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Parkinson's Disease-linked protein α-synuclein: do small-molecule inhibitors of protein aggregation also prevent its accumulation with lipids?

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Abstract

Parkinson's Disease (PD), a neurodegenerative disorder, is characterized by the pathological aggregation of α-synuclein (αS) into Lewy bodies, primarily composed of fibrillar and lipid/membrane-rich αS inclusions. Recent studies suggest that both fibrillar and lipid-rich αS aggregates may contribute to PD pathology. This study investigates how lipid-rich αS inclusions are affected by small molecules that had previously been shown to disaggregate fibrillar αS protein aggregation. Using a live-cellular assay based on YFP-tagged membrane-accumulating, engineered αS-3K (E35K+E46K+E61K) in M17D neuroblastoma cells, we assessed the effects of fasudil, Epigallocatechin gallate (EGCG), anle138b, and ethanol compared to the positive control trifluoperazine (TFP), which is known to disassemble lipid-rich αS aggregates. fasudil, EGCG, and anle138b had previously been shown to prevent protein aggregation of αS. In our experiments, fasudil and anle138b significantly decreased inclusion formation in a dose-dependent manner in both αS lipid-rich inclusion prevention and reduction assays, while EGCG and ethanol exhibited either negligible or inclusion-increasing effects. These findings indicate that fasudil and anle138b may effectively target lipid-rich αS inclusions, supporting further exploration of their therapeutic potential in PD. However, fasudil's observed associated toxicity necessitates careful evaluation. This study underscores the therapeutic relevance of targeting diverse αS aggregate types in PD.

Keywords

α-synuclein, Protein aggregation, Parkinson's Disease, Lewy bodies, Lipids, Cellular assay, Fasudil, Trifluoperazine, anle138b, Epigallocatechin gallate

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Introduction

Parkinson's Disease (PD) is a neurodegenerative disorder that impacts the nervous system, causing stiffness, slowing of movement, tremors and other symptoms. It is believed that the aggregation of the 14 kDa protein α-synuclein (αS), composed of 140 amino acids, into neuronal inclusions (Lewy bodies) is the key pathological event that causes PD and other so-called synucleinopathies (2-8). The exact mechanism of how αS converts from a physiological, native to a pathological, aggregated state is not fully understood. Early research has suggested that Lewy bodies mainly comprise beta-sheet structured fibrillar αS (9). However, more recent insight highlights the presence of lipid/membrane-rich αS aggregates, in which fibrillar αS is not a dominant species and other folding states of αS prevail (5). The relative contribution of each type of αS aggregate to pathology is currently under debate, and it seems possible that both aggregates are relevant (10).

Previous research has indicated that αS features an 11-amino acid repeat, with the core consensus sequence KTKEGV appearing imperfectly 6-9 times in the first two-thirds of the protein (2). Strategically mutating certain amino acids in the repeats causes αS to readily form lipid/membrane-rich aggregates that share similarities with lipid-rich Lewy bodies. One example is the αS 3K model (E35K+E46K+E61K) which technically amplifies PD-linked αS E46K (8). These lipid-rich models are considered to be complementary to approaches that lead to fibrillar αS inside of cells, most importantly the application of "pre-formed fibrils" (PFFs) (3 – 9).

Currently, there are no treatments for PD. Several research groups have identified small molecules capable of disaggregating αS fibrils, and such compounds are being developed as PD therapeutics. However, their impact on lipid-rich αS aggregates has not yet been tested. Consequently, this study explores the effect of potential small molecules in an αS-3K::YFP live-cell assay, monitoring the compounds' ability to interfere with lipid-rich αS aggregation. The reporter cells were treated with different small molecules at increasing concentrations, fasudil (7), Epigallocatechin gallate (EGCG) (6) and anle138b (Emrusolmin) (13), all of which have been shown to disaggregate αS protein fibrils (3). We sought to determine whether these compounds have the same effect on lipid-rich inclusions as they reportedly have on fibrillar αS aggregates. The compounds' effects were also compared to a published positive control, trifluoperazine (1) and to the vehicle, DMSO. Ethanol at increasing concentration percentages was also screened to determine whether the liquid alcohol would impact the inclusions generated.

