3
Diclofenac Induces Proteasomal Dysfunction and Engenders Apoptosis Through Mitochondrial Pathway

The 26S proteasome is a multi-subunit intracellular proteolytic machinery, which eliminates non-native proteins and prevents cell death by the clearance of multifactorial cytotoxic load of protein aggregation [Nandi *et al.*, 2006; Voges *et al.*, 1999]. Interestingly, it has been shown that inhibition of proteasome function can be useful strategy in cancer therapy [Moreau *et al.*, 2012; Orlowski and Kuhn, 2008; Rastogi and Mishra, 2012]. NSAIDs chemopreventive action has been recently shown in the suppression of tumor formation and cancer prevention; this effect of NSAIDs could be due to induction of apoptosis [Gurpinar *et al.*, 2013; Leibowitz *et al.*, 2014; Liggett *et al.*, 2014]. However, the mechanism by which NSAIDs induce apoptosis in different cancers and inhibit tumorigenesis is not well understood. Diclofenac is one of the commonly prescribed medications for pain, fever and inflammation. Its name is derived from its chemical name 2-(2,6-dichloranilino) phenylacetic acid and was initially discovered in 1973. The anti-inflammatory action of diclofenac involves both inhibition of COX-1 and COX-2 enzymes affecting prostaglandins synthesis. Diclofenac has also been found to induce apoptosis and several mechanisms have been proposed for diclofenac mediated apoptosis, such as ER stress, oxidative stress and mitochondrial abnormalities [Albano *et al.*, 2013; Franceschelli *et al.*, 2011; Hickey *et al.*, 2001]. In the present study, the effect of Diclofenac on proteasome activity has been characterized, as inhibition of proteasome can also lead to apoptosis. Following section shows the results obtained through various experiments conducted.

3.1 RESULTS

3.1.1 Diclofenac Treatment Causes Stabilization of Ubiquitylated Misfolded or Abnormal Proteins

Despite previous studies indicated important roles of NSAIDs in oxidative [Adachi *et al.*, 2007] and ER stress [Tsutsumi *et al.*, 2004] responses, the mechanism of NSAIDs or diclofenac mediated cell death program is not well understood. Interestingly, studies in past have found that functional impairment of proteasome leads to stress responses in cells [Bush *et al.*, 1997; Chhangani and Mishra, 2013; Mazroui *et al.*, 2007]. Therefore, to explore the possible effect of diclofenac on proteasomal function, Cos-7 cells were treated with diclofenac in concentration dependent manner (skeletal formula Figure 3.1a), and immunoblot analysis was done to check the accumulation of ubiquitylated proteins. As shown in Figure 3.1b and Figure 3.1c, enhanced buildup of cellular ubiquitylated protein derivatives occurred, a possible characteristic of proteasomal malfunction. The results indicated that diclofenac might disturb proteasome function; therefore, to further extend this observation, EGFP-HDQ23 and EGFP-HDQ74 plasmids transient transfection was done in Cos-7 cells and then treated with diclofenac and MG132. Cell lysates were immunoblotted to confirm the expression of polyglutamine constructs in cells as indicated in Figure 3.2a. Similar sets of diclofenac and MG132-treated samples indicated increased accumulation of ubiquitylated higher molecular weight derivatives of polyglutamine proteins in the stacking gel of immunoblots as shown in Figure 3.2b.

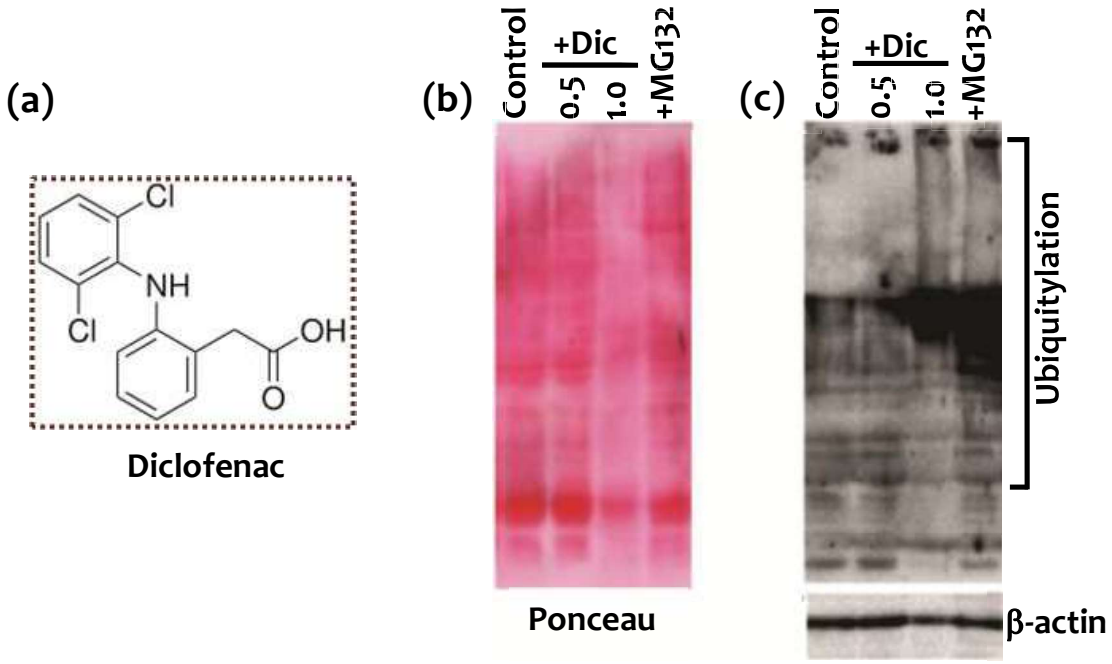

Figure 3.1 : *Diclofenac exposure leads to enhanced buildup of ubiquitylated proteins:* As depicted in section, Cos-7 cells were treated with various concentrations of diclofenac, skeletal formula (a), for 6 hrs and MG132 (10 μM) and cell lysates were made and processed for ponceau (b), immunoblots were probed with anti-ubiquitin and anti-β-actin antibodies (c)

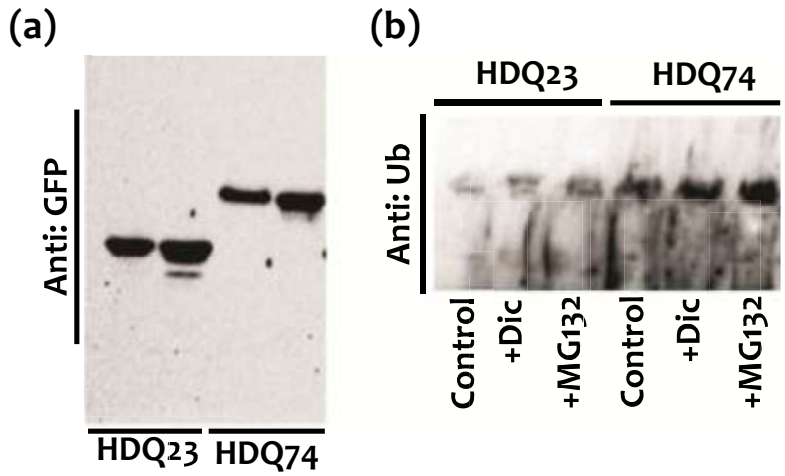

Figure 3.2 : *Diclofenac causes increased accumulation of ubiquitinated polyglutamine proteins:* (a) Some sets of Cos-7 cells were used for EGFP-HDQ23 and EGFP-HDQ74 plasmids transient transfection and were processed for immunoblotting; blots were developed with anti-GFP antibody. (b) Few different sets of cells transfected with EGFP-HDQ23 and EGFP-HDQ74 were left with or without treatment of Dic (0.5 mM) and MG132 (10 μM). Cell lysates were made to detect higher molecular weight derivatives for which stacking gel was subjected to immunoblotting using anti-ubiquitin antibody.

Next, to further confirm diclofenac mediated effects on proteasome, accumulation of ubiquitylated proteins around microtubule organizing center (MTOC) was checked. As shown in Figure 3.3, in double immunofluorescence labeling analysis ubiquitin positive-aggresomes formation around MTOC were observed after diclofenac and MG132 treatment. In this experiment, γ-tubulin was used as a marker for MTOC, where the bulk of cytoplasmic abnormal proteins are eliminated. The nocodazole treatment which inhibits the formation of aggresomes around MTOC, strengthened the observed outcomes of diclofenac and MG132 treatment on proteasome dysfunction and aggresomes formation in cells.

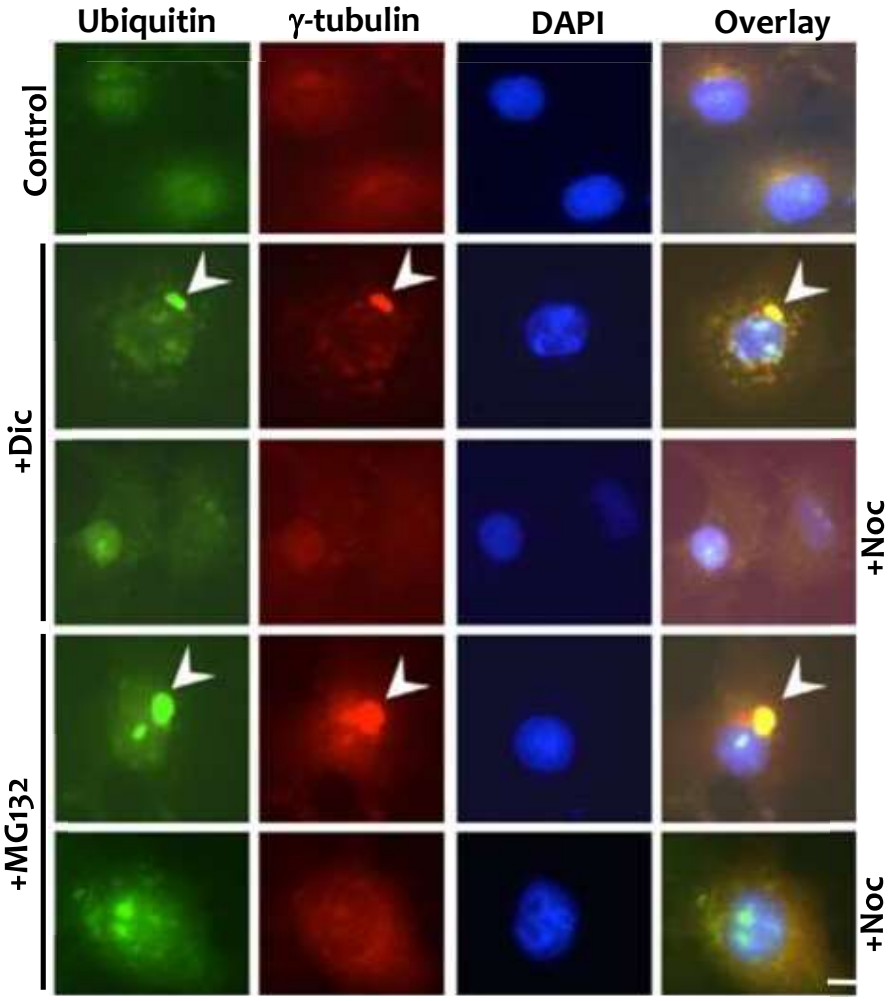

Figure 3.3 : *Diclofenac treatment leads to accumulation of ubiquitylated proteins around MTOC region:* Two well chamber slides seeded with Cos-7 cells were treated with Dic (0.5 mM) and MG132 (10 μM) and post treated cells were used double labeled immunofluorescence analysis with anti-ubiquitin (green) and anti-γ-tubulin (red) antibody, few sets of cells were treated with 10 μM of nocodazole (Noc) in the absence or presence of Dic and MG132. Fluorescein isothiocyanate-conjugated secondary antibody was used to label ubiquitin and rhodamine-conjugated secondary antibody was used to stain γ-tubulin. Nuclear staining was performed by using 4',6-diamidino-2-phenylindole (DAPI). The arrow heads represent redistribution and accumulation of various protein, Scale bar 20μm.

