Hypoxia-Selective Antitumor Agents. 3. Relationships between Structure and Cytotoxicity against Cultured Tumor Cells for Substituted *N,N*-Bis(2-chloroethyl)anilines

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A series of aniline mustards with a wide range of electron-donating and -withdrawing substituents in the 3- and 4-positions has been synthesized and evaluated for cytotoxicity in cell culture to examine the potential of using nitro group deactivated nitrogen mustards for the design of novel hypoxia-selective anticancer drugs (Denny, W. A.; Wilson, W. R. *J. Med. Chem.* **1986**, *29*, 879). Hydrolytic half-lives in tissue culture media, determined by bioassay against a cell line (UV4) defective in the repair of DNA interstrand cross-links showed the expected dependence on the Hammett electronic parameter, σ, varying from 0.13 h for the 4-amino analogue to >100 h for analogues with strongly electron-withdrawing substituents. Cytotoxic potencies in aerobic UV4 cultures showed a similar dependence on σ. This dependence predicted that the 4-nitroaniline mustard would be 7200-fold less potent than its potential six-electron reduction product, the 4-amino compound, in growth inhibition assays using a 1-h drug exposure. The measured differential was much lower (225-fold) because of the instability of the latter compound, but a differential of 17 500-fold was observed in the initial rate of killing by using a clonogenic assay. The potential for formation of reactive mustards by reduction to the amine or hydroxylamine was demonstrated by the 4-nitroso compound, which had an aerobic toxicity similar to that of the amine. Although these features confirmed the original rationale, the 3-nitro- and 4-nitroaniline mustards had only minimal hypoxic selectivity against UV cells. Toxicity to hypoxic cells appears to be limited by the low reduction potentials of these compounds and consequent lack of enzymatic nitroreduction. However, this study has demonstrated that nitro groups can be used to latentiate aromatic nitrogen mustards and indicates that examples with higher reduction potentials could provide useful hypoxia-selective therapeutic agents.

There is much evidence available that solid tumors possess significant numbers of hypoxic cells[1] and that these cells may limit the clinical efficacy of both radiotherapy[2] and chemotherapy.[3] Since most normal tissues are relatively well-oxygenated, compounds designed to be activated to cytotoxic species only in hypoxic environments would be expected to provide tumor selectivity. We have recently discussed the design of a novel class of hypoxia-selective agents (HSA) based on nitrophenyl mustards.[4] In such compounds, cellular reduction of the nitro group to more electron-donating species is designed to activate the alkylating moiety, by electron release through the aromatic ring to the nitrogen of the mustard, generating species with greatly increased cytotoxicity.[4] It is well-established that the reactivity of aromatic nitrogen mustards toward hydrolysis[5,6] and alkylation[7] reactions is increased by electron-donating substituents, which increase electron density at the nitrogen. In addition, several quantitative structure–activity relationship studies[6,8,9] have shown that the in vivo potency of substituted aniline mustards is also dominated by the electronic properties of substituent groups. This dependence of toxicity on substituent electronic properties has been assumed to be directly due to the rate at which the compounds alkylate cellular DNA, but the complexity of in vivo systems and the possible roles of drug transport and metabolism also have to be considered. Surprisingly, there is virtually no quantitative data available concerning substituent electronic effects on cytotoxicity and stability for these compounds.

We therefore wished to study structure–activity relationships for simple substituted aniline mustards in cell-culture systems, to firmly establish the validity of the above assumptions, as part of a program[4] on the rational design of nitrophenyl mustards as HSA. In this paper, we report the synthesis and biological evaluation against mammalian tumor cells in culture of a series of meta- and para-substituted aniline mustards, where the substituent groups have been carefully chosen to span π and σ parameter space.[8]

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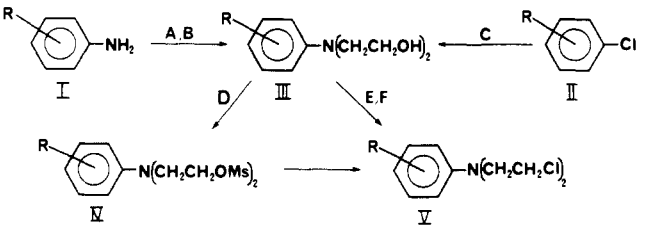
Scheme I



Chemistry

Most of the nitrogen mustards (V) of Table II were prepared from the corresponding diols (III), either by direct chlorination[10] with $SOCl_2$ or $POCl_3$ (Methods E and F, Scheme I) or via the mesylates (IV) by displacement with sodium halides in DMF at 160 °C (method D, Scheme I). The diols (III) in turn were usually prepared by reacting the corresponding amines (I) with ethylene oxide (method A, Scheme I) or by reaction of activated aryl halides (II) with diethanolamine at elevated temperatures (method C, Scheme I).

The amine required for preparation of the sulfone (11) was obtained by nitration of methyl phenyl sulfone, followed by reduction of the nitro group (H_2/Pd/EtOAc). To obtain 4-isomer **10**, 4-nitrothiophenol[11] was methylated and

(1) Moulder, J. E.; Rockwell, S. *Int. J. Radiat. Oncol. Biol. Phys.* **1984**, *10*, 695.
(2) Coleman, C. N. *J. Natl. Cancer Inst. (U.S.)* **1988**, *80*, 310.
(3) Sartorelli, A. C. *Cancer Res.* **1988**, *48*, 775.
(4) Denny, W. A.; Wilson, W. R. *J. Med. Chem.* **1986**, *29*, 879.
(5) Everett, J. L.; Ross, W. C. J. *J. Chem. Soc.* **1953**, 2386.
(6) Panthananickal, A.; Hansch, C.; Leo, A.; Quinn, F. R. *J. Med. Chem.* **1978**, *21*, 16.
(7) Bardos, T. J.; Datta-Gupta, N.; Heborn, P.; Triggle, D. J. *J. Med. Chem.* **1965**, *8*, 167.
(8) Lien, E. J.; Tong, G. L. *Cancer Chemother. Rep.* **1973**, *57*, 251.
(9) Panthananickal, A.; Hansch, C.; Leo, A. *J. Med. Chem.* **1979**, *22*, 1267.
(10) Ross, W. C. J. *J. Chem. Soc.* **1949**, 183.
(11) Price, C. C.; Stacy, G. W. *J. Am. Chem. Soc.* **1946**, *68*, 498.

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the nitro group was reduced (Sn/HCl) to give 4-aminothioanisole.[12] Reaction of this amine with ethylene oxide (method A) did not give the bis(2-hydroxyethyl)amine cleanly, but reaction with an excess of 2-chloroethanol in aqueous Na_2CO_3 under reflux (method B, Scheme I) did give the required diol. Halogenation using $POCl_3$ gave sulfide **14**, which was oxidized to the corresponding sulfone (**10**) with 3-chloroperoxybenzoic acid.

Due to the low reactivity of 4-nitroaniline to both ethylene oxide and 2-chloroethanol, 4-nitrophenyl mustard **5** was obtained by the displacement of 4-chloronitrobenzene with diethanolamine at 120 °C (method C, Scheme I), followed by mesylation of the resulting diol and displacement with NaCl in DMF. Reactive amino mustards **19** and **20** were obtained by reduction of the corresponding nitro compounds using Sn/HCl and were handled as their hydrochloride salts. 4-Nitrosophenyl mustard **21** was prepared by nitrosation of aniline mustard **5** by the procedure of Ross.[13] Attempts to prepare the corresponding 3-nitrosophenyl mustard by oxidation of 3-amino compound **20** were unsuccessful.

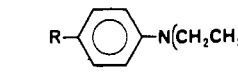
Chemical and Metabolic Stability

Preliminary experiments demonstrated that the cytotoxicities of the diols were very much less than the corresponding mustards, as expected. The differential was particularly pronounced with the AA8-derived Chinese hamster cell line UV4, which is defective in its ability to repair DNA interstrand cross-links formed by nitrogen mustards and other bifunctional alkylating agents and is thus markedly sensitive to such agents.[14] This observation suggested that hydrolysis of the nitrogen mustards to the corresponding diols could be conveniently monitored under physiological conditions by bioassay against UV4 cells. To this end, drugs were incubated in Alpha MEM culture medium at 37 °C, pH 7.2, in the presence and absence of fetal calf serum (10% v/v) and AA8 cells (10^5/mL). At various times residual cytotoxic activity was assayed by titration of medium against log-phase UV4 cells in 96-well microplate cultures as described previously,[15] with growth inhibition being assessed by staining with methylene blue and measuring absorbance with a microplate photometer. The fraction of cytotoxic activity remaining was calculated by comparing the IC_{50} (concentration for inhibition of growth to 50% of controls) with that for fresh drug determined concurrently.

Cytotoxicity Assays

The cytotoxicities of the compounds were determined, under both aerobic and hypoxic conditions, against both AA8 and UV4 cells. The marked hypersensitivity of the latter (typically ca. 50-fold) enabled comparison of cytotoxic potencies of many agents which were too insoluble for evaluation against AA8. Two cytotoxicity assays were used. In the first, log-phase cultures of AA8 or UV4 cells were exposed to drugs in 96-well microplates for 18 h under an atmosphere of 5% CO_2 in air or nitrogen. Drug was then removed by washing the adherent cells with fresh medium, and cultures were grown aerobically for a further 72–75 h before determination of cell numbers as above. This semiautomated assay has a high sample throughput and high sensitivity due to the long drug-contact times.

