Hypoxia-Selective Antitumor Agents. 13. Effects of Acridine Substitution on the Hypoxia-Selective Cytotoxicity and Metabolic Reduction of the Bis-bioreductive Agent Nitracrine *N*-Oxide

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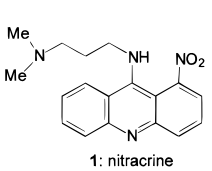
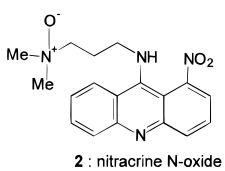
A series of nuclear-substituted derivatives of nitracrine *N*-oxide (**2**; a bis-bioreductive hypoxia-selective cytotoxin) were prepared and evaluated, seeking analogues of lower nitroacridine reduction potential. Disubstitution with Me or OMe groups at the 4- and 5-positions did not provide analogues with one-electron reduction potentials significantly lower than those of the corresponding monosubstituted derivatives ($E(1)$ ca. −350 mV for both the 4-OMe and 4,5-diOMe compounds). This appears not to be due to a concomitant raising of the acridine pK_a but to a lack of direct electronic effect of substituents in the ring not bearing the nitro group. Conversely, placing two OMe groups in the nitro-bearing ring does result in a substantial further lowering of reduction potential (the 2,4-diOMe analogue has an $E(1)$ of −401 mV). The mono- and disubstituted *N*-oxides have substantially lower cytotoxicities than the parent nitracrine *N*-oxide **2** but generally retain very high hypoxic selectivity. The OMe-substituted *N*-oxides all showed greater metabolic stability than **2** in hypoxic AA8 cell cultures, and the 4-OMe compound **6** had improved activity in EMT6 multicellular spheroids suggesting that this metabolic stabilization may allow more efficient diffusion in tumor tissue. The parent compound **2** was selectively toxic to hypoxic cells in KHT tumors *in vivo* and clearly superior to nitracrine itself (although only at doses which would eventually be lethal to the host). The analogues of lower $E(1)$, including **6**, were not superior to **2** *in vivo*, indicating that metabolic stabilization of the nitro group is not alone sufficient to improve therapeutic utility.

The acridine derivative 9-[(3-(dimethylamino)propyl)-amino]-1-nitroacridine (nitracrine, **1**), originally developed as a classical anticancer drug,[1,2] is also a potent hypoxic cell radiosensitizer[3,4] and hypoxia-selective cytotoxin[5] in cell culture. However, it does not show significant activity against hypoxic cells in solid tumors. Primary reasons for this appear to be a sufficiently high nitro group reduction potential (−303 mV)[6] to allow rapid metabolic reduction of the nitro group, together with a DNA binding constant high enough[5,7] to markedly limit its rate of diffusion into hypoxic areas.[8,9]

The tertiary amine *N*-oxide derivative **2** of this compound was therefore evaluated, in the expectation that the dipolar but formally neutral side chain would provide a prodrug form with weaker DNA binding and therefore lower toxicity and improved extravascular drug transport properties.[7] Since the metabolic reduction of tertiary amine *N*-oxides has long been known to be inhibited by oxygen,[10] such a prodrug was also expected to be activated selectively in hypoxic regions. This compound was found to show exceptional (ca. 1300-fold) hypoxic selectivity toward AA8 cells in culture.[7]

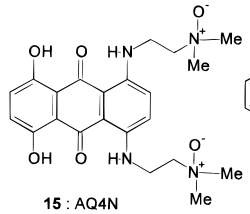


1: nitracrine

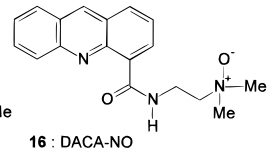


2 : nitracrine N-oxide

The very high selectivity of **2** appears to be due to a requirement for reduction of both the nitro and *N*-oxide moieties for full activation. This compound was the first reported example of such a "bis-bioreductive" agent, with two independent oxygen-sensitive redox centers (Scheme 1).[7] Reduction of the nitro group appears to generate reactive intermediates responsible for DNA alkylation, as deduced earlier for nitracrine,[11,12] while oxygen-inhibitable reduction[13] of the *N*-oxide of **2** (to give **1**), generating a cationic side chain, increases intercalative DNA binding affinity by 15-fold at low ionic strength.[7] The potential of *N*-oxides to act as hypoxia-activated prodrugs of DNA intercalators has since been demonstrated by the *in vitro* hypoxic selectivity of the anthraquinone di-*N*-oxide AQ4N (**15**)[14,15] and the acridine mono-*N*-oxide DACA-NO (**16**)[15] and the activity of AQ4N against hypoxic cells in some tumors.[15,16]



15 : AQ4N



16 : DACA-NO

The ability of nitracrine *N*-oxide (**2**) to kill cells in EMT6 multicellular spheroids has been shown[7] to be improved relative to that of **1**. However, intact spheroids were still appreciably more resistant than single-cell suspensions, suggesting that extravascular diffusion of **2** is still restricted, possibly because of high rates of drug metabolism.[7] An avenue for further development of this interesting lead was considered to be stabilization

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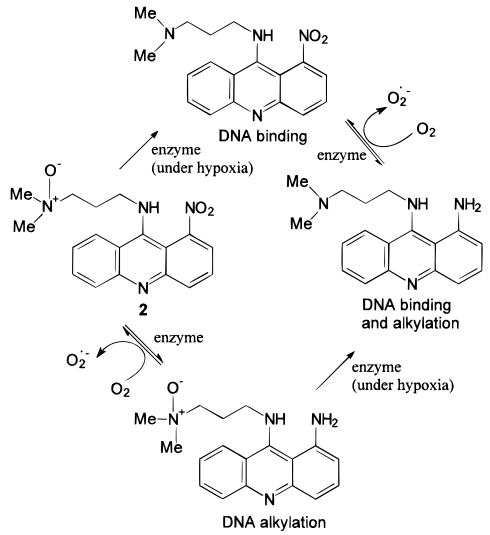
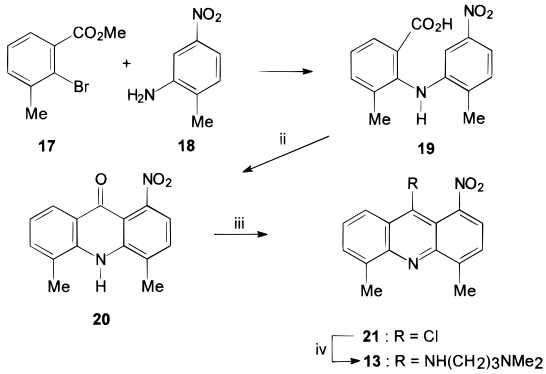
Scheme 1. Alternative Reductive Pathways for the Bis-bioreductive Agent Nitracrine *N*-Oxide (**2**)



Scheme 2[a]



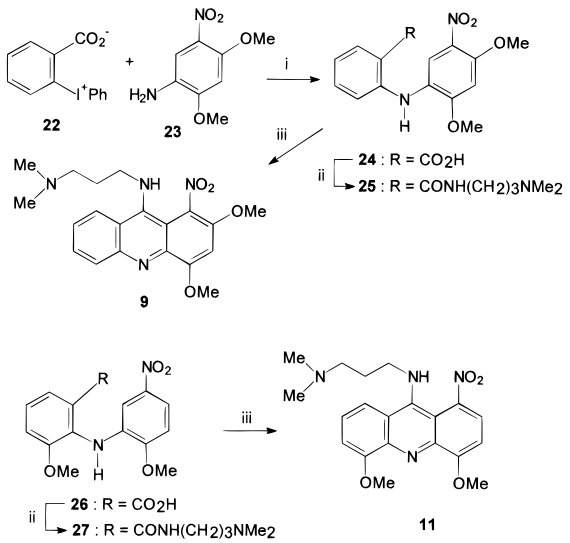
[a] (i) K_2CO_3/CuI/Cu/HMPA/170 °C/2 h, then aqueous KOH/reflux/45 min; (ii) 90% H_2SO_4/80−85 °C/3.5 h; (iii) $SOCl_2$/DMF/reflux/1 h; (iv) $H_2N(CH_2)_3NMe_2$/DMF/80 °C/3.5 h.

of one or both of the nitroaromatic and aliphatic *N*-oxide redox centers against reductive metabolism. In this paper we report the preparation and evaluation of a series of analogues of nitracrine substituted in the acridine ring with electron-donating Me and OMe groups and their corresponding *N*-oxides (compounds **3**−**14**). Previous work[6,17,18] has shown that monosubstitution of nitracrine (**1**) in the 4-position with such groups does provide analogues with lower reduction potentials and improves metabolic stability of the nitro group, with the 4-methoxy derivative **5** showing limited activity against hypoxic cells in EMT6 tumors *in vivo*.[17]

