To the cold solution of this urea (1.2 g, 4.5 mmol) in formic acid (8 mL) was added, portionwise, sodium nitrite (2.5 g, 36 mmol). After the addition and stirring for 30 min, water was added, and after additional stirring for 30 min, the mixture was poured into ethyl acetate (100 mL) and the organic layer was washed, dried (Na_2SO_4), and concentrated under reduced pressure. Column chromatography with CH_2Cl_2–MeOH (9:1) as eluent gave 600 mg (45%) of **22**.

In Vivo Tests. The experimental procedures, the type of mice used (B6D2F1 or C57B1/6), and the calculation of the median survival times of the groups of control and treated animals were in accordance with the protocols of the National Cancer Institute (Instr. 271F November 1983).

Test against L1210 Leukemia in Oily Suspension (Table I). On day 0, mice (B6D2F1) were inoculated intraperitoneally with 10^5 L1210 leukemia cells. On days 1, 5, 9, the mice (15 for each group treated) received intraperitoneally the dose of 20 mg/kg of compounds **22–26** in 0.2 mL of neutralized and sterilized olive oil suspension.

The control group (30 mice) received only the same volume of solvent. The mortality of the mice was monitored daily, and autopsies were performed to find out whether deaths were due to leukemia or to a toxic action of the drug. The observation period lasted at least 60 days.

Test against Lewis Lung Carcinoma (3LL) (Table II) and B16 Melanocarcinoma (Table III) in Oily Suspension. On day 0, mice (C57BL/6) were inoculated intraperitoneally either with 2×10^6 Lewis lung carcinoma (3LL) cells or with 2×10^6 B16 melanocarcinoma cells. The treatment protocol, the dose used and the observation period are similar to the above procedure. Twenty mice for each treated group and 40 mice for control groups were used.

Tests against L1210 Leukemia in Aqueous Solution (Tables IV–VI). The protocol of mice leukemia inoculation is the same as in Table I. Ten mice for each treated group and 20 mice for control group were used (Tables IV and VI). Eight mice for each treated group and 16 mice for control group were used (Table V). In Table IV, the mice received intraperitoneally on days 1, 5, 9 the dose of 5 mg/kg of each drug in 0.2 mL of water. In Table V, the mice received on days 1, 5, 9 various doses (0.62 to 10 mg/kg) of each drug in 0.2 mL of water. In Table VI, various doses of compound **24** (2.5 to 30 mg/kg) were administered intraperitoneally in a single injection on day 1 in 0.2 mL of water. The observation period was for all antitumor testings as above at least 60 days.

Tests against B16 Melanocarcinoma in Aqueous Solution (Table VII). On day 0, mice (C57B1/6) were inoculated intraperitoneally with 2×10^6 B16 melanocarcinoma cells. On day 1, various doses of compound **24** (5 to 30 mg/kg) were administered intraperitoneally in a single injection in 0.2 mL of water.

Acknowledgment. We thank R. Leroy, G. Bretou, E. Faure, and P. Ardouin for technical assistance. Also, the secretarial assistance of Bretou, Billon-Galland was greatly appreciated.

Registry No. 1, 6386-19-2; **2**, 18933-62-5; **3**, 98383-20-1; **4**, 98383-21-2; **5**, 67693-33-8; **5** [*N*-(*N'*-(2-chloroethyl)ureido) derivative], 98383-15-4; **6**, 98383-22-3; **7**, 20379-53-7; **7** (4,6-di-*O*-acetyl derivative), 20379-54-8; **8**, 16697-56-6; **9**, 98383-24-5; **10**, 98383-25-6; **11**, 98383-26-7; **12**, 98383-28-9; **13**, 98383-29-0; **14**, 98383-30-3; **15**, 98383-31-4; **15**·picrate, 116724-65-3; **16**, 72075-77-5; **17**, 116724-59-5; **17** (4,6-di-*O*-acetyl derivative), 116724-64-2; **18**, 116836-60-3; **19**, 116724-60-8; **20**, 54623-23-3; **21**, 85439-77-6; **22**, 98383-16-5; **23**, 98383-23-4; **24**, 98383-18-7; **25**, 98383-27-8; **26**, 98383-32-5; **27**, 116724-61-9; **28**, 116724-62-0; **29**, 98383-36-9; **30**, 116724-63-1; $ClCH_2CH_2NCO$, 1943-83-5.

Hypoxia-Selective Antitumor Agents. 1. Relationships between Structure, Redox Properties and Hypoxia-Selective Cytotoxicity for 4-Substituted Derivatives of Nitracrine

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The nitroacridine derivative 9-[[3-(dimethylamino)propyl]amino]-1-nitroacridine (nitracrine) is selectively cytotoxic to hypoxic tumor cells in culture. However, the compound undergoes reductive metabolism too rapidly, with the reduction not being sufficiently inhibited by molecular oxygen in aerobic tissues, for it to demonstrate the same activity in vivo. In a search for derivatives with lower reduction potentials, we have synthesized and evaluated a series of derivatives bearing 4-substituents with a wide range of electronic properties. The one-electron reduction potentials ($E(1)$) of these compounds, when compared under conditions of equivalent ionization, were highly correlated with σ_p values. However, at pH 7 the influence of substituent electronic properties was modified by prototropic equilibria, with the basic nature of the acridine limiting the extent to which ring substituent electronic effects can be used to modulate reduction potential of the 1-nitro group. Nevertheless, comparison of the kinetics of the killing of AA8 cells under hypoxia suggests that some metabolic stabilization of the compounds can be achieved by the use of electron-donating substituents, with such compounds retaining the hypoxia-selective toxicity of nitracrine in cell culture. However, the 4-substituted nitracrines show no clear relationship between $E(1)$ and cytotoxic potency, in distinct contrast to simpler nitroheterocycles such as nitroimidazoles.

The nitroacridine nitracrine (**4**) shows antitumor activity in some experimental systems and has been used clinically in Poland for the treatment of mammary, lung, ovarian, and colon tumors.[1,2] Nitracrine was briefly investigated by the National Cancer Institute in 1975 (as NSC 247561), but was not advanced to clinical trial because of its restricted antitumor spectrum and high toxicity.[3] Since then we have shown[4] nitracrine to be an extremely potent hy-

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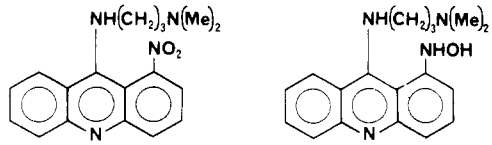
poxia-selective cytotoxic agent in vitro, with a selectivity for hypoxic tumor cells similar to that of simple nitroheterocycles such as misonidazole, but with a potency greater by many orders of magnitude.

