Published in final edited form as:

J Alzheimers Dis. 2012 ; 32(4): 949–967. doi:10.3233/JAD-2012-120178.

Latrepirdine (Dimebon™) enhances autophagy and reduces intracellular GFP-A β 42 levels in yeast

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Abstract

Latrepirdine (Dimebon™), an anti-histamine, has shown some benefits in trials of neurodegenerative diseases characterized by accumulation of aggregated or misfolded protein such as Alzheimer's disease (AD) and has been shown to promote the removal of α -synuclein protein aggregates *in vivo*. An important pathway for removal of aggregated or misfolded proteins is the autophagy-lysosomal pathway, which has been implicated in AD pathogenesis, and enhancing this pathway has been shown to have therapeutic potential in AD and other proteinopathies. Here we use a yeast model *Saccharomyces cerevisiae*, to investigate whether latrepirdine can enhance

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autophagy and reduce levels of A β 42 aggregates. Latrepirdine was shown to up-regulate yeast vacuolar (lysosomal) activity and promote transport of the autophagic marker (Atg8) to the vacuole. Using an *in vitro* GFP tagged A β yeast expression system, we investigated whether latrepirdine-enhanced autophagy was associated with a reduction in levels of intracellular GFP-A β 42. GFP-A β 42 was localized into punctate patterns compared to the diffuse cytosolic pattern of GFP and the GFP-A β 42 (19:34), which does not aggregate. In the autophagy deficient mutant (Atg8 Δ), GFP-A β 42 showed a more diffuse cytosolic localization, reflecting the inability of this mutant to sequester GFP-A β 42. Similar to rapamycin, we observed that latrepirdine significantly reduced GFP-A β 42 in wild-type compared to the Atg8 Δ mutant. Further, latrepirdine treatment attenuated A β 42-induced toxicity in wild-type cells but not in the Atg8 Δ mutant. Together, our findings provide evidence for a novel mechanism of action for latrepirdine in inducing autophagy and reducing intracellular levels of GFP-A β 42.

Keywords

Alzheimer's disease (AD); Beta Amyloid (A β); Latrepirdine; Yeast Model; Autophagy

INTRODUCTION

Initially marketed in Russia as a treatment for hayfever in the 1980's, latrepirdine (DimebonTM, dimebolin) has shown to have neuroactive, neuroprotective and cognitive enhancing properties in a number of *in vitro/in vivo* studies [1–7]. It's mechanism of action in the CNS are still poorly understood, however despite this, latrepirdine was taken into clinical trials for Huntington's disease [8] and Alzheimer's disease (AD) [9]. Despite promising results for AD in phase II trials [9], latrepirdine did not show benefits in the short 6 month phase III (CONNECTION) trial and in the recently concluded phase III (CONCERT) AD trial.

Blame for the failed clinical trial has been attributed, in part, to the fact that the mechanism action was not known and a number of recent editorials in *Science* [10], *Nature Medicine* [11], and *Nature Reviews Drug Discovery* [12] have outlined the importance of understanding the mechanism of action. Although there have been a number of proposed mechanisms of action for latrepirdine, its ability to improve cognition and its effects on hallmarks of AD pathogenesis are yet to be determined. Interest in latrepirdine rebounded recently a study showing that it enhances neuronal survival [13] and another outlining the similarity of the latrepirdine scaffold to that of a new, neuroprotective class of drugs [14]. Recent findings have also demonstrated that latrepirdine causes an acute elevation in extracellular levels of A β *in vitro*, and promote the secretion of A β into the interstitial fluid (ISF) *in vivo* [15]. A study of synucleinopathy in a mouse model has found that latrepirdine reduces levels of α -synuclein protein deposits and associated neurodegeneration, suggesting improved clearance of protein aggregates [1]. A more recent report has shown latrepirdine to slow progression of proteinopathy in a transgenic mouse model of amyotrophic lateral sclerosis (ALS) overexpressing γ -synuclein [16]. These studies suggest that latrepirdine may mediate its effects by modulating cellular clearance pathways.

A major degradation/recycling pathway in cells is macroautophagy (also referred to as autophagy-lysosomal pathway (reviewed in [17])). Autophagy is a lysosomal catabolic pathway that is responsible for the degradation of long-lived proteins and damaged cellular organelles. In neurodegenerative diseases, autophagy is considered to be important in the removal of aggregated or misfolded proteins [18–20]. Autophagy has been shown to be an active pathway for APP/A β turnover and clearance [21–23] and impaired clearance of autophagic vesicles is observed in the brains of AD mice models and patients [21, 23, 24].

Enhancing autophagy by treating with the mTOR inhibitor rapamycin has been shown to protect SH-SY5Y cells from A β 42 toxicity [25], and recently has been shown to improve cognition and reduce cerebral amyloid load in a mouse model of AD [26].

The data presented here identifies a novel mechanism of action for latrepirdine in modulating autophagy. We have shown that latrepirdine modulates autophagy in the budding yeast *Saccharomyces cerevisiae*, which has proven to be a valuable model organism for studying fundamental cellular processes in human disease pathologies, especially neurodegenerative diseases featured by protein misfolding [27–29]. Yeast models have been engineered to study proteins involved in misfolding disorders such as polyglutamine repeats [30], α -synuclein [31], SOD-1 [32], A β 42 [33–35] and autophagy is one of the most well studied and characterized intracellular clearance pathways in yeast [36]. In a yeast model expressing GFP-tagged A β 42 fusion protein, we show that enhancing autophagy with latrepirdine reduces levels of intracellular A β 42. In addition, we show that latrepirdine's ability to activate autophagy protects these cells from oligomer A β 42 mediated toxicity. Latrepirdine's ability to enhance autophagy and modulate A β accumulation and associated toxicity may underlie its cognitive benefits in AD.

MATERIALS AND METHODS

Yeast, Bacterial Strains and Plasmids

The yeast strains used in this study were as follows: *Candida glabrata*; *Saccharomyces cerevisiae* KYY55 wild type (*MAT α leu2 ura3 trp1 lys2 his3 suc2- Δ 9*), and Atg8 Δ (*MAT α leu2 ura3 trp1 lys2 his3 suc2- Δ 9 Δ apg8::HIS3*) were kind gifts from Dr. Kuninori Suzuki, Integrated Research Institute, Tokyo Institute of Technology [37]. For plasmid amplification, *E-coli* DH5 α strain (*fhuA2 Δ (argF-lacZ)U169 phoA glnV44 Φ 80 Δ (lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*) was used.

