Hypoxia-Selective Antitumor Agents. 2. Electronic Effects of 4-Substituents on the Mechanisms of Cytotoxicity and Metabolic Stability of Nitracrine Derivatives

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The mechanism of cytotoxicity of a series of 4-substituted derivatives of 9-[[3-(dimethylamino)propyl]amino]-1-nitroacridine (nitracrine) has been studied, using a panel of DNA repair-defective mutants of the Chinese hamster ovary cell line AA8. Cell lines UV-4 and UV-5 were hypersensitive to nitracrine, with sensitivities approximately 10-fold greater than that of AA8, while EM-9 showed a hypersensitivity factor (HF) of about 2-fold. This pattern suggests the major cytotoxic lesions induced by nitracrine are bulky DNA monoadducts, rather than DNA interstrand cross-links as previously suggested. The desnitro analogue of nitracrine, which retains the intercalative potential of the latter but cannot be metabolically activated by nitro reduction, showed no hypersensitivity, indicating the specificity with which this panel of cell lines can discriminate different types of DNA damage. Several of the highly cytotoxic 4-substituted nitracrine derivatives showed HFs similar to that of the parent, but the less potent 4-dialkylamino and 4-COOMe derivatives showed much lower HFs for UV-4, suggesting that different mechanisms of cytotoxicity contribute. All compounds showed similar HFs under both aerobic and hypoxic conditions, indicating that hypoxia-selective toxicity in this series is due to a quantitative rather than qualitative change in the presence of oxygen. Rates of metabolic consumption of the compounds were measured under both aerobic and hypoxic conditions by bioassay against the sensitive UV-4 cell line. The results agreed well with previous inferences on metabolic stability derived from cell-killing kinetics and showed that electron-donating 4-substituents can be used to increase metabolic stability in vitro. Such stabilization may enhance the therapeutic utility of the nitroacridines in cancer therapy since rapid metabolism of nitracrine appears to prevent its activity against hypoxic cells in solid tumors.

Hypoxic cells in solid tumors are an important target for cancer chemotherapy.[1,2] They are not only refractory to radiation and to some cytotoxic drugs but may also be targeted specifically by drugs designed to be activated only in the absence of oxygen, providing selective bioactivation within tumor tissue.[3] A compound of current interest in this regard is the 1-nitroacridine derivative nitracrine (4; 9-[[3-(dimethylamino)propyl]amino]-1-nitroacridine), which has been shown to be a hypoxia-selective cytotoxic agent[4,5] and radiation sensitizer.[6] In possessing these biological effects, nitracrine resembles well-known electron-affinic nitroheterocycles such as misonidazole, but its in vitro potency is greater by many orders of magnitude, probably because of efficient targeting to DNA via intercalative binding.[4,6] Despite this high potency, nitracrine lacks activity against hypoxic tumor cells in vivo, apparently because of its high rate of reductive metabolism.[5] Although the reduction of the 1-nitro group of nitracrine is inhibited by oxygen, studies with multicellular spheroids suggest that the rate of nitro reduction even in well-oxygenated tumor tissue is sufficiently high to restrict its penetration into hypoxic regions.[5] Thus, if 1-nitroacridines are to be used successfully for eliminating hypoxic cells in solid tumors, an essential step is the development of analogues with improved metabolic stability.

In the preceding paper,[7] we reported the synthesis of a series of 4-substituted derivatives of nitracrine, with the objective of determining whether substituent electronic effects could be used to modulate reduction potential and hence control rates of metabolic nitro reduction. The one-electron reduction potentials ($E(1)$) of these compounds were shown to vary with σ, although the effects of prototropic equilibria tended to oppose these changes. Despite this, $E(1)$ values at pH 7 varied by over 100 mV in the series.[7] Furthermore, comparison of the kinetics of in vitro cell killing by these agents suggested that electron-donating functionality provided the desired trend toward higher metabolic stability. In the present study, quantitative information on rates of drug metabolism in aerobic and hypoxic cultures of the Chinese hamster ovary cell line AA8 have been obtained, by using a bioassay for residual cytotoxic activity.

For simple (neutral, non-DNA-binding) nitroheterocycles, aerobic or hypoxic cytotoxicity increases by about 10-fold for every 100-mV rise in $E(1)$,[8] with this single parameter accounting for almost all the variance in quantitative structure–activity relationship studies.[9] In contrast, the cytotoxic potency of the 4-substituted nitracrines varies by over 1000-fold and shows no significant correlation with $E(1)$.[7] Although insufficient analogues are available to attempt a full multivariate analysis, it seems unlikely from the data that variations in DNA binding, lipophilicity, or steric bulk can account for this marked variability in potency.[7] This observation, coupled with the fact that not all members of the series display hypoxia selectivity,[7] raises the possibility that more than one mechanism of cell killing may be operative.

Although the mechanism of cytotoxicity of nitracrine is not understood in detail, cell killing appears due to metabolic generation of reactive alkylating or arylating species, rather than to the demonstrated intercalative binding of

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Table I. Activity of Nitracrine against Repair-Defective Cell Lines Derived from AA8: Comparison of Assay Methods

culture method	exposure time, h	endpoint	potency against AA8, nM	hypersensitivity factor[a]		
				UV-4	UV-5	EM-9
suspension[b]	1	clonogenicity	D_{10}[c] = 570 ± 90[d]	10.8 ± 1.0	9.3 ± 1.4	1.8 ± 0.1
24-well[e]	4	growth inhibition[f]	IC_{50}[g] = 49 ± 9	12.8 ± 3.9	8.0 ± 1.5	1.8 ± 0.3
96-well[h]	18	growth inhibition[i]	IC_{50} = 24 ± 2	11.4 ± 2.9	8.0 ± 2.4	1.9 ± 0.3

[a] D_{10}(AA8)/D_{10}(mutant cell line), or IC_{50}(AA8)/IC_{50}(mutant cell line). [b] Method as described in Figure 1. [c] Drug concentration required to reduce surviving fraction to 10% of controls. [d] All values are means ± SE for three to four independent experiments. [e] Miniassay (see the Experimental Section). [f] Determined with electronic cell counter. [g] Drug concentration required to reduce cell density to 50% of controls. [h] Microassay (see The Experimental Section). [i] Determined with methylene blue staining.

the drug to DNA.[4,5,10,11] We present here a study of the contribution of DNA-reactive metabolites to cell killing and an investigation of the nature of the lesions responsible, using a panel of repair-defective mutants which have been selected from the AA8 cell line.[12,13]

Chemistry

Nitracrine and its 4-substituted derivatives (**1–9**) were synthesized as previously reported.[7] *N*-[[3-(Dimethylamino)propyl]amino]acridine (**15**) was prepared by phenol-mediated coupling of 9-chloroacridine and *N*,*N*-dimethylpropane-1,3-diamine at 120 °C.

