well) for 10 min, then stained with a solution of 0.1% crystal violet (250 μL/well) for 20 min. The medium was decanted, and the plates were gently rinsed with water and air-dried. The cells were counted under an inverted phase-contrast microscope. The surviving fractions were calculated, dose–response curves constructed, and the IC_{50} values (the drug concentrations that inhibited colony formation by 50%) determined. Each value represents the average of duplicate determinations.

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Hypoxia-Selective Antitumor Agents. 5. Synthesis of Water-Soluble Nitroaniline Mustards with Selective Cytotoxicity for Hypoxic Mammalian Cells

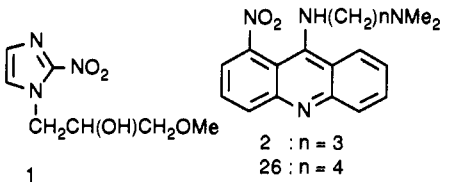
Brian D. Palmer, William R. Wilson,† Stephen Cliffe,† and William A. Denny*

Cancer Research Laboratory and Section of Oncology, Department of Pathology, University of Auckland School of Medicine, Private Bag, Auckland, New Zealand. *Received December 26, 1991*

Nitroaniline mustards have potential as hypoxia-selective cytotoxic agents, with reductive metabolism activating the nitrogen mustard by converting the electron-withdrawing nitro group to an electron-donating hydroxylamine or amine. However, the parent compounds have poor aqueous solubility, and their potencies are limited by low reduction potentials ($E_{1/2}$ ca. −600 mV versus the normal hydrogen electrode) and corresponding slow rates of nitro reduction. To address these limitations, a series of 4-nitroaniline mustards bearing hydrophilic side chains attached via an electron-withdrawing carboxamide group was prepared and evaluated for hypoxia-selective cytotoxicity against Chinese hamster cell lines. The *N*-[(*N*,*N*-dimethylamino)ethyl]carboxamide derivatives proved to have excellent aqueous solubility and improved cytotoxic potency, but their reduction potentials, while higher than the non-carboxamide compounds, were still low and little selectivity for hypoxic cells were observed. A series of carboxamides of 2,4-dinitroaniline mustard was also prepared. These compounds had reduction potentials in the desired range ($E_{1/2}$ ca. −450 mV by cyclic voltammetry) and were more toxic to hypoxic than aerobic UV4 cells. The most selective compounds were 5-[*N*,*N*-bis(2-chloroethyl)amino]-2,4-dinitrobenzamide (**20**, SN 23862) and its water-soluble *N*-[(*N*,*N*-dimethylamino)ethyl]carboxamide analogue. These showed selectivities of 60- to 70-fold for hypoxic UV4 cells. The selectivity of **20** was much superior to that of its aziridine analogue (**23**, CB 1954), which was only 3.6-fold more toxic to hypoxic than oxic cells in the same system. Compound **20** is a much less efficient substrate than CB 1954 for the major aerobic nitroreductase from rat Walker tumor cells, NAD(P)H:quinone oxidoreductase (DT diaphorase). Lack of aerobic bioactivation of **20** by DT diaphorases may be responsible for its higher hypoxic selectivity than that of **23**.

Solid tumors contain a proportion of cells which are either transiently or chronically hypoxic.[1,2] Because of their low proliferative activity[3] and inaccessibility to blood-borne drugs,[4,5] these cells represent a potential clinical problem in the chemotherapy of solid tumors. At the same time, the hypoxic microenvironment offers an attractive target, since nearly all normal tissues are well-perfused, and drugs activated only in hypoxic regions (hypoxia-selective cytotoxins, HSCs) may be truly specific for solid tumors.

The nitro aromatics most thoroughly-studied as HSCs are 2-nitroimidazoles, such as misonidazole (1), which undergo metabolic one-electron reduction of the nitro group to the radical anion. In well-oxygenated cells this transformation is efficiently reversed by oxygen, but in hypoxic cells the radical anion is reduced further to form reactive, cytotoxic products.[6] While showing significant hypoxic selectivity, these compounds are not very potent, and it has proven difficult to achieve sufficiently high concentrations in tumors to show therapeutic benefit in vivo, except with 2-nitroimidazoles carrying additional DNA-reactive alkylating functionality.[7,8] While we[9,10] and others[11] have shown that DNA-affinic nitro aromatics (e.g., nitracrine, 2) show greatly-increased potency as HSCs, the high DNA binding affinity of such compounds appears to hinder their extravascular diffusion.[4,12]



1

2 : n = 3
26 : n = 4

As an alternative approach to the design of nitro aromatics as potent HSCs, we have discussed in detail[13] the

† Section of Oncology, Department of Pathology.

(1) Urtasun, R. C.; Chapman, J. D.; Raleigh, J. A.; Franko, A. J.; Koch, C. J. Binding of ^{3}H-misonidazole to solid human tumors as a measure of tumor hypoxia. *Int. J. Radiat. Oncol. Biol. Phys.* **1986**, *12*, 1263–1267.
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(5) Jain, R. K. Delivery of novel therapeutic agents in tumors: physiological barriers and strategies. *J. Natl. Cancer Inst.* **1989**, *81*, 570–576.
(6) Rauth, A. M. Pharmacology and toxicology of sensitizers: mechanism studies. *Int. J. Radiat. Oncol. Biol. Phys.* **1984**, *10*, 1293–1300.
(7) Adams, G. E.; Stratford, I. J. Hypoxia-mediated nitroheterocyclic drugs in the radio- and chemotherapy of cancer. *Biochem. Pharmacol.* **1986**, *35*, 71–76.

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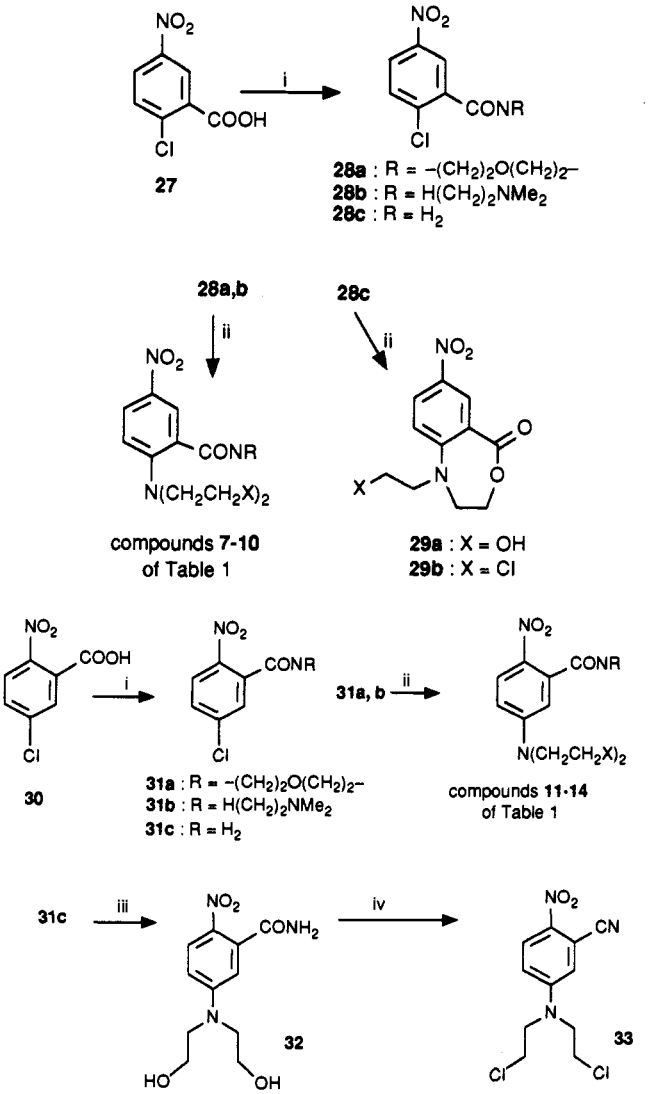
design of a novel class of compounds, nitroaniline *N*-mustards, where 4- or 6-electron reduction of the nitro group triggers increased electron release to the nitrogen of the mustard moiety, greatly increasing its reactivity. We have reported[14] the in vitro cytotoxicity of a series of substituted aniline mustards, in which biological half-lives and aerobic cytotoxicities were dominated (as expected) by the electronic properties of the substituents. *N,N*-Bis(2-chloroethyl)-4-nitroaniline (**4**) was 1800-fold less cytotoxic than its potential reduction product, the 4-amino compound. Differential reduction of the nitro group of **4** in hypoxic cells was implied by the demonstration of hypoxia-selective cytotoxicity by this compound, although the observed selectivity was trivial (1.6-fold) in comparison with the large differential cytotoxicity between the nitro and amino compounds noted above.