The yeast shuttle plasmid, p416.GPD [38] was used for the expression of GFP, GFP-A β 42 and the mutant GFP-A β 42 (19:34) in yeast. It is a centromeric plasmid stably maintained at one or two copies per cell, and has the strong constitutive GPD promoter to direct heterologous gene expression throughout the yeast life cycle. Standard molecular biology techniques were used for the construction of recombinant plasmids. Briefly, a GFP coding fragment was isolated from pAS1N [39] and inserted into p416.GPD via a shuttle vector to assist in manipulations. The GFP-A β 42 fragment was obtained from pAS1N.GFP-A β 42 from our previous work [34]. To create the GFP-A β 42 (19:34) mutant, site-directed mutagenesis was carried out on a sub-fragment cloned into pBluescript to avoid spurious changes in coding sequences. The changes effected were F19S, L34P, with numbering referring to A β amino acid residue number. Sequencing was carried out to confirm that the required changes had been effected and that no additional mutations were introduced into the A β coding sequence. Mutated fragments were subsequently cloned back into p416.GPD.

The plasmids used in this study are as follows: p416 (with URA3 selectable marker) expressing 1) GFP or N-terminal A β fusions 2) GFP-A β 42, 3) GFP-A β 42 (19:34); pRS306 expressing GFP-Atg8 fusion containing the *GFP-ATG8* gene with the endogenous *ATG8* promoter [40] was a generous gift from Prof. Daniel Klionsky, University of Michigan.

Yeast cells were grown in YEPD (1% yeast extract; 2% peptone, 2% dextrose) or yeast nutrient broth (YNB medium; 0.067% yeast nitrogen base, 2% glucose with different mixtures of auxotrophic amino acids [uracil, adenine, histidine, tryptophan, leucine]). Synthetic minimal medium containing 2% glucose without ammonium sulfate or amino acids (YNB (-N) medium) was used for nitrogen starvation experiments.

Reagents

Latrepirdine (Dimebolin dihydrochloride MW: 392.37) was purchased from Biotrend AG, Zurich. Latrepirdine was dissolved into sterile double distilled water to 30 mM concentration. Working stock aliquots (1 mM) were prepared in water from the 30 mM stock. All solutions were stored at -80°C . Freshly thawed solutions were used for all experiments.

Synthetic human A β 42 was purchased from the W. M. Keck Laboratory (Yale University, New Haven, CT). A β 42 with substitutions at positions [F19S] and [L34P] [A β 42 (19:34)] [41] was purchased from Biomatik Corporation (Wilmington, USA). Rapamycin (MW: 914.17, Sigma, Cat no. R0395) was a generous gift from Mr. Jay Steer, University of Western Australia. 1 mM stocks in DMSO were prepared. Working stocks of 100 μM rapamycin were prepared in DMSO. All solutions were stored at -80°C . FM 4-64 stain was purchased from Molecular Probes, Invitrogen (Cat no. T-3166). α -naphthyl phosphate disodium salt (Cat no. N7255) and D-sorbitol (S1876) were purchased from Sigma.

Assessing autophagy in yeast

Physiological or pharmacological induced autophagy was determined by three different methods as described below.

i.) FM 4-64 staining—Vacuolar membrane specific lipophilic dye, FM 4-64 staining in yeast was done as described in Journo et al, 2008 [42]. *Saccharomyces cerevisiae* (BY4743, KUY55, and Atg8 Δ) yeast cells were cultured to mid exponential phase in YEPD media. The cells were later washed in 50 mM HEPES (pH 7) twice and resuspended in YNB media (-trp) or YNB (-N) media containing 1 mM PMSF. Latrepirdine or rapamycin treatment was done in YNB (-trp) media. Following 6h incubation at 30°C , the cells are washed and resuspended in YNB (-trp) media containing 10 mM sodium citrate (pH 4.3). FM 4-64 stain was added to the cells to a final concentration of 1 μM . The solutions were incubated for 30 min @ RT. The cells were washed and resuspended in YNB (-trp) media containing 10 mM sodium citrate (pH 4.3) and observed under the fluorescence microscope. The levels of intravacuolar staining and multivesicular bodies in cells were quantified by microscopy.

ii.) Vacuolar Alkaline phosphatase activity: Pho8 assay—Vacuolar alkaline phosphatase activity in yeast cell lysates were measured as described in Noda et al, 2008 [43]. *Saccharomyces cerevisiae* (KUY55, Atg8 Δ) yeast cells were cultured to mid exponential phase in YEPD media. The cells were later washed in sterile water twice and resuspended in YNB (complete) media or YNB (-N) media. Latrepirdine or rapamycin treatment were done in YNB complete) media. Following 6h incubation at 30°C , the cells are washed and resuspended in ice-cold assay buffer (250 mM; Tris-HCl, pH 9.0; 10 mM MgSO $_4$, and 10 mM ZnSO $_4$). The cells were lysed using glass beads vortexing. The samples were centrifuged at $14,000\times g$ for 5 min. The supernatant was used for the assay. Protein concentration of each sample was measured using Micro BCA protein assay kit (Thermo Scientific). A total of 100 μg of cell lysate was incubated with 55 mM α -naphthyl phosphate disodium salt for 20 min at 30°C and diluted in the lysis buffer to a final volume of 0.5 mL. The reaction was stopped by adding 0.5 mL stop buffer (2M glycine-NaOH, pH 11). The fluorescence was measured at 345 nm excitation and 470 nm emission wavelengths. After correcting for blank, the activity was calculated Units/ μg of sample.

iii.) GFP-Atg8 Transport assay—For the GFP-Atg8 processing assay, yeast strains harbouring the GFP-Atg8 plasmid were grown to mid exponential phase in YNB (-ura). Cells were then treated with latrepirdine or rapamycin for 6h at 30°C . Following incubation

the samples were observed under the fluorescence microscope [44]. The levels of diffuse vacuolar GFP fluorescence in cells were quantified by microscopy.

Yeast Transformation

Yeast cells were transformed with p416 (GFP-A β fusion expressing) and pRS306 (GFP-Atg8 expressing) using electroporation. A single colony of the untransformed yeast strain was inoculated in 5 mL YEPD media and incubated at 30°C overnight with shaking. The overnight culture was transferred into fresh YEPD media and grown until mid-exponential phase (OD at 600 nm, 1). Cells were washed in ice-cold sterile water two times and then in 1M sorbitol. The cells were then resuspended in 1M sorbitol. 50 μ L of the yeast suspension was mixed with ~5 μ g DNA in a pre-chilled electroporation cuvette (0.2 cm) and pulsed once at V=1.5 kV, 25 μ F, 200 Ohms. 500 μ L of 1M sorbitol was added to the yeast suspension and plated out on selective media agar plates (YNB –ura for p416 and pRS306 expression). The plates were then incubated at 30°C for 5–6 days. Yeast colonies from the transformant plates were then re-streaked onto fresh selective media agar plates (YNB –ura) and stored at 4°C after formation of yeast colonies.