Table I. Variation of the Leaving Group X in Aniline Mustards



no.	R	X	π	L[a]	aerobic IC_{50},[b] (μM) AA8	UV4	HF[c]
1	H	Cl	0.71	−1.61	10.5 ± 1.2	0.33 ± 0.06	52
2	H	Br	0.86	0.00	3.4	0.14 ± 0.04	41
3	H	I	1.12	−0.04	0.54	0.02 ± 0.008	42
4	H	OMs	−0.88	1.57	13.6	0.59 ± 0.09	30
5	NO_2	Cl			>100	22 ± 3	>5
6	NO_2	Br			*d*	2.8 ± 0.2	>1
7	NO_2	I			*d*	5.3 ± 0.8	>1
8	NO_2	OMs			*d*	1.39 ± 0.02	>1

[a] Leaving group constants L for group X: see the text and ref 19. [b] IC_{50}: the concentration of drug to reduce cell numbers to 50% of controls by using the microassay (see the text). [c] HF: hypersensitivity factor = IC_{50} AA8/IC_{50} UV4 intraexperiment ratios only (see the text). [d] Compound inactive at solubility limit.

Under these conditions the viability of hypoxic cells can be maintained for much longer periods than in a stirred-suspension culture (see below). However, full deoxygenation of the cultures is slow, because of the large reservoir of oxygen dissolved in the polystyrene tissue-culture plasticware.[16] Thus hypoxia-selective cytotoxicity tends to be underestimated by this technique, especially for labile drugs.[15]

In the second assay, stirred suspensions of late log-phase cells were incubated with drug at 10^6 cells/mL under an atmosphere of 5% CO_2 in air or nitrogen, and samples were withdrawn at intervals to assay cell killing by clonogenic assay as described previously.[17] The concentration times time to reduce the surviving fraction of 10% (CT_{10}) was determined by interpolation as an representative measure of cytotoxic potency. This assay has the advantage of providing access to early drug/cell contact times, yielding information on initial rates of cell killing by unstable drugs.

Results and Discussion

Selection of the Alkylating Moiety. The first question considered was the nature of the leaving group X in the mustard $ArN(CH_2CH_2X)_2$ and its effect on biological activity. In order to compare the four most commonly used leaving groups (Cl, Br, I, and OMs), compounds **1–8** were prepared and evaluated. The most relevant physicochemical properties of the groups were lipophilicity and chemical reactivity, and data relating to these are given in Table I. Since the aniline mustards are rather lipophilic molecules with low water solubility, hydrophilic groups would be desirable, with the OMs group (π = −0.88) being the most favored, followed by Cl (π = 0.71). The reactivity of the mustard is largely controlled by the leaving ability of the groups X, a property measured by the leaving-group constant L, which is defined by the ratio of the rates of S_N2 reaction by a nucleophile for a compound containing group X compared to that of a standard compound (X = Br) for which L is defined as zero and b, which is a constant characteristic of the nucleophile.[18]

$$\log (K_X/K_{Br}) = bL \quad (1)$$

Due to solubility, a full data set could only be obtained in the more sensitive UV4 cell line, but the biological data

(12) Gilman, H.; Gainer, G. C. *J. Am. Chem. Soc.* **1949**, *71*, 1747.
(13) Everett, J. L.; Ross, W. C. J. *J. Chem. Soc.* **1949**, 1972.
(14) Hoy, C. A.; Thompson, L. H.; Mooney, C. L.; Salazar, E. P. *Cancer Res.* **1985**, *45*, 1737.
(15) Wilson, W. R.; Thompson, L. H.; Anderson, R. F.; Denny, W. A. *J. Med. Chem.* **1989**, *32*, 31.
(16) Chapman, J. D.; Sturrock, J.; Boag, J. W.; Crookall, J. O. *Int. J. Radiat. Biol.* **1970**, *17*, 305.
(17) Wilson, W. R.; Denny, W. A.; Twigden, S. J.; Baguley, B. C.; Probert, J. C. *Br. J. Cancer* **1984**, *49*, 215.
(18) Price, C. C. In *Antineoplastic and Immunosuppressive Agents*; Sartorelli, A. C., Johns, D. G., Eds.; Springer-Verlag: New York, 1975; Part II, p 1.

does not follow this chemical reactivity. For unsubstituted mustards 1–4, OMs (the best leaving group[18]) results in the least toxic compound, while Cl substitution gives the least toxic of the halogen derivatives. This accords with earlier data for the in vivo potency of N-substituted mustards, where bromo mustards were found to be on average 3-fold more potent than the corresponding chloro mustards.[6] The nitro compounds (5–8) were generally much less toxic (20–200-fold), with the exception of OMs derivative 8, where the difference was only 2-fold (Table I).

The cytotoxicity of compound 1 could also be determined against the wild-type AA8 line, and a difference in potency (hypersensitivity factor, HF) between the two cell lines of 52-fold was seen (Table I), indicating that the mechanism of toxicity is likely to be DNA cross-linking.[14] (In this and subsequent tables, HF values are based on intraexperiment ratios only and therefore do not necessarily correspond to the ratios of means for the AA8 and UV4 IC50s, which include all data for the cell line.) The lower aqueous solubility and lower potency of 4-nitro compound 5 meant that only approximate toxicity data could be obtained for the AA8 cell line. It showed variable toxicity to AA8 cells near the solubility limit of 100 μM, but it is probable that the true IC_{50} is higher than this, and that the true HF is probably greater than the 5-fold suggested by this data.

Although the OMs is the most hydrophilic group, corresponding compounds 4 and 8 did not show any marked advantage in aqueous solubility, and the relatively small differential toxicity between the two compounds weighed against selection of this alkylating group for further studies, since it implied a lack of senstivity to changes to the electronic character of ring substituents. Although Cl compounds 1 and 5 were the least potent of the halogen compounds, their superior solubility led to the selection of this alkylating moiety for the remainder of this work.

Substitution Effects on the Stability of Aniline Chloro Mustards. In order to study the effects of substituent groups on the stability and biological potency of aniline chloro mustards, a carefully selected set of substituted derivatives were made and evaluated. The substituent groups were positioned at the 3- and 4-positions only, to avoid the steric effects which are known to complicate relationships for ortho substituents. The substituents were chosen to have approximately similar size, to minimize possible variations caused by nonspecific binding to serum proteins (a property which has been correlated to molecular size[19]), but with varying lipophilicity, to determine whether this variable would be significant. However, the main aim was to provide a series where the electronic properties of the substituent groups evenly spanned as wide a range as possible, for it is this property which is considered to be the primary modulator of biological activity among the aniline mustards.[6] The substituents chosen have σ values ranging from 0.72 (4-SO_2Me) to −0.66 (4-NH_2) in addition to the nitro group (4-NO_2, 0.78), with the other substituents evenly spanning this range. The 3-substituted compounds were included not so much to evaluate positional effects, but for their differing σ values.

Figure 1 shows the results of experiments where unsubstituted mustard 1 and 4-amino mustard 19 were incubated in culture medium at 37 °C either with or without the addition of lethally irradiated UV-4 cells. Although

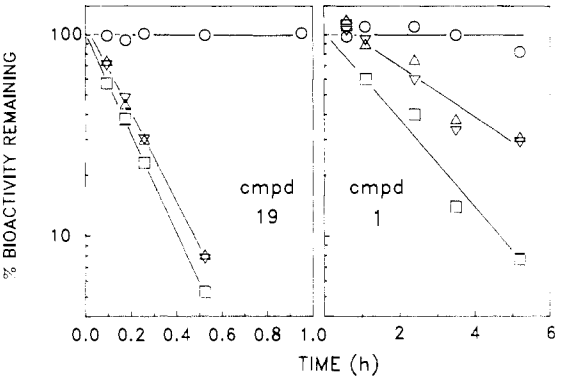


Figure 1. Rate of inactivation of compounds 19 (left-hand panel) and 1 (right-hand panel) as assessed by bioassay against UV4. The compounds were incubated at 10 μM (compound 19) and 60 μM (compound 1) at 37 °C in Alpha MEM (□), Alpha MEM containing 10% fetal calf serum either with (▽) or without (△) lethally irradiated UV4 cells at 10^5 cells/mL. Stock solutions of drugs in 0.01 N HCl (compound 19) or acetone (compound 1) were similarly assayed at intervals while holding at 0 °C (○). The percentage bioactivity remaining was calculated by dividing the average IC_{50} for the stock solutions held at 0 °C by the measured IC_{50} for the test solutions.

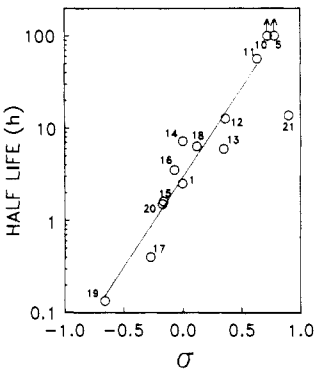


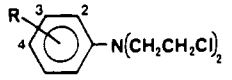
Figure 2. Biological half-life of aniline mustards in culture medium, assessed by bioassay of the culture supernatant against UV4 cells. Drugs were incubated at 37 °C in Alpha MEM with 10% fetal calf serum. The compounds are numbered as in Tables I and II. A linear-regression line was fitted to the data, excluding compounds 5, 10, and 21 (see the text).

such cells will not divide, they retain full metabolic capacity over the period of the experiments. The amount of bioactivity remaining in the solutions was measured at intervals by the methods described above, and the results are plotted in Figure 1. Fetal bovine serum (FBS) in the medium provided some protection against loss of bioactivity (possibly because of the drug binding to serum proteins). However, the presence of metabolically viable cells made no difference to the rate of loss of bioactivity, indicating that the stability of these compounds in culture is dependent entirely on nonmetabolic processes (presumably hydrolysis of the mustard).

