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RESEARCH ARTICLE

Inhibition of acetylpolyamine and spermine oxidases by the polyamine analogue chlorhexidine

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Abstract

Acetylpolyamine and spermine oxidases are involved in the catabolism of polyamines. The discovery of selective inhibitors of these enzymes represents an important tool for the development of novel anti-neoplastic drugs. Here, a comparative study on acetylpolyamine and spermine oxidases inhibition by the polyamine analogue chlorhexidine is reported. Chlorhexidine is an antiseptic diamide, commonly used as a bactericidal and bacteriostatic agent. Docking simulations indicate that chlorhexidine binding to these enzymes is compatible with the stereochemical properties of both acetylpolyamine oxidase and spermine oxidase active sites. In fact, chlorhexidine is predicted to establish several polar and hydrophobic interactions with the active site residues of both enzymes, with binding energy values ranging from −7.6 to −10.6 kcal/mol. In agreement with this hypothesis, inhibition studies indicate that chlorhexidine behaves as a strong competitive inhibitor of both enzymes, values of K_i being 0.10 μM and 0.55 μM for acetylpolyamine oxidase and spermine oxidase, respectively.

Keywords: Spermine oxidase, acetylspermine oxidase, chlorhexidine, K_i

Introduction

The polyamines (PA) putrescine (Put), spermidine (Spd), and spermine (Spm) are natural aliphatic polycations ubiquitous in living organisms and essential for cell growth, proliferation, and differentiation[1,2]. In animals, the polyamine catabolism is a recycling pathway that converts Spm to Spd and Spd to Put, with the production of toxic aldehydes and H_2O_2. Polyamine catabolism is achieved via the concerted action of spermidine/spermine N^1-acetyltransferase and acetylpolyamine oxidase (APAO), or alternatively, via spermine oxidase (SMO) with no need of the acetylation step[3–6]. In the last decade, APAO and SMO catabolic enzymes have been extensively characterized and well documented to play an essential role in maintaining vertebrate PA homeostasis, the latter being mandatory for cellular life[1,2,7–10]. The recombinant protein expression of the mammalian APAO and SMO in different heterologous systems provided the basis for a deeper understanding of the structure/function relationships of these enzymes[11–16]. At present, only the three-dimensional structures of two members of the polyamine oxidase family are available, namely the maize polyamine oxidase (ZmPAO[17,18]) and the yeast polyamine oxidase (FMS1[19]). Molecular modeling of mouse SMO based on the three-dimensional structure of ZmPAO and sequence analysis of animal polyamine oxidases indicated that the general features of the ZmPAO three-dimensional structure are conserved in the mammalian enzymes[11,20,21]. Furthermore, molecular modeling, docking and site-directed mutagenesis studies demonstrated that Spm binds to mammalian SMO in the same fashion as observed in the yeast FMS1, which shares with SMO and APAO the substrate oxidation mode[21].

Several inhibitors of APAO and SMO have been studied, the most prominent example being MDL 72527 (N^1,N^4-bis(2,3-butadienyl)-1,4-butanediamine)

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(Figure 1), that selectively inhibits FAD-containing polyamine oxidases[14,16,20,22]. In particular, APAO and SMO inhibition by 1,8-diaminooctane, 1,12-diaminododecane, *N*-prenylagmatine and guazatine has been reported[20]. Since selective mammalian APAO and SMO inhibitors represent important tools for studying the involvement of this class of enzymes in PA homeostasis and in designing new antineoplastic drugs, the search for new PA analogues with enzyme inhibitory activity results to be an exacting research field. Among potential inhibitor candidates, the present study has focused on the diamide chlorhexidine, N′,N‴″-hexane-1,6-diylbis[N-(4 chlorophenyl-)(imidodicarbonimidic diamide)], hereafter referred to as CHX (Figure 1).

CHX is mainly used in dental applications as an active ingredient in mouthwash, designed to kill dental plaque and other oral bacteria. Actually, CHX based products are usually utilized for the prevention and treatment of periodontal diseases. CHX has been demonstrated to inhibit several host cell-derived proteolytic matrix metallo-proteinases (MMPs), as well as some gingipains (HRgpA, RgpB, and Kgp), which play a major role in periodontitis[23,24]. However, CHX is also used in non-dental applications, for example in general skin cleaning, as a surgical scrub, and in pre-operative skin preparation.

