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ORIGINAL RESEARCH ARTICLE

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Polyphenolic flavonoid (Myricetin) upregulated proteasomal degradation mechanisms: Eliminates neurodegenerative proteins aggregation

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Abstract

Major neurodegenerative disorders are characterized by the formation of misfolded proteins aggregates inside or outside the neuronal cells. Previous studies suggest that aberrant proteins aggregates play a critical role in protein homeostasis imbalance and failure of protein quality control (PQC) mechanism, leading to disease conditions. However, we still do not understand the precise mechanisms of PQC failure and cellular dysfunctions associated with neurodegenerative diseases caused by the accumulation of protein aggregates. Here, we show that Myricetin, a flavonoid, can eliminate various abnormal proteins from the cellular environment via modulating endogenous levels of Hsp70 chaperone and quality control (QC)-E3 ubiquitin ligase E6-AP. We have observed that Myricetin treatment suppresses the aggregation of different aberrant proteins. Myricetin also enhances the elimination of various toxic neurodegenerative diseases associated proteins from the cells, which could be reversed by the addition of putative proteasome inhibitor (MG132). Remarkably, Myricetin can also stabilize E6-AP and reduce the misfolded proteins inclusions, which further alleviates cytotoxicity. Taken together these findings suggested that new mechanistic and therapeutic insights based on small molecules mediated regulation of disturbed protein quality control mechanism, which may result in the maintenance of the state of proteostasis.

KEYWORDS

chaperone, E6-AP, misfolded proteins, Myricetin, neurodegeneration, proteasome

1 | INTRODUCTION

Presence of abnormal or misfolded proteins assemblies is a major clinical hallmark of neurodegenerative diseases and ageing-associated disorders (David, 2012; Ross & Poirier, 2004). Intracellular accumulation of misfolded proteins also represents a possible failure of protein quality control (PQC) mechanisms (Chhangani & Mishra, 2013; Goldberg, 2003). Previous reports indicate that the accumulation of aberrant proteins suppresses the functions of PQC machinery, but the precise molecular pathomechanism responsible for the lack of functions of protein degradation machinery is not clear (Bence, Sampat, & Kopito, 2001; McNaught, Perl, Brownell, & Olanow, 2004). Major function of chaperones is to fold various proteins into their native structures; therefore, they can accomplish designated conformational flexibility based functions (Craig, Weissman, & Horwich, 1994; Hartl, Bracher, & Hayer-Hartl, 2011). Overall, these efforts establish the stringent functional integrity of the proteome to face the routine course of chronic cellular stress conditions (Hightower, 1991; Kültz, 2005). Cells always try to maintain the state of proteostasis and its failure may lead to the progression of several

neurodegenerative diseases and protein conformational disorders (Amanullah et al., 2017).

Earlier findings indicate that heat-shock factor-1 (HSF-1)-regulated pathways play critical roles in the reestablishment of deregulated proteostasis to cope up from proteotoxic-stress conditions inside the cells (Anckar & Sistonen, 2007; Dai & Sampson, 2016). HSF-1, the transcription factor of many chaperones including Hsp70 has been explored for targeting misfolded proteins in neurodegenerative disorders (Heller, 2010; Neef, Jaeger, & Thiele, 2011). Huntington's disease mouse model R6/2, when crossed with transgenic mice expressing active HSF-1, showed efficient suppression of the formation of the polyglutamine inclusions and prolonged life span (Fujimoto et al., 2005). Further studies observed that the activation of HSF-1 causes an increased expression of Hsp70 or Hsp40; this coexpression of two chaperones finally results in suppression of aggregates formed by α-synuclein and polyQ proteins (Muchowski et al., 2000; Neef et al., 2011).

Similar results were also obtained on activation of HSF-1 by 17-(allylamino)-17-demethoxygeldanamycin (17AAG) in *Drosophila* model of spinocerebellar ataxia (Fujikake et al., 2008). It has also been reported that HSF-1 degradation by NEDD4, an E3 ubiquitin ligase, results in enhanced α-synuclein aggregates mediated toxicity (Kim et al., 2016). Further, the role of HSF-1 was also explored in upregulating the Hsp70 expression under stress conditions generated by proteasome inhibition or downregulation of the proteolytic pathway (Pirkkala, Alastalo, Zuo, Benjamin, & Sistonen, 2000). So far various modulators of HSF-1 have been identified to target this master regulator of molecular chaperones to explore its potential as a therapeutic agent in multiple neurodegenerative diseases (Mu et al., 2008; Neef et al., 2011). All these findings suggest that the search of new natural or pharmacological compounds based enhancement of PQC functions can open new promising approaches for the therapeutic treatment of numerous diseases linked with the aberrant proteins accumulation and loss of proteostasis.

Flavonoids are plant's secondary metabolites synthesized from primary metabolites those functions to establish the interaction with the environment and to provide the strength of survival under extreme conditions (Kliebenstein, 2013; Yang et al., 2018). The genetic, ontogenetic, morphogenetic, other biotic and abiotic factors that influence the biosynthesis and accumulation of plant secondary metabolites majorly include transcription factors, pathogens or parasites infection, temperature, light, availability of water, chemicals, and many more (Verma & Shukla, 2015; Yang et al., 2018). Different classes of plant secondary metabolites have been identified that include flavonoids, coumarins, tannins, alkaloids, terpenes, glucosinolates, and polyketides which have been explored for their biological roles and pharmaceutical significance (Hartmann, 2007). Flavonoids are the group of secondary metabolites having phenylbenzopyran or C_6–C_3–C_6 structure, which primarily give pigmentation to flowers, fruits, and seeds (Bohm, 1998; Yang et al., 2018). The ubiquitously present flavonoids are divided in different classes namely flavones, flavonols, flavanones, flavanonol, isoflavones, anthocyanidin, chalcone, and flavan-3-ol, grouped on the basis of different oxidation level and C ring substitution pattern (Falcone Ferreyra, Rius, & Casati, 2012; Kumar & Pandey, 2013). Synthesis of these benzo-γ-pyrone containing polyphenolic flavonoids is initiated by coumaroyl-CoA and malonyl-CoA which yield naringenin chalcone in the end, under tight regulation of multiple enzymes (Hahlbrock & Grisebach, 1979; Quattrocchio, Baudry, Lepiniec, & Grotewold, 2006). The medical importance of flavonoids in complex diseases like cancer, cardiovascular diseases, neurodegeneration, and bacterial or viral infections highlight their significance and also further research is needed to obtain maximum therapeutic advantages (Havsteen, 2002; Spencer, Abd-el-Mohsen, & Rice-Evans, 2004).

Myricetin (3,3′,4′,5,5′,7-hexahydroxyflavone) is the flavonoid of subclass flavonols, which has been well explored for its bioactivities like pro/antioxidant property, antiviral/bacterial, antidiabetic activities, and DNA degradation induction abilities under in vivo or in vitro conditions (el-Gammal & Mansour, 1986; Ong & Khoo, 1997). Myricetin was first extracted as yellow color crystals from the bark of *Myrica nagi* and was reported to have dyeing properties; further, in the year 1924, the method of its synthesis was given by Jan Kalff and Robert Robinson (Kalff & Robinson, 1925; Perkin & Hummel, 1896). Later, it has been reported that Myricetin and its structurally related compounds are metabolized by the intestinal microflora under in vitro conditions (Griffiths & Smith, 1972). In last two decades, Myricetin has been widely explored for its therapeutic potential in complex disorders like diabetes, cancer, neurodegeneration, as well as for antiageing properties (Buchter et al., 2013; Ma, Wang, Jiang, Liu, & Xie, 2007aa; Maggiolini et al., 2005; Ong & Khoo, 2000).

In previous studies, neuroprotective role of Myricetin was thought to be due to its antioxidant properties, which helps in reduction of oxidative stress generated during multiple brain pathologies (Barzegar, 2016; Dajas et al., 2003). Here, in present study, we reported that Myricetin treatment induces the cellular levels of Hsp70 chaperone and QC E3 ubiquitin ligase E6-AP and most importantly it also decrease the aggregation of crucial misfolded proteins. In addition, our study also suggests that Myricetin treatment enhances the clearance of neurodegeneration-linked proteins that could be reversed by the addition of proteasome inhibitor. Interestingly, use of Myricetin also stabilizes the endogenous levels of E6-AP protein. These results suggest that small molecules based modulation of PQC mechanism at different levels can improve the survival of cells under proteotoxic-stress conditions engendered by the accumulation of aggregate-prone proteins.

2 | MATERIALS AND METHODS

2.1 | Materials

Myricetin, tunicamycin, cycloheximide, dimethyl sulfoxide (DMSO), MG132, chloroquine, and all other cell culture reagents were purchased from Sigma-Aldrich Corp. (St. Louis, MO). Antifade 4′, 6-diamidino-2-phenylindole (DAPI), Lipofectamine®2000, and Opti-MEM were procured from Life Technologies (Carlsbad, CA). Primary antibodies of E6-AP, Hsp70, HSF-1, ubiquitin, luciferase, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Antirabbit and antimouse secondary antibodies conjugated with horseradish peroxidase (HRP), fluorescein isothiocyanate, and rhodamines have been obtained from Vector Laboratories (Burlingame, CA). Dual Luciferase® Reporter Gene Assay kit was procured from Promega (Madison, WI). pF141 pAcGFP1 SOD1WT (Addgene-26402), pF148 pSOD1G37RAcGFP1 (Addgene-26409), pAAV asyn WT (Addgene-36055), pAAV asyn S87A (Addgene-36056), luciferase-pcDNA3 (Addgene-18964), pEGFP-C1-Ataxin3Q28Q (Addgene-22122), and pEGFP-C1-Ataxin3Q84Q (Addgene-22123) plasmids were procured from Addgene (Cambridge, MA).

2.2 | Cell culture and plasmids transfection

Dulbecco's modified Eagle's medium (DMEM) supplemented with 1X antibiotic antimycotic (Gibco Thermo Fisher Scientific, Waltham, MA), and 10% heat-denatured fetal bovine serum was used to culture kidney derived fibroblast like Cos-7 and lung epithelial carcinoma A549 cells. The optimal culture conditions of 37°C and 5% CO_2 were given to cells. For experiments, cells were either plated into six-well tissue culture plates or two-well chamber slides. On appropriate confluency, cells were transfected with desired plasmids with Lipofectamine®2000 according to given manufacturer's protocol. Transfection efficiency of Cos-7 cells was about 80–90% and 60–70% for A549 cells.

2.3 | Bright field images, immunofluorescence staining, MTT analysis, and aggregate counting

A549 cells were grown into six-well tissue culture plates and given a treatment of MG132, chloroquine, and tunicamycin. Posttreatment images were obtained with bright field microscope. Cells were grown on 96-well plates and were exposed to different stress-inducing agent. After treatment, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reagent (5 mg/ml in phosphate-buffered saline [PBS]) is added in a ratio 1:10 with media. After giving an incubation of 4 hours in CO_2 incubator at 37°C, media was removed, and 100 µl of acidic isopropanol was added for lysis. Formazan particles were formed after mixing the plates, and absorbance at 595 nm was measured via microplate reader. Experiments were conducted in triplicated and results were presented as percent of the control. For immunofluorescence staining, cells were plated into two-well chamber slides at appropriate density. After transfection with appropriate plasmids or drug doses, cells were rinsed with PBS two times. Cells fixing was performed with paraformaldehyde (4% in PBS) for 30 min and then rinsed with PBS two times. Permeabilization of cells was done by 0.5% Triton X-100 for the duration of 5 min and then again rinsed three times with PBS. Standard 2% horse serum in PBS was used for 30 min blocking. Appropriate primary antibody (1:200 dilution) was used for overnight incubation at 4°C and then given 4 hr incubation at room temperature (RT) with the rhodamine-conjugated secondary antibody. Mounting of slides was performed by antifade with DAPI that gives stain to the nucleus. Slides were viewed for images under a fluorescence microscope and similar slides were also used to count aggregates manually under a microscope (approximately 400 transfected cells in each set). Because of counting, more than one aggregate in a single cell was considered as one big inclusion.

2.4 | Western blot analysis, cycloheximide chase, and statistical analysis

Six-well tissue culture plates were seeded with Cos-7 cells for western blot analysis. Posttransfection cells treated as per the requirement, cells were rinsed with PBS and lysates were prepared by centrifugation. Samples were sonicated after adding sample buffer and run in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Bio-Rad Laboratories, Hercules, CA) to separate them according to their molecular weights. Proteins were then transferred to nitrocellulose membrane and blocked for 45 min at room temperature with 5% skimmed milk (in tris-buffered saline and Tween 20 [TBST]). For primary antibody (1:500 diluted in TBST) incubation, membranes were kept overnight (at 4°C). Next day, cells were rinsed with TBST three times and incubated with HRP-conjugated secondary antibody in a dilution of 1:10,000 for 45 min at RT. After several TBST washes luminescence signals were detected by Luminata Crescendo Western HRP substrate (EMD Milipore, MA). In cycloheximide chase experiment, cells after treatment with Myricetin, were exposed to cycloheximide (15 µg/ml) for different time intervals, up to 20 hr. On completion of incubation, western blot analysis was done by E6-AP and β-actin primary antibodies. Statistical analysis in all experiments was done by Student's *t*-test, with $p < 0.05$ statistical significance and the values represent the mean ± standard deviation (*SD*) in all experiments.

2.5 | Reporter gene assay and docking studies

Cells were transfected with luciferase plasmid and treated with Myricetin under normal 5% CO_2 condition. For 30 min, plates were kept at 43°C and then again kept at 37°C in CO_2 incubator for 2 hr recovery period. Collected samples from these cells were used for dual luciferase reporter gene assay performed according to the manufacturer's (Promega, Madison, WI) given protocol. For docking studies, E6-AP HECT catalytic domain structure was taken from protein databank ID: 1D5F. Similarly, the structure of Myricetin was obtained from PDB ID: 2O63. Autodock vina was used for docking study (Trott & Olson, 2010) with grid dimensions of 56 Å × 64 Å × 111 Å was made that included entire Homologous to the E6-AP Carboxyl Terminus (HECT) catalytic domain centered on 91.019, 90.02, and 49.614 coordinates. The depicted image in the figure was generated using Chimera tool (Pettersen et al., 2004).

3 | RESULTS

3.1 | Myricetin treatment enhances the endogenous levels of protein quality control components

The mechanisms underlying misfolded proteins-related proteotoxicity and the interrelationships between the aggregation of abnormal

proteins, their degradation, and the establishment of neurodegeneration remain poorly understood. How intervention of small molecules in these mechanisms can improve the overall functionality of PQC mechanism linked with the refolding, ubiquitination, and degradation of misfolded proteins is a challenging problem. To answer this question, in our preliminary experiments we treated cells with Myricetin and then checked the endogenous levels of various molecular chaperones and QC-E3 ubiquitin ligases. As shown in immunofluorescence analysis, we found that endogenous levels of E6-AP, a putative QC E3 ubiquitin ligase and molecular chaperone Hsp70 get elevated in response to Myricetin treatment in cells (Figure 1a).

The increased expression of Hsp70 and E6-AP bound us to check the endogenous levels of HSF-1 transcription factor, as it has previously

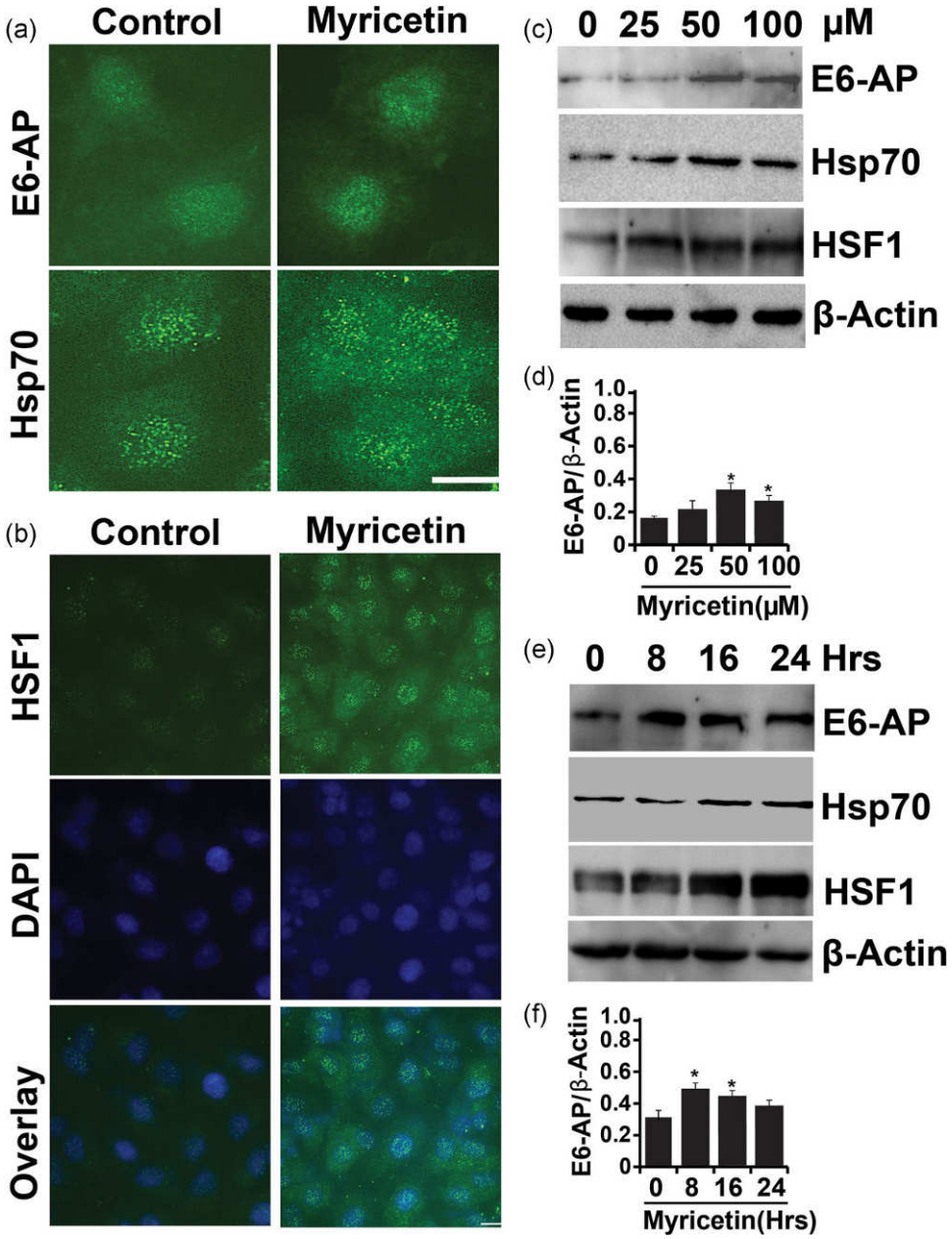
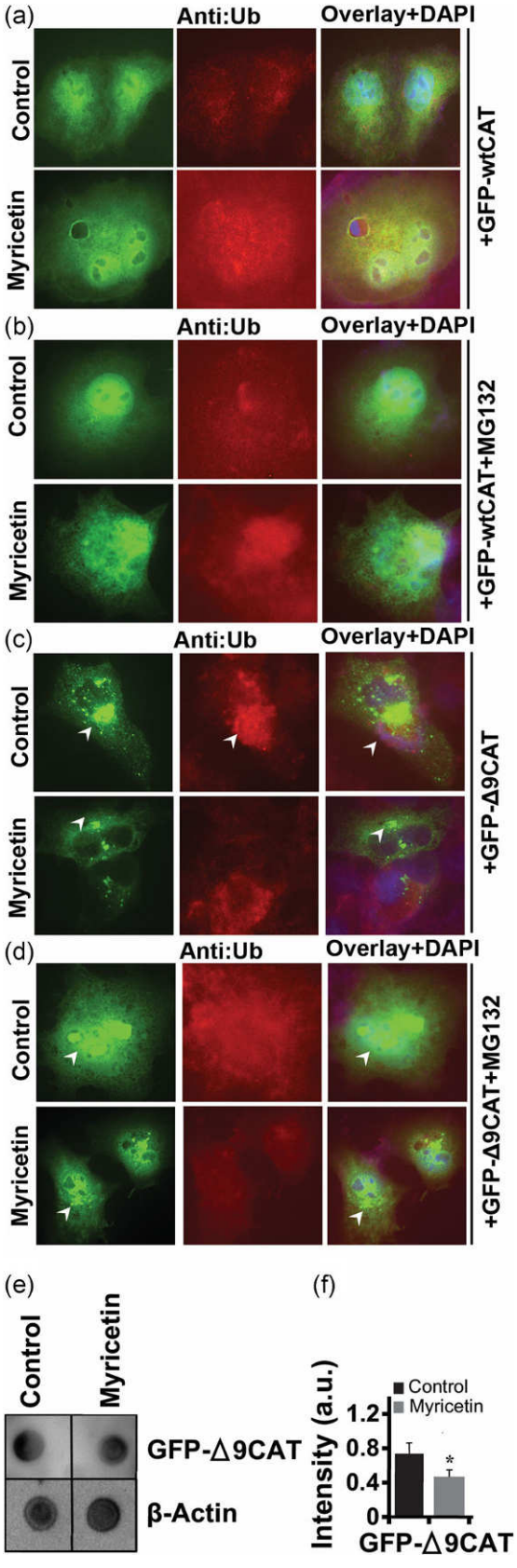


FIGURE 1 Myricetin elevated endogenous levels of QC E3 ubiquitin ligase E6-AP, transcription factor HSF-1, and chaperone Hsp70. (a) Cos-7 cells were seeded into chamber slides (two-well) and processed for immunofluorescence staining with primary antibodies of E6-AP and Hsp70 in presence or absence of Myricetin 20 μM for 24 hr. (b) Upstream regulator of heat-shock proteins, HSF1, was also probed with the anti-HSF1 antibody. The nucleus was stained with antifade DAPI. (c) Cells seeded into six-well tissue culture plates were exposed with Myricetin in concentration dependent manner as 0, 25, 50, and 100 μM for 24 hr. Expression levels of E6-AP, Hsp70, and HSF1 were analyzed by western blot analysis using whole cell lysates, collected from these cells using β-actin as loading control. (d) Quantification of bands in (c) was performed using NIH image analysis software. (e) Myricetin (20 μM) was given to Cos-7 cells at different time intervals of 0, 8, 16, and 24 hr and cell lysates were used for E6-AP, Hsp70, and HSF1 expression level analysis by western blot analysis. (f) NIH image analysis software was used for quantification of bands in (e). *$p < 0.05$ in comparison with the control. DAPI: 4′,6-diamidino-2-phenylindole; HSF1: heat-shock factor-1 [Color figure can be viewed at wileyonlinelibrary.com]

been observed that the 5′-untranslated region of *E6-AP* gene retains numerous consensus HSF-1 binding sequences (Mishra, Godavarthi, Maheshwari, Goswami, & Jana, 2009). Immunofluorescence analysis of HSF-1, after the treatment of Myricetin, clearly indicated the enhanced expression of HSF-1 in treated cells (Figure 1b). To further confirm these preliminary results, we again exposed the cells with Myricetin in both concentration (Figure 1c), time dependent (Figure 1e) manner. As depicted in Figure 1c,e, immunoblot analysis represents the increased levels of E6-AP, Hsp70, and HSF-1 under the concentration and time dependent effect of the Myricetin treatment. These data indicate that treatment of Myricetin can induce the expression of HSF-1, Hsp70, and E6-AP inside the cells.