Immunoblotting of cell extracts for A β and GFP

Total cell protein extracts from yeast were used for immunoblotting. Frozen cell pellets were resuspended in 150 μ L cold lysis buffer (50 mM Tris pH 8, 1 mM EDTA, 150 mM NaCl, 1 mM DTT, 2% SDS) with freshly added 1X protease inhibitor cocktail (Roche) and then incubated at 70°C for 15–20 min. Following centrifugation at 18000 $\times$ g for 10 min, the supernatant was collected and protein concentration of each sample was measured using Micro BCA protein assay kit (Thermo Scientific). A total of 50 μ g protein of each sample was loaded and electrophoretically resolved on 4–12% Bis-Tris gels. The proteins were transferred from the polyacrylamide gel to a nitrocellulose membrane using iBlot™ (Invitrogen) dry transfer. Membranes were blocked in 5% casein Tris buffered saline (50 mM Tris, 150 mM NaCl), pH 7.4 (TBS) for 1h. Primary antibodies WO2 (mouse anti-A β residues 3-10) or rabbit anti-GFP (sigma) were diluted in 0.5% casein, 0.05% tween, TBS (TBST) at concentrations of 1/3000 and 1/5000 respectively. Incubation was performed at RT for 1–2h followed by three washes in TBST. Horseradish peroxidase (HRP) conjugated secondary antibodies anti-mouse or anti-rabbit were diluted in 0.5% casein TBST at concentrations of 1/5000 and incubated with the membranes for 1h. After washing with TBST and TBS, the membranes were incubated for 2 min with HRP reactive ECL (Enhanced chemiluminescence) reagent (GE Healthcare). The membranes were developed on films which were scanned using a densitometer. The immunoreactive bands were later quantified using Quantity One 1-D analysis software (version 4.6.8).

Blue Native Polyacrylamide Gel Electrophoresis (BN-PAGE) analysis of yeast cell extracts

Yeast cells (*Saccharomyces cerevisiae*, KUY55, wild type) expressing GFP-A β 42 and GFP-A β 42 (19:34) were grown until exponential phase (OD 0.7–0.8) in YNB (-ura) media. The cells were pelleted and stored frozen at –80°C. The cells were lysed by glass bead homogenization (2 min X 3) on ice in 1X phosphate buffered saline (PBS) only or containing 1% SDS, 1% TritonX100 (TX100) or 1% Dodecylmaltoside (DDM). The crude extracts were analysed by Blue Native Polyacrylamide Gel Electrophoresis (BN-PAGE) [45–47]. The yeast cell extracts were mixed with 3 $\times$ sample buffer containing 100 mM morpholinepropanesulfonic acid (MOPS), 100 mM Tris-HCl, pH 7.7, 40% glycerol, and 0.1% Coomassie blue. Samples were then loaded onto a 4-to-12% Bis-Tris NuPAGE gel (Invitrogen), using High Molecular Weight (HMW) calibration kit (Amersham, GE) as a size standard, and were electrophoresed at 4°C for 3h at 100 V with 50 mM MOPS-50 mM Tris, pH 7.7, containing 0.002% Coomassie blue as the cathode buffer and the same buffer without Coomassie blue as the anode buffer. The proteins were transferred from the

polyacrylamide gel to a polyvinylidene difluoride (PVDF) membrane using iBlot™ (Invitrogen) dry transfer. Excess Coomassie blue dye was removed after blotting by washing with 30% methanol/10% acetic acid followed by 100% methanol. The blots were air dried and transferred to blocking buffer 5% casein Tris buffered saline (50 mM Tris, 150 mM NaCl), pH 7.4 (TBS) for 1h. Following blocking the membranes were analysed by immunoblotting with anti-GFP or anti-A β (WO2) as described above.

Microscopic quantification

Yeast cell suspensions were collected, washed and resuspended in sterile distilled water. A total of 4–5 μ L of the suspension was loaded on microscopic slide for imaging using oil immersion at 100X magnification. Images were taken using similar fluorescence and bright field exposure levels using Olympus DP71 digital camera. A number of 15 image frames each containing approximately 50 cells were quantified for each sample.

A β 42 toxicity assay

Oligomeric A β 42 was prepared according to the method of Bharadwaj et al, 2008 with some modifications [33, 48, 49]. Briefly, the A β 42 peptide was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) and incubated overnight at RT. The HFIP was then evaporated and the resulting peptide film dissolved in sterile double-distilled water. The solution was sonicated on ice for 5 min and centrifuged for 10 min at 14000 rpm. The supernatant was incubated at room temperature for 20h to form oligomers which was used for the toxicity experiments. The A β 42 peptide concentration was determined by measuring absorbance at 214 nm. The final concentration was calculated using the formula $A\beta_{42} (M) = \text{Abs}_{214} \times \text{DF}/75887$. All solvents used for the preparation of A β 42 solutions were filtered using 0.2 μ m filters and the entire procedure was performed in laminar air flow hoods. A β 42 (19:34) peptide solution was also prepared in a similar fashion. For inducing autophagy, cells were pre-incubated with indicated concentrations of rapamycin or latrepirdine or incubated in YNB (-N) for 6h. Cells were diluted in sterile water and aliquoted into 96-well microtitre plates for peptide treatments. Peptide preparations were added to the diluted cell suspension to required concentrations. The final volume in each well was made up to 125 μ L. The microtitre plate was then sealed with gas permeable membrane (Diversified Biotech Inc.) and incubated at 30°C constantly shaken at 150 rpm for 20h. Cell survival was determined by plating aliquots of peptide treated cell suspensions onto YEPD agar plates to measure the number of colony-forming units (cfu) [33].

Statistical Analysis

Where indicated, results were compared using Student's *t* test or one-way ANOVA analysis and considered statistically significant when *p* was < 0.05. When indicating significance, an *asterisk* indicates *p* < 0.05, a *double asterisk* indicates *p* < 0.01. All experiments were done independently at least three times unless otherwise indicated.

RESULTS

Latrepirdine enhances autophagy in yeast

We first compared latrepirdine's ability to enhance autophagy with known activators of autophagy, nitrogen starvation and rapamycin. Incubating cells in growth media depleted of any form of nitrogen source or treating with rapamycin are well known methods of inducing macroautophagy in yeast [50]. Wild-type and Atg8 Δ mutant cells were either nitrogen starved, treated with various doses of latrepirdine or 0.2 μ M rapamycin. Autophagy was then monitored by measuring the increased vacuolar alkaline phosphatase (Pho8) activity in cell extracts using the fluorimetric α -naphthyl phosphate assay [43] and by following the

bulk transport of cytosolic material for degradation in the vacuole using a vacuolar specific lipophilic dye FM 4-64 [42].

