Annexure A
Materials and Methods

A.1 MATERIALS

Cycloheximide, MG132, NAC, lactacystin, curcumin, ibuprofen, MTT, and all cell culture reagents were purchased from Sigma Aldrich. Lipofectamine® 2000 and OptiMEM™ were purchased from Life Technologies. FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen™) and Mitochondrial Membrane Potential Detection JC-1 Kit (BD™ MitoScreen) were obtained from BD Biosciences. JC-1 was obtained from Molecular Probes. Proteasome-Glo™, TUNEL Assay Kit, and Dual Luciferase Reporter Gene Assay Kit were purchased from Promega. Anti-mouse IgG-fluorescein isothiocyanate (IgG-FITC) (FI-2000) and IgG-rhodamine (TI-2000); anti-rabbit IgG-FITC (FI-1000) and IgG-rhodamine (TI-1000); and the horseradish peroxidase-conjugated anti-mouse (PI-2000), anti-rabbit-(PI-1000), and anti-goat IgG (PI-9500) secondary antibodies were purchased from *Vector Laboratories, Inc.*(*Burlingame, CA, USA*).

Table A.1 : Antibodies Used in the Present Study

S. N.	Antibodies	Company	Catalogue Number
1.	p62/SQSTM1	Sigma Aldrich	WH0008878M1
2.	GFP	Roche Applied Science	12600500
3.	HA	Thermo Fisher Scientific	26183
4.	Lamp-2	Thermo Fisher Scientific	51-2200
5.	LC3	Thermo Fisher Scientific	PA1-16930
6.	Synuclein	Invitrogen	32-8100
7.	p53	Santa Cruz Biotechnology	sc-6243
8.	GFP	Santa Cruz Biotechnology	sc-8334
9.	p27	Santa Cruz Biotechnology	sc-528
10.	p21	Santa Cruz Biotechnology	sc-756
11.	ubiquitin	Santa Cruz Biotechnology	sc-9133
12.	ubiquitin	Santa Cruz Biotechnology	sc-58448
13.	IκB	Santa Cruz Biotechnology	sc-847
14.	20S proteasom	Santa Cruz Biotechnology	sc-166073
15.	Cytochrome c	Santa Cruz Biotechnology	sc-13561
16.	Bax	Santa Cruz Biotechnology	sc-23959
17.	CHIP	Santa Cruz Biotechnology	sc-66830
18.	Luciferase	Santa Cruz Biotechnology	sc-74548
19.	SOD1	Santa Cruz Biotechnology	sc-11407
20.	β-actin	Santa Cruz Biotechnology	sc-81178

Table A.2 : List of Plasmids Constructs Used in the Present Study

S.N.	Construct name	Source
1.	pcDNA™ 3.1	Life Technologies
2.	pcDNA3-EGFP	Addgene 13031
3.	Luciferase-pcDNA3	Addgene 18964
4.	pEGFP-C1-Ataxin3Q28	Addgene 22122
5.	pEGFP-C1-Ataxin3Q84	Addgene-22123
6.	pF141 pAcGFP1 SOD1WT	Addgene 26402
7.	pF148 pSOD1G37RAcGFP1	Addgene 26409
8.	pAAV asyn WT	Addgene 36055
9.	pAAV asyn S87A	Addgene 36056
10.	pRK5-HA-ubiquitin-WT	Addgene 17608
11.	GFP-Ub	Addgene 11928
12.	EGFP-HDQ74	Gifted
13.	EGFP-HDQ23	Gifted
14.	pEGFP-C1 GFP-wtCAT	Gifted
15.	pEGFP-C1 GFP- Δ9CAT	Gifted
16.	EGFP-C1 CFTRΔF508	Gifted
17.	pd1EGFP	Gifted

A.1.1 Cell Lines

All the experiments were conducted in A549 and Cos-7 cells.

A.2 METHODS

To conduct the present research work and to confirm the validity of proposed hypotheses, multiple experimental techniques have been used according to experimental requirements. Several cell system based assays were also conducted, for which the detailed methodologies and protocols are as follow.

A.2.1 Large Scale Plasmid Extraction

In accordance with requirements of various cell transfection based experiments, different plasmid DNA constructs were extracted as per instructions provided with QIAGEN midi prep kit.