3.1.2 Diclofenac Disturbs Proteasome Function and Sensitizes Cells towards Apoptosis

As shown in previous experiments, that diclofenac treatment stabilizes ubiquitylated proteins, therefore interaction of diclofenac with proteasome subunits were predicted using *in silico* approach using Swissdock web server, as described in methods section, which is a freely available protein ligand docking web interface. Full Fitness of 1033.69 and 1224.64 along with

8.22 and 7.70 kcal/mol docked free energy with β1 and β5 subunits were observed at active sites respectively. In docking images surrounding and interacting residues of β1 and β5 subunit with diclofenac are represented and green lines are used to represent hydrogen bonds as shown in Figure 3.4a and Figure 3.4b.

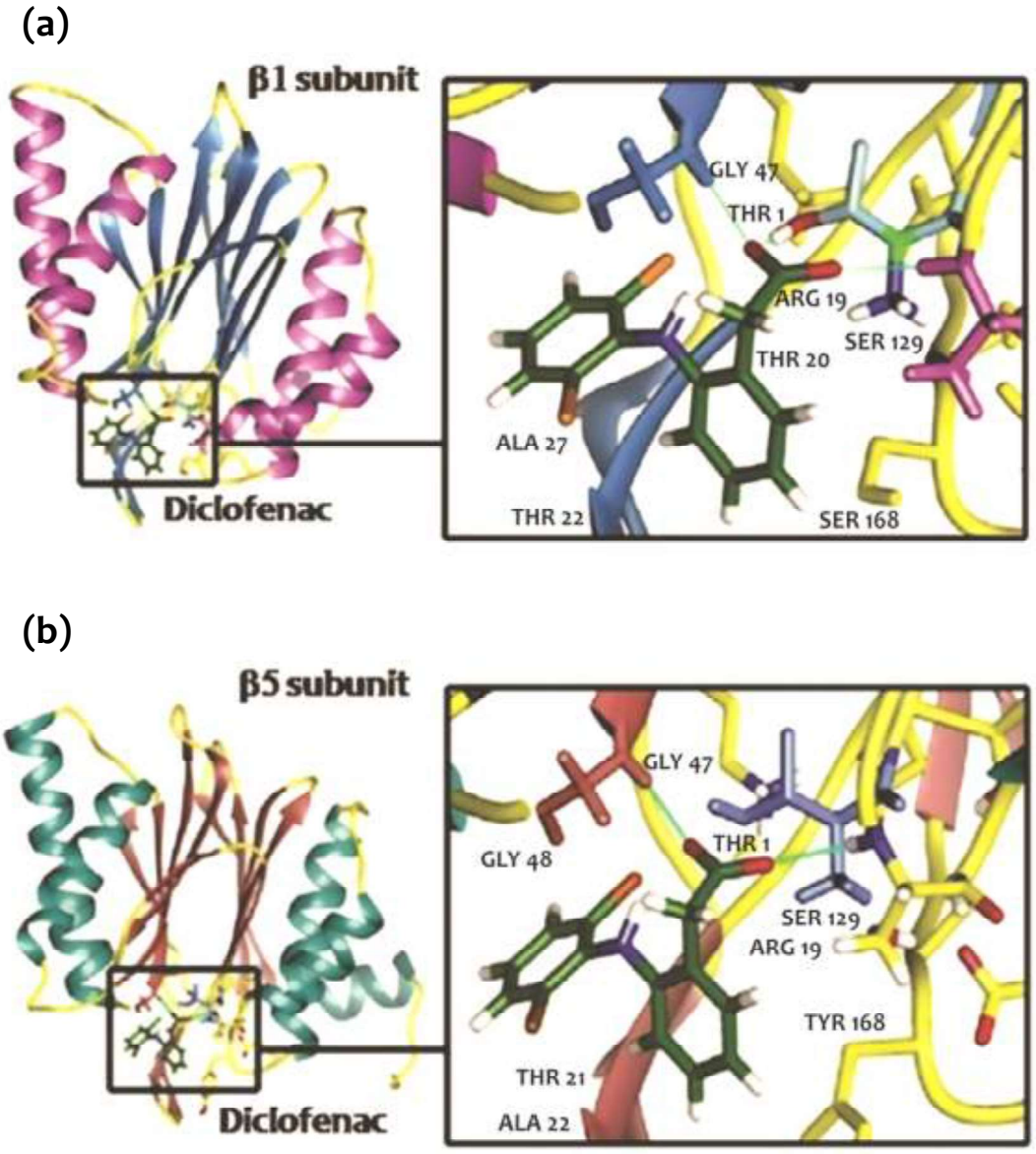

Figure 3.4 : *Diclofenac demonstrates in silico interaction with proteasome subunits:* (a-b) *In silico* docking of proteasome subunits (β1 and β5) with diclofenac. Swiss docking online web interface was used for docking, and the best binding pose obtained are shown as β1 (a) and β5 (b) subunit with diclofenac in green (ZINC ID: 1281) . The side panels provides a close-up view of the protein ligand interface labeled with surrounding residues. The hydrogen bonds formed between diclofenac and proteasome subunits are represented as green lines.

To further ascertain these initial observations, cells were treated known proteasome inhibitors MG132 and lactacystin, and few samples were exposed with diclofenac in a dose-dependent manner. The results of treatments were than observed under bright field microscope. As shown in the Figure 3.5a, diclofenac-treated cells showed morphological changes that are characteristics of apoptosis such as rounded and shrinked shape with membrane blebbing when compared with control cells. Because earlier results indicated a

possible role of diclofenac in sensitizing the cells towards disturbance of proteasome induced apoptosis, the effect of diclofenac was further examined by its treatment at different periods and observed under bright field microscope. Figure 3.5b shows a marked increase in morphological characteristics of apoptosis, after time dependent exposure of diclofenac in cell.

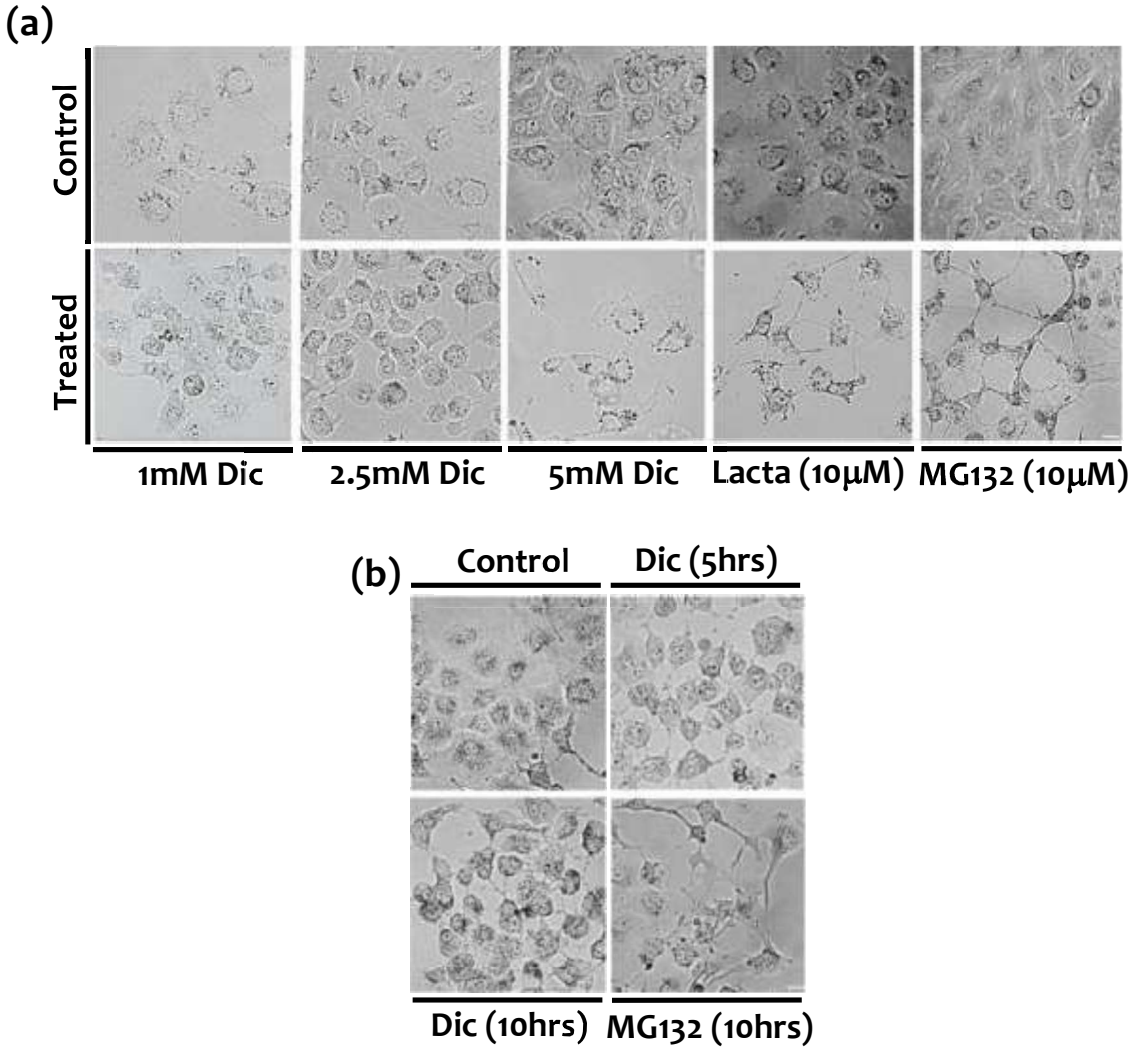

Figure 3.5 : *Diclofenac treatment exhibited apoptosis like characteristics:* (a) Cos-7 cells were seeded into chamber slides and treated with different doses of diclofenac (Dic) for 6 hrs and with proteasomal inhibitors (MG132 and lactacystin) for 12 hrs. After treatment, cells were observed using a brightfield microscope as shown in section, Scale bar 20μm (b) Cos-7 cells were incubated without or with 2.5 mM diclofenac for different time intervals as represented and 10 μM MG132 for 10 hrs, Scale bar 20μm.