Mutant AA8 Cell Lines

The cell lines UV-4 and UV-5 are representative of the two main phenotypes observed among the range of UV-sensitive mutants selected from AA8.[14–17] Both mutants resemble human xeroderma pigmentosum cell lines in their inability to perform the incision step of excision repair and in their consequent hypersensitivity (2–10-fold) to the cytotoxic and mutagenic effects of UV photoproducts and to DNA adducts formed by a variety of bulky carcinogens. The two lines differ in their sensitivities to DNA interstrand cross-linking agents, with UV-4 demonstrating dramatic hypersensitivity (8–200-fold) to a wide variety of such agents while UV-5 shows hypersensitivity factors (HFs) in the range 1–3-fold for the same compounds.[13,14]

The ethyl methanesulfonate (EMS) sensitive mutant EM-9 detects a different class of DNA lesions. This line,[12] also selected from AA8, shows hypersensitivity to some methylating and ethylating agents and to a wide variety of agents which induce DNA single strand breaks, including ionizing radiation,[12] bleomycin,[13] some genotoxic metals,[17] and neocarzinostatin.[18] In several respects EM-9 appears to be a phenocopy of cells treated with inhibitors of poly(ADPR) polymerase,[16] although no specific lesion in poly(ADPR) metabolism[19] or in any other aspect of the biochemistry of DNA repair has yet been identified. None of the three lines show any hypersensitivity toward classical DNA intercalating agents such as doxorubicin or to a variety of nonmutagenic chemicals.[13]

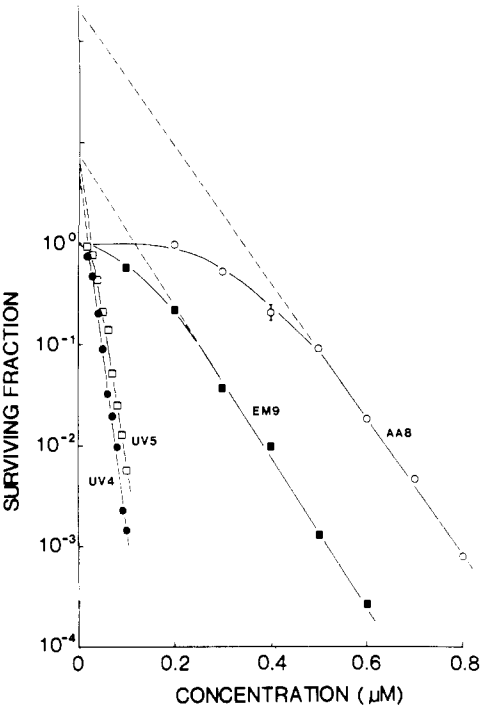

Figure 1. Clonogenic survival curves for 1-h exposure of log-phase AA8 cells (○) and the repair-defective mutants UV-4 (●), UV-5 (□) and EM-9 (■) to nitracrine (**4**). Control plating efficiencies were greater than or equal to 84% for all cell lines.

Human genes have now been cloned that correct each of the three mutant cell lines used in this study. Gene ERCC1 (Excision Repair Cross Complementing) corrects mutant UV4[20–22] and gene ERCC2 corrects mutant UV5.[23] The gene XRCC1 (X-ray Repair Cross Complementing) corrects mutant EM9.[24] These human genes function well

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Table II. Growth Inhibition of AA8 Cells, and the Repair-Defective Mutants UV-4, UV-5, and EM-9, after Exposure to Genotoxic Chemicals for 4 h[a]

agent	formula	AA8 IC_{50}, μM	hypersensitivity factor[b]		
			UV-4	UV-5	EM-9
mitomycin C	10	1.08 ± 0.18	86 ± 28	2.35 ± 0.05	0.78 ± 0.08
chlorambucil	11	36 ± 1	43 ± 7	1.4 ± 0.3	0.69 ± 0.13
4NQO[d]	12	0.33 ± 0.19	17 ± 3	16 ± 4	0.8 ± 0.2
EMS[e]	13	6800 ± 2400	1.3 ± 0.4	1.2 ± 0.3	6.3 ± 1.5
amsacrine	14	0.060 ±	0.9 ± 0.2	0.9 ± 0.3	0.68 ± 0.05
f	15	4.3 ± 0.7	1.03 ± 0.03	0.87 ± 0.05	0.78 ± 0.03
nitracrine[g]	4	0.049 ± 0.009	12.8 ± 3.8	8.0 ± 1.5	1.8 ± 0.3

[a] Assayed in 24-well dishes (miniassay). [b] IC_{50}(AA8)/IC_{50}(mutant). [c] Mean ± SE for two to four experiments. [d] 4-Nitroquinoline *N*-oxide. [e] Ethyl methanesulfonate. [f] Desnitro nitracrine. [g] Data from Table I.

in the hamster system, suggesting strong evolutionary conservation of repair proteins.

This panel of repair-defective mutants thus offers a possible approach to the detection of genotoxic activity arising from formation of DNA-reactive metabolites on metabolic reduction of the 1-nitroacridines and for the preliminary characterization of cytotoxic DNA adducts. Growth inhibition assays in 24-well culture dishes have been previously used[25] for determining differential cytotoxicity against these mutants. The present study extends this technique to 96-well microcultures and provides a quantitative end point for the determination of cell density with a microplate photometer.

