Synopsis of the Ph.D. Thesis on

Targeting Mutant Huntingtin Toxicity for Development of Novel Therapeutics for Huntington's Disease

To be submitted to
The Maharaja Sayajirao University of Baroda, Vadodara

The Department of Biochemistry,
The Maharaja Sayajirao University of Baroda.

For the degree of
Doctor of Philosophy in Biochemistry

By

Dabhi Rajubhai Khimabhai

Under the Supervision of
Dr. Ravi Vijayvargia
Department of Biochemistry,
Faculty of Science,
The Maharaja Sayajirao University of Baroda,
Vadodara – 390 002.

INTRODUCTION

Definition

Huntington's disease (HD) is a fatal autosomal dominant neurodegenerative disorder caused by a polymorphic CAG triplet repeat expansion in the Huntingtin gene, leading to an abnormal polyQ expansion in the huntingtin (HTT) protein **(The Huntington's Disease Collaborative Research Group (1993))**. At a cellular level, HD is defined by the preferential degeneration and death of striatal medium spiny neurons (MSNs) as well as cortical pyramidal neurons **(Nicolas Arbez *et al.,* 2017)**. Basically, it is a class of tri-nucleotide repeat disorder.

What role does it play in physiology?

The neuropathology of HD is characterized by the dysfunction and death of specific neurons within the brain **(Ross and Tabrizi *et al.,* 2011)**. The symptoms vary between individuals but are usually characterized by a triad of motor, cognitive, and psychiatric symptoms. Motor symptoms can be divided into the choreiform movements with gait disturbances that tend to appear early in the course of the disease and motor impairments such as bradykinesia and rigidity that are observed in later stage patients. Cognitive symptoms can be detected up to a decade before diagnosis, and decline progresses as the disease progresses. The deficits include cognitive slowing and decreases in both attention and mental flexibility. Psychiatric symptoms and/or emotional deficits are also observed early in HD patients. HD patients are frequently depressed and show signs of apathy, irritability, impulsivity, and social disinhibition **(Humbert *et al*., 2016)**. Some reports have been also suggested that patients with HD show peripheral symptoms like, muscle atrophy, osteoporosis, difficulty in swallowing, weight loss, impaired immune system, slurred speech, heart failure, testicular atrophy etc. other than neuronal symptoms **(The Huntington's Disease Collaborative Research Group (1993))**.

Incidence and prevalence

The prevalence of HD varies with ethnic origin, with Caucasian populations of North America and Western Europe having 5-10 subjects affected by HD per 100,000 people but that figure is on rise as genetic testing is becoming more prevalent. Some juvenile forms exist but are rare, accounting for 5% of the cases **(Humbert *et al.,* 2016)**. Approximately 90% of HD cases are inherited from a person's parents, with 10% of cases due to a new mutation and transmitted in an autosomal dominant fashion **(Lars Bertram *et al.,* 2005)**. While HD prevalence is 5-10 patients per 100,000

people in the western population, it is not clearly established for Indian population due to lack of large scale population studies. Only few population studies to determine prevalence of HD in Indian population have been carried out **(Pramanik *et al.,* 2000; Shiwach *et al.,* 1990; Saleem *et al.,* 2003)**.

Pathophysiology of condition

The mutation responsible for HD is an abnormal expansion of a CAG repeat in the *HTT* gene that encodes for huntingtin (HTT), a large protein of 3,144 amino acids **(Humbert *et al.,* 2016)**. The *HTT* gene encodes a 348-kDa protein well conserved from flies to mammals, the highest identity being found between mammals. Huntingtin (HTT) is found in all mammalian cells with highest concentrations are found in the brain and testes, with moderate amounts in the liver, heart, and lungs **(DiFiglia M *et al.,* 1995)**. The role of the wild-type protein is, as yet, poorly understood, as is the underlying pathogenesis of Huntington's disease **(Walker FO *et al.,* 2007)**. It has been reported that absence of the HTT gene is associated with embryonic lethality in animals **(Walker FO *et al.,* 2007)**. Huntingtin up regulates the expression of Brain Derived Neurotrophic Factor (BDNF) at the transcription level, but the mechanism of regulation of gene expression is not known **(Gauthier LR *et al.,* 2004)**. Huntingtin is thought to function as a scaffolding protein that mediates a variety of protein-protein interactions involved in vesicular trafficking, signaling, and transcription **(Gu *et al.*, 2009)**. Huntingtin has been shown to interact with polycomb repressive complex -2 (PRC2) and facilitates its activity **(Seong *et al.,* 2010)**. PRC2 complex plays a vital role as transcriptional regulator and mediates gene silencing by Histone H3-Lysine-27-tri-methylation. Mutant huntingtin facilitates this activity significantly higher compared to the normal Huntingtin **(Seong *et al.,* 2010)**. The pathological hallmark of HD is the accumulation of nuclear and cytoplasmic protein aggregates containing mHTT N-terminal polyQ fragments in the cortex and striatum **(Gu *et al.*, 2009)**. The very N-terminal region of HTT has been extensively studied, as it contains the expandable polyQ stretch **(Humbert *et al.,* 2016)**. The longer polyQ domain seems to induce conformational changes in the protein, which causes it to form intracellular aggregates that, in most cases, manifest as nuclear inclusions. However, aggregates can also form outside the nucleus **(Gillian Bates *et al.,* 2003)**.

