Rejuvenation of Proteostasis Defects Based on Proteasomal Functions Regulates Improper Cellular Proliferation and Induces Misfolded Proteins Clearance

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Conclusion

Proteostasis is an essential process ensuring the optimal functioning of proteins as well as cellular homeostasis, involving maintenance of protein synthesis and degradation. The cellular PQC machinery components are essential for both proteostasis and homeostasis and any imbalance, either stress-induced or natural (as with ageing), in this machinery can make the cellular and living system vulnerable to a host of diseases, like neurodegenerative conditions, metabolic disorders, immune system-related disorders, and cancer [Chhangani and Mishra, 2013; Wang and Hill, 2015; Barrera et al., 2018]. The disturbed cellular proteostasis as well as pathological protein aggregation are considered hallmarks of neurodegenerative diseases [Wilson et al., 2023]. Previous findings have suggested the common molecular signs, like presence of ubiquitin-positive inclusions, molecular chaperones, proteasome, and autophagic pathway components in these disorders. Although abnormal protein aggregation is common to many neuronal degeneration diseases, still it is difficult to comprehend the complex mechanism of proteostasis failure and how that can lead to accumulating toxic protein species in neurons? The rejuvenation of the cellular proteostasis network and PQC machinery, including proteasome proteolytic complex, has the potential to clear the harmful protein aggregates and establish cellular homeostasis, which can be beneficial against a wide array of disease conditions, including neuronal degeneration. In contrast to this, the high proliferation rate of cancer cells necessitates high rates of transcription and translation. The increased synthesis of proteins, in turn, places higher demand on PQC machinery of cells, including molecular chaperones and UPS. In this context, inhibiting the proteasome has the potential to target cancer cells, decreasing their proliferation and inducing cell death.

5.1 SUMMARY

The natural compound lanosterol and small molecule drug valproate are reported in this work as modulators of proteasomal activity. In this work (Chapter 3), we studied effect of tetracyclic triterpenoid lanosterol on cellular PQC machinery. Specifically, increased proteasomal levels and activity upon lanosterol treatment with cells was observed. Furthermore, this increase in proteasome levels and activity is also associated with increased degradation of misfolded proteins heat-denatured luciferase and Δ9CAT and proteasomal substrate d1EGFP. The proteasome proteolytic activities chymotrypsin-like and PGPH-like, both increased upon lanosterol treatment. Lanosterol-mediated induced proteasomal function in cells also enhanced clearance of aggregates of exogenously expressed neurotoxic mutants SOD1-G37R (Amyotrophic lateral sclerosis), ataxin-3 (Q84) (spinocerebellar ataxia), and HD74Q (Huntington's disease). Lanosterol also increased solubility of mutant misfolded proteins and provided cytoprotection in response to proteotoxic, endoplasmic reticulum as well as oxidative stress.

In this study (Chapter 4), valproate treatment to cells increased levels of ubiquitinylated proteins and aggregation of mutant model proteins. Moreover, valproate reduced the chymotrypsin- and PGPH-like proteolytic activities of proteasomes. The inhibited proteasome function is also linked with increased stabilisation of many proteasomal substrates, which include cell cycle and cell death-associated proteins, such as p53, Bax, p21, and p27. Moreover, NF-κB transcription factor activity was also reduced upon valproate treatment, another indication for its

cell death inducing and anti-proliferative potential. The cell death induction by valproate treatment is possibly based on dysfunction of mitochondria, which involves enhanced cytochrome *c* release from mitochondria and depolarisation of mitochondrial membrane. The HDAC inhibition property of valproate along with its proteasome inhibition property indicates its potential for combination therapies for cancer treatment.

5.2 CONCLUDING REMARKS

This work has reported lanosterol and valproate as proteasome activator and inhibitor respectively. The enhanced proteasome activity upon lanosterol treatment leads to improved clearance of pathological protein aggregates from cells, which is cytoprotective and has potential for alleviation of disorders involving protein misfolding and aggregation. Valproate treatment to cells, in contrast, inhibits proteasome activity, leads to stabilisation of various proteasomal substrates, including p53 and p27, and disturbs mitochondrial physiology, ultimately, inducing cell death. Such strategies targeting PQC machinery of the cells holds great potential for a wide array of disease conditions, including prolonging healthspan and lifespan.

