3
Lanosterol Elevates Cytoprotective Response Through Induced-Proteasomal Degradation of Aberrant Proteins

3.1 RESULTS

Proteasomes are an essential component of quality control mechanism of UPS and clear many abnormal proteins from the cells to prevent any of their cytotoxic effects [Mishra et al., 2018]. In fact, proteasome impairment is shown in many neuronal degenerative conditions, resulting in excessive proteotoxic stress and ultimately, reduced cellular fitness and survival [Keller et al., 2000; McNaught and Jenner, 2001]. Increasing the levels or activity of proteasome by different approaches, including small molecules, are being investigated for their therapeutic potential against diseases with abnormal protein folding and aggregation [Njomen and Tepe, 2019].

3.1.1 Lanosterol Treatment Increases Endogenous Levels of Proteasome and Prevents Aggregation of Model Proteins

In our initial experiments involving treatment with lanosterol of the cells, we noticed increase in endogenous levels of proteasomes in comparison to control (DMSO) (Figure 3.1 (a, b)).

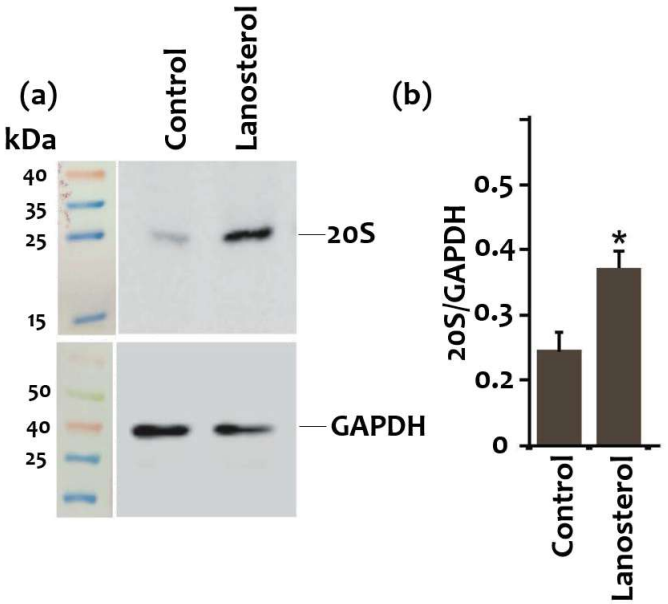


Figure 3.1: Effect of lanosterol on 20S proteasome levels: (a) A549 cells in 6-well plates were treated with control (DMSO) or lanosterol (20 μM, 48 h). Immunoblotting analysis was done with anti-20S and anti-GAPDH antibodies. (b) Band intensity quantification was performed by ImageJ software. Values in plot are mean ± SD from three experiments done independently in triplicate. *p < 0.05 when compared to control indicating statistical significance.

Moreover, varying the exposure of cells to lanosterol for different time periods also resulted in increased levels of proteasomes upon treatment for 12 and 24 hours (Figure 3.2 (a, b)). In light of this finding, we asked whether increased proteasome levels can also be associated with improved clearance of different model misfolded proteins.

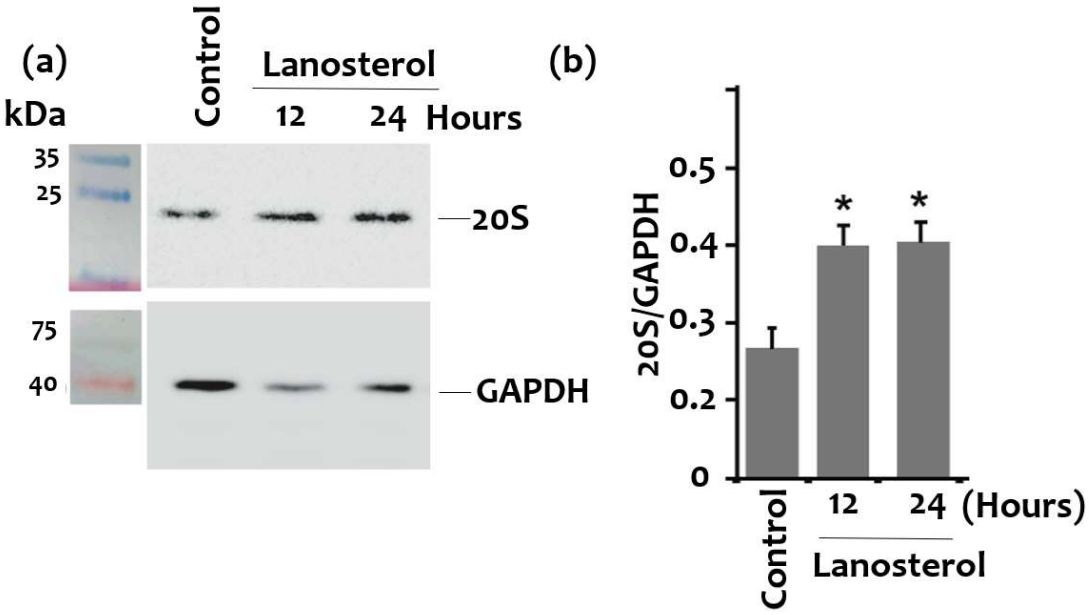


Figure 3.2: Effect of time-dependent lanosterol treatment on 20S proteasome levels: (a) A549 cells were treated with control (DMSO) or lanosterol (20 μM) at various time points. Immunoblotting was done with anti-20S and anti-GAPDH antibodies. (b) Band intensity quantification was performed by ImageJ software. Values in plot are mean ± SD from three experiments done independently in triplicate. *p < 0.05 when compared to control indicating statistical significance.

For this, we briefly transfected cells with luciferase plasmid and utilised heat-denatured luciferase as a modeled misfolded protein. We treated transfected cells with DMSO or lanosterol with or without proteasome inhibitor MG132 and autophagy inhibitor chloroquine as indicated in Figure 3.3 (a). The heat denaturation of luciferase was achieved by giving heat shock to cells and then allowed them to recover at normal temperature. We noticed decreased luciferase activity in the samples collected post-heat stress and co-treated with lanosterol (Figure 3.3 (a)). The luciferase activity assay results suggested increased degradation of luciferase in presence of lanosterol compared to DMSO and this effect was decreased with addition of MG132 and chloroquine. Similarly, lanosterol also improved the clearance of ubiquitin-positive heat-denatured luciferase aggregates (Figure 3.3 (b)).

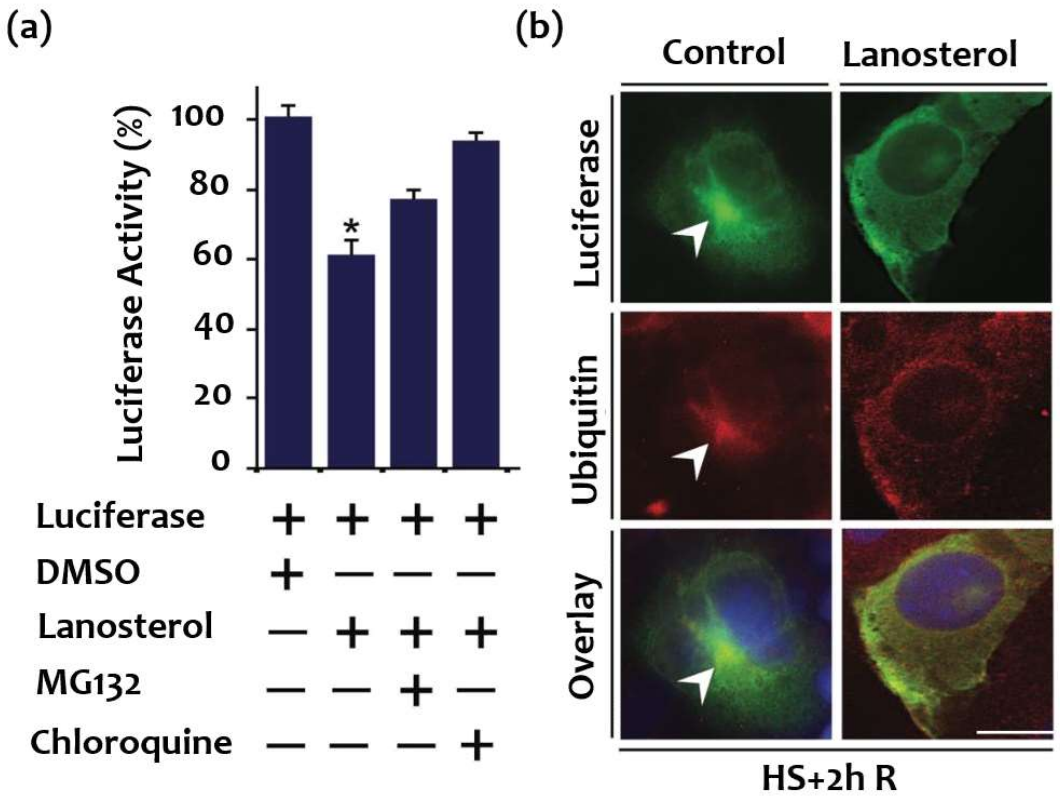


Figure 3.3: Lanosterol promotes heat denatured luciferase degradation: (a) Cells were transfected transiently with firefly luciferase plasmid. Post-transfection, different sets of cells were exposed to lanosterol (20 μM, 48 h), DMSO, MG132 (10 μM, 12 h), and chloroquine (20 μM, 12 h) as shown. Next, cells were incubated at 43 °C for 30 min and made to have recovery for 2 h at 37 °C. These cells were harvested and lysed and luciferase activity assay was executed. Values in figure are mean ± SD from three experiments done independently in triplicate. *$p < 0.05$ when compared to control indicating statistical significance. (b) Another set of luciferase-transfected and lanosterol- or control-treated cells were subjected to heat and allowed to recover as in section (a). These cells were processed for immunocytochemistry experiments, probed for luciferase (green) and ubiquitin (red), and imaged with epifluorescence microscopy. Nuclear staining was performed with DAPI (blue). Arrowheads represent luciferase aggregates and ubiquitin localization. *Scale bar: 20 μm.*

To further study proteasomal involvement in possible cytoprotective effect of lanosterol, next, we transiently transfected cells with plasmid construct of d1EGFP, since it is a substrate of proteasome, as it consists of sequences of proline, glutamate, serine, and threonine at its carboxyl terminal. Treatment of cells with lanosterol improved the clearance of d1EGFP aggregates (Figure 3.4 (b)) compared to control (Figure 3.4 (a)) and MG132 reversed this effect. These results indicate that lanosterol-induced proteasome levels can improve the clearance of abnormal protein inclusions from cells, which can alleviate proteotoxicity.

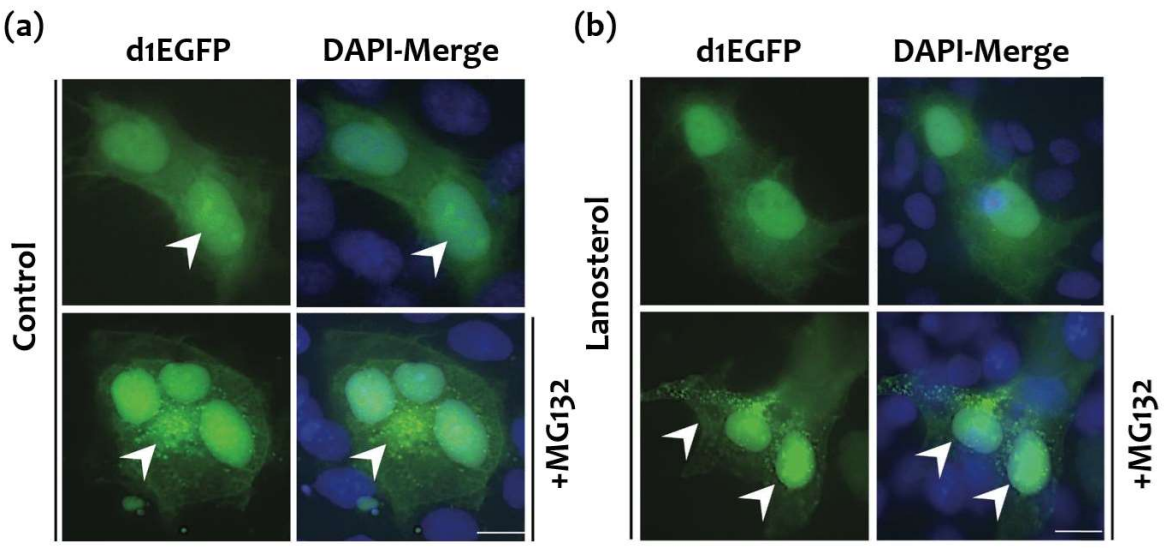


Figure 3.4: Lanosterol induces clearance of proteasomal substrate d1EGFP in cells: Cells grown in chambered slides were transfected with pd1EGFP plasmid and exposed to DMSO (a) or lanosterol (20 μM, 48 h) (b) with or without MG132. d1EGFP inclusions (green) were then imaged with epifluorescence microscopy. Nuclei were stained with DAPI (blue). Arrowheads represent perinuclear aggresomes-like structures. *Scale bars: 20 μm.*

3.1.2 Treatment of Lanosterol Facilitates Proteasomal Degradation of Bona Fide Misfolded Proteins

It is essential to selectively target misfolded proteins for intracellular elimination, which can minimize disturbances in proteostasis maintenance and reduce proteotoxicity [Chen et al., 2011]. Our current findings demonstrate the proteasome-enhancing potential of lanosterol against abnormal protein accumulation. Therefore, it is conceivable though still remains to be formally analyzed that exposure to lanosterol can improve proteasome-mediated degradation of aberrant proteins in cells. In our next experiment, we examined effect of lanosterol on the accumulation of GFP-tagged Δ9CAT, a bona fide misfolded protein. To test the significance of the anti-aggregation impact of lanosterol against bona fide misfolded proteins, we transiently expressed both wild-type (GFP-wtCAT) and mutant (GFP-Δ9CAT) vector constructs.

We treated transfected cells with or without lanosterol; some cells were subjected to MG132. Once treated, cells were processed for immunofluorescence analysis by antibody anti-20S (Red). As indicated in Figure 3.5, GFP-wtCAT expressing cells do not form apparent inclusions, which were increased after treatment with MG132, and the same phenomenon was reverted by the exposure of lanosterol (Figure 3.6). This sequence of analyses prompted us to observe the lanosterol effect on the aggregation of mutant (GFP-Δ9CAT) proteins (Figure 3.7) representing the elimination of Δ9CAT aggregates in the presence of lanosterol. In similar sets of experiments, the anti-aggregation impact of lanosterol against misfolded proteins was reverted by adding MG132 as shown in Figure 3.8. These observations suggest that lanosterol facilitates the proteasomal-mediated degradation of bona fide abnormally folded proteins from the cells.

