Biochemical Pharmacology, Vol. 60, pp. 969–978, 2000.

ISSN 0006-2952/00/$–see front matter
PII S0006-2952(00)00420-2

Comparison of Aromatic and Tertiary Amine *N*-Oxides of Acridine DNA Intercalators as Bioreductive Drugs

CYTOTOXICITY, DNA BINDING, CELLULAR UPTAKE, AND METABOLISM

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ABSTRACT. Some *N*-oxide derivatives of DNA intercalators are bioreductive prodrugs that are selectively toxic under hypoxic conditions. The hypoxic selectivity is considered to result from an increase in DNA binding affinity when the *N*-oxide moiety is reduced. This study investigated whether differences in DNA binding affinity between *N*-oxides and their corresponding amines, measured by equilibrium dialysis, can account for the hypoxic cytotoxicity ratios (HCR) of tertiary amine *N*-oxide (-tO) and aromatic *N*-oxide (-aO) derivatives of the 1-nitroacridine nitracrine (NC) and its non-nitro analogue 9-[3-(*N*,*N*-dimethylamino)propylamino]acridine (DAPA). Cytotoxicity was measured in aerobic and hypoxic suspensions of Chinese hamster ovary (CHO) AA8 cells by clonogenic assay. HCR were much greater for NC-tO (820-fold) than for NC (5-fold) or NC-aO (4-fold), whereas DAPA and its *N*-oxides lacked hypoxic selectivity (1-fold). DNA binding measurements demonstrated that binding affinity is lowered more by aromatic than tertiary amine (side-chain) *N*-oxides, an observation that does not correlate with HCR. Compounds were accumulated in cells to high concentrations ($C_i/C_e \approx 10–200$), with the exception of the tertiary amine *N*-oxides, for which the ratio of intracellular to extracellular drug was less than unity. For NC-tO this probably resulted from low pK_a values for both the acridine chromophore and the side-chain, whereas DAPA-tO may be too hydrophilic for efficient membrane permeation. Bioreductive drug metabolism, assessed by HPLC, was faster for the NC than the DAPA *N*-oxides. The high HCR of NC-tO relative to NC-aO is ascribed to the rapid and selective reduction of its *N*-oxide moiety, followed by activation of the NC intermediate by O_2-sensitive reduction of its 1-nitro group to the corresponding 1-amine. The metabolism studies suggest that unmasking of DNA binding affinity by reductive removal of the *N*-oxide moiety, although not the only determinant, is important and needs to occur before nitroreduction for optimal effect. BIOCHEM PHARMACOL **60**;7:969–978, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. N-oxides; nitroacridines; bioreductive drug metabolism; DNA binding; log *P*; cellular uptake

Many human tumors differ from normal tissues in that they contain substantial numbers of hypoxic cells [1, 2]. It is known that these cells can contribute to radiation resistance of tumors [3, 4] and to neoplastic progression within unirradiated tumors [5, 6]. These features make hypoxic cells important targets in cancer chemotherapy; there is currently much interest in the development of hypoxia-selective cytotoxins capable of being activated by enzymatic reduction (bioreductive drugs) under hypoxic conditions to exploit this feature of tumors [7–9].

The 1-nitroacridine derivative NC§ (Fig. 1) was one of the first bioreductive drugs to be evaluated as an anticancer agent in humans, but in initial clinical studies in Poland it showed only modest activity as a single agent [10]. NC is activated by reduction of the 1-nitro group to form a reactive species, with inhibition of this reduction by oxygen, making NC a hypoxia-selective cytotoxin in culture [11, 12]. These features suggested that NC might complement agents whose activity is limited by hypoxia, such as ionising radiation, but no increase in radiation response of murine tumors was seen when NC was administered either before or after radiation [13].

The rapid reductive metabolism of NC under hypoxic conditions [12], and its reversible binding to DNA by intercalation [14], might be expected to compromise its extravascular diffusion into hypoxic zones in tumors. DNA

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Received 15 November 1999; accepted 16 March 2000.

§ *Abbreviations*: AC, 1-amino-9-[3-(*N*,*N*-dimethylamino)propylamino]-lacridine; AC-aO, aromatic *N*-oxide of AC; Ac-tO, tertiary amine *N*-oxide of AC; DAPA, 9-[3-(*N*,*N*-dimethylamino)propylamino]acridine; DAPA-aO, aromatic *N*-oxide of DAPA; DAPA-tO, tertiary amine *N*-oxide of DAPA; HCR, hypoxic cytotoxicity ratio; NC, nitracrine; NC-aO, aromatic *N*-oxide of NC; NC-tO, tertiary amine *N*-oxide of NC; and Rt, retention time.

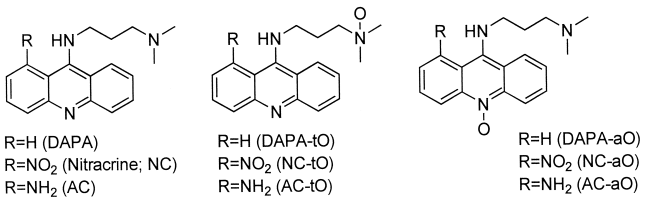

FIG. 1. Structures of compounds investigated in this study.

intercalators with basic side-chains are also trapped frequently in acidic endosomes [15–17], which interferes with extravascular diffusion of basic cytotoxic drugs, including DAPA, the des-nitro analog of NC [18, 19]. Studies with multicellular spheroids, confirming the poor diffusion of NC into hypoxic tissue, have suggested that this might be responsible for its disappointing activity in tumors [14]. This led us to investigate the corresponding NC-tO (SN 24030, NSC 672819, Fig. 1) in the expectation that masking the cationic charge of the tertiary amine side-chain in this way would lower DNA binding affinity and lysosomal uptake, and thereby enhance extravascular diffusion [14]. The resistance of cells in spheroids (relative to single-cell suspensions) is not as marked for NC-tO as for NC itself [14], suggesting that the *N*-oxide does have superior diffusion properties.