Results and Discussion

Mechanism of Toxicity of Nitracrine (4). The ability of the panel of repair-defective mutants of AA8 to detect nitracrine-induced DNA lesions was assessed by measuring clonogenic survival after 1-h exposure to the drug under aerobic conditions (Figure 1). The drug concentration required to reduce the surviving fraction to 10% (D10) was used to quantitate the hypersensitivity of each line. With this end point, UV-4 and UV-5 showed pronounced hypersensitivity relative to AA8 (approximately 10-fold), while EM-9 was 1.8 times more sensitive than the parental cell line (Table I).

For all three mutant cell lines the survival curve extrapolation number, *n* (intercept on ordinate of the extrapolated exponential region), for 1-h exposure to nitracrine was reduced relative to that for AA8. For three experiments values of *n* were 270 ± 50 for AA8, 5 ± 1 for UV-4, 7.5 ± 0.5 for UV-5, and 13 ± 5 for EM-9 (means ± SE). For EM-9, hypersensitivity to nitracrine was due to the change in *n* only, while for UV-4 and UV-5 large decreases in D_0 (by about 6-fold) were also evident. The lowering of *n* in the repair-defective cell lines suggests that the threshold on the AA8 survival curve is largely due to DNA-repair processes. This finding is in distinct contrast to that for misonidazole where the threshold appears to be due to the protective effect of glutathione which must be consumed during nitro reduction before cell killing ensues.[26]

Since determination of drug sensitivity by clonogenic assay is an expensive procedure not well suited to the screening of large numbers of compounds, the differential cytotoxicity of nitracrine was also assessed by using a growth-inhibition assay. Cells were exposed to drug for

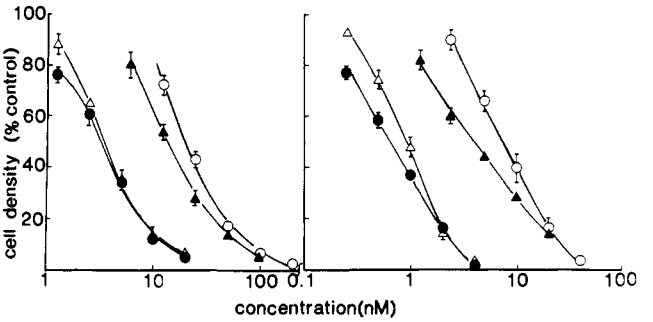

Figure 2. Comparison of the sensitivity of AA8 (○), UV-4 (●), UV-5 (△), and EM-9 (▲) cells to growth inhibition by nitracrine (4). Left panel: 4-h drug exposure in 24-well tissue culture dishes with measurement of cell density 30 h later with an electronic particle counter (miniassay). Right panel: 18-h drug exposure in 96-well tissue culture plates with measurement of cell density 75 h later by methylene blue staining (microassay).

4 h in 24-well tissue culture plates as described previously,[25] and subsequent growth inhibition was assessed by visual scoring of the staining intensity of the cell monolayer 72 h later. By this technique the hypersensitivity factors of UV-4 and UV-5 were both approximately 8, while EM-9 showed no apparent hypersensitivity. The growth inhibition assay was modified by determining cell numbers with an electronic particle counter to provide a more objective end point. This assay (miniassay) confirmed the marked hypersensitivity of UV-4 and UV-5 and the less pronounced effect on EM-9 (Figure 2). With use of the drug concentrations needed to reduce cell densities to 50% of controls (IC_{50}), HFs in good agreement with those obtained from clonogenic survival curves were found.

The growth-inhibition assay was also adapted for use in 96-well tissue culture plates to take advantage of a staining technique using a microplate photometer (96-well plate reader) to determine cell numbers.[27] This assay (microassay) uses 20-fold less drug than the miniassay (0.05-mL cultures compared to 1-mL cultures) and allows faster sample handling by using eight-channel pipettors. Using a method which measures total cell mass (staining) rather than cell number required some additional modifications. In particular, low seeding densities were required to allow a sufficiently long posttreatment growth period to minimize the effects of transient enlargement of cells due to reversible division delay or giant cell formation. For AA8, UV-4, UV-5, and EM-9, seeding densities of 200, 300, 300, and 450 cells per well were used, respectively, to compensate for the differences in growth rate, providing in each case late log-phase cultures 75 h after the treatment period. Under these conditions, nitracrine again provided steep dose–response curves for growth inhibition (Figure

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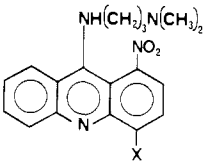
Table III. Effect of Nitracrine and 4-Substituted Derivatives on the Growth Inhibition of AA8 Cells and the Repair-Defective Mutant UV-4 in 96-Well Cultures under Aerobic and Hypoxic Conditions

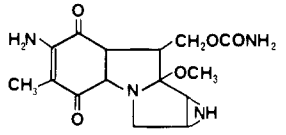


		AA8 IC_{50}[a]		UV-4 IC_{50}		HF[b]	
no.	X	air (nM)	air/N_2	air (nM)	air/N_2	air	N_2
1	COOMe	2900 ± 90[c]	1.20 ± 0.05	1950 ± 200	1.2 ± 0.3	1.5 ± 0.1	1.5 ± 0.1
2	Cl	33 ± 2	3.7 ± 0.5	3.3 ± 0.3	4.9 ± 0.9	10 ± 1	13 ± 1
3	F	250 ± 20	1.3 ± 0.2	12 ± 1	1.4 ± 0.1	20 ± 3	21 ± 1
4	H	26.3 ± 1.4	3.54 ± 0.3	2.0 ± 0.3	3.8 ± 0.3	12.9 ± 1.5	14.9 ± 1.7
5	Me	76 ± 2	5.0 ± 0.9	8.0 ± 0.7	4.6 ± 0.6	9.5 ± 0.7	9.0 ± 1
6	$N(Me)_2$	18900 ± 2400	3.3 ± 0.2	6200 ± 470	2.3 ± 0.1	3.0 ± 0.2	2.1 ± 0.1
7	OMe	616 ± 73	5.3 ± 1.0	63 ± 5	4.2 ± 0.9	9.8 ± 0.6	7.8 ± 1.2
8	$N(CH_2CH_2OH)_2$	62000 ± 16000		19400 ± 2000		3.2 ± 0.2	
9	$N(CH_2CH_2OAc)_2$	43000 ± 10000	4.5 ± 0.1	11600 ± 3000	4.8 ± 1.7	3.7 ± 0.1	4.8 ± 1.8

[a] See footnote *g*, Table I. [b] Hypersensitivity factor (IC_{50}(AA8)/IC_{50}(UV-4)). Values are means of intraexperiment ratios. [c] All values are means ± SE for three independent experiments.

