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ORIGINAL ARTICLE

Synthetic polyamines as potential amine oxidase inhibitors: a preliminary study

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Abstract In the last few decades, medicinal chemists have carried out extensive research on synthetic polyamines for use as anticancer drugs and multitarget-directed ligands in neurodegenerative diseases. The aim of this study was to evaluate the effect of some synthetic polyamines as inhibitors of two new potential targets, human semicarbazide-sensitive amine oxidase/vascular adhesion protein-1 (SSAO/VAP-1) and monoamine oxidases B (MAO B), enzymes involved in various multi-factorial diseases such as Alzheimer's disease. *N*,*N*′-Dibenzyl-dodecane-1,12-diamine (Bis-Bza-Diado), a newly synthesised compound, and ELP 12, a muscarinic cholinergic M_2 receptor antagonist, were found to behave as reversible and mixed non-competitive inhibitors of both amine oxidases (dissociation constants of about 100 μM). ELP 12 was found to be more selective for SSAO/VAP-1. Combining kinetic and structural approaches, the binding mode of ELP 12 to SSAO/VAP-1 was investigated. ELP 12 may bind at the entrance of the active site channel by ionic interactions with ASP446 and/or ASP180; one end of the polyamine may be accommodated inside the channel, reaching the TPQ cofactor area. The binding of ELP 12 induces rearrangement of the secondary structure of the enzyme and impedes substrate entry and/or product release and catalysis. These structural data reveal that the entrance and the first part of the SSAO/VAP-1 channel may be considered as a new target area, or a "secondary binding site", for modulators of human SSAO/VAP-1 activity. These results indicate ELP 12 and Bis-Bza-Diado as new "skeletons" for the development of novel SSAO/VAP-1 and MAO B inhibitors.

Keywords Synthetic polyamines · Semicarbazide-sensitive amine oxidases · Monoamine oxidase B · Amine oxidase inhibitors · Structure–function relationships

Abbreviations

AChE	Acetylcholine esterase
AD	Alzheimer's disease
BD6	*N*-(3-Amino-propyl)-*N*′-(3-benzylamino-propyl)-butane-1,4-diamine
BVT 128	1,4-Bis-{6-[ethyl-(2-methoxybenzyl)amino]hexyl} piperazine-2,5-dione
Bis-Bza-Diado	*N*,*N*'-Dibenzyl-dodecane-1,12-diamine
CD	Circular dichroism
E	Free enzyme

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ELP 12	6,6′-(4,4′-(Ethane-1,2-diyl)bis(piperidine-4,1-diyl)bis(*N*-(2-methoxybenzyl)hexan-1-amine)
ES	Enzyme–substrate complex
Et_2O	Diethyl ether
EtOH	Ethanol
Hepes	(4-(2-Hydroxyethyl)-1-piperazineethanesulphonic acid)
I	Inhibitor
IES	(Enzyme–substrate)-inhibitor complex
KIE	Dissociation constant for the free enzyme–inhibitor complex
KIES	Dissociation constant for the (enzyme–substrate)-inhibitor complex
$K_{m\ app}$	Apparent Michaelis–Menten constant
MeOH	Methanol
MTDL	Multitarget-directed ligand
MS (ESI*)	Mass spectrometry (electron spray ionization)
r-MAO B	Human recombinant monoamine oxidase B
r-SSAO/VAP-1	Human recombinant semicarbazide-sensitive amine oxidase/vascular adhesion protein-1
TLC	Thin layer chromatography
S	Substrate
TPQ	2,4,5-Trihydroxyphenylalanine quinone.
$V_{max\ app}$	Apparent maximum velocity

Introduction

Polyamine analogues are currently being extensively researched, thanks to their potential pharmacological applications. Polyamine molecular structures can bind to various targets and their affinity and selectivity may be fine-tuned by inserting appropriate substituents and varying the methylene chain lengths on the polyamine backbone (Minarini et al. 2010). As well as the development of synthetic polyamines as antitumor agents (Casero and Woster 2009), these compounds have been studied as multitarget-directed ligands (MTDLs) for a new therapeutic approach in neurodegenerative diseases (Melchiorre et al. 2010). The MTDLs approach in Alzheimer's disease (AD) is based on the use of a single molecule that hits multiple targets which are significant for the pathology (Cavalli et al. 2008; Youdim and Buccafusco 2005). This seems more appropriate for addressing the complexity of AD and may provide new drugs for tackling the multifactorial nature of AD, stopping its progression. In particular, the MTDLs developed so far for neurodegenerative diseases, act as acetylcholinesterase (AChE), Aβ aggregation and β-secretase inhibitors, muscarinic M_2 receptor antagonists, nicotinic acetylcholine receptor agonists, α_2-adrenergic agonists, or monoamine oxidase inhibitors.

Generally, amine oxidases catalyse the oxidative deamination of biogenic amines, to produce aldehyde, hydrogen peroxide and ammonia. First two may contribute to the oxidative damage involved in neurodegeneration and other pathologies (e.g., diabetes, cardiovascular disorders and inflammatory diseases (Cohen and Tong 2010; Yu et al. 2003; Valko et al. 2007; Gubisne-Haberle et al. 2004; Vavilova et al. 2009).

In particular, monoamine oxidases (MAOs) are "old" and well-known targets of anti-neurodegenerative drugs (particularly MAO B, Youdim 2006; Carreiras and Marco 2004) and anti-depressant therapies (MAO A), because they catalyse the oxidative deamination of neurotransmitters (Mutsuhiko and Yasuhara 2004; Bortolato et al. 2008). In the past few years, much important information on the molecular properties of MAOs have been obtained, thanks to the resolution of human MAO A and B crystal structures (Edmonsons et al. 2009) and many efforts have been oriented towards the development of reversible MAOs inhibitors, to avoid the adverse side-effects of early MAO inhibitors (hydrazine derivatives). Many compounds have been proposed as MAO B inhibitors, such as pyrazoline-based compounds (Sahoo et al. 2010), safinamide and coumarin analogues (Binda et al. 2007) and indole and benzofurane derivatives (Prins et al. 2010).

Semicarbazide-sensitive amine oxidase, known also as vascular adhesion protein–1 (SSAO/VAP-1), is another type of amine oxidase emerging as a new potential target in neurodegenerative and other multi-factorial diseases.

Human SSAO/VAP-1 (SSAO, EC 1.4.3.6) is a dual-function membrane-bound glycoprotein involved in inflammatory processes. It is mainly expressed in endothelial cells, hepatic sinusoidal endothelial cells, adipocytes and smooth muscle cells. It has an adhesive domain, mediating leucocyte adhesion to endothelial cells and an amine oxidase enzyme domain (Jalkanen and Salmi 2001). Its enzymatic activity is fundamental in inflammatory processes, in both leucocyte anchoring and the inflammatory signalling pathway (Jalkanen and Salmi 2008). Increased levels of the circulating/plasmatic form of SSAO/VAP-1 has also been found in AD patients (Unzeta et al. 2007), in patients affected by diabetes and its complications (rethinopathy and nephropathy), and in stroke, inflammatory liver diseases and atherosclerosis (Boomsma et al. 2003). In post-mortem AD brains, increased SSAO/VAP-1 expression has been found to co-localise with abnormal amyloid deposition (Ferrer et al. 2002). Thus, SSAO/VAP-1 has been recognised as a good therapeutic target for new drug molecules, which could open up the

possibility of new treatments in various diseases (Kinemuchi et al. 2004; Dunkel et al. 2008). Several SSAO/VAP-1 inhibitors have been identified so far: they are generally small molecules, most of which are hydrazine derivatives, such as BTT2052 (Smith et al. 2003; Nurminen et al. 2010) able to inactivate its carbonyl cofactor, 2,4,5-trihydroxyphenylalanine quinone, (TPQ). Other types of SSAO/VAP-1 inhibitors are halo-phenylallylamines, such as LJP-1207 (Wang et al. 2006), some carboxamide and sulphonamide derivatives (Clauzel et al. 2007; Olarte et al. 2006), 4-amino-methylpyridazine derivatives (Haider et al. 2010) and some reversible guanidine derivatives, such as guanabenz (Holt et al. 2008). However, a really effective and selective compound for clinical application is still lacking, and the search for new lead structures is a challenge.

No information about the possible inhibitory effect of synthetic polyamines on human SSAO/VAP-1 and MAO activities has been reported so far. On these bases, the aim of this work was to evaluate the effect of six synthetic polyamines as potential inhibitors of human SSAO/VAP-1 and MAO B. We found that some of these compounds behave as mixed non-competitive inhibitors, with dissociation constants for the free enzyme of about 100 μM. In particular, ELP 12 (a muscarinic cholinergic M_2 receptor antagonist) appeared to be more selective for SSAO/VAP-1, whereas Bis-Bza-Diado (a newly synthesised compound) inhibited both amine oxidases. Kinetic studies were performed and integrated with docking and structural studies to understand the binding mode of ELP 12 to human SSAO/VAP-1. A "secondary binding site" for novel modulators of human SSAO/VAP-1 was revealed. Our results suggest that these compounds might be good "skeletons" for the development of a new type of amine oxidase inhibitors.

Materials and methods

Materials and enzymes

All chemicals were of analytical grade and purchased from Fluka-Sigma-Aldrich s.r.l. (Milan, Italy) with the exception of Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine), purchased from Molecular Probes/Invitrogen (Invitrogen s.r.l, San Giuliano Milanese (MI, Italy). Recombinant human SSAO/VAP-1 (r-VAP-1) was a kind gift from Biotie Therapies Corp. (Turku, Finland); it was stored at 4°C at 0.1 mg/ml, in 10 mM sodium phosphate (pH 7.4) containing Triton X-100 (0.1% v/v). Human SSAO/VAP-1 from adipocyte membranes was prepared from isolated fat cells according to a previously reported procedure (Bonaiuto et al. 2010). SSAO/VAP-1 activity of solubilised membranes was determined after pre-incubation of the sample in 0.5 mM pargyline (for 30 min, at 37°C) to ensure that any monoamine oxidase activity, if present, was completely inactivated.

Human recombinant MAO B expressed in baculovirus-infected (BTI-TN-5B1-4) cells and horseradish peroxidases were purchased from Fluka-Sigma-Aldrich s.r.l. (Italy).