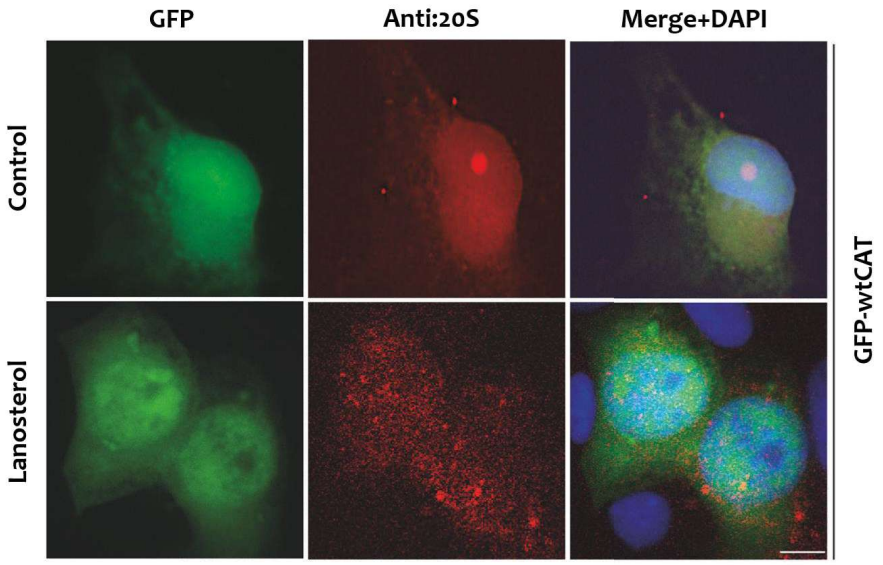


Figure 3.5: Lanosterol treatment effect on GFP-wtCAT protein: Cos-7 cells in chambered slides were transfected with GFP-tagged wtCAT plasmid construct. Once transfected, cells were given DMSO or lanosterol (20 μM) for 48 h. Cells were prepared for immunofluorescence experiments to be examined with anti-20S antibody and secondary antibody conjugated with Texas Red with fluorescence microscope. DAPI (blue) was utilized for confirming nuclear locations. *Scale bar: 20 μm.*

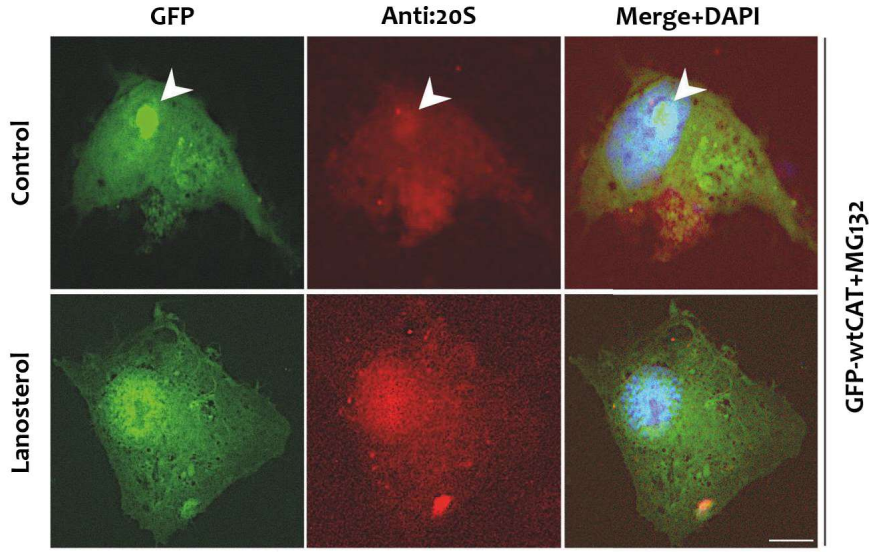


Figure 3.6: Lanosterol treatment effect on GFP-wtCAT in presence of MG132: Cos-7 cells in chambered slides were transfected with GFP-wtCAT plasmid construct. Post-transfection, cells were given DMSO or lanosterol (20 μM) for 48 h along with proteasome inhibitor MG132 (10 μM,12 h). Anti-20S antibody was utilised and imaging was done via fluorescence microscope. The cells were imaged to view CAT aggregates via fluorescence microscope. The nuclear locations were confirmed with DAPI (blue). The arrowheads represent perinuclear aggregates of CAT protein co-localized with proteasome machinery. *Scale bar: 20 μm.*

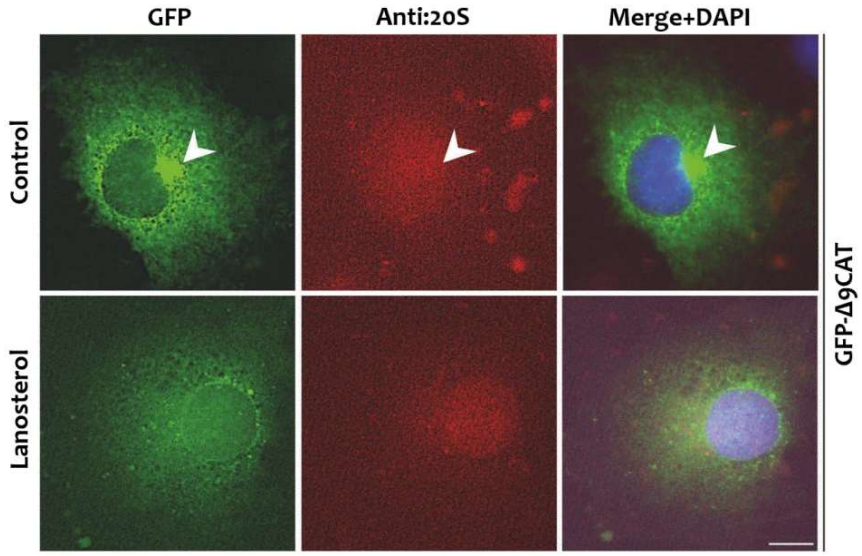


Figure 3.7: Lanosterol treatment promotes proteasomal degradation of bona fide misfolded Δ9CAT protein: Cos-7 cells were transfected with mutant GFP-Δ9CAT plasmid. Once transfected, cells were given DMSO or lanosterol (20 μM) for 48 h. Post-treatment, immunofluorescence analysis was done with anti-20S antibody and cells were imaged with fluorescence microscope (Δ9CAT – green; 20S proteasome – red). The nuclear locations were confirmed with DAPI (blue). The arrowheads represent perinuclear aggregates of Δ9CAT protein co-localized with proteasomes. *Scale bar: 20 μm.*

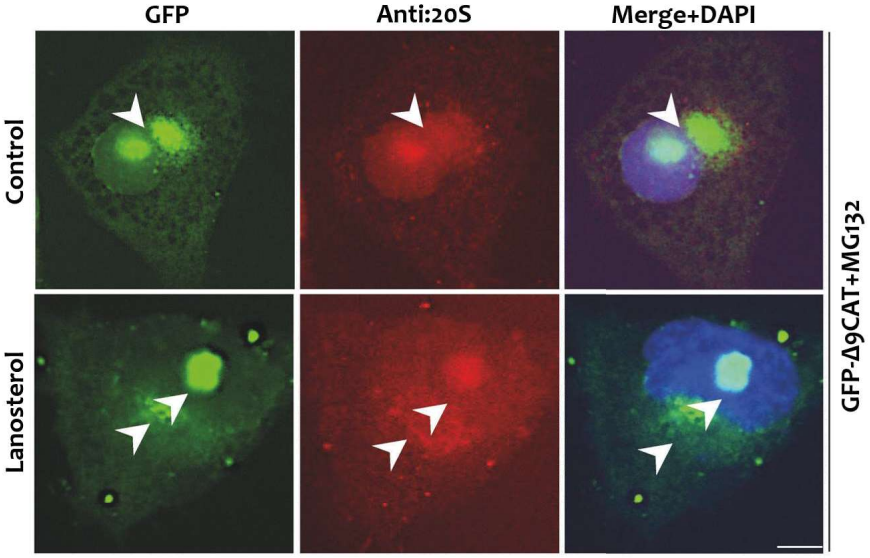


Figure 3.8: Lanosterol-induced clearance of Δ9CAT protein aggregates is reverted by MG132: Cos-7 cells were transfected with GFP-Δ9CAT plasmid. After transfection, cells were exposed to DMSO or lanosterol (20 μM) for 48 h in presence of MG132 (10 μM,12 h). Immunofluorescence imaging was done with anti-20S antibody to observe co-localization of proteasome (red) with the CAT protein aggregates (green) represented by arrowheads. DAPI (blue) was utilized for confirming nuclear locations. *Scale bar: 20 μm.*

3.1.3 Lanosterol Suppresses the Accumulation of Neurotoxic Proteins

The clearance of massive inclusions of toxic proteins appears to be a sensitive and tedious task for cells [Rao and Bredesen, 2004], therefore to avoid unwanted proteotoxic insults most likely under such conditions, cells prefer to store them into a separate compartment [Kaganovich et al., 2008]. How can we improve the clearance of massive aggregation of intracellular abnormal proteins from the cells is a critical challenge. Here, in our next experiment to further analyze the proteasomal-mediated potential of lanosterol against neurotoxic proteins, we transiently expressed wild-type (SOD1-WT) and mutant SOD1-G37R (ALS-linked motor neuron toxic

mutant) in cells. Post-transfected cells were subjected to control or lanosterol; few sets of cells were also exposed with MG132. After treatment, cells were used for immunocytochemistry experiments utilizing anti-20S antibody. As shown in Figure 3.9, SOD1-WT expression forms negligible aggregates, but the exposure of MG132 produces few aggregates, which were reduced after the addition of lanosterol (Figure 3.10).

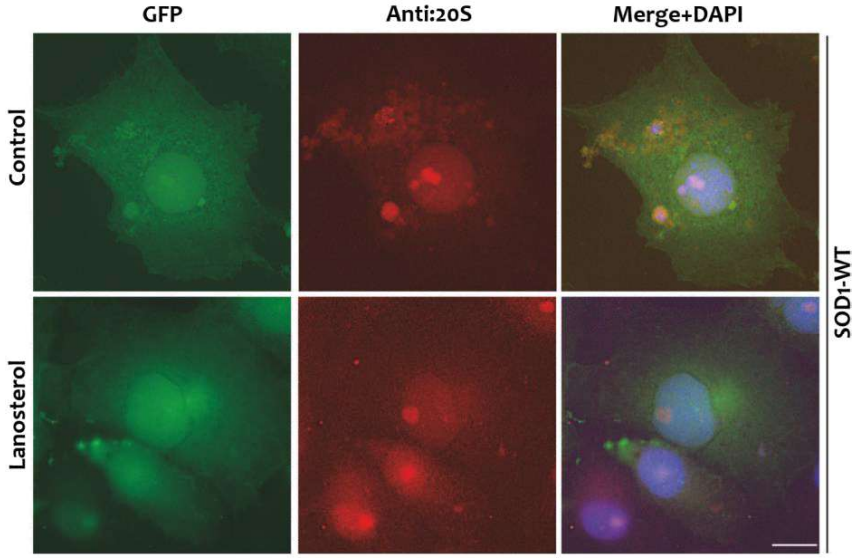


Figure 3.9: Effect of lanosterol on WT SOD1 protein: Cos-7 cells transfected with SOD1-WT and GFP fusion construct plasmid. After transfection, cells were exposed to control or lanosterol (20 µM) for 48 h. Cells were processed for immunocytochemistry experiments and examined with anti-20S antibody and Texas Red-conjugated secondary antibody for imaging under epifluorescence microscopy (red). SOD1 imaging was done under green channel of fluorescence microscope. To stain nuclei, DAPI was used (blue). *Scale bar: 20 µm.*

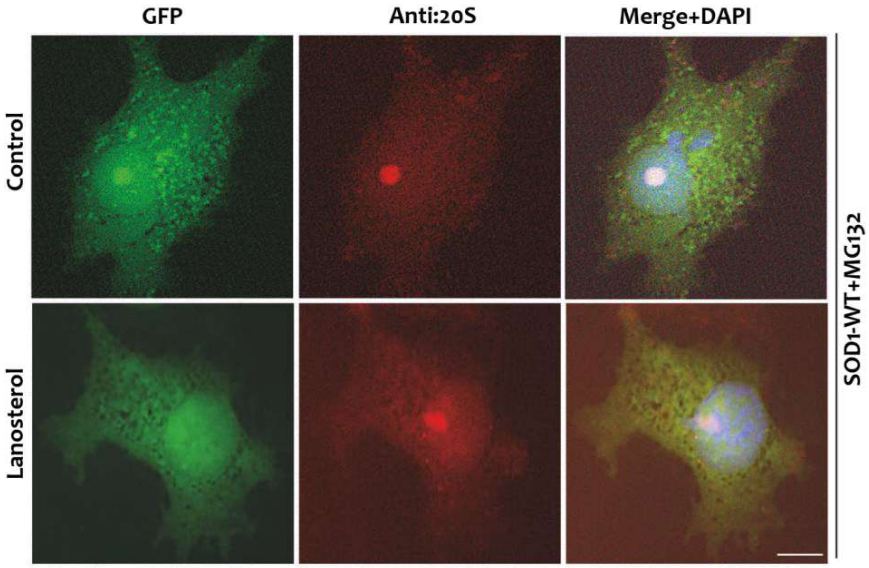


Figure 3.10: Effect of lanosterol on WT SOD1 protein in presence of MG132: Cos-7 cells, transfected with SOD1-WT GFP fusion construct plasmid, were exposed to control or lanosterol (20 µM) for 48 h along with MG132 (10 µM, 12 h). Anti-20S antibody was utilised for immunofluorescence analysis and imaging done with epifluorescence microscope. DAPI was used to stain nuclei (blue). *Scale bar: 20 µm.*

As shown in Figure 3.11, exposure to lanosterol resulted in degradation of mutant SOD1-G37R aggregates; parallel exposure of MG132 diminished the effect of lanosterol (Figure 3.12). This infers that lanosterol promotes the efficiency of proteasome, which may improve clearing of

the misfolded protein inclusion from the cells. This might be the primary route helpful to alleviate neurotoxic insults of mutant SOD1 proteins.

