Pharmacology of Hsp70 Activation

by

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Dedication

This dissertation is dedicated to my family. To my parents Kimberly and Carl Bolles, thank you for nurturing my love of science and making sacrifices to provide me with opportunities that have been instrumental to my career. To my husband Sean Davis, thank you for being a source of unwavering support.

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Abstract

The heat shock protein 90 and 70 (Hsp90 and Hsp70) chaperone system maintains protein quality control for over one hundred client proteins, including many implicated in neurodegenerative disorders, by surveilling substrate binding clefts and selectively targeting damaged proteins for degradation. Hsp90 stabilizes client proteins in their native state and prevents degradation, whereas Hsp70 facilitates ubiquitination by E3 ligases and promotes proteasomal degradation. Traditionally the chaperone system has been targeted with Hsp90 inhibitors, and while many Hsp90 inhibitors have entered clinical trials for cancer they have failed to progress due in part to toxicity. Activation of Hsp70 has recently emerged as an alternative approach, with numerous genetic studies demonstrating that activation of Hsp70-dependent ubiquitination increases the degradation of disease-causing client proteins in cellular and animal models. Studies on the pharmacological activation of Hsp70 have been limited by the small number of compounds known to activate Hsp70-dependent ubiquitination. Thus, an innovative workflow was developed to discover novel small molecule activators of Hsp70. A high-throughput Hsp70 thermal shift assay and Hsp90 counter screen were utilized to identify compounds that bind and thermostabilize Hsp70. To test the functional effect of Hsp70 thermostabilizers *in vitro*, a purified protein system for Hsp70-dependent ubiquitination of neuronal nitric oxide synthase (nNOS) and a highly sensitive ELISA for the measurement of nNOS ubiquitination were established. With the use of this workflow we successfully identified one novel small molecule activator of Hsp70-dependent ubiquitination. Moreover, in the course of these studies we discovered pharmacological activation of Hsp70 can selectively increase the ubiquitination of damaged nNOS over nNOS in its native

state. The small molecule identified by this workflow increases the structural diversity of compounds known to activate Hsp70-dependent ubiquitination and may provide a valuable tool to advance the study of pharmacological activation of Hsp70 as a therapeutic strategy.

Chapter 1 : Targeting Hsp70 facilitated protein quality control for treatment of polyglutamine diseases

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Abstract

The polyglutamine (polyQ) diseases are group of nine fatal, adult-onset neurodegenerative disorders characterized by the misfolding and aggregation of mutant proteins containing toxic expansions of CAG/polyQ tracts. The heat shock protein 90 and 70 (Hsp90/Hsp70) chaperone machinery is a key component of cellular protein quality control, playing a role in the regulation of folding, aggregation, and degradation of polyQ proteins. The ability of Hsp70 to facilitate disaggregation and degradation of misfolded proteins makes it an attractive therapeutic target in polyQ diseases. Genetic studies have demonstrated that manipulation of Hsp70 and related co-chaperones can enhance the disaggregation and/or degradation of misfolded proteins in models of polyQ disease. Therefore, the development of small molecules that enhance Hsp70 activity is of great interest. However, it is still unclear if currently available Hsp70 modulators can selectively enhance disaggregation or degradation of misfolded proteins without perturbing other Hsp70 functions essential for cellular homeostasis. This review discusses the multifaceted role of Hsp70 in protein quality control and the opportunities and challenges Hsp70 poses as a potential therapeutic target in polyQ disease.

Introduction

Age-dependent protein aggregation disorders are characterized by the accumulation of damaged or mutant proteins in the central nervous system, most often in neurons. The preferential accumulation of misfolded proteins in neurons underscores the importance of protein quality control mechanisms, such as the heat shock protein 90 and 70 (Hsp90/Hsp70) chaperone machinery, in neuronal homeostasis. The polyglutamine (polyQ) diseases are an example of hereditary protein aggregation disorders and include Huntington disease (HD), spinal bulbar muscular atrophy (SBMA/Kennedy disease), dentatorubral pallidoluysian atrophy (DRPLA), and six autosomal dominant forms of spinocerebellar ataxia (SCAs) [1]. These diseases are caused by the expansion of CAG/polyQ tracts in a collection of unrelated proteins, and penetrance only occurs once polyQ length exceeds disease-specific thresholds. Additional disorders in which expanded CTG repeats are associated with antisense transcripts containing long CAG repeats have also been described [2]. Proteins containing disease length polyQ tracts are prone to misfolding and can self-assemble into soluble oligomers and microaggregates that coalesce into insoluble, amyloid-like fibrils. The polyQ length threshold for disease penetrance is strikingly correlated to the threshold for aggregation of polyQ proteins *in vitro* [3]. It is well established the misfolding of polyQ containing proteins drives disease. While the exact specie(s) of polyQ proteins that mediate toxicity is unclear, the accumulation and aggregation of misfolded polyQ proteins in neurons implies that aggregate formation overwhelms protein quality control as an underlying basis of disease.

The role of the Hsp90/Hsp70 chaperone machinery in protein quality control has been well studied in the context of HD, SBMA, and SCA3. Therefore, this review will focus on these three polyQ diseases as representatives of the whole group, with additional examples provided when

relevant. HD, the most prevalent of the polyQ diseases, is caused by mutant huntingtin (Htt) harboring an expanded polyQ tract [4]. The normal function of Htt is incompletely understood, however recent work suggests it acts as a scaffold in retrograde transport, vesicle trafficking, and selective autophagy [5]. PolyQ Htt-dependent neuron dysfunction and death causes progressive motor, cognitive, and psychiatric manifestations in HD patients. SBMA is a neuromuscular degenerative disorder that is characterized by progressive weakness of proximal limb and bulbar muscles [6]. SBMA is caused by a polyQ expansion in the androgen receptor (AR) and pathogenesis is dependent on circulating levels of androgen; therefore, the disease only affects males. Steroid hormone-dependent translocation of polyQ AR to the nucleus leads to ligand-dependent misfolding and formation of nuclear inclusions. PolyQ proteins cause degeneration of the cerebellum in six forms of SCA [7]. The most common polyQ SCA, SCA3, is caused by the expansion of a polyQ tract in ataxin-3 (ATXN3), a deubiquitinating enzyme [8]. All polyQ diseases are ultimately fatal, with disease onset typically occurring in mid-life and disease progression occurring over the next 10 to 30 years [9]. Even though the genetic cause and progression of these diseases are well understood, there are currently no FDA-approved disease-modifying treatments.

A large body of work has demonstrated that genetic manipulation of the Hsp90/Hsp70 chaperone machinery is therapeutically beneficial in cellular and animal models of polyQ disease [10, 11], making the Hsp90/Hsp70 chaperone machinery an attractive therapeutic target. There are two main pharmacological strategies for targeting this system: inhibition of Hsp90 and activation of Hsp70. While it is well established that cycling into complexes with Hsp90 stabilizes Hsp90 client proteins, such as the AR and Htt and that specific inhibition of Hsp90 enhances client protein degradation, this approach has the potential to cause on-target side effects due a global decrease

in the hundreds of proteins reliant on Hsp90 for stabilization [10]. Activation of Hsp70, on the other hand, is focused on selectively enhancing the disaggregation and degradation of already misfolded proteins and should not affect properly folded Hsp90-dependent proteins, thus minimizing side effects. Hsp70 selectively facilitates the degradation of misfolded proteins, thus activation of Hsp70-facilitated degradation may provide a strategy to eliminate misfolded proteins while leaving native proteins untouched. However, targeting Hsp70 is not without problems. To be therapeutically beneficial a small molecule must selectively enhance the anti-aggregation or pro-degradative activity of Hsp70, without disrupting other Hsp70 functions critical for cellular homeostasis. Hsp70 activity is dependent on a conformational cycle determined by nucleotide binding, hydrolysis, and release, and this cycle is adapted to specific functions through regulation by co-chaperones. Indeed, co-chaperone binding sites may provide targets for small molecules that alter Hsp70 function. Designing small molecules that selectively alter one Hsp70 function while leaving others untouched poses a challenge. This review will focus on the role of Hsp70 in protein quality control and the opportunity and obstacles it presents as a therapeutic target in polyQ disease; Hsp90 will be discussed only in context of its cooperation with Hsp70, as comprehensive reviews on Hsp90 are available.

Hsp70 function is driven by nucleotide cycling and regulated by co-chaperones

Heat shock proteins (Hsp) were first discovered due to their increased expression upon heat exposure [12, 13]. These proteins were originally named based on their molecular weight and include Hsp110 (HSPH), Hsp90 (HSPC), Hsp70 (HSPA1), Hsp60 (chaperonins), Hsp40 (J-domain/DNAJ), and small heat shock proteins (sHsp/HspB) [14]. It is now recognized that several environmental and pathological conditions, not just heat, can induce the expression of Hsps, underscoring their importance in the response to cellular stress. The transcription factor HSF1

(heat shock factor 1) is the master regulator of the heat shock response (HSR). Under conditions of stress, localization of HSF1 to the nucleus and binding to the heat shock element in promoters leads to the activation of genes encoding inducible members of the Hsp90/Hsp70 chaperone machinery [15, 16]. Hsp families include constitutively expressed members, such as Hsc70 in the Hsp70 family, and therefore also play a role in protein quality control under basal conditions. To further add to the complexity, Hsp families contain numerous members with distinct subcellular localization and unique functions. The Hsp70 family alone contains 13 members in humans, including the cytoplasmic variants Hsp70 (HSPA1) and Hsc70 (HSPA8), the mitochondrial variant mtHsp70 (mortalin/HSPA9), and the endoplasmic reticulum variant BIP (GRO78/HSPA5) [17]. In this review the term Hsp70 will be used broadly to refer to all family members, and when pertinent, specific family members will be defined.

Interaction of Hsp70 with client proteins is not dependent on a specific sequence or structural motif; instead, Hsp70 interacts with proteins containing exposed hydrophobic stretches [18]. These hydrophobic residues are exposed in proteins during their synthesis, in protein folding (i.e. ligand binding) clefts, in domains regulating protein-protein interactions, or in misfolded proteins. Hsp70 is comprised of two domains, a nucleotide binding domain (NBD) and a substrate binding domain (SBD), connected by a flexible linker. The action of Hsp70 is facilitated by a nucleotide-dependent conformational cycle. In the ATP state, the SBD is docked to the NBD, locking Hsp70 into a conformation that has low affinity for unfolded, hydrophobic substrate (Fig. 1-1a). Upon hydrolysis of ATP to ADP a large conformational change allows the SBD to move freely, and in the ADP state Hsp70 exhibits high affinity for unfolding proteins, reviewed in [19] (Fig. 1-1b). However, the intrinsic ATPase activity of Hsp70 is very low, with ATP hydrolysis being the rate limiting step [20].

ATP hydrolysis is stimulated by the J-domain family of co-chaperones (Fig. 1-1b) [21]. Nucleotide exchange factors (NEFs), including the Hsp110 and Bcl-2-associated athanogene (BAG) families, are co-chaperones that promote the release of ADP from Hsp70 and allow for the binding of a new ATP molecule and the continuation of the cycle (Fig. 1-1c) [22]. The nucleotide cycle of Hsp70 is further regulated by the co-chaperone HIP (Hsp70 interacting protein). HIP binds to the NBD of Hsp70 in the ADP-bound state via a tetratricopeptide repeat (TPR) domain, and HIP binding antagonizes NEF activity (Fig. 1-1c). Therefore, HIP stabilizes the ADP state of Hsp70 and increases the amount of time Hsp70 stays bound to a misfolded protein [23]. Through the regulation of nucleotide cycling these co-chaperones adapt Hsp70 function to different roles in protein quality control.

Multifaceted role of Hsp70 in protein quality control

Hsp70 plays a multifaceted role in protein quality control and is adapted to different pathways through regulation by co-chaperones (Table 1-1).

Folding of newly synthesized proteins

While the Hsp70 family was first recognized for its role in stress response, the constitutive members of the family also play a critical role in protein quality control during basal conditions, including the folding of newly synthesized protein. *In vitro* many proteins can fold spontaneously [24]; however, larger proteins and proteins with a high abundance of disordered regions require the assistance of chaperones to properly fold in the crowded cellular environment [25]. The constitutive form of Hsp70, Hsc70, works in concert with specialized J-domain proteins and NEFs to prevent the misfolding of newly synthesized proteins by cycling with the nascent chain as it exits the ribosome, a process known as co-translational folding [26–29]. Binding of Hsc70 stabilizes the unfolded state of the newly synthesized protein and can even reverse misfolded states

[30]. Subsequent release of the stabilized protein from Hsc70 allows folding to proceed in the correct order, starting with secondary structure [31]. Under conditions of proteotoxic stress, such as proteasome inhibition, Hsc70 is sequestered from the ribosomes by a build-up of misfolded proteins, resulting in the misfolding of newly synthesized proteins and inhibiting global protein synthesis [32]. The expression of a dominant-negative Hsp70 (K71M), incapable of nucleotide cycling, or treatment with the Hsp70 inhibitors VER-155008 (VER) or Pifithrin-μ (PES) also decreases global protein synthesis [32], demonstrating the importance of Hsc70 for protein quality control under homeostatic conditions.

Maturation of Hsp90 client proteins

Along with Hsp90 itself, Hsp70 is an essential member of the multiprotein chaperone machinery that facilitates the maturation of Hsp90 client proteins. Several hundred Hsp90 client proteins have been identified, including many signaling proteins such as protein kinases and steroid receptors [10, 33]. Hsp90 interacts with client proteins at regions where hydrophobic amino acids are exposed to solvent, such as where ligand binding clefts fuse with the charged protein surface, stabilizing the cleft in an active conformation for ligand binding and stabilizing the protein [34]. For steroid receptors, such as AR, proper folding of the ligand binding cleft, insertion of steroid (Fig. 1-2a), translocation to the nucleus (Fig. 1-2b) and therefore transcriptional activity requires the Hsp90 chaperone machinery. This machinery has been studied in detail with relationship to the glucocorticoid receptor (GR), as reviewed in [35]. In the first step of this machinery's function, Hsp70 in the ATP state [36] interacts with the closed (folded) ligand binding cleft of the steroid receptor and subsequent ATP hydrolysis (promoted by DNAJ proteins) partially unfolds the cleft, priming it for interaction with Hsp90 [37, 38]. The co-chaperone HOP (Hsp Organizing Protein) forms a chaperone machinery by facilitating the interaction of Hsp70 and

Hsp90. This interaction occurs through binding of an N-terminal and central TPR domain to EEVD motifs on the C-terminus of Hsp70 and Hsp90 [39], promoting the transfer of the steroid receptor from Hsp70 to Hsp90 [40]. Subsequent ATP dependent cycling of Hsp90 leaves the cleft in an open state and binding of p23 to Hsp90 [41] stabilizes the cleft in an open conformation, allowing for the insertion of steroid [33]. The ATPase dead Hsp70 mutant K71M fails to facilitate the assembly of the multichaperone machinery with the progesterone receptor (PR) [42], and inhibition of Hsp70 by methylene blue (MB) inhibits steroid binding activity of GR, demonstrating Hsp70 is necessary for the proper function of the Hsp90 multichaperone machinery [43]. Steroid insertion leads to rapid retrograde transport of steroid receptors to the nucleus by an Hsp90-immunophilin-dynein complex and passage through nuclear pores in an importin-dependent process, as reviewed in [44]. For polyQ AR, the conformational change induced by steroid insertion causes misfolding and translocation of the misfolded protein to the nucleus, leading to the formation of nuclear aggregates. Either genetic or pharmacological inhibition of Hsp90 reduces ligand-dependent nuclear translocation of polyQ AR and decreases polyQ AR nuclear aggregates [45].

