Supplementary Material Available: Experimental procedures for the synthesis of hydroxyethylamine inhibitors **4a,b** (20 pages). Ordering information is given on any current masthead page.

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Articles

Hypoxia-Selective Antitumor Agents. 4. Relationships between Structure, Physicochemical Properties, and Hypoxia-Selective Cytotoxicity for Nitracrine Analogues with Varying Side Chains: The "Iminoacridan Hypothesis"

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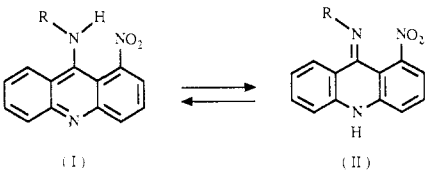
The nitroacridine derivative nitracrine is a potent hypoxia-selective cytotoxin for mammalian cells in culture. In an attempt to modulate the degree of hypoxia selectivity among this class of compounds, we have studied a series of side-chain analogues of nitracrine. Both the electronic and steric properties of the side chain are shown to be important in determining the hypoxia selectivity of the compounds, by controlling the degree of aminoacridine/iminoacridan tautomerism. Studies with the repair-defective Chinese hamster cell line UV4 indicate that the cytotoxicity of all the compounds is due to nitro group reduction and subsequent macromolecular adduct formation. However, compounds such as the 9-amino derivative, which exist totally as the aminoacridine tautomer, form much less lethal lesions than the 9-alkylamino derivatives, which exist to varying degrees in the iminoacridan conformation. For the whole set of compounds, the degree of hypoxia-selective cytotoxicity correlates well with the proportion of iminoacridan tautomer present.

Nitracrine (**2**) is a nitroacridine DNA-intercalating ligand that has been used clinically as an anticancer drug.[1,2] We have previously shown[3] that nitracrine has potent hypoxia-selective cytotoxicity against tumor cells in culture. This property is of considerable interest, since hypoxic cells are relatively resistant to ionizing radiation and to most chemotherapeutic agents. Nitracrine represents a novel approach to the selective targeting of hypoxic tumor cells since it combines a readily reduced nitro group (the source of hypoxia-selective cytotoxicity via reductive bioactivation[4]) with a DNA-intercalating chromophore, which may enhance cytotoxicity by targeting reduced metabolites to DNA.

Although the net metabolic reduction of nitracrine is partially inhibited by oxygen (due to back-oxidation of the initial radical anion[4]), absolute rates of metabolism are high even in well-oxygenated tissue.[4] This leads to two problems which appear to limit activity against hypoxic cells in solid tumors. Firstly, metabolic activation in normal tissues with high nitroreductase content, such as liver,[4] probably contributes to host toxicity. Secondly, studies with multicellular spheroids indicate that metabolic consumption of drug in relatively well-oxygenated tissue is one of the important determinants restricting diffusion to the target hypoxic microenvironments.[4] The development of nitroacridines with clinical utility against hypoxic tumor cells will require analogues with improved metabolic stability and/or more effective inhibition of metabolism (and cytotoxicity) by oxygen.

Scheme I



Improved metabolic stability has been sought by using a series of acridine-substituted analogues of nitracrine, where the nitro group reduction potentials are modulated

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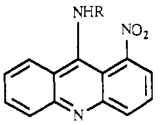
Table I. Physicochemical Properties of Side-Chain Analogues of Nitracrine



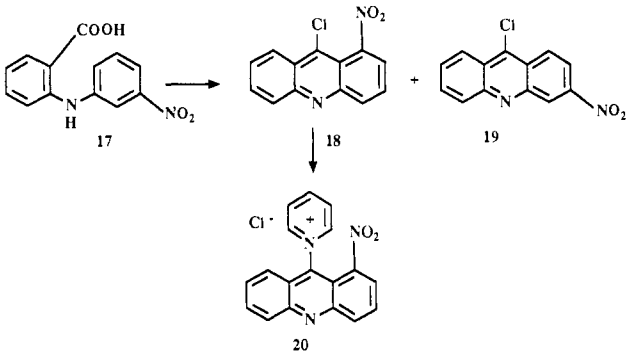
no.	R	Rm[a]	pK_a[b]	% un-ionized[c]	K[d]	$E(1)$,[e] mV
1[f]	$(CH_2)_2NMe_2$	−0.99	4.9	99	3.64 ± 0.36	−347 ± 10
2[g]	$(CH_2)_3NMe_2$	−0.85	6.2	86	18.0 ± 3.7	−303 ± 11
3[g]	$(CH_2)_4NMe_2$	−0.79	6.9	56	92.5 ± 10.0	−261 ± 11
4[h]	$(CH_2)_5NMe_2$	−0.71	7.4	28	112 ± 6	−256 ± 10
5[i]	$(CH_2)_6NMe_2$	−0.64	(7.4)[j]	28	85 ± 7	−263 ± 10
6[k]	$(CH_2)_3NHMe$	−0.90	6.2	86	194 ± 25[l,r]	−312 ± 10
7[m]	$(CH_2)_3COOH$		*n*	28	35.2 ± 2.4	−289 ± 10
8[o]	H	−0.30	7.3	33	121 ± 2	−254 ± 10
9[i]	CH_3	−0.13	7.2	39	57 ± 7	−273 ± 11
10[i]	$(CH_2)_2CH_3$	0.30	7.2	39	34.0 ± 4.9	−288 ± 11
11[m]	$(CH_2)_2CH(Me)_2$	0.58	(7.4)[j]	28	6.5 ± 1.5[l]	−329 ± 12
12[p]	CH_2Ph	0.37	(7.4)[j]	28		(−331)[q]
13[m]	$(CH_2)_2Ph$	0.39	(7.4)[j]	28	224 ± 51[l,r]	−309 ± 12
14[s]	$(CH_2)_2OH$	−0.30	7.2	39	50.4 ± 2.3	−278 ± 10
15[s]	$(CH_2)_3OH$	−0.15	7.55	22	35.3 ± 7.3	−287 ± 12

