3
Lanosterol Suppresses the Aggregation and Cytotoxicity of Misfolded Proteins Linked with Neurodegenerative Diseases

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 [Haynes *et al.*, 2004; Pan *et al.*, 2011]. 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, including autophagy and UPS [Hartl *et al.*, 2011]. How mutations or lack of functions of protein folding and degradation machinery induce aggregation and unsolicited pathogenic problems in cells is still not well known. Recent advances on the effectiveness 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.

Lanosterol, a precursor molecule of steroid hormones in our body also regulates the activity of HMGCR in cholesterol biosynthesis pathway; whereas mutation in the gene lanosterol synthase is reported in cataratctous rat strain [Mori *et al.*, 2006; Song *et al.*, 2005]. Interestingly, it has also been reported to protect dopaminergic neurons from MPTP-induced neurotoxicity via induction of cellular autophagy [Lim *et al.*, 2012]. In a recent study based on the dogs and rabbits, it has been reported that treatment of lanosterol suppresses aggregation of eye lens crystallins and provides transparency to the cataractous eyes [Zhao *et al.*, 2015]. However, the precise molecular mechanism behind lanosterol-mediated disaggregation of already formed protein aggregates is not reported so far.

3.1 RESULTS

Multiple studies in the past have elaborated the possible therapeutic advantages of plethora of natural molecules in many disease-linked pathological conditions. In the present study, effects of lanosterol treatment on the cellular PQC machinery; and hence its implications in ameliorating the toxic effects of various neurodegenerative disease associated aggregatory proteins have been observed. The results obtained are presented below:

3.1.1 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 [Zhao *et al.*, 2015]. 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, expression levels of various cellular QC components (i.e., UPS and autophagy) have been examined inside the cells in presence of lanosterol.

It has been found that various critical components of the autophagy-lysosomal pathway, such as LC3 and Lamp2, were highly upregulated during lanosterol treatment in cells (Fig. 3.1a, b). Previously, it was reported that autophagy inhibition leads to the accumulation of Sequestosome 1 (SQSTM1 or p62); and when autophagy is induced, p62 endogenous levels might get decreased representing increased autophagic flux [Bjørkøy *et al.*, 2009; Komatsu and Ichimura, 2010].

In the present study, a reduced intracellular level of p62 was observed after lanosterol treatment that indicates the selective and effective participation of p62 to transport ubiquitinated proteins into lysosomes via protective autophagy pathway (Fig. 3.1c). Immunoblotting also confirmed that p62 protein level was decreased after lanosterol treatment in cells. Effect of lanosterol on different crucial components of QC pathway were also monitored; and surprisingly it has been observed that lanosterol exposure dramatically induces endogenous levels of co-chaperone CHIP inside the cells (Fig. 3.1d) that was further confirmed by immunoblot analysis using anti-CHIP antibody (Fig. 3.1e). It was important to know how lanosterol treatment dramatically elevates endogenous levels of CHIP protein inside the cells.

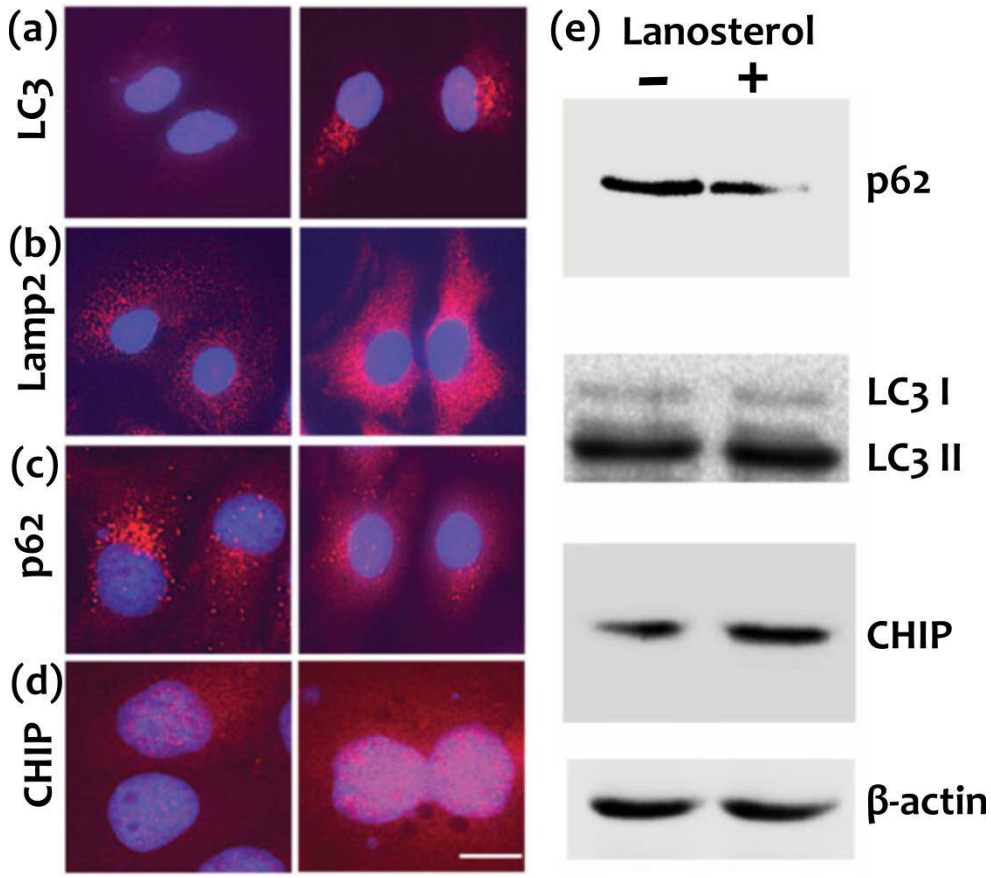

Figure 3.1 : *Lanosterol treatment upregulates cellular autophagy and induces co-chaperone CHIP expression:* (a-d) Cos-7 cells were plated into two-well chamber slides and treated with lanosterol (10 μM) for 24 h. After treatment, cells were processed for fluorescence immunocytochemistry labeled with 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. *Scale bar*, 20 μm. (Figure published in Upadhyay et al. 2018; Molecular Neurobiology)

Therefore, to test this notion, cells were treated with different concentrations of lanosterol and for varying time intervals; and then checked messenger RNA (mRNA) levels of CHIP by RT-PCR analysis (Fig. 3.2a). An approximate 0.2-fold increase in CHIP mRNA levels was observed in the lanosterol-treated cells; as compared to dimethyl sulfoxide (DMSO) treated cells (Fig. 3.2b, c).

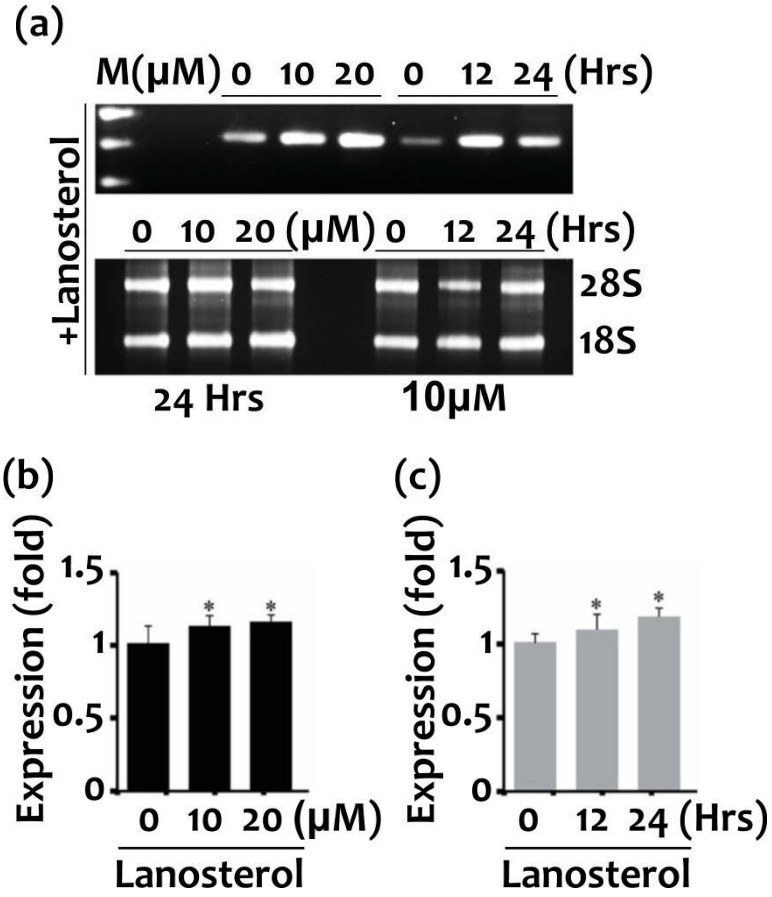

Figure 3.2 : *Treatment of lanosterol enhances the mRNA expression level of CHIP:* **(a)** A549 cells were plated into six-well tissue culture plates and treated in concentration- (lanosterol for 24 h) and time-dependent manner (lanosterol 10 μM). Twenty-four hours later, cells were then processed for 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. (b-c) 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) (b) and time-dependent approach (lanosterol 10 μM) (c) after lanosterol treatment. (Figure published in Upadhyay et al. 2018; Molecular Neurobiology)

These results indicate that mRNA expression levels of endogenous CHIP were upregulated in lanosterol-treated cells. To further ascertain the above-described interesting finding, next protein levels of CHIP were checked following the lanosterol exposure to cells; and it was confirmed by immunoblot analyses that total CHIP protein levels were also elevated in time-dependent manner in response to lanosterol treatment (Fig. 3.3).

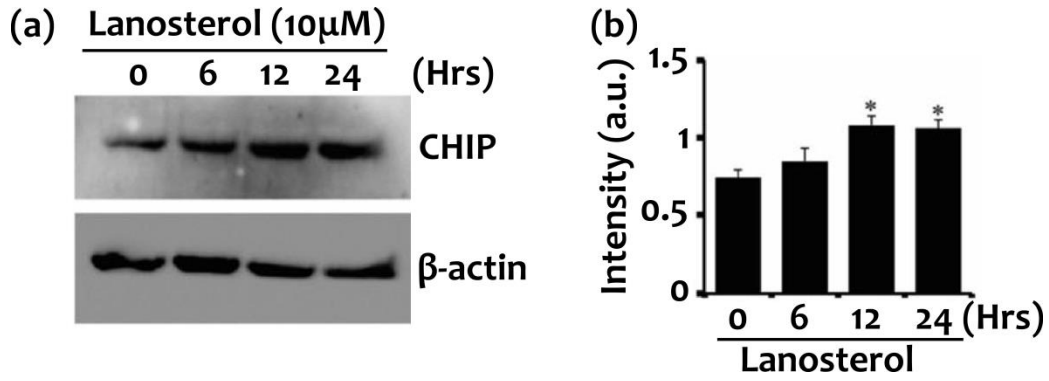

Figure 3.3 : *Time-dependent treatment of lanosterol increases cellular level of CHIP protein:* Cos-7 cells were treated with different time intervals (lanosterol 10 μM) and following cell lysis, anti-CHIP, and β-actin antibodies were used for immunoblot analysis (a). Quantification of the band intensities was obtained from three different experiments with NIH Image analysis software (b). The β-actin protein levels in each were used for the normalization of each sample. (Figure published in Upadhyay et al. 2018; Molecular Neurobiology)

In another set of experiments, cells were administered with lanosterol in concentration-dependent manner and a similar kind of upregulation in levels of CHIP was observed (Fig. 3.4). Altogether, these results suggest that lanosterol treatment increases the cytoplasmic expression of CHIP; and also induces cytoprotective autophagy signaling. Probably, it could be one of the critical survival response strategies adapted by eukaryotic cells against the deleterious effects of multifactorial abnormal protein aggregation.