3.2 | Exposure of myricetin suppresses the aggregation of misfolded proteins

To determine whether the induced expression of heat-shock protein and QC-E3 ubiquitin ligase based on the Myricetin treatment can suppress the aggregation of misfolded proteins, we have used green fluorescence protein (GFP) fusions of wild-type chloramphenicol-acetyltransferase (wtCAT) and mutant GFP-Δ9CAT proteins in our experiments (Arslan, Chikina, Csermely, & Soti, 2012; Chhangani, Upadhyay, Amanullah, Joshi, & Mishra, 2014). Mutant GFP-Δ9CAT forms intracellular misfolded proteinaceous inclusion bodies; however wild-type GFP-wtCAT remains diffusely distributed inside the cells. Transient transfection was performed with GFP-wtCAT and GFP-Δ9CAT plasmids, posttransfected cells were treated with or without Myricetin in the presence or absence of MG132 a putative proteasome inhibitor.

Treated cells were prepared for detailed immunofluorescence analysis; and were probed with ubiquitin antibody. As shown in Figure 2a, overexpression of wild-type GFP-wtCAT diffusely localized in the cells and accumulated in the form of inclusions after the MG132 treatment (Figure 2b). The effect of Myricetin on the

FIGURE 2 Enhanced clearance of model misfolded protein aggregates after treatment of myricetin. (a,b) Cos-7 cells grown in two-well chamber slides and subsequently transfected with the GFP-wtCAT construct. Posttransfection, cells were given a treatment of DMSO and Myricetin 10 μM for 48 hr with or without MG132 10 μM for 12 hr and processed for immunocytochemistry with anti-ubiquitin antibody. (c,d) Cells, transfected with GFP-Δ9CAT, were also treated with Myricetin in presence and absence MG132 10 μM for 12 hr. Posttreatment, immunostaining was done using ubiquitin as a primary antibody and with rhodamine-conjugated secondary antibody for microscopic analysis of cells. Fluorescence microscope was used to obtain images and DAPI was used for nuclear localization and overlay. Arrowheads indicate the colocalization of ubiquitin with GFP-Δ9CAT aggregates at perinuclear space in the cytoplasm. Scale bar 20 μm. (e,f) Cos-7 cells, seeded into six-well tissue culture dishes, transfected with GFP-Δ9CAT plasmid with or without Myricetin (10 μM for 48 hr). Posttreatment samples were collected and subjected to filter trap assay with primary antibodies of anti-GFP and β-actin. Quantification of dots intensity was obtained via NIH image analysis software. In comparison with control $^*p < 0.05$. DAPI, 4′,6-diamidino-2-phenylindole; DMSO; dimethyl sulfoxide [Color figure can be viewed at wileyonlinelibrary.com]

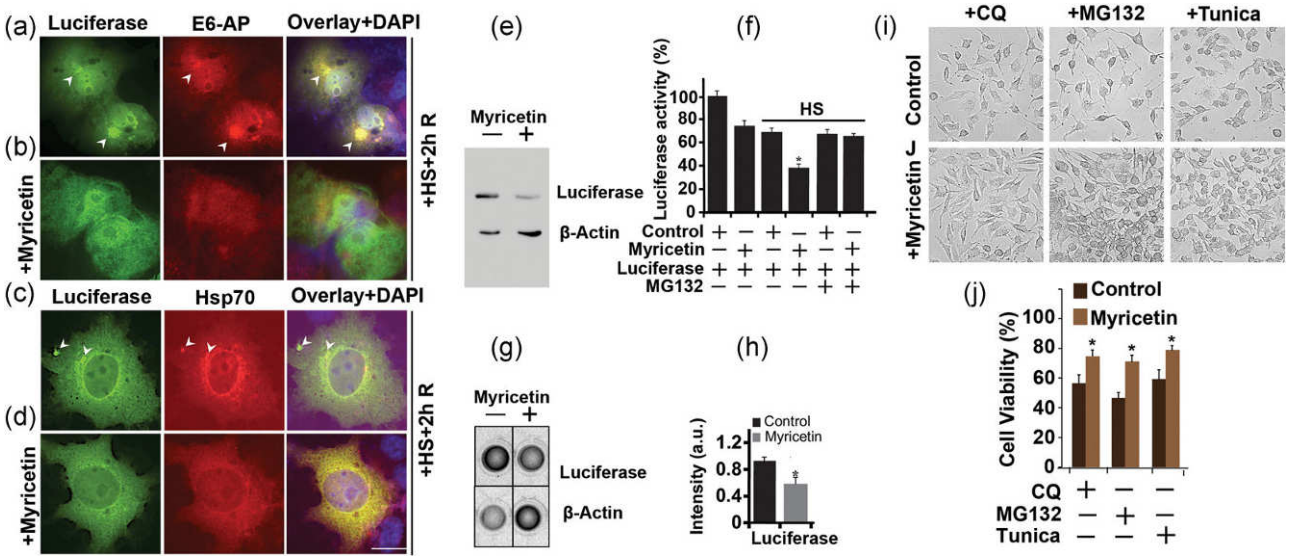


FIGURE 3 Myricetin suppresses the aggregation of heat-denatured luciferase and reduces cytotoxicity under stress conditions. (a–d) Cells seeded in chamber slides were transfected with constructs of firefly luciferase and treated with or without Myricetin 10 μM for 48 hr. Posttreatment, cells were given 30 min heat-shock at 43°C, followed by a recovery period of 2 hr at 37°C and then further used for immunofluorescence staining with primary antibodies of luciferase, E6-AP, and Hsp70. FITC conjugated secondary (green) antibody was used for luciferase, whereas, E6-AP and Hsp70 were stained using rhodamine conjugated (red) secondary antibody. Arrowheads indicate the colocalization of E6-AP and Hsp70 with luciferase aggregates respectively in (a,c). (e) Similar set of cells, transfected with luciferase and administered with or without Myricetin 30 μM for 24 hr, were collected and subjected for western blot analysis with luciferase antibody. β-actin was used as loading control. (f) Luciferase activity assay was also performed according the give manufacturer's protocol in cells treated with DMSO and Myricetin. (g,h) Similar set of cells as used for western blot analysis also subjected for direct measurement of luminescence by dot blot and graph was generated for quantitative analysis. (i) A549 cells were plated into six-well tissue culture plates and treated with chloroquine (20 μM for 10 hr), MG132 (10 μM for 12 hr), and tunicamycin (10 μg/ml for 12 hr) with or without Myricetin 10 μM for 48 hr and bright field images were obtained to visualize the effects of Myricetin on cellular morphology under stress conditions. (j) Cell viability was also calculated among different stress conditions by MTT analysis. DAPI: 4′,6-diamidino-2-phenylindole; DMSO: dimethyl sulfoxide; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide [Color figure can be viewed at wileyonlinelibrary.com]

aggregates of overexpressed mutant GFP-Δ9CAT proteins was shown in Figure 2c. Treatment of Myricetin significantly suppresses the aggregation of misfolded GFP-Δ9CAT mutant protein, which can be reverted by the addition of proteasome inhibitor MG132, as represented in Figure 2d. Analysis by immunofluorescence microscopy revealed that treatment of Myricetin was able to reduce the accumulation of large ubiquitin-positive perinuclear misfolded inclusions of mutant GFP-Δ9CAT protein and the effect of Myricetin can be overcome by the addition of MG132 proteasome inhibitor. Above findings were also confirmed by filter trap assay as shown in Figure 2e,f that there is less aggregates formation on treatment of Myricetin.

3.3 | Myricetin reduces aggregation of misfolded luciferase proteins and protects stress-induced cell death

In this study, for the first time, we observed that use of Myricetin reduces the aggregation of misfolded proteins and most likely mediates the aggregate clearance via proteasome pathway. To further ascertain the effects of Myricetin on misfolded proteins, we used thermally denatured misfolded protein based study. After transfection of intracellular expressing luciferase constructs, cells were exposed with Myricetin and kept at 43°C for 30 min and then provided a 2 hr recovery period at 37°C. Thereafter, cells were subjected to immunofluorescence microscopy analysis by using luciferase and E6-AP (Figure 3a,b) or Hsp70 (Figure 3c,d) antibodies in different experimental sets. The results obtained from immunofluorescence staining clarify that Myricetin reduces the aggregation of heat-denatured luciferase perinuclear bodies.

Further, we performed immunoblot analysis on similar set of cells (Figure 3e), luciferase activity assays according to manufacturer's protocol (Figure 3f). Both results support the previous observations that most likely, Myricetin triggers a cytoprotective response in cells via inducing the intracellular degradation of various misfolded proteins. Our above results likely present the cytoprotective proficiency of Myricetin against aberrant proteins. To further confirm these results, we check the luminescence of above described samples in the presence and absence of Myricetin by dot blot quantitative analysis and further confirmed that Myricetin treatment reduces misfolded proteins (Figure 3g,h). But, to test the influence of Myricetin on cellular protection under various stress conditions, we treated cells with different stress-inducing agents. Chloroquine (CQ) for autophagic dysfunction, MG132 for proteasomal inhibition and tunicamycin for ER-dysfunctions and some cells were also exposed with Myricetin. As shown in Figure 3i,j, (MTT assay) use of Myricetin

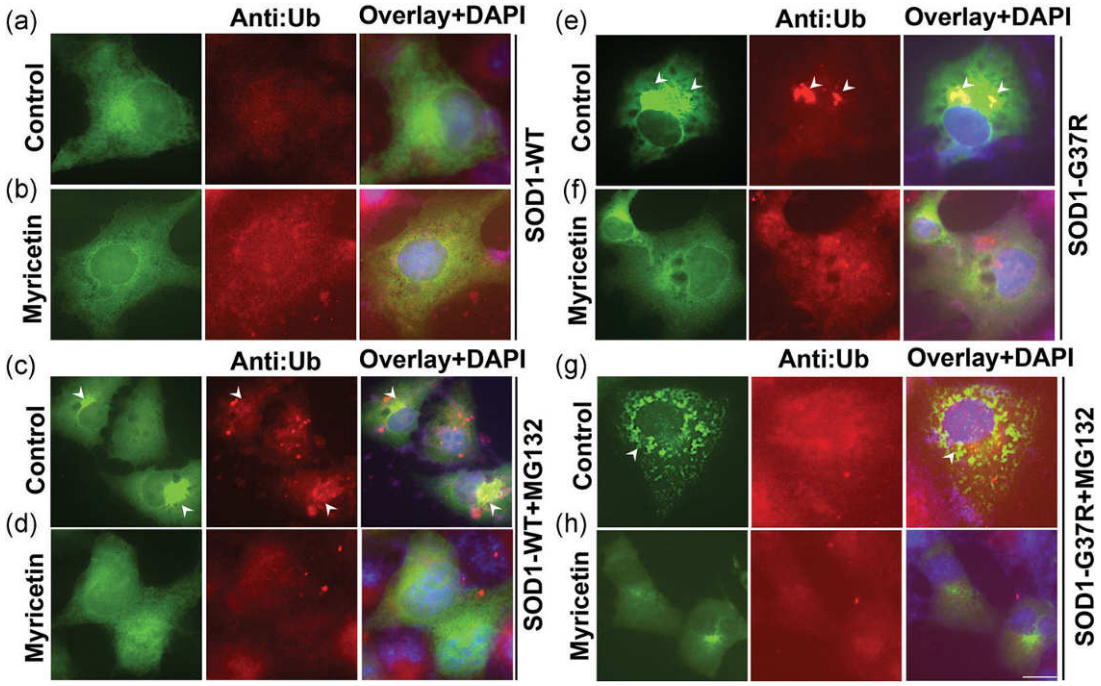


FIGURE 4 Myricetin reduces the intracellular accumulation of mutant SOD1 inclusions. (a–d) Immunocytochemistry analysis of Cos-7 cells, transfected with SOD1-WT constructs with or without Myricetin 10 μM for 48 hr, was performed. Few sets of cells were also given a treatment of MG132 10 μM for 12 hr. Ubiquitin primary antibody was used with rhodamine conjugated secondary antibody for detecting the localization of the ubiquitinated protein fraction in the cytoplasm and DAPI was used as a nuclear stain. (e–h) SOD1-G37R constructs transfected cells were also analyzed after Myricetin treatment with or without MG132 and stained for visualization of ubiquitin colocalization with aggregates. Arrowheads show the presence of highly accumulated protein aggregates. Scale bar 20 μm. DAPI: 4′,6-diamidino-2-phenylindole; SOD1: superoxide dismutase-1 [Color figure can be viewed at wileyonlinelibrary.com]

significantly improves the survival of cells compared with control (DMSO treated) cells under similar stress conditions. Together these observations suggested that most probably, Myricetin alleviates cytotoxicity and provides protection due to the enhanced elimination of misfolded proteins, which could be over accumulated during different stress conditions.

3.4 | Aggregation inhibitory effects of myricetin on intracellular mutant SOD-1 inclusions

Although the precise mechanism, by which Myricetin can inhibit the accumulation of misfolded proteins is not still clear, yet our current observations provide some intriguing clues linked with elevation of PQC components. To further determine the function of Myricetin against aberrant proteins accumulation, next, we used neurodegenerative disease, Amyotrophic Lateral Sclerosis (ALS) associated mutant superoxide dismutase-1 (SOD-1) construct. Cells were transfected with both wild-type SOD1 (SOD1-WT) and mutant SOD1 (SOD1-G37R) plasmids as a ALS cellular model in the absence or presence of Myricetin.

To assess the efficacy of Myricetin in the clearance of neurodegeneration-linked protein, we performed immunofluorescence staining, as described in above experimental set of cells. In this cellular model, we observed that wild-type SOD1 (SOD1-WT) does not exhibit profound aggregate formation (Figure 4a,b) but the inhibition of proteasome leads to overaccumulation of expressed SOD1-WT proteins and produce perinuclear aggresome (Figure 4c,d). Expression of mutant SOD1 (SOD1-G37R) protein also forms clear aggresome-like structures in cells; and exposure of Myricetin decreases the intracellular aggregation of ubiquitin-positive mutant SOD1 (SOD1-G37R) proteins (Figure 4e,f). Addition of MG132 up to some extent reduces the antiaggregation effect of Myricetin in the cells (Figure 4g,h). Overall, these results suggest that Myricetin improves the clearance of misfolded SOD1 aggregates from the cells, which may also contribute to the survival of cells under proteotoxic disturbances.

3.5 | Inhibition of mutant α-synuclein intracellular aggregation by myricetin

Interestingly, during the experiments with nonpathogenic modeled misfolded proteins, we observed that Myricetin suppresses their aggregation. Consequently, we examined that treatment of Myricetin alleviates cytotoxic effects of various stress conditions and also reduce the aggregation of mutant SOD1 protein associated with ALS neurodegenerative disease. Next, it was necessary for us to further verify the aggregation inhibitory effect of Myricetin on another neurodegenerative disease (Parkinson's diseases)-linked proteins. For this, we used mutant α-Synuclein (S87A) proteins; and cells were transfected with both normal and mutant (S87A) encoding

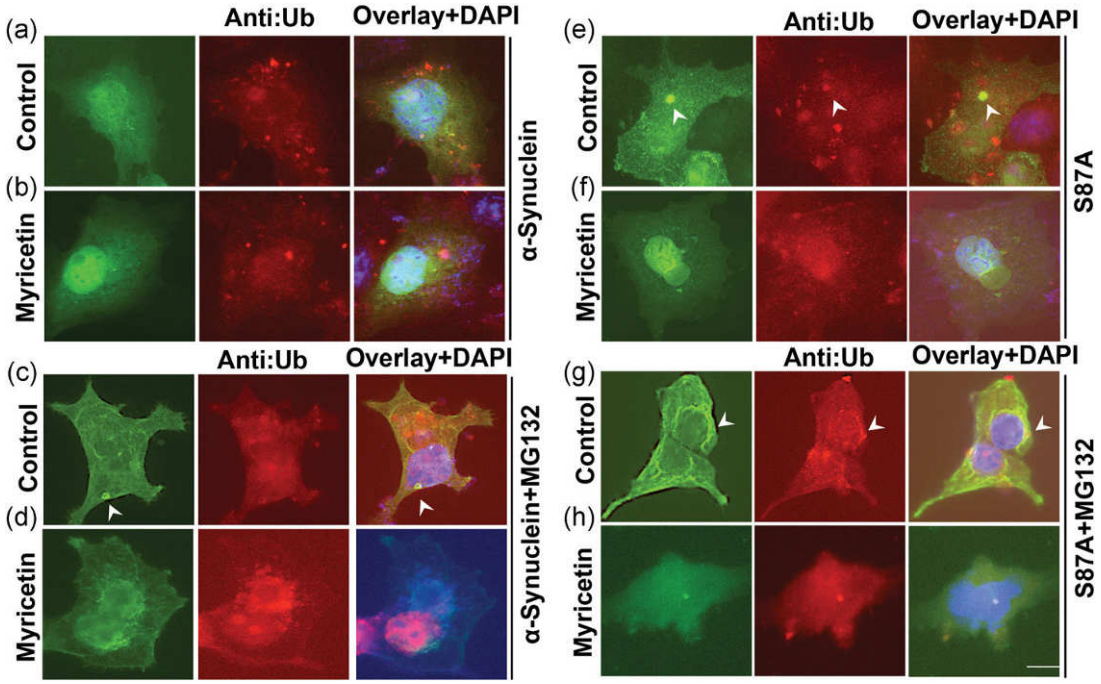


FIGURE 5 Myricetin mediates intracellular suppression of α-Synuclein aggregates. (a–d) Cos-7 cells were grown in chamber slides (having two-wells), were transfected with α-synuclein plasmid constructs with or without treatment of Myricetin 10 μM for 48 hr and few sets of cells were then given a treatment of MG132, 10 μM for 12 hr. Posttreatment, immunofluorescence staining was performed using ubiquitin antibody along with rhodamine conjugated secondary antibody to visualize the localization of ubiquitin with α-Synuclein aggregates as shown by arrowheads. DAPI was used for nuclear staining. (e–h) Cells were transfected with α-Synuclein mutant S87A constructs and treated with DMSO and Myricetin. Few sets of cells were also treated with MG132 to monitor the effect of Myricetin following proteasome inhibition. Scale bar 20 μm. DAPI: 4′,6-diamidino-2-phenylindole [Color figure can be viewed at wileyonlinelibrary.com]

