Journal of Medicinal Chemistry

Volume 29, Number 6 June 1986

Perspective

Considerations for the Design of Nitrophenyl Mustards as Agents with Selective Toxicity for Hypoxic Tumor Cells

William A. Denny*† and William R. Wilson‡

Cancer Research Laboratory and Section of Oncology, Department of Pathology, University of Auckland, School of Medicine, Private Bag, Auckland, New Zealand. *Received September 9, 1985*

The presently used clinical anticancer drugs all display relatively poor selectivity for neoplastic cells. Despite recent advances in the molecular biology of tumorigenesis and the identification of oncogenes, no useful chemotherapy at this biochemical level (e.g., based on unique enzymes or metabolic pathways of tumor cells) has yet been devised. Much of the current success of cancer chemotherapy probably lies in utilizing differences in cell kinetics between tumor and normal tissue, for most drugs do show some selective toxicity toward rapidly-dividing cells compared to noncycling cells.[1] Thus the leukemias and solid tumors with high growth fractions have generally been the most responsive to chemotherapy.

Exploitation of kinetic differences is less successful with the common solid tumors, which generally have much lower growth fractions, and other approaches are needed. While there may be few useful differences between solid tumor cells and normal tissue at the biochemical and kinetic levels, there are important differences at the level of tissue organization that create microenvironments unique to solid tumors.[2]

Although tumor growth is supported by an active program of neovascularization, tumor cell proliferation eventually outstrips that of the vascular endothelium, and the blood supply in large solid tumors becomes disorganized and inefficient.[3] Large and variable intercapillary distances,[4,5] fluctuating blood flow,[6] and occlusion of blood vessels due to infiltration or compression by the growing mass[7] may all contribute to the formation of hypoxic regions in tumors. Radiobiological studies indicate that for murine tumors, typically 5–50% of the clonogenic cells are hypoxic.[8] The radioresistance of these cells probably limits the clinical efficacy of radiotherapy.[9]

More recently hypoxic cells have also been recognized as a potential problem in chemotherapy. Hypoxic regions of tumors may be relatively inaccessible to drugs that are rapidly metabolized or that bind avidly to tissue components.[10,11] In addition, chronically hypoxic cells are also starved of other nutrients and appear to be predominantly noncycling,[12,13] limiting their sensitivity to cycle-selective agents. Further, the cytotoxicity of some drugs, such as bleomycin, is dependent upon generation of active oxygen radicals. These agents show reduced activity against hypoxic cells in culture.[14,15]

While hypoxic cells thus represent a therapeutic obstacle, they also offer the potential for development of a new class of tumor-selective drugs. Since the presence of large numbers of hypoxic cells is a characteristic of tumors not shared by most normal tissues, the targeting of these cells could provide a novel basis for tumor selectivity. Those normal tissues that are somewhat hypoxic (e.g., cartilage or the germinal epithelium of the testis) are not those that are normally dose limiting in chemotherapy. Prodrugs capable of bioactivation only in the absence of oxygen would be expected to provide agents with useful antitumor activity when used in combination with treatments likely to be limited by hypoxic cells. If the active cytotoxic species generated in hypoxic areas were capable of limited diffusion within the tumor, then killing of surrounding oxygenated cells could also be achieved. Thus there has been an increasing interest[2,16–20] in designing

† Cancer Research Laboratory.
‡ Section of Oncology, Department of Pathology.

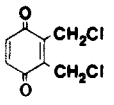
(1) Tannock, I. F. *Cancer Treat. Rep.* **1978**, *62*, 1117.
(2) Kennedy, K. A.; Teicher, B. A.; Rockwell, S.; Sartorelli, A. C. *Biochem. Pharmacol.* **1980**, *29*, 1.
(3) Warren, B. A. In "Tumor Blood Circulation. Angiogenesis, Vascular Morphology and Blood Flow in Experimental and Human Tumors"; Peterson, H-I., Ed; CRC Press: Boca Raton, FL, 1978; pp 1–47.
(4) Thomlinson, R. H.; Gray, L. H. *Br. J. Cancer* **1955**, *9*, 539.
(5) Vaupel, P. W.; Frinak, S.; Bicher, H. I. *Cancer Res.* **1981**, *41*, 2008.
(6) Brown, J. M. *Br. J. Radiol.* **1979**, *52*, 650.
(7) Falk, P. *Eur. J. Cancer* **1980**, *16*, 203.
(8) Moulder, J. E.; Rockwell, S. *Int. J. Radiat. Oncol. Biol. Phys.* **1984**, *10*, 695.
(9) Denekamp, J. In "The Biological Basis of Radiotherapy"; Steel, G. G., Adams, G. E., Peckham, M. J., Eds.; Elsevier: Amsterdam, 1983; pp 139–155.
(10) Ozols, R. F.; Locker, G. Y.; Doroshow, J. H.; Grotzinger, K. R.; Myers, C. E.; Young, R. C. *Cancer Res.* **1979**, *39*, 3209.
(11) Tannock, I. F. *Cancer Res.* **1982**, *42*, 4921.
(12) Tannock, I. F. *Br. J. Cancer* **1968**, *22*, 258.
(13) Hirst, D. G.; Denekamp, J. *Cell Tissue Kinet.* **1979**, *12*, 31.
(14) Tannock, I. F.; Guttman, P. *Br. J. Cancer* **1981**, *43*, 245.
(15) Teicher, B. A.; Lazo, J. S.; Sartorelli, A. C. *Cancer Res.* **1981**, *111*, 73.
(16) Teicher, B. A.; Sartorelli, A. C. *J. Med. Chem.* **1980**, *23*, 955.

0022-2623/86/1829-0879$01.50/0

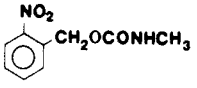
drugs for activation in hypoxic environments.

Such hypoxia-selective agents (HSA) are required to be transformed, only in hypoxic cells, to an activated form of much greater toxicity than the parent. The most widely considered such transformation is reduction. Two phenomena are available to restrict reductive activation to hypoxic cells; their reportedly[21] greater capacity for reductive reactions and the lack of molecular oxygen to carry out back-oxidation of the reduced species. Since most oxygenated cells still retain reductive capacity,[21] the most appropriate compounds are those where the initially reduced species can be rapidly reoxidized by molecular oxygen, to avoid the build up of reduced metabolites in normal oxygenated tissues. Two classes of compound with these general properties have been studied as HSA: quinones and nitroaromatics.

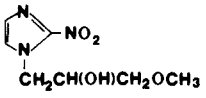
Some selectivity toward hypoxic cells has been shown in vitro for both quinones (1)[17,22] and nitrophenyl derivatives (2)[15,19] bearing leaving groups ortho to the reducible function, but the general suitability of both of these classes of compounds can be questioned on theoretical grounds. In the quinones, the initially reduced species can disproportionate to form the active metabolite, and such (irreversible) disproportionation may compete with back-oxidation in oxygenated cells. For the nitrophenyl carbamates,[16] complete (six-electron) reduction to the amine is necessary to activate the molecule, and it is probable that much compound will be diverted at the four-electron reduction (hydroxylamine) stage. The most widely studied HSA are the imidazole derivatives (e.g., 3 and 5). The



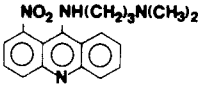
1



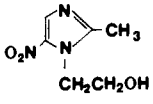
2



3



4



5

2-nitroimidazole derivative misonidazole (3) was initially developed as a hypoxia-selective radiosensitizer and subsequently found to be selectively cytotoxic to hypoxic cells in culture.[23,24] Reduction is known to occur preferentially in hypoxic cells, probably to the hydroxylamine, which

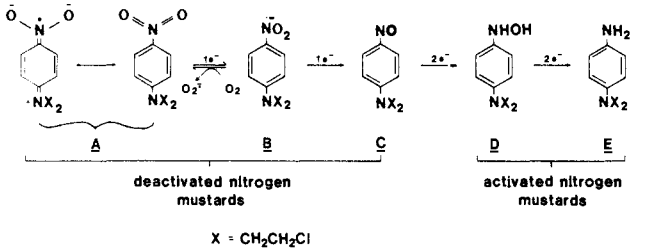


Figure 1. Metabolic reduction and consequent activation of nitrophenyl mustards.

