CHARACTERIZATION OF THE INTERACTION BETWEEN A STABLE G-QUARTET FORMING OLIGONUCLEOTIDE AND HUNTINGTIN WITH AN EXPANDED POLY-GLUTAMINE REGION

by

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ABSTRACT

Huntington's Disease is a progressive neurodegenerative disorder that is inherited in an autosomal dominant fashion. The disease is the result of an expanded CAG repeat in exon 1 of the HD gene which encodes an elongated poly-glutamine tract in the mutant form of the protein, huntingtin. The expanded poly-glutamine region causes the mutant protein to mis-fold and oligomerize, initiating a complex aggregation process. Disease pathogenesis is linked to the formation of these intracellular aggregates. It has been proposed that the aggregates themselves are toxic due to their physical presence which may block intracellular trafficking and signaling processes. A concurrent possibility is the sequestration of important proteins such as transcription factors during the aggregation process, disrupting their function resulting in cellular stress and death. In addition, the mechanistic steps that lead to aggregate formation may be central to HD pathology. A viable approach for HD therapeutics is to block the aggregation process of mutant huntingtin.

It has been previously reported that guanosine rich oligonucleotides(ODN) that fold into a G-quartet formation are effective inhibitors of the aggregation process of a huntingtin protein fragment with an elongated polyglutatmine tract, Htt 1-171 (Q58)(Skogen, Roth, Yerkes, Parekh-Olmedo, & Kmiec, 2006). The most robust ODN inhibitor of aggregation is a single stranded oligonucleotide composed of 20 guanosine

residues termed, G20. It is unique in its propensity to form a secondary structure known as a G-quadruplex. For this thesis, analysis was done on the G-quadruplex forming G20 to determine if its structure is central to its aggregation inhibition activity. Circular dichroism and Atomic Force Microscopy(AFM) experiments were carried out to investigate the secondary structure and it was determined that G20 most likely forms a "G-wire" formation of a G-quadruplex and that this structure is important for the interaction necessary for inhibition of mutant huntingtin aggregation. The interaction between G20 and huntingtin was investigated in several aggregation models of huntingtin by both *in vitro* and *in vivo* assays. The results indicate that G20 acts by directly binding to huntingtin, and that this interaction is dependent on the unique structure formed by G20 and on the process of mutant huntingtin aggregation. *We hypothesize that G20 inhibits mutant huntingtin aggregation through a direct and structurally specific interaction.*

Chapter 1

INTRODUCTION

1.1 Background on Huntington's Disease

The first definitive account of Huntington's Disease was published by the physician, Dr. George Huntington in 1872 in *The Medical and Surgical Reporter.* He described it as a hereditary disease that manifested in emotional, cognitive and motor symptoms (Martin & Gusella, 1986). We now know that Huntington's Disease is a true autosomal dominant disorder that is characterized by progressive and selective neurodegeneration over the course of the disease. The genetic cause of Huntington's Disease is a triplet repeat (CAG) expansion in exon 1 of the HD gene (IT15) which was discovered in 1993 by the Huntington's Disease Collaborative Research Group (MacDonald, Gines, Gusella, & Wheeler, 2003). The CAG expansion encodes an elongated poly-glutamine tract in the N-terminus of the resulting protein, huntingtin. Normal individuals possess a poly-glutamine repeat of 35 residues or less, whereas the disease threshold is 36 or more repeats (Hatters, 2008). Huntingtin is a large (350 kDa) protein that has widespread expression in the brain and peripheral tissues (MacDonald et al., 2003). The polyglutamine expansion that occurs in mutant huntingtin causes the protein to mis-fold and undergo a deleterious process of aggregation. This process is thought to confer a gain of function toxicity to mutant huntingtin which may be linked to pathogenesis.

HD is a late onset disorder, where the age of onset is inversely proportional to the number of poly-glutamine repeats (Kremer et al., 1993). Symptoms appear in diseased individuals on average from age 40-50, with a significantly higher number of CAG repeats corresponding to juvenile onset (Foroud, Gray, Ivashina, & Conneally, 1999; Kremer et al., 1993; Foroud et al., 1999). It affects one out of every 10,000 Americans, with an estimated 250,000 individuals at risk to inherit this disorder. The disease is marked by psychiatric, cognitive and motor symptoms that are mild at first, and progress over the course of the disease. These symptoms include involuntary movements of limbs and face, sadness, depression, irritability, anxiety and an overall marked change in personality (Kirkwood et al., 2000; Penney et al., 1990). Symptoms gradually worsen over a 15-20 year period, while the patient becomes more and more disabled. Death usually ensues due to a side effect such as choking, pneumonia or falling rather than directly from HD (Kirkwood et al., 2000). The molecular link between the gene mutation and disease pathology has not been fully elucidated, however cognitive and motor dysfunction in HD is mostly attributed to selective neuronal loss primarily in the striatum of the basal ganglia (Lee, Chen, Lu, & Hsi, 1986). There are currently no treatments to stop or slow the progression of the disease.

1.2 Huntingtin function

The Huntington gene, known as IT15 is located on the short arm of chromosome 4 (Gusella et al., 1983). It encodes a 350 kDa protein known as huntingtin, which is expressed throughout the body, with elevated levels in the brain and testes. The function of wild type huntingtin has not yet been fully elucidated; however it is thought to be a neurotrophic protein that plays a role in axonal trafficking and transcriptional regulation (Conforti et al., 2008; Kazantsev, Preisinger, Dranovsky, Goldgaber, & Housman, 1999; Zuccato et al., 2007)(Duyao et al., 1995). It is primarily localized to the cytoplasm, specifically to the plasma membrane, the mitochondria, liposomes and the endoplasmic reticulum (Reddy, Mao, & Manczak, 2009). Huntingtin shuttles in and out of the nucleus which is believed to be necessary for it to play a role in transcription (Jiang et al., 2006; Zuccato & Cattaneo, 2009). There are many known interacting partners of huntingtin which may be related to its function (Gusella & MacDonald, 1998; Kazantsev et al., 1999). There has also been a proposed role as a scaffolding protein in dynamic cellular processes (Marcora, Gowan, & Lee, 2003). A role has been suggested for huntingtin in neurogenesis during development from knock-out studies in several mouse models for HD that reveals an embryonic lethal phenotype (Duyao et al., 1995; Duyao et al., 1995; Schmitt et al., 1995).

Huntingtin is comprised of several HEAT repeat elements, which may play a role in protein-protein interactions (Takano & Gusella, 2002). HEAT elements are repetitive 38 amino acid motifs that are oriented in tandem arrays. Each motif is

composed of two helical domains and a non helical domain, they are believed to facilitate protein-protein interactions. The structural function of HEAT repeats is unknown, however they are often found in proteins involved in cell transport (Takano & Gusella, 2002). The HEAT elements in huntingtin are concentrated in its N-terminal region, and are the only structurally distinct components in the entire protein (Takano & Gusella, 2002). Taking into consideration the function of other proteins containing HEAT elements, it has been proposed that Huntingtin may function as a molecular scaffold that guides protein interactions (Takano & Gusella, 2002). Both mutant and wild type huntingtin have a multitude of interacting partners, and the disruption to these interactions may inhibit wild type function and play a role in disease pathology.

Huntingtin is highly expressed in neurons during embryonic development, and may be necessary for neurogenesis (Schmitt et al., 1995). Conversely, there is evidence that Huntingtin expression is downregulated in non-neuronal tissues during development (Schmitt et al., 1995). The essential role of huntingtin in development is clear in results of studies of three different lines of htt knockout mice, which do not survive past embryonic days 6-10 (Duyao et al., 1995; Nasir et al., 1995). Studies with conditional knockouts reveal that reducing total amount of huntingtin expressed leads to brain abnormalities and underdevelopment (Dietrich, Shanmugasundaram, Shuyu, & Dragatsis, 2009). At this time there is no evidence indicating that the presence of mutant huntingtin during development is detrimental, rather it is the stress of mutant huntingtin expression over a long time that contributes to cellular stress and

death. The fact that homozygous individuals do not exhibit worse symptoms than heterozygous also supports the prediction that mutant huntingtin can perform wild type huntingtin's role during development, and is no more toxic during the disease process (Penney et al., 1990). Interestingly enough, removal of huntingtin expression in cell culture does not result in cell death. Huntingtin's function may be related to a developmental process rather than a precise function in a single cell. This is also further evidence that disease pathogenesis is due to a toxic gain of function of mutant huntingtin rather than ablation of wild type function. Reintroducing wild type huntingtin expression ameliorates pathology, indicating a haploinsufficency effect and reiterating the protective role of wild type huntingtin (Dietrich et al., 2009).

1.3 Disease Pathology

The genetic cause of Huntington's Disease is the expansion of the CAG repeat in the disease causing gene, IT15. However the link between the expanded poly-glutamine region in the resulting protein, huntingtin and disease pathology remains unclear. The mechanism by which the mutant protein causes selective neuronal death which results in disease symptoms is the focus of intense study and investigation.

One hypothesis is that the aggregation process of mutant huntingtin is toxic and causes cellular stress and eventual death. This is supported by the fact that the threshold of polyglutamine repeats for aggregation of mutant huntingtin to occur both *in vivo* and *in vitro* is the same for HD penetrance (Ross & Poirier, 2004; Scherzinger et al., 1999). Aggregates are also observed in post-mortem brains of Huntington's

Disease patients, as well as many animal models and almost all cell culture models of Huntington's Disease (Ross & Poirier, 2004). How aggregation causes cellular toxicity is under significant debate and there are several possibilities. The aggregates themselves may cause cellular stress. It has been predicted that the presence of large aggregates block cellular transport down axons and inhibit other cellular processes. There is also evidence that these large aggregates are protective to the cell by sequestering the toxic, mutant protein and that smaller oligomeric species may be toxic. During the process of aggregation, mutant huntingtin sequesters wild type huntingtin, transcription factors and other proteins into aggregates (Jiang et al., 2006; Kremer, Weber, & Hayden, 1992). The loss of function of wild type huntingtin and deregulation of transcription has been shown to play a role in disease pathology. Another cause of cellular stress is proteasome malfunction, which may be caused by mutant huntingtin aggregation (Hatters, 2008). One of the cellular responses to mutant huntingtin expression is to attempt to degrade the toxic protein by tagging it with ubiquitin and marking it for degradation by the proteasome. It is thought that aggregation in HD overwhelms the cellular machinery to clear the mutant protein, which leads to its destruction and eventually cell toxicity. Mutant huntingtin aggregation may also impair cellular organelles such as the mitochondria and endoplasmic reticulum (Hatters, 2008).

There is a fair amount of evidence that implicates Huntinton's Disease as a metabolic disorder. For example; weight loss is a significant problem for HD patients. Striatal neurons from post-mortem HD patients revealed reduced activity in

mitochondria, and several mouse models of HD have been found to have decreases in mitochondrial enzyme activity. Calcium-induced mitochondrial permeability has been found as a factor in many studies and finally mitochondrial trafficking may be impaired (Browne, 2008; Orr et al., 2008; Phan, Hickey, Zhang, Chesselet, & Reue, 2009). How these pathologies occur as the result of mutant huntingtin expression is not very clear. Trushina et al. completed studies in 2004 on the effect of mutant huntingtin on axonal trafficking. They found that mitochondrial trafficking is impaired very early in the disease course in HD models (Trushina et al., 2004). There is evidence from post-mortem HD brains of mitochondrial components sequestered into aggregates, which may indicate that mutant huntingtin aggregation leads to decreased movement and eventually results in mitochondrial impairment (Reddy et al., 2009; Trushina et al., 2004). This mitochondrial impairment generates free oxygen radicals, or reactive oxygen species(ROS) which in turn can damage the mitochondria, lead to DNA damage and overall cellular stress. This toxic circle may eventually be too much for the cell to handle, leading to eventual death (Reddy et al., 2009).

Huntingtin is post-translationally modified in several ways including caspase cleavage. Caspase 1 is upregulated early in the disease process, and may begin a cascade that activates several caspases including some known to cleave huntingtin (Ona et al., 1999; Zhang et al., 2006). Caspase-1, -3 and -6 cleave huntingtin, but may not all be associated with pathogenesis (Graham et al., 2006). Cleaved N-terminal fragments of mutant huntingtin can act in a feed back manner, which further increases expression of caspase-1 (Li, Lam, Cheng, & Li, 2000). A progressive increase in

caspase generated cleavage fragments occurs over the course of the disease and correlates to a depletion of wild type huntingtin (Ona et al., 1999). Caspase inhibitors relieve toxicity and cell death in cultured cells expressing mutant huntingtin (G. H. Wang et al., 1999). One inhibitor; minocycline is even in clinical trials for the treatment of HD. It has also been predicted that mutant huntingtin cleavage fragments are absolutely necessary for aggregation to occur. Prevention of the caspase cleavage cascade may be a viable therapeutic target for Huntington's Disease (Graham et al., 2006).

There is some evidence that HD pathology is linked to the presence of nuclear aggregates. It is thought that wild type huntingtin shuttles in and out of the nucleus where it plays a role in transcription regulation, however its nuclear localization is very transient (Zuccato et al., 2007; Zuccato & Cattaneo, 2009). Mutant huntingtin may become trapped in the nucleus when it aggregates and can no longer be transported back to the cytoplasm (J. Wang, Gines, MacDonald, & Gusella, 2005). It is then able to aberrantly disrupt transcription and other processes. Nuclear inclusions may also be the result of post-translational processing. Caspase cleavage fragments are able to diffuse into the nucleus where they nucleate inclusion formation, recruiting transcription factors and other important cellular proteins (Ratovitski et al., 2007). There is a correlation between poly-glutamine number and the amount of huntingtin cleavage that occurs, which results in increased amounts of mutant protein in the nucleus which may contribute to disease pathology (Ross & Poirier, 2004).

We know the molecular mechanisms of disease pathology result in selective neuronal cell death, which can account for the many visible symptoms of Huntingtin's Disease (Douaud et al., 2009). These symptoms present themselves in a broad combination of cognitive impairment, emotional disturbances and motor abnormalities which can appear different from patient to patient. The length of CAG repeat expansion is the best explanation for phenotypic variation among patients; however it is only attributed to variation in age of onset, and not symptom manifestation (Cardoso, 2009). HD pathology is attributed to the selective loss of (GABA)ergic medium spiny neurons in the striatum (Cicchetti et al., 2009; Douaud et al., 2009). These medium spiny neurons carry the output signals from the striatum to the globus pallidus and substantia nigra. They make up almost 95 percent of the cells in the striatum and are GABA ergic in nature, meaning they respond to the neurotransmitter, γ-aminobutyric acid (Douaud et al., 2009). At the time of death, HD brains weigh up to 10-20 percent less than those of age matched controls (Douaud et al., 2009). The direct link between mutant huntingtin aggregation and striatal vulnerability and neuronal loss is unclear, as is the direct cause of neuronal death. Motor abnormalities in HD reflect striatal dysfunction, and it is becoming apparent that cognitive changes are the result of disruption to striatal circuitry. This may indicate that neuronal loss in the neo-striatum accounts for cognitive as well as motor symptoms (Douaud et al., 2009).

Huntington's Disease belongs to a class of poly-glutamine expansion disorders along with nine other diseases, including spinal bulbar muscular atrophy, fragile x

syndrome and Creutzfeldt-Jakob's Disease. These diseases all have an underlying mutation of a poly-Q expansion which may indicate toxicity associated with poly-glutamine repeats themselves (Ross & Poirier, 2004). Common traits of all nine diseases include selective neuro-pathology, disease protein misfolding and aggregation associated with a threshold poly-Q number and an autosomal dominant inheritance pattern.(Ross & Poirier, 2004) Each disease is associated with selective neuronal death in different regions of the brain.

We know that Huntington's Disease is caused by a specific mutation, which results in the formation of a mutant protein. That protein must be responsible for the physical symptoms of the disease; however the exact mechanism has yet to be elucidated. There are several possibilities, discussed above, all of which may be connected and interrelated. We do not know the function of wild type huntingtin, or how the expression of mutant huntingtin causes neuronal death only in select regions of the brain. Until we find the missing link, concentrating on inhibiting the pathogenic processes we do know about, such as mutant huntingtin aggregation, is of great interest.

1.4 **Aggregation Characteristics**

As previously stated, caspase cleavage events result in N-terminal fragments of mutant huntingtin that undergo a process of aggregation which is somehow deleterious to the cell (Ratovitski et al., 2007). Mutant huntingtin eventually forms amyloid-like aggregates that are rich in β-sheets, however elucidating the process of aggregation has proved to be a tricky feat (Hatters, 2008; Wetzel, 2006). There is a lot of debate

surrounding the role of aggregates in Huntington's Disease pathology. Some hypothesize that a specific conformation of aggregate is necessary for toxicity, while there is evidence that specific fragments from post-translational processing are the causative agent (Ratovitski et al., 2007; Scherzinger et al., 1999). A significant amount of work is being done to elucidate what this toxic species is, what causes it to emerge, and how to prevent it from forming. Studying the mechanism of mutant huntingtin aggregation is necessary to answer many of those questions.

The large size of mutant huntingtin makes it prohibitive to elucidate its exact structure. The propensity of polyglutamine fragments and peptides to aggregate makes them difficult to work with and to study their monomer structure. For this reason, it has been very challenging and has taken careful work with purified fragments of mutant huntingtin and specially made peptides in an attempt to model huntingtin aggregation (Thakur et al., 2009).

In a very interesting study, Nekooki-Machida et al. directly introduced different amyloid conformations of mutant huntingtin into mammalian cells. They found that amyloid aggregates rich in densely packed ß-sheets were less toxic to the cells than amyloid structures that had looser loops and turns with exposed poly-glutamines (Nekooki-Machida et al., 2009). They predicted that the protective effect of macro-aggregates reported by some is due to their densely packed β-sheet structure. According to their conclusions, the poly-glutamine region is responsible for all toxicity associated with mutant huntingtin aggregates due to its ability to recruit other cellular proteins and disrupt their function. Interestingly enough, a significant amount

of proteins found sequestered in aggregates have a poly-glutamine region themselves, such as the transcription factor CREB binding protein(CBP) (Jiang et al., 2006). They found the highest level of toxicity associated with aggregates of any conformation that had a significant amount of exposed poly-glutamines, including oligomer species and mutant huntingtin monomers (Nekooki-Machida et al., 2009). This is very consistent with the fact that there are nine other poly-glutamine expansion disorders, it is logical to conclude that there is toxicity associated with the poly-glutamine region itself. They also found a correlation between their structural studies and the selective toxicity in a HD brain. They proposed that the heterogeneity of aggregates found in the brain are responsible, indicating the possibility that aggregates formed in the striatum are less densely packed and have more exposed poly-glutamine regions. So in fact, it may be a possibility to promote the formation of a certain aggregate structure, or prevent the formation of the toxic structure as a therapeutic target (Nekooki-Machida et al., 2009).

Another group studies the aggregation of specially designed peptides to mimic the first exon of mutant huntingtin through a variety of imaging and thermodynamic techniques (Thakur et al., 2009). They determined that the first 17 amino acids of mutant huntingtin play a role in aggregate formation. The structure of the first 17 amino acids in a peptide with a poly-glutamine number in the non-pathogenic range is in a “compact coil” state, which is a relatively unstructured but stable conformation. The unstructured nature of this region is believed to aid in dynamic protein-protein interactions, which is consistent with proposed roles of huntingtin as a scaffolding

protein for cellular transport (Takano & Gusella, 2002). The elongation of the poly-glutamine region past the pathogenic threshold results in a destabilization of this "compact coil" conformation, and an unstable "mis-folded monomer" is formed. The mis-folded monomer then reacts with other mis-folded monomers in a rate limiting step of aggregation to form a micelle-like oligomer where the poly-glutamine region is very exposed. This oligomer then converts into an amyloid like intermediate which can nucleate the rapid addition of other monomers, and presumably wild-type monomers into an amyloid aggregate formation. These amyloid aggregates can rapidly expand by monomer addition and by the addition of other amyloid oligomers to quickly become a macro aggregate (Thakur et al., 2009). This model is consistent with the lag phase we see in the disease process, where a rate limiting step precedes the formation of toxic oligomers and eventually aggregates that may play a role in disease pathology. So prolonging this rate-limiting conformation change, stabilizing the native form of the "collapsed coil" and thereby inhibiting the aggregation process are all logical targets for therapeutic intervention.