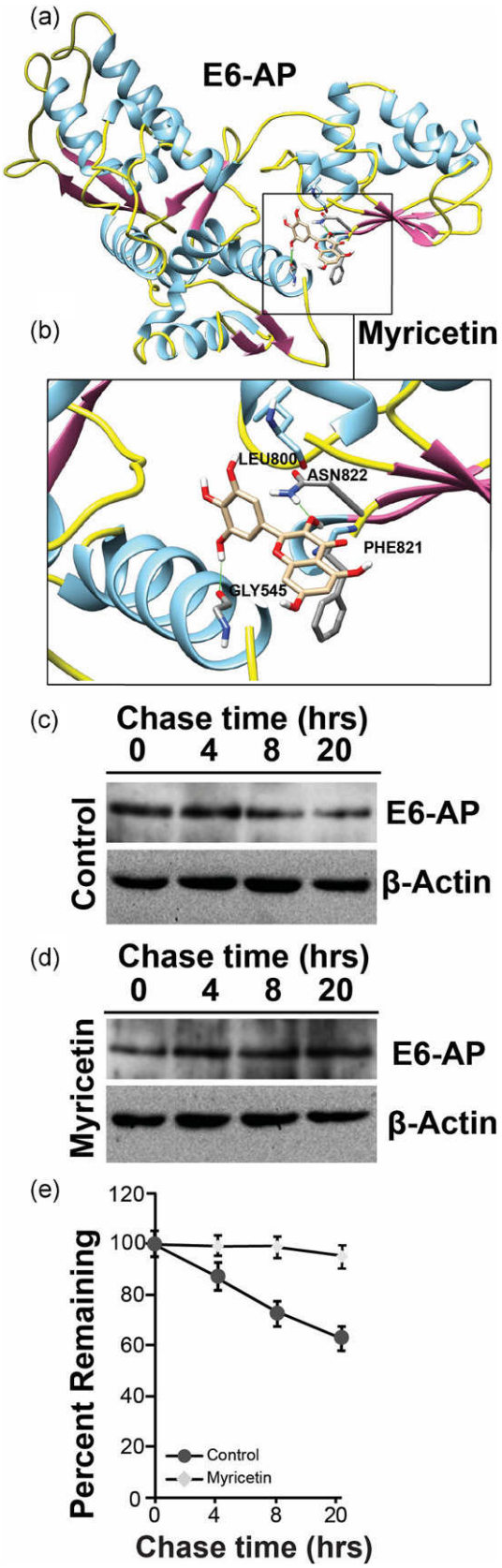
α-Synuclein constructs. Transfected cells were treated with Myricetin and subjected to immunofluorescence analysis by using α-Synuclein and ubiquitin antibodies.

As shown in Figure 5a,b the expression of normal α-Synuclein represents an overall diffused pattern in cells, whereas inhibition of proteasome with MG132 induces the overaccumulation of normal α-Synuclein proteins which produces marginal ubiquitin-positive aggregates in cells (Figure 5c,d). Immunofluorescence analysis of cells expressing mutant (S87A) α-Synuclein proteins revealed ubiquitin-positive aggregates formation in cells (Figure 5e). Exposure of Myricetin reduces the accumulation of mutant (S87A) α-Synuclein aggregates (Figure 5f). The antiaggregation effect of Myricetin was reversed after the addition of MG132 (Figure 5g,h). These findings strongly suggest that Myricetin may serve as a useful compound against the aggregation of neurodegenerative disease associated toxic proteins in cells.

3.6 | Myricetin enhances the stability of E3 ubiquitin ligase E6-AP

Our results suggest that Myricetin increases the endogenous levels of E3 ubiquitin ligase E6-AP and previously, it has also been observed that Hsp70 interaction with E6-AP assists the degradation of misfolded proteins (Mishra et al., 2009). Here, we have observed that treatment of Myricetin reduces the intracellular aggregation of aberrant proteins. Therefore, we thought that most likely there might be a possible interaction between E6-AP and Myricetin, which can provide an effective functional stability to E6-AP. To address this notion, we have performed detailed docking studies. In silico analysis showed a − 7.0 kcal/mol binding affinity between best confirmation of Myricetin with E6-AP HECT catalytic domain. Two hydrogen bonds were obtained involving amino acid GLY545 and ASN822 of E6-AP HECT catalytic domain with Myricetin (Figure 6a,b).

Since, Myricetin influences the endogenous expression levels of E6-AP protein; therefore, it was important for us to examine the effects of Myricetin on the stability of E6-AP. Cells were exposed to control (DMSO), or Myricetin and posttreated cells were processed for cycloheximide chase experiment. As depicted in Figure 6c,d administration of Myricetin slows down the rate of degradation of E6-AP as compared with control. Earlier observations and present results suggest that probably treatment of Myricetin not only upregulates the expression of E6-AP, but also stimulates the progressive intracellular stabilization of E6-AP (Figure 6c–e); which could serve as an additional contributing factor affecting the elimination of misfolded proteins from the cells.



3.7 | Myricetin reduces the formation of insoluble expanded polyglutamine proteins inclusion bodies

Intracellular expression of expanded polyglutamine stretches generates misfolded protein aggregates and such CAG repeats/polyglutamine-linked inclusion bodies were earlier reported in Huntington's disease (HD; Chen, Ferrone, & Wetzel, 2002). Previously, it has also been shown that E6-AP promotes the degradation of expanded polyglutamine proteins (Mishra et al., 2008). As our current observations indicate that Myricetin provides intracellular stability to E6-AP; therefore, next we tried to understand whether Myricetin could decrease the load of misfolded inclusions formation in cells and alleviate cytotoxicity associated with another neurodegenerative disease (HD).

We used the cellular model of HD in our next course of experiments; cells were then transfected with normal (EGFP-HDQ23) and expanded (EGFP-HDQ74) polyglutamine expression constructs and some set of cells were treated with or without Myricetin in the presence or absence of MG132. As shown in Figure 7a–d, expressed normal glutamine repeats EGFP-HDQ23 does not exhibit significant aggregate formation in comparison with expanded (EGFP-HDQ74) polyglutamine proteins. Addition of Myricetin dramatically reduces the toxic aggregates of expanded polyglutamine proteins (Figure 7e,f). Proteasome inhibitor (MG132) prevents the clearance of EGFP-HDQ74 expanded polyglutamine proteins (Figure 7g,h). These observations suggest that probably Myricetin accelerates clearance of expanded polyglutamine protein aggregates via proteasome pathway.

3.8 | Effect of myricetin on different cellular model of neurodegenerative diseases

Previously, it has been observed that the formation of intracellular inclusions sequester components of PQC pathways and further increases accumulation of aggregates may lead to the impairment of proteasomal dysfunction and cell death (Schipper-Krom, Juenemann, & Reits, 2012; Zheng et al., 2016). In neurodegenerative diseases, including spinocerebellar ataxias, formation of similar intracellular inclusions serves as the major pathological hallmark (Koeppen, 2005; Koyano et al., 1999). To overcome these multifactorial complex pathogenic issues, it was important to find new molecules that can improve the clearance efficiency of PQC pathways against the aggravated deposition of misfolded proteinaceous inclusions. In our study, we found that Myricetin

FIGURE 6 Treatment of Myricetin stabilizes E6-AP and in silico analysis. (a,b) Myricetin-Ube3a HECT catalytic domain interaction obtained by in silico docking approach along with a closeup view of the interacting regions marked with interacting amino acids. (c,d) Cos-7 cells were seeded on six-well tissue culture plates and treated with control and Myricetin 10 μM for 48 hr on a subsequent day and chased for 20 hr by cyclohexamide (15 μg/ml) treatment at different time intervals. E6-AP and β-actin were used for band detection in western blot analysis. (e) Quantification of bands obtained in (c,d) was done by NIH image analysis software, where $^{*}p < 0.05$ compared with the control [Color figure can be viewed at wileyonlinelibrary.com]

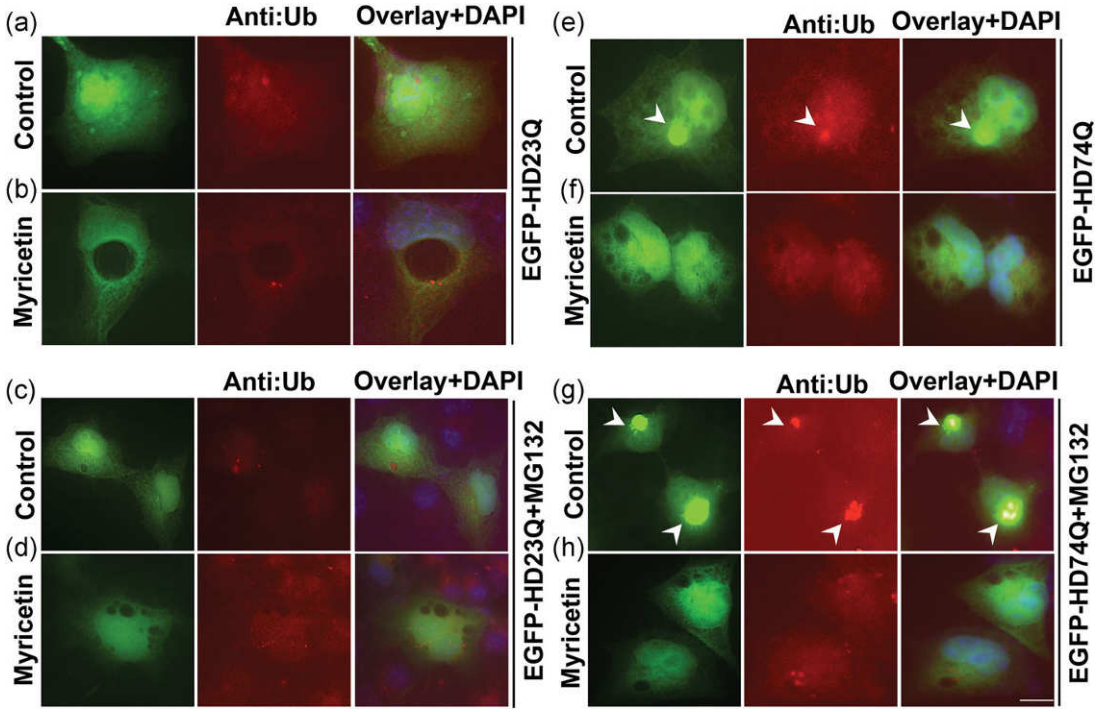


FIGURE 7 Myricetin treatment in Cos-7 cells reduces the Huntingtin expanded polyglutamine aggregates. (a–d) Cells were transfected with wild-type EGFP-tagged huntingtin (23Q) plasmids and were exposed with control and Myricetin 10 μM for 48 hr. Some sets of cells were also treated with proteasome inhibitor MG132, 10 μM for 12 hr. Immunofluorescence staining was done by ubiquitin primary antibody with rhodamine conjugated secondary antibody and cells were analyzed under fluorescence microscope. (e–h) Another set of cells were transfected with extended polyglutamine huntingtin (74Q) plasmids and processed for immunofluorescence in an above described manner to observe ubiquitin colocalization with huntingtin polyglutamine aggregates, indicated by the arrowheads. Antifade with DAPI was used to stain nucleus in all sets of cells. Scale bar 20 μm. DAPI: 4′,6-diamidino-2-phenylindole [Color figure can be viewed at wileyonlinelibrary.com]

treatment was effective against the aggregation of various misfolded proteins. Therefore, it was crucial for us to understand the effect of Myricetin on the rate of formation of misfolded protein aggregate.

We transiently-transfected cells with expanded-polyglutamine ataxin-3(84Q), HD (74Q), SOD1-G37R, and S87A mutant plasmids; and posttransfected cells were treated with or without Myricetin. Collected cell lysates were then subjected to filter trap assay, as shown in Figure 8a,b. We observed that use of Myricetin reduces the aggregation of misfolded inclusions of mutant proteins. Fluorescence microscopic analysis revealed that treatment of Myricetin suppresses the intracellular aggregation of ataxin-3(84Q) expanded polyglutamine aggregates (Figure 8c). Few sets of similar samples were processed for immunoblot analysis by using GFP and β-actin antibodies, as shown in Figure 8d, treatment of Myricetin enhances the degradation of expanded polyglutamine ataxin-3 repeats. Next, we found the direct effect of Myricetin on the number of aggregates of other misfolded proteins. Therefore, as explained earlier, we exogenously expressed different expanded-polyglutamine EGFP-HDQ74, mutant GFP-Δ9CAT and mutant SOD1 (SOD1-G37R) proteins in cells in the presence or absence of Myricetin. Effect of Myricetin was observed on various intracellular aggregates of different misfolded proteins. As shown in Figure 8e,f, the total number of aggregates were reduced in the presence of Myricetin. Taken together the above observations suggested that the Myricetin treatment can increase endogenous levels of the chaperone (Hsp70) and QC-E3 ubiquitin ligase E6-AP, which can further significantly improve the survival of cells via enhanced degradation of misfolded proteins.

4 | DISCUSSION

The integrity of cellular fitness and health get often compromised during neurodegenerative diseases and ageing, which is linked with the accumulation of misfolded proteins. In this study, we have shown that treatment of Myricetin increases the intracellular levels of few crucial proteins (HSF-1, Hsp70, and E6-AP) associated with PQC mechanism, which maintain cellular survival under proteotoxic insults, generated by aggregated proteins. How defects in cellular PQC machinery can cause the onset of such a diverse range of neurodegenerative diseases remains poorly understood. Recent research provides a diverse and emerging insight that insufficient removal of misfolded proteins, PQC dysfunctions, and loss of their clearance generate prominent pathogenic features of many neurodegenerative disorders (Chhangani & Mishra, 2013; Gestwicki & Garza, 2012; Kabashi & Durham, 2006). Identification of molecules, which can improve cellular PQC functions and can act as novel inhibitors of abnormal protein accumulation, might increase our understanding for the treatment of various neurodegenerative

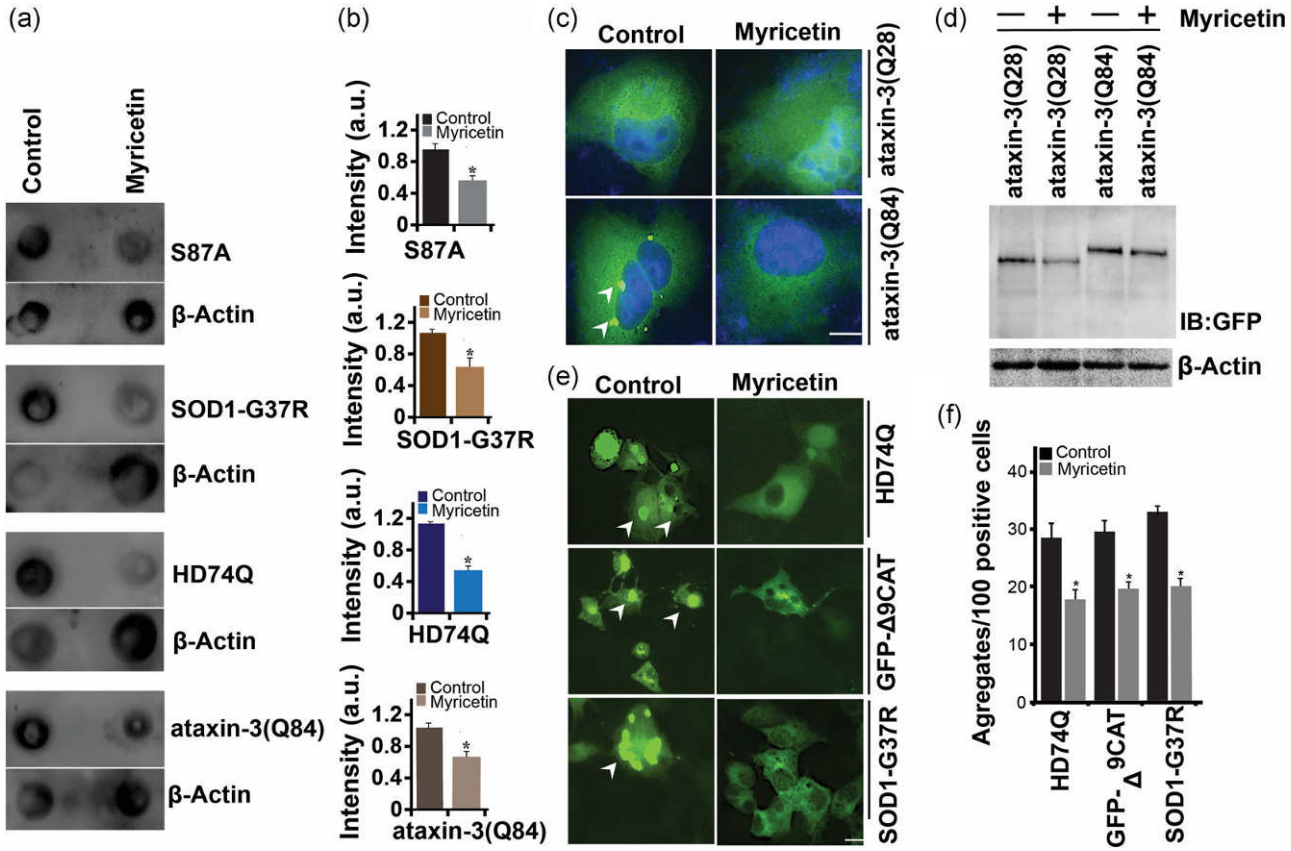


FIGURE 8 Myricetin treatment suppressed the aggregation of neurodegenerative associated abnormal proteins. (a,b) Cos-7 cells, seeded into six-well tissue culture dishes, transfected with ataxin-3 (84Q), S87A, HD (74Q), and SOD1-G37R plasmids with or without Myricetin (10 μM for 48 hr). Posttreatment samples were collected and subjected to filter trap assay with primary antibodies of anti-GFP and β-actin. Quantification of dots intensity was obtained via NIH image analysis software. In comparison with control $^*p < 0.05$. (c,d) Cells transfected with plasmid constructs of normal polyglutamine repeats containing ataxin-3 (28Q) and extended polyglutamine repeats containing ataxin-3 (84Q) were subsequently treated with or without Myricetin to visualize under a fluorescence microscope. Similar sets of cell lysates were processed for western blot analysis by anti-GFP and β-actin antibodies. (e,f) Cos-7 cells, after transfection with plasmid constructs of HD74Q, GFP-Δ9CAT, and SOD1-G37R were given Myricetin and control DMSO treatment and numbers of aggregates were counted manually, under a fluorescence microscope. Quantification of a number of aggregates was performed in triplicates of three independent experiments, represented as ± SD with a value of $^*p < 0.05$ in comparison with control. DMSO: dimethyl sulfoxide [Color figure can be viewed at wileyonlinelibrary.com]

diseases. Chaperone machinery is an integral for protein folding and associated functions of QC-E3 ubiquitin ligases result in the misfolded proteins degradation (Barral, Broadley, Schaffar, & Hartl, 2004; Chhangani, Jana, & Mishra, 2013). Therefore, it could be crucial to find out PQC mechanism-inducing compounds without having any toxicity and we searched for them in flavonoids. Earlier reports have suggested the medical importance of flavonoids in complex diseases like cancer, cardiovascular diseases, neurodegeneration, and bacterial or viral infections, highlighting their significance and need of further research to obtain maximum therapeutic advantages (Havsteen, 2002; Spencer et al., 2004).

Here, we have demonstrated that use of Myricetin induces the endogenous levels of intracellular Hsp70 chaperone. This result strongly prompted us to observe the direct effects of Myricetin on HSF-1 levels and in our preliminarily experiments, we observed that the induction of Hsp70 by Myricetin is most likely mediated via activation of HSF-1. Despite the fact that alone up regulation of HSF-1 and Hsp70 could not significantly induce the clearance of misfolded proteins from the dense cellular pool, therefore, we extended our search for QC-E3 ubiquitin ligases. Previous reports have indicated that QC-E3 ubiquitin ligases can recognize aberrant proteins via several molecular strategies based on the need of cells under stressed conditions (Chhangani et al., 2013; Houck, Singh, & Cyr, 2012). We have noticed in our current observations that E6-AP E3 ubiquitin ligase was also induced after Myricetin treatment and this might explain the important role of Myricetin in the positive modulation of PQC system against misfolded proteins aggregation. But, how does Myricetin functionally perform the anti-aggregatory functions by activation of HSF-1, Hsp70, and E6-AP levels is not known. To answer this question, we further ascertain the concentration and time dependent manner effect of Myricetin on the above PQC molecules. Our findings suggest that Myricetin retains the similar inductive effect under different concentrations and time intervals.

