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Ibuprofen Induces Mitochondrial-Mediated Apoptosis through Proteasomal Dysfunction

Genetic mutations and several kinds of intra- or extracellular stresses mount severe damage to the genomic and proteomic content of the cells [Amanullah *et al.*, 2017]. To cope up these unwanted alterations, cells try to incorporate multiple protective strategies, including the induction of several stress-response pathways [Fulda *et al.*, 2010]. Failing to do so, sometimes cells initiate cascade of sequential molecular changes leading towards programmed cell death, commonly known as apoptosis [Elmore, 2007]. Mitochondrial depolarization and nucleosomal DNA damage are the major hallmark changes that induce the apoptotic cell death [Saraste and Pulkki, 2000]. Treatment of a well-known NSAID, ibuprofen, generates enormous oxidative and ER stress along with upregulation of p53 levels leading to suppression of cellular growth and unfolded protein responses inside the cells [Adachi *et al.*, 2007; Gomez-Olivan *et al.*, 2014; Ikegaki *et al.*, 2014; Tsutsumi *et al.*, 2004]. Negative effects of ibuprofen on overall mitochondrial health have also been observed in previous studies [Al-Nasser, 2000; Moorthy *et al.*, 2008].

Other studies have also reported the involvement of many NSAIDs in the induction of various cytotoxic stresses and hence in the generation of enormous cytotoxicity [Tsutsumi *et al.*, 2004]. Higher accumulation of inhibitor of kappa B (IκB-α) and subsequent inhibition of NF-κB are among other cellular effects mounted by ibuprofen and other putative NSAIDs [Pierce *et al.*, 1996; Scheuren *et al.*, 1998; Yin *et al.*, 1998]. Inhibition of the functional activities of the proteasome subunits promotes the mitochondrial membrane disruption and release of cytochrome *c* from mitochondria to cytosol followed by induction of apoptosis in various cells [Goldbaum *et al.*, 2006; Qiu *et al.*, 2000]. Till now, few known NSAIDs have been shown to present inhibitory potential against various proteasomal activities that tend to mount a heightened stress response inside the cells leading to initiation of apoptotic cell death [Huang *et al.*, 2002].

4.1 RESULTS

The current study suggests that ibuprofen treatment may disturb the proteasome function, which can induce apoptosis by altered mitochondrial permeability transition and cytochrome c release into cytosol. Ibuprofen treatment induces aggregation of misfolded ubiquitylated proteins and elevates aggregation of proteasome substrates. Most likely, this study provides a better prospect to understand the potentially helpful functions and adverse reactions of NSAIDs, which may be effective for treating a range of diseases.

4.1.1 Ibuprofen Treatment Causes Accumulation of Ubiquitylated Proteins and Induces Time-Dependent Morphological Apoptotic Changes

In the present study, the abilities of ibuprofen to induce apoptosis along with the associated molecular mechanisms to provide the possible clues of how the disturbances of this delicate balance overall affect the intracellular protein degradation machinery have been explored. Cells were transiently transfected with HA-ubiquitin expression construct, and post-transfected cells were treated with varying doses of ibuprofen. Samples were immunoblotted

with anti-hemagglutinin (HA) and anti-β-actin antibodies as shown in Fig. 4.1. Ibuprofen treatment exhibited accumulation of HA-ubiquitylated protein derivatives. However, the molecular mechanism by which ibuprofen induces apoptosis is not well known.

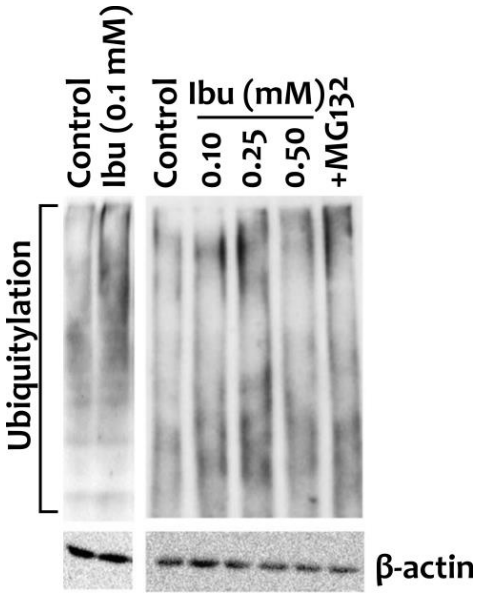

Figure 4.1 : *Ibuprofen treatment causes accumulation of ubiquitinated proteins:* Cells were transiently transfected with HA-ubiquitin expression plasmid. After 24 h of transfection, cells were exposed with 10 μM MG132 and varying doses of ibuprofen, as specified in figure. Cell lysates were used for immunoblotting by using anti-HA and anti-β-actin antibodies. (Figure published in Upadhyay et al. 2016; Molecular Neurobiology)

Next, the effects of ibuprofen on cells were monitored in a time-dependent experiment. As shown in Fig. 4.2, ibuprofen treatment induced apoptotic morphological changes in cells, such as, loss of contact with the neighboring cells, membrane blebbing, and shrinkage of cells in comparison with DMSO treated control cells.

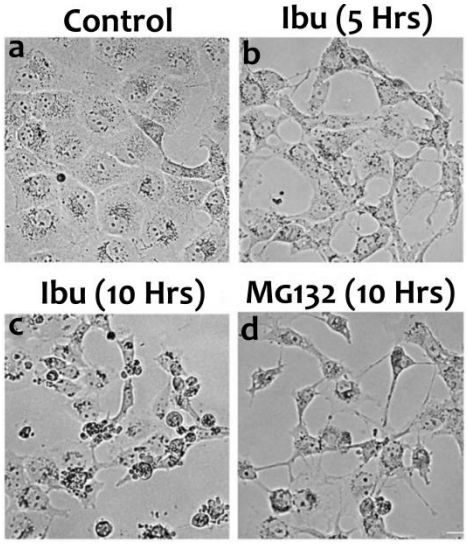

Figure 4.2 : *Visualization of morphological changes caused by ibuprofen treatment:* Cos-7 cells were treated with (a) DMSO, ibuprofen (*Ibu* 5 mM) for different time periods (b) 5 hours and (c) 10 hours and (d) MG132 (10 μM, 10 hours). Post-treated cells were observed under bright field microscope as shown in the figure. *Scale bar*, 20 μm. (Figure published in Upadhyay et al. 2016; Molecular Neurobiology)

Since, in the preliminary results, it has been observed that treatment of ibuprofen aggravated the aggregation of ubiquitylated protein derivatives in cells; therefore it became necessary to check whether ibuprofen treatment could affect the proteasome function or not. Hence, the cells were exposed to ibuprofen and other known proteasome inhibitors and subjected to protease activity assay for chymotrypsin-like activity of the proteasome. As depicted in Fig. 4.3a, ibuprofen treatment reduced the proteasome's protease activity in a similar profile as of other known proteasome inhibitors.

Previously, it has been observed that inhibition of proteasome function induces cell death via apoptosis in cells [Chauhan *et al.*, 2005]. Therefore, the effects of ibuprofen on cell viability were examined. For this, cells were treated as described in previous experiment and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed. As shown in Fig. 4.3b, ibuprofen exposure reduced cell viability and similar patterns were observed for other proteasome inhibitors. Consequently, it was crucial to understand if the effect of ibuprofen-induced cell death is due to the elevation of proteotoxic insults in cells. NSAIDs have been found to generate oxidative stress. Thus, apoptosis due to ibuprofen might be because of stimulation of stress responses. N-acetylcysteine (NAC) restores natural levels of antioxidant glutathione, which helps cell to fight against damage caused by oxidative stress. To address this question, the role of antioxidant NAC on ibuprofen-induced cell death were determined. As shown in Fig. 4.3c, treatment of NAC alleviates ibuprofen-induced cell death monitored by MTT assay.

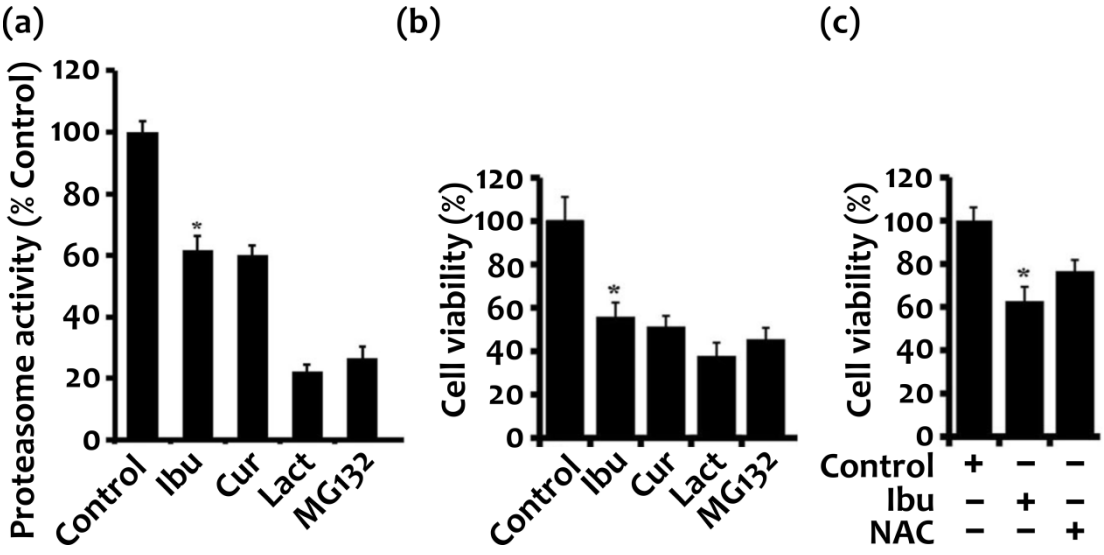

Figure 4.3 : *Treatment of ibuprofen disturbs proteasome function and induces cytotoxicity:* Cells were exposed with ibuprofen (*Ibu* 1 mM) and with the known proteasome inhibitors curcumin (*Cur*, 100 μM), lactacystin (*Lact*, 10 μM), and MG132 (10 μM). After treatments, cells were subjected to proteasome activity assay (chymotrypsin). As explained in the previous section, similar sets of treated cells were used for MTT assay to measure cell viability (b), few similar set of cells were then exposed to either ibuprofen (1 mM Ibu) alone or along with NAC (5 mM) and cell viability was measured by MTT assay (c). Values are shown as means±SD from triplicates of three independent experiments. **p*<0.05 compared with control. (Figure published in Upadhyay et al. 2016; Molecular Neurobiology)