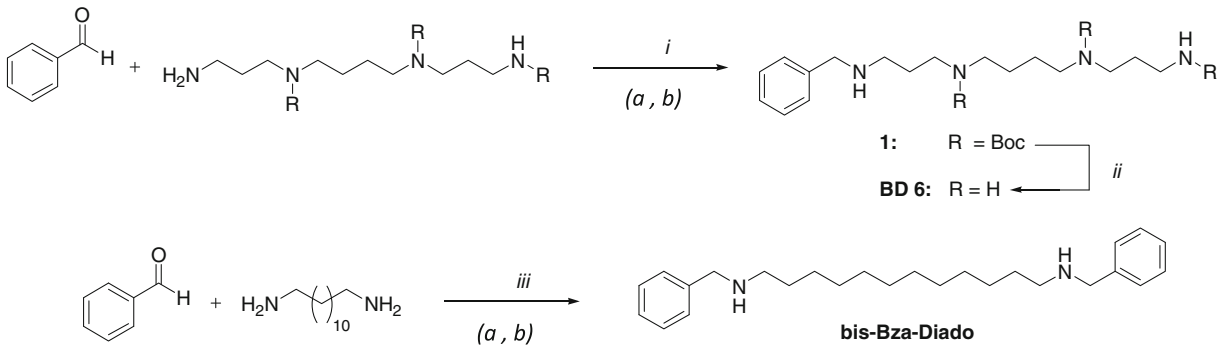
The protein concentration of the samples was determined according to the method of Bradford (1976) with bovine serum albumin as standard protein. The molar concentration of SSAO/VAP-1 was calculated assuming a molecular mass of 180 kDa.

Chemistry

^{1}H and ^{13}C NMR spectra were recorded on Varian VXR 400 and 200 spectrometer in $CDCl_3$ or D_2O as solvents. Chemical shifts are given in ppm, *J* values are given in Hz, and spin multiplicities are given as s (singlet), brs (broad singlet), d (doublet), t (triplet), q (quartet), or m (multiplet). Electron spray ionization (ESI) mass spectra were recorded on VG 7070E instrument. Chromatographic separations were performed on silica gel columns by flash (Kieselgel 40, 0.040–0.063 mm; Merck), or gravity column (Kieselgel 60, 0.063–0.200 mm; Merck) chromatography. Reactions were followed by thin-layer chromatography (TLC) on Merck (0.25 mm) glass-packed precoated silica gel plates (60 F_{254}) that were visualised in an iodine chamber, UV lamp and bromocresol green. The term "dried" refers to the use of anhydrous Na_2SO_4.

Tetraamines Methoctramine (Minarini et al. 1989) and ELP-12 (Tumiatti et al. 2007) and diamine-diamides Caproctamine (Melchiorre et al. 1998) and BVT-128 (Tumiatti et al. 2008) were synthesised as previously reported.

{4-[(3-Benzylamino-propyl)-*tert*-butoxycarbonyl-amino]-butyl}-(3-*tert*-butoxycarbonylamino-propyl)-carbamic acid *tert*-butyl ester (1, Scheme 1) A solution of tri-Boc-spermine (Wellendorph et al. 2003) (0.502 g, 1 mmol) and benzaldehyde (0.117 g, 1.1 mmol) in toluene (50 mL) was refluxed in a Dean-Stark apparatus for 5 h and on cooling solvent was removed in vacuo. $NaBH_4$ (0.151 g, 4 mmol) was added in portions to a cooled (0°C) solution of the residue in EtOH (30 mL) and stirring was continued at room temperature for 4 h. The solvent was then removed and the residue was dissolved in CH_2Cl_2 (30 ml) and washed with H_2O (3 × 20 ml). Removal of the dried solvent in vacuo gave a residue that was purified by flash chromatography with a mixture of CH_2Cl_2/MeOH/33% aq. NH_4OH (9:1:0.05) as eluent, yielding the desired product **1** (0.440 g, 76%) as yellow oil; ^{1}H NMR ($CDCl_3$) 1.39–1.47 (m, 29H, 3 × $C(CH_3)_3$ + CH_2), 1.64–1.75 (m, 4H, 2 × CH_2), 2.83 (brs, 1H exchangeable with D_2O, NH



Scheme 1 (*i*) (*a*) toluene/Δ/3 h, (*b*) $NaBH_4$/EtOH/rt/4 h, (*ii*) HCl 3 M/rt/overnight, (*iii*) (*a*) toluene/Δ/6 h, (*b*) $NaBH_4$/EtOH/rt/6 h

amine), 2.64–2.68 (m, 2H, CH_2), 3.09–3.24 (m, 12H, 6 × CH_2–N), 3.80 (s, 2H, CH_2–Ph), 5.27 (brs, 1H exchangeable with D_2O, NH Boc), 6.86–6.89 (m, 2H, 2 × Ph–H), 7.27–7.29 (m, 3H, 3 × Ph–H).

***N*-(3-Amino-propyl)-*N*′-(3-benzylamino-propyl)-butane-1,4-diamine tetrahydrochloride salt (BD 6,** Scheme 1) A solution of **1** (0.400 g, 0.69 mmol) in 30 ml of MeOH and 20 ml of aq. HCl (10 mL, 3 M) was stirred overnight at room temperature. Following solvent removal, the residue was washed with Et_2O (5 × 20 ml). The resulting solid was filtered and dried to afford the desired product **BD 6** tetrahydrochloride as a white solid (0.278 g, 91%); ^{1}H NMR (D_2O) 1.62–1.66 (m, 4H, 2 × CH_2), 1.93–2.00 (m, 4H, 2 × CH_2), 2.94–3.06 (m, 12H, 6 × CH_2–N), 4.13 (s, 2H, CH_2–Ph), 7.34–7.38 (m, 5H, 5 × Ph–H); ^{13}C NMR (D_2O) 22.5, 22.6, 22.7, 36.5, 43.8, 44.4, 44.5, 46.9, 51.2, 129.2, 129.7, 129.8, 130.3; MS (ESI^+) $m/z = 147$ $(M + 2H)^{2+}$.

***N,N*′-Dibenzyl-dodecane-1,12-diamine dihydrochloride salt (Bis-Bza-Diado,** Scheme 1) A solution of 1,12-diaminododecane (1.00 g, 5 mmol) and benzaldehyde (1.06 g, 10 mmol) in toluene (50 mL) was refluxed in a Dean-Stark apparatus for 6 h and after cooling the solvent was removed in vacuo. $NaBH_4$ (0.38 g, 10 mmol) was added in portions to a solution of the residue in EtOH (30 mL), stirring was continued at room temperature for 6 h, aq. HCl (10 mL, 3 M) was added and the solvents were evaporated to dryness in vacuo. The residue was dissolved in H_2O (30 ml), the resulting solution was washed with Et_2O (3 × 20 ml), made basic with aq. NaOH (10 mL, 2 M), and extracted with CH_2Cl_2 (3 × 20 ml). Combined CH_2Cl_2-extracts were subsequently washed with water (20 ml), brine (20 ml) and dried. Solvent was distilled off in vacuo, the residue was dissolved in EtOH (5 ml), treated with saturated HCl/Et_2O (5 ml), precipitated with Et_2O (5 ml) and after drying in vacuo resulted in Bis-Bza-Diado tetrahydrochloride (2.11 g, 93%) as a white solid. ^{1}H NMR (D_2O) 1.12–1.22 (m, 16H, 8 × CH_2), 1.49–1.55 (m, 4H, 2 × CH_2), 2.90 (t, 4H, $J = 7.8$, 2 × CH_2), 4.08 (s, 4H, 2 × CH_2–Ph), 7.31–7.37 (m, 10H, 10 × Ph–H). ^{13}C NMR (D_2O) δ 25.3, 25.6, 28.0, 28.3, 28.5, 46.9, 50.8, 129.2, 129.5, 129.7, 134.2. MS (ESI^+) $m/z = 382$ $(M + H)^+$.

Kinetic assays of enzymatic activity

Amine oxidase activity was determined by measuring hydrogen peroxide generation rate (one of the reaction products) by a peroxidase-coupled continuous assay. The Amplex Red reagent was used as fluorogenic substrate for horseradish peroxidase (Zhou and Panchuck-Voloshina 1997). All experiments were carried out in "physiological Hepes": Hepes 50 mM, KCl 50 mM, $CaCl_2$ 2 mM, $MgCl_2$ 1.4 mM, NaCl 120 mM, pH 7.4, (Holt et al. 2007) unless otherwise specified.

After a defined pre-incubation time, the solutions containing SSAO/VAP-1 or MAO B (in the presence or absence of the various polyamines), were used to perform assays of amine oxidase activity. Assays were carried out in the presence of Amplex Red and type II horseradish peroxidase, to give final concentrations of 100 μM and 5 Uml^{-1}, respectively. The reaction was run, continuously, for 5 min after the addition of the amine substrate (benzylamine).

Initial velocities were determined by measuring the increase in fluorescence intensity detected with a Cary-Eclipse fluorimeter (Varian Inc., Palo Alto, CA, USA) ($\lambda_{exc} = 563$ nm and $\lambda_{em} = 586$ nm). In some experiments, the reaction rate was measured by detecting the fluorescence intensity with a Fluoroscan ASCENT FL plate reader (excitation 544 nm; emission 590, fluorescence readings every 20 s). H_2O_2 generation rate was calculated from the change in fluorescence intensity, using calibration curves obtained by serial dilutions of stock solution of H_2O_2. For each measurement, the corresponding blank, obtained in the absence of the substrate, was subtracted. No interference of the various compounds (synthetic polyamines) on fluorescence intensity was observed.

All experiments were carried out, in air-equilibrated solutions and at least in triplicate. The incubation and the kinetic assays of SSAO/VAP-1 were performed at 37°C. In the case of MAO B, experiments were carried out at 30°C, as generally reported for monoamine oxidases (Wang and Edmondson 2007; Lühr et al. 2010).

Kinetic analysis and kinetic parameter determinations

Steady state kinetic parameters (V_{max} and K_m) were calculated by fitting an appropriate kinetic equation to the experimental data (initial rate of reactions vs. substrate concentrations), with Sigma Plot software, version 9.0 (Jandel Scientific, San Rafael, CA, USA) or GraphPad 5.0 software.

In particular, for MAO B, the Michaelis–Menten equation was applied to determine the apparent V_{max} and K_m values in the presence of the various polyamines. The mode of inhibition was determined by global fit analysis (GraphPad 5.0 software) of the initial rate of reaction versus substrate concentration curves, in the presence and absence of inhibitor, to fit equations for competitive, mixed, non-competitive and uncompetitive inhibition models. The fit giving the highest r^2 value was selected to calculate the inhibition constant value.

