Nitrobenzyl Mustard Quaternary Salts: A New Class of Hypoxia-Selective Cytotoxins Showing Very High in Vitro Selectivity

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There is growing interest in the development of compounds (hypoxia-selective cytotoxins; HSCs) which are selectively toxic to the (hypoxic) cell populations which exist at very low oxygen concentrations in solid tumors, and which appear to limit the effectiveness of both radiotherapy and conventional chemotherapy.[1] We report here that nitrobenzyl mustard quaternary salts are a new class of HSCs which show very high hypoxic selectivities *in vitro*.

Examples of this class of HSCs and their proposed mechanism of action are shown in Scheme I. The one-electron reduction of nitroaromatic compounds by cellular enzymes to give the nitro radical anions is controlled primarily by the reduction potential of the compound, and can occur in both oxygenated and hypoxic cells. In the former, reoxidation by molecular oxygen can regenerate the parent compound. However, in the absence of oxygen, fragmentation of the initial radical anion can occur, generating, from *N*,*N*-bis(2-chloroethyl)-*N*-methyl-*N*-(2-nitrobenzyl)ammonium chloride (**1**), a benzyl radical and releasing the reactive nitrogen mustard mechlorethamine (**4**). The proposed mechanism is supported by much published evidence that closely related nitrobenzyl halides and nitrobenzyl quaternary ammonium salts do fragment following one electron reduction,[2] and that the nitro radical anions derived from such compounds are readily reoxidized by oxygen *in vitro*.[3]

This design for HSCs has a number of advantages. The cationic charge on the quaternary salt ensures a high degree of deactivation of the mustard and excellent water solubility for the compound. The large positive Hammett substituent parameter ($\sigma_{o,p}$) of the benzyl quaternary function (expected to be similar to the value of +0.67 reported[4] for $CH_2N^+(Me)_3$) equates to a calculated[5,6] reduction potential of −330 mV for the 4-isomer (**3**), while cyclic voltammetry measurements (Table I) indicate a value of −350 mV. These estimates fall within the window (−300 to −450 mV) suggested[5] as desirable for HSCs. A possible disadvantage is that the charge may slow the rate of cellular uptake of the compounds.

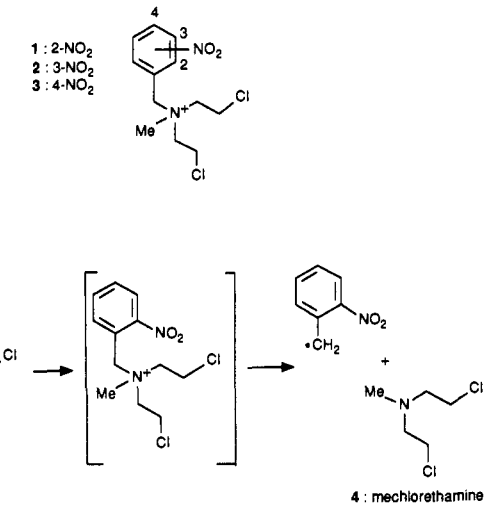
Fragmentation of the prodrug following one-electron reduction then releases a reactive aliphatic mustard of well-defined chemistry. Mechlorethamine not only shows activity against noncycling cells[7] but also has a half-life sufficiently long (ca. 15 min)[8] that it would be expected to back-diffuse to surrounding tumor cells at higher oxygen concentration.[9] The rates of the fragmentation reaction can be controlled over several orders of magnitude by appropriate structural changes (in a series of nitrobenzyl halides),[10] allowing in principle for this fragmentation to be competitive with the back-oxidation process at particular oxygen concentrations.

