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Structure–Activity Relationships of SSAO/VAP-1 Arylalkylamine-Based Substrates

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Semicarbazide-sensitive amine oxidase/vascular adhesion protein-1 (SSAO/VAP-1) substrates show insulin-mimetic effects and are therefore potentially valuable molecules for the treatment of diabetes mellitus. Herein we review several structural and electronic aspects of SSAO arylalkylamine-based substrates. Two main modifications directly affect amine oxidase (AO) activity: 1) variation in ring substitution modulates the biological activity of the arylalkylamine ligand by converting a substrate into a substrate-like inhibitor, and 2) variation in the number of methylene units between the aromatic ring and the ammonium groups of the arylalkylamine substrates dramatically alters the oxidation rate between species. Furthermore, we review relevant information about mammalian SSAO/VAP-1 substrate selectivity and specificity over monoamine oxidases (MAOs).

Introduction

Semicarbazide-sensitive amine oxidase/vascular adhesion protein-1 (SSAO/VAP-1) substrates show insulin-like effects and may serve as potential agents for the treatment or prevention of diabetes mellitus. SSAO is responsible for metabolizing endogenous amines such as methylamine and aminoacetone, with the concomitant production of hydrogen peroxide and ammonia. SSAO is highly expressed in mammalian adipocytes. White adipose tissue (WAT) participates in the regulation of glucose homeostasis, and alterations to WAT function are found patients with obesity, diabetes mellitus, metabolic syndrome, and associated complications.[1–3] Interestingly, the abundant SSAO activity present in adipocytes may contribute to the regulation of glucose metabolism, as SSAO substrates such as benzylamine or tyramine, in combination with vanadate, activate glucose uptake in isolated fat cells.[4,5] In fact, the hydrogen peroxide byproducts of SSAO substrates are crucial for the insulin-mimetic properties of SSAO substrates.[6] SSAO substrates exhibit in vitro insulin-like properties, and the chronic administration of benzylamine in combination with low doses of vanadate decreases hyperglycemia in streptozotocin-induced diabetic rats and in Goto–Kakizaki diabetic rats, animal models that resemble human type 1 and type 2 diabetes, respectively.[7–9] SSAO substrates alone or in combination with vanadate exert other insulin-like effects such as inhibition of lipolysis, improvement of glucose tolerance in insulinopenic rats, and stimulation of hexose uptake in human adipocytes.[10–13]

In isolated rat adipocytes, the combination of arylalkylamine substrates of SSAO with ineffective vanadate concentrations causes a potent stimulation of glucose transport, which is inhibited by semicarbazide or by catalase.[5,14] This combination of compounds also translocates GLUT-4 glucose transporters to the cell surface, thereby stimulating lipogenesis and the inhibition of lipolysis.[5,12,14,15] Available data indicate that the SSAO-dependent generation of hydrogen peroxide is responsible for these effects through a chemical interaction with vanadate, which forms peroxovanadate, a powerful insulin-mimetic agent.[8,16]

SSAO may be responsible for the metabolism of methylamine and aminoacetone, thus generating formaldehyde or methylglyoxal as side products.[17] These highly reactive side products contribute to the formation of advanced-glycation end products (AGEs), which are associated with the vascular complications of diabetes.[18] In this regard, therapies with SSAO substrates may generate angiotoxic molecules with the potential development of vascular complications associated with diabetes.[19,20] However, a recent study by Visentin et al. has challenged this hypothesis.[21] In fact, the administration of

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SSAO substrates may have beneficial effects, as they decrease methylamine oxidation in diabetes.

On the basis of these data, we conclude that the use of SSAO substrates promotes glucose use by peripheral tissues. This effect is not linked to oxidative stress caused by hydrogen peroxide formation and represents a valuable strategy for the treatment of diabetes.[21] Therefore, research that addresses the identification of pharmaceutically efficient SSAO/VAP-1 substrates is a good starting point that can lead to the identification of potential candidates for the oral treatment of diabetes and its complications.[22]

Herein we review information on the structure–activity relationships (SARs) of substrates metabolized by mammalian SSAO enzymes. At present, arylalkylamines are the best SSAO substrates, and potent antidiabetic effects have been obtained both in vitro and in vivo. Because there is a certain amount of overlap in substrate specificity among amine oxidases (AOs), this review also analyzes SAR data regarding enzyme selectivity relative to monoamine oxidase (MAO).

1. SSAO is an amine oxidase

SSAO/VAP-1 is a bifunctional copper-containing protein with AO activity (EC 1.4.3.6) that converts primary amines to aldehydes, with concomitant production of hydrogen peroxide and ammonia (Figure 1).[23,24] AOs are classified by the nature of the active site cofactor. The first group includes well-known FAD-containing AOs such MAO A/MAO B and polyamine oxidase (PAO).[25,26] The second group is formed by copper-containing SSAOs, which include diamine oxidase (DAO), lysyl oxidase (LO), and SSAO proteins as soluble or membrane-associated forms.[27] The members of this second group share a common feature: they all have a reactive carbonyl group in their cofactor, which is sensitive to semicarbazide. All these enzymes differ not only in their cofactor active site but also in their localization, structural form (membrane or soluble), functions, substrates, and inhibitors. Localized in the outer mitochondrial membrane, MAOs metabolize neurotransmitters and are inhibited by chlorgyline and deprenyl.[25] Intracellular PAO metabolizes multiply charged polyamines and could be involved in cell growth.[28] Intracellular DAO preferentially selects diamines as substrates, such as putrescine and cadaverine.[29] Extracellular LO deaminates specific lysine residues of extracellular matrix proteins.[30] Soluble or extracellular SSAOs have distinct substrates (e.g., methylamine or benzylamine) and are sensitive to semicarbazide.[31]

