4
Valproate Mediated Proteasome Dysfunctions Induce Apoptosis

4.1. RESULTS

Cells target abnormal proteins for clearance, primarily, with the aid of UPS [Eisele and Wolf, 2008; Amm et al., 2014]. Compromised functions of UPS lead to accumulating aberrant proteins, which are a clinical hallmark for various known neurodegenerative diseases [Ross and Poirier, 2004; Huang and Figueiredo-Pereira, 2010]. Still, it is not well understood how depleted functions of the proteasome contribute to disturbance of cellular homeostasis resulting into cell death? How we can modulate the functions of proteasome for its potential therapeutic interventions in restricting the aberrant cellular proliferation and induce cancer cell death is a challenge. To find the answer to these questions, we executed a few preliminary experiments with valproate (VPA), a known antiepileptic drug that also retains anti-inflammatory properties [Ximenes et al., 2013].

4.1.1 Valproate Induces the Intracellular Accumulation of Ubiquitin Positive Inclusions

Cells were given valproate and known proteasome inhibitor MG132, and post-treated cells were incubated with γ-tubulin and ubiquitin antibodies. As shown in Figure 4.1 (a, b), we noticed that the treatment of valproate induces the accumulation of inclusions positive for both ubiquitin and γ-tubulin at microtubule-organizing center (MTOC), and similar effect was observed in MG132 treated cells.

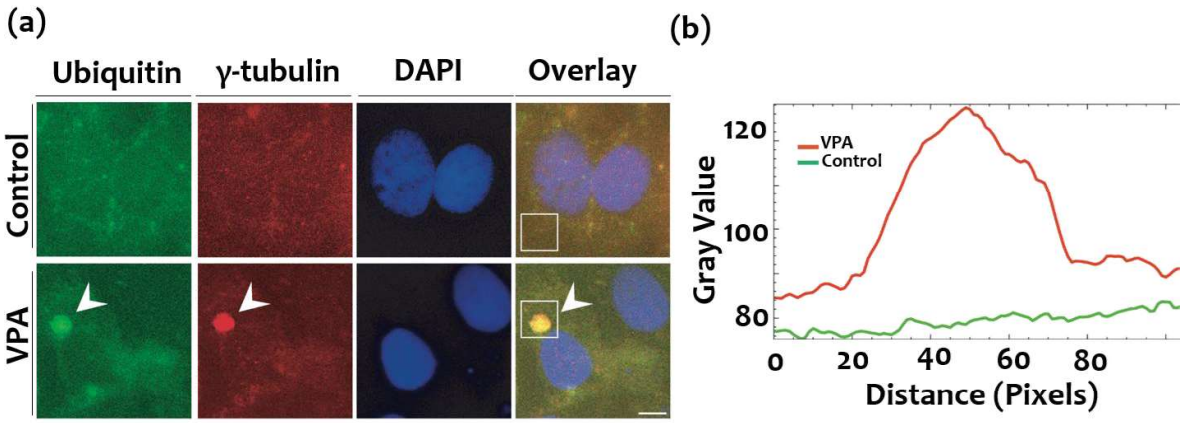

Figure 4.1: Valproate promotes accumulation of ubiquitinated proteins at MTOC (Microtubule-organizing center) sites: (a) Cos-7 cells were exposed to control (distilled water) and valproate (VPA, 5 mM, 24 h) and processed for immunocytochemistry analysis with dual labelling with antibodies for ubiquitin (green) and γ-tubulin (red). Imaging was done with epifluorescence microscope. *Scale bar: 20 μm*. (b) Section (a) micrographs were used for the analysis of fluorescence intensity plots of VPA (red) and Control (green) through ImageJ software analysis.

Here, we also assessed the effect of nocodazole (Noc), a microtubule polymerization-disrupting drug, on cells treated with valproate and MG132; immunofluorescence analysis suggests that use of nocodazole abolished the effect of valproate and MG132 on ubiquitinated protein accumulation as shown in Figure 4.2 (a, b). These preliminary findings indicate that valproate may diminish the functions of proteasome, potentially leading to induced accumulation of abnormal ubiquitin-positive inclusions.

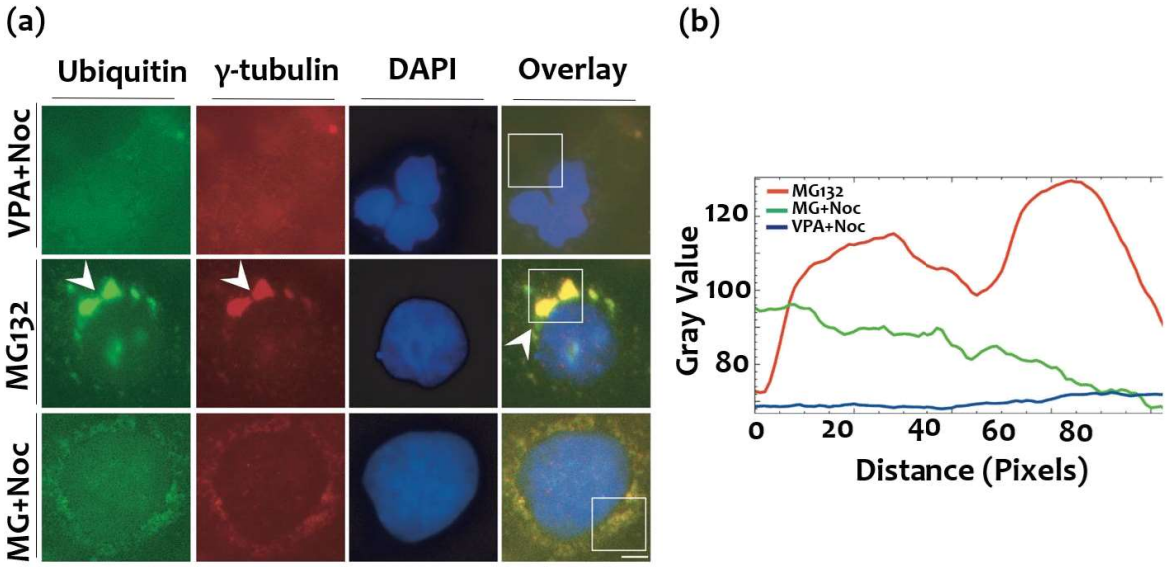

Figure 4.2: Effect of valproate on ubiquitinated protein inclusions at MTOC in presence of nocodazole and MG132: (a) A few sets of Cos-7 cells were given combination of valproate (5 mM, 24 h) with nocodazole (10 µM), MG132 (10 µM, 12 h) with nocodazole (10 µM), and MG132 (10 µM) alone for 12 h; incubation with nocodazole was performed for 12 h before immunocytochemistry processing. Post-exposure, cells were processed for immunocytochemistry analysis; labelling was done with both ubiquitin and γ-tubulin antibodies. DAPI utilised to stain nuclei. Arrowheads in overlay images indicate co-localization of ubiquitin and γ-tubulin. *Scale bar: 20 µm.* (b) Section (a) micrographs were used for fluorescence intensity profile analysis: VPA+Noc (blue), MG132 (red), and MG132+Noc (green) obtained using ImageJ software.

Next, it was imperative for us investigating effect of valproate on cellular proteolytic machinery; thus, we conducted an additional set of experiments. Here, cells were transiently transfected with normal (EGFP-HD23Q) and expanded (EGFP-HD74Q) polyQ plasmids, and subsequent immunoblot analysis of same sets of samples using anti-GFP antibody confirmed their exogenous expression (Figure 4.3 (a)). Furthermore, immunofluorescence analysis (Figure 4.3 (b, c)) also represents the formation of polyQ inclusions.

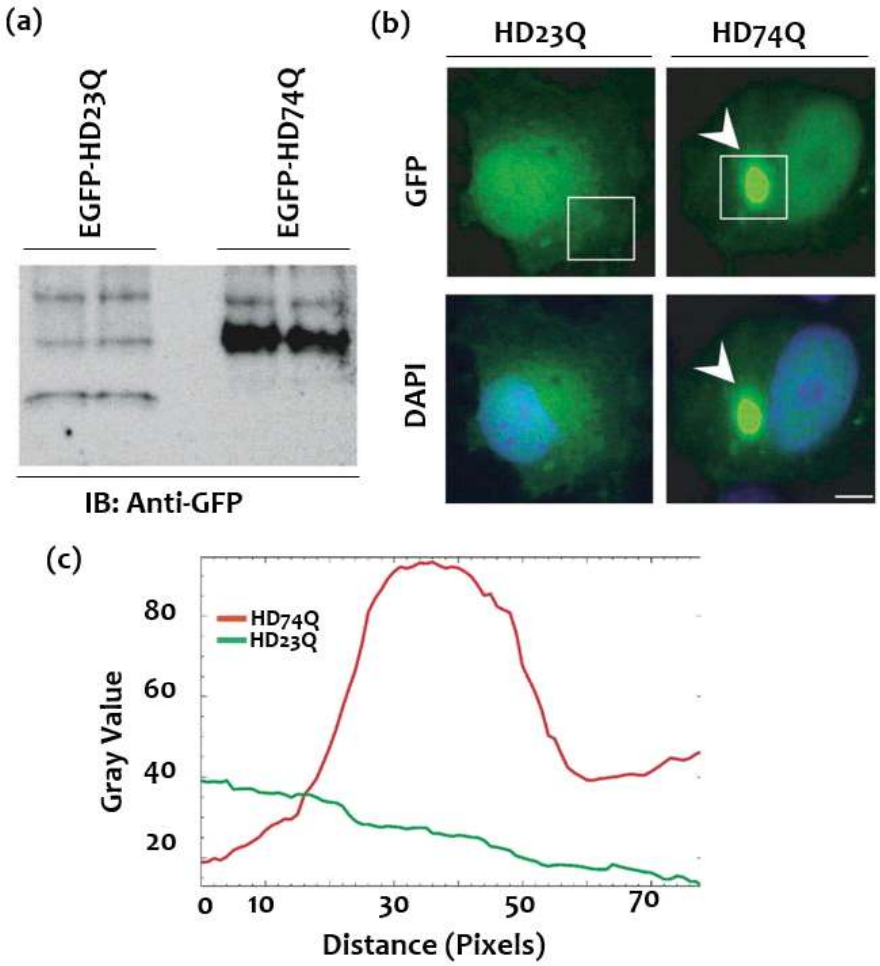

Figure 4.3: Transfection of cells with EGFP-HD23Q and EGFP-HD74Q plasmids: (a) Transfected Cos-7 cells with EGFP-HD23Q and EGFP-HD74Q plasmids and, processed for immunoblot, and probed with anti-GFP antibody. (b) Another set of cells transfected with EGFP-HD23Q and EGFP-HD74Q plasmids were fixed and imaged with a fluorescence microscope. *Scale bar: 20 µm.* (c) The fluorescence intensity plot of section (b) HD23Q (green) and HD74Q (red) obtained via ImageJ software analysis.

In similar sets of experiments, treatment of valproate and MG132 both induces accumulation of ubiquitylated higher molecular weight derivatives of polyglutamine proteins in the stacking gel portion as compared to control cells (Figure 4.4 (a, b)). Altogether, our preliminary results suggest that use of valproate may affect the proteasome functions, which further aggravates formation of intracellular ubiquitin-positive inclusions as also seen in MG132-treated cells.

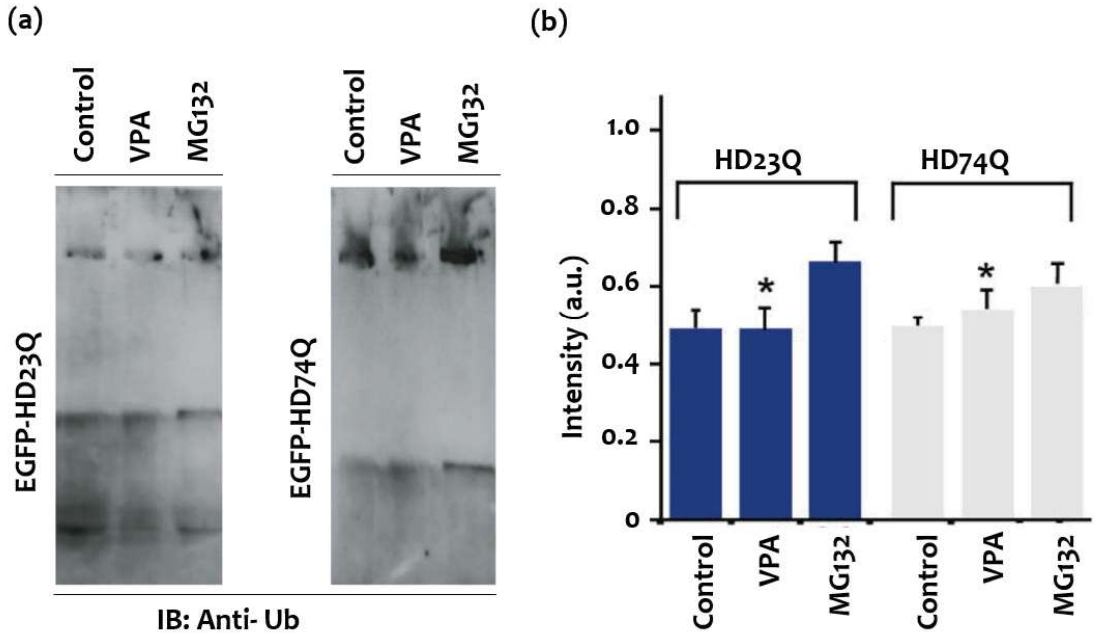

Figure 4.4: Valproate and MG132 treatment induce accumulation of ubiquitinylated high molecular weight derivatives: (a) The EGFP-HD23Q and EGFP-HD74Q plasmids transfected cells were treated with control, valproate (5 mM, 24 h), and MG132 (10 µM, 12 h), were processed for immunoblotting, and probed with ubiquitin antibody. (b) Band quantification of blots from (a) was done with ImageJ software. *p < 0.05 compared with controls.

