Hypoxia-Selective Antitumor Agents. 9. Structure–Activity Relationships for Hypoxia-Selective Cytotoxicity among Analogues of 5-[*N*,*N*-Bis(2-chloroethyl)amino]-2,4-dinitrobenzamide

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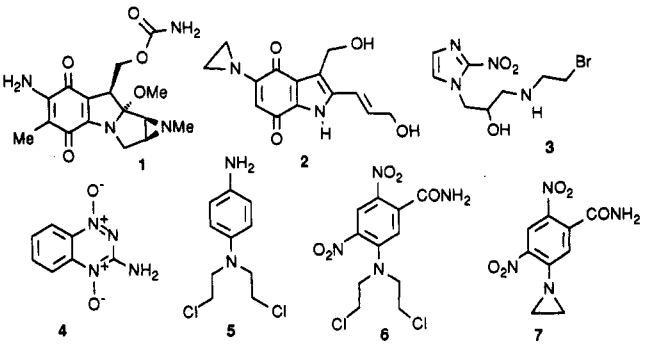
A series of analogues of the novel hypoxia-selective cytotoxin 5-[*N*,*N*-bis(2-chloroethyl)amino]-2,4-dinitrobenzamide (**6**) have been prepared and evaluated, in a search for compounds which retain high hypoxic selectivity but have increased potency and/or aqueous solubility. Several analogues with ionizable or dipolar carboxamide side chains showed improved solubility but generally had reduced cytotoxic potency and hypoxic selectivity. Modification of the mustard leaving groups or replacement of the carboxamide moiety provided some compounds with superior potency, but only the mixed chloro/mesylate mustard **20** provided a gain in potency relative to solubility while retaining the hypoxic selectivity of **6**. These nitrogen mustards did not show the remarkable activity demonstrated by the related aziridine **7** [CB 1954, 5-(*N*-aziridinyl)-2,4-dinitrobenzamide] in Walker 256 adenocarcinoma cells and are not efficient substrates for the DT-diaphorase which activates the latter compound by aerobic nitroreduction in Walker cells. Variations in hypoxic selectivity within the dinitrobenzamide mustards appear not to be due to differences in sensitivity to activation by this enzyme. Walker cells showed intermediate sensitivity to the mono(2-chloroethyl) analogue **26** but not to the related half-mustard **27**, suggesting that the inhibition of DT-diaphorase activity is due to steric effects in the 5-position. The preferred compound overall with respect to solubility, potency, and *in vitro* hypoxic cell selectivity was the (dimethylamino)-ethyl derivative **11**. DNA elution studies and comparison of the sensitivity of AA8 and UV4 cells to this compound indicated reductive activation to form a DNA cross-linking agent under hypoxia. Radiobiological studies indicated **11** to be equally active against both aerobic and hypoxic cells in KHT tumors. It is not clear whether this reflects efficient killing of aerobic cells as a result of diffusion of reduced metabolites from hypoxic regions or whether cytotoxicity in tumors is independent of hypoxia.

There is considerable evidence that hypoxic cells in human tumors can limit the effectiveness of fractionated radiotherapy.[1-3] The same cells may also be resistant to many chemotherapeutic drugs.[4] The development of bioreductive drugs, which are activated to cytotoxic species only in the absence of oxygen (hypoxia-selective cytotoxins, HSCs), is thus of increasing interest, and clinical studies with several compounds [porfiromycin (**1**),[5] EO-9 (**2**),[6] RB 6145 (**3**),[7] and SR 4233 (**4**, tirapamazine)[8]] are pending or in progress.

Many bioreductive drugs appear to be fully active only at extremely low oxygen concentrations (<0.1 μM O_2).[9-11] Since much higher oxygen concentrations (about 3 μM) are required for half-maximal radiosensitization,[12] it is likely that many hypoxic cells in tumors will be resistant to both radiation and bioreductive drugs.[9] As we have discussed in detail recently,[13] this problem might be circumvented by developing HSCs which generate relatively stable cytotoxic products on reduction. If these activated cytotoxins were capable of diffusing several cell diameters (i.e., have half-lives in the range 0.1–10 min), then the killing of relatively well-oxygenated cells surrounding severely hypoxic zones might be achieved. The diffusible cytotoxic species should ideally be effective against all major tumor cell subpopulations (including noncycling cells).



These criteria appear to be met by aliphatic or activated aromatic nitrogen mustards. For example, the 4-aminoaniline mustard **5** is very reactive, with a half-life of ca. 8 min in tissue culture medium at 37 °C,[14] but is capable of effective diffusion into EMT6 tumor spheroids and kills both cycling and noncycling cells efficiently in the tumor-like spheroid microenvironment.[13] We are therefore interested in the development of bioreductively activated prodrugs of such mustards and have previously discussed nitro-deactivated aniline mustards as HSCs.[15] The reactivity and cytotoxicity of these compounds are very dependent on the electronic properties of the substituents on the aromatic ring;[14,16] bioreduction of an electron-withdrawing nitro group in a ring position resonant to the mustard (to give the resultant hydroxylamine or amine) is likely to dramatically activate the mustard.

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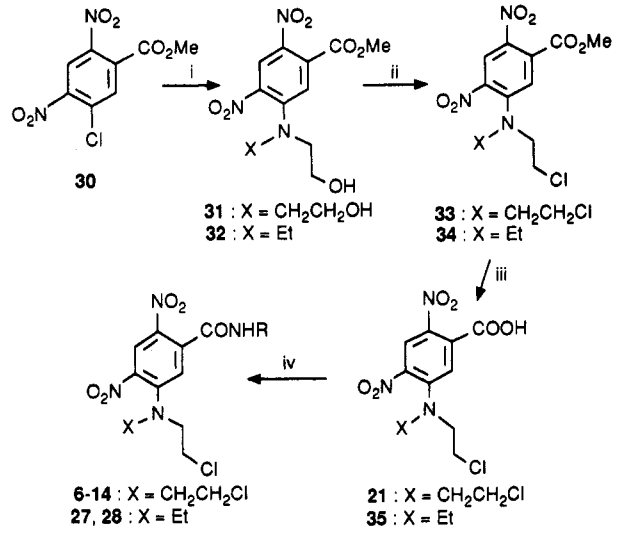
Scheme 1[a]



[a] (i) $XNH(CH_2)_2OH$/heat; (ii) MsCl/Et_3N, then LiCl; (iii) KOH/dioxane/H_2O; (iv) $SOCl_2$, then RNH_2.

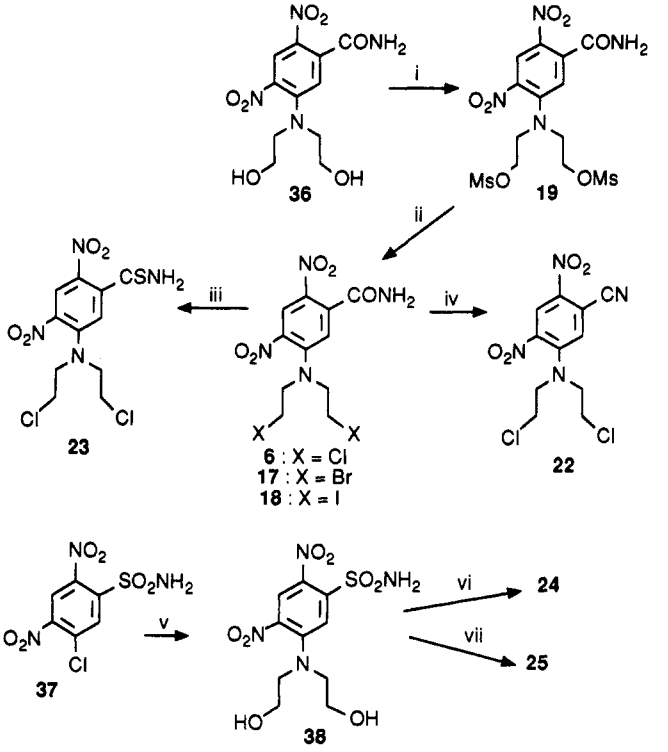
We have previously shown that the nitro-deactivated mustard 5-[*N*,*N*-bis(2-chloroethyl)amino]-2,4-dinitrobenzamide (**6**, SN 23862) is 60-fold more potent against anoxic than aerobic Chinese hamster UV4 cells in culture and that this compound is much more selective than the corresponding aziridine **7** (CB 1954).[17] The superiority of **6** over **7** as an HSC has been confirmed in human, rat, and mouse cell lines[18] and has been suggested to result from the lower sensitivity of the mustard, than that of the aziridine, to oxygen-insensitive activation by the obligate 2-electron reductase DT-diaphorase (NAD(P)H:quinone oxidoreductase). The latter enzyme is known to be responsible for aerobic activation of **7** (at least in rat cell lines) by reduction of the 4-nitro group to the hydroxylamine.[19,20]

Compound **6** is therefore an important new lead for further development. In this paper, we describe the synthesis and the biological evaluation of a series of analogues of **6**, seeking compounds which retain the high hypoxic selectivity of the lead compound and will give rise to diffusible cytotoxins on reduction but with increased potency and/or aqueous solubility. In view of the importance of avoiding oxygen-insensitive activation by 2-electron reductases, the compounds were also evaluated for their sensitivity to reduction by rat DT-diaphorase.

Chemistry

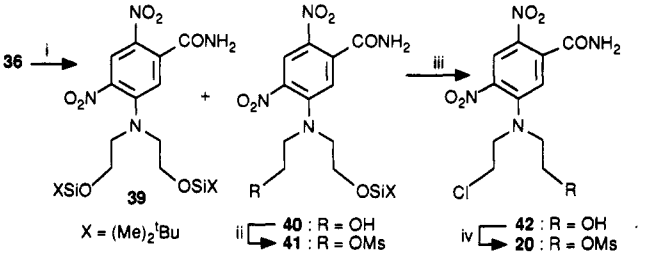
Analogues of the lead compound **6** with variations in the carboxamide side chain were conveniently prepared from the acid mustard **21** (Scheme 1). Chloride displacement by diethanolamine from the known[21] methyl-5-chloro-2,4-dinitrobenzoate (**30**) gave the diol **31**, which was elaborated to the mustard **33** by mesylation and treatment with lithium chloride. Basic hydrolysis then gave the acid **21** in good overall yield, and this was coupled (*via* the acid chloride) with the appropriate amines. A similar route using *N*-ethylethanolamine gave the half-mustards **27** and **28** *via* intermediates **32**, **34**, and **35** (Scheme 1). Selective oxidation of the *N*,*N*-dimethylamino group of **11** with 2-(phenylsulfonyl)-3-phenyloxaziridine[22] gave the corresponding *N*-oxide **13**. Compounds **17**–**19**, with different mustard functionalities, were prepared from the diol **36**[17] *via* mesylation and displacement (Scheme 2).