Table I. Cytotoxicity of Benzyl Quaternary Mustard Derivatives 1–3 and Mechlorethamine (4)

no.	$E(1)_{est}$[a]	IC_{50}[b]	HF[c]	C_{10}[d]	air/N_2 ratio[e]
1	−320	2.7 ± 1.3	300 ± 70	8.0	200
2	−370	20.9 ± 2.8	ND[f]	>50	*g*
3	−350	6.1 ± 1.7	2900 ± 810	6.0	8–9
4		0.0022 ± 0.001	28 ± 8		

[a] Half-wave potentials ($E(1/2)$ values) were measured by cyclic voltammetry for aqueous solutions of approximately 0.1 mM concentration, containing 0.1 mM $NaClO_4$ as electrolyte. $E(1)$ values were then estimated from a linear correlation equation (correlation coefficient r^2 = 0.93) established between measured $E(1/2)$ and known[16] $E(1)$ values for a series of 10 nitro aromatic compounds. These had $E(1)$ values covering a range from −175 to −530 mV. [b] Growth inhibition assay. IC_{50} = concentration of drug (mM) for 50% inhibition of growth of AA8 cells, under aerobic conditions, 18-h exposure. [c] HF (hypersensitivity factor) = IC_{50}(AA8)/IC_{50}(UV4). [d] Clonogenic assay, using stirred suspensions of UV4 cells. C_{10} = concentration of drug (mM) to give 10% cell survival, under aerobic conditions, 1-h exposure. [e] Ratio = C_{10}(aerobic)/C_{10}(hypoxic) for UV4 cells. [f] Not done. [g] Nontoxic at 50 mM under both aerobic and hypoxic conditions.

Scheme I



Compounds **1** and **3** and the 3-nitro analogue **2** were prepared from the corresponding nitrobenzyl chlorides by displacement with *N*-methyldiethanolamine and chlorination with $SOCl_2$. **1** and **3** have previously been prepared and tested for activity against experimental rodent tumors,[11] with the weak activity found for the 4-nitro isomer (**3**) being attributed to release of mechlorethamine via nucleophilic displacement (e.g., by thiols). The hypoxic selectivity of the compounds was not considered.

We have evaluated the cytotoxicity and hypoxic selectivity of compounds **1–3** in cultures of Chinese hamster ovary cells (Table I). The 3-isomer **2** showed by far the lowest cytotoxicity, using either the AA8 subline in growth inhibition assays or the UV4 cell line in clonogenic assays. The greater potencies of the 2- and 4-isomers **1** and **3** support the proposed mechanism of action, since the radical anions of 3-nitrobenzyl halides are known to fragment more than 10^3 times more slowly than the 2- or 4-nitro isomers.[10] The release of mechlorethamine from **1** following incubation with hypoxic EMT6 cells has been confirmed by trapping the released mustard with diethyl dithiocarbamate and HPLC analysis of the adduct.[12]

The selectivity of **1** for hypoxic cells was also assessed by determining clonogenic survival after exposure for 1 h

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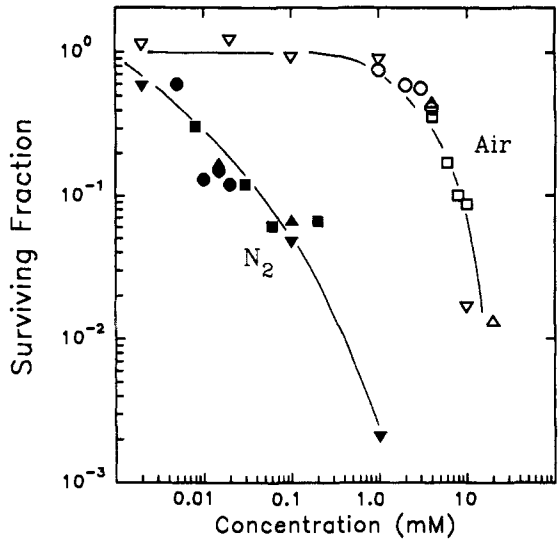