To determine the potential of Myricetin against misfolded proteins aggregation, we treated cells expressing misfolded proteins with it. We

observed that Myricetin reduces the aggregation of mutant GFP-Δ9CAT protein and also degrade the heat-denatured luciferase misfolded protein. In previous years, neuroprotective roles of Myricetin were well explored because of its antioxidant properties, which helps in reduction of oxidative stress generated during multiple brain pathologies (Barzegar, 2016; Dajas et al., 2003). Another study reported that Myricetin provides neuroprotection by reducing glutamate induced neuronal death and excitotoxicity by affecting three different pathways that are by inhibiting reactive oxygen species production, reducing Ca^{2+} overload generated by phosphorylation of N-methyl D-aspartate receptor and finally by preventing caspase-3 activation (Shimmyo, Kihara, Akaike, Niidome, & Sugimoto, 2008).

Therefore, it was crucial to check the cytoprotective function of Myricetin against various stress conditions. Cells were treated with multiple stress-inducing agents (autophagic dysfunction, proteasomal inhibition, and ER stress) in the presence or absence of Myricetin. Our findings suggest that exposure of Myricetin protect cells from proteotoxic insults that could be due to the stabilization of QC E3 ubiquitin ligase E6-AP, which we have confirmed by cycloheximide chase experiment and docking analysis also suggest the possible interaction of Myricetin with E6-AP. Earlier, it has also been observed that Myricetin can inhibit cytotoxicity and DNA damage generated by peroxynitrite; and thus provides neuroprotection in multiple neurodegenerative disorders (Chen et al., 2011; Peng & Kuo, 2003).

Neurodegenerative disorders encompass a broad range of pathologies showing a common clinical feature of the presence of aggregates or inclusion bodies in neuronal cells that suggests the lack of PQC functions (Gestwicki & Garza, 2012; Nedelsky, Todd, & Taylor, 2008). Although the functional role of molecular chaperone Hsp70 and E6-AP haas been studied against misfolded proteins accumulation, but their precise enhanced cellular physiological roles based on the use of natural molecules or Myricetin are not well understood (Mishra et al., 2009; Upadhyay, Amanullah, Mishra, Kumar, & Mishra, 2018). To answer this question, we have used various neurodegeneration associated cellular models in our present study. We have observed that administration of Myricetin significantly reduces the aggregations of mutant SOD1 (SOD1-G37R), mutant α-Synuclein (S87A) protein, and expanded (EGFP-HDQ74; ataxin-3(84Q)) polyglutamine proteins. Overall, these results suggest the beneficiary potential of Myricetin to improve the survival of cells against the proteotoxic conditions mediated by the accumulation of misfolded proteins. Myricetin also inhibits the release of glutamate, a neurotransmitter that influences the various chronic brain diseases like epilepsy, stroke, and few others (Chang, Chang, Wang, & Huang, 2015; Meldrum, 2000). Further, studies on the role of Myricetin and other flavonoids in diseases like Alzheimer's, Parkinson's, Huntington's, and multiple sclerosis revealed their higher therapeutic significance in protection from neurodegeneration (Dajas et al., 2003; Solanki, Parihar, Mansuri, & Parihar, 2015).

Quercetin is another flavonoid that affects expression of Hsp70 and its functionally associated protein IRE1α in tumor cells and help in protection from death by endoplasmic reticulum stress (Storniolo, Raciti, Cucina, Bizzarri, & Di Renzo, 2015). However, dihydroquercetin does not have the same effect on Hsp70 as quercetin shows (Budagova, Zhmaeva, Grigor'ev, Goncharova, & Kabakov, 2003). There is one flavonoid luteolin that disrupts the binding of human papillomavirus early protein E6 with the E6-AP and cause increase in p53 and p21, which decreases the proliferation (Cherry et al., 2013; Malecka et al., 2014). These studies further support our results about possible modulations of E6-AP and Hsp70 by flavonoids may help in targeting pathological conditions caused due to perturbed PQC inside the cells.

In Parkinson's model Myricetin reduces the dopamine neuron degeneration, induced by 6-hydroxydopamine and 1-methyl-4-phenyl-pyridinium in substantia nigra-striatum (Ma, Wang, Jiang, Liu & Xie, 2007bb; K. Zhang, Ma, Wang, Xie, & Xie, 2011). Recent investigation of Myricetin activity in HD and Spinocerebellar ataxia reported its interaction with RNA, specifically CAG motif, thus reduces the huntingtin protein translation and sequestration; also reduces the cytotoxicity in Huntington's and other polyQ disease models (Khan et al., 2018). Moreover, the role of Myricetin was also observed in demyelinated mice model of multiple sclerosis as a potential molecule for alleviating motor defects (Q. Zhang et al., 2016). To find a possible cure against protein misfolding and aggregation problem is a challengeable task. The current study suggests that search of new modulators based on PQC mechanisms could provide new insights to design possible therapeutic strategies. Taken together our current work suggested that the Myricetin treatment can improve the molecular physiological functions of Hsp70 molecular chaperone and QC-E3 Ubiquitin ligase E6-AP linked to the PQC mechanism; which might provide a new therapeutic approach for neurodegenerative diseases.

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CONFLICT OF INTERESTS

The authors declare that they have no conflicts of interests.

AUTHORS CONTRIBUTIONS

VJ, RM, AU, and AA executed the experiments. KM and SS performed critical analysis of results, analytic calculations, framework of draft, and analyzed the data of manuscript. AM planned the experiments and wrote the manuscript. All authors reviewed the results and contributed to the final manuscript.

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REFERENCES

Amanullah, A., Upadhyay, A., Joshi, V., Mishra, R., Jana, N. R., & Mishra, A. (2017). Progressing neurobiological strategies against proteostasis failure: challenges in neurodegeneration. *Progress in Neurobiology*, *159*, 1–38. https://doi.org/10.1016/j.pneurobio.2017.08.005

Anckar, J., & Sistonen, L. (2007). Heat shock factor 1 as a coordinator of stress and developmental pathways, *Molecular Aspects of the Stress Response: Chaperones, Membranes and Networks* (78–88). Palo Alto, CA: Springer.

Arslan, M. A., Chikina, M., Csermely, P., & Soti, C. (2012). Misfolded proteins inhibit proliferation and promote stress-induced death in SV40-transformed mammalian cells. *FASEB Journal*, *26*(2), 766–777. https://doi.org/10.1096/fj.11-186197

Barral, J. M., Broadley, S. A., Schaffar, G., & Hartl, F. U. (2004). Roles of molecular chaperones in protein misfolding diseases. *Seminars in Cell and Developmental Biology*, *15*(1), 17–29. https://doi.org/10.1016/j.semcdb.2003.12.010

Barzegar, A. (2016). Antioxidant activity of polyphenolic myricetin in vitro cell- free and cell-based systems. *Molecular Biology Research Communications*, *5*(2), 87–95.

Bence, N. F., Sampat, R. M., & Kopito, R. R. (2001). Impairment of the ubiquitin-proteasome system by protein aggregation. *Science*, *292*(5521), 1552–1555. https://doi.org/10.1126/science.292.5521.1552

Bohm, B. A. (1998). *Introduction to flavonoids*. Australia: Harwood Academic Publishers.

Buchter, C., Ackermann, D., Havermann, S., Honnen, S., Chovolou, Y., Fritz, G., … Watjen, W. (2013). Myricetin-mediated lifespan extension in *Caenorhabditis elegans* is modulated by DAF-16. *International Journal of Molecular Sciences*, *14*(6), 11895–11914. https://doi.org/10.3390/ijms140611895

Budagova, K. R., Zhmaeva, S. V., Grigor'ev, A. N., Goncharova, A. Y., & Kabakov, A. E. (2003). Flavonoid dihydroquercetin, unlike quercetin, fails to inhibit expression of heat shock proteins under conditions of cellular stress. *Biochemistry (Mosc)*, *68*(9), 1055–1061.

Chang, Y., Chang, C. Y., Wang, S. J., & Huang, S. K. (2015). Myricetin inhibits the release of glutamate in rat cerebrocortical nerve terminals. *Journal of Medicinal Food*, *18*(5), 516–523. https://doi.org/10.1089/jmf.2014.3219

Chen, S., Ferrone, F. A., & Wetzel, R. (2002). Huntington's disease age-of-onset linked to polyglutamine aggregation nucleation. *Proceedings of the National Academy of Sciences of the United States of America*, *99*(18), 11884–11889. https://doi.org/10.1073/pnas.182276099

Chen, W., Li, Y., Li, J., Han, Q., Ye, L., & Li, A. (2011). Myricetin affords protection against peroxynitrite-mediated DNA damage and hydroxyl radical formation. *Food and Chemical Toxicology*, *49*(9), 2439–2444. https://doi.org/10.1016/j.fct.2011.06.066

Cherry, J. J., Rietz, A., Malinkevich, A., Liu, Y., Xie, M., Bartolowits, M., … Androphy, E. J. (2013). Structure based identification and characterization of flavonoids that disrupt human papillomavirus-16 E6 function. *PLoS One*, *8*(12), e84506. https://doi.org/10.1371/journal.pone.0084506

Chhangani, D., Jana, N. R., & Mishra, A. (2013). Misfolded proteins recognition strategies of E3 ubiquitin ligases and neurodegenerative diseases. *Molecular Neurobiology*, *47*(1), 302–312. https://doi.org/10.1007/s12035-012-8351-0

Chhangani, D., & Mishra, A. (2013). Protein quality control system in neurodegeneration: A healing company hard to beat but failure is fatal. *Molecular Neurobiology*, *48*(1), 141–156. https://doi.org/10.1007/s12035-013-8411-0

Chhangani, D., Upadhyay, A., Amanullah, A., Joshi, V., & Mishra, A. (2014). Ubiquitin ligase ITCH recruitment suppresses the aggregation and cellular toxicity of cytoplasmic misfolded proteins. *Scientific Reports*, *4*, 5077. https://doi.org/10.1038/srep05077

Craig, E. A., Weissman, J. S., & Horwich, A. L. (1994). Heat shock proteins and molecular chaperones: Mediators of protein conformation and turnover in the cell. *Cell*, *78*(3), 365–372.

Dai, C., & Sampson, S. B. (2016). HSF1: Guardian of proteostasis in cancer. *Trends in Cell Biology*, *26*(1), 17–28.

Dajas, F., Rivera-Megret, F., Blasina, F., Arredondo, F., Abin-Carriquiry, J. A., Costa, G., … Morquio, A. (2003). Neuroprotection by flavonoids. *Brazilian Journal of Medical and Biological Research*, *36*(12), 1613–1620.

David, D. C. (2012). Aging and the aggregating proteome. *Frontiers in Genetics*, *3*, 247. https://doi.org/10.3389/fgene.2012.00247

el-Gammal, A. A., & Mansour, R. M. (1986). Antimicrobial activities of some flavonoid compounds. *Zentralblatt für Mikrobiologie*, *141*(7), 561–565.

Falcone Ferreyra, M. L., Rius, S. P., & Casati, P. (2012). Flavonoids: Biosynthesis, biological functions, and biotechnological applications. *Frontiers of Plant Science*, *3*, 222. https://doi.org/10.3389/fpls.2012.00222

Fujikake, N., Nagai, Y., Popiel, H. A., Okamoto, Y., Yamaguchi, M., & Toda, T. (2008). Heat shock transcription factor 1-activating compounds suppress polyglutamine-induced neurodegeneration through induction of multiple molecular chaperones. *Journal of Biological Chemistry*, *283*(38), 26188–26197. https://doi.org/10.1074/jbc.M710521200

Fujimoto, M., Takaki, E., Hayashi, T., Kitaura, Y., Tanaka, Y., Inouye, S., … Nakai, A. (2005). Active HSF1 significantly suppresses polyglutamine aggregate formation in cellular and mouse models. *Journal of Biological Chemistry*, *280*(41), 34908–34916.

Gestwicki, J. E., & Garza, D. (2012). Protein quality control in neurodegenerative disease. *Progress in Molecular Biology and Translational Science*, *107*, 327–353. https://doi.org/10.1016/B978-0-12-385883-2.00003-5

Goldberg, A. L. (2003). Protein degradation and protection against misfolded or damaged proteins. *Nature*, *426*(6968), 895–899.

Griffiths, L. A., & Smith, G. E. (1972). Metabolism of myricetin and related compounds in the rat. Metabolite formation in vivo and by the intestinal microflora in vitro. *Biochemical Journal*, *130*(1), 141–151.

Hartl, F. U., Bracher, A., & Hayer-Hartl, M. (2011). Molecular chaperones in protein folding and proteostasis. *Nature*, *475*(7356), 324–332.

Hartmann, T. (2007). From waste products to ecochemicals: Fifty years research of plant secondary metabolism. *Phytochemistry*, *68*(22), 2831–2846. https://doi.org/10.1016/j.phytochem.2007.09.017

Havsteen, B. H. (2002). The biochemistry and medical significance of the flavonoids. *Pharmacology and Therapeutics*, *96*(2-3), 67–202.

Heller, K. (2010). Targeting misfolded proteins to fight neurodegenerative diseases. *PLoS Biology*, *8*(1), e1000290.

Hightower, L. E. (1991). Heat shock, stress proteins, chaperones, and proteotoxicity. *Cell*, *66*(2), 191–197.

Houck, S. A., Singh, S., & Cyr, D. M. (2012). Cellular responses to misfolded proteins and protein aggregates. *Methods in Molecular Biology*, *832*, 455–461. https://doi.org/10.1007/978-1-61779-474-2_32

K Hahlbrock, a, & Grisebach, H. (1979). Enzymic controls in the biosynthesis of lignin and flavonoids. *Annual Review of Plant Physiology*, *30*(1), 105–130. https://doi.org/10.1146/annurev.pp.30.060179.000541

Kabashi, E., & Durham, H. D. (2006). Failure of protein quality control in amyotrophic lateral sclerosis. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease*, *1762*(11-12), 1038–1050.

Kalff, J., & Robinson, R. (1925). XXVIII.-A synthesis of myricetin and of a galangin monomethyl ether occurring in galanga root. *Journal of the Chemical Society, Transactions*, *127*(0), 181–184. https://doi.org/10.1039/CT9252700181

Khan, E., Tawani, A., Mishra, S. K., Verma, A. K., Upadhyay, A., Kumar, M., … Kumar, A. (2018). Myricetin reduces toxic level of CAG repeats RNA in Huntington's disease (HD) and spino cerebellar ataxia (SCAs). *ACS Chemical Biology*, *13*(1), 180–188. https://doi.org/10.1021/acschembio.7b00699

Kim, E., Wang, B., Sastry, N., Masliah, E., Nelson, P. T., Cai, H., … Liao, F. F. (2016). NEDD4-mediated HSF1 degradation underlies alpha-synucleinopathy. *Human Molecular Genetics*, *25*(2), 211–222. https://doi.org/10.1093/hmg/ddv445

Kliebenstein, D. J. (2013). Making new molecules—evolution of structures for novel metabolites in plants. *Current Opinion in Plant Biology*, *16*(1), 112–117.

Koeppen, A. H. (2005). The pathogenesis of spinocerebellar ataxia. *Cerebellum*, *4*(1), 62–73. https://doi.org/10.1080/14734220510007950

Koyano, S., Uchihara, T., Fujigasaki, H., Nakamura, A., Yagishita, S., & Iwabuchi, K. (1999). Neuronal intranuclear inclusions in spinocerebellar ataxia type 2: Triple-labeling immunofluorescent study. *Neuroscience Letters*, *273*(2), 117–120.

Kültz, D. (2005). Molecular and evolutionary basis of the cellular stress response. *Annual Review of Physiology*, *67*, 225–257.

Kumar, S., & Pandey, A. K. (2013). Chemistry and biological activities of flavonoids: An overview. *The Scientific World Journal*, *2013*, pp.16. Article ID: 162750. https://doi.org/10.1155/2013/162750

Ma, Z.-G., Wang, J., Jiang, H., Liu, T.-W., & Xie, J.-X. (2007a). Myricetin reduces 6-hydroxydopamine-induced dopamine neuron degeneration in rats. *Neuroreport*, *18*(11), 1181–1185.

Ma, Z. G., Wang, J., Jiang, H., Liu, T. W., & Xie, J. X. (2007b). Myricetin reduces 6-hydroxydopamine-induced dopamine neuron degeneration in rats. *Neuroreport*, *18*(11), 1181–1185. https://doi.org/10.1097/WNR.0b013e32821c51fe

Maggiolini, M., Recchia, A. G., Bonofiglio, D., Catalano, S., Vivacqua, A., Carpino, A., … Ando, S. (2005). The red wine phenolics piceatannol and myricetin act as agonists for estrogen receptor alpha in human breast cancer cells. *Journal of Molecular Endocrinology*, *35*(2), 269–281. https://doi.org/10.1677/jme.1.01783

Malecka, K. A., Fera, D., Schultz, D. C., Hodawadekar, S., Reichman, M., Donover, P. S., … Marmorstein, R. (2014). Identification and characterization of small molecule human papillomavirus E6 inhibitors. *ACS Chemical Biology*, *9*(7), 1603–1612. https://doi.org/10.1021/cb500229d

McNaught, K. S., Perl, D. P., Brownell, A. L., & Olanow, C. W. (2004). Systemic exposure to proteasome inhibitors causes a progressive model of Parkinson's disease. *Annals of Neurology*, *56*(1), 149–162. https://doi.org/10.1002/ana.20186

Meldrum, B. S. (2000). Glutamate as a neurotransmitter in the brain: Review of physiology and pathology. *Journal of Nutrition*, *130*(4S Suppl), 1007S–1015S. https://doi.org/10.1093/jn/130.4.1007S

Mishra, A., Dikshit, P., Purkayastha, S., Sharma, J., Nukina, N., & Jana, N. R. (2008). E6-AP promotes misfolded polyglutamine proteins for proteasomal degradation and suppresses polyglutamine protein aggregation and toxicity. *Journal of Biological Chemistry*, *283*(12), 7648–7656. https://doi.org/10.1074/jbc.M706620200

Mishra, A., Godavarthi, S. K., Maheshwari, M., Goswami, A., & Jana, N. R. (2009). The ubiquitin ligase E6-AP is induced and recruited to aggresomes in response to proteasome inhibition and may be involved in the ubiquitination of Hsp70-bound misfolded proteins. *Journal of Biological Chemistry*, *284*(16), 10537–10545. https://doi.org/10.1074/jbc.M806804200

Mu, T.-W., Ong, D. S. T., Wang, Y.-J., Balch, W. E., Yates, J. R., III, Segatori, L., … Kelly, J. W. (2008). Chemical and biological approaches synergize to ameliorate protein-folding diseases. *Cell*, *134*(5), 769–781.

Muchowski, P. J., Schaffar, G., Sittler, A., Wanker, E. E., Hayer-Hartl, M. K., & Hartl, F. U. (2000). Hsp70 and hsp40 chaperones can inhibit self-assembly of polyglutamine proteins into amyloid-like fibrils. *Proceedings of the National Academy of Sciences of the United States of America*, *97*(14), 7841–7846. https://doi.org/10.1073/pnas.140202897

Nedelsky, N. B., Todd, P. K., & Taylor, J. P. (2008). Autophagy and the ubiquitin-proteasome system: Collaborators in neuroprotection. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease*, *1782*(12), 691–699.

Neef, D. W., Jaeger, A. M., & Thiele, D. J. (2011). Heat shock transcription factor 1 as a therapeutic target in neurodegenerative diseases. *Nature Reviews Drug Discovery*, *10*(12), 930–944. https://doi.org/10.1038/nrd3453

Ong, K. C., & Khoo, H. E. (1997). Biological effects of myricetin. *General Pharmacology*, *29*(2), 121–126.

Ong, K. C., & Khoo, H. E. (2000). Effects of myricetin on glycemia and glycogen metabolism in diabetic rats. *Life Sciences*, *67*(14), 1695–1705.