Scheme 2[a]



[a] (i) MsCl/Et_3N; (ii) NaX; (iii) $P_4S_{10}/NaHCO_3$; (iv) $SOCl_2$/reflux; (v) $NH(CH_2CH_2OH)_2$/heat; (vi) MsCl (2 equiv)/Et_3N/0 °C, then LiCl; (vii) MsCl (4 equiv)/Et_3N/20 °C, then LiCl.

Scheme 3[a]

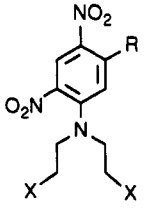


[a] (i) *tert*-Bu$(Me)_2$SiCl/imidazole/DMF/20 °C/18 h; (ii) MsCl/Et_3N/CH_2Cl_2/20 °C;10 min; (iii) LiCl/DMF/125 °C/20 min; (iv) MsCl/THF/20 °C/10 min.

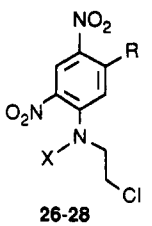
The nitrile **22** was prepared by direct dehydration of **6** in refluxing $SOCl_2$, while thiation of **6** with $P_4S_{10}/NaHCO_3$ gave the thioamide **23** (Scheme 2). The benzenesulfonamide **24** was prepared from 5-chloro-2,4-dinitrobenzenesulfonamide[23] (**37**) by an analogous procedure (formation of the diol **38** with diethanolamine, mesylation, and chloride displacement) (Scheme 2). However, use of an excess of MsCl in this process provided the *N*-(methylsulfonyl)benzenesulfonamide **25** as the major product.

Treatment of the diol **36** with *tert*-butyldimethylsilyl chloride and imidazole in DMF gave a 44% yield of the mono(*tert*-butyldimethylsilyl)oxy derivative **40**, together with 38% of the bis(*tert*-butyldimethylsilyl)oxy derivative **39** (Scheme 3). This reagent achieves better selectivity than the high-temperature mesyl chloride reaction reported previously.[24] The asymmetric mustard **20** was readily prepared from **40** by mesylation to give **41** followed by chlorination (with concomitant deprotection) on treatment with LiCl in DMF at 120 °C to give the chloride **42** followed by mesylation (Scheme 3).

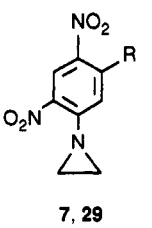
Table 1. Analytical and Physicochemical Data for Analogues of 5-[*N*,*N*-Bis(2-chloroethyl)amino]-2,4-dinitrobenzamide



6, 8-25



26-28



7, 29

no.	X	R	mp (°C)	formula	analyses[a]
6	Cl	$CONH_2$	109–111	ref 17	
8	Cl	CONHMe	180.5	$C_{12}H_{14}Cl_2N_4O_4$	C, H, N, Cl
9	Cl	$CONMe_2$	130.5	$C_{13}H_{16}Cl_2N_4O_5$	C, H, N
10	Cl	COX[b]	140–142	ref 17	
11	Cl	$CONH(CH_2)_2NMe_2$·HCl	85–90	ref 17	
12	Cl	$CONH(CH_2)_2X$[b]·HCl	119–120	$C_{17}H_{23}Cl_2N_5O_6$·HCl	C, H, N, Cl
13	Cl	$CONH(CH_2)_2N(O)Me_2$·HCl	65–169	$C_{15}H_{21}Cl_2N_5O_6$·HCl	C, H, N, Cl
14	Cl	$CONH(CH_2)_2COOH$	150–153	$C_{14}H_{16}Cl_2N_4O_7$	C, H, N
15	Cl	$CONHCH_2CH_2OH$	157–158	$C_{13}H_{16}Cl_2N_4O_6$	C, H, N, Cl
16	Cl	$CONHCH_2CH(OH)CH_2OH$	gum	$C_{14}H_{18}Cl_2N_4O_7$	C, H, N, Cl
17	Br	$CONH_2$	126–128	$C_{11}H_{12}Br_2N_4O_5$	C, H, N
18	I	$CONH_2$	170–172 dec	$C_{11}H_{12}I_2N_4O_5$	C, H, N, I
19	OMs[c]	$CONH_2$	142–144	$C_{13}H_{18}N_4O_{11}S_2$	C, H, N, S
20	Cl/OMs	$CONH_2$	103–104	$C_{12}H_{15}ClN_4O_8S$	C, H, N
21	Cl	COOH	125–127	$C_{11}H_{11}Cl_2N_3O_6$·0.5EtOAc	C, H, N
22	Cl	CN	127	$C_{11}H_{10}Cl_2N_4O_4$	C, H, N
23	Cl	$CSNH_2$	166–167	$C_{11}H_{12}Cl_2N_4O_4S$	C, H, N, Cl
24	Cl	SO_2NH_2	153–154	$C_{10}H_{12}Cl_2N_4SO_6$	C, H, N, S
25	Cl	SO_2NHSO_2Me	206–208	$C_{11}H_{14}Cl_2N_4S_2O_8$·0.5$H_2O$	C, H, N, S
26	H	$CONH_2$	225	$C_9H_9ClN_4O_5$	C, H, N
27	CH_2CH_3	$CONH_2$	151–152	$C_{11}H_{13}ClN_4O_5$	C, H, N, Cl
28	CH_2CH_3	$CONH(CH_2)_2NMe_2$·HCl	133–134	$C_{15}H_{22}ClN_5O_5$·HCl	C, H, N, Cl
7		$CONH_2$	189	ref 40	
29		COX[b]	198–200	ref 17	

[a] Analyses for all listed elements were within ±0.4%. [b] X = morpholide. [c] OMs = OSO_2CH_3.

Biological Evaluation

Aerobic cytotoxicity was assessed in a growth inhibition assay,[25] using log-phase cultures of the Chinese hamster ovary fibroblast lines AA8 and UV4. The UV4 mutant derived from AA8 is defective in the repair of bulky DNA monoadducts or cross-links.[26,27] The ratio of the IC_{50} values of the compounds in the wild-type and mutant lines can provide evidence for their mode of action; DNA interstrand cross-linking agents show hypersensitivity factors [HF = IC_{50}(AA8)/IC_{50}(UV4)] in the range 8–200-fold.[27] DNA cross-linking by compound **11** was also examined using alkaline elution methods.[28]

Aerobic cytotoxicity against the rat Walker 256 adenocarcinoma was also assessed by growth inhibition assay since this cell line is exquisitely sensitive to **7**, being ca. 10^5-fold more sensitive than Chinese hamster cells,[29] primarily because of the efficient activation of this drug by the high levels of DT-diaphorase in Walker cells.[19,20] The ratio of IC_{50} values for AA8 and Walker cells was therefore used as an indicator of the sensitivity of these compounds to activation by the Walker cell DT-diaphorase. Reduction of a subset of compounds by highly purified DT-diaphorase from this cell line was also examined by HPLC.

The selective toxicity of the compounds toward hypoxic cells was determined by comparing the rate of killing in aerobic and hypoxic stirred cell suspensions, using a clonogenic assay as described previously.[25,30] The sensitive UV4 cell line was used, which allowed hypoxic selectivity to be quantitated even for the less soluble analogues. The concentration × time to reduce the surviving fraction to 10% (CT_{10}) was 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. The activity of compound **11** against aerobic and hypoxic cells in KHT tumors was assessed by clonogenic assay, using ionizing radiation to kill oxygenated tumor cells and thus reveal the hypoxic subpopulation.

Results and Discussion

The first part of this study focused on close analogues of **6** (compounds **8–16**), seeking variations in the carboxamide chain which would provide analogues with better solubility while retaining high selectivity for hypoxic cells in culture. Solubilities were measured in culture medium containing 5% fetal calf serum and are recorded in Table 2. The parent compound **6** has only moderate solubility (0.19 mM), and substituents on the carboxamide in compounds **8–16** provided large variations in this. The CONHMe and $CONMe_2$ derivatives **8** and **9** were little different from the parent, and the morpholide **10** was even less soluble. The hydroxyethyl derivative **15** was also (unexpectedly) less soluble than the parent, but the dihydroxypropyl compound **16** was 40-fold more soluble. As expected, ionic side chains provided high solubility (compounds **11**, **12**, and **14**), as did the formally neutral but dipolar *N*-oxide group of compound **17**. The cytotoxicities of most of these compounds against aerobic AA8 cells in the growth inhibition assay did not differ greatly from that of **6**, with the exception of the acid **14** and the *N*-oxide **13** (Table 2). The much lower potencies of these compounds are likely due, at least in part, to low cell uptake. Most of these nitrogen mustards were significantly more active against the UV4 than the AA8 cell line, although wide variations in the HF values were observed (Table 2).

