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# Proteasomal inhibition hypersensitizes differentiated neuroblastoma cells to oxidative damage

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**Abstract**

Parkinson's disease (PD) is a multifactorial disease caused by both genetic and environmental factors. Alpha-synuclein is of particular interest in PD since it is a major component of Lewy bodies and mutations in the alpha-synuclein gene were identified in familial PD. Oxidative stress and proteasomal dysfunction are implicated in the pathogenesis of PD but their interactions as well as their effect on aggregates formation are not yet clear. We therefore examined the roles of oxidative stress and proteasomal inhibition on protein aggregates induction in naïve and neuronally differentiated neuroblastoma SH-SY5Y cells. Neuroblastoma cells were stably transfected with wild type (WT) and A53T mutant alpha-synuclein. Naïve and transfected cells were exposed to oxidative stress induced by rotenone, SIN-I, $FeCl_2$, and to proteasomal inhibition by lactacystin. Proteasomal inhibition caused a dose-dependent decrease in viability and induced protein aggregates formation containing alpha-synuclein and ubiquitin. Proteasomal inhibition induced significantly increased alpha-synuclein aggregation in cells expressing mutant alpha-synuclein. Exposure to reactive oxygen species (ROS) combined with proteasomal inhibition increased aggregates formation. Inclusion body formation and cell death of differentiated neuroblastoma cells overexpressing alpha-synuclein can serve as a valuable model for elucidating the molecular components that cause neurodegeneration in PD as well as evaluating pharmacological interventions.


*Keywords:* Parkinson's disease; Alpha-synuclein; Ubiquitin–proteasome system; Protein aggregates

Parkinson's disease (PD) is the most common neurodegenerative movement disorder. The pathologic hallmark of PD is the appearance of cytoplasmic inclusion bodies, named Lewy bodies [4]. All forms of familial and idiopathic PD have Lewy bodies with the exception of the autosomal recessive juvenile-onset type [19]. Lewy bodies contain a heterogeneous mixture of insoluble, filamentous proteins and lipids, including alpha-synuclein, ubiquitin, neurofilaments, and oxidized/nitrated proteins [5,7,21,26]. Brain stem and cortical Lewy bodies have numerous features in common including the diameter of their filaments (7–12 nm) and a large proportion of their proteins are alpha-synuclein and ubiquitin [19]. Neuronal pathology in PD is associated with oxidative stress, mitochondrial dysfunction, and excitotoxicity but it is not clear how these events contribute to the neurodegenerative process [9].

Recent evidence suggests that failure of the ubiquitin–proteasome system (UPS) leading to protein accumulation, contributes to the degeneration of dopaminergic neurons and Lewy body formation in the substantia nigra pars compacta (SNc) in both familial and sporadic forms of PD [17]. The UPS is primarily responsible for degradation of damaged proteins in eukaryotic cells. Impairment of proteolytic activities of 26/20S proteasomes in the SNc of patients with sporadic PD was demonstrated in the post mortem examination of their midbrains [14,15]. In addition, various deletions and point mutations found in patients with familial PD are also related to the UPS: mutations in the parkin gene and ubiquitin C-terminal hydrolase L1 (UCH-L1) [11,24].

Alpha-synuclein is a major component of Lewy bodies in sporadic PD. In addition, in cases of autosomal dominant PD, missense mutations in the gene encoding alpha-synuclein (4q21–q23) have been shown to produce proteins that are prone to misfold and aggregate [6,22]. Mutant alpha-synuclein resists and inhibits proteolysis, and increases the sensitivity of cells to a variety of toxic insults [2,10]. In vitro experiments demonstrated

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that the formation of filamentous aggregates of alpha-synuclein was promoted in the presence of iron or other heavy metals [8]. Moreover, reactive oxygen species (ROS) were suggested to promote alpha-synuclein aggregation.

The aim of this study is to construct a model which will enable elucidation of the roles of oxidative stress and proteasomal inhibition and their mutual interactions, in protein aggregates formation and neurodegeneration in PD.