Controversy over the role aggregates play in Huntington's Disease pathology has sparked a great deal of research to try and elucidate what the "toxic species" of aggregate is. As evident by the work of Nekooki-Machida et al., emerging evidence has shed light on the confusion by revealing that different aggregate conformations may be more toxic to the cell. Several groups have begun to develop antibodies that specifically recognize different species of aggregates, some of which have been shown to elicit cellular death (Legleiter et al., 2009; Miller et al., 2005). These antibodies

may be useful in identifying the toxic form of aggregate in different HD models, and also in developing therapeutics to stop the formation of those species. Overall, these results indicate that a soluble, oligomer form of mutant huntingtin is the most harmful to the cell. The formation of macro-aggregates serves a protective role by sequestering these toxic forms and keeping them from participating in aberrant protein interactions (Legleiter et al., 2009; Miller et al., 2005).

Many labs have focused their work on inhibiting mutant huntingtin aggregation as a means to prevent the pathogenic process of the disease. Previous work in our lab has identified a molecule that is effective in inhibiting the aggregation in a purified mutant huntingtin system (Parekh-Olmedo, Wang, Gusella, & Kmiec, 2004; Skogen et al., 2006). Characterizing that molecule and the mechanism by which it is able to inhibit mutant huntingtin aggregation is the focus of this thesis work.

1.5 G20 as an inhibitor of aggregation

Like many others, our lab identified the process of mutant huntingtin aggregation to be central to the pathology of Huntington's Disease. Previous work sought to identify a compound that would inhibit the aggregation process by establishing a screen of single stranded oligonucleotides that would prevent aggregation in a biochemical assay (Parekh-Olmedo et al., 2004; Skogen et al., 2006; J. Wang et al., 2005). They found that G-rich oligonucleotides were the most potent at inhibiting aggregation in that purified system. The most robust oligonucleotide was a 20-mer composed of entirely guanine residues, called "G20". Comparison studies were conducted to determine the selectiveness of the all guanosine oligonucleotide's

ability to inhibit aggregation. Single stranded oligoncucleotides composed of all adenine bases, all thymine bases and all cytosine bases were tested for their ability to inhibit aggregation in the same biochemical assay. G20 remained the only molecule able to effectively block the formation of macro aggregates tested by the assay, indicating it must possess unique properties that promote this activity.

G-rich oligonucleotides are known to assemble into unique secondary structures known as G-quartets or G-quadruplexes (Dapic et al., 2003; Fogolari et al., 2009; Fogolari et al., 2009). The guanine bases are able to interact in a unique hydrogen bonding relationship called Hoogsteen bonds as evident in Figure 1 (Dapic et al., 2003). G-quartet forming DNA is found endogenously in promoter regions of many oncogenes where it is thought to play a role in gene expression and the telomeres of human chromosomes where it is protective (Chang et al., 2004; Verma, Yadav, Basundra, Kumar, & Chowdhury, 2009). Quartet forming oligonucleotides are also being investigated as therapeutic aptamers for several diseases including certain types of cancer (Bates, Choi, & Nayak, 2009; Schwartz, Vasta, Bauer, Parekh-Olmedo, & Kmiec, 2008). Circular dichroism analysis was performed on G20 to determine if it was forming a G-quartet type of secondary structure. The results revealed a unique spectra for G20 in comparison to other 20-mer oligonucleotides tested that was consistent with reported spectral patterns for G-quartet structures (Skogen et al., 2006). When compared with other G-quartet forming oligonucleotides, G20 seemed to have a higher propensity to form G-quartets and in a more stable

conformation indicated by higher amplitude at the maximum and minimum of the spectra (Schwartz et al., 2008).

In all likelihood, the unique structure formed by G20 is central to its ability to block mutant huntingtin aggregation. The mechanism by which this happens has not yet been elucidated. The focus of this thesis work was to further characterize the secondary structure of G20 and to examine the relationship between G20 and purified mutant huntingtin to establish the mechanism by which aggregation inhibition is taking place.

1.6 Systems

1.6.1 GST Fusion Protein

The systems used in this thesis work were chosen to best characterize the interaction between mutant huntingtin and the single stranded oligonucleotide, G20. The difficulty of working with a very large, full length huntingtin led us to seek a system in which the aggregation of mutant huntingtin could be controlled and managed in a relatively short amount of time. In 2005, Wang et al. established a system in which the first exon of either mutant (Q58) or wild type (Q23) huntingtin is tagged with a glutathione S-transferase(GST) tag (J. Wang et al., 2005). The fusion protein can be expressed in E.coli and purified via affinity to glutathione in a batch purification method. The tag is useful for affinity purification, but also prevents the mutant protein from aggregating prematurely. The tag can be cleaved via a thrombin cleavage site and aggregation will commence in a concentration and temperature

dependent manner. This purified protein system is very useful in biochemical experiments, and will be employed in numerous assays to analyze aggregate inhibition and the specific interaction between G20 and huntingtin.

1.6.2 Cell lines engineered to express huntingtin

There are many cell lines available expressing different forms of mutant and wild type huntingtin. There are advantages and disadvantages to every system. We chose four rat pheochromocytoma (PC12) cell lines for our study. The first, Htt14A2.6, expresses a randomly integrated exon one fragment (Httex1tr-103Q-EGFP) of mutant (Q103) huntingtin fused to green fluorescent protein (GFP). The second, Htt17A2 is the same parental line expressing an exon 1 fragment (Httex1tr-25Q-EGFP) of wild type (Q25) huntingtin fused to GFP. Both of these lines were a gift from Dr. L. Thompson (UC Irvine). We chose these cell lines because, the expression of the huntingtin fragments in these cell lines are under the control of an inducible promoter which can be activated by treatment with the hormone, muristerone. Also, aggregation occurs rapidly (within 24 hours) and can be monitored over time by fluorescent microscopy.

The third and fourth cell lines were given as a gift by Dr. R. Hirschhorn, Department of Biology, Hood College, Frederick, MD 21701, USA and Dr C. Ross, Department of Neurology, The Johns Hopkins University School of Medicine, Baltimore, MD 21287, USA. Cell line, S6 is a Tet-off PC12 line expressing full length mutant huntingtin with 126 poly-glutamines. S6-5 is identical to S6 except it expresses full length wild type huntingtin with 21 poly-glutamines (Ratovitski et al.,

2007). Expression in these cell lines is maintained in a traditional Tet-off system; the cells are cultured in media containing doxycycline, a tetracycline derivative, to maintain the absence of expression. They are induced by the removal of doxycycline from the media. Due to the size of huntingtin (350 kDa) expression and subsequent aggregation in this cell line takes much longer than huntingtin fragment models, up to 14 days in culture. For this reason, the cells are differentiated during huntingtin expression with murine nerve growth factor in able to keep them viable in culture for a long time. These cells exhibit caspase cleavage events previously described for huntingtin. They also show decreased viability with mutant huntingtin expression over the course of 12 -14 days (Ratovitski et al., 2007). These cell lines were chosen for study because we wanted to study the interaction of G20 and huntingtin in a full-length model system. They are also useful for viability studies because of the effect of mutant huntingtin over time.

1.6.3 *Caenorhabditis Elegans* as a model for Poly-glutamine toxicity

To begin to test the effect of G20 on an *in vivo* model of Huntington's Disease we sought to obtain a *C. Elegans* line expressing mutant and wild type huntingtin. We were unable to get those lines, but did receive as a gift from Dr. Morimoto, Rice Institute for Biomedical Research, Northwestern University, 2153 North Campus Drive, Evanston, Illinois 60208, several lines of *C. Elegans* expressing different lengths of poly-glutamine fused to yellow fluorescent protein (YFP) (Brignull, Moore, Tang, & Morimoto, 2006; Brignull, Morley, Garcia, & Morimoto, 2006). These lines have been established as a model for poly-glutamine toxicity and are relevant to all

poly-glutamine expansion disorders in addition to Huntington's Disease (Brignull et al., 2006; Brignull, Morley et al., 2006). *C. Elegans* is an excellent *in vivo* model because its entire genome has been sequence and characterized, they are easy to maintain in the laboratory, they have a transparent body, are small in size and reproduce quickly. They also have the ability to reproduce as hermaphrodites, making them very useful for genetic experiments. Aggregation in these lines occurs in a poly-glutamine and age dependent manner, closely mirroring the human disease process. The phenotype of aggregation is easily visualized under a fluorescent microscope due to the YFP expression. Experiments can be performed at each developmental stage by synchronizing nematodes at the first stage, L1 and then following their development over time (Figure2). With an extreme poly-glutamine expansion these organisms exhibit a disabled movement phenotype; where, with increased aggregation, they move less often and more slowly (Brignull, Morley et al., 2006). This system was utilized as a starting point for establishing the effect of G20 in a true *in vivo* model of poly-glutamine disorders.

Studies will be conducted to examine the structure-function relationship between huntingtin and a G-quartet forming oligonucleotide, G20. Several systems and assays will be used to characterize the extent of the relationship between G20 and mutant huntingtin during the aggregation process, and the effect of that relationship in vivo.

Figure 1

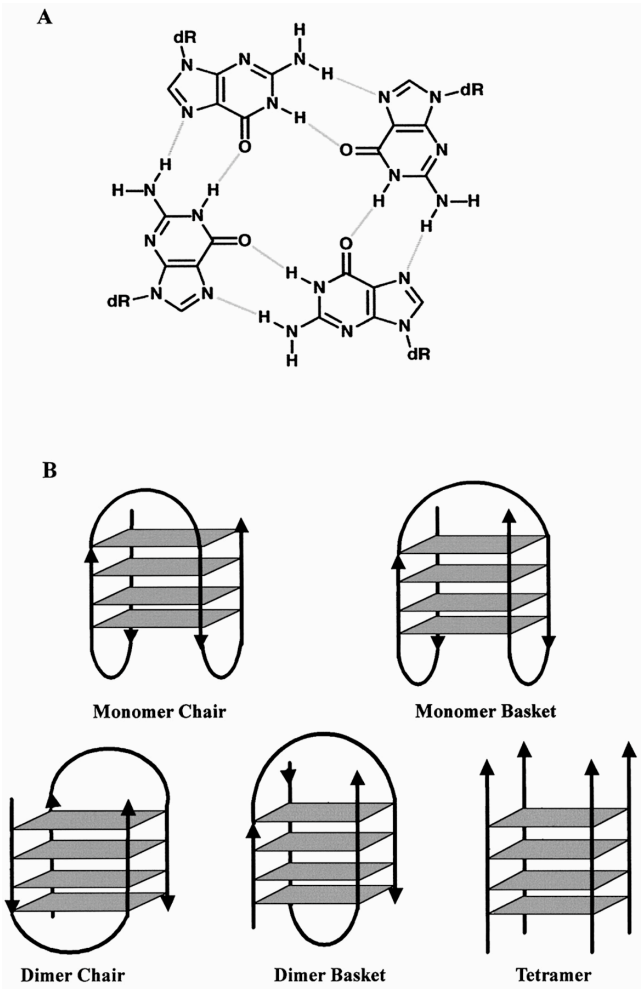


Figure 1: Illustration of Hoogsteen Binding and Possible G-quartet Conformations

A. Illustration of how guanine bases can interact via Hoogsten bonds. B. Possible conformations of G-quartet forming oligonucleotides.
(Virna api , Vedra Abdomerovi , Rachel Marrington, Jemma Peberdy, Alison Rodger, John O. Trent and Paula J. Bates Biophysical and biological properties of quadruplex oligodeoxyribonucleotides. Nucleic Acids Research, 2003, Vol. 31, No. 8 2097-2107.
(FIGURE 1). HTTP://NAR.OXFORDJOURNALS.ORG. © 2003 OXFORD UNIVERSITY PRESS)

Figure 2

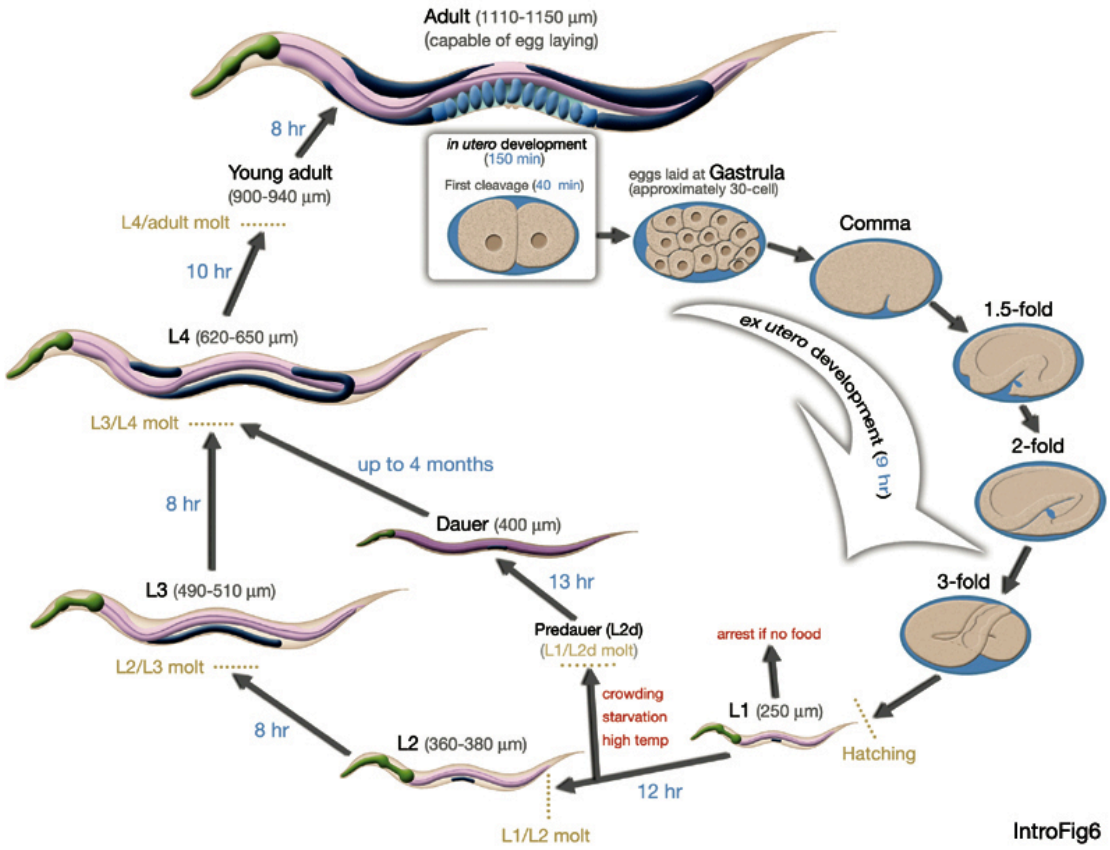


Figure 2: Life Cycle of *Caenorhabditis Elegans*

Illustration depicting the life cycle of *C. elegans* and the stages at which growth can be arrested for experimental purposes. www.nematodeatlas.org

Chapter 2

INVESTIGATING THE MECHANISM BY WHICH G20 INHIBITS MUTANT HUNTINGTIN AGGREGATION.

2.1 Introduction:

There is still a great deal to be learned about the molecular mechanism of Huntington's Disease pathology. The direct link between the mutation of a poly-glutamine expansion beyond 36 residues and the selective neuronal loss of medium spiny striatal neurons has yet to be fully elucidated. There are several hypotheses relating to this phenomenon that were discussed in chapter one. Even in unrelated proteins, expansions of poly-glutamine regions beyond a certain threshold results in mis-folding and aggregation of the disease protein (Bauer & Nukina, 2009). In the case of Huntington's Disease the poly-glutamine threshold for aggregation is the same for disease penetrance (Ross & Poirier, 2004; Scherzinger et al., 1999). There are several predictions for how aggregation may cause cellular dysfunction and eventual neuronal death.

Mutant huntingtin aggregation may confer a loss of wild type huntingtin function by recruiting it and other important cellular proteins into insoluble aggregates. There is evidence of wild type huntingtin and several transcription factors being found in aggregates in both animal models and cell culture models of Huntington's Disease(Jiang et al., 2006). It is also possible that mutant huntingtin is

toxic in a gain of function manner. For example, wild type huntingtin is thought to be neuroprotective by upregulating the expression of the protein, Brain Derived Neurotrophic Factor(BDNF) (Zuccato et al., 2007). BDNF is a small protein that is highly expressed in the hippocampus and cerebral cortex of adult brains. BDNF is necessary for striatal neuron maintenance and activity, so although it is produced in the cerebral cortex, high levels are transported to the striatum. BDNF expression is regulated by transcription factor binding to its neuron restrictive silencer element (NRSE) (Zuccato et al., 2007). Wild type huntingtin as been found to bind to the transcription factor, repressor element-1 transcription factor/neuron restrictive silencer factor (REST/NRSF) and sequester it in the cytoplasm where it cannot bind to NRSE and silence BDNF expression. Most likely due to conformation changes in mutant huntingtin, it does not correctly bind to REST/NRSF leading to its accumulation in the nucleus and aberrant transcription regulation of many neuronal genes, including BDNF (Zuccato et al., 2007). Therefore the gain of function of mutant huntingtin is the aberrant expression of neuronal genes caused by the inadequate regulation of NRSE. Another possibility is that the aggregates themselves are toxic to the cells. Huntingtin is predicted to play a role in membrane transport and vesicle trafficking down the axons of neurons. The large macro aggregates may block this essential transport process, preventing essential signals and nutrients from traveling to the necessary locations (Bauer & Nukina, 2009; Hatters, 2008).

As discussed in chapter 1, there is disagreement on the role of mutant huntingtin aggregates in the disease process. A new idea that is emerging is the

possibility of a "toxic aggregate species" contributing to the disease process. During the process of aggregation a species emerges that has the greatest detrimental effect on the cell. Predictions have been made that this is a soluble, oligomeric, "micro-aggregate" species that possibly contains exposed glutamine residues (Legleiter et al., 2009; Li et al., 2000; Nekooki-Machida et al., 2009; Scherzinger et al., 1999; Thakur et al., 2009). This concept brings together evidence that the presence of mutant huntingtin aggregates causes HD pathology and the notion that macro aggregates serve a protective role to the cell. It is actually a species along the aggregation pathway that is toxic, while sequestration of that toxic species into macro aggregates is protective.

Mutant huntingtin aggregation is a pathological hallmark of Huntington's Disease. The presence of aggregates in models of HD is the most apparent phenotypic difference between wild type and mutant huntingtin. Aggregates occur in several cell culture models, mouse models, yeast and *C. elegans* models and has been seen in post-mortem brains of human patients (Kremer et al., 1992; MacDonald et al., 2003). Mutant huntingtin aggregation has been linked to mitochondrial dysfunction, decreased cellular transport and signaling, transcription dysregulation and loss of wild type huntingtin function (Jiang et al., 2006; Lim et al., 2008; Ross & Poirier, 2004; Scherzinger et al., 1999; Trushina et al., 2004; Zuccato et al., 2007). Blocking the process of aggregation may prevent these aberrant effects, which would hopefully eliminate the gradual increase in cellular stress and eventual death.

It has been previously shown that single stranded oligonucleotides that fold into a stable G-quartet structure block mutant huntingtin aggregation in a HD cell

culture model and in a purified system containing an exon 1 fragment of mutant huntingtin(GST-Htt 1-171) (Skogen et al., 2006). The next logical focus of study is to determine the mechanism by which the stable G-quartet molecule acts to inhibit mutant huntingtin aggregation.

2.2 Materials and Methods

2.2.1 Rationale for using a GST-exon 1 fragment of mutant huntingtin fusion protein:

Mutant huntingtin aggregation is a dynamic process that occurs at different rates and frequencies *in vivo* (Hatters, 2008). To study the effect of G20 on mutant huntingtin aggregation, we needed an assay that would allow us to have some control over the overall process. To analyze how G20 is interacting with huntingtin, and at what point during aggregation it exhibits its inhibitory effect, it would be beneficial to have a system in which a uniform population of huntingtin is moving through the aggregation process. The GST fusion protein system developed by Wang et al.(2005) is composed of a glutathione S. transferase (GST) tag fused to an exon one fragment (amino acids 1-171) of mutant huntingtin containing 58 glutamine residues (J. Wang et al., 2005). The GST tag is used for batch purification of the protein after expression in E.Coli, and also serves to maintain the mutant huntingtin fragment in a monomer form until it is cleaved with thrombin. Once the GST tag has been cleaved, the process of aggregation is initiated. The time at which thrombin incubation is complete can be considered a “zero” time point, and the protein will continue to aggregate in a

fairly uniform, temperature and concentration dependent manner. The fusion protein is easily purified and can be readily characterized for aggregation experiments.

