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A Yeast Chronological Lifespan Assay to Assess Activity of Proteasome Stimulators

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Abstract

Aging is characterized by changes in several cellular processes, including dysregulation of proteostasis. Current research has shown long-lived rodents display elevated proteasome activity throughout life and proteasome dysfunction is linked to shorter lifespans in a transgenic mouse model. The Ubiquitin Proteasome System (UPS) is one of the main pathways leading to cellular protein clearance and quality maintenance. Reduction in proteasome activity is associated with aging and its related pathologies. Small molecule stimulators of the proteasome have been proposed to help alleviate cellular stress related to unwanted protein accumulation. Here we have described the development of techniques to monitor the impact of proteasome stimulation in wild-type yeast and a strain that has impaired proteasome expression. We validated our chronological lifespan assay using both types of yeast with a variety of small molecule stimulators at different concentrations. By modifying the media conditions for the yeast, molecules can be evaluated for their potential to increase chronological lifespan in five days. Additionally, our assay conditions can be used to monitor the activity of proteasome stimulators in modulating the degradation of a YFP-alpha-synuclein fusion protein produced by yeast. We anticipate these methods to be valuable for those wishing to study the impact of increasing proteasome-mediated degradation of proteins in a eukaryotic model organism.

TOC

A chronological lifespan assay using various strains of yeast to monitor the effect of proteasome stimulation with small molecules is described.

Graphical Abstract

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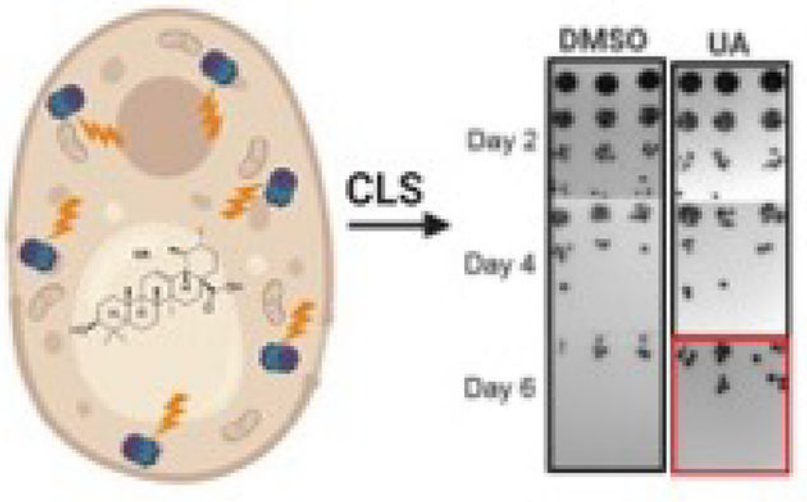
Conflict of interest

Prof. Trader is a shareholder and consultant for Booster Therapeutics, GmbH. Other authors declare no conflict of interest.



Keywords

CLS; Proteasome; Stimulator; Yeast

Introduction

Aging and its related pathologies pose a significant global health and economic burden, as humans are living longer, and a large portion of the population moves into later stages of life. Currently, there are no specific medical interventions to slow or reverse the signs of aging, making the discovery of new therapeutics critical to improving the quality of life of an aging population. Aging onset is driven by several changes in cellular processes.[1] This includes decline in the autophagy pathway and protein clearance by the proteasome.[2,3] This is further exacerbated by dysregulation of the transcriptome and proteome, which leads to accumulation of unwanted proteins in the cell.[4] Moreover, protein accumulation in regions of the brain and spinal cord can lead to the onset of neurodegenerative diseases.[5,6] The link between decline of protein clearance pathways and aging has warranted investigation of the proteasome as a therapeutic target for aging.

Recent studies have demonstrated that an increase in proteasomal activity is associated with extended healthspan or lifespan of organisms. For example, the naked mole rat, one of the longest-lived mammals, exhibits increased proteasomal activity compared to other rodents of similar size.[7] Although rodents studied showed similar amounts of protein damage, naked mole rats had lower amounts of ubiquitinated proteins in various tissues than other rodents. This suggests that increased activity of the proteasome facilitates better clearance of damaged proteins and results in a longer lifespan. Studies performed with yeast models, in which expression of the proteasome was manipulated using genetically modified strains, revealed that overexpression of proteasomal subunits can enhance lifespan, while reduction in expression yields shorter lifespans.[8] A recent report also found that the proteasome is especially important for yeast lifespan extension under caloric restricted conditions.[9] Conversely, studies with transgenic mice knocked down for proteasomal

subunit expression exhibited earlier onset of aging and shorter lifespans than normal mice.[10] This phenomenon also extends to humans. Decline in proteasomal activity has been observed in several aged human tissues including the brain[11], heart[12], and fibroblasts[13]. Interestingly, fibroblasts harvested from centenarians demonstrated increased activity of the proteasome compared to other aged individuals.[13] Not only was proteasomal activity higher in centenarian tissues, but was also similar to that fibroblasts taken from younger humans.

