Nonantioxidant Tetramethoxystilbene Abrogates α-Synuclein-Induced Yeast Cell Death but Not That Triggered by the Bax or βA4 Peptide

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S Supporting Information

ABSTRACT: The overexpression of α-synuclein (α-syn) and its aggregation is the hallmark of Parkinson's disease. The α-syn aggregation results in the formation of Lewy bodies that causes neuronal cell death. Therefore, the small molecules that can protect neuronal cells from α-syn toxicity or inhibit the aggregation of α-syn could emerge as anti-Parkinson agents. Herein, a library of methoxy-stilbenes was screened for their ability to restore the cell growth from α-syn toxicity, using a yeast strain that stably expresses two copies of a chromosomally integrated human α-syn gene. Tetramethoxy-stilbene **4s**, a nonantioxidant, was the most capable of restoring cell growth. It also rescues the more toxic cells that bear three copies of wild-type or A53T-mutant α-syn, from cell growth block. Its EC_{50} values for growth restoration of the 2-copy wild-type and the 3-copy mutant α-syn strains are 0.95 and 0.35 μM, respectively. Stilbene **4s** mitigates mitochondrial membrane potential loss, negates ROS production, and prevents nuclear DNA-fragmentation, all hallmarks of apoptosis. However, **4s** does not rescue cells from the death-inducing effects of Bax and βA4, which suggest that **4s** specifically inhibits α-syn-mediated toxicity in the yeast. Our results signify that simultaneous use of multiple yeast-cell-based screens can facilitate revelation of compounds that may have the potential for further investigation as anti-Parkinson's agents.
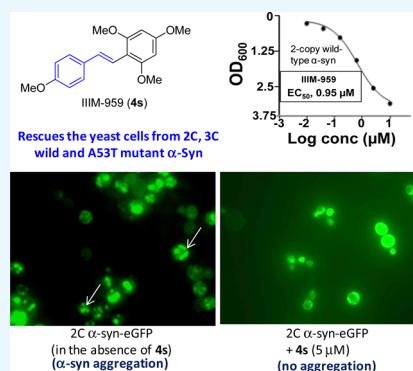
1. INTRODUCTION

Parkinson's disease (PD) is caused by the loss of neuronal cells in the brain which leads to a reduction in dopamine that plays a vital role in regulating the body movement. The manifestations of PD include bradykinesia (slow movement), postural instability, muscular rigidity, and resting tremors. The overexpression of α-synuclein (α-syn) and βA4 proteins causes accumulation of aggregated or mis-folded proteins which are thought to be the key to the pathogenesis of PD and Alzheimer's disease. Aggregation results in the formation of insoluble α-syn and βA4 deposits, referred to as Lewy bodies that lead to neuronal cell death (i.e., neuronal apoptosis).[1] Gene duplication, multiplication of the α-syn gene locus,[2] and point mutations in the α-syn gene that incorporate single amino acid changes, such as Ala53Thr (A53T), lead to overexpression of the 140-amino acid α-syn protein.[3] Proteolytic cleavage of the precursor amyloid precursor protein releases the 42-amino acid βA4 peptide which is overproduced, mainly because of genetic reasons, in individuals suffering from Alzheimer's disease.[4] The expression of human α-syn in the baker's yeast, *Saccharomyces cerevisiae*, results in the accumu-

lation of ROS followed by the manifestation of apoptosis.[5] *S. cerevisiae* has also been used to target a green fluorescent protein (GFP)-tagged βA4 peptide to yeast's secretory pathway, with the help of a signal sequence upstream of the βA4-GFP fusion gene. The secreted βA4 fusion protein manifests toxicity in yeast.[6] A number of studies have now confirmed that yeast is a suitable system for studying the pathogenesis of both human α-syn and βA4.[5,7]

Bax is a proapoptotic protein that belongs to the Bcl-2 family.[8] It manifests its apoptotic function when bound to mitochondrial membranes.[9] It is very likely that Bax has a much broader role than α-syn in neuronal cell death and plays a major part in the overall regulation of neurodegenerative processes that lead to neuronal apoptosis.[10] More specifically, in a mouse model of PD, Bax takes part in the destruction of neurons that produce dopamine. Hence, it has been suggested that down-regulation of Bax can be an attractive and novel
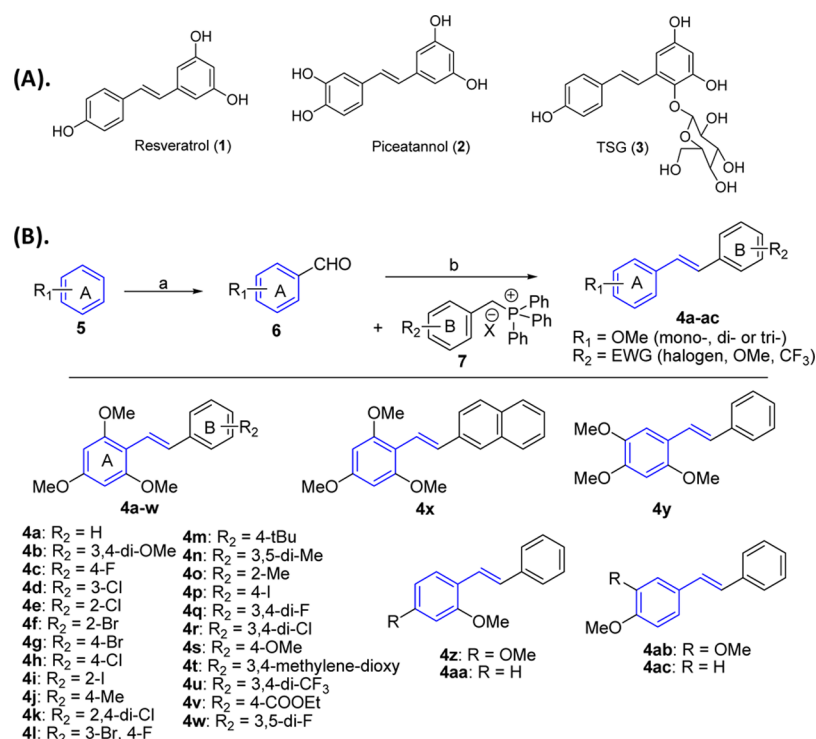
Figure 1. (A) Structures of naturally occurring stilbenes **1−3** which inhibit α-syn aggregation. (B) Synthesis of methoxy-stilbenes **4a−4ac**. Reagents and conditions: (a) POCl$_3$, dimethylformamide, room temp., 70−80%; (b) **7** (1 mmol), **6** (1 mmol), KOtBu (1 equiv), dry THF, 80 °C, 30 min, 60−95%.

therapeutic target for restricting the progression of PD.[11] In the yeast *S. cerevisiae*, the expression of Bax also generates ROS which precedes apoptosis that is prevented by the anti-apoptotic proteins of the Bcl-2 family, Bcl-2, and Bcl-xL.[12] This is similar to what has been observed in mammalian cells.[13]

We have cloned the gene coding for the human proteins α-syn, its more toxic A53T mutant, Bax and βA4, under the control of the galactose-inducible GAL1 promoter, in plasmids that allow integration of gene expression cassettes at chromosomal locations of a yeast strain (i.e., W303-1a; ATCC 208352), via homologous recombination.[14] The human α-syn and the mutant A53T α-syn were cloned in such a way that one to three copies of the gene could stably be expressed from yeast chromosomes. For human βA4, yeast strains were constructed that allow secretion of the peptide from one copy or two copies of the gene. We have observed that, with the increase of gene dosage or copy number (i.e., from one copy to two or three copies), the toxicity manifested by α-syn and βA4 also increases. The human Bax protein was expressed only from a single chromosomally integrated gene where it showed enough toxicity to stop cell growth and induce cell death. The two-copy wild-type α-syn expressing yeast strain was used for the initial screening of a chemical library of methoxy-stilbenes, to identify compounds which can overcome the α-syn protein toxicity.

Because deposition of α-syn as neuronal inclusions, synonymous to Lewy bodies, is likely to be the causative agent of the disease suffered by most PD patients,[15] targeting toxicity manifested by α-syn overexpression is considered an important approach for discovering new anti-PD drugs.[16] Reported inhibitors of α-syn aggregation include N-methylated derivatives of peptides from the α-syn protein sequence,[17] single-chain antibodies,[18] small-molecule antioxidant com-pounds such as curcumin,[19] epigallocatechin gallate (EGCG),[20] and 8-hydroxyquinolines.[21] A large number of naturally occurring polyphenolic compounds also have been reported to inhibit the aggregation of α-syn and prevent its toxicity.[22] Among these polyphenols, the antioxidant stil-benes[23] viz. resveratrol (**1**), piceatannol (**2**), and 2,3,5,4′-tetrahydroxystilbene-2-*O*-β-D-glucoside (TSG, **3**) have been shown to inhibit α-syn aggregation, although it has never been shown that these compounds can overcome α-syn's toxicity in yeast, perhaps because of their relatively low permeability across the yeast cell wall. Guo et al.[24] have reported that resveratrol ameliorates motor deficits and pathological changes in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated mice via activation of SIRT1 and subsequent LC3 deacetylation-mediated autophagic degradation of α-syn. Another phenolic stilbene, piceatannol, a constituent of vine, inhibits the formation of α-syn fibrils by destabilizing preformed filaments.[25] The glycosylated stilbene (TSG, **3**, Figure 1A), extracted from *Polygonum multiflorum*, inhibits α-syn aggrega-tion and apoptosis in A53T mutant α-syn-transfected cells.[26]

In the present paper, the in silico-designed series of methoxy-stilbenes were synthesized and studied for their ability to protect yeast cells from α-syn mediated toxicity. The tetramethoxy stilbene **4s** (IIIM-959) was identified as the unique compound, which is a nonantioxidant, and rescues the more toxic cells that bear 3-copies of wild-type or A53T-mutant α-syn, from cell growth block. However, it does not rescue cells from the death-inducing effects of Bax and βA4. The effect of **4s** on inhibition of α-syn aggregation was also studied. The results are presented in this paper.
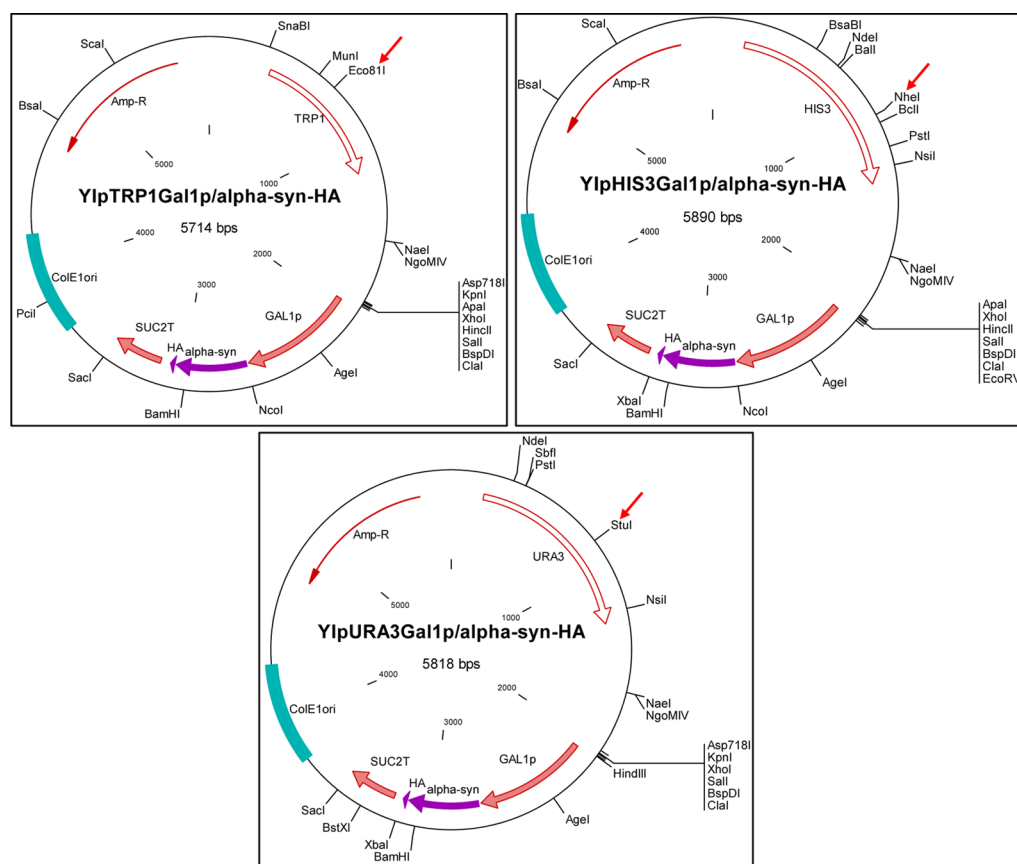
Figure 2. Three integrative plasmids used to introduce HA-tagged human α-syn gene expression cassettes, under the control of the GAL1 promoter, into three different chromosomal locations (i.e., where the TRP1, HIS3, and URA3 genes lie) of the yeast genome. The arrows show the restriction sites at which the plasmids were linearized for genomic (i.e., chromosomal) integration via homologous recombination.[14]

2. RESULTS AND DISCUSSION

2.1. Design and Synthesis of Methoxy-Stilbenes. The structure of the α-syn monomer[27] and its fibrils[28] has been studied by several groups. Preclinical studies have shown that the α-syn fibrils cause Parkinson-like neurodegeneration in nontransgenic mice models.[29] Furthermore, recently Mach and co-workers[30] have shown that small molecules binds to various pockets of the β-sheet structure (residue K34 to K97) of α-syn fibrils which is rich in hydrophobic residues; thus it was speculated that methoxystilbenes would display strong interactions with the β-sheet structure. Thus, a series of mono-, di-, tri-, and tetramethoxy-stilbenes were synthesized as depicted in Figure 1B. The synthesis involved Wittig reaction of aryl aldehydes **6** with phosphine salts **7** in the presence of potassium *tert*-butoxide under anhydrous conditions. The aldehydes which are not commercially available were synthesized by Vilsmeier−Haack reaction. Using the synthetic scheme as shown in Figure 1B, the stilbenes **4a−4ac** were obtained in excellent yields. A total of 29 stilbenes were prepared and were characterized by NMR and MS analysis.

2.2. Construction of Yeast Strains That Contain One Copy, Two Copies, and Three Copies of the Human α-Syn Gene. At first, the plasmids were constructed that allow the expression of 1-copy and 2- and 3-copies of the α-syn gene in the yeast. A BglII-XbaI fragment of the coding sequence of the human α-syn gene [NCBI Accession # NM_000345.3], containing a 3′-end DNA sequence that codes for the HA-tag peptide (consisting of the nine amino acid residues YPYDVPDYA),[31] was isolated from a human hippocampal cDNA library (BioCat) by PCR and was cloned in yeast integration vectors downstream of the GAL1 promoter [*Saccharomyces* Genome Database ID S000000224] and upstream of the SUC2 gene terminator signal [*Saccharomyces* Genome Database ID S000001424]. The GAL1 promoter is repressed in the presence of glucose and induced in the presence of the sugar and galactose. After cloning of the HA-tagged wild-type α-syn gene in appropriate yeast integrative vectors, the following plasmids were obtained: YIpTRP1-Gal1p/α-syn-HA, YIpHIS3Gal1p/α-syn-HA, and YIpURA3-Gal1p/α-syn-HA (Figure 2). These plasmids contain the α-syn gene sandwiched between the GAL1 promoter and the SUC2 terminator signal and allow integration of one copy, two copies, and three copies of the α-syn gene into chromosomal locations where the auxotrophic markers, *TRP1*, *HIS3*, and *URA3* genes, reside on the yeast genome.