Table 2. Biological Properties of Analogues of 5-[*N*,*N*-Bis(2-chloroethyl)amino]-2,4-dinitrobenzamide

		aerobic growth inhibition			clonogenic assay (UV4)	
			IC_{50} ratios			
no.	solubility (mM)[a]	IC_{50} (μM)[b] AA8	AA8/Walker[c]	AA8/UV4 (HF)[d]	hypoxic CT_{50}[e]	air/N_2[f]
6[g]	0.19	470 ± 50	4.1	6.8 ± 1.1	95 ± 30	58
8	0.20	>100[h]	[i]	[i]	>360[h]	
9[g]	0.13	310 ± 40	<0.3	1.4 ± 0.1	250 ± 80	>20
10[g]	0.03	240 ± 87	4.1	23 ± 5	125 ± 13	1.3 ± 0.3
11	35	220 ± 33	5.6	3.9 ± 0.7	21 ± 4	60 ± 8
12[g]	4.7	920 ± 430	3.7	3.0 ± 0.1	83	45
13[g]	>42	7290 ± 1470	2.3	1.7 ± 0.1	4500 ± 600	8
14	11	6100 ± 1040	1.5	1.1 ± 0.1	12 000 ± 3000	2.3 ± 0.3
15	0.10	>100[h]	[i]	[i]	[i]	
16	7.4	1930 ± 140	1.6	4.4 ± 1.3	465 ± 39	8.6 ± 1.8
17	0.042	57 ± 1	3.1	7.5 ± 1.0	8.0	20
18	0.035	34 ± 2	5.2	2.6 ± 1.1	4.4 ± 0.7	42 ± 2
19	0.53	>200[h]	>3	>4	70	17
20	0.84	945 ± 90	6.0	13 ± 1	48 ± 3	60
21	18	10 600 ± 500	4.7	1.4 ± 0.1	19 000	>1.2
22	0.12	24 ± 1.4	3.2	6.8 ± 1.0	10 ± 1	8.5 ± 1.5
23	0.038	>25[h]	>1	>2	19	>3
24	0.16	2.0 ± 0.7	1.7	1.5 ± 0.5	5.7	2.1
25	32	466 ± 75	1.5	1.0 ± 0.1	980 ± 330	5.2
26		748 ± 172	380	1.4 ± 0.4		
27	0.16	>97	>1	[h]	>300[h]	
28	>38	570 ± 215	5.0	1.9 ± 0.8	390 ± 40	3.5 ± 0.7
7[j]	7.9[k]	563 ± 52	37 000	50 ± 11	225 ± 30	3.6
29[l]		694 ± 122	800	330 ± 110	30	1.7

[a] Solubility (mM) in α-MEM culture medium containing 5% fetal calf serum, determined at ambient temperature by UV spectroscopy. [b] IC_{50} (μM) determined against aerobic AA8 cells as described in the text, using an exposure time of 18 h. Values are means ± SEM. [c] IC_{50} ratio = IC_{50}(AA8)/IC_{50}(Walker cells). Walker cell IC_{50} values were determined using continuous drug exposure under aerobic conditions for 4 days. Ratios are interexperiment means. [d] HF: hypersensitivity factor = IC_{50}(AA8)/IC_{50}(UV4), using an 18-h drug exposure under aerobic conditions for both lines. Values are intraexperiment means ± SEM. [e] CT_{10}: the product of the drug concentration (μM) and exposure time (h) required to reduce cell survival to 10% of that of controls under hypoxic conditions, using UV4 cells at 10^6/mL in the clonogenic assay (see text). Mean ± SEM for separate cultures. [f] Ratio of CT_{10} values in air and N_2 [CT_{10}(air)/CT_{10}(nitrogen)]. Mean ± SEM for separate experiments. [g] Some values taken from ref 17. [h] Inactive at solubility limit. [i] IC_{50} > solubility limit in both lines. [j] Aziridine 7 (CB 1954). [k] Solubility in water, taken from ref 40. [l] Aziridine 29.

The hypoxic selectivities of these compounds also varied widely (from 1.3- to 60-fold) in UV4 cell cultures. As reported previously,[17] the parent mustard 6 shows much higher selectivity than the corresponding aziridine 7. The cationic analogues 11 and 12 retained the high selectivity of 6 for hypoxic cells, but the other readily soluble compounds (13, 14, and 16) showed lower selectivity.

The second series of compounds studied (17–28) explored the effects of more extensive changes in structure, primarily altering mustard reactivity (by changing either the leaving group or the electronic properties of the side-chain substituent). It is well known that the use of better leaving groups on the mustard increases alkylating potency, and we have shown this to be the case for the *in vitro* cytotoxicities of simple aniline mustards.[14] In the present study, the bromo and iodo mustard derivatives 17 and 18 were about 10-fold more potent than 6 in both the growth inhibition and clonogenic assays, but this gain was essentially offset by a drop in solubility of similar magnitude. The low potency of the dimesylate 19 was unexpected, since this should be a more reactive species than the chloro mustard 6. Following a report[24] that mixed chloro/mesylate mustards show greater potency and higher solubility than the corresponding bischloro compounds, we prepared and evaluated the analogues compound 20. This proved to be about 4-fold more soluble than the dichloro analogue 6 and twice as potent in hypoxic UV4 cultures, with equivalent hypoxic selectivity.

The nitrile derivative 22 was 20-fold more potent than 6 in the growth inhibition assay, with little loss in solubility, but had considerably lower hypoxic selectivity. The high potency of this compound is unexpected, given the strong electron-withdrawing effects of the nitrile group. The thioamide 23 proved very insoluble, so that full biological data could not be obtained. The sulfonamide 24 was of particular interest, since this group is both more hydrophilic and more electron-withdrawing than a carboxamide.[31] However, although 24 had only similar aqueous solubility to 6 and surprisingly poor hypoxic selectivity (which was not attributable to enhanced reduction by DT-diaphorase), it was extremely cytotoxic (by far the most potent of all the compounds studied). This potency is very sensitive to cell density, being substantially reduced at 2×10^6 cells/mL (a property not shared by other members of the series). The related *N*-(methylsulfonyl)-benzenesulfonamide 25 was much more soluble, due to the ionizable NH group, but lost potency and did not regain hypoxic selectivity. The mono(2-chloroethyl)amine analogues 26–28 showed similar potencies to those of the corresponding full mustards against AA8 cells. Only 28 was sufficiently soluble to evaluate in the clonogenic assay; its selectivity (3.5-fold) was similar to that of CB 1954 (7).

The relative sensitivity of the compounds to reductive activation by DT-diaphorase was evaluated by determining the ratios of their IC_{50}s against the AA8 and Walker cell lines. The latter is very sensitive to 7, as shown by the AA8/Walker IC_{50} differential of 37 000-fold. It also showed high (but lesser) sensitivity to 29, the only other aziridine tested. In contrast, the mustard analogues of these aziridines (6 and 10) showed ratios close to unity, suggesting they are much less efficient substrates for the Walker cell DT-diaphorase. The mono(2-chloroethyl)amine analogues with an *N*-ethyl group replacing the second *N*-2-chloroethyl function (27 and 28) also did not show significant selectivity for Walker cells, although 27 was not sufficiently

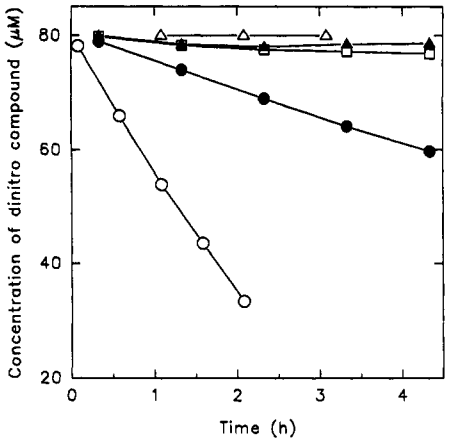


Figure 1. Reduction of dinitrobenzenecarboxamides (0.08 mM) by purified rat (Walker cell) DT-diaphorase (10 IU/mL) with 1.6 mM NADH under aerobic conditions in sodium phosphate buffer, pH 7.0, 20 °C. Concentrations of the parent nitro compounds (○, 7; ●, 29; □, 26; △, 11; ▲, 12) were monitored by HPLC.

soluble to determine its potency in the AA8 cell line. The secondary amine with a single 2-chloroethyl function (26) was 380-fold more toxic against Walker cells than AA8 cells. The intermediate sensitivity of Walker cells to 26 suggests that the steric effect of the bis(2-chloroethyl) substitution of 6 is responsible for inhibition of 2-electron reduction by DT-diaphorase.

Factors other than reduction by DT-diaphorase may influence the relative sensitivities of Walker and AA8 cells to nitro compounds. Rates of reduction by highly purified DT-diaphorase from Walker cells were therefore compared for a subset of compounds (Figure 1). We have previously shown[17] that 6 is reduced by the purified enzyme at a rate at least 100-fold slower than is the corresponding aziridine 7. In the present work, 7 was again used as a standard for evaluating the rates of reduction of selected compounds of Table 1 (Figure 1). The only other compound to show appreciable reduction by the enzyme was the other aziridine (29); the half-mustard 26 was not reduced significantly (Figure 1). These results parallel the sensitivities of Walker cells to these agents (7 > 29 > 26; Table 2). The two full mustards 11 and 12 were also not reduced by the purified enzyme, consistent with the low sensitivity of Walker cells to these compounds.

On the basis of the sensitivity of Walker cells, none of the nitrophenyl mustards 6–25 are activated appreciably by DT-diaphorase. While there may be some species differences in DT-diaphorase substrate specificity, as demonstrated[32] for 7, it appears unlikely that the large differences in hypoxic selectivity observed in UV4 cultures (1.3–60-fold) are due to variations in sensitivity to aerobic nitroreduction by this enzyme.

The hypersensitivity of UV4 cells to CB 1954 (7; HF value = 50) was in the range (8–200-fold) expected for DNA cross-linking agents, consistent with its proposed mechanism of toxicity.[20,29] In contrast, almost all of the dinitrophenyl mustards gave HF values <8 (e.g., 3.9 ± 0.7 for 11), suggesting that DNA cross-links may not be responsible for the aerobic toxicity of these compounds. However, rates of killing by 11 under hypoxic conditions by clonogenic assay indicated an 18-fold greater sensitivity of UV4 than AA8 cells (CT_{10} 375 ± 34 μM h for AA8 versus 21 ± 4 for UV4), consistent with activation of 11 to a DNA cross-linking agent under hypoxic conditions.