Review of literature

The gene *HTT* codes for Huntingtin protein (HTT) is located on the short arm of chromosome 4 and is associated with an expanded trinucleotide repeat **(Walker *et al.,* 2007)**. In normal population CAG length ranges from 9 to 35. Intermediary alleles, which have 27-35 CAG repeats, are unstable and can be transmitted as an expanded allele to offspring; nevertheless, carriers of the intermediary allele type also have a normal phenotype. Alleles with 36-39 CAG units have reduced penetrance and generate both a normal phenotype as well as rare cases of HD. Alleles with >39 CAG copies show complete penetrance and inevitably cause, at some stage of life, the HD phenotype. The age of onset of HD is inversely correlated to CAG repeat length. In patients with HD, the onset of clinical symptoms usually starts between the ages of 35-55, although the disease can manifest after the age of 80, or much earlier, in teenagers or children, because of the phenomenon of anticipation, which occurs in 20% of cases of HD. Approximately 10% of patients with HD have onset of clinical manifestations before the age of 20, and 5% before the age of 14. Current therapeutic strategies are aimed at selectively targeting mutant huntingtin associated SNPs and eliminating the mutant huntingtin allele in mouse models of HD. Different methods including RNAi **(Hu *et al.,* 2012; Yu *et al.,* 2012)**, Zinc-finger nucleases **(Garriga-Canut *et al.,* 2012)**, and antisense oligonucleotides **(Gagnon *et al.,* 2010)** have been used and are reviewed extensively **(Sah *et al.,* 2011)**. Small molecule screening to alter mutant huntingtin toxicity by preventing its aggregation or even reverse pathogenic processes have been carried out but clinical testing has yet not been initiated. 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 **(Warby *et al.,* 2009; Zala *et al.,* 2008; Warby *et al.,* 2005; Luo *et al.,* 2005; Schilling *et al.,* 2006; Gafni *et al.,* 2004; Anne *et al.,* 2007)**. Most compelling evidence for protective nature of huntingtin phosphorylation was presented in a recent study where constitutive phosphorylation at Ser13 and Ser16 was shown to revert the toxic phenotype of mutant huntingtin in BAC mouse model **(Gu *et al.,* 2009)**. The mechanism of this effect is not very clear and needs further examination. Further, reduced ATP/ADP ratio in *STHdh*$^{Q111/Q111}$ knock-in mouse striatal cells expressing mutant huntingtin was reported **(Seong *et al.,* 2005)** but the mechanism and regulation warrants further investigation.

Problem addressed

Phosphorylation of many proteins, at serine (S), threonine (T) or tyrosine (Y) residues, regulates their functional activities, turnover **(Tozser *et al.,* 2003)** and nuclear transport **(Poon *et al.,* 2005)**. Several phosphorylation sites have been predicted in huntingtin and some sites have been confirmed; Thr3 and Ser13 and Ser16 **(Aiken *et al.,* 2009; Thompson *et al.,* 2009)**, as well as Ser421, which is phosphorylated by AKT **(Humbert *et al.,* 2002)**. Some of these sites have been implicated in toxicity of mutant huntingtin while others are demonstrated to be of protective nature. Some of the most compelling evidence for the protective nature of huntingtin phosphorylation comes from tests of full-length human huntingtin Ser13 and Ser16 phosphorylation in BAC transgenic mice **(Gu *et al.,* 2009)**. Expression of phosphomimetic huntingtin, where serine was changed to aspartate (SD) or, alternatively, expression of nonphosphorylatable alanine (SA) residues-huntingtin, demonstrated that while, both SA and SD mutant huntingtin proteins retained essential huntingtin function in rescuing Hdh knockout mouse phenotypes, only the SD mutant protein was associated with a striking absence of the motor, psychiatric and neuropathological phenotypes and decrease in mutant huntingtin aggregates **(Gu *et al.,* 2009)**. This result strongly predicts that huntingtin phosphorylation at Ser13/Ser16 can directly or indirectly prevents the toxic consequences associated with expanded polyQ huntingtin. Secondly, $STHdh^{Q111/Q111}$ knock-in mouse striatal cells expressing mutant huntingtin showed significant reduction in ATP/ADP ratio and was confirmed in several human lymphoblasts expressing at least one mutant huntingtin allele **(Seong *et al.,* 2005)**.

Key question aimed in this study

Currently, there is no cure available for HD but some of the strategies like silencing of the mutant allele by siRNA, RNAi and targeting huntingtin post-translational modifications are being employed in animal models of HD, with limited success. Thus in this study, we propose to probe mutant huntingtin phosphorylation as a therapeutic target by analyzing the effect on phosphorylation on PRC2 activity assay as well as by screening for kinase and phosphatase inhibitors that enhance phosphorylation at Ser13 and Ser16 of mHTT and can rescue low ATP/ADP ratio, mitochondrial function and abrogate PRC2 activation in a mutant huntingtin cell line model system.

AIM of this investigation

This study is aimed to examine whether mutant huntingtin phosphorylation status can modulate its toxicity by alleviating ATP/ADP ratio, improving mitochondrial function and suppressing over-activation of PRC2 activity in mutant huntingtin expressing *STHdh*^{Q111} knock-in mouse striatal cells.

Hypothesis of the study:

From the above mentioned studies, we hypothesize that Ser13 / Ser16 phosphorylation alleviates mutant huntingtin (mHTT) toxicity by normalization of dysregulated pathways.

Significance of the study:

It has been 25 years since the mutation in huntingtin gene that causes HD was identified **(HDCRG, 1993)** and still there is no cure available. Several research groups are investigating different aspect including selective deletion or depletion of mutant huntingtin allele/protein with some success in animal models. Targeting mutant huntingtin phosphorylation as potential therapeutic is an attractive avenue and some studies have shown promising results **(Gu et al., 2009; Atwal et al., 2011)** but the mechanism is unclear. This study was an attempt to decipher the mechanism behind ablation of toxic effects of mutant huntingtin demonstrated in these studies utilizing an *in vitro* and *in vivo* PRC2 assay as well as by measuring rescue of ATP/ADP ratio. Thus, this study paves the way for the identification of novel therapeutics to treat HD.