4.1.2 Treatment of Ibuprofen Disturbs Proteasomal Function and Induces Rapid Accumulation of Misfolded Proteins

Since it has been observed that ibuprofen promotes the apoptotic morphological appearance of cells and reduces proteasome activity, further the detailed characterization of ibuprofen treatment on proteasome function were performed. Cells were treated with varying concentrations of ibuprofen, and samples were processed for chymotrypsin-like (Fig. 4.4) protease activity assay of the proteasome.

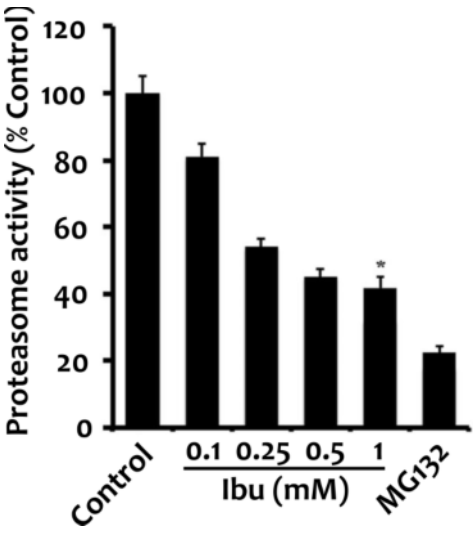

Figure 4.4 : *Exposure of ibuprofen induces loss of chymotrypsin-like proteasome activity in dose-dependent manner:* A549 cells were treated with varying doses of ibuprofen as represented in the figure; and proteasome activity assay for chymotrypsin-like protease activity was performed in post-treated cells. Values represented here as mean±SD. *Columns* are mean of representatives of three independent experiments performed in triplicates. **p*<0.05 compared with control. (Figure published in Upadhyay et al. 2016; Molecular Neurobiology)

In other set of experiments, chymotrypsin-like protease activities were measured in A549 cells following time dependent treatment of ibuprofen (Fig. 4.5).

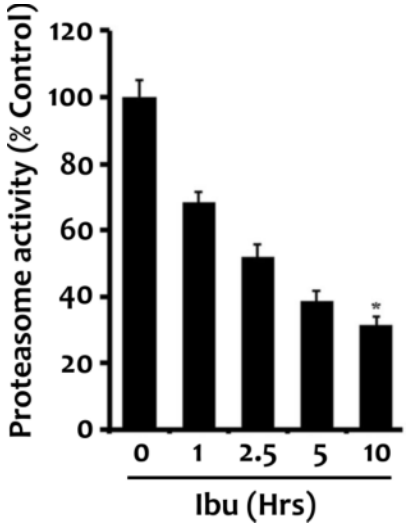

Figure 4.5 : *Time-dependent loss of chymotrypsin-like proteasomal activity in response to ibuprofen administration:* Time-dependent treatments of A549 cells were followed by assay for chymotrypsin-like proteasomal activity. Values are shown as mean±SD. *Columns*, mean of representative of three independent experiments in triplicate. **p*<0.05 compared with control. (Figure published in Upadhyay et al. 2016; Molecular Neurobiology)

The results demonstrate reduced protease activities of proteasome, after ibuprofen treatment in a dose-dependent manner. To further elucidate this experiment, cells were exposed with ibuprofen at different concentrations (Fig. 4.6) and time-intervals (Fig. 4.7); and then the processed for post-glutamyl peptide hydrolase (PGPH)-like protease activity assay of the proteasome.

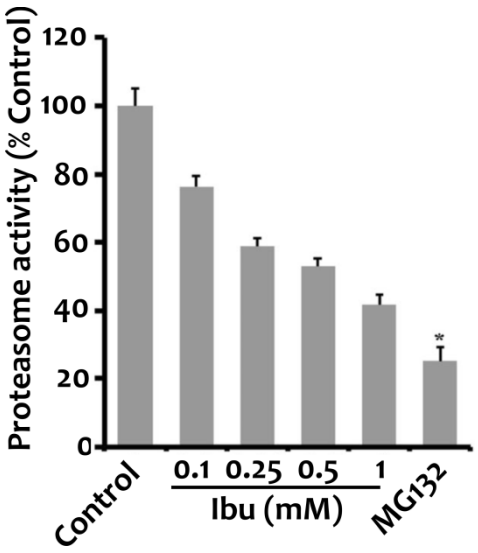

Figure 4.6 : *Ibuprofen also suppresses post-glutamyl peptide hydrolase-like activity of proteasome:* A549 cells were grown in 96-well tissue culture plates and treated with different doses of ibuprofen and post-glutamyl peptide hydrolase-like protease activity assays were performed. Values obtained from three independent assays performed in triplicates were presented for percent of remaining proteasomal activity. Values are shown as mean±SD. **p*<0.05 compared with control. (Figure published in Upadhyay et al. 2016; Molecular Neurobiology)

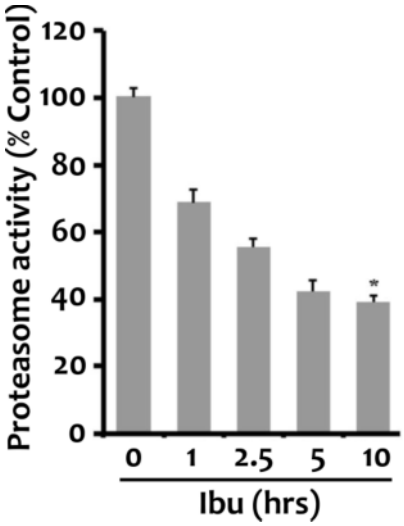

Figure 4.7 : *Time-dependent inhibition of PGPH-like activity of 20S proteasome:* Cells cultured and treated with ibuprofen for different time intervals were subjected to PGPH-like protease activity assays and values were collected from three independent assays that were performed in triplicates. Here, the values represent mean±SD, where **p*<0.05 compared with control. (Figure published in Upadhyay et al. 2016; Molecular Neurobiology)

As shown in above figures, treatment of ibuprofen led to a decrease in both chymotrypsin-like as well as PGPH-like protease activities of 20S proteasome. Previously, it has been shown that ibuprofen treatment induces neuronal damages at higher concentration and also enhances bilirubin toxicity in embryonic neuronal cortical cultures [Berns *et al.*, 2009]. However, the mechanism by which ibuprofen induces toxicity in various cells is not well-known. Preliminary results also suggest that ibuprofen treatment reduces cell viability that were in-line with few other studies, which indicate that ibuprofen and NSAID exposure leads to hepatotoxicity [O'Connor *et al.*, 2003; Riley and Smith, 1998].

To understand if ibuprofen-induced proteasome dysfunction contributes in the accumulation of ubiquitylated proteins and generate proteotoxicity inside the cells, transient transfections of EGFP-HDQ23 and EGFP-HDQ74 plasmids were performed followed by treatment with ibuprofen (Fig. 4.8).

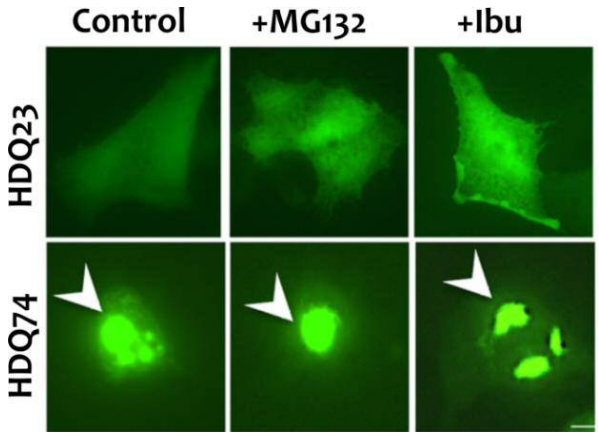

Figure 4.8 : *Ibuprofen treatment leads to stabilization of misfolded proteins in cells:* A549 cells were transiently transfected with EGFP-HDQ23 and EGFP-HDQ74 plasmids and then treated with ibuprofen (0.5 mM) and MG132 (10 µM) for 24 hours. Treated cells were observed under fluorescence microscope as shown. *Arrowheads* indicate aggregate formation. Scale bar, 20 µm. (Figure published in Upadhyay et al. 2016; Molecular Neurobiology)

As shown in Fig. 4.8, ibuprofen-treated cells follow MG132 treatment-like profile of expanded polyglutamine protein aggregate formation in perinuclear regions. To further ascertain these results, few similar sets of cells were used for immunoblot analysis using ubiquitin, GFP, and β-actin antibodies. Exposure of ibuprofen elevated the accumulation of ubiquitylated derivatives of expanded polyglutamine proteins as shown in Fig. 4.9a. As depicted in Fig. 4.9b, expressions of normal and expanded polyglutamine plasmids were confirmed in the above-described experiment by using immunoblot analysis and blots were probed with GFP antibody.