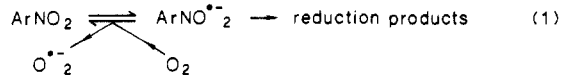


4 4a

This hypoxia-selective cytotoxicity justifies a renewed evaluation of the therapeutical potential of the nitroacridines, since hypoxic cells represent important targets for cancer chemotherapy and radiotherapy. Not only are these cells refractory to ionizing radiation and to many chemotherapeutic agents but hypoxia is more pronounced in solid tumors than in normal tissue, providing an environmental difference which might be exploitable in the design of novel antitumor agents.[5] The hypoxia-selective cytotoxicity of nitracrine is due to oxygen-sensitive metabolic nitroreduction.[6] The major cellular metabolite under hypoxic conditions has been tentatively identified as the hydroxylamine (**4a**), but this end product has very low cytotoxic activity, suggesting that some reactive intermediate in the reduction pathway is responsible for the observed cytotoxicity, via the generation of macromolecular adducts.[6] The aerobic toxicity of nitracrine appears to be also due to metabolic generation of reactive species,[6] with formation of DNA interstrand cross-links suggested to be the key toxic lesion.[7] Although intercalative DNA binding does not appear to be directly responsible for cytotoxicity,[8] it may be a key factor in the high potency of nitracrine by serving to target reactive reduced metabolites to the DNA.

Despite high selectivity and high potency in vitro, nitracrine is ineffective against hypoxic cells in solid tumors in vivo, probably because metabolic activation is too rapid to allow efficient distribution through tumor tissue, and because inhibition of this reductive metabolism by oxygen in normal tissues is incomplete.[6] In this paper we take up the search for analogues with improved metabolic stability and oxygen sensitivity.

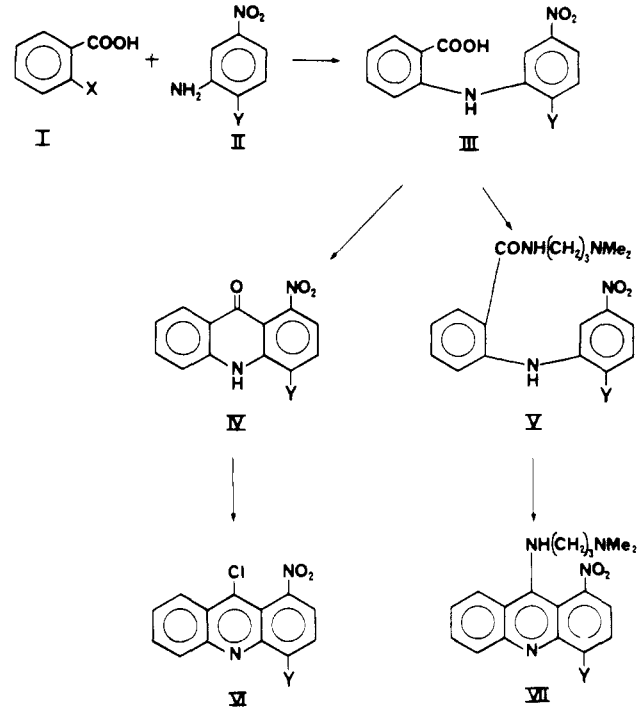
As discussed in detail elsewhere,[5] the critical oxygen-sensitive step in the reduction of nitroaromatics is the first one-electron reduction to the nitro radical anion:



(1)

While the net rate of nitro reduction and the hypoxia selectivity of nitroaromatics will depend on the relative rates of the forward and backward reactions of the radical anion, the reduction potential for the $ArNO_2/ArNO^{\bullet-}{}_2$ couple is expected to be the major determinant, with theoretical considerations suggesting that there may be an optimum reduction potential somewhere in the range from −200 to −500 mV.[5] At values which are too positive, the absolute rate of nitro reduction will be too high and its sensitivity to oxygen too low, while values too negative will not provide sufficiently rapid generation of active metabolites, and thus will lead to loss of potency.

Scheme I



The one-electron reduction potential ($E(1)$) of nitracrine (**4**) at pH 7 has been measured as −303 mV by pulse radiolysis,[9] while nonequilibrium values for net nitroreduction of −320 and −360 mV have been obtained by polarography[10] and cyclic voltammetry,[11] respectively. The reduction potential of nitroaromatics can be altered predictably by use of electron-withdrawing or -donating substituents,[5,12] as shown by eq 2, derived for substituted nitrobenzenes.[5]

$$E(1)\ (\text{mV}) = 163\sigma^- - 492 \qquad (2)$$

$$n = 13,\ r = 0.952$$

In order to study the relationship between reduction potential and biological properties in the 1-nitroacridines, we have synthesized a series of 4-substituted derivatives of nitracrine, since substituents at this position are expected to have the largest electronic effects on the 1-nitro group without steric interference with nitro reduction. Further, by analogy with amsacrine analogues,[13] substitution in the 4-position of the acridine nucleus should have minimal influence on intercalative DNA binding. We have measured the reduction potentials of these compounds by pulse radiolysis, since this provides a true equilibrium determination for the one-electron reduction, and have explored the effect of varying pH on the reduction potential of the parent nitracrine in order to assess likely pK_a dependence in the series. The hypoxia-selective cytotoxicity of these compounds has been assessed in culture against a tumorigenic Chinese hamster ovary cell line