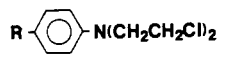
then further fragments to give DNA alkylating species, including glyoxal.[25-27] We have recently reported[28] that the clinical antitumor drug nitracrine (4: a nitroacridine) also possesses selectivity for hypoxic mammalian cells in vitro. The mechanism of action of nitracrine is not clear but again probably involves some type of reductively triggered rearrangement to form an alkylating species. However, despite marked selectivity for hypoxic cells in culture, neither misonidazole or nitracrine appears likely to be useful as an HSA in vivo. Therapeutically achievable doses of misonidazole are, in most animal models, inadequate to provide useful cytotoxicity against hypoxic tumor cells.[29] Although nitracrine is 100 000 times more potent against hypoxic cells in culture than is misonidazole, recent studies indicate that it too lacks activity against hypoxic cells in murine or human xenografted tumors.[30]

An alternative and more deliberate approach to increasing the selective cytotoxicity of HSA would be to introduce suitable alkylating moieties into the molecule, rather than relying on undefined reductively triggered molecular rearrangements to provide alkylating species. Proper theoretical considerations could then be used to design HSA where reduction would activate these alkylating functions to give species of known structure and physicochemical properties such as half-life. The activated form should have a sufficiently short half-life that the toxic effect is exerted only within the tumor; judicious choice of the half-life, together with a knowledge of drug diffusion rates, could allow the drug to be activated in the hypoxic region and diffuse outward to also affect adjacent oxygenated cells.

The activation of most nitrogen-based alkylating functions (such as mustards) is best achieved by increasing the electron density at the nitrogen,[31] which leads to the consideration of nitrophenyl carrier molecules for these alkylating functions. With such compounds the conjugated nitro group provides a deactivated nitrogen mustard, while activation upon reduction is assured by efficient transmission of electron release from the amine (or hydroxylamine) function (Figure 1). Microsomal reduction of nitrophenyl compounds, via the nitro radical anion as an obligate intermediate, has been demonstrated, as has the inhibition of nitro reduction by oxygen resulting from back-oxidation of the free-radical intermediate.[32]

(17) Lin, Y-S.; Teicher, B. A.; Sartorelli, A. C. *J. Med. Chem.* **1980**, *23*, 1237.
(18) Moore, H. W. *Science* **1977**, *197*, 527.
(19) Teicher, B. A.; Sartorelli, A. C. In "Design of Models for Testing Cancer Chemotherapeutic Agents"; Fidler, I. J., White, R. J., Eds.; van Nostrand Reinhold: New York, 1982; pp 19–36.
(20) Workman, P. L. *Cancer Topics* **1983**, *4*, 54.
(21) Carter, D. B.; Phillips, A. F. *Nature (London)* **1954**, *174*, 121.
(22) Lin, A. J.; Cosby, L. A.; Sartorelli, A. C. In "Cancer Chemotherapy"; Sartorelli, A. C., Eds.; American Chemical Society: Washington, D.C., 1976; p 71.
(23) Hall, E. J.; Roizin-Towle, L. *Radiology* **1975**, *117*, 153.
(24) Moore, B. A.; Palcic, B.; Skarsgard, L. D. *Radiat. Res.* **1976**, *67*, 459.
(25) Raleigh, J. A., Liu, S. F. *Biochem. Pharmacol.* **1983**, *32*, 1444.
(26) Rauth, A. M. *Int. J. Radiat. Oncol. Biol. Phys.* **1984**, *10*, 1293.
(27) McClelland, R. A.; Fuller, J. R.; Seaman, N. E.; Rauth, A. M.; Battistella, R. *Biochem. Pharmacol.* **1984**, *33*, 303.
(28) Wilson, W. R.; Denny, W. A.; Twigden, S. J.; Baguley, B. C.; Probert, J. C. *Br. J. Cancer* **1984**, *49*, 215.
(29) Rauth, A. M. In "Radiation Sensitizers"; Brady, L. W., Ed.; Masson: New York, 1980; pp 207–214.
(30) Wilson, W. R.; Tannock, I. F., unpublished results.
(31) Wilman, D. E. V.; Connors, T. A. In "Molecular Aspects of Anticancer Drug Action"; Neidle, S., Waring, M. J., Eds.; MacMillan: London, 1983; pp 233–282.
(32) Mason, P. P.; Holtzman, J. L. *Biochemistry* **1975**, *14*, 1626.

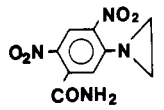
In the present paper we examine the theoretical basis for the rational design of nitrophenyl mustards as potential HSA. No examples of such drugs have yet been reported, although *N,N*-bis(2-chloroethyl)-4-nitroaniline (**6**) has been suggested (but not evaluated) as an HSA.[33,34] Also, 5-(1-Aziridinyl)-2,4-dinitrobenzamide (**9**; CB 1954), which has been shown to be selectively toxic to hypoxic cells,[35] may be activated in an analogous manner to that shown in Figure 1.



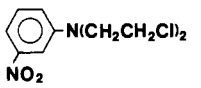
6 R = NO_2

7 R = NH_2

8 R = H



9



10

Discussion

Three fundamental properties are necessary for nitrophenyl alkylators to act successfully as HSA: sufficient solubility of the compound in aqueous media; selective activation by reduction of the nitro group to an electron-donating moiety only in hypoxic cells; and greatly enhanced cytotoxicity of the reduced species over the parent drug. These factors will be considered in detail. Since the acid–base properties of HSAs may have an important influence on their ability to accumulate selectively within cells that experience a hypoxic environment, this feature of drug design is also discussed.

Aqueous Solubility. Although an obvious requirement, this is not always adequately considered in drug design. Even for model compounds, sufficient water solubility is necessary so that the hypothesized biological properties can be measured and the theory tested. The *N,N*-bis(2-chloroethyl) mustard group is a very lipophilic moiety (π value of 1.00[36]), and the resulting simple phenyl derivatives are very insoluble in water; the octanol–water partition coefficient (log *P*) for *N,N*-bis(2-chloroethyl)-aniline (**8**) is 2.90.[36] The previously proposed[33,34] HSA *N,N*-bis(2-chloroethyl)-4-nitroaniline (**6**) was prepared by us and found to have a solubility in water at 20 °C of only 20 μM [presumably because of its high lipophilicity (calculated log *P* 2.62) and low base strength]. The compound was not cytotoxic at the solubility limit. 5-(1-Aziridinyl)-2,4-dinitrobenzamide (**9**; CB 1954) is much more soluble (measured log *P* is 0.14)[35] and has been shown[35] to have activity as an HSA in vitro. A significant factor in the much greater water solubility of this compound is the use of an aziridine rather than a bis(2-chloroethyl)-amine function as the alkylating moiety. However, the aziridine, as a monofunctional alkylating agent, is much less desirable than the difunctional bis(2-chloroethyl)-amine, since cross-linking of DNA by such difunctional agents is thought to be the main mechanism for their cytotoxicity.[37] For compounds based on the mustard **6**, additional solubilizing moieties will have to be added. The most effective way of providing water solubility is to use either a weak base or a weak acid; when fully charged, such moieties contribute 3–4 log *P* units of hydrophilicity,[36] while allowing a proportion of neutral species to facilitate drug diffusion.

