Pergamon

Biochemical Pharmacology, Vol. 48, No. 8, pp. 1593–1604, 1994.
Elsevier Science Ltd
Printed in Great Britain.
0006-2952/94 $7.00 + 0.00

0006-2952(94)00328-9

METABOLIC AND RADIOLYTIC REDUCTION OF 4-ALKYLAMINO-5-NITROQUINOLINE BIOREDUCTIVE DRUGS

RELATIONSHIP TO HYPOXIA-SELECTIVE CYTOTOXICITY

BRONWYN G. SIIM,* GRAHAM J. ATWELL† and WILLIAM R. WILSON

Section of Oncology, Department of Pathology, and †Cancer Research Laboratory, University of Auckland School of Medicine, Auckland, New Zealand

(*Received* 25 *February* 1994; *accepted* 16 *June* 1994)

Abstract—The 4-alkylamino-5-nitroquinolines (5NQs) are a new series of bioreductive drugs that exhibit varying degrees of selective toxicity (up to 60-fold) under hypoxic conditions in cell culture. This study tested the hypothesis that differences in hypoxia-selective cytotoxicity in this series reflect differences in the efficiency with which oxygen inhibits metabolic reduction. The products of reduction of six 5NQs were characterized and rates of reduction compared in aerobic and hypoxic AA8 cells. The major stable products of both radiolytic and metabolic reduction under anoxic conditions were the corresponding amines, which were not responsible for the toxicity of the parent nitro compounds. Metabolism of each compound was inhibited completely in aerobic cells, indicating that differences in hypoxia-selective toxicity in this series are not due to variations in efficiency as substrates for oxygen-insensitive nitro reduction. Rates of hypoxic metabolism correlated broadly with hypoxia-selective cytotoxicity; the 5NQ derivatives with high rates of hypoxic metabolism had good hypoxia-selective cytotoxicity, whereas the compounds with low rates of reduction (the 3,6-dimethyl and 8-methylamino compounds; 3,6diMe-5NQ and 8NHMe-5NQ) were non-selective. Low rates of drug-induced oxygen consumption by 3,6diMe-5NQ and 8NHMe-5NQ in respiration-inhibited cells confirmed that these compounds are poor substrates for enzymatic nitro reduction. While there was an overall correlation between one-electron reduction potential at pH 7 (E_7^1) and rate of metabolic reduction, the relatively high E_7^1 of 3,6diMe-5NQ (−367 mV) indicates that rates of reduction, and hypoxic selectivity of cytotoxicity, cannot be predicted from reduction potential alone. 3,6diMe-5NQ and 8NHMe-5NQ are cytotoxic through a non-bioreductive mechanism, the variable contribution of which may underlie the differences in hypoxia-selective cytotoxicity within this series of bioreductive drugs.

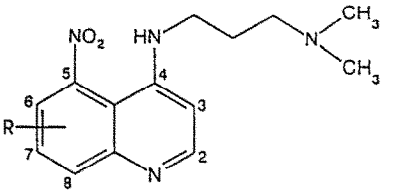
Key words: nitroquinoline; bioreductive drugs; hypoxia-selective cytotoxicity; radiolytic reduction; metabolic reduction; HPLC

Hypoxic cells in solid tumours are resistant to ionizing radiation and to some cytotoxic drugs, and are considered to contribute to treatment failure in both radiotherapy and chemotherapy regimens [1–3]. There is current interest in developing drugs that are selectively toxic under hypoxic conditions and can thus be used to eliminate these resistant tumour cells [4–6]. Most agents of interest are activated by reduction and are hence termed bioreductive drugs. Several types of functional groups are known to undergo oxygen-sensitive reduction in tumour cells, examples being provided by nitro compounds, quinones, aromatic and aliphatic *N*-oxides, and cobalt complexes. Examples of each of these classes are being examined as potential clinical agents for use in combination with radiation or other treatment modalities that might be limited by the presence of hypoxic cells (e.g. chemotherapy, photodynamic therapy, radioimmunotherapy).

A key parameter in bioreductive drug development, although not the only one relevant to activity *in vivo*, is the magnitude of the selective toxicity of the drugs for hypoxic versus oxygenated cells as measured in cell culture. Wide variations in hypoxia-selective cytotoxicity are observed between different bioreductive drugs, often even within a closely related series [7–10], although only in a few cases has it been possible to establish the reasons for this variation. For quinone bioreductive drugs, hypoxia-selective cytotoxicity appears to be determined largely by the relative contribution of oxygen-sensitive and oxygen-insensitive activation, the latter by enzymes such as NAD(P)H:quinone oxidoreductase (DT diaphorase) [11–13]. It is clearly important to understand the determinants of hypoxia-selective cytotoxicity of bioreductive drugs if further progress towards their rational development is to be made.

* Corresponding author. Current address: Department of Radiation Oncology, Stanford University, Stanford, CA 94305-5468, U.S.A. Tel. (415) 723-7439; FAX (415) 723-7382.

Table 1. Structures, one-electron reduction potentials at pH 7 (E_7^1), and *in vitro* cytotoxicity data for the 5NQs



Compound R	Abbreviation	E_7^1 (mV)	Aerobic CT_{10} (μM.hr)	Hypoxic selectivity CT_{10} (air/N_2)
H	5NQ	−286 ± 11*	660 ± 60*	14 ± 2*
8Me-	8Me-5NQ	−316 ± 11*	2100 ± 380*	61 ± 10*
3Me-	3Me-5NQ	−369 ± 10*	1870 ± 170*	47 ± 11*
3,8diMe-	3,8diMe-5NQ	−334 ± 10†	3790 ± 260†	20 ± 8†
3,6diMe-	3,6diMe-5NQ	−367 ± 8†	6630 ± 1900†	1.1 ± 0.0†
8NHMe-	8NHMe-5NQ	−520 ± 12*	2100 ± 600*	1.2 ± 0.1*

* Data from [14].
† Data from [15], with additional determinations.

A series of 5NQ* bioreductive drugs has been reported recently to display large variations in hypoxia-selective cytotoxicity towards the Chinese hamster AA8 cell line [14]. The 8-methyl-substituted congener (8Me-5NQ; Table 1) shows high selectivity (61-fold) for hypoxic cells, the parent compound (5NQ) shows moderate selectivity (14-fold), and the 8-methylamino derivative (8NHMe-5NQ) is essentially non-selective (1.2-fold), this pattern being observed in three tumour cell lines from different species [16]. These and related compounds therefore provide an opportunity to investigate systematically the determinants of hypoxia-selective cytotoxicity within a congeneric drug series. The two dimethyl-substituted compounds, 3,8diMe-5NQ and 3,6diMe-5NQ, are of particular interest; they possess similar E_7^1s values (−334 and −367 mV, respectively) yet display quite different selective toxicities for hypoxic AA8 cells with the 3,8diMe-derivative having a selectivity of 20-fold while the 3,6diMe-derivative is non-selective (Table 1).

The hypothesis that the observed variations in hypoxia-selective cytotoxicity reflect differences in the efficiency with which oxygen inhibits metabolic reduction of these compounds was tested in the present study. The chemical (radiolytic) and metabolic reduction of six representative 5NQs was investigated to characterize their reduction products and to compare rates of nitro reduction in aerobic and hypoxic AA8 cell cultures. Rates of oxygen consumption due to oxidation of nitro reduction intermediates in aerobic cultures were also assessed to provide further information on the rates of cellular metabolism of these compounds.

* Abbreviations: 5AQ, 4-alkylamino-5-aminoquinoline; CM, culture medium (α-minimal essential medium containing 5% (v/v) heat-inactivated FBS plus 100 IU/mL penicillin and 100 μg/mL streptomycin); CT_{10}, concentration·time to reduce cell survival to 10%; E_7^1, one-electron reduction potential at pH 7; $E_{\frac{1}{2}}$, polarographic half-wave reduction potential; EtOAc, ethyl acetate; FBS, fetal bovine serum; MeCN, acetonitrile; 80% MeCN, 80% acetonitrile/20% H_2O (v/v); MeOH, methanol; MISO, misonidazole; 5NQ, 4-alkylamino-5-nitroquinoline; 8NQL, 8-nitroquinoline; R_t, retention time.

† Atwell GJ, Pullen SM, Wilson WR and Denny WA, Manuscript in preparation.