Proteasomal Degradation

The chaperone machinery plays a critical role in protein quality control by selectively recognizing misfolded proteins and targeting them for degradation by the ubiquitin proteasome system (UPS) [46]. Ubiquitination is a post translational modification by which an 8.6 kDa ubiquitin protein is covalently added to a protein; ubiquitination can target a protein to numerous cellular pathways including degradation by the proteasome. Hsp90 and Hsp70 play opposing roles in the triage of misfolded proteins, as reviewed in [46]. Hsp90 stabilizes proteins in a near-native state, protecting them from degradation. In contrast, if a protein is too misfolded to be stabilized

by Hsp90, then Hsp70 facilitates ubiquitination by chaperone dependent E3 ubiquitin ligases, such as CHIP (C-terminus of Hsp70 interacting protein), a TPR domain containing protein that binds the EEVD domain on the C-terminus of Hsp70 (Fig. 1-2c) [47, 48]. CHIP interacts with E2 ubiquitin conjugating enzymes, through a C-terminal U box domain, and ubiquitinates proteins bound to Hsp70 [49]. Numerous co-chaperones promote Hsp70-mediated proteasomal degradation of misfolded proteins. For example, the J-domain protein DNAJB2 (HSJ1) recruits misfolded proteins to Hsp70 and promotes ubiquitination by CHIP [50], then DNAJB2 targets the ubiquitinated proteins to the proteasome via ubiquitin interaction motifs (UIMs) [50, 51]. The NEF BAG1 also promotes Hsp70-mediated proteasomal degradation. BAG1 contains a ubiquitin like domain that allows it to associate with the proteasome and promote the release of Hsp70-bound ubiquitinated proteins to the proteasome [52, 53]. Hsp70-mediated ubiquitination and degradation has been shown to regulate the turnover of polyQ disease-related proteins, including AR, Htt, and ATXN3 as well as non-polyQ proteins implicated in age dependent neurodegenerative disorders such as tau and α-synuclein [10, 11]

Autophagy

Hsp70 can also target misfolded proteins to degradation through autophagy, a process by which proteins are degraded in lysosomes. Proteins destined for degradation by autophagy are delivered to the lysosome by different mechanisms. In macroautophagy, cargo is transported to lysosomes inside double membrane vesicles known as autophagosomes [54]. Chaperone-assisted selective autophagy (CASA) is a form of macroautophagy which utilizes Hsp70 to target proteins to the autophagosome. In microautophagy the lysosomal membrane invaginates and pinches off to form vesicles that internalize autophagic cargo [55]. Proteins can also enter the lysosome through a protein translocation system embedded in the lysosomal membrane in a process known as

chaperone-mediated autophagy (CMA) [56]. Autophagy has been shown to play an important role in the degradation of polyQ proteins including AR, Htt, and ATXN3 [57]. Autophagy can facilitate the degradation of protein species that are not amenable to degradation by the proteasome, such as insoluble polyQ aggregates and proteolytic fragments of polyQ proteins. For example, N-terminal fragments of polyQ AR are toxic species that lack the ligand binding domain, the site of recognition by the Hsp90/Hsp70 chaperone machinery, and therefore are not readily targeted to the proteasome, being preferentially degraded by autophagy [43]. N-terminal fragments of polyQ Htt also contribute to toxicity in HD and are preferentially degraded by autophagy [57, 58].

Chaperone-mediated autophagy (CMA). In CMA, proteins containing a KFERQ-like pentapeptide motif, present in approximately 40% of the mammalian proteome, are targeted to degradation in the lysosome by Hsp70 [59, 60]. Hsp70 binds to the KFERQ domain and targets the protein to the lysosomal membrane, where the protein then binds to LAMP2A (lysosome associated membrane protein type 2A). The target protein is subsequently unfolded and then translocated into the lysosomal lumen [61–63] through the CMA translocon complex, which consists of a trimer of LAMP2A [64]. CMA is observed at low levels under homeostatic conditions in most cells, but upon stress, CMA activity significantly increases, highlighting the importance of this pathway as a compensatory mechanism for proteotoxic stress [61]. Upregulation of CMA activity has been observed in cellular and animal models of polyQ disease [65] and has been shown to promote the clearance of polyQ Htt [66] and polyQ Atxn7, the disease-causing protein in SCA7 [67].

Chaperone-assisted endosomal microautophagy (eMI). Proteins containing the KFERQ-like pentapeptide motif can also be targeted by Hsc70 to the late endosome for degradation by microautophagy. In eMI proteins do not enter the late endosome through a translocon; instead, the

endosomal membrane invaginates to trap cytosolic proteins for degradation [61]. Therefore, unlike CMA, which requires proteins to be unfolded before translocation, chaperone assisted eMI can facilitate the degradation of proteins that are folded, in early stages of misfolding and aggregation, or assembled into higher order aggregation complexes [68]. Hsp70 binds the pentapeptide-containing protein and recruits the protein to the endosomal membrane by binding of a unique site on the lid of Hsp70 to phosphatidylserine moieties [69]. Hsp70 and its cargo are then internalized in ESCRT-mediated microvesicles [68].

Chaperone-assisted selective autophagy (CASA). Hsp70 also plays a role in assembly of the presentation complex responsible for selectively targeting proteins to the forming autophagosome for degradation by macroautophagy (Fig. 1-2e) [52]. BAG3 and HspB8 connect Hsp70 to the macroautophagy pathway [70, 71]. First, BAG3 binds Hsp70 in complex with CHIP and ubiquitinated substrate via its BAG domain and then binds the small Hsp, HspB8, through an IPV motif [72]. BAG3 then interacts with dynein to facilitate the transport of the misfolded protein containing CHIP-Hsp70-BAG3-HspB8 complex to the aggresome at the MTOC (microtubule organization center), a region highly enriched in autophagy/lysosomal pathway components. This complex then interacts with the macroautophagy receptor protein p62, which targets the ubiquitinated, misfolded protein to the autophagosome via the autophagosome membrane-associated protein LC3 [73]. The autophagosome then fuses to the lysosome where the misfolded protein is degraded. In a motoneuronal cell model of SBMA it was demonstrated that polyQ AR-induced proteotoxic stress leads to an upregulation of HspB8, promoting the clearance of cytosolic polyQ AR aggregates by autophagy [74].

Disaggregation

Hsp70 in collaboration with specialized J-domain proteins and the Hsp110 family of NEFs can disaggregate and solubilize misfolded proteins (Fig. 1-2d) [75, 76]. While the structure and composition of the DNAJ-Hsp70-Hsp110 disaggregase complex is still debated, the current 'nucleation' model suggests that complex formation is initiated by the recruitment of multiple Hsp70s to the surface of aggregates by J-domain proteins [77]. J-domain proteins belonging to class A and B, such as DNAJB1 (HDJ1), have been implicated in the targeting of Hsp70 to aggregates [78, 79]. Both class B and A J-domain proteins have high homology to the canonical E. coli J-domain protein, DNAJ, and contain a conserved N-terminal J-domain responsible for promoting ATPase activity [17]. However, their C-terminal domains differ in structure and confer unique substrate specificity [21]. In addition to recruiting Hsp70 to the aggregate surface, J-domain proteins also stimulate ATPase activity. This allows for the binding of Hsp70 to the exposed hydrophobic residues on the ends of aggregated proteins and, through a process known as entropic pulling, liberation of polypeptides from the protein aggregate. Members of the Hsp110 family then reset the disaggregase machinery by promoting dissociation of ADP, allowing for a new round of ATP hydrolysis [80]. It appears that only members of the HSP110 family of NEFs participate in Hsp70-mediated disaggregation [79]. Ubiquilin-2 (UBQLN2) interacts with misfolded proteins liberated from aggregation, through an ubiquitin-associated domain, and targets them to the proteasome via an ubiquitin-like domain [81]. UBQLN2 has been shown to work together with the DNAJ-Hsp70-Hsp110 disaggregase complex to promote the proteasomal degradation of polyQ Htt in cellular and mouse models of HD [82]. Interestingly some J-domain proteins, including DNAJB6 and DNAJB8, have been shown to directly interact with and prevent aggregation of

polyQ proteins in a mechanism independent of the DNAJ-Hsp70-Hsp110 disaggregase complex [83, 84].

When it comes to aggregation, most research has focused on Hsp70's ability to disassemble toxic inclusions of polyQ proteins. However, it is important to keep in mind that controlled sequestration of polyQ proteins can be cytoprotective and Hsp70 also plays a regulatory role in the formation of polyQ aggregates. For example, in a mechanism overlapping with CASA, Hsp70 interacts with dynein motors, in a BAG3-dependent manner [73], to direct retrograde transport of misfolded proteins along microtubules to an aggresome, located at the MTOC [85]. Sequestration of misfolded proteins into the aggresome promotes their clearance by autophagy.

Dysregulation of protein quality control in polyglutamine disease

Dysregulation of the Hsp90/Hsp70 chaperone machinery, the ubiquitin proteasome system, and autophagy leads to diminished protein quality control and accumulation of misfolded proteins in polyQ disease [58, 86]. Disease progression correlates with a decrease in key members of the Hsp90/Hsp70 chaperone machinery in HD, SBMA, and SCA17 [87–89]. One mechanism by which polyQ proteins decrease chaperone levels is through the sequestration of chaperones and related transcription factors into aggregates. For example, levels of soluble DNAJB1 decline over time in the brains of an HD mouse model due to direct sequestration of DNAJB1 into nuclear polyQ Htt aggregates [90]. Consistent with these observations, DNAJB1 also co-localizes with aggregates in a cellular model of SCA3 [91]. In yeast, it has been demonstrated that sequestration of DNAJB1 into aggregates prevents the co-chaperone from targeting misfolded proteins to the proteasome [92]. Hsp70 levels also progressively decline in mouse models of HD. However, this decline is not due to direct sequestration of Hsp70: instead, polyQ Htt aggregates sequester the transcription factor NF-Y leading to decreased expression of Hsp70 [93]. Sequestration of NF-Y

by aggregates was also shown to decrease Hsp70 expression in a cellular model of SCA17 [94]. These findings suggest that sequestration of chaperones, and the transcription factors that regulate their expression, is a shared mechanism by which polyQ proteins disrupt the function of the chaperone machinery.

Accumulation and aggregation of misfolded proteins activates the HSF1-driven HSR, inducing expression of the Hsp90/Hsp70 chaperone machinery. However, in HD, the HSR is greatly diminished in large part, by polyQ Htt-dependent dysregulation of HSF1. For example, cells expressing polyQ Htt have decreased expression of HSF1 and impaired HSR [95]. Consistent with these findings, HSF1 and Hsp70 levels are reduced in the striata of HD knock-in mice compared to wildtype mice [95]. One mechanism by which polyQ Htt decreases HSF1 levels is through the upregulation of CK2α' kinase and Fbxw7 E3 ligase levels, increasing phosphorylation and proteasomal degradation of HSF1 [14]. In contrast, other studies in both cellular and animal models of HD have not observed changes in HSF1 levels, and instead conclude that altered chromatin structure leads to decreased binding of HSF1 to heat shock response elements and impaired HSR [96, 97]. It is unclear if diminished HSR is a common mechanism amongst all polyQ diseases, but HSR impairment has been observed in other protein folding diseases such as Parkinson disease [98, 99]. Activation of HSR by HSF1 modulators has been shown to alleviate toxicity in both cellular and animal models of polyQ-disease and is therefore being investigated as a potential therapeutic strategy, as reviewed in [11]. Further work is needed to evaluate the long term consequences of pharmacological activation of HSR, as some studies have found that chronic activation of HSR can exacerbate toxicity in cellular models of protein misfolding disease including HD [100] and Alzheimer's disease [101].

Dysregulation of the ubiquitin proteasome system is also observed in polyQ disease. The Hsp90/Hsp70 chaperone machinery selectively targets misfolded proteins for degradation by the proteasome; therefore, polyQ proteins have an accelerated turnover. This concept is highlighted by cellular experiments where proteasome inhibition causes a greater build-up of ubiquitinated proteins in cells expressing disease length versus wildtype AR and Htt [102, 103]. In polyQ disease it is thought that the increase in proteins targeted for degradation overwhelms the capacity of the proteasome, thus contributing to the accumulation of polyQ proteins [104]. Consistent with this hypothesis ubiquitinated polyQ Htt has been shown to accumulate in the brains of both HD mouse models and patients [105]. This build-up of polyQ proteins has also been shown to compete with other proteasome substrates for a limited degradation capacity. For example, expression of polyQ Htt and Atxn7 in cells has been shown to decrease the degradation of other non-polyQ proteasome substrates [106, 107]. Therefore, the cell has decreased ability to respond to proteotoxic stress [108]. PolyQ proteins can also decrease function of the ubiquitin proteasome system by decreasing the expression of key proteasome components. For example, in a mouse model of SBMA, polyQ AR diminished activity of the proteasome transcription factor NRF1 (nuclear factor erythroid 1) in skeletal muscle in a hormone- and polyQ-tract length-dependent manner, resulting in decreased degradation capacity and a build-up of dysfunctional proteasomes in mutant muscle [109]. Taken together these findings demonstrate that dysregulation of the ubiquitin proteasome system is a compounding factor in the accumulation of misfolded proteins in polyQ disease.

The proteotoxic stress induced by the accumulation of misfolded proteins has been shown to alter autophagic pathways. For example, expression of misfolded proteins or pharmacological inhibition of the proteasome causes a robust increase in HspB8 expression. In contrast, other stressors such as serum starvation and heat shock do not alter HspB8 levels, demonstrating this

compensation is specific to proteasome impairment [70, 71]. Both HspB8 and BAG3, co-chaperones that facilitate CASA, are upregulated in polyQ AR knock-in mice [110]. It has been demonstrated that upregulation of HspB8 increases the clearance of cytosolic polyQ AR and reduces toxicity in a motorneuron cellular model of SBMA [74], consistent with the hypothesis that enhancement of autophagy is a beneficial compensatory mechanism to restore proteostasis. However other studies have demonstrated that activation of autophagy is in fact a maladaptive response in SBMA. For example, the activity of the transcriptional factor EB (TFEB) is upregulated in the muscle of polyQ AR knock-in mice leading to an increase in autophagic flux [110, 111]. Activation of autophagy has been shown to increase muscle atrophy in the same mouse model of SBMA [112] whereas genetic inhibition of autophagy decreased muscle atrophy and expanded lifespan [112]. In contrast to findings in SBMA, it has been demonstrated that down regulation of autophagy contributes to diminished protein quality control in HD [11, 113]. For example, transcriptional dysregulation of autophagy-related genes, including TFEB, has been observed in the brains of HD mouse models and patients [114, 115]. PolyQ Htt has also been shown to directly impair cargo recognition and engulfment of cytosoplamic cargo [116, 117].

Not all cell types expressing polyQ proteins experience a decrease in quality control. In HD, polyQ Htt is expressed ubiquitously in the brain but accumulates preferentially in neurons [118]. Interestingly, glial cells such as astrocytes do not experience the same accumulation and are therefore resistant to the toxicity associated with polyQ proteins [119]. This is thought to be related to the capacity of each cell type to degrade misfolded proteins, as astrocytes have been shown to degrade polyQ Htt faster than neurons [120]. What differences between neurons and astrocytes determines their capacity to degrade misfolded proteins? One explanation is differential expression of Hsp70-binding protein 1 (HspBP1) a co-chaperone known to antagonize the action of CHIP

[121]. HspBP1 decreases CHIP-dependent ubiquitination of polyQ Htt and subsequent proteasomal degradation. Knockdown of HspBP1 enhances degradation of polyQ Htt in cultured neurons and reduces polyQ Htt-dependent neuropathology in a mouse model of HD [121]. These findings highlight the importance of co-chaperone composition in determining the capacity of the protein quality control system in a cell and susceptibility to polyQ protein-mediated toxicity.

Mutations in Hsp70 co-chaperones lead to neurodegenerative disease

Is the disruption of Hsp70-mediated protein quality control observed in polyQ disease a driving mechanism of toxicity? Rare familial neurodegenerative disorders caused by mutations in Hsp70 co-chaperones may shed light on this question. Numerous disease-causing mutations have been identified in genes encoding Hsp70 co-chaperones including J-domain, NEF, and HspB family members, as reviewed in [100–102]. These rare chaperonopathies can manifest with similar muscular and neurodegenerative phenotypes to those observed in polyglutamine disease, suggesting that dysfunction of the Hsp90/Hsp70 chaperone machinery may be a common underlying mechanism. For example, multiple chaperonopathies are linked to mutations in DNAJB2, a J-domain protein shown to work in concert with Hsp70 to shuttle ubiquitinated proteins to the proteasome for degradation [50, 51]. A single amino acid substitution in the J-domain of DNAJB2 causes Charcot Marie Tooth disease type 2 resulting in progressive axonal degeneration involving sensory and spinal cord motoneurons [122]. Splicing mutations in DNAJB2 that result in either decreased DNAJB2 expression or loss of the J-domain lead to distal hereditary motor neuropathies and spinal muscular atrophy, respectively [122, 123]. Mutations in another J-domain protein, DNAJB6 (MRJ), shown to potently inhibit the aggregation of misfolded proteins [124, 125], cause limb-girdle muscular dystrophy type 1 (LGMD1), a disease characterized by the accumulation of abnormal protein aggregates in skeletal muscle fibers and

progressive distal greater than proximal muscle atrophy [125, 126]. When expressed in Drosophila, human mutations leading to LGMD1 result in a loss of DNAJB6-mediated anti-aggregation [127]. These studies suggest that disrupting DNAJ protein function can cause muscular and neurodegeneration through dysregulation of pro-degradative and disaggregation functions.

Mutations in co-chaperones essential to Hsp70-mediated autophagy have also been linked to chaperonopathies. Mutations in BAG3 located in the BAG domain responsible for binding Hsp70 or in an isoleucine-proline-valine motif responsible for binding HspB8 cause a dominant form of myopathy that is characterized by protein aggregation in skeletal and cardiac muscle [128]. Characterization of human mutations *in vitro* demonstrated mutant BAG3 was still capable of binding Hsp70, but surprisingly inhibited Hsp70 ATPase activity. Expression of these mutants in cells caused aggregation of the mutant BAG3 itself, Hsp70, and ubiquitinated Hsp70 clients and impaired CASA [129]. Mutations in HspB8 cause distal hereditary motor neuropathy type II (dHMNII), a disease characterized by neuron degeneration in the peripheral nervous system [130, 131] These HspB8 mutants show decreased interaction with BAG3 [70]. Over-expression of wild type HspB8 in cells promotes association of the autophagosome with lysosomes, whereas expression of dHMNII HspB8 mutants prevents interaction of the autophagosome and lysosome. Consistent with these findings, impaired autophagy was also observed in cells isolated from dHMNII patients [131]. These studies demonstrate disruption of a pro-autophagy Hsp70 co-chaperone leads to muscular and neuronal toxicity.