In the case of SSAO/VAP-1, for which inhibition by substrate was found, the reaction scheme 1 developed by Holt et al. (2008) was considered. Briefly, this model takes into account both substrate inhibition and the presence of an inhibitor; it considers the interactions of a single substrate molecule (S) or a single inhibitor molecule (I) with the active site on the free (and oxidized) form of SSAO/VAP-1 (E) and on the reduced form of the enzyme or, in general, with an enzyme–substrate complex (ES). In addition, this model does not exclude the possibility of the residual activity of the (enzyme–substrate)-inhibitor complex (IES). Since in our experimental conditions [(benzylamine) <10 mM], the term "$\alpha \times [S]/K_{IS}$" in Eq. 2, from Holt et al. (2008), was negligible and the following simplified equation was obtained and applied to analyse our kinetic data by Sigma Plot software:

$$v = \frac{V_{maxapp}[S]}{\left(K_{mapp} + [S] + \frac{[S]^2}{\left(K_i + \frac{K_i \times [I]}{K_{IES}}\right)}\right)} \quad (1)$$

In this equation, K_i is the dissociation constant for the ES–S complex.

The best fits of the SSAO/VAP-1 apparent kinetic parameters ($V_{max\ app}$ and $K_{m\ app}$) versus inhibitor concentrations were obtained with the following "classical equations" for mixed non-competitive inhibition (Leidler and Bunting 1973) (for details, see Supplementary materials):

$$\frac{1}{V_{maxapp}} = \frac{1}{V_{max\,0}} \times \left(1 + \frac{[I]}{K_{IES}}\right) \quad (2)$$

$$\frac{K_{mapp}}{V_{maxapp}} = \frac{K_{m0}}{V_{max\,0}} \times \left(1 + \frac{[I]}{K_{IE}}\right). \quad (3)$$

In Eqs. 2 and 3, V_{max0} and K_{m0} are kinetic parameters in the absence of inhibitors (I); K_{IE} and K_{IES} are dissociation constants for the free enzyme–inhibitor complex (EI) and for the (enzyme–substrate)–inhibitor complex (IES), respectively.

The ionic strength effect on SSAO/VAP-1 activity was analysed according to the Debye Hückel equation (Atkins 1986), i.e.:

$$\mathrm{Log}\left(\frac{1}{K_{IE}}\right) = \mathrm{Log}\left(\frac{1}{K_{IE}}\right)_0 + 2 \times C \times Z_a \times Z_b \times I^{\frac{1}{2}} \quad (4)$$

where $(1/K_{IE0})$ is the association constant at $I = 0$, and Z_a and Z_b are the electrostatic charges of the interacting ionic species (involved in the formation of the enzyme–inhibitor complex). Constant C has a value of ≈ 0.509 in water solutions (at 298 K) (Atkins 1986) and slope $2 \times C \times Z_a \times Z_b$ therefore represents the product of the interacting charges, as $2 \times C \approx 1$.

Computational methods

Docking simulation was performed with AutoDock 4.2 software with the Lamarckian genetic algorithm (Morris et al. 1998; Huey et al. 2007). The crystallographic structure of r-SSAO/VAP-1 with Protein Data Bank code 2C10 (Jakobsson et al. 2005) was employed. Polar hydrogens were added with the Molprobity server (Chen et al. 2010). Gasteiger charges were added to the protein and the ligand with ADT version 1.5.4 (Scripps Research Institute, La Jolla, CA, USA). The unbound ligand was assumed to be in the extended conformation, and the corresponding parameter file was used. The number of individuals in the population was 300, the maximum number of energy evaluations was 25,000,000, and the number of runs was 200. The grid had dimension 90 × 90 × 90 points and spacing 0.375 Å, and was centred approximately halfway between the side-chains of Tyr394 and Leu469, covering the entire active site with side-pocket (Nurminen et al. 2010), the channel and the protein surface around the channel entrance. Only the atoms of the ligand were allowed to move during simulations. The rotamers of residues Tyr394 and Leu469 were modified with Coot (Emsley et al. 2010) to the "m-30" and "tp" states (Lovell et al. 2000), respectively (Fig. 5), in order to allow the inhibitor to enter the active site. The rotamers of Met211 and Ser496 were also modified to the "mtm" and "p" states (Lovell et al. 2000) respectively (Fig. 5), to allow

access to the side pocket flanking the active side (Nurminen et al. 2010).

Circular dichroism spectra

CD spectra were obtained on a Jasco J-810 spectrapolarimeter (Jasco Europe Ltd., Cremella (LC), Italy), at 25°C. Recombinant h-SSAO/VAP-1 sample was extensively dialysed against potassium phosphate 5 mM, pH 7.4 (at 4°C), to reduce the amount of Triton X-100 present in the stock solution of the enzyme. The protein concentration of the dialysed sample was determined according to the Bradford method and, on this basis, the residual Triton concentration of the sample was estimated by absorption spectra (220–450 nm). SSAO/VAP-1 samples (0.09 mg/ml in buffer containing 0.0002% of residual Triton X-100), in the presence and absence of ELP 12 300 μM, were placed in a 1-mm quartz cuvette and CD spectra were measured between 260 and 190 nm, with the following set-up: bandwidth = 1 nm, time constant = 0.5 s, scan rate = 100 nm min^{-1}, N_2 purging rate = 10 l min^{-1}. Twenty-five spectra were averaged, corrected for background (buffer and ELP 12) and then smoothed with a 15 point "adaptative smoothing" procedure. The final CD spectra were normalized as $\Delta\varepsilon$ units, i.e., "per residue molar circular-dichroism absorption coefficient".

Analysis of the secondary structure content was performed with the CDSSTR analysis program, available online on the Dichroweb site (http://www.public-1.cryst.bbk.ac.uk/cdweb/html, Compton and Johnson 1986; Sreerama and Woody 2000; Lobley et al. 2002). The protein reference set number 4, containing 43 proteins, was used to perform analysis.

Results and discussion

Selection of some synthetic polyamines as potential amine oxidase inhibitors

Molecular structures of selected polyamines are shown in Table 1. The choice of methoctramine, ELP 12, caproctamine and BVT-128 in the present study was driven by their already investigated activities on different targets involved in AD. The drug design and synthesis of the new compounds BD6 and Bis-Bza-Diado was based on our recent results with SSAO/VAP-1 (Bonaiuto et al. 2010).

The "one-molecule-multiple-targets" paradigm suggests that a single molecule could hit several targets responsible for the onset and/or progression of AD. The design and synthesis of polyamine structure-based MTDLs useful for combating neurodegenerative diseases have been extensively published. In particular, since AD is characterized by a pronounced degradation of the cholinergic system, AChE inhibition together with muscarinic M_2 presynaptic autoreceptor blockade remain the most important strategies for designing new potential anti-AD agents.

The polymethylene tetramine methoctramine is a potent M_2 muscarinic receptor antagonist with a significant AChE inhibitory activity (Melchiorre et al. 1998). The replacement of the inner flexible polymethylene chain of methoctramine with a dipiperidine moiety as in ELP 12 preserves the muscarinic antagonist activity and enhances AChE inhibitory activity in comparison to the lead compound. In addition, in ACh release assays ELP 12 enhances the ACh release in rat parietal cortex caused by muscarinic M_2 presynaptic autoreceptor blockade (Tumiatti et al. 2007). Transforming the inner amine functions of methoctramine into amide groups and inserting methyl groups on its terminal nitrogens as in caproctamine decreases the affinity for muscarinic M_2 receptors but produces an increase in the affinity for AChE. In particular, caproctamine acts as AChE inhibitors (Melchiorre et al. 1998) and antagonizes muscarinic M_2 receptors. The constrained analogue of caproctamine, BVT 128, shows also an additional activity as Aβ aggregation inhibitor (Tumiatti et al. 2008) emerging as a promising MTDLs in AD.

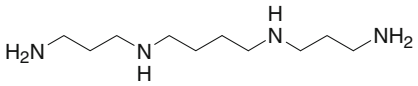
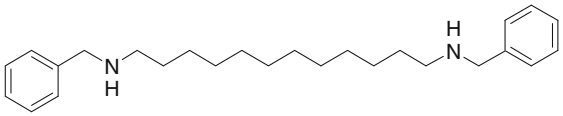
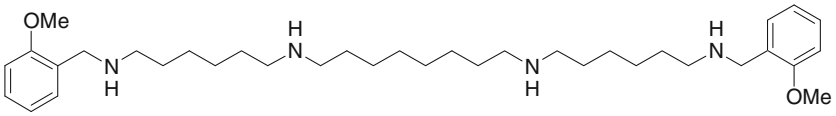
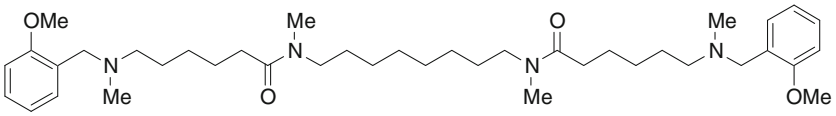
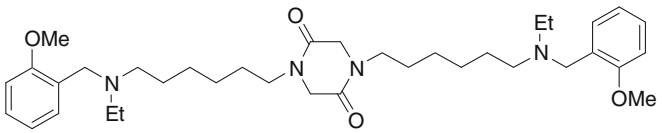
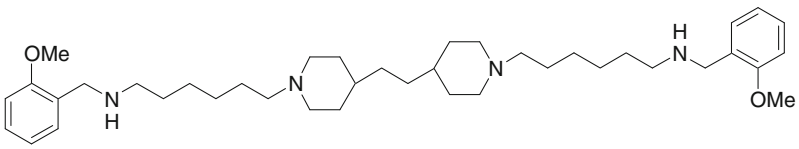
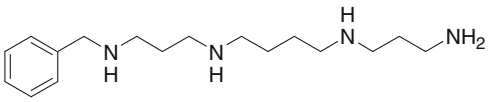
In addition, starting from the very high affinity of human SSAO/VAP-1 for 1,12-diaminedodecane (K_m = 13 μM, Bonaiuto et al. 2010), the reactive amino groups of this substrate were modified by adding benzyl moieties to obtain *N*,*N*′-dibenzyl-dodecane-1,12 diamine (Bis-Bza-Diado). A benzyl moiety was also added to spermine, a physiological polyamine, to obtain BD6. We verified that neither spermine nor BD6 were amine oxidase substrates (0.1–1 mM range concentrations).