MATERIALS AND METHODS

Chemicals

5NQ [17], 8Me-5NQ, 8NHMe-5NQ, 3Me-5NQ [14], 3,8diMe-5NQ, 3,6diMe-5NQ† and 5AQ [18] were synthesized as dihydrochlorides by methods detailed elsewhere. Stock solutions were prepared in 50% aqueous EtOH. A stock solution of 8NQL (purchased from Aldrich-Chemie GMBH & Co.) was prepared in dimethyl sulfoxide. MISO, a gift from the National Cancer Institute (Bethesda, MD, U.S.A.), was dissolved in CM and filter sterilized. All stock solutions were stored at −80°. Concentrations were checked in each experiment by dilution in 0.01 N HCl using extinction coefficients of 5890 $M^{-1}cm^{-1}$ at 350 nm for 5NQ; 7110 $M^{-1}cm^{-1}$ at 352 nm for 8Me-5NQ; 15,400 $M^{-1}cm^{-1}$ at 438 nm for 8NHMe-5NQ; 6120 $M^{-1}cm^{-1}$ at 376 nm for 3,8diMe-5NQ; 7470 $M^{-1}cm^{-1}$ at 350 nm for 3,6diMe-5NQ; 5760 $M^{-1}cm^{-1}$ at 376 nm for 3Me-5NQ; 7750 $M^{-1}cm^{-1}$ at 326 nm for MISO; and 9830 $M^{-1}cm^{-1}$ at 316 nm for 8NQL. [U-^{14}C]Sucrose (137 MBq/mmol) and ^3H_2O (47 MBq/mmol) were purchased from NEN Research Products (Boston, MA, U.S.A.).

Synthesis of amines

Preparation of 5-amino-4-[[3-(dimethylamino)-propyl]amino]-8-methylquinoline (8Me-5AQ). A solution of 8Me-5NQ (0.04 g, 1.1 mmol) in MeOH (20 mL) was hydrogenated over 5% Pd/C (50 mg)

at 4 atmospheres for 1 hr. The catalyst was filtered off, the solvent was evaporated *in vacuo* below 30°, and the residue was recrystallized three times from MeOH/EtOAc (decolorizing charcoal) to give 0.28 g (76%) of 8Me-5AQ as the dihydrochloride salt: m.p. 245–246°; ^{1}H NMR (400 MHz, $(CD_3)_2SO$) δ 12.85 (bs, 1H, NH), 11.04 (bs, 2H, NH_2), 8.25 (d, J = 7.1 Hz, 1H, H-2), 7.50 (d, J = 8.1 Hz, 1H, H-7), 7.04 (d, J = 7.9 Hz, 1H, H-6), 6.80 (d, J = 7.27 Hz, 1H, H-3), 3.63 (t, J = 4.2 Hz, 2H, $NHCH_2CH_2C\underline{H_2}$), 3.22 (t, J = 5.0 Hz, 2H, $NHC\underline{H_2}$), 2.75 (s, 6H, $N(CH_3)_2$), 2.48 (s, 3H, 8-CH_3), 2.12 (quintet, J = 7.3 Hz, 2H, $CH_2C\underline{H_2}CH_2$). Anal. ($C_{15}H_{22}N_4 \cdot 2HCl \cdot \frac{1}{2}H_2O$) C,H,N,Cl.

The following compounds were prepared by the above general procedure.

5-Amino-4-[[3-(dimethylamino)propyl]amino]-3-methylquinoline (3Me-5AQ) was prepared from 3Me-5NQ and obtained as the dihydrochloride salt: m.p. 270–271° (from MeOH/EtOAc); ^{1}H NMR (400 MHz, $(CD_3)_2SO$) δ 8.22 (s, 1H, H-2), 7.54 (t, J = 8.0 Hz, 1H, H-7), 7.22 (d, J = 8.1 Hz, 1H, H-8), 6.98 (d, J = 7.7 Hz, 1H, H-6), 3.80 (t, J = 7.1 Hz, 2H, $NHCH_2CH_2C\underline{H_2}$), 3.10 (t, J = 7.6 Hz, 2H, $NHC\underline{H_2}$), 2.71 (s, 6H, $N(CH_3)_2$), 2.47 (s, 3H, 3-CH_3), 2.07 (quintet, J = 7.4 Hz, 2H, $CH_2C\underline{H_2}CH_2$). Anal. ($C_{15}H_{22}N_4.2HCl.H_2O$) C,H,N,Cl.

5-Amino-3,6-dimethyl-4-[[3-(dimethylamino)-propyl]amino]quinoline (3,6diMe-5AQ) was prepared from 3,6diMe-5NQ and obtained as the dihydrochloride salt: m.p. 275–276° (from MeOH/EtOAc); ^{1}H NMR (400 MHz, $(CD_3)_2SO$) δ 8.22 (s, 1H, H-2), 7.52 (d, J = 8.4 Hz, 1H, H-7), 7.19 (d, J = 8.4 Hz, 1H, H-8), 3.75 (t, J = 6.8 Hz, 2H, $NHC\underline{H_2}$), 3.08 (t, J = 7.7 Hz, 2H, $C\underline{H_2}N(CH_3)_2$), 2.71 (s, 6H, $N(CH_3)_2$), 2.46 (s, 3H, 3-CH_3), 2.30 (s, 3H, 6-CH_3), 2.05 (quintet, J = 7.4 Hz, 2H, $CH_2C\underline{H_2}CH_2$). Anal. ($C_{16}H_{24}N_4.2HCl.H_2O$) C,H,N,Cl.

5-Amino-3,8-dimethyl-4-[[3-(dimethylamino)-propyl]amino]quinoline (3,8diMe-5AQ) was prepared from 3,8diMe-5NQ and obtained as the dihydrochloride salt: m.p. 280–281° (from MeOH/EtOAc); ^{1}H NMR (400 MHz, $(CD_3)_2SO$) δ 12.7 (bs, 1H, NH), 10.9 (vbs, 2H, NH_2), 8.10 (s, 1H, H-2), 7.44 (d, J = 8.1 Hz, 1H, H-7), 6.95 (d, J = 7.8 Hz, 1H, H-6), 3.83 (t, J = 6.6 Hz, 2H, $NHCH_2CH_2C\underline{H_2}$), 3.12 (t, J = 7.7 Hz, 2H, $NHC\underline{H_2}$), 2.73 (s, 6H, $N(CH_3)_2$), 2.51 (s, 3H, 8-CH_3), 2.45 (s, 3H, 3-CH_3), 2.07 (quintet, J = 4.9 Hz, 2H, $CH_2C\underline{H_2}CH_2$). Anal. ($C_{16}H_{24}N_4.2HCl.2H_2O$) C,H,N,Cl.

Radiolytic reduction

Compounds were reduced with controlled stoichiometry by steady-state radiolysis of solutions in sodium formate, essentially as described by Whillans and Whitmore [19]. Drug solutions (50 μM) were prepared in 0.085 M sodium phosphate buffer (pH 7.0) containing 0.1 M sodium formate. Samples (5 mL) were irradiated in glassware consisting of a modified Schlenk tube fitted with a B24 cone. The solution was deaerated by evacuation and irradiated at room temperature using a ^{60}Co source. The dose rate (1.28 ± 0.02 Gy/sec) was determined by Fricke dosimetry [20] under identical conditions. The irradiation time, t_n, required to deliver n reducing equivalents is given by:

$$t_n = \frac{nN}{GRw}$$

where N is the number of moles of drug irradiated, G is the radiation chemical yield (0.61 μmol/J; [19]), R is the dose rate in Gy/sec, and w is the weight of the solution irradiated in kg. After irradiation, a 0.25-mL sample was analysed immediately by HPLC.

HPLC analysis

The HPLC system was a Waters MR600 programmable solvent delivery system with a refrigerated (4°) Waters WISP autosampler and a C18 μBondapak column (8 × 100 mm). The eluate was monitored with an HP1040A diode array absorbance detector and analysed using HP 79996A software on a Hewlett–Packard 310 computer. The mobile phase consisted of formate buffer, pH 4.5 (0.44 M ammonium formate, 68 mM formic acid) as the aqueous component and 80% MeCN as the organic component, both continuously sparged with helium. Compounds were eluted isocratically for 10 min at a flow rate of 1.8 mL/min with the mobile phase composition shown in Table 2 followed by a linear gradient over 5 min to 40:60 (v/v) formate buffer/80% MeCN. Quantitation was based on peak areas using absorbance at the wavelength ranges specified in Table 2.

Cells

The Chinese hamster cell lines AA8 and UV4 [21] were maintained in logarithmic-phase growth in tissue culture flasks with weekly subculture by trypsinization using antibiotic-free CM. Bulk cultures of late log-phase AA8 cells (1.0–1.2 × 10^6 cells/mL) were grown for experiments in spinner flasks in CM containing 10% (v/v) FBS by adjusting the cell density to 5 × 10^5 cells/mL 18 hr before use. Cells were harvested by centrifugation and resuspended to the required density in CM.