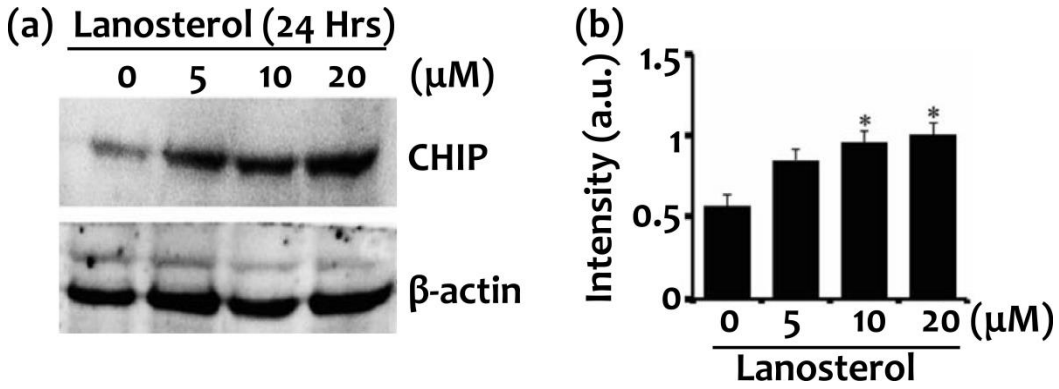

Figure 3.4 : *Lanosterol modulates CHIP levels in concentration-dependent manner:* Cells treated with lanosterol in different concentrations (for 24 hours) were collected and lysed. Immunoblot analysis was performed using anti-CHIP, and β-actin antibodies (a). Intensities of each band ware obtained from three different experiments using NIH Image analysis software (b). The β-actin blots were used as loading controls in each set of experiments. (Figure published in Upadhyay et al. 2018; Molecular Neurobiology)

3.1.2 Lanosterol Prevents Cytoplasmic Aggregation of Mutant Bona-fide Misfolded Proteins in Cells

These observations showing increased mRNA and protein levels of CHIP in the lanosterol-treated cells led 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, previously described [Arslan *et al.*, 2012] green fluorescent protein (GFP) fusions of wild-type chloramphenicol-acetyltransferase (wtCAT) and mutant GFP-Δ9CAT constructs were overexpressed 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. 3.5); but overexpressed mutant GFP-Δ9CAT bona-fide misfolded proteins form distinct cytoplasmic perinuclear aggregates or inclusion bodies in cells, which were also positive for ubiquitin staining (Fig. 3.7).

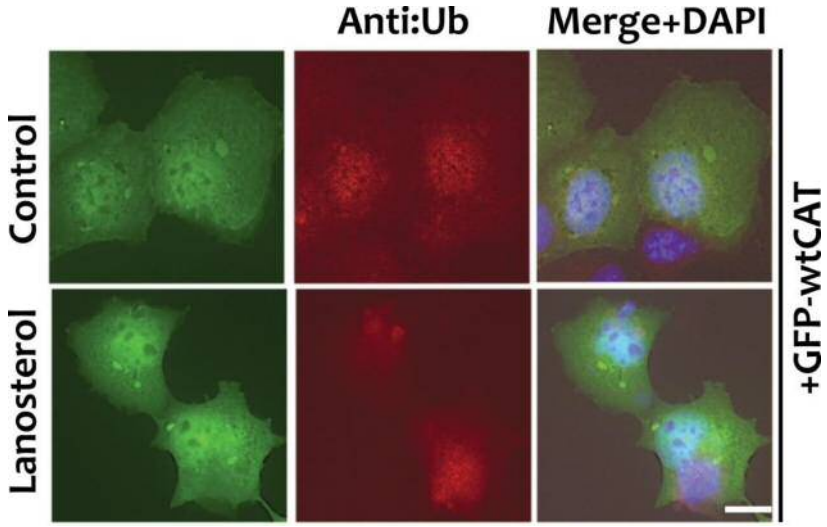

Figure 3.5 : *Expression of GFP-wtCAT plasmid constructs:* Cos-7 cells were transiently transfected with GFP-wtCAT for 24 hours; and treated with DMSO or lanosterol (10 µM). Immunocytochemistry analysis was performed using anti-ubiquitin (Ub) antibody. A rhodamine-conjugated secondary antibody was used to stain ubiquitin and nucleus localization was confirmed by using DAPI (blue) staining. *Scale bar*, 20 µm. (Figure published in Upadhyay et al. 2018; Molecular Neurobiology)

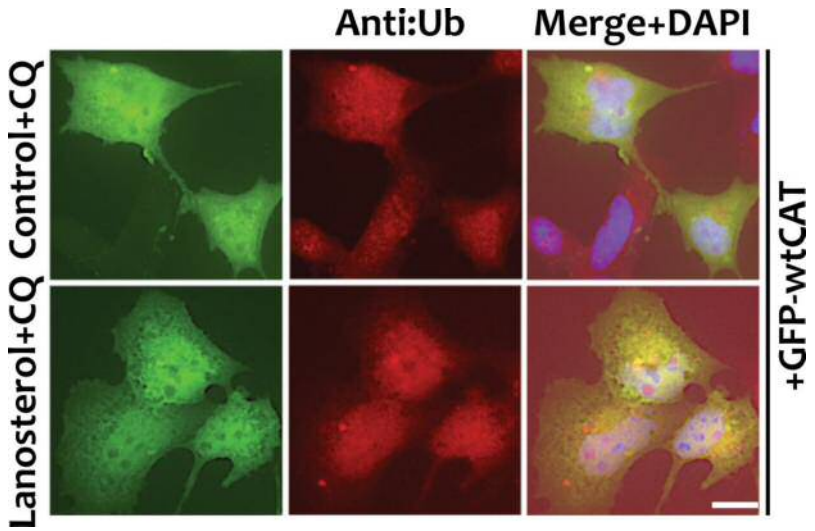

Figure 3.6 : *Effect of chloroquine treatment on the expression of GFP-wtCAT:* After transfection of Cos-7 cells with GFP-wtCAT, 24 hours treatment of lanosterol was given. Few sets of transfected cells were treated with 20 µM chloroquine (CQ) for 12 hours and immunocytochemistry analysis was performed using ubiquitin (Ub) antibody. DAPI was used to counterstain nuclei. *Scale bar*, 20 µm. (Figure published in Upadhyay et al. 2018; Molecular Neurobiology)

It was observed that the exposure of lanosterol noticeably reduces ubiquitin-positive perinuclear aggregates or inclusion bodies of GFP-Δ9CAT misfolded proteins in cells (Fig. 3.7).

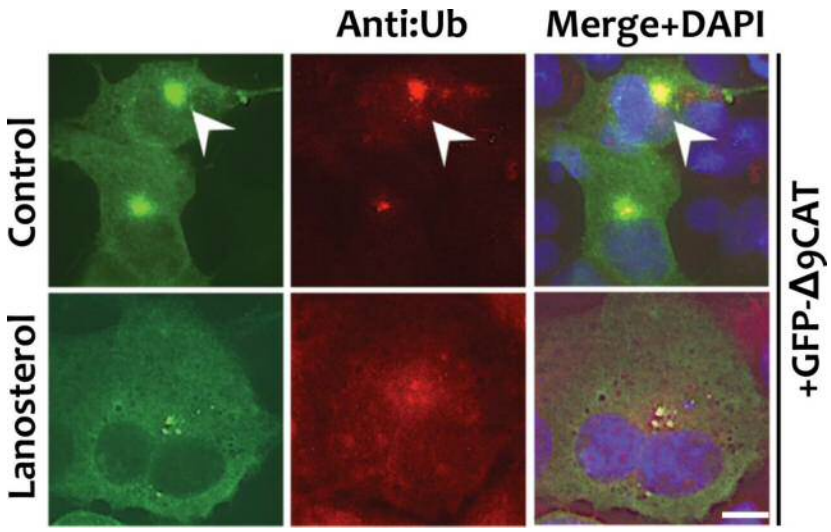

Figure 3.7 : *Lanosterol enhances the clearance of a mutant bona-fide misfolded proteins:* Cos-7 cells were transfected with mutant GFP-Δ9CAT plasmids. Post-transfection, cells were administered with DMSO or lanosterol. Immuno-micrographs were taken after ubiquitin staining, while DAPI was used to counterstain nuclei. *Scale bar*, 20 µm. (Figure published in Upadhyay et al. 2018; Molecular Neurobiology)

In the preliminary experiments, it has been observed that lanosterol treatment increases the endogenous levels of microtubule-associated protein 1A/1B-light chain 3 (LC3); lysosome-associated membrane protein 2 (Lamp2); and expression of co-chaperone CHIP, along with the decreased p62 levels, which clearly represents high autophagic flux inside the cells. Therefore, it was presumed that probably addition of lanosterol might stimulate the clearance of abnormal protein aggregation via activation of selective autophagy pathway. To answer this interpretation, few similar sets of above-described cells transiently transfected with GFP-wtCAT (Fig. 3.6) and GFP-Δ9CAT plasmids (Fig. 3.8) were simultaneously exposed to chloroquine to prompt autophagy dysfunction in cells.

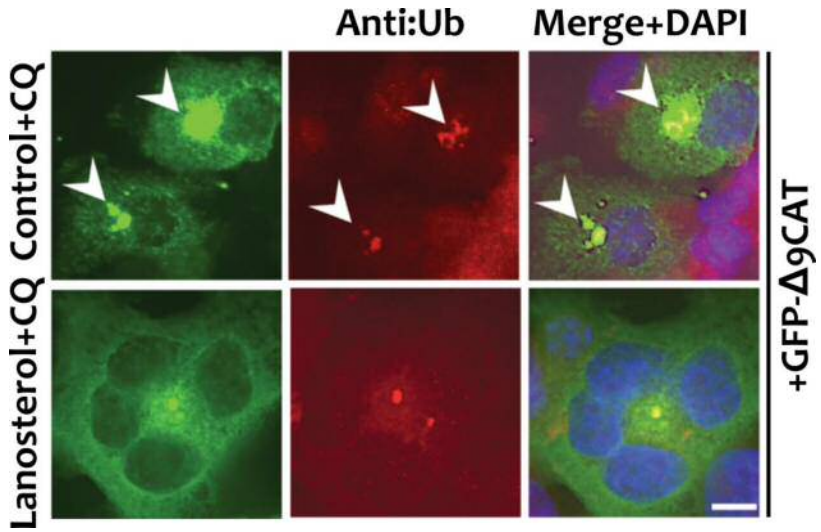

Figure 3.8 : *Chloroquine treatment enhances the aggregation propensity of modeled misfolded protein:* After Cos-7 cells were transfected with GFP-Δ9CAT, few sets were treated with DMSO or lanosterol (10 µM). Additionally, 20 µM choloroquine (CQ) was given to these cells for 12 hours. Anti-ubiquitin primary and rhodamine-conjugated secondary antibodies were used to perform immunocytochemistry analysis. DAPI was used to stain the nuclei. *Scale bar*, 20 µm. (Figure published in Upadhyay et al. 2018; Molecular Neurobiology)

Expected treatment of chloroquine aggravates the accumulation of GFP-Δ9CAT misfolded aggregates (Fig. 3.8), which were earlier prevented by the addition of lanosterol in the absence of chloroquine (Fig. 3.7). These findings indicate that most probably lanosterol treatment may influence the clearance of abnormal proteins via induction of selective autophagy pathway.