Figure 1. Survival of UV4 cells (stirred suspension at 10^6 cells/mL in α-MEM containing 5% fetal bovine serum) exposed to compound 1 for 1 h under aerobic (open symbols) or hypoxic (filled symbols) conditions. Different symbol shapes refer to separate experiments.

in stirred suspensions of UV4 cells gassed continuously with 5% CO_2 or N_2 (Figure 1).[13] The concentration of 1 required to reduce survival to 10% of controls (C_{10}) was 200-fold less under hypoxic than aerobic conditions, showing the benzyl quaternary mustard (1) to be one of the most hypoxia-selective mono-bioreductive agents yet reported. This property is not restricted to repair-deficient cell lines, since recent studies show that the compound has pronounced hypoxic selectivity (>100-fold) with EMT6 mouse mammary carcinoma cells in similar assays,[12] although mechlorethamine itself shows no significant selectivity with these cells (ca. 1.3-fold; unpublished data, this laboratory). The marked difference in hypoxic selectivity between 1 and 3 could be due to different rates of fragmentation (the half-life of the radical anion derived from 2-nitrobenzyl chloride is about half that derived from the 4-isomer),[10] or to differences in rates of enzymic reduction.

The UV4 cell line, which is defective in DNA cross-link repair,[14] is hypersensitive to DNA cross-linking agents, as shown by the HF value (ratio of IC_{50} values in the wild-type AA8 and UV4 lines) of 28 for mechlorethamine (Table I). The high HF values also shown by 1 and 3 suggest that the mechanism of cytotoxicity is via DNA alkylation. The fact that these HF values are much higher than that of mechlorethamine (a typical cross-linking agent) may be due to limited cellular uptake of these fully-cationic compounds (possibly by a saturable, transport-mediated process). This would serve to exaggerate the ratio, since very high extracellular drug levels might be needed to attain a cytotoxic intracellular concentration in the repair-competent cells. Studies of drug uptake of these compounds are in progress.

The high selectivity of 1 for hypoxic cells suggests that it is not activated primarily by nucleophilic displacement by thiols, as was originally proposed.[11] In fact, the compounds proved surprisingly stable in the presence of thiols; a solution of 1 exposed to a large excess of diethyl dithiocarbamate in HEPES buffer at pH 7.5 for 2 days at 37 °C showed only traces of reaction, as monitored by HPLC. Further, use of an enzymic assay for GSH[15] demonstrated little depletion (ca. 10%) of GSH in AA8 cells incubated with 1 at 2 mM under hypoxic conditions for 2.5 h.

Further chemical development and *in vivo* biological studies of compounds of this class are in progress.

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References

(1) (a) Wilson, W. R. Tumour hypoxia: challenges for cancer chemotherapy. In, *Cancer Biology and Medicine. Vol. 3. The Search for Anticancer Drugs*; Waring, M. J., Ponder, B. A. J., Eds.; Kluwer: Lancaster, 1992; pp 87–132. (b) Rockwell, S. Use of hypoxia-selective drugs in therapy of solid tumors. *Semin. Oncol.* **1992**, *19*, 29–40. (c) Adams, G. E. *Radiat. Res.* **1992**, *132*, 129–139.

(2) (a) Russell, G. A.; Danen, W. C. Electron transfer processes. VIII. Coupling reactions of radicals with carbanions. *J. Am. Chem. Soc.* **1968**, *90*, 347–353. (b) Stock, L. M.; Wasielewski, M. R. The electron paramagnetic resonance of spectra of α-substituted nitrotoluene anion radicals. The influence of electron-withdrawing substituents on the coupling constants for β-hydrogen atoms. *J. Am. Chem. Soc.* **1975**, *97*, 5620–5622.

(3) Moreno, S. N. J.; Schreiber, J.; Mason, R. P. Nitrobenzyl radical metabolites from microsomal reduction of nitrobenzyl chlorides. *J. Biol. Chem.* **1986**, *261*, 7811–7815.