The *N*-oxide side-chain not only confers potentially advantageous physicochemical properties in relation to extravascular diffusion, but also provides a less toxic prodrug form that can be reduced to NC in hypoxic cells [14]. NC-tO, therefore, can be considered a "bis-bioreductive" drug, with reduction of both the nitro and *N*-oxide moieties required for full activation. NC-tO is approximately 100-fold less toxic than NC in aerobic cell cultures, but has a similar potency to NC under hypoxia; it thus shows very marked hypoxic selectivity in culture, with an HCR (ratio of concentrations for 90% cell kill under aerobic and hypoxic conditions) in the order of 1000-fold [13, 14]. Unlike NC, NC-tO shows selective toxicity towards hypoxic (radioresistant) cells in KHT tumours, although this activity is seen only at doses that would eventually be lethal to the host [13]. It is not clear whether this improvement in *in vivo* activity (relative to NC) is due to the higher HCR of the *N*-oxide, its improved tissue penetration properties, or both.

The use of *N*-oxide derivatives as prodrugs is potentially applicable to other DNA intercalators with tertiary amine side-chains. This approach has been used independently to develop the anthraquinone bis-*N*-oxide AQ4N, 1,4-bis{[2-(dimethylamino)-*N*-oxide)ethyl]amino}5,8-dihydroxyanthracene-9,10-dione, as a bioreductive drug [20, 21], which currently is scheduled for clinical trial, and the tertiary amine *N*-oxide of the DNA intercalator DACA, *N*-[2-(dimethylamino)ethyl]acridine-4-carboxamide [22]. However, there are many unresolved questions concerning *N*-oxide derivatives as bioreductive prodrugs. In particular, there is little information as to the magnitude of the differential in DNA binding affinity between *N*-oxides and the corresponding amines. Earlier studies in this laboratory [14] have demonstrated that the binding affinity of NC-tO is 15-fold lower than NC under non-physiological conditions (0.01 M salt, 22°). It is not clear whether this differential is sufficient to account for the very large difference in aerobic cytotoxic potency between NC-tO and NC, or whether other factors such as differences in cellular uptake also contribute. In addition, the intercalator *N*-oxides evaluated to date are all aliphatic (side-chain tertiary amine) *N*-oxides; it is unknown whether aromatic *N*-oxides (e.g. the acridine *N*-10 *N*-oxide derivative NC-aO, Fig. 1) of intercalators would be similarly effective in lowering DNA binding or lysosomal uptake, and whether these also have potential as hypoxia-selective cytotoxins. Some (non-DNA-binding) aromatic *N*-oxides are known to be reduced to DNA-reactive radicals in hypoxic cells [23–25], and one bioreductive drug of this type (tirapazamine) currently is in Phase III clinical trials [26].

The present study sought to clarify the mechanism(s) responsible for the very high HCR of NC-tO, and to determine whether aromatic *N*-oxides of nitroacridines might also have potential for bioreductive drug design. To assess the contribution of the *N*-oxide moieties to hypoxic cytotoxicity, DNA binding, and cell uptake, the corresponding des-nitro compounds (i.e. the *N*-oxide derivatives of the aminoacridine DAPA, Fig. 1) also were investigated.

MATERIALS AND METHODS

Drugs

All drugs were synthesized in the Auckland Cancer Society Research Centre using published methods [14, 27], and were >99% pure by HPLC. The tertiary amine *N*-oxides were purified rigorously so that they contained <0.02% of the corresponding amine [13]. Stock solutions of drugs were prepared in 50% aqueous ethanol and stored at −80°.

Cells and Drug Exposure

All studies used Chinese hamster AA8 cells maintained as reported previously [28]. Bulk cultures of cells for experiments were grown to late log phase (10^6 cells/mL) in spinner flasks, harvested by centrifugation, and resuspended in fresh culture medium (α-minimal essential medium, 5% v/v heat-inactivated fetal bovine serum, 100 IU/mL of penicillin, and 100 μg/mL of streptomycin). All drug exposures were performed using continuously stirred and gassed single-cell suspensions (10^6 cells/mL) at 37°, equilibrated with 5% CO_2 in air or N_2 for 60 min before drug addition, as detailed previously [28].

Cytotoxicity

Clonogenic survival was determined using aerobic and hypoxic AA8 cell suspensions as above; after a 60-min drug exposure, cells were washed by centrifugation and plated to determine colony formation [28]. Cytotoxicity was assessed as the drug

concentration required to reduce plating efficiency to 10% of controls (C_{10}). The HCR was determined as the ratio of the C_{10} values under aerobic and hypoxic conditions.