In this study, we first explored the hypothesis that CHX could act as a polyamine oxidase inhibitor by docking simulations. Docking of CHX in APAO and SMO active sites indicates that CHX binding to these enzymes is compatible with their stereochemical properties. Indeed, CHX is predicted to establish polar and hydrophobic interactions with the active site residues of both enzymes, with binding energy values in the 7.6–10.6 kcal/mol range, which correspond to K_d values ranging from 0.02 μM to 0.6 μM. In agreement with the docking studies, enzyme inhibition analyses indicate that CHX behaves as a strong competitive inhibitor of both enzymes, K_i values being 0.10 μM and 0.55 μM for APAO and SMO, respectively.

Methods

Chemicals

Chlorhexidine (CHX) digluconate solution, spermine (Spm) tetrahydrochloride, N^1-acetyl-spermine (N^1-acetyl-Spm) trihydrochloride, 4-aminoantipyrine, 3,5-dichloro-2-hydroxybenzenesulfonic acid and horseradish peroxidase were from Sigma-Aldrich (St. Louis, MO, USA). Other compounds were from Sigma-Aldrich (St. Louis, MO, USA), Bio-Rad (Bio-Rad Italia, Milano, Italy) and J.T. Baker (Baker Italia, Milano, Italy). All chemicals were of analytical or reagent grade and used without further purification.

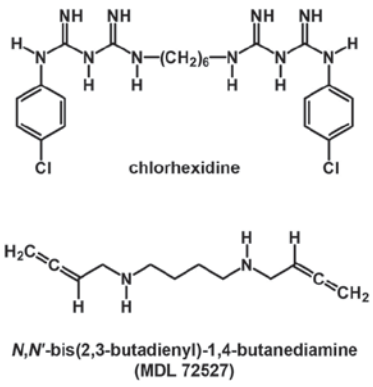

Figure 1. Chemical structures of chlorhexidine and MDL 72527.

Molecular docking simulations of APAO- and SMO-CHX complexes

Docking of CHX onto the three-dimensional models of APAO and SMO was carried out using AutoDock Vina[25]. The maximum number of binding modes to generate (maximum number of poses to be returned) was kept to the default value of 9, while the exhaustiveness of the search was increased from default value 8 to 30. The three-dimensional models of APAO and SMO were obtained as described previously[20,21].

Expression and purification of APAO and SMO proteins in E. coli cells

The recombinant APAO and SMO proteins were expressed in *E. coli* BL21 DE3 cells and purified as previously reported by Bianchi et al.[20] and Cervelli et al.,[11] respectively. SDS/PAGE electrophoretic analysis was performed on purified recombinant proteins to assess the grade of purity. Protein concentration was calculated by means of the 460 nm molar extinction coefficient ($\varepsilon_{460} = 9000\ M^{-1}\cdot cm^{-1}$) which accounts for FAD absorption.

Enzyme activity and inhibition assays

The APAO and SMO activities were measured spectrophotometrically by following the formation of a pink adduct ($\varepsilon_{515\ nm} = 2.6 \times 10^4\ M^{-1}\ cm^{-1}$) as the result of the H_2O_2-dependent oxidation of 4-aminoantipyrine catalyzed by horseradish peroxidase, and the subsequent condensation of oxidized 4-aminoantipyrine with 3,5-dichloro-2-hydroxybenzenesulfonic acid, as described in Bianchi et al.[20] APAO activity was assayed at pH 7.5 (0.1 M Tris-HCl buffer) and 25°C with N^1-acetyl-Spm as the substrate in the presence and absence of CHX. SMO activity was assayed at pH 8.3 (0.1 M Tris-HCl buffer) and 25°C using Spm as the substrate in the presence and absence of CHX. Data reported are the average of three different experiments, each with two replicates, standard deviation (SD) was 5%. Under all the experimental conditions, the initial reaction rate for the APAO- and SMO-catalyzed conversion of the substrate was unaffected by the enzyme/inhibitor (*i.e.* APAO/ and SMO/CHX) incubation time, prior to substrate addition.

To diagnose the inhibition type, both Lineweaver–Burk (1/v versus 1/s) and Dixon (1/v versus [I]) plots were scrutinized. Estimates of *K*i were obtained by second plot of slopes from Lineweaver–Burk versus CHX concentrations and from Dixon plots. In the latter case, two sets of equations were sequentially solved; one equation for each of the straight lines, as obtained by linear least-squares regression, was equated with the line that represented

the highest substrate concentration, that line having the smallest slope. The point of intersection of these pairs of lines represents the value of Ki, reported as means.