[a] Rm values (a measure of lipophilicity) were determined chromatographically as in ref 16. [b] Acridine pK_a values were determined in aqueous solution at 25 °C by the spectrophotometric method detailed in ref 16. [c] Percent acridine free base at pH 7, calculated from the measured pK_a. [d] K: equilibrium constant for the equation $BV^{\bullet+} + ArNO_2 \rightleftharpoons ArNO_2^{\bullet-} + BV^{2+}$, where V is benzylviologen ($BV^{2+}/BV^{\bullet+}$ = −380 ± 10 mV) and the solvent is 0.2 M 2-propanol unless otherwise indicated. [e] Reduction potential at pH 7, corrected for ionic strength effects. [f] Reference 18. [g] Reference 19. [h] Reference 30. [i] Reference 31. [j] Free base too insoluble; pK_a assumed. [k] Reference 15. [l] Solvent was 1 M 2-propanol/1 M acetone. [m] See the Experimental Section. [n] pK_a value not determined. [o] Reference 32. [p] Reference 33. [q] $E(1)$ value extrapolated from the value obtained at pH 6 ($E(1)$ = −303 ± 11 mV), where the compound was more soluble. Solvent 2 M 2-propanol. [r] Value determined with methylviologen ($MV^{2+}/MV^{\bullet+}$) = −447 ± 7 mV). [s] Reference 34.

by the electronic properties of the substituent groups.[5,6] Several derivatives containing electron-donating groups did show lower redox potentials and had substantially slower rates of metabolism by cultured cells than nitracrine, while retaining similar levels of hypoxia selectivity.[5]

The modulation of hypoxic selectivity presents greater difficulties, since details of the radical chemistry relating to oxygen inhibition of nitroreduction in the 1-nitroacridines are not well-understood at the molecular level. Structure–activity relationships (SAR) for this type of biological activity have not been studied in the 1-nitroacridines (except for the acridine-substituted compounds mentioned above) or compared with the more well-defined[7,8] SAR for antitumor activity in this series. The (dimethylamino)propyl side chain of nitracrine plays a critical role in its biological activity, by influencing not only lipophilicity and DNA binding but also the structure of the chromophore. Thus, while 9-amino-1-nitroacridine (8) exists as the aminoacridine tautomer (I, Scheme I), as shown[9] by a C9–N10 bond length of 1.33 Å typical for aromatic amines, nitracrine (2) and other 9-(alkylamino)-1-nitroacridines exist as the iminoacridan tautomer (II), with a C9–N10 bond of ca. 1.28 Å, indicative of much double-bond character and a severely distorted nonplanar chromophore, due to steric interactions between the 1- and 9-substituents.[10,11]

Scheme II



In this paper we evaluate a series of nitracrine analogues with varying side chains to study the influence of side-chain structure on their reduction potentials and hypoxia-selective cytotoxicity. Compounds bearing four different types of side chains (cationic, anionic, neutral hydrophobic, and neutral hydrophilic) were studied, and the results are recorded in Table I.

Chemistry

The compounds of Table I were prepared by phenol-mediated coupling of the appropriate amines with 9-chloro-1-nitroacridine (18), which in turn is conveniently prepared in small quantities by flash chromatography of the mixture of 1- and 3-nitro isomers 18 and 19 formed by $POCl_3$ cyclization of *N*-(3-nitrophenyl)anthranilic acid (17, Scheme II).[12] For larger quantities, a more convenient method is fractional crystallization of the 9-(*N*-pyridinium) chlorides,[13,14] followed by conversion to 9-chloro compound

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Table II. Biological Properties of Side-Chain Analogues of Nitracrine

		growth inhibition		clonogenicity		HF[e] (AA8/UV4)	
no.	R	IC_{50}(air),[a] nM	IC_{50} ratio[b] (air/N_2)	CT_{10}(air),[c] μM h	CT_{10} ratio[d] (air/N_2)	air	N_2
1	$(CH_2)_2NMe_2$	47 ± 5	7.4 ± 0.9	1.22 ± 0.04	14 ± 2	19 ± 2	15 ± 5
2	$(CH_2)_3NMe_2$	26.3 ± 1.4	3.5 ± 0.3	0.47 ± 0.02	9.9 ± 1.0	12.9 ± 1.5	14.9 ± 1.7
3	$(CH_2)_4NMe_2$	26 ± 1	2.6 ± 0.4	0.46 ± 0.13	11.9 ± 0.7	20 ± 1	29 ± 8
4	$(CH_2)_5NMe_2$	15.5 ± 1.0[f]	1.65 ± 0.3	0.32	7.0		
5	$(CH_2)_6NMe_2$	11.5 ± 3.5[f]	1.55 ± 0.2	0.38	6.8		
6	$(CH_2)_3NHMe$	34 ± 7	4.8 ± 0.9	0.38 ± 0.04	5.9 ± 0.4	14 ± 3	11 ± 3
7	$(CH_2)_3COOH$	18000 ± 2000	4.2 ± 0.8	50	2.5	6.1 ± 0.7	6.6 ± 0.2
8	H	3600 ± 1000	1.02 ± 0.01	3.9[g]	1–2[h]	4.5 ± 0.2	5.8 ± 1.2
9	CH_3	3.8	1.3	0.01[g]	0.8–1.5[g,h]	11	11
10	$(CH_2)_2CH_3$	32 ± 3	1.42 ± 0.04	0.62	3.4	11 ± 3	20 ± 3
11	$(CH_2)_2CH(Me)_2$	120 ± 28	1.13 ± 0.05	3.2	2.4	12 ± 2	16 ± 4
12	CH_2Ph	380	1.2	9.0	1.9	12	17
13	$(CH_2)_2Ph$	122	1.3	2.8	2.3	14	15
14	$(CH_2)_2OH$	11.5 ± 1.2	1.1 ± 0.2	0.13	3.2	15	18
15	$(CH_2)_3OH$	27 ± 3	1.45 ± 0.03	0.43	3.0	15 ± 2	13 ± 3

[a] IC_{50}: the concentration of drug to reduce cell numbers to 50% of controls, using AA8 cells in the microassay (see the text). [b] IC_{50} ratio = IC_{50}(air)/IC_{50}(N_2). [c] CT_{10}: the product of the drug concentration times the exposure time needed to reduce cell survival to 10% of controls, using AA8 cells at 10^6/mL in the clonogenic assay (see the text and ref 6). [d] CT_{10} ratio = CT_{10}(air)/CT_{10}(N_2). [e] HF: hypersensitivity factor = IC_{50}(AA8)/IC_{50}(UV4). [f] IC_{50} values for these compounds measured against V79 cells using the miniassay (ref 6): values are comparable with those determined with AA8 cells and the microassay (see the text). [g] Cell density 10^5/mL. Rapid drug metabolism at 10^6 cells/mL precluded measurement of rate of killing. [h] Ratio varies with drug concentration. See Figures 2 and 3.