Proposed Objectives:

- **<u>Objective 1</u>: Cloning, Expression and Purification of Recombinant full-length huntingtin proteins with SA or SD mutations.**

- Generation of full-length huntingtin baculovirus constructs (pFastBacQ23Htt and pFastBacQ78Htt) with Ser13 and Ser16 substituted to alanine (SA) or aspartate (SD) by site-directed mutagenesis.

    - Design of mutagenesis primers to generate Serine to Alanine and Aspartate mutants at position 13 and 16 as reported previously **(Gu *et al.*, 2009)**.

- Carry out mutagenesis as per the kit instructions (QuikChange II XL SDM kit from Agilent technologies).
- Confirm the clones by sequencing.

- Expression and purification of the huntingtin proteins with SA or SD mutation using Sf-9 insect cell Baculovirus system and affinity-gel filtration chromatography.
  - Generation of recombinant bacmid DNA carrying huntingtin gene with SA or SD mutations by transformation of pFastBac constructs into *E.coli* DH10Bac cells.
  - Transfection of recombinant bacmid DNA into Sf9 insect cells using Cellfectin reagent (Invitrogen).
  - Isolation of recombinant baculovirus and their amplification.
  - Infection of large culture of Sf9 insect cells with high titer recombinant baculovirus to express recombinant huntingtin.
  - Purify the recombinant huntingtin using FLAG affinity chromatography followed by gel filtration chromatography.

❖ **<u>Objective 2</u>: To Study the impact of phosphorylation status of Ser13 and Ser16 of normal and mutant huntingtin on cell-free and cell-based models.**

- Analyzing the impact of these substitutions on huntingtin's facilitation of reconstituted PRC2 histone H3K27-tri-methylase activity *in vitro*.
  - Set-up the *in vitro* assay as described previously **(Seong *et al.*, 2010)**.
  - Determine the levels of Histone-H3-Lysine-27-tri-methylation using specific antibody (Cell Signaling Technology) by immunoblotting and quantify with ImageJ (NIH).

- Analyzing effect of specific kinase and phosphatase inhibitors on PRC2 activity in cell line models of HD.

- Treat cultured ST*Hdh*$^{Q7/7}$ and ST*Hdh*$^{Q111/Q111}$ cells with different kinase and phosphatase inhibitors along with vehicle.

- Determine the effect of PRC2 mediated Histone-H3-K27-tri-methylation using specific antibody (Cell Signaling Technology) by western blotting and quantify with ImageJ (NIH).

- Studying the peripheral effects of mutant huntingtin (mHTT) in cell line models of HD.

  - Checking the expression of different cytokines e.g., *TNFα, IL6, IL8* etc. by Real Time PCR (RT-qPCR).

❖ **<u>Objective 3</u>: Deciphering the global impact of phosphorylation status of Ser13 and Ser16 on mutant huntingtin (mHTT) toxicity.**

- Studying mutant huntingtin (mHTT) toxicity in Mitochondrial Dysfunction.

- To study the neuroprotective effects of specific kinase and phosphatase inhibitors to prevent mHTT aggregation in HD 150Q cells.

- Identification of differentially expressed proteins associated with mHTT toxicity using quantitative proteomic approaches.

- Analysis of PRC2 targets and ER stress markers playing role in mHTT toxicity by Real Time PCR and Western Blotting.

Results:

<u>Objective1</u>: Cloning, Expression and Purification of Recombinant full-length huntingtin proteins with SA or SD mutation.

pFastBac vectors containing *HTT* gene with 23, 46 and 78 CAG repeats (HttQ23, HttQ43 and HttQ78) were isolated using Plasmid Miniprep Kit (QIAGEN) and run on 0.8% Agarose gel. After successfully isolation of the plasmids, they were checked for the presence of gene of interest using PCR. The plasmids were also transformed into competent cells of *E.coli* DH10Bac and plated on Luria agar (LA) plates containing Kanamycin, Gentamycin, Tetracycline, X-gal and IPTG and

incubated in dark at 37^0C for 24-48 hours. The bacmids containing the gene of interest were identified using blue-white screening upon transformation. Recombinant bacmids with the *HTT* gene disrupt the reading frame of LacZα promoter, thus giving white colonies on addition of X-gal and IPTG to the medium as opposed to wild type *E.coli* DH10Bac cells giving blue colonies. The white colonies were streaked again on medium containing the required antibiotics and X-gal and ITPG to select true positive colonies. True white colonies were then inoculated into Luria broth (LB) containing the required antibiotics to get bacmids. The bacmids were isolated using alkaline lysis method from true white colonies. PCR was also carried out to confirm the presence of gene of interest in these bacmids using *HTT* gene specific primers.

Sf9 insect cells were revived from previously available cell stock and maintained in Sf-900 II SFM (serum-free media). These *Sf9* cells were transfected with bacmids using a cationic lipid, cellfectin II reagent. After 72 hours post incubation, media was collected as P1 virus stock and infected cells were processed for protein expression by western blotting using anti-huntingtin antibody. Expression of huntingtin (HTT) Q23 protein was observed. After confirmation of protein expression at P1 stage, Sf-9 cells were infected with P1 baculovirus stock to get P2 virus stock. Protein expression was again checked by western blotting. Finally, high titer P3 baculovirus stock was obtained by transfecting P2 stock to Sf-9 insect cells.