Genetic manipulation of Hsp70 and co-chaperones in polyQ disease

Numerous studies have shown that genetic manipulation of Hsp70 and associated co-chaperones can ameliorate toxicity in models of polyQ disease by decreasing aggregation or increasing degradation of polyQ proteins (reviewed in detail by Reis et al. [11] and Zarouchlioti

et al. [126]). These proof-of-concept studies demonstrate that Hsp70 is an attractive therapeutic target in polyQ disease. For example, over-expression of Hsp70 reduced aggregation of polyQ AR and increased survival in cells [132]. Hsp70 over-expression also reduced aggregation and enhanced degradation of polyQ AR in mice, leading to improved motor function [133]. Additionally, Hsp70 over-expression improved motor function in a mouse model of SCA1 and ameliorated neuropathology in mouse models of both SCA1 and SCA17 [134, 135]. Hsp70 over-expression shows beneficial but less consistent results in models of HD, and most studies focus solely on correlating effects of Hsp70 on polyQ Htt aggregation to toxicity. Over-expression of the ER localized Hsc70 homolog BIP [136] and Hsc70 [137], but not Hsp70, reduced polyQ Htt aggregation in Neuro2a cells. However, while both BIP and Hsc70 decreased aggregation, only over-expression of BIP increased survival. In contrast, over-expression of Hsp70 in HEK293 cells had no effect on Htt aggregation but did increase cell survival [138]. Each Hsp70 family member plays a unique role in protein quality control, and these experiments highlight the need for further work to investigate if specific Hsp70 isoforms offer better therapeutic targets in different polyQ diseases. Effects of Hsp70 expression have also been mixed in mouse models of HD. In one experiment utilizing R6/2 mice, a transgenic model expressing a small N-terminal fragment of the human HD gene containing ~150 CAG repeats, Hsp70 over-expression had no effect on polyQ Htt aggregation but delayed weight loss [139], while in another study Hsp70 over-expression caused a delay in polyQ Htt aggregation but did not affect disease related phenotypes [90]. However, depletion of Hsp70 in R6/2 mice significantly decreased survival and worsened motor dysfunction, re-affirming the importance of Hsp70 in the protein quality control of polyQ Htt [140]. This group of studies also demonstrates that the effect of Hsp70 on polyQ Htt aggregation is not always correlated with survival. While our understanding of the role aggregates play in polyQ protein

toxicity is incomplete, these studies suggest that over-expression of Hsp70 may decrease toxicity through a mechanism independent of aggregation.

The role of DNAJ proteins in polyQ diseases has been studied extensively, and family members DNAJB1, DNAJB2, and DNAJB6 have been identified as possible therapeutic targets [126]. Over-expression of DNAJB1 in Neuro2a cells decreased polyQ Htt inclusion formation, and co-expression with Hsp70 reduced toxicity [137, 141]. Similarly, DNAJB1 increased solubility and proteasomal degradation of polyQ AR in cells, and co-expression of Hsp70 further enhanced DNAJB1 action [132, 142]. Over-expression of the Drosophila DNAJB1 homolog, dHDJ1, suppressed polyQ Htt- and ATXN3-dependent toxicity [143, 144], and the effect of dHDJ1 on ATXN3-mediated toxicity was enhanced by over-expression with wildtype Hsp70 but inhibited by co-expression with a dominant negative mutant of Hsp70. DNAJB2 reduced ATXN3 levels by promoting proteasomal degradation or stabilizing ATXN3 in an Hsp70-independent manner in a cellular model of SCA3 [145]. DNAJB2 decreased polyQ AR inclusions in cells by increasing ubiquitination and proteasomal degradation of polyQ AR [51]. DNAJB2 over-expression decreased polyQ Htt aggregation in R6/2 mice and improved neurological performance [146]. These findings suggest that activation of Hsp70-facilitated disaggregation or degradation by upregulation of DNAJB1 and DNAJB2 is beneficial in polyQ disease. Similarly, DNAJB6 over-expression reduced polyQ Htt inclusion formation in the brain of R6/2 mice, and improved both neurological function and lifespan [124]. The extent to which Hsp70 is required for the therapeutic effect of DNAJB6 is uncertain, however these results suggest decreased aggregation is beneficial in both cellular and animal models of polyQ disease.

Over-expression of co-chaperones that promote degradation of polyQ proteins also has beneficial effects. For example, over-expression of CHIP in models of SBMA decreases polyQ

AR aggregation, increases polyQ AR ubiquitination, and enhances proteasomal degradation, leading to improved motor function [147, 148]. CHIP over-expression preferentially enhanced the proteasomal degradation of polyQ AR over wildtype, demonstrating the selectivity of Hsp70-CHIP-facilitated ubiquitination for misfolded proteins. CHIP over-expression also increased polyQ Htt ubiquitination in cells [149] and increased survival in both zebrafish and cellular models of HD [149, 150]. Additionally, over-expression of CHIP has been shown to increase ubiquitination and proteasomal degradation of polyQ proteins in cellular models of SCA3 and SCA1, and to decrease toxicity in a Drosophila model of SCA1 [151]. Surprisingly, CHIP over-expression readily increases the ubiquitination of both wild type and polyQ ATXN1 and ATXN3 [145, 151, 152]. These findings contrast with studies utilizing other polyQ proteins that have demonstrated Hsp70-CHIP-mediated ubiquitination preferentially targets misfolded proteins. With respect to ATXN3, these findings may reflect its role as a deubiquitinating enzyme. Wild type ATXN3 forms a complex with CHIP, regulating its activity by modulating CHIP-autoubiquitination [153]. Over-expression of the co-chaperone HIP, known to stabilize Hsp70 in the ADP state, also appears to enhance proteasomal degradation of polyQ proteins. HIP over-expression increased polyQ AR proteasomal degradation in cells and decreased toxicity in a Drosophila model of SBMA [154]. Over-expression of HspB8 in cellular models of SBMA increased autophagy of polyQ but not wild type AR [74]. Over-expression of HspB8 and BAG3 also enhanced the autophagic degradation of polyQ Htt in cellular models [155]. As a whole, this body of literature demonstrates that the development of pharmacological chaperones that enhance Hsp70 mediated degradation through the UPS or autophagy may be therapeutically beneficial in polyQ disease.

Small molecules targeting Hsp70

Numerous small molecule modulators of Hsp70 have been discovered (reviewed in detail in [156]); however, only a select few have been evaluated in cellular and animal models of polyQ disease (Table 1-2). Two major strategies have been evaluated thus far: activation of Hsp70-facilitated disaggregation and proteasomal degradation.

Activation of disaggregase activity

The role of polyQ protein aggregation in disease pathogenesis is a subject of ongoing debate, as some evidence suggests that the formation of large aggregates protects cells from the toxic effects of soluble polyQ proteins [157]. For several polyQ diseases there is good evidence small oligomers are the toxic species [10]. It is also proposed that later stages of aggregation can contribute to toxicity through the sequestration of proteins critical to the maintenance of proteostasis [141]. Nevertheless, as discussed in the previous section, over-expression of co-chaperones that promote Hsp70's disaggregase activity has been shown to reduce toxicity in models of polyQ disease. Therefore, one therapeutic strategy for the treatment of neurodegenerative disease is to enhance the disaggregase activity of Hsp70 [158, 159], a function that is dependent on nucleotide cycling [160]. Nadler and colleagues discovered that the immunosuppressant 15-deoxyspergualin (15-DSG), a natural product isolated from Bacillus laterosporus, binds to Hsp70 [161, 162] at the EEVD domain on the C-terminus [163]. It was later determined by Brodsky and co-workers that 15-DSG enhanced the basal rate of Hsp70 ATPase activity but did not affect the stimulation of ATPase activity by J-domain proteins [164]. While the immunosuppressant activity of 15-DSG appeared to be due to Hsp70-dependent dysregulation of NF-κB signaling [165], numerous off target effects including binding to Hsp90 [166] made it difficult to use 15-DSG as a tool to investigate the impact of enhanced Hsp70 ATPase activity.

Brodsky and co-workers utilized an *in vitro* assay for Hsp70 ATPase activity to study the effect of compounds structurally similar to 15-DSG. Groups lead by Brodsky and Gestwicki have discovered that certain dihydropyrimidine compounds activate Hsp70 ATPase activity while others inhibit. Further adding to the complexity, some of the compounds require the presence of co-chaperones for their action [167–170].

The dihydropyrimidine SW02 activates Hsp70 ATPase activity [170] and decreases Aβ aggregation measured by Thioflavin T in an *in vitro* assay for Aβ aggregation [171]. In contrast, treatment with an inhibitor of ATPase activity, the dihydropyrimidine SW08, enhances Aβ aggregation. 115-7c, a compound structurally similar to SW02, was shown to bind the NBD of Hsp70 in close proximity to the binding site of Hsp40 [172]. Treatment of yeast and PC12 cells expressing polyQ Htt with SW02 decrease aggregation of polyQ Htt, while treatment with the ATPase inhibitor CE12 increased polyQ Htt aggregation [173]. However, in the same model, SW02 enhanced and CE12 decreased polyQ related toxicity [173]. This toxicity correlated with the effect each compound had on the level of proteins, with SW02 increasing and CE12 decreasing soluble polyQ protein. Consistent with these results, treatment of HeLa cells expressing tau with the Hsp70 ATPase activators SW02 and 115-7c increased tau levels, whereas ATPase inhibitors decreased tau levels by enhancing the proteasomal degradation of tau [174]. As a whole these studies suggest that enhancing the disaggregation activity of Hsp70 leads to toxicity by increasing levels of toxic soluble polyQ species.

Enhancing proteasomal degradation

Studies with dihydropyrimidine-containing inhibitors of Hsp70 ATPase demonstrated that pharmacological enhancement of Hsp70-facilitated proteasomal degradation was feasible. Most Hsp70 ATPase inhibitors have been discovered by use of *in vitro* ATPase activity assays. These

assays look at the ability of Hsp70 to hydrolyze nucleotide with or without the presence of J-domain and NEF co-chaperones. The benzothiazine containing compound methylene blue (MB) and it's analogue azure C (AC) were discovered to inhibit ATPase activity of the Hsp70 bacterial homolog DnaK [170]. MB inhibits Hsp70 through oxidation of Cys306 (a residue not present on Hsc70), which prevents nucleotide binding [175] These compounds were first evaluated for their effect on tau. MB and AC were shown to decrease tau levels in HeLa cells, an action that is enhanced by the over-expression of Hsp70 and decreased by inhibition of the proteasome [174], and MB reduced tau aggregation and soluble tau levels in a mouse model of human tauopathy [176]. While it has been demonstrated that MB enhances the proteasomal degradation of tau, this effect has not translated to polyQ-containing Hsp90 client proteins. For example, inhibition of Hsp70 by MB decreases proteasomal degradation of polyQ AR in HeLa cells [43]. Inhibition of Hsp70-mediated proteasomal degradation by MB also induces autophagy in HeLa cells, enhancing the autophagy of N-terminal polyQ AR fragments lacking the ligand binding domain. Additionally, MB and AC increased levels of ATXN3 in a cellular model of SCA3 [145]. MB demonstrated a clinical benefit in an extrapolated phase II clinical trial of patients with moderate and mild Alzheimer's disease [177]. However, MB has numerous actions unrelated to Hsp70, including inhibition of tau-tau interactions [178] and induction of tau autophagy [179]. Therefore, it is difficult to determine the extent to which inhibition of Hsp70 contributed to clinical benefit. MB has also been studied in models of HD; MB reduced aggregation and prevented neurotoxicity in a Drosophila model and decreased levels of insoluble Htt in R6/2 mice [180]. In both models MB also increased levels of BDNF. BDNF levels have been shown to be protective in HD, thus it is unclear if the positive effects of MB were related to its action on Hsp70.

MKT-077 is a rhodacyanine dye derivative discovered by the Fuji-Chemical Company to have anti-cancer activity [181–184]. A lipophilic cation imparts a positive charge on MKT-077 causing the compound to be retained in cancer cell mitochondria, leading to mitochondrial dysfunction and contributing to its anti-cancer activity [183]. MKT-077 was taken into a phase I clinical trial, however it caused renal toxicity in patients and its clinical development was halted [185]. It was later discovered that MKT-077 binds to numerous species of the Hsp70 family including Hsc70 [186] and mtHsp70 [187]. In search of more clinically applicable compounds, numerous MKT-077 derivatives have been created [182, 188–191]. The MKT-077 derivative YM-01 has decreased retention in the mitochondria and increased localization to the cytosol, factors that are thought to contribute to improved potency in various cancer cells [182, 192]. MKT-077 and its derivatives bind selectively to the ADP state of Hsp70 at an allosteric site on the NBD, located within the interface of subdomains IA and IIA [191, 193, 194]. MKT-077 does not compete with nucleotide binding to Hsp70 [193, 195] and does not appear to alter basal ATPase activity [188, 196]. However, MKT-077 significantly decreases J-protein stimulated ATPase activity [188, 196].

In addition to anti-cancer activity, MKT-077 and YM-01 have also been shown to enhance the degradation of proteins implicated in neurodegenerative disease including polyQ AR and tau (reviewed in [10, 11, 46]). For example, MKT-077 and YM-01 reduced tau levels in primary neurons and brain slices [196]. YM-01 also reduced the levels of polyQ AR in cells and alleviated polyQ AR-mediated toxicity in a Drosophila model of SBMA [154]. YM-01 was shown to substitute for HIP in an *in vitro* assay of Hsp70-dependent activation of neuronal nitric oxide synthase [197], suggesting that, like HIP, it may stabilize the ADP-bound conformation of Hsp70. Consistent with this hypothesis, HIP competes with YM-01 for binding to Hsp70, and partial

trypsin proteolysis experiments have demonstrated YM-01 promotes an ADP-like conformation of Hsp70 [154]. Notably, preliminary studies suggest YM-01 does not inhibit other functions of the Hsp90/Hsp70 chaperone machinery, including GR-Hsp90 heterocomplex assembly and steroid binding [154]. These findings suggest that stabilization of the ADP state of Hsp70 may promote the degradation of damaged client proteins without abolishing other functions of the Hsp90 and Hsp70 chaperone complex.

Conclusion

Hsp70 has been established as an attractive therapeutic target in polyQ disease, and numerous studies, in both cellular and animal models, demonstrate that the enhancement of Hsp70 activity by the over-expression of co-chaperones can decrease polyQ protein aggregation and increase polyQ protein degradation. Therefore, small molecules that target Hsp70 are being investigated in hopes of discovering a "pharmacological co-chaperone" that can enhance Hsp70's protein quality control functions. Compounds that increase Hsp70 ATPase activity have been shown to reduce polyQ aggregation, but are also associated with increased toxicity. Compounds that inhibit Hsp70 ATPase activity have given mixed results, with some compounds increasing and others decreasing proteasomal degradation of polyQ proteins. Additionally, some inhibitors of Hsp70 ATPase activity have been shown to perturb other functions of the Hsp90/Hsp70 chaperone machinery, including the maturation of steroid receptors, and this approach has not been pursued. These findings demonstrate that a major obstacle in development of Hsp70 modulators for the treatment of polyQ disease will be designing compounds that selectively enhance Hsp70's protein quality control functions while leaving other functions critical to cell survival untouched. Preliminary results suggest that compounds that stabilize the ADP state of Hsp70, such as YM-01,

may be able to selectively promote proteasomal degradation of already unfolded proteins, including polyQ proteins, and alleviate toxicity without abolishing other Hsp70 functions.

