Characterization of Human Hsp70 Chaperone Complexes and Chemical Control Over Their Formation

by

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To my family

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Table of Contents

List of Figures

List of Tables

List of Abbreviations

Hsp70	Heat shock protein 70
Hsp40	Heat shock protein 40
NEF	Nucleotide exchange factor
NBD	Nucleotide Binding Domain
SBD	Substrate Binding Domain
TPR	Tetratricopeptide region
BSA	bovine serum albumin
CD	circular dichroism
DMSO	dimethyl sulfoxide
ELISA	enzyme-linked immunosorbent assay
FCPIA	Flow Cytometry Protein Interaction Assay
BAG	Bcl associated Athanogene
HTS	High Throughput Screens
PB	Pancuronium Bromide
MB	Methylene Blue
CE	Capillary Electrophoresis
CHIP	Carboxy-terminal Hsc70 Interacting Protein

ABSTRACT

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Heat shock protein 70 (Hsp70) is a molecular chaperone that plays a central role in cellular protein homeostasis by assisting in the folding and/or degradation of protein substrates. In these tasks, Hsp70 is assisted by associated co-chaperones, each of which is thought to contribute to specialized chaperone functions. However, it isn't yet clear why specific co-chaperones are recruited to the Hsp70 complex and how each distinct complex is able to carry out specific tasks. Further, imbalances in this system are thought to be involved in human diseases, such as cancer and neurodegenerative disorders. Thus, chemically controlling the formation/function of Hsp70-based chaperone complexes

could provide new insights into the logic of protein quality control and also provide new avenues for drug discovery.

The goals of this work are (a) biochemically characterize distinct Hsp70 complexes to permit insights into their specialized functions and (b) identify and characterize chemical compounds that promote the formation of specific Hsp70 complexes. Towards these goals, we devised a new approach termed “gray-box” screening, in which high throughput chemical screens are performed against reconstituted components of the Hsp70 chaperone family, facilitating the identification of compounds that target specific protein-protein interfaces in the Hsp70 complex. Using this approach, we found unexpected allosteric mechanisms for some of these compounds. For example, we found that 115-7c stimulates the ATPase and protein-folding activities of Hsp70 by promoting the action of the co-chaperone Hsp40. To further extend this screening method, we biochemically characterized ~12 distinct Hsp70 complexes formed from reconstitution of recombinant, human co-chaperones. These findings are expected to facilitate a large number of additional “gray box” screens. Finally, we extended this work to the Hsp70 complex with CHIP, a co-chaperone that is emerging as a critical regulator of the pro-degradation activities of Hsp70. Together, these studies have provided important new insights into the molecular mechanisms of Hsp70 chaperone system, including key differences between the prokaryotic and human chaperones. This work has also provided chemical probes that will be useful in understanding the roles of protein-protein interactions in the Hsp70 complexes.

Chapter 1

Introduction to the Hsp70 Chaperone Complexes and Their Use As Potential Drug Targets

1.1 Abstract

The molecular chaperone, heat shock protein 70 (Hsp70), is involved at multiple steps in a protein's life cycle, including during the processes of folding, trafficking, remodeling and degradation. To accomplish these various tasks, the activity of Hsp70 is shaped by a host of co-chaperones, which bind to the core chaperone and influence its functions. Genetic studies have strongly linked Hsp70 and its co-chaperones to numerous diseases, including cancer, neurodegeneration and microbial pathogenesis, yet the potential of this chaperone as a therapeutic target remains largely underexplored. Here, we review the current state of Hsp70 as a drug target, with a special emphasis on the important challenges and opportunities imposed by its co-chaperones, protein-protein interactions and allostery.

1.2 Structure and Function of Hsp70 Family

1.2.1 Introduction

Heat-shock protein 70 (Hsp70) is a molecular chaperone that interacts with exposed hydrophobic residues in nascent polypeptides and guides these substrates into one of several distinct fates [1-4]. This chaperone assists newly synthesized proteins during their entry into the folding pathway, but it also mediates trafficking, quality control and degradation [5-8]. In addition to its activities on nascent proteins, Hsp70 interacts with folded and partially folded substrates; for example, it is involved in nuclear hormone signaling, mediates the remodeling of multi-protein complexes and is required for clathrin re-organization during endocytosis [9-12]. Thus, Hsp70 is able to intersect with a protein during virtually every stage of its life cycle: from primary folding to function and degradation. Moreover, Hsp70 is thought to bind promiscuously to non-polar sequences, so it is able to serve as a core chaperone for the proteome [13, 14]. Because of this diversity and breadth of functions, Hsp70 is considered a central mediator of protein homeostasis.

Hsp70 is abundant (~ 1-2% of total cellular protein) and highly conserved, with approximately 50% sequence identity between prokaryotic and mammalian family members. In addition, many organisms express multiple Hsp70s (*e.g.* 13 in humans) and members are found in all the major subcellular compartments. One of the functions of Hsp70 in these regions is to protect against cellular stress. Accordingly, its expression, like that of many other heat shock proteins, is controlled by the stress-responsive

transcription factor, heat shock factor 1 (HSF1). To add functionality to this system, the Hsp70s also interact with a variety of co-chaperones. These associated factors help Hsp70's substrates find their fates and, together, this network regulates protein processing and makes the cell responsive to changes in the folding environment. However, the complexity of Hsp70's functions also creates numerous challenges in designing safe and effective therapeutics that target this chaperone [15]. In this review, we will discuss Hsp70's roles in disease and specifically focus on how structure and function studies might assist identification of therapeutic leads.

1.2.2 Domain Architecture

Hsp70 is a 70 kDa, dynamic molecular machine that hydrolyzes ATP and binds exposed hydrophobic peptides. These diverse tasks are accomplished by a relatively minimal structure composed of three major domains: a ~44 kDa N-terminal nucleotide binding domain (NBD), a ~15 kDa substrate binding domain (SBD) and a ~10 kDa C-terminal alpha helical, "lid" domain (Figure 1.1). The NBD contains the important

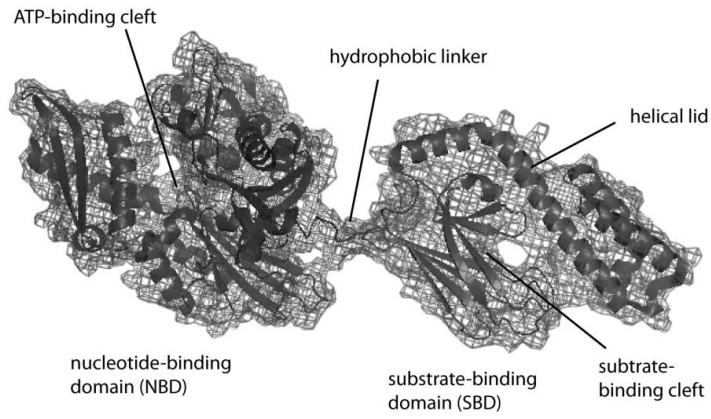

Figure 1.1 Domain architecture of heat shock protein 70 (Hsp70). An N-terminal nucleotide-binding domain (NBD) contains the nucleotide binding and hydrolysis center. The substrate-binding domain (SBD), which binds to exposed hydrophobic polypeptides, is attached through a short linker. The C-terminus of the SBD is a mostly helical subdomain, which is termed the "lid". The prokaryotic Hsp70, DnaK, is shown (PDB code #2HKO), but the general architecture appears to be conserved amongst prokaryotic and eukayrotic family members.

site of ATP binding and hydrolysis, which is critical to Hsp70's chaperone functions. When ATP is bound, the SBD and NBD exhibit coupled motion, suggestive of their tight association [16, 17]. Also in this ATP-bound form, the lid domain remains open, which facilitates transient interactions with substrates (Figure 1.2). Following ATP hydrolysis, a conformational change releases the SBD, resulting in closure of the lid and a ~10-fold increase in the affinity for substrate [18, 19]. The conformation change associated with ATP hydrolysis is communicated through a key proline switch and involves the conserved, hydrophobic linker that connects the NBD to the SBD [20]. Together, these structural and biochemical studies have begun to reveal the dynamic changes in Hsp70 that accompany nucleotide hydrolysis and substrate binding [21]. However, the intrinsic ATPase rate of Hsp70 is remarkably slow (on the order of 0.2 nmol/μg/min) [22]. This situation allows for regulation by multiple factors, including substrates and associated co-chaperones, which bind Hsp70 and modify nucleotide turnover. Because the ATPase activity of Hsp70 is central to controlling its structure and function, these regulatory interactions are likely key to understanding chaperone biology.

1.2.3 J-domain co-chaperones

The J-domain proteins are a large class of Hsp70-associated co-chaperones; there are six of these proteins in *Escherichia coli*, ~20 in *Saccharomyces cerevisiae* and ~40 in humans [23, 24]. These factors are characterized by a conserved ~70 amino acid J-domain, which is named after the founding member of the class, *E. coli* DnaJ. The main role of this domain is to stimulate the intrinsically slow ATPase activity of Hsp70 [25, 26] and the key region involved in this process is an invariant histidine-proline-aspartic

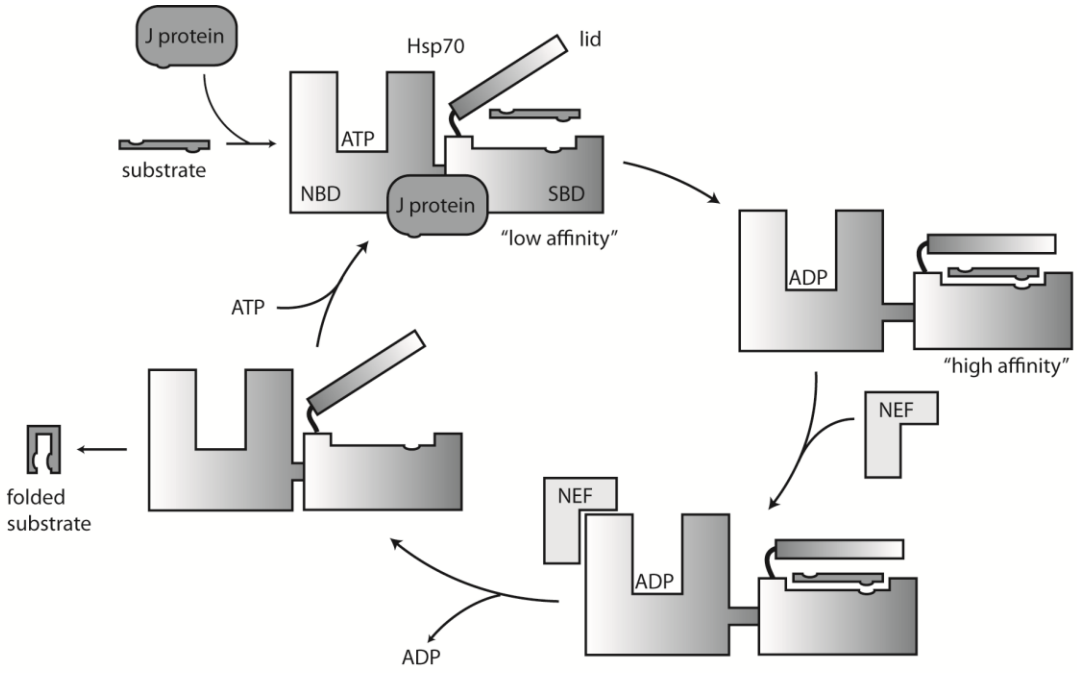

Figure 1.2 Nucleotide hydrolysis, allostery and substrate binding in the Hsp70 complex. Substrate binding in the SBD, coupled with J-domain co-chaperone interactions in the NBD, promote ATP hydrolysis. Conformational changes associated with ATP conversion close the lid and enhance affinity for the substrate. To complete the cycle, nucleotide exchange factor interacts with the NBD and assists with ADP release. The released substrate can adopt one of many fates, including proper folding (as shown).

acid (HPD) motif, which resides in a loop between helix 2 and 3 of the J-domain [27-29]. Interactions between the J-domain and Hp70's NBD stimulate ATPase activity by approximately 5- to 10-fold [22, 30], resulting in enhanced substrate affinity.

In addition to the J-domain at their N-termini, members of this co-chaperone family contain a wide variety of distinct domains at their C-termini. The identity of this C-terminal domain is used for classification; briefly, proteins in class I and II contain domains involved in dimerization and substrate binding [31, 32], while the class III members have domains with a variety of predicted functions [33]. Consistent with this diversity of functions, deletion studies have suggested that individual J protein co-

chaperones play distinct cellular roles. For example, complementation studies involving thirteen cytosolic J domain proteins revealed that at least four examples (Sis1, Jjj1, Jjj3, Cwc23) fulfill unique functions in yeast [34]. For Sis1, its C-terminal region was responsible for its specificity because fusing it to the J-domain of Ydj1 was sufficient to suppress the loss-of-function phenotype [35]. Similarly, the mammalian J-domain protein, DJA2, is competent for refolding of denatured proteins *in vitro*, but the related co-chaperone, DJA1, is not [36]. These and other studies have repeatedly shown non-overlapping roles for the J-proteins [24, 37, 38]; however, how these factors assemble and exchange on Hsp70 *in vivo* remains unclear.

1.2.4 Nucleotide exchange factors

Completion of Hsp70's ATPase cycle requires release of ADP, which is a process that is catalyzed by another class of co-chaperones, the nucleotide exchange factors (NEFs). These co-chaperones accelerate ADP release through interactions with the NBD and they can be categorized into four distinct, evolutionarily unrelated families named after representative members: GrpE, Bag1, Hsp110 and HspBP1. Prokaryotic GrpE was shown to increase the rate of release of nucleotide from the bacterial Hsp70, DnaK, without affecting the rate of hydrolysis [39]. Similar findings were shown with the mammalian counterparts [40]. Together with DnaJ, GrpE stimulates the overall ATPase activity of DnaK by ~50 fold [41], which suggests that the active, physiological chaperone is minimally composed of this multi-protein complex [42, 43]. Co-crystallization revealed that a dimer of GrpE binds asymmetrically to a single molecule of DnaK and forces the lobes of the NBD open by 14° [44]. In addition to its roles as a NEF, GrpE has also been

shown to function as a thermosensor; it reversibly unfolds in response to heat shock, thus decreasing the rate of nucleotide exchange and favoring substrate retention until conditions improve [43, 45, 46]. The second class of NEFs is named after Bag-1 (Bcl-2-associated athanogene-1), which was the first eukaryotic exchange factor to be identified [47]. This family contains 6 members in humans, characterized by a C-terminal BAG domain that interacts with Hsp70. Like GrpE, the BAG domain induces a 14° outward rotation in the NBD [48, 49]. The conservation of this mechanism is interesting because BAG domains are structurally and evolutionarily unrelated to GrpE. Importantly, the BAG family members typically possess a variety of additional protein-protein interaction motifs and, like the J-domain co-chaperones, these regions are thought to bring unique capabilities into the Hsp70 machinery. For example, Bag1 has an ubiquitin-like domain through which it interacts with the proteasome, stimulating degradation of Hsp70 substrates [50]. Although GrpE and Bag-1 share mechanistic features, the other major classes, HspBP1 and Hsp110, are both structurally and functionally distinct. For example, the co-crystal structure of HspBP1 revealed that this NEF reduces the affinity for nucleotide by displacing one lobe of the NBD [51]. Members of the Hsp110 family are structurally similar to Hsp70 and, consistent with this observation, they protect against aggregation of heat-denatured proteins in addition to their NEF functions [52-56]. Importantly, the substrate-binding activity of Hsp110 is strictly associated with ‘holdase’ outcomes and these factors are not competent in the refolding of denatured substrates. Together, these four classes of NEFs comprise a diverse array of important co-chaperones that interact in only partially overlapping regions on the Hsp70 surface. Moreover, like J-domain proteins, they possess separate, intrinsic capabilities and/or

interact with other cellular pathways to recruit Hsp70 into a variety of tasks.

1.2.5 TPR-domain proteins

The tetratricopeptide repeat (TPR) is a 34 amino acid motif that binds to a region at the extreme C-terminus of Hsp70's lid domain, which is characterized by four conserved amino acids, EEVD. Interestingly, the C-terminal EEVD motif is found in both Hsp70 and Hsp90, but not the prokaryotic DnaK or the mitochondrial or ER-resident Hsp70 isoforms. The structure of a representative TPR reveals that it interacts with the negatively charged side chains of the linear EEVD peptide [57]. In addition, residues upstream of the conserved EEVD are also recognized and these sites have been hypothesized to provide specificity [58, 59]. Binding of some TPR domains appears to be dependent on the nucleotide state of Hsp70, for example, the TPR-domain protein, Hop, binds tighter to the ADP-bound form [60]. Moreover, yeast Sti1 can actively stimulate the ATPase activity of the yeast Hsp70 Ssa1 [61], suggesting crosstalk between the TPR co-chaperones and nucleotide turnover [62]. In addition to their ability to bind Hsp70 and regulate ATP hydrolysis, the various TPR-containing proteins exhibit an array of additional activities, provided via their other domains. For example, Hop has two TPR domains and it mediates the association between Hsp70 and Hsp90 [63]. Hop-mediated substrate transfer between these major chaperone networks is thought to be critical to some functions, such as nuclear hormone receptor signaling [10, 64]. Conversely, CHIP (carboxyl terminus of Hsc70 interacting protein) is a TPR-domain protein that both inhibits J-domain-stimulated ATPase activity and also contains a U-box domain associated with E3 ubiquitin ligase activity. Through this modality, CHIP has been shown

to ubiquitinate Hsp70 substrates, diverting them to the proteasome [65-68]. Thus, CHIP functions as a chaperone-associated, quality control monitor that can tag substrates for degradation. Knockouts of CHIP are viable and show distinct phenotypes [69], suggesting that this co-chaperone plays roles that are not entirely redundant with other TPR family members.

1.2.6 Hsp70 as a Combinatorially Assembled Machine

Mammalian cells contain approximately 13 Hsp70s, over 40 J-domain proteins, at least 4 distinct types of NEFs and (conservatively) dozens of proteins with TPR-domains. At any given time, an individual Hsp70 molecule can only interact with a single representative of each major class; therefore, in theory, tens of thousands of possible complexes might be formed (Figure 1.3). Thus, a major question in chaperone biology is how these various components are assembled and whether different combinations always lead to specific outcomes. The answer to this question might lead to a better understanding of how Hsp70 can engage in its dizzying array of biological process, from folding to trafficking, endocytosis and protein turnover. Moreover, understanding the emergent properties of each complex might provide insight into how Hsp70 can act on such a wide range of substrates. Moreover, this 'combinatorial assembly' hypothesis creates a number of important challenges and opportunities for drug discovery. Although perturbation of protein-protein interactions has become increasingly common [70-72], the path to these compounds remains less straightforward. Given that Hsp70 is central to many cellular processes, effective and safe compounds might be required to act on specific Hsp70 complexes in order to avoid toxicity associated with global disruption of proteostasis.