We observed marked reductions of both αS inclusion formation and total protein levels for fasudil and anle138b treatment while EGCG had no αS inclusion- or level-reducing activity. Ethanol increased αS inclusions and apparent levels in the prevention paradigm.

Materials and Methods

Cell Culture

M17D cells were grown in Dulbecco's Modified Eagle's Medium (DMEM), enriched with 10% fetal bovine serum (FBS), 50 units/mL penicillin, 50 µg/mL streptomycin, and 2 mM L-glutamine.

To induce the expression of αS-YFP, doxycycline was added to a final concentration of 1 µg/mL.

Compounds

The positive control utilized in this experiment as a modulator of αS homeostasis was trifluoperazine (TFP); Sigma-Aldrich, St. Louis, MO, T8516, 2019). Ethanol was purchased from Sigma-Aldrich (catalog number: 493546-500ML), EGCG from Fisher Scientific, Walthan, MA (50-163-8008), fasudil from Fisher Scientific (50-187-2031), and anle138b from Fisher Scientific (50-196-7890).

Drug treatments

In the prevention assay, cells were plated on Day 1, treated with compounds on Day 2, induced on Day 3 to express *α*S-3K::YFP and read on Day 5 utilizing live-cell microscopy (Figure 1A). In the clearance assay, cells were plated on Day 1, induced on Day 2 to express *α*S-3K::YFP, treated on Day 4 with drugs, and read on Day 5-6 after ensuring similar inclusion populations (Figure 2A).

Imaging

Inclusion formation was recorded at 4-hour intervals in αS-3K::YFP cells on a 96-well plate using an IncuCyte S3-C2 machine (Essen Bioscience, Ann Arbor, MI). The following parameters were used in the processing definition "Inclusions6". Segmentation: Surface Fit; Threshold (GCU): 2.0000; Edge Split On; Edge Sensitivity: 100; Hole Fill (μm^2): 0.0000; Adjust Pixel Size: -2; Area (μm^2) - min: (none), max: 40.000; Eccentricity - min: (none), max: 0.8500; Mean Intensity - min: 120.00, max: (none); Integrated Intensity: (none).

Results

Screening of molecular compounds in prevention assay

Given their effectiveness against αS fibrillar aggregates, we tested the effects of fasudil (7), EGCG (6) and anle138b (13) on lipid-rich αS-inclusion in a "prevention" paradigm. Inducible αS-3K::YFP inclusion-forming cells were plated in a 96-well microtiter plate at 5000 cells per well. Subsequently, the cells were treated with the drugs in a gradient format (0 µM, 3 µM, 10 µM, 30 µM) for 24 hours, and induced for αS expression using doxycycline for another 24 hours. Inclusions were quantified and imaged 48 hours after initiation of treatment. All values were analyzed relative to the DMSO vehicle (0 µM).

Our positive control, TFP, had previously demonstrated positive effects in preventing inclusion formation on lipid-rich αS aggregates (8). As expected, a pre-prepared constant concentration of 8 µM TFP prevented a significant portion of inclusion development (Figure 1B).

The most compelling results were observed with increasing fasudil and anle138b concentrations, which both showed dose-dependent inclusion lowering. Cells pretreated with 3 µM fasudil showed little to no change in the number of inclusions. However, increasing the concentration to 10 µM fasudil reduced inclusions compared to the DMSO vehicle, while 30 µM fasudil almost completely eliminated them (Figure 1B, C). Similarly, anle138b demonstrated a decrease in inclusion development as drug concentration increased. At 3 µM anle138b, there was a slight reduction in inclusion development, while 10 µM and 30 µM further

decreased the number of inclusions (Figure 1B, C).