Cell extracts from untreated cells and those that underwent nitrogen starvation or were treated with rapamycin (0.2 μ M) or latrepirdine (0.25–5 μ M) underwent analysis of vacuolar alkaline phosphatase (Pho8) activity (Figure 1A, B). Compared to untreated wild-type cells, nitrogen starvation and rapamycin treatment resulted in a 5 and 4 fold, respectively, increase in alkaline phosphatase activity (Figure 1A). Treatment of cells with latrepirdine also resulted in an increase in alkaline phosphatase activity (Figure. 1B), however, 2–3 fold less than rapamycin and nitrogen starvation (activity for nitrogen starvation = 4.5 U/ μ g, highest activity for latrepirdine = 1.7 U/ μ g). Treatment of Atg8 Δ mutant cells did not result in any increase in vacuolar phosphatase activity (Figure. 1A, B).

Activation of autophagy in wild-type cells was also shown by intravacuolar staining of FM4-64 and the presence of multivesicular bodies following nitrogen starvation, rapamycin or latrepirdine treatment (Figure. 1D–F). In the absence of autophagy in either untreated wild-type cells or Atg8 Δ mutant cells, staining of the vacuole perimeter was observed (Figure. 1C, K–N). The percentage of cells showing intravacuolar staining was estimated and shown in Figure 1S. As expected, nitrogen starvation and rapamycin treatment resulted in almost 90% of cells showing intense intravacuolar staining, indicating strong activation of autophagy. Although latrepirdine also showed significantly more intravacuolar staining, it was ~2 fold less than nitrogen starvation and rapamycin. The Atg8 Δ mutant showed an abnormal vacuolar phenotype compared to wild-type even in the absence of autophagy. As expected nitrogen starvation, rapamycin or latrepirdine treatment of Atg8 Δ mutant cells did not result in significant intravacuolar staining, reflecting the inability of these cells to undergo autophagy.

To investigate a more specific autophagic marker, we studied the localization of Atg8p, which is up-regulated and transported to vacuole for degradation during autophagy. Atg8 Δ mutant cells were transformed with the GFP-Atg8p plasmid (pRS306), to examine the transport of Atg8p to the vacuole [44]. Starting yeast culture at OD 0.2 was grown until exponential phase (OD 0.4) and then left untreated, nitrogen starved, treated with rapamycin (0.2 μ M) or latrepirdine (2.5 μ M) (Figure. 2A–D) for 6h. Following treatment, the cells were observed by fluorescent microscopy. With 2.5 μ M latrepirdine treatment ~25% of the cell population showed diffuse GFP fluorescence in the vacuole compared to ~90% with nitrogen starvation or rapamycin treatment (Figure 2I), indicating activation of autophagy. Data from all three assays indicates that although not to same extent as starvation or rapamycin treatment, latrepirdine induces autophagic like characteristics in yeast.

Latrepirdine reduces GFP-A β 42 levels in yeast

We previously developed an *in vitro* constitutive GFP tagged A β yeast expression system as a cellular model for studying the aggregation and localization of intracellular A β [34]. In this system, yeast cells were transformed with p416 plasmid construct harboring GFP tagged A β and a uracil synthesis gene (URA3) for selective growth in media lacking uracil YNB + 2% glu (-ura), thus allowing for only stable transformants to be selected. In this study, cells expressing GFP-A β 42 (19:34) were also generated. This mutant form of A β 42 is modified at positions [F19S] and [L34P] and has been shown to have reduced tendency to aggregate compared to A β 42. In our study, the synthetic A β 42 (19:34) peptide was found to be completely monomeric and significantly less toxic compared to A β 42 peptide preparations which formed oligomers (7–17 kDa and >50 kDa range) (Figure 3A, B). The A β 42 (19:34) mutant produced as a fusion with GFP was shown to have increased solubility and reduced aggregation compared to A β 42-GFP fusion in a bacterial expression system [41]. In addition, *in vitro* structural modeling and yeast two hybrid analysis also indicate that

positions 19 (phenylalanine) and 34 (leucine) in A β 42 sequence play an important role in self interaction of the peptide monomers [51–53].

Wild-type yeast cells were transformed with GFP, GFP-A β 42 or GFP-A β 42 (19:34). Cells lacking Atg8p (Atg8 Δ) were also transformed with GFP, GFP-A β 42 or the GFP-A β 42 (19:34) mutant. In wild-type cells expressing GFP-A β 42, fluorescence was found to localize into punctate like patterns (Figure. 3C) within the cell compared to diffuse cytosolic fluorescence in cells expressing GFP only (Figure. 3E). Cells expressing the GFP-A β 42 (19:34) mutant showed a similar diffuse cellular pattern of expression to those expressing GFP only (compare Figure. 3D with 3E). These observations indicated that aggregated GFP-A β 42 was compartmentalized into inclusion bodies, whereas the non-aggregating GFP-A β 42 (19:34) mutant was distributed throughout the cytoplasm. In addition to the differential distribution, GFP-A β 42 expression was shown previously to induce a strong heat shock in yeast compared to GFP indicating a stress response to protein misfolding and aggregation in the cell [34]. Notably, expression of GFP-A β 42 (19:34) in yeast showed a significantly reduced heat shock response indicating its reduced tendency to aggregate compared to GFP-A β 42 in the yeast cells [54]. In Atg8 Δ mutant cells expressing GFP-A β 42, fluorescence was more diffuse and cytosolic compared to the punctate staining observed in wild-type cells expressing GFP-A β 42, reflecting the inability of the mutant to sequester A β aggregates (compare Figure 3F with 3C).

GFP-A β 42 in yeast is found to be largely associated with the insoluble pellet fraction and present in low levels in the soluble cytosol (Supplementary Figure 1, [34]). Thereby, to further characterize the aggregation states of the GFP-A β fusion proteins, the transformant wild-type cells were lysed in non-ionic detergents, 1% TritonX100 (TX100) and 1% Dodecylmaltoside (DDM) and protein extracts analyzed by Blue Native-PAGE (Figure 3I) [45–47]. A protein smear at >43 kDa representing monomeric GFP tagged A β was prominent in the extracts from yeast cells expressing GFP-A β 42 or GFP-A β 42 (19:34). Large molecular weight aggregate material ranging from 158 kDa–669 kDa were prominent in 1% TX100, 1% DDM extracts of GFP-A β 42 expressing cells (Figure 3I, lanes 1 and 3). A very faint smear was detected in 1% DDM extracts of cells expressing GFP-A β 42 (19:34) (Figure 3I, lane 4), which was absent in 1% Triton X-100 extracts (Figure 3I, lane 2), indicating under certain conditions, small amount of aggregates could be detected but the vast majority is monomeric GFP-labeled A β 42 (19:34). Taken together with the previous studies [51–53] these findings show GFP-A β 42 (19:34) protein's reduced tendency to aggregate in the yeast cell compared to GFP-A β 42.