The basic yeast strain used for integration was W303-1a (*Mata, ade2, his3, leu2, trp1, ura3*) (ATCC #208352). Herein, it is referred to as BC300. The yeast strain obtained after integration of the plasmid YIpTRP1Gal1p/α-syn-HA in BC300 was named BC300::α-syn-HA(TRP1); it contained a single copy of the α-syn gene integrated at the *TRP1* chromosomal locus. The plasmid YIpHIS3Gal1p/α-syn-HA was integrated into the strain BC300::α-syn-HA(TRP1) to obtain the strain BC300::α-syn-HA(TRP1), α-syn-HA(HIS3); it contained two copies of the α-syn gene integrated at the *TRP1* and *HIS3* chromosomal loci. The plasmid YIpUR-A3Gal1p/α-syn-HA was integrated into the strain BC300::α-syn-HA(TRP1), α-syn-HA(HIS3) to obtain the strain
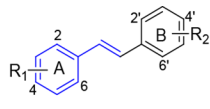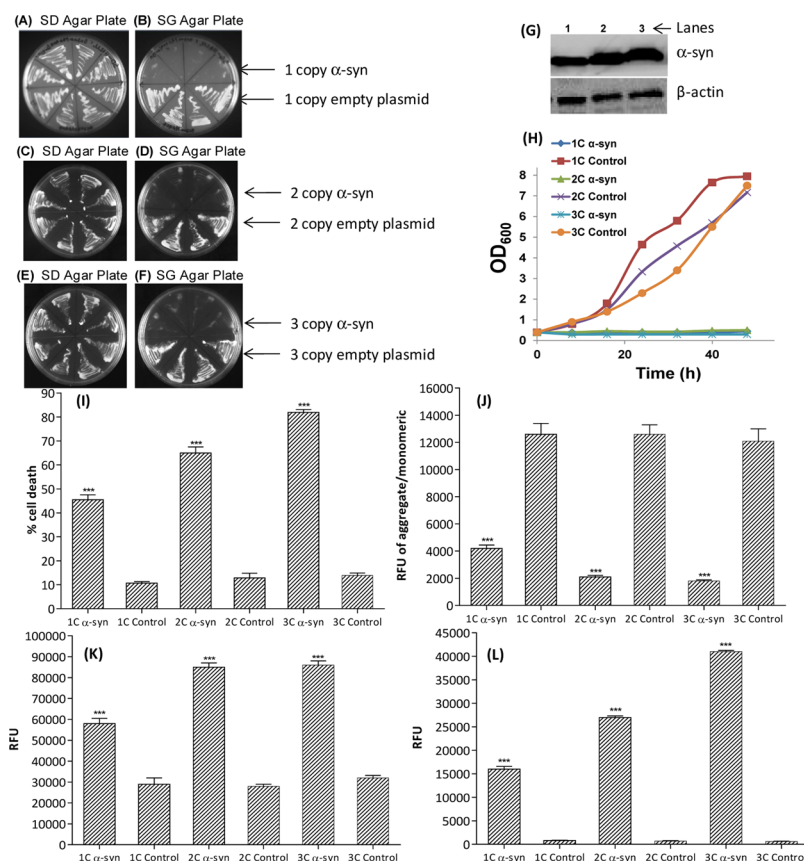
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Figure 3. Growth of yeast strains containing two and three copies of the human α-syn gene, and analysis of cell death, MMP loss, ROS activity, and nuclear DNA fragmentation. (A−F) Growth of yeast cells harboring one copy, two copies, and three copies of the human α-syn gene on minimal medium solid agar plates that contained either glucose (SD) or galactose (SG). Panels (A−F), upper half (4 clones): strains containing one copy of the α-syn gene or two and three copies of the α-syn gene. Panels (A−F), lower half (4 clones): strains containing an empty plasmid or empty plasmids, without any α-syn gene, under the control of the GAL1 promoter. The solid agar plates were incubated at 30 °C for 96 h. (G) Western blot analyses of cells expressing one to three copies of HA-tagged α-syn protein. On lanes 1, 2, and 3 were loaded total protein (10 μg) obtained after lysis of cells that express one copy, two copies, and three copies of α-syn. The upper panel was probed with an antibody that recognizes the HA epitope (Proteintech, 51064-2-AP) and the lower panel with a β-actin antibody (Proteintech, 60008-1-Ig); levels of β-actin were used as a loading control, β-actin being a housekeeping gene. (H) Growth of yeast strains that contain one copy, two copies, and three copies of the α-syn gene in liquid minimal medium yeast cell culture that contained galactose as a carbon source. OD_{600} represents the optical density of cells at 600 nm. The OD_{600} values at each time point represent the average of three independent experiments. (I) Analysis of cell death—percentage of yeast cells undergoing death after culturing strains expressing one copy, two copies, and three copies of α-syn for 20 h in galactose. Cells were stained with Phloxine B for detection of dead cells.[33] The data represent mean ± SD of five independent fields of observation. Around 200 cells were counted in each field; (J) comparing the MMP of cells expressing one copy, two copies, and three copies of α-syn with cells containing empty plasmids as controls. Cells were stained with the JC-10 dye to detect MMP loss.[34] The data represent mean ± SD of three independent experiments. (K) Total ROS activity of yeast strains containing one copy, two copies, and three copies of α-syn compared with strains than contain empty plasmids. Cells were stained with dihydroethdium to detect ROS.[35b] The data represent mean ± SD of three independent experiments. (L) Comparative levels of nuclear DNA fragmentation in yeast strains which express one copy, two copies, and three copies of α-syn, using the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, compared with strains that contain empty plasmids.[35b] The data represent mean ± SD of three independent experiments. For all four sets of analyses (I−L), cells were cultured for 20 h in galactose, for induction of α-syn protein expression, before treatment of cells, as required by the assay [statistical analysis was performed in (I−L) for comparison between the 1C, 2C, and 3C α-syn with the respective control group, ***p < 0.01].

BC300::α-syn-HA(TRP1), α-syn-HA(HIS3), α-syn-HA-(URA3); it contained three copies of the α-syn gene integrated at the *TRP1*, *HIS3* and *URA3* chromosomal loci. To generate negative controls, the strain BC300 was integrated with empty vectors (i.e., basic integrating vectors which do not contain the α-syn gene) in three successive steps to obtain the three strains: (a) BC300::—(TRP1); (b) BC300::—(TRP1), —(HIS3); and (c) BC300::—(TRP1), —(HIS3), —(URA3); they contain one copy, two copies, and three copies of an empty plasmid at the (i) *TRP1*, (ii) *TRP1&HIS3*, and (iii) *TRP1, HIS3&URA3* chromosomal loci, respectively.

2.3. Growth of Yeast Strains That Contain (a) Two and Three Copies of the Human α-Syn Gene and (b) Two and Three Copies of Empty Plasmids, on Sold Agar Minimal Medium Plates, Containing Glucose or Galactose.

Four clones from each of the three strains BC300::α-syn-HA(TRP1), BC300::α-syn-HA(TRP1), α-syn-HA(HIS3), and BC300::α-syn-HA(TRP1), α-syn-HA(HIS3), α-syn-HA-(URA3), containing 1-copy, 2-copies, and 3-copies of the human α-syn gene, respectively, were streaked out on solid agar synthetic minimal medium plates that contained either galactose (SG) or glucose (SD) as the carbon source. Cells were grown at 30 °C for 96 h. In galactose-containing medium,

Table 1. Rescue from the Block in the Growth of Yeast Cells That Express Two Copies of α-Syn, by Methoxy-Stilbenes, at 10 μM Concentration

Entry	R_1	R_2	Percentage Growth (mean ± SD)[a]
4a	2,4,6-tri-OMe	H	-6.23 ± 1.15
4b	2,4,6-tri-OMe	3,4-di-OMe	-9.74 ± 0.95
4c	2,4,6-tri-OMe	4-F	-13.46 ± 1.22
4d	2,4,6-tri-OMe	3-Cl	1.28 ± 0.32
4e	2,4,6-tri-OMe	2-Cl	5.92 ± 0.76
4f	2,4,6-tri-OMe	2-Br	7.24 ± 1.85
4g	2,4,6-tri-OMe	4-Br	-23.08 ± 1.76
4h	2,4,6-tri-OMe	4-Cl	-8.97 ± 1.02
4i	2,4,6-tri-OMe	2-I	-2.69 ± 0.62
4j	2,4,6-tri-OMe	4-Me	-6.92 ± 1.18
4k	2,4,6-tri-OMe	2,4-di-Cl	-5.64 ± 0.93
4l	2,4,6-tri-OMe	3-Br, 4-F	4.41 ± 1.43
4m	2,4,6-tri-OMe	4-tert-Bu	-6.55 ± 1.11
4n	2,4,6-tri-OMe	3,5-di-Me	-23.08 ± 2.83
4o	2,4,6-tri-OMe	2-Me	-8.72 ± 1.52
4p	2,4,6-tri-OMe	4-I	5.13 ± 0.84
4q	2,4,6-tri-OMe	3,4-di-F	62.89 ± 9.93
4r	2,4,6-tri-OMe	3,4-di-Cl	59.86 ± 7.48
4s	2,4,6-tri-OMe	4-OMe	79.64 ± 9.42
4t	2,4,6-tri-OMe	3,4-methylene-dioxy	-19.67 ± 1.96
4u	2,4,6-tri-OMe	2,4-di-CF$_3$	44.41 ± 4.43
4v	2,4,6-tri-OMe	4-COOEt	0 ± 0.08
4w	2,4,6-tri-OMe	3,5-di-CF$_3$	-7.08 ± 0.66
4x	2,4,6-tri-OMe	Ring B is 'naphth-2-yl'	-7.44 ± 0.82
4y	3,4,6-tri-OMe	H	-67.95 ± 6.5
4z	2,4-di-OMe	H	-26.92 ± 1.94
4aa	2-OMe	H	-9.31 ± 1.78
4ab	3,4-di-OMe	H	20.42 ± 5.94
4ac	4-OMe	H	0.96 ± 0.31

[a]The data represent mean ± SD of four independent experiments.

the α-syn gene is induced by the GAL1 promoter and, hence, the protein must be produced, whereas in glucose, the α-syn protein should not be produced because the gene expression is repressed. The strains (a) BC300::—(TRP1),(b) BC300::—(TRP1), —(HIS3), and (c) BC300::—(TRP1), —(HIS3), —(URA3) [containing one copy, two copies, and three copies of empty plasmids that do not contain the α-syn gene] were used as negative controls. The results show that expression of 1-copy, 2-copies, and 3-copies of the α-syn gene from different chromosomal loci block the growth of cells in

galactose, but not in glucose. Control strains containing 1-copy, 2-copies, and 3-copies of empty plasmids grow, as expected, both in SG and SD media (Figure 3A−F). It was inferred that the human α-syn protein must be toxic to the yeast, as has been reported earlier.[32] Cells were grown in minimal SG liquid medium containing galactose. Total cellular proteins were obtained via lysis of cells, and they were examined for the expression of the human α-syn protein. Western blot results show that the levels of α-syn protein, expressed in the yeast increase as the copy number increases
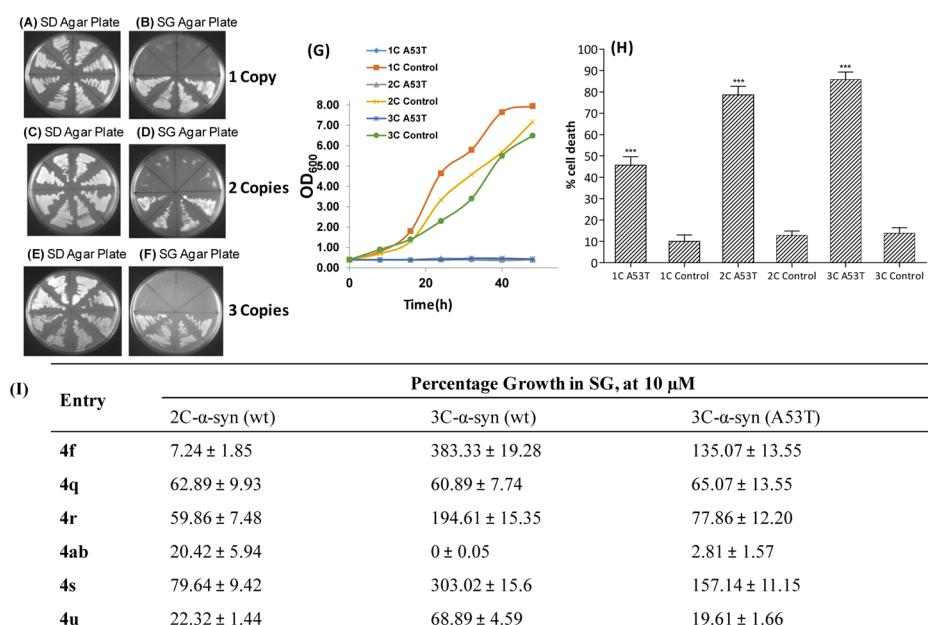
Figure 4. (A−F), Growth of yeast cells harboring one copy, two copies, and three copies of the human α-syn (A53T) gene, on minimal medium solid agar plates that contained either glucose (SD) or galactose (SG). Panels (A−F), upper half (4 clones): strains containing one copy, two copies, or three copies of the α-syn(A53T) gene, under the control of the GAL1 promoter that induces the expression of α-syn(A53T) protein, in the presence of galactose. Panels (A−F), lower half (4 clones): strains containing an empty plasmid or empty plasmids, without any α-syn (A53T) gene. The solid agar plates were incubated at 30 °C for 96 h. (G) Growth of yeast strains that contain one copy, two copies, or three copies of the α-syn (A53T) mutant gene and empty plasmids (1 to 3-copies) in liquid yeast cell culture minimal medium that contained galactose. The OD_{600} values at each time point represent the average of three independent experiments. (H) Percentage of cells undergoing death in yeast strains expressing one copy, two copies, and three copies of α-syn (A53T), after cells were cultured for 20 h in SG medium. Dead cells were stained with Phloxine B. The data represent mean ± SD of five independent fields of observation (statistical significance, ***$p < 0.01$). Around 200 cells were counted in each field. (I) Rescue from the block in growth of yeast cells that express (a) three copies of wild-type α-syn(wt) [3C-α-syn(wt)] and (b) three copies of mutant α-syn(A53T) [3C-α-syn(A53T)], by methoxy-stilbenes, at 10 μM concentration. This is compared with the growth that had been seen in the two-copy wild-type α-syn strain. The data represent mean ± SD of four independent experiments.