Chemistry

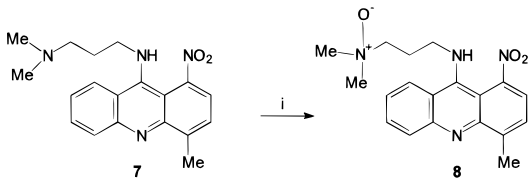
The new compounds of Table 1 were prepared by one of two different methods. The classical synthesis of 9-(aminoalkyl)acridines from 9-chloroacridine and alkylamines, in the presence of excess phenol as reagent and solvent, proceeds through 9-phenoxy intermediates.[19] In the present case, the nitro group was sufficiently activating for the methyl-substituted chloroacridines to react directly with excess *N*,*N*-dimethylpropane-1,3-diamine at 80 °C in DMF to give derivatives **7** and **13** (Scheme 2). The monomethoxy compounds **3** and **5** gave only low yields under these conditions and required reaction *via* the phenoxy compounds. However, treatment of the dimethoxy-9-chloroacridines under either

Scheme 3[a]



[a] (i) $Cu(OAc)_2$/DMF/90 °C/7 h; (ii) CDI/DMF/40 °C/10 min, then $H_2N(CH_2)_3Me_2$; (iii) PPE/100 °C/2 h.

Scheme 4[a]



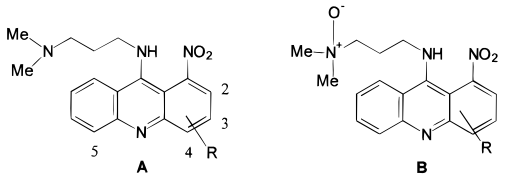
[a] (i) 2-(Phenylsulfonyl)-3-phenyloxaziridine/CH_2Cl_2/20 °C/30 min.

of these conditions resulted in contamination with considerable amounts of products resulting from methoxy group displacement (a problem noted previously in a similar synthesis of methoxynitroquinolines).[20] To prepare these compounds, the side chain was attached before ring closure by condensation of the *N*-phenylanthranilic acids with *N*,*N*-dimethylpropane-1,3-diamine in the presence of 1,1′-carbonyldiimidazole[21] to give the amides (e.g., **25**, Scheme 3). Subsequent cyclodehydration of these with polyphosphate ester (PPE) at 100 °C gave the desired dimethoxynitracrine analogues **9** and **11** in moderate yields.

3-Methoxy-2-[(2-methoxy-5-nitrophenyl)amino]benzoic acid (**26**) for the preparation of **11** was made *via* the classical Jourdan−Ullman synthesis, and 2-[(2,4-dimethoxy-5-nitrophenyl)amino]benzoic acid (**24**) was prepared by coupling of diphenyliodonium-2-carboxylate[22] (**22**) and 1-amino-2,4-dimethoxy-5-nitrobenzene[23] (**23**) (Scheme 3).

Selective *N*-oxidation of the side chain tertiary aliphatic amine was achieved in high yield using 2-(phenylsulfonyl)-3-phenyloxaziridine,[24] enabling synthesis of the *N*-oxide derivatives essentially free of the starting amines (<0.02% amine by HPLC) (Scheme 4). Such high purity was essential because the corresponding amines are the products of hypoxia-selective reduction and are much more potent than the *N*-oxides (Table 1). Contamination of the *N*-oxides with very small amounts of the corresponding amines (<0.5%) markedly lowered their hypoxic selectivity.[7] Reaction of 9-chloroacridines with N^1,N^1-dimethylpropane-1,3-diamine N^1-oxide[25] failed to provide compounds of sufficient purity, due to difficulties in rigorously purifying the starting *N*-oxide.

Table 1. Structural, Physicochemical, and Biological Data for Acridine-Substituted Nitracrine Derivatives and Their Tertiary Amine *N*-Oxides



					aerobic growth inhibition[c]		clonogenic assay (AA8)[d]		
no.	formula	R	pK_a[a]	$E(1)$[b] (mV)	IC_{50}(AA8) (μM)	HF(UV4) ratio	C_{10}(air) (μM)	air/N_2 ratio	mouse toxicity[e] (MTD) (μmol/kg)
1	A	H	6.21	−297	0.024 ± 0.001	15 ± 1	0.40	5.1	0.3
2	B	H	7.10	−283	2.7 ± 0.5	15 ± 1	61	820	15
3	A	2-OMe	5.48	−352	2.9 ± 0.3	4.6 ± 0.3	>60	200	
4	B	2-OMe			84 ± 19		>300		300
5	A	4-OMe	6.61	−355	0.47 ± 0.07	14 ± 5	16	11	15
6	B	4-OMe			130 ± 20	43 ± 14	15 700	1250	100
7	A	4-Me	6.29	−315	0.053 ± 0.009	13	1.5	6.9	0.2
8	B	4-Me			5.4 ± 0.6	29 ± 2	47	150	15
9	A	2,4-diOMe	5.85	−401	47 ± 17	1.3 ± 0.1	500	2.3	300
10	B	2,4-diOMe			1960	0.9	20 000	1.2	800
11	A	4,5-diOMe	6.53	−344	0.51 ± 0.02	11 ± 3	47	14	100
12	B	4,5-diOMe			150 ± 20	14 ± 1	59 000	2770	450
13	A	4,5-diMe	6.01	−317	0.19 ± 0.0300	22 ± 12	9.6	11	20
14	B	4,5-diMe			6.8 ± 1.5	32 ± 6	60	54	15

[a] Acridine pK_a values were literature values (ref 6) or determined in aqueous solution at 25 °C by spectrophotometry (see ref 36). [b] $E(1)$ values (mV) for the $ArNO_2/ArNO_2^{\bullet-}$ were literature values (ref 6) or determined at pH 7 by pulse radiolysis, using benzylviologen as the reference standard (see ref 6). [c] Growth inhibition assay as described in the text. IC_{50} value determined against aerobic AA8 or UV4 cells, using an exposure time of 18 h. HF (hypersensitivity factor = IC_{50}(AA8)/IC_{50}(UV4)), using 18 h drug exposures under aerobic conditions. [d] Clonogenic assay as described in the text. C_{10} is the concentration needed to reduce cell survival to 10% of control values, using 1 h exposure of plateau-phase AA8 cells at 10^6 cells/mL. Air/N_2 ratio = C_{10}(air)/C_{10}(nitrogen). [e] Maximum tolerated ip dose for male C_3H/HeN mice.

Results and Discussion

Physicochemical and *in vitro* biological data for substituted nitracrines and their corresponding *N*-oxides are given in Table 1. One-electron reduction potentials for the non-*N*-oxides were determined by pulse radiolysis. As reported previously,[6] substitution of **1** with a 4-Me group lowers $E(1)$ by about 15 mV, compared to about 55 mV for either a 2- or 4-OMe group. However, a second electron-donating group on the other ring had no additional effect on $E(1)$ as demonstrated by the 4,5-diOMe and 4,5-diMe compounds **11** and **13** which have $E(1)$ values similar to those of the corresponding 4-substituted derivatives **5** and **7**. Determination of the direct electronic effects of ring substituents on reduction potential in this series is complicated by the concomitant effects on the acridine pK_a.[6] These determine the relative proportions of cationic and free base forms, which have different reduction potentials.[12] However, in the present study the second substituents have no net effect on pK_a, presumably because their additional electron-donating properties are counteracted by steric inhibition of protonation, a phenomenon previously noted with 4,5-disubstituted acridines.[26]

The 2,4-diOMe derivative **9** was therefore prepared to investigate the effects of disubstitution in the same ring as the nitro group. This compound had an $E(1)$ 100 mV lower than that of **1**, suggesting essentially full electronic contributions by each substituent, although its pK_a (5.85) did not reflect any additive effects of the two groups, being intermediate between those of the 2-OMe and 4-OMe compounds **3** and **5**. Unfortunately both this compound and the related monosubstituted 2-OMe derivative **3**, together with their respective *N*-oxides **4** and **9**, proved to be comparatively insoluble. The reason for this does not appear to be pK_a-related but may be due to a considerably different conformation of the side chain due to the additional steric compression provided by the 2-OMe substituent.

