ARTICLE IN PRESS

Biochemical and Biophysical Research Communications xxx (2018) 1–7

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Suppression of tau propagation using an inhibitor that targets the DK-switch of nSMase2

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A R T I C L E I N F O

Article history:
Received 18 March 2018
Accepted 27 March 2018
Available online xxx

Keywords:
Tauopathy
nSMase2
Extracellular vesicles
Cambinol
Tau biosensor
Molecular modeling

A B S T R A C T

Targeting of molecular pathways involved in the cell-to-cell propagation of pathological tau species is a novel approach for development of disease-modifying therapies that could block tau pathology and attenuate cognitive decline in patients with Alzheimer's disease and other tauopathies. We discovered cambinol through a screening effort and show that it is an inhibitor of cell-to-cell tau propagation. Our *in vitro* data demonstrate that cambinol inhibits neutral sphingomyelinase 2 (nSMase2) enzyme activity in dose response fashion, and suppresses extracellular vesicle (EV) production while reducing tau seed propagation. Our *in vivo* testing with cambinol shows that it can reduce the nSMase2 activity in the brain after oral administration. Our molecular docking and simulation analysis reveals that cambinol can target the DK-switch in the nSMase2 active site.

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1. Introduction

A number of tauopathies, including the most prevalent form of dementia – Alzheimer's disease (AD)– have been described, but there are no approved therapies targeting tauopathies that are currently available [1]. The common feature of this group of neurodegenerative disorders is accumulation of tau protein aggregates in neurons and/or glial cells [2]. Notably, propagation of tau starts from a single disease-specific site resulting in the well-defined stereotypic pattern of progression in AD [3], and in other tauopathies, such as argyrophilic grain disease (AGD), progressive supranuclear palsy (PSP), Pick's disease also known as Frontotemporal dementia (FTD), chronic traumatic encephalopathy (CTE) and a subtype of globular glial tauopathies [2,4]. Onset and severity of clinical symptoms in AD, AGD, FTD and CTE have been shown to correlate with the degree/stage of tau pathology [5–7], making modulation of tau propagation pathways an approach for therapeutic development.

Recent reports suggest a “prion-like” mode of tau propagation [8], including distinct pathological phenotypes induced by disease-specific stable tau strains [9,10]. Pathological tau oligomers released from affected neurons/glial cells or injected into the brains of mice, can be internalized by healthy cells wherein they act as proteopathic seeds by templating abnormal protein conformations - inducing further spread of pathology, both *trans*-synaptically [11–14] and through microglia-mediated propagation [15]. The proposed cell-to-cell transfer mechanisms for tau include release/uptake mechanisms [16–18], transfer through tunneling nanotubes [19] and transport in extracellular vesicles (EVs) such as plasma membrane-derived ectosomes and multivesicular body (MVB)-derived exosomes [20–24].

Our screening efforts for tau propagation inhibitors led us to the identification of a brain permeable small molecule – cambinol – that is a known inhibitor of the neutral sphingomyelinase 2 (nSMase2) [25]. In our *in vitro* model, using HEK293T Tau RD P301S biosensor (tau biosensor) cells [26], cambinol was shown to inhibit the spread of tau oligomers derived from synaptosomes from Alzheimer's disease patients. Further evaluation of cambinol, as described herein, led to the discovery of a novel binding mode for this inhibitor at the active site of nSMase2, that is a known gatekeeping enzyme involved in ceramide-mediated exosome production [27]. There is significant evidence in the literature on the involvement of nSMase2 in brain exosome biogenesis and

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https://doi.org/10.1016/j.bbrc.2018.03.209
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Please cite this article in press as: T. Bilousova, et al., Suppression of tau propagation using an inhibitor that targets the DK-switch of nSMase2, Biochemical and Biophysical Research Communications (2018), https://doi.org/10.1016/j.bbrc.2018.03.209

exosome-mediated transfer of proteopathic seeds [15,28–30] through a non-canonical pathway independent of the endosomal sorting complexes required for transport (ESCRT). A noncompetitive inhibitor of this enzyme GW4869 [31,32], has been previously reported to reduce exosome biogenesis and suppresses pathology propagation in tauopathy mouse models [15]. Mammalian nSMase2 is an integral membrane protein that is highly expressed in the brain; it is a Mg^{2+} dependent enzyme with optimal activity at neutral pH [33].

The recently published crystal structure of the active-site domain of the enzyme (pdb: 5UVG) [32] was used in our molecular docking and simulation studies with cambinol which reveals its interaction with the "DK-switch" of the nSMase2. In this enzyme the DK-switch involves the interaction between residues Asp430 (D430) and Lys435 (K435). The enzyme backbone around this region can adopt a loop conformation that directs the Asp residue away from the active site, or an α-helical conformation that directs the Asp residue into the active site to form a salt bridge with the Lys residue [32]. Switching between the loop and helical conformation would reposition the Asp and Lys residues at the active site center and modulate nSMase2 enzymatic activity.