2. Active site of SSAO/VAP-1

The three-dimensional structures of the active sites of CuAOs of diverse origin (bacterial, plant, and mammalian) have been extensively studied. Bovine AO (BAO) was the first mammalian enzyme structure published and shares 83% identity with the human enzyme.[32] The X-ray crystal structure of human SSAO/VAP-1 has been reported, and the resolution showed that it includes an organic cofactor TPQ (topaquinone) and a copper

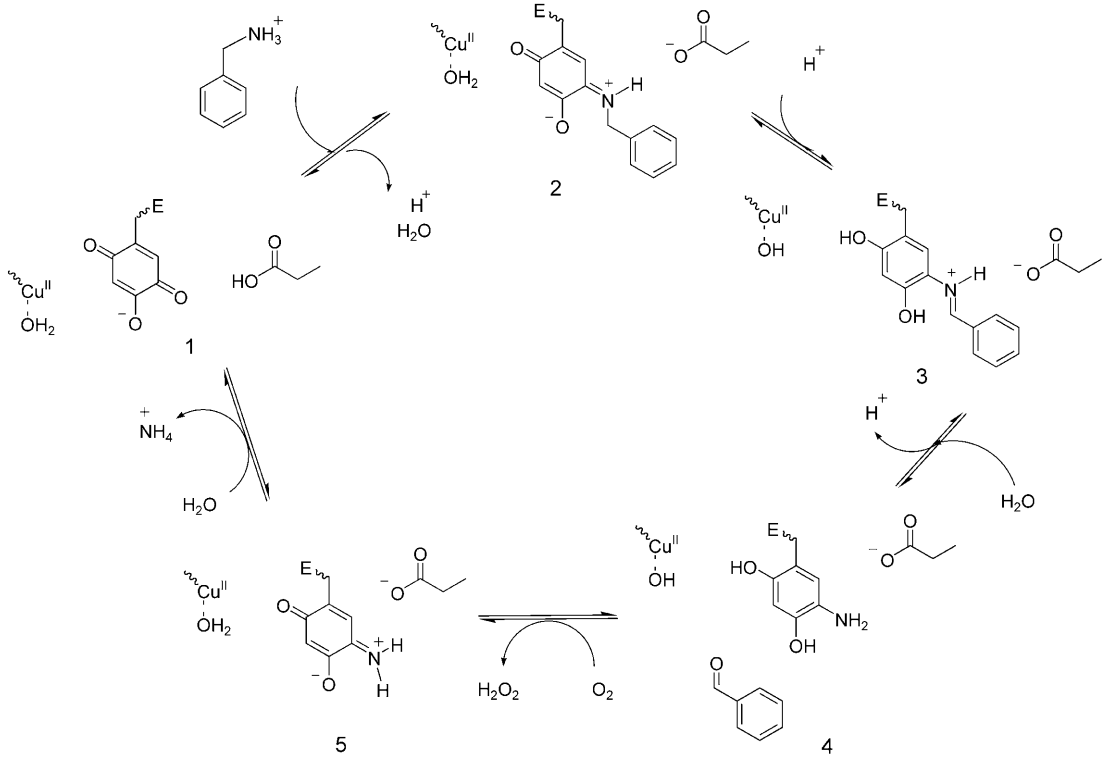


Figure 1. SSAO catalytic cycle: 1) The benzylamine analogue is recognized by the enzyme; 2) rate-limiting step in which the α-hydrogen is extracted by a carboxylate residue; 3) aldehyde byproduct is released with the formation of an aminoquinol cofactor; 4) and 5) concomitant production of H_2O_2 and regeneration of the TPQ cofactor with ammonia production in the presence of water.

center.[33] X-ray analysis revealed that TPQ is a highly mobile residue that adopts two main conformations, as observed for *E. coli* AO and bovine serum AO (BSAO). These two conformations are correlated with the enzymatic activity of TPQ. The "on-copper" TPQ conformation is its inactive form, and it establishes direct contact with the metal center through O4 (Figure 2 A). TPQ is generated by an autocatalytic copper-assisted reaction from a Tyr residue in the active site. The "off-copper" TPQ conformation is the active form, and O2 forms a hydrogen bond with water.[34] This change is caused by exposure of C=O5 to nucleophilic attack by the amine substrate. This cofactor acts in the reductive and oxidative half-reaction through a "ping-pong" mechanism (Figure 2 B). In the human enzyme, the carboxylate form of Asp 386 acts as base, and the copper ion is surrounded by three His residues and one oxygen atom (either from water or TPQ) in a tetrahedral geometry. The same elements have been found for other AOs, indicating that the TPQ surroundings are highly conserved.[35]

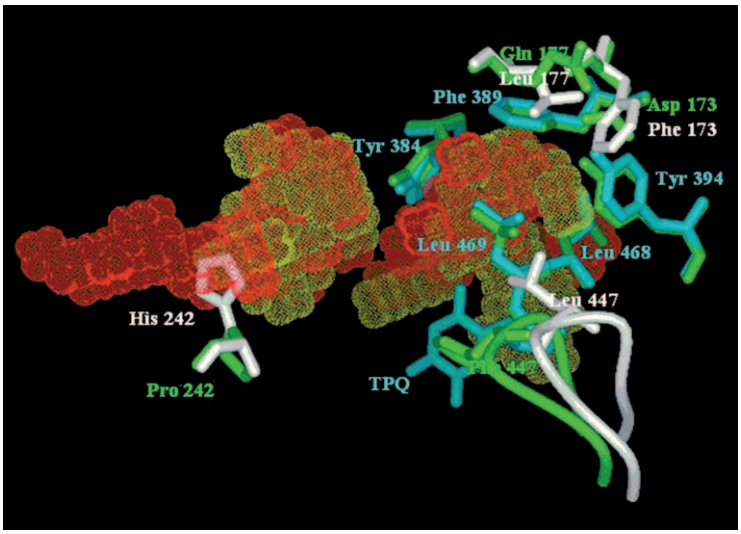


Figure 3. Comparison of mouse and human SSAO active sites. Active site residues in human SSAO are shown in cyan. Residues near the human SSAO active site that are not conserved in mouse SSAO are in white. The equivalent residues in mouse SSAO are in green. Binding cavities are shown as dots (human in yellow, mouse in red). Image used from reference [38] with permission; Copyright 2006, American Chemical Society.