The SH-SY5Y neuroblastoma cells were stably transfected with pcDNA3.1(-) plasmid containing wild type (WT) or A53T mutant alpha-synuclein, using lipofectamine reagent (Invitrogene). In order to establish non-dividing, neuronally differentiated cells overexpressing a high level of alpha-synuclein, we used an in vitro differentiation-based cellular model. Increased vulnerability to insults by neuronally differentiated dopaminergic cells is supported by Hasegawa et al. in their recently published study [8].

Overexpression of WT and mutant alpha-synuclein was determined using Western blot and immunohistochemistry. Twenty-five micrograms of total protein from each sample was separated by 12% SDS-PAGE gels and transferred to a nitrocellulose membrane. The membranes were probed with monoclonal anti-alpha-synuclein antibody (LB509, 1:2000; Zymed Laboratories), polyclonal rabbit anti alpha-synuclein antibodies (1:5000, Chemicon), or mouse anti actin (1:10000; Sigma), followed by horseradish peroxidase conjugated secondary antibody (1:10000; Sigma) and developed with the ECL plus detection system (Amersham Pharmacia Biotech).

For immunocytochemistry, cells were grown on poly-L-lysine coated coverslips were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100. The cells were then incubated in a blocking solution followed by overnight incubation at 4 °C with the following primary antibodies: LB509 monoclonal anti alpha-synuclein antibody (1:500; Zymed Laboratories), polyclonal anti alpha-synuclein antibodies (1:5000; Chemicon International), polyclonal anti ubiquitin antibodies (1:50; Sigma). After washing with PBS, the cells were incubated with fluorescent dye (Alexa 568 or Alexa 488) conjugated goat anti-mouse or anti-rabbit antibodies (1:1000 and 1:5000, respectively; Molecular Probes) for 1 h, at room temperature. For thioflavin S staining, fixed cells were incubated with 0.05% thioflavin S (Sigma) for 10 min and washed with 80% ethanol before the immunocytochemical procedures. The nuclei were counterstained with 4,6-diamidino-2-penyl indole dihydrochloide (DAPI) 5 μg/ml (Sigma).

Neuroblastoma cells as well as WT and A53T mutant alpha-synuclein stably transfected cells, were exposed to various ROS in order to induce aggregates formation. The cells were treated with rotenone (4–100 nM for 6 to 24 h), SIN-I chloride (0.1–0.3 mM for 3 days) and $FeCl_2$ (0.1–5 mM for 3 days) (Rotenone ICN Biomedical; SIN-I and $FeCl_2$ Sigma). In order to determine the role of misfolded protein removal systems, the UPS system was inhibited using lactacystin (Sigma) (1–10 μM for 6–24 h). The incidence of aggregation-positive cells was evaluated in five independent fields using a confocal laser-scanning microscope. Aggregates quantification was done using the Image Pro Plus software. At least 30 cells were counted in each field. The quantification of aggregates was done blind, without knowledge of the treatment used.

Cell viability was determined by the MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay. Cells were plated in 96 well plates and viability after pharmacological treatment was analyzed by adding MTT solution to each well followed by incubation at 37 °C for 3 h. The medium was then removed and the formazan crystals were dissolved in DMSO. Absorbance was determined at 564 nm in a microplate reader. Cell viability was evaluated in triplicates for each treatment.

All experiments were repeated at least three times. Significance of differences between two groups was determined by Student's *t*-test. For multiple comparisons one-way ANOVA followed by LSD test was used.

After achieving stable transfection with WT or A53T mutant alpha-synuclein, immunocytochemical examination demonstrated diffuse staining of alpha-synuclein in the entire cytoplasm without any aggregations (Fig. 1A–C). There were no obvious changes in the growth or morphology of the transfected cells compared to controls. To investigate the effect of oxidative stress on alpha-synuclein overexpressing cells or A53T mutant alpha-synuclein expressing cells, the pro-oxidants rotenone, SIN-I or $FeCl_2$ were added to the culture media, which resulted in the formation of intracytoplasmic proteinaceous aggregates (Fig. 1D–F). A few untransfected cells also contained aggregates after ROS exposures, but the size and number of aggregates were much smaller than those in WT or mutant alpha-synuclein overexpressing cells. At higher concentrations or longer exposure times, pro-oxidants exposure caused noxious effects (Fig. 2). However, under the conditions shown in this study to promote aggregates formation, these treatments did not show apparent cytotoxic influences, and cellular viability was not affected.