18. Although *N*-pyridinium chloride **20** has been coupled directly with amines to prepare nitracrine analogues,[15] in our hands this method gave products of lower purity.

Physicochemical Studies

Compound lipophilicities were measured by liquid–liquid chromatography[16] for the drug cations and are recorded in Table I. The pK_a values for the acridine chromophores were measured by UV spectrophotometry as previously described.[16] Nitro group reduction potentials were determined by pulse radiolysis, by establishing a reversible equilibrium[5,17] against benzylviologen (where the value of the redox couple $BV^{2+}/BV^{\bullet+}$ is −380 ± 10 mV) in pH 7 phosphate-buffered aqueous 2-propanol solutions.

$$ArNO_2 + BV^{\bullet+} \rightleftharpoons ArNO_2^{\bullet-} + BV^{2+}$$

The equilibrium constant K for the reaction was determined spectrophotometrically for each compound, and the $E(ArNO_2/ArNO_2^{\bullet-})$ redox couples were calculated from this[5,18] and are recorded in Table I.

Biological Studies

The cytotoxicity of the compounds was determined under both aerobic and hypoxic conditions against Chinese hamster ovary cells (CHO, subline AA8) in two assays, and the results are listed in Table II. In the growth inhibition microassay,[6] 0.05-mL cultures were grown and exposed to drugs for 18 h in 96-well tissue-culture dishes under aerobic or hypoxic conditions. Drugs were then removed by washing with fresh medium and cultures were grown for 72–78 h before cell numbers were determined by staining with methylene blue and measuring absorbance in a microplate photometer.[6,19] The IC_{50} was defined as the drug concentration which reduced cell numbers to 50% of those in control cultures on the same 96-well dish. In the clonogenic assay, AA8 cells in early unfed plateau phase were exposed to drugs in continuously gassed, stirred suspension cultures, and clonogenic survival was assessed at various times by determining plating efficiency as described previously.[3,5] The drug concentration multiplied by the time required to reduce cell survival to 10% of controls (the CT_{10}) was determined, under both aerobic and hypoxic conditions. This technique uses clonogenic potential as the end point and has the advantage over the growth inhibition assay of providing essentially complete anoxia throughout the period of drug exposure.

Results

Physicochemical Properties. The first group of compounds are the *N,N*-dimethylaminoalkyl homologous series (**1–5**), to which nitracrine itself (**2**) belongs. As expected, these show increasing lipophilicity with longer chain length. For the early members of the series, pK_a values vary markedly due to the proximity effect of the second charged center. The homologues with shorter charged side chains (**1–3**) have relatively low acridine pK_as, and these increase with chain length until the C5 and C6 analogues (**4** and **5**) have pK_a values similar to that of **8**, with an uncharged alkyl side chain.

For these compounds (**1–5**) there is a steady increase in $E(1)$ at pH 7 with chain length, presumably due to the increase in pK_a across the series resulting in an increasing proportion of the more electrophilic charged chromophore at pH 7. In fact the $E(1)$ values correlate well with the percentage of charged chromophore present at pH 7 (determined from the measured pK_a values for compounds **1–4** and **6**), as shown in eq 1.

$$E(1) = 1.19\ (\pm 0.38)\ (\%\ \text{ionized}) - 327\ (\pm 14) \quad (1)$$
$$n = 5,\ r = 0.87,\ s = 21$$

From this it is possible to calculate the $E(1)$ value expected for fully charged (−220 mV) and noncharged (−335 mV) chromophores, with the difference of 115 mV being attributable to the increased electrophilicity due to a cationic charge on the acridine. This simple analysis yields a $\Delta E(1)$ value of approximately $^1/_2$ of that (ca. 250 mV) observed[5] for nitracrine itself as the pH is varied from 3 to 9. The reason for this is that, at high pH, deprotonation of the side chain in both the ground state (pK_a 9.7) and

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radical (pK_a ca. 10.2) forms significantly lowers the potential of the acridine ring.

Some of the compounds with neutral side chains were too insoluble for their pK_a values to be determined directly (see the Experimental Section). However, all the compounds which could be directly assessed had similar pK_as (7.2–7.6), allowing a broad assessment of the influence of side chain structure on $E(1)$ independent of pK_a effects. Generally, increasing steric demand in the side chain results in a lower reduction potential. This is well-illustrated by compounds (8–13), which bear hydrocarbon side chains varying from H to $(CH_2)_2Ph$ and where the $E(1)$ values range from −254 mV for the smallest to about −330 mV for the largest side chain.

Aerobic Cytotoxicity. Table II records data for the cytotoxicities of nitracrine (2) and the 14 analogues against AA8 cells, using both the growth inhibition and clonogenicity assays. For compounds 4 and 5, the growth inhibition assay was carried out with V79-171b, another Chinese hamster fibroblast cell line, in 24-well tissue culture dishes. However, the two cell lines gave virtually identical results for nitracrine (2), with an aerobic IC_{50} of 26.3 ± 1.4 nM and an IC_{50} ratio (air/N_2) of 3.5 ± 0.3 (n = 18) for AA8 cells, compared to an aerobic IC_{50} of 27.5 ± 1.1 nM and an IC_{50} ratio of 4.0 ± 0.4 (n = 14) for V79-171b cells (Table II).