2), with UV-4, UV-5, and EM-9 showing HFs of approximately 10, 10, and 2, respectively. Thus good agreement is obtained with all three assays, each of which employs a different end point (Table I).

The miniassay and microassay were then used to compare HFs for several cytotoxic agents which give rise to well-characterized DNA lesions, and representative data for the miniassay are shown in Table II. The DNA interstrand cross-linking agents mitomycin C (**10**) and chlorambucil (**11**) showed the dramatic selective activity against UV-4 which is characteristic of this class of agents.[13] In contrast, 4-nitroquinoline *N*-oxide (**12**) showed high HFs against both UV-4 and UV-5, typical of agents which form bulky monoadducts.[13] The DNA methylating agent ethyl methanesulfonate (**13**) showed hypersensitivity only against EM-9, and the topoisomerase II poison amsacrine (**14**), although an efficient inducer of enzyme-mediated DNA breaks, did not show hypersensitivity in any of the mutant lines. This agrees with earlier observations that the cytotoxic lesions caused by DNA intercalating agents are not detected by this panel of mutants.[13]



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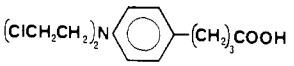

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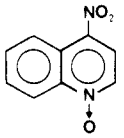

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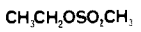

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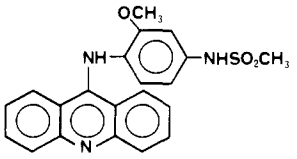

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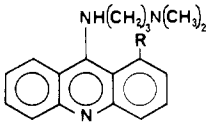

15 R = H
16 R = NHOH

The hypersensitivity pattern shown by nitracrine thus suggests that its cytotoxicity is due to the formation of bulky DNA monoadducts, and that these lesions are repaired efficiently in the parental AA8 line. The pattern is not consistent with previous suggestions[10,28] that DNA interstrand cross-linking is the critical cytotoxic lesion. It was of particular interest to evaluate the desnitro analogue of nitracrine in the panel of cell lines, since this compound (**15**) retains the DNA-intercalating chromophore and basic side chain of the latter but cannot be metabolized by nitro reduction. This compound was shown by HPLC to be metabolically stable in AA8 cultures at 10^6 cells/mL, under which conditions nitracrine undergoes extensive metabolism (data not shown). The lack of hypersensitivity to **15** (Table II) suggests that nitro reduction is necessary for the generation of the cytotoxic DNA lesions induced by nitracrine. Furthermore, the clear distinction between nitracrine and the desnitro analogue **15** demonstrates the utility of this panel of cell lines in discriminating classical DNA intercalators from intercalators capable of also forming covalent DNA adducts.

Mechanism of Cytotoxicity of 4-Substituted Nitracrines. The above panel of AA8 mutants was used to compare the mechanisms of cytotoxicity of the 4-substituted nitracrine analogues (**1–3, 5–9**), under both aerobic and hypoxic conditions. The microassay was used for this work, since its high throughput (eight drugs/96-well plate) allowed direct intra-experiment comparison of all agents. The experimental conditions were designed to minimize problems of slow deoxygenation in unstirred monolayer cultures in polystyrene plates by using long drug contact times (18 h) under an atmosphere of air or nitrogen and by using lethally irradiated AA8 cells to facilitate induction of hypoxia by metabolic scavenging of residual oxygen diffusing out of the plastic substrate, and will be reported in detail elsewhere.[29] Under these conditions, nitracrine had a hypoxic selectivity of about 3.5-fold (Table III), as compared to 10-fold in stirred suspension cultures.[7] In the latter assay, all the 4-substituted derivatives demonstrated similar hypoxic selectivity to nitracrine, with the exception of the 4-COOMe compound 1, while in the microassay all derivatives except 1 and 3 showed similar selectivity (Table III). The lack of hypoxic selectivity of 3 in the microassay

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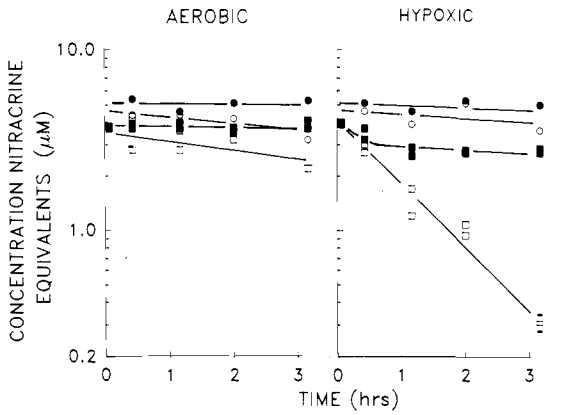

Figure 3. Assay of extracellular medium from stirred AA8 cultures (10^6 cells/mL) after addition of [^{3}H]nitracrine to 0.5 μM: (□) bioactivity against UV-4, (■) total extracellular radioactivity. Parallel incubations were performed with tissue culture medium in the absence of cells: (○) bioactivity, (●) radioactivity. Left hand panel: equilibrated with 5% CO_2 in air. Right hand panel: equilibrated with 5% CO_2 in N_2.

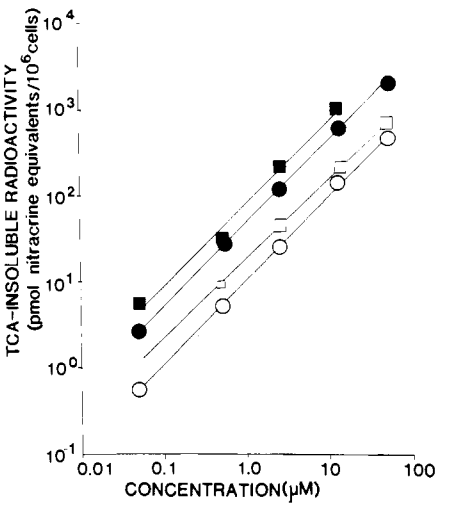

Figure 4. Formation of macromolecular (TCA-insoluble) adducts in AA8 cells on incubation with [^{3}H]nitracrine in stirred suspension cultures under aerobic or hypoxic conditions. The abscissa indicates nitracrine concentration at zero time. (○) 30 min aerobic incubation, (□) 60 min aerobic; (●) 30 min hypoxic, (■) 60 min hypoxic.

is readily explained by its pronounced metabolic instability under aerobic conditions[8] (vide infra), since loss of drug during the deoxygenation period would reduce the apparent selectivity. Against UV-4, each compound showed similar hypoxic selectivity to that against AA8 (Table III).

