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HPLC-UV assays for evaluation of inhibitors of mono and diamine oxidases using novel phenyltetrazolylalkanamine substrates

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ABSTRACT

Recently, we have described an HPLC-UV assay for the evaluation of inhibitors of plasma amine oxidase (PAO) using 6-(5-phenyl-2*H*-tetrazol-2-yl)hexan-1-amine (**4**) as a new type of substrate. Now we studied, whether this compound or homologues of it can also function as substrate for related amine oxidases, namely diamine oxidase (DAO), monoamine oxidase A (MAO A) and monoamine oxidase B (MAO B). Among these substances, **4** was converted by DAO with the highest rate. The best substrate for MAO A and B was 4-(5-phenyl-2*H*-tetrazol-2-yl) butan-1-amine (**2**). To validate the new assays, the inhibition values of known enzyme inhibitors were determined and the data were compared with those obtained with the substrate benzylamine, which is often used in amine oxidase assays. For the DAO inhibitor 2-(4-phenylphenyl)acetohydrazide an about 10fold lower IC_{50}-value against DAO was obtained when benzylamine was applied instead of **4**, indicating that **4** binds to the enzyme with higher affinity than benzylamine. The IC_{50}-values of clorgiline and selegiline against MAO A and B, respectively, also decreased (two- and 30fold) replacing **2** by benzylamine. The discrepancies largely disappeared, when the enzymes were pre-incubated with the inhibitors for 15 min. This can be explained with the covalent inhibition mechanism of the inhibitors.

Introduction

Amine oxidases are enzymes, which catalyze the oxidative degradation of primary amines to aldehydes with the concomitant formation of ammonia and hydrogen peroxide [1,2]. They can be categorized into two subclasses, the copper-containing amine oxidases [3] and the flavin-adenine-dinucleotide (FAD)-containing amine oxidases [4]. The copper-containing amine oxidase family consists of the diamine oxidase (DAO, EC 1.4.3.22), the retina amine oxidase, the plasma amine oxidase (PAO, EC 1.4.3.21) and the lysyl oxidase. A deficiency of the histamine degrading DAO can result in an intolerance against histamine ingested with food [5]. For this reason, DAO is used as food supplement with the intention to decrease the histamine levels in the gastrointestinal tract. Plasma amine oxidase (PAO), also known as semicarbazide-sensitive amine oxidase (SSAO), copper containing amine oxidase 3 (AOC3), or vascular adhesion protein-1 (VAP-1), is localized to the extracellular surface of certain cells such as vascular cells and adipocytes. Besides it exists as a soluble enzyme in blood plasma, generated from proteolytic cleavage of the vascular PAO. The enzyme is involved in physiological as well as pathophysiological processes [6,7]. In particular, it acts as a vascular adhesion protein that regulates leukocyte extravasation from the blood into tissues [8,9]. Since an increased transmigration of leukocytes through the vascular endothelium occurs during inflammatory responses, inhibitors of PAO could be useful in the therapy of inflammation-related diseases [10]. The mitochondrial monoamine oxidases A and B (MAO A and MAO B, EC 1.4.3.4) and the cytosolic polyamine oxidase belong to the group of amine oxidases containing FAD. MAO A and MAO B play important roles in the metabolism of neurotransmitters such as dopamine, noradrenaline and serotonin. Inhibitors of MAO A are marketed drugs against depression, MAO B inhibitors are therapeutically used for the treatment of Parkinson's disease [11].

In literature, various methods have been reported for the determination of the activity of amine oxidases and for screening of inhibitors of these enzymes. PAO assays mostly employ the non-physiological substrate benzylamine. The enzyme product benzaldehyde is quantified by UV-spectrophotometry [12–18] or by HPLC and UV-detection after derivatization with dinitrophenylhydrazine [19]. Alternatively, the conversion of ^{14}C-benzylamine to ^{14}C-benzaldehyde is determined radiometrically [20–22]. Moreover, peroxidase-coupled colorimetric [23–30], fluorometric [31–34] or luminometric [35] assays are applied, which measure the amount of H_2O_2 produced during benzylamine oxidation. Besides, a (naphthalene-2-yl)methylamine substrate was used in a fluorometric non-coupled assay [36].