4.1.2 Cellular Treatment of Valproate Promotes Aggregation of Polyglutamine Proteins by Impairing their Proteasomal Degradation

Previously reported findings indicate formation of aggresomes, characterized by deposition of aggregated misfolded proteins during cellular stress conditions, including altered proteasomal functions [Johnston et al., 1998; Dehvari et al., 2012]. Additionally, the accumulation of mutant huntingtin proteins at perinuclear region upon its impaired degradation through UPS is also suggested [Waelter et al., 2001]. In our initial experiments, we detected that valproate induces accumulation of ubiquitylated proteins toward MTOC sites. Most likely, impaired proteasomal functions contribute to the accumulation of abnormal ubiquitin-positive proteins at MTOC. To answer this notion in detail, it was important for us to check the possible interaction among valproate and proteasome subunits. To this end, we conducted detailed molecular *in silico* analysis to examine the interaction of valproate with β1 (Figure 4.5), β2 (Figure 4.6), and β5 (Figure 4.7) 20S proteasome subunits. After the blind molecular docking of valproic acid with catalytic subunits of 20S proteasome, β1, β2, and β5, best poses with minimum binding energy of −4.3, −4.4, and −4.3 kcal/mol, respectively were observed as shown in Figures 4.5-4.7. To visualize the 2D ligand-protein interaction and analyze interaction between proteins and small molecules, findings of *in silico* docking output were used as input together with 3D coordinates (Figures 4.5 (b), 4.6 (b), and 4.7 (b)).

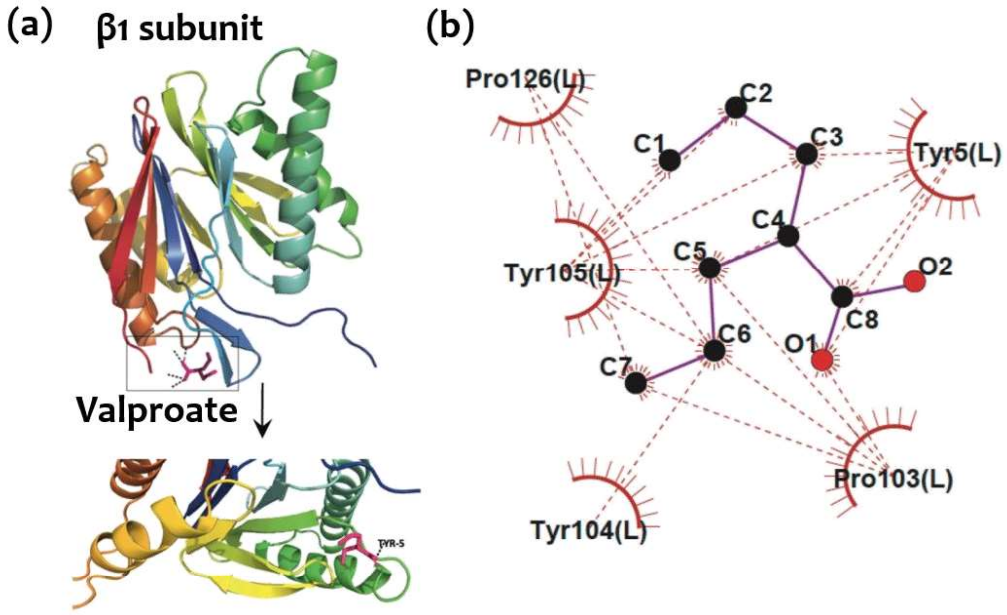

Figure 4.5: Valproate possibly interacts with proteasome β1 subunit: (a) The shown image represents the best pose having minimum binding energy after *in silico* docking of valproic acid (PubChem CID 3121) with catalytic subunit β1 of 20S proteasome (PDB 5LE5). (b) The resulting output of docking was analyzed through LigPlot+. Here, 2D ligand-protein interactions are represented, hydrophobic interactions are represented by dashed lines. Residues depicted by spoked arcs make nonbonded contacts with ligands.

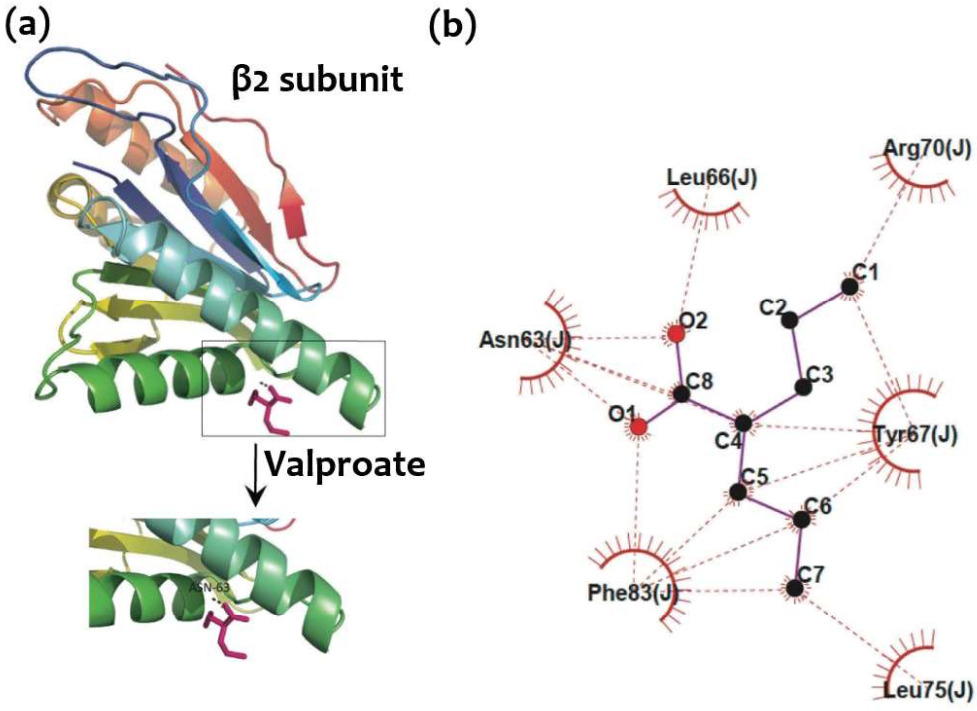

Figure 4.6: Valproate possibly interacts with β2 subunit of proteasome: (a) The shown image represents the best pose having minimum binding energy after *in silico* docking of valproic acid (PubChem CID 3121) with 20S proteasome catalytic subunit β2 (PDB 5LE5). (b) The resulting output of docking was analyzed through LigPlot+. Here, 2D ligand-protein interactions are represented, hydrophobic interactions are represented by dashed lines. Residues depicted by spoked arcs make nonbonded contacts with ligands.

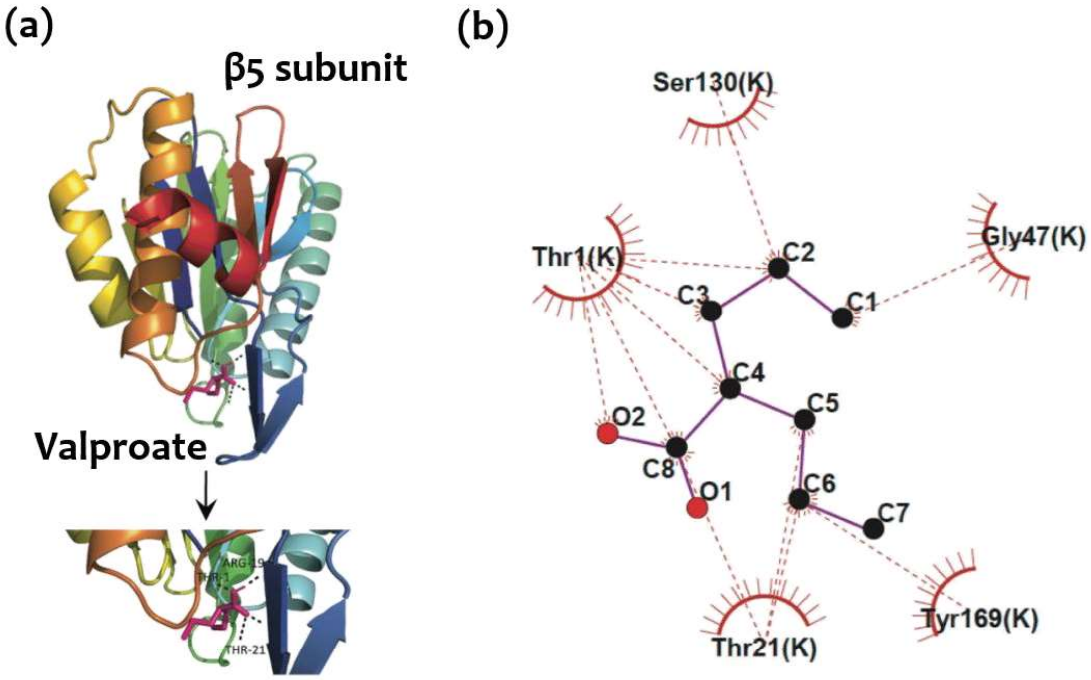

Figure 4.7: Valproate possibly interacts with β5 subunit of proteasome: (a) The shown image represents the best pose having minimum binding energy after *in silico* docking of valproic acid (PubChem CID 3121) with 20S proteasome catalytic subunit β5 (PDB 5LE5). (b) The resulting output of docking was analyzed through LigPlot+. The 2D ligand-protein interactions are represented, hydrophobic interactions are represented by dashed lines. Residues depicted by spoked arcs make nonbonded contacts with ligands.

To further confirm that valproate can induce aggregation of abnormal proteins because of proteasome dysfunction, we transiently expressed normal (EGFP-HD23Q) and expanded polyglutamine (EGFP-HD74Q) plasmid constructs in cells (Figure 4.8). Post-transfected cells were treated with valproate, and a few sets of similar cells were treated with MG132.

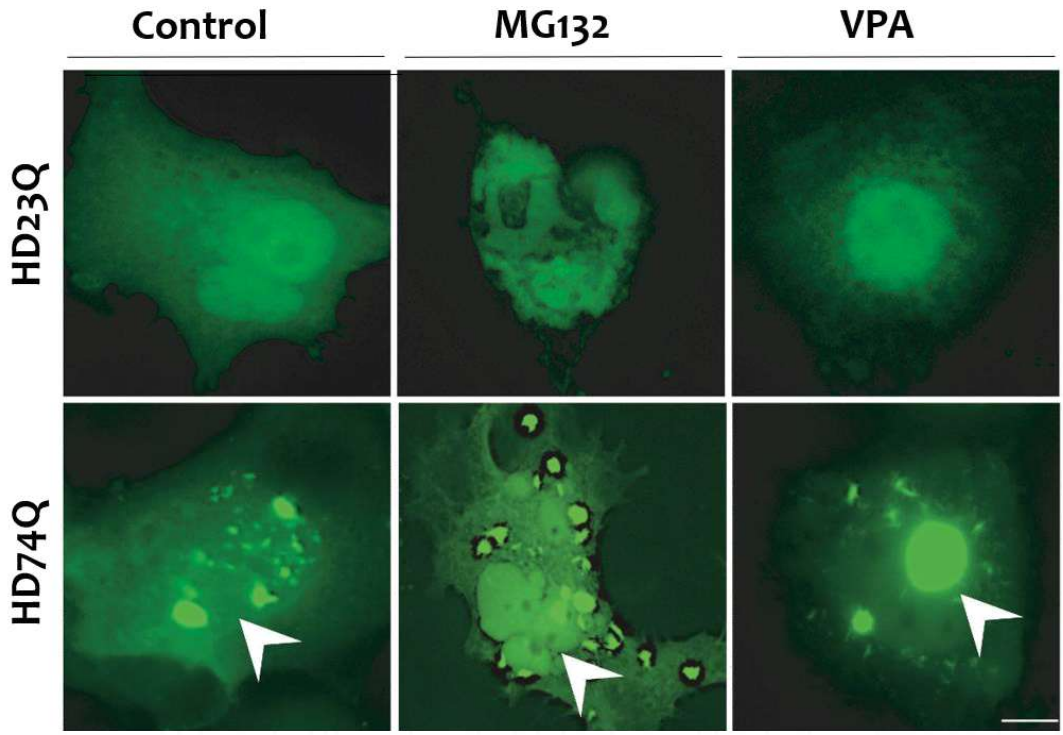

Figure 4.8: Valproate increases protein aggregate number *in vivo*: Transfected Cos-7 cells with HD74Q and HD23Q plasmids were exposed to valproate (5 mM, 24 h), control, and MG132 (10 µM, 12 h), fixed post-treatment, and were imaged with a fluorescence microscope. Arrowheads represent accumulations of indicated proteins. *Scale bar: 20 µm.*

As shown in Figure 4.9, quantitative fluorescence micrograph analysis suggests that use of valproate induces the accumulation of Huntington's disease associated expanded polyglutamine proteins actively compared to normal glutamine repeat. The same was observed in MG132-treated cells. Earlier studies suggest proteasome machinery have unique capability for degrading misfolded proteins [Meacham et al., 2001; Ciechanover and Kwon, 2015]; proteasomes also play a critical role in polyglutamine protein degradation [Chai et al., 1999; Jana et al., 2001; Jana et al., 2005]. Here, we also observed that use of valproate induces expanded polyglutamine protein accumulation, as observed in proteasomal inhibitor MG132-treated cells. These results suggest that valproate exposure induces accumulation of polyglutamine proteins, possibly via inhibiting proteasomal activities.

