Chapter 6:

Results and Discussion 3

Deciphering the global impact of phosphorylation status of Ser13 and Ser16 on mutant huntingtin (mHTT) toxicity

This objective of our study was to investigate the neuroprotective effects of Kinetin, BMS 345541, and Bay 11-7082 in preventing mutant huntingtin aggregation and thus it's associated pathology in HD150Q cells. Huntington's Disease (HD) is characterized by the dysregulation of multiple cellular pathways, including mitochondrial dysfunction, which leads to the generation of reactive oxygen species (ROS), oxidative stress, and protein misfolding. This cascade of events results in endoplasmic reticulum (ER) stress and ultimately neuronal cell death [1].

The post-translational modifications (PTMs) of huntingtin, including phosphorylation, are crucial in regulating its function and toxicity [2], intracellular localization [3], as well as protein degradation resulting their clearance from cells [4]. Multiple phosphorylation sites in huntingtin have been predicted, with several experimentally confirmed, including Ser13 and Ser16 [4,5]. Phosphorylation at these sites has been shown to reverse the toxic phenotype of mutant huntingtin in a BAC mice model, presenting strong evidence for the protective nature of huntingtin phosphorylation [6]. Additionally, Ser-13 and Ser-16 phosphorylation inhibits fibril formation and promotes HTT clearance by proteasomes and lysosomes [3,7]. The mechanism behind this effect remains unclear and requires further investigation. It is hypothesized that altered protein solubility and protein-protein interactions, due to the addition of phosphate groups by casein kinase 2 (CK2) [3] and the inflammatory kinase, IκB kinase (IKK) [4,8], play a role.

Based on these observations, interventions that prevent or reverse the toxicity of mutant huntingtin, particularly through the restoration of N17 phosphorylation, are potential therapeutic avenues for HD. We, therefore, employed Kinetin, BMS 345541 and Bay 11-7082 to modulate N17 phosphorylation in the HD150Q cell line model. Kinetin (N6-furfuryladenine), a plant cytokine, exhibits promising capabilities in hindering polyglutamine-based mutant huntingtin aggregation within neuronal cells [9]. This effect is attributed to its conversion by adenine phosphoribosyltransferase (APRT) into Kinetin triphosphate (KTP), an ATP analogue that facilitates the phosphorylation of N17 by casein kinase 2 [10]. This mechanism restores N17 phosphorylation levels and enhances the clearance of mutant huntingtin inclusions, underscoring Kinetin's role as a pivotal protective agent in various Huntington's disease models cells [9].

In addition to Kinetin, we also evaluated the neuroprotective potential of BMS 345541 and Bay 11-7082, both of which inhibit IKKβ, a kinase involved in the dephosphorylation of the N17 domain [11-14]. These compounds were included in our study to determine their ability to increase

N17 phosphorylation in HD150Q cells, reduce the number of aggregates, improve mitochondrial functions, decrease ER stress and oxidative stress, and restore ATP levels.

To investigate the pathways responsible for the protective effect of N17 phosphorylation by these compounds, the HD150Q cell line, a well-established Huntington's disease model system, was employed. HD150Q is a mutant mouse neuroblastoma cell line (N2a) expressing a truncated N-terminal huntingtin protein with 150 glutamine residues, fused to enhanced green fluorescent protein (e-GFP) under an ecdysone promoter. These cells were cultured in DMEM supplemented with 10% fetal bovine serum, 1X penicillin-streptomycin, and antibiotics (0.4 mg/mL of Zeocin and G418) at 37°C in a 5% CO_2 incubator. To induce the expression and then the aggregation of the mutant huntingtin protein, 1 µM Ponasterone A was administered. Aggregates of mutant huntingtin appear as green puncta under a fluorescence microscope, and the cells typically die within 7−8 days due to the cytotoxicity of the aggregated mutant huntingtin. By utilizing this model system, our study aims to provide further insights into the potential therapeutic effects of Kinetin, BMS 345541, and Bay 11-7082 in modulating the pathological processes associated with Huntington's disease.

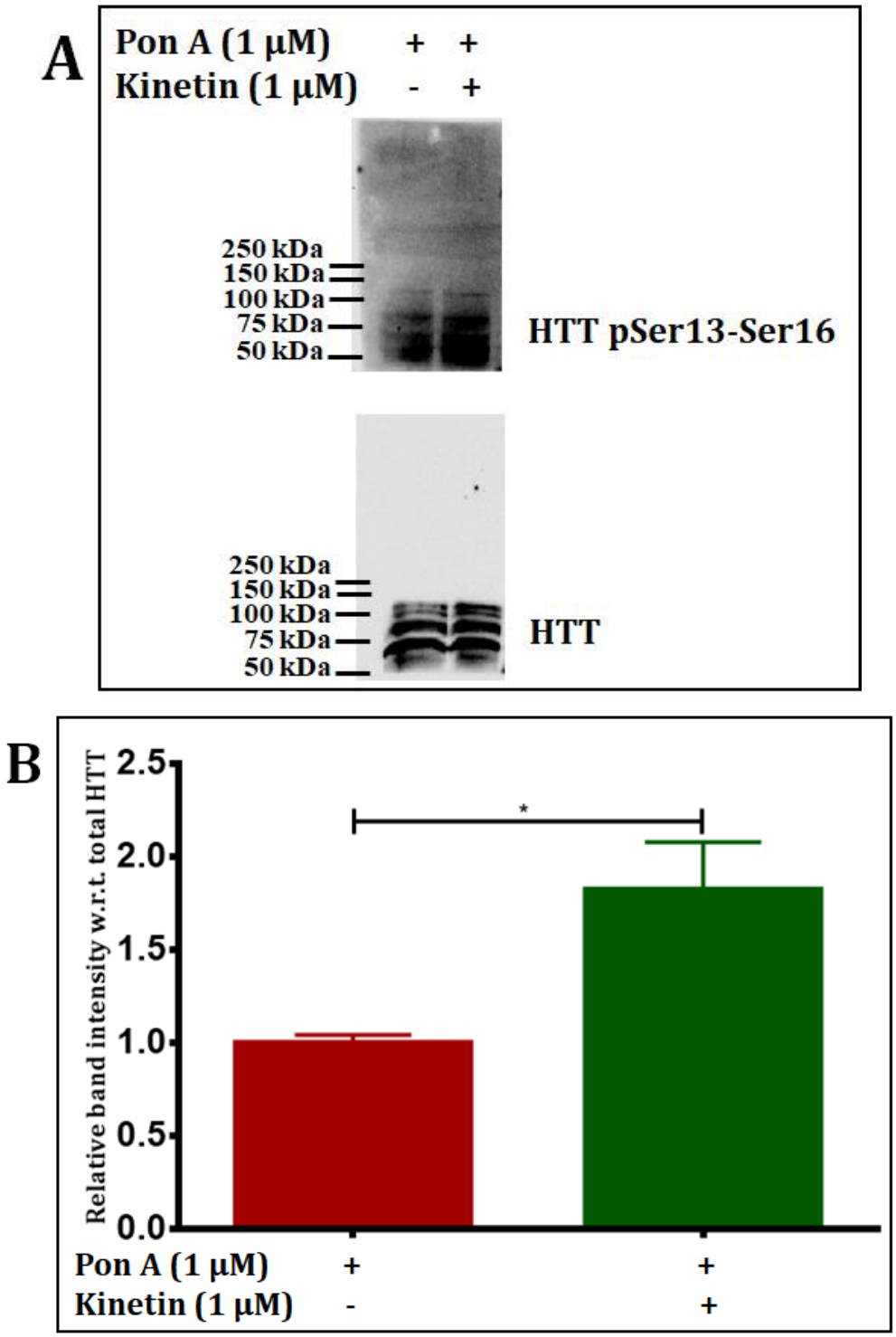
6.1 Kinetin, BMS 345541, and Bay 11-7082 Increase Phosphorylation of Ser13/Ser16 in HD150Q Cells Expressing Mutant Huntingtin

The phosphorylation of huntingtin at Ser13 and Ser16 within the N17 region has been identified as a protective post-translational modification in the context of HD [6]. The N17 sequence of huntingtin functions as a nuclear export signal, and its PTMs are believed to play a crucial role in regulating the subcellular localization and clearance of mutant huntingtin [15]. To explore the effects of Kinetin, BMS 345541, and Bay 11-7082 on the phosphorylation of Ser13 and Ser16 in mHTT, we treated HD150Q cells with these compounds.

In our experimental procedure, HD150Q cells were induced with 1 µM Ponasterone A to promote mHTT expression, followed by treatment with 1 µM Kinetin, 300 nM BMS 345541, and 1 µM Bay 11-7082 for 48 hours. After treatment, the cells were harvested, and protein extracts were analyzed by western blot using an anti-N17-S13pS16p antibody (Coriell Institute for Medical Research, USA), which specifically detects phosphorylation of huntingtin at Ser13 and Ser16.

The results demonstrated that treatment with Kinetin significantly elevated the phosphorylation levels of Ser13/Ser16 in HD150Q cells compared to untreated controls. Similar increases in

phosphorylation were observed with treatments of BMS 345541 and Bay 11-7082, indicating that all three compounds effectively enhanced the protective phosphorylation of the N17 region of mHTT. These findings are illustrated in **Fig. 6.1.1A, B, C** and **D**, which shows a statistically significant increase in phosphorylation levels upon treatment with each of these compounds.



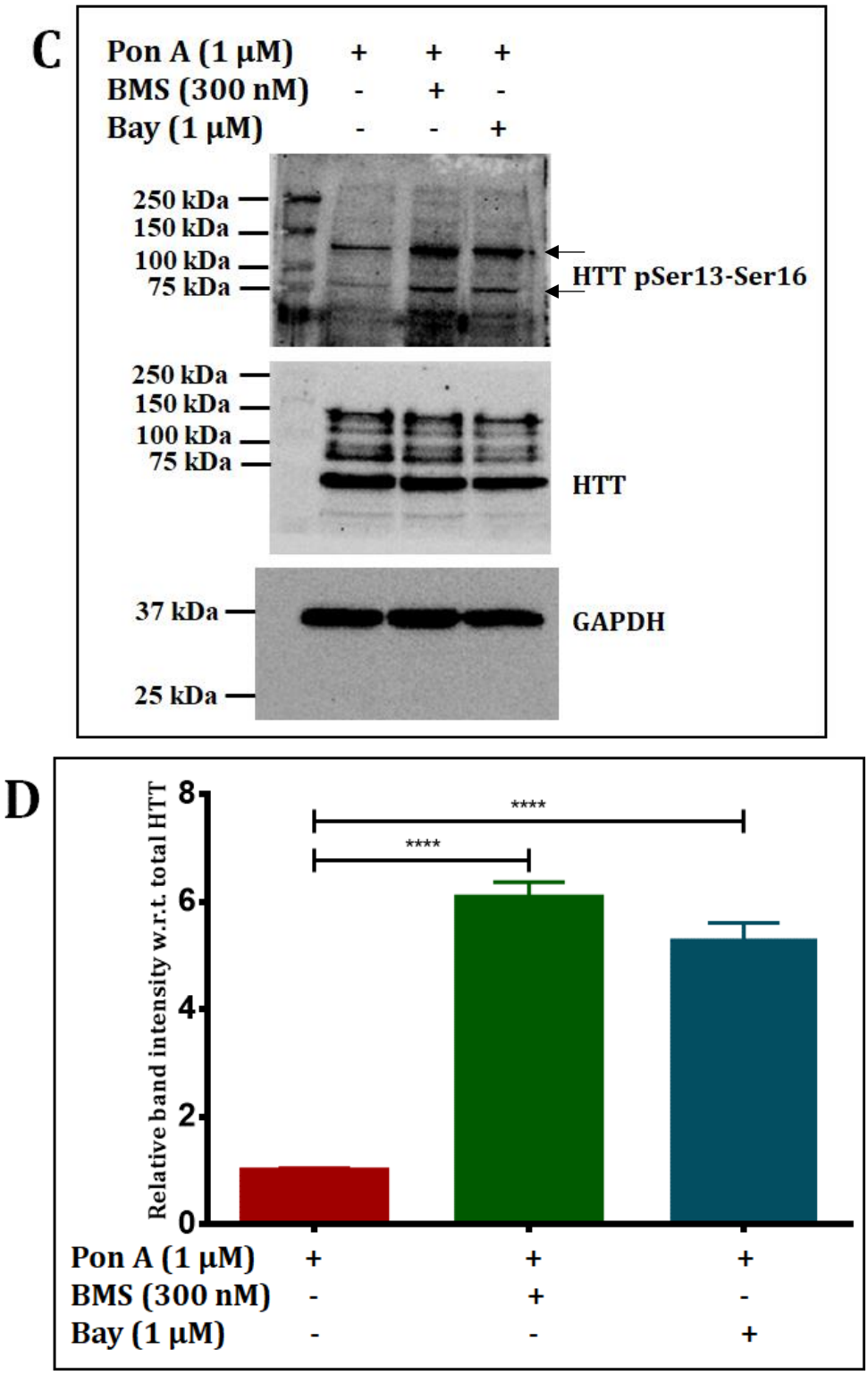


Figure 6.1.1: Western Blot images (A, C) and graphical representations for densitometric analysis (B, D) show pSer13-pSer16 levels in HD150Q cells treated with Kinetin (A, B), BMS 345541, and Bay 11-7082 (C, D). **(**n=3; $p > 0.05$ (ns), $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***)**.

6.2 Kinetin, BMS 345541, and Bay 11-7082 Prevent and/or Reduce mHTT Aggregates

The proteolysis of mutant full-length mHTT results in the formation of toxic N-terminal fragments bearing polyglutamine (polyQ) expansions, which play a crucial role in Huntington's Disease pathogenesis. These mHTT fragments form cytoplasmic aggregates and intranuclear inclusion bodies, contributing significantly to the neurotoxicity observed in HD [7,16]. Previous studies have demonstrated the presence of such aggregates in cell models of HD and post-mortem brain slices from HD patients [17].

To investigate the aggregation kinetics of GFP-tagged mHTT in HD150Q cells, we induced mHTT expression with 1 μM Ponasterone A and analyzed the cells at 24, 48, 72, and 96 hours using fluorescence microscopy. As shown in **Fig. 6.2.1A**, the number and size of mHTT aggregates, visualized as green puncta, increased over time. The average number of puncta per field significantly increased at each time point tested (**Fig. 6.2.1B**), consistent with the findings of Weiss *et al.*, who characterized mHTT aggregates using agarose gel electrophoresis and demonstrated that cytoplasmic, but not nuclear, HTT aggregates enlarge as the disease progresses in transgenic mice brains [18].

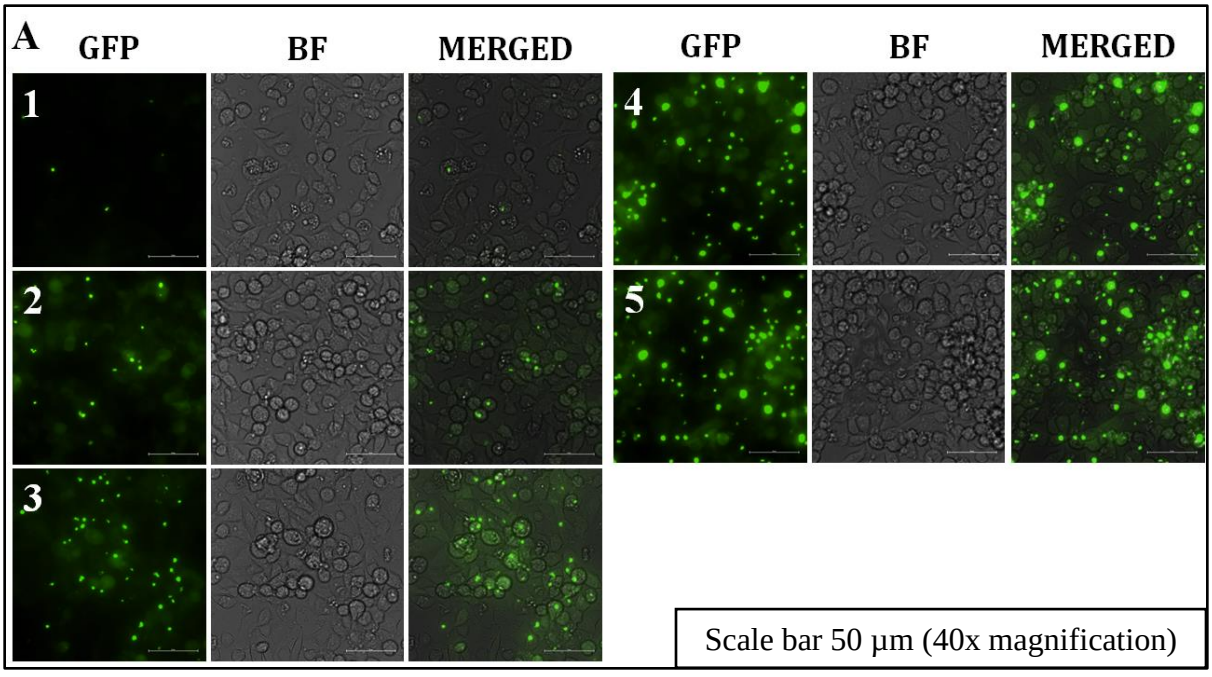


Figure 6.2.1A: 40X Fluorescence microscopic images showing GFP tagged mHTT aggregates in HD150Q cells after Pon A treatments for 24, 48, 72 and 96 hours, respectively. 1. HD150Q control 2. 24 h Ponasterone A 3. 48 h Ponasterone A 4. 72 h Ponasterone A 5. 96 h Ponasterone A.

