Hypoxia-Selective Antitumor Agents. 16. Nitroarylmethyl Quaternary Salts as Bioreductive Prodrugs of the Alkylating Agent Mechlorethamine

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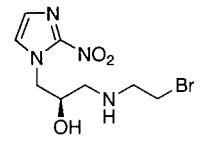
Nitrobenzyl quaternary salts of nitrogen mustards have been previously reported as hypoxia-selective cytotoxins. In this paper we describe the synthesis and evaluation of a series of heterocyclic analogues, including pyrrole, imidazole, thiophene, and pyrazole examples, chosen to cover a range of one-electron reduction potentials (from −277 to −511 mV) and substitution patterns. All quaternary salt compounds were less toxic in vitro than mechlorethamine, and all were more toxic under hypoxic than aerobic conditions, although the differentials were highly variable within the series. The most promising analogue, imidazole **2**, demonstrated DNA cross-linking selectively in hypoxic RIF-1 cells, and was active in vivo in combination with radiation or cisplatin. However, **2** also produced unpredictable toxicity in vivo, suggestive of nonspecific nitrogen mustard release, and this has restricted further development of these compounds as hypoxia-selective cytotoxins.

Introduction

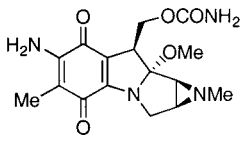
Hypoxia-selective cytotoxins are designed to be preferentially activated to toxic species in the low-oxygen microenvironments present in many solid tumors.[1] This hypoxic cell subpopulation has been directly linked to tumor resistance to radiotherapy,[2] and these slower cycling cells at a greater distance from blood vessels are also considered more likely than well-oxygenated cells to be resistant to various forms of chemotherapy. Further, there is increasing evidence that hypoxia is involved in several aspects of tumor progression, as a consequence of upregulation of the expression of angiogenic factors,[3] selection for apoptosis-resistant cells,[4] and stimulation of metastasis.[5] Hence, a successful hypoxia-selective cytotoxin should offer a considerable therapeutic benefit if combined with other treatments that eliminate the major aerobic portions of a tumor.

Several classes of compounds have been considered for development as hypoxia-selective cytotoxins, such as the quinone porfiromycin (under clinical evaluation in combination with radiotherapy[6]) and the nitroimidazole CI-1010 (highly effective against hypoxic cells in murine tumors,[7] but withdrawn from clinical evaluation due to retinal toxicity[8]). Two very promising and more recently investigated analogues are the amine *N*-oxides AQ4N[9] and tirapazamine,[10] the latter of which has progressed to phase III evaluation and appears particularly effective in combination with cisplatin[11] or as an adjunct to cisplatin/radiotherapy treatment.[12]

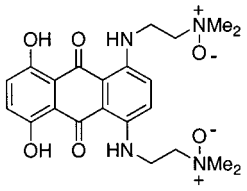
We have previously reported a further class of compounds that have potential as hypoxia-selective cytotoxins, namely, nitrobenzyl quaternary salts of nitrogen mustards.[13] The prototype member of this class, *N*,*N*-bis(2-chloroethyl)-*N*-methyl-*N*-(2-nitrobenzyl)ammonium chloride (**1**) combines a number of attractive features: very strong deactivation of the mustard and high water solubility by virtue of the permanent positive charge, and a reduction potential (−358 mV) within the range expected to be suitable for metabolic activation. **1** was found to display extremely high hypoxic selectivities in vitro (up to several thousand fold), with the mechanism of action being established as reductive fragmentation to release mechlorethamine.[14] Release of a diffusible cytotoxin was also inferred from the much greater cytotoxicity of **1** against intact versus dissociated EMT6 spheroids.[14] A number of benzyl-substituted, naphthyl, and mustard analogues of **1** were prepared and investigated.[15] Although the various substituents affected the reduction potential in a reasonably predictable manner, and there was general evidence for DNA alkylation as the common cytotoxic event, the observed hypoxic selectivities were highly variable, ranging from no selectivity up to several thousand fold, i.e., up to the levels observed for the parent compound **1**. However, when investigated in vivo in combination with radiation,



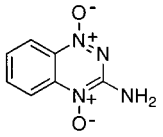
CI-1010



Porfiromycin



AQ4N



Tirapazamine

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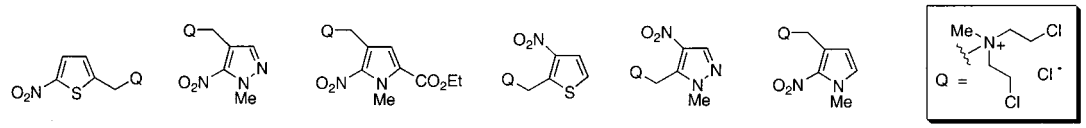
Table 1. One-Electron Reduction Potentials, *E*(1), Determined by Pulse Radiolysis, and in Vitro Cytotoxicity of Nitroarylmethyl Quaternary Nitrogen Mustards under Aerobic and Hypoxic Conditions



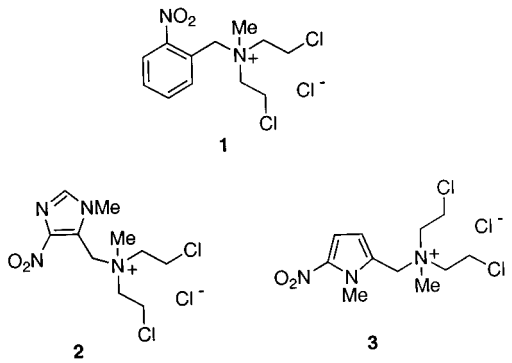
4 **5** **6** **7** **8** **9**

		IC$_{50}$ (μM), aerobic 4 h exposure[a]				***C***$_{10}$ (μM), 3 h exposure, EMT6[b]		
compd	*E*(1) (mV)	AA8	UV4	EMT6	Skov3	air	N_2	HCR[c]
4	−277 ± 10	47 ± 18	0.19 ± 0.02	2.7 ± 0.8	29 ± 11	22 ± 5	1.3 ± 0.2	16 ± 2
5	−287 ± 8	4260 ± 760	6.5 ± 2.6	200 ± 120	2400 ± 800	3450 ± 450	2.2 ± 0.4	2500 ± 500
6	−344 ± 8	32 ± 7	0.36 ± 0.08	3.0 ± 0.4	32 ± 4	30 ± 2	4.5 ± 2.5	14 ± 9
1	−358 ± 10	24600 ± 4400	61 ± 14	300 ± 70	10000 ± 3600	12300 ± 540	66 ± 30	400 ± 290
7	−361 ±10	140 ± 90	0.38 ± 0.12	5.1 ± 1.0	43 ± 8	30 ± 19	6 ± 2	6 ± 4
2	−397 ± 8	150 ± 90	1.9 ± 0.2	14 ± 2	140 ± 25	57 ± 22	1.2 ± 0.1	46 ± 15
8	−433 ± 8	330 ± 60	1.4 ± 0.2	15 ± 6	250 ± 130	85 ± 20	3.5 ± 1.0	17 ± 7
9	−500 ± 8	550 ± 170	1.6 ± 0.1	17 ± 2	215 ± 30	245 ± 40	18 ± 10	16 ± 6
3	−511 ± 10	1000 ± 400	1.8 ± 0.6	9.9 ± 1.3	120 ± 40	320 ± 140	27 ± 9	12 ± 5
HN2[d]		2.5 ± 0.3	0.029 ± 0.004	0.15 ± 0.02	1.09 ± 0.04	1.0 ± 0.1	0.85 ± 0.07	1.17 ± 0.02

[a] Drug concentration to reduce cell density to 50% of that of the controls. Values are mean ± SEM for 2−7 experiments. [b] Drug concentration to reduce surviving fraction to 10% of that of the controls. Values are mean ± SEM for 2−3 experiments. [c] Hypoxic cytotoxicity ratio = C_{10}(air)/C_{10}(N_2). [d] Mechlorethamine.

1 was found to exhibit only marginal activity against hypoxic cells in the KHT tumor.[15]

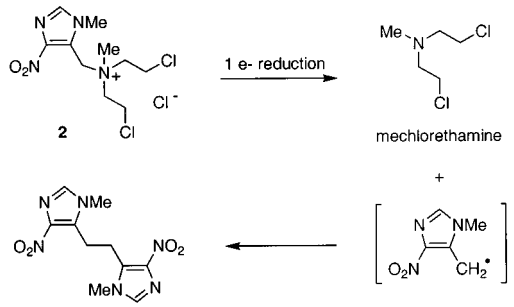
The detailed mechanism of the reductive activation step was initially assumed to involve fragmentation at the one-electron (nitro radical anion) stage, as previously documented for nitrobenzyl halides.[16] However, investigation by steady-state[17] and pulse[18] radiolysis has conclusively shown that multielectron reduction of **1** is required before fragmentation occurs, and is accompanied by the production of many aromatic byproducts in low yield. This is true for all nitrobenzyl and nitronaphthyl derivatives investigated, but is in sharp contrast to the results obtained with two nitro*heterocyclic* analogues—the nitroimidazole **2** and the nitropyrrole **3**.[17] For these compounds clean one-electron reductive



1 **2** **3**

fragmentation occurs, with high-yielding release of mechlorethamine, along with the product derived from arylmethyl radical dimerization (Scheme 1). As previously noted, **2** and **3** also display significant hypoxic selectivity in vitro, although not to the same extreme levels as observed for **1**.[19] The observation that these compounds fragment efficiently on radiolytic reduction also suggests the possibility, ultimately, of activating nitroarylmethyl quaternary salts under hypoxic conditions using radiotherapy rather than enzymes to effect reduction, although this would probably require release of more potent cytotoxic amines than mechlorethamine to be therapeutically useful.[19]

Scheme 1. Reductive Fragmentation of Compound **2**

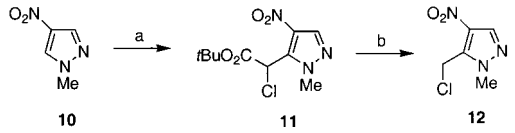


Given the distinctive difference between the reductive chemistry of **2** and **3** compared to previous benzyl examples, and the much higher yielding release of mechlorethamine from these compounds, we have undertaken a detailed study of their hypoxia-selective activity. We also report the synthesis and activity of a series of further heterocyclic analogues with a variety of substitution patterns and reduction potentials. This study seeks to optimize the nitroheterocyclic system in relation to masking of mustard toxicity in the prodrug form, enzymatic activation by hypoxic cells in tissue culture, and activity against hypoxic cells in tumors.