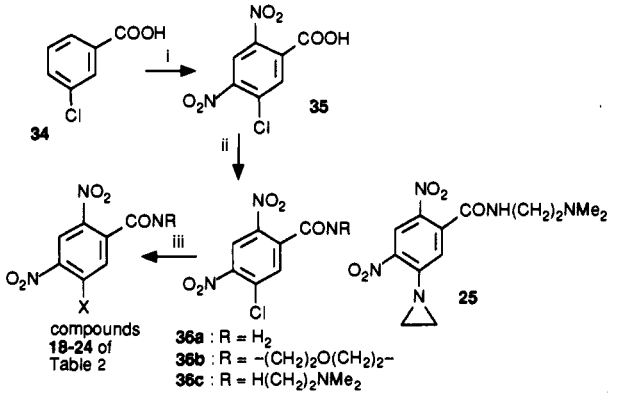
The inefficient hypoxic bioactivation of **4** was attributed[14] to its low reduction potential ($E_{1/2}$ = ca. −620 mV vs the normal hydrogen electrode, NHE). The limited aqueous solubility of the nitroaniline mustards also made testing difficult. Nevertheless, the results constituted the first experimental evidence for suggestions[13,15,16] that use of cellular nitroreduction to activate a nitrogen mustard could provide alkylating agents with hypoxia-selective cytotoxicity. To improve on these modest results, it will be necessary to increase both aqueous solubility and the rate of nitro reduction. A small series of nitroaniline mustards related to chlorambucil provided enhanced solubility but had very low reduction potentials and did not provide useful hypoxic selectivity.[17] In the present paper we address the requirements for higher solubility and rates of reduction through the synthesis and evaluation of a number of substituted mono- and dinitroaniline mustards bearing water-solubilizing side chains attached to the benzene ring via an electron-withdrawing carboxamide group.

Scheme I[a]



[a] (i) $SOCl_2$ or 1,1′-carbonyldiimidazole; RNH_2 or R_2NH; (ii) diethanolamine/Δ; $SOCl_2$/1,2-dichloroethane; (iii) diethanolamine/Δ; (iv) $SOCl_2$/1,2-dichloroethane.

Scheme II[a]



[a] (i) fuming HNO_3/concentrated H_2SO_4; (ii) $SOCl_2$ or 1,1′-carbonyldiimidazole; RNH_2 or R_2NH; (iii) diethanolamine/Δ; $SOCl_2$/1,2-dichloroethane.

Chemistry

Preparations of the simple nitroaniline compounds (**3** and **4**) of Table I have been reported.[14] Synthesis of the

(8) Cole, S.; Stratford, I. J.; Adams, G. E.; Fielden, E. M.; Jenkins, T. C. Dual-function 2-nitroimidazoles of hypoxic cell radiosensitizers and bioreductive cytotoxins: in vivo evaluation in KHT murine sarcomas. *Radiat. Res.* **1990**, *124*, S38–S43.
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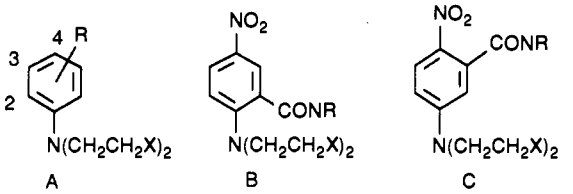
(17) Palmer, B. D.; Wilson, W. R.; Denny, W. A. Nitro analogues of chlorambucil as potential hypoxia-selective anti-tumour drugs. *Anti-cancer Drug Design* **1990**, *5*, 337–349.

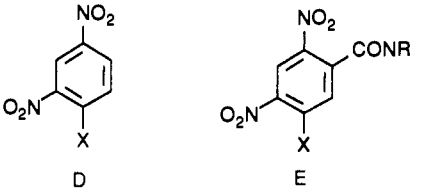
Table I. Physicochemical and Biological Properties of Mononitroaniline Mustards



no.	form.	X	R	R_m[a]	$E_{1/2}$[b] (mV)	IC_{50}[c] (μM) AA8	IC_{50}[c] (μM) UV4	HF[d]
3	A	OH	4-NO_2	0.21	−620	3200 ± 180[e]	2950	1.1 ± 0.2
4	A	Cl	4-NO_2	0.87		102 ± 24	17 ± 3	12
5	A	Cl	3-$CONH(CH_2)_2NMe_2$			11 ± 2	0.27 ± 0.03	44 ± 7
6	A	Cl	3-$CONMe_2$			12 ± 3	1.2 ± 0.4	29 ± 9
7	B	OH	$-(CH_2)_2O(CH_2)_2-$	−0.05	−560	10400 ± 1500	8400 ± 2700	1.3 ± 0.1
8	B	OH	$H(CH_2)_2NMe_2$	−0.65	−490	6700 ± 1500	4000 ± 300	1.1 ± 0.1
9	B	Cl	$-(CH_2)_2O(CH_2)_2-$	0.65		>500	68 ± 9	>7
10	B	Cl	$H(CH_2)_2NMe_2$	−0.15		208 ± 20	5.7 ± 0.6	40 ± 8
11	C	OH	$-(CH_2)_2O(CH_2)_2-$	−0.14	−620	11200 ± 900	9200 ± 1600	1.0 ± 0.2
12	C	OH	$H(CH_2)_2NMe_2$	−1.09	−570	13200 ± 400	8000 ± 800	1.6 ± 0.1
13	C	Cl	$-(CH_2)_2O(CH_2)_2-$	0.54		243 ± 57	15 ± 2	19 ± 4
14	C	Cl	$H(CH_2)_2NMe_2$	−0.24		1140 ± 290	72 ± 28	12 ± 3

[a] R_m = chromatographic measure of drug lipophilicity, measured as detailed in ref 22. [b] $E_{1/2}$: redox potential in millivolts, versus the normal hydrogen electrode, measured by cyclic voltammetry as described in the text. [c] IC_{50} determined against aerobic AA8 or UV4 cells as described in the text, using an exposure time of 18 h. [d] Hypersensitivity factor = IC_{50}(AA8)/IC_{50}(UV4), using an 18 h drug exposure under aerobic conditions. Values are intraexperiment ratios and are therefore not identical to the ratio of the preceding two columns, which include data from experiments with one cell line only. [e] Values are mean ± SEM for 3–6 independent experiments.

Table II. Physicochemical and Biological Properties of Dinitroaniline Mustards and Aziridines



no.	form.	X	R	R_m[a]	$E_{1/2}$[b] (mV)	IC_{50}[c] (μM) AA8	IC_{50}[c] (μM) UV4	HF[d]
15	D	diol		0.22	−450	295 ± 60[e]	280 ± 90	0.9 ± 0.1
16	D	must				>50[f]	44 ± 6	>1
17	E	diol	H_2	0.41		6200 ± 1000	5900 ± 800	1.1 ± 0.1
18	E	diol	$-(CH_2)_2O(CH_2)_2-$	0.12	−420	2950	2600	1.1
19	E	diol	$H(CH_2)_2NMe_2$	−0.76	−460	4110 ± 180	3100 ± 200	1.4 ± 0.1
20	E	must	H_2	−0.28		470 ± 50	108 ± 22	6.8 ± 1.1
21	E	must	$-(CH_2)_2O(CH_2)_2-$	0.50		260 ± 70	12 ± 3	23 ± 5
22	E	must	$H(CH_2)_2NMe_2$	−0.17		260 ± 26	68 ± 9	4.2 ± 0.8
23	E	azir	H_2	−0.36	−430	560 ± 50	11.7 ± 1.4	50 ± 11
24	E	azir	$-(CH_2)_2O(CH_2)_2-$		−400	690 ± 120	2.7 ± 0.7	330 ± 110

[a-e] As for Table I. [f] Compound too insoluble for accurate determination. IC_{50} for UV4 = 44 ± 6 μM.

mononitrocarboxamide mustards of Table I was achieved from the mononitrochloro acids (**27** and **30**) (Scheme I). Amide formation was followed by chloride displacement with diethanolamine to give the diols, which were converted into the mustards either directly with $SOCl_2$ or via the dimesylates (see Experimental Section). The mononitro amides **28** and **31** were not sufficiently reactive to undergo direct displacement of the chlorine with *N,N*-bis(2-chloroethyl)amine. The strongly-activated chloro groups of the amides **36** (prepared from 5-chloro-2,4-dinitrobenzoic acid[18,19] (**35**)) could be displaced with either diethanolamine, aziridine, or *N,N*-bis(2-chloroethyl)amine to give directly the diols, aziridines, and mustards of Table II in good yields (Scheme II).

Measurement of Physicochemical Properties. The most biologically relevant measure of nitro group reduction potential is that for the thermodynamically-reversible addition of the first electron, $E(1)$, as measured by pulse radiolysis.[20] The one-electron reduction species, the radical anion, is a key intermediate, since its reoxidation by molecular oxygen is responsible for inhibition of net nitro reduction under aerobic conditions. Although half-wave reduction potentials ($E_{1/2}$ values) determined in aqueous solution by cyclic voltammetry or polarography are not usually reversible reactions, and for nitro aromatics may involve the addition of up to four electrons, addition

(18) Goldstein, H.; Stamm, R. *Helv. Chim. Acta* **1952**, *35*, 1330–1332.