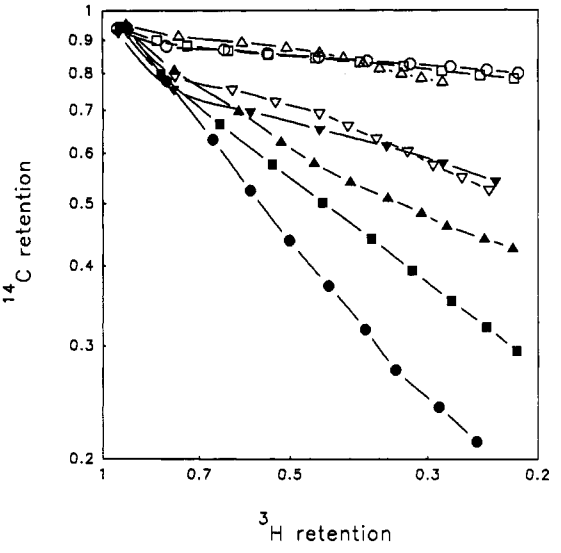


Figure 2. Demonstration of DNA interstrand cross-links in UV4 cells treated with 11 for 5 h under hypoxic conditions. [^{14}C]-TdR-labeled cells received no drug treatment (○, ●) or were treated with 11 at 20 μM (□, ■), 100 μM (△, ▲), or 500 μM (od, sd) before assaying by alkaline elution. Filled symbols represent samples which received cobalt-60 irradiation (3 Gy) immediately before elution. The abscissa indicates retention of irradiated (3 Gy) [^{3}H]TdR-labeled cells used as internal standard.

The ability of 11 to generate DNA interstrand cross-links in hypoxic AA8 cells was tested more directly using alkaline elution (Figure 2). Treatment with 11 at 500 μM for 5 h resulted in DNA breakage as demonstrated by an increased elution rate, although no breakage was observed at ≤100 μM. Treatment with 11 markedly retarded elution of irradiated DNA, providing clear evidence for concentration-dependent DNA cross-linking. Thus, despite the low HF value for this compound under aerobic conditions, it does act as a DNA cross-linking agent after reductive activation under hypoxia.

Overall, 11 was the best of the analogues evaluated with respect to solubility, potency, and hypoxic cell selectivity *in vitro* and was therefore evaluated *in vivo*. The maximum tolerated dose (MTD) of 11 in C_3H/HeN mice was 240 μmol/kg when given as a single ip injection. Figure 3 shows the effect of 11 on KHT tumors, assayed by excision and clonogenic assay 18 h after treatment, when administered alone or 5 min after γ-irradiation (15 Gy). It caused substantial tumor cell killing only at doses above the MTD. Killing due to the drug was about 5-fold greater in combination with radiation than for the drug alone, suggesting some selectivity for hypoxic cells, although this difference was not statistically significant. Cell killing by 11 in the absence of radiation indicates activity against oxygenated as well as hypoxic cells. We are currently investigating whether this reflects efficient release of a diffusible activated mustard from hypoxic microenvironments or whether the observed cell killing is independent of tumor hypoxia.

Conclusions

This work sought to identify analogues of the novel HSC 5-[*N*,*N*-bis(2-chloroethyl)amino]-2,4-dinitrobenzamide (6) which retained its high selectivity for hypoxic cells *in vitro* but had increased potency and/or aqueous solubility. To this end, the composition of the carboxamide chain (compounds 8–16) or the reactivity of the mustard (compounds 17–25) was varied. The aqueous solubilities

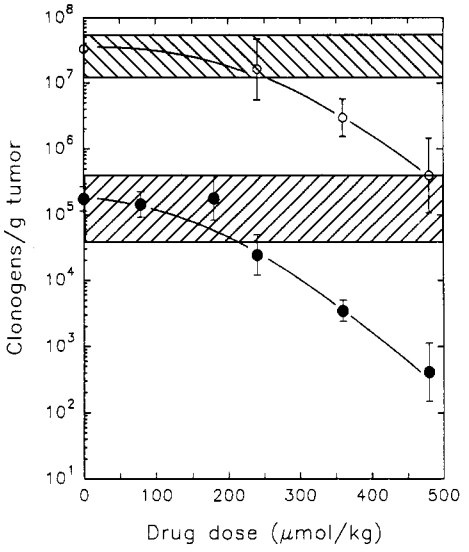


Figure 3. Activity of 11 against KHT tumors in C_3H/HeN mice. The drug was administered ip alone (○) or 5 min after irradiation (●; 15 Gy, ^{60}Co). The ordinate is the product of cell yield/g of tumor and plating efficiency, determined 18 h after treatment. Points are geometrical means, and error bars are SEM for two to four separate experiments, each experiment using two to four pooled tumors per dose. The upper and lower hatched bands represent the 95% confidence limits for historical control data for untreated tumors and non-drug-treated irradiated tumors, respectively.

of the compounds studied were broadly predictable from structure, with ionizable or very dipolar groups providing the most soluble analogues (e.g., **11–14**, **21**, and **25**). The neutral diol **16** also had acceptable solubility. However, many of these solubilizing groups also lowered cytotoxic potency. The hypoxic cell selectivity of the compounds varied markedly. No general structure–activity relationships for hypoxic selectivity could be discerned.

Despite marked variations in hypoxic selectivities, the mustard compounds all appeared to be poor substrates for Walker cell DT-diaphorase, with IC_{50}(AA8)/IC_{50}(Walker) ratios of 1.5–6-fold [compared with 37 000 for **7**, 800 for the dinitroaziridine **29**, and 380 for the mono(2-chloroethyl)amine **26**]. The lack of sensitivity of the mustards to reduction by DT-diaphorase, at least in rat Walker cells, is conceptually a desirable feature of these compounds, relative to the (dinitrophenyl)aziridines, in the context of their development as HSCs. However, it appears that this factor alone cannot account for the large differences in hypoxic selectivity observed within the dinitrophenyl mustard series. The low efficiency of DT-diaphorase in reducing nitro compounds, relative to quinones, quinoneimines, and nitroso compounds,[32–34] makes it unclear whether this enzyme contributes significantly to the aerobic activation of **7** in other than rat cells. The hypoxic selectivity of quinone bioreductive drugs is markedly influenced by their susceptibility to reductive activation by DT-diaphorase,[35] but for nitro compounds, the contribution of toxicity from non-bioreductive mechanisms may be a more important determinant.[36] Although not systematically investigated in the present study, the apparently lower UV4 HF factor for **11** under aerobic conditions compared to that under hypoxic conditions suggests that a non-bioreductive mechanism of toxicity quite distinct from cross-link formation may contribute to aerobic toxicity. It is possible that this phenomenon (rather than oxygen-insensitive bioreduction) might be responsible for the observed variations in hypoxic selectivity.

Compound **11** showed the best *in vitro* profile of the analogues evaluated and was active against KHT tumors in mice when given as a single dose (Figure 3). However, activity was seen only at doses which would eventually be lethal to the host, and the similarity of effect with and without radiation makes it unclear whether its (limited) activity *in vivo* is dependent on bioreduction. Thus, despite the favorable biological profile of **11** in cell culture, there remains a clear need for analogues with higher therapeutic ratios *in vivo*.

Experimental Section

Chemistry. Analyses indicated by symbols of the elements were within ±0.4% of theoretical and were carried out in the Microchemical Laboratory, University of Otago, Dunedin, NZ. Melting points were determined on an Electrothermal 2300 melting point apparatus. NMR spectra were obtained on Bruker AC-200 or AM-400 spectrometers and are referenced to Me_4Si. Thin-layer chromatography was carried out on aluminum-backed silica gel plates (Merck 60 F_{254}). Flash column chromatography was carried out on Merck silica gel (230–400 mesh). Petroleum ether refers to the fraction boiling at 40–60 °C. All compounds were checked for purity by reverse-phase HPLC with diode array detection.

5-[*N,N*-Bis(2-chloroethyl)amino]-2,4-dinitrobenzoic Acid (21) and Derivatives. Methyl 5-chloro-2,4-dinitrobenzoate[21] (**30**) (1.24 g, 4.76 mmol) was treated with diethanolamine (1.00 g, 9.52 mmol) in dioxane (50 mL) at 50 °C for 3 h, and the residue after workup was chromatographed on silica gel. Elution with EtOAc gave methyl 5-[*N,N*-bis(2-hydroxyethyl)amino]-2,4-dinitrobenzoate (**31**) (1.52 g, 97%), mp (EtOAc/petroleum ether) 104–106 °C. ^1H NMR ($CDCl_3$): δ 8.48 (s, 1 H, H-3), 7.45 (s, 1 H, H-6), 3.95 (s, 3 H, OCH_3), 3.81 (t, J = 5.0 Hz, 4 H, CH_2OH), 3.60 (t, J = 5.0 Hz, 4 H, CH_2N), 2.20 (br, 2 H, OH). ^{13}C NMR: δ 165.89 (*C*OOMe), 147.53 (C-5), 138.67, 136.21 (C-2,4), 133.30 (C-1), 124.22, 121.65 (C-3,6), 59.34 (CH_2OH), 54.59 (CH_2N), 53.74 (OCH_3). Anal. ($C_{12}H_{15}N_3O_8$) C, H, N.

A solution of **31** (1.50 g, 4.56 mmol) and Et_3N (1.33 mL, 9.57 mmol) in dry CH_2Cl_2 (60 mL) was treated with MsCl (0.73 mL, 9.34 mmol) at 0 °C and poured into saturated aqueous $NaHCO_3$ after 15 min. Dilution with CH_2Cl_2 and workup gave crude dimesylate, which was treated directly with LiCl (10.0 g) in DMF (40 mL) at 120 °C for 30 min. The mixture was poured into brine, extracted with EtOAc, worked up, dried over Na_2SO_4, and chromatographed on silica gel. Elution with EtOAc/petroleum ether (4:1) gave methyl 5-[*N,N*-bis(2-chloroethyl)amino]-2,4-dinitrobenzoate (**33**) (1.34 g, 80%), mp (EtOAc/petroleum ether) 136.5–138 °C. ^1H NMR ($CDCl_3$): δ 8.54 (s, 1 H, H-3), 7.34 (s, 1 H, H-6), 3.97 (s, 3 H, OCH_3), 3.68 (br s, 8 H, NCH_2CH_2Cl). ^{13}C NMR: δ 165.30 ($COOCH_3$), 147.22 (C-5), 139.81, 137.82 (C-2,4), 133.39 (C-1), 124.33, 121.77 (C-3,6), 53.86 (OCH_3), 53.78 (CH_2N), 40.69 (CH_2Cl). Anal. ($C_{12}H_{13}Cl_2N_3O_6$) C, H, N, Cl.