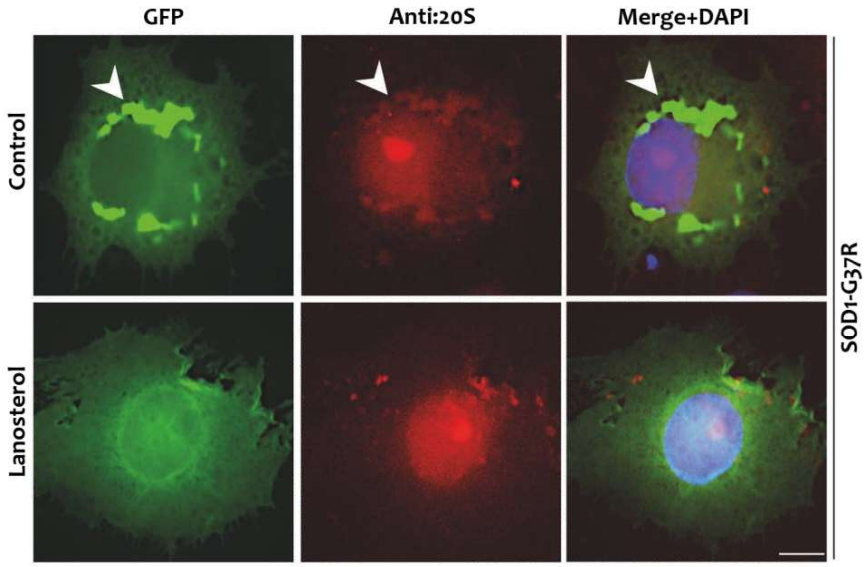


Figure 3.11: Lanosterol suppresses accumulation of ALS-linked SOD1 mutant protein: Cos-7 cells, transfected with mutant SOD1-G37R GFP fusion construct plasmid, were exposed to control or lanosterol (20 μM) for 48 h. Immunocytochemistry experiments were performed with anti-20S antibody to observe colocalization of proteasome with the aggregates under epifluorescence microscopy (red). The mutant SOD1-G37R aggregates were imaged under green channel. To stain nuclei, DAPI was used (blue). The arrowheads represent aggregates of SOD1-G37R mutant co-localized with recruited proteasomes. *Scale bar: 20 μm.*

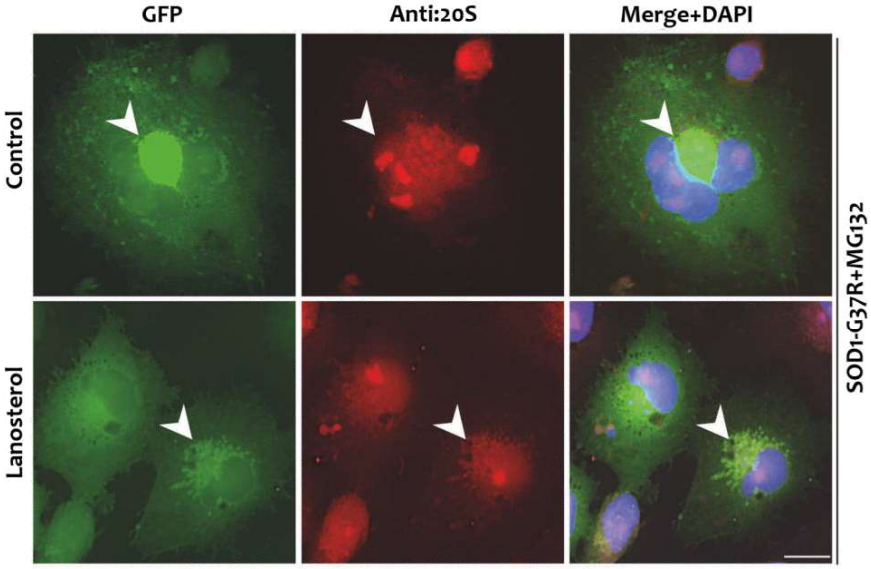


Figure 3.12: Aggregation suppressive effect of lanosterol on SOD1 mutant protein is diminished by MG132: Cos-7 cells were transfected with SOD1-G37R GFP fusion construct plasmids. Post transfection, cells were given control or lanosterol (20 μM) for 48 h in MG132 presence (10 μM; 12 h). Immunocytochemistry experiments were performed with antibody anti-20S and co-localisation was observed between mutant SOD1 aggregates (green) and 20S proteasome (red). To stain nuclei, DAPI was used (blue). The arrowheads represent aggregates of SOD1-G37R mutant co-localized with recruited proteasomes. *Scale bar: 20 μm.*

3.1.4 Lanosterol Induces the Protein Quality Control Components, Including Proteasome

Earlier, it has been observed that failure of UPS is linked to the establishment of several neuronal degenerating disorders [Dennissen et al., 2012]. But still, developing drugs-based proteasome-dependent activation of the proteolytic pathway remains a critical challenge. We have observed in our preliminary findings that treatment with lanosterol can elevate the endogenous levels of proteasomes. Initially, we treated cells with different compounds apart from lanosterol that are well-known proteasomal inhibitors (aspirin, MG132, lactacystin) [Kisselev and Goldberg, 2001; Dikshit et al., 2006]. Post-treatment, lysates of cells were prepared and proteasome activity assays were executed, as described in the Method section. The treatment of lanosterol significantly enhances both chymotrypsin-like (Figure 3.13 (a)) and post-glutamyl peptide hydrolyzing (PGPH)-like (Figure 3.13 (b)) activities.

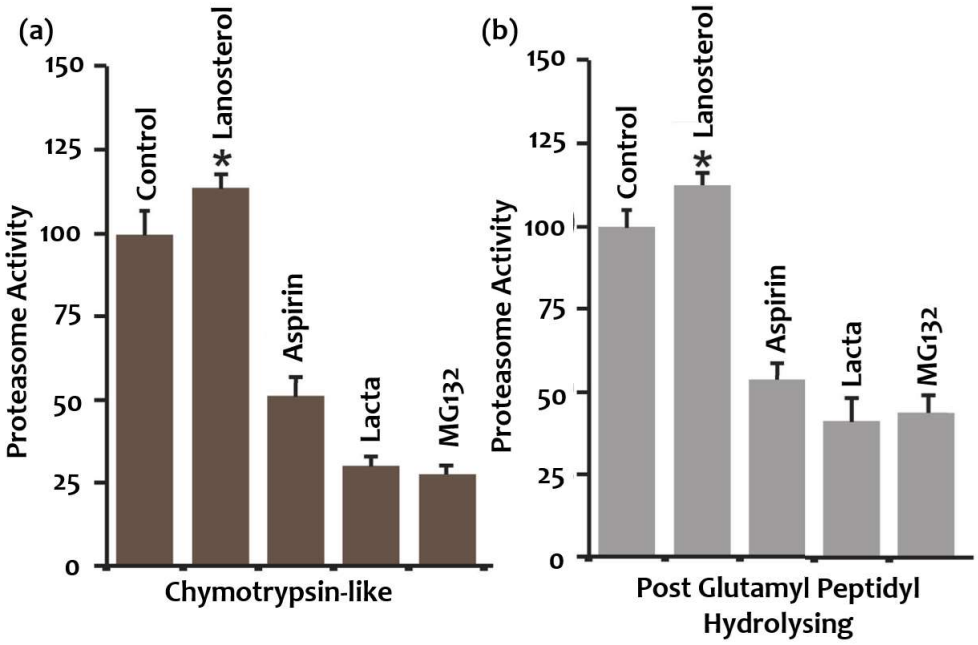


Figure 3.13: Lanosterol induces the proteasome activities: A549 cells in 6 well plates were exposed to control and lanosterol (20 µM, 48 h) as well as proteasome inhibitors aspirin (2.5 mM, 12 h), lactacystin (10 µM, 12 h), and MG132 (10 µM, 12 h). Cells were harvested and processed to measure proteasome activities chymotrypsin-like (a) and PGPH-like (b). Values are shown as mean ± SD from three experiments done independently in triplicate. *$p < 0.05$ presents statistical significance on comparison to control.

Next, we needed to examine the exogenous effect of lanosterol treatment on overall cellular health and survival. As shown in Figures 3.14 and 3.15, exposure to lanosterol showed a very mild cellular insults in both time-dependent (Figure 3.14) and concentration-dependent (Figures 3.15) manner as compared to the use of proteasomal inhibitor MG132 in a similar set of experiments.

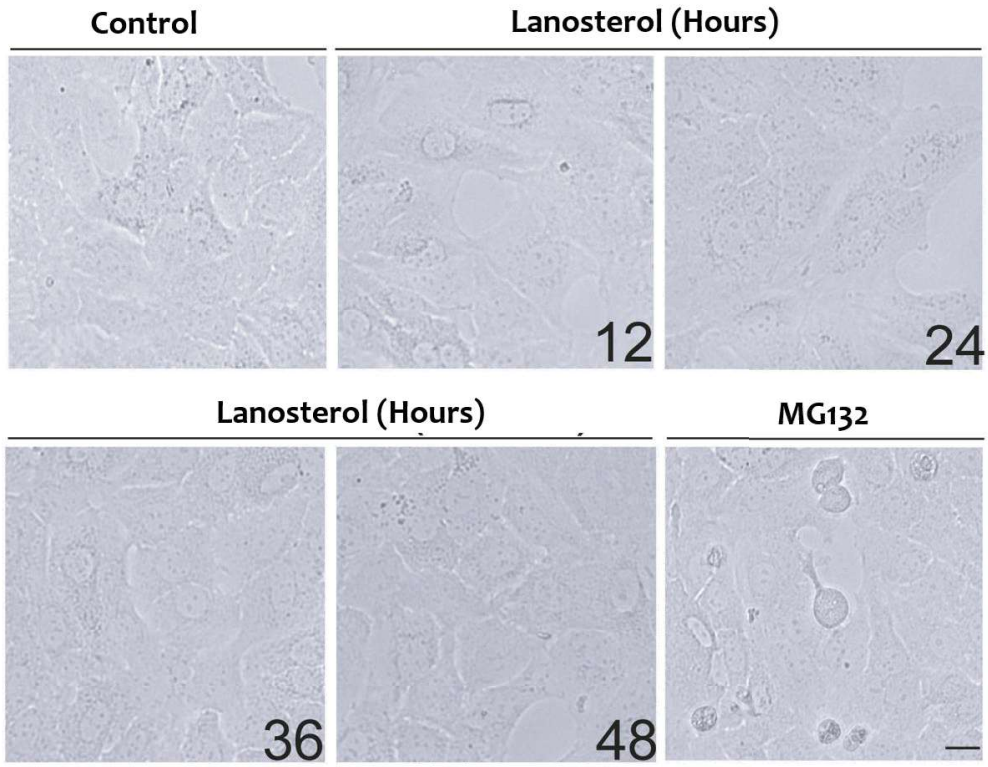


Figure 3.14: Effect of lanosterol in time-dependent manner on morphological aspects of cells: A549 cells were administered for different time points with lanosterol (20 µM) and imaged with brightfield microscopy, compared with control and MG132 (10 µM, 12 h). *Scale bar: 20 µm.*

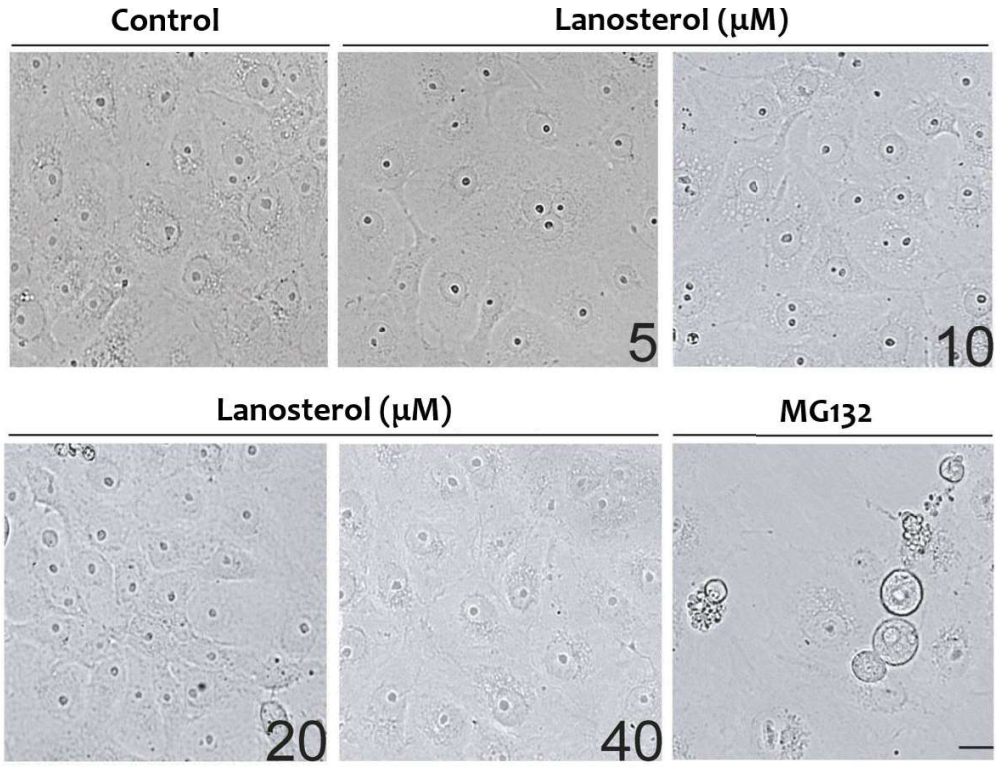


Figure 3.15: Concentration-dependent effect of lanosterol on morphological aspects of cells: A549 cells were given indicated concentrations of lanosterol and imaged with brightfield microscopy post treatment completion, compared with control and MG132 (10 µM, 12 h). *Scale bar: 20 µm.*

We have shown the elevation of proteasome activities in the presence of lanosterol without cellular toxicity. Therefore, we thought to analyze the endogenous localization of proteasome and other PQC components (ubiquitin and p62) with or without lanosterol treatment. As shown in the immunofluorescence analysis in Figure 3.16, exposure to lanosterol significantly induced the endogenous levels of proteasome as well as various PQC-associated chief molecules such as ubiquitin and p62/SQSTM1.

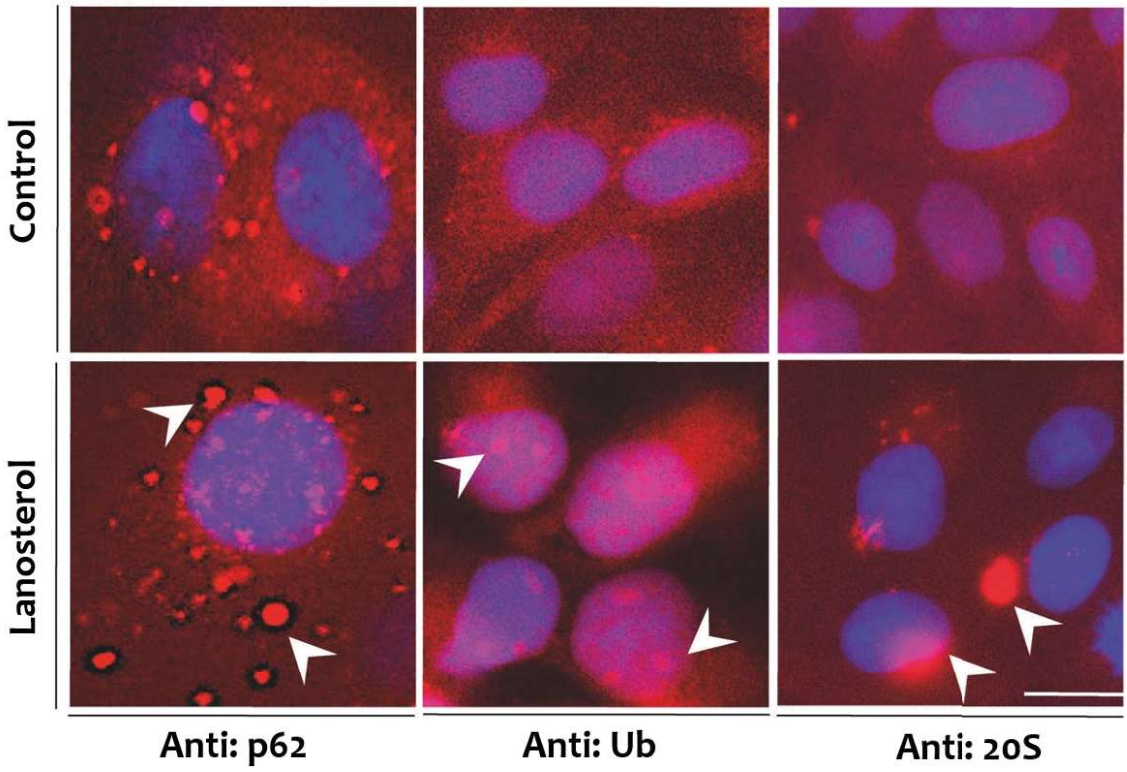


Figure 3.16: Lanosterol induces the endogenous levels of PQC elements: A549 cells were exposed to control or lanosterol (20 μM). These cells were prepared for immunocytochemistry experiments and tested with anti-20S, anti-ubiquitin, and anti-p62 antibodies, and Texas Red-conjugated secondary antibody to analyze effect on their endogenous levels with epifluorescence microscopy. Nuclear localization was done with DAPI (blue). *Scale bar: 20 μm.*

Together, these data suggest that lanosterol can induce proteasome and PQC-based misfolded protein proteolytic degradation. Our results indicate lanosterol treatment can induce proteasome activities without generating adverse effects on cellular health, and lanosterol may retain a PQC-associated therapeutic potential against protein-conformational disorders.