Drug metabolism in cell suspensions

Magnetically stirred AA8 cell suspensions (typically 50 mL) at 10^6–10^7 cells/mL were equilibrated for 90 min at 37° with continuous gassing under humidified air or nitrogen containing 5% CO_2. Drug exposure was initiated by addition of a small volume of a concentrated drug solution to give a final concentration of 40 μM unless otherwise specified. Samples containing 10^7 cells were centrifuged, and the extracellular medium and cell pellet were precipitated with MeCN to prepare samples for HPLC as described previously [22].

All drug concentrations were corrected for measured recovery efficiency. Recovery of intracellular drug was estimated by lysing non-drug-treated cell pellets with water containing the analyte in an appropriate concentration range (determined by preliminary estimates of intracellular drug concentrations), with preparation for HPLC as above. Recovery of drug from the extracellular fluid was determined by sampling 50-μL aliquots of

solutions of known concentration in CM with sample preparation as above.

Intracellular and extracellular water volumes in AA8 cell pellets

Cell pellets (10^7 cells) were prepared by sampling 1-mL aliquots from suspensions of late log-phase AA8 cells as above 10 min after addition of [U-^{14}C]-sucrose to 17 kBq/mL and ^3H_2O to 28 kBq/mL to mark the extracellular and total water volume, respectively [23]. Radioactivity in aliquots of the supernatant (30 μL) was determined by scintillation counting using a water-accepting cocktail. Activity in the cell pellets was determined by counting MeCN extracts prepared as above. The volume of extracellular medium trapped in the cell pellet (V_e) was calculated from its ^{14}C activity, the total water volume of the pellet (V_t) from its ^{3}H activity, and the intracellular water volume (V_i) from the difference between these two values. These values (mean ± SEM, N = 20), determined in three independent experiments, were $V_t = 12.7 \pm 0.3$ μL, $V_e = 1.1 \pm 0.1$ μL, and $V_i = 11.6 \pm 0.3$ μL.

Cell growth inhibition

Aerobic growth inhibition was assessed by methods described previously [24, 25] by exposing log-phase monolayers to drugs for 18 hr in 96-well tissue culture plates, and then growing without drugs for 3 days. The IC_{50} was the drug concentration required to reduce cell protein, assessed by staining with methylene blue, to 50% of that in controls on the same 96-well plate.

Drug-induced oxygen consumption in respiration-inhibited AA8 cells

Measurement of drug-induced oxygen consumption in cyanide-inhibited cells was based on the method of Biaglow *et al.* [26]. Aliquots (7 mL) of cell suspension at 10^7/mL in CM containing 3 mM KCN were transferred to a glass respiration vial fitted with a ceramic spin bar and lid through which an oxygen electrode [27] was inserted. The vial was equilibrated with magnetic stirring in a 37° waterbath until the background rate of oxygen consumption was linear. A small volume of a concentrated drug solution was added to give a final concentration of 200 μM, and the initial rate of oxygen consumption was recorded. Before each experiment the oxygen electrode was calibrated using CM equilibrated with 20% O_2 at 37°, in which the concentration of dissolved oxygen was assumed to be 202 μM.

RESULTS

Radiolytic reduction

Reduction of 8Me-5NQ by $CO_2^{\cdot-}$ was investigated by radiolysis of anaerobic sodium formate solutions. HPLC showed good resolution of the parent drug ($R_t = 8.0$ min) and the major reduction product ($R_t = 9.7$ min). The latter reached its highest concentration after addition of six reducing equivalents, suggesting it to be the corresponding amine, 8Me-5AQ (Fig. 1). Its identity was confirmed by comparison of retention time (R_t) and spectral characteristics with synthetic 8Me-5AQ (Table 2, Fig. 2). Other minor reduction products were observed, including one ($R_t = 6.7$ min) that was detected in highest concentration after addition of four reducing equivalents, and may thus represent the corresponding hydroxylamine. The absorbance spectrum of this product showed maxima similar to those of the amine (Fig. 2); since both $-NH_2$ and $-NHOH$ are electron-donating, this also suggests it to be the hydroxylamine. The presence of the hydroxylamine as a distinct intermediate is also consistent with the initial slope of the curve for the parent nitro compound in Fig. 1, which extrapolates

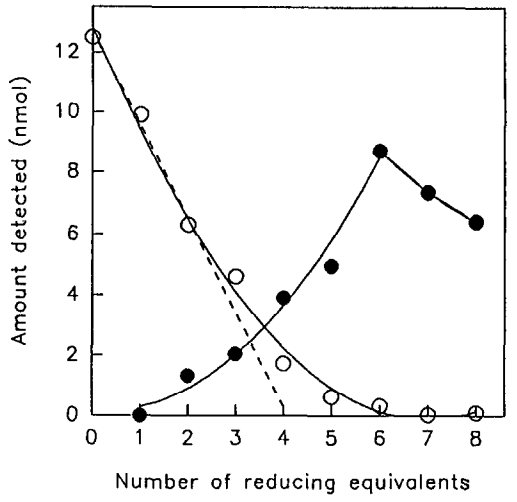


Fig. 1. Amount of 8Me-5NQ (○) and 8Me-5AQ (●) detected by HPLC as a function of reducing equivalents after radiolytic reduction of 8Me-5NQ (50 μM). The dashed line shows the initial slope for loss of 8Me-5NQ (linear regression fitted from 0–2 equivalents).

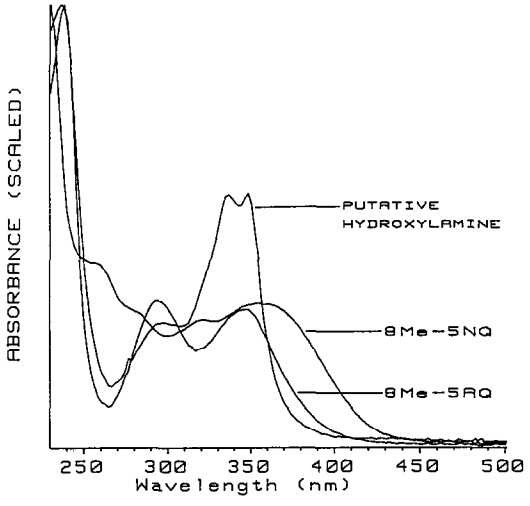


Fig. 2. Absorbance spectra of 8Me-5NQ and major products after radiolytic reduction with four reducing equivalents, determined with a diode array HPLC detector.

Table 2. HPLC conditions, retention times (R_t), peak areas/nmol, yield of amines following radiolytic reduction, and recovery efficiencies

Compound	Mobile phase* (first 10 min)	R_t (min)	Signal† (nm)	Peak area‡ (mAU·sec/nmol)	Yield of amine§ (%)	Recovery %‖ Intracellular	Extracellular
5NQ	90:10	10.2	345	188		57–79¶	69 ± 9
5AQ		11.0	345	301	76	41 ± 1	61 ± 2
8Me-5NQ	85:15	8.0	281	208		50–89¶	100 ± 2
8Me-5AQ		9.7	281	135	70	57 ± 1	66 ± 4
8NHMe-5NQ	70:30**	10.2	268	207	ND††	61–90¶	68 ± 5
3Me-5NQ	85:15	9.8	376	190		48–88¶	80 ± 3
3Me-5AQ		6.2	376	157	23	48 ± 3	43 ± 4
3,8diMe-5NQ	82:18	9.9	376	223		62–86¶	77 ± 8
3,8diMe-5AQ		5.8	376	186	50	44 ± 2	47 ± 2
3,6diMe-5NQ	75:25	8.1	350	226		78 ± 1	75 ± 2
3,6diMe-5AQ		4.4	350	196	26	49 ± 4	66 ± 2
MISO	60:40	8.7	326	268	ND	84 ± 6	84 ± 2
8NQL	90:10	8.8	316	129	ND	49 ± 5	59 ± 5

* Formate buffer/80% MeCN, v/v.
† Signal used for quantitation referenced to absorbance at 550 nm. A bandwidth of 10 nm was used in each case.
‡ Peak area/nmol, pathlength of 10 mm.
§ Yield of amine after radiolytic reduction of parent nitro compound with six reducing equivalents.
‖ Recovery efficiency was determined from HPLC analysis. Values are means ± SEM for triplicate samples.
¶ Range for recovery efficiency for analyte concentrations between 20–3000 μM.
** HPLC analysis of cellular metabolism of 8NHMe-5NQ was carried out using an initial mobile phase composition of 80:20.
†† Not determined.

to four reducing equivalents. A peak at R_t = 18.3 min had a retention and spectrum consistent with hydrolysis of the 4-sidechain of a nitroreduction product to give the corresponding quinolone. Attempts to confirm this by radiolytic reduction of 8-methyl-5-nitroquinolone were unsuccessful due to the insolubility of the latter in aqueous solutions.