A positively charged group (e.g., a weak base such as a tertiary aliphatic amine, pK ~ 9) would have the advantage of helping to localize the drug at the DNA target, thus increasing absolute potency. On the other hand, weak acids (such as an aliphatic carboxylic acid, pK ~ 5) may provide enhanced accumulation of the drug in hypoxic cells, due to the low pH environment of these cells relative to normal tissue (see below).

Selective Activation in Hypoxic Cells. Activation of the alkylating function is designed to occur by electron release, triggered by reduction of the nitro group. Reduction of nitroaromatics (A) occurs in a stepwise fashion, with at least four well-recognized intermediate species in the pathway (Figure 1).[38] The ease of initial one-electron reduction to the radical anion (B) is governed by the first reduction potential E^1_7 ($ArNO_2/ArNO_2^{-\cdot}$). Nitroaromatics that act as radiosensitizers work by taking the place of oxygen, accepting an electron from radiation-created DNA radicals by forming the nitro radical anion (B).[39] A good correlation is seen between sensitization efficiency and the first reduction potential E^1_7 ($ArNO_2/ArNO_2^{-\cdot}$) over the range −155 to −500 mV.[40,41] The maintenance of this correlation to −200 mV and above suggests that the reduction potential of the target DNA radicals is greater than about −700 mV.[39] However, for nitroaromatics acting as HSA, reduction must be carried out in the absence of external radiation by cellular nitroreductase enzymes, whose reduction potentials are not as negative as this.[42]

For such electron-transfer processes, the rate of reaction is related to the difference in reduction potentials ΔE between the nitroaromatic and the cellular nitroreductase by

$$K_{red} = Z \exp(-\Delta F^*/RT) \quad (1)$$

where Z is a collision number ($\sim 10^{10}$ dm^3 mol^{-1} s^{-1}) and ΔF^*, the free energy of activation, is related to $\Delta G°$ and thus to ΔE.[43,44] An increase of 100 mV in the reduction potential of the nitroaromatic will increase the rate of reduction about 10-fold, but if ΔE is greater than about 500 mV, the reaction becomes essentially diffusion controlled and no longer dependent on the nitroaromatic reduction potential.[39] Thus, to ensure rapid reduction of

(33) Alston, T. A.; Porter, D. J. T.; Bright, H. J. *Acc. Chem. Res.* **1983**, *16*, 418.
(34) 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.
(35) Stratford, I. J.; Williamson, C.; Hoe, S.; Adams, G. E. *Radiat. Res.* **1981**, *88*, 502.
(36) Hansch, C.; Leo, A. J. "Substituent Constants for Correlation Analysis in Chemistry and Biology"; Wiley: New York, 1979.
(37) Kohn, K. W., in ref 31, pp 315–361.
(38) Greenstock, G. L. In "Chemistry of Functional Groups"; Patai, S., Ed.; Wiley: New York, 1982; Suppl F, Part 1, pp 291–318.
(39) Wardman, P. *Curr. Topics Radiat. Res.* **1977**, *11*, 347. Wardman, P. *Radiat. Phys. Chem.* **1984**, *24*, 293.
(40) Adams, G. E.; Flockart, I. R.; Smithen, C. E.; Stratford, I. J.; Wardman, P.; Watts, M. E. *Radiat. Res.* **1976**, *67*, 9.
(41) Adams, G. E.; Clarke, E. O.; Flockhart, I. R.; Jacobs, R. S.; Sehmi, D. S.; Stratford, I. J.; Wardman, P.; Watts, M. E.; Parrick, J.; Wallace, R. G.; Smithen, C. E. *Int. J. Radiat. Biol.* **1979**, *35*, 133.
(42) Porras, A. G.; Palmer, G. *J. Biol. Chem.* **1981**, *256*, 9096.
(43) Marcus, R. A. *Ann. Rev. Phys. Chem.* **1964**, *15*, 155.
(44) Wardman, P.; Clarke, E. D. "Electron Transfer and Radical-Addition in the Radiosensitization and Chemotherapy of Hypoxic Cells", Proceedings of the 4th International Symposium on Hypoxic Cell Radiosensitizing Drugs, Fermo, Italy, Aug 1984.

nitroaromatics to the radical anions by hypoxic mammalian cells, E^1_7 for the first one-electron reduction should be up to 500 mV more positive than the reduction potential for the cellular nitroreductases, and preferably at the more positive end of the range.

A competing requirement is that the nitro radical anion (B) formed in *oxygenated* cells (as it will be at an increasing rate as E^1_7 is made more positive) be efficiently back-oxidized by molecular oxygen, to avoid build up of this metabolite.

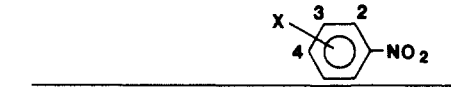
$$ArNO_2^{-}\cdot + O_2 \rightarrow ArNO_2 + O_2^{-}\cdot$$

This reaction needs to compete favorably with the alterative of conversion of B to the nitroso compound C, for once C is formed further reduction to activated species D, E is irreversible (Figure 1). For most radical anions, this conversion is via proton-assisted disproportionation reactions, and the rate of this reaction has also to be considered. Several studies[45-48] have suggested second-order rate constants of ca. 10^4–10^5 M^{-1} s^{-1} for the reaction, which may allow this to compete with the back-oxidation reaction. However, this will depend in each individual case on the steady-state concentration of radical anion achieved, the rate of the back-oxidiation reaction, and the propensity for unimolecular decay.

As E^1_7 ($ArNO_2/ArNO_2^{-}\cdot$) approaches −155 mV (the potential of the $O_2/O_2^{-}\cdot$ couple), then this back-reaction is greatly slowed[48,49] leading to possible loss of hypoxic selectivity. Thus, to demonstrate selective activity under ideal conditions (in cell culture) the "window" for the reduction potential governing the first one-electron reduction (E^1_7 $ArNO_2/ArNO_2^{-}\cdot$) of nitroaromatic HSA is probably in the range −400 to −200 mV. For HSA to be active in vivo, where the difference in oxygen concentration is between about 50 μM in "normal" tissues and less than 10 μM in radiobiologically hypoxic cells, the acceptable range of E^1_7 values may be even smaller. At the lower end of the range, reduction will be slow and the back-oxidation by oxygen rapid, leading to sensitivity of the phenomenon of selective reduction to trace amounts of oxygen, while at the higher end reduction will also proceed rapidly in normal, oxygenated tissues. In addition to a loss of sensitivity to oxygen, the enhanced *absolute* rate of nitro reduction expected at high reduction potentials will also impose an upper limit on the acceptable range of E^1_7. An HSA will have therapeutic utility in vivo only if it has a diffusion range in tissue considerably greater than that of oxygen, and rapid metabolic consumption of highly electron affinic HSA by nitro reduction will limit this diffusion. The upper limit on E^1_7 will be difficult to predict on theoretical grounds, as it depends on rates of various redox reactions, the effective diffusion coefficient of the compound in tissue and the distribution of oxygen. Ideally, a range of E^1_7 values should be explored for any new class of HSA to determine these bounds empirically.

It should be noted that reductive activation of nitrophenyl alkylators requires at least a four-electron reduction (see below), and the reduction potentials of the $ArNO_2^{-}\cdot/ArNO$ and $ArNO/ArNHOH$ couples need to be considered.[39,48] While it is accepted that aromatic nitroso compounds are more electron affinic (and thus more easily reduced) than the corresponding nitro compounds, less is known about the comparative electron affinity of the nitro radical anions. However, for most two-electron reduction systems, the potential for addition of the second electron is more positive than that for the first.[48] Thus the value of E^1_7 ($ArNO_2/ArNO_2^{-}\cdot$) will largely determine the selectivity of reductive activation, since the necessary subsequent steps (at least to the hydroxylamine D) are likely to be more facile.

