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Chemoproteomic, biochemical and pharmacological approaches in the discovery of inhibitors targeting human α/β-hydrolase domain containing 11 (ABHD11)

Dina Navia-Paldanius [a,*], Jayendra Z. Patel [b], Miriam López Navarro [a], Hermina Jakupović [a], Steffi Goffart [d], Sanna Pasonen-Seppänen [a], Tapio J. Nevalainen [b], Tiina Jääskeläinen [a,c], Tuomo Laitinen [b], Jarmo T. Laitinen [a], Juha R. Savinainen [a]

[a] School of Medicine, Institute of Biomedicine, University of Eastern Finland, P.O Box 1627, 70211 Kuopio, Finland
[b] School of Pharmacy, University of Eastern Finland, P.O Box 1627, 70211 Kuopio, Finland
[c] Institute of Dentistry, University of Eastern Finland, P.O Box 1627, 70211 Kuopio, Finland
[d] Department of Biology, University of Eastern Finland, P.O Box 111, 80101 Joensuu, Finland

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ABSTRACT

ABHD11 (α/β-hydrolase domain containing 11) is a non-annotated enzyme belonging to the family of metabolic serine hydrolases (mSHs). Its natural substrates and products are unknown. Using competitive activity-based protein profiling (ABPP) to identify novel inhibitors of human (h)ABHD11, three compounds from our chemical library exhibited low nanomolar potency towards hABHD11. Competitive ABPP of various proteomes revealed fatty acid amide hydrolase (FAAH) as the sole off-target among the mSHs. Our fluorescent activity assays designed for natural lipase substrates revealed no activity of hABHD11 towards mono- or diacylglycerols. A broader profiling using *para*-nitrophenyl (pNP)-linked substrates indicated no amidase/protease, phosphatase, sulfatase, phospholipase C or phosphodiesterase activity. Instead, hABHD11 readily utilized *para*-nitrophenyl butyrate (pNPC4), indicating lipase/esterase-type activity that could be exploited in inhibitor discovery. Additionally, a homology model was created based on the crystal structure of bacterial esterase YbfF. In contrast to YbfF, which reportedly hydrolyze long-chain acyl-CoA, hABHD11 did not utilize oleoyl-CoA or arachidonoyl-CoA. In conclusion, the present study reports the discovery of potent hABHD11 inhibitors with good selectivity among mSHs. We developed substrate-based activity assays for hABHD11 that could be further exploited in inhibitor discovery and created the first homology-based hABHD11 model, offering initial insights into the active site of this poorly characterized enzyme.

1. Introduction

The family of serine hydrolases (SH) is a large group of enzymes that represent approximately 1% of all human protein genes. They are subdivided to proteases and metabolic serine hydrolases (mSHs). Nearly half of these proteins are poorly characterized, including many of those that are linked to different human diseases (Bachovchin and Cravatt, 2012). α/β-hydrolase domain (ABHD) containing mSHs comprises at least 19 members that seem to be specialized in catalyzing lipid modifications in cells, but the majority of ABHDs, however, is still without known function (Lord et al., 2013). ABHD11 belongs to the class of orphan ABHDs; those with no identified endogenous substrates or products. As predicted, ABHD11 contains both nucleophile and acyltransferase motifs (Lord et al., 2013). It also represents one of the genes (chromosomal locus 7q11.23) deleted in Williams-Beuren syndrome. This syndrome causes developmental disorders, *e.g.* intellectual disability, distinctive facial features and cardiovascular problems (Merla et al., 2002). However, any causal connection of ABHD11 to these symptoms remains to be established. In addition, ABHD11 has been suggested as a potential cancer biomarker in lung adenocarcinoma (Wiedl et al., 2011) and is linked to breast cancer malignancy, being a potential target gene for the cancer-overexpressed transcription factor HOXB7 (Heinonen et al., 2015). Yet, unrelated to the coding sequence, overexpression of the intergenic non-protein coding RNA, called an opposite strand of ABHD11, has shown to slightly increase the ABHD11 expression in mice striatum (Francelle et al., 2015). However, whether this *anti*-sense regulates the expression of ABHD11 remains to be proven. ABHD11 has a broad tissue distribution (Merla et al., 2002) with highest expression in metabolically active tissues such as brown adipose tissue, heart and skeletal muscle (Lord et al., 2013). Proteomic studies have predicted that ABHD11 is a mitochondrial enzyme (Lefort et al., 2009).