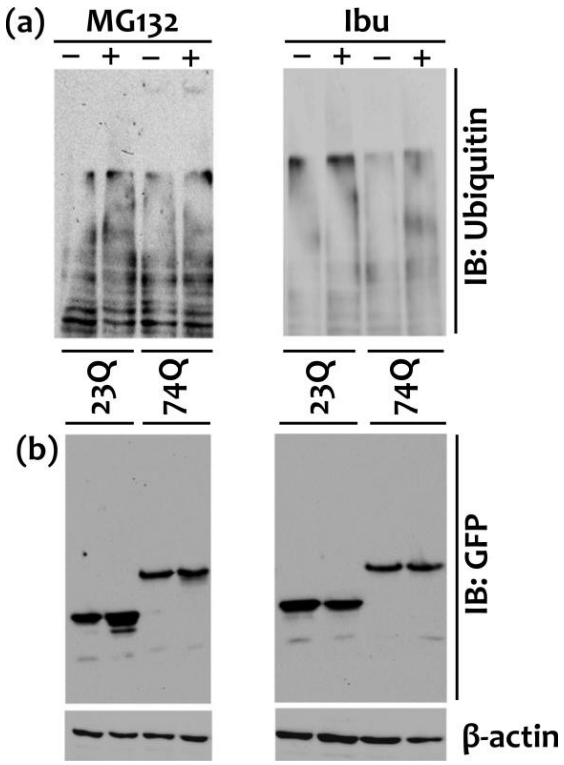

Figure 4.9 : *Effect of ibuprofen treatment on soluble fraction of pathogenic huntingtin protein:* Few sets of EGFP-HDQ23 and EGFP-HDQ74-transfected Cos-7 cells were treated with ibuprofen (Ibu 0.5 mM) and MG132 (10 μM). These cells were collected, and lysates were processed for immunoblotting using anti-ubiquitin (a), anti-GFP, and anti-β-actin (b) antibodies. (Figure published in Upadhyay et al. 2016; Molecular Neurobiology)

Next, it was important to reconfirm if ibuprofen-induced proteasomal dysfunction plays a role in the accumulation of ubiquitylated proteins in cells. Ibuprofen treatment in a dose-dependent manner was used in GFP-ubiquitin transfected cells, and lysates were immunoblotted with anti-GFP, anti-ubiquitin, and anti-β-actin antibodies. As represented in Fig. 4.10, ibuprofen-induced accumulation of higher molecular weight derivatives of ubiquitylated exogenously expressed GFP-ubiquitin proteins; this might be due to malfunction of proteasome.

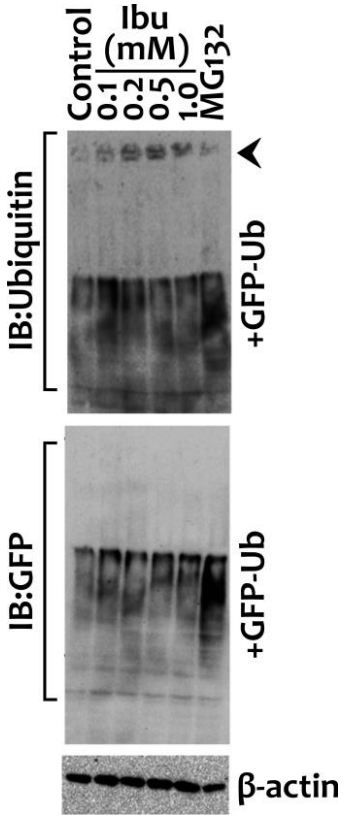

Figure 4.10 : *Ibuprofen treatment stabilizes ubiquitinated proteins inside the cells:* Cos-7 Cells were transiently transfected with GFP-ubiquitin expression plasmid, and after 24 h of transfection, cells were treated with varying concentrations of ibuprofen and MG132 (10 μM) as shown in the figure. Cell lysates were prepared and then immunoblotted with ubiquitin, GFP, and β-actin antibodies. (Figure published in Upadhyay et al. 2016; Molecular Neurobiology)

To test whether ibuprofen-mediated proteasomal dysfunction contributes to cellular toxicity or not, cells were treated with different concentrations of ibuprofen and cells were used for MTT assay to determine cell viability (Fig. 4.11).

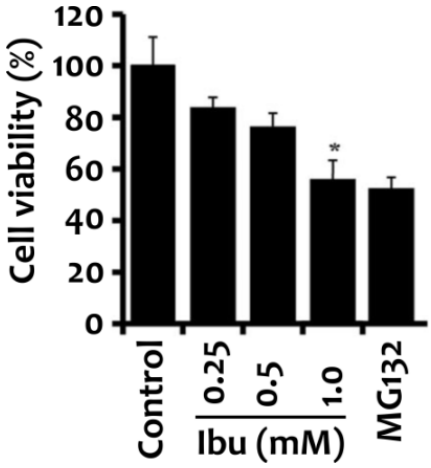

Figure 4.11 : *Concentration-dependent cytotoxicity conferred by ibuprofen:* A549 cells were treated with varying concentrations of ibuprofen as represented in figure, and after treatments, cells were used for MTT assay to measure cell viability. **p*<0.05 compared with control. (Figure published in Upadhyay et al. 2016; Molecular Neurobiology)

4.1.3 Ibuprofen-Mediated Interference Can Contribute in Proteasomal Inhibition-Induced Cytotoxicity

To obtain clues related to the mechanism by which ibuprofen alters or disturbs proteasomal function and how these events can contribute in cellular stress and cell death, *in silico* studies were performed on the basis of the preliminary findings. Interaction of ibuprofen with β1 and β5 subunit (containing PGPH and chymotrypsin like activities respectively) of proteasome was predicted by using in silico approach. At the active sites, the docked free energy with β1 and β5 was observed to be −7.72 and −7.38 kcal/mol, respectively. As shown in Fig. 4.12, docking images depicted hydrogen bonds formed between ibuprofen and proteasome subunits as shown in green lines along with surrounding residues.

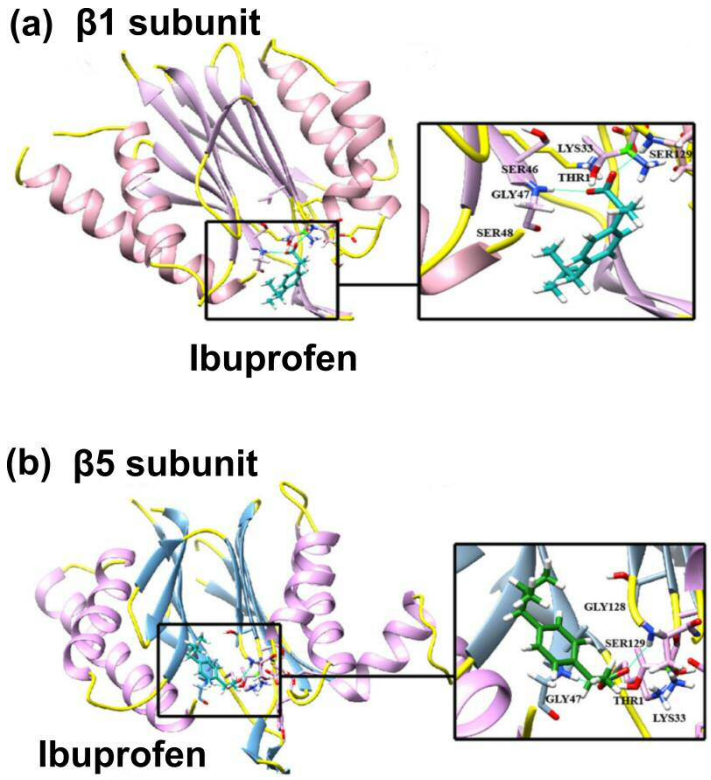

Figure 4.12 : *Ibuprofen presents in silico interaction with proteasomal subunits:* Protein ligand docking was performed using the web interface of Swiss docking server. (a) General view of the molecular docking of β1 subunit (PDB 1JD2) with ibuprofen (ZINC ID 2647); (b) Molecular docking of β5 subunit with ibuprofen demonstrates formation of hydrogen bonds, which are depicted as *green lines*. Close-up view of the protein-ligand interface is shown in the *side panels*. (Figure published in Upadhyay et al. 2016; Molecular Neurobiology)

Further experiments were conducted to examine whether ibuprofen treatment affects the overall ubiquitylation profile for cellular proteins. As demonstrated in Fig. 4.13, the exposure of ibuprofen caused a dose-dependent elevation in the accumulation of ubiquitylated derivatives of different cellular proteins.