We next determined if latrepirdine's ability to enhance autophagy contributed to reducing levels of intracellular GFP-A β 42. Exponentially growing yeast cells (OD 0.4) were treated with different concentrations of latrepirdine ranging from 1–5 μ M. After incubating at 30°C till OD 0.8 for ~4–5h, aliquots were collected and underwent fluorescent microscopy (Figure 4A–F) for a quantitative analysis of GFP-A β levels. Compared to vehicle, latrepirdine treatment resulted in a significant reduction in GFP fluorescence in wild type cells expressing GFP-A β 42 (Figure 4G). A decrease of ~10% with 1 μ M and up to ~40–50% was observed with 2.5–5 μ M latrepirdine treatment in wild type expressing GFP-A β 42 (Figure 4G). However, latrepirdine treatment did not significantly alter the fluorescence in wild-type yeast cells expressing GFP-A β 42 (19:34) or Atg8 Δ mutant cells expressing GFP-A β 42 (Figure 4G). Levels of GFP-A β protein was also determined by A β immunoblotting of the cell extracts (Figure 4H, I). Compared to untreated cells, latrepirdine treatment showed significant reduction (~40%) in GFP-A β 42 levels in wild-type cells. Latrepirdine treatment of wild-type yeast cells expressing GFP-A β 42 (19:34) mutant or Atg8 Δ mutant cells expressing GFP-A β 42 did not alter the A β levels (Figure. 4H, I). The ability of latrepirdine to reduce GFP-A β 42 levels but lack of effect in cells expressing the non-

aggregated A β 42 (19:34) mutant, indicates a novel mechanism of action for this drug at removal of intracellular A β 42. The lack of an effect in cells deficient in Atg8p indicates that autophagosome formation is important in this process.

Evidence presented above showed that although latrepirdine activated autophagy, it was not as efficient as rapamycin. To determine if rapamycin would also reduce GFP-A β 42 levels in yeast, we treated wild type and Atg8 Δ mutant yeast cells expressing GFP-A β 42 with rapamycin [36]. Compared to vehicle, rapamycin treatment (0.1 and 0.2 μ M) resulted in a significant reduction of GFP fluorescence in all cells. However, wild-type cells expressing GFP-A β 42 were more sensitive to rapamycin where treatment resulted in an 80–95% decrease. Rapamycin treatment of Atg8 Δ mutant yeast, resulted in a 55–80% decrease in fluorescence levels, whilst treatment of wild type cells expressing non aggregating GFP-A β 42 (19:34) resulted in a 40–75% decline (Figure 5G). Cell extracts were also analyzed by western immunoblotting for A β 42 (Figure. 5H, I). Compared to untreated cells, rapamycin treatment resulted in a significant reduction in GFP-A β 42 in all cell lines. Similar to GFP fluorescence quantification, wild-type cells expressing GFP-A β 42 were found to be more sensitive to rapamycin where treatment resulted in an 80–95% decrease in A β 42 levels. Rapamycin treatment of Atg8 Δ mutant yeast, resulted in a 65–80% decrease in A β 42 levels, whilst treatment of wild type cells expressing non aggregating GFP-A β 42 (19:34) resulted in a 40–80% decline in GFP-A β 42 levels.

Rapamycin's potent autophagy enhancing properties via inhibition of mTOR signalling may account for the decrease in GFP-A β 42 in Atg8 Δ and GFP-A β 42 (19:34) in wild type yeast. Rapamycin has been demonstrated to attenuate cell growth and inhibit protein synthesis in many cell lines [55, 56]. Results presented in Figure 5J show that cell viability was not significantly altered, however inhibition of protein synthesis cannot be ruled out which may have contributed to the decreased A β levels even in the absence of autophagy in Atg8 Δ cells. Similar to our findings in yeast expressing GFP-A β 42, rapamycin was shown to reduce α -synuclein in both wild-type and Atg8 Δ mutant cells [57]. In this study, the mutant cells were less sensitive to rapamycin treatment (ie, compared to wild-type cells, mutant cells expressed more α -synuclein). Another study using a mammalian cellular model of HD, found that rapamycin significantly reduced aggregation-prone polyglutamine Q, expanded Huntington protein and its inclusion bodies in both autophagy active and deficient (Atg 5-/-) cells [58]. It was shown that the amount of eGFP labelled Huntington protein was reduced via a decrease of protein synthesis following rapamycin treatment (0.2 μ M). This reduction in protein synthesis was mimicked following cyclohexamide treatment. A reduction in protein synthesis could also explain the reduction of the non-aggregating GFP-A β 42 (19:34) protein levels in the yeast cell.

Latrepirdine attenuates A β 42 mediated toxicity in *S cerevisiae*

Studies have shown that both rapamycin and latrepirdine protect cerebellar neuron cultures against A β induced toxicity [59, 60]. Here we have determined whether enhancing autophagy by latrepirdine can reduce A β 42 mediated toxicity in yeast. We had previously described oligomeric A β 42 mediated toxicity in yeast cells [33]. Exponentially growing wild type and Atg8 Δ mutant cells (OD 0.7) were incubated with latrepirdine (1–5 μ M), rapamycin (0.2 μ M), or nitrogen depleted media for 6h. After treatment, cells were treated with oligomer A β 42 (2 μ M) for 20h followed by cell viability analysis (Figure 6). A β 42 (2 μ M) treatment caused significant cell death (~80%) in vehicle treated wild type cells. However in nitrogen starved or rapamycin pre-treated wild type cells, A β 42 caused only ~30–40% cell death. Similarly with latrepirdine pre-treatment (2.5 and 5 μ M) of wild type cells, A β 42 caused only 40–50% cell death. The data showed that rapamycin, latrepirdine or nitrogen starvation pre-treatment reduced A β 42 mediated toxicity in wild type yeast (Figure 6A). Interestingly, Atg8 Δ cells showed increased cell viability compared to wild type cells

with A β 42 treatment (compare Figure 6B, A, ** $p < 0.01$). Treatment of cells with 2 μ M A β 42 caused cell death of only ~40% in the Atg8 Δ cells compared to ~80% in the wild type. However unlike wild type, the cell survival did not increase in Atg8 Δ cells pre-treated with latrepirdine (1–5 μ M), rapamycin (0.2 μ M) or nitrogen starved following A β 42 treatment (Figure 6B).

