Ibuprofen Induces Mitochondrial-Mediated Apoptosis Through Proteasomal Dysfunction

Arun Upadhyay[1] · Ayeman Amanullah[1] · Deepak Chhangani[1] · Vibhuti Joshi[1] ·
Ribhav Mishra[1] · Amit Mishra[1]

Abstract In routine course of life, nonsteroidal anti-inflammatory drugs (NSAIDs) are widely prescribed antipyretic, analgesic, and anti-inflammatory drugs. It is a well-proposed notion that treatment of NSAIDs may induce antiproliferative effects in numerous cancer cells. Ibuprofen from isobutylphenylpropanoic acid is NSAID and used to relieve fever, pain, and inflammation. It is also used for juvenile idiopathic arthritis, rheumatoid arthritis, patent ductus arteriosus, and for pericarditis. Despite few emerging studies have expanded the fundamental concept that the treatment of NSAIDs influences apoptosis in cancer cells, however the NSAID-mediated precise mechanisms that determine apoptosis induction without producing adverse consequences in variety of cancer cells are largely unknown. In our present study, we have observed that ibuprofen reduces proteasome activity, enhances the aggregation of ubiquitylated abnormal proteins, and also elevates the accumulation of crucial proteasome substrates. Ibuprofen treatment causes mitochondrial abnormalities and releases cytochrome c into cytosol. Perhaps, the more detailed study is needed in the future to elucidate the molecular mechanisms of NSAIDs that can induce apoptosis without adverse effects and produce effective anti-tumor effects and consequently help in neurodegeneration and ageing.

Keywords NSAIDs · Ibuprofen · Proteasome ·
Mitochondrial dysfunction · Neurodegeneration · Ageing ·
Apoptosis

✉ Amit Mishra
amit@iitj.ac.in

1 Cellular and Molecular Neurobiology Unit, Indian Institute of Technology Jodhpur, Rajasthan, India 342011

Introduction

Previous studies have demonstrated that use of diclofenac and sulindac sulfide induces apoptosis in human acute myeloid leukemia cells [1]. It has been observed that NSAID-mediated endoplasmic reticulum (ER) and oxidative stress events could activate the intrinsic pathways of pro-apoptosis events [2, 3]. Few other studies have observed that exposure of NSAIDs and acetaminophen inhibits nuclear factor-kappaB (NF-κB activity) [4, 5], deregulates the mitochondrial function including abnormal mitochondrial membrane permeability [6, 7], and disturbs proteasome function that accumulates pro-apoptotic proteasomal substrates [8, 9]; this may further lead to initiation of programmed cell death. It is noteworthy that DNA damage and mitochondrial abnormalities induce apoptosis; ibuprofen treatment also induces DNA fragmentation and promotes apoptosis in cultured neuronal cells [10]. S(+)-ibuprofen treatment increases p53 expression levels which results in inhibition of cell growth and increases the unfolded protein responses in neuroblastoma cell lines [11]. Recently, few studies have shown the effects of ibuprofen on mitochondrial dysfunctions; treatment of ibuprofen induces the loss of inner mitochondrial membrane potential and release of cytochrome c into cytosol [12, 13].

Previous observations indicate that ibuprofen enhances oxidative stress in *Daphnia magna* [14]; use of ibuprofen and other NSAIDs induces ER stress response in cells [3]. Another observation indicates that NSAIDs including ibuprofen treatment lead to accumulation of IκB-α and subsequently inhibit activation of NF-κB [15–17]. Inhibiting the function of proteasomes promotes mitochondrial membrane disruption, release of cytochrome c from mitochondria to cytosol, and induces apoptosis in various cells [18, 19]. However, the detailed molecular mechanisms of ibuprofen-mediated effects on mitochondrial dysfunction, oxidative and ER stress

response, progression of cell cycle inhibition, and apoptosis induction are unknown. Our 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.

Results

Ibuprofen Treatment Causes Accumulation of Ubiquitylated Proteins and Induces Time-Dependent Morphological Apoptotic Changes In our current study, we have tried to define the effects of ibuprofen for its ability to induce apoptosis and also tried to find out how the disturbances of this delicate balance overall affect intracellular protein degradation machinery. We transiently transfected cells with HA-ubiquitin expression construct, and post-transfected cells were treated with varying doses of ibuprofen. Samples were immunoblotted with anti-HA and anti-β-actin antibodies as shown in Fig. 1a. Ibuprofen treatment exhibited accumulation of HA-ubiquitylated protein derivatives. However, the molecular mechanism by which ibuprofen induces apoptosis is not well known. Next, we examined the effect of ibuprofen on cells; in a time-dependent experiment, we observed that ibuprofen treatment induced apoptotic morphological changes in cells such as loss of contact with the neighboring cells, membrane blebbing, and shrinkage of cells as compared to control cells (Fig. 1b–e). Because, in our preliminary results, we observed that treatment of ibuprofen aggravated the aggregation of ubiquitylated protein derivatives in cells, therefore it became important for us to check whether ibuprofen treatment could affect the proteasome function or not. Hence, we exposed cells with ibuprofen and other known proteasome inhibitors and subjected them to chymotrypsin-like protease activity assay of proteasome. As depicted in Fig. 1f, 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 [20]. We, therefore, examined the effects of ibuprofen on cell viability. For these experiments, cells were treated as described in previous experiment and 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed. As shown in Fig. 1g, ibuprofen exposure reduced cell viability and similar patterns were observed for other proteasome inhibitors. Consequently, it was crucial for us 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, we determined the role of antioxidant NAC on ibuprofen-induced cell death. As shown in Fig. 1h, treatment of NAC alleviates ibuprofen-induced cell death monitored by MTT assay.

Treatment of Ibuprofen Disturbs Proteasomal Function and Induces Rapid Accumulation of Misfolded Proteins
Since we have observed that ibuprofen promotes the apoptotic morphological appearance of cells and reduces proteasome activity, we further performed the detailed characterization of ibuprofen treatment on proteasome function. Cells were treated with varying concentrations of ibuprofen, and samples were processed for chymotrypsin-like and post-glutamyl peptide hydrolase-like protease activity assay of the proteasome. Figure 2a, b demonstrates reduced protease activities of proteasome, after ibuprofen treatment in a dose-dependent manner. To further elucidate this experiment, we exposed cells with ibuprofen at different intervals and samples were used for chymotrypsin-like and post-glutamyl peptide hydrolase-like protease activity assay of the proteasome. As shown in Fig. 2c, d, treatment of ibuprofen decreased proteasome activities.