5.3 CLOSING COMMENTS AND FUTURE PROSPECTS

Rejuvenation of proteostasis by small molecules can improve the health of cells in the presence of various stressors, whereas interfering with proteostasis can make cells, such as tumor cells, susceptible to death. Proteostasis is central to many different cellular processes and is an essential homeostasis mechanism. Investigating the modulators of proteostasis, including proteasome, chaperones, and autophagy can be highly beneficial for healthcare as well as research. Different diseases even apart from neuronal degenerative diseases and cancer can be benefited from proteostasis modulation, such as infectious diseases, cystic fibrosis, and ageing-related diseases. Overall, the present work extends and add to the knowledge of proteostasis mechanisms and modulation and opens avenues for searching natural compounds as well as already approved drugs for their potential for PQC modulation.

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Annexure A
Materials and Methods

A.1 MATERIAL

Lanosterol, Valproate, dimethyl sulfoxide (DMSO), β-mercaptoethanol (BME), MG132, aspirin, lactacystin, curcumin, chloroquine, tunicamycin, thapsigargin, cycloheximide, nocodazole (Noc), propidium iodide (PI), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), N-acetylcysteine, and other cell culture-related reagents were purchased from Sigma. TRIzol™ (15596018) and 4′,6-diamidino-2-phenylindole (DAPI) were purchased from Invitrogen. Hydrogen peroxide solution (30%) was bought from Fisher Scientific and ethidium bromide from Merck. Protein molecular weight biomarkers were obtained from Bio Rad and Thermo Scientific (26634). Lipofectamine™ 2000 and OptiMEM were purchased from Life Technologies and 5,5′, 6,6′-tetrachloro-1,1′,3,3′ tetraethylbenzimidazol-carbocyanine iodide (JC-1) from Molecular Probes. Proteasome-Glo™ Assays (G8622, G8642), Dual-Luciferase® Reporter (DLR™) Assay System (E1910), and DeadEnd™ Fluorometric TUNEL System (G3250) were bought from Promega. Annexin V-FITC and Annexin V-binding buffer were procured from BioLegend. The primary and secondary antibodies utilized in the present work are listed in Table A.1 and the various plasmid constructs used are listed in Table A.2.

Table A.1: List of primary and secondary antibodies used in the present work

Serial Number	Antibody	Manufacturer	Catalog Number
Primary Antibodies			
1	20S proteasome	Santa Cruz Biotechnology	sc-58412, sc-166073
2	Ubiquitin	Santa Cruz Biotechnology	sc-8017, sc-9133, sc-58448
3	p62	Sigma	WH0008878M1
4	Luciferase	Santa Cruz Biotechnology	sc-74548
5	GFP	Santa Cruz Biotechnology	sc-8334
6	p53	Santa Cruz Biotechnology	sc-6243
7	Bax	Santa Cruz Biotechnology	sc-493
8	Procaspase-3	Santa Cruz Biotechnology	sc-7148
9	p21	Santa Cruz Biotechnology	sc-756
10	p27	Santa Cruz Biotechnology	sc-528
11	Cytochrome c	Santa Cruz Biotechnology	sc-13561
12	CHIP	Thermo Scientific	PA1-015
13	CHIP	Santa Cruz Biotechnology	sc-66830
14	γ-tubulin	Sigma	T6557
15	GAPDH	Santa Cruz Biotechnology	sc-47724
Secondary Antibodies			
17	Anti-Rabbit IgG Antibody (H+L), Peroxidase	Vector Laboratories	PI-1000
18	Anti-Mouse IgG Antibody (H+L), Peroxidase	Vector Laboratories	PI-2000
19	Anti-rabbit IgG-Texas Red™	Vector Laboratories	TI-1000

20	Anti-mouse IgG-Texas Red™	Vector Laboratories	TI-2000
21	Anti-rabbit IgG-Fluorescein	Vector Laboratories	FI-1000
22	Anti-mouse IgG-Fluorescein	Vector Laboratories	FI-2000