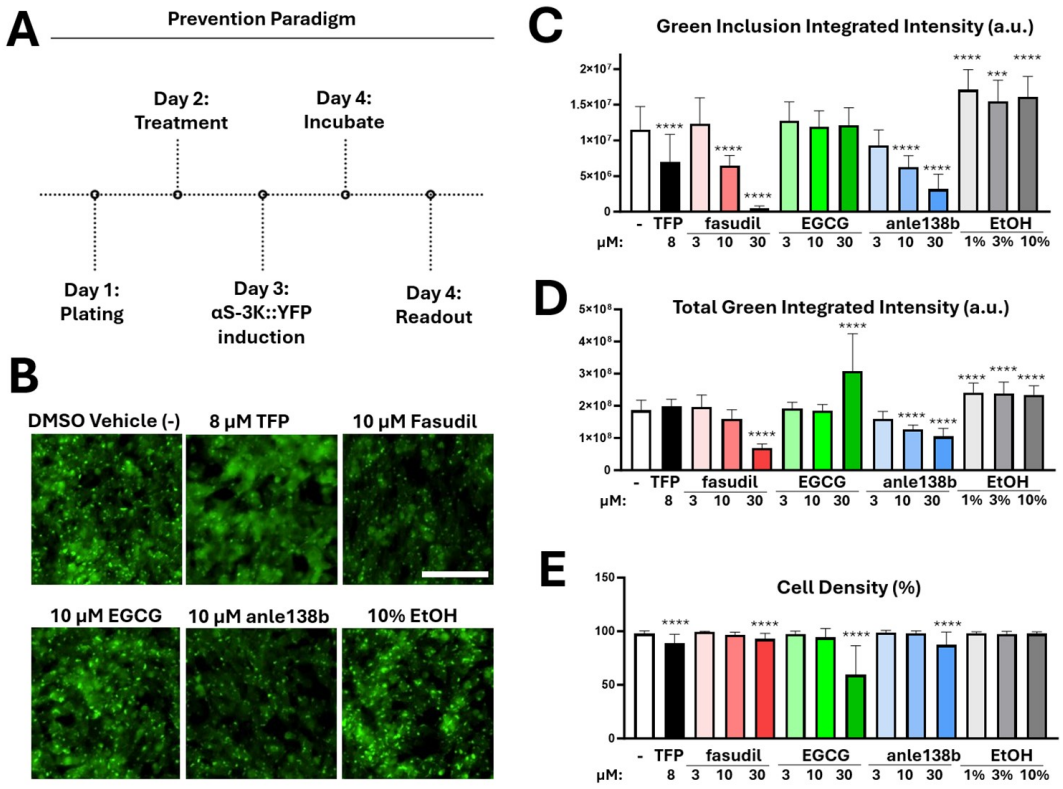

Figure 1. Effect of select drugs on the number of αS inclusions developed relative to DMSO vehicle. **(A)** Timeline of Prevention Assay. **(B)** Representative images (YFP signal) of DMSO vehicle control, 8 µM TFP, 10 µM fasudil, 10 µM anle138b, 10 µM EGCG, and 10% EtOH. Scale bar, 100 µm. Cells were pretreated with drugs and then induced 24 hours later to express αS-3K::YFP, imaging 24 h post-induction. **(C)** Quantification of inclusions, dose-response. Cells were treated with trifluoperazine (TFP, 8 µM), fasudil (3, 10, 30 µM), EGCG (3, 10, 30 µM), anle138b (3, 10, 30 µM), and ethanol (EtOH, 1, 3, 10%). Criteria for significance relative to DMSO vehicle; n = 36; n.s., not significant; ***$P < 0.001$ and ****$P < 0.0001$, Kruskal-Wallis test *(C, top)* Response from concentration dose of 8 µM trifluoperazine relative to DMSO vehicle (0 µM). **(D)** Quantification of total green signal in the same experiment. **(E)** Quantification of cell density in the same experiment.