The cytochemical features of the aggregates were essentially indistinguishable using all the treatments described. The aggregates were distributed throughout the cytoplasm, some in the perinuclear region. They were immunopositive for alpha-synuclein and ubiquitin, some with double staining for both (Fig. 1G). Some of the aggregates were also positive for thioflavin S staining, indicating that they contain proteins with β-sheet conformation (Fig. 1H and I).

Proteasomal inhibition by lactacystin treatment induced cytoplasmic alpha-synuclein and ubiquitin positive aggregates (Fig. 3B). Neuroblastoma cells expressing A53T mutant alpha-synuclein were significantly more prone to develop alpha-synuclein aggregates when exposed to proteasomal inhibition ($p = 0.009$), as compared to neuroblastoma cells overexpressing WT alpha-synuclein (Fig. 3C), indicating that the UPS system is important in clearing this aberrant protein and preventing its aggregation in the cells. After exposure to ROS, the incidence of aggregates-positive cells was low among untransfected cells, significantly increased in neuroblastoma cells transfected with WT alpha-synuclein ($p = 0.002$), and significantly higher in the A53T mutant alpha-synuclein transfected cells ($p < 0.001$ compared to untransfected neuroblastoma cells, and $p = 0.046$ compared to neuroblastoma cells transfected with WT alpha-synuclein) (Fig. 3D). Moreover, combined exposure to ROS