* Corresponding author.
E-mail address: dina.navia-paldanius@uef.fi (D. Navia-Paldanius).

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Even though there are several organophosphates targeting multiple mSH including ABHD11 (Medina-Cleghorn and Nomura, 2014; Nomura et al., 2008), very few inhibitors have been described for ABHD11: one carbamate-based (WWL222) (Bachovchin et al., 2010) and three triazole urea-based inhibitors (ML211 (Adibekian et al., 2010b), ML226 (Adibekian et al., 2010a) and AA44-2 (Adibekian et al., 2011)). From these, ML226 was reported to selectively inhibit hABHD11 and AA44-2 to inhibit mouse ABHD11 *in vitro* and *in situ* in murine T-cells (Adibekian et al., 2010a; Adibekian et al., 2011). Interestingly, WWL222 was reported to exhibit nanomolar potency against mouse ABHD11, while it was inactive against hABHD11 (Adibekian et al., 2010a). On the other hand, ML211 was reported to inhibit hABHD11 with nanomolar potency but it additionally inhibited the lysophospholipases LYPLA1 and LYPLA2 (also known as acyl-protein thioesterases 1/2; APT1/APT2), two mSHs that are thought to catalyze the depalmitoylation of membrane-anchored proteins bearing this reversible acyl modification (Duncan and Gilman, 1998; Kong et al., 2013).

In this study, we describe three potent and *in vitro*-selective hABHD11 inhibitors that were discovered by screening over 200 in-house synthesized compounds, designed to target serine hydrolases. Screening was facilitated by competitive ABPP utilizing a serine hydrolase targeting fluorescent probe TAMRA-FP using various tissue and cell proteomes. To further characterize these inhibitors, we established a colorimetric substrate-based activity assay suitable for hABHD11 as well as for two small mSHs, namely LYPLA2 and arylformamidase (AFMID; EC3.5.1.9). While no mono- or diacylglycerol lipase activity was detected, more systematic substrate screening with chromogenic substrates disclosed that pNP (*para*-nitrophenyl) butyrate (pNPC4) and pNP-acetate (pNPC2) were readily utilized, suggesting that ABHD11 likely serves as a lipase/esterase-type enzyme. We utilized the substrate-based activity assay for further pharmacological characterization of the ABHD11 inhibitors initially discovered using the ABPP approach. In addition, we present the first homology-based molecular ABHD11 model offering initial insights into the active site. Finally, by utilizing competitive ABPP we have evaluated both *in vitro* and *in situ* the selectivity of the newly discovered inhibitors against the mSHs using various cell and tissue proteomes.

2. Material and methods

2.1. HEK293 cells with transient overexpression of ABHD11, AFMID and LYPLA2

The procedures were as previously described (Navia-Paldanius et al., 2012). Briefly, HEK293-cells were cultured as monolayers in DMEM containing 10% fetal bovine serum, under antibiotics (penicillin/streptomycin) at 37 °C in a humidified atmosphere of 5% CO2/95% air. Full-length of pCMV6-XL5-hABHD11, pCMV6-Entry-hAFMID, pCMV6-XL5-hLYPLA2 and pCMV6-XL5-hMAGL, all fully sequenced cDNAs obtained from Origene Technologies Inc. (Rockville, MD), were introduced to cells by a standard (transient) transfection procedure using X-tremeGENE Hp DNA Transfection reagent (Roche, Mannheim, Germany) following manufacturer's instructions. Cellular lysates were prepared and protein concentrations determined as previously described (Navia-Paldanius et al., 2012).

