Annexure A
Materials and Methods

A.1 MATERIALS

MG132, Cycloheximide, Diclofenac, Indomethacin, Curcumin, Lactacystin, NAC, MTT, Nocodazole and all other cell culture reagents were obtained from Sigma. Transfection reagent Lipofectamine® 2000, OptiMEM and staining dye JC-1 were purchased from Life Technologies and Molecular Probes, respectively. JC-1 Mitochondrial Membrane Potential Detection Kit (BD™ MitoScreen) and FITC Annexin-V-Apoptosis Detection kit I (BD Pharmingen™) were purchased from BD Biosciences. TUNEL assay kit, Dual luciferase reporter gene assay kit and ProteasomeGlo™ assay reagents were purchased from Promega. Other general laboratory chemicals utilized were obtained from Himedia, Sigma-Aldrich and Merck.

Table A.1: List of primary antibodies used in the study

Serial Number	Antibodies	Company	Catalogue Number	Host animal
1.	Ubiquitin	Santa Cruz	Sc-58448	Mouse
2.	Cytochrome c	Santa Cruz	Sc-13561	Mouse
3.	β-actin	Santa Cruz	Sc-47778	Mouse
4.	γ-tubulin	Sigma	T6557	Mouse
5.	HA	Thermo Fischer	26183	Mouse
6.	p53	Santa Cruz	Sc-65226	Mouse
7.	p21	Santa Cruz	Sc-756	Rabbit
8.	p27	Santa Cruz	Sc-1641	Mouse
9.	Bax	Santa Cruz	Sc-23959	Mouse
10.	IκB-α	Santa Cruz	Sc-847	Rabbit
11.	20S-proteasome	Santa Cruz	Sc-166073	Mouse
12.	GFP	Santa Cruz	Sc-8334	Rabbit
13.	VDAC	Life Technologies	710606	Rabbit

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

Serial Number	Name of the Construct	Source
1.	pcDNA3-EGFP	(Addgene-13031)
2.	pRK5-HA-Ubiquitin-WT	(Addgene-17608)
3.	pEGFP-Hsp70	(Addgene-15215)
4.	Luciferase-pcDNA3	(Addgene-18964)
5.	pCMX IκB	(Addgene-12331)
6.	pcDNA^{TM} 3.1	Life Technologies
7.	GFP-wtCAT plasmid	Gifted by Dr. Csaba Soti (Department of Medical Chemistry, University, Budapest, Hungary)
8.	pEGFP-C1 CFTR delta-F508	Gifted by Dr. Ron R. Kopito (Stanford University, Biology Department, Stanford, CA)
9.	pd1EGFP plasmid	Gifted by Dr. Nihar Ranjan Jana (National Brain Research Centre, Manesar, Gurgaon, India)
10.	NF-κB-Luc plasmid	Gifted by Dr. Aleem Siddiqui (UC San Diego, Gilman Dr. La Jolla, CA)
11.	EGFP-HDQ23	Gifted by Dr. A. Tunnacliffe (Department of Chemical Engineering and Biotechnology, University of Cambridge, Cambridge, UK)
12.	EGFP-HDQ74	Gifted by Dr. A. Tunnacliffe (Department of Chemical Engineering and Biotechnology, University of Cambridge, Cambridge, UK)

Anti-mouse-IgG and anti-rabbit-IgG secondary antibodies conjugated with horse radish peroxidase, fluorescein isothiocyanate and rhodamine were purchased from Vector Laboratories.

A.1.1 Cell Lines

Cos-7(fibroblast like monkey kidney tissue derived cell line), A549 (human adenocarcinoma derived cell line) and Neuro2a (mouse neuroblastoma derived cell line) were used for conducting different experiments. These cells were obtained from national repository of animal cell cultures located at National Centre for Cell Science (NCCS) Pune, India.

A.2 METHODS

Different methods were used in order to understand the effect of NSAIDs on the proteasomal activity and associated cellular apoptosis. The details are mentioned in the following sub-sections.

A.2.1 Cell Culture, Treatment and Transfection

Cos-7, A549 and Neuro2a cell lines were maintained in DMEM supplemented with 10% heat inactivated FBS along with 100 U/ml penicillin and 100 μg/ml streptomycin antibiotics. In general, for different treatment and transfection experiments, cells were seeded into two-well chamber slides, six and ninety six-well tissue culture plates at sub-confluent density. Following day, 60-75% confluent cells were treated with diclofenac, indomethacin and other known proteasome inhibitors in time and concentration dependent manner. Lipofectamine® 2000

reagent was used for transient transfection of expression vectors in cells according to manufacturer's instructions.

