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ORIGINAL ARTICLE

A novel yeast-based screening system for potential compounds that can alleviate human α-synuclein toxicity

Anyaporn Sangkaew[1] | **Thanaporn Kojornna**[1] | **Ryoya Tanahashi**[2] | **Hiroshi Takagi**[2] | **Chulee Yompakdee**[1]

[1]Department of Microbiology, Faculty of Science, Chulalongkorn University, Bangkok, Thailand

[2]Division of Biological Science, Graduate School of Science and Technology, Nara Institute of Science and Technology, Nara, Japan

Correspondence
Chulee Yompakdee, Department of Microbiology, Faculty of Science, Chulalongkorn University, Phayathai Road, Patumwan, Bangkok 10330, Thailand.
Email: chulee.y@chula.ac.th

Hiroshi Takagi, Division of Biological Science, Graduate School of Science and Technology, Nara Institute of Science and Technology, ma, Nara 630-0192, Japan.
Email: hiro@bs.naist.jp

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Abstract

Aims: This study aimed to establish a yeast-based screening system for potential compounds that can alleviate the toxicity of α-synuclein (α-syn), a neuropathological hallmark of Parkinson's disease, either inhibition of α-syn aggregation or promotion of ubiquitin-mediated degradation of α-syn.

Methods and Results: A powerful yeast-based screening assay using the *rsp5*^{A401E}-mutant strain, which is hypersensitive to α-syn aggregation, was established by two-step gene replacement and further overexpressed the GFP-fused α-syn in the drug-sensitive yeast strain with a galactose-inducible multicopy plasmid. The *rsp5*^{A401E}-mutant strain treated with baicalein, a known α-syn aggregation inhibitor, showed better α-syn toxicity alleviation than the same background wild type strain as accessed by comparison on the reduction kinetics of viable dye resazurin fluorometrically ($\lambda_{ex}540/\lambda_{em}590$ nm). The *rsp5*^{A401E}-mutant yeast-based assay system showed high sensitivity as it could detect as low as 3.13 μmol l^{-1} baicalein, the concentration that lower than previously report detected by the *in vitro* assay.

Conclusions: Our yeast-based system has been effective for screening potential compounds that can alleviate α-syn toxicity with high sensitivity and specificity.

Significance and Impact of the Study: Yeast-based assay system can be used to discover novel neuroprotective drug candidates which may be either efficiently suppress-α-syn aggregation or enhance ubiquitin-dependent degradation.

KEYWORDS

Parkinson's disease, resazurin reduction, *Saccharomyces cerevisiae*, ubiquitin ligase Rsp5, yeast-based assay, α-synuclein aggregation

INTRODUCTION

More than 10 million people worldwide suffer from Parkinson's disease (PD). The pathological hallmark of PD is the loss of dopamine neurons in substantia nigra par compacta associated with the accumulation of misfolded and aggregated α-synuclein (α-syn) into intraneuronal inclusions (called Lewy bodies) (Kalia, 2019; Kim et al., 2014; Stefanis, 2012). These days, due to the absence of disease-modifying treatments for PD, there is a great desire to develop new drugs for the management of PD that are not only therapeutic but can also prevent the initiation of the disease or either delay or stop its progression (Davis et al., 2019; DeMaagd & Philip, 2015). One of the current

approaches to prevent disease progression include targeting the formation and promoting intracellular degradation of α-syn aggregates. Multiple lines of evidence suggested that the modulation of α-syn aggregation, oligomerization, fibrillation and propagation to reduce its toxicity emerged as an important therapeutic target for slowing or halting disease progression (Brundin et al., 2017; Kalia, 2019). Moreover, the overexpression of E3 ubiquitin ligase Nedd4, which enhance α-syn ubiquitination and leading to α-syn clearance, is sufficient to reduce neurotoxicity *in vivo* (Boland et al., 2018; Davies et al., 2013; Yuan et al., 2019).

The budding yeast *Saccharomyces cerevisiae* is one of the organisms used in drug discovery. It can be highly useful in the first-line screening of potential active compounds (Franssens et al., 2013; Tenreiro & Outeiro, 2010; Zimmermann et al., 2018). It is a powerful model organism for PD because its cellular processes are highly conserved with those of higher eukaryotes. Other advantages are its rapid growth and the existence of comprehensive genetic tools (Franssens et al., 2013; Tenreiro & Outeiro, 2010). As described above, *S. cerevisiae* has been widely used as a model organism with which to gain insights into α-syn toxicity. Previous reports suggested that α-syn-related effects, such as proteasome impairment, vesicle trafficking dysfunction, and reactive oxygen species generation, can be efficiently mimicked in *S. cerevisiae* cells, which do not endogenously express the α-syn ortholog. Additionally, the heterologous expression of human α-syn results in intracellular α-syn accumulation and growth defect in an α-syn dose-dependent manner (Franssens et al., 2009; Petroi et al., 2012; Soper et al., 2011). These findings have made *S. cerevisiae* a valuable cell model for studying the physiological and pathological features of α-syn and for screening active compounds that alleviate α-syn toxicity.

Saccharomyces cerevisiae Rsp5 is an essential homolog to the E6-AP carboxyl terminus (HECT)-type E3 ubiquitin ligase that is highly conserved with the mammalian Nedd4-family E3 ubiquitin ligases (Wang et al., 1999). Rsp5 was recently shown to affect a variety of cellular events, such as intracellular trafficking, signal transduction and quality control of the plasma membrane and cytosolic proteins through the interaction and ubiquitination of diverse substrate proteins (Dunn & Hicke, 2001; Jarmoszewicz et al., 2012; Shiga et al., 2014). Rsp5 is structured in three domains: an amino-terminal C2 domain, three substrate-recognizing WW domains and a carboxyl-terminal catalytic HECT domain (Wang et al., 1999). It was previously reported that the *rsp5*^{A401E} mutant, having a point mutation in the nonconserved Ala residue at position 401 with Glu of the WW3 domain, showed much more sensitivity to stresses that cause protein misfolding, such as toxic amino acid analogues, high growth temperature in a rich medium and ethanol, than the wild-type strain (Hoshikawa et al., 2003). Wijayanti et al. (2015) showed that the overexpression of α-syn in *S. cerevisiae* cells led to growth inhibition, especially in the *rsp5*^{A401E} mutant, indicating that the *rsp5*^{A401E} mutant is also hypersensitive to α-syn accumulation. These results suggested that Rsp5 is involved in the maintenance of stress-induced abnormal proteins through a degradation or repair system (Haitani et al., 2006; Haitani & Takagi, 2008; Hiraishi et al., 2009). Therefore, biological/chemical agents or compounds that could complement the stress sensitivity of the *rsp5*^{A401E} mutant might be promising as drug candidates, which exert their effect via the ubiquitin/proteasome system.

