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Targeting alpha-synuclein oligomers by protein-fragment complementation for drug discovery in synucleinopathies

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Abstract

Objective—Reducing the burden of alpha-synuclein oligomeric species represents a promising approach for disease-modifying therapies against synucleinopathies such as Parkinson's disease and dementia with Lewy bodies. However, the lack of efficient drug discovery strategies that specifically target alpha-synuclein oligomers has been a limitation to drug discovery programs.

Research design and methods—Here we describe an innovative strategy that harnesses the power of bimolecular protein-fragment complementation to monitor synuclein–synuclein interactions. We have developed two robust models to monitor alpha-synuclein oligomerization by generating novel stable cell lines expressing alpha-synuclein fusion proteins for either fluorescent or bioluminescent protein-fragment complementation under the tetracycline-controlled transcriptional activation system.

Main outcome measures—A pilot screen was performed resulting in the identification of two potential hits, a p38 mitogen-activated protein kinase inhibitor and a casein kinase 2 inhibitor, demonstrating the suitability of our protein-fragment complementation assay for the measurement of alpha-synuclein oligomerization in living cells at high-throughput.

Conclusions—The application of the strategy described herein to monitor alpha-synuclein oligomer formation in living cells with high-throughput will facilitate drug discovery efforts for disease-modifying therapies against synucleinopathies and other proteinopathies.

Keywords

alpha-synuclein; high-throughput screening; Parkinson's disease; protein-fragment complementation; oligomers; synucleinopathy

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Declaration of interest

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1. Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disorder and currently there are no disease-modifying treatments. The disease is pathologically characterized by the loss of dopaminergic neurons within the midbrain and the abnormal presence of Lewy bodies which are intra-cellular aggregates of α -synuclein protein (α syn) ¹⁻³. α Syn aggregation is also commonly found in related neurodegenerative disorders such as Parkinson's disease with dementia, dementia with Lewy bodies and multiple system atrophy ⁴. Moreover, duplication, triplication and missense mutations in the α syn gene (*SNCA*) are responsible for familial forms of parkinsonism ⁵⁻¹¹ and common polymorphisms in the non-coding region of the *SNCA* gene are strongly associated with PD susceptibility ¹². In mice ¹³⁻¹⁶, rats ¹⁷⁻¹⁹ and fruit flies ²⁰, overexpression of the human α syn protein leads to α syn aggregation and dopaminergic denervation.

α Syn aggregation is a significant target for the development of therapeutic strategies against synucleinopathies. Although the exact mechanisms by which α syn aggregation induces toxicity remains unclear, there is a body of evidence that points to the prefibrillar α syn oligomers as a source of α syn-induced toxicity ²¹⁻²⁷. α Syn oligomers are submicroscopic, soluble, discrete polymers that are extremely challenging to detect. α Syn residues do not form covalent bonds and their oligomerization is highly dynamic and sensitive to external conditions such as concentration, temperature and medium composition ^{21, 22, 28-30}. For these reasons, α syn oligomers are only detectable using time-consuming biochemical techniques such as native gel electrophoresis, density gradient centrifugation and size exclusion chromatography, or microscopic methods such as atomic force microscopy and fluorescence intensity distribution analysis ²². A major drawback of these techniques is that none enable the study of α syn oligomerization in a live cellular environment in real-time. Furthermore these approaches are time-consuming, labor intensive and not scalable for use in a high-density microtiter plate format. Consequently the difficulty in assaying α syn oligomerization and the challenges of finding small molecules that specifically and potently target protein-protein interactions, such as those mediating α syn oligomerization, have prevented serious consideration of α syn as viable target for drug discovery.

Here, we report on the development of two assays in which a bimolecular protein-fragment complementation assay (PCA) enables rapid and non-destructive report of α syn oligomerization in living cells. Moreover, we demonstrate that this elegant approach is amenable to high-throughput screening (HTS) to identify inhibitors of α syn oligomerization.

2. Methods

2.1. Plasmids and construct generation

A tetracycline-driven bi-directional expression plasmid containing the flippase recognition target (*FRT*) sites was constructed by combining the pTRE3G-BI (Clontech, USA) with the pcDNA5/*FRT* (Life technologies, USA) plasmid. The pcDNA5/*FRT* fragment 1603-2963 was amplified by high-fidelity polymerase chain reaction (KOD Hot start, EMD Millipore, Germany) with the following primers (forward: 5'-GCGGCATGTGAAGTTCCTATTCCGAAG-3' and reverse: 5'-

GCGACATGCGGTCGACGGTATACAG-3') that incorporate NspI restriction enzyme sites for sub-cloning in pTRE3G-BI. The α syn fusion protein constructs, α syn + N-terminal half of *Gaussia* luciferase (= SL1) and α syn + C-terminal half of *Gaussia* luciferase (= SL2), N-terminal half of Venus YFP + α syn (= V1S), α syn + C-terminal half of Venus YFP (= SV2) were generated previously^{23, 31, 32}. SL1 and SL2 were sub-cloned into pTRE3G-BI-*FRT*Hygro at the NotI/EcoRV and SmaI/Acc65I restriction sites, respectively. SV2 and V1S were inserted into pTRE3G-BI-*FRT*-Hygro at the SmaI/Acc65I and PspOMI/SgrAI restriction sites, respectively. The control fusion constructs with leucine zipper motives, leucine zipper + N-terminal half of *Gaussia* luciferase (= LzL1), leucine zipper + C-terminal half of *Gaussia* luciferase (= LzL2), N-terminal half of Venus YFP + leucine zipper (= V1Lz) and leucine zipper + C-terminal half of Venus YFP (= LzV2) were generated previously^{33, 34}. LzL1 or V1Lz were sub-cloned into pTRE3G-BI-*FRT*-Hygro at the NotI/EcoRV restriction sites and LzL2 or LzV2 at the SmaI restriction site. All plasmids were verified by sequencing.