The stability of the substituted chloro mustards in culture medium at 37 °C (in the absence of cells) was then similarly determined, and the results (expressed as half-lives, $T_{1/2}$) are recorded in Table II and Figure 2. The data of Table II show that the protective effect of FCS is general, resulting in a 1.3–2.5-fold increase in half-life. The effect does not seem to be related to any substituent parameters, suggesting that it is due to nonspecific binding to serum proteins. In support of this, the solubilities of many of the compounds are increased in the presence of serum, to the extent that $T_{1/2}$ data can only be obtained for the full data set under these conditions. This data

(19) Hansch, C. In *Drug Design*; Ariens, E. J., Ed.; Academic: London, 1971; Vol. 1, p 271.

Table II. Physiochemical and Biological Data for 3- and 4-Substituted *N,N*-Bis(2-chloroethyl)anilines



			growth inhibition assay (UV4)						SSC assay (UV4)	
			$T_{1/2}$,[b] h		aerobic IC_{50},[c] μM		IC_{50} ratio[d]	HF AA8/UV4[e]	aerobic	CT_{10}
no.	R	σ[a]	MEM	+10% FCS	1 h	18 h	(air/N_2)	(air, 18 h)	CT_{10},[f] μM/h	ratio[g]
5	4-NO_2	0.78	*h*	>100	15.8 ± 2.6	21.5 ± 2.9	3.2 ± 0.3	>5	750 ± 100	1.7 ± 0.2
9	3-NO_2	0.71	*h*		96 ± 13	13.6 ± 2.5	1.5 ± 0.3	>1		
10	4-SO_2Me	0.72	*h*	>100	174 ± 25	42 ± 9	0.88 ± 0.12	3.5	1120	0.84
11	3-SO_2Me	0.63	*h*	56.6	76 ± 4	6.8 ± 1.1	0.98 ± 0.18	52		
12	4-$CONMe_2$	0.36	12.1	12.6 ± 0.9	14.6 ± 1.2	1.58 ± 0.2	0.95 ± 0.17	60	30	1
13	3-$CONMe_2$	0.35	*h*	5.9 ± 0.3	7.6 ± 0.1	1.5 ± 0.6	0.98 ± 0.15	>20		
1	H	0	1.3 ± 0.1	2.5 ± 0.3	1.2 ± 0.2	0.33 ± 0.06	1.11 ± 0.01	52	3	1
14	4-SMe	0.00	2.4 ± 0.1	7.2	1.62 ± 0.04	0.40 ± 0.11	0.67 ± 0.03	ND[j]	3.1 ± 1.0	1.2
15	4-CH_3	−0.17	1.08	1.5	0.40 ± 0.01	0.21 ± 0.13	0.79 ± 0.16	46	2.9	1.9
16	3-CH_3	−0.07	1.42	3.5	1.2 ± 0.15	0.15 ± 0.10	0.94 ± 0.03	50		
17	4-OCH_3	−0.27	0.27	0.40	0.45 ± 0.17	0.41 ± 0.16	0.80 ± 0.02	42	0.23	1.4
18	3-OCH_3	0.12	2.9	6.3	2.7 ± 0.2	0.57 ± 0.06	0.97 ± 0.10	42		
19	4-NH_2·HCl	−0.66	0.121 ± 0.004	0.134 ± 0.009	0.071 ± 0.001	0.083 ± 0.02	0.66 ± 0.06	25	0.043 ± 0.004	1.27 ± 0.0
20	3-NH_2·HCl	−0.16	1.25	1.6	0.38 ± 0.02	0.16 ± 0.02	0.98 ± 0.13	18	0.80	1.18
21	4-NO	0.90		13.7	<0.1	0.04 ± 0.03	0.98 ± 0.11	8	<0.1[i]	1.0

[a] σ_m for 3-substituted compounds, σ_p for 4-substituted compounds. [b] $T_{1/2}$: mean ± SE for two or three determination. If no SE is given, values are for a single determination. [c] IC_{50} determined against UV-4 cells under aerobic conditions as described in the text, with a drug exposure of either 1 or 18 h. [d] Ratio of IC_{50} values determined against UV-4 cells with an 18-h exposure under either aerobic or hypoxic conditions. Ratio = aerobic IC_{50}/hypoxic IC_{50}. [e] Hypersensitivity factor = IC_{50} AA8/IC_{50} UV-4. Values are intraexperiment ratios. [f] CT10 = concentration x time to reduce the surviving fraction to 10% of controls in the stirred suspension culture assay. [g] Ratio of CT10 values = aerobic CT10/hypoxic CT10. [h] Limited drug solubility means $T_{1/2}$ cannot be determined by this method. [i] Extreme metabolic instability precludes measurement of initial rates of killing (see the text). [j] ND = not determined.

(excluding that for compounds **5** and **9**, which could not be accurately determined) can be fitted to the expression

$$\log T_{1/2} = 1.94\ (\pm 0.34)\sigma + 0.46\ (\pm 0.14) \qquad (2)$$

$$n = 11 \qquad r = 0.96 \qquad s = 0.24$$

The slope of the line (1.94) shows that there is a dependence of half-life in culture medium on substituent electronic properties (σ) that is very similar to that seen for the dependence of the chemical hydrolysis of substituted aniline chloro mustards, which is best described[6] by eq 3.

$$\log K = -1.84\sigma - 4.02 \qquad (3)$$

The similarity of the magnitude of these slopes suggests that the primary determinant of the half-life of the compounds of Table II in culture medium is the rate of hydrolysis of the mustard and that this is dominated by the degree of electron release. These results also show that several of the derivatives have half-lives of only 1–2 h (or even less for amino compounds **19** and **20**). Thus the stability of these compounds in culture has to be considered when determining their cytotoxicity, for unless very short exposures are used, there will be appreciable drug breakdown during the experiment, which will lead to an underestimation of the biological potency of the more unstable compounds.

Data for 4-nitroso compound **21** was not used in the derivation of eq 2. It has a predicted half-life of 174 h but an observed half-life of only 13.7 h (Table II). Unlike the other compounds investigated, it showed maximal stability in PBS and lower stability in medium. These results, currently under further investigation, suggest that reaction of the nitroso moiety with species such as thiols may generate a less stable aniline mustard. The loss of bioactivity is greatly accelerated by addition of cells. It has not yet been determined whether this represents rapid reaction with intracellular constituents such as thiols or enzymatic nitrosoreduction. The latter possibility is suggested by the observation[20] that compound **21** is an excellent substrate for xanthine oxidase.

Substituent Effects on the Cytotoxicity of Aniline Chloro Mustards. Growth-inbhibitory potencies of the compounds against UV4 cells were determined in two different assays. In the 96-well plate microassay, drugs were incubated with cells for either 1 h or 18 h, with the drug being washed out after the required time and replaced by fresh medium, the cultures being incubated for a total of 96 h after drug addition. IC_{50} values are shown in Table II and in Figure 3. For the 1-h exposure, there is a clear relationship between log IC_{50} values and substituent electronic parameters, except for 4-nitro compound **5**. This compound appears to bind to plastic surfaces, which may reduce the efficiency of drug washout after the 1-h exposure. The rest of the data is fitted well by the relationship

$$\log IC_{50/1} = 2.50\ (\pm 0.41)\sigma + 0.21\ (\pm 0.25) \qquad (4)$$

$$n = 13 \qquad r = 0.97 \qquad s = 0.35$$

The cytotoxicity of 4-amino derivative **19** (which has a half-life of only 0.13 h, Table II) is expected to be underestimated in this assay, even with a 1-h exposure.

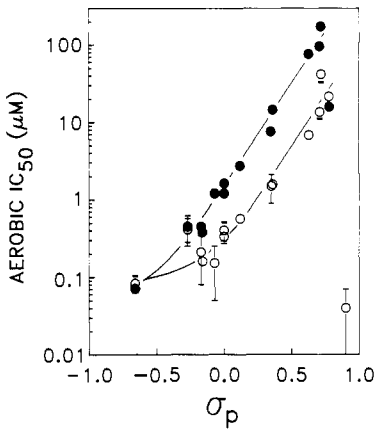


Figure 3. Growth inhibitory potencies of aniline mustards against aerobic UV4 cells which were exposed to drug for 1 h (●) or 18 h (○) before replacement with fresh medium. Cell densities were determined by staining attached cells with methylene blue 4 days after initiation of drug treatment. Values are means of three independent experiments in which all drug exposure times were compared directly. Error bars represent the standard error of the mean.

(20) Sutton, B. M. Unpublished results, this laboratory.

Omitting this compound from the analysis improves the correlation.

$$\log IC_{50/1} = 2.68\ (\pm 0.45)\sigma + 0.15\ (\pm 0.17) \quad (5)$$
$$n = 12 \quad r = 0.99 \quad s = 0.21$$

The coefficient of dependency of cytotoxicity upon σ is large (2.68) but similar in magnitude to that observed[6] for the dependence of the alkylating ability of substituted aniline chloro mustards as measured by their relative rates of alkylation of (4-nitrobenzyl)pyridine (NBP). For this data set (compounds XI-1 to XI-9 of ref 6), the following equation can be computed:

$$\log K_{rel} = -2.16\ (\pm 0.92)\sigma - 2.18\ (\pm 0.36) \quad (6)$$
$$n = 9 \quad r = 0.87 \quad s = 0.53$$

The fact that substituted aniline chloro mustards show a very similar dependency on electronic properties for both cytotoxicity and ability to alkylate NBP suggests that these compounds express their cytotoxicity primarily as alkylating agents. This is further supported by the fact that all the compounds show large HF factors (generally 20–60-fold) for cytotoxicity against the DNA interstrand cross-link repair-deficient UV4 cell line compared to the wild-type AA8 (Table II).