The data showed that pre-treatment with known activators of autophagy, rapamycin and nitrogen starvation enhanced survival against A β 42 toxicity. In a similar fashion, latrepirdine pre-treatment also enhanced survival against A β 42 toxicity. The increase in cell survival with rapamycin, nitrogen starvation or latrepirdine pre-treatment was evident only in wild type cells and was absent in the autophagy deficient mutant (Atg8 Δ). This result indicated that stimulation of autophagy in wild type cells increased its resistance against A β 42 toxicity, whereas due to the lack of autophagy Atg8 Δ cells did not show any increase in cell survival with rapamycin, nitrogen starvation or latrepirdine. Supporting this data is the previous observations where Atg8 Δ cells did not show any increase in autophagy with rapamycin, nitrogen starvation or latrepirdine treatment (Figures 1 and 2). Interestingly, the Atg8 Δ cells displayed an increased resistance to A β 42 toxicity (Figure 6B). Whether this phenomenon is associated with a deficiency in autophagy in the mutant or due to a secondary effect of Atg8 gene deletion remains to be determined.

DISCUSSION

In this study we investigated the ability of latrepirdine to activate the autophagy-vacuolar pathway in yeast and its contribution to reducing intracellularly expressed A β 42 and attenuate oligomer A β 42 mediated toxicity. There were a number of findings that indicated that latrepirdine activated autophagy. Latrepirdine was shown to increase the levels of vacuolar alkaline phosphatase activity and the intravacuolar uptake of FM-464, indicators that vacuolar activity (lysosomes of yeast) was up-regulated. This did not occur in cells deficient in Atg8 (yeast homologue to the mammalian LC3 and required for autophagosome formation). Further analysis of the distribution of Atg8 indicated that latrepirdine promoted the uptake of Atg8 into the vacuole. This process occurs under conditions of autophagy where the Atg8-phosphatidylethanolamine (PE) complex, together with the autophagic body, enters the vacuole and is degraded [61]. These features of autophagy were also observed following treatment with the known activators of autophagy, nitrogen starvation and rapamycin treatment.

Further, similar to rapamycin, latrepirdine reduced levels of GFP-A β 42 expressed in yeast cell, a model developed previously to investigate intracellular accumulation of A β 42 [34]. The aggregating nature and localization pattern of GFP-A β 42 in the yeast cell compared to GFP-A β 42 (19:34) mutant supports the notion that the punctate distribution observed with GFP-A β 42 may result from its ability to aggregate and indicates that these aggregates are sequestered into cytoplasmic inclusion bodies/vesicles. Notably, immunoelectron microscopy of GFP-A β 42 expressing cells has shown that A β 42 is localized into vesicular structures in yeast [34]. Although the precise identity of these vesicles in yeast was not determined in the Caine et al., study, the diffuse cytoplasmic expression observed with GFP-A β 42 expression in Atg8 Δ mutant cells suggested that intracellular GFP-A β 42 was not sequestered due to the inability of these cells to generate autophagosomes. Together, these results indicate that aggregating GFP-A β 42 is sequestered into autophagic-like vesicles and may be targeted for degradation. This process may be enhanced by latrepirdine and rapamycin as these agents were shown to activate the autophagy-vacuole pathway in yeast and reduce GFP-A β 42 levels in wild-type cells and not in Atg8 Δ mutant cells. Overall, the results indicate that latrepirdine mediated decrease in GFP-A β 42 levels in the yeast cell is a result of an intracellular degradation pathway, possibly autophagy mediated.

Activation of autophagy has been shown to be protective against a variety of stresses to cells [62, 63]. The pro-survival function of autophagy has been demonstrated in cells and animal models during nutrient and growth factor deprivation, endoplasmic reticulum stress, development, microbial infection, and diseases characterized by the accumulation and toxicity of protein aggregates [64, 65]. Our data showed that activation of autophagy by latrepirdine, rapamycin and nitrogen starvation enhanced survival against oligomer A β 42 toxicity. The increase in cell survival was evident only in wild type cells and was absent in the Atg8 Δ cells. These results indicated that stimulation of autophagy increased resistance against A β 42 toxicity and perhaps due to its inability to undergo autophagy, Atg8 Δ mutant cells did not show any increase in cell survival with latrepirdine, rapamycin and nitrogen starvation. The observations showing that Atg8 Δ cells do not respond to rapamycin, nitrogen starvation or latrepirdine treatment further supported the notion that activation of autophagy enhanced survival against oligomer A β 42 toxicity.

Interestingly, under normal conditions (without any pre-treatment) Atg8 Δ cells displayed an increased resistance to A β 42 toxicity compared to the wild type. Ideally, the Atg8 Δ mutant would have expected to be more susceptible to oligomeric A β 42 treatment due to its inability to undergo autophagy. However in the yeast A β 42 toxicity assay, Atg8 Δ cells showed almost ~2 fold increase in cell survival comparing the wild type. Studies indicate that autophagic related (ATG) genes can act both in cellular protection and also in cell death [62, 63]. Notably, many examples of ATG-gene dependent death occur in mammalian cells [66, 67]. Whether A β 42 mediated cell death in yeast is associated with Atg8 gene expression in cells is yet to be determined. Another likely possibility is that the knock-out of Atg8 gene in yeast may be associated with effects beyond autophagy which may contribute to this phenomenon. It is therefore unclear whether the increased resistance of Atg8 Δ cells to oligomer A β 42 toxicity is autophagy dependent or a result of a secondary effect of Atg8 gene deletion. Overall the results show that, in addition to reducing levels of intracellular GFP-A β 42, latrepirdine's ability to promote autophagy may underlie its protective role against oligomer A β 42 toxicity.