Radiolytic reduction of 5NQ (R_t = 10.2 min) gave an essentially identical result with the major product, the corresponding amine 5AQ (R_t = 11.0 min), present in highest concentration after addition of six reducing equivalents (Table 2). A minor reduction product (R_t = 7.9 min) present after one reducing equivalent had an absorbance spectrum similar to that of the four-electron reduction product of 8Me-5NQ. A number of products observed at late retention times (17–19 min) probably represent reduction products that have lost the 4-alkylamino sidechain by hydrolysis.

Radiolytic reduction of 3,8diMe-5NQ, 3,6diMe-5NQ and 3Me-5NQ at a single dose of six reducing equivalents gave the corresponding amines as major products, but in lower yield than for 8Me-5NQ and 5NQ (Table 2). For both dimethyl compounds a second reduction product was observed at a R_t of 20.9 min, although these two products had different spectra. Reduction of 8NHMe-5NQ (R_t = 10.2 min) gave two products (R_t = 3.1 and 3.4 min), the latter of which was maximal after addition of between six to seven reducing equivalents. Attempts to synthesize 8NHMe-5AQ gave mixtures of products, including the two species observed on radiolytic reduction of 8NHMe-5NQ, suggesting that this triaminoquinoline is oxidatively unstable.

Metabolism

MeCN extraction from centrifugally packed cell pellets and from CM spiked with parent nitro compounds and amines indicated acceptable recoveries from both the intracellular and extracellular compartments (Table 2). Extracellular recovery was independent of analyte concentration over the concentration range investigated (4–40 μM). However, the recovery of some nitro compounds from cells decreased at low analyte concentration (Table 2). For such compounds an iterative calculation was used to determine the appropriate recovery efficiency.

8Me-5NQ concentrations decreased slowly in both the intracellular and extracellular compartments of hypoxic AA8 cell cultures at 10^6 cells/mL (Fig. 3A). The amine 8Me-5AQ, identified by R_t and spectrum, was the sole metabolite detected. Uptake factors (intracellular/extracellular drug concentrations, C_i/C_e) for the nitro compound were calculated by extrapolating to the time of drug addition. Under aerobic conditions the ratio of C_i/C_e was 50 for 8Me-5NQ, 2-fold higher than under hypoxia (Table 3). The intracellular concentration of amine was also higher than in the extracellular medium with a C_i/C_e ratio of 40 at 5 hr (Fig. 3B). However, since the extracellular volume was 10^3 times greater than the intracellular volume, most of the 8Me-5AQ (and 8Me-5NQ) was in the extracellular compartment.

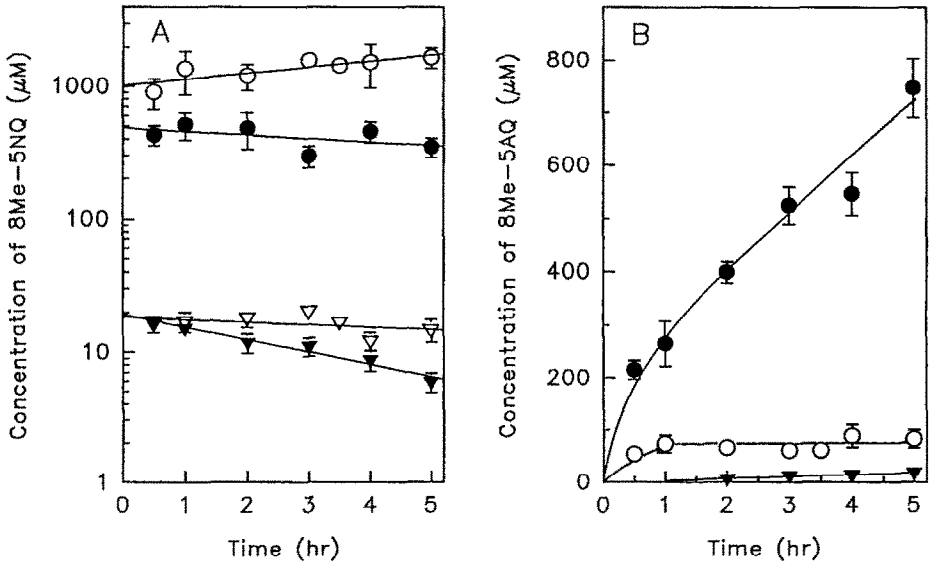


Fig. 3. Metabolism of 8Me-5NQ (A) and formation of 8Me-5AQ (B) in AA8 cultures (10^6 cells/mL) exposed to 40 μM 8Me-5NQ under aerobic (open symbols) and hypoxic conditions (filled symbols); circles, intracellular concentrations; triangles, extracellular concentrations. Values are means ± range for two experiments.

Summing the two compartments gave a 96% yield of amine at 5 hr relative to the amount of nitro compound consumed.

Under aerobic conditions metabolism of 8Me-5NQ was inhibited, with no appreciable loss of parent compound with time and only low concentrations of the corresponding amine observed (detected only in the intracellular compartment), which did not increase with time (Fig. 3B). Further experiments indicated that the amine detected in aerobic cells was the result of metabolism occurring during preparation of HPLC samples, presumably the result of centrifugally packed cell pellets rapidly becoming hypoxic. Investigation of alternative methods of sample preparation, such as filtration of cells onto glass fiber filters followed by lysis with H_2O and rapid washing with MeCN, gave lower ratios of 8Me-5AQ/8Me-5NQ, but results were too variable for routine use of this method.

Preliminary investigation of the other compounds indicated that, with the exception of 5NQ, the rates of hypoxic metabolism were much slower than that of 8Me-5NQ. Raising the cell density from 10^6 to 10^7 cells/mL decreased the half-life for loss of 8Me-5NQ from 2.8 to 0.45 hr in hypoxic cultures and increased the initial rate of amine formation from

Table 3. Summary of drug uptake and metabolism

Compound	Aerobic C_i/C_e*	Hypoxic C_i/C_e†	T_i‡ (hr at 10^6 cells/mL)	% Parent metabolized at 5 hr§	% Detected as amine at 5 hr‖	Rate of O_2 consumption¶ (nmol O_2/min/10^6 cells)
5NQ	60	35	1.5	91	76	1.47 ± 0.04
8Me-5NQ	50	25	2.8	74	96	0.96 ± 0.07
3Me-5NQ	40	25	10.2	100	83	0.28 ± 0.03
3,8diMe-5NQ	55	40	11.7	100	76	0.18 ± 0.01
3,6diMe-5NQ	25	18	165	11	17	<0.025
8NHMe-5NQ	25	21	190	20	ND**	0.05 ± 0.01
MISO	1.1	1.2	43.3	55	nd††	ND
8NQL	2.6	2.6	30.0	69	nd	ND

* Ratio of intracellular to extracellular drug concentrations under aerobic conditions at $t \approx 0$.
† Ratio of intracellular to extracellular drug concentrations under hypoxic conditions at $t \approx 0$.
‡ First half-life for loss of parent nitro compound.
§ Percentage of the total initial concentration of parent drug consumed after 5 hr in hypoxic AA8 cell cultures.
‖ Yield of corresponding amine as a percentage of parent nitro compound consumed at 5 hr.
¶ Rate of oxygen consumption by cyanide-inhibited AA8 cells induced by 200 μM drug. Values are means ± range for two independent measurements.
** Not determined.
†† Not detected.

