CHAPTER TWENTY-FIVE

Detection of Compounds That Rescue Rab1-Synuclein Toxicity

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Abstract

Recent studies implicate a disruption in Rab-mediated protein trafficking as a possible contributing factor to neurodegeneration in Parkinson's disease (PD). Misfolding of the neuronal protein α-synuclein (asyn) is implicated in PD. Overexpression of asyn results in cell death in a wide variety of model systems, and in several organisms, including yeast, worms, flies, and rodent primary

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neurons, this toxicity is suppressed by the overproduction of Rab proteins. These and other findings suggest that asyn interferes with Rab function and provide new avenues for PD drug discovery. This chapter describes two assay formats that have been used successfully to identify small molecules that rescue asyn toxicity in yeast. The 96-well format monitors rescue by optical density and is suitable for screening thousands of compounds. A second format measures viable cells by reduction of the dye alamarBlue, a readout that is compatible with 96-, 384-, and 1536-well plates allowing the screening of large libraries (>100,000 compounds). A secondary assay to eliminate mechanistically undesirable hits is also described.

1. Introduction

Defects in Rab GTPase function underlie a variety of inherited diseases (Olkkonen and Ikonen, 2006; Seabra *et al.*, 2002). Examples include mutations in Rab proteins (Rab7a/Charcot–Marie–Tooth disease type 2B and Rab27a/Griscelli syndrome) and mutations in proteins that regulate Rab activity (Rab escort protein-1/choroideremia, Rab5 GTPase activating protein/tuberous sclerosis, and Rab GDP dissociation inhibitor α/X-linked mental retardation). A deficiency of Rab function has been implicated in Parkinson's disease (PD), a movement disorder caused by the loss of dopamine-producing neurons in the substantia nigra. In the case of PD, the deficiency of Rab function is caused not by mutations in the Rab regulatory machinery, but rather by overexpression (Cooper *et al.*, 2006) of the aggregation-prone protein α-synuclein (asyn), a critical determinant in familial (Eriksen *et al.*, 2005) and sporadic PD (Goedert, 2001).

Overexpression of wild-type asyn in several model systems (Maries *et al.*, 2003), including yeast (Outeiro and Lindquist, 2003), worms (Lasko *et al.*, 2003), flies (Whitworth *et al.* 2006), rodents (Kirik *et al.*, 2002; Lo Bianco *et al.*, 2002), and primates (Kirik *et al.*, 2003), induces cell death. To investigate the mechanism of asyn toxicity, Cooper *et al.* (2006) identified genetic modifiers of asyn toxicity in yeast. Especially noteworthy was the recovery of a number of genes involved in endoplasmic reticulum (ER) to Golgi protein trafficking, including the ER Rab *YPT1*, suggesting that asyn inhibits ER to Golgi protein trafficking, a hypothesis that was confirmed in yeast by demonstrating the asyn-dependent accumulation of ER forms of secreted proteins (Cooper *et al.*, 2006). To test whether asyn inhibits ER to Golgi trafficking in dopaminergic neurons, the *YPT1* ortholog, Rab1a, was overexpressed in three different models of PD (transgenic worm and fly and rat primary neurons) and found to rescue asyn-mediated toxicity in all. Thus, the mechanism of asyn toxicity discovered in yeast is conserved in dopaminergic neurons.

Current PD therapies achieve their clinical benefit by restoring dopamine levels but ultimately fail, as they do not address the underlying cause of the disease, the degeneration of dopaminergic neurons. To identify candidate neuroprotective agents, we used the yeast PD model to screen for small molecules that rescue asyn toxicity. Because overexpression of *YPT1*/Rab1a rescues asyn toxicity, our screen can identify small molecules that rescue asyn toxicity by stimulating Rab function and/or increasing Rab levels.