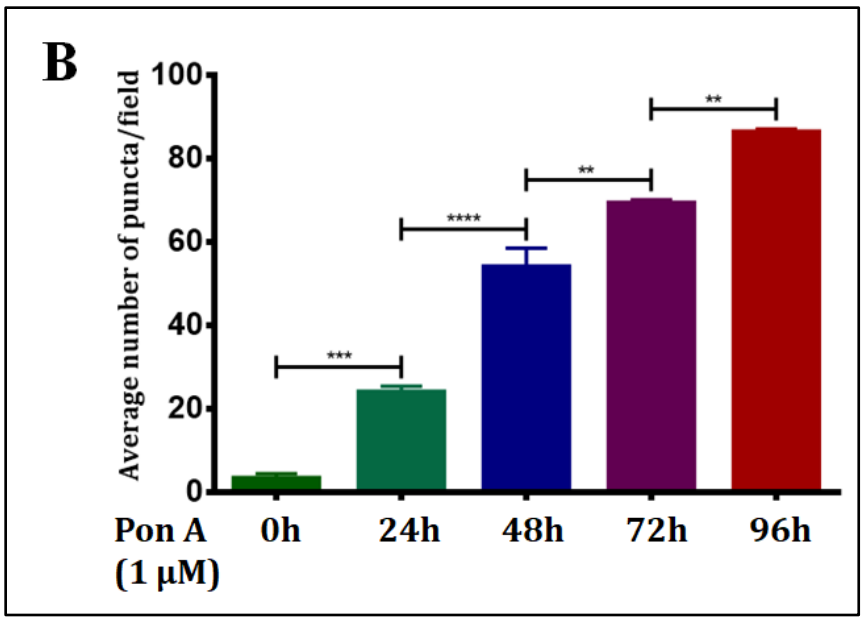


Figure 6.2.1B: Graphical representation showing average number of puncta/field in HD150Q cells after Ponasterone A treatment for 24, 48, 72 and 96 hours, respectively (n=3; $p > 0.05$ (ns), $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***).

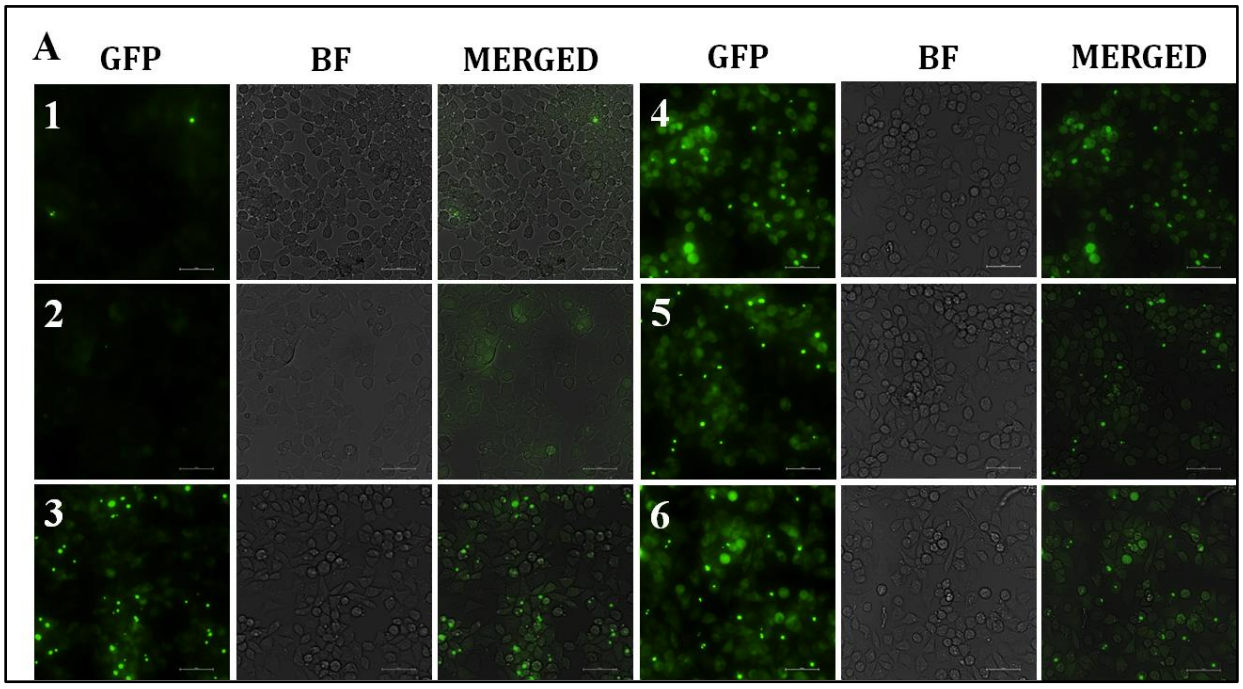


Figure 6.2.2A: 40X Fluorescence microscopic images showing GFP tagged mHTT aggregates in HD150Q cells after different concentrations of Kinetin with/without Ponasterone A for 24 hours. 1. HD150Q control 2. Kinetin alone 3. Ponasterone A alone 4. Pon A + 0.5 µM Kinetin 5. Pon A + 1 µM Kinetin 6. Pon A + 2 µM Kinetin. Scale bar 50 µm (40x magnification).

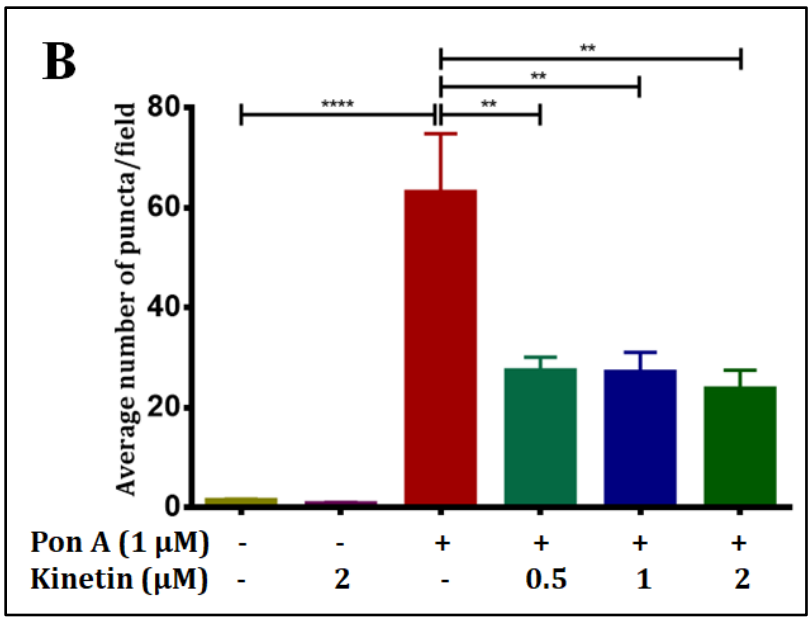
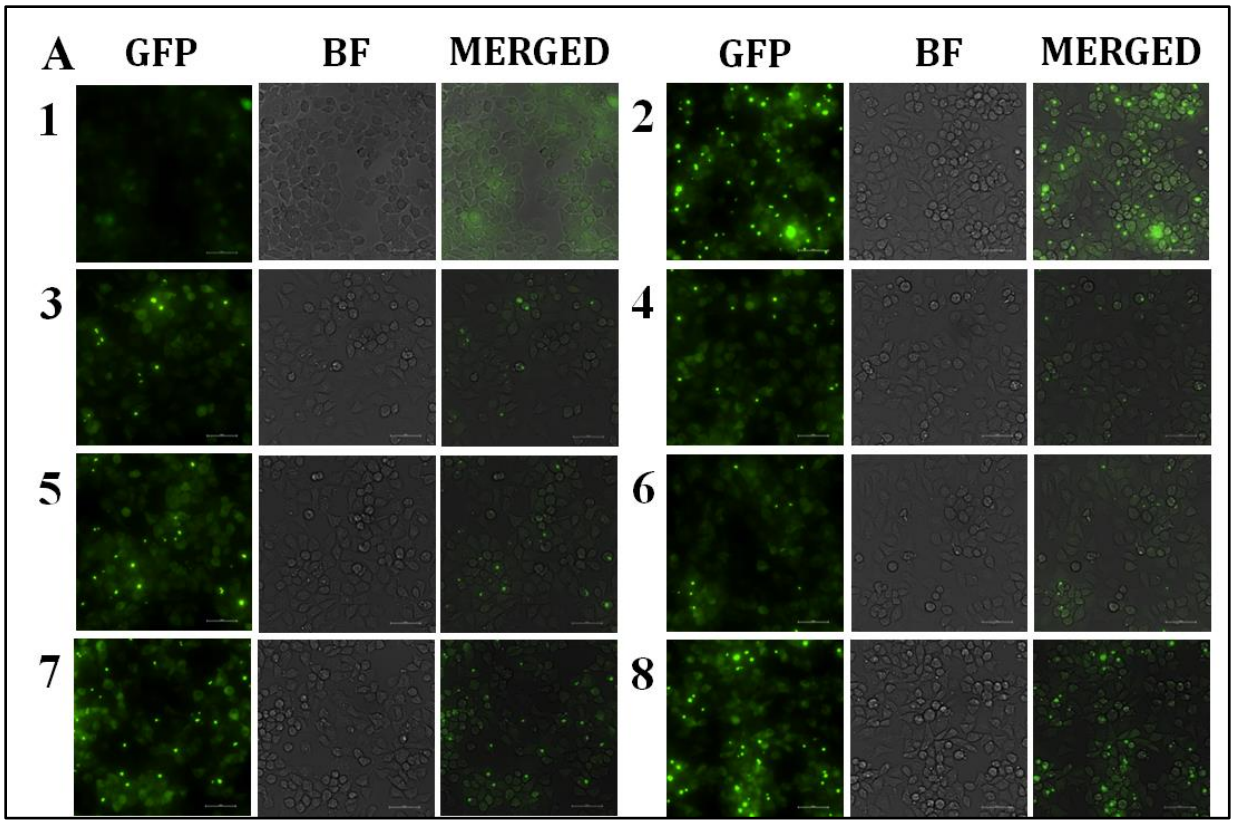


Figure 6.2.2B: Graphical representation showing average number of puncta/field in HD150Q cells after different concentrations of Kinetin with/without Ponasterone A for 24 hours (n=3; $p > 0.05$ (ns), $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***).

Given the protective effect of Ser13 and Ser16 phosphorylation on mHTT, we next assessed the impact of Kinetin on mHTT aggregation levels. HD150Q cells were treated with various concentrations of Kinetin (0.5 μM, 1 μM, and 2 μM), known to enhance HTT Ser13 and Ser16 phosphorylation), either alone or in combination with 1 μM Ponasterone A for 24 hours. Compared to uninduced cells (DMSO control), Ponasterone A significantly increased mHTT expression and aggregation, visualized as puncta. However, Kinetin co-treatment at all tested concentrations significantly reduced mHTT aggregate formation (**Fig. 6.2.2A** and **6.2.2B**).

To determine whether Kinetin could prevent mHTT aggregation or eliminate preformed aggregates, we pre-treated HD150Q cells with Kinetin (1 μM and 2 μM) for 24 hours before inducing mHTT with Ponasterone A for an additional 24 hours. Pre-treatment with Kinetin was as effective as co-treatment in reducing mHTT aggregates (**Fig. 6.2.3A** and **6.2.3B**). Furthermore, Kinetin treatment (1 μM and 2 μM) 24 hours post-induction with Ponasterone A significantly resolved pre-formed mHTT aggregates (**Fig. 6.2.3A** and **6.2.3B**). Uninduced HD150Q cells served as a negative control, and Ponasterone A-induced cells served as a positive control. The fluorescence microscopy images were manually analyzed, and the average number of puncta per

field was determined and plotted (**Fig. 6.2.3B**). These quantitative results were consistent with the observed trends.



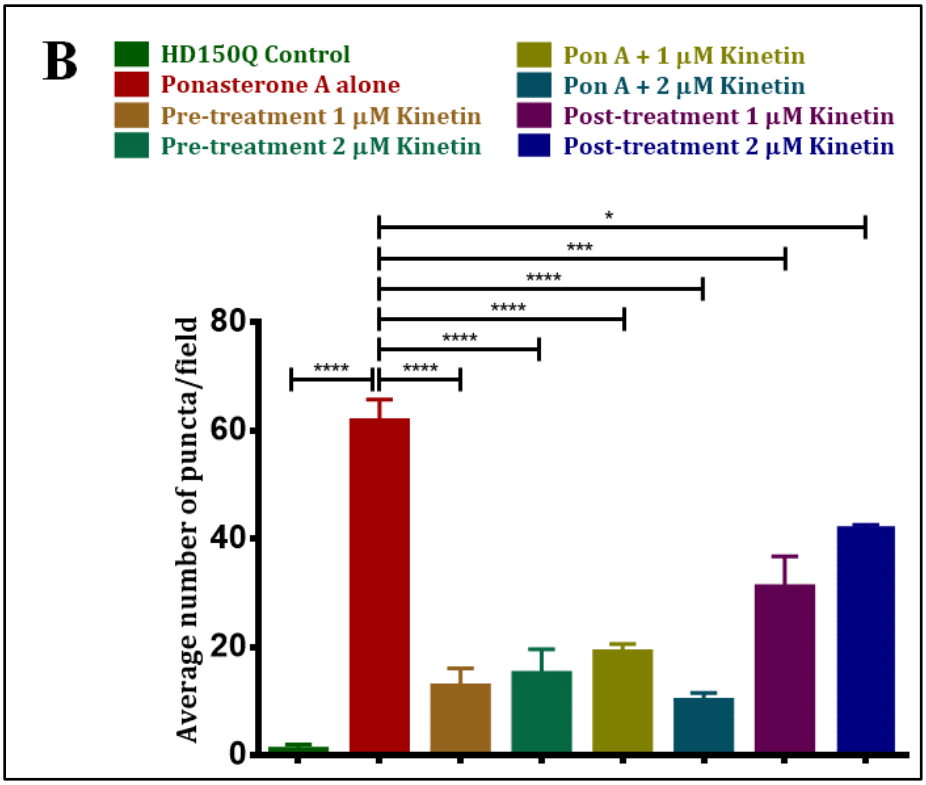
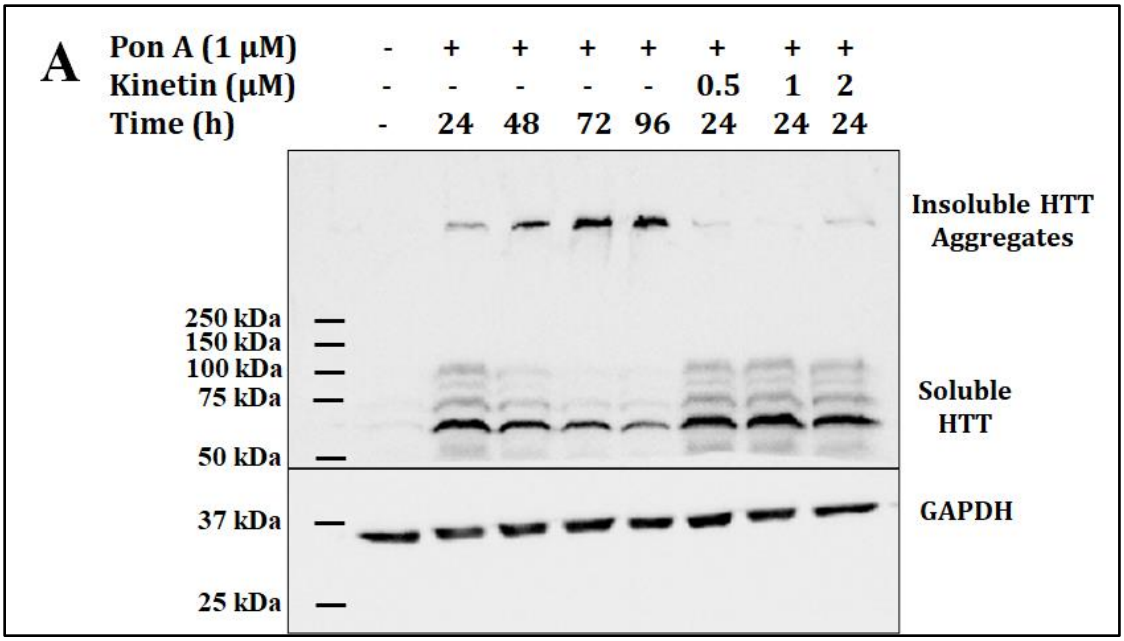


Figure 6.2.3A: 40X Fluorescence microscopic images showing GFP tagged mHTT aggregates in HD150Q cells after different concentrations of Kinetin with/without Ponasterone A for 48 hours. 1. HD150Q control 2. Ponasterone A alone 3. Pre-treatment 1 µM Kinetin 4. Pre-treatment 2 µM Kinetin 5. Co-treatment 1 µM Kinetin 6. Co-treatment 2 µM Kinetin 7. Post-treatment 1 µM Kinetin 8. 7. Post-treatment 2 µM Kinetin. Scale bar 50 µm (40x magnification).

Figure 6.2.3B: Graphical representation showing average number of puncta/field in HD150Q cells after different concentrations of Kinetin with/without Ponasterone A for 48 hours (n=3; $p > 0.05$ (ns), $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***).

To confirm the role of Kinetin in reducing insoluble GFP-tagged mHTT aggregates, we performed western blot analysis on uninduced, Ponasterone A-induced, and Kinetin-treated (co-, pre-, and post-treatment with Ponasterone A) HD150Q cells. Cells were lysed, and protein extracts were subjected to SDS-PAGE and transferred to nitrocellulose membranes. Membranes were probed with an anti-huntingtin antibody. As shown in **Fig. 6.2.4A** and **6.2.4B**, insoluble mHTT aggregates increased with Ponasterone A treatment duration (upper panel), while soluble HTT decreased (lower panel). Kinetin co-treatment at various concentrations (0.5 µM, 1 µM, and 2 µM) abolished mHTT aggregates and increased soluble mHTT levels, consistent with microscopy results (**Fig. 6.2.4A** and **6.2.4B**). Pre- and post-treatment with Kinetin also demonstrated similar effects, confirming Kinetin's ability to prevent and resolve mHTT aggregates in a dose-dependent manner, likely via N17 phosphorylation (**Fig. 6.2.4C** and **6.2.4D**).