The growth inhibition assay indicated a wide range (>1000-fold) of cytotoxic potencies. There was generally a close correlation between aerobic cytotoxic potencies in both the growth inhibition and clonogenic assays, with the ratio of the magnitudes of the end points (CT_{10} and IC_{50}, defined in Table I) being relatively constant at about 20 for most of the drugs. The exceptions were the 9-amino (8) and 9-methylamino (9) analogues, which had relatively low potencies in the growth inhibition assay compared to the clonogenic assay. This can be accounted for by the metabolic instability of these compounds, since the former assay uses an 18-h drug-contact time, while the latter measures a true initial rate of cell killing. Rapid metabolic depletion of compounds 8 and 9 is suggested by the rapid decrease in rate of killing by these compounds compared to nitracrine (2) as illustrated in Figure 1. The decreasing rate of cell kill by nitracrine in hypoxic, stirred suspension culture is known[3,6] to be due to metabolism of the drug. Further evidence for rapid metabolism of 8 and 9 is provided by measurements of macromolecular adduct formation (vide infra).

For the (*N*,*N*-dimethylamino)alkyl series (1–5), there is a steady increase in aerobic cytotoxicity with increasing lipophilicity, which is summarized by eqs 2 and 3. Al-

$$\log IC_{50} = -1.74\ (\pm 0.17)\mathrm{Rm} - 0.03\ (\pm 0.14) \quad (2)$$

$$n = 5,\ r = -0.98,\ s = 0.05$$

$$\log CT_{10} = -1.50\ (\pm 0.34)\mathrm{Rm} - 1.51\ (\pm 0.42) \quad (3)$$

$$n = 5,\ r = -0.90,\ s = 0.11$$

though similar correlations exist between cytotoxicity and both pK_a and $E(1)$, due to the covariance of these physicochemical variables within the homologous series, the independent variable that best predicts the cytotoxicity of the monomethylamino analogue 6 is lipophilicity (observed log IC_{50} = 1.53, calculated from eq 1, 1.59).

However, none of the relationships can be used to explain the enormous variations in aerobic cytotoxicity among the other compounds. The low potency of acid derivative 7 is largely accounted for by its inefficient uptake into AA8 cells. HPLC studies using the conditions of the clonogenic assays reported here indicate an uptake

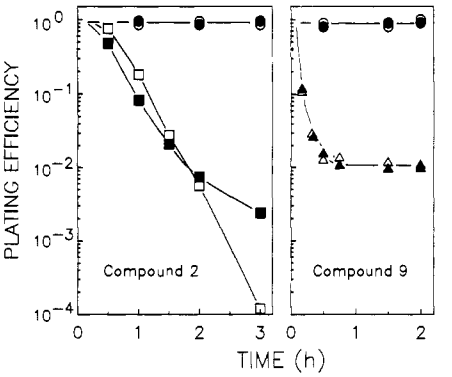

Figure 1. Cytotoxicity of 2 (left panel) and 9 (right panel) against AA8 cells in aerobic (open symbols) or hypoxic (filled symbols) stirred suspension cultures at 10^6 cells/mL: (○, ●) controls, (■) 0.25 μM compound 2, (□) 0.4 μM compound 2, (△, ▲) 0.1 μM compound 9.

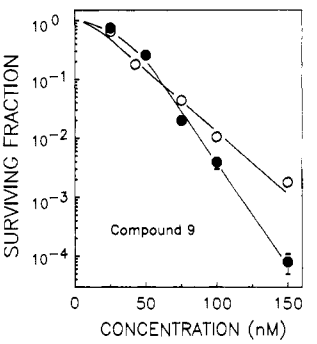

Figure 2. Survival curve for 1-h exposure of AA8 cells to compound 9 under aerobic (○) or hypoxic (●) conditions at 10^6 cells/mL. Survival is expressed relative to untreated controls (mean plating efficiency 0.85). Points are means of four determinations. Error bars (standard errors) are smaller than the plotted points when not shown.

factor (intracellular/extracellular concentration ratio) for 7 of only 2.1 ± 0.1, compared to an uptake factor of 117 ± 6 for 2 under the same conditions (P. van Zijl and W. R. Wilson, unpublished). In addition, the same studies indicated extensive hydrolysis of 7 to 1-nitroacridone, which is taken up efficiently by AA8 cells (uptake factor of approximately 36), so that intracellular concentrations of 1-nitroacridone were higher than those of 2. For this reason, the biological data obtained for 7 were not used in calculation of any of the following equations. Hydroxyethyl compound 14 was evaluated since it is reported[7] to be one of the most antitumor-active analogues of nitracrine and is undergoing clinical trial.[10] This compound was significantly more potent than nitracrine, while its propyl analogue (15) had IC_{50} and CT_{10} values similar to that of nitracrine (2).

Hypoxia-Selective Cytotoxicity. The magnitude of hypoxic selectivity for individual compounds also showed substantial variation (Table II). Figure 1 illustrates representative clonogenic assay data for compounds 2 and 9, which have CT_{10} ratios of about 10 and 1, respectively, and which demonstrate the difficulties in reducing the clonogenicity data to a single parameter. One problem is that apparent hypoxic selectivity is not independent of drug concentration, so that a full dose–response curve for 9 at 1 h (Figure 2) indicates a slightly higher activity under aerobic conditions at low concentration and a slight hypoxic selectivity at higher concentrations. A second difficulty arises in attempting to use the product of concentration × time as a single index of drug effect. Although not analyzed in detail for the present series, in cases where

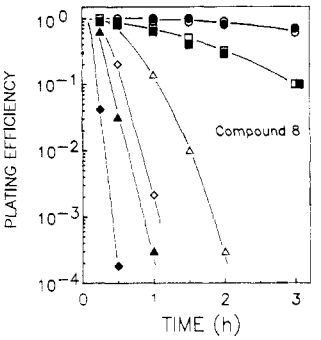

Figure 3. Cytotoxicity of compound 8 against AA8 cells in aerobic (open symbols) or hypoxic (filled symbols) cultures at 10^6 cells/mL: (○, ●) 1 μM, (□, ■) 2 μM, (△, ▲) 3 μM, (◇, ◆) 4 μM. Controls, omitted for clarity, had plating efficiencies in the range 0.8–1.0.