Healthy Sf-9 cells were infected with HTTQ23 P3 virus stock for protein purification. After 48 hours post incubation, cells were lysed as described in methodology section and centrifuged at 12000 rpm for 10 minutes at 4^0C. Supernatant was collected and subjected to protein purification. Wild type HTTQ46 and HTTQ78 proteins were also expressed and purified as HTTQ23. After confirmation of protein expression at P1 stage, equal no. of *Sf9* cells were infected with P1 baculovirus stock to get P2 virus stock. *Sf9* cells were observed under inverted microscope after 48 hours and the morphology of the cells was different as well as growth of the cells retarded compare to negative control clearly indicating the successful infection. Finally, high titer P3 baculovirus stock was obtained by infecting P2 stock to Sf-9 insect cells and HTT expression was confirmed by western blotting. Healthy *Sf9* cells were infected with HTTQ46 and HTTQ78 P3 virus stocks for protein purification. After 48 hours post incubation, cells were lysed and centrifuged at 12000 rpm for 10 minutes at 4^0C. Supernatant was collected and subjected to protein purification using FLAG affinity purification. Before purification, small volume of supernatants were used to check HTT expression by western blotting. The purified huntingtin protein samples

were subjected to SDS-PAGE followed by Coomassie Brilliant Blue (CBB) staining to check purity and integrity of the protein. There were protein bands observed of around 350 kDa. This result was also verified through immunoblotting where band patterns were observed same as SDS-PAGE.

PCR based Site directed mutagenesis (SDM) was carried out to modify serine 13 and 16 residues to alanine and aspartate, respectively. Site-directed mutagenesis is a crucial and widely used tool in molecular biology to generate specific changes in the DNA sequence of a given gene/genome. As a result, 14 kb PCR products were found on 0.8% Agarose Gel for HttQ23 S13A and HttQ23 S13D but didn't get HttQ23 S16A and HttQ23 S16D. The PCR products for HttQ23 S16D were obtained using gradient PCR but still HttQ23 S16A not found. The PCR products for HttQ46 S13A, S13D and S16D were also produced but HttQ46 S16A didn't find. These PCR products were then subjected to DpnI restriction digestion as DpnI cleaves only methylated DNA. After 2 hours incubation with DpnI, small volume of Plasmids were run on 0.8% Agarose gel to check digestion efficiency. To produce more copy numbers of plasmids, these recombinant plasmids were transformed into *E.coli* DH5α competent cells. These recombinant plasmids were then isolated using Plasmid Miniprep Kit and run on 0.8% Agarose gel. They were checked for the presence of gene of interest using PCR with two different sets of primers. These plasmids were also sent for sequencing for confirmation. The plasmids were then transformed into competent cells of *E.coli* DH10Bac and bacmids containing the gene of interest were identified using antibiotic selection and blue-white screening. The bacmids were isolated using alkaline lysis method from true white colonies. PCR was also carried out to confirm the presence of gene of interest for these recombinant bacmids.

<u>Objective 2</u>: To Study the impact of phosphorylation status of Ser13 and Ser16 of normal and mutant huntingtin on cell-free and cell-based models.

Polycomb Repressive Complex 2 (PRC2) is an epigenetic gene silencer having a Histone Methyltransferase (HMT) enzymatic activity. PRC2 trimethylates Lysine 27 on Histone H3 resulting in gene silencing and chromatin compaction. Previous studies have shown that interaction of PRC2 with Huntingtin protein enhances its HMT activity in a polyQ dependent manner, thereby leading to abnormal gene silencing.

Expression and purification of PRC2 protein was also carried out simultaneously. Briefly, the donor plasmid pFastBac contains the gene for each individual subunit of PRC2 complex. Thus, there were four sets of pFastBac carrying the four core subunit genes (*Ezh2*, *EED*, *Suz12* and *RbAp48*). Each set of donor plasmids was introduced into *E.coli DH10Bac* host cells by transformation. The bacmids of PRC2 subunits gene were isolated by alkaline lysis method from respective plasmids**.** Presence of gene of interest was also confirmed through PCR. The bacmids were transfected into *Sf9* cells, an insect cell line derived from the pupal ovarian tissue of *Spodoptera frugiperda*. Transfection of *Sf9* cells with recombinant bacmids gave rise to P1 viral stock. Subsequent viral amplification was carried out by infecting *Sf9* cells with P1 stock to generate P2 stock, which was further used to infect the cells and generate high-titre P3 viral stock. Protein expression was also checked at each stage by western blotting using anti-EZH2 antibody. Protein expressions for remaining subunits could not be checked by immunoblotting because of unavailability of antibodies.

PRC2 is a multi-subunit complex with two larger subunits, EZH2 and SUZ12 and two smaller subunits, EED and RbAp48. *Sf9* cells were co-infected with high-titre P3 viral stocks containing baculovirus particles for the individual subunits. The P3 stock for all the subunits were used for co-infecting *Sf9* cells. This allowed expression of the PRC2 complex in *Sf9* cells. Subsequently the complex was purified by FLAG affinity chromatography. The eluted fractions were subjected to SDS-PAGE and western blotting to check protein purity. There was a protein band seen at around 85 kDa for EZH2/SUZ12 and two bands at around 50-55 kDa for EED and RbAp48 respectively. Protein expression of EZH2 was also confirmed by western blotting.