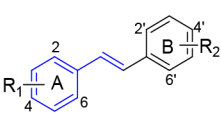| Entry	Percentage Growth in SG, at 10 μM		
	2C-α-syn (wt)	3C-α-syn (wt)	3C-α-syn (A53T)
4f	7.24 ± 1.85	383.33 ± 19.28	135.07 ± 13.55
4q	62.89 ± 9.93	60.89 ± 7.74	65.07 ± 13.55
4r	59.86 ± 7.48	194.61 ± 15.35	77.86 ± 12.20
4ab	20.42 ± 5.94	0 ± 0.05	2.81 ± 1.57
4s	79.64 ± 9.42	303.02 ± 15.6	157.14 ± 11.15
4u	22.32 ± 1.44	68.89 ± 4.59	19.61 ± 1.66

from 1 to 3 (Figure 3G). This would suggest that manifestation of α-syn protein's toxicity (i.e., block in cell growth) would increase with the increasing copy number from 1-copy to 2- and 3-copies of the α-syn gene.

Cell growth of the strains (a) BC300::α-syn(TRP1), (b) BC300::α-syn(TRP1), α-syn(HIS3), and (c) BC300::α-syn-(TRP1), α-syn(HIS3), α-syn(URA3) containing one copy, two copies, and three copies of the human α-syn gene, was compared in minimal liquid SG medium containing galactose (Figure 3H). The graphs show that the strains containing 1-copy, 2-copies, and 3-copies of the human α-syn gene do not grow in SG liquid medium, whereas the strains containing the empty plasmids do grow in the same medium. The strains were grown at 30 °C over a period of 48 h in liquid culture. The results again indicate that the human α-syn gene, when expressed in a galactose-containing yeast cell culture medium, completely blocks cell growth. The strain BC300::α-syn-(TRP1), containing a single copy of the α-syn gene, was not used any further in these studies. Although the expression of a single copy also blocked cell growth on agar plates, it was clear from Figure 3G that the BC300::α-syn(TRP1) expressed the least amount of α-syn protein.

To identify compounds in a screen of a library of methoxy-stilbenes, we wished to identify a compound that would overcome the maximum levels of toxicity mediated by α-syn, in yeast cells. Hence, it was thought that yeast strains which express 2-copies and 3-copies of the α-syn gene may be more appropriate for screening of chemical libraries to see if compounds could be identified that would overcome cell growth. Before embarking on such a screen, it was necessary to clarify the levels of cell death that the newly constructed yeast strains undergo, upon the expression of human α-syn. Cells, after the expression of α-syn in galactose, were treated with the dye Phloxine B which stains dead cells red.[33] The percentage of cell death, in the one- to three-copy α-syn expressing strains and the corresponding empty strains (i.e., cells which do not harbor the α-syn gene), is shown in Figure 3I. Increasing cell death was observed with the increasing α-syn copy number. Next, the loss of mitochondrial membrane potential (MMP) in yeast cells expressing one copy, two copies, and three copies of human α-syn was assessed. It has been suggested that α-syn aggregation causes mitochondrial dysfunction and thereby affects MMP[34] which allows release of ROS[35] that leads to neuronal apoptosis, a form of programmed cell death. It was, therefore, necessary to find out if these newly constructed α-syn-expressing yeast strains suffer from loss of MMP, generate ROS, and undergo apoptosis. Yeast strains expressing one copy, two copies, and three copies of α-syn do show gradual loss of MMP with the increasing copy number (Figure 3J). Concomitantly, yeast strains expressing one copy, two copies, and three copies of α-syn generate increasing levels of ROS with the copy number increase (Figure 3K). It was also seen that the three-copy α-syn-bearing cells undergo the most nuclear DNA fragmentation which mirrors the degree of apoptosis, that is, the two-copy and one-copy strains suffer less apoptosis than the three-copy strain (Figure 3L).[35]

2.4. Screening of the Methoxy-Stilbene Library in the Two-Copy Wild-Type α-Syn Strain. The 29-compound

Table 2. EC$_{50}$ Values of the Five Best Methoxy-Stilbenes in Their Ability to Rescue Growth of Cells That Express Two Copies of Wild-Type α-Syn

Entry	R$_1$	R$_2$	EC$_{50}$ (μM) [a]
4f	2,4,6-tri-OMe	2-Br	15.3 ± 1.3
4q	2,4,6-tri-OMe	3,4-di-F	1.2 ± 0.04
4r	2,4,6-tri-OMe	3,4-di-Cl	1.0 ± 0.8
4s	2,4,6-tri-OMe	4-OMe	0.95 ± 0.3
4u	2,4,6-tri-OMe	2,4-di-CF$_3$	1.4 ± 0.5

[a]The determined EC$_{50}$ values represent mean ± SD of three independent experiments.

chemically synthesized methoxy-stilbene library was screened using a yeast strain that expresses two copies of HA-tagged human α-syn from chromosomal loci. Compounds, at 10 μM concentration in 5% dimethyl sulfoxide (DMSO), were incubated at 30 °C with two-copy α-syn-expressing cells in minimal SG medium (that contained 2% galactose and 0.2% glucose) in 96-well microtiter plates. The plates were shaken at 200 rpm for 72 h. The growth of cells (measured by monitoring the optical density of cells at 600 nm; OD$_{600}$), in the absence of compounds but in the presence of 5% DMSO, was used as the base for calculating the percentage growth in the presence of compounds. The results are shown in Table 1. We have used another chemical library consisting of 20 "quinazolinones"[36] to show that one does not obtain "hits" in this yeast-based α-syn screen from any chemical library. One may refer to this as a negative control for such a screen. Results are shown in the Supporting Information, Section S1. The five best compounds **4f**, **4q**, **4r**, **4s**, and **4u** that rescue growth of the two-copy α-syn expressing yeast cells, in liquid culture, also rescue growth of the same cells on solid agar plates (Supporting Information, Section S2).

2.5. Yeast Cells Expressing One to Three Copies of the A53T α-Syn Mutant Protein Block Cell Growth and Induce Cell Death. The hits identified in the yeast strain that expresses two copies of α-syn were further tested for their cell rescuing ability in strains that express three copies of wild type α-syn, and also three copies of mutant α-syn (A53T); the plasmids encoding the A53T mutant α-syn gene are shown in Section S3 of the Supporting Information. The A53T amino acid change in the primary protein sequence of α-syn is the result of a familial mutation in the α-syn DNA sequence that is linked to PD.[37] It has been claimed that it is the most amyloidogenic α-syn mutant protein, implying that it is also more prone to aggregation than the other known α-syn mutants. Hence, it has been predicted to be the most toxic among all α-syn protein variants. Similar to the wild type α-syn protein, the expression of the A53T mutant blocks yeast cell growth in solid agar and liquid culture media that contain galactose (Figure 4A−G). Again, similar to wild type α-syn, expression of one to three copies of the A53T α-syn mutant induces cell death after the growth of cells, for 20 h, in liquid culture medium containing galactose (Figure 4H). Cell death increases with the copy number.

All 29 compounds in the methoxy-stilbene library were separately tested, at 10 μM concentration, in the strains that

express (a) three copies of wild-type α-syn(wt) [i.e., 3C-α-syn(wt)] and (b) three copies of mutant α-syn(A53T) [i.e., 3C-α-syn(A53T)]. Cells were incubated, at 30 °C, in the presence or absence of compounds, in 96-well plates in SG medium (that contained 2% galactose and 0.2% glucose); the plates were shaken at 200 rpm. It was observed that the compounds, which had been identified in the screen using the two-copy wild-type α-syn strain, also restore cell growth in these two new strains, in the presence of 2% galactose and 0.2% glucose (Figure 4I). Percentage growth was again calculated based on the growth of the respective cells in galactose (SG medium) and in the presence of only 5% DMSO, but no compound, which was considered as zero percent or base line. It should be noted that the cells expressing three copies of α-syn and three copies of the mutant A53T α-syn hardly grew in SG, over 72 h, compared to the strain that expresses two copies of α-syn which had been used for the initial screen. This may be one of the reasons why the calculated percentage growth appears to be relatively higher, for some of the compounds, in the three-copy wild-type α-syn and mutant α-syn strains than the strain bearing two-copy wild-type α-syn. The two compounds, **4f** and **4s**, proved to be the best in rescuing growth of three-copy α-syn expressing yeast cells, in liquid culture (Figure 4I). They also rescued growth of the same cells on solid agar plates (Supporting Information, Section S4).

2.6. Determination of EC$_{50}$ Values of Selected Methoxy-Stilbenes That Restore Growth in α-Syn-Expressing Yeast Cells. We have determined the EC$_{50}$ values of the five compounds which restored growth the best, in liquid culture and on solid agar, in cells blocked in growth by expression of two copies of wild-type α-syn. For determination of EC$_{50}$ values, cells were grown for 48 h at 30 °C, in the presence of different concentrations of compounds in wells of 6-well plates. The OD$_{600}$ values were plotted against compound concentrations to calculate the EC$_{50}$ values. Results are shown in Table 2. The EC$_{50}$ value for compound **4s**, in the strain expressing three copies of A53T mutant α-syn, which has been found to be the most toxic among α-syn variants,[38] was determined to be 0.35 μM.

2.7. Methoxy-Stilbenes 4q, 4r, and 4s Prevent Cell Death and Restore Life in Cells Expressing α-Syn. The three best compounds, **4q**, **4r**, and **4s**, identified in the initial screen of the methoxy-stilbene library using the two-copy wild-type α-syn-bearing yeast strain, were then tested for their
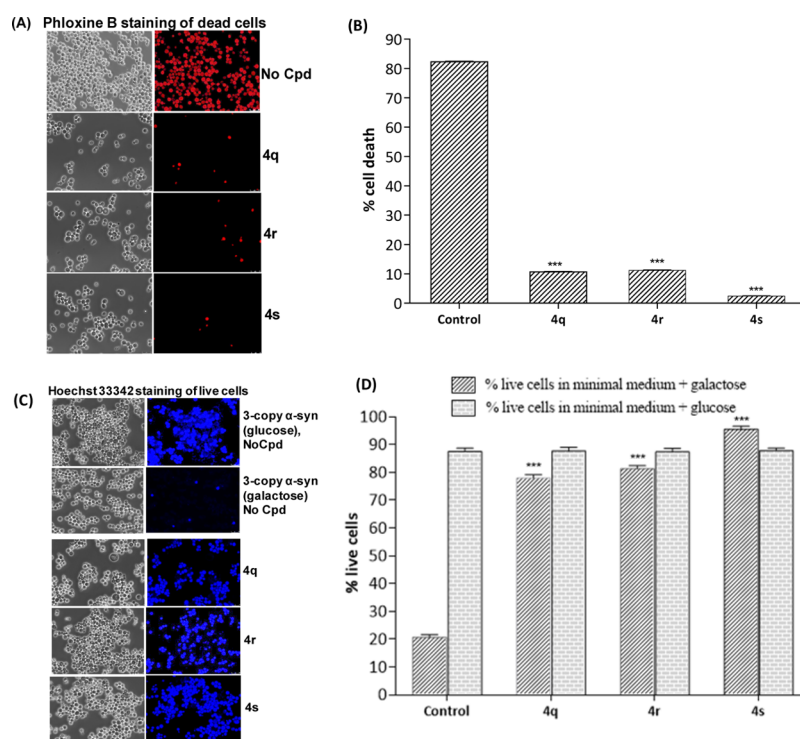
Figure 5. Effect of three best compounds, **4q**, **4r**, and **4s**, which restore cell growth in the two-copy α-syn screen, on α-syn-induced cell death. Cells, harboring three copies of α-syn, were grown in SG minimal medium, containing galactose, for 20 h at 30 °C, before further treatment with Phloxine B or Hoechst 3342 dye. (A) Cells grown in the absence or presence of compounds, **4q**, **4r**, and **4s** stained with Phloxine B. (B) Percentage of dead cells in the absence of any compound and in the presence of the three compounds, **4q**, **4r**, and **4s**. The data represent mean ± SD of five independent fields of observation (statistical significance, ***$p < 0.01$). Around 150 cells were counted in each field. (C) Cells stained with the Hoechst 33342 dye. The upper two panels are the controls showing that most cells are alive in glucose-containing SD medium (because there is no α-syn expression), whereas they are dead in SG medium that contains galactose. In the presence of compounds **4q**, **4r**, and **4s**, cells grown in galactose-containing SG medium are resistant to the toxic effects of α-syn. (D) Percentage of live cells, in SG medium, in the absence of any compound and in the presence of the three compounds, **4q**, **4r**, and **4s**. The data represent mean ± SD of five independent fields of observation (statistical significance, ***$p < 0.01$). In (D), the statistical significance was determined by comparison of compound-treated live cells vs control cells containing galactose.

ability to prevent cell death and also restore life in yeast cells which express three copies of α-syn; the reason for choosing the three-copy strain was because it manifests more toxicity than the two-copy cells (see Figure 5).

The results, obtained after treatment of three-copy cells with the compounds, are shown in Figure 5. Death of yeast cells, caused by the expression of human α-syn, was visualized by staining with the dye, Phloxine B. Dead cells are stained red. The observations (in Figure 5A,B) indicate that, in the presence of the three compounds, **4q**, **4r**, and **4s**, there is a remarkable decrease in cell death mediated by α-syn. In parallel, we show that the compounds provide once again life to the cells which are being inflicted by the toxic human α-syn by staining cells with the Hoechst 33342 dye (Figure 5C,D). The Hoechst dye is used for fluorescent staining of DNA and nuclei of live cells, including that of the yeast.[39]

2.8. Apoptosis Induced by the Expression of Human α-Syn in Yeast Is Prevented by Treatment with Compounds 4q, 4r, and 4s. In eukaryotic cells, ROS emanates from the mitochondrion, an intracellular compartment responsible for respiration. Perpetual increase in the production of ROS production, because of mitochondrial dysfunction, leads to accumulation of ROS within cells which is likely to result in damage of DNA, proteins, and lipids. This ROS-perpetrated damage prevents proper functioning of the cells. It has been demonstrated that the consequence of

mitochondrial dysfunction and increase in ROS levels, depending on the degree of ROS production and the loss of mitochondrial function, is the induction of apoptosis.[40] Mitochondrial dysfunction usually implies opening up the mitochondrial permeability transition pore (mtPTP) which induces depolarization of the mitochondrial transmembrane potential with attendant release of apoptogenic factors and loss of mitochondria's innate oxidative phosphorylation capacity. The mtPTP has been recognized as a potential pharmacological target for the treatment of neurodegenerative diseases, like PD, in which excessive cell death occurs because of mitochondrial dysfunction.[41]

