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Active-site directed irreversible inhibition of diamine oxidase by a homologous series of aziridinylalkylamines

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Abstract—Three electrophilic homologous aminoalkylaziridine analogues of putrescine, cadaverine, and 1,3-diaminopropane were synthesized and found to represent a mechanistically distinct class of irreversible inhibitors of diamine oxidase. The putrescine analogue, *N*-(4-aminobutyl)aziridine gave the lowest calculated IC_{50} value, whereas *N*-(3-aminopropyl)aziridine, an analogue of the poorest substrate of the series, showed the highest IC_{50}. The findings suggest that the aziridinylalkylamines tested are site-directed agents that form irreversible complexes at the active site of diamine oxidase. Affinity of the inhibitors for the active site appeared to be dependent on alkyl chain length, suggesting that binding promotes the reactivity of the aziridinyl group.

Aziridine (ethylenimine) has been known to be an electrophilic alkylating agent for over 100 years [1]. Aziridine-containing probes of biological systems have been described frequently in the literature [2–5]. To illustrate, a number of anticancer drugs including thiotepa, triethylenemelamine, diaziquone, and mitomycin C contain aziridine as a functional group as do several new enzyme inhibitors or receptor binding agents [6–11]. Series of aziridine-substituted cyclophosphazenes [12], nitroimidazole aziridines [13], and phenylaziridines [14] have been shown recently to have tumor inhibitory activity.

The role of polyamine biochemistry in cell growth and proliferation has attracted much interest [15]. A major pathway for the catabolic processing of polyamines has been assigned to diamine oxidase [diamine:oxygen oxidoreductase (deaminating) (copper containing), EC 1.4.3.6]. Putrescine (1,4-diaminobutane), in addition to being one of the better substrates for diamine oxidase [16], is also taken up into cells by a polyamine uptake system [17]. Monosubstitution of an amino group of putrescine with aziridine produces the analogue, *N*-(4-aminobutyl)aziridine, which is a cytotoxic [18, 19], potent reversible inhibitor of polyamine uptake [17, 20]. *N*-(4-Aminobutyl)aziridine has been evaluated as a potential chemotherapeutic agent for prostatic cancer [19, 21].

Diamine oxidase is inhibited by a variety of guanidine [22–25] and hydrazine derivatives [26, 27]. Irreversible inhibition has been observed with aminoguanidine [25] and substituted hydrazides [27], presumably by a mechanism involving nucleophilic attack on the carbonyl cofactor [28]. Since aziridinyl substituents react as electrophiles, a series of aziridinyl derivatives of diamines was prepared and evaluated as novel inhibitors of diamine oxidase and as potential covalent inactivators at active-site regions distanced from the cofactor by an alkyl chain.

Methods

PMR and mass spectra were consistent with assigned structures of synthesized compounds. Freshly distilled pure compounds were used for IC_{50} determinations. The mutagenicity, carcinogenicity, and toxicity of the synthesized aziridinyl compounds have not been assessed.

N-(3-*aminopropyl)aziridine*. Aziridine (8.6 g, 0.2 mol), prepared from 2-aminoethyl hydrogen sulfate [29], was added dropwise to stirred acrylonitrile (12.8 g, 0.22 mol) heated to 35–40°. The temperature was maintained throughout the addition period and for an additional 1 hr. The reaction mixture stood at room temperature overnight and then the product was distilled (b.p. 35–36°/0.15 mm). The resulting nitrile was reduced with $LiAlH_4$ by a modification of the procedure of Amundsen and Nelson [30]. To a cooled (ice-bath) solution of $LiAlH_4$ (3.8 g, 0.1 mol) in 200 mL of anhydrous ethyl ether was slowly added the nitrile (9.6 g, 0.1 mol) dissolved in 20 mL of anhydrous ethyl ether. With continued cooling and vigorous stirring, 4 mL of water, 3 mL of 20% sodium hydroxide and 14 mL of water were carefully added in succession. The ether solution was decanted from the white, granular solid and the residue was then washed twice with ether. The ether layers were combined and evaporated to give the product (6.9 g, 70% yield); b.p. 61–62°/3 mm.