To discover novel drug candidates, it is essential to establish a simple, robust and inexpensive assay method. The assay should also serve as a tool for high-throughput screening to assess large sets of compound libraries (Inglese et al., 2007; Szymanski et al., 2012). Resazurin (7-hydroxy-3H-phenixazin-3-one-10-oxide) is a tetrazolium-based, weakly fluorescent, nontoxic and water-soluble dye that can be reduced by cellular metabolic activity to the highly fluorescent resorufin (Bowling et al., 2012; Csepregi et al., 2018). Several intracellular oxidoreductases, such as NADH dehydrogenase and diaphorase, are responsible for the reduction of resazurin to resorufin during cell viability assays (Bowling et al., 2012; Rolón et al., 2006). Therefore, resazurin has been successfully used to determine cell viability and proliferation of micro-organism for drug discovery (Lescat et al., 2019; Sangkaew et al., 2020; Uzarski et al., 2017).

In this study, we developed a novel yeast-based assay system to search for molecules that could alleviate the human α-syn-induced cytotoxicity of multidrug-sensitive strains with the *rsp5*^{A401E} background. A yeast-integrating plasmid carrying the *rsp5*^{A401E} gene was introduced into a drug-sensitive strain, allowing us to observe the sensitivities of the resultant strains to various stresses, such as high temperature, toxic amino acid analogue and α-syn. Subsequently, the yeast-based assay system with resazurin was optimized to be used as a high-throughput platform. This system was also validated using two known compounds, baicalein, and ampicillin, for sensitivity and specificity.

MATERIALS AND METHODS

Strains, plasmids, culture media and chemicals

Yeast strains used in this study are listed in Table 1. All yeasts strains were *S. cerevisiae* with the CAY29 (Andreasson & Ljungdahl, 2002) or BY25929 (obtained from Yeast Genetic Resource Center, Japan) background. The *rsp5*^{A401E}-mutant strains (HT01) in the CAY29 background were previously constructed (Haitani et al., 2009).

A multidrug-sensitive yeast strain BY25929, in which four multiple drug resistance genes (*YRS1*, *YRR1*, *PDR1* and *PDR3*) were deleted, was obtained from Yeast Genetic Resource Center, Osaka, Japan. To construct strain TK01 (the *rsp5*^{A401E} mutant with the BY25929 background) (Figure 1), the integrating plasmid pRS406 (*URA3*) was used for two-step replacements of the wild-type *RSP5* with *rsp5*^{A401E}-mutant allele (Gray et al., 2005). For overexpression of human α-syn, a galactose-inducible multicopy plasmid pYES2 α-syn GFP was used (Wijayanti et al., 2015).

The culture medium used for the growth of *S. cerevisiae* was a nutrient-rich yeast extract-peptone-dextrose (YPD) medium (1% yeast extract, 2% peptone and 2% glucose) and synthetic complete (glucose or raffinose) medium lacking uracil [SC-Ura or SR-Ura) (2% glucose or raffinose, 0.67% yeast nitrogen base without amino acids (Difco Laboratories) and 1× amino acid mix lacking uracil]. An alternative synthetic complete medium, synthetic complete (galactose) medium lacking uracil (SG-Ura) that contains 2% galactose as the carbon source instead of glucose was used to induce the overexpression of α-syn.

TABLE 1 Yeast strains used in this study

Strain	Mating type	Genotype	Source or reference
CAY29	*MATa*	*ura3-52*	Andreasson and Ljungdahl (2002)
BY25929	*MATa*	*trp1-1 leu2-3, 112 his3-11, 15 ura3-1 ade2-1 can1-100 yrs1::HIS3 yrr1::TRP1 pdr1::hisG pdr3::hisG*	Yeast Genetic Resource Center, Japan
HT01	*MATa*	CAY29 *rsp5*^{A401E}	Haitani et al. (2009)
TK01	*MATa*	BY25929 *rsp5*^{A401E}	This study

