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Hypoxia-selective antitumor agents derived from 1,9-diazaanthracene

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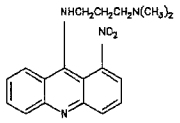
Summary – The nitroacridine derivative 9-[3-(*N*,*N*-dimethylamino)propylamino]-1-nitroacridine (nitracrine) is a potent hypoxia-selective cytotoxin for tumor cells in culture. Modifications of the acridine ring result in altered DNA binding properties. This has suggested the possibility of preparing new nitracrine analogues retaining the nitro group and the alkylamino lateral chain. In this paper we describe the synthesis of the new diazaanthracenes **4**, **5**, **6** in order to study the relationships between the heterocyclic system and the hypoxia-selective cytotoxicity. This class of new bioreductive agents may constitute a group of potential interest for the design of new cytotoxins.

nitroheterocycle / diazaanthracene / hypoxia-selective cytotoxicity

Introduction

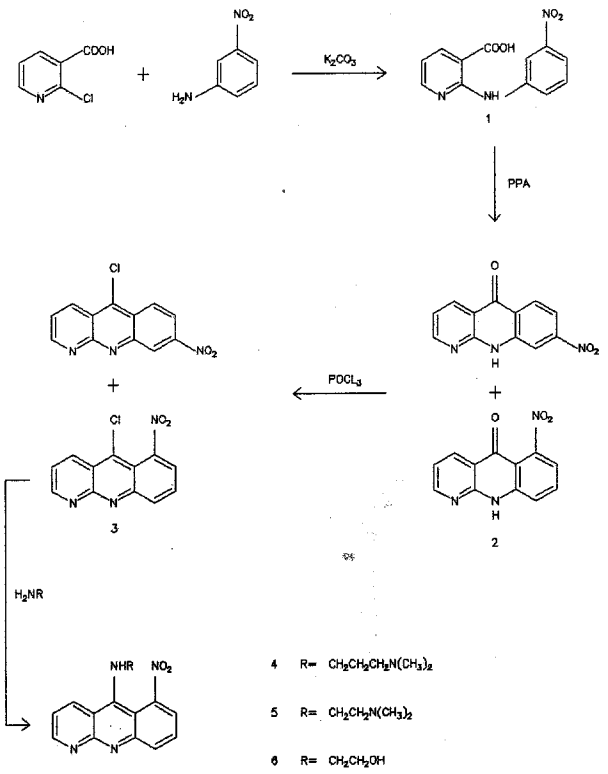
Hypoxic cells in solid tumors are an important target for cancer chemotherapy [1, 2]. They are not only refractory to radiation and to some cytotoxic drugs but may also be targeted specifically by drugs designed to be activated only in the absence of oxygen, providing selective bioactivation within tumor tissue [3, 4]. A compound that is of current interest in this respect is the 1-nitroacridine derivative, nitracrine, which has been shown to be a hypoxia-selective cytotoxic agent [5, 6] and a radiation sensitizer [7]. In possessing these biological effects, nitracrine resembles known electron-affinic nitroheterocycles, such as misonidazole, but its *in vitro* potency is greater by many orders of magnitude, probably because of efficient targeting to DNA *via* intercalative binding [5, 7]. It is known that the combination of the 1-nitro group and the alkyl side chain on the nitrogen atom of C-9 is responsible for the antitumor activity of nitracrine [8]. On the other hand, modifications of the acridine ring result in altered DNA binding properties [9]. In order to find new heterocyclic systems related to acridines with improved potency and selectivity, we have prepared a family of 1,9-diazaanthracenes bearing a nitro group and an alkylamino lateral chain. The influence of the nitrogen N9 on hypoxia-selective cytoxicity has been evaluated.