The cellular toxicity seen on the expression of human α-syn in mammalian cells has precisely been reproduced in the baker's yeast, *S. cerevisiae*.[42] Yeast cells, expressing human α-syn, do undergo apoptosis because of oxidative stress originating from generation of excess ROS, triggered by misfolding of α-syn, and accompanied by mitochondrial dysfunction.[43] We show in Figure 6B that nuclear DNA fragmentation in three-copy α-syn-bearing yeast cells is mostly abrogated by compounds **4q**, **4r**, and **4s**. We then investigated if the compounds, **4q**, **4r**, and **4s**, would also mitigate loss of MMP and negate production of ROS. The compounds indeed prevent MMP loss and increased levels of ROS formation caused by expression of three copies of human α-syn in yeast (Figure 6C,D).
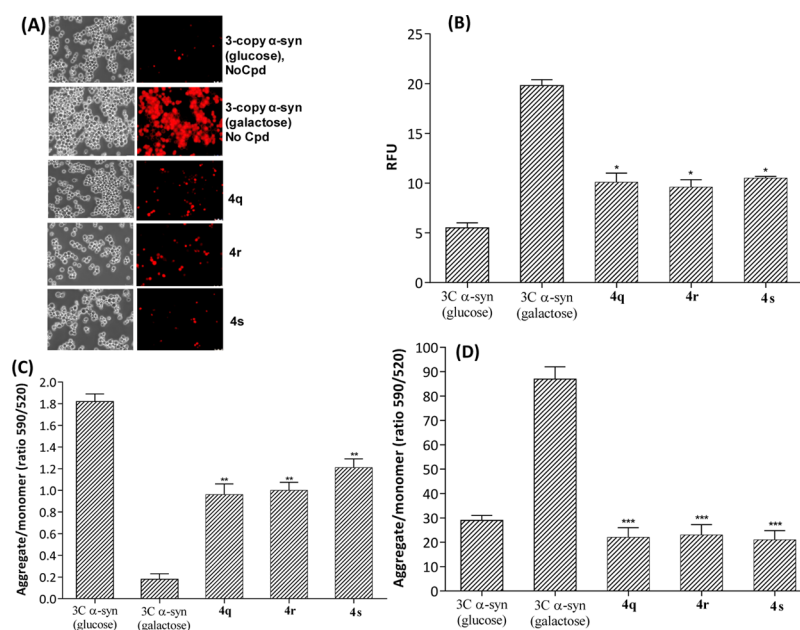
Figure 6. Effect of the three best compounds, **4q**, **4r**, and **4s**, which restore cell growth in the two-copy α-syn screen, on α-syn-induced nuclear DNA fragmentation which occurs only in apoptotic cells. Cells, harboring three copies of α-syn, were grown in SG minimal medium, containing galactose, for 20 h at 30 °C, before assessing for nuclear DNA fragmentation with the TUNEL assay. (A) Cells, grown in SG medium, in the absence or presence of compounds, **4q**, **4r**, and **4s**, which incorporate the fluorescence dye TF3 and modified deoxyuridine 5′-triphosphate (TF3-dUTP) at the 3′-OH ends of apoptotic DNA fragments. The uppermost panel shows that there is hardly any DNA fragmentation in cells that do not express α-syn, in the presence of glucose in contrast to cells which express α-syn, in the presence of galactose. The levels of DNA fragmentation in cells grown in galactose, in the presence of compounds, are much less than in cells expressed in galactose, in the absence of compound. (B) Comparison of the relative levels of nuclear DNA fragmentation in cells grown in galactose, in the presence of compounds, with cells grown in the absence of compounds, in galactose. Labeled DNA of cells which do not express α-syn, in medium containing glucose, acted as a positive control. DNA labeling is expressed as relative fluorescence units (statistical significance, * $0.05 > p < 0.1$). (C) Effect of the three best compounds, **4q**, **4r**, and **4s**, on α-syn-induced loss of MMP (statistical significance, *$p < 0.25$). (D) Effect of the three best compounds, **4q**, **4r**, and **4s** on α-syn-induced increase of levels of ROS in the three-copy α-syn-expressing yeast cells (statistical significance, ***$p < 0.01$).

2.9. Antioxidant Activity of Identified Hits in the 1,1-Diphenyl-2-picrylhydrazyl (DPPH) and 2,2′-Azino-bis-3-ethylbenzthiazoline-6-sulphonic Acid (ABTS) Assays.

The antioxidant potential of selected methoxy-stilbenes was evaluated in two assays that allow determination of the compounds' ability to scavenge free radicals generated by (a) DPPH[44] and (b) ABTS[45] (Figure 7). Among the compounds identified as rescuers of human α-syn's toxicity in the yeast, only one compound, **4r**, has some antioxidant activity when compared with trolox, a vitamin E analogue. None of the other methoxy-stilbenes seem to be antioxidants, although resveratrol and piceatannol (on the basis of which the methoxy-stilbene library was synthesized) have been found to be strong antioxidants using the same DPPH and ABTS assays.[23] We have confirmed that resveratrol is an antioxidant in both the DPPH and ABTS assays and piceatannol is an antioxidant in the ABTS assay (Section S5, Supporting Information).

2.10. Testing of Methoxy-Stilbenes for Rescue of Human Bax-Expressing Yeast Cells.

All 29 methoxy-stilbenes were then tested in the yeast strain that expresses a single copy of the human Bax gene with the aim of identifying compounds that may overcome Bax-mediated block of cell growth and to see if there were compounds that nullified the toxicity of both α-syn and Bax. The synthetic Bax gene, synthesized on the basis of the published protein sequence for Bax-α (NCBI Accession # AAA03619), was cloned with a 3′-end DNA that codes for the c-myc tag (peptide sequence, EQKLISEEDL) in a yeast integrative plasmid (see Section S6, Supporting Information). A chromosomally integrated copy of

the human Bax gene in the yeast strain BC300 (as described before) was expressed under the control of the galactose-inducible GAL1 promoter.[12] Because the yeast cells that express Bax do not grow in galactose, it would indicate that the expression of the Bax protein (Figure 8C) is toxic to the yeast (Figure 8A,B). The Bax expression also causes yeast cell death (Figure 8D). Bax-expressing yeast cells were incubated in SG medium, at 30 °C, in the presence or absence of compounds in 96-well plates; the plates were shaken at 200 rpm. The results obtained in the screen of methoxy-stilbenes with Bax-expressing yeast cells are summarized in Figure 8E.

Compound **4aa** stands out because it rescues only Bax's toxicity in the yeast but not α-syn's. It is possible that it prevents cell death at a juncture, perhaps via a protein or a protein, which is not shared by the biochemical pathways used by Bax and α-syn to inflict death on yeast cells. On the other hand, the compounds **4f**, **4r**, and **4s** are unable to overcome Bax-induced yeast cell death, triggered by mitochondrial dysfunction,[12] but still can rescue α-syn-mediated apoptosis which, similar to the Bax expression in yeast, also occurs via loss of MMP and generation of excess ROS. It is, therefore, very likely that compounds **4f**, **4r**, and **4s** are the ones that prevent aggregation of α-syn and thereby prevent α-syn-mediated apoptosis (for visualization of concept, see Figure 8). Hence, we asked the question whether compounds **4f**, **4r**, and **4s** would also prevent the aggregation of the βA4 peptide. We have found that, like in human cells, overexpression of βA4 causes yeast cell death. It has earlier been shown that the overexpression of a GFP-tagged βA4 in the yeast leads to its
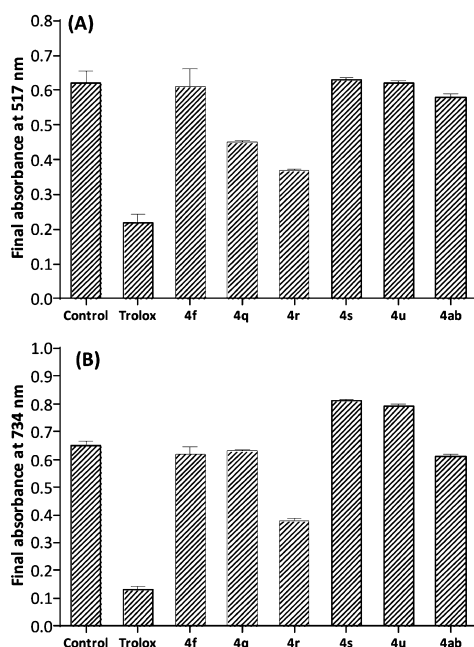
Figure 7. Antioxidant activity of methoxy-stilbenes at 10 μM concentration. The antioxidant capacity of the compounds is compared with the standard Trolox, a soluble vitamin E equivalent (also at 10 μM concentration). (A) Antioxidant potential in the scavenging of the free radicals generated by DPPH. The "Control" contained DPPH and 5% DMSO but no compound. (B) Antioxidant potential in the scavenging of the free radicals generated by ABTS. The antioxidant capacity of the compounds is again compared with the standard Trolox. The "Control" contained ABTS and 5% DMSO but no compound. The data in both (A,B) represent mean ± SD of three independent experiments (statistical significance, ns, $p > 0.25$).

spontaneous aggregation, thereby causing toxicity but not cell death.[6b,46]

2.11. Testing of Methoxy-Stilbenes for Rescue of Human βA4-Expressing Yeast Cells.

The seven methoxy-stilbenes depicted in Figure 8E were next tested in the yeast strain that secretes two copies of the 42-amino acid human βA4 peptide gene (i.e., in cells where the βA4 peptide is targeted to yeast's secretory pathway with the help of a signal sequence), to see if any of these compounds overcome βA4-mediated block in cell growth. It has been reported that, like in human cells, βA4 is only toxic to yeast cells when it enters the pathway that allows secretion of a protein/peptide from eukaryotic cells.[6] The synthetic human βA4 gene, with the 5'-end invertase signal sequence (Inv$_{ss}$),[47] was synthesized on the basis of the published βA4 protein sequence (NCBI Accession # P05067). The Inv$_{ss}$-βA4 gene and the βA4 gene (without the Inv$_{ss}$) were cloned in two yeast integrative plasmids that allow integration at the HIS3 and URA3 chromosomal loci (see Section S7, Supporting Information). Chromosomally integrated copies (i.e., 1-copy and 2-copies) of the Inv$_{ss}$-βA4 gene and the βA4 gene (without the Inv$_{ss}$) were expressed, in the yeast strain BC300, under the control of galactose-inducible GAL1 promoter. Because the yeast cells that express secreted βA4 do not grow in galactose (Figure 9I,II), which is in contrast to cytosolic the expression of βA4 (i.e., without the Inv$_{ss}$), where cells do grow, it would indicate that the expression of only the secreted βA4 peptide (Figure 9III) is toxic to the yeast. Secretion of the βA4 peptide, but not its cytosolic expression, causes yeast cell death (Figure 9IV).

The selected methoxy-stilbenes were tested to see if they would overcome secreted human βA4-mediated cell death in the yeast. Yeast cells, expressing two copies of Inv$_{ss}$-βA4, were incubated in 96-well plates, at 30 °C, in the presence or absence of compounds; the plates were shaken at 200 rpm. The results, depicted in Figure 10A, obtained using these cells,
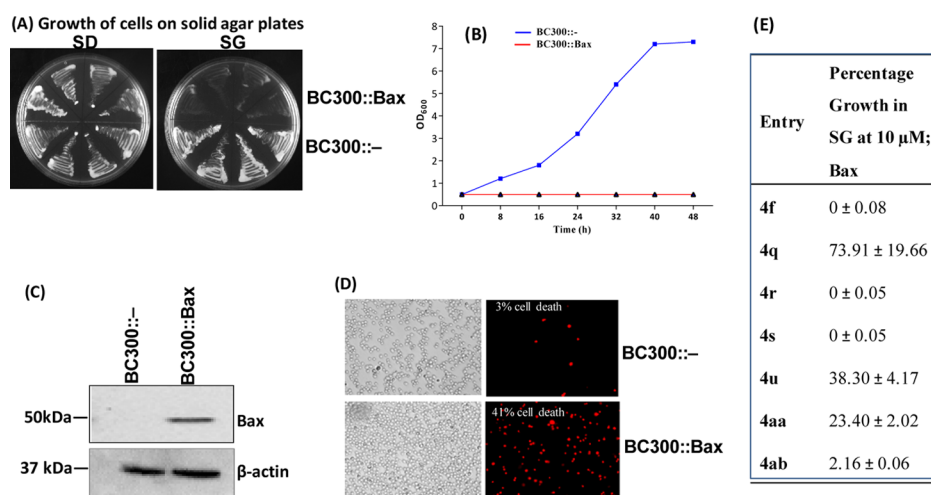

Figure 8. (A) Solid agar plates depicting the growth of yeast cells that express human Bax protein and cells that do not contain Bax, in minimal medium containing either glucose (SD) or galactose (SG). (B) Growth of one set of clones from the strains, as in (A), in liquid minimal medium containing galactose (SG); the OD$_{600}$ values at each time point represent the average of three independent experiments (*$P < 0.01$; one-tailed Student's t-test). (C) Western blot of total cellular proteins obtained after lysis of cells that do not express Bax (BC300::—) and do express c-myc-tagged Bax (BC300::Bax), probed with a c-myc antibody (Thermo Scientific, MA 1-980), and a β-actin antibody (Proteintech, 60008-1-Ig) as a loading control, β-actin being a housekeeping gene. (D) Staining of cells that express BC300::— and BC300::Bax, with Phloxine B, under the fluorescent microscope. The left-hand panels show cells in phase contrast microscopy. (E) Rescue from the block in growth of yeast cells that express human Bax, by methoxy-stilbenes, at 10 μM. The data represent mean ± SD of four independent experiments where optical density at 600 nm (OD$_{600}$) was measured for each cell culture.
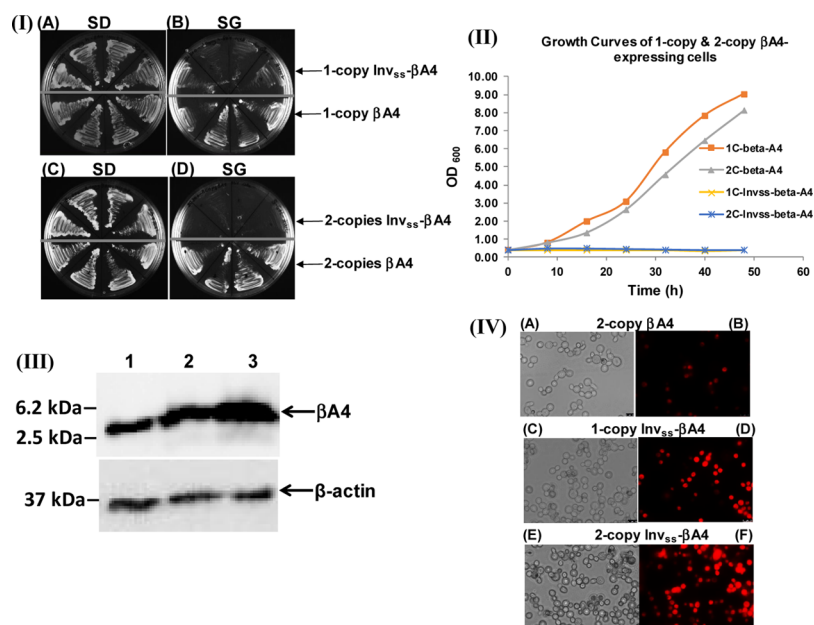
Figure 9. (I) Growth of yeast cells harboring one copy and two copies of the human βA4 gene on minimal medium solid agar plates that contained either glucose (SD) or galactose (SG). Panels (A−F), upper half (4 clones): strains containing one copy or two copies of the Inv$_{ss}$-βA4 gene, under the control of the GAL1 promoter that induces the expression of βA4 protein. Panels (A−D), lower half (4 clones): strains containing one copy and two copies of the βA4 gene, without the invertase signal sequence (Inv$_{ss}$). The solid agar plates were incubated at 30 °C for 96 h. (II) Growth of yeast strains that contain one copy and two copies of the Inv$_{ss}$-βA4 gene and the βA4 gene (without the Inv$_{ss}$), in liquid yeast cell culture minimal medium that contained galactose. The OD$_{600}$ values at each time point represent the average of three independent experiments. (III) Western blot of total cellular proteins obtained after lysis of cells that express cytosolic βA4 (lane 1), one copy of secreted Inv$_{ss}$-βA4 (lane 2) and two copies of Inv$_{ss}$-βA4 (lane 3), probed with a βA4 antibody (Abcam, ab12267); the same proteins were probed with a β-actin antibody (Proteintech, 60008-1-Ig), which acted as a loading control. (IV) Percentage of cells undergoing death in yeast strains expressing one copy (panels C,D) and two copies of secreted Inv$_{ss}$-βA4 (panels E,F), after cells were cultured for 20 h. Cells expressing two copies of cytosolic βA4, without the Inv$_{ss}$ signal sequence, are represented in panels A and B. Dead cells were stained red with Phloxine B. The data represent mean ± SD of five independent fields of observation. Around 200 cells were counted in each field. The left-hand panels show cells in phase contrast microscopy.

were compared with those obtained from human Bax-expressing yeast cells and the three different strains that express human α-syn. None of the methoxy-stilbenes that had restored cell growth suppressed by α-syn had any ability to rescue cells that were blocked in growth by two copies of secreted βA4. This was also confirmed by attempting to form yeast colonies on solid agar plates, in the presence of compounds **4f**, **4q**, **4r**, **4s**, and **4u** (10, 5, and 2.5 μM concentrations), from cells that express two copies of secreted βA4 in SG minimal medium containing galactose (Section 8, Supporting Information). EGCG was used as a positive control in this set of experiments.[20,48]

The results, presented in Figure 10A, strongly suggest that the compounds **4f**, **4r**, and **4s** specifically target the aggregation of α-syn, but surprisingly not that of βA4. These three compounds are also unable to prevent Bax-mediated block in cell growth and induction of cell death. It could be inferred that, only by preventing α-syn aggregation, the compounds **4f**, **4r**, and **4s** inhibit the toxic manifestations of the over-expression of human α-syn in the yeast that result in loss of MMP, ROS generation, and resultant apoptosis (Figure 10B).