Autophagy-lysosome and ubiquitin-proteasome pathways represent the main intracellular degradation pathways for protein and organelle clearance in eukaryotic cells. Modulating these cellular degradation pathways to enhance clearance of protein aggregates is gaining interest as a therapeutic strategy in several neurodegenerative disorders featuring abnormal protein accumulation. It is suggested that enhancing the autophagy-lysosome pathway is a more favorable target to promote clearance of protein aggregates compared to the ubiquitin-proteasome system [68]. One reason is the substrate limitations for proteasome mediated degradation. The proteasome degrades proteins that are ubiquitin tagged and only unfolded proteins which can enter the proteasomal core. By contrast, autophagy has the capacity to degrade folded proteins, large aggregates and even entire organelles. Studies from a variety of models have shown that up-regulation of autophagy; both pharmacologically and physiologically can promote clearance of protein aggregates and attenuate neurodegeneration [63, 69–72]. In mammalian AD models, activators of autophagy have been shown to be neuroprotective, improve cognition and reduce amyloid deposition [25, 26]. Rapamycin has been widely reported to enhance clearance of aggregate-prone proteins [69, 70, 73]. We show that rapamycin reduced levels of GFP-A β 42 in wild-type cells and although not to the same extent, led to a reduction in GFP-A β 42 in Atg8 Δ cells and GFP-A β 42 (19:34) in wild-type cells. Rapamycin's ability to inhibit mTOR may also account for its ability to attenuate cell growth and protein synthesis [55, 56], which may, in part, account for reductions in GFP-A β in all cells lines. Together with the effects of mTOR signaling cell growth, proliferation and protein synthesis [56], rapamycin has also been associated with immunosuppressant effects [74]. Therefore, synthetic small molecule derivatives of rapamycin are being developed and have shown to have benefits at reducing toxicity.

induced by Huntington protein aggregates, whilst minimizing rapamycin's adverse effects [70, 75]. A recent study has shown that a small molecule enhancer of rapamycin (SMER28) decreases levels of A β in neuronal cells [22]. Further, derivatives of known agents shown to have benefits in AD models, such as resveratrol, have been developed and shown to modulate mTOR signaling and facilitate autophagy and A β degradation [76].

Latrepirdine is another small molecule that appears to mediate a number of activities associated with receptor inhibition [2], neuronal survival [13, 59], intracellular Ca⁺² stabilization [60], protection against A β -induced toxicity [60], A β secretion and metabolism [15] and clearance of α -synuclein protein aggregates [4, 77]. Some recent studies have also reported latrepirdine's cognitive enhancing properties [5, 6]. Latrepirdine was not as efficient as the known potent activators of autophagy like rapamycin or nitrogen starvation. The degree of activation of autophagy by latrepirdine correlates with its ability to reduce A β levels. Findings from this study indicate that latrepirdine can enhance autophagy and can still significantly reduce A β 42 levels without exhibiting the undesirable effects of rapamycin on cell growth or protein synthesis. In collaborative studies, a protective role of latrepirdine in the clearance of α -synuclein in mouse brain and neuronal cells and reducing intracellular A β in mouse brain, in parallel with elevation of the levels of autophagic activity was observed [78, 79]. These findings represent latrepirdine's ability to enhance autophagy which may account for its action in promoting the clearance of protein aggregates [4, 77] and may also be the underlying mechanism of action for its benefits in Huntington's disease where, autophagy has been shown to be the major clearance pathway of misfolded Huntington protein [4, 70].

Overall, our findings have provided support for the use of yeast as an *in vitro* model to study autophagy and the intracellular degradation of A β 42. Assessing A β 42 clearance in yeast cells deficient in essential components involved in the initiation of the pathway (through inhibition of mTOR), autophagosome formation and transport or autophagosome-vacuolar fusion, will provide insight into possible targets for latrepirdine, which will require further validation in mammalian neuronal cells. The identification and characterization of all components of the autophagy-vacuolar (lysosomal) pathway in yeast provides a simple *in vitro* model to address the contribution of this and other intracellular clearance mechanisms involved in the removal of aggregated A β . Similar to other yeast models for studying protein aggregates such as Huntington poly Q aggregates [30] or synuclein aggregates [57] the GFP-A β 42 yeast model can also be used to identify/screen agents that can enhance A β 42 intracellular clearance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors acknowledge the generous support of NH&MRC (APP1009295 to RM, GV, SG), McCusker Alzheimer's Research Foundation (RM, GV), Cure Alzheimer's Fund (S.G.) and NIH 12 (AG10491 to S.G.), and the Fidelity Biosciences Research Initiative (SJ, JL, DR, GAP), Deutsche Forschungsgemeinschaft (MLL). J.W.S is a trainee in the Integrated Pharmacological Sciences Training Program supported by grant T32M062754 from the NIGMS.

M.E.E. holds research grant support from Medivation, Inc. S.G. holds research grant support from Amicus Pharmaceuticals and is a consultant to the Pfizer-Janssen Alzheimer's Immunotherapy Alliance.

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Figure 1. Latrepirdine increases vacuole activity

Exponentially growing wild type and Atg8Δ yeast cells were collected for Pho8 activity assay or stained with FM 4-64 after rapamycin (0.2 μM), latrepirdine treatment or incubation in YNB (-N) media for 6h. **(A)** Pho8 activity was significantly increased (**, $p < 0.01$, $n=3$) in wild type compared to Atg8Δ cells that underwent N-starvation or treatment with 0.2 μM rapamycin. **(B)** Activity was significantly increased dose dependently in wild type compared to Atg8Δ cells treated with latrepirdine (**, $p < 0.01$, $n=3$). FM 4-64 staining was observed by fluorescence microscopy in wild type (**C–F**) and Atg8Δ (**K–N**) cells. The number of cells showing intravacuolar staining and multivesicular morphology were counted as percentage of total cell population (**S**). Significant increase in intravacuolar or multivesicular staining was observed in wild-type cells (marked by white arrows) compared to Atg8Δ cells that underwent N-starvation, rapamycin treatment (0.2 μM) or latrepirdine treatment (1 μM, 2.5 μM) (**, $p < 0.01$).

Figure 2. Latrepirdine promotes the uptake of GFP-Atg8p into the yeast vacuole

Cells expressing GFP-Atg8p plasmid (pRS306) were left untreated (**A, E**), treated with rapamycin (**B, F**) (0.2 μ M), nitrogen starved (**C, G**) or latrepirdine (2.5 μ M) (**D, H**) for ~6h and observed by microscopy. The number of cells showing diffuse vacuolar green fluorescence was counted and represented as a percentage in the total cell population (**G**). Data is represented as mean $\pm$ SEM (n=4). Rapamycin (0.2 μ M), N-starvation or latrepirdine treatment (2.5 μ M) significantly increased the levels of diffuse GFP fluorescence in the vacuoles (marked by white arrows, **, p<0.01, n=4) indicating the increased transport of GFP-Atg8 to the vacuole.