Cellular Uptake and Metabolism

Cellular uptake and rates of metabolism were determined in stirred aerobic cultures of AA8 cells as above with or without 10 mM ammonium chloride (added 15 min prior to drug). Intracellular (C_i) and extracellular (C_e) drug concentrations were determined, 60 min after addition of a 30 μM concentration of drug, by HPLC following rapid centrifugation of duplicate 10-mL samples. Extracellular concentrations were determined from samples of the extracellular medium (300 μL) injected directly onto the C_{18} μBondapak column (8 × 100 mm), with the eluate monitored using an HP1040A diode array absorbance detector. Intracellular concentrations were determined from cell pellets (10^7 cells) lysed on ice using 90 μL H_2O followed by 1 M $MgCl_2$ (10 μL) and MeOH (500 μL). After rapid centrifugation, the supernatant was dried and dissolved in mobile phase (250 μL) before injection of 200 μL onto the column. The mobile phase consisted of a linear gradient of MeCN (typically 14–50% MeCN over 25 min) in formate buffer, pH 4.5 (0.44 M ammonium formate adjusted with formic acid), at a flow rate of 1.8 mL/min. Quantitation was based on peak areas using absorbance at the longest wavelength maximum. All drug concentrations were corrected for measured recovery efficiency, determined by spiking cell pellets as detailed previously [29], using drug concentrations in the lysate equivalent to C_i values in the range 3–10,000 μM. Recoveries for all compounds were not less than 80%, with the exception of NC-tO, for which recovery efficiency averaged 60%. Intracellular drug concentrations were calculated after correcting for trapped extracellular medium, using volumes for intracellular and extracellular water in AA8 cell pellets as reported elsewhere [29]. Metabolites were identified either by comparison of retention time and absorbance spectrum with authentic standards (NC, DAPA), or by on-line mass spectrometry using a Hewlett Packard HP1100 LC/MS system. The mass detector used N_2 as the nebulizing and drying gas (flow rate 10 L/min, 350°) for positive mode electrospray ionization (nebulizer pressure 25 psi, capillary voltage 3500 V, fragmentor voltage 80 V). Quantitation of the 1-aminoacridine metabolite AC was based on the absorbance signal determined using acridine-ring tritiated NC [30], assuming unchanged specific activity on reduction to the amine (area/nmol 1194 for AC vs 998 for NC). The absorbance area/nmol of AC-tO was assumed to be the same as that of AC, and the area/nmol of AC-aO was assumed to be the same as that of NC-aO. Recovery efficiency of AC, AC-tO, and AC-aO was assumed to be the same as that measured for NC, NC-tO, and NC-aO, respectively. Rates of drug metabolism were determined by summing intracellular and extracellular drug, determined as above, with samples taken over 5 hr of drug exposure.

Partition Coefficients

Octanol/water partition coefficients at pH 7.4 were determined by a low-volume shake flask method, with drug concentrations in both the octanol and buffer phases analysed by HPLC [31, 32]. Drug was diluted from stock solutions to give 100 μM in PBS pre-saturated with octan-1-ol (BDH). Five hundred microliters of this solution was placed into a 1-mL Eppendorf tube containing 500 μL octan-1-ol pre-saturated with PBS, and the remainder was retained for determination of initial drug concentration and pH (range 7.36 to 7.46). The tubes were tumbled on a rotary mixer (25 rpm) for 3 hr at ambient temperature (21°). The mixture then was centrifuged at 2500 *g* for 5 min, and the drug concentration in both phases was determined by HPLC as above by injecting 10–200 μL of the PBS phase and 50–100 μL of a 10-fold dilution of the octanol phase. Log *P* (pH 7.4) was determined by the ratio of the peak areas in the octanol and PBS phases and checked by a mass balance calculation based on the initial and final PBS concentrations (agreement was within ± 0.08 log units for all determinations). Log *P* (dibase) for the uncharged species was calculated from the formula

$$\text{Log } P \text{ (dibase)} = \text{Log } P \text{ (pH 7.4)} + \text{Log} \cdot (1 + 10^{(pK_{a1} - pH)} + 10^{(pk_{a1} + pk_{a2} - 2pH)})$$

adapted from [33], where $pK_{a1} > pK_{a2}$. This assumes that the protonated forms have negligible distribution into the octanol phase.

DNA Binding Affinity

Binding to calf thymus DNA (Sigma Type I) was measured by equilibrium dialysis (3 hr, 37°) as previously described [15] in KHE buffer (2 mM HEPES, 10 μM EDTA, pH 7.0, with KCl as required to raise the ionic strength) using a dialysis membrane with MW cutoff of 10,000. Drug concentrations on both sides of the membrane were determined by spectrophotometry at the isosbestic point, except for NC and NC-tO, which were available in ^{3}H-labelled form [30] and were assayed by scintillation counting. Loss of drug through binding to the dialysis membrane was <10% for all compounds. DNA association constants (K_{DNA}) were determined from Scatchard plots of ν/C_f versus ν where ν is the binding ratio (C_b/D), D is the DNA concentration in base pairs, and C_b and C_f are the bound and free drug concentrations, respectively. Scatchard plots were fitted using the neighbouring site exclusion model of McGhee and von Hippel [34].

RESULTS

Cytotoxicity

Survival curves for exposure of AA8 cells to DAPA, NC, and their *N*-oxides for 60 min, under aerobic and hypoxic conditions, are compared in Fig. 2, and C_{10} values are

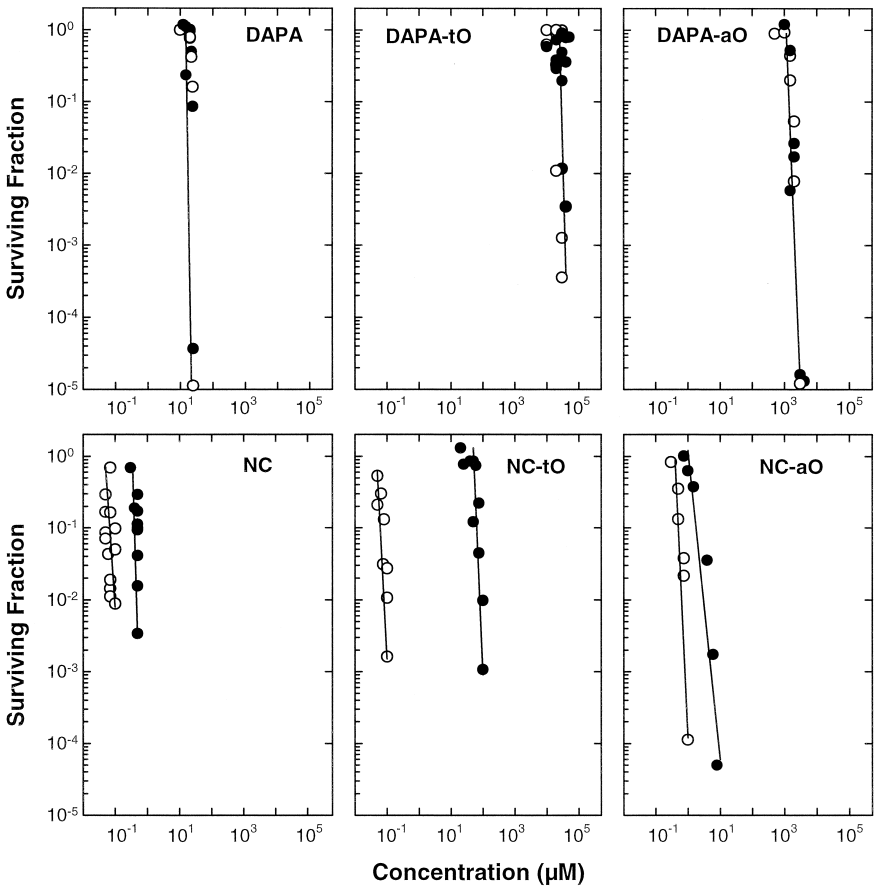

FIG. 2. Cytotoxicity towards aerobic (filled symbols) and hypoxic (open symbols) AA8 cells (10^6 cells/mL) after 60-min drug exposure, determined by clonogenic assay. Data points for each curve are from at least two independent experiments.

