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Research article

Prolyl oligopeptidase inhibition attenuates the toxicity of a proteasomal inhibitor, lactacystin, in the alpha-synuclein overexpressing cell culture

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HIGHLIGHTS

- Decreased proteasomal activities have been connected to Parkinson's disease.
- Lactacystin increased alpha-synuclein aggregation in a cell culture.
- Prolyl oligopeptidase inhibition by KYP-2047 attenuated lactacystin toxicity.
- KYP-2047 decreased alpha-synuclein oligomers and enhanced autophagy.

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ABSTRACT

Lewy bodies, the histopathological hallmarks of Parkinson's disease (PD), contain insoluble and aggregated α-synuclein (aSyn) and many other proteins, proposing a role for failure in protein degradation system in the PD pathogenesis. Proteasomal dysfunction has indeed been linked to PD and aSyn oligomers have been shown to inhibit proteasomes and autophagy. Our recent studies have shown that inhibitors of prolyl oligopeptidase (PREP) can prevent the aggregation and enhance the clearance of accumulated aSyn, and therefore, we wanted to study if PREP inhibition can overcome the aSyn aggregation and toxicity induced by lactacystin, a proteasomal inhibitor. The cells overexpressing human A30P or A53T mutated aSyn were incubated with lactacystin and a PREP inhibitor, KYP-2047, for 48 h. Theafter, the cells were fractioned, and the effects of lactacystin with/without 1 μM KYP-2047 on aSyn aggregation and ubiquitin accumulation, cell viability and on autophagic markers (p62, Beclin1 and LC3BII) were studied. We found that KYP-2047 attenuated lactacystin-induced cell death in mutant aSyn overexpressing cells but not in non-overexpressing control cells. KYP-2047 reduced significantly SDS-insoluble high-molecular-weight aSyn oligomers that were in line with the cell viability results. In addition, significant reduction in protein accumulation marker, p62, was seen in SDS fraction while LC3BII, a marker for autophagosome formation, was increased, indicating to enhanced autophagy. Our results further streghten the possibilities for PREP inhibitors as a potential drug therapy against synucleinopathies and other protein aggregating diseases.

1. Introduction

In Parkinson's disease (PD), degeneration of dopaminergic neurons in *substantia nigra* and *striatum* leads to progressing movement deficits. The cause for this neurodegeneration is not known but histopathological findings of PD are insoluble inclusions, Lewy bodies, mainly consisting of an α-synuclein (aSyn) [1].

aSyn is a natively unfolded protein that can misfold, leading to protein aggregation, formation of toxic oligomers and cell-to-cell propagation of aSyn pathology [2–4]. Moreover, genome-wide association studies have pointed aSyn gene, *SNCA*, as a risk factor for PD and mutations in *SNCA* are associated with familial forms

Abbreviations: aSyn, alpha-synuclein; [A53T]aSyn, cell lines stably overexpressing human A53T mutated form of aSyn; [A30P]aSyn, cell lines stably overexpressing human A30P mutated form of aSyn; HMW, high-molecular-weight; LC3BI-II, microtubule-associated protein light chain 3B I-II; NC, negative control; OD, optical density; PD, Parkinson's disease; PREP, prolyl oligopeptidase; UPS, ubiquitin-proteasome system; WB, Western blot; Wt, Wild type.

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of PD, supporting its participation in PD pathology (for review, see Ref. [5]). The most studied point mutations in *SNCA* are A30P and A53T, causing familial PD with early onset. Both of them enhance the fibrillization of aSyn, leading to aSyn oligomers and cellular damages, but A30P is more prone for membrane-induced aggregation (for review, see, [6]). In addition to damaging several cellular organelles, aSyn overexpression and aSyn oligomers have been shown to inhibit ubiquitin-proteasome system (UPS) [7,8], and decreased proteasomal activity in the *substantia nigra* has been identified in PD patients [9]. Moreover, aSyn can inhibit and impair chaperone-mediated autophagy [10] and macroautophagy (referred as autophagy hereafter) [11], thus inducing its own accumulation. In addition, several reports show that proteasomal inhibition induces the aggregation of aSyn in cells and *in vivo* [12–14], stressing the importance of proteolytic balance in protein aggregation diseases.

Several factors can induce and enhance the aggregation process of aSyn (for review, see [4]). On of them is prolyl oligopeptidase (PREP), a serine protease that can increase the aggregation of aSyn via direct protein-protein interaction [15,16]. PREP is also colocalized with aSyn in substantia nigra of *post mortem* samples of PD patients, supporting the role of this interaction in PD pathology [17]. Recently, we have shown that inhibition of PREP by a small-molecule inhibitor reduces aSyn protein aggregation *in vitro* and *in vivo* by modifying the interaction between PREP and aSyn [15,18–20]. Moreover, we have shown that PREP is a negative regulator of autophagy and that PREP inhibition enhances the autophagic flux via beclin1 dependent manner [19], leading to decreased amount of high-molecular weight (HMW) aSyn oligomers in cells and in aSyn transgenic mouse models.