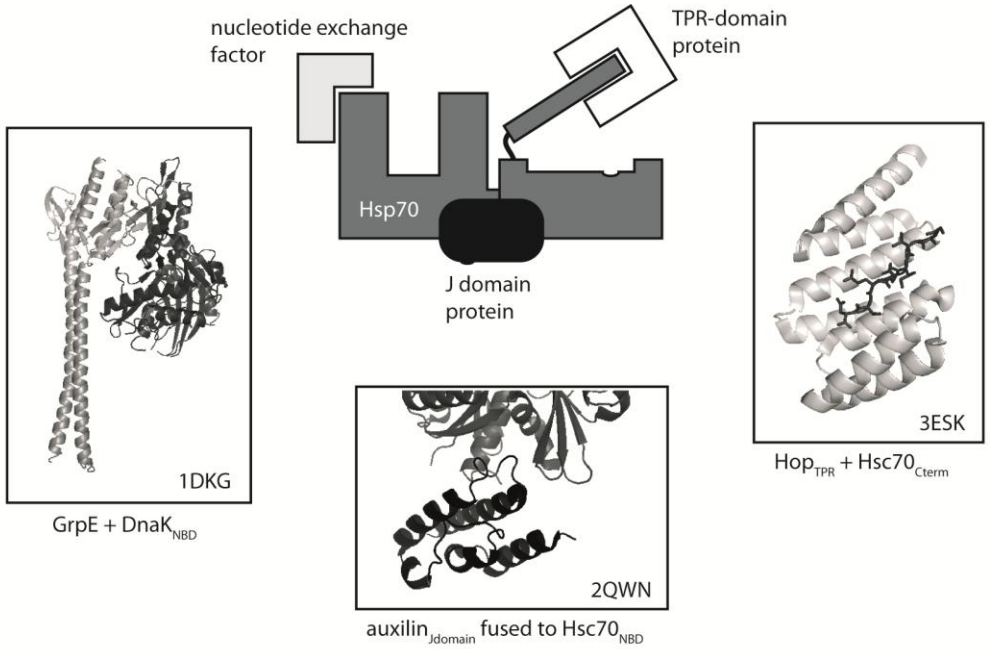

Figure 1.3 Hsp70 forms the core of a multi-protein machine. A least three distinct classes of co-chaperones interact with Hsp70 and regulate its activity, its localization and the fate of its associated substrates. Representative structures are shown to highlight the macro-molecular interactions and protein-protein surfaces involved in these regulatory conduits. The J domain co-chaperones and NEFs interact with the NBD, while the TPR-domain proteins bind the C-terminal region in the lid. In addition, each cell is thought to harbor multiple co-chaperones from each class, which suggests that combinatorial assembly can occur at these distinct surfaces. The identity of the specific structures and the corresponding PDB code numbers are included. As noted in the text, there are multiple classes of NEFs and only the GrpE interaction is pictured. All images were prepared in PyMol.

1.3 Roles of Hsp70 in Disease

Consistent with Hsp70's many roles in the maintenance of the proteome, genetic studies have implicated this chaperone in numerous diseases. In some of these instances, disease pathology is associated with "too much" Hsp70 activity (e.g. aberrant stabilization of a specific substrate). In other examples, defects appear to arise from a failure of Hsp70 to properly recognize and remove misfolded substrates. Thus, disease might arise from disruption of the proteostasis network in either direction. In this section, we will briefly outline some of the molecular roles for Hsp70 in disease and point out some of the

remarkably diverse opportunities for therapeutic intervention.

1.3.1 Cancer

Hsp72, a stress inducible Hsp70, is known to directly inhibit several steps in apoptotic signaling, including both intrinsic and extrinsic pathways [73]. Hsp72 inhibits lysosomal membrane permeabilization [74], activation and translocation of pro-apoptotic factors such as JNK, BID, BAX, or AIF, and release of cytochrome c from mitochondria. In addition, Hsp72 has been shown to suppress cellular senescence pathways [75, 76]. Consequently, its levels are elevated in several cancer cell lines and overexpression of this chaperone is correlated with poor prognosis [77] and resistance to chemotherapies [78]. Important roles have also been attributed to the mitochondrial Hsp70 isoform, mtHsp70 or Grp75, which was identified as a mortality factor in cancer and thus named "mortalin". mtHsp is over-expressed in breast, colon and colorectal cancer cells and its upregulation has been shown to induce malignant transformation in a mouse model [79, 80]. Moreover, RNAi knockdown of the chaperone causes growth arrest in human immortalized cells [81]. Finally, the ER-localized BiP has also been implicated in cancer [82]. It is highly expressed in glioblastomas and its expression exhibited a negative relationship with patient survival [83]. Recently, BiP was found to promote angiogenesis and assist proliferation of tumor cells [84]. Together, these studies illustrate Hsp70's anti-apoptotic roles, consistent with a general capacity as a stress-inducible, pro-survival factor.

Based on these studies, Hsp70 has been proposed to be a potential drug target in cancer.

In part, this model is based on parallel studies on Hsp90. Briefly, Hsp90 is thought to both enhance the cellular capacity to accommodate otherwise toxic proteins, while also preventing the onset of apoptosis by stabilizing pro-survival substrates, such as Akt [85-90]. Inhibitors of Hsp90 show selective toxicity against cancer cells *in vitro* and they are currently in multiple clinical trials [91]. Interestingly, Hsp90 in tumor cells is predominantly complexed with its co-chaperones, whereas, normal cells contain higher levels of free (or latent) Hsp90 [92]. Consistent with this concept, the Hsp90 inhibitor, 17-AAG, binds with higher affinity to the Hsp90 complex than to the latent chaperone, suggesting a mechanism for specific toxicity in cancer cells. The analogous studies have not been thoroughly performed for Hsp70, but, as discussed above, this chaperone is also known to interact with numerous substrates and co-chaperones. Moreover, Hsp90 and Hsp70 appear to share common substrates in anti-apoptotic signaling and both chaperones are over-expressed in tumor cells, presumably because of the higher demand for protein folding and the stressful microenvironments that these cells encounter. Thus, Hsp70 may also be a good target for anti-cancer strategies in much the same way as Hsp90.

One interesting aspect of Hsp90-based treatments, which has yet to be thoroughly explored for Hsp70, is the ability of some Hsp90 inhibitors to induce a compensatory stress response. Hsp90 inhibitors lead to over-expression of Hsp72 and many other heat shock proteins, through release of HSF1[93, 94]. Briefly, the transcription factor, HSF1, is held in an inactive state by the Hsp90 complex. Accumulation of misfolded substrates diverts the chaperone, triggers nuclear localization of HSF1 and upregulates targets

involved in protection from cellular stress. This feedback loop has been hypothesized to decrease the overall effectiveness of Hsp90 inhibition by enhancing the overall chaperone pool[95, 96]. Moreover, these observations suggest that the efficacy of Hsp90 inhibitors may be improved by co-administration with Hsp70 inhibitors. Consistent with this idea, Guo *et al.* used siRNA against Hsp70 to increase the efficacy of 17-AAG in cancer cells [97]. Recently, Powers *et al.* showed that simultaneous suppression of two cytosolic Hsp70s, Hsc70 and Hsp72, also sensitized cancer cells to 17-AAG [98]. Together, these findings highlight the inter-connectivity of the chaperone networks and the ability of the basic biological knowledge to assist in design of therapeutic strategies.

1.3.2 Neurodegenerative and Protein Misfolding Diseases

Many neurodegenerative disorders, such as Alzheimer's and Parkinson's diseases, are characterized by abnormal protein misfolding and accumulation [99-101]. In these disorders, misfolded proteins are not properly cleared by the chaperone / quality control system and they self-associate into cytotoxic oligomers. Conversely, indications such as cystic fibrosis and Gaucher disease involve premature protein degradation [102]. For example, certain point mutations in the cystic fibrosis transmembrane receptor (CFTR) cause misfolding and premature degradation in the secretory pathway, leading to pathology consistent with CFTR loss-of-function. Thus, many important and challenging diseases involve alterations in protein homeostasis, but some diseases might involve "too little" chaperone function while others appear to arise from "too much" of this activity. Recently, the "proteostasis boundary" hypothesis has been put forward to describe the concept of cellular protein folding capacity and its relationship with disease [103].

Briefly, the folding capacity of the cell (e.g. the chaperone network, the proteasome and related quality control systems) is normally able to catalyze the folding or timely degradation of proteins within the proteome. However, aging, mutation or environmental stress can disrupt this balance and place individual substrates beyond the boundary of the cellular capacity. According to this model, accumulation of misfolded proteins during neurodegeneration might overwhelm the network and lead to widespread failures. Alternatively, boundaries that are too restrictive might trigger loss-of-function phenotypes via premature clearance. Because of its central and diverse roles in protein processing, it isn't surprising that Hsp70 has been linked to both types of disorders [104, 105]. For example, Hsp70 has been over-expressed in *S. cerevisiae* [106], mouse [107, 108], *Caenorhabditis elegans* [109] and *Drosophila melanogaster* [110-112] models of neurodegenerative disease. In each of these experiments, high levels of Hsp70 were found to partially restore normal physiology. Similarly, pharmacological induction of Hsp70 has been found to mirror the beneficial effects of chaperone over-expression in some models. Compounds such as 17-AAG or celasterol, which stimulate a stress response and enhance expression of heat shock proteins, are sometimes protective in protein misfolding disease [113, 114]. This recovery is thought to result, in part, from enhanced capacity to process proteins, combined with enhanced pro-survival signaling. Conversely, reduction of chaperone function has been found to be beneficial in cases involving premature protein degradation; or example, mutation of either Hsp70 or the J-domain co-chaperones, *ydj1* and *hlj1*, reduces aberrant degradation of CFTR in models of cystic fibrosis [115, 116]. Thus, the relationship between Hsp70 and disease is complex, and the desired properties of a therapeutic are expected to be dependent on the specific

damage to the proteostasis boundary.

Consistent with this idea, Hsp70's regulatory co-chaperones are also thought to be important in disease. For example, the interaction of Hsp70 with the TPR-domain proteins CHIP[117-119] and BAG2[120] are important for clearance of tau, a protein implicated in Alzheimer's disease and frontotemporal dementia. Over-expression of the J-domain co-chaperone, Hsp40 (E6-AP), has been found to be sufficient to inhibit polyglutamine aggregation[106, 121]. Similarly, RNA interference of the Hsp90 co-chaperone, Aha1, also partially recovers CFTR stability [122]. These genetic findings suggest that both Hsp70 and its co-chaperones belong to a defense system that normally counteracts neurodegenerative disease. Together, these findings point to the Hsp70 complex (e.g. the core chaperone and its associated factors) as potential targets for pharmacologic intervention in neurodegenerative and protein misfolding disorders.

1.3.3 Microbial Pathogenesis

The prokaryotic Hsp70, DnaK, has been strongly linked to bacterial survival under stress [123-126]. In addition, Hsp70s have also been linked to malarial infection [127] and host-pathogen interactions [128]. Consistent with these roles, knockouts of *E. coli* DnaK are viable under normal laboratory conditions, but they are sensitive to elevated temperature [129] or addition of antibiotics [125]. Moreover, null mutations in DnaK make *Staphalococcus aureus* less efficient in mouse infection models [126] and *Streptococcus mutans dnakΔ* strains have impaired biofilm formation [124]. Consistent with the idea that the entire Hsp70 chaperone system is important in disease, knockouts of the

regulatory co-chaperone, DnaJ, also show growth defects under stress conditions [125, 130]. Together, these observations have led to the hypothesis that inhibitors of DnaK and its co-chaperones might have antibiotic applications. Importantly, the expected mechanism would be to sensitize pathogens towards additional stresses.

1.3.4 Other Diseases

In addition to being implicated in cancer, protein misfolding disorders and infection, Hsp70 and its co-chaperones have been associated with numerous other diseases, including Crohn's disease [131] and ALS [132], and this chaperone plays roles in normal aging [133, 134]. The breadth and diversity of these findings highlight the chaperone's central role in protein homeostasis. Moreover, these findings suggest that chemical modulators of Hsp70 might find use in a wide variety of indications.

1.4 Targeting the Nucleotide Binding Domain

As mentioned above, Hsp70 is composed of multiple domains, including the NBD and SBD. These domains each harbor specific activities and interact with key co-chaperones. Thus, Hsp70 is a dynamic, multi-component machine, with numerous potential locations for chemical manipulation. Accordingly, the known chemical modulators of Hsp70 share little structural similarity and they bind multiple regions of the Hsp70 protein. In addition, there are a few compounds whose binding sites have not been explored [135]. Before discussing our efforts to characterize the human Hsp70 complexes (see Chapters 3 and 4) and develop new screening methods for the identification of new compounds (see Chapter 2), it is likely useful to provide an overview of the known chemical ligands for

Hsp70 and what is known about their mechanisms of action.

1.4.1 Nucleotide mimetics

Compounds that compete with ATP for binding to Hsp70 would be expected to have significant effects on chaperone function, as this turnover is required for many of its activities. By analogy, many of the successful Hsp90 inhibitors, which have been informative in mechanistic and therapeutic studies [136, 137], are ATP-competitive. Thus, compounds that displace ATP from Hsp70 are also expected to be powerful tools [73]. Unlike Hsp90, Hsp70 binds to nucleotide using the actin-like fold, so compounds that bind Hsp90's ATP-binding sites are not cross-reactive. To solve this problem, Williamson *et al.* recently designed and synthesized adenosine-derived molecules based on the X-ray crystal structure of a commercially available ATP analog in complex with Hsc70/Bag1 [138]. Several rounds of SAR studies afforded molecules with affinity for Hsp70 that are comparable to that of ATP; for example, compound 1 showed an IC_{50} of 0.5 μM by a fluorescent polarization assay and a K_D of 0.3 μM by surface plasmon resonance (Figure 1.4). Consistent with a role for Hsp70 in cancer cell viability, this compound also exhibited a GI_{50} of 5 μM against human colon tumor (HCT) 116 cells and caused degradation of the Hsp90 clients, Her2 and Raf-1. Following the logic that Hsp90 inhibitors have been useful probes, it seems likely that these Hsp70-directed compounds will also be important reagents in the arsenal. Of course, further studies on their selectivity and potency are needed – which will be a recurring theme in these discussions, as the development of Hsp70-targeted compounds is a field in its relative infancy.

1.4.2 (-)-epigallocatechin gallate (EGCG)

EGCG is a naturally occurring polyphenolic flavonoid that has various biological activities, including its functions as an antioxidant. Among these functions, it has also been shown to interact with the ER-resident Hsp70, BiP, by affinity chromatography [139]. Subsequently, the binding site was localized to the NBD using deletion constructs and it was shown to compete with ATP for binding. Although the selectivity of this compound is uncertain, it was shown to enhance the apoptotic activity of etoposide, consistent with a role for BiP in pro-survival signaling. Thus, one of the pleiotropic activities of EGCG in cancer cells might involve partial inactivation of Hsp70 function. In support of this model, another flavonoid, myrectin, has recently been reported as an inhibitor of this chaperone[140]. This compound inhibits Hsp70's ATPase activity (IC_{50} ~ 10 μM) and initiates Hsp70-mediated degradation of the Alzheimer's disease-related substrate, tau. Over-expression of Hsp70 significantly enhanced the potency of myrectin in cell-based models, consistent with this chaperone being a target. It remains to be seen whether more selective compounds can be developed, based on these studies and the polyphenol structure.

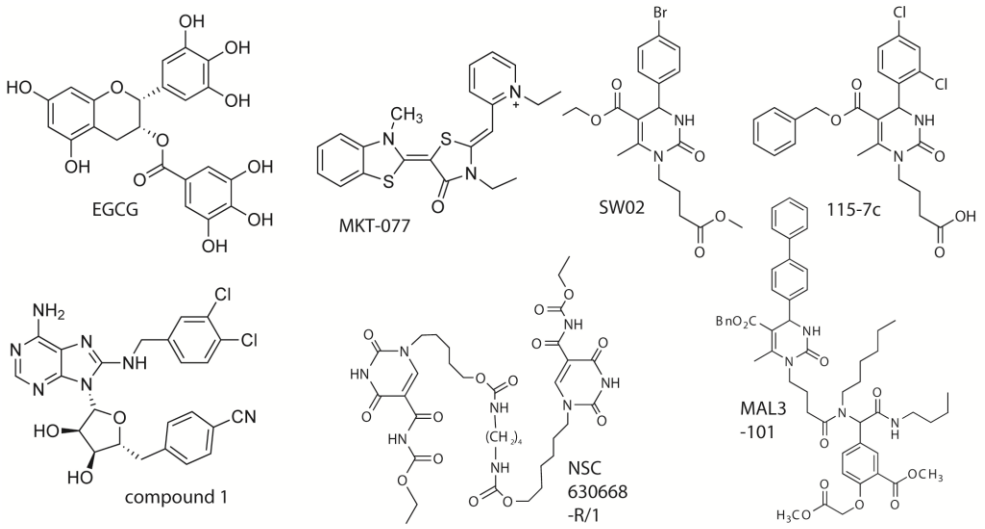

Figure 1.4 NBD Modulators. Structures of Hsp70 modulators that are thought to interact with the nucleotide-binding domain (NBD). In some cases, representative structures from a series are shown.

1.4.3 MKT-077

The NBD is also thought to be the site of action of MKT-077, a cationic dye with selective toxicity against cancer cells. Using a pull down assay, Wadhwa, *et al.* first showed that the molecule binds to mtHsp70 [141]. The same group also localized the binding site to the NBD using deletion mutants [142]. Interestingly, the binding site of MKT-077 overlapped with that of p53, suggesting that it might disrupt p53-mtHsp70 interactions and influence apoptotic signaling [143]. Selectivity wasn't reported, but the 'drug-like' nature of this scaffold warrants further investigation.

1.4.4 Sulfoglycolipids

In binding assays, sulfogalactoglycerolipid (SGG) and sulfoglactosylceramide (SGC) were identified as having affinity for the testis-specific Hsc70 [144]. Using domain deletions and site-directed mutagenesis, the binding site was determined to reside in the NBD [145]. These compounds were also found to bind Hsc70s from numerous

organisms, highlighting the potential problem of isoform selectivity in this highly conserved family. Interestingly, these compounds did not affect the K_m of ATP turnover but they did decrease V_{max}, suggesting a noncompetitive mechanism. Further structural studies will be required to understand whether these compounds operate by an allosteric binding site. However, such studies seem worthwhile, as a derivative of SGC, adamantylSGC, inhibited the ATPase activity of bovine brain Hsc70, a potential target in neurodegenerative disease [146]. Because of the similarity of these compounds to endogenous glycolipids, it is also interesting to hypothesize that they might be taking advantage of intrinsic regulatory mechanisms (*e.g.* mimicking interactions between Hsp70 and signaling lipids) [147].