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Tables & Figures

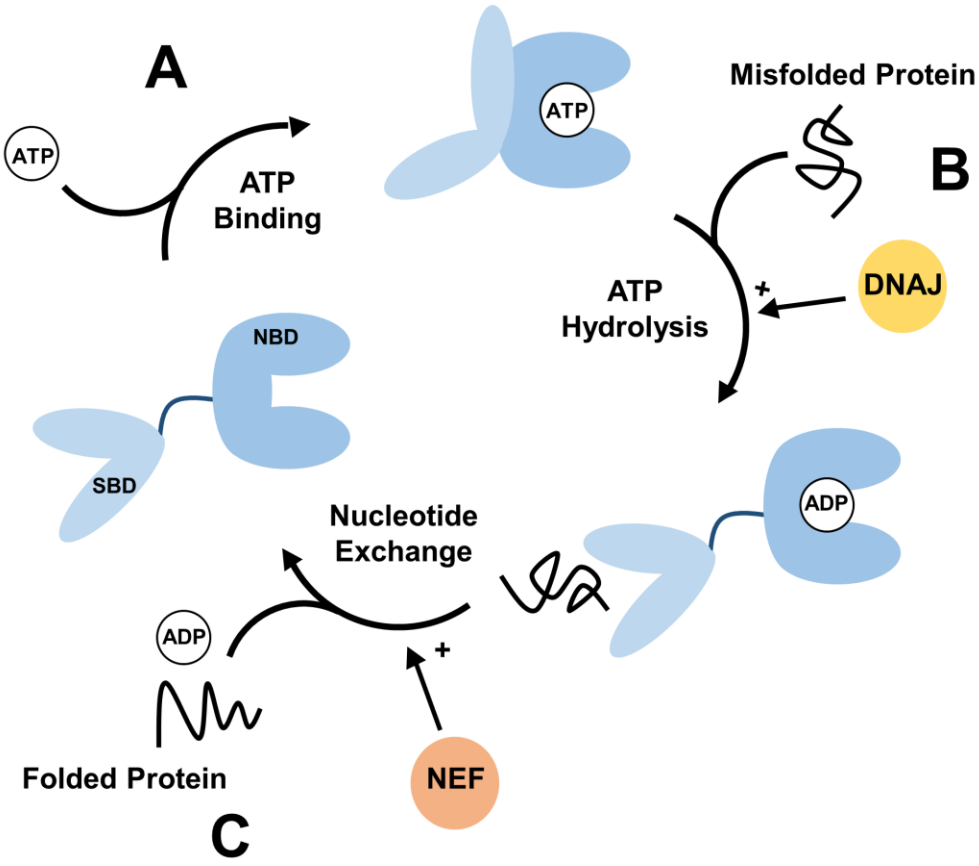


Figure 1-1. The activity of Hsp70 is driven by a nucleotide-dependent conformational cycle and is regulated by co-chaperones.

(A) Binding of ATP promotes a closed state of Hsp70 where the SBD is docked to the NBD, locking Hsp70 into a conformation with low affinity for misfolded protein. (B) ATP hydrolysis to ADP induces a large conformational change, increasing the affinity of Hsp70 for misfolded protein. DNAJ binds to and recruits substrate to Hsp70, in addition to stimulating Hsp70 ATP hydrolysis. (C) Nucleotide exchange promotes the release of protein from Hsp70, and allows the cycle to occur again. NEFs promote nucleotide exchange.

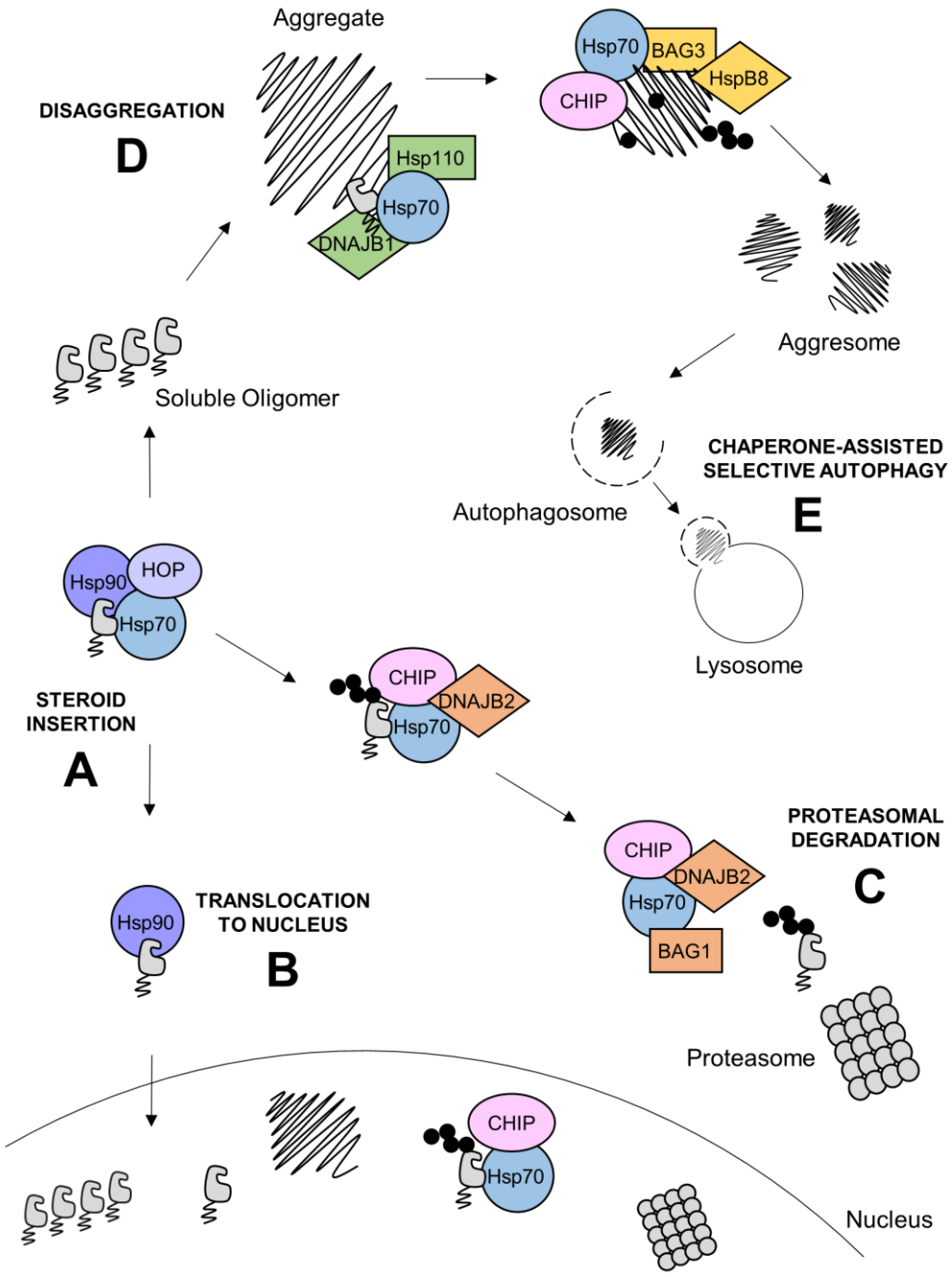


Figure 1-2. Proposed mechanism for regulation of polyQ AR protein quality control by Hsp90/70 chaperone machinery in SBMA.

Hsp90 and Hsp70, in cooperation with a multi-protein machinery including HOP, facilitate steroid insertion (A), promoting misfolding and subsequent aggregation of polyQ AR. PolyQ AR is translocated to the nucleus by an Hsp90-immunophilin-dynein complex (B), where it forms nuclear aggregates. Hsp70 facilitates the CHIP-dependent ubiquitination of poly QAR, targeting the protein for degradation by the proteasome (C); the co-chaperones DNAJB2 and BAG3 promote

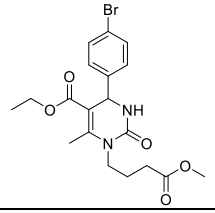
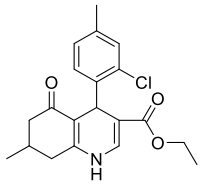
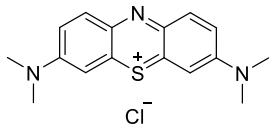
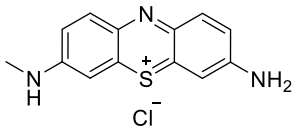
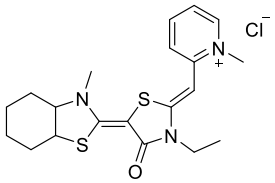
proteasomal degradation of ubiquitinated proteins. PolyQ AR that escapes Hsp70/CHIP-dependent ubiquitination and degradation via (C) forms small oligomers that coalesce into aggregates that are subject to disaggregation by the Hsp70-Hsp110-J-domain complex (D). The CHIP-Hsp70-BAG3-HspB8 complex targets polyQ AR to the aggresome leading to degradation of polyQ AR in the lysosome by chaperone-assisted selective autophagy (E)

Table 1-1. Proposed effect of co-chaperones on Hsp70-facilitated protein quality of polyQ proteins.

Co-chaperone	Effect	Ref.
J-domain containing protein (DNAJ)		
DNAJB1	Decreases aggregation	[80, 133, 77]
	Targets Hsp70 substrate to the proteasome	[93, 143]
DNAJB2	Recruits substrate to Hsp70	[51]
	Targets Hsp70 substrate to the proteasome	[51,52]
DNAJB6	Decreases aggregation*	[84, 85]
Nucleotide exchange factor (NEF)		
BAG1	Releases Hsp70 substrate to the proteasome	[53, 54]
BAG3	Facilitates the degradation of Hsp70 substrate by autophagy	[72, 111]
Hsp110	Decreases aggregation	[76, 77]
Tetratricopeptide repeat (TPR) containing proteins		
CHIP	Ubiquitinates Hsp70 substrate and promotes degradation by the proteasome or autophagy	[50, 53, 150]
HIP	Stabilizes ADP state of Hsp70 and promotes degradation by the proteasome	[155]
HOP	Coordinates the interaction of Hsp70 and Hsp90	[40]
Small heat shock protein		
HspB8	Facilitates the degradation of Hsp70 substrate by autophagy	[71, 75]

*Effect may be independent of cooperation with Hsp70

Table 1-2. Small molecules targeting Hsp70 and their effects in cellular and animal models of polyQ disease.

Compound	Structure	Mechanism of action	PolyQ protein	Model system	Outcome	Ref.
SW02		ATPase activator	Htt (46/103Q)	Yeast	↑ Toxicity ↑ Levels ↓ Aggregation	[174]
				PC12 cells	− Toxicity ↓ Aggregation	[174]
CE12		ATPase inhibitor	Htt (46/103Q)	Yeast	↓ Toxicity ↓ Levels ↑ Aggregation	[174]
				PC12 cells	↓ Toxicity ↑ Aggregation	[174]
Methylene Blue (MB)		ATPase inhibitor	AR (112Q)	HEK293T cells	↑ Levels ↓ Degradation	[43]
			ATXN3 (22/71Q)	HEK293T cells	↑ Levels	[146]
			Htt (103Q)	Primary mouse neurons	↓ Toxicity	[181]
				R6/2 mice	↓ Toxicity	
				Drosophila	↓ Toxicity	
Azure C (AC)		ATPase inhibitor	ATXN3 (Q22/71)	HEK293T Cells	↑ Levels	[146]
YM-01		Stabilizes ADP state	AR (112Q)	PC12 cells	↓ Aggregation ↓ Total levels ↑ Degradation	[155]
			AR (52Q)	Drosophila	↓ Toxicity	

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Chapter 2 : Activation of hsp70 leads to selective ubiquitination of dysfunctional forms of nNOS by CHIP

Abstract

Heme containing enzymes such as neuronal nitric oxide synthase (nNOS) are highly susceptible to damage by xenobiotics, and metabolism-based inactivation by specific environmental toxins or drugs leads to the rapid degradation of nNOS. In the course of studies on nNOS inactivation, we discovered that covalent alteration of the substrate-binding cleft leads to misfolding of the cleft and specific recognition by the heat shock protein 90 and 70 (Hsp90 and Hsp70) chaperone system. Hsp70 recruits CHIP (c-terminus of Hsp70 interacting protein), an E3 ubiquitin ligase, leading to the selective ubiquitination and proteasomal degradation of the inactivated nNOS. Conversely, stabilization of the substrate-binding cleft by slowly reversible inhibitors protects nNOS from degradation. The ability of the Hsp90 and Hsp70 chaperone system to selectively recognize and degrade dysfunctional proteins makes it an attractive therapeutic target. We have shown that pharmacological activation of Hsp70 increases the ubiquitination of nNOS in cell-based systems. However, it has yet to be investigated if activation of Hsp70 selectively increases the ubiquitination of dysfunctional forms nNOS. We developed a highly sensitive ELISA to measure nNOS ubiquitination and utilized this assay to establish a minimal purified protein system for the study of Hsp70-dependent ubiquitination of nNOS by CHIP *in vitro*. Utilizing well established pharmacological tools for manipulation of the substrate-binding cleft, we demonstrated that this purified protein system recapitulates the selective ubiquitination of dysfunctional forms of nNOS by Hsp70 that is observed *in vivo.* Activation of Hsp70 by the small molecule JG-98 increased the ubiquitination of dysfunctional nNOS, but not nNOS in its

native state. These findings demonstrate that pharmacological activation maintains the natural selectivity of Hsp70-dependent ubiquitination for dysfunctional proteins.

Introduction

Guanabenz (Wytensin) causes a dose- and time-dependent loss of neuronal nitric oxide synthase (nNOS) protein in penile tissue *in vivo* (1). Guanabenz causes the inactivation of nNOS and triggers the ubiquitination and proteasomal degradation of the enzyme in cell-based systems (2). Studies with purified nNOS show that guanabenz acts in a metabolism-based manner leading to oxidation and loss of the cofactor tetrahydrobiopterin (BH4) present in the substrate-binding cleft of the enzyme (2,3). Moreover, supplementation of BH4 abrogates the inactivation and the loss of nNOS protein *in vivo* as well as in cell-based systems (3). In the course of studies on how guanabenz causes this highly selective loss of nNOS *in vivo*, we discovered that nNOS is a client protein of Hsp90 (4) and is ubiquitinated by the Hsp70 associated E3-ligase CHIP (c-terminus of Hsp70 interacting protein) (5). We postulated that the Hsp90- and Hsp70-based quality control machinery recognizes conformational changes in the substrate-binding cleft leading to ubiquitination and proteasomal degradation, thus removing the BH4-depleted and dysfunctional nNOS (6). It is noteworthy that depletion of BH4 from cells can trigger nNOS ubiquitination (7). Interestingly, the depletion of BH4 from nNOS leads to a deleterious form of the enzyme that can cause oxidative stress (8) and the removal of such a species by ubiquitination and proteasomal degradation would be beneficial. Other metabolism-based inactivators that covalently alter the substrate-binding cleft through heme alkylation (9) as well as removal of the heme prosthetic group to form apo-nNOS (10,11) or a C331A mutation of nNOS with an altered active site conformation (12) are known to enhance the ubiquitination and degradation of nNOS. These findings further support the notion that changes in the conformation of the substrate-binding cleft trigger

recognition and the selective ubiquitination and degradation of the dysfunctional nNOS. Based in part on these studies, we wondered if activation of the Hsp70 might enhance the selective removal of deleterious client proteins. We were able to show that Hip, an Hsp70 co-chaperone that promotes client protein binding, as well as a small molecule mimic of Hip, YM-01, enhances the ubiquitination of nNOS in transfected HEK293 cells (13). However, it is not known if activation of Hsp70 leads to ubiquitination of only the dysfunctional forms of nNOS. In other words, does activation of Hsp70, which has been postulated as a potential therapeutic target (14), maintain the protein quality control function of the chaperone machinery. In the current study, we apply our knowledge of altering the active-site cleft of nNOS to probe the selectivity of ubiquitination when Hsp70 is activated.

In order to study how Hsp70 activation affects nNOS ubiquitination, we developed a facile and reproducible *in vitro* system based on ELISA detection of ubiquitin conjugates that was more sensitive and reproducible than previously established western blotting techniques. We utilized this method to detect nNOS ubiquitination from a defined reaction mixture containing purified proteins, specifically optimized to ensure that we could reproducibly measure Hsp70:CHIP-dependent ubiquitination. With the use of this system, we validated that known Hsp70 modulators such as Bag-1 and the YM-01 derivative JG-98, decreased and enhanced nNOS ubiquitination, respectively, as expected from their known actions on Hsp70. The depletion of BH4 by auto-inactivation of holo-nNOS or treatment with guanabenz lead to selective ubiquitination of the dysfunctional nNOS. Conversely, stabilization of the substrate-binding cleft by slowly reversible inhibitors prevents nNOS ubiquitination. Treatment with JG-98 increased the ubiquitination of auto-inactivated nNOS but not nNOS in its native state. Thus, activation of Hsp70 maintains the

quality control function of the chaperone machinery and selects for dysfunctional nNOS while leaving the native protein intact.

Materials and Methods

Materials

Adenosine 5′-triphosphate disodium salt hydrate (ATP), NADP+, glucose-6-phosphate, and glucose-6-phosphate-dehydrogenase and guanabenz were purchased from Sigma (St. Louis, MO). NG-Nitro-L-arginine was purchased from Cayman Chemical (Ann Arbor, MI). Sodium citrate was purchased from Fisher (Hampton, NH). Tetrahydrobiopterin (BH4) was purchased from Schircks Laboratories (Jona, Switzerland); BH4 was prepared in an aqueous solution containing equal molar dithiothreitol (DTT). NG-Nitro-D-arginine and the mono- and polyubiquitinylated conjugates monoclonal antibody (FK2) (HRP conjugate) was purchased from Enzo Life Sciences (Farmingdale, NY). DYKDDDDK-tag (FLAG) Antibody Plate (Clear, 8X12 strip) was purchased from GenScript (Piscataway, NJ). Ubiquitin activating enzyme (UBE1) was purchased from R&D Systems (Minneapolis, MN). The cDNA for expressing the GST-tagged UbcH5a (E2, ubiquitin carrier protein) was kindly provided by C. M. Pickart (Johns Hopkins Medical School, Baltimore, MD). cDNA for His-CHIP was kindly provided by C. Patterson (University of North Carolina, Chapel Hill). The cDNA for rat nNOS was kindly provided by Dr. Solomon Snyder (The Johns Hopkins Medical School, Baltimore). The cDNA for His-HA-tagged ubiquitin was from Dr. Yi Sun (University of Michigan, Ann Arbor). Human Hsp70 cDNA was kindly provided by Dr. David Toft (Mayo Clinic, Rochester, MN). Anti-nitric oxide synthase, brain (1409-1429) antibody produced in rabbit was purchased from Sigma (St. Louis, MO) and IRDye® 680RD goat anti-rabbit IgG secondary antibody was purchased from LI-COR Biosciences (Lincoln, Nebraska).