When the compounds were incubated for 18 h before drug washout in the growth inhibition assay, the data recorded in Table II and Figure 3 were obtained. Over the longer time period, the deviation from linearity in the plot of log IC_{50} versus σ is more pronounced, because of the increasing effects of drug loss. However, for the eight compounds with the longest half-lives, the cytotoxicity for an 18-h exposure is fitted well by eq 7. The slope of the dependency is very similar to that for eq 5 describing the 1-h data.

$$\log IC_{50/18} = 2.46\ (\pm 0.52)\sigma - 0.53 \quad (7)$$
$$n = 8 \quad r = 0.97 \quad s = 0.20$$

This analysis suggests that an important criterion for the design of HSA (differential cytotoxicity of parent and reduced-drug forms[4]) can be adequately met by the substituted aniline chloro mustards. Earlier work analyzing in vivo data (summarized in ref 4) suggested that differential potencies of between 200- and 400-fold could be obtained between the 4-nitro mustard and its potential six-electron reduction product, the 4-amine. For UV-4 cells in culture, the measured IC_{50}s of the compounds studied here range over 2450-fold, with a 220-fold difference between the 4-nitro and 4-amino compounds **5** and **19** in the 1-h experiment. However, the cytotoxicity of the 4-amino compound is underestimated and that of the 4-nitro compound may be overestimated as mentioned above, and the theoretical differential cytotoxicity between compounds **5** and **19** (computed by substitution of their σ values into eq 5) is 7200-fold.

The compounds were also assayed for cytotoxicity using the stirred suspension culture assay, with cell survival being determined at various times by measuring plating efficiency and drug cytotoxicity being evaluated as the concentration times time to reduce cell survival to 10% (CT_{10} value). Values for the entire set of compounds are summarized in Table II and Figure 4, with representative results for 4-nitro and 4-amino compounds **5** and **19** shown in Figure 5. This assay, by measuring initial rates of cell killing, is less likely to underestimate the cytotoxicity of unstable compounds of short half-life, and CT_{10} data are fitted excellently by an equation in σ (eq 8). The observed

$$\log CT_{10} = 3.07\ (\pm 0.21)\sigma + 0.60 \quad (8)$$
$$n = 8 \quad r = 0.99$$

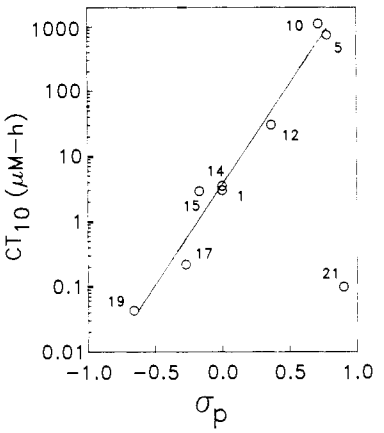


Figure 4. Initial rates of killing by aniline mustards of late log-phase aerobic UV4 cells in stirred suspension cultures (10^6 cells/mL). The ordinate is the concentration × time required to reduce cell survival (plating efficiency) to 10% of the control value.

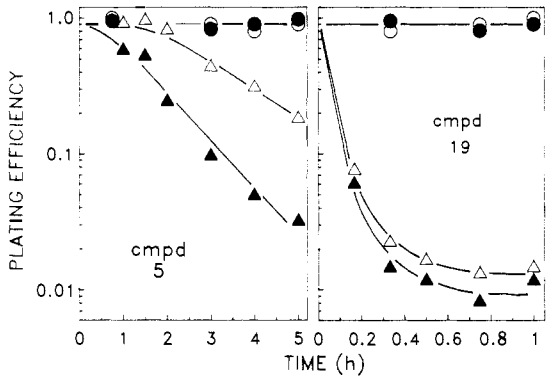


Figure 5. Toxicity of aniline mustards against late log-phase UV4 cells in stirred suspension culture (10^6 cells/mL), assessed by measurement of plating efficiency. ○: aerobic, no drug. ●: hypoxic, no drug. △: aerobic, compound **5**, 100 μM (left-hand panel), or compound **19**, 0.3 μM (right-hand panel). ▲: hypoxic, compound **5**, 100 μM (left-hand panel) or compound **19**, 0.3 μM (right-hand panel).

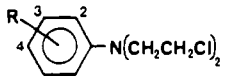
difference in potency of the 4-nitro and 4-amino compounds (**5** and **9**) in this assay is 17 500-fold, which is even greater than the theoretical difference in $IC_{50/1}$ values (7200-fold) computed from eq 5.

Hypoxia-Selective Cytotoxicity of Nitro-Substituted Aniline Chloro Mustards. The large differential in cytotoxicity of 4-nitroaniline mustard **5** and the corresponding amine (**19**) indicates that a correspondingly large increase in cytotoxic potency should occur on metabolic nitroreduction. Even if reduction proceeds only with four-electron stoichiometry to give the hydroxylamine (σ_p = −0.34), a 2750-fold increase in potency would be predicted from eq 8.

The observation that compounds **5** and **19** have IC_{50} values broadly expected from their σ values indicates that, in aerobic UV4 cultures, cytotoxicity is due to the parent compounds. This suggests that little net nitro reduction occurs, either because the reduction potentials of the nitro groups are too low or because of efficient back-oxidation of the initially formed nitro radical anion by molecular oxygen.

In contrast, 4-nitroso compound **21** is 3–4 orders of magnitude more potent in aerobic UV4 cultures than expected by virtue of the σ value of the substituent NO group (Figures 3 and 4). Thus, after two-electron reduction of a nitro group to the highly electron-affinic nitroso, further rapid and irreversible reduction presumably occurs even

Table III. Biological Data for Isomeric Nitro- and Aminoaniline Mustards



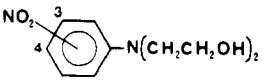
		growth inhibition							
		AA8 IC_{50}, μM		UV IC_{50}, μM		HF[a]		clonogenicity: UV4 CT_{10}	
no.	R	air	air/N_2	air	air/N_2	air	N_2	air (μM h)	air/N_2 ratio
22	2-NO_2	21 ± 5	1.0 ± 0.1	0.52 ± 0.14	1.0 ± 0.2	65 ± 18	64 ± 11	14.3 ± 0.7	1.19 ± 0.09
23	2-NH_2	13.5	0.53	0.48 ± 0.13	0.6 ± 0.2	~39	~28		
9	3-NO_2	c	-	13.6 ± 2.5[b]	1.5 ± 0.3[b]	>1	>1	c	-
20	3-NH_2	2.9	0.90	0.16 ± 0.02	1.0 ± 0.1	18	16	0.80	1.18
5	4-NO_2	⩾100[b]	~1[b]	22 ± 3	3.2 ± 0.3	⩾5[b]	⩾5[b]	760 ± 99	1.64 ± 0.13
21	4-NH_2	2.0 ± 0.2	0.61	0.08 ± 0.02	0.66 ± 0.06	25	19	1.004 ± 0.004	1.27 ± 0.09

[a] Hypersensitivity factors are for *intra*experiment ratios (IC_{50} AA8/IC_{50} UV-4), and are thus not identical with the ratios of the means in the preceding columns. [b] Data unreliable. Endpoint near the solubility limit. [c] Inactive (nontoxic) at the solubility limit.

in aerobic cells. Therefore, it appears that slow reduction to the nitroso compound limits the potency of nitroaniline mustards. If this slow nitroreduction were due to inhibition by oxygen, then very pronounced hypoxia-selective toxicity would be expected for compounds such as **5**. To test for such selectivity, IC_{50} values against UV4 cells were compared following an 18-h drug exposure under aerobic or hypoxic conditions (Table III). The 3-nitro and 4-nitro compounds (**9** and **5**) demonstrated a slight but significant increase in cytotoxicity under hypoxia, with IC_{50} ratios (air/N_2) of 1.5 and 3.2, respectively. Compound **5** also showed slight but reproducible hypoxia selectivity in the clonogenic assay, while compound **9** could not be evaluated due to lack of activity at its solubility limit. Representative data on rates of cell killing by **5** under aerobic and hypoxic conditions (Figure 5) contrast its hypoxic selectivity with the relative oxygen insensitivity of 4-amine **19**. However, the hypoxic selectivities of **5** and **9** are clearly much less than would be expected if these compounds served as good substrates for nitroreductases in hypoxic cells. 2-Nitro derivative **22** was also prepared and evaluated for comparison but did not show any hypoxia-selective cytotoxicity (Table III). A possible reason for this is that the ortho positions of the nitro and mustard groups in this compound make it likely that intramolecular interception reactions to form dihydropyrazines will occur on reduction.[21] However, chemical reduction of **22** with $SnCl_2$ followed by chromatography of the product on silica gel gave only the corresponding amino mustard (**23**). The structure of this compound was quite clear from its ^1H and ^{13}C NMR spectra. Even at 400 MHz, the ^1H spectrum showed only two chemically-distinct methylene groups at δ 3.42 and 3.58 due to the CH_2Cl and CH_2N protons, respectively. Similarly, the ^{13}C spectrum contained only two methylene carbon resonances at δ 59.13 and 44.73, consistent with the existence of a bis(2-chloroethyl) moiety.