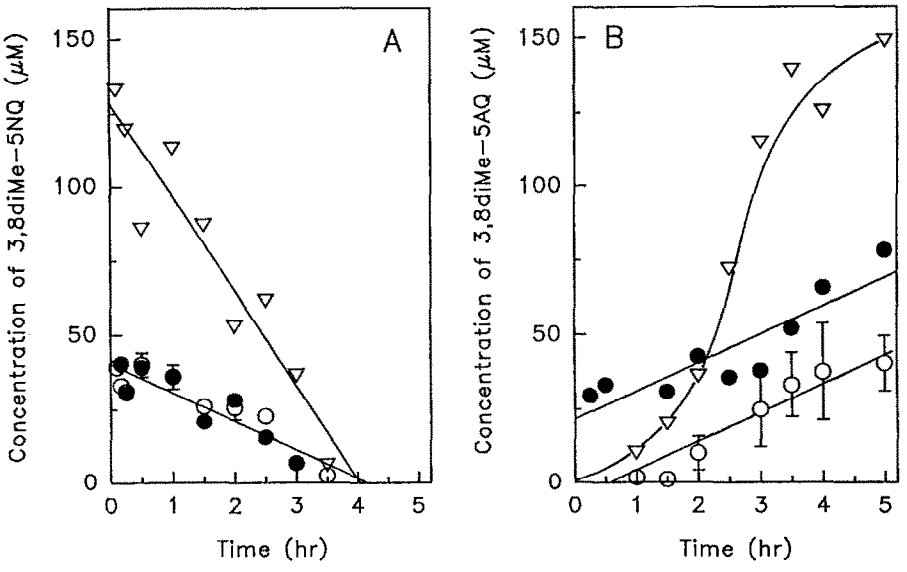


Fig. 4. Metabolism of 3,8diMe-5NQ (A) and formation of 3,8diMe-5AQ (B) in hypoxic AA8 cultures (5×10^6 cells/mL) exposed to: (○) 40 μM 3,8diMe-5NQ, values are means ± range for two experiments, (▽) 150 μM 3,8diMe-5NQ, or (●) 40 μM 3,8diMe-5NQ + 30 μM 3,8diMe-5AQ. The ordinate represents total concentration (extracellular plus intracellular drug).

3.7 to 33 μM/hr, indicating that rates of metabolism are approximately proportional to cell density. Evaluation of the more slowly reducing nitro compounds was thus undertaken at higher cell densities (5×10^6/mL, 3Me-5NQ, 3,8diMe-5NQ, 3,6diMe-5NQ; 10^7/mL, 8NHMe-5NQ, MISO, 8NQL) so that measurable rates could be obtained under hypoxic conditions.

Loss of parent drugs did not appear to follow first-order kinetics in all cases. For example, metabolism of 3,8diMe-5NQ in hypoxic AA8 cultures at 5×10^6 cells/mL showed a linear rather than an exponential decrease in total drug concentration (calculated by summing the intracellular and extracellular drug) at both concentrations investigated (Fig. 4A). One major metabolite, identified as the amine 3,8diMe-5AQ, was detected in yields of 76 and 100% at 5 hr following hypoxic metabolism of initial concentrations of 40 and 150 μM 3,8diMe-5NQ, respectively (Fig. 4B). Addition of 3,8diMe-5AQ (30 μM) to hypoxic AA8 cells at the same time as 3,8diMe-5NQ (40 μM) had no effect on the kinetics of disappearance of the nitro compound or formation of amine (Fig. 4, A and B).

The rates of metabolism of the other nitro compounds were determined in hypoxic cultures, again by summing the drug detected in the intra- and extracellular compartments. Based on the first half-life for loss of the parent compound, the rate of metabolism in hypoxic cultures (corrected for differences in cell density during drug exposure) decreased in the order 5NQ > 8Me-5NQ > 3,8diMe-5NQ ≈ 3Me-5NQ > 3,6diMe-5NQ ≈ 8NHMe-5NQ, with both MISO and 8NQL having rates intermediate between 3,8diMe-5NQ and 3,6diMe-5NQ (Table 3).

Uptake factors were high for all the 5-nitroquinolines under aerobic conditions, average intracellular concentration being 25- to 60-fold higher than extracellular concentrations (Table 3). These uptake factors were slightly lower under hypoxic conditions (Table 3). For MISO and 8NQL, ratios of C_i/C_e, *ca.* 1.1 and 2.6, respectively, were essentially the same in aerobic and hypoxic AA8 cultures (Table 3).

In hypoxic cultures, the major metabolite of each of the 4-alkylamino-5-nitroquinolines detected by HPLC was the corresponding amine (from comparison of R_t and spectrum with authentic amines). For 8NHMe-5NQ, a single metabolite, observed in the intracellular compartment only, was identical to the (unidentified) product observed at $R_t = 3.4$ min on radiolytic reduction of the nitro compound. Hypoxic metabolism of 3,8diMe-5NQ and 3,6diMe-5NQ (Fig. 5) yielded a second metabolite in addition to the amine. These metabolites had R_t values and spectra identical to those of the non-polar radiolytic reduction products and are presumably azo or azoxy dimers. For the four 5NQ derivatives with relatively rapid rates of metabolism in hypoxic cultures (5NQ, 8Me-5NQ, 3Me-5NQ, 3,8diMe-5NQ), the yield of amine by 5 hr was a high proportion (76–96%) of the parent nitro compound consumed (Table 3). The rates of amine formation from metabolism of the 5NQs in hypoxic AA8 cells, corrected for differences in cell density during drug exposure (Fig. 6), were in the same rank order as the rates of loss of parent nitro compound, i.e. 5NQ > 8Me-5NQ > 3,8diMe-5NQ ≈ 3Me-5NQ > 3,6diMe-5NQ.

For each of the drugs investigated, metabolic loss was inhibited completely under aerobic exposure conditions. In addition, with the exception of 8Me-5NQ as noted above, and 3Me-5NQ for which a low constant concentration of the corresponding amine

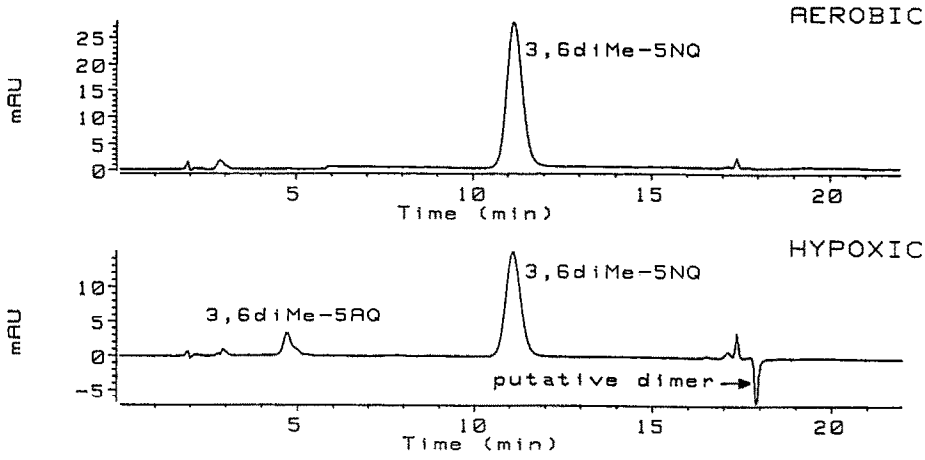


Fig. 5. HPLC chromatograms of cellular extracts from AA8 cultures exposed to 40 μM 3,6diMe-5NQ for 4 hr under aerobic (top panel) and hypoxic (lower panel) conditions. The ordinate is in units of mAU; absorbance units·10^{-3}. Chromatograms show absorbance at 350 nm referenced to absorbance at 550 nm. The negative absorbance for the putative dimer is due to greater absorbance at the latter wavelength.

(*ca.* 50 μM) was also observed in the intracellular samples (which presumably results from metabolism occurring during sample preparation), no metabolites were detected at any times in aerobic cultures. The inhibition of amine formation by oxygen is illustrated for 3,6diMe-5NQ in Fig. 5.

Cell growth inhibition

The aerobic cytotoxic potencies of the 5-nitroquinolines and the corresponding amines were compared in AA8 and UV4 cells using growth inhibition after an 18-hr exposure as the endpoint (Table 4). The amines varied in potency with 5AQ being less toxic than 5NQ, while the dimethyl substituted amines were more toxic than the corresponding nitro compounds. The UV4 cell line, which is defective in the repair of bulky DNA monoadducts and DNA interstrand crosslinks [21, 28], was more sensitive to 5-nitroquinolines with relatively fast rates of hypoxic metabolism (5NQ, 8Me-5NQ, 3Me-5NQ and 3,8diMe-5NQ) than was the parental AA8 line, while the potencies of the slowly metabolized 3,6diMe-5NQ and 8NHMe-5NQ were similar in both cell lines. All the amines were equally potent in both the AA8 and UV4 lines.

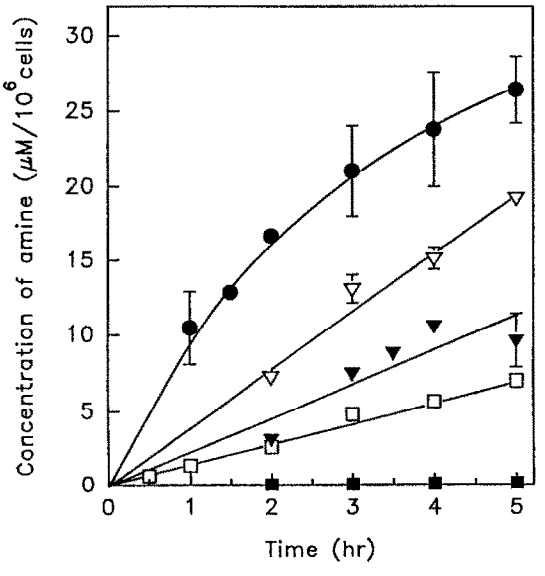


Fig. 6. Rates of amine formation from hypoxic metabolism of 5NQs corrected for differences in cell density during drug exposure: (●) 5AQ, (▽) 8Me-5AQ, (▼) 3,8diMe-5AQ, (□) 3Me-5AQ, and (■) 3,6diMe-5AQ. Values are means ± range for two experiments when error bars are shown.