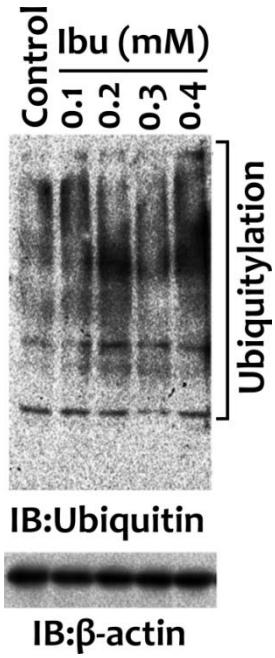

Figure 4.13 : *Enhanced accumulation of cytoplasmic ubiquitinated proteins following ibuprofen-mediated proteasomal inhibition:* A549 cells were seeded into 6-well tissue culture plates and treated with different concentrations of ibuprofen and MG132 (10 µM). After treatment, cells were collected, and then, lysates were immunoblotted with anti-ubiquitin and anti-β-actin antibodies. (Figure published in Upadhyay et al. 2016; Molecular Neurobiology)

Higher dose of ibuprofen might lead to accumulation of abnormal proteins in cells, which seems to induce stress events in cells and can affect their survival. To validate this assumption, cells were treated with varying concentrations of ibuprofen and observed under bright field microscope. Interestingly, as shown in Fig. 4.14, noticeable morphological changes in cells were also observed, which indicate that cells are more susceptible for apoptosis in the presence of ibuprofen higher dose treatments.

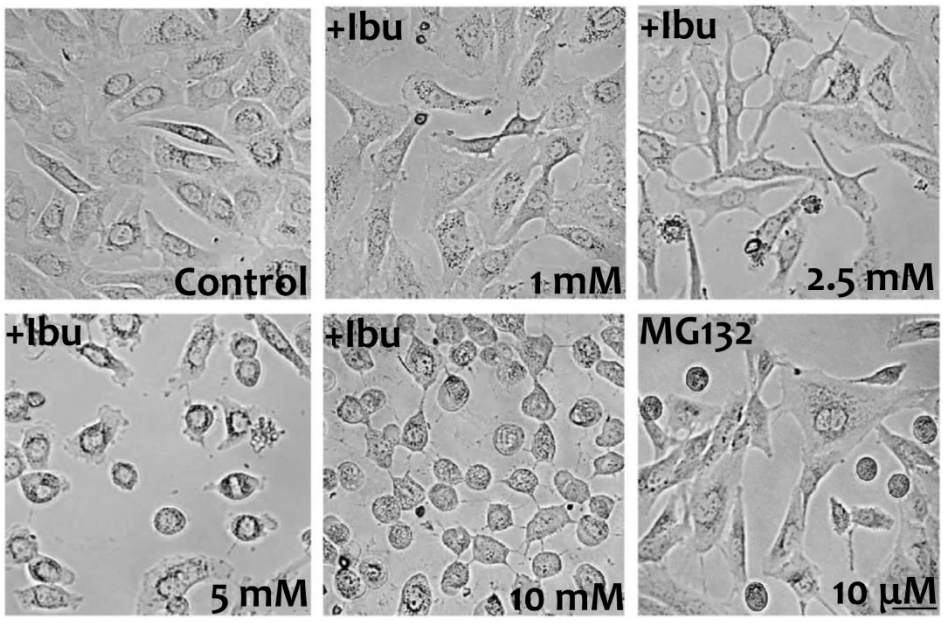

Figure 4.14 : *Bright-field image micrographs of ibuprofen treated cells:* A549 cells were treated with or without varying concentration of ibuprofen and MG132, as represented in the figure; post-treated cells were observed under bright field microscope. Scale bar, 20 µm. (Figure published in Upadhyay et al. 2016; Molecular Neurobiology)

4.1.4 Ibuprofen Induces Proteasomal Dysfunction, Facilitates Formation of Inclusions of Misfolded Proteins, and Elevates Aggregation of Proteasomal Substrates

To further confirm if ibuprofen-induced proteasomal dysfunction causes accumulation of its substrates or abnormal proteins in cells, three different kinds of proteins were used for next course of analysis. Cells were transiently transfected with CFTRΔF508, pd1EGFP, and GFP-wtCAT plasmids, and then treated with ibuprofen. Results presented in Fig. 4.15 show that ibuprofen treatment increases the propensity of inclusions of formation in perinuclear region. This overburden of misfolded protein aggregation might be due to altered proteasomal function [Chhangani and Mishra, 2013a].

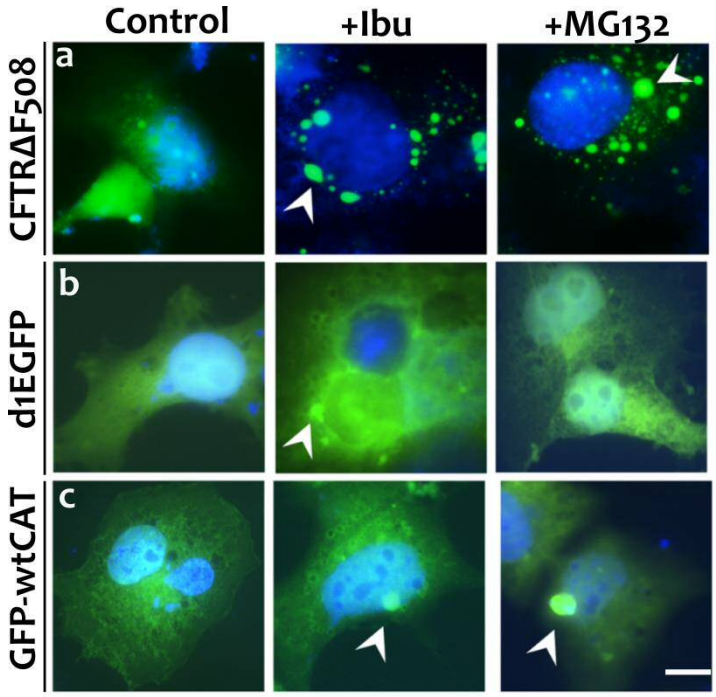

Figure 4.15 : *Formation of aggresomes of misfolded proteins response to ibuprofen treatment in cells:* Cos-7 cells were transiently transfected with CFTRΔF508 (**a**), pd1EGFP (**b**), and GFP-wtCAT (**c**) as represented in the figure. After transfection, cells were exposed with ibuprofen (Ibu 0.5 mM) and MG132 (10 μM) and observed under fluorescence microscope. The *arrowheads* denote perinuclear cytoplasmic aggresome-like structures of misfolded proteins in cells. Scale bar, 20 μm. (Figure published in Upadhyay et al. 2016; Molecular Neurobiology)

Previously, it has been shown that treatment of NSAID-induced proteasome impairment inhibits the activation of NF-κB that might be due to the accumulation of IκB-α [Dikshit *et al.*, 2006; Pierce *et al.*, 1996]. To validate these findings with ibuprofen, cells were administered with ibuprofen in different concentrations, as shown in Fig. 4.16. After treatment, lysates were prepared and subjected to immunoblot analyses using anti-IκB-α and anti-β-actin antibodies. Interestingly, an accumulation of IκB-α inside the cells following the treatment of ibuprofen has also been observed in the present studies.

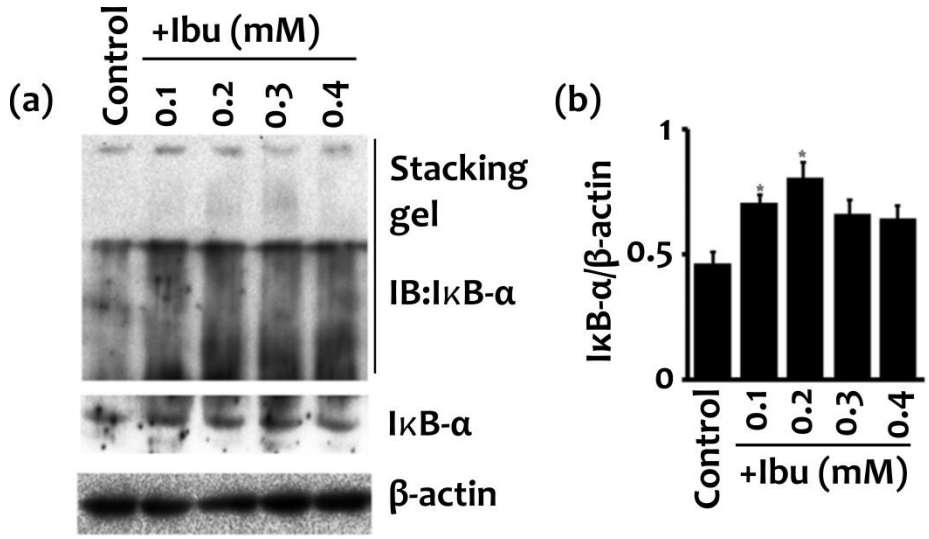

Figure 4.16 : *Ibuprofen mediates stabilization of IκB-α*: (a) A549 cells treated with various concentrations of ibuprofen; cell lysates were prepared and used for immunoblotting with IκB-α and β-actin antibodies. (b) Blot intensities were quantified and normalized against β-actin. (Figure published in Upadhyay et al. 2016; Molecular Neurobiology)

As shown in Fig. 4.17, a downregulation of NF-κB-dependent transcriptional activity was also observed in another set of experiments that could probably be due to the elevated levels of IκB-α. Surprisingly, ibuprofen-mediated NF-κB inhibition was high as compared to proteasomal inhibition. Earlier study has also observed that ibuprofen exposure has inhibited the constitutive activation of NF-κB in prostate cancer cells [Palayoor *et al.*, 1999].