2.2. Cell line generation

Human H4 neuroglioma cells (HTB-148, ATCC, USA) were transfected with the pTet-Off plasmid (Clontech, USA) and a stable clone was selected by geneticin resistance. The resulting H4 Tet-Off cell line was subsequently transfected with the p*FRT*/LacZeo plasmid (Life tech) to generate H4 TetOff *FRT* cell lines. A stable single integrant clone with high beta-galactosidase activity and zeocin resistance was then selected. H4 TetOff *FRT* empty cells were cotransfected with the pOG44 plasmid (Life tech), for transient expression of the Flp recombinase, and with either pTRE3G-BI-*FRT*-Hygro-V1S&SV2, pTRE3G-BI-*FRT*-Hygro-SL1&SL2, pTRE3G-BI-*FRT*-Hygro-V1Lz&LzV2 or pTRE3G-BI-*FRT*-Hygro-LzL1&LzL2 for generation of the H4 V1S&SV2, H4 SL1&SL2, H4 V1Lz&LzV2 or H4 LzL1&LzL2 cell lines, respectively. Stable clones resistant to hygromycin B but not to zeocin were then selected, confirming the recombinase-conducted insertion of our genes of interest at the *FRT* site, thus guaranteeing the isogenicity of our cell lines.

2.3. Cell maintenance

All cell lines were maintained at 37°C in a 95% air / 5% CO₂ humidified incubator in Opti-MEM medium with 10% fetal bovine serum supplemented with 200µg/ml geneticin, 300µg/ml zeocin, 200µg/ml hygromycin B and 1µg/ml tetracycline as necessary (Life tech). Cells were split once a week and frequently tested for mycoplasma contamination.

2.4. Immunofluorescence

Cells were plated on 15mm coverslips. After 48h incubation with or without 1µg/ml tetracycline, cells were washed with phosphate-buffered saline (PBS) and subsequently fixed for 20min using a 2% formaldehyde solution in PBS. After washing, cells were permeabilized and blocked using a solution containing 0.05% saponin, 1% bovine serum albumin and 5% goat serum in PBS. Cells were incubated overnight at 4°C with anti- α syn antibodies (1:500; syn-1, 610787, Becton Dickinson, USA), followed by 1h incubation at room temperature (RT) with Alexa Fluor®647-conjugated secondary anti-mouse IgG antibodies (1:1000; A21235, Life tech) and 4µM of Hoechst 33342 dye (Life tech). Control

stainings were performed with secondary antibodies alone. Coverslips were mounted on microscope slides and cells were visualized using an inverted confocal microscope (LSM 510 META, Carl Zeiss, Germany).

2.5. Flow cytometry

Flow cytometry was used to correlate α syn expression to the fluorescence resulting from the Venus YFP protein-fragment complementation. After 48h incubation with or without 1 μ g/ml tetracycline, cells were suspended in PBS supplemented with 1% bovine serum albumin and 2.5% goat serum and subsequently fixed and permeabilized following supplier's instructions (Leucoperm, AbD serotec, USA). Cells were incubated for 1h at RT with anti- α syn antibodies (1:500; syn-1, 610787, BD) followed by 1h at RT with Alexa Fluor®647-conjugated anti-mouse IgG secondary antibodies (1:1000; A21235, Life tech). Unstained cells and control stainings with isotype control antibodies were also performed. The samples were measured on a flow cytometer (Accuri™C6, BD) and data analysis was performed using FlowJo software (Tree Star Inc., USA).