Peng, I. W., & Kuo, S. M. (2003). Flavonoid structure affects the inhibition of lipid peroxidation in Caco-2 intestinal cells at physiological concentrations. *Journal of Nutrition*, *133*(7), 2184–2187. https://doi.org/10.1093/jn/133.7.2184

Perkin, A. G., & Hummel, J. J. (1896). LXXVI.-The colouring principle contained in the bark of Myrica nagi Part I. *Journal of the Chemical Society, Transactions*, *69*(0), 1287–1294. https://doi.org/10.1039/CT8966901287

Pettersen, E. F., Goddard, T. D., Huang, C. C., Couch, G. S., Greenblatt, D. M., Meng, E. C., … Ferrin, T. E. (2004). UCSF Chimera--a visualization system for exploratory research and analysis. *Journal of Computational Chemistry*, *25*(13), 1605–1612. https://doi.org/10.1002/jcc.20084

Pirkkala, L., Alastalo, T. P., Zuo, X., Benjamin, I. J., & Sistonen, L. (2000). Disruption of heat shock factor 1 reveals an essential role in the ubiquitin proteolytic pathway. *Molecular and Cellular Biology*, *20*(8), 2670–2675.

Quattrocchio, F., Baudry, A., Lepiniec, L., & Grotewold, E. (2006). The regulation of flavonoid biosynthesis, *The Science of Flavonoids* (97–122). New York, NY: Springer Science Business Media, Inc..

Ross, C. A., & Poirier, M. A. (2004). Protein aggregation and neurodegenerative disease. *Nature Medicine (New York, NY, United States)*, *10*(Suppl), S10–S17. https://doi.org/10.1038/nm1066

Schipper-Krom, S., Juenemann, K., & Reits, E. A. (2012). The ubiquitin-proteasome system in Huntington's disease: Are proteasomes impaired, initiators of disease, or coming to the rescue? *Biochemistry Research International*, *2012*, pp.12. Article ID: 837015. https://doi.org/10.1155/2012/837015

Shimmyo, Y., Kihara, T., Akaike, A., Niidome, T., & Sugimoto, H. (2008). Three distinct neuroprotective functions of myricetin against glutamate-induced neuronal cell death: Involvement of direct inhibition of caspase-3. *Journal of Neuroscience Research*, *86*(8), 1836–1845. https://doi.org/10.1002/jnr.21629

Solanki, I., Parihar, P., Mansuri, M. L., & Parihar, M. S. (2015). Flavonoid-based therapies in the early management of neurodegenerative diseases. *Advances in Nutrition*, *6*(1), 64–72. https://doi.org/10.3945/an.114.007500

Spencer, J. P., Abd-el-Mohsen, M. M., & Rice-Evans, C. (2004). Cellular uptake and metabolism of flavonoids and their metabolites: Implications for their bioactivity. *Archives of Biochemistry and Biophysics*, *423*(1), 148–161.

Storniolo, A., Raciti, M., Cucina, A., Bizzarri, M., & Di Renzo, L. (2015). Quercetin affects Hsp70/IRE1-3b1; mediated protection from death induced by endoplasmic reticulum stress. *Oxidative Medicine and Cellular Longevity*, *2015*, 11. https://doi.org/10.1155/2015/645157

Trott, O., & Olson, A. J. (2010). AutoDock Vina: Improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. *Journal of Computational Chemistry*, *31*(2), 455–461. https://doi.org/10.1002/jcc.21334

Upadhyay, A., Amanullah, A., Mishra, R., Kumar, A., & Mishra, A. (2018). Lanosterol suppresses the aggregation and cytotoxicity of misfolded proteins linked with neurodegenerative diseases. *Molecular Neurobiology*, *55*(2), 1169–1182. https://doi.org/10.1007/s12035-016-0377-2

Verma, N., & Shukla, S. (2015). Impact of various factors responsible for fluctuation in plant secondary metabolites. *Journal of Applied Research on Medicinal and Aromatic Plants*, *2*(4), 105–113. https://doi.org/. https://doi.org/10.1016/j.jarmap.2015.09.002

Yang, L., Wen, K. S., Ruan, X., Zhao, Y. X., Wei, F., & Wang, Q. (2018). Response of plant secondary metabolites to environmental factors. *Molecules*, *23*(4), E762. https://doi.org/10.3390/molecules23040762

Zhang, K., Ma, Z., Wang, J., Xie, A., & Xie, J. (2011). Myricetin attenuated MPP(+)-induced cytotoxicity by anti-oxidation and inhibition of MKK4 and JNK activation in MES23.5 cells. *Neuropharmacology*, *61* (1-2), 329–335. https://doi.org/10.1016/j.neuropharm.2011.04.021

Zhang, Q., Li, Z., Wu, S., Li, X., Sang, Y., Li, J., … Ding, H. (2016). Myricetin alleviates cuprizone-induced behavioral dysfunction and demyelination in mice by Nrf2 pathway. *Food & Function*, *7*(10), 4332–4342. https://doi.org/10.1039/c6fo00825a

Zheng, Q., Huang, T., Zhang, L., Zhou, Y., Luo, H., Xu, H., … Wang, X. (2016). Dysregulation of ubiquitin-proteasome system in neurodegenerative diseases. *Frontiers in Aging Neuroscience*, *8*, 303. https://doi.org/10.3389/fnagi.2016.00303

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Lanosterol Suppresses the Aggregation and Cytotoxicity of Misfolded Proteins Linked with Neurodegenerative Diseases

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Abstract Accumulation of misfolded or aberrant proteins in neuronal cells is linked with neurodegeneration and other pathologies. Which molecular mechanisms fail and cause inappropriate folding of proteins and what is their relationship to cellular toxicity is not well known. How does it happen and what are the probable therapeutic or molecular approaches to counter them are also not clear. Here, we demonstrate that treatment of lanosterol diminishes aberrant proteotoxic aggregation and mitigates their cytotoxicity via induced expression of co-chaperone CHIP and elevated autophagy. The addition of lanosterol not only reduces aggregation of mutant bonafide misfolded proteins but also effectively prevents accumulation of various mutant disease-prone proteotoxic proteins. Finally, we observed that lanosterol mitigates cytotoxicity in cells, mediated by different stress-inducing agents. Taken together, our present results suggest that upregulation of cellular molecular chaperones, primarily using small molecules, can probably offer an efficient therapeutic approach in the future against misfolding of different disease-causing proteins and neurodegenerative disorders.

Keywords Lanosterol · CHIP · Misfolded proteins · Neurodegeneration · Cell death

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Introduction

A major hallmark of neurodegenerative diseases is the formation of insoluble misfolded intracellular inclusions of disease-linked proteins, which often are controlled by multifarious cellular factors, including transcription factors, molecular chaperones, and other essential cellular proteins [1–3]. Abnormal or damaged proteins are targeted by cellular protein quality control (QC) mechanism for their refolding, elimination, or deposition into distinct cellular compartments [4, 5]. However, when these aberrant proteins tend to accumulate due to the exponential load, the overall efficiency of QC system suffers severely [6]. Since QC system has limitations, such as insufficient chaperone capacity against the massive accumulation of aberrant proteins, this may result in high unrecoverable costs such as impairment of ubiquitin-proteasome system [7]. Therefore, it is critical to find an efficient strategy for the elimination of over-accumulated non-native proteins, based on the use of small molecules or pharmacological chaperone that may assist in the folding of misfolded proteins, improve their solubility, and reduce aggregation [8–11]. Interestingly, in a review, it has been summarized that pharmacological chaperone therapy, based on the use of chaperone molecules, is now in clinical translational application stage for lysosomal disorders (Fabry, Gaucher, and Pompe diseases) [12].

In living cells, generation of stress may cause misfolding of normal proteins or induce deregulation of cellular protein degradation machinery and may lead to cell death [13–15]. Now, it is a well-established fact that chaperones and co-chaperones play a central role in the folding of misfolded proteins and failure of these attempts leads to their removal via cellular degradative pathways [16]. How mutations or lack of functions of protein folding machinery induce aggregation and unsolicited pathogenic problems in cells is still not well

known. Recent advances in a synergistic effect of pharmacological chaperones on the activity of proteolytic systems prompt us to understand the strengths and therapeutic potential of this approach to target abnormal protein accumulation disorders. In a recent study, it has been shown that structurally different small molecule nuclear factor erythroid 2-related factor 2 (NRF2) activators induce Hsp70 chaperone in heat shock transcription factor-1 (HSF-1)-dependent manner [17]. Among the various molecular approaches, based on small molecules, the strategy to improve heat shock response may be useful to target protein conformation disorders. Treatment of 4-phenylbutyrate induces Hsp70 and heat shock protein HSPH1 in IB-3 bronchial epithelial cell line [18]. A previous study suggests that heat shock gene expression was induced by arachidonate treatment in a dose-dependent manner, which can collectively influence HSF-1 activation [19]. In another study, it has been demonstrated that curcumin (diferuloylmethane) is the chief active ingredient of turmeric (*Curcuma longa*) elevates HSF-1, binds with heat shock regulatory element (HSE), and consequently induces heat shock response in human leukemia cells [20].

Lanosterol is an intermediate of cholesterol synthesis and use of lanosterol induces ubiquitination and degradation of a rate-controlling enzyme of cholesterol synthesis, i.e., HMG CoA reductase; mutations in lanosterol synthase cause cholesterol deficiency in a hereditary Shumiya cataractous rat strain [21, 22]. Another report indicates that oxidosqualene: lanosterol cyclase (OSC) enzyme catalyzes cyclization of the linear 2,3-monoepoxysqualene to lanosterol and its inhibition can be useful to reduce cholesterol biosynthesis and consequently in the treatment of atherosclerosis [23]. Interestingly, it has been shown that lanosterol is significantly reduced in nigrostriatal regions of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-treated mice and exogenous usage of lanosterol rescued dopaminergic neurons from MPTP-mediated toxicity. Collectively, this study also suggests that lanosterol treatment induces mild mitochondrial uncoupling and promotes autophagy [24]. Recently, it has been shown that treatment of lanosterol dramatically decreases intracellular aggregates of different crystallin mutant proteins and also alleviates cataract severity, in vivo, in dogs [25]. However, the molecular mechanism by which lanosterol reduces abnormal intracellular protein aggregation is not well known. Here, we demonstrate that lanosterol induces cytoprotective function of QC mechanism (ubiquitin-proteasome system (UPS) and autophagy) via increased expression and stabilized levels of co-chaperone CHIP and thus also reduces aggregation of modeled and bonafide misfolded proteins. Treatment of lanosterol diminished cytotoxic aggregation of various neurodegenerative disease-associated aberrant proteins. We found that lanosterol treatment not only suppresses aggregation of misfolded proteins but also mitigates proteotoxicity of abnormal mutant proteins in cells. Our results suggest the role of lanosterol in cytoprotection through elimination of mutant misfolded-mediated toxicities in cells.

Materials and Methods

Materials

Bafilomycin, chloroquine, 2-mercaptoethanol (BME), cycloheximide, lanosterol, MG132, tunicamycin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and all cell culture reagents were obtained from Sigma. Distinct antibodies used in this study were obtained from following sources. Anti-CHIP, anti-ubiquitin, anti-luciferase, anti-GFP, anti-SOD1, and anti-β-actin were purchased from Santa Cruz Biotechnology. Anti-GFP was obtained from Roche Applied Science. Anti-p62/SQSTM1 and antibodies were obtained from Sigma. Anti-LC3 was obtained from Pierce Antibodies. Anti-rabbit and anti-mouse (IgG-fluorescein isothiocyanate and IgG-rhodamine), horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG, were procured from Vector Laboratories. Anti-synuclein and anti-Lamp2 were purchased from Invitrogen and Thermo Fisher Scientific. Lipofectamine® 2000 and OptiMEM were purchased from Life Technologies. iQ SYBR green super mix was obtained from Bio-Rad. The dual luciferase reporter gene assay kit was purchased from Promega. TRIzol reagent and Plasmid pcDNA™ 3.1 was obtained from Life Technologies. Luciferase-pcDNA3 (Addgene plasmid 18964), pEGFP-C1-Ataxin3Q28 (Addgene-22122), pF141 pAcGFP1 SOD1WT (Addgene 26402) plasmids, pAAV asyn WT (Addgene plasmid 36055), pAAV asyn S87A (Addgene plasmid 36056), pF148 pSOD1G37RAcGFP1 (Addgene 26409), pEGFP-C1-Ataxin3Q84 (Addgene-22123), and pcDNA3-EGFP (Addgene 13031) were purchased from Addgene.

Cell Culture, Transfection, Drug Treatment, Cell Viability and Statistical Analysis

Cos-7 and A549 cell lines were maintained in Dulbecco's modified Eagle's medium (Sigma-Aldrich), supplemented with 10% heat-inactivated fetal bovine serum with 100 mg/ml streptomycin and 100 U/ml penicillin and cells were grown at 37 °C in 5% CO_2. Cells were cultured into 6-well and 96-well tissue culture plates, 1 day prior to transfection at a subconfluent density for different experiments. Cells were trasiently transfected with an equimolar ratio in combination with various expression vectors, using Lipofectamine® 2000 transfection reagent, according to the manufacturer's instructions. Bafilomycin, 2-mercaptoethanol, chloroquine, DMSO (control), lanosterol, MG132, and tunicamycin were added to the

culture medium, according to the need of various experiments (with or without constructs), as represented in different figures and explained in their respective legend sections. For cell viability experiments, cells were grown on 96-well plates and were exposed to different stress-inducing agents, as shown in the figure with doses of lanosterol, MG132, and bafilomycin. After treatment, these cells were subjected to cell viability assay, as described previously [26]. Statistical values are depicted as mean ± SD; statistical analysis was performed using the Student's *t* test, and $p < 0.05$ indicated statistical significance.

Immunofluorescence Techniques and Counting of Aggregates

Cos-7 cells were maintained in two-well chamber slides and were transiently transfected with the appropriate plasmids. Post-transfected cells were washed two times with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde in PBS for 20 min, and permeabilized in the presence of 0.5% Triton X-100 in PBS for 5 min. Cells were extensively washed four times and then blocked with 5% nonfat skimmed milk in Tris-Buffered Saline Tween 20 (TBST) [50 mM Tris, pH 7.5, 0.15 M NaCl, 0.05% Tween 20] for 1 h. After blocking and incubating with appropriate primary and secondary (rhodamine-conjugated secondary antibody (1:500 dilution)) antibody, finally cells were processed for mounting, as described previously [27]. Mounted immunofluorescence slides were visualized under a fluorescence microscope with 4′,6-diamidino-2-phenylindole (DAPI) for nuclear staining. Same sets of cells were used for various misfolded protein aggregate counting; cells retaining more than one aggresome were considered to have a single big inclusion. Fluorescence microscope (~400 transfected cells in each case) was used to count and analyze aberrant protein intracellular aggregates and they were manually counted under a microscope. Docking calculation was performed using Autodock Vina [28].

Docking Studies

The structure of CHIP TPR domain (PDB ID: 3Q49) was obtained from protein databank. Lanosterol structure used in our study was acquired from PDB ID: 1W6K. Protein and ligand were modified for docking, using MGL tools (http://mgltools.scripps.edu/). Search space box of 46 Å × 54 Å × 50 Å covering entire TPR domain was prepared and was centered on −16.846, −9.814, and 16.5. The best binding mode image was generated using Chimera [29]. The interactions present in the predicted binding mode were determined by PoseView [30].

Reverse Transcriptase PCR Analysis, Quantitative Real-Time RT-PCR Analysis, and Reporter Gene Assay

A549 cells were plated into 6-well plates and treated with lanosterol in varying concentration and time dependent manner. Post-treated cells were used to isolate total RNA using TRIzol reagent and semiquantitative RT-PCR was carried out by using CHIP specific primers. The quantitative real-time PCR for CHIP and β-actin was performed via complemetary DNA (cDNA) synthesis from total RNA isolated from treated cells, by using iQ SYBR green super mix (Bio-Rad). An iCycler iQ Real-Time Thermocycler Detection System (Bio-Rad) was used for real-time PCRs. To normalize all reactions, ribosomal 18S RNA was used as internal control and all reactions were performed in triplicates including negative control in the absence of template DNA. PCR conditions were same for both, CHIP as well as β-actin with 35 and 25 cycles, respectively. The PCR conditions were 30 min at 50 °C for reverse transcription step, followed by an initial denaturation at 94 °C for 2 min. Then, cycling through 94 °C for 50 s of denaturation, 55 °C for 50 s of annealing, and at 72 °C for 1 min were done; thereafter, final extension was done at 72 °C for 10 s and cooled down to 4 °C. The primer sequences used were as follows: CHIPF, 5′-AGGCCAAGCACGACAAGTACAT-3′; CHIPR, 5′-TATACTCGAGTCAGTAGTCCTCCACCCAGCCATT-3′; β-actinF, 5′-ATCGTCCACCGCAAATGCTTCTA-3′; and β-actinR, 5′-AGCCATGCCAATCTCATCTTGTT-3′. As described in previous section, lanosterol-treated cells transiently transfected with luciferase expression plasmid were used for luciferase activity assay. After treatment, cells were exposed to 43 °C for 30 min and then returned to 37 °C normal incubator for 2 h of the recovery period. Few experiments were carried out in the presence of bafilomycin and MG132 before heat stress. Cells were then subjected to luciferase activity assay according to the manufacturer's protocol of Promega (dual luciferase reporter gene assay kit) as previously described [3].

Immunoblotting and Degradation Assay

Cells were seeded into 6-well cultures plates. After appropriate treatment with lanosterol at different concentration and time intervals. Whole cell extracts were prepared and subjected to SDS-polyacrylamide gel electrophoresis (Bio-Rad Laboratories) and transferred to nitrocellulose membranes; blocking buffer (5% skimmed milk in Tris-Buffered Saline Tween 20 (TBST) [50 mM Tris; pH 7.4, 0.15 M NaCl, 0.05% Tween]) was used for incubations of blots for 1 h, and subsequently, blots were incubated with the appropriate primary antibody diluted (1:1000) overnight at 4 °C. The next day, after several washings with TBST buffer, secondary antibody (horseradish peroxidase-conjugated) was applied and blot signal was detected by using Luminata Crescendo

Western horseradish peroxidase (HRP) substrate (EMD Millipore). For chase experiment, Cos-7 cells were treated with lanosterol as represented in figure and cells were incubated with 15 μg/ml of cycloheximide for various time intervals. After treatment, cells were washed twice with PBS and extracted cell lysates were processed for immunoblotting analysis by using anti-CHIP and anti-β-actin antibodies.

Results

Lanosterol Induces Protective Autophagy and Upregulation of Endogenous Co-Chaperone CHIP Expression in Cells

Recently, it has been shown that exposure of lanosterol reduces preformed lens protein aggregates under in vitro as well as in cells; it also improves cataract severity, in vivo, in dogs [25]. However, to date, only lanosterol effects on reversal of protein aggregation in cataracts have been reported. But, how the addition of lanosterol reduces preformed protein aggregates is not well known. To explore the role of lanosterol in misfolded protein elimination, we examined expression levels of various cellular QC components (i.e., UPS and autophagy) in cells in the presence of lanosterol. We found that various critical components of the autophagy-lysosomal pathway, such as LC3 and Lamp2, were highly upregulated during lanosterol treatment in cells (Fig. 1a, b).

Previously, it has been reported that autophagy inhibition leads to p62/SQSTM1 accumulation, and when autophagy is induced, p62 endogenous levels may be decreased which represent autophagic flux [31, 32]. In our present study, we also observed reduced intracellular levels of p62 after lanosterol treatment, which indicate the selective and effective participation of p62 to transport ubiquitinated proteins into lysosomes via protective autophagy pathway (Fig. 1c). Immunoblotting also confirmed that p62 protein level was decreased after lanosterol treatment in cells (Fig. 1e). We further examined the effect of lanosterol on different crucial components of QC pathway and surprisingly observed that lanosterol exposure dramatically induces endogenous levels of co-chaperone CHIP in cells (Fig. 1d), which was further confirmed by immunoblot analysis using anti-CHIP antibody as shown in Fig. 1e.

It was important for us to know how lanosterol treatment dramatically elevates endogenous levels of CHIP protein in cells. We thought that the expression of CHIP might be influenced by exposure of lanosterol in cells. Therefore, to test this notion, we treated cells with different concentrations and for varying time intervals with lanosterol and then checked messenger RNA (mRNA) levels of CHIP by RT-PCR analysis (Fig. 1f). We also observed approximately a 0.2-fold increase in CHIP mRNA levels in the lanosterol-treated cells as compared with control (Fig. 1g, h). These results indicate that mRNA expression levels of endogenous CHIP were upregulated in lanosterol-treated cells. To further ascertain the above-described interesting finding, we next checked protein levels of CHIP after lanosterol exposure in cells and it was confirmed by immunoblot that total CHIP protein levels were also elevated in time-and dose-dependent manner in response to lanosterol treatment (Fig. 1i–l). Together, these results suggest that lanosterol treatment increases expression of CHIP and also induces cytoprotective autophagy signaling; this could be one of the critical survival response strategies against the deleterious effects of multifactorial abnormal protein aggregation in cells.