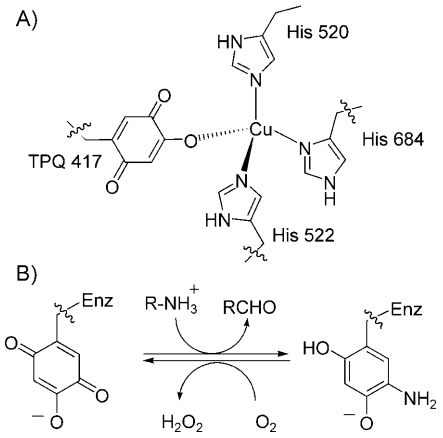


Figure 2. A) Tetrahedral copper environment Cu(N3-His)O in TPQ "on-copper"-type conformation.[47] B) Oxidative-reductive half-reaction of AOs.[38]

More recently, the active center residues that interact directly with the substrates have been described. Marti et al. reported a model of the mouse AO active site in which the aromatic residues Tyr 384, Phe 389, and Tyr 394 define a pocket of stable width (7 Å), which may participate in the binding of nonpolar substrates.[36] In the case of human SSAO, the active site is more restrictive than other mammalian enzymes.[37] Restriction is attributed to differences in the distribution of human catalytic site residues.[38] AO substrate recognition may be influenced by the amino acid composition of the channel near the junction of domains D3 and D4 of the protein, which form the entrance for the substrate from the protein surface to the active site. Because of their critical position within the catalytic site, Tyr 384 or Leu 468 and Leu 469 may affect the substrate selectivity of the human enzyme (Figure 3). It has been described that a β-ribbon arm stretches along the surface of the protein to the channel entrance. This arm could be involved in substrate specificity, as the residues at the end of the arm differ in each protein. For instance, Leu 447 in this loop in human SSAO is substituted by Phe 447 in the mouse enzyme. This loop differs between human and bovine SSAOs at only two residues (Leu 447, Tyr 448 in human versus Phe 446, Leu 447 in bovine). Indeed, the side chain of Leu 469 plays a key role in the recognition process because it is situated at the entrance of the active site and interferes with substrate recognition (Figure 3).[38]

3. SSAO substrates

Although there are many AOs and a broad range of substrates, some general characteristics such as molecular charge, size, and hydrophobicity have a strong influence on enzymatic activity. In the case of the mammalian enzyme, the work published by Lunelli et al. defined key features of substrate recognition by BSAO.[39] Among a broad range of substrates recognized by AOs, BSAO shows a preference for long-chain diamines and polyamines (such as spermine and spermidine) rather than aromatic amines (like benzylamine) or long aliphatic amines (such as nonylamine). In the case of SSAO, benzylamine is traditionally considered the best substrate, but SSAO also recognizes aliphatic amines (compounds **1** and **2**, Figure 4) and some aromatic amines that are neither polyamines nor diamines.[40–42] In 1996, Lyles summarized that benzylamine (**3**), phenethylamine (**4**), tyramine (**5**), dopamine (**6**), tryptamine (**7**) and histamine (**8**) are good SSAO substrates in rat or human tissue, with benzylamine being the best (Figure 4).[27]

Findings by Marti et al. marked a considerable breakthrough.[36] These authors performed homology modeling of the catalytic domain of mouse SSAO/VAP-1 and described the

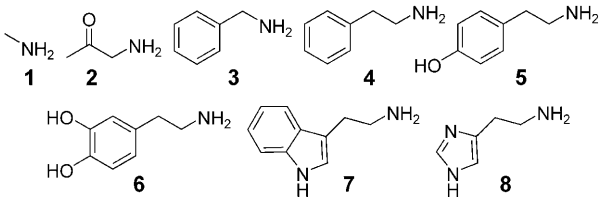


Figure 4. Classic aliphatic and aromatic SSAO substrates reported by G. A. Lyles.[27]

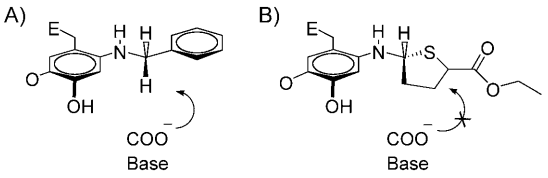


Figure 6. A) Pro-*S* α-proton extraction of the coplanar TPQ–benzylamine adduct compared with B) the forbidden α-proton extraction from the pro-*R* TPQ–4-amino-4,5-dihydrothiophene-2-carboxylate complex.[43,45]

hydrophobic properties of the mouse cavity (Tyr 348, Phe 389, and Tyr 394 in Figure 3). A pharmacophoric motif based on benzylamine was constructed and used to identify new SSAO substrates by searching chemical databases using the homology model. Five new SSAO substrates were found (Figure 5).[36]