3.1.3 Diminished Proteasome Activity After Diclofenac Treatment Blocks the Elimination of Aggregation-Prone Ubiquitylated Proteins

The mechanism, through which diclofenac affects proteasomal function and could lead to several molecular changes implicated in apoptosis are not well known. Therefore, to further explore the mechanism and effect of diclofenac on proteasome activity; diclofenac was further tested for its possible roles in affecting proteasomal function. To understand the effect of diclofenac on the proteasomal chymotrypsin-like, Figure 3.6a, and PGPH-like Figure 3.6b, activities, cell free proteasome activity assay were performed by incubating purified proteasomes with different concentrations of diclofenac. MG132 and lactacystin were used as reference controls.

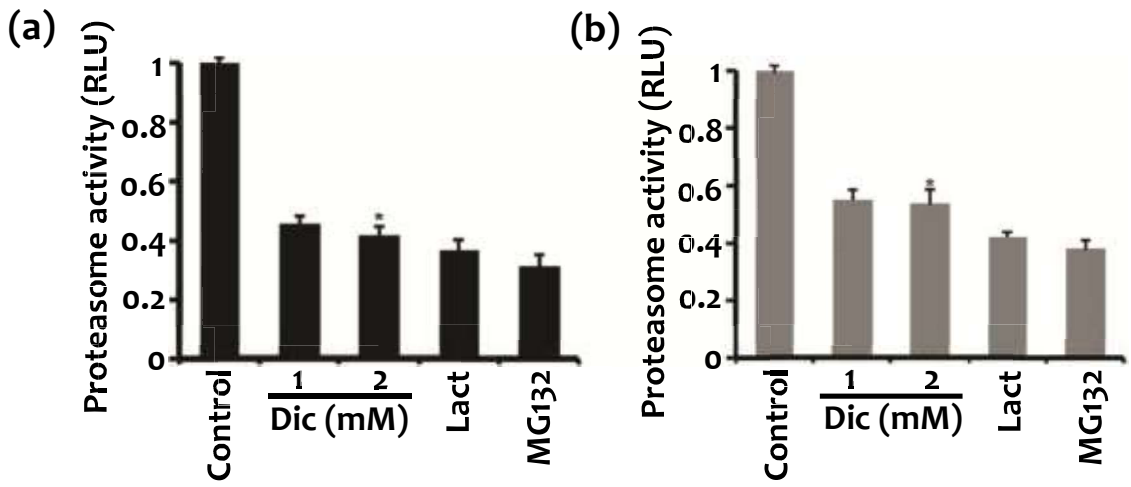

Figure 3.6 : *Diclofenac inhibits purified proteasome activities:* Purified proteasome were incubated with the denoted concentrations of diclofenac for 1 hr and after incubation proteasomal chymotrypsin-like (a) and PGPH-like activities (b) were measured as stated in methods section.

Next, cells were plated into tissue culture plates and incubated with diclofenac in concentration dependent manner. After treatment, cells were processed for chymotrypsin-like and PGPH-like activity assay of the proteasome. As can be observed from Figure 3.7a and 3.7b, diclofenac treatment reduced the protease activities of proteasome in a dose-dependent manner.

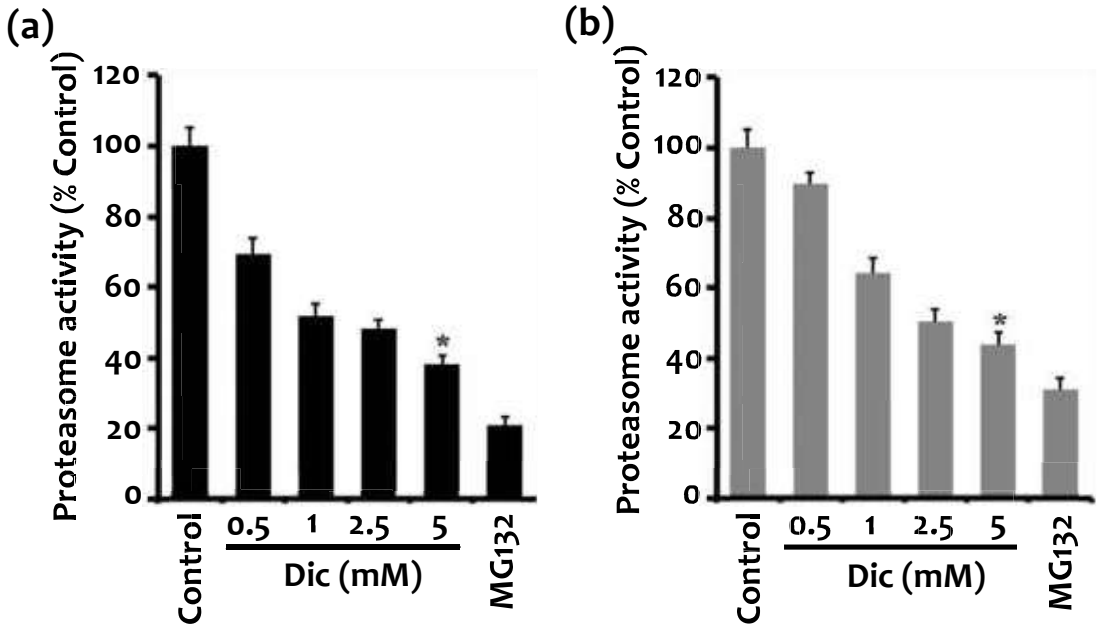

Figure 3.7 : *Diclofenac treatment resulted in inhibiton of cellular proteasome activites in dose dependent manner:* A549 cells were exposed with different dose of diclofenac for 6 hrs and 10 μM MG132 for 12 hrs. After treatment, cells were collected and subjected to proteasome activity ((a), chymotrypsin and (b), post glutamyl peptide hydrolase-like protease activity) assays as explained under the section methods.

To further confirm the chymotrypsin-like and post-glutamyl peptide hydrolase-like assay results, a detailed time-dependent experiment with diclofenac were performed, as represented in Figure 3.8a and 3.8b. Here, it was found that exposure of diclofenac decreased the protease activity of proteasomes in time-dependent manner as compared with untreated-control cells.

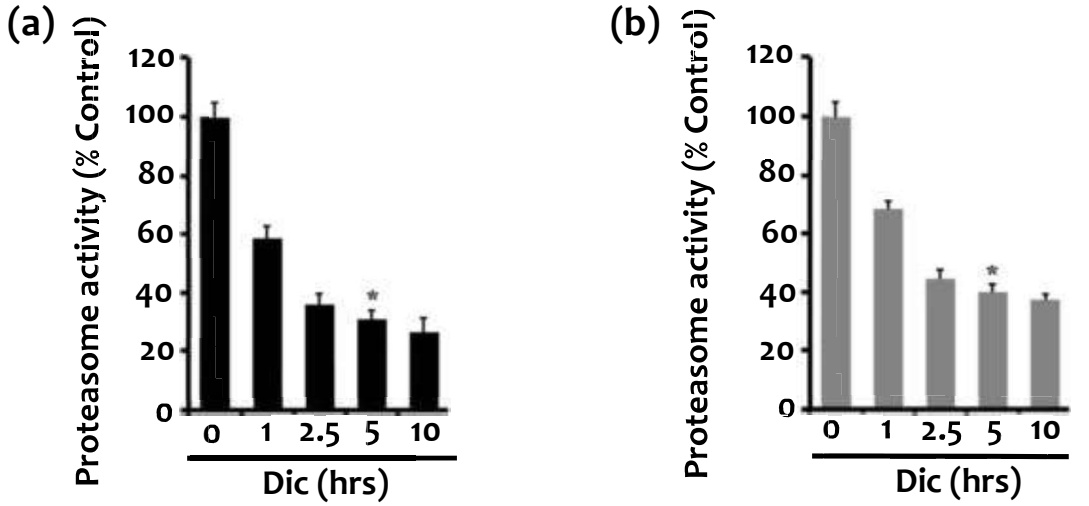

Figure 3.8 : *Diclofenac exposure resulted in inhibiton of cellular proteasome activites in time dependent manner:* Proteasome acitivity assay was performed on cells treated with diclofenac (1 mM) in time dependent manner, ((a), chymotrypsin and (b), PGPH like acitivity assays). *p < 0.05 compared to control.

Further, with the help of fluorescence microscope, it was also noticed that diclofenac exposure leads to accumulation of aggregation prone ubiquitylated proteins, Figure 3.9a. The transient transfection of HA-ubiquitin plasmid in Cos-7 cells followed by treatment with diclofenac showed dose-dependent increased accumulation of HA-tagged ubiquitylated proteins as represented in Figure 3.9b.