Results and discussion

Structural bases for inhibition of APAO and SMO by CHX

To probe the hypothesis that CHX could act as a strong competitive inhibitor of APAO and SMO, the molecule was docked inside the two enzymes active sites using the previously published three-dimensional models of SMO and APAO[20,21] and the AutoDock Vina molecular docking software[25]. Analysis of the best docking poses highlights a general stereochemical compatibility of CHX with the active sites of both enzymes. Indeed, CHX displays the proper size to fit in the tunnel-shaped active site of animal polyamine oxidases, occupying their entire length (Figure 2). Several polar and hydrophobic interactions can be observed between CHX and active site residues. In detail, in APAO, one end of the CHX molecule binds in the innermost part of the catalytic tunnel, in the vicinity of the FAD cofactor, thus establishing hydrophobic interactions with Trp62 (Figure 2). Moving towards the solvent accessible side of the active site, CHX is further stabilized by a charge-charge interaction with Glu89, hydrogen bonds with Asn91 and Arg124, and aromatic stacking interactions with Tyr127 (Figure 2). The CHX binding mode in SMO is very similar to that observed in APAO, as far as the innermost part of the molecule is concerned. However, moving towards the active site entrance, differences between APAO and SMO active site composition lead to a distinct set of interactions. Glu216 forms a charge-charge interaction with one of the carbonimidic groups and Gln199 establishes a hydrogen bond with one of the imide groups of CHX (Figure 2). Furthermore, at variance with the APAO-CHX putative complex, the second chlorophenyl group of CHX is solvent inaccessible and bound in a hydrophobic pocket made up by Met197 and Tyr147 (Figure 2).

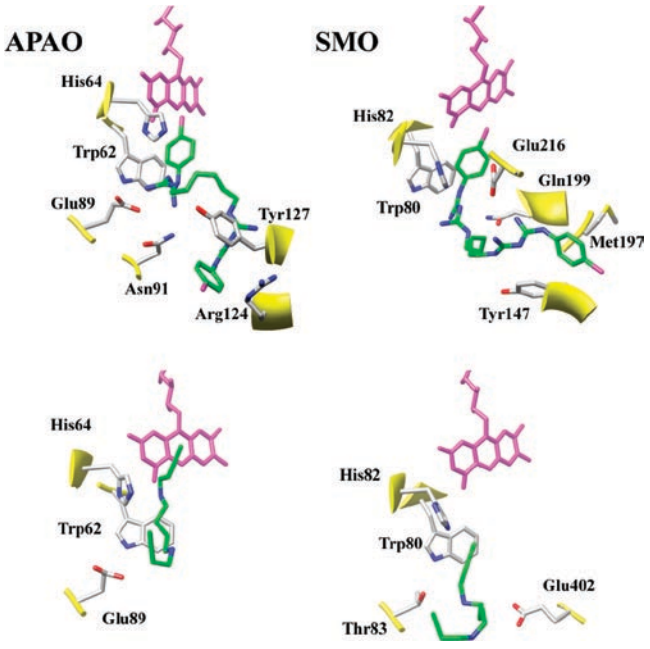

Figure 2. Schematic representation of the APAO and SMO best docking complexes with CHX (top panels) and MDL 72527 (bottom panels) showing proteins residues interacting with the inhibitors. The FAD cofactor (colored in magenta) and the active site His residue (His64 and His82 in APAO and SMO, respectively) are shown for reference. (See colour version of this figure online at www.informahealthcare.com/enz)

Binding energy values calculated by AutoDock force field for the best nine poses (the default number of poses returned) range from –10.6 to –7.6 kcal/mol. This binding energy values translate in K_d values ranging from 0.02 μM to 0.6 μM, thus suggesting fairly strong inhibition properties of CHX towards animal polyamine oxidases.