To test all these compounds on amine oxidase activity, kinetic parameters V_{max}, K_m and V_{max}/K_m of human SSAO/VAP-1 and of MAO B were determined in the absence and presence of the various polyamines (at fixed 200 μM concentration). Amine oxidase activity measurements were carried out after 15 min of pre-incubation, with benzylamine as substrate. Results are shown in Tables 2 and 3; some examples of initial rate of reaction versus substrate concentration plots are shown in Fig. 1.

Table 2 shows that all the tested polyamines decrease the V_{max} values of SSAO/VAP-1 (examples in Fig. 1a). In particular, the lowest residual V_{max} values of about 60–65% with respect to control were found in the presence of ELP 12, Bis-Bza-Diado and BVT 128, and the lowest V_{max}/K_m values were found with ELP 12, caproctamine and BD6 (about 60–66% relative to control; last column in Table 2).

Unlike SSAO/VAP-1, the main effect of these polyamines on MAO B was on its catalytic efficiency, as shown in Table 3. In particular, Bis-Bza-Diado was the most

Table 1 Chemical structure of synthetic polyamines

Compound	Chemical Structure
Spermine[a]	
N,N'-Dibenzyl-dodecane-1,12-diamine (bis-Bza-Diado)[b]	
Methoctramine[a]	
Caproctamine[c]	
BVT 128[d]	
ELP 12[d]	
BD 6[a]	

[a] Tetrahydrochloride salts

[b] Dihydrochloride salt

[c] Dioxalate salt

[d] Tetraoxalate salts

Table 2 Kinetic parameters of human r-SSAO/VAP-1 in presence of synthetic polyamines

Compound (200 μM)	$V_{max\ app}$ (nmol H_2O_2 min^{-1} mg^{-1})[a]	K_m (μM)	$(V_{max}/K_m)_{app}$ (min^{-1} ml mg^{-1})	V_{max} relative to control	V_{max}/K_m relative to control
Control	212 ± 21[b]	313 ± 19	0.68	1	1
Bis-Bza-Diado	127 ± 8	234 ± 36	0.54	0.60	0.8
Metoctramine	175 ± 14	300 ± 59	0.58	0.82	0.85
ELP 12	126 ± 12	307 ± 41	0.41	0.60	0.60
BVT 128	125 ± 9	260 ± 48	0.48	0.59	0.70
Caproctamine	175 ± 6	396 ± 31.8	0.44	0.82	0.64
Spermine	164 ± 4	206 ± 16	0.70	0.77	1
BD6	169 ± 7	357 ± 37	0.45	0.77	0.66

[a] Kinetic measurements were performed after 15 min pre-incubation, at 37°C, with potential inhibitor; r-SSAO/VAP-1 concentration was 1.4 nM (0.37 μg/ml); benzylamine was used as substrate

[b] This V_{max} value corresponds to a catalytic constant value of 0.96 s^{-1}

Table 3 Kinetic parameters of human r-MAO B in presence of synthetic polyamines

Compound (200 μM)	$V_{max\ app}$ (nmol H_2O_2 min^{-1} mg^{-1})	K_m (μM)	$(V_{max}/K_m)_{app}$ (min^{-1} ml mg^{-1}) × 10^3	V_{max} relative to control	V_{max}/K_m relative to control
Control	5.37 ± 0.10	513 ± 46	10.48	1.00	1.00
Bis-Bza-Diado	4.55 ± 0.96	1,108 ± 98	4.08	0.85	0.39
Metoctramine	5.47 ± 0.13	732 ± 64	7.44	1.01	0.71
ELP 12	4.70 ± 0.10	748 ± 65	6.24	0.88	0.60
BVT 128	4.35 ± 0.10	688 ± 18	6.32	0.81	0.60
Caproctamine	4.20 ± 0.09	728 ± 58	5.76	0.78	0.55
Spermine	5.22 ± 0.11	446 ± 72	11.68	0.97	1.12
BD6	5.20 ± 0.33	681 ± 47	7.44	0.97	0.71

Kinetic measurements were performed after 15 min pre-incubation, at 30°C, with potential inhibitor; r-MAO B concentration was 12.5 μg/ml; benzylamine was used as substrate

efficient compound, with residual V_{max}/K_m of about 39% respect to control, whereas caproctamine, ELP 12 and BVT 128 reduced V_{max}/K_m values to about 55–60% (last column, Table 3). As regards V_{max}, only Bis-Bza-Diado, BVT 128 and caproctamine had slight effects, reducing V_{max} values by about 20% maximum (78–85% of residual V_{max} values).

On the basis of these preliminary results, we focused our kinetic studies on Bis-Bza-Diado and ELP 12: the former because it appears to be the most efficient compound in inhibiting MAO B (giving the lowest residual V_{max}/K_m value) and the latter because it is the most efficient compound in inhibiting SSAO/VAP-1 (reducing both V_{max}/K_m and V_{max} values).

Kinetic characterisation of ELP 12 and Bis-Bza-Diado inhibitory behaviour

At first, the effect of the pre-incubation time of enzymes with inhibitors was evaluated. SSAO/VAP-1 and MAO B were incubated for different times in the presence and absence of 200 μM of ELP 12 or Bis-Bza-Diado. The kinetic parameters of SSAO/VAP-1 were determined after 2, 15, 30, 60 and 120 min of pre-incubation time, and, in the case of MAO B, after 2, 15, 30 and 60 min only, because longer incubation times reduced the MAO activity of the control samples. The inhibitory effect of the compounds on MAO B was not found to be significantly dependent on incubation time: the residual V_{max} and V_{max}/K_m values with respect to control samples were unvaried, within experimental error. Instead, in the case of SSAO/VAP-1, pre-incubation time was found to be an important parameter on kinetic parameters determined in the presence of inhibitors. Figure 2 shows the residual V_{max}/K_m and V_{max} determined in the presence of ELP 12 (relative to control samples) versus time as an example. Figure 2 also shows that relative V_{max}/K_m and V_{max} values (with respect to control samples) reach asymptotic values of (0.28 ± 0.06) and (0.47 ± 0.03), respectively, after an incubation time of at least 30 min. This result suggests that

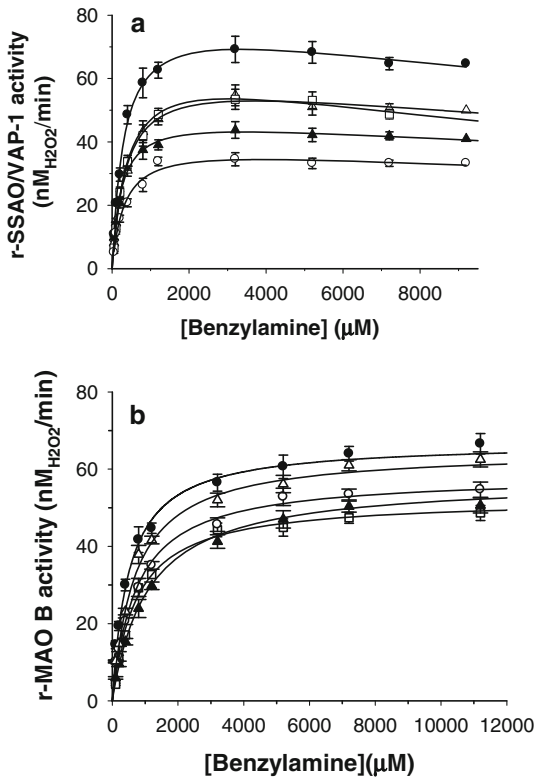


Fig. 1 Effect of some synthetic polyamines on human r-SSAO/VAP-1 and r-MAO B activity Benzylamine oxidase activity was measured in presence of 200 μM of following compounds: (*open square*) caproctamine, (*open circle*) ELP 12, Bis-Bza-Diado (*filled triangle*), BD6 (*open triangle*) and control sample (*filled circle*). Measurements carried out after 15 min pre-incubation with tested compound. **a** SSAO/VAP-1 activity and incubation carried out at 37°C, in presence of 0.37 μg/ml of enzyme. *Continuous lines* results of best fitting of Eq. 1 to experimental data. **b** MAO B activity and incubation carried out at 30°C, in presence of 12.5 μg/ml of enzyme. *Continuous lines* results of best fitting of Michaelis–Menten equation to experimental data

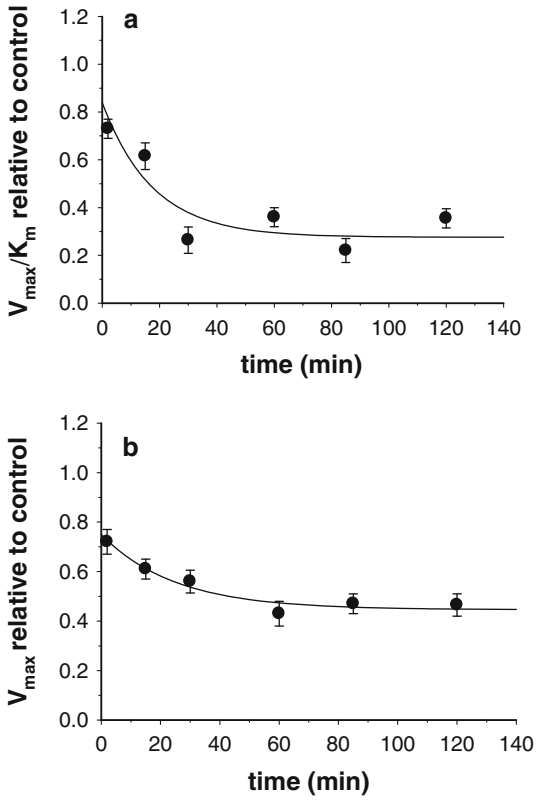


Fig. 2 Effect of pre-incubation time on inhibition of SSAO/VAP-1 by ELP 12. Relative V_{max}/K_m (**a**) and V_{max} (**b**) values of SSAO/VAP-1 with ELP 12 (200 μM), determined after various incubation times, at 37°C

the formation of the enzyme–inhibitor complex needs some "slow" conformational rearrangement of SSAO/VAP-1. On this basis, the inhibition mode of ELP 12 and Bis-Bza-Diado was characterised by performing enzyme activity measurements after 30 min of pre-incubation. The apparent V_{max}, K_m and V_{max}/K_m were calculated, at various polyamine concentrations, fitting the experimental data of the initial reaction rate versus substrate concentration to Eq. 1 (for SSAO/VAP-1) or the Michaelis–Menten equation (for MAO B). The effects of ELP 12 on SSAO/VAP-1 and of Bis-Bza-Diado on MAO B are shown, as examples, in Figs. 3 and 4, respectively. These compounds differently affect both the apparent K_m and V_{max} values. Thus, the apparent kinetic parameters were analysed with various inhibition models, by global fit analysis with GraphPad Prism software 5.0 in the case of MAO B, and according to Eqs. 2–3 in the case of SSAO/VAP-1 (for details of kinetic analysis, see Supplementary materials). The best fits were obtained with the mixed inhibition model. The plots of $(K_m/V_{max})_{app}$ and $(1/V_{max})_{app}$ versus inhibitor concentration shown in Figs. 3 and 4 (panels "b" and "c") clearly show the mixed mode of inhibition; the calculated dissociation constants for binding of the inhibitor to the free enzyme (K_{IE}) and to the enzyme–substrate complex (K_{IES}), respectively, are listed in Table 4.