2. Strains

Strains suitable for primary screening (rescue of asyn toxicity) and a secondary assay (elimination of mechanistically undesirable hits using induction of *lacZ*) are constructed using standard methods for *Saccharomyces cerevisiae* (Rothstein, 1991) essentially as described (Outeiro and Lindquist, 2003). Briefly, DNA constructs containing asyn or *lacZ* under the control of the galactose promoter (Schneider and Guarente, 1991) are integrated into the genome of a Gal^+ host strain. Plasmid-borne asyn expression constructs are unstable under inducing conditions and give inconsistent and/or weak toxicity. Asyn toxicity is strongly dose dependent, and two constructs per haploid genome must be integrated to obtain growth inhibition. The site of integration appears to modestly affect asyn expression. This observation can be used to generate strains with different levels of growth inhibition. We have found that asyn integrated at *URA3* and *TRP1* gives stronger toxicity than integration at *HIS3* and *TRP1*. To test whether positives from the primary screen rescue by reducing transcription or translation, we developed a secondary assay that monitors the induction of *lacZ*. This reporter assay uses a congenic strain containing vector inserts at *URA3* and *TRP1* and *lacZ* under the control of the galactose promoter integrated at *HIS3*.

3. Methods for Detecting Compounds That Rescue α-Synuclein-Induced Toxicity

To identify molecules that rescue the toxicity of asyn we developed two methods of compound screening. The first is a medium throughput 96-well assay that uses optical density (OD) as a measure of yeast growth. The second, a high throughput assay configurable to 96-, 384-, and 1536-well formats, employs alamarBlue reduction to readout cell growth. Both methods make use of the asyn screening strain. As this strain contains integrated copies of asyn at both *URA3* and *TRP1* loci, both protocols use synthetic complete media lacking the nucleoside uracil (-Ura) and the

Table 25.1 Buffered synthetic complete medium

Component	Vendor	Amount per liter	Final concentration
Yeast nitrogen base without amino acids	Difco	6.7 g	0.67% (w/v)
Carbon source: one of glucose, galactose, raffinose (see Table 25.2)	See Table 25.2	See Table 25.2	2 or 4% (w/v)
CSM -Trp -Ura or CSM -His -Trp -Ura	Qbiogene	≈0.8 g[a]	
MOPS (mol. biol. grade)	EMD	20.9 g[b,c]	0.1 *M*
Milli Q water	—	1 liter	—

[a] According to manufacturer's instructions.
[b] If required.
[c] If making media containing MOPS, pH to 6.0 with 1 *M* NaOH.

Table 25.2 Carbon sources

Component	Vendor	Amount per liter	Final concentration
Glucose (also known as dextrose)	Fisher	20 g	2% (w/v)
Galactose	Sigma	20 g	2% (w/v)
Raffinose	Difco	40 g	4% (w/v)

amino acid tryptophan (-Trp, see Tables 25.1 and 25.2 and Section 4). Expression of asyn is toxic in yeast (Outeiro and Lindquist, 2003). Therefore, the copies of asyn in the screening strain are under control of the inducible galactose promoter (Schneider and Guarente, 1991). This promoter is controlled by the carbon source supplied in the media. Growth in glucose-containing media represses transcription from the galactose promoter. Growth in raffinose neither induces nor represses transcription of the galactose promoter, whereas growth in galactose-containing media results in high expression. The screening strain is maintained on glucose media. When growing cells for the rescue assay, raffinose media are used so that simple dilution of the cells into galactose media results in the induction of asyn. Four percent instead of the usual 2% raffinose is used in the media as

our experiments demonstrate that the asyn screening strain grows optimally under these conditions (data not shown).

When screening compounds in either format, negative and positive controls are tested in each screening plate. This allows for per plate quality control metrics and a way to compare the extent of compound rescue between plates (or experiments) by expressing the extent of rescue as a function of the positive control for that plate. As compounds are dissolved in dimethyl sulfoxide (DMSO), it alone is used as the negative control. A sublethal concentration of the topoisomerase poison daunorubicin (Sigma) is used as a positive control in these assays. In addition to causing double-stranded DNA breaks, daunorubicin inhibits transcription in yeast. Daunorubicin binds preferentially to GC-rich regions of DNA. As the galactose promoter is GC rich in an AT-rich *S. cerevisiae* genome, sublethal concentrations of daunorubicin preferentially inhibit transcription from this promoter (Marin *et al.*, 2002). This inhibition of galactose-promoted transcription rescues the asyn screening strain by lowering the expression of the toxic asyn protein.