A.2.2 Cell Culture, Transfection, and Drug Treatments

Cos-7 and A549 cell lines were maintained in Dulbecco's modified Eagle's medium (Sigma-Aldrich), supplemented with 10% heat-inactivated fetal bovine serum with 100 mg/ml streptomycin and 100 U/ml penicillin and cells were grown at 37 °C in 5% CO_2. Cells were cultured into six-well and 96-well tissue culture plates, 1 day prior to transfection at a subconfluent density for different experiments. Cells were trasiently transfected with an equimolar ratio in combination with various expression vectors, using Lipofectamine® 2000 transfection reagent, according to the manufacturer's instructions. Bafilomycin, 2-mercaptoethanol, chloroquine, DMSO, ibuprofen, lanosterol, MG132, and tunicamycin were added to the culture medium, according to the need of various experiments (with or without constructs), as represented in different figures and explained in their respective legend sections.

A.2.3 Cell Viability Assay

Cells were grown on 96-well plates and were exposed to different stress-inducing agents and drugs of intrest. After treatment, MTT reagent (5mg/ml in PBS) is added to the wells containing media in a ratio 1:10. Afetr an incubation of four hours in CO_2 incubator at 37°C, media was removed, and 100 µl of lysis buffer, i.e., acidic isopropanol was added. Soon, formazan particles were formed after mixing the plates properly, and small amounts of each sample (100 µl) were subjected to microplate reader for measurements of absorbance at 595 nm. Experiments were conducted in triplicated and results were presented as percent of the control.

A.2.4 SDS-PAGE and Immunoblotting

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

A.2.5 Cycloheximide Chase Experiment

Cos-7 cells were treated with lanosterol or transiently transfected with pd1EGFP plasmid followed with exposure of ibuprofen. On the following day, treatment with cycloheximide (15 µg/ml) at different time intervals was performed. After treatment, cells were washed twice with phosphate-buffered saline (PBS), and prepared cell lysates were subjected to immunoblotting analysis by using appropriate antibodies. β-actin blots were using as loading control in all the experiments.

A.2.6 Immunofluorescence Techniques and Counting of Aggregates

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

A.2.7 Reverse Transcriptase PCR Analysis, Quantitative Real-Time RT-PCR Analysis

A549 cells were plated into 6-well plates and treated with lanosterol in varying concentration and time dependent manner. Post-treated cells were used to isolate total RNA using TRIzol reagent and semiquantitative RT-PCR was carried out by using CHIP specific primers. The quantitative real-time PCR for CHIP and β-actin was performed via complemetary DNA (cDNA) synthesis from total RNA isolated from treated cells, by using iQ SYBR green super mix (Bio-Rad). An iCycler iQ Real-Time Thermocycler Detection System (Bio-Rad) was used for real-time PCRs. To normalize all reactions, ribosomal 18S RNA was used as internal

control and all reactions were performed in triplicates including negative control in the absence of template DNA. PCR conditions were same for both, CHIP as well as β-actin with 35 and 25 cycles, respectively. The PCR conditions were 30 minutes at 50 °C for reverse transcription step, followed by an initial denaturation at 94 °C for 2 minutes. Then, cycling through 94 °C for 50 seconds of denaturation, 55 °C for 50 seconds of annealing, and at 72 °C for 1 minute were done; thereafter, final extension was done at 72 °C for 10 seconds and cooled down to 4 °C. The primer sequences used were as follows:

Table A.3 : Details of Primer Sequences Used to Perform PCR Analysis

S.N.	Primer Name	Primer Sequesnce
1.	CHIP-F	5'-AGGCCAAGCACGACAAGTACAT-3';
2.	CHIP-R	5'- TATACTCGAGTCAGTAGTCCTCCACCCAGCCATT-3'
3.	β-actin-F	5'-ATCGTCCACCGCAAATGCTTCTA-3'
4.	β-actin-R	5'-AGCCATGCCAATCTCATCTTGTT-3'

A.2.8 Proteasome Assay

Cos-7 cells were treated with ibuprofen and MG132 at different concentrations and time intervals. Treated cells were used to determine proteasome activity by using Proteasome-GloTM systems (Promega), according to manufacturer's instructions.