To probe for the reliability of AutoDock Vina, docking simulations were performed also with the reference polyamine oxidases inhibitor MDL 72527, known to be a weak, non selective inhibitor of both APAO and SMO [K_i values being 21.0 and 63.0 μM, respectively[20];]. Analysis of the best docking poses indicates that binding of MDL 72527 to both APAO and SMO takes place essentially only through a hydrophobic interaction with Trp62 side-chain, with the inhibitor binding deeper in the active site in the case of APAO with respect to SMO. In both cases, weak electrostatic interactions are observed between the amino groups of MDL 72527 and polar residues of the enzymes active site. In detail, in APAO, Glu89 is at 4.5 Å distance from one of the MDL 72527 amino group, while in SMO the hydroxyl groups of Thr83 and Glu402 are at 3.6 and 4.5 Å distance from one of the amino groups of MDL 72527, respectively. Binding energy values calculated by AutoDock force field for the best nine poses range from –4.6 to –4.1 kcal/mol, significantly higher than those obtained for CHX, pointing to stronger inhibition properties of the latter compound with respect to MDL 72527.

Inhibition of APAO and SMO by chlorexidine

Purified recombinant murine APAO and SMO follow classical Michaelis–Menten kinetics. Values of K_m and k_{cat} for N^1-acetyl-spermine (N^1-acetyl-Spm) oxidation by APAO are 2.0 μM and 2.0 s^{-1}, respectively. Values of K_m and k_{cat} for Spm oxidation by SMO are 90 μM and 4.5 s^{-1}, respectively. The polyamine analogue CHX was tested as *in vitro* modulator of APAO and SMO activities.

CHX impairs both APAO and SMO activities competitively. Lineweaver–Burk and Dixon plots of APAO and SMO activity inhibition by CHX, as shown in Figure 3, indicate that CHX behaves as a strong competitive inhibitor of both enzymes. The K_i values were obtained by secondary plotting of Lineweaver–Burk data (slopes versus CHX concentrations) and by Dixon plots, yielding similar results. The obtained values were calculated to be 0.10 ± 0.05 μM and 0.55 ± 0.02 μM for APAO and SMO, respectively (Figure 3).

In agreement with docking simulations results, K_i values for APAO and SMO reported in Table 1, clearly