Hydrolysis of **33** (4.51 g, 12 mmol) with 4 N KOH (20 mL) in dioxane (60 mL) at 20 °C for 15 min followed by acidification with 3 N HCl and extraction with EtOAc gave a quantitative yield of 5-[*N,N*-bis(2-chloroethyl)amino]-2,4-dinitrobenzoic acid (**21**), mp (EtOAc/petroleum ether) 125–127 °C. ^1H NMR [$(CD_3)_2CO$]: δ 8.56 (s, 1 H, H-3), 7.72 (s, 1 H, H-6), 3.89–3.86 (br, 8 H, NCH_2CH_2Cl) (COOH too broad to observe). ^{13}C NMR: δ 166.02 (COOH), 148.52 (C-5), 140.25, 138.09 (C-2,4), 134.72 (C-1), 124.87, 122.58 (C-3,6), 54.12 (CH_2N), 42.12 (CH_2Cl). Anal. ($C_{11}H_{11}Cl_2N_3O_6 \cdot {}^1/_2EtOAc$) C, H, N, Cl (signals for $^1/_2$EtOAc seen in ^1H and ^{13}C NMR spectra).

Heating of **21** (0.40 g, 1.14 mmol) in $SOCl_2$ (20 mL) containing a drop of DMF under reflux for 20 min followed by concentration to dryness and azeotroping with benzene gave crude 5-[*N,N*-bis(2-chloroethyl)amino]-2,4-dinitrobenzoyl chloride, which was dissolved in Me_2CO (20 mL), cooled to 0 °C, and treated with excess aqueous methylamine (10 mL of a 40% solution in water). After 15 min, the mixture was extracted with EtOAc, and the

product after workup was purified by chromatography on silica gel. Elution with EtOAc/petroleum ether (1:1) gave 5-[*N,N*-bis(2-chloroethyl)amino]-*N*-methyl-2,4-dinitrobenzamide (**8**) (0.31 g, 75%), mp (EtOAc/petroleum ether) 180.5 °C. ^{1}H NMR [$(CD_3)_2CO$]: δ 8.53 (s, 1 H, H-3), 7.75 (br, 1 H, CONH), 7.56 (s, 1 H, H-6), 3.88–3.80 (m, 8 H, NCH_2CH_2Cl), 2.90, 2.89 (2 s, 3 H, CONHMe). ^{13}C NMR: δ 165.89 (CONH), 148.43 (C-5), 140.05, 138.26 (C-2,4), 138.94 (C-1), 125.05, 122.45 (C-3,6), 54.20 (CH_2N), 42.10 (CH_2Cl), 26.69 ($CONHCH_3$). Anal. ($C_{12}H_{14}Cl_2N_4O_5$) C, H, N, Cl.

Similar treatment of the acid chloride with the appropriate amines gave the following compounds:

5-[*N,N*-Bis(2-chloroethyl)amino]-*N,N*-dimethyl-2,4-dinitrobenzamide (9) (65% yield), mp ($CHCl_3$/petroleum ether) 130.5 °C. ^{1}H NMR ($CDCl_3$): δ 8.61 (s, 1 H, H-3), 7.07 (s, 1 H, H-6), 3.62 (s, 8 H, NCH_2CH_2Cl), 3.07, 2.78 (2 × 3 H, $CON(CH_3)_2$). ^{13}C NMR: δ 166.12 ($CON(CH_3)_2$), 148.33 (C-5), 138.88, 135.75 (C-2,4), 137.82 (C-1), 124.92, 120.00 (C-3,6), 53.55 (CH_2N), 40.98 (CH_2Cl), 38.06, 34.85 ($CON(CH_3)_2$). Anal. ($C_{13}H_{16}Cl_2N_4O_5$) C, H, N.

5-[*N,N*-Bis(2-chloroethyl)amino]-*N*-[2-(4-morpholino)ethyl]-2,4-dinitrobenzamide (12) (72% yield) as a gum. The hydrochloride salt crystallized from MeOH/^{i}Pr$_2$O, mp 119–120 °C. ^{1}H NMR (D_2O): δ 8.81 (s, 1 H, H-3), 7.59 (s, 1 H, H-6), 4.07 (br, NH and HCl), 3.91 (t, *J* = 6.0 Hz, 4 H, $N^+CH_2CH_2O$), 3.86–3.81 (m, 10 H, NCH_2CH_2Cl and $CH_2CH_2N^+$), 3.56 (t, *J* = 6.0 Hz, 6 H, $N^+CH_2CH_2O$ and $CONHCH_2$). ^{13}C NMR: δ 171.17 (CONH), 151.69 (C-5), 140.61, 137.28 (C-2,4), 138.12 (C-1), 128.53, 123.48 (C-3,6), 66.30 ($N^+CH_2CH_2O$), 58.07 (CH_2N^+), 55.48 (CH_2N), 54.65 ($N^+CH_2CH_2O$), 44.09 (CH_2Cl), 36.84 ($CONHCH_2$). Anal. ($C_{17}H_{23}Cl_2N_5O_6$·HCl·0.5H_2O) C, H, N, Cl.

5-[*N,N*-Bis(2-chloroethyl)amino]-*N*-(2-hydroxyethyl)-2,4-dinitrobenzamide (15) (85% yield), mp (EtOAc/petroleum ether) 157–158 °C. ^{1}H NMR [$(CD_3)_2SO$]: δ 8.73 (t, *J* = 5.5 Hz, 1 H, CONH), 8.53 (s, 1 H, H-3), 7.45 (s, 1 H, H-6), 4.76 (t, *J* = 5.5 Hz, 1 H, OH), 3.82 (t, *J* = 6.0 Hz, 4 H, CH_2Cl), 3.68 (t, *J* = 6.0 Hz, 4 H, CH_2N), 3.54 (dt, *J* = 6.1, 5.5 Hz, 2 H, CH_2OH), 3.31 (dt, *J* = 6.1, 5.5 Hz, 2 H, $CONHCH_2$). ^{13}C NMR: δ 164.44 (CONH), 147.11 (C-5), 137.87, 136.27 (C-2,4), 137.32 (C-1), 124.24, 121.01 (C-3,6), 59.32 (CH_2OH), 52.44 (CH_2N), 41.93 ($CONHCH_2$), 41.65 (CH_2Cl). Anal. ($C_{13}H_{16}Cl_2N_2O_6$) C, H, N, Cl.

5-[*N,N*-Bis(2-chloroethyl)amino]-*N*-(2,3-dihydroxypropyl)-2,4-dinitrobenzamide (16) (59% yield) as a hygroscopic yellow foam. ^{1}H NMR [$(CD_3)_2SO$]: δ 8.70 (t, *J* = 5.7 Hz, 1 H, CONH), 8.53 (s, 1 H, H-3), 7.44 (s, 1 H, H-6), 4.83 (d, *J* = 4.9 Hz, 1 H, CHO*H*), 4.56 (t, *J* = 5.7 Hz, 1 H, CH_2OH), 3.83 (t, *J* = 5.9 Hz, 4 H, CH_2Cl), 3.68 (t, *J* = 5.9 Hz, 4 H, CH_2N), 3.50–3.06 (m, 5 H, $CONHCH_2CHOHCH_2OH$). ^{13}C NMR: δ 164.61 (CONH), 147.12 (C-5), 37.94, 136.40 (C-2,4), 137.40 (C-1), 124.29, 121.18 (C-3,6), 70.08 (CHOH), 63.77 (CH_2OH), 52.55 (CH_2N), 42.79 ($CONHCH_2$), 41.72 (CH_2Cl). Anal. ($C_{14}H_{18}Cl_2N_4O_7$) C, H, Cl; N: calcd, 12.6; found, 13.2.

5-[*N,N*-Bis(2-chloroethyl)amino]-*N*-(2-carboxyethyl)-2,4-dinitrobenzamide (14). Treatment of crude 5-[*N,N*-bis(2-chloroethyl)amino]-2,4-dinitrobenzoyl chloride (0.90 g, 2.50 mmol) in CH_2Cl_2 (20 mL) with a slurry of β-alanine methyl ester hydrochloride (0.70 g, 5.00 mmol) and Et_3N (1.04 mL, 7.50 mmol) in CH_2Cl_2 (20 mL) at 20 °C for 2 h gave crude 5-[*N,N*-bis(2-chloroethyl)amino]-*N*-[2-(methoxycarbonyl)ethyl]-2,4-dinitrobenzamide as a yellow oil. This was immediately dissolved in THF (25 mL) and treated with aqueous KOH (10 mL of 2 N) at 20 °C for 3 h. The solution was then acidified with concentrated HCl, extracted with EtOAc, and worked up as usual. Chromatography on silica gel, eluting with EtOAc, gave 5-[*N,N*-bis(2-chloroethyl)amino]-*N*-(2-carboxyethyl)-2,4-dinitrobenzamide (**14**) (0.72 g, 68%), mp (EtOAc/petroleum ether) 150–153 °C. ^{1}H NMR [$(CD_3)_2SO$]: δ 12.27 (br, 1 H, COOH), 8.79 (t, *J* = 5.6 Hz, 1 H, CONH), 8.54 (s, 1 H, H-3), 7.42 (s, 1 H, H-6), 3.83 (t, *J* = 6.0 Hz, 2 H, CH_2Cl), 3.68 (t, *J* = 6.0 Hz, 4 H, CH_2N), 3.44 (dt, *J* = 6.9, 5.6 Hz, 2 H, $CONHCH_2$), 2.53 (t, *J* = 6.9 Hz, 2 H, CH_2COOH). ^{13}C NMR: δ 172.74 (COOH), 164.53 (CONH), 147.21 (C-5), 137.94, 136.13 (C-2,4), 137.29 (C-1), 124.37, 120.93 (C-3,6), 52.58 (CH_2N), 41.72 (CH_2Cl), 35.24, 33.19 ($CONHCH_2CH_2$). Anal. ($C_{14}H_{16}Cl_2N_4O_7$) C, H, N, Cl.

