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Aromatic diamidines are reversible inhibitors of porcine kidney diamine oxidase

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Abstract—The inhibitory ability of aromatic diamidines has been studied on porcine kidney diamine oxidase. The reversibility of drug–protein interactions has been tested by means of exhaustive dialysis experiments, showing in all cases a reversible binding pattern. K_i values obtained by means of Lineweaver–Burk plots were: stilbamidine 12 μM, 2-OH-stilbamide 8.5 μM, phenamidine 4 μM, propamidine 8 μM, dibromopropamidine 4.9 μM and amicarbalide 12 μM.

Polyamine (PA‡) metabolism in mammalian tissues involves: (1) a highly regulated biosynthetic pathway constituting two key regulatory enzymes, L-ornithine decarboxylase and *S*-adenosyl-L-methionine decarboxylase, and two constitutive synthases, one for spermidine and a second for spermine [1]. (2) An interconversion pathway used by mammalian cells to synthesize spermidine and putrescine from spermine and spermidine, respectively, through an inducible acetyl transferase enzyme that uses either spermidine or spermine as substrate and acetylCoA as acetyl donor, and a constitutive polyamine oxidase [2]. (3) Finally, PAs are degraded by an oxidative pathway called terminal oxidation, where putrescine undergoes an oxidative deamination giving γ-aminobutyric acid and ultimately succinate [3]. The first enzyme of the putrescine terminal oxidative pathway is diamine oxidase (DAO), a copper enzyme that uses a wide range of normobiotic diamines as substrate, including PAs. DAO is involved in the reduction of PA (especially putrescine) levels in mammalian cells, particularly in rapid proliferation [4].

Amongst DAO inhibitors, diguanidine and diamidine compounds are PA analogs [5], whose therapeutic relevance is related to antitumoral and antiparasitic chemotherapy, respectively [5, 6]. Aromatic diamidines are a family of antiparasitic compounds used widely in human and veterinary medicine mostly as antiprotozoan drugs [7, 8], whose mechanisms of action on target cells and side effects on hosts cells have been studied very little. Two of the most important compounds of this family, diminazene aceturate (Berenil) and pentamidine, have been shown to be non-competitive inhibitors of DAO from mammalian sources at micromolar concentrations [9]. Furthermore, a widely used DNA dye, whose chemical structure belongs to the aromatic diamidine family: 4′,6-diamidino,2-phenylindole (DAPI), has recently shown identical behaviour against porcine kidney DAO [10]. Therefore, a screening of different aromatic diamidines, whose basic chemical structure is maintained, is required in order to understand the inhibitory kinetic mechanism of this important group of compounds. The results presented in this communication are a comprehensive approach for this purpose, using six different diamidines other than Berenil, pentamidine or DAPI, on porcine kidney DAO (Fig. 1).

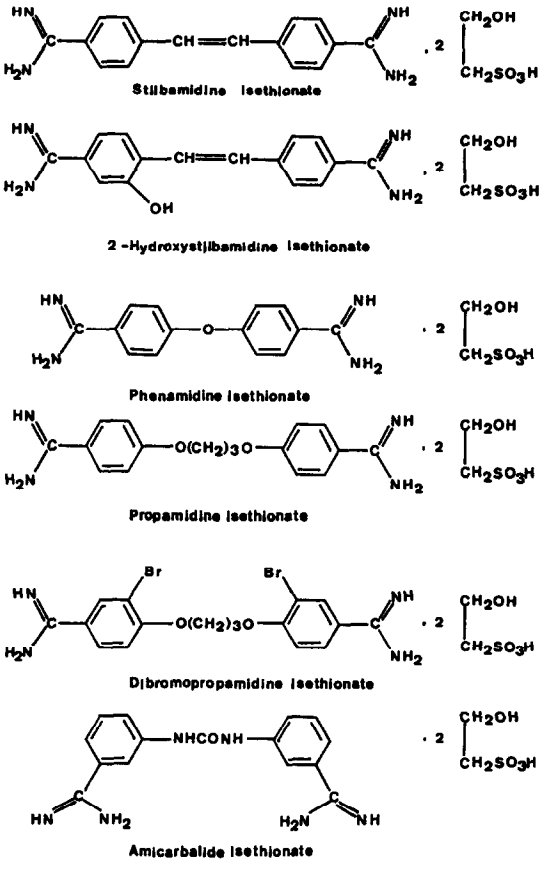

Fig. 1. Chemical structure of the selected aromatic diamidines used in the present study.

