Medical implications from the crystal structure of a copper-containing amine oxidase complexed with the antidepressant drug tranylcypromine

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Abstract The X-ray crystal structure of the copper-containing quinoprotein amine oxidase from *E. coli* has been determined in complex with the antidepressant drug tranylcypromine to 2.4 Å resolution. The drug is a racemic mix of two enantiomers, but only one is seen bound to the enzyme. The other enantiomer is not acting as a substrate for the enzyme as no catalytic activity was detected when the enzyme was initially exposed to the drug. The inhibition of human copper amine oxidases could be a source of side-effects in its use as an antidepressant to inhibit the flavin-containing monoamine oxidases in the brain.

Keywords: Amine oxidase; Copper metalloprotein; Quinoprotein; Tranylcypromine; X-ray crystallography

1. Introduction

Tranylcypromine (TCP) is used in the treatment of depression (brand name, Parnate (SmithKline Beecham)). TCP belongs to the class of antidepressants that inhibit the flavin-containing monoamine oxidases found in the brain (MAOIs). These drugs have been used clinically for over 30 years, along with the tricyclic antidepressants (TCAs). Multiple side-effects have been recorded, and this led to the development of new classes of antidepressants, for example the serotonin reuptake inhibitors [1]. However, these new drugs are less effective and many people who do not respond to the newer drugs can be successfully treated with MAOIs or TCAs. TCP is therefore still used to treat major depression in patients where the side-effects can be closely supervised.

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Abbreviations: TCP, tranylcypromine; MAOI, flavin-containing monoamine oxidase inhibitor; TCA, tricyclic antidepressant; CuAO, copper-containing amine oxidase; HuPAO, human plasma amine oxidase; AGE, advanced glycation endproduct; TPQ, 2,4,5-trihydroxyphenylalanine quinone; ECAO, *Escherichia coli* amine oxidase; 2-HP, 2-hydrazinopyridine

TCP is a reversible inhibitor of the flavin monoamine oxidases, and is administered as a racemic mixture of the two *trans* enantiomers, 1*S*,2*R*-(+)-*trans*-2-phenylcyclopropylamine ((+)-TCP) and 1*R*,2*S*-(−)-*trans*-2-phenylcyclopropylamine ((−)-TCP) (Fig. 1(a)) [2]. Differences in the efficacy and side-effects of administering the two enantiomers separately have been reported, with (+)-TCP being ten times more potent a MAOI, and (−)-TCP exhibiting a 3–4 times greater inhibition of catecholamine uptake [3,4]. The racemic TCP has also been shown to inhibit the copper-containing amine oxidases (CuAOs), for which there are three homologues in human tissues [5]. Interest in inhibitors of CuAOs has increased recently due to the observation that the elevated levels of human plasma amine oxidase (HuPAO) observed in diabetes mellitus patients [6] correlate to the degree of late-diabetic vascular disease [7,8]. Aldehydes resulting from HuPAO activity lead to advanced glycation endproducts (AGEs) [9], the major cause of late-diabetic complications, including neuropathy, nephropathy and retinopathy [10,11]. Additionally, the hydrogen peroxide produced during CuAO catalysis may enhance oxidative damage [12] and play a role in vasoconstriction [13]. However, the true physiological roles and substrates of the human CuAOs remain elusive.

The CuAOs catalyse the conversion of primary amines to aldehydes in an aerobic redox reaction involving both the copper ion and a quinone cofactor (TPQ) formed by post-translational modification of a constituent tyrosine residue (Fig. 1(b)) [14]. The catalytic mechanism of the enzyme has been extensively studied kinetically and spectroscopically [15], and through X-ray crystallography of the *Escherichia coli* enzyme (ECAO) [16–19]. During catalysis the amine substrate forms a Schiff base with the TPQ (Fig. 1(b)), and a proton is abstracted from the methylene carbon bonded to the primary amine group by a conserved aspartate base in the enzyme. This abstraction is stereospecific, but varies with enzyme source [20]. ECAO always abstracts the pro-*S* proton from its preferred substrates, aromatic monoamines [17]. The much studied irreversible hydrazine derivative inhibitors of CuAOs trap the enzyme in a covalent adduct analogous to the Schiff base intermediate of the reaction [14]. The 2.0Å X-ray crystal structure of ECAO complexed with 2-hydrazinopyridine (2-HP) showed the TPQ cofactor covalently linked to 2-HP via position 5 of its ring (Fig. 2) [17]. The 2-HP moiety lay next to Asp 383, the catalytic base of the reaction, with the pyridine ring directed away from the copper ion. The TPQ is indirectly linked to the copper ion via a hydrogen bond