Table 4 clearly shows that both ELP 12 and Bis-Bza-Diado behave as "mixed" non-competitive inhibitors, since the K_{IE} and K_{IES} values are different: ELP 12 and Bis-Bza-Diado show better affinity for the free enzyme than for the enzyme–substrate complex ($K_{IE} < K_{IES}$). It is also to be noted that the affinity of these compounds for the enzyme–substrate complex of MAO B is lower by a factor of about 4 than that for SSAO/VAP-1 ($K_{IES(MAO)} > K_{IES(VAP)}$). Table 4 also shows that Bis-Bza-Diado is poorly selective: K_{IE} values for MAO B and SSAO/VAP-1 are very similar (K_{IE} about 100 μM) and the selectivity for SSAO/VAP-1, calculated from K_{IES} values, is about 4 ($K_{IES\ (MAO\ B)}/K_{IES(VAP)} \approx 4$). Differently, ELP 12 exhibits a better selectivity than Bis-Bza-Diado for SSAO/VAP-1, being $K_{IE\ (MAO\ B)}/K_{IE(VAP)} \approx 3$ and $K_{IES\ (MAO\ B)}/K_{IES\ (VAP)} \approx 6$.

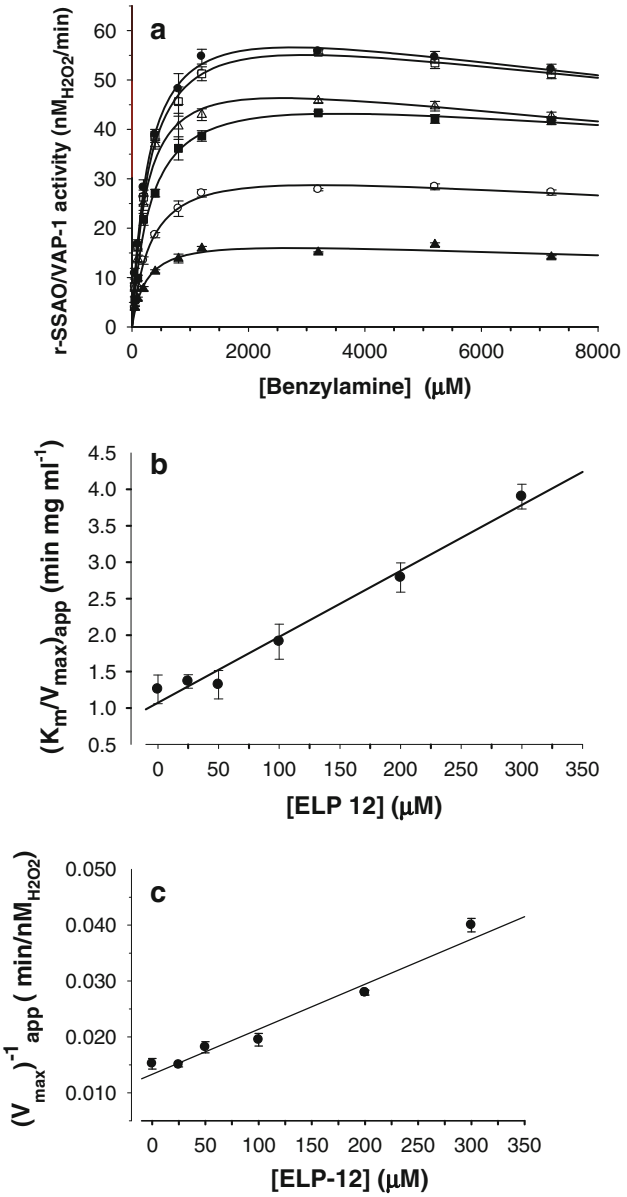


Fig. 3 Inhibition of r-SSAO/VAP-1 activity by ELP 12 (**a**) Benzylamine oxidation assays carried out after 30 min of pre-incubation of r-SSAO/VAP-1 (0.312 μg/ml) at 37°C, with or without ELP 12, at these concentrations: 0 (*filled circle*), 25 (*open square*), 50 (*open triangle*), 100 (*filled squares*), 200 (*open circles*) and 300 (*filled triangles*) μM. *Continuous lines* results of best fitting of Eq. 1 to experimental data. **b**, **c** Plots of apparent K_m/V_{max} and $1/V_{max}$ values versus ELP 12 concentration and best fitting with equations of mixed non-competitive inhibition model (Eqs. 2 and 3)

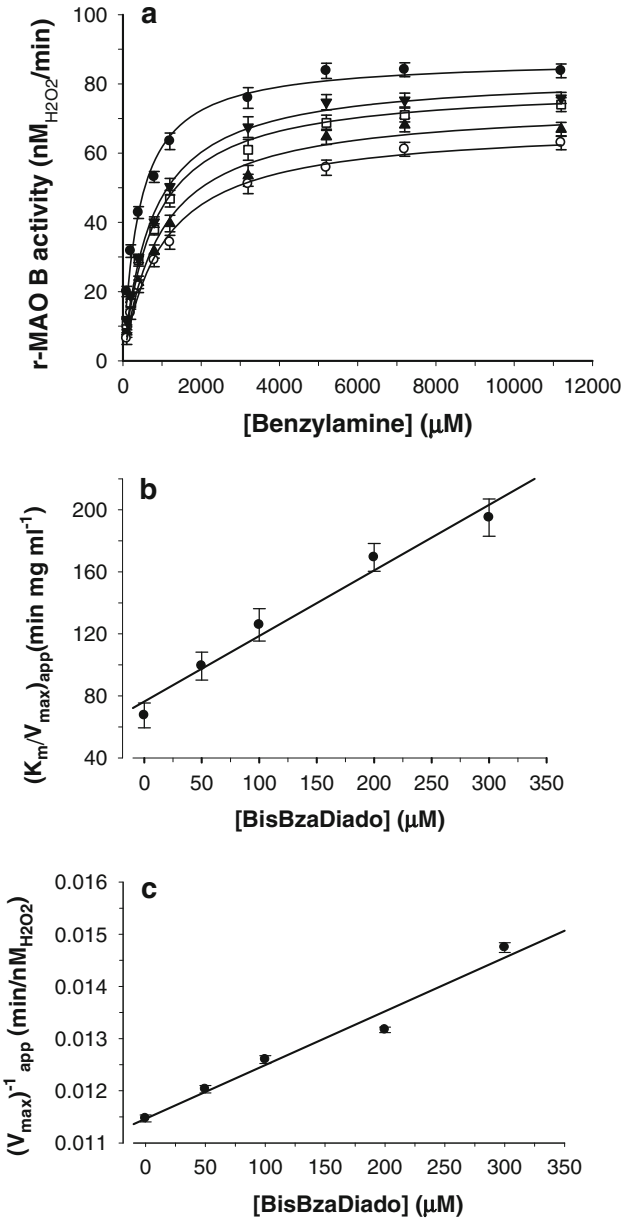


Fig. 4 Inhibition of r-MAO B activity by Bis-Bza-Diado. **a** Benzylamine oxidation assays carried out after 30 min of pre-incubation of r-MAO B (12.5 μg/ml) at 30°C, with or without Bis-Bza-Diado, at these concentrations: 0 (*filled circles*), 50 (*filled inverted triangles*), 100 (*open squares*), 200 (*filled triangles*) and 300 (*open circles*) μM. *Continuous lines* best fitting of Michaelis–Menten equation to experimental data, by nonlinear regression analysis (global fit analysis according to mixed non-competitive inhibition model, by GraphPad 5.0 software). **b**, **c** Plots of apparent K_m/V_{max} and $1/V_{max}$ values versus Bis-Bza-Diado concentration and best fitting with equations of mixed non-competitive inhibition model

Additionally, we found that these polyamines behave as "reversible" inhibitors, because the amine oxidase activity was completely restored after removal of the inhibitor from enzyme–polyamine complexes (prepared at 300 μM of polyamine concentration), by dilution (data not shown).

On the basis of the above results, we conclude that the reversible binding of these long polyamines to SSAO/VAP-1 and MAO B affects their catalytic steps by impeding substrate entry and/or product release. The effects on apparent V_{max}/K_m consequently depend on changes in the substrate binding site of the enzyme, which would directly result from enzyme–inhibitor interaction.

Table 4 Inhibition constants of Bis-Bza-Diado and ELP 12 for human r-SSAO/VAP-1 and r-MAO B determined after 30 min pre-incubation with compound

Compound	r-SSAO/VAP-1		r-MAO B	
	$K_{i_{EI}}$ (μM)[a]	$K_{i_{ESI}}$ (μM)[b]	$K_{i_{EI}}$ (μM)[a,c]	$K_{i_{ESI}}$ (μM)[b,c]
Bis-Bza-Diado	97 ± 31	271 ± 39	129 ± 20	1,048 ± 151
ELP 12	111 ± 13	163 ± 45	323 ± 54	1,050 ± 200

[a] From $(K_m/V_{max})_{app}$ versus inhibitor concentration plots ($n = 3$ determinations)

[b] From $(1/V_{max})_{app}$ versus inhibitor concentration plots ($n = 3$ determinations)

[c] Same inhibition constant values were found by global fit analysis with GraphPad Prism software 5.0

ELP 12 and SSAO/VAP-1: effect of ionic strength and substrate structure

On the basis of the higher specificity of ELP 12 than that of Bis-Bza-Diado for SSAO/VAP-1, we focused on obtaining information on the binding mode of the former to SSAO/VAP-1. The K_{IE} and K_{IES} values of ELP 12 for SSAO/VAP-1 were determined at different ionic strengths (I) (44–210 mM). A linear dependence of the logarithm of ELP 12-SSAO/VAP-1 association constant ($1/K_{IE}$) on $I^{1/2}$ was found and a slope $(2 \times C \times Z_a \times Z_b) = -1.8$ was calculated according to Eq. 4 (data not shown). No significant effect of ionic strength was found on K_{IES} values (slope ≈ 0.08). According to the Debye Hückel theory (Atkins 1986), we deduce that the formation of the enzyme–inhibitor complex depends on ionic interactions between positive charges of ELP 12 (Z_a) and negative charges of SSAO/VAP-1 (Z_b) and that the product of interacting charges ($Z_a \times Z_b$) is about −1.8.