However, comparison of IC_{50} values for AA8 and UV-4 in the microassay showed that HFs for the repair-deficient cell line were not the same for all drugs in the series (Table III). The highest HF (20-fold) was consistently obtained with the 4-F compound (**3**), while compounds **2**, **4**, **5**, and **7** all gave HFs of about 10. The two dialkyamines (**6** and **9**) gave significantly lower HFs of about 3 while the related diol (**8**), although not included in the set of matched microassay experiments, gave an HF for UV-4 of 3.2 (Table III). The low HFs for the substituted amines thus appear to be a common feature, while even lower HFs (about 1.5) were found for the 4-COOMe derivative **1**.

The marked variation in HFs for UV-4 among the 4-substituted nitracrines suggests that the compounds do not all have an identical mechanism of cytotoxicity and that the compounds therefore do not represent a true congeneric series. One possible interpretation is that at the high concentrations required for the less dose-potent analogues (**1**, **6**, **8**, and **9**), which have IC_{50} values equal to or greater than that for the nonmetabolized desnitro analogue **14**, toxicity is mediated more by the consequences of reversible DNA intercalation (perhaps via topoisomerase II) than by metabolically activated DNA alkylation or arylation. However, the observation that compounds **6** and **9** retain the full level of hypoxic selectivity shown by nitracrine argues against this interpretation, since such activity implies that metabolic activation by nitroreduction is occurring for these compounds. An alternative possibility, not able to be examined here, is that all compounds (except **1**) act via reductive alkylation, but give different types of DNA lesions. Whatever the details of the mechanisms, they appear to be the same under both aerobic and hypoxic conditions, since for each compound the aerobic and hypoxic HFs are similar (Table III). Thus, oxygen appears to act as a quantitative rather than a qualitative modifier of cytotoxicity in the 4-substituted nitracrines.

Metabolism of Nitracrine. Previous studies[4] showed rapid loss of nitracrine bioactivity in hypoxic AA8 cultures at 10^6 cells/mL, presumably due to drug metabolism, although no quantitative estimate of rate was reported. The availability of semiautomated growth-inhibition techniques used in the microassay above and sensitive indicator cell lines such as UV-4 suggested the possibility of monitoring drug loss from the extracellular medium by bioassay. An experiment using these techniques is illustrated in Figure 3. Titration of culture supernatant against log-phase UV 4 cultures in 96-well microculture plates showed a pseudo-first-order decrease in biological activity (expressed as nitracrine equivalents) in hypoxic AA8 cultures at 10^6 cells/mL, with a half life of 0.85 h. This loss of bioactivity was dependent on the presence of cells (Figure 3), suggesting that it was due to metabolism rather than chemical instability. Inhibition by oxygen of this loss of bioactivity from the extracellular medium (Figure 3) indicates the involvement of metabolic nitroreduction rather than cell uptake per se, since the latter process is oxygen-independent.[6] Tritium-labeled nitracrine was used in this experiment to allow monitoring of total extracellular nitracrine plus biotransformation products (Figure 3). The total nitracrine-derived extracellular radioactivity showed only a small initial decrease (presumably due to cellular uptake and intracellular binding[6]) followed by a plateau, clearly demonstrating the extracellular accumulation of biologically inactive metabolites. Radiochromatography by HPLC showed one major extracellular metabolite, more polar than the parent and tentatively identified[6] as the 1-hydroxylamine **16**. Thus, for nitracrine, bioassay techniques appear to be suitable for quantitating rates of metabolic conversion to a biologically less active end product.

The large differences in IC_{50}s for the 4-substituted nitracrines presented potential difficulties in attempting to compare rates of metabolic depletion with identical drug concentrations for all compounds. To determine whether the kinetics of metabolism were likely to vary with drug concentration, rates of formation of macromolecular (TCA-insoluble) metabolites[5] were compared over a 1000-fold range of tritium-labeled nitracrine concentrations under both aerobic and hypoxic conditions (Figure 4). Formation of macromolecular adducts was inhibited by oxygen to an equal extent over this wide concentration range, and the relationship between initial rate and drug concentration was linear, indicating pseudo-first-order kinetics. Thus the rate of drug metabolism and its hypoxic selectivity appeared to be constant over a wide range of drug concentrations. This conclusion is also supported by

Table IV. Metabolic Consumption of 4-Substituted Nitracrine Derivatives in Stirred Aerobic and Hypoxic AA8 Suspension Cultures As Assessed by Bioassay[a]

				aerobic[e]		hypoxic[e]	
no.	$E(1)$ at pH 7,[b] mV	UV-4 IC_{50},[c] nM	Co,[d] μM	t,[f] h	A_0[g]	t,[f] h	A_0[G]
1	−244 ± 11	590 ± 50	10	NS[h]	0.47 ± 0.05	3.9 ± 0.4	0.45 ± 0.04
2	−325 ± 11	0.62 ± 0.08	0.04	4.3 ± 0.7	0.79 ± 0.07	0.68 ± 0.06	0.56 ± 0.12
3	−354 ± 11	2.0 ± 0.33	0.1	0.91 ± 0.13	0.64 ± 0.11	0.77 ± 0.08	0.84 ± 0.09
4	−303 ± 11	0.66 ± 0.13	0.04	7.3 ± 1.3	0.86 ± 0.08	0.84 ± 0.08	0.98 ± 0.04
5	−321 ± 11	1.65 ± 0.17	0.2	NS[h]	0.65 ± 0.09	0.99 ± 0.06	1.12 ± 0.05
6	−334 ± 11	410 ± 59	30	4.8 ± 0.2	0.78 ± 0.02	2.3 ± 0.2	0.68 ± 0.04
7	−361 ± 11	14.5 ± 1.9	3.0	14 ± 4	0.76 ± 0.07	5.2 ± 1.0	0.78 ± 0.07
9	−314 ± 10	3450 ± 440	50	18 ± 6	1.11 ± 0.01	8.4 ± 1.7	1.03 ± 0.16