2.6. Immunoblotting

Cells were washed with PBS and then lysed (150mM NaCl, 1mM EDTA, 1mM EGTA, 20mM Tris, 0.5% IGEPAL CA-630, pH 7.4) supplemented with inhibitor cocktail for phosphatases (Halt phosphatase, Thermo Fisher Scientific, USA) and proteases (cOmplete Mini, Roche Diagnostics). Protein concentration was determined by bicinchoninic acid assay (Thermo Fisher Sci). Equal amounts of protein were separated on Bis-Tris polyacrylamide gradient gels (NuPAGE Novex 4-12% Bis-Tris Gel, Life tech) and transferred to polyvinylidene difluoride membranes (EMD Millipore). Membranes were then blocked for 1h at RT in TBS-T (500mM NaCl, 20mM Tris, 0.1% Tween 20, pH 7.4) supplemented with 10% nonfat dried milk. Subsequently membranes were incubated overnight at 4°C with primary antibodies followed by 1h at RT with HRP-conjugated secondary antibodies (1:1000; 1010-05 or 4010-05, Southern biotech, USA). Tagged and untagged α syn (Fig. 2 A-E, 4 B and Supplemental Fig. 1) were detected using a mouse monoclonal antibody against human α syn (1:2000; 4B12, SIG-39730, Covance, USA), whereas LzL1 and LzL2 (Fig. 2 G and 4 C) and V1Lz and LzV2 (Fig. 2 F) were detected using antibodies against *Gaussia* luciferase (1:2500; 401P, NanoLight tech, USA), or against GFP (1:2500; ab6556, Abcam, UK). Anti-GFP and anti-luciferase are rabbit polyclonal antibodies raised against the recombinant full length proteins and can consequently recognize both halves of the split protein. Loading accuracy was controlled by actin detection (1:10000; rabbit polyclonal, A2668, Sigma-Aldrich, USA) and 17-AAG treatment efficiency by heat shock protein 70 (Hsp70) induction (Fig. 4 B and C) (1:5000; rabbit polyclonal, ADI-SPA-812, Enzo Life Sciences, USA). Detection was performed using an enhanced chemiluminescent detection system (EMD Millipore) and a CCD imaging system (LAS-4000, Fujifilm, Japan).

2.7. Protein crosslinking

Prior to cell lysis, cells were washed with PBS and incubated 30min in PBS supplemented with 1mM dithiobis(succinimidyl propionate) (Thermo Fisher Sci) for chemical crosslinking

of the intracellular proteins. After crosslinking, cells were washed and the reaction was quenched by addition of PBS supplemented with 20mM Tris, pH 7.4. Thereafter cells were lysed and protein concentrations were determined as described above. Equal amounts of protein were loaded on polyacrylamide gels (NuPAGE Novex 4-12% Bis-Tris Gel, Life tech). In parallel, decrosslinking was performed by incubation of the samples with 50mM of the reducing agent DL-1,4-dithiothreitol (Sigma-Aldrich) 15min at RT followed by 5min at 95°C prior to loading on the gel.

2.8. Bioluminescent protein-complementation assay

H4 SL1&SL2 and LzL1&LzL2 cells were plated into 96-well microtiter plates at 30000 cells/well. Cells were incubated with or without 1µg/ml tetracycline, 100nM 17-allylamino-17-demethoxygeldanamycin (17-AAG, PubChem CID: 6440175, #A8476, Sigma-Aldrich), and/or dimethyl sulfoxide (DMSO). After 48h at 37°C, cells were washed three times with PBS followed by addition of 100µl of fresh Opti-MEM media minus phenol red and fetal bovine serum. Cytotoxicity (ToxiLight™, Lonza, Switzerland) and bioluminescence produced by luciferase complementation was measured on a plate reader (Victor³V, PerkinElmer) after injection of 100µl per well of 40µM coelenterazine, the substrate of the *Gaussia* luciferase (NanoLight tech). For HTS, the assay was optimized and miniaturized into 384-well plates. H4 SL1&SL2 cells were plated at 8000 cells/well and incubated for 48h at 37°C with or without 1µg/ml tetracycline and in the presence of compounds or DMSO. Media was removed and 40µM coelenterazine was added in the dark immediately followed by luminescence measurement with 0.1s integration time using an EnVision plate reader (PerkinElmer). In a separate experiment under the same treatment conditions, cytotoxicity was assessed using the ATPlite assay (PerkinElmer) as described previously ³⁵.

2.9. Miniaturization and optimization of the fluorescent protein-complementation assay

H4 V1S&SV2 cells were plated into 384- or 1536-well microtiter plates at 8000 and 2000 cells/well, respectively. To assess assay performance and stability in HTS format, cells were incubated with or without 1µg/ml tetracycline and in the presence of varying concentrations (0-2.0%, v/v) of DMSO for 24 to 96h at 37°C. After incubation, the plates were placed at RT for 10 minutes, the assay media was removed and fluorescence (Ex485/Em535nm) was measured using an EnVision plate reader (PerkinElmer). Immediately following the fluorescence read, cytotoxicity was assessed using the ATPlite luminescence assay (PerkinElmer) ³⁵.

2.10. Implementation of α syn-PCA assays for HTS

After miniaturization and optimization for HTS, pilot testing was conducted on the H4 V1S&SV2 cells employing a library of pharmacologically active compounds (LOPAC, Sigma-Aldrich). H4 V1S&SV2 cells were plated as described above (2000 cells/well) in media lacking tetracycline in columns 3-48 of a 1536-well plate. Negative control (high signal) wells (columns 3 and 4) included the addition of DMSO (1% final concentration v/v). Positive control (low signal) wells included cells plated in columns 1 and 2, in media containing 1µg/ml tetracycline and 1% DMSO. Compounds were dispensed in columns 5-48

of a 1536-well assay plate, at a final concentration of 5 and 10 μ M and incubated for 24h at 37°C. The screening window coefficient, Z' factor, was calculated for wells containing H4 V1S&SV2 in the presence and absence of 1 μ g/ml tetracycline. Results from the pilot screen were formatted and plotted as a histogram. Hits were selected using a cut-off value 50% of maximum average signal obtained in the absence of tetracycline (high signal) that was 6 times greater than the average standard deviation. Active compounds were immediately triaged for tetracycline-like structures using automated structure clustering. Compounds that were not cytotoxic, and were not tetracycline-like were subsequently retested on the H4 V1S&SV2 and SL1&SL2 cells in a 10-pt concentration range (0.1-50 μ M). Concentration response data were plotted with log inhibitor (M) on the x-axis versus percent DMSO control activity on the y-axis. Analysis was performed using a four point logistic nonlinear regression analysis.