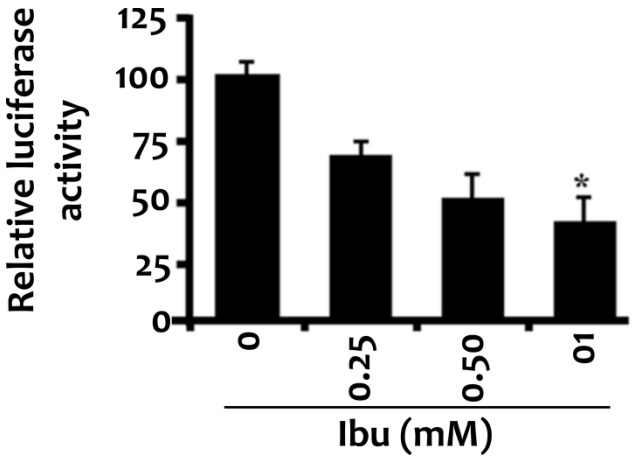

Figure 4.17 : *Downregulation of NF-κB transcriptional activity in response to ibuprofen treatment:* Cells were transiently transfected with NF-κB luciferase and pRL-SV40 constructs, and after 24 hours of transfection, cells were treated with different doses of ibuprofen; and then, luciferase activity assay was performed. (Figure published in Upadhyay et al. 2016; Molecular Neurobiology)

Next experimental sets were used to monitor if ibuprofen-mediated proteasomal disturbance might generate an impact on a model substrate for proteasome via UPS pathway. In a different set of experiments, cells were transiently transfected with d1EGFP plasmid, which encodes a destabilized enhanced green fluorescent protein (d1EGFP), with 1 hour of half-life. After transfection, cells were chased with cycloheximide with or without treatment of ibuprofen and collected cell lysates were used for immunoblotting analysis with anti-GFP and anti-β-actin antibodies. Exposure of ibuprofen led to an increased half-life of d1EGFP proteins in cells as represented in Fig. 4.18.

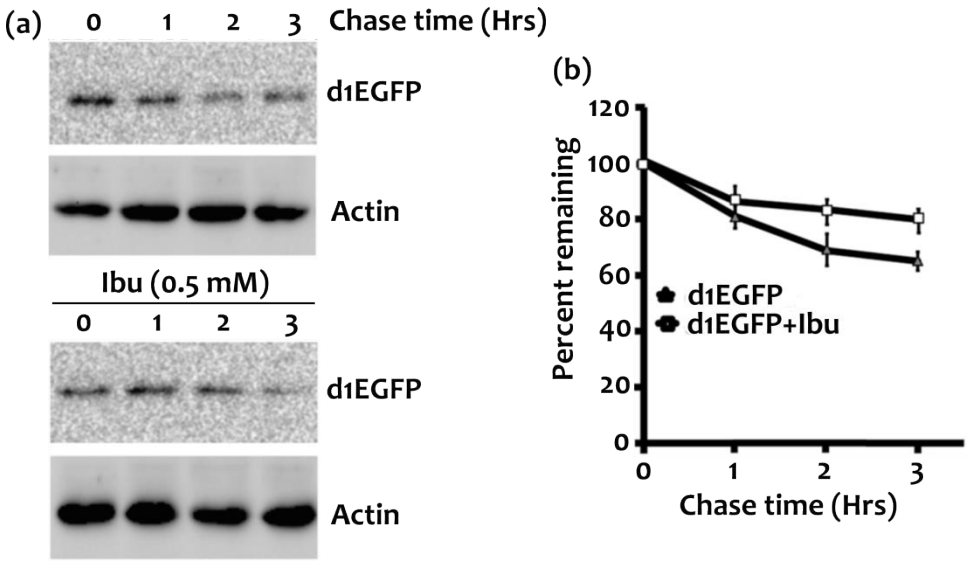

Figure 4.18 : *Ibuprofen causes stabilization of aggregation-prone misfolded protein species:* (a) Cells were transiently transfected with pd1EGFP plasmid, and after 24 hours of transfection, cells were treated with 0.5 mM ibuprofen and chased in the presence of cycloheximide (15 µg/ml). (b) Protein d1EGFP levels were quantified from three independent chase experiments. β-actin was used for normalization, and values are represented as mean±S.D. of three independent experiments, performed in triplicates. (Figure published in Upadhyay et al. 2016; Molecular Neurobiology)

4.1.5 Ibuprofen Compromises the Clearance of Pro-Apoptotic Ubiquitinated Proteins Destined for Proteasomal Degradation

In order to understand the effect of ibuprofen on short-lived regulatory proteins, thorough immunocytochemistry analyses were conducted. After the cells were treated with ibuprofen and MG132, antibodies against 20S proteasome and ubiquitin were used to perform the immunocytochemistry, as shown in Fig. 4.19. To further ascertain the effects of suppression of proteasomal activities on cell cycle regulatory proteins p27 and p53, which are putative proteasomal substrates, immunofluorescence analysis were performed using respective antibodies Fig. 4.20.

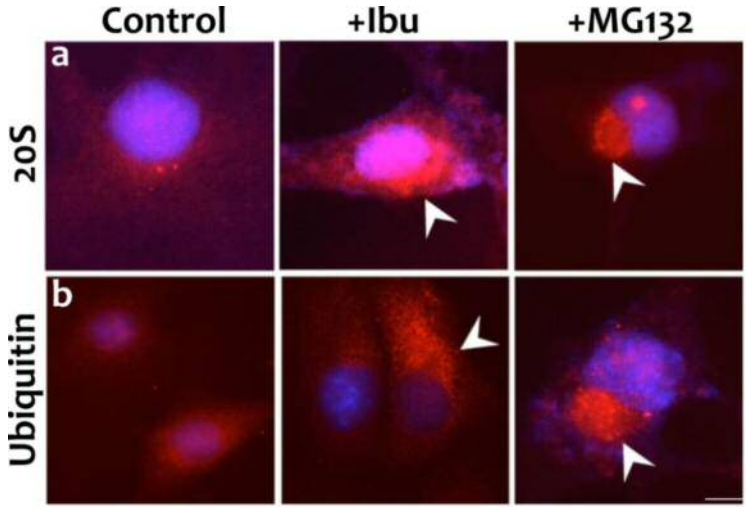

Figure 4.19 : *Treatment of ibuprofen accelerates the accumulation of proteasome and ubiquitin-positive protein aggregates:* Cos-7 cells were treated with ibuprofen (Ibu 1 mM) and MG132 (10 µM). After treatment, cells were processed for immunofluorescence analysis and were probed with 20S (a) and ubiquitin (b) antibodies; and observed under fluorescence microscope. *Arrows* indicate the redistribution and accumulation of different pro-apoptotic proteasome target proteins. Images were obtained using a fluorescence microscope. *Scale bar*, 20 µm. (Figure published in Upadhyay et al. 2016; Molecular Neurobiology)

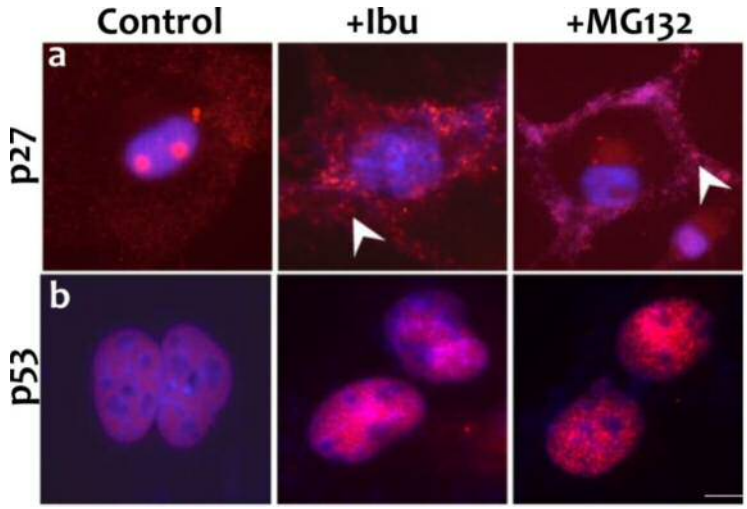

Figure 4.20 : *Proteasomal inhibition by ibuprofen leads to increased accumulation of cell cycle regulatory proteins p53 and cyclin-dependent kinase inhibitor p27:* Few sets of Cos-7 cells, treated with ibuprofen and MG132, were subjected to immunofluorescence analyses by using anti-p27 (a) and anti-p53 (b) antibodies. Drug treatements lead to increased accumulation of proteasomal substrate proteins along with total ubiquitin present inside the cytoplasm. *Scale bar*, 20 µm. (Figure published in Upadhyay et al. 2016; Molecular Neurobiology)

Similarly, fluorescence analyses of other putative proteasomal substrates, which are also known for their pro-apoptotic functions, like Bax and cyclin-dependent kinase inhibitor p21 were conducted to monitor the alterations in expression level and subcellular locallization following treatments of ibuprofen and MG132 Fig. 4.21.