Table 4. Cytotoxicity data for 5NQs and corresponding amines

Compound	IC_{50}^{*} (μM) AA8	UV4
5NQ	12.4 ± 0.8†	1.0 ± 0.1
5AQ	128 ± 49	107 ± 41
8Me-5NQ	29 ± 5†	2.6 ± 0.3
8Me-5AQ	26 ± 14	19 ± 7
3Me-5NQ	33 ± 3†	2.0 ± 0.5
3Me-5AQ	52 ± 13	61 ± 2
3,8diMe-5NQ	277 ± 28	12 ± 4
3,8diMe-5AQ	13.0 ± 2.6	12.4 ± 1.9
3,6diMe-5NQ	202 ± 21	155 ± 16
3,6diMe-5AQ	52 ± 1	59 ± 4
8NHMe-5NQ	98 ± 9†	43 ± 4

* Values are means ± SEM (N ≥ 3) for nitro compounds and mean ± range (N = 2) for amines.
† Data from [14].

Drug-induced stimulation of oxygen consumption in respiration-inhibited cells

The background rate of oxygen consumption in AA8 cells treated with 3 mM KCN was 0.143 ± 0.003 nmol O_2/min/10^6 cells (mean ± SEM, N = 70), 6.7 ± 0.2% the rate of oxygen consumption in control cells. Stimulation of oxygen consumption in respiration-inhibited cells by the 5-nitroquinolines (200 μM) varied over a range of about 50-fold (Table 3). The rates of nitro group redox cycling of the 5NQ analogues under aerobic conditions, as measured by this method, paralleled those for net metabolism under hypoxic conditions (loss of nitro compound and formation of amine) with rates being highest for 5NQ and 8Me-5NQ and lowest for 8NHMe-5NQ. No drug-induced stimulation in the rate of cellular oxygen consumption was detected for 3,6diMe-5NQ.

DISCUSSION

Radiolytic and metabolic formation of amines

Radiolytic reduction of the 5NQs gave one major reduction product in each case (with the exception of 8NHMe-5NQ), these products being detected at the highest concentration after addition of six reducing equivalents, suggesting them to be the corresponding amines. This identification was confirmed by comparison of chromatographic and spectral properties with the authentic amines. Two major reduction products were observed for 8NHMe-5NQ, both of which were present in the mixture of products obtained from attempts to synthesize 8NHMe-5AQ. Under identical conditions the nitroacridine analogue of 5NQ, nitracrine, was reduced with three-electron stoichiometry to give at least ten unidentified products.* MISO is reduced under similar conditions with four-electron stoichiometry, suggesting the unstable hydroxylamine to be the predominant product [7, 19].

In hypoxic AA8 cell cultures, each of the 5-nitroquinolines, with the possible exception of 8NHMe-5NQ, was metabolized to the corresponding amine. High yields of the amines (76–96%), relative to the amount of parent nitro compound consumed, were observed for the 5-nitroquinolines with the fastest rates of hypoxic metabolism (5NQ, 8Me-5NQ, 3Me-5NQ and 3,8diMe-5NQ). The products of hypoxic metabolism of other nitro(hetero)arenes are generally either the hydroxylamino or amino reduction products. Although no cellular metabolites were detected for MISO or 8NQL in the present study, others [29] have reported that the hydroxylamine is the major metabolite of MISO in hypoxic Chinese hamster ovary cells. Metabolic reduction of 4-nitroquinoline *N*-oxide in both aerobic and anoxic cells also gives the corresponding hydroxylamino derivative as the major product [30], as does the nitrothiazole niridazole in anaerobic rat liver microsomes [31]. Reduction of the 5NQs in hypoxic cells to give the corresponding amines in high yields as stable products is in contrast to many other nitro compounds, although reductive metabolism to the amine has also been reported for benznidazole [32], nitrofurazone [33] and 1- [34] and 2-nitropyrene [35] in anaerobic rat liver microsomes.

The 5-nitroquinolines with high hypoxia-selective cytotoxicity ratios (5NQ, 8Me-5NQ, 3Me-5NQ, 3,8diMe-5NQ, see Table 1) were markedly more potent toward UV4 cells than AA8 cells, suggesting that their cytotoxicity is due to the formation of DNA adducts. The lack of hypersensitivity of the UV4 line to the corresponding amines (Table 4) suggests a different mechanism of toxicity for the amines and indicates that some intermediate in the reduction pathway, rather than the amine itself, is responsible for cytotoxicity of the nitro compounds. UV4 cells are no more sensitive to 3,6diMe-5NQ and 8NHMe-5NQ than is the repair proficient AA8 line, suggesting an alternative mechanism of cytotoxicity for these non-hypoxia-selective compounds.

Rates of hypoxic metabolism

Apparent differences in the kinetics of metabolic reduction of the parent nitro compounds make an adequate quantitative comparison of the rates of reduction difficult. For some compounds (5NQ, 3,6diMe-5NQ, 8NHMe-5NQ) metabolic consumption of the parent appeared to follow first-order kinetics. Metabolic consumption of 3,8diMe-5NQ appeared to be linear at initial concentrations of either 40 or 150 μM (Fig. 4A). However, the rates of parent drug loss were very different at the two starting concentrations, indicating that the kinetics are not zero order. Half-order kinetics, as have been reported for the binding of radiolabelled MISO to cellular macromolecules under hypoxic conditions [36, 37], also do not fit the observed rates of metabolism. The possibility that formation of the amine end-product influenced the kinetics of metabolic reduction of 3,8diMe-5NQ in hypoxic AA8 cells was excluded as 3,8diMe-5AQ (30 μM) did not modify either the rate of loss of the nitro compound (Fig. 4A) or the rate of formation of the amine (Fig. 4B). Despite uncertainty as to the kinetic order of metabolic reduction, the identical rank orders of loss of parent compound and formation of amine gives confidence that the use of a first half-life for loss of parent drug is an appropriate index of nitro reduction rates.

There was a significant linear relationship (r = 0.756, P = 0.03) between the first half-life for loss of total parent drug (correcting for differences in cell density during drug exposure) and the E_7^1 for all nitro compounds investigated in this study according to Equation 1:

$$-\log T_{1/2} = aE_7^1 + b \qquad (1)$$

with slope a = 8.0 V^{-1} and intercept b = 1.62 (Fig. 7). Despite this correlation, 3,6diMe-5NQ appears to be an anomaly in that the observed rate of hypoxic metabolism was very slow relative to its E_7^1 of −367 mV. Excluding 3,6diMe-5NQ improved the correlation (r = 0.855, P = 0.01) and provided a slope and intercept of 7.9 V^{-1} and 1.70, respectively, for Equation 1.

* O'Connor CJ, Denny WA, Gamage RSKA, Sutton BM, van Zijl PL and Wilson WR, Manuscript in preparation.

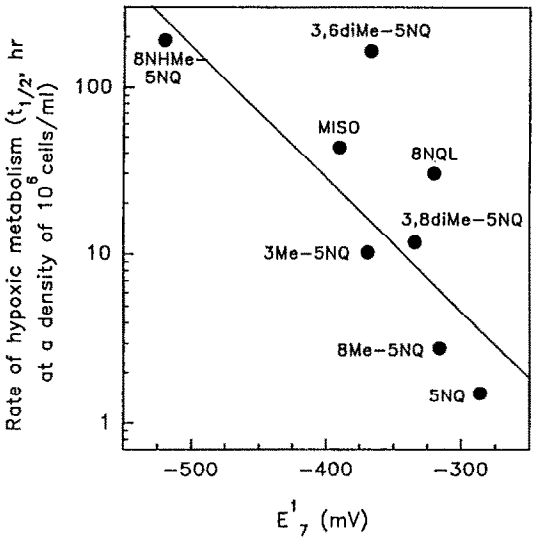


Fig. 7. Relationship between rate of hypoxic metabolism ($T_{\frac{1}{2}}$ for loss of nitro compound) and E^1_7 for 5NQs, MISO and 8NQL. (The E^1_7 of MISO is −389 mV [38] and the E^1_7 for 8NQL was assumed to be the same as its $E_{\frac{1}{2}}$ of −320 mV [39].) Rates of metabolism have been corrected for differences in cell density during drug exposure. The solid line represents linear regression through the data, excluding 3,6diMe-5NQ.