Previously, it has been shown that ibuprofen treatment induces neuronal damages at higher concentration and also enhances bilirubin toxicity in embryonic neuronal cortical cultures [10]. However, the mechanism by which ibuprofen induces toxicity in various cells is not well known. Our previous results also suggest that ibuprofen treatment reduces cell viability. Similarly, another report indicates that ibuprofen and NSAID exposure leads to hepatotoxicity [21, 22]. To understand if ibuprofen-induced malfunction of proteasome contributes in the accumulation of ubiquitylated proteins and generates proteotoxicity in cells, we transiently transfected cells with EGFP-HDQ23 and EGFP-HDQ74 plasmids and then treated them with ibuprofen. As shown in Fig. 3a, ibuprofen-treated cells follow MG132 treatment-like profile of expanded polyglutamine protein aggregate formation in perinuclear region. To further ascertain these results, few similar sets of cells were used for immunoblot analysis by using anti-ubiquitin, anti-green fluorescent protein (GFP), and anti-β-actin antibodies. Exposure of ibuprofen elevated the accumulation of ubiquitylated derivatives of expanded polyglutamine proteins as shown in Fig. 3b. As depicted in Fig. 3c, expression of normal and expanded polyglutamine plasmids was confirmed in the above-described experiment by using immunoblot analysis and blots were probed with GFP antibody.
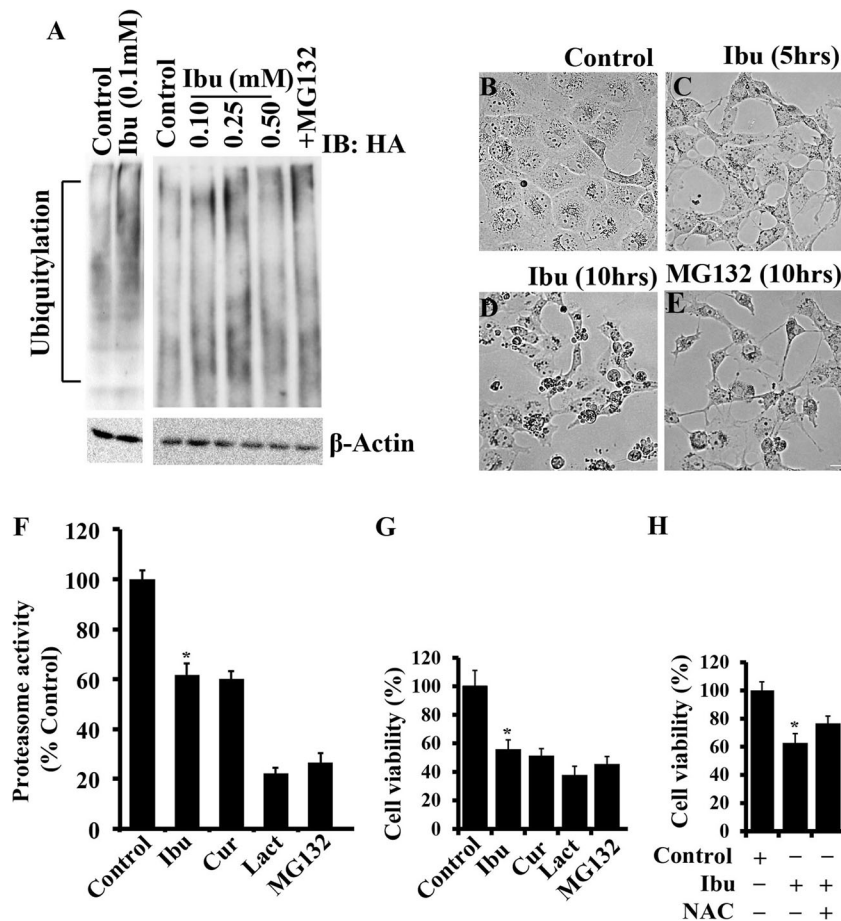
Fig. 1 Ibuprofen treatment causes accumulation of ubiquitinated proteins and disturbs proteasome function. **a** Cells were transiently transfected with HA-ubiquitin expression plasmid. After 24 h of transfection, cells were exposed with 10 μM MG132 and varying doses of ibuprofen, as specified in figure; cell lysates used for immunoblotting by using anti-HA and anti-β-actin antibodies. **b–e** Cos-7 cells were untreated (**b**) or treated with ibuprofen (*Ibu* 5 mM) for different time periods (**c** 5 h and **d** 10 h) and MG132 (10 μM) (**e**). Post-treated cells were observed under bright field microscope as shown in the figure. *Scale bar*, 20 μm. **f** 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 described under "Methods". **g–h** As explained in the previous section, similar sets of treated cells were used for MTT assay to measure cell viability (**g**), 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 (**h**). Values are shown as means±SD from triplicates of three independent experiments. *$p < 0.05$ compared with control

Next, it was important for us 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. 3d, ibuprofen-induced accumulation of higher molecular weight derivatives of ubiquitylated exogenously expressed GFP-ubiquitin proteins; this might be due to malfunction of proteasome. 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. The results in Fig. 3e shows that ibuprofen treatment reduces cell viability as compared to control cells.