Lanosterol Prevents Cytoplasmic Aggregation of Mutant Bonafide Misfolded Proteins in Cells

Our observation of increased mRNA and protein levels of CHIP in the lanosterol-treated cells led us to evaluate the effect of lanosterol on mutant bonafide misfolded proteins in cells. To test whether lanosterol exposure directly reduces misfolded protein aggregation in cells, we overexpressed previously described [33] green fluorescent protein (GFP) fusions of wild-type chloramphenicol-acetyltransferase (wtCAT) and mutant GFP-Δ9CAT constructs in cells with or without lanosterol. Exogenously expressed GFP-wtCAT proteins diffusely localized in cells and do not form fine aggregates or inclusion-like structures (Fig. 2a); but overexpressed mutant GFP-Δ9CAT bonafide misfolded proteins form distinct cytoplasmic perinuclear aggregates or inclusion bodies in cells, which were also positive for ubiquitin staining (Fig. 2c).

We found that exposure of lanosterol noticeably reduces ubiquitin-positive perinuclear aggregates or inclusion bodies of GFP-Δ9CAT misfolded proteins in cells (Fig. 2c). In our preliminary experiments, we observed that lanosterol treatment increases the endogenous levels of LC3, Lamp2, and co-chaperone CHIP and also decreases p62 levels, which clearly represents high autophagic flux in cells. Therefore, we presumed that, most probably, addition of lanosterol stimulates the clearance of abnormal protein aggregation via activation of selective autophagy pathway. To answer this interpretation, we exposed few similar sets of above-described cells GFP-wtCAT (Fig. 2b) and GFP-Δ9CAT (Fig. 2d) with chloroquine to prompt autophagy dysfunction in cells. Expected treatment of chloroquine aggravates the accumulation of GFP-Δ9CAT misfolded aggregates (Fig. 2d), which were earlier prevented by the addition of lanosterol in the absence of chloroquine (Fig. 2c). These findings indicate that most probably lanosterol treatment may influence the clearance of abnormal proteins via induction of selective autophagy pathway.

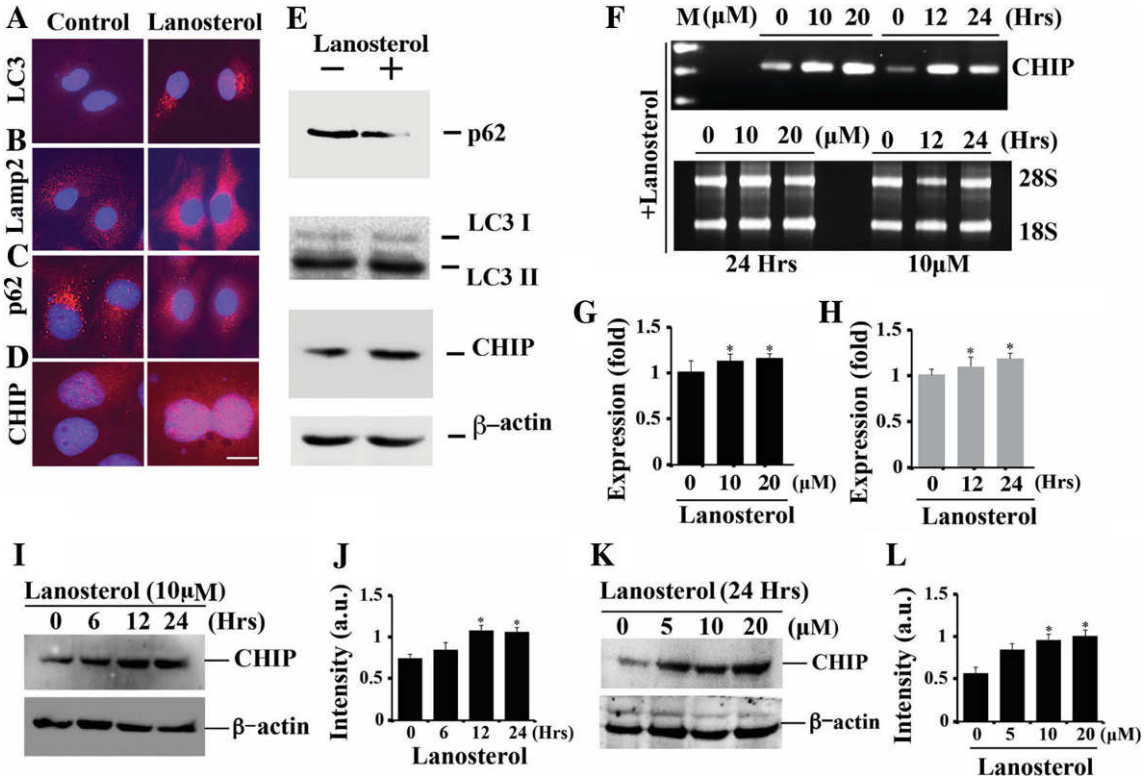

Fig. 1 Lanosterol treatment induces co-chaperone CHIP expression and elevates the levels of CHIP in cells. (a–d) Cos-7 cells were plated into different two-well chamber slides and treated with lanosterol (10 μM) for 24 h. After treatment, cells were processed for fluorescence immunocytochemistry labeled with LC3 (**a**); *Lamp2* (**b**); *p62* (**c**), and *CHIP* (**d**) antibodies as indicated in micrographs. DAPI was used to counterstain nuclei. **e** Similar sets of cells were used for immunoblot analysis and blots were probed with various antibodies as shown in the **f**. A549 cells were plated into 6-well tissue culture plates and treated in concentration- (lanosterol for 24 h) and time-dependent manner (lanosterol 10 μM). Twenty-four hours later, cells were then processed for RNA extraction followed by reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of CHIP and 18S RNA as the internal control, and all reactions were carried out in triplicate with negative controls lacking the template DNA. **g**–**h** Quantitation of CHIP mRNA levels using quantitative real-time RT-PCR in the experiment as described in the previous section in a concentration- (lanosterol for 24 h) (**g**) and time-dependent approach (lanosterol 10 μM) (**h**) after lanosterol treatment. **i**–**l** Cos-7 cells were treated with different time intervals (lanosterol 10 μM) (**i**); and varying concentrations (lanosterol for 24 h) (**k**); following cell lysis, anti-CHIP, and β-actin antibodies were used for immunoblot analysis; and their respective quantification of the band intensities (time (**j**) and concentration (**l**)) was obtained from three different experiments with NIH Image analysis software. The β-actin protein levels in each were used for the normalization of each sample

Lanosterol Enhances the Degradation of Heat-Denatured Luciferase

Regular proteolysis is an important and assured mechanism for regulating the turnover of abnormal or denatured proteins in cells. In our earlier experiment, we noticed that lanosterol enhanced autophagic degradation of mutant bonafide misfolded GFP-Δ9CAT protein. Therefore, now it was important for us to assess if lanosterol treatment also induces the intrinsic protein degradation systems for other misfolded proteins; we next demonstrate the effect of lanosterol in the degradation of the thermally denatured luciferase protein. We overexpressed luciferase plasmid in cells. As shown in Fig. 3a, b, transfected cells were treated with lanosterol and exposed to 43 °C for 30 min and then returned to 37 °C for 2 h of recovery and used for immunofluorescence staining by using antibodies luciferase (green) and CHIP (red).

In few experiments, cells were treated with MG132 (putative proteasome inhibitor) and bafilomycin (a selective autophagy inhibitor) with or without lanosterol before the heat shock exposure. Cells were subjected to luciferase activity assay (Fig. 3c) and immunoblotting analysis (Fig. 3d). As shown in Fig. 3b, lanosterol significantly reduces the intracellular perinuclear accumulation of heat-denatured luciferase protein, which was efficiently prevented by the MG132 and bafilomycin-mediated inhibition of UPS and autophagy, respectively (Fig. 3c). Degradation of heat-denatured luciferase protein was also confirmed by performing an immunoblot using a luciferase antibody; addition of MG132 and bafilomycin prevents the clearance of denatured luciferase protein (Fig. 3d, e). Next, it was critical for us to examine whether the addition of lanosterol in cells affects overall cellular health/fitness or generates cellular toxicity during a course of action in the inhibition of misfolded proteins aggregation. As represented in Fig. 3f, increase in a concentration-

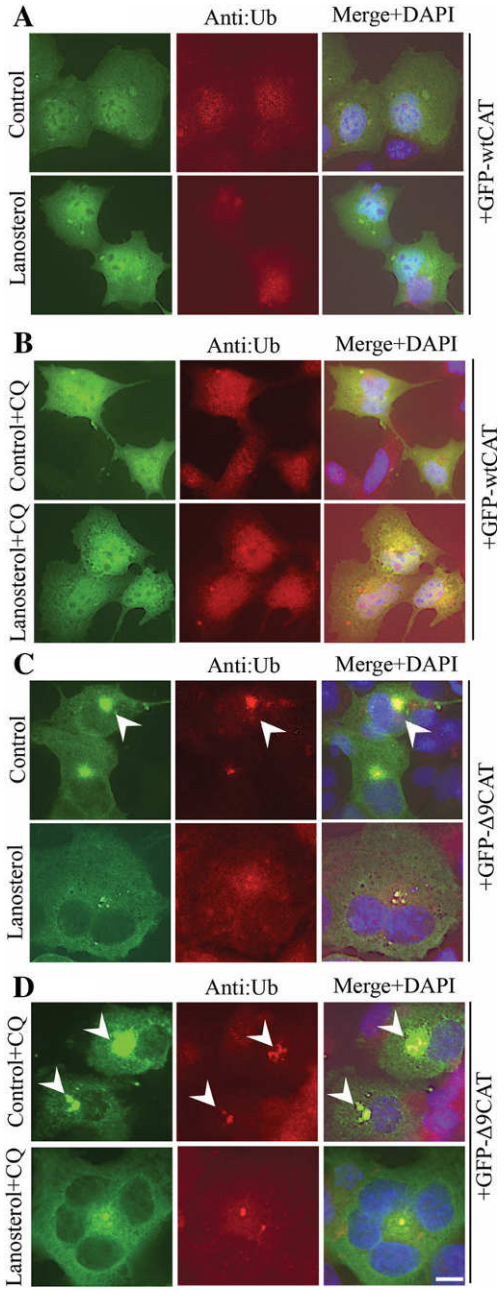

Fig. 2 Lanosterol enhances the clearance of a mutant bonafide misfolded protein. **a**–**d** Cos-7 cells were transiently transfected with GFP-wtCAT (**a**, **b**) and GFP-Δ9CAT (**c**, **d**) for 24 h and subsequently treated with control (DMSO) and lanosterol (10 μM) for 24 h; few sets of cells (**b**, **d**) were also exposed to 20 μM chloroquine (CQ) for 12 h. Post-treated cells were used for immunocytochemistry analysis stained with an ubiquitin antibody. A rhodamine-conjugated secondary antibody was used to stain ubiquitin and nucleus localization was confirmed by using DAPI (blue) staining. Cells were observed with conventional immunofluorescence microscope. The *arrows* indicate the colocalization of ubiquitin with perinuclear cytoplasmic aggregates of GFP-Δ9CAT misfolded proteins. *Scale bars* 20 μm

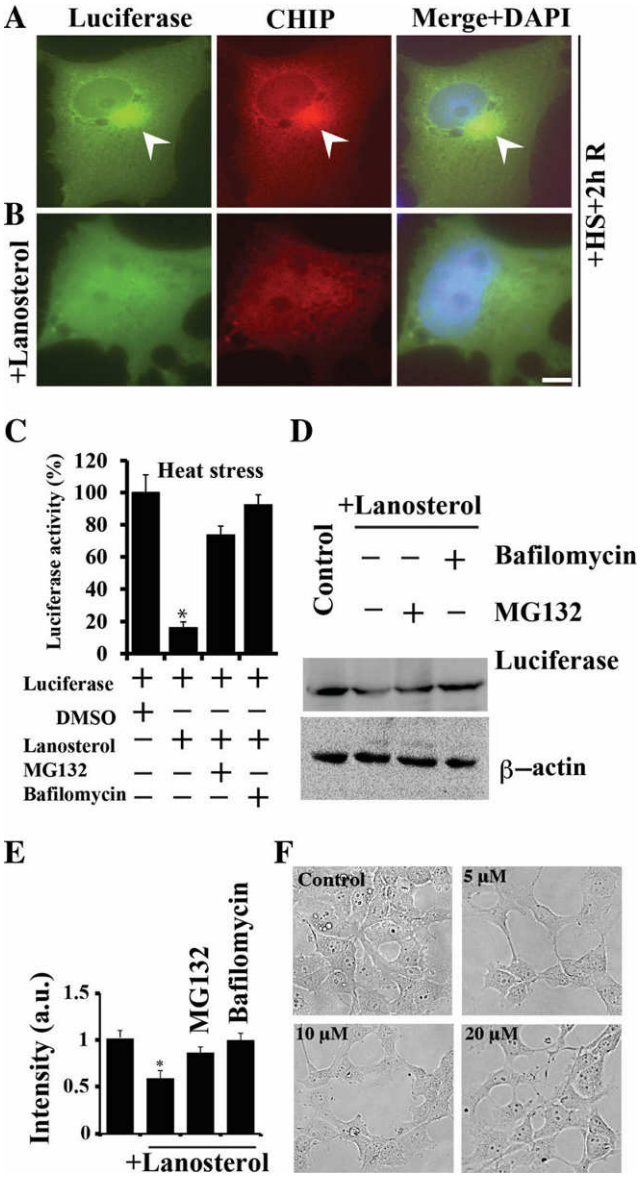

Fig. 3 Effect of lanosterol on the degradation of heat-denatured luciferase protein. **a**–**b** Cells were plated on two-well chamber slides and transient transfection was performed with a firefly luciferase expression construct. After transient transfection, cells were treated with lanosterol (10 μM) for 24 h. Post-treated cells were exposed to 43 °C for 30 min and then returned to 37 °C for 2 h of recovery and processed for immunofluorescence analysis by using antibodies; luciferase (*green*) and CHIP (*red*). Arrowheads represent nuclear peripheral colocalization of misfolded luciferase aggresomes positive for CHIP staining. **c**–**d** As described in above section, few same sets of cells were treated with MG132 (10 μM) and bafilomycin (50 nM) for 12 h and then collected cells were subjected to a luciferase activity assay (**c**) and immunoblot analysis using (**d**) luciferase and β-actin antibodies. **e** As shown in **c**, quantification of band intensities collected from three different experiments was performed by using NIH image analysis software. *$P < 0.05$ compared with the control. **f** Cos-7 cells were treated with DMSO (control) and different concentration of lanosterol for 24 h and after treatment, cells were visualized using a bright-field microscope as shown in micrographs

dependent manner treatment of lanosterol generally do not generate any morphological or apoptotic changes in cells, e.g., shrinkage of cells, loss of contact with the adjacent neighboring cells and membrane blebbing compared to control cells. These results indicate that lanosterol retains a

capability to prevent accumulation of exogenously expressed misfolded protein aggregation towards UPS and autophagic pathway and also do not represent cellular toxicity profile at a lower concentration.

Effects of Lanosterol on Neurodegenerative Disease-Linked Proteins

The process of protein QC is crucial for the degradation of misfolded proteins and molecular chaperones also contribute to the folding mechanism of abnormal proteins and subsequently reduce a load of damaged proteins in cells [16]. Previous results suggest that elevated levels of CHIP and induced autophagy after lanosterol treatment plays a significant role in the elimination of bonafide and modeled misfolded proteins. If lanosterol assists in the prevention of non-disease associated inert misfolded (GFP-Δ9CAT and denatured luciferase) protein aggregation, most probably, it can also effectively suppress toxic protein aggregation in various cell culture models of neurodegenerative diseases. To directly test this important assumption, we used mutant SOD1G37R protein expressing cells as a cellular model of amyotrophic lateral sclerosis (ALS) neurodegenerative disease with or without lanosterol treatment profile.

Cells were transiently transfected with wild-type SOD1 construct (control) and SOD1G37R (mutant) constructs, and after transfections, cells were treated with or without lanosterol and post-treated cells were processed for immunofluorescence staining using anti-ubiquitin antibody. Microscopy analysis results suggest that use of lanosterol dramatically enhances the reduction of mutant SOD1G37R aggregates (Fig. 4c) in cells, and treatment with chloroquine partially blocks the clearance of abnormal intracellular aggregates (Fig. 4d). As clearly seen in Fig. 4b, exposure to chloroquine disturbs autophagy function and even wild-type SOD1 protein is prone to accumulate in near nuclear peripheral region; however, in the absence of chloroquine, wild-type SOD1 protein do not accumulate as an inclusion but diffusely localized in the cytoplasm (Fig. 4a).

Above findings suggest that inhibiting the autophagy-lysosomal mechanism might interfere with lanosterol-mediated effective clearance of mutant SOD1 protein aggregates into cells. Since usage of lanosterol diminishes the aggregation of ALS-linked mutant SOD1 proteins, we next wanted to examine if induction of autophagy and elevated levels of co-chaperone CHIP mediated by lanosterol treatment had the same effect on other neurodegenerative disease-linked mutant α-synuclein (S87A) protein. Treatment with lanosterol resulted in a decrease in the proportion of mutant α-synuclein (S87A) aggregate-containing cells (Fig. 5c). Indeed, chloroquine abrogated the effect of lanosterol on these cells (Fig. 5b, d); consistent with the observation that lanosterol causes a drastic reduction in the aggregation of neurodegenerative disease-linked mutant α-Synuclein (S87A) protein via generating high co-chaperone CHIP availability and elevation of autophagy-mediated protein clearance.

