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# Characterization of the interaction of the novel antihypertensive etamicastat with human dopamine-β-hydroxylase: Comparison with nepicastat

Maria João Bonifácio [a], Filipa Sousa [a], Marco Neves [a], Nuno Palma [a], Bruno Igreja [a], Nuno Miguel Pires [a], Lyndon C. Wright [a], Patrício Soares-da-Silva [a,b,c,*]

[a] Department of Research & Development, BIAL, 4745-457 São Mamede do Coronado, Portugal
[b] Department of Pharmacology & Therapeutics, Faculty of Medicine, University Porto, Porto, Portugal
[c] MedInUP – Center for Drug Discovery and Innovative Medicines, University of Porto, Porto, Portugal

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ABSTRACT

The interaction of etamicastat, a novel peripherally acting dopamine-β-hydroxylase (DBH) inhibitor, with the enzyme was studied using a classical kinetic approach and the pharmacodynamics effect of the compound upon administration to rats was also evaluated. SK-N-SH cell homogenates convert tyramine into octopamine with a $K_m$ value of 9 mM, and a $V_{max}$ of 1747 nmol/mg protein/h. The $K_m$ value for ascorbate was 3 mM. The inhibition of DBH by etamicastat and nepicastat, a known centrally acting DBH inhibitor, with $IC_{50}$ values of 107 and 40 nM, respectively, was fully reversed by dilution. Non-linear fitting of the velocities, determined at various concentrations of substrate (tyramine) and co-substrate (ascorbic acid), and of etamicastat and nepicastat, indicated that the inhibition of DBH by both compounds follows a mixed-model inhibition mechanism, approaching competitive behavior with regards to the substrate tyramine, with $K_i$ values of 34 and 11 nM, respectively. Relatively to ascorbate, both compounds followed a mixed-model inhibition mechanism, approaching uncompetitive behavior. Oral administration of both compounds (at 30 mg/kg) inhibited adrenal DBH activity over time and significantly decreased noradrenaline levels in the heart. Nepicastat also decreased noradrenaline levels in the parietal cortex, but not etamicastat. Both compounds significantly decreased systolic and diastolic blood pressure in spontaneously hypertensive rats. In conclusion, etamicastat and nepicastat behave as multisubstrate DBH inhibitors, binding reversibly and preferentially to the reduced form of the enzyme, and simultaneously at the substrate and oxygen binding sites. Etamicastat, in contrast to nepicastat, offers the advantage of peripheral selectivity without central effects.



## 1. Introduction

Dopamine-β-hydroxylase (DBH; EC 1.14.17.1), the enzyme responsible for the conversion of dopamine to noradrenaline in the catecholamine biosynthetic pathway, has been considered a promising therapeutic target for the treatment of hypertension and chronic heart failure (Gomes and Soares-da-Silva, 2008; Grassi et al., 2008, 2010; Soares-da-Silva, 1986, 1987; Stanley et al., 1997). DBH inhibition has the merit to cause gradual sympathetic slow-down instead of acute inhibition, thus decreasing the hemodynamic negative impact (Hegde and Friday, 1998), sometimes observed with adrenergic blockade. Several inhibitors of this enzyme have been developed (Beliaev et al., 2009), none achieving marketing approval because of weak potency, poor DBH selectivity and/or significant adverse effects (Beliaev et al., 2009; Kruse et al., 1986b). Etamicastat (Fig. 1) is a novel DBH inhibitor under development by BIAL – Portela & Cª, SA (S. Mamede do Coronado, Portugal) as a new putative drug therapy for cardiovascular disorders. Etamicastat has limited access to the brain acting mainly in the periphery by decreasing noradrenaline levels in sympathetically innervated tissues (Beliaev et al., 2006; Bonifácio et al., 2009). Etamicastat was also shown to reduce both systolic (SBP) and diastolic (DBP) blood pressure, alone or in combination with other antihypertensive drugs, and to reduce noradrenaline urinary excretion in spontaneously hypertensive rats (SHR), while no

*Abbreviations:* Cu, copper; DBH, dopamine-ß-hydroxylase; DBP, diastolic blood pressure; HEPES, N-2-hydroxyethylpiperazine-N-2-ethanosulfonic acid; PCA, perchloric acid; SBP, systolic blood pressure; SHR, spontaneously hypertensive rats; WKY, normotensive Wistar-Kyoto rats

* Corresponding author at: Department of Research and development, BIAL – Portela & Cª, S.A., 4745-457 S. Mamede do Coronado, Portugal.
Tel.: +351 229866100; fax: +351 229866102.
*E-mail address:* psoares.silva@bial.com (P. Soares-da-Silva).

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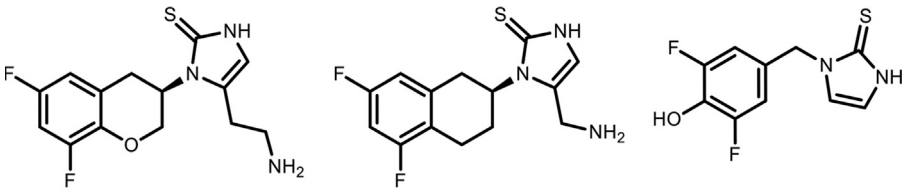


**Fig. 1.** Structures of DBH inhibitors. (1) Etamicastat, (2) Nepicastat, (3) 1-(3,5-difluoro-4-hydroxybenzyl)imidazole-2-thione.

changes in blood pressure were reported in normotensive Wistar-Kyoto rats (WKY) (Igreja et al., 2015). The safety and pharmacokinetic profiles of etamicastat investigated in healthy subjects revealed that etamicastat was well tolerated and showed approximate linear pharmacokinetics following single oral doses (Rocha et al., 2012) and multiple once-daily oral doses (Nunes et al., 2010), with no significant differences being observed in elderly versus young healthy subjects (Nunes et al., 2011). Recently, etamicastat was demonstrated with blood pressure lowering effects in hypertensive patients (Almeida et al., 2013).