2.2.2 Production of GST fusion protein for use in biochemical analysis:

The GST fusion protein is produced by a construct using the pGEX vector system which was engineered to encode the first 171 amino acids of huntingtin, and contains a poly-glutamine repeat of 58 (mutant) or 23 (wild type). The protein is expressed in *E. Coli* and then isolated and purified via a batch purification method using Glutathione Sepharose 4B beads (Amersham Biosciences; Uppsala, Sweden). The resulting protein is quantified using BCA reagents and method (Pierce; Perbio Science UK Ltd., UK) and purity and concentration is verified via SDS PAGE and western blotting.

2.2.3 Biochemical aggregation assay

To analyze the inhibition of aggregation by GROs, an immunoblot assay developed by Wang et al. (2005) was employed. The fusion protein GST-Htt1-171(Q58) was incubated for 45 minutes at room temperature with thrombin (1 U/1 μg protein) at a concentration of 10 μg/ml in a buffer of 50 mM Tris-HCl, pH 8, 100 mM NaCl, 2.5 mM $CaCl_2$, and 1 mM EDTA, to cleave between the huntingtin fragment and GST. As indicated by Wang et al. this fragment consists of the amino terminal 171 amino acids with a tract of 58 glutamine residues fused to GST. The protein mix was then centrifuged at 13,000 × g at 4°C for 35 minutes to remove any aggregates that had already formed. The protein was added to wells containing 0.5–60 μM GROs or

control ODNs or 10 μM Congo Red in the buffer detailed above with 100 mM KCl replacing NaCl. The 0-hour control was stopped immediately; after 24 hours incubation at room temperature the remaining reactions were stopped by adding 10% SDS/50 mM 2-mercaptoethanol and heating to 99°C for five minutes. The mixture was diluted in 1X PBS and then filtered through a cellulose acetate membrane (Osmonics) using the Easy-Titer ELIFA system (Pierce) followed by a 2% SDS wash. After blocking in 5% milk/1X PBS-0.05% Tween, the membrane was incubated with a specific anti-huntingtin antibody (HP1, 1:1000 dilution), followed by incubation with a peroxidase-conjugated goat anti-rabbit antibody (Sigma,1:40,000 dilution) and chemiluminescence reagent (ECL Plus, Amersham).

2.2.4 Native Gel Electrophoresis

Samples of purified fusion protein GST-Htt 1-171(Q58) were incubated for 45 minutes at room temperature with thrombin (0.2 U/1 μg protein) at decreasing concentrations in a buffer of 50 mM Tris-HCl, pH 8, 100 mM NaCl, 2.5 mM CaCl2, and 1 mM EDTA. Samples were centrifuged at 13,000 × g for 35 minutes at 4°C and further incubated with G20 or T20 for 18 hrs at RT. Reactions were placed in native 2X sample buffer (500mM Tris-HCL pH 6.8, 100% glycerol) and loaded on a 10% non-denaturing acrylamide gel. The gel was run for 1 hour in non-SDS running buffer (12.4 mM Tris, 125.2 mM Glycine), the samples were transferred to a pdf membrane in a wet transfer apparatus (BioRad). The membrane was blocked in 5% milk/ 1X TBS-.05% Tween, then incubated with an anti-huntingtin antibody (HP1 1:1000

dilution) and followed by incubation with a peroxidase-conjugated goat anti-rabbit antibody (Sigma, 1:20,000 dilution) and chemiluminescence reagent (ECLPlus, Amersham). The blot was then exposed on x-ray film(Kodak) and developed.

2.2.5 Agarose Gel Electrophoresis for Resolving Aggregates (AGERA)

Pure GST-Htt1-171(Q58) samples were prepared at a concentration of 0.25 mg/ml in a buffer of 50 mM Tris-HCl, pH 8, 100 mM NaCl, 2.5 mM $CaCl_2$, and 1 mM EDTA. They were incubated with thrombin (0.2 U/1 μg protein) for 45 minutes to cleave the GST tag and then centrifuged (at 13,000 × g) for 35 minutes to remove any preformed aggregates. They were then incubated with increasing concentrations of G20 or 100 μM Congo Red for 18 hours at room temperature. Samples were run on a 2% agarose gel in SDS running buffer for 2-3 hours at 4°C. The blot was transferred to a pdf membrane with a bio-rad dry transfer apparatus for 1.5 hours at 30 mAmps. The membrane was blocked in 5% milk/1X TBS-.05% Tween, then incubated with an anti-huntingtin antibody(HP1 1:1,000) followed by incubation with peroxidase conjugated goat anti-rabbit antibody (Sigma, 1:20,000 dilution) and chemiluminescence reagent (ECLPlus, Amersham). The blot was exposed on x-ray film (Kodak) and developed. A more detailed description of the AGERA protocol can be found in (Weiss et al., 2008)

2.2.6 Biotinylated Oligonucleotide Pulldown Assay

Pure GST-Htt1-171(Q58) samples were prepared at specified concentration in a buffer of 50 mM Tris-HCl, pH 8, 100 mM NaCl, 2.5 mM $CaCl_2$, and 1 mM EDTA and

incubated with thrombin (0.2U/μg protein) for 45 minutes and then centrifuged to remove pre-formed aggregates. Samples were allowed to aggregate (incubated at room temperature) for 0, 2, 4, 6, 10 or 18 hrs before addition of oligonucleotide. They were then incubated with 15 μM biotinylated G20 for 18 hours at room temperature in buffer (50 mM Tris-HCl, pH 8, 100 mM NaCl, 2.5 mM $CaCl_2$, and 1 mM EDTA). Streptavidin beads (Invitrogen, Carlsbad, CA) were added in a 50% slurry and incubated at room temperature for 45 minutes while shaking. The beads were recovered by low speed centrifugation, and washed with TE buffer, binding buffer (10 mM Tris-HCL pH 8.0, 40 mM KCL, 1 mM DTT, 6% glycerol, 0.05% NP-40) and 1X PBS respectively. Bound G20 and protein were eluted by boiling in 2X sample buffer at 95°C for 5 minutes. Samples were run on a 10% SDS polyacrylamide gel, and then transferred to a pdf membrane in a wet transfer apparatus. The membrane was blocked with 5% milk/TBS-.05% Tween, incubated with an anti-huntingtin antibody (HP1, 1:1,000) and incubated with a peroxidase conjugated goat anti-rabbit antibody (Sigma, 1:20,00 dilution) and chemiluminescence reagent (ECLPlus, Amersham). The blot was exposed to x-ray film(Kodak) and developed.

2.2.7 Cell Culture

The PC12 lines, Htt14A2.6 and Htt17A2 were grown at 37° C in 5% CO_2. They were maintained in cell culture media composed of; high glucose Dulbecco's Modified Eagle media(DMEM), 10% horse serum, 5% fetal bovine serum(FBS), 1% penicillin/streptomycin and under the selection of 50mg/mL Geneticin and 100 mg/mL Zeocin (Invitrogen). The Tet-off PC12 lines, S6 and S6-5 were also grown at

37° C in 5% CO_2. They were maintained in media composed of high glucose Dulbecco's Modified Eagle media(DMEM), 10% horse serum, 5% fetal bovine serum(FBS), 1% L-glutamine, 1% penicillin/streptomycin, and under selction of 50mg/mL Geneticin and hygromycin B. To maintain the system in a non-expressing state the cell culture media was supplemented with 200ng/mL doxycycline (dox, Invitrogen) every 48 hours.

2.2.8 *In vivo* Biotinylated Oligonucleotide Pull Down Assay

Htt14A2.6(Q103) and Htt17A2(Q25) PC12 cell lines were plated in 6-well dishes at 60% confluency in normal media and allowed to adhere to the plate for up to 20 hours. The cells were transfected with 1µg oligonucleotide/well with lipofectamine 2000 transfection reagent(Invitrogen) at a ratio of 1.25µl per µg oligonucleotide in optimem(Gibco). The cells were simultaneously induced to express huntingtin with 5µM muristerone at the time of transfection and allowed to incubate/aggregate for 24 hours. The cells were harvested and protein was extracted using the MPER protocol from Pierce. Total protein was quantified with BCA reagents from Bradford and read on a plate reader. The protein concentration from each sample was standardized and then the biotinylated oligonucleotide pull down procedure was followed as described above. To maintain the integrity of cellular proteins the samples were incubated with the streptavidin beads for two hours at 4°C instead of 45 minutes at room temperature.

2.2.9 Tet-Off PC12 Expression and Differentiation

For expression of full length huntingtin the S6 and S6-5 Tet-off PC12 cell lines are plated in expression and differentiation media; low glucose Dulbecco's Modified

Eagle media(DMEM), 1% horse serum which is supplemented with 50 ng/mL nerve growth factor every 48 hours and maintained on rat tail type 1 collagen coated plates for the length of expression time(up to 12 days.)

2.3 Results

2.3.1 Analysis of G20 activity in inhibiting the formation of macro-aggregates:

The biochemical assay used to screen G-rich oligonucleotides utilizes a nitrocellulose filter upon which huntingtin aggregates can be detected by immunoblotting.(J. Wang et al., 2005) There is a limitation to this technique, because although macro-aggregates can be detected on the filter, smaller oligomers or microaggregates may pass through. To further elucidate that aggregation inhibition ability of G20 we employed an assay to detect intermediate sized aggregates or oligomers, termed Agarose Electrophoresis for Resolving Aggregates (AGERA). This technique utilizes an agarose gel run in an SDS PAGE manner and has previously been determined to detect oligomers that may pass through nitrocellulose membrane of the immunoblot assay developed by Wang et al(2005).(Weiss et al., 2008) We see that G20 inhibits the formation of macro-aggregates as evident by the immunoblot assay (Figure 3), and upon utilizing the AGERA assay we see a reduction in intermediate sized aggregates with increasing concentrations of G20 (Figure 4). A known aggregation inhibitor, Congo Red, was used as a positive control and the lane containing that reaction mixture shows no intermediate aggregates of GST-Htt 1-171(Q58). This tells us that Congo Red prevented the formation of aggregates as

expected, and that this assay is a valid way of measuring aggregate inhibition. The level of intermediate-aggregate inhibition by G20 does not quite match that of the Congo Red positive control. This might indicate a discrepancy in their mechanisms of aggregation inhibition. G20 may target an intermediate species in the aggregation pathway, therefore halting the progression into a macro-aggregate, whereas Congo Red may act at the monomer level.

Another limitation of the immunoblot assay and the AGERA assay as is the inability to visualize mutant huntingtin monomers that have been prevented from aggregating. In an attempt to ameliorate this, we performed native gel electrophoresis with samples of GST-htt 1-171 and G20. The presence of protein on the native gel indicates aggregate inhibition, because insoluble aggregates would not be resolved (Kazantsev et al., 1999; Weiss et al., 2008). We see aggregate inhibition with the presence of G20 compared to control samples as evident by the appearance of monomers, dimers and trimers on the blot (Figure 5). Of notable interest is the most activity occurs at one particular concentration of GST-htt 1-171, which may indicate specificity in the mechanism of G20 inhibition.

2.3.2 Investigation of direct binding of huntingtin by G20.

Up until this point we have gained further evidence that G20 effectively inhibits mutant huntingtin aggregation in a dose dependent manner. However we have yet to gain insight on the actual mechanism of G20 activity. By utilizing the affinity of streptavidin for biotin we developed a "pull-down" assay which would allow us to detect if there was a direct interaction between GST 1-171 and G20. We obtained

biotinylated G20, which was incubated with mutant huntingtin and then "pulled down" with streptavidin coated beads. The bound protein product was eluted and run on a standard SDS-PAGE and blotted for the presence of huntingtin. As evident by Figure 6 we determined that G20 was in fact binding to mutant huntingtin and that this interaction is the strongest at a particular concentration of GST-Htt 1-171(Q58). We also wanted to ask the question if G20 has an affinity for mutant huntingtin alone, or if it would bind to wild type as well. We expressed and purified samples of GST-htt 1-171 with a polyglutamine tract of 23 residues, and tested them in the biotinylated pull down assay. We found that G20 binds to this wild type form of huntingtin as well, and in contrast to GST-Htt 1-171 (Q58), the binding occurs strongly at several concentrations.

Next we wanted to determine if the interaction between G20 and huntingtin occurred *in vivo* as well as in the biochemical assays. We performed the biotinylated pull-down assay in four different Huntington's Disease cell culture models. The first two, Htt14A2.6(Q103) and Htt17A2(Q25) PC12 express an integrated exon 1 fragment of huntingtin fused to green fluorescent protein (GFP) with either a poly-glutamine repeat of 103 residues (mutant) or 25 residues (wild type) in a rat pheocychroma neuronal-like cell line. The results indicate that binding occurs in both the mutant and wild type lines. The blot presented in Figure 7 A. is a representative result from studies done in the mutant (Q103) cell line where a control oligonucleotide composed of 20 "T" bases was used in comparison to G20. The lack of binding with the control oligonucleotide represents the specificity of G20 interaction, which reflects

its ability to specifically inhibit aggregation of mutant hungtingin by Skogen et al. 2007. Evident in Figure 7 B, another *in vivo* binding experiment was conducted to determine if G20 binds to wild type in addition to mutant huntingtin. In this experiment, we also wanted to test if this interaction is dependent on the secondary structure formed by G20. Another G-quartet forming molecule composed of 26 residues, 5′-GGTGGTGGTGGTTGTGGTGGTGGTGG and called "AS1411" was used as a positive control. AS1411 is in clinical trials as an anti-cancer aptamer, and was found to form secondary structure indicative of a G-quartet, however it is less stable and less robust than G20 (Bates, Laber, Miller, Thomas, & Trent, 2009; Schwartz et al., 2008). Positive binding with both oligonucleotides may be a clue that the G-quartet structure is necessary for binding to huntingtin *in vivo,* further characterization of the specific G-quartet structure formed by G20 is of value.

We see similar results in the Tet-off PC12 cell lines, S6 and S6-5 that express full length wild type(Q23) and mutant(Q126) huntingtin respectively. These cell lines take a significantly longer time to express huntingtin and to form intracellular aggregates, up to 14 days in culture. For this reason, they must be differentiated once they are induced to express huntingtin which gives us the ability to maintain viable cells for that amount of time. Once differentiated, it is difficult to transfect cells via lipofection. We still thought it was valuable to see if G20 could bind to full length huntingtin, so we performed binding experiments in this cell line in total protein extracts as described above. We do get confirmation that G20 has the ability to interact with intact, full length huntingtin in both wild type and mutant lines as evident

in Figure 8A and Figure 8B. AS1411 was once again used as a positive control. We see higher levels of binding to G20 than AS1411, which may be related to the stability of G-quartet structure. We also see that binding in the wild type(Q21) cell line, S6 exhibits robust binding at every time point tested. Binding in the mutant line, S6-5 is more selective, revealing a binding profile that is limited to the middle time points.

2.4 Discussion:

Of therapeutic interest is the ability of G-rich oligonucleotides to inhibit aggregation in a purified mutant htt fragment. To investigate the mechanism of inhibition we employed two electrophoresis assays, one to visualize intermediate oligomer species of aggregation and another to visualize lower molecular weight soluble monomer and dimer species.

Evident by previous work, G20 inhibits the formation of macro-aggregates in our system (Skogen et al., 2006). The AGERA assay gave us the ability to test whether it also has an effect on intermediate aggregates. The results in Figure 4 indicate that there is a reduction in intermediate sized aggregates when compared to control wells. A complete disruption of aggregation is evident in the Congo Red control. This might mean that G20 and Congo Red inhibit aggregation by different mechanisms. Where G20 seems to have an effect on oligomeric species of intermediate molecular weight, Congo Red seems to act before the formation of an intermediate species. The precise delineation of a known “toxic species” of aggregate

would help in determining where and when it is necessary to block aggregation for a compound to be therapeutically viable. The native gel electrophoresis results in Figure 5 confirm the ability of G20 to inhibit mutant huntingtin aggregation. There is evidence of dimer and trimer formation which might be another clue that G20 does not act at the monomer stage, but at a later stage in the aggregation pathway. G20 also seems to have maximal activity at a specific concentration of mutant huntingtin. The dependence on concentration may indicate a possible requirement for a stoichiometric relationship between G20 and mutant huntingtin for aggregation inhibition to take place. Since we know that mutant huntingtin aggregation is concentration dependent, and it seems that the inhibition of aggregation is dependent on mutant huntingtin concentration, then this relationship could be indicative that G20 interacts with mutant huntingtin in an aggregation dependent manner.

The biotinylated oligonucleotide pull-down assays revealed that G20 does in fact exhibit a direct interaction with both mutant and wild-type huntingtin. This reveals the possibility that G20 binds to mutant huntingtin and retards or prevents aggregation. The robust interaction of G20 and mutant huntingtin at a specific concentration as illustrated in Figure 6, mirrors the aggregate inhibition seen at a specific concentration in the native gel electrophoresis assay, Figure 5. This is a good indication that the binding activity of G20 to mutant huntingtin (Figure6) is closely related to and possibly necessary for its ability to inhibit aggregration. Again, there is a dependency on mutant huntingtin concentration, which may be related to the aggregation pathway, and the possibility of the presence of a specific aggregate

species necessary for G20 activity. We also see binding of G20 to wild type GST-Htt 1-171 (Q23) at a larger range of concentrations. This raises the possibility that the structure or motif that is necessary for G20 binding is present in the wild type protein at a wider range of concentrations. This is consistent with the fact that a moving target of mutant huntingtin aggregation would only have small window of opportunity for the necessary interaction to take place.

The fact that we see pull down of mutant and wild type huntingtin in cell culture models of HD gives us *in vivo* evidence of a binding relationship and verifies the interaction between G20 and Huntingtin. We are able to show that G20 binds to mutant and wild type huntingtin in a complex cellular environment. AS1411 is another G-quartet forming oligonucleotide that is less stable than G20 evident by CD analysis (Schwartz et al., 2008). AS1411 was also tested for its ability to inhibit mutant huntingtin aggregation in the immunoblot assay developed by Wang et al. and was not effective (unpublished data by Michael Skogen). The use of AS1411 as a positive control reveals that the secondary structure of G20 may be central to its ability to bind huntingtin and inhibit its aggregation.

The Tet-off PC12 cell lines S6 and S6-5 give us a full-length huntingtin model to test. In this case, G20 still robustly binds to both mutant and wild type full length huntingtin in a total protein extract. Again, the wild type seems to be preferentially bound by G20 at all time points tested, while mutant Htt is bound for just an intermediate time point before falling off. This could be due to decreased stability of the mutant, full length protein or it could indicate that binding of G20 to

mutant huntingtin is related to aggregation of mutant huntingtin, which begins around day 8 (Ratovitski et al., 2007). This reflects the experiment conducted with the purified GST-Htt 1-171 (Q58) where only a specific concentration is preferentially bound, indicating that the binding profile may be dependent on aggregation of the mutant protein. AS1411 was used as a control in these experiments as well. Here, we also see that AS1411 binds mutant and wild type huntingtin to a lesser extent (Figure 8), indicating that the stability of G-quartet secondary structure may be important for G20 activity.

The development of the biotinylated oligonucleotide pull down assay allowed us to test if there was a direct binding of huntingtin by G20. We found in a purified system of an exon one fragment of wild type (Q23) or mutant (Q58) huntingtin that G20 exhibits a direct interaction with GST-Htt 1-171 (Q58) in a concentration specific manner, and with GST-Htt 1-171 (Q23) at several concentrations. We see a concurrent trend with the concentration dependency of aggregation inhibition as seen in the native gel assay and the binding relationship between G20 and GST-Htt 1-171 (Q58). This is a good indication that the binding interaction is related to the ability of G20 to effectively inhibit mutant huntingtin aggregation. Characterizing the specific species that is preferentially bound is the next logical focus of work.