[a] As described in Figure 5. [b] Data from ref 7. [c] See footnote *g*, Table I. Cells were exposed continuously to the drug for 4 days. Values are mean ± SE for six determinations. [d] Total drug concentration in AA8 suspension cultures at zero time. [e] Values are mean ± SE for three independent experiments in each of which all eight compounds were assayed simultaneously. [f] Half-life of extracellular bioactivity assessed by bioassay of culture supernatants against UV-4. Half-lives were determined by log-linear regression analysis. [g] Extrapolated fraction of biological activity remaining at time zero. [h] No statistically significant trend with time.

the observation of identical bioassay half-lives for nitracrine when initial drug concentrations differed by 12-fold (compare Figure 3 and Table IV). The pseudo-first-order kinetics of nitracrine metabolism contrasts with the half-order kinetics (square root concentration dependence) observed for the formation of macromolecular adducts on nitro reduction of misonidazole.[30]

Metabolism of 4-Substituted Nitracrines. Rates of metabolic consumption of nitracrine analogues in both aerobic and hypoxic AA8 cultures were assessed by using drug concentrations approximately 20–50 times greater than the continuous exposure IC_{50} values against UV-4 (Table IV). Culture supernatants were then assayed against microcultures of log-phase UV-4 cells to provide the data of Figure 5. In all cases where metabolic depletion of bioactivity was large enough to measure the kinetics appeared to be first order, although in most cases the fitted log-linear regression line extrapolated to less than 100% at zero time, suggesting that a rapid uptake component may be present. These extrapolated zero time values were similar under aerobic and hypoxic conditions (Table IV).

Bioassay data for the 4-Cl and 4-Me derivatives (**2** and **5**) were similar to those for nitracrine, although the electron-withdrawing 4-Cl appeared to decrease metabolic stability somewhat while the electron-donating 4-Me increased it. Compounds **6**, **7**, and **9**, with more strongly electron donating 4-substituents, had substantially increased metabolic stabilities. This apparent correlation of metabolic rate with electronic parameters was broken by the 4-COOMe compound **1**, which appears to have greater metabolic stability than nitracrine despite a strongly electron donating substituent. The other surprising finding was the oxygen independence of depletion rates for the 4-F compound **3**. However, the results agree well with inferences about metabolic stability in this series derived from comparisons of cell-killing kinetics.[7] It should be noted that the bioassay technique used here provides valid estimates of sensitivity to nitroreduction only if (a) cell uptake factors (intracellular/extracellular concentration ratios) are similar for all compounds and (b) the end products of nitro reduction have much lower bioactivity than the starting compounds. Thus the apparent slow metabolism of **1** could be due to lack of cellular accumulation or conversion to a stable metabolite with growth inhibitory potency similar to that of the original drug. True rates of nitro reduction can only be assessed by more compound-specific methods such as HPLC. However, the present technique has the virtue of biological relevance since it measures residual bioactivity directly.

Conclusions

The hypersensitivity pattern shown by nitracrine (**4**) against the panel of AA8 mutant cell lines UV-4, UV-5, and EM-9 (HFs of 11, 8, and 2, respectively) suggests that its primary cytotoxic effect is due to the formation of bulky DNA monoadducts rather than DNA interstrand cross-links, as previously suggested,[10,28] since this would be expected to give very high HFs (8–200) against UV-4 but much lower HFs (1–3) against UV-5. Similar patterns were also seen with several of the highly cytotoxic 4-substituted derivatives (**2**, **3**, **5**, and **7**). However, the less potent 4-dialkylamino compounds (**6**, **8**, and **9**) had much lower HFs against UV-4, suggesting possibly different modes of cytotoxicity. The lack of hypersensitivity shown by the desnitro compound **15** supports previous suggestions[5] that metabolic nitro reduction is necessary for the cytotoxicity of nitracrine.

While nitracrine (**4**) has significant hypoxia selectivity against a range of tumor cell lines in vitro,[4,5,7] it shows no activity against hypoxic cells in vivo, probably due to excessively rapid nitro reduction.[5] Later work[7] showed that significant modulation of the nitro group reduction potential could be achieved by acridine substitution, with electron-donating groups lowering the potential by up to 60 mV. These derivatives retained hypoxia-selective cytotoxicity while appearing to have greater metabolic stability, as judged by their cell-killing kinetics.[7] This has now been confirmed by bioassay using the sensitive UV-4 cell line to measure directly the kinetics of drug loss. While nitracrine (**4**) has a half-life of 0.85 h in hypoxic AA8 cultures and the 4-F, 4-Cl, and 4-Me derivatives (**2**, **3**, and **5**) have similar or even lower stability, the analogues with strongly electron-donating substituents (**6**, **7**, and **9**) have half-lives up to 10-fold longer under the same conditions.

These studies show that nitracrine analogues with electron-donating 4-substituents may avoid the problem of rapid loss of bioactivity due to rapid metabolism. If this process prevents the penetration of nitracrine into the hypoxic regions of tumors as suggested,[5] then more stable analogues such as the 4-OMe derivative **6** would be expected to show improved activity as hypoxia-selective cytotoxic agents in vivo. Preliminary studies with advanced subcutaneous EMT-6 tumors suggest that **6**, but not **4**, does have selective activity against hypoxic tumor cells in vivo.