Huntingtin has been shown to interact with polycomb repressive complex -2 (PRC2) and promotes its activity. PRC2 complex plays a vital role as transcriptional regulator and mediates gene silencing by Histone H3-Lysine-27-tri-methylation. Mutant huntingtin facilitates this activity significantly higher compared to the normal huntingtin **(Seong *et al*., 2010)**.In cell-free assay for the H3K27 trimethylation activity of PRC2, to wells containing nucleosomal array, purified PRC2 is added. In the presence of the methyl group donor S-adenosyl methionine (SAM) histone 3 is trimethylated at the lysine 27 residue. We conducted an assay in the absence/presence of purified wild type huntingtin. PRC2 is expected to interact with huntingtin and increase the level of H3K27 trimethylation in a polyQ dependent manner. The extent of methylation in each case was checked by Western blotting using Anti-H3K27me3 specific antibodies against H3 trimethylated at K27

residue. Mutant huntingtin **(**HTT Q78) with expanded Poly-Q repeats exhibits greater PRC2 mediated H3K27me3 signal compared to HTT Q23 and HTT Q46. This confirms already established fact that HTT interacts with PRC2 in a Poly-Q dependent manner and facilitates PRC2 H3K27me3 activity.

Mouse striatal cell lines (*STHdh*$^{Q7/Q7}$ and *STHdh*$^{Q7/Q111}$) were maintained in DMEM with 10% FBS and 1X pen strep at 33°C in a 5% CO2 incubator. In order to analyze effect of Kinetin (N6-furfuryladenine) on huntingtin phosphorylation, these cells were treated with Kinetin and DMSO (as a vehicle control) in DMEM with 0.2% FBS and incubated for 24 hours. After 24 hours post incubation, cells were washed with 1X ice-cold PBS and processed to analyze effect of Kinetin as described in methodology section.

The level of Histone-H3-Lysine-27-tri-methylation was determined using immunoblotting to investigate the effect of kinetin on modulation of PRC2 activity. Huntingtin has been shown to interact with polycomb repressive complex -2 (PRC2) and promotes its activity. PRC2 complex plays a vital role as transcriptional regulator and mediates gene silencing by Histone H3-Lysine-27-tri-methylation. Mutant huntingtin facilitates this activity significantly higher compared to the normal huntingtin **(Seong *et al.*, 2010)**. Histone-H3-Lysine-27-tri-methylation level was significantly reduced in kinetin treated *STHdh*$^{Q7/Q111}$ cells as compared to control *STHdh*$^{Q7/Q7}$ cells. The level of Histone-H3-Lysine-27-tri-methylation was also reduced with increasing concentration of kinetin in kinetin treated *STHdh*$^{Q7/Q111}$ cells (showing HD phenotype) as compared to control *STHdh*$^{Q7/Q7}$ cells. However, histones were extracted and used for western blotting, normalization should be against H3 histone concentration which yet to be done. Thus, no comment can be made on the concentration dependence of the effect of kinetin treatment until normalization is done.

The expression of some key pro and anti-inflammatory marker genes were checked by real time PCR to investigate the peripheral effect of mHTT in HD. While the conditioned media from un-induced cells (not expressing mHTT) showed no immune response, the conditioned media from HD150Q cells treated with Ponasterone A for 48h and expressing mHTT showed robust induction of both pro-inflammatory (IL6, TNF-α and IL-1β) as well as anti-inflammatory (IL-10 and TGF-β1) cytokines in normal PBMCs and to a smaller extent in THP-1 cells.

<u>Objective 3</u>: Deciphering the global impact of phosphorylation status of Ser13 and Ser16 on mutant huntingtin (mHTT) toxicity.

This objective comprised of studying the neuroprotective effects of Kinetin, BMS 345541 and Bay 11-7082 to prevent mutant Huntingtin (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 **(Anand Goswami *et al*.)**. Kinetin (N6-furfuryladenine) is a plant cytokine able to inhibit polyglutamine-based mutant huntingtin aggregation inside neuronal cells. It has been also shown that N6FFA restores N17 phosphorylation levels by being salvaged to a triphosphate form by adenine phosphoribosyltransferase (APRT) and used as a phosphate donor by casein kinase 2 (CK2) **(Laura E. Bowie *et al*.)**. Several studies have also shown that kinetin plays a very crucial role in prevention of mutant huntingtin aggregation and a protective precursors molecules in many HD models.

Here, we used HD150Q cell line, which is a commonly used Huntington's disease model system. It is a mutant mouse neuroblastoma cell line (N2a) expressing a truncated N-terminal huntingtin protein containing 150 glutamine residues, fused to an enhanced green fluorescent protein. These cells were maintained in DMEM with 10% FBS, 1X pen strep and antibiotics containing 0.4 mg/mL of Zeocin and G418 at 37°C in a 5% CO2 incubator. Ponasterone A (1 μM) was used to induce aggregation of the mutant huntingtin protein. Aggregates of mutant huntingtin appears as green particles under fluorescence microscope, and the cells die within 7−8 days due to the toxicity of aggregated mutant huntingtin.

Phosphorylation of huntingtin N17 at Ser13 and Ser16 has been found to be a protective modification in the context of HD. The N17 sequence serves as a nuclear export signal, and its PTMs are thought to regulate the subcellular localization and the clearance of mHTT **(Gu *et al.*)**. In order to investigate the role of kinetin on mHTT phosphorylation of Ser13/Ser16, HD150Q cells were treated with 1μM of Ponasterone A and 1μM of kinetin for 24 h. After post incubation, cells were harvested and performed western blotting using anti-N17-S13pS16p antibody (Coriell Institute for Medical Research, USA). Interestingly, HD150Q cells treated with kinetin showed statistically significant elevated expression compare to untreated cells as shown in.