Figure 3

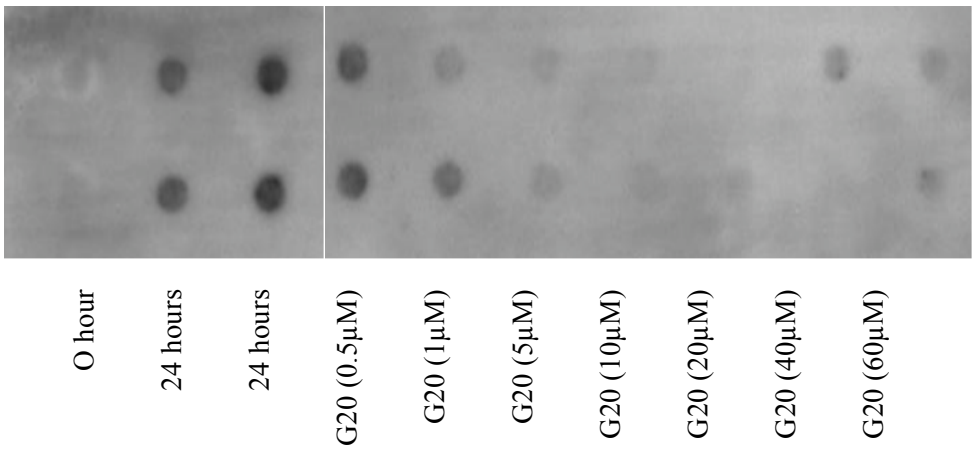


Figure 3: Immunoblot analysis of the effect of increasing concentrations of G20 on aggregation of GST-Htt 1-171 (Q58).

The zero(0) hour control represents samples that were stopped immediately after addition of protein, the twenty four (24) hour samples were incubated in the absence of oligonucleotide and the reaction was stopped 24 hours later.

Figure 4

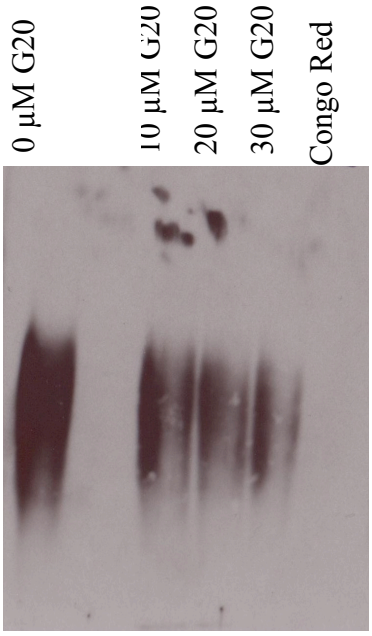


Figure 4: Agarose Gel Electrophoresis for Resolving Aggregates(AGERA) analysis for the inhibition of macro-aggregate formation by G20.

GST-Htt 1-171 (Q58) Samples were incubated in the presence or absence of oligonucleotide for 20 hours. Congo Red (CR) is a positive control for the inhibition of aggregation. Gel resolves aggregates of intermediate molecular weight. Decreased amount of sample with increased amounts of G20 indicates a decrease in the amount of intermediate molecular weight aggregates.

Figure 5

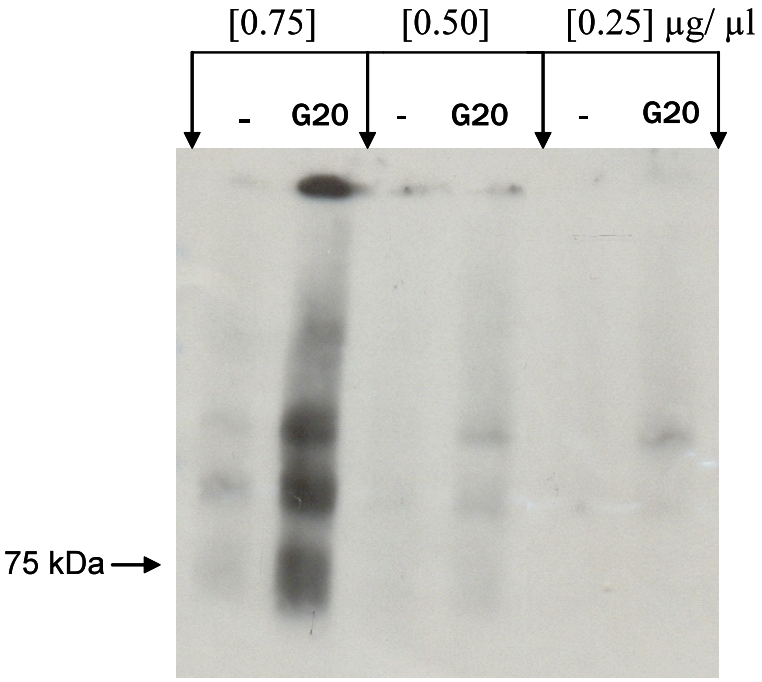


Figure 5: Native gel electrophoresis analysis of the effect of G20 on aggregation of GST-Htt 1-171 (Q58).

Samples of decreasing concentration of GST-Htt 1-171 (Q58) were incubated for 20 hours in the presence or absence of oligonucleotide. Disruption of aggregation is evident by sample entering the gel during electrophoresis.

Figure 6

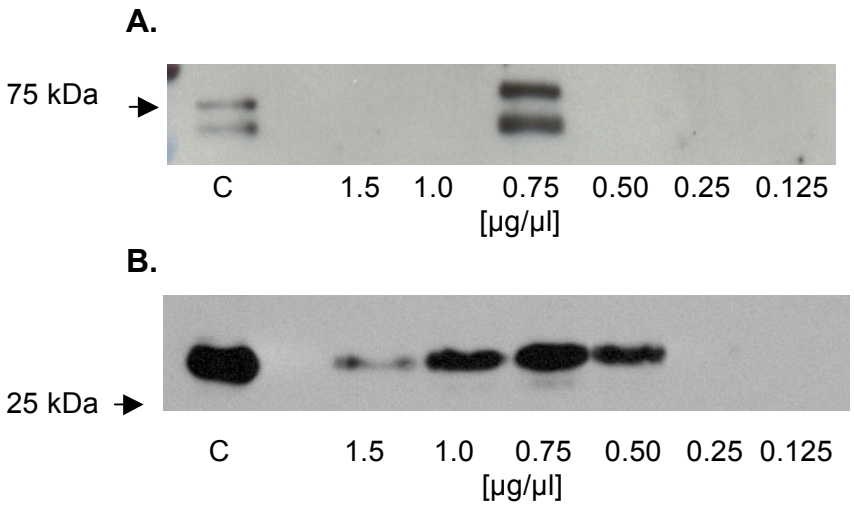


Figure 6: Analysis of G20 –GST-Htt 1-171 interaction by a biotinylated oligonucleotide pull-down assay.

A. Samples of decreasing concentration of Htt 1-171(Q58) were incubated for 20 hours in the presence of biotinylated G20. Interaction with G20 was assayed by "pulling down" the biotinylated oligonucleotide with streptavidin beads and then analyzed by Western blotting for Htt 1-171. The presence of protein on the blot indicates a direct interaction with G20. **B.** Analysis of G20 – Htt 1-171 (Q23) interaction by a biotin pull-down assay. Analyzed in the same way as Figure 4 A.

Figure 7

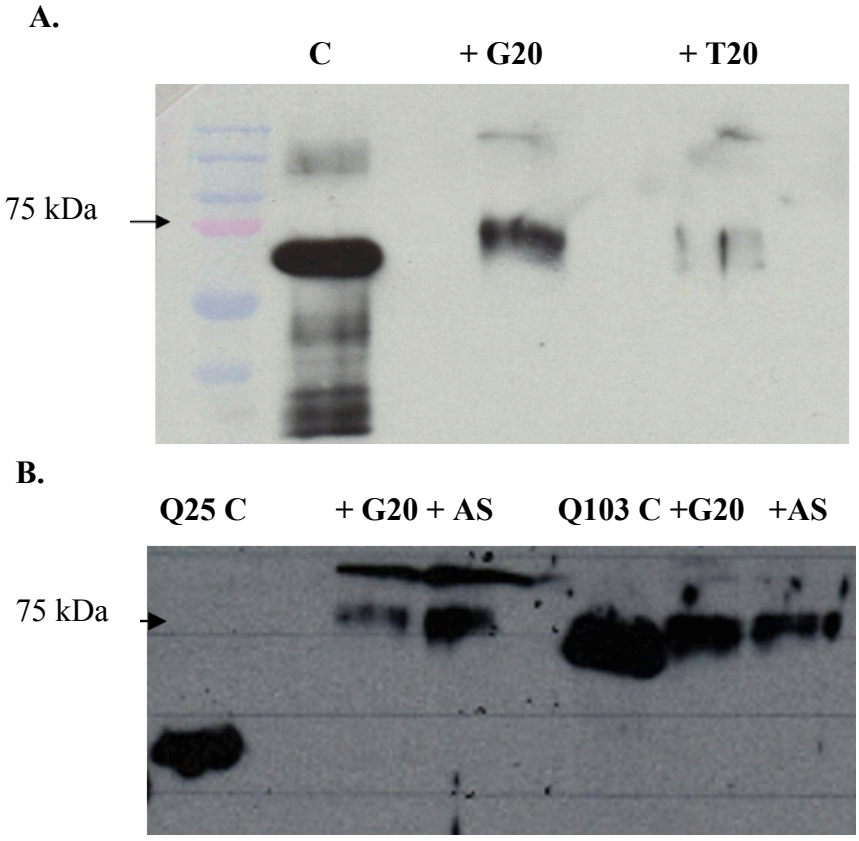
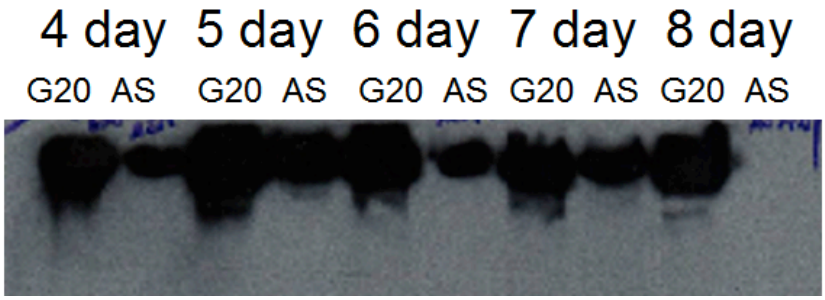


Figure 7: *In vivo* biotinylated oligonucleotide pull-down analysis in a PC12 cell line expressing an exon 1 fragment of mutant huntingtin

A. Cells were transfected with biotinylated oligonucleotide(ODN) and allowed to incubate for 24 hours. Total protein was extracted, and the biotinylated oligonucleotide was pulled down with streptavidin beads. Bound protein was analyzed by Western Blotting. T20 is a control oligonucleotide composed of 20 thymine bases. **B.** The experiment was performed the same way with a wild type(Q25) and mutant(Q25) PC12 cell line. AS stands for AS1411 another G-quartet forming oligonucleotide that is used as a control.

Figure 8

A.



B.

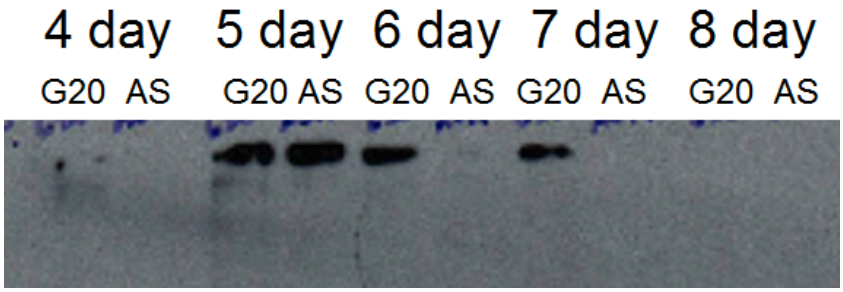


Figure 8: Biotinylated oligonucleotide pull down analysis in an inducible PC12 full length huntingtin cell line

A. Wild type Tet-off PC12(Q21) and **B.** Mutant Tet-off PC12 cells were differentiated and induced to express huntingtin for the amount of time specified(up to 8 days). Total protein was extracted, and quantified. Equal amounts of protein were incubated with biotinylated oligonucleotide for 18 hours at 4°C, and then the nucleo-protein complexes were pulled down with streptavidin beads. The protein was eluted and the bound product was analyzed by Western Blotting. AS stands for AS1411 and is another G-quartet forming oligonucleotide that is used as a control.

Chapter 3

CHARACTERIZING THE INTERACTION BETWEEN G20 AND HUNTINGTIN

3.1 Introduction

G20 is a single stranded oligonucleotide that is a robust inhibitor of mutant huntingtin aggregation. We have presented evidence that G20 exhibits a direct relationship with huntingtin, in a purified assay system and cell culture models of Huntington's Disease. The ability of G20 to inhibit mutant huntingtin aggregation is most likely directly linked to its binding relationship. To develop G20 as a therapeutic for Huntington's Disease it is necessary to gain more information about how and why this binding interaction takes place. The next logical step is to characterize the interaction and to find out what region or conformation of huntingtin is necessary for it to take place. G20 is designed as a single stranded oligonucleotide composed of 20 guanosine residues, a molecule that would not be predicted to have any sort of specificity for a protein binding partner. However, we have learned from previous data that G20 forms a unique structure known as a G-quartet, which may be central to its binding activity. Further analysis of the structure – function relationship between mutant huntingtin aggregates and G20 is of great interest. We performed circular dichroism analysis on G20 to confirm its G-quartet structure, and in conjunction with

Dr. James Vesenka at University of New England, atomic force microscopy experiments were performed to further investigate the specific G-quartet structure formed by G20.

Another observation to investigate is the apparent specificity of the G20-htt interaction. As evident by the native gel electrophoresis experiment (Figure 5) and the biotinylated pull down assay conducted with purified GST-Htt 1-171 (Q58) (Figure 6A); G20 exhibits a higher level of activity at a specific concentration of protein. This may indicate that a stoichiometric relationship between G20 and mutant huntingtin is necessary for an interaction to take place. Mutant huntingtin aggregation is a dynamic process which is influenced by protein concentration, so there might also be the possibility that the presence of a specific species that appears along the aggregation pathway is necessary for G20 activity. To study this phenomenon, we utilized the GST- fusion system to perform experiments to examine the interaction of G20 at specific time points along the aggregation course.

3.2 Materials and Methods

3.2.1 Circular Dichroism Spectroscopy

Circular dichroism spectra of 15 μM oligonucleotide samples in 10 mM KCl were recorded on an Aviv model 202 spectrometer. Measurements were performed at 24°C using a 0.1 cm path-length quartz cuvette (Hellma). The CD spectra were obtained by taking the average of two scans made at 1 nm intervals from 200 to 320 nm and subtracting the baseline value corresponding to that of buffer alone. Spectral data are expressed in units of millidegree.

3.2.2 Atomic Force Microscopy – Performed by Dr. James Vesenka

Samples of G20 were prepared at different concentrations in deionized water, adsorbed onto freshly cleaved mica, incubated for 10 minutes and flash dried without rinsing with dry nitrogen. No reaction buffer was used in their preparation for imaging. Htt samples were prepared according to the same protocol as biochemical assays. 0.75 μg/μL Htt was incubated in 0.2 U thrombin/μg Htt for 30 minutes in reaction buffer (50 mM Tris HCl pH8.0, 100 mM KCl, 2.5 mM $CaCl_2$ and 1 mM EDTA) and centrifuged at 15000xg for 30 minutes and incubated at room temperature. Samples of the supernatant were isolated after different incubation times (t=0, 2, 4, 6, 8, 16, 24, 48 and 96 hrs) with and without the addition G20. Samples were prepared for AFM imaging according to the following procedure. A few microliters of supernatent were diluted 1/10 in reaction buffer and adsorbed onto freshly cleaved mica for 10 minutes at room temperature, rinsed with 1 mL deionized water and flash dried with dry nitrogen for AFM imaging. Htt and G20 interactions were imaged after diluting G20 down to 15 μM for ten minutes in Htt (final concentration 0.71 μg/μL). An aliquot of the mixture was diluted 1/10 in reaction buffer and adsorbed onto freshly cleaved mica for 10 minutes at room temperature, rinsed with 1 mL deionized water and flash dried with dry nitrogen for AFM imaging. The samples were imaged on a Digital Instruments (Santa Barbara, CA) Nanoscope IIIa controller and Multimode™ atomic force microscope with a variety of general purpose and sharp AFM probes (probe and scanning details for each image described in corresponding figure caption). AFM probes used included Nanoprobe (www.veeco.com),

Micromasch (http://www.spmtips.com/) "Hi-ResC" diamond-like carbon probes, and Nanoworld cvd single wall carbon nanotubes (www.nanoworld.com). Images were captured in dry helium after at least six hours of equilibration of fresh probe at room temperature and "false engagement" scanning to reduce piezoelectric hysteresis and thermal drift. Off line analysis was accomplished with Nanoscope software or Image SXM 1.82 (http://www.ImageSXM.org.UK).

3.2.3 Aggregation Time Course Experiments in Conjunction with Biotinylated Pull Down Assay

Samples of GST-Htt 1-171(Q58) were prepared in reaction buffer (50 mM Tris HCl pH8.0, 100 mM KCl, 2.5 mM $CaCl_2$ and 1 mM EDTA) at the concentrations described in Figure 12. They were incubated with thrombin (0.2U/μg Htt) for forty five minutes at room temperature and then spun down for thirty minutes at 15,000 x g. The samples were then allowed to incubate at room temperature in the absence of oligonucleotide for the specified amount of time. At the designated time 15 μM of G20 was added to each sample, which incubated for another 18 hours at room temperature. The samples were then incubated with streptavidin beads (Invitrogen, Carlsbad, CA) which were added in a 50% slurry and incubated at room temperature for 45 minutes while shaking. The beads were recovered by low speed centrifugation, and washed with TE buffer, binding buffer (10 mM Tris-HCL pH 8.0, 40 mM KCL, 1 mM DTT, 6% glycerol, 0.05% NP-40) and 1X PBS respectively. Bound G20 and protein were eluted by boiling in 2X sample buffer at 95°C for 5 minutes. Samples were run on a 10% SDS polyacrylamide gel, and then transferred to a pdf membrane

in a wet transfer apparatus. The membrane was blocked with 5% milk/TBS-.05% Tween, incubated with an anti-huntingtin antibody (HP1, 1:1,000) and incubated with a peroxidase conjugated goat anti-rabbit antibody (Sigma, 1:20,00 dilution) and chemiluminescence reagent (ECLPlus, Amersham). Blots were exposed to x-ray film (Kodak) and developed.

3.3 Results

3.3.1 Rationale

It is evident from our *in vitro* pull-down analysis (Figure 6) that the interaction between G20 and GST-Htt 1-171 (Q58) is dependent on concentration. Aggregation of GST-Htt 1-171 (Q58) is time and concentration dependent. So the prediction can be made that there is a dependency on a specific aggregate species for this interaction to take place. It is also evident from previous work, (Skogen et al., 2006) that the unique G-quartet structure form by G-rich oligonucleotides is important for aggregation inhibition to take place. For this reason a more in depth analysis of the structure of G20 was made, as well as an attempt to characterize the specific aggregate species preferentially bound by G20.

3.3.2 Circular Dichroism Analysis

G20 is designed as a single stranded oligonucleotide composed of a sequence of 20 guanosine residues. However, the prediction that it forms a unique secondary structure led us to perform analysis by circular dichroism. The results reveal a spectral profile with a maximal ellipticity found at 264 nm and a minimum at 240 nm.

Evidence from published data indicates this is a standard spectral pattern for a secondary structure often formed by G-rich DNA, known as a G-quartet conformation (Chang et al., 2004; Dapic et al., 2003). The stability of the G20 was tested by increasing the temperature from 24°C to 99°C. Samples were measured in an Aviv model 202 spectrometer. Circular dichroism (CD) spectra and the elliptical profile were obtained at each temperature respectively. As evident in Figure 9, the G20 molecule exhibits a high degree of stability since the spectral pattern changes only minimally as the temperature rises, indicating that the integrity of secondary structure formation does not change.

