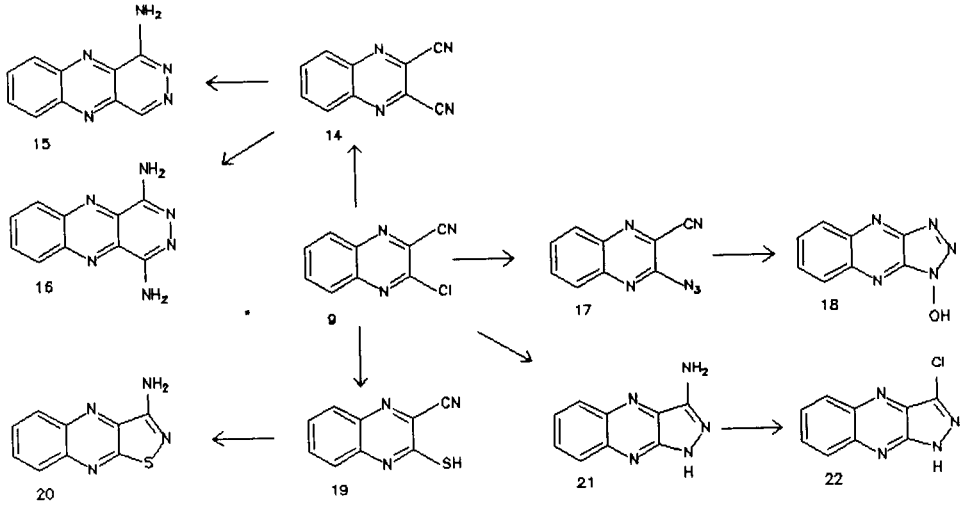
Our interest in the development of novel cytotoxic agents encouraged us to synthesize new quinoxaline derivatives structurally related to benzotriazines. A series of benzotriazines described by Brown's group have shown significant toxicity for hypoxic mammalian cells both *in vitro* and *in vivo* [7-10].

Compounds were obtained, as illustrated in Schemes 1 and 2, starting with 3-amino-2-quinoxalinecarbonitrile 1,4-dioxide **1** [11] and 3-amino-2-quinoxalinecarbonitrile **3**, obtained from **1** as previously reported [12]. The 4-oxide **2** was synthesized by heating **1** in ethanolic hydrochloric acid. The carboxamide **4** was prepared by treating **3** with hydrogen peroxide/potassium hydroxide. The carbohydrazide **5** was obtained by refluxing **4** in hydrazine hydrate. Nitration of 3-amino-2-quinoxalinecarbonitrile **3** afforded the 7-nitroquinoxaline **6**. 3-Chloro derivatives **7** and **9** were prepared by diazotization of the corresponding aro-

Scheme 1



Scheme 2



matic amines **6** and **3**. Removal of the chlorine atom with ethyl aminoacetate hydrochloride yielded **8** and **10**.

Attempts to reoxidize the reduced compound **9** in trifluoroacetic acid/hydrogen peroxide at room temperature for 2 hours gave **11**. However, when **9** was stirred and heated at 60° for 5 hours a mixture of **12** and **13** was obtained. Flash chromatography eluting with toluene gave **12** and eluting with ethyl acetate afforded **13**.

2,3-Quinoxalinedicarbonitrile **14** was obtained by replacing the chlorine atom of **9** in the presence of sodium cyanide in dimethyl sulfoxide at room temperature. Heating under reflux the dicarbonitrile **14** in the presence of hydrazine hydrate a 1-aminopyridazino[4,5-*b*]quinoxaline **15** was prepared. 1,4-Diaminopyridazino[4,5-*b*]quinoxaline (**16**) is readily obtained by heating **14** at 70°.

The azide **17**, which in turn is conveniently prepared from **9**, afforded 1-hydroxytriazolo[4,5-*b*]quinoxaline **18**. Replacement of the chlorine in **9** in the presence of sodium hydrosulfide at mild temperatures yielded the 3-mercaptoquinoxaline **19**. 3-Aminoisothiazolo[4,5-*b*]-quinoxaline **20** was obtained from **19** by cyclization with hydroxylamine-*o*-sulfonic acid. 3-Amino-1*H*-pyrazolo[3,4-*b*]quinoxaline **21** was readily obtained from **9** by refluxing in hydrazine hydrate. Diazotization of **21** afforded the 3-chloro derivative **22**.