A.2.2 Proteasome Activity Assay (In Cells and Purified Proteasome)

Proteasome activity was determined both in cells and in purified proteasome. For detection in cells, A549 cells were seeded in ninety six-well tissue culture plates and following day were treated with diclofenac or indomethacin in concentration and time dependent manner. DMSO was used as control along with positive control MG132 or Lactacystin. Following treatment, proteasome activity assay were carried out using Proteasome-Glo™ systems (Promega) according to manufacturer's instructions. Briefly, cell samples are incubated with substrate reagent and the contents were mixed with the help of plate shaker for few minutes. The proteasomal substrates; contain amino acid sequences labelled with 7-amino-4-methylcoumarin (AMC) which are specific for chymotrypsin like and post-glutamyl peptide hydrolase like active site of proteasome respectively. Following shaking, 10 minutes of incubation was provided at room temperature and cellular proteasome activity were measured on the basis of signals generated due to AMC release from peptide substrates cleaved by proteasome, with the help of GloMax Multi Jr detection system.

In case of cell free assay, purified proteasomes (0.5 μg/ml) were diluted in 10mM HEPES buffer (pH 7.6) and proteasome substrates (Proteasome-GloTM systems, Promega) were prepared as mentioned in manufacturer's instructions. These proteasomal substrates were then incubated in the presence of DMSO (control) and various concentrations of diclofenac or indomethacin to check their effect on proteasome activity. MG132 and Lactacystin, well known proteasome inhibitors, were used as positive controls. After 1 hour of incubation at 37 °C, inhibition of each proteasomal activity were measured by the release of hydrolyzed 7-amino-4-methylcoumarin (AMC) from the substrate peptide sequences (i.e. low signal means less cleavage of substrate due to interference in proteasome activity and vice-versa) using GloMax Multi Jr detection system, Promega.

A.2.3 Immunoblotting Experiment

For immunoblotting, post treated and/or transfected cell lysates were separated using SDS polyacrylamide gel electrophoresis and transferred onto nitro cellulose membranes. Membranes were incubated in blocking buffer (5% skimmed milk in TBST [50 mM Tris; pH 7.4. 0.15 M NaCl, 0.05% Tween]) and successively probed with primary antibody in TBST for overnight period at 4°C. Next day, after TBST washing of 3-4 times for 5 minutes each, incubation with appropriate secondary antibody conjugated with horse radish peroxidase in TBST was performed. Secondary antibodies were used at 1:2000 dilutions for 30 minutes. Blots were then again washed with TBST 3-4 times for 5 minutes each and later were developed using Luminata® crescendo western HRP substrate (EMD Millipore).

A.2.4 Immunofluorescence Technique

For immunofluorescence staining, Cos-7 cells were seeded into two well chamber slides and treated with diclofenac or indomethacin. MG132 was used as positive control. Some set of cells were transiently transfected with appropriate plasmids. Cells were then washed with phosphate buffered saline (PBS) and were fixed with 4% paraformaldehyde prepared in PBS for 15 min. Cells were then permeabilized using 0.5% Triton X-100 in PBS for 5 min and further washed extensively with PBS. After blocking for an hour appropriate primary antibody at in TBST were used for overnight incubations at 4°C. Following incubations, cells were extensively washed with TBST and suitable rhodamine-conjugated/FITC-conjugated secondary antibodies were used for 1 hr incubation at 1:1000 dilutions. After washing several times, nuclear staining was done with DAPI mounting and images were captured using fluorescence microscope.

A.2.5 Quantification of Immunoblots and Statistical Analysis

Densitometric analysis of immunoblots for quantification of band intensities were performed using NIH image analysis software Image J. Student's t test was used to test statistical significance of data and were considered significant if p values obtained were <0.05 .

A.2.6 Docking Studies

SwissDock server was used to perform docking. This server is based on EADock DSS software that first generates large number of binding modes along with estimation of CHARMM energies on a grid. The most favorable binding modes are then clustered and calculated with FACTS implicit solvation model. Using default parameters (blind and rigid docking) of swiss dock server, docking conformations of diclofenac or indomethacin with proteasome subunits (β1 and β5) of yeast 20S proteasome (pdb 1JD2) were obtained. Structure of diclofenac and indomethacin were acquired from Zinc database; code 1281 and code 27643987 respectively. UCSF chimera was used for showing favorable binding modes.