Table I. Electronic Parameters for Substituted Nitro Compounds Used To Derive Equation 1b (Data from Ref 51)



X	σ	σ^-	E^1_7
2-NO_2	0.78	1.24	−287
H	0	0	−486
3-NO_2	0.71	0.71	−345
4-NO_2	0.78	1.24	−257
3-$COCH_3$	0.38	0.38	−437
4-$COCH_3$	0.50	0.87	−355
2-CHO	0.42	1.03	−355
4-CHO	0.42	1.03	−322
2-CN	0.66	1.00	−308
4-CH_2OH	0.0	0.0	−477
4-COOH	0.45	0.73	−396
3-COOH	0.37	0.37	−433
2-COOH	0.45	0.73	−412

Enough E^1_7 values have now been measured for substituted nitrobenzenes (by pulse radiolysis) to allow quantitative determination of the effects of substituent groups. The best correlation has been found to be with σ_p^- values, which are a measure of electron donation, taking into account "through resonance" effects,[36] and eq 1a has been determined for six 4-substituted nitrobenzenes.[50]

$$E^1_7\ (mV) = 168\sigma_p^- - 484 \qquad (1a)$$

Use of σ^- values for a larger number of monosubstituted nitrobenzenes (listed in Table I)[51] allows us to calculate a very similar equation (1b). This equation (1b) can then

$$E^1_7\ (mV) = 163\sigma^- - 492 \qquad (1b)$$
$$n = 13,\ r = 0.952$$

be used to calculate approximate E^1_7 values for other substituted nitrobenzenes.

There are no reliable data for the electronic substituent parameters of either the bis(2-chloroethyl)amine or bis[2-[(methylsulfonyl)oxy]ethyl]amine substituents, but comparison with the related bis(2-hydroxyethyl)amine suggests a σ_p value of about −0.15.[52] However, since the σ_p^- parameter is to be used, a value of −0.10 will not be much in error, as the scale terminates at −0.16 for the most electronegative substituents.[36] Use of eq 1b then gives a value of −508 mV for the 4-nitrophenyl mustard **6**. Thus, even if this compound had sufficient water solubility, its low reduction potential makes it unlikely that intracellular reduction would occur at a useful rate. Since the σ_m^- ($\cong \sigma_m$) substituent values are similar to σ_p^- values,[36] the 3-nitro isomer **10** will have a comparable E^1_7. Clearly, additional electron-withdrawing substituents are necessary to make the nitro group of **6** or **10** more easily reducible. The obvious approach would be to use such groups (e.g.,

(45) Rauth, A. M.; McClelland, R. A.; Michaels, H. B.; Battistella, R. *Int. J. Radiat. Oncol. Biol. Phys.* **1984**, *10*, 1323.
(46) Wardman, P. *Life Chem. Rep.* **1985**, *3*, 22.
(47) Biaglow, J. E.; Jacobson, B.; Greenstock, C. L.; Radleigh, J. *Mol. Pharmacol.* **1977**, *13*, 269.
(48) Wardman, P. *EHP, Environ. Health Perspect.*, in press.
(49) Wardman, P.; Clarke, E. D. *Biochem. Biophys. Res. Commun.* **1976**, *69*, 942.
(50) Wardman, P. A. In, "Advanced Topics on Radiosensitizers of Hypoxic Cells"; Breccia, A., Rinaldi, C., Adams, G. E., Eds.; Plenum Press: New York, 1982; p 49.
(51) Wardman, P. A., Unpublished data.
(52) Leo, A., personal communication.

Table II. Correlation Equations for the in Vivo Activity of Substituted Phenyl Mustards

eq	ref	tumor system[a]	equation[b]	n[c]	r	ΔC[d] (NH_2)	ΔC[e] (NHOH)
4	56	P388 leukemia: log $1/C$ for 80% ILS	$\log(1/C) = -1.39\sigma - 0.34\pi + 4.15$	16	0.91	211-fold	83-fold
5	56	L1210 leukemia: log $1/C$ for 25% ILS	$\log(1/C) = -0.96\sigma - 0.31\pi + 4.07$	19	0.93	48-fold	25-fold
6	54	Walker 256 carcinoma: log $1/C$ for ED90	$\log(1/C) = -1.30\sigma^- + 4.12$	14	0.89	64-fold	64-fold
7	56	B16 melanoma: log $1/C$ for 25% ILS	$\log(1/C) = -1.71\sigma - 0.16\pi + 4.27$	15	0.94	411-fold	121-fold
8	54	L1210 leukemia: log $1/C$ for 25% ILS (methanesulfonates)	$\log(1/C) = -0.60\sigma^- + 4.37$	16	0.92	7-fold	7-fold

[a] C is the drug dose (in moles/kilogram) given on each treatment. Protocols vary slightly but usually are daily dosage until death. ILS is the percentage increase in lifespan for groups of tumor-bearing drug-treated animals compared to tumor-bearing untreated control groups. [b] Some of the original equations contain additional dummy variables to account for special subgroups of compounds. These have been omitted for clarity and do not alter the calculated differentials. [c] Number of compounds (sets of compounds not identical). [d] Differential potency (ΔC) calculated from the equation for the 4-NO_2 and 4-NH_2 mustards (although these compounds not necessarily in the data set). [e] Differential potency (ΔC) calculated from the equation for the 4-NO_2 and 4-NHOH mustards (although these compounds not necessarily in the data set).

CONHR or SO_2NHR) to at the same time attach the water-solubilizing functionality that will also be needed.

Differential Cytotoxicity of Parent and Reduced Drug Forms. A third essential requirement for HSA based on nitroaromatic alkylating agents is that the reduced form have a greatly increased cytotoxicity. Ideally the lifetime and diffusibility of this reduced active species would be such that the oxygenated tumor cells surrounding hypoxic regions are also affected. The aromatic nitrogen mustards exert their cytotoxic effect by alkylating DNA, probably via an S_N1 mechanism.[31] The rate of alkylation is thus dependent only on the concentration of alkylating agent and the rate constant for the reaction.

The rates of hydrolysis of a number of substituted phenyl mustards have been studied in 1:1 acetone/water at 66 °C,[53] and eq 2 has been formulated.[54] For these

$$\log K = -1.84\sigma - 4.02 \quad (2)$$
$$n = 11, r = 0.96$$

compounds, the rate of hydrolysis is seen to depend almost totally on the electronic properties of the substituent group, specifically on its degree of electron release to the alkylating function (measured by Hammett σ values).[36]

On six-electron reduction of a nitro group to an amino function, the concomitant electron release to a para-substituted mustard is represented by the difference in σ_p values of the two functions (0.78 and −0.66). Substitution of this difference (1.44 Hammett units) into eq 2 predicts the rate of hydrolysis of the amino derivative **7** to be 445-fold faster than that of the parent nitro compound **6**. These calculations show that a para-substituted mustard group (as in **6**) is to be greatly preferred to a meta-substituted one (such as **10**), where the electron release on reduction to the amine is only 0.87 Hammett units.

Similar results have been found[55] for the rates of alkylation of 4-(nitrobenzyl)pyridine by substituted mustards and are summarized by eq 3.[54] In this work the best

$$\log K = -1.92\sigma^- - 1.17 \quad (3)$$
$$n = 14, r = 0.97$$

fit of the data is to the σ^- parameter. However, the difference between the nitro and amino functions is similar (1.39 Hammett units), and use of eq 3 shows that the fully reduced form is expected to alkylate 4-(nitrobenzyl)-pyridine about 470-fold faster than the parent compound. Again, this is much superior to the differential to be expected with a meta-substituted mustard such as **10**. Even four-electron reduction of the nitro group to the hydroxylamine triggers a large electron release; 1.12 Hammett units if σ_p values are used, and 1.39 units if σ_p^- values are used (assuming σ_p^- for NHOH to be ca. −0.16 by analogy with the value for OCH_3[36]).