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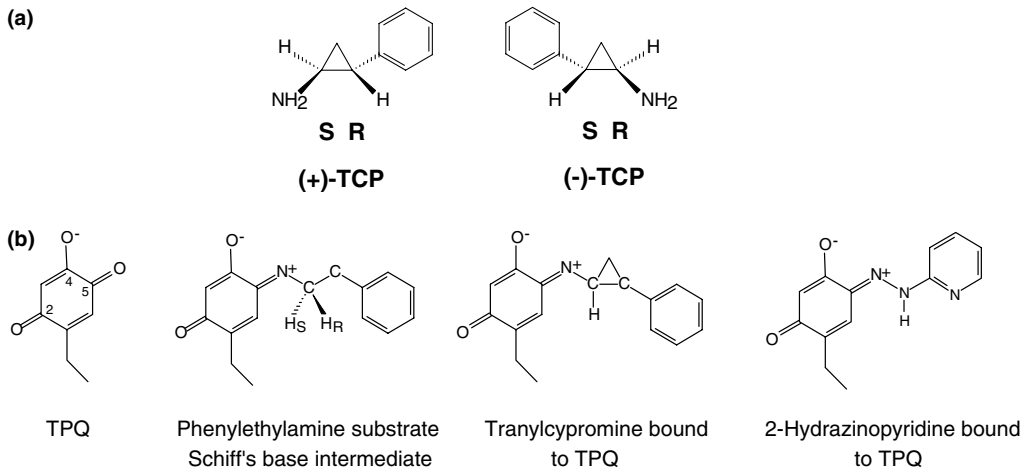

Fig. 1. (a) The *trans* enantiomers of TCP; 1*S*,2*R*-(+)-*trans*-2-phenylcyclopropylamine ((+)-TCP) and 1*R*,2*S*-(−)-*trans*-2-phenylcyclopropylamine ((−)-TCP). (b) Comparison of covalent adducts of TPQ. The substrate Schiff base adduct with the ECAO substrate, β-phenylethylamine is shown with the inhibitor adducts formed with TCP and 2-HP, an example of a hydrazine based inhibitor. Labelling of atoms corresponds to the text, except for H_S and H_R which correspond to the pro-*S* and pro-*R* protons, respectively.

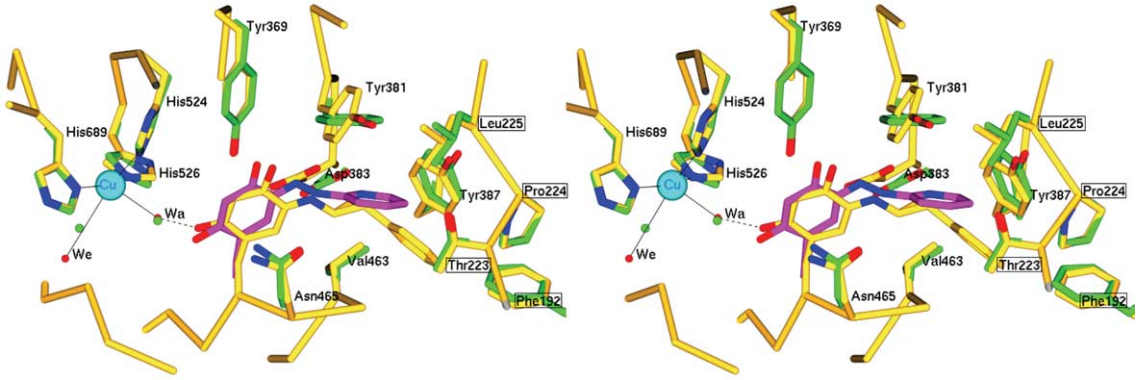

Fig. 2. Comparison of the active site of the (+)-TCP complex (carbon atoms yellow) and the 2-HP complex (carbon atoms green and purple; waters green). Residues in the peripheral domain (D3) that forms part of the substrate entry channel are indicated by boxed residue identifiers, whilst those of the catalytic domain (D4) are unboxed. Figure produced using SPOCK [39].