(19) We note that under IUPAC rules, **35** is named 3-chloro-4,6-dinitrobenzoic acid rather than 5-chloro-2,4-dinitrobenzoic acid. However, to be consistent with previous literature on the related compound CB 1954 (**23**), we have chosen to use the former numbering for **35** and its analogues in this paper.

(20) Wardman, P.; Clarke, E. D. One-electron reduction potentials of substituted nitroimidazoles measured by pulse radiolysis. *J. Chem. Soc., Faraday Trans. 1* **1976**, 1377–1390.

of the first is usually the most difficult.[21] We have therefore used $E_{1/2}$ values (determined by cyclic voltammetry against the normal hydrogen electrode) as an approximation of $E(1)$ values and as a relative measure of ease of reduction of the nitro group.

Relative lipophilicities were measured by liquid–liquid thin-layer chromatography at pH 1–2 as previously described[22] and are recorded in Tables I and II as R_m values relative to 4′-(9-acridinylamino)methanesulfonanilide as internal standard.

Measurement of Selective Cytotoxicity. Aerobic cytotoxic potencies of the nitro aromatic compounds were initially assessed in a growth inhibition assay, using cell cultures of the Chinese hamster ovary fibroblast lines AA8 and UV4. The latter is a UV complementation group 1 mutant derived from AA8 with a defect in the incision step of excision repair[23,24] and is highly sensitive to agents which form bulky DNA monoadducts or cross-links.[25] The ratio of the IC_{50}s in these two lines, termed the hypersensitivity factor HF (HF = IC_{50}(AA8)/IC_{50}(UV4)), therefore provides a guide to mode of action. Interstrand cross-linking agents show hypersensitivity factors in the range 8–200-fold.[25] We have previously shown[10] the utility of these cell lines in determining the nature of DNA adducts formed by nitro aromatic drugs, and the assay used has been described in detail.[26] The ability of drugs to inhibit cell growth was evaluated after an 18-h exposure of log-phase monolayers in 96-well microtiter trays under aerobic conditions. Drugs were then washed out, the cultures were grown for a further 78 h, and cell numbers were determined by staining with methylene blue.[27] The IC_{50} was determined as the drug concentration required to reduce cell density to 50% of the controls.

The selectivity of compounds for hypoxic cells was assessed using aerobic and hypoxic stirred suspensions of late log phase UV4 cells, gassed continuously with 5% CO_2 in air or N_2, with measurement of cell killing by clonogenic assay as described previously.[26] This assay provides essentially complete anoxia throughout the period of drug exposure (up to 8 h) in the N_2-equilibrated cultures.[28] The concentration times time to reduce the surviving fraction to 10% (CT_{10}) was determined and used as an inverse measure of cytotoxic potency and the ratio of CT_{10} values under aerobic and hypoxic conditions as a measure of hypoxic selectivity.

Results and Discussion

Table I gives data on the lipophilicity, reduction potentials, and cytotoxic potency of the set of mononitroaniline mustards and their diol precursors. The very lipophilic nature of the mustard group is reflected in the difference in the chromatographic parameter R_m for the simple mustard 4 and the corresponding diol 3. The difference of 0.66 R_m units corresponds to a log P[29] difference of approximately 2.5 units,[30] and this is reflected in their widely-differing aqueous solubilities. While the diol 3 is soluble in water to >10 mM, the solubility limit of the mustard 4 is only 20 μM. Sufficient solubility for testing (ca. 120 μM) could just be achieved by dissolving it in acetone and diluting into tissue culture medium (Alpha MEM plus 10% fetal calf serum); the increase in solubility appears to be due to binding to serum proteins.

An $E_{1/2}$ value could not be obtained for 4 due to insolubility. However, although no reliable σ values are available for the $N(CH_2CH_2Cl)_2$ group, it is considered[13] to have similar electronic properties to the $N(CH_2CH_2OH)_2$ substituent, and thus it is reasonable to assume the simple mustards will have reduction potentials similar to those of their corresponding diols.

To provide compounds with both improved water-solubility and higher reduction potentials, the addition of hydrophilic side chains to the phenyl ring of 4 via a suitable electron-withdrawing link group was explored. The carboxamide moiety was selected as the link group because of its powerful yet position-independent electron-withdrawing capacity (σ_m = 0.35, σ_p = 0.36), its high hydrophilicity,[29] and its synthetic accessibility. Three hydrophilic side chains of differing chemistry and physicochemical properties were used: the primary carboxamide, $CONH_2$; the secondary *N*-[(*N*,*N*-dimethylamino)ethyl]-carboxamide, $CONH(CH_2)_2NMe_2$; and the tertiary *N*-morpholinocarboxamide, $CON(CH_2CH_2)_2O$.

The cationic (*N*,*N*-dimethylamino)ethyl side chain (pK_a ca. 9.9) was expected to have a large solubilizing effect, lowering log P by 2–3 units.[29] A basic side chain should also increase cellular uptake of the mustard because of the lower intracellular than extracellular pH under typical tissue culture conditions[14] and possibly also target it to DNA. Either process would be expected to increase absolute drug potency, and indeed the model compound 5 was found to have an IC_{50} of 0.27 μM against UV4 (Table I), considerably greater than that of the isoelectronic but neutral carboxamido mustard 6[14] (1.2 μM) or that calculated for 5 (2.3 μM) from eq 1. This equation was derived

$$\log (IC_{50}) = 2.46\sigma - 0.53 \quad (1)$$

previously (eq 7 of ref 14) to relate the cytotoxicity of substituted aniline mustards to the electronic effects of the substituent groups, where IC_{50} is the inhibitory potency against UV4 cells, measured as in the present study. The enhancement in potency is similar to that achieved by use of basic side chain functionality in the 2-nitroimidazole radiosensitizers.[31]

(21) Page, J. E.; Smith, J. W.; Waller, J. G. Polarographic behaviour of aromatic nitro compounds. *J. Phys. Colloid Chem.* **1949**, *53*, 545–560.

(22) Denny, W. A.; Cain, B. F. Potential Antitumor Agents. 27. QSAR for the 4′-((9-acridinylamino)phenyl)alkanoic acids. *J. Med. Chem.* **1978**, *21*, 430–436.

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(28) Whillans, D. W.; Rauth, A. M. An experimental and analytical study of oxygen depletion in stirred cell suspensions. *Radiat. Res.* **1980**, *34*, 97–103.

(29) Hansch, C.; Leo, A. *Substituent Constants for Correlation Analysis in Chemistry and Biology*; Wiley and Sons Ltd.: New York, 1979.

(30) Cain, B. F.; Atwell, G. J.; Denny, W. A. Potential Antitumor Agents. 17. 9-Anilino-10-methylacridinium salts. *J. Med. Chem.* **1976**, *19*, 772–777.

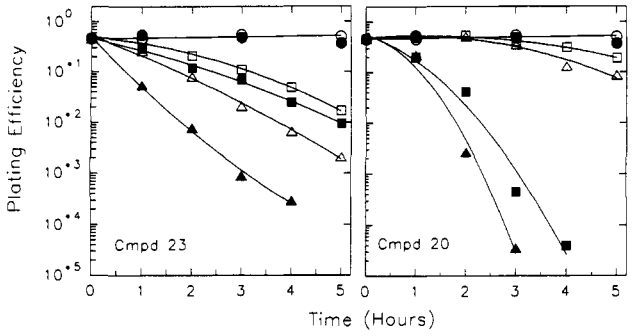

Figure 1. Plating efficiency of UV4 cells incubated with compounds **23** (CB 1954) and **20** (SN 23862) under aerobic (open symbols) or hypoxic (filled symbols) conditions in stirred suspension cultures. Controls: aerobic (○), hypoxic (●). Drug treated: 240 μM aerobic (□), 400 μM aerobic (△), 80 μM hypoxic (■), 160 μM hypoxic (▲).

The largest differences in Hammett σ values between nitro and reduced forms (and therefore the highest differential cytotoxicity[13]) occurs for compounds with an ortho or para relationship between the nitro and mustard groups, and experimental studies[14] showed that the 4-nitroaniline mustard **4** showed greater hypoxic selectivity than its 2- or 3-nitro isomers. In the present work we therefore retained this basic configuration, focusing on derivatives of **4**. The carboxamide side chain can be placed in one of two different positions (ortho or meta to the mustard), and both of these configurations were explored. In both series, unexpected chemistry of the primary $CONH_2$ side chain precluded the preparation of this example. In the ortho series, reaction of the appropriate chloro compound (**28c**) with diethanolamine gave the *N*-hydroxyethyl lactone **29a** as sole product, and in the meta series all attempts to convert the primary amide diol **32** to the corresponding mustard resulted in dehydration to the nitrile **33**. Thus in each case only the (*N*,*N*-dimethylamino)ethyl and morpholide compounds could be prepared.