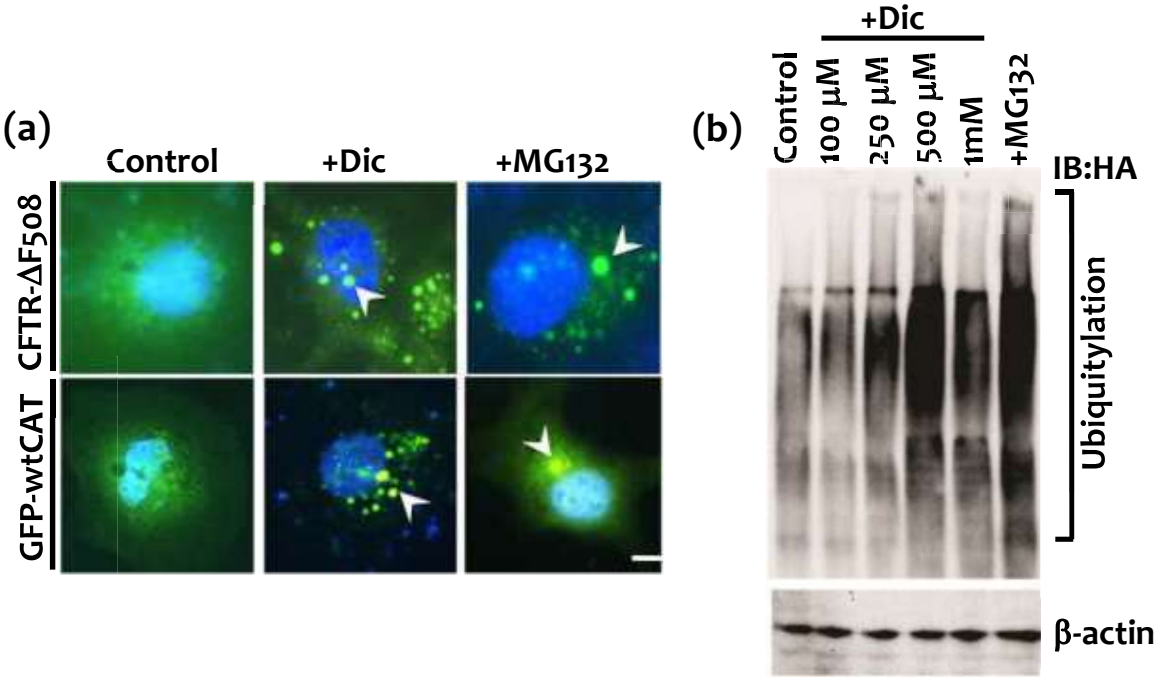

Figure 3.9 : *Diclofenac application aggravates aggresome-like structures:* (a) Cos-7 cells were seeded into chamber slides and were transfected with plasmids pEGFP-C1 CFTRΔF508 and GFP-wtCAT. Post-transfected cells were treated with diclofenac (500 μM) for 12 hrs and 10 μM MG132 for 24 hrs, nuclear staining was done using DAPI. The arrow heads indicate aggresome-like structures of GFP tagged proteins, Scale bar 20μm (b) Cos-7 cells were transiently transfected with HA-ubiquitin expression plasmid. Forty-eight hours later, cells were treated with indicated doses of diclofenac and MG132 (10 μM) as shown in figure. The cell lysates were subjected to immunoblotting using anti-HA and anti-β-actin antibodies.

Next, the effect of diclofenac on cell viability was checked. For that, ninety six-well tissue culture plates were used to seed cells, and further given concentration dependent treatment of diclofenac on following day, along with proteasome inhibitors MG132, Lactacystin, curcumin and processed for cell viability assay using MTT assay, as shown in Figure 3.10a and 3.10b. It was also important to understand the role of stress on diclofenac-induced apoptosis. To answer this question, the effect of molecular chaperones and antioxidant NAC on diclofenac-induced cell death was checked. As represented in Figure 3.10c, diclofenac-induced cell death was suppressed after the treatment of NAC and upon overexpression of Hsp70 chaperones.

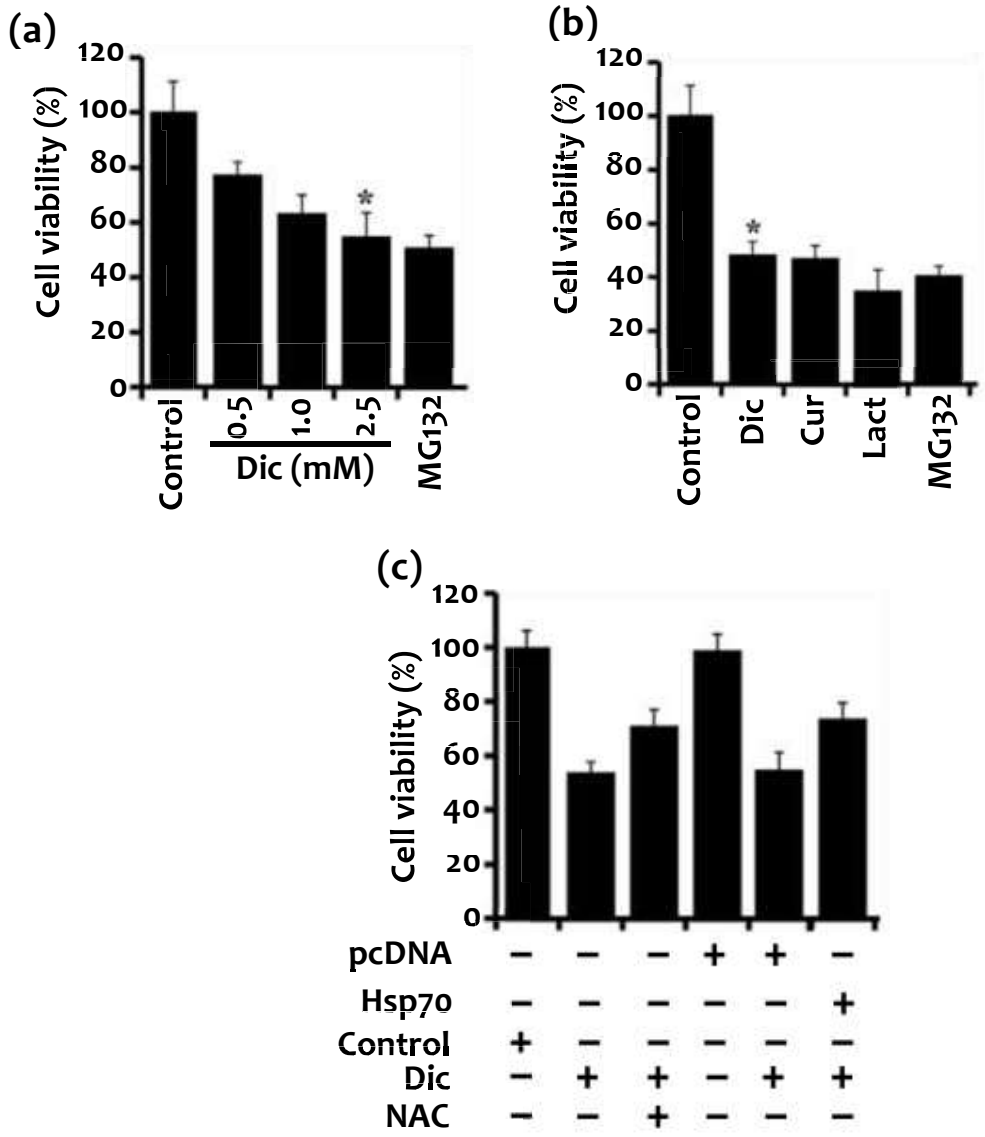

Figure 3.10 : *Diclofenac treatment reduces cell viability* : As exhibited in section (a-b) A549 cells were treated with various concentrations of diclofenac. Some Cells were exposed with Dic (2.5 mM) and with the known proteasome inhibitors Cur, curcumin (100 μM); Lact, lactacystin (10 μM), and MG132 (10 μM). Cells were used for cell viability experiment and cell viability was measured by MTT assay. *$p < 0.05$ compared with control. (c) Cos-7 cells were plated into 24-well tissue culture plates and transiently transfected with Hsp70-EGFP and pcDNA-EGFP (control) plasmids. After 48 hrs, few cells were treated with NAC (5 mM) and Dic (2.5 mM) as represented in figure. After treatment, cell viability was measured by MTT assay. pcDNA-EGFP (control)-transfected with diclofenac-treated experiments.

3.1.4 Diclofenac Treatment Stabilizes the Degradation of Model Substrate of Proteasome

The results obtained suggest that diclofenac treatment induces aggregation of ubiquitylated proteins and reduces cell viability. Further, the effect of diclofenac on cellular proteasome targeted substrate, IκB-α were investigated. After 24 hrs of transient transfection with IκB plasmid both IκB over-expressed and normal cells were treated with diclofenac in a dose-dependent manner, as shown in Figure 3.11a. Treatment of diclofenac led to increase in the levels of IκB-α which can be understood from Figure 3.11b. In the next sequence of experiment, effect of diclofenac on down-regulation of transcriptional activity of NF-κB was checked, which might be due to the elevated levels of IκB-α, as shown in Figure 3.11c.