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, we performed an in silico study on the basis of our preliminary findings. Interaction of ibuprofen with β1 and β5 subunit 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. 4a–d, docking images depicted hydrogen bonds formed between ibuprofen and proteasome subunits as shown in green lines along with surrounding residues. We have further examined whether ibuprofen treatment affects overall ubiquitylation profile for cellular pool proteins. As demonstrated in the results (Fig. 4e), the exposure of ibuprofen caused a dose-
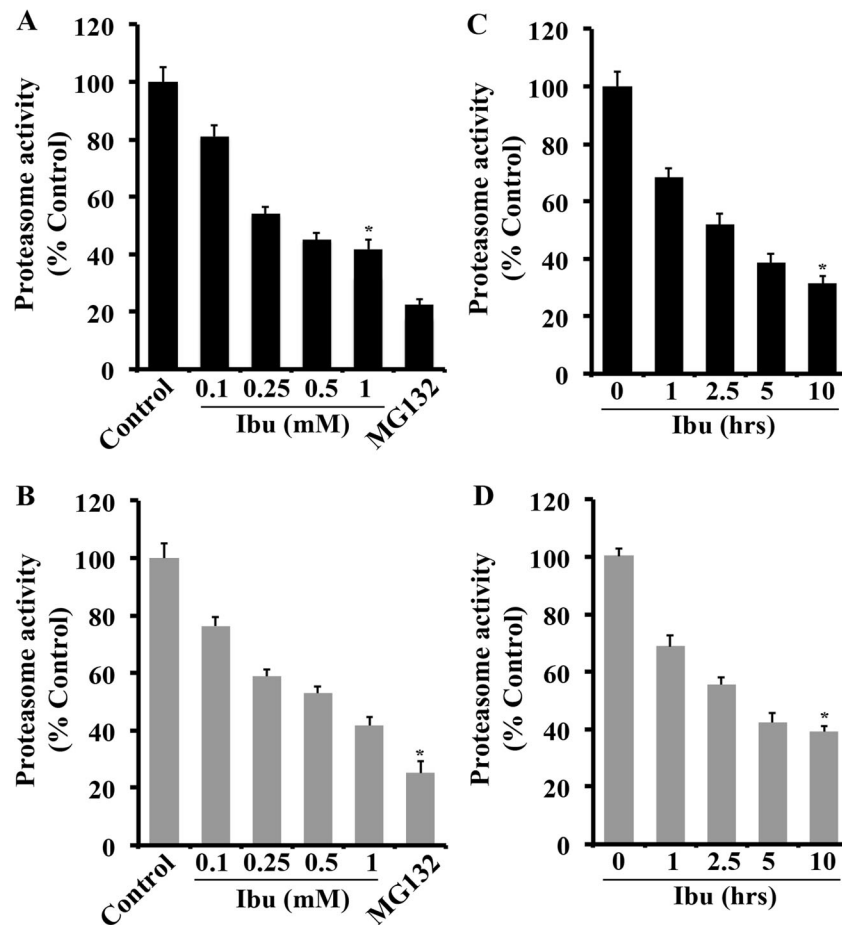
Fig. 2 Exposure of ibuprofen induces loss of proteasome activity. **a–b** A549 cells were treated with varying doses of ibuprofen as represented in the figure, proteasome activity assays (**a** chymotrypsin-like and **b** post-glutamyl peptide hydrolase-like protease activity) in post-treated cells. **c–d** Cells were exposed with ibuprofen (1 mM) at different time intervals as depicted in sections; after treatment, cells were subjected to proteasome activity (**c** chymotrypsin-like and **d** post-glutamyl peptide hydrolase-like protease activity) assays. Values are shown as mean±SD. *Columns*, mean of representative of three independent experiments in triplicate. *$p<0.05$ compared with control

dependent elevated accumulation of ubiquitylated derivatives of different cellular proteins. 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, we treated cells with varying concentrations of ibuprofen and observed under bright field microscope. Interestingly, we have observed noticeable morphological changes in cells, which indicate that cells are more susceptible for apoptosis in the presence of ibuprofen higher dose treatments (Fig. 4f).

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, we used three different kinds of proteins for next course of analysis. Cells were transiently transfected with CFTRΔF508 (A), pd1EGFP (B), and GFP-wtCAT (C) plasmids, and transfected cells were then treated with ibuprofen. Results presented in Fig. 5a–c 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 [23].

However, it was important for us to further test our assumption; hence, we treated cells with ibuprofen in different concentrations as shown in Fig. 5d. After treatment, cells were collected and subjected to immunoblot analysis using anti-IκB-α and anti-β-actin antibodies. Previously, it has been shown that treatment of NSAID-induced proteasome impairment inhibits the activation of NF-κB, which may be due to the accumulation of IκB-α [9, 16]. Similarly, we observed that ibuprofen treatment facilitates the accumulation of IκB-α in cells. We have also observed the downregulation of NF-κB-dependent transcriptional activity (Fig. 5e), probably due to the elevated levels of IκB-α. Surprisingly, ibuprofen-mediated NF-κB inhibition was high as compared to proteasomal
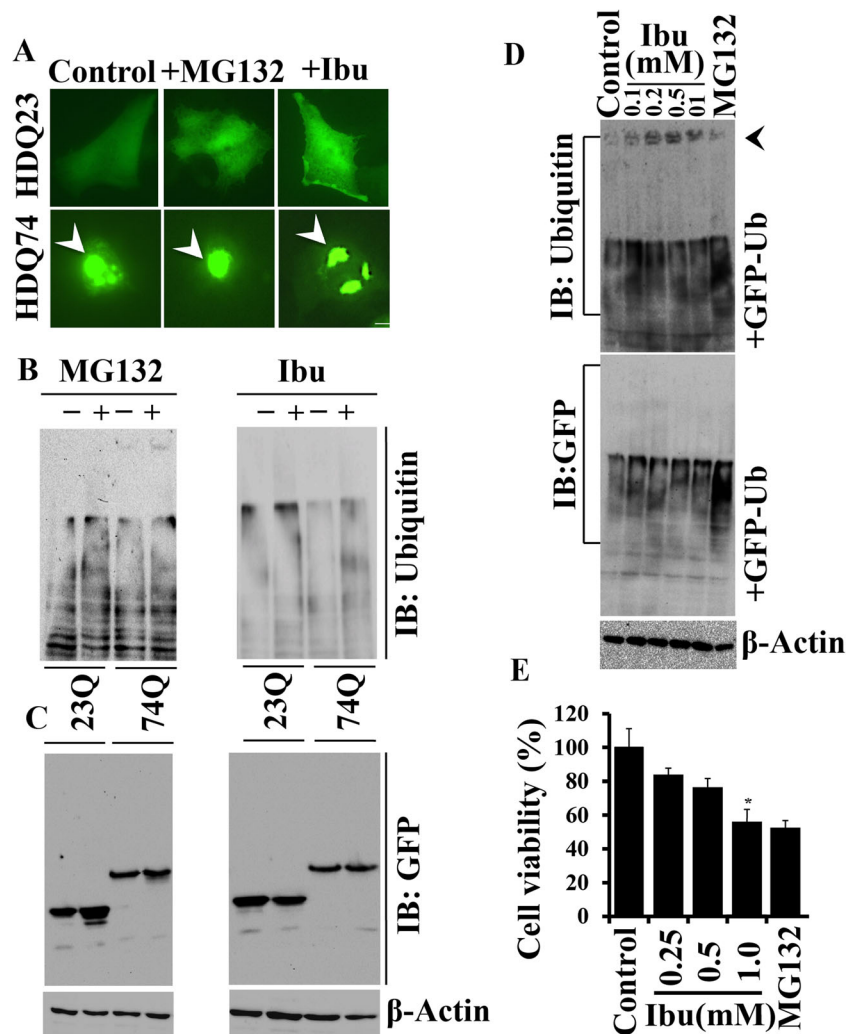
Fig. 3 Ibuprofen treatment leads to stabilization of misfolded and ubiquitinated proteins in cells. **a** A549 cells were transiently transfected with EGFP-HDQ23 and EGFP-HDQ74 plasmids and then treated with ibuprofen (Ibu 0.5 mM) and MG132 (10 μM) for 24 h. Treated cells were observed under fluorescence microscope as shown in section **a**; *arrowheads* indicate aggregate formation. *Scale bar*, 20 μm. **b**–**c** 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 then, lysates were processed for immunoblotting using anti-ubiquitin (**b**), anti-GFP, and anti-β-actin antibodies (**c**). **d** 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. **e** 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