The aerobic cytotoxicities of the substituted nitracrines (IC_{50} values) in AA8 cells show a trend toward decreasing cytotoxic potency with lower $E(1)$. The 4-Me derivative **7** was 2-fold less potent, and the 4-OMe and 4,5-diOMe compounds **5** and **11** were 10−20-fold less potent. The 2-OMe derivative was very much less potent than **1** (120-fold). The reduction potential of this compound was similar to that of the 4-OMe derivative, so the further decrease in potency may be due to the additional structural distortion caused by the 2-substituent. Thus, although the 2,4-diOMe derivative **9** does have the lowest $E(1)$, the very marked drop in potency seen (2000-fold less than that of **1**) is likely not due entirely to this factor. This observation is reminiscent of the steric inhibition of nitroreduction by substituents *ortho* to the nitro group in the structurally-related 4-(alkylamino)-5-nitroquinolines.[27]

The relative potencies of the compounds against AA8 cells and a DNA repair-defective mutant (UV4) selected from AA8 were determined as hypersensitivity values (HF = IC_{50}(AA8)/IC_{50}(UV4)). The UV4 mutant is defective in the incision step of excision repair of DNA interstrand crosslinks or bulky monoadducts[28] and is consequently hypersensitive to the cytotoxic effects of DNA-alkylating or -arylating agents. We have shown previously[17] that nitracrine (**1**) is significantly more cytotoxic (11−13-fold) in this line than in AA8, suggesting that the principal cytotoxic lesions it forms are DNA adducts. All of the derivatives (both the amines and corresponding *N*-oxides) had high HF values (10−30-

fold) except for the much less potent 2-OMe and 2,4-diOMe derivatives **3** and **9**. The similarity of the HF values of the *N*-oxides and corresponding amines in each case is consistent with cytotoxicity being due to reduction of the *N*-oxides to the amines. The low HF values for the relatively nontoxic 2-OMe-substituted compounds **3** and **9** indicate that, when nitroreduction is severely depressed, a nonbioreductive mechanism of toxicity becomes dominant.

Cytotoxic potencies determined in clonogenic assays with aerobic AA8 cell suspensions, assessed as the concentration required to lower cell survival to 10% (C_{10}), showed similar trends to the IC_{50} data (Table 1) with the substituted compounds all less potent than **1** and the 2-OMe derivatives **3** and **9** being the least potent. The *N*-oxides, as expected,[7] were considerably less cytotoxic than the parent amines in both the growth inhibition and clonogenic assays, but there were significant differences between chromophore substitution patterns (Table 1). Since the *N*-oxides are designed as nontoxic prodrugs which will liberate the tertiary amines on reduction, the preferred compounds are those showing a large deactivation relative to the corresponding amine. By this criterion the best are the 4-OMe and 4,5-diOMe 1-nitroacridines (C_{10} ratio *N*-oxide/amine ca. 1000) followed by the parent 1-nitroacridine (ratio 150) with the 2,4-diOMe- and methyl-substituted compounds showing lower ratios.

The hypoxic selectivities of all compounds were determined by comparing C_{10} values in AA8 cell suspensions gassed with 5% CO_2 in air or N_2. The survival curves for the key *N*-oxide prodrugs are shown in Figure 1, and the results are summarized for all compounds in Table 1. The C_{10} ratios for nitracrine itself (**1**) and its 4-OMe and 4-Me derivatives **5** and **7** were similar to the differentials reported previously using slightly different methods.[6] The 4,5-diOMe and 4,5-diMe compounds **11** and **13** had ratios of 11−14, similar to those for the corresponding monosubstituted derivatives. The 2,4-diOMe derivative **9** showed only low selectivity (2-fold), and a value could not be determined for the 2-OMe compound **3**.

With the exception of the 2-OMe-substituted compounds, the *N*-oxide derivatives all showed very high selectivity for hypoxia (Figure 1), with differentials much greater than those of the corresponding tertiary amines (Table 1). The hypoxic differential for the parent compound **2** (820) was similar to that reported previously,[7] while the 4-OMe derivative **6** gave a ratio of 1250 and the 4,5-diOMe derivative **12** a ratio of 2800. The 4-Me (**8**) and 4,5-diMe (**14**) analogues were less selective than the parent, with ratios of 150 and 54, respectively. Surprisingly, the 2,4-diOMe compound **10** showed essentially no hypoxic selectivity. Excluding the 2-OMe-substituted compounds, these hypoxic differentials parallel the differences in aerobic potency between *N*-oxide and tertiary amine, indicating that a key design feature for hypoxic selectivity in this series is that the prodrug (*N*-oxide) and active effector (tertiary amine) show a large difference in cytotoxic potency.

The ability of electron-donating OMe substituents to stabilize the 1-nitroacridine tertiary amines, and their corresponding *N*-oxide prodrugs, against reductive metabolism in hypoxic AA8 cultures was assessed by HPLC of samples of extracellular medium (Figure 2). Loss of

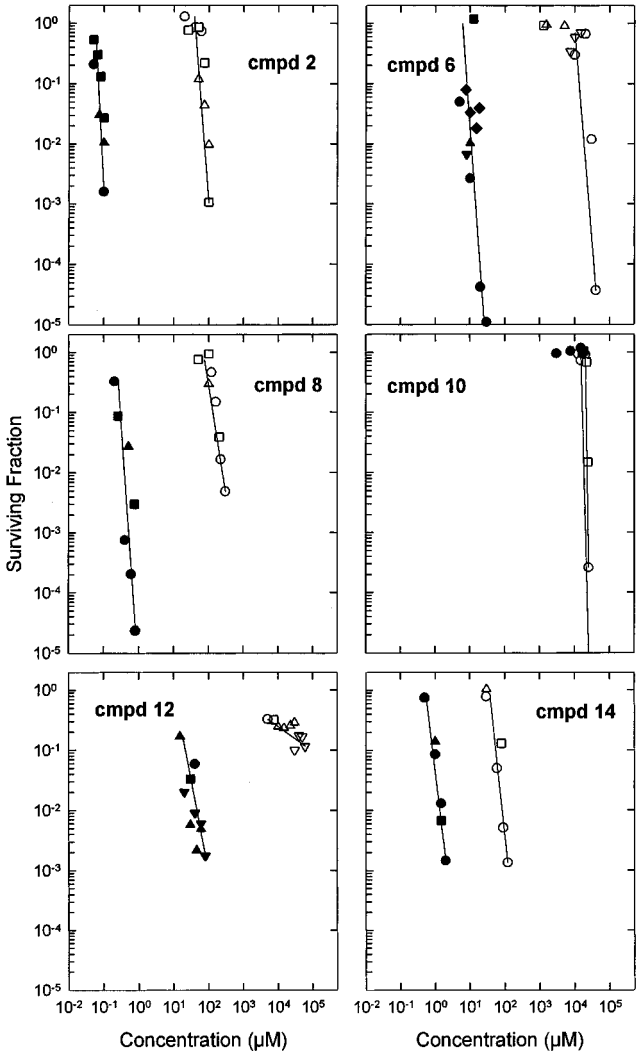


Figure 1. Survival curves for treatment of plateau-phase AA8 cells with acridine-subsituted 1-nitroacridine *N*-oxides for 60 min at the indicated concentrations. Open symbols: aerobic. Filled symbols: hypoxic. Different symbol shapes refer to separate experiments.

the parent nitracrine *N*-oxide **2** (Figure 2B) was slower than that of nitracrine (**1**) itself (Figure 2A) but was extensive. Since tissue cell densities are 100−1000-fold higher than those used in the *in vitro* experiments (10^6 cells/mL), very rapid metabolism would be expected in the hypoxic regions of tumors. The observed loss from the extracellular compartment is primarily due to metabolism (rather than uptake of drugs into cells) since little loss was observed under aerobic conditions except during the first 30 min (data not shown), and loss of the parent compounds in hypoxic cultures was accompanied by appearance of reductive metabolites. Thus incubation with nitracrine (**1**) under the conditions shown in Figure 2A gave the nitroreduction product previously tentatively identified as the hydroxylamine,[9] and the *N*-oxide **2** gave the amine **1** as an intermediate as reported previously[7] (see also Figure 4).

All four OMe-substituted tertiary amines showed dramatically slower rates of metabolism (Figure 2A) with the compound of lowest *E*(1) value (the 2,4-diOMe compound **9**) being the most stable. The corresponding OMe-substituted *N*-oxides also showed slower rates of metabolism than the parent **2** (Figure 2B), although the extent of protection was not as marked. The lesser

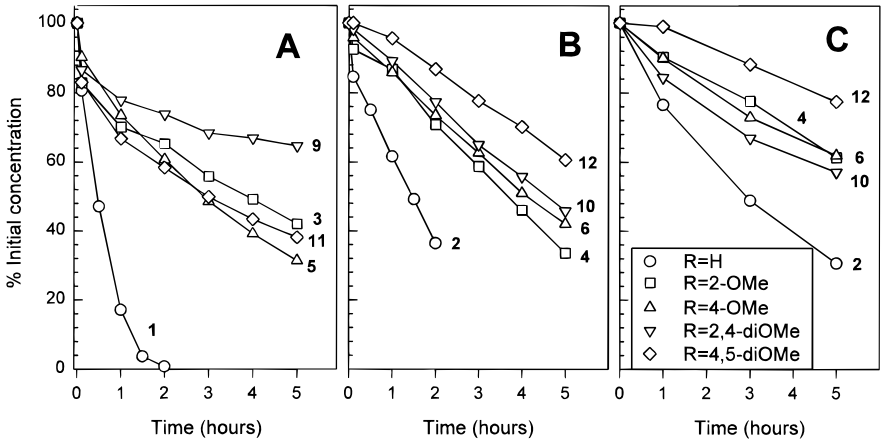


Figure 2. Loss of acridine-substituted nitracrine derivatives (initial concentration 30 μM) from extracellular medium (αMEM with 5% FBS) under hypoxic conditions as determined by HPLC. A: Tertiary amine derivatives in AA8 cell cultures (10^6 cells/mL). B: Tertiary amine *N*-oxides in AA8 cell cultures (10^6 cells/mL). C: Tertiary amine *N*-oxides in medium without cells.