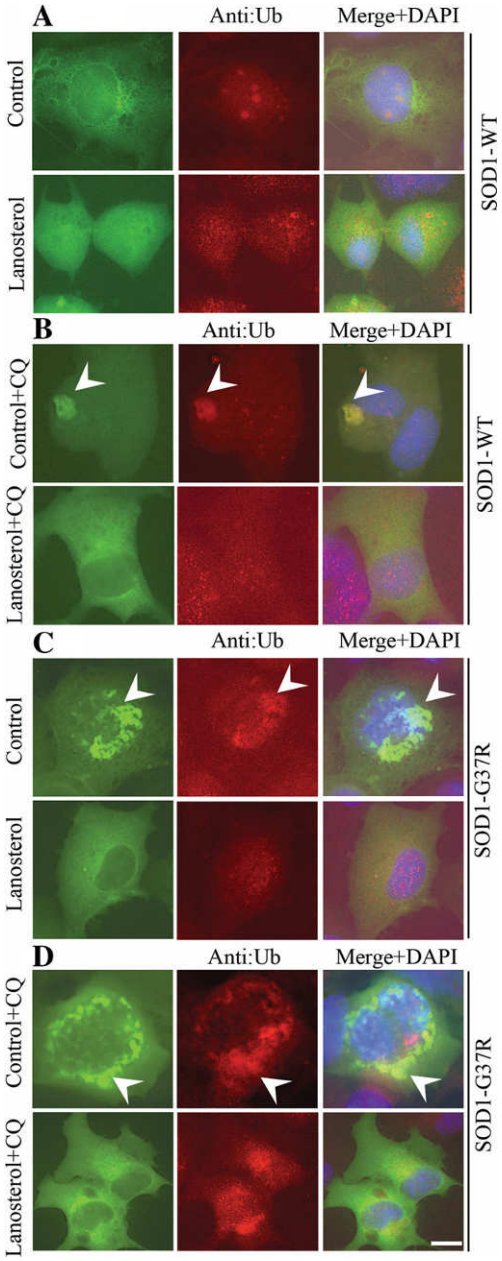

Fig. 4 Lanosterol suppresses the mutant superoxide dismutase 1 (SOD1) aggresomes formation in cells. **a**–**d** Representative micrographs of Cos-7 cells transiently transfected with SOD1-WT (*upper panels* **a**–**b**) and SOD1-G37R (*lower panels* **c**–**d**). Few sets of transfected cells were treated with lanosterol (10 μM) for 24 h and some sets were also exposed to 20 μM chloroquine (CQ) for 12 h, as shown in the micrograph. After treatment, cells were subjected to immunofluorescence analysis by using ubiquitin antibody. DAPI was used for nuclear staining, and the rhodamine-conjugated secondary antibody was used to probe endogenous ubiquitin. *Arrows* indicate the recruitment of ubiquitin to the accumulated misfolded mutant SOD1 inclusions. *Scale bars* 20 μm

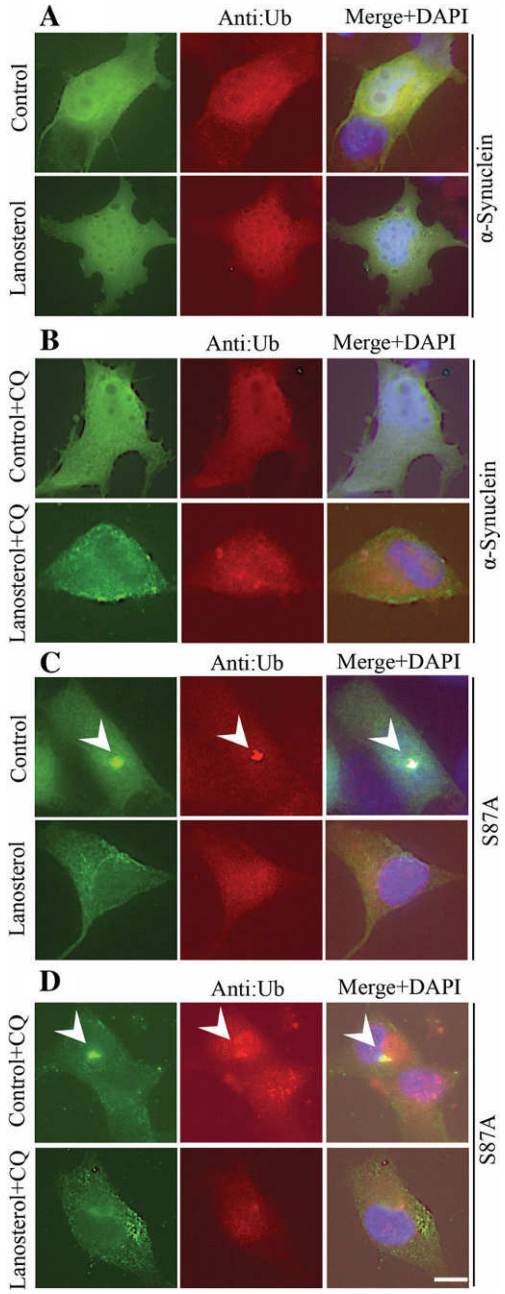

Fig. 5 Treatment of lanosterol reduces aggregation of mutant α-synuclein. (A-B) Wild-type and (C-D) mutant (S87A) alpha-synuclein expression were performed in Cos-7 cells via transient transfection and some post-transfected sets of cells were treated with lanosterol (10 μM) for 24 h with or without 20 μM chloroquine (CQ) for 12 h. Photomicrographs showing the immunofluorescence staining of ubiquitin (red) in cells and nuclei were stained with DAPI (blue). Ubiquitin-positive mutant (S87A) alpha-synuclein inclusion was demonstrated as indicated with white arrowheads at the nuclear peripheral region. *Scale bars* 20 μm

Lanosterol Increased the Stability of Co-Chaperone CHIP

We have observed that lanosterol treatment induces the endogenous expression of CHIP and also reduces the aggregation of bonafide and misfolded proteins in cells. Since CHIP level was increased after lanosterol treatment; therefore, we presumed that there might be a possible interaction of lanosterol with CHIP, which may cause an additive effect on the stability of CHIP. To address this question, we analyzed the possible interaction of lanosterol with CHIP by docking analysis. Binding affinity obtained for the best binding mode was −9.2 kcal/mol. As shown in Fig. 6a–b, docking images that depicted residues involved in hydrophobic interaction were Leu72, Lys73, Leu69, Phe99, and Phe132; hydrogen bond was obtained between OE1 of Glu107 and oxygen of lanosterol. To obtain a possible clue, related to the effect of lanosterol on endogenous levels of CHIP, now it was important for us to observe the direct influence of lanosterol on endogenously available CHIP. Therefore, we next treated Cos-7 cells with control (DMSO), either alone or along with lanosterol. After treatment, cells were chased with cycloheximide. As shown in Fig. 6c, d, in the presence of lanosterol, CHIP is degraded at a much slower rate compared to the control. It was an interesting observation that treatment of lanosterol induced stabilization of CHIP (Fig. 6c–e). All the above findings suggest that use of lanosterol enhanced a progressive stability into the endogenous levels of CHIP and which may probably, additionally contributes in the clearance of aberrant proteins from the cells.

Lanosterol Diminishes Aggregation of Expanded Polyglutamine Proteins in Cells

A previous report [25] and our current experiments clearly indicate that lanosterol has a central role in reducing the aggregation of misfolded or disease-causing aggregated proteins. We next analyzed if this compound could affect the accumulation propensity of pathogenic-expanded polyglutamine inclusions implicated in the pathomechanisms of neurodegenerative diseases. To test this notion and observe the direct effect of lanosterol on polyglutamine aggregates, we performed a detailed immunofluorescence analysis with ataxin-3-expanded polyglutamine aggregates in cells. For this experiment, we used normal ataxin-3(28Q) (Fig. 7a, b) and expanded polyglutamine ataxin-3(84Q)-expressing cells (Fig. 7c, d) in the presence or absence of lanosterol. To further clarify the proactive effect of lanosterol on selective autophagy degradation mechanism, we carried out some experiments under autophagy inhibitory conditions (20 μM chloroquine (CQ) for 12 h).

We found that use of lanosterol potently diminished the occurrence of cytoplasmic inclusions of expanded polyglutamine ataxin-3(84Q) (Fig. 7c); which was partially reverted in the form of numerous small cytoplasmic inclusions near the periphery of nucleus by the dysfunction of autophagy with the use of chloroquine (Fig. 7d). In contrast to ubiquitin-positive aggregates of ataxin-3(84Q) inclusions, in respective control, normal glutamine repeat expressing ataxin-3(28Q) protein, they were

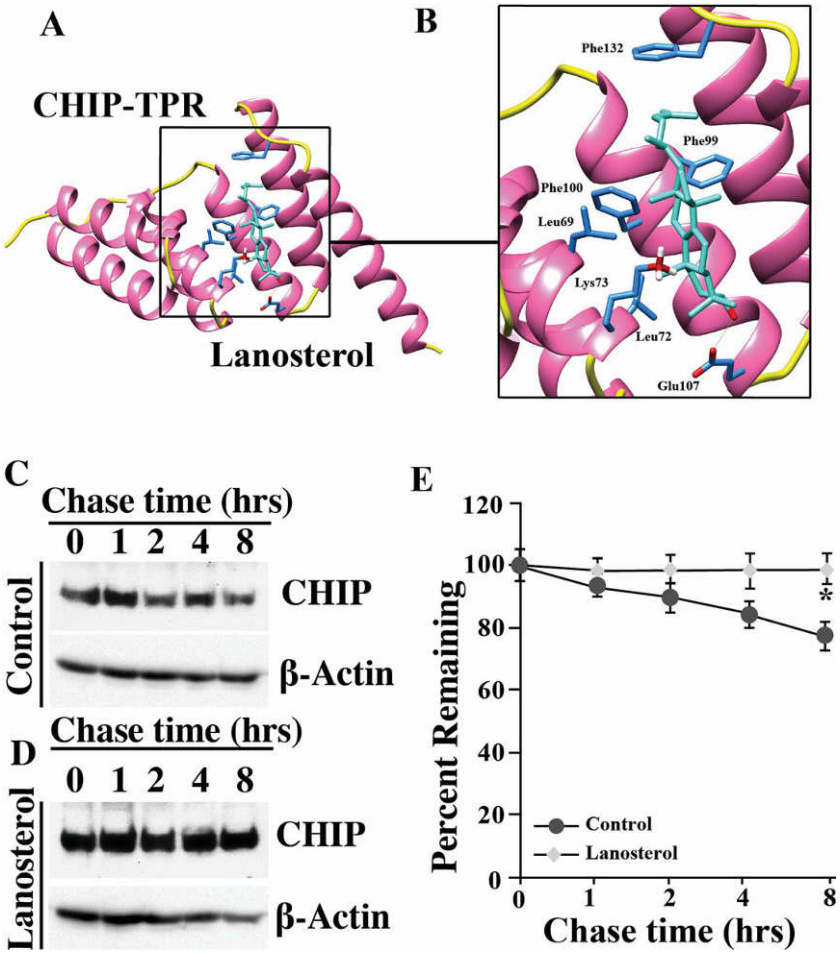

Fig. 6 Lanosterol stabilizes co-chaperone CHIP. **a**–**b** Representation of interaction of lanosterol with CHIP TPR domain; *right panel* shows **b** a close-up view of this interaction. Lanosterol is shown in cyan color. Amino acid residues of TPR domain interacting with lanosterol are also depicted. **c**–**d** Cos-7 cells were plated into 6-well culture plates. In the next day, cells were treated with control (DMSO), lanosterol (10 μM) for 24 h and cycloheximide (15 μg/ml) and chased for different periods. Blots were detected with CHIP and β-actin antibodies. **e** Blot band intensities from three independent experiments were quantified using NIH Image analysis software. Values are the mean ± SD

soluble and diffusely localized throughout cytoplasmic and nuclear compartments. These observations are consistent with our findings that lanosterol induces loss of specifically misfolded cytosolic proteins in cells.

Although the above result was promising but till now, at least, nine polyglutamine- expansion containing neurodegenerative diseases have been reported [34]. In our current work, we found that treatment of lanosterol efficiently diminished aggregation of expanded polyglutamine non-native intracellular inclusions of ataxin-3 protein. To evaluate whether the addition of lanosterol can also prevent aggregation of another externally applied polyglutamine expansion protein; we used huntingtin-normal (EGFP-HDQ23) (Fig. 8a, b) and expanded EGFP-HDQ74 (Fig. 8c, d) polyglutamine proteins for immunofluorescence staining.

When we immunocytochemically examined lanosterol-treated EGFP-HDQ74 cells for inclusion formation by using ubiquitin antibody, which specifically recognized robust aggresomes like proteinaceous bodies, we observed a marked reduction in ubiquitin-positive intracellular accumulation of expanded EGFP-HDQ74 polyglutamine proteins (Fig. 8c). The effect of lanosterol was partially reverted after disturbing autophagy mechanism by the use of chloroquine (Fig. 8d). As a control of expanded glutamine repeats, normal glutamine repeats (EGFP-HDQ23) represent diffused GFP fluorescence pattern (Fig. 8a, b). Overall, these results suggest that lanosterol contributes in the mitigation of neurodegenerative disease-causing polyglutamine proteins.

Lanosterol Rescues Stress-Induced Cell Death and Suppresses Aggregation of Deleterious Intracellular Protein Aggregation

Cellular health is compromised primarily due to the formation of insoluble proteinaceous inclusions, which serve as pivotal hallmark of different neurodegenerative diseases. However, the mechanistic solution to get rid of the multifactorial toxic accumulation of unwanted proteins remains unknown. It was crucial for us to directly observe the effects of lanosterol on the deposition of various protein aggregates under cellular conditions and to perform quantitative analysis of the insoluble forms of the mutant proteins, after lanosterol treatment. Therefore, next, we performed a dot-blot analysis with cellular extracts from cells expressing different misfolded proteins, as depicted in Fig. 9a. Quantification of these dot-blot analyzes suggested that exogenously applied lanosterol dramatically

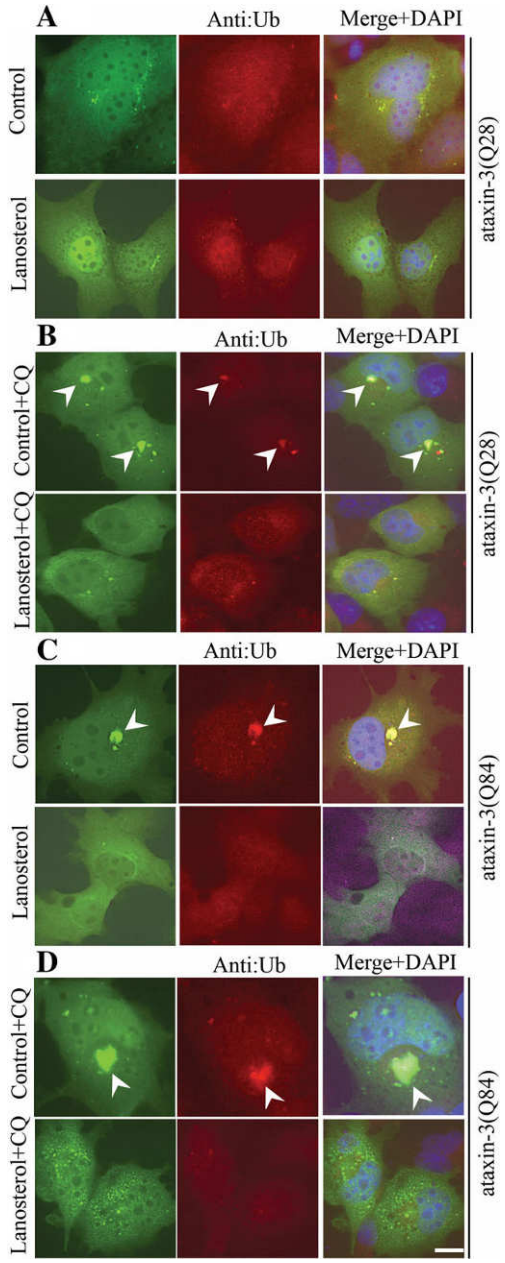

Fig. 7 Lanosterol induces suppression of pathogenic ataxin-3 polyglutamine protein aggregation in cells. **a**–**d** Cos-7 cells were plated into two-well chamber slides and transiently transfected with ataxin-3-GFP fusion with normal (ataxin-3(Q28)) (**a**–**b**) or expanded polyglutamine repeats (ataxin-3(Q84)) (**c**–**d**) constructs. After transfection, cells were exposed with lanosterol (10 μM) and 20 μM chloroquine (CQ), as indicated in the micrograph. To detect colocalization of ubiquitin with ataxin-3(Q28) and ataxin-3(Q84) aggregates, cells were probed with anti-ubiquitin during immunofluorescence analysis and DAPI (*blue*) was used to stain nuclei in cells. *Arrowheads* indicate the recruitment of ubiquitin at the site of ataxin-3-GFP polyglutamine aggregate formation. *Scale bars* 20 μm

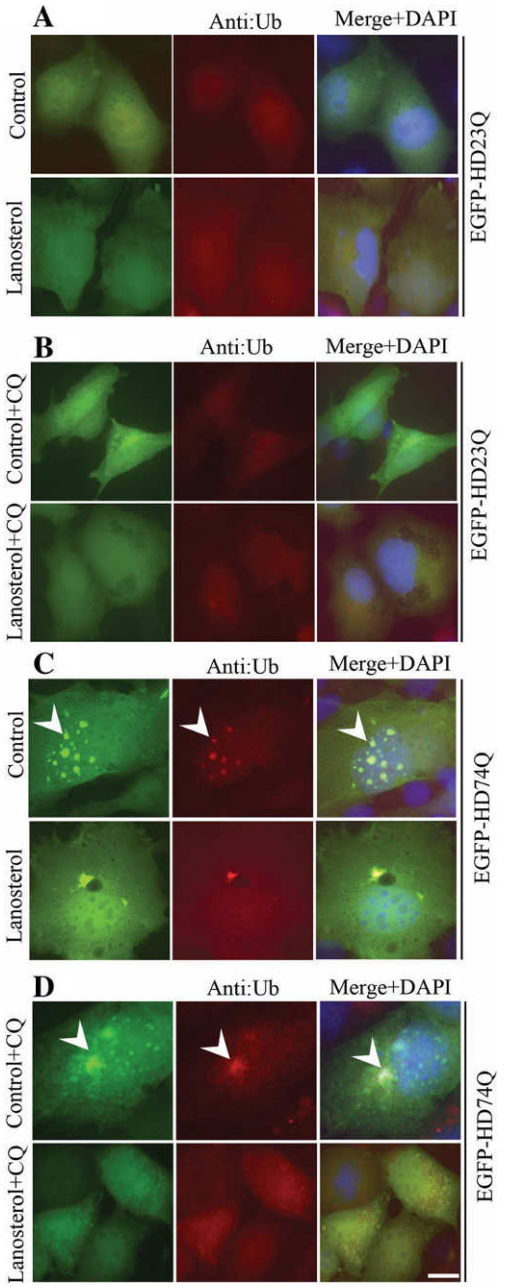

Fig. 8 Treatment of lanosterol results in decrease of cytoplasmic misfolded inclusions of expanded huntingtin polyglutamine aggregates. **a**–**d** Immunofluorescence microscopic images of the Cos-7 cells expressing the EGFP-HD23Q (**a**–**b**) and EGFP-HD74Q protein (**c**–**d**), treated with lanosterol (10 μM) and 20 μM chloroquine (CQ) as shown in the figure and stained with an anti-ubiquitin antibody to detect the expanded polyglutamine ubiquitin-positive protein aggregates (*red*) and DAPI for nuclear staining (*blue*). *Scale bars* 20 μm

reduced the aggregation profile of various misfolded proteins and they became more soluble in nature as shown in Fig. 9b. Using this approach, we analyzed the ability of the lanosterol to modify the aggregation state of misfolded proteins under cellular conditions.

It was previously reported that misfolded proteins generate different cellular stress conditions in cells and consequently produce a massive buildup of abnormal and damaged proteins and in response; cells try to cope with such cytotoxic conditions [2, 1]. However, recent study [25] and our current results suggest that lanosterol usage reduces aggregation of misfolded

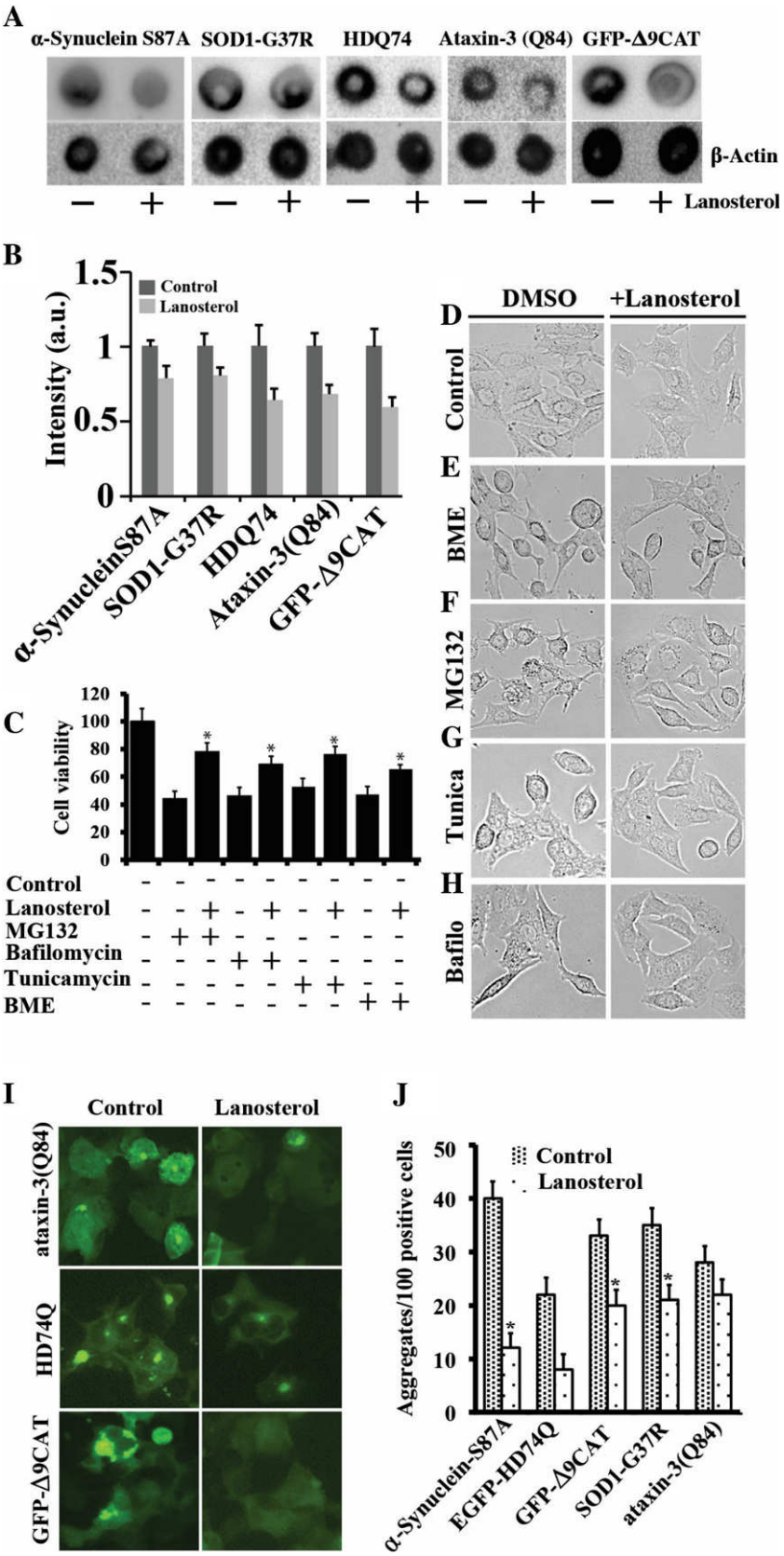

Fig. 9 Lanosterol reduces misfolded protein aggregation and also alleviates stress-induced cytotoxicity. **a** As shown in the figure, Cos-7 cells were transiently transfected with various expression constructs to generate misfolded and aggregated proteins in cells with or without lanosterol (10 μM). After treatment, the dot-blot analysis was subjected with the extracts from the cells and blots were detected by immunoassays with anti-synuclein, anti-SOD1, anti-GFP, and anti-β-actin antibodies. **b** Quantification of the dot-blot results as represented in **a**. Different aggregated protein inclusions were quantified by densitometric analysis with NIH image analysis software. **c** Cos-7 cells were treated with 10 μM MG132 for 10 h, 50 nM bafilomycin for 12 h, 10 μg/ml tunicamycin for 10 h, and 5 mM β-mercaptoethanol (BME) for 3 h. Few sets of similar experiments were carried out in the presence of lanosterol (10 μM) for 24 h. After treatment cell viability was measured by MTT assay. Values are the means ± SD of three independent experiments, each performed in triplicate. $^*P < 0.05$ as compared with control (DMSO). **d**–**h** As described previously in **c**, cells were treated with the various stressors in the presence and absence of lanosterol (10 μM) and observed using a bright-field microscope. **i** Cells were treated with lanosterol (10 μM) as shown in the figure; after 24 h, aggregation and quantitation were monitored for ataxin-3(Q84), EGFP-HDQ74 and GFP-Δ9CAT under a fluorescence microscope. **j** Quantitation of various misfolded protein aggregates in cells, transfected with their respective mutant plasmids and treated with lanosterol (10 μM). The results are presented ±SD of three independent experiments each performed in triplicate. $^*P < 0.05$ compared with the control treatment

proteins and thus may provide cytoprotection against misfolded protein-mediated stress-induced toxic insults. Therefore, we tested the effect of lanosterol under various cellular stress conditions; we treated cells with stress-inducing agents (MG132-

proteasomal dysfunction, bafilomycin-autophagy dysfunction, tunicamycin, and β-mercaptoethanol-ER dysfunction) that caused the reduction in cell viability. After treatment, cells were subjected to MTT assay (Fig. 9c) and some cells were visualized using bright-field microscopy (Fig. 9d–h). These results strongly substantiate that lanosterol contributes in cellular defense mechanisms in response to the multifactorial toxic effects caused by various stress-inducing agents.