2.12. Prevention of α-Syn Aggregation by Compound 4s. The compound **4s** was further tested in yeast cells expressing two copies of α-syn-eGFP fusion protein. As shown in the fluorescence picture of cells expressing two copies of α-syn-eGFP fusion protein in the absence of compounds (Figure 11C), the cells marked with white arrows shows α-syn aggregation, whereas the ones with yellow arrows show dead cells. However, when the cells were treated with compound **4s**

at 5 μM, cells do not show aggregation of α-syn (Figure 11D). The results show that compound **4s** is likely to have the ability to prevent α-syn aggregation (Figure 11A−D).

3. CONCLUSIONS

The screening of a library of methoxy-stilbenes, in parallel, using yeast cells that express human α-syn, Bax, and βA4, has resulted in the identification of compound **4s** as a likely inhibitor of α-syn aggregation. The compound **4s** does not possess antioxidant properties. It prevents yeast cell death brought about by expression of two copies and three copies of the wild-type α-syn gene as well as three copies of its A53T mutant. However, it does not rescue yeast cells from Bax- and βA4-induced death. The in silico docking studies indicated that it binds to the two sites on the β-sheet of the α-syn fibrils. The results presented in this paper suggest that compound **4s** could be used for further exploration in preclinical studies related to PD. Results also illustrate that simultaneous use of three sets of recombinant yeast cells, which express human α-syn, Bax, and βA4, could rapidly reveal compounds from a chemical library that may specifically prevent the aggregation of α-syn.

4. EXPERIMENTAL SECTION

4.1. General. ^{1}H, ^{13}C, and DEPT and 2D-NMR spectra were recorded on Brucker-AVANCE DPX FT-NMR 500 and 400 MHz instruments. NMR experiments were carried out in the indicated solvent. Chemical data for protons are reported in parts per million downfield from tetramethylsilane and are referenced to the residual proton in the NMR solvent
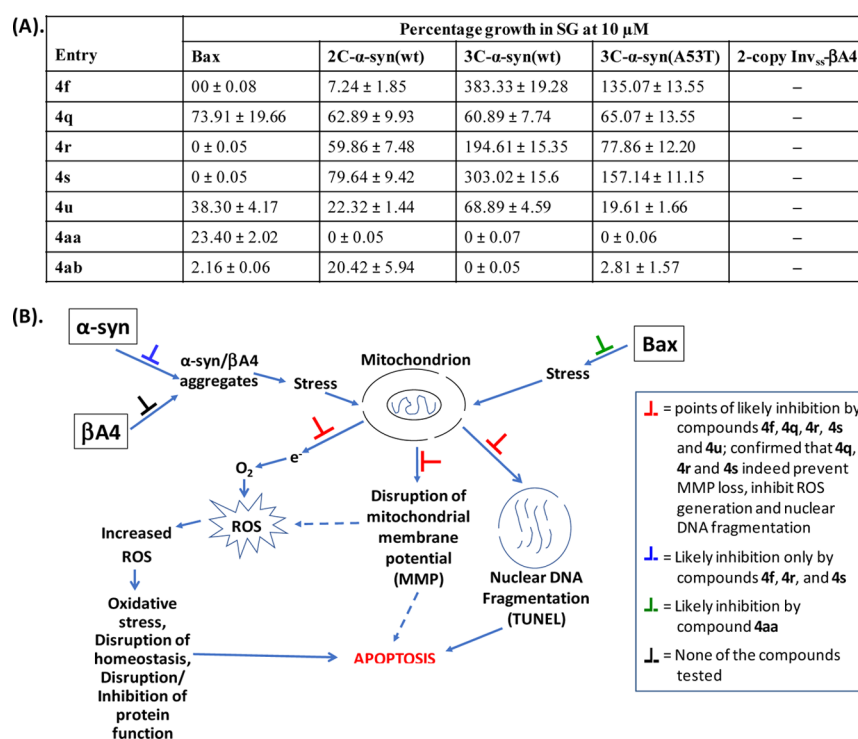
(A).

Entry	Percentage growth in SG at 10 μM				
	Bax	2C-α-syn(wt)	3C-α-syn(wt)	3C-α-syn(A53T)	2-copy Inv$_{ss}$-βA4
4f	00 ± 0.08	7.24 ± 1.85	383.33 ± 19.28	135.07 ± 13.55	–
4q	73.91 ± 19.66	62.89 ± 9.93	60.89 ± 7.74	65.07 ± 13.55	–
4r	0 ± 0.05	59.86 ± 7.48	194.61 ± 15.35	77.86 ± 12.20	–
4s	0 ± 0.05	79.64 ± 9.42	303.02 ± 15.6	157.14 ± 11.15	–
4u	38.30 ± 4.17	22.32 ± 1.44	68.89 ± 4.59	19.61 ± 1.66	–
4aa	23.40 ± 2.02	0 ± 0.05	0 ± 0.07	0 ± 0.06	–
4ab	2.16 ± 0.06	20.42 ± 5.94	0 ± 0.05	2.81 ± 1.57	–

(B).

Figure 10. (A) Summary of the methoxy-stilbenes effect on the growth of human α-syn, βA4, and Bax-bearing yeast cells at 10 μM. The two-copy Inv$_{ss}$-βA4 strain was used, in the presence of 10, 5, and 2.5 μM compounds, on SG agar plates. The data represent mean ± SD of four independent experiments where optical density at 600 nm (OD$_{600}$) was measured for each cell culture. (B) Cartoon depicts some of the possible pathways that can lead to apoptosis, when the human proteins α-syn, βA4, and Bax are expressed in the yeast. It shows the points in the pathways that certain methoxy-stilbenes can act and prevent apoptosis.

(CD$_3$OD 3.31 ppm). Carbon nuclear magnetic resonance spectra (^{13}C NMR) were recorded at 125 or 100 MHz: chemical data for carbons are reported in parts per million (δ scale) downfield from tetramethylsilane and are referenced to the carbon resonance of the solvent (CD$_3$OD, 49.0 ppm). ESI-MS and HRMS spectra were recorded on Agilent 1100 LC-Q-TOF and HRMS-6540-UHD machines. All chromatographic purifications were performed on Sephadex and Dianion HP-20 resin. Thin layer chromatography was performed on precoated silica gel 60 GF$_{254}$ aluminium sheets (Merck). All solvents used were of analytical grade and purchased from Merck. The HPLC purity analysis of all samples was carried out on a UPLC Shimadzu system (LC20ADXR), connected with a Lichrosphere RP18e (5 μ, 4.6 mm × 250 mm) column, and was eluted with water (0.1% formic acid): ACN gradient at the flow rate of 0.8 mL/min. The gradient elution was set with the increasing concentration of ACN in following format: 5% ACN (0−3 min), 5−80% ACN (3−8 min), 80% ACN (8−12 min), 80−20% ACN (12−14 min), and 5% ACN (14−16 min). The detection wavelength used was 254 nm. All compounds comply the purity requirement of >95% (HPLC purity).

4.2. General Procedure for Preparation of (E/Z)-1-Styryl Methoxybenzene 4a.
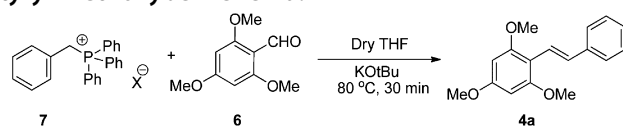
To the stirred solution of benzyl triphenylphosphine halide salt 7 (2.8 mmol) in anhydrous THF (10 mL) under N$_2$ atmosphere was added potassium *tert*-butoxide (3.0 mmol) in small portions at 0−5 °C, and the resulting mixture was

stirred at 80 °C for 30 min. Formation of orange color was observed in the reaction. Reaction was cooled to the room temperature, and the respective 2-methoxy benzaldehyde 6 (0.5 g, 2.5 mmol) was added. The resulting reaction mixture was again refluxed at 80 °C for 30 min. The reaction mixture was quenched with ethyl acetate (10 mL), and the solvent was evaporated under vaccum, followed by partitioning between water and ethyl acetate (50 mL × 2). The organic layer was collected, dried on anhydrous sodium sulphate, and evaporated on a rotary evaporator to get the crude product. The crude product was purified by silica gel (#100-200) column chromatography using 20% EtOAc/hexane to get (E/Z)-1-styryl methoxybenzene **4a** in 95% yield. The ratio of cis/trans isomer in isolated products was determined by HPLC. All other stilbenes **4b−4ac** were synthesized using a similar procedure.

4.2.1. (E/Z)-1-Styryl-2,4,6-trimethoxybenzene (4a). Mixture of cis/trans isomers (43:56 ratio, determined based on HPLC); Light yellow oil; ^{1}H NMR (CDCl$_3$, 400 MHz) of trans-isomer: δ 7.51 (d, J = 7.6 Hz, 2H), 7.43 (d, J = 12.4 Hz, 1H), 7.33 (t, J = 7.6 Hz, 2H), 7.19 (m, 2H), 6.16 (s, 2H), 3.87 (s, 6H), 3.83 (s, 3H); ^{1}H NMR (CDCl$_3$, 400 MHz) of cis-isomer: δ 7.48 (m, 1H), 7.43 (m, 1H), 7.36 (m, 1H), 7.19 (d, J = 7.6 Hz, 1H), 7.12 (m, 1H), 6.67 (d, J = 12 Hz, 1H), 6.42 (d, J = 12.4 Hz, 1H), 6.10 (s, 2H), 3.82 (s, 3H), 3.57 (s, 6H); ^{13}C NMR (CDCl$_3$, 125 MHz): δ 160.8, 160.2, 159.5, 158.3, 139.6, 139.0, 131.1, 129.9, 128.4, 127.8, 127.6, 126.5, 126.1, 120.9, 119.8, 108.1, 90.7, 90.6, 55.8, 55.4, 55.3; IR (CHCl$_3$) ν$_{max}$: 3436, 3079, 3056, 3000, 2959, 2936, 2533, 2155, 2109, 1955, 1726, 1605, 1594, 1584, 1514, 1497, 1466, 1455, 1436, 1377, 1331, 1271, 1214, 1205, 1190, 1155, 1140, 1122, 1072, 1060, 1038 cm^{-1}; ESI-MS *m/z*: 271.1 [M + H]$^+$.[49]
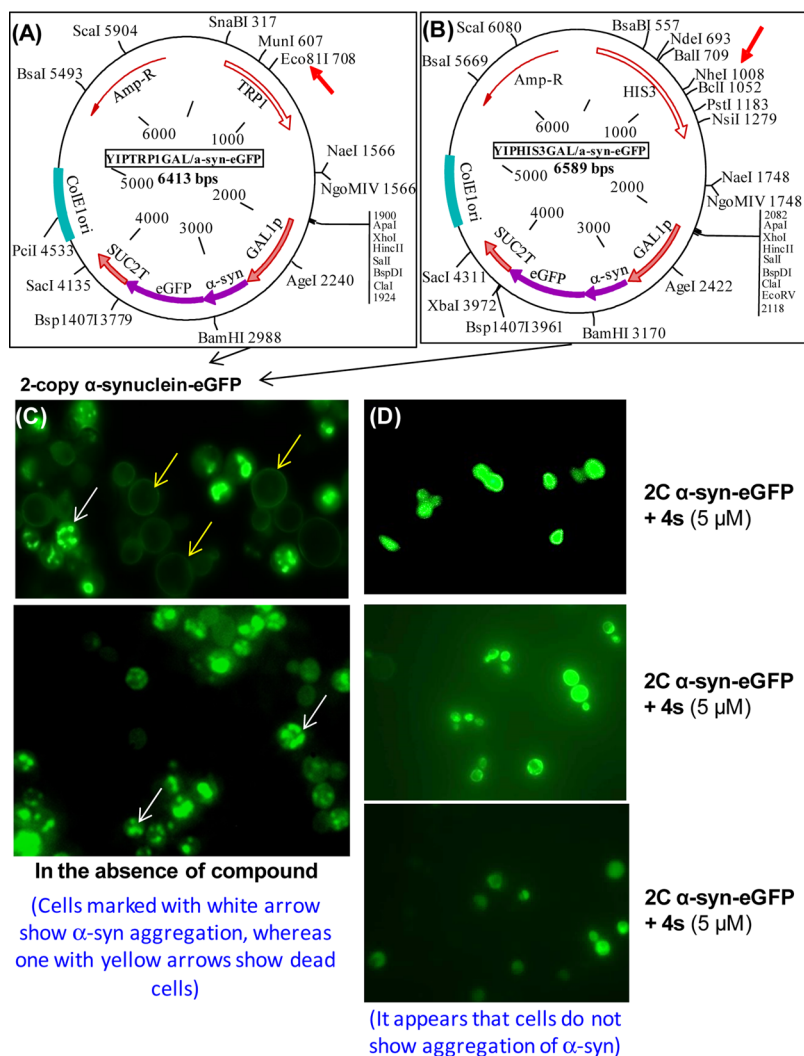
Figure 11. Analysis of aggregation of α-syn-eGFP fusion protein. (A,B) Maps of the plasmids used for integration of the α-syn-eGFP gene expression cassettes into the yeast strain BC300. The red arrows indicate the restriction sites that were used for linearizing the plasmids for integration. (C) Fluorescence pictures of cells expressing two copies of α-syn-eGFP fusion protein in the absence of compounds. (D) Fluorescence pictures of cells expressing two copies of α-syn-eGFP fusion protein in the presence of compound **4s** (5 μM concentration).