between O2 of cofactor and a water molecule (Wa) coordinated to the copper. The copper ion appears sensitive to the state of the quinone, as the equatorial water ligand (We) increases its distance from the copper by 1.0 Å in the 2-HP complex compared to the native ECAO structure. Unlike substrate, these hydrazine based inhibitors have a nitrogen adjacent to the primary amine group, and the enzyme is unable to catalyse the chemistry required to break the N–N bond to release a product. In this respect, TCP resembles substrate in having a carbon atom attached to the amine group, but it is a tertiary rather than a secondary carbon with only one proton (Fig. 1(b)). The inhibitory effects of TCP on copper amine oxidases in solution studies have been reported recently [21,22]. The differing stereospecificity of CuAOs in mammals could potentially lead to differential binding of the two enantiomers of TCP and could contribute to the enantiomeric specific side-effects in treating depression. To investigate whether binding of TCP to an amine oxidase is stereospecific and to examine the inhibitory action of this drug on a CuAO, we have determined the X-ray crystal structure of ECAO following exposure to racemic TCP.

2. Materials and methods

2.1. Enzyme activity

ECAO protein was prepared as detailed previously [16]. Enzyme activity was assayed by measuring oxygen consumption during catalysis using an oxygen electrode (Rank Brothers). The baseline for the reaction was the enzyme buffer; air saturated 100 mM phosphate buffer, pH 7.0. ECAO was added to the reaction mix (2.5 mM racemic TCP in air saturated 100 mM phosphate buffer, pH 7.0) to a final concentration of 0.035 mg/ml. Monitoring continued for 60 min with no evidence of oxygen consumption. The control of ECAO added to air saturated buffer alone showed no oxygen consumption, whilst ECAO added to 2.5 mM β-phenylethylamine in air saturated buffer (the "classic" substrate for the enzyme) led to >90% complete oxygen consumption in 2 min.

2.2. X-ray crystallography

ECAO crystals were prepared as detailed previously [16]. The crystal used for data collection was left soaking in a 10:1 ratio of racemic TCP hemisulfate (Sigma) solution to protein in crystal-stabilising liquor (1.4 M sodium citrate solution, 0.1 M HEPES buffer at pH 7.2) for 20 days to give high occupancy of binding at the ECAO active site [17]. Data were collected at the Daresbury Synchrotron Radiation Source, station 9.6, on a MAR 300 mm image plate (wavelength 0.87 Å; 1° and 1.2° oscillations). The data were processed with MOSFLM/ROTAV-

ATA/AGROVATA [23,24] and converted to structure factors ($|F_o|$) using the program TRUNCATE from the CCP4 package of programs [23,24]. Modelling was performed using O [25], and using the native ECAO model (resolution 2.0 Å; PDB code 1DYU) [18] with the TPQ side-chains and all active site waters removed as the initial model. All electron density map calculations and refinement were carried out in CNS [26], in cycles of positional Powell minimisation and individual temperature factor refinement, with a maximum likelihood function target and an overall anisotropic temperature factor correction. Copper ion restraints were weakened by adjustment of the non-bonded parameters, to enable the copper ion/ligand distances to reflect the data more accurately. Models for the derivatised cofactor with the two TCP enantiomers were built by using standard geometry to link TPQ with TCP coordinates derived from the crystal structure of *N*,*N*-dimethyl-2-phenylcyclopropylamine HCl [27]. Dictionaries for CNS and O were produced with the help of HIC-Up [28] and incorporated ideal geometries. Refinement was ceased when the largest $F_o - F_c$ peaks appeared to represent noise and the R_{free} no longer decreased.

3. Results and discussion

3.1. Structure of ECAO complexed with TCP

The structure was solved by difference Fourier methods and refined to 2.4 Å resolution. The final model has good stereochemistry and an *R* factor = 18.5% (R_{free} = 22.9%, Table 1).