sufficient data were generated the product CT for a given end point was not strictly constant, and the term CT_n where $n < 1$ provided a better fit to the data. This problem was minimized by calculating CT_{10} at drug concentrations which gave similar rates of cell kill under aerobic and hypoxic conditions. These features are illustrated for compound 8 in Figure 3. In this instance, drug metabolism was so rapid that it was necessary to lower cell densities from 10^6 to 10^5/mL to provide measurable initial rates of cell killing. As for compound **9**, hypoxic selectivity was observed only at high drug concentrations. Comparison of CT_{10} values at 6 μM in air and 4 μM in N_2 indicates a differential of about 2-fold.

The growth inhibition and clonogenicity assays gave quantitative estimates of hypoxic selectivity which were consistent across the series, as shown in Figure 3 and eq 4.

$$CT_{10}[\text{ratio}] = 1.73\ (\pm 0.39) IC_{50}[\text{ratio}] + 1.36\ (\pm 1.13) \quad (4)$$
$$n = 14,\ r = 0.79,\ s = 2.63$$

This equation indicates a significant linear correlation between IC_{50} and CT_{10} ratios, with the clonogenic assay the more sensitive of the two, giving ratios about 1.7-fold larger on average. The lower sensitivity of the growth inhibition assay presumably reflects the slow deoxygenation kinetics in these unstirred cultures growing on a polystyrene substrate, which releases its dissolved oxygen only slowly. Recent studies in this laboratory indicate that induction of full (radiobiological) hypoxia requires approximately 12 h using this system, so that hypoxic selectivity is expressed only during part of the 18-h drug-contact period.

The large variation in hypoxic selectivity of the 1-nitroacridines with changes in the side chain was unexpected. Inhibition of toxicity by oxygen was most pronounced for compounds with basic side chains, particularly the lower homologues (**1**–**3**). These showed hypoxic/oxic potency differentials of 3–4-fold in the growth inhibition assay and 10–14-fold in the clonogenic assay. In contrast, the compounds with neutral side chains showed little or no selectivity, with ratios of only 1.0–2.1 in the former and 1.0–3.4 in the latter assay.

Hypersensitivity of a DNA Repair-Defective Mutant Cell Line. The relative potencies of the compounds against AA8 cells and a DNA repair-defective mutant (UV4) selected from AA8 were determined. This mutant is defective in the incision step of excision repair of DNA interstrand cross-links or bulky monoadducts.[20] The UV4

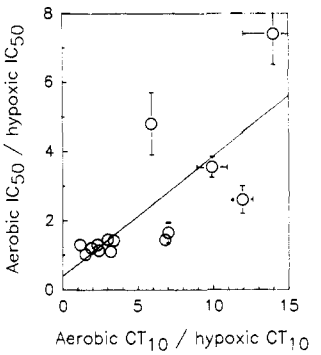

Figure 4. Hypoxic selectivity of nitracrine analogues as assessed with stirred suspension cultures with clonogenicity as the end point (abscissa) or substrate-attached cells in 96-well dishes with growth inhibition as the end point (ordinate).

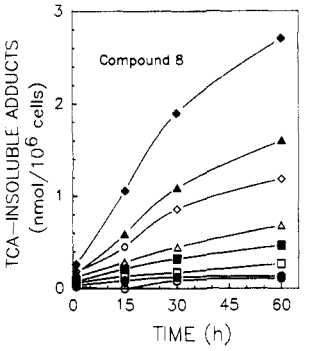

Figure 5. Formation of macromolecular adducts from [^{3}H-acridinyl]-9-amino-1-nitroacridine (**8**) in aerobic (open symbols) and hypoxic (filled symbols) AA8 cultures at 10^5 cells/mL. The yield of adducts was calculated assuming the same specific activity as that of the parent compound. Symbols as for Figure 3.

cell line is consequently hypersensitive to the cytotoxic effects of DNA alkylating or arylating agents, and its pronounced hypersensitivity (11–13-fold) to nitracrine (**2**) has been shown.[6] This level of hypersensitivity does not allow unambiguous classification of nitracrine as a cross-linking agent (ratios 8–200-fold) or as forming bulky monoadducts (ratios 2–17-fold).[21] However, the similar hypersensitivity of UV4 and UV5 to nitracrine[6] strongly suggests that the critical cytotoxic lesions formed by this compound are DNA monoadducts, since the UV5 cell line displays the same sensitivity as UV4 to the latter but is much less sensitive than UV4 to cross-linking agents.[21]

All the 9-(alkylamino)-1-nitroacridines tested in the present study showed UV4 hypersensitivity factors similar to that of nitracrine (Table II), under both aerobic and hypoxic conditions. This strongly suggests that the compounds share a common broad mechanism of cytotoxicity, with oxygen acting to modify only the rate of formation of the DNA lesions and not their type. The lower values for 9-amino compound 8 suggest a different mechanism for this compound (vide infra).