Expression and purification of proteins

GST-tagged UbcH5a was bacterially expressed and purified by GSH-Sepharose affinity chromatography as previously described (15). His-CHIP and His-HA-ubiquitin were bacterially expressed and purified by nickel-nitrilotriacetic acid affinity chromatography as previously described (16). Murine BAG-1 was expressed in E. coli and purified as previously described (17). Hsp70 was bacterially expressed and purified by ATP-agarose chromatography, adapted from (18). Hsp90 was purified from rabbit reticulocyte lysate by sequential chromatography on DE52, hydroxylapatite, and ATP-agarose as previously described (18). Rat untagged and c-terminal FLAG-tagged apo-nNOS (19) was expressed in Sf9 insect cells using a recombinant baculovirus and purified by 2′,5′-ADP-sepharose and gel-filtration chromatography as described previously (4). The same method was utilized for the generation of FLAG-tagged holo-nNOS except that exogenous heme was added to Sf9 cells (4). The FLAG-tagged and untagged nNOS was ubiquitinated to the same degree by a purified protein system, as determined by western blot analysis (data not shown). The specific activity of the FLAG-tagged and untagged nNOS were equivalent, as determined by an oxyhemoglobin assay for the measurement of nitric oxide (19).

in vitro ubiquitination reaction with purified proteins

A purified protein ubiquitination reaction containing purified FLAG-tagged nNOS (0.2 μM), Hsp70 (1.0 μM), his-CHIP (1.0 μM), E2 ubiquitin conjugating enzyme (1 μM), UBE1 (0.1 μM), His-HA-ubiquitin (100 μM), and ATP (100 μM) in a total volume of 20 μl of 50 mM Hepes pH 7.4, 100 mM KCl, and 5 mM dithiothreitol (DTT) was incubated at 22 ˚C for the specified time. Ubiquitination reactions were then analyzed by ELISA or western-blotting as described below.

ELISA for nNOS ubiquitination

Ubiquitination reactions were diluted in RIPA buffer to a final FLAG-nNOS concentration of 0.03 µM and 100 µl was added per well to an anti-FLAG 96-well plate. The plate was incubated for 4 hrs at 4 °C and then washed three times with RIPA buffer and once with PBS. Wells were treated with 100 µl anti-ubiquitin antibody conjugated to HRP (1:2,000). The plate was incubated for 1 hr at RT and then washed three times with PBS with 1% Tween20 and once with PBS. To measure HRP activity wells were treated with 100 µl TMB and absorbance at 370 nM was read every 7 seconds for 120 seconds. Raw values are shown in Fig. 2-2, but in all other figures the ELISA assay values were corrected for the non-nNOS-specific HRP signal, which is shown in Fig. 2-2, and HRP activity was reported relative to an apo-nNOS untreated control. Statistical analysis was performed with GraphPad Prism (San Diego, CA); comparisons were made by one-way anova and curves were fit with linear regression or non-linear sigmoidal 4PL.

Western blotting

Ubiquitination reactions were treated with 4X laemmli buffer and heated for 5 min at 100 ˚C. A total of 0.48 µg of FLAG-nNOS was loaded per well, resolved on a 5% (w/v) SDS-PAGE gel, and transferred to a polyvinylidene fluoride membrane for 2 h at 100 V. The blot was probed with an anti-nNOS primary antibody and a fluorescent anti-rabbit IgG secondary antibody. Higher molecular weight nNOS ubiquitin conjugates were quantified in Image Studio from LI-COR Biosciences (Lincoln, Nebraska) and values were corrected for background and reported as absorbance (arbitrary units).

Alteration of the substrate-binding cleft of nNOS

nNOS (1 µM) was incubated with or without an NADPH regenerating system containing NADP+ (400 µM), glucose-6-phosphate (10 mM), glucose-6-phosphate-dehydrogenase (1

unit/ml), and sodium citrate (10 μM), calmodulin (1 μM), and calcium (100 μM) in 50 mM Hepes pH 7.4, 100 mM KCl, and 5 mM dithiothreitol DTT for 30 min at 30 ºC. The pre-incubation was then diluted 5-fold into the purified protein *in vitro* ubiquitination reaction as described above.

Results

Purified protein system for the study of Hsp70-dependent ubiquitination of nNOS by CHIP

We sought to establish an *in vitro* method to study Hsp70-dependent ubiquitination of nNOS by CHIP. In previous studies, western blotting was utilized to measure nNOS ubiquitination as shown in Fig. 2-1. Thus, based on previous studies we incubated FLAG-tagged apo-nNOS with a purified protein mixture containing the E1 ubiquitin activating enzyme, an E2 ubiquitin-conjugating enzyme, the E3-ligase CHIP, and Hsp70 for increasing amounts of time at 22 ºC and quenched the reaction with sample buffer. Sample buffer treated reactions were then resolved by SDS-PAGE and visualized by western blotting with an anti-nNOS antibody (Fig. 2-1A). Higher molecular weight bands corresponding to nNOS-ubiquitin conjugates were then quantified (Fig. 2-1B). Due to the large amount of nNOS used for visualization by western blotting, the unmodified nNOS band tended to obscure bands with one or a few ubiquitin added, favoring the measurement of higher molecular weight poly-ubiquitin bands (Fig. 2-1A). Additionally, there was a large error associated with the quantification of higher molecular weight poly-ubiquitin bands (Fig. 2-1B).

To help overcome these limitations, we developed an ELISA to measure nNOS ubiquitination (Fig. 2-2). In this assay, FLAG-tagged apo-nNOS was incubated with the purified protein ubiquitination reaction mixture and then immobilized on an anti-FLAG 96-well plate. An anti-ubiquitin antibody conjugated to HRP, capable of detecting both mono- and poly-ubiquitin chains, was then used to probe for nNOS-ubiquitin conjugates and HRP activity was measured. A shown in Fig. 2-2A, a time course was performed with ubiquitin reaction mixtures containing

FLAG-tagged (closed circles) or untagged apo-nNOS (open circles). The signal for the mixture containing FLAG-tagged apo-nNOS increased over time while the signal for the mixture containing untagged apo-nNOS did not, demonstrating the signal is dependent on FLAG-tagged nNOS. To assess the dependence of the signal on CHIP-mediated ubiquitination, FLAG-tagged apo-nNOS was incubated for 15 min with a complete ubiquitination reaction mixture or mixtures lacking CHIP or ubiquitin (Fig. 2-2B). Omission of CHIP or ubiquitin significantly decreased HRP activity. These results demonstrate that the signal generated by the ELISA is representative of CHIP-dependent ubiquitination of FLAG-tagged nNOS. Moving forward nNOS ubiquitination was calculated by correcting for the non-nNOS-specific signal, shown in Fig. 2-2A, and reported as HRP activity relative to untreated apo-nNOS control.

One potential pitfall of utilizing a purified protein system to study Hsp70-dependent ubiquitination *in vitro* is that, in the absence of the spatial regulation present in the cell, Hsp70-independent ubiquitination by CHIP may occur (20). Simple omission of Hsp70 from the mixture does not provide an accurate measure of Hsp70-depedence as it leads to direct ubiquitination by CHIP, independent of Hsp70. To overcome this experimental limitation, we utilized the Hsp70 co-chaperone BAG-1M and the small molecule activator of Hsp70 JG-98 to probe the dependence of nNOS ubiquitination of Hsp70 function. Hsp70 function is driven by a conformational change resulting from the hydrolysis of ATP to ADP and the ADP-bound conformation of Hsp70 has high affinity for client proteins (21). BAG1-M can act as a nucleotide exchange factor, promoting the release of ADP from Hsp70 (22). While BAG1-M plays many roles *in vivo* (23), *in vitro* BAG1-M has been shown to cause the dissociation of Hsp70 from client proteins (24). As shown in Fig. 2-3A, the ubiquitination reaction mixture was treated with BAG1-M. Addition of BAG1-M caused a concentration-dependent decrease in apo-nNOS ubiquitination. The small molecule JG-98 is

thought to stabilize Hsp70 in an ADP-like state and has been shown to antagonize the effect of BAG proteins (25). Treatment of the mixture with JG-98 (closed circles) caused a concentration-dependent increase in nNOS ubiquitination (Fig. 2-3B). Treatment of the ubiquitination reaction mixture with the JG-258 (open circles), an inactive analog of JG-98, had no effect on nNOS ubiquitination (Fig. 2-3B). These findings demonstrate that the nNOS ubiquitination observed in this system is dependent on Hsp70 function.

Hsp70 preferentially ubiquitinates dysfunctional nNOS

We have demonstrated that alteration of the substrate-binding cleft by auto-inactivation and BH4 depletion triggers the ubiquitination and proteasomal degradation of nNOS (7,10,11), and that stabilization of the substrate-biding cleft by a slowly-reversible inhibitors protects nNOS from degradation (26). As shown in Fig. 2-4, we utilized well established conditions and pharmacological tools to manipulate the substrate-binding cleft of nNOS in order to determine if the minimal purified protein system for Hsp70-dependent ubiquitination preferentially ubiquitinates dysfunctional nNOS.

Auto-inactivation increases the ubiquitination of nNOS in cells and stabilization of the substrate-binding cleft by L-NNA has been shown block this effect (26). Studies *in vitro* have demonstrated that incubation of holo-nNOS with NADPH and calmodulin, in the absence of substrate, causes auto-inactivation through the generation of reactive oxygen species within the heme-containing substrate-binding cleft (8). Under these previously established conditions, we investigated the effect of auto-inactivation of nNOS on its ubiquitination (Fig. 2-4A). Flag-tagged apo-nNOS or holo-nNOS was subjected to 30 min pre-incubation at 30 ºC with and without NADPH and calmodulin. In the absence of NADPH and calmodulin the ubiquitination of apo-nNOS was significantly greater than holo-nNOS. This finding is consistent with studies in cell-

based systems that demonstrate depletion of heme from the substrate-binding cleft of nNOS significantly increases ubiquitination (10). Pre-incubation with NADPH and calmodulin significantly increased the ubiquitination of holo-nNOS. To determine if this increase in ubiquitination reflects changes within the substrate-binding cleft, holo-nNOS was pre-incubated with NADPH and calmodulin in the presence of L-NNA (Fig. 2-4A). Treatment with L-NNA blocked the effect of NADPH and calmodulin on the ubiquitination of holo-nNOS. Treatment with the inactivate stereoisomer D-NNA had no effect on the increase in ubiquitination caused by NADPH and calmodulin. These findings demonstrate that alteration of the substrate-binding cleft through auto-inactivation of increases ubiquitination of holo-nNOS.

As another way to alter the substrate-binding cleft we utilized the metabolism-based inactivator guanabenz. Guanabenz causes the oxidation and ensuing loss of BH4 from the substrate-binding cleft of nNOS (2,3), leading to rapid ubiquitination and proteasomal degradation *in vivo* (1). As shown in Fig. 2-4B, FLAG-tagged holo-nNOS was pre-incubated with or without NADPH, calmodulin, and 100 μM guanabenz. Pre-incubation with NADPH, calmodulin, and guanabenz significantly increased ubiquitination of holo-nNOS. Additionally, co-treatment with BH4 abolished the effect of guanabenz (Fig 2-4B). These findings demonstrate that depletion of BH4 from the substrate-binding cleft increases the ubiquitination of holo-nNOS. These findings demonstrate that the minimal purified protein system for Hsp70-dependent ubiquitination successfully recapitulates the selective ubiquitination of dysfunctional nNOS that is observed *in vivo.*

Pharmacological activation of Hsp70 maintains the selectivity for ubiquitination of dysfunctional nNOS

It appears that we have recapitulated the selective ubiquitination observed in cells with the minimal purified protein system we developed for Hsp70-dependent ubiquitination of nNOS by CHIP. We next sought to investigate if the activation of Hsp70 would alter the selectivity of the ubiquitination such that native functional forms of nNOS would also be ubiquitinated. In order to address this, we assessed the effect of JG98 on holo-nNOS as our representative of native protein to that of the auto-inactivated holo-nNOS obtained by redox cycling with NADPH in the presence of calmodulin as shown above in Fig. 2-4A. As shown in Fig. 2-5, JG-98 caused a marked concentration-dependent increase in the ubiquitination of auto-inactivated holo-nNOS (closed circles) as would be expected from activation of Hsp70 shown earlier for apo-nNOS. Interestingly, the native holo-nNOS was unaffected by JG98 (open circles). These results demonstrate that activation of Hsp70 by JG-98 does not abolish the selectivity of Hsp70-dependent ubiquitination.

Discussion

The Hsp90 and Hsp70 chaperone system recognizes and selectively targets dysfunctional forms of nNOS for ubiquitination and proteasomal degradation (27). We have previously demonstrated that pharmacological activation of Hsp70 can increase the ubiquitination of nNOS (13). Therefore, activation of Hsp70 may provide a therapeutic strategy to remove disease-causing forms of nNOS, such as BH4-depleted nNOS which is implicated in cardiovascular disease. However, it had yet to be investigated if pharmacological activation abolishes the natural selectivity of Hsp70 ubiquitination for dysfunctional nNOS. This is an important area of investigation, as increased ubiquitination and degradation of functional nNOS in its native state could lead to toxicity. In this study we have established a minimal purified protein system for

Hsp70-dependent ubiquitination of nNOS by CHIP. We have demonstrated that alteration of the substrate-binding cleft by auto-inactivation and BH4 depletion increases nNOS ubiquitination in this system (Fig. 2-4). Conversely, we have shown stabilization of the substrate-binding cleft by the slowly reversible inhibitor L-NNA decreases nNOS ubiquitination in this system (Fig. 2-4A). Thus, we have successfully recapitulated the selective ubiquitination of dysfunctional nNOS by Hsp70 that is observed *in vivo.* Moreover, we have demonstrated that pharmacological activation of Hsp70 by the small molecule JG-98 increases the ubiquitination of auto-inactivated nNOS but does not affect nNOS in its native state (Fig. 2-5). These findings demonstrate that pharmacological activation by JG-98 maintains the natural selectively of Hsp70-dependent ubiquitination for dysfunctional nNOS.

The Hsp90 and Hsp70 chaperones act as a protein quality control system for several 100 client proteins, many of which are implicated in disease. As with nNOS, it is hypothesized that the chaperone system surveils unstable protein-folding clefts and selectively targets damaged proteins for degradation. This hypothesis is supported by studies demonstrating mutations affecting the steroid-binding cleft of the glucocorticoid receptor (GR) (28) or the nucleotide binding-cleft of the receptor tyrosine kinases ErbB-1 and ErbB-2 (HER1 and HER2) (29,30) have been shown to alter chaperone binding. Additionally, covalent alteration of the nucleotide-binding cleft of HER2 by an irreversible inhibitor was shown to increase HER2 degradation (31). Many proteins prone to misfolding and subsequent aggregation in neurons such as tau, α-synuclein, and huntingtin are Hsp90 and Hsp70 client proteins (27). Thus, the therapeutic potential of Hsp70 activation is not limited to nNOS as it may also provide an approach to selectively degrade misfolded proteins for the treatment of neurogenerative disease.

Figures

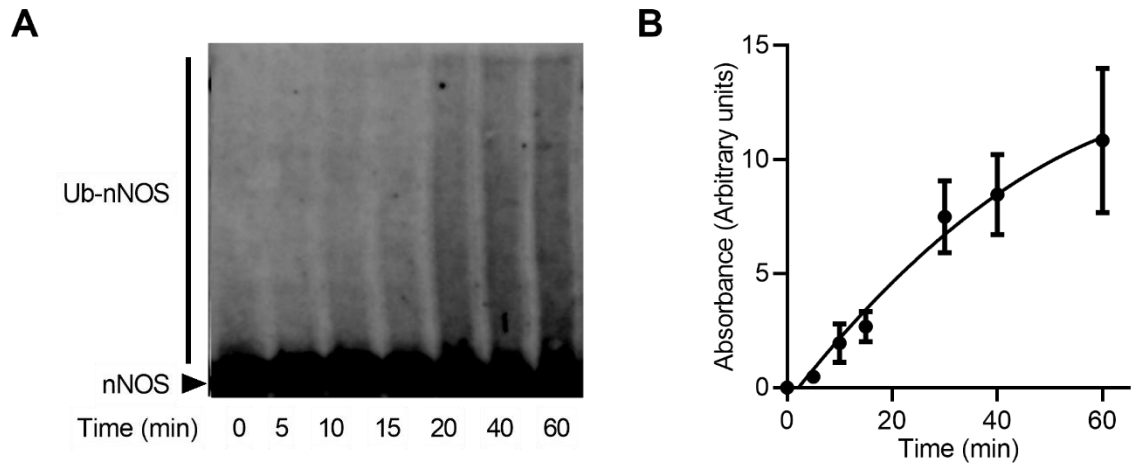

Figure 2-1. Measurement of nNOS ubiquitination by western blot.