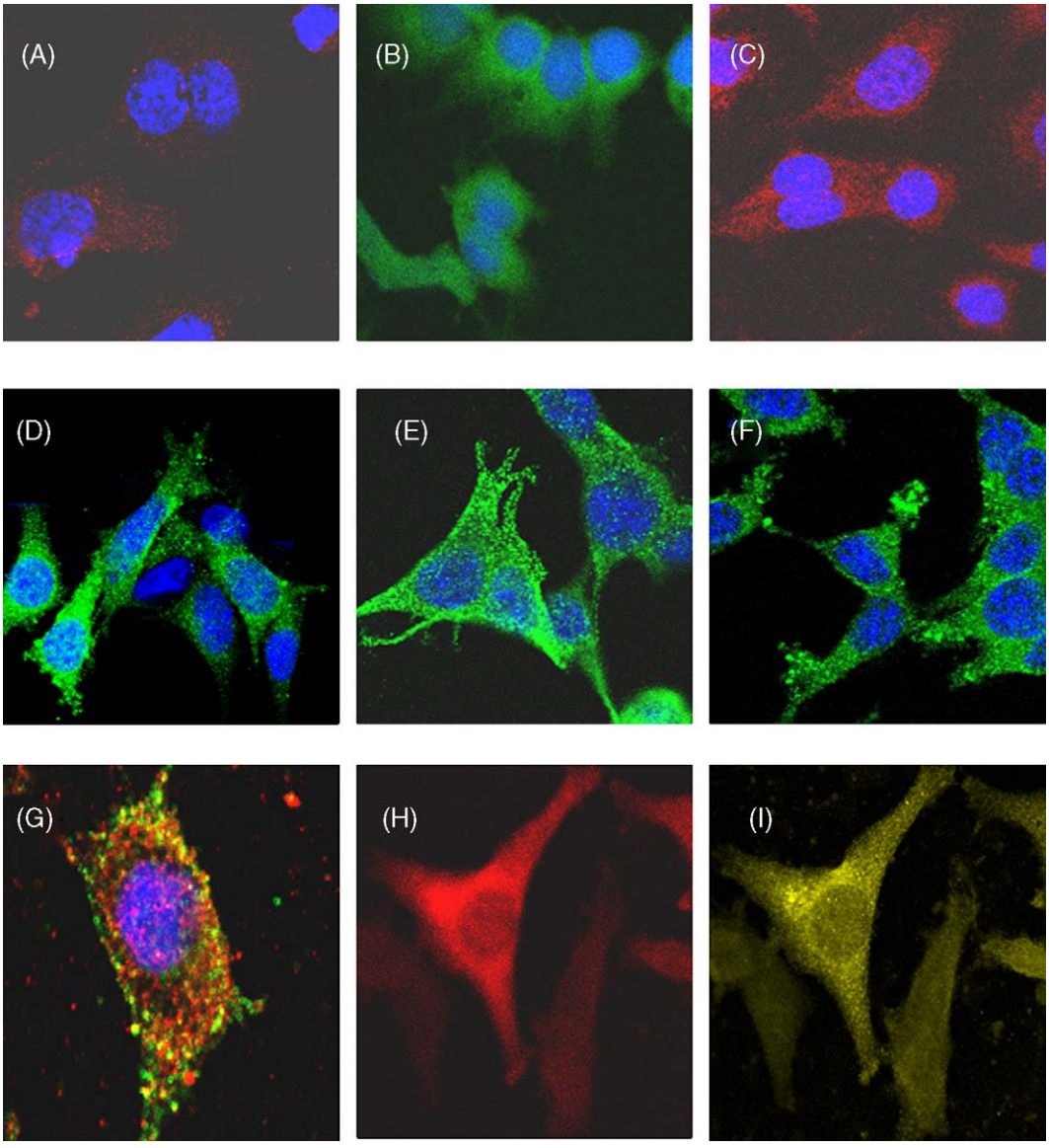


Fig. 1. Oxidative stress leads to formation of cytoplasmic aggregates that contain alpha-synuclein and ubiquitin. Upper panel show alpha-synuclein staining in control neuroblastoma cells (A), neuroblastoma cells transfected with WT alpha-synuclein (B) and neuroblastoma cells transfected with A53T mutant alpha-synuclein (C). Nuclei were stained by DAPI (A–G). Diffuse cytoplasmic alpha-synuclein is demonstrated by mouse (B) or rabbit (A, C) anti-alpha-synuclein antibodies followed by Alex-488 (green, B), or Alexa-568 (red, A, C) conjugated antibodies. Exposure to pro-oxidants (rotenone 100 nM (D); SIN-I 0.1 mM (E); $FeCl_2$ 5 mM (F)) resulted in intracytoplasmic alpha-synuclein positive aggregates. The cytoplasmic aggregates were immunopositive for alpha-synuclein (green) and ubiquitin (red), some with double staining (yellow) (G). Thioflavin S (I, yellow) staining of alpha-synuclein aggregates (H, red) indicates that they contain β-sheet conformation.

and proteasomal inhibition resulted in significantly increased levels of aggregates (Fig. 3D). Aggregates formation was significantly increased in neuroblastoma cells expressing mutated alpha-synuclein when exposed to combined treatment with ROS generators and proteasomal inhibition ($p = 0.005$) compared to untransfected neuroblastoma cells, and compared to neuroblastoma cells transfected with WT alpha-synuclein ($p = 0.023$).

These series of experiments indicate that oxidative stress induces cytoplasmic alpha-synuclein aggregates, which are also immunopositive for ubiquitin and thioflavin S staining. Overexpression of WT alpha-synuclein increases the rates of aggregates formation. Neuroblastoma cells transfected with A53T mutant alpha-synuclein are hypersensitive to oxidative stress and have increased rate of aggregates formation when exposed to ROS. Proteasomal inhibition promotes formation of alpha-synuclein and ubiquitin aggregates, more when aberrant alpha-synuclein (A53T) is expressed. Protesomal inhibition increases the rate of aggregates formation in response to oxidative stress, emphasizing the synergistic effect of both insults.