inhibition. Earlier study has also observed that ibuprofen exposure has inhibited the constitutive activation of NF-κB in prostate cancer cells [24].

We used the next experimental paradigm to test 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 h 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. We found that

exposure of ibuprofen increased the half-life of d1EGFP proteins in cells as represented in Fig. 5f, g.

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, like p53, as well as on pro-apoptotic proteins such as Bax and p27[kip1], we treated cells with ibuprofen and MG132; after treatment, cells were subjected to immunocytochemistry by using different antibodies as shown in Fig. 6a–f. 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
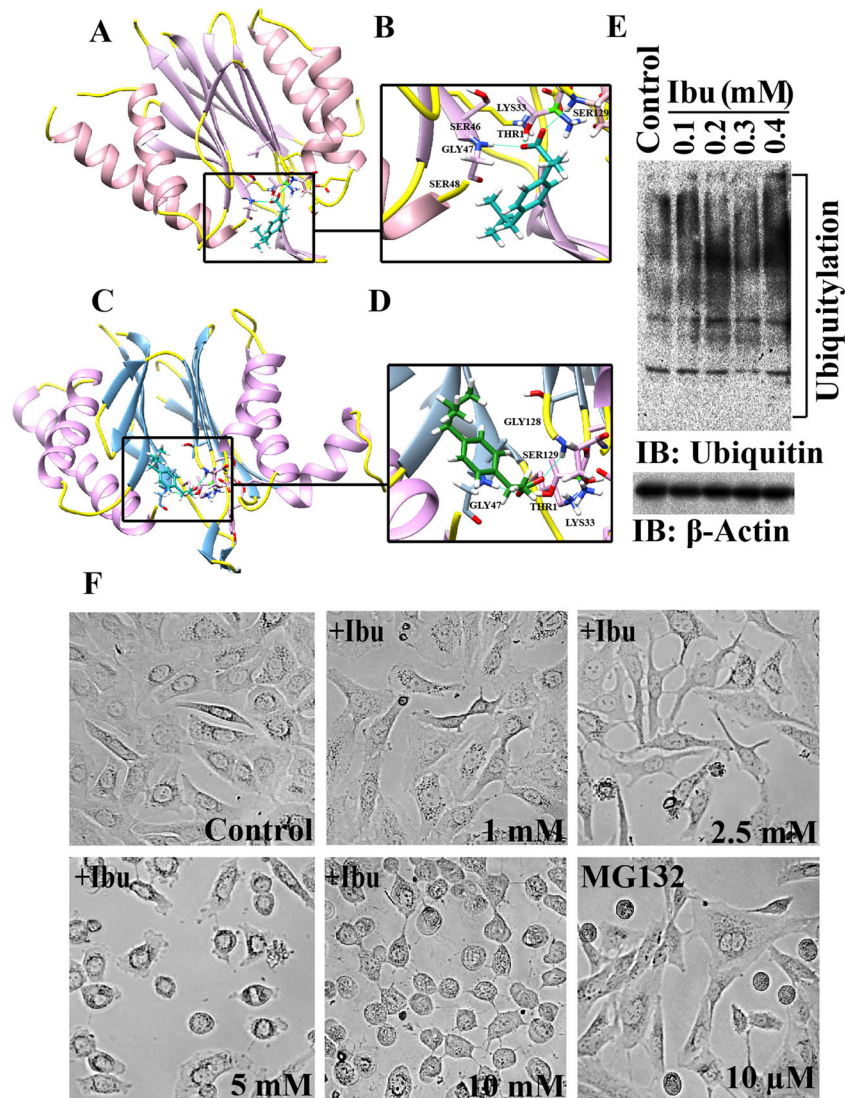
Fig. 4 Ibuprofen represents in silico interaction and proteasome impairment effects. Protein ligand docking was performed using the web interface of Swiss docking server. **a–b** General view of the molecular docking of β1 subunit (PDB 1JD2) with ibuprofen (ZINC ID 2647); close-up view of the protein ligand interface is represented in the *side panel*. **c–d** Molecular docking of β5 subunit with ibuprofen demonstrates hydrogen bonds, which are depicted as *green lines*. These docking models were obtained as described in "Methods". Close-up view of the protein ligand interface is shown in the *side panel*. **e** 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. **f** A549 cells were treated with or without varying concentration of ibuprofen as represented in the figure; post-treated cells were observed under bright field microscope. *Scale bar*, 20 μm

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 [25, 26]. Most likely, accumulation of these ubiquitinated substrates makes a direct consequence on other pro-apoptotic and apoptosis-linked proteins.