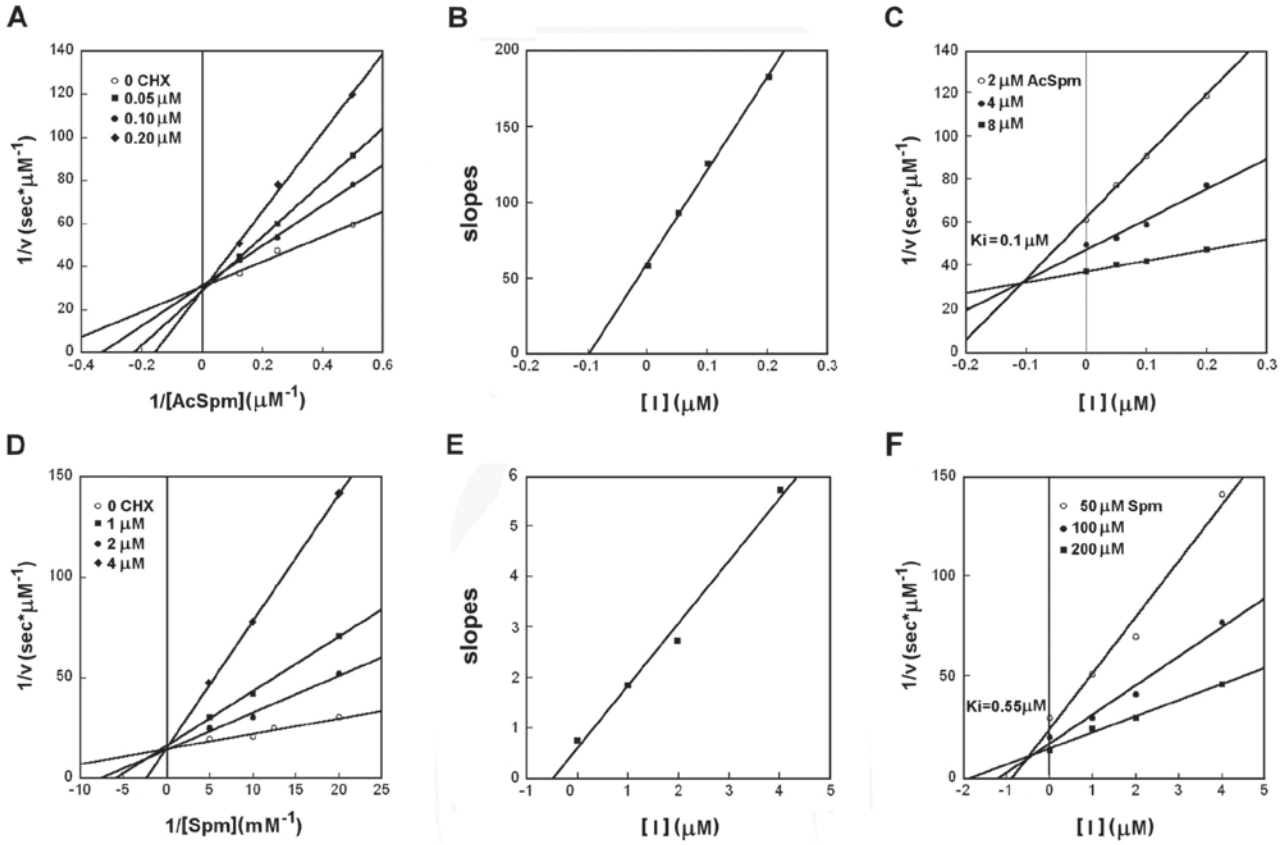

Figure 3. Lineweaver–Burk plots reporting reciprocal rate oxidation of N^1-acetyl-Spm by APAO (A) and Spm by SMO (D) versus reciprocal substrate concentrations, in the presence and absence of CHX. To calculate Ki for the inhibition by CHX, the data are also reported by second plot of slopes from Lineweaver–Burk plot versus CHX concentrations (B, APAO; E, SMO), and by Dixon plots reporting reciprocal rate substrate oxidation as a function of CHX concentrations (C, APAO; F, SMO)

demonstrate that CHX is a stronger inhibitor than the reference polyamine oxidases inhibitor MDL 72527. Analysis of the best docking complexes indicates that this is due to the ability of CHX to establish a higher number of interactions with the active site residues of both APAO and SMO. In fact, interaction of MDL 72527 with both enzymes is essentially mediated only by a non-specific hydrophobic interaction with Trp62 (Figure 2, numbering of APAO) and weak electrostatic interactions with one (in APAO) or two (in SMO) polar residues of the active site. On the contrary, CHX is able to establish a network of interactions both of hydrophobic and electrostatic nature, due to the presence of the chlorophenyl ring and of positively charged carbonimidic and imidic groups. According to docking simulations results, the different binding mode of CHX to APAO with respect to SMO is related to the presence of a hydrophobic cavity at the entrance of the active site in the former enzyme (made up by Tyr147 and Met197) which is absent in the latter. This difference could be exploited to design more effective and more selective inhibitors for APAO and SMO. In so much so that the results of this study suggest that an increase in the polarity of one of the CHX phenyl groups (for instance, substituting one chlorophenyl group with a nitrophenyl one) may very likely improve the inhibition properties of the resulting inhibitor for APAO (strengthening the interaction with Arg124); while a decrease in the polarity of one of the phenyl groups (for instance, substituting one chlorophenyl group with a phenyl one) may result in a stronger inhibitor for SMO.

Table 1. Values of K_i for CHX and MDL 72527 binding to APAO and SMO.

	K_i (μM)[a]	
Inhibitor	APAO	SMO
Chlorhexidine	0.10	0.55
MDL72527[b]	21.0[b]	63.0[b]

[a]The standard deviation is ± 5%.
[b]Bianchi et al[20].

Conclusions

Inhibition studies of related enzymes can provide a deeper insight of the structural basis for substrate and/or inhibitor specificity, and help in designing novel and more selective inhibitors. This is of utmost importance in the case of animal polyamine oxidases, in that the availability of selective inhibitors for APAO and SMO would allow the precise role of these enzymes in polyamine metabolism to be dissected. In this regard, here we show that CHX behaves as the strongest known competitive inhibitor of APAO, the previously known as the best inhibitor being

guazatine with a K_i value of 0.45 μM[20]. In addition, while guazatine displays a K_i value for SMO comparable to that for APAO [0.40 μM[20];], CHX is a weaker inhibitor for SMO, K_i value for the latter protein being over five times higher than that for APAO (Table 1). Thus, CHX is a promising lead compound to develop highly efficient and selective inhibitors for animal polyamine oxidases. From this viewpoint, docking studies provide some clues on how to modify CHX moiety so as to increase selectivity for APAO or SMO. In fact, it appears that increasing the polarity of one of the CHX chlorophenyl rings may turn out in a better inhibitor for APAO, while a poorer inhibitor for SMO.

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Declaration of interest

The authors report no declarations of interest.

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