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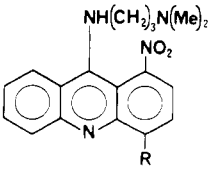
Table I. Physicochemical and Biological Data for 4-Substituted Nitracrines



no.	R	σ_p^- [a]	pK_a [b]	π [c]	Rm [d]	K, [e] M^{-1}	$E(1)$ [f] at pH 7, mV	$E(1)$ [g] at pK_1, mV	log K [h] M^{-1}	clonogenicity: CT_{10} (air), [c] $\mu M \cdot h$	clonogenicity: CT_{10} ratio [d] (air/N_2)
1	COOMe	0.64	5.35	0.01	k	175 ± 49	−244 ± 11	−164	6.06	8	0.32
2	Cl	0.27	5.00	0.71	−0.25	7.0 ± 1.0	−325 ± 11	−225	5.95	0.68	13.6
3	F	0.05	5.41	0.14	−0.46	2.4 ± 0.5	−354 ± 11	−277	6.15	2.0	5.3
4	H	0.0	6.21	0.0	−0.85	18 ± 4	−303 ± 11	−270	6.92	0.47 ± 0.02	9.9 ± 1.0
5	Me	−0.15	6.29	0.56	−0.76	8.8 ± 1.1	−321 ± 11	−292	7.23	2.0	10
6	$N(Me)_2$	−0.12	6.47	0.18	−0.63	5.3 ± 0.2	−334 ± 11	−314	7.11	720 ± 150	16 ± 1
7	OMe	−0.16	6.61	−0.02	−0.88	1.9 ± 0.2	−361 ± 11	−346	6.76	22 ± 1	16 ± 2
8	$N(CH_2CH_2OH)_2$	(−0.1) [i]	6.53		−0.74	ND [m]	ND	ND	6.73	540 ± 100	6.0 ± 1.3
9	$N(CH_2CH_2OAc)_2$	(−0.1) [i]	6.20		−0.29	11.7 ± 1.1	−314 ± 10	−280	6.50	575	11.5

[a] σ_p^- values were taken from ref 22. [b] Acridine pK_a values were determined in aqueous solution at 25 °C by the spectrophotometric method detailed in ref 21. [c] π values were taken from ref 22. [d] Rm values were determined as in ref 21. [e] Equilibrium constants for the reaction between the redox couple ($ArNO_2/ArNO_2^{\bullet-}$) and benzylviologen were measured by pulse radiolysis at pH 7 (see text). [f] $E(1)$ values for the $ArNO_2/ArNO_2^{\bullet-}$ couple were determined from the above K values as detailed in the text. [g] $E(1)$ at pK_1 calculated as in the text. [h] Logarithm of association constant for binding to poly[d(A-T)], determined by ethidium displacement. Quenching errors were small and quenching corrections were not made; see ref 25. [i] CT_{10}: the product of the drug concentration times the exposure time needed to reduce cell survival to 10% of controls in the clonogenic assay (see text). [j] CT_{10} ratio = $CT_{10}(air)/CT_{10}(N_2)$. [k] Value could not be determined due to drug instability. [l] Estimated value; see ref 5. [m] Not determined.

(AA8) by clonogenic assay of stirred, gassed single cell suspensions.

Chemistry

The 4-substituted nitracrines were prepared by the method outlined in Scheme I. The classical Jourdan–Ullmann condensation of 2-halobenzoic acids (I) with amines proceeds poorly with electron-deficient amines, and the 2-methyl- and 2-methoxy-5-nitroanilines (II) are reported[14] to give only 10–20% yields of the corresponding phenylanthranilic acids (III). Reaction with even less basic amines such as (II: Y = Cl) is even poorer, and yields of no more than 5–10% could be achieved by this method. Since diphenyliodonium-2-carboxylate (I: X = IPh) has been shown[15] to give good yields of phenylanthranilic acids with a variety of amines, this method was investigated. Yields of 75–85% were achieved by use of this reagent, even with the very weakly basic 2-chloro-5-nitroaniline (II), and this became the method of choice for preparation of the phenylanthranilic acids (III).

Cyclodehydration of the methyl and methoxy derivatives (III) with H_2SO_4 gave the corresponding acridones (IV). Reaction of these with $SOCl_2$ then provided the 9-chloroacridines (VI), which were converted to the 4-substituted nitracrines (VII) by phenol-mediated reaction with 3-(dimethylamino)propylamine.[16]

This procedure gave good yields, but could not be used for derivatives bearing electron-withdrawing groups, since these labilized the 1-nitro group to replacement by Cl during the reaction with $SOCl_2$. An alternative procedure for these compounds was to form the amides (V) and cyclize these with polyphosphate ester (PPE) to give the nitracrine derivatives directly. Although the yields were only moderate, the desired products could be easily purified by flash chromatography on silica gel.

Nitracrine itself is susceptible to hydrolytic cleavage of the side chain in aqueous conditions,[16] and electron-withdrawing groups on the ring accelerate this reaction. The 4-COOMe derivative (1) is significantly more unstable, and original intentions to extend the series to derivatives bearing even more powerful electron-withdrawing groups were not pursued.

Pulse Radiolysis

Rapid one-electron reduction of the compounds $ArNO_2$ was achieved in deaerated aqueous solution containing either 2-methylpropan-2-ol (0.2 M) or propan-2-ol (0.2 M). The e_{aq}^- species, formed upon the radiolysis of water (eq 3), is scavenged directly by $ArNO_2$, and the oxidizing $H^\bullet$

$$H_2O \rightarrow e_{aq}^-, OH^\bullet, H^\bullet, H_2O_2, H_2, H_3O^+ \quad (3)$$

and $OH^\bullet$ radicals are scavenged by 2-methylpropan-2-ol or converted to the propan-2-oxyl radical, which also reduces $ArNO_2$.

$$e_{aq}^- + ArNO_2 \rightarrow ArNO_2^{\bullet-} \quad (4)$$

$$OH^\bullet(H^\bullet) + (CH_3)_2CHOH \rightarrow (CH_3)_2COH + H_2O(H_2) \quad (5)$$

$$(CH_3)_2COH + ArNO_2 \rightarrow ArNO_2^{\bullet-} + (CH_3)_2CO + H^+ \quad (6)$$

The pulse radiolysis method of establishing reversible equilibria[17,18] within 100 μs of the pulse was used to determine the redox couple $E(ArNO_2/ArNO_2^{\bullet-})$ at pH 7 against benzylviologen (BV^{2+}) for each of the compounds.

$$ArNO_2 + BV^{+\bullet} \rightarrow ArNO_2^{\bullet-} + BV^{2+} \quad (7)$$

The equilibrium constant, K, can be estimated from the absorption of the equilibrium concentration of $ArNO_2^{\bullet-}$ and $BV^{\bullet+}$ (at 605 nm) relative to their maximal absorption before significant bimolecular decay occurs. Since

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$[ArNO_2^{\bullet-}] + [BV^{\bullet+}]$ = a constant at a fixed radiation dose, the ratio $[ArNO_2^{\bullet-}]/[BV^{\bullet+}]$, and hence K, can be calculated for various substrate concentrations. From the result of

$$E(ArNO_2/ArNO_2^{\bullet-})\ (mV) = -59 \log K(E(BV^{2+}/BV^{\bullet+})) \quad (8)$$

eq 8, it is possible[19] to calculate $E(ArNO_2/ArNO_2^{\bullet-})$ using 9. A small correction of 4 ± 1 mV to allow for ionic

$$E(BV^{2+}/BV^{\bullet+}) = -380\ (\pm 10)\ mV \quad (9)$$

strength effects on K was applied. Typically, a mixture of both BV^{2+} (0.1–0.2 mM) and each compound $ArNO_2$ (0.05–0.2 mM) were used to produce the data presented in Table I.