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. 7a, ibuprofen-treated cells exhibited cytoplasmic shrinkage; apoptotic nuclei stained with DAPI became progressively pyknotic and were extensively fragmented compared to untreated cells. To further understand and ascertain the underlying mechanism by which ibuprofen induces apoptosis in cells, we next seeded cells in tissue culture plates and treated them 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. 7b).
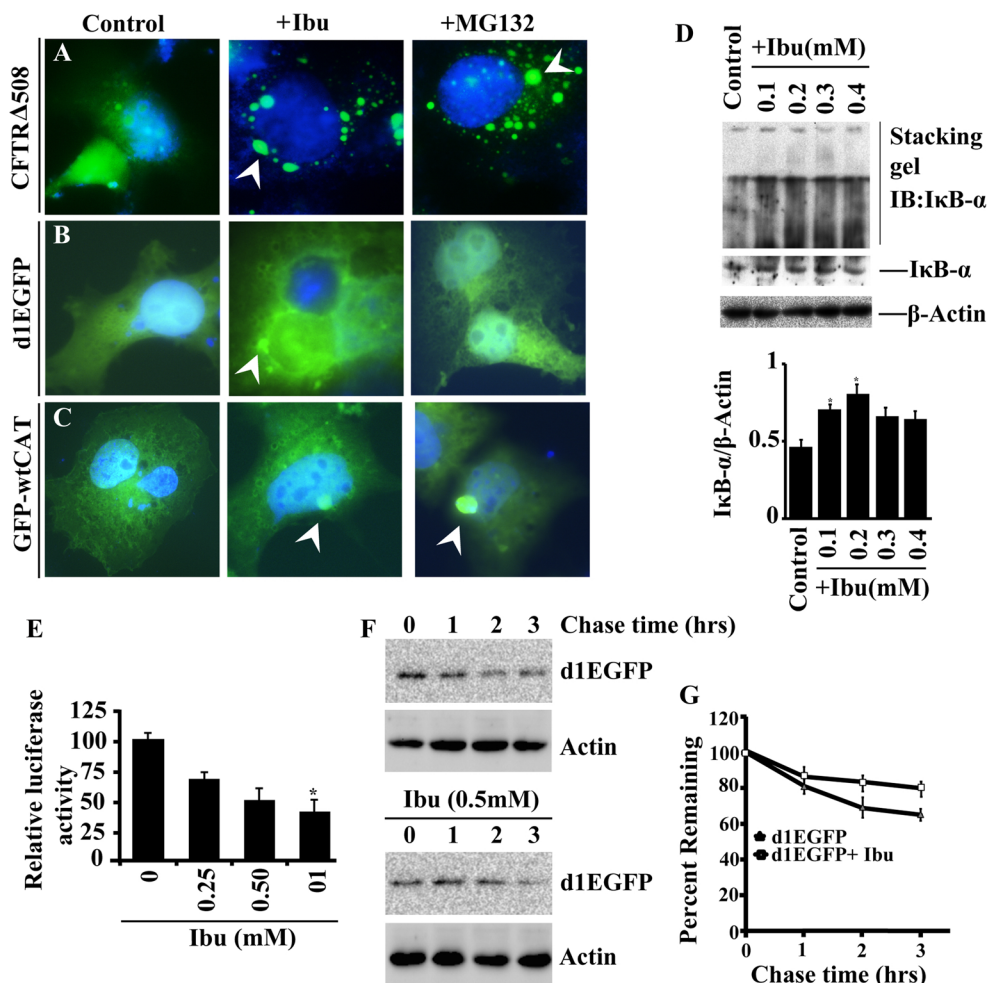
Fig. 5 Formation of aggresomes of misfolded proteins and stabilization of IκB-α in response to ibuprofen treatment in cells. **a–c** Cos-7 cells were transiently transfected with CFTRΔF508 (**a**), pd1EGFP (**b**), and GFP-wtCAT (**c**) as represented in the figure. After transfection, cells were exposed 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. **d** A549 cells treated with various concentrations of ibuprofen and MG132; cell lysates were prepared and used for immunoblotting with IκB-α and β-actin antibodies. **e** Cells were transiently transfected with

NF-κB luciferase and pRL-SV40 constructs, and after 24 h of transfection, cells were treated with different doses of ibuprofen, and then, luciferase activity assay was performed as described in "Methods". **f** Cells were transiently transfected with pd1EGFP plasmid, and after 24 h of transfection, cells were treated with 0.5 mM ibuprofen and chased in the presence of cycloheximide (15 μg/ml). **g** Protein d1EGFP levels were quantified from three independent chase experiments. β-actin was used for normalization, and values are represented as mean±SD of three independent experiments, performed in triplicates

Quantification of FACS results represents that the apoptotic cell fraction was increased after the treatment of ibuprofen in cells compared to control cells (Fig. 7c).

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, we treated cells with ibuprofen and performed agarose gel electrophoresis, as shown in Fig. 7d. Ibuprofen-exposed samples demonstrated the oligonucleosomal laddering pattern linked with apoptotic cells. To substantiate our 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 7e 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 [3, 27].

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 [7, 28–30]. Even treatment of ibuprofen also disturbs
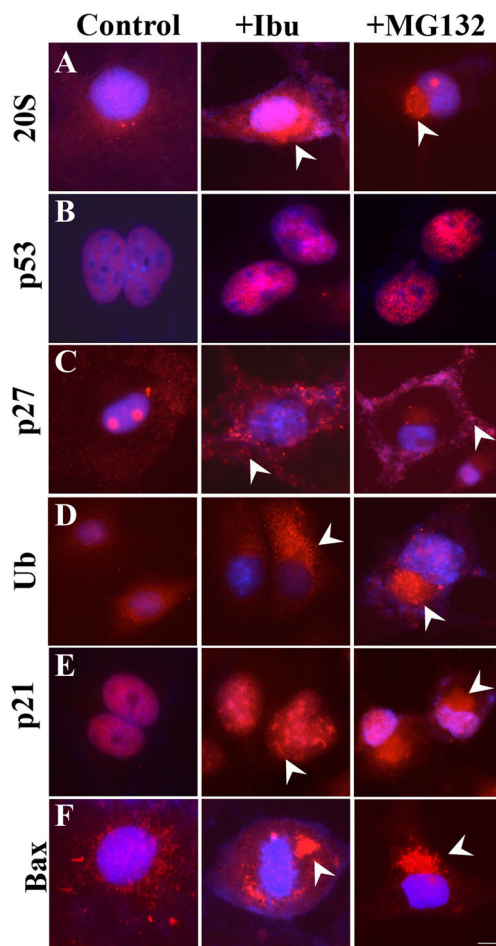
Fig. 6 Treatment of ibuprofen accelerates the accumulation of pro-apoptotic proteasome target proteins. Cos-7 cells were treated with ibuprofen (Ibu 1 mM) and MG132 (10 μM). After treatment, cells were processed for immunofluorescence analysis and were probed with 20S (**a**), p53 (**b**), p27 (**c**), Ub (**d**), p21 (**e**), and Bax (**f**) 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