Compounds were subjected to preliminary cytotoxic evaluation on V79 cells in hypoxic and aerobic conditions at 20 μM determining the survival fraction (SF). The most interesting compounds were: **3** ($SF_{hypoxia}$: 68, SF_{air}: 45), **8** (99, 90), **9** (66, 43), **13** (63, 33), **18** (98, 25), **19** (64, 62) and **21** (66, 46).

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 60-80°). Infrared spectra were recorded on a Perkin-Elmer 1600 series FTIR 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 about 0.1 g/ml and with dimethyl sulfoxide-d_6 as the solvent; the chemical shifts are reported in ppm of tetramethylsilane in δ units. Thin layer chromatography (tlc) was carried out on silica gel (HF, 254-266, Merck or DSF-5, Cammaga) with the indicated solvents and the plates were scanned under ultraviolet length 254 and 366 nm. Column chromatography was carried out with silica gel 60 Merck (70-230 mesh ASTM) and indicated solvents. Elemental analyses were within $\pm 0.4\%$ of the theoretical values.

3-Amino-2-quinoxalinecarbonitrile 1,4-Dioxide (**1**).

Powdered benzofuroxane (1.36 g, 10.00 moles) and malononitrile (0.70 g, 10.60 mmoles) were dissolved in dry dimethylformamide (4 ml). The resulting solution was stirred at 0° in an ice-bath. Another solution of triethylamine (5 drops) in dry dimethylformamide (3 ml) was added dropwise. The mixture was stirred at 10° for 4 hours. The red solid which precipitated was filtered off and washed with diethyl ether. Finally the product was recrystallized from dioxane giving **1**, (1.51 g, 75%), mp 190°; ir (potassium bromide): 3350-3251 (NH_2), 2236 (CN), 1350 (NO) cm^{-1}; ^1H nmr (dimethyl sulfoxide-d_6): δ 7.64 (t, 1H, H_6, J_{6-7} = 7.6 Hz), 7.91 (t, 1H, H_7), 8.08 (s, 2H, NH_2), 8.25 (d, 1H, H_5, J_{5-6} = 8.2 Hz), 8.26 (d, 1H, H_8, J_{8-7} = 7.8 Hz).

Anal. Calcd. for $C_9H_6N_4O_2$: C, 53.47; H, 2.97; N, 27.72. Found: C, 53.22; H, 3.03; N, 27.42.

3-Amino-2-quinoxalinecarbonitrile 4-Oxide (**2**).

3-Amino-2-quinoxalinecarbonitrile 1,4-dioxide **1** (4.04 g, 20.00 mmoles) was added to a mixture of ethanol (50 ml) and 6% hydrochloric acid (20 ml) and the suspension was heated under reflux for 24 hours. The cooled suspension was filtered, washed with water and dried to afforded a brown solid. Flash chromatography

(ethyl acetate) gave an orange solid, **2**, (2.80 g, 75%), mp 266°; ir (potassium bromide): 3348-3265 (NH_2), 2236 (CN), 1350 (NO) cm^{-1}; ^1H nmr (dimethyl sulfoxide-d_6): δ 7.66 (t, 1H, H_6, J_{6-7} = 7.4 Hz), 7.88 (t, 1H, H_7), 7.97 (d, 1H, H_5, J_{5-6} = 7.6 Hz), 8.05 (s, 2H, NH_2), 8.25 (d, 1H, H_8, J_{8-7} = 8.6 Hz).

Anal. Calcd. for $C_9H_6N_4O$: C, 58.06; H, 3.23; N, 30.11. Found: C, 57.67; H, 3.30; N, 29.66.

3-Amino-2-quinoxalinecarbonitrile (**3**).