Structural changes following TCP binding are localised to the active sites of the homodimeric ECAO. Although exposed to a racemic mixture of the two TCP enantiomers, the electron density clearly indicates that only one of the enantiomers, (+)-TCP (Fig. 1(a)), is covalently bound to the enzyme at high, although not 100%, occupancy (Fig. 3(a)). Meaningful occupancy refinement was not possible due to the medium resolution of the data, and the complexity of underlying electron density from ordered waters, Tyr 381 that "gates" off the active site, and unbound TPQ from the native structure. Extensive modelling with the second enantiomer, (−)-TCP, always led to either the cyclopropyl or phenyl ring lying outside the electron density (Fig. 3(b)).The residues are tightly packed through most of the active site, particularly surrounding Asp 383, the catalytic base, whose hydrophobic environment is critical for elevating its pK_a [15]. There was no evidence in the electron density that could be associated with a (−)-TCP model, nor that any of the active site residues had an alternate position that could be accommodating this enantiomer.

Table 1
Crystallographic data collection and refinement statistics

Space group	$P2_12_12_1$
Unit cell (Å)	$a = 135.2$, $b = 166.5$, $c = 79.6$
Resolution (Å)	20–2.4
Number of observed reflections	163 856
Number of unique reflections	64 108
Completeness (%) overall (final shell)	90.1 (80.6)
$I/\sigma(I)$ overall (final shell)	7.5 (2.9)
R_{sym} (%) overall (final shell)[a]	7.5 (25.1)
Wilson *B*-factor (Å^2)	36.6
R_{cryst} (%)[b]	18.5
R_{free} (%)[c]	22.6
Number of atoms	12 909
Average overall *B*-factor (Å^2)	30.4
rms bond lengths (Å)	0.008
rms bond angles (°)	1.6

[a] $R_{sym} = \sum_{hkl}(\sum_I(|I_{hkl,I} - \langle I_{hkl}\rangle|))/\sum_{hkl,I} I_{hkl,I}$, where $I_{hkl,I}$ is the intensity of an individual reflection and $\langle I_{hkl}\rangle$ is the mean intensity of that reflection.

[b] $R_{cryst} = \sum_{hkl}(|F_{o,hkl}| - |F_{c,hkl}|)/|F_{o,hkl}|$, where $|F_{o,hkl}|$ and $|F_{c,hkl}|$ are the observed and calculated structure factor amplitudes for reflections used during refinement (working set).

[c] R_{free} is equivalent to R_{cryst} but calculated with reflections omitted from the refinement process (test set). The R_{free} dataset contained equivalent reflections to a reference R_{free} dataset for this crystal form of ECAO [18].

In wild-type ECAO, the electron density for the TPQ cofactor ring shows a high degree of rotational mobility [18]. In contrast, the electron density for the Schiff base formed at ring position 5 of the TPQ with the (+)-TCP indicates a well-defined position for the adducted cofactor. Tyr 381, that has been described as a "gating" residue blocking off the back of the active site from solvent in the wild type enzyme [29], has swung out of the way in the complex to make a side to face ring/ring interaction with the cyclopropyl ring of (+)-TCP (Fig. 2) [30]. The cyclopropyl ring of (+)-TCP also makes van der Waals contacts with Val 463 and the catalytic base, Asp 383. The phenyl ring of the (+)-TCP lies in the narrow substrate entry/exit channel formed between the ~440 amino acid C-terminal catalytic domain (D4) and a ~100 amino acid peripheral domain (D3) [17]. It lies in the hydrophobic concave roof of this channel, making van der Waals contacts with Phe 192, Thr 223, Pro 224, Leu 225 (D3), Val 463, and Gly 464 (D4) and stacking with the ring of Tyr 387 (D4) (Fig. 2). As observed in the 2-HP complex with ECAO, the copper ion is sensitive to the state of the nearby quinone and the equatorial water ligand distance in the (+)-TCP/ECAO complex lengthens from 2.0 to 4.0 Å. The catalytic base, Asp 383, occupies its wild type position. The lone hydrogen on the tertiary carbon of (+)-TCP, adjacent to the nitrogen covalently linked to position 5 of the TPQ ring, is pointing away from Asp 383. Therefore, the geometry is incorrect for proton abstraction.

3.2. Enantioselectivity of ECAO for TCP

The (−)-TCP enantiomer can be modelled so that the phenyl ring lies in the correct position within the entry channel, although the cyclopropyl ring would sterically clash with Asn 465 (green model, Fig. 3(b)). This would lead to the lone hydrogen on the tertiary carbon pointing towards the base for possible abstraction to yield a ketone product. Within the confines of the substrate binding pocket, the groups attached to a tertiary carbon would be expected to sterically prevent the amine group of such a molecule from getting close enough to TPQ to form the Schiff base. However, the first report of a CuAO ketone product has been provided in the enantioselective oxidation of (*R*,*S*)-1-phenyl-2-aminopropane (amphetamine) by ECAO and *Klebsiella oxytoca* CuAO [31]. This shows that the chemistry for ketone formation can be performed by CuAOs.