There has been well establish fact that toxic poly(Q)-expanded HTT fragments are formed from full length HTT via proteolysis. Mutant HTT has been observed to form cytoplasmic aggregates as well as intranuclear inclusion bodies in neurons both *in vivo* and in cell cultures. These mHTT aggregates play a very crucial role in the neurotoxicity observed in HD. In order to check the induction of GFP tagged mutant huntingtin (mHTT) aggregates in HD150Q cells, Ponasterone A (1 μM) was added after 24 hours of cell seeding. Induction was successfully achieved after 48 hours of 1μM Ponasterone A using Fluorescence microscopy. Further it was also checked that aggregation of GFP tagged mHTT was time dependent or independent. To determine this effect, HD150Q cells were incubated with 1μM of Ponasterone A for 24, 48, 72 and 96 hours and images were taken using Fluorescence microscopy. mHTT aggregates increased as incubation time increased resulting 72 and 96 hours of Ponasterone A treatment produced maximum protein aggregates. To elucidate the neuroprotective role of Kinetin, HD150Q cells were treated with different concentrations of Kinetin (0.5μM, 1μM and 2μM) with/without Ponasterone A. In comparison to the Ponasterone A control cells, the number and size of aggregates decreased at all doses of kinetin treated cells but 1 μM and 2 μM of Kinetin successfully prevented mHTT aggregation in HD150Q cells, indicating the maximum action by kinetin. After that, using fluorescence microscopy images of several fields, the average number of puncta per field was determined and plotted in the graph. These findings are also consistent with the microscopy data. Additionally, Kinetin could also reduce mHTT aggregates for 48 hours.

In order to confirm the reduction of insoluble GFP tagged mutant huntingtin (mHTT) aggregates after Kinetin treatment, HD150Q cells were incubated with 1μM of Ponasterone A for 24, 48, 72 and 96 hours with/without Kinetin. After post incubation, cells were washed with 1X ice-cold PBS and lysed on ice using Lysis Buffer (0.1% Triton X-100 in 50mM Tris-Cl pH-8.0 with complete protease inhibitors cocktail). Protein concentrations were quantified using Bradford method and equal amount of protein was subjected to SDS-PAGE. Post SDS-PAGE, the proteins were transferred onto nitrocellulose membrane and the membrane was blocked with 5% fat free milk in 1X TBST. Protein expression was checked using anti-huntingtin antibody. Insoluble mHTT aggregates increased as incubation time increased resulting 72 and 96 hours of Ponasterone A treatment produced maximum insoluble protein aggregates (observed at upper side) whereas soluble HTT significantly decreased gradually (observed lower side). Interestingly, after treatment with different concentrations of kinetin (0.5μM, 1μM and 2μM), there was a completely reversal

effect observed. Further, we also wanted to check whether this effect was up to 48 hours or not. For this, HD150Q cells were incubated with 1µM of Ponasterone A and 1µM and 2µM of Kinetin for 48 hours. Surprisingly, the same neuroprotective effect of Kinetin observed as 24 hours by western blotting.

Mitochondrial dysfunctions affect a number of cellular pathways leading to cell death; hence, we monitored mitochondrial functions in HD conditions. It is shown that *STHdh*$^{Q111/Q111}$ knock-in mouse striatal cells expressing mutant huntingtin alters cellular metabolism and showed significant reduction in ATP/ADP ratio. It was also confirmed in several human lymphoblasts expressing at least one mutant huntingtin allele **(Seong *et al*., 2005)**. The impact of kinetin treatment on cellular ATP production was also investigated using luminescent based ATP determination kit as per the manufacturer's instructions. In order to analyze effect of Kinetin (N6-furfuryladenine) on cellular ATP production, these cells were treated with Ponasterone A (for different time period e.g. 24h, 48h, 72h, and 96h) and different concentrations of Kinetin (1µM and 2µM) in DMEM with 10% FBS and incubated. After post incubation, cells were washed with 1X ice-cold PBS and processed to analyze the effect of Kinetin treatment on cellular ATP production. The ATP concentrations were divided with respective protein concentrations to obtain normalized ATP concentrations. The level of ATP was observed significantly lower in Ponasterone A treated HD 150Q cells compared to uninduced HD150Q cells. Interestingly, after treatment with different concentrations of kinetin (1µM and 2µM), the ATP level in Ponasterone A treated HD 150Q was significantly restored.

In HD, transcriptional dysregulation and mitochondrial deterioration are interconnected processes guided by the crosstalk between *p53* and the mitochondrial biogenesis factor *PGC-1α* (peroxisome proliferator-activated receptor γ co-activator 1α). *PGC-1α* expression is reduced by mHTT, resulting in reduced transcription of mitochondrial respiration factors **(Intihar *et al.*)**. For RT-qPCR, RNA was isolated and cDNA were synthesized using PrimeScript cDNA synthesis kit (Clontech) as per the manufacturer's instructions. The expressions of mitochondrial target genes were evaluated using TB Green Master mix (TaKaRa) in QuantStudio™ 3 System (Applied Biosystem). Fold change of gene expression was calculated using $2^{-\Delta\Delta Ct}$ method. The levels of *BDNF*, *PGC1α* and *NRF-1* were significantly lower in HD150Q cells. After kinetin treatment, there was completely reversal effect observed.

One of the major factors contributing to the development of Huntington's disease is oxidative stress, according to conventional wisdom. 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. Here, we examined mitochondrial ROS by MitoSOX^{TM} Red using fluorescence microscope. Like mHTT aggregation data, mitochondrial ROS increases as incubation time increases resulting 72h and 96h produced highest red fluorescence signals. However, different concentrations of kinetin treatment caused decreased levels of mitochondrial ROS significantly. Combining all the results, it can be inferred that kinetin can rescue some of the mitochondrial dysfunctions in HD condition.