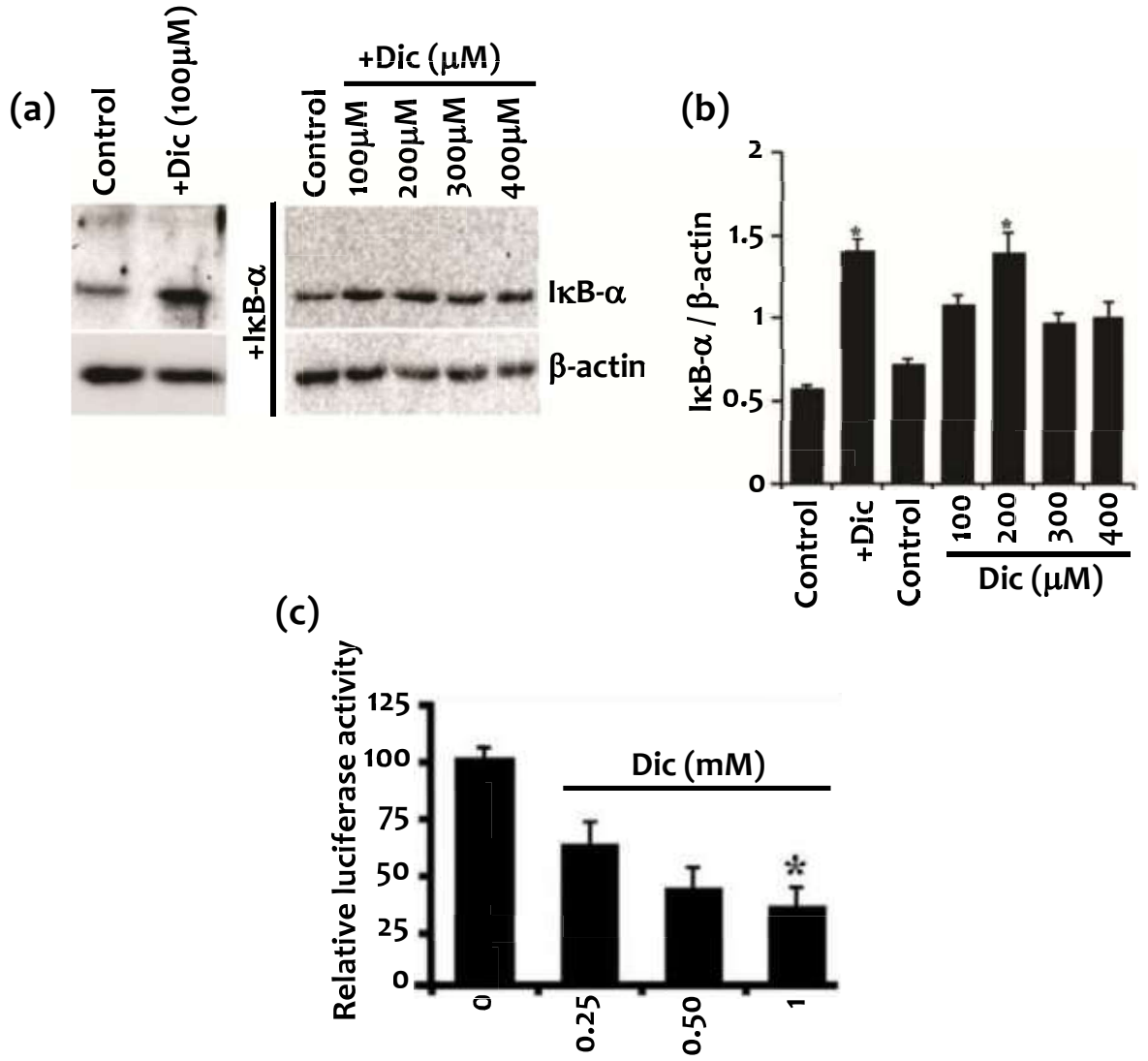

Figure 3.11 : *Treatment with diclofenac increases level of proteasomal substrate IκB-α and reduces NF-κB activity:* A549 cells were treated with diclofenac and few sets of Cos-7 cells were transiently transfected with pCMX IκB plasmid and treated with diclofenac in concentration dependent manner as represented in section (a). Cells were collected and lysates were processed for immunoblotting using IκB-α and β-actin antibodies. Quantification of endogenous IκB-α levels after diclofenac treatment was done using NIH Image analysis software (b). Data were normalized using β-actin. Values are the mean ± SD. *$p < 0.05$ with respect to control cells. (c) Cos-7 cells were plated into 6-well tissue culture plates and transiently transfected with NF-κB luciferase and pRL-SV40 plasmids. After 24 hrs of transfection, cells were treated with varying doses of diclofenac and subjected to luciferase activity assay as explained in the Section Methods. Values are presented as mean ± SD of three independent experiments. *$p < 0.05$ compared with control.

Next, the effect of diclofenac on d1EGFP protein degradation, a model substrate for proteasome was checked. Cells were transfected with d1EGFP constructs that can be recognized for proteasomal degradation due to the presence of signal sequence containing serine, proline, glutamic acid and threonine at its C terminus, and treated with diclofenac. After transfection, cells were used for cycloheximide chase experiment with or without diclofenac and samples were immunoblotted with anti-GFP antibody. As shown in Figure 3.12a and 3.12b, treatment of diclofenac decreased the turnover of d1EGFP proteins. Further presence of diclofenac lead to accumulation of d1EGFP as evident from fluorescence micrographs shown in figure 3.12c

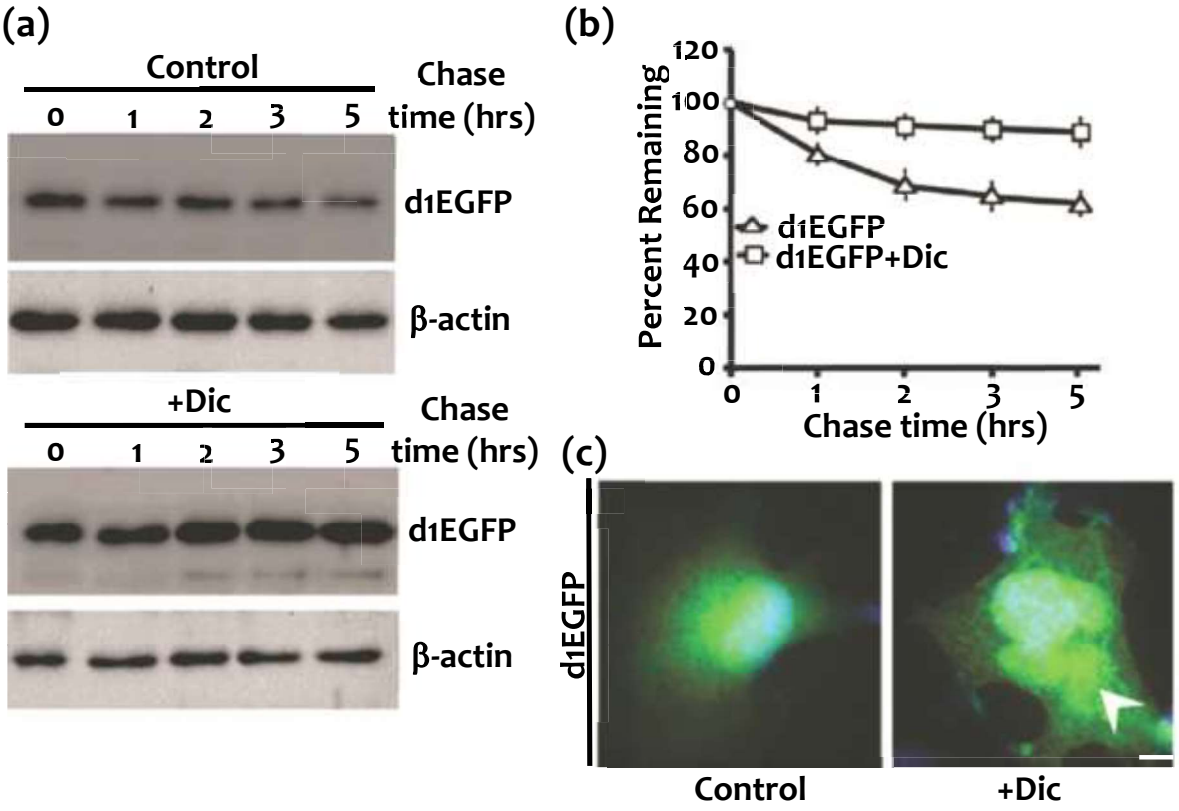

Figure 3.12 : *Diclofenac treatment decreases turnover of model proteasomal substrate:* After 24 hrs of transient transfection with pd1EGFP plasmid, Cos-7 cells were exposed with 2.5 mM diclofenac and chased in the presence of cycloheximide at concentration of 15 μg/ml, for different time intervals as shown in the section (a). Data from three independent experiments were used for quantification of d1EGFP protein levels in the chase experiments. Data normalization was done against β-actin and values are presented as mean ± SD of three independent experiments (b). Fluorescence microscopic analysis of Cos-7 cells having transient transfection with pd1EGFP plasmid showed accumulation of d1EGFP in response to diclofenac treatment as shown in section (c). Arrows indicate aggresome like structures of d1EGFP protein in presence of diclofenac , Scale bar 20μm.

To further ascertain these results, the complete ubiquitylation profile of overexpressed GFP-Ub protein was checked, because disturbance of UPS function leads to the accumulation of ubiquitylated higher molecular weight derivatives of cellular proteins. Thus, transiently transfected cells with GFP-Ub plasmid were treated with diclofenac in dose-dependent manner. Cell lysates were prepared and immunoblot analysis was performed with anti-GFP, anti-ubiquitin, and anti-β-actin antibodies. As shown in Figure 3.13, accumulation of ubiquitylated higher molecular weight derivatives of GFP-Ub enriched misfolded proteins after diclofenac treatment in cells were observed.

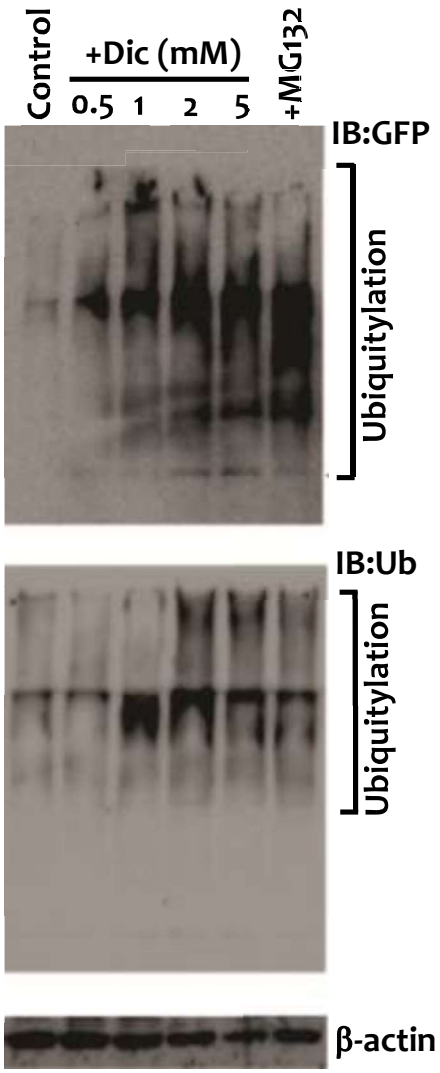

Figure 3.13 : *Diclofenac exposure showed increased accumulation of ubiquitylated proteins : Cos-7* cells were transiently transfected with GFP-Ub expression plasmid, treated with varying doses of diclofenac and after treatment processed for immunoblotting using anti-GFP, anti-ubiquitin, and anti-β-actin antibodies**.**

3.1.5 Diclofenac Treatment Disturbs Levels of Pro-Apoptotic Proteasome Target Proteins and Apoptotic Events in Cells

To examine the morphological effects of proteasome inhibition in cells, the localization and distribution of other known substrates of proteasome and pro-apoptotic proteasome target proteins such as Bax, p53, and $p27^{kip1}$ were assessed using fluorescence microscope. In addition, the 20S proteasome, ubiquitin and p21 localization after diclofenac treatment in cells were also checked. As shown in various micrographs Figure 3.14 to Figure 3.16, exposure of diclofenac accumulated the levels of above described proteins in cells and few of them make aggresomes and small inclusion-like structures in cells. As shown in Figure 3.17, to further confirm earlier observation similar sets of diclofenac or MG132 treated samples were used for immunoblot analysis using ubiquitin, p53, Bax, $p27^{kip1}$, p21, and anti-β-actin antibodies. It was observed that treatment of diclofenac or MG132 increased the levels of known substrates of proteasome and pro-apoptotic proteasome targeted proteins.