Results and Discussion

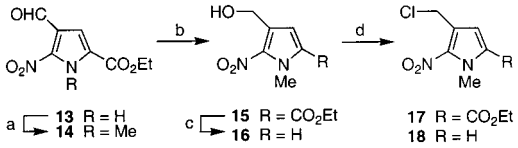
Chemistry. The reference compounds **1**−**3** and newly synthesized quaternary salts are presented in Table 1. Heterocyclic systems investigated include imidazole, pyrrole, thiophene, and pyrazole, in which the nitro group and methyl quaternary substituent are in conjugated positions. The general synthetic plan follows from the route previously described,[15] i.e., displacement of the leaving group from a nitro(halomethyl)heterocycle with *N*-methyldiethanolamine, and conversion of the resulting quaternary salt diol to the mustard using $SOCl_2$. In several cases the required (chloromethyl)- or (bromomethyl)heterocycles were known compounds: 5-(chloromethyl)-1-methyl-4-nitroimidazole[20] for the synthesis of **2**, 2-(chloromethyl)-5-nitrothiophene[21] for the synthesis of **4**, and 2-(bromomethyl)-3-nitrothiophene[22] for the synthesis of **7**. In two other cases the (chloromethyl)-

Scheme 2[a]



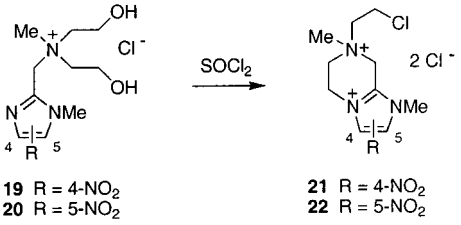
[a] Conditions: (a) Cl_2CHCO_2-*t*-Bu, *t*-BuOK; (b) AcOH, 110 °C.

Scheme 3[a]



[a] Conditions: (a) Me_2SO_4, K_2CO_3; (b) $NaBH_4$; (c) NaOH and then Cu, quinoline, 180 °C; (d) Ph_3PCl_2.

Scheme 4



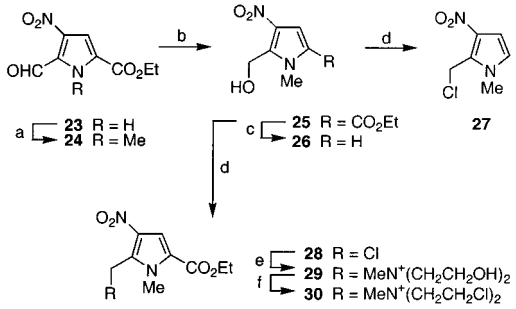
heterocycles were readily obtained from the corresponding aldehyde or acid: 1-methyl-5-nitropyrrole-2-carboxaldehyde[23] and 1-methyl-5-nitropyrazole-4-carboxylic acid[24] were each reduced to the hydroxymethyl analogues ($NaBH_4$[25] or BH_3·DMS) and then converted to the chlorides with $MsCl/Et_3N$, thus leading to the preparation of **3** and **5**, respectively.

The route to a further compound (**8**) made use of the vicarious nucleophilic substitution (VNS) strategy reported by Makosza and Bialecki[20] (Scheme 2). Reaction of 1-methyl-4-nitropyrazole (**10**) with the anion derived from *tert*-butyl dichloroacetate gave, as anticipated,[26] the product of VNS reaction at the 5-position (**11**). The *tert*-butyl ester was cleaved with AcOH and the resulting acid decarboxylated to give the desired chloromethyl intermediate **12**.

A common intermediate for the final two analogues, **6** and **9**, was ethyl 4-formyl-5-nitropyrrole-2-carboxylate[27] (**13**) (Scheme 3). This pyrrole was *N*-methylated and the aldehyde **14** reduced with $NaBH_4$. Hydrolysis of the ester **15** and decarboxylation using Cu/quinoline at elevated temperature gave the unsubstituted analogue **16**. Both **15** and **16** were converted to their chloromethyl analogues, and these were carried through to the quaternary mustard compounds **6** and **9** as described above.

Unsuccessful attempts were also made to prepare a number of other analogues. Two isomers of the imidazole **2**, with the quaternary group in the 2-position, were successfully carried through to the diol stage (Scheme 4), but all attempts to convert **19** and **20** to the mustard forms led to isolation of the products of intramolecular quaternization. Presumably other imidazole isomers (2-nitro-4-substituted and 5-nitro-4-substituted) would show the same self-quaternization behavior. Isomers of the pyrroles **6** and **9** in which the substituents at the 2- and 3-positions were reversed were also investigated. The chemistry follows that

Scheme 5[a]



[a] Conditions: (a) Me_2SO_4, K_2CO_3; (b) $NaBH_4$; (c) NaOH and then Cu, quinoline, 180 °C; (d) Ph_3PCl_2; (e) $MeN(CH_2CH_2OH)_2$; (f) $SOCl_2$.

described above and is illustrated in Scheme 5. With this isomer pattern, although the chloromethyl compound **27** could be isolated in a pure state, it was found to be very sensitive to nucleophilic displacement and hydrolysis. The quaternary salt analogues of **27**, containing an even better leaving group, were anticipated to be more unstable than **27**, and their synthesis was not pursued. However, with **28**, the addition of the extra electron-withdrawing ester substituent was sufficiently deactivating so that both **28** and the quaternary salt diol **29** could be isolated without difficulty. A crude sample of the mustard **30** was also prepared, but was found to decompose on attempted purification.

Reduction Potentials. One-electron reduction potentials, $E(1)$, were determined in aqueous solution by measuring the equilibrium constant for electron transfer between the radical anions of the compounds and viologen reference standards.[28] Although reduction potentials for compounds **1**−**3** have been recorded previously,[18] the reported value for **3** (−561 mV) was found to require revision as a consequence of the very fast breakdown of its radical anion, and the relatively slow electron transfer from the reducing 2-propanyl radical to **3**. Using fast reduction by the aquated electron, and high substrate concentrations to establish equilibrium before any significant breakdown, a revised $E(1)$ value of −511 mV was determined. The reduction potentials of all the compounds are collected in Table 1, with the compounds arranged in order of decreasing electron affinity. The compounds span an $E(1)$ range from −277 to −511 mV, with the previously reported examples (**1**−**3**) lying in the middle and at the bottom of the range.

Although there are only a limited number of structurally diverse heterocycles, some SAR is apparent: for a given substitution pattern, nitropyrroles are considerably harder to reduce than either nitrothiophenes or nitropyrazoles (**3** vs **4**, **9** vs **5**), while the addition of an electron-withdrawing ester substituent raises the reduction potential as anticipated (**6** vs **9**, where the ester is conjugated to the nitro group). We have previously noted that the quaternary methyl substituent is itself strongly electron withdrawing (the Hammett σ_p value for the $CH_2N^+Me_3$ substituent has been variously reported as +0.44 and +0.67[29]) and were therefore interested in comparing the reduction potentials in Table 1 with those of analogues bearing a more neutral "benzyl" substituent. Where the hydroxymethyl analogues were available for comparison (i.e., analogues of **2**−**4**, **6**, and **9**),[30] the change from CH_2OH (σ_p 0.00[29]) to

methyl quaternary salt was found to raise the reduction potential, as expected. However, $\Delta E(1)$ was quite variable (+94 mV for **3**, +180 mV for **2**), suggesting that there is a complex electronic perturbation on introducing a methyl quaternary substituent, which likely varies with both the heterocycle and substitution pattern.

Aerobic Toxicity. Aerobic cytotoxicities were determined as IC_{50} values after 4 h of drug exposure in four cell lines: the Chinese hamster lines AA8 and UV4, the murine mammary carcinoma EMT6, and the human ovarian carcinoma Skov3. The UV4 cell line is an ERCC1 mutant defective in nucleotide excision repair[31] and is hypersensitive to DNA cross-linking agents such as nitrogen mustards.[32] Striking variations in toxicity were observed among the different cell lines and compounds (Table 1). Although all the quaternary salts were less toxic than mechlorethamine, the ratio varied from 7 (**4** in UV4) to nearly 10000 (**1** in AA8). Despite these large variations some patterns do emerge: the relative cell line sensitivity is in all cases UV4 > EMT6 > Skov3 > AA8, the same order as observed for mechlorethamine itself. Further, it can be seen that **1** is the least toxic compound to all cell lines, while **4** (with a single exception) is the most toxic. The extent to which toxicity is masked in the prodrugs is clearly not directly related to reduction potential, as, among the heterocycles, **4** and **5** are at the extremes of toxicity but their $E(1)$ values are separated by only 10 mV. These observations suggest that aerobic toxicity is unlikely to be a consequence of reductive activation (unless there is a great variation in the stability of the reduced intermediates); an alternative possibility is that there is a slow release of mechlorethamine by direct nucleophilic displacement (either chemical or enzymatic) during the drug exposure phase.[33]

Release of mechlorethamine, whether via reduction or nucleophilic displacement, is implicated in the aerobic toxicity by the large hypersensitivity factor [$HF = IC_{50}(AA8)/IC_{50}(UV4)$] between the AA8 and UV4 cell lines. Typical HF values for cross-linking agents fall in the range 10−70[32] (in Table 1 mechlorethamine gives a value of 86), while the results for the quaternary salts lie between 50 and 660. Such abnormally high HF values were also previously observed with several nitrobenzyl quaternary mustards, and in those cases it was suggested that saturable uptake of the quaternary salts might magnify the toxicity differential.[15] A further potential contributor to aerobic toxicity is the generation of reactive oxygen species through redox cycling, which would be expected to be more pronounced with the more easily reduced (higher reduction potential) compounds, and was in fact implicated in the aerobic toxicity of nitrobenzyl analogues.[15]

Clearly, there are a number of factors potentially involved in the aerobic toxicity of mustard quaternary salts, and quaternizing a mustard does not always eliminate its toxicity. However, in most of the examples in Table 1, the toxicity differential compared to mechlorethamine is large enough that substantial hypoxia-selective cytotoxicity should still be achievable if these prodrugs are mainly reduced by oxygen-sensitive reductases.

Hypoxia-Selective Cytotoxicity. The ability of endogenous reductases to activate the nitroarylmethyl quaternary salts was investigated by determining dose−response relationships for clonogenic killing of EMT6 cells, obtained by dissociation of multicellular spheroids, under aerobic and anoxic conditions. In these experiments, stirred and continuously gassed cell suspensions (10^6 cells/mL) were exposed to drugs for 3 h and washed by centrifugation, and the plating efficiency determined. Typical results are illustrated for mechlorethamine, for **2**, and for the corresponding chloromethyl compound [5-(chloromethyl)-1-methyl-4-nitroimidazole] in Figure 1. This shows that **2** is 60-fold less toxic than mechlorethamine under aerobic conditions (similar to the 90-fold differential against EMT6 cells in the IC_{50} assay), but is almost as potent as mechlorethamine under hypoxic conditions. This suggests almost quantitative release of mechlorethamine from **2** under hypoxia. The chloromethyl analogue was slightly less potent than **2** under aerobic conditions, and showed no significant hypoxic selectivity. This is consistent with the proposed mechanism for the hypoxic toxicity of **2**, in which cytotoxicity is due not to reactive species derived from the reduced nitro group (e.g., nitroso or hydroxylamine), but to intramolecular electron transfer leading to fragmentation and release of mechlorethamine.

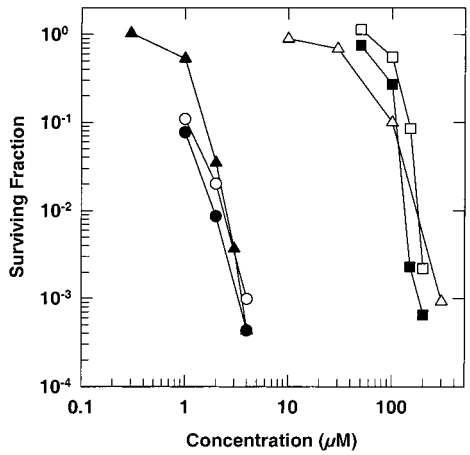


Figure 1. Clonogenic survival curves for EMT6 cells (10^6 cells/mL) exposed to mechlorethamine (circles), **2** (triangles), or 5-(chloromethyl)-1-methyl-4-nitroimidazole (squares) for 3 h under aerobic (open symbols) or hypoxic (filled symbols) conditions. Representative data from a single experiment for each compound. The control plating efficiencies were 80−92% under aerobic conditions and 69−82% under hypoxia.