1.4.5 Dihydropyrimidines

Because ATPase activity appears to be important for many chaperone functions, screens for this activity have been useful tools for identifying new inhibitors and activators [15]. In one screen of a subset of the NCI drug collection, NSC 630668-R/1 (R/1) (Figure 1.4) was identified. R/1 inhibits the endogenous and J-domain protein stimulated ATPase activity of both Ssa1p and BiP and it also blocked Hsp70-dependent protein translocation [148]. R/1 contains a keto-functionalized pyridine core and, based this general heteroaromatic structure, a more focused cheminformatic search was conducted. These efforts led to the testing of 31 small molecules, mostly peptoid-functionalized 2-dihydropyrimidinones generated by sequential Biginelli and Ugi reactions. This pilot library was screened for the ability to modulate the endogenous and J-protein stimulated ATPase activity of recombinant Ssa1p. From these efforts, compounds such as MAL3-

101 (Figure 1.4) were identified as specific inhibitors of J-domain protein stimulated Hsp70 activity. Conversely, MAL3-90 and related compounds enhance the endogenous ATPase activity but inhibit J-domain co-chaperone activity [149]. Thus, compounds from this class are able to modulate ATP turnover in either direction. Given the complexity of Hsp70 biology, access to both classes of compounds may be fortuitous. In support of this idea, dihydropyrimidines are able to tune Hsp70-mediated luciferase folding activity in either direction [150]. Following these initial studies, a focused library of functionalized dihydropyrimidines was assembled by microwave-assisted, *in situ* Biginelli cyclocondensation at the terminus of a resin-bound β-peptide. A series of di and tripeptides with a range of hydrophobic and polar side chains were utilized to explore SAR in a DnaK ATPase assay [22, 150]. These results confirmed that dihydropyrimidines operate as either stimulators or inhibitors, depending on their context. Members of this collection also showed some homolog-specific modulators, as compound 14 inhibited bovine Hsc70 but not DnaK [151]. Finally, hydrophobic groups pendant to the dihydropyrimidinone core were found to be important for activity.

Although the potency of these compounds is not yet optimized (variable IC_{50} ~ 10-200 μM), representatives of the class have been shown to possess interesting biological activities. Compound SW02 (Figure 1.4), which stimulates ATPase activity, was shown to enhance the ability of Hsp70 to block aggregation of amyloid beta, an important target in Alzheimer's disease target [152]. In another model, analogs of MAL3-101 were shown to inhibit proliferation of SK-BR-3 cell cancer cells, presumably by modulating the anti-apoptotic functions of Hsp70 [153]. A similar derivative, MAL2-11B, was shown to

inhibit the activity of a viral J-domain protein, T antigen. This activity was associated with a block in viral replication in a plaque assay, suggesting that MAL2-11B may represent a new class of polyomavirus inhibitors [154]. Select pyrimidinone-peptoid hybrid compounds also exhibit potent effects on the malaria parasite, *Plasmodium falciparum*, and were shown to inhibit its replication in human red blood cells [155]. Recently, compounds from this class have also been shown to alter processing of the microtubule-associated protein, tau. Dihydropyrimidines SW02 and 115-7c (Figure 1.4) stimulate ATPase activity *in vitro* and they also promote Hsp70-dependent tau accumulation in models of Alzheimer's disease [156]. These compounds share a halogen-modified phenyl group and a simple, pendant carboxyl associated with the dihydropyrimidine core. While further inquiries likely await synthesis of more potent derivatives, the first-generation probes have been successfully used in a diverse collection of applications.

1.5 Targeting the Substrate Binding Domain

1.5.1 Substrate mimetics

A number of short, antibacterial peptides, including drosocin, pyrrhocoricin and apidaecin, have been shown to bind *E. coli* DnaK [157]. These peptides are interesting because an analog of pyrrhocoricin exhibits broad-spectrum antibacterial activity against both gram-positive and gram-negative species. Competition experiments suggest that pyrrhocoricin (Kd ~ 50 μM) binds to DnaK at two different sites, including the substrate-binding cleft of the SBD. Using fragments of the chaperone, another binding site was

identified within the C-terminal "lid" domain. The interaction between this peptide and DnaK inhibits ATP turnover *in vitro* and folding of model substrates *in vivo* [158], but it isn't clear if these particular activities are responsible for the observed anti-bacterial function. Interestingly, pyrrhocoricin, failed to bind DnaK from *S. aureus* and it also had no anti-bacterial activity against this species, consistent with this chaperone as a target. In addition to these natural antibacterial peptides, Haney *et al.* synthesized peptides that were patterned after DnaK's model substrate, NRLLLTG [159]. One of these peptides activated the ATPase activity of yeast Hsp70 Ssa1p by 10% at 300 μM and modeling confirmed that this compound could occupy the SBD cleft. The stereochemistry of this peptide was important for function, as the opposite diasteromer was inactive. Together, these studies indicate that targeting the substrate-binding pocket is an effective strategy for manipulating chaperone function. Another interesting observation in these studies was that pyrrhocoricin had no activity against human Hsc70, suggesting that this binding site might be leveraged to gain selectivity.

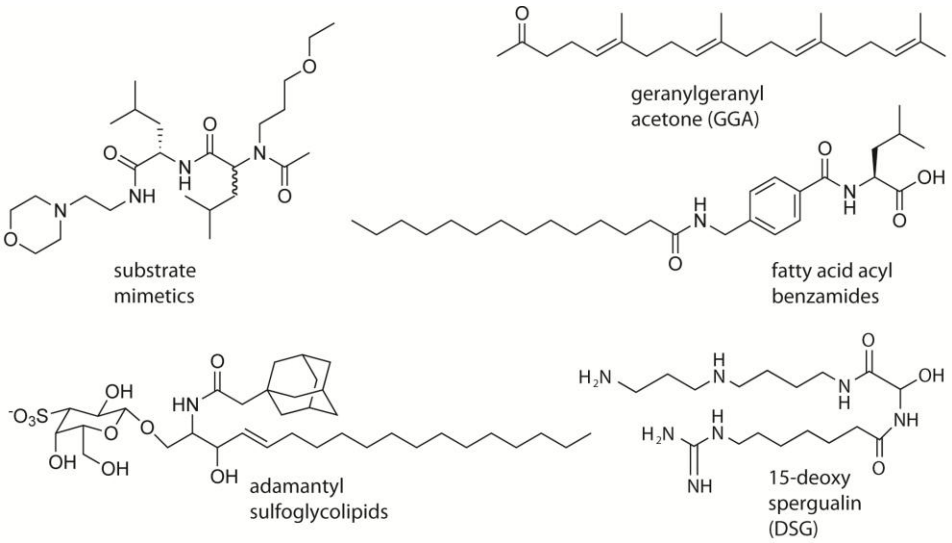

Figure 1.5 SBD Modulators. Structures of Hsp70 modulators that are thought to interact with the substrate-binding domain (SBD). In some cases, representative structures from a series are shown.

1.5.2 Geranylgeranyl acetone (GGA)

GGA (Figure 1.5) induces the expression of heat shock proteins and this compound has been used to activate the stress response [160, 161]. For many of the pharmacological inducers of the stress response, such as celasterol, their molecular mechanisms are only beginning to emerge [162, 163]. In an attempt to explore how GGA operates, this compound was immobilized onto Sepharose and potential cellular targets identified by mass spectrometry. Interestingly, the major protein was found to be Hsp70 itself [164]. Subsequent experiments using truncated domains of Hsp70 suggest that the binding site of GGA is within the SBD, but it still isn't clear why this interaction leads to a stress response. The similarity between GGA and the endogenous lipid modifications found on some proteins, such as farnesyl and geranylgeranyl groups, raises the interesting possibility that Hsp70 might natively recognize these post-translationally modified substrates, but this hypothesis has not been thoroughly explored. Moreover, like many of the scaffolds, there has not been much chemistry performed on GGA or its derivatives, so its utility as a drug lead is largely unexplored.

1.5.3 Acyl benzamides

In addition to their ATPase activity, Hsp70s are known to possess amide peptide bond cis/trans isomerase (APIase) activity [165]. This activity was specifically targeted by Liebscher *et al.*, who designed and synthesized fatty acyl benzamides, consisting of three chemical moieties; a fatty acid, an aromatic linker and an amino acid residue (Figure 1.5) [166]. One of these compounds inhibited the APIase activity of DnaK with an IC_{50} value of 2.7 μM and bound with a K_d of 2.6 μM. The compound also inhibited the chaperone-

mediated refolding of denatured firefly luciferase with an IC_{50} value of 9.5 μM. This compound was shown to compete with a model peptide for binding to DnaK, suggesting that it binds to the SBD. This compound was also tested for its antibacterial activity against *E. coli* and it was shown to decrease viability at a nonpermissive temperature (42 °C) with an MIC of ~380 μg/mL. This action mirrors the failure of *dnakΔ* strains to grow at elevated temperatures, consistent with this chaperone as a cellular target. Also consistent with anti-Hsp70 functions, SDS-PAGE experiments showed a significant increase in the amount of insoluble protein in the treated cells. To explore the potential of the acyl benzamide scaffold as a drug lead, the SAR was explored using both DnaK's APIase activity and antibacterial potency as indicators. Variations in the fatty acid moiety, including the number of carbons and unsaturations, impacted inhibition of DnaK *in vitro*, but the SAR did not correlate well with antibacterial activity. Although substitution on the benzoic acid linker did not influence the activity against DnaK, positional changes in the substituent significantly increased its MIC value, suggesting that antibacterial activity may involve targets in addition to DnaK. However, it is important to note that Hsp70 is a complex machine and the relationships between APIase activity and other *in vivo* functions are uncertain. To better understand this relationship, the focused collection was examined for effects on chaperone-mediated refolding of firefly luciferase, but again changes in the acyl benzamide structure had a dramatic influence on antibacterial activity but dissimilar effects on substrate folding efficiency. Together, these data show that acyl benzamides inhibit the refolding and APIase activities of DnaK and the growth of *E. coli* although their correlation remains to be elucidated. However, despite some mechanistic questions, these acyl benzamides appear

to be promising anti-bacterial leads. It will be interesting to see if they have selectivity for prokaryotic Hsp70 isoforms and whether they might be used in other systems to manipulate APIase and chaperone functions.

1.5.4 Spergualin Derivatives

The natural product spergualin was first identified as an antibiotic from culture filtrates of *Bacillus laterosporus* BMG162-aF2 [167]. Subsequently its structure was determined to be (15S)-1-amino-19-guanidino-11,15-dihydroxy-4,912-triazanonadecane-10,13-dione and only the (-)-spergualin enantiomer was found to be active. The Umezawa group accomplished the first total synthesis of spergualin and its analogues by the acid-catalyzed condensation of ω-guanidino alkanamides with glyoxyloylspermidine. They showed that both the carbon chain length in the polyamine region and the 15-hydroxyl group affected activity [168, 169]. Using these compounds in T-cell assays, they focused on (-)-15-deoxyspergualin (DSG) (Figure 1.5) as a potent immunosuppressive agent [170]. To identify the cellular targets of DSG, human T-cell lysates were analyzed by affinity chromatography using Sepharose beads covalently coupled to methoxy-DSG [171]. Using Western blotting and peptide sequencing, the interacting protein was identified to be Hsc70. This interaction was further characterized through affinity capillary electrophoresis to obtain binding affinities for purified Hsc70 (K_d = 4 μM). Further studies revealed that DSG and its analogs stimulate the ATPase activity of Hsc70 (~2-fold; Km = 3 μM), but had no effect on stimulation by J-domain co-chaperones or on the release of substrate [172]. To further clarify the binding site of the compound, ^{14}C-DSG was cross-linked to purified bovine Hsc70 by addition of EDC (1-ethyl-3-(3-

dimethoxyaminopropyl)-carbodiimide). Interestingly, mass spectrometry analysis of the resulting peptide fragments localized the binding site to the C-terminal amino acids, EEVD, which is the same site of binding to the TPR-domain co-chaperones. Consistent with this binding site, DSG was also found to bind Hsp90 with an affinity of 5 μM [173].

DSG is likely the Hsp70 modulator with the best-characterized clinical utility. Although DSG was originally identified as a potential anti-bacterial agent, it also has anti-tumor activity and it has found clinical use in organ transplantation and autoimmune diseases [174-176]. DSG prolongs survival time after allogenic graft and minimizes acute rejection. Unfortunately, its mechanism-of-action is poorly understood and the potential roles played by its Hsp70/90 binding are unclear. One possibility arises from observations that DSG interferes with antigen processing/presentation by monocytes, which is an Hsp70-dependent process [177]. Alternatively, DSG has been found to block B cell development by inhibiting the nuclear translocation of transcription factor NF-κB [178], another activity that may involve Hsp70 and Hsp90. Similarly, it was shown that DSG kills the malaria parasite by interfering with the trafficking of essential proteins [179]. Unlike other immunosuppressive agents, such as FK506 and cyclosporin A, DSG does not suppress cytokine production (IL-1, IL-2) but does inhibit cytotoxic lymphocyte (CTL) induction [180]. Consistent with this model, DSG prophylaxis with FK506 improves long-term graft survival in renal transplant patients [181]. Unfortunately, DSG has a very low bioavailability (<5%) and is very unstable in aqueous solution because of its labile hydroxylglycine group. To address this issue, Renaut *et al*., developed an alternative synthetic route in which this group was substituted by a malonyl unit. In a

graft-versus-host disease (GVHD) model in mice, these derivatives were found to be significantly more stable, although the SAR around the spermidine moiety is restricted [182]. One of the lead compounds, LF 15-0195, was less toxic and more potent than DSG in a renal allograft rejection primate model, suggesting that further chemical optimization might improve the performance of these compounds [183]. Thus, the strong biological data around these compounds make a case for further development. At the same time, there are many uncertainties in their molecular mechanism-of-action and there are pharmacological hurdles to overcome.

1.6 Targeting the Co-Chaperones

1.6.1 J-protein substrate mimetics

As discussed above, J-domain proteins deliver substrate peptides to Hsp70 and stimulate ATP turnover. Consistent with the importance of these functions, Bischofberger *et al.* reported that D-peptides targeting *E. coli* DnaJ inhibit chaperone-dependent luciferase refolding *in vitro* [184]. The peptides were further shown to inhibit DnaJ stimulated ATPase activity of DnaK, which genetic studies have shown to be critical under physiological conditions. These compounds are thought to compete with natural substrates for binding to DnaJ, thus preventing its capacity to engage in productive interactions with the chaperone complex. These results highlight one of the interesting aspects of Hsp70 as a target; compounds might impact Hsp70 functions indirectly via the essential co-chaperones. Such approaches might be expected to have lower toxicity and greater selectivity, although this model remains to be tested.

1.6.2 Inhibitors of the TPR domain

Another strategy that has been reported recently is direct targeting of the TPR-domain. Using an AlphaScreen high-throughput approach, Yi and Regan identified pyrimidotriazinediones that interfere with the Hop-Hsp90 interaction [185]. These compounds were toxic to WST-1 cells *in vitro*, consistent with an important role for this contact. In theory, a similar route could be used to develop selective inhibitors that decouple this class of co-chaperones from their contact with Hsp70.

1.7 Concluding Remarks

Hsp70 regulates multiple aspects of protein homeostasis. As such, it has been linked to numerous diseases and it has been suggested as a potential therapeutic target for many indications. Genetic and biochemical studies support this model and limited pharmacological findings have also provided intriguing insights. However, the discovery of potent, selective and well-characterized Hsp70 modulators remains an on-going task. Given recent clinical success with other proteins in the proteostasis network, such as Hsp90 or the proteasome, aggressive pursuit of Hsp70 seems worthwhile. The challenge will be to identify potent and selective chemical scaffolds.

In this Chapter, we have focused on the interesting structural biology of Hsp70 and its many co-chaperones and how these complexes might provide unique opportunities for drug discovery. Specifically, Hsp70 operates as part of a combinatorial, multi-protein complex, with protein-protein interfaces, allosteric sites, a catalytic center and a distinct substrate-binding cleft. Partly owing to this structural and regulatory complexity, many

different chemical scaffolds have been reported to interact with Hsp70 and their binding sites are spread across the chaperone's surface. Importantly, many of these first-generation compounds have biological activities and both activators and inhibitors appear to have useful applications. While the complexity of Hsp70 makes it a challenging target, the same complexity also creates opportunities to design molecules with interesting capabilities. Based on the limited evidence collected thus far, we suggest that the ultimate toolbox of Hsp70-targeted compounds will include compounds that operate at distinct sites. Moreover, each disease system might require a specific Hsp70 modulator that tunes the proteostasis boundary to the desired and appropriate level. Thus, the goal of this pharmacological intervention will be to restore balance to the system and the number and diversity of chemical probes may need to mirror the complexity of the biology. In the remaining Chapters of this thesis, we will explore efforts towards this goal.

Notes

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Chapter 2

‘Gray Box’ Screening Strategies For Targeting Hsp70 Chaperone Complexes

2.1 Abstract

As discussed in Chapter 1, Heat shock protein 70 (Hsp70) is a molecular chaperone that plays multiple roles in protein homeostasis. In these tasks, the activity of Hsp70 is shaped by its interactions with co-chaperones, such as the J-domain containing Hsp40 proteins and nucleotide exchange factor (NEF’s). To identify chemicals that target these critical protein-protein interactions, we devised a novel approach termed “gray-box” screening, in which we performed high throughput chemical screens against reconstituted components of the prokaryotic Hsp70 chaperone family (DnaK/DnaJ/GrpE). Using this approach we identified myricetin, which specifically inhibits the DnaK/DnaJ complex, and pancuronium bromide, which selectively inhibits the DnaK/GrpE complex. We also identified a series of dihydropyrimidines that either promote or inhibit the interaction between Hsp70 and a J protein co-chaperones. Based on this interesting activity, we further characterized one representative dihydropyrimidine, **115-7c**. Both *in vitro* and in yeast, we found that the activities of **115-7c** mirrored those of a J-domain co-chaperone; it stimulated ATPase and protein-folding activities and partially compensated for a loss-

of-function mutation. NMR and mutagenesis studies suggest that the **115-7c** binding site is adjacent to the region required for J-domain-mediated stimulation. Interestingly, the artificial and natural partners do not compete for binding and, rather, they act in concert, suggesting that **115-7c** accesses a parallel allosteric conduit at the protein-protein interface. The unusual mechanisms of this suite of pharmacological agents may provide unexpected opportunities for exploring the logic of protein quality control. Further, these results show how "gray box" screening can be used to identify compounds that act on allosteric pathways in multi-protein complexes.