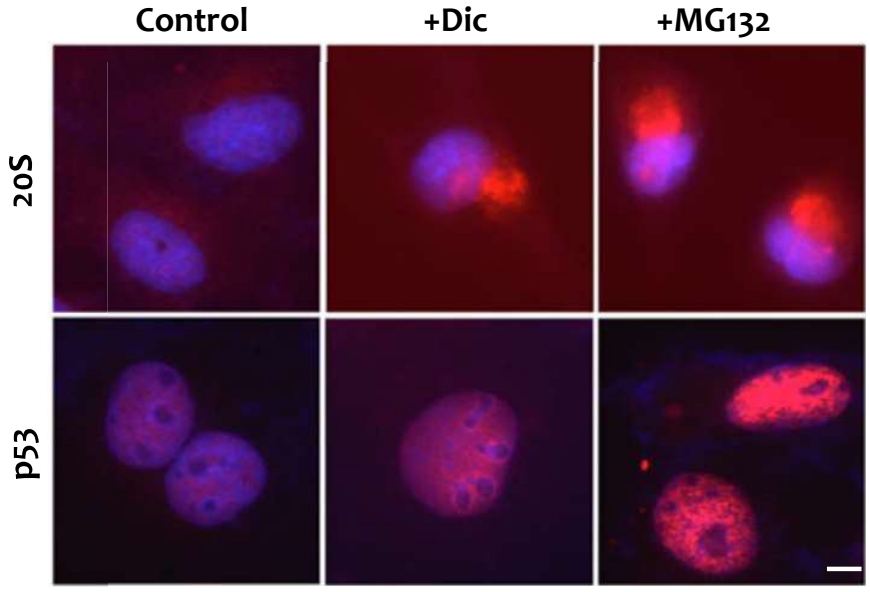

Figure 3.14 : *Diclofenac treatment induces accumulation of proteasome targeted proteins 20S and p53*: Cells were seeded into two-well chamber slides and treated with diclofenac (Dic 0.5 mM for 10 hrs) and MG132 (10 μM for 24 hrs). After treatment, cells were processed for immunofluorescence staining with 20S and p53 antibodies; Nuclear staining was performed by using 4',6-diamidino-2-phenylindole (DAPI), and observed under fluorescence microscope , Scale bar 20μm.

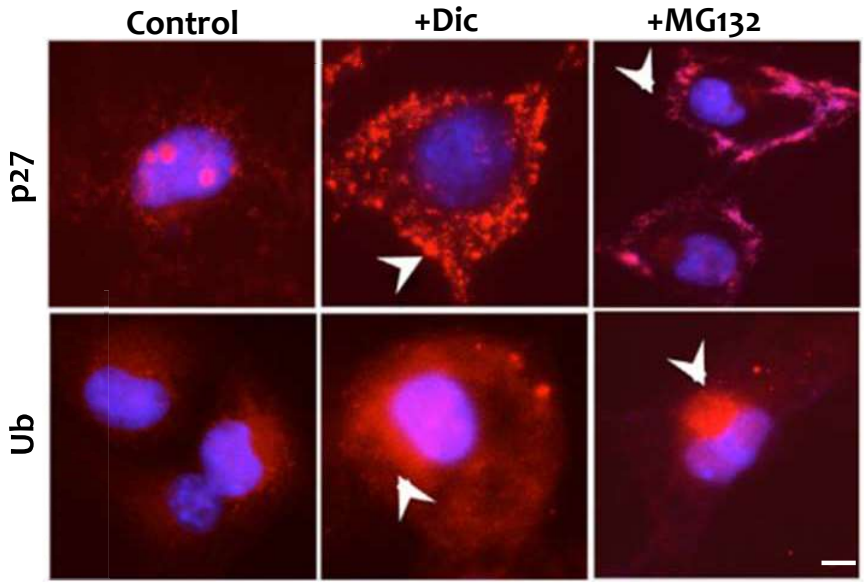

Figure 3.15 : *Diclofenac application induces accumulation of proteasome targeted proteins p27 and Ub*: Cells were seeded into two-well chamber slides and treated with diclofenac (Dic 0.5 mM for 10 hrs) and MG132 (10 μM for 24 hrs). After treatment, cells were processed for immunofluorescence staining with p27 and ubiquitin (Ub) antibodies. Nuclear staining was performed by DAPI. The arrow heads in overlay images represent the redistribution and accumulation of various proteins in cells. Scale bar 20μm.

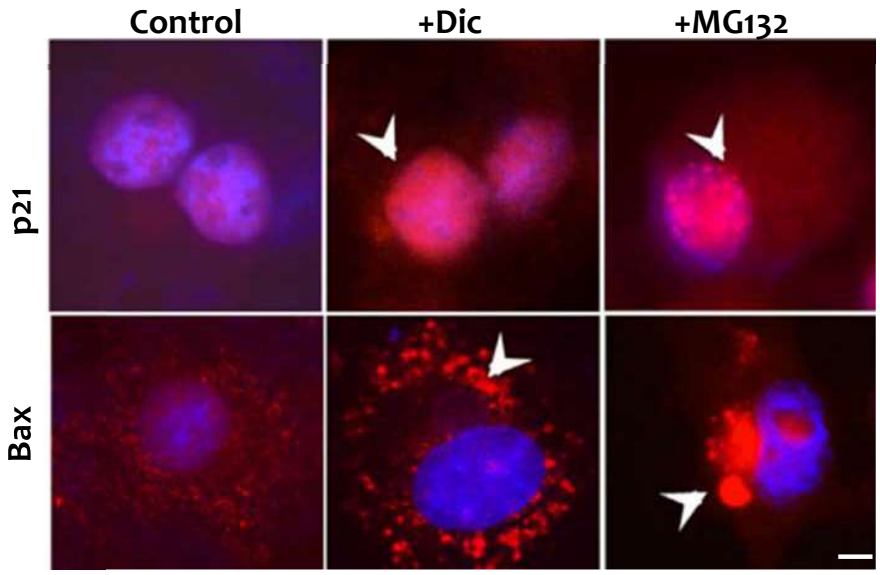

Figure 3.16 : *Diclofenac treatment induces accumulation of proteasome targeted proteins p21 and Bax*: Cells were seeded into two-well chamber slides and treated with diclofenac (Dic 0.5 mM for 10 hrs) and MG132 (10 μM for 24 hrs). After treatment, cells were processed for immunofluorescence staining with p21 and Bax antibodies. Nuclear staining was performed by using DAPI. The arrows in overlay images represent the redistribution and accumulation of various proteins in cells. Scale bar 20μm.

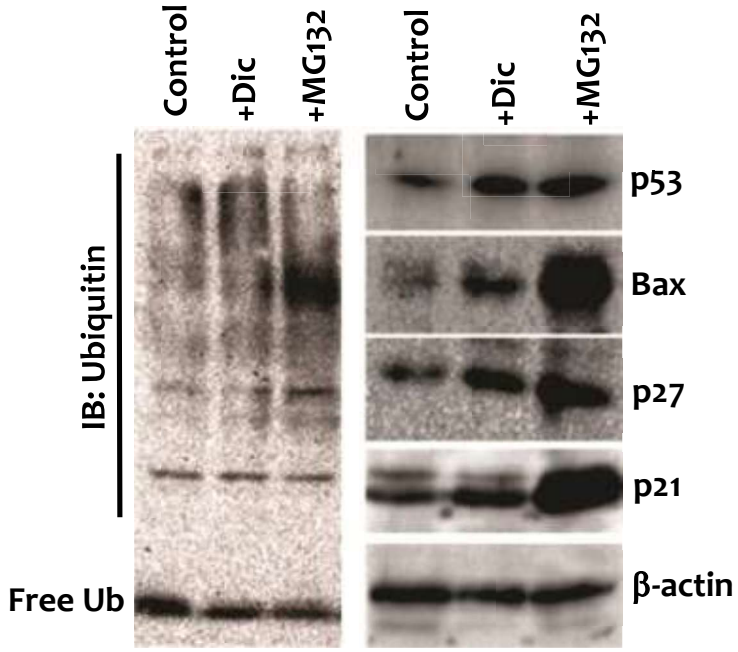

Figure 3.17 : *Diclofenac exposure induces accumulation of various proteasome targeted pro-apoptotic proteins*: Cos-7 cells treated with diclofenac (Dic 0.5 mM for 10 hrs) and MG132 (10 μM for 24 hrs) and after treatment lysates were prepared and used for immunoblotting with ubiquitin, p53, Bax, p27, p21, and β-actin antibodies.

Earlier, it has been observed that exposure of NSAIDs induces apoptosis in cells [Shureiqi et al., 2000; Jana, 2008]. Therefore, to explore the role of diclofenac in apoptosis

induction, diclofenac or MG132 were given to cells at various time intervals and stages of apoptosis were determined by Annexin-V FITC/PI assay, using flow cytometry analysis. As shown in Figure 3.18a and 3.18b, treatment of diclofenac resulted in time-dependent increase in apoptosis .