3.3.3 Atomic Force Microscopy of G20 and GST-Htt 1-171 (Q58)

To analyze the G-quartet formation by G20, the molecule was imaged by Atomic Force Microscopy (AFM) by our collaborator at University of New England, Dr. James Vesenka. The imaging was conducted on mica with the oligonucleotide samples being flash-dried after being resuspended in deionized water. Highly concentrated G20 DNA is pictured in Figure 10 A, revealing details of complex structures with an average height of 1.9±0.1 nm. Of significant note is that G20 spontaneously forms into a G-quartet structure without the need of stabilizing ions, which are necessary for other G-quadruplex forming molecules (Marsh, Vesenka, & Henderson, 1995; Neaves, Huppert, Henderson, & Edwardson, 2009). At these concentrations, the G20 quartets exhibit a higher level of interaction, forming higher order structures (horizontal thick arrow). The vertical thin arrow points out species representing incomplete quadruplex formation. Under these conditions, the apical

AFM probe asperities can detect subtle details while the sample is supported by the surrounding structure (Mou, Czajkowsky, Zhang, & Shao, 1995). The cross section of Figure 10 B (black line, Figure 10 A) reveals how uniform the height of the G-quadruplex structures is (1.9 nm). At these high concentrations it is not possible to identify individual G20 strands.

At lower G20 concentrations, individual G-quartet formations become clear as evident in Figure 10 C. It is important to note that these lower concentrations are consistent with experimental values. The quartets have an average length of 70±30nm still have an average, uniform height of 1.9±0.1nm. The width of the G-wire images appear wider due to the geometry of the AFM probe (Vesenka et al., 1992). Though some higher order structures (thick arrow) are formed the majority of the G-quadruplex structures appear to take on the more common rod-like appearance (thin arrow). The cross section in Figure 10 D (black line with colored arrows in Figure 10 C again illustrates the uniformity of height in all G-quadruplex formations. Based on previously published work, we can predict that the G-quadruplex structure being formed by G20 is most likely a G-wire (Marsh et al., 1995). G-wires can be easily identified by their rod-like structure and consistent length and diameters. Our measurements are consistent with a G-wire confirmation, so we can conclude that the G20 molecule adopts a G-quartet configuration and can assemble into structures most closely resembling G-wires, see Figure 11 for a schematic diagram of the predicted structure.

To start to elucidate structural characteristics of the G20-Htt interaction, AFM experiments were performed on GST-Htt 171 (Q58) alone and mixed with G20. Evident in Figure 14, samples of GST-Htt1-171(Q58) appear as small unorganized structures when prepared and visualized according to the protocol outlined in materials and methods. These small, irregular structures that appear immediately after samples were prepared can be visualized in Figure 14 A. A cross section (Figure 14 B) indicates that these structures present themselves with a uniform height (0.5±0.1nm) but with a variety of shapes; elongation being typical for larger molecules. A volume analysis macro was developed for Image SXM (a variation of NIH Image designed to handle data files from SPM manufacturers). This software was used to measure 283 molecules, the distribution of which can be seen in Figure 14 C. Samples present without any incubation time (immediately following centrifugation) reveal a volume analysis with a mean value of $23nm^3$ with standard deviation of $23nm^3$ (N=283). No species were recorded larger than $200nm^3$.

The samples were then allowed to incubate for 48 hours at room temperature before imaging. The 48 hour samples appear very different. These complexes are larger and have a more "raft-like" appearance on the substrate in a variety of sizes that is evident in Figure 14 D**.** A cross section (Figure 14 E) reveals that these aggregates continue to have a consistent height (0.5±0.1nm). The volume analysis was performed on these samples as well, and the results evident in Figure 14 F, reveal that the aggregate structures have volumes with a mean size of $69nm^3$ and standard deviation of $71nm^3$ (N=283). Nine aggregates had a volume larger than $200nm^3$, the largest

being 583nm^3. These species on average are three times the size of the protein sample taken from the supernatant immediately after centrifugation and have a variety in size. The self-assembly of the GST-Htt1-171(Q58) is likely driven by presence of polyglutamine tracts within the protein and reflects mutant huntingtin aggregation. We predict that the images underestimate the actual sizes of the aggregate species due to possible disruption during AFM sample preparation. Figure 15 C is an image of a sample reaction in which G20 DNA was added at a concentration of 15 μM and allowed to incubate for 10 minutes with GST-Htt1-171(Q58) at the same concentration used in the biochemical assay. The sample was then diluted 1/10 in reaction buffer and immediately adsorbed onto freshly cleaved mica for AFM imaging. Again, the dilution aids in the identification of distinct aggregates and G-wires. Of interest is that every G-wire has a GST-Htt1-171(Q58) clump surrounding it, but not all protein "aggregates" are associated with G-wires. This image provides visual evidence of an interaction between GST-Htt 1-171 (Q58) and G20.

The cross section in Figure 15 B reveals a consistent height of the huntingtin fragment protein (0.5±0.1nm) contained within the aggregates. The cross section in Figure 15 D supports the argument that there is an interaction between G20 and Htt because the cross sections of the G-wires reveal an approximate height of 2.4±0.1nm which differs from the previous experiment in figure which show that the G20 quadruplexes alone have a uniform height of 1.9±0.1nm. The protein alone seen in Figure 15 B also has a height of 0.5±0.1nm (cross section not shown). We can predict that G20 forms its G-wire conformation first and then attracts the aggregating

huntingtin fragment, because the orientation of the G-quadruplex DNA on mica is identical to that of previous experiments.

3.3.4 Aggregation Time Course

To assess if G20 interacts with a unique species that is transient during the aggregation process we conducted an aggregation time course before performing biotinylated olignucleotide pull down analysis. The samples were incubated for various times up to 18 hours, enabling aggregation to take place in the absence of G20. At the specified time point, 15 μM G20 was added to the reaction mixtures. The samples continued to incubate in the presence of G20 for an additional 18 hours. By design, these reaction mixtures varied only in the time that fragment protein aggregation took place while the incubation time with G20 was the same for each point. The biotinylated oligonucleotide pull down assay was continued at that point by adding streptdavidin beads to the samples, which was followed by protein elution, SDS PAGE and Western blotting. Figure 13 illustrates that at the middle concentration of GST-Htt 1-171, G20 preferentially binds a protein species that is present at the four hour time point. This may indicate that the interaction between G20 and mutant huntingtin is specific to a species along the pathway of aggregation that occurs at the four hour time point.

We performed this experiment at three different concentrations; (0.5, 1.0 and 1.5 μg/μl) because aggregation of GST-Htt 1-171(Q58) is a dynamic process that is concentration dependent. The controls for the experiments presented in Figure 13 were samples that were incubated for 18 hours not in the presence of oligonucleotide,

so they can be considered representative of aggregate species that are present in solution at the time when the oligonucleotide is presented to the reaction mixtures at 18 hours. As discussed in chapter 1, mutant huntingtin becomes insoluble by SDS and boiling as it oligomerizes and continues to aggregate (Kazantsev et al., 1999). The absence of protein in a control well on the blot indicates that the sample has aggregated past the SDS soluble state. Evident in Figure 12 at the two lower concentrations, soluble control proteins are identifiable in the blot. At the highest concentration (1.5 μg/μl); no soluble protein is visible in the control lane. This is most likely due to an accelerated rate of aggregation in the samples containing a higher concentration of protein. Whereas the apparent increase in sample size in the lower concentration (0.05 μg/μl) control is due to a lag in aggregation rate. At the 1.0 μg/μl concentration we see a preferential binding by G20 of a species present in the four hour time point sample, and degeneracy in binding from that point on. However, in both the lower (0.5 μg/μl) and higher (1.5 μg/μl) concentrations we do not see binding of GST-Htt 1-171 (Q58) by G20 at any time point, evident by the lack of protein on the blot.

3.4 Discussion

The circular dichroism analysis of G20 confirms what was already suspected from previous work (Schwartz et al., 2008; Skogen et al., 2006) that this G-rich oligonucleotide forms a very stable secondary structure characteristic of a G-quadruplex. Increasing the temperature of the sample, even to the point of boiling,

does not disrupt the stability of the quartet structure. This may indicate that there is a very strong driving force for G-rich oligonucleotides, G20 in particular to preferentially form into that type of secondary structure. This is supported by the fact that upon sample preparation for Atomic Force Microscopy (AFM) experiments we discovered that G20 spontaneously forms G-quadruplex structures without the need for stabilizing ions provided by sample buffer which is unique from other G-quadruplex forming molecules (Marsh et al., 1995).

The AFM analysis of G20 alone reveals a great deal about the structural nature of this molecule. Not only does it confirm that a G-quadruplex structure is being formed, but we can make predictions that it is most likely forming what is known as a "G-wire" based on the uniformity of the measurements and cross sections taken. The G-wire structure involves the complexation of 4 separate oligonucleotides into a long tube like quartet structure with the guanosine bases of each strand participating in Hoogsteen bonds with an adjacent strand (Marsh et al., 1995; Neaves et al., 2009). Figure 11 provides a schematic of the predicted structure of G20. At high concentrations, it most likely forms complexes with other G-wire strands so you get branching such as that depicted in Figure 11 A. Based on cross section measurements, and the appearance of the molecule in the AFM images, at the experimental (lower) concentrations we can predict that single G-wire conformations are being formed by complexes of four strands of G20. These strands would stack in a linear fashion, to appear like the schematic on Figure 11 B. Delineating the precise structure of G20 as a G-wire is very important for possible therapeutic applications down the line. If we

know the structure of the active molecule, it may be possible to design drugs through structural chemistry to take on a similar conformation, which will be active but easier to deliver to therapeutic targets. Determining G20 stability is also important for future applications. If the specific G-wire secondary structure is necessary for its physiological activity, then it is vital that it is maintained.

The AFM analysis of G20 with GST-Htt 1-171(Q58) supports our hypothesis that an interaction is taking place between the protein and oligonucleotide. The orientation of G20 on mica is the same as it is when imaged alone, this tells us that the G-wires probably form first and are not impeded by the presence of GST-Htt 1-171 (Q58). It appears that GST-Htt 1-171(Q58) is clumping around the G-wire DNA and that in representative images there seems to be less visible protein in the field. This may be an indication that aggregate inhibition has taken place, or that all soluble GST-Htt 1-171 (Q58) clumps around the G-wire molecule. It is also important to note that the protein we see interacting with the G-wires is not in a monomeric form, further support for the possibility that an oligomeric species may be preferentially bound by G20. In regards to all of the AFM data, it is necessary to remember that we are taking a static glance at a dynamic process and that all imaging is conducted in the presence of mica. So although this information has value in itself, it is necessary to evaluate this interaction in solution over time.

The biotinylated oligonucleotide pull down experiment discussed in Chapter 2 and shown in Figure 6 revealed that G20 does exhibit a direct binding relationship to GST-Htt 1-171(Q58). The fact that the binding occurs at a specific concentration of

protein reproducibly was interesting. It might indicate that the binding interaction is dependent on the process of mutant huntingtin aggregation, meaning that a specific species along the course of aggregation is preferentially bound by G20. To investigate this further we performed an aggregation time course of GST-Htt 1-171(Q58) in conjunction with the biotinylated G20 pull down assay. The results show that the preferential binding species occurs reproducibly in the four hour time point. This is additional evidence that G20 does not intervene at the monomer stage, but at a later time point of aggregation, possibly an oligomeric species where it is able to bind and prevent the complexation into a macro aggregate. It is also interesting to note that G20 interacts with wild type huntingtin at most concentrations and time points tested as evident in the *in vitro* and *in vivo* experiments conducted. This might possibly mean that G20 actually has an affinity for the native structure of huntingtin, and that during the process of aggregation it is able to block the conversion from a "native – like" structure to a toxic oligomer. There is also the possibility that G20 binds preferentially to a specific region of huntingtin, such as the poly-glutamine tract. This region may be available in the native conformation of wild type huntingtin, but in mutant huntingtin is only exposed early on in the aggregation process and then is masked as that process continues, which is consistent with several models of mutant huntingtin aggregation (Nekooki-Machida et al., 2009; Thakur et al., 2009). As discussed previously, it has been proposed that the toxic species of mutant huntingtin is a soluble, oligomeric species or "micro aggregate" (Legleiter et al., 2009; Miller et al., 2005). Other than identifying certain "toxic species" with recently developed

antibodies; the ability to isolate and study this species has so far eluded the field. It may now be possible to use G20 as a tool to "trap" and purify these intermediate species so that their conformation and toxicity can be studied.

The time course experiments conducted at different concentrations shown in Figure 12 support the specificity of G20 binding to a intermediate aggregate species. At the lower concentration (0.5µg/µl), a lot of protein is seen on the blot in the control lane, representing that after 18 hours of incubation the majority of the population is not highly aggregated. At the higher concentration (1.5 µg/µl), no protein appears in the control lane, indicating that after 18 hours of incubation, all of the sample has aggregated past the point of being SDS soluble. In both of these cases we do not see binding by G20 over an 18 hour time course, and a possible explanation may be that in the lower concentration samples, the protein has not aggregated enough to hit the "optimal binding species". The exact opposite has happened in the higher concentration samples, the aggregation commenced too quickly for the "optimum binding species" to be trapped by G20. This is further evidence that the interaction of G20 and huntingtin is dependent on the aggregate species. A schematic diagram illustrating this point is presented in Figure 13.

Figure 9

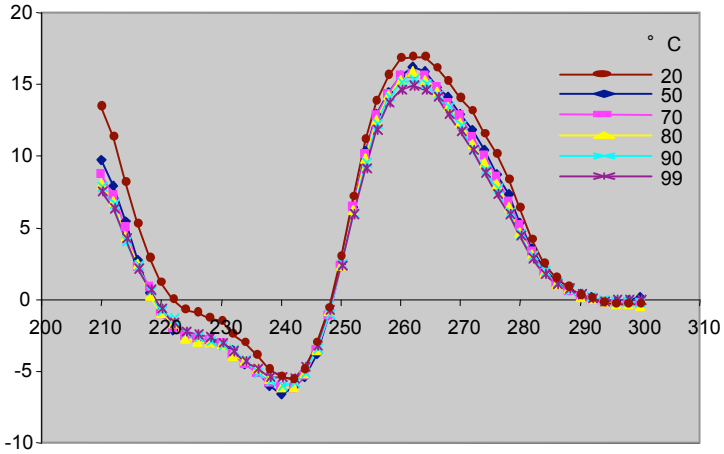


Figure 9: Analysis of G20 stability by circular dichroism

The spectral pattern of G20 at 15 μM was examined over a temperature range of 24°C to 99°C. The standard spectral pattern for a G-quartet is found with a maximal ellipticity at 264nm and a minimum ellipticity at 240nm and the pattern remains stable as the temperature increases.

Figure 10

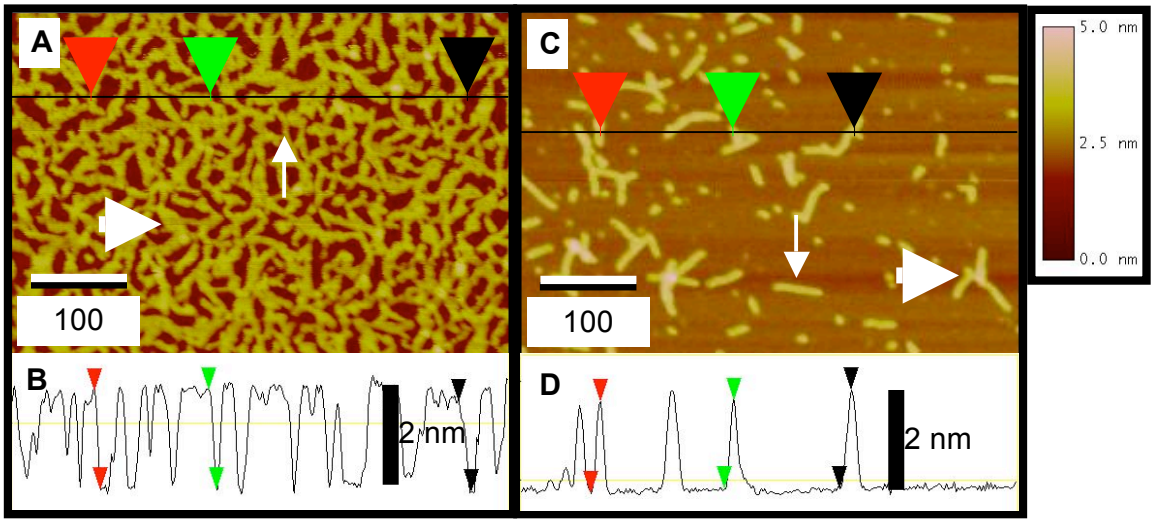


Figure 10: Analysis of G20 by atomic force microscopy.

A. 225µM G20 DNA in water, adsorbed onto freshly cleaved mica, incubated for 10 minutes and flash dried with dry nitrogen. Average height 1.9±0.1nm. G20 samples at this concentration can interact to form higher order structures (horizontal thick arrows). The vertical thin arrow represents incomplete quadruplex formation. Tip speed 0.5(m/s, diamond-like carbon whisker tip, resonant frequency 165kHz. **B**. Cross section of typical G-wires (black line Figure c) indicating uniform height of 1.9nm. **C**. Representative image of 22.5µM G20 DNA in water, adsorbed onto freshly cleaved mica, incubated for 10 minutes and flash dried with dry nitrogen. Average length 70±30nm, average height 1.9±0.1nm. Some higher order structures (thick arrow) are formed with a rod-like appearance (thin arrow). False color height scale at far right is same for all figures. Tip speed 2.0(m/s, diamond-like carbon whisker tip, resonant frequency 306kHz. **D**. Cross section of typical G-wires (black line Figure 1 E) indicating uniform height of 1.9nm.

Figure 11

A. **B.**

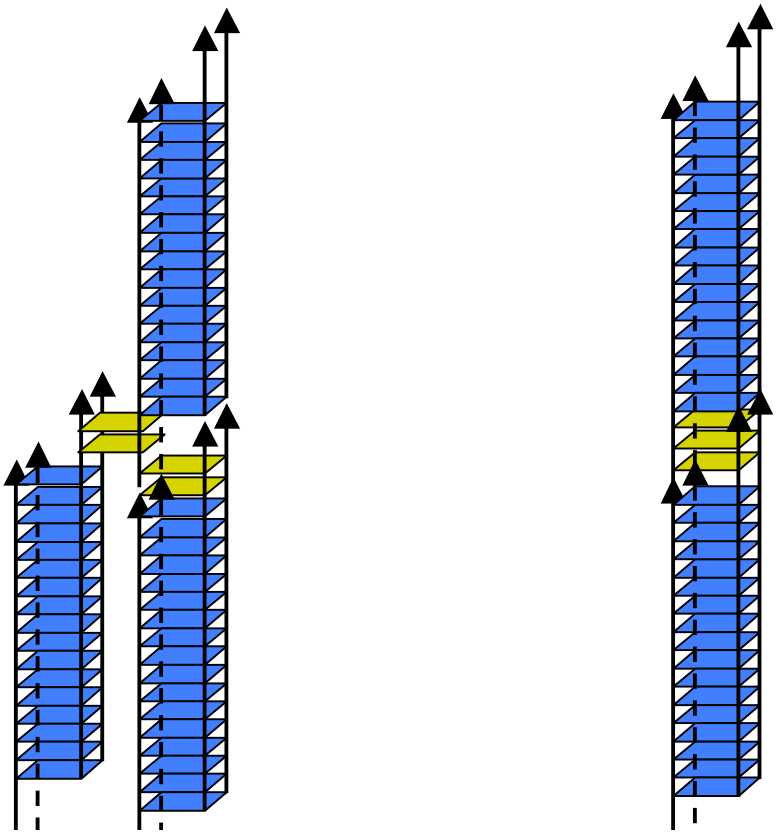


Figure 11: Schematic illustration of proposed G-wire structure formed by G20.

A. At higher concentrations, G20 can form branched structures illustrated here. **B.** At lower concentrations, AFM evidence is consistent with the G-wire depicted here.