The in vivo antitumor activity of a variety of substituted phenyl mustards in a number of different animal tumor systems have also been measured, and their potencies are similarly influenced by the electronic properties of the substituents (and to a lesser extent by the lipophilicity of the compounds).[54-56] The equations resulting from several different studies (using nonidentical sets of compounds) are summarized in Table II, and the differential potencies between the 4-NO_2 and 4-NH_2 phenyl mustards (**6** and **7**) and also between the 4-NO_2 and 4-NHOH mustards are calculated from the various equations. While the effects are not as great as the differential rates of hydrolysis and alkylation, they are substantial. For the P388 and L1210 leukemias, the differential (assisted by a small π term) is about 2 orders of magnitude. For the Walker 256 carcinoma and the B-16 melanoma, the data (eq 6 and 7, Table II) also suggest large differentials. Interestingly, a series of (methylsulfonyl)oxy mustards gives an equation (Table II, eq 8) with a much smaller coefficient. This results in a differential for the corresponding 4-NO_2 and 4-NH_2 compounds of only 7-fold.

These data show that for substituted *N,N*-bis(2-chloroethyl)anilines, rates of hydrolysis, alkylation, and in vivo potency against various tumor models are heavily dependent on the degree of electron release to the mustard group. The resulting equations suggest that six-electron reduction of **6** to the amino derivative **7** results in a 500-fold increase in the rate of alkylation and a 50- to 200-fold increase in potency. The in vivo data must be treated with some caution, as the compounds are insoluble and many are unstable, but the reasonably consistent results are encouraging. No reliable in vitro cytotoxicity data is available for substituted phenyl mustards, but it seems likely the six-electron reduction of **6** to **7** would result in an increase in toxicity of at least 2 orders of magnitude.

The linear relationship between log potency and σ values in eq 2–8 further suggests that this *differential* toxicity will remain the same for the six-electron reduction of substituted *N,N*-bis(2-chloroethyl)-4-nitroanilines, although the *absolute* potencies will of course differ depending on the total electron release to the mustard due to all substituents.

Acid–Base Properties and Selective Accumulation in Hypoxic Cells. Other features of the microenvironment of hypoxic tumor cells, in addition to the absence

(53) Everett, J. L.; Roberts, J. J.; Ross, W. C. J. *J. Chem. Soc.* **1953**, 2386.
(54) Panthananickal, A.; Hansch, C.; Leo, A. *J. Med. Chem.* **1978**, *21*, 16.
(55) Bardos, T. J.; Datta-Gupta, N.; Hepborn, P.; Triggle, D. J. *J. Med. Chem.* **1965**, *8*, 167.
(56) Panthananickal, A.; Hansch, C.; Leo, A. *J. Med. Chem.* **1979**, *22*, 1267.

of oxygen, may also be exploitable in the design of HSA. For example, it has repeatedly been demonstrated by microelectrode measurements that the pH in both experimental and human tumors is lower than in normal tissues.[57-60] More recent high-resolution measurements obtained using glass microelectrodes with small pH-sensitive tips indicate a marked heterogeneity of pH within a single tumor,[5] presumably reflecting inefficient lactate clearance from poorly vascularized (hypoxic) regions. The low pH in the environment of tumor cells, particularly in hypoxic regions, suggests drug design considerations that could be used to enhance further the tumor selectivity of HSA.

The implications of low tumor pH depend critically on which compartment is acidified. It has often been assumed that the high rate of glycolysis in cancer cells leads to *intracellular* acidosis via lactic acid production,[61,63] although it has also been suggested that it might be the extracellular rather than intracellular compartment in solid tumors that is at lower pH than in normal tissues.[60,64,65]

While there have been surprisingly few direct comparisons of these two compartments in solid tumors, the available data do appear to indicate that acidification of tumor tissue is primarily restricted to the extracellular compartment. Microelectrode measurements of tumor pH tend to reflect extracellular pH (pH_e), with a small and variable contribution from the intracellular contents of those cells damaged by the electrode.[59,60,66,67] Recent noninvasive measurements of pH_e in the Walker 256 carcinoma by inductive loop telemetry,[68] or using pH electrodes in micropore chambers within the tumor,[69] indicate pH_e values much lower than in normal tissues, with pronounced further depression of pH_e (to ca. 0.8 pH unit less than in normal tissues) on infusion of glucose.[69] In contrast, noninvasive measurements of intracellular pH (pH_i) in the same tumor by ^{31}P NMR spectroscopy provided a value only 0.07 pH unit less than in muscle, with no change on administration of glucose.[70] Similar results have been obtained in studies of pH_i based on distribution of the weak acid 5,5-dimethyl-2-oxazolidine-2,4-dione in this tumor.[65] The latter investigation also showed that tumor pH_i is relatively insensitive to extracellular acidosis caused by elevation of respiratory CO_2 levels. A more direct comparison of pH_e and pH_i has been made in the Yoshida sarcoma in rats,[71] again demonstrating a pH_i value similar to normal tissues. It is significant that glucose administration, which markedly decreased blood flow in this tumor,[71,72] resulted in a pronounced depression of pH_e with no change in pH_i.[71] These observations suggest that pH_e will be selectively depressed in poorly vascularized hypoxic regions in tumors.

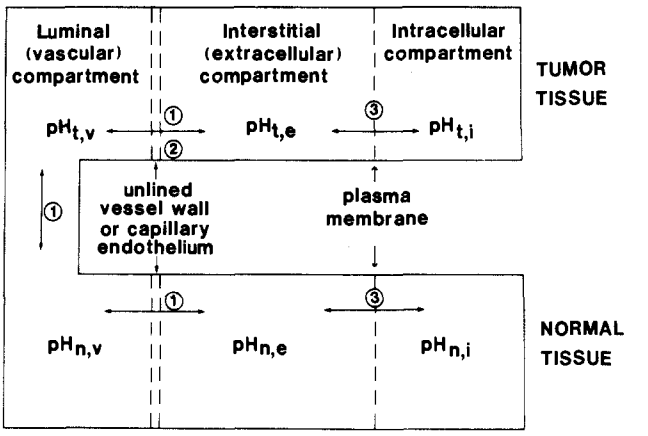


Figure 2. A schematic representation of the luminal, interstitial, and intracellular compartments in tumor and normal tissue. Movement of drugs between compartments is identified by numbered arrows as follows: (1) bulk transport (e.g., blood flow, pinocytosis), (2) pH-independent diffusion (leaky capillaries), and (3) pH-dependent partitioning across a semipermeable membrane (charged species considered impermeant).

The above interpretation implies that protons are actively pumped from tumor cells, especially in hypoxic environments. In view of the sensitivity of so many biochemical processes to pH, it would indeed be surprising if viable cells in poorly vascularized regions of solid tumors did not attempt to maintain pH_i within relatively narrow limits despite acidification of their environment. It is now widely accepted[73,74] that H^+ is not in electrochemical equilibrium across the plasma membrane, pH_i being regulated at a higher value than predicted for passive diffusion through the action of widely distributed proton pumps.[73-75] Regulation of pH_i on acidification of the medium has been demonstrated for tumor cells in culture,[66,76,77] with the decrease in pH_i being less than the decrease in pH_e. In these examples the transmembrane pH gradient typical for mammalian cells (pH_i slightly less than pH_e) was observed to reverse in sign when pH_e was reduced below about 7.0. Thus observations on cultured tumor cells are consistent with the suggestion that viable clonogenic cells in hypoxic regions of solid tumors maintain a pH_i significantly higher than the local pH_e. The transmembrane pH gradient in such regions will favor uptake of weak acids rather than weak bases, in contrast to the converse preference by cells in normal tissue. Thus basic functions on HSA would be anticipated to be dystherapeutic and acidic functions protherapeutic.