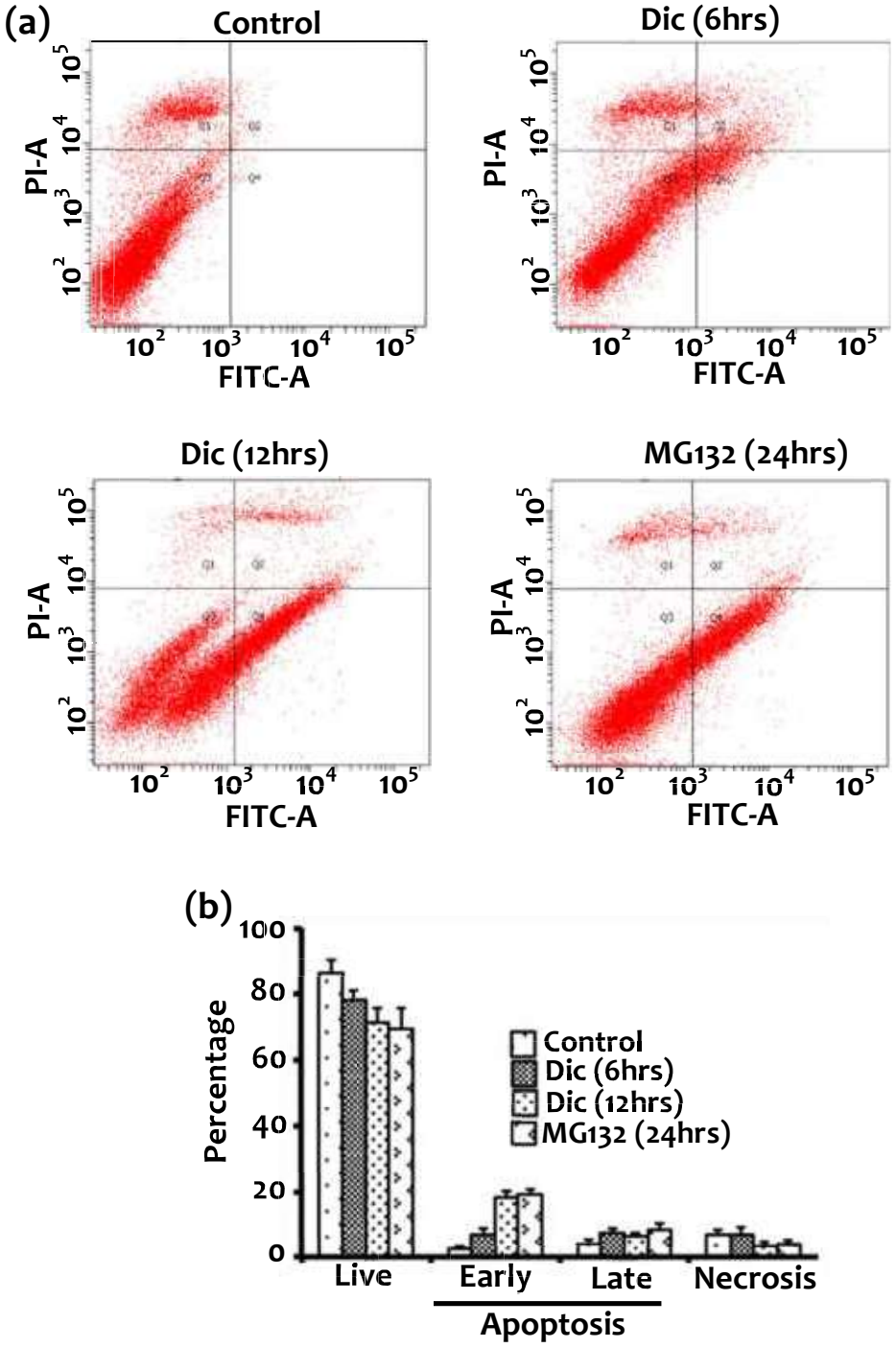

Figure 3.18 : *Diclofenac treatment leads to activation of apoptosis:* (a) A549 cells were treated with Diclofenac (500 μM) and MG132 (10 μM) at indicated time periods. After treatment, cells were processed to assess apoptosis by flow cytometry using Annexin V-FITC and propidium iodide (PI) double staining assay. (b) As described in previous experiment, bar graph represents increase in apoptosis after diclofenac treatment and values are presented as mean ± SD of three independent experiments.

Furthermore, the apoptotic changes in cells were evaluated by observing chromatin condensation using DAPI, Figure 3.19a and TUNEL analysis, Figure 3.19b. After diclofenac treatment in concentration dependent manner, cells were stained with DAPI and analyzed under the fluorescence microscope; as shown in Figure 3.19a cells were observed with condensed chromatin. From TUNEL assay, DNA fragmentation a cytological characteristic of apoptotic cells was also examined after diclofenac treatment in concentration dependent manner. Labeling cells by TUNEL technique demonstrated, diclofenac treatment induces apoptosis in cells as shown in Figure 3.19b

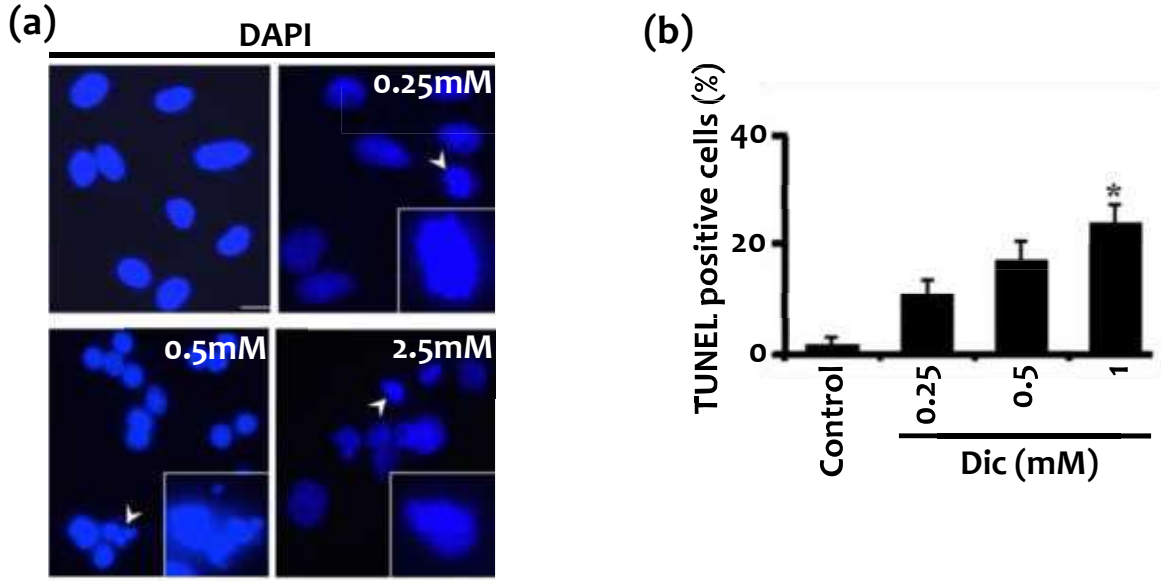

Figure 3.19 : *Application of diclofenac leads to chromatin condensation and DNA fragmentation:* (a) Cells were seeded into two-well chamber slides and treated with varying concentration of diclofenac (12 hrs) as shown in the figure. After treatment, cells were observed under fluorescence microscope by using 4',6-diamidino-2-phenylindole (DAPI) for nuclear staining. In photomicrographs, arrowheads indicate nuclear condensation in cells, Scale bar 20μm (b) Cells were treated with diclofenac in dose-dependent manner; post-treated cells were used for TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) staining as described in the Section Methods. In bar graph, values are presented as mean ± SD of three independent experiments, performed in triplicate *$p < 0.05$ compared with control.

3.1.6 Mitochondrial Dysfunction and Cytochrome Release After Treatment of Diclofenac in Cells

Cytosolic release of Cytochrome *c* from mitochondria causing increase of cytochrome *c* content in cytosolic fraction act as a crucial apoptogenic factor in the intrinsic apoptotic pathway [Hengartner, 2000; Tsujimoto, 2003]. Since it was observed that diclofenac disturbed proteasome function, hence it was important to examine whether diclofenac-induced proteasomal dysfunction contributes to disruption of mitochondrial functions and subsequently releases apoptogenic proteins such as cytochrome *c* into cytosol. For that, cells were incubated with varying concentrations of diclofenac. Following treatment, cells were stained with JC-1, a sensitive fluorescent dye that stains polarized mitochondria with its monomeric form red color. As shown in Figure 3.20b-d, treatment of diclofenac induced the disruption of the mitochondrial membrane potential ($\Delta\psi m$) and that can be observed by the loss of the red-dotted color in mitochondria as compared to control cells Figure 3.20a. Disruption of mitochondrial membrane potential and apoptotic morphological changes on diclofenac treatment likely due to proteasomal dysfunction suggested to check the cytochrome *c* release from the cells. Cells were exposed to different concentrations of diclofenac and

immunofluorescence staining, Figure 3.20e-h were carried out by using anti-cytochrome *c* antibody.

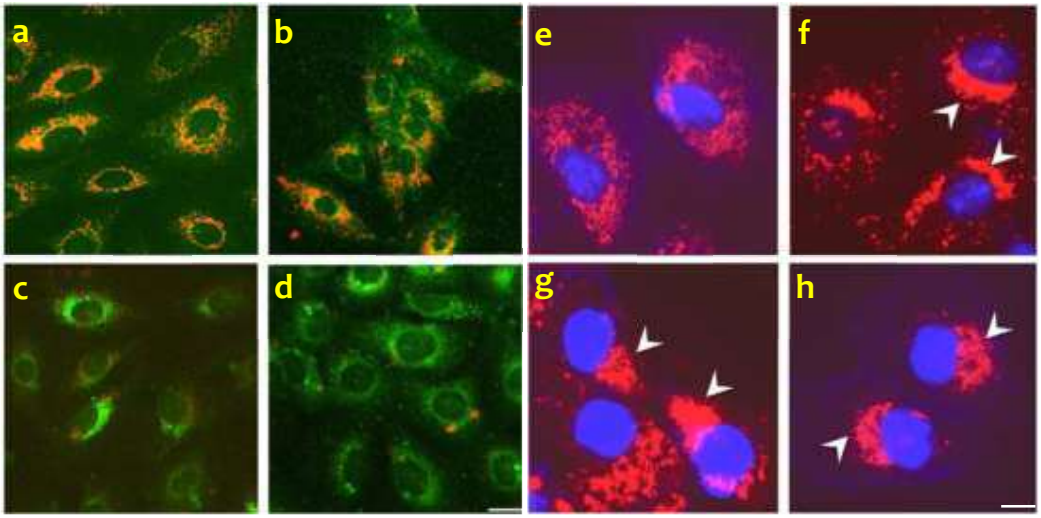

Figure 3.20 : *Diclofenac treatment results in changes in the mitochondrial potential and induces cytochrome c release:* (a-d) Cells were plated into two-well chamber slides and treated with control (a), diclofenac (b, 1 mM; c, 02 mM), and 10 μM MG132 (d); after treatment to detect changes in mitochondrial membrane potential, cells were processed for 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1) staining. (e–h) As described in previous section, few sets of cells were used for immunofluorescence staining probed with cytochrome c antibody. (e), Control; (f), diclofenac (0.5 mM); (g), diclofenac (1 mM); (h), MG132 (10 μM) , Scale bar 20μm.

Few set of diclofenac-treated samples were used for JC-1 staining, analyzed by flow cytometry Figure 3.21a. Earlier it has been reported that decreased levels of procaspase-9 or activation of caspase-9 is associated with mitochondrial release of cytochrome c into cytosol or induction of apoptotic signaling pathways [Qiu *et al.*, 2000]. The apoptotic effect of diclofenac was further confirmed using Cos-7 cells, which were treated with diclofenac or MG132, cell lysates were prepared and then processed for immunoblotting; treatment of diclofenac decreased the endogenous levels of procaspase-9, Bcl-2, procaspase-3 and, followed by the cleavage of lamin B as shown in Figure 3.21b.