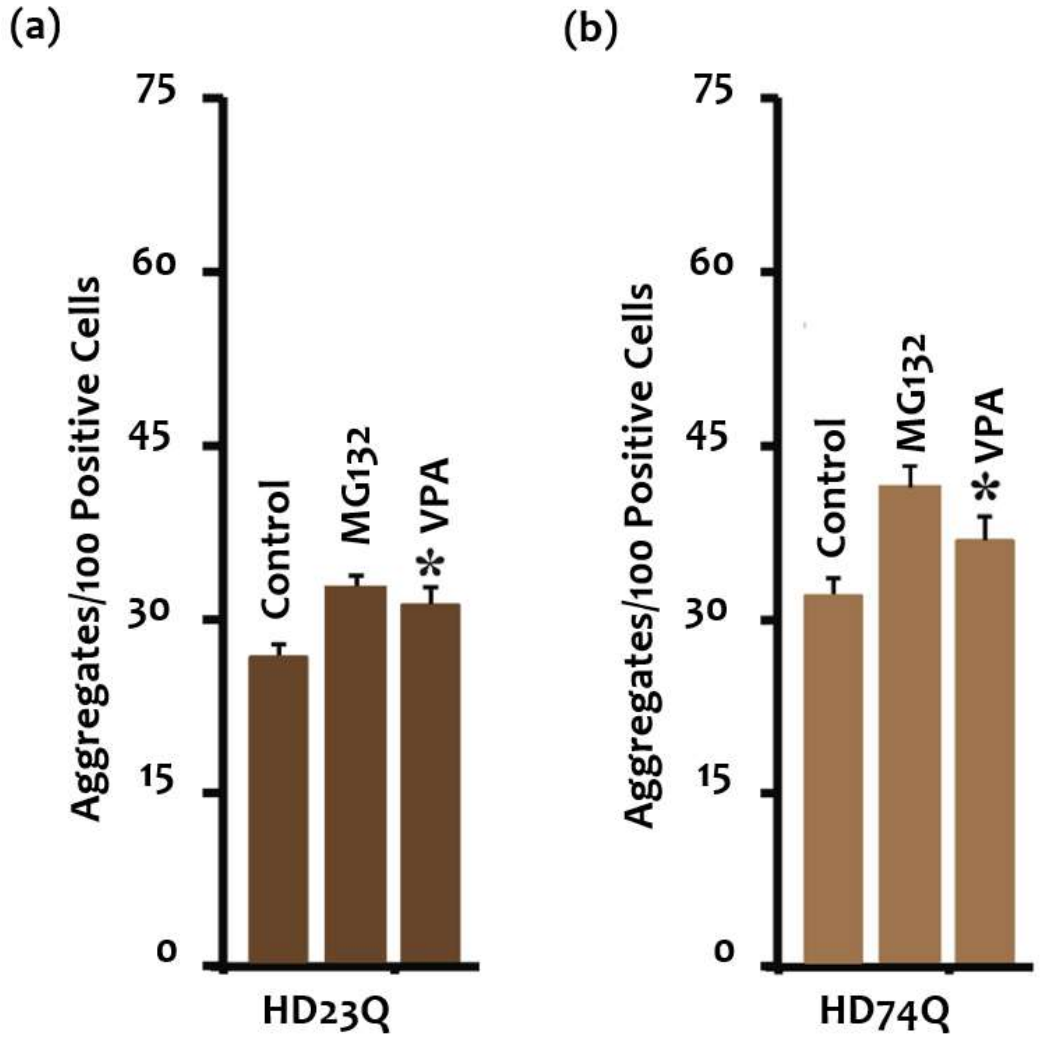

Figure 4.9: Increased aggregation of polyglutamine proteins upon valproate and MG132 treatment: Aggregates in GFP-positive cells were counted manually for EGFP-HD23Q (a) and EGFP-HD74Q (b) proteins and represented as mean ± SD obtained from three independent experiments. *p < 0.05 shows statistical significance compared with their controls.

4.1.3 Exposure of Cells to Valproate Generates Cytotoxic Effects Associated with Proteasome Dysfunction

The coordinated function of PQC mechanisms, including UPS, is critical for normal cellular health and fitness. Previous studies suggest that deregulation of PQC [Sannino and Brodsky, 2017] and impaired proteasome functions contribute to cell death. Following the analysis of possible role of valproate-mediated proteasomal dysfunction in aggregation of polyQ protein, it was important for us to assess impact of valproate on cellular viability. Therefore, we treated cells with valproate at varying time periods and concentrations, as shown in Figures 4.10 and 4.11.

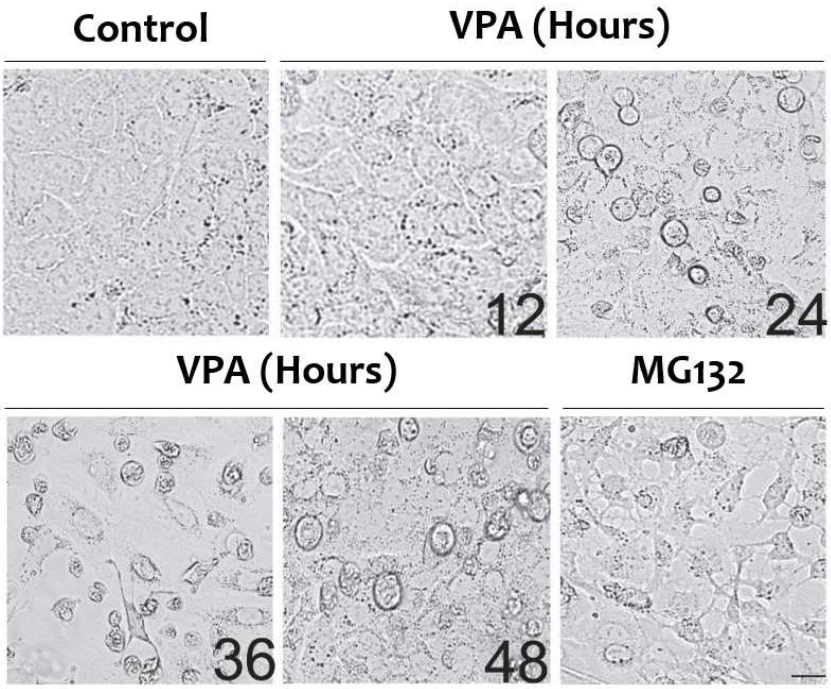

Figure 4.10: Valproate promotes cytotoxicity in time-dependent manner: Cells were given valproate for different time points, control, and MG132 (10 μM, 12 h), and images acquired with brightfield microscopy. *Scale bar: 20 μm.*

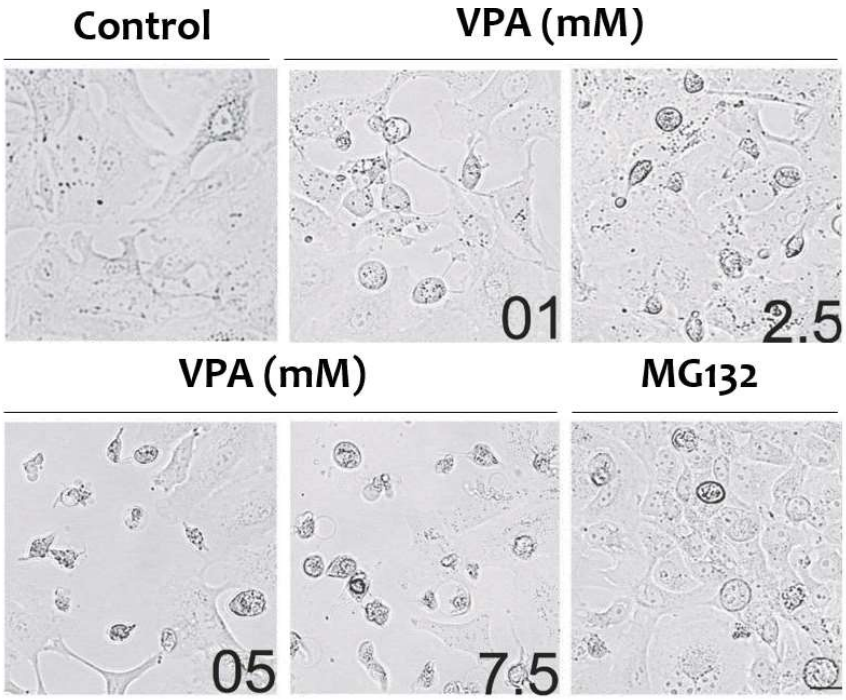

Figure 4.11: Valproate promotes cytotoxicity in concentration-dependent manner: Cells were given valproate at various concentrations, control, and MG132 (10 μM, 12 h), and imaging was done with brightfield microscopy. *Scale bar: 20 μm.*

Brightfield micrographs quantitative analysis indicates that use of valproate induces cytotoxicity at different time intervals (Figure 4.12 (a)) and various concentration levels (Figure 4.12 (b)). Earlier findings also support our current observations, which demonstrated cell death after the treatment with valproate [Phillips et al., 2003; Dragunow et al., 2006].

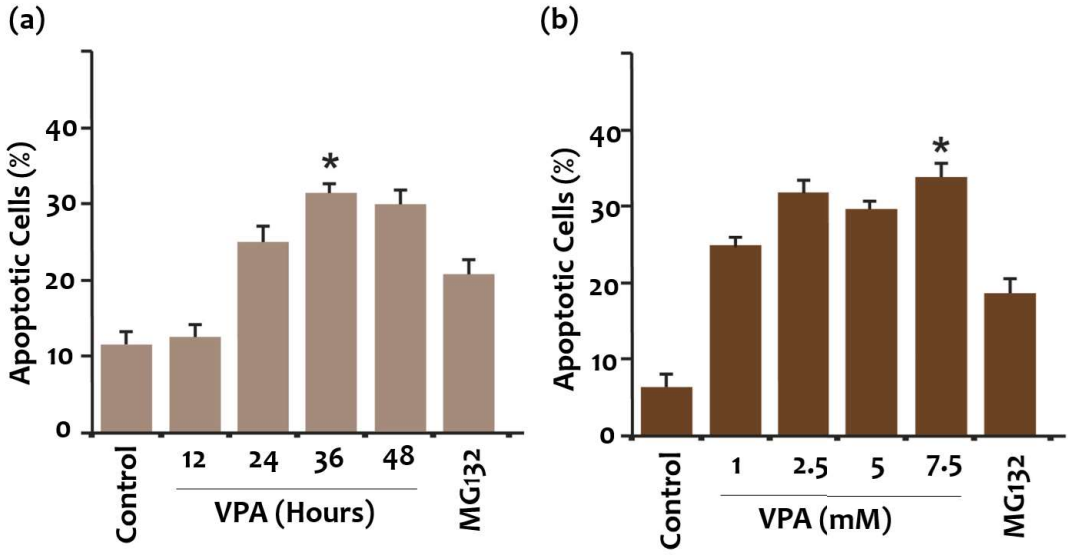

Figure 4.12: Valproate treatment enhances apoptotic cells number: Brightfield micrographs images obtained from time-dependent (a) and concentration-dependent (b) treatment of valproate were used for the apoptotic bodies quantification. Value represents mean ± SD of three experiments performed independently. *p < 0.05 represents a significant difference comparison to control.

Upon confirming the cytotoxic effect of valproate, we were motivated to explore the molecular mechanism of cellular insults mediated by valproate. Prior reports indicate that disrupted proteasome catalytic activities contribute to proteotoxic insults and elevate cell death [Obeng et al., 2006; Amanullah et al., 2017a; Nunes and Annunziata, 2017]. Subsequently, we investigated whether valproate treatment leads to proteasome inhibition by analyzing its impact on both chymotrypsin-like (Figure 4.13 (a)) and post glutamyl peptide hydrolysing (PGPH)-like catalytic (Figure 4.13 (b)) activities.