In addition, for information about the inhibitory effect of ELP 12 on substrate structure, we used two substrates with different steric hindrance, benzylamine and the "short" methylamine. We found that the relative V_{max}/K_m values (with respect to control samples) differed in the two substrates, as shown in Table 5. In particular, ELP 12 was less effective in the presence of the smallest substrate (methylamine), residual activity being about twice higher than that measured with benzylamine. No differences between the residual V_{max} values of SSAO/VAP-1 for the two substrates were found. These results suggest that, when ELP 12 is bound to free SSAO/VAP-1, a small substrate may reach the enzyme cofactor more easily than a larger one.

ELP 12 and SSAO/VAP-1: recombinant enzyme versus "native" enzyme

The inhibitory behaviour of ELP 12 was tested on the human "native enzyme". Human adipocyte membranes were used as a source of "native" SSAO/VAP-1 and inhibition experiments were performed in the same experimental conditions reported for the recombinant enzyme. We found that, also with the "native" enzyme, pre-incubation time is important: the maximum inhibitory effect was reached after about 2 h of incubation. Also in the case of the "native" SSAO/VAP-1, ELP 12 showed mixed non-competitive behaviour: $K_{IE} = 95$ μM and $K_{IES} = 248$ μM were calculated. These data match the inhibition constant values found with the recombinant enzyme very well.

ELP 12 and SSAO/VAP-1: docking studies

The search for possible binding sites of ELP 12 was focused on the region around the active site and around the channel conducting to it. It seems reasonable to assume that the inhibitor is attracted preferentially towards this region of the enzyme, because ELP 12 is highly charged at physiological pH, like the amine substrates. The most stable binding mode found by AutoDock showed the inhibitor ELP 12 bound on the protein surface at the channel entrance, about 10 Å from TPQ cofactor (Fig. 5).

Table 5 Effect of substrate structure on inhibition of r-SSAO/VAP-1 activity by ELP 12

Substrate	V_{max} (nmoli H_2O_2 min^{-1} mg^{-1})[a]		V_{max}/K_m (min^{-1} ml mg^{-1})[a]		V_{max} (% with respect to control)	V_{max}/K_m (% with respect to control)
	Control	ELP 12	Control	ELP 12		
Methylamine	91 ± 3	45 ± 5	0.054 ± 0.002	0.026 ± 0.001	49 ± 3	49 ± 1
Bza	223 ± 15	109 ± 12	1.052 ± 0.150	0.305 ± 0.035	48 ± 5	28 ± 3

[a] Kinetic experiments were carried out after 30 min pre-incubation, in presence of 200 μM ELP 12, at 37°C

The estimated free energy of binding was −14.3 kcal/mol. The amino groups of ELP 12 form salt bridges with the carboxylates of Asp180 and Asp446 and hydrogen bonds with the side-chain of Tyr448 and with the backbone carbonyl of Thr424 (yellow pose in Fig. 5a). An aromatic ring of ELP 12 is stacked to the ring of Tyr176, and various hydrophobic residues contribute to stabilize the central rings and the long carbon chain: Phe173, Tyr394, Pro397, Ile425, and Leu447. Since we had modified the rotamers of Leu469 and Tyr394 to unblock access to the cofactor (see "Materials and methods"), AutoDock also found a pose of ELP 12 partially inside the active site (green pose in Fig. 5a). One of the aromatic rings is about 5 Å from TPQ, sandwiched between the side-chains of Leu469 and Phe389. One amino group of the ligand is stabilized by a hydrogen bond with the side-chain of Tyr394. As in the most stable pose, the other amino groups are stabilised by the interaction with the backbone of Thr424 and with the side-chain of Asp446, and the hydrophobic residues at the channel entrance stabilise the carbon chain and the central rings of the inhibitor. However, the estimated free energy of binding was −12.6 kcal/mol, and this binding mode is therefore predicted to be less stable than the inhibitor bound at the channel entrance. Other binding modes found by AutoDock, with the inhibitor occupying the active site, were estimated to be even less stable. For example, ELP 12 can enter the active site, get in contact with the TPQ and also occupy with an aromatic ring the side pocket flanking the active site recently reported (Nurminen et al. 2010), but its estimated binding energy was only −11.2 kcal/mol.

These docking results indicate that ELP 12 binds both at the entrance of the active site channel and may partially enter the channel: in all cases, the presence of ELP 12 may partially or totally occlude the channel in such a way that it impedes the amine substrate entry and/or product release.

These docking results match the kinetic data. In particular, the possible binding poses of ELP 12 explain the reduction in inhibitory effect of ELP 12 in the presence of the small substrate (methylamine), which can reach the TPQ cofactor more easily, thanks to its low steric hindrance. The role of aspartic residues in the binding of ELP 12 also matches the effect of ionic strength on K_{IE} for ELP 12, which indicates the presence of one or two negative charges on the enzyme involved in binding the inhibitor.

In addition, the docking poses indicate that, apart from the aspartic residues involved in ionic interactions with ELP 12, other residues may contribute to inhibitor binding by various types of interactions (apolar interactions with the hydrophobic groups of ELP 12 and hydrogen bonds). The important role of apolar interactions had already been reported in the binding of a long aliphatic substrate, 1,12-diaminododecane, to SSAO/VAP-1 (Bonaiuto et al. 2010) and it is reasonable to presume that such interactions also play a role in the case of apolar Bis-Bza-Diado or other types of SSAO/VAP-1 activity modulators.

Circular dichroism of SSAO/VAP-1 in the presence of ELP 12

To understand the possible effect of ELP 12 on the secondary structure of SSAO/VAP-1, UV CD spectra of the enzyme were obtained in the absence and presence of ELP 12 300 μM, after an incubation time of 120 min. At this ELP 12 concentration, the enzyme is predominantly present as an enzyme–inhibitor complex.

Figure 6 shows that the secondary structure of the native enzyme is affected by ELP 12 and that the overall lineshapes of the CD spectra fall within the range of α/β proteins. In particular, quantitative analysis of secondary structure contents revealed that SSAO/VAP-1 bound to ELP 12 is characterised by a higher β strand contents than the native enzyme (36% in comparison with 32%) and lower contents of α-helixes and turns, which fall from 10 to 8% and from 26 to 24%, respectively.

Similar effects on β strands and α-helix contents caused by binding of an inhibitor have already been reported for bovine serum amine oxidase (Di Paolo et al. 2007) and pig kidney amine oxidase (Padiglia et al. 2001).

Our results suggest that the binding of ELP 12, a long molecule, containing two "large" intra-chain piperidine moieties, may induce structural rearrangements of the secondary structure of SSAO/VAP-1; they also suggest that SSAO/VAP-1 has a flexible, "mobile" secondary structure which can adapt itself to molecules interacting with its active site, as already suggested by molecular dynamic studies performed on SSAO/VAP-1 (Nurminen et al. 2010).

Conclusion and perspectives

In this study, some synthetic polyamines were evaluated as inhibitors of human SSAO/VAP-1 and MAO B activity. The main findings are that these compounds can act as a new type of reversible, mixed non-competitive inhibitors, for both SSAO/VAP-1 and MAO B. To the best of our knowledge, this is the first study in the field of polyamines and human SSAO/VAP-1 and MAO B. Until now, only the effect of some polyamine analogues (1,8-diaminooctane, 1,12-diaminododecane, *N*-prenylagmatine, guazatine and *N*,*N*1-bis(2,3-butadienyl)-1,4-butanediamine, known as MDL72527) on murine polyamine oxidases and on spermine oxidase have been reported (Bianchi et al. 2006).

In particular, ELP 12, a muscarinic cholinergic M_2 receptor antagonist, is more specific for SSAO/VAP-1 than for MAO B, whereas Bis-Bza-Diado, a newly synthesised