3.1. Synthetic Media

Based on the genotype of the strain to be tested, the appropriate supplementation for synthetic medium is chosen. Strains containing integrated constructs should be grown in medium that maintains selection for the construct (see later). CSM (Qbiogene) is a commercially available amino acid mix for growing *S. cerevisiae*. It can be obtained lacking one or more amino acids as required.

To make liquid synthetic medium, mix the components listed in Tables 25.1 and 25.2. After the components have dissolved, adjust the pH to 6.0 if necessary (when using alamarBlue as a readout, see later) and sterilize by filtration (Millipore Stericup) into a sterile bottle.

4. Primary Assays

4.1. Medium throughput format (96 well)

The medium throughput protocol allows for screening of compounds in 96-well plates without the need of robotics or detection equipment more sophisticated than a plate reader that can measure absorbance. We perform OD_{600} measurements on a Wallach Victor2 plate reader (Perkin Elmer) with a 600-nm (10-nm band-pass) filter. Flat-bottom tissue culture-treated polystyrene plates are used for assaying yeast growth. Tissue culture-treated flat bottom plates allow for easier mixing of yeast. Compounds

are dispensed from V-bottom polypropylene plates. Polypropylene plates are used for compounds, as DMSO and polystyrene are not compatible during long-term storage.

Plates are mixed immediately before OD_{600} reads. This step was incorporated because yeast cells settling in the wells result in increased OD_{600} measurements. This cell settling effect begins shortly after mixing and plateaus at 1 h; final values for settled cells typically exceed initial values by more than 2.5-fold (data not shown). Reading plates in which the yeast cells have been allowed to settle is not advisable, as the movements of the plate in the reader generate foci of yeast at the bottom of wells, which increases the variability and extent of the OD_{600} read (data not shown).

4.2. Medium throughput protocol

1. Inoculate an appropriate volume of SC -Trp -Ura 4% raffinose medium with the asyn screening strain at OD_{600} of 0.05, 0.025, and 0.0125. By inoculating dilutions of the strain, we ensure that at least one culture is in log phase growth the next day.
2. Grow strains overnight 14 to 18 h at 30° with shaking.
3. Measure OD_{600} of the cell cultures and identify one in log phase growth (OD_{600} 0.1–1.0). *Note*: In our spectrophotometer, an OD_{600} of 1.0 equals $\approx 2 \times 10^7$ cells/ml. This ratio varies depending on the spectrophotometer used.
4. Dilute cells into SC -Trp -Ura 2% galactose media to OD_{600} 0.003.
5. Transfer 100 μl of galactose culture to each well of a 96-well flat bottom microtiter assay plate.
6. Transfer 1 μl of compound in DMSO at 100× testing concentration to each well. Add 1 μl DMSO alone to negative controls and 1 μl 2.5 m*M* daunorubicin in DMSO to positive control wells. The final concentration of DMSO should be $\leq$1%. Mix the compound in wells by either pipetting or vortexing the plate. *Note*: Compounds can be added by hand quickly using a multichannel pipettor that can dispense 1 μl accurately.
7. Incubate plates for 48 h at 30°.
8. At the end of the incubation period, mix the plate to suspend yeast and immediately read OD_{600} on a plate reader.

We employed this protocol to screen a 2000 compound library for compounds that rescue asyn toxicity. At a cutoff of 6 SD above the mean value for the DMSO-only samples, we were able to identify 23 compounds that rescue at 12.5 μM (1.2% hit rate). We rescreened hits under identical conditions and found a 70% confirmation rate.