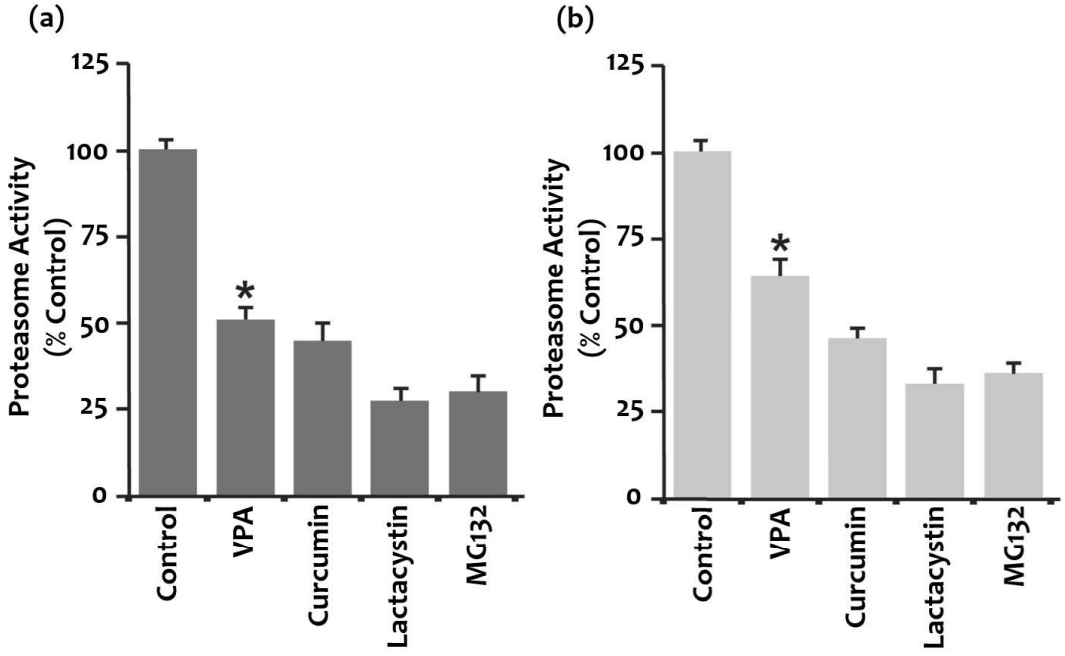

Figure 4.13: Valproate promotes proteasome dysfunction: Sets of A549 cells were given valproate (5 mM) or control for 24 h, and known proteasome inhibitors lactacystin (10 µM, 12 h), curcumin (100 µM, 8 h), and MG132 (10 µM, 12 h). Following treatment, cells were lysed and proteasome assay was done. Values in bar graphs for chymotrypsin-like (a) and PGPH-like (b) activities are mean ± SD and obtained from three independent experiments. *p < 0.05 represents a significant difference in comparison to control.

Next, we observed treatment of valproate induces p27 (Kip1) accumulation and reduction in levels of procaspase-3, indicating possible induction of apoptotic signaling pathway (Figure 4.14). Overall, our proteasome activity assay results suggest that use of valproate inhibits proteasome catalytic activities. Furthermore, a similar proteasome inhibition effect was observed when using various other known proteasome inhibitors in the same experiment. Thus, these observations indicated that valproate can induce proteasome inhibition, possibly contributing to cell death and proteotoxic insults.

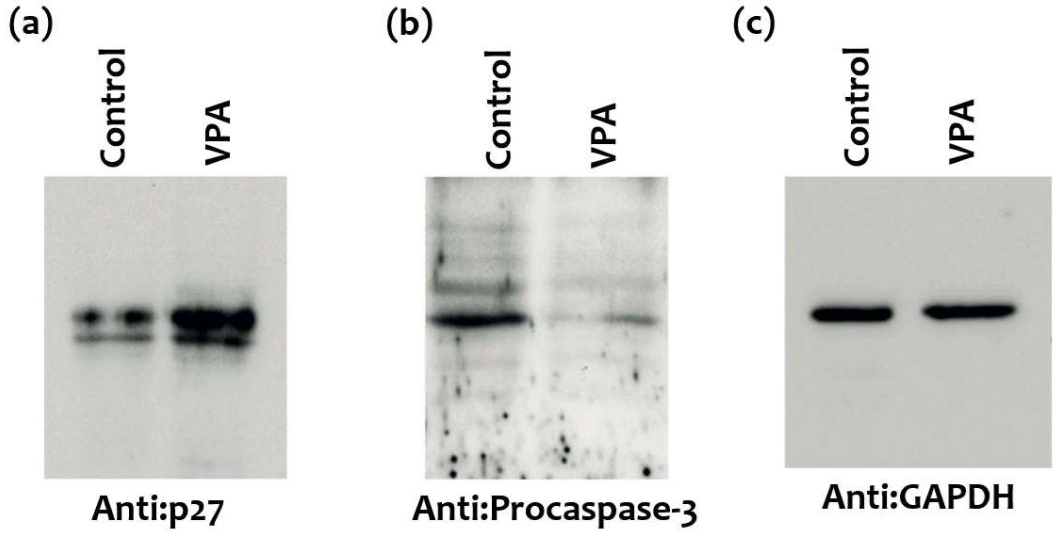

Figure 4.14: Valproate promotes p27 accumulation and triggers apoptosis signaling: Cos-7 cells were valproate (30 mM) or control treated for 36 h. Following completion of the treatment period, cell samples were utilized for immunoblot via anti-p27 (a), anti-procaspase-3 (b), and anti-GAPDH (c) antibodies.

4.1.4 Valproate Inhibits Proteasome Catalytic Activities

Cellular proteostasis requires tight control of synthesis of protein and degradation of abnormal proteins without affecting routine course of cellular activities [Jayaraj et al., 2020]. Improper degradation of abnormal proteins induces stress response and can also indicate proteasome functional impairment in the cells [Bush et al., 1997; Sherman and Goldberg, 2001]. Our present findings suggest that exposure to valproate inhibits proteasome function and generates cellular toxicity. After confirming comparable proteasome inhibitory effects of valproate to other known proteasome inhibitors, we decided to analyze time and concentration-dependent effect of valproate on the catalytic activities of proteasomes. For this, we treated cells with valproate at various concentrations and for different time points, as shown in Figures 4.15 and 4.16; post-treated cell samples were used to perform proteasome activity assay. Our results indicate that exposure of valproate in a concentration-dependent manner suppressed both chymotrypsin-like (Figure 4.15 (a)) and PGPH-like (Figure 4.15 (b)) protease activities.

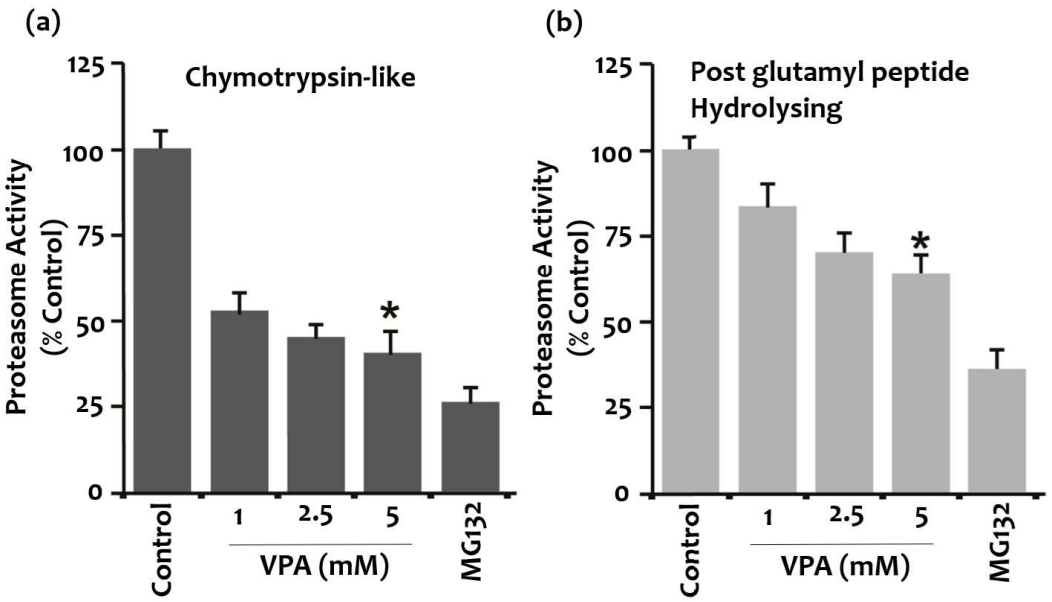

Figure 4.15: Valproate treatment depletes proteasome activities in concentration-dependent manner: A549 cells were given valproate at different concentrations (1, 2.5, 5 mM for 24 h). Sets of cells were given control or MG132 (10 μM for 12 h) as indicated. Post-treatment, cells were harvested and processed in preparation for proteasome activity assay. Effect of valproate on chymotrypsin-like (a) and PGPH-like (b) activities of proteasome were measured. Values represent mean ± SD and obtained from three independent experiments. *p < 0.05 compared with control.

To further confirm the effect of valproate, we also treated cells for different time intervals and performed both chymotrypsin-like (Figure 4.16 (a)) and PGPH-like (Figure 4.16 (b)) proteasome activity assays. These results suggest that treatment of valproate reduces proteasome catalytic activities at different concentrations and exposed time periods.

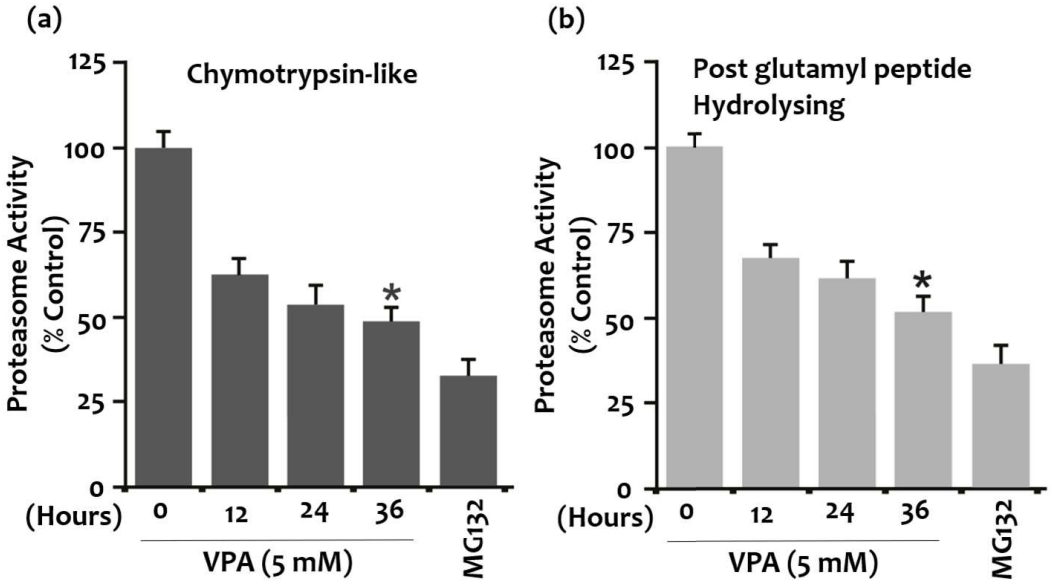

Figure 4.16: Valproate treatment depletes proteasome activities in time-dependent manner: A549 cells were given valproate for different time points (12, 24, 36 h at 5 mM). Sets of cells were also exposed to control or Valproate or MG132 (10 μM for 12 h). Post-treatment, cells were harvested and proteasome activity assay was executed. Effect of valproate on chymotrypsin-like (a) and PGPH-like (b) proteasomal activities were measured. Values represent mean ± SD and obtained from three independent experiments. *p < 0.05 compared with control.

Next, we further verify the effect of valproate through propidium iodide staining, and observed that treatment of valproate induces apoptosis in cells (Figure 4.17 (a)), overexpression of chaperone-dependent E3 ligase CHIP alleviates stress conditions against valproate treatment in cells (Figure 4.17 (a, b)). Depleted functional activities of proteasome after valproate treatment indicate that it is most likely linked with induced accumulation of ubiquitylated proteins and cell death.

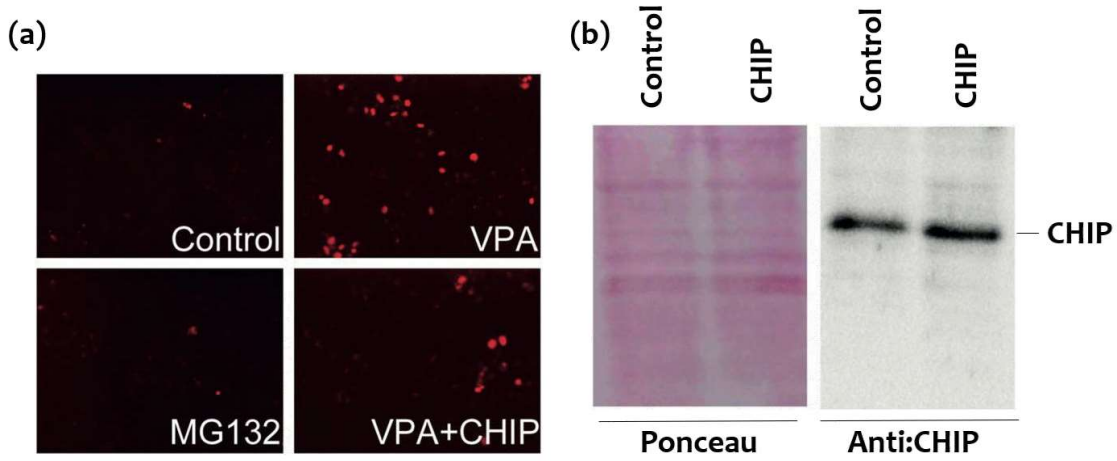

Figure 4.17: Valproate treatment can promote cell death: (a) Cos-7 cells exposed to control, valproate (10 mM, 24 h), or MG132 (10 μM, 12 h). Some sets of cells were transfected with CHIP plasmid, and post 24 h of transfection, cells underwent treatment with valproate (10 mM, 24 h). Following treatment period completion, propidium iodide staining was done and cells visualized with fluorescence microscope. (b) To confirm exogenous expression of CHIP, 24 h post-transfection, Cos-7 cells were harvested and lysed for immunoblot analysis utilizing antibody anti-CHIP. The left panel shows ponceau staining of corresponding blot at the right.