2.1.1 Co-chaperones regulate the activity of Hsp70

Heat shock protein 70 (Hsp70) is a member of a ubiquitously expressed family of molecular chaperones that are involved in protein homeostasis. In its role as a central mediator of protein fate, this chaperone has been linked to multiple tasks, including primary protein folding, subcellular trafficking, protein aggregation, proteasome-mediated degradation and autophagy[1]. In these various tasks, Hsp70 physically interacts with the exposed hydrophobic residues of peptides via its C-terminal substrate-binding domain (SBD). In turn, Hsp70's N-terminal nucleotide-binding domain (NBD) regulates this interaction; hydrolysis of ATP in the NBD propagates an allosteric change in the SBD, which controls the position of a helical "lid" and enhances substrate affinity ~ 10-fold [2]. Thus, although the molecular mechanisms are still unclear, ATPase activity is believed to be a critical determinant of chaperone structure and function.

A family of associated co-chaperones, including nucleotide exchange factors (NEFs) and

J-domain containing proteins (also called Hsp40s), regulate the ATPase activity of Hsp70. For example, the ATP turnover rate of bacterial DnaK (Hsp70 family) is stimulated ~7-fold by the J-domain containing factor, DnaJ [3, 4]. Recent structural and mutagenesis studies have suggested that J-domains control enzymatic activity via interactions within the IA and IIA sub-domains in the NBD of DnaK [5]. More specifically, the residues important for this partnership are ~ 18-20 Å removed from the nucleotide-binding cleft, suggesting that the J-domain controls ATPase activity through allostery[6]. In the presence of DnaJ, ADP release becomes rate-limiting and GrpE (a prokaryotic NEF) assists nucleotide exchange via protein-protein interactions with the IB and IIB sub-domains [7]. Thus, the combination of DnaK, DnaJ and GrpE efficiently turns over ATP and allows control over chaperone activity [4]. As detailed in Chapter 1, members of both co-chaperone families (Hsp40/NEFs) possess separate, intrinsic capabilities and/or interact with other cellular pathways to recruit Hsp70 into a variety of tasks. Further, members of the Hsp70, J protein and NEF families are found in virtually all organisms and they are highly conserved (*E. coli* DnaK is ~50% identical and 75% similar to human Hsp70).

As discussed in Chapter 1, these observations suggest that one powerful path towards better understanding Hsp70 biology is to identify small molecules that selectively block allosteric communication between the co-chaperones and Hsp70. Based on strong genetic findings, such molecules would be expected to be able to tune chaperone activities, regulate ATP cycling and provide a way to understand the logic of Hsp70-mediated quality control.

2.1.2 Few selective inhibitors of Hsp70 are known

Despite the central role of the Hsp70 chaperone family in protein homeostasis and its implications in various human diseases, only a handful of pharamocological targets have been reported in literature (summarized in Chapter 1). More importantly, the mechanism of these compounds is rarely well characterized. For example, one of the earliest compounds identified was the natural product spergualin and its derivatives, such as 15-deoxyspergualin (DSG), which bind Hsc70 and stimulate ATPase activity [8, 9]. Based on structural similarity to DSG, the Brodsky group identified NSC 630668-R/1 (R/1), which is a polyamine that inhibits ATPase activity and also blocks Hsp70-mediated trafficking of polypeptides [10]. A more focused cheminformatic search based on R/1 led to the testing of 31 related, peptoid-functionalized 2-dihydropyrimidinones. This pilot library was screened for the ability to modulate the endogenous and J-protein stimulated ATPase activity of recombinant Ssa1p (a yeast homolog of Hsp70). From these efforts, compounds such as MAL3-101 were identified as specific inhibitors of J-domain protein stimulated Hsp70 activity. Conversely, MAL3-90 and related compounds were found to *enhance* the endogenous ATPase activity but inhibit J-domain co-chaperone activity [11]. Based on these initial studies, our laboratory assembled a focused library of functionalized dihydropyrimidines by microwave-assisted, *in situ* Biginelli cyclocondensation at the terminus of a resin-bound β-peptide. A series of di and tripeptides with a range of hydrophobic and polar side chains were utilized to explore SAR in a DnaK ATPase assay [12]. These results confirmed that dihydropyrimidines operate as either stimulators or inhibitors, depending on their context. Members of this collection also showed some homolog-specific modulators, as compound 14 inhibited

bovine Hsc70 but not DnaK [13]. Thus, this scaffold appeared to have interesting activities on the Hsp70-J protein complex, but the mechanism was not clear.

More broadly, one of the major goals of the Gestwicki laboratory (and primary long-term goal of my thesis work) is to produce a suite of small molecules that target each of the various activities of Hsp70. As discussed in Chapter 1, a full complement of Hsp70 modulators should include compounds that inhibit ATPase activity and those that stimulate it. Moreover, compounds should be able to either inhibit or promote interactions between Hsp70 and NEFs and Hsp70 and J proteins. Finally, one would like to understand the molecular mechanisms, binding sites and structure-activity relationships of these ligands. The challenge is that traditional high throughput methods for identifying chemical inhibitors do not typically have the versatility required to reveal compounds across this spectrum of activities. One might readily identify competitive inhibitors of ATP binding (for example), but the same screen is unlikely to reveal activators or compounds that act on co-chaperone activities. Thus, a major goal of this Chapter is to develop high throughput methods for (a) identifying compounds that act on various aspects of Hsp70 complexes and (b) develop ways of understanding the molecular mechanisms of the resulting compounds.

2.1.3 Design of high throughput screens for Hsp70

Early attempts at developing a high-throughput screen (HTS) against the ATPase activity of Hsp70 failed due to the relatively low ATP turnover rate and high signal to noise (from spontaneous nucleotide hydrolysis) ratio. In 2008, our laboratory successfully developed

a robust, colorometric method in 96-well plates, using malachite green reagent (MG) to measure the ATPase activity of DnaK. The key design optimizations were the use of high concentrations of ATP to reduce discovery of nucleotide-competitive inhibitors and the signal was enhanced by the inclusion of co-chaperone DnaJ [4]. Two graduate students in the Gestwicki group, Lyra Chang and Yoshi Miyata, later adopted an energy transfer strategy to convert the assay to 384-well format. Briefly, white 384-well plates emit fluorescence when irradiated at 430nm. This intrinsic fluorescence can be quenched by energy with the quinalidine red (QR) chromophore. Using this more sensitive assay, we tested 55,400 compounds against the DnaK/DnaJ complex and identified at least one new inhibitor [14]. This compound (3c) favored high-affinity binding of DnaK to a model substrate (luciferase) and it specifically blocked stimulation by DnaJ. Using this platform, we also screened a small natural product library (assembled from the organic extracts of natural spices and crude plant materials) against the DnaK/DnaJ complex. Much like the screen of synthetic molecules, this approach resulted in the identification of a series of flavinoids, including myricetin, which selectively inhibited J-stimulated ATPase activity by up to 75%. Interestingly, myricetin had minimal impact on either DnaK's intrinsic turnover rate or its stimulation by another co-chaperone, GrpE. Together, these findings suggest that this approach was able to identify compounds that inhibit the DnaK/DnaJ complex, without affecting other complexes or activities. Finally, NMR studies revealed that myricetin binds DnaK at an unanticipated site between the IB and IIB subdomains [15]. This mechanism is unexpected because myricetin's binding site is at least 20 Å from where DnaJ interacts with DnaK, in a region not previously implicated in DnaJ-mediated allostery. Thus, this work identified an unanticipated allosteric site in the

DnaK/DnaK complex that might be suitable for targeting [15].

2.1.4 'Gray-Box' screening strategy to selectively target specific Hsp70 complexes

We have termed this general screening approach "gray box" screening. This name comes from the colloquial name that is used for cell-based or animal-based phenotypic screens, which are called 'black box" screens because the identification of the key biological target is challenging. However, the great strength of 'black box' screens is that they include intact pathways and protein complexes in their native cellular environment. The reductionist approach is to use purified proteins (usually a recombinant enzyme) and directly screen for the inhibition of its activity. Obviously, target identification in this format is trivial, but purified enzymes are often not a good representation of the physiological activity in the cell. This strategy has proven to be extremely successful, but the attrition rate is very high when the "hits" are taken from the *in-vitro* screening conditions into the biologically relevant environment in the cells/animals.

Based on this terminology, we developed the idea of "gray-box" screening as a term to describe screens performed against reconstituted protein complexes. As discussed above, the aim of the approach is to design screens to identify small molecules that target a specific protein-protein interaction in the context of its native complex. Indeed, other members of the Gestwicki group have shown the advantages of this feature and identified myricetin, dihydropyrimidines and other scaffolds that have selective activity on the DnaK/DnaJ complex [12]. In this Chapter, I have explored whether this concept could be extended to the DnaK/GrpE complexes. In addition, I have explored the binding partners

and mechanisms of a number of compounds identified through DnaK/DnaJ and DnaK/GrpE 'gray box' screens. Importantly, I found that this project required a number of steps that are not needed in reductionist ('white box') screens. For example, the biochemical activity of the reconstituted complexes must be carefully characterized prior to the HTS effort and additional deconvolution steps are needed following the primary screens to understand the mechanisms of the active compounds. The major findings of this Chapter are that the concept of 'gray box' screening appears general and that compounds identified through this process have interesting activities at allosteric sites in the DnaK/DnaJ/GrpE complex.

More generally, we propose that the concept of 'gray box' screens might have general use in other biological systems. The key design feature of the screen would be multiple components of the target protein complex are purified and re-constituted *in vitro*. The target protein would usually have the measurable enzymatic activity, while the other components would regulate the readout. In addition, protein-protein interactions between the protein target and its partners might mask some potential drug-binding sites, and at the same time induce conformation changes that might reveal other, latent sites.

2.2 Results

2.2.1 Concentration of the components in the reconstituted complex influences the identity of the "hit compounds"

As a model system, we focused on the *Escherichia coli* DnaK protein. As discussed

earlier, the nucleotide turnover by DnaK is tightly controlled by its co-chaperones, including DnaJ and GrpE and also by the binding of its client substrate. Because 'gray box' screens require the reconstitution of a multi-protein complex, we first purified each of the three components, synthesized a model DnaK substrate, NRLLLTG (termed NR peptide), and characterized the effects of these factors on ATP turnover [16] (Appendix 2.1). Specifically, we were interested in the co-chaperone concentration that yielded half-maximal stimulation (J'_m , E'_m , Pep'_m) and maximal stimulation (J'_{sat} , E'_{sat} , Pep'_{sat}) in the malachite green ATPase (MG) assay. In most reductionist high throughput screens, the assay conditions are chosen to maximize the signal:noise ratio to ensure an optimal Z' score (statistical measure of the quality of the screen). However, we hypothesized that the relative concentration of the regulatory co-chaperone should instead be tuned to permit identification of compounds that could either activate or inhibit activity, which might be best accomplished by setting the co-chaperone level to near the half-maximal level instead of saturation. To test this idea directly, we screened the natural product (NP)-library against the DnaK complexes under two different concentrations of the co-chaperone: the apparent half-maximal and saturating concentrations (Figure 2.1).

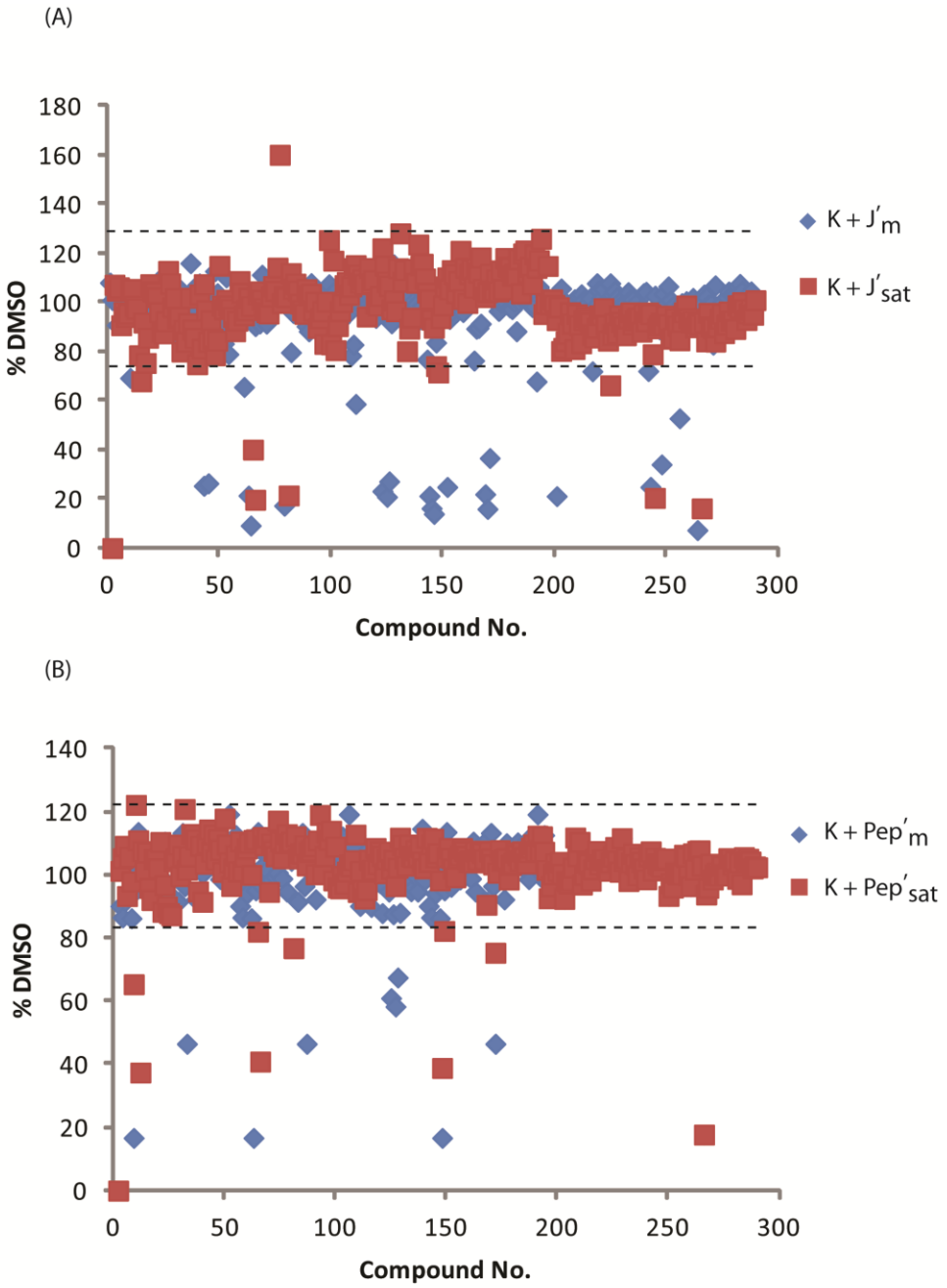


Figure 2.1 Hit compound identification is dependent on concentration of co-chaperone. DnaK concentration was 0.5μM and the concentration of DnaJ and NRLLLTG peptide (Pep) are as shown in Appendix 2.1. Compounds were screened in 96 well plates.

The ATPase HTS effort was performed as previously described [14]. We found that the number of active compounds (± 3 SD) varied depending on the concentration of both

DnaJ (J'_m - 23 hits, J'_{sat} - 11 hits) and peptide substrate (Pep'_m – 19 hits, Pep'_{sat} – 9 hits) (Table 2.1). We could not achieve a good Z' for the screen using the half-maximal concentration for GrpE (E'_m) so we could not analyze the concentration dependence for GrpE. Thus, consistent with our model, more active compounds were identified when the co-factor was added near its most sensitive value. This same concept will be re-explored in Chapter 3 when I study the more complex human chaperone system.

Table 2.1 Number of hit compounds depend on the concentration and identity of the individual reconstitued proteins in the screen. All screens were performed in 96 well plates using the MR reagent.

Screening conditions	Number of Hits	Screening conditions	Number of Hits
$K + J'_m$	23	$K + J'_m + E'_m$	19
$K + J'_{sat}$	11	$K + J'_m + Pep'_m$	7
$K + Pep'_m$	19		
$K + Pep'_{sat}$	9		
$K + E'_m$	0 (Bad Z score)		
$K + E'_{sat}$	12		

2.2.2 Identity of the components in the reconstituted complex also influences screening outcomes

Our next question is whether the identity of the co-chaperone would impact the selectively of the resulting active compounds. In other words, does a screen against the DnaJ/DnaK system produce compounds that only affect this complex and not the DnaK/substrate or DnaK/GrpE combinations? Our rationale is that protein-protein interactions are expected to mask potential

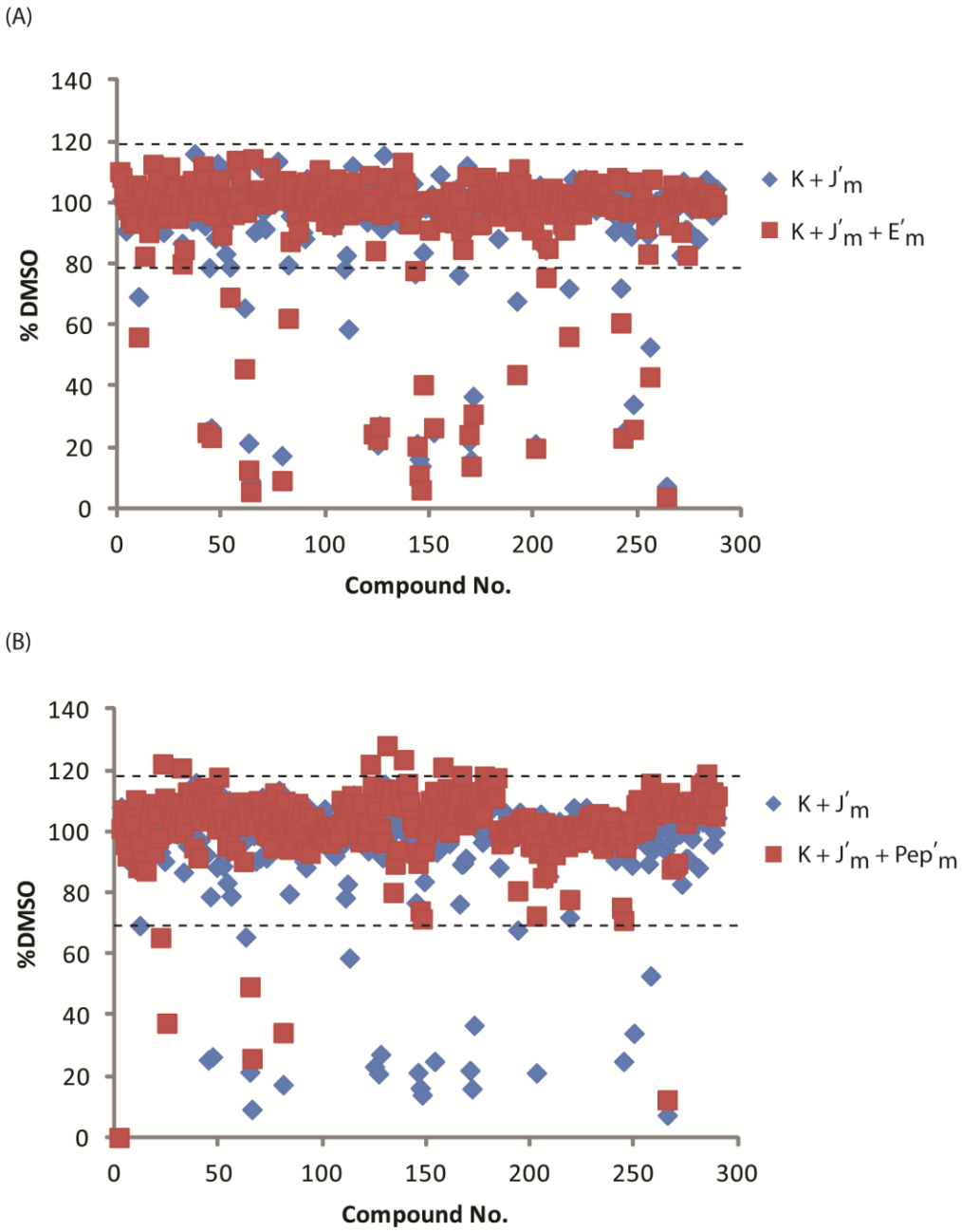


Figure 2.2 Hit compound identification is dependent on composition of reconstituted proteins in the screening complex. DnaK concentration was 0.5μM and the concentration of DnaJ, GrpE and NRLLLTG peptide (Pep) are as shown in Appendix 2.1. Compounds were screened in 96 well plates.

binding sites, while also inducing conformations in the target protein that might reveal new sites. Further, we considered it quite possible that some compounds would bind the co-chaperone, permitting selective inhibition. To formally test this concept, we screened

the NP library against the DnaK + DnaJ'$_m$ + GrpE'$_m$. We found that, in contrast to the 23 compounds which acted on the K + J'$_m$ system, only 19 compounds acted on the K + J'$_m$ + E'$_m$ system. Further, the magnitude of inhibition was often reduced by adding GrpE. We found similar results when we added apparent half-maximal concentration of peptide (Table 2.1). These findings suggest that the composition of the reconstituted protein complex directly influences the identity and quality of the resulting active compounds.