Figure 12

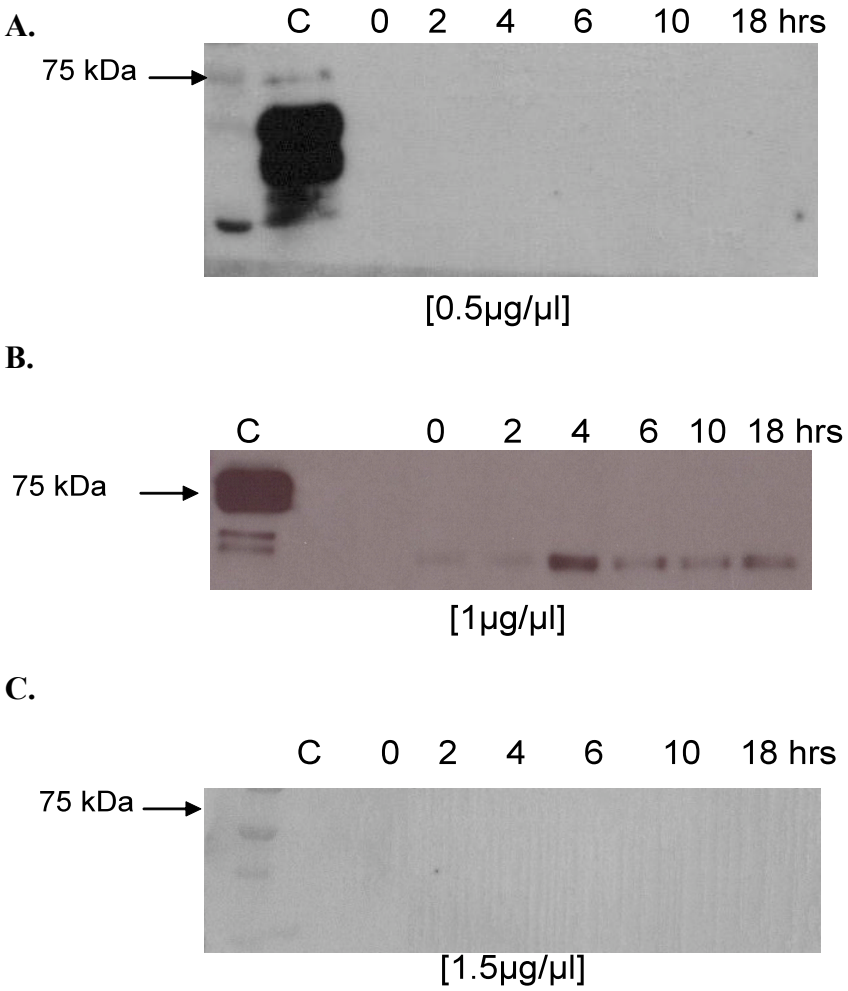


Figure 12: Time course analysis of G20-Htt 1-171 (Q58) interaction by a biotinylated oligonucleotide pulldown assay.

Samples of Htt 1-171 (Q58) at three concentrations were allowed to incubate in the absence of oligonucleotide for the time specified, and then in the presence of biotinylated G20 for 20 hours. The controls represent samples incubated in the absence of oligonucleotide for 18 hours and then not subject to streptavidin bead incubation. Interaction with G20 was assayed by "pulling down" the biotinylated oligonucleotide with streptavidin beads and then analyzed by Western blotting for Htt 1-171. The presence of protein on the blot indicates a direct interaction with G20.

Figure 13

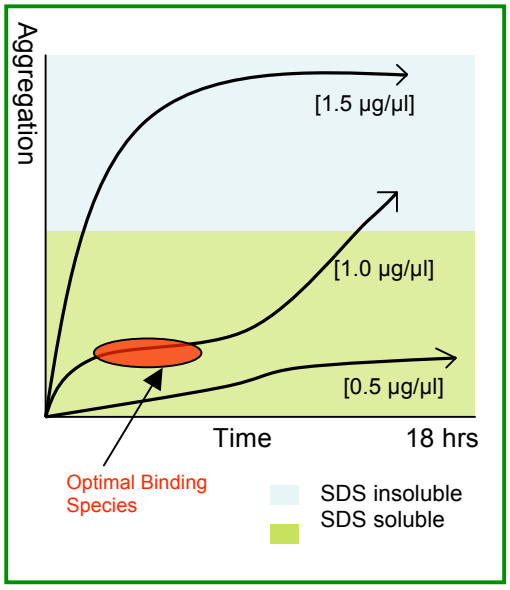


Figure 13: Schematic overview of G20 binding to GST-Htt 1-171 (Q58)

As evident in Figure 12, G20 binds preferentially to a species of Htt-1-171 (Q58) that is present after approximately 4 hours of aggregation with samples at a 1.0µg/µl concentration. G20 does not exhibit a binding interaction in identical experiments conducted with higher (1.5µg/µl) and lower (0.5µg/µl) concentrations of Htt-1-171 (Q58). We propose that the preferred binding substrate of G20-Htn binding is that an early species of Htt-1-171(Q58) along the pathway of aggregation. This "optimal binding species" appears at approximately four hours of Htt-1-171 (Q58) aggregation when the concentration is 1.0 µg/µl. At the higher concentration, huntingtin aggregation occurs too quickly for G20 binding to occur and the species is hidden; and at lower concentrations aggregation occurs too slowly, and the "optimal binding species" may never form during the 18 hour time period. Data from Figure 5 indicate that aggregation in the [1.5µg/µl] samples, occurs quickly because the control sample is SDS insoluble after 18 hours of incubation. This observation may indicate that larger aggregate complexes have formed, whereas in the lower concentration samples the controls still remain SDS soluble.

Figure 14

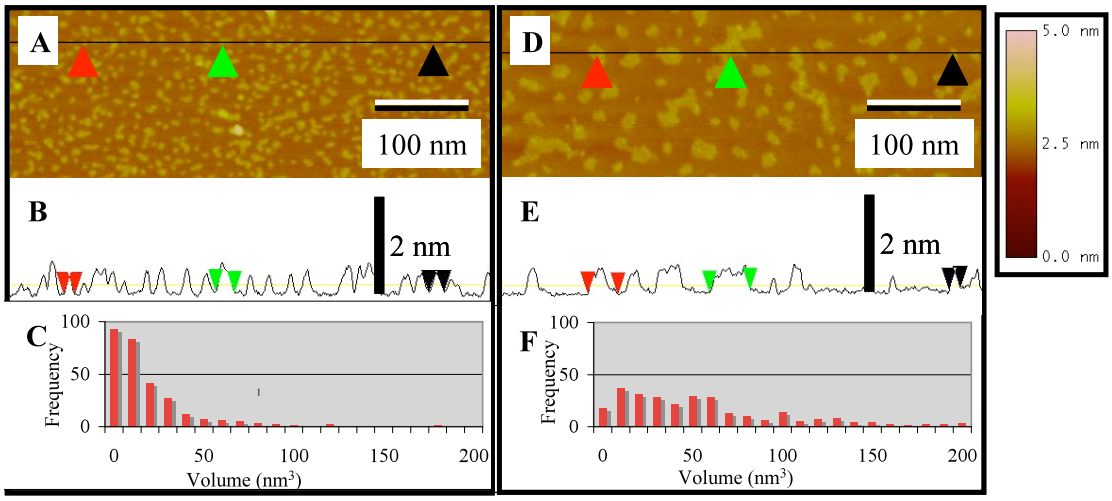


Figure 14: AFM analysis of GST-Htt 1-171 (Q58) at 0 and 48 hours

A. AFM image of samples of GST-Htt 1-171 prepared at 0.75µg/µl and diluted at room temperature 1/10 in reaction buffer and adsorbed onto freshly cleaved mica for 10 minutes, rinsed with 1mL deionized water and flash dried with dry nitrogen for AFM imaging. Tip speed 1.0(m/s, diamond-like carbon whisker tip, resonant frequency 316kHz. **B**. Cross section of the black line in Figure 6 A. Note consistent height (0.5±0.1nm) and small widths of Htt early in aggregation process. **C**. Volume distribution of aggregates at 0hr incubation at room temperature. The Htt 1-171(Q58) aggregates into volumes with a mean size of 23nm3 and standard deviation of 23nm3 (N=283). No aggregates were recorded larger than 200nm3 . **D**. AFM image of Htt incubated for 48hr at room temperature before AFM sample preparation. Tip speed 1.0(m/s, diamond-like carbon whisker tip, resonant frequency 275kHz. **E**. Cross section of the black line in Figure 6 D. Note consistent height (0.5±0.1nm) and larger widths of Htn later in aggregation process. **F**. Volume distribution of aggregates at 48hr incubation at room temperature. The Htt 1-171 (Q58) aggregates into volumes with a mean size of 69nm3 and standard deviation of 71nm3 (N=283). Nine aggregates had a volume larger than 200nm3, the largest being 583nm3.

Figure 15

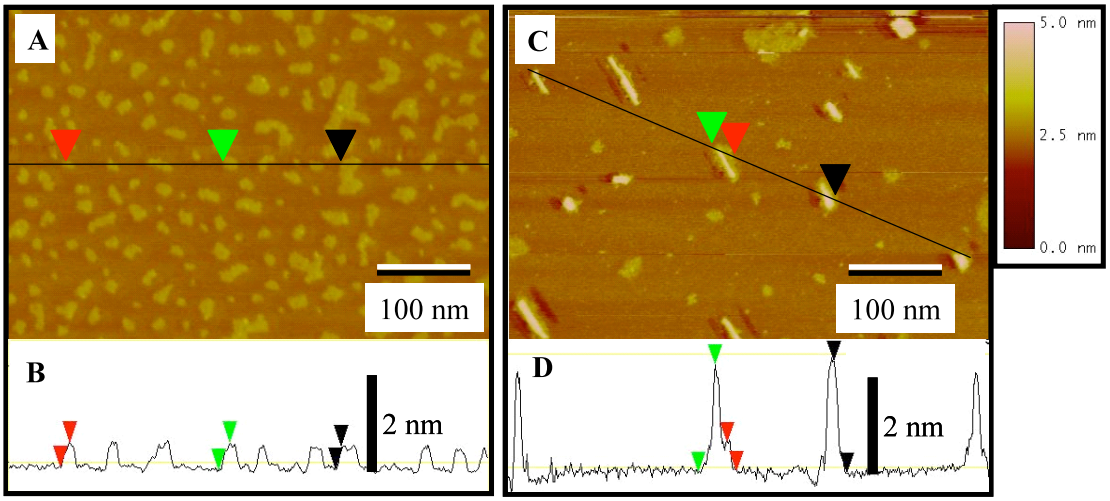


Figure 15 AFM Analysis of GST-Htt 1-171 (Q58) and G20

A. AFM image of GST-Htt 1-171 (Q58) aggregates. Samples prepared at a concentration of 0.75μg/μL and diluted 1/10 in reaction buffer and adsorbed onto freshly cleaved mica for 10 minutes, rinsed with 1mL deionized water and flash dried with dry nitrogen. Tip speed 1.0μm/s, diamond-like carbon whisker tip, resonant frequency 275kHz. **B.** A cross section of Figure a (black line with colored arrows) indicates an average height of about 0.5±0.1nm for the aggregates. Three randomly selected aggregates are identified. **C.** AFM image of Htt 1-171 (Q58) and G20 G-wire DNA. After eight hours of incubation the supernatent was diluted to 0.71 μg/μL in 15μM G20 in reaction buffer for ten minutes. This sample was then diluted 1/10 in reaction buffer and adsorbed onto freshly cleaved mica for 10 minutes, rinsed with 1mL deionized water and flash dried with dry nitrogen. All G-wire DNA is found to interact with Htt 1-171 (Q58) aggregates, as can be seen from the cross sections. Free aggregates also found visible over the surface and look like the "mats". Tip speed 2.0μm/s, single wall carbon nanotube tip, resonant frequency 298kHz. **D.** Total height of G-wire DNA 2.4±0.2nm (green and black arrows). Shoulders (red arrows) of G-wires show aggregates of height 0.5±0.1nm.

Chapter 4

IN VIVO IMPLICATIONS OF HUNTINGTIN-G20 INTERACTION

4.1 Introduction

Huntington's Disease has a fairly complex pathogenic process. Although the exact disease causing mutation is known, the direct link from mutation to cell death is unknown. For this reason, it is important to have good *in vivo* models to test potential therapeutic molecules on specific targets. The S6 and S6-5 Tet-off PC12 cell lines used previously serve as a good starting point to test the effect of G20 *in vivo*. These lines can be simultaneously differentiated and induced to express full length mutant (Q126) and wild type (Q21) huntingtin over a period of 12-14 days in culture. During this time period, the expression of mutant huntingtin exhibits a lot of the characteristics associated with the pathogenic process of Huntington's Disease, such as caspase mediated cleavage of mutant huntingtin, aggregation of resulting N-terminal fragments and reduced cell viability (Ratovitski et al., 2007). *C. elegans* is also a very good *in vivo* model for poly-glutamine toxicity because of their small size, ease of maintenance and quick reproduction time. We received several lines expressing increasing lengths of a poly-glutamine fusion protein to yellow-fluorescent protein (YFP) as a gift from Dr. Morimoto, Rice Institute for Biomedical Research, Northwestern University, 2153 North Campus Drive, Evanston, Illinois 60208. These

lines exhibit poly-glutamine aggregation in an age and poly-glutamine dependent manner. They also exhibit a reduced motility phenotype at very high poly-Q number (80) (Brignull, Morley et al., 2006). For the purpose of this work, we sought to establish some preliminary *in vivo* evidence of the effect of a possible G20-huntingtin interaction. There are several mouse models available for Huntington's Disease, each with their own advantages and disadvantages. The ultimate and future goals of this work would involve establishing the effect of G20-huntingtin interaction on HD mouse development, HD pathogenesis and subsequent viability.

To test cell viability after treatment with G20, we utilized Tet-off PC12 cell lines, S6 and S6-5 expressing full length wild type (Q23) or mutant (Q126) huntingtin respectively. As discussed previously, work has been published that demonstrates that after 12 days of huntingtin expression the S6-5 (Q126) cell line exhibits decreased viability when compared to the S6 (Q21) cell line (Ratovitski et al., 2007). We transfected both the S6-5 (Q126) and the S6 (Q21) Tet-off PC12 cell lines with G20 and monitored viability over a period of 12 days of full length huntingtin expression and cell differentiation.

We also wanted to establish a true *in vivo* system for testing the effects of G20 treatment on viability, aggregation and phenotype. For this, we utilized several lines of *C. elegans* expressing increasingly expanded poly-glutamine tracts fused to yellow fluorescent protein, in order to establish a system in which G20 activity can be monitored. Aggregation in these *C. elegans* lines is age dependent and correlates with a threshold poly-glutamine expansion of 40 (Brignull, Morley et al., 2006). These

nematode lines exhibit a toxic effect of poly-glutamine expression with a decreased motility phenotype that can be measured by the nematodes' movement across an agar plate (Brignull, Morley et al., 2006). This phenotype is worse with an increased aggregation count and an expanded poly-glutamine number indicating there may be toxicity associated with the expression of the poly-glutamine protein itself. In the absence of the total protein context of huntingtin, we asked the question whether or not G20 would bind to the poly-glutamine fusion protein. To test if G20 would bind to the expanded poly-glutamine proteins expressed in this model, we performed biotinylated oligonucleotide pull down experiments in an *in vitro* protein extract assay in a total *C. elegans* population, and later in each line at various points of development. We then established transfection parameters for this model system and monitored binding *in vivo.*

4.2 Material and Methods

4.2.1 Tet-off PC12 Cell Viability Assay

S6 and S6-5 Tet-off PC12 cell lines were plated at low density in normal growth media(high glucose DMEM, 10% horse serum, 5% fetal bovine serum (FBS), 1% L-glutamine, 1% penicillin/streptomycin, 50mg/mL Geneticin, 50mg/mL hygromycin B) on rat tail collagen I coated plates (Invitrogen). The cells were allowed to adhere to the plate for 24 hours, and then were transfected with 1µg oligonucleotide per well with lipofectamine 2000 transfection (Invitrogen) reagent following their protocol, or mock transfected with just lipofectamine treatment. The cells incubated in the transfection media (lipofectamine 2000 + optimem) for

approximately 12 hours. The transfection media was removed, and the cells were placed on either expression and differentiation media (low glucose DMEM, 1% horse serum) or just differentiation media (low glucose DMEM, 1% horse serum, 200ng/mL doxycycline) and supplemented with 50ng/mL nerve growth factor every 48 hours. The cells were maintained in the differentiation media for the amount of time specified (up to 12 days). To measure cell viability, an MTT assay is performed. The media is removed from the cells and a 1X concentration of MTT working solution is added to each well, and the cells are incubated at 37°C for 2 hours. After 2 hours, the MTT working solution is removed from the cells, and they are treated with DMSO for 30 minutes while shaking. Once complete, 200 µl of solution from each well is transferred to a 96-well plate, and the absorbance is read on a Wallac 1420 Victor micro-plate reader. The experiments are done in triplicate, and read on the plate reader in triplicate.

4.2.2 *C. elegans in vitro* binding experiments

The *C. elegans* were maintained on 2% agar plates that contained nematode growth media (NGM) that were seeded with *E. coli* strain OP50 for nutrients at 20°C. For the *in vitro* binding experiments mixed stage populations of nematodes were harvested from 100 mm NGM agar plates by rinsing with water. Total protein was extracted by suspending the nematode populations in hypotonic buffer + 6.8% sucrose (10 mM HEPES, pH 7.9 @ 4°C. 1.5 mM MgCl2. 10 mM KCl. 0.5 mM DTT) for 15 minutes on ice. The nematodes were spun down in 15 mL conical tubes and small glass beads were added to the pellet. The pellet was frozen at -20°C for 5 minutes,

and then manually crushed, frozen again and crushed three times. The pellets were resuspended in hypotonic buffer (10 mM HEPES, pH 7.9 @ 4°C. 1.5 mM MgCl2. 10 mM KCl. 0.5 mM DTT) and allowed to incubate on ice for 30 minutes. The samples were centrifuged at 13,000 rpm for 10 minutes to remove cellular debris. Total protein was quantified with BCA reagents and read on a micro-plate reader.

Equivalent amounts of total protein were incubated with 20 µM biotinylated oligonucleotide for 18 hours at 4°C while shaking. The samples were incubated with a 50% slurry of streptavidin beads(Invitrogen) for 2 hours at 4°C. The beads were recovered by low speed centrifugation, and washed with Tris-EDTA buffer, 1X binding buffer(10 mM Tris-HCL pH 8.0, 40 mM KCL, 1 mM DTT, 6% glycerol, 0.05% NP-40) and 1X Phosphate buffered saline respectively. Oligonucleotide and bound protein was eluted by boiling in 2X sample buffer for 5 minutes. The samples were analyzed by Western Blotting.

4.2.3 *C. elegans in vivo* experiments

C. elegans mixed stage populations were maintained on NGM agar plates for several days until they contained a lot of adult worms and laid eggs. The populations were harvested and bleached with a solution of 15% 5 N NaOH, 10% bleach and monitored until all adult worms had disintegrated. The resulting eggs were washed twice in M9 buffer (3% KH_2PO_4,6% Na_2PO_4, 5% NaCl, 1 mM $MgSO_4$) and incubated overnight in M9 buffer while shaking. The L1 stage nematodes were cultured in liquid Nematode Growth Medium approximately 14 hours until L2 stage was reached. The L2 nematodes were washed twice with water and resuspended in trehalose buffer

(272mM trehalose, 7mM KH_2PO_4, 1 mM $MgSO_4$). The samples were placed in electroporation cuvettes with 10 μL, 100 mM oligonucleotide and electroporated for 20 ms at 250 volts. The surviving nematodes were allowed to recover on agar plates containing NGM and OP50. They were harvested at the L4 developmental stage and either imaged by confocal microscopy or the total protein was extracted as described previously. The biotinylated oligonucleotide pull down assay was then performed, or the agarose gel electrophoresis for resolving aggregates (AGERA) assay as described previously.

4.3 Results

4.3.1 Viability in Tet-off PC12 cell lines expressing full length huntingtin with G20 treatment

We have established that G20 can bind to full length huntingtin in an *in vitro* total protein extract of a Tet-off PC12 system expressing full length huntingtin. We wanted to see if treating the cells with G20 would improve cell viability over time with the expression of mutant huntingtin. After differentiation, the Tet-off PC12 cells are resistant to transfection by lipofection. Before performing viability assays, we needed to establish transfection conditions under which G20 could be efficiently introduced into the cells. We experimented with several different parameters, and found that transfecting the cells before they were differentiated produced the most robust results. To establish how long G20 is present in the cell after transfection, we transfected cells with lipofectamine 24 hours before they were placed in induction and differentiation media, and monitored the presence of a HEX labeled G20 in the cell

lines over the time course of 10 days. We found that G20 remained within the cells for 6 days at robust levels. After the 6th day the HEX signal diminished slowly, indicating that the oligonucleotide was being processed out, or the fluorescent label had faded. For the purpose of this study, we considered that G20 was present in the cells up to 6 days after transfection.