A quantitative prediction of the magnitude of such effects can be made with reference to a model of the relevant tissue compartments and transport processes, as depicted

(57) Ashby, B. S. *Lancet* **1966**, *2*, 312.
(58) Gullino, P. M.; Grantham, F. H.; Smith, S. H.; Haggerty, A. C. *J. Natl. Cancer Inst.* **1965**, *34*, 857.
(59) Kahler, H.; Moore, B. *J. Natl. Cancer Inst.* **1962**, *28*, 561.
(60) Voegtlin, C.; Kohler, H.; Fitch, R. H. *Natl. Inst. Health Bull.* **1935**, *164*, 15.
(61) Ross, W. C. J. "Biological Alkylating Agents"; Butterworth: London, 1962.
(62) Wilson, D. W.; Grier, D.; Reimer, R.; Bauman, J. D.; Preston, J. F.; Gabbay, E. J. *J. Med. Chem.* **1976**, *19*, 381.
(63) Sinkula, A. A.; Yalkowsky, S. H. *J. Pharm. Sci.* **1975**, *64*, 181.
(64) Friedkin, M. *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **1973**, *32*, 2148.
(65) Schloerb, P. R.; Blackburn, G. L.; Grantham, J. J.; Mullard, D. S.; Cage, G. K. *Surgery* **1965**, *58*, 5.
(66) Poole, D. T.; Butler, T. C.; Waddell, W. J. *J. Natl. Cancer Inst.* **1964**, *32*, 939.
(67) Waddell, W. J.; Bates, R. G. *Physiol. Rev.* **1969**, *49*, 285.
(68) Hinsall, S. M.; Colson, R. H.; Franklin, A.; Watson, B. W.; Bellamy, D. *J. Natl. Cancer Inst.* **1984**, *73*, 463.
(69) Jain, R. K.; Shah, S. A.; Finney, P. L. *J. Natl. Cancer Inst.* **1984**, *3*, 429.
(70) Griffiths, J. R.; Stevens, A. N.; Iles, R. A.; Gordon, R. E.; Shaw, D. *Biosci. Rep.* **1981**, *1*, 319.
(71) Dickson, J. A.; Calderwood, S. K. *J. Natl. Cancer Inst.* **1979**, *63*, 1371.
(72) Dickson, J. A.; Calderwood, S. K. *Ann. N.Y. Acad. Sci.* **1980**, *335*, 180.
(73) Roos, A.; Boron, W. F. *Physiol. Rev.* **1981**, *61*, 296.
(74) Moody, W. J. In "Intracellular pH: Its Measurement, Regulation and Utilization in Cellular Functions"; Nuccitelli, R., Deamer, D. W., Eds.; Alan R. Liss: New York, 1982; p 283.
(75) Boron, W. F. *J. Membrane Biol.* **1983**, *72*, 1.
(76) Navon, G.; Ogawa, S.; Schulman, R. G.; Yamane, T. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 87.
(77) Dennis, M. F.; Stratford, M. R. L.; Wardman, P.; Watts, M. E. *Int. J. Radiat. Biol.* **1985**, *47*, 629.

in Figure 2. In the simplest version of this model, only one pH-dependent partitioning process, that between the interstitial and intracellular compartments, is considered to be of significance. Ignoring possible partitioning across the capillary barrier (i.e., between the luminal and interstitial compartments) implies either that there is no significant pH gradient across this barrier or that partitioning is restricted by a relatively high permeability for the charged species (dissociated acid or undissociated base). Both of these assumptions may be reasonable given the leaky nature of tumor capillaries,[78] the absence of an endothelial lining in some tumor vessels,[3] and the possible importance of convective rather than diffusive interstitial transport of some drugs, especially if protein bound.[79] Even if these conditions are not fulfilled and pH-dependent partitioning dominates transport from the intraluminal compartment, the analysis presented below still applies except that pH_v substitutes for pH_e.

The distribution of a weak base across a semipermeable membrane (when the charged species is impermeant is described[80] by

$$\frac{C_1}{C_2} = \frac{10^{-pH_1} + 10^{-pK}}{10^{-pH_2} + 10^{-pK}} \quad (9)$$

where C_1 and C_2 are the total drug concentrations (base and conjugate acid) in compartments at pH_1 and pH_2, respectively. Thus, the ratio of intracellular drug in tumor tissue to that in normal tissue is given by

$$\frac{C_{t,i}}{C_{n,i}} = \frac{C_{t,i}}{C_{t,e}}\frac{C_{n,e}}{C_{n,i}}\frac{C_{t,e}}{C_{n,e}} = \left(\frac{10^{-pH_{t,i}} + 10^{-pK}}{10^{-pH_{t,e}} + 10^{-pK}}\right)\left(\frac{10^{-pH_{n,e}} + 10^{-pK}}{10^{-pH_{n,i}} + 10^{-pK}}\right)\left(\frac{C_{t,e}}{C_{n,e}}\right) \quad (10)$$

where C_x is the total drug concentration and pH_x is the pH of compartment x as defined in Figure 2. Similarly, for a weak acid

$$\frac{C_1}{C_2} = \left(\frac{10^{-pH_1} + 10^{-pK}}{10^{-pH_2} + 10^{-pK}}\right)10^{[pH_1 - pH_2]}$$

Thus

$$\frac{C_{t,i}}{C_{n,i}} = \left(\frac{10^{-pH_{t,i}} + 10^{-pK}}{10^{-pH_{t,e}} + 10^{-pK}}\right) \times \left(\frac{10^{-pH_{n,e}} + 10^{-pK}}{10^{-pH_{n,i}} + 10^{-pK}}\right)\left(\frac{10^{[pH_{t,i} - pH_{t,e}]}}{10^{[pH_{n,i} - pH_{n,e}]}}\right)\left(\frac{C_{t,e}}{C_{n,e}}\right) \quad (11)$$

The dependence of $C_{t,i}/C_{n,i}$ on pK_a, as predicted by eq 10 and 11, is illustrated in Figure 3 for representative pH values, with the simplifying assumption that $C_{t,e} = C_{n,e}$ (see below). The chosen value of $pH_{t,e}$ (6.4) is toward the lower end of the range of values found by high-resolution microelectrode measurements[5] and might be representative of hypoxic microenvironments. The corresponding value of $pH_{t,i}$ (6.8) is that determined for Ehrlich ascites tumor cells in vitro when pH_e is decreased to 6.4 by lowering the bicarbonate concentration in Krebs-Ringer bicarbonate/CO_2 buffer.[66,76] A similar pH_i at pH_e 6.4 is implied by recent data for hypoxic V79 cells in culture.[77] Values for normal tissue ($pH_{n,e}$ 7.4, $pH_{n,i}$ 6.9) were chosen as representative of estimates of whole-body values in healthy humans.[67]

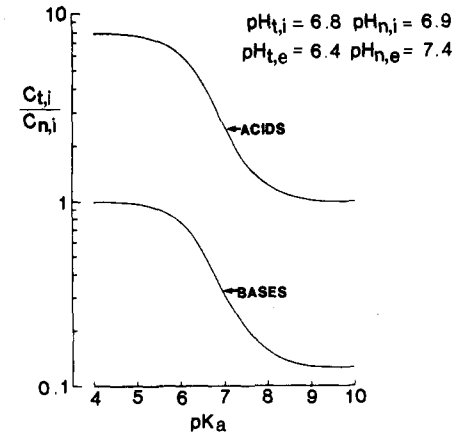


Figure 3. Dependence of the ratio of intracellular drug concentration in tumor tissue to normal tissue ($C_{t,i}/C_{n,i}$) on pK_a for weak acids and bases. Values are calculated from eq 10 (weak bases) and 11 (weak acids) with the assumption that $C_{t,e} = C_{n,e}$. Assumed pH values of each compartment are $pH_{t,e}$ 6.4, $pH_{t,i}$ 6.8, $pH_{n,e}$ 7.4, $pH_{n,i}$ 6.9.