4.3. High throughput format

To screen a large compound library, we devised a high throughput asyn rescue assay that allows for screening in a 96-, 384-, or 1536-well format. Using OD as a readout at this scale is problematic because it is prone to cell settling effects that create highly variable values, especially in small wells that are difficult to mix. As an alternative readout for growth, we have used alamarBlue to assess cell proliferation (Ahmed *et al.*, 1994; To *et al.*, 1995). In the presence of metabolically active cells, alamarBlue (resazurin) is reduced to resorufin, which can be monitored either colormetrically or fluorometrically. As it is more sensitive, we opted to use fluorometric detection of alamarBlue reduction. AlamarBlue is not fluorescent, wheres resorufin excites at 530 to 560 nm and emits at 590 nm. We assayed resorufin production on a Wallach Victor2 plate reader (Perkin Elmer) using a 540-nm filter for excitation and a 590-nm filter to read emission. The fluorescence of resorufin is pH dependent (Bueno *et al.*, 2002). Growing yeast cells acidify the media, and the pH of rescued wells is not optimal for fluorescent detection of resorufin. Therefore, to enhance the signal and reproducibility in the assay we use media buffered with 0.1 *M* 3-(*N*-morpholino)propanesulfonic acid (MOPS) to pH 6.0. *Note*: There is a point with large numbers of yeast cells where resorufin is further reduced to a nonfluorescent product (hydroresorufin). This can be determined by visual inspection of wells or by absorbance reading, as hydroresorufin is clear and colorless.

4.4. High throughput protocol (384 well)

1. Inoculate cultures and grow overnight as described in steps 1 and 2 of Section 5.2 except that medium containing 0.1 *M* MOPS and pH adjusted to 6.0 is used.
2. Measure OD_{600} of the cell cultures and identify one in log phase growth (OD_{600} 0.1–1.0).
3. Dilute cells into SC -Trp -Ura 2% galactose 0.1 *M* MOPS, pH 6.0, medium to OD_{600} 0.03.
4. Transfer 25 μl of galactose culture to each well of a 384-well flat bottom microtiter assay plate.
5. Transfer 0.25 μl of compound at 100× testing concentration to each well. Add 0.25 μl DMSO alone to negative controls and 0.25 μl 2.5 m*M* daunorubicin in DMSO to positive control wells. Mix the compound in wells by either pipetting or vortexing.
6. Incubate plates for 24 h at 32°.
7. At the end of the incubation period, add 2.5 μl of alamarBlue to each well. Mix alamarBlue in wells by either pipetting or vortexing.
8. Incubate plates for 5 h at 32°.
9. After incubation, measure resorufin fluorescence on a Victor2 Plate reader.

The high throughput protocol is employed to screen a 280k compound library at 20 μM. The Z' factor describes the separation of positive and negative controls and is employed as a common screening metric (Zhang *et al.*, 1999). The mean Z' value for the screen (0.8) was greater than the commonly accepted criteria of $Z' \geq 0.5$. Compounds with alamarBlue signal greater than 3 standard deviations above the mean of the DMSO controls were designationd hits, 0.84% of compound passed this criterion, 77% of the hits test positive in confirmation assays.

5. Secondary Assays

Compounds identified in the high throughput screen are tested in secondary assays to assess the potency and mechanism of action (MOA). Potency is assessed by examining the activity of the compound in a dose–response fashion. Limited MOA testing is performed to identify compounds with an undesirable mechanism of action (see later). *Note*: The majority of compounds identified in the compound screens described previously rescue in both buffered and unbuffered media. However, subsets have been identified that are exclusively active in either condition. In addition, we have discovered that compounds can have differential activity dependent on the final concentration of DMSO in the assay. Therefore, when testing hits from the screening protocols in either secondary assay, it is important to keep these parameters consistent.

5.1. Dose–response testing

Compounds that rescue asyn toxicity are retested in a dose–response format to assess potency. In this protocol, compounds are serially diluted 1:1 (v:v) in DMSO in rows of a 96-well V-bottom polypropylene microtiter plate and then added to yeast in the assay plate. Addition and dilution of compounds in medium give unsatisfactory results. Certain compound classes appear prone to sticking to the sides of tips in the presence of medium and leech off during subsequent dilutions, which leads to falsely high compound potencies. The compound potency is assessed by the extent of rescue compared to the positive control (daunorubicin), by the minimal concentration that results in significant rescue above background, and by the concentration of compound that results in half-maximal rescue (EC_{50}). Example dose–responses are shown in Fig. 25.1.