A surprising feature of the data is the apparent slight hypoxic selectivity of some other 4-substituted aniline mustards (e.g. **14**, **15**, **17**, **19**) in the clonogenic assay (although not in the growth inhibition assay, Table II). Although the cytotoxicity of simple nitrogen mustards has generally been considered to be oxygen-independent, a recent study[22] has demonstrated the hypoxic selectivity of melphalan against EMT6 spheroid cells. The mechanism of such selectivity is not clear and may reflect secondary metabolic changes in hypoxic cells rather than lack of molecular oxygen per se. Thus, not only is the hypoxic selectivity of the nitroaniline mustards much less than that theoretically achievable, but caution is required in interpreting the slight observed selectivity as being due to oxygen-sensitive nitroreduction.

Table IV. Physiochemical Data for the Isomeric Nitroaniline Diols



no.	NO_2 position	half-wave potential (E,[a] mV)	^{17}O NMR shift, ppm[b]	NO_2 twist angle,[c] deg	IC_{50} AA8, μM
24	2	-720	616	56	7970 ± 100
25	3	-790	578	6	3060 ± 90
26	4	-860	564	-11	3200 ± 200
nitrobenzene		-860[d]	575	0	

[a] E values: half-wave potential measured by cyclic voltammetry and referenced to NHE (see the text). [b] ^{17}O NMR resonance position of the NO_2 group (of the corresponding mustards) in parts per million downfield from the water resonance (see the text). [c] Torsion angle of the nitro group (of the corresponding mustards) determined by ^{17}O NMR by the method of ref 24. [d] $E(1)$ at pH 7 (determined by pulse radiolysis) is -490 mV (ref 33).

Reduction Potentials of Nitroanilines. Since reduction of nitro aromatics in hypoxic cells is largely controlled by the reduction potential of the nitro group, it was of interest to determine these values for the nitro compounds in this series. The expected value can be computed by the equation established for substituted nitrobenzenes.[4]

$$E(1)\ (\text{mV}) = 163\sigma - 492 \qquad (9)$$

With use of this equation and the best available estimate[4] of ca. -0.10 for the σ_{p^-} and σ_{m^-} ($=\sigma_m$) values of the $N(CH_2CH_2Cl)_2$ and the $N(CH_2CH_2OH)_2$ groups, a value of ca. -508 mV can be calculated for the $E(1)$ of both the 4- and 3-nitro compounds **5** and **9** and their corresponding diols **24–26**. The reduction potentials of the three nitro diols were measured experimentally by determination of their half-wave potentials ($E(^1/_2)$) at pH 7 by cyclic voltammetry (Table IV). The value of -860 mV for 4-nitro diol **26** is identical with that obtained for nitrobenzene by the same method. The $E(1)$ of **26** is therefore presumably similar to the value of -490 mV for nitrobenzene measured by pulse radiolysis.[23] This is well toward the low end of the probably useful range[4] and would be expected to provide very low rates of metabolic nitro reduction. As expected from substituent effects, 3-substituted diol **25** has a higher half-wave reduction potential (-790 mV). The half-wave reduction potential of 2-substituted compound **24** should be similar to that of the 4-isomer if electronic effects alone were involved, but is in fact considerably

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(22) Chaplin, D. J.; Acker, B.; Olive, P. L. *Int. J. Radiat. Oncol. Biol. Phys.* **1989**, *16*, 1131.
(23) Meisel, D.; Neta, P. *J. Am. Chem. Soc.* **1975**, *97*, 5198.

higher at −720 mV. The chloro mustards (**5**, **9**, and **22**) corresponding to diols **24–26** were too insoluble for their redox potentials to be measured directly in aqueous solution. However, from the available data concerning the electronic parameters of the diol and chloro mustard groups[4] it seems likely that the reduction potentials for the two species will be similar.

The higher reduction potential of 2-substituted diol **24** can be attributed to the steric effects of the ortho bis(2-hydroxyethyl)amine group. Even an adjacent methyl group has a significant conformational effect; in 2-nitrotoluene the nitro group is twisted 35° out of the plane of the aromatic ring.[24] Recently, ^{17}O NMR shifts have been shown to be diagnostic of the nitro group torsion angles in nitrobenzenoid systems,[24] and we have used this technique to determine the nitro group torsion angles of isomeric aniline mustards **5**, **9**, and **22** (Table IV). The para and meta isomers **5** and **9** have ^{17}O resonance positions of 564 and 578 ppm, respectively, indicative of nitro groups lying close to coplanar with the aromatic ring (angles of 6° and 11°, respectively). However, the resonance position of ortho isomer **22** is 616 ppm, which is consistent with a nitro group torsion angle of 55°. The resultant loss of conjugation of the nitro group with the electron-rich aniline ring [the $N(CH_2CH_2Cl)_2$ group is an electron donor, σ_p ca. −0.10] is sufficient to explain the higher reduction potential of the ortho isomer.

Conclusions

The above results show that both the biological half-lives and the cytotoxic potencies of the substituted aniline chloro mustards are dominated by the electronic properties of the substituent group. The half-lives of the compounds vary over 3 orders of magnitude, with electron-withdrawing groups stabilizing the compounds by deactivating the mustard, and depend on nonmetabolic processes (presumably hydrolysis of the mustard to the nontoxic diol species). In the growth inhibition assay, cytotoxicities are similarly dependent on substituent electronic effects but are somewhat underestimated for the more unstable compounds. Consistent with this hypothesis is the fact that initial rates of cell killing (CT_{10} values) determined by the clonogenic assay, where drug instability is a less important factor, show a much larger range of values between the most and least stable compounds.

The equations describing dependence of both the cytotoxicities and the alkylating abilities of substituted aniline chloro mustards on σ have similar slopes, suggesting that these compounds express their cytotoxicity primarily by alkylation. It is evident that the critical biological alkylation site is DNA and that cytotoxicity is due to formation of DNA interstrand cross-links, since the UV4 mutant defective in repair of these lesions[14] shows marked hypersensitivity to all the aniline mustards. Several lines of evidence indicate the nitroaniline mustards exert their cytotoxicity via the mustard moiety, even though this is deactivated, rather than through the nitro group: (1) Nitromustards **5** and **9** are much more potent than the corresponding diols. (2) The DNA repair cross-link defective mutant shows hypersensitivity to the nitroaniline mustards but not to the diols or other simple nitrobenzenoid compounds. (3) Their cytotoxicities are similar to those predicted for aniline mustards with strongly electron-withdrawing functionality.

While the majority of the aniline mustards showed no hypoxia-selective cytotoxicity in the growth inhibition assay (Table II), the corresponding 3-NO_2 and 4-NO_2 compounds (**9** and **5**) were more toxic under hypoxic conditions against UV4 cells, with the 4-NO_2 derivative showing a ratio of 3.2 (Table III and Figure 5). The result is encouraging, since it provides the first direct evidence that this approach to the design of hypoxia-selective compounds (the use of cellular nitro reduction to activate an alkylating moiety)[4,25,26] is valid. However, the ratio of 3.2-fold is small compared to the measured difference in aerobic toxicities (260-fold) between 4-NO_2 compound **5** and its potential end-stage metabolite, 4-NH_2 compound **19**. In the clonogenic assay, the difference in potency (CT_{10} ratio) between these compounds is even larger (17 500-fold), yet the hypoxia-selectivity of the 4-NO_2 compound in this assay is only 1.5-fold (Table III). Further, weak apparent hypoxic selectivity is seen with some non-nitroaniline mustards, suggesting that the selectivity of the nitro compounds might not be due to nitro reduction.

While the cytotoxicity ratios between the NO_2 and NH_2 derivatives must be considered as a theoretical maximum for the hypoxia-selective toxicity of the corresponding nitro compound (it is unlikely that there will be complete reduction to the six-electron product), the ratios shown by compounds **9** and **5** are very small fractions of this maximum. The main reason for this is almost certainly the low nitro group reduction potentials of these compounds. The 4-NO_2 derivative has a half-wave reduction potential of −860 mV (corresponding to an $E(1)$ of about −500 mV), which is likely to be too low for efficient enzymic nitro reduction, and that of the 3-NO_2 compound ($E(^1/_2)$ −790 mV) is similar. 2-Isomer **22** does have a higher redox potential due to steric crowding, and this may well be the reason for its considerably higher cytotoxicity ($IC_{50/18}$ 0.52 μM in UV4 cf. 22 μM for the 4-NO_2), since significant reduction may be occurring even under aerobic conditions.

Future work in this area is therefore being directed toward the development of more soluble nitrophenyl mustards with higher nitro group reduction potentials.

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 by the Microchemical Laboratory, University of Otago, New Zealand. Melting points were determined on an Electrothermal apparatus using the supplied, stem-corrected thermometer and are as read. Routine NMR spectra were obtained on a Bruker WP-60 spectrometer. Mass spectra were determined on a Varian CH 7 11/250 spectrometer.

Preparation of Unreported Anilines. Methyl 3-Aminophenyl Sulfone. Methyl phenyl sulfone (8.7 g, 56 mmol) was added in portions to a well-stirred mixture of fuming HNO_3 (8.7 mL) and concentrated H_2SO_4 (12.2 mL) at 0 °C. After 5 min at 20 °C, the mixture was warmed to 80 °C for 15 min, cooled, and poured into ice/water. The precipitate was collected and recrystallized from EtOH to give methyl 3-nitrophenyl sulfone as glistening, cream plates (7.9 g, 70%), mp 145 °C (lit.[27] mp 146 °C). This nitro compound was hydrogenated (Pd/C/EtOAc/60 psi) for 2 h to give the required amine as a viscous, yellow oil.