3.1.5 *In Silico* Docking Analysis of Lanosterol with Proteasome Subunits

To investigate the possibility of lanosterol interaction with proteasome, we performed detailed docking analysis of proteasome subunits with lanosterol as shown in Figures 3.17-3.19. Blind docking was performed to identify high-probability binding sites. The free energy associated with lanosterol interaction at the possible binding sites of β1, β2, and β5 subunits were observed to be −7.4, −7.4, and −6.7 kcal/mol, respectively.

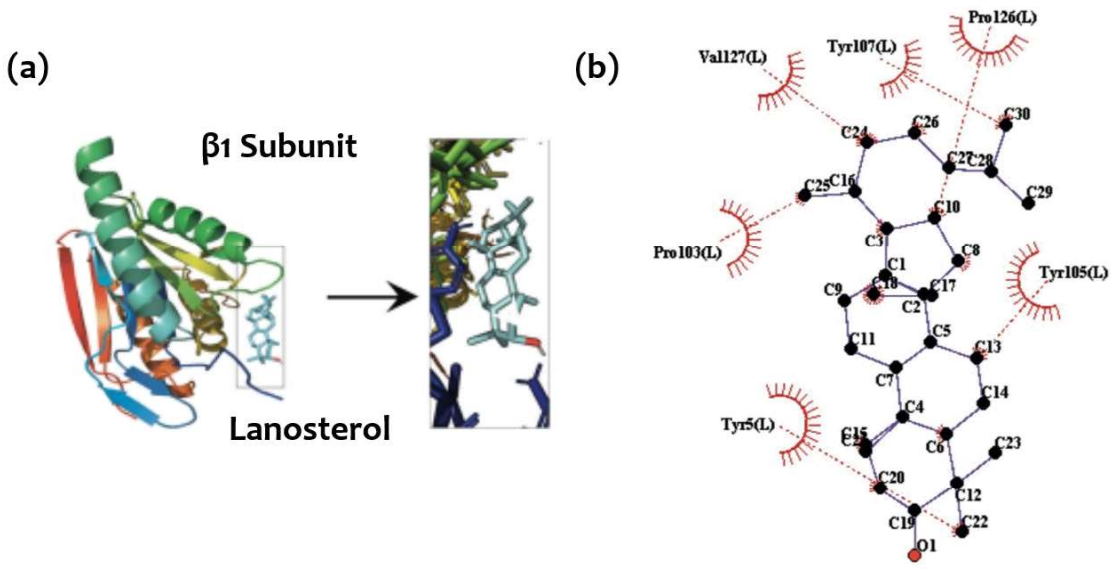


Figure 3.17: *In silico* docking of lanosterol with proteasome proteolytic activity subunit β1: (a) Pose with minimum binding energy after *in silico* docking of lanosterol with catalytic subunit β1 of 20S proteasome (PDB 5LE5) is represented. (b) The 2D ligand-protein interactions via LigPlot+ are represented, hydrophobic interactions are shown by red dashed lines and residues depicted by spoked arcs make nonbonded contacts with ligands.

Next, to examine the interaction between proteins and small molecules and to show the 2D ligand-protein interaction, the results of the *in silico* docking output were utilised as input, along with 3D coordinates. Dashed lines in green indicate the hydrogen bond, while dashed lines in red show the hydrophobic interaction at the possible binding sites of β1, β2, and β5 subunits respectively as shown in Figures 3.17 (b), 3.18 (b), and 3.19 (b). In the future, further detailed biophysical analysis, NMR studies and detailed experiments are required to understand in-depth the possible interaction of lanosterol with various proteasome subunits.

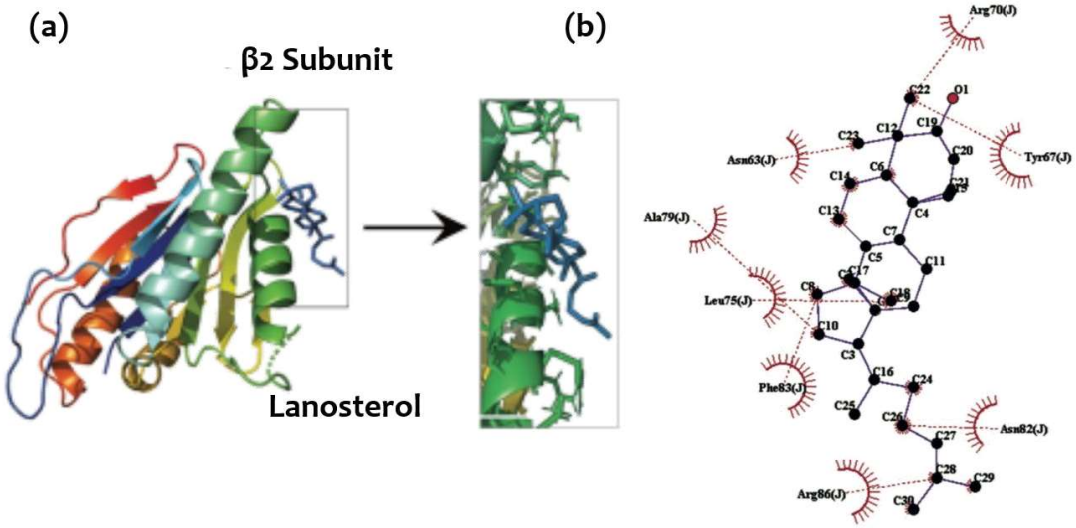


Figure 3.18: *In silico* docking of lanosterol with proteasome catalytic subunit β2: (a) After *in silico* docking of lanosterol with catalytic subunit β2 of 20S proteasome (PDB 5LE5), pose with minimum binding energy is represented. (b) The 2D ligand-protein interactions featuring, hydrophobic interactions are shown by red dashed lines and residues depicted by spoked arcs make nonbonded contacts with ligands.

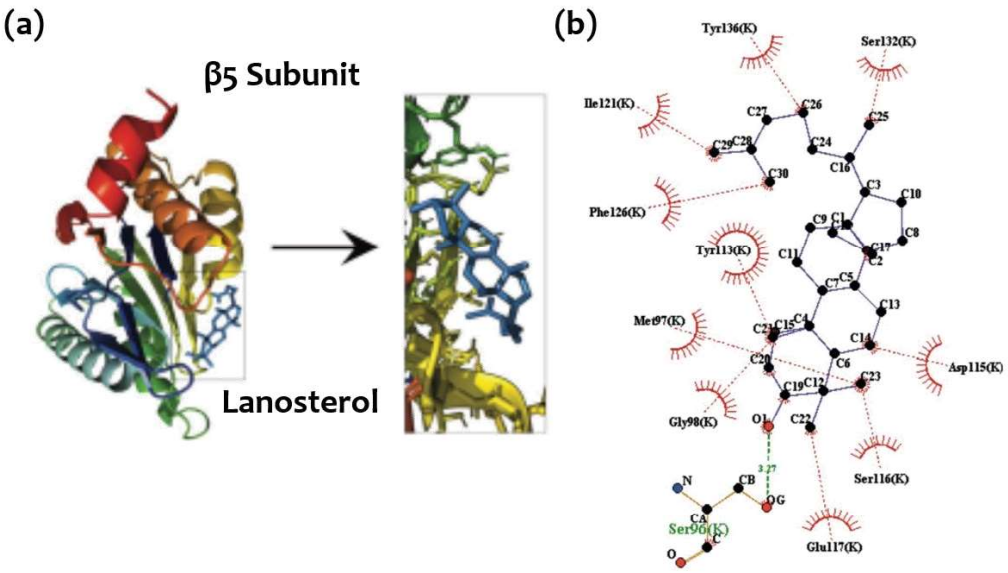


Figure 3.19: *In silico* docking of lanosterol with catalytic subunit β5 of proteasome: (a) Pose with minimum binding energy after *in silico* docking of lanosterol with catalytic subunit β5 of 20S proteasome (PDB 5LE5) is depicted. (b) The 2D ligand-protein interactions via LigPlot+ are represented, hydrophobic interactions are represented by red dashed lines, hydrogen bonds by green dashed lines, and residues depicted by spoked arcs make nonbonded contacts with ligands.

3.1.6 Lanosterol Treatment Elevates Proteolytic Activities of Proteasome

The formation of abnormal inclusions or misfolded protein aggresomes is a well-reported hallmark of numerous neurodegenerating conditions [Olzmann et al., 2008]. The presence of ubiquitinated proteins is commonly associated with such inclusions, and previously, it has been reported that over-accumulation of aggresomes can block the proteasome functions or activities [Kristiansen et al., 2007]. Based on previous results in this study, we thought that treatment of lanosterol could modulate the proteasome function. This strategy can improve clearance of over-accumulated abnormal proteins. To investigate this question, we executed detailed proteasome activities assays; cells were given lanosterol at multiple concentrations (Figure 3.20) and time periods (Figure 3.21). These cells were used for the measurement of both chymotrypsin-like (Figures 3.20 (a) and 3.21 (a)) and PGPH-like (Figures 3.20 (b) and 3.21 (b)) activities; some cells sets were exposed to MG132. Our observations suggest that the treatment of lanosterol significantly elevated both chymotrypsin-like (Figures 3.20 (a) and 3.21 (a)) and PGPH-like (Figures 3.20 (b) and 3.21 (b)) activities of proteasome in concentration and time-dependent fashion.

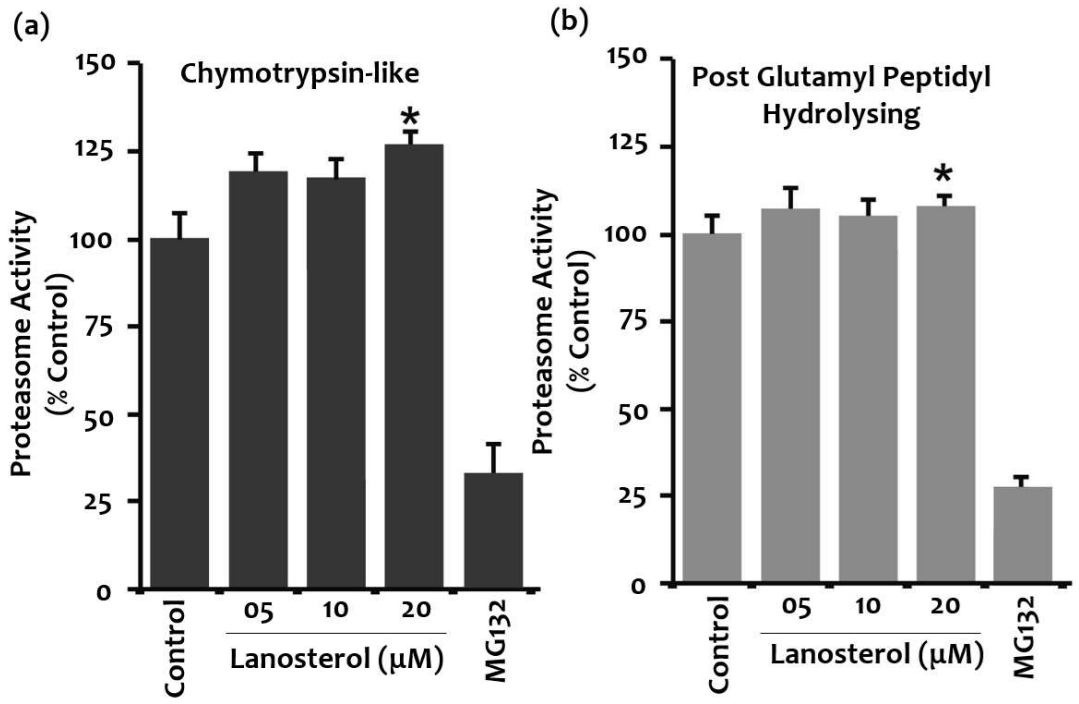


Figure 3.20: Effect of lanosterol concentration-dependent treatment on proteasome proteolytic activities: A549 cells grown in 96-well plates were given control (DMSO) or multiple concentrations of lanosterol for 36 h or MG132 (10 µM) for 12 h. Cells were harvested, processed, and subjected to proteasome assay to measure chymotrypsin-like (a) and PGPH-like (b) activities. Values shown in figure are shown as mean ± SD from three experiments done independently in triplicate. *p < 0.05 when compared to control.

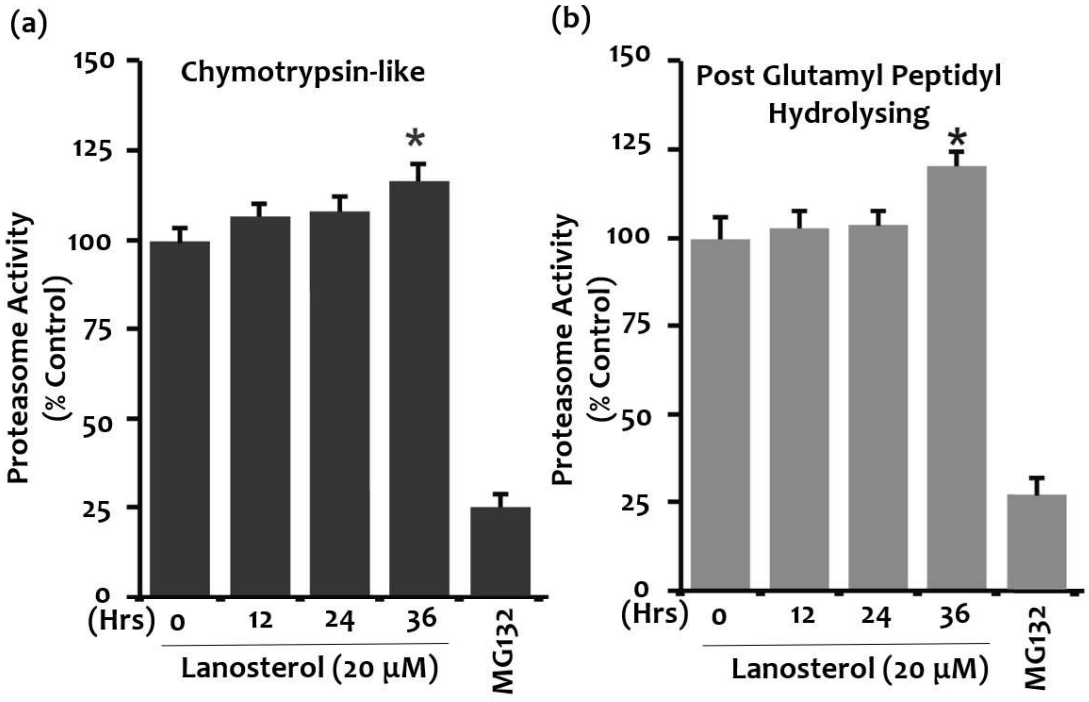


Figure 3.21: Effect of lanosterol time-dependent treatment on proteasome proteolytic activities: A set of A549 cells were treated with DMSO, MG132 (10 µM), and lanosterol (20 µM) for multiple indicated time periods. Cells were harvested and processed for proteasome assay to measure chymotrypsin-like (a) and PGPH-like (b) activities. Values shown in figure are shown as mean ± SD from three experiments done independently in triplicate. *p < 0.05 when compared to control.