summarised in Table 1. As previously reported [11], the nitroacridine NC showed potent cytotoxicity, which was enhanced 5-fold under hypoxic conditions. The corresponding tertiary amine *N*-oxide (NC-tO) showed an aerobic cytotoxic potency *ca.* 150-fold lower than NC and an HCR *ca.* 150-fold greater than NC. The aromatic (*N*-10) nitroacridine *N*-oxide NC-aO showed a modest (6-fold) decrease in aerobic cytotoxicity relative to NC, but its HCR was similar to NC. The non-nitro aminoacridine DAPA had aerobic cytotoxic potency 50-fold lower than NC and showed no hypoxic selectivity. The tertiary amine *N*-oxide DAPA-tO was dramatically (1300-fold) less toxic than DAPA under aerobic conditions, and the aromatic (*N*-10) *N*-oxide was also less toxic (by a factor of 85-fold). However, despite these large protection factors, neither of the DAPA *N*-oxides were more toxic under hypoxic than aerobic conditions.

TABLE 1. Cytotoxicity of acridine (DAPA) and nitroacridine (NC) *N*-oxides

Compound	C_{10}Air* (μM)	HCR† (C_{10}Air/N_2)
DAPA	21	1
DAPA-tO	28,000	1
DAPA-aO	1800	1
NC	0.4	5
NC-tO	61	820
NC-aO	2.5	4

*C_{10} is the drug concentration required to reduce survival to 10% of untreated controls, using 1-hr exposure of AA8 cells at 10^6 cells/mL.

†Hypoxic cytotoxicity ratio.

DNA Binding Affinity

Binding to calf thymus DNA was determined as a function of ionic strength at pH 7 for each compound, using equilibrium dialysis at 37°. Representative Scatchard plots for DAPA-tO (Fig. 3) demonstrated the expected marked change in association constant (K_{DNA}, intercept on ordinate) with ionic strength. The binding site size (reciprocal of intercept on the abscissa), though less precisely defined, showed no consistent trend with ionic strength. Values of log K_{DNA} are plotted as a function of ionic strength for each compound in Fig. 4. The number of ion pairs involved in the interaction with DNA can be determined from these plots, with a gradient of -0.88 $\{d(\log K_{DNA})/d[K^+]\}$ expected for binding of a monocation to the DNA polyanion [35]. The inferred numbers of ion pairs are summarised in Table 2. DAPA and NC showed similar ionic strength dependence, consistent with *ca.* 2 ion pairs, which indi-

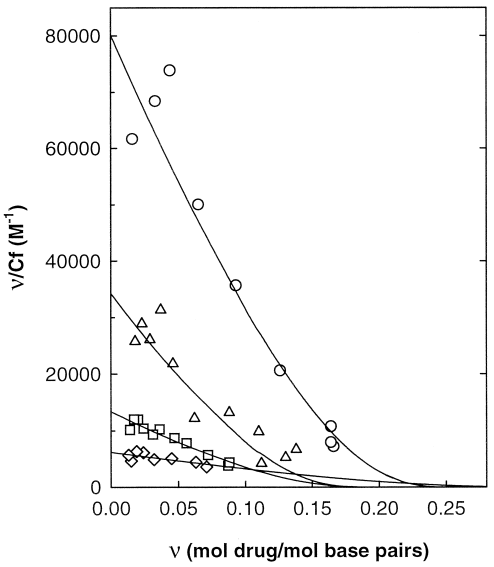

FIG. 3. Scatchard plots for the binding of DAPA-tO to calf thymus DNA as a function of ionic strength at 37° determined by equilibrium dialysis. Key: 0.02 M K^+ (○), 0.04 M K^+ (Δ), 0.08 M K^+ (□), and 0.16 M K^+ (◇). Binding curves were fitted using the neighbouring site exclusion model of McGhee and von Hippel [34].

cated that it is the diprotonated species that binds to DNA. In contrast, the *N*-oxides DAPA-tO, DAPA-aO, and NC-tO had slopes that equated to approximately 1.4 ion pairs in the drug:DNA complex, suggesting that they behave more like monocations.

Binding affinities at physiological ionic strength (0.15 M) were estimated by interpolation of the data of Fig. 4 and are summarised as $K_{DNA,0.15}$ in Table 2. The binding of NC-aO was too weak to measure, except at low ionic strength, and was extrapolated assuming the same ionic strength dependence as for DAPA-aO. Within each series DNA binding at 0.15 M ionic strength was strongest for the parent tertiary amines (DAPA and NC) and weakest for the aromatic *N*-oxides (DAPA-aO and NC-aO), with the tertiary amine *N*-oxides (DAPA-tO and NC-tO) having intermediate binding affinity (Table 2).