3.1.3 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 the earlier experiment, it was noticed that lanosterol enhanced autophagic degradation of mutant bona-fide misfolded GFP-Δ9CAT protein. Therefore, it was important to assess if lanosterol treatment also induces the intrinsic protein degradation systems for other misfolded proteins; next the effect of lanosterol in the degradation of the thermally denatured luciferase protein was demonstrated. Luciferase plasmids were overexpressed in cells. As shown in (Fig. 3.9), transfected cells were treated with lanosterol and exposed to 43 °C for 30 minutes and then returned to 37°C for 2 hours of recovery and used for immunofluorescence staining by using antibodies luciferase (green) and CHIP (red).

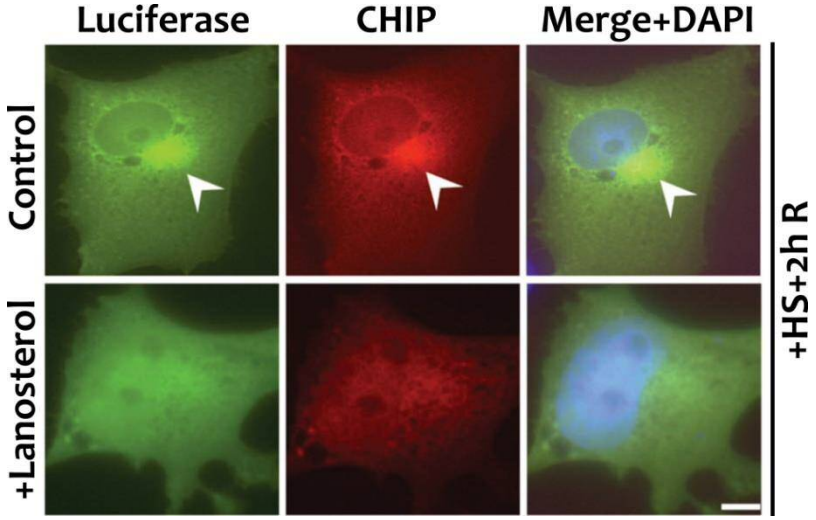

Figure 3.9 : *Lanosterol reduces the intracellular perinuclear accumulation of heat-denatured luciferase protein:* Cells were plated on two-well chamber slides and transient transfection was performed with a firefly luciferase expression construct. After transient transfection, cells were treated with lanosterol (10 μM) for 24 hours. Post-treated cells were exposed to 43 °C for 30 minutes for heat shock (HS) and then returned to 37 °C for 2 hours of recovery (R) 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. *Scale bar*, 20 μm. (Figure published in Upadhyay et al. 2018; Molecular Neurobiology)

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. 3.10a) and immunoblotting analysis (Fig. 3.10b). As shown in (Fig. 3.9), 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. 3.10a). 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. 3.10b, c).

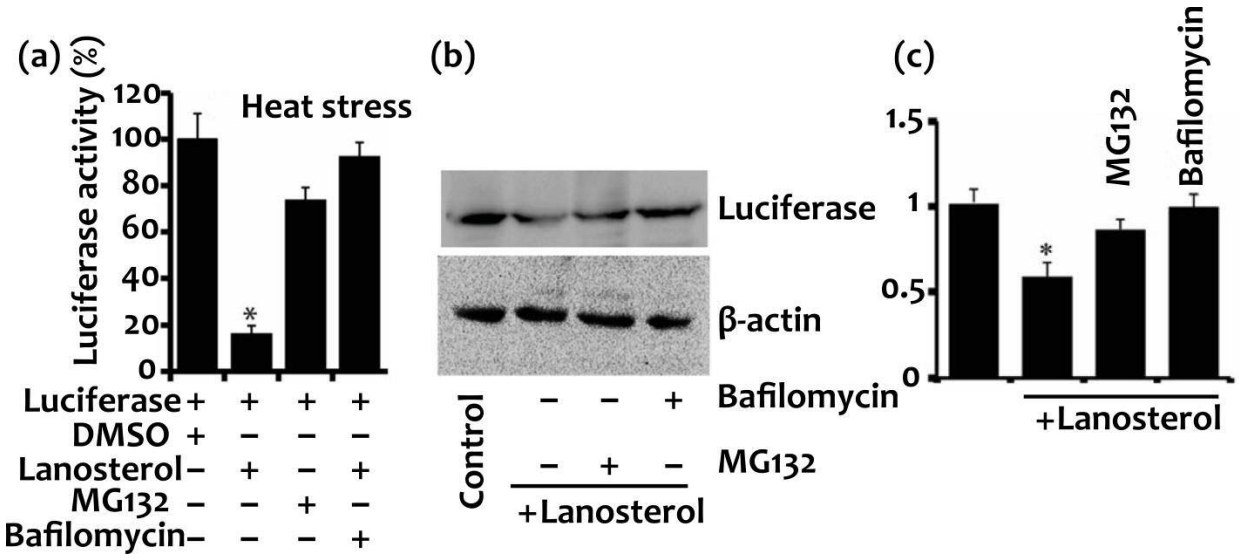

Figure 3.10 : *Effect of lanosterol on the degradation of heat-denatured luciferase protein:* As described in earlier experiments, few similar sets of cells were treated with MG132 (10 μM) and bafilomycin (50 nM) for 12 h and then collected cells were subjected to luciferase activity assay (a) and immunoblot analysis using (b) luciferase and β-actin antibodies. 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. (Figure published in Upadhyay et al. 2018; Molecular Neurobiology)

Next, it was critical to examine whether the addition of lanosterol in cells affects overall cellular health/fitness or generates cellular toxicity during the course of action in the inhibition of misfolded proteins aggregation. As represented in Fig. 3.11, increase in a concentration-dependent manner, 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.

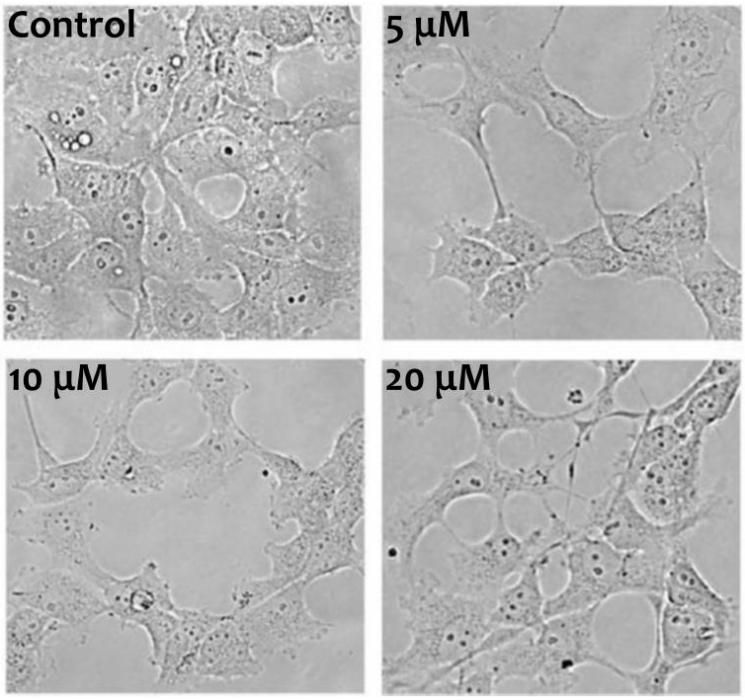

Figure 3.11 : *Effects of lanosterol treatment on overall cellular health:* Cos-7 cells were treated with DMSO (control) and different concentration of lanosterol for 24 hours; and after treatment, cells were visualized using a bright-field microscope as shown in micrographs. (Figure published in Upadhyay et al. 2018; Molecular Neurobiology)

3.1.4 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 [Bukau *et al.*, 2006; Hartl *et al.*, 2011]. Previous results suggest that elevated levels of CHIP and induced autophagy after lanosterol treatment plays a significant role in the elimination of bona-fide 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, cells were transiently transfected with mutant SOD1G37R plasmid constructs as a cellular model of amyotrophic lateral sclerosis (ALS) neurodegenerative disease with or without lanosterol treatment. Cells were transfected with both wild-type SOD1 (Fig. 3.12) and mutant SOD1G37R (Fig. 3.14) constructs. After transfection, 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 enhanced the reduction of mutant SOD1G37R aggregates in cells. Few sets of wild-type (Fig. 3.13) and mutant SOD1 (Fig. 3.15) constructs expressing cells were treated with 20 µM chloroquine for 12 hours that partially blocks the clearance of abnormal intracellular aggregates.

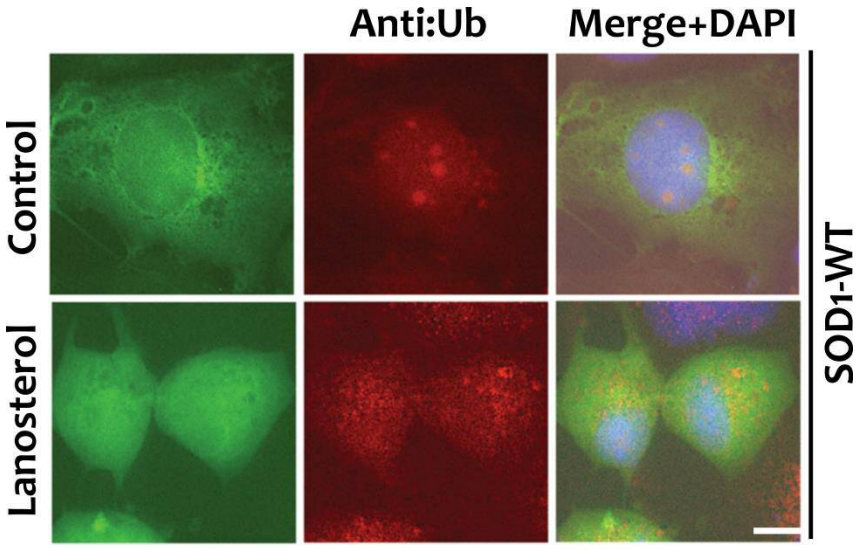

Figure 3.12 : *Expression of SOD1-WT plasmid constructs:* Representative micrographs of Cos-7 cells transiently transfected with SOD1-WT and treated with DMSO or lanosterol. *Scale bar*, 20 µm. (Figure published in Upadhyay et al. 2018; Molecular Neurobiology)