The same assay was used to compare the hypoxic selectivity (quantified as the hypoxic cytotoxicity ratio, HCR, as defined in Table 1) of the series of heterocyclic analogues. As noted previously with **1**,[15] cytotoxicity was quite variable in repeat experiments, as indicated by the large standard errors for cytotoxic potency (C_{10}) and HCR in Table 1. Despite considerable investigation of possible sources of variability (differences in cell density, growth stage, medium age, ascorbate concentration, exposure to light, etc.), we have not been able to circumvent this problem. Nevertheless, it is clear that there are substantial differences in hypoxic selectivity between the different heterocycles, with the pyrazole **5** (HCR 2500) being the most selective and the thiophene **7** (HCR 6) the least selective. Since many of the analogues are almost as toxic as mechlorethamine under hypoxic conditions, these differences in HCR largely reflect variations in the aerobic toxicity of the

Table 2. In Vivo Toxicity (Maximum Tolerated Dose in C3H/HeN Mice) and Activity against Aerobic and Hypoxic Cells in RIF-1 Tumors

		activity against RIF-1 tumor						
					log(cell kill), drug + radiation (15 Gy)			
compd[a]	MTD, single ip dose (μmol/k g)	dose (μmol/k g)	log(cell kill), drug only[b]	p[c]	drug 30 min before[b]	p[c]	drug 5 min after[b]	p[c]
TPZ	300	225	0.1 ± 0 (2)	ns	0.53 ± 0.15 (4)	ns	0.52 ± 0.07 (22)	0.014
CI-1010	940	705	0.07 ± 0.02 (2)	ns	0.72 ± 0.13 (4)	0.0170	1.08 ± 0.11[d] (38)	<0.001
PORF	75	56.2	0.63 ± 0.15 (4)	<0.001	1.21 ± 0.22 (6)	<0.001	0.86 ± 0.23 (6)	<0.001
AQ4N	450	450	0.08 ± 0.13 (3)	ns	−0.17 ± 0.09 (3)	ns	0.09 ± 0.19 (5)	ns
4	56.2	56.2	0.54 ± 0.10 (2)	<0.001	−0.16 ± 0.24 (5)	ns	−0.55 ± 0.22 (5)	ns
5	31.6	31.6	−0.04 ± 0.05 (4)	ns	−0.16 ± 0.16 (6)	ns	0.17 ± 0.15 (6)	ns
6	31.6	31.6	0.03 ± 0.00 (2)	ns	−0.55 ± 0.07 (5)	ns	−0.73 ± 0.09 (5)	ns
1	100	100	0.24 ± 0.05 (2)	ns	−0.01 ± 0.08 (5)	ns	0.92 ± 0.11 (5)	<0.001
7	56.2	56.2	0.23 ± 0.03 (2)	ns	0.16 ± 0.13 (5)	ns	0.11 ± 0.19 (5)	ns
2	100	100	0.38 ± 0.12 (4)	<0.001	0.39 ± 0.09 (6)	ns	1.01 ± 0.13 (41)	<0.001
8	23.7	23.7	−0.12 ± 0.02 (4)	ns	−0.17 ± 0.21 (6)	ns	0.10 ± 0.13 (6)	ns
9	56.2	56.2	0.24 ± 0.06 (2)	ns	−0.38 ± 0.03 (5)	ns	−0.47 ± 0.17 (5)	ns
3	42.1	42.1	0.25 ± 0.02 (2)	ns	0.48 ± 0.09 (5)	ns	0.23 ± 0.21 (5)	ns
HN2	17.8	17.8	−0.18 ± 0.06 (2)	ns	0.04 ± 0.14 (8)	ns	−0.03 ± 0.13 (8)	ns

[a] TPZ, tirapazamine; PORF, porfiromycin; HN2, mechlorethamine. [b] Numbers in parentheses refer to the number of mice per group. [c] Significance of drug-induced cell killing, assessed as the decrease in clonogens relative to the appropriate control (untreated, or radiation only). Note that because of differences in the number of animals per group the magnitude of the effect provides a more useful basis for comparing compounds than does the statistical significance. [d] One out of thirty-eight tumors below the limit of detection (10^3 clonogens/g) was converted to 10^3 clonogens/g for this analysis.

compounds. There is also, however, a trend to lesser hypoxic potency at lower $E(1)$ values (particularly if the single nonheterocyclic analogue **1** is excluded from the comparison). Presumably this reflects less facile metabolic reduction of these analogues, but even so, the lowest potential compound (pyrrole **3**, $E(1) = -511$ mV) still shows appreciable hypoxic selectivity (HCR 12) in this assay.

DNA Cross-Linking in Hypoxic RIF-1 Cells. The hypoxia-selective cytotoxicity of **2** was investigated further in RIF-1 tumor cells, under the same conditions as used above for EMT6 cells, but with 1 h of drug exposure. This demonstrated an HCR of 59, with C_{10} values of 200 μM under aerobic conditions and 3.4 μM under hypoxia (data not shown). The ability of **2** to form DNA cross-links in these cells was tested using the comet assay (single-cell gel electrophoresis) following 1 h of drug exposure at 3.5 μM. In the comet assay,[34] DNA breakage in individual cells is detected by alkaline lysis in gels on microscope slides, followed by electrophoresis, which draws DNA fragments out of nuclei to form comet-like tails. Comets are stained with propidium, and the tail moment is determined using an image analysis system. Exposure of RIF-1 cells to **2** under either aerobic or hypoxic conditions did not cause DNA breakage (Figure 2A−C), whereas γ irradiation (10 Gy) under aerobic conditions gave extensive breakage (Figure 2D). Pretreatment with **2** under aerobic conditions had no effect on subsequent DNA breakage by radiation (Figure 2E), whereas breakage was strongly suppressed by pretreatment with **2** under hypoxia before irradiation (Figure 2F). These results are similar to the effect of known DNA cross-linking agents, including mechlorethamine, in preventing radiation-induced changes in DNA breakage in this assay,[35] and demonstrate hypoxia-selective induction of DNA interstrand cross-links in RIF-1 cells.

In Vivo Toxicity. The maximum tolerated doses (MTDs) of the quaternary nitrogen mustard derivatives in mice were evaluated using single ip doses at 1.33-fold dose increments (Table 2). The MTD of mechlorethamine was 17.8 μmol/kg with deaths occurring 2−8

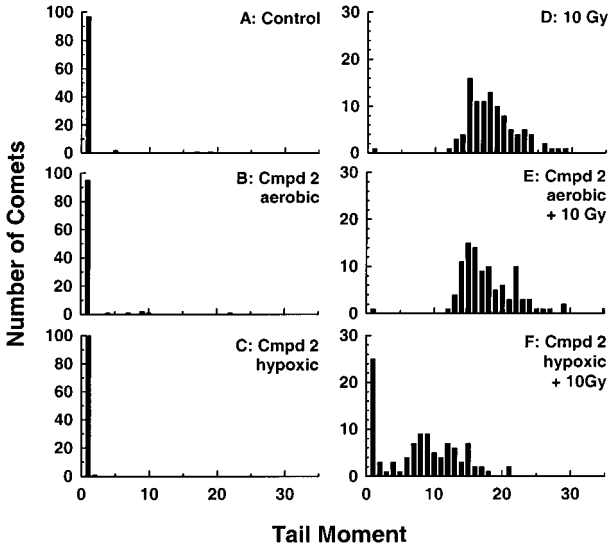


Figure 2. Comet analysis of RIF-1 cell suspensions (10^6 cells/mL) following exposure to **2** at 3.5 μM for 1 h under aerobic and hypoxic conditions. Cells were washed, and then half were irradiated on ice with 10 Gy γ irradiation. Cells were analyzed for DNA breakage or cross-linking, with the histograms showing the distibution of tail moments from 100 comets per treatment condition.

days later at the next two dose levels. The prodrugs all showed lower host toxicity than mechlorethamine, although the change was only modest, with MTD values ranging from 23.7 μmol/kg for **8** to 100 μmol/kg for **1** and **2**. The dose−response curve was shallow, with only moderate toxicity (1−2 deaths/group of six, and weight loss <10% at 2−3 dose levels above the MTD). This makes the MTD an imprecise measure of toxicity in this series. Nonetheless, it is obvious that the prodrugs protect against the toxicity of the mustard effector much less well in vivo than in aerobic cell cultures (Table 1), and that the MTD does not correlate with the aerobic IC_{50} or C_{10} values. At doses above the MTD, the median day of death was 5 days after treatment, and signs of gut toxicity (diarrhea and/or bloody stools) were sometimes seen. These findings are consistent with nitrogen

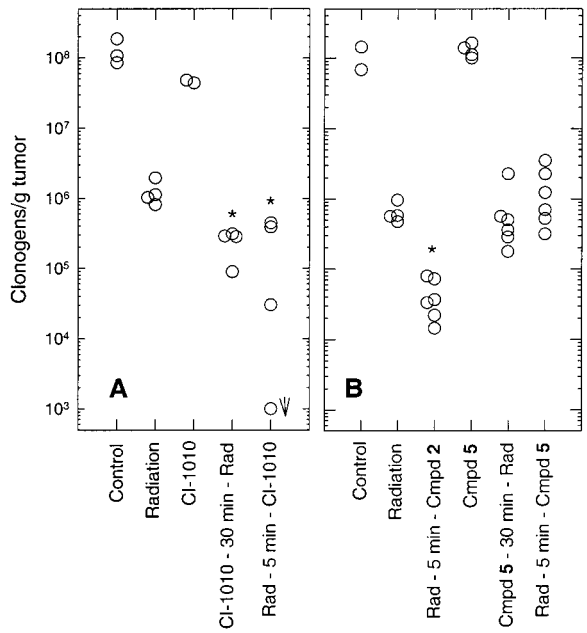


Figure 3. Activity of the reference hypoxia-selective cytotoxin CI-1010 (A) and the nitroarylmethyl quaternary nitrogen mustards **2** and **5** (B) against aerobic and hypoxic cells in RIF-1 tumors. The radiation dose was 15 Gy, and the drug doses were 705 μmol/kg for CI-1010, 100 μmol/kg for **2,** and 31.6 μmol/kg for **5**. Data points are individual tumors. The downward arrow indicates that no colonies were detected. Groups showing a statistically significant drug effect ($p < 0.05$) relative to the appropriate controls (untreated, or radiation only) are marked with an asterisk.