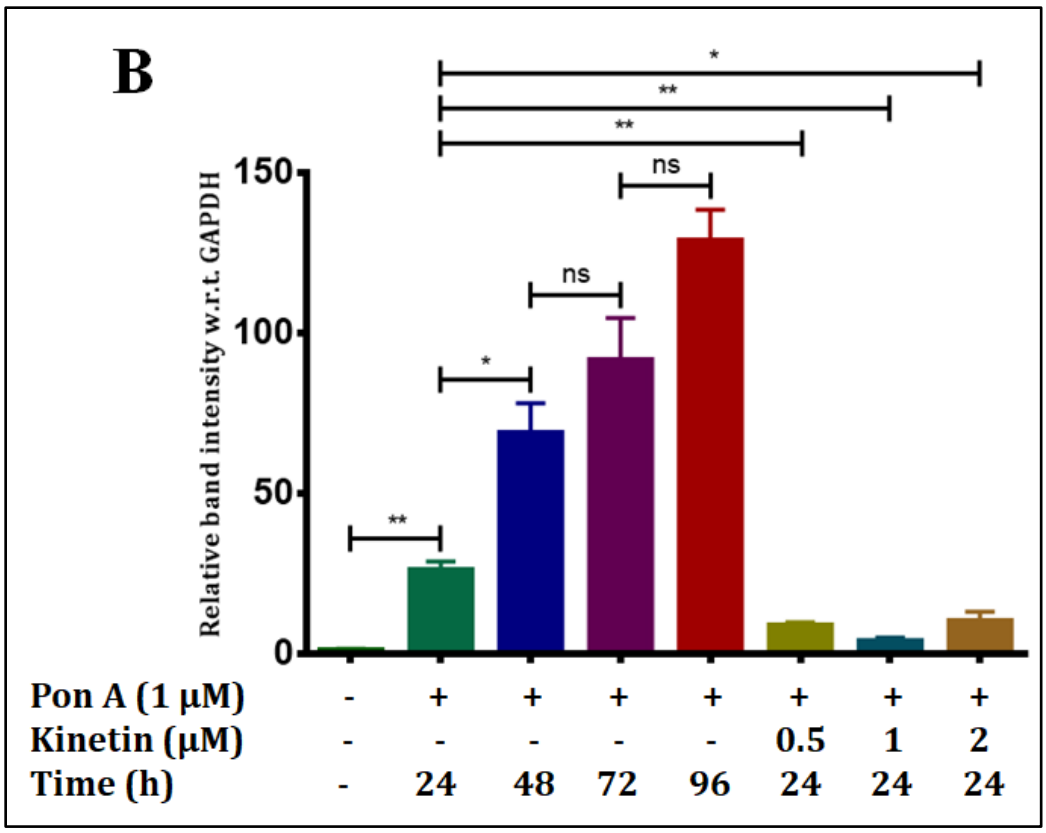
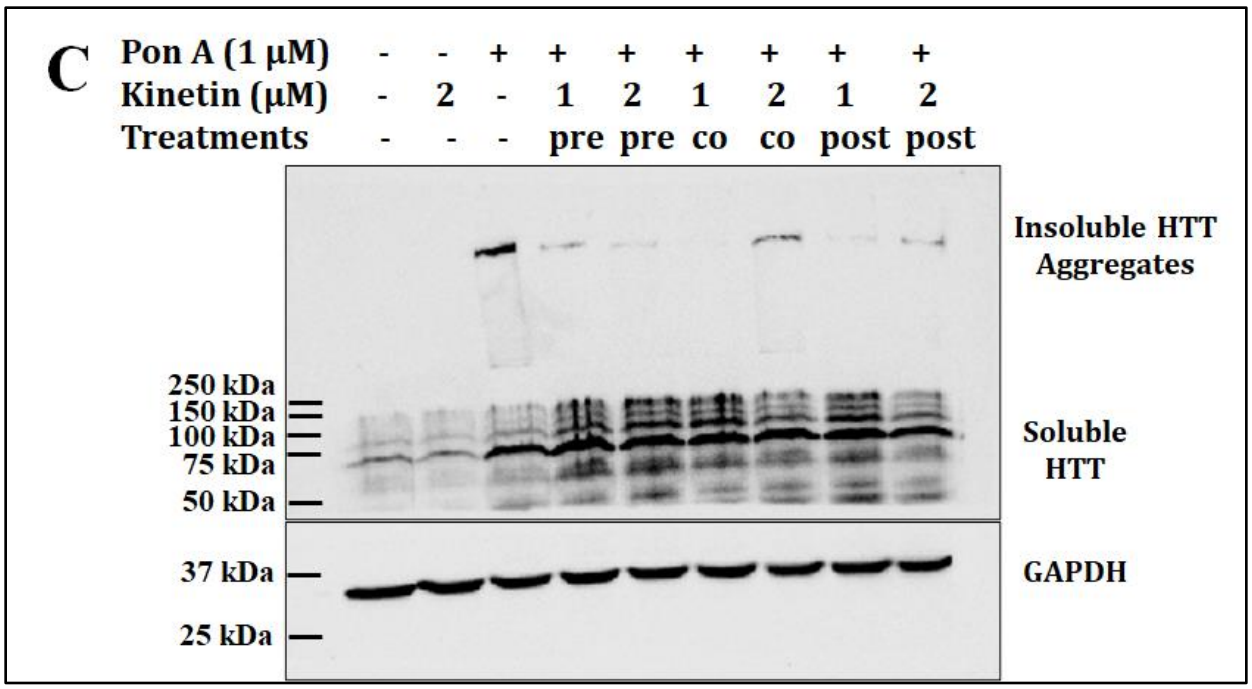


Figure 6.2.4: (A) Western Blot image and **(B)** Graphical representation for densitometric analysis showing mHTT aggregates in HD150Q cells after different concentrations of Kinetin with/without Ponasterone A for 24 hours (n=3; $p > 0.05$ (ns), $p \leq 0.05$ (*), $p \leq 0.01$ (**).



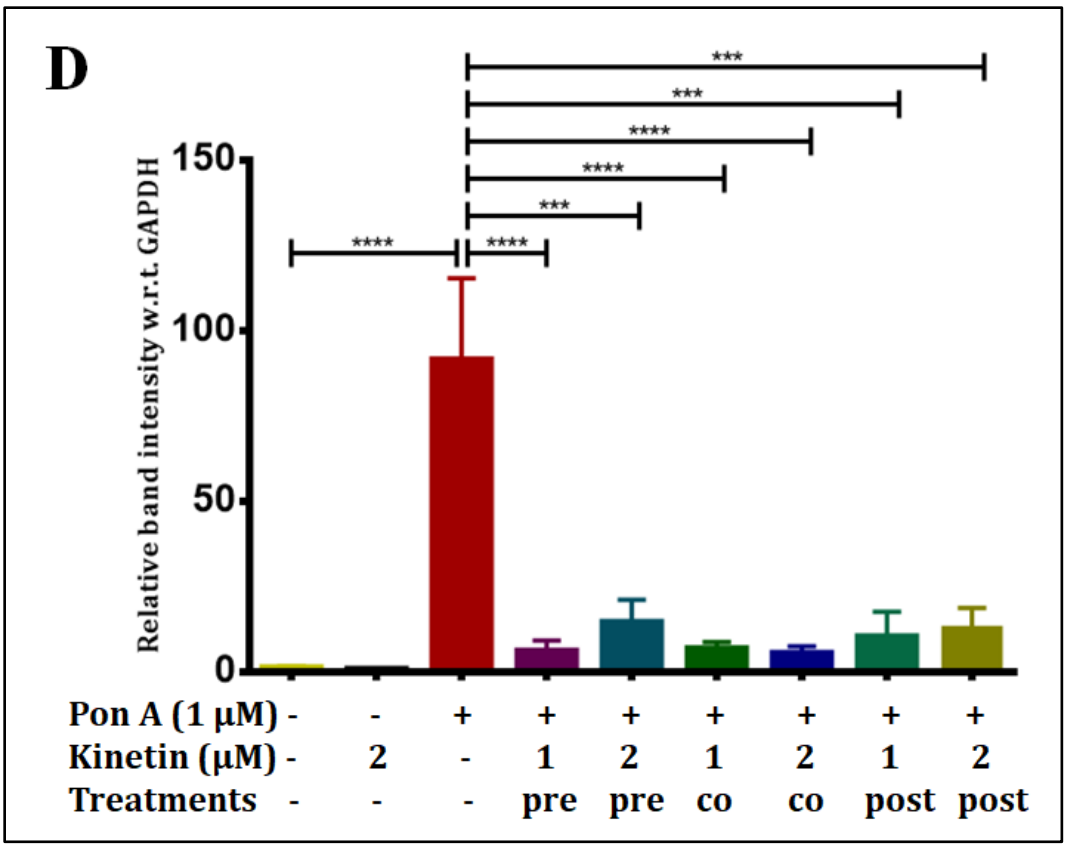


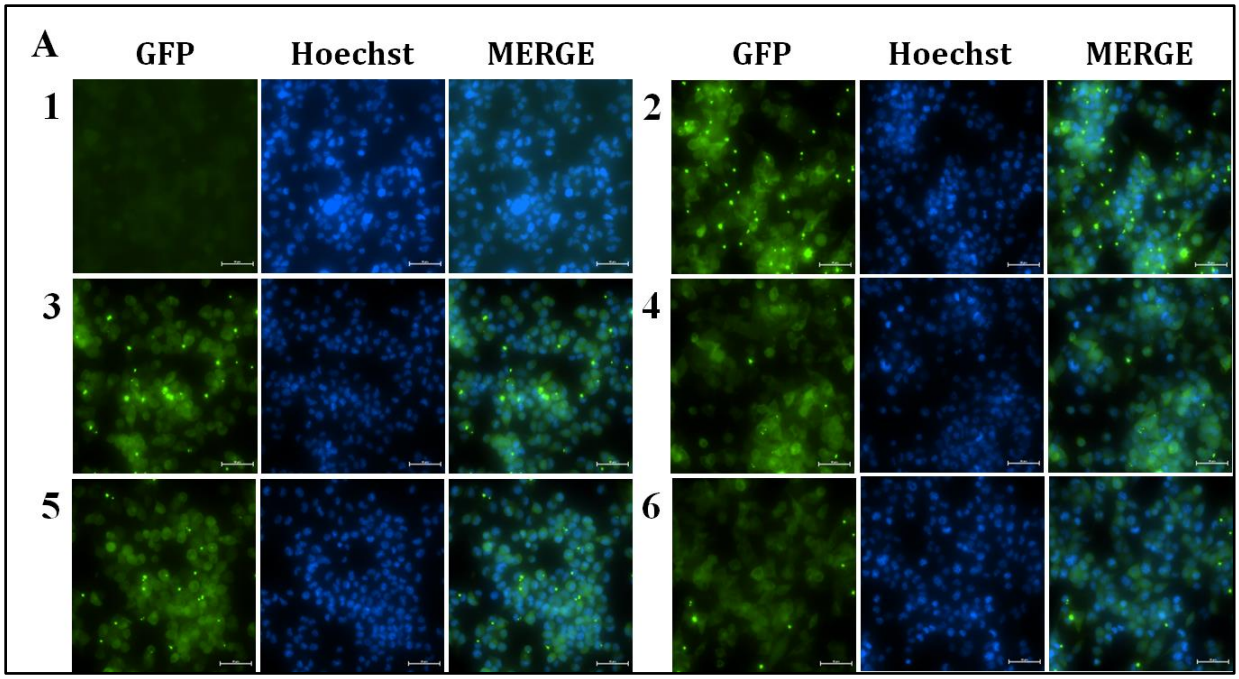
Figure 6.2.4: (C) Western Blot image and **(D)** Graphical representation for densitometric analysis showing mHTT aggregates in HD150Q cells after different concentrations of Kinetin with/without Ponasterone A for 48 hours (n=3; $p > 0.05$ (ns), $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***).

We also investigated the effects of BMS 345541 and Bay 11-7082 on mHTT aggregation. HD150Q cells were treated with Ponasterone A and BMS 345541 at concentrations of 100 nM and 300 nM. Fluorescence microscopy after 48 hours revealed that 300 nM BMS 345541 had the greatest effect in reducing mHTT aggregates (**Fig. 6.2.5A**). Quantification of puncta per field supported these findings (**Fig. 6.2.5B**). HD150Q cells treated with Bay 11-7082 at 500 nM and 1000 nM final concentration resulted in significant reduction in aggregates compared to Ponasterone A cells, with 1000 nM showing the maximum reduction (**Fig. 6.2.5A**). This pattern was consistent in puncta per field analysis (**Fig. 6.2.5B**).

Western blotting confirmed the fluorescence microscopy results. BMS 345541 treatment reduced insoluble mHTT aggregates at doses of 50 nM, 100 nM, 200 nM, and 300 nM, while increasing soluble HTT levels (**Fig. 6.2.6A** and **6.2.6B**). Similarly, Bay 11-7082 treatment at concentrations ranging from 100 nM to 1000 nM decreased mHTT aggregation compared to Ponasterone A alone

(**Fig. 6.2.6C** and **6.2.6D**). Densitometric analysis of blots corroborated these findings, with 1000 nM showing the least aggregation, indicating the highest efficacy (**Fig. 6.2.6D**).

These results collectively demonstrate that Kinetin, BMS 345541, and Bay 11-7082 effectively prevent and/or reduce mHTT aggregation in HD150Q cells, potentially offering therapeutic avenue for HD via modulation of N17 phosphorylation.



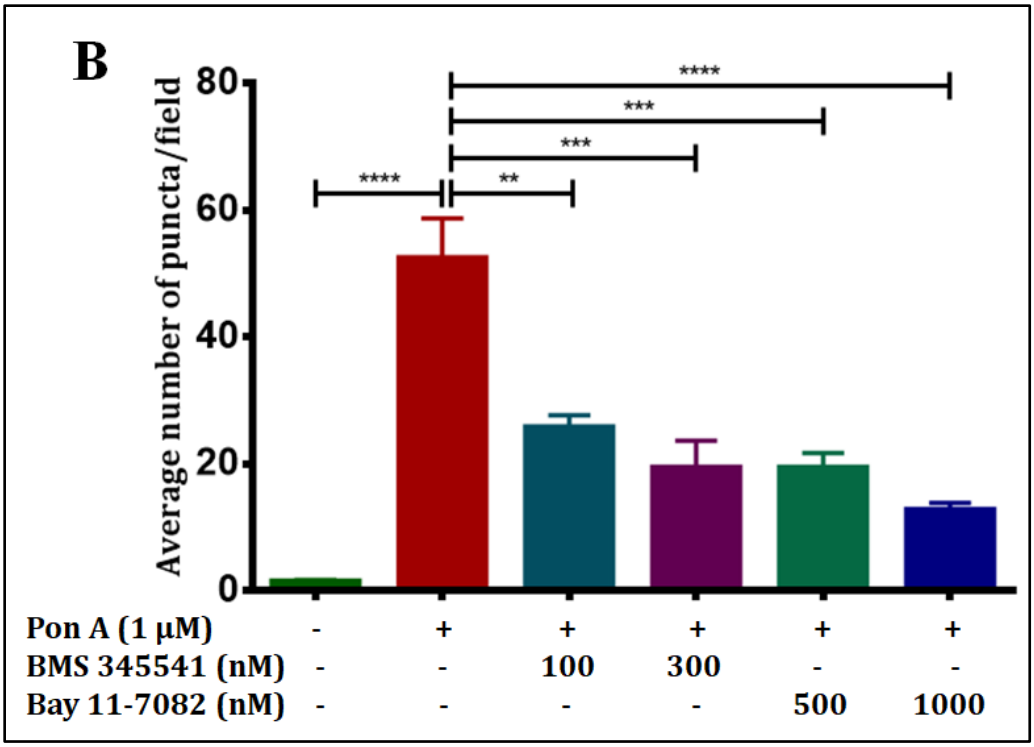


Figure 6.2.5A: 40X Fluorescence microscopic images showing GFP tagged mHTT aggregates in HD150Q cells after Ponasterone A with/without different concentrations of BMS 345541 and Bay 11-7082 for 48 hours. 1. HD150Q control 2. Ponasterone A alone 3. Pon A + 100 nm BMS 345541 4. Pon A + 300 nm BMS 345541 5. Pon A + 500 nm Bay 11-7082 6. Pon A + 1000 nm Bay 11-7082. Scale bar 50 µm (40x magnification).

Figure 6.2.5B: Graphical representation showing Average number of puncta/field in HD150Q cells after Ponasterone A with/without different concentrations of BMS 345541 and Bay 11-7082 for 48 hours (n=3; $p > 0.05$ (ns), $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***).