Bioreductive Activation in AA8 Cells. Nitracrine undergoes extensive oxygen-inhibited nitro reduction in AA8 cells to form macromolecular adducts.[4] These adducts correlate with cytotoxicity and presumably include the DNA lesions responsible for cell killing.[4,6] In the present study, the rates of bioreductive activation of a subset of three compounds (**2**, **8**, **9**) were compared, to determine if this could account for their widely different potencies and

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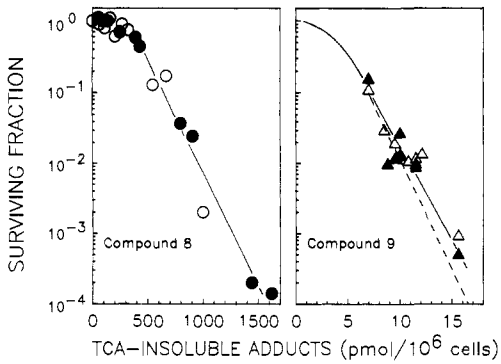

Figure 6. Relationship between surviving fraction and macromolecular adducts in AA8 cells after exposure to compound 8 (left panel) or compound 9 (right panel) for various times up to 1 h. Cell survival and adducts were assayed on samples from the same cultures (Open symbols, aerobic exposure; filled symbols, hypoxic exposure). The dashed line represents the data for nitracrine (2), redrawn from ref 4.

hypoxia selectivities. Tritiated versions of these compounds were synthesized,[12] and the formation of macromolecular adducts in AA8 cells was assessed and compared to cell killing, with both end points measured in the same experiment, as described previously for nitracrine.[4,6]

Rates of formation of total intracellular macromolecular adducts by 8 showed modest hypoxic selectivity (Figure 5), with a decrease in the initial rate from about 400–500 pmol/10^6 cells per μM drug per h under hypoxic conditions to about 200 in air. When the cell survival data (Figure 4) was plotted with the level of intracellular adducts (Figure 5) as an index of the delivered dose, the data for hypoxic and aerobic cells fell on the same curve (Figure 6), with a threshold followed by exponential killing.

Comparable experiments were performed with tritiated nitracrine (2) and 9-(methylamino) compound **9**. Rates of formation of macromolecular adducts under hypoxia were similar to compound 8, being approximately 160 and 400 pmol/10^6 cell per μM drug per h, respectively. Both compounds displayed a similar relationship between cell killing and adduct formation, but cytotoxicity was much greater than for 9-amino compound 8 when compared on this basis. One decade of cell killing corresponded to about 640 pmol of adducts/10^6 cells for compoun 8 but only about 8 pmol of adducts/10^6 cells for **2** and **9**.

Discussion

A large number of 1-nitroacridine derivatives have been synthesized, and their enhanced cytotoxicity and in vivo antitumor activity relative to that of the isomeric 2-, 3-, or 4-nitroacridines is considered due to reductive metabolism resulting in covalent attachment to DNA.[22] Structure–activity relationships for the in vivo antitumor activity of 1-nitroacridines against sarcoma 180 have been established,[7,8] and correlations between this activity and DNA cross-linking ability have been discussed.[7] The nature of the side chain is of critical importance, and early work showed that in vivo activity was confined to compounds (e.g. 1–6) bearing a cationic (alkylamino)alkyl side chain. More recently, the activity of compounds with neutral, hydrophilic hydroxyalkyl side chains was discovered and one compound of this type, hydroxyethyl derivative 14, is presently in clinical trial.[10] In contrast, compounds with neutral lipophilic or anionic side chains show no significant in vivo activity.[8]

This class of compound can exist as either the aminoacridine (I) or the iminoacridan (II) tautomer (Scheme I). A number of crystallographic studies have shown that 1-nitroacridines bearing a variety of alkyl side chains exist as the iminoacridan tautomer (II),[9,10,11] whereas the corresponding 2-nitro isomers adopt the aminoacridine configuration (I).[10] A more recent comparative NMR study[23] of nitracrine and its three nitro positional isomers shows these relationships also hold in both $CDCl_3$ and D_2O solution.

Although steric crowding is apparent in the 1-isomers, with the nitro group being twisted considerably out of the coplanar state it adopts in the 2-isomers, this cannot be the primary reason for adoption of the iminoacridan configuration since 9-amino-1-nitroacridine (8) also has a severely twisted 1-nitro group, yet it exists as the aminoacridine.[10] It appears that electronic effects are important, with an electron-withdrawing 1-nitro group, a side chain bearing an alkyl group, and an unprotonated acridine all being necessary for adoption of the iminoacridan form. Thus both the free base and the monosalt (with the charge on the dimethylamino side chain) of nitracrine adopt the iminoacridan form, while nitracrine disalt (with a charged acridine) is in the aminoacridine form.[10] Although crystal structures of the acid salts of hydroxyethyl analogue 14 and the propionic acid related to **7** show them to be in the aminoacridine conformation,[10] it seems likely that the free bases of these compounds would also adopt the iminoacridan form.

It has been suggested[10] that the SAR for antitumor activity in the nitroacridines (activity restricted to the 1-nitro derivatives) is associated with formation of the the iminoacridan configuration, which facilitates activation and subsequent covalent binding of the molecules to DNA. The present work extends this hypothesis, by showing that, although both the aerobic and hypoxic cytotoxicity of all derivatives appears to be due to nitroreduction and subsequent DNA alkylation as indicated by the hypersensitivity of UV4 cells, the cytotoxicity of these lesions is not identical for all compounds. Thus both nitracrine itself (2) and 9-(methylamino) derivative **9** form macromolecular adducts of similar average cytotoxicity (Figure 6b) and are potent cytotoxins. In contrast 9-amino compound 8, which cannot exist in the iminoacridan configuration even as the free base,[10] forms much less cytotoxic macromolecular adducts and is also much less potent (by 1 order of magnitude) than the other derivatives. Significant hypoxia-selective cytotoxicity is confined to the lower members of the (alkylamino)alkyl series (**1–3, 6**). As noted earlier, these are the compounds with the lowest acridine pK_as, allowing the highest proportions of free base (and therefore iminoacridan species) under physiological conditions. For the compounds of Table I, there is a good correlation between the degree of hypoxia selectivity measured in the growth inhibition assay (IC_{50} ratio) and the proportion of iminoacridan form present, as shown by eq 5. For 9-amino-

$$IC_{50}\ \text{ratio} = 0.06\ (\pm 0.008)(\%\ \text{iminoacridan}) - 0.31\ (\pm 0.41) \quad (5)$$

$$n = 14,\ r = 0.91,\ s = 0.82$$

acridine 8 the percent of iminoacridan form is taken as zero,[10] while for all other compounds it is assumed to be the same as the percent of free base present at pH 7.1 (the most likely mean intracellular pH under the experimental conditions used).