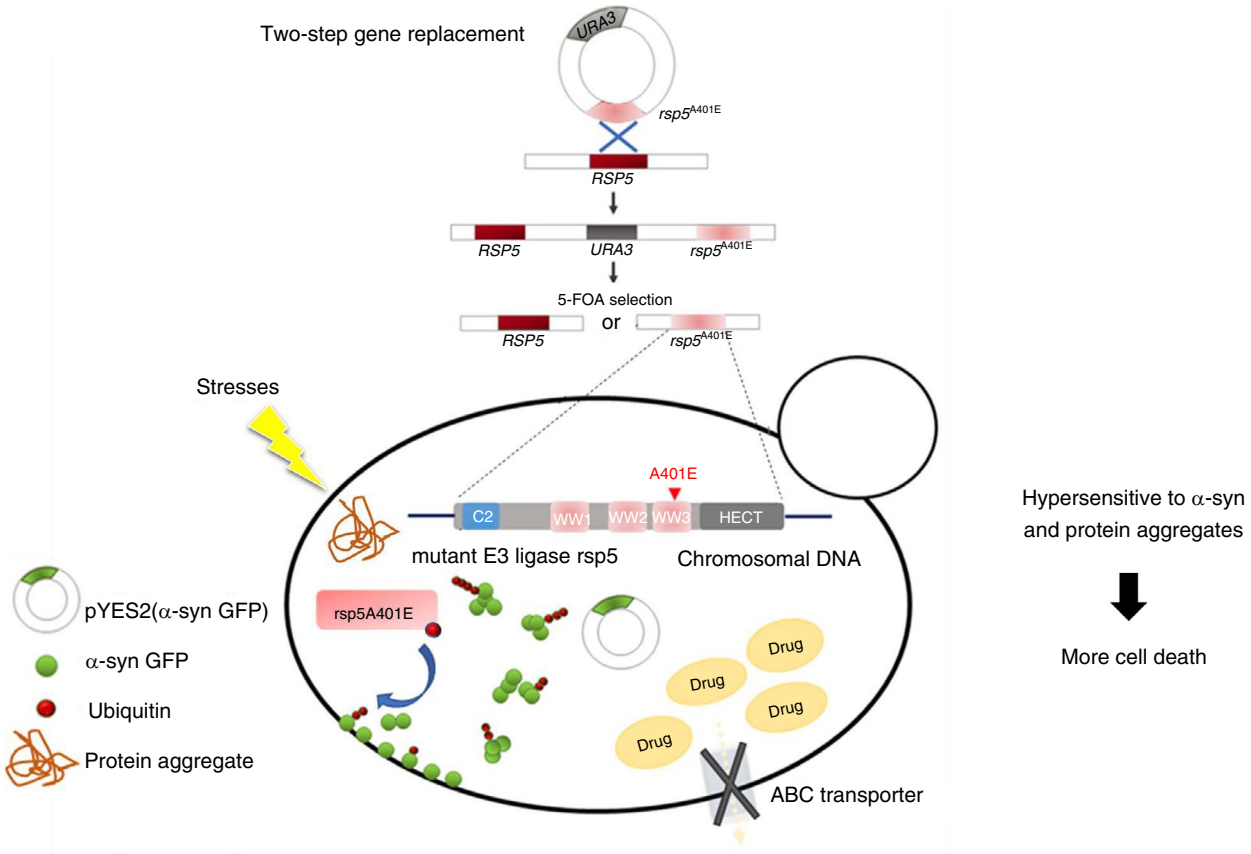

FIGURE 1 Schematic diagram of the genetically modified strain TK01 (pYES2 α-syn GFP). Strain TK01 was hypersensitive to various drugs and stresses since the ABC transporter genes were deleted, leading to the accumulation of drug in the cell, and the *rsp5*^{A401E} mutant allele was introduced in its chromosomal *RSP5* locus by two-step gene replacement method. In order to construct yeast strain expressing human α-syn, strain TK01 was transformed with pYES2 α-syn GFP. Generally, human α-syn is a plasma membrane-bound protein, which can be ubiquitinated by Rsp5 and finally degraded via the ubiquitin-mediated protein degradation. Accumulation of aggregated α-syn triggers cytotoxicity, leading to cell death. The effect was more obvious in the yeast *rsp5*^{A401E} mutant than in the *RSP5* strain

For L-azetidine-2-carboxylic acid (AZC) sensitivity, a synthetic minimal (glucose) medium (SD) (0.67% yeast nitrogen base without amino acids, 2% glucose and only essential amino acids) with or without 1 mmol l^{-1} AZC (TCI Chemicals) was used.

All chemicals used were of analytical grade or higher. Resazurin sodium salt (Sigma-Aldrich) was dissolved in 1× phosphate buffer saline to make a stock solution, and subsequently filtered using a syringe with 0.22-μm membrane. The prepared resazurin solution was covered to prevent exposure to light and was stored at 4°C for no longer than 2 weeks. For compounds used in system validation, baicalein (kindly provided by Dr. Boon-ek Yingyongnarongkul, Ramkhamhaeng University) and ampicillin sodium salt (Sigma-Aldrich) were freshly prepared in 100% dimethyl sulfoxide, water respectively.

Yeast transformation

Yeast transformation was performed by lithium acetate method (Schiestl & Gietz, 1989). To enhance the transformation efficiency, 50 μg of deoxyribonucleic acid sodium salt from salmon testes (Sigma-Aldrich) was added. For two-step gene replacement method, strain BY25929 was transformed with 5 μg of linearized pRS406-*rsp5*^{A401E} (Haitani & Takagi, 2008). Yeast transformants were selected on SC-Ura and then some of them were subjected to counter selection in the medium containing uracil and 5-fluoroorotic acid (5-FOA) (Zymo Research) (Akada et al., 2006). Only cells which have popped out the *URA3* marker by recombination between homologous duplicated sequences could survive and grow. In order to identify transformants containing the *rsp5*^{A401E} mutation, DNA sequencing was performed. For construction of the strain overexpressing α-syn, the *rsp5*^{A401E} mutant was transformed with pYES2 α-syn GFP.

Growth assay

Spot-dilution assays were performed using overnight culture grown in SR medium, a synthetic minimal (raffinose) medium (0.67% yeast nitrogen base without amino acids, 2% raffinose and only essential amino acids). Aliquots from such cultures were adjusted to $A_{660\ nm}$ =0.08 or 1 × 10^6 cells per ml with sterilized water. Five-fold serial dilutions were made in sterilized water, and aliquots (5 μl) of all yeast suspensions were spotted onto the assay medium. After the liquid in each spot was absorbed, the plates were incubated under assay conditions, and then the resulting plates were photographed. For assay of AZC sensitivity, the cell suspensions were spotted onto SD medium supplemented with 1 mmol l^{-1} AZC, and the plates were incubated at 30^{0}C for 2–3 days. In parallel, high temperature sensitivity of yeast cells was tested on YPD medium. After spotting cell suspensions, the plates were incubated at 37°C for 30 h and further incubated at 30°C for 2–3 days. The sensitivity against α-syn was assessed using yeast cells overexpressing α-syn. Overnight cultured cells in SR-Ura medium were spotted onto SG-Ura (for α-syn overexpression) and SC-Ura (for α-syn shutoff) media. After 2–3 days incubation at 30°C, the plates were photographed.

Resazurin yeast-based cell viability assay

Resazurin yeast-based assay was performed using black polystyrene, nontissue cultured treated, sterile, low binding 96-well microtiter plates (Thermo Fisher Scientific) and fluorescent signal of resorufin was measured using an Ensight Multimode Microplate Reader (PerkinElmer). Excited wavelength was set on 540 with 590 nm as emission wavelength. The experimental parameters were optimized, including resazurin concentration, initial cell densities and length of incubation of yeast indicator strain with resazurin, for assessing cell viability of the yeast strain. The resazurin reduction test was carried out as follows. Briefly, a total of 50 μl of yeast cultured SR-Ura at twice the desired final yeast density was added to wells of a 96-well black microtiter plate. Subsequently, 50 μl of inducible SG-Ura medium supplemented with or without test compound at twice the desired concentration was added to each well for a final volume of 100 μl. To correct for autofluorescence, the blank was prepared for all treatments by substitution of the cell suspensions with the growth medium. The assay plate was incubated at 30°C for 24 h. After incubation, 10 μl of resazurin at a desired concentration in 1× PBS was added to each well. Yeast cell viability was expressed as relative fluorescence unit (RFU) with top scanning mode. RFU of yeast-generated resorufin was recorded immediately after the resazurin dosing to all wells, and then again in 30 min period until RFU in each well without the test compound decreased. To define the relationship between the compound concentration in the sample suspension and intensity of the resorufin fluorescence, resazurin reduction curves were plotted. The minimal effective concentration was defined as the lowest concentration of the test compound that alleviates human α-syn toxicity and so accelerated the resazurin reduction.

Statistical analysis

All assays were performed triplicates on at least two independent occasions. Two-way ANOVA analysis was

performed for the appropriate data. All statistical data were analysed using the statistical and graphical functions of the GRAPHPAD PRISM 5.02 package (GraphPad Software). $p < 0.05$ was accepted as the level of statistical significance.

RESULTS

Effects of AZC and high temperature on the growth phenotype of the *rsp5*^{A401E}-mutant strains

In a previous study, the *rsp5*^{A401E} mutant strain showed hypersensitivity to various stresses that induce protein misfolding in the cell, such as the toxic proline analogue AZC and high temperature (Hoshikawa et al., 2003). To enhance sensitivity to test compounds, the drug-hypersensitive strain BY25929 lacking the multidrug-resistant genes was used to replace the *rsp5*^{A401E}-mutant allele with its chromosomal *RSP5* by a two-step gene replacement method (Figure 1). The resultant strain (the *rsp5*^{A401E} mutant of BY25929) was designated as strain TK01 (Figure 1). To further examine its growth phenotype, serially diluted cell suspensions of strains BY25929 (*RSP5*) and TK01 (*rsp5*^{A401E}) were spotted onto SD medium supplemented with or without 1 mmol l^{-1} AZC. High growth sensitivity to 1 mmol l^{-1} AZC on the assay medium was clearly observed in strain TK01 in comparison with strain BY25929 (Figure 2a). To assess sensitivity to high temperature, both strains were pretreated to high temperature at 37°C for 30 h, and further incubated at 30°C for 48 h. Strain TK01 grew on YPD medium at 30°C at a rate similar to that of strain BY25929; however, after pretreatment at 37°C, this mutant (TK01) showed a high temperature growth defect compared to the wild-type strain (BY25929) (Figure 2b). Both of the growth phenotypes observed indicated the characteristics of the mutant (Hoshikawa et al., 2003).