4.1.5 Valproate Triggered Proteasome Inhibition Induces Accumulation of Proteasome Substrates

The inhibition of proteasome functions demonstrates potential for possible anti-tumor therapeutic strategy. However, the discovery of effective proteasome inhibitors presents a critical challenge. In this study, we show that valproate exhibits proteasome inhibition capability and also induces the accumulation of ubiquitylated abnormal proteins. Next, it was vital for us to check impact of valproate on cellular health and stress response after its treatment. Since we observed the cytotoxic effects of valproate through cellular morphological insults in a concentration and time-dependent manner, we conducted another experiment to compare and verify the effects of valproate with those of other known proteasome inhibitors on cell viability. As shown in Figure 4.18 (a), use of valproate induces cell death, similarly observed with other known proteasomal inhibitors. Further, to explore any potential impact of valproate on genomic DNA integrity, we performed an experiment where valproate was observed to increase DNA fragmentation in a concentration-dependent manner (Figure 4.18 (b)).

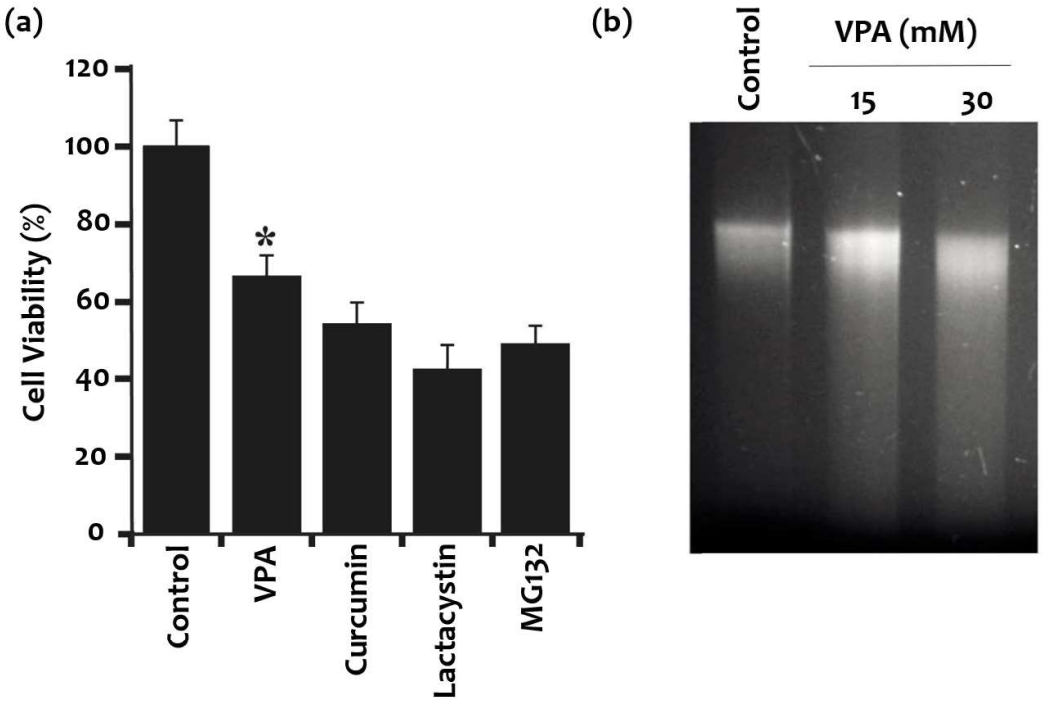

Figure 4.18: Valproate treatment reduces cellular viability and promotes DNA fragmentation: (a) Cells treated with control, valproate (5 mM for 24 h), and known inhibitors of proteasome, lactacystin (10 μM for 12 h), curcumin (100 μM for 8 h), and MG132 (10 μM for 12 h) and cell viability assay was executed. Cell viability plot values are mean ± SD and obtained from three independent experiments done in triplicates. *$p < 0.05$ compared with control. (b) Sets of Cos-7 cells were given different doses (15 and 30 mM) of valproate and control for 36 h. Genomic DNA was isolated from collected cells and electrophoresed on agarose gel, and DNA fragmentation pattern was observed and imaged.

To further validate the possible inhibitory action of valproate on proteasomal catalytic activities, we did immunofluorescence experiment to assess valproate effect on levels of proteasome substrates (Figure 4.19). Cells were transfected transiently with various plasmid constructs (encoding CFTR-ΔF508, d1EGFP, and GFP-wtCAT). Post-transfected cells were treated with valproate and MG132; immunofluorescence analysis revealed that treatment of valproate elevates accumulation of exogenous proteins in inclusions form near nuclear peripheral sites. These results suggest that disrupted proteasome activity potentially leads to the induced aggregation of critical substrates of proteasome.

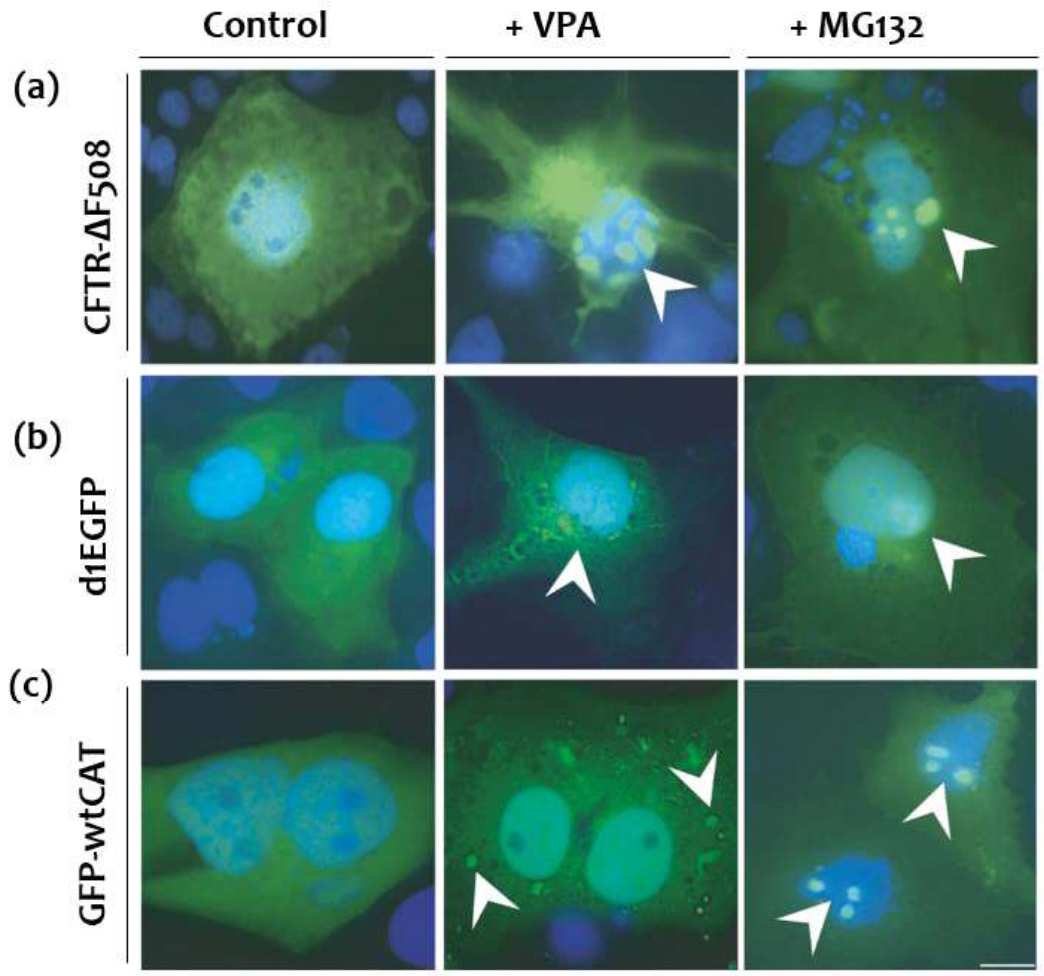

Figure 4.19: Valproate treatment enhances cellular protein accumulation in proteasome-dependent manner: Separate sets of Cos-7 cells were transfected with pEGFP-C1-CFTR-ΔF508 (a), pd1EGFP (b), and GFP-wtCAT (c) plasmids and afterward exposed to valproate (5 mM for 24 h), MG132 (10 μM for 12 h), and control. Post-treatment, cells were fixed, and nuclei were stained with DAPI. Imaging was done with epifluorescence microscope where arrowheads represent accumulations of indicated proteins. *Scale bar: 20 μm.*

Next, with the help of cell viability assay, we observed induced cell death in presence of valproate, and the effect was reverted by the addition of N-acetylcysteine (an antioxidant) or exogenous expression of molecular chaperone Hsp70 (Figure 4.20 (a)). To understand the possible role of valproate that can trigger proteasome inhibition and induce apoptosis, we used valproate in a concentration-dependent manner and performed a TUNEL assay. As represented in Figure 4.20 (b), results agree with our observations earlier for valproate treatment in a concentration-dependent manner elevates apoptosis, possibly because of depleted proteasome catalytic activities.

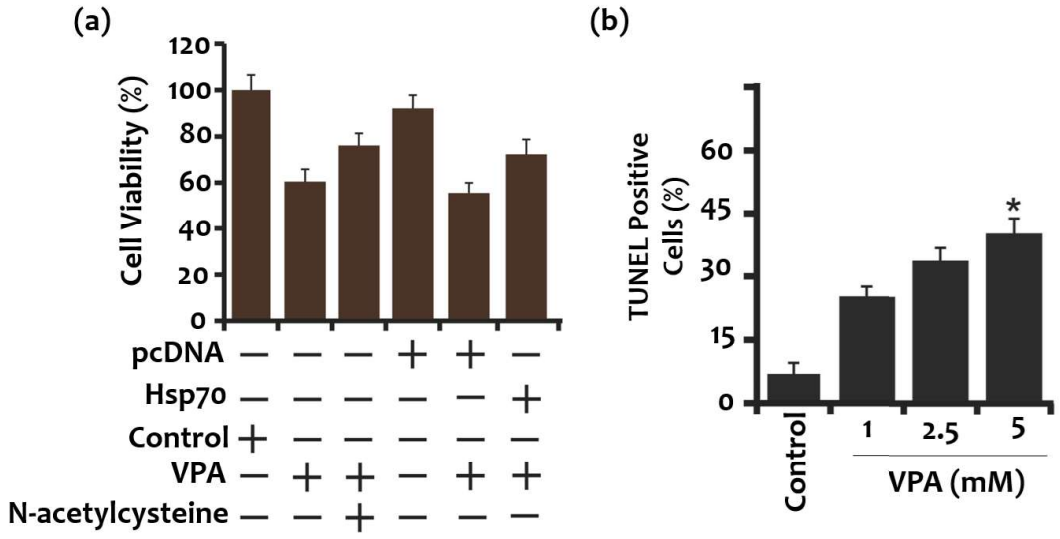

Figure 4.20: Valproate promotes cell death: Sets of cells were treated with valproate (5 mM) with or without N-acetylcysteine (5mM) treatment as well as with or without Hsp70 transfection. pcDNA was used as control for Hsp70 plasmid. Cell viability assay was done on treatment time completion, and values represented are mean ± SD obtained from three independent experiments done in triplicates. (b) A549 cells treated with control and valproate in a dose-dependent fashion for 24 h and were subjected to TUNEL assay. Plot values of TUNEL positive cells are mean ± SD obtained from three independent experiments. *$p < 0.05$ compared with control.

4.1.6 Accumulation of Pro-Apoptotic Proteins During Valproate-Induced Proteasome Dysfunction

Cellular homeostasis is a critical mechanism and it maintains a balance between forming new proteins and degrading older proteins [Jayaraj et al., 2020]. Moreover, disturbed cellular homeostasis leads to several critical disorders, including improper cellular proliferation [Panieri and Santoro, 2016]. Cancer cells require increased protein production, and their rapid degradation is also essential, making proteasome-dependent proteolysis a critical target for designing new molecules capable of inhibiting proteasome functions. Earlier studies suggested that because of depleted proteasome activities, disturbed degradation of proteins related to cell cycle (p53, p27, and p21) leads to apoptosis [Almond and Cohen, 2002; Nunes and Annunziata, 2017; do Patrocinio et al., 2022]. Still, the mechanistic understanding of apoptosis induction in tumor cells through proteasome inhibition is still challenging, necessitating further deep investigation. After examining the inhibitory action of valproate on proteasome activity, we were further interested in exploring its effect on the different proteasome substrates, including cell cycle and apoptosis-associated proteins. So, we treated cells with valproate and MG132; post-treated cells were utilised for detailed immunocytochemistry experiments via various antibodies (Figures 4.21 and 4.22). These results suggest that use of valproate induced accumulation of various pro-apoptotic proteins, most likely due to valproate-mediated proteasome dysfunction. Results in similar vein were observed upon cells treatment with MG132. Earlier reports also suggest that treatment of valproate modulates levels of p53 [Di Renzo et al., 2010], p27 [Tung and Winn, 2011], and p21 [Paradis and Hales, 2015]. These results and earlier studies support that proteasome inhibition can serve as a potential anti-tumor agent because their apoptosis-inducing capability may emerge as a useful therapeutic strategy against deregulated cell proliferation.