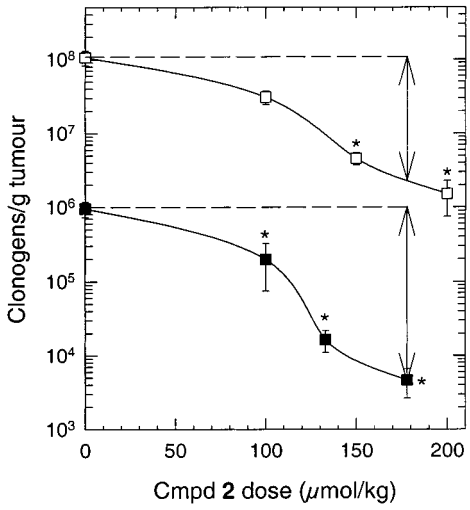


Figure 4. Dose−response activity of **2** either alone (open symbols) or in combination with radiation (closed symbols) against RIF-1 tumors. In the combination treatment **2** was administered 5 min after a 15 Gy radiation dose. Values are the mean ± SEM for 3−9 mice. Groups showing a statistically significant drug effect ($p < 0.05$) relative to the appropriate controls (untreated, or radiation only) are marked with an asterisk.

mustard toxicity. Taken together, these results suggest that the prodrugs are activated extensively, either by nitroreduction or by nucleophilic displacement of mechlorethamine, in non-tumor-bearing mice.

Antitumor Activity. Antitumor activity was assessed by excising RIF-1 tumors 18 h after treatment of mice and measuring cell survival by assaying clonogenic cells. Each quaternary salt compound was administered at the MTD, either alone or in combination with a dose of ionizing radiation (15 Gy) sufficient to sterilize all except the most hypoxic cells in the tumors. In this assay, selective activity against hypoxic cells manifests as a greater increase in killing due to the drug in combination with radiation than for the drug alone.

Typical results are shown for the reference bioreductive drug CI-1010 at 75% of its MTD in Figure 3A, which provided statistically significant additional cell killing when administered 30 min before or 5 min after radiation, but lacked activity as a single agent. The mean log(cell kill) due to CI-1010 under each of these conditions is shown in Table 2, where its activity is compared to three other hypoxic cytotoxins: tirapazamine, porfiromycin, and AQ4N. Tirapazamine at 75% of its MTD was not quite as active as CI-1010, but also showed selective toxicity in combination with radiation. Porfiromycin at 75% of its MTD showed activity comparable to that of CI-1010, but this was not restricted to its combination with radiation, indicating that it is not highly selective for hypoxic cells in this tumor. AQ4N was inactive at its MTD against aerobic or hypoxic cells in RIF-1 tumors, although Patterson et al. have reported this compound to enhance the radiation response of RIF-1 tumors using a growth delay end point.[36]

The above assay was used to evaluate the nitroarylmethyl quaternary nitrogen mustards, using the experimental design illustrated in Figure 3B. The latter experiment, for example, demonstrates the lack of activity of the pyrazole **5** in comparison with the imidazole **2**, which was used as an internal standard in all experiments. The calculated log(cell kill) for each of the prodrugs alone or in combination with radiation is summarized in Table 2. Mechlorethamine itself showed no significant activity either alone or in combination with radiation in this assay. The most active of the nitroarylmethyl quaternary mustards was compound **2**, which provided an additional log(cell kill) when administered 5 min after radiation, while analogue **1** showed slightly less activity. Using this schedule of administration, **2** was at least as active as CI-1010 and porfiromycin (at 75% of their MTD), and significantly better than both tirapazamine (75% MTD) and AQ4N (100% MTD). Both **1** and **2** were less active in the absence of radiation, thus demonstrating some selectivity for hypoxic cells in tumors. They also appeared to be less active when administered 30 min before irradiation, suggesting possible interference with tumor blood flow (and consequent radioresistance via induction of hypoxia). In contrast, none of the other nitroarylmethyl quaternary mustards showed any significant activity either pre- or postirradiation. There was also no obvious relationship between the in vitro and in vivo results, with the compound having the highest hypoxic selectivity in culture (**5**) lacking activity in tumors.

Given the encouraging results with **2**, further studies of its antitumor activity were undertaken. Investigation of the time course of interaction with radiation showed statistically significant activity at most postirradiation times investigated (up to 90 min), with no clear optimum (data not shown). The dose−response relationship was also investigated (Figure 4), with the drug administered 5 min after radiation, using drug doses at and above the MTD. Cytotoxicity due to the drug in combi-

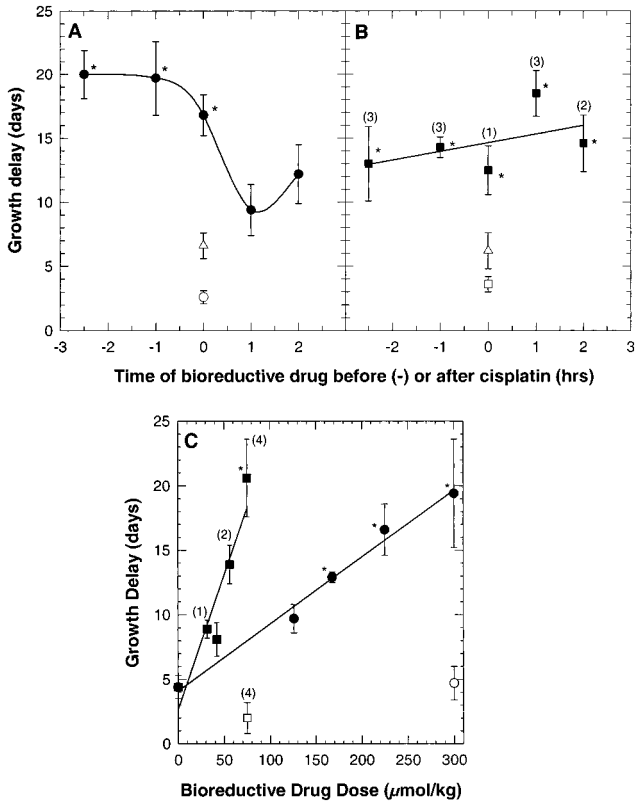


Figure 5. Activity of **2** and tirapazamine in combination with cisplatin (27 μmol/kg) against RIF-1 tumors, determined by measurement of tumor growth delay. All drugs were administered as single ip doses. (A) Time course of interaction between tirapazamine (270 μmol/kg) and cisplatin. (B) Time course of interaction between **2** (75 μmol/kg) and cisplatin. (C) Bioreductive drug dose response, with tirapazamine or **2** administered 1 h before cisplatin. Cisplatin (triangles), tirapazamine (circles), and **2** (squares), either alone (open symbols) or in combination with cisplatin (closed symbols). Each point is the mean ± SEM for a group of seven mice, with the number of deaths from drug toxicity shown in parentheses. An asterisk indicates that the time to the end point (1.2 g of tumor) is significantly different from that of cisplatin alone.

nation with radiation was greater than for the drug alone, with, for example, a log(cell kill) at 178 μmol/kg of 2.4 log units following radiation versus an interpolated value of 1.8 log units for drug alone at this dose. This confirms that **2** is a hypoxia-selective cytotoxin in RIF-1 tumors, at least at high doses, although the notable cell killing in the absence of radiation also implicates significant toxicity to aerobic cells in vivo.

Tirapazamine has been reported to be highly active against the RIF-1 tumor when combined with cisplatin,[10,37] probably because it inhibits the repair of cisplatin-induced DNA cross-links.[38] We compared tirapazamine and **2** in this model, using tumor growth delay as the end point (Figure 5). Varying the time between administration of the two agents confirmed the large enhancement of cisplatin activity when tirapazamine was given before (but not after) cisplatin (Figure 5A). Statistically significant increases in tumor growth delay relative to that of cisplatin alone were also observed when **2** was administered either before or after cisplatin, at each time tested, with no obvious time dependence (Figure 5B). However, unlike tirapazamine, enhancement of cisplatin antitumor activity by **2** was accompanied by substantial toxicity, with 13 deaths in the 35 animals treated with the combination. Evaluation of the bioreductive drug dose response (Figure 5C) confirmed these results; both drugs gave marked responses when administered 1 h before cisplatin, but **2** was more toxic than tirapazamine in the combination. In this experiment, **2** was toxic by itself (four deaths in seven mice) at only 75% of its nominal MTD, so it is not clear that the toxicity in the combination is due to an interaction with cisplatin.

The variable toxicity of **2** in tumor-bearing mice confounded further attempts to assess its therapeutic activity using tumor growth delay assays. A protracted series of experiments (data not shown) demonstrated statistically significant activity of **2** at 75 μmol/kg in combination with radiation against RIF-1 and MDAH-MCa-4-tumors in some groups, but severe toxicity (up to fives deaths in seven mice) in other groups under identical treatment conditions. Reduction of the dose of **2** to 56 μmol/kg did not completely eliminate this toxicity, and generally provided nonsignificant activity. Thus, **2** appears to have activity as a hypoxia-selective cytotoxin in tumors, but its variable toxicity is a serious problem for further development of this series of compounds as bioreductive drugs.

Conclusions

On the basis of the observed clean one-electron reduction chemistry of a nitroimidazole and a nitropyrrole quaternary salt of mechlorethamine, we have prepared and analyzed a series of nitroheterocyclic analogues as hypoxia-selective cytotoxins. Although all quaternary salt compounds were less toxic than mechlorethamine in vitro, and all were more toxic under hypoxic than aerobic conditions (with HCR ratios as much as several thousand fold), the differentials were quite variable and there was no obvious correlation with reduction potential or structure. The 4-nitroimidazole **2** was found to become as toxic as mechlorethamine under hypoxic conditions in EMT6 cell suspensions (Figure 1), while the corresponding chloromethyl analogue showed no significant differential. Compound **2** also demonstrated hypoxia-selective DNA cross-linking in RIF-1 cells (Figure 2). Both of these observations strongly support the proposed mechanism of activation, involving reductive release of mechlorethamine. In vivo, in a RIF-1 excision assay, **2** showed comparable or superior activity to hypoxia-selective cytotoxins of current clinical interest, when combined with radiation or cisplatin treatment. Unfortunately, this activity was accompanied by unpredictable host toxicity, and the accumulated evidence suggests that these nitroarylmethyl quaternary salts are too unstable with regard to nonspecific release of mechlorethamine to be of use as bioreductive agents.

Experimental Section

Chemistry. Analyses were carried out in the Microchemical Laboratory, University of Otago, NZ. Melting points were determined using an Electrothermal Model 9200 digital melting point apparatus and are as read. NMR spectra were obtained on a Bruker AM-400 spectrometer at 400 MHz (^{1}H) or 100 MHz (^{13}C). Mass spectra were obtained on a Varian VG 7070 mass spectrometer at nominal 5000 resolution.