Preparation of *N*,*N*-Bis(2-hydroxyethyl)anilines. Method A. Preparation of *N*,*N*-Bis(2-hydroxyethyl)-3-nitroaniline (25) from Amines and Ethylene Oxide. A mixture of 3-nitroaniline (5.0 g, 36 mmol) and ethylene oxide (10 mL) in 1 N aqueous AcOH (100 mL) was stirred vigorously at 20 °C for 24 h. The resulting solution was poured into brine and extracted

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(26) Connors, T. A. In *Structure–Activity Relationships of Antitumor Agents*; Reinhoudt, D. N., Connors, T. A., Pinedo, H. M., van der Poll, K. W., eds.; Nijhoff: The Hague, 1983; pp 47–59.
(27) Baldwin, W. A.; Robinson, R. *J. Chem. Soc.* **1932**, 1445.

Table V. Physical and Analytical Data for the Compounds of Tables I–IV

no.	cryst solvent	mp, °C	formula	anal. or lit. mp	method of prepn
1	hexane	44–45	$C_{10}H_{13}Cl_2N$	45[a]	A,E
2	hexane	52–53	$C_{10}H_{13}Br_2N$	53–55[a]	A,D
3		oil	$C_{10}H_{13}I_2N$	oil[b]	A,D
4	CH_2Cl_2/ hexane	59–60	$C_{12}H_{19}NO_6S_2$	C,H,N	A,D
5	EtOH	94–95	$C_{10}H_{12}Cl_2N_2O_2$	C,H,N	C,D
6	EtOH	98–100	$C_{10}H_{12}Br_2N_2O_2$	C,H,N,Br	C,D
7	EtOH	126–127	$C_{10}H_{12}I_2N_2O_2$	C,H,N,I[c]	C,D
8	EtOH/EtOAc	137–139	$C_{12}H_{18}N_2O_8S_2$	C,H,N	C,D
9	EtOH	110–111	$C_{10}H_{12}Cl_2N_2O_2$	C,H,N,Cl[d]	A,E
10	CH_2Cl_2/ pet. ether	88	$C_{11}H_{15}Cl_2NSO_2$	C,H,N	*e*
11	CH_2Cl_2/ pet. ether	117	$C_{11}H_{15}Cl_2NSO_2$	C,H,N,Cl	A,F
12	pet. ether	76–77	$C_{13}H_{18}Cl_2N_2O$	C,H,N,Cl	A,F
13		oil	$C_{13}H_{18}Cl_2N_2O$	C,H[f]	A,F
14	pet. ether	62–64	$C_{11}H_{15}Cl_2NS$	C,H,N	B,F
15	pet. ether	42–44	$C_{11}H_{15}Cl_2N$	43–45[a]	A,F
16	pet. ether	30	$C_{11}H_{15}Cl_2N$	33[a]	A,F
17	pet. ether	49–50	$C_{11}H_{15}Cl_2NO$	52[a]	A,E
18	pet. ether	28–30	$C_{11}H_{15}Cl_2NO$	C,H,N,Cl	A,F
19	MeOH/Et_2O	250–260	$C_{10}H_{14}Cl_2N_2$·HCl	250–260[b]	*e*
20	MeOH/Et_2O	206–210	$C_{10}H_{14}Cl_2N_2$·HCl	C,H,N,Cl	*e*
21	Et_2O	76–78	$C_{10}H_{12}Cl_2N_2O$	79–80[b]	*b*
22		oil	$C_{10}H_{12}Cl_2N_2O_2$	C,H,N,Cl	C,E
23	MeOH/Et_2O	175–178	$C_{10}H_{14}Cl_2N_2$·HCl	C,H,N	*e*
24		oil	$C_{10}H_{14}N_2O_4$	oil	C
25	$CHCl_3$/ pet. ether	100–102	$C_{10}H_{14}N_2O_4$	C,H,N	A
26	aqueous EtOH	103–104	$C_{10}H_{14}N_2O_4$	103–104[g]	C

[a] Reference 10. [b] Reference 13. [c] I out by 1%. [d] Cl out by 0.5%. [e] See the Experimental Section for details. [f] Determined by High-Resolution Mass spectrum. [g] Reference 28.

with EtOAc, and the organic layer was washed with saturated $NaHCO_3$, dried, and evaporated. The resulting red oil was chromatographed on SiO_2; elution with EtOAc giving diol 25 (5.16 g, 63%), which crystallized from $CHCl_3$/petroleum ether as yellow needles: mp 100–102 °C; ^1H NMR (CD_3COCD_3) δ 7.64–6.90 (m, 4 H, ArH), 4.30 (t, J = 5 Hz, 2 H, CH_2O), 3.75 (m, 7 H, CH_2O, CH_2N and OH), 3.05 (s, 1 H, OH). See Table V for analysis.

Method B. Preparation of *N,N*-Bis(2-hydroxyethyl)-4-aminothioanisole from Amines and 2-Chloroethanol. A mixture of 4-aminothioanisole[11,12] (4.1 g, 29 mmol), 2-chloroethanol (9.80 mL, 146 mmol and $CaCO_3$ (4.12 g, 41 mmol) in water (200 mL) was heated under reflux with vigorous stirring for 20 h, cooled, and partitioned between EtOAc and water. The organic layer was separated, dried, and concentrated to give an oil, which was chromatographed on SiO_2. Elution with EtOAc/petroleum ether (1:1) gave forerunners containing the monoalkylated amine, while EtOAc eluted the required diol (2.8 g, 42%), which was used without further characterization.

Method C. Preparation of *N,N*-Bis(2-hydroxyethyl)-4-nitroaniline from Halides and Diethanolamine. A mixture of 4-chloronitrobenzene (78.8 g, 0.5 mol) and diethanolamine (105 g, 1 mol) was heated with stirring at 115–120 °C for 6 h. The mixture was poured into boiling water and mixed well, and the aqueous layer was decanted while hot. The residue was recrystallized from aqueous EtOH to give the desired product as orange crystals (38.4 g, 34%): mp 103–104 °C (lit.[28] mp 103–104 °C). ^1H NMR ($CDCl_3$/CD_3COCD_3) δ 8.03 (d, 2 H, J = 10 Hz, ArH3,5), 6.80 (d, J = 10 Hz, ArH2,6), 3.83 (br t, 8 H, NCH_2CH_2OH), 3.47 (s, 2 H, OH).

Preparation of *N,N*-Bis(2-haloethyl)anilines. Method D. Preparation of *N,N*-Bis(2-chloroethyl)-4-nitroaniline (5) from the Diols via the Dimesylates. *N,N*-Bis(2-hydroxyethyl)-4-nitroaniline (4 g, 13.7 mmol) was suspended in EtOAc (60 mL) and the mixture was gently warmed to effect dissolution. Methanesulfonyl chloride (3.0 mL, 39 mmol) was added, followed by dropwise addition of Et_3N (6.2 mL, 44 mmol). After a further 5 min, the Et_3N·HCl was filtered off and washed with Me_2CO. The combined filtrates were evaporated, and the residue was crystallized from EtOH/EtOAc to give *N,N*-bis[2-[(methylsulfonyl)oxy]ethyl]-4-nitroaniline (8) as yellow plates (5.4 g, 80%): mp 137–139 °C; ^1H NMR ($CDCl_3$) δ 8.05 (d, J = 10 Hz, 2 H, ArH3,5), 6.54 (d, J = 10 Hz, 2 H, ArH2,6), 4.36 (m, 4 H, CH_2OSO_2), 3.67 (m, 4 H, NCH_2), 2.95 (s, 6 H, OSO_2CH_3). See Table V for analysis.

This dimesylate (8, 4 g, 10.5 mmol) was dissolved in dry DMF (25 mL) containing NaCl (5 g) and heated at 160 °C for 10 min. The solvent was removed under reduced pressure, and the residue was partitioned between EtOAc and water. The organic phase was worked up and the resulting solid was recrystallized from EtOH to give *N,N*-bis(2-chloroethyl)-4-nitroaniline (5, 2.2 g, 80%) as yellow needles: mp 94–95 °C; ^1H NMR ($CDCl_3$) δ 8.11 (d, 2 H, J = 9 Hz, ArH3,5), 6.65 (d, 2 H, J = 9 Hz, ArH2,6), 4.0–3.5 (m, 8 H, CH_2N and CH_2Cl). See Table V for analysis.

***N,N*-Bis(2-bromoethyl)-4-nitroaniline (6).** Similar treatment of the mesylate (4) with NaBr in DMF at 160 °C for 30 min gave the dibromide (6) in 65% yield, mp 98–100 °C (EtOH). See Table V for analysis.

***N,N*-Bis(2-iodoethyl)-4-nitroaniline (7).** Similar treatment of mesylate 4 with NaI in DMF at 160 °C for 2 h gave a 40% yield of diiodide 7, mp 126–127 °C (EtOH). See Table V for analysis.

***N,N*-Bis[2-[(methylsulfonyl)oxy]ethyl]aniline (4).** A solution of *N,N*-bis(2-hydroxyethyl)aniline[10] (15 g, 83 mmol) in CH_2Cl_2 and Et_3N (34 mL, 3 equiv) was treated dropwise at 0 °C with methanesulfonyl chloride (16 mL, 2.5 equiv) over 30 min. After a further 30 min at 20 °C, the solution was washed well with water, and the residue was crystallized from CH_2Cl_2/hexane to give 4 as needles: mp 59–60 °C; ^1H NMR ($CDCl_3$) δ 7.5–6.5 (m, 5 H, ArH), 4.38 (t, J = 6 Hz, 4 H, CH_2OSO_2), 3.76 (t, J = 6 Hz, 4 H, CH_2N), 2.96 (s, 6 H, OSO_2CH_3).