2.2.3 Selective inhibitors for a particular chaperone complex can be identified using a 'gray box' screening strategy

The NP library that we used in these studies is enriched with modulators for the DnaK-DnaJ chaperone system, but we also wanted to identify synthetic compounds that are selective to a particular protein complex. Towards that goal, we screened the MS2000 collection of bioactive compounds against two distinct chaperone complexes, DnaK/DnaJ and DnaK/GrpE, using the more sensitive QR-based, low volume, ATPase HTS assay [14]. Because we wanted to increase our odds of finding both inhibitors and activators, we chose to screen the library at the apparent half-maximal

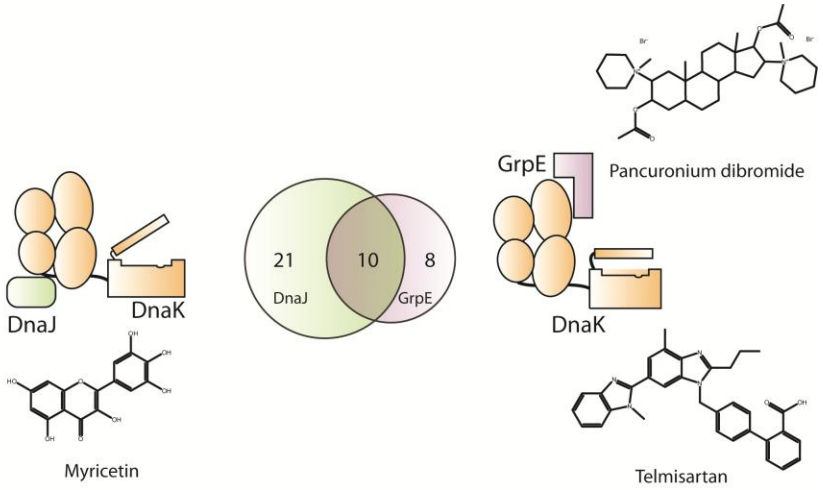


Figure 2.3 Gray-Box screens against the prokaryotic chaperone complexes. Screening results from the screens against the complexes shown above. The compound library was the MS2000 library.

concentrations of either GrpE or DnaJ ($Z^{'}$ > 0.5 for both assays). From this collection, we identified 31 "hits" for the DnaK/DnaJ complex and 18 hits for the DnaK/GrpE complex (Figure 2.3). Of these hits, 10 hits were common to both screens. Excitingly, we also found 21 unique hits for the DnaK/DnaJ complex and 8 unique hits for the DnaK/GrpE complex.

We purchased these compounds from commercial sources (Appendix 2.2) and confirmed them in the malachite green ATPase assay in a 96 well format. Specifically, we focused on three confirmed compounds Myricetin (IC_{50} = 35 ± 4.2 µM) against the DnaK/DnaJ complex and Pancuronium Bromide (IC_{50} = 42 ± 3.7µM) and Telmisartan (IC_{50} = 56 ± 7.3 µM) against the DnaK/GrpE complex (Figure 2.4). Myricetin had previously been identified by another graduate student, Lyra Chang, in her DnaK/DnaJ screens, so it was reassuring that it was re-identified in my studies. Consistent with her report, this

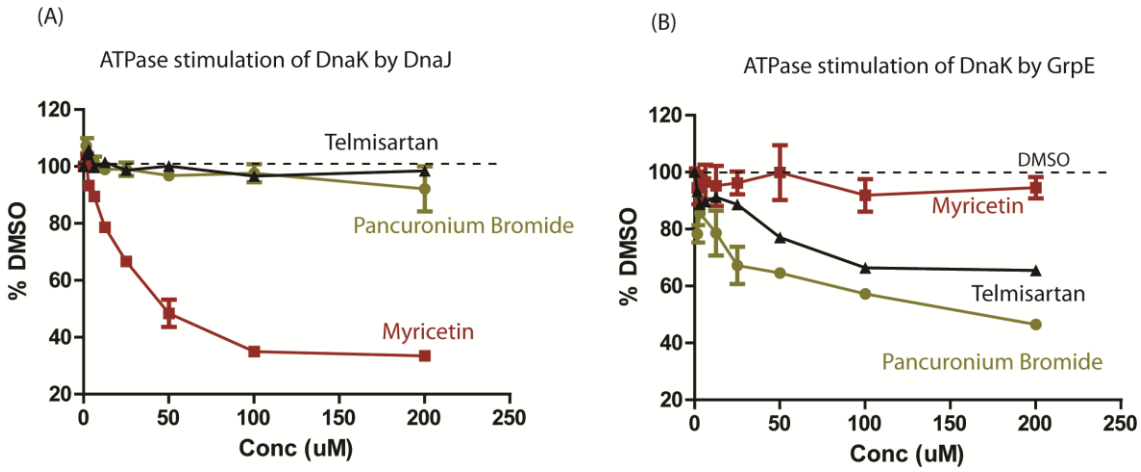


Figure 2.4 Hit compounds are specific inhibitors against specific complexes. A) Myricetin inhibits only the DnaK/DnaJ complex . (B)Telmisartan and Pancuronium Bromide inhibit the DnaK/GrpE complex. ATPase assays were performed in 96 well plates using the malachite green (MG) reagent.

compound only inhibits the DnaK/DnaJ complex with little affect on other DnaK complexes [15]. Pancuronium Bromide and Telmisartan inhibit the GrpE stimulation of

the ATPase activity of DnaK only upto 50%. One possible explanation maybe that these compounds may be inhibiting the complex allosterically.

2.2.4 Pancuronium bromide selectively inhibits only the DnaK/GrpE complex

Although previous efforts by the group had identified selective inhibitors of the DnaK/DnaJ complex, we hadn't yet identified compounds with activity on the DnaK/GrpE complex. Thus, I focused on better understanding and validating these compounds. To characterize the mechanism of inhibition of pancuronium bromide (PB), we performed a series of ATPase stimulation assays.

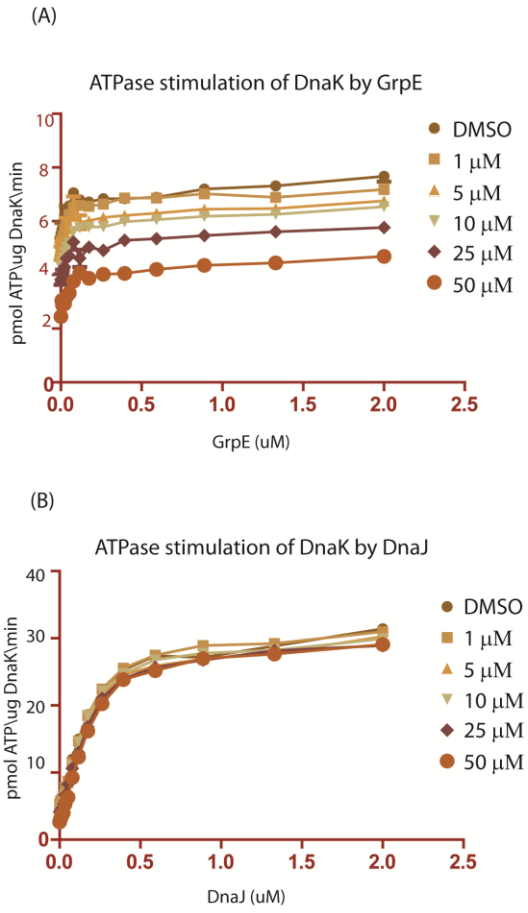


Figure 2.5 Pancuronium Bromide (PB) inhibits only the GrpE stimulation of DnaK's ATPase activity in a dose-dependent manner. PB does affect the stimulatory effect of DnaJ.

In the absence of compound, GrpE stimulates the ATPase activity of DnaK by approximately 2-fold, with a half maximal stimulation ($GrpE'_m$) of about 0.025 ± 0.002 uM. PB inhibited this stimulatory activity in a dose-dependent manner, and at a concentration of 50 uM increased the apparent half maximal concentration of $GrpE'_m$ 3-fold to ~ 0.1 ± 0.005uM (Figure 2.5). The compound did not have any effect on the ability of DnaJ to stimulate the ATPase activity of DnaK

(Figure 2.5), the 'mirror image' of what was previously observed with myricetin. PB also did not affect the intrinsic ATPase activity of DnaK. Together, these results suggest that pancuronium bromide can specifically inhibit the DnaK/GrpE complex, without impacting the function of the other co-chaperones. This is an exciting finding because it suggests that (at least for the DnaK system) 'gray box' screens are indeed able to identify selective compounds and that the user-directed choice of the identity of the co-chaperone directly impacts the selectivity of the resulting compounds. Additional work is underway by other members of the Gestwicki group to more fully characterize the binding site and activity of PB.

2.2.5 Characterizing 115-7c, a small molecule that modulates the DnaK/DnaJ protein complex

Although we have shown that a 'gray box' screening strategy is a powerful way to identify compounds that act on multi-protein complexes, it does (as mentioned above) create a number of unique challenges as well. Principle among these challenges is the need to perform an extra round of deconvolution to understand which target is being bound. This issue is not commonly a problem in reductionist screens, because there is only one protein in the system. In 'gray box', however, the target could theoretically include the enzyme or the co-chaperone and the binding site could be far from the active site (as Lyra Chang had shown previously with myricetin). In this Chapter, one of my goals was to better characterize one of the most interesting Hsp70 modulators that has been identified, the dihydropyrimidines. As mentioned above, the dihydropyrimidines had been previously identified by the Brodsky group as acting on the DnaK/DnaJ system

(and the yeast orthologs; [11, 17]). Moreover, our group had shown that these compounds can either suppress or active J protein activity in vitro [12]. Because target identification and compound's mechanism are proposed to be important aspects of 'gray box' screens, I wanted to better understand the activity of this class of compounds.

Previous structure-activity studies by a postdoctoral fellow, Susanne Wisen, led to the synthesis of **115-7c** (Figure 2.6A), which she found to stimulate the ATPase activity of the *Escherichia coli* DnaK ($DnaK_{FL}$), by ~ 2-fold (Figure 2.6B). Binding of peptide substrates to DnaK is known to stimulate ATP hydrolysis, so our first hypothesis was that **115-7c** might enact its activity by interacting with the SBD. To test this model, we determined the activity of **115-7c** against a truncated DnaK that lacks the SBD ($DnaK_{NBD}$). These experiments revealed that **115-7c** retained full activity against $DnaK_{NBD}$ (turnover increased ~3-fold), suggesting that it might bind somewhere in the NBD. We next studied the effects of **115-7c** on the chaperone-mediated refolding of denatured firefly luciferase.

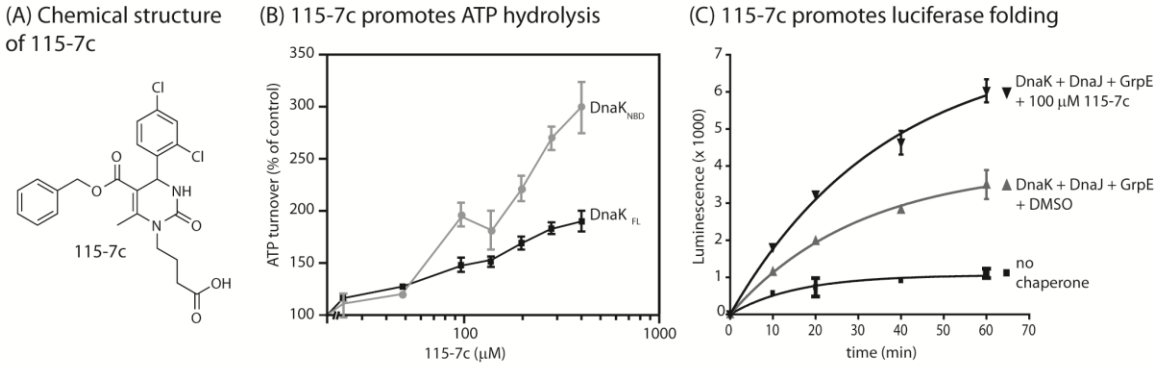


Figure 2.6 Compound 115-7c stimulates the ATPase and folding activities of Hsp70-family chaperones. (A) Chemical structure of the dihydropyrimidine, 115-7c. (B) 115-7c promotes the ATPase activity of E. coli DnaK ($DnaK_{FL}$) and a truncated DnaK containing only the nucleotide binding domain ($DnaK_{NBD}$). Results are the average of triplicates and the error is standard deviation. (C) 115-7c promotes the luciferase-folding activity of the DnaK-DnaJ-GrpE complex. Results are the average of triplicates and the error is standard deviation.

One of the major functions of the Hsp70 complex is to assist in protein folding and the chaperone-mediated re-folding of chemically denatured firefly luciferase is a common assay for studying this function *in vitro* [18, 19]. In this system, firefly luciferase was denatured with guanidinium hydrochloride, diluted and then treated with the prokaryotic Hsp70 system, comprised of DnaK with its two stimulatory co-chaperones, DnaJ and the nucleotide exchange factor GrpE. In this system, **115-7c** potently stimulated chaperone-mediated refolding activity by ~2-fold (Figure 2.6C). Together, these results indicate that the dihydropyrimidine, **115-7c**, stimulates both ATP turnover and protein folding activities. Thus, this compound appeared to be a good probe for my mechanistic studies.

2.2.6 Compound 115-7c is an artificial co-chaperone in yeast

Because **115-7c** displayed activities that were reminiscent of J-domain functions, we reasoned that it might act as an artificial co-chaperone in cells. To explore this idea, we turned to a budding yeast model in which a role for J-domain co-chaperones has been clearly documented[20]. Ydj1 is one of the DnaJ orthologs in yeast and the growth of a *ydj1*Δ mutant is severely compromised at elevated temperature [21, 22]. Thus, we hypothesized that **115-7c** might be able to compensate for the loss of Ydj1 function. To investigate this possibility, we treated *ydj1*Δ cells with **115-7c** (100 μM) by applying this compound to the solid growth media. As expected, the *ydj1*Δ cells grew normally at 30 °C but failed to thrive at 34 °C. Application of **115-7c** to *ydj1*Δ cells, however, greatly enhanced growth at elevated temperature (34 °C; Figure 2.7A). As an independent test of this idea, we also treated *ydj1*Δ cells with calcoflour white. This treatment impairs cell wall biogenesis and preferentially decreases the viability of *ydj1*Δ mutants[23]. Mirroring

the results of the thermal stress experiments, **115-7c** (100 μM) partially rescued this phenotype (Figure 2.7B). We found these cell-based results particularly striking because we are unaware of similar genetic loss-of-function alleles that can be functionally recovered by synthetic mimetics. However, it is worth noting that **115-7c** was incompletely capable of replacing Ydj1p and relatively high quantities of the small molecule were required, as might be expected because of the small size of the compound (~400 Da) compared to the full length co-chaperone (40 kDa).