Effect of α-syn on the growth phenotype of the *rsp5*^{A401E}-mutant strain

To evaluate whether the *rsp5*^{A401E} mutant is more sensitive to α-syn than the wild-type *RSP5*, strains BY25929 and TK01 were transformed with either empty vector pYES2 or a plasmid harbouring α-syn (pYES2 α-syn GFP). The α-syn was expressed under the control of a strong inducible *GAL1* promoter. The transformants were grown onto either SC-Ura (no α-syn expression) or SG-Ura (induction of α-syn expression). As clearly shown in Figure 3 (right panel), the overexpression of α-syn from the 2μ plasmid led to a severe growth defect. Similar results were obtained in the case of another genetic background yeast strains, CAY29 and HT01 (Figure 3). It appears that the α-syn sensitivity of strain TK01 was not as clear as that of strain HT01. However, both strains TK01 (pYES2 α-syn GFP) and HT01 (pYES2 α-syn GFP) were further evaluated in order to set up a resazurin yeast-based assay system, and were compared for drug sensitivity.

Setting up the resazurin yeast-based assay to search for compounds alleviating α-syn toxicity

Metabolic activity may differ among yeast strains, so the concentration of resazurin should be optimized. Strains HT01 (pYES2 α-syn GFP) and TK01 (pYES2 α-syn GFP)

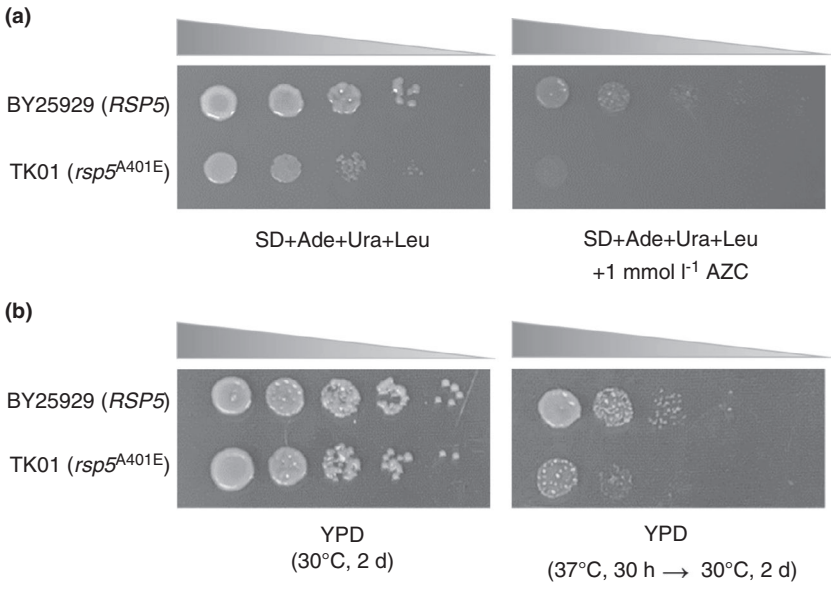

FIGURE 2 Effects of AZC and high temperature on the growth phenotype of the *rsp5*^{A401E} mutant strain. After cultivation in SC liquid medium at 30°C for 18 h, approximately 1×10^6 cells per ml of BY25929 (wild-type *RSP5*) or TK01 (mutant *rsp5*^{A401E}) and their serial dilutions were spotted and incubated on SD+Ade+Ura+Leu medium (a) containing 1 mmol l^{-1} AZC at 30°C for 2 days and on YPD medium (b) at following conditions, at 30°C for 2 days (left), or at 37°C for 30 h and then at 30°C for 2 days (right)

were plated in 96-well plates at various cell concentrations (between 1.6×10^3 and 1×10^6 cells per ml). Different concentrations of resazurin were added, starting from 0.05 to 0.4 mmol l^{-1}. The results showed that resazurin reduction was positively proportional to the resazurin concentration (from 0.05 until 0.2 mmol l^{-1}) (Figure 4). However, this gradual increase in fluorescence intensity was limited to 0.2 mmol l^{-1} since, at 0.4 mmol l^{-1}, the resazurin reduction showed a greater decrease than that of 0.2 mmol l^{-1}, indicating that the optimal resazurin concentration of these strains was 0.2 mmol l^{-1}.

Because resazurin reduction is affected by time and cell concentration, it is important to determine the initial cell concentration and resazurin incubation time. Strains HT01 and TK01, carrying either empty vector pYES2-GFP or pYES2 α-syn GFP, were cultured in SG-Ura at different initial cell concentrations and were then incubated for 16–18 h. Resazurin solution was added according to the optimal concentration obtained. The reduction of resazurin was recorded over 300 min. The kinetics of resazurin conversion to resorufin in cell suspensions of the four strains are summarized in Figure 5, which depicts the resazurin reduction curves for various initial cell concentrations. In this experiment, strains HT01 (pYES2 α-syn GFP) (Figure 5a) and TK01 (pYES2 α-syn GFP) (Figure 5c) were used as assay strains to determine the effect of compound on alleviating α-syn toxicity. Yeast strains harbouring the empty vector (Figure 5b,d), which showed faster reduction kinetics than the assay strains, served as positive growth controls. Figure 5a shows that assay strain HT01, at a starting cell concentration of less than 5×10^4 cells per ml, displayed significant lags in RFUs. Cell concentrations of

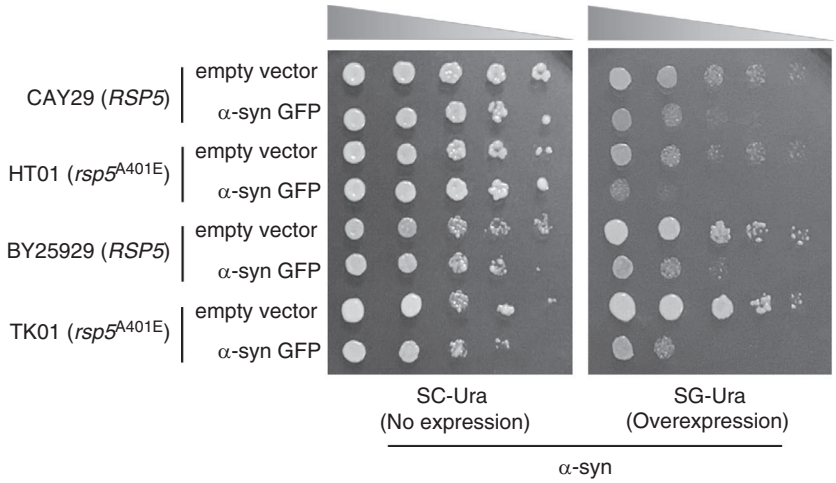

FIGURE 3 Effect of α-syn on the growth phenotype of the *rsp5*^{A401E} mutant strains. Spotting assay was performed to assess the effect on growth following overexpression of α-syn. Cell suspension of five-fold serial dilutions of the yeast transformants harbouring plasmid either α-syn GFP or empty vector were spotted onto selective medium plates containing 2% glucose (no α-syn expression) or 2% galactose (α-syn overexpression) starting with equal number of cells (1×10^6 cells per ml), and the plates were incubated at 30°C for 2 days