Nitracrine

Chemistry

Compounds were prepared by the method outlined in scheme 1. Condensation of 2-chloronicotinic acid with 3-nitroaniline [10, 11] in the presence of copper-bronze as a catalyst and potassium carbonate gave nicotinic acid **1** (59%). Attempts to cyclize **1** with $POCl_3$ or concentrated H_2SO_4 were unsuccessful. Cyclocondensation with polyphosphoric acid [12] (PPA) afforded the anthracenone **2** in a mixture of isomers (5- and 7-nitro). Chlorination by reaction with $POCl_3$ gave a mixture of 10-chloro-isomers which, in turn, were conveniently separated by flash chromatography. Due to the instability of the 10-chloro-5-nitro compound **3**, it immediately underwent phenol-mediated coupling with different selected amines, yielding the 10-alkylamino-5-nitro-1,9-diazaanthracenes **4**, **5**, and **6**. Final compounds **4**, **5** and **6** were



Scheme 1.

stored at –20°C before testing in V79 cells. They were hygroscopic and, in that state, difficult to work up and analyze.

Biological results and discussion

Three new diazaanthracenes derivatives **4**, **5** and **6** structurally related to nitracrine, and 2 intermediates **2**, **3** were subjected to preliminary cytotoxic evaluation on V79 cells in hypoxic and aerobic conditions. Nitracrine was chosen as a standard due to its selective toxicity to hypoxic cells.

Intermediate **2**, a diazaanthracenone, and compound **3**, 10-chloro-5-nitro-1,9-diazaanthracene, did not cause significant cytotoxicity either in air or nitrogen at 10 μM.

Diazaanthracenes **4**, **5** and **6** showed an interesting selective cytotoxicity effect, while in the screening they seemed to be at least 10-fold less potent than nitracrine (table I). Compound **4**, the structure of which resembles nitracrine (both possess a dimethylamino-propylamino lateral chain), was active and selective at 5 μM but less potent than **5** and **6**, which were also toxic in nitrogen at 1 μM. Compound **5** has a dimethylaminoethylamino chain in position 10 and **6** bears a 2-hydroxyethylamino group. These structure–activity relationships are similar to those observed in the related 1-nitroacridines [4], where the nature of the side chain was of critical importance in the *in vivo* activity of compounds bearing a cationic (alkylamino)alkyl side chain. The activity of compounds with hydrophilic hydroxyalkyl side chains was discovered and one compound of this type, a hydroxyethyl derivative, is presently undergoing clinical trials. In contrast, compounds with neutral lipophilic or anionic side chains did not show any significant *in vivo* activity [13].

The effect of different durations of drug exposure was measured for compounds **5** and **6**, and nitracrine. These results are presented in figures 1 and 2. In hypoxia, diazaanthracenes and nitracrine have a similar response at different doses: a very drastic toxic effect in the first 30 min. Compounds **5** and **6** are approximately 10-fold less potent than nitracrine. With respect to selectivity, Wilson *et al* [4] quoted hypoxic selectivity for nitracrine of about 3–10-fold in stirred suspension cultures of AA8 cells. We have obtained a similar value in our system with V79 cells (data not shown). Compounds **5** and **6** show hypoxic selectivity of approximately 5-fold because time-response in air at 5 μM is similar to time-response in hypoxia at 1 μM (fig 1a and b).

The introduction of a new nitrogen atom in 9 position, N9, does not affect the selectivity of this group of nitroheterocycles (1-nitroacridines and 5-nitro-1,9-diazaanthracenes). However, the potency of diazaanthracenes is approximately 10 times lower than nitracrine. In our opinion, although these preliminary results do not improve the nitracrine *in vitro* profile, this approach may be interesting for the design of new selective cytotoxins.

Table I. Survival fraction (SF) in aerobic and hypoxic conditions after 2 h of treatment of V79 suspension cultures with the diazaanthracenes derivatives at the doses indicated.

Compound	*Dose (μM)*	*Aerobic SF*	*Hypoxic SF*
4	5	26.8 ± 5.3	0
	1	104.5 ± 10.3	109.6 ± 8.3
5	5	0	0
	1	66.0 ± 5.5	10.8 ± 1.3
6	5	4.9 ± 0.8	0
	1	80.2 ± 6.6	16.9 ± 1.3
Nitracrine	0.06	23.4 ± 2.6	0.04 ± 0.01

Mean of 3 data points ± standard deviation.