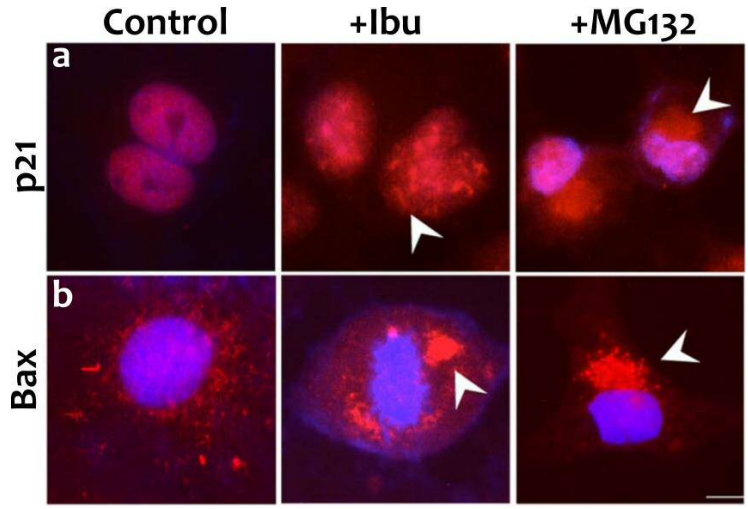

Figure 4.21 : *Ibuprofen induces cytoplasmic accumulation of pro-apoptotic proteasomal target proteins:* Few sets of Cos-7 cells were grown on two-well chamber slides and after treatment of ibuprofen and MG132, anti-p21 (a) and anti-Bax (b) antibodies were used for immunocytochemistry staining. Micrographs were obtained using fluorescence microscope. *Scale bar*, 20 µm. (Figure published in Upadhyay et al. 2016; Molecular Neurobiology)

These results suggest that ibuprofen-mediated malfunction in proteasome causes an increase in levels of those pro-apoptotic proteins. Few of these proteins were even positive for aggregosomes like inclusions in nuclear peripheral regions of cells. Earlier studies demonstrated that NSAID exposure inhibits the proteasomal degradation of p53 and p27 proteins in cells [Dey *et al.*, 2008; Hung *et al.*, 2000]. Most likely, accumulation of these ubiquitinated substrates makes a direct consequence on other pro-apoptotic and apoptosis-linked proteins.

4.1.6 Ibuprofen-Mediated Proteasomal Disturbance Triggers Chromatin Condensation, Nuclear Disassembly, and DNA Fragmentation and Activates Cell Death Program

Cells were exposed to different doses of ibuprofen, and post-treated cells were subjected to nuclear staining with 4′,6-diamidino-2-phenylindole (DAPI). As shown in Fig. 4.22, ibuprofen-treated cells exhibited cytoplasmic shrinkage; apoptotic nuclei stained with DAPI that became progressively pyknotic and were extensively fragmented compared to untreated cells.

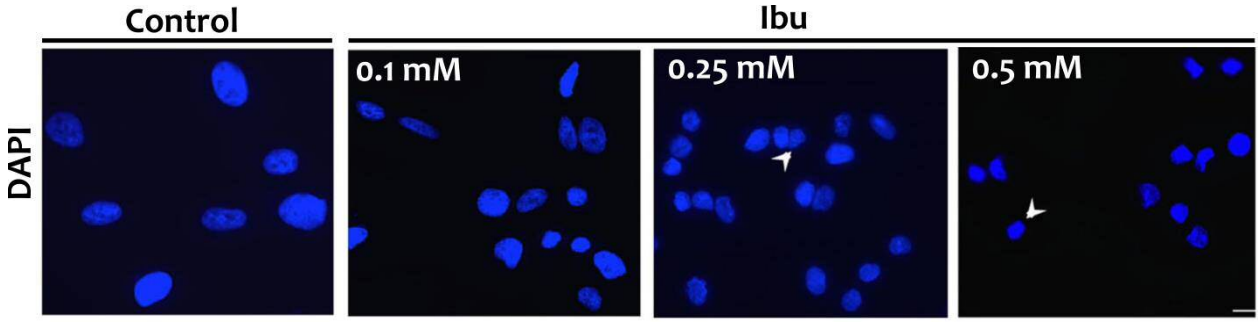

Figure 4.22 : *Ibuprofen treatment induces apoptosis and causes nuclear morphological changes (condensation):* Cos-7 cells were exposed with different concentrations of ibuprofen and MG132. Nuclear morphology of cells was observed with DAPI. Scale bar, 20 µm. (Figure published in Upadhyay et al. 2016; Molecular Neurobiology)

To further understand and ascertain the underlying mechanism by which ibuprofen induces apoptosis, cells were seeded in tissue culture plates and were treated with ibuprofen at different time periods. After treatment, cells were subjected to the assessment of apoptosis using annexin V staining and fluorescence-activated cell sorting (FACS) analysis (Fig. 4.23a). Quantification of FACS results represents that the apoptotic cell fraction was increased after the treatment of ibuprofen in cells compared to control cells (Fig. 4.23b).

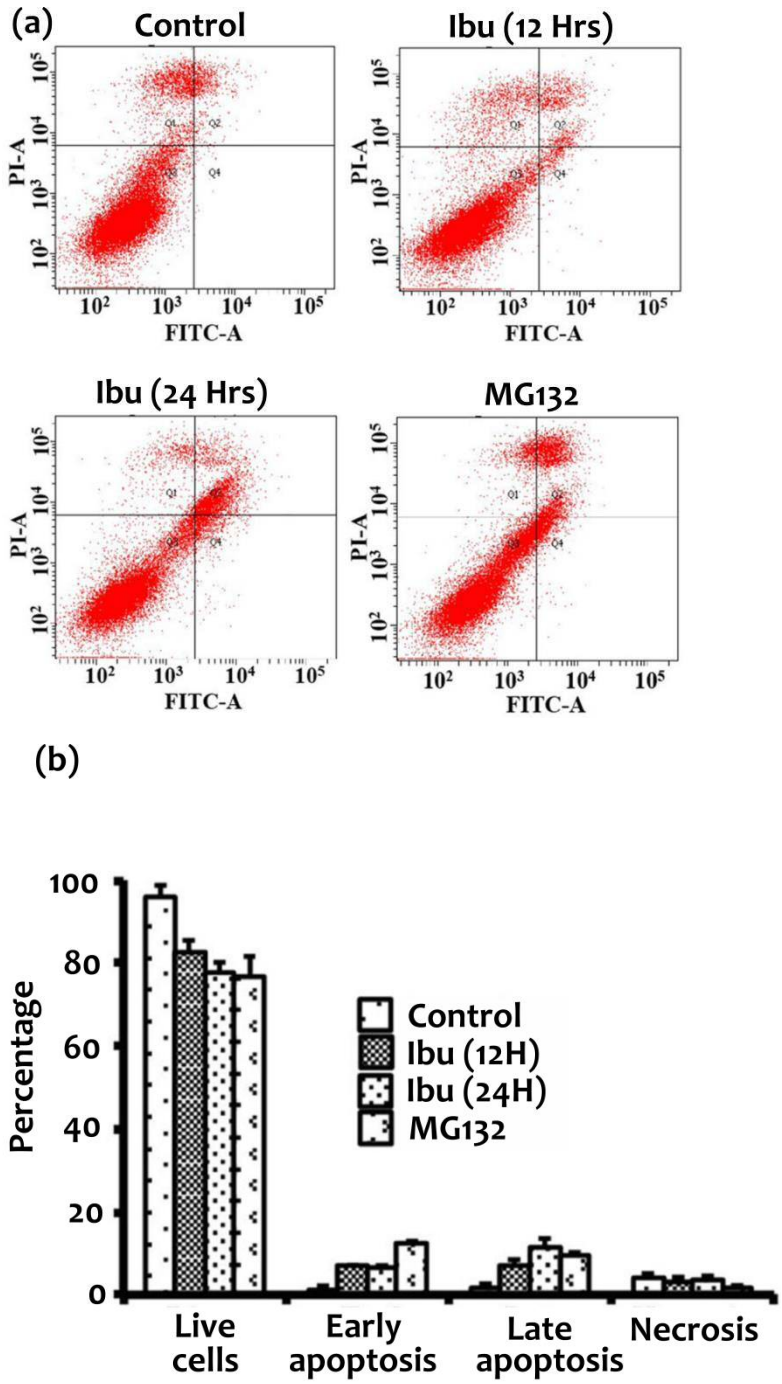

Figure 4.23 : *Time-dependent assessment of apoptosis by flow cytometry using annexin V-FITC and propidium iodide double staining:* A549 cells were treated with ibuprofen (1 mM) for 12 and 24 hours; MG132 (10 µM) treated cells were used as positive control for these experiments. (b) Values are mean±S.D. of three independent experiments, as shown using bar graph. (Figure published in Upadhyay et al. 2016; Molecular Neurobiology)

Apoptosis is a distinct form of cell death; apart from chromatin condensation, DNA fragmentation is another parallel pivotal hallmark, which can also be used as one of the most important criteria to recognize apoptotic cells. In order to gain insight into the mechanism of ibuprofen-induced apoptosis, cells were treated with ibuprofen; and performed agarose gel electrophoresis, as shown in Fig. 4.24a. Ibuprofen-exposed samples demonstrated the oligonucleosomal laddering pattern linked with apoptotic cells.