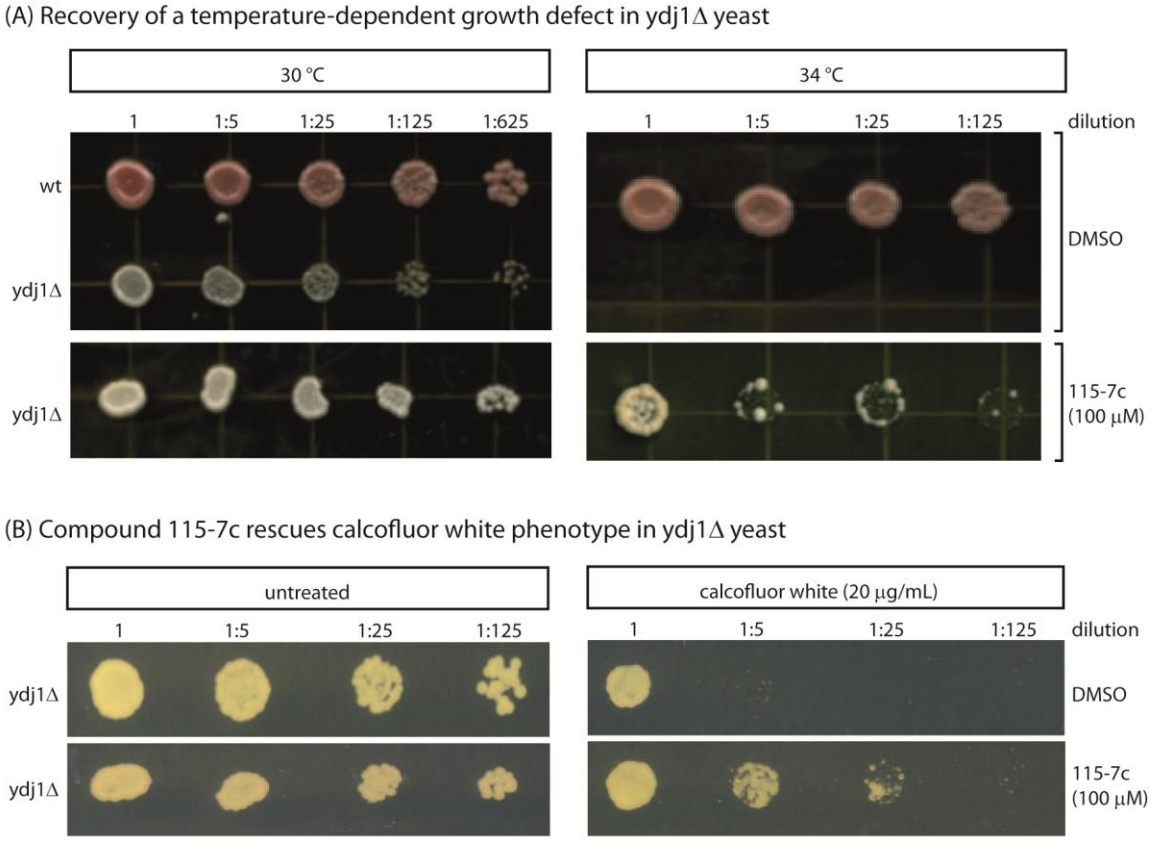


Figure 2.7 Compound 115-7c partially recovers loss-of-function mutations in the native co-chaperone, Ydj1, in yeast. (A) Addition of 115-7c (100 μM), but not the solvent control, partially restores growth at elevated temperatures in a ydj1Δ strain. (B) Deletion of Ydj1 sensitizes cells to treatment with calcoflour white and application of 115-7c (100 μM) partially recovers this phenotype.

2.2.7 115-7c binds to the IIA subdomain of DnaK

To better understand the molecular mechanisms by which **115-7c** stimulates ATP turnover and partially compensates for *ydj1*Δ phenotypes, we collaborated with Prof. Erik Zuiderweg (Dept. Biol. Chem.) to perform nuclear magnetic resonance (NMR) experiments on $DnaK_{FL}$. In these experiments, ^{1}H-^{15}N labeled $DnaK_{FL}$ (500 µM) was titrated with **115-7c** and the TROESY spectra were collected (Figure 2.8A). In this assay, we observed a small number of chemical shifts in the presence of **115-7c** and these signals clustered to the IIA subdomain of the NBD (Figure 2.8B). No significant changes were observed in the SBD.

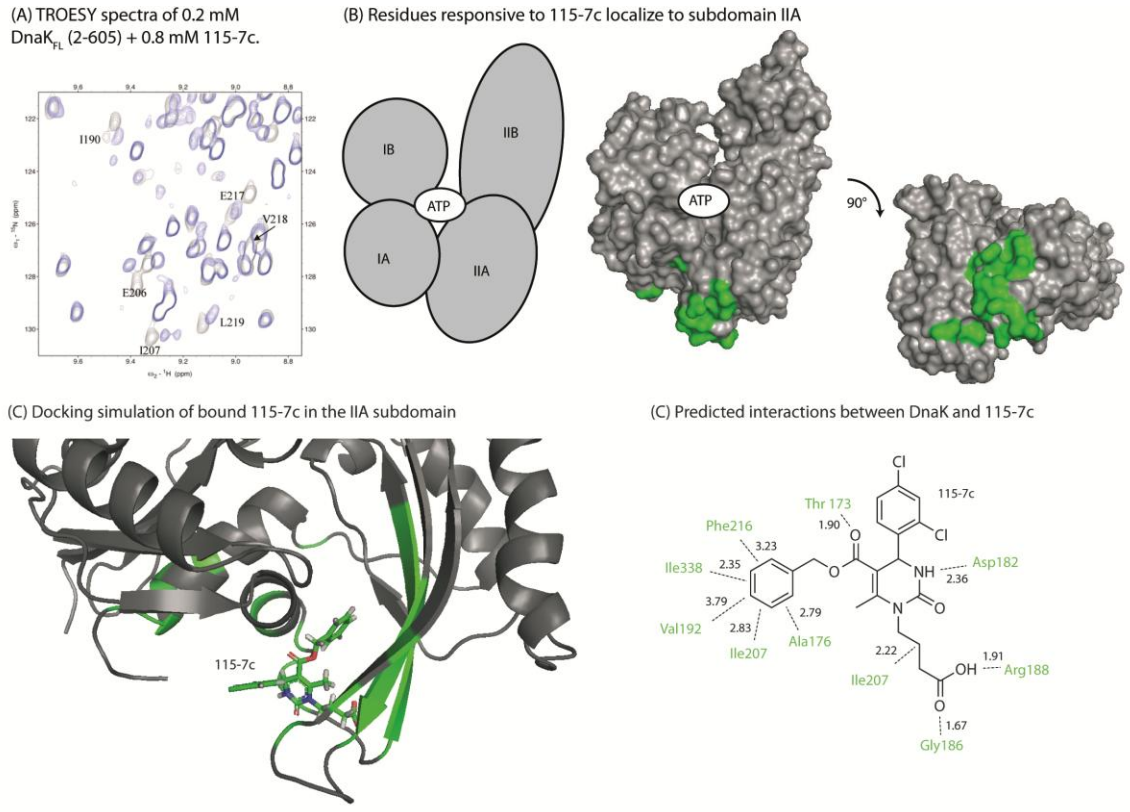


Figure 2.8 Compound 115-7c interacts with the subdomain IIA site on DnaK's NBD. (A) A subset of the 2D spectra of nearly full length DnaK (+ 1 mM ATP) is shown in the presence (blue) and absence (gray) of four equivalents of 115-7c. (B) Mapping the chemical shift above 0.2 ppm revealed a localized region at the IIA subdomain. The subdomains are shown in the schematic and the responsive residues shown in green. No significant chemical shifts were observed in the substrate-binding domain. (C) Model for binding of 115-7c to the IIA subdomain. See the Methods section for details. (D) Based on the docked configuration, the key interactions between DnaK and 115-7c are predicted. Approximate distances are in Angstroms.

Based on these NMR results, we collaborated with Prof. Heather Carlson's group to use Langevin dynamics simulations and induced fit docking in GLIDE to build a model of the **115-7c**-bound complex. The lowest energy configuration inserted the compound into a hydrophobic cleft at the bottom of the IIA subdomain (Figure 2.8C). This model included a number of favorable interactions, including the juxtaposition of the carboxylic acid with a charged surface composed of R188 and the amide nitrogen of G186 (Figure 2.8D). Moreover, the phenyl group was placed into a deep hydrophobic pocket composed of F216, I207, V192, I338 and A176. This orientation provides a model for how **115-7c** might bind the IIA subdomain.

2.2.8 115-7c and DnaJ access parallel allosteric mechanisms

Intriguingly, the IIB and IIA regions of the NBD have been implicated in allosteric, interdomain communication and the stimulatory activity of J-domains [5, 24, 25]. To examine potential overlap between physiologically important residues in this region and those responsive to **115-7c**, we first mapped both sets onto the crystal structure of $DnaK_{NBD}$. This analysis suggested that the artificial and natural co-chaperones might share only partially overlapping allosteric sites (Figure 2.9A). To test the relevance of this model, we made point mutations in three residues (E170S, E217A and Y193A) and measured their responsiveness to ATPase stimulation. The E170S mutation was chosen because it was previously reported to be important for stimulation by J-domains [5, 6] and, consistent with that idea, we confirmed that $Hsc70_{NBD}$ (E170S) was resistant to DnaJ (Figure 2.9B). In contrast, **115-7c** retained full activity against this mutant. Similarly, we

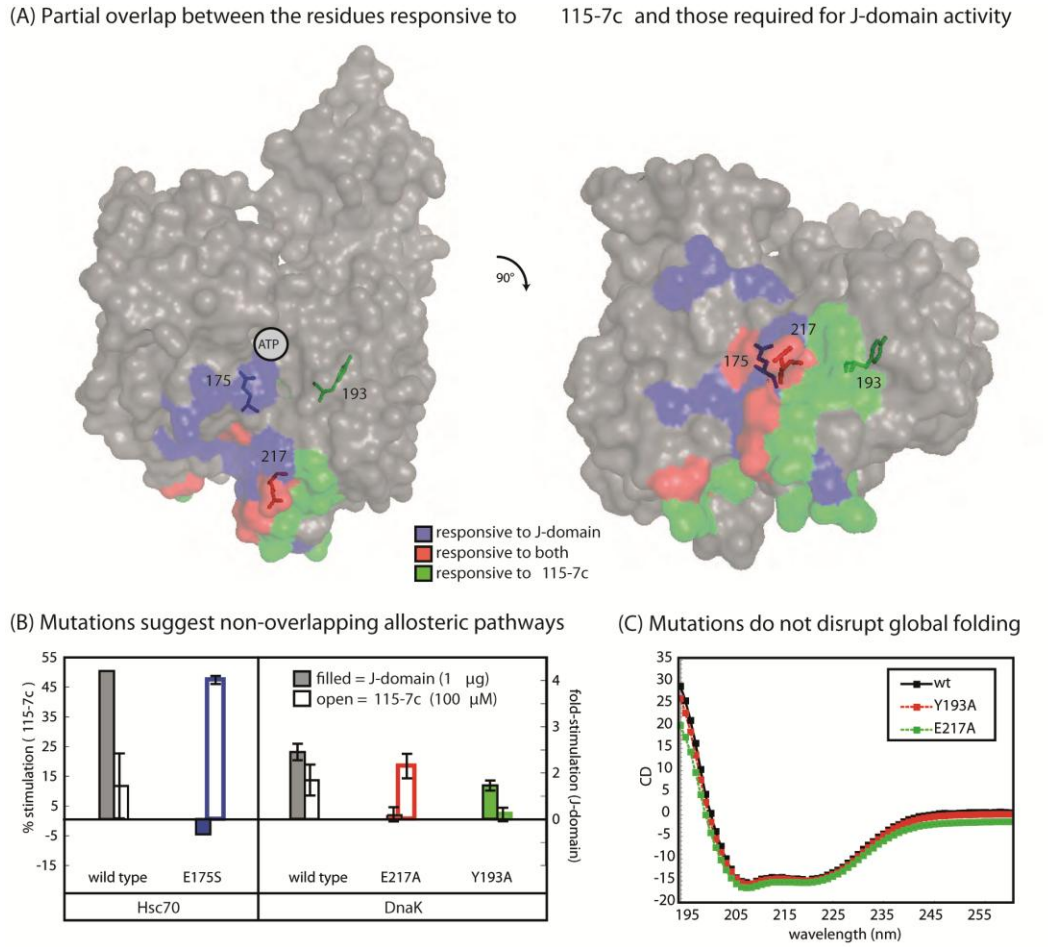


Figure 2.9 Site-directed mutagenesis suggests atleast two allosteric pathways in DnaK. (A) Overlay of the residues identified by NMR as responsive to 115-7c (green) , those important for J-domain activity (purple) and those amino acids that fit in both categories (red). Two view are shown to highlight that some of the responsive residues such as E175 and Y193, are found in the interior, while others, such as E217 are surface residues. They overlay was generated in Pymol. (B) Either wt $Hsc70_{NBD}$ or the known mutant $Hsc70_{NBD}$(E175S) were examined for stimulation by J-domain (filled bars) or 115-7c (open bars). Both factors enhanced ATP turnover of the wild type, but only 115-7c stimulated the E175S mutant. Point mutants were also generated in $DnaK_{FL}$ at either E217A or Y193A. The Y193A mutation blocked 115-7c mediated stimulation, but did not completely prevent J-domain activity. Results are the average of (atleast) triplicates and error is standard deviation. (C) Point mutants Y193A and E217A did not disrupt global folding, as measured by CD.

found that $DnaK_{FL}$ (Y193A) retained responsiveness to DnaJ but not **115-7c** (Figure 2.9B). Finally, we explored the influence of a residue that was predicted to be important for both J-domains and **115-7c**. This mutant, $DnaK_{FL}$ (E217A), could be stimulated by the small molecule but not DnaJ, suggesting that only a subset of the residues from the NMR study are critical for mediating the activity of **115-7c**. Importantly, although some

of these mutations altered the intrinsic hydrolysis rate [5], none of them impacted global structure, as measured by circular dichroism (Figure 2.9C). Together, these mutagenesis results suggest that there are at least two allosteric possibilities in $DnaK_{NBD}$ and that the artificial and native co-chaperones access parallel, but non-overlapping conduits.

2.2.9 Compound 115-7c binds better to the DnaK-DnaJ complex than to DnaK alone

Based on the structural and mutagenesis studies, we wanted to understand whether **115-7c** and DnaJ could both bind to DnaK at the same time. Towards this goal, we first measured **115-7c** binding by isothermal calorimetry (ITC) and found that it binds weakly to both $DnaK_{FL}$ (K_d ~ 220 µM) and $DnaK_{NBD}$ (K_d ~ 211 µM; Figure 2.10A). Next, we attempted to block interactions with **115-7c** using saturating levels of DnaJ. Surprisingly, we found that **115-7c** bound ~ 2-fold better to the DnaK-DnaJ complex than the isolated chaperone (K_d 113 µM). Because **115-7c** has no appreciable affinity for DnaJ these results suggest that **115-7c** has a more favorable binding site on the composite DnaK-DnaJ complex than on the DnaK protein alone.

2.2.10 Converting 115-7c into an inhibitor by preventing synergy with the J-domain

Based on these results, we envisioned a simple ternary model in which both **115-7c** and DnaJ bind DnaK in the cleft between the IIA and IIB subdomains. Because the residues important for their functions are in close proximity (see Figure 2.10A), we next explored whether adding bulk to the small molecule could create steric clashes. In these studies, we focused on the dicholorobenzyl substitution on **115-7c**, because this region was

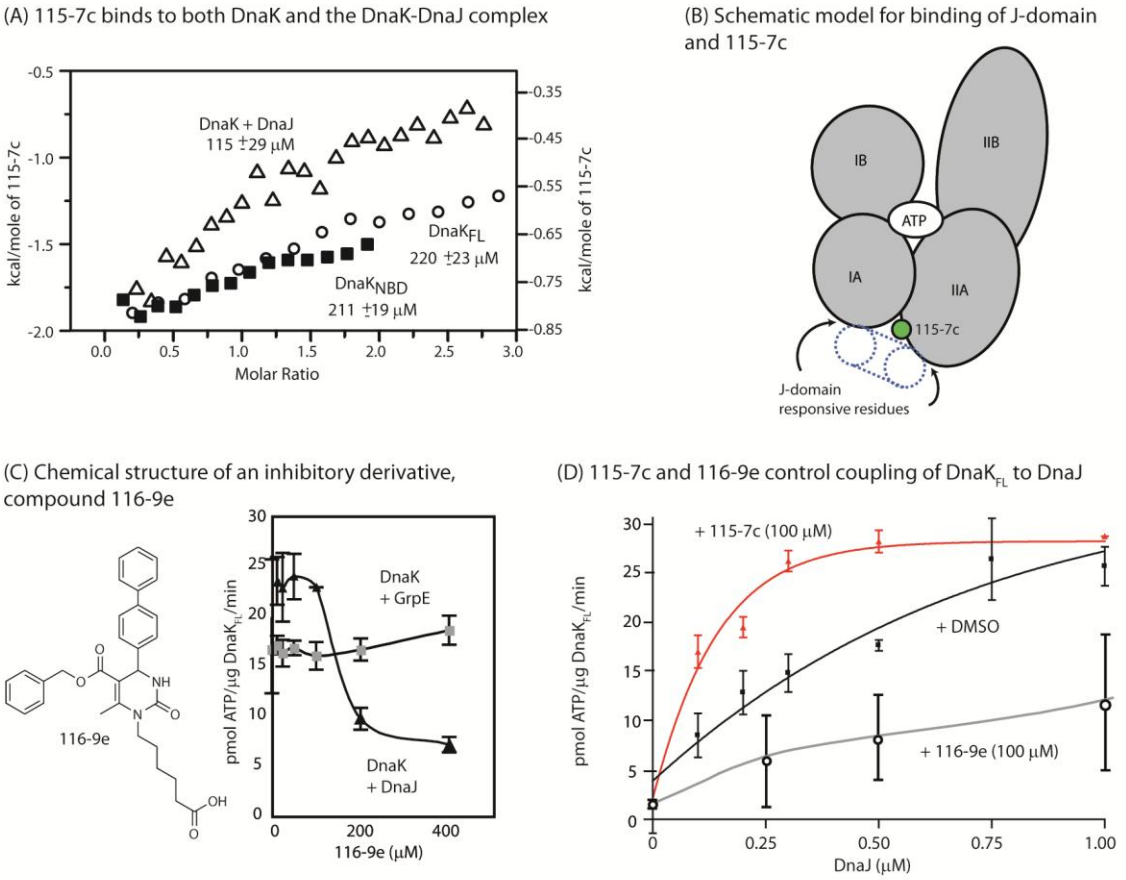


Figure 2.10 Compound 115-7c and DnaJ are able to share binding to DnaK. (A) Isothermal calorimetry reveals that 115-7c binds approximately 2-fold tighter to the DnaK-DnaJ complex than to DnaK alone. Results are representative of duplicate experiments. Also, note that 115-7c does not bind to DnaJ (B) Based on the bound model of 115-7c and the ITC results, both DnaJ and 115-7c might both bind to DnaK. (C) Chemical structure of compound 116-9e, which has a diphenyl substitution in place of the solvent-exposed dichlorobenzyl group. This replacement is predicted to sterically interfere with J-domain interactions. 116-9e inhibits ATP turnover by the DnaK-DnaJ complex but does not impact GrpE-mediated ATPase activity. Results are the average of triplicates and the error is standard deviation. (D) In the ATPase assay, 115-7c promotes DnaJ-mediated stimulation, while 116-9e interferes with this coupling. Results are the average of triplicates and the error is standard deviation.

predicted to be relatively solvent exposed in the docked model and, moreover, this group pointed towards the J-domain responsive residues in the adjacent IIB subdomain (see Figure 2.8C and 2.9A). This analysis led to the design and synthesis of **116-9e**, which is similar to **115-7c** except that the dichlorobenzyl functionality has been replaced with a bulkier di-phenyl group. In the ATPase assay, **116-9e** was a mild (~20%) stimulator of

the DnaK-GrpE complex, but it potently inhibited the DnaK-DnaJ complex, suggesting that it selectively interfered with functional coupling to the J-domain (Figure 2.10D). To further explore this relationship, we titrated DnaJ into $DnaK_{NBD}$ in the presence of **115-7c** or **116-9e** and measured ATP turnover. In these experiments, we found that **115-7c** enhanced coupling of DnaJ to DnaK; the half-maximal potency of DnaJ was approximately 2-fold better in the presence of **115-7c** (Figure 2.10D). Conversely, **116-9e** strongly suppressed J-domain-mediated stimulation of ATPase activity (Figure 2.10D). Together, these results suggest that **115-7c** and **116-9e** tune chaperone activity in the context of the DnaK-DnaJ complex by regulating interactions with the native co-chaperone. Thus, a compound identified in a 'gray box' screen was found to have an unusual (and largely unprecedented) activity at a protein-protein interface.