There has recently been interest in the development of small molecules that can increase the endogenous activity of the proteasome to degrade proteins through a ubiquitin-independent mechanism. This has led to the discovery of several small molecules that can help the core particle of the proteasome (20S CP) degrade proteins such as α-synuclein, tau, ODC, and others that contain a significantly disordered regions.[14,15] In conclusion, there is strong interest in proteasomal activation as a therapeutic approach to target proteotoxicity which is associated with neurodegenerative disorders and aging. While there are activity probes and assays developed to monitor the increase of activity of the proteasome in immortalized human cells, there are a lack of methods to study how proteasomal stimulators impact aging through rescuing proteasomal activity.

With technical and cost limitations, it is difficult to test multiple stimulators at multiple concentrations in a mammalian animal model. However budding yeast (*Saccharomyces cerevisiae*) has emerged as an important tool for aging studies.[16] There are two main approaches to study the aging of yeast. The first is to study their replicative lifespan (RLS), where the ability of a single yeast cell to produce daughter cells is monitored over time.[17,18] As yeast age, they begin to produce fewer offspring. The ability of yeast to continue to produce daughters in response to treatments with different compounds is sometimes used to evaluate the impact of small molecules on aging. The second approach is to analyze their chronological lifespan (CLS).[17,18] CLS is monitored by determining the time that yeast cells remain viable in a senescent state in response to a treatment by monitoring outgrowth capacity in liquid media or on an agar plate. Both RLS and CLS studies have been used to study yeast lifespan in response to changes in proteasomal activity.[4,16]

Mutant strains of yeast are available where the expression of proteasomal subunits is decreased. One such strain is the *rpn4Δ* BY4741, in which the RPN4 transcription factor, which controls expression of proteasomal subunits is knocked down.[8,19] Reduced expression of these genes results in fewer proteasomes, leading to reduced activity as compared to the wildtype (WT) BY4741 strain. Here, we describe the application of these two strains of yeast to study the effect of small molecule stimulators of the proteasome on CLS. We developed a system that could provide anti-aging yeast data in only six days and analyze the degradation of an α-synuclein fusion protein from yeast. We were able to show that one of our proteasomal stimulators, ursolic acid, helped prolong the CLS of the *rpn4Δ* BY4741 yeast strain. We expect these assays will find utility for those that are interested in the therapeutic benefit of increasing the ability of proteasome-mediated protein degradation.

Results and Discussion

Using a FRET-peptide to detect proteasomal activity, we discovered that ursolic acid stimulated the proteasome.[14] We first wanted to confirm that ursolic acid (UA) and two structurally similar derivatives to UA, betulinic acid (BA) and oleanolic acid (OA) stimulated the proteasome (Figure 1A).[14] Since purified yeast proteasome is not commercially available, we compared the stimulatory capabilities of these three molecules with human proteasome at 10 μM, Figure 1B. As a control, we used 25 μM of bortezomib (BTZ), an inhibitor of the proteasome. We also included 4'4-dimethyoxychalcone (DMC), which has been previously described to prolong the lifespan of yeast through increasing the activity of the autophagy pathway.[20] We planned on using this molecule as a positive control in our CLS yeast assays and wanted to confirm it did not impact proteasomal activity. We utilized a proteasomal activity probe designed by our lab called TAS-1 and it was added to the samples at a final concentration of 10 μM (Supporting Information Figure S1).[21] After normalizing proteasomal activity to samples treated with DMSO, it was apparent that UA, BA, and OA significantly stimulated the proteasome, while DMC did not. Dosing with 25 μM of BTZ significantly reduced proteasome activity as expected. While these stimulators are not especially potent, these steroid scaffolds are stable and are known to be permeable to yeast.[22,23] To ensure these small molecules also stimulate yeast proteasomes, we dosed both *rpn4Δ* and WT lysate with each compound or BTZ and a commercially available proteasome reporter probe. We observed a significant increase in the activity of the proteasomes from both samples (Supporting Information Figure S2).

To develop assays to elucidate the impact of proteasomal stimulators on yeast lifespan, we selected the WT and the *rpn4Δ* BY4741 strain of *S. cerevisiae*. We tested the lysate of each yeast type for endogenous proteasomal activity (Supporting Information Figure S3). As expected, this revealed a striking difference in the activity of proteasomes in the lysates. The result demonstrated that the *rpn4Δ* strain has a 40% reduction in activity when normalized to the WT strain (Figure 1C), validating that these strains could be used for lifespan extension studies.

After establishing that these compounds could stimulate purified proteasome, we next sought to determine if these small molecules could prolong the CLS of yeast with less endogenous proteasomal activity. For our initial CLS assay, cultures of yeast were dosed with proteasomal stimulators and incubated in media with aeration. Every seven days, samples were diluted in sterile water and spotted on agar plates to monitor outgrowth. Since it is well established that it can be difficult for yeast to take up and retain small molecules, we first sought to utilize a strain of yeast with reduced expression for drug efflux pumps. For this purpose, we selected the BY4741 WT strain and an isogenic strain with gene deletions for eight drug resistance-associated genes (*Δ8*).[24] The WT yeast strain used in this assay was able to survive for several weeks before it produced measurable changes in lifespan (data not shown). This is consistent with previous reports indicating that the BY4741 strain has a long lifespan compared to other yeast strains.[25] The *Δ8* strain demonstrated a shorter lifespan than the WT but still required several weeks of incubation to generate measurable effects. However, dosing the *Δ8* strain with 20 μM of UA did show an increase in lifespan (Figure 2).