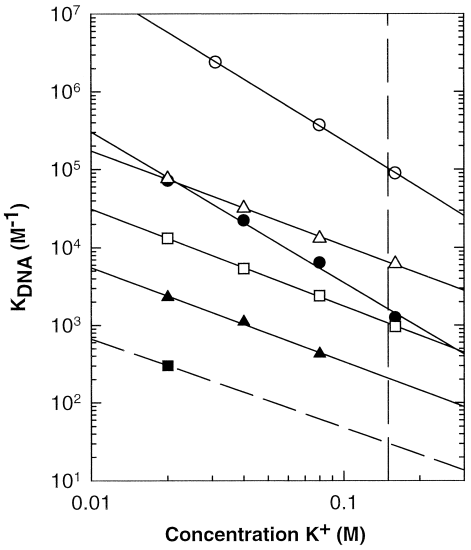

FIG. 4. Ionic strength dependence of DNA association constants. Key: DAPA (○), DAPA-tO (Δ), DAPA-aO (□), NC (●), NC-tO (▲), and NC-aO (■). The vertical dashed line indicates physiological ionic strength.

TABLE 2. Binding of DAPA, NC, and their corresponding *N*-oxides to calf thymus DNA

Compound	Ion pairs*	$K_{DNA,\,0.15}$† (M^{-1})	$\frac{K_{DNA}\text{ amine}}{K_{DNA}\ N\text{-oxide}}$‡
DAPA	2.2	100,000	—
DAPA-tO	1.4	6400	16
DAPA-aO	1.4	1000	100
NC	2.2	1600	—
NC-tO	1.4	200	8
NC-aO	—	≈30§	≈50

*Number of ion pairs in ligand:DNA complex, determined from gradient of Fig. 4, assuming $d(\log K_{DNA})/d[K^+] = -0.88$ for a single ion pair [35].

†DNA binding affinity in HEPES buffer at 0.15 M K^+, 37°, estimated from Fig. 4.

‡Ratio of DNA binding affinities for the parent compound and *N*-oxide at 0.15M K^+.

§Extrapolated assuming the same ionic strength dependence as DAPA-aO.

The extent to which metabolic reduction of the *N*-oxides to the corresponding amines would increase DNA binding affinity was calculated as the ratio K_{DNA} amine/K_{DNA} *N*-oxide (Table 2). Reduction of the aromatic *N*-oxide would result in an increase in binding affinity of 100-fold for DAPA–aO and *ca.* 50 for the corresponding nitroacridine NC-aO. Reduction of the tertiary amine *N*-oxides would result in a smaller increase in DNA binding affinity of 16- and 8-fold for DAPA-tO and NC-tO, respectively.

Lipophilicity

The lipophilicity of the *N*-oxides and corresponding amines was determined from HPLC analysis of drug distribution between octanol and aqueous buffer at pH 7.4. Partition coefficients for the neutral species log *P* (dibase), calculated for the free bases by assuming that the charged forms have very low lipophilicity, showed the same pattern in both the DAPA and nitroacridine series, with the parent amines (DAPA, NC) being more lipophilic than the aromatic *N*-oxides (DAPA-aO, NC-aO), and the tertiary amine *N*-oxides (DAPA-tO, NC-tO) having lowest lipophilicity (Table 3). The nitroacridines have higher log *P* (dibase) values than the DAPA analogues, which may reflect iminoacridan tautomerisation of the nitroacridines [37]. At pH 7.4, log *P* values for the nitroacridine series were highest for NC, with a similar value for NC-tO, whereas NC-aO had the lowest lipophilicity (Table 3). In the DAPA series the aromatic *N*-oxide DAPA-aO was the most lipophilic, followed by DAPA itself, with DAPA-tO being the least lipophilic.

TABLE 3. pK_a, log *P*, and cellular uptake factors for DAPA, NC, and their corresponding *N*-oxides

Compound	pKa		log *P*		Uptake factor (Ci/Ce)*	
	side-chain	chromophore	pH 7.4 (measured)	dibase (calc.)	$-NH_4Cl$	$+NH_4Cl$†
DAPA	9.7‡	8.8‡	−0.16	3.6	204 ± 16	104 ± 7
DAPA-tO	5.1‡	8.8‡	−1.00	0.4	0.4 ± 0.2	0.3 ± 0.1
DAPA-aO	9.7‡	6.2‡	0.13	2.5	53 ± 2	7 ± 0.1
NC	9.7‡	6.2§	1.5	3.8	138 ± 1	27 ± 1
NC-tO	5.1‡	7.1§	1.3	1.4	0.7 ± 0.5	ND‖
NC-aO	9.7‡	<6.2‡	0.77	3.1	8.6 ± 0.2	3.9 ± 0.1

*Ratio of intracellular (Ci) to extracellular (Ce) drug concentrations using an input concentration of 30 μM. Errors are ranges for duplicate determinations.
†Ci/Ce determined in the presence of 10 mM NH_4Cl.
‡Estimated values based on data for structurally similar compounds in [36].
§From [13].
‖Not determined.

Cellular Uptake and Metabolism

Cell uptake and metabolism of compounds in AA8 cell suspensions, under the same conditions as the cytotoxicity assays, was determined by HPLC using 30 μM as the initial drug concentration, with rapid centrifugation to separate cells from extracellular medium. All of the compounds except the tertiary amine *N*-oxides were accumulated to high concentrations in cells under aerobic conditions (Table 3). Ratios of intracellular to extracellular drug (C_i/C_e) were highest for the non-*N*-oxides (204 and 138 for DAPA and NC, respectively), lower for the aromatic *N*-oxides, and were less than unity for the tertiary amine *N*-oxides ($C_i < C_e$). Addition of the lysosomotropic agent ammonium chloride (10 mM) inhibited uptake of the four compounds that were accumulated in cells to high concentrations (DAPA, DAPA-aO, NC, NC-aO), although the extent of inhibition varied from *ca.* 50% for DAPA and NC-aO to 90% for DAPA-aO (Table 3).