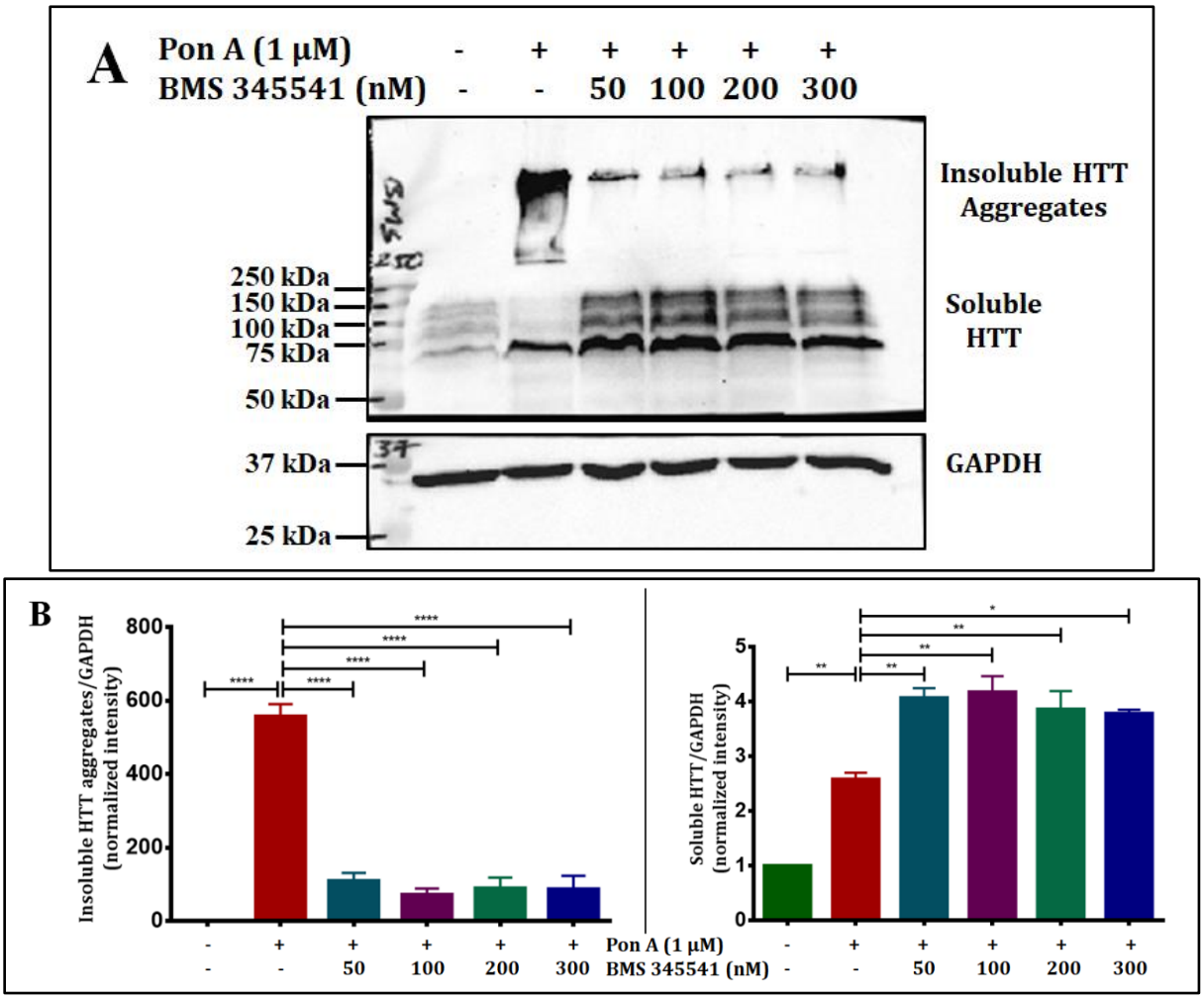


Figure 6.2.6: (A) Western Blot image and **(B)** Graphical representation for densitometric analysis showing mHTT aggregates and soluble HTT expression in HD150Q cells after Ponasterone A with/without different concentrations of BMS 345541 for 48 hours (n=3; $p > 0.05$ (ns), $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***).

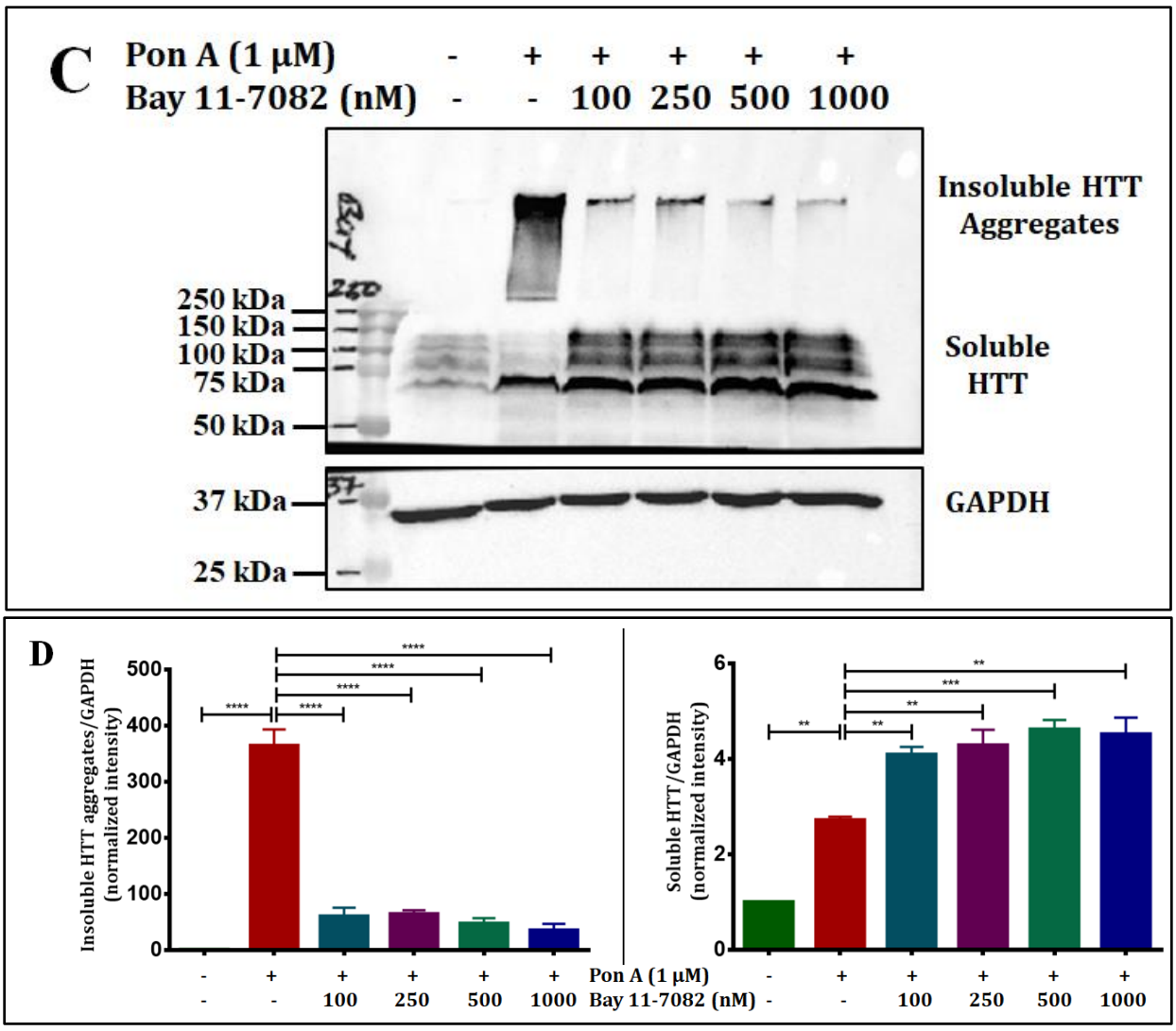


Figure 6.2.6: (C) Western Blot image and **(D)** Graphical representation for densitometric analysis showing mHTT aggregates and soluble HTT expression in HD150Q cells after Ponasterone A with/without different concentrations of Bay 11-7082 for 48 hours (n=3; $p > 0.05$ (ns), $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***).

6.3 Kinetin, BMS 345541, and Bay 11-7082 Rescues mHTT-Induced Mitochondrial Dysfunction

Mitochondrial dysfunction is a key contributor to cellular pathologies leading to cell death in Huntington's Disease. This dysfunction manifests as altered mitochondrial morphology and biogenesis, reduced electron transport chain activity, impaired oxygen consumption, decreased Ca^{2+} buffering, and lower ATP and NAD^{+} production production [19]. To investigate the potential of Kinetin in rescuing cellular ATP production, HD150Q cells were induced with Ponasterone A

for various durations (24h, 48h, 72h, and 96h) and treated with 1µM Kinetin for 24 h and 48 h. ATP levels, measured using a luminescence-based ATP determination kit, showed a significant reduction within 24h of Ponasterone A induction, with further declines observed at 48h, 72h, and 96h. However, co-treatment with Kinetin restored ATP levels to normal (**Fig. 6.3.1**).

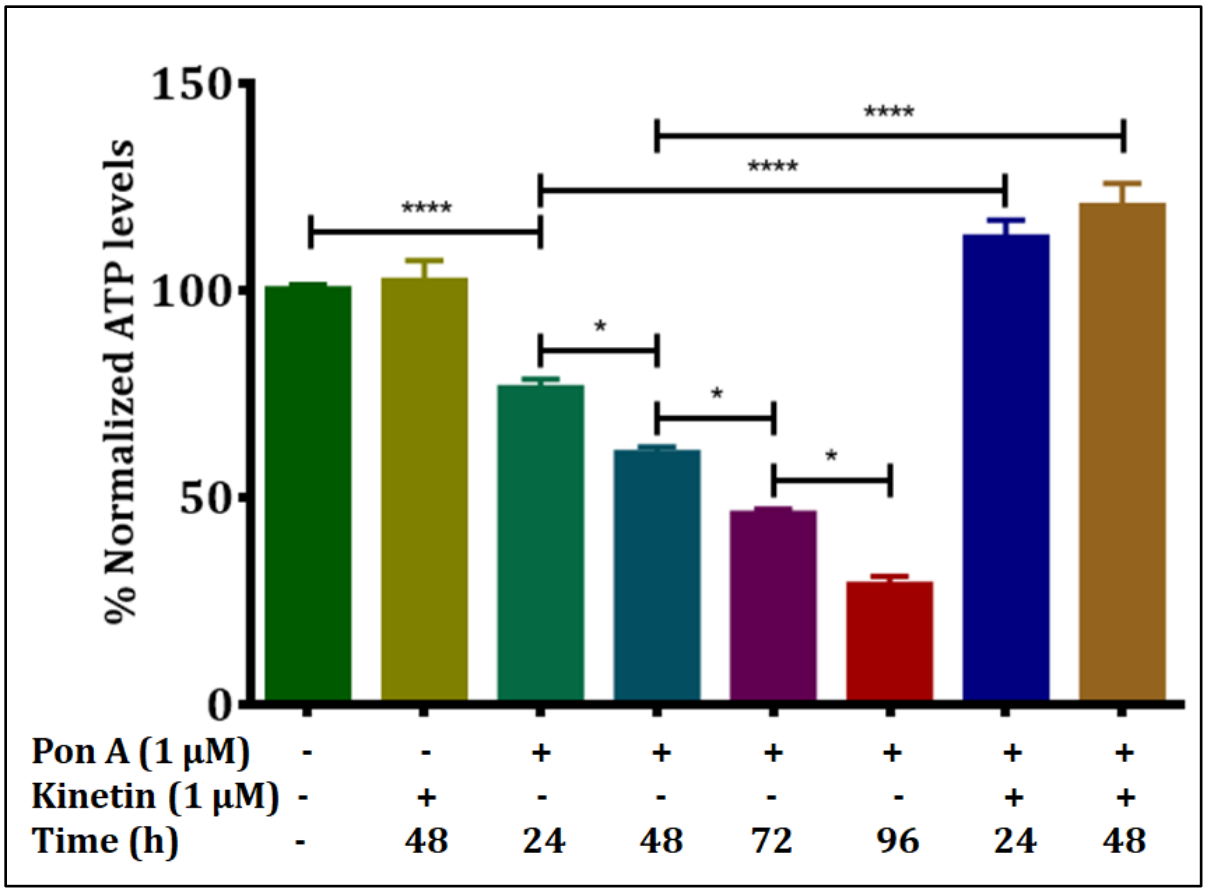


Figure 6.3.1: Graphical representation of normalized ATP levels in HD150Q cells after different concentrations of Kinetin with/without Ponasterone A obtained through ATP determination kit (n=3; $p > 0.05$ (ns), $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***).

HD pathogenesis involves transcriptional dysregulation and mitochondrial deterioration, processes linked by interactions between *p53* and the mitochondrial biogenesis factor *PGC-1α* (peroxisome proliferator-activated receptor γ co-activator 1α) [20]. mHTT reduces *PGC-1α* expression, thereby decreasing the transcription of mitochondrial respiration factors [21]. To determine whether Kinetin can rescue transcriptional downregulation of key mitochondrial genes, HD150Q cells were induced with Ponasterone A (1µM) alone or along with Kinetin (1µM) for 24h. RNA was isolated from harvested cells and cDNA was synthesized. The expressions of mitochondrial target genes

were evaluated by real time PCR using TB Green Master mix in QuantStudio™ 3 System (Applied Biosystem). Fold change of gene expression was calculated using $2^{-\Delta\Delta Ct}$ method. As expected, mHTT induction by Ponasterone A led to dramatic reduction in transcript levels of *Bdnf*, *Pgc1α* and *Nrf-1*. However, co-treatment with Kinetin resulted in significant rescue (**Fig. 6.3.2**).

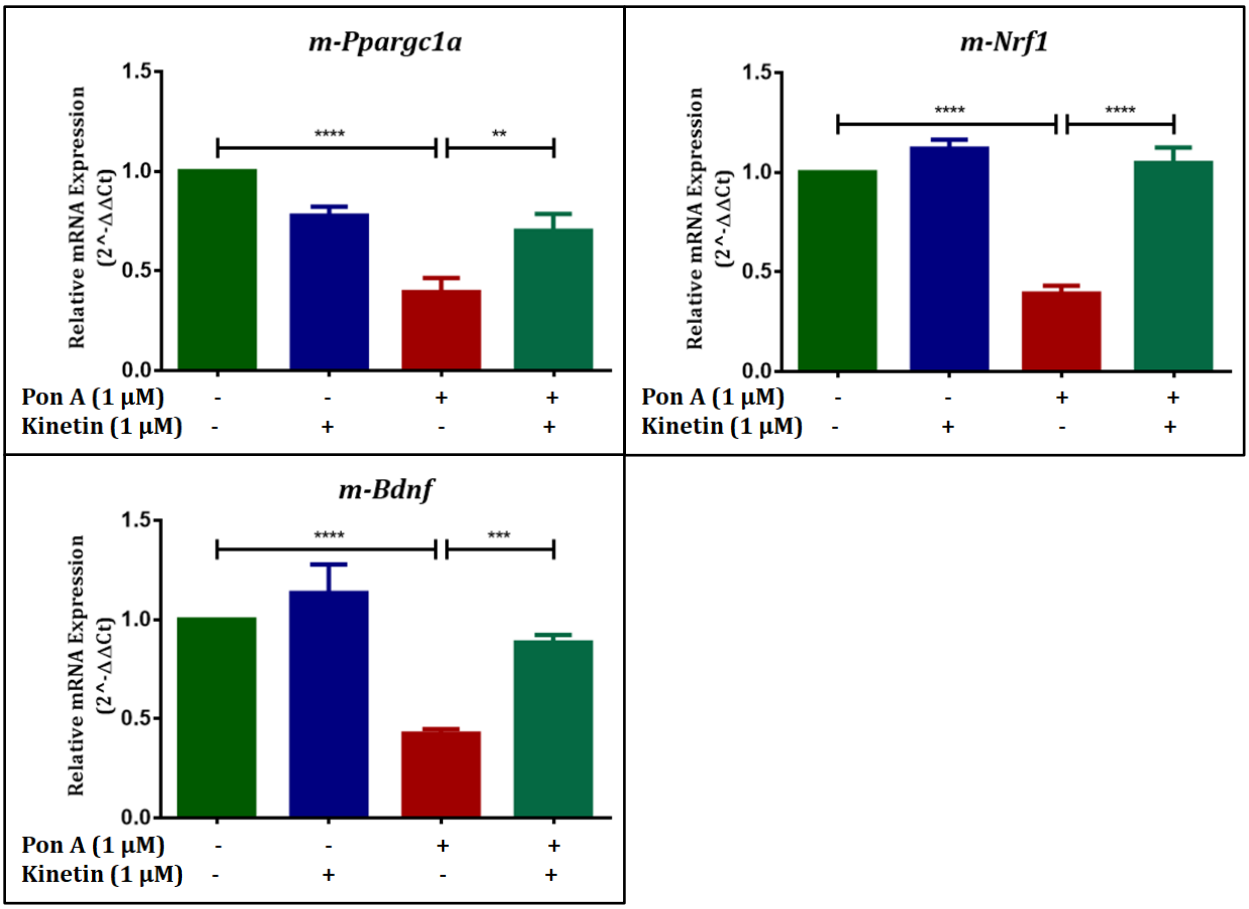


Figure 6.3.2: Expression profile of some of the key mitochondrial genes (*Pgc1α* and *Nrf1*) and *Bdnf* obtained through RT-qPCR (n=3; $p > 0.05$ (ns), $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***).

One of the major factors contributing to the development of Huntington's disease is oxidative stress. Multiple lines of evidence indicate that mitochondrial dysfunction, excessive production of reactive oxygen species (ROS), and oxidative stress—a balance between pro-oxidant and antioxidant systems that causes oxidative damage to proteins might play significant roles in the mechanisms underlying the selective neuronal death [22]. Here, we examined mitochondrial ROS levels by MitoSOX Red using fluorescence microscopy. Mitochondrial ROS levels significantly increased upon Pon A induction in HD150Q cells as function of duration of incubation as evidenced by enhancement of red fluorescence (**Fig. 6.3.3A** and **6.3.3B**). However, co-treatment

of induced HD150Q cells with Kinetin (1µM) dramatically reduced mitochondrial ROS levels at both 24h and 48h (**Fig. 6.3.3C** and **6.3.3D**). These results suggest that mHTT N17 domain phosphorylation can alter it deleterious impact on mitochondrial gene transcription, ATP levels as well as oxidative stress.