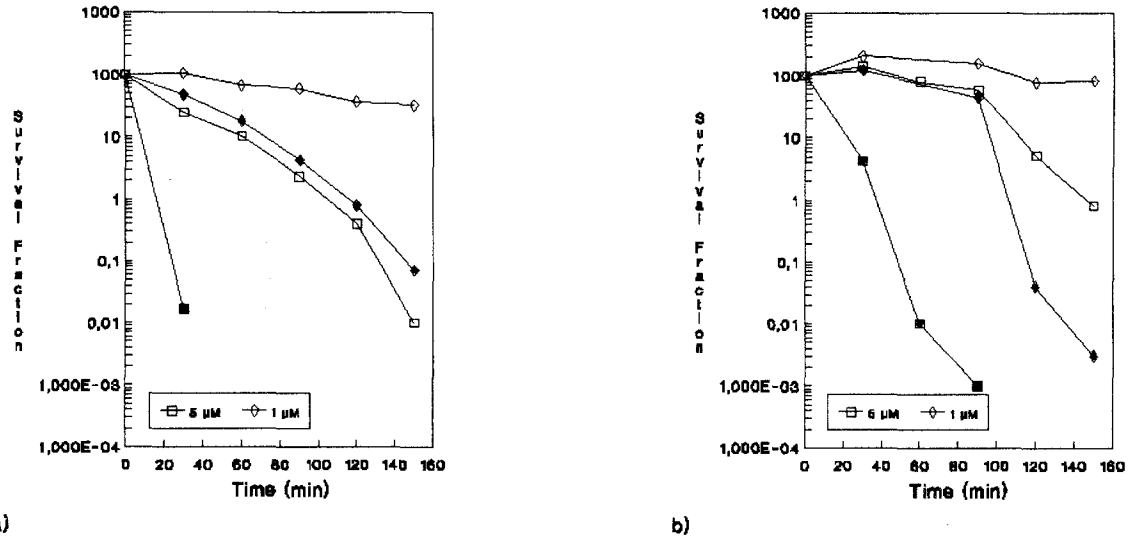


Fig 1. Survival fraction of V79 cells exposed to compound **6** (a) or compound **5** (b) under aerobic (open symbols) or hypoxic (filled symbols) conditions at concentrations of 1 and 5 µM.

Experimental protocols

Chemistry

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 about 60–80°C). 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 ^{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 dimethylsulfoxide-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 (HF, 254-266, Merck or DSF-s, Cammaga) with the indicated solvents and the plates were scanned under ultraviolet light at 254 and 366 nm. Column chromatography was carried out with silica gel 60 Merck (70-230 mesh ASTM) and indicated solvents.

Analyses indicated by the symbols of the elements or functions were within ± 0.4% of theoretical values.

Nitracrine (positive control) was prepared by phenol-mediated coupling of 3-(*N,N*-dimethylamino)propylamine with 9-chloro-1-nitroacridine, which in turn was conveniently prepared in small amounts by flash chromatography of the mixture of 1- and 3-nitro-isomers formed by $POCl_3$ cyclization of *N*-(3-nitrophenyl)-anthranilic acid [14]. For larger quantities, a more convenient method was fractional crystallization of the 9-(*N*-pyridinium)chlorides [15, 16], followed by conversion to 9-chloro derivatives.