Table A.2: List of plasmid constructs used in the present work

Serial Number	Name of Plasmid Construct	Source Details
1	pF141 pAcGFP1 SOD1WT	Addgene: 26402
2	pF148 pSOD1G37RAcGFP1	Addgene: 26409
3	Luciferase-pcDNA3	Addgene: 18964
4	pEGFP-C1-Ataxin3Q28	Addgene: 22122
5	pEGFP-C1-Ataxin3Q84	Addgene: 22123
6	pcDNA3-c-myc	Addgene: 16011
7	pcDNA3-EGFP	Addgene: 13031
8	pEGFP-Hsp70	Addgene: 15215
9	pd1EGFP	Dr. Nihar Ranjan Jana (National Brain Research Centre, Manesar, Gurgaon, India)
10	CHIP	Dr. Nihar Ranjan Jana (National Brain Research Centre, Manesar, Gurgaon, India)
11	pEGFP-C1 CFTRΔF508	Dr. Ron R. Kopito (Stanford University, Biology Department Lorry Lokey Bldg Campus Drive Stanford CA)
12	GFP-wtCAT	Dr. Csaba Soti (Department of Medical Chemistry, Semmelweis University, Budapest, Hungary)
13	GFP-Δ9CAT	Dr. Csaba soti (Department of Medical Chemistry, Semmelweis University, Budapest, Hungary)
14	EGFP-HDQ23	Dr. A Tunnacliffe (Department of Chemical Engineering and Biotechnology, University of Cambridge, Cambridge, UK)
15	EGFP-HDQ74	Dr. A Tunnacliffe (Department of Chemical Engineering and Biotechnology, University of Cambridge, Cambridge, UK)
16	NF-κB-Luc plasmid	Dr. Aleem Siddiqui (UC San Diego, Gilman Dr. La Jolla, CA)

A.1.1 Cell Lines Used in the Study

The cell lines Cos-7 (African green monkey kidney fibroblast-like cell line) and A549 (human lung epithelial cell adenocarcinoma) were used in this work and were obtained from the National Centre for Cell Science (NCCS), Pune, India.

A.2 METHODS

A.2.1 Cell Culture, Plasmid Transfection, and Drug Treatment

A549 and Cos-7 cell lines were seeded and maintained in Dulbecco's Modified Eagle's medium (DMEM), enriched with FBS (10 %), at 37 °C and 5 % CO_2 under humid conditions. Penicillin and streptomycin (100 U/ml and 100 µg/ml, respectively) were also added to DMEM. Cell lines were maintained in appropriate culture vessels, including 100 mm plates, 06-, 96-well plates, and 2-well chamber slides, depending on the experiment. They were grown till sub-confluent density, and were transiently transfected with various plasmids used in this work, such as those encoding model misfolded proteins, proteasomal substrates, mutant aberrant folded proteins [Ward et al., 1995; Arslan et al., 2012; Dogra and Mukhopadhyay, 2012], and chaperones, like Hsp70 and CHIP, using Lipofectamine™ 2000 transfection reagent, following instructions of the maker. Post-transfection, cells were treated with compounds being studied lanosterol or valproate, various stressors, such as tunicamycin, thapsigargin, BME, H_2O_2, or known proteasome inhibitors, like MG132, lactacystin, aspirin, and curcumin, and autophagy inhibitor chloroquine at different concentrations and for different time points, on the basis of experiment. Brightfield imaging of cells was done using FLoid™ Cell Imaging Station (Life Technologies) to observe cellular morphology upon treatment with different drugs and chemicals, including lanosterol and valproate, under different conditions.

A.2.2 Immunofluorescence Analysis and Aggregate Counting

Cos-7 cells transfection with required plasmids coding for different wild type and mutant disease-linked proteins as well as bona fide misfolded proteins at sub-confluent density (mentioned in corresponding figure legends of chapters 3 and 4). After successful transfection and completion of drug treatment time, cells were rinsed with phosphate-buffered saline (PBS) and fixation was done with paraformaldehyde (PFA, 4% in PBS) for 20 min at room temperature (RT) under static condition, which was followed by three PBS washes of 5 min each on rotator to remove excess PFA. Permeabilization was done with Triton X-100 (0.5% in PBS) for 5 min at room temperature under static conditions and then washing was done with PBS on rotator three times for 5 min each. Next, for blocking, rabbit serum (2% in PBS with Tween 20, 0.05%) was used for 45 min. Afterward, incubation of these cells with appropriate primary antibodies (1:500 dilutions) was done for overnight at 4 °C, followed by three washings with PBST (PBS with Tween 20, 0.05%) was done to remove unbound antibody. In next step, incubation was performed with appropriate fluorescein or Texas Red-conjugated secondary antibodies (1:5000 dilutions) for 4 h, after which PBST washes were given. Nuclei were stained with 4',6-diamidino-2- phenylindole (DAPI) and mounting was done with anti-fade solution. For fluorescence imaging, Leica DM6000B epifluorescence microscope was used. Manual counting of polyglutamine protein aggregates in control- and VPA-treated Cos-7 cells and GFP-Δ9CAT, SOD1-G37R, and ataxin-3(Q84) in control and lanosterol-treated Cos-7 cells with or without MG132 was done from fluorescence images. Cells with >1 aggregates together were considered to have 1 large inclusion. The statistical analysis was performed via Student's t-test with p-value less than 0.05 representing statistical significance. The fluorescent intensity profile graphs have been plotted using ImageJ software, representing fluorescent intensity along the selected region, shown as internal box in the fluorescence image.