To investigate whether the absence of (−)-TCP in the active site of the complex might be due to (−)-TCP actually being a substrate for the enzyme, we looked for evidence, following initial exposure to the drug, for the consumption of oxygen. There was no measurable oxygen consumption, as monitored by an oxygen electrode, upon addition of racemic TCP. This suggests that (−)-TCP does not act as a substrate for ECAO, and that the enzyme can not rearrange to alleviate the steric clash between Asn 465 and the cyclopropyl ring of (−)-TCP to allow Schiff base formation with the TPQ.

3.3. Comparison with 2-HP binding

Comparing the TCP and 2-HP complexes with ECAO (Fig. 2), the structures are startlingly similar, which is

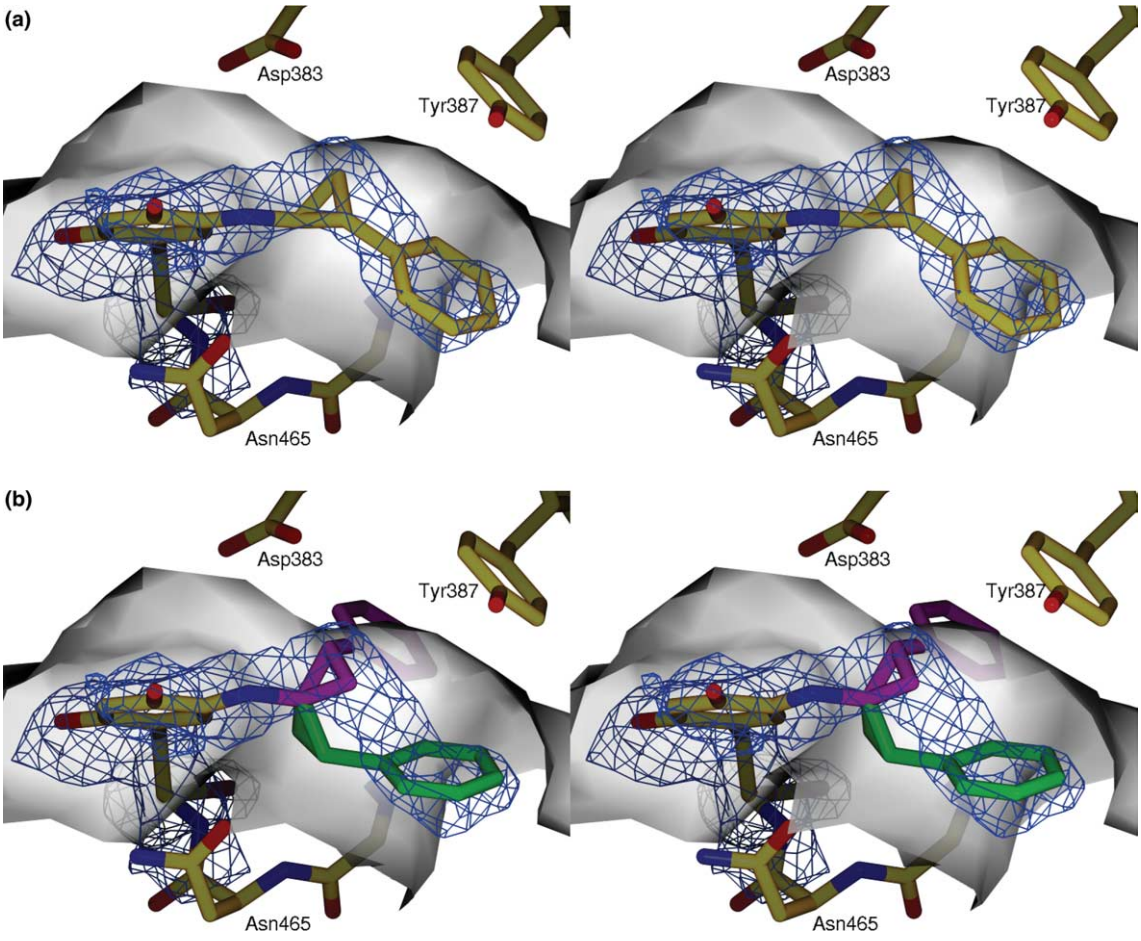

Fig. 3. (a) View of the final $2F_o - F_c$ electron density contoured at 1.0σ for the (+)-TCP adducted to the TPQ cofactor. The relationship between the substrate entry channel and the active site is represented by the molecular surface created by the protein atoms with the adducted TPQ, the copper ion and its ligands removed. (b) View of the final $2F_o - F_c$ electron density contoured at 1.0σ with best fits for a (−)-TCP adduct model based on fitting into the cyclopropyl (pink model) or phenyl ring (green model) electron density, respectively. The molecular surface is shown (defined as in (a)) to aid in visualisation of the steric clashes. Figure produced using SPOCK [39].

surprising considering how different the two inhibitors appear at first glance. The majority of the residues lining the substrate binding pocket are in identical positions and the space occupied by the adducts are superimposable. The "gate" residue, Tyr 381, takes up a different ring orientation in the two complexes, and Tyr 387 is pushed slightly away by the presence of the extra carbon atom in TCP causing the phenyl ring to protrude further out of the substrate pocket than the pyridine ring of 2-HP. The only amino acid whose position is significantly different between the two is that of the base, Asp 383. In the TCP complex, the position of Asp 383 is superimposable with its position in wild type. The presence of nitrogen (N1) in the numerous hydrazine based inhibitors at this position provides a hydrogen bonding opportunity for Asp 383 and this "pulls" the residue towards the adduct. In the case of the 2-HP complex, there is an additional hydrogen bond between Asp 383 and the pyridine ring nitrogen, probably displacing the position of the base even further.

The proportion of the 2-HP complex is very high in its crystal structure, as 2-HP is an irreversible inhibitor of CuAOs. In contrast, TCP is a reversible competitive inhibitor, with a μM K_i [21]. The electron density for the TCP moiety is weaker than that of the surrounding protein, and indicates less than 100% occupancy, which is consistent with the reversible nature of the inhibitor.