Since we wished to develop a CLS assay that could efficiently monitor multiple proteasomal stimulators at multiple concentrations, we needed conditions for which the CLS of the WT and *rpn4Δ* yeast was shorter. It is well established that several factors in the growth media impact the ability of yeast to survive, such as amino acid composition, nitrogen concentration, and glucose concentration.[26] We tested three base medias that each differed in amino acid composition (SD, SC and CSM media). Each media was supplemented with a combination of low, high or normal concentrations of nitrogen and glucose. WT yeast grew in each of the fifteen media compositions and outgrowth was monitored by a spot assay (Supplemental Information Figure S4). Excitingly, supplementing CSM media with a normal nitrogen source concentration and high glucose concentration (referred to as aging media) was successful in reducing WT yeast lifespan to just six days, thirty-four days shorter than our initial design. The same experiment was performed with the *rpn4Δ* yeast and yielded a similar result (Supplemental Information Figure S5). We also confirmed that the shortening of the yeast lifespan was not due to acidification of the media (Supplemental Information Figure S6). High glucose conditions have previously been demonstrated to reduce CLS possibly by increasing stress and superoxide generation.[17,25] These increased stress conditions lead to increased proteotoxic effects that are detrimental to lifespan.[27,28] This modified media conditions allowed us to acquire data more rapidly as compared to our initial experimental design.

With this new aging media in hand, we first tested the ability of DMC and BTZ to extend or shorten the lifespan of both the WT and *rpn4Δ* yeast. Cultures of each strain were dosed with each compound or DMSO and samples were plated every day.(Figure 3). Samples diluted 1:100 were also further diluted in YPD media and allowed to grow for 16 hours, then the OD_{600} was measured and graphed (Supplemental Information Figure S7). As expected, DMC prolonged the survival of both yeast strains, as evidenced by improved outgrowth of the more dilute samples on the agar plates after five days. The impact of lifespan extension by DMC was more apparent in the *rpn4Δ* strain than the WT strain. Conversely, BTZ successfully reduced the lifespan of both strains of yeast. This is reflected by the decreased outgrowth of the BTZ dosed yeast at each dilution compared to the DMSO control.

Next, we sought to determine the optimal concentration of UA to prolong yeast lifespan. Although 10 μM had successfully stimulated purified human 20S proteasomes, it is more difficult for the small molecule to elicit the desired effect in yeast as it can be challenging to pass through the cell wall and remain internalized because of numerous drug transporters. As a result, we dosed both yeast strains with varying concentrations of UA to elucidate the optimal concentration to monitor its impact on CLS. Cultures of yeast dosed with each UA concentration were spotted on agar plates every day to monitor outgrowth(Supporting Information Figure S8). Yeast growth was also assessed with a quantitative outgrowth assay by diluting 1:100 in YPD media and cultured for 18 hours, then the OD_{600} was measured and graphed (Figure 4). Surprisingly, dosing with higher concentrations of UA did not appear to greatly enhance lifespan as evidenced by the OD_{600} being relatively similar to that of the DMSO control. Similarly, UA did not significantly increase the OD_{600} of WT yeast each day, suggesting that its impact on the lifespan of this strain is not large. This suggests that UA may not increase WT proteasomal activity possibly because the proteasome is already functioning optimally.

However, dosage of the *rpn4Δ* yeast with 10 μM of UA led to a significant increase of the OD_{600} on day 1 and day 3, suggesting that this concentration is sufficient to extend CLS of this strain. Excitingly, it enhanced the OD_{600} to a similar extent as dosing the yeast with 200 μM of DMC, our positive control. This data suggests that chemical stimulation of proteasomes in yeast with reduced proteasome activity could result in an extended lifespan. From this experiment, we also concluded 10 μM was the optimal concentration for dosing yeast in future experiments.

We next tested the derivatives of UA to determine if they too could extend the CLS of yeast. Cultures of WT or *rpn4Δ* yeast were prepared as previously described and dosed with 200 μM DMC or BTZ (positive and negative controls) or 10 μM of UA, BA, or OA, or DMSO. None of the proteasome stimulators affected the WT yeast lifespan compared to the DMSO control, while the DMC-treated had a marginal effect similar to what was observed in Figure 3. The BTZ, however, shortened lifespan with outgrowth almost completely inhibited after four days (Figure 5). Excitingly, dosage of *rpn4Δ* yeast with both UA and BA resulted in improved outgrowth of the yeast as compared to the DMSO control, indicating lifespan extension (Figure 5). In fact, the extent of outgrowth of UA and BA-dosed yeast was similar to that of 200 μM DMC. OA, however, was not successful in extending lifespan, although it was previously demonstrated to stimulate human 20S proteasomes. There are many factors that may explain the lack of activity for this UA derivative including insufficient drug entry, expulsion through efflux pumps or lack of direct action on the yeast proteasome. These results suggest that dosing yeast with proteasome stimulators is effective at prolonging lifespan of yeast with compromised proteasomal activity.