For the viability experiments, the cells were transfected with G20, 24 hours before being induced to express huntingtin and placed in differentiation media where they remained for up to 12 days. An MTT assay was performed, and the results indicate that G20 does improve the cell viability of PC12 cells expressing mutant huntingtin over those mock transfected with G20, with the biggest effect occurring at 6 days of mutant huntingtin expression. That time point is significant because it is the last time point at which G20 is known to be present in the cell. It is also a reasonable amount of time for mutant huntingtin expression to begin to have a toxic effect on the cell as evident by the control samples. However, at this time point there is an overall increase in cell viability in samples treated with G20, with or without huntingtin expression. This may indicate that the positive effect of G20 is not directly associated with mutant huntingtin. There is also a significant positive effect on cell viability at the 12 day time point on cells treated with G20 when compared to mock transfected cells expressing huntingtin. This positive effect by G20 may be related to lasting effects of mutant huntingtin-G20 interaction. Viability in cells expressing wild type (Q21) full length huntingtin did not vary significantly from cells transfected with G20 and those mock transfected. This indicates that at least in this system there doesn't

appear to be toxicity associated with G20 alone. The overall trend of decreased viability over time, even in non-induced cells is due to maximum confluency being reached around day 8 or 10 of plating.

4.3.2 Establishing G20 binding in a *C. elegans* model of poly-glutamine toxicity

C. elegans is emerging as an excellent model for many genetic diseases for several reasons. They are small, easy to maintain and reproduce quickly. They have many human homologs, their cell fate mapped and are great for doing genetic experiments (Brignull, Morley et al., 2006). *In vitro* biotinylated oligonucleotide binding experiments were performed in total protein extracts in each poly-glutamine nematode line in a mixed population of developmental stages. There appeared to be robust binding in each line, with a decreased amount in Q40 nematodes. The binding experiments were then performed in each nematode line at each developmental stage (data not shown), which revealed ambiguous results. There was not a significant amount of binding in the early developmental stages. This could be due to the fact that it was difficult to extract enough protein from nematodes at earlier stages in development. There is also less expression of the poly-Q fusion protein at these stages, so a decrease in the amount of material available to bind. There was robust binding at later stages of development; however we also obtained a positive binding result in the Q0 nematode lines, which were supposed to be a negative control.

The next step was to perform binding experiments *in vivo*, but first we needed to establish a transfection protocol for introducing G20 into *C. elegans.* Several different parameters were tested, but the most robust transfection as screened by

fluorescence microscopy resulted from electroporation at 250 volts for 20 milliseconds. Representative images are presented in Figure 18. The *in vivo* binding experiments were performed at all the same stage of development in each nematode line, because electroporation of G20 was the most robust at the L2 stage. The results presented in Figure 16 indicate that *in vivo* binding occurs in all cell lines except Q0, with less binding as poly-glutamine number increases. However, these could not be reciprocated, and we later had the same issue with G20 binding in Q0 lines.

To correlate binding of poly-glutamine proteins *in vivo* with a disruption of aggregation, we sought to perform aggregation inhibition experiments in *C. elegans.* We did not see a robust inhibition of aggregation by just observing the expression of Q40-YFP in the presence of G20 over time. So we performed an AGERA assay on the extract of Q40 nematodes that had been electroporated with G20. We did see a decrease in the amount of intermediate aggregates via the AGERA gel, indicating a global effect on aggregate formation in poly-Q (40) *C. elegans.*

4.4 Discussion

The ability of G20 to inhibit mutant huntingtin aggregation could be of great therapeutic value. To utilize that value, the ability of G20 to bind mutant huntingtin and to inhibit aggregation must be correlated *in vivo.* There is significant debate about the toxic nature of aggregates, so just inhibiting aggregation may not be beneficial in an HD patient. The nature of aggregates formed, or prevented from formation and the mechanism of interaction between G20 and huntingtin might not be what is necessary

to alleviate mutant huntingtin toxicity. For this reason, any potential therapeutic must be validated and re-validated in several models of Huntington's Disease.

In the Tet-off PC12 S6 and S6-5 cell lines we see a significant increase in cell viability in cells treated with G20 while expressing mutant huntingtin. This is a positive, but preliminary result. It would be beneficial to repeat this experiment with repeated treatments of G20 to see if it could ameliorate toxicity over an even more extended period of time.

To further validate the activity of G20, we sought an *in vivo* system in which phenotypic effects could be measured. We obtained several lines of *C. elegans* expressing increasing repeats of a poly-glutamine stretch fused to YFP. These lines produce visible aggregates that form at a threshold poly-Q repeat of 40 residues. They exhibit toxicity in an age and poly-glutamine dependent manner which mirrors poly-glutamine expansion disorders in humans. We were able to deliver G20 to these nematode lines via specific electroporation conditions. However, there is higher levels of toxicity associated with increased concentrations of G20, more viable delivery methods would be of great value. We also, established binding of G20 to the poly-glutamine fusion proteins by an *in vitro* biotinylated oligonucleotide pull down assay. This validated the use of the model in further studies of G20 activity, and gave us an indication that G20 has an affinity for the poly-glutamine region. It is unlikely that the poly-Q fusion proteins have the exact same mechanism of aggregation as a fragment or full length mutant huntingtin (Thakur et al., 2009). The common motif in both huntingtin and the *C. elegans* poly-glutamine fusion proteins is the poly-glutamine

region itself. This reveals the possibility that an exposed poly-glutamine region may be necessary for G20-htt binding, rather than a specific conformer of aggregate. Studies with purified poly-glutamine peptides would be valuable to elucidate the exact structure or motif necessary for binding.

Establishing binding in the poly-glutamine *C. elegans* model is encouraging, however further, *in vivo* and *in vitro* binding experiments conducted in these lines revealed ambiguous results. It is of concern that the control line (Q0), exhibited positive binding to G20 in several experiments, and not consistently. This may indicate a non-specific interaction of G20 with the YFP protein, or possibly the Q0 lines were contaminated with another poly-Q expressing line. There is also a slim chance that the fusion gene was integrated in frame with a protein that has an affinity for G20. For future experiments, it might be of value to create or obtain a true HD model expressing increasing lengths of poly-glutamines within the context of the huntingtin protein.

To correlate the binding of G20 to a poly-glutamine expansion with aggregation inhibition, we followed aggregation of Q40 expressing *C. elegans* that had been electroporated with G20. We did not observe a robust difference in aggregation between treated and non treated samples by fluorescence microscopy experiments. This could be due to continuous over-expression of the Q40 protein that overwhelmed the amount of G20 present in the nematode. It could also be the inability to follow the amount of G20 present in individual nematodes. We know that under our electroporation conditions, there was heterogeneity in the amount of G20 taken up by

each individual nematode, so we could be missing a correlation between amount of G20 present and aggregation dynamics. To bypass that issue, we performed an AGERA assay to see if we could see an effect at the biochemical level. Evident from those results (Figure 18) we do see an effect on amount and size of intermediate aggregates present in samples from nematodes at two different stages in development. This may indicate that G20 has a global effect on inhibiting the aggregation of expanded poly-glutamine proteins. This is a positive sign for not only Huntington's Disease, but also for the eight other poly-glutamine expansion disorders discussed previously.

Figure 16

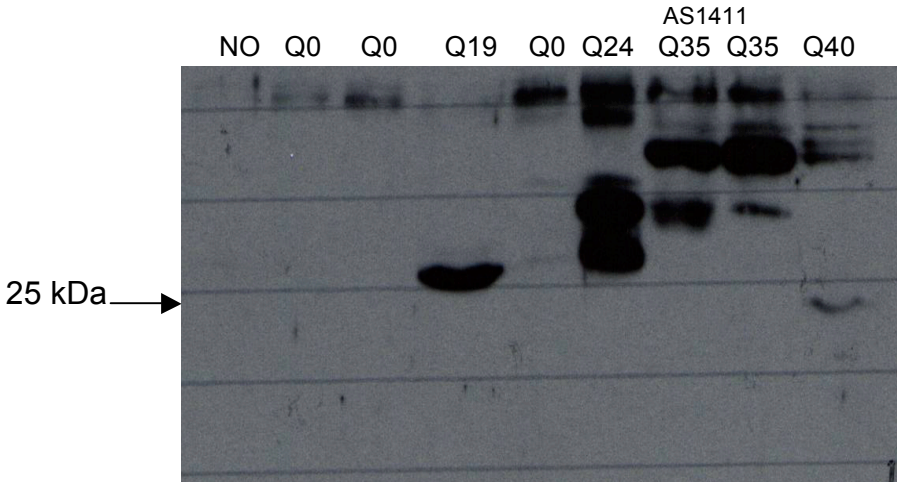


Figure 16: *In vitro* binding of G20 to poly-glutamine fusion proteins in *C. elegans*

Nematodes were plated on agar plates streaked with OP50 for nutrients and grown to confluency. The total nematode population of each strain was harvested and total protein was extracted. Equal amounts of total protein was incubated with 15 μM biotinylated olignoculeotide for 18 hours at 4°C. The biotinylated olignucleotides were pulled down in the pull down assay, and the bound protein was analyzed by Western Blot.

Figure 17

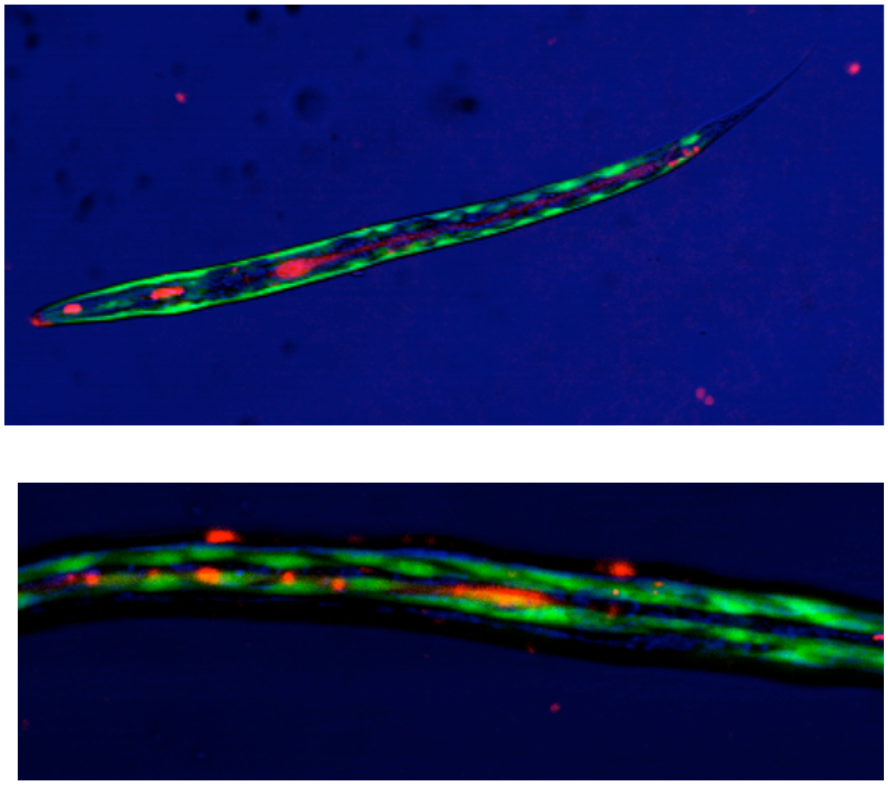


Figure 17: Representative images of Q0 *C. elegans* electroporated with HEX labeled G20

Q0 *C. elegans* were synchronized by bleaching. The L1 nematodes were plated on new agar plates with OP50 for nutrients, and allowed to develop to L2 stage. They were electroporated with 100 µM HEX-G20 in trehalose buffer for 20 ms at 250 volts. Transfected nematodes were imaged immediately by confocal microscopy(Green = YFP, Red = HEX).

Figure 18

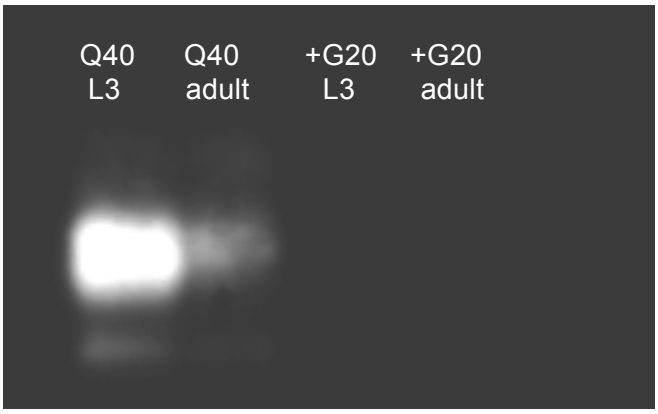


Figure 18: AGERA analysis of Q40 *C. elegans* electroporated with G20.

Q40 *C. elegans* were synchronized by bleaching. L1 nematodes were plated on new agar plates with OP50 for nutrients and allowed to develop to L2. At L2 stage the nematodes were harvested and electroporated with 100 μM G20 or mock transfected in trehalose buffer for 20 ms at 250 volts. The nematodes were allowed to develop to stage L3 and adulthood, and then harvested and total protein was extracted. The total protein was run on an AGERA gel as described previously and blotted for YFP.

Figure 19

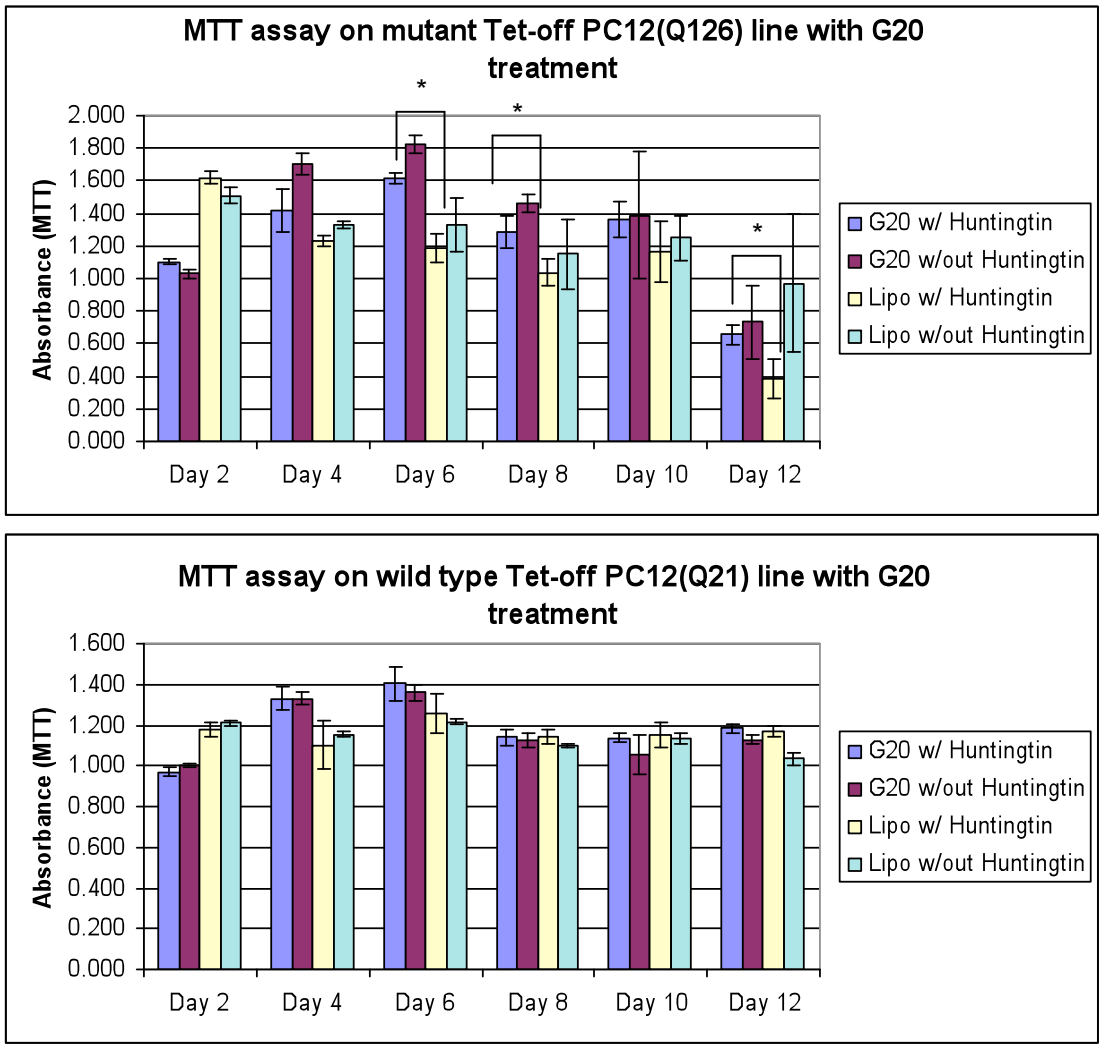


Figure 19: Tet-off PC12 viability with G20 treatment

Tet-off PC12 cell lines expressing full length huntingtin transfected with oligonucleotide before induction of protein expression. Expression was maintained up to 12 days, cell viability was measured by MTT assay. Lipofectamine treatment alone(lipo) was used as a positive control.
* $P < .005$ by T-test

Chapter 5

SUMMARY AND PERSPECTUS

Huntington's Disease is a devastating neurodegenerative disorder caused by an expanded CAG repeat in exon 1 of the disease causing gene, IT15. The resulting mutant protein, huntingtin contains an elongated poly-glutamine region that has a propensity to mis-fold and undergo a deleterious process of aggregation. This disease is genetic and is passed down in an autosomal dominant fashion. Approximately one out of every 10,000 Americans is affected with HD and thousands more are at-risk for inheriting the disorder (Kremer et al., 1992; MacDonald et al., 2003; Martin & Gusella, 1986). Symptoms appear later in life, and progressively worsen over the course of 15-20 years. Death is usually the result of a complication of the disease, not HD itself (MacDonald et al., 2003). This disorder can destroy families, negatively effecting generations at a time. Living at-risk for HD is a unique situation in which an individual must deal with the progressive illness and de-habilitation of an ailing parent while making their own life decisions about the 50 percent possibility they will inherit the disease as well. There is currently no treatment or cure for Huntington's Disease, so even though there is a genetic test available the outcomes of having a positive result can be devastating.

Symptoms of Huntington's Disease include cognitive dysfunction, personality changes, psychiatric disturbances and motor abnormalities. The most characteristic

symptom of HD is the dance-like movement and gait disturbances termed "chorea" (Cardoso, 2009). Motor symptoms persist over time, and eventually render the patient disabled and bound to a wheel chair. The most disruptive aspects of this disease early in the process are the vast personality changes that occur in the majority of patients (MacDonald et al., 2003). Instances of depression and suicide are elevated in HD patients as well. The motor abnormalities and cognitive changes are linked to the specific loss of medium spiny neurons in the striatum. The specificity of this cell death and the molecular pathways leading to death are unknown (Bauer & Nukina, 2009).

Although the genetic cause of Huntington's Disease was discovered in 1993 (MacDonald et al., 2003) there still exists a huge gap in knowledge about the molecular mechanisms of disease pathology. The function of wild type huntingtin remains to be elucidated, although there is evidence that it is a neurotrophic protein that is essential for neurogenesis during development (Duyao et al., 1995). It has also been linked to play a role as a molecular scaffold in cellular transport and possibly gene regulation. It is thought that loss of wild type function may play a role in disease pathogenesis (Zuccato et al., 2007). The most obvious pathology in post-mortem brains of HD patients as well as animal and cell culture models of Huntington's Disease is the presence of intracellular aggregates composed of mutant huntingtin and other cellular proteins (Ross & Poirier, 2004; Scherzinger et al., 1999; Wetzel, 2006).