The carboxamido diol **8** had an $E_{1/2}$ of −490 mV, while that of the isomeric carboxamide **12** was lower, at −570 mV (Table I). In each case, the corresponding morpholides (**7** and **11**) had slightly lower values. By comparison with the reduction potential of the parent diol **3**, it appears that the carboxamido sidechain has a lesser influence on reduction potential when placed at the 3-position (compounds **11** and **12**) than when at the 2-position (compounds **7** and **8**). However, the measured $E_{1/2}$ value (−490 mV) suggests that even **8**, the most easily-reduced compound, has a reduction potential which is probably still too low for efficient bioreduction.[14]

The diols **7**, **8**, **11**, and **12** were weakly cytotoxic, with low HFs against UV4 as expected, since these compounds cannot cross-link DNA. Conversion to the corresponding mustards (**9**, **10**, **13**, **14**) generally provided a large increase in cytotoxicity, although the ortho morpholide **9** proved too insoluble to be evaluated in the AA8 cell line. High HFs (ranging from 11- to 50-fold) against UV4 suggest DNA cross-linking to be the critical toxic event with these compounds. The high potency and enhanced UV4 sensitivity relative to the diol precursors strongly suggests that the toxicity of these nitrobenzamide mustards is due to the nitrogen mustard rather than to the reduction of the nitro group per se. In addition, the ca. 100-fold decrease in aerobic toxicity caused by the 4-NO_2 group (compare **5** and **14**) indicates the potential for a large increase in activity on reduction of the nitro group to an electron-donating moiety.

Table III. Hypoxic Selectivity of Substituted 4-Nitroaniline Mustards in Stirred Suspension Cultures of UV4

no.	CT_{10}[a] (air) (μM/h)	CT_{10} ratio[b] (air/N_2)	no.	CT_{10}[a] (air) (μM/h)	CT_{10} ratio[b] (air/N_2)
4[c]	760	1.6	**17**	>35000[d]	
9	2325	1.15	**20**	2700	60
10	78	1.5	**21**	156	1.3
12	11300	0.95	**22**	1080	70
13	450	1.00	**23**	960	3.6
14	465	2.3	**24**	50	1.7

[a] CT_{10}: the product of the drug concentration (μM) and exposure time (h) needed to reduce cell survival to 10% of controls, using UV4 cells at 10^6/mL in the clonogenic assay (see text). [b] CT_{10} ratio = CT_{10}(air)/CT_{10}(nitrogen). [c] Value taken from ref 14. [d] Nontoxic at the solubility limit (7000 μM) under aerobic or hypoxic conditions.

Rates of killing by the mononitrobenzamide mustards under aerobic and hypoxic conditions were determined in stirred UV4 cultures (see Figure 1 for examples) and CT_{10} values were compared with **4** which we previously reported[14] to be weakly selective in the same assay (Table III). Oxygen had no effect on the rate of killing by the morpholide **9** or its more potent isomer **13**. The corresponding *N*-[(*N*,*N*-dimethylamino)ethyl]carboxamides **10** and **14** were of greater interest by virtue of their higher aqueous solubilities and higher estimated reduction potentials. Both showed slight hypoxic selectivity, with CT_{10} ratios of 1.5 and 2.3, respectively. The diol corresponding to **14**, compound **12**, was sufficiently soluble to evaluate in this assay despite its low potency but showed no hypoxic selectivity. The low hypoxic selectivities of these compounds may reflect reduction potentials which are too low for efficient bioactivation even in the absence of oxygen.

In an attempt to raise reduction potentials, a series of related dinitro carboxamide mustards was also evaluated (Table II). The dinitro diol **15** had an $E_{1/2}$ 170 mV more positive than that of the mononitro diol **3**, showing the significant effect of the additional nitro group in increasing the reduction potential. Compound **15** was 1 order of magnitude more cytotoxic than **3** against the AA8 and UV4 cell lines, suggesting that the toxicity of these compounds is mediated through nitroreduction. However, cell killing is probably due to one-electron redox cycling rather than DNA alkylation, since neither hypoxic selectivity (data not shown) nor UV4 hypersensitivity was seen. The corresponding mustard **16** was too insoluble to evaluate in AA8 cells but did show some activity against UV4 cells in the growth inhibition assay when tested near the solubility limit of 50 μM, indicating an HF value greater than one.

The three side chain-bearing dinitro carboxamide diols (**17**–**19**) were surprisingly nontoxic compounds with low HFs, but did have nitro group $E_{1/2}$ values of ca. −460 mV, which are in the desired range.[13] The corresponding dinitro mustards (**20**–**22**) were at least 10-fold more potent in the AA8 cell line and were 50–200-fold more potent than the diols in the UV4 line. The morpholide **21** showed a hypoxia selectivity of only 1.3-fold, but the other dinitro carboxamide mustards (**20** and **22**) showed very high selectivities for hypoxic cells (Table III and Figures 1 and 2).

Compound **20** (SN 23862) is the nitrogen mustard analogue of the (dinitroaziridinyl)benzamide CB 1954 (**23**), which first attracted interest because of its exceptional therapeutic activity against the Walker carcinoma in rats[32]

(31) Adams, G. E.; Ahmed, I.; Clarke, E. D.; O'Neill, P.; Parrick, J.; Stratford, I. J.; Wallace, R. G.; Wardman, P.; Watts, M. E. Structure–activity relationships in the development of hypoxic cell radiosensitisers. *Int. J. Radiat. Oncol.* **1980**, *38*, 613–626.

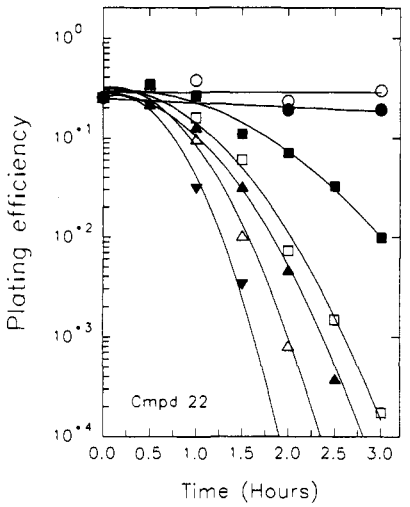

Figure 2. Plating efficiency of UV4 cells incubated with compound **22** in stirred suspension cultures. Controls: aerobic (○), hypoxic (●). Drug treated: 600 μM aerobic (□), 900 μM aerobic (△), 5 μM hypoxic (■), 10 μM hypoxic (▲), 15 μM hypoxic (▼).

but which is also known to be selectively toxic to hypoxic V79 cells in culture.[33] This compound has an $E(1)$ of −385 mV as measured directly by pulse radiolysis,[33] and its reductive bioactivation is considered to result from the generation of a DNA cross-linking agent,[34-36] with reduction of the 4-NO_2 group to the hydroxylamine providing a second reactive center (after acylation[37]) to convert the monofunctional aziridine into a much more toxic bifunctional alkylating agent.[36] Nitro reduction is expected to enhance the reactivity of the aziridine,[38] and this could also contribute to bioactivation, essentially as proposed for **20**.

A direct comparison of the hypoxic selectivies of **20** and **23** was made in the same experiment (Figure 1). The selectivity of **23**, assessed by comparing the CT_{10} values at similar rates of killing (i.e. average of 240 and 400 μM aerobic compared with 80 μM hypoxic), was only 3.6-fold (Table III). A similarly low selectivity was also observed for the morpholino analogue **24** (Table III). At the same concentrations used for **23**, the mustard derivative **20** was clearly much more selective, showing higher potency under hypoxic conditions and lower potency under aerobic conditions than **23**. Further studies with concentrations up to the solubility limit of 1600 μM (data not shown) indicate a selectivity of approximately 60-fold (Table III). The excellent selectivity of **20** was retained in the much more water-soluble *N*-[(*N*,*N*-dimethylamino)ethyl]carboxamide **22**, which showed a rate of killing at 10 μM under hypoxia which was intermediate between that at 600 and 900 μM under aerobic conditions, indicating a 70-fold hypoxic differential (Figure 2 and Table III). Recent studies in this laboratory (Wilson and Pullen, unpublished results) indicate that the hypoxic selectivities of **20** and **22** are much superior to those of **23** in all cell lines tested (AA8, EMT6 mouse mammary carcinoma, FME human melanoma, and Walker rat carcinoma). The (dimethylamino)ethyl analogue **25** was also prepared, but unexpectedly proved too unstable to be evaluated biologically, decomposing rapidly at room temperature.

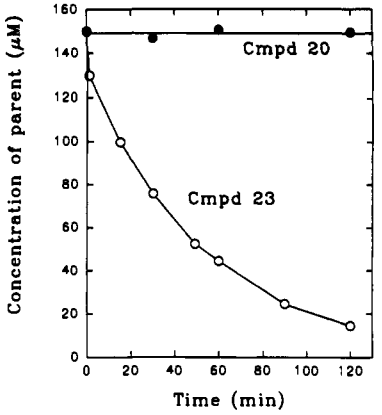

Figure 3. Reduction of compounds **23** and **20** by NADPH (1 mM) catalysed by purified rat (Walker cell) DT diaphorase (100 μg/mL) under aerobic conditions.