(4) Hoefnagel, A. J.; Hoefnagl, M. A.; Wepster, B. M. Substituent effects. 6. Charged groups: a simple extension of the Hammett equation. *J. Org. Chem.* **1978**, *43*, 4720–4745.

(5) Denny, W. A.; Wilson, W. R. Considerations for the design of nitrophenyl mustards as agents with selective toxicity for hypoxic tumor cells. *J. Med. Chem.* **1986**, *29*, 879–887.

(6) The reduction potential of 2 is calculated as *ca.* −330 mV, using the empirical equation $E(1)$ (mV) = $163\sigma_p^-$ − 492 (from ref 5), and estimating σ_p^- for $CH_2N^+CH_3(CH_2CH_2Cl)_2$ as *ca.* 1.0 from the known value of σ_p for $CH_2N^+(CH_3)_3$ of 0.67. (See ref 4 and Hansch, C.; Leo, A. J. *Substituent Constants for Correlation Analysis in Chemistry and Biology*; Wiley and Sons Ltd: New York, 1979.)

(7) Tannock, I. Cell kinetics and chemotherapy; a critical review. *Cancer Treatment Rep.* **1978**, *62*, 1117–1133.

(8) Niculescu-Duvaz, I.; Baracu, I.; Balaban, A. T. Alkylating agents. In, *Chemistry of Antitumor Agents*; Wilman, D. E. V., Ed.; Blackie: Glasgow, 1990.

(9) Denny, W. A.; Wilson, W. R. Bioreducible mustards; a paradigm for hypoxia-selective prodrugs of diffusible cytotoxins (HPDCs). *Cancer Met. Rev.* In press.

(10) (a) Neta, P.; Behar, D. Intramolecular electron transfer in the anion radicals of nitrobenzyl halides. *J. Am. Chem. Soc.* **1980**, *102*, 4798–4802. (b) Bays, J. P.; Blumer, S. T.; Baral-Tosh, S.; Behar, D.; Neta, P. Intramolecular electron transfer and dehalogenation of nitroaromatic anion radicals. *J. Am. Chem. Soc.* **1983**, *105*, 320–324. (c) Norris, R. K.; Barker, S. D.; Neta, P. Steric effects on dehalogenation of anion radicals derived from substituted nitrobenzyl halides. *J. Am. Chem. Soc.* **1984**, *106*, 3140–3144.

(11) Papanastassiou, Z. B.; Bruni, R. J.; White, E. V. Potential carcinolytic Agents. VI. New deactivated biological alkylating agents. *Experienta* **1968**, *24*, 325.

(12) Denny, W. A.; Wilson, W. R.; Tercel, M., Van Zijl, P.; Pullen, S. M. Benzylic mustard quaternary salts, a new class of hypoxia-selective cytotoxins capable of releasing diffusible cytotoxins on bioreduction. Manuscript submitted to *Int. J. Radiat. Biol. Oncol. Phys.*

(13) Wilson, W. R.; Anderson, R. F.; Denny, W. A. Hypoxia-selective antitumor agents. 1. Relationship between structure, redox properties and hypoxia-selective cytotoxicity for 4-substituted derivatives of nitracrine. *J. Med. Chem.* **1989**, *32*, 23–30.

(14) Hoy, C. A.; Thompson, L. H.; Mooney, C. L.; Salazar, E. P. Defective DNA crosslink removal in Chinese hamster cell mutants hypersensitive to bifunctional alkylating agents. *Cancer Res.* **1985**, 1737–1743.

(15) Baker, M. A.; Cerniglia, G. J.; Zaman, A. Microtiter plate assay for the measurement of glutathione and glutathione disulfide in large numbers of biological samples. *Anal. Biochem.* **1990**, *190*, 360–365.

(16) Wardman, P. Some reactions and properties of nitro radical anions important in biology and medicine. *Environ. Health Perspect.* **1985**, *63*, 309–320.