2.2. Generation of the SH-triplet preparation

The SH-triplet preparation was generated by pooling cell lysates of hAFMID, hABHD11 and hLYPLA2. The total protein concentration in the final combination was 3 mg/ml containing 1 mg/ml of each HEK293 cell lysate overexpressing the SH of interest. This pool was further diluted as indicated in the results.

2.3. Competitive activity-based protein profiling (ABPP)

A competitive ABPP was used for enzyme inhibitor screening and to label active mSH as previously described (Navia-Paldanius et al., 2012) using a fluorophosphonate probe TAMRA-FP (ActivX Fluorophosphonate Probes, Thermo Fisher Scientific, Rockford, IL). Briefly, 3.75 μg (SH triplet) or 100 μg protein of cell lysates and tissue homogenates were pre-incubated with 0.5 μl of vehicle (DMSO) or inhibitor with indicated concentrations for 1 h at room temperature (RT), followed by labeling of the SHs with TAMRA-FP (1 μM final concentration) for 1 h at RT. The reaction was stopped by adding 2× SDS-loading buffer, and the proteins were separated in SDS-PAGE (82 × 82 mm and 170 × 200 mm) together with molecular weight standards. Protein bands were visualized after in-gel fluorescent scanning using a fluorescence scanner (λ_{ex} 532 nm, λ_{em} 580 nm) and quantified by an image analysis software ImageJ.

2.4. Fluorescent-based glycerol assays

The assay is based on fluorescent glycerol detection in 96-well format and was conducted as previously described (Aaltonen et al., 2013; Navia-Paldanius et al., 2012). We used 0.3 μg of protein of HEK293 cell lysates overexpressing serine hydrolase of interest.

2.5. Functional pNP assays

The pNP assay was adapted and modified from previous reports (Kakugawa et al., 2015; Muccioli et al., 2008). Briefly, enzyme preparations were diluted (2 μg/well) in assay buffer (50 mM Tris-HCl, pH 7.4; 5 mM $MgCl_2$; 100 mM NaCl, 1 mM EDTA, containing 0.1% (w/v) fatty acid free BSA) followed by incubation with the indicated chromogenic substrates at the concentrations indicated in the Results and discussion section. The enzymatic hydrolysis was performed in 96-well-plate (total volume 200 μl/well) and the absorbance of pNP (λ 405 nm) was kinetically monitored for 90 min using a colorimetric plate reader. The amount of pNP was calculated based on pNP standard.

2.6. Preparation of mouse brain membrane (Mbm)

Mbm was prepared as previously described (Navia-Paldanius et al., 2015). Briefly, the teflon/glass pestle homogenized brains were centrifuged at low speed (1000 ×*g*, 10 min, +4 °C) and the supernatant was collected, followed by centrifugation at high speed (145,000 ×*g*, 45 min at +4 °C). The resulting pellet was re-suspended in Tris-buffer (50 mM Tris–HCl [pH 7.4], 150 mM NaCl), sonicated and centrifuged at high speed. The washing was repeated and the supernatant was discarded. The pellet was re-suspended in Tris-buffer and aliquoted for storage at −80 °C.

2.7. Isolating mitochondria from mice tissues

Mitochondria were purified from fresh mouse tissues by homogenization, differential centrifugation and density gradient purification as described (Reyes et al., 2005). The mitochondrial pellets were re-suspended in PBS and the protein concentration determined as previously described (Navia-Paldanius et al., 2012). Enrichment of mitochondrial outer membrane transporter (TOMM20) in the pellets was verified using Western Blot (Fig. 3d).

2.8. Acyl-CoA hydrolase assays

The protocol was adapted and modified from a previous publication (Hunt et al., 2006). Briefly, lysates of HEK293 cells and hABHD11-HEK293 cells (2 μg/well) were incubated in assay buffer (50 mM Tris-HCl, pH 7.4; 5 mM $MgCl_2$; 100 mM NaCl, 1 mM EDTA, containing additionally 0.1% (w/v) fatty acid free BSA) together with oleoyl-CoA or arachidonoyl-CoA (25, 50 or 100 μM final concentration, 96-well-