General Method for the Conversion of Nitro(halomethyl)heterocycles to their Quaternary Salt Diol and Mustard Analogues. Preparation of *N,N*-Bis(2-chloroethyl)-*N*-methyl-*N*-[(1-methyl-4-nitro-5-imidazolyl)methyl]ammonium Chloride (2). A solution of 5-(chloromethyl)-1-methyl-4-nitroimidazole[20] (1.22 g, 6.9 mmol) and *N*-methyldiethanolamine (1.24 g, 10.4 mmol) in CH_3CN (40 mL) was stirred at reflux for 18 h and then cooled to 20 °C and the supernatant decanted. The oily residue was crystallized from EtOH/Et_2O to give *N,N*-bis(2-hydroxyethyl)-*N*-methyl-*N*-[(1-methyl-4-nitro-5-imidazolyl)methyl]ammonium chloride as a cream solid (1.51 g, 74%): mp 177−178 °C; ^{1}H NMR [$(CD_3)_2$SO] δ 8.13 (s, 1 H, H-2), 5.59 (t, J = 4.6 Hz, 2 H, 2 × OH), 5.25 (br s, 2 H, $ArCH_2N$), 3.98−3.86 (m, 4 H, NCH_2CH_2O), 3.90 (s, 3 H, NCH_3), 3.85−3.75 (m, 2 H, NCH_2CH_2O), 3.58−3.48 (m, 2 H, NCH_2CH_2O), 3.12 (s, 3 H, $^+NCH_3$); ^{13}C NMR δ 147.5, 139.8, 120.6, 63.5, 55.4, 55.0, 47.6, 33.8. Anal. ($C_{10}H_{19}N_4ClO_4$) C, H, N, Cl.

This diol (1.04 g, 3.5 mmol) was added in portions to $SOCl_2$ (5 mL) and the mixture stirred at 20 °C for 3 days and then evaporated at 20 °C. The residue was crystallized from MeOH to give **2** as a white solid (0.77 g, 66%): mp 173−176 °C dec; ^{1}H NMR [$(CD_3)_2$SO] δ 8.15 (s, 1 H, H-2), 5.22 (s, 2 H, $ArCH_2N$), 4.26−4.12 (m, 4 H, NCH_2CH_2Cl), 4.06−3.97 (m, 2 H, NCH_2CH_2Cl), 3.95−3.86 (m, 2 H, NCH_2CH_2Cl), 3.89 (s, 3 H, NCH_3), 3.18 (s, 3 H, $^+NCH_3$); ^{13}C NMR δ 147.8, 140.2, 119.4, 61.4, 54.9, 47.3, 36.0, 33.8. Anal. ($C_{10}H_{17}N_4Cl_3O_2$) C, H, N, Cl.

The following compounds were prepared by the same general method.

***N,N*-Bis(2-chloroethyl)-*N*-methyl-*N*-[(1-methyl-5-nitro-2-pyrrolyl)methyl]ammonium Chloride (3).** 2-(Chloromethyl)-1-methyl-5-nitropyrrole and *N*-methyldiethanolamine (1.0 equiv) at reflux for 3 h gave on cooling *N,N*-bis(2-hydroxyethyl)-*N*-methyl-*N*-[(1-methyl-5-nitro-2-pyrrolyl)methyl]ammonium chloride as a cream solid (76%); mp 180−181 °C; ^{1}H NMR [$(CD_3)_2$SO] δ 7.34 (d, J = 4.5 Hz, 1 H, H-3 or H-4), 6.73 (d, J = 4.5 Hz, 1 H, H-3 or H-4), 5.59 (t, J = 5.0 Hz, 2 H, OH), 4.93 (s, 2 H, $ArCH_2N$), 3.99 (s, 3 H, NCH_3), 3.93−3.88 (m, 4 H, NCH_2CH_2O), 3.67−3.60 (m, 2 H, NCH_2CH_2O), 3.49−3.42 (m, 2 H, NCH_2CH_2O), 3.06 (s, 3 H, $^+NCH_3$); ^{13}C NMR δ 139.6, 127.8, 115.8, 113.0, 62.7, 57.5, 54.8, 47.8, 34.8. Anal. ($C_{11}H_{20}N_3ClO_4$) C, H, N.

This diol in $SOCl_2$ for 3 days gave **3** as a very pale yellow powder (0.84 g, 92%): mp 140−141 °C (MeOH/Et_2O); ^{1}H NMR [$(CD_3)_2$SO] δ 7.36 (d, J = 4.5 Hz, 1 H, H-3 or H-4), 6.66 (d, J = 4.5 Hz, 1 H, H-3 or H-4), 4.98 (s, 2 H, $ArCH_2N$), 4.24−4.12 (m, 4 H, NCH_2CH_2Cl), 3.99 (s, 3 H, NCH_3), 3.97−3.89 (m, 2 H, NCH_2CH_2Cl), 3.84−3.76 (m, 2 H, NCH_2CH_2Cl), 3.13 (s, 3 H, $^+NCH_3$); ^{13}C NMR δ 140.0, 126.7, 115.9, 113.1, 61.2, 57.5, 47.7, 36.4, 35.3. MS (^{35}Cl, FAB) m/z 294 (100, M^+ for ammonium ion); HRMS calcd for $C_{11}H_{18}N_3Cl_2O_2$ 294.0776, found 294.0766.

***N,N*-Bis(2-chloroethyl)-*N*-methyl-*N*-[(5-nitro-2-thienyl)methyl]ammonium Chloride (4).** 2-(Chloromethyl)-5-nitrothiophene[21] and *N*-methyldiethanolamine (1.0 equiv) at reflux for 5 h gave crude *N,N*-bis(2-hydroxyethyl)-*N*-methyl-*N*-[(5-nitro-2-thienyl)methyl]ammonium chloride as a dark oil that was not purified but was treated directly with $SOCl_2$ for 17 h to give **4** (27% for two steps): mp (EtOAc/MeOH) 161−163 °C dec; ^{1}H NMR [$(CD_3)_2$SO] δ 8.23 (d, J = 4.2 Hz, 1 H, H-4), 7.58 (d, J = 4.2 Hz, 1 H, H-3), 5.13 (s, 2 H, $ArCH_2$), 4.24−4.13 (m, 4 H, NCH_2CH_2Cl), 3.94−3.76 (m, 4 H, NCH_2CH_2Cl), 3.21 (s, 3 H, CH_3). Anal. ($C_{10}H_{15}N_2Cl_3O_2S$) C, H, N.

***N,N*-Bis(2-chloroethyl)-*N*-methyl-*N*-[(1-methyl-5-nitro-4-pyrazolyl)methyl]ammonium Chloride (5).** 4-(Chloromethyl)-1-methyl-5-nitropyrazole and *N*-methyldiethanolamine (1.0 equiv) at reflux for 26 h gave on cooling *N,N*-bis(2-hydroxyethyl)-*N*-methyl-*N*-[(1-methyl-5-nitro-4-pyrazolyl)methyl]ammonium chloride as a cream solid (76%): mp 151−153 °C; ^{1}H NMR [$(CD_3)_2$SO] δ 8.02 (s, 1 H, H-3), 5.52 (t, J = 4.9 Hz, 2 H, OH), 4.87 (s, 2 H, $ArCH_2N$), 4.18 (s, 3 H, NCH_3), 3.95−3.84 (m, 4 H, NCH_2CH_2O), 3.63−3.54 (m, 2 H, NCH_2CH_2O), 3.50−3.42 (m, 2 H, NCH_2CH_2O), 3.06 (s, 3 H, $^+NCH_3$). Anal. ($C_{10}H_{19}N_4ClO_4$) C, H, N, Cl.

This diol in $SOCl_2$ for 4 days gave **5** as a white solid (88%): mp 155−156 °C (*i*-PrOH); ^{1}H NMR [$(CD_3)_2$SO] δ 7.98 (s, 1 H, H-3), 4.91 (s, 2 H, $ArCH_2N$), 4.22−4.14 (m, 4 H, NCH_2CH_2Cl), 4.18 (s, 3 H, NCH_3), 3.91−3.77 (m, 4 H, NCH_2CH_2Cl), 3.12 (s, 3 H, $^+NCH_3$). Anal. ($C_{10}H_{17}N_4Cl_3O_2$) C, H, N, Cl.

***N,N*-Bis(2-chloroethyl)-*N*-methyl-*N*-[(2-(ethoxycarbonyl)-1-methyl-5-nitro-4-pyrrolyl)methyl]ammonium Chloride (6).** Ethyl 4-(chloromethyl)-1-methyl-5-nitropyrrole-2-carboxylate (**17**) and *N*-methyldiethanolamine (1.0 equiv) at reflux for 13 h gave on cooling and dilution with EtOH *N,N*-bis(2-hydroxyethyl)-*N*-methyl-*N*-[(2-(ethoxycarbonyl)-1-methyl-5-nitro-4-pyrrolyl)methyl]ammonium chloride as yellow prisms (61%): mp 78−82 °C (MeOH/Et_2O); ^{1}H NMR [$(CD_3)_2$SO] δ 7.31 (s, 1 H, H-3), 5.47 (t, J = 4.9 Hz, 2 H, OH), 4.88 (s, 2 H, $ArCH_2N$), 4.35 (q, J = 7.1 Hz, 2 H, CH_2CH_3), 4.18 (s, 3 H, NCH_3), 3.94−3.84 (m, 4 H, NCH_2CH_2O), 3.65−3.57 (m, 2 H, NCH_2CH_2O), 3.49−3.42 (m, 2 H, NCH_2CH_2O), 3.05 (s, 3 H, $^+NCH_3$), 1.33 (t, J = 7.0 Hz, 3 H, CH_2CH_3). Anal. ($C_{14}H_{24}N_3ClO_6$·MeOH) C, H, N, Cl.

This diol in $SOCl_2$ for 5 days gave **6** as a pale green powder (85%): mp 125−127 °C (EtOH/Et_2O); ^{1}H NMR [$(CD_3)_2$SO] δ 7.27 (s, 1 H, H-3), 4.90 (s, 2 H, $ArCH_2N$), 4.35 (q, J = 7.1 Hz, 2 H, CH_2CH_3), 4.22−4.11 (m, 4 H, NCH_2CH_2Cl), 4.17 (s, 3 H, NCH_3), 3.92−3.76 (m, 4 H, NCH_2CH_2Cl), 3.12 (s, 3 H, $^+NCH_3$), 1.32 (t, J = 7.1 Hz, 3 H, CH_2CH_3). Anal. ($C_{14}H_{22}N_3Cl_3O_4$·H_2O) C, H, N, Cl.

***N,N*-Bis(2-chloroethyl)-*N*-methyl-*N*-[(3-nitro-2-thienyl)methyl]ammonium Chloride (7).** 2-(Bromomethyl)-3-nitrothiophene[22] and *N*-methyldiethanolamine (1.0 equiv) at reflux for 2 h gave on cooling and dilution with EtOAc the crude quaternary bromide salt. This was dissolved in MeOH/H_2O (1:1) and stirred with a large excess of Biorad AG 1-X4 resin (HCl form) at 20 °C for 1 h. The resin was filtered and washed with MeOH/H_2O, the filtrate was evaporated, and the residue was recrystallized to give *N,N*-bis(2-hydroxyethyl)-*N*-methyl-*N*-[(3-nitro-2-thienyl)methyl]ammonium chloride as a pale yellow solid (70%): mp 149−151 °C (MeOH/EtOAc); ^{1}H NMR [$CD_3)_2$SO] δ 8.40 (d, J = 5.6 Hz, 1 H, H-5), 7.84 (d, J = 5.6 Hz, 1 H, H-4), 5.47 (t, J = 4.8 Hz, 2 H, OH), 5.34 (s, 2 H, $ArCH_2$), 3.98−3.86 (m, 4 H, NCH_2CH_2O), 3.71−3.63 (m, 2 H, NCH_2CH_2O), 3.58−3.50 (m, 2 H, NCH_2CH_2O), 3.13 (s, 3 H, CH_3). Anal. ($C_{10}H_{17}N_2ClO_4S$) C, H, N.