Since none of the UA derivatives extended lifespan to a greater extent than UA, we further evaluated its ability to assist proteasomes in clearing proteins prone to aggregation. α-synuclein is known to aggregate in the cells of brains of people with Parkinson's Disease (PD), leading to neurodegeneration.[27,28] These aggregates are difficult for the proteasome to clear, resulting in a buildup of proteins and eventually cell death. A previous study demonstrated that chemical modulation of upstream pathways resulting in increased 26S proteasomal activity can alleviate α-synuclein toxicity in human cells.[29] Since reduced proteasome activity is associated with aging and neurodegenerative diseases, we sought to determine if chemical stimulation could help the proteasome clear α-synuclein in yeast. To test this hypothesis, we utilized a strain of yeast that can be induced to express high levels of α-synuclein fused to Yellow Fluorescent Protein (YFP).[30] Although α-synuclein is not natively expressed in yeast, previous studies demonstrated that overexpression of α-synuclein in yeast has relevant activity to human cells because α-synuclein forms cytoplasmic foci and detectable aggregates in lysates due to its propensity to form oligomers.[30] Induced expression of a single copy of α-synuclein results in plasma membrane localization, but induced expression of two gene copies results in the formation of cytoplasmic foci. After yeast were induced to express the YFP-α-synuclein fusion protein, samples were lysed and dosed with 10 μM of UA or DMC, 50 μM MG-132, or DMSO for four hours. Protein concentration between the samples was normalized, and samples were subjected to analysis by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a membrane for western blot analysis. Samples were blotted with an antibody that recognizes the YFP and imaged to compare

the amounts of the α-synuclein-YFP fusion that remained (Figure 6 and Supplemental Information Figure S9). As a loading control, membranes were blotted for Glucose-6-phosphate 1-dehydrogenase (G6PDH), a house keeping protein. We were expecting to find that dosage of yeast lysate with UA would result in better degradation of the α-synuclein portion of the protein fusion, corresponding to less intense bands after blotting. Conversely, dosage with MG-132, a proteasome inhibitor, should result in accumulation of α-synuclein and result in a more intense signal from the western blot. Excitingly, the results obtained from this experiment were as expected. Dosage of lysate with UA resulted in a statistically significant reduction in YFP-fused α-synuclein, while dosing with MG-132 resulted in the accumulation of the fusion protein. This is reflected by the lower or higher fluorescence intensities than the DMSO control, respectively. DMC did not appear to impact clearance of α-synuclein. Although some autophagic processes can be recapitulated in cell-free systems, [31] the complete autophagic process may not be functioning sufficiently to degrade α-synuclein. We also investigated the levels of G6PDH enzyme as a control protein and found that G6PDH was not appreciably affected by UA treatment (Supplemental Information Figure S10). This data indicates that chemical stimulation of the proteasome could be a viable strategy to prevent accumulation of undesirable proteins such as α-synuclein, potentially increasing the health and lifespan of eukaryotic cells.

Conclusion

Pathologies that result from protein accumulation will continue to pose a significant medical and economic burden as a large portion of the global population is aging. Unfortunately, few therapies exist that can prevent or reverse the effects of protein accumulation, making the discovery of novel targets and drugs critical to improving the quality of life of an aging population. In this work, we present a methodology to study the impact of stimulation of the proteasome to prolong the lifespan of yeast with compromised proteasome activity. While it has been well established that the proteasome plays an important role in the progression of aging through the studies of long-lived rodents and yeast models in which proteasome expression can be manipulated, evaluation of the proteasome as a target for small molecule intervention has remained understudied. Here, we have described two assays that can be utilized to study the effects of proteasomal stimulators on the CLS of wild-type yeast, as compared to the strain that has less proteasomal activity. The experiments described here can be expanded as an in vivo screening method for assessment of potential proteosome activators that need validation such as structure-based drug design molecules. In addition, further mechanistic studies and interplay of pharmacological stimulation of the proteasome with biological pathways that affect aging are facilitated by the plethora of available yeast genetic strains. While the proteasomal stimulators described here are not especially potent and may have multiple targets, UA did have a positive outcome in aiding the *rpn4Δ* yeast strain to survive longer and increase the degradation of an α-synuclein protein fusion. Since there is a clear link between aging, protein accumulation diseases, and reduced proteasomal activity, the results of this study provide a way to study proteasomal stimulators quickly in a model organism. Delaying and reversing the pathologies of aging associated with unwanted protein accumulation through modification of activity of the proteasome is an exciting mechanism of geroprotection to pursue.

Materials and Methods

Biochemical Assay to Monitor Stimulation of Human 20S Proteasome in Response to UA, BA, and OA.