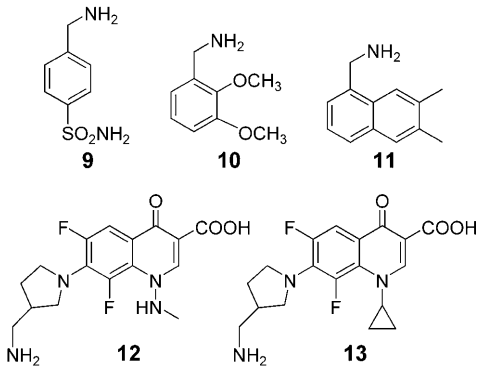


Figure 5. Substrates **9**, **10**, **11** (arylalkylamines), **12**, and **13** (aminomethylpyrrolidines).[36]

The substrates **9**–**13** identified are oxidized by SSAO, and: 1) compounds **10**, **11**, and **12** are rat SSAO substrates; 2) **11** shows the highest affinity for human SSAO adipose tissue; and 3) **9** and **11** are the only substrates oxidized by SSAO from rat adipose tissue. Human SSAO recognizes four substrates, except 2,3-dimethoxybenzylamine (**10**), with K_M values higher than rat and mouse SSAOs, but in general, with the inconvenience that the substrates were found to have turnover values (V_{max}/K_M) low enough to be considered suitable substrates. Marti and co-workers also found that SSAO recognizes hydrophobic substrates, with V_{max} being the major index that indicates its capacity to activate glucose transport by adipose cells.[36] The same study also concluded that there are small structural differences between mouse, rat, and human SSAO proteins and that these structural differences could define the substrate specificity among species.[36]

Other structural factors should also be considered in ligand recognition by SSAO. One is the abstraction of the α-hydrogen atom of benzylamine, which is reported as a stereospecific pro-*S* process in several mammalian species.[43] Alton et al. were the first to report that the coplanarity between the aromatic ring of the substrates and that of the TPQ cofactor was in concordance with a unique active site base, as well as the need for an aromatic cage that promotes this relative positioning (Figure 6 A).[43] Therefore, factors that prevent the abstraction of the α-hydrogen directly affect substrate oxidation. This is the case for α-methylamines, which are not accepted structural motifs for SSAO substrates. In most cases, α-carbon branched amines have been described as inhibitors of SSAO. Specifically, α-methylbenzylamine is recognized as a non-substrate, and in the case of the amphetamine derivatives FLA(±)336 ((±)-4-dimethylamino-2,α-dimethylphenethylamine) that have been reported as SSAO and MAO A inhibitors, SSAO shows stereospecificity with respect to FLA(+)336.[44]

Quiao et al., however, recently provided the first report on the recognition of a branched amine by bovine plasma AO. In that study, a new type of inhibition based on a reversible mechanism was presented, thereby demonstrating that AOs have the capacity to recognize other types of pharmacophores.[45] Preferential metabolism of ethyl-(*S*)-4-amino-4,5-dihydrothiophene-2-carboxylate is consistent with pro-*S* α-proton extraction and thus branched amine recognition (Figure 6 B). Therefore, aminothiophene derivatives represent a new scaffold, as they are recognized by AOs. This was the first evidence that dihydrothiophene and thiophene rings interact with the active site of a mammalian enzyme.[45]

As mentioned above, SSAO is a TPQ-containing enzyme, and therefore putative substrates should not have reactive functional groups. SSAO small-molecule inhibitors have been classified on the basis of their reactive groups (e.g., hydrazine derivatives, haloallylamine derivatives, arylpropargylic amines).[46–48] Although all these structures are highly hydrophobic small arylalkylamine molecules, all of them are potent irreversible inhibitors that interact with the TPQ cofactor through a covalent bond.

4. SSAO arylalkylamine-based substrates

Benzylamine is the most representative arylalkylamine substrate for SSAO. When this ligand interacts with TPQ, the invariant carboxylate group of Asp/Glu extracts the α-proton of the substrate in the Schiff TPQ–substrate complex and promotes tautomerization (Figure 7). This is an enzymatic reaction that is influenced significantly by isotopic effects because it involves electron transfer from the amine substrate to molecular oxygen. In the case of AO, kinetic isotope experiments performed by Olsson et al. demonstrated that this step in the reaction is rate limiting.[49] The data reported are consistent with a deamination mechanism in which the α-proton abstraction is strongly dependent on the electronic properties of the substituents (Figure 7). Benzylamine substrate analogues contribute to substrate oxidation by stabilizing the enzyme–substrate

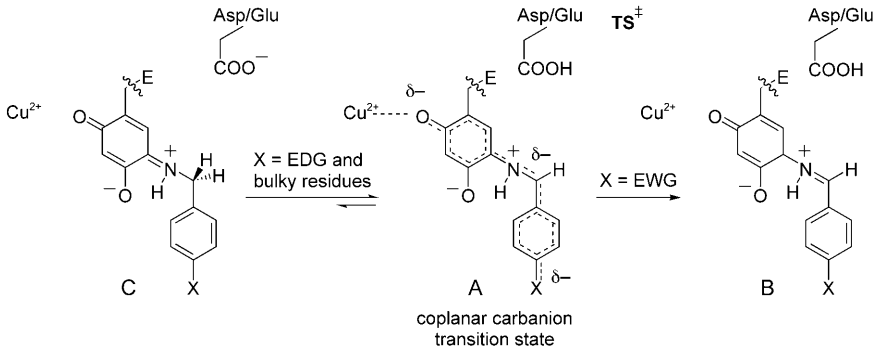


Figure 7. Rate-limiting reaction for TPQ-containing AOs. (A): Transition state ($TS^{\ddagger}$) after α-hydrogen extraction by a carboxylate residue; (A→B): EWG (F, CF_3 or Br) accelerates C–H cleavage, stabilizing the carbanion intermediate; (A→C): EDG such as OCH_3 or NCH_3 increases the electron density at the α-carbon by inductive or resonance effects, or a bulky residue at the *para* position such as *n*Bu or *t*Bu prevents TPQ–benzylamine coplanarity.