This diol in $SOCl_2$ for 20 h gave **7** as a cream solid (87%): mp 145−147 °C (MeOH/EtOAc); ^{1}H NMR [$(CD_3)_2$SO] δ 8.90 (d, J = 5.7 Hz, 1 H, H-5), 7.86 (d, J = 5.7 Hz, 1 H, H-4), 5.39 (s, 2 H, $ArCH_2$), 4.24−4.12 (m, 4 H, NCH_2CH_2Cl), 3.99−3.85 (m, 4 H, NCH_2CH_2Cl), 3.22 (s, 3 H, CH_3). Anal. ($C_{10}H_{15}N_2Cl_3O_2S$) C, H, N, Cl.

***N,N*-Bis(2-chloroethyl)-*N*-methyl-*N*-[(1-methyl-4-nitro-5-pyrazolyl)methyl]ammonium Chloride (8).** 5-(Chloromethyl)-1-methyl-4-nitropyrazole (**12**) and *N*-methyldiethanolamine (1.0 equiv) at reflux for 16 h gave on cooling and dilution with Et_2O *N,N*-bis(2-hydroxyethyl)-*N*-methyl-*N*-[(1-methyl-4-nitro-5-pyrazolyl)methyl]ammonium chloride as a white solid (80%): mp 135−137 °C; ^{1}H NMR [$(CD_3)_2$SO] δ 8.54 (s, 1 H, H-3), 5.52 (t, J = 4.7 Hz, 2 H, OH), 5.28 (s, 2 H, $ArCH_2N$), 4.10 (s, 3 H, NCH_3), 3.95−3.87 (m, 4 H, NCH_2CH_2O), 3.86−3.71 (m, 2 H, NCH_2CH_2O), 3.56−3.48 (m, 2 H, NCH_2CH_2O), 3.13 (s, 3 H, $^+NCH_3$). Anal. ($C_{10}H_{19}N_4ClO_4$·$^1/_4H_2O$) C, H, N, Cl.

This diol in $SOCl_2$ for 3 days gave **8** as a white solid (90%): mp 147−150 °C (MeOH/Et_2O); ^{1}H NMR [$(CD_3)_2$SO] δ 8.58 (s, 1 H, H-3), 5.27 (s, 2 H, $ArCH_2N$), 4.26−4.14 (m, 4 H, NCH_2CH_2Cl), 4.12 (s, 3 H, NCH_3), 4.09−4.01 (m, 2 H, NCH_2CH_2Cl), 3.97−3.88 (m, 2 H, NCH_2CH_2Cl), 3.20 (s, 3 H, $^+NCH_3$). Anal. ($C_{10}H_{17}N_4Cl_3O_2$) C, H, N, Cl.

***N,N*-Bis(2-chloroethyl)-*N*-methyl-*N*-[(1-methyl-2-nitro-3-pyrrolyl)methyl]ammonium Chloride (9).** 1-Methyl-3-(chloromethyl)-2-nitropyrrole (**18**) and *N*-methyldiethanolamine (1.0 equiv) at 20 °C for 3 days gave on cooling *N,N*-bis(2-hydroxyethyl)-*N*-methyl-*N*-[(1-methyl-2-nitro-3-pyrrolyl)methyl]ammonium chloride as a cream solid (73%): mp 147−150 °C; ^{1}H NMR [$(CD_3)_2$SO] δ 7.45 (d, J = 2.8 Hz, 1 H,

H-4 or H-5), 6.62 (d, $J = 2.9$ Hz, 1 H, H-4 or H-5), 5.42 (t, $J = 4.9$ Hz, 2 H, OH), 4.89 (s, 2 H, $ArCH_2N$), 3.98 (s, 3 H, NCH_3), 3.92−3.86 (m, 4 H, NCH_2CH_2O), 3.63−3.55 (m, 2 H, NCH_2CH_2O), 3.48−3.41 (m, 2 H, NCH_2CH_2O), 3.03 (s, 3 H, $^+NCH_3$). Anal. ($C_{11}H_{20}N_3ClO_4$) C, H, N.

This diol in $SOCl_2$ for 3 days gave **9** as a pale yellow powder (90%): mp 145−146 °C (MeOH/Et_2O); ^{1}H NMR [$(CD_3)_2SO$] δ 7.48 (d, $J = 2.9$ Hz, 1 H, H-4 or H-5), 6.57 (d, $J = 2.8$ Hz, 1 H, H-4 or H-5), 4.92 (s, 2 H, $ArCH_2N$), 4.22−4.12 (m, 4 H, NCH_2CH_2Cl), 3.98 (s, 3 H, NCH_3), 3.90−3.75 (m, 4 H, NCH_2CH_2Cl), 3.10 (s, 3 H, $^+NCH_3$). Anal. ($C_{11}H_{18}N_3Cl_3O_2$) C, H, N, Cl.

2-(Hydroxymethyl)-1-methyl-5-nitropyrrole. $NaBH_4$ (0.19 g, 5.03 mmol) was added in portions to a stirred solution of 1-methyl-5-nitropyrrole-2-carboxaldehyde[23] (0.78 g, 5.07 mmol) in MeOH (40 mL) at 20 °C under N_2. After addition was complete, the reaction mixture was stirred for a further 20 min, then water (40 mL) was added, and the mixture was saturated with solid K_2CO_3. Extraction with EtOAc gave 2-(hydroxymethyl)-1-methyl-5-nitropyrrole (0.77 g, 97%): mp 76−77 °C (EtOAc/petroleum ether) (lit.[25] mp 83−86 °C); ^{1}H NMR ($CDCl_3$) δ 7.16 (d, $J = 4.3$ Hz, 1 H, H-3 or H-4), 6.17 (d, $J = 4.3$ Hz, 1 H, H-3 or H-4), 4.68 (s, 2 H, CH_2), 4.02 (s, 3 H, CH_3). Anal. ($C_6H_8N_2O_3$) C, H, N.

2-(Chloromethyl)-1-methyl-5-nitropyrrole. A stirred solution of 2-(hydroxymethyl)-1-methyl-5-nitropyrrole (0.13 g, 0.83 mmol) in dry CH_2Cl_2 (5 mL) at 0 °C was treated with MsCl (0.36 mL, 1.25 mmol), followed by Et_3N (0.50 mL). After being stirred at 0 °C for 15 min, the mixture was evaporated to dryness under reduced pressure at 20 °C, and the resulting brown solid was partitioned between water and EtOAc. The organic layer was dried (Na_2SO_4), evaporated, and purified by chromatography (1:1 CH_2Cl_2/petroleum ether) to give 2-(chloromethyl)-1-methyl-5-nitropyrrole (0.13 g, 88%): mp 82.5−83.5 °C (EtOAc/petroleum ether); ^{1}H NMR ($CDCl_3$) δ 7.16 (d, $J = 4.4$ Hz, 1 H, H-3 or H-4), 6.26 (d, $J = 4.4$ Hz, 1 H, H-3 or H-4), 4.60 (s, 2 H, CH_2Cl), 4.02 (s, 3 H, CH_3). Anal. ($C_6H_7N_2ClO_2$) C, H, N.

4-(Hydroxymethyl)-1-methyl-5-nitropyrazole. Borane dimethyl sulfide (2 M solution in THF, 4.2 mL, 8.4 mmol) was added to a solution of 1-methyl-5-nitropyrazole-4-carboxylic acid[24] (1.11 g, 6.5 mmol) in dry THF (50 mL) under nitrogen, and the mixture was stirred at reflux for 80 min and then cooled. MeOH, then water, and then 2 N HCl were added, and the THF was evaporated. The residue was diluted with water and extracted with EtOAc. The extracts were dried (Na_2SO_4), evaporated, and purified by chromatography (1:1 EtOAc/petroleum ether) to give 4-(hydroxymethyl)-1-methyl-5-nitropyrazole as a white solid (0.52 g, 51%): mp 78−80 °C (PhH); ^{1}H NMR ($CDCl_3$) δ 7.58 (s, 1 H, H-3), 4.82 (d, $J = 3.4$ Hz, 2 H, CH_2), 4.25 (s, 3 H, NCH_3), 2.39 (br s, 1 H, OH). Anal. ($C_5H_7N_3O_3$) C, H, N.

4-(Chloromethyl)-1-methyl-5-nitropyrazole. MsCl (0.46 mL, 6.0 mmol) was added to a solution of 4-(hydroxymethyl)-1-methyl-5-nitropyrazole (468 mg, 3.0 mmol) and Et_3N (1.25 mL, 9.0 mmol) in CH_2Cl_2 (10 mL), and the mixture was stirred at reflux for 1 h. The mixture was cooled, diluted with water, and extracted with CH_2Cl_2. The extracts were dried (Na_2SO_4) and evaporated, and the residue was purified by chromatography (1:3 EtOAc/petroleum ether) to give 4-(chloromethyl)-1-methyl-5-nitropyrazole as a very pale yellow liquid (374 mg, 71%): ^{1}H NMR ($CDCl_3$) δ 7.64 (s, 1 H, H-3), 4.77 (s, 2 H, CH_2Cl), 4.25 (s, 3 H, NCH_3). MS (^{35}Cl, EI) m/z 175 (23, M^+), 140 (100, M − Cl); HRMS calcd for $C_5H_6N_3ClO_2$ 175.01485, found 175.01421.

5-(Chloromethyl)-1-methyl-4-nitropyrazole (12). A solution of 1-methyl-4-nitropyrazole (**10**)[39] (1.07 g, 8.45 mmol) and *tert*-butyl dichloroacetate (2.35 g, 12.7 mmol) in dry DMF (6 mL) was added dropwise over 30 min to a suspension of *t*-BuOK (3.32 g, 30 mmol) in dry DMF (20 mL) under nitrogen, keeping the internal temperature in the range −25 to −35 °C. The deep purple solution was stirred at −25 °C for a further 20 min, then poured into cold HCl (0.5 N, 80 mL), and extracted with EtOAc (3×). The extracts were dried (Na_2SO_4) and evaporated, and the residue was purified by chromatography (1:3 EtOAc/petroleum ether) to give recovered 1-methyl-4-nitropyrazole (0.18 g, 17%) and *tert*-butyl chloro(1-methyl-4-nitro-5-pyrazolyl)acetate (**11**) as a pale yellow solid (1.39 g, 60%): ^{1}H NMR ($CDCl_3$) δ 8.11 (s, 1 H, H-3), 6.54 (s, 1 H, $CHCO_2$-*t*-Bu), 3.98 (s, 3 H, NCH_3), 1.48 (s, 9 H, *t*-Bu).