Rates of metabolism of the *N*-oxides in aerobic and hypoxic AA8 cell cultures under these same conditions were determined by summing intracellular and extracellular drug. Figure 5 shows loss of the *N*-oxides (initial concentration 30 μM) in the upper panels, and formation of the corresponding amines in the lower panels. Both DAPA-tO

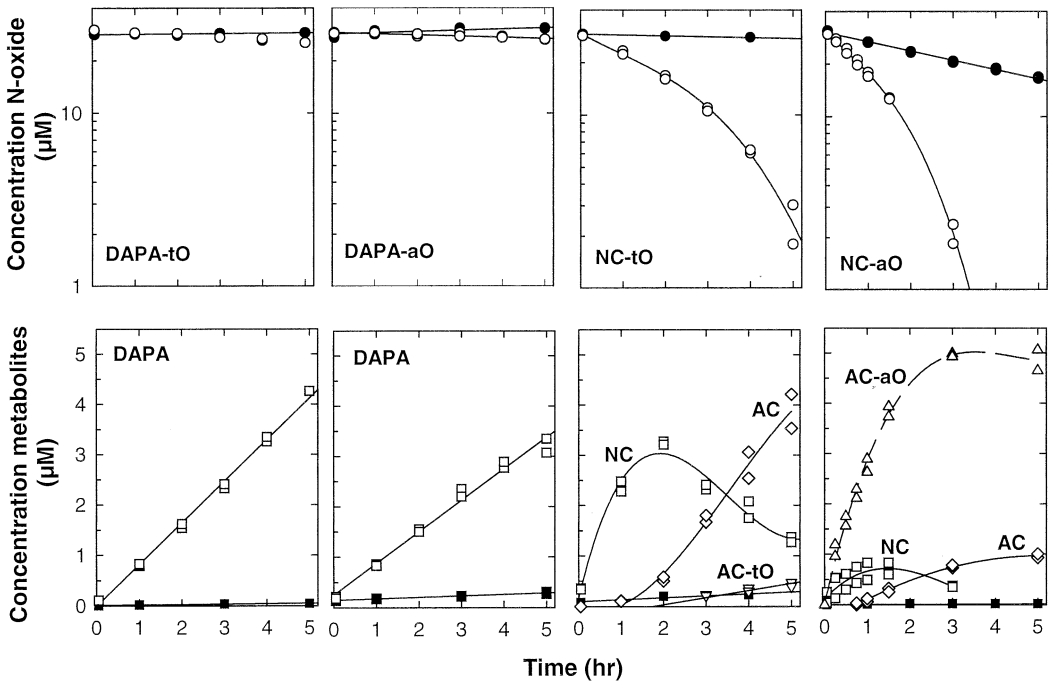

FIG. 5. Metabolism of DAPA and NC *N*-oxides in AA8 cell cultures (10^6 cells/mL) exposed to a 30 μM concentration of drug under aerobic (filled symbols) and hypoxic (open symbols) conditions showing loss of parent *N*-oxide (top panel) and formation of metabolites (lower panel). Key: (□ , ■): Products resulting from reduction of the *N*-oxide moiety (DAPA, NC). Other symbols: products from reduction of the 1-nitro group (◇: AC; ▽: AC-tO; Δ: AC-aO). The ordinate represents total concentration calculated by summing intracellular plus extracellular drug. Data are from duplicate experiments.

TABLE 4. HPLC retention times and absorbance maxima for DAPA, NC, the corresponding *N*-oxides and 1-amine metabolites

Compound	Retention time (min)	Absorbance maxima (nm)
NC	13.1	276, 324(s), 436
AC	8.5	272, 336, 436
NC-tO	14.8	276, 322(s), 436
AC-tO	13.8	272, 336, 436
NC-aO	16.7	284, 460
AC-aO	11.2	276, 336, 448

and DAPA-aO were reduced to DAPA selectively in hypoxic cell cultures, with loss of the DAPA *N*-oxides (approximately 0.7 μM/hr) accompanied by quantitative formation of DAPA. Further investigation indicated that the conversion of DAPA-tO and DAPA-aO to DAPA under these conditions is not the result of intracellular metabolism but occurs primarily in the extracellular medium. Thus incubation of 30 μM DAPA-tO for 1 hr in hypoxic α-minimal essential medium containing 5% fetal bovine serum with or without AA8 cells (10^6/mL) gave identical concentrations of 0.6 ± 0.1 μM DAPA. Similar results were obtained for DAPA-aO, with 0.2 ± 0.1 μM DAPA without cells and 0.3 ± 0.1 μM DAPA with cells. At a higher cell density (10^7 cells/mL) the contribution of cellular metabolism became evident, with 2- to 3-fold higher concentrations of DAPA (1.3 ± 0.1 and 0.9 ± 0.2 μM for DAPA-tO and DAPA-aO, respectively).

The nitroacridine tertiary amine *N*-oxide NC-tO was consumed more rapidly than the DAPA *N*-oxides in hypoxic cell cultures, with a first half-life of 2.4 hr (Fig. 5), accompanied by formation of NC as the major metabolite at early times. Incubation of 30 μM NC-tO in hypoxic culture medium for 1 hr in the absence of cells resulted in loss of 2.90 ± 0.24 μM NC-tO compared with 2.5-fold greater metabolism in hypoxic cell cultures [7.20 ± 0.48 μM NC-tO lost after 1 hr (Fig. 5)]. In the absence of cells NC was the only metabolite detected, indicating that extracellular metabolism reduces only the *N*-oxide moiety and not the nitro group. As with the DAPA *N*-oxides, there was almost complete inhibition of metabolism under aerobic conditions. Under hypoxia, NC was metabolised subsequently by cells to a number of other products, including a more polar derivative (Rt 8.5 min, 4.6 min ahead of NC) that increased in concentration throughout the experiment. On-line mass spectrometry (electrospray, positive mode) of this peak gave a parent molecular ion ($[M+H]^+$) with m/z = 295, identifying it as the 1-amine, AC (Fig. 1, Table 4). This assignment is consistent with the change in absorbance spectrum relative to NC (Table 4), indicating metabolism of the chromophore rather than the side-chain. A minor peak, reaching a maximum at 5 hr, was also observed with retention time 13.8 min (1.0 min earlier than NC-tO) and had an absorbance spectrum essentially identical to AC (Table 4). Mass spectrometry of this peak gave $[M+H]^+$ = 311, identifying this metabolite as AC-tO, the 1-amine of NC-tO.