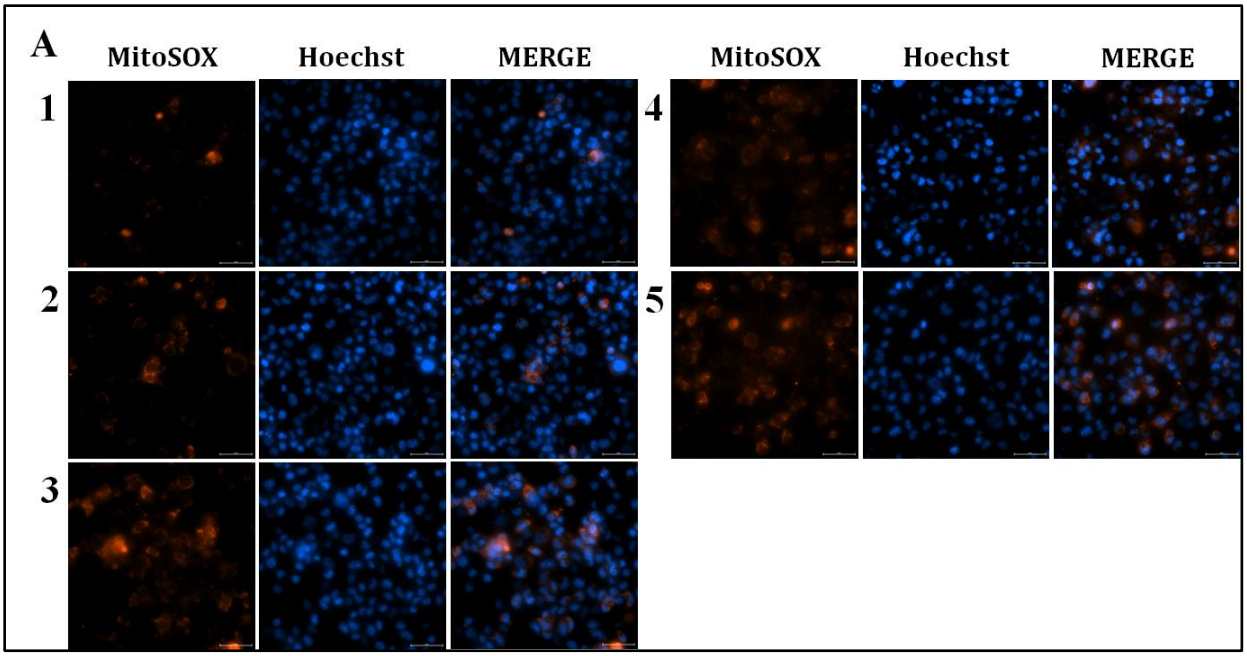


Figure 6.3.3A: 40X Fluorescence microscopic images showing mitochondrial ROS levels using MitoSOX Red in HD150Q cells after Ponasterone A treatment for 24, 48, 72 and 96 hours, respectively. 1. HD150Q control 2. 24 h Ponasterone A 3. 48 h Ponasterone A 4. 72 h Ponasterone A 5. 96 h Ponasterone A. Scale bar 50 µm (40x magnification).

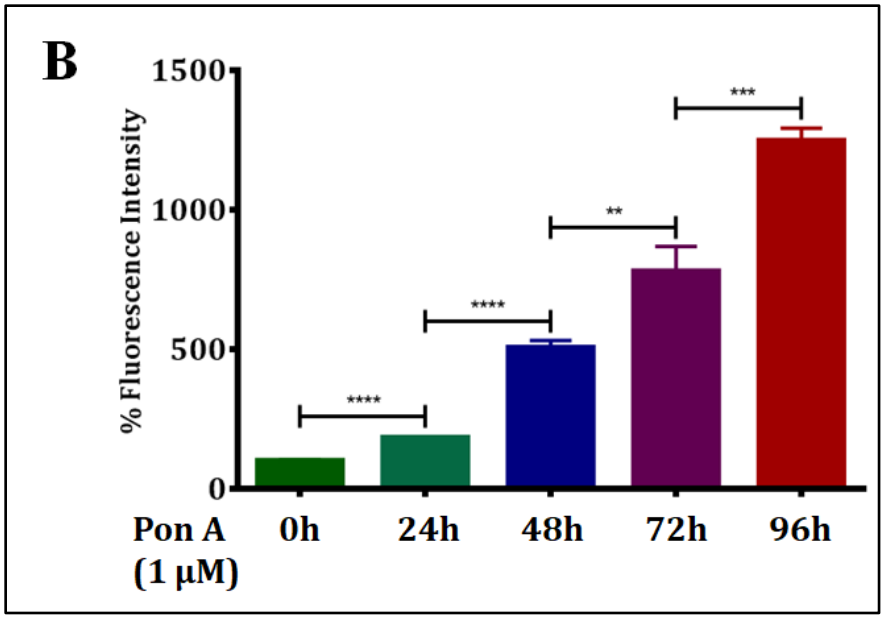


Figure 6.3.3B: Graphical representation showing % fluorescence intensity of MitoSOX Red in HD150Q cells after Ponasterone A treatment for 24, 48, 72 and 96 hours, respectively (n=3; $p > 0.05$ (ns), $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***).

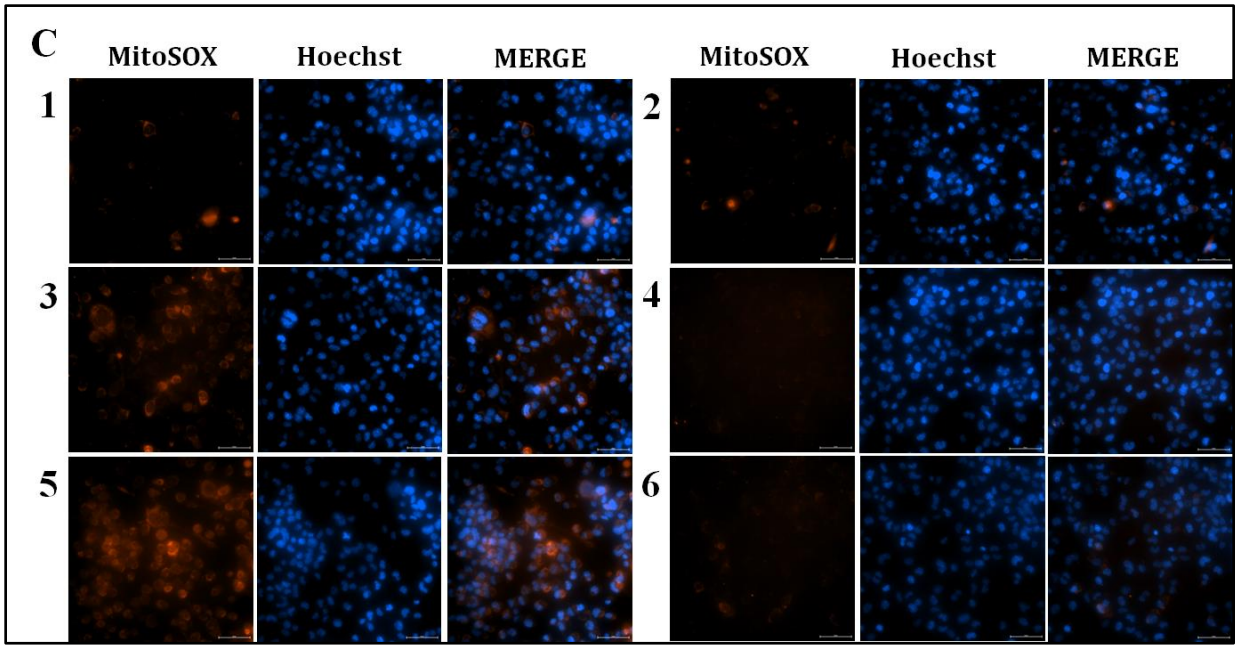


Figure 6.3.3C: 40X Fluorescence microscopic images showing mitochondrial ROS levels using MitoSOX Red in HD150Q cells after different concentrations of Kinetin with/without Ponasterone A for 24 and 48 hours. 1. HD150Q control 2. Kinetin alone 48 h 3. Ponasterone A alone 24 h 4. Pon A + 1 µM Kinetin 24 h 5. Ponasterone A alone 48 h 4. Pon A + 1 µM Kinetin 48 h.

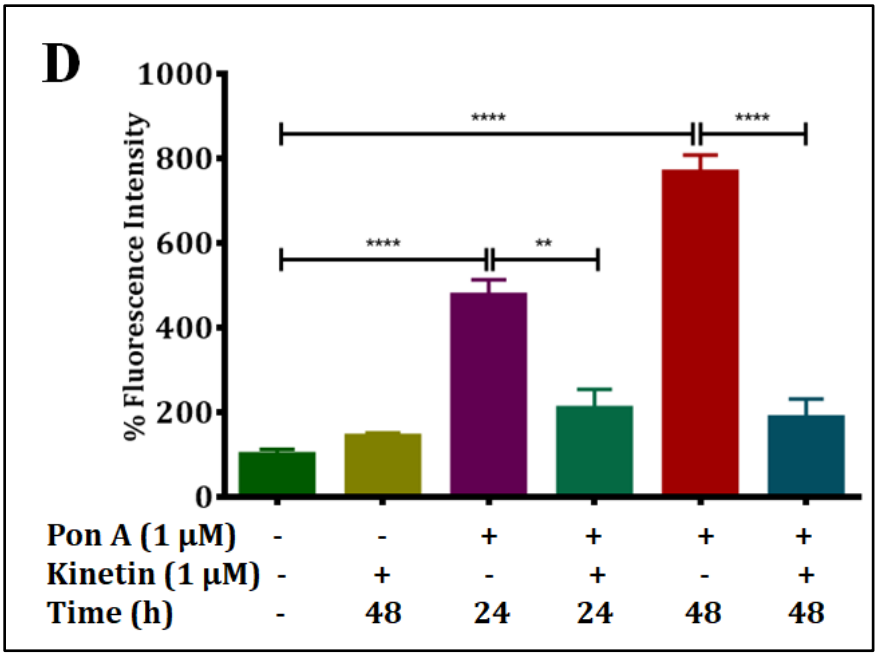


Figure 6.3.3D: Graphical representation showing % fluorescence intensity of MitoSOX Red in HD150Q cells after treatment of Kinetin with/without Ponasterone A for 24 and 48 hours (n=3; p > 0.05 (ns), p≤ 0.05 (*), p ≤ 0.01 (**), p ≤ 0.001 (***).

The effects of BMS 345541 and Bay 11-7082 on cellular ATP production were also examined using a luminescence-based ATP determination kit. Results indicated that 48-hour treatments with BMS 345541 or Bay 11-7082 restored ATP levels in HD150Q cells (**Fig. 6.3.4**). Additionally, RT-qPCR analysis showed that Ponasterone A treatment significantly reduced the expression of *Bdnf*, *Pgc1α*, and *Nrf-1*, which was completely reversed by treatment with BMS 345541 and Bay 11-7082 (**Fig. 6.3.5**). These findings suggest that BMS 345541 and Bay 11-7082 are promising therapeutic candidates for restoring mitochondrial dysfunction that is beneficial in HD.

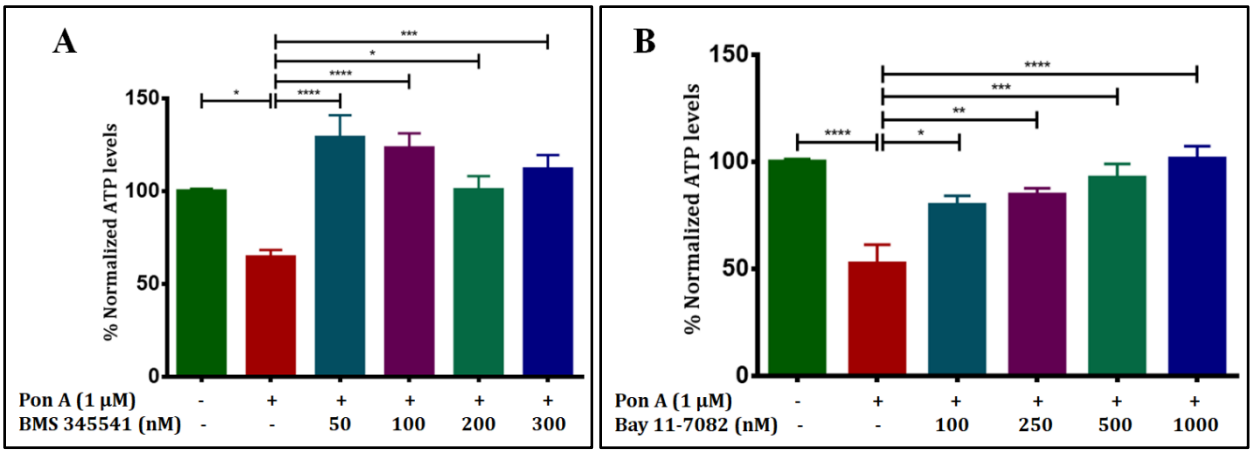
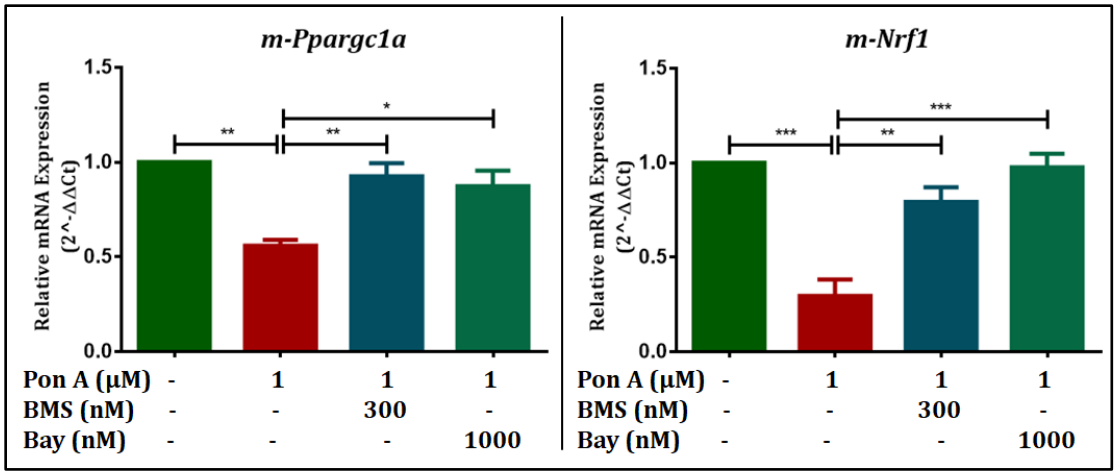


Figure 6.3.4: Graphical representation of normalized ATP levels in HD150Q cells after Ponasterone A with/without different concentrations of **(A)** BMS 345541 and **(B)** Bay 11-7082 for 24 hours (n=3; p > 0.05 (ns), p≤ 0.05 (*), p ≤ 0.01 (**), p ≤ 0.001 (***).



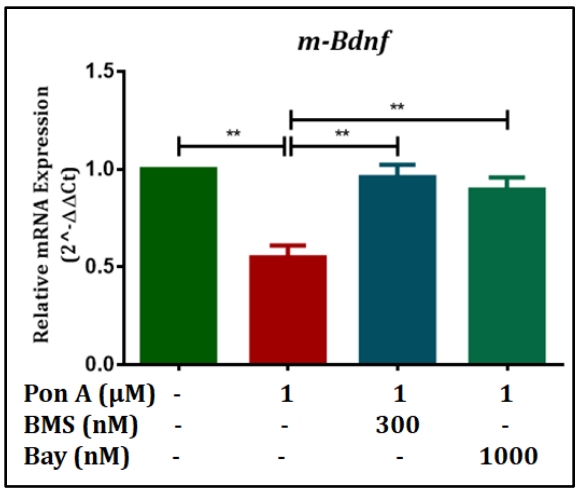


Figure 6.3.5: Expression profile of some of the key mitochondrial genes (*Pgc1α* and *Nrf1*) and *Bdnf* after Ponasterone A with/without BMS 345541 and Bay 11-7082 treated HD150Q cells for 24 hours obtained through RT-qPCR (n=3; $p > 0.05$ (ns), $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***).

Mitochondrial ROS levels were further assessed using MitoSOX Red. Similar to the effects on mHTT aggregation, various concentrations of BMS 345541 and Bay 11-7082 significantly reduced mitochondrial ROS levels (**Fig. 6.3.6A** and **6.3.6B**). Collectively, these results demonstrate that BMS 345541 and Bay 11-7082 can alleviate mitochondrial dysfunction associated with HD, supporting their potential as therapeutic agents in the disease's management.

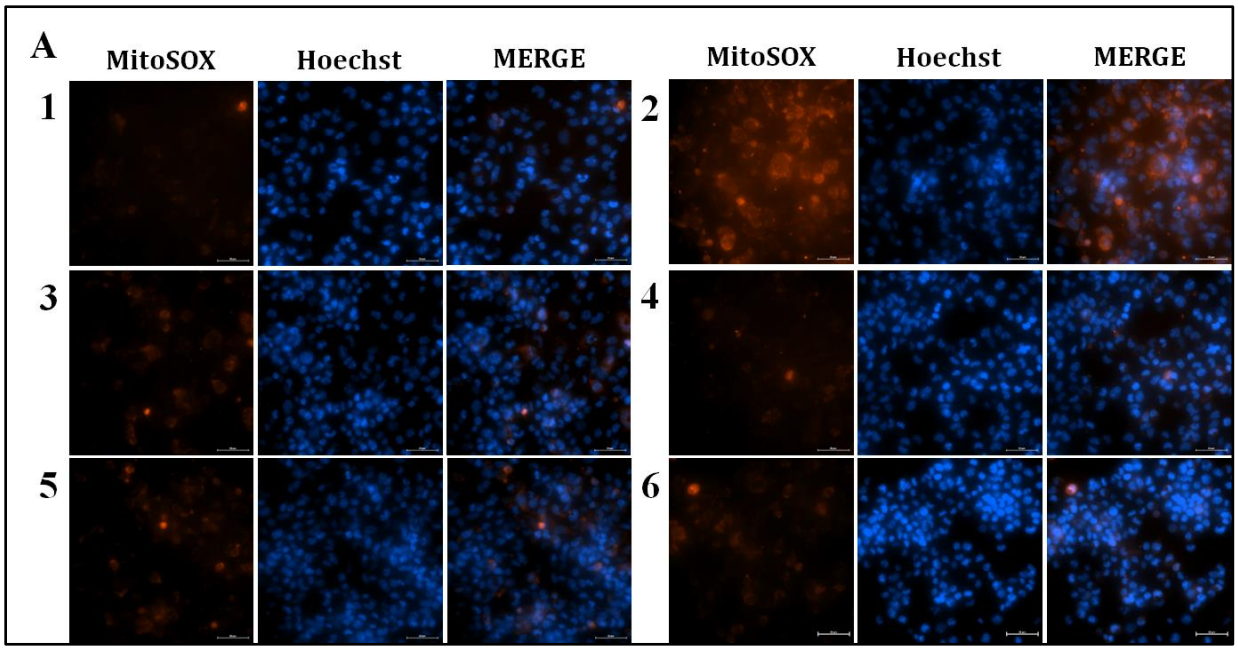


Figure 6.3.6A: 40X Fluorescence microscopic images showing mitochondrial ROS levels using MitoSOX Red in HD150Q cells after Ponasterone A with/without different concentrations of BMS 345541 and Bay 11-7082 for 48 hours. Scale bar 50 µm (40x magnification).