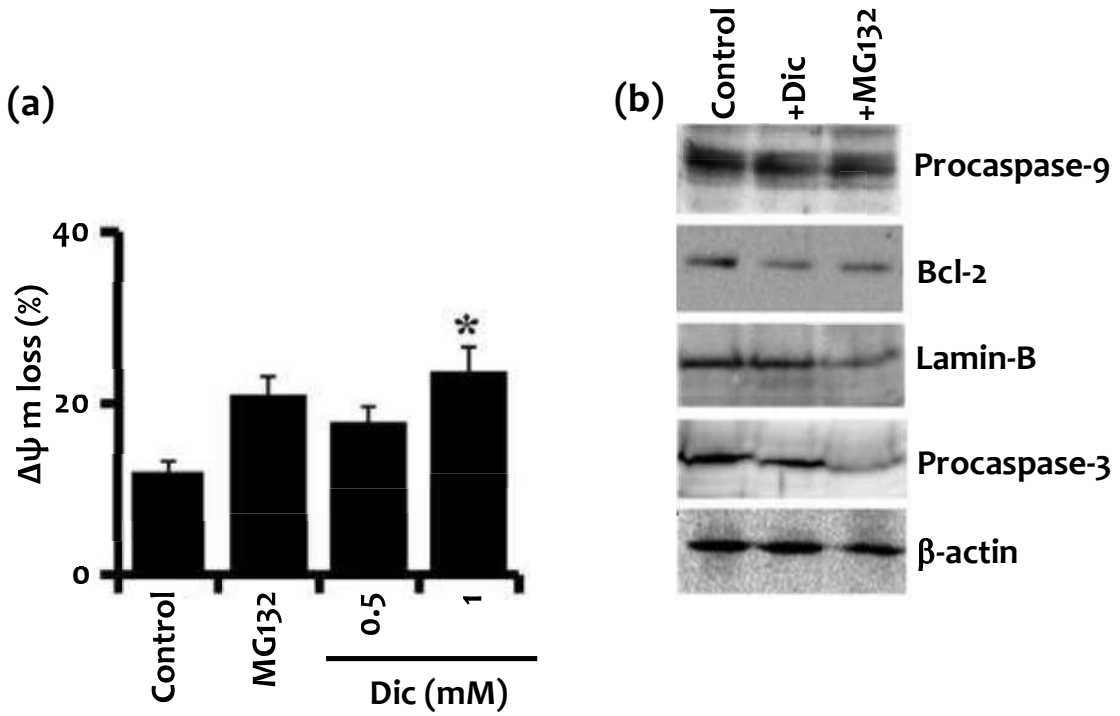

Figure 3.21 : *Diclofenac exposure results in mitochondrial membrane depolarization and activates apoptosis signaling:* (a) Cells were seeded into 6-well tissue culture plates and subsequent day treated with various concentrations of diclofenac; after treatment, cells were stained with JC-1 and analyzed by flow cytometry. Represented values are the mean ± SD of three independent experiments, each performed in triplicate. *p < 0.05 compared with the control (b) As described in previous section cells were treated with diclofenac (Dic 0.5 mM for 10 hrs) and MG132 (10 μM for 24 hrs) and post-treated cells were used for lysates preparation and immunoblot analysis were performed. β-Actin was used as loading control and blots were incubated with procaspase-9, Bcl-2, lamin-B, and procaspase-3 antibodies.

3.2 Discussion

It was unclear how NSAIDs-mediated proteasome impairment is involved in apoptosis of cells. To answer this question, *in silico* docking analysis was performed and potential interaction of diclofenac with β1 and β5 subunits of proteasome were obtained. Based on the previous observations, the next aim of the study was to determine whether diclofenac exposure alters proteasome function and how it can affect cell viability. The results demonstrated that diclofenac treatment decreases the proteasome function as well as cell viability and behaves in a similar profile of other known putative proteasome inhibitors. In addition, it was observed that time-dependent treatment of diclofenac dramatically induces apoptotic morphological changes in cells. Moreover, using intact cells as well as purified proteasomes, it was demonstrated that diclofenac treatment reduced chymotrypsin-like and post-glutamyl peptide hydrolase-like activities of proteasome in a concentration-dependent as well as a time-dependent manner but with the highest potency to the chymotrypsin-like activity.

Overall, the study suggests that diclofenac treatment cause apoptosis, which might be due to the generation of mitochondrial abnormalities in cells induced by proteasomal disturbance. Here, additional results have been provided that shows diclofenac-induced proteasomal malfunction inhibits proteasome degradation of substrates and disturbs the localization of pro-apoptotic proteasome target proteins. It has been observed that diclofenac treatment in cells leads to the high levels of p21 and p53 proteins. In addition, the formation of

ubiquitin positive aggresomes-like perinuclear inclusions of 20S proteasome, Bax, and p27kip1 were also observed. Treatment of NSAIDs including diclofenac, increases the levels of p21 and p27 cyclin-dependent kinase (CDK) inhibitors and inhibit the proliferation of rat A10 abnormal vascular smooth muscle cell (VSMC) [Brooks *et al.*, 2003]. Earlier findings have indicated that NSAIDs including diclofenac and indomethacin inhibit cell cycle progression and may cause apoptosis in ovarian cancer cells [Valle *et al.*, 2013]; in rabbit corneal epithelial cells, under in vitro conditions diclofenac also inhibits cell cycle and induces apoptosis [Wu and Du, 2010]. Previous reports have demonstrated that NSAIDs inhibit the activation of NF-κB [Dikshit *et al.*, 2006; Kopp and Ghosh, 1994; Yamamoto *et al.*, 1999]. Treatment of ibuprofen in T cells inhibits activation of NF-κB [Scheuren *et al.*, 1998].

In the current study, it was also observed that diclofenac treatment elevated IκB levels and also reduces the NF-κB transcriptional activity in cells. Recently, a report showed that ACS15 and ACS32 are the members of NSAIDs and derivatives of H_2S-releasing diclofenac (S-DCF), treatment of these derivatives inhibit IκB phosphorylation [Frantzias *et al.*, 2012]. Another study demonstrates that 100 μM diclofenac exposure also decreases the NF-κB transcriptional activity monitored by using NF-κB luciferase reporter plasmid [Fredriksson *et al.*, 2011]. Above studies also support the current findings that diclofenac treatment decreases NF-κB transcriptional activity likely through the disturbance in proteasome-mediated degradation mechanism. Interestingly, it has been observed that inhibition of NF-κB function increases amyloid β–mediated neuronal apoptosis [Kaltschmidt *et al.*, 1999]. Cumulative treatment of both diclofenac with NF-κB inhibitors induced apoptosis in ovarian cancer cells [Zerbini *et al.*, 2011].

In the current study, it was noticed that treatment of diclofenac in cells disturbs UPS function and possibly elevates the load of misfolded proteins aggregation in cells, which might contribute in the altered NF-κB function. It have been observed that diclofenac treatment affects normal proteasome function in cells; and the results demonstrate that diclofenac treatment induces the formation of aggresomes-like structures of various (pEGFP-C1 CFTR-ΔF508, pd1EGFP, and GFP-wtCAT) aggregate-prone proteins. In the present work, the apoptotic effect of diclofenac were assessed via various experimental approaches using Annexin V-FITC/PI flow cytometry analysis, TUNEL assay, chromatin condensation, and nuclear fragmentation analysis.

These results are in agreement with above described earlier findings and also support preliminary observations of morphological apoptotic changes in cells and clearly demonstrate that diclofenac treatment promotes apoptosis in cells. Since diclofenac treatment disturbs proteasome function, diclofenac was tested for the possible involvement in abnormal mitochondrial function and subsequent events such as depolarization of mitochondrial membrane potential and cytochrome c release. The results obtained suggest that diclofenac exposure promotes mitochondrial dysfunction and also induces cytochrome c release from the mitochondria too. Proteasome inhibition has been reported to initiate caspase-dependent apoptotic signaling, involving mitochondrial membrane depolarization, cytochrome c release into cytosol, and caspase-3-like proteases activation [Mitsiades *et al.*, 2002; Qiu *et al.*, 2000]. It has been observed that diclofenac-induces apoptosis through mitochondrial pathway involving Bcl-2, procaspase-9, procaspase-3 reduction followed by cleavage of lamin B.

Previously it has been reported that NSAIDs including diclofenac promote a central event of apoptosis by the mitochondrial release of cytochrome c into cytosol and affect mitochondrial membrane potential [Cecere *et al.*, 2010; Inoue *et al.*, 2005; Leibowitz *et al.*, 2014; Piqué *et al.*, 2000]. The current study was undertaken to observe the death pathway induced in cells by proteasomal malfunction. The current findings suggests that treatment of diclofenac

disturb proteasome functions, which induces accumulation of different ubiquitylated misfolded proteins in cells and most probably activate mitochondrial abnormalities linked with apoptotic molecular mechanism. Recent studies on NSAIDs and the present observation of diclofenac mediated outcomes provide a better understanding which may be useful in defining molecular mechanisms and new strategies implicated in diseases like cancer. Altogether, the present study has been undertaken to find the possible role of diclofenac-induced apoptosis through proteasome dysfunction, which could contribute to the cells undergoing cell death via the mitochondrial pathway.

3.3 Concluding Remarks

Several epidemiological studies have demonstrated anti-proliferative activity of diclofenac and identified various mechanisms involved in its apoptosis inducing outcomes in different cancer cell lines. It has been reported that NSAIDs including diclofenac promote a central event of apoptosis by the mitochondrial release of cytochrome c into cytosol and affect mitochondrial membrane potential [Cecere *et al.*, 2010; Inoue *et al.*, 2005; Leibowitz *et al.*, 2014; Piqué *et al.*, 2000]. The current study was undertaken to observe proteasomal malfunction involvement in induction of cell death pathway. In this study, it has been observed that diclofenac treatment induces proteasomal malfunction. This observation was supported by identification of accumulation of different proteasomal targeted substrates that also included few pro-apoptotic proteins like Bax which has been previously found to be involved in mitochondrial depolarization and cytochrome *c* release [Finucane *et al.*, 1999; Tikhomirov and Carpenter, 2005]. Inhibition of proteasome has also been reported to induce caspase-dependent apoptotic signaling pathways, consisting of involvement and disruption of the mitochondrial membrane potential, release of cytochrome c into cytosol, and activation of caspase-3-like proteases [Mitsiades *et al.*, 2002; Qiu *et al.*, 2000]. Here, it has been observed that diclofenac-induced apoptosis through mitochondrial pathway, involving Bcl-2, procaspase-9, procaspase-3 reduction and cleavage of lamin B. Altogether, the present study has been undertaken to find the possible role of diclofenac-induced apoptosis through proteasome dysfunction, which could contribute to the cells undergoing cell death via the mitochondrial pathway.

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