The steps that lead to alpha-synuclein aggregation in Lewy bodies are key to understanding the pathogenesis of PD. Although the discovery of alpha-synuclein mutations in patients suffering from familial PD has improved our understanding of its importance in the disease pathogenesis, the vast majority of parkinsonian patients suffers from sporadic disease and does not harbor alpha-synuclein mutations. This notion indi-

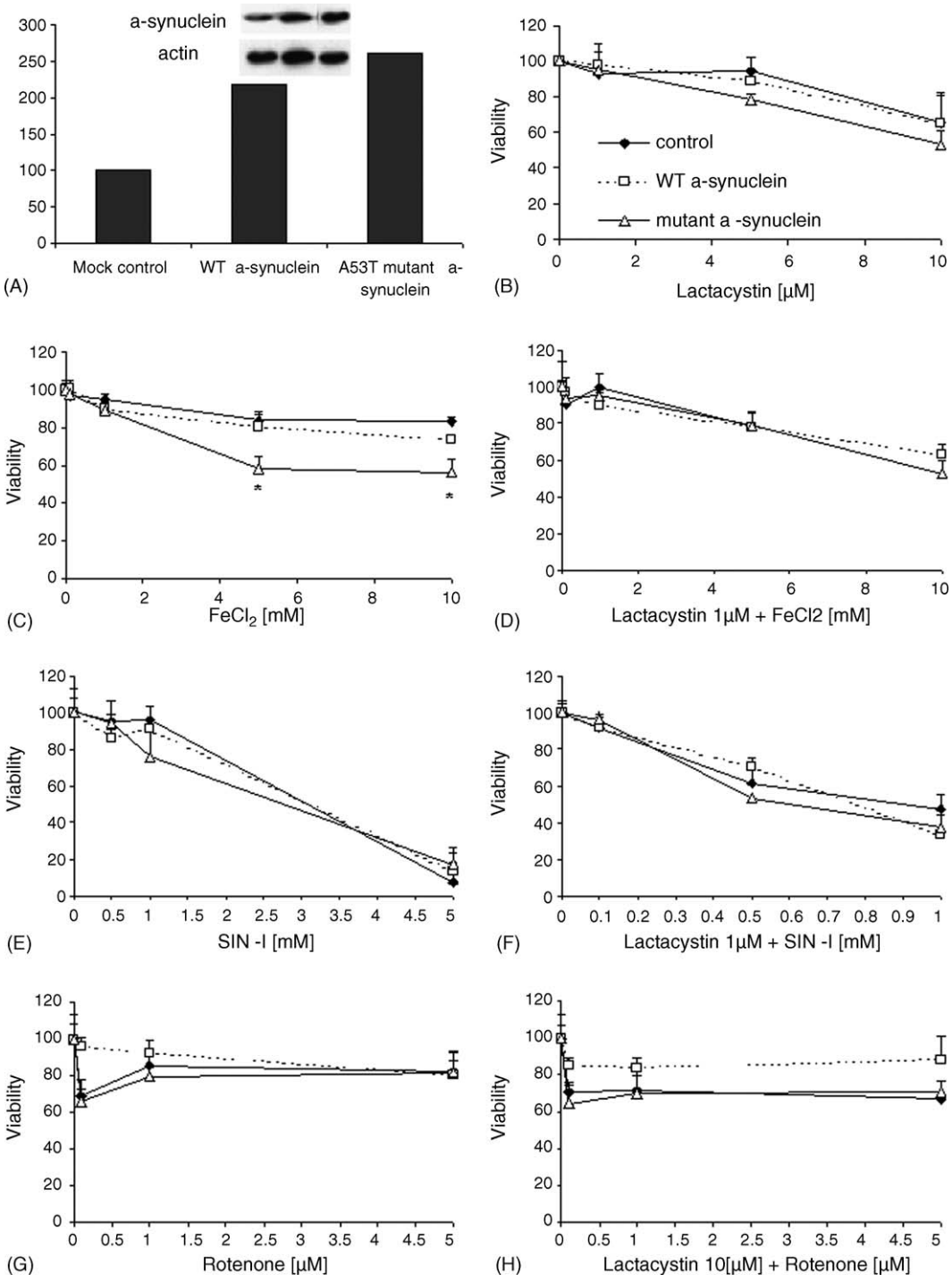


Fig. 2. Reactive oxygen species and proteasomal inhibition induce neuroblastoma cell death. Neuroblastoma cells stably transfected with WT alpha-synuclein and A53T mutant alpha-synuclein demonstrated elevated levels of alpha-synuclein indicated by Western blot (A). Cells (control, WT and mutated alpha-synuclein) were exposed to increasing concentrations of Lactacystin (0–10 μM) (B), $FeCl_2$ (0–10 mM) (C), lactacystin 1 μM + $FeCl_2$ (0–10 mM) (D), SIN-I (0–5 mM) (E), lactacystin 1 μM + SIN-I (0–1 mM) (F), rotenone (0–5 μM) (G), and lactacystin 10 μM + rotenone (0–5 μM) (H). Cell survival was measured with MTT viability assay. Bars are means ± S.E.M., expressed as percent of untreated controls. (*) indicates $p < 0.05$.

cates that a complex interaction between genetic susceptibility and environmental factors as well as age related factors are of supreme importance in the pathogenesis of PD. We found that oxidative stress as well as proteasomal inhibition promotes alpha-synuclein aggregation, and that the effects are dose dependent.

Oxidative stress is considered one of the major environmental insults leading to neurodegeneration, and to PD in particular [12]. Environmental factors such as pesticides, herbicides, and industrial chemicals have been identified as potential risk factors for PD, primarily through their oxidative stress induction [12,25]. The role of alpha-synuclein as a potential target of intra-