20 μM stocks of UA, DMC, and BTZ were prepared by diluting 1 mg in DMSO. 10 μM stocks of BA and OA were prepared by diluting 1 mg in DMSO. 500 μM stocks of DMC, UA, BA, and OA were prepared by further diluting each stock in DMSO. A 2.5 mM stock of BTZ was prepared by diluting the 20 mM stock in DMSO. A 20 mM aliquot of TAS-1 was thawed and diluted to a final concentration of 11.4 μM in 50 mM Tris-HCl pH 7.4 (tris buffer). Master mixes of TAS-1 and each compound were prepared by aliquoting the TAS-1 solution into 1.5 mL eppitubes and adding 4.5 μL of each compound. As a control, 4.5 μL of DMSO was also added to a tube containing the TAS-1 solution. 45 μL of the master mix was added to the wells of a black 96-well plate that was placed on ice. Edge wells were excluded from use. A 50 nM stock of human 20S proteasome was prepared by diluting a 2 mM stock in tris buffer. 5 μL was added quickly to each well. The plate was gently struck to mix then briefly centrifuged. The final concentration of DMC, UA, and BA was 10 μM. The final BTZ concentration was 25 μM. The final 20S concentration was 5 nM. The final volume in the well was 50 μL. The final DMSO concentration in each well was 2%. Fluorescence was measured every two minutes for one hour on a plate reader preheated to 37 °C. The resulting data was graphed with GraphPad Prism software and the slopes of the resulting lines were calculated and graphed as bar graphs as shown in the manuscript body. The experiment was conducted in technical triplicate and experimental duplicate.

Biochemical Assay to Monitor Proteasome Activity in WT and rpn4Δ Lysate

Two days prior the experiment, WT and *rpn4Δ* yeast were streaked on agar plates and grew at 30 °C. Single colonies were then inoculated into 8 mL of YPD media. After overnight growth at 30 °C, yeast were pelleted and media was poured off. Yeast were resuspended in 300 μL of ice-cold lysis buffer (10 mM Tris HCl, 150 mM NaCl and 0.5 mM EDTA with no protease inhibitor). 300 μL of 0.5 mm glass beads were added to the samples. To lyse, seven cycles of vortexing yeast at max speed for 25 seconds followed by incubation on ice for 25 seconds were conducted. Lysate was clarified by centrifuging at 4 °C in a prechilled centrifuge. Lysate was transferred to a new tube and the protein concentration was determined by a BCA assay. A 40 μM stock of suc-LLVY-AMC was prepared by diluting a 20 mM stock in tris buffer. 25 μL of the reporter probe solution was added to the nonedged wells of a black 96-well plate on ice. Next, 2 ng/μL stocks of WT and *rpn4Δ* lysate were prepared by diluting the lysate in tris buffer. 25 μL of lysate was added to each well. The plate was gently struck to mix and briefly spun in a plate centrifuge. The final concentration of suc-LLVY-AMC in each well was 20 μM. The final amount of lysate was 50 μg and the final volume in the well was 50 μL. Fluorescence was measured every 2 minutes for 40 minutes on a plate reader preheated to 30 °C. The data was graphed with GraphPad Prism software the slopes of the resulting lines were calculated and graphed as bar graphs as shown in the manuscript body. The experiment was conducted in technical triplicate and experiment triplicate.

CLS Assay to Monitor Lifespan of Yeast.

Frozen glycerol stocks of haploid WT (BY4741) or the isogenic *rpn4Δ* yeast were plated on YPD agar plates and allowed to grow at 30 °C 4 days before the start of an experiments. Single colonies were inoculated into liquid YPD media and grew overnight at 30°C. Aging media was prepared by autoclaving yeast nitrogen base dissolved in water and adding CSM powder while the liquid was very hot. Immediately before the experiment, a 30% solution of glucose that had been sterile filtered was added to media to a final concentration of 10%. A 30% solution of ammonium sulfate that had been sterile filtered was added to final concentration of 0.5%. The OD_{600} of the yeast cultures was determined by nanodrop and yeast were diluted in CSM aging media such that the final OD_{600} was 0.2. 250μL of yeast was added to wells of a sterile deep well 96-well plate. Each compound was diluted in aging media to achieve the desired concentration in the well and 250μL of each diluted compound was added to wells in replicates of five. The final volume in each well was 500μL and the wells on the edges of the plates were filled with 500μL of sterile water to prevent evaporation of the samples. As a control, five wells of yeast were also dosed with DMSO to a final concentration of 0.2%. Plates were sealed with sterile gas exchange membranes, then a lid was placed on top and sealed with medical tape. Plates were then placed in clean plastic containers with wet paper towels and a secured with a lid to prevent evaporation of the samples. Yeast grew at 30°C for six days. Every day, aliquots of each sample were removed from the stock plate and serially diluted in sterile water (final dilutions of 1:10, 1:100, 1:1,000, and 1:10,000). Each dilution was spotted on YPD agar plates to monitor yeast outgrowth and allowed to incubate at 30°C for two days prior to imaging the plates. Samples of the 1:100 diluted yeast were further diluted in liquid YPD media in a sterile 96-well plate and allowed to grow at 30°C for 18 hours. The OD_{600} of these samples was then measured with a TecanInfinite F200 Pro plate reader system. The values of each replicate were graphed with GraphPad Prism software and analyzed to determine how dosing with proteasome stimulators impacted lifespan. Each CLS experiment was performed in triplicate.

Monitoring Clearance of YFP-Fused α-Synuclein in Yeast Lysate.