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Diamine oxidase (DAO) is stored in epithelial cells and secreted into the blood stream upon stimulation. Preferred substrates of this enzyme are histamine, 1-methylhistamine, various diamines such as cadaverine (pentane-1,5-diamine) and putrescine (butane-1,4-diamine), and the polyamine spermidine. Despite benzylamine was reported to be only a poor substrate for DAO, it also can be applied for determination of DAO activity [37–40]. The most widely used DAO substrate is putrescine. This diamine is converted to γ-aminobutyraldehyde, which undergoes a spontaneous and rapid internal cyclization to Δ^1-pyrroline. Some methods detect this compound by colorimetry, GC or HPLC after its derivatization with ninhydrin, *o*-aminobenzaldehyde, or *o*-aminobenzaldehyde/CrO_3 [41–45]. Alternatively, enzyme activity is monitored by measuring the decrease of putrescine or histamine by ion mobility spectrometry [46] or fluorometry after derivatization [47], respectively. In the same way as described for PAO above, in several DAO assays the formation of H_2O_2 formed during the oxidation of an amine substrate is determined by using coupled reactions of peroxidase and a dye leading to coloured [26,48–51] or luminescence [35,40] products. Furthermore, the aromatic aldehydes produced by the enzyme from *p*-nitrobenzylamine or *p*-dimethylaminomethylbenzylamine are quantified directly by UV/Vis-spectrophotometry [52,53]. Besides, a variety of other methods has been reported, such as radiometric assays, which use ^{14}C-putrescine or ^{14}C-cadaverine as substrates [54,55], or NADH-coupled spectrophotometric methods, which detect the aldehyde or the ammonia released by DAO from amine substrates [56,57].

A large number of assays are described for the determination of MAO A and/or MAO B activity using kynuramine as substrate. The aldehyde formed by enzymatic oxidation of this compound undergoes a complete cyclization to 4-hydroxyquinoline, which can be detected by UV/Vis-spectrophotometry, fluorometry or mass spectrometry directly or after LC separation [58–67]. A variety of different amine substrates such as tyramine are used in MAO assays, in which the produced H_2O_2 is detected by generation of a chromogenic or fluorogenic compound in a peroxidase-coupled reaction [25,26,34,68–71]. Tyramine is also used as substrate in assays, in which the aldehyde produced by MAO is derivatized with semicarbazide and dinitrophenylhydrazine before detection by UV/Vis-spectrophotometry [72,73]. Some established test systems for MAO A and MAO B inhibitor screening or for comparative determination of activity use different substrates for both enzymes, e.g. serotonin for MAO A and benzylamine, 3-methoxy-4-hydroxybenzylamine or 2-phenylethylamine for MAO B. The generated aldehyde products are measured directly by UV-spectrophotometry, by HPLC with UV, MS or electrochemical detection, respectively, by peroxidase-coupled reactions, or after derivatization with dinitrophenylhydrazine by colorimetry [74–79]. Although benzylamine is described to be only a poor substrate for MAO A [64], it is also used for the determination of MAO A activity [58,80]. Furthermore, a luciferase-coupled MAO assay [81], methods, in which the aldehyde produced by MAO is oxidized to the corresponding carboxylic acid, which is quantified by HPLC or capillary electrophoresis [82–85], and several test systems applying radiolabelled MAO substrates [86–89] have been published. Moreover, the neurotoxic illicit drug 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is applied as substrate in a MAO B assay [90]. In contrast to the water soluble PAO and DAO, MAO A and B are obligate membrane-associated enzymes embedded in the mitochondrial membranes. Thus, for solubilization and stabilization of these enzymes, often detergents are added to the reaction mixtures in MAO A and B assays [58,59,91–96].

Recently, we have published a method for the detection of PAO inhibitors, which monitors the conversion of the new substrate 6-(5-phenyl-2*H*-tetrazol-2-yl)hexan-1-amine (**4**) to the enzyme product 6-(5-phenyl-2*H*-tetrazol-2-yl)hexanal (**11**) by HPLC with UV-detection [97]. Since this aldehyde product only eluted with poor peak shape due to hydrate formation in the aqueous mobile phase, a pre-column derivatization with tris(hydroxymethyl)aminomethane (TRIS) leading to the formation of an oxazolidine was carried out before analysis (Fig. 1). In the present work, we describe HPLC-UV assays for the determination of the activity of the amine oxidases DAO, MAO A and MAO B using novel ω-(5-phenyl-2*H*-tetrazol-2-yl)alkan-1-amine substrates. The procedures are suitable for screening inhibitors of these important enzymes, and for testing the specificity of agents found to be active towards PAO.