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(23) Boyd, M.; Denny, W. A. Manuscript submitted to *J. Med. Chem.*

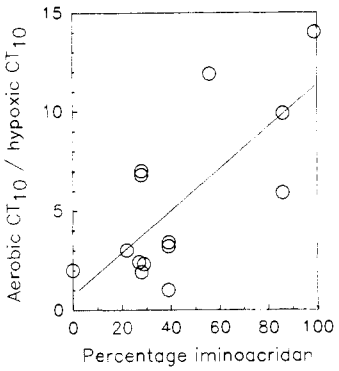

Figure 7. Relationship between the hypoxic selectivity of nitracrine analogues as assessed with stirred suspension cultures with clonogenicity as the end point and the percentage of iminoacridan tautomer present at physiological pH.

Use of the other measure of hypoxia selectivity (the CT_{10} ratio) gives a similar result (eq 6 and Figure 7), which is to be expected given the degree of correlation of these measures noted above in eq 4 and Figure 3.

$$CT_{10}\ \text{ratio} = 0.11\ (\pm 0.03)(\%\ \text{iminoacridan}) + 0.63\ (\pm 0.44) \quad (6)$$

$$n = 14,\ r = 0.75,\ s = 2.84$$

These equations imply that the hypoxia selectivity of 9-(aminoalkylamino)-1-nitroacridines depends on the amount of iminoacridan tautomer present, that the nature of the alkyl chain serves purely to control this, and that the aminoacridine tautomer has no selectivity (IC_{50} and CT_{10} ratios ca. 1 when % = 0), while a compound exclusively in the iminoacridan form would have an IC_{50} ratio of ca. 5 and a CT_{10} ratio of ca. 12).

The mechanism(s) underlying the apparently greater hypoxic selectivity of the iminoacridan form of the 1-nitroacridines has not yet been explored. It is possible that the iminacridan gives rise to a radical anion which is efficiently back-oxidized by O_2 relative to its rate of disproportionation, thus providing good inhibition of cytotoxicity by O_2. However, the high pK_a (ca. 10) of the nitracrine radical anion[5] suggests it would exist as the aminoacridine tautomer, and it is unlikely that the differences in tautomerism within the series would persist in the corresponding one-electron radicals. Alternatively, the aminoacridine tautomer may be preferentially reduced by two-electron systems such as DT-diaphorase, a known oxygen-insensitive reductase present in AA8 cells.[24]

Conclusions

The above studies on the hypersensitivity of the repair-defective cell line UV4 to 1-nitroacridines with varying 9-alkylamino side chains indicate that both the aerobic and hypoxic cytotoxicity of these compounds are due to nitro reduction and subsequent DNA adduct formation. However, the numbers of total macromolecular adducts in cells treated to equivalent cytotoxicity show large variations, indicating that the adducts formed by different members of the series are not identical. This factor, together with varying metabolic stability, goes some way toward explaining the observed wide variation in aerobic cytotoxicities. However, while a great deal of work has gone into the development of nitracrine (**2**) and its analogues,[7,8] their activity against all but the sarcoma 180 tumor system remain very modest, and it seems unlikely that further development in this series will provide compounds with significantly better activity as classical antiproliferative antitumor drugs.

The recently discovered in vitro hypoxia-selective cytotoxicity of nitracrine[3] and analogues[5,6] is more interesting, and it is worthwhile to explore further the basis for this phenomenon and to develop and compare structure–activity relationships (SAR) for this type of activity with the demonstrated SAR for in vivo sarcoma 180 activity. We have shown here that, for a series of compounds with identical chromophores, the degree of hypoxia selectivity varies in a manner consistent with the hypothesis that the iminoacridan tautomer of the chromophore is responsible. Variations in the side chain serve only to determine the proportion of iminoacridan by controlling the pK_a.

Since all 9-(alkylamino)-1-nitroacridines appear to form the iminoacridan tautomer when unprotonated,[10,23] eqs 5 and 6 suggest that compound 1, which has a pK_a low enough to exist almost entirely in the iminoacridan form, has close to the maximum degree of hypoxia selectivity to be expected from the unsubstituted 1-nitroacridine chromophore. One goal for future drug design in this area will therefore be to seek related compounds which are permanently held in the iminoacridan configuration while satisfying the other requirements, such as improved extravascular transport properties, necessary for improved activity against hypoxic cells in tumors.

Experimental Section

Chemistry. Analyses indicated by the symbols of the elements were within ±0.4% of the theoretical value and were carried out in the Microchemical Laboratory, University of Otago, Dunedin, New Zealand. Melting points were determined on an Electrothermal apparatus with a stem-corrected thermometer and are as read. NMR spectra were obtained on a Bruker WP-60 spectrometer (Me_4Si).

9-Chloro-1-nitroacridine (18). The mixture of isomers (ca. 75% 1-nitro and 25% 3-nitro by NMR) obtained by $POCl_3$-induced cyclization of *N*-(3-nitrophenyl)anthranilic acid (**17**, 75 %) was dissolved in $CHCl_3$ (100 mL) and poured into rapidly stirred petroleum ether (1 L) to obtain a finely divided precipitate. This was collected and suspended in dry pyridine (400 mL), and the suspension was stirred vigorously for 10 min at 20 °C. The undissolved solid was filtered off and washed with pyridine (2 × 70 mL), and the combined filtrates were heated to 60 °C for 20 min and then cooled to 0 °C for 20 h. The precipitate was collected, washed with ice-cold pyridine, and recrystallized from MeOH to give 1-nitro-9-(N-pyridinio)acridine hydrochloride (**20**, 36%), mp 150–152 °C (lit.[14] mp 152–153 °C).

The pyridinium complex (10 g) was heated with $POCl_3$ (30 mL) at 120 °C for 2 h, the excess $POCl_3$ was removed under reduced pressure, and the residue was dissolved in CH_2Cl_2 and poured into ice-cold excess NH_4OH. Separation and evaporation of the organic layer gave a solid, which was filtered through Al_2O_3 in CH_2Cl_2 to give pure 9-chloro-1-nitroacridine (**18**) as a bright yellow solid (6 g, 78% yield).