Interestingly, cells pretreated with EGCG, despite its reported association with disaggregating αS protein fibrils (6), did not inhibit inclusion development (Figure 1B, C). Instead, it had no statistical effect, as inclusion values remained consistent across the 3–30 µM range. Interestingly, EtOH had a negative impact on the αS-3K cells, drastically increasing the number of inclusions compared to the DMSO vehicle (Figure 1B, C). Analysis of total green intensity, as a proxy for αS-3K::YFP protein levels, revealed a significant reduction in 30 µM fasudil, 10 and 30 µM anle138b. 30 µM EGCG were accompanied by an apparent increase in total green signal (Figure 1D). Lastly, quantification of cell density as a proxy for cell health suggested slight toxicity for fasudil and anle138b at 30 µM, while 30 µM EGCG led to pronounced cell loss (Figure 1E).

Screening of molecular compounds in reduction assay

While some drugs yielded positive results and others negative when used in pretreatment,

the compounds were additionally tested for their ability to reduce the size/number of pre-existing inclusions in a reduction assay. In this paradigm, cells were induced for αS-3K::YFP expression and started developing inclusions 24 hours before being treated with individual drugs. Unlike in pretreated αS-3K cells, TFP, when applied to cells in the reduction assay, did not significantly decrease pre-existing inclusions relative to the DMSO vehicle 24 h after treatment (Figure 2B, C).