To substantiate the above findings that the ibuprofen-mediated proteasomal dysfunction contributes in cell death mechanism, terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling (TUNEL) analysis was performed in order to determine the ibuprofen-mediated apoptosis. Figure 4.24b shows the quantification of cell death by counting TUNEL-positive cells; treatment of ibuprofen increased the number of apoptotic cells in a dose-dependent manner. It has also been established that NSAIDs retain an ability to stimulate the ER stress response and are responsible for increased apoptotic cell death [Pyrko *et al.*, 2007; Tsutsumi *et al.*, 2004].

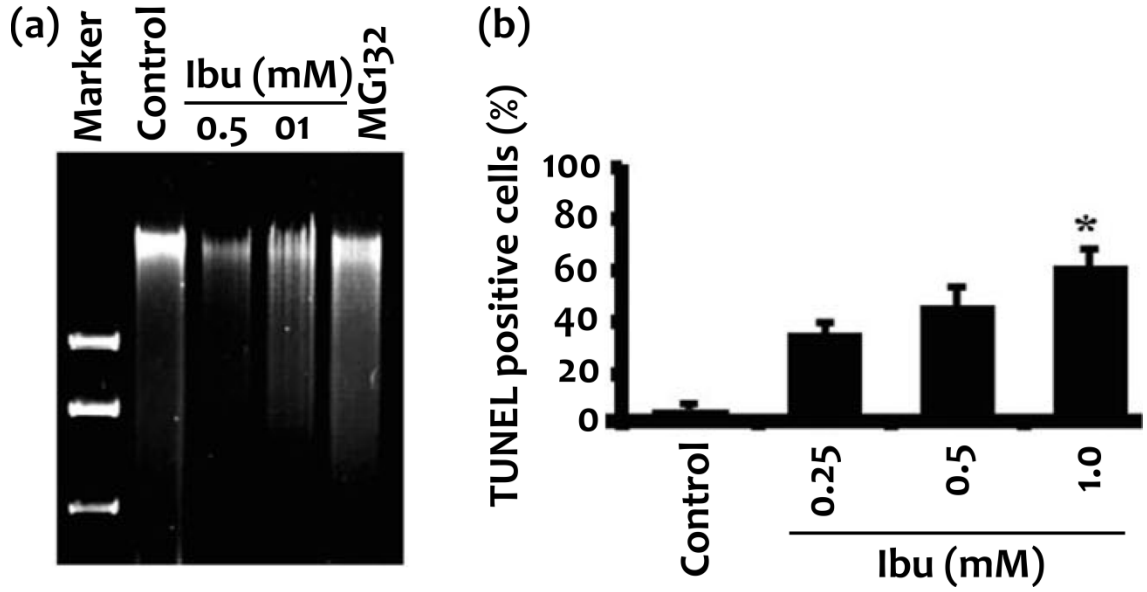

Figure 4.24 : *Representation of DNA fragmentation following treatment of inbuprofen:* After treatment of Cos-7 cells with ibuprofen and MG132, total DNA was isolated from different experimental sets and agarose gel analysis was performed. Detection of late-stage apoptosis-associated DNA degradation in ibuprofen- and MG132-treated cells was also confirmed by TUNEL assay; quantification values are presented as mean±S.D. of three independent experiments. *p<0.05 compared with control. (Figure published in Upadhyay et al. 2016; Molecular Neurobiology)

4.1.7 Ibuprofen Induces Loss of Mitochondrial Membrane Potential and Release of Cyctochrome *c*

Earlier, it has been established that NSAIDs trigger mitochondrial dysfunction and affect the mitochondrial membrane permeability and also release cytochrome c from mitochondria into the cytosol [Lal *et al.*, 2009; Pique *et al.*, 2000; Zimmermann *et al.*, 2000]. Even treatment of ibuprofen also disturbs mitochondrial permeability [Al-Nasser, 2000]. But, the molecular mechanism by which ibuprofen treatment deregulates mitochondrial functions and induces apoptosis is not clear. Therefore, now it was important to determine whether the ibuprofen-mediated proteasomal dysfunction and apoptosis are due to mitochondrial functional loss. To examine whether cytochrome *c* is released during ibuprofen-induced apoptosis, cells were treated with different doses of ibuprofen and treated cells were used for immunofluorescence staining (Fig. 4.25).

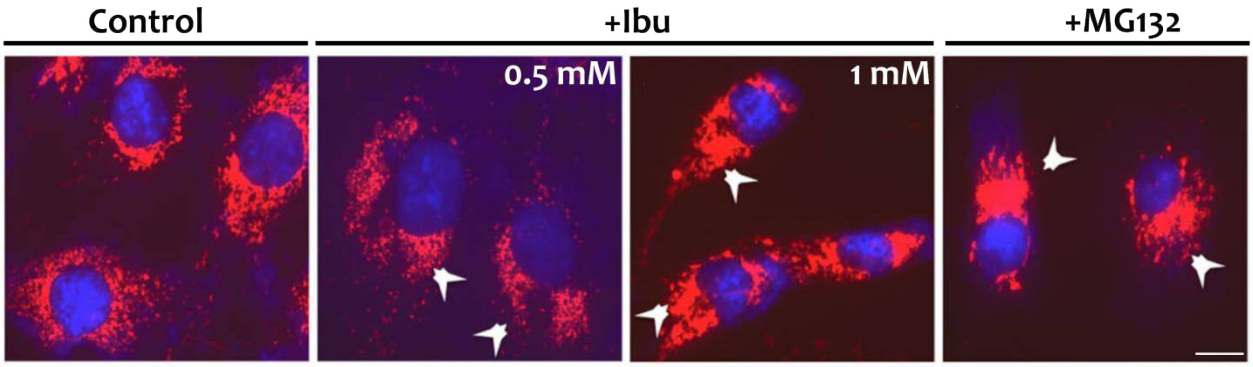

Figure 4.25 : *Ibuprofen treatment induces cytochrome c release during apoptosis:* Cos-7 cells were plated into 2-well chamber slides, and cells were treated with different concentrations of ibuprofen (0.5 mM and 1 mM) and d MG132 (10 μM). After treatment, cells were used for immunofluorescence analysis using cytochrome c antibody. *Scale bar*, 20 μm. (Figure published in Upadhyay et al. 2016; Molecular Neurobiology)

Few sets of Cos-7 cells, treated with ibuprofen and MG132 in similar manner, as described above, were subjected to immunoblotting (Fig. 4.26) using antibody against cytochrome *c*. Ibuprofen treatment released cytochrome *c* into the cytosol from mitochondria, as observed by immunofluorescence analysis and western blotting.

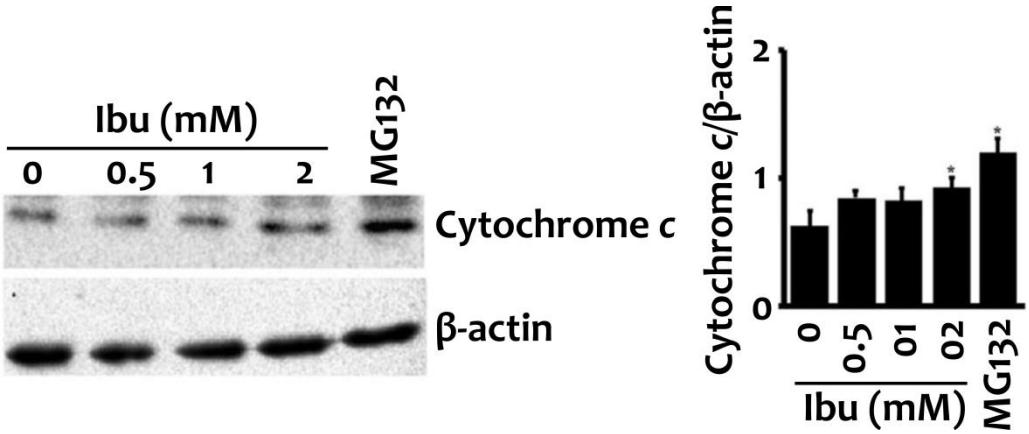

Figure 4.26 : *Higher accumulation of cytochrome c following the treatment of cells with ibuprofen:* Cells were treated with various concentrations of ibuprofen and MG132 (10 μM) and collected after 24 hours of treatment. Cell lysates were prepared and used for immunoblot analysis using cytochrome c and β-actin antibodies. (Figure published in Upadhyay et al. 2016; Molecular Neurobiology)

Next, to determine whether ibuprofen treatment disturbs the mitochondrial membrane potential, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide (JC-1) fluorescence dye was used. JC-1 constitutes a good voltage-sensitive mitochondrial membrane potential (Ψmt) indicator, loss of membrane potential shift fluorescence from red (polarized) to green (depolarized) emission, in accordance with reduction in membrane potential (Fig. 4.27).