The pharmacological activity of a drug is dependent on pharmacokinetic characteristics such as plasma half-life, fraction of free drug, distribution and metabolism, but also on the affinity of the drug for the target. There has been increasing evidence suggesting that the kinetics of drug–target interaction should also be carefully considered, as it influences the clinical activity of the compound. It is thus essential to study and characterize the interaction of the drugs with its target (Nunez et al., 2012).

DBH is an ascorbate dependent copper-bound enzyme, whose three-dimensional structure is yet to be solved, although preliminary in silico models of the human and rat enzyme have been described (Prigge et al., 2000). The active unit of the enzyme is a tetramer constituted by two dimers held together by noncovalent interactions; the subunits forming the dimers being connected through interchain disulfide linkages. There are two mononuclear copper centers per subunit with distinct properties; one of the Cu is the site of dioxygen binding and activation (CuB), while the other works as an electron transfer site (CuA) in the reaction mechanism. Reduction of the enzyme is performed in two sequential, one-electron transfer steps involving two ascorbate molecules, which leads to the generation of reduced enzyme and two equivalents of semi-dehydroascorbate. It is proposed that ascorbate interacts at CuA, followed by inter-site electron transfer and reduction of the catalytic copper site (CuB), from Cu(II) to Cu(I). Return to the oxidized state is obtained in the presence of the substrate and dioxygen.

The aim of this study was first to characterize the interaction of etamicastat with human DBH using an enzymatic kinetic approach and nepicastat (Fig. 1) as reference compound, and evaluate the in vivo effects of both compounds in the rat.

## 2. Materials and methods

### 2.1. Reagents and other materials

Eagle's minimum essential medium, N-2-hydroxyethylpiperazine-N-2-ethanosulfonic acid (HEPES), glutamine and antibiotic–antimycotic solution were obtained from Sigma-Aldrich (St Louis, MO). Gibco® fetal bovine serum was obtained from Life Technologies (Paisley, UK). Tyramine hydrochloride, DL-octopamine hydrochloride, N-ethylmaleimide (NEM), copper sulfate, sodium fumarate, sodium acetate, pargyline, bovine liver catalase, ascorbic acid, ammonium hydroxide, sodium metabisulfite and sodium periodate were obtained from Sigma-Aldrich (St Louis, MO). Perchloric acid (PCA) was obtained from Merck (Darmstadt, Germany). SPE cartridges ISOLUTE SCX-3 were obtained from Biotage (Uppsala, Sweden). 96-well plates UV-transparent were obtained from Corning Incorporated (Corning, NY). Nepicastat and etamicastat (Fig. 1) were synthesized in BIAL's Chemical Research Laboratory with purities above 95%.

### 2.2. Animal treatment

Male Wistar rats, obtained from Harlan (Spain) and Spontaneously Hypertensive Rats (SHR), obtained from Charles River Laboratories (Germany), were maintained under controlled environmental conditions in a colony room (12 h light/dark cycle, room temperature $22 \pm 1$ °C and humidity $55 \pm 15\%$) with food and water provided ad libitum. Animals were quarantined for 1 week before dosing. Before killing the animals were anaesthetized with pentobarbital (60 mg/kg) intraperitoneally. All animal procedures were conducted in accordance with the 2010/63/EU European Directive on the protection of animals used for scientific purposes and the Portuguese law on animal welfare (Decreto-Lei 113/2013).

### 2.3. Pharmacodynamic evaluation

Wistar rats were orally administered with 30 mg/kg etamicastat or nepicastat with a dose volume of 10 ml/kg, in sterile water or 0.5% carboxymethylcellulose. In the experiments designed for adrenal DBH evaluation, adrenal glands were collected from anaesthetized animals at 1, 4, 8 and 15 h after dosing and were stored frozen in 50 mM Tris–HCl pH 7.4 until analysis. In experiments designed to evaluate catecholamine levels, heart left ventricle and parietal cortex fragments were collected from anaesthetized Wistar rats at 8 and 15 h post-administration and put in tubes containing PCA (0.2 M).

### 2.4. Catecholamine quantification

Tissues collected in PCA were stored at 4 °C for 24 h after which solution was filtered by centrifugation (15,000*g*, 4 min, 4 °C) through 0.22 μm pore size filters (COSTAR SPIN-X from Corning Inc., USA). Catecholamines were quantified in filtrates by directly injecting 50 μl of sample volume on a Gilson High Pressure Liquid Chromatography system with electrochemical detection. The chromatographic system consisted on a Spheri-5 RP-18 5 μm column measuring 25 cm in length and 4.6 mm in diameter (Perkin-Elmer), a pump (Gilson 307), an automatic sampling injector (Gilson 231 XL), a dilutor (Gilson 402) and an electrochemical detector (Gilson 142) with a glassy carbon electrode and an Ag/AgCl reference electrode. The detector cell was operated at 0.75 V. Mobile phase consisted on a solution containing 0.1 M Citric acid, 0.1 M sodium acetate, 0.15 mM EDTA, 1 mM dibutylamine, 1 mM octylsulfate, 5% methanol adjusted to pH 3.5 with PCA.