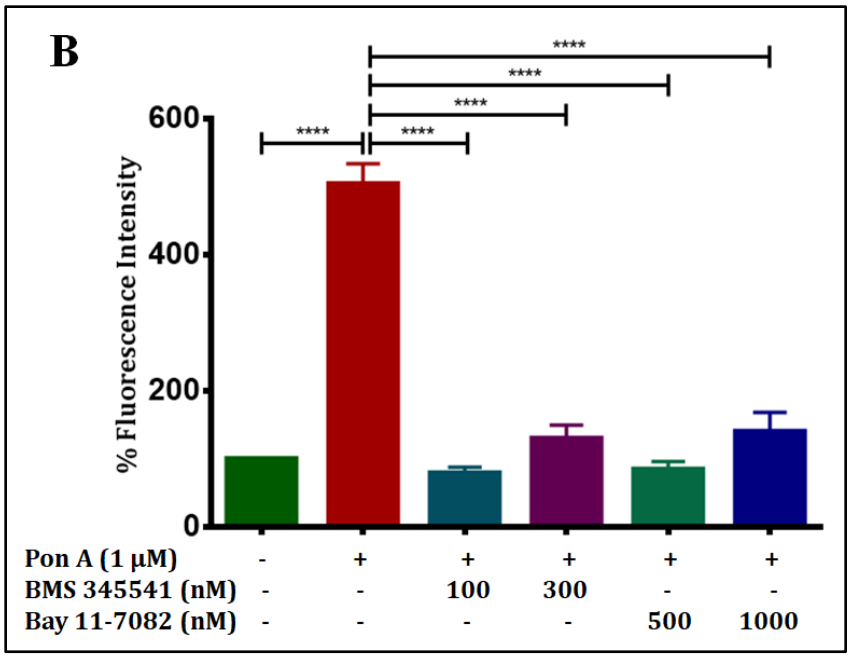


Figure 6.3.6B: Graphical representation showing % fluorescence intensity of MitoSOX Red in HD150Q cells after Ponasterone A with/without different concentrations of BMS 345541 and Bay 11-7082 for 48 hours (n=3; $p > 0.05$ (ns), $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***).

6.4 Kinetin, BMS 345541, and Bay 11-7082 Play a Vital Role in the Regulation of ER Stress Induced by mHTT

The inhibition of the ER-associated degradation (ERAD) pathway by mutant huntingtin (mHTT) leads to endoplasmic reticulum (ER) stress and triggers the unfolded protein response (UPR), a critical cellular stress response. The UPR aims to restore cellular homeostasis or induce cell death to prevent the accumulation of damaged cells [23]. This response is mediated by three major ER-resident transmembrane proteins: PKR-like endoplasmic reticulum kinase (PERK), activating transcription factor-6 (ATF6), and inositol-requiring enzyme-1 (IRE1) [24].

To evaluate the effect of Kinetin on the expression of ER stress-associated genes and proteins, RT-qPCR and western blotting were employed. As expected, transcription levels of key ER stress regulatory genes, such as *Perk*, *Chop*, *Xbp1s*, and *Atf6*, were significantly elevated in Ponasterone A-treated HD150Q cells compared to untreated controls. Co-treatment with Kinetin, however,

restored the expression of these genes to control levels (**Fig. 6.4.1**). Protein level analysis showed similar trends. Ponasterone A-induced mHTT expression increased the levels of IRE1α, BiP, XBP1s, GADD34, and CHOP proteins, along with enhanced phosphorylation of PERK and eIF2α, indicative of ER stress (**Fig. 6.4.2**). Kinetin co-treatment normalized these levels and phosphorylation states to those of control cells (**Fig. 6.4.2**). Quantitative analysis of western blots further supported these observations (**Fig. 6.4.3**).

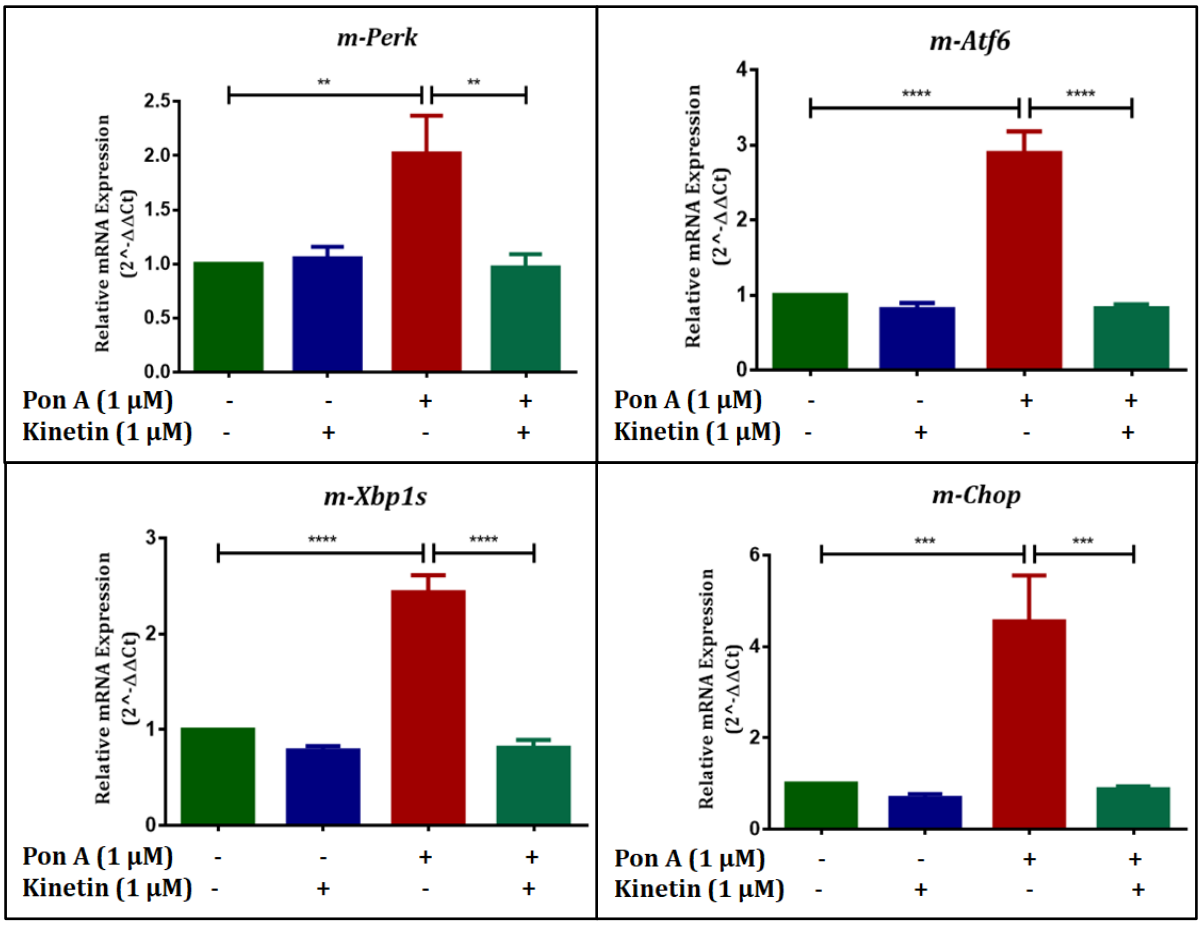
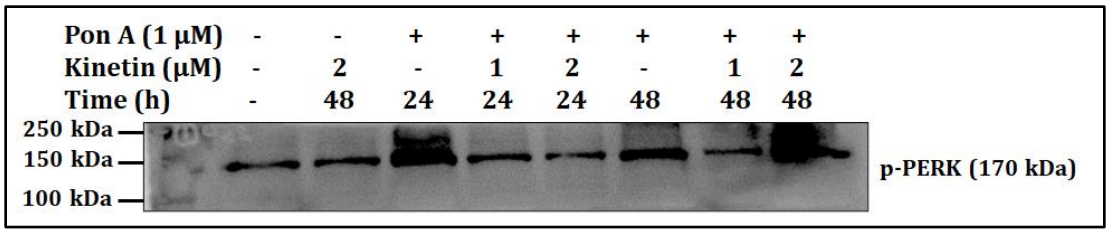


Figure 6.4.1: Expression profile of some of the crucial genes involved in ER stress in HD150Q cells after treatment of Kinetin with/without Ponasterone A obtained through RT-qPCR (n=3; $p > 0.05$ (ns), $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***).



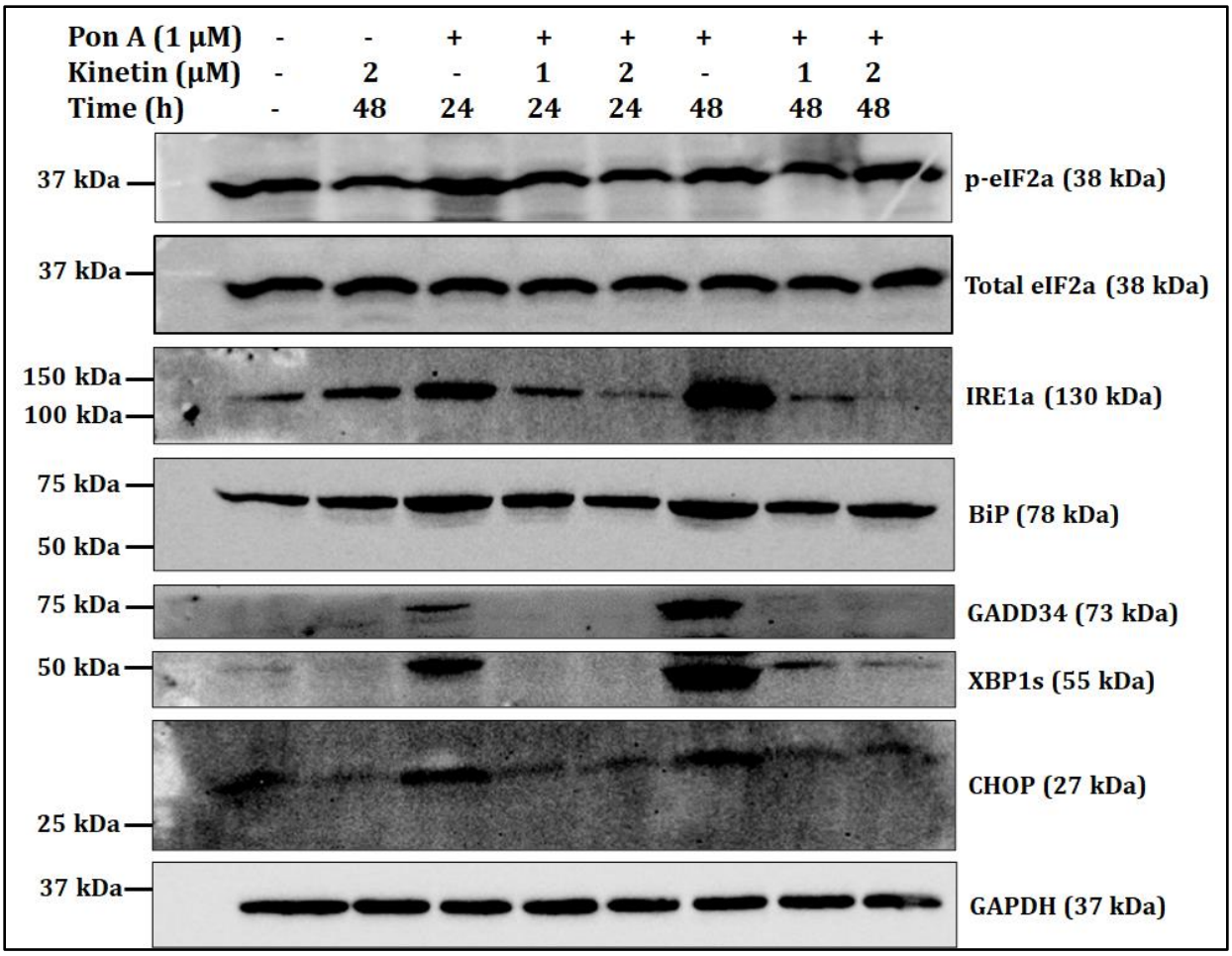
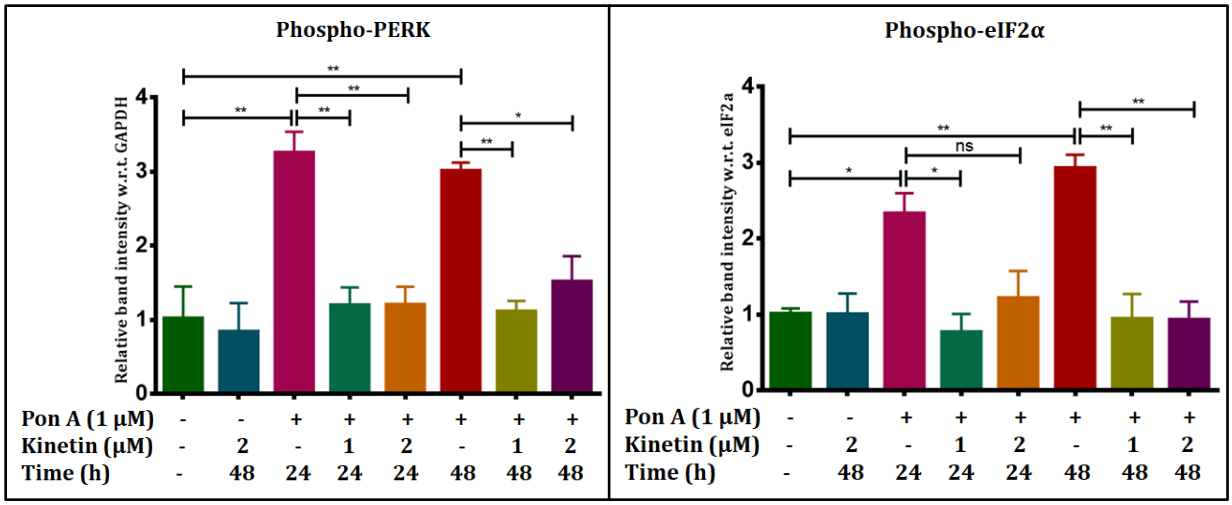


Figure 6.4.2: Western Blot image showing some of the key proteins involved in ER stress in HD150Q cells after different concentrations of Kinetin with/without Ponasterone A treatment for 24 and 48 hours.



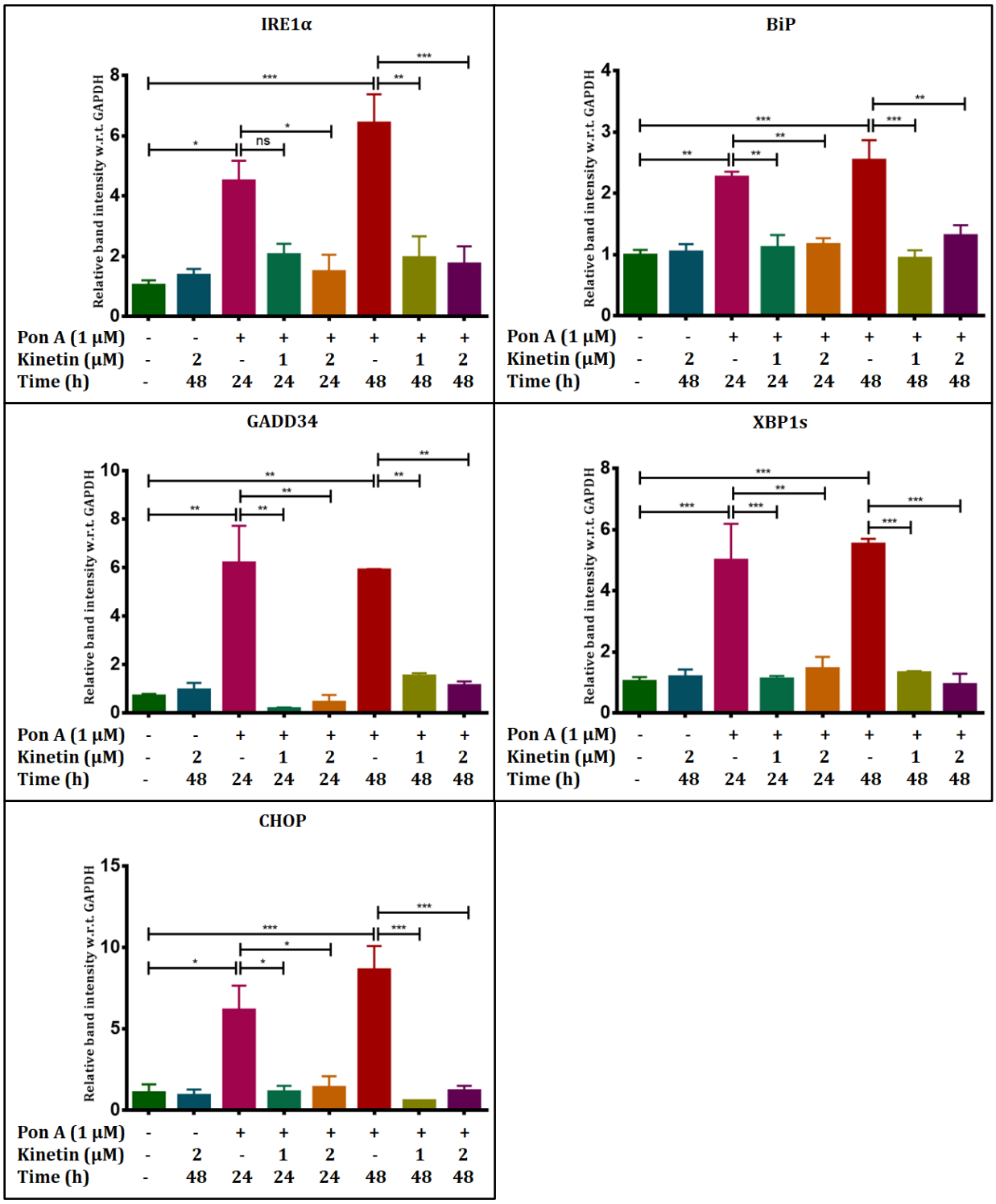


Figure 6.4.3: Graphical representations for densitometric analysis of some of the key proteins involved in ER stress in HD150Q cells after different concentrations of Kinetin with/without Ponasterone A for 24 and 48hours (n=3; $p > 0.05$ (ns), $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***).