A suspension of 3-amino-3-quinoxalinecarbonitrile 1,4-dioxide **1** (2 g, 9.90 mmoles) in methanol (18 ml) was stirred at 50° for 2 hours. Then, a solution of sodium ditionate (4.8 g, 27.58 mmoles) in distilled water (18 ml) was added dropwise over a period of 15 minutes. The mixture was stirred at 50° for 3 hours. The product was recrystallized from dimethylformamide/water. A bright yellow solid was obtained, **3**, (1.47 g, 87%), mp 201°; ir (potassium bromide): 3412-3322 (NH_2), 2220 (CN) cm^{-1}; ^1H nmr (dimethyl sulfoxide-d_6): δ 7.45 (s, 2H, NH_2), 7.51-7.86 (m, 4H, H_5, H_6, H_7, H_8).

Anal. Calcd. for $C_9H_6N_4$: C, 63.53; H, 3.53; N, 32.94. Found: C, 63.95; H, 3.67; N, 32.76.

3-Amino-2-quinoxalinecarboxamide (**4**).

A mixture of 3-amino-2-quinoxalinecarbonitrile **3** (0.30 g, 1.76 mmoles) and hydrogen peroxide (110 vol, 3 ml) was added over a solution of potassium hydroxide (1.40 g, 25.00 mmoles) in ethanol (15 ml), the resulting solution was stirred at 40° for 2 hours. The mixture was allowed to stand at room temperature. The precipitate was filtered off and recrystallized from methanol giving **4**, (0.23 g, 72%), mp 265°; ir (potassium bromide): 3440-3380 (NH), 1670 (CO) cm^{-1}; ^1H nmr (dimethyl sulfoxide-d_6): δ 7.42-8.00 (m, 6H, H_5, H_6, H_7, H_8, NH_2), 8.49 (s, 2H, NH_2).

Anal. Calcd. for $C_9H_8N_4O$: C, 57.45; H, 4.26; N, 29.79. Found: C, 56.98; H, 4.32; N, 29.65.

3-Amino-2-quinoxalinecarbohydrazide (**5**).

3-Amino-2-quinoxalinecarboxamide **4** (3.79 g, 20.16 mmoles) was added over hydrazine hydrate (20 ml) and the mixture was refluxed for 2 hours. The mixture was poured into water. The precipitate was filtered off and recrystallized from ethanol/water (4/1) to give a yellow solid, **5**, (2.80 g, 68%), mp 201°; ir (potassium bromide): 3391-3261 (NH), 1672 (CO) cm^{-1}; ^1H nmr (dimethyl sulfoxide-d_6): δ 7.37-7.84 (m, 7H, H_5, H_6, H_7, H_8, NH_2, NH), 10.24 (s, 2H, NH_2).

Anal. Calcd. for $C_9H_9N_5O$: C, 53.20; H, 4.43; N, 34.48. Found: C, 53.44; H, 4.48; N, 35.00.

3-Amino-7-nitro-2-quinoxalinecarbonitrile (**6**).

3-Amino-2-quinoxalinecarbonitrile **3** (2.00 g, 11.76 mmoles) was dissolved in concentrated sulfuric acid (15 ml). The solution was stirred in an ice-bath and 60% nitric acid (4 ml) was added dropwise. The final mixture was stirred for 30 minutes. The crude compound was precipitated by adding over cold water. The solid was recrystallized from ethanol giving **6**, (1.95 g, 77%), mp >300°; ir (potassium bromide): 3406-3315 (NH_2), 2240 (CN), 1347 (NO_2) cm^{-1}; ^1H nmr (dimethyl sulfoxide-d_6): δ 7.65 (d, 1H, H_7, J_{7-8} = 9.2 Hz), 8.11 (s, 2H, NH_2), 8.37 (d, 1H, H_8), 8.61 (s, 1H, H_5).

Anal. Calcd. for $C_9H_5N_5O_2$: C, 50.23; H, 2.32; N, 32.56. Found: C, 50.41; H, 2.23; N, 32.24.

3-Chloro-6-nitro-2-quinoxalinecarbonitrile (**7**).