***N,N*-Bis(2-bromoethyl)aniline (2).** A mixture of dimesylate 4 (1.2 g, 4.4 mmol) and NaBr (2 g) in dry DMF (10 mL) was heated to 80 °C for 20 min with stirring. The mixture was cooled and diluted with water, and the resulting solid was filtered through SiO_2 in hexane to give dibromide 2 (1.05 g, 78%), which was crystallized from hexane as plates: mp 52–53 °C (lit[10] mp 53–55 °C); ^1H NMR ($CDCl_3$) δ 7.5–6.5 (m, 5 H, ArH), 3.60 (t, J = 6 Hz, 4 H, CH_2N), 3.45 (t, J = 6 Hz, 4 H, CH_2Br).

Method E. Preparation of *N,N*-Bis(2-chloroethyl)-3-nitroaniline (9) from the Diols with $SOCl_2$. A solution of *N,N*-bis(2-hydroxyethyl)-3-nitroaniline (26, 1.77 g, 7.83 mmol) and $SOCl_2$ (1.20 mL, 16.4 mmol) in CH_2Cl_2 (30 mL) was heated under reflux for 30 min and then diluted with CH_2Cl_2. The solution was washed with saturated aqueous $NaHCO_3$ and worked up to give an oil, which was chromatographed on SiO_2. Elution with EtOAc/petroleum ether (1:5) gave *N,N*-bis(2-chloroethyl)-3-nitroaniline (9), which was crystallized from absolute EtOH as orange plates (1.46 g, 71%): mp 110–111 °C; ^1H NMR ($CDCl_3$) δ 7.73–6.80 (m, 4 H, ArH), 3.78 (br t, 8 H, CH_2N and CH_2Cl). See Table V for analysis.

***N,N*-Bis(2-chloroethyl)aniline (1).** A solution of *N,N*-bis(2-hydroxyethyl)aniline[10] (1.6 g, 8.9 mmol) in 1,2-dichloroethane (10 mL) and $SOCl_2$ (1.73 mL, 2.2 equiv) was heated under reflux for 10 min. The cooled mixture was diluted with CH_2Cl_2 and washed well with water. The residue, after removal of solvent, was chromatographed on SiO_2, and hexane/EtOAc (9:1) eluted dichloride 1 as a colorless solid (1.6 g, 83% yield), which was crystallized from hexane as plates, mp 44–45 °C (lit.[13] mp 45 °C).

***N,N*-Bis(2-chloroethyl)-2-nitroaniline (22).** Similar treatment of *N,N*-bis(2-hydroxyethyl)-2-nitroaniline (24)[29] followed by chromatography on SiO_2 gave 2-nitro isomer 22 as a yellow oil. ^1H NMR ($CDCl_3$) δ 7.73–7.02 (m, 4 H, ArH), 3.50 (s, 8 H, CH_2N and CH_2Cl). See Table V for analysis.

Method F. Preparation of *N,N*-Bis(2-chloroethyl)-4-(methylthio)aniline (14) from the Diols with $POCl_3$. Crude *N,N*-bis(2-hydroxyethyl)-4-(methylthio)aniline (see above, 2.00 g, 8.79 mmol) was added with stirring to $POCl_3$ (1.80 mL, 19 mmol), and the mixture was stirred at 100 °C for 30 min. Ice/water was then added, followed by EtOAc, and the organic layer was washed well with water and worked up to give a brown oil. Chromatography on SiO_2 and elution with EtOAc/petroleum ether

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(1:9) gave *N,N*-bis(2-chloroethyl)-4-(methylthio)aniline (14, 2.16 g, 93%), which crystallized from petroleum ether as white plates: mp 62–64 °C; ^{1}H NMR ($CDCl_3$) δ 7.24 (d, 2 H, J = 8.7 Hz, ArH3,5), 6.59 (d, 2 H, J = 8.7 Hz, ArH2,6), 3.61 (br s, 8 H, CH_2N and CH_2Cl), 2.38 (s, 3 H, SMe). See Table V for analysis.

Miscellaneous Preparations. Methyl *N,N*-Bis(2-chloroethyl)-4-aminophenyl Sulfone (10). A solution of 3-chloroperoxybenzoic acid (1.22 g of 80% material, 5.68 mmol) in CH_2Cl_2 (15 mL) was added to a solution of sulfide **14** (0.5 g, 1.89 mmol) in CH_2Cl_2 (15 mL). After 30 min at 20 °C, the solution was diluted with CH_2Cl_2 and washed with 10% aqueous Na_2SO_3, saturated $NaHCO_3$, and water. Workup gave sulfone mustard **10**, which was crystallized from CH_2Cl_2/petroleum ether as needles (0.46 g, 82%): mp 88 °C; ^{1}H NMR ($CDCl_3$) δ 7.78 (d, 2 H, J = 8.6 Hz, ArH2,6), 6.75 (d, 2 H, J = 8.6 Hz, ArH3,5), 3.75 (br t, 8 H, CH_2N and CH_2Cl), 3.03 (s, 3 H, SO_2Me). See Table V for analysis.

***N,N*-Bis(2-chloroethyl)-1,3-phenylenediamine Hydrochloride (20).** Stannous chloride (1.44 g, 7.6 mmol) was added to a solution of nitro mustard **9** (0.50 g, 1.9 mmol), and the mixture was heated under reflux for 3 h. The mixture was cooled, diluted with water, and washed with EtOAc. The aqueous layer was basified with concentrated NH_4OH and extracted with EtOAc, to give the amino mustard as a green oil, which was percolated rapidly through SiO_2 in EtOAc/petroleum ether (1:1) to give a colorless oil. This was immediately dissolved in MeOH and treated with concentrated HCl (1 equiv). The solution was evaporated to dryness under reduced pressure, and the residue was crystallized from MeOH/Et_2O to give hydrochloride **20** as cubes, mp 206–210 °C. See Table V for analysis.

***N,N*-Bis(2-chloroethyl)-1,2-phenylenediamine Hydrochloride (23).** A solution of mustard **22** (1.59 g, 6.04 mmol) in concentrated HCl (30 mL) was treated with $SnCl_2$ (4.58 g, 24 mmol), and the resulting suspension was heated under reflux for 1 h. After dilution with water and washing with EtOAc, the aqueous portion was basified with concentrated NH_4OH and extracted with EtOAc. The residue on evaporation was percolated through a column of silica gel in EtOAc, and the appropriate fractions were collected. The residue was immediately treated as above for compound **20** to give hydrochloride **23** (0.61 g, 35%), which crystallized from MeOH/Et_2O as cubes: mp 175–178 °C dec; ^{1}H NMR (D_2O) δ 7.67 (m, 1 H, ArH6), 7.61 (m, 1 H, ArH4), 7.51 (m, 1 H, ArH3), 7.48 (m, 1 H, ArH5), 3.58 (t, J = 5.4 Hz, 4 H, CH_2N), 3.42 (t, J = 5.4 Hz, 4 H, CH_2Cl); ^{13}C NMR (D_2O) δ 144.55 (C1), 133.15, 130.77 (C2), 130.43, 129.10, 125.48 (aromatic C), 59.13 (CH_2N), 44.73 (CH_2Cl).

NMR Spectroscopy. The ^{17}O NMR spectra of compounds **5**, **9**, and **22** were measured at 54.2 MHz on a Bruker AM-400 spectrometer, using ca. 0.1 M solutions in CH_3CN containing 10% CD_3CN to provide a lock signal. Spectra were aquired at 70 °C over a spectral width of 8000 Hz, with 4K data points zero-filled to 8K, a pulse width of 15.0 μs, and an acquisition time of 0.25 s. After 40–50K scans and the application of an exponential line broadening of 20 Hz, the signal-to-noise ratio was typically 2–3 to 1, and the peak width was 200–500 Hz (4–10 ppm). Spectra were referenced to external D_2O at 70 °C. Under these conditions, the ^{17}O resonance of nitrobenzene occurred at 575 ppm (lit.[24] 576 ppm).

Cyclic Voltammetry. Reduction potentials were determined for aqueous solutions (ca. 5×10^{-4} M) containing 0.1 M $NaClO_4$ as electrolyte and 5×10^{-3} M phosphate buffer (pH 7). The compounds (ca. 5×10^{-5} mol) were dissolved in 100 mL of a standard electrolyte (0.1 mol of $NaClO_4 \cdot H_2O$ and 100 mL of phosphate buffer (pH 7) diluted to 1 L with deionized water). The solutions were deoxygenated immediately before use by bubbling N_2 through them for 15 min. Cyclic voltammograms were determined over the range +5 to −1.5V at a scan rate of 50 mV/s, with a stationary, wax-impregnated, carbon electrode. Values are accurate to ±30 mV and are referenced to the standard calomel electrode.

Cell Culture. AA8 cells, a subline of CHO, were grown in Alpha MEM containing 10% v/v FBS without antibiotics in 25 cm^2 tissue culture flasks as described previously.[30] The doubling time in log phase was about 12 h. The UV4 cell line,[31] a repair-defective mutant selected from AA8, was grown in the same manner with a doubling time of about 13 h. Both cell lines were free of mycoplasma as judged by cytochemical staining for cytoplasmic DNA.[32] Bulk cultures were prepared for experiments by growth in 200-mL spinner flasks. The growth medium (GM) for experiments was Alpha MEM containing 10 v/v FBS, 100 IU/mL penicillin and 100 μg/mL streptomycin.