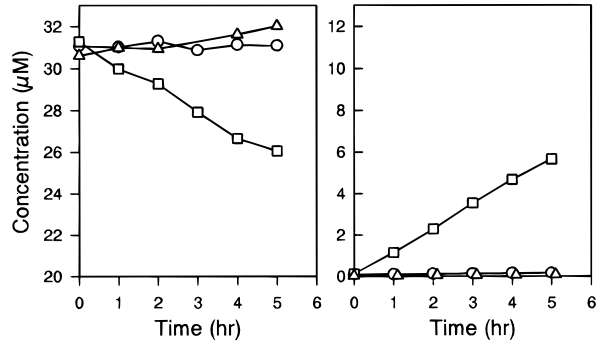


Figure 3. Reduction of DACA *N*-oxide (**16**) in αMEM culture medium (without cells) as determined by HPLC. Left panel: loss of parent *N*-oxide. Right panel: formation of DACA free amine (○, 20% O_2, 5% FBS; □, N_2, 5% FBS; △, N_2, no FBS).

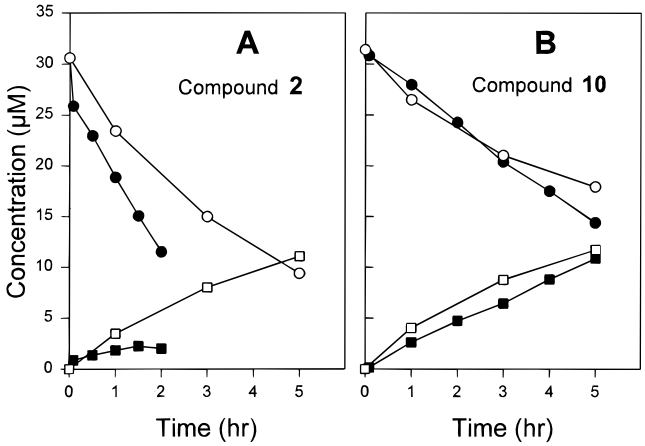


Figure 4. Reduction of the parent *N*-oxide **2** (left panel) and its 2,4-diOMe derivative **10** (right panel) in hypoxic culture medium (αMEM with 5% FBS) alone (open symbols) and with AA8 cells at 10^6 cells/mL (filled symbols) as determined by HPLC. Circles: *N*-oxide prodrug. Squares: tertiary amine reduction product.

effect in the *N*-oxides was expected, since reduction of the *N*-oxide moiety itself provides an alternate route for compound loss which would not be expected to be influenced by substitution of the acridine nucleus.

The analysis of the reduction of the *N*-oxide moiety is complicated by the finding that the *N*-oxides are also reduced in hypoxic (but not aerobic) culture medium (Figure 2C), although at lower rates than when AA8 cells are present (Figure 2B). The corresponding tertiary amines were stable under these conditions (data not shown). To explore this phenomenon, a variety of other tertiary amine *N*-oxides, including AQ4N (**15**),[14] the desnitro analogue of **2**, and the acridine carboxamide derivative DACA *N*-oxide (**16**),[15] were also examined in hypoxic culture medium. All the compounds were reduced to the corresponding amines, at broadly similar rates. As illustrated for DACA *N*-oxide (**16**), the loss of the *N*-oxide was accompanied by quantitative formation of the corresponding amine DACA, a reaction which was inhibited by O_2 and completely dependent on the fetal bovine serum (FBS) component of the medium (Figure 3). The rate of reduction of **16** was ca. 3-fold higher using a batch of FBS with a hemoglobin concentration of 0.16 mg/mL than it was with a batch containing only 0.11 mg/mL, suggesting that the known[29] reduction of tertiary amine *N*-oxides by denatured hemoglobin may partially account for this phenomenon.

The interplay of cellular and extracellular reduction is illustrated by comparison of **2** (with a readily-metabolized 1-nitro group) and **10** (with a stabilized 1-nitro group) in Figure 4. The *N*-oxide **2** is slowly reduced to **1** in hypoxic culture medium. The loss of **2** is faster in the presence of cells, but the concentrations of **1** are lowered because the latter is reduced further by cellular nitroreduction (Figure 4A). In contrast, **10** is lost at essentially the same rate in the presence or absence of cells, and the concentrations of the product **9** are unaffected by cells (Figure 4B). The other OMe-substituted compounds gave results intermediate between those of **2** and **10** but more similar to the latter (data not shown). The above data are consistent with two major hypoxia-selective routes of reduction of the 1-nitroacridine tertiary amine *N*-oxides: reduction of the *N*-oxide moiety to provide the tertiary amine (probably mainly by hemoglobin in the extracellular medium) and intracellular reduction of the 1-nitro group. When the latter process is slowed (e.g., in the 2,4-diOMe derivative **10**), only the *N*-oxide reduction is significant in cell culture. Thus the use of electron-donating functionality can be used to slow nitroreduction as expected.

The relative ability of the metabolically-stabilized 4-OMe *N*-oxide **6**, and its unsubstituted parent **2**, to diffuse into spheroids was evaluated by comparing killing in intact EMT6 multicellular spheroids with

Table 2. Sensitivity of EMT6 Cells in Intact and Dissociated Spheroids[a] to Selected Nitracrine *N*-Oxides

no.	C_{10}[b] (μM)	SRF[c]
1	0.11 ± 0.04 (9)[d]	10.7 ± 0.9 (9)
2	1.9 ± 0.6 (3)	7.0 ± 1.0 (3)
5	1.7 ± 0.3 (5)	7.7 ± 2.0 (5)
6	300 ± 10 (3)	1.9 ± 0.4 (3)

[a] Spheroids of ca. 1200 μm diameter were exposed to drugs under aerobic conditions (5% CO_2 in air) at 37 °C with stirring for 60 min, either before or immediately after dissociation with a pronase/DNAase enzyme cocktail (see the Experimental Section). [b] Concentration resulting in 10% survival in dissociated spheroid cell suspensions. [c] SRF, spheroid resistance factor = C_{10}(intact)/C_{10}(dissociated). See text for further definition and comments. [d] Values are means ± SEM for the number of independent experiments indicated in parentheses.

killing of single cells from freshly-dissociated spheroids exposed under equivalent ambient conditions (Table 2). The differential was quantitated as the spheroid resistance factor (SRF), defined as the ratio of C_{10} values for intact and dissociated spheroids. As reported previously[7] (although now based on a larger data set), the SRF of **2** against intact spheroids is somewhat less than that of nitracrine itself (7.0 compared to 10.7), but for the 4-OMe compounds the SRF for the *N*-oxide **6** is 4-fold lower than that for the amine **5** (SRF = 1.9 compared with 7.7). These ratios cannot be directly related to drug penetration since the SRF will also depend on the ability of the drug to kill cells under the microenvironmental conditions pertaining in spheroids. However, since both the *N*-oxides show similar hypoxic selectivity in AA8 single-cell suspensions and have otherwise similar physicochemical properties, it is likely that the low SRF for the 4-OMe compound reflects improved distributive properties because of lowered rates of metabolic reduction.

The host toxicities of the *N*-oxide prodrugs and their tertiary amines were compared in mice, using the maximum tolerated (nonlethal) dose as the end point (Table 1). The parent *N*-oxide **2** was 50-fold less toxic than nitracrine (**1**). Of the analogues, only the 4-Me derivatives **5** and **6** provided a differential as large as this. The 4-OMe- and 4,5-diOMe-substituted chromophores also gave substantial differences (ca. 5-fold) between prodrug and tertiary amine toxicity, but the other compounds showed little differential. The activity of compounds **2**, **6**, **8**, **10**, **12**, and **14** was examined against the KHT tumor, at a dose corresponding to 75% of the MTD, by excising tumors 18 h after treatment and determining clonogen number *in vitro*. None of these compounds showed activity when administered alone or provided increased killing when given either before or after radiation (data not shown). At doses above the MTD, mouse deaths due to drug occurred sufficiently late with **2**, **6**, and **14** so that it was possible to evaluate doses up to 3 times the MTD. Under these conditions, both the parent *N*-oxide **2** and the 4-OMe derivative **6** (but not the 4,5-diMe derivative **14**) showed activity when administered after irradiation (Figure 5). This activity was somewhat variable, but the *N*-oxides were clearly more active than the corresponding tertiary amines **1** and **5** at equivalent multiples of the MTD (Figure 5). The parent *N*-oxide **2** was more active in combination with radiation than when administered alone, indicating selective toxicity for hypoxic cells. Selectivity for hypoxic cells was less clear for the 4-OMe compound **6**, and there appears to be no advantage of this derivative relative to **2**.