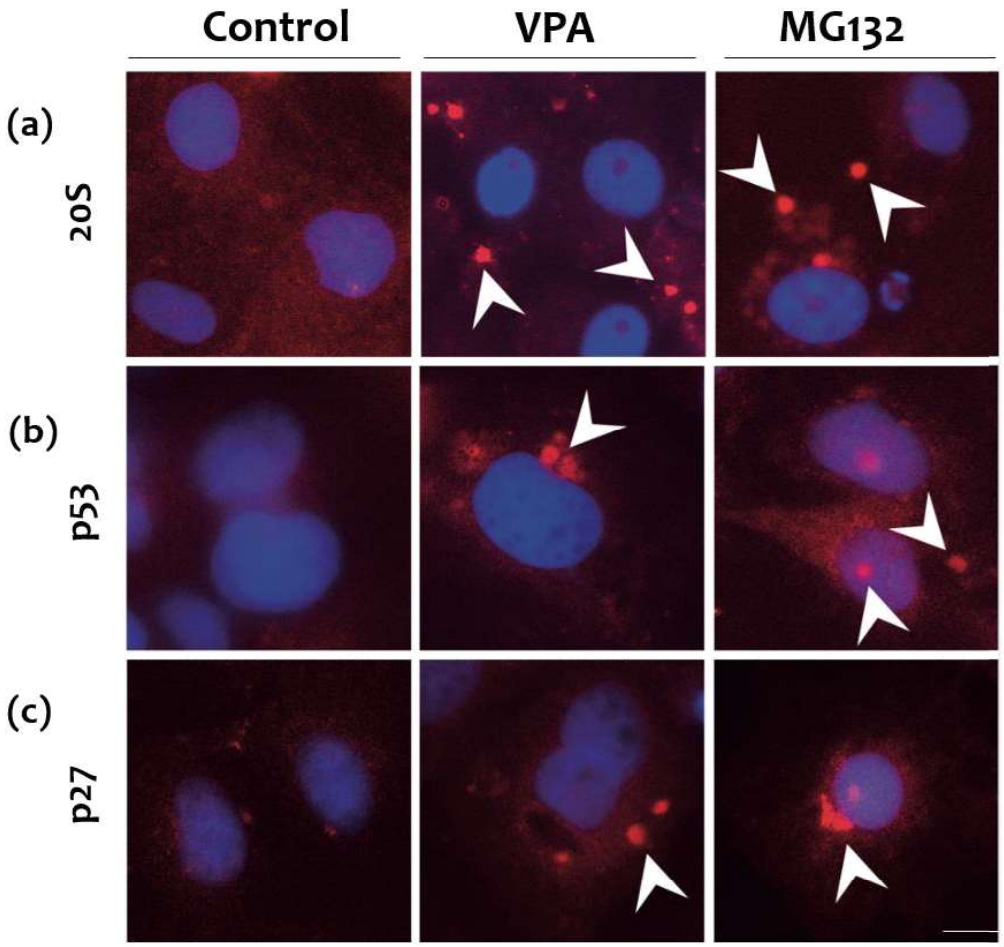

Figure 4.21: Exposure of cells to valproate induces accumulation of cell cycle and cell death-linked proteins p53 and p27: Cos-7 cells were exposed to control, valproate (5 mM for 24 h), and MG132 (10 μM for 12 h). Treated cells were processed for immunocytochemistry and probed with antibodies against (a) 20S proteasome, (b) p53, and (c) p27. DAPI utilized to stain nuclei (blue). Imaging was done with epifluorescence microscope. Arrowheads represent accumulation of indicated proteins at specific cellular locations. *Scale bar: 20 μm.*

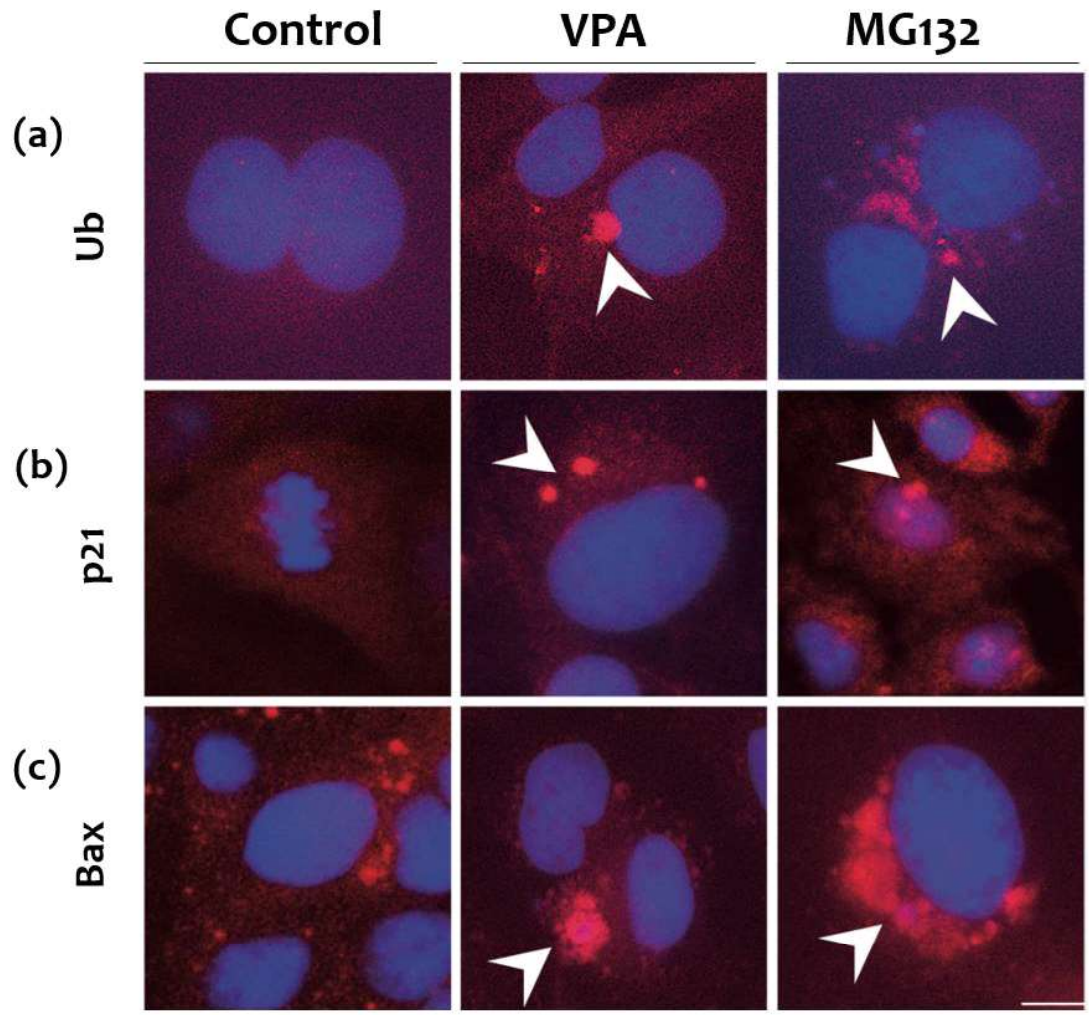

Figure 4.22: Exposure of cells to valproate induces accumulation of cell cycle and cell death-linked proteins p21 and Bax: Cos-7 cells were exposed to control, valproate (5 mM for 24 h), and MG132 (10 μM for 12 h). Treated cells were processed for immunocytochemistry and probed with antibodies for (a) Ub, (b) p21, and (c) Bax. DAPI utilized to stain nuclei (blue). Imaging with epifluorescence microscope was executed. Arrowheads represent accumulation of indicated proteins at specific cellular locations. *Scale bar: 20 μm.*

4.1.7 Valproate Triggered Proteasome Inhibition Leads to Mitochondrial Dysfunction and Cellular Insults

In contrast to normal cells, tumor cells necessitate substantial clearance and degradation of aberrant proteins produced by dysregulated signaling pathways and gene transcription [Lin et al., 2012; Chen et al., 2017], which makes cancer cells more dependent on intracellular proteolytic machinery such as proteasome complex. Despite the long-standing recognition of proteasome complex as a promising therapeutic target for inducing apoptosis in cancer cells, effective targeting of this complex remains challenging. Prior studies have reported disturbed mitochondrial homeostasis resulting from proteasome inhibition [Sullivan et al., 2004; Maharjan et al., 2014]. In our study, we observed depleted proteasome catalytic activities following valproate treatment, prompting us to investigate any potential mitochondrial abnormalities resulting from valproate-mediated proteasomal disturbances. To check this notion, we treated cells with valproate and MG132, and as shown in Figure 4.23 (a), immunofluorescence observations and quantitative analysis (Figure 4.23 (b)) using antibody anti-cytochrome *c*, we demonstrated release of cytochrome *c* into cytoplasmic compartments in valproate and MG132-exposed cells compared to control samples. The cytosolic release of cytochrome *c* suggests

potential involvement of impaired mitochondria-mediated apoptosis cell death resulting from valproate treatment.

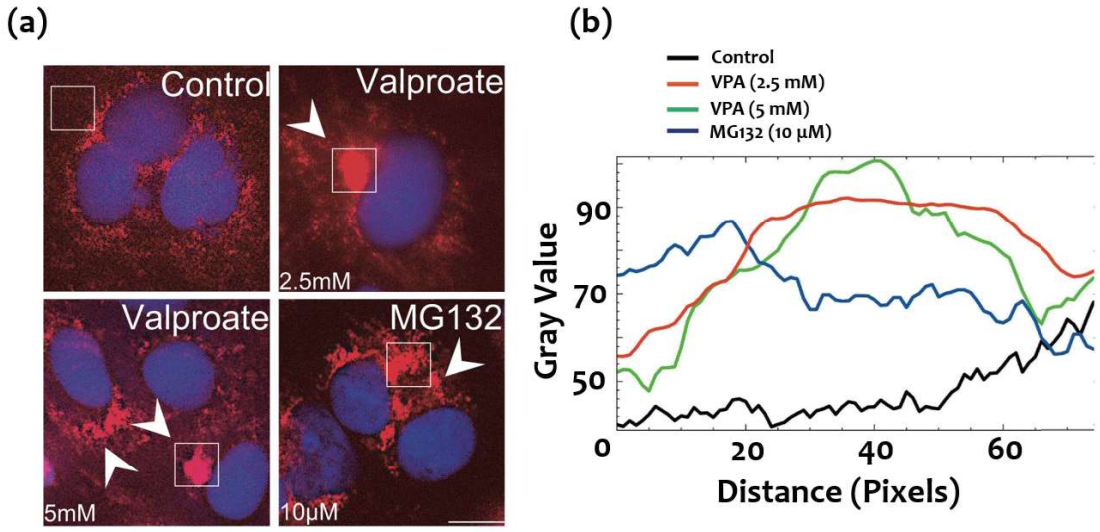

Figure 4.23: Valproate treatment promotes cytochrome c release: (a) Concentration-dependent treatment of Cos-7 cells was done with valproate for 24 h, and some sets of cells were given control or MG132 (10 µM for 12 h). These cells were prepared for immunocytochemistry, treated with anti-cytochrome *c* antibody (red), and imaged with epifluorescence microscope. DAPI utilized to stain nuclei (blue). *Scale bar: 20 µm*. (b) Fluorescence intensity profile graphs of section (a): control (black), valproate 2.5 mM (red), valproate 5 mM (green), and MG132 10 µM (blue) obtained using ImageJ software. Represented fluorescence intensity graphs were plotted along the line of internal box.

These findings prompt us to observe other possible proapoptotic morphological hallmarks in the valproate-treated cells. Staining of nuclei with DAPI of valproate and MG132-treated cells indicates cytoplasmic shrinkage, and DAPI-stained progressive apoptotic and fragmented nuclei (Figure 4.24).

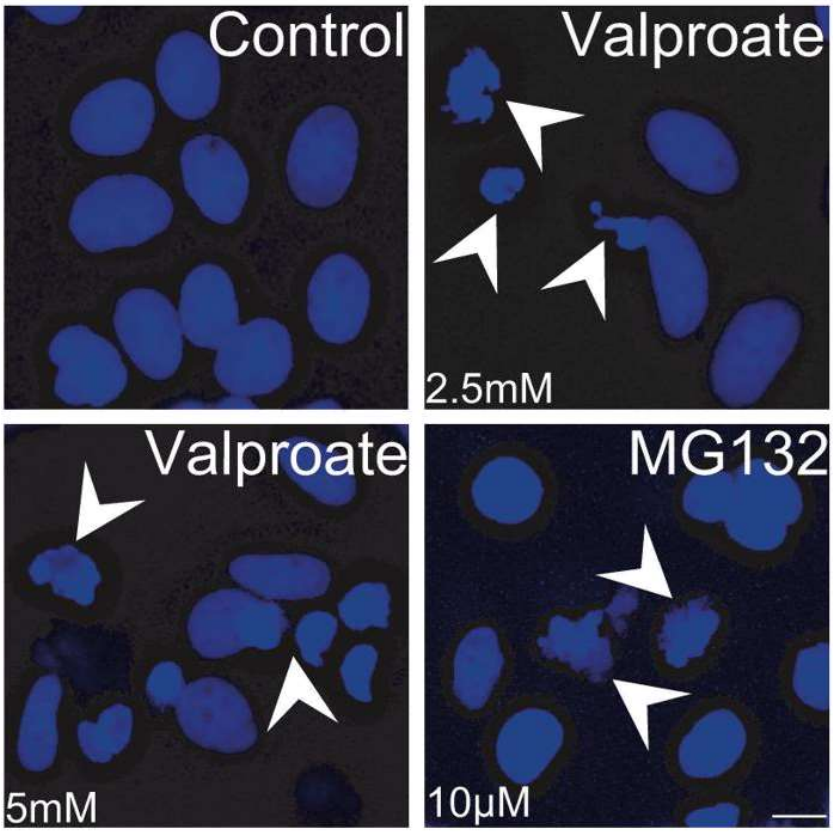

Figure 4.24: Valproate treatment damages nuclear architecture: A549 cells treated similar as Figure 4.23 (a) were fixed after treatment and stained with DAPI, and mounted. Imaging was done with fluorescence microscope where arrowheads indicate nuclear morphological changes. *Scale bar: 20 μm*.