This crude ester (1.38 g, 5.0 mmol) was stirred at reflux in AcOH (15 mL) for 21 h, and the solvent was evaporated. TLC analysis showed a mixture of the acid and decarboxylated product. The residue was dissolved in EtOAc, washed with water, and extracted with aqueous $NaHCO_3$. The organic layer was dried (Na_2SO_4) and evaporated to give a brown oil (0.62 g). The aqueous layer was acidified and extracted with EtOAc, and the extracts were dried (Na_2SO_4) and evaporated. The residue (0.22 g) was dissolved in DMF (5 mL) and the solution heated for 10 min to 110 °C to complete the decarboxylation. The solvent was evaporated, the residue was combined with the above brown oil, and the product was purified by chromatography (1:3 EtOAc/petroleum ether) to give **12** as a very pale yellow liquid (0.71 g, 81%). A sample was crystallized from PhH/petroleum ether: mp 67−69 °C; ^{1}H NMR ($CDCl_3$) δ 8.09 (s, 1 H, H-3), 5.01 (s, 2 H, CH_2Cl), 4.00 (s, 3 H, NCH_3). MS (^{35}Cl, EI) m/z 175 (25, M^+), 140 (100, M − Cl); HRMS calcd for $C_5H_6ClN_3O_2$ 175.01485, found 175.01482. Anal. ($C_5H_6N_3ClO_2$) C, H, N.

Ethyl 4-Formyl-1-methyl-5-nitropyrrole-2-carboxylate (14). Dimethyl sulfate (0.31 mL, 3.2 mmol) was added to a mixture of ethyl 4-formyl-5-nitropyrrole-2-carboxylate (**13**) (0.57 g, 2.7 mmol) and K_2CO_3 (0.56 g, 4.0 mmol) in DMSO (4 mL), and the brown suspension was stirred at room temperature for 1 h. The mixture was diluted with water, acidified with HCl (2 N), and extracted with EtOAc (2×). The extracts were dried (Na_2SO_4), evaporated, and purified by chromatography (1:9 EtOAc/petroleum ether) to give **14** as a pale green solid (0.53 g, 86%). A sample was recrystallized from PhH/petroleum ether: mp 59−60.5 °C; ^{1}H NMR ($CDCl_3$) δ 10.32 (s, 1 H, CHO), 7.42 (s, 1 H, H-3), 4.37 (q, $J = 7.1$ Hz, 2 H, CH_2CH_3), 4.33 (s, 3 H, NCH_3), 1.39 (t, $J = 7.1$ Hz, 3 H, CH_2CH_3). Anal. ($C_9H_{10}N_2O_5$) C, H, N.

Ethyl 4-(Hydroxymethyl)-1-methyl-5-nitropyrrole-2-carboxylate (15). $NaBH_4$ (0.33 g, 8.7 mmol) was added in portions to a solution of **14** (3.96 g, 17.5 mmol) in EtOH (100 mL), and the mixture was stirred at 20 °C for 20 min. Water was slowly added, the EtOH was evaporated, and the residue was diluted with aqueous NaCl and extracted with EtOAc (2×). The extracts were washed with aqueous NaCl, dried (Na_2SO_4), and evaporated, and the resulting solid was recystallized from PhH to give **15** (1.40 g, 35%) as white needles: mp 95.5−96.5 °C. The mother liquor was evaporated and purified by chromatography (1:9 EtOAc/petroleum ether) to give more **15** (1.46 g, 37%): ^{1}H NMR ($CDCl_3$) δ 7.01 (s, 1 H, H-3), 4.80 (br s, 2 H, CH_2OH), 4.36 (q, $J = 7.1$ Hz, 2 H, CH_2CH_3), 4.31 (s, 3 H, NCH_3), 2.49 (br s, 1 H, OH), 1.38 (t, $J = 7.1$ Hz, 3 H, CH_2CH_3). Anal. ($C_9H_{12}N_2O_5$) C, H, N.

Ethyl 4-(Chloromethyl)-1-methyl-5-nitropyrrole-2-carboxylate (17). Dichlorotriphenylphosphorane (1.66 g, 5.14 mmol) was added to a solution of **15** (390 mg, 1.71 mmol) in CH_3CN (20 mL), and the mixture was stirred at 20 °C for 2 h. The mixture was evaporated, the residue purified by chromatography (1:9 EtOAc/petroleum ether), and the crude product crystallized from PhH/petroleum ether to give **17** as colorless needles (267 mg, 63%): mp 82−83.5 °C; ^{1}H NMR ($CDCl_3$) δ 7.07 (s, 1 H, H-3), 4.81 (br s, 2 H, CH_2Cl), 4.36 (q, $J = 7.2$ Hz, 2 H, CH_2CH_3), 4.29 (s, 3 H, NCH_3), 1.39 (t, $J = 7.2$ Hz, 3 H, CH_2CH_3). Anal. ($C_9H_{11}N_2ClO_4$) C, H, N.

3-(Hydroxymethyl)-1-methyl-2-nitropyrrole (16). A solution of NaOH (2.7 g, 68 mmol) in water (15 mL) was added to a solution of **15** (1.02 g, 4.47 mmol) in EtOH (30 mL), and the mixture was stirred at 20 °C for 1 h. The EtOH was evaporated, and the aqueous phase was washed with EtOAc and then acidified (HCl). The aqueous mixture was extracted with EtOAc (3×), and the extracts were dried (Na_2SO_4) and evaporated to give crude 4-(hydroxymethyl)-1-methyl-5-nitropyrrole-2-carboxylic acid (0.84 g, 96%) as a red-brown solid.

This acid was suspended in quinoline (6 mL) with Cu powder (0.44 g), and the mixture was heated at 170−180 °C for 50 min. The cooled mixture was diluted with HCl (2 N) and extracted with EtOAc (3×), and the extracts were dried (Na_2SO_4) and evaporated. The residue was purified by chromatography (2:3 EtOAc/petroleum ether) to give **16** as a pink solid (0.45 g, 64% from **15**). A sample was recrystallized from PhH: mp 79−80.5 °C; ^{1}H NMR ($CDCl_3$) δ 6.78 (d, J = 2.8 Hz, 1 H, H-4 or H-5), 6.27 (d, J = 2.5 Hz, 1 H, H-4 or H-5), 4.80 (d, J = 6.8 Hz, 2 H, CH_2OH), 4.00 (s, 3 H, NCH_3), 2.75 (t, J = 6.8 Hz, 1 H, OH). Anal. ($C_6H_8N_2O_3$) C, H, N.

3-(Chloromethyl)-1-methyl-2-nitropyrrole (18). Dichlorotriphenylphosphorane (3.9 g, 12.1 mmol) was added to a solution of **16** (0.63 g, 4.0 mmol) in CH_3CN (50 mL), and the mixture was stirred at 20 °C for 30 min. The mixture was evaporated and the residue purified by chromatography (1:4 EtOAc/petroleum ether) to give **18** as a pale yellow solid (0.60 g, 85%). A sample was recrystallized from PhH/petroleum ether: mp 80−81 °C; ^{1}H NMR ($CDCl_3$) δ 6.75 (d, J = 2.8 Hz, 1 H, H-4 or H-5), 6.37 (d, J = 2.7 Hz, 1 H, H-4 or H-5), 4.86 (s, 2 H, CH_2Cl), 4.00 (s, 3 H, NCH_3). Anal. ($C_6H_7N_2ClO_2$) C, H, N.

***N,N*-Bis(2-hydroxyethyl)-*N*-methyl-*N*-[(1-methyl-4-nitro-2-imidazolyl)methyl]ammonium Chloride (19).** Reaction of 2-(chloromethyl)-1-methyl-4-nitroimidazole[40] and *N*-methyldiethanolamine according to the general method above gave **19** as a light brown foam (90%): ^{1}H NMR [$(CD_3)_2SO$] δ 8.58 (s, 1 H, H-5), 5.58 (t, J = 4.9 Hz, 2 H, OH), 4.95 (s, 2 H, $ArCH_2N$), 3.98−3.85 (m, 4 H, NCH_2CH_2O), 3.88 (s, 3 H, NCH_3), 3.75−3.65 (m, 4 H, NCH_2CH_2O), 3.24 (s, 3 H, $^+NCH_3$); ^{13}C NMR δ 145.7, 137.2, 124.9, 63.6, 56.4, 54.9, 48.9, 34.5. MS (FAB) m/z 259 (100, M^+ for ammonium ion); HRMS (FAB) calcd for $C_{10}H_{19}N_4O_4$ 259.1406, found 259.1399.

7-(2-Chloroethyl)-1,7-dimethyl-3-nitro-5,6,7,8-tetrahydro-1*H*-imidazo[1,2-a]pyrazinium Dihydrochloride (21). Reaction of **19** with $SOCl_2$ gave **21** as white prisms (70%): mp 150−155 °C dec (MeOH/Et_2O); ^{1}H NMR [$(CD_3)_2SO$] δ 9.35 (s, 1 H, H-2), 5.83 (AB coupling, J = 17.1 Hz, 2 H, $ArCH_AH_BN$), 5.11 (dt, J = 15.6, 4.7 Hz, 1 H, NCH_2CH_2N), 4.98−4.90 (m, 1 H, NCH_2CH_2N), 4.39−4.30 (m, 1 H, NCH_2CH_2N), 4.31 (t, J = 6.9 Hz, 2 H, NCH_2CH_2Cl), 4.27−4.18 (m, 1 H, NCH_2CH_2N), 4.18 (t, J = 6.9 Hz, 2 H, NCH_2CH_2Cl), 3.96 (s, 3 H, NCH_3), 3.48 (s, 3 H, $^+NCH_3$); ^{13}C NMR [$(CD_3)_2SO$] δ 138.4, 137.1, 126.3, 64.4, 55.6, 54.3, 42.1, 49.2, 36.0, 35.4. MS (FAB) m/z 259 [100, M^{2+} (bisammonium ion) − H^+], 196 (80%, M^{2+} − H^+ − CH_2-CH_2Cl). Anal. ($C_{10}H_{17}N_4Cl_3O_2\cdot H_2O$) C, H, N, Cl.

***N,N*-Bis(2-hydroxyethyl)-*N*-methyl-*N*-[(1-methyl-5-nitro-2-imidazolyl)methyl]ammonium Chloride (20).** Reaction of 2-(chloromethyl)-1-methyl-5-nitroimidazole[41] and *N*-methyldiethanolamine according to the general method above gave **20** as cream plates (74%): mp 162−163 °C (EtOH); ^{1}H NMR [$(CD_3)_2SO$] δ 8.22 (s, 1 H, H-4), 5.61 (t, J = 5.6 Hz, 2 H, OH), 5.04 (s, 2 H, $ArCH_2N$), 4.03 (s, 3 H, NCH_3), 3.98−3.90 (m, 4 H, NCH_2CH_2O), 3.77−3.64 (m, 4 H, NCH_2CH_2O), 3.26 (s, 3 H, $^+NCH_3$); ^{13}C NMR δ 141.9, 140.0, 132.0, 63.7, 56.6, 54.9, 49.2, 34.2. Anal. ($C_{10}H_{19}N_4ClO_4$) C, H, N, Cl.