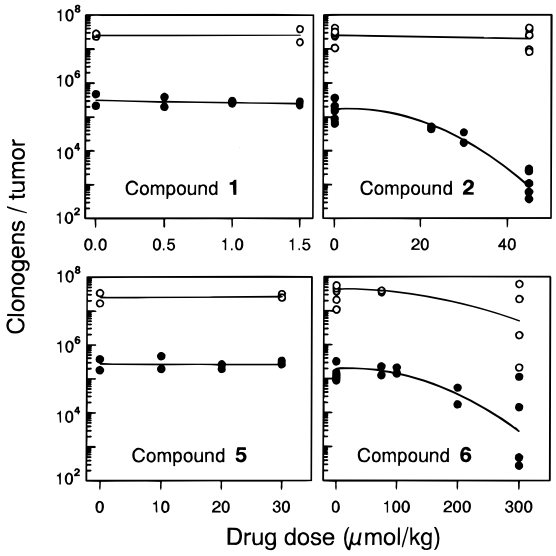


Figure 5. Activity of 1-nitroacridine tertiary amines (left panels) and their *N*-oxide derivatives (right panels) against the KHT tumor as determined by excision and clonogenic assay 18 h after treatment. Drugs were administered ip alone (○) or 5 min after γ-irradiation at 15 Gy (●). In each case the highest drug dose tested corresponds to 3 times the maximum tolerated dose (as determined using a 28-day observation period). Each point represents two pooled tumors from separate mice. Multiple points at each dose are from separate experiments.

Conclusions

The main aims of this work were to provide analogues of nitracrine *N*-oxide with lower nitroacridine reduction potentials, and consequently lowered rates of metabolic activation, and to test whether this would lead to improved distributive properties and enhanced therapeutic activity against hypoxic cells in tumors. The results show that 4,5-disubstitution of nitracrine (**1**) with Me or OMe groups does not provide analogues with lower reduction potentials than the corresponding monosubstituted compounds. This appears not to be due to a concomitant raising of the acridine pK_a but to a lack of direct electronic effect of substituents not in the ring bearing the nitro group. However, placing two OMe groups in the nitro-bearing ring does result in a substantial further lowering of $E(1)$ (from −355 mV in **5** to −401 mV in **9**). With all of the Me- or OMe-substituted compounds, a significant metabolic stabilization was observed in hypoxic AA8 cell cultures. This effect was greatest for the tertiary amine **9**, which had the lowest $E(1)$, although lesser protection against overall metabolic loss was achieved with the corresponding *N*-oxide **10** (presumably because reduction of the *N*-oxide moiety now becomes significant).

While the 2,4-diOMe-substituted *N*-oxide **10** showed poor hypoxic selectivity in culture, the *N*-oxides of the 4-substituted and 4,5-disubstituted derivatives of nitracrine generally retained the very high selectivity of the parent *N*-oxide **2**. For the 4-OMe derivative **6** and the 4,5-diOMe derivative **12**, this selectivity was >1000-fold, although absolute potency was much lower than that of **2**. The 4-OMe compound also showed activity in intact multicellular spheroids almost as great as that in single-cell suspensions, with a SRF of only 1.9 (cf. 7

for the parent *N*-oxide **2**), suggesting that it is better able to diffuse in a multicellular environment. These apparent improvements *in vitro* did not translate into improved activity against hypoxic cells in KHT tumors. However, this work has shown that the unsubstituted nitracrine *N*-oxide **2** and its 4-methoxy derivative **6** have sufficient selectivity as hypoxia-selective cytotoxins to warrant further development. Significant improvement of therapeutic activity *in vivo* may require lowering of the rates of reduction of the *N*-oxide center as well as the nitro group, and future work in this series will therefore focus on stabilization of the *N*-oxide center toward hypoxic metabolism.

Experimental Section

Analyses indicated by symbols of the elements were within ±0.4% of theoretical. Analyses were carried out in the Microchemical Laboratory, University of Otago, Dunedin, New Zealand. Melting points were determined on an Electrothermal 2300 melting point apparatus. pK_a values were determined by UV spectrophotometry. NMR spectra were obtained on a Bruker AC-200 or AM-400 spectrometer and are referenced to Me_4Si (organic solvents) or sodium 2,2-dimethyl-2-silapentane-5-sulfonate (D_2O solvent). Mass spectra were determined on a VG-70SE mass spectrometer using an ionizing potential of 70 eV at a nominal resolution of 1000. High-resolution spectra were obtained at nominal resolutions of 3000, 5000, or 10 000 as appropriate. Spectra were obtained using the ionization mode specified, with PFK as the reference unless otherwise stated. Thin-layer chromatography was carried out on aluminum-backed silica gel plates (Merck, 60 F_{254}) and preparative column chromatography on silica gel (Merck, 230−400 mesh).

Compounds for biological evaluation were analyzed by HPLC using a Waters μBondapak C18 column (8 × 100 mm) with a mobile phase comprising a 25 min linear gradient of MeCN (typically 14−50% MeCN) in 0.45 M ammonium formate, pH 4.5, at a flow rate of 1.8 mL/min. All compounds were >99% pure based on absorbance at 254 nm. The 1-nitroacridine *N*-oxides contained <0.02% of the corresponding tertiary amines as impurities. The absence of biologically significant amine impurities was confirmed in each case by bioassay of the column eluate after injecting 0.6−40 μmol of the *N*-oxide. Fractions (1 mL) were collected, diluted 200-fold into 96-well plate cultures of UV4 cells (360 cells/well in 0.2 mL of culture medium) and grown for 4 days before staining with methylene blue to assess cell growth.[30] In all cases the only bioactive peak corresponded to the parent *N*-oxide.

4,5-Dimethyl-9-[(3-(dimethylamino)propyl)amino]-1-nitroacridine (13) (Scheme 2): Example of General Method A. A mixture of methyl 2-bromo-3-methylbenzoate (**17**) (prepared from 2-bromo-3-methylbenzoic acid[31] with $SOCl_2$/MeOH) (3.46 g, 15.1 mmol), 2-methyl-5-nitroaniline (**18**) (4.59 g, 30.2 mmol), anhydrous K_2CO_3 (6.25 g, 45.3 mmol), CuI (0.75 g, 3.93 mmol), and copper powder (0.23 g, 3.62 mmol) in HMPA (15 mL) was stirred at 170 °C under N_2 for 2 h. The cooled mixture was filtered, and the solids were washed with aqueous MeOH. The combined filtrates were basified with KOH, heated under reflux for 45 min, and then concentrated under reduced pressure, diluted with 1 N aqueous KOH, and filtered. The filtrate was acidified to pH 1−2 with concentrated HCl to give 3-methyl-2-[(2-methyl-5-nitrophenyl)amino]-benzoic acid (**19**) (2.7 g, 63%), sufficiently pure for the next step. A sample crystallized from MeOH had mp 203−205 °C: ^{1}H NMR ($CDCl_3$) δ 8.36 (br s, 1 H, exhangeable with D_2O, COOH), 8.01 (dd, J = 7.7, 1.0 Hz, 1 H, H-6), 7.69 (dd, J = 8.2, 2.3 Hz, 1 H, H-4′), 7.50 (dd, J = 7.7, 1.0 Hz, 1 H, H-4), 7.29 (d, J = 8.2 Hz, 1 H, H-3′), 7.17 (t, J = 7.7 Hz, 1 H, H-5), 7.12 (d, J = 2.3 Hz, 1 H, H-6′), 3.5 (s, 1 H, exchangeable with D_2O, NH), 2.50 (s, 3 H, CH_3), 2.05 (s, 3 H, CH_3). Anal. ($C_{15}H_{14}N_2O_4$) C, H, N.