5-[*N,N*-Bis[2-[(methylsulfonyl)oxy]ethyl]amino]-2,4-dinitrobenzamide (19) and Analogues. MsCl (5.17 mL, 0.067 mol) was added dropwise to a cooled (0 °C) solution of 5-[*N,N*-bis(2-hydroxyethyl)amino]-2,4-dinitrobenzamide (**36**)[17] (10.00 g, 0.032 mol) in dry pyridine (150 mL). After a further 10 min at 0 °C, the solution was stirred at 20 °C for 30 min and then volatiles were removed under reduced pressure at below 40 °C. The residue was partitioned between EtOAc and water, and the organic layer was washed with water and worked up to give a low-melting solid which was chromatographed on silica gel. Elution with EtOAc gave foreruns followed by 5-[*N,N*-bis[2-[(methylsulfonyl)oxy]ethyl]amino]-2,4-dinitrobenzamide (**19**) (11.05 g, 74%), mp (EtOAc/petroleum ether; sealed tube) 140–144 °C. ^{1}H NMR [$(CD_3)_2CO$]: δ 8.54 (s, 1 H, H-3), 7.65 (s, 1 H, H-6), 4.49 (t, *J* = 5.3 Hz, 4 H, $CH_2OSO_2CH_3$), 3.89 (t, *J* = 5.3 Hz, 4 H, CH_2N), 3.09 (s, 6 H, SO_2CH_3), 2.90 (br s, NH_2). ^{13}C NMR: δ 167.01 ($CONH_2$), 148.56 (C-5), 140.45, 138.44 (C-2,4), 138.57 (C-1), 124.92, 123.06 (C-3,6), 67.65 ($CH_2OSO_2CH_3$), 52.02 (CH_2N), 37.23 (SO_2CH_3). Anal. ($C_{13}H_{18}N_4O_{11}S_2$) C, H, N, S.

A solution of **19** (1.00 g, 2.12 mmol) in dry DMF (50 mL) was treated with solid NaBr (10.0 g), warmed to 120 °C with vigorous stirring for 15 min and then concentrated to dryness under reduced pressure. The residue was partitioned between EtOAc and water, and the organic portion was washed well with 3 N HCl and worked up. Chromatography of the residue on silica gel and elution with EtOAc/petroleum ether (1:1) gave 5-[*N,N*-bis(2-bromoethyl)amino]-2,4-dinitrobenzamide (**17**) (0.73 g, 78%), mp (EtOAc/petroleum ether) 126–128 °C. ^{1}H NMR [$(CD_3)_2CO$]: δ 8.53 (s, 1 H, H-3), 7.58 (s, 1 H, H-6), 3.89 (t, *J* = 6.6 Hz, 4 H, CH_2N), 3.73 (t, *J* = 6.6 Hz, 4 H, CH_2Br), 2.93 (br, 2 H, $CONH_2$). ^{13}C NMR: δ 167.20 ($CONH_2$), 147.89 (C-5), 140.06, 138.26 (C-2,4), 138.75 (C-1), 124.95, 122.30 (C-3,6), 54.11 (CH_2N), 29.89 (CH_2Br). Anal. ($C_{11}H_{12}Br_2N_4O_5$) C, H, N. A similar reaction using NaI gave 5-[*N,N*-bis(2-iodoethyl)amino]-2,4-dinitrobenzamide (**18**) in 92% yield, mp (EtOAc/petroleum ether) 170–172 °C. ^{1}H NMR [$(CD_3)_2CO$]: δ 8.52 (s, 1 H, H-3), 7.55 (s, 1 H, H-6), 3.84 (t, *J* = 7.2 Hz, 4 H, CH_2N), 3.50 (t, *J* = 7.2 Hz, 4 H, CH_2I), 2.88 (br, 2 H, $CONH_2$). ^{13}C NMR: δ 167.09 ($CONH_2$), 147.17 (C-5), 140.08, 138.33 (C-2,4), 138.74 (C-1), 124.94, 122.31 (C-3,6), 54.89 (CH_2N), 1.72 (CH_2I). Anal. ($C_{11}H_{12}I_2N_4O_5$) H, N; C: calcd, 24.7; found, 25.5; I: calcd, 47.5; found, 46.9.

5-[*N*-(2-Chloroethyl)-*N*-[2-[(methylsulfonyl)oxy]ethyl]amino]-2,4-dinitrobenzamide (20). A solution of *tert*-butyldimethylsilyl chloride (1.00 g, 6.68 mmol) in DMF (10 mL) was added to a solution of **36** (1.00 g, 3.18 mmol) and imidazole (0.54 g, 7.95 mmol) in DMF (60 mL). After stirring at 20 °C for 18 h, the solution was concentrated to dryness under reduced pressure, the residue was partitioned between EtOAc and brine and worked up, and the oily residue was chromatographed on silica gel. After foreruns with EtOAc/petroleum ether (1:9), elution with EtOAc/petroleum ether (1:1) gave 5-[*N,N*-bis[2-[(*tert*-butyldimethylsilyl)oxy]ethyl]amino]-2,4-dinitrobenzamide (**39**) (0.65 g, 38%), mp (EtOAc/petroleum ether) 127–129 °C. ^{1}H NMR ($CDCl_3$): δ 8.57 (s, 1 H, H-3), 7.26 (s, 1 H, H-6), 5.95, 5.65 (2 × br, 2 H, $CONH_2$), 3.79 (t, *J* = 5.4 Hz, 4 H, CH_2O), 3.51 (t, *J* = 5.4 Hz, 4 H, CH_2N), 0.82 (s, 18 H, $SiC(CH_3)_3$), 0.00 (s, 12 H, $Si(CH_3)_2$). ^{13}C NMR: δ 167.36 (CONH), 148.72 (C-5), 137.66 (C-2), 136.31 (C-1), 133.97 (C-4), 125.09 (C-3), 119.44 (C-6), 60.55 (CH_2O), 54.89 (CH_2N), 25.70 ($C(CH_3)_3$), 18.06 ($C(CH_3)_3$), −5.57 ($SiCH_3$). Anal. ($C_{23}H_{42}N_4O_7Si_2$·0.5H_2O) C, H, N.

Elution with EtOAc gave 5-[*N*-(2-hydroxyethyl)-*N*-[2-[(*tert*-butyldimethylsilyl)oxy]ethyl]amino]-2,4-dinitrobenzamide (**40**) (0.60 g, 44%), mp (EtOAc/petroleum ether) 152–154 °C. ^{1}H NMR ($CDCl_3$): δ 8.53 (s, 1 H, H-3), 7.32 (s, 1 H, H-6), 6.27, 6.12 (2 × br, 2 H, $CONH_2$), 3.82 (br, 2 H, CH_2OH), 3.75 (t, *J* = 5.0 Hz, 2 H, CH_2OSi), 3.63 (t, *J* = 5.0 Hz, 2 H, CH_2N), 3.49 (t, *J* = 5.0 Hz, 2 H, CH_2N), 0.80 (s, 9 H, $SiC(CH_3)_3$), −0.01 (s, 6 H, $Si(CH_3)_2$). ^{13}C NMR: δ 167.69 (CONH), 148.37 (C-5), 138.09 (C-2), 136.44 (C-1), 134.59 (C-4), 124.85 (C-3), 120.46 (C-6), 60.40, 59.50 (CH_2O), 54.94, 54.60 (CH_2N), 25.67 ($C(CH_3)_3$), 18.09 ($C(CH_3)_3$), −5.65 ($SiCH_3$). Anal. ($C_{17}H_{28}N_4O_7Si$) C, H, N.

A solution of **40** (0.40 g, 0.93 mmol) and Et_3N (195 μL, 1.40 mmol) in CH_2Cl_2 (5 mL) was treated with MsCl (79 μL, 1.03 mmol) at 20 °C. After 10 min, the solution was washed with water and worked up, and the resulting crude mesylate (**41**) was dissolved in DMF (20 mL) containing LiCl (5.0 g) and heated to 125 °C for 20 min. Solvent was then removed under reduced pressure, the residue was partitioned between EtOAc and water,

and the organic residue was chromatographed on silica gel. Elution with EtOAc/MeOH (19:1) gave 5-[*N*-(2-chloroethyl)-*N*-(2-hydroxyethyl)amino]-2,4-dinitrobenzamide (**42**) (0.27 g, 87%) as a waxy solid. A solution of this crude product (0.40 g, 0.93 mmol) and Et_3N (175 μL, 1.21 mmol) in dry THF (10 mL) was treated with MsCl (69 μL, 0.89 mmol) for 10 min at 20 °C and then worked up as usual, and the residue was chromatographed on silica gel. Elution with EtOAc gave 5-[*N*-(2-chloroethyl)-*N*-[2-[(methylsulfonyl)oxy]ethyl]amino]-2,4-dinitrobenzamide (**20**) (0.26 g, 78%), mp (EtOAc/petroleum ether) 103–104.5 °C. ^{1}H NMR [$(CD_3)_2SO$]: δ 8.51 (s, 1 H, H-3), 8.13, 7.82 (2 × br, 2 H, $CONH_2$), 7.46 (s, 1 H, H-6), 4.33 (t, *J* = 5.0 Hz, 2 H, CH_2OMs), 3.81 (t, *J* = 6.0 Hz, 2 H, CH_2Cl), 3.72 (t, *J* = 5.0 Hz, 2 H, CH_2N), 3.64 (t, *J* = 6.0 Hz, 2 H, CH_2N), 3.12 (s, 3 H, SO_2CH_3). ^{13}C NMR: δ 165.99 ($CONH_2$), 147.04 (C-5), 137.92, 137.47, 136.20 (C-1,2,4), 124.17, 120.84 (C-3,6), 66.70 (CH_2OMs), 52.90, 49.68 (CH_2N), 41.45 (CH_2Cl), 36.51 (SO_2CH_3). Anal. ($C_{12}H_{15}ClN_4O_8S$) C, H, N.