Experimental Section

Chemistry. Synthesis of *N*-[[3-(Dimethylamino)propyl]amino]acridine (15). A mixture of 9-chloroacridine (1 g, 4.7 mmol), phenol (1.5 g), and *N*,*N*-dimethylpropane-1,3-di-

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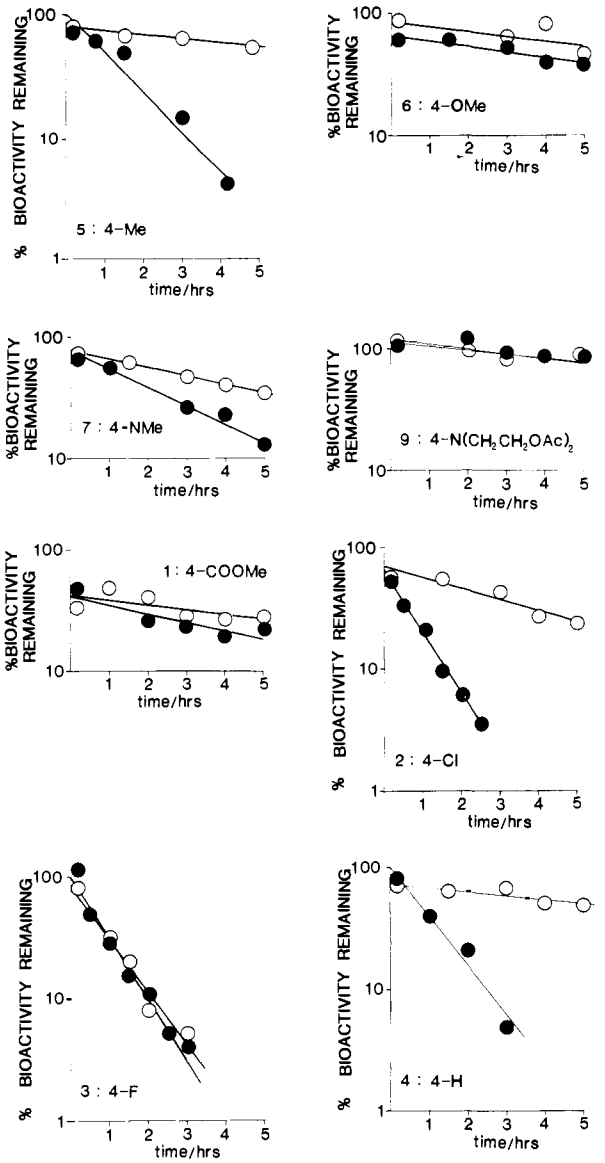

Figure 5. Bioassay of cytotoxic activity in the extracellular medium of aerobic (○) or hypoxic (●) AA8 cultures at 10^6 cells/mL. Biological activity remaining was assessed by assaying growth inhibition of UV-4. Drug concentrations at zero time are indicated in Table IV and were the same for aerobic and hypoxic cultures. Curves were fitted to the data by log-linear regression analysis.

amine (0.6 g, 6 mmol) was dissolved in dry benzene (5 mL) and heated at 120 °C (allowing evaporation of the solvent) for 1 h. The cooled mixture was partitioned between CH_2Cl_2 and 2 N NaOH, and the organic layer was washed well with water and evaporated to give the solid free base (1.05 g, 80%), which was crystallized from MeOH/EtOAc/HCl as the dihydrochloride, mp 263–265 °C.

[^{3}H-G-*acridinyl*]Nitracrine (specific activity 145 mCi/mmol) was synthesized from randomly tritiated sodium *N*-(3-nitrophenyl)anthranilate as previously described.[31] The radiochemical purity at time of use was >98%, as determined by scintillation counting of the HPLC eluate with a reverse-phase chromatographic system.[6]

Cell Culture. AA8, UV-4, UV-5, and EM-9 cells were maintained as mycoplasm-free exponential-phase cultures (doubling times 14, 15, 15, and 18 h, respectively) in 25-cm^2 tissue culture flasks as described previously[7] for AA8. Bulk cultures were prepared by growing cells in spinner flasks with Alpha MEM containing fetal calf serum (10% w/v), penicillin (100 IU/mL), and streptomycin (100 μg/mL).

Survival Curves. Log-phase spinner cultures (grown to (2–3) × 10^5 cells/mL) were diluted to 10^5 cells/mL, and aliquots of this cell suspension were placed into polystyrene culture tubes (10 mL) which were flushed with 5% CO_2 and equilibrated in a 37 °C water bath. Cultures were exposed to nitracrine by adding sterile, aqueous solutions of the drug (0.1 mL). Cells were kept in suspension by frequent mixing by inversion. After 60 min, cultures were centrifuged and resuspended in fresh medium, and clonogenicity was determined as described previously.[4]

Growth Inhibition (Miniassay). Cultures of AA8, UV-4, UV-5, and EM-9 cells were initiated at 2 × 10^4 cells/mL in 24-well culture dishes (0.5 mL/well). After 24 h, drugs were added in an equal volume of medium. Two drugs were assayed at five concentrations (serial 2-fold dilutions) in duplicate, with four untreated control cultures in each dish. After incubation at 37 °C for 4 h, cultures were washed three times with fresh medium and incubated for a further 30 h. Single-cell suspensions were then prepared by trypsinizing with 0.07% trypsin in citrate–saline (trisodium citrate dihydrate, 4.4 g/L; KCl, 10 g/L; pH 7.3), and cell numbers were determined with an electronic particle counter (Coulter Electronics). The IC_{50} was defined as the drug concentration required to reduce the cell density to 50% of the mean control value.

Growth Inhibition (Microassay). Cultures were initiated in 96-well flat-bottomed tissue culture plates with viable AA8, UV-4, UV-5, or EM-9 cells (200, 300, 300, and 450 cells/well, respectively) plus 5000 lethally irradiated (35 Gy, ^{60}Co) AA8 cells per well, in 0.05 mL of medium. After 24 h, drugs were added by making serial 2-fold dilutions directly into the 96-well plate, with an eight-channel air-displacement pippet. Plates were then transferred to anaerobic chambers submerged in a water bath at 20 °C. The chambers were evacuated three times to 160 mmHg and flushed for 10 min with 5% CO_2 in air (aerobic exposure) or N_2 (hypoxic exposure) after each evacuation before sealing. The latter gas mixture included 1% H_2 to provide scavenging of residual O_2 by a Pd catalyst in the chamber (Gas Pak charge, BBL Microbiology Systems). Following the final evacuation, the water bath temperature was raised to 37 °C. After 18 h, cultures were washed three times with fresh medium and grown in a CO_2 incubator for a further 72–78 h before staining with methylene blue as described previously.[27] Absorbance was determined at 620 nm, with subtraction of absorbance at 405 nm, with a 96-well microplate photometer (Titertek Multiscan MC, Flow Laboratories) with data capture on a Sharp PC 1500 computer. The average absorbance of the blanks, seeded with irradiated cells only, was subtracted from all values but was typically only 1–3% of the untreated controls. The IC_{50} value was determined as above with absorbance in place of cell density, with eight controls/plate. Calibration curves obtained by seeding known numbers of AA8 or UV-4 cells into 96-well plates 4 h before staining established the linearity of the staining method in determining cell numbers.