The neuroprotective effects of BMS 345541 and Bay 11-7082 on ER stress-related genes and proteins were also investigated using RT-qPCR and western blotting. As shown in **Fig. 6.4.4**, Ponasterone A-treated HD150Q cells exhibited elevated levels of *Perk*, *Chop*, *Xbp1s*, and *Atf6* compared to untreated cells. Treatment with BMS 345541 and Bay 11-7082 reversed these effects. Western blot analysis mirrored these findings; 48 hours post-Ponasterone A treatment, HD150Q cells showed increased expression of p-PERK, p-eIF2α, IRE1α, and BiP. Treatment with BMS 345541 and Bay 11-7082 significantly reduced the expression of these proteins, suggesting a protective role against ER stress (**Fig. 6.4.4A** and **6.4.4B**). This indicates that both compounds, like Kinetin, can mitigate ER stress effectively.

These results collectively demonstrate that Kinetin, BMS 345541, and Bay 11-7082 significantly modulate ER stress pathways, highlighting their potential as therapeutic agents in the treatment of Huntington's Disease by mitigating mHTT-induced ER stress.

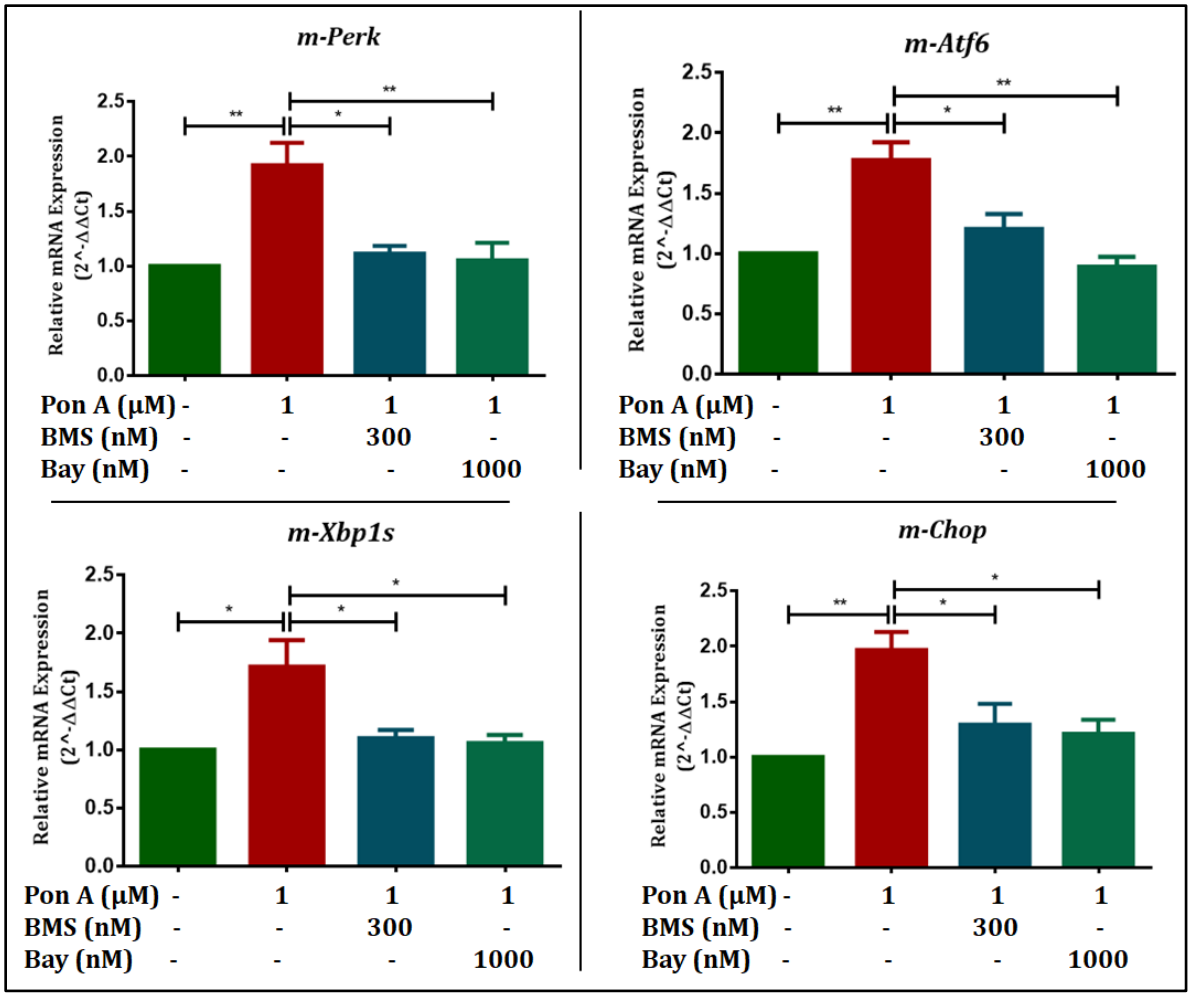
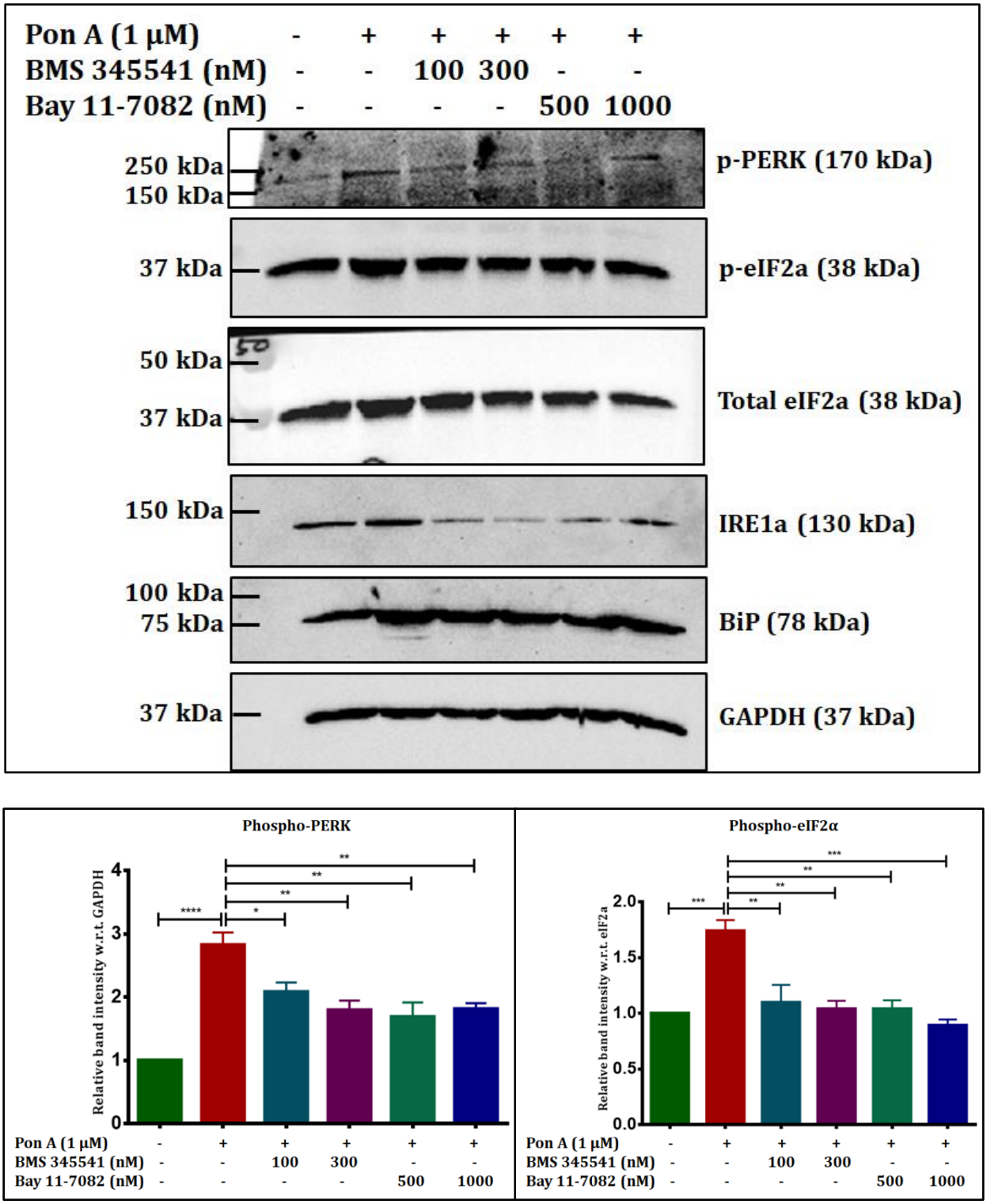


Figure 6.4.4: Expression profile of some of the crucial genes involved in ER stress after Ponasterone A with/without BMS 345541 and Bay 11-7082 treated HD150Q cells for 24 hours obtained through RT-qPCR (n=3; $p > 0.05$ (ns), $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***).



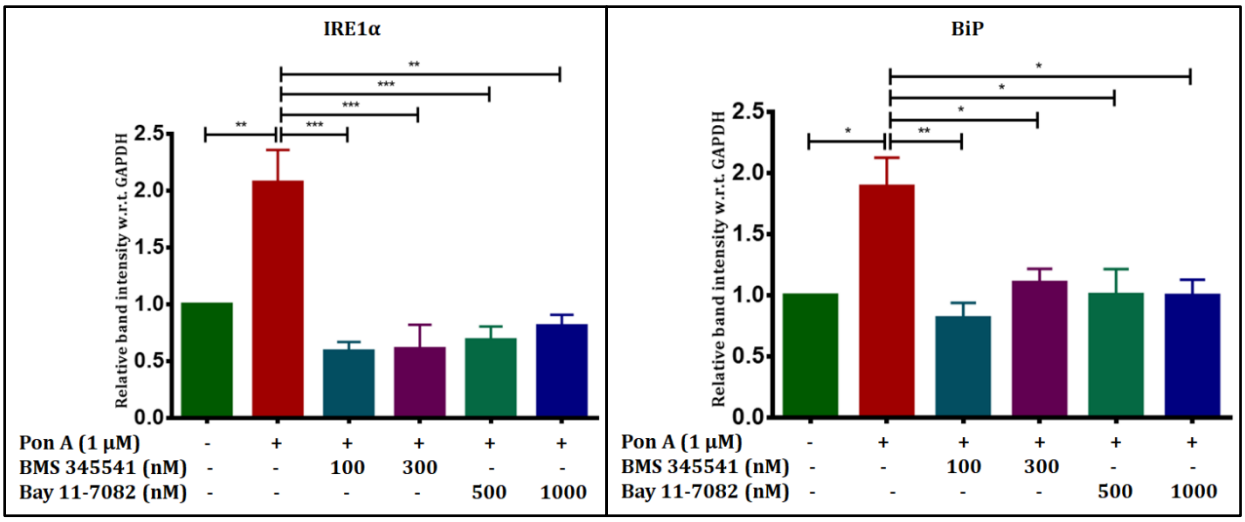


Figure 6.4.5A: Western Blot image showing some of the key proteins involved in ER stress after Ponasterone A with/without different concentrations of BMS 345541 and Bay 11-7082 treated HD150Q cells for 48 hours.

Figure 6.4.5B: Graphical representations for densitometric analysis of some of the key proteins involved in ER stress after Ponasterone A with/without different concentrations of BMS 345541 and Bay 11-7082 treated HD150Q cells for 48 hours (n=3; $p > 0.05$ (ns), $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***).

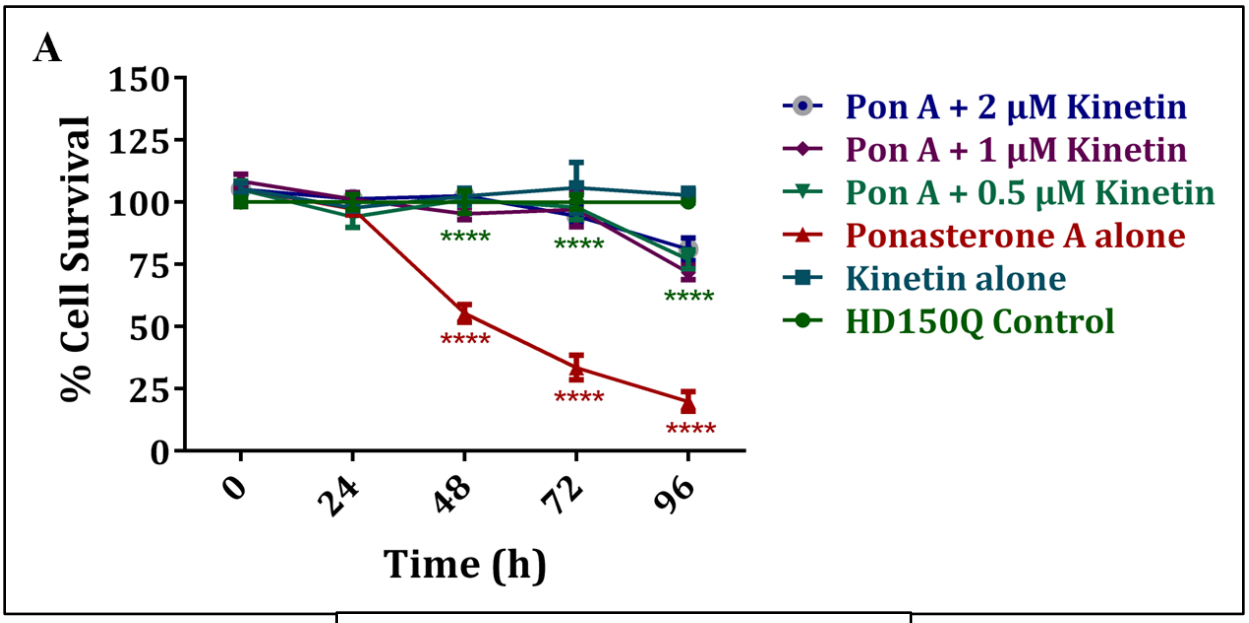
6.5 Kinetin, BMS 345541, and Bay 11-7082 Improve Cell Viability

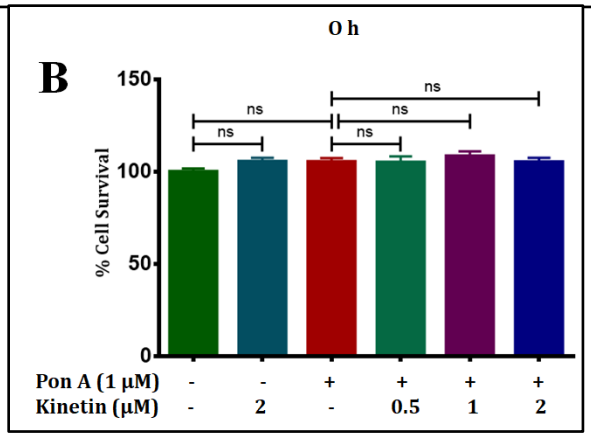
Given that the treatments with Kinetin, BMS 345541, and Bay 11-7082 successfully reduced mHTT aggregation, improved mitochondrial function, and alleviated ER stress, it became essential to explore their impact on cell viability. This aspect is crucial because the overall effectiveness of these treatments hinges not just on their ability to address specific molecular dysfunctions, but also on their capacity to sustain cell survival. To thoroughly assess this, HD150Q cells were subjected to Ponasterone A to induce mHTT expression, either alone or in combination with defined concentrations of Kinetin, BMS 345541, and Bay 11-7082. Cell viability was meticulously monitored at 24, 48, 72, and 96 hours using the well-established MTT assay.