The above acid **19** (2.34 g, 8.18 mmol) was powdered and added slowly to stirred, ice-cold 90% sulfuric acid (50 mL). The resulting mixture was stirred at 80−85 °C for 3.5 h, cooled, and poured onto ice. The precipitate was filtered, washed with water, and dried to give 4,5-dimethyl-1-nitro-9-oxoacridan (**20**) (1.26 g, 58%), mp (CH_2Cl_2/MeOH) 349−351 °C: ^{1}H NMR [$(CD_3)_2SO$] δ 8.87 (br s, 1 H, exchangeable with D_2O, NH), 8.03 (dd, J = 8.0, 1.0 Hz, H-8), 7.75 (d, J = 8.0 Hz, 1 H, H-2), 7.69 (d, J = 8.0 Hz, 1 H, H-6), 7.43 (d, J = 8.0 Hz, 1 H, H-3), 7.28 (t, J = 8.0 Hz, 1 H, H-7), 2.74 (s, 3 H, CH_3), 2.69 (s, 3 H, CH_3). Anal. ($C_{15}H_{12}N_2O_3$) C, H, N.

The above acridone **20** (1.19 g, 4.44 mmol) was suspended in $SOCl_2$ (5 mL) and DMF (1 drop), and the mixture was heated under reflux for 1 h. Excess reagent was evaporated, and the residue was coevaporated with toluene and then dissolved in CH_2Cl_2 and washed with ice-cold aqueous Na_2CO_3. Workup gave a quantitative yield of crude 9-chloro-4,5-dimethyl-1-nitroacridine (**21**), which was dissolved in dry DMF (5 mL) and treated with *N*,*N*-dimethylpropane-1,3-diamine (0.83 mL, 6.7 mmol) at 80 °C for 3.5 h. The cooled mixture was partitioned between CH_2Cl_2 and cold aqueous Na_2CO_3. Workup of the organic layer followed by chromatography of the residue on silica gel (elution with EtOAc) gave 4,5-dimethyl-9-[(3-(dimethylamino)propyl)amino]-1-nitroacridine (**13**) (0.35 g, 23%). This was converted to the dihydrochloride salt, mp (MeOH) 170 °C dec: ^{1}H NMR (D_2O) δ 8.15 (d, J = 8.2 Hz, 1 H, H-2), 8.02 (d, J = 8.2 Hz, 1 H, H-3), 7.87 (d, J = 7.8 Hz, 1 H, H-8), 7.81 (d, J = 7.8 Hz, 1 H, H-6), 7.49 (t, J = 7.8 Hz, 1 H, H-7), 3.77 (t, J = 7.6 Hz, 2 H, NHCH_2), 3.11 (t, J = 7.6 Hz, 2 H, CH_2N$(CH_3)_2$), 2.88 (s, 6 H, N$(CH_3)_2$), 2.80 (s, 3 H, CH_3), 2.70 (s, 3 H, CH_3), 2.20 (quintet, J = 7.6 Hz, 2 H, $CH_2CH_2CH_2$). Anal. ($C_{20}H_{24}N_4O_2$·2HCl·H_2O) C, H, N, Cl.

Similarly prepared, from 4-methyl-1-nitro-9-oxoacridan,[6] was 9-[(3-(dimethylamino)propyl)amino]-4-methyl-1-nitroacridine (**7**) (63%), dihydrochloride salt, mp 259−261 °C dec (lit.[12] mp 256−257 °C): ^{1}H NMR (D_2O) δ 7.50−8.20 (m, 6 H, ArH), 3.73 (t, J = 7.2 Hz, 2 H, NHCH_2), 3.10 (t, J = 7.9 Hz, 2 H, CH_2N$(CH_3)_2$), 2.88 (s, 6 H, N$(CH_3)_2$), 2.71 (s, 3 H, CH_3), 2.20 (m, 2 H, $CH_2CH_2CH_2$).

9-[(3-(Dimethylamino)propyl)amino]-4-methoxy-1-nitroacridine (**5**) was prepared from 4-methoxy-1-nitro-9-oxoacridan,[6] *via* phenol-mediated reaction of the corresponding 9-chloro compound, in 48% yield, dihydrochloride salt, mp 190−192 °C dec (lit.[12] mp 190−191 °C): ^{1}H NMR [$(CD_3)_2SO$] δ 7.40−8.80 (m, 6 H, ArH), 4.25 (s, 3 H, OCH_3), 3.60 (br s, 2 H, NHCH_2), 3.00 (br s, 2 H, CH_2N$(CH_3)_2$), 2.65 (s, 6 H, N$(CH_3)_2$), 2.20 (br s, 2 H, $CH_2CH_2CH_2$).

9-[(3-(Dimethylamino)propyl)amino]-2-methoxy-1-nitroacridine (**3**) was similarly prepared from 9-chloro-2-methoxy-1-nitroacridine[32] in 82% yield: ^{1}H NMR ($CDCl_3$) δ 7.30−8.30 (m, 6 H, ArH), 6,10 (br s, 1 H, exchangeable with D_2O, NH), 3.45 (br s, 2 H, NHCH_2), 2.48 (t, J = 6 Hz, 2 H, $NHCH_2CH_2CH_2$), 2.30 (s, 6 H, N$(CH_3)_2$), 1.80 (quintet, J = 6.5 Hz, 2 H, $NHCH_2CH_2$). The dihydrochloride salt crystallized from EtOAc/MeOH, mp 170−172 °C dec (lit.[24] mp 167−168 °C).

2,4-Dimethoxy-9-[(3-(dimethylamino)propyl)amino]-1-nitroacridine (9) (Scheme 3): Example of General Method B. A suspension of 1,5-dimethoxy-2,4-dinitrobenzene[33] (20.1 g, 90.0 mmol) in water (117 mL) was stirred under reflux and treated dropwise (over 30 min) with a solution of sodium polysulfide, prepared by heating Na_2S·$9H_2O$ (28.6 g, 119 mmol) and sulfur (6.8 g, 213 mmol) in water (120 mL). The reaction mixture was stirred under reflux for a further 3 h and then cooled and evaporated under reduced pressure at 45 °C to give a dark residue. This was extracted several times with warm EtOAc, and the combined extracts were filtered through a short column of silica gel. The eluates were washed with water and evaporated to dryness, and the residue was dissolved in 1 N HCl, filtered, and basified with aqueous NaOH to give 1-amino-2,4-dimethoxy-5-nitrobenzene (**23**) (11.0 g, 63%), mp 126−128 °C (lit.[19] mp 136−137 °C): ^{1}H NMR ($CDCl_3$) δ 7.40 (s, 1 H, H-6), 6.49 (s, 1 H, H-3), 3.95 (s, 3 H, OCH_3), 3.93 (s, 3 H, OCH_3), 3.73 (br s, 2 H, exchangeable with D_2O, NH_2).

A mixture of **23** (6.0 g, 30.9 mmol), diphenyliodonium-2-carboxylate[22] (**22**) (20.0 g, 61.4 mmol), and copper(II) acetate (0.4 g, 2.2 mmol) in dry DMF (180 mL) was stirred at 90 °C (bath temperature) for 7 h and then at 20 °C overnight. Most

of the DMF was evaporated under reduced pressure, and the residue was taken up into CH_2Cl_2, washed twice with water, and extracted into 0.5 N KOH. The alkaline layer was back-extracted with CH_2Cl_2 and filtered, and the filtrate was acidified at 0 °C to give 2-[(2,4-dimethoxy-5-nitrophenyl)amino]-benzoic acid (**24**) (9.7 g, 99%), mp ($CHCl_3$/MeOH) 283−285 °C: ^{1}H NMR [$(CD_3)_2SO$] δ 9.50 (s, 1 H, exchangeable with D_2O, COOH), 7.96 (s, 1 H, H-6′), 7.91 (d, J = 7.3 Hz, 1 H, H-6), 7.41 (t, J = 7.3 Hz, 1 H, H-4), 7.07 (d, J = 7.3 Hz, 1 H, H-3), 6.97 (s, 1 H, H-3′), 6.80 (t, J = 7.3 Hz, 1 H, H-5), 4.00 (s, 6 H, 2 × OCH_3). Anal. ($C_{15}H_{14}N_2O_6$) C, H, N.