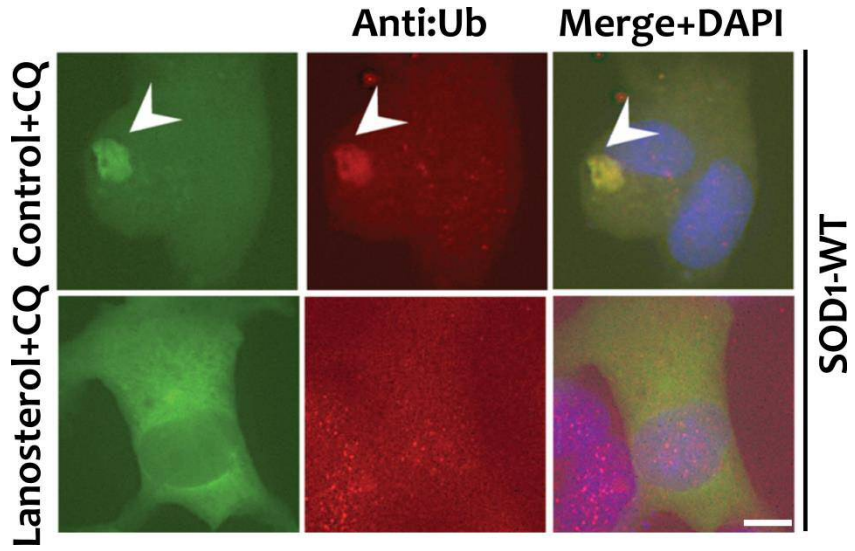

Figure 3.13 : *Effects of autophagy inhibition on the aggregation of ubiquitinated cellular proteins:* Cos-7 cells, after transfection with SOD1-WT, were treated with chloroquine (CQ) for 12 hours to inhibit autophagic flux inside the cells. Immunocytochemistry analyses were performed using ubiquitin antibody in cobination with rhodamine-conjugated secondary antibody. DAPI (blue) was used to stain nuclei. *Scale bar*, 20 µm. (Figure published in Upadhyay et al. 2018; Molecular Neurobiology)

As clearly seen in Fig. 3.13, exposure to chloroquine disturbs autophagy function and even wild-type SOD1 protein is prone to accumulate near nuclear periphery region. However, in the absence of chloroquine, wild-type SOD1 protein does not accumulate into cytoplasmic inclusions; instead these are localized diffusely into the cytoplasm (Fig. 3.12).

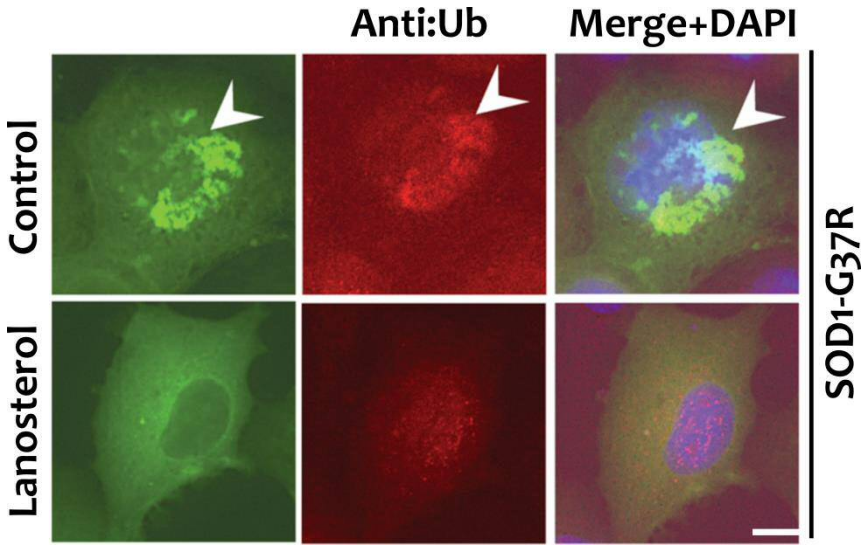

Figure 3.14 : *Lanosterol suppresses the mutant SOD1 aggresomes formation in cells:* After transient transfection with mutant SOD1-G37R plasmid constructs, cells were administered with DMSO or lanosterol (10 µM) for 24 hours. Thereafter, immunocytochemistry analysis was performed using ubiquitin antibody. *Scale bar*, 20 µm. (Figure published in Upadhyay et al. 2018; Molecular Neurobiology)

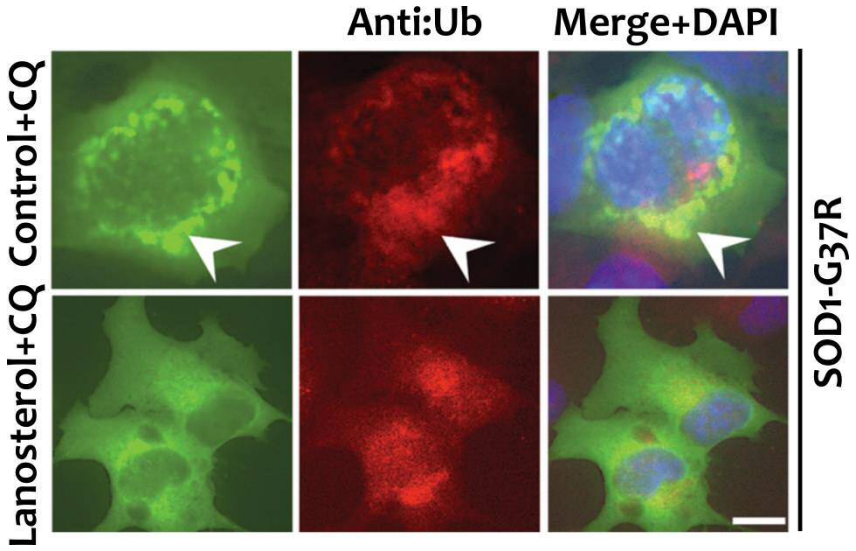

Figure 3.15 : *Higher accumulation of mutant SOD1-G37R protein following autophagy inhibition:* Mutant SOD1-G37R expressing Cos-7 cells were treated with 20 µM chloroquine (CQ) to block the cellular autophagy pathway and then cells were stained using ubiquitin antibody to observe the localization of ubiquitin with cytoplasmic aggregated proteins. *Scale bar*, 20 µm. (Figure published in Upadhyay et al. 2018; Molecular Neurobiology)

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, next, effects of induced autophagy and elevated levels of co-chaperone CHIP that was mediated by lanosterol treatment were examined for other neurodegenerative disease-linked protein, i.e., mutated α-synuclein S87A. It has been observed that wild-type α-synuclein (Fig. 3.16) has a very low aggregation propensity as compared to its mutant form α-synuclein (S87A). Treatment with lanosterol resulted in a decrease in the proportion of mutant α-synuclein (S87A) aggregate

containing cells (Fig. 3.18). Indeed, chloroquine abrogated the effect of lanosterol on witd-type (Fig. 3.17) and mutant α-synuclein (Fig. 3.19) expressing cells.

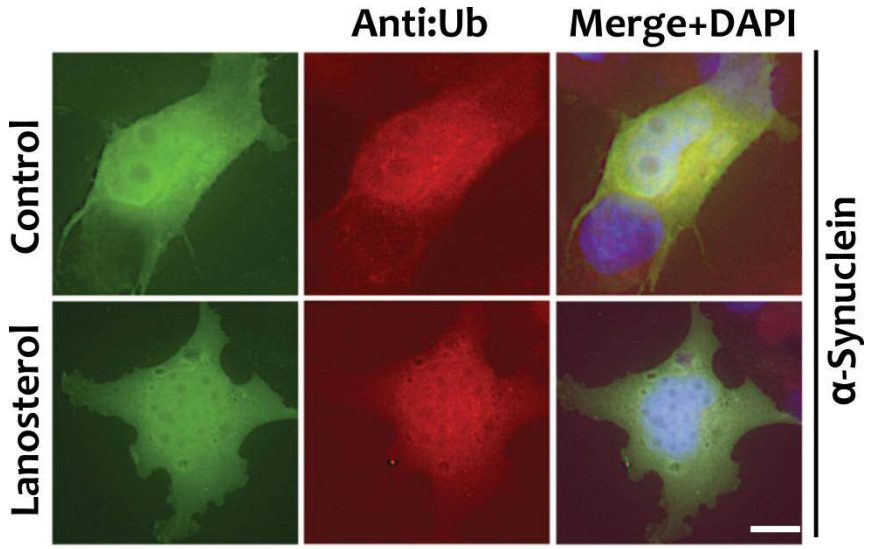

Figure 3.16 : *Wild-type alpha-synuclein expression analysis:* Cos-7 cells were transiently transfected with plasmids expressing wild-type alpha-synuclein and some post-transfected sets of cells were treated with lanosterol (10 µM) for 24 hours. Thereafter, immunocytochemistry staining was done by anti-synuclein and anti-ubiquitin antibodies and analyses were performed to visualize the protein inside the cells. *Scale bar*, 20 µm. (Figure published in Upadhyay et al. 2018; Molecular Neurobiology)

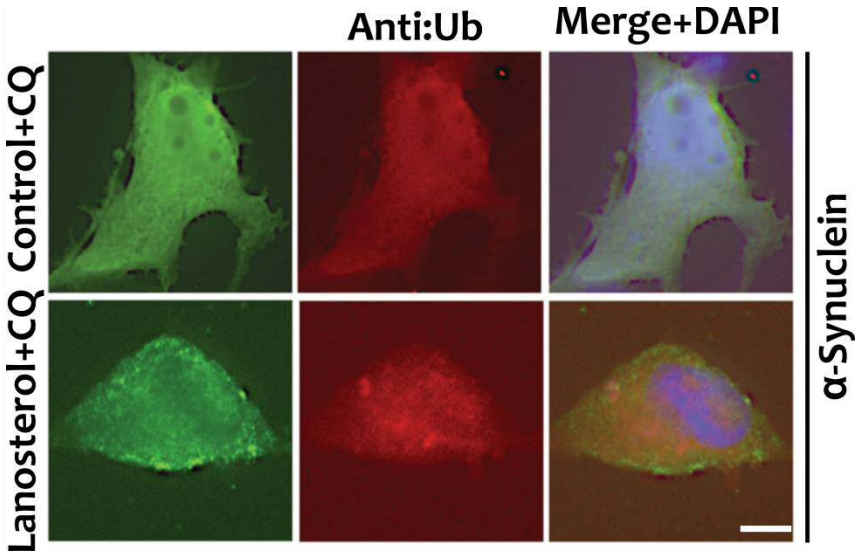

Figure 3.17 : *Effects of administration of chloroquine on the expression of α-synuclein:* Few sets of alpha-synuclein expressing cells were co-treated with chloroquine (CQ) along with DMSO or lanosterol (10 µM, 24 hours). After completion of treatment hours, cells were processed for immunocytochemistry analysis by using anti-synuclein and anti-ubiquitin antibodies. DAPI (blue) was used for staining the nuclei. *Scale bar*, 20 µm. (Figure published in Upadhyay et al. 2018; Molecular Neurobiology)

The results were 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.

