Biochimica et Biophysica Acta 1854 (2015) 1078–1089

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbapap

Discovery of compounds that protect tyrosine hydroxylase activity through different mechanisms☆

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ARTICLE INFO

Article history:
Received 26 February 2015
Received in revised form 21 April 2015
Accepted 24 April 2015
Available online 8 May 2015

Keywords:
Dopamine synthesis
Pharmacological chaperone
Tyrosine hydroxylase deficiency
Iron coordination
Enzyme stabilization

ABSTRACT

Pharmacological chaperones are small compounds that correct the folding of mutant proteins, and represent a promising therapeutic strategy for misfolding diseases. We have performed a screening of 10,000 compounds searching for pharmacological chaperones of tyrosine hydroxylase (TH), the tetrahydrobiopterin (BH_4)-dependent enzyme that catalyzes the rate-limiting step in the synthesis of catecholamines. A large number of compounds bound to human TH, isoform 1 (hTH1), but only twelve significantly protected wild-type (hTH1-wt) and mutant *TH-R233H* (hTH1-p.R202H), associated to the rare neurological disorder TH deficiency (THD), from time-dependent loss of activity. Three of them (named compounds 2, 4 and 5) were subjected to detailed characterization of their functional and molecular effects. Whereas compounds 2 and 4 had a characteristic pharmacological chaperone (stabilizing) effect, compound 5 protected the activity in a higher extent than expected from the low conformational stabilization exerted on hTH1. Compounds 4 and 5 were weak competitive inhibitors with respect to the cofactor BH_4 and, as seen by electron paramagnetic resonance, they induced small changes to the first coordination sphere of the catalytic iron. Molecular docking also indicated active-site location with coordination to the iron through a pyrimidine nitrogen atom. Interestingly, compound 5 increased TH activity in cells transiently transfected with either hTH1-wt or the THD associated mutants p.L205P, p.R202H and p.Q381K without affecting the steady-state TH protein levels. This work revealed different mechanisms for the action of pharmacological chaperones and identifies a subtype of compounds that preserve TH activity by weak binding to the catalytic iron. This article is part of a Special Issue entitled: Cofactor-dependent proteins: Evolution, chemical diversity and bio-applications.

1. Introduction

Tyrosine hydroxylase (TH) catalyzes the conversion of L-tyrosine (L-Tyr) to L-3,4-dihydroxyphenylalanine (L-Dopa), using iron and tetrahydrobiopterin (BH_4) as cofactors and oxygen (O_2) as an additional substrate. This reaction is the rate-limiting step in the biosynthesis of dopamine and the other catecholamines [1], which function as neurotransmitters and hormones involved in the regulation of motor coordination, behavior, sleep–wake cycle regulation, learning and memory [2,3].

Human TH (hTH) is a homo-tetrameric enzyme which exists as four different isoforms produced from alternative splicing [4,5]. Isoforms 1 and 2 (hTH1 and hTH2) are the most abundant in the central nervous system, mainly in dopaminergic neurons [1,5]. Mutations in the *TH* gene cause TH deficiency (THD), an autosomal recessive neurological disorder which leads to deficiency of catecholamine synthesis [6]. THD is a rare disease with onset within the first year of life. The patients can be categorized into two subgroups: type A, which manifests as a progressive extrapyramidal movement disorder with onset in infancy or childhood, and type B, which is associated to a more severe, complex encephalopathy with onset in the neonatal period or early infancy [7]. Most patients are type A and generally respond well to L-Dopa

Abbreviations: BH_4, tetrahydrobiopterin; DSF, differential scanning fluorimetry; L-Dopa, (S)-3,4-dihydroxyphenylalanine; PAH, phenylalanine hydroxylase; PKU, phenylketonuria; TH, tyrosine hydroxylase; THD, TH deficiency; TPH2, tryptophan hydroxylase isoform 2; PD, Parkinson's disease

☆ This article is part of a Special Issue entitled: Cofactor-dependent proteins: Evolution, chemical diversity and bio-applications