2.11. Statistics and data analysis

Data analysis and statistics were performed with GraphPad Prism (GraphPad software, USA). The statistical significance of differences between experimental groups was calculated by one-way analysis of variance test followed by Dunnett's post-test. Differences were considered to be statistically significant with * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. Results are given as mean $\pm$ S.E.M. unless otherwise stated.

3. Results

3.1. Generation of bimolecular protein-fragment complementation assay cell lines

Bimolecular protein-fragment complementation assay (PCA) is a powerful tool to study protein-protein interactions³⁶⁻³⁸. We have previously demonstrated that PCA can be applied to visualize α syn oligomerization in living cells using a fluorescent α syn-PCA, based on the venus yellow fluorescent-protein (Venus YFP)^{23, 31, 33, 39, 40} or a bioluminescent α syn-PCA, based on humanized *Gaussia princeps* luciferase (Luc)^{23, 32, 34, 41} (Fig. 1). In the present study, we have generated stable cell lines that co-express the α syn-PCA constructs to enable high throughput experiments. H4 neuroglioma cells were chosen for their human neuronal background. Following generation of an H4 TetOff *FRT* mother cell line, we generated an H4 TetOff *FRT* Venus1- α syn + α syn-Venus2 stable cell line (=H4 V1S&SV2), which expresses both moieties required for the fluorescent α syn-PCA and a H4 TetOff *FRT* α syn-Luc1 + α syn-Luc2 stable cell line (=H4 SL1&SL2) that expresses both moieties needed for the bioluminescent α syn-PCA (Fig. 1). Using the same Flp-*FRT* recombinase and tetracycline-driven bi-directional systems, we also generated two control cell lines (H4 V1Lz&LzV2 and H4 LzL1&LzL2) in which α syn is replaced by leucine zipper motives (Lz). Additionally an H4 TetOff cell line that overexpress untagged human wild-type α syn (=H4 wt α syn) and the H4 TetOff *FRT* mother cell line (=H4 empty) were used as controls.

3.2. Characterization of the cell lines

Induction and expression of the transgenes were measured by western blotting (Fig. 2). As expected, robust expression of either untagged α syn or the fusion proteins V1S + SV2, SL1 + SL2, V1Lz + LzV2 or LzL1 + LzL2 was observed upon withdrawal of tetracycline (Fig. 2). Expression of V1Lz + LzV2 and LzL1 + LzL2 was monitored using polyclonal

antibodies directed against full length GFP and *Gaussia* luciferase, respectively (Fig. 2 F and G), whereas an anti- α syn antibody was used to detect tagged and untagged α syn proteins (Fig. 2 A-E). Expression was induced at tetracycline concentrations below 10ng/ml and was readily detectable 16h after removal of tetracycline and increased with time (Fig. 2 A, B). Conversely, re-addition of tetracycline repressed expression (Fig. 2 C). A half-life of approximately 24h was observed for the SL1 and SL2 fusion constructs in the H4 SL1&SL2 cells, consistent with the half-life of untagged wt α syn (Fig. 2 C). However, in the H4 V1S&SV2 cells, SV2 and V1S fusion proteins had longer half-lives, with V1S still detectable 120h after addition of tetracycline (Fig. 2 C). Finally, protein crosslinking revealed the presence of dynamic α syn species of high molecular weight in the H4 wt α syn cell line as well as in the H4 SL1&SL2 and the H4 V1S&SV2 cells, indicating that the addition of the small tags necessary for PCA does not block α syn polymerization (Fig. 2 D and E).

Expression of the α syn fusion constructs was also monitored by confocal microscopy and flow cytometry (Fig. 3). Immunofluorescence labelling demonstrated basal α syn immunoreactivity in the H4 wt α syn, V1S&SV2, SL1&SL2 cell lines, similar to endogenous α syn levels in the presence of tetracycline and pronounced α syn immunoreactivity in response to tetracycline removal (Fig. 3). Confocal imaging of the H4 wt α syn, V1S&SV2 and SL1&SL2 cells showed a similar cytoplasmic and nuclear α syn staining (Fig. 3 E-G), suggesting that the presence of the small tags necessary for PCA does not interfere with the normal distribution of α syn.

In addition to the α syn immunoreactivity, fluorescent protein-fragment complementation was monitored in H4 V1S&SV2 and V1Lz&LzV2 cells. As expected, green fluorescence appeared after removal of tetracycline and was easily detected using fluorescent microscopy and flow cytometry (Fig. 3). Comparison of the α syn immunoreactivity (red fluorescence) with the protein-fragment complementation signal (green fluorescence) showed an overall co-localization of both signals in the H4 V1S&SV2 cells using flow cytometry and confocal microscopy (Fig. 3). Interestingly, a minority of cells displayed little or no green fluorescence despite their high levels of α syn, suggesting these cells may only contain monomeric α syn (Fig. 3 F and N).