3.4. Stereospecificity and substrate preferences of copper-containing amine oxidases

The "restricted" conformational space available to inhibitor/substrate in the substrate pocket and entry channel would explain the strictly controlled stereospecificity of proton abstraction and substrate preferences of CuAOs. Viewed from above, the substrate binding pocket in ECAO bends 120° around Asn 465 (a conserved residue) and Val 463 ensuring that the pro-*S* hydrogen of substrate is pointing towards Asp 383 as defined by the required position of the aromatic ring of substrate in the curve of the pocket (Fig. 3(a)). Thus, as observed, ECAO always abstracts the pro-*S* hydrogen from its substrates. These pockets and entry channels vary significantly in the known crystal structures of CuAOs [16,29,32–34], and rationally account for the differing stereospecificities and substrate preferences observed in CuAOs from different sources.

3.5. Medical implications

ECAO has between 24% and 25% amino acid sequence identity with each of the three human CuAO sequences

within the three C-terminal domains (D2–D4) conserved in all CuAOs [35]. The N-terminal domain (D1) is variable or absent in different CuAOs. The human CuAOs have between 41% and 64% sequence identity with each other in these regions [35]. The observation that only one of the enantiomers of TCP can bind to ECAO may be considered in the context that the differing stereospecificities of proton abstraction are probably ''fine-tuned'' by the different characteristics of the substrate binding pockets and entry channels. This leads to the reasonable supposition that the human CuAOs with their expected different stereospecificites could selectively bind different enantiomers of TCP and could explain some of the differences in side-effects observed from administering the enantiomers separately, rather than the racemate, in the treatment of depression. Although it is unlikely that these drugs could be refined to preclude binding to all human CuAOs to help alleviate the side-effects involved in their use as antidepressants acting on flavin-containing amine oxidases, the exquisite substrate specificity of the CuAOs could allow these types of drugs to act as lead compounds in the rational design of drugs against HuPAO, whose action is linked to the vascular changes in late diabetic complications and congestive heart disease. As the human CuAOs are further studied, links to other diseases are likely to emerge, and the inherent differing specificities of these enzymes may allow a new palette of drugs to be designed from this original class of MAOI antidepressants to complement other mechanism-based compounds [36,37] or peptides [38].

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