Reasons for the greater hypoxic selectivity of **20** and **22** than the aziridine **23** are not yet clear. Comparison of the measured $E_{1/2}$ values of the morpholino derivatives **18** and **24** suggests a slightly lower one-electron redox potential for the aziridine compound compared to the bis(2-hydroxyethyl) derivative, as expected from the respective electron-donating capacities of these two groups.[13] However, on this basis the reduction potential of **20** is expected to be little different from that of **23**, and recent experiments (Thompson and Wilson, unpublished results) show a similar rate of reduction of both compounds in hypoxic UV4 cells.

Compound **23** is known to be bioactivated under aerobic conditions by NAD(P)H:quinone oxidoreductase (DT diaphorase) which can act as an oxygen-insensitive nitroreductase.[36,39] The abilities of **23** and **20** to act as substrates for purified DT diaphorase from rat Walker cells were compared (Figure 3). The rate of reduction of **20** was <1% of that of **23**, suggesting that the high hypoxic selectivity of the former compound in UV4 cells may be due to its insensitivity to aerobic reduction by DT diaphorases which are known to be present in this cell line.[40]

(32) Cobb, L. M.; Connors, T. A.; Elsan, L. A.; Khan, A. H.; Mitchley, B. C. V.; Ross, W. C. J.; Whisson, M. E. 2,4-Dinitro-5-ethyleneiminobenzamide (CB 1954): a potent and selective inhibitor of the growth of the Walker carcinoma 256. *Biochem. Pharmacol.* **1969**, *18*, 1519–1527.

(33) Stratford, I. J.; Williamson, C.; Hoe, S.; Adams, G. E. Radiosensitizing and cytotoxicity studies with CB 1954 (2,4-dinitro-5-aziridinylbenzamide). *Radiation Res.* **1981**, *88*, 502–509.

(34) Venitt, S. The differential response of resistant and sensitive strains of *Escherichia coli* to the cytotoxic effects of 5-aziridino-2,4-dinitrobenzamide (CB 1954). *Chem.-Biol. Int.* **1971**, *3*, 177–191.

(35) Roberts, J. J.; Friedlos, F.; Knox, R. J. CB 1954 (2,4-dinitro-5-aziridinylbenzamide) becomes a DNA interstrand crosslinking agent in Walker tumour cells. *Biochem. Biophys. Res. Commun.* **1986**, *140*, 1073–1078.

(36) Knox, R. J.; Friedlos, F.; Jarman, M.; Roberts, J. J. A new cytotoxic DNA interstrand crosslinking agent agent, 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide, is formed from 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB 1954) by a nitroreductase enzyme in Walker carcinoma cells. *Biochem. Pharmacol.* **1988**, *37*, 4661–4667.

(37) Knox, R. J.; Friedlos, F.; Marchbank, T.; Roberts, J. J. The bioactivation of CB 1954: Reaction of the active 4-hydroxylamino derivative with thioesters to form the ultimate DNA-DNA interstrand crosslinking species. *Biochem. Pharmacol.* **1991**, *42*, 1691–1697.

(38) Lewis, D. F. V. Molecular orbital calculations on tumour-inhibitory phenyl aziridines: QSARs. *Xenobiotica* **1989**, *19*, 241–356.

(39) Knox, R. J.; Boland, M. P.; Friedlos, F.; Coles, B.; Southan, C.; Roberts, J. J. The nitroreductase enzyme in Walker cells that activates 5-(aziridin-1-yl)-2,4-dinitrobenzamide (CB 1954) to 5-(aziridin-1-yl)-4-hydroxylamino-2-nitrobenzamide is a form of NAD(P)H dehydrogenase (quinone) (EC 1. 6.99.2). *Biochem. Pharmacol.* **1988**, *37*, 4671–4677.

Preliminary studies suggest that 21 is also a poor substrate for Walker cell DT diaphorase, indicating that DT diaphorase sensitivity may not be the only determinant of hypoxic selectivity in this series. Reasons for the lack of hypoxic selectivity of 21 require further investigation.

The marked hypersensitivity of UV4 to 23 (Table II) provides further evidence[34-36] that the cytotoxicity of this compound is due to DNA cross-linking. The morpholide analogue 24 shows an even higher HF ratio than 23, again suggesting that DNA cross-linking is the predominant mode of toxicity. It is of interest that the dinitro carboxamide mustards show lower HF values than the corresponding aziridines. The two nitrogen mustards with greatest hypoxic selectivity (20 and 22) have HF values under aerobic conditions which are below the range usually considered diagnostic of DNA interstrand cross-linking.[25] This may indicate that mechanisms of toxicity other than DNA alkylation contribute under aerobic conditions. Further studies will be required to determine to what extent the cytotoxicity of these promising new bioreductive agents is due to DNA cross-linking under either aerobic or hypoxic conditions.

Most bioreductive agents give rise to proximal cytotoxins which are either short-lived radical species or very reactive electrophiles, expected to have short diffusion ranges and to kill only those cells in which they are generated. In contrast, the dinitrobenzamide mustards are expected to give rise to much more stable cytotoxic products, which should be capable of diffusing from the cell of origin. Reduction of one nitro group to the hydroxylamine or amine will greatly lower the reduction potential and probably preclude further reduction. The reactivity of such a nitrogen mustard would probably be less than that of classical aromatic nitrogen mustards such as melphalan or chlorambucil since the electron-withdrawing effect of the remaining nitro group would outweigh the electron-donating effect of the hydroxylamine or amine. Efficient diffusion of the active reduction product is thus expected. Preliminary studies with compound 22 indicate a much greater activity against intact than dissociated EMT6 spheroids, suggesting that bioactivation in the hypoxic core may result in killing which includes surrounding oxic cells.

Conclusions

The carboxamide analogues of 4, described above were designed to have both higher reduction potentials and improved aqueous solubility than the parent compound, which had been shown previously[14] to have limited hypoxia-selective cytotoxicity against mammalian cells in culture. The dinitro mustards 20 and 22 were highly selective for hypoxic cells, with CT_{10} ratios of 60–70-fold. Preliminary studies with purified DT diaphorase suggest that 20 differs from 23 in not being a substrate for activation by this oxygen-insensitive reductase. A loss of sensitivity to activation by DT diaphorases may be responsible for the much greater inhibition of toxicity by oxygen in the case of 20.

The selectivities of the dinitrobenzamide mustards are comparable with those of the benzotriazine di-*N*-oxide SR 4233[17,41] and the alkylating 2-nitroimidazole RB 6145,[42] both of which are scheduled for clinical evaluation as hypoxia-selective bioreductive drugs in combination with radiotherapy. They are certainly more selective than nitroheterocycles such as misonidazole and nitracrine, which have CT_{10} ratios (air/N_2) of approximately 10 for AA8 and UV4 cells in the same experimental system as employed here.[10,16,26]

The dinitrobenzamide mustards are expected to give rise to cytotoxic products sufficiently stable to be capable of diffusing from the cell of origin. The release of such diffusible cytotoxins within hypoxic foci provides an opportunity for exploiting tumor hypoxia to achieve killing of more than just the hypoxic fraction and is an important goal for further development of this new class of bioreductive agents.

Experimental Section

Chemistry. Where analyses are indicated by the symbols of the elements, results obtained were within ±0.4% of the theoretical value. Analyses were carried out in the Microchemical Laboratory, University of Otago, NZ. Melting points were determined on an Electrothermal apparatus using the supplied, stem-corrected thermometer and are as read. ^1H NMR spectra were obtained on a Bruker WP-60 spectrometer (Me_4Si), and ^{13}C NMR spectra on a Bruker AM-400. Mass spectra were obtained on an AEI MS-30 spectrometer at nominal 3000 resolution.

***N*-(2-Chloro-5-nitrobenzoyl)morpholide (28a).** A mixture of 2-chloro-5-nitrobenzoic acid (27) (5.0 g, 25 mmol) and $SOCl_2$ (20 mL) was heated under reflux under N_2 for 1 h and evaporated to dryness. The residue was dissolved in Me_2CO (20 mL), cooled to 0 °C, and treated dropwise with a solution of morpholine (4.7 mL, 62 mmol) in Me_2CO (20 mL). After 15 min the solution was evaporated to dryness and the residue partitioned between EtOAc and water. The organic layer on workup gave the morpholide 28a, which was recrystallized from aqueous EtOH as cubes (4.64 g, 73%): mp 104.5 °C; ^1H NMR ($CDCl_3$) δ 8.2 (m, 2 H, ArH4, ArH6), 7.63 (d, J = 10 Hz, 1 H, ArH3), 3.83 (br, $W_{1/2}$ = 8 Hz, 4 H, CH_2O), 3.67 (t, J = 6 Hz, 2 H, CH_2N), 3.24 (t, J = 6 Hz, 2 H, CH_2N). Anal. ($C_{11}H_{11}ClN_2O_4$) C, H, N, Cl.