In H4 SL1&SL2 and LzL1&LzL2 cells, protein-fragment complementation was assessed by measuring the activity of *Gaussia* luciferase. As expected, after removal of tetracycline and addition of coelenterazine, robust bioluminescence was measured in H4 LzL1&LzL2 and SL1&SL2 cells (Fig. 4). In H4 SL1&SL2 cells, the bioluminescence was detected for tetracycline concentration below 10ng/ml with an IC_{50} of 1.5ng/ml tetracycline (Fig. 4 A) which is in line with the western blot results (Fig. 2 B).

To investigate whether our cell lines can be used to screen for α syn oligomer inhibitors, we treated them with the geldanamycin derivative 17-AAG which we and others have previously shown to inhibit α syn oligomerization by induction of Hsp70^{23, 32, 42-45}. As expected from previous results on transiently transfected cells^{23, 32}, treatment with 100nM 17-AAG induced Hsp70 expression and significantly reduced the bioluminescence signal in

the H4 SL1&SL2 cells but not in the control H4 LzL1&LzL2 cells (Fig. 4 B and C), demonstrating that we can discriminate compounds that block α syn oligomerization.

3.3. Optimization for screening conditions

Miniaturization of the α syn-PCAs was performed by plating H4 V1S&SV2 and SL1&SL2 cells in 1536-well or 384-well microtiter plates (Fig. 5). Multiple cell densities and incubation times with and without tetracycline were tested to determine the optimal culture conditions. Fluorescence and luminescence were read on an EnVision multilabel HTS plate reader (PerkinElmer). 24h after tetracycline removal, H4 V1S&SV2 cells produced a robust fluorescence with a signal to background ratio of 6.3 (Fig. 5 A). With time, this value continued to increase to finally reach a plateau around 14. DMSO concentrations up to 2% did not significantly affect the fluorescence intensity (Fig. 5 C). Cell viability was assessed as a luminescence readout in H4 V1S&SV2 cells for multiple cell densities and incubation times with or without tetracycline (Fig. 5 B and Supplemental Fig. 2). No significant change was observed in viability at 24h and 48h, however decreased cell viability was observed at later time points (Fig. 5 B and Supplemental Fig. 2), a finding that could be exploited to screen for neuroprotective compounds in future studies. However, the 24h time point was chosen to read the primary fluorescence signal because although the signal is not maximal at this time point, it is nonetheless robust with sufficient signal-to-background and has the greatest separation from any observable effect on cell viability. The bioluminescent α syn-PCA was also miniaturized to a 384-well plate format as a high-throughput counter assay (Fig. 5 D). After 48h without tetracycline, H4 SL1&SL2 cells produced a bioluminescence burst with an intensity peak at 4min after addition of coelenterazine and a signal to background ratio around 7 (Fig. 5 D).

3.4. Validating α syn-PCA for high-throughput assay platform

As a final validation step, we conducted a preliminary screen of a library of 1280 pharmacologically active compounds (LOPAC, Sigma-Aldrich). Due to its superior simplicity and cost effectiveness, the fluorescent α syn-PCA was chosen as our primary assay and multiplexed with a cell viability measurement. H4 V1S&SV2 cells were plated at 2000 cells/well in 1536-well plates and incubated 24h with the 1280 LOPAC compounds at 5 μ M and 10 μ M in the absence of tetracycline. The screen performed better at 5 μ M, with lower toxicity, a signal to background ratio of 5.3, a Z' factor greater than 0.5 which is equivalent to a signal to background ratio greater than 6 times the sum of the standard deviations. Moreover at 5 μ M, 22 compounds were able to reduce fluorescence by more than 50% and 20 by more than 60%. Of the 22 compounds that reduced fluorescence by more than 50% at 5 μ M, 9 were below the 50% toxicity threshold (Table 1). Among these 9 hits, 5 were considered as top candidates due to their low toxicity and dissimilarity to tetracycline (Table 1, in white). The top five candidates included a potent and selective p38 mitogen-activated protein kinase (p38 MAPK) inhibitor (PD 169316, 4-(4-fluorophenyl)-2-(4-nitrophenyl)-5-(4-pyridyl)-1H-imidazole, PubChem CID: 4712, #P9248, Sigma-Aldrich), a human fibroblast growth factor-1 receptor tyrosine kinase (FGF-1R TK) inhibitor (PD 161570, 1-tert-butyl-3-[6-(2,6-dichlorophenyl)-2-[4-(diethylamino)butyl]amino]pyrido[2,3-d]pyrimidin-7-yl]urea, PubChem CID: 5328135, #PZ0109, Sigma-Aldrich), a casein kinase 2 (CK2) inhibitor (TBBz, 4,5,6,7-tetrabromobenzimidazole, PubChem CID: 5149739,