A suspension of **24** (5.0 g, 15.9 mmol) in dry DMF (25 mL) was treated with CDI[21] (3.87 g, 23.9 mmol) at 40 °C for 10 min and then cooled and treated with *N*,*N*-dimethylpropane-1,3-diamine (6.0 mL, 47.7 mmol) at 20 °C for 17 h. The mixture was partitioned between CH_2Cl_2 and 0.5 N aqueous NaOH, and the organic layer was washed twice with water, dried, and evaporated to give *N*-[3-(dimethylamino)propyl]-2-[(2,4-dimethoxy-5-nitrophenyl)amino]benzenecarboxamide (**25**) (5.3 g, 84%), mp (EtOAc) 88−90 °C: ^{1}H NMR ($CDCl_3$) δ 9.69 (s, 1 H, exchangeable with D_2O, NH), 8.55 (br t, 1 H, exchangeable with D_2O, NHCO), 8.08 (s, 1 H, H- 6′), 7.39 (d, J = 7.8 Hz, 1 H, H-6), 7.32 (m, 2 H, H-3,4), 6.81 (ddd, J = 7.8, 5.6, 2.8 Hz, 1 H, H-5), 6.57 (s, 3 H, H-3′), 3.99 (s, 6 H, 2 × OCH_3), 3.54 (q, J = 5.9 Hz, collapsed to t after D_2O, 2 H, CONHCH_2), 2.50 (t, J = 5.9 Hz, 2 H, $NHCH_2CH_2CH_2$), 2.30 (s, 6 H, $N(CH_3)_2$), 1.76 (quintet, J = 5.9 Hz, 2 H, $CH_2CH_2CH_2$). Anal. ($C_{20}H_{26}N_4O_5$) C, H, N.

The above amide **25** (5.1 g, 12.8 mmol) was added to stirred, neat polyphosphate ester (PPE), and the mixture was heated at 100 °C for 2 h, allowing volatile solvents to evaporate. A further portion of PPE (50 mL) was then added, and the mixture was stirred at 100 °C for another 2 h. The cooled mixture was poured into cold 2 N aqueous Na_2CO_3 and extracted with EtOAc. The crude product from the organic layer was chromatographed on silica gel, eluting with CH_2Cl_2/Et_3N (99:1), to give 2,4-dimethoxy-9-[(3-(dimethylamino)-propyl)amino]-1-nitroacridine (**9**) (2.4 g, 49%), mp (dihydrochloride salt from EtOAc/CH_3OH) 158 °C dec: ^{1}H NMR (D_2O) δ 8.17 (d, J = 7.7 Hz, 1 H, H-8), 7.96 (t, J = 7.7 Hz, 1 H, H-6), 7.90 (d, J = 7.7 Hz, 1 H, H-5), 7.60 (t, J = 7.7 Hz, 1 H, H-7), 7.22 (s, 1 H, H-3), 4.27 (s, 3 H, OCH_3), 4.18 (s, 3 H, OCH_3), 3.74 (t, J = 7.2 Hz, 2 H, NHCH_2), 2.90 (m, 8 H, $CH_2N(CH_3)_2$), 2.19 (m, 2 H, $CH_2CH_2CH_2$). Anal. ($C_{20}H_{24}N_4O_4$·2HCl·H_2O) C, H, N.

Similar reaction of 3-methoxy-2-[(2-methoxy-5-nitrophenyl)-amino]benzoic acid (**26**) [prepared in 64% yield by Jourdan−Ullmann condensation of methyl 2-bromo-3-methoxybenzoate and 2-methoxy-5-nitroaniline by Method A: mp ($CHCl_3$/MeOH) 261−263 °C; ^{1}H NMR ($CDCl_3$) δ 6.90−7.98 (m, 6 H, ArH), 4.06 (s, 3 H, OCH_3), 3.81 (s, 3 H, OCH_3). Anal. ($C_{15}H_{14}N_2O_6$) C, H, N] with CDI and *N*,*N*-dimethylpropane-1,3-diamine gave *N*-[3-(dimethylamino)propyl]-3-methoxy-2-(2-methoxy-5-nitrophenyl)benzenecarboxamide (**27**) (55% yield), mp (EtOAc/MeOH) 161−162 °C: ^{1}H NMR ($CDCl_3$) δ 8.55 and 8.14 (2 × br s, 2 H, exchangeable with D_2O, 2 × NH), 7.74 (dd, J = 8.9, 2.7 Hz, 1 H, H-4′), 7.22 (d, J = 2.7 Hz, 1 H, H-6′), 7.07−7.20 (m, 3 H, H-4,5,6), 6.84 (d, J = 8.9 Hz, 1 H, H-3′), 4.03 (s, 3 H, OCH_3), 3.82 (s, 3 H, OCH_3), 3.45 (q, J = 6.2 Hz, 2 H, collapsed to a t after D_2O, NHCH_2), 2.40 (t, 2 H, J = 6.2 Hz, C$H_2N(CH_3)_2$), 2.23 (s, 6 H, $N(CH_3)_2$), 1.67 (quintet, J = 6.2 Hz, 2 H, $CH_2CH_2CH_2$). Anal. ($C_{19}H_{26}N_4O_5$) C, H, N.

Cyclodehydration of **27** with PPE as above gave 4,5-dimethoxy-9-[(3-(dimethylamino)propyl)amino]-1-nitroacridine (**11**) (0.85 g, 64%). A solution of this in a mixture of CH_2Cl_2 and *i*-PrOH was treated with anhydrous HCl gas, followed by addition of EtOAc, to give the dihydrochloride salt, mp 170 °C dec: ^{1}H NMR (D_2O) δ 8.15 (d, J = 8.8 Hz, 1 H, H-2), 7.34−7.55 (m, 3 H, H-6,7,8), 7.27 (d, J = 8.8 Hz, 1 H, H-3), 4.28 (s, 3 H, OCH_3), 4.18 (s, 3 H, OCH_3), 3.65 (t, J = 7.0 Hz, 2 H, $NHCH_2$), 3.11 (t, J = 8.1 Hz, 2 H, C$H_2N(CH_3)_2$), 2.89 (s, 6 H, $N(CH_3)_2$), 2.18 (m, 2 H, $CH_2CH_2CH_2$). Anal. ($C_{20}H_{24}N_4O_4$·2HCl·$2H_2O$) C, H, N.

9-[(3-(Dimethylamino)propyl)amino]-4-methyl-1-nitroacridine N^3-Oxide (8) (Scheme 4): Example of General Method C. A stirred solution of the free base of 9-[(3-(dimethylamino)propyl)amino]-4-methyl-1-nitroacridine (**7**) (1.08 g, 3.19 mmol) in dry CH_2Cl_2 (40 mL) was treated dropwise over 30 min at 20 °C with a solution of 2-(phenylsulfonyl)-3-phenyloxaziridine[20] (1.00 g, 3.83 mmol) in the same solvent. The reaction mixture was stirred at 20 °C for a further 30 min and then evaporated to dryness under reduced pressure. The residue was applied directly to a column of silica gel. Elution with EtOAc/Et_3N (99:1) followed by $CHCl_3$/EtOH/Et_3N (90:9:1) gave 9-[(3-(dimethylamino)propyl)amino]-4-methyl-1-nitroacridine N^3-oxide (**8**) (1.1 g, 97%). A solution of this in a mixture of CH_2Cl_2 and *i*-PrOH was treated with anhydrous HCl gas, followed by addition of EtOAc, to give the dihydrochloride salt, mp 251 °C dec: ^{1}H (D_2O) δ 7.50−8.10 (m, 6 H, ArH), 3.79 (t, J = 7.2 Hz, 2 H, NHCH_2), 3.60 (t, J = 7.2 Hz, 2 H, C$H_2N(CH_3)_2$), 3.48 (s, 6 H, $N(CH_3)_2$), 2.72 (s, 3 H, CH_3), 2.35 (quintet, J = 7.2 Hz, 2 H, $CH_2CH_2CH_2$). Anal. ($C_{19}H_{22}N_4O_3$·2HCl·$0.5H_2O$) C, H, N.

Similarly prepared, from **1**, was 9-[(3-(dimethylamino)-propyl)amino]-1-nitroacridine N^3-oxide (**2**) (100% yield), dihydrochloride salt, mp (MeOH/EtOAc) 194−196 °C (lit.[25] mp 196 °C): ^{1}H NMR (D_2O) δ 8.60−7.50 (m, 7 H, ArH), 3.79 (t, J = 7.2 Hz, 2 H, NHCH_2), 3.62 (t, J = 7.2 Hz, 2 H, $NHCH_2CH_2CH_2$), 3.47 (s, 6 H, NMe_2), 2.37 (quintet, J = 7.2 Hz, 2 H, $CH_2CH_2CH_2$).

Similarly prepared, from **3**, was 9-[(3-(dimethylamino)-propyl)amino]-2-methoxy-1-nitroacridine N^3-oxide (**4**) (92% yield), dihydrochloride salt, mp (EtOAc/MeOH) 195 °C dec: ^{1}H NMR (D_2O) δ 8.20 (d, J = 8.6 Hz, 1 H, H-4), 8.03 (dd, J = 8.0, 1.0 Hz, 1 H, ArH), 8.01 (dd, J = 8.0, 1.0 Hz, 1 H, ArH), 7.96 (td, J = 8.0, 1.0 Hz, 1 H, ArH), 7.79 (d, J = 8.6 Hz, 1 H, H-3), 7.58 (td, J = 8.0, 1.0 Hz, 1 H, ArH), 4.13 (s, 3 H, OCH_3), 3.86 (t, J = 7.3 Hz, 2 H, NHCH_2), 3.63 (t, J = 7.3 Hz, 2 H, $NHCH_2CH_2CH_2$), 3.46 (s, 6 H, $N(CH_3)_2$), 2.35 (quintet, J = 7.3 Hz, 2 H, $NHCH_2CH_2$). Anal. ($C_{19}H_{22}N_4O_4$·2HCl·$0.5H_2O$) C, H, N, Cl.