Materials and Methods

Chemicals. Pig kidney DAO (EC 1.4.3.6) (0.05 U/mg) and unlabelled putrescine were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). [1,4^{14}C]Putrescine (60 mCi/mmol) was from New England Nuclear (Du Pont de Nemours, Germany). Compounds listed on Fig. 1: propamidine, phenamidine, 2-hydroxy-stilbamidine, dibromopropamidine and amicarbalide were generous gifts from Rhône Poulenc Rorer (Dagenham, U.K.). Stilbamidine was a gift from Merrel Dow (Cincinnati, OH, U.S.A.). All other chemicals were analytical grade products.

Enzyme assays. Porcine kidney DAO dissolved in 0.1 M HEPES buffer pH 7.5 was measured following the procedure described by Snyder and Hendley [11] with some modifications. Standard incubation mixtures, at a final volume of 0.5 mL, contained: 100 μL of 0.5 M HEPES buffer pH 7.5 (Na^+/K^+ phosphate buffer was substituted by HEPES buffer because diamidines precipitated with phosphate salts), 0.014 μCi of [1,4^{14}C]putrescine (60 mCi/mmol), 50–500 μM unlabelled putrescine and 0.083

‡ Abbreviations: PA, polyamine; DAO, diamine oxidase; DAPI, 4′,6-diamidino-2-phenylindole.

enzymatic units. After incubation for 30 min at 37° in a shaking bath, reactions were stopped adding 0.2 mL acetone, and the amount of putrescine oxidized to δ^1-pyrroline was determined by extraction twice into a toluene-based scintillation cocktail (4 g 2,2'-(1,4-phenylene)-bis(5-phenyloxazole), 0.1 g bis MSB in 1 L toluene). After vortexing vigorously for 30 sec, test tubes were frozen at −20° and the upper phase was decanted into a scintillation vial and extracted again. Radioactivity was determined in a Beckman LS-188 liquid scintillation counter. DAO assay blanks were prepared by incubation of the same assay mixture in which DAO had been inactivated by boiling for 2 min. For each determination three test assays and three corresponding blanks were carried out. Results were expressed as μmoles of δ^1-pyrroline formed per hour and per milligram of protein. One unit of DAO activity was defined as the capacity of the enzymatic mixture to oxidize 1 μmole of putrescine per hour and per milligram of protein.

Reversibility analysis. The reversibility of aromatic diamidine interaction with DAO protein was analysed by means of exhaustive dialysis against 500 vol. of enzyme buffer. DAO was incubated together with 50–200 μM of each diamidine at 37° for 30 min. Then, mixtures were introduced into dialysis tubing and dialysed overnight. Finally, DAO activity was assayed as above and compared with control assays.

Protein determinations. Protein measurements were carried out according to the Bradford method [12], using bovine serum albumin as standard.

Table 1. Reversible inhibition of porcine kidney DAO by aromatic diamidines

Inhibitor (μM)	% DAO activity	
	Dialysed	Undialysed
None	100	100
Stilbamidine		
50	92.2	11.5
200	80.1	4.8
2-Hydroxystilbamidine		
50	97.2	9.5
200	72.6	6.9
Phenamidine		
50	97.3	7.8
200	93.5	5.2
Propamidine		
50	97.0	11.8
200	99.3	0.8
Dibromopropamidine		
50	100.8	5.6
200	88.8	2.9
Amicarbalide		
50	96.8	13.5
200	90.4	9.9

Samples were assayed as described in Materials and Methods. Initial specific activities (100%) were 0.97 μmol/min/mg protein on undialysed fractions. Untreated controls cost less than 10% of activity during dialysis.