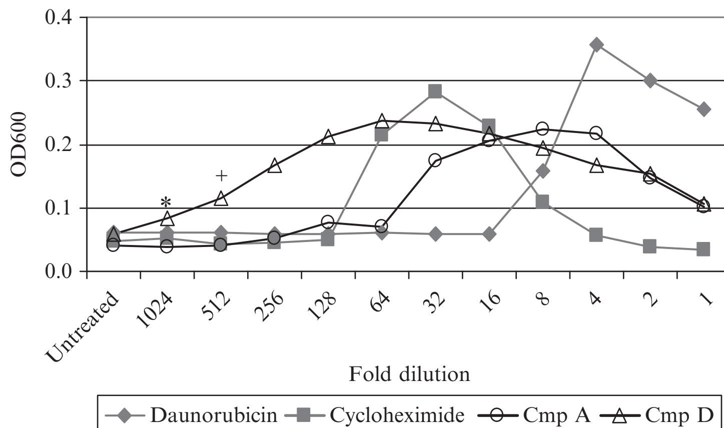

Figure 25.1 Dose-dependent rescue of asyn toxicity. Daunorubicin, cycloheximide, and two hits from the asyn screen [compound (Cmp) A and Cmp D] were examined for rescue using the asyn dose–response protocol. The starting concentrations of compound (fold dilution of 1) were daunorubicin, 50 μM; cycloheximide, 500 ng/ml; compound A, 150 μM; and compound D, 50 μM. Compound concentration increases left to right. The minimal rescue concentration is the lowest concentration that increases the OD_{600} above the DMSO control (indicated by an asterisk for compound D). The EC_{50} is the concentration that gives 50% of the maximal OD_{600} (indicated by a plus sign for compound D).

5.2. Dose–response protocol

1. Inoculate cultures and grow overnight as described in steps 1 and 2 of Section 5.2.
2. Prepare compound plate by adding 25 μl of DMSO to columns 2 to 12 of a 96-well V-bottom microtiter plate.
3. Add 50 μl of compounds at 100× the highest testing concentration to column 1 of the microtiter plate. *Note*: We routinely start at 5 m*M* compound (50 μM after dilution with cells) and always run daunorubicin as a positive control in the assay.
4. Using a multichannel pipettor, aspirate 25 μl of compound in DMSO from the first column of each row, dispense into the second column, and mix.
5. Repeat step 4 nine additional times on each subsequent set of columns. After mixing in column 11, aspirate 25 μl of the DMSO solution-containing compound and discard. The final plate should contain an approximately 1000-fold dilution of compound in columns 1 to 11 and DMSO alone in column 12.
6. Go to step 3 in Section 5.2 and proceed as described.

5.3. Eliminating compounds that rescue by inhibition of transcription/translation

As exemplified by the use of daunorubicin as a positive control, inhibitors of transcription can rescue in the asyn toxicity assay. Additionally, translational inhibitors such as cycloheximide rescue (see Fig. 25.1) via the same underlying mechanism (lowering asyn expression). To eliminate compounds identified in the screen with either of these MOAs we examined the ability of compounds to inhibit the expression of a reporter gene under control of the galactose promoter. This secondary screen uses a strain that is congenic with the screening strain; it does not express asyn but instead has a galactose promoter *lacZ* fusion (Rupp, 2002) integrated at the *HIS3* locus. Expression of *lacZ* is induced in the presence of the compound, and activity is assessed at 1 and 4 h. β-Galactosidase activity is measured using the gal-Screen kit for yeast (T1030) supplied by Applied Biosystems. Compounds that inhibit transcription and translation result in lowered activity of the reporter (Fig. 25.2). Because the galactose-promoted *lacZ* reporter is integrated at the *HIS3* locus, the strain is grown in synthetic complete media minus uracil, tryptophan, and histidine. We use white microtiter assay plates for this experiment, as they result in a better luminescence signal and minimize signal bleed into adjacent wells. We typically test compounds at concentrations 2- to 10-fold above the EC_{50} of rescue to ensure those with undesirable MOA are identified. Figure 25.2 shows *lacZ* reporter data for two