Since lanosterol produced the decrease in insoluble mutant misfolded proteins and also provide cytoprotection against misfolded protein aggregation, we further analyzed the effects of lanosterol on the propensity of aberrant protein aggregate formation. As shown in Fig. 9i, ataxin-3(84Q), EGFP-HDQ74, and GFP-Δ9CAT expressing cells were treated with or without lanosterol and visualized under fluorescence microscope. This observation suggests that the addition of lanosterol intensified the reduction of misfolded protein aggregation in cells. To further validate this observation, we performed another detailed experiment including other non-native proteins and found that addition of lanosterol seems effective to reduce overall aggregation load in cells generated by various misfolded proteins (Fig. 9i–j). Our findings indicate that lanosterol promotes the reduction of cytotoxic aggregate formation in cells, which also provides protection against intracellular proteotoxic effects mediated by over accumulation of misfolded proteins.

Discussion

Our present study demonstrates that treatment of lanosterol not only induces expression of co-chaperone CHIP and autophagy pathway but the presence of lanosterol can also efficiently stabilized CHIP endogenous levels. Therefore, most likely the elevated and stabilized levels of CHIP may promote degradation of misfolded proteins with the help of proteasome system; simultaneously, lanosterol-mediated induced autophagy mechanism can also contribute to the clearance of misfolded proteins aggregation. These results provide compelling evidence that lanosterol treatment plays a crucial and pivotal cytoprotective function to alleviate mutant misfolded protein-mediated proteotoxicity.

Interestingly, there are accumulating evidences addressing the importance of small molecular approaches, application of chaperones, and co-chaperones for the degradation of misfolded or damaged proteins generated in cells [9, 35, 36]. Previously, it has been shown that CHIP performs dual function: as an ubiquitin ligase of the U-box family and as a co-chaperone, which also recognizes various chaperone substrates and misfolded proteins for their degradation [37–40]. Treatment of carbenoxolone (CBX), a glycyrrhizic acid derivative, upregulates Hsp70 chaperone and decreases α-synuclein aggregation in neuroglioma cells [41]. Similarly, YM-1, a stable and soluble analog of MKT-077, promotes Hsp70 binding to unfolded substrates and this synthetic co-chaperone induces client protein degradation [42].

Recently, it has been shown that the use of lanosterol reduces aggregation of mutant crystallin proteins and also improves dissolution of preformed amyloid-like fibrils of crystallin proteins, which in turn reduces cataract formation [25]. Another report in similar context demonstrates that treatment of pharmacological chaperones binds with α-crystallins (cryAA and cryAB), which reversed their aggregation in vitro and partially repairs transparency in cataract models [43]. These studies suggest a functional link between small molecules; chaperone systems and cellular proteolytic machinery which can also open new promising horizons and wide prospects in neurodegenerative and late age-of-onset diseases that are caused by protein aggregation. Taken together, the findings of all the above studies, which also support our current results that lanosterol treatment elevates the endogenous levels of co-chaperone CHIP, improve its stability that most likely contributes to the degradation of bonafide and denatured misfolded proteins.

In our current study, we also observed that lanosterol treatment dramatically induces and stabilizes CHIP levels. We also observed that addition of lanosterol decreases pathogenic aggregation of mutant (SOD1, α-Synuclein, and expanded polyglutamine) proteins in cells. Earlier studies also suggest that CHIP is responsible for the proteasomal degradation of ALS-linked mutant SOD1 proteins [44]. CHIP also promotes α-Synuclein degradation by two discrete mechanisms, i.e., (1) proteasomal-dependent degradation and (2) lysosomal pathway; this study also suggests that CHIP acts as a molecular switch between two proteolytic mechanisms [45]. Subsequently, it was found that CHIP interacts with expanded polyglutamine proteins and degrades polyglutamine proteins, which suppresses polyQ aggregation and associated cytotoxicity [46, 47]. Overall, these studies and current results support our present observation that most likely, in the presence of lanosterol, elevation, and stabilization of CHIP acts as a critical molecular switch for both proteasomal and lysosomal mechanisms, which can efficiently reduce aggregation of misfolded proteins.

Previous study showed that cooperative function of BAG-3 and CHIP facilitates ubiquitin-mediated selective autophagy and provide muscle maintenance via degradation of filamin [48]. Interestingly, another study suggest that oral administration of a disaccharide, i.e., trehalose, reduces the formation of truncated huntingtin protein aggregates and alleviates the polyglutamine-induced pathology in a transgenic mouse model of Huntington's disease [49]. In our present study, we observed that treatment of lanosterol not only reduces aggregation of various misfolded proteins but also provides cytoprotection against deleterious effects of proteotoxic misfolded proteins and protects against stress-induced cell

death. Taken together, all these studies along with our present results suggest that selective and proficient elimination of damaged proteins, chiefly by the use of small molecules, can offer a well-tractable therapeutic approach against protein misfolding and neurodegenerative diseases in the future.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interests.

References

1. Woerner AC, Frottin F, Hornburg D, Feng LR, Meissner F, Patra M, Tatzelt J, Mann M et al (2016) Cytoplasmic protein aggregates interfere with nucleocytoplasmic transport of protein and RNA. Science 351(6269):173–176. doi:10.1126/science.aad2033
2. Chhangani D, Mishra A (2013) Protein quality control system in neurodegeneration: a healing company hard to beat but failure is fatal. Mol Neurobiol 48(1):141–156. doi:10.1007/s12035-013-8411-0
3. Chhangani D, Mishra A (2013) Mahogunin ring finger-1 (MGRN1) suppresses chaperone-associated misfolded protein aggregation and toxicity. Sci Rep 3:1972. doi:10.1038/srep01972
4. Kaganovich D, Kopito R, Frydman J (2008) Misfolded proteins partition between two distinct quality control compartments. Nature 454(7208):1088–1095. doi:10.1038/nature07195
5. Olzscha H, Schermann SM, Woerner AC, Pinkert S, Hecht MH, Tartaglia GG, Vendruscolo M, Hayer-Hartl M et al (2011) Amyloid-like aggregates sequester numerous metastable proteins with essential cellular functions. Cell 144(1):67–78. doi:10.1016/j.cell.2010.11.050
6. Ciechanover A, Kwon YT (2015) Degradation of misfolded proteins in neurodegenerative diseases: therapeutic targets and strategies. Exp Mol Med 47:e147. doi:10.1038/emm.2014.117
7. Bennett EJ, Bence NF, Jayakumar R, Kopito RR (2005) Global impairment of the ubiquitin-proteasome system by nuclear or cytoplasmic protein aggregates precedes inclusion body formation. Mol Cell 17(3):351–365. doi:10.1016/j.molcel.2004.12.021
8. Cohen FE, Kelly JW (2003) Therapeutic approaches to protein-misfolding diseases. Nature 426(6968):905–909. doi:10.1038/nature02265
9. Chaudhuri TK, Paul S (2006) Protein-misfolding diseases and chaperone-based therapeutic approaches. FEBS J 273(7):1331–1349. doi:10.1111/j.1742-4658.2006.05181.x
10. Yang YP, Hu LF, Zheng HF, Mao CJ, Hu WD, Xiong KP, Wang F, Liu CF (2013) Application and interpretation of current autophagy inhibitors and activators. Acta Pharmacol Sin 34(5):625–635. doi:10.1038/aps.2013.5
11. Sarkar N, Kumar M, Dubey VK (2011) Exploring possibility of promiscuity of amyloid inhibitor: studies on effect of selected compounds on folding and amyloid formation of proteins. Process Biochem 46(5):1179–1185. doi:10.1016/j.procbio.2011.02.010
12. Parenti G (2009) Treating lysosomal storage diseases with pharmacological chaperones: from concept to clinics. EMBO Mol Med 1(5):268–279. doi:10.1002/emmm.200900036
13. Haynes CM, Titus EA, Cooper AA (2004) Degradation of misfolded proteins prevents ER-derived oxidative stress and cell death. Mol Cell 15(5):767–776. doi:10.1016/j.molcel.2004.08.025
14. Pan JA, Ullman E, Dou Z, Zong WX (2011) Inhibition of protein degradation induces apoptosis through a microtubule-associated protein 1 light chain 3-mediated activation of caspase-8 at intracellular membranes. Mol Cell Biol 31(15):3158–3170. doi:10.1128/MCB.05460-11
15. Upadhyay A, Amanullah A, Chhangani D, Joshi V, Mishra R, Mishra A (2015) Ibuprofen induces mitochondrial-mediated apoptosis through proteasomal dysfunction. Mol Neurobiol. doi:10.1007/s12035-015-9603-6
16. Hartl FU, Bracher A, Hayer-Hartl M (2011) Molecular chaperones in protein folding and proteostasis. Nature 475(7356):324–332. doi:10.1038/nature10317
17. Zhang Y, Ahn YH, Benjamin IJ, Honda T, Hicks RJ, Calabrese V, Cole PA, Dinkova-Kostova AT (2011) HSF1-dependent upregulation of Hsp70 by sulfhydryl-reactive inducers of the KEAP1/NRF2/ARE pathway. Chem Biol 18(11):1355–1361. doi:10.1016/j.chembiol.2011.09.008
18. Wright JM, Zeitlin PL, Cebotaru L, Guggino SE, Guggino WB (2004) Gene expression profile analysis of 4-phenylbutyrate treatment of IB3-1 bronchial epithelial cell line demonstrates a major influence on heat-shock proteins. Physiol Genomics 16(2):204–211. doi:10.1152/physiolgenomics.00160.2003
19. Jurivich DA, Sistonen L, Sarge KD, Morimoto RI (1994) Arachidonate is a potent modulator of human heat shock gene transcription. Proc Natl Acad Sci U S A 91(6):2280–2284
20. Teiten MH, Reuter S, Schmucker S, Dicato M, Diederich M (2009) Induction of heat shock response by curcumin in human leukemia cells. Cancer Lett 279(2):145–154. doi:10.1016/j.canlet.2009.01.031
21. Mori M, Li G, Abe I, Nakayama J, Guo Z, Sawashita J, Ugawa T, Nishizono S et al (2006) Lanosterol synthase mutations cause cholesterol deficiency-associated cataracts in the Shumiya cataract rat. J Clin Invest 116(2):395–404. doi:10.1172/JCI20797
22. Song BL, Javitt NB, DeBose-Boyd RA (2005) Insig-mediated degradation of HMG CoA reductase stimulated by lanosterol, an intermediate in the synthesis of cholesterol. Cell Metab 1(3):179–189. doi:10.1016/j.cmet.2005.01.001

23. Huff MW, Telford DE (2005) Lord of the rings—the mechanism for oxidosqualene:lanosterol cyclase becomes crystal clear. Trends Pharmacol Sci 26(7):335–340. doi:10.1016/j.tips.2005.05.004
24. Lim L, Jackson-Lewis V, Wong LC, Shui GH, Goh AX, Kesavapany S, Jenner AM, Fivaz M et al (2012) Lanosterol induces mitochondrial uncoupling and protects dopaminergic neurons from cell death in a model for Parkinson's disease. Cell Death Differ 19(3):416–427. doi:10.1038/cdd.2011.105
25. Zhao L, Chen XJ, Zhu J, Xi YB, Yang X, Hu LD, Ouyang H, Patel SH et al (2015) Lanosterol reverses protein aggregation in cataracts. Nature 523(7562):607–611. doi:10.1038/nature14650
26. Chhangani D, Upadhyay A, Amanullah A, Joshi V, Mishra A (2014) Ubiquitin ligase ITCH recruitment suppresses the aggregation and cellular toxicity of cytoplasmic misfolded proteins. Sci Rep 4:5077. doi:10.1038/srep05077
27. Chhangani D, Endo F, Amanullah A, Upadhyay A, Watanabe S, Mishra R, Yamanaka K, Mishra A (2015) Mahogunin ring finger 1 confers cytoprotection against mutant SOD1 aggresomes and is defective in an ALS mouse model. Neurobiol Dis 86:16–28. doi:10.1016/j.nbd.2015.11.017
28. Trott O, Olson AJ (2010) AutoDock Vina: improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. J Comput Chem 31(2):455–461. doi:10.1002/jcc.21334
29. Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, Meng EC, Ferrin TE (2004) UCSF chimera—a visualization system for exploratory research and analysis. J Comput Chem 25(13): 1605–1612. doi:10.1002/jcc.20084
30. Stierand K, Maass PC, Rarey M (2006) Molecular complexes at a glance: automated generation of two-dimensional complex diagrams. Bioinformatics 22(14):1710–1716. doi:10.1093/bioinformatics/btl150
31. Bjørkøy G, Lamark T, Pankiv S, Øvervatn A, Brech A, Johansen T (2009) Chapter 12 Monitoring autophagic degradation of p62/SQSTM1. In: Methods in Enzymology, vol 452. Academic Press, New York, p 181–197. doi:10.1016/S0076-6879(08)03612-4
32. Komatsu M, Ichimura Y (2010) Physiological significance of selective degradation of p62 by autophagy. FEBS Lett 584(7):1374–1378. doi:10.1016/j.febslet.2010.02.017
33. Arslan MA, Chikina M, Csermely P, Soti C (2012) Misfolded proteins inhibit proliferation and promote stress-induced death in SV40-transformed mammalian cells. FASEB journal: official publication of the Federation of American Societies for Experimental Biology 26(2):766–777. doi:10.1096/fj.11-186197
34. Sisodia SS (1998) Nuclear inclusions in glutamine repeat disorders: are they pernicious, coincidental, or beneficial? Cell 95(1):1–4
35. Suzuki Y (2014) Emerging novel concept of chaperone therapies for protein misfolding diseases. Proceedings of the Japan Academy Series B, Physical and biological sciences 90(5):145–162
36. Perlmutter DH (2002) Chemical chaperones: a pharmacological strategy for disorders of protein folding and trafficking. Pediatr Res 52(6):832–836. doi:10.1203/00006450-200212000-00004
37. Connell P, Ballinger CA, Jiang J, Wu Y, Thompson LJ, Hohfeld J, Patterson C (2001) The co-chaperone CHIP regulates protein triage decisions mediated by heat-shock proteins. Nat Cell Biol 3(1):93–96. doi:10.1038/35050618
38. Meacham GC, Patterson C, Zhang W, Younger JM, Cyr DM (2001) The Hsc70 co-chaperone CHIP targets immature CFTR for proteasomal degradation. Nat Cell Biol 3(1):100–105. doi:10.1038/35050509
39. Xu W, Marcu M, Yuan X, Mimnaugh E, Patterson C, Neckers L (2002) Chaperone-dependent E3 ubiquitin ligase CHIP mediates a degradative pathway for c-ErbB2/Neu. Proc Natl Acad Sci U S A 99(20):12847–12852. doi:10.1073/pnas.202365899
40. McDonough H, Patterson C (2003) CHIP: a link between the chaperone and proteasome systems. Cell Stress Chaperones 8(4):303–308
41. Kilpatrick K, Novoa JA, Hancock T, Guerriero CJ, Wipf P, Brodsky JL, Segatori L (2013) Chemical induction of Hsp70 reduces alpha-synuclein aggregation in neuroglioma cells. ACS Chem Biol 8(7): 1460–1468. doi:10.1021/cb400017h
42. Wang AM, Miyata Y, Klinedinst S, Peng HM, Chua JP, Komiyama T, Li X, Morishima Y et al (2013) Activation of Hsp70 reduces neurotoxicity by promoting polyglutamine protein degradation. Nat Chem Biol 9(2):112–118. doi:10.1038/nchembio.1140
43. Makley LN, McMenimen KA, DeVree BT, Goldman JW, McGlasson BN, Rajagopal P, Dunyak BM, McQuade TJ et al (2015) Pharmacological chaperone for alpha-crystallin partially restores transparency in cataract models. Science 350(6261):674–677. doi:10.1126/science.aac9145
44. Urushitani M, Kurisu J, Tateno M, Hatakeyama S, Nakayama K, Kato S, Takahashi R (2004) CHIP promotes proteasomal degradation of familial ALS-linked mutant SOD1 by ubiquitinating Hsp/Hsc70. J Neurochem 90(1):231–244. doi:10.1111/j.1471-4159.2004.02486.x
45. Shin Y, Klucken J, Patterson C, Hyman BT, McLean PJ (2005) The co-chaperone carboxyl terminus of Hsp70-interacting protein (CHIP) mediates alpha-synuclein degradation decisions between proteasomal and lysosomal pathways. J Biol Chem 280(25): 23727–23734. doi:10.1074/jbc.M503326200
46. Miller VM, Nelson RF, Gouvion CM, Williams A, Rodriguez-Lebron E, Harper SQ, Davidson BL, Rebagliati MR et al (2005) CHIP suppresses polyglutamine aggregation and toxicity in vitro and in vivo. J Neurosci 25(40): 9152–9161. doi:10.1523/JNEUROSCI.3001-05.2005
47. Jana NR, Dikshit P, Goswami A, Kotliarova S, Murata S, Tanaka K, Nukina N (2005) Co-chaperone CHIP associates with expanded polyglutamine protein and promotes their degradation by proteasomes. J Biol Chem 280(12):11635–11640. doi:10.1074/jbc.M412042200
48. Arndt V, Dick N, Tawo R, Dreiseidler M, Wenzel D, Hesse M, Furst DO, Saftig P et al (2010) Chaperone-assisted selective autophagy is essential for muscle maintenance. Curr Biol 20(2):143–148. doi:10.1016/j.cub.2009.11.022
49. Tanaka M, Machida Y, Niu S, Ikeda T, Jana NR, Doi H, Kurosawa M, Nekooki M et al (2004) Trehalose alleviates polyglutamine-mediated pathology in a mouse model of Huntington disease. Nat Med 10(2):148–154. doi:10.1038/nm985