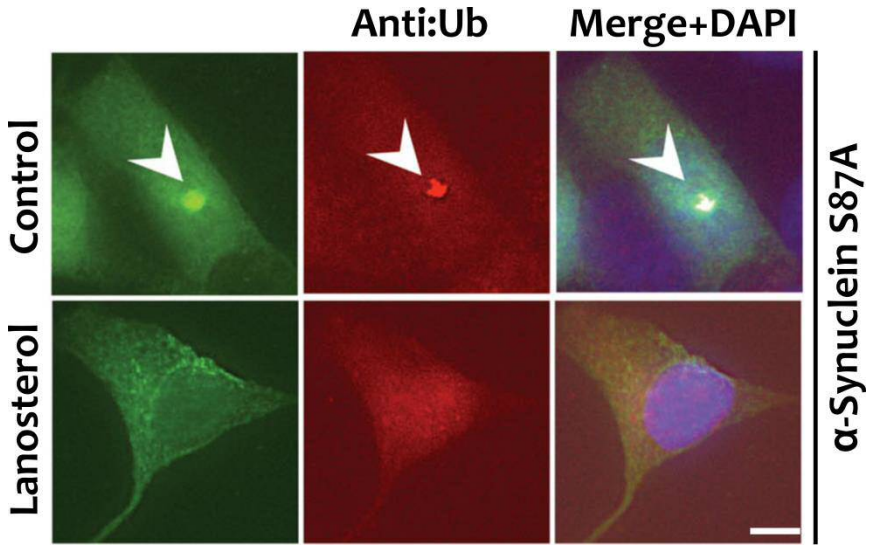

Figure 3.18 : *Treatment of lanosterol reduces aggregation of mutant α–synuclein:* Mutant α-synuclein (S87A) plasmids were transiently transfected in Cos-7 cells and were later treated with DMSO or lanosterol. Following day, cells were stained with synuclein and ubiquitin antibodies using rhodamine-conjugated secondary antibody. *Scale bar*, 20 µm. (Figure published in Upadhyay et al. 2018; Molecular Neurobiology)

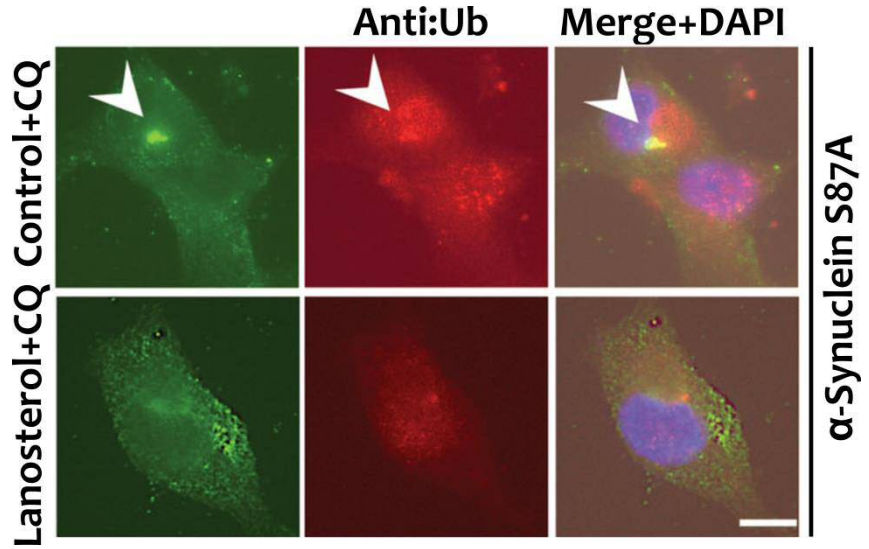

Figure 3.19 : *Inhibition of autophagy overturns the anti-aggregatory effects of lanosterol:* Few sets of mutant α-synuclein (S87A) expressing Cos-7 cells were treated with chloroquine (CQ) to inhibit cellular autophagy pathway following DMSO or lanosterol treatments. Immunocytochemistry was performed using synuclein and ubiquitin antibodies. DAPI was used to stain nuclear components of the cells. Ubiquitin-positive mutant alpha-synuclein (S87A) inclusions were demonstrated as indicated with white arrowheads at the nuclear peripheral region. *Scale bar* 20 µm. (Figure published in Upadhyay et al. 2018; Molecular Neurobiology)

3.1.5 Lanosterol Increases the Stability of Co-Chaperone CHIP

It has been observed that lanosterol treatment induces the endogenous expression of CHIP and also reduces the aggregation of bona-fide 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, the possible interactions of lanosterol with CHIP were analyzed by docking analysis. Binding affinity obtained for the best binding mode was −9.2 kcal/mol. As shown in Fig. 3.20, 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.

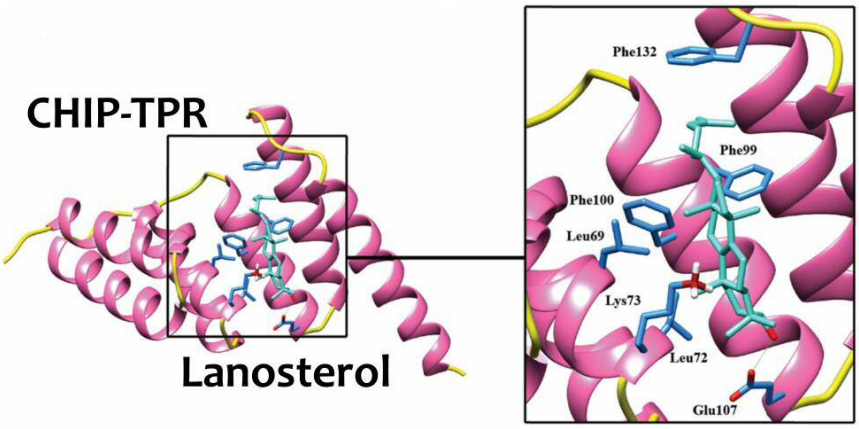

Figure 3.20 : *Representation of interaction of lanosterol with CHIP TPR domain:* Right panel shows a close-up view of this interaction. Lanosterol is shown in cyan color. Amino acid residues of TPR domain interacting with lanosterol are also depicted. CHIP TPR Domain PDBID: 3Q49. (Figure published in Upadhyay et al. 2018; Molecular Neurobiology)

To obtain a possible clue related to the effects of lanosterol on endogenous levels of CHIP, now it was important to observe the direct influence of lanosterol on endogenously available CHIP. Therefore, treated Cos-7 cells were treated with DMSO or lanosterol. After treatment, cells were chased with cycloheximide. As shown in Fig. 3.21a 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. 3.21b). 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.

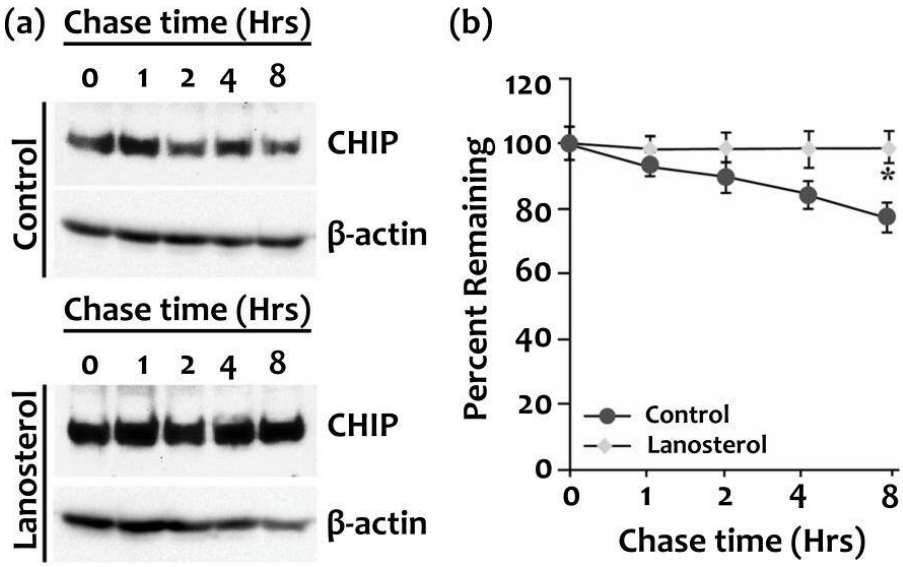

Figure 3.21 : *Lanosterol stabilizes co-chaperone CHIP:* (a) Cos-7 cells were plated into six-well culture plates. In the next day, cells were treated with control (DMSO), lanosterol (10 µM) for 24 hours and cycloheximide (15 µg/ml) and chased for different periods. Blots were detected with CHIP and β-actin antibodies. (b) Blot band intensities from three independent experiments were quantified using NIH Image analysis software. Values are the mean ± SD. (Figure published in Upadhyay et al. 2018; Molecular Neurobiology)

3.1.6 Lanosterol Diminishes Aggregation of Expanded Polyglutamine Proteins in Cells

A previous report [Zhao *et al.,* 2015] and current experiments clearly indicate that lanosterol has a central role in reducing the aggregation of misfolded or disease-causing aggregated proteins. To check if this compound could affect the accumulation propensity of pathogenic-expanded polyglutamine inclusions implicated in the pathomechanism of neurodegenerative diseases, detailed immunofluorescence analyses were performed with ataxin-3-expanded polyglutamine aggregates inside the cells.

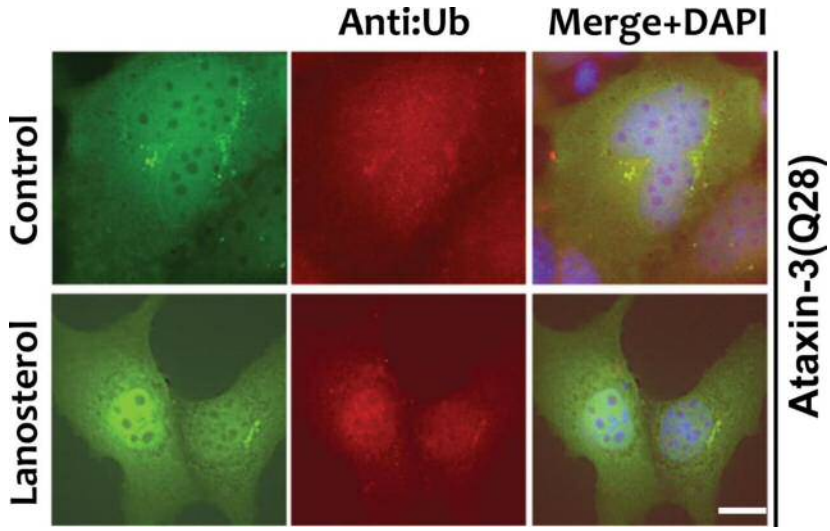

Figure 3.22 : *Expression of ataxin-3(Q28) plasmid constructs:* Cos-7 cells were plated into two-well chamber slides and transiently transfected with ataxin-3-GFP fusion with normal ataxin-3(Q28) glutamine repeats. After transfection, cells were exposed with lanosterol (10 µM) and to detect colocalization 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. *Scale bar,* 20 µm. (Figure published in Upadhyay et al. 2018; Molecular Neurobiology)

For this experiment, normal ataxin-3(28Q) (Fig. 3.22 and Fig. 3.23) and expanded polyglutamine repeats containing ataxin-3(84Q)-expressing plasmids (Fig. 3.24 and Fig. 3.25) were transfected in cells, in the presence or absence of lanosterol.