A.2.7 Flow Cytometry Analysis

To check the effect of diclofenac or indomethacin on cellular apoptosis, A549 cells were seeded into 06-well culture plates and treated with diclofenac or indomethacin and MG132. FITC Annexin-V-Apoptosis Detection kits I (BD Pharmingen™) were used according to the manufacturer's instructions by using BD FACSAria Cell-Sorting System BD, Biosciences, San Jose, CA. Briefly following treatment, cells were washed with cold PBS buffer, trypsinized and suspended in a binding buffer solution. 5µl of FITC Annexin V and 5µl of Propidium Iodide was then added to 100µl of the each tube of above cell sample and vortexed gently. Then incubation for 15 min was provided at room temperature in dark. Finally, 400ul of binding buffer was added to each tube and analysed with help of flow cytometer. Analyses of data collected were performed by using FACSDiva software. Principally, cells undergoing apoptosis, their membrane phospholipid phosphatidylserine (PS) is flipped from the inner side of the plasma membrane to the outer side, causing PS to get exposed to the external environment. Annexin V is a calcium-dependent phospholipid-binding protein which has a high affinity for phosphatidylserine, and thus is used for identifying those apoptotic cells. On the other hand Propidium Iodide (PI) is used to distinguish viable from nonviable cells as viable cells that have intact membranes are impermeable to PI, whereas the membranes of dead cells are permeable to it. Thus those cells which stain positive for only FITC Annexin V and negative for PI are considered to be undergoing apoptosis. Whereas, cells that stain positive for both FITC Annexin V and PI are either in later stage of apoptosis or are undergoing necrosis or are dead already. Cells that have negative stain are alive or are not undergoing measurable apoptosis. .

For mitochondrial membrane potential detection, JC-1 Mitochondrial Membrane Potential Kit (BDTM MitoScreen) was used. In brief, JC-1 working solution was made as per manufacturer's instruction and was incubated for 10min at 37^0C in CO_2 incubator with treated cell samples. Sample were then washed and resuspended in assay buffer for flow cytometric analysis. Analysis of collected data was done using FACSDiva software. Basically, live cells having polarized mitochondria can be identified by the presence of red fluorescence of J-aggregates whereas the depolarized mitochondria can be identified by green fluorescence of monomeric form of JC-1. All experiments were executed three times independently.

A.2.8 TUNEL Assay

For detection of apoptosis the terminal deoxynucleotidyltransferase dUTP nick-end labeling (TUNEL) assay was carried out in control (DMSO), diclofenac or indomethacin-treated A549 cells, on the basis of available instructions from manufactures of TUNEL assay kit (Promega). In this assay terminal deoxynucleotidyl transferase recombinant, enzyme (rTdT) catalytically incorporates fluorescein-12-dUTP at 3´-OH DNA ends, formed due to fragmentation of DNA; a characteristic feature of cells undergoing apoptosis. These labeled

DNA can then be visualized directly under fluorescence microscope to observe apoptotic cells. In short, treated cells were washed with PBS twice and were fixed with 4% paraformaldehyde. Post fixation, cells were again washed with PBS twice and were incubated with 0.5% triton X-100 for 5 min. After washing with PBS thrice, samples were first incubated with equilibration buffer for 10min and then with incubation buffer containing rTdt and nucleotide mix for 4hrs at 37^0C. Reaction was then stopped by adding saline-sodium citrate (SSC) buffer. After washing with PBS thrice, samples were mounted with DAPI and images were then acquired using fluorescence microscope. TUNEL positive cells were counted manually from the images obtained.

A.2.9 MTT Assay

To determine cell viability MTT assay was used; viable cells can convert MTT to colored formazan crystals. Cells were seeded into 96 well plates and following day were treated with appropriate doses of drugs. Some cells were transiently transfected with pEGFP-Hsp70 plasmids. MTT solution was prepared by diluting 5mg MTT reagent in 1 ml of PBS and added to cell samples following incubation of 4 hrs at 37°C in dark. Further, 1 hr incubation at 37°C in dark was given after addition of acidic isopropanol and proper mixing to dissolve formazan crystals. Absorbance was measured at 570nm wavelength in microplate reader.