Discovery of this novel molecular mechanism of interaction between cambinol and nSMase2 has implications in the rational design and synthesis of novel nSMase2 inhibitors with superior drug-like properties that could inhibit propagation of pathologic forms of tau.

2. Methods

2.1. nSMase2 enzyme assay

nSMase2 activity was measured in the homogenates of confluent tau biosensor cells or mouse cortex using Amplex red sphingomyelinase assay kit according to manufacturer protocol (ThermoFisher, A12220), using either cell lysate from confluent tau biosensor cells or mouse brain lysate as a source of nSMase2. Detailed protocol in the Supplementary Materials.

2.2. Preparation of human brain derived synaptosomes

Brain autopsy samples were obtained from the University of California Irvine and University of Southern California AD Research Centers. Detailed information about individual cases presented in Supplementary Table S1. Brain tissue was cryopreserved and synaptosomal fractions (P2-fractions, or P2) were prepared as previously described [34]. In order to prepare P2-extracts, aliquots were quickly defrosted at 37 °C and centrifuged at 10,000 g for 10 min at 4 °C to remove P2 from sucrose. After aspirating the supernatant, cold PBS was added to each sample in a 1:5 weight/volume ratio. Samples were then sonicated in 10-second intervals three times, incubated on ice for 30 min and centrifuged at 20,000 g for 20 min at 4 °C. P2 extracts were collected and stored at −80 °C.

2.3. Functional assays for tau propagation inhibitors

Our functional assays are based on the previously published protocol for flow cytometry detection of tau seeds using HEK293T Tau RD P301S biosensor (tau biosensor) cells [35]. In contrast to the original protocol, two distinct cell populations of tau biosensor cells were employed: donor cells (D), which were seeded with P2-extracts; and recipient cells (R), which never were in direct contact with human brain derived material and could only receive tau seeds from donor cells. Cell viability was assessed with LDH assay (Promega, G1780). The experimental scheme is presented in Fig. 1A. Detailed protocol is provided in the Supplementary Materials.

2.4. EV purification and characterization

Donor cells were grown in medium with exosome-depleted FBS (ThermoFisher, A2720803) for 48 h with or without compounds, cell culture medium was collected and EVs purified using ExoQuick-TC kit (SBI biosciences). For quality control, small amounts of purified EVs were fixed on a copper mesh in glutaraldehyde/paraformaldehyde solution, stained with 2% uranyl acetate solution and imaged on a JEOL 100CX electron microscope at 29,000 times magnification. The remaining samples were either sent for nanoparticle tracking analysis (NTA) at Alpha Nanotech or used for biochemical characterization. Immunoblot analysis was done by 10–20% Tris-Glycine gel in non-reduced conditions, transferred to PVDF membrane and probed with antibodies against CD63 (ThermoFisher, 10628D), CD9 (ThermoFisher, 10626D), and syntenin-1 (sc-48742), followed by HRP-conjugated secondary antibodies. Chemiluminescent signals were obtained with Super Signal West Femto substrate (Thermo Scientific Pierce 34,095) and detected using a BioSpectrum 600 imaging system and quantified using VisionWorks Version 6.6A software (UVP; Upland, CA).

2.5. In vivo treatment with cambinol

Brain permeability and target engagement studies were conducted in C57BL/6J mice. Cambinol stock solution (60 mg/ml) was prepared in DMSO. Four hours after oral gavage treatment with cambinol as a single 100 mg/kg dose, or vehicle (2 mice per group), mice were sacrificed and tissue samples (brain and plasma) collected. Drug level evaluation in brain and plasma samples was performed by LC-MS/MS analysis. Brain tissue was homogenized and nSMase2 activity measured as described above.

2.6. Modeling of cambinol binding to nSMase2

Molecular docking and molecular dynamic simulation studies were conducted with cambinol using recently published crystal structure of nSMase2 catalytic domain (pdb: 5UVG) [32]. Detailed protocol provided in the Supplementary Materials.

2.7. Statistical analysis

All the data was expressed as the mean ± SEM. Significant differences were determined by one-way ANOVA with post hoc Tukey multiple comparisons test using online web statistical calculator (http://astatsa.com/OneWay_Anova_with_TukeyHSD). P-values of #, *<0.05 and ##, **<0.01 were considered statistically significant.

3. Results

3.1. Cell-free assay for nSMase2 activity

As previously reported, nSMase2 is upregulated during confluence induced growth arrest [36]. We similarly found that nSMase2 activity was elevated in confluent tau biosensor cells and in confluent SH-SY5Y human neuroblastoma cells (data not shown). Cambinol shows dose-dependent inhibition of nSMase2 activity in cell extracts from tau biosensor cells with an IC_{50} of ~7.7 μM (Fig. 1D) similar to the previously reported value of 5 μM [25].

3.2. Cambinol inhibits EV-mediated cell-to-cell tau propagation

We show that cambinol inhibits tau seed spread from donor to recipient cells in the "D + R" functional assay. Pooled synaptosomal (P2) extracts from cryopreserved human AD brains (Table S1) were used to seed tau aggregation in donor cells. We found high levels of

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