A mixture of **6** (6.20 g, 28.84 mmoles), glacial acetic acid (100 ml) and concentrated hydrochloric acid was cooled below 0°. Then a freshly solution of sodium nitrite (7.40 g, 107.25 mmoles) in distilled water (40 ml) was added dropwise over the first solution. Temperature was maintained at 0° for 4 hours. A brown solid was filtered off and washed with distilled water giving **7** which was recrystallized from ethanol giving **7**, (5.61 g, 83%), mp >300°; ir (potassium bromide): 3076 (CH), 1534 and 1349 (NO_2) cm^{-1}; ^1H nmr (dimethyl sulfoxide-d_6): δ 8.35-9.06 (m, 3H, H_5, H_7, H_8).

Anal. Calcd. for $C_9H_3ClN_4O_2$: C, 46.05; H, 1.28; N, 23.88. Found: C, 46.17; H, 1.08; N, 24.06.

Ethyl 2-[*N*-(3-Cyano-6-nitro-2-quinoxalinyl)amino]acetate (**8**).

A solution of 3-chloro-6-nitro-2-quinoxalinecarbonitrile **7** (2.56 g, 10.92 mmoles) in dimethyl sulfoxide (40 ml) was stirred at room temperature. Potassium carbonate (1.40 g, 10.14 mmoles) was added. A second solution of ethyl 2-aminoacetate hydrochloride (1.40 g, 10.03 mmoles) in dimethyl sulfoxide (15 ml) was added over the first solution. The mixture was heated at 80° for 5 hours. The cooled suspension was poured into ice-water. The solid was recrystallized from ethanol/water (4/1) giving a yellow compound, **8**, (1.80 g, 55%), mp 139°; ir (potassium bromide): 3255 (NH), 2240 (CN), 1727 (CO), 1564-1341 (NO_2) cm^{-1}; ^1H nmr (dimethyl sulfoxide-d_6): δ 1.21 (t, 3H, CH_3), 4.17 (m, 4H, NCH_2, OCH_2), 7.67-8.68 (m, 3H, H_5, H_6, H_8), 8.80 (t, 1H, NH).

Anal. Calcd. for $C_{13}H_{11}N_5O_4$: C, 51.83; H, 3.65; N, 23.25. Found: C, 51.87; H, 3.46; N, 23.47.

3-Chloro-2-quinoxalinecarbonitrile (**9**).

A mixture of **3** (2.40 g, 14.12 mmoles), 35% hydrochloric acid (50 ml) and acetic acid (40 ml) in an ice-salt bath (below 4°) was stirred for 20 minutes. Over the cooled solution was added sodium nitrite (3.00 g, 43.48 mmoles) and water (25 ml). The reaction mixture was allowed to stand at room temperature for 6 hours. The white solid was filtered and washed with water. Recrystallization from methanol gave **9**, (1.47 g, 55%), mp 200°; ir (potassium bromide): 3039 (CH), 2234 (CN) cm^{-1}; ^1H nmr (dimethyl sulfoxide-d_6): δ 7.30-8.30 (m, 4H, H_5, H_6, H_7, H_8).

Anal. Calcd. for $C_9H_4ClN_3$: C, 56.99; H, 2.11; N, 22.16. Found: C, 56.88; H, 2.21; N, 22.15.

Ethyl 2-[*N*-(3-Cyano-2-quinoxalinyl)amino]acetate (**10**).

A solution of 3-chloro-2-quinoxalinecarbonitrile **9** (0.37 g, 1.95 mmoles) in dimethyl sulfoxide (10 ml) was stirred at room temperature. A mixture of ethyl aminoacetate hydrochloride (0.28 g, 2.01 mmoles) and potassium carbonate (0.14 g, 1.01 mmoles) in dimethyl sulfoxide (3 ml) was added over the first solution and heated at 80° for 2 hours. The resulting suspension was poured into distilled water. The product was isolated by filtration and recrystallized from dioxane/water (4/1) giving **10**, (0.25 g, 50%), mp 123°; ir (potassium bromide): 3369 (NH), 2234 (CN), 1744 (CO) cm^{-1}; ^1H nmr (dimethyl sulfoxide-d_6): δ 1.20 (t, 3H, CH_3), 4.08-4.18 (m, 4H, NCH_2, OCH_2), 7.50-8.30 (m, 5H, H_5, H_6, H_7, H_8, NH).