N-(4-*Aminobutyl)aziridine*. A solution of 4-bromobutyronitrile (10.4 g, 0.07 mol) in 5 mL of anhydrous tetrahydrofuran was added dropwise to a stirred solution of aziridine (8.6 g, 0.2 mol) and triethylamine (20 g, 0.2 mol) in 40 mL of anhydrous tetrahydrofuran preheated to 35–40°. An hour later, the mixture was cooled to room temperature and then stirred overnight. The product was purified by distillation (b.p. 45–46°/0.15 mm). The nitrile was reduced to the amine by the same procedure as described above (4.1 g, 51% yield, b.p. 54–55°/0.15 mm), literature [31] b.p. 79–80°/20 mm.

N-(5-*Aminopentyl)aziridine*. A solution of 5-bromovaleronitrile (5.7 g, 0.035 mol) was added to a stirred solution of aziridine (4.3 g, 0.1 mol) and benzyltriethylammonium chloride (23 g, 0.1 mol) in 50 mL of 30% sodium hydroxide. After stirring the mixture for 12 hr, the product was extracted with ether and purified by distillation (b.p. 53–54°/0.15 mm). The resulting nitrile was reduced with $LiAlH_4$ by the procedure described above to give the product (2.2 g, 35% yield) with a boiling point of 64–65°/0.15 mm.

Enzyme activity. Pig kidney diamine oxidase and *o*-aminobenzaldehyde were purchased from the Sigma Chemical Co. (St. Louis, MO). Enzyme activity was determined using the method of Holmstedt *et al.* [32] as modified by Tamura *et al.* [25]. The activity was revealed using a coupling of *o*-aminobenzaldehyde with 1-pyrroline, the deamination product of putrescine as substrate. Only freshly prepared solutions of *o*-aminobenzaldehyde were used to avoid contamination by polymerized reagent [33]. The assay was linear for up to 1 hr and with changing substrate concentration. Control incubations were performed by omitting putrescine or inhibitors. Solutions were incubated at 37° in a covered water bath. All experiments were run in duplicate. Calculation of IC_{50} values was made on the means of duplicate runs following the method of Chou and Talalay [34].

Co-incubation of putrescine at 0.25 and 2.25 mM with inhibitors in concentrations equal to their IC_{50} values for

40 min was used to determine if diamine oxidase activity could be protected from inhibition by the presence of substrate.

To determine the reversibility of inhibition, diamine oxidase was preincubated with inhibitor for 30–80 min. Control incubations were carried out with no inhibitor. The mixtures were dialyzed overnight at 4° with two exchanges of 0.1 M phosphate dialysis buffer (pH 7.4) at 60–100 times the sample volume to determine the degree of enzyme reactivation.

Results and Discussion

To determine if a new class of inhibitors of diamine oxidase could be generated based on the incorporation of an electrophilic aziridinyl functional group into the substrate structure, a homologous series of monoaziridinyl diamines was synthesized and evaluated. As shown in Fig. 1, three aziridinylalkylamines inhibited diamine oxidase with different median inhibitory concentrations. The inhibition was irreversible for all test compounds since prolonged dialysis did not regenerate active enzyme and complete inactivation could be obtained. A Lineweaver–Burk plot of the initial rate assays for putrescine and the inhibitors, *N*-(3-aminopropyl)aziridine, *N*-(4-aminobutyl)aziridine and *N*-(5-aminopentyl)aziridine, at their respective IC_{50} concentrations resulted in intersecting lines at the $1/v$ axis (Fig. 2), indicating that the inhibitors compete with substrate for interaction at the active site.

As a means of assessing the specificity of the inhibition, a homologous series approach was taken where the structures of the inhibitors differed only by the number of methylene groups separating the amino moiety from the aziridinyl functionality. *N*-(5-Aminopentyl)aziridine is a close analogue of cadaverine (1,5-diaminopentane), which is one of the best substrates of diamine oxidase as measured by efficiency of oxygen uptake [16]. Putrescine (1,4-diaminobutane) is 96% as efficient a substrate for diamine oxidase as cadavarine [16], and is represented by the analogue *N*-(4-aminobutyl)aziridine. The shorter chain diamine, 1,3-diaminopropane, is a poorer substrate for diamine oxidase than either putrescine or cadaverine [16]. Bardsley *et al.* [16] reported that 1,3-diaminopropane at its optimum concentration (5 mM) is oxidized at about 8% the rate of 1,4-diaminobutane or 1,5-diaminopentane when incubated with diamine oxidase. The analogue of