A.2.3 Proteasome Activity Assay

A549 cells were treated with lanosterol or valproate in concentration- and time-dependent manner; some cell sets were treated with DMSO or distilled water (solvents for lanosterol and valproate, respectively) and MG132 (10 µM for 12 h), lactacystin (10 µM for 12 h), aspirin (2.5 mM, 12 h), and curcumin (100 µM for 8 h) as positive controls for proteasome assay. The statistical analysis was performed via Student's t-test with p-value less than 0.05 representing statistical significance. These treated cells were lysed by sonication and used for Proteasome-Glo™ Assay (Promega), following maker's protocol. This assay is based on generation of bioluminescence

from the cleavage of luminogenic substrates by proteasome catalytic activities. Specifically, Suc-LLVY-aminoluciferin and Z-nLPnLD-aminoluciferin substrates can undergo proteolytic cleavage by proteasome subunits β5 (chymotrypsin-like) and β1 (caspase-like), respectively. The freed aminoluciferin undergoes luciferase reaction leading to bioluminescence. The bioluminescence was measured with GloMax®-Multi Jr Single-Tube Multimode Reader (Promega), and graph was plotted for proteasome activity for different conditions.

A.2.4 MTT Cell Viability Assay

To understand the impact of lanosterol or valproate on viability of cells, they were treated with control (DMSO or distilled water) and lanosterol or valproate in accordance with experiment design and subjected to MTT assay. MTT reagent (5 mg/ml dissolved in PBS) was added in a ratio of 1:10 with respect to DMEM and incubated in dark at 37 °C and 5 % CO_2 for 4 h. Next, media was removed and acidified isopropanol was added to dissolve formazan crystals via incubation in dark at 37 °C and 5 % CO_2 for 1 h. The absorbance was measured at 595 nm. Viable cells can reduce MTT to formazan via cellular oxidoreductase enzymes in a NAD(P)H-dependent manner, producing more purple color.

A.2.5 Immunoblotting Analysis

Cells were exposed to control and lanosterol or valproate under varying conditions (mentioned in corresponding figure legends of Chapters 3 and 4), after which they were harvested. The sonication-lysed and heated whole cell extracts were electrophoresed in SDS-polyacrylamide gel and transferred onto nitrocellulose membrane via Trans-Blot® SD Semi-Dry Transfer Cell (Bio-Rad). The membrane was next blocked with skimmed milk (5 %) in Tris-buffered saline Tween 20 (TBST; 50 mM Tris, pH 7.4; 150 mM NaCl; Tween 20, 0.1 %) followed by incubation with primary antibodies (1:1000; overnight at 4 °C) in TBST. Next, multiple washings of membrane with TBST buffer were performed and then, it was incubated with HRP-conjugated secondary antibody (1:10000; 40 min). The dilutions of both primary and secondary antibodies were prepared in TBST buffer (pH 7.4). Next, the membrane (blot) was washed multiple times with TBST buffer. After washing, membrane was treated with Luminata™ Crescendo HRP substrate (Merck, Millipore), and chemiluminescence was detected and imaged in Gel Doc XR+ Gel Documentation System (Bio-Rad).

A.2.6 Molecular Docking

For molecular docking purposes, the receptor structure of 20S subunits was obtained from Protein Data Bank (PDB 5LE5) [Schrader et al., 2016]. The structure of ligands lanosterol (PubChem CID: 246983) and valproic acid (PubChem CID: 3121) were generated through UCSF Chimera (version 1.15) [Pettersen et al., 2004]. AutoDock Tools (version 1.5.6) was utilised for ligand and receptor molecule preparation [Morris et al., 2009].