4.2.2. (E)-1-(3′,4′-Dimethoxystyryl)-2,4,6-trimethoxyben-zene (4b). Trans-isomer (93.7% determined based on HPLC); white amorphous solid; mp 124−126 °C; ^{1}H NMR (CDCl₃, 400 MHz): δ 7.41 (d, J = 16.4 Hz, 1H), 7.25 (d, J = 16.4 Hz, 1H), 7.06 (d, J = 13.6 Hz, 2H), 6.84 (d, J = 8.0 Hz, 1H), 6.17 (s, 2H), 3.94 (s, 3H), 3.88 (s, 9H), 3.83 (s, 3H); ^{13}C NMR (CDCl₃, 100 MHz): δ 159.9, 159.3, 148.9, 148.1, 132.8, 129.8, 119.1, 118.1, 111.1, 108.8, 108.2, 90.8, 55.9, 55.88, 55.81, 55.3; IR (CHCl₃) ν_{max}: 3435, 2998, 2932, 2835, 1604, 1577, 1513, 1464, 1418, 1330, 1263, 1231, 1203, 1155, 1117, 1060, 1025 cm⁻¹; ESI-MS m/z: 331.1 [M + H]⁺.[49]

4.2.3. (E,Z)-1-(4′-Fluorostyryl)-2,4,6-trimethoxybenzene (4c). Mixture of cis/trans isomers (17.1:82.8 ratio determined based on HPLC); brown crystalline solid; mp 70−72 °C; ^{1}H NMR (CDCl₃, 400 MHz) of trans-isomer: δ 7.47 (m, 2H), 7.43−7.39 (d, J = 16.4 Hz, 1H), 7.31−7.27 (d, J = 16.4 Hz, 1H), 7.0 (t, J = 8.8 Hz, 2H), 6.16 (s, 2H), 3.86 (s, 6H), 3.82 (s, 3H); ^{13}C NMR (CDCl₃, 100 MHz) of mixture of cis/trans isomers: δ 163.0 (¹J_CF = 243.8 Hz), 160.3, 159.4, 158.4, 135.9, 135.8, 129.8, 129.46, 129.38, 128.7, 127.54, 127.46, 120.7, 119.64, 115.3 (²J_CF = 21.3 Hz), 114.5 (²J_CF = 21.3 Hz), 108.0, 90.8, 55.8, 55.4, 55.3; ^{19}F NMR (CDCl₃, 376.5 MHz): δ

−115.63 (m, 1F), −116.29 (m, 1F); IR (CHCl₃) ν_{max}: 3400, 2919, 2850, 1904, 1733, 1605, 1579, 1508, 1455, 1417, 1331, 1223, 1205, 1155, 1118, 1094, 1038, 1019 cm⁻¹; ESI-MS m/z: 289.1 [M + H]⁺.[49]

4.2.4. (E)-1-(3′-Chlorostyryl)-2,4,6-trimethoxybenzene (4d). Trans-isomer (98.6% determined based on HPLC); white crystalline solid; mp 81−83 °C; ^{1}H NMR (CDCl₃, 400 MHz): δ 7.49 (s, 1H), 7.39 (s, 2H), 7.36 (d, J = 7.6 Hz, 1H), 7.23 (d, J = 8.0 Hz, 1H), 7.15 (d, J = 8.0 Hz, 1H), 6.16 (s, 2H), 3.88 (s, 6H), 3.84 (s, 3H); ^{13}C NMR (CDCl₃, 100 MHz): δ 160.6, 159.7, 141.7, 134.4, 129.6, 128.3, 126.3, 125.9, 124.4, 121.3, 107.7, 90.8, 55.8, 55.3; IR (CHCl₃) ν_{max}: 3000, 2937, 2837, 1605, 1587, 1491, 1468, 1455, 1410, 1330, 1212, 1156, 1120, 1061, 1038 cm⁻¹; ESI-MS m/z: 305.0 [M + H]⁺.[49]

4.2.5. (E,Z)-1-(2′-Chlorostyryl)-2,4,6-trimethoxybenzene (4e). Mixture of cis/trans isomers (5.8:94.1 ratio based on HPLC); white amorphous solid; mp 94−95 °C; ^{1}H NMR (CDCl₃, 400 MHz) of trans-isomer: δ 7.87 (d, J = 16.4 Hz, 1H), 7.72 (d, J = 8.0 Hz, 1H), 7.38 (m, 2H), 7.25 (t, J = 7.6 Hz, 1H), 7.13 (t, J = 7.6 Hz, 1H), 6.16 (s, 2H), 3.88 (s, 6H), 3.84 (s, 3H); ^{13}C NMR (CDCl₃, 100 MHz): δ 160.6, 159.7, 137.8, 133.0, 129.5, 127.4, 126.7, 126.1, 125.8, 122.2, 108.1,

90.8, 55.8, 55.3; IR (CHCl$_3$) ν_{max}: 3785, 2923, 2850, 1737, 1604, 1585, 1491, 1468, 1437, 1416, 1384, 1330, 1214, 1205, 1191, 1155, 1119, 1060, 1033 cm^{-1}; ESI-MS: 305.0 [M + H]$^+$.[49]

4.2.6. (E)-1-(2′-Bromostyryl)-2,4,6-trimethoxybenzene (4f). Trans-isomer (98.2% determined based on HPLC); white crystalline solid; mp 109−111 °C; ^{1}H NMR (CDCl$_3$, 400 MHz): δ 7.84 (d, *J* = 16.8 Hz, 1H), 7.71 (d, *J* = 7.6 Hz, 1H), 7.55 (d, *J* = 8.0 Hz, 1H), 7.35−7.26 (m, 2H), 7.06 (m, 1H), 6.18 (s, 2H), 3.90 (s, 6H), 3.85 (s, 3H); ^{13}C NMR (CDCl$_3$, 100 MHz): δ 160.6, 159.7, 139.4, 132.8, 128.5, 127.7, 127.4, 126.3, 123.9, 122.3, 107.9, 90.7, 55.8, 55.3; IR (CHCl$_3$) ν_{max}: 3400, 3058, 3000, 2959, 2935, 2837, 2532, 1956, 1822, 1742, 1604, 1582, 1491, 1467, 1435, 1416, 1346, 1330, 1277, 1258, 1191, 1177, 1156, 1120, 1061, 1038, 1021 cm^{-1}; ESI-MS *m/z*: 350.8 [M + H]$^+$.[49]

4.2.7. (E)-1-(4′-Bromostyryl)-2,4,6-trimethoxybenzene (4g). Trans-isomer (96.9% determined based on HPLC); brown crystalline solid; mp 95−96 °C; ^{1}H NMR (CDCl$_3$, 400 MHz): δ 7.41−7.33 (m, 6H), 6.13 (s, 2H), 3.84 (s, 6H), 3.80 (s, 3H); ^{13}C NMR (CDCl$_3$, 100 MHz): δ 160.7, 159.6, 138.7, 131.4, 128.4, 127.6, 120.6, 120.0, 107.8, 90.8, 55.7, 55.3; IR (CHCl$_3$) ν_{max}: 3368, 3000, 2924, 2850, 1740, 1602, 1580, 1492, 1466, 1455, 1435, 1330, 1214, 1205, 1189, 1175, 1156, 1120, 1062, 1038, 1006 cm^{-1}; ESI-MS *m/z*: 350.8 [M + H]$^+$.[49]

4.2.8. (E)-1-(4′-Chlorostyryl)-2,4,6-trimethoxybenzene (4h). Trans-isomer (96.4% determined based on HPLC); white crystalline solid; mp 98−100 °C; ^{1}H NMR (CDCl$_3$, 400 MHz): δ 7.43 (d, *J* = 8.8 Hz, 2H), 7.38 (d, *J* = 4.4 Hz, 2H), 7.27 (d, *J* = 8.8 Hz, 2H), 6.16 (s, 2H), 3.87 (s, 6H), 3.83 (s, 3H); ^{13}C NMR (CDCl$_3$, 100 MHz): δ 160.4, 159.6, 138.2, 131.9, 128.5, 128.4, 127.3, 120.5, 107.8, 90.8, 55.7, 55.3; IR (CHCl$_3$) ν_{max}: 3400, 2922, 2851, 1741, 1603, 1581, 1494, 1385, 1331, 1205, 1118, 1156, 1020 cm^{-1}; ESI-MS *m/z*: 305.0 [M + H]$^+$.[49]

4.2.9. (E)-1-(2′-Iodostyryl)-2,4,6-trimethoxybenzene (4i). Trans-isomer (99.2% determined based on HPLC); white crystalline solid; mp 121−122 °C; ^{1}H NMR (CDCl$_3$, 400 MHz): δ 7.83 (d, *J* = 8.0 Hz, 1H), 7.74 (d, *J* = 16.5 Hz, 1H), 7.67 (d, *J* = 8.0 Hz, 1H), 7.32 (m, 2H), 6.88 (t, *J* = 7.5 Hz, 1H), 6.16 (s, 2H), 3.89 (s, 6H), 3.83 (s, 3H); ^{13}C NMR (CDCl$_3$, 100 MHz): δ 160.6, 159.7, 142.5, 139.4, 133.5, 128.8, 128.0, 125.7, 122.4, 107.8, 100.7, 90.7, 55.9, 55.4; IR (CHCl$_3$) ν_{max}: 3436, 2921, 2850, 1603, 1578, 1457, 1432, 1416, 1384, 1330, 1216, 1155, 1119, 1020 cm^{-1}; ESI-MS *m/z*: 396.9 [M + H]$^+$.[49]

4.2.10. (E/Z)-1-(4′-Methylstyryl)-2,4,6-trimethoxy-benzene (4j). Mixture of cis/trans isomers (6.4:88.8 ratio determined based on HPLC); brown crystalline solid; mp 90−92 °C; ^{1}H NMR (CDCl$_3$, 400 MHz) of trans-isomer: δ 7.45−7.31 (m, 4H), 7.13 (d, *J* = 8.0 Hz, 2H), 6.16 (s, 2H), 3.87 (s, 6H), 3.83 (s, 3H), 2.33 (s, 3H); ^{13}C NMR (CDCl$_3$, 100 MHz): δ 159.3, 158.7, 136.2, 135.6, 129.2, 128.5, 125.4, 118.1, 107.6, 90.1, 55.1, 54.7, 20.6; IR (CHCl$_3$) ν_{max}: 3785, 3436, 2923, 2850, 1604, 1585, 1491, 1437, 1416, 1384, 1330, 1214, 1205, 1191, 1155, 1119, 1060, 1033 cm^{-1}; ESI-MS *m/z*: 285.1 [M + H]$^+$.[49]

4.2.11. (E)-1-(2′,4′-Dichlorostyryl)-2,4,6-trimethoxyben-zene (4k). Trans-isomer (99.2% determined based on HPLC); white crystalline solid; mp 129−131 °C; ^{1}H NMR (CDCl$_3$, 400 MHz): δ 7.79 (d, *J* = 16.4 Hz, 1H), 7.64 (d, *J* = 8.8 Hz, 1H), 7.36 (m, 2H), 7.20 (d, *J* = 8.4 Hz, 1H), 6.15 (s, 2H), 3.88 (s, 6H), 3.83 (s, 3H); ^{13}C NMR (CDCl$_3$, 100 MHz): δ 160.8, 159.7, 136.4, 133.3, 132.0, 129.2, 127.0, 126.8,

124.6, 122.7, 107.7, 90.7, 55.8, 55.3; IR (CHCl$_3$) ν_{max}: 3435, 2922, 2851, 1743, 1603, 1579, 1513, 1466, 1414, 1384, 1327, 1249, 1215, 1204, 1156, 1118, 1099, 1019 cm^{-1}; ESI-MS *m/z*: 339.0 [M + H]$^+$.[49]

4.2.12. (E)-1-(3′-Bromo-4′-fluorostyryl)-2,4,6-trimethoxy-benzene (4l). Trans-isomer (90% determined based on HPLC); white amorphous solid; mp 110−112 °C; ^{1}H NMR (CDCl$_3$, 400 MHz): δ 7.67 (d, *J* = 7.0 Hz, 1H), 7.39−7.25 (m, 3H), 7.07 (t, *J* = 8.5 Hz, 1H), 6.16 (s, 2H), 3.88 (s, 6H), 3.84 (s, 3H); ^{13}C NMR (CDCl$_3$, 100 MHz): δ 159.7, 158.7, 157.8 (d, $^1J_{CF}$ = 244 Hz), 136.7, 129.7, 126.3, 125.6, 120.0, 115.5 (d, $^2J_{CF}$ = 22.2 Hz), 108.2 (d, $^2J_{CF}$ = 21 Hz), 106.6, 89.8, 54.9, 54.5; ^{19}F NMR (CDCl$_3$, 376.5 Hz): δ −111.06 (m, 1F); IR (CHCl$_3$) ν_{max}: 3436, 3003, 2923, 2838, 1726, 1608, 1581, 1496, 1469, 1455, 1436, 1420, 1344, 1260, 1247, 1216, 1202, 1192, 1158, 1118, 1061, 1039 cm^{-1}; ESI-MS *m/z*: 366.9 [M + H]$^+$.[49]

4.2.13. (E)-1-(4′-(tert-Butyl)styryl)-2,4,6-trimethoxyben-zene (4m). Trans-isomer (99.7% determined based on HPLC); white crystalline solid; mp 83−84 °C; ^{1}H NMR (CDCl$_3$, 400 MHz): δ 7.45 (t, *J* = 8.4 Hz, 3H), 7.35 (m, 3H), 6.16 (s, 2H), 3.86 (s, 6H), 3.83 (s, 3H), 1.32 (s, 9H); ^{13}C NMR (CDCl$_3$, 100 MHz): δ 160.0, 159.4, 149.5, 136.9, 129.8, 125.8, 125.3, 119.2, 108.3, 90.8, 55.8, 55.3, 34.5, 31.3; IR (CHCl$_3$) ν_{max}: 2960, 1837, 1729, 1601, 1581, 1514, 1465, 1416, 1363, 1330, 1270, 1217, 1204, 1155, 1116, 1061, 1039 cm^{-1}; ESI-MS *m/z*: 327.1 [M + H]$^+$.[49]

4.2.14. (E)-1-(3′,5′-Dimethylstyryl)-2,4,6-trimethoxyben-zene (4n). Trans-isomer (98.3% determined based on HPLC); white crystalline solid; mp 145−147 °C; ^{1}H NMR (CDCl$_3$, 400 MHz): δ 7.42−7.31 (dd, *J* = 12.4, 16.8 Hz, 2H), 7.13 (s, 2H), 6.83 (s, 1H), 6.16 (s, 2H), 3.87 (s, 6H), 3.83 (s, 3H), 2.32 (s, 6H); ^{13}C NMR (CDCl$_3$, 100 MHz): δ 160.1, 159.5, 139.5, 137.7, 130.3, 128.3, 124.1, 119.4, 108.4, 90.9, 55.8, 55.3, 21.3; IR (CHCl$_3$) ν_{max}: 3001, 2920, 2850, 1737, 1603, 1580, 1490, 1457, 1411, 1329, 1222, 1200, 1156, 1113, 1063, 1031 cm^{-1}; ESI-MS *m/z*: 299.1 [M + H]$^+$.[49]