***N*-[(*N*,*N*-Dimethylamino)ethyl]-2-chloro-5-nitrobenzamide (28b).** A solution of 1,1′-carbonyldiimidazole (5.22 g, 0.032 mol) in CH_2Cl_2 (25 mL) was added in one portion to a stirred solution of 2-chloro-5-nitrobenzoic acid (27) (5.00 g, 0.025 mol) in THF (30 mL). After 15 min, *N*,*N*-dimethylethylenediamine (2.97 mL, 0.027 mol) was added, and the mixture was stirred at 20 °C for 1 h and poured into brine. Extraction with EtOAc gave a gummy solid which was triturated with petroleum ether and crystallized from $CHCl_3$/petroleum ether to give the amide 28b (5.87 g, 87%): mp 110–111 °C; ^1H NMR ($CDCl_3$) δ 8.46 (d, J = 2 Hz, 1 H, ArH6), 8.19 (dd, J = 10, 2 Hz, 1 H, ArH4), 7.55 (d, J = 10 Hz, 1 H, ArH3), 6.89 (br, 1 H, NH), 3.51 (dt, J = 6, 5 Hz, 2 H, CH_2NHCO), 2.47 (t, J = 6 Hz, 2 H, CH_2NMe_2), 2.22 (s, 6 H, NMe_2). Anal. ($C_{11}H_{14}ClN_3O_3$) C, H, N, Cl.

***N*-[2-[*N*,*N*-Bis(2-chloroethyl)amino]-5-nitrobenzoyl]morpholide (9).** A mixture of the above morpholide (28a) (4.44 g, 17 mmol) and diethanolamine (4.03 g, 38 mmol) was warmed at 90 °C for 4 h, diluted with MeOH, and adsorbed onto SiO_2. Chromatography on SiO_2 and elution with MeOH/EtOAc (3:7) gave the diol 7 (3.02 g, 53%) as a viscous orange oil: ^1H NMR (CD_3COCD_3) δ 7.97 (dd, J = 9, 2 Hz, 1 H, ArH4), 7.88 (d, J = 2 Hz, 1 H, ArH6), 7.07 (d, J = 9 Hz, 1 H, ArH3), 3.65 (br m, 16 H, CH_2O, CH_2N), 2.92 (br, 2 H, OH). Anal. ($C_{15}H_{21}N_3O_6$) C, H. A solution of this crude diol (0.4 g, 1.22 mmol) and $SOCl_2$ (1 mL)

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(41) Zeman, E. M.; Brown, J. M.; Lemon, M. J.; Hirst, V. K.; Lee, W. W.; SR 4233, a new bioreductive agent with high selective toxicity for hypoxic mammalian cells. *Int. J. Radiat. Oncol. Biol. Phys.* **1986**, *12*, 1239–1242.

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(43) Ross, W. C. J. Aryl-2-halogenoalkylamines. Part 1. *J. Chem. Soc.* **1949**, 183–191.

in 1,2-dichloroethane (50 mL) was refluxed under N_2 for 1 h and then concentrated to dryness. The residue was dissolved in EtOAc, washed with saturated aqueous $NaHCO_3$, and worked up to give an oil. Chromatography on SiO_2 and elution with EtOAc/petroleum ether (3:7) gave the mustard **9** (0.37 g, 80%), which was crystallized from $CHCl_3$/petroleum ether: mp 112–114 °C; ^1H NMR ($CDCl_3$) δ 8.13 (dd, J = 9, 2 Hz, 1 H, ArH4), 8.05 (d, J = 2 Hz, 1 H, ArH6), 7.01 (d, J = 9 Hz, 1 H, ArH3), 3.72 (m, 14 H, CH_2N, CH_2Cl and CH_2O), 3.37 (m, 2 H, CH_2N). Anal. ($C_{15}H_{19}Cl_2N_3O_4$) C, H, N, Cl.

***N*-[(*N*,*N*-Dimethylamino)ethyl]-2-[*N*,*N*-bis(2-chloroethyl)amino]-5-nitrobenzamide (10).** A mixture of the amide **28b** (4.18 g, 0.015 mol), Cu powder (0.10 g), and diethanolamine (3.39 g, 0.032 mol) was heated at 70 °C for 2 h. The product was dissolved in MeOH and subjected to flash chromatography on SiO_2. EtOAc eluted some nonpolar material, and EtOAc/MeOH (3:2) gave the diol **8** (4.45 g, 85%) as a yellow oil: ^1H NMR ($CD_3COCD_3/CDCl_3$) δ 8.45 (d, J = 2.5 Hz, 1 H, ArH6), 8.13 (dd, J = 8, 2.5 Hz, 1 H, ArH4), 7.27 (d, J = 8 Hz, 1 H, ArH3), 4.0–3.2 (m, 8 H, CH_2NHCO, CH_2OH and OH), 2.91 (t, J = 7 Hz, 4 H, CH_2N), 2.51 (m, 3 H, CH_2NMe_2 and NH), 2.21 (s, 6 H, NMe_2). Anal. ($C_{15}H_{24}N_4O_5$ requires mass spectrum M^+ 340.1737, found M^+ 340.1741). A solution of this diol (1.40 g, 4.11 mmol) and Et_3N (2.29 mL, 16.45 mmol) in CH_2Cl_2 (30 mL) was treated at 0 °C with MsCl (0.95 mL, 12.34 mmol). After 2 h the mixture was diluted with CH_2Cl_2, washed with saturated aqueous $NaHCO_3$, and evaporated to give the crude dimesylate as an oil. This was dissolved in DMF (20 mL) containing NaCl (2 g), and the mixture was held at 160 °C for 30 min and evaporated to dryness. The residue was dissolved in EtOAc, washed with brine, and concentrated, and the residue was chromatographed on SiO_2. Elution with EtOAc gave foreruns, while EtOAc/MeOH/Et_3N (9:1:0.1) gave the mustard **10** (0.81 g, 52%) as a yellow oil: ^1H NMR ($CDCl_3$) δ 8.66 (d, J = 2 Hz, 1 H, ArH6), 8.20 (dd, J = 10, 2 Hz, 1 H, ArH4), 7.19 (d, J = 10 Hz, 1 H, ArH3), 3.62 (br s, 10 H, NCH_2CH_2Cl and CH_2NHCO), 2.52 (t, J = 6 Hz, 2 H, CH_2NMe_2), 2.25 (s, 6 H, NMe_2). Anal. ($C_{15}H_{22}Cl_2N_4O_3$ requires mass spectrum M^+ 379.0932, 366.0961, 375.0991, found M^+ 379.0945, 377.0998, 375.1044).

Lactones 29. Treatment of 2-chloro-5-nitrobenzamide (**28c**; prepared as above for **28a**) with diethanolamine gave, after chromatography on SiO_2, only the hydroxy lactone **29a** (63%), which crystallized from EtOAc/petroleum ether: mp 132.5 °C; ^1H NMR (CD_3SOCD_3) δ 8.58 (d, J = 3 Hz, 1 H, ArH6), 8.10 (dd, J = 10, 3 Hz, 1 H, ArH4), 7.12 (d, J = 10 Hz, 1 H, ArH3), 4.76 (br, 1 H, OH), 4.53 (m, 2 H, CH_2OCO), 3.90 (m, 2 H, CH_2OH), 3.65 (m, 4 H, CH_2N). Anal. ($C_{11}H_{12}N_2O_8$) C, H, N. Reaction of the hydroxy lactone with $SOCl_2$ as above gave the chloro lactone **29b** (71%): mp (EtOAc) 195 °C; ^1H NMR (CD_3SOCD_3) δ 8.67 (d, J = 3 Hz, 1 H, ArH6), 8.33 (dd, J = 10, 3 Hz, 1 H, ArH4), 6.97 (d, J = 10 Hz, 1 H, ArH3), 4.55 (m, 2 H, CH_2O), 4.00–3.60 (m, 6 H, CH_2N and CH_2Cl). Anal. ($C_{11}H_{11}ClN_2O_4$) C, H, N, Cl.

***N*-[5-Chloro-2-nitrobenzoyl]morpholide (31a).** Treatment of 5-chloro-2-nitrobenzoic acid (**30**) with $SOCl_2$ followed by morpholine gave the morpholide **31a**, which crystallized from aqueous EtOH: mp 158–160 °C; ^1H NMR ($CDCl_3$) δ 8.16 (d, J = 9 Hz, 1 H, ArH3), 7.60 (d, J = 2 Hz, 1 H, ArH6), 7.37 (dd, J = 9, 2 Hz, 1 H, ArH4), 3.81 (br, $W_{1/2}$ = 8 Hz, 4 H, CH_2O), 3.62, 3.21 (2 t, 4 H, CH_2N). Anal. ($C_{11}H_{11}ClN_2O_4$) C, H, N, Cl.

***N*-[(*N*,*N*-Dimethylamino)ethyl]-5-chloro-2-nitrobenzamide (31b).** Treatment of 5-chloro-2-nitrobenzoic acid (**30**) with 1,1-carbonyldiimidazole followed by *N*,*N*-dimethylethylenediamine gave the amide **31b** (57% yield): mp 70–72 °C (after distillation at 100 °C/1 mmHg); ^1H NMR ($CDCl_3$) δ 7.99 (d, J = 9 Hz, 1 H, ArH3), 7.46 (dd, J = 9, 2 Hz, 1 H, ArH4), 7.46 (d, J = 2 Hz, 1 H, ArH6), 6.71 (br, 1 H, NH), 3.50 (dt, J = 6, 5 Hz, 2 H, CH_2NHCO), 2.51 (t, J = 6 Hz, 2 H, CH_2NMe_2), 2.24 (s, 6 H, NMe_2). Anal. ($C_{11}H_{14}ClN_3O_3$) C, H, N, Cl.