Anal. Calcd. for $C_{13}H_{12}N_4O_2$: C, 60.94; H, 4.69; N, 21.88. Found: C, 60.70; H, 4.27; N, 22.03.

3-Chloro-2-quinoxalinecarbonitrile 1-Oxide (**11**).

A combined mixture of 2-chloro-3-cyanoquinoxaline **9** (2.00 g, 10.55 mmoles), trifluoroacetic acid (20 ml) and hydrogen peroxide (60 vol, 3 ml) was stirred at room temperature for 2 hours. Then, the solution was poured into crushed ice. The precipitate was filtered off and chromatographed eluting with toluene. The

1-oxide, **11**, was eluted (0.89 g, 41%), mp 269°; ir (potassium bromide): 2235 (CN), 1346 (NO) cm^{-1}; ^1H nmr (dimethyl sulfoxide-d_6): δ 7.90-9.50 (m, 4H, H_5, H_6, H_7, H_8).

Anal. Calcd. for $C_9H_4ClN_3O$: C, 52.55; H, 1.95; N, 20.44. Found: C, 52.83; H, 1.85; N, 20.70.

3-Chloro-2-quinoxalinecarbonitrile 4-Oxide (**12**) and 3-Hydroxy-2-quinoxalinecarbonitrile 4-Oxide (**13**).

A combined mixture of **9** (1.60 g, 8.44 mmoles), trifluoroacetic acid (10 ml) and hydrogen peroxide (60 vol, 8 ml) was stirred and heated at 60° for 5 hours. The final solution was allowed to stand at room temperature. The precipitate was filtrated. Flash chromatography eluting with toluene gave a yellow compound, **12**, (1.04 g, 60%), mp 259°; ir (potassium bromide): 2240 (CN), 1361 (NO) cm^{-1}; ^1H nmr (dimethyl sulfoxide-d_6): δ 7.93-8.43 (m, 4H, H_5, H_6, H_7, H_8).

Anal. Calcd. for $C_9H_4ClN_3O$: C, 52.55; H, 1.95; N, 20.44. Found: C, 52.18; H, 2.15; N, 20.04.

Compound **13** was eluted with ethyl acetate (0.50 g, 32%), mp 283°; ir (potassium bromide): 2229 (CN), 1398 (NO) cm^{-1}; ^1H nmr (dimethyl sulfoxide-d_6): δ 7.39-8.11 (m, 4H, H_5, H_6, H_7, H_8), 12.96 (s, 1H, OH).

Anal. Calcd. for $C_9H_5N_3O_2$: C, 57.75; H, 2.67; N, 22.46. Found: C, 57.87; H, 2.63; N, 22.44.

2,3-Quinoxalinedicarbonitrile (**14**).

Powdered **9** (0.40 g, 2.11 mmoles) was dissolved in dimethyl sulfoxide (20 ml). The resulting mixture was added over a solution of sodium cyanide (0.20 g, 4.08 mmoles) in dimethyl sulfoxide (20 ml). The mixture was stirred at room temperature for 10 minutes. Then the resulting solution was diluted with distilled water. The grey solid was recrystallized from ethanol giving **14**, (0.20 g, 53%), mp 218°; ir (potassium bromide): 2250 (CN), 763 (2,3-quinoxaline) cm^{-1}; ^1H nmr (dimethyl sulfoxide-d_6): δ 8.20-8.36 (m, 4H, H_5, H_6, H_7, H_8).

Anal. Calcd. for $C_{10}H_4N_4$: C, 66.67; H, 2.22; N, 31.11. Found: C, 66.77; H, 2.22; N, 31.12.

1-Aminopyridazino[4,5-*b*]quinoxaline (**15**).