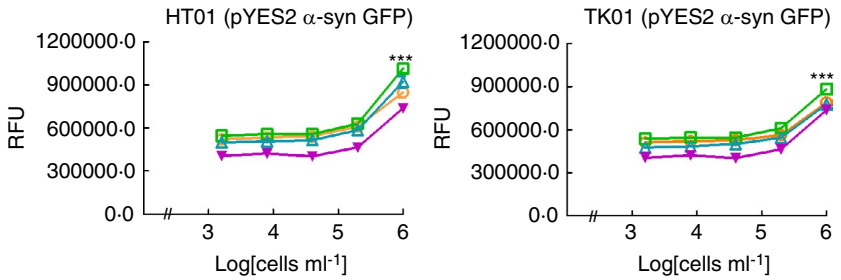

FIGURE 4 Effects of resazurin and cell concentrations on fluorescence intensity in the *rsp5*^{A401E}-mutant strains. Various cell concentrations (1×10^6, 2×10^5, 4×10^4, 8×10^3 and 1.6×10^3 cells per ml) of strains HT01 (pYES2 α-syn GFP) (a) and TK01 (pYES2 α-syn G*FP*) (b) were added into wells of a 96-well plate. Different resazurin concentrations were then added, ranging from 0.05 to 0.4 mmol l^{-1}. After 90 min incubation, fluorescence intensity was measured and expressed as relative fluorescence unit (RFU). The values are means ± standard deviations (SD) of results from three independent experiments. Symbols indicate the resazurin concentrations at 0.05 (▾), 0.1 (Δ), 0.2 (□) and 0.4 (○) mmol l^{-1}. Statistical significances are indicated by the *p*-value (***$p < 0.001$, 0.2 mmol l^{-1} vs. 0.05, 0.1, 0.4 mmol l^{-1})

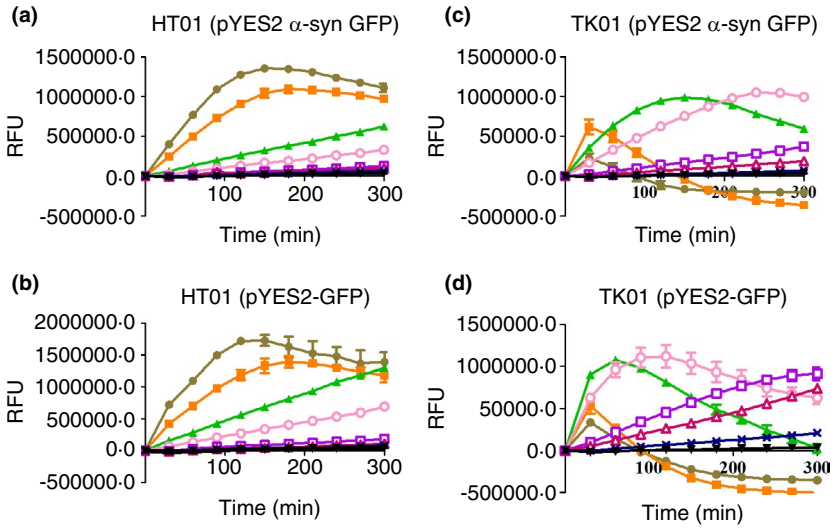

FIGURE 5 Kinetics of resazurin reduction in the *rsp5*^{A401E} mutant strains. Strains HT01 (pYES2 α-syn GFP) (a), HT01 (pYES2-GFP) (b), TK01 (pYES2 α-syn GFP) (c) and TK01 (pYES2-GFP) (d) at various initial cell concentrations were grown in SG-Ura at 30°C for 16–18 h. The kinetics were assessed immediately after addition of resazurin at every 30 min for 300 min. The relationship between RFU and time was plotted. The values are means ± SD of replicate cultures ($n = 3$) for each cell concentration tested. Symbols indicate the initial cell concentrations at 1×10^3 (*), 5×10^3 (Δ), 1×10^4 (□), 5×10^4 (○), 1×10^5 (▲), 5×10^5 (■) and 1×10^6 (●) cells per ml

5×10^5 and 1×10^6 cells per ml produced maximal RFU relatively quickly (180 and 150 min respectively). However, the resazurin kinetics of strain HT01 (pYES2 α-syn GFP) showed almost no difference compared to that of strain HT01 (pYES2-GFP) (Figure 5a,b). Therefore, starting cell concentrations of 5×10^4 to 1×10^5 cells per ml already showed significant RFUs in the presence of any growth. Also, the optimal cell concentrations of the HT01 background strains were between 5×10^4 and 1×10^5 cells per ml, and the optimal incubation time with resazurin was longer than 300 min.

The TK01 background strains showed faster reduction kinetics than the HT01 background strains (Figure 5c, 5d vs 5a, 5b). Initial cell concentrations of theTK01 background strains between 5×10^5 and 1×10^6 cells per ml almost immediately reduction resazurin to resorufin, with a steep increase in fluorescence intensity, whereas lower cell concentrations started to increase the amount of resorufin with slower kinetics of resazurin reduction. However, starting cell concentrations of less than 5×10^4 cells per ml displayed significant lags in growth. Starting cell concentrations between 5×10^4 and 1×10^5 cells per ml yielded a significant increase in RFUs over time compared to the other cell concentrations and thus were chosen as the optimal range of cell concentrations (Figure 5c,d). According to the optimal concentrations chosen, the incubation time with resazurin should be monitored for up to 240 min for strain TK01 (pYES2 α-syn GFP) as the yeast indicator strain for the assay (Figure 5c).

Validation of the resazurin yeast-based assay system

Usually test compounds are dissolved in 100% (v/v) DMSO solvent, so the DMSO concentration must be optimized in order to avoid the cytotoxic effect on yeast cells. This study, a final concentration of DMSO at 0.5% (v/v) was chosen to minimize its cytotoxicity (Figure S1).