Next, we performed cycloheximide chase analysis; cells were transfected transiently with pd1EGFP, which codes for a proteasome modeled substrate, and after transfection, cells were exposed with or without lanosterol. Post-treated cells were chased with cycloheximide, and collected samples were used for GFP antibody probed immunoblot analysis, as shown in Figure 3.22 (a). We observed that the treatment of lanosterol induced the degradation rate of d1EGFP, and a few sets of similar cells were observed via fluorescence microscope (Figure 3.22 (b)). The proactive function of the proteasome in the PQC mechanism is essential for maintaining proteostasis [Kumar Deshmukh et al., 2019]. These results suggest that lanosterol can induce proteasome activities and, therefore, also help in removal of long-lived aberrant proteins from the cells, aiding their rejuvenation against proteotoxic insults.

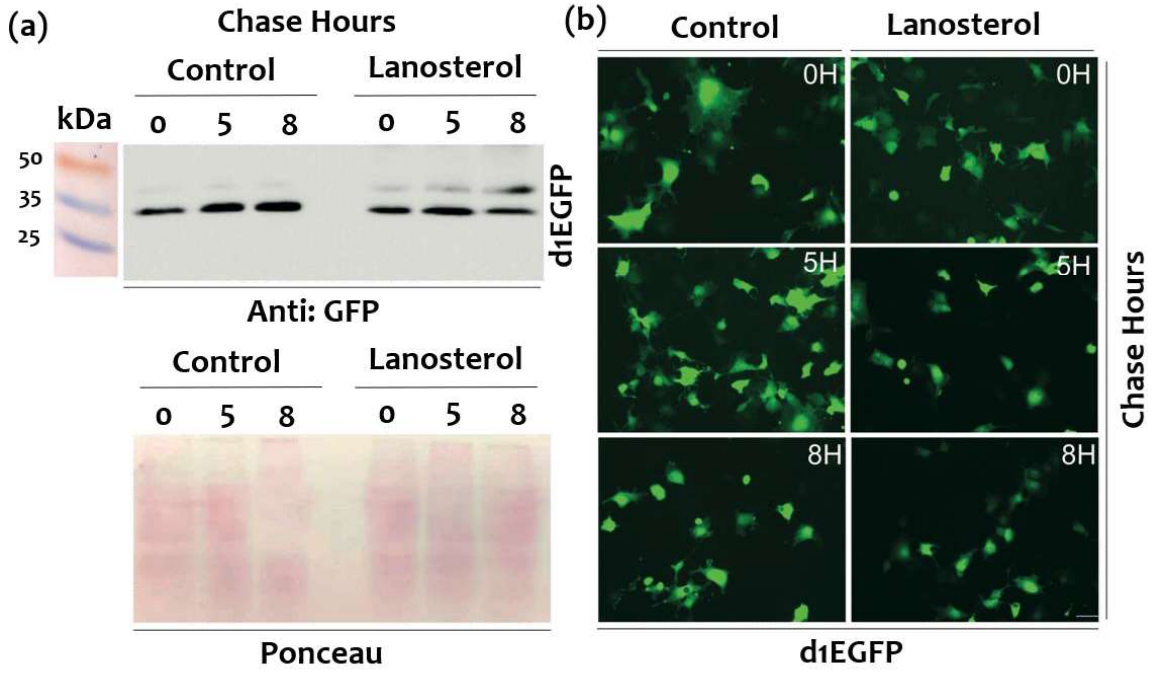


Figure 3.22: Lanosterol treatment effect on d1EGFP upon cycloheximide chase: Cos-7 cells were transfected transiently with pd1EGFP construct and post-transfected cells were given lanosterol (20 μM, 24 h) or control and post-treatment chased with cycloheximide (10 μM) for shown time points. Post-chase, cells were utilised for immunoblot experiment via GFP antibody (a) and few sets of similar cells were observed via fluorescence microscope (b).

3.1.7 Lanosterol-mediated Proteasomal Upregulation Promotes the Degradation of Ataxin-3 Expanded Polyglutamine Proteins

The dominant mechanism of selective intracellular misfolded protein degradation in eukaryotic cells is UPS [Lilienbaum, 2013]. Aberrant proteins are marked by the ubiquitin chain linkages and delivered to proteasome machinery for degradation [Komander and Rape, 2012]. In our previous experiments, we demonstrated that exposure to lanosterol induces proteasome function; therefore, we intended to check impact of lanosterol on proteasomal-based clearance of ataxin-3 (Q84) with polyglutamine expansion. To perform the above-described experiment, we transiently transfected cells with GFP-tagged normal repeat ataxin-3 (Q28) and polyglutamine expanded repeat ataxin-3 (Q84) expressing plasmids. Post-transfection cells were treated with lanosterol and few sets of experiments were executed with or without MG132.

After exposure, cells were prepared for immunocytochemistry investigation by utilizing anti-20S antibody (Red). As shown in Figure 3.23, cells expressing normal repeat ataxin-3 (Q28) form almost negligible aggregates compared to cells that were also expressing ataxin-3 (Q28) but treated with MG132 (Figure 3.24); simultaneously in another set, treatment of lanosterol

diminished the aggregates as compared with control cells (Figure 3.24). As shown in Figure 3.25, exposure to lanosterol enhances the clearance of abnormal inclusions of ataxin-3 (Q84) proteins, which was prevented by the addition of MG132 (Figure 3.26). These results suggest lanosterol to have a proteasomal-mediated anti-aggregation capacity against aberrant intracellular inclusions linked with neurodegeneration.

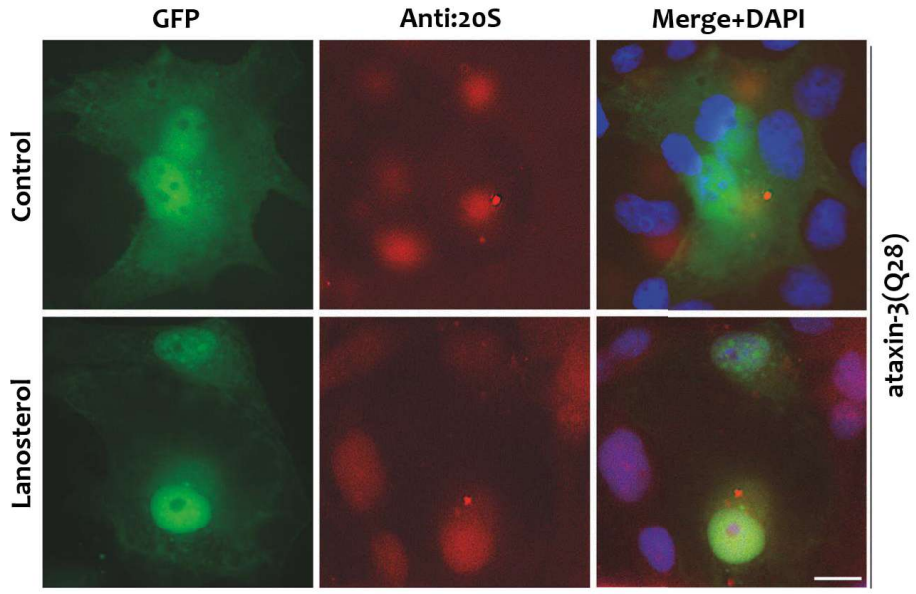


Figure 3.23: Effect of lanosterol treatment on ataxin-3 (Q28) polyglutamine protein: Cos-7 cells transfected with GFP-ataxin- 3 (Q28) were treated with control (DMSO) or lanosterol (20 μM) for 48 h. Cells were examined with anti-20S antibody via immunocytochemistry through fluorescence microscope. The ataxin-3 proteins were imaged via GFP fluorescence in green channel. DAPI was utilized for nuclear staining (blue). *Scale bar: 20 μm.*

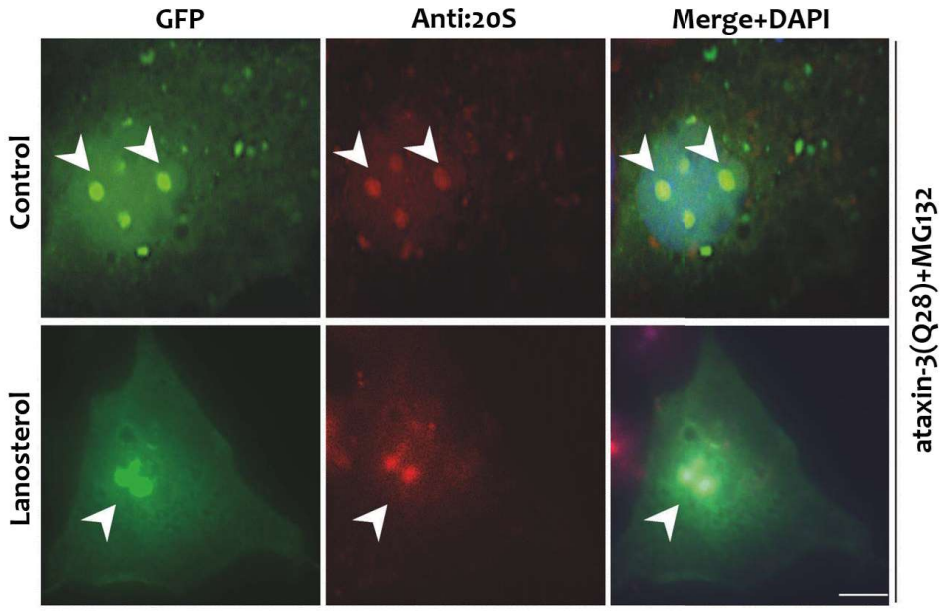


Figure 3.24: Lanosterol treatment to cells with ataxin-3 (Q28) in presence of MG132: Cos-7 cells transiently transfected with GFP-ataxin- 3 (Q28) fusion construct were treated with control (DMSO) or lanosterol (20 μM) for 48 h with MG132 (10 μM) for 12 h. The anti:20S micrograph represents PQC element proteasome (red) co-localising with ataxin-3 aggregates (green). Nuclear staining was done with DAPI (blue). *Scale bar: 20 μm.*

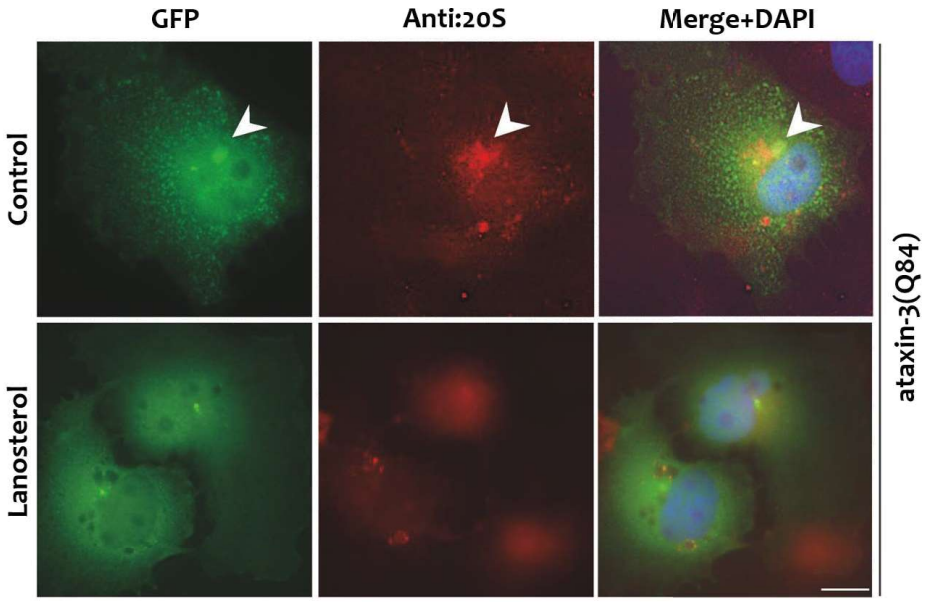


Figure 3.25: Lanosterol-mediated proteasomal upregulation facilitates the degradation of ataxin-3 polyglutamine expanded protein: Transfected Cos-7 cells with GFP-ataxin- 3 (Q84) fusion construct were given control (DMSO) or lanosterol (20 μM) for 48 h. The ataxin-3 aggregates were imaged via GFP fluorescence and 20S via immunofluorescence. Nuclear staining was done with DAPI (blue). The arrowheads represent aggregates of ataxin-3 with proteasome. *Scale bar: 20 μm.*

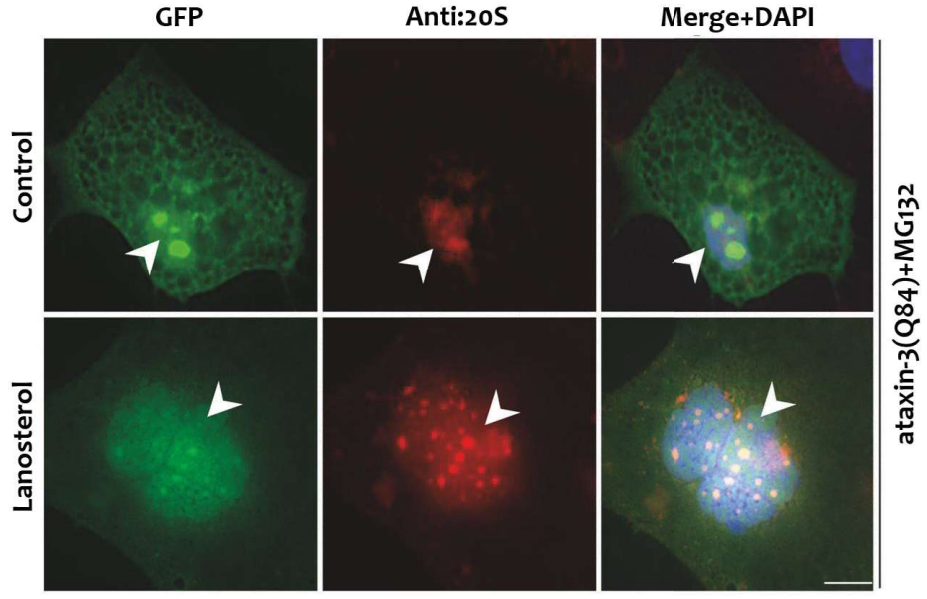


Figure 3.26: Anti-aggregation effect of lanosterol on ataxin-3 (Q84) is diminished by MG132: Cos-7 cells transfected with GFP-ataxin- 3 (Q84) plasmid were given control (DMSO) or lanosterol (20 μM) for 48 h in MG132 (10 μM, 12 h) presence. Cells were examined with anti-20S antibody via immunocytochemistry investigation to observe co-localization of PQC element proteasome with the aggregates in red channel in fluorescence microscope. The ataxin-3 aggregates were imaged via GFP fluorescence in green channel. Nuclear staining was done with DAPI (blue). The arrowheads represent aggregates of ataxin-3 with proteasome. *Scale bar: 20 μm.*

3.1.8 Lanosterol Promotes the Clearance of Huntington Disease Associated Proteins via Proteasomal Pathway

Targeted protein degradation is challenging for cells, specifically when proteins are mutant or toxic [Hanna et al., 2019]. Cells must apply the PQC mechanisms against the abnormal aggregation and protect themselves through depletion of the unwanted load of abnormal proteins

[Fredrickson and Gardner, 2012]. In this experiment, we monitored the proteasomal-associated anti-aggregation effect of lanosterol against another polyglutamine expansion-associated neurodegenerative disease-linked protein. Therefore, we used normal (EGFP-HD23Q) and Huntington's disease-linked expanded (EGFP-HD74Q) polyQ proteins in our immunofluorescence experiments using anti-20S antibody. Cells were transiently transfected with normal (EGFP-HD23Q) and expanded (EGFP-HD74Q) polyQ proteins expressing plasmids and exposed them with or without lanosterol. As shown in Figures 3.27 and 3.28, normal polyglutamine protein repeats (EGFP-HD23Q) expressing cells generate the marginal number of aggregates upon co-addition of MG132, which were depleted with the addition of lanosterol (Figure 3.28).