fluorescence dye. 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. 8f). Micrographs (Fig. 8g–j) represent that ibuprofen treatment reduces mitochondrial membrane potential in a dose-dependent manner as depicted from the change in fluorescence emission from red mitochondrial staining to green appearance.
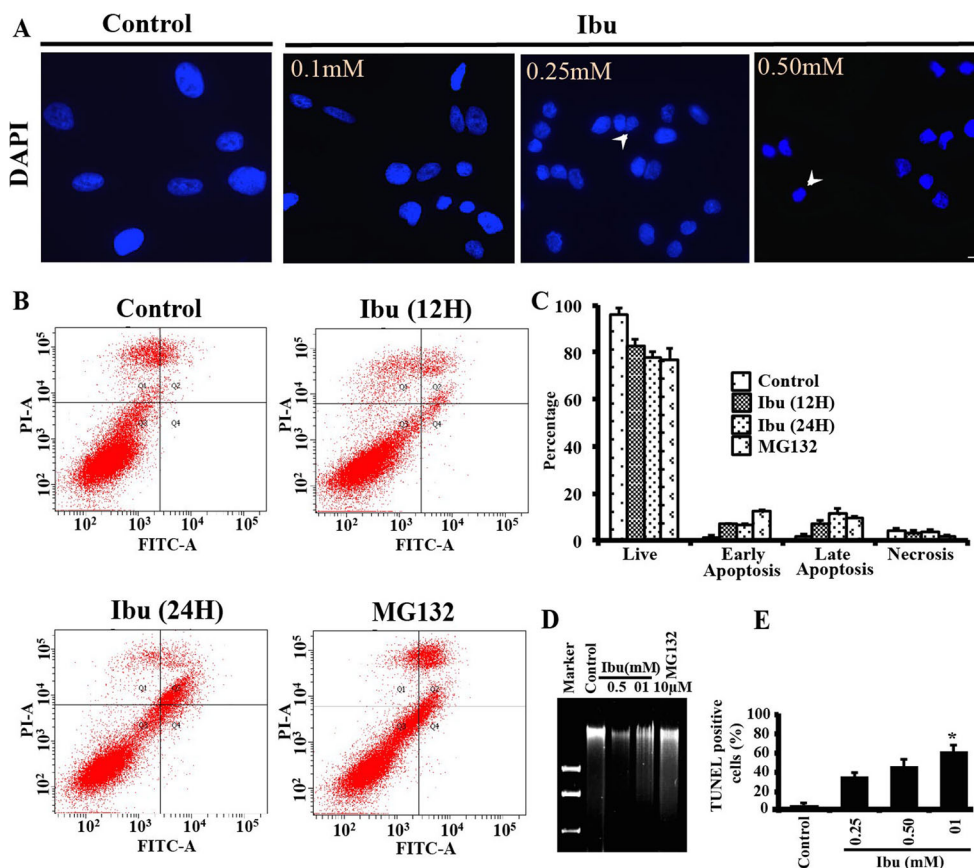
Discussion

We noticed that treatment of ibuprofen induces apoptosis, most likely due to proteasome malfunction mediated by mitochondrial abnormalities. Treatment of ibuprofen disturbed proteasome function and induced morphological apoptotic changes in a time-dependent manner. Ibuprofen-mediated reduced cell viability was recovered by the use of antioxidant NAC. In our current study, we observed that ibuprofen treatment in both time as well as in concentration-dependent manner promotes suppression of proteasome function in cells. Interestingly, our findings suggest that ibuprofen treatment induces aggregation of expanded polyglutamine proteins and their inclusions in nuclear peripheral region. Ibuprofen treatment also increases the accumulated levels of ubiquitylated derivatives of polyglutamine expansion proteins; similar effects were observed in cells when treated with MG132, an established proteasome inhibitor.

To further examine whether ibuprofen-induced proteasome dysfunction is involved in apoptosis, we demonstrated that concentration-dependent treatments of ibuprofen promotes the accumulation of ubiquitylated GFP protein. Simultaneously, we monitored reduced cell viability in a time-dependent manner with the exposure of ibuprofen in cells. Several studies have shown that NSAID treatment causes impaired proteasome function and generates stress events in cells [2, 31, 32]. In our present study, we demonstrated a possible interaction of ibuprofen with β1 and β5 subunits of proteasome by performing in silico docking analysis. In addition, our results suggest that dose-dependent exposure of ibuprofen accumulates ubiquitylated proteins in cells, which further develops apoptotic morphological changes in cells comparable to that of MG132, a putative proteasome inhibitor.

Emerging evidence demonstrates that treatment of NSAIDs leads to various stresses such as oxidative and ER stress [3, 11, 14, 31, 33]. It has been shown that celecoxib, an NSAID, upregulated ER chaperones in human gastric cells. But at present, the molecular basis for this inhibitory action of NSAIDs on proteasome is largely unknown and the mechanism behind upregulation of ER chaperons with use of NSAIDs remains obscure. We, therefore, further performed

mitochondrial permeability [12]. But still, the molecular mechanism by which ibuprofen treatment deregulates mitochondrial functions and induces apoptosis is not clear. Therefore, in our current study, it was important for us 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, we treated cells with different doses of ibuprofen and treated cells were used for immunofluorescence staining (Fig. 8a–d) and immunoblotting (Fig. 8e) of cytochrome c. Ibuprofen treatment released cytochrome c into the cytosol from mitochondria, as observed by immunofluorescence analysis and western blotting. Next, to determine whether ibuprofen treatment disturbs the mitochondrial membrane potential, we used 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide (JC-1)

Fig. 7 Ibuprofen treatment induces apoptosis and causes nuclear morphological changes (condensation, marginalization, or fragmentation). **a** Cos-7 cells were exposed with different concentrations of ibuprofen and MG132. Nuclear morphology of cells was observed with DAPI. *Scale bar*, 20 μm. **b–c** Time-dependent assessment of apoptosis by flow cytometry analysis using annexin V-FITC and propidium iodide double staining. A549 cells were treated with ibuprofen (1 mM) for 12 and 24 h, and MG132 (10 μM) treated cells were used as positive control (**b**); values are mean±standard deviation of three independent experiments, as shown using bar graph (**c**). **d** Representation of DNA fragmentation by agarose gel analysis after ibuprofen and MG132 treatment in cells. **e** Detection of apoptosis in ibuprofen- and MG132-treated cells by TUNEL assay; quantification values are presented as mean±SD of three independent experiments. *$p < 0.05$ compared with control

experiments in the presence of various aggregation-prone proteins (CFTRΔF508, pd1EGFP, and GFP-wtCAT). Since expression of these proteins is confirmed by fluorescence microscopy analysis, it was observed 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. Ibuprofen treatment also increases the accumulation of IκB-α, another cellular substrate of proteasome. In addition, our results suggest that treatment of ibuprofen downregulates NF-κB-dependent transcriptional activity in cells. Few studies suggest that NSAIDs inhibit the NF-κB activation, treatment of ibuprofen inhibits the degradation of IκB-α in PC-3 and LNCaP cells, and even in T cells, ibuprofen treatment inhibits NF-κB activation [4, 15, 17, 24, 34, 35].