transition state throughout the substitution of the aromatic ring. Deprotonation of the α-carbon would formally create a negative charge in the proximity of the benzylamine ring which would promote an increase or decrease in resonance energy during the tautomerization process, depending on the nature of the substituents. The presence of *para* electron-withdrawing groups *(p*-EWG) in the aromatic ring produces an increase in AO activity as a result of the stabilization of the electron density at the α-carbon by inductive or resonance effects. Compounds with *para* electron-donating groups (*p*-EDG) decrease the tautomerization reaction (Figure 7). Hartman and Klinman observed the same effect with the BSAO enzyme.[50] They studied the oxidation mechanism of substituted *p*-benzylamines to define a correlation between the kinetic parameters and electronic effect. These authors demonstrated a transition state with a carbanion species. A QSAR equation was defined: $\log k = \sigma A + \pi B + Es(\mathrm{vdW})C + D$, in which the Taft parameters and van der Waals interactions increase in importance when the size and hydrophobicity of *para* substituents increase, in detriment to enzymatic activity. The electronic effects are correlated with the EWG or EDG behavior with a positive slope, for which $\log k$ $CF_3 > F > Br > CH(CH_3)_2 \approx CH_3 > N(CH_3)_2$, according to Olson et al.[49]

Recently, Yraola et al. published SSAO/VAP-1 SAR studies that reached a similar conclusion.[38] The in vitro data for the percentage of mouse or human SSAO activity show that an increase in the size of *para* residues decreases the percentage of SSAO residual activity. A comparison of serial compounds on the basis of *para* substituents on the phenyl ring of *p*-benzylamines, the percentage of SSAO activity is highest for *p*-F, and $p\text{-}CH_3/m\text{-}F > p\text{-}CF_3 > p\text{-}C_2H_5 > p\text{-}C_4H_9$, with the *p*-F derivative being the most active compound for the human enzyme. In addition, SSAO activity was not observed for arylalkylamines with *ortho* substituents such as OCH_3 or OH.[38]

Bertini et al. and others have described how the aromatic ring substituents modulate the catalytic behavior of arylalkylamine derivatives. B-24 (**14**) is an inhibitor because it forms a stable complex that slowly hydrolyzes to produce the aldehyde byproduct (Figure 8).[51] The inhibitory effect of derivates of **14** are decreased when they are pre-incubated with SSAO, thereby showing that they act as weak substrates. Moreover, the study showed that compound **14** acts as a competitive site-directed inhibitor by increasing K_M and decreasing V_{max}, yet acts as a noncompetitive inhibitor when it is pre-incubated at a lower concentration than the enzyme. All these results suggest that the inhibitory effect is not only due to affinity factors but that it is also related to the electronic properties of aromatic ring substitution. For example, benzylamine alkoxy derivatives yield SSAO-selective inhibitors such as **15**. The same SARs were observed with pyridoxamine (**17**) and **18**, which are derivates of the mammalian SSAO substrate **16** (Figure 8).[52] Compound **17** inhibits plasma and tissue SSAO, and the ethoxy derivative B-24 is described as a plasma- and tissue-selective reversible inhibitor in the low-micromolar concentration range.[53,54]

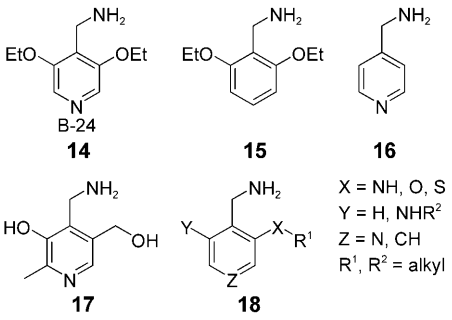


Figure 8. Some SSAO arylalkylamine substrate-like inhibitors.

These results point to the possibility of modulating activity through ring substitution by converting SSAO arylalkylamine substrates into inhibitors. Bertini et al. have tested this hypothesis for BSAO.[51] Pycolylamine (**16**) has a significant substrate inhibition effect because of its pyridine ring. Compound **16** (127 $nmol\,mL^{-1}\,h^{-1}$ and 16 μmol) alone has a greater affinity for BAO than the benzene ring of benzylamine (1653 $nmol\,mL^{-1}\,h^{-1}$ and 124 μmol) for BSAO. It is clear that **16** has an electronic contribution through the pyridine ring, and a nonstructural contribution to affinity because it is structurally related to the benzene ring of benzylamine.

The extent of the substitution effect in arylalkylamines has also been observed with other alkylthio, alkylamino- and alkoxybenzylamine, and pyridine derivatives **18**, which are also defined as "substrate-like inhibitors".[51] The results from several 2,6-alkoxy-, 2,6-alkylthio-, and 2,6-alkylaminopyridine derivatives indicate that 2,6-alkoxy compounds such as **19**, **20**, and **21** are more potent, selective (BAO versus MAO, DAO, and LO), and fully reversible than 2,6-alkylthio, with 2,6-alkylamino derivatives showing the poorest performance (Figure 9).

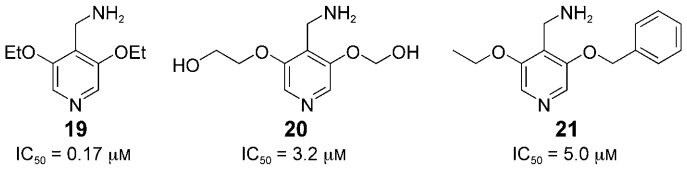


Figure 9. BAO-selective substrate-like inhibitors, with selectivity over DAO, LO, and MAO A and B.