A combined mixture of **14** (1.00 g, 5.55 mmoles) and hydrazine hydrate (20 ml) was heated under reflux for 3 hours. After cooling some red-brown crystals were obtained, **15**. A sample was recrystallized from ethanol (0.57 g, 52%), mp 290°; ir (potassium bromide): 3455-3262 (NH_2), 760 (2,3-quinoxaline) cm^{-1}; ^1H nmr (dimethyl sulfoxide-d_6): δ 7.68 (s, 2H, NH_2), 8.12-8.37 (m, 4H, H_5, H_6, H_7, H_8), 9.19 (s, 1H, H_4).

Anal. Calcd. for $C_{10}H_7N_5$: C, 60.91; H, 3.55; N, 35.53. Found: C, 60.79; H, 3.73; N, 35.37.

1,4-Diaminopyridazino[4,5-*b*]quinoxaline (**16**).

A mixture of **14** (0.25 g, 1.39 mmoles) and hydrazine hydrate (10 ml) was stirred and heated at 70° for 90 minutes. The solution was allowed to cool and the solid was filtered off. Finally **16** was recrystallized from dioxane (0.17 g, 58%), mp 289°; ir (potassium bromide): 3437 (NH_2), 770 (2,3-quinoxaline) cm^{-1}; ^1H nmr (dimethyl sulfoxide-d_6): δ 6.41 (s, 4H, NH_2, NH_2), 8.14-8.33 (m, 4H, H_5, H_6, H_7, H_8).

Anal. Calcd. for $C_{10}H_8N_6$: C, 56.60; H, 3.77; N, 39.62. Found: C, 56.92; H, 3.82; N, 39.89.

3-Azido-2-quinoxalinecarbonitrile (**17**).

3-Chloro-2-quinoxalinecarbonitrile **9** (0.80 g, 4.22 mmoles) was dissolved in dimethyl sulfoxide (8 ml), and the mixture was stirred and heated at 80°. Another solution of sodium azide (0.40 g, 6.15 mole) in dimethyl sulfoxide (8 ml) was added dropwise over the first solution. The mixture was stirred for additional 3 hours giving **17** as a white solid which was recrystallized from ethanol (0.43 g, 52%), mp 268°; ir (potassium bromide): 3091 (CH), 769 (2,3-quinoxaline) cm^{-1}; ^1H nmr (dimethyl sulfoxide-d_6): δ 8.06-8.78 (m, 4H, H_5, H_6, H_7, H_8).

Anal. Calcd. for $C_9H_4N_6$: C, 55.10; H, 2.04; N, 42.86. Found: C, 55.14; H, 1.93; N, 43.07.

1-Hydroxytriazolo[4,5-*b*]quinoxaline (**18**).

A suspension of 3-azido-2-quinoxalinecarbonitrile **17** (1.90 g, 9.69 mmoles) in 6% hydrochloric acid (6 ml) was heated under reflux for 2 hours. The white solid was filtered off and washed with water. Recrystallization from dioxane/toluene (6/1) afforded **18**, (0.95 g, 52%), mp 288°; ir (potassium bromide): 1338 (NO), 763 (2,3-quinoxaline) cm^{-1}; ^1H nmr (dimethyl sulfoxide-d_6): δ 7.41-7.67 (m, 3H, H_5, H_6, H_7), 8.27 (d, 1H, H_8), 12.58 (s, 1H, OH).

Anal. Calcd. for $C_8H_5N_5O$: C, 51.34; H, 2.67; N, 37.43. Found: C, 51.75; H, 2.67; N, 37.89.

3-Mercapto-2-quinoxalinecarbonitrile (**19**).

A solution of sodium hydrosulfide (0.40 g, 7.14 mmoles) in dimethyl sulfoxide (20 ml) was heated at 40-50° for 10 minutes. Another solution of 3-chloro-2-quinoxalinecarbonitrile **9** (0.60 g, 3.17 mmoles) in dimethyl sulfoxide (5 ml) was added over the first solution and the mixture was stirred for 1 minute. Water (200 ml) and 6% hydrochloric acid (8 ml) were added appearing a solid, **19**, which was filtrated and recrystallized from ethanol (0.17 g, 29%), mp 115° dec; ir (potassium bromide): 2226 (CN), 765 (2,3-quinoxaline) cm^{-1}; ^1H nmr (dimethyl sulfoxide-d_6): δ 7.55-7.06 (m, 3H, H_5, H_6, H_7, H_8), 14.99 (bs, 1H, SH).

Anal. Calcd. for $C_9H_5N_3S$: C, 57.75; H, 2.67; N, 22.46. Found: C, 57.55; H, 2.63; N, 22.71.

3-Aminoisothiazolo[4,5-*b*]quinoxaline (**20**).

3-Mercapto-2-quinoxalinecarbonitrile **19** (2.00 g, 10.69 mmoles) was added over an aqueous solution of potassium carbonate (13.80 g/100 ml) (25 ml). Another solution of hydroxylamine-*o*-sulfonic acid (2.00 g, 17.70 mmoles) in distilled water (15 ml) was added over the first solution. The mixture was stirred and heated at 80° for 1.5 hours. The solid, **20**, was filtered off and recrystallized from toluene (1.86 g, 86%), mp 266°; ir (potassium bromide): 3353-3295 (NH_2), 759 (2,3-quinoxaline) cm^{-1}; ^1H nmr (dimethyl sulfoxide-d_6): δ 7.46 (s, 2H, NH_2), 8.32-7.93 (m, 4H, H_5, H_6, H_7, H_8).

Anal. Calcd. for $C_9H_6N_4S$: C, 53.46; H, 2.97; N, 27.72. Found: C, 53.80; H, 2.91; N, 27.48.

3-Amino-1*H*-pyrazolo[3,4-*b*]quinoxaline (**21**).

A combined mixture of **9** (0.80 g, 4.22 mmoles) and hydrazine hydrate (18.00 g, 360.00 mmoles) was heated under reflux for 1 hour. After cooling the red crystals, **21**, were filtered off and recrystallized from methanol (0.70 g, 90%), mp 285°; ir (potassium bromide): 3385-3321 (NH_2), 742 (2,3-quinoxaline) cm^{-1}; ^1H nmr (dimethyl sulfoxide-d_6): δ 6.16 (s, 2H, NH_2), 7.64-8.18 (m, 4H, H_5, H_6, H_7, H_8), 12.23 (s, 1H, NH).

Anal. Calcd. for $C_9H_7N_5$: C, 58.38; H, 3.78; N, 37.84. Found: C, 58.76; H, 4.08; N, 38.28.

3-Chloro-1*H*-pyrazolo[3,4-*b*]quinoxaline (**22**).

3-Amino-1*H*-pyrazolo[3,4-*b*]quinoxaline **21** (2.00 g, 10.81 mmoles) was dissolved in 6*N* hydrochloric acid (20 ml). The solution was cooled in a ice-bath system. The mixture was stirred at 0° and a fresh solution of sodium nitrite (1.50 g, 21.74 mmoles) in distilled water (10 ml) was added dropwise. After stirring for 60 minutes an orange precipitated was obtained. Recrystallization from toluene yielded **22**, (1.17 g, 53%), mp >300°; ir (potassium bromide): 765 (2,3-quinoxaline) cm^{-1}; ^1H nmr (dimethyl sulfoxide-d_6): δ 7.79-8.26 (m, 4H, H_5, H_6, H_7, H_8), 14.27 (s, 1H, NH).

Anal. Calcd. for $C_9H_5N_4Cl$: C, 52.81; H, 2.44; N, 27.38. Found: C, 53.27; H, 2.46; N, 27.60.

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 $1\text{-}2\cdot10^4$ 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 erlenmeyers: $2\cdot10^4$ cells/ml in 30 ml of EMEM containing 10% v/v FBS and HEPES 10nM. The erlenmeyers were tightly closed with rubber caps which were perforated with two needles of 19G•40 mm to provide gas inlet and outlet. Erlenmeyers 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 (dimethyl sulfoxide). 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 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 suplemented 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 μ*M* in duplicate flasks both in aerobic and hypoxic conditions.

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