As described in methods, FLAG-tagged apo-nNOS was incubated with a purified protein ubiquitination reaction mixture for increasing amounts of time. Resulting ubiquitin-nNOS conjugates were visualized by western blot utilizing an anti-nNOS antibody (A), and higher molecular weight mono- and poly-ubiquitin-nNOS conjugates were quantified (B). Values are reported as mean + SD, n = 3.

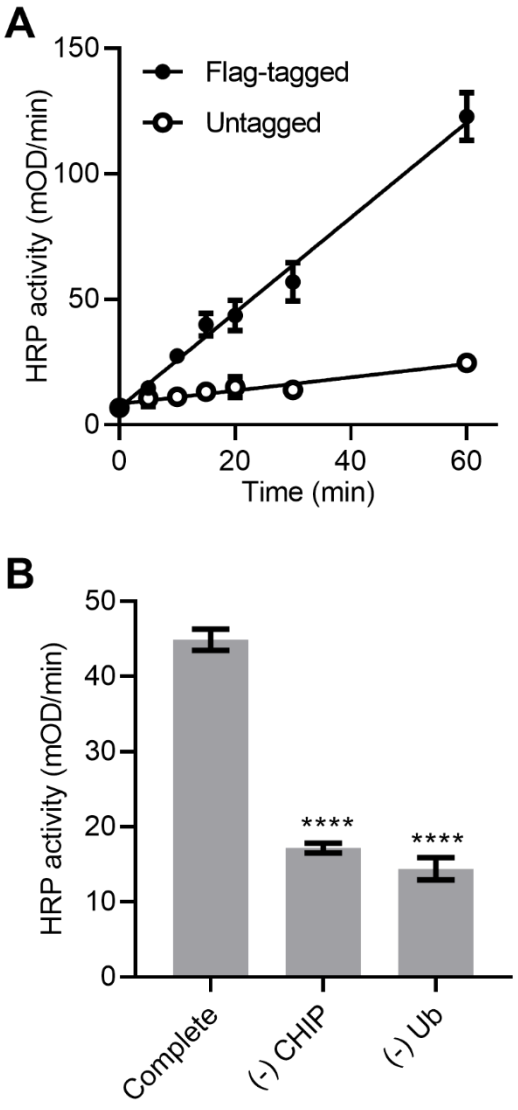

Figure 2-2. Development of an ELISA to measure CHIP-dependent ubiquitination of FLAG-tagged nNOS.

As described in methods, FLAG-tagged apo-nNOS was incubated in a purified protein ubiquitination reaction mixture and immobilized on an anti-FLAG 96-well plate. The plate was probed with an anti-ubiquitin antibody conjugated to HRP and HRP activity was quantified. A) FLAG-tagged and untagged apo-nNOS were compared to assess the nNOS-dependence of the signal. FLAG-tagged (closed circles) and untagged (open circles) apo-nNOS were incubated for increasing amounts of time with the ubiquitination reaction mixture. B) To evaluate the dependence of the signal on CHIP-mediated ubiquitination, FLAG-tagged apo-nNOS was incubated for 15 min with the ubiquitination reaction mixture and mixtures lacking CHIP or ubiquitin (Ub). Values represent mean + SD, n= 4.

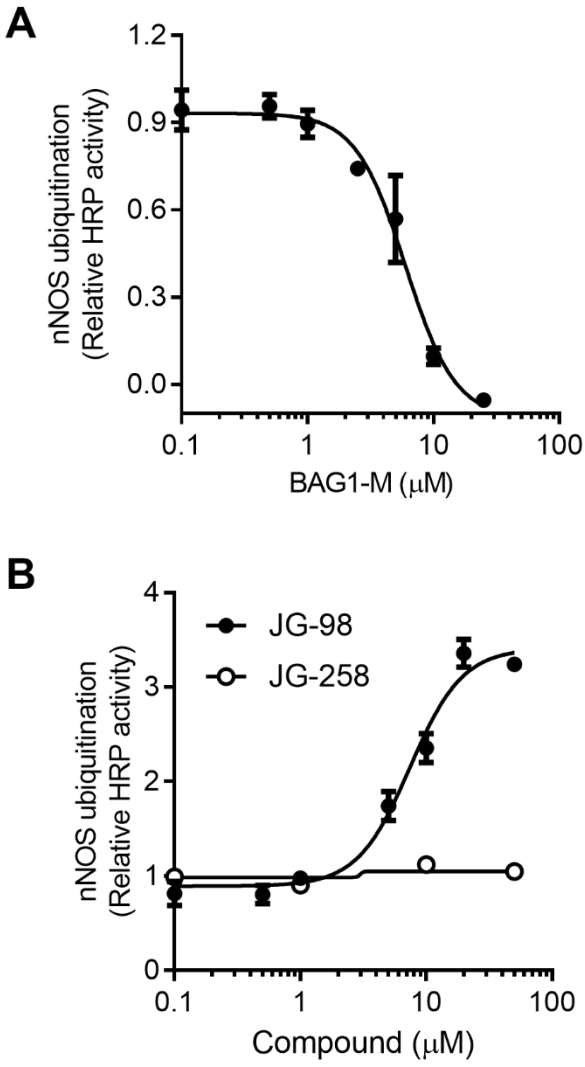

Figure 2-3. nNOS ubiquitination is dependent on Hsp70 function.

FLAG-tagged apo-nNOS was incubated with the purified protein ubiquitination reaction mixture for 15 min in the presence increasing concentrations of the Hsp70 co-chaperone BAG1-M (A), or the small molecule modulator of Hsp70 JG-98 (closed circles) and the structurally similar inactive analog JG-258 (open circles) (B). Values reported relative to untreated apo-nNOS and represent mean + SD, n= 4.

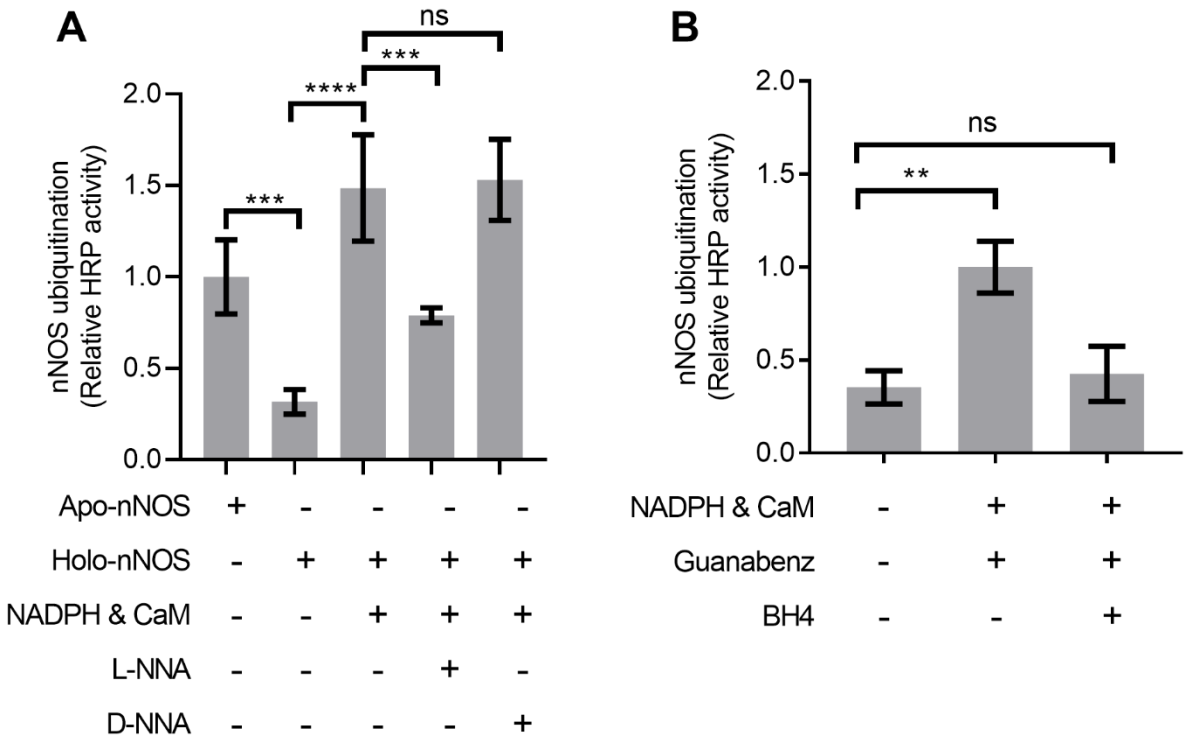

Figure 2-4. Alteration of the substrate-binding cleft by auto-inactivation and BH4 depletion increases Hsp70-dependent ubiquitination of holo-nNOS.

To investigate the effect of substrate-binding cleft alteration on nNOS ubiquitination, pre-incubations promoting autoinactivation and BH4 depletion of holo-nNOS were performed. FLAG-tagged nNOS was pre-incubated for 30 min at 30 ºC and then incubated with the purified protein *in vitro* ubiquitination mixture for an additional 30 minutes. A) The effect of auotinactivation was investigated by pre-incubating FLAG-tagged apo- or holo-nNOS with NADPH and calmodulin with and without the slowly reversible inhibitor L-NNA or its inactive stereoisomer D-NNA. B) The effect of BH4 depletion was investigated by pre-incubating holo-nNOS with the mechanism based inactivator guanabenz, NADPH, and calmodulin with and without BH4. Values reported relative to untreated apo-nNOS and represent mean + SD, n=3-6.

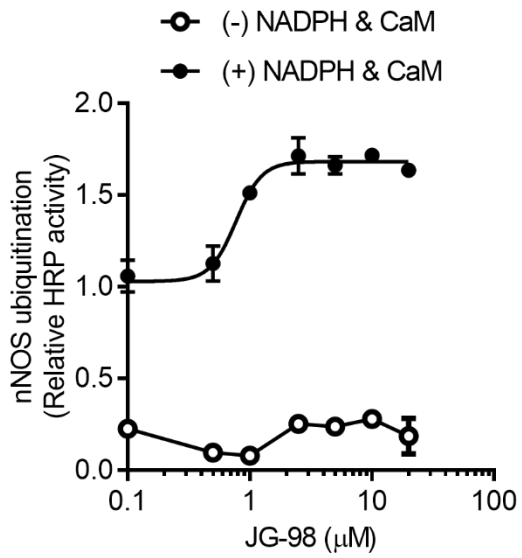

Figure 2-5. Pharmacological activation of Hsp70 selectively increases the ubiquitination of auto-inactivated nNOS.

To determine if pharmacological activation alters the selectivity of Hsp70-dependent ubiquitination, FLAG-tagged holo-nNOS was pre-incubated with and without NADPH and calmodulin. FLAG-tagged holo-nNOS was then transferred to the purified protein ubiquitination reaction mixture treated with increasing amounts of JG-98 and incubated for 15 min. Values reported relative to untreated apo-nNOS and represent mean + SD, n =3.

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Chapter 3 : Development of a workflow for the discovery of small molecule activators of Hsp70-dependent ubiquitination

Abstract

The Hsp90 and Hsp70 chaperones act as a protein quality control system for over several 100 client proteins, including many implicated in neurodegenerative disorders. We have demonstrated that activation of Hsp70 by the co-chaperone HIP, which is known to stabilize Hsp70 in the ADP-bound state, or the HIP like compound YM-01 increases ubiquitination of neuronal nitric oxide synthase (nNOS) and promotes degradation of the polyglutamine androgen receptor (polyQ-AR). Additionally, activation of Hsp70 ameliorated disease phenotype in a Drosophila model of Kennedy's disease, a rare muscular and neurodegenerative disorder caused by the misfolding and aggregation of polyQ-AR. Further pharmacological studies have been limited by the small number of compounds known to activate Hsp70. Thus, an innovative three-part workflow was developed to discover novel Hsp70 activators. In the first step of this workflow, a high-throughput thermal shift assay was utilized to identify compounds that bind and thermostabilize Hsp70. Binding of ADP greatly thermostabilizes Hsp70, therefore we hypothesized this approach could detect compounds that promote the ADP-bound state. In the second step, a Hsp90 thermal shift counter screen was utilized to evaluate the selectivity of the Hsp70 thermostabilizers. In the third step, the functional effect of the Hsp70 thermostabilizers was investigated in an *in vitro* assay for Hsp70-dependent ubiquitination of nNOS by CHIP. Of the 44,447 small molecules screened one, compound #15, was shown to thermostabilize Hsp70 and activate Hsp70-dependent ubiquitination of nNOS. The discovery of compound #15 increases the structural diversity of small

molecules known to activate Hsp70 and may provide a valuable tool for the advancement of Hsp70 activation as a therapeutic strategy.

Introduction

The heat shock protein 90 and 70 (Hsp90 and Hsp70) chaperone system regulates protein quality control for over 100 client proteins, including those implicated in neurodegenerative disease (1,2). Hsp90 and Hsp70 play opposing roles in the regulation of client protein degradation; Hsp90 stabilizes and protects proteins from degradation, whereas Hsp70 facilitates the ubiquitination of client proteins by chaperone associated E3 ligases such as CHIP (c-terminus of Hsp70 interacting protein) and subsequent degradation. We have demonstrated that overexpression of the Hsp70 co-chaperone HIP (Hsp70 interacting protein), known to stabilize Hsp70 in the ADP-bound state which has high affinity for client protein, increases Hsp70-dependent ubiquitination of nNOS by CHIP in cells (3). Overexpression of HIP also increased clearance of the poly-glutamine (polyQ) containing androgen receptor (AR) in cells and improved a disease phenotype in a Drosophila model of Kennedy's disease, a rare muscular and neurodegenerative disorder caused by the misfolding and aggregation of polyQ-AR (3). These findings, along with other genetic studies on a variety of client proteins (1), demonstrate that co-chaperone activation of Hsp70-dependent ubiquitination and degradation is therapeutically beneficial in animal models. Thus, there is great interest in the development of small molecule co-chaperones that can activate Hsp70-dependent ubiquitination.

YM-01 is a small molecule shown to bind competitively with HIP and stabilize Hsp70 in an ADP-like state *in vitro* (3). We have demonstrated that YM-01 increases the ubiquitination of nNOS and enhances the clearance of polyQ-AR in cell-based systems (3). YM-01 also increased polyQ-AR clearance and ameliorated a disease phenotype in a Drosophila model of Kennedy's

disease (3). Additionally, YM-01 has been shown to decrease tau levels both *in vitro* and *ex vivo* (4). These studies suggest that pharmacological activation of Hsp70-dependent ubiquitination and subsequent degradation of disease-causing proteins is a promising therapeutic strategy. However, YM-01 and further derivatives have failed to translate in other animal models due in part to a short half-life and mitochondrial toxicity. Thus, to advance the study of this therapeutic strategy there is a critical need for new small molecules that can enhance Hsp70-dependent ubiquitination.

We found that ADP causes thermostabilization of Hsp70 and hypothesized this could be the basis of finding compounds that bind to Hsp70 and favor the ADP-bound state. To our knowledge this is the first time a thermal shift assay has been used for the discovery of Hsp70 modulators. Therefore, we sought to design a thermal shift-based workflow that would allow us to identify novel Hsp70 modulators and directly probe their effect on Hsp70-dependent ubiquitination *in vitro*. We developed a high-throughput Hsp70 thermal shift assay and an Hsp90 thermal shift counter screen to identify compounds that bind and thermostabilize Hsp70. To test the functional effect of Hsp70 thermostabilizers we established a purified protein system for the study of Hsp70-dependent ubiquitination of nNOS by CHIP, as previously described in chapter 2. Through the use of this workflow we successfully screened 44,447 small molecules from a structurally diverse library and identified, a novel Hsp70 modulator that selectively thermostabilizes Hsp70 and activates Hsp70-dependent ubiquitination *in vitro.*

Materials and methods

Materials

1-Step™ slow TMB (3,3',5,5'-tetramethylbenzidine)-ELISA substrate solution, RIPA lysis and extraction buffer, and SYPRO™ Orange protein gel stain (5,000X Concentrate in DMSO) were purchased from Thermo Fisher Scientific (Waltham, MA). Adenosine 5′-triphosphate

disodium salt hydrate (ATP) and Adenosine 5′-diphosphate sodium salt (ADP) were purchased from Sigma (St. Louis, MO). Radicicol and VER-155008 were purchased from Cayman Chemical (Ann Arbor, MI). Mono- and polyubiquitinylated conjugates monoclonal antibody (FK2) (HRP conjugate) was purchased from Enzo Life Sciences (Farmingdale, NY). DYKDDDDK-tag (FLAG) Antibody Plate (Clear, 8X12 strip) was purchased from GenScript (Piscataway, NJ). Untreated rabbit reticulocyte lysate was purchased from Green Hectares (Oregon, WI). Ubiquitin activating enzyme (UBE1) was purchased from R&D Systems (Minneapolis, MN). The cDNA for expressing the GST-tagged UbcH5a (E2, ubiquitin carrier protein) was kindly provided by C. M. Pickart (Johns Hopkins Medical School, Baltimore, MD). cDNA for His-CHIP was kindly provided by C. Patterson (University of North Carolina, Chapel Hill). The cDNA for rat nNOS was kindly provided by Dr. Solomon Snyder (The Johns Hopkins Medical School, Baltimore). The cDNA for His-HA-tagged ubiquitin was from Dr. Yi Sun (University of Michigan, Ann Arbor). Human Hsp70 cDNA was kindly provided by Dr. David Toft (Mayo Clinic, Rochester, MN). JG-98 was kindly Provided by Jason Gestwicki (University of California, San Francisco, CA).