A lot of controversy surrounds the role of aggregates in the disease process. There is evidence that neurons containing the most aggregates survive longer over the

course of the disease (Weiss et al., 2008). It has been proposed that aggregates are a defense mechanism of neurons to sequester mutant huntingtin and therefore reduce its toxic effects. Other hypotheses predict that aggregates are the disease causing culprit (Nekooki-Machida et al., 2009; Ratovitski et al., 2007; Thakur et al., 2009). It is possible that their large size causes a physical barrier in neurons that disrupts cellular transport and other vital processes. Aggregates are also known to sequester wild type huntingtin, transcription factors and other important proteins, whose loss of function might cause cellular stress and eventual death. There is a large amount of data which indicates aberrant transcription and gene deregulation in Huntington's Disease models (Jiang et al., 2006; Zuccato et al., 2007). The most likely story is that a little bit of everything is going on. Increasing evidence points towards the fact that a specific species of aggregate may be toxic to the cell and that this species is possibly an oligomer that occurs somewhere along the process of aggregation (Graham et al., 2006; Hatters, 2008; Legleiter et al., 2009). In this case, a macro-aggregate would have a protective effect by recruiting the toxic species and masking its effects, yet toxicity is still related to the process of aggregation.

Mutant huntingtin aggregation is a dynamic process that has proven difficult to study (Thakur et al., 2009; Wetzel, 2006). Huntingtin is a 350 kDa protein, and its large size has made it prohibitive to isolate and purify in its native form; making structural and functional studies almost impossible. Studies have shown that the first exon, containing the poly-glutamine region of the HD gene is enough to cause toxicity and symptoms mirroring HD pathology in several mouse models (Graham et al., 2006;

Ona et al., 1999; Schmitt et al., 1995). Also, a great deal of work indicates that the production of N-terminal fragments by caspase cleavage events is absolutely necessary for the aggregation process to ensue and for mut htt toxicity (Graham et al., 2006; Ona et al., 1999). Almost every HD model from cell culture, to yeast, *C. elegans* and mice exhibit aggregates with a huntingtin fragment reaching the threshold poly-Q number. For this reason, exon 1 and other fragments of the huntingtin protein have been used in aggregation modeling and toxicity studies (Brignull et al., 2006; Hatters, 2008; Hoffner, Soues, & Djian, 2007; Thakur et al., 2009).

The lab of R. Wetzel has used pure populations of synthetic poly-glutamine peptides to study aggregation for a long time. From previous data they have proposed a nucleated growth mechanism of mutant huntingtin aggregation (Wetzel, 2006). In this model, the expanded poly-glutamine repeat causes the rate limiting step of a mis-folded monomer, which is then able to interact with another monomer species and nucleate aggregation (Wetzel, 2006). Once the nucleation step has taken place, rapid aggregation ensues with the addition of native and mis-folded monomers as well as oligomeric complexes. However, this model was based strictly on studies conducted with a pure poly-glutamine repeat. As is the case for all poly-glutamine expansion disorders, the aggregation process takes place within a specific protein context. For this reason, Wetzel et al. began work on peptides containing the first 17 amino acids of huntingtin, and the poly-proline region immediately following the poly-Q region (Thakur et al., 2009). They found that the aggregation process was influenced by the structure of the amino-terminus. An expanded poly-glutamine region destabilizes the

compact nature of the first 17 amino acids. The destabilized N-terminus can then interact with the N-terminus of other peptides causing the formation of an oligomeric amorphous aggregate species. The poly-glutamine stretches then compact and form into a β-sheet type structure, which can rapidly recruit native and misfolded monomers and aggregate into an amyloid type macro aggregate. Although this work was done with synthetic peptides, it can easily represent the aggregation process of an exon one fragment, because further studies confirmed the N-terminus and poly-glutamine region to have the most influence on the aggregation process (Thakur et al., 2009).

Another interesting study was done by Nekooki-Machida et al on the toxic nature of different conformations of aggregates both *in vitro* and *in vivo.* They utilized thermodynamic techniques to promote the formation of different conformations of aggregates out of an exon 1 fragment of huntingtin, and then injected them into mammalian cells and monitored toxicity. They found that the conformation with less compact β-sheets and exposed poly-glutamine residues were more toxic to the cell than the more compact β-sheet conformation that masked the poly-glutamine regions. They contributed the toxicity to the availability of the glutamine residues to interact with other important cellular proteins. They also found that aggregates found in different regions of the R6/2 mouse brains had different conformations evident by thermal stability experiments. Interestingly enough, the region of the brain most affected by HD contained aggregates that were the least thermally stable, whereas aggregates found in brain regions less affected were more thermally stable. They predicted that the thermal stability was due to the fact that aggregates from the

striatum had more loops and turns, whereas the other regions of the brain contained aggregates composed of compact β-sheets.

This investigation supports the idea that a particular species, or conformation of mutant huntingtin aggregate is more toxic to the cell than others. In this study, they do not consider the possible toxicity of oligomeric or micro-aggregates, however they do provide evidence that the exposure of the poly-glutamine region may be responsible for aberrant protein interactions and cellular toxicity. This notion is also supported by studies done on antibodies predicted to bind to toxic conformers of mutant huntingtin. The majority of which are raised against and bind specifically to the poly-glutamine region of huntingtin (Legleiter et al., 2009; Miller et al., 2005). This is a further indication that blocking formation of the toxic aggregate may be therapeutically viable.

Previous data from our lab identified a molecule that was an effective inhibitor of mutant huntingtin aggregation in a biochemical screen (Skogen et al., 2006). The molecule was designed to be a single stranded oligonucleotide composed of 20 guanosine residues, termed G20. G20 is robust in inhibiting the aggregation of a GST fusion protein consisting of an exon 1 fragment of mutant huntingtin (Q58) as tested in an immunoblot assay developed by Wang et al.(J. Wang et al., 2005) Its activity was compared to other single stranded oligonucleotides composed of 20 adenine bases, 20 thymine bases and 20 cytosine bases respectively, which all exhibited no ability to inhibit aggregation. To determine if the specificity of G20 was related to secondary structure formation, circular dichroism analysis was performed on all four

oligonucleotides. Analysis of the results indicated that G20 presented a CD spectra characteristic of a G-quartet formation, which was thought to be necessary for its ability to inhibit aggregate formation (Skogen et al., 2006). G-quartet structures have been found in promoter regions of several genes, and are known to have various biological functions (Chang et al., 2004; Dapic et al., 2003; Verma et al., 2009). G-quartet forming oligonucleotides are currently being developed as aptamers for a variety of diseases, including several types of cancer (Bates, Laber et al., 2009; Schwartz et al., 2008; Soundararajan et al., 2009). In order to develop G20 as a possible aptamer therapeutic for Huntington's Disease, the purpose of this thesis work is to elucidate the mechanism by which G20 inhibits mutant huntingtin aggregation and to characterize that interaction.

We decided to start our investigation with the same system in which the activity of G20 was identified. This system utilizes an *E. coli* expression system to express a fragment of huntingtin consisting of the first 171 amino acids and containing either 58 glutamine repeats or 23 glutamine repeats. This fragment is fused to a glutathione S-transferase tag, which is used in batch purification of the protein by affinity to glutathione. The GST tag also maintains the mutant protein in a monomer form until cleavage via a thrombin cleavage site (J. Wang et al., 2005).

The first goal of this thesis was to examine the mechanism by which G20 inhibits aggregate formation in GST Htt 1-171(Q58) samples. Evident in Figure 3, a dose response of G20 demonstrates its ability to effectively block aggregation in the immunoblot essay developed by Wang et al. A downfall of this assay is that only

macro-aggregates can be visualized, as monomers and smaller molecular weight species pass through the membrane filter. We sought to visualize the effect of G20 on lower molecular weight aggregates by an Agarose Electrophoresis for Resolving Aggregates(AGERA) assay recently developed by Weiss et al. This technique utilizes an agarose gel run in the manner of an SDS-PAGE. As demonstrated by Weiss et al. it can be used to visualize aggregate species of an intermediate molecular weight that pass through the filter of the immunoblot assay (Weiss et al., 2008). Our results reveal a reduction in the amount of intermediate aggregates formed in samples incubated in the presence of G20. This is further evidence that G20 is able to inhibit mutant huntingtin aggregation. The positive control used was Congo Red, a compound that is known to inhibit aggregation. Evident by the AGERA gel, no intermediate aggregates were seen in the positive control. This may indicate the Congo Red prevents the formation of aggregates at the monomer level, whereas G20 may be acting at a later stage in the aggregation process. We also visualized the presence of monomers, dimers and trimers in samples incubated with G20 compared to non-treated samples in a Native Gel Electrophoresis experiment. The results confirm the ability of G20 to inhibit aggregation formation. Of interest is the observation that G20 had more of a robust effect at a specific concentration of GST-Htt 1-171 (Q58), indicating a possible dependency on a stoichiometric relationship.

We still wanted to determine the mechanism in which G20 was exerting its effect on mutant huntingtin. Our first logical hypothesis was that a physical interaction might be occurring. It is possible that G20 is binding directly to the protein

and preventing the aberrant mis-folding required for nucleation of the aggregate process. To see if a direct interaction was occurring, we adapted a biotinylated-oligonucleotide pull-down assay. We had G20 synthesized with a biotin tag, which could be "pulled-down" via its affinity to streptavidin along with any bound protein product. After eluting the nucleo-protein complexes we could visualize the bound protein by standard western blotting techniques. Our results indicate that G20 is in fact binding directly to GST-Htt 1-171 (Q58), which is most likely connected to blocking the aggregation process. Of notable interest again is the specific concentration of protein at which G20 exhibits the most binding, mirroring the native gel electrophoresis assay. We predicted that if the interaction is concentration specific, it may be dependent on the aggregation process. We wanted to see if this interaction was specific to mutant huntingtin, or if G20 would bind to wild type huntingtin as well. We performed the same pull-down assay with GST-Htt 1-171(Q23) and found that G20 bound wild type huntingtin in a more degenerate fashion. Instead of one concentration, the binding profile exhibited that a less specific interaction was going in. This was interesting to us and we thought it could be another indication that the aggregation process had an effect on how mutant huntingtin and G20 interact.

To validate the pull-down assay, and to affirm the binding activity of G20 we performed the biotinylated oligonucleotide pull-down assay in two different cell lines. The rat pheochromocytoma(PC12) cell lines expressing a mutant(Q103) or wild type(Q25) exon 1 fragment of huntingtin fused to green fluorescent protein, Htt14A2.6

and Htt17A2 respectively, were transfected with biotinylated G20 as described in chapter 2. The protein was extracted from the cells and the pull-down assay performed. We see binding occurring in the Htt14A2.6 and Htt17A2 cell lines *in vivo* to G20. Another G-quartet forming oligonucleotide (AS1411) was used as a positive control, and we see positive binding in both cell lines. This is further evidence that the G-quartet secondary structure formed by G20 may be required for activity. In the mutant cell line (Q103), Htt14A2.6 we see binding that is specific to G20 when compared to an all thymine oligonucleotide control, further verifying the specificity of G20. We also tested binding in total protein extract of G20 to full length mutant (Q126) and wild type (Q21) huntingtin in a Tet-off PC12 cell model of HD. We see a robust binding of G20 and AS1411 to wild type huntingtin over time, with more stable binding occurring with G20. We see binding to full length mutant huntingtin as well, although the binding occurs later in time of expression (5days) and drops of by day 7, which is when aggregation has started to occur, supporting the idea that a specific interaction between G20 and huntingtin may be aggregation dependent. Taken together, this data validates the binding interaction we saw *in vitro* but also gives us a clue that the secondary structure formed by G20 may be central to its ability to interact with huntingtin. This led us to design experiments to characterize the specific secondary structure formed by G20, as well as examine the binding of G20 to mutant huntingtin during the aggregation process.

After observing that the secondary structure formed by G20 may be important for its activity, and because determining the exact structure of G20 could be important

for defining the mechanism of aggregation inhibition; we sought a way to better define the exact conformation of G20. Also, if there is a specific activity related to structure, it may be possible to design a compound or small drug that mimics that activity. Through collaboration with Dr. James Vesenka at University of New England, we performed atomic force microscopy analysis of G20 alone, and of samples of G20 and huntingtin together. The images revealed that G20 is forming a specific G-quartet conformation known as a G-wire, evident by the rod-like appearance and consistent height of the structures (Marsh et al., 1995). At high concentrations, the G-wire structures form branches and networks, and may resemble the schematic pictured in Figure 11 A. At concentrations consistent with our experimental design, G20 forms un-branched G-wires which can be depicted by Figure 11 B. Unlike other G-quartet forming oligonucleotides, G20 spontaneously forms secondary structure without the need for stabilizing ions, indicating a very stable conformation (Fogolari et al., 2009; Marsh et al., 1995). This is reinforced by circular dichroism analysis on G20 stability. Increasing the temperature from 20°C to 99°C has virtually no effect on the amplitude or pattern of G20 CD spectra, indicating stability at even high temperatures.

AFM analysis was also done on samples of G20 and GST-Htt 1-171 (Q58) mixed together. These images reveal that G20 forms stable G-wires even in the presence of protein, indicating that the protein does not have an effect on G20 structure. The GST-Htt 1-171 forms complexes around G20, and in this representative image (Figure 15 C) there seems to be less visible aggregation in the sample at the same concentration as a sample without G20 (Figure 15 A). This could be due to a

disruption of aggregate formation by G20. We must keep in mind that these experiments are static pictures of a dynamic process, and that they are done in the presence of mica. Imaging this interaction over time and in solution would be an excellent future direction to take.

The binding profile of G20 to mutant huntingtin in the biotinylated oligonucleotide pull down experiment was specific to a single concentration of GST-Htt 1-171 (Q58). We predicted that this might reflect a dependency on aggregation. We designed an experiment to characterize the binding relationship between G20 and GST-Htt 1-171 (Q58) during the process of aggregation. Samples of protein were allowed to aggregate for different amounts of time from 2-24 hours *before* the addition of G20. Each sample was then in the presence of G20 for 18 hours to allow binding to take place. The biotin pull-down assay was performed in the same way as before, and the bound product was analyzed by western blot. The results revealed robust binding at the four hour time point, and slightly less bound protein at later time points. This experiment was repeated several times, with the same time point, four hours, being the crucial binding period. From these results (Figure 12 B) we can conclude that G20 has a preference for a specific species along the aggregation time course. Either, a unique structure is required for G20 interaction, or a specific region of the protein is exposed at that time and bound preferentially by G20. Further studies to characterize this specific species are of great interest.

Aggregation is both time and concentration dependent, so after identifying that the specific binding species at 1μg/μl occurs at four hours, we sought to identify the

same species at a higher and lower concentration. The same time course experiment was performed at 0.5 μg/μl and 1.5 μg/μl concentrations. Evident by Figure 12, no binding was identified at either of these concentrations at the time points tested. We can predict the reasoning for this by looking at the control samples. The controls were samples prepared and allowed to aggregate for 18 hours without the addition of oligonucleotide, so they are representative of the species available in the samples at 18 hours. At the high concentration, no sample appears in the control lane, indicating that the sample was aggregated past the point of being SDS soluble. There is a lot of sample present in the lower concentration at 18 hours, indicating that a very small amount of aggregation occurred over those 18 hours. We can predict that, if a specific species of aggregate is required for G20 binding, at the higher concentration the aggregation occurred too quickly for binding to occur. At the lower concentration, aggregation did not proceed until the point necessary for binding to occur. This analysis is depicted in the schematic illustrated in Figure 13.

Our next goal was to determine the effect of G20 on an *in vivo* system. We obtained two PC12 cell lines, S6 and S6-A expressing full length huntingtin in either a mutant (Q126) or wild type (Q25) form under the control of a Tet-off expression system. These cells exhibit decreased viability after several days of mutant huntingtin expression (Ratovitski et al., 2007). We wanted to see if G20 would ameliorate mutant huntingtin toxicity as measured by an MTT viability assay. Our results reveal that after 6 days of mutant huntingtin expression we see a decrease in cell viability when compared to cells expressing wild type huntingtin. Cells transfected with G20

before being induced to express mutant huntingtin have a significant improvement in cell viability compared to cells without G20 treatment. This may indicate that binding to mutant huntingtin by G20 does ameliorate some of the toxic effects associated its expression. The same experiment in wild type cells reveals that G20 binding to wild type huntingtin does not have a toxic effect in this system.

To further our studies on the effect of G20 *in vivo* we obtained a *C. elegans* model for poly-glutamine toxicity. *C. elegans* is an excellent genetic model, they are very small, easy to maintain and reproduce quickly. The *C. elegans* lines we received as a gift from Dr. Morimoto express poly-glutamine fusion proteins to yellow fluorescent protein with increasing poly-glutamine numbers (Brignull, Morley et al., 2006). They exhibit aggregation in an age and poly-glutamine expansion dependent manner, and toxic phenotypes associated with aggregation. Our goal for this system was to observe binding to a poly-glutamine protein by G20, study inhibition of poly-glutamine aggregation and ameliorate the toxic phenotype with G20 treatment.

We were able to establish binding in this model system, which again validates the binding interaction between G20 and huntingtin. It also tells us that G20 may bind specifically to the poly-glutamine region because this model system lacks the total protein context of huntingtin. Unfortunately, we obtained ambiguous results for the rest of our binding experiments with the *C. elegans* model. We were unable to consistently observe a pattern of binding that revealed conclusive results. Further work needs to be completed to determine if contamination was an issue or something unique was happening with the integration of the Q0 lines. We did establish a protocol for *in*

vivo delivery of G20 via electroporation and obtained positive results for *in vivo* binding of G20. We also were able to correlate binding of G20 to inhibition of aggregation evident by the AGERA assay (Figure 18)

As discussed previously, the poly-glutamine region is absolutely central to the aggregation process. Work done by Nekooki-Machida et al. predicts that it is exposure of the poly-glutamine region that causes aberrant protein interactions and contributes to toxicity and cell death (Nekooki-Machida et al., 2009). This hypothesis complements the aggregation model proposed by Thakur et al. that predicts that along the process of aggregation the poly-glutamine region becomes exposed in oligomer species (Thakur et al., 2009). It has also been proposed that intermediate, oligomeric aggregates are the toxic species to the cell, and that blocking their formation is of therapeutic value (Legleiter et al., 2009; Ross & Poirier, 2004). Experiments with antibodies specific to the toxic aggregate identify the polyglutamine region specifically, adding further evidence that is the region central to HD toxicity (Legleiter et al., 2009). We have a molecule, G20 that binds to mutant huntingtin and prevents its aggregation. G20 forms a unique secondary structure known as a G-wire that is necessary for its ability to directly interact with huntingtin. Characterizing this interaction has enabled us to determine that G20 preferentially binds to a specific species along the aggregation pathway, that is most likely oligomeric and intermediate. Evidence from studies in a *C. elegans* model of poly-glutamine toxicity indicates that G20 binds to the poly-glutamine region itself. Studies from several labs propose that a soluble, oligomer species of mutant huntingtin is responsible for

aberrant protein interactions that lead to cellular stress, other toxic effects and eventual death. These studies also indicate that the poly-glutamine region may be largely responsible for the damaging effect of this toxic species (Legleiter et al., 2009; Miller et al., 2005; Nekooki-Machida et al., 2009; Thakur et al., 2009). Considering this evidence, there is great potential that G20 masks the toxic effects of the exposed poly-glutamine region by binding it and preventing further aggregation. Further studies characterizing this interaction and the specific region of huntingtin that is bound by G20 is of great interest. From preliminary *in vivo* studies, we see that G20 treatment has a positive effect on viability of cells expressing mutant huntingtin. A great deal of work should focus on this interaction *in vivo* in several models of Huntington's Disease. Characterizing the mechanism of aggregate inhibition *in vivo* and the effect on cellular viability and disease phenotypes of whole organisms are absolutely necessary.

Future work should continue to focus on determining the specific region of huntingtin that interacts with G20. This could be done with synthetic peptides designed to mimic different regions of the protein, specifically concentrating on the N-terminus and exon 1 fragment like those designed by Thakur et al (Thakur et al., 2009). We could also perform selected mutagenesis on the plasmid used to express our GST fusion protein. Binding experiments could be done with the resulting fusion proteins that could determine the necessary region for G20 interaction to occur. Analysis of the aggregate species trapped by G20 with conformation specific antibodies would also be beneficial in characterizing the toxic nature of that species.

Another issue with developing G20 as a viable therapeutic for Huntington's Disease is delivery to the specific region of the brain affected by mutant huntingtin expression. Learning more about the structural properties of G20 could aid in chemically developing a structural compound that could be delivered easily as a Huntington's Disease Therapeutic.

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