As seen in Figure 3, the calculated ratios of intracellular drug concentrations (tumor/normal tissue) never exceed unity for weak bases, while for weak acids this ratio reaches 7.94 at sufficiently low pK_a. The optimum pK_a can be predicted to be in the vicinity of 5–6. Once pK_a values drop much below this, kinetic restraints on membrane diffusion may be imposed by the low concentration of neutral species, even though the equilibrium ratio will remain high.

The relationship between the maximum achievable selective concentration of weak acids in tumor tissue vs. normal tissue (i.e., when $pK_a << pH$) to the transmembrane pH gradients is obtained from eq (11) as

$$\log C_{t,i}/C_{n,i} = \Delta pH_t - \Delta pH_n$$

where $\Delta pH = pH_i - pH_e$. This relationship indicates that any further increase in the transmembrane pH gradient in tumor tissue, which may be achievable by glucose infusion to increase extracellular acidosis,[57,72] will induce a corresponding logarithmic increase in selective drug accumulation if this acidosis is restricted to tumor tissue. Conversely, for weak bases the minimum ratio (i.e., when $pK_a >> pH$) is obtained from eq 10 as

$$\log C_{t,i}/C_{n,i} = \Delta pH_n - \Delta pH_t$$

indicating the reversal of dependence on the sign of the transmembrane pH gradients in normal and tumor tissue relative to that for weak acids.

The above assumption that $C_{t,e} = C_{n,e}$ (efficient bulk transport) is, of course, an unrealistic one for poorly vascularized regions of solid tumors. However, the same term $C_{t,e}/C_{n,e}$ enters both eq 10 and 11. Thus, in the simplest version of the model of Figure 1, where drug delivery to the extracellular compartment is assumed to be independent of acid–base properties, $C_{t,e}$ will be the same time-dependent function of $C_{n,e}$ for both acids and bases.

Recent in vitro studies have provided experimental support for enhanced uptake of weak acids at low pH_e in cell culture: both the uptake and cytotoxicity of chlorambucil (pK_a = 5.8) is enhanced on lowering of extracellular pH,[81] as is the uptake[77] and hypoxic cell radiosensitizing potency[82] of azomycin (pK_a = 7.2).

(78) Peterson, H-I., in ref 3, pp 77–85.
(79) Swabb, E. A.; Wei, J.; Guillino, P. M. *Cancer Res.* **1974**, *34*, 2814.
(80) Jacob, M. H. *Cold Spring Harbor Symp. Quant. Biol.* **1940**, *8*, 30.
(81) Brophy, G. T.; Sladek, N. E. *Biochem. Pharmacol.* **1983**, *32*, 79.

The role of such pH-driven drug transport within solid tumors is much less clear, and deserves further investigation. Most work has failed to discriminate between intra and extracellular drug, or between well-vascularized and poorly vascularized regions of tumors. One approach toward obtaining such data is exemplified by studies with the basic hypoxic cell radiosensitizer Ro-03-8799 (17) where efficiency of radiosensitization can be used as an indicator of drug concentrations in the vicinity of the DNA target in hypoxic cells. Williams et al.[83] have shown that Ro-03-8799 is 2–4 times more potent than misonidazole as a hypoxic cell radiosensitizer when the two drugs are compared at equivalent average tumor concentration, which is approximately equal to the increased sensitization efficiency expected for Ro-03-8799 on the basis of its higher E^1_7. This observation implies that concentrations in hypoxic cells may not be very different from the average tumor value, in contrast to our expectation that the weak base would be excluded from hypoxic cells. However, lack of information of pH values in the tumor in question precludes quantitative evaluation of the model, and a more detailed understanding of tumor pH is required. The analysis presented here suggests that acidic solubilizing groups will be preferable to basic ones in terms of favorable drug transport, but until firm data are available it would appear desirable to evaluate both acidic and basic functions as solubilizing moieties for HSA.

Conclusions

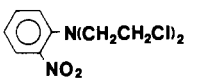
The above discussion outlines the three basic properties necessary for a nitrophenyl mustard to act as a HSA (adequate aqueous solubility, rapid and selective reduction of the nitro group in hypoxic cells, and a high differential cytotoxicity for the parent and reduced form) and suggests that weak acids rather than weak bases may show favorable distributive properties. It also sets limits on the values of certain molecular parameters and provides, from existing data, equations that can be used to make approximate calculation of these parameters for any molecular species.

Thus it is clear from the difference in Hammett σ (or σ^-) values that a para- (or ortho-)substituted mustard provides a much greater differential electron release on reduction than does a meta-substituted one and that designs based on compound **10** need not be considered. Apart from the insolubility of compounds such as **6** and **11**, a major problem is the inherently low reduction potentials of nitrophenyl compounds. Nitrobenzene has an E^1_7 of −486 mV,[41] and the mustard group lowers this further to a calculated value of −508 mV for compound **6**.

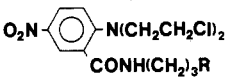
If a charged water-solubilizing group R is attached via an electron-withdrawing link group (e.g., $CONH(CH_2)_3R$) to **6**, this chain can be added at either of two positions: ortho to the mustard to give **12**, calculated E^1_7 −463 mV, or meta to give **13**, calculated E^1_7 −405 mV. While providing the higher E^1_7, compound **13** also gives greater total electron release to the mustard ($\sum\sigma$ 1.52 compared to 1.87).

These reduction potentials are still at the low end of the desirable range (see above). A further increase in E^1_7 could only be achieved by addition of another electron-withdrawing function at a suitable free position (preferably ortho to the mustard, where σ^- values will be maximized, ignoring ortho effects and assuming $\sigma_o^- = \sigma_p^-$). An existing example of this effect is seen in compound **9** (CB 1954), where a second nitro group has been added ortho to the (aziridine) alkylating moiety. The calculated E^1_7 value for either nitro group of this molecule is −213 mV (assuming that eq 1b holds for polysubstituted nitrobenzenes and that the aziridine has a σ_p^- of −0.16: the maximum value). Since the measured E^1_7 for compound **9** is −385 mV, it is clear that these assumptions do not hold very well. It is noteworthy that eq 1b was derived from data on electron-withdrawing groups only, and it is possible the σ^- parameter does not give sufficient weight to very electron-donating groups (σ_p^- for aziridine is −0.16, but σ_p is estimated to be −0.7 or lower).

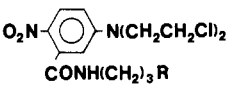
The difunctional alkylating agent analogous to **9** with a suitable solubilizing side chain would be **14**. The E^1_7



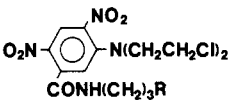
11



12



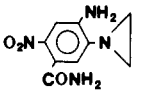
13



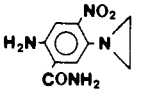
14

calculated for this compound by eq 1b is −203 mV; while this will clearly be in error given the measured value for compound **9**, the much weaker electron-donating ability of the bis(2-chloroethyl)amine compared to the aziridine substituents suggests a value considerably higher than −385 mV.