To validate the resazurin yeast-based assay for the isolation of compounds that alleviate α-syn toxicity, we determined the effects of baicalein, which inhibits α-syn oligomerization (Hu et al., 2016), at various concentrations (25–0.04 μmol l^{-1}). For strain HT01 (pYES2 α-syn GFP), no protective effect of baicalein was found against α-syn toxicity at any concentrations tested (Figure 6a). In contrast, strain TK01 (pYES2 α-syn GFP) treated with 5 μmol l^{-1} baicalein showed a significant increase in RFUs compared to the untreated control (0 μmol l^{-1}) (Figure 6c). However, RFUs from cells treated with lower concentrations (<5 μmol l^{-1}) of baicalein were not statistically different (Figure 6c). The strains harbouring the empty vector treated with any concentration of baicalein served as indicator cells for the cytotoxicity of the compound at the concentrations tested. At 25 μmol l^{-1}, but not at 5 μmol l^{-1}, baicalein caused toxicity to all tested strains (Figure 6a–d).

Furthermore, another compound, ampicillin, a β-lactam antibiotic that inhibit bacterial cell wall synthesis, was also tested for the specificity of the assay system. Strain TK01, carrying either pYES2 α-syn GFP or the empty

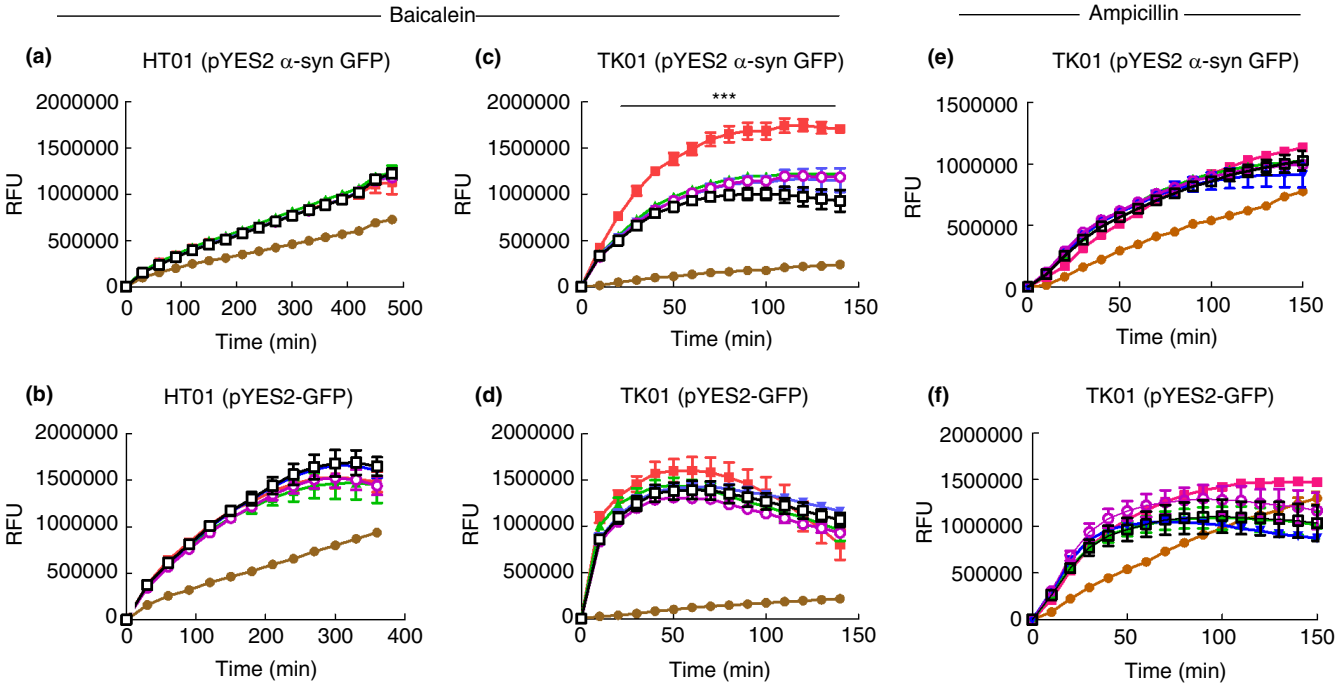

FIGURE 6 Effects of baicalein and ampicillin on alleviating α-syn toxicity in the resazurin yeast-based assay system. Strains HT01 (pYES2 α-syn GFP) (a), HT01 (pYES2-GFP) (b), TK01 (pYES2 α-syn GFP) (c) and TK01 (pYES2-GFP) (d) were grown in SG-Ura supplemented with baicalein at final concentrations 0 (□), 0.04 (○), 0.2 (▼), 1 (▲), 5 (■) and 25 (●) μmol l^{-1} as indicated. Strains TK01 (pYES2 α-syn GFP) (e) and TK01 (pYES2-GFP) (f) were grown in SG-Ura supplemented with ampicillin at final concentrations 0 (□), 1.56 (○), 3.13 (▼), 6.25 (▲), 12.5 (■) and 25 (●) mmol l^{-1} as indicated. The 96-well plates were incubated at 30°C for 16–18 h. Resazurin was added to a final concentration at 0.2 mmol l^{-1}. Fluorescence intensity was measured over 100 min. The values are means ± standard deviations (SD) of results from three independent experiments. For (c), statistical significances are indicated by the *p*-value (***$p < 0.001$, 5 μmol l^{-1} vs. 0 μmol l^{-1})

vector, were incubated in assay medium containing ampicillin at concentrations ranging from 25 to 1.56 mmol l^{-1} for 24 h. The kinetic of resazurin reduction were monitored. The results showed that ampicillin treatments did not exhibit any statistically significant differences from the untreated control, suggesting that ampicillin does not have a role in alleviating α-syn toxicity. Treatment of cells with a high concentration of ampicillin (25 mmol l^{-1}) resulted in a cytotoxic effect in both yeast strains (Figure 6e and f; Figure S2).

Next, to compare the sensitivity of strains TK01 (pYES2 α-syn GFP) and BY25929 (pYES2 α-syn GFP) was incubated in assay medium containing the desired concentrations of baicalein. The kinetics of resazurin conversion into resorufin in cell suspensions of both strains are summarized in Figure 7, which depicts the resazurin reduction curves for strains TK01 and BY25929 in various concentrations of baicalein. The cytotoxicity of baicalein was observed at 12.5–25 μmol l^{-1} with low resazurin reduction rates. Strain BY25929 (pYES2 α-syn GFP), treated with either 3.13 or 6.25 μmol l^{-1} baicalein, exhibited a significantly higher RFU than the untreated control (Figure 7a). Interestingly, strain TK01 (pYES2 α-syn GFP), treated with either 3.13 or 6.25 μmol l^{-1} baicalein, revealed much higher reduction kinetics than strain BY25929 (pYES2 α-syn GFP), treated with the same amount of baicalein, showing a significantly higher RFU than the untreated control (Figure 7c). These findings indicate that in the BY25929 background strain, the *rsp5*^{A401E} mutant strain (TK01) was more sensitive to a compound that alleviate α-syn toxicity than the wild-type *RSP5* strain (BY25929) (Figure 7c vs 7a).