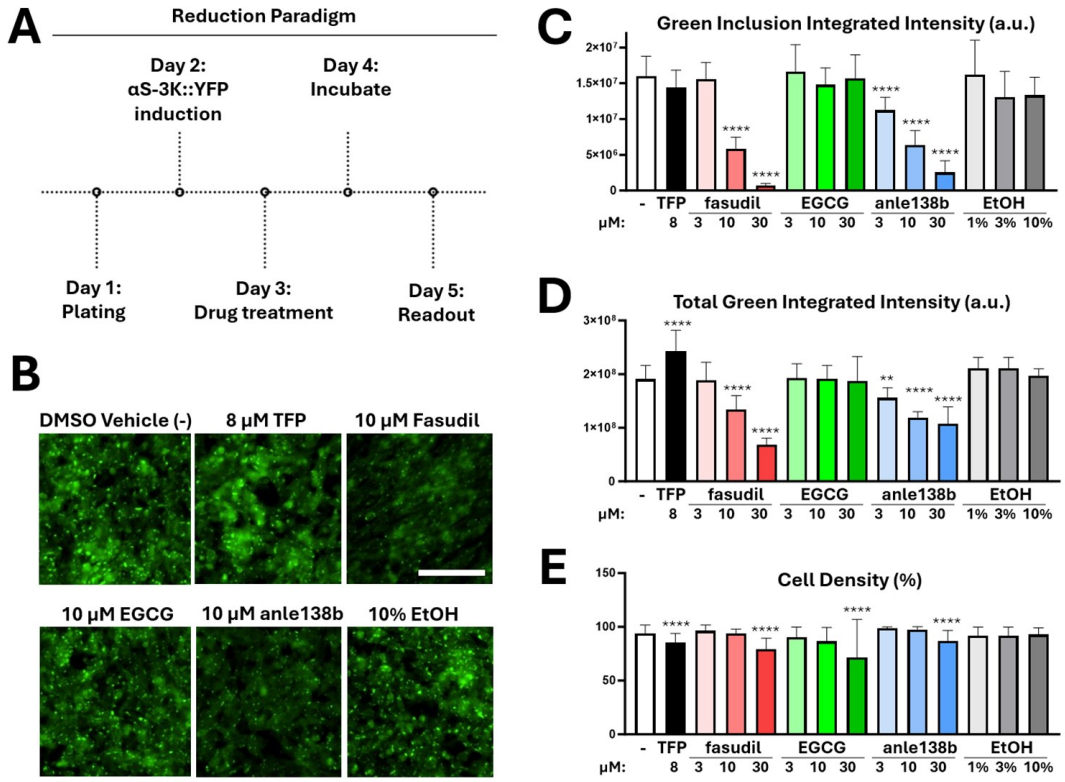

Figure 2. Effect of select drugs on the number of αS inclusions developed relative to DMSO vehicle. **(A)** Timeline of Reduction Assay. **(B)** Representative images (YFP signal) of DMSO vehicle control, 8 µM TFP, 10 µM fasudil, 10 µM anle138b, 10 µM EGCG, and 10% EtOH. Scale bar, 100 µm. Cells were induced to express αS-3K::YFP and treated 24 hours later with the indicated drugs, imaging 24 h post-treatment. **(C)** Quantification of inclusions, dose-response. Cells were treated with trifluoperazine (TFP, 8 µM), fasudil (3, 10, 30 µM), EGCG (3, 10, 30 µM), anle138b (3, 10, 30 µM), and ethanol (EtOH, 1, 3, 10%). Criteria for significance relative to DMSO vehicle; n = 36; n.s., not significant; $^{**}P < 0.001$, $^{***}P < 0.001$, and $^{****}P < 0.0001$, Kruskal-Wallis test. **(D)** Quantification of total green signal in the same experiment. **(E)** Quantification of cell density in the same experiment.

Similar to the pretreated cell cultures, increasing fasudil and anle138b concentrations each lowered inclusion-integrated intensities after treatment. At 3 µM, Fasudil had no effect relative to the DMSO vehicle. However, increasing the concentration to 10 µM fasudil significantly reduced inclusion development, and 30 µM fasudil reduced it even further, almost completely eliminating inclusions (Figure 2B, C). In the case of anle138b, 3 µM mildly reduced pre-existing inclusions, while increasing the concentration to 10 µM and 30 µM led to more pronounced decreases in inclusion burden (Figure 2B, C). EGCG and EtOH had no measurable effect under these

conditions (Figure 2B, C) Analysis of total green signal suggested αS-lowering effects of fasudil (10 µM, 30 µM) and anle138b (3 µM, 10 µM, 30 µM) while TFP increased αS levels under these conditions (Figure 2D). Cell density was found to be reduced by 8 µM TFP and the highest concentrations (30 µM) of fasudil, EGCG and anle138b, in line with cytotoxicity.

For further mechanistic insight, we studied αS inclusions, total levels and cell density only 4 h after drug application in the reduction paradigm (Figure 3A). In this setting, 8 µM TFP, 10 µM fasudil and all tested anle138b concentrations significantly reduced αS inclusions while only 10 µM fasudil slightly reduced total αS levels (Figure 3B, C, D). Lastly, only 30 µM fasudil had a (very slight) effect on cell density.