Previously, it is observed that proteasome inhibitors suppressed NF-κB activation, which may possibly be due to overaccumulation of inhibitor of nuclear factor kappa B (IκBα) [Palombella et al., 1994; Almond and Cohen, 2002]. Our current findings demonstrate that valproate treatment can affect proteasome functions, therefore it was critical for us to know, whether it can also affect NF-κB activation. To check this, we performed a detailed luciferase assay as described in Methods section, and we found that use of valproate can also reduce the activation of NF-κB (Figure 4.25). Interestingly, earlier reports on effect of valproate on NF-κB activity also exist, where valproate is indicated to reduce NF-κB activity, possibly involving phosphorylated IκBα and STAT1 in different cell types [Schwertheim et al., 2014; Chen et al., 2018].

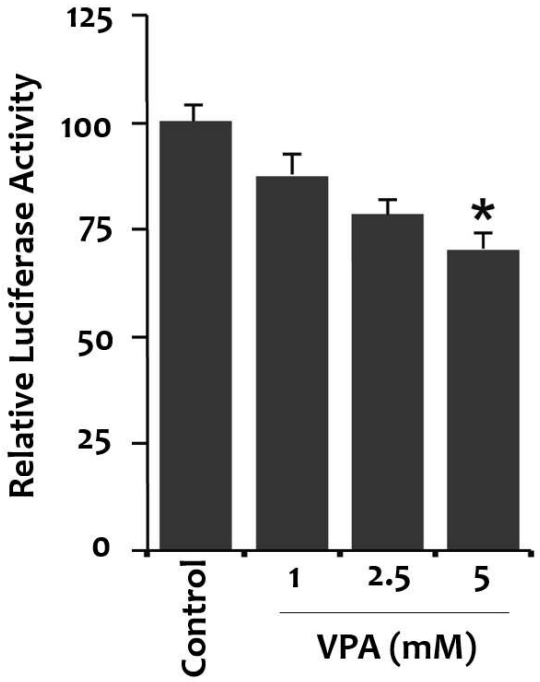

Figure 4.25: Valproate treatment reduces NF-κB activity: A549 cells co-transfected with NF-κB-Luc and pRL-SV40 plasmids, treated with valproate in a dose-dependent (1, 2.5, and 5 mM) manner for 24 h, and subjected to luciferase activity assay. Values in plot are mean ± SD and obtained from three independent experiments. *p < 0.05 compared with control.

Most studied proteasome inhibitors demonstrate anti-tumor activity and potential to induce cytotoxicity and apoptosis in cancer cells [Adams, 2004]. We also observed that the treatment of valproate reduces cell viability in both concentration (Figure 4.26 (a)) and time-dependent (Figure 4.26 (b)) manner. These findings underscore the potential of valproate to induce cell death, and as hypothesized earlier, use of a combination of proteasome inhibition with other anti-proliferative agents can play a significant role in overcoming the high survival and resistance in tumor cells [Coronel et al., 2011; Scherpereel et al., 2011].

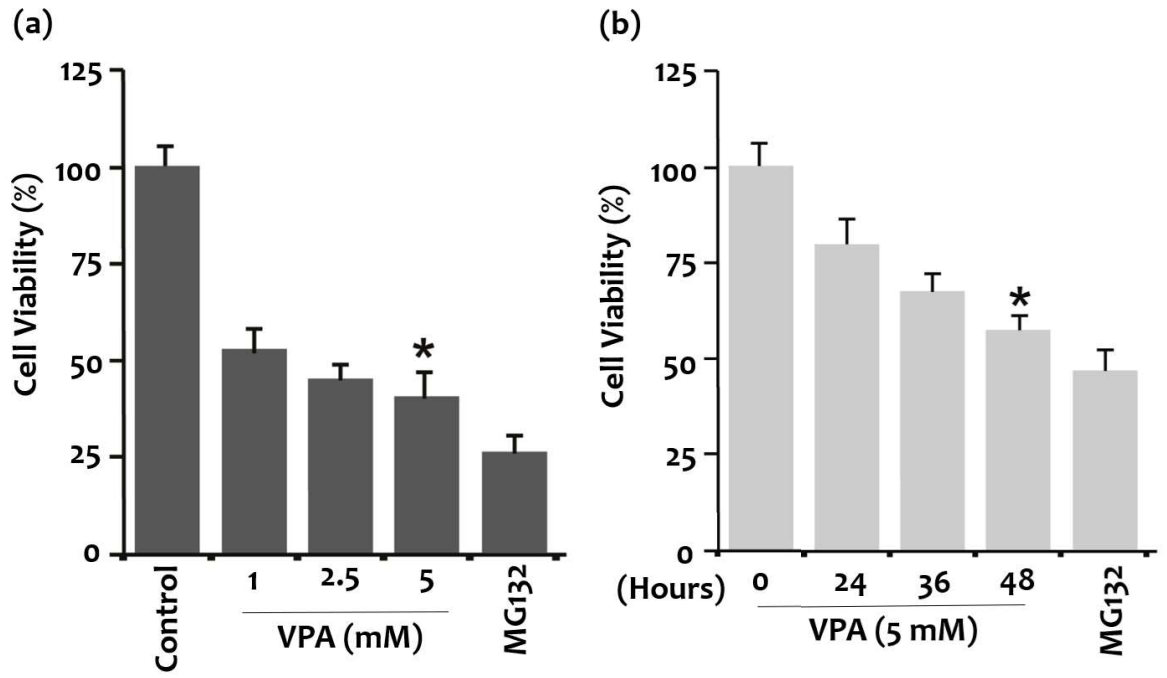

Figure 4.26: Valproate reduces viability of cells in concentration- and time-dependent fashion: Cell viability assay was performed on cells treated with valproate (indicated concentrations for 24 h and indicated time points at 5 mM), control, and MG132 (10 µM, 12 h). Values in plots (a-b) are mean ± SD and obtained from three independent experiments. *p < 0.05 compared with control.

4.1.8 Valproate Associated Mitochondrial Abnormalities and Apoptosis Induction

New proteasome inhibitor development is required to inhibit proteasome functions in tumor cells with higher effectiveness to face their molecular resistance against many existing chemotherapeutic drugs [Holohan et al., 2013; Bo Kim, 2021]. Use of proteasome inhibitors with other approved anti-proliferative drugs can be a promising therapeutic strategy against improper cellular proliferation. Our results suggest that valproate can affect proteasome catalytic activities that may cause cytotoxicity and can be useful to induce proapoptotic events in cells. Following our findings of cytochrome *c* release into the cytoplasmic compartment in the presence of valproate, we aim to further validate the mitochondrial damage caused by valproate treatment. To check this, we executed another experiment, where cells were given valproate at multiple concentrations and then JC-1 staining was executed, which indicates mitochondrial membrane potential [Smiley et al., 1991]. As shown in Figure 4.27A–E, exposure of valproate in manner depending upon concentration demonstrates the shifting of red fluorescence (J aggregates) to green fluorescence (JC-1 monomers) emission, which suggests the depolarization of mitochondrial membranes in cells and overall indicates depleted cellular health. The release of cytochrome *c* and mitochondrial membrane potential changes (depolarization) suggest the potential cytotoxic and pro-apoptotic effect of valproate, possibly mediated through the impaired proteasome functions.

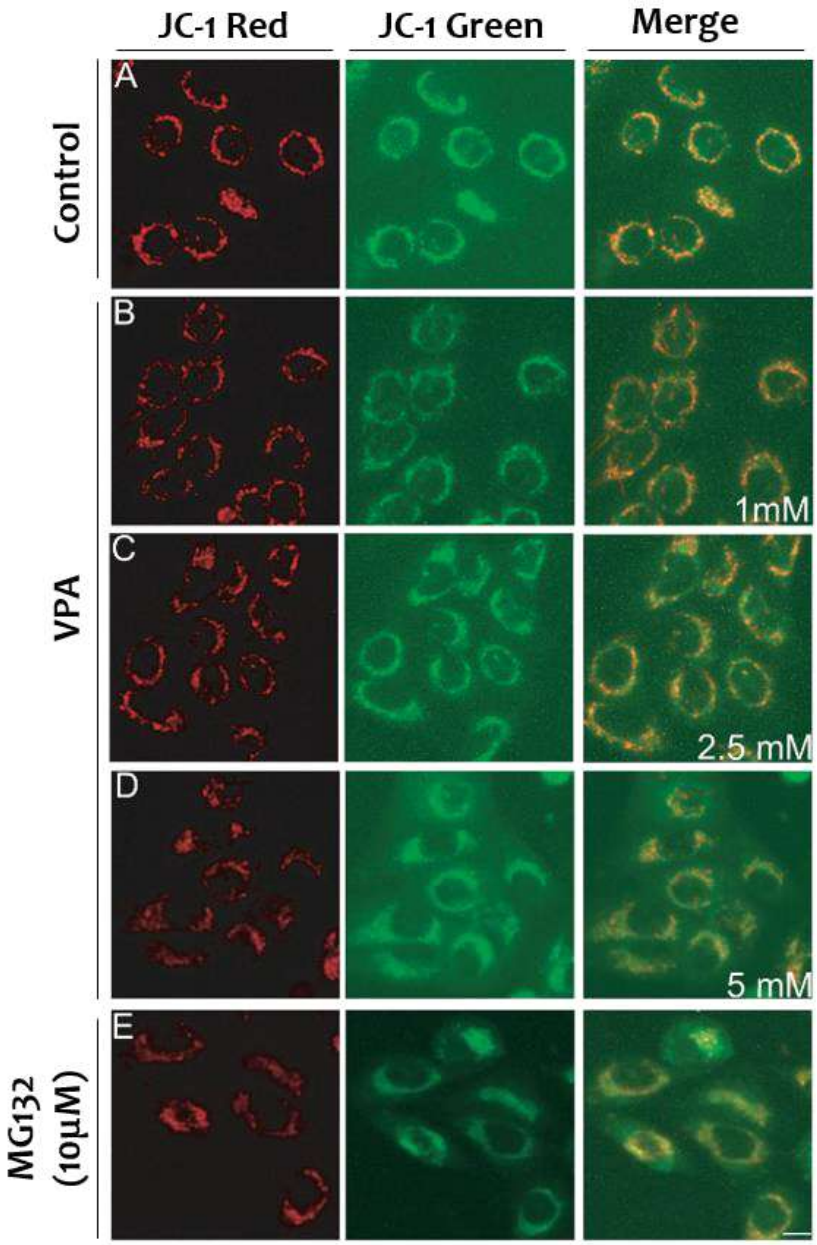

Figure 4.27: Treatment with valproate disturbs mitochondrial membrane potential: (A–E) A549 cells were given valproate with shown concentrations for 24 h; separate sets of cells were given MG132 (10 μM for 12 h) or control. Post-treatment, these cells were incubated with JC-1 (5 μM, 45 min) for mitochondria staining and imaged with fluorescence microscope. *Scale bar: 20 μm.*

To further confirm valproate effect on viability of cells, we performed Annexin V-FITC / PI assay. Here, we observe the role of valproate on apoptosis induced cell death; this assay was performed on A549 cells following treatment of 5 mM valproate. As shown in Figures 4.28 and 4.29, valproate treatment for 24 h did not exhibit much impact on cell death. However, following 48 h of treatment, there is an enhanced apoptosis of A549 cells compared with control cells, which was evident from the significantly increased percentage of late apoptotic cells (Annexin V-FITC + PI +) in comparison to control cells.