To compare the sensitivity of our developed assay system with that of the conventional growth monitoring method using optical density measurement at 660 nm, a 96-well clear microplate containing either strain BY25929 (pYES2 α-syn GFP) or TK01 (pYES2 α-syn GFP) treated with various concentrations of baicalein (25–0.39 μmol l^{-1}) was incubated at 30°C for 24 h. Then, the growth of yeast cells in each well was measured at OD_{660} nm. There were no statistically significant differences in growth between the cells treated with 0.39–6.25 μmol l^{-1} baicalein and the untreated control (Figure 7b and 7d). However, a significant decrease in the growth of cells treated with 12.5 and 25 μmol l^{-1} baicalein were observed, indicating the cytotoxic effect of baicalein at such concentrations (Figure 7b and 7d). These results showed that the assay based on a fluorescent signal of

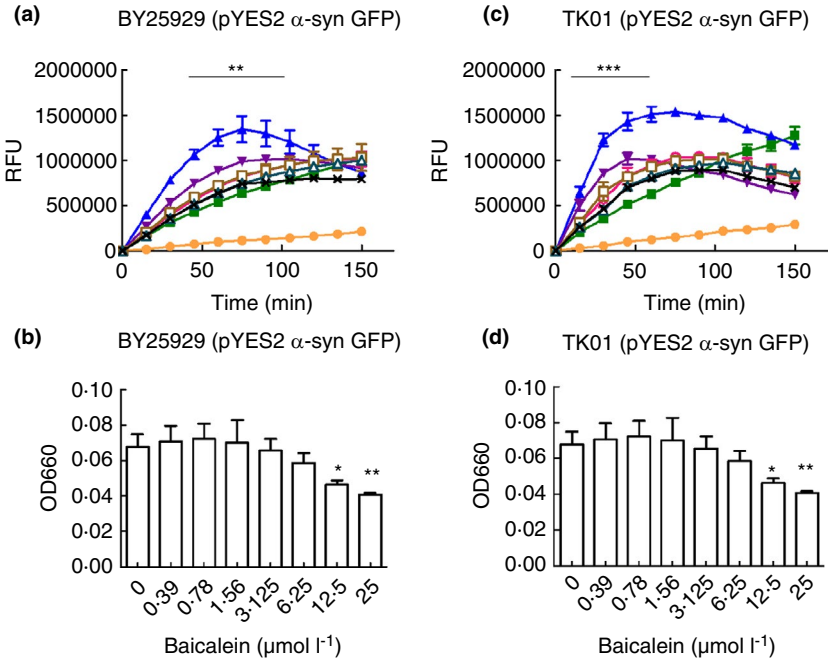

FIGURE 7 Comparison of sensitivity to various baicalein concentrations in the assay systems. Strains BY25929 (pYES2 α-syn GFP) (a and b) and TK01 (pYES2 α-syn GFP) (c and d) were grown in SG-Ura supplemented with baicalein at final concentrations 0–25 μmol l^{-1}. The cultures were incubated at 30°C for 16–18 h. For (b) and (d), their growth was assessed by measuring the optical density at 660 nm (OD_{660}). For (a) and (c), resazurin was added to a final concentration at 0.2 mmol l^{-1}. Fluorescence intensity was measured over 100 min. Results represent the mean ± standard deviations (SD) of three independent experiments. For (a) and (c), statistical significances are indicated by the *p*-values (**$P < 0.01$; ***$P < 0.001$, 3.13 μmol l^{-1} vs 0 μmol l^{-1}). For (b) and (d), statistical significances are indicated by the *p*-values (*$p < 0.05$, 12.5 μmol l^{-1} vs. 0 μmol l^{-1}; **$p < 0.01$, 25 μmol l^{-1} vs. 0 μmol l^{-1}). Symbols indicate the final concentration of baicalein at 0 (×), 0.39 (Δ), 0.78 (□), 1.56 (○), 3.13 (▼), 6.25 (▲), 12.5 (■) and 25 (●) μmol l^{-1}

resorufin was much more sensitive than the cell turbidity determination (Figure 7a, 7c vs. 7b, 7d).

Together, these findings supported that the resazurin yeast-based assay system using the *rsp5*^{A401E} mutant strain TK01 (pYES2 α-syn GFP) was an effective assay system with high sensitivity and specificity.

DISCUSSION

Saccharomyces cerevisiae is one of the most fantastic model organisms due to its easy handling and genetic availability. Expression of α-syn in yeast cells disrupted vesicle trafficking, ion homeostasis and mitochondrial function (as in neurons), leading to growth inhibition (Flight, 2013). This finding was based on the assumption that compounds that protect yeast cells from α-syn-mediated toxicity would be "positively" detectable by their ability to promote the growth of cells suffering from α-syn toxicity. We thus developed a novel yeast-based assay to search for compounds alleviating α-syn toxicity by utilizing resorufin, a highly sensitive fluorescent growth reduced product, to measure the output in 96-well plate format. This is based on the finding that *S. cerevisiae* cells have thick cell walls and bear ABC transporters with xenobiotic efflux capacity, which are obstacles to drug screening for test compounds entering and accumulating inside the cells (Sangkaew et al., 2018). To overcome this problem, in this study a multidrug-sensitive yeast strain BY25929, from which were deleted four so-called *MDR1* genes encoding for ABC transporters, was used to facilitates the uptake of compounds in the drug screening (Sangkaew et al., 2018).

Rsp5p, the Nedd4 ortholog in *S. cerevisiae* is also able to bind α-syn and efficiently ubiquitinates α-syn leading to the promotion of α-syn degradation and cell protection from α-syn-induced toxicity in *S. cerevisiae* (Braun, 2015). Interestingly, Hoshikawa et al. (2003) reported that the *rsp5*^{A401E} mutant showed much higher sensitivity to the toxic proline analogue AZC compared to the wild-type strain. This AZC-associated phenotype was involved in the deficient ubiquitination of the general amino acid permease Gap1, leading to the stabilization of Gap1 on the plasma membrane (Hoshikawa et al., 2003). After α-syn expression was shut off in the *rsp5*^{A401E} mutant, the intracellular level of α-syn was found to decrease, suggesting that the mutant still could ubiquitinate α-syn (unpublished results). When α-syn was overexpressed, the *rsp5*^{A401E} mutants (HT01 and TK01 strains) were more sensitive to α-synuclein than their respective *RSP5*^{WT} strains (Figure 3).

To determine the viability of yeast cells expressing α-syn in a high-throughput format, we set up a resazurin reduction assay, which is a reliable, sensitive and easy-to-use fluorescent assay, using two different background yeast strains, HT01 (pYES2 α-syn GFP) and TK01 (pYES2 α-syn GFP). Resazurin is an oxidation-reduction dye that turns into a fluorescent compound (resorufin) after a reduction process caused by viable cells (Bowling et al., 2012; Zare et al., 2015). A previous study suggested that the assay using resazurin to monitor both viability and growth of cells was more sensitive than the agar plate assay (Sangkaew et al., 2018). The fluorescence intensity measured is proportional to cell growth and can be quantified by fluorescence spectroscopy (Ibáñez Escribano et al., 2012). Several experimental parameters, such as the resazurin concentration, starting cell concentration and time of incubation with the redox dye, were optimized as follows. A concentration of 0.2 mmol l^{-1} of resazurin was chosen for further experiments because this concentration yielded the highest RFU signal and because higher concentrations might exhibit antifungal activity (Figure 4).