The dependence of $E(ArNO_2/ArNO_2^{\bullet-})$ for nitracrine on pH was determined by pulse radiolysis. The results were fitted to the expression

$$E(1) = -303 + 59 \log \left[\frac{([H^+]/K_{r_1} + K_{r_2}/[H^+] + 1)(10^{-7}/K_1 + K_2/10^{-7} + 1)}{([H^+]/K_1 + K_2/[H^+] + 1)(10^{-7}/K_{r_1} + K_{r_2}/10^{-7} + 1)} \right] mV \quad (10)$$

where the equilibrium constants are calculated from the pK_a values of the ground state for the acridine ring (pK_1) and the side chain (pK_2) and from the corresponding pK_a values (pK_{r_1}, pK_{r_2}) of the one-electron reduced form.

Biological Studies

The cytotoxicity of the compounds was determined by clonogenic assay against Chinese hamster ovary cells (CHO, subline AA8). In this assay, AA8 cells in early unfed plateau phase were exposed to drugs under aerobic or hypoxic conditions as stirred cell suspensions. Clonogenic cell survival was assessed at various times by determining plating efficiency. The drug solutions and cell suspensions were equilibrated separately under nitrogen for 45 min prior to mixing to provide essentially complete anoxia throughout the period of drug exposure. Plateau-phase cultures were used to minimize cytokinetic changes which could influence drug sensitivity during prolonged incubation under hypoxia. This also provides an appropriate model for hypoxic cells in solid tumors, most of which have a drug sensitivity better represented by plateau-phase (predominantly noncycling) than exponential-phase cultures. Drug concentrations were chosen to provide similar rates of killing under aerobic and hypoxic conditions, and the concentration × time required to reduce survival to 10% (CT_{10}) was estimated.

Results and Discussion

Physicochemical Properties. Table I gives the physicochemical properties of nitracrine (4) and eight 4-substituted derivatives (1–3, 5–9). The substituents were chosen to provide a wide range of electronic properties and vary from the electron-withdrawing COOMe to the electron-donating NMe_2 and other groups. The compounds are dicationic and hence very hydrophilic, with Rm values (determined by liquid–liquid chromatography for the dications[21]) between −0.88 and −0.25, in rough correlation with the π values[22] of the substituent groups (Table I) The lipophilicity of the COOMe derivative (1) could not be determined due to hydrolysis during the experiment to the corresponding acridone, demonstrating the sensitivity of this compound to acid hydrolysis. For the compounds of Table I, there is an approximate relationship between pK_a and substituent electronic parameters, but the relationship is complicated by steric effects, as noted previously with 4-substituted amsacrines.[23]

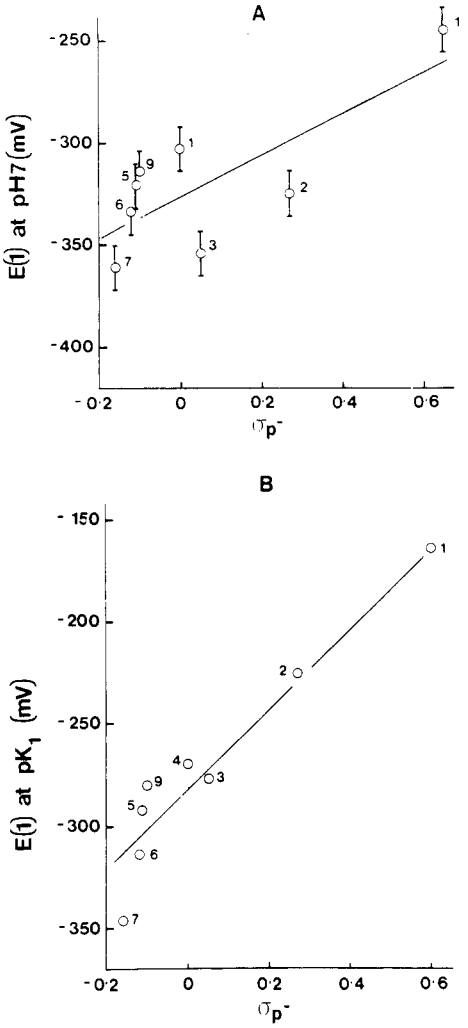

Figure 1. Dependence of the nitro group redox potential of nitracrine analogues upon the electronic properties of substituent groups. (A) $E(1)$ values measured by pulse radiolysis at pH 7. (B) $E(1)$ values at pK_1 for each compound calculated by eq 10.

The one-electron reduction potentials ($E(1)$ at pH 7) ($ArNO_2/ArNO_2^{\bullet-}$) of the 4-substituted series were in the range from −244 to −361 mV (Table I), and could thus all be determined with benzylviologen as the redox indicator. The relationship between $E(1)$ at pH 7 and σ_p^- is illustrated in Figure 1a and can be summarized by eq 11.

$$E(1)\ (mV) = 104\sigma_p^- - 325 \quad (11)$$

$$n = 8,\ r = 0.75$$

Although this correlation is statistically significant, it is much poorer than that seen with substituted nitrobenzenes (eq 2). A likely complicating factor is the effect of prototropic equilibria, since the nitroacridine derivatives have pK_a values in the vicinity of the experimental pH. To assess the effect of pK_a, the pH dependence of $E(1)$ was

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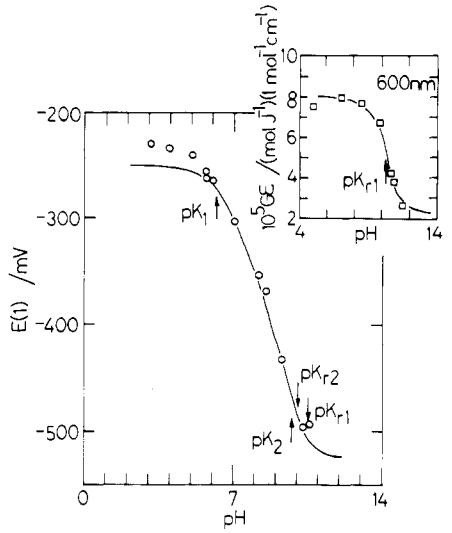