Figure 3. Toxicity and aggregation state of Aβ42 and Aβ42 (19:34)

The Aβ42 and Aβ42 (19:34) peptides were prepared as described in the experimental procedures. (A) The peptide solutions were analyzed by SDS-PAGE (4–12% Bis-Tris gels). 100 ng of Aβ42 (lane 1) and Aβ42 (19:34) (lane 2) were analyzed by immunoblotting (anti-Aβ, WO2). 5 μg of Aβ42 (lane 3) and Aβ42 (19:34) (lane 4) of the same peptide stock were analyzed by coomassie staining. (B) Aβ42 toxicity in yeast was performed as described before [33]. Exponentially growing yeast cells (*Candida glabrata*) were treated with Aβ42 or Aβ42 (19:34) peptide (5 μM and 10 μM) for 20h at 30°C. Cell viability was determined by counting the number of colonies formed and expressed as a percentage of vehicle treated control. Compared to vehicle treated cells, cell viability was significantly reduced (**, $p < 0.01$) in those cells treated with 5 or 10 μM Aβ42. Treatment with Aβ42 (19:34) did not significantly alter cell viability. Data is expressed as mean $\pm$ SEM ($n = 4$). (C–H) GFP-Aβ42, GFP-Aβ42 (19:34) and GFP expressing wild-type or Atg8Δ yeast cells were collected for microscopy. Wild-type cells expressing GFP-Aβ42 showed punctate staining (C). Wild-type cells expressing GFP-Aβ42 (19:34) (D) or GFP (E), or Atg8Δ mutant cells expressing GFP-Aβ42 (F), GFP-Aβ42 (19:34) (G) or GFP only (H) showed diffuse cytoplasmic staining. To characterize the aggregation states of the GFP-Aβ fusion proteins, the transformant wild-type cells were lysed in non-ionic detergents, 1% TritonX100 (TX100) and 1% Dodecylmaltoside (DDM) and protein extracts analyzed by Blue Native-PAGE (I). Total protein (150 μg) from the 1% TX100 and 1% DDM extraction were analyzed by 8% Bis-Tris gels (Blue Native PAGE-MOPS buffering). Monomeric bands (<43 kDa size) were

observed in GFP-A β 42 and GFP-A β 42 (19:34) extracts (lanes 1–4). Higher aggregates ranging from 158 kDa–669 kDa were observed with GFP-A β 42 extracts (lanes 1 and 3), whereas present in significantly low levels in GFP-A β 42 (19:34) extracts (lanes 2 and 4).

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Figure 4. Latrepirdine reduces levels of GFP-Aβ42

GFP fluorescence images of untreated (A–C) and latrepirdine treated (2.5 μM) (D–F) wt-GFP-Aβ42 (A, D), Atg8Δ-GFP-Aβ42 (B, E) and wt-GFP-Aβ42 (19:34) (C, F) expressing cells are shown. Cells expressing green fluorescence following treatment was quantified and expressed as a percentage of untreated control (G). Compared to untreated cells, latrepirdine treatment resulted in significant reduction in fluorescence levels in the wild type-GFP-Aβ42 (*p<0.05). Cell extracts (50 μg) from untreated and latrepirdine treated (1–5 μM) wild-type and Atg8Δ cells expressing GFP-Aβ42 or wild-type cells expressing GFP-Aβ42 (19:34) were prepared and probed with WO2 (H). Levels of Aβ42 were quantified and expressed as a percentage of untreated control (I). Compared to untreated cells, latrepirdine treatment

resulted in significant reduction in A β levels only in wild type GFP-A β 42 cells (*p<0.05, **p<0.01). Cell viability was measured following latrepirdine treatment (**J**). All data is represented as mean $\pm$ SEM (n=3)

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Figure 5. Rapamycin treatment reduces levels of GFP-A β 42

GFP fluorescence images of untreated (A–C) and rapamycin treated (0.2 μ M) (D–F) wt-GFP-A β 42 (A, D), Atg8 Δ -GFP-A β 42 (B, E) and wt-GFP-A β 42 (19:34) (C, F) expressing cells are shown. Cells expressing green fluorescence following treatment was quantified and expressed as a percentage of untreated control (G). Compared to untreated control, the percentage of cells expressing green fluorescence were significantly reduced in all cell lines treated with rapamycin (# $p < 0.01$). Compared to wild-type cells expressing GFP-A β 42 (19:34) or atg8 Δ mutant cells expressing GFP-A β 42, the fluorescence levels were significantly reduced in wild-type cells expressing GFP-A β 42 (* $p < 0.01$ at 0.1 and 0.2 μ M rapamycin treatment). Cell extracts (100 μ g) from untreated and rapamycin treated (0.1 μ M or 0.2 μ M) wild-type and Atg8 Δ cells expressing GFP-A β 42 or wild-type cells expressing GFP-A β 42 (19:34) were analyzed by immunoblotting (H). Levels of A β 42 were quantified and expressed as a percentage of untreated control (I). Compared to untreated control, levels

of A β 42 were significantly reduced in all cell lines treated with rapamycin (#, $p < 0.01$). Compared to wild-type cells expressing GFP-A β 42 (19:34) or Atg8 Δ cells expressing GFP-A β 42, A β 42 levels were significantly reduced in wild-type cells expressing GFP-A β 42 (**, $p < 0.01$ at 0.2 μ M) and data represented as mean $\pm$ SEM (n=3). Cell viability was measured following treatment (**J**). All data is represented as mean $\pm$ SEM (n=3)

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Figure 6. Latrepirdine attenuates oligomer Aβ42 mediated toxicity in yeast cells

Exponentially growing *Saccharomyces cerevisiae* (KVY55) wild type cells were incubated with rapamycin (0.2 μM), nitrogen depleted media or increasing concentrations of latrepirdine (1–5 μM) for 6h. After treatment, cells were treated with oligomer Aβ42 (2 μM) for 20h. Cell viability was measured by viable colony count and represented as percent of untreated (A). Aβ42 treatment caused significant loss of viability in vehicle treated cells (# p<0.001). However cell viability following Aβ42 treatment was significantly higher in nitrogen starved, rapamycin treated or latrepirdine (2.5 and 5 μM) treated wild type cells compared to vehicle treated (* p<0.05, ** p<0.01). (B) Similar to wild type, exponentially growing *Saccharomyces cerevisiae* (KVY55) Atg8Δ mutant cells were incubated with rapamycin (0.2 μM), nitrogen depleted media or increasing concentrations of latrepirdine (1–5 μM) for 6h followed by oligomer Aβ42 (2 μM) treatment for 20h. Aβ42 treatment caused significant loss of viability compared to vehicle treated cells (#, p<0.001), however lower compared to the wild type treated with similar amount of Aβ42 (**p<0.01). No significant change in cell viability following Aβ42 treatment was observed in nitrogen starved, rapamycin treated or latrepirdine (2.5 and 5 μM) treated Atg8Δ cells compared to vehicle treated. All data expressed as mean ± SEM (n=5).