Marti et al. reported the possibility of improving the binding affinity of the substrates by filling the cavity of SSAO/VAP-1 and exploring other hydrophobic interactions with the residues of the active site. In this regard, naphthalene derivatives could be a good choice to improve these interactions.[36] In fact, **11** is a high-affinity substrate for rat and human SSAOs (10.2 ± 2.0 and 72 ± 34 μM, respectively).[36] However, naphthalene rings can be considered a benzylamine analogue and thus the same electronic effect could be expected. This result has been corroborated by the biological behavior of 1-aminomethylnaphthalen-2-ol, which inhibits mouse and human SSAO activity when administered at 100 μM.[55] The inhibitory activity of this compound showed that aromatic rings fused to benzylamine interact with the catalytic site of the enzyme, but that the electronic properties of ring substitution are critical in defining the enzymatic activity of arylalkylamine-based substrates.

5. MAO arylalkylamine-based substrates

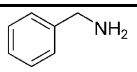
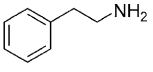
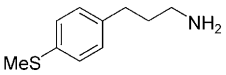
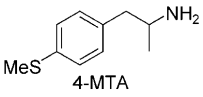
Having reviewed the chemical features that may be determinant in arylalkylamines for SSAO recognition, it is also relevant to discuss which substrate features could guide SSAO selectivity over MAO. In general, most of the SSAO substrates show poor selectivity relative to MAO. A similar scenario can be found for substrate recognition and metabolism by MAO. In general, results based on the arylalkylamine substrates and inhibitors demonstrate that: 1) the active sites of both MAO isoforms are highly hydrophobic (without acidic or basic residues inside the pocket); 2) *para*-substituted benzylamines with EWG are preferred because of the nature of the FAD cofactor; 3) substrate affinity increases with hydrophobicity of the residues on the benzylamine phenyl ring; 4) affinity decreases with increasing size of the substituents; and 5) deprotonated substrates are preferred over protonated ones.[56–59] From a structural point of view, small differences have been found between two MAO isoforms. Recently, De Colibus et al. published the three-dimensional structure of human MAO A and compared it with the B isoform; hMAO A was shown to have a single cavity which is shorter and wider (~500 Å^3) than hMAO B, which is longer and narrower (~700 Å^3).[60] Therefore, the cavity of hMAO B decreases ligand mobility in terms of steric clashes inside the active site, and the ligand disposition inside the hMAO B cavity is more restricted than it is for hMAO A.[60] This effect was previously observed in 1994, when a QSAR analysis of the oxidation of *para*-substituted benzylamines by MAO B demonstrated that the rate of ligand oxidation depends more on the *Es* Taft steric constant than is the case with MAO A.[56] In contrast, oxidation of *para*-substituted benzylamines by MAO A is strongly dependent on the σ Hammett parameter.[56] The structural differences between these isoforms represent a valuable starting point for future studies by homology or by ligand screening. Some discrepancy has arisen regarding benzylamine-based arylalkylamines because *para*-substituted benzylamines with EWG are good substrates for SSAO and MAO A.

6. AOs: aminomethyl, -ethyl, -propyl, and -butyl arylalkylamine-based substrates

Unzeta and co-workers studied the effect of rearranging the carbon chain between the phenyl ring and the NH_2 group in 4-methylthioamphetamine (4-MTA) to (4-methylthiophenyl)propylamine on substrate oxidation.[61] 4-MTA is a highly potent and reversible MAO A inhibitor, a less potent inhibitor of SSAO, and a weak inhibitor of MAO B. A lack of potency was observed relative to MAO A when the rearrangement was carried out from 4-MTA to (4-methylthiophenyl)propylamine, increasing its affinity relative to MAO B (Table 1). When (4-methylthiophenyl)propylamine was assayed as a substrate, the results showed that the molecule had 319- or 320-fold SSAO catalytic efficiency versus 19-fold for MAO B, and was not oxidized by MAO A. The authors proposed that differences in substrate recognition between SSAO and MAO B are because (4-methylthiophenyl)propylamine probably fits into the cavity of SSAO much better than MAO B. They based their observation on crystallographic data, which indicated that entry of the ligand was restricted and the carbon chain could not fully interact with the FAD cofactor in the active site.[61]

The effect of methylene chain elongation on benzylamine substrate has also been studied for MAO isoforms.[62] A number of observations were quantified and correlated with the MAO activity of human liver and later observed by Yraola et al. for mouse and human SSAO/VAP-1.[38] The results of Nandigama and Edmondson on the interaction of phenethylamine

Table 1. K_M/V_{max} of arylalkylamines for SSAO and MAO enzymes.[61]

Entry	Substrate	SSAO[a] (K_M/V_{max})	MAO A[b]	MAO B[c] (K_M/V_{max})
1		50.25[d]	substrate	substrate
2		3.14[d]	22 μM	522[d]
3		320[d]	5.8 μM	19[d]
4		inhibitor	0.2 μM	weak inhibitor

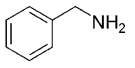
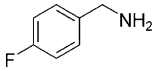
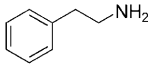
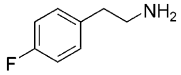
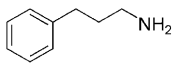
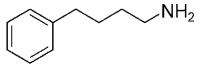
Entry 1: [a] bovine lung microsomes, [b,c] see text.[38] Entries 2 and 3: [a] bovine lung microsomes, [b] see text,[62] [c] rat liver mitochondria. Entry 4: [a,b,c] see text.[38] [d] K_M/V_{max} units: μM (pmol min^{-1} mg^{-1})$^{-1}$.

analogues with MAO A do not show an electronic effect. Only steric effects on binding and rates were observed.[62] In addition, the affinity of substrates recognized by MAO increases linearly with an increase in van der Waals volume of the *para* substituent.[62] The results demonstrate that 3-phenylpropylamine is oxidized 2.5-fold more slowly and binds 75-fold more tightly than phenethylamine and 4-phenylbutylamine, which is a competitive inhibitor with $K_i = 31 \pm 5$ μM. QSAR showed that the methylene chain by itself correlates with Taft steric values.[62]