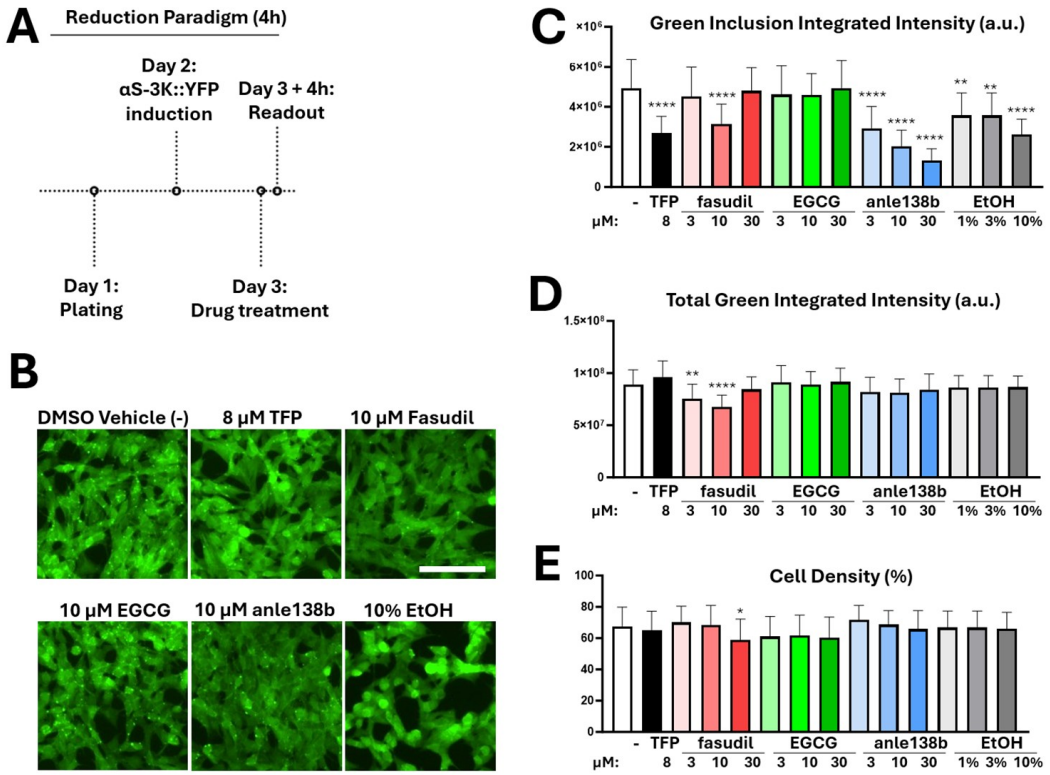

Figure 3. Effect of select drugs on the number of αS inclusions developed relative to DMSO vehicle. **(A)** Timeline of Short-term (4h) Reduction Assay. **(B)** Representative images (YFP signal) of DMSO vehicle control, 8 µM TFP, 10 µM fasudil, 10 µM anle138b, 10 µM EGCG, and 10% EtOH. Scale bar, 100 µm. Cells were induced to express αS-3K::YFP and treated 24 hours later with the indicated drugs, imaging 4h post treatment. **(C)** Quantification of inclusions, dose-response. Cells were treated with trifluoperazine (TFP, 8 µM), fasudil (3, 10, 30 µM), EGCG (3, 10, 30 µM), anle138b (3, 10, 30 µM), and ethanol (EtOH, 1, 3, 10%). Criteria for significance relative to DMSO vehicle; n = 36; n.s., not significant; $*P < 0.001$, $**P < 0.001$, $***P < 0.001$, and $****P < 0.0001$, Kruskal-Wallis test. **(D)** Quantification of total green signal in the same experiment. **(E)** Quantification of cell density in the same experiment.

Discussion

The ongoing debate about whether αS aggregation is predominantly fibrillar or lipid-rich led to the research question of whether established drugs and compounds previously shown to disaggregate αS fibrils (typically in reduction assays) would have the same effect on lipid-rich αS aggregates cell

culture. The present cell model utilized in this experiment, M17D neuroblastoma cells inducibly expressing αS-3K::YFP, robustly and consistently produce lipid-rich αS aggregates in a cell-autonomous manner (8).