There have been few studies on the relationship between rates of metabolic reduction of bioreductive drugs and their reduction potentials using intact cells. Olive [39] showed a logarithmic correlation between $E_{\frac{1}{2}}$ and rate of hypoxic metabolism in mouse L-929 cells for a number of nitro(hetero)arenes, including MISO and 8NQL (slope = 10.2 V^{-1}, r = 0.890, $P < 0.001$). The rates of metabolic reduction determined for the 5NQs in the present study thus show a similar dependence on reduction potential. However, the absolute rates of hypoxic reduction of MISO and 8NQL were approximately 10-fold higher in the present study, extrapolating to an equivalent cell density, than those reported by Olive [39]. Although cell line differences could be responsible, the high cell density used in the Olive study (10^8 cells/mL) may have depleted extracellular glucose and compromised reduction by lowering the supply of NADPH via the hexose monophosphate pathway [40].

The observed rate of metabolism of MISO in hypoxic AA8 cell cultures was comparable to that of 5-nitroquinolines with similar reduction potentials despite the much lower (20- to 60-fold) average intracellular concentration of MISO (Table 3). This suggests that for the 5NQs not all of the drug in the intracellular compartment is available for nitro reduction, consistent with recent studies demonstrating that 5-nitroquinolines are accumulated in lysosomes to high concentrations [15]. Intralysosomal accumulation probably contributes to the high mean intracellular concentrations of the 5NQs relative to extracellular concentrations. The higher ratios of C_i/C_e observed in aerobic cells presumably result from the lower extracellular pH of the hypoxic cultures (6.8 to 6.9) than the aerobic cultures (pH 7.1 to 7.2) observed in these uptake experiments, which would be expected to inhibit intracellular accumulation of these basic drugs [41, 42].

The hypoxia-selective cytotoxicity of the 5NQs appears to be broadly determined by the rate of hypoxic metabolism. The compounds with high rates of metabolism (5NQ, 8Me-5NQ, 3Me-5NQ and 3,8diMe-5NQ) exhibit much greater toxicity towards hypoxic than aerobic AA8 cells, while the slowly metabolized 3,6diMe-5NQ and 8NHMe-5NQ lack significant hypoxic selectivity. However, the rate of hypoxic metabolism is not the sole determinant of hypoxia-selective toxicity for the 5-nitroquinolines as 5NQ, which has the fastest rate of metabolism, was less selective than 8Me-5NQ and 3Me-5NQ (Tables 1 and 3).

Aerobic nitro reduction

Drug-induced stimulation of oxygen consumption in respiration-inhibited cells is diagnostic of redox cycling of radicals formed by one-electron reduction. No drug-induced stimulation in the rate of cellular oxygen consumption was observed for 3,6diMe-5NQ, providing further evidence that this compound is not a good substrate for enzymatic nitro reduction. 8NHMe-5NQ, which is also metabolized slowly in hypoxic cultures ($T_{\frac{1}{2}} \approx 190$ hr), induced a low rate of oxygen consumption in cyanide-inhibited cells consistent with its low E^1_7 of −520 mV. There was a linear correlation ($r = 0.980$, $P < 0.01$) between log rates of loss of parent drug in hypoxic cultures (log $T_{\frac{1}{2}}$) and log rates of drug-induced consumption in respiration-inhibited cells. Since the latter involves a one-electron redox cycle, the correlation implies that one-electron reduction is the rate-limiting step in net reduction of 5-nitroquinolines.

The observed lack of net reduction (loss of nitro compound and amine formation) under aerobic conditions indicates that the 5-nitroquinolines are not substrates for oxygen-insensitive nitroreductases and that this is not the reason for the observed variations in hypoxia-selective cytotoxicity within the series. It appears that most nitro compounds investigated to date are poor substrates for DT-diaphorase [10, 43], relative to quinones and quinoneimines [44], and therefore reductive activation by this enzyme is not likely to be a major determinant of the hypoxic selectivity of nitro(hetero)arenes. This is contrast to the hypoxia-selective cytotoxicity of quinone bioreductive drugs, which appears to be determined largely by their efficiency as substrates for the obligate two-electron reductase DT-diaphorase [11–13].

Non-bioreductive mechanism of toxicity

Although 8NHMe-5NQ and 3,6diMe-5NQ were reduced slowly in hypoxic AA8 cultures, this reduction was clearly inhibited by O_2 (e.g. Fig. 5). The lack of hypoxia-selective cytotoxicity of these compounds therefore suggests that their toxicity is not due to net nitro reduction but results from a non-bioreductive mechanism. This is consistent with the lack of hypersensitivity of the UV4 line to these compounds (Table 4), which clearly distinguishes

them from the 5-nitroquinolines with high hypoxic selectivity ratios for which reduction to a DNA alkylating species is the toxigenic mechanism. The variable contribution of the unreduced nitro compound to the observed toxicity may underlie the differences in hypoxic selectivity in this series. Although the structure–activity relationships for this non-bioreductive toxicity are unclear, there is presumably no dependence on reduction potential. The possible contribution of reactive oxygen species, formed by redox cycling, to the aerobic cytotoxicity of the compounds with high rates of nitro reduction cannot be excluded and might contribute to the greater aerobic toxicity (and lower hypoxic selectivity) for 5NQ than 8Me-5NQ [16]. Within this series of 5NQs there may therefore exist a window for the rate of metabolic nitro reduction; if reduction occurs slowly then cytotoxicity is dominated by the non-bioreductive non-hypoxia-selective mechanism, while if reduction is too rapid then reactive oxygen species contribute to toxicity, enhancing aerobic potency and lowering hypoxic selectivity. While the one-electron reduction potential gives some indication as to the rates of reduction, and hence hypoxic selectivity of toxicity, the exception provided by 3,6diMe-5NQ shows that the E_7^1 value is not the only determinant of this rate and that direct measures of metabolic reduction are preferable.

We have identified previously the very rapid metabolism of the 5NQs *in vivo* as a potential problem [18], and have developed analogues bearing electron-donating functionality in an attempt to lower rates of nitro reduction [14]. The present study demonstrates that compounds that are poor substrates for enzymatic nitro reduction have little selectivity for hypoxic cells, and thus indicates that further lowering of reduction potential is unlikely to be beneficial.

Acknowledgements—This work was supported by the Health Research Council of New Zealand, the Cancer Society of New Zealand and the National Cancer Institute, U.S.A. (Contract CM 07321). B.G.S. was the recipient of a Postgraduate Scholarship from the University Grants Committee of New Zealand.