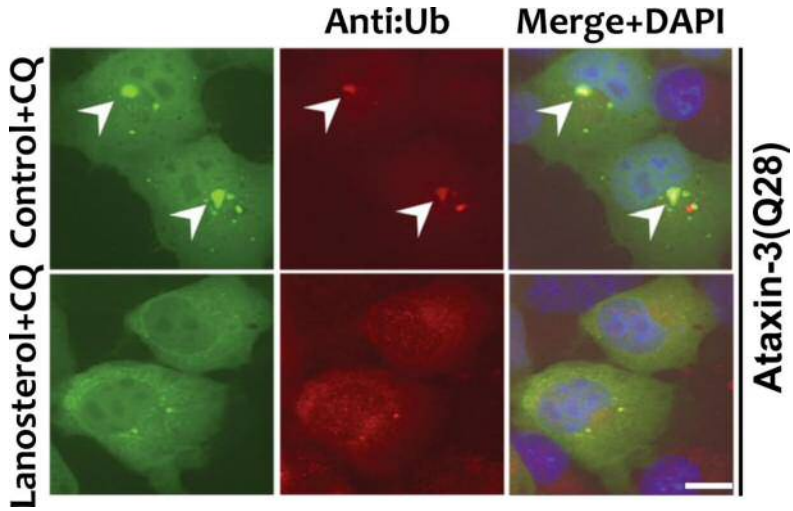

Figure 3.23 : *Inhibition of autophagy leads to increased accumulation of normal ataxin-3(Q28) containing proteins:* Normal polyglutamine repeats containing (ataxin-3(Q28)) construct expressing cells were treated with DMSO or lanosterol in presence of 20 µM chloroquine (CQ). Further, immunomicrographs were analyzed after staining of the cells with ubiquitin primary antibody in combination with rhodamine-conjugated secondary antibody. *Scale bar*, 20 µm. (Figure published in Upadhyay et al. 2018; Molecular Neurobiology)

To further clarify the proactive effects of lanosterol on selective autophagy degradation mechanism, few experiments were carried out under autophagy inhibitory conditions (20µM chloroquine (CQ) for 12 h). It was observed that the use of lanosterol potently diminished the occurrence of cytoplasmic inclusions of expanded polyglutamine ataxin-3(84Q) (Fig. 3.24).

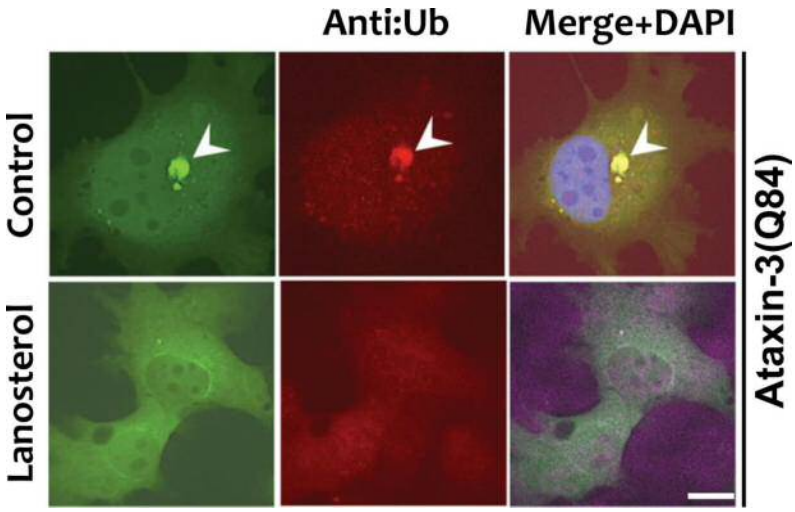

Figure 3.24 : *Lanosterol induces suppression of pathogenic ataxin-3 polyglutamineprotein aggregation in cells:* After transfection with polyglutamine containing ataxin-3-GFP plasmids, cells were exposed with DMSO or lanosterol (10 µM), as indicated in the micrographs. To detect colocalization of ubiquitin with 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 bar*, 20 µm. (Figure published in Upadhyay et al. 2018; Molecular Neurobiology)

The effects were partially reverted in the form of numerous small cytoplasmic inclusions near the periphery of the nucleus by the use of chloroquine that led to the dysfunction of autophagy (Fig. 3.25).

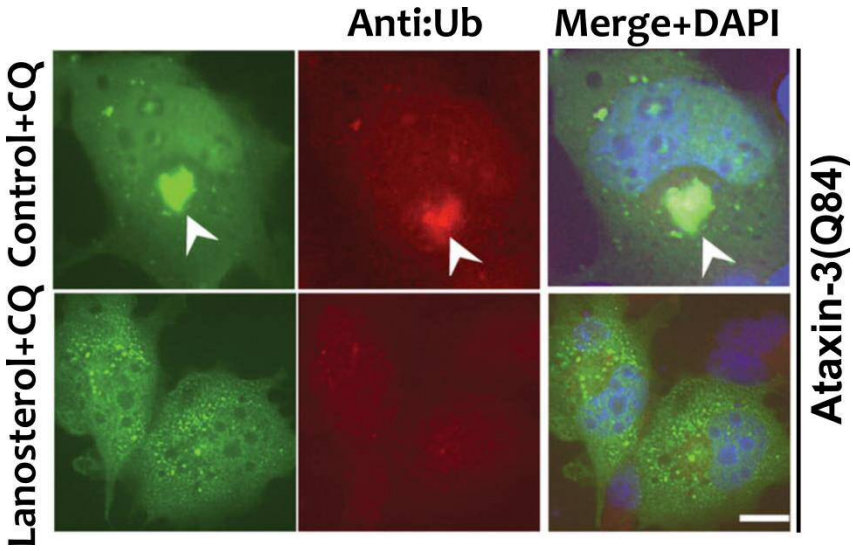

Figure 3.25 : *Administration of chloroquine ameliorates the cytoprotective effects of lanosterol:* Polyglutamine containing ataxin-3-GFP expressing cells were additionally treated with chloroquine (CQ) in combination with DMSO or lanosterol, following which immunocytochemistry staining was performed to visualize the colocalization (arrowheads) of ubiquitin with ataxin-3(Q84) aggregates. *Scale bar*, 20 µm. (Figure published in Upadhyay et al. 2018; Molecular Neurobiology)

In contrast to ubiquitin-positive aggregates of ataxin-3(84Q) inclusions, normal glutamine repeats expressing ataxin-3(28Q) protein, in the respective control cells were soluble and diffusely localized throughout cytoplasmic and nuclear compartments. These observations are consistent with earlier findings that lanosterol induces clearance of specifically misfolded cytosolic proteins in cells.

Results obtained so far were promising and indicate that the treatment of lanosterol efficiently diminishes aggregation of non-native expanded polyglutamine intracellular inclusions of ataxin-3 protein. However, around nine polyglutamine expansion-containing neurodegenerative diseases have been reported so far [Sisodia, 1998]. Therefore, to evaluate whether the addition of lanosterol can also prevent aggregation of other exogenously expressed polyglutamine expansion-containing protein or not, the expression constructs of HD-associated huntingtin protein were used for immunofluorescence studies. As a control of expanded polyglutamine repeats, normal glutamine (EGFP-HDQ23) proteins represent diffused GFP fluorescence pattern (Fig. 3.26 and 3.27)

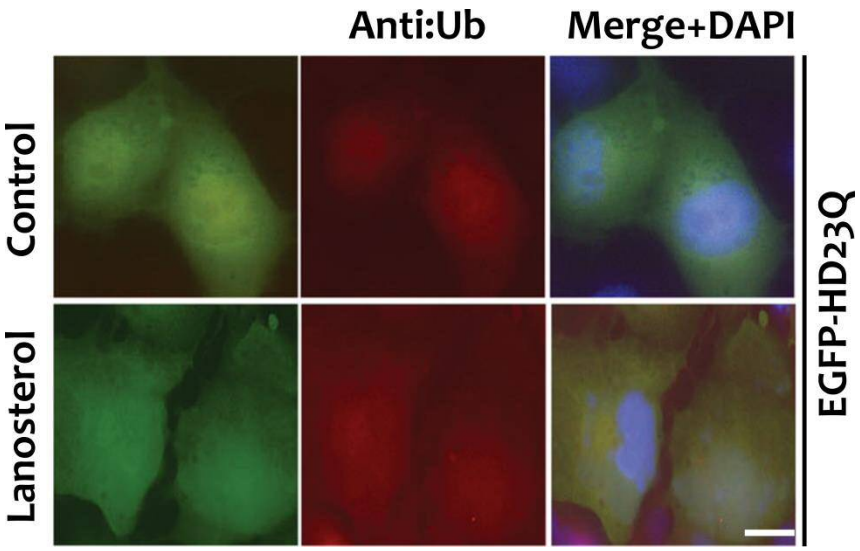

Figure 3.26 : *Visualization of normal huntingtin protein expression in Cos-7 cells:* Immunofluorescence microscopic images of the Cos-7 cells expressing the EGFP-HD23Q plasmids and treated with DMSO and lanosterol (10 µM). Post-treatment, cells were stained with ubiquitin antibody to perform immunocytochemistry analysis. *Scale bar,* 20 µm. (Figure published in Upadhyay et al. 2018; Molecular Neurobiology)

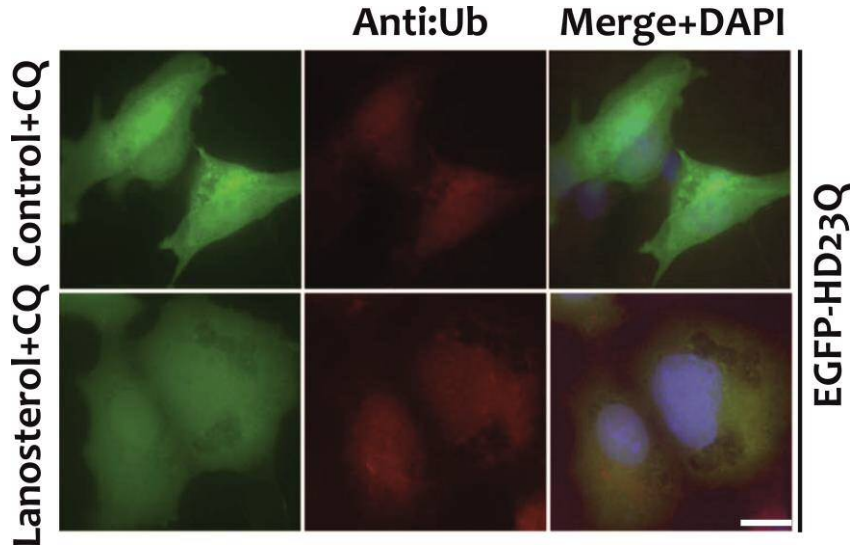

Figure 3.27 : *Effects of inhibition of cellular autophagic flux on the expression of normal huntingtin protein:* Cos-7 cells expressing the EGFP-HD23Q plasmids were treated with autophagy inhibitor chloroquine (20 µM) in addition to DMSO or lanosterol (10 µM) . Thereafter, immunocytochemistry analysis was performed by staining ubiquitin positive cellular proteins by using anti-ubiquitin antibody. *Scale bar,* 20 µm. (Figure published in Upadhyay et al. 2018; Molecular Neurobiology)