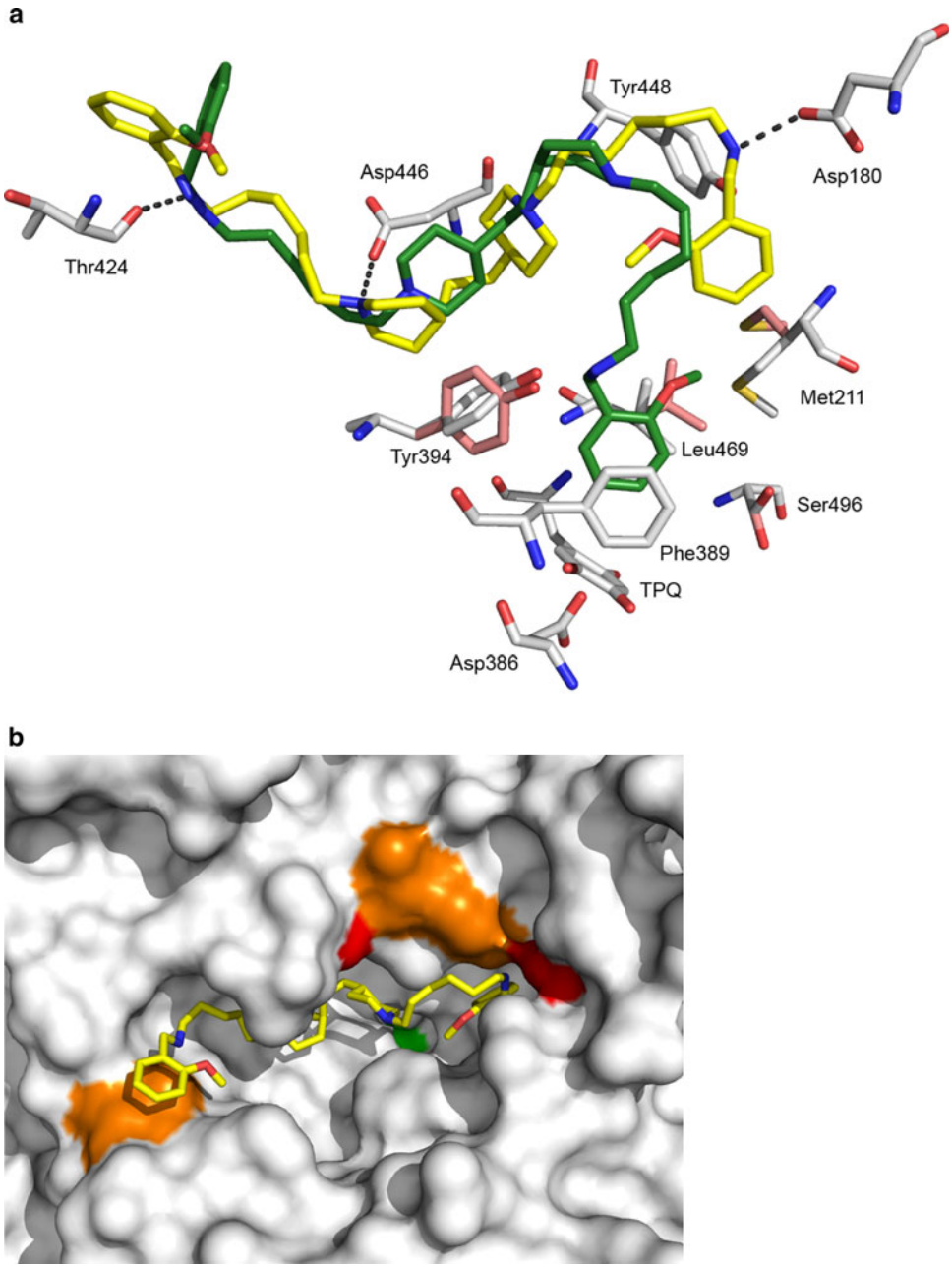


Fig. 5 Predicted binding modes of ELP 12 to human SSAO/VAP-1. **a** *Yellow* most stable pose of ELP 12. *Grey* some residues lining active site and those involved in hydrogen bonds or salt bridges with ligand. *Dotted lines* polar contacts between inhibitor and protein. *Green* most stable pose of inhibitor partially inside active site. *Pink* side-chains of residues modified to grant access to active site and side pocket (see text for more detailed description). **b** View of surface of active site entrance with ELP 12 bound in most stable pose (*yellow* in **a**). Residues involved in polar contacts shown in red (Asp180 and Asp446 and hydrogen bonds with backbone carbonyl of Thr424). Residues involved in salt bridges and hydrogens bonds with inhibitor are shown in red and orange, respectively (Asp180, Asp446, Tyr448 and Thr424). *Green* visible surface of the active site

compound, may inhibit both enzymes, with similar K_{IE} values. The inhibition constant values of ELP 12 and Bis-Bza-Diado are not very low (about 100 μM), but it should be noted that potent, reversible, specific inhibitors of human SSAO/VAP-1 are still subject of much research. The known reversible inhibitors of SSAO/VAP-1 do have dissociation constants in the mid-μM range and lack of selectivity, e.g., guanabenz (Holt et al. 2008). Conversely, for MAO B, various reversible inhibitors with IC_{50} at submicromolar concentration have been already developed (see, e.g., Lühr et al. 2010).

Our kinetic and structural studies showed that ELP 12 may bind at the entrance of the SSAO/VAP-1 active site channel, interacting by ionic interactions with aspartic residues (ASP446 and ASP180), and that one end of ELP 12 may even be accommodated inside the channel reaching the TPQ area. Binding of ELP 12 induces rearrangement of the secondary structure of the enzyme impeding substrate entry and catalysis. This binding mode is quite different from that of other SSAO/VAP-1 inhibitors (most of which are irreversible, targeting the TPQ cofactor) and indicates that the entrance and this first part of the SSAO/VAP-1 channel may be considered as a new target area or a "secondary binding site" for modulators of human SSAO/VAP-1 activity. A "secondary binding site" for modulators of amine oxidase have already been reported for bovine

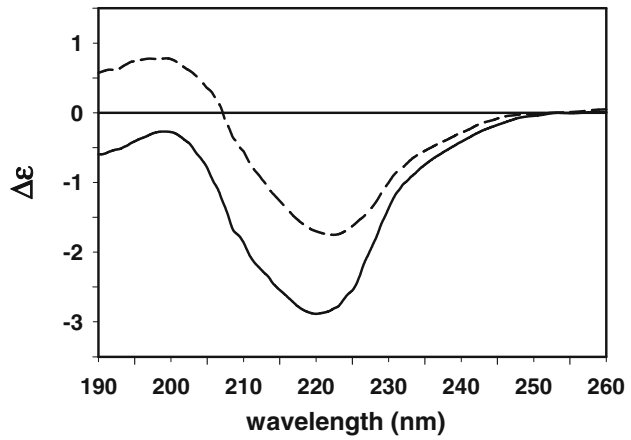


Fig. 6 Effect of ELP 12 on r-SSAO/VAP-1 secondary structure. Continuous line: UV-CD spectra of free enzyme; dashed line: enzyme after incubation with 300 μM ELP 12. Spectra measured in 5 mM potassium phosphate buffer, pH 7.4, normalized to Δε units by standard procedure

serum amine oxidase (Di Paolo et al. 2004; Holt et al. 2008) and for MAO B (an imidazoline binding site distinct from the substrate-binding cavity; Bonivento et al. 2010), but no data have been reported so far for the human enzyme. Thus, this finding may be important and useful for the development of novel human SSOA/VAP-1 inhibitors.

Further studies will be necessary to improve both the affinity and specificity of ELP 12 and Bis-Bza-Diado, by adding more specific moieties and/or by specific substitution, and also to evaluate their specificity versus other human amine oxidases and polyamine oxidases.

At present, we conclude that ELP 12 and Bis-Bza-Diado may be considered as new "skeletons" for developing a novel type of reversible, mixed non-competitive inhibitor of SSAO/VAP-1 and MAO B, two pharmacological targets in various disorders, such as inflammatory and neurodegenerative diseases.

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Conflict of interest The authors declare no conflict of interest.

References

Atkins PW (1986) Equilibrium electrochemistry: ions and electrodes. In physical chemistry, 3rd edn. Oxford University Press, Oxford, pp 242–243

Bianchi M, Polticelli F, Ascenzi P, Botta M, Federico R, Mariottini P, Cona A (2006) Inhibition of polyamine and spermine oxidases by polyamine analogues. FEBS J 273:1115–1123. doi:10.1111/j.1742-4658.2006.05137.x

Binda C, Wang J, Pisani L, Caccia C, Carotti A, Salvati P, Edmondson DE, Mattevi A (2007) Structures of human monoamine oxidase B complexes with selective noncovalent inhibitors: safinamide and coumarin analogs. J Med Chem 50:5848–5852. doi:10.1021/jm070677y

Bonaiuto E, Lunelli M, Scarpa M, Vettor R, Milan G, Di Paolo ML (2010) A structure- activity study to identify novel and efficient substrates of the human semicarbazide-sensitive amine oxidase/VAP-1 enzyme. Biochimie 92:858–868. doi:10.1016/j.biochi.2010.03.006

Bonivento D, Milczek EM, McDonald GR, Binda C, Holt A, Edmondson DE, Mattevi A (2010) Potentiation of ligand binding through cooperative effects in monoamine oxidase B. J Biol Chem 285:36849–36856. doi:10.1074/jbc.M110.169482

Boomsma F, Bhaggoe UM, van der Houwen AMB, van den Meiracker AH (2003) Plasma semicarbazide-sensitive amine oxidase in human (patho)physiology. Biochim Biophys Acta 1647:48–54. doi:10.1016/51570-9639(03)00047-5

Bortolato M, Chen K, Shih JC (2008) Monoamine oxidase inactivation: from pathophysiology to therapeutics. Adv Drug Deliv Rev 60:1527–1533. doi:10.1016/j.addr.2008.06.002

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Carreiras MC, Marco JL (2004) Recent approaches to novel anti-Alzheimer therapy. Curr Pharm Des 10:3167–3175

Casero RA, Woster PM (2009) Recent advances in the development of polyamine analogues as antitumor agents. J Med Chem 52:4551–4573. doi:10.1021/jm900187v

Cavalli A, Bolognesi ML, Minarini A, Rosini M, Tumiatti V, Recanatini M, Melchiorre C (2008) Multi-target-directed ligand to combact neurodegenerative diseases. J Med Chem 51:347–372. doi:10.1021/jm7009364

Chen VB, Arendall WB 3rd, Headd JJ, Keedy DA, Immormino RM, Kapral GJ, Murray LW, Richardson JS, Richardson DC (2010) MolProbity: all-atom structure validation for macromolecular crystallography. Acta Crystallogr D66:12–21. doi:10.1107/S0907444909042073

Clauzel LM, Font FY, Vicente SG, Exposito MR, Palomera FA, Olarte AZ (2007) Carboxamides and related compounds for inhibiting copper-containing amine oxidases and their preparation, pharmaceutical compositions and use in the treatment of diseases US. Pat Appl Publ US 20070066646 A1 20070322

Cohen RA, Tong XY (2010) Vascular oxidative stress: the common link in hypertensive and diabetic vascular disease. J Cardiovasc Pharmacol 55:308–316

Compton LA, Johnson WC Jr (1986) Analysis of protein circular dichroism spectra for secondary structure using a simple matrix multiplication. Anal Biochem 155:155–167. doi:10.1016/0003-2697(86)90241

Di Paolo ML, Lunelli M, Scarpa M, Rigo A (2004) Phosphonium compounds as new and specific inhibitors of bovine serum amine oxidase. Biochem J 384:551–558. doi:10.1042/BJ20031883

Di Paolo ML, Pesce C, Lunelli M, Scarpa M, Rigo A (2007) *N*-alkanamines as substrates to probe the hydrophobic region of bovine serum amine oxidase: a kinetic and spectroscopic study. Arch Biochem Biophys 465:50–60. doi:10.1016/j.abb.2007.04.031

Dunkel P, Gelain A, Barlocco D, Haider N, Gyires K, Perlagh B, Magyar K, Maccioni E, Fadda A, Matyus P (2008) Semicarbazide-sensitive amine oxidase/vascular adhesion protein 1: recent developments concerning substrates and inhibitors of a

promising therapeutic target. Curr Med Chem 15:1827–1839. doi:0929-8673/08

Edmonsons DE, Binda C, Wang J, Upadyay AK, Mattevi A (2009) Molecular and mechanistic properties of mitochondrial monoamine oxidases. Biochemistry 48:4220–4230. doi:10.1021/bi900413g