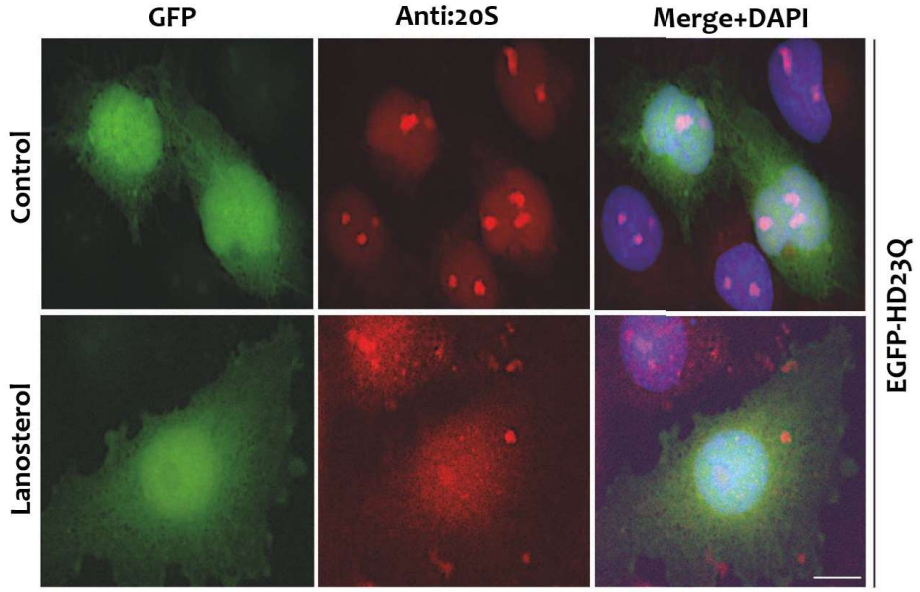


Figure 3.27: Effect of lanosterol on EGFP-HD23Q: Transfected Cos-7 cells with EGFP-HD23Q plasmid construct were administered control or lanosterol (20 μM) for 48 h. The anti-20S antibody was utilised for immunofluorescence analysis of treated cells to observe colocalization of proteasome (red). DAPI utilized to stain nuclei (blue). *Scale bar: 20 μm.*

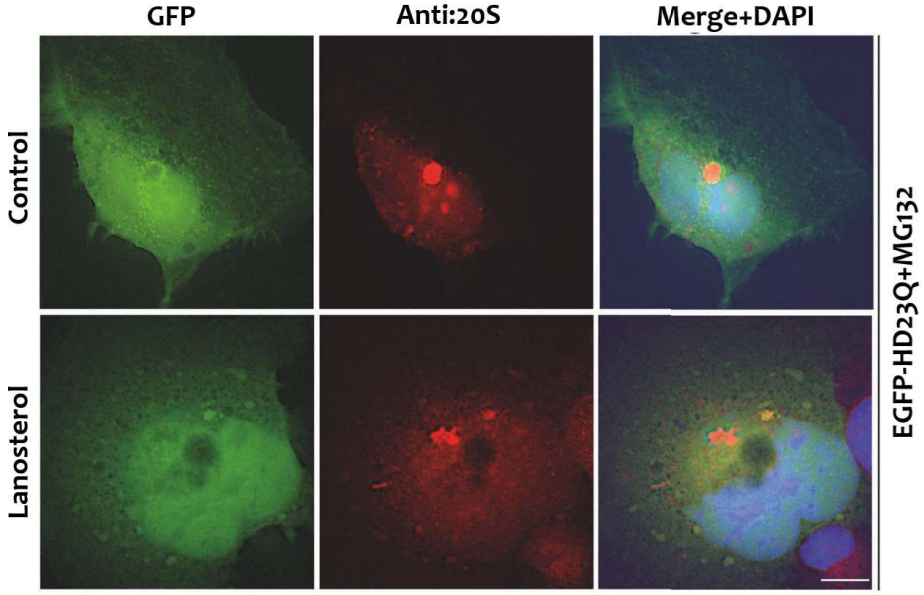


Figure 3.28: Effect of lanosterol on EGFP-HD23Q in presence of MG132: Cos-7 cells transfected as in Figure 3.27 were administered control or lanosterol (20 μM) for 48 h with MG132 (10 μM) for 12 h. For immunofluorescence analysis, cells were prepared and tested with anti-20S antibody and imaged via fluorescence microscopy. DAPI utilized for staining nuclei (blue). *Scale bar: 20 μm*

We next examined the expanded EGFP-HD74Q polyglutamine proteins expressing cells and observed administration of lanosterol to effectively reduce the aggregation of expanded proteins (Figure 3.29). Further, co-addition of MG132 (Figure 3.30) inhibits the proteasome and diminishes the effect of lanosterol to reduce the proteotoxic accumulation of expanded (EGFP-HD74Q) polyglutamine protein.

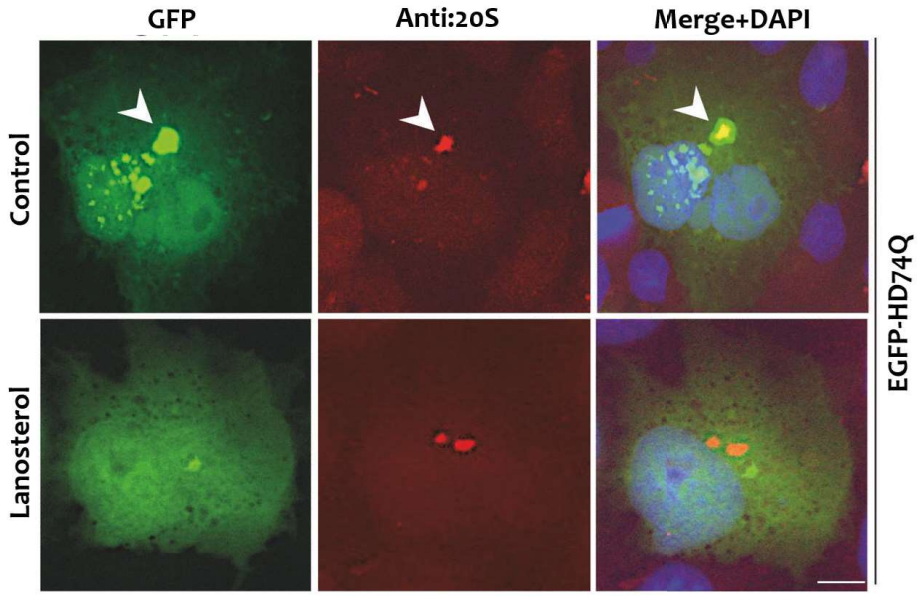


Figure 3.29: Lanosterol induces proteasome-mediated degradation of Huntington's disease-associated aggresomes: Transfected Cos-7 cells with EGFP-HD74Q were given control or lanosterol (20 µM) for 48 h. The immunofluorescence analysis was performed on treated cells with anti-20S antibody to observe colocalization of proteasome (red) with polyQ expanded HD74Q aggregates (green) as indicated by arrowheads. DAPI utilized for staining nuclei (blue). *Scale bar: 20 µm.*

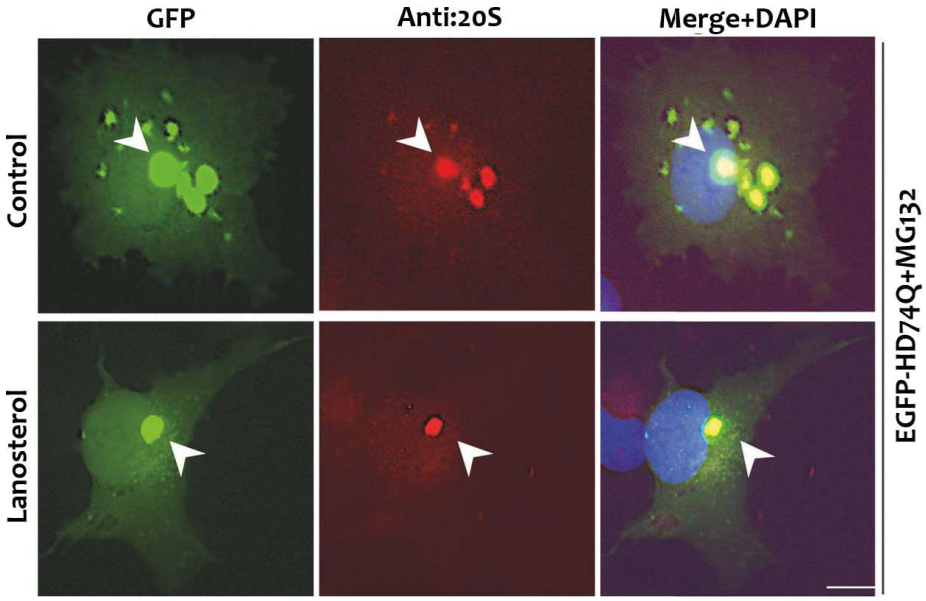


Figure 3.30: MG132 diminished the effect of lanosterol induced proteasome-mediated degradation of Huntington disease associated aggresomes: Transfected Cos-7 cells as in Figure 3.29 were given control or lanosterol (20 µM) for 48 h in MG132 presence (10 µM) for 12 h. Treated cells were processed for immunofluorescence and tested with anti-20S antibody to observe colocalization of proteasome (red) and polyQ expanded HD74Q aggregates (green) as shown by arrowheads. DAPI utilized for staining nuclei (blue). *Scale bar: 20 µm.*

3.1.9 Lanosterol Reduces Misfolded Proteins and Improves Cell Survival Under Stress Conditions

In current work, we observed treatment of lanosterol elevates endogenous levels of proteasome and induces proteasome activities. Therefore, we next tested the direct impact of lanosterol on the aggregation propensity of misfolded proteins. We collected lanosterol-treated samples and used them in immunoblot experiment using an antibody anti-ubiquitin. In Figure 3.31, treatment of lanosterol promotes degradation of proteins; overall, we observed less accumulated ubiquitin proteins in lanosterol-exposed cells.

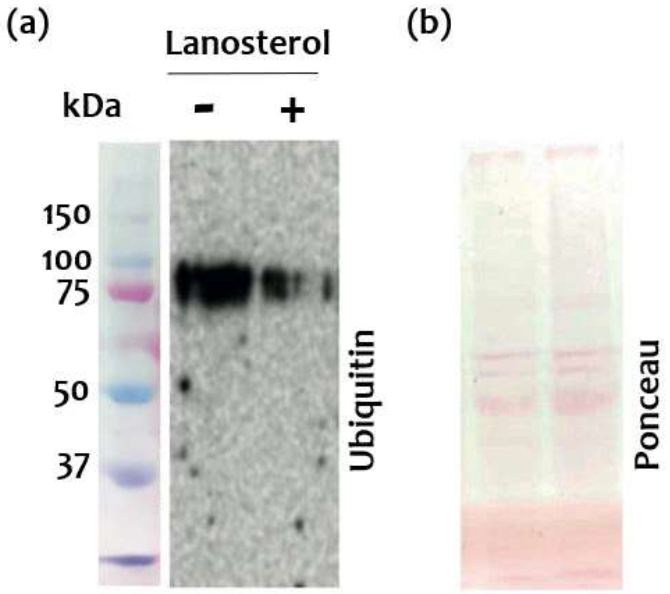


Figure 3.31: Effect of lanosterol on ubiquitinated conjugates: (a) Cos-7 cells were given lanosterol or control, and afterwards, cell lysates were utilised for immunoblotting via anti-ubiquitin antibody. (b) This panel represents the ponceau staining of the blot shown in section (a).

Next, we performed dot-blot analysis using cell lysates expressing various abnormal proteins. As shown in Figures 3.32 and 3.33, exposure to lanosterol promotes the clearance of aberrant proteins, and our findings suggest that using lanosterol promotes the solubility of abnormal proteins.

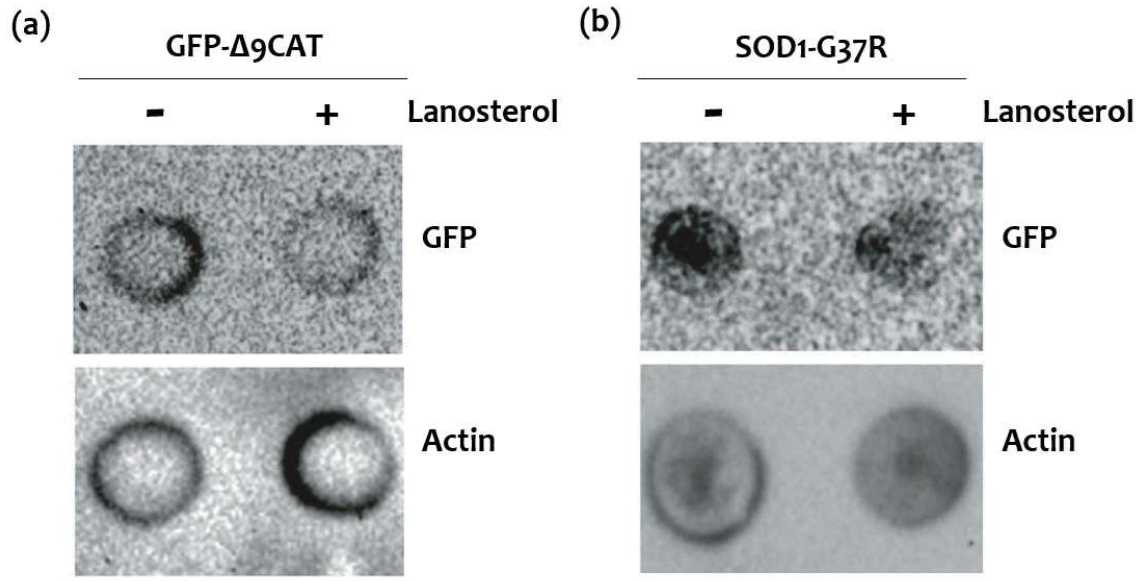


Figure 3.32: Lanosterol reduces aberrant proteins aggregation: Cells were transfected transiently with plasmids of GFP-Δ9CAT (a) and SOD1-G37R (b). Post-transfection, cells were given lanosterol (20 μM) or control; collected cell extracts were utilised for analysis by dot-blot, and blots were incubated with antibodies anti-GFP and anti-actin.