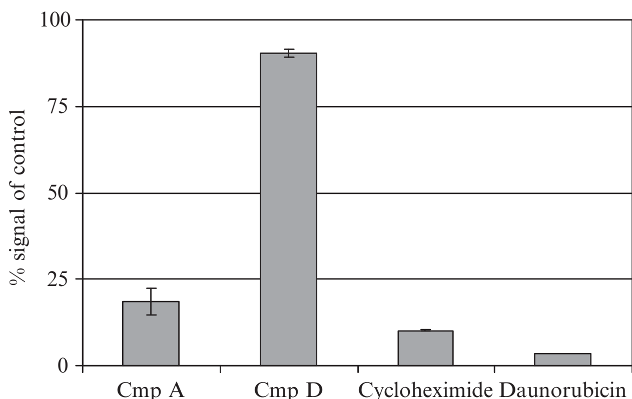

Figure 25.2 Identifying compounds that inhibit transcription or translation. Two novel compounds that rescue asyn toxicity (Cmp A and Cmp D) and two controls (daunorubicin, a transcriptional inhibitor; and cycloheximide, a translational inhibitor) were tested for effects in the *lacZ* reporter activity assay as described in the text. The concentrations tested were Cmp A, 150 μM; Cmp D and daunorubicin, 100 μM; and cycloheximides, 100 ng/ml. Values are the average of four replicates per condition. Data are expressed as the percentage of the DMSO control; this allows for comparison of compound activities across multiple experiments.

compounds identified in the asyn high throughput screen that have distinct effects on β-galactosidase expression.

5.4. lacZ protocol

1. Inoculate an appropriate volume of SC -His -Trp -Ura 4% raffinose medium with the *lacZ* reporter strain.
2. Grow strains overnight at 30° with shaking.
3. Measure OD_{600} of the cell cultures and identify one in log phase growth (OD_{600} 0.1–1.0).
4. Dilute cells into SC -His -Trp -Ura 2% galactose media to OD_{600} 0.03.
5. Transfer 100 μl of galactose culture to each well of a 96-well flat bottom white microtiter assay plate.
6. Transfer 1 μl of compound at 100× testing concentration to each well. Add 1 μl DMSO alone to negative controls and 1 μl 5.0 m*M* daunorubicin in DMSO to positive control wells. Mix the compound in wells by either pipetting or vortexing. *Note*: Because of the variability intrinsic to reporter assays, we normally test each concentration of compound in triplicate.
7. Incubate plates for 1 to 4 h at 30°.
8. During incubation, prepare the detection reagent by diluting the gal-Screen substrate 1:24 (v:v) into the gal-Screen lysis buffer and allow to warm to room temperature.
9. At the end of the incubation period, add 100 μl of detection reagent to each well of the microtiter plate.
10. Incubate plates for 1 h at room temperature and measure total luminescence for 1.0 s/well on a Wallach Victor2 plate reader (Perkin Elmer).

Nine hundred and eight hits from the 280k screen described earlier were tested for activity in the *lacZ* reporter activity assay. The compounds were tested at 40 μM (twofold the concentration used as described in the primary screen). Nine percent of the compounds tested demonstrated a >50% inhibition of β-galactosiadase reporter activity and were not pursued further.

6. Conclusion

FoldRx Pharmaceuticals has performed the aforementioned primary screens on approximately 350,000 compounds in an effort to identify Parkinson's disease therapeutics. These screens have identified multiple compound series that are active not only in the yeast model, but in worms overexpressing asyn, primary neurons expressing the familial PD asyn mutation A53T (Cooper *et al.*, 2006), and in H4 neurogliomal

cells overexpressing wild-type asyn. The ER to Golgi trafficking block induced by asyn can be overcome by Rab overexpression. Therefore, among the compounds that result asyn toxicity may be modulators of Rab function. Experiments are underway to address this possibility.

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