Figure 2. The dependence of the redox couple $E(ArNO_2/ArNO_2^{\bullet-})$ for nitracrine on pH. The solid line is calculated using the expression and pK_a values given in the text. Insert: The dependence of the radical band absorbance, produced upon one-electron reduction of nitracrine, on pH. The solid line corresponds to the best fit of the data which yields $pK_{r_1} = 10.5$.

determined for nitracrine (Figure 2). The data could be fitted adequately by a model (eq 10) which assumed two partcipating ionizable centers, using measured pK_a values of $pK_1 = 6.2$ for the acridinyl nitrogen and $pK_2 = 9.7$ for the side-chain tertiary amine. The radical pK_a for the acridine ring (pK_{r_1}) was estimated as 10.5 from the absorbance change with pH of the transient one-electron reduced species (Figure 2, insert), and a value of 10.0 was assumed for the radical pK_a of the side chain (pK_{r_2}) to obtain the fit shown in Figure 2. The observed dependence of $E(1)$ on pH is consistent with the expectation that protonation of the chromophore will facilitate electron acceptance, while protonation of the radical anion will stabilize it to oxidation, both effects tending to raise $E(1)$. Thus nitracrine analogues with lowered pK_a values (compounds **1–3**) would be expected to have $E(1)$ values more negative than predicted in the absence of such prototropic equilibria.

A quantitative estimate of the magnitude of pK_a effects on $E(1)$ was obtained by using eq 10 to calculate the expected $E(1)$ at a pH corresponding to pK_1 (Table I). At this pH, pK_a effects should disappear in the comparison of the 4-substituted nitracrines since the proportion of acridine ring charged and neutral species will be the same for each drug, while all the side chains will be fully ionized ($pK_2 \gg pK_1$). For the purpose of this calculation the side-chain pK_a values (pK_2 and pK_{r_2}) were assumed to be the same for all derivatives, while pK_{r_1} was assumed to be 4.3 pH units higher than pK_1 as measured for **4**. While the latter will obviously be a crude approximation, the calculated $E(1)$ at pK_1 is independent of pK_{r_1} provided that the latter is at least 2 pH units higher than pK_1.

The relationship between $E(1)$ at pK_1 and σ_p^- is illustrated in Figure 1b and is described by the relationship

$$E(1) \text{ at } pK_1 \text{ (mV)} = 194\sigma_p^- - 282 \qquad (12)$$

$$n = 8,\ r = 0.953$$

Comparison with eq 11 shows that the correction for pK_a effects greatly improves the correlation with σ_p^-, then adequately accounting for substituent effects on $E(1)$.

The compounds were evaluated for hypoxia-selective cytotoxicity in stirred suspension culture by clonogenic assay, with full cell survival curves being obtained for all compounds under both hypoxic and aerobic conditions (Table II and Figure 3). As noted earlier,[4] nitracrine is a very potent cytotoxic agent, with a CT_{10} against AA8 cells under aerobic conditions of 0.47 μM·h. It also showed considerable hypoxia selectivity, with a 10-fold higher potency under hypoxic conditions, as determined by the ratio of CT_{10} values under air and N_2 (Table II).

Table II. Analytical Data for the Compounds of Table I

no.	mp, °C	formula	anal. or reported mp (°C)
1	133–134	$C_{20}H_{22}N_4O_4$	C, H, N
2	121–122	$C_{18}H_{19}ClN_4O_2$	C, H, N, Cl
3	76–78	$C_{18}H_{19}FN_4O_2$	C, H, N
4	222–225	$C_{18}H_{20}N_4O_2 \cdot 2HCl$	223–224[a]
5	256–258	$C_{19}H_{22}N_4O_2 \cdot 2HCl$	254–255[b]
6	98–100	$C_{20}H_{25}N_5O_2$	C, H, N
7	189–191	$C_{19}H_{22}N_4O_3 \cdot 2HCl$	190–191[b]
8	gum	$C_{22}H_{29}N_5O_4$	*c*
9	101	$C_{26}H_{33}N_5O_6$	101[c]

[a] Reference 33. [b] Reference 14. [c] Reference 34.

Evaluation of the nitracrine analogues of Table I demonstrated a striking range of cytotoxic potencies, extending over approximately 3 orders of magnitude. The 4-Cl derivative (**2**) was the most potent analogue, with a CT_{10} value almost as low as that of nitracrine, while the alkylamino derivatives (**6**, **8**, **9**) were much less potent. Hypoxic selectivity also showed substantial variation. Most compounds had values similar to those of nitracrine, although the 4-COOMe derivative (**1**) was consistently more toxic to aerobic than hypoxic cells.

Inspection of the data of Table I shows that neither absolute potency nor hypoxic selectivity show clear trends with reduction potential, with no statistically significant correlation between $E(1)$ at pH 7 (or at pK_1) and CT_{10}. This result is in marked contrast to that for neutral monocyclic nitroheterocycles, where $E(1)$ is the dominant variable determining cytotoxic potency.[24] Thus it was of interest to investigate other variables, unrelated to redox properties and not relevant in the case of the simple nitroheterocycles. Firstly, as weak bases the pH-dependent partitioning of the 1-nitroacridines into the cell would be expected to increase as pK_a increases. However, this effect should represent only a 30% change in uptake factors between the highest and lowest pK_a values in this series (6.61 and 5.00) assuming intra- and extracellular pH values of 6.9 and 7.4, respectively.[5] Secondly, the high cytotoxic potency of nitracrine has been suggested to reflect targeting of reactive reduction products to the DNA by intercalative binding of the acridine chromophore.[4] Association constants for the interaction of the 4-substituted nitracrines with calf thymus DNA were therefore determined by using the ethidium displacement assay,[25] and the results are listed in Table I. While there is significant variation in association constants, there is no correlation with cytotoxicity, and all the compounds have values high enough to suggest that virtually all the drug will be DNA-bound. The marked differences in potency and hypoxic selectivity among the 4-substituted nitracrines not accounted for by $E(1)$, pK_a, or intercalative DNA binding raise the possibility that members of the series might have differing mechanisms of action. Further biological studies supporting this conclusion are reported in the accompanying paper.[26]