Similarly prepared, from **5**, was 9-[(3-(dimethylamino)-propyl)amino]-4-methoxy-1-nitroacridine N^3-oxide (**6**) (92% yield), dihydrochloride salt, mp (MeOH/EtOAc) 190 °C dec: ^{1}H NMR (D_2O) δ 8.21 (d, J = 8.8 Hz, 2 H, H-2), 7.47−8.05 (m, 4 H, ArH), 7.32 (d, J = 8.8 Hz, 1 H, H-3), 4.28 (s, 3 H, OCH_3), 3.78 (t, J = 7.0 Hz, 2 H, NHCH_2), 3.61 (t, J = 7.0 Hz, 2 H, C$H_2N(CH_3)_2$), 3.49 (s, 6 H, $N(CH_3)_2$), 2.35 (quintet, J = 7.0 Hz, 2 H, $CH_2CH_2CH_2$). Anal. ($C_{19}H_{22}N_4O_4$·2HCl·$0.5H_2O$) C, H, N.

Similarly prepared, from **9**, was 2,4-dimethoxy-9-[(3-(dimethylamino)propyl)amino]-1-nitroacridine N^3-oxide (**10**) (76% yield), dihydrochloride salt, mp (EtOAc/MeOH) 182 °C dec: ^{1}H NMR (D_2O) δ 8.02 (d, J = 8.0 Hz, 1 H, H-8), 7.92 (t, J = 8.9 Hz, 1 H, H-6), 7.81 (d, J = 8.0 Hz, 1 H, H-5), 7.53 (t, J = 8.0 Hz, 1 H, H-7), 7.20 (s, 1 H, H-3), 4.28 (s, 3 H, OCH_3), 4.21 (s, 3 H, OCH_3), 3.75 (t, J = 7.3 Hz, 2 H, NHCH_2), 3.61 (t, J = 7.3 Hz, 2 H, $NHCH_2CH_2CH_2$), 3.49 (s, 6 H, $N(CH_3)_2$), 2.32 (quintet, J = 7.3 Hz, 2 H, $NHCH_2CH_2CH_2$). Anal. ($C_{20}H_{24}N_4O_5$·2HCl·H_2O) C, H, N, Cl.

Similarly prepared, from **11**, was 4,5-dimethoxy-9-[(3-(dimethylamino)propyl)amino]-1-nitroacridine N^3-oxide (**12**) (66% yield), dihydrochloride salt, mp (EtOAc/MeOH) 160 °C dec: ^{1}H NMR (D_2O) δ 8.20 (d, J = 8.7 Hz, 1 H, H-2), 7.40−7.59 (m, 3 H, H-6,7,8), 7.31 (d, J = 8.7 Hz, 1 H, H-3), 4.27 (s, 3 H, OCH_3), 4.17 (s, 3 H, OCH_3), 3.78 (br s, 2 H, NHCH_2), 3.60 (br s, 2 H, C$H_2N(CH_3)_2$), 3.46 (s, 6 H, $N(CH_3)_2$), 2.33 (br s, 2 H, $CH_2CH_2CH_2$). Anal. ($C_{20}H_{24}N_4O_5$·2HCl·$1.5H_2O$) C, H, N, Cl.

Similarly prepared, from **13**, was 4,5-dimethyl-9-[(3-(dimethylamino)propyl)amino]-1-nitroacridine N^3-oxide (**14**) (100% yield), dihydrochloride salt, mp (CH_2Cl_2/EtOH/EtOAc) 227 °C dec: ^{1}H NMR (D_2O) δ 8.18 (d, J = 7.9 Hz, 1 H, H-2), 7.93 (d, J = 7.8 Hz, 1 H, H-8), 7.87 (d, J = 7.9 Hz, 1 H, H-3), 7.77 (d, J = 7.8 Hz, 1 H, H-6), 7.42 (t, J = 7.8 Hz, 1 H, H-7), 3.81 (t, J = 7.0 Hz, 2 H, NHCH_2), 3.61 (t, J = 7.0 Hz, 2 H, C$H_2N(CH_3)_2$), 3.47 (s, 6 H, $N(CH_3)_2$), 2.80 (s, 3 H, CH_3), 2.67 (s, 3 H, CH_3), 2.36 (quintet, J = 7.0 Hz, 2 H, $CH_2CH_2CH_2$). Anal. ($C_{20}H_{24}N_4O_3$·2HCl·$0.5H_2O$) C, H, N.

Cytotoxicity Assays. AA8 and UV4 cells were maintained in logarithmic-phase growth at 37 °C as monolayers and grown to plateau phase in spinner flasks as described previously.[34] All drug exposures were performed in αMEM with 5% fetal calf serum under an atmosphere containing 5% CO_2. Growth

inhibition studies were performed as described in detail elsewhere,[30] by seeding 200 log-phase AA8 or 300 UV4 cells in 96-well tissue culture dishes. One day later, cells were exposed to drugs for 18 h under aerobic conditions. The IC_{50} was determined as the drug concentration needed to reduce the cell mass (protein content, measured 72−78 h after removal of drug by staining with methylene blue) to 50% of the mean value for eight control cultures on the same 96-well plate. Clonogenic assays with magnetically-stirred 10 mL suspension cultures (plateau-phase AA8 cells, 10^6/mL) were performed using continuous gassing with 5% CO_2 in air or N_2 as detailed elsewhere.[34] Both cell suspensions and drug solutions in growth medium were preequilibrated under the appropriate gas phase for 60 min prior to mixing, to ensure essentially complete anoxia (in hypoxic cultures) throughout the drug exposure period. Cell killing was quantitated as the drug concentration required to lower the plating efficiency to 10% of that of concurrent non-drug-treated controls.

Metabolic Reduction in Cell Culture. Plateau-phase AA8 cells were incubated with drugs (30 μM) under identical conditions to those used for cytotoxicity (clonogenic) assays. Cells were routinely stained with trypan blue at the end of drug exposures; in all studies it was confirmed that the drug did not compromise metabolic integrity of the cells (>90% viability). The gassed and stirred cell suspensions (or solutions of drugs in media without cells) were sampled at intervals and centrifuged quickly, and the cell-free supernatant was stored at −80 °C. Thawed samples were centrifuged briefly and assayed by HPLC, without further workup, using the method outlined above. Samples were held in a WISP autosampler at 4 °C until injection, with interspersed standards to check analyte stability, and samples of up to 0.25 mL were injected. Detection was by diode array absorbance, with data capture and analysis using a Hewlett Packard Chemstation instrument. Identification of tertiary amine products was by absorbance spectrum and retention time.

Spheroid Assays. EMT6/Ak spheroids were grown in αMEM with 10% FBS in spinner flasks to diameters of 1000−1400 μm, and the activities of drugs against intact and dissociated spheroids were compared by determining clonogenic survival curves. Spheroids were dissociated using an enzyme cocktail of pronase (0.5 mg/mL)/DNAase I (1 mg/mL) for 30 min at 37 °C. The resulting single-cell suspension was washed by centrifugation in αMEM containing 5% FBS and exposed to drugs for 1 h before determination of plating efficiency. The sensitivity of this single-cell suspension was compared with that of cells in intact spheroids which were exposed in magnetically-stirred vials containing 1 spheroid/mL for 1 h immediately before dissociation and plating. The mean cell density during the intact and dissociated exposures (typically 2×10^5 cells/mL) and ambient conditions (5% CO_2/20% O_2, 37 °C) were identical. The C_{10} value was interpolated using at least four drug concentrations for each survival curve in each independent experiment.

***In Vivo* Toxicity and Antitumor Activity.** Drugs were formulated in water and injected ip at 0.01 mL/g of body weight using 20−25 g male C_3H/HeN mice. Toxicity was determined using groups of six mice with 1.33-fold dose increments, with an observation time of 28 days. The maximum tolerated dose (MTD) was defined as the highest dose which did not cause deaths or unacceptable morbidity. Antitumor activity was assessed using subcutaneous KHT tumors in the size range 0.5−1 g as determined by calipers, with excision of tumors 18 h after treatment, pooling of tumors from two mice, and determination of clonogenic survivors by plating in agar as described previously.[35] Drugs were combined with whole-body γ-radiation (^{60}Co) at a dose of 15 Gy to test activity against hypoxic (radioresistant) tumor cells.

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