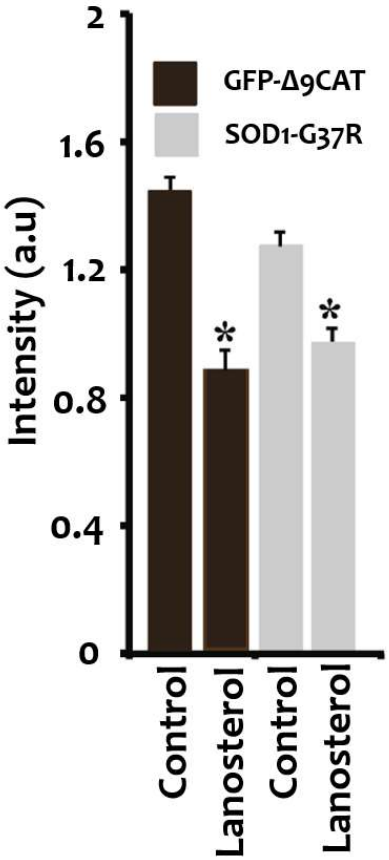


Figure 3.33: Quantification analysis of lanosterol effect on mutant protein aggregation: The dot-blots shown in Figure 3.32 were used for quantification analysis. NIH ImageJ densitometric method used to analyze the accumulated protein inclusions.

Earlier reports indicated that the formation of abnormal proteins induces cellular stress and impacts overall cellular survival. We examined lanosterol effect on cell survival under oxidative and ER stress-induced conditions. Cells were exposed to H_2O_2 and tunicamycin, and a few sets of cells were transfected with Hsp70 or CHIP plasmid constructs. Our results suggest that treatment of lanosterol provides cytoprotection against cellular stress conditions, and the cumulative effect of lanosterol and expression of molecular chaperones (CHIP and Hsp70) is more beneficial for the cells for their survival, as represented in Figure 3.34. We also confirmed the exogenous expression of CHIP and Hsp70 in the above experiments, as shown in Figure 3.35. Overall, these results indicate that using small molecules such as lanosterol with PQC components can improve the survival of cells under stress conditions, which is most likely due to the elevated functions of PQC and proteasome-based degradation of abnormal proteins.

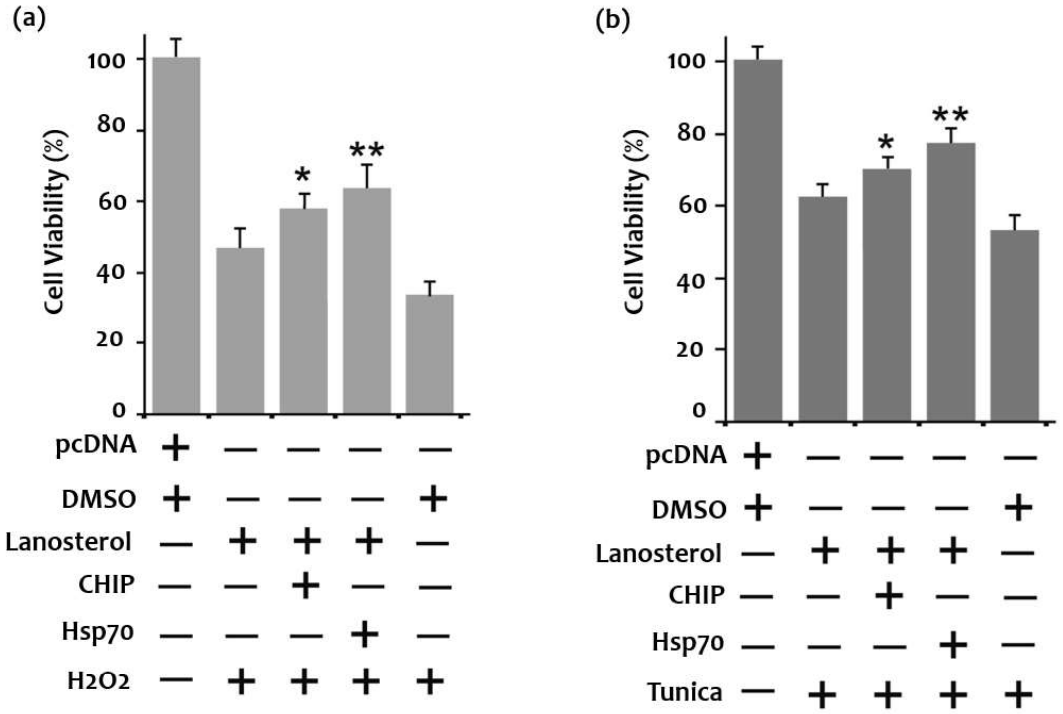


Figure 3.34: Use of lanosterol alleviates cytotoxicity: Cells were transiently transfected with pcDNA, Hsp70 and CHIP constructs, few sets of similar cells were exposed to H2O2 (0.2 mM for 3 h) (a) and tunicamycin (10 µg/ml for 12 h) (b) with or without lanosterol (20 µM). Post treatment time completion, cells were subjected to MTT assay; each experiment was performed in triplicate, and values are means±SD of three independent experiments.

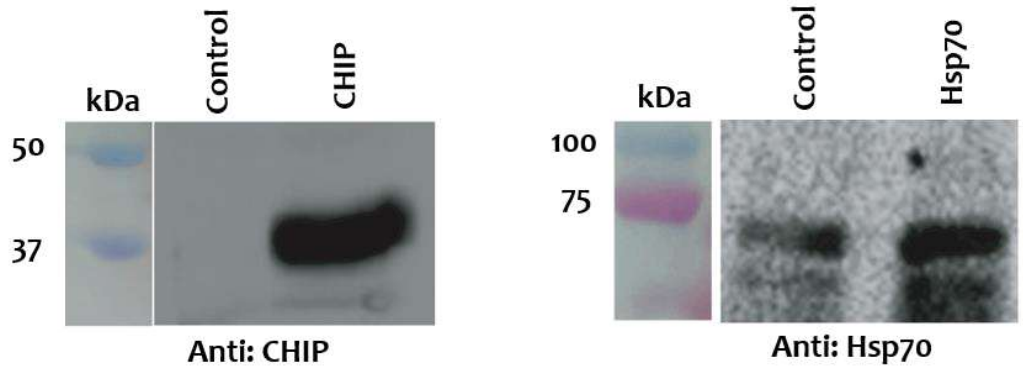


Figure 3.35: Confirmation of exogenous expression of CHIP and Hsp70: A few samples collected from Figure 3.34 were used for immunoblotting analysis using CHIP and Hsp70 antibodies to confirm the exogenous expression of constructs.

3.1.10 Lanosterol-mediated Enhancement of Proteasome Functions Alleviates Cytotoxicity

We need to understand more about the small compounds as modulators of proteasome-based therapeutic strategies and the cytoprotective aspects of their nature. Our results, till now, have demonstrated that lanosterol treatment can induce proteasome activities and improve the clearance of neurodegeneration-linked proteins. To understand how the treatment of lanosterol can be helpful against proteotoxic insults, we exposed cells with various stress-inducing agents (Figure 3.36). Treatment of H_2O_2, BME, MG132, and thapsigargin indicated increased cellular toxicity and decreased fitness in comparison to control cells. In contrast, lanosterol treated cells showed proportionally better health compared to the corresponding stress-exposed cell population.

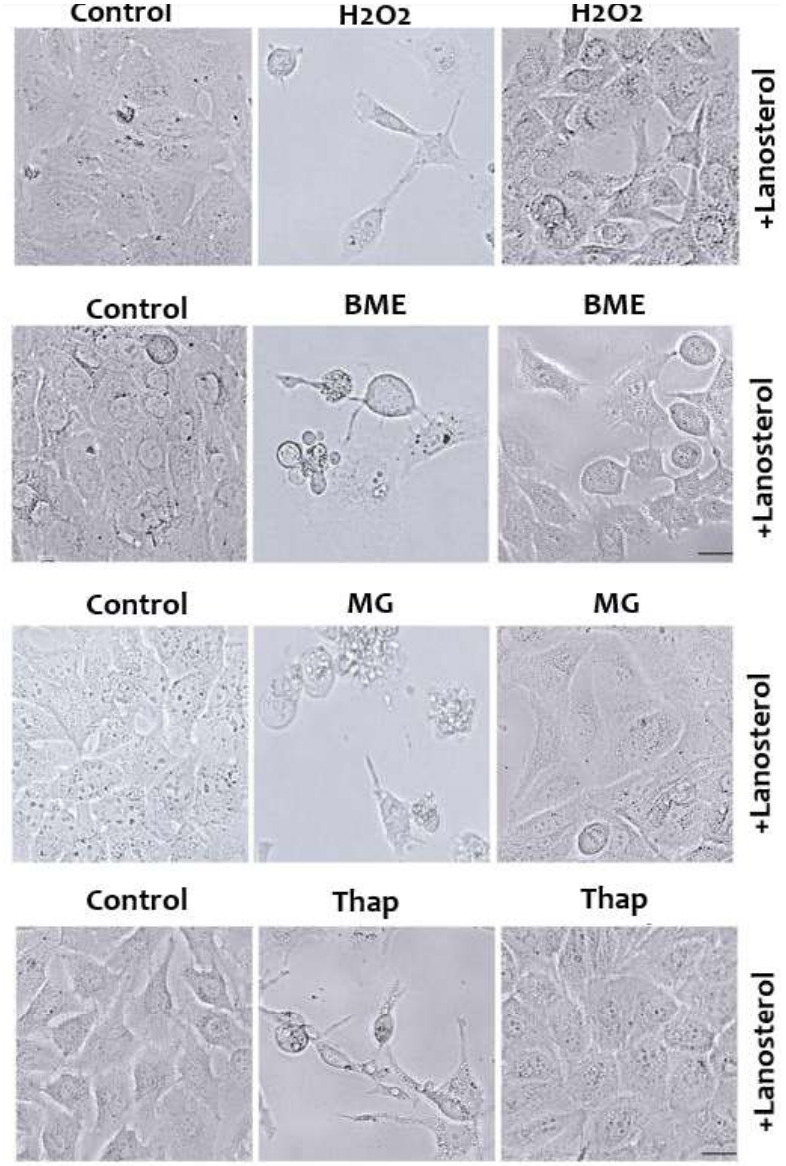


Figure 3.36: Lanosterol provides cytoprotection against toxicity by stressors: A549 cells exposed to multiple stressors in 6-well plates with or without lanosterol (20 μM) and compared to control (DMSO). These stressors were H_2O_2 (0.2 mM for 3 h), BME (5 mM for 3 h), MG132 (10 μM for 12 h), and thapsigargin (5 μg/ml for 8 h). These cells were imaged with brightfield microscopy. *Scale bar: 20 μm.*

Because lanosterol seems to provide overall cellular health protection under stress conditions, whether the exposure of lanosterol itself affects cellular viability needs to be assessed. To confirm this question, we performed a detailed cell viability assay under both concentration-

(Figure 3.37 (a)) and time-dependent (Figure 3.37 (b)) manner and noticed that, as such, there is no direct adverse effect of lanosterol on cell viability.

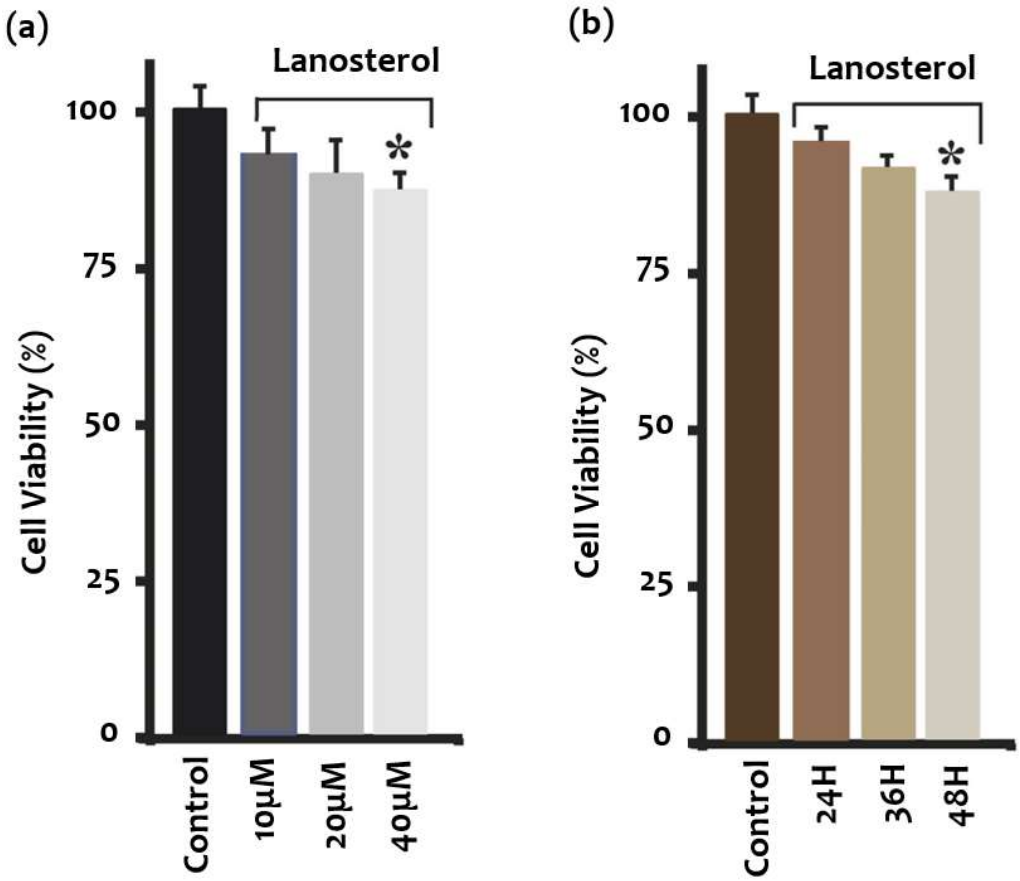


Figure 3.37: Effect of lanosterol on cellular viability: (a-b) Cells given variable concentrations (for 48 h) and for time points (at 20 μM) of lanosterol and viability was assessed with MTT assay. Values are shown in figure as mean ± SD from three experiments done independently in triplicate. *$p < 0.05$ when compared with control.