Bioassay Studies. Plateau-phase AA8 cells were exposed to nitracrine analogues using stirred suspension cultures at 10^6 cells/mL under an atmosphere of 5% CO_2 in air or N_2, as described previously.[7] At various times, samples were withdrawn and centrifuged rapidly (1300*g* × 1 min) to pellet cells and suitable dilutions of culture supernatants were assayed for growth-inhibitory activity against log-phase UV-4 cultures, essentially as described for the microassay above. UV-4 cultures were initiated for this purpose at 3 × 10^3 cells/mL approximately 24 h before addition of drugs. Cultures were exposed continuously to the drugs until staining 4 days later. The fraction of bioactivity remaining was calculated as the ratio of IC_{50} values at time zero (determined by assaying samples of culture media containing drugs immediately before addition of cells) to the apparent IC_{50} at subsequent times.

Metabolism Studies with [^{3}H]Nitracrine. Stirred plateau-phase AA8 cultures at 10^6 cells/mL were treated with [^{3}H]nitracrine under aerobic or hypoxic conditions as described previously.[7] Samples (1 mL) were withdrawn at 1, 30, and 60 min and centrifuged at 2 °C (150*g* × 5 min), and the cell pellet was resuspended in ice-cold saline (1 mL) containing 1% calf serum. An equal volume of trichloroacetic acid (10% w/v) was added, and after holding on ice for 15 min, the macromolecular precipitate

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was collected on Whatman GF/C glass fiber filters and washed extensively with 1 N HCl. Filters were dried, and radioactivity was determined by scintillation counting in toluene containing 2,5-diphenyloxazole (4 g/L) and 1,4-bis(5-phenyloxazol-2-yl)-benzene (0.1 g/L). Counting efficiencies were determined by drying known amounts of [^{3}H]nitracrine onto blank filters prepared with an equivalent number of nonradiolabeled cells.

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Registry No. 1, 116374-64-2; **2**, 116374-65-3; **3**, 116374-66-4; [^{3}H]-**4**, 116405-65-3; **5**, 24400-01-9; **6**, 116374-67-5; **7**, 25799-70-6; **8**, 107210-40-2; **9**, 107210-39-9; **10**, 50-07-7; 11, 305-03-3; **12**, 56-57-5; **13**, 62-50-0; 14, 51264-14-3; **15**, 51264-14-3; **15**·2HCl, 1092-03-1; **16**, 87061-35-6; 9-chloroacridine, 1207-69-8; *N,N*-dimethylpropane-1,3-diamine, 109-55-7; sodium *N*-(3-nitrophenyl)-anthranilate, 118-92-3.

Stereoelectronic Study of Zetidoline, a Dopamine D2 Receptor Antagonist

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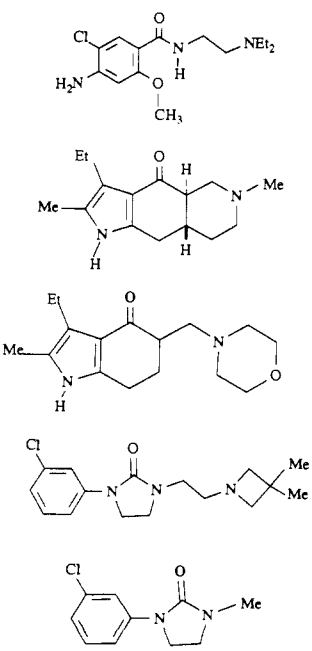
A combination of experimental and theoretical methods were used to investigate the stereoelectronic structure of zetidoline, a dopamine D2 receptor antagonist showing Na^+-dependent binding. The solid-state conformation of zetidoline is characterized by synplanarity (coplanarity of the two rings with the chloro substituent and the carbonyl group on the same side). The side chain in the crystal adopts a folded conformation which places the azetidine nitrogen atom at about 8 Å from the center of the aromatic ring. Quantum mechanical calculations indicate the synperiplanar and antiperiplanar conformations of the ring system to be of approximately equal energies. The molecular electrostatic potential of zetidoline in a nearly extended conformation shows a remarkable similarity with that of orthopramides (e.g. metoclopramide) and indolones (e.g. piquindone), i.e. two groups of drugs displaying the same D2 selectivity and Na^+-dependent binding. We postulate that the close stereoelectronic similarity between zetidoline, orthopramides, and indolones accounts for their identical mechanism of action in the molecular level.

Among dopamine receptor antagonists, a number of compounds are known to act selectively on the subgroup of D2 receptors and to display a Na^+-dependent binding.[1-4] These compounds (see Scheme I) include orthopramides (e.g. metoclopramide (**1**), sulpiride, and tropapride), indolones (e.g. piquindone (**2**) and molindone (**3**)), and zetidoline (**4**).[5-7]

In previous studies, some of us have demonstrated close structural analogies among orthopramides. In particular, their molecular electrostatic potential (MEP) could be rationalized in terms of a pharmacophore[8] which has been found to be similar to that displayed by molindone and piquindone.[9] Recently, this pharmacophoric model has received independent validation by being proven congruent with the results of a traditional quantitative structure-activity relationship (QSAR) analysis of 20 orthopramide derivatives.[10,11]

The pharmacological analogy (i.e. their high, selective, and Na^+-dependent affinity for dopamine D2 receptors) between zetidoline, orthopramides, and indolones[12] suggests a structural resemblance which is not obvious when the chemical formulae are examined (Scheme I). In the present study, we report the crystallographic structure, conformational behavior, and MEP of zetidoline, showing that its stereoelectronic structure is indeed similar to that of other Na^+-dependent D2 receptor antagonists.

Scheme I



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