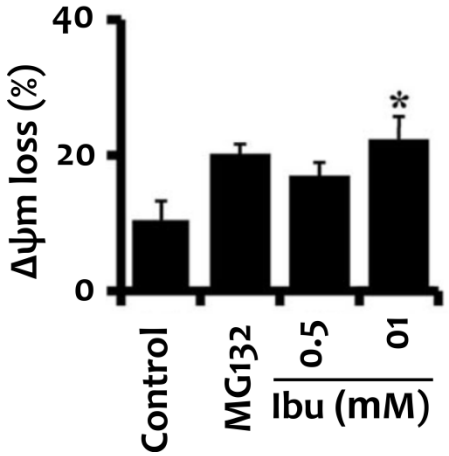

Figure 4.27 : *Ibuprofen treatment leads to mitochondrial depolarization:* Cells were treated with different doses of ibuprofen and stained with JC-1 dye and subjected to flow cytometric analyses. Values are the mean±S.D. of three independent experiments, each performed in triplicate. *p<0.05 compared with the control. (Figure published in Upadhyay et al. 2016; Molecular Neurobiology)

As depicted Fig. 4.28, micrographs represent that ibuprofen treatment reduces mitochondrial membrane potential in a dose-dependent manner, from the change in fluorescence emission from red mitochondrial staining to green appearance.

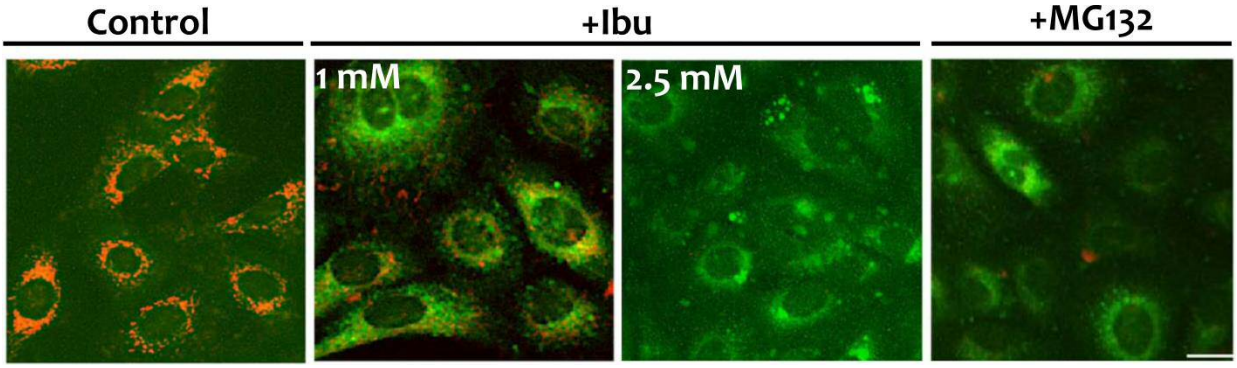

Figure 4.28 : Analysis of ibuprofen-mediated mitochondrial alterations using JC-1 staining and fluorescence analysis: Cells were seeded for JC-1 staining and analyzed by fluorescence analysis. Mitochondrial depolarization is indicated with reduction in red fluorescence. Cells were treated with DMSO, Ibu 1 mM, and 2.5 mM and MG132. *Scale bar*, 20 μm. (Figure published in Upadhyay et al. 2016; Molecular Neurobiology)

4.2 DISCUSSION

Treatment of ibuprofen induces apoptosis, most likely due to proteasomal dysfunction and induced mitochondrial abnormalities. In the current study, it has been observed that ibuprofen treatment in both time- and concentration-dependent manner promotes suppression of proteasomal activities leading to morphological apoptotic alterations in the cells. Results of cell viability assays confirmed that ibuprofen leads to a reduction in viability of cellular population; however the effects can be suppressed by the use of antioxidant NAC. Several previous studies have also shown that NSAID treatment causes impaired proteasome function and generates stress events inside the cells [Adachi *et al.*, 2007; Galati *et al.*, 2002; Jana, 2008].

Emerging evidence demonstrates that treatment of NSAIDs leads to various stresses such as oxidative and ER stress [Gomez-Olivan *et al.*, 2014; Ikegaki *et al.*, 2014; Tsutsumi *et al.*, 2004; Tsutsumi *et al.*, 2006]. Indomethacin treatment causes gastric mucosal injury due to oxidative stress and epithelial cell apoptosis, which further induces gastropathy [Naito and

Yoshikawa, 2006]. Idiosyncratic NSAID drug leads to oxidative stress, and treatment of NSAIDs also promotes small bowel injury and mitochondrial dysfunctions [Galati *et al.*, 2002; Nagano *et al.*, 2012; Watanabe *et al.*, 2011]. It has been shown that celecoxib, an NSAID, upregulated ER chaperones in human gastric cells that in turn relieved cells from celecoxib induced cytotoxicity [Tsutsumi *et al.*, 2006]. However, the molecular basis for this inhibitory action of NSAIDs over proteasome functions and effects of such inhibition on overall cellular health and associated molecular pathways remains obscure and need detailed analysis.

In the current study, possible interactions of ibuprofen with β1 and β5 subunits of proteasome have been demonstrated by performing *in silico* docking analysis. In addition, these results suggest that dose-dependent exposure of ibuprofen accumulates ubiquitylated proteins in cells, which further develops apoptotic morphological changes in cells in a manner similar to that of MG132, a putative proteasome inhibitor. An earlier study has demonstrated that ibuprofen treatment in mice exhibits severe adverse effects in a murine prion model; however, the detailed molecular pathomechanism of ibuprofen-induced adverse effects could not have been shown [Riemer *et al.*, 2008]. Interestingly, these findings also suggest that ibuprofen treatment induces aggregation of expanded polyglutamine proteins and their inclusions in nuclear peripheral region. Ibuprofen also increases the accumulated levels of ubiquitylated derivatives of polyglutamine expansion proteins; similar effects were also observed in cells when treated with MG132.

To further examine whether ibuprofen-induced proteasome dysfunction could be the underlying cause of increased rates of apoptosis, concentration-dependent treatments of ibuprofen has been performed inside the cells that promotes the accumulation of ubiquitylated GFP protein. Further, immunocytochemistry experiments were also performed to monitor the effects of ibuprofen on the levels and subcellular localization of various aggregation-prone proteins (CFTRΔF508, pd1EGFP, and GFP-wtCAT). The effects of proteasome inhibition on the expression and localization of these proteins were confirmed by fluorescence microscopy analysis, which shows that treatment of ibuprofen in these cells markedly increased the accumulation of inclusion-like structures in the nuclear peripheral region. Ibuprofen exposure reduces the turnover of d1EGFP, a model substrate for proteasome.

Few studies suggest that NSAIDs inhibit the NF-κB activation, treatment of ibuprofen inhibits the degradation of IκB-α in PC-3, LNCaP cells, and even in T cells, leading to inhibition of NF-κB activation [Grilli *et al.*, 1996; Kazmi *et al.*, 1995; Kopp and Ghosh, 1994; Palayoor *et al.*, 1999; Scheuren *et al.*, 1998; Yin *et al.*, 1998]. In the current study, similar effects of ibuprofen treatment on the accumulation of IκB-α, and downregulation NF-κB-dependent transcriptional activity in cells were observed. Previous reports have demonstrated that NSAID treatment modulates nuclear translocation of NF-κB and also leads to accumulation of pro-apoptotic proteins like Bax, p21waf1/Cip1, and p27kip1 and promotes cell cycle arrest, which may cause apoptosis in cells [Bock *et al.*, 2007; Marra *et al.*, 2000; Scheuren *et al.*, 1998; Zhou *et al.*, 2001]. Here, it has also been shown that treatment of ibuprofen leads to accumulation or mislocalization of few pro-apoptotic proteins (Bax, p53, p27kip1, and p21) from their native cellular compartments. In rat, exposure of NSAIDs in A10 abnormal vascular smooth muscle cell induces the levels of cyclin-dependent kinase inhibitors p21waf1/Cip1 and p27kip1 [Brooks *et al.*, 2003]. Apoptotic effects of ibuprofen were observed with DAPI nuclear staining and cytofluorimetric dot plot analysis of annexin V versus propidium iodide staining in cells.

4.3 CONCLUDING REMARKS

A recent finding has suggested that in metabolically compromised microenvironments, induction of mitochondrial dysfunction can be a possible way to target tumor cells for better cancer treatment [Zhang *et al.*, 2014]. Mitochondrial membrane depolarization and deregulated permeability leading to the release of cytochrome *c* into the cytosol were also observed after ibuprofen exposure suggesting a compromised mitochondrial health [Al-Nasser, 2000; Endo *et al.*, 2014]. The present observations are consistent with earlier described studies, which indicate that ibuprofen posses an anti-tumorigenic therapeutic potential via induction of cell death program in various tumor cells. Results presented in this study also suggest that ibuprofen, most likely, alters proteasomal function and triggers apoptosis in cells due to defective mitochondrial permeability. These results could be helpful in the designing new strategies to encourage further research efforts to aid in the development of therapeutic concepts and possible involvements of ibuprofen in various diseases.

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