Here, we have shown that treatment of ibuprofen leads to accumulation or mislocalization of few pro-apoptotic proteins (Bax, p53, p27^{kip1}, and p21) from their native cellular

compartments. In rat, A10 abnormal vascular smooth muscle cell (VSMC) exposure of NSAIDs induces the levels of cyclin-dependent kinase (CDK) inhibitors p21$^{waf1/Cip1}$ and p27^{kip1} [36]. Previous reports have demonstrated that NSAID treatment modulates nuclear translocation of NF-κB and also leads to accumulation of pro-apoptotic proteins like Bax, p21$^{waf1/Cip1}$, and p27^{kip1} and promotes cell cycle arrest, which may cause apoptosis in cells [17, 37–39]. Effect of ibuprofen was observed with DAPI nuclear staining and cytofluorimetric dot plot analysis of annexin V versus propidium iodide staining in cells. Our results suggest that ibuprofen affects cell viability and induces apoptosis in cells, which is also being confirmed by nuclear condensation, DNA fragmentation, and TUNEL assay analysis. Mitochondrial membrane depolarization and deregulated permeability was observed after ibuprofen exposure, which also represents the loss of inner mitochondrial membrane potential [12, 40].

In our current study, we observed that treatment of ibuprofen produces loss of mitochondrial membrane
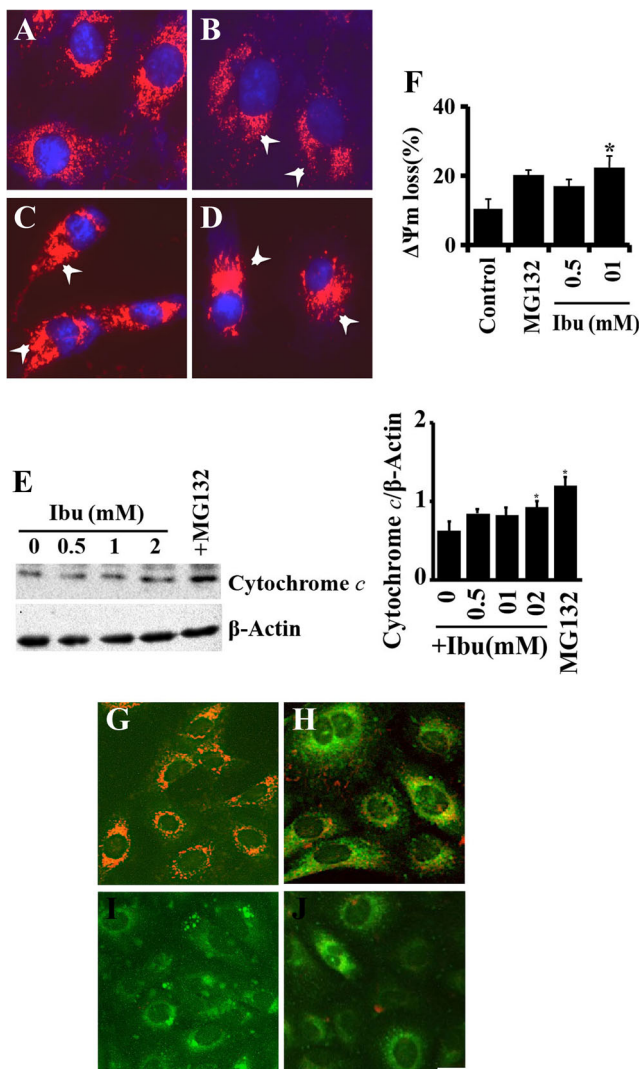
Fig. 8 Ibuprofen treatment induces cytochrome c release and mitochondrial depolarization during apoptosis. **a–d** Cos-7 cells were plated into 2-well chamber slides, and cells were left untreated (**a**) or treated with different concentrations of ibuprofen (**b** Ibu 0.5 mM and **c** Ibu 1 mM) and **d** MG132 (10 μM). After treatment, cells were used for immunofluorescence analysis using cytochrome c antibody. **e** Cells were treated with various concentrations of ibuprofen and MG132 (10 μM) and collected after treatment; cell lysates were used for immunoblot analysis using cytochrome c and β-actin antibodies. **f** Cells were treated with different doses of ibuprofen and stained with JC-1 and analyzed by flow cytometry. Values are the mean±SD of three independent experiments, each performed in triplicate. *$p<0.05$ compared with the control. **g–j** Cells were seeded for JC-1 staining and analyzed by fluorescence analysis. Mitochondrial depolarization is indicated with reduction in red fluorescence. Cells treated with ibuprofen (**g** control, **h** Ibu 1 mM, and **i** 2.5 mM) and **j** MG132 (10 μM)

potential and causes release cytochrome c into cytosol, which was confirmed by the use of JC-1, a voltage-sensitive fluorescence dye. Interestingly, 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 [41]. Our 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. Indomethacin treatment causes gastric mucosal injury due to oxidative stress and epithelial cell apoptosis, which further induces gastropathy [42]. Idiosyncratic NSAID drug leads to oxidative stress, and treatment of NSAIDs also promotes small bowel injury and mitochondrial dysfunctions [6, 31, 43]. Another study has demonstrated that ibuprofen treatment in mice exhibits severe adverse effects in a murine prion model [44]. However, the detailed molecular pathomechanism of ibuprofen-induced adverse effects in prion disease mice model is not clear. Together, above-described studies and our present observation suggests that ibuprofen, most likely, alters proteasomal function and triggers apoptosis in cells due to defective mitochondrial permeability. Our results may be helpful in designing of new strategies to encourage further research efforts to aid in the development of therapeutic concept and effect of ibuprofen in diseases.