***N*-[5-[*N*,*N*-Bis(2-chloroethyl)amino]-2-nitrobenzoyl]morpholide (13).** Treatment of the morpholide **31a** as above with excess diethanolamine gave the diol **11**: mp (Me_2CO/petroleum ether) 188–191 °C; ^1H NMR (CD_3SOCD_3) δ 7.90 (d, J = 9 Hz, 1 H, ArH3), 6.73 (dd, J = 9, 3 Hz, 1 H, ArH4), 6.50 (d, J = 3 Hz, 1 H, ArH6), 4.95 (br, 2 H, OH), 3.55 (br s, 14 H, CH_2N, CH_2O), 3.00 (m, 2 H, CH_2N). Anal. ($C_{15}H_{21}N_3O_6$) C, H, N. Treatment of this diol with $SOCl_2$ as above gave the mustard **13**: mp ($CHCl_3$/petroleum ether) 146–148 °C; ^1H NMR ($CDCl_3$) δ 8.10 (d, J = 9 Hz, 1 H, ArH3), 6.67 (dd, J = 9, 2 Hz, 1 H, ArH4), 6.48 (d, J = 2 Hz, 1 H, ArH6), 3.70 (m, 14 H, CH_2N, CH_2Cl and CH_2O), 3.15 (t, 2 H, CH_2N). Anal. ($C_{15}H_{19}Cl_2N_3O_4$) C, H, N.

***N*-[(*N*,*N*-Dimethylamino)ethyl]-5-[*N*,*N*-bis(2-chloroethyl)amino]-2-nitrobenzamide (14).** Similar treatment of the amide **31b** with excess diethanolamine gave the diol **12** in 42% yield as a yellow oil: ^1H NMR (CD_3SOCD_3) δ 7.92 (d, J = 8 Hz, 1 H, ArH3), 6.82 (dd, J = 8, 2.5 Hz, 1 H, ArH4), 6.61 (d, J = 2.5 Hz, 1 H, ArH6), 4.25 (br s, 2 H, OH), 3.55 (m, 6 H, CH_2O and CH_2NHCO), 2.94 (t, J = 7 Hz, 4 H, CH_2N), 2.50 (m, 2 H, CH_2NMe_2), 2.22 (s, 6 H, NMe_2). Anal. ($C_{15}H_{24}N_4O_5$ requires mass spectrum M^+ 340.1737, found M^+ 340.1737). Treatment of this with MsCl followed by NaCl as detailed above gave the corresponding mustard (**14**, 59% yield): mp (EtOAc/petroleum ether) 143–145 °C; ^1H NMR ($CDCl_3$) δ 8.02 (d, J = 10 Hz, 1 H, ArH3), 6.67 (dd, J = 10, 2 Hz, 1 H, ArH4), 6.62 (d, J = 2 Hz, 1 H, ArH6), 3.71 (m, 8 H, NCH_2CH_2Cl), 3.46 (m, 2 H, CH_2NHCO), 2.47 (t, J = 6 Hz, 2 H, CH_2NMe_2), 2.20 (s, 6 H, NMe_2). Anal. ($C_{15}H_{22}Cl_2N_4O_3$) C, H, N, Cl.

5-[*N*,*N*-Bis(2-hydroxyethyl)amino]-2-nitrobenzamide (32). A mixture of 5-chloro-2-nitrobenzamide (**31c**; made from **30** as above) (3.32 g, 16 mmol) and diethanolamine (3.82 g, 36 mmol) was stirred at 100 °C for 4 h and adsorbed onto SiO_2. Elution with MeOH/EtOAc (1:9) gave the diol **32** (2.63 g, 59%): mp (EtOAc/MeOH) 210–212 °C; ^1H NMR (CD_3SOCD_3) δ 7.90 (d, J = 9 Hz, 1 H, ArH3), 7.73–7.74 (br, 2 H, $CONH_2$), 6.70 (dd, J = 9, 3 Hz, 1 H, ArH4), 6.53 (d, J = 3 Hz, 1 H, ArH6), 4.74 (br, 2 H, OH), 3.50 (br s, 8 H, CH_2N, CH_2O). Anal. ($C_{11}H_{15}N_3O_5$) C, H, N.

Attempted Preparation of 5-[*N*,*N*-Bis(2-chloroethyl)amino]-2-nitrobenzamide. Treatment of the diol **32** with MsCl and Et_3N in 1,2-dichloroethane at 0 °C, followed by treatment with NaCl in DMF at 160 °C gave, after chromatography on silica gel, only 5-[*N*,*N*-bis(2-chloroethyl)amino]-2-nitrobenzonitrile (**33**) (15% yield): mp ($CHCl_3$/petroleum ether) 127 °C; ^1H NMR ($CDCl_3$) δ 7.03 (br s, 1 H, ArH6), 6.98 (br d, J = 9 Hz, 1 H, ArH4), 6.60 (d, J = 9 Hz, 1 H, ArH3), 4.05–3.62 (m, 8 H, CH_2N and CH_2Cl). Anal. ($C_{11}H_{11}Cl_2N_2O_3$ requires mass spectrum M^+ 291.0169, 289.0199, 287.0228, found M^+ 291.0173, 289.0237, 287.0243). No other product was eluted, and no trace of the desired 5-[*N*,*N*-bis(2-chloroethyl)amino]-2-nitrobenzamide could be detected.

***N*-[2-(*N*,*N*-Dimethylamino)ethyl]-5-chloro-2,4-dinitrobenzamide (36c).** Treatment of 5-chloro-2,4-dinitrobenzoic acid[18] (**35**) with $SOCl_2$ followed by *N*,*N*-dimethylethylenediamine as above gave **36c** (59% yield) as an unstable solid: ^1H NMR (CD_3COCD_3) δ 8.78 (s, 1 H, ArH3), 8.10 (s, 1 H, ArH6), 4.30 (br, 1 H, NHCO), 3.51 (m, 2 H, CH_2NHCO), 2.61 (t, J = 6 Hz, 2 H, CH_2NMe_2), 2.30 (s, 6 H, NMe_2). The hydrochloride salt crystallized from MeOH/Et_2O, mp 143–145 °C. Anal. ($C_{11}H_{13}ClN_4O_5$.HCl) C, H, N, Cl.

Similarly was prepared 5-chloro-2,4-dinitrobenzamide (**36a**): mp 210 °C dec (lit.[18] mp 212 °C); ^1H NMR (CD_3COCD_3) δ 8.68 (s, 1 H, ArH3), 8.02 (s, 1 H, ArH6), 7.50 (br, 2 H, $CONH_2$); and (5-chloro-2,4-dinitrobenzoyl)morpholide (**36b**): mp (EtOAc/petroleum ether) 184 °C (lit.[44] mp 180 °C); ^1H NMR (CD_3COCD_3) δ 8.83 (s, 1 H, ArH3), 7.98 (s, 1 H, ArH6), 3.73 (m, 6 H), 3.40 (m, 2 H). Anal. ($C_{11}H_{10}ClN_3O_6$) C, H, N, Cl.

5-[*N*,*N*-Bis(2-chloroethyl)amino]-2,4-dinitrobenzamide (20). A mixture of 5-chloro-2,4-dinitrobenzamide (**36a**) and diethanolamine was treated as above to give the diol **17** (45% yield): mp (EtOAc) 176–178 °C; ^1H NMR (CD_3SOCD_3) δ 8.47 (s, 1 H, ArH3), 8.07, 7.73 (2 br, 2 H, $CONH_2$), 7.35 (s, 1 H, ArH6), 4.80 (m, 2 H, OH), 3.83–3.20 (m, 8 H, CH_2N, CH_2O). Anal. ($C_{11}H_{14}N_4O_7$) C, H, N. Treatment of this with MsCl followed by NaCl/DMF as above followed by chromatography on SiO_2 gave (elution with EtOAc) the mustard **20** (0.47 g, 20%): mp (EtOAc/petroleum ether) 109–111 °C; ^1H NMR ($CDCl_3$) δ 8.58 (s, 1 H, ArH3), 7.33 (s, 1 H, ArH6), 6.53 (br, 2 H, $CONH_2$), 3.72 (br s, 8 H, CH_2N and CH_2Cl); ^{13}C NMR (CD_3COCD_3) δ 167.10 (CO-

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NH_2), 148.31 (C-5), 140.08 (C-2), 138.84 (C-1), 138.20 (C-4), 124.94, 122.24 (C-3,6), 54.25 (CH_2N), 42.04 (CH_2Cl). Anal. ($C_{11}H_{12}Cl_2N_4O_5$) C, H, N, Cl.