5-[*N*,*N*-Bis(2-chloroethyl)amino]-2,4-dinitrobenzonitrile (22) and 5-[*N*,*N*-Bis(2-chloroethyl)amino]-2,4-dinitrothiobenzamide (23). A solution of 5-[*N*,*N*-bis(2-chloroethyl)amino]-2,4-dinitrobenzamide (**6**)[17] (0.20 g, 0.57 mmol) in $SOCl_2$ (5 mL) was heated at reflux under N_2 for 84 h. Excess $SOCl_2$ was removed under reduced pressure, and the residue was chromatographed on silica gel. Elution with EtOAc/petroleum ether (3:7) gave 5-[*N*,*N*-bis(2-chloroethyl)amino]-2,4-dinitrobenzonitrile (**22**) (0.16 g, 87%), mp ($CHCl_3$) 127 °C. ^{1}H NMR [$(CD_3)_2CO$]: δ 8.79 (s, 1 H, H-3), 8.19 (s, 1 H, H-6), 3.93 (s, 8 H, NCH_2CH_2Cl). ^{13}C NMR: δ 148.55 (C-5), 140.98, 139.07 (C-2,4), 129.50, 126.10 (C-3,6), 115.05 (C-1), 112.84 (CN), 54.18 (CH_2N), 42.20 (CH_2Cl). Anal. ($C_{11}H_{10}Cl_2N_4O_4$) C, H, N.

A solution of **6** (0.50 g, 1.42 mmol) in dioxane (20 mL) was treated with P_2S_5 (0.63 g, 2.84 mmol) and $NaHCO_3$ (0.24 g, 2.84 mmol) and then heated under reflux with stirring for 3 h. The residue after workup was chromatographed on silica gel, and elution with EtOAc/petroleum ether (1:4) gave 5-[*N*,*N*-bis(2-chloroethyl)amino]-2,4-dinitrothiobenzamide (**23**) (0.48 g, 92%), mp (EtOAc/petroleum ether) 166–167 °C. ^{1}H NMR [$(CD_3)_2SO$]: δ 9.50, 9.34 (2 × br, 2 H, $CSNH_2$), 8.50 (s, 1 H, H-3), 7.45 (s, 1 H, H-6), 3.87, 3.82 (2 × m, 8 H, NCH_2CH_2Cl). ^{13}C NMR: δ 199.46 ($CSNH_2$), 148.08 (C-5), 143.87 (C-1), 139.51, 136.99 (C-2,4), 125.23, 121.36 (C-3,6), 54.23 (CH_2N), 42.02 (CH_2Cl). Anal. ($C_{11}H_{12}Cl_2N_4O_4S$) C, H, N, S.

5-[*N*,*N*-Bis(2-chloroethyl)amino]-*N*-[(*N*^{1},*N*^{1}-dimethylamino)ethyl]-2,4-dinitrobenzamide *N*^{1}-Oxide (13). A solution of the free base of 5-[*N*,*N*-bis(2-chloroethyl)amino]-*N*-[(*N*^{1},*N*^{1}-dimethylamino)ethyl]-2,4-dinitrobenzamide (**11**)[17] (0.50 g, 1.18 mmol) in CH_2Cl_2 (15 mL) was treated with a solution of 2-(phenylsulfonyl)-3-phenyloxaziridine[22] (0.32 g, 1.24 mmol) in CH_2Cl_2 (3 mL). After 10 min, petroleum ether (10 mL) was added, the solution was cooled at −30 °C overnight, and the resulting solid was dried under high vacuum to give 5-[*N*,*N*-bis(2-chloroethyl)amino]-*N*-[(*N*^{1},*N*^{1}-dimethylamino)ethyl]-2,4-dinitrobenzamide *N*^{1}-oxide (**13**) as a hygroscopic foam. Treatment with MeOH/HCl gave the hydrochloride salt (0.36 g, 64%), mp (MeOH/^{i}Pr_2O) 165–169 °C. ^{1}H NMR [D_2O/$(CD_3)_2SO$]: δ 8.82 (s, 1 H, H-3), 7.46 (s, 1 H, H-6), 3.98, 3.93 (2 t, *J* = 5.1 Hz, 2 × 2 H, CH_2CH_2), 3.82 (t, *J* = 4.7 Hz, 4 H, CH_2Cl), 3.77 (t, *J* = 4.7 Hz, 4 H, CH_2N), 3.57 (s, 6 H, $N(CH_3)_2$). ^{13}C NMR: δ 170.71 (CONH), 151.63 (C-5), 140.68, 137.31 (C-3,6), 69.20 ($CH_2N(O)Me_2$), 59.03 ($N(O)Me_2$), 55.40 (CH_2N), 44.06 (CH_2Cl), 36.45 ($CONHCH_2$). Anal. ($C_{15}H_{21}Cl_2N_5O_6$·HCl) C, H, N, Cl.

5-[*N*,*N*-Bis(2-chloroethyl)amino]-2,4-dinitrobenzenesulfonamide (24). A solution of 5-chloro-2,4-dinitrobenzenesulfonamide[23] (**37**) (1.55 g, 5.5 mmol) and diethanolamine (1.16 g, 11 mmol) in dioxane (100 mL) was held at 60 °C for 1.5 h and then adsorbed directly onto silica gel by concentration under reduced pressure. Chromatography on silica gel, eluting with EtOAc/MeOH (19:1) gave 5-[*N*,*N*-bis(2-hydroxyethyl)amino]-2,4-dinitrobenzenesulfonamide (**38**) (1.91 g, 99%), mp (EtOAc/petroleum ether) 138–139 °C. ^{1}H NMR [$(CD_3)_2SO$]: δ 8.53 (s, 1 H, H-3), 7.97 (s, 1 H, H-6), 7.93 (br s, 2 H, SO_2NH_2), 4.88 (t, *J* = 5.1 Hz, 2 H, OH), 3.65 (dt, *J* = 5.2, 5.1 Hz, 4 H, CH_2OH), 3.49 (t, *J* = 5.1 Hz, 4 H, NCH_2). ^{13}C NMR: δ 146.84 (C-5), 140.53 (C-1), 136.84, 134.39 (C-2,4), 125.70 (C-3), 120.88 (C-6), 58.03 (CH_2OH), 54.27 (NCH_2). Anal. ($C_{10}H_{14}N_4O_8S$) C, H, N, S.

A stirred solution of **38** (1.90 g, 5.43 mmol) and Et_3N (1.90 mL, 14 mmol) in dry THF (60 mL) was treated dropwise at 0 °C with MsCl (0.89 mL, 11 mmol). After a further 15 min, the solution was diluted with EtOAc, washed well with water, and worked up to give the crude dimesylate. This was immediately dissolved in DMF (50 mL) containing LiCl (20 g) and the mixture stirred at 130 °C for 15 min before solvent was removed under reduced pressure. The residue was partitioned between EtOAc and water, and the organic layer was worked up and chromatographed on silica gel. Elution with EtOAc gave 5-[*N*,*N*-bis(2-chloroethyl)-amino]-2,4-dinitrobenzenesulfonamide (**24**) (1.47 g, 64%), mp (EtOAc/petroleum ether) 153–154 °C. ^{1}H NMR [$(CD_3)_2CO$]: δ 8.54 (s, 1 H, H-3), 7.99 (br s, 2 H, SO_2NH_2), 7.91 (s, 1 H, H-6), 3.84 (t, *J* = 6.0 Hz, 4 H, CH_2Cl), 3.67 (t, *J* = 6.0 Hz, 4 H, CH_2N). ^{13}C NMR: δ 146.13 (C-5), 140.64 (C-1), 138.86, 136.93 (C-2,4), 124.95, 121.77 (C-3,6), 52.48 (CH_2N), 41.62 (CH_2Cl). Anal. ($C_{10}H_{12}Cl_2N_4S_2O_6$) C, H, N, S.

Treatment of **38** with 4 equiv of MsCl and Et_3N in THF at 20 °C followed by reaction with LiCl as above gave 5-[*N*,*N*-bis(2-chloroethyl)amino]-*N*-(methylsulfonyl)-2,4-dinitrobenzenesulfonamide (**25**), mp (EtOAc/petroleum ether) 206–208 °C. ^{1}H NMR [$(CD_3)_2SO$]: δ 8.33 (s, 1 H, H-3), 7.87 (s, 1 H, H-6), 3.79 (t, *J* = 6.2 Hz, 4 H, CH_2Cl), 3.59 (t, *J* = 6.2 Hz, 4 H, CH_2N), 2.82 (s, 3 H, SO_2CH_3). ^{13}C NMR: δ 144.14 (C-5), 142.39 (C-1), 139.66, 139.04 (C-2,4), 123.79, 122.78 (C-3,6), 53.05 (CH_2N), 43.02 (SO_2CH_3), 41.54 (CH_2Cl). Anal. ($C_{11}H_{14}Cl_2N_4S_2O_8$·0.5$H_2O$) C, H, N, S.

5-[*N*-(2-Chloroethyl)amino]-2,4-dinitrobenzamide (26). A mixture of 5-chloro-2,4-dinitrobenzamide[21] (1.23 g, 5.01 mmol), 2-chloroethylamine hydrochloride (0.58 g, 5.01 mmol), and Et_3N (1.54 mL, 0.011 mmol) in THF (20 mL) was held at 40 °C for 6 h and then partitioned between water and EtOAc. The residue from workup of the organic layer was chromatographed on silica gel, EtOAc eluting 5-[*N*-(2-chloroethyl)amino]-2,4-dinitrobenzamide (**26**) (0.39 g, 27%), mp (EtOAc/petroleum ether) 225 °C. ^{1}H NMR [$(CD_3)_2SO$]: δ 8.93 (br s, 1 H, NH), 8.77 (s, 1 H, H-3), 8.13, 7.82 (2 × br s, 2 H, $CONH_2$), 7.20 (s, 1 H, H-6), 3.90 (m, 4 H, NCH_2CH_2Cl). ^{13}C NMR: δ 166.30 ($CONH_2$), 147.00 (C-5), 139.86 (C-1), 133.23, 129.22 (C-2,4), 124.69, 114.35 (C-3,6), 44.05, 42.65 (NCH_2CH_2Cl). Anal. ($C_9H_9ClN_4O_5$) C, H, N.