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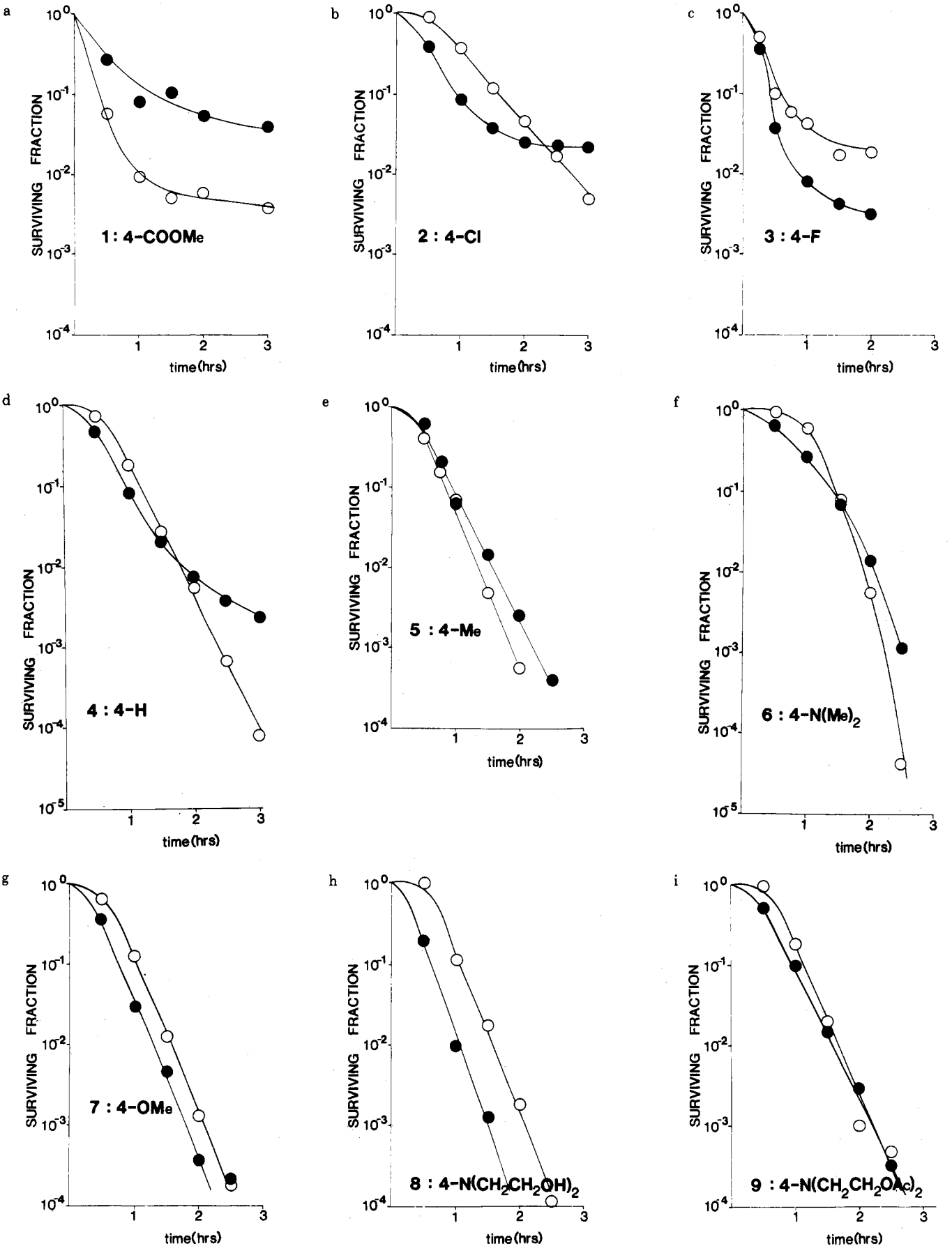

Figure 3. Toxicity of nitracrine analogues against plateau-phase AA8 cells in stirred suspension culture (10^6 cells/mL) (clonogenic assay): (○) aerobic, drug-treated; (●) hypoxic, drug-treated. (a) **1**; 20 μM in air, 20 μM in N_2; (b) **2**; 0.4 μM in air, 0.05 μM in N_2; (c) **3**; 4 μM in air, 0.8 μM in N_2; (d) **4**; 0.4 μM in air, 0.05 μM in N_2; (e) **5** μM in aiar, 0.2, μM in N_2; (f) **6**; 600 μM in air, 40 μM in N_2; (g) **7**; 20 μM in air, 1.5 μM in N_2; (h) **8**; 400 μM in air, 100 μM in N_2; (i) **9**; 500 μM in air, 50 μM in N_2.

The data of Figure 3 show an upward curvature in the semilogarthmic plots of surviving fraction versus time for some of the compounds. In the case of nitracrine (4) this curvature has been shown to reflect disappearance of drug due to rapid metabolism under hypoxic conditions.[4] It is thus of interest that the rate of killing under hypoxic conditions appears to remain constant with time (after an initial threshold) for the compounds with electron-donating functionality (5–9; Figure 3), while for compounds with electron-withdrawing substituents (1–3; Figure 3), the curvature of these semilogarithmic plots is even more pronounced than for nitracrine itself. This observation suggests that the use of electron-donating 4-substituents may provide the metabolic stabilization sought. Quantitative studies investigating rates of metabolic depletion of the compounds of Table I under aerobic and hypoxic conditions are reported in the accompanying paper.[26]

Conclusions

In a search for derivatives of nitracrine (4) with slower reductive metabolism, we have shown that substituents in the 4-position of the acridine ring modulate the reduction potentials of the 1-nitro group. When the effects of substituent-induced variation in pK_a of the compounds are eliminated, there is a similar correlation (eq 12) between $E(1)$ and group σ_p^- values to that found earlier for substituted nitrobenzenes (eq 2). However, several factors indicate that the goal of metabolic stabilization of nitroacridines through the use of electron-donating substituents will be difficult to achieve.

Firstly, the 1-nitroacridines have $E(1)$ values about 200 mV more positive than nitrobenzenes with the same substituents (compare eq 2 and 12). Thus the highly electron deficient 1-nitroacridine ring system is intrinsically sensitive to metabolic reduction. Secondly, attempts to modulate nitroacridine $E(1)$ by nuclear substitution are resisted by the effects of substituents on pK_a, these effects tending to counter electronic effects on $E(1)$. Thus the dependence of $E(1)$ on σ_p^- is less steep for the 1-nitroacridines than for nitrobenzenes (compare eq 2 and 11) although it is possible to predict that without the interfering effects of pK_a this σ_p^- dependence would be at least as pronounced (eq 2 and 12). Thirdly, $E(1)$ is best modelled by σ_p^- rather than σ_p. Because the σ_p^- scale terminates at −0.16, there is relatively little prospect for substantial lowering of $E(1)$ by ring substitution.