***N*-[5-[*N*,*N*-Bis(2-chloroethyl)amino]-2,4-dinitrobenzoyl]morpholide (21).** The morpholide **36b** similarly gave the diol **18** (1.31 g, 39%), which crystallized from EtOAc/petroleum ether: mp 178–180 °C; ^{1}H NMR (CD_3SOCD_3) δ 8.62 (s, 1 H, ArH3), 7.37 (s, 1 H, ArH6), 4.79 (br, 2 H, OH), 3.83–3.16 (m, 16 H, CH_2O, CH_2N). Anal. ($C_{15}H_{20}N_4O_8$) C, H, N. This was treated with $SOCl_2$ in 1,2-dichloroethane as above, and the product was chromatographed on SiO_2. Elution with EtOAc gave **21** (0.94 g, 71%), which crystallized from $CHCl_3$/hexane: mp 140–142 °C; ^{1}H NMR ($CDCl_3$) δ 8.66 (s, 1 H, ArH3), 7.55 (s, 1 H, ArH6), 3.68 (br, 10 H, NCH_2CH_2Cl and CH_2O), 3.60 & 3.34 (2 m, 2 × 2 H, CH_2NCO); ^{13}C NMR δ 165.22 (CON), 149.34 (C-5), 139.88, 137.00 (C-2,4), 138.26 (C-1), 125.59, 121.17 (C-3,6), 66.92, 66.79 (CH_2O), 54.22 (CH_2N), 47.88, 42.80 ($CONCH_2$), 42.26 (CH_2Cl). Anal. ($C_{15}H_8Cl_2N_4O_6$) C, H, N.

***N*-(2,4-Dinitro-5-aziridinobenzoyl)morpholide (24).** A solution of the morpholide **36b** (0.91 g, 2.88 mmol) in EtOAc (100 mL) was added dropwise at 10 °C to a stirred solution of ethylene imine (0.27 mL, 5.19 mmol) and Et_3N (0.72 mL, 5.19 mmol) in EtOAc (100 mL). The mixture was stirred for a further 4 h and then chromatographed directly on silica gel. Elution with EtOAc gave foreruns, followed by the imine **24** (0.63 g, 68%), which crystallized from $CHCl_3$/petroleum ether: mp 198–200 °C dec (lit.[44] mp 177 °C); ^{1}H NMR (CD_3COCD_3) δ 8.87 (s, 1 H, ArH3), 7.38 (s, 1 H, ArH6), 3.75 (s, 4 H, CH_2O and CH_2N), 3.58 (m, 2 H, CH_2O), 3.33 (m, 2 H, CH_2N), 2.57 (br s, 4 H, aziridine CH_2). Anal. ($C_{13}H_{14}N_4O_6$) C, H, N.

Similar treatment of the dinitrobenzamide **36c** gave *N*-[(*N*,*N*-dimethylamino)ethyl]-5-aziridino-2,4-dinitrobenzamide (**25**) as an unstable, pale yellow solid (0.31 g, 52%): ^{1}H NMR ($CDCl_3$) δ 8.93 (s, 1 H, ArH3), 7.33 (s, 1 H, ArH6), 3.56 (m, 2 H, CH_2NHCO), 2.53 (br s, 4 H, aziridine CH_2), 2.20 (m, 2 H, CH_2NMe_2), 2.07 (s, 6 H, NMe_2). This compound could not be characterized further due to its instability and decomposed to a red polymer over a few hours at 20 °C.

***N*-[(*N*,*N*-Dimethylamino)ethyl]-5-[*N*,*N*-bis(2-chloroethyl)amino]-2,4-dinitrobenzamide (22).** A solution of bis(2-chloroethyl)amine hydrochloride (2.79 g, 0.016 mol) in water (10 mL) was neutralized with $NaHCO_3$ (2.62 g, 0.031 mol), extracted with Et_2O, and worked up to give crude bis(2-chloroethyl)amine as a viscous oil. To this was added powdered dinitrochlorobenzamide (**36c**) (0.94 g, 0.031 mol), and the mixture was stirred at 50 °C for 1 h and adsorbed onto SiO_2 from a solution in MeOH for flash chromatography. Elution with EtOAc gave foreruns, while elution with EtOAc/MeOH (20:1) gave the mustard **22** (0.74 g, 56%) as a yellow solid: ^{1}H NMR (CD_3COCD_3) δ 8.50 (s, 1 H, ArH3), 8.00 (br, 1 H, CONH), 7.62 (s, 1 H, ArH6), 3.88 (br s, 8 H, NCH_2CH_2Cl), 3.50 (m, 2 H, CH_2NHCO), 2.53 (t, J = 6 Hz, 2 H, CH_2NMe_2), 2.40 (s, 6 H, NMe_2). The hydrochloride salt crystallized from MeOH/Et_2O, mp 85–90 °C. Anal. ($C_{15}H_{21}Cl_2N_5O_5$.HCl) C, H, N.

Similar treatment of **36c** with diethanolamine gave the corresponding diol **19** as a viscous oil. Anal. ($C_{15}H_{23}N_5O_7$ requires mass spectrum M^+ 385.1597, found M^+ 385.1589).

Determination of Half-Wave Reduction Potentials by Cyclic Voltammetry. Reduction potentials were determined for aqueous solutions of approximately 5 × 10^{-4} M concentration, containing 0.1 M $NaClO_4$ as electrolyte and 5 × 10^{-3} M phosphate buffer (pH 7). The nitro aromatic compound (ca. 5 × 10^{-6} mol) was dissolved in 100 mL of a standard electrolyte solution, itself prepared by diluting 0.1 mol of $NaClO_4 \cdot H_2O$ and 100 mL of 0.05 M phosphate buffer (pH 7) to 1 L with deionized water. The solutions were deoxygenated immediately prior to use by bubbling N_2 through them for 15 min. Cyclic voltammograms were determined over the range +0.5 to −1.5 V at a scan rate of 50 mV/s, using a stationary wax-impregnated carbon electrode and a platinum counter electrode. Values are accurate to ±30 mV and are referenced to the normal hydrogen electrode.

Rates of Reduction by Rat DT Diaphorase. Reduction by highly purified DT diaphorase (NAD(P)H:quinone oxidoreductase) from Walker cells[39] was assessed by monitoring loss of parent compound by HPLC. The activity of the enzyme was determined to be 22 μkat/mg at 37 °C in 0.1 M sodium phosphate buffer using 10 μM menadione as substrate and 70 μM cytochrome C (ϵ_{550} = 29 500 M^{-1} cm^{-1}) as terminal electron acceptor. The drugs (0.15 mM) were incubated with enzyme (100 μg/mL) and NADH (1 mM) in 0.1 M sodium phosphate buffer at 37 °C under aerobic conditions in reaction volumes of 0.4 mL. At intervals aliquots (0.05 mL) were diluted into 25% MeOH (0.2 mL), frozen, thawed, centrifuged, and analyzed using a C18 μBondapak column (8 × 100 mm) and a mobile phase comprising a linear gradient of 25–50% MeOH (**23**) or 50–80% MeOH (**20**) in water. The eluate was monitored using a diode array absorbance detector (Hewlett-Packard 1040A) at 298 nm (bandwidth 10 nm) for **23** and 380 nm (bandwidth 20 nm) for **20**.

Cell Culture. AA8 and UV4 cells were maintained in exponential phase growth (doubling times 14 and 15 h, respectively) using Alpha MEM containing fetal calf serum (10% v/v) without antibiotics. Cells were subcultured twice weekly by trypsinization. Cultures were regularly shown to be free of mycoplasma contamination by fluorescence staining for cytoplasmic DNA.[45] Bulk cultures were prepared for experiments by seeding cells in spinner flasks at 10^4 cells/mL in the above medium with addition of penicillin (100 IU/mL) and streptomycin (100 μg/mL).

Measurement of Cytotoxicity. Growth Inhibition Assay. Cultures were initiated in 96-well microtiter trays to give 200 (AA8) or 300 (UV4) cells in 0.05 mL per well. After growth in a CO_2 incubator for 24 h, drugs were added in culture medium, using serial 2-fold dilutions to provide duplicate cultures at five different concentrations for each of eight drugs (plus eight controls) per tray. After 18 h drugs were removed by washing cultures three times with fresh medium, and the trays were incubated for a further 78 h. Cell density was then determined by staining with methylene blue as described previously.[27] The IC_{50} was calculated as the drug concentration providing 50% inhibition of growth relative to the controls.

Stirred Suspension Culture Assay. Clonogenic assays with magnetically-stirred 10-mL suspension cultures (late log phase UV4 cells, 10^6/mL) were performed by removing samples periodically during continuous gassing with 5% CO_2 in air or N_2 as detailed elsewhere.[10] 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 throughout the period of drug contact in hypoxic cultures. Several drug concentrations were investigated to identify those concentrations which gave, under both aerobic and hypoxic conditions, approximately the same rate of cell kill. The ratios of the concentration × time for a surviving fraction of 10% (CT_{10}) for these two survival curves was used as the measure of hypoxic selectivity.

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