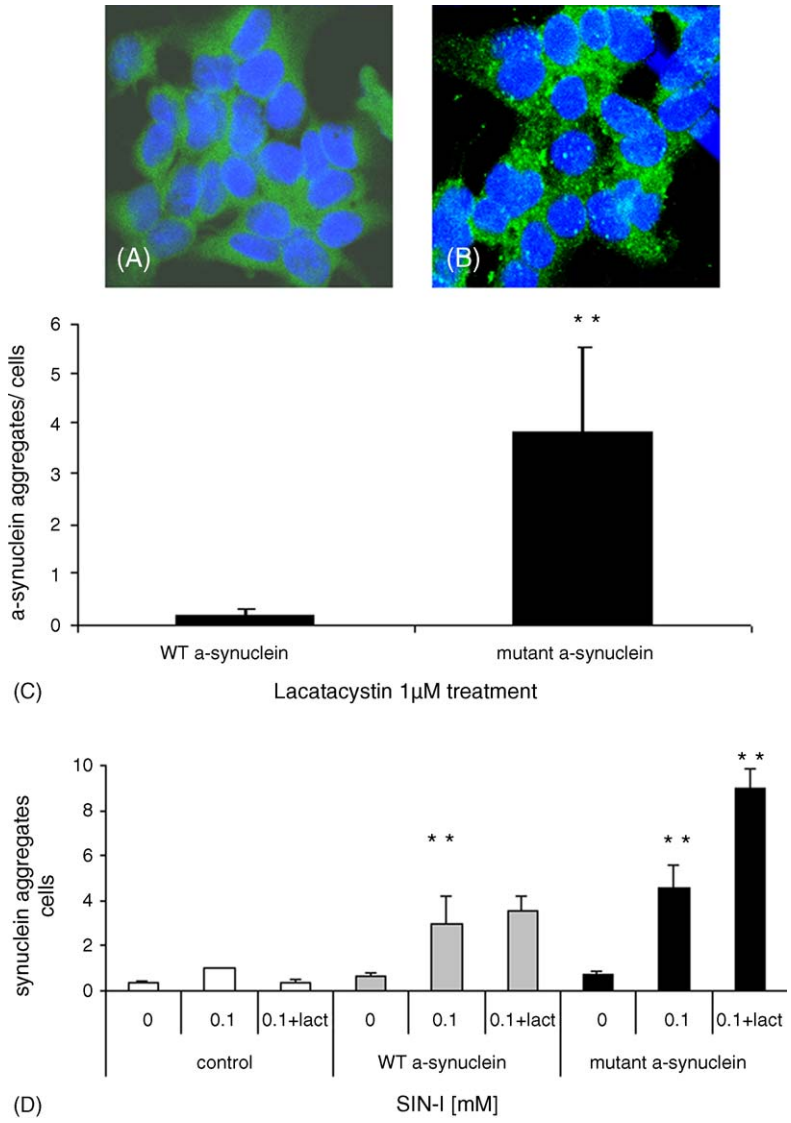


Fig. 3. Proteasomal inhibition induces alpha-synuclein positive cytoplasmic inclusions, and acts synergistically with oxidative stress on aggregates induction. Lactacystin induces high level of cytoplasmic alpha-synuclein aggregates in cells expressing A53T mutant alpha-synuclein (B) as compared to neuroblastoma cells expressing WT (A) alpha-synuclein ($^{**}p < 0.01$). Aggregates quantification using Image Pro Plus software is shown for the treatments with lactacystin (C), SIN-I (D), and combined treatments with SIN-I and lactacystin (D) ($^{**}p < 0.01$). Accelerated formation of alpha-synuclein-positive aggregates induced by SIN-I and lactacystin in neuroblastoma cells overexpressing WT or A53T mutant alpha-synuclein as compared to control ($^{**}p < 0.01$) (D). Significantly increased aggregates are found in neuroblastoma cells expressing mutated alpha-synuclein exposed to combined treatments with ROS generators and proteasomal inhibition compared to cells exposed only to oxidative stress ($^{**}p < 0.01$), and compared to combined treatments of untransfected neuroblastoma cells ($^{**}p < 0.01$), and neuroblastoma cells transfected with WT alpha-synuclein ($^{**}p < 0.01$) (D).

cellular oxidants has been demonstrated by the identification of posttranslational modifications of alpha-synuclein within intracellular aggregates that accumulate in PD brains, as well as the ability of a number of oxidative insults to induce alpha-synuclein oligomerization [3].

Rideout et al. [23] were the first to show that PC12 cells form discrete cytoplasmic inclusions that are immunopositive for ubiquitin when treated with proteasomal inhibition. Additionally, a proportion of the inclusions also showed synuclein-1 immunoreactivity (the rat homologous protein to alpha-synuclein) [23]. McNaught et al. [16] demonstrated in primary ventral mesencephalic cultures that inhibition of normal proteasomal function induced the degeneration of dopaminergic neurons and the formation of inclusion bodies that stain positively for alpha-synuclein and ubiquitin. Ardley et al. [1] demonstrated that over expression of UCH-L1 also forms aggregates in response to proteasomal inhibition. Disease associated mutations in UCH-L1, which affect