When lanosterol-treated EGFP-HDQ74 cells were examined immunocytochemically for inclusion formation using ubiquitin antibody that specifically recognizes robust aggresomes like proteinaceous bodies, a marked reduction in ubiquitin-positive intracellular accumulation of expanded EGFP-HDQ74 polyglutamine proteins were observed (Fig. 3.28)

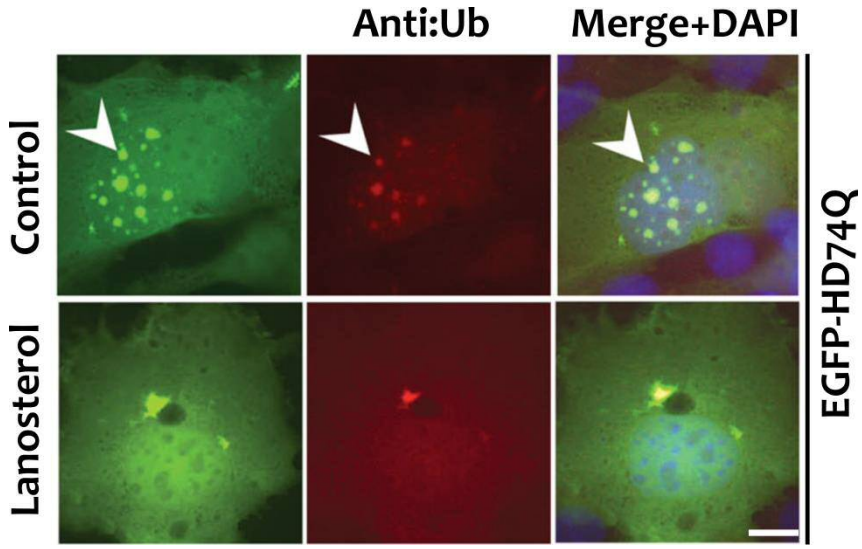

Figure 3.28 : *Treatment of lanosterol results in decrease of cytoplasmic misfolded inclusions of expanded huntingtin polyglutamine aggregates:* Polyglutamine repeats-containing EGFP-HD74Q plasmids were transiently transfected into Cos-7 cells and after 24 hours cells were treated with DMSO or lanosterol (10 µM). On the following day, cells were processed for immunocytochemistry analysis by using anti-ubiquitin antibody to monitor accumulation of ubiquitin positive aggregates (arrowheads). DAPI was used to stain cell nuclei. *Scale bar*, 20 µm. (Figure published in Upadhyay et al. 2018; Molecular Neurobiology)

Interestingly, similar to earlier results, the effect of lanosterol was partially reverted after disturbing autophagy mechanism by the use of chloroquine (Fig. 3.29).

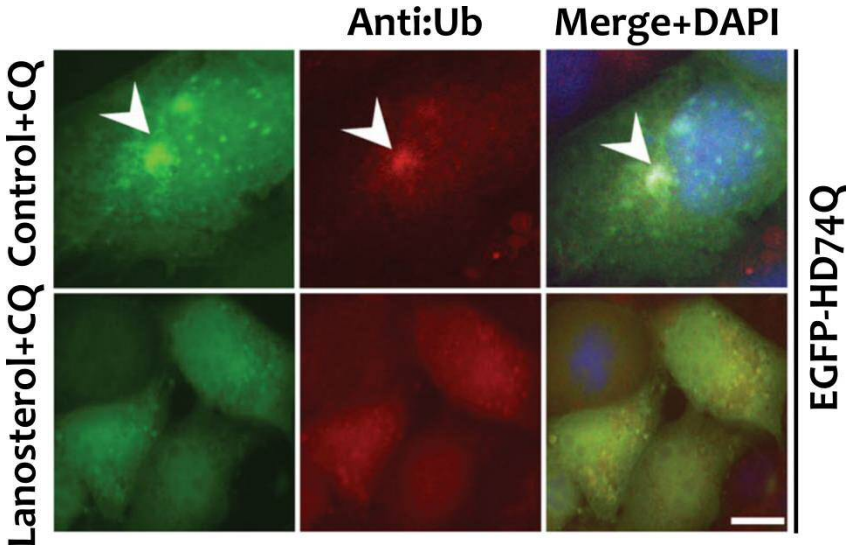

Figure 3.29 : *Monitoring of impact of chloroquine treatment on the formation of perinuclear inclusions of polyglutamine-containing huntingtin inside the cells:* Cos-7 cells expressing polyglutamine constructs of EGFP-HD74Q were treated with DMSO or lanosterol (10 µM) after 24 hours of transfection. In addition, chloroquine (CQ) was added to inhibit autophagic flus inside the cells. Immunomicrographs were obtained from cells that were stained with anti-ubiquitin antibody to detect the expanded polyglutamine ubiquitin-positive protein aggregates (*red*) and DAPI for nuclear staining (*blue*). *Scale bar*, 20 µm. (Figure published in Upadhyay et al. 2018; Molecular Neurobiology)

Overall, these results suggest that lanosterol contributes in the mitigation of neurodegenerative disease-causing polyglutamine proteins.

3.1.7 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. Now, it was crucial 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, dot-blot analyses were performed with cellular extracts of cells expressing different misfolded proteins, as depicted in Fig. 3.30a. As presented in Fig. 3.30b, quantification of the intensities of the obtained dot-blots suggests that exogenously applied lanosterol dramatically reduced the aggregation of various misfolded proteins; also they were potentially more soluble in nature. Using this approach, the ability of the lanosterol to modify the aggregation state of misfolded proteins under cellular conditions was studied.

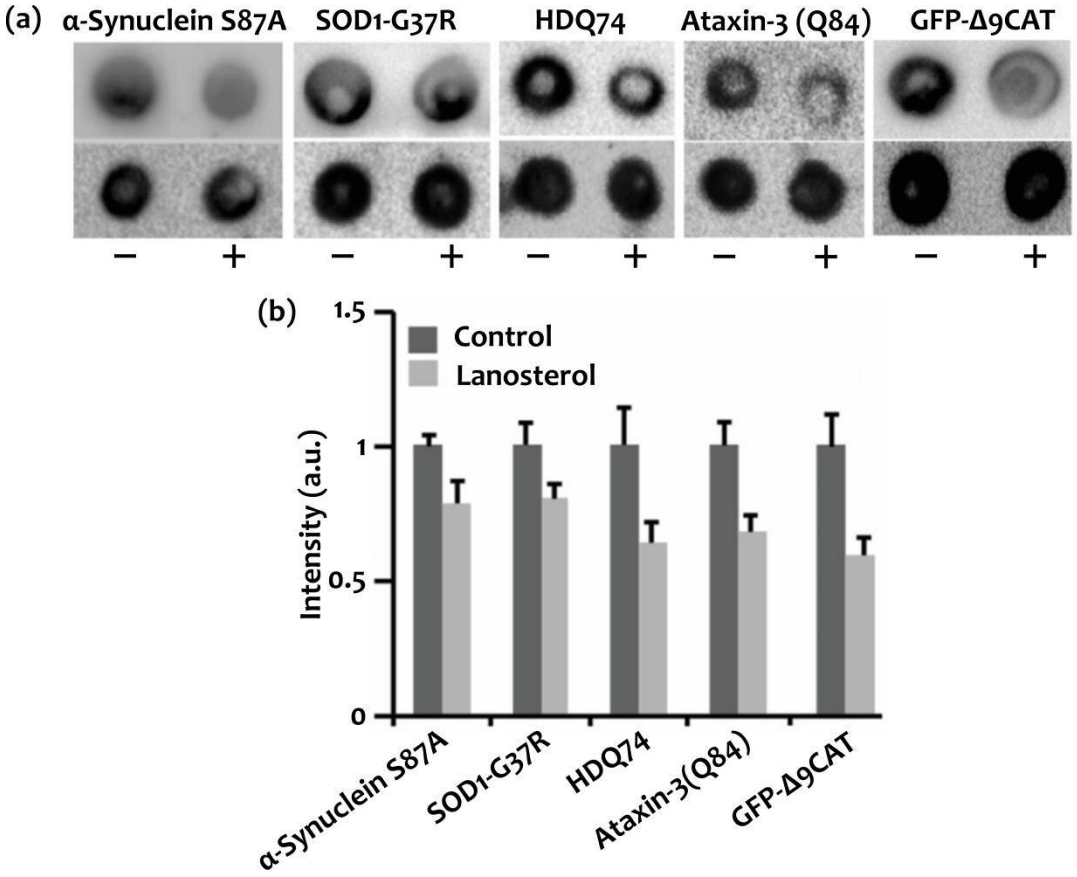

Figure 3.30 : *Lanosterol reduces misfolded protein aggregation:* Cos-7 cells were transiently transfected with various expression constructs to generate misfolded and aggregated proteins in cells with or without lanosterol (10 μM). (a) After treatment, the dot-blot analysis was subjected with the extracts 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. (Figure published in Upadhyay et al. 2018; Molecular Neurobiology)

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 [Chhangani and Mishra, 2013a; Woerner *et al.*, 2016]. However, one recent study [Zhao *et al.*, 2015] and also the current results suggest that application of lanosterol reduces aggregation of misfolded proteins; and thus may provide cytoprotection against misfolded protein-mediated stress-induced toxic insults.

Therefore, the effects of lanosterol under various cellular stress conditions were monitored. Cells treated 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. 3.31a), while some cells were also visualized under bright-field microscopy (Fig. 3.31b). These results strongly substantiate that lanosterol contributes in cellular defense and protection mechanisms in response to the multifactorial toxic effects caused by various stress-inducing agents.