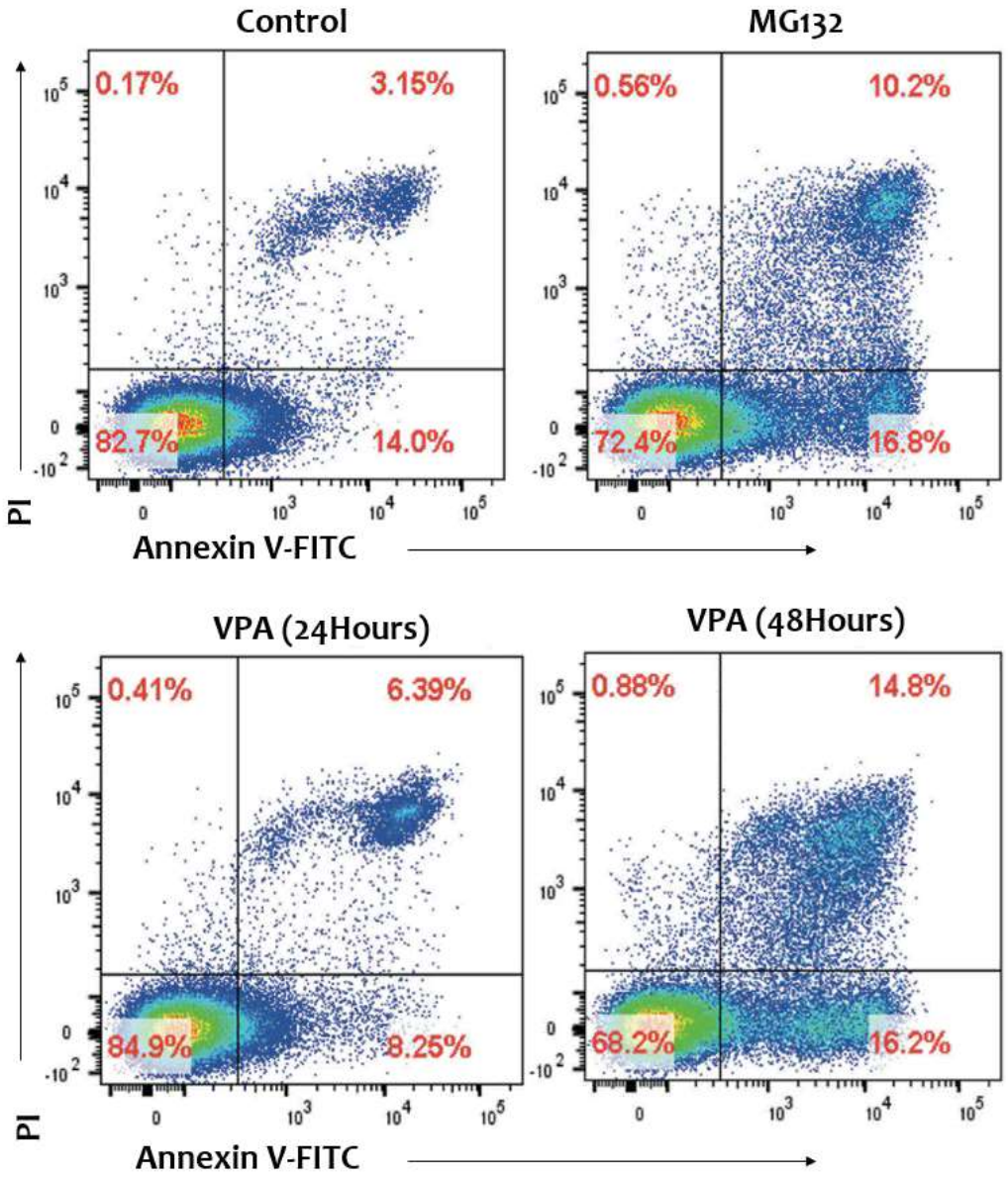

Figure 4.28: Valproate treatment promotes cellular apoptosis: Treatment of A549 cells with valproate (5 mM for 24 and 48 h), control, or MG132 (10 μM, 24 h) was done and were analyzed by FACS. The distribution of treated cells into live, early, and late apoptosis, and necrosis are represented as color density plot.

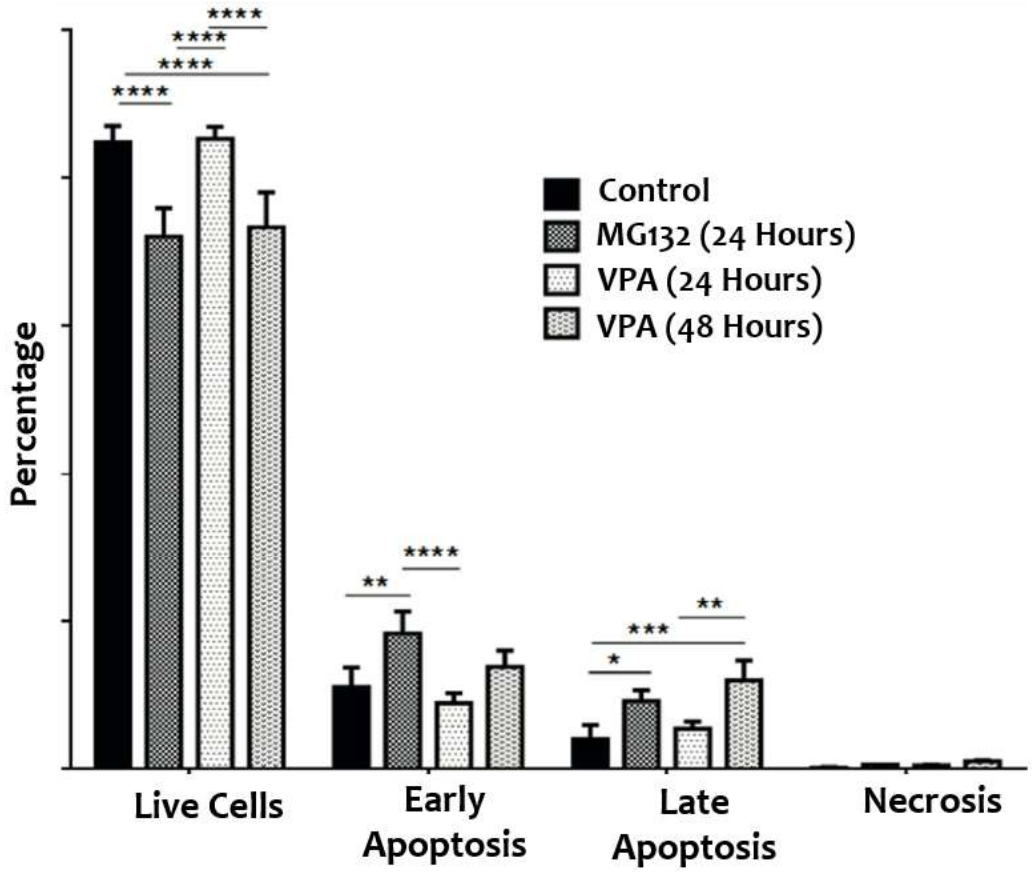

Figure 4.29: Valproate treated A549 cells show apoptosis: FACS analysis results distribution of treated cells into live, early, and late apoptosis, and necrosis are represented as bar graph. Values in bar graph are mean ± SD obtained from three independent experiments performed in triplicate. *p < 0.05; represents a significant difference as compare to control.

These results demonstrated valproate to have a pro-apoptotic potential on A549 cells in a time-dependent fashion. Overall, our results indicate that use of valproate can disturb proteasome activities with adverse cellular effects leading to apoptosis. This molecular interference of valproate with proteasome subunits may serve as a possible anti-cancer therapeutic intervention combined with other known anti-tumor agents.

4.2 DISCUSSION

Many of the cellular homeostatic mechanisms are exploited by cancer cells to their survival advantage. A robust PQC machinery, including proteasome, is one of these mechanisms that facilitates tumor growth and progression. The inhibition of proteasome as an anti-cancer therapeutic approach has already been approved, though instances of resistance and limitation to only a few types of cancers are vital challenges. In this study, we observed valproate exposure promotes accumulation of ubiquitylated proteins in cells, which suggests the disturbance of proteolytic machinery. Further, our *in silico* findings indicate a possible interaction of valproate with proteasome subunits. Next, we demonstrated that use of valproate induced polyglutamine protein accumulation and cytotoxic effects. Moreover, inhibition of proteasome by valproate also induced mitochondrial dysfunction and apoptosis. Overall, our finding elucidates the potential of valproate as a proteasome inhibitor, which can be beneficial in combination with other anti-tumor agents for more effective therapeutic interventions. Furthermore, the earlier known HDAC inhibition property of valproate, along with its possible proteasome inhibition capacity, can be explored in depth for a better understanding of PQC mechanism associated signaling cascade.

Proteolysis is a key mechanism for survival of tumor cells [Kampmeyer et al., 2017]. Earlier findings report that proteasome inhibitors can disturb proteolytic machinery of tumor cells and act as promising therapeutic anti-proliferative agents [Adams, 2004]. Still, we are far from understanding precise molecular mechanism of proteasome inhibitors mediated apoptosis in tumor cells. Here, we observed that treatment of valproate aggravates accumulation of ubiquitylated proteins near MTOC regions, which presented an indication of proteasome inhibition. Previous findings indicate the potential benefit of proteasome inhibition as they can induce apoptosis in cancer cell line models [Kisselev et al., 2012]. Next, to further examine the impact of valproate on cellular proteolytic machinery, we treated both normal and expanded polyglutamine transfected cells, and a few sets of samples were also treated with MG132. These results suggest that most likely, disturbance of proteasome functions via the treatment of valproate induces aggregation of expanded polyglutamine proteins near the perinuclear region, and the same sets of samples also demonstrate the over-accumulation of higher molecular weight derivatives of expanded polyglutamine proteins in stacking gels. MG132 (proteasome inhibitor) treated cells also exhibit a similar profile of expanded polyglutamine protein accumulation. Earlier findings suggest that failure of proteasome functions is linked to accumulation of ubiquitin-positive inclusions of expanded polyglutamine proteins [Verhoef et al., 2002; Holmberg et al., 2004]. These studies also support our current findings that disturbance of proteasome functions induced by valproate contributes to the over-accumulation of abnormal inclusions of expanded polyglutamine proteins.

Proteasome-dependent clearance of abnormal proteins facilitates the cellular physiological processes and maintains cellular survival dynamics [Adams, 2003]. Previous findings indicate that cellular stress, abnormal protein aggregation, and proteasome dysfunction represent proteostasis abnormalities and can induce apoptosis [Kannan and Jain, 2000; Almond and Cohen, 2002]. To understand the cellular physiological effects of valproate on overall cell viability, we first observed cell morphological changes after treatment in both concentration and time dependent manner. These results prompted us to observe the cell viability via MTT assay where we noted depleted cell viability in the above-described same set of samples. Earlier studies indicate the dysregulation of proteasome functions is also linked with cell death [Adams, 2004].

Interestingly, our current finding represents that treatment of valproate induces proteasome dysfunctions and inhibits both chymotrypsin-like and PGPH-like protease activities under varying concentration and time-dependent fashion. Suppression of proteasome activities is one of the critical approaches against tumor progression [Crawford et al., 2011]. We, therefore, analyzed the role of valproate linked with cell viability and proteasome dysfunction. As expected, treatment of valproate depleted cell viability, which was also confirmed by propidium iodide staining, induced DNA fragmentation, and promoted the accumulation of exogenously expressed proteins, leading to proteasome substrate d1EGFP protein accumulation. Remarkably, the use of valproate in cells disrupted proteasome functions and induced apoptosis, which can be alleviated upon exogenous expression of chaperone dependent E3 ligase CHIP in cells. Interestingly, treatment with N-acetylcysteine and exogenous expression of Hsp70 mitigated cell death up to some extent and improved cell viability by imparting protective effects against valproate-induced stress conditions.

Apart from eliminating abnormal proteins, proteasome machinery is a vital regulator of transcription factors, cell growth, mitochondrial health, and apoptosis [Mishra et al., 2018]. But still, it is a challenge to know how the abnormal functions of proteasome machinery induce cytotoxic insults and apoptosis in cells. To answer these questions, we further treated cells with valproate and performed immunofluorescence staining using anti-cytochrome *c* to demonstrate release of cytochrome *c*, which represents a disturbance of mitochondrial functions. Subsequently, our JC-1 mitochondrial membrane potential voltage-sensitive dye-based fluorescence shift analysis further clarified that treatment of valproate leads to depletion of mitochondrial

transmembrane potential in cells. We also confirmed the apoptotic effects of valproate in cells by using Annexin V-FITC/PI assay, which showed significant apoptosis upon longer drug treatment period.

4.3 CONCLUDING REMARKS

For maintenance of cellular homeostasis, precise regulation of different signaling processes such as cellular proliferation, oncogenic mechanisms, apoptosis, signal transduction, antigen presentation, and expression of various transcription factors is crucial [Jakobisiak et al., 2003; Spitz and Furlong, 2012; Galluzzi et al., 2018]. Cancer cells often exploit these cellular homeostasis mechanisms to sustain rapid cellular proliferation and develop resistance against chemotherapeutics and stress conditions. Designing of novel proteasome inhibitors and targeting proteasome machinery for therapeutic purposes emerged as a promising molecular strategy for restricting the cancer cell proliferation. With this perspective, proteasome inhibitors represent significant anti-tumor potential against persisting molecular resistance in cancer cells. Numerous findings suggest that cancer cells require robust homeostasis for their survival [Basanta and Anderson, 2017] and still, it is a challenge to develop proteasome inhibitors that can specifically target proteasome machinery to make cells more susceptible to apoptosis.

The results in the present work indicate that valproate can lead to apoptosis in cells, most likely due to the disturbance in cellular proteostasis. Proteasome-mediated modulations are highly relevant for the therapeutic interventions of neurodegenerative diseases, but in recent decades, they have received huge attention for treatment of cancer. In conclusion, our current study demonstrates that valproate can inhibit proteasome functions and induce cytotoxic events in the cells; these findings overall represent the significance of proteasome inhibition based new strategies, and possible therapeutic interventions for uncontrolled tumor growth.

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