Metabolism of NC-aO in hypoxic cultures was even more rapid than for NC-tO, with a first half-life for disappearance of the *N*-oxide of 1.2 hr. Unlike NC-tO, there was no loss of NC-aO in the absence of cells; incubation of 30 μM NC-aO in hypoxic culture medium for 1 hr gave 31.1 ± 0.3 μM NC-aO. Cellular metabolism of NC-aO under hypoxia gave three metabolites. The major species (Rt = 11.2 min) was identified by on-line electrospray mass spectrometry ($[M+H]^+$ = 311) as the 1-amino reduction product AC-aO. This assignment is consistent with the shift in absorbance spectrum of the chromophore (Table 4). AC-aO reached a steady-state concentration of 5 μM by 3 hr. Two minor metabolites were observed: NC (Rt = 13.2 min), which reached a maximum concentration of *ca.* 0.75 μM at about 1.5 hr, and AC (Rt = 8.5 min), which accumulated throughout the exposure period. Thus, in the case of NC-aO, reduction of the 1-nitro group in hypoxic cells was more rapid than that of the *N*-oxide.

Unlike the tertiary amine *N*-oxide, there was also significant loss of NC-aO in aerobic cell cultures ($t_{1/2}$ = 5.7 hr), indicating incomplete inhibition of metabolism by O_2. In the absence of cells there was no significant loss of NC-aO in aerobic culture medium, indicating that the observed metabolism in aerobic cultures was cell-dependent. NC and its corresponding 1-amine AC were the only metabolites observed in aerobic cell cultures, and were detected only in trace amounts.

DISCUSSION

Derivatisation of acridine DNA intercalators as *N*-oxides, in either the aliphatic side-chain (tertiary amine *N*-oxides) or the acridine ring (aromatic *N*-oxides), markedly lowered aerobic cytotoxic potency relative to the parent amines. The extent of suppression of aerobic toxicity was less for the aromatic than the tertiary amine *N*-oxides in both the acridine (DAPA) and nitroacridine (NC) series (Table 1). By this criterion the tertiary amine *N*-oxides would be expected to have greater potential as bioreductive prodrugs, but utility as hypoxia-selective cytotoxins will also depend on whether metabolic reduction is facile, and whether it is inhibited by oxygen. The present study confirms the excellent selectivity of NC-tO as a hypoxic cytotoxin (HCR = 820), and demonstrates that the corresponding aromatic *N*-oxide NC-aO has an HCR no greater than NC itself. Neither of the DAPA *N*-oxides demonstrated any differential in cytotoxicity between aerobic and hypoxic cells.

DNA binding, lipophilicity, cell uptake, and metabolism of these compounds was investigated to clarify the biochemical basis for the large variations in aerobic and hypoxic cytotoxicities. Both the tertiary amine *N*-oxide and the aromatic *N*-oxide of NC had low DNA binding affinity relative to the parent amine, and bound with an average ligand charge equivalent to +1.4, rather than as a dication like NC. The same pattern was observed in the

DAPA series. The suppression of DNA binding affinity was greater when the *N*-oxide was on the acridine chromophore rather than the basic alkylamino side-chain. This presumably resulted from specific interference with bonding interactions between the chromophore and DNA for the aromatic *N*-oxides. The greater suppression of DNA binding affinity for NC-aO (50-fold) than NC-tO (8-fold), despite the much higher HCR for the latter (Table 1), indicates that masking of DNA binding is not the only determinant of hypoxic selectivity of the acridine *N*-oxides.

The parent amines DAPA and NC were accumulated in cells to high concentrations (Table 3), consistent with their high K_{DNA} values and high pK_a values, these features being expected to drive sequestration in cells via reversible binding to DNA and entrapment in acidic vesicles, respectively. Endosomal uptake of DAPA in AA8 cells was confirmed by the observed inhibition of net cellular uptake by the lysosomotropic base NH_4Cl. This agrees with previous fluorescence microscopy studies with V79 cells [18]. The larger effect of NH_4Cl on uptake factors of NC than of DAPA is consistent with the expected greater contribution of DNA binding for the latter (60-fold higher $K_{DNA,\ 0.15}$, Table 2). Both aromatic *N*-oxides also showed substantial accumulation in cells, and inhibition of this accumulation by NH_4Cl, indicating endosomal accumulation, as expected from their high side-chain pK_a values. In distinct contrast, both tertiary amine *N*-oxides were excluded from cells ($C_i < C_e$), despite having higher DNA binding affinities than the aromatic *N*-oxides. In the case of NC-tO, the lack of basicity of both chromophore and side-chain (Table 3) may account for its failure to accumulate in acidic endosomes, but the lack of accumulation of DAPA-tO (which has a relatively basic chromophore, pK_a 8.8) is surprising.

These results raised the possibility that DAPA-tO (and possibly NC-tO) are sufficiently hydrophilic that passive diffusion through the plasma membrane is compromised. Measurement of log *P* at pH 7.4 demonstrated that DAPA-tO is indeed considerably more hydrophilic than the other compounds (Table 3), suggesting that this may account for its exclusion from cells. This analysis ascribes the lack of uptake of the two tertiary amine *N*-oxides by cells to different mechanisms; in the case of NC-tO, pK_a values (and DNA binding affinity) are not high enough to drive accumulation, whereas DAPA-tO is too hydrophilic for efficient membrane permeation. This model suggests that the determinants of cellular uptake of DNA intercalator *N*-oxides are complex, with independent effects of DNA binding, pK_a, and lipophilicity. While the model is consistent with observation, given its complexity, a more extensive series of compounds would need to be investigated to clarify the contributions of the proposed determinants.