There has been some evidences suggest that inhibition of ER-associated degradation (ERAD) of mHTT, which leads to endoplasmic reticulum (ER) stress and the onset of the unfolded protein response (UPR), a conserved stress response is one of the significant consequences of the gradual accumulation of misfolded mHTT in neurons. The main purpose of the UPR is to restore cellular homeostasis or, if it is unsuccessful, to cause cell death to stop further accumulation of damaged, non-functional cells. In this study, we checked the neuroprotective effect of kinetin on ER stress associated genes and proteins by RT-qPCR and western blotting, respectively.

The levels of *PERK*, *CHOP*, *XBP1s* and *ATF6* were showed elevated in Ponasterone A treated HD150Q cells compare to untreated cells. After kinetin treatment, there was a completely reversal effect observed. The same phenomenon was also observed in western blotting. The proteins like p-PERK, p-eIF2a, IRE1a, BiP, XBP1s, GADD34 and CHOP were overexpressed in 24h and 48h Ponasterone A treated HD150Q cells. But Kinetin could easily reverse the expression of all the proteins mentioned here suggesting that kinetin could rescue ER stress successfully.

As mutant huntingtin is hypophosphorylated at the N17 domain and phosphorylation is beneficial in HD models, the effects we observed with 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 successfully prevention and/or rescue of mHTT aggregates. These data suggest that Kinetin could be a potential therapeutic target for prevention of mHTT aggregates as helpful in Huntington’s disease.

Additionally, we also evaluated BMS 345541 and Bay 11-7082 as neuroprotective compounds that prevent the pathways that are dysregulated in HD. Both BMS 345541 and Bay 11-7082 are chemical compounds that have been shown to inhibit IKKβ, a kinase involved in the dephosphorylation of the N17 domain. These compounds have been chosen for this study to see if they can reduce the number of aggregates and restore ATP levels by increasing the protein's N17 phosphorylation in HD150Q cells. To assess the effects of BMS 345541 and Bay 11-7082 on these cells, fluorescence microscopy and western blot were used. The cells' ATP levels were then assessed using an ATP chemiluminescence assay.

The 6 well plates of both the BMS 345541 and Bay 11-7082 treated cells were observed under fluorescence microscopy after 24 hours treatments using the same compounds, respectively. HD150Q cells were treated with Ponasterone A and/or BMS 345541 at concentrations of 50 nM, 100 nM, 200 nM, and 300 nM. Among all the concentrations, 50 nM BMS 345541 showed the greatest effect. After that, using microscopy images of several fields, the average number of puncta per field was counted. These findings are also consistent with the microscopy data. HD150Q cells were also treated with Bay 11-7082 at varied doses such as 100 nM, 250 nM, 500 nM, 750 nM, and 1000 nM. In comparison to the Ponasterone A control cells, the number of aggregates decreased at all doses of Bay 11-7082 treated cells. The cells treated with 750 nM had the fewest aggregates, indicating the maximum action by Bay 11-7082. When the number of puncta per field was determined and plotted in the graph, a similar pattern was observed.

The fluorescence microscopy results were also confirmed through western blotting using HTT specific antibody. Western blot data revealed reduction of insoluble mHTT aggregates in BMS 345541 treated cells with doses of 50 nM, 100 nM, and 200 nM. At the same time, it was also discovered that as the number of insoluble aggregates decreased, soluble HTT increased. These findings are consistent with microscopy results and show that the highest effect was seen at a dosage of 50 nM BMS 345541. HD150Q cells were then treated with Bay 11-7082 concentrations of 100 nM, 250 nM, 500 nM, 750 nM, and 1000 nM, it was observed that mHTT aggregation was diminished at all concentrations when compared to the Ponasterone A control alone. The results of the densitometric analysis of the blot are consistent when comparing the various concentrations. Additionally, it was also shown that 750 nM had the maximum impact because it has the least aggregation.

The impact of BMS 345541 and Bay 11-7082 treatments on cellular ATP production was also investigated using luminescent based ATP determination kit. Interestingly, the ATP chemiluminescent assay showed that after 24 and 48 hours, the cells were able to restore ATP levels with treatment of BMS 345541 or Bay 11-7082. These findings suggest that BMS 345541 and Bay 11-7082 could be a promising therapeutic candidate for preventing mHTT aggregates, which is beneficial in Huntington's disease.

Conclusions:

With the rise in life expectancy over the years, there has been an unanticipated increase in the frequency of age-related illnesses, particularly neurodegenerative diseases like Huntington's disease (HD). Currently, there is no effective treatment available for HD however some of the strategies such as silencing of the mutant allele by siRNA, RNAi, Zinc-finger nucleases and targeting huntingtin post-translational modifications are being employed in animal models of HD, with limited success. There has been well established that post-translational modifications (PTMs) of huntingtin, including phosphorylation, have been shown to regulate its function and toxicity, intracellular localization, as well as protein degradation resulting their clearance from cells. Based on these observations, any intervention that might prevent or totally reverse the toxicity of mutant huntingtin should be pursued, with N17 phosphorylation restoration being a potential therapeutic target for HD drug development.

We therefore set out to employ N6-furfuryladenine (N6FFA), also known as "kinetin" that could modulate N17 phosphorylation in an attempt to restore the hypophosphorylation of N17 in HD150Q cell line model of Huntington's disease. Kinetin (N6-furfuryladenine) may phosphorylate Ser13-Ser16 of mutant huntingtin resulted in elevated ATP production, reduction of insoluble mHTT aggregates and restoration of soluble huntingtin, rescuing some of the mitochondrial dysfunctions as well as ER stress. From the data obtained, it was further concluded that treatment with kinetin showed successfully prevention of mHTT aggregates in dose dependent manner. Thus, Kinetin could be a promising neuroprotective compound as a potential therapeutic target for Huntington's diseases.