2-(m-Nitrophenylamino)nicotinic acid ***1***

A mixture of 2-chloronicotinic acid (66.00 g, 0.42 mol), *m*-nitroaniline (70.00 g, 0.51 mol), potassium carbonate (33.00 g), copper-bronze (6.60 g) and *N,N*-DMF (1 L) was stirred and heated under reflux for 15 h. The cooled mixture was poured into water, and acidified with 2 N HCl to pH 1. The precipitate was filtered off and washed with hot water (500 mL) until total decoloration of the water was obtained, in order to eliminate copper salts and the excess *m*-nitroaniline. Finally, the solid was washed with methanol (100 mL), recrystallized from EtOH/H_2O and dried to give **1** (63.50 g, 56% yield): R_f (EtOAc) = 0.5; mp: 245°C; NMR (DMSO-d_6) δ = 6.99 (t, 1 H, H_5, Py, $J_{4,5,6}$ = 5.7 Hz); 7.58 (t, 1 H, $H_{5'}$, Py, $J_{4',5',6'}$ = 7.8 Hz); 7.84 (d, 1 H, $H_{6'}$, Ph, $J_{5',6'}$ = 8.0 Hz); 7.94 (d, 1 H, $H_{4'}$, Ph, $J_{4',5'}$ = 7.9 Hz), 8.31 (d, 1 H, H_4, Py, $J_{4',5'}$ = 7.7 Hz); 8.48 (m, 1 H, H_6,

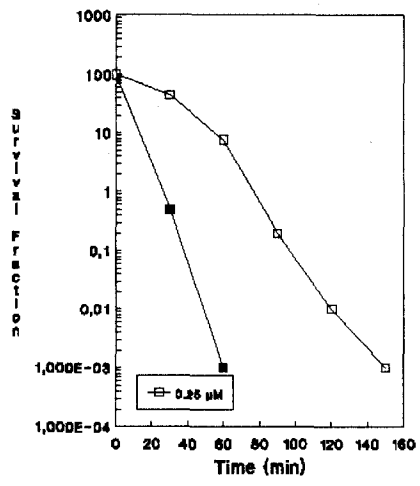


Fig 2. Survival fraction of V79 cells exposed to nitracrine (0.25 µM) under aerobic (open symbols) or hypoxic (filled symbols) conditions.

Py); 8.91 (s, 1 H, H_2, Ph); 10.76 (s, 1 H, NH); 13.76 (s, 1 H, COOH), MS (EI) m/e (70 eV) = 259 (M, 100); 240 (41); 214 (14); 122 (28); anal $C_{12}H_9N_3O_4 \cdot 0.5H_2O$ (C, H, N).

5-Nitro-1,9-diazaanthracene-10-one (isomer A) and 7-nitro-1,9-diazaanthracene-10-one (isomer B) **2**

A mixture of 2-(*m*-nitrophenylamino)nicotinic acid **1** (10.00 g, 38.60 mmol) and polyphosphoric acid (400 g) was stirred and heated at 140°C for 16–18 h. The resulting solution was diluted in water (700 mL), the precipitate was filtered off and washed with 2 N NaOH (300 mL) in order to eliminate the excess polyphosphoric acid. It was then dried at 130°C (P_2O_5), giving **2** (7.83 g, 84% yield): mp > 300°C; NMR (DMSO-d_6) δ = 7.43 (m, 2 H, H_3 isomer A, H_3 isomer B); 7.55 (m, 2 H, H_7 isomer A, H_5 isomer B); 7.90 (m, 4 H, H_6 H_8 isomer A, H_6 H_8 isomer B); 8.54 (d, 2 H, H_2 isomer A, H_2 isomer B, $J_{2,3}$ = 7.6 Hz); 8.87 (m, 2 H, H_4 isomer A, H_4 isomer B); 12.75 (bs, 2 H, NH isomer A, NH isomer B); MS (EI) m/e (70 eV)= 241 (M, 100); 195 (36); 183 (12); 167 (18); 140 (23); anal $C_{12}H_7N_3O_3$ (C, H, N).