Several studies suggest that when proteasomal processing of the proteins is impaired, autophagy becomes important in degradation of accumulated proteins (for review, see [21]). Moreover, induction of autophagy is shown attenuate the toxicity of aSyn aggregation (for review, see Ref. [21]). Thus, based on our previous findings, we wanted to test whether inhibition of PREP is protective against a proteasomal inhibitor, lactacystin, induced toxicity and protein accumulation in aSyn overexpressing cell culture.

2. Materials and methods

2.1. Chemicals

Chemicals used were purchased form Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified. Ethanol was purchased from Altia (Helsinki, Finland). Lactacystin was purchased from A.G. Scientific (#L-1147; San Diego, CA, USA) and dissolved in PBS in concentration of 2 mg/ml. The PREP inhibitor, KYP-2047 (4-phenylbutanoyl-L-prolyl-2(*S*)-cyanopyrrolidine), was synthesized in School of Pharmacy, University of Eastern Finland, as previously described [22]. KYP-2047 has been shown to be highly potent, brain and cell penetrating and specific for PREP [23,24]. KYP-2047 was dissolved in DMSO at a concentration of 100 mM for stock.

2.2. Cell lines

Stable cell lines overexpressing human A53T ([A53T]aSyn) or A30P aSyn ([A30P]aSyn) were generated using a lentiviral vector as described in [25]. SH-SY5Y human neuroblastoma cell line without aSyn overexpression (Wt cells) was purchased from ATCC (LGC Standards; #CRL-2266, Middlesex, UK). Cells were cultured with Dulbecco's modified eagle medium (DMEM-Glutamax, #31966-021; ThermoFisher, Waltham, MA, USA) containing 15% fetal bovine serum (FBS, #16000-044; ThermoFisher), 1% non-essential amino acids (NEAA, #11140; ThermoFisher) and 50 μg/ml Gentamycin (#15750-094; ThermoFisher). With aSyn overexpressing cells 1 μg/ml puromycin was added to medium for selection [18].

2.3. Induction of aSyn aggregation by lactacystin, a proteasomal inhibitor

For Western blot (WB) and proteasomal activity experiments, the cells were seeded in a 6-well plate with the density 400,000 cells/well and allowed to attach overnight. Thereafter, the cells were incubated for 24 h on 10 μM lactacystin with 1 μM KYP-2047 or 0.001% DMSO (vehicle control). The concentration of KYP-2047 was chosen based on earlier studies [18,19] and on dose-response experiment (Fig. S1A in Supplementary material). Lactacystin dose was chosen based on cell viability results on dose-response experiments (Fig. 1B–D). After the incubation, the cells were homogenized as described below.

2.4. Proteasome activity assay

For determining chymotrypsin-like 20S proteasomal activity, the protocol based on Suc-Leu-Leu-Val-Tyr-AMC (#I-1395, Bachem, Bubendorf, Switzerland) substrate was used as described in [19]. The fluorescence was read with Victor 2 well-plate reader (PerkinElmer, Waltham, MA, USA) and all assays were done in triplicate.

2.5. Cell viability assay

Cells were plated with the density of 50,000 cell/well in 96-well plate and the next day incubated for 24 h with variable concentrations of lactacystin (10 nM, 100 nM, 1 μM, 10 μM and 100 μM) in the presence of 1 μM KYP-2047 or DMSO vehicle (0.001% DMSO; 150 μL/well). To assess cell viability a standard LDH release assay was performed as previously described [18].

2.6. Cell fractionation and WB

WB was used to detect aSyn, ubiquitin accumulation and autophagy markers from cellular fractions. The cells were fractioned to TBS soluble (soluble aSyn), Triton X-100 soluble (membrane-bound aSyn) and SDS soluble (containing SDS soluble and insoluble aSyn conformations) fractions as described in [26]. Before WB, the protein concentrations of TBS and Triton X-100 fractions were measured by using BCA method (Bio-Rad, Hercules, CA, USA), and the samples were loaded to 4–20% TGX gels (Bio-Rad) with equal protein amounts. The loading of SDS-fraction was based on protein amount of triton X-100 soluble fraction. Standard SDS-PAGE techniques were used, and the membranes were incubated +4 °C overnight in 5% skim milk in 0.05% Tween20 in TBS. Following primary antibodies were used: mouse anti-aSyn (1:1000; #ab1903, AbCam), mouse anti-ubiquitin (1:1000; #3936S, Cell Signaling Technology, Danvers, MA, USA), mouse anti-SQSTM1/p62 (p62, 1:5000; #ab56416, AbCam), rabbit anti-microtubule associated protein light chain 3 B I–II (LC3BI-II, 1:1000; #L7843), rabbit anti-beclin1 (1:2000; #ab16998, AbCam) and rabbit anti-β-actin (loading control, 1:2000; #4967S, Cell Signaling Technology). After overnight incubation, the membranes were washed and incubated with appropriate HRP-conjugated secondary antibodies for 2 h in room temperature [for aSyn and SQSTM1/p62, goat anti-mouse HRP (dilution 1:2000 in 5% milk, #31430; Thermo Fischer); for ubiquitin, LC3BI-II, beclin1, parkin and β-actin, goat anti-rabbit (dilution 1:2000; Product #31463, Thermo Fisher Scientific)]. The images were captured using the C-Digit imaging system (Licor, Lincoln, NE, USA). Three independent WB experiments were performed. ImageJ was used for analysing bands, and the optical

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