Materials and Methods

Materials

Cycloheximide, MG132, NAC, lactacystin, curcumin, ibuprofen, MTT, anti-ubiquitin, and all cell culture reagents were purchased from Sigma. Anti-GFP was obtained from Roche Applied Science. Anti-HA was purchased from Thermo Fisher Scientific. Anti-p21, anti-p53, anti-p27, anti-ubiquitin, anti-IκB, anti-20S proteasome, anti-cytochrome c, anti-β-actin, and anti-Bax were purchased from Santa Cruz Biotechnology. Anti-rabbit and anti-mouse (IgG-fluorescein isothiocyanate and IgG-rhodamine), horseradish peroxidase-conjugated anti-mouse, anti-rabbit-IgG, were purchased from Vector Laboratories. 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 respectively. Proteasome-Glo™, TUNEL Assay Kit, and Dual Luciferase Reporter Gene Assay Kit were purchased from Promega. Plasmid pcDNA™ 3.1 was obtained from Life Technologies. pRK5-HA-ubiquitin-WT (Addgene plasmid 17608), Luciferase-pcDNA3 (Addgene plasmid 18964), plasmids pEGFP-Hsp70 (Addgene plasmid 15215), GFP-Ub (Addgene plasmid 11928), and pcDNA3-EGFP (Addgene 13031) were purchased from Addgene.

Primary and Secondary Antibodies

The green fluorescent protein (GFP-11814460001) antibody was purchased from *Roche Applied Science*. The anti-ubiquitin (sc-58448), anti-GFP (sc-8334), ubiquitin (sc-9133), β-actin (sc-81178), IκB-α (sc-847), p53 (sc-6243), p27 (sc-528), p21 (sc-726), Bax (sc-493), cytochrome c (sc-13561), and 20S proteasome α-1 (sc-166073) were purchased from *Santa Cruz Biotechnology, Inc.* (*Santa Cruz, CA, USA*). Anti-HA (H6908) and IκB-α (SAB4501995) were obtained from *Sigma-Aldrich Co. LLC*. Polyclonal anti-ubiquitin (Z0458) was purchased from *Dako* (*Glostrup, Denmark*). Anti-mouse IgG-fluorescein isothiocyanate (FITC) (FI-2000) (IgG-FITC) 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 IgGs (PI-9500) were purchased from *Vector Laboratories, Inc.* (*Burlingame, CA, USA*). The Zenon® Secondary Detection-Based Zenon® Alexa Fluor® 350 Mouse IgG$_1$ Labeling Kit (Z-25000) was obtained from *Life Technologies Corporation*.

Cell Culture, Transfection, Treatment, Reporter Gene Assay, and Cell Viability Assay

A549 and Cos-7 cells were grown in Dulbecco's modified Eagle's medium (Life Technologies, Gaithersburg, MD, USA) containing 10 % fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were seeded into different tissue culture plates for various transfection and treatment experiments and, on subsequent day at 60–70 % confluence, were transfected using Lipofectamine® 2000 reagent according to manufacturer's instructions. Cells were treated with different concentrations as well as various time periods with ibuprofen and other known proteasome inhibitors, and few sets of similar cells were transiently transfected with NF-κB luciferase and pRL-SV40 plasmids. Plasmid transfections were performed by Lipofectamine® 2000 reagent. After treatment, cell lysates were prepared and used for luciferase activity assay, which was carried out according to the manufacturer's protocol (Dual Luciferase Reporter Gene Assay Kit). Renilla expression vector was used as an internal control to normalize the data. Data were represented as relative luciferase activity, which was calculated by taking ratio of firefly to renilla values. NF-κB activity was reflected by firefly luciferase activity. Luciferase activity was measured using Luminometer. For cell viability experiments, cells were seeded into 96-well plates and were treated with ibuprofen and MG132 with different doses, and these cells were used for cell viability assay, medium was replaced, and cells were exposed with ibuprofen alone or along with NAC. Cell viability was determined by MTT assay. Statistical analysis was performed using the Student's *t* test, and $p < 0.05$ was considered to represent statistical significance.

Immunoblotting and Cycloheximide Chase Experiment

The Cos-7 cells were transiently transfected with an appropriate plasmid, and few similar sets of samples were treated with or without ibuprofen and MG132. Twenty-four hours' post-transfection or applicable treatment hours, the cells were collected and lysates were used to separate them through sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. Blocking buffer (5 % skim milk in Tris-buffered saline and 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, blots were probed with appropriate primary antibody diluted (1:1000) in TBST for overnight incubation at 4 °C. After several washings with TBST buffer, appropriate horseradish peroxidase-conjugated secondary antibody was applied to detect the blots by using Luminata Crescendo Western horseradish peroxidase (HRP) substrate (EMD Millipore) [45]. For chase experiments, Cos-7 cells were transiently transfected with pd1EGFP plasmid, and after 24 h of transfection, cells were exposed with ibuprofen and treated with cycloheximide (15 μg/ml) at different time intervals. After treatment, cells were washed twice with phosphate-buffered saline (PBS), and prepared cell lysates were subjected to immunoblotting analysis by using anti-GFP and anti-β-actin antibodies.

Immunofluorescence Technique and Proteasome Assay

Cos-7 cells were seeded into 2-well chamber slides and cultured for 24 h where represented cells were transiently transfected with an appropriate plasmid and few sets of cells were treated with different doses of ibuprofen and MG132. After treatment, cells were washed twice with PBS, fixed with 4 % paraformaldehyde in PBS for 20 min, and permeabilized with 0.5 % Triton X-100 in PBS for 5 min. The cells were then blocked with using 5 % nonfat-dried milk in TBST for 60 min. Cells were then immunolabeled with an appropriate primary antibody that was diluted (1:1000) in TBST for overnight incubation at 4 °C followed by with suitable fluorescein isothiocyanate-conjugated/rhodamine-conjugated secondary antibody (1:1000 dilutions) for 1 h [46]. Coverslips were mounted on glass slides using antifade solution with or without DAPI. For 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-Glo™ systems (Promega), according to manufacturer's instructions.

Docking Studies and TUNEL Assay

Molecular docking calculations were performed with default parameters using SwissDock webserver [47]. To determine structural visualization of favorable binding modes and hydrogen bond calculation, UCSF Chimera was utilized in current study [48]. Structure of ibuprofen was obtained by ZINC database (code 2647) [49] and yeast 20S proteasome from Protein Data Bank (PDB 1JD2) [50]. 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 instructions and explained elsewhere [51].

Flow Cytometry Analysis for Apoptosis and Measurement of Mitochondrial Membrane Potential

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 Pharmingen™, 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). To measure alterations in mitochondrial membrane potential, the cationic fluorescent dye, JC-1, was used. Where represented cells were exposed with different concentrations of ibuprofen and MG132 and after treatment, cells were labeled with the JC-1 reagent for staining at 20 min in CO_2 incubator at 37 °C. After washing, fluorescence was observed under fluorescence microscope. 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.

Compliance with Ethical Standards

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