To determine the impact of UA, DMC, and MG-132 on clearance of protein aggregates, a strain of yeast expressing YFP-fused α-synuclein (W303 based parent strain) was selected for study. Frozen glycerol stocks of yeast were plated on YPD agar plates and allowed to incubate at 30°C or two days. Single colonies were then inoculated into 8mL of YPD broth and grew overnight at 30°C. The OD_{600} was determined by nanodrop the following morning and yeast were pelleted such that resuspension in 20mL of media would result in and OD_{600} of 0.2. Induction media that would induce the expression of the fluorescent α-synuclein protein was prepared by supplementing SC media with 2% raffinose and 2% galactose to a final concentration of 2%. This was achieved by adding a 20% solution of both sugars to the media that had been previously sterile filtered. Yeast were resuspended in 20mL of induction media and allowed to agitate at 30°C for six hours to induce α-synuclein expression. Yeast were pelleted and resuspended in 50μL of ice-cold lysis buffer, then lysed according to the procedure described above. Protein concentration of the lysate was determined by BCA. Protein concentration was normalized by diluting lysate in phosphate-buffered saline (PBS). 650μL of a 0.75μg/μL stock of lysate was prepared and

100μL was added to the wells of clear 96-well plate in replicates of six to achieve a final lysate amount of 75μg per sample. The plate was sealed with parafilm and incubated for four hours at 30°C. Protein concentration was then re-checked and normalized if necessary (MG-132 lead to a slight accumulation of protein) by diluting lysate in PBS. Samples were then run on SDS PAGE and transferred to a membrane. The membrane was blocked with 5% milk in PBS for 40 minutes at room temperature. An anti-GFP antibody (Novus Biologicals) was diluted 1:1,000 in protein blocking buffer and added to each blot and incubated overnight at 4 °C. The following day, the antibody was collected, and blots were washed 3X with PBS. Next, an anti-rabbit 800 CW antibody (LI-COR) was diluted 1:10,000 in protein blocking buffer and added to the membrane. This incubated for 40 minutes at room temperature, protected from light. The antibody was then collected, and the blots were washed 3X with PBS. Blots were imaged with a LI-COR CLx Odyssey Imaging System. Fluorescence intensities of bands corresponding to the molecular weight of YFP-fused α-synuclein were quantified using ImageStudio software from LI-COR and graphed and analyzed for statistical significance using GraphPad Prism software. Each compound was tested in experimental and technical triplicate.

Please see the Supporting Information for full protocols and experimental procedures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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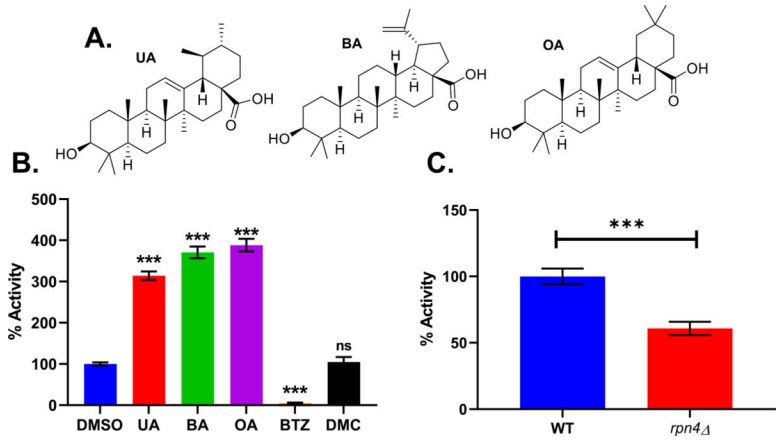

Figure 1.

(**A**) Structures of UA, BA, and OA. (**B**) 5 nM of human proteasome was dosed with 10 μM of UA, BA, OA or DMC or 25 μM BTZ as a control. TAS-1 was added to a final concentration of 10 μM and fluorescence was monitored over one hour. The resulting slopes of the lines produced by cleavage of the probe were calculated, normalized to the DMSO control, and graphed. UA and all its derivatives stimulated the purified proteasome to a similar extent. Conversely, DMC did not stimulate. BTZ was successful in inhibiting the proteasome. This experiment was repeated in experimental duplicate and technical triplicate. (**C**) Proteasome activity of WT and *rpn4Δ* yeast strains was evaluated by dosing 50 μg of lysate with 20 μM of the commercially available suc-LLVY-AMC proteasome probe. Fluorescence was monitored over one hour. The change in RFU per minute was calculated, normalized to the WT strain, and graphed. This revealed significantly reduced proteasome activity in the *rpn4Δ* strain, which was expected since this strain is knocked down for proteasome subunit expression. The plotted data represents the combination of experimental triplicate and technical triplicate. A column analysis T-test (Prism v9.0) was used was used to determine significant changes as compared to the DMSO (**B)** control or wild-type (**C**). * $p<0.05$, ** $p<0.01$, *** $p<0.001$