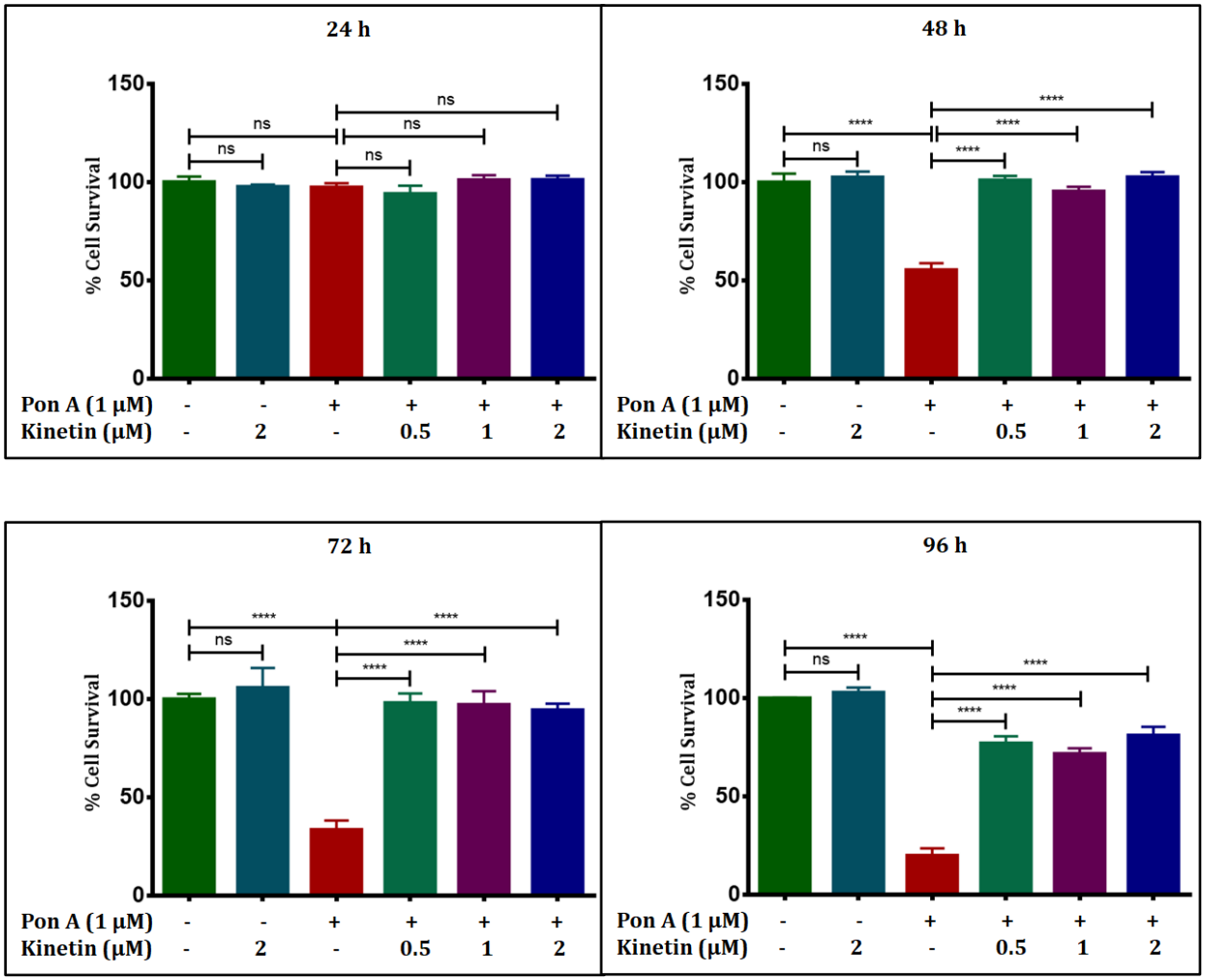
The induction of mutant HTT led to a notable decline in cell viability starting at the 48-hour mark (**Fig. 6.5.1A** and **6.5.1C**), reflecting the toxic impact of mHTT on cellular health. However, when co-treated with Kinetin, BMS 345541, and Bay 11-7082, cell viability was remarkably maintained at levels comparable to untreated control cells up to 72 hours. Although a slight reduction in

viability was observed at 96 hours (**Fig. 6.5.1A, B, C** and **D**), the overall preservation of cell viability indicates a significant protective effect imparted by these compounds. These results clearly suggest that Kinetin, BMS 345541, and Bay 11-7082 enhance the survival of HD150Q cells, positioning them as potential therapeutic agents for Huntington's disease.

The ability of these compounds to maintain cell viability in the face of mHTT induction is particularly noteworthy. It underscores their comprehensive role in mitigating the multiple detrimental effects associated with Huntington's disease, which include not only mHTT aggregation but also mitochondrial dysfunction and ER stress. By addressing these key pathological features, Kinetin, BMS 345541, and Bay 11-7082 offer a multi-pronged therapeutic approach that holds promise for alleviating the cellular dysfunctions characteristic of Huntington's disease. This broad-spectrum protective capability highlights the therapeutic potential of these compounds and warrants further investigation into their clinical applicability for HD treatment.






24 h
48 h
72 h
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% Cell Survival
150
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Pon A (1 µM) - - + + + +
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25
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24
48
72
96
Time (h)
****
Pon A + 1000 nm Bay 11-7082
Pon A + 500 nm Bay 11-7082
Pon A + 300 nM BMS 345541
Pon A + 100 nM BMS 345541
Ponasterone A alone
HD150Q Control

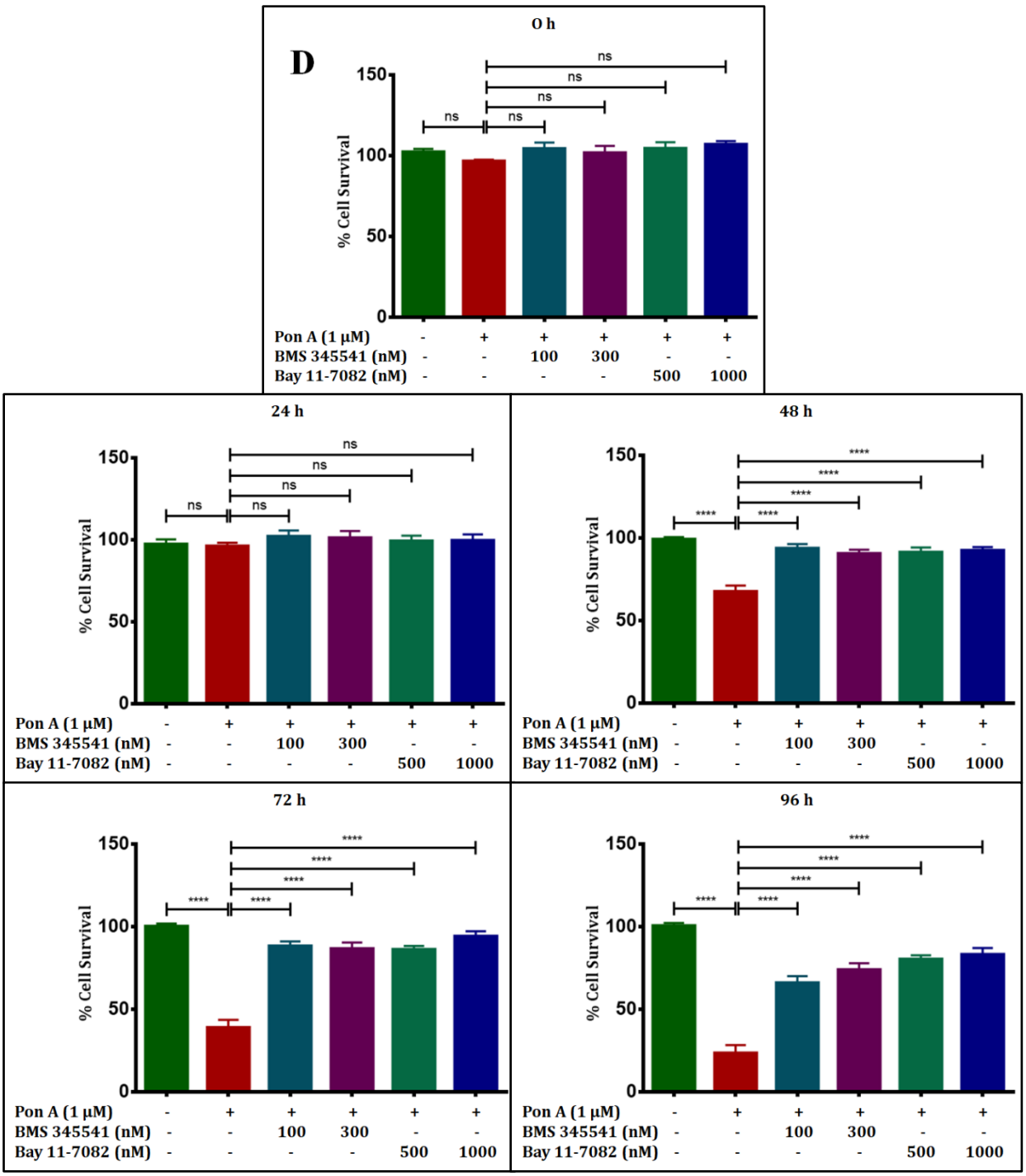

Figure 6.5.1A: Graphical representation of Ponasterone A and/or Kinetin treated HD150Q cells for cell survivability by MTT assay (n=3; $p > 0.05$ (ns), $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***).

Figure 6.5.1B: Graphical representation of Ponasterone A and/or Kinetin treated HD150Q cells for cell survivability by MTT assay for 24, 48, 72 and 96 hours, respectively (n=3; $p > 0.05$ (ns), $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***).

Figure 6.5.1C: Graphical representation of Ponasterone A with/without different concentrations of BMS 345541 and Bay 11-7082 treated HD150Q cells for cell survivability by MTT assay (n=3; $p > 0.05$ (ns), $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***).

Figure 6.5.1D: Graphical representation of Ponasterone A with/without different concentrations of BMS 345541 and Bay 11-7082 treated HD150Q cells for cell survivability by MTT assay for 24, 48, 72 and 96 hours, respectively (n=3; $p > 0.05$ (ns), $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***).

6.6 Discussion

Worldwide collaborative efforts have led to the progression of HD research from pre-clinical development to several ongoing clinical trials. However, the cure for HD is still elusive. The development and testing of therapeutics that target the HD pathogenesis, namely by targeting huntingtin gene, RNA, and protein, is currently a major area of focus. These strategies eventually seek to lower mHTT levels and, consequently, ameliorate its numerous and diverse downstream pathogenic effects [25]. The highly conserved first 17 amino acids of huntingtin (N17) have been extensively studied as a region that leads huntingtin to the endoplasmic reticulum (ER), late endosomes, and autophagic vesicles [26]. In-depth research on this small domain has revealed that several of its residues are subjected to post-translational modifications like as SUMOylation, phosphorylation, and acetylation, all of which seem to impact the toxicity of this small amino terminal fragment of HTT with expanded polyQ repeats [3]. Post-translational modifications (PTMs) of proteins regulate a variety of cellular processes through modification of protein structure and functions. Since many PTMs are reversible and regulated by specific enzymes that can be modified by small molecules, making them potential therapeutic targets [2].

Post-translational modifications of huntingtin, including phosphorylation, have been shown to regulate its function and toxicity. Huntingtin is heavily phosphorylated and several phosphorylation sites have been reported. Phosphorylation at several sites including Ser421, Ser434, Ser513, Ser536, Ser1181 and Ser1201 were shown to be protective in nature and reduce the aggregation and toxicity of the mutant protein [27-33]. There is considerable accumulated evidence for the impact of huntingtin phosphorylation on pathogenic consequences of expanded polyglutamine (polyQ) region. Phosphorylation at Ser421, by Akt or serum and glucocorticoid induced kinase (SGK) was neuroprotective against cellular toxicity [34,35]. Ser421

phosphorylation decreased the accumulation of both full-length huntingtin and huntingtin fragments (including those generated by caspase 6) in the nucleus [27] and restored anterograde and retrograde axonal transport in neurons [28]. The regional pattern of phosphorylation at Ser421 in the brain paralleled the graded pathology of HD, with highest phospho-huntingtin levels in the cerebellum, less in the cortex and least in the striatum [29]. Phosphorylation of mutant huntingtin at Ser434 by the serine-threonine kinase CDK5 reduced huntingtin cleavage at 513 amino acid by caspase 3 and inhibited polyQ aggregation and cytotoxicity [30]. The aggregation and cellular toxicity of mutant huntingtin was decreased upon phosphorylation at Ser536, which inhibited the cleavage of huntingtin by calpain at this amino acid [31,32]. Phosphorylation of polyQ huntingtin at Ser1181 and Ser1201 by CDK5 protected against toxicity, whereas the absence of phosphorylation of normal huntingtin at the same sites conferred toxic properties similar to the expanded polyQ protein [33]. Most compelling evidence for protective nature of huntingtin phosphorylation was presented in a study where constitutive phosphorylation at Ser13 and Ser16 was shown complete ablation of motor, psychological and psychiatric symptoms in BAC transgenic mice expressing human mutant huntingtin with serine residues at position 13 and 16 substituted with phosphomimetic aspartate [36]. This was followed by a kinase screen wherein it was shown that inhibiting the kinase responsible for phosphorylation of serine residues at position 13 and 16 had dramatic effects on nuclear and sub-nuclear localization of huntingtin [3]. However, the mechanism by which mutant huntingtin toxicity is rescued remains unclear.

This study comprised of studying the neuroprotective effect of Kinetin to prevent mHTT aggregation in HD150Q cells. It has been reported that multiple pathways are dysregulated in HD which includes mitochondrial dysfunction leading to generation of ROS, oxidative stress and protein misfolding leading to ER stress resulting neuronal cell death [1]. Unfortunately, there is currently no effective treatment for HD or other polyQ diseases that can have neuroprotective effects or alter the course of the disease. Novel synthetic medications also have severe side effects and are extremely toxic. It's interesting that using phytochemicals instead of target-specific, synthetic drugs to treat neurodegenerative diseases has a number of benefits, including almost no side effects and little toxicity to normal cells. It is anticipated that natural compounds that target several cellular processes would be crucial in developing novel treatment leads for polyQ diseases. One such phytochemical curcumin, frequently used ingredient in Asian food has a variety of anti-oxidant, anti-inflammatory, and anti-fibrilogenic characteristics. Chongtham *et al* reported that

curcumin significantly reduces disease symptoms in a *Drosophila* HD model by preventing cell death and may hold the secret to reversing the course of Huntington's disease with the fewest adverse effects [37]. One more study by Chakraborty *et al*. shown the effect of different doses of quercetin for protecting a neuronal cell line HD150Q that expresses mutant huntingtin. They observed that quercetin increased cell survival, balanced ubiquitin-proteasomal complex activity, and prevented the development of mutant protein aggregates when used in low dosages [38]. Apart from phytochemicals, researchers have also reported the neuroprotective effect of different kinase and phosphatase inhibitors viz. Bay 11-7082, piceatannol, DMAT, BMS-345541, N6-furfuryladenine (N6FFA), also known as "Kinetin" for HD [3,9].

Kinetin is a plant cytokine that has been reported to inhibit polyglutamine-based mutant huntingtin aggregation inside neuronal cells. Adenine phosphoribosyltransferase (APRT) salvages Kinetin to create Kinetin triphosphate (KTP), an ATP analogue. It was discovered that Kinetin is the precursor to KTP. In the context of HD, when ATP levels are considerably decreased, this nucleotide salvaging is crucial, especially in neurons, which heavily rely on nucleotide salvage as opposed to *de novo* nucleotide synthesis pathways. Additionally, ATP synthesis may halt during DNA damage repair, and the amount of free ATP decreases considerably during the ER stress response [9]. Here, we demonstrate that casein kinase 2 (CK2) can employ KTP to phosphorylate N17, which is produced from kinetin, and is protective in cell line model of HD. Mutant huntingtin is hypophosphorylated in the N17 domain and its phosphorylation has been shown to be beneficial in HD models. The protective effect of kinetin treatment connect DNA-damage repair, altered bioenergetics, and mutant huntingtin hyper-phosphorylation. From the data obtained, it was concluded that treatment with Kinetin showed successful prevention and/or rescue of mHTT aggregates. These data suggest that Kinetin could be a potential therapeutic target for prevention of mHTT aggregates and helpful in Huntington's disease.

BMS 345541, an IκB kinase (IKK) inhibitor, has shown promise in mitigating the deleterious effects of mutant huntingtin aggregation [3]. By inhibiting IKK, BMS 345541 interferes with the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathway, which is implicated in the inflammatory response and neurodegeneration seen in Huntington's disease [11,12]. The treatment of HD150Q cells with BMS 345541 not only reduced mHTT aggregation but also improved mitochondrial function and alleviated ER stress. These improvements are crucial, as mitochondrial dysfunction and ER stress are hallmarks of Huntington's disease

pathology, contributing to neuronal death and the progression of the disease. Thus, BMS 345541's ability to preserve cellular homeostasis by reducing stress responses and maintaining ATP levels highlights its therapeutic potential.

Similarly, Bay 11-7082, another inhibitor targeting the NF-κB pathway, has demonstrated significant efficacy in reducing mHTT aggregates and restoring mitochondrial function. By inhibiting IκBα phosphorylation, Bay 11-7082 prevents NF-κB activation, thereby reducing inflammation and cellular stress responses [13,14]. Treatment with Bay 11-7082 resulted in the normalization of ATP levels and reduced ROS production, indicative of improved mitochondrial health. Furthermore, Bay 11-7082 alleviated ER stress markers, suggesting a comprehensive protective effect against mHTT-induced cellular dysfunction. These findings underscore Bay 11-7082's potential as a therapeutic agent in Huntington's disease, offering a multi-faceted approach to mitigating the molecular and cellular disruptions caused by mHTT.

In summary, we can conclude that Kinetin, BMS 345541, and Bay 11-7082 phosphorylate mutant huntingtin at Ser13-Ser16 resulting in increased ATP production, decreased insoluble mHTT aggregates, and restored soluble huntingtin, alleviation of mitochondrial dysfunction and ER stress. These compounds may therefore represent a promising class of neuroprotective drugs for treat Huntington's disease.

6.7 References

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