Emsley P, Lohkamp B, Scott WG, Cowtan K (2010) Features and development of coot. Acta Crystallogr D66:486–501. doi:10.1107/S0907444910007493

Ferrer I, Lizcano JM, Hernández M, Unzeta M (2002) Overexpression of semicarbazide sensitive amine oxidase in the cerebral blood vessels in patients with Alzheimer's disease and cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy. Neurosci Lett 321:21–24. doi:10.1016/S0304-3940(01)02465-X

Gubisne-Haberle D, Hill W, Kazachkov M, Richardson JS, Yu PH (2004) Protein cross-linkage induced by formaldehyde derived from semicarbazide-sensitive amine oxidase-mediated deamination of methylamine. J Pharmacol Exp Ther 310:1125–1132

Haider N, Hochholdinger I, Matyus O, Wobus A (2010) Synthesis of *ortho*-functionalized 4-Aminomethylpyridazines as substrate-like semicarbazide-sensitive amine oxidase inhibitors. Chem Pharm Bull 58:964–970. doi:10.1248/cpb.58.964

Holt A, Degenhardt OS, Berry PD, Kapty JJ, Mithani S, Smith DJ, Di Paolo ML (2007) The effects of buffer cations on interactions between mammalian copper-containing amine oxidases and their substrates. J Neural Transm 114:733–741. doi:10.1007/s00702-007-0680-1

Holt A, Smith DJ, Cendron L, Zanotti G, Rigo A, Di Paolo ML (2008) Multiple binding sites for substrates and modulators of semicarbazide-sensitive amine oxidases: kinetic consequences. Mol Pharm 73:525–538. doi:10.1124/mol.107.040964

Huey R, Morris GM, Olson AJ, Goodsell DS (2007) A semiempirical free energy force field with charge-based desolvation. J Comput Chem 28:1145–1152. doi:10.1002/jcc.20634

Jakobsson E, Nilsson J, Ogg D, Kleywegt GJ (2005) Structure of human semicarbazide-sensitive amine oxidase/vascular adhesion protein-1. Acta Crystallogr D61:1550–1562. doi:10.1107/s1744309105002678

Jalkanen S, Salmi M (2001) Cell surface monoamine oxidases: enzymes in search of a function. EMBO J 20:3893–3901. doi:10.1093/emboj/20.15.3893

Jalkanen S, Salmi M (2008) VAP-1 and CD73, endothelial cell surface enzymes in leukocyte extravasation. Arterioscler Thromb Vasc Biol 28:18–26. doi:10.1161/ATVBAHA.107.153130

Kinemuchi H, Sugimoto H, Obata T, Satoh N, Ueda S (2004) Selective inhibitors of membrane-bound semicarbazide-sensitive amine oxidase (SSAO) activity in mammalian tissues. Neurotoxicology 25:325–335. doi:10.1016/S0161-813X(03)00118-9

Leidler K, Bunting P (1973) The chemical kinetics of enzyme action, 2nd edn. Clarendon Press, Oxford, pp 89–138

Lobley A, Withmore L, Wallace BA (2002) DICHROWEB: an interative Website for the analysis of protein secondary structure from Circular Dichroism Data. Bioinformatics 18:211–212

Lovell SC, Word JM, Richardson JS, Richardson DC (2000) The penultimate rotamer library. Proteins 40:389–408. doi:10.1002/1097-0134(20000815)

Lühr S, Vilches-Herrera M, Fierro A, Ramsay RR, Edmonson DE, Reyes-Parada M, Cassels BK, Iturriaga-Vasquez P (2010) 2-Arylthiomorpholine derivatives as potent and selective monoamine oxidase B inhibitors. Bioinorg Med Chem 18:1388–1395. doi:10.1016/j.bmc.2010.01.029

Melchiorre C, Andrisano M, Bolognesi ML, Budriesi R, Cavalli A, Cavrini V, Rosini M, Tumiatti V, Recanatini M (1998) Acetylcholinesterase noncovalent inhibitors based on a polyamine backbone for potential use against Alzheimer's disease. J Med Chem 41:4186–4189. doi:10.1021/jm9810542

Melchiorre C, Bolognesi ML, Minarini A, Rosini M, Tumiatti V (2010) Polyamines in drugs discovery: from the universal template approach to the multitarget-directed ligand design strategy. J Med Chem doi:10.1021/jm100293f

Minarini A, Quaglia W, Tumiatti V, Melchiorre C (1989) An improved synthesis of *N*,*N*′-Bis[6-[[(2-methoxyphenyl) methyl]amino]hexyl]-1,8octanediamine (Methoctramine). Chem Ind 652–653

Minarini A, Milelli A, Tumiatti V, Rosini M, Bolognesi ML, Melchiorre C (2010) Synthetic polyamines: an overview of their multiple biological activities. Amino Acids 38:383–392. doi:10.1007/s00726-009-0430-9

Morris GM, Goodsell DS, Halliday RS, Huey R, Hart WE, Belew RK, Olson AJ (1998) Automated docking using a Lamarckian genetic algorithm and empirical binding free energy function. J Comput Chem 19:1639–1662

Mutsuhiko Y, Yasuhara H (2004) Clinical pharmacology of MAO inhibitors: safety and future. Neurotoxicol 25:215–221. doi:10.1016/S0161-813X(03)00097-4

Nurminen EM, Pihlavisto M, Lázár L, Szakonyi Z, Putikäinen U, Fülöp F, Putikäinen OT (2010) Synthesis, in vitro activity, and three-dimensional quantitative structure-activity relationship of novel hydrazine inhibitors of human vascular adhesion protein-1. J Med Chem 53:6301–6315. doi:10.1021/jm100337z

Olarte AZ, Mian A, Clauzel LM, Exposito MR, Fonte FY, Palomera FA (2006) Compounds for inhibiting copper-containing amine oxidases and their use in inflammatory disease. Int Appl WO 2006013209, A2 20060209

Padiglia A, Medda R, Lorrai A, Paci A, Pedersen JZ, Boffi A, Bellelli A, Finazzi Agro' A, Floris G (2001) Irreversible inhibition of pig kidney copper-containing amine oxidase by sodium and lithium ions. Eur J Biochem 268:4686–4697. doi:10.1046/j.1432-1327.2001.02390.x

Prins LH, Petzer JP, Malan SF (2010) Inhibition of monoamine oxidase by indole and benzofuran derivatives. Eur J Med Chem 45:4458–4466. doi:10.1016/j.ejmech.2010.07.005

Sahoo A, Yobanoglu S, Sinha BN, Ucar G, Basu A, Jayaparkash V (2010) Towards development of selective and reversibile pyrazoline based MAO-inhibitors: synthesis, biological evaluation and docking studies. Bioorg Med Chem Lett 20:132–136. doi:10.1016/j.bmcl.2009.11.015

Smith DJ, Fulop F, Pihlavisto M, Lazar L, Alaranta S, Vainio PJ, Szakonyi ZS (2003) Carbocyclic hydrazino inhibitors of copper-containing amine oxidases. Int Pat Appl WO 03/006003 A1; Chem Abstr 138, 57890

Sreerama N, Woody RW (2000) Estimation of protein secondary structure from CD spectra: comparison of CONTIN, SELCON and CDSSTR methods with an expanded reference set. Anal Biochem 287:252–260. doi:10.1006/abio.2000.4880

Tumiatti V, Minarini A, Milelli A, Rosini M, Buccioni M, Marucci G, Ghelardini C, Bellucci C, Melchiorre C (2007) Structure-activity relationships of methoctramine-related polyamines as muscarinic antagonist: effect of replacing the inner polymethylene chain with cyclic moieties. Bioorg Med Chem 15:2312–2321. doi:10.1016/j.bmc.2007.01.022

Tumiatti V, Milelli A, Minarini A, Rosini M, Bolognesi ML, Micco M, Andrisano V, Bartolini M, Mancini F, Recanatini M, Cavalli A, Melchiorre C (2008) Structure-activity relationships of acetylcholinesterase noncovalent inhibitors based on a polyamine backbone. 4. Further investigation on the inner spacer. J Med Chem 5:7308–7312. doi:10.1021/jm8009684

Unzeta M, Solè M, Boada M, Hernandez M (2007) Semicarbazide-sensitive amine oxidase (SSAO) and its possible contribution to

vascular damage in Alzheimer's disease. J Neural Transm 114:857–862. doi:10.1007/s00702.007.07010

Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J (2007) Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol 39:44–84

Vavilova T, Ostrovskaya I, Axenova L, Buneeva O, Medvedev A (2009) Monoamine oxidase and semicarbazide sensitive amine oxidase activities in normal and inflamed human dental pulp. Med Sci Monit 15:BR289–BR292

Wang J, Edmondson DE (2007) Do monomeric vs dimeric forms of MAO-A make a difference? A direct comparison of the catalytic properties of rat and human MAO-A's. J Neural Transm 114:721–724. doi:10.1007/s00702-007-0678-8

Wang EY, Gao A, Salter-Cid L, Zhang J, Huang L, Podar EM, Miller A, Zhao J, O'Rourke A, Linnik MD (2006) Design, synthesis and biological evaluation of semicarbazide-sensitive amine oxidase (SSAO) inhibitors with anti-inflammatory activity. J Med Chem 49:2166–2173. doi:10.1021/jm050538l

Wellendorph P, Jaroszewski JW, Hansen SH, Franzyk H (2003) A sequential high-yielding large-scale solution-method for synthesis of philanthotoxin analogues. Eur J Med Chem 38:117–122. doi:10.1016/S0223-5234(02)00003-X

Youdim MB (2006) The path from anti Parkinson drug selegiline and rasagiline to multifunctional neuroprotective anti Alzheimer drugs ladostigil and m30. Curr Alzheimer Res 3:541–550

Youdim MB, Buccafusco JJ (2005) CNS targets for multifunctional drugs in the treatment of Alzheimer's and Parkinson's diseases. J Neural Transm 112:519–537. doi:10.1007/s00702-004-0214-z

Yu PH, Wright S, Fan EH, Lun ZR, Gubisne-Harberle D (2003) Physiological and pathological implications of semicarbazide-sensitive amine oxidase. Biochim Biophys Acta 11(1647): 193–199

Zhou M, Panchuck-Voloshina NA (1997) A one-step fluorimetric method for the continuous measurement of monoamine oxidase activity. Anal Biochem 253:169–174. doi:10.1006/abio.1997.2392