10-Chloro-5-nitro-1,9-diazaanthracene **3**

A complete mixture of 5-nitro-1,9-diazaanthracene-10-one **2** (1.50 g, 6.22 mmol) and phosphorus oxychloride (30 mL) was refluxed for 6 h. After removal of the excess phosphorus oxychloride, the dark oil was dissolved in dichloromethane (100 mL) and poured over ice/NH_4OH (250 g/5 mL). The organic layer was separated and the organic compound was extracted with additional dichloromethane (5 x 100 mL) and dried over Na_2SO_4. After removal of the solvent, a yellow solid was obtained. HPLC analysis showed the mixture of the 2 isomers: 5-nitro and 7-nitro in a ratio of 81:19. Flash chromatography by eluting with a gradient of CH_2Cl_2/EtOAc afforded 5-nitro-isomer **3** first and then, the 7-nitro-isomer (0.90 g, 56% yield): R_f (dioxane)= 0.7; mp > 300 °C; NMR (DMSO-d_6) δ = 7.40 (m, 1 H, H_3); 7.52 (m, 1 H, H_7); 7.86 (m, 2 H, H_6, H_8); 8.52 (d, 1 H, H_2, $J_{2,3}$ = 7.7 Hz); 8.84 (m, 1 H, H_4); MS (EI) m/e (70 eV) = 259 (M, 100); 241 (53); 224 (58); 178 (40); anal $C_{12}H_6ClN_3O_2$ (C, H, N).

10-[3-(N,N-Dimethyl)aminopropylamino]-5-nitro-1,9-diazaanthracene **4**

A mixture of 10-chloro-5-nitro-1,9-diazaanthracene **3** (0.90 g, 3.47 mmol) and phenol (2.70 g, 28.72 mmol) was heated at 100°C for 45 min. After cooling to below 50°C, 3-(*N,N*-dimethylamino)propylamine (0.36 g, 3.53 mmol) was added. The resulting solution was heated at 70°C for 2 h and then it was set aside at room temperature. The mixture was dissolved in dichloromethane (75 mL) and washed with 2 N NaOH (800 mL) and water (150 mL). The organic layer was dried over Na_2SO_4 and filtered off. The solvent was eliminated giving a reddish oil which was dissolved in methanol (30 mL) and passed through celite–charcoal. Methanol was removed *in vacuo* and the oil was dissolved in ethyl acetate (15 mL). Compound **4** was precipitated by the addition of methanol (1 mL) and a few drops of concentrated HCl. Due to the precipitate's strong hygroscopic properties, it became oil-like when filtration was carried out. A more convenient method consisted of treating the oil *in vacuo* at 80°C (P_2O_5) (53 mg, 3% yield): R_f (MeOH) = 0; NMR (DMSO-d_6) δ = 1.73 (m, 2 H, CH_2); 2.30 (s, 6 H, 2 CH_3); 2.50 (m, 2 H, NCH_2); 3.68 (m, 2 H, NCH_2); 7.15 (c, 1 H, H_3, $J_{2,3,4}$ = 4.9 Hz); 7.35 (m, 1 H, H_7); 7.55 (m, 2 H, H_4, H_8); 8.25 (d, 1 H, H6, $J_{6,7}$ = 7.6 Hz); 8.47 (d, 1 H, H_2, $J_{2,3}$ = 4.0 Hz); 11.38 (bs, 1 H, NH); MS (EI) m/e (70 eV)= 325 (M, 2); 253 (34); 241 (96); 58 (100); anal $C_{17}H_{19}N_5O_2 \cdot 2HCl \cdot 2.5H_2O$ (C, H, N).