#T6951, Sigma-Aldrich), a calcineurin phosphatase inhibitor (cyclosporin A from *Tolypocladium inflatum*, PubChem CID: 5280754, #C3662, Sigma-Aldrich), and a Src family kinase inhibitor (PD 173952, 6-(2,6-dichlorophenyl)-8-methyl-2-(4-morpholin-4-ylphenylamino)-8H-pyrido[2,3-d]pyrimidin-7-one, PubChem CID: 5328733, #PZ0113, Sigma-Aldrich). These compounds were reordered as powder and retested in a dose response manner followed by cytotoxicity assessment in H4 V1S&SV2 and SL1&SL2 cells for hit confirmation (Fig. 6). Inhibition of α syn oligomerization was confirmed in both fluorescent and bioluminescent α syn-PCAs for all compounds except for the calcineurin phosphatase inhibitor, cyclosporin A. Similar potencies and toxicities were measured in both α syn-PCAs and all compounds, displayed sigmoidal dose-response curves beyond the range of cytotoxicity (Fig. 6 A-D), except for the p38 MAPK inhibitor for which no curve fit for an IC_{50} value was calculable despite a clear inhibition. Selectivity was tested by counter-screening of the hit compounds at 0.25, 1, 5 and 10 μ M in the H4 LzL1&LzL2 cells and multiplexed with measurement (Fig. 6 E and Supplemental Fig. 3). Compounds with inhibitory effect >50% at 5 μ M on the analogue bioluminescent Lz-PCA were considered as false positive (Fig. 6 E). This was the case for the Src family kinase and the FGF-1R TK inhibitors that down-regulated the signal by 76 and 55%, respectively. In contrast, the p38 MAPK and calcineurin inhibitors showed no inhibitory effect and even slightly up-regulated the signal by 38% and 39%, respectively. The effect of the CK2 inhibitor was less clear. At 5 μ M it reduced the signal of 44%, just below the 50% cut off, and at 1 μ M it increased the signal by 32% (Supplemental Fig. 3). Furthermore, no clear effect was seen in the α syn-PCA using another commercially available CK2 inhibitor (TBCA, (E)-3-(2,3,4,5-tetrabromophenyl)acrylic acid, PubChem CID: 16760346, #SML0854, Sigma-Aldrich), suggesting that the effect of TBBz may be CK2 independent. In contrast, two additional p38 MAPK inhibitors, one (SB 239063, trans-1-(4-hydroxycyclohexyl)-4-(4-fluorophenyl)-5-(2-methoxypyridimidin-4-yl)imidazole, PubChem CID: 5166, #S0569, Sigma-Aldrich) with a similar chemical scaffold than PD 169316, the other (JX401, 1-[2-methoxy-4-(methylthio)benzoyl]-4-benzylpiperidine, PubChem CID: 1126109, #J4774, Sigma-Aldrich) with a different chemical scaffold than PD 169316 (Fig. 7) were potent in the α syn-PCA. These results suggest that p38 MAPK pathways, probably through p38 α/β , might have legitimate activity in α syn oligomerization. However follow up studies to exactly determine the mechanisms of action of these compounds and further validation using other methods to quantify α syn oligomerization are needed before being considered relevant for synucleinopathies.

Taken together, these results clearly demonstrate that the fluorescent α syn-PCA can be used as high-throughput primary assay to screen large libraries and that the bioluminescent α syn- and Lz-PCAs can be subsequently used as secondary assays for compound triage and target validation.

4. Discussion

α Syn aggregation is a key mechanism in the development of PD and related synucleinopathies. Molecules that prevent α syn oligomerization or enable the selective disruption or clearance of α syn oligomers, may halt or even reverse the pathological process

and be used as disease-modifying therapeutics^{46, 47}. However, α syn oligomers are challenging to detect and quantify due to their highly evanescent and dynamic nature.

Here, we report on the development and validation of two versatile cell-based assays that use protein-fragment complementation (PCA) for *in situ* monitoring of α syn oligomerization using either fluorescence microscopy, flow cytometry, or multiwell plate reading (Fig. 1).

By combining the Flp-*FRT* system with a tetracycline-driven bi-directional expression system, we generated isogenic stable cell lines with controllable expression of the fusion proteins necessary for PCA. The addition of small tags for PCA did not alter α syn cellular distribution, as shown by confocal imaging (Fig. 3), or block α syn propensity to oligomerize, as shown by protein crosslinking (Fig. 2 E), native, and semi-native gel electrophoresis (Supplemental Fig. 1). In comparison to H4 wt α syn cells, no changes in α syn half-life and oligomerization were observed in the H4 SL1&SL2 cells (Fig. 2). In contrast, in the H4 V1S&SV2 cells, we observed an increased α syn half-life and oligomer stability probably due to the irreversible nature of the venus YFP protein-fragment complementation^{33, 34, 48}. This is consistent with our earlier study showing that V1S and SV2 had a longer half-life when transiently co-expressed rather than expressed individually³¹. However, by implementing two different PCAs (fluorescent and bioluminescent), we avoid reliance on a single read-out or single assay. In addition, we have also generated two analog Lz-PCA cell lines to validate the specificity of any compound identified with the α syn-PCAs and rule out any false positive that may act by blocking the expression of the constructs or interacting with the protein-complementation tags rather than with α syn. Consequently possible false positives can be distinguished from compounds that specifically modulate α syn oligomerization. For instance, the geldanamycin derivative 17-AAG which we and others have previously shown to inhibit α syn oligomerization by induction of Hsp70^{23, 32, 42-45}, reduced the signal in the α syn-PCA but not in the control Lz-PCA (Fig. 4 B and C).