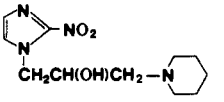
While such electron-deficient benzene rings solve the problem of the reducibility of the nitro group, total electron release to the alkylating function is then low, even after six-electron reduction. Although the *differential* electron release is the same for both compounds **13** and **14** on reduction of the 4-nitro group (as signified by a value of 1.44 Hammett units in each case), the *total* electron release to the alkylating function is greater for compound **13** than for **14** (estimated by $\sum\sigma$ values of −0.38 and +0.40 Hammett unit, respectively). In the case of dinitro-substituted compounds such as **9** and **14**, it cannot be expected that both nitro groups will be reduced, since electron release from the first reduction will greatly lower the E^1_7 for the second group. Thus a study of the metabolism of **9** showed only products of monoreduction (e.g., **15**) as urinary me-



15



16



17

tabolites.[84] If our assumption of ignoring ortho effects in the calculation of the reduction potentials of the nitro groups in **9** were correct, then both would have the same E^1_7 and one might expect to see *both* monoreduction products **15** and **16** as metabolites. In fact, only **15** was detected,[84] although it is possible that **16** is the active

(82) Watts, M. E.; Jones, N. R. *Int. J. Radiat. Biol.* **1985**, *47*, 645.
(83) Williams, M. W.; Denekamp, J.; Minchinton, A. I.; Stratford, M. R. L. *Br. J. Cancer* **1982**, *46*, 127.
(84) Jarman, M.; Melzack, D. H.; Ross, W. C. J. *Biochem. Pharmacol.* **1976**, *25*, 2475.

species but is too unstable to be isolated.

Since many of the postulated structures are very lipophilic, the question of using a charged side chain for maximum water solubility was examined in detail. Our reevaluation of the influence of pH-dependent partitioning on drug accumulation by the target cells takes into account the fact that the transmembrane pH gradient in normal tissue is likely to be reversed in hypoxic regions of tumors. Thus weakly acidic HSA may be therapeutically superior to neutral or basic analogues.

In conclusion, nitrophenyl mustards can be seen as having potential as HSA if a number of design criteria are considered simultaneously. A study of weakly basic and weakly acidic derivatives of the dinitro compound 14, the corresponding mononitro derivatives 12 and 13, and various other isomeric compounds is now under way.

Acknowledgment. This work was supported by the Auckland Division of the Cancer Society of New Zealand and by the Medical Research Council of New Zealand. We thank Dr. P. Wardman for his constructive criticism of the manuscript.

Registry No. 1, 100858-17-1; 2, 74109-34-5; 3, 13551-87-6; 4, 4533-39-5; 5, 443-48-1; 6, 55743-71-0; 7, 2067-58-5; 8, 553-27-5; 9, 21919-05-1; 10, 24813-13-6; 11, 100858-18-2; 15, 61837-23-8; 16, 61837-26-1; 17, 70132-50-2; oxygen, 7782-44-7.

Communications to the Editor

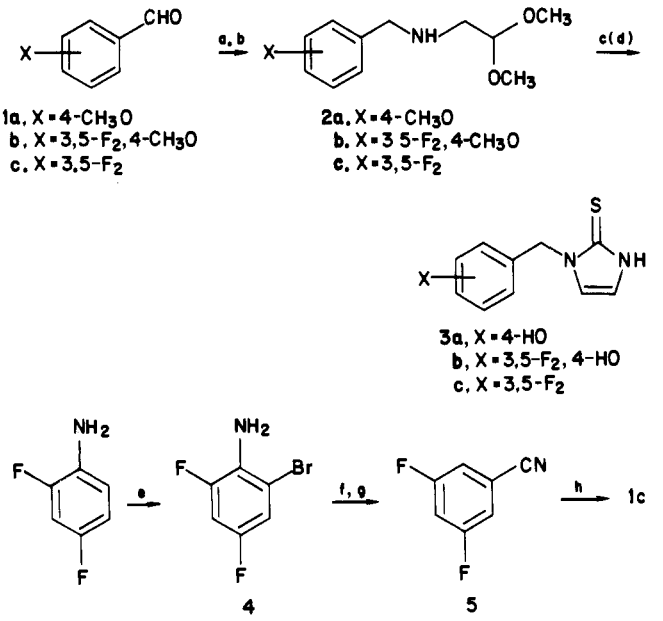
Substituted 1-Benzylimidazole-2-thiols as Potent and Orally Active Inhibitors of Dopamine β-Hydroxylase[1]

Sir:

Dopamine β-hydroxylase (DBH; EC 1.14.17.1), a copper-containing mixed-function oxidase, catalyzes the penultimate step in the catecholamine biosynthetic pathway, the benzylic oxidation of dopamine (DA) to norepinephrine (NE).[2] This enzymatic transformation affords a particularly attractive point for biochemical modulation of catecholamine levels in vivo. A selective DBH inhibitor would be expected to lower NE levels to blunt noradrenergic drive while simultaneously elevating levels of DA to provide the beneficial effects of peripheral dopamine receptor activation (i.e., renal vasodilation). An additional advantage of modulating this biochemical transformation derives from the known, limited potential for DBH upregulation.[3] This suggests a low probability of tachyphylaxis upon chronic administration of inhibitor, an important concern in the development of enzyme inhibitors as new therapeutic modalities.

The catalytic activity of DBH, hydroxylation of an unactivated C–H bond, is contingent upon the intermediacy of a powerful oxidizing species, presumably an activated copper–oxygen complex. The chemical reactivity of this species requires a proximity between the active site copper atom(s) and the enzyme-bound phenethylamine substrate to facilitate catalysis rather than single turnover autoxidation of enzyme. This rationale suggests compounds that are capable of binding to both the phenethylamine site and the active site copper atom(s) should be inhibitors of considerable potency. We have found that certain 1-benzylimidazole-2-thiols inhibit DBH by binding in such a fashion.[1,4] Here we report the synthesis and pharmacological evaluation of several of these, e.g., **3a–c**, which are extraordinarily potent inhibitors of DBH. Indeed, **3b** binds DBH approximately 10^6-fold more tightly than the phenethylamine (dopamine, tyramine) substrates do. The preparation of compounds **3a–c** is illustrated in Scheme I. Condensation of the appropriate benzaldehydes **1a–c** with aminoacetaldehyde dimethyl acetal followed by $NaBH_4$ reduction afforded (70–80%) the (*N*-benzylamino)acetaldehyde dimethyl acetals **2a–c** as oils. Cyclization of the crude oils **2a–c** with acidic thiocyanate and subsequent BBr_3 demethylation of the anisoles afforded imidazole-2-thiols **3a** (65%, mp 188–190 °C), **3b** (48%, mp 213–215 °C) and **3c** (76%, mp 142–144 °C). The benzaldehyde **1c**, a known compound that is difficult to pre-

Scheme I[a]



[a] Reagents and conditions: (a) $NH_2CH_2CH(OCH_3)_2$, ethanol; (b) $NaBH_4$, ethanol; (c) KSCN, HCl, H_2O, ethanol, reflux; (d) BBr_3, CH_2Cl_2; (e) Br_2, acetic acid; (f) $NaNO_2$, HBr, then $NaH_2PO_2 \cdot xH_2O$; (g) CuCN, *N,N*-dimethylacetamide, reflux; (h) Raney Ni alloy, formic acid, reflux.

(1) Presented in part at the 190th National Meeting of the American Chemical Society: Chicago, IL, Sept. 1985; American Chemical Society: Washington, DC, 1985; MEDI 65, MEDI 66.

(2) For recent monographs, see: (a) Skotland, T.; Ljones, T. *Inorg. Perspect. Biol. Med.* **1979**, *2*, 151. (b) Rosenberg, R. C.; Lovenberg, W. In *Essays in Neurochemistry and Neuropharmacology*; Youdin, M. B. H., Lovenberg, W., Sharman, D. F., Lagnado, J. R., Eds.; Wiley: New York, 1980; Vol. 4, p 163. (c) Villafranca, J. J. In *Copper Proteins*; Spiro, T. G., Ed.; Wiley: New York, 1981; p 264. (d) Ljones, T.; Skotland, T. In *Copper Proteins and Copper Enzymes*; Lontie, R., Ed.; CRC Press: Boca Raton, 1984; Vol. 2, p 131.

(3) Ungar, A.; Phillips, J. H. *Physiol. Rev.* **1983**, *63*, 787.

(4) Kruse, L. I.; DeWolf, W. E., Jr.; Flaim, K. E.; Frazee, J. S., unpublished results.

0022-2623/86/1829-0887$01.50/0