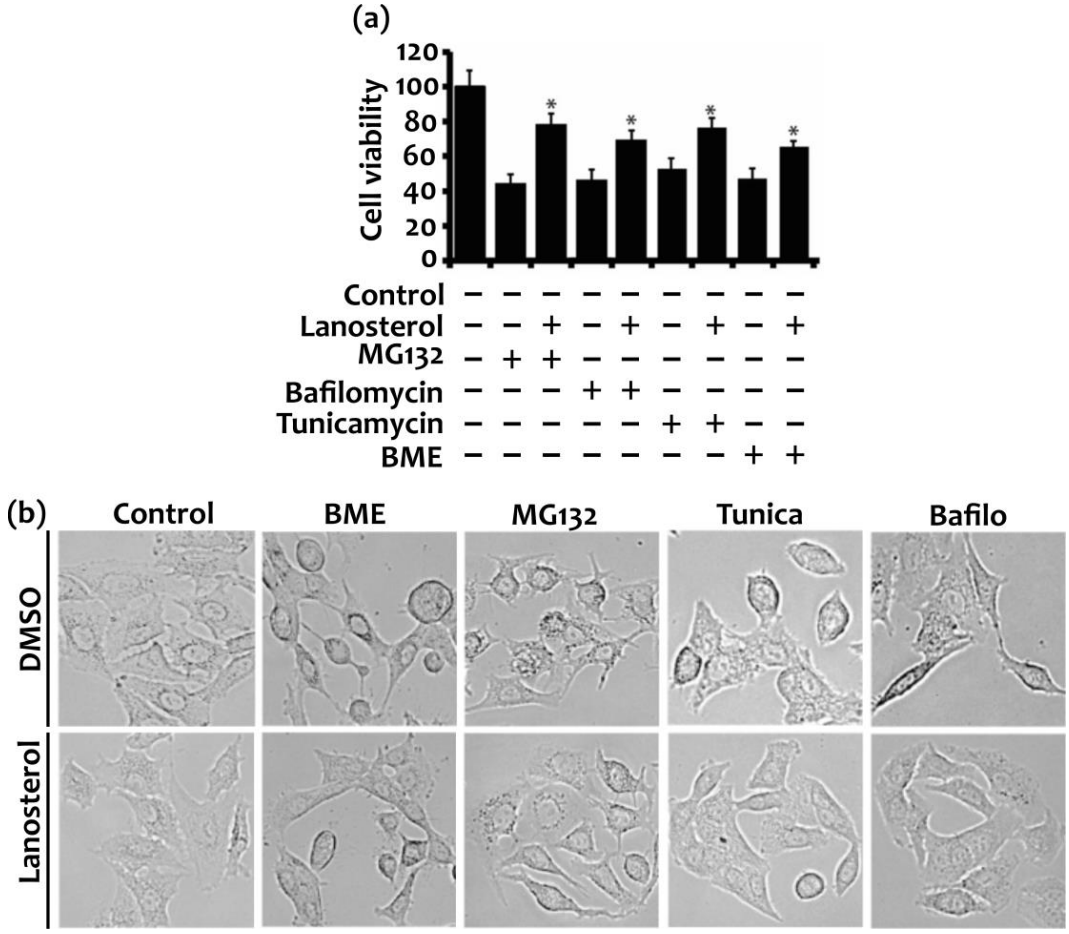

Figure 3.31 : *Lanosterol alleviates stress-induced cytotoxicity:* Cos-7 cells were treated with 10 μM MG132 for 10 h, 50 nM bafilomycin for 12 hours, 10 μg/ml tunicamycin for 10 h, and 5 mM β-mercaptoethanol (BME) for 3 hours. Few sets of similar experiments were carried out in the presence of lanosterol (10 μM) for 24 hours. (a) 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). (b) As described previously, cells were treated with the various stressors in the presence and absence of lanosterol (10 μM) and observed using a bright-field microscope. (Figure published in Upadhyay et al. 2018; Molecular Neurobiology)

Since lanosterol causes a decrease in insoluble mutant misfolded proteins; and also provides cytoprotection against misfolded protein aggregation, further the effects of lanosterol were studied on the propensity of aberrant protein aggregate formation. As shown in Fig. 3.32a, 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, another detailed experiment including several non-native proteins were performed and it was found that addition of lanosterol seems effective in reducing the overall aggregation load of the cells that is generated due to various types of misfolded or aberrant proteins (Fig. 3.32b).

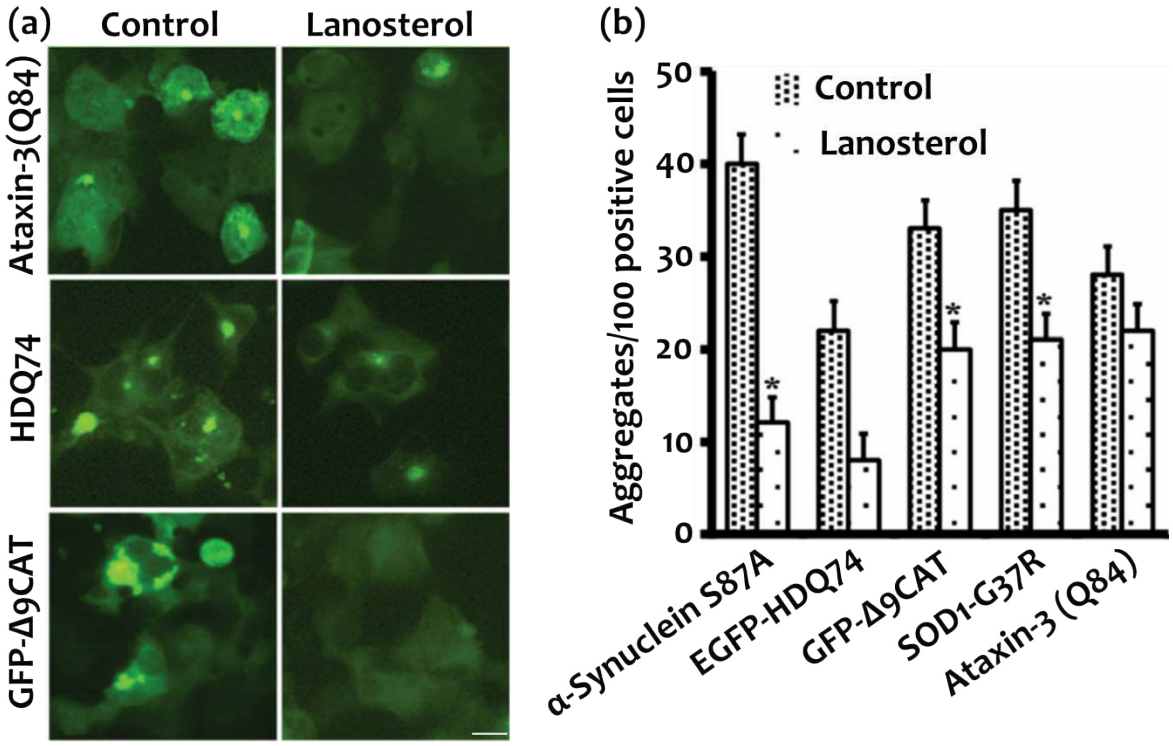

Figure 3.32 : *Suppression of overall cytoplasmic aggregate formation following lanosterol treatment.* Cells were treated with lanosterol (10 µM) as shown in the figure; after 24 h, aggregation and quantitation were monitored for ataxin-3(Q84), EGFP-HDQ74 and GFP-Δ9CAT under a fluorescence microscope. Quantitation of various misfolded protein aggregates in cells, transfected with their respective mutant plasmids and treated with lanosterol (10 µM). The results are presented as mean ±SD of three independent experiments each performed in triplicate. **P* < 0.05 compared with the control treatment. *Scale bar*, 20 µm (Figure published in Upadhyay et al. 2018; Molecular Neurobiology)

The above 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.

3.2 DISCUSSION

In the cellular system, CHIP performs dual functions: as an E3 ubiquitin ligase of the U-box family and also as a co-chaperone that can recognize various chaperone substrates and misfolded proteins for their degradation via proteasomal or lysosomal pathways [Connell *et al.*, 2001; McDonough and Patterson, 2003; Meacham *et al.*, 2001; Xu *et al.*, 2002]. Previous study showed that cooperative function of BAG-3 and CHIP facilitates ubiquitin-mediated selective autophagy and provide muscle maintenance via degradation of filamin [Arndt *et al.*, 2010]. Another study suggests that oral administration of a disaccharide trehalose reduces the formation of truncated huntingtin protein aggregates and alleviates the polyglutamine-induced pathology in a transgenic mouse model of Huntington's disease [Tanaka *et al.*, 2004].

Interestingly, there are accumulating evidence addressing the importance of small molecular approaches, application of chaperones, and co-chaperones for the degradation of misfolded or damaged proteins generated in cells [Chaudhuri and Paul, 2006; Perlmutter, 2002; Suzuki, 2014]. Treatment with carbenoxolone (CBX) a glycyrrhizic acid derivative, upregulates Hsp70 chaperone and decreases α-synuclein aggregation in neuroglioma cells [Kilpatrick *et al.*, 2013]. 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 [Wang *et al.*, 2013].

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 [Zhao *et al.*, 2015]. Another report demonstrates that some pharmacological chaperones binds with α-crystallins (cryAA and cryAB) and reversed their aggregation in vitro and partially render transparency in cataract models [Makley *et al.*, 2015]. These studies suggest a functional link between small molecules, chaperone systems and cellular proteolytic machinery, which can also open promising horizons and wide prospects in neurodegeneration and late-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 denatured misfolded proteins.

In the current study, it was observed that lanosterol treatment dramatically induces and stabilizes CHIP levels. Effect of lanosterol treatment on the induction of cellular autophagic flux has also been observed. It has also been shown that addition of lanosterol decreases pathogenic aggregation of bona-fide and disease-linked (mutant SOD1, α-Synuclein, and expanded polyglutamine) proteins inside the cells. Earlier, it was found that CHIP interacts with expanded polyglutamine proteins and degrades its high molecular weight inclusions and suppresses associated cytotoxicity [Jana *et al.*, 2005; Miller *et al.*, 2005]. Previous study also suggests that CHIP is responsible for the proteasomal degradation of ALS-linked mutant SOD1 proteins [Urushitani *et al.*, 2004].

CHIP also promotes α-Synuclein degradation by two discrete mechanisms, i.e., (1) proteasomal-dependent degradation and (2) lysosomal pathway [Shin *et al.*, 2005]. In fact, these studies suggest that CHIP acts as a molecular switch between the two proteolytic mechanisms. Therefore, most likely the elevated and stabilized levels of CHIP, following the treatment of lanosterol, may promote degradation of misfolded proteins with the help of proteasome system and induced autophagy pathway.

These results provide compelling evidence that lanosterol treatment plays a crucial and pivotal cytoprotective function to alleviate mutant misfolded protein-mediated proteotoxicity. In the present study, it has been established that treatment of lanosterol proficiently reduces the aggregation of various misfolded proteins; and thus provides cytoprotection against deleterious effects of the aggregated proteins and protects the cells from stress-induced death. Current results and observations suggest that most likely, the presence of lanosterol causes elevation, and stabilization of CHIP, which acts as an important molecular switch between proteasomal and lysosomal degradation. 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 suitable therapeutic approach against protein misfolding and neurodegenerative diseases in the future.

3.3 CONCLUDING REMARKS

Lanosterol plays crucial roles in the induction of cytoprotective functions of QC mechanism (UPS and autophagy) via up-regulating the expression and stabilizing the levels of co-chaperone CHIP; and hence suppresses the overall aggregation of different types of modeled and disease-associated misfolded proteins. An overall decrease in cytotoxic aggregation of multiple kinds of neurodegenerative disease-linked aberrant forms of proteins have also been observed following lanosterol treatment. Lanosterol treatment simultaneously reduces the inherent propensity of aggregation of these proteins as well as ameliorates proteotoxicity generated by abnormal accumulation of mutant proteins inside the cells. Therefore, current findings propose a novel role of lanosterol in cytoprotection of stressed cells via removal of mutant misfolded proteins and also by lowering the cytotoxic burden inside the cells.

...