Despite these problems, significant modulation of $E(1)$ has been achieved in the present series, and the derivatives with lowest $E(1)$ values, the 4-NMe_2 and 4-OMe compounds (6 and 7), retain the hypoxic selectivity of the parent compound. Comparison of the rates of cell killing in hypoxic AA8 cultures suggests that these analogues may have improved metabolic stability. These agents are now being evaluated against hypoxic tumour cells in vivo.

Experimental Section

Chemistry. Analyses indicated by the symbols of the elements were within ±0.4% of the theoretical value. Analyses were carried out in the Microchemical Laboratory, University of Otago, Dunedin. 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).

2-(Dimethylamino)-5-nitroaniline. A mixture of $Na_2S·9H_2O$ (77.6 g, 320 mmol) and S (10.3 g, 320 mmol) in water (200 mL) was added over 10 min to a refluxing solution of *N,N*-dimethyl-2,4-dinitroaniline (52.5 g, 250 mmol) in EtOH (200 mL). After 30 min the solvents were removed under vacuum, and the residue was taken up in EtOAc. The organic layer was washed with water, dried, and evaporated to give crude product. Chromatography (SiO_2) and elution with CH_2Cl_2 gave 2-(dimethylamino)-5-nitroaniline (19.0 g, 42%), mp (Et_2O/hexane) 60–61 °C (lit.[18] mp 63 °C). The *N*-acetate had mp 165–166 °C (lit.[27] mp 162 °C). Later column fractions gave a small amount of the isomeric noncrystalline 5-(dimethylamino)-2-nitroaniline.

***N*-(2-Fluoro-5-nitrophenyl)anthranilic Acid (III; Y = F).** A suspension of 2-fluoro-5-nitroaniline (2.4 g, 15 mmol), diphenyliodonium-2-carboxylate[15] (4.9 g, 15 mmol) and copper(II) acetate (100 mg) in dry DMF (15 mL) was heated with stirring at 100 °C for 10 h. Most of the solvent was evaporated under vacuum, and the residue was partitioned between water and EtOAc. After filtration to remove suspended solids, the EtOAc layer was washed with 2 N HCl and then extracted with 2 N NH_4OH. Acidification of this extract gave a yellow precipitate, which was crystallized from EtOH to give the acid (III; Y = F) as yellow needles (3.4 g, 79%), mp 250–251 °C. Anal. ($C_{13}H_9FN_2O_4$) C, H, N.

Similar reactions with the appropriate 2-substituted 5-nitroanilines gave *N*-(2-chloro-5-nitrophenyl)anthranilic acid (III; Y = Cl, 82%), mp (EtOH) 259–260 (lit.[28] mp 260–261 °C), *N*-(2-methyl-5-nitrophenyl)anthranilic acid (III; Y = CH_3, 84%), mp (EtOH) 221–223 °C (lit.[14] mp 221–222 °C); *N*-(2-methoxy-5-nitrophenyl)anthranilic acid (III; Y = OCH_3, 76%), mp (EtOH) 242–243 °C (lit.[14] mp 241–243 °C); *N*-[2-(dimethylamino)-5-nitrophenyl]anthranilic acid (III; Y = $N(CH_3)_2$), 83%), mp 203–205 °C (aqueous Me_2CO). Anal. ($C_{15}H_{15}N_3O_4$) C, H, N.

4-(Dimethylamino)-9-[[3-(dimethylamino)propyl]amino]-1-nitroacridine (6). A mixture of *N*-[2-(dimethylamino)-5-nitrophenyl]anthranilic acid (2.42 g, 8.03 mmol), $SOCl_2$ (0.87 mL, 1.5 equiv), and one drop of DMF in 1,2-dichloroethane (20 mL) was heated under reflux for 30 min. Solvents were removed under vacuum, and CH_2Cl_2 (20 mL) was added, followed by an excess (2 mL) of 3-(dimethylamino)propylamine. The mixture was stirred for 5 min, diluted with water, and dried. Removal of solvent gave the crude amide (V) in quantitative yield. A mixture of this amide (0.52 g, 1.35 mmol) and $POCl_3$ (10 mL) was heated under reflux for 4 h. Excess $POCl_3$ was removed under vacuum, and the residue was partitioned between CH_2Cl_2 and 1 N HCl. The aqueous layer was adjusted to pH 10 (NaOH) and extracted with CH_2Cl_2 to give the crude acridine free base. This was chromatographed (SiO_2), eluting with a gradient (0–3% of Et_3N in EtOAc), to give the pure product (0.17 g, 34%), which was crystallized from Et_2O/hexane as red needles, mp 98–100 °C. Anal. ($C_{20}H_{25}N_5O_2$) C, H, N.

4-Fluoro-9-[[3-(dimethylamino)propyl]amino]-1-nitroacridine (3). *N*-(2-Fluoro-5-nitrophenyl)anthranilic acid (III; Y = F) (1 g, 3.6 mmol) was converted to the corresponding amide (V) as detailed above. This was dissolved in PPE,[29] the solvent was allowed to evaporate, and the mixture was heated at 100 °C for 96 h. The cooled mixture was diluted with water, basified with Na_2CO_3, and extracted into EtOAc. The residue after evaporation of solvent was chromatographed on silica, eluting with a gradient of Et_3N (0–3%) in EtOAc. Initial fractions gave starting material, followed by the desired 4-fluoronitracrine (0.41 g, 32% overall). Crystallization from aqueous Me_2CO gave yellow needles, mp 76–78 °C.

A similar reaction sequence gave 4-chloronitracrine (2) in 36% yield.