Expression and purification of proteins

GST-tagged UbcH5a was bacterially expressed and purified by GSH-Sepharose affinity chromatography as previously described (5). His-CHIP and His-HA-ubiquitin were bacterially expressed and purified by nickel-nitrilotriacetic acid affinity chromatography as previously described (6). Hsp70 was bacterially expressed and purified by ATP-agarose chromatography, adapted from (7). Hsp90 was purified from rabbit reticulocyte lysate by sequential chromatography on DE52, hydroxylapatite, and ATP-agarose as previously described (7). Rat FLAG-nNOS (8)

was expressed in Sf9 insect cells using a recombinant baculovirus and purified by 2′,5′-ADP-sepharose and gel-filtration chromatography as described previously (9).

Fluorescence thermal shift assay

Medium-throughput 96-well format: The melting temperature (Tm) of Hsp70 was determined by a fluorescence thermal shift assay utilizing SYPRO orange dye (λex 470 nm /λem 570 nm). A mixture of Hsp70 (1.5 µM), SYPRO orange (10X), and the small molecule to be tested was prepared in a buffer containing 50 mM potassium phosphate pH 7.4, 300 mM KCl, and 10% glycerol in a total volume of 25 µl. A CFX96 Real-Time PCR System (Biorad, Hercules, CA) was utilized to heat samples from 25 °C to 99 °C with a temperature increase of 0.3°C per minute while simultaneously measuring fluorescence. The Tm of the resulting melting curve was determined utilizing CFX Manager (Biorad). The Hsp90 thermal shift assay was carried out under the same conditions except that purified Hsp90 (1.5 µM) was used in place of Hsp70.

High-throughput 384-well format: The thermal shift assay was adapted to a high throughput 384-well plate format and 45,000 compounds from the ChemDiv and Maybridge collection at the Center for Chemical Genomics were screened at 50 µM, under the same conditions described above except that the final volume was 10 µl. A QuantStudio 7 Flex Real-Time PCR System (ThermoFisher, Waltham, MA) was utilized to heat samples from 25 °C to 80 °C with a temperature increase of 0.03 °C per second while simultaneously measuring fluorescence. Protein Thermal Shift Software 1.3 (ThermoFisher, Waltham, MA) was utilized to determine the Tm of the resulting melting curves. Samples treated with DMSO or 1 mM ADP were utilized as negative and positive controls, respectively. A Z-factor was calculated as previously described in (10). An increase in Tm greater than 3 SDs was considered a positive hit.

in vitro ubiquitination of nNOS

An ubiquitination reaction mixture containing purified FLAG-tagged nNOS (0.2 μM), Hsp70 (1.0 μM), his-CHIP (1.0 μM), E2 ubiquitin conjugating enzyme (1 μM), UBE1 (0.1 μM), His-HA-ubiquitin (100 μM), and ATP (100 μM) in a total volume of 20 μl of 50 mM Hepes pH 7.4, 100 mM KCl, and 5 mM dithiothreitol (DTT) was incubated for 15 minutes, unless specified otherwise, at 22 ˚C. Reactions were then diluted in RIPA buffer to a final FLAG-nNOS concentration of 0.03 μM and 100 μl was added per well to an anti-FLAG 96-well plate. The plate was incubated for 4 hrs at 4 °C and then washed three times with RIPA buffer and once with PBS. Wells were treated with 100 μl anti-ubiquitin antibody conjugated to HRP (1:2,000). The plate was incubated for 1 hr at RT and then washed three times with PBS containing 1% Tween20 and once with PBS. Wells were treated with 100 μl TMB and absorbance at 370 nM was measured every 15 seconds for 120 seconds. HRP activity (mOD/min) was calculated by linear regression. HRP activity was corrected for the nNOS-independent signal, normalized to untreated control, and reported as nNOS ubiquitination.

Statistical analysis

Statistical analysis was performed with GraphPad Prism (San Diego, CA); comparisons were made by one-way anova and curves were fit with a non-linear sigmoidal 4PL.

Results

High-throughput screening for small molecule thermostabilizers of Hsp70

We rationally designed a workflow for the identification of compounds that activate Hsp70-dependent ubiquitination, as outlined in Fig. 3-1. In the first step of this workflow, we utilized a thermal shift assay to screen for compounds that bind and thermostabilize Hsp70. In the

thermal shift assay purified Hsp70 is mixed with the fluorescent dye SYPRO™ orange and the temperature of the mixture is increased over time, resulting in an Hsp70 melting curve (Fig. 3-2A). As the mixture is heated Hsp70 begins to unfold exposing hydrophobic residues. Binding of SYPRO™ orange to these hydrophobic residues increases the fluorescence of the dye, which had previously been quenched by the aqueous buffer. Consequently, as the mixture is heated and Hsp70 unfolds an increase in fluorescence is observed. Upon further heating Hsp70 begins to aggregate, leading to a decrease in fluorescence. The inflection point of the increasing portion of this curve, known as the melting temperature (Tm), is a measure of Hsp70 thermostability. Binding of small molecules can cause either an increase or decrease in the thermostability of a protein. To validate the Hsp70 thermal shift assay, we investigated the effect of ADP on Hsp70 thermostability. Addition of ADP caused a rightward shift in the melting curve of Hsp70 compared to untreated control (Fig. 3-2A). The effect was dependent on the concentration of ADP as shown by an increase in the Tm of Hsp70 (EC50 = 56.45 μM, Fig. 3-2B). These results are consistent with the substantial conformational change induced by the binding of ADP to Hsp70 (11).

The Hsp70 thermal shift assay was adapted to a high-throughput platform (Fig. 3-2C). A Z-factor is a statistical characteristic for the evaluation of signal dynamic range and variation (10). Using 1 mM ADP as a positive control a Z-factor of 0.80 was obtained, this Z-score is well within the acceptable range. In collaboration with Drug Discovery and the Center for Chemical Genomics at the University of Michigan (Ann Arbor, MI), we screened 44,447 compounds from a collection of structurally diverse small molecules (Fig. 3-2C). Compounds that caused an increase in Tm greater than three standard deviations were considered positive in this assay; 2,126 positives were identified resulting in a hit ratio of 4.8% (Fig. 3-1). The hits were re-run at 5 different concentrations and 1,056 were found to produce a concentration-dependent response (Fig. 3-1).

Compounds with high EC50s and properties known to interfere with biochemical assays (such as known aggregators) were excluded, resulting in a collection of 105 high priority compounds (Fig. 3-1). These compounds were then grouped by structure similarity and 35 representative commercially available compounds were chosen for re-purchase (Fig. 3-1). For validation, the re-purchased compounds were tested at increasing concentrations, up to 500 µM, in the medium-throughput Hsp70 thermal shift assay. Of the 35 re-purchased compounds 16 caused an increase in Tm greater than three standard deviations with the change in Tm ranging from 0.9 to 2.5 ºC (Fig. 3-1, Table 3-1). For 14 of the validated compounds an EC50 was successfully calculated with the lowest and highest EC50 being equal to 9.9 µM and 258 µM, respectively (Table 3-1). Two of the validated compounds resulted in a concentration-dependent increase in Tm that did not plateau by 500 µM and therefore an EC50 could not be determined for these compounds.

Hsp90 counter screen to evaluate Hsp70 thermostabilizer selectivity

An Hsp90 counter screen was utilized to investigate the selectivity of the Hsp70 thermostabilizers identified by high-throughput screening. This assay is identical to the medium-throughput Hsp70 thermal shift assay, except that purified Hsp90 is used in place of purified Hsp70. To validate the counter screen, we utilized the known Hsp90 inhibitor radicicol (12). Radicicol binds to the N-terminal nucleotide domain of Hsp90, inhibiting Hsp90 activity, but does not bind to Hsp70. As expected, addition of radicicol caused a rightward shift in the melting curve of Hsp90 (Fig. 3-3A) but not Hsp70 (Fig. 3-3B). Radicicol caused a significant increase in the Tm of Hsp90 and not Hsp70 (Fig. 3-3C) demonstrating the assay could identify compounds that bind and thermostabilize Hsp90. The 16 validated Hsp70 thermostabilizers were tested in the Hsp90 counter screen, and the maximum change in Hsp90 Tm and corresponding EC50 were determined (Table 3-1). Seven of the validated Hsp70 thermostabilizers caused an increase in Hsp90 Tm

greater than three standard deviations, ranging from 2.3 to 15.6 ºC. As with the thermostabilization of Hsp70, the EC50 for the thermostabilization of Hsp90 by these compounds was in the mid-micromolar range, 4.1 to 35.4 µM. Thus, only 9 of the 16 validated high-throughput screening positive demonstrated selectivity for Hsp70 over Hsp90 (Fig. 3-1, Table 3-1 highlighted in grey). It is noteworthy that the effects in this counter screen do not necessarily indicate a specific effect as non-specific effects on fluorescence would also be triaged at this step.

Effect of Hsp70 thermostabilizers on Hsp70-dependent ubiquitination of nNOS by CHIP in vitro

To assess the functional effect of the Hsp70 thermostabilizers, we utilized the minimal purified protein system for Hsp70-dependent ubiquitination of nNOS by CHIP and ELISA for the measurement of nNOS ubiquitination, which was developed in chapter 2. As shown in Fig. 3-4A, FLAG-tagged apo-nNOS was incubated at 22 ºC for 15 minutes with a purified protein ubiquitination reaction mixture that was treated with 50 µM of the Hsp70 thermostabilizers and nNOS ubiquitination was measured by ELISA. JG-98 (10 µM) was utilized as a positive control and resulted in a 2-fold increase in nNOS ubiquitination (Fig. 3-4A). Of the 9 selective Hsp70 thermostabilizers identified by high-throughput screening only one, compound #15, caused a significant increase in nNOS ubiquitination at 50 µM (Fig. 3-1, Fig 3-4A). Moreover, we determined compound #15 caused a concentration-dependent 1.5-fold increase in nNOS ubiquitination with an EC50 of 11.26 µM (Fig. 3-4B). In comparison, JG-98 causes a 2-fold increase in nNOS ubiquitination with an EC50 of 7.44 µM (Chapter 2). For comparison, previously established thermal shift data (Table 3-1) were plotted in Fig. 3-4. Compound #15 caused a concentration-dependent increase in the Tm of Hsp70 (EC50 = 29.62 µM, Fig. 3-4C) but had no effect on the Tm of Hsp90 (Fig. 3-4D). Taken together, these results demonstrate that compound

#15 is a selective Hsp70 thermostabilizer that activates Hsp70-dependent ubiquitination on nNOS *in vitro.*

Discussion

Numerous genetic and limited pharmacological studies have demonstrated activation of Hsp70 increases the ubiquitination and subsequent degradation of disease-causing client proteins in cellular and animal models (1). However, further studies have been hindered by the limited number of small molecule activators of Hsp70-dependent ubiquitination available. Traditionally, high-throughput ATP hydrolysis assays have been utilized to identify novel Hsp70 modulators. However, effect on ATP hydrolysis does not always translate to activation of Hsp70-dependent ubiquitination. Additionally, compounds that affect ATP hydrolysis have the potential to interfere with critical Hsp70 functions dependent on nucleotide cycling. Thus, we utilized a high-throughput Hsp70 thermal shift assay and Hsp90 counter screen to identify compounds that bind Hsp70, independent of their effect on ATP hydrolysis. This high-throughput screening approach may provide a way to identify compounds with little or no effect on nucleotide cycling that are therefore, less likely to cause on-target side effects. Further studies will be needed to determine if Hsp70 thermostabilizers affect ATP hydrolysis.

While the thermal shift assay provides an unbiased screening approach to identify compounds that that bind Hsp70, it offers no information on the effect of a compound on Hsp70-dependent functions. In this workflow we directly probed the effect of the Hsp70 thermostabilizers identified by high-throughput screening on Hsp70-dependent ubiquitination *in vitro* utilizing the nNOS ubiquitination assay developed in chapter 2. The Hsp70 thermostabilizer compound #15 caused an increase in Hsp70-dependent ubiquitination of nNOS by CHIP. Further studies are required to determine if compound #15 also increases the ubiquitination of nNOS in cells and if

this effect translates to other Hsp70 client proteins. Elucidation of the mechanism of action by which compound #15 activates Hsp70-dependent ubiquitination may shed light on the structural-characteristics required for Hsp70 activation. These findings demonstrate that the workflow described in this study can be utilized to discover novel activators of Hsp70-dependent ubiquitination. Small molecules, like compound #15, identified by this workflow enhance the number of structurally diverse tools available to advance the study of Hsp70 activation as a therapeutic strategy.

Figures & Tables

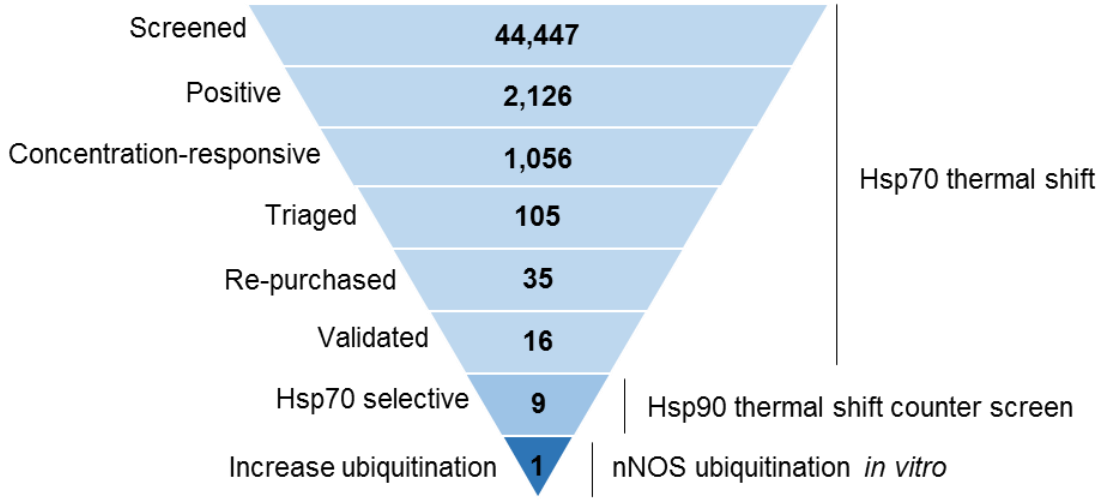

Figure 3-1. Workflow for the discovery of small molecules that activate Hsp70-dependent ubiquitination of nNOS.

In the first step of this workflow, a high-throughput thermal shift assay was utilized to screen for small molecules that bind and thermostabilize Hsp70. Next, a Hsp90 thermal shift counter screen was utilized to evaluate the selectivity of Hsp70 thermostabilizers. In the third step, the effect of selective Hsp70 thermostabilizers on Hsp70-dependent ubiquitination was investigated in an *in vitro* assay for nNOS ubiquitination. One activator of Hsp70-dependent ubiquitination was identified by this workflow.