With the exception of ethanol and EGCG, three of the five drugs tested—TFP, anle138b, and fasudil—showed promise in reducing inclusion intensity. Notably, fasudil and anle138b, which have previously been shown to disaggregate pre-formed αS fibrils, also demonstrated this potential in lipid-rich cell cultures, where αS tends to accumulate at membranes. These results suggest that, regardless of whether αS pathology is driven by beta-sheet aggregation or lipid-rich accumulation, these two drugs could positively decrease inclusion formation and benefit potential patients treated with these compounds. Unlike TFP, both fasudil and anle138b may act by a dual mechanism: TFP prevented (Figure 1) and dissolved existing inclusions upon short-term treatment (Figure 3) while not affecting total αS levels (total green signal). In contrast, fasudil and anle138b reduced both inclusions and total αS levels in the prevention (Figure 1) and in the reduction (Figure 2) assays. In the short-term (4h) reduction paradigm (Figure 3), however, anle138b only reduced inclusions and left total green levels unchanged. This may indicate that the compound first dissolves lipid-rich aggregates and then promotes their degradation by an unknown mechanism. Fasudil (10 μM), in contrast, reduced both αS total levels and inclusions already after 4 hours in the reduction paradigm, indicating that dissolution of inclusions and degradation may occur simultaneously.

Of note, the overall lack of total green reduction in the 4h reduction paradigm (Figure 3) suggests that none of the drugs creates artifacts by quenching YFP signals: such a physical effect on fluorescence would be expected to be immediate. Our data also indicate that our dose range (3, 10, 30 μM) was relatively well selected: 30 μM treatments were invariably associated with toxicity for all the drugs tested (lower cell densities in Figure 1E and 2E), suggesting that lower doses should also be used in *in vivo* testing. 10 μM fasudil and anle138b both reduced αS inclusions and levels (even in the 4h reduction paradigm). 3 μM, however, was only effective in the case of anle138b. While we did not formally determine IC_{50} values, our data suggest that 5-15 μM fasudil or anle138b can dissolve at least 50% of αS inclusions. In the case of anle138b, prior research indicates the drug's capability to penetrate the blood-brain barrier, BBB (13). With fasudil, past findings indicate that the drug has BBB-enhancing and protecting properties as well as increased cell impedance (14).

Although previous research has shown that anle138b is non-toxic and can be implemented as a drug (11), further studies are required to mitigate or bypass the toxic effects if cells encounter doses > 1 μM *in vivo*. The same can be said about fasudil before it can be considered for clinical use (12). Overall, our study suggests that these two compounds might act on different aspects of αS misfolding, being capable of disrupting both beta-sheet and lipid-rich aggregates, and possibly also triggering αS degradation. A possible interpretation of our data, however, is that the lipid-rich aggregates are more fibrillar than previously thought - and that is why fasudil and anle138b dissolve them.

Future studies should further characterize the disruption of lipid-rich and fibrillar αS aggregates by these and other drugs using biochemical and biophysical methods.

Additionally, we did not explicitly measure αS redistribution—that is, αS levels in the cytosol versus in the membrane. This could be achieved through sequential protein extraction followed by Western blotting (8). In past studies, a reduction in lipid-rich αS inclusions was concurrently associated with decreased αS membrane binding. The observed reduction in αS protein levels following fasudil and anle138b treatment in our degradation assays would generally suggest a proportional decrease in both cytosolic and membrane-associated αS, given their equilibrium. However, our experimental setup did not rule out the possibility that either cytosolic or membrane-bound αS was preferentially degraded. Moreover, we did not measure αS multimer-to-monomer (αS60:14) ratios, where monomer accumulation is typically linked to neurotoxicity, as this was outside the scope of the project.

Conclusion

In this study, multiple compounds previously shown to disaggregate proteinaceous αS fibrils were tested to determine whether they could also disaggregate lipid-rich αS inclusions, using both prevention and reduction assays. The assays utilized a doxycycline-inducible system to express YFP-tagged αS-3K (E35K, E46K, and E61K mutations, with an emphasis on the "amplified E46K") in neuroblastoma cells. The primary data obtained were inclusion counts, measured using the Incucyte system. The results indicate that fasudil and anle138b significantly decreased inclusion intensity with increasing concentrations in both assays. In contrast, EGCG and ethanol showed no change and an increase in inclusion development, respectively. These findings suggest that regardless of the exact nature of αS aggregation, certain drugs are capable of reducing inclusion intensity, with important implications for PD drug development.

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