Determination of Stability by Bioassay. Stock solutions of drugs were prepared at 10 mL in ice-cold acetone (or 0.01 N HCl in the case of compound **19**) and diluted into GM with or without FBS (10% v/v) at 37 °C. Initial concentrations were 100 μM in the presence of FBS or 20 μM in its absence. At various times, aliquots were removed and diluted in GM to a concentration approximately 8 times the estimated IC_{50} and 0.1-mL samples were immediately added to 96-well tissue culture plates containing 0.1-mL log-phase cultures of UV4 initiated 1 day previously at 3×10^3 cells/mL in GM. At each time a dilution of the original stock solution of drug stored at 0 °C was also assayed. Five serial 2-fold dilutions were made in the 96-well plate and cultures were incubated for 4 days. Cell densities were then determined by staining fixed monolayers with methylene blue, solubilizing the stain with 1% sarkosyl in phosphate-buffered saline, and determining absorbance with a 96-well microplate photometer (Dynatek MR600) as described by Finlay et al.[33] The IC_{50} was calculated as the nominal drug concentration required to reduce the cell density to 50% of control values, using eight control cultures on each microplate. The fraction of drug bioactivity remaining was calculated as the ratio of the IC_{50} for fresh drug to that from the reaction mixture. Since the IC_{50} values for fresh drug (stock solution at 0 °C) showed no trend with time the mean of these values was used.

Cytotoxicity Assays. Growth-inhibitory potencies were determined using log-phase AA8 or UV4 cells in 96-well microculture plates as above except that drugs were removed after 1 or 18 h by washing wells three times with 0.2 mL of fresh GM and culturing for a further 72–75 h before measuring cell densities. Hypoxic selectivities were determined in similar experiments as described in detail elsewhere.[15] Briefly, 0.05 mL cultures were initiated containing 200 AA8 cells or 300 UV4 cells plus 5000 lethally irradiated (35 Gy, cobalt 60) AA8 cells, the high total cell density and low volume serving to assist in deoxygenation of cultures. After the addition of drugs, plates were incubated in sealed chambers containing 5% CO_2 in air or N_2. The latter gas phase also contained 1% H_2 and a palladium catalyst to facilitate deoxygenation, and the chambers were evacuated three times to 20 mmHg and flushed with this gas mixture for 30 min before closing and immersing in a 37 °C water bath. After 18 h the chambers were opened and drugs were removed by washing with fresh growth medium. Cultures were grown for a further 72–75 h and cell densities determined as above.

Rates of cell killing under aerobic and hypoxic conditions were determined by measuring the plating efficiency of late log-phase populations of UV4 cells grown to 5×10^5 cells/mL in spinner flasks. Cells were concentrated to 5×10^6/mL by centrifugation and resuspension in fresh GM containing 5% v/v FBS. Cell suspensions and drugs at 1.25 times the final concentration in the same medium (8 mL) were equilibrated at 37 °C with magnetic stirring in glass universal bottles under a gas phase of 5% CO_2 in humidified air or N_2 flowing at a rate of 50 mL/min. After 60 min, aliquots of the cell suspension (2 mL) were added to the drug solutions and samples were withdrawn at intervals to determine plating efficiency as described previously.[17]

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Registry No. 1, 553-27-5; **2**, 2045-19-4; **3**, 29523-51-1; **4**, 22964-38-1; **5**, 55743-71-0; **6**, 122567-47-9; **7**, 122567-48-0; **8**, 23721-18-8; **9**, 24813-13-6; **10**, 1669-15-4; **11**, 122567-49-1; **12**, 122567-50-4; **13**, 24813-06-7; 14, 64977-17-9; **15**, 1204-68-8; **16**, 1204-57-5; **17**, 1448-52-8; **18**, 6636-74-4; **19**, 945-68-6; **20**, 13686-10-7; **21**, 779-28-2; **22**, 100858-18-2; **23**, 5321-00-6; **24**, 7334-82-9; **25**, 24812-82-6; **26**, 18226-17-0; methyl 3-aminophenyl sulfone, 35216-39-8; methyl phenyl sulfone, 3112-85-4; methyl 3-nitrophenyl sulfone, 2976-32-1; 3-nitroaniline, 99-09-2; ethylene oxide, 75-21-8; 4-aminothioanisole, 104-96-1; 2-chloroethanol, 107-07-3; 4-chloronitrobenzene, 100-00-5; diethanolamine, 111-42-2; *N,N*-bis(2-hydroxyethyl)aniline, 120-07-0; *N,N*-bis(2-hydroxyethyl)-4-(methylthio)aniline, 122567-51-5; 3-chloroperoxybenzoic acid, 937-14-4.

Synthesis and in Vivo Antitumor Activity of 2-Amino-9*H*-purine-6-sulfenamide, -sulfinamide, and -sulfonamide and Related Purine Ribonucleosides

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A number of 6-sulfenamide, 6-sulfinamide, and 6-sulfonamide derivatives of 2-aminopurine and certain related purine ribonucleosides have been synthesized and evaluated for antileukemic activity in mice. Amination of 6-mercaptopurine ribonucleoside (**7a**) and 6-thioguanosine (**7b**) with chloramine solution gave 9-β-D-ribofuranosylpurine-6-sulfenamide (**8a**) and 2-amino-9-β-D-ribofuranosylpurine-6-sulfenamide (sulfenosine, **8b**), respectively. Selective oxidation of **8a** and **8b** with 3-chloroperoxybenzoic acid (MCPBA) gave (*R,S*)-9-β-D-ribofuranosylpurine-6-sulfinamide (**9a**) and (*R,S*)-2-amino-9-β-D-ribofuranosylpurine-6-sulfinamide (sulfinosine, **9b**), respectively. However, oxidation of **8a** and **8b** with excess of MCPBA gave 9-β-D-ribofuranosylpurine-6-sulfonamide (**10a**) and 2-amino-9-β-D-ribofuranosylpurine-6-sulfonamide (sulfonosine, **10b**), respectively. Similarly, amination of 5′-deoxy-6-thioguanosine (**7c**) afforded the 6-sulfenamide derivative (**8c**), which on controlled oxidation gave (*R,S*)-2-amino-9-(5-deoxy-β-D-ribofuranosyl)purine-6-sulfinamide (**9c**) and the corresponding 6-sulfonamide derivative (**10c**). Treatment of 6-thioguanine (**12**) with aqueous chloramine solution gave 2-amino-9*H*-purine-6-sulfenamide (**13**). Oxidation of **13** with 1 molar equiv of MCPBA afforded (*R,S*)-2-amino-9*H*-purine-6-sulfinamide (14), whereas the use of 4 molar equiv of MCPBA furnished 2-amino-9*H*-purine-6-sulfonamide (**15**). The resolution of *R* and *S* diastereomers of sulfinosine (**9b**) was accomplished by HPLC techniques. The structures of (*R*)-**9b** and **10b** were assigned by single-crystal X-ray diffraction studies. (*R*)-**9b** exists in the crystal structure in four crystallographically independent conformations. Of the 18 compounds evaluated, 13 exhibited very significant anti-L1210 activity in mice. Sulfenosine (**8b**) at 22 mg/kg per day × 1 showed a T/C of 170, whereas sulfinosine (**9b**) at 173 mg/kg per day × 1 showed a T/C of 167 against L1210 leukemia. The 5′-deoxy analogue of sulfinosine (**9c**) at 104 mg/kg per day also showed a T/C of 172. A single treatment with **8b**, **9b**, and **9c** reduced body burdens of viable L1210 cells by more than 99.8%.

The development of sulfonamides is one of the most fascinating and informative chapters in medical science, highlighting the roles of skillful planning and serendipity in drug research.[1] The introduction of prontosil over 50 years ago as an antibacterial agent (discovered by Gerhard Domagk, for which he was awarded the Nobel Prize in 1939) marked the beginning of a tempestuous development of other sulfonamides, and from the sulfonamide era developed the age of effective chemotherapy for infectious diseases. Since the initial dramatic results obtained with sulfonamides in the treatment of streptococcal infections, studies with compounds containing the sulfonamide group have been extended to viruses. The compounds of particular interest are 2-amino-5-(2-sulfamoylphenyl)-1,3,4-thiadiazole (G413)[2] and sodium 5-sulfamoyl-2,4-dichlorobenzoate (M12325),[3] both of which are found to be effective in inhibiting the replication of a broad spectrum of viruses in vitro as well as in vivo at concentrations which do not exhibit any toxic effects on host cells.

There is probably no class of compounds, as characterized by a single functional group, that exceeds sulfonamides in their influence on medical practice and treatment of human disease.[4] The sulfamoyl group has been extensively utilized as an activity-modifying substituent in many different classes of drugs. The purine derivatives and analogues have played a very magnificent role in cancer chemotherapy since the introduction of 9*H*-purine-6(1*H*)-thione (6-mercaptopurine, 6-MP) by Elion and Hitchings[5] in 1952, to treat lymphoblastic leukemia in children.[6] Medicinal chemists have continued to synthesize novel purine derivatives with the hope of obtaining greater potency and selective toxicity in compounds with a broader spectrum of antitumor activity. 9*H*-Purine-6-sulfonamide (1) and its 6-*N*-alkyl derivatives, first synthesized and reported from our laboratory,[7] exhibited significant antitumor activity against Adenocarcinoma 755 and L1210 leukemia in mice at several dosage levels.[8,9] 9*H*-Purine-6-sulfonamide (1) at 75 mg/kg per day × 5

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