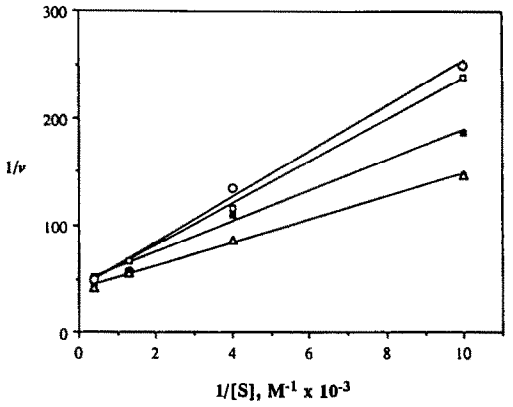
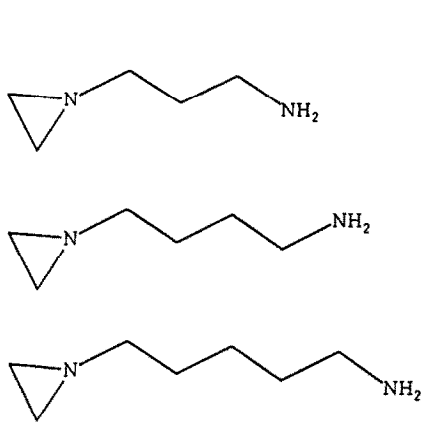


Fig. 2. Inhibition oxidase activity against putrescine by aziridinylalkylamines. Key: (■) *N*-(3-aminopropyl)-aziridine, 1.6 mM; (□) *N*-(4-aminobutyl)aziridine, 0.05 mM; (○) *N*-(5-aminopentyl)aziridine, 0.11 mM; and (△) uninhibited control. Incubation mixtures were as described in the legend of Fig. 1.

1,3-diaminopropane synthesized and tested was *N*-(3-aminopropyl)aziridine.

As indicated by their IC_{50} values (Fig. 1), the putrescine and cadavarine analogues, *N*-(4-aminobutyl)aziridine and *N*-(5-aminopentyl)aziridine, respectively, were 35- and 15-fold more effective as inhibitors of diamine oxidase than the 1,3-diaminopropane analogue, *N*-(3-aminopropyl)-aziridine. This dependence on chain length is consistent with the substrate specificity of diamine oxidase in that the analogue of the poorest substrate gave the highest IC_{50}. If the inhibition of diamine oxidase by these aziridines was based solely on the reactivity of the aziridinyl functional group, then approximately equal inhibitory concentrations would be anticipated throughout the homologous series, which was not observed. A likely explanation for differences in reactivity of the aziridinylalkylamines may be related to an association of the inhibitors with specific sites on the enzyme. An aziridinyl compound that has an affinity for binding to an enzyme surface will have a lower activation



	IC_{50}
N-(3-Aminopropyl)aziridine	1.6×10^{-3} M
N-(4-Aminobutyl)aziridine	4.6×10^{-5} M
N-(5-Aminopentyl)aziridine	1.1×10^{-4} M

Fig. 1. Median inhibitory concentrations of homologous aziridinylalkylamine inhibitors of diamine oxidase. Incubation mixtures consisted of diamine oxidase (0.4 to 2.0 mg, nominally 0.06 U/mg), putrescine (1.0 mM), *o*-aminobenzaldehyde (2.0 mM), and 0.1 M potassium phosphate buffer, pH 7.4, to bring the volume to 5 mL. Values were generated from averages of the results of duplicate runs of at least four inhibitor concentrations with a 1-hr incubation time.

energy for an alkylation reaction with a nearby nucleophile than that of an aziridine-containing compound that does not bind well. In the series tested, the observed large increase in inhibitory potency resulting from extending the chain from three to four carbons suggests that the topology of the enzyme active site favors interactions with internitrogen chain-length distances longer than three methylenes.

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