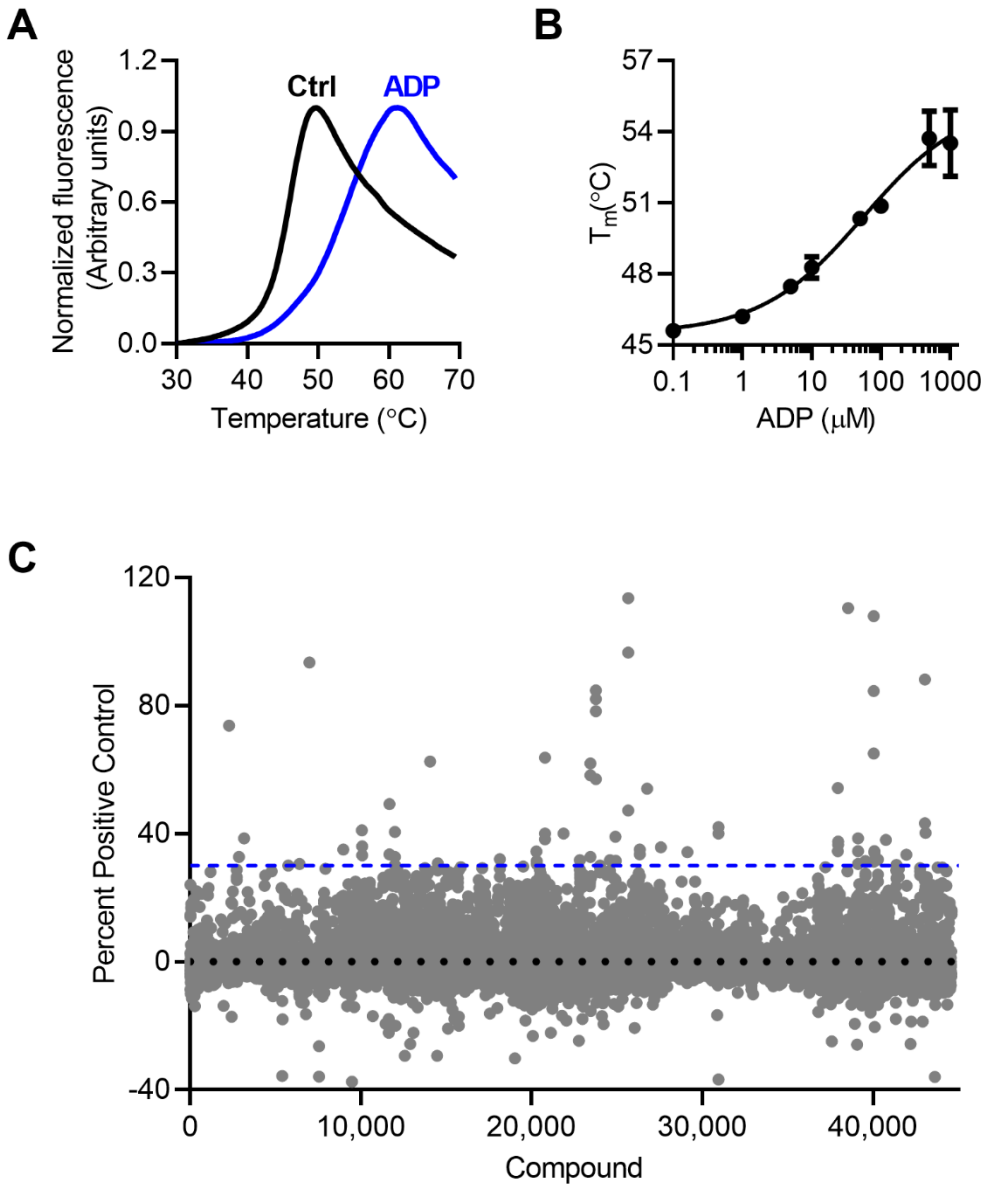

Figure 3-2. High-throughput screen for small molecules that thermostabilize Hsp70.

A thermal shift assay utilizing purified Hsp70 and the fluorescent dye SYPRO™ Orange was developed to identify compounds that bind and thermostabilize Hsp70. ADP, which causes a large conformational change in Hsp70 upon binding, was utilized to validate the assay. A) The effect of 1 mM ADP on the melting curve of Hsp70. Values reported for representative control (Ctrl, black) and ADP (blue) treated melting curves. B) . The effect of ADP on the melting temperature (Tm) of Hsp70. Values reported as mean + SD, n= 3. C) The Hsp70 thermal shift assay was adapted to a high-throughput platform and 44,447 small molecules were screened; ADP (1 mM) was utilized as a positive control. Compounds that caused a change in Tm greater than three standard deviations (blue dashed line) were considered positive.

Table 3-1. Small molecule Hsp70 thermostabilizers identified by high-throughput screening.

	Hsp70 thermal shift assay		Hsp90 counter screen	
Compound #	ΔT_m (˚C)	EC50 (µM)	ΔT_m (˚C)	EC50 (µM)
1	1.0	68.0	2.6	22.6
2	1.8	15.1		
4	2.2	> 500		
5	1.9	12.1	15.2	35.4
6	0.8	24.4		
10	0.9	11.9	2.3	4.1
12	1.0	50.7		
15	1.8	29.6		
16	1.1	51.9		
24	1.0	25.8	8.8	11.9
26	0.7	139.7	5.6	15.8
30	2.5	15.7	7.7	4.1
31	0.7	9.9		
32	1.3	> 500	7.7	33.5
33	1.6	87.7		
35	1.1	10.2		

Thermostabilizers selective for Hsp70 are highlighted in grey

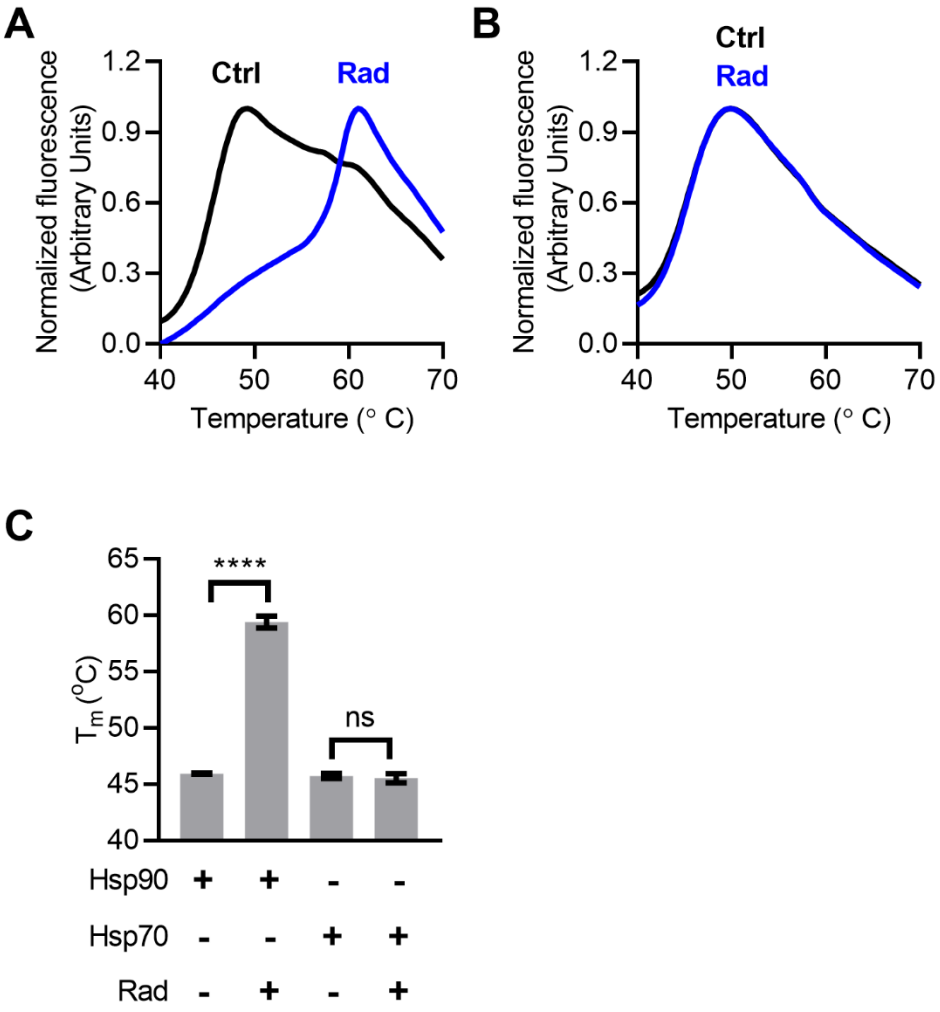

Figure 3-3. Hsp90 thermal shift counter screen to investigate selectivity of Hsp70 thermostabilizers.

A thermal shift counter screen utilizing purified Hsp90 and the fluorescent dye SYPRO™ Orange was developed to investigate the selectivity of the Hsp70 thermostabilizers identified by high-throughput screening. The counter screen was validated using the known Hsp90 inhibitor radicicol. The effect of radicicol (10 µM) on the melting curve of Hsp90 (A) or Hsp70 (B) was investigated. Values reported for representative control (Ctrl, black) and plus radicicol (Rad, blue) melting curves. C) Effect of radicicol (10 µM) on the melting temperature (Tm) of Hsp90 and Hsp70. Values reported as mean + SD, n= 3.

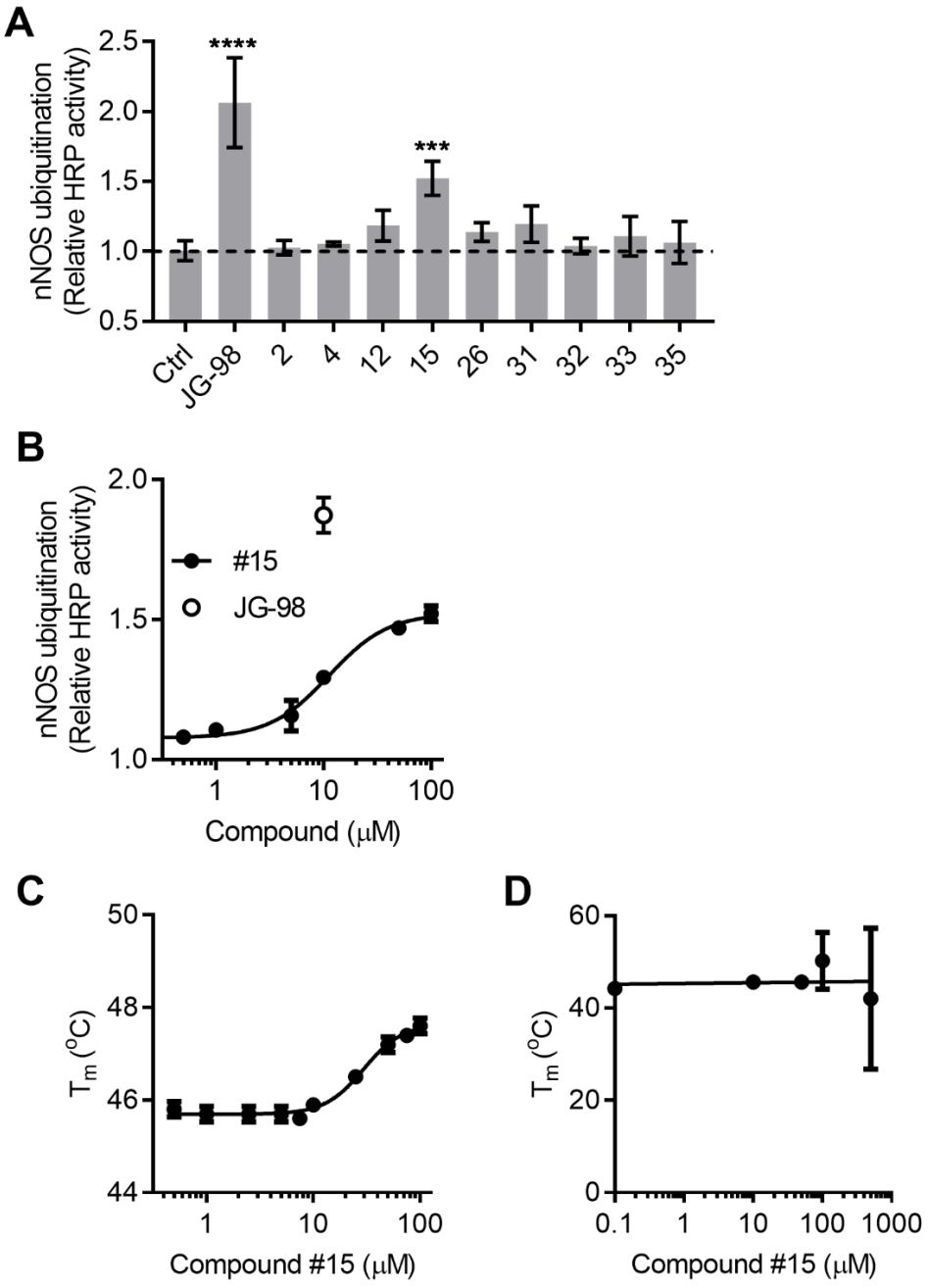

Figure 3-4. A small molecule Hsp70 thermostabilizer activates Hsp70-dependent ubiquitination of nNOS by CHIP.

The effect of the selective Hsp70 thermostabilizers on Hsp70-dependent ubiquitination was tested *in vitro*. FLAG-tagged nNOS was incubated with a purified protein ubiquitination reaction mixture and nNOS ubiquitination was measured by ELISA. A) The ubiquitination reaction mixture was treated with 50 μM of each Hsp70 selective thermostabilizers or JG-98 (10 μM) as a positive control. Compound #15 was the only selective Hsp70 thermostabilizer to significantly increase

nNOS ubiquitination. Values reported as mean + SD, n = 3-6. B) To further investigate the effect of compound #15 on Hsp70-depnent ubiquitination, the ubiquitination reaction mixture was treated with compound #15 at increasing concentrations (closed circles) or 10 µM JG-98 (open circle) as a positive control. Values reported as mean + SD, n = 3 Previous experiments demonstrated, compound #15 caused a concentration-dependent increase in the melting temperature (Tm) of Hsp70 (B) but not Hsp80 (C). Values represent mean + SD, n = 3.

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Chapter 4 : Discussion

This thesis discusses the pharmacology of heat shock protein 70 (Hsp70) activation. In chapter one, a comprehensive literature review establishes activation of Hsp70 as a promising therapeutic strategy in the treatment of polyglutamine (polyQ) disease, a group of diseases caused by the misfolding and aggregation of proteins containing abnormal polyQ expansions. Numerous genetic and limited pharmacological studies have demonstrated that activation of Hsp70 promotes the ubiquitination and subsequent proteasomal degradation of polyQ-containing proteins. This review also reveals a need for more structurally diverse small molecule activators of Hsp70, in order to further the study of this therapeutic strategy. Thus, in chapter two and three an innovative workflow for the discovery of novel Hsp70 activators was developed as illustrated in chapter 3, figure 1.

In chapter two, a method was established to study activation of Hsp70-dependent ubiquitination *in vitro.* A highly sensitive ELISA was designed to measure nNOS ubiquitination, and this assay was critical to the development of a purified protein system for Hsp70-dependent ubiquitination of neuronal nitric oxide synthase (nNOS) by c-terminus of Hsp70-interacting protein (CHIP). To our knowledge this is the first purified protein system for solely Hsp70-dependent ubiquitination of nNOS (free of non-specific ubiquitination by CHIP). Through the study of nNOS inactivation, pharmacological tools have been established manipulate the folding of the nNOS substrate biding cleft. With the use of these tools, it was demonstrated that this purified protein system re-capitulates the selective ubiquitination of misfolded nNOS observed *in vivo.* Moreover, it was shown for the first time that pharmacological activation of Hsp70

increases the ubiquitination of misfolded nNOS while leaving nNOS in its native state untouched. This method allows for the direct testing of compounds on Hsp70-dependent ubiquitination, and therefore played a key role in the discovery of novel Hsp70 activators as described in chapter 3.

In chapter three, a high-throughput Hsp70 thermal shift assay and Hsp90 counter-screen were developed to identify small molecules that bind and thermostabilize Hsp70. Thermal shift assays have previously been used to characterize conformational changes in Hsp70, therefore it was hypothesized that compounds which promote an ADP-like conformation, known to have high affinity for misfolded proteins, could be identified by this approach. Traditional high-throughput approaches have utilized ATP hydrolysis assays to identify novel Hsp70 modulators. However, compounds that alter ATP hydrolysis have the potential to interfere with critical housekeeping functions dependent on Hsp70 nucleotide cycling. Therefore, the thermal shift assay, which identifies Hsp70 modulators independent of their effect on ATP hydrolysis, may allow for the discovery of less toxic compounds. The purified protein system for Hsp70-dependent ubiquitination of nNOS was then utilized to test the functional effect of the Hsp70 thermostabilizers. Of the 44,447 compounds screened one compound, referred to as compound #15, was shown to activate Hsp70-dependent ubiquitination of nNOS.

The effect of compound #15 on Hsp70 is previously undescribed and the compound is structurally distinct from known Hsp70 activators. Structure activity relationship studies have identified the structural determinants for binding of compound #15 to Hsp70. Therefore, the structure of compound #15 has not been disclosed in this thesis to protect potential intellectual property. Further studies in cellular and animal models of disease will investigate the therapeutic potential of compound #15. These will include further studies with nNOS, which is implicated in

heart disease, as well as other client proteins of the Hsp90 and Hsp70 chaperone system, including polyQ-containing proteins.

In summary, this thesis has established an innovative workflow for the discovery of Hsp70 activators and this workflow has successfully identified a novel small molecule activator of Hsp70, compound #15. This compound increases the structural diversity of small molecules shown to activate Hsp70. Thus, compound #15, and future compounds discovered by this workflow, may provide crucial tools to advance the development of Hsp70 activation as a therapeutic strategy.