REFERENCES

1. Bush RS, Jenkin RDT, Allt WEC, Beale FA, Bean H, Dembo AJ and Pringle JF, Definitive evidence for hypoxic cells influencing cure in cancer therapy. *Br J Cancer* **37** (Suppl III): 302–306, 1978.
2. Gatenby RA, Kessler HB, Rosenblum JS, Coia LR, Moldofsky PJ and Hartz WH, Oxygen distribution in squamous cell carcinoma metastases and its relationship to outcome of radiation therapy. *Int J Radiat Oncol Biol Phys* **14**: 831–838, 1988.
3. Höckel M, Knoop C, Schlenger K, Vorndran B, Baußmann E, Mitze M, Knapstein PG and Vaupel P, Intratumoral pO_2 predicts survival in advanced cancer of the uterine cervix. *Radiother Oncol* **26**: 45–50, 1993.
4. Adams GE, Stratford IJ, Bremner JCM, Cole S, Edwards HS and Fielden EM, Nitroheterocyclic compounds as radiation sensitizers and bioreductive drugs. *Radiother Oncol* **20** (Suppl): 85–91, 1991.
5. Wilson WR, Tumour hypoxia: Challenges for cancer chemotherapy. In: *Cancer Biology and Medicine* (Eds. Waring MJ and Ponder BAJ), Vol. 3, pp. 87–131. Kluwer Academic Publishers, Lancaster, 1992.
6. Workman P and Stratford IJ, The experimental development of bioreductive drugs and their role in cancer therapy. *Cancer Metastasis Rev* **12**: 73–82, 1993.
7. Rauth AM, McClelland RA, Michaels HB and Battistella R, The oxygen dependence of the reduction of nitroimidazoles in a radiolytic model system. *Int J Radiat Oncol Biol Phys* **10**: 1323–1326, 1984.
8. Zeman EM, Baker MA, Lemmon MJ, Pearson CI, Adams JA, Brown JM, Lee WW and Tracy M, Structure–activity relationships for benzotriazene di-*N*-oxides. *Int J Radiat Oncol Biol Phys* **16**: 977–981, 1989.
9. Jenkins TC, Naylor MA, O'Neill P, Threadgill MD, Cole S, Stratford IJ, Adams GE, Fielden EM, Suto MJ and Stier MA, Synthesis and evaluation of α-[[(2-haloethyl)amino]methyl] -2-nitro-1*H*-imidazole-1-ethanols as prodrugs of α-[(1-aziridinyl)methyl] -2-nitro-1*H*-imidazole-1-ethanol (RSU-1069) and its analogues which are radiosensitizers and bioreductively activated cytotoxins. *J Med Chem* **33**: 2603–2610, 1990.
10. Palmer BD, Wilson WR, Cliffe S and Denny WA, Hypoxia-selective antitumor agents. 5. Synthesis of water-soluble nitroaniline mustards with selective cytotoxicity for hypoxic mammalian cells. *J Med Chem* **35**: 3214–3222, 1992.
11. Dulhanty AM and Whitmore GF, Chinese hamster ovary cell lines resistant to mitomycin C under aerobic but not hypoxic conditions are deficient in DT-diaphorase. *Cancer Res* **51**: 1860–1865, 1991.
12. Marshall RS, Paterson MC and Rauth AM, Studies on the mechanism of resistance to mitomycin C and porfiromycin in a human cell strain derived from a cancer-prone individual. *Biochem Pharmacol* **41**: 1351–1360, 1991.
13. Plumb JA, Gerritsen M, Milroy R, Thomson P and Workman P, Relative importance of DT-diaphorase and hypoxia in the bioactivation of EO9 by human lung tumor cell lines. *Int J Radiat Oncol Biol Phys* **29**: 295–299, 1994.
14. Denny WA, Atwell GJ, Roberts PB, Anderson RF, Boyd M, Lock CJL and Wilson WR, Hypoxia-selective antitumor agents. 6. 4-(Alkylamino)nitroquinolines: A new class of hypoxia-selective cytotoxins. *J Med Chem* **35**: 4832–4841, 1992.
15. Siim BG, Denny WA and Wilson WR, Does DNA targeting affect the cytotoxicity and cell uptake of basic nitroquinoline bioreductive drugs? *Int J Radiat Oncol Biol Phys* **29**: 311–315, 1994.
16. Siim BG, Atwell GJ and Wilson WR, Oxygen dependence of the cytotoxicity and metabolic activation of 4-alkylamino-5-nitroquinoline bioreductive drugs. *Br J Cancer*, in press.
17. Stefanska B, Zirra JA, Peryt J, Kaminski K and Ledochowski A, Research on tumour inhibiting compounds. Part LII. 5- and 8-Nitro-4-aminoquinoline derivatives. *Rocz Chem* **47**: 2339–2343, 1973.
18. Wilson WR, Siim BG, Denny WA, van Zijl PL, Taylor ML, Chambers DM and Roberts PB, 5-Nitro-4-(*N*,*N*-dimethylaminopropylamino)quinoline (5-nitraquine), a new DNA-affinic hypoxic cell radiosensitizer and bioreductive agent: Comparison with nitracrine. *Radiat Res* **131**: 257–265, 1992.
19. Whillans DW and Whitmore GF, The radiation reduction of misonidazole. *Radiat Res* **86**: 311–324, 1981.
20. Fricke H and Hart EJ, Chemical dosimetry. In: *Radiation Dosimetry* (Eds. Attix FH and Roesch WC), pp. 167–239. Academic Press, New York, 1969.
21. Thompson LH, Rubin JS, Cleaver JE, Whitmore GF and Brookman K, A screening method for isolating DNA repair-deficient mutants of CHO cells. *Somat Cell Genet* **6**: 391–405, 1980.
22. Roberts PB, Denny WA, Wakelin LPG, Anderson RF

and Wilson WR, Radiosensitization of mammalian cells *in vitro* by nitroacridines. *Radiat Res* **123**: 153–164, 1990.
23. Paterson ARP and Cass CE, Transport of nucleoside drugs in animal cells. In: *Membrane Transport of Antineoplastic Agents* (Ed. Goldman ID), pp. 309–329. Pergamon Press, Oxford, 1986.
24. Finlay GJ, Baguley BC and Wilson WR, A semiautomated microculture method for investigating growth inhibitory effects of cytotoxic compounds on exponentially growing carcinoma cells. *Anal Biochem* **139**: 272–277, 1984.
25. Wilson WR, Thompson LH, Anderson RF and Denny WA, Hypoxia-selective antitumor agents. 2. Electronic effects of 4-substituents on the mechanisms of cytotoxicity and metabolic stability of nitracrine derivatives. *J Med Chem* **32**: 31–38, 1989.
26. Biaglow JE, Jacobson B, Greenstock CL and Raleigh J, Effect of nitrobenzene derivatives on electron transfer in cellular and chemical models. *Mol Pharmacol* **13**: 269–282, 1977.
27. Koch CJ, Polarographic oxygen sensor. US Patent 5030036, July 1991.
28. Hoy CA, Salazar EP and Thompson LH, Rapid detection of DNA-damaging agents using repair-deficient CHO cells. *Mutat Res* **130**: 321–332, 1984.
29. Josephy PD, Palcic B and Skarsgard LD, *In vitro* metabolism of misonidazole. *Br J Cancer* **43**: 443–450, 1981.
30. De Flora S, Bennicelli C, Camoirano A, Serra D and Hochstein P, Influence of DT diaphorase on the mutagenicity of organic and inorganic compounds. *Carcinogenesis* **9**: 611–617, 1988.
31. Feller DR, Morita M and Gillette JR, Enzymatic reduction of niridazole by rat liver microsomes. *Biochem Pharmacol* **20**: 203–215, 1971.
32. Walton MI and Workman P, Nitroimidazole bioreductive metabolism. Quantitation and characterisation of mouse tissue benznidazole nitroreductases *in vivo* and *in vitro*. *Biochem Pharmacol* **36**: 887–896, 1987.
33. Tatsumi K, Kitamura S and Yoshimura H, Reduction of nitrofuran derivatives by xanthine oxidase and microsomes. Isolation and identification of reduction products. *Arch Biochem Biophys* **175**: 131–137, 1976.
34. El-Bayoumy K and Hecht SS, Identification and mutagenicity of metabolites of 1-nitropyrene formed by rat liver. *Cancer Res* **43**: 3132–3137, 1983.
35. Roy AK, Upadhyaya P, Fu PP and El-Bayoumy K, Identification of the major metabolites and DNA adducts formed from 2-nitropyrene *in vitro*. *Carcinogenesis* **12**: 475–479, 1991.
36. Chapman JD, Baer K and Lee J, Characteristics of the metabolism-induced binding of misonidazole to hypoxic mammalian cells. *Cancer Res* **43**: 1523–1528, 1983.
37. Koch CJ, Stobbe CC and Baer KA, Metabolism induced binding of ^{14}C-misonidazole to hypoxic cells: Kinetic dependence on oxygen concentration and misonidazole concentration. *Int J Radiat Oncol Biol Phys* **10**: 1327–1331, 1984.
38. Wardman P, The use of nitroaromatic compounds as hypoxic cell radiosensitizers. *Curr Top Radiat Res Q* **11**: 347–398, 1977.
39. Olive PL, Correlation between metabolic reduction rates and electron affinity of nitroheterocycles. *Cancer Res* **39**: 4512–4515, 1979.
40. Ling LL and Sutherland RM, Modulation of the hypoxic toxicity and binding of misonidazole by glucose. *Br J Cancer* **54**: 911–917, 1986.
41. Dennis MF, Stratford MRL, Wardman P and Watts ME, Cellular uptake of misonidazole and analogues with acidic or basic functions. *Int J Radiat Biol* **47**: 629–643, 1985.
42. Denny WA and Wilson WR, Considerations for the design of nitrophenyl mustards as agents with selective toxicity for hypoxic tumor cells. *J Med Chem* **29**: 879–887, 1986.
43. Boland MP, Knox RJ and Roberts JJ, The differences in kinetics of rat and human DT diaphorase result in a differential sensitivity of derived cell lines to CB 1954 (5-(aziridin-1-yl)-2,4-dinitrobenzamide). *Biochem Pharmacol* **41**: 867–875, 1991.
44. Lind C, Cadenas E, Hochstein P and Ernster L, DT-diaphorase: Purification, properties and function. *Methods Enzymol* **186**: 287–301, 1990.