Yraola et al. described the effect of methylene chain elongation on SSAO/VAP-1 activity (Table 2). The in vitro activity data were also compared with docking studies.[38] Preliminary results demonstrated a correlation between the activity of human SSAO/VAP-1 and methylene chain modification with the energy/k_{cat} relation of this substrate. Compounds such as benzylamine, 2-phenylethylamine, and 2-(4-fluorophenyl)ethylamine have slightly larger computed binding energy values than those expected for the similar compounds 4-fluorobenzylamine, 3-phenylpropylamine, and phenylbutylamine.[38] This effect is basically due to the interaction of the aromatic ring inside the SSAO active site. Several differences have been reported between AOs with regard to methylene chain elongation. While ethylene or propylene chains produce an increase in activity with MAO A, they abolish activity in the case of SSAO/VAP-1 and MAO B (Table 2). However, the 4-aminobutyl chain changes the enzymatic profile, yielding a potent SSAO/VAP-1 substrate and a MAO A-competitive inhibitor.[38,62]

A similar scenario has been observed when the 4-aminomethyl chain is analyzed as a lysine motif. In 1968, Pinnell and Martin described LAO as an enzyme that deaminates lysyl ε-amino groups of the extracellular matrix.[30] However, LO differs from the other SSAOs because it has an LTQ cofactor and lacks the $(H_2O)CuN(His)_3$ coordination environment.[63,64] Twenty years later, Salmi and Jalkanen reported that extracellular membrane SSAO is involved in the leukocyte–endothelial cell interaction related to the VAP-1 function of SSAO.[65] VAP-1 activity suggests that leukocyte adhesion and rolling are directly related to terminal deamination of lysine or aminoglycane residues of leukocytes via a Schiff base intermediate.[66] Yegutkin et al. designed a lysine-containing peptide (GGGGKGGGG) that inhibits recombinant SSAO/VAP-1 ($IC_{50} = 534.0 \pm 28.2$ μM), demonstrating again the importance of the lysine ε-NH_2 group in blocking lymphocyte rolling through the endothelial cell.[67] Molecular modeling studies with the peptide showed an extensive interaction of the V-shaped poly-G peptide conformation filling the groove on the VAP-1 surface. Furthermore, GGGGKGGGG did not block other AOs such as RAO, DAO from porcine kidney, and FAD-containing MAO A/B from human liver.[67] In addition, lysine is not a human or rat SSAO substrate by itself, and 4-phenylbutylamine is an active substrate ($k_{cat}/K_M = 2938\ s^{-1}\ M^{-1}$) for human SSAO/VAP-1 (Figure 10).[36,38] Docking studies have demonstrated that the ammonium group of 4-phenylbutylamine is located in the same spatial coordinates as in benzylamine, and its aromatic ring is closer to the active site entry, without major atomic clashes with surrounding protein atoms.[38] In this context, the four methylene units of a lysine side chain show modular behavior toward SSAO.

Table 2. Catalytic rate constant values of arylalkylamine substrates and inhibitors for SSAO and MAO isoforms from human tissues.[38,62]

Entry	Substrate	SSAO k_{cat} [sec^{-1}][a]	MAO A k_{cat} [min^{-1}][b]	MAO B k_{cat} [min^{-1}][c]
1		0.45	2.54	640
2		0.71	7.55	390
3		0.43	64	228.9
4		0.41	111.5	–
5		0.49	25.1	–
6		0.82	$IC_{50} = 31$ μM	–

Entries 1 and 2: [a] human recombinant SSAO, [b] liver MAO, [c] bovine lung mitochondrial MAO B. Entry 3: [a] human recombinant SSAO, [b] human liver MAO A, [c] human MAO B. Entry 4: [a] human recombinant SSAO, [b] human liver MAO A, [c] no data. Entries 5 and 6: [a] human recombinant SSAO, [b] liver MAO A, [c] no data.

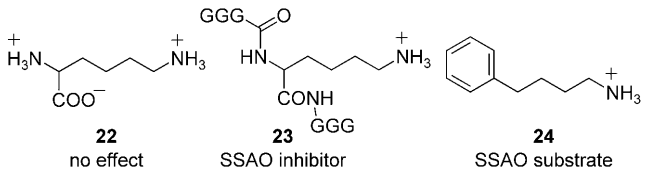


Figure 10. Lysine (**22**), lysine-containing peptide **23,** and 4-phenylbutylamine (**24**).

The 4-aminobutyl chain of **22** is unable, per se, to interact with SSAO, but when this chain is included in extensive peptides such as **23**, it interacts with the catalytic center and acts as an SSAO inhibitor. However, if a 4-aminobutyl side chain is included in a small molecule such as **24**, the resulting compound is a potent SSAO substrate (Figure 10).