The grid parameters with spacing 1.000 Å were designed using ADT and were as follows: (a) Lanosterol: For proteasome subunits, grid size used were 64 × 42 × 68 with center dimensions (1.595, 210.500, 31.091) xyz for β1, 48 × 54 × 56 with center (38.556, 169.880, 36.476) xyz for β2, and 48 × 54 × 56 with center (7.569, 180.850, 32.567) xyz for β5 subunit, for considering their complete structures for docking analysis.

(b) Valproate: For proteasome subunits, grid size used were 58 × 46 × 60 with center dimensions (0.466, 211.614, 26.753) xyz for β1, 40 × 48 × 56 with center (35.966, 173.082, 36.622) xyz for β2, and 48 × 50 × 60 with center (5.226, 180.85, 28.935) xyz for β5 subunit, for considering their complete structures for docking analysis.

The protocols as per Forli et al. were followed and AutoDock Vina (Version 1_1_2) was used for blind docking of the molecules [Trott and Olson, 2010; Forli et al., 2016]. For visualization purposes, PyMOL (The PyMOL Molecular Graphics System, version 1.7.4 Schrödinger, LLC https://www.pymol.org/) was utilized [DeLano, 2002]. LigPlot+ (Version 2.2.8) was used for the

analysis of the molecular docking result in flattened-out 2D ligand-protein interaction graphs [Wallace et al., 1995; Laskowski and Swindells, 2011].

A.2.7 Luciferase Activity Assay

Cells were grown to sub-confluent state and transfected with luciferase (firefly) plasmid, 24 h after which separate sets of such cells were administered DMSO, lanosterol (20 µM, 48 h), lanosterol and MG132 (10 µM, 12 h; known proteasome inhibitor), and lanosterol and chloroquine (20 µM, 12 h; known autophagy inhibitor). The treated cells were subjected to heat stress (43 °C; 30 min), after which recovery was allowed (37 °C; 2 h). Cells were collected and protein isolate from cell lysis were subjected to luciferase activity assay. Luciferase activity of cell lysates was measured via Promega's GloMax®-Multi Jr. Single-Tube Multimode Reader using Dual-Luciferase® Reporter (DLR™) Assay, following makers instructions.

A.2.8 Cycloheximide Chase Assay

For cycloheximide chase analysis, Cos-7 cells were transfected transiently with pd1EGFP and 24 h after transfection were exposed to control or lanosterol (20 µM, 24 h). Cycloheximide (10 µM) was added at different time points to chase d1EGFP proteasome modelled substrate (destabilised EGFP variant with 1 h half-life enriched in PEST sequences at its carboxyl terminal region). After completion of treatment time with lanosterol, cells were harvested and immunoblotting was executed via antibodies against GFP. Cells were also imaged with fluorescence microscope (FLoid™ Cell Imaging Station; Life Technologies) corresponding to different time intervals of cycloheximide treatment of control and lanosterol-treated cells.

A.2.9 Dot Blot Analysis

Cells were transfected in a transient manner with plasmid constructs encoding bona fide mutant misfolded protein GFP-Δ9CAT and ALS-linked mutant misfolded protein SOD1-G37R. Post-transfection, cells were given control or lanosterol (20 µM). After treatment, cells were collected and cell lysate extracts were subjected to membrane filter trap assay for dot blot analysis. The dot blots were analyzed with antibodies against GFP and actin. The dot blots quantification was done using NIH image analysis software ImageJ.

A.2.10 Luciferase Reporter Gene Assay

A549 cells were co-transfected with NF-κB-luciferase (firefly) and pRL-SV40 (*Renilla*) plasmids together [Dikshit et al., 2006] and 24 h afterward treated with control and different valproate concentrations (1, 2.5, and 5 mM) for 24 h. The NF-κB promoter firefly luciferase plasmid expression is dependent on NF-κB activity in cells and luciferase activity represents NF-κB activity. pRL-SV40 act as internal control. The treated cells were collected and processed for luciferase activity analysis, using Dual-Luciferase Reporter® (DLR™) Assay System (Promega). The firefly luciferase reaction is started by adding Luciferase Assay Reagent II (LARII) to cell lysate, mixing. and immediately bioluminescence was measured with GloMax®-Multi Jr. Single-Tube Multimode Reader (Promega). Next, quenching of the above reaction and *Renilla* luciferase reaction is started through adding Stop & Glo® reagent to above-mentioned mixture, mixed, and again immediately bioluminescence was measured. The graph between relative luciferase activity (ratio of values of firefly to *Renilla* luciferase) on Y axis and valproate concentrations on X axis was plotted.