4.2.15. (E)-1-(2′-Methylstyryl)-2,4,6-trimethoxy-benzene (4o). Trans-isomer (95.4% determined based on HPLC); white crystalline solid; mp 96−98 °C; ^{1}H NMR (CDCl$_3$, 400 MHz): δ 7.68 (m, 2H), 7.26−7.09 (m, 4H), 6.17 (s, 2H), 3.87 (s, 6H), 3.83 (s, 3H), 2.38 (s, 3H); ^{13}C NMR (CDCl$_3$, 100 MHz): δ 160.1, 159.4, 138.7, 135.3, 130.1, 128.1, 126.5, 126.0, 124.9, 120.7, 108.5, 90.8, 55.8, 55.3, 20.0; IR (CHCl$_3$) ν_{max}: 3000, 2937, 2837, 1605, 1587, 1491, 168, 1455, 1418, 1330, 1212, 1191, 1156, 1120, 1061, 1038 cm^{-1}; ESI-MS *m/z*: 285.1 [M + H]$^+$.[49]

4.2.16. (E)-1-(4′-Iodostyryl)-2,4,6-trimethoxybenzene (4p). Trans-isomer (95.1% determined based on HPLC); brown crystalline solid; mp 91−92 °C; ^{1}H NMR (CDCl$_3$, 400 MHz): δ 7.63 (d, *J* = 8.4 Hz, 2H), 7.38 (d, *J* = 3.2 Hz, 2H), 7.24 (d, *J* = 8.4 Hz, 2H), 6.16 (s, 2H), 3.88 (s, 6H), 3.84 (s, 3H); ^{13}C NMR (CDCl$_3$, 100 MHz): δ 160.4, 159.6, 139.2, 137.4, 128.5, 127.9, 120.6, 107.6, 91.3, 90.7, 55.7, 55.3; IR (CHCl$_3$) ν_{max}: 3435, 2924, 2851, 1744, 1603, 1578, 1455, 1416, 1204, 1155, 1121, 1059, 1017, 1002 cm^{-1}; ESI-MS *m/z*: 396.9 [M + H]$^+$.[49]

4.2.17. (E,Z)-1-(3′,4′-Difluorostyryl)-2,4,6-trimethoxyben-zene (4q). Mixture of cis/trans isomers (6.8:93.1 ratio determined based on HPLC); white crystalline solid; mp 79−80 °C; ^{1}H NMR (CDCl$_3$, 400 MHz) of trans-isomer: δ 7.34−7.25 (m, 3H), 7.15 (m, 1H), 7.10−7.04 (m, 1H), 6.16 (s, 2H), 3.88 (s, 6H), 3.83 (s, 3H); ^{13}C NMR (CDCl$_3$, 125 MHz): δ 160.5, 159.5, 151.5 ($^1J_{CF}$ = 176 Hz), 150.1 ($^1J_{CF}$ =

176 Hz), 137.1, 127.5, 122.2, 120.9, 117.0 (d, $^2J_{CF}$ = 17.1 Hz), 114.1 (d, $^2J_{CF}$ = 17.2 Hz), 107.4, 90.7, 55.7, 55.3; ^{19}F NMR (CDCl$_3$, 376.5 Hz): δ −138.73 (m, 1F), −141.08 (m, 1F); IR (CHCl$_3$) ν_{max}: 3008, 2939, 2840, 2153, 1581, 1514, 1463, 1432, 1414, 1349, 1323, 1293,1201, 1271, 1223, 1150, 1120,1061, 1037 cm^{-1}; ESI-MS m/z: 307.1 [M + H]$^+$.[49]

4.2.18. (E,Z)-1-(3′,4′-Dichlorostyryl)-2,4,6-trimethoxybenzene (4r). Mixture of cis/trans isomers (10.6:89.3 ratio determined based on HPLC); white amorphous solid; mp 104−105 °C; ^{1}H NMR (CDCl$_3$, 400 MHz) of trans-isomer: δ 7.56 (d, J = 2.0 Hz, 1H), 7.37−7.29 (m, 4H), 6.16 (s, 2H), 3.88 (s, 6H), 3.84 (s, 3H); ^{13}C NMR (CDCl$_3$, 125 MHz): δ 160.7, 159.7, 140.0, 132.3, 130.2, 129.6, 127.6, 127.0, 125.3, 121.7, 107.3, 90.6, 55.7, 55.3; IR (CHCl$_3$) ν_{max}: 3436, 3003, 2936, 2839, 2483, 1898, 1605, 1587, 1577, 1507, 1469, 1457, 1437, 1420, 1384, 1331, 1258, 1219, 1204, 1188, 1156, 1121, 1062, 1039, 1016 cm^{-1}; ESI-MS m/z: 339.0 [M + H]$^+$.[49]

4.2.19. (E)-2-(4-Methoxystyryl)-1,3,5-trimethoxybenzene (4s). White solid; mp 61−64 °C; ^{1}H NMR (CDCl$_3$, 400 MHz) of trans-isomer: 7.45−7.43 (m, 3H), 7.26−7.23 (m, 2H), 6.87 (d, J = 8.4 Hz, 2H), 6.16 (s, 2H), 3.87 (s, 3H), 3.83 (s, 3H), 3.75 (s, 3H); ^{13}C NMR (CDCl$_3$, 100 MHz): 160.23, 159.34, 158.37, 158.12, 132.55, 130.21, 129.51, 129.31, 127.30, 117.93, 114.23, 113.98, 113.06, 108.30, 90.82, 55.76, 55.55, 55.29, 55.28; IR (CHCl$_3$) ν_{max}: 3437, 2912, 2847, 2312, 1724, 1600, 1501, 1422, 1300, 1265, 1112, 1013 cm^{-1}; ESI-MS (−ve) m/z: 299.0 [M − H]$^-$.[50]

4.2.20. (E)-5-(2,4,6-Trimethoxystyryl)benzo[d][1,3]dioxole (4t). Off-white solid; Mixture of cis/trans isomers (28:72 based on ^{1}H NMR); ^{1}H NMR (CDCl$_3$, 400 MHz) of trans isomer: 7.32 (m, 1H), 7.22 (m, 1H), 7.17 (s, 1H), 6.92 (d, J = 8 Hz, 1H), 6.77 (d, J = 8 Hz, 1H), 6.31 (s, 2H), 6.12 (s, 2H); 3.87 (s, 6H), 3.83 (s, 3H); IR (CHCl$_3$) ν_{max}: 3435, 3002, 2938, 2638, 1919, 1725, 1601, 1516, 1490, 1468, 1457, 1437, 1419, 1270, 1217, 1206, 1193, 1184, 1156, 1119, 1108, 1065, 1038, 1014; ESI-MS m/z: 316.0 [M + H]$^+$.[49]

4.2.21. ((E)-2-(2,4-Bis(trifluoromethyl)styryl)-1,3,5-trimethoxybenzene (4u). Mixture of cis/trans isomers (5:95 ratio based on HPLC); white solid; ^{1}H NMR (CDCl$_3$, 400 MHz) of trans-isomer: δ 7.95−7.85 (m, 3H), 7.72 (d, J = 8.4 Hz, 1H), 7.50 (d, J = 16.0 Hz, 1H), 6.17 (s, 2H), 3.89 (s, 6H), 3.85 (s, 3H); IR (CHCl$_3$) ν_{max}: 3435, 2926, 2849, 2318, 1726, 1603, 1503, 1458, 1417, 1343, 1305, 1277, 1224, 1207, 1124, 1082, 1053, 1016 cm^{-1}; ESI-MS m/z: 407.0 [M + H]$^+$.[49]

4.2.22. (E)-Ethyl 4-(2,4,6-Trimethoxystyryl)benzoate (4v). Mixture of cis/trans isomers (10:90 based on ^{1}H NMR); brown solid; ^{1}H NMR (CDCl$_3$, 400 MHz) of trans-isomer: δ 8.02−7.97 (m, 2H), 7.58−7.50 (m, 4H), 6.17 (s, 2H), 4.38 (q, J = 2.8, 4.0 Hz, 2H), 3.89 (s, 6H), 3.85 (s, 3H), 1.40 (t, J = 2.8 Hz); ^{13}C NMR (CDCl$_3$, 100 MHz) 166.75, 160.23, 159.23, 158.34, 144.38, 129.93, 129.80, 128.34, 128.94, 128.58, 125.85, 122.45, 107.67, 90.72, 60.75, 55.79, 55.38, 55.38, 14.40; IR (CHCl$_3$) ν_{max}: 3401, 2938, 2840, 1605, 1507, 1456, 1419, 1331, 1257, 1218, 1203, 1156, 1121, 1062, 1039, 1016 cm^{-1}; ESI-MS m/z: 343.2 [M + H]$^+$.[49]

4.2.23. (Z)-1-(3′,5′-Di(trifluoromethyl)styryl)-2,4,6-trimethoxybenzene (4w). Brown crystalline solid; ^{1}H NMR (CDCl$_3$, 400 MHz): δ 7.87 (s, 2H), 7.65 (s, 1H), 7.54 (d, J = 13.2 Hz, 1H), 7.50 (d, J = 13.2 Hz, 2H), 6.18 (s, 2H), 3.91 (s, 6H), 3.86 (s, 3H); ^{13}C NMR (CDCl$_3$, 125 MHz): δ 161.2, 159.9, 141.9, 131.5 (q, $^1J_{C-F}$ = 27.7 Hz), 126.4, 125.7, 124.6, 123.5, 122.5, 119.5 (t, $^2J_{C-F}$ = 3.0 Hz), 106.9, 90.6, 55.8, 55.4; IR (CHCl$_3$) ν_{max}: 3432, 3073, 3052, 2893, 2923, 2853, 1732, 1600, 1503, 1412, 1323, 1112 cm^{-1}; ESI-MS m/z: 407.0 [M + H]$^+$.[49]

4.2.24. (E,Z)-2-(2,4,6-Trimethoxystyryl)naphthalene (4x). Mixture of cis/trans isomers (33:67 ratio determined based on HPLC); white amorphous solid; mp 93−94 °C; ^{1}H NMR (CDCl$_3$, 400 MHz): δ 7.81 (m, 6H), 7.72 (m, 2H), 7.61 (s, 1H), 7.55 (m, 2H), 7.44−7.36 (m, 4H), 7.31 (d, J = 8.8 Hz, 1H), 6.82 (d, J = 12.0 Hz, 1H), 6.51 (d, J = 12.4 Hz, 1H), 6.19 (s, 2H), 6.12 (s, 2H), 3.91 (s, 6H), 3.84 (s, 6H), 3.53 (s, 6H); ^{13}C NMR (CDCl$_3$, 125 MHz): δ 160.9, 160.2, 159.5, 158.4, 137.2, 136.8, 133.8, 133.2, 132.6, 132.4, 130.9, 129.8, 128.0, 127.9, 127.8, 127.6, 127.4, 127.0, 126.7, 126.0, 125.9, 125.7, 125.6, 125.4, 125.2, 123.7, 121.3, 120.2, 108.0, 90.7, 90.5, 55.8, 55.4, 55.3, 53.4; IR (CHCl$_3$) ν_{max}: 3436, 2923, 2849, 1737, 1603, 1455, 1415, 1330, 1224, 1204, 1156, 1060, 1038 cm^{-1}; ESI-MS m/z: 321.1 [M + H]$^+$.[49]

4.2.25. (E,Z)-1-Styryl-2,4,5-trimethoxybenzene (4y). Mixture of cis/trans isomers (20:80 ratio, determined by ^{1}H NMR); light yellow oil; ^{1}H NMR (CDCl$_3$, 400 MHz) of mixture of cis/trans-isomer: δ 7.52 (d, J = 7.6 Hz, 1H), 7.46 (d, J = 16.4 Hz, 1H), 7.35 (m, 2H), 7.28 (m, 2H), 7.21 (m, 3H), 7.16 (m, 3H), 6.99 (d, J = 16.4 Hz, 1H), 6.70 (m, 2H), 6.55 (m, 3H), 3.91 (s, 12H), 3.81 (s, 3H), 3.44 (s, 3H); ^{13}C NMR (CDCl$_3$, 100 MHz): δ 151.8, 149.6, 149.1, 143.5, 142.4, 138.1, 137.7, 128.8, 128.6, 128.1, 127.0, 126.8, 126.3, 125.0, 123.0, 118.3, 117.4, 113.3, 109.5, 97.8, 97.3, 56.7, 56.6, 56.1, 56.0, 55.9; IR (CHCl$_3$) ν_{max}: 3436, 3077, 3053, 2999, 2935, 2832, 1734, 1608, 1583, 1512, 1491, 1465, 1410, 1328, 1163, 1110 cm^{-1}; ESI-MS m/z: 271.1 [M + H]$^+$.[49]

4.2.26. (E,Z)-1-Styryl-2,4-dimethoxybenzene (4z). Mixture of cis/trans isomers (73:25 ratio, determined based on HPLC), light yellow oil; ^{1}H NMR (CDCl$_3$, 400 MHz): δ 7.50 (d, J = 8.4 Hz, 2H), 7.41 (d, J = 16.4 Hz, 1H), 7.32−7.03 (m, 10H), 7.01 (d, J = 16.4 Hz, 1H), 6.63 (d, J = 12.4 Hz, 1H), 6.54−6.43 (m, 4H), 6.28 (d, 8.4 Hz, 1H), 3.82 (s, 3H), 3.79 (s, 3H), 3.76 (s, 3H), 3.74 (s, 3H); ^{13}C NMR (CDCl$_3$, 100 MHz): δ 160.6, 160.4, 158.3, 158.1, 138.3, 137.7, 130.6, 129.0, 128.8, 128.6, 128.1, 127.2, 127.0, 126.7, 126.3, 125.4, 123.3, 119.5, 118.8, 105.0, 104.2, 98.5, 98.3, 55.55, 55.50, 55.44, 55.34; IR (CHCl$_3$) ν_{max}: 3053, 3003, 2957, 2936, 2835, 2592, 1950, 1882, 1608, 1576, 1503, 1490, 1463, 1438, 1417, 1288, 1208, 1183, 1159, 1118, 1107, 1072 cm^{-1}; ESI-MS m/z: 241.1 [M + H]$^+$.[49]

4.2.27. (E,Z)-1-Styryl-2-methoxybenzene (4aa). Mixture of cis/trans isomers (92.2:7.8 ratio, determined based on HPLC), colourless oil; ^{1}H NMR (CDCl$_3$, 400 MHz) of cis-isomer (92%): δ 7.23−7.12 (m, 7H), 6.89 (d, J = 8.0 Hz, 1H), 6.76−6.60 (m, 3H), 3.81 (s, 3H); ^{13}C NMR (CDCl$_3$, 100 MHz): δ 157.2, 137.3, 130.2, 130.1, 128.8, 128.6, 128.0, 126.9, 126.2, 125.8, 120.2, 110.7, 55.4; IR (CHCl$_3$) ν_{max}: 3436, 2938, 2850, 1590, 1513, 1462, 1446, 1418, 1311, 1259, 1224, 1153, 1138, 1025 cm^{-1}; ESI-MS m/z: 211.1 [M + H]$^+$.[49]