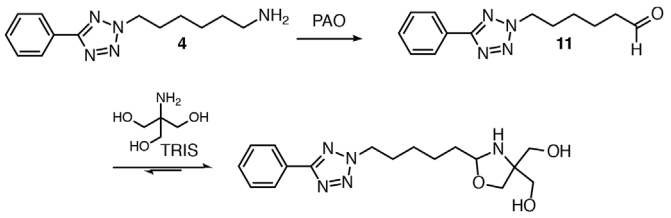

Fig. 1. Conversion of 6-(5-phenyl-2*H*-tetrazol-2-yl)hexan-1-amine (**4**) to 6-(5-phenyl-2*H*-tetrazol-2-yl)hexanal (**11**) by PAO and derivatization of the aliphatic aldehyde product to an 1,3-oxazolidine by TRIS.

Material and methods

Reagents

Phosphate buffered saline tablets (one tablet dissolved in 200 mL of deionized water yields 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4, at 25 °C), glycerol, sucrose, EDTA-Na_2, dimethyl sulfoxide, benzylamine, berenil, clorgiline, selegiline (Sigma-Aldrich, Steinheim, Germany); potassium dihydrogen phosphate, dibasic potassium phosphate, tris(hydroxymethyl)aminomethane (TRIS) base, benzaldehyde, ammonium acetate LC/MS-grade, Triton X-100 (VWR, Darmstadt, Germany); acetonitrile HPLC-grade, Brij35 (Fischer Scientific, Loughborough, UK); bovine plasma amine oxidase (PAO) purified from bovine plasma (lyophilized powder, activity: 32 Tabor units/mg protein) (Abnova, Taipei, Taiwan delivered via Biozol, Eching, Germany); diamine oxidase (DAO) from porcine kidney (powder, 0.11 units/mg protein, Cat. No. D7876), monoamine oxidase A (MAO A) (human recombinant, expressed in baculovirus infected BTI insect cells, 2.5 mg or 0.45 units/0.5 mL buffer solution, Cat. No. M7317), monoamine oxidase B (MAO B) (human recombinant, expressed in baculovirus infected BTI insect cells, 2.5 mg or 0.19 units/0.5 mL buffer solution, Cat. No. M7441) (Sigma-Aldrich, Steinheim, Germany); ω-(5-phenyl-2*H*-tetrazol-2-yl) alkanamines **2–6**, the corresponding aldehydes **9–13** and the PAO inhibitor 2-(4-phenylphenyl) acetohydrazide were synthesized as published recently [97]; for the synthesis of 3-(5-phenyl-2*H*-tetrazol-2-yl)propan-1-amine (**1**) and 3-(5-phenyl-2*H*-tetrazol-2-yl)propanal (**8**) see *Supplementary data*.

Determination of the conversion rates of different amine substrates by PAO, DAO, MAO A and MAO B

Preparation of enzyme dilutions

PAO: The purchased enzyme (600 Tabor units [18], 19 mg) was dissolved in 1 mL phosphate buffered saline (pH 7.4); an aliquot of this solution was diluted 1:100 with phosphate buffered saline; final concentration: 0.19 mg/mL.

DAO: An aliquot of the purchased enzyme (27.5 units, 250 mg) was dissolved in potassium phosphate buffer (0.1 M, pH 7.2); final concentration: 10 mg/mL.

MAO A: An aliquot of the purchased enzyme solution (0.45 units or 2.5 mg/0.5 mL) (40 µL) was diluted with potassium phosphate buffer (0.1 M, pH 7.4, containing 0.25 M sucrose, 0.1 mM EDTA-Na_2 and 5% (v/v) glycerol) (104 µL); final concentration: 1.4 mg/mL.

MAO B: An aliquot of the purchased enzyme solution (0.19 units or