4-Methoxy-9-[[3-(dimethylamino)propyl]amino]-1-nitroacridine (7). 4-Methoxy-1-nitroacridine[14] (IV) (2 g, 7.4 mmol) was suspended in $SOCl_2$ (30 mL) with a drop of DMF, and the mixture was heated under reflux until homogeneous (about 30 min) and for a further 1 h. Excess $SOCl_2$ was evaporated, and the residue was dissolved in CH_2Cl_2 and poured into excess ice-cold 2 N NH_4OH. The organic layer was separated, dried, and evaporated to give crude 9-chloro-4-methoxy-1-nitroacridine (VI) (1.9 g, 89%). This was heated to 100 °C for 15 min in dry phenol

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(5 g), the melt was cooled to 20 °C, and 3-(dimethylamino)-propylamine (0.8 g, 1.2 equiv) was added. This mixture was heated at 90 °C for 45 min and then dissolved in CH_2Cl_2. The organic layer was washed with excess 2 N aqueous NaOH and water, dried, and evaporated to give crude 4-methoxynitracrine (1.6 g, 67%). This was dissolved in MeOH, concentrated HCl was added to pH 2, and EtOAc was added slowly to the boiling solution to give the dihydrochloride salt as yellow needles, mp 189–191 °C (lit.[15] mp 190–191 °C).

The 4-methyl derivative was similarly prepared and crystallized from MeOH/EtOAc as the dihydrochloride salt, mp 256–257 °C (lit.[14] mp 254–255 °C).

4-(Methoxycarbonyl)-9-[[3-(dimethylamino)propyl]-amino]-1-nitroacridine (1). *N*-[2-(Methoxycarbonyl)-5-nitrophenyl]anthranilic acid[14] was converted to the corresponding amide (V) as detailed above, and this compound (0.77 g) was cyclized by heating in PPE at 100 °C for 100 h. The cooled mixture was dissolved in water, made alkaline at 20 °C with 5 N NaOH, and quickly extracted into EtOAc. The residue after solvent evaporation was chromatographed on silica gel, using a gradient of Et_3N (0–3%) in EtOAc. Initial eluates gave the (methoxycarbonyl)nitracrine 1 (320 mg, 43%), which was crystallized from Et_2O/hexane as orange needles, mp 133–134 °C. Anal. ($C_{20}H_{22}N_4O_4$) C, H, N. This compound was stable as the free base, but a solution in 0.01 N HCl became cloudy in 5 min, due to formation of 1-nitro-4-(methoxycarbonyl)acridone, identical with an authentic sample.

Determination of Reduction Potentials by Pulse Radiolysis. Pulse radiolysis experiments were carried out with a 1.8 MV Linac (ca. 3 Gy in 0.2 μs). The associated charge monitoring device and optical detection system have been described.[30] The dose delivered to the solutions (21 °C) contained in a 2-cm pathlength cell was determined by measurement of the optical density at 472 nm in aerated KSCN (10 mM), assuming[31] an extinction coefficient E 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 7612D digitizer interfaced to a PDP 11/34 computer for data analysis.

All pulse radiolysis solutions contained buffer at a concentration of 2 mM. Solutions at or near pH 7 were buffered with mixtures of sodium dihydrogen orthophosphate and disodium hydrogen orthophosphate. For the $E(1)$ vs pH study with nitracrine, solutions were buffered at low pH with either citric acid or succinic acid and at high pH with mixtures of tetrasodium pyrophosphate and trisodium orthophosphate. Data at pH >9 were obtained with solutions containing both propan-2-ol (1 M) and acetone (1 M) to aid the solubility of nitracrine.

Biological Assays. AA8 cells were maintained in exponential-phase growth (doubling time 14 h) with alpha MEM containing fetal calf serum (10% w/v) without antibiotics. Cells were subcultured twice weekly by trypsinization. Cultures were regularly shown to be free of mycoplasma contamination by fluorescence staining for cytoplasmic DNA.[32] Bulk cultures were prepared for experiments by seeding cells in spinner flasks at 10^4 cells/mL in the above medium with addition of penicillin (100 IU/mL) and streptomycin (100 μg/mL).

Cells were grown to (1.0–1.3) × 10^6 cells/mL in spinner flasks. These unfed plateau-phase cultures were harvested by centrifugation and resuspended to 5 × 10^6 cells/mL in medium containing 5% FCS. Magnetically stirred cell suspensions and drug solutions (in 8 mL of medium at 1.25 times final intended concentration) were equilibrated at 37 °C for 45 min with flowing 5% CO_2 in air or N_2. Aliquots (2 mL) of the cell suspension were transferred to the drug solutions, with a syringe purged with the appropriate gas mixture. Samples were subsequently withdrawn to assess plating efficiency as described previously.[4] No decrease in plating efficiency was observed in non-drug-treated aerobic or hypoxic controls over the course of the experiments (up to 3 h), and all such control plating efficiencies were in the range 80–100%. The concentration × time required to reduce the surviving cell fraction to 10% (CT_{10}) was determined as an (inverse) measure of cytotoxic potency. This parameter was not strictly a constant for most of the drugs studied because of the presence of a threshold region in the dose–response relationship. For this reason drug concentrations were chosen, on the basis of preliminary experiments, such that rates of cell killing were similar for each drug under aerobic and hypoxic conditions.

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Registry No. **1**, 116374-64-2; **2**, 116374-65-3; **3**, 116374-66-4; **4**, 4533-39-5; **4**·2HCl, 6514-85-8; **5**, 24400-01-9; **5**·2HCl, 21193-46-4; **6**, 116374-67-5; **7**, 25799-70-6; **7**·2HCl, 22670-66-2; **8**, 107210-40-2; **9**, 107210-39-9; **II** (Y = NMe_2), 5367-52-2; **II** (Y = F), 369-36-8; **II** (Y = Cl), 6283-25-6; **II** (Y = Me), 99-55-8; **II** (Y = OMe), 99-59-2; **III** (Y = F), 116748-07-3; **III** (Y = Cl), 107210-27-5; **III** (Y = CH_3), 21193-40-8; **III** (Y = OCH_3), 21193-39-5; **III** (Y = $N(CH_3)_2$), 116748-08-4; **III** (Y = CO_2Me), 100038-85-5; **V** (Y = $N(CH_3)_2$), 116748-09-5; **V** (Y = F), 116748-10-8; **V** (Y = CO_2Me), 116748-11-9; **VI** (Y = OMe), 21193-43-1; **VII** (Y = Me), 24400-01-9; **VII** (Y = Me)·2HCl, 21193-46-4; *N*,*N*-dimethyl-2,4-dinitroaniline, 1670-17-3; *N*-acetyl-2-(dimethylamino)-5-nitroaniline, 5367-36-2; diphenyliodonium-2-carboxylate, 92011-62-6; copper(II) acetate, 142-71-2; 3-(dimethylamino)propylamine, 109-55-7; 4-methoxy-1-nitroacridine, 116748-12-0; 4-methyl-1-nitroacridine, 116748-13-1.

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