enzymatic activities, significantly increased the number of inclusions [1]. Systemic administration of proteasomal inhibitors into rats caused both clinical parkinsonian characteristics and neurodegeneration of the nigrostriatal pathway, locus coeruleus, dorsal motor nucleus of the vagus, and the nucleus basalis of Meynert accompanied with alpha-synuclein inclusion bodies [18]. These observations suggest that impairment of proteasomal function in SNc neurons could play an important role in the neurode-

generative process occurring in both familial and sporadic PD [13].

Since neurons do not regenerate, it is of great importance that adequate UPS function be maintained to degrade unwanted proteins and facilitate neuronal viability. Our findings demonstrate that proteasomal inhibition augments aggregate formation induced by oxidative stress. Our results are in agreement with a recently published study by Mytilineou et al. [20], who found that conditions promoting protein damage and misfolding such as oxidative stress, heat shock, and canavanine also induce neuronal degeneration with preferential loss of dopamine neurons and that cell death is markedly increased when any of these is combined with a proteasome inhibitor [20].

These observations may shed new light on the pathogenesis of PD as a disease caused by a double hit sequence: proteasomal inhibition on one hand and an environmental insult causing protein damage, such as increased oxidative stress, on the other. The combination of these events may lead to a proteinopathy and sequentially to cell death. Proteinaceous aggregates formation may be a result of a failing protective system or may be directly toxic to the dopaminergic neurons. However, since aggregates are detected in cells exposed to sub-lethal doses of ROS generators, as well as to sub-lethal levels of the proteasomal inhibitor lactacystin, it may suggest that aggregation is an earlier event. The appearance of these proteinaceous aggregates can be looked upon as a marker for cellular stress. Our cellular model is a convenient and informative model for studying the molecular mechanisms of the disease process in PD and can be used as a tool for examining future drug therapies.

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