7-(2-Chloroethyl)-1,7-dimethyl-2-nitro-5,6,7,8-tetrahydro-1*H*-imidazo[1,2-a]pyrazinium Dihydrochloride (22). Reaction of **20** with $SOCl_2$ according to the general method above gave **22** (6%): mp 135 °C dec; ^{1}H NMR [$(CD_3)_2SO$] δ 9.42 (s, 1 H, H-3), 5.92 (AB coupling, J = 17.0 Hz, 2 H, $ArCH_AH_BN$), 4.89 (dt, J = 15.3, 4.6 Hz, 1 H, NCH_2CH_2N), 4.83−4.73 (m, 1 H, NCH_2CH_2N), 4.46−4.31 (m, 2 H, NCH_2-CH_2N), 4.34 (t, J = 6.9 Hz, 2 H, NCH_2CH_2Cl), 4.23 (t, J = 6.9 Hz, 2 H, NCH_2CH_2Cl), 4.05 (s, 3 H, NCH_3), 3.52 (s, 3 H, $^+NCH_3$); ^{13}C NMR δ 138.8, 138.4, 124.6, 64.8, 55.8, 54.6, 41.5, 49.4, 35.5, 35.3; MS (FAB) m/z 259 [30, M^{2+} (bisammonium ion) − H^+], 196 (100, M^{2+} − H^+ − CH_2CH_2Cl). Anal. ($C_{10}H_{17}N_4$-$Cl_3O_2\cdot 2H_2O$) C, H, N, Cl.

By the methods described above (for the synthesis of **14**−**18**), the following compounds were prepared starting from ethyl 5-formyl-4-nitropyrrole-2-carboxylate[27] (**23**).

Data for Ethyl 5-Formyl-1-methyl-4-nitropyrrole-2-carboxylate (24): pale green solid (89%); mp 70.5−71.5 °C (PhH/petroleum ether); ^{1}H NMR ($CDCl_3$) δ 10.55 (s, 1 H, CHO), 7.49 (s, 1 H, H-3), 4.38 (q, J = 7.2 Hz, 2 H, CH_2CH$_3$), 4.33 (s, 3 H, NCH_3), 1.40 (t, J = 7.2 Hz, 3 H, CH_2CH_3). Anal. ($C_9H_{10}N_2O_5$) C, H, N.

Data for Ethyl 5-(Hydroxymethyl)-1-methyl-4-nitropyrrole-2-carboxylate (25): cream solid (81%); mp 119−120.5 °C (PhH); ^{1}H NMR ($CDCl_3$) δ 7.48 (s, 1 H, H-3), 4.97 (d, J = 6.8 Hz, 2 H, CH_2OH), 4.32 (q, J = 7.1 Hz, 2 H, CH_2CH$_3$), 4.06 (s, 3 H, NCH_3), 2.72 (t, J = 7.1 Hz, 1 H, OH), 1.37 (t, J = 7.1 Hz, 3 H, CH_2CH_3). Anal. ($C_9H_{12}N_2O_5$) C, H, N.

Data for 2-(Hydroxymethyl)-1-methyl-3-nitropyrrole (26): pale yellow solid (54%); mp 63−64 °C (PhH); ^{1}H NMR ($CDCl_3$) δ 6.73 (d, J = 3.4 Hz, 1 H, H-4 or H-5), 6.50 (d, J = 3.4 Hz, 1 H, H-4 or H-5), 4.89 (d, J = 7.2 Hz, 2 H, CH_2OH), 3.73 (s, 3 H, NCH_3), 2.83 (t, J = 7.2 Hz, 1 H, OH). Anal. ($C_6H_8N_2O_3$) C, H, N.

Data for 2-(Chloromethyl)-1-methyl-3-nitropyrrole (27): white flakes (91%); mp 70−71 °C (PhH/petroleum ether); ^{1}H NMR ($CDCl_3$) δ 6.76 (d, J = 3.2 Hz, 1 H, H-4 or H-5), 6.57 (d, J = 3.2 Hz, 1 H, H-4 or H-5), 5.07 (s, 2 H, CH_2Cl), 3.74 (s, 3 H, NCH_3). Anal. ($C_6H_7N_2ClO_2$) C, H, N.

Data for Ethyl 5-(Chloromethyl)-1-methyl-4-nitropyrrole-2-carboxylate (28): cream crystalline solid (83%); mp 92−83 °C (PhH/petroleum ether); ^{1}H NMR ($CDCl_3$) δ 7.50 (s, 1 H, H-3), 5.08 (s, 2 H, CH_2Cl), 4.33 (q, J = 7.1 Hz, 2 H, CH_2-CH_3), 4.06 (s, 3 H, NCH_3), 1.38 (t, J = 7.1 Hz, 3 H, CH_2CH_3). Anal. ($C_9H_{11}N_2ClO_4$) C, H, N.

***N,N*-Bis(2-hydroxyethyl)-*N*-methyl-*N*-[(2-(ethoxycarbonyl)-1-methyl-4-nitro-5-imidazolyl)methyl]ammonium Chloride (29).** Reaction of **28** and *N*-methyldiethanolamine according to the general method above gave **29** as a cream solid (21%): mp 124−126 °C (MeOH/Et_2O); ^{1}H NMR [$(CD_3)_2SO$] δ 7.57 (s, 1 H, H-3), 5.7−4.9 (v br m, 4 H, $ArCH_2N$ and 2 × OH), 4.31 (q, J = 7.0 Hz, 2 H, CH_2CH$_3$), 4.05 (s, 3 H, NCH_3), 3.95−3.75 (m, 6 H, NCH_2CH_2O), 3.55−3.46 (m, 2 H, NCH_2CH_2O), 3.08 (s, 3 H, $^+NCH_3$), 1.32 (t, J = 7.1 Hz, 3 H, CH_2CH_3). Anal. ($C_{14}H_{24}N_3ClO_6$) C, H, N, Cl.

***N,N*-Bis(2-chloroethyl)-*N*-methyl-*N*-[(2-(ethoxycarbonyl)-1-methyl-4-nitro-5-imidazolyl)methyl]ammonium Chloride (30).** Reaction of **29** with $SOCl_2$ according to the general method above gave **30**, which was isolated in an impure state as a white solid (EtOH/Et_2O): ^{1}H NMR [$(CD_3)_2SO$] δ 7.59 (s, 1 H, H-3), 5.6−5.0 (v br m, 2 H, $ArCH_2N$), 4.32 (q, J = 7.1 Hz, 2 H, CH_2CH$_3$), 4.28−3.87 (m, 8 H, NCH_2CH_2Cl), 4.06 (s, 3 H, NCH_3), 3.16 (s, 3 H, $^+NCH_3$), 1.33 (t, J = 7.0 Hz, 3 H, CH_2CH_3).

One-Electron Reduction Potentials. The pulse radiolysis equipment used in these studies has been described.[18] Thermodynamic reversible equilibria were established between the one-electron-reduced compounds and reference viologen standards at pH 7 in deaerated aqueous solutions containing 2-propanol or 2-methyl-2-propanol (0.2 M) and phosphate buffer (10 mM). The equilibrium constants were used to calculate ΔE values and hence $E(1)$ values for the compounds.[28] The following are the reference compounds used and their reduction potentials $E(1)$ (mV): for **4**, duroquinone, −260 ± 10;[28] for **1**, **5**, and **7**, benzyl viologen, −375 ± 10;[28,42] for **2**, **8**, and **6,** methyl viologen, −447 ± 7;[43] for **3** and **9,** triquat, −548 ± 7.[43]

In Vitro Cytotoxicity. Inhibition of proliferation of log-phase cell monolayers was assessed in 96-well plates as described previously,[44] using 4 h exposure to compounds under aerobic conditions followed by sulforhodamine B staining 3 days later. The IC_{50} was determined by interpolation as the drug concentration required to inhibit cell density to 50% of that of thecontrols on the same plate. Cytotoxic potency was compared under aerobic (20% O_2) and hypoxic (<20 ppm O_2) conditions using continuously gassed and stirred supensions of EMT6 cells.[45] Cells for this assay were obtained by enzymatic dissociation of EMT6 spheroids (ca. 800 μm diameter), and cells were plated to determine clonogenicity after exposure to drugs for 3 h. The C_{10} was determined as the interpolated drug concentration required to give 10% cell survival relative to the controls.

Comet Assay for DNA Cross-Linking. A single cell suspension was obtained from two RIF-1 tumors by enzymatic dissociation at 37 °C for 30 min using Pronase (0.5 mg/mL), collagenase (0.2 mg/mL), and DNAase I (0.2 mg/mL). The cells and drug solution were equilibrated under aerobic or hypoxic conditions as for the clonogenicity experiments above. After 1 h of exposure to 3.5 μM **2**, 2×10^5 cells were washed in cold phosphate-buffered saline (PBS) by centrifugation and resuspended in fresh cold PBS at the same density. Aliquots were irradiated on ice (10 Gy, cobalt-60, 1.5 Gy/min), and 0.5 mL samples of irradiated or unirradiated cell suspensions (10^4 cells) were added to 1.5 mL of a 1% solution of low-gelling-temperature agarose (Sigma Type VII), mixed, and pipetted onto a microscope slide. Slides were placed in lysis solution (30 mM sodium hydroxide, 1 M sodium chloride, 0.1% *N*-lauroylsarcosine) for 1 h and then washed for 3×20 min in rinse solution (30 mM sodium hydroxide, 2 mM EDTA), followed by electrophoresis at 0.5 V/cm for 25 min in a fresh solution of 30 mM sodium hydroxide and 2 mM EDTA. Slides were rinsed with distilled water for 15 min and then stained with propidium iodide (2.5 μg/mL) for 15 min. The DNA from individual cells was examined using a Nikon epifluorescence microscope with a 10× objective, attached to a JVC TK-C1360 digital $^1/_2$ in. CCD color video camera and image analysis system using VComet software (Digital Science Consulting, Auckland). DNA damage was quantitated as the tail moment, the product of the percentage of DNA in the tail and the distance between the means of the head and tail distributions. DNA cross-links were detected as inhibition of radiation-induced single-strand breaks.

Toxicity and Antitumor Activity in Mice. Nitroarylmethyl quaternary nitrogen mustards, mechlorethamine, porfiromycin, AQ4N, and cisplatin were formulated in 0.9% saline, tirapazamine was formulated in 10% DMSO/saline, and CI-1010 was formulated in 0.05 M lactate buffer, pH 4. The compounds were administered as single ip doses at 0.01 mL/g of body weight. The toxicity in nontumor-bearing mice was determined using dose increments of 1.33-fold on a fixed scale with a 28 day observation time. The MTD was defined as the highest dose causing no deaths in 6 mice and <15% mean body weight loss on day 5.

RIF-1 cells were maintained by alternating in vivo and in vitro passage as described.[46] Tumors were grown by injecting 10^5 cells im in 20 μL into the gastrocnemius muscle. Mice were individually ear-tagged and randomized to treatment groups when the tumor size reached 0.5 g (leg plus tumor diameter 10 mm). For clonogenic assays, mice received drugs with or without whole body cobalt-60 radiation (2.5 Gy/min) using the experimental design illustrated in Figure 3B. Each of the compounds **1**−**9** was tested either in one experiment with five mice per group or in two experiments with three mice per group, which were then pooled. Tumors were removed by dissection 18 h after treatment, and dissociated enzymatically to assess the number of clonogenic cells as described previously.[47] For tumor growth delay assays, tumors were irradiated by restraining unanesthetized mice in jigs allowing local irradiation of the leg (cobalt-60, 1.7 Gy/min). Leg diameters were measured three times per week until the end point (13 mm leg diameter corresponding to ca. 1.2 g of tumor). The growth delay was calculated as the difference in time to the end point for treated versus control groups. The statistical significance of drug effects was evaluated using the log of clonogens per gram of tumor for the clonogenic assay, and the time to the end point for the growth delay assay, by ANOVA followed by Dunnett's test to determine *p* values for individual groups.

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