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http://dx.doi.org/10.1016/j.bbapap.2015.04.030
1570-9639/

treatment, in fact presenting a Dopa-responsive dystonia phenotype. However, L-Dopa treatment is often associated with low long-term efficiency and secondary effects [7,8]. Furthermore, type B patients are often poor- or non-responders to L-Dopa [7], and new therapeutic alternatives are therefore necessary for these severely affected patients. A reduction of TH activity and dopamine synthesis is also a characteristic of Parkinson's disease (PD) [9,10], Alzheimer's disease [11,12] and of various neuropsychiatric disorders such as manic depressive illness, schizophrenia and ADHD [13]. Novel therapies based on stimulation of TH activity will also have a valuable potential in these disorders.

THD-associated mutations correlate with loss-of-function-misfolding and instability of TH [14–16], and the mutants are rapidly degraded by intracellular proteases, leading to decreased TH and dopamine levels in THD patients. Increasing evidence points to pharmacological chaperones as a promising therapeutic strategy for loss-of-function misfolding diseases [17–20]. Pharmacological chaperones, which are small molecular weight compounds that exert their effect either by accelerating folding or by stabilizing the native conformation, could potentially be used to halt and reverse loss-of-function misfolding disorders [21–23]. In the case of phenylalanine hydroxylase (PAH), another BH_4-dependent aromatic amino acid hydroxylase with high sequence and functional similarity to TH, it has been shown that one of the mechanisms operating in BH_4 responsive phenylketonuria (PKU) is the pharmacological chaperone effect of the cofactor [24]. A certain chaperone effect of BH_4 has also been shown for TH [25], although the mechanism is still not clear, as other studies have also shown that BH_4 may cause TH aggregation [26]. Furthermore, a number of studies have revealed several small molecular weight compounds with pharmacological chaperone potential for PKU [17,27]. Some of these compounds, such as compounds III (3-amino-2-benzyl-7-nitro-4-(2-quinolyl)-1,2-dihydroisoquinolin-1-one) and IV (5,6-dimethyl-3-(4-methyl-2-pyridinyl)-2-thioxo-2,3-dihydrothieno[2,3-d]pyrimidin-4(1H)-one) from Pey et al. [17], have also been tested on TH in vitro and supplemented to mice [28]. Whereas compound III behaved as a classical pharmacological chaperone that increased both the thermal stability of TH in vitro and the steady state TH protein level in mice brain, compound IV leads to higher TH activity in brain extracts without apparently affecting the conformational stability of the enzyme [28]. Despite the potential effect of compounds III and IV for the treatment of TH deficiencies, these compounds are not TH-specific and compound IV also inhibits isoform 2 of tryptophan hydroxylase (TPH2) [28], the BH_4-dependent aromatic amino acid hydroxylase that catalyzes the rate-limiting step in the synthesis of serotonin. We thus aimed to obtain more effective stabilizing compounds with higher specificity for TH.

In this study we have searched for potential pharmacological chaperones for the treatment of TH deficiencies. We performed an experimental screening for compounds that bound to TH, based on differential scanning fluorimetry (DSF), followed by an assay to identify those that protected TH from time-dependent loss of activity. Five of these compounds were tested for protection of TH towards limited proteolysis by trypsin, and three were subsequently selected. These three compounds were further studied, characterizing their enzyme kinetics, electron paramagnetic resonance (EPR) properties and inhibition of the other aromatic amino acid hydroxylases. Their effects on TH protein level and activity were also measured in cells transiently expressing TH (hTH1-wt and THD-associated mutants). All together, the screening and validation assays revealed that protection from inactivation is not only related to conformational stabilization of TH. More specifically, we have shown that compounds that bind competitively to the cofactor BH_4 binding site with low affinity and little conformational effect on TH upon binding are effective to protect TH activity. As shown by EPR and molecular docking, weak iron-binding is the most likely mechanism for the catalytic activity protection. In addition to their potential to correct THD, activity-protective compounds might also be promising in the treatment of other neurological diseases where dysfunction of TH has been shown, and notably PD.