Optimization and miniaturization adapted α syn-PCAs for high-throughput compound profiling and robotic screening (Fig. 5). Following validation, the fluorescent α syn-PCA was chosen as principal assay for its superior simplicity and cost effectiveness to conduct a pilot screen of the LOPAC1280 library. This pilot screen verified assay robustness and reproducibility with excellent statistics and resulted in the identification of 5 hit compounds (Table 1 and Fig. 6). The high hit rate of 1 potential modulator out of 256 tested compounds was not surprising considering that the LOPAC1280 library only contains bioactive compounds and that our screening strategy also detects compounds that modulate α syn oligomerization via secondary targets and not only compounds that directly interfere in the α syn- α syn interaction.

The 5 hit compounds were all either kinase or phosphatase inhibitors which is particularly interesting given that abnormal phosphorylation of α syn is observed in Lewy bodies in PD and is implicated in the pathological process of synucleinopathies^{49, 50}. The kinases and pathways involved in pathogenic α syn phosphorylation have yet to be elucidated however studies have linked CK2, calcineurin, and p38 MAPK to cellular stress, PD, and α syn⁵¹⁻⁵⁶. Follow up screens of additional kinase inhibitors eliminated calcineurin, src family kinase,

FGF-1R TK, and CK2 families of inhibitors as having activity in our assay, but suggested p38 MAPK inhibitors as putative targets for α syn oligomerization.

5. Conclusions

In this study, we have shown that bioluminescent and fluorescent protein-fragment complementation are well suited for simple, rapid and high-throughput profiling of modulators of α syn oligomerization and consequently represent an important tool for target validation and discovery of new inhibitors of α syn oligomerization. Our innovative strategy may also be applicable to other aggregation-prone proteins such as amyloid beta for drug discovery in other proteinopathies such as Alzheimer's disease ⁵⁷.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of abbreviations

αsyn	alpha-synuclein protein
CK2	casein kinase 2
DMSO	dimethyl sulfoxide
FGF-1R TK	human fibroblast growth factor-1 receptor tyrosine kinase
FRT	flippase recognition target
Hsp70	heat shock protein 70
HTS	high-throughput screening
LOPAC	library of pharmacologically active compounds
Luc	humanized <i>Gaussia princeps</i> luciferase
Lz	leucine zipper motives
LzL1	leucine zipper + N-terminal half of <i>Gaussia</i> luciferase
LzL2	leucine zipper + C-terminal half of <i>Gaussia</i> luciferase
LzV2	leucine zipper + C-terminal half of Venus YFP
p38 MAPK	p38 mitogen-activated protein kinase
PBS	phosphate-buffered saline
PCA	bimolecular protein-fragment complementation assay

PD	Parkinson's disease
RT	room temperature
SNCA	α syn gene
SL1	α syn + N-terminal half of <i>Gaussia</i> luciferase
SL2	α syn + C-terminal half of <i>Gaussia</i> luciferase
V1Lz	N-terminal half of Venus YFP + leucine zipper
V1S	N-terminal half of Venus YFP + α syn
SV2	α syn + C-terminal half of Venus YFP
venus YFP	venus yellow fluorescent protein
wt	wild-type

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Figure 1. The α -synuclein bimolecular protein-fragment complementation assays (PCA)
Both α syn-PCAs are based on the fusion of α syn molecules with complementary halves of either venus yellow fluorescent protein (venus YFP) to generate the non-fluorescent fusion proteins V1S and SV2 (**A**) or the *Gaussia princeps* luciferase to form the non-bioluminescent fusion proteins SL1 and SL2 (**B**). When α syn- α syn interactions occur, complementary halves of the reporter proteins are in close enough proximity to restore the reporter function. Values are given as mean $\pm$ S.E.M., n = 4.

Figure 2. Expression dynamic of the tetracycline-regulated cell lines

Protein expression was visualized in the H4 wt α syn, V1S&SV2, SL1&SL2, V1Lz&LzV2 and LzL1&LzL2 cells by western blotting using anti-actin antibodies and either anti- α syn (A-E), anti-GFP (F) or anti-*Gaussia* luciferase antibodies (G) in the presence (Tet +) or absence (Tet -) of 1 μ g/ml tetracycline. Expression of α syn constructs V1S, SV2, SL1 and SL2 was measured over time after removal of tetracycline (A). Sensitivity to tetracycline was tested by incubating cells 72h with decreasing concentrations of tetracycline (B). Repression of expression of the α syn constructs V1S, SV2, SL1 and SL2 was monitored over time after re-addition of 1 μ g/ml tetracycline on cells that were previously turned-on for 96h and compared to untagged α syn (C). To detect dynamic α syn species of high molecular weight, proteins were chemically crosslinked prior to cell lysis (E) or not (D).