Our previous research has suggested that treating cells with certain natural compounds minimizes accumulation of aberrant proteins and improves cellular survival [Upadhyay et al., 2018; Joshi et al., 2019; Dubey et al., 2022]. The cytoprotective effect of lanosterol prompted us to determine its rationale; we thought, most likely, treatment of lanosterol depletes aggregation load of misfolded proteins in cells, which possibly re-establishes proteostasis and other pathways of cellular homeostasis possibly also play an effective role, which improves cellular survival. We analyzed this possibility by observing the overall aggregation propensity in presence or absence of lanosterol in cells expressing bona fide misfolded or mutated neurodegenerative diseases associated proteins. The administration of lanosterol lowers the aggregation of various such proteins (Δ9CAT, SOD1-G37R, ataxin-3 Q84) (Figures 3.38 and 3.39). Therefore, we can conclude that lanosterol exposure induces intracellular proteasome activities, which act as a critical factor in removing excess load of abnormal proteins from the cells.

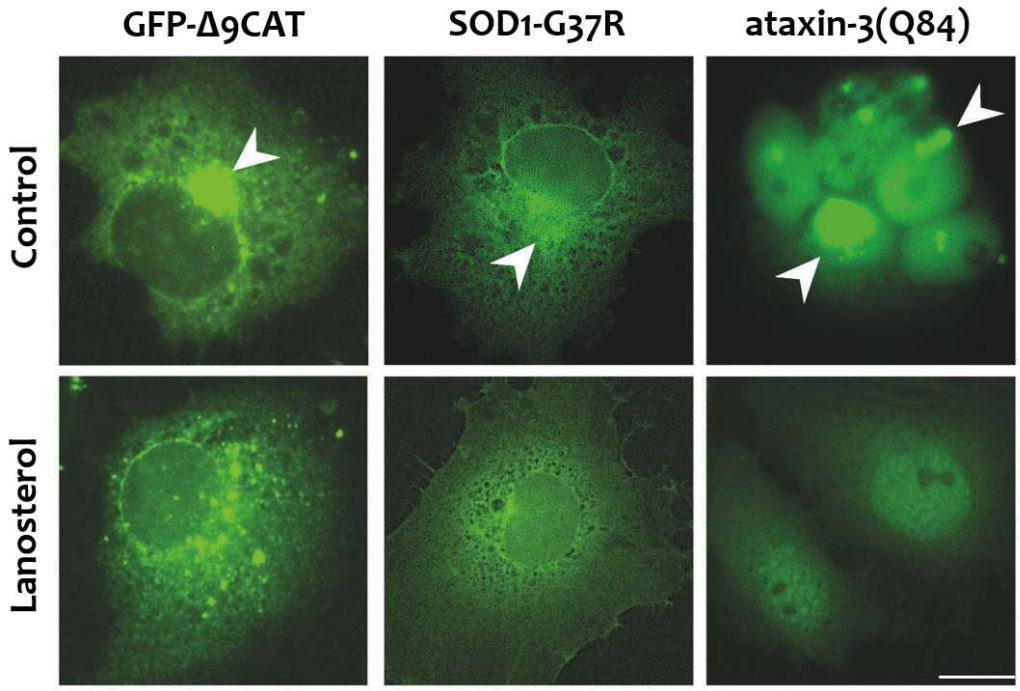


Figure 3.38: Lanosterol treatment reduces aberrant protein aggregate: Cos-7 cells transfected transiently with mutant protein plasmids tagged with GFP (Δ9CAT, SOD1-G37R, ataxin-3 (Q84)) and treated with control or lanosterol (20 μM). The effect on their aggregation was observed via fluorescence microscopy. *Scale bar: 20 μm.*

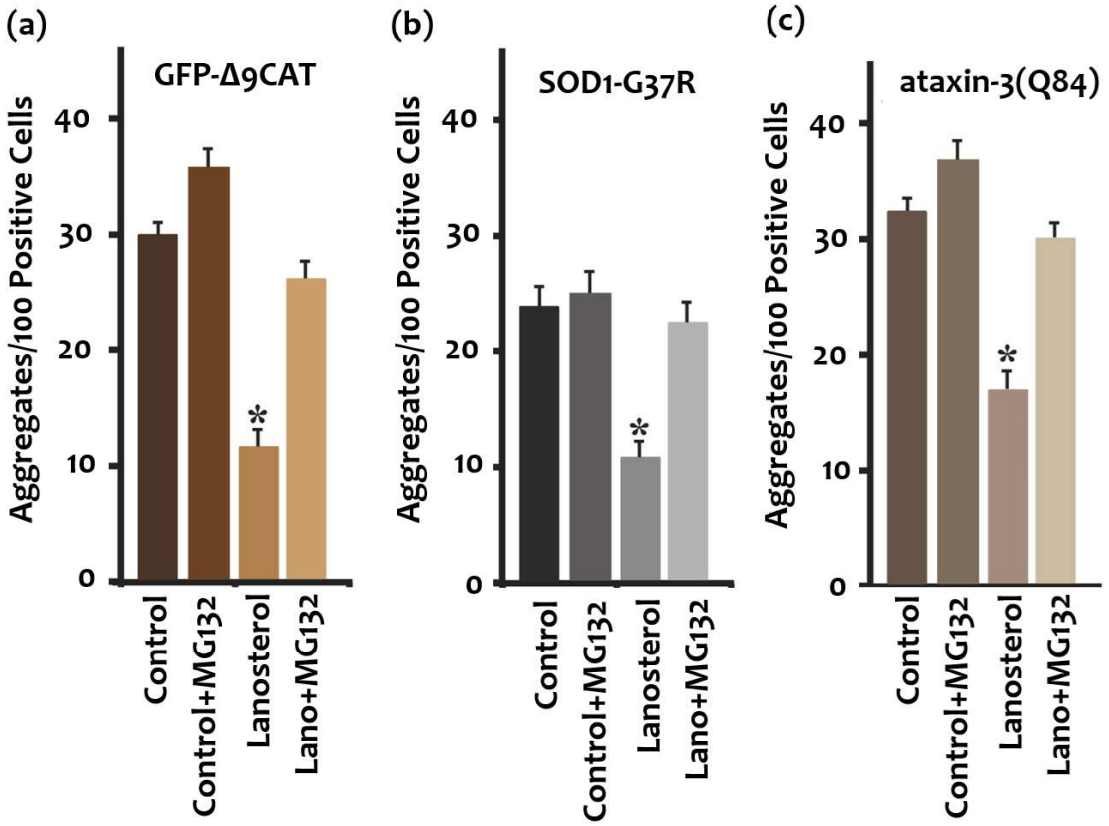


Figure 3.39: Lanosterol treatment reduces aggregation count of aberrant proteins: (a-c) Quantification of the indicated aggregates was done with different sets of treatments to cells, namely control and lanosterol (20 μM); some sets of control or lanosterol-treated cells were exposed to MG132 (10 μM). Values are shown in figure as mean ± SD from three experiments done independently in triplicate. *$p < 0.05$ when compared to control.

3.2 DISCUSSION

Proteasome-associated degradation is a crucial element for normal cellular health and an essential need of the cells' PQC mechanism [Schmidt and Finley, 2014]. Intracellular proteasomal degradation can ensure the removal of an excess of abnormal proteins, and inhibition of proteasome is linked with many neuronal degenerative conditions and protein conformational disorders [Zheng et al., 2014]. Effective degradation and clearance of aberrant proteins are necessary for a functional proteome, important for cellular fitness and survival under stress conditions [Amanullah et al., 2017b]. Earlier findings suggest that using different natural compounds, we can target cellular proteolytic machinery to effectively eliminate misfolded proteins from the cells [Huang et al., 2007; Rusmini et al., 2020]. Still, a critical question in this respect is whether we can induce the removal of abnormal proteins without generating proteostasis imbalance. We used both bona fide model misfolded proteins and several neuronal degenerating condition-linked proteins in the current study to approach this question. We observed that the treatment of lanosterol, an intermediate of the cholesterol synthesis pathway [Huff and Telford, 2005] elevates the proteasome activities. Further, our finding suggests that lanosterol-mediated induction of proteasome functions promotes the clearance of bona fide model misfolded proteins and neuronal degenerating condition-linked proteins. We noticed that lanosterol facilitates proteasomal-mediated intracellular removal of misfolded protein aggregates and possibly the other cellular pathways also play a critical role to improve cellular survival.

To maintain proteostasis, cells always replace old or defective proteins with new ones [Pratt et al., 2002]. Overaccumulation of abnormal proteins leads to development of pathological aggregates [Fandrich et al., 2009; Soto and Pritzkow, 2018]. However, effective maintenance of cellular proteome, specifically under proteotoxic insults, still needs to be thoroughly examined. Here we report that lanosterol can improve proteasome activities and promote degradation of abnormal proteins. One of our first findings of current study indicated an increase in endogenous levels of 20S proteasome upon treatment with lanosterol, even in a time-dependent fashion. Next, we questioned whether lanosterol-based proteasome elevated proteolytic function can improve the degradation of a bona fide protein. Cells expressing luciferase construct were treated with heat stress with or without lanosterol; after heat recovery, those cells were utilised for immunocytochemistry analysis with antibody against ubiquitin to target polyubiquitinated inclusions of thermally denatured luciferase protein. Our results suggest the proactive degradation of ubiquitinated-abnormal inclusions of heat-denatured luciferase proteins in presence of lanosterol. Similarly, our experiments with cells transfected with the plasmid of d1EGFP, a modeled proteasomal substrate, indicated lanosterol to improve its clearance and promote its faster degradation. These results suggest that lanosterol exhibits proteasome activity modulation capability, which seems helpful in reducing the aggregation of model proteins.

Our study indicates a beneficiary effect of lanosterol on cells under proteotoxic insults. It was essential for us to check whether the treatment of lanosterol induces proteasome-mediated degradation of bona fide misfolded proteins, which have no loss or gain of function in the cells. To confirm this notion, we used fusions of GFP with wild-type wtCAT and mutant Δ9CAT proteins for immunofluorescence analysis. Interestingly, lanosterol also reduces the aggregation of mutant GFP-Δ9CAT proteins; co-addition of MG132 reverted the effect of lanosterol. Our following experiment results suggest that lanosterol also depletes the aggregation of mutant SOD1-G37R ALS disease-linked misfolded protein. We have observed that exposure to lanosterol can induce proteasome-mediated degradation of aberrant proteins. The vital effect of lanosterol for both neurodegenerative disease-associated proteins and bona fide misfolded proteins indicates that lanosterol has proteasome-mediated potential to reduce the aggregation of over-accumulated proteins. To further understand these findings, it was critical for us to analyze detailed effect of lanosterol on various proteasome activities. Collected cell lysates with or without lanosterol treatment were processed for the quantitative analysis of proteasome assays based on chymotrypsin-like and PGPH-like activities.

Previous findings suggest that in normal conditions, cell maintains proteostasis via mutual functions of autophagy and UPS, and it is difficult to challenge the endogenous modulation in proteolytic machinery during homeostasis. Earlier finding [Hu et al., 2020] also observed a slight increase in proteosome activity utilising lanosterol under MG132-treated conditions, which seems to be consistent with the present study. When cells continuously produce abnormal proteins, the aggravated condition of misfolding blocks proteasome functions and exhausts the chaperone capacity to produce poor proteins. The effect of lanosterol or other proteasome modulators appears more effective during stress conditions as compared to normal conditions. Previous reports also summarize drugs that can increase proteasome activities have therapeutic potential against imperfect aging and neurodegeneration [Chondrogianni et al., 2015; Njomen et al., 2018]. The data shown here are the first to represent lanosterol as a proteasome activator that affects proteasome activities; lanosterol was found to selectively elevate the chymotrypsin- and PGPH-like proteasome activities. For understanding the effect of lanosterol that possibly enhance proteasome activities, we next performed molecular docking analysis. The docking between the subunits of 20S proteasome and lanosterol molecule shows that a pose having minimum energy of −7.4 kcal/mol is possible where no polar interactions were seen, which indicates that there may be a possibility of other hydrophobic non-polar interactions to occur between lanosterol and β subunits.

The molecular docking-based mechanistic details represent a possibility of interactions between lanosterol and proteasome, but how the treatment overall impacts cellular health and fitness is an interesting question and an open alternative interpretation in the PQC pathway. We treated cells with lanosterol in concentration-dependent and time-dependent manners; brightfield micrograph image analysis suggests that lanosterol treatment does not affect cellular health adversely and maintains overall cellular fitness. Since lanosterol enhances proteasome activities, it can also have possible additional roles in PQC mechanisms. In one of our immunofluorescence analyses, we observed that lanosterol also possess the capability to induce the endogenous levels of PQC key regulators such as ubiquitin, proteasome, and p62 (also called SQSTM1). Many findings suggest that proteasome agonists can play a critical role and possible new therapeutic strategy that can ameliorate proteotoxic insults linked with the aggregation of redundant proteins [Leestemaker et al., 2017; Coleman and Trader, 2018]. Further, our findings suggest that lanosterol induces both chymotrypsin-like and PGPH-like activities under different concentrations and time-dependent manner.

Earlier, a result indicated lanosterol to suppress Aβ peptides self-assembly and also induces unfolding of Aβ peptides [Zhou et al., 2019]. Such anti-aggregation properties of lanosterol against Aβ peptides made us check its effect on various neurodegenerative diseases associated proteins. We performed transient over-expression of expanded polyglutamine constructs associated with spinocerebellar ataxia and Huntington's disease. We noticed treatment of lanosterol diminished the aggregation of polyglutamine expanded structures and reduced the perinuclear aggresomes-like inclusions of both ataxin-3 (Q84) and EGFP-HD74Q from the cells. The misfolded proteins formation and aggregation are known for the disturbance of the proteome and could potentially contribute to the failure of PQC mechanisms [Olzscha et al., 2011]. Our work examines lanosterol's effect on proteasome and demonstrates that the treatment of lanosterol can promote degradation of neurodegeneration disease-associated aberrant proteins from cells. We monitored that using lanosterol improves the clearance of abnormal proteins, which provides cytoprotection against oxidative stress and ER stress-mediated proteotoxic conditions. The cytoprotection against stress conditions is more effective in the cumulative presence of both lanosterol and exogenous expression of chaperones.

3.3 CONCLUDING REMARKS

Cells always tolerate the threat of protein misfolding, though up to a limit, and overaccumulation of these proteins can cause proteome complexity, and ultimately, cell death [Nakamura and Lipton, 2009]. Promoting protein folding and clearing the over-aggregation of aberrant proteins is a critical choice and challenge for a cell [Chen et al., 2011]. To overcome such molecular hurdles, cells trigger proactive functions of PQC mechanism comprising molecular chaperones, UPS, and autophagy pathway [Chhangani and Mishra, 2013; Ciechanover and Kwon, 2017]. Because the accumulation of abnormal proteins causes various neurodegenerative diseases and complex disorders; therefore, effective removal of these inclusions can provide newer insights on mechanism and therapeutics based on small molecules or natural compounds.

In this current study, we observed administration of the endogenous natural compound and cholesterol synthesis pathway intermediate lanosterol diminishes the aggregation of many mutant neurodegenerative diseases associated proteins and provides cytoprotection against cellular insults by different stress-inducing agents. Overall, the above results indicate the positive role of lanosterol against cellular stress conditions via elevation of proteasome activities, which help in degrading misfolded proteins. Our findings highlight the possible therapeutic potential of proteasome activities by lanosterol, which can be improved and achieved through more research that can open new possibilities for small molecules and drug repurposing.

…