2. Materials and methods

2.1. Materials

The MyriaScreen Diversity Collection from Sigma Aldrich/TimTec (Newark, DE) was used for screening. It consists of 10,000 compounds with an averaged purity of 95% at 2 mg/mL in 100% DMSO. The hit compounds from the screening were consecutively ordered from TimTec and Sigma Aldrich (purity >90% for the different batches) prepared at concentrations of 4 mg/mL in 100% DMSO, and stored at −20 °C. (6R)-L-erythro-5,6,7,8-tetrahydrobiopterin (BH_4) was purchased from Schircks Laboratories (Jona, Switzerland), compound IV (5,6-dimethyl-3-(4-methyl-2-pyridinyl)-2-thioxo-2,3-dihydrothieno[2,3-d]pyrimidin-4(1H)-one) was obtained from Maybridge Ltd. (Cambridge, UK), and other reagents, including dopamine, were purchased from Sigma Aldrich, unless otherwise stated.

2.2. Expression and purification of TH, PAH and TPH2

Wild-type (wt) recombinant human TH, isoform 1 (isoform b; NCBI accession code NP_000351.2; hTH1) and the mutant *TH-R233H* (hTH1-p.R202H) were expressed in *Escherichia coli* and purified to homogeneity either by heparin-Sepharose (Amersham Biosciences) chromatography as described [29] or using the construct $(His)_6$-maltose binding protein (MBP)-hTH1 (Bezem et al., manuscript in preparation). $(His)_6$-MBP-hTH1 codes for a fusion protein with a Tobacco etch virus (TeV) protease-cutting site and after expression in *E. coli* (BL21 Codon Plus (DE3), Stratagene, CA, US) in LB medium, 28 °C for 6 h, bacteria were lysed [29] and the fusion protein was purified using amylose resin (New England Biolabs) as reported [30]. The fusion tag was removed by proteolytic cleavage using TeV protease (1:25 TeV protease:hTH1 by mass) in 15 mM NaHepes, pH 7.4, 150 mM NaCl, 1 mM dithiothreitol (DTT), and 5% glycerol, for 4 h on ice, followed by gel filtration (Superdex 200 HR10/30, GE Healthcare, UK) in the same buffer but without DTT. Recombinant human PAH and recombinant human TPH2 were expressed in *E. coli* and purified as described previously [30,31].

2.3. Screening for ligand binding

For the first screening step, we applied differential scanning fluorimetry (DSF) [32], where a fluorescent dye (SYPRO Orange) interacts with the hydrophobic areas that expose in the proteins upon temperature-induced unfolding, providing a fluorescent signal (λ_{exc} = 465 nm, λ_{em} = 610 nm) that represents the fraction of unfolded protein. Compounds that increase the midpoint melting temperature (T_m) are considered to bind and stabilize the protein [17,32,33]. The experiments were performed on a LightCycler 480 Real-Time PCR System from Roche Applied Science. The enzyme was diluted to 0.05 mg/mL in 20 mM NaHepes, pH 7.0, and 200 mM NaCl with 5× SYPRO Orange, and compounds dissolved in DMSO were then added to a final concentration of 0.08 mg/mL (corresponding to an averaged concentration of 200 μM) and 4% DMSO. Compounds from the MyriaScreen diversity collection, containing 10,000 different compounds with molecular weight varying from 220 to 547 Da, provided by Sigma Aldrich, were screened. The assay was performed with a total volume of 25 μL on 384-well microplates (from Roche Applied Science). The samples were incubated at room temperature for approximately 30 min before loading them into the PCR-instrument. Controls with 4% DMSO were performed on each plate. The unfolding curves were registered from 20 °C to 95 °C with a scan rate of 2 °C/min, with measurements approximately every 0.2 °C. The samples were analyzed using homemade software. We recorded the midpoint T_m-values from the unfolding curves in the presence and absence of the compounds and calculated the shift in T_m ($\Delta T_m = T_m - T_{m,ref}$) for each compound. Concentration-dependent DSF was performed with an initial compound concentration of 0.04 mg/mL