A.2.10 Reporter Gene Assay

Cos-7 cells were plated into 6-well culture plates and transiently transfected with NF-κB luciferase (firefly luciferase) and pRL-SV40 (Renilla luciferase) plasmids. After transfection, cells were treated with appropriate doses of diclofenac or indomethacin and luciferase acitivity assay was performed according to manufacturer's protocol (Dual luciferase reporter gene assay kit-Promega). Briefly, post transfected and treated cells were washed with PBS. Cells were then incubated with passive lysis buffer on rocking platform for 15 min at room temperature. 20µl of cell lysates prepared were then transferred to tube containing 100µl LAR-II (Luciferase Assay Reagent II) and signals produced from firefly luciferase activity were recorded using luminometer. Sample tube is then removed from luminometer and 100µl of Stop & Glo® Reagent was added and again placed in luminometer for recording Renilla luciferase activity induced signals. The transcription of firefly luciferase is driven by presence of active NF-KB. Firefly luciferase enzyme acts on its substrate LAR-II to give luminescence signal which can be quantified for NF-KB activity. pRL-SV40 plasmid (Renilla luciferase) acts on substrate present in Stop & Glo® Reagent to give luminescence signal, used for normalizing transfection.

A.2.11 JC-1 staining

Cos-7 cells were plated into two well chamber slides and treated with diclofenac or indomethacin. Some cells were treated with MG132. Post treated cells were incubated with 5µM JC-1 dye for 45 minutes in CO_2 incubator. Cells were then washed using prewarmed PBS at 37°C several times. JC-1 staining was then detected under fluorescence microscope at 568 nm filter to identify mitochondrial membrane depolarization. Live cells having polarized mitochondria can be identified by the presence of red fluorescence of J-aggregates whereas the depolarized mitochondria can be identified by green fluorescence of monomeric form of JC-1.

A.2.12 STD-NMR Experiment

To observe interaction between the indomethacin and the proteasome, Saturation Transfer Difference (STD) NMR experiments were performed. Purified proteasomes (purchased from Ubiquitin-Proteasome Biotechnologies, Aurora, CO) were dissolved in 20 mM Tris–HCl pH 2, 150 mM KCl, 1 mM β-Mercaptoethanol, and 10% glycerol (v/v) buffer up to a final concentration of 0.1 µM. A stock solution of 65 mM indomethacin was prepared in DMSO-d6. The final NMR sample was prepared by adding indomethacin to proteasome at 10:1

stoichiometry. STD experiments were carried out at 25°C on a Bruker Ascend 800 NMR spectrometer equipped with a cryogenic probe.

Selective saturation of the receptor was achieved by a train of Gaussian-shaped pulses of about 50 ms each, saturating a bandwidth of about 5Hz at 1ppm. Off-resonance irradiation was set at 30 ppm. Data were collected with 32 k complex points in the direct dimension for 1.2s. A recycling delay of 2 s was applied between each free induction decays (FID) to ensure complete relaxation of the ligand. A total of 128 scans are accumulated for the STD experiments. The FID for on-resonance and off-resonance saturation was collected in an interleaved fashion. The data was processed and plotted in TopSpin.

A.2.13 Senescence Associated Beta Galactosidase Experiment

Presence of senescence associated beta galactosidase enzyme is considered as biomarker for cells undergoing senescence. The galactosidase enzyme cleaves X-gal substrate to give blue color staining in senescent cells. For β-galactosidase activity staining for senescence detection, after 72 hrs of treatment and recovery with indomethacin, hydrogen peroxide and MG132, A549 cells were washed with PBS and fixed with 4% formaldehyde. Fixed cells were then washed with PBS and incubated with staining solution (1 mg/ml X-gal [5-bromo-4-chloro-3-indolyl β-D-galactopyranoside], 5 mM $K_4Fe[CN]_6$, 5 mM $K_3Fe[CN]_6$, 2 mm $MgCl_2$ and citrate acid/sodium phosphate buffer [pH = 6.0]) at 37°C overnight. Images of unstained and senescence associated stained cells were acquired using bright field microscope.

A.2.14 Neurite Outgrowth Experiment

Neuro2a cells were seeded into 2 well chamber slides and were subsequently treated with indomethacin at different concentrations. MG132 was used as positive control. Changes in Neuro2a cellular morphology for formation of neurite outgrowth were then monitored using microscope.

A.2.15 Cell Fractionation

A549 cells were sonicated in HEPES-KOH (20 mM/L, pH 7.5) buffer containing 0.25 mol/L sucrose. Lysates were first centrifuged at 1,000g for 5 min to separate nuclei and unbroken cells. The supernatants obtained were again centrifuged at 10,000g, for 15 min to obtain cytosolic supernatant and enriched mitochondrial pellets. The enriched mitochondrial pellets formed after centrifugation was then used to prepare crude mitochondrial lysates for cytochrome *c* release experiment.

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