Conclusions and Future Prospects

Herein we have analyzed several features of SSAO arylalkylamine-based substrates. We have also reviewed the data related to ligand steric and electronic factors that modulate the enzymatic behavior of AOs. Modifications on the benzylamine ring can improve the substrate affinity for each AO. In addition, the effect of exploring cavity size by methylene chain elongation between the aromatic ring and amino function in SSAO arylalkylamine-based substrates has been discussed. Figure 11 summarizes the current state of the art. For cases A and B, we have highlighted the importance of *para* substitution in SSAO

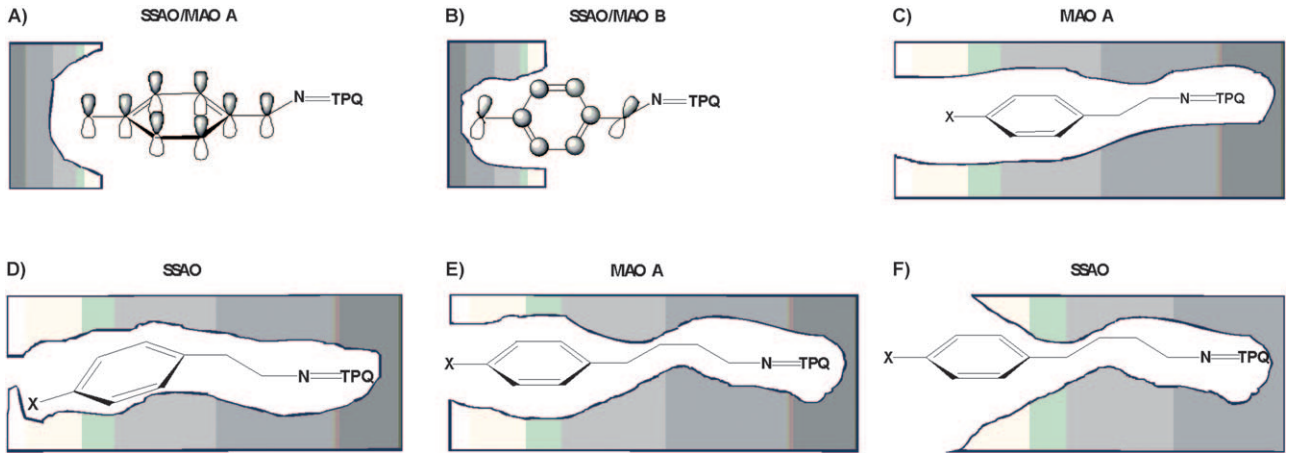


Figure 11. A) Coplanar *para*-substituted benzylamines in the SSAO/MAO A cavity, allowing transmission of electronic effects at the α-carbon; B) size limitation of bulky *para*-substituted benzylamines in SSAO and MAO B that prevent the transmission of electronic effects at the α-carbon; C) *p*-phenethylamine analogues in MAO A fit well, but D) *p*-phenethylamine analogues interact with hydrophobic residues in SSAO, and the entrance residues interact with the ligand; E) phenylbutylamine interacts well with the MAO A active site as a competitive inhibitor; F) the aromatic ring of the phenylbutylamine substrate sticks out of the SSAO/VAP-1 cavity, preventing α-interactions. Panels A) and B) adapted from references [57, 58].

arylalkylamine-based substrates, where the electronic properties of *para* substituents and the size of each one are crucial. However, for model A, in which benzylamine-based structures are recognized by SSAO or MAO B, EWG substituents are far from yielding selective substrates toward specific AOs. For model B, representing the cavity for hMAO B, the cavity residues could diminish substrate recognition as a result of steric clashes and hinder substrate orientation inside the cavity. This situation can also be found for SSAO with bulky *para* residues such as *n*Bu or *t*Bu, where the loss of coplanarity between the TPQ cofactor and the arylalkylamine prevent the correct substrate metabolism.[38,43,50] A new situation is presented upon comparison of the size and shape of each enzyme cavity together with the effect of methylene chain elongation on enzymatic activity. Molecular dynamics simulations were performed with the X-ray crystal structure of human SSAO/VAP-1. These show that SSAO has an active site with a stable volume of ~185 Å^3.[38] In the case of MAO isoforms, hMAO A has single cavity that is shorter and wider (~500 Å^3) than hMAO B, which is longer and narrower (~700 Å^3).[60] The limited size of the SSAO active site contrasts with the cavity size of both MAO isoforms. A plausible explanation could be the side chain of Leu 469, situated in the entrance of the SSAO cavity, but which is not present for the MAOs (see also Figure 3). The conformation of this residue limits the orientation of the aromatic ring of arylalkylamines when situated inside active site of SSAO. The conformation of Leu 469 affects compounds such as phenylethylamine derivatives by blocking the ligand inside the cavity (models C and D, Figure 11). Additionally, it has been demonstrated that phenylethylamines are better substrates for MAO A and are metabolized faster than the benzylamine analogues (model C, Figure 11). This is not the case for arylalkylamines with a more extended methylene chain such as 3-phenylpropylamine or 4-phenylbutylamine (models E and F, Figure 11). The 4-phenylbutylamine ligand interacts extensively with the active site and acts as good competitive inhibitor of MAO A (model E, Figure 11). For the last case, its phenyl ring sticks out of the SSAO/VAP-1 cavity, thus avoiding π-interactions (model F, Figure 11).

In summary, we present two main situations for the future design of SSAO/VAP-1 substrates. First, it has been shown that the appropriate balance between size and electronic properties of each substituent of benzylamine derivatives determines the enzymatic profile (V_{max}/K_M) of each substrate. Second, the methylene chain elongation between the amino function and the aromatic ring of benzylamines can modulate ligand behavior, thereby yielding substrates and inhibitors, depending on the particular AO. The application of this rationale in future ligand design will introduce novel approaches to develop new SSAO substrates as insulin-mimetic compounds.

Abbreviations

AO, amine oxidase; BAO, bovine amine oxidase; BSAO, bovine serum amine oxidase; CuAO, copper-containing amine oxidase; DAO, diamine oxidase; EDG, electron-donating group; EWG, electron-withdrawing group; GLUT-4, glucose transporter isoform 4; LO, lysyl oxidase; MAO, monoamine oxidase; PAO, polyamine oxidase; QSAR, quantitative structure–activity relationship; SAR, structure–activity relationship; SSAO, semicarbazide-sensitive amine oxidase; VAP-1, vascular adhesion protein-1.

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