Results and Discussion

The important findings of the chemotherapeutic use of pentamidine against *Pneumocystis carinii* pneumonia in acquired immunodeficient syndrome patients have increased the clinical relevance of this compound and aromatic diamidine analogs [13]. However, the biochemical mode of action of this group of molecules has hardly been investigated and the undesirable side effects on host tissues are also unknown at the molecular level.

Table 1 shows the ability of selected aromatic diamidines to inhibit porcine kidney DAO on both undialysed and dialysed extracts. The diamidines tested at 50 and 200 μM were able to reduce DAO activity by more than 85% in undialysed extracts. However, when enzyme protein was incubated together with each compound in the absence of putrescine for 30 min, at 37° and then dialysed overnight at 4°, in all the cases studied more than 70% of DAO activity was recovered with respect to control tubes subjected to the same procedure (less than 10% of initial DAO activity was lost during the dialysis procedure).

Once the reversibility interaction of aromatic diamidines with DAO had been studied, kinetic parameters were determined by means of varying the putrescine concentration at different inhibitor levels. Table 2 shows the results obtained by means of the reciprocal plots of putrescine concentrations vs DAO activity. These plots show a very uniform pattern of unambiguously non-competitive inhibition. The K_i values determined by means of slope vs inhibitor concentration replots were: stilbamidine 12 μM; 2-hydroxystilbamidine 8.5 μM; phenamidine 4 μM; propamidine 8 μM; dibromopropamidine 4.9 μM; amicarbalide 12 μM.

As expected, selected aromatic diamidines were powerful reversible non-competitive inhibitors of porcine kidney DAO. K_i values obtained ranged from 4.9 to 12 μM. These values resemble those obtained previously with diminazene aceturate (Berenil) [14] and with pentamidine in rat small intestine [9] and porcine kidney [15], respectively. DAPI, a DNA dye whose trypanocide activity has also been reported [16], showed a competitive inhibitory pattern using pig kidney extracts, but with a K_i value (13 μM) slightly higher than the average found for aromatic diamidines obtained in this study [10].

It is difficult to determine the molecular mechanism of DAO inhibition by aromatic diamidines with the results so far reported. Mondovi *et al.* [17] have described the

Table 2. Inhibitory patterns obtained by means of Lineweaver–Burk plots of aromatic diamidines on porcine kidney DAO

Stilbamidine isethionate	Non-competitive	12
2-Hydroxystilbamidine isethionate	Non-competitive	8.5
Phenamidine isethionate	Non-competitive	4
Propamidine isethionate	Non-competitive	8
Dibromopropamidine isethionate	Non-competitive	4.9
Amicarbalide isethionate	Non-competitive	12

In the presence of 1,2,5,10 and 20 μM of each inhibitor 0.083 enzymatic units were assayed. K_i values where obtained from slope vs inhibitor concentration replots.

Results are the averages of duplicated experiments.

existence of three sites on the mammalian DAO active centre: (a) a carbonylic centre with a pyridoxal phosphate residue as prostetic group, where the oxidative deamination takes place; (b) a fixation site for substrates and (c) an anionic centre, essential for enzyme activity but not for substrate oxidation. Binding of these compounds to the c site may explain the non-competitive inhibitory behaviour found herein. This theory has been suggested previously to explain the mode of action of methylglyoxal bis-(guanylhydrazone) and phenylated analogs [18], and Berenil [14], although it cannot explain DAPI induction due to the competitive kinetic pattern of this compound [10]. However, the existence of a hypothetical critical distance between diamidino groups [15, 18] is not supported by the results obtained in this study because no quantitative inhibitory differences were found amongst the compounds studied and reported. All these findings and the possible ability of this family of compounds to inhibit *S*-adenosyl-L-methionine decarboxylase as has been reported for their counterparts Berenil and pentamidine, not only from mammalian sources [14, 19] but also from parasitic organisms [20, 21], and their ability to intercalate into the DNA double helix [22] make this kind of compound of therapeutic relevance on proliferative processes.

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