Our results in Figure 5 showed that the resazurin reduction rate was accelerated as the amount of yeast cells increased. When the amount of resorufin reached its maximum and entered a stationary phase, fluorescence became stable for a certain time period, while in the final part of each curve the RFU value was decreased. The decline of the curve was probably attributable to a secondary reduction of resorufin to a colourless and nonfluorescent product, hydro-resorufin (Travnickova et al., 2019).

Overall, strain TK01 showed faster reduction kinetics than strains HT01 (Figure 5c, 5d vs 5a, 5b). Low-level RFU peaks from initial cell concentrations between 5×10^5 and 1×10^6 cells per ml of TK01 (pYES2 α-syn GFP) were observed due to the faster resazurin reduction to resorufin and hydro-resorufin (Sangkaew et al., 2018). Consequently, the optimal initial cell concentrations of strains HT01 (pYES2 α-syn GFP) and TK01 (pYES2 α-syn GFP) were 5×10^4–1×10^5 cells per ml (Figure 5a and 5c). Furthermore, it is important to determine the optimal resazurin incubation periods since resazurin reduction is affected by time and cell density. In order to prevent the over-reduction of resazurin, the time periods needed to reach a maximum level of RFU were also recorded. At the optimal initial cell concentration of strain HT01 (pYES2 α-syn GFP), the kinetics of resazurin at the time periods required to determine the effect of the test compound should be monitored more than 300 min (Figure 5a), while that of strain TK01 (pYES2 α-syn GFP) could be observed within 300 min (Figure 5c). The resazurin reduction rate in a yeast strain overexpressing α-syn treated with a candidate compound is expected to be faster than that of an untreated control and may be, in some cases, close to that of a respective yeast strain without α-syn expression, HT01 (pYES2-GFP) and TK01 (pYES2-GFP), indicating the alleviation of α-syn toxicity by the candidate compound. However, to avoid a false-negative screening, it is important to monitor the kinetics of resazurin reduction of each test compound due to the different behaviors of each individual test compound.

To determine the reliability of this resazurin yeast-based assay for drug screening, two known compounds, baicalein and ampicillin, were tested for their influence on α-syn toxicity. To minimize the toxic effect of DMSO, these compounds were dissolved in DMSO at a final concentration of 0.5% (v/v) (Figure S1). Baicalein is a typical flavonoid compound isolated from traditional Chinese herb *Scutellaria baicalensis Georgi*. It exerts neuroprotective effects in PD model through antioxidant and anti-inflammatory activity (Mu et al., 2009; Wang et al., 2013). Simultaneously, previous research reported that baicalein could inhibit the formation of α-syn oligomers, and disaggregate existing oligomer of α-syn *in vitro* (Zhu et al., 2004). In PD cellular model, baicalein is demonstrated to be an effective inhibitor of α-syn aggregation and protect cells from α-syn oligomer-induced cytotoxicity (Hu et al., 2016; Lu et al., 2011). Our results showed that baicalein prevented α-syn oligomer-induced cytotoxicity only in strain TK01 (pYES2 α-syn GFP) (Figure 6c) and not in strain HT01 (pYES2 α-syn GFP) (Figure 6a). These results indicate that the drug-sensitive BY25929 background showed higher sensitivity to baicalein than the CAY29 background. This may be due to greater accumulation of baicalein inside TK01 (pYES2 α-syn GFP) cells than the other. Baicalein concentrations of 0.04–5 μmol l^{-1} showed no cytotoxicity except at the concentration of 25 μmol l^{-1} (Figure 6a–d). The irrelevant compound ampicillin, an antibiotic used to prevent and treat bacterial infections via inhibition of bacterial cell wall synthesis (Chambers & Sachdeva, 1990), did not significantly increase the RFU signal of strain TK01 (pYES2 α-syn GFP) (Figure 6e) including HT01(pYES2 α syn GFP) (Figure S2) when compared to that of the untreated control (0 μmol l^{-1}). However, high concentrations of ampicillin showed cytotoxicity with significantly lower RFU signals (Figure 6e and f and Figure S2). These results assured the specificity of the developed assay system.

Baicalein was also used to compare the sensitivity to α-syn toxicity between the *rsp5*^{A401E} and *RSP5*^{WT} strains with the BY25929 background. Strain TK01 (pYES2 α-syn GFP) treated with baicalein exhibited faster and higher reduction kinetics than strain BY25929 (pYES2 α-syn GFP) treated at 3.13 μmol l^{-1} baicalein (Figure7c vs 7a). In contrast, when baicalein-treated cells were measured for growth using conventional optical density at OD_{660}, no positive effect of baicalein was detected (Figure 7b and

d). The highly sensitive assay of our resazurin yeast-based screening system was further confirmed by using strain TK01 (pYES2 α-syn GFP) as an indicator strain could detect baicalein at concentrations as low as 3.13 μmol l^{-1}, which was lower than that in previous report that showed that baicalein inhibited α-syn fibrillation at 5–100 μmol l^{-1} using an *in vitro* Thioflavin T (ThT) assay (Zhu et al., 2004).

Taken together, these results suggest that TK01 (pYES2 α-syn GFP) is sensitive and specific enough to serve as an indicator strain for this resazurin yeast-based assay system. Furthermore, the toxicity of the test compound was confirmed during screening using strain TK01 (pYES2-GFP). This study has successfully established a novel yeast-based screening system for compounds that can restore growth inhibition of *rsp*5^{A401E} cells overexpressing α-syn. Baicalein alleviated α-syn toxicity by restoring the growth of the indicator strain. For pharmacological application, our method can be used as a primary drug screening system for the discovery of potential active compounds for neurodegenerative diseases especially PD. The candidate compounds may either efficiently suppress α-syn aggregation or enhance ubiquitin-dependent degradation. However, to validate the compounds identified in our yeast-based system, a human cell PD model need to be further used.

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CONFLICT OF INTEREST

The authors confirm that there are no conflicts of interest associated with the present work.

AUTHOR CONTRIBUTIONS

A.S., H.T. and C.Y. conceived the study and designed the experiments. A.S., T.K. and R.T. performed the experiments. A.S., H.T. and C.Y. analysed the data and wrote the manuscript. All authors read and approved the final manuscript.

ETHICAL APPROVAL

This article does not contain any studies with human participants or animals by the authors.

ORCID

Hiroshi Takagi https://orcid.org/0000-0002-1786-3174
Chulee Yompakdee https://orcid.org/0000-0003-4690-4809

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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