2.3 Discussion

Our major goals in this Chapter were to better understand the design principles governing successful completion of 'gray box' screens. More specifically, we wanted to test whether this approach could also be used to identify inhibitors of the DnaK/GrpE system and whether the ratio of the co-chaperones was important in determining the screen results. Further, we wanted to explore biochemical and structural methods for rapidly performing follow-up studies that would reveal the binding partner, its binding site and its potential mechanisms.

In this Chapter, we found that the identity of the co-chaperone does impact the chemical scaffolds that are identified and we collected evidence that inhibitors of the DnaK/GrpE

combination can be found with this approach. Together with previous work from the group, these findings support a model in which 'gray box' screens can be used to identify compounds that tune the activity of this complex chaperone system. More work is needed to improve the potency of these compounds, but the efforts described here provide a basis for further work in this area. In Chapter 3, we will discuss related efforts to characterize the human Hsp70 system and enable parallel HTS efforts.

Another goal of this project was to fully characterize one promising product of 'gray box' screens: the dihydroyrimidines. We found that the stimulatory activity of **115-7c** was not competitive with DnaJ and, in fact, these factors appeared to work together to stimulate ATP turnover. Based on this model, we converted **115-7c** into a context-specific inhibitor, **116-9e**, by appending steric bulk. These results help clarify how compounds like **115-7c** might be re-assigned as either activators or inhibitors depending on the presence of J-domains and their chemical substitution patterns. More broadly, these results also establish that **115-7c** and its derivatives might be used as chemical probes to explore Hsp70-mediated protein homeostasis.

Another interesting and unexpected aspect of this study is that the small molecules appear to operate at a protein-protein interface to either stimulate or inhibit allosteric pathways. A growing number of studies have reported potent inhibitors of protein-protein interactions [26, 27]. In those studies, the molecules typically bind to one protein partner and either directly [28] or allosterically [29] block formation of the protein-protein complex. Our findings provide evidence that inhibitory activities at protein-protein

contacts are not predestined; **115-7c** accesses a native protein-protein interface and mimics functional aspects of the natural co-factor. Thus, this chemical probe operates in a previously unanticipated mode at a natural interface, suggesting new avenues for regulating the function of multi-protein complexes.

2.4 Experimental Procedures

2.4.1 Protein expression and purification

DnaK, DnaJ and GrpE proteins were expressed in *E. coli* BL21 (DE3) using T7-based vectors. DnaK and GrpE were expressed at 37 °C, while DnaJ was expressed at 25 °C in order to increase the fraction of soluble protein. All purification steps were carried out at 4 °C. Protein concentration was estimated by Bradford assay, using BSA as the standard. Following purification, proteins were frozen on liquid nitrogen and stored at –80 °C until use.

Purification of DnaK was accomplished by a modification of established procedures. Briefly, cell pellets were suspended in buffer A (25 mM Tris, 10 mM KCl, 5 mM $MgCl_2$, pH 7.5) containing 0.01 mM PMSF and 1 mM DTT, and disrupted using a microfluidizer (Microfluidics). Cleared extracts were applied to a Q-Sepharose fast-flow column (GE Healthcare), and the protein was eluted with a 10-500 mM gradient of KCl in buffer A. Fractions containing DnaK were pooled and applied to ATP-agarose (Sigma). After extensive washing with Buffer A and Buffer A containing 1 M KCl, the protein was eluted with Buffer A containing 3 mM ATP. The pure protein was concentrated and exchanged to buffer A for storage.

DnaJ was purified using a streamlined version of established methodology. Briefly, cell pellets were resuspended in buffer B (25 mM Tris, 2 M urea, 0.1% Brij-58, 2 mM DTT, pH 7.5) containing 0.01 mM PMSF and disrupted using a microfluidizer. Cleared extracts were applied to a Source SP (GE Healthcare) column, and the protein was eluted with a gradient of 0-350 mM KCl in buffer B. Fractions containing DnaJ were pooled and applied to a hydroxyapatite (BioGel HTP, Bio-Rad Laboratories) column that had been equilibrated with buffer C (buffer B containing 50 mM KCl and no detergent). After extensive washing with buffer C and buffer C containing 1.0 M KCl, the protein was eluted with a 0-350 mM gradient of potassium phosphate, pH 7.4. Fractions containing DnaJ were pooled, diluted two-fold with 25 mM Tris, 50 mM KCl, 2 M urea, pH 9.0, and applied to a Q-Sepharose fast-flow column. Pure DnaJ was collected in the flow-through, concentrated, and exchanged into buffer A containing 150 mM KCl for storage.

GrpE was purified as follows: Cell pellets were resuspended in buffer D (25 mM Tris, pH 7.5) containing 0.01 mM PMSF and disrupted using a microfluidizer. Cleared extracts were applied to a Q-Sepharose fast-flow column, and protein was eluted with a 0-500 mM gradient of KCl in buffer D. Fractions containing GrpE were pooled, supplemented with 1 M ammonium sulfate, and applied to a phenyl-Sepharose high-performance 16/10 column (GE Healthcare). Protein was eluted with a 1.0-0 M gradient of ammonium sulfate in buffer D. Fractions containing GrpE were pooled, concentrated, and applied to a Superdex 200 26/60 column (GE Healthcare). Protein was eluted at 0.5 ml/min in buffer D. Fractions containing GrpE were pooled, concentrated, and the protein was exchanged into buffer A for storage. The protein was greater than 90% pure as judged by SDS-PAGE and Coomassie staining.

2.4.2 Screening against DnaK complexes in 384- well Format

The QR reagent was prepared exactly as indicated above. All components other than compounds were added by a Multidrop dispensor (Thermo Fisher Scientific, Inc., Waltham, MA). The DnaK-DnaJ stock solution was prepared so that the final concentration of DnaK was 0.4 μM and DnaJ was 0.7 μM (unless noted). This solution (5 μL) was then added to each well of a 384-well opaque, white, low-volume, non-sterile, polystyrene 384-well plates (Greiner Bio-One North America Inc., Monroe, NC). To this solution, 0.2 μL of either compound (1.5 mM) or DMSO was added to each well by Biomek HDR (Beckman, Fullerton, CA). Finally, 2 μL of a 3.5 mM ATP solution was added to begin the reaction. The plates were then incubated for 3 hrs at 37 °C. After incubation, each well received 15 μL of the QR reagent, allowing 2 min of reaction time, and then quenched by addition of 32% w/v solution of sodium citrate (2 μL). These plates were incubated for 15 min at 37 °C and the fluorescence intensity measured (excitation 430 nm, emission 530 nm) on a PHERAstar plate reader. Standard curves were obtained using stock solutions of dibasic potassium phosphate.

2.4.3 Yeast growth experiments

Wild-type yeast in the W303 background (*MAT***a**, *leu2-3,112, his3-11, trp1-1, ura3-1, can1-100, ade2-1*) and a *ydj1*Δ strain (DYJ1) (*MAT***a**, *ura3-52, lys2-801, ade2-101, trp1-Δ63, his3-Δ200, leu2-Δ1, ydj1::TRP1*) were used. These cells were grown in rich media (YPD) at 30 °C over night, followed by dilution to equal concentrations (OD_{600} of 0.5). For the experiments performed at elevated temperature, cells were spotted in four 5-fold dilutions on YPD agar plates. These plates were pretreated with compound (50 or 100

µM, 0.5% DMSO) or 0.5% DMSO alone. The plates were grown at 34 °C for 4 days. We noted more dramatic growth recovery on YPD (compared to SC) but the origin of this difference is not clear. Alternatively, the YPD plates were pretreated with calcofluor white to a final concentration of 20 µg/ml, alone or in combination with **115-7c**, followed by incubation at 30 °C for four days.

2.4.4 NMR spectroscopy

^{15}N-labeled $DnaK_{2\text{-}605}$ and $DnaK_{2\text{-}388}$ were expressed in standard minimal medium containing $^{15}NH_4Cl$ as the sole nitrogen source, using T7-based expression vectors. Following purification, proteins were concentrated to approximately 0.5 mM, exchanged into NMR buffer (25 mM Tris, 10 mM $MgCl_2$, 5 mM KCl, 10% ^2H_2O, 0.01% sodium azide, pH 7.1), and stored at –80 °C until use. Prior to NMR analysis, $DnaK_{2\text{-}388}$ samples were thawed and supplemented with 5 mM ATP (ATP). Samples of $DnaK_{2\text{-}605}$ were additionally supplemented with 2.5 mM of a high affinity peptide (NRLLLTG). After collecting reference spectra, 115-7C was titrated to a final concentration of 1 mM from a 100 mM stock in DMSO. Control experiments showed that DMSO alone had had no detectable effects on the spectrum of $DnaK_{2\text{-}388}$ at concentrations up to 1%, and that it had minimal effects on the spectra of $DnaK_{2\text{-}605}$ at the same concentration. 2D HSQC-TROSY NMR spectra were collected at 30 ºC on a cryoprobe-equipped Varian Inova 800 MHz spectrometer, with data collection times of approximately 2 hours per spectrum. NMR data were processed using NMRPipe and analyzed with Sparky (UCSF). Combined ^1H and ^{15}N chemical shift changes were measured using a weighted function and the assignments generated previously.

2.4.5 Induced Fit Docking (IFD)

The crystal structure of *E. coli* NBD (PDB: 1DKG) (Harrison 1997) was used after removal of the GrpE and rebuilding of the missing loops in the NBD using PyMOL (DeLano) and MOE (2005.06). Langevin dynamics simulations of NBD with Amber 10 (Case 2008) and FF99SB force field (Hornak 2006) in various nucleotide-binding states were performed to sample relaxed, alternative conformations of the protein (manuscript in preparation). A snapshot of the simulations was used to perform docking. Using this snapshot, an IFD protocol (Sherman 2006) developed by Schrödinger (2008) was used. Briefly, this procedure involved four core steps: 1) generating ligand poses into the defined binding site by softened-potential docking; 2) refining residue side-chains near each ligand pose from the first step; 3) re-docking ligand into the optimized induced-fit structure and 4) scoring the poses by docking energy (GlideScore) and receptor energetic terms (Prime energy). The ligand and protein were prepared in Maestro (2007). IFD was initiated with a constrained minimization of the receptor. Prime loop prediction function of IFD was used to generate a maximum of 5 alternative loop (D208-T215) conformations for Glide docking. Side-chain refinement was performed to residues within 5.0 Å of the defined loop segment. Predicted loop structures with energy 30 kcal/mol above the lowest-energy structure were rejected. In the first stage of docking, Glide docked ligand into the rigid receptor with a softened van der Waals potential. The grid box was centered at residue E206 (based on the NMR results) and the box size was determined automatically. 10 top scoring ligand poses were kept. For each initial docking pose, Prime performed side-chain refinement to residues that were within 5.0 Å of the ligand, allowing the side-chains to adopt optimal conformation to interact with the ligand.

Glide then re-docked the ligand into the induced-fit receptor with standard parameters and the final ligand poses were scored using a combination of Glide SP score and Prime energy. Ligand poses with ligand-receptor interaction matching the observed NMR chemical-shifts were accepted.

2.4.6 Isothermal calorimetry

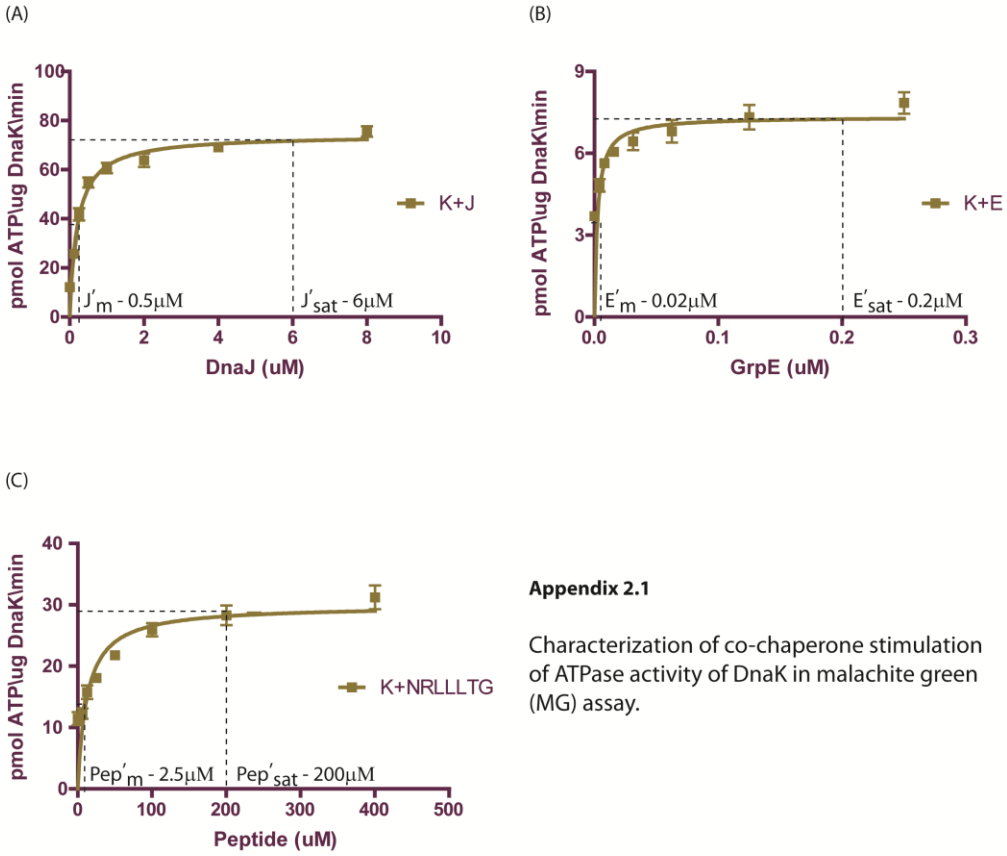
Isothermal calorimetric(ITC) titrations were performed with a VP-ITC MicroCalorimeter (MicroCal, Inc., Northampton, MA) at 37 °C. Titration experiments were performed in 25 mM PBS (pH 7.4), 5 mM $MgCl_2$, 150 mM KCl, 5% DMSO. $DnaK_{FL}$ (60 µM), $DnaK_{NBD}$ (60 µM) or $DnaJ_{FL}$ (1 mM) were extensively dialyzed and then introduced into the calorimetric cell (cell volume = 1.43 mL). Protein samples were then titrated with 500 µM **115-7c** in 15 µL steps until saturation was achieved. Injections were performed at 2 µL/s. Data was analyzed using Microcal Origin (v2.9) assuming independent binding sites to yield enthalpy, stoichiometry of binding and association constant. As a control experiment, we confirmed that 115-7c does not bind to DnaJ even at 1mM concentrations. The weak affinities of 115-7c to DnaK and DnaK-DnaJ complex observed in the ITC platform need to be confirmed by NMR experiments.

Notes

Srikanth Patury and Jason Gestwicki designed the experiments. Chemical synthesis and yeast cell studies were done by Susanne Wisen and Chris Evans. Mutational analysis of DnaK was done by Andrea Thompson. NMR experiments were performed by Eric Bertelsen and Prof. Erik Zuiderweg. Characterization of the mechanism of 115-7c has

been published as ‘Binding of a small molecule at a protein-protein interface regulates chaperone activity in the Hsp70-Hsp40 complex.’ (2010) *ACS Chemical Biology* 5:611-622.

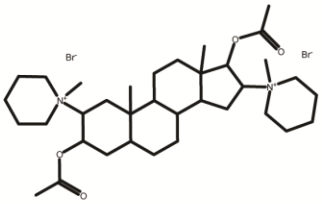
2.5 Appendices



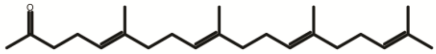
Appendix 2.1

Characterization of co-chaperone stimulation of ATPase activity of DnaK in malachite green (MG) assay.

Appendix 2.2 The 18 hit compounds purchased for reconfirmation.