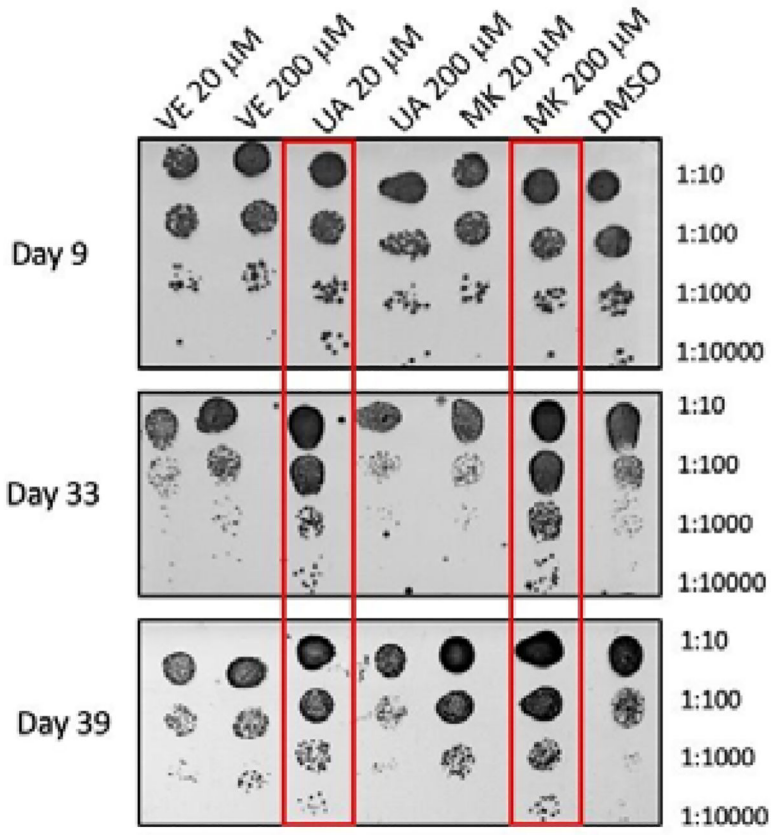

Figure 2. Establishing Conditions for CLS Assay with a Drug Transporter Deficient Yeast Strain.

A CLS assay was conducted after dosing yeast with various proteasomal stimulators. Every 7 days until day 21 samples were spotted on agar plates. Then the frequency of spotting was increased to every 3 days to efficiently monitor aging. The 20 μM ursolic acid (UA) and 200 μM MK886 (MK) prolonged the lifespan of *Δ8* yeast (highlighted by a red box), while vitamin E succinate (VE) did not. Dosing with these small molecules resulted in better outgrowth of higher dilutions of yeast compared to the DMSO-dosed control after 39 days.

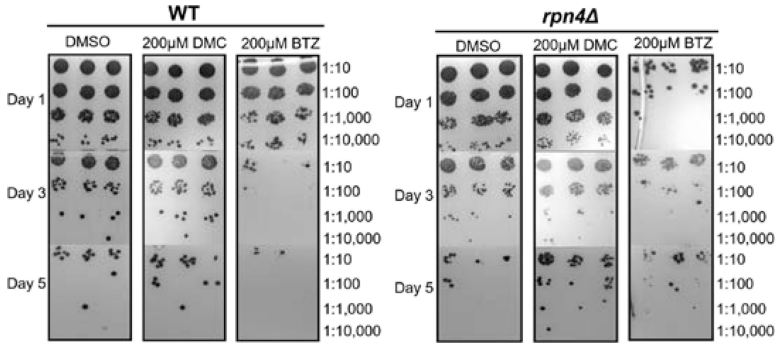

Figure 3. DMC and BTZ Successfully Prolongs or Reduce Yeast Lifespan.

A CLS assay was carried out in which yeast were dosed with DMSO, DMC, or BTZ as previously described. DMC had a neglible impact on prolonged lifespan of the WT strain, but had a bigger impact on the *rpn4Δ* strain as evidenced by better outgrowth of more dilute yeast compared to the DMSO control. Likewise, BTZ greatly reduced the outgrowth of both yeast strains, reflecting a shortening in CLS. This experiment solidified our CLS assay as a valid way to monitor yeast lifespan and established the controls used in the remaining experiments.

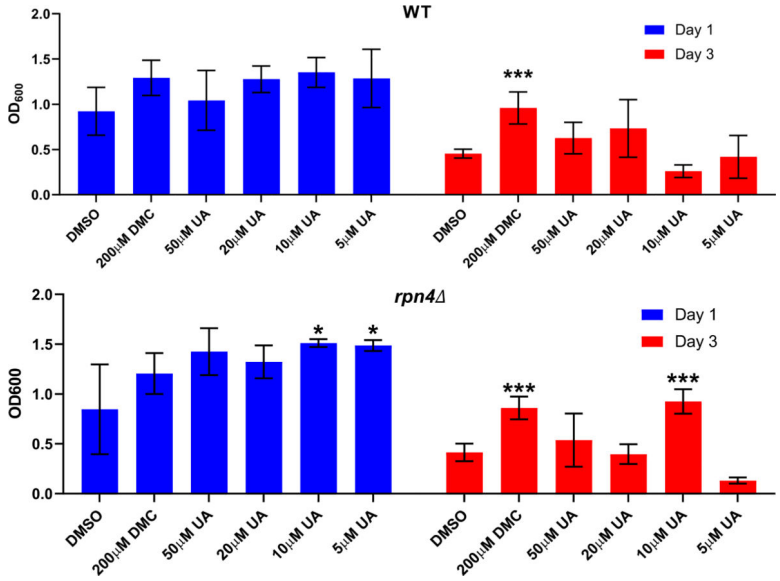

Figure 4. *rpn4Δ* CLS is Extended by Dosing Yeast with UA.