A.2.11 Propidium Iodide Staining

For propidium iodide staining analysis, Cos-7 cells were subjected to treatment with valproate (10 mM, 24 h) and MG132 (10 µM, 12 h). Additionally, certain sets of cells were transfected with plasmid of CHIP, which has functions as a co-chaperone and an E3 enzyme, and post 24 h, cells underwent treatment of valproate (10 mM, 24 h). After completion of treatment period, cells were washed with PBS and exposed to propidium iodide/RNase A staining buffer

for 30 min at RT, maintaining dark conditions. Subsequently, cells were visualized under a fluorescence microscope (FLoid™ Cell Imaging Station; Life Technologies).

A.2.12 DNA Fragmentation and Nucleus Architecture Analysis

A set of Cos-7 cells was treated with valproate in concentration-dependent fashion (15 and 30 mM) and control for 36 h. From treated cells, genomic DNA was isolated via TRIzol™, following maker's protocol. It was then electrophoresed on agarose gel and imaged on Gel Doc XR+ Gel Documentation System (Bio-Rad), using ethidium bromide (Merck) staining. A set of A549 cells were treated with control, valproate (2.5 and 5 mM, 24 h), and MG132 (10 µM, 12 h). Post-treatment, cells were fixed with PFA 4%, washed, and stained with DAPI. The nuclear-stained cells were imaged with fluorescence microscope (FLoid™ Cell Imaging Station; Life Technologies) to observe nuclear morphology.

A.2.13 TUNEL Assay

A549 cells were given different concentrations (1, 2.5, and 5 mM) of valproate or control for 24 h. Afterward, cells were fixed with PFA (4% in PBS) for 15 min, and next, washing with PBS was done. Cell permeabilization was done with Triton X-100 (0.5% in PBS) for 5 min, followed by washing with PBS. Terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling (TUNEL) reaction was performed at 37 °C in dark according to maker's protocol and then imaged with fluorescence microscope (FLoid™ Cell Imaging Station; Life Technologies). The apoptotic cells were counted manually and plotted. The nuclear fragmented DNA, which acts a marker of apoptotic cells, is acted upon by TdT to add fluorescein-12-dUTP at 3'-OH of DNA.

A.2.14 JC-1 Ratiometric Fluorescent Dye Staining

A549 cells were given control, MG132 (10 µM, 12 h), or concentrations of valproate (1, 2.5, and 5 mM) for 24 h. After treatment, JC-1 (5 µM) ratiometric fluorescent dye for mitochondrial membrane potential was added to media with cells and incubated at 37 °C, 5% CO_2 for 45 min in dark. Next, media was removed, PBS (prewarmed) was added and used for washing, and cells were imaged with fluorescence microscope (FLoid™ Cell Imaging Station; Life Technologies). The JC-1 dye can enter healthy and polarised mitochondria and form J aggregates there, which have fluorescence emission wavelength at 590 nm, and imaged in red channel. JC-1 enters unhealthy and depolarised mitochondria to a lesser extent, thereby, remains as monomers, which have fluorescence emission at 530 nm, and imaged in green channel [Sivandzade et al., 2019].

A.2.15 Flow Cytometry and Statistical Analysis

A549 cells were treated with control or valproate (5 mM for 24 and 48 h) or MG132 (10 µM for 24 h). Afterward, cells were collected via trypsinization and incubated with Annexin V-FITC and PI for 15 min at RT in Annexin V binding buffer. Next, the apoptosis data were acquired by flow cytometry in BD FACSCanto II. The statistical analyses were performed using GraphPad Prism software (Version 6.0.1; GraphPad Software Inc, San Diego, California). Means of different groups were compared using two-way ANOVA. Annexin V-FITC and PI, both are impermeable to live cells and stain cells on the basis of different principles – binding to phosphatidylserine on outer leaflet of plasma membrane (a biochemical feature of apoptosis) and binding DNA upon damage to membrane integrity, respectively. This can aid in differentiation of cell death pathways.

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