The other key determinant of the utility of *N*-oxides as bioreductive prodrugs is their sensitivity to bioreductive removal of the *N*-oxide moiety, and the ability of O_2 to inhibit this process. Overall metabolic consumption of the prodrugs under hypoxia was much faster for the NC *N*-oxides than the DAPA *N*-oxides (Fig. 5). For the latter compounds, loss of the parent compounds (ca. 0.7 μM/hr) corresponded within error to formation of the metabolically stable product DAPA. If this rate (2% of initial prodrug concentration/hr) applied at the much higher concentrations of the cytotoxicity experiments, the exposure to the DAPA metabolite would be 36 μM·hr for DAPA-aO, which is of the same order as the DAPA C_{10} for 1-hr exposure of AA8 cells (21 μM). In the case of DAPA-tO, the prodrug C_{10} is much higher (Table 1), and a DAPA concentration well in excess of the C_{10} would be expected by linear extrapolation. Concentration dependence experiments (data not shown) indicated that the metabolic reduction of DAPA-aO is linear over a wide range, but that formation of DAPA from DAPA-tO saturates at approximately 10 μM/hr. Thus, the rate of metabolic reduction of the DAPA *N*-oxides to DAPA, although strongly inhibited by O_2, is probably too slow for the reduction product to provide a large increase in cytotoxicity under hypoxic conditions.

The metabolic reduction of the nitroacridine *N*-oxides is qualitatively and quantitatively different from the non-nitro *N*-oxides. Assignment of the metabolites indicates that rapid loss of NC-tO in hypoxic cultures is largely due to reduction of the *N*-oxide moiety to form NC, which subsequently is removed by reduction of the 1-nitro group. The present study identifies the major metabolite of NC as the corresponding 1-amine AC, whereas we previously [12] suggested this species to be the 1-hydroxylamine on the grounds that it was distinct from the product of catalytic hydrogenation of NC (assumed to be the 1-amine). Radiolytic reduction of NC in deoxygenated sodium formate gives the same reduction product as in cells [12], but with a stoichiometry of 4 reducing equivalents [38], which is more consistent with the hydroxylamine than the amine. However, subsequent catalytic hydrogenation of NC failed to provide the product originally reported, yielding the same species as in hypoxic cells, and high resolution mass spectrometry, infrared spectrometry, and NMR spectrometry of the purified product were all consistent with the 1-amine [38]. Metabolic reduction to the amine in AA8 cells is also consistent with the well-characterised reduction of the nitroquinoline analog of NC to the corresponding amine in this cell line [29]. Thus, the balance of evidence favors the current assignment of the major stable NC metabolite as the 1-amine.

The reduction of the *N*-oxide moiety of NC-tO occurs in preference to reduction of its 1-nitro group, as judged by the low concentrations of the 1-amine derivative that retains the tertiary amine *N*-oxide, AC-tO. In contrast, metabolic reduction of NC-aO occurs preferentially at the 1-nitro group rather than the *N*-oxide, with the major metabolite (AC-aO) containing a reduced nitro group but an intact *N*-oxide. This is a less favorable metabolic profile, since the retention of the *N*-oxide in the metabolite would be expected to hinder DNA intercalation and, therefore,

compromise cytotoxicity of reactive nitro reduction products such as the nitroso or hydroxylamine intermediates. A further difference between NC-tO and NC-aO is the less complete inhibition of overall metabolic consumption of the latter by O_2 (Fig. 5), which might also compromise its suitability as a hypoxia-selective cytotoxin.

The above prodrug reduction characteristics provide an explanation for the differential cytotoxicities of the acridine *N*-oxides in AA8 cell cultures under aerobic and hypoxic conditions. In all cases metabolic reduction of the *N*-oxide is inhibited by O_2, but for the non-nitro acridines the rate of reduction under hypoxia is relatively slow, and the resulting reduction product (DAPA) is not very potent. Thus, even under hypoxic conditions, cytotoxicity of the DAPA *N*-oxides is primarily due to the unreduced prodrugs rather than their hypoxic metabolites, and the HCR is not significantly different from unity. For NC-aO, net reduction is more rapid, but this occurs preferentially at the 1-nitro group rather than at the *N*-oxide moiety, resulting in only low concentrations of the potent nitroacridine NC (and its subsequent nitroreduction products). The major metabolite arising from nitroreduction retains an aromatic *N*-oxide moiety, which would be expected to compromise its cytotoxicity by interfering with DNA intercalation (as demonstrated in this study for the aromatic *N*-oxides of DAPA and NC, Table 2).

The large hypoxic cytotoxicity ratio of NC-tO thus is ascribed to the facile and selective reduction of its *N*-oxide moiety in hypoxic AA8 cultures to generate NC, which then is reduced further by nitroreduction under hypoxia to a potent DNA-targeted alkylating agent [14]. This identifies the sequence of reduction events as being important in the development of bis-bioreductive agents. In the context of mixed-function bis-bioreductive agents such as the nitroacridine *N*-oxides, unmasking of DNA intercalative binding (through reductive removal of the *N*-oxide moiety) should precede formation of chemically reactive intermediates via nitroreduction. Controlling the sequence of activation steps will require a more detailed understanding of both reduction pathways, including the structure–activity relationships for key reductive enzymes. While the present results provide a reasonable description of reductive activation and its relation to hypoxic cytotoxicity in one experimental model (AA8 cells in culture), much of the *N*-oxide reduction in this system is extracellular (largely mediated by denatured hemoglobin [13]). It will be important to characterise more physiological systems, including tumors with higher levels of cytochrome P450 and other potential *N*-oxide reductases, particularly if poor cell uptake by relatively hydrophilic *N*-oxides compromises access to intracellular reductases. In this regard, the high octanol/water partition coefficient of NC-tO, which results in part from its relatively non-basic nitroacridine chromophore, is a feature that should be retained.

This study was funded by a contract from the National Cancer Institute (NO1-CM-47019), with additional support from the Health Research Council of New Zealand (W.R.W.) and the Wellcome Trust (LC/MS equipment grant). We thank Dr Ho Lee for synthesis of compounds used in this study, and Dianne Ferry for assistance in identifying the metabolites of NC-tO and NC-aO.

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