4.2.28. (E)-1-Styryl-3,4-dimethoxybenzene (4ab). Trans-isomer (100% determined based on HPLC); white amorphous solid; mp 121−122 °C; ^{1}H NMR (DMSO-d_6, 400 MHz): δ 7.44 (d, J = 7.2 Hz, 2H), 7.30 (t, J = 7.2 Hz, 2H), 7.20 (d, J = 7.2 Hz, 1H), 7.01−6.89 (m, 4H), 6.74 (d, J = 8.4 Hz, 1H), 3.84 (s, 3H), 3.77 (s, 3H); ^{13}C NMR (DMSO-d_6, 100 MHz): δ 149.2, 149.0, 137.6, 130.5, 128.7, 128.5, 127.3, 126.8, 126.4, 120.0, 111.3, 109.0, 55.9, 55.8; IR (CHCl$_3$) ν_{max}: 3436, 2938, 2839, 1590, 1513, 1462, 1446, 1418, 1311, 1259, 1224, 1154, 1138, 1025 cm^{-1}; ESI-MS m/z: 241.1 [M + H]$^+$.[49]

4.2.29. (E,Z)-1-Styryl-4-methoxybenzene (4ac). Mixture of cis/trans isomers (1.3:98.6 ratio determined based on HPLC); white amorphous solid; mp 106−108 °C; ^{1}H NMR (CDCl$_3$, 400 MHz) of cis/trans isomers: δ 7.49 (dd, *J* = 7.2, 8.8 Hz, 3H), 7.34 (t, *J* = 7.6 Hz, 2H), 7.28−7.16 (m, 9H), 7.08−6.95 (m, 2H), 6.90 (d, *J* = 8.8 Hz, 2H), 6.76 (d, *J* = 8.8 Hz, 2H), 6.51 (d, *J* = 1.6 Hz, 2H), 3.82 (s, 3H), 3.77 (s, 3H); ^{13}C NMR (CDCl$_3$, 100 MHz): δ 159.3, 158.6, 137.6, 130.1, 129.7, 129.6, 128.8, 128.7, 128.69, 128.67, 128.25, 128.22, 127.7, 127.2, 126.9, 126.6, 126.5, 126.2, 114.1, 113.5, 55.3, 55.2; IR (CHCl$_3$) ν_{max}: 3745, 3436, 3078, 3053, 3021, 2961, 2934, 2836, 1605, 1510, 1463, 1446, 1421, 1297, 1252, 1179, 1150, 1112, 1072 cm^{-1}; ESI-MS *m/z*: 211.1 [M + H]$^+$.[49]

4.3. Cloning of α-Syn, Bax, and βA4 Genes in Yeast Integrating Vectors. The gene coding for human α-syn, its A53T mutant, Bax, and βA4 was cloned using standard molecular biology techniques as either BglII-XbaI or BamHI-XbaI fragments in appropriate yeast integration vectors. The maps of the resultant plasmids have been shown either in the manuscript or in the Supporting Information.

4.4. Construction of Yeast Strains That Bear Integrated Copies of the α-Syn, Bax, and βA4 Genes. The integrative plasmids, obtained after cloning of the human genes, were linearized with appropriate restriction enzymes, before transformation into the yeast strain W303-1a (*Mata, ade2, his3, leu2, trp1, ura3*; ATCC 208352). It has been referred to here as the strain, BC300. Integration of plasmids into the yeast genome at specific chromosomal sites of BC300 occurred via homologous recombination.[14]

4.5. Growth of Recombinant Yeast Cells in Liquid and Solid-Agar Media. The recombinant cells were grown either in synthetic dextrose minimal medium (SD; contained 0.67% bacto-yeast nitrogen base, with no supplements, and 2% glucose which is also known as dextrose) or in synthetic galactose minimal medium (SG; contained 0.67% bacto-yeast nitrogen base, with no supplements, and 2% galactose). SD and SG media are selected for the presence of the chromosomally integrated plasmid. The solid agar SD, SG, and YPD plates contained the same ingredients plus 2% agar. For growing pre-cultures of cells, complete YPD medium, containing 2% bacto-peptone and 1% yeast extract, was used.[51]

4.6. Microtiter Plate Assays for Identifying Compounds That Rescue Yeast Cells from the Toxicity of α-Syn, Bax, and βA4. α-syn, Bax, or βA4-expressing yeast cells were initially grown in SD medium at 30 °C, for 24 h. Cells were harvested, washed with sterile water, and then resuspended in SG medium. Cells (10^3) were inoculated in 195 μL SG medium (that contained 2% galactose and 0.2% glucose, with appropriate nutrients); for the initial screen, 5 μL of individual compounds in the methoxy-stilbene chemical library was added to cell suspension such that the final concentration of compounds was 10 μM and a DMSO concentration <0.5%. Control cells (containing no α-syn, Bax or βA4), in the absence of compounds but with a DMSO concentration <0.5%, were grown in SG in the same manner. The two flavonoids, fisetin and quercetin, which have been identified as compounds with the therapeutic potential in a yeast-based model of α-synucleopathy, were used as positive controls.[52] Unfortunately, resveratrol (1) and piceatannol (2) (Figure 1A) did not restore cell growth blocked by two copies of α-syn; rescue of α-syn's toxicity in yeast by resveratrol (1) and piceatannol (2) has not been reported in the literature. Cell growth was determined via measurement of optical density at the wavelength of 600 nm (OD$_{600}$). The OD$_{600}$ was measured, before the plates were shaken, using a Synergy HT BioTek plate reader, and the parameters were set using the KC4 software. The OD$_{600}$ was measured once again after 48 and 72 h of shaking at 30 °C on a rotating platform set at 200 rpm. The growth of each set of cells incubated with different compounds is calculated by subtraction of the OD$_{600}$ at 72 h from the initial OD$_{600}$ at 0 h and is then expressed as a percentage growth compared to the control cells (that contained just DMSO and no compound) using Excel. Compounds showing a repetitive high percentage cell growth of 20% and over in the following yeast strains were identified: two-copy α-syn, three-copy α-syn, three-copy mutant A53T α-syn strains, and Bax. The identified compounds were then further tested in the two-copy βA4-expressing yeast cells.

4.7. Detection of Dead Cells with Phloxine B Dye. Cell death was measured by staining with Phloxine B which is a fluorescent dye. Live (i.e., viable) cells expel Phloxine B dye; the dye accumulates only in dead cells. This can be observed using a fluorescent microscope. Phloxine B (Sigma, P-4030-25G) was added to both test yeast cells (bearing the human gene) and control yeast cells (that contain empty plasmid) to a final concentration of 5 μg/m. After incubation at 30 °C for 30 min in the dark, the cells were washed with sterile phosphate-buffered saline (PBS) three times. Cells were then mounted on a hemocytometer for cell counting and on a glass slide for microscopic examination with an Olympus BX-51 fluorescent microscope (at excitation/emission 570/650 nm). Image capturing was done using a Leica digital imaging color camera.

4.8. Yeast Colony Formation. The method was used to distinguish between the numbers of live cells in control cells and cells expressing α-syn, Bax, or βA4 in the presence and absence of "hit" compounds. Yeast strains of interest were grown in 10 mL of YPD liquid medium as pre-cultures. Cells were centrifuged, washed twice with SG, and resuspended in a final volume of 10 mL. The OD$_{600}$ was determined, and the cells were also counted using a hemocytometer. Three hundred cells were spread on a fresh SG agar plate (with appropriate amino acids), containing 10, 5, and 2.5 μM concentration of a compound of interest. Control cells contained no compound. The plates were incubated at 30 °C for 3−4 days. The colonies formed were counted manually, and the difference in colony numbers from different cultures was recorded.

4.9. Detection of ROS with Dihydroethidium. An AAT Bioquest Fluorimetric Intracellular Total ROS Activity Assay Kit (Catalog #22901) was used to measure ROS. After expression of protein(s), OD$_{600}$ was again measured and cells were counted, and 100 000 cells were pipetted into wells of a 96-well microtiter plate (Corning, # 04815027) in triplicate. The plate was centrifuged at 800 rpm for 2 min, and supernatants were discarded. Cells in wells of a microtiter plate were then resuspended in yeast cell culture medium and centrifuged for 2 min at 800 rpm. DMSO (40 μL, component C of the kit) was added to ROS red (component A of the kit) and mixed well. ROS red is cell-permeable and generates red fluorescence when it reacts with ROS. The ROS red/DMSO mixture (20 μL) was added to 10 mL of assay buffer (component B of the kit) and mixed well. This is regarded as the working solution. Working solution (100 μL) was added per well of cells. The plate containing cells was then incubated for 90 min at 30 °C. Fluorescence was then bottom read with a

Bio-Tek Synergy HT plate reader at excitation/emission 520/605 nm.

4.10. Measurement of MMP in Yeast Cells. An AAT Bioquest JC-10 MMP Assay kit (Catalog #22800) was used for measuring MMP. The kit uses JC-10 dye and can selectively enter into the mitochondria. JC-10 changes color from green to orange as the polarization of the mitochondrial membrane increases. The reaction is reversible because of the formation of monomeric (green; emission at 520 nm) and aggregated (orange or red; emission at 570 nm) forms of JC-10, as MMP increases. In healthy cells, JC-10 accumulates in the mitochondrial matrix and forms orange (or red) fluorescent aggregates, but in necrotic or apoptotic cells, JC-10 disperses out of the mitochondria and changes to the monomeric form. Cells are then stained with green fluorescence. After expression of human proteins in yeast, OD_{600} was measured and cells were counted, and 100 000 cells were pipetted in triplicate into wells of a 96-well microtiter plate (Corning, 04815027). Cells were centrifuged at 800 rpm for 2 min. Cells were then resuspended in yeast cell culture medium and centrifuged for 2 min at 800 rpm. All kit components were thawed at room temperature. Component A (50 μL, 100x JC-10) was added into 5 mL of assay buffer A (component B) and mixed well (this is the working solution). The working solution (50 μL) was added to wells of a microtiter plate, and the plate was incubated for 90 min at 30 °C (in the dark). Assay buffer B (50 μL, component C) was added to each well already containing cells and JC-10. The plate was centrifuged for 2 min at 800 rpm before loading the plate on to the Bio-Tek Synergy HT plate reader so that excitation/emission 490/525 nm and 540/590 nm could be read from the bottom. The ratio of aggregated/monomeric JC-10 was plotted.

4.11. TUNEL Assay to Detect Nuclear DNA Fragmentation of Yeast Cells. An AAT Bioquest TUNEL Apoptosis Assay kit (Catalog #22844) was used for the detection of DNA fragmentation. The kit works by incorporating the fluorescence dye TF3-dUTP at the 3′-OH ends of apoptotic DNA fragments. After the expression of the protein of interest, OD_{600} was measured, and cells were counted, and 30 000 cells were pipetted on to wells of a 96-well microtiter plate (Corning, 04815027) in triplicate. The plate was then centrifuged for 2 min at 800 rpm. Supernatants were removed, and 100 μL of 4% formaldehyde fixative buffer was added to each well and incubated for 30 min at room temperature. The fixative buffer was removed and cells were washed twice with PBS. Component A (6 μL, 100× TF3-dUTP) was added to 600 μL of component B (reaction buffer). The above-mentioned mixture (50 μL) was added to wells of a 96-well microtiter plate and incubated for 120 min. Cells were then washed and fluorescence was monitored from the bottom with the help of a Bio-Tek Synergy HT plate reader at excitation/emission 550/590 nm. An Olympus BX-51 fluorescence microscope with a Leica digital imaging camera was used to take the microscopic images.

4.12. Staining with Hoechst Dye for Monitoring Live Cells. Hoechst 33258 (pentahydrate (bisbenzimide) FluoroPure grade; Cat #H21491; Thermo Fisher Scientific) is a nucleic acid stain that is widely used to detect live cells. When bound to double-stranded DNA, the Hoechst dye emits blue fluorescence. Yeast cells expressing a protein of interest were counted, and 1×10^6 cells were pipetted into an Eppendorf tube. Cells were incubated with 5-bromodeoxyuridine for 30 min, and 500 μL of 10 μg/mL of Hoechst 33258 was added to the tube and incubated for 10 min. Cells were washed with PBS and viewed under a fluorescence setting using an Olympus BX-51 fluorescence microscope with a GXCAM-FLUOMAX fluorescence digital camera, with magnification 60× and Image J software version 1.48.

4.13. DDPH Antioxidation Assay. It is a simple and quick method for evaluating the antioxidant potential of a compound, an extract from biological sources. The assay is based on measuring the decrease in the absorption at 517 nm of DPPH, whence the compound or extract is mixed with DPPH solution. DPPH is a stable free radical which changes color from red to yellow when scavenging. The DPPH concentration in a sample is reduced when it reacts with antioxidants, hence the absorbance decreases. The scavenging potential of the antioxidant is proportional to the degree of discoloration of DDPH. Initially, 0.1 mM DPPH (Sigma, D9132) solution was prepared in methanol. For dilution of DPPH, a PBS/methanol solution (20% PBS, 80% methanol) was used. The testing was done in 96-well microtiter plates, in triplicates. Each microtiter plate is setup so that final volume in each well is 300 μL. Each well has 3 components (volumes in μL): PBS-methanol (75)/DPPH stock (150)/Compound stocks(75), and they are added in that order, in triplicate.

4.14. ABTS Antioxidation Assay. ABTS free-radical scavenging assay was used to determine the total antioxidant activity of compounds of interest. It is based on the measurement of ABTS radical cation formation in the presence of an unknown compound. Trolox [6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid], a water-soluble vitamin E analog, served as a positive control for inhibiting the formation of the radical cation. The ABTS-solution, 7 mM ABTS, 2.45 mM potassium peroxodisulfate in PBS (pH 7.4), was freshly made and stored in the dark at room temperature for 4 h to stabilize. The ABTS solution was diluted 1:50 in PBS to provide an absorbance of ~0.7 when read at 734 nm. In a 96-well microtiter plate, 20 μL of compounds (from 200 μM stock solutions) was pipetted in triplicate. For the positive control, 20 μL of 200 μM Trolox was used. For the negative control, 20 μL of 10% DMSO was used, and 180 μL of diluted ABTS solution was added to all wells. The absorbance was read at 734 nM, in 30 s intervals, over 60 min. The results were analyzed using Excel.

4.15. Statistical Analyses. Data from experiments were analyzed by Microsoft Excel 2010 or GraphPad Prism. Student's t tests were performed to compare EC_{50} values using Excel.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b01154.

^{1}H, ^{13}C, and DEPT NMR, data scans of all compounds, and additional biology results (PDF)

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Author Contributions

A.D. and R.M. contributed equally to this work as a first author. S.B.B. and B.C. designed, executed, and coordinated this whole study; R.M. performed synthesis of compounds; M.A. helped in synthesis of a few compounds; A.D. constructed all yeast strains related to βA4 and performed all screening assays. D.A. provided all yeast strains related to α-synuclein and Bax. I.S.W. constructed the plasmids related to βA4. B.C. and S.B.B. wrote the manuscript.

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

α-syn, α-synuclein; PD, Parkinson's disease; MMP, mitochondrial membrane potential; ROS, reactive oxygen species; APP, amyloid precursor protein; AD, Alzheimer's disease; GFP, green fluorescent protein; PICALM, phosphatidylinositol binding clathrin assembly protein; EGCG, epigallocatechin gallate; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; mtPTP, mitochondrial permeability transition pore; DPPH, 2,2-diphenyl-1-picrylhydrazyl; ABTS, 2,2′-azino-bis-3-ethyl-benzthiazoline-6-sulphonic acid

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