Figure 3. Fluorescent PCAs

Confocal microscopy (A-H, scale bars = 20μm) and fluorescence activated cell sorting (I-N) of tetracycline regulated stable cell lines. Cells were cultured 48h in the presence (+ Tet, A-D, blue traces J-L, and M) or absence (– Tet, E-H, red traces J-L and N) of 1μg/ml tetracycline and immunostained using anti-αsyn antibodies (red). Nucleii were detected using Hoechst staining (blue). Green fluorescence in H4 V1S&SV2 and V1Lz&LzV2 cells is due to venus YFP complementation. H4 empty cells (I) were used as control and were either unstained (I, blue trace), stained with the secondary antibodies alone (I, green trace) or in presence of anti-αsyn antibodies (I, red trace). αSyn immunoreactivity (red fluorescence) was correlated with protein-fragment complementation of the venus YFP (green fluorescence) in the H4V1S&SV2 cultured with (M) or without tetracycline (N).

Figure 4. Bioluminescent PCAs

Bioluminescence was measured in H4 SL1&SL2 cells cultured for 48h in a range of tetracycline concentrations (**A**). Values are given as mean $\pm$ S.E.M., $n = 3$. H4 SL1&SL2 (**B**) or LzL1&LzL2 (**C**) cells were cultured in fresh media supplemented with either 1000ng/ml tetracycline (+ Tet), 0.005% DMSO or 100nM of the Hsp70 inducer 17-AAG, a potent inhibitor of α -syn oligomerization (**B and C**). Values are given as mean $\pm$ S.E.M., $n = 4$. After 48h incubation, bioluminescence was measured in cells and levels of actin, Hsp70, and transgenes were monitored by western blotting using anti-actin antibodies (**B and C**, blue boxes), anti-Hsp70 antibodies (**B and C**, green boxes) and anti- α -syn (**B**, red box) or anti-*Gaussia* luciferase antibodies (**C**, red box), respectively.

Figure 5. Miniaturization and optimization of assay conditions for HTS

H4 V1S&SV2 cells were plated into 1536-well microtiter plates at 2000 cells per well and incubated in the presence (+ Tet, white bars) or absence (– Tet, black bars) of 1µg/ml tetracycline for 24, 48 and 72h followed by fluorescence and luminescence cell viability (ATPlite) measurement using an EnVision plate reader (**A and B**). H4 V1S&SV2 cells at 2000 cells per well were incubated for 24h with varying % of DMSO to test the assay sensitivity (**C**). Bioluminescence was measured at different time points after coelenterazine injection in H4 SL1&SL2 cells cultured with or without tetracycline at 8000 cells per well in 384-well plates (**D**). Values are given as mean ± S.E.M., n = 3.

Figure 6. Candidate validation

The best hits of the pilot screen were retested for confirmation in H4 V1S&SV2, SL1&SL2 and LzL1&LzL2 cells in a dose response manner followed by cytotoxicity assessment. Example of dose-toxicity curves (**red dashed lines, A-D**) superimposed to dose-response curves from the fluorescent asyn-PCA (**green line, A and C**) or the bioluminescent asyn-PCA (**blue line, B and D**) for the CK2 inhibitor (**A, B**) or the FGF-1 receptor tyrosine kinase inhibitor (**C, D**). Values are given as mean $\pm$ S.E.M., n = 6. Potency and cytotoxicity IC₅₀ for the fluorescent and bioluminescent asyn-PCAs as well as % of inhibition for the bioluminescent Lz-PCA at 5μM of the 5 best hits are summarized in table **E**.

Figure 7. Target validation

Two additional p38 MAPK inhibitors, SB 239063 (**A**) and JX401 (**B**), were tested in H4 V1S&SV2 cells. Cells were plated into 1536-well microtiter plates at 2000 cells per well and incubated for 24h in the presence of the compound or DMSO and in absence of tetracycline followed by fluorescence and cell viability (ATPlite) measurement. Dose-response curves from the fluorescent asyn-PCA (green lines) in response to treatment were superimposed to their dose-toxicity curves (red dashed lines). Values are given as mean $\pm$ S.E.M., n = 3.

Table 1

Hit list from the LOPAC1280 pilot screen.

Structure	Compound information		% of inhibition		Cell viability in %	
	Comments	Sigma #	at 5µM	at 10µM	at 5µM	at 10µM
PD 169316 = p38 MAPK inhibitor	P9248	62	62	128	189
Tetracycline antibiotic interferes with protein synthesis	D6140	101	97	120	133
PD 161570 = inhibitor of human FGF-1 receptor tyrosine kinase	PZ0109	77	89	106	81
Basement membrane protease inhibitor inhibits endothelial cell proliferation and angiogenesis	M9511	101	102	105	151
RepSox = TGFbeta receptor inhibitor	R0158	82	89	98	141
6-deoxytetracycline antibiotic interferes with protein synthesis	D9691	99	99	91	124
TBBz = cell-permeable casein kinase 2 inhibitor	T6951	88	94	68	51
Cyclosporin A = calcineurin phosphatase inhibitor immunosuppressant	C3662	64	59	64	41

Structure	Compound information	Sigma #	% of inhibition		Cell viability in %	
	Comments		at 5µM	at 10µM	at 5µM	at 10µM
PD 173952 = a Src family kinase inhibitor	PZ0113	97	93	53	69