Pancuronium dibromide



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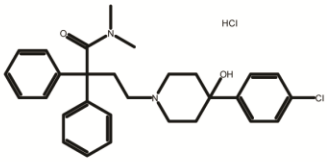

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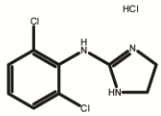

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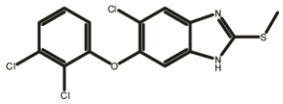
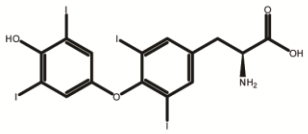
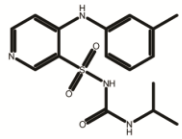


Triclabendazole



L-Thyroxine



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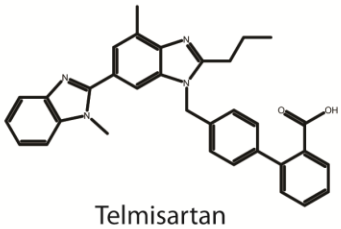
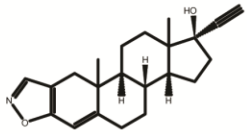
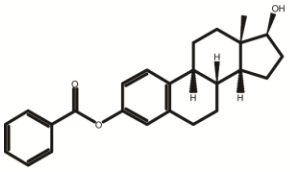


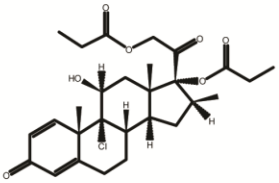
Telmisartan



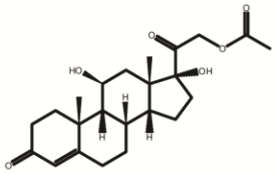
Danazol



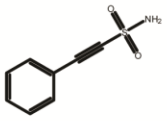
beta-Estradiol 3-benzoate



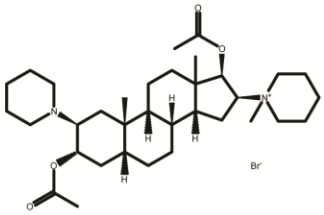
Beclomethasone dipropionate



Hydrocortisone 21-acetate



Pifithrin-u



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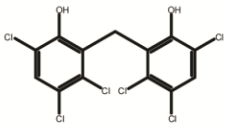
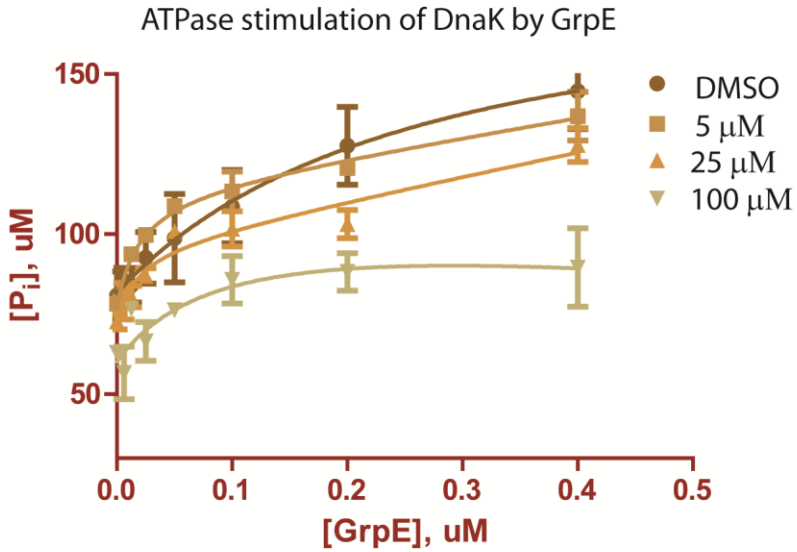
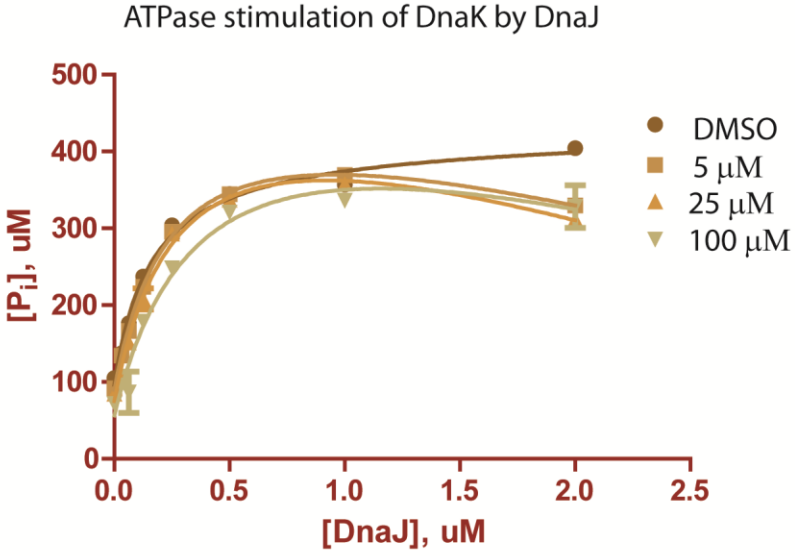


Hexachlorophene

(A)



(B)



Appendix 2.3 Telmisartan also inhibits only the GrpE stimulation of DnaK's ATPase activity in a dose-dependent manner. It does affect the stimulatory effect of DnaJ.

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Chapter 3

Biochemical characterization of the Human Hsp70 chaperone complexes

3.1 Abstract

As described in Chapter 1, heat shock protein 70 is an ATPase that cooperates with its co-chaperones, Hsp40s (or J proteins) and nucleotide exchange factors (NEFs), to regulate protein quality control. Although there are only one Hsp40 (DnaJ) and NEF (GrpE) in most prokaryotes, these classes of co-chaperones are greatly expanded in higher organisms. For example, there are at least 20 J proteins in yeast and more than 40 in humans. Based on the knowledge gained in Chapter 2, the main goal of this Chapter is to identify chemical probes that target the human Hsp70 chaperone complexes. Towards that goal, we first needed to biochemically characterized a number of distinct Hsp70 complexes, composed of 16 combinations of two human Hsp70 isoforms (Hsc70 and Hsp72), three representative Hsp40s (DJA1, DJA2 and Hsj1) and three major NEFs (BAG1, BAG2, BAG3). This part of the study provided unexpected insights into the specialized, biochemical functions of these human Hsp70 complexes *in vitro* and they identified previously unknown and dramatic differences between the eukaryotic and prokaryotic systems. For example, we found that the ATPase activity of human Hsp70s is not stimulated by substrates and that human NEFs, such as BAG1 and BAG2, do not

dramatically stimulate nucleotide exchange in steady state ATPase assays, despite both of these factors having good affinity for Hsp70s. Moreover, we show that the Hsp40 DJA2, but not its highly homologous ortholog DJA1, is able refold denatured luciferase, even though both DJA1 and DJA2 have similar effects on ATP stimulation. Finally, a major goal of this thesis is to characterize the Hsp70 systems in order to enable "gray box" screening (as described in Chapter 2). Thus, in the last part of this Chapter, we performed a high-throughput screen of 100,000 diverse compounds against a reconstituted human chaperone complex (Hsp72/DJA2/BAG2) and successfully identified primary "hit compounds". Interestingly, some of these compounds appear to inhibit Hsp40-stimulated ATP turnover, suggesting that (like we saw in Chapter 2) a "gray box" strategy may be an effective way to find new chemical probes for this system. Together, these results provide promising, new chemical probes and the most systematic biochemical study of human Hsp70s to date.

3.1.1 The human HSPA (Hsp70) family

The human Hsp70 family consists of at least eight homologous proteins with a high degree of sequence conservation (~85%) [1]. Isoforms of Hsp70s are found in every subcellular organelle. For example, a family member is found in both the endoplasmic reticulum (HSPA5 or Bip) and mitochondria (HSPA9 or mtHsp70) [2, 3]. In the cytosol, the major isoforms are the stress-inducible HSPA1 (Hsp72) and the constituitively expressed HSPA8 (Hsc70). Hsc70 is considered a 'housekeeping' member of the family that is involved in folding of nascent polypeptides, protein translocation, chaperone mediated autophagy and other tasks and it is constitutively expressed in all cell types [4].

Hsp72 is the major stress inducible isoform and it functions in enabling the cell to cope with aggregation denatured proteins during and after the stress [5].

3.1.2 Hsp70 chaperone complexes can select from large numbers of potential co-chaperones in eukaryotes

In addition to having multiple Hsp70 isoforms, mammalian cells contain over 40 Hsp40s (J-proteins) and at least 4 distinct types of NEFs [6]. At any given time, an individual Hsp70 molecule can only interact with a single representative of each major class; therefore tens of thousands of possible complexes are possible (Figure 1.3). Why do mammalian cells have so many chaperone complexes? Do they serve specific functions? Understanding the biochemical properties of these complexes might provide insight into Hsp70 biology.

3.1.3 DNAJ (Hsp40s, J-proteins) family co-chaperones are diverse in humans and enable the multifunctionality of Hsp70 chaperones

In the human genome, at least 41 different DNAJ-encoding genes have been identified [7]. The various members of this family are classified into 3 groups (A-, B- and C-type) based on their sub-domain architectures (described in Chapter 1 and extensively reviewed in [8]). The defining feature of these proteins is the highly conserved J-domain, which interacts with the Hsp70 and stimulates its ATPase activity [9]. In addition to the J domain, many DNAJ co-chaperones have other domains with distinct functions. For example, some A-type J-proteins have also been shown to bind client proteins and deliver them to Hsp70 [10]. The diversity of structures in these non-J domain regions suggests

that different Hsp40s may play distinct roles in Hsp70 biology. This model has been partially verified by genetic studies in yeast systems, in which J proteins are found to have some redundant and some unique activities [11, 12]. However, biochemical analyses and comparisons of human J proteins have been rather limited.

As a first step in better understanding the biochemistry of human Hsp70 chaperone systems, we chose to focus on three cytosolic J-proteins, DJA1, DJA2 and Hsj1, which are closely related to the best-characterized yeast cytosolic protein, Ydj1 [13]. In part, we chose these J proteins because DJA2 has been previously shown to stimulate the ATPase activity of Hsp72 [14], but DJA2 was found to stimulate Hsc70-mediated refolding of denatured luciferase to much greater extent than DJA1 [15]. Hsj1 is an interesting J-protein because it contains two ubquitin-interacting motifs (UIMs) in its C terminal region, suggesting a role in turnover. Moreover, Hsj1 can target misfolded proteins such as polyQ for degradation [16, 17]. Together, we hoped that this suite of J proteins would provide a good overview of interactions between these co-chaperones and Hsp70.

3.1.4 Nucleotide Exchange Factors (NEFs) also give chaperone complexes specific functions

There are four distinct classes of NEFs in the human genome and although they all interact primarily with the NBD of Hsp70, nucleotide release activity is achieved by mechanistically distinct ways (as described in Chapter 1). Briefly, the GrpE-type and HSP-binding protein 1 (HSPBP1) type NEF families are primarily involved in only nucleotide exchange [18]. In contrast, the Hsp110 class both binds to client proteins and

facilitates nucleotide exchange [19]. The final class of NEFs, the Bcl2-associated athanogene (BAG) family of proteins, appears to offer the most divergence in cellular functions. These six NEFs contain a variety of other domains thought to be involved in guiding the fate of bound substrates. For example, BAG1 contains an ubiquitin-like domain and it has been shown to mediate interactions between Hsp70 and the proteasome [20], helping to degrade raf-1 protein kinase, the glucocorticoid hormone receptor and mutant huntington [21]. In contrast, the related NEF, BAG2, has been identified as an inhibitor of CHIP that blocks proteasomal degradation [22], while BAG3 specifically helps with Hsp70-mediated aggresome-targeting and autophagy [23]. In this Chapter, we focused on BAG1-3 to better understand how they bind Hsp70s and how they impact ATP cycling.

3.1.5 Combinatorial assembly of Hsp70 complexes and "gray box" screening

One of our major goals is to identify small molecules that target the activity of specific Hsp70 chaperone functions. As discussed in Chapter 2, the design of 'gray box' screens requires informed choices about the identity and relative ratio of Hsp70 and its co-chaperones used in the screen. In contrast to the prokaryotic DnaK-DnaJ-GrpE system, as detailed above, the diversity of the co-chaperones in the human is more complex. Thus, in this Chapter, we sought to characterize the biochemical properties of reconstituted human chaperones using the "modules" of Hsc70, Hsp72, DJA1, DJA2, Hsj1, BAG1, BAG2, and BAG3. Specifically, we characterized their ATP turnover rates, binding constants of the components for each other and explored how they differ in their ability to refold a model substrate *in vitro*. Guided by this knowledge, we designed and

implemented a HTS campaign against Hsp72-DJA2-BAG2 and found new inhibitors.

3.2 Results

3.2.1 Human Hsp70s (Hsp72 and Hsc70) have similar ATPase and holdase activities compared to *E. coli* DnaK

Towards our goal of characterizing the human Hsp70 chaperone system, we recombinantly expressed and purified the Hsc70 and Hsp72 chaperones and the DJA1, DJA2, Hsj1, BAG1, BAG2 and BAG3 co-chaperones. In the first set of characterization studies, we measured the intrinsic ATPase activity of the human Hsp70s by malachite green (MG) assays and compared them to the well-known DnaK protein (see Chapter 2). These results suggested that the human chaperones had similar intrinsic ATP hydrolysis rates to each other (~1.4 ± 0.3pmolP_i/µg chaperone/min) (Figure 3.1A) and that both rates were similar (or slightly reduced) compared to DnaK (~1.7 ± 0.23pmolP_i/µg DnaK/min). We also wanted to verify our recombinant Hsp70s bound to luciferase

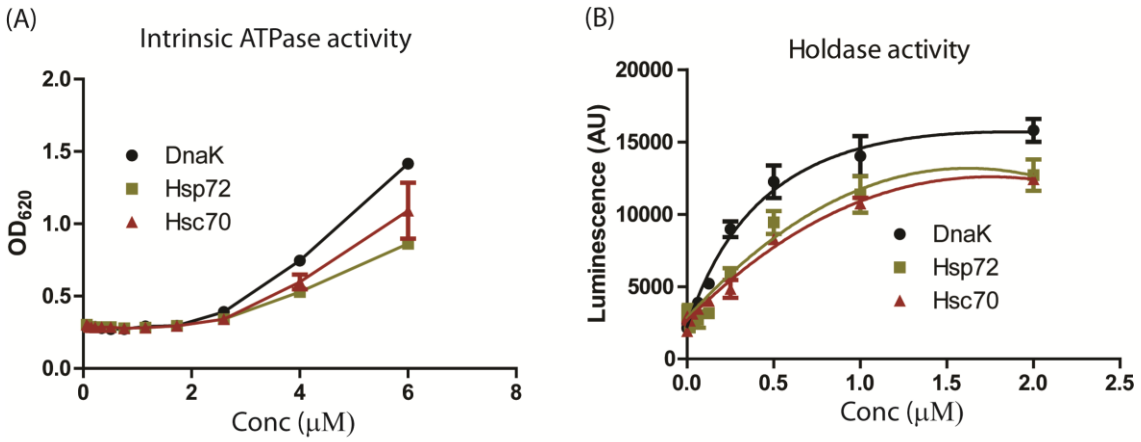

Figure 3.1 Comparison prokaryotic DnaK and human Hsp70s. (A) intrinsic ATPase activity of DnaK and human isoforms Hsp72 and Hsc70. (B) Holdase activity of the chaperones. DnaK has similar intrinsic ATPase and holdase activity compared to the human isoforms. Experiments were repeated in triplicate.

using a "holdase" assay. In this assay, the ability of a chaperone to protect luciferase from heat denaturation is measured (see Experimental Procedures). We found that both Hsp72 (12.7×10^3 lum) and Hsc70 (12.1×10^3 lum) were able to protect firefly luciferase from unfolding, but that these activities were slightly reduced compared to DnaK (15.1×10^3 lum) (Figure 3.1B). These rather modest differences between DnaK and the human Hsp70s was not statistically significant and could be due to small differences in folding.

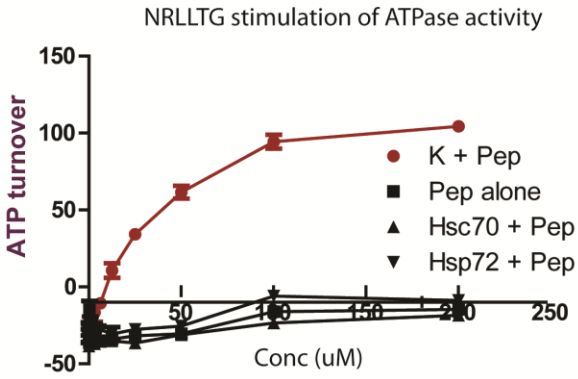

Figure 3.2 NR-peptide does not stimulate ATPase activity of human isoforms in the steady state MG assay.

3.2.2 Substrate peptide does not stimulate the ATPase activity of human Hsp70s

Binding of substrate in the substrate binding domain (SBD) of DnaK induces an allosteric conformational change that stimulates ATP hydrolysis in the NBD [24]. In addition, it has also been shown that ATPase rates correlate to substrate binding affinity [25]. These findings have been used to propose that substrates

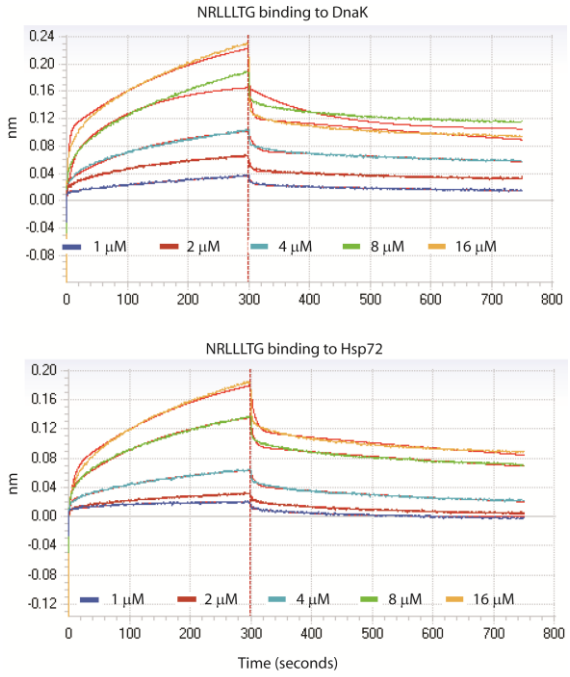

Figure 3.3 NR-peptide binds to both DnaK(K_d - 3.76 ± 0.36µM) and Hsp72 (K_d - 17.74 ± 1.48µM). It binds 4 fold weaker to Hsp72 and may explain the lack of stimulation of ATPase activity.

control, in part, their own fate in the DnaK-DnaJ-GrpE system by allosterically stimulating nucleotide hydrolysis. To test if this same model holds true in human Hsp70s, we performed ATPase assays in the presence of the well-characterized chaperone substrate, NRLLLTG (NR) peptide. We were surprised to find that NR-peptide clearly did not stimulate the ATPase activity of either Hsp72 and Hsc70. This is a major and potentially significant difference between the eukaryotic and prokaryotic systems

To verify this unexpected result, we wanted to confirm that the NR peptide binds to the human chaperones. Using immobilized Hsp70s in a bio-layer interferometry (Octet Red) system, we found that NR-peptide binds to the human chaperones (K_d = 17.7 ± 1.4 μM) at only a 4-fold weaker affinity compared to DnaK (K_d=3.76 ± 0.3 μM). Satisfyingly, the affinity of NR peptide for DnaK by Octet Red was similar to the affinity published previously (K_d = 11 μM) by ITC [24, 26]. Thus, differences in affinity are not sufficient to describe why substrate doesn't stimulate ATP hydrolysis in human Hsp70s. Rather, differences in allostery may be involved.

3.2.3 Different co-chaperones are able to support different levels of ATPase stimulation in human Hsp70s

As discussed in Chapters 1 and 2, DnaK's co-chaperones DnaJ and GrpE stimulate steady state ATPase activity through their ability to stimulate hydrolysis and exchange, respectively. Using parallel logic, we wanted to characterize the human J-proteins (DJA1, DJA2 and Hsj1) and NEFs (BAG1, BAG2 and BAG3). Specifically, we tested ~12 distinct Hsp70 complexes formed from combinatorial reconstitution of these co-

chaperones in MG assays (Table 3.1 and Appendix 3.1). These results are plotted as surface diagrams in which the Hsp70 concentration is held constant and the levels of one J protein and one NEF is varied.