5-[*N*-(2-Chloroethyl)-*N*-ethylamino]-2,4-dinitrobenzamide (27) and 5-[*N*-(2-Chloroethyl)-*N*-ethylamino]-*N*-[2-(dimethylamino)ethyl]-2,4-dinitrobenzamide (28). A mixture of methyl 5-chloro-2,4-dinitrobenzoate (**30**)[21] (3.39 g, 12 mmol) and *N*-ethylethanolamine (2.85 g, 32 mmol) in dioxane (20 mL) was kept at 20 °C for 8 h and then diluted with water. The resulting solid was chromatographed on silica gel, and elution with CH_2Cl_2/EtOAc (2:1) gave methyl 2,4-dinitro-5-[*N*-(2-hydroxyethyl)-*N*-ethylamino]benzoate (**32**) (3.64 g, 89%), mp (MeOH/H_2O) 87 °C. ^{1}H NMR ($CDCl_3$): δ 8.54 (s, 1 H, H-3), 7.26 (s, 1 H, H-6), 3.95 (s, 3 H, $COOCH_3$), 3.82 (t, *J* = 5.2 Hz, 2 H, CH_2OH), 3.56 (t, *J* = 5.2 Hz, 2 H, NCH_2CH_2), 3.35 (q, *J* = 7.1 Hz, 2 H, NCH_2CH_3), 1.95 (br s, 1 H, OH), 1.23 (t, *J* = 7.1 Hz, 3 H, CH_3). Anal. ($C_{12}H_{15}N_3O_7$) C, H, N. A stirred solution of **32** (3.76 g, 12 mmol) and Et_3N (1.93 mL, 14 mmol) in CH_2Cl_2 (20 mL) was treated dropwise at 0 °C with MsCl (1.09 mL, 14 mmol). The reaction mixture was stirred at 20 °C for a further 30 min and then diluted with CH_2Cl_2 (20 mL) and worked up to give the crude mesylate, which was dissolved in dry DMF (15 mL) and treated with LiCl (1.5 g, 35 mmol) at 80 °C for 15 min. Solvent was removed under reduced pressure, and the residue was partitioned between CH_2Cl_2 and dilute aqueous $KHCO_3$. The organic layer was washed with water, and the residue obtained after removal of the solvent was extracted with boiling petroleum ether to give methyl 5-[*N*-(2-chloroethyl)-*N*-ethylamino]-2,4-dinitrobenzoate (**34**) (3.26 g, 82%), mp (benzene/petroleum ether) 77–77.5 °C. ^{1}H NMR ($CDCl_3$): δ 8.52 (s, 1 H, H-3), 7.20 (s, 1 H, H-6), 3.97 (s, 3 H, $COOCH_3$), 3.65 (t, *J* = 5.9 Hz, 2 H, NCH_2CH_2), 3.59 (t, *J* = 5.9 Hz, 2 H, CH_2Cl), 3.45 (q, *J* = 7.1 Hz, 2 H, NCH_2CH_3), 1.23 (t, *J* = 7.1 Hz, 3 H, CH_3). Anal. ($C_{12}H_{14}ClN_3O_6$) C, H, N, Cl.

A solution of **34** in MeOH was treated with aqueous NaOH (1.5 equiv) and heated under reflux for 1 h and then diluted with water and acidified with HCl to give 5-[*N*-(2-chloroethyl)-*N*-ethylamino]-2,4-dinitrobenzoic acid (**35**) (88%), mp (benzene) 139.5–140.5 °C (light-sensitive). ^{1}H NMR [$(CD_3)_2SO$]: δ 14.10

(s, 1 H, COOH), 8.53 (s, 1 H, H-3), 7.51 (s, 1 H, H-6), 3.81 (t, J = 6.2 Hz, 2 H, NCH_2CH_2), 3.64 (t, J = 6.2 Hz, 2 H, CH_2Cl), 3.42 (q, J = 7.1 Hz, 2 H, NCH_2CH_3), 1.94 (t, J = 7.1 Hz, 3 H, CH_3). Anal. ($C_{11}H_{12}ClN_3O_6$) C, H, N, Cl. Treatment of this acid with $SOCl_2$/DMF followed by removal of excess reagent and azeotroping with benzene gave the crude acid chloride, which was dissolved in cold, dry Me_2CO and treated with excess concentrated ammonia to give 5-[*N*-(2-chloroethyl)-*N*-ethylamino]-2,4-dinitrobenzamide (**27**) (71% yield), mp (benzene) 151–152 °C. ^1H NMR [$(CD_3)_2SO$]: δ 8.53 (s, 1 H, H-3), 8.10, 7.78 (2 × s, 2 H, $CONH_2$), 7.40 (s, 1 H, H-6), 3.82 (t, J = 6.3 Hz, 2 H, NCH_2CH_2), 3.64 (t, J = 6.3 Hz, 2 H, CH_2Cl), 3.39 (q, J = 7.1 Hz, 2 H, NCH_2CH_3), 1.13 (t, J = 7.1 Hz, 3 H, CH_3). Anal. ($C_{11}H_{13}ClN_4O_5$) C, H, N, Cl.

Similar treatment of the acid chloride with a solution of excess *N*,*N*-dimethylethylenediamine in CH_2Cl_2 followed by chromatography of the product on alumina (activity II–III) and elution with EtOAc gave 5-[*N*-(2-chloroethyl)-*N*-ethylamino]-*N*-[2-(dimethylamino)ethyl]-2,4-dinitrobenzenecarboxamide (**28**) as an oil (77% yield). ^1H NMR ($CDCl_3$): δ 8.61 (s, 1 H, H-3), 7.13 (s, 1 H, H-6), 6.50 (br s, 1 H, CONH), 3.5–3.7 (3 × m, 3 × 2 H, NCH_2CH_2Cl and $CONHCH_2$), 3.42 (q, J = 7.1 Hz, 2 H, NCH_2CH_3), 2.55 (t, J = 5.9 Hz, 2 H, $CH_2CH_2N(CH_3)_2$), 2.24 (s, 6 H, $N(CH_3)_2$), 1.25 (t, J = 7.1 Hz, 3 H, NCH_2CH_3). The hydrochloride salt crystallized from EtOAc as yellow needles, mp 133–134 °C. Anal. ($C_{15}H_{22}ClN_5O_5$·HCl) C, H, N, Cl.

Formulation of Compounds for Testing. The purity of all compounds was evaluated by HPLC with diode array aborbance detection. All had a purity of >97%, based on absorbance at 250 nm (bandwidth 80 nm). Stock solutions were prepared in α-MEM culture medium containing 5% fetal bovine serum and filter sterilized (**11–14**, **16**, **21**, **25**, and **28**); all other compounds were dissolved in dimethyl sulfoxide and diluted at least 200-fold into cell cultures. Stock solutions were stored at −80 °C, and concentrations were determined in each experiment by spectrophotometry in 0.01 N HCl. Compound **11** was dissolved in phosphate-buffered saline for administration to mice.

Rates of Reduction by Rat DT-Diaphorase. Reduction of selected compounds by highly purified DT-diaphorase (NAD(P)H:quinone oxidoreductase) from Walker cells[19] was assessed by monitoring loss of parent compound by HPLC, essentially as described previously.[17] Drugs (0.08 mM) were incubated with enzyme [10 IU; determined at 20 °C using menadione as substrate and cytochrome c (ϵ_{550} = 29 500 M^{-1} cm^{-1}) as terminal electron acceptor] and NADH (1.6 mM) in 0.1 M sodium phosphate buffer at 20 °C under aerobic conditions in reaction volumes of 0.3 mL. Aliquots were removed at intervals and analyzed by HPLC, using direct injection onto a C18 μBondapak column (8 × 100 mm). The mobile phase for compounds **9**, **26**, and **29** was a linear gradient of MeOH and water and for **11** and **12** a linear gradient of formate buffer (pH 4.5) and acetonitrile. The eluate was monitored by diode array absorbance spectrophotometry.

Microculture Assays for Aerobic Growth Inhibition. AA8 or UV4 cell cultures[17] 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.[37] The IC_{50} was calculated as the drug concentration providing 50% inhibition of growth relative to the controls. The alkylating agent-sensitive (WS) Walker 256 adenocarcinoma cell line was obtained from Dr. Richard Knox, Institute of Cancer Research, Sutton, U.K. Inhibition of the growth of this cell line was determined as for AA8, except that the seeding cell density was 225 cells/0.15 mL, drug exposure was continuous throughout the growth period of 4 days, and cell densities were assessed using the MTT method.[38]

Determination of Hypoxia-Selective Cytotoxicity *in Vitro*. 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.[25] Both cell suspensions and drug solutions in growth medium were pre-equilibrated 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 were used as the measure of hypoxic selectivity.

Elution Assay for DNA Cross-Linking. AA8 cells were grown to 5 × 10^5/mL and labeled with either [^{14}C]thymidine (TdR; 37 kBq/mL) or [^{3}H]TdR (3.7 kBq/mL) for 20 h. [^{14}C]-TdR-labeled cells were exposed to **11** at 10^6 cells/mL for 5 h under hypoxic conditions as above and washed by centrifugation. An aliquot of 5 × 10^5 cells from each culture was irradiated on ice under aerobic conditions (3 Gy, cobalt-60), and cross-links were assayed using the method of Kohn et al.[28] with irradiated (3 Gy, cobalt-60) [^{3}H]TdR-labeled cells (5 × 10^5/channel) as internal standard.

***In Vivo* Tumor Excision Assay.** The maximum tolerated dose of **11** was estimated by treatment of groups of six male C_3H/HeN mice with single ip doses (0.01 mL/g of body weight) at 1.5-fold dose intervals, with an observation time of 30 days. Groups of two mice bearing bilateral KHT tumors in the gastrocnemius muscle were treated with ip drug either alone or 5 min after whole body irradiation (cobalt 60, 15 Gy; ca. 2.5 Gy/min) of unanesthesized, unrestrained mice. Three to four tumors within the size range 0.5–1.0 mL at treatment were removed from each group 18 h later, pooled, and assayed for clonogenicity in agar essentially as described,[39] except that tumors were dissociated using an enzyme cocktail (0.5 mg/mL pronase, 0.2 mg/mL collagenase, 0.2 mg/mL DNase I) for 40 min and total nucleated cells were counted with an electronic particle counter.

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