References:

1. Aiken *et al.* Phosphorylation of Threonine 3 IMPLICATIONS FOR HUNTINGTIN AGGREGATION AND NEUROTOXICITY. *J Biol Chem.* 2009, 284; 29427-29436.
2. Gauthier LR *et al.* Huntingtin controls neurotrophic support and survival of neurons by enhancing BDNF vesicular transport along microtubules. *Cell.* 2004, 118(1); 127-38.
3. Gillian Bates *et al.* Huntingtin and the molecular pathogenesis of Huntington's disease. *EMBO reports.* 2004, 5; 958–963.
4. Gu *et al.* Serines 13 and 16 Are Critical Determinants of Full-Length Human Mutant Huntingtin Induced Disease Pathogenesis in HD Mice. *Neuron.* 2009, 64; 828-840.
5. Humbert *et al*. The Biology of Huntingtin. *Neuron.* 2016, 89; 910-926.
6. Nicolas Arbez *et al.* Posttranslational modifications clustering within proteolytic domains decrease mutant huntingtin toxicity. *J Biol Chem.* 2017, 292(47); 19238-19249.
7. Ross and Tabrizi *et al.* Huntington's disease: from molecular pathogenesis to clinical treatment. *Lancet Neurol.* 2011, 10(1); 83-98.
8. Saleem *et al.* Molecular analysis of Huntington's disease and linked polymorphisms in the Indian population. *Acta Neurol Scand.* 2003, 108(4); 281-6.
9. Schilling *et al.* Huntingtin Phosphorylation Sites Mapped by Mass Spectrometry MODULATION OF CLEAVAGE AND TOXICITY. *J Biol Chem.* 2006, 281; 23686-23697.
10. Seong *et al.* HD CAG repeat implicates a dominant property of huntingtin in mitochondrial energy metabolism. *Hum Mol Genet.* 2005, 14(19); 2871-80.
11. Seong *et al.* Huntingtin facilitates polycomb repressive complex 2. *Hum Mol Genet.* 2010, 19; 573-583.
12. The Huntington's Disease Collaborative Research Group. *Cell.* 1993, 72; 971-983.
13. Thompson *et al.* IKK phosphorylates Huntingtin and targets it for degradation by the proteasome and lysosome. *J Cell Biol.* 2009, 187; 1083-1099.
14. Walker FO *et al.* Huntington's disease. *Lancet.* 2007, 369; 218–28.
15. Warby *et al.* Huntingtin phosphorylation on serine 421 is significantly reduced in the striatum and by polyglutamine expansion *in vivo. Hum Mol Genet.* 2005, 14; 1569-1577.

Achievements:

1. Got awarded Senior Research Fellowship of Indian Council of Medical Research (Ref. No.: 3/1/2/127/Neuro/2019-NCD-1).

Publications:

1. **Raju Dabhi** and Ravi Vijayvargia. Kinetin inhibits mutant huntingtin aggregation and rescues ATP level in cell line model of Huntington's disease. (Submitted)

2. **Raju Dabhi**, Suvorit Bhowmick, Hitarthi Vyas, Julie Tahilramani, Rakesh Shah and Ravi Vijayvargia. Genotype-Phenotype Correlation of Huntingtin CAG Repeat Expansion and Associated Symptoms in a Large Indian Family Carrying Huntington's Disease Mutation. (Under preparation)

3. **Raju Dabhi**, Anwesha Biswas, Pawan Tagadghar and Ravi Vijayvargia. Role of Polycomb repressive complex 2 (PRC2) in neurodegenerative diseases. (Under preparation)

Oral/ Poster presentations:

1. Roshni Bhatt, Suvorit Bhowmick, Hitarthi Vyas, **Raju Dabhi**, Julie Tahilramani, Rakesh Shah and Ravi Vijayvargia. "Genotype-Phenotype Correlation of Huntingtin CAG Repeat Expansion and Associated Symptoms in a Large Indian Family Carrying Huntington's Disease Mutation" presented at the European Huntington's Disease Network (EHDN) plenary meeting at the Austria Centre Vienna in Vienna, Austria, 14-16 September, 2018.

Conferences/Workshops attended

- **"1 day Workshop on Biological Applications of Magnetic Nanoparticles"** conducted at The M. S. University of Baroda, Vadodara, Gujarat, India on 27th March, 2019.
- **"10th Annual Meeting of the Proteomics Society, India (PSI) and International Conference on Proteomics for Cell Biology and Molecular Medicine"** held at National Centre for Cell Science, Pune during 12th to 14th December, 2018.
- **"The Hands-on Training Workshop on Basic Cell Culture Technology"** conducted by NCCS Cell Repository at National Centre for Cell Science, Pune during 29th October to 1st November, 2018.

Date: 07/09/2022

Place: Vadodara

Dabhi Rajubhai Khimabhai

Ph.D. candidate

Dr. Ravi Vijayvargia

Ph.D. Guide

DR. RAVI VIJAYVARGIA
Assistant Professor
Department of Biochemistry
Faculty of Science
The M. S. Univ. of Baroda
Vadodara-390 002 (Guj) India

C. Ratna Prabha
9/9/22

Head

Department of Biochemistry

HEAD
Biochemistry Department

Dean

Faculty of Science

DEAN
FACULTY OF SCIENCE
THE M.S. UNIVERSITY OF BARODA