A.2.9 Flow Cytometry Analysis

Cells were treated with ibuprofen and MG132 for represented time periods. After treatment, cells were used for flow cytometry analysis to identify cells undergoing apoptosis using FITC Annexin-V-Apoptosis Detection Kit I (BD PharmingenTM, http://www.bdbiosciences.com/pharmingen) according to the manufacturer's procedures. For all the analyses, three independent experiments were performed and each condition was tested in triplicates through BD FACSAria III Cell-Sorting System BD, Biosciences, San Jose, CA, USA, and results were analyzed by using the FACS Diva software (Becton Dickinson, USA).

A.2.10 Molecular Docking Studies

Molecular docking calculations were performed with default parameters using SwissDock webserver [Grosdidier *et al.*, 2011]. To determine structural visualization of favorable binding modes and hydrogen bond calculation, UCSF Chimera was utilized [Pettersen *et al.*, 2004]. Structure of ibuprofen and lanosterol were obtained from ZINC database (code 2647) and PDBID: 1W6K , respectively [Irwin and Shoichet, 2005]. The structure of CHIP TPR domain (PDBID: 3Q49) and yeast 20S proteasome (PDBID: 1JD2) were also obtained from protein databank. from Protein Data Bank [Groll *et al.*, 2001]. Protein and ligand were modified for docking, using MGL tools (http://mgltools.scripps.edu/). In CHIP TPR domain, search space box of 46 Å × 54 Å × 50 Å covering entire domain was prepared and was centered on −16.846, −9.814, and 16.5. The best binding mode image was generated using Chimera. The interactions present in the predicted binding mode were determined by PoseView [Stierand *et al.*, 2006].

A.2.11 JC-1 Staining

To measure alterations in mitochondrial membrane potential, the cationic fluorescent dye, JC-1, was used. Cos-7 cells, seeded into two-well chamber slides, were exposed with different concentrations of ibuprofen and MG132. After treatment, cells were labeled with the JC-1 (5 mM) reagent for staining at 20 min in CO_2 incubator. After washing with pre-warmed PBS at 37 °C, fluorescence was observed under fluorescence microscope using 568-nm filter. To further ascertain the changes in mitochondrial membrane potential, above-described few sets of

cells were collected and fluorescence properties were monitored with flow cytometry over time by using the JC-1 Mitochondrial Membrane Potential Kit according to the manufacturer's procedures.

A.2.12 Reporter Gene Assay and Statistical Analyses

Lanosterol-treated cells, transiently transfected with luciferase expression plasmids, were used for luciferase activity assay. After treatment, cells were exposed to 43 °C for 30 min and then returned to 37 °C normal incubator for 2 hours of the recovery period. Few experiments were carried out in the presence of bafilomycin and MG132 before heat stress. Cells were then subjected to luciferase activity assay according to the manufacturer's protocol of Promega (dual luciferase reporter gene assay kit) as previously described. All the experiments were done in multiple sets and values obtained from multiple sets were subjected to statistical analyses using Student's t test**.** Statistical values are depicted as mean ± SD; and $p<0.05$ indicated statistical significance.

A.2.13 TUNEL Assay and Filter Trap Assay

For TUNEL analysis, cells were seeded into tissue culture plates, and on the following day, cells were treated with different concentrations of ibuprofen and MG132. After treatment, cells were used for TUNEL staining as per manufacturer's (Promega) instructions and counting of TUNEL-positive cells was performed on a fluorescence microscope. For filter trap assay, Cos-7 cells were transiently transfected with various plasmids and extraction of protein samples was done using RIPA buffer. The lysates were allowed to pass through the nitrocellulose membrane under controlled suction pressure using the Bio-Rad Filter Trap Assay equipment. Thereafter, by using different antibodies, immunoblots analyses were performed to detect the higher molecular weight insoluble protein aggregates and inclusions.

A.2.14 Extraction of Genomic DNA and Agarose Gel Electrophoresis

Cos-7 cells, after treatment with different doses of ibuprofen and MG132, were collected and total genomic DNA content were extracted using phenol-choloroform method. Extracted DNA samples were subjected to agarose gel electrophoresis to monitor fragmentation of the cellular DNA. In PCR analyses, densitometric analyses of the band intensities of agarose gels were performed on NIH image analysis software platform Image J.

…