Every day, samples of yeast dosed with varying concentrations of UA were diluted 1:100 in sterile water. This sample was diluted 1:100 again in YPD media and allowed to grow for 18 hours, then the OD_{600} was measured and graphed. DMC successfully increased the OD_{600} of the WT strain for the first few days of the experiment. None of the concentrations of UA tested resulted in significant enhancement of WT OD_{600}, suggesting little impact on the CLS of WT yeast. However, dosage of *rpn4Δ* yeast with 10 μM UA significantly increased the OD_{600} during the first days of the experiment. The increase was similar to that of dosing with 200 μM DMC. This data reveals that dosing yeast with compromised proteasome activity with a stimulator may provide a novel way to reverse or delay the onset of aging. A column analysis T-test (Prism 9.0v) was used was used to determine significance between the DMSO control and each compound for each day.

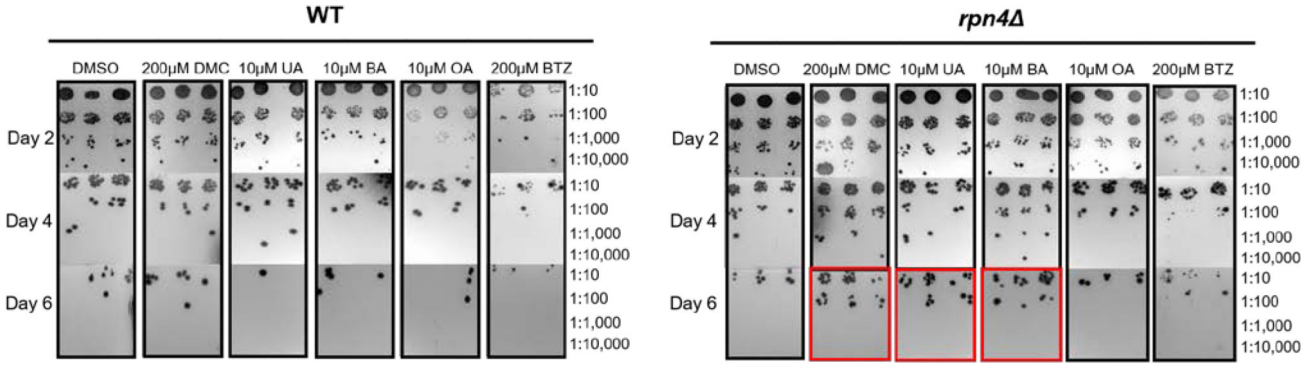

Figure 5. UA Derivatives Prolong the Lifespan of Yeast with Reduced Proteasome Activity.A CLS assay was conducted as described above and samples of yeast were diluted in sterile water. Yeast were dosed with each compound and diluted sterile water. Samples from each dilution were spotted on agar plates and outgrowth was monitored. Outgrowth in response to dosage with all compounds was compared to the DMSO control. None of the compounds, including DMC prolonged the lifespan of the WT yeast, as outgrowth of yeast at each dilution is similar to that of dosage with DMSO. As expected, the BTZ was successful in shortening lifespan, as outgrowth was almost completely inhibited four days after dosing. Both UA and BA were successful in prolonging the lifespan of *rpn4Δ* yeast, as evidenced by better outgrowth of lower dilution yeast compared to the DMSO control. The extent of outgrowth was similar to that of DMC, the positive control. OA, however, did not impact the outgrowth of the yeast. As expected BTZ reduced yeast lifespan, as evidenced by reduced outgrowth four days after dosing.

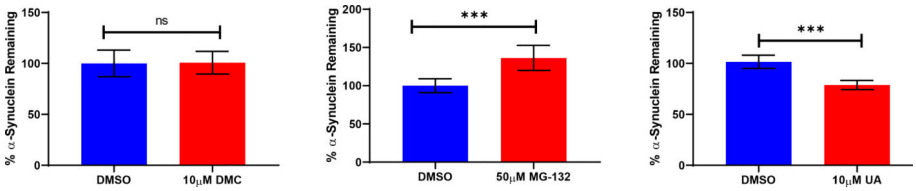

Figure 6. UA Aids the Proteasome in Clearing α-Synuclein.

Lysate from yeast induced to express YFP-fused α-synuclein was subjected to western blot analysis after dosage with each compound. The fluorescence intensities of the bands corresponding to molecular weight of YFP-fused α-synuclein were quantified and graphed as shown above. DMC did not significantly alter levels of α-synuclein, as expected since this compound acts through promoting autophagy which does not fully function in lysate. Dosage with MG-132 lead to an increase in α-synuclein, indicating the proteasome was unable to effectively clear the protein. Conversely, dosing with UA resulted in reduction of α-synuclein, revealing that chemical stimulation of the proteasome could be a viable strategy to reduce the impact of protein accumulation on the cell. A student's T-test was used to determine significance.