Preparation of Compound 11 of Table I: Typical Example. A mixture of 9-chloro-1-nitroacridine (**18**, 1 g, 3.9 mmol) and phenol (2 g, 21 mmol) was heated to 100 °C for 15 min. The melt was cooled to 50 °C, 3-methylbutylamine (0.35 g, 4 mmol) was added, and the mixture was heated at 70–75 °C for 30 min. The melt was then diluted with CH_2Cl_2 and washed successively with 2 N NaOH and water. The residue after removal of solvent was dissolved in MeOH (15 mL), treated with charcoal/Celite, and acidified to pH 2 with concentrated HCl. Addition of EtOAc precipitated **11** as the hydrochloride salt (1.15 g, 78%), mp 148–152 °C. Anal. ($C_{18}H_{19}N_3O_2$·HCl) C, H, N, Cl. Other new compounds prepared similarly were **7** (mp 125–128 °C. Anal. ($C_{17}H_{15}N_3O_4$) C, H, N.) and **13** (mp 200–201 °C. Anal. ($C_{21}H_{17}N_3O_2$·HCl) C, H, N, Cl.). All other compounds of Table I had melting points

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which broadly agreed with those recorded in the original references cited in Table I.

Redox Potentials. Pulse radiolysis experiments were carried out on a 1.8 MV Linac, delivering ca. 3 Gy in 0.2 μs to a 2-cm pathlength cell. The dose was determined by measurement of the optical density at 472 nm in aerated KSCN (10 mM), assuming[25] an extinction coefficient ϵ of 7580 L mol^{-1} cm^{-1} and a radiation chemical yield G of 0.29 μmol J^{-1}. Transients were recorded on a Tektronic 7621D digitizer interfaced to a PDP 11/34 computer for data analysis. Drug solutions were 2 mM in phosphate buffer at pH 7, using either 2-propanol or 2-propanol/acetone mixtures as cosolvent (see Table II).

Metabolic Bioactivation. [^{3}H-Acridinyl]-9-amino-1-nitroacridine (specific activity 184 mCi/mmol) and [^{3}H-acridinyl]-9-(methylamino)-1-nitroacridine (specific activity 177 mCi/mmol) were synthesized from randomly tritiated sodium *N*-(3-nitrophenyl)anthranilate as described previously.[12] Radiochemical purities of both compounds were >95% at the time of use, as determined by HPLC.[4] Stirred suspensions of AA8 cells were exposed to radiolabeled drugs as described for cytotoxicity assays below, and samples containing 10^5–10^6 cells were withdrawn at intervals to determine total acid-insoluble radioactivity. Cells were centrifuged, resuspended in 0.1 mL of 0.15 M NaCl containing 1% fetal calf serum, and 10% trichloroacetic acid (1 mL) was added. Precipitates were collected on Whatman GF/C glass-fiber filters, washed extensively with ice-cold 1 H HCl, and dried. Radioactivity was determined by scintillation counting, with counting efficiencies (ca. 35%) determined by spotting known amounts of labeled drugs onto blank filters.

Biology. AA8, UV4, and V79-171b cells were maintained in logarithmic-phase growth in 25-mL tissue culture flasks with subculture twice weekly by trypsinization. The growth medium was antibiotic-free Alpha MEM with 10% v/v heat inactivated (56 °C, 40 min) fetal calf serum. Doubling times were approximately 14 h for AA8, 15 h for UV4, and 9 h for V79-171b cells. Cultures were tested for mycoplasma contamination frequently, by using a cytochemical staining method.[26] Bulk cultures of AA8 cells were prepared in spinner flasks, with the above growth medium plus penicillin (100 IU/mL) and streptomycin (100 μg/mL).

Growth inhibition studies were performed as described in detail elsewhere,[5,19] using 200 viable AA8 or 300 viable UV4 cells plus 5000 lethally irradiated AA8 feeder cells per well in 96-well tissue-culture dishes. The IC_{50} was determined as the drug concentration needed to reduce the cell mass (protein content) to 50% of the mean value for eight control cultures on the same 96-well plate. Similar experiments using V79-171b in 24-well dishes employed 5000 cells in 0.5 mL of growth medium, with assay being performed 45 h after drug washout by counting trypsinized cell suspensions with a Coulter counter as described previously.[27]

Clonogenic assays with magnetically stirred 10-mL suspension cultures (plateau-phase AA8 cells, 10^6/mL) were performed by removing samples periodically during continuous gassing with 5% CO_2 in air or N_2 as detailed elsewhere.[5] Both cell suspensions and drug solutions in growth medium were preequilibrated under the appropriate gas phase for 45 min prior to mixing, to ensure essentially complete anoxia throughout the period of drug contact in hypoxic cultures. Several drug concentrations were investigated for each agent, and the concentration × time required to reduce the surviving cell fraction to 10% (CT_{10}) was determined at each concentration. CT_{10} values were not strictly constant, with a trend toward lower values at higher drug concentrations. To minimize this, comparisons between aerobic and hypoxic cytotoxicity were based on CT_{10} values at concentrations which gave similar rates of cell killing (usually 1 log in about 1 h).

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Registry No. 1, 15539-41-0; **2**, 4533-39-5; **3**, 6237-29-2; **4**, 32987-50-1; **5**, 19395-53-0; **6**, 69514-89-2; **7**, 125685-71-4; **8**, 21914-54-5; **9**, 19395-61-0; **10**, 19395-63-2; **11**, 125685-72-5; **12**, 125685-73-6; **13**, 125685-74-7; **14**, 81483-73-0; **15**, 125685-75-8; **17**, 27693-70-5; **18**, 17431-90-2; **20**, 20141-88-2; $NH_2(CH_2)_3CO_2H$, 56-12-2; $NH_2(CH_2)_2CH(CH_3)_2$, 107-85-7; $NH_2(CH_2)_2Ph$, 64-04-0.

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