10-[2-(N,N-Dimethyl) aminoethylamino]-5-nitro-1,9-diazaanthracene **5**

A mixture of 10-chloro-5-nitro-1,9-diazaanthracene **3** (1.01 g, 3.89 mmol) and phenol (3.10 g, 32.98 mmol) was heated at 100°C for 45 min. After cooling to below 50°C, 3-(*N,N*-dimethylamino)ethylamine (0.35 g, 3.98 mmol) was added. The resulting solution was heated at 55°C for 1 h and set aside at room temperature. The mixture was dissolved in dichloromethane (75 mL) and washed with 2 N NaOH (800 mL) and water (150 mL). The organic layer was dried over Na_2SO_4 and filtered off. The solvent was eliminated, giving a reddish oil which was dissolved in methanol (30 mL) and passed through celite–charcoal. Methanol was removed *in vacuo* and the oil was dissolved in ethyl acetate (15 mL). Compound **5** was precipitated by adding methanol (1 mL) and a few drops of concentrated HCl. The resulting oil was solidified by heating *in vacuo* at 80°C (P_2O_5) (0.53 g, 38% yield): R_f (MeOH) = 0; mp: 240–245°C (d); NMR (DMSO-d_6) δ = 2.74 (s, 6 H, $N(CH_3)_2$); 3.23 (t, 2 H, CH_2N, J = 5.8 Hz), 4.07 (t, 2 H, NCH_2, J = 5.9 Hz); 7.20 (m, 1 H, H_7); 7.40 (m, 1 H, H_3); 7.59 (m, 2 H, H_4, H_8); 8.28 (d, 1 H, H_6, $J_{6,7}$ = 8.0 Hz); 8.52 (d, 1 H, H_2, $J_{2,3}$ = 4.5 Hz); 10.25 (bs, 1 H, NH); 11.57 (s, 1 H, NH); MS (EI) m/e (70 eV)= 311 (M, 0.2); 294 (2); 179 (4); 58 (100); anal $C_{16}H_{17}N_5O_2 \cdot HCl \cdot 0.5H_2O$ (C, H, N); N: calc 19.63; found 19.13.

10-(2-Hydroxyethylamino)-5-nitro-1,9-diazaanthracene **6**

A mixture of 10-chloro-5-nitro-1,9-diazaanthracene **3** (0.67 g, 2.58 mmol) and phenol (2.10 g, 22.34 mmol) was heated at 100°C for 1 h. After cooling to below 50°C, 2-aminoethanol (0.16 g, 2.62 mmol) was added. The resulting solution was heated at 65°C (rigorous control of temperature was necessary) for 2 h and set aside at room temperature. The mixture was dissolved in dichloromethane (100 mL) and washed with 2 N NaOH (800 mL) and water (300 mL). The organic layer was dried over Na_2SO_4 and filtered off. The solvent was eliminated giving a reddish oil which was dissolved in methanol (70 mL) and passed through celite–charcoal. Methanol was removed *in vacuo* and the oil was dissolved in ethyl acetate (15 mL). Compound **6** was precipitated by adding methanol (1 mL) and a few drops of concentrated HCl. The yellow solid was filtered off and dried at 80°C (P_2O_5). (0.11 g, 11% yield): R_f (MeOH) = 0; mp: 172°C. NMR (DMSO-d_6) δ = 2.84 (m, 2 H, NCH_2); 3.68 (m, 2 H, CH_2OH); 5.74 (bs, 1 H, OH); 7.07 (d, 1 H, H_8, $J_{7,8}$ = 8.3 Hz); 7.58 (m, 1 H, H_4); 8.08 (m, 2 H, H_3, H_7); 8.93–9.12 (m, 2 H, H_2, H_6); 10.62 (bs, 1 H, NH); 13.76 (bs, 1 H, NH); MS (EI) m/e (70 eV) = 284 (M, 6); 253 (100); 223 (13); 207 (21); 180 (34); anal $C_{14}H_{12}N_4O_3 \cdot 2HCl \cdot 2.2H_2O$ (C, H, N).

Biology

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

Cells

V79 cells (Chinese hamster lung fibroblasts) [17] 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 x 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 erlenmeyers: 2 x 10^4 cells/mL in 30 mL of EMEM-containing 10% v/v FBS and HEPES 10 mM. The erlenmeyers were tightly closed with rubber caps which were perforated with 2 needles of 19G·40 mm to provide gas inlet and outlet. Erlenmeyers were submerged and stirred in a water bath at 37°C, 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 dimethylsulfoxide (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 with 0.2 mL DMSO (negative control) and another with nitracrine 0.06 μM (positive control) for screening, treatment lasted 2 h, during which time, 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°C in 5% CO_2 for 7 d 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 surviving fraction (SF) is the percentage of PE of treated cultures with respect to the control.

Screening assays

Compounds were tested at 10 μM in duplicate flasks in both aerobic and hypoxic conditions. Compounds active at 10 μM were tested at 5 and 1 μM.

Time-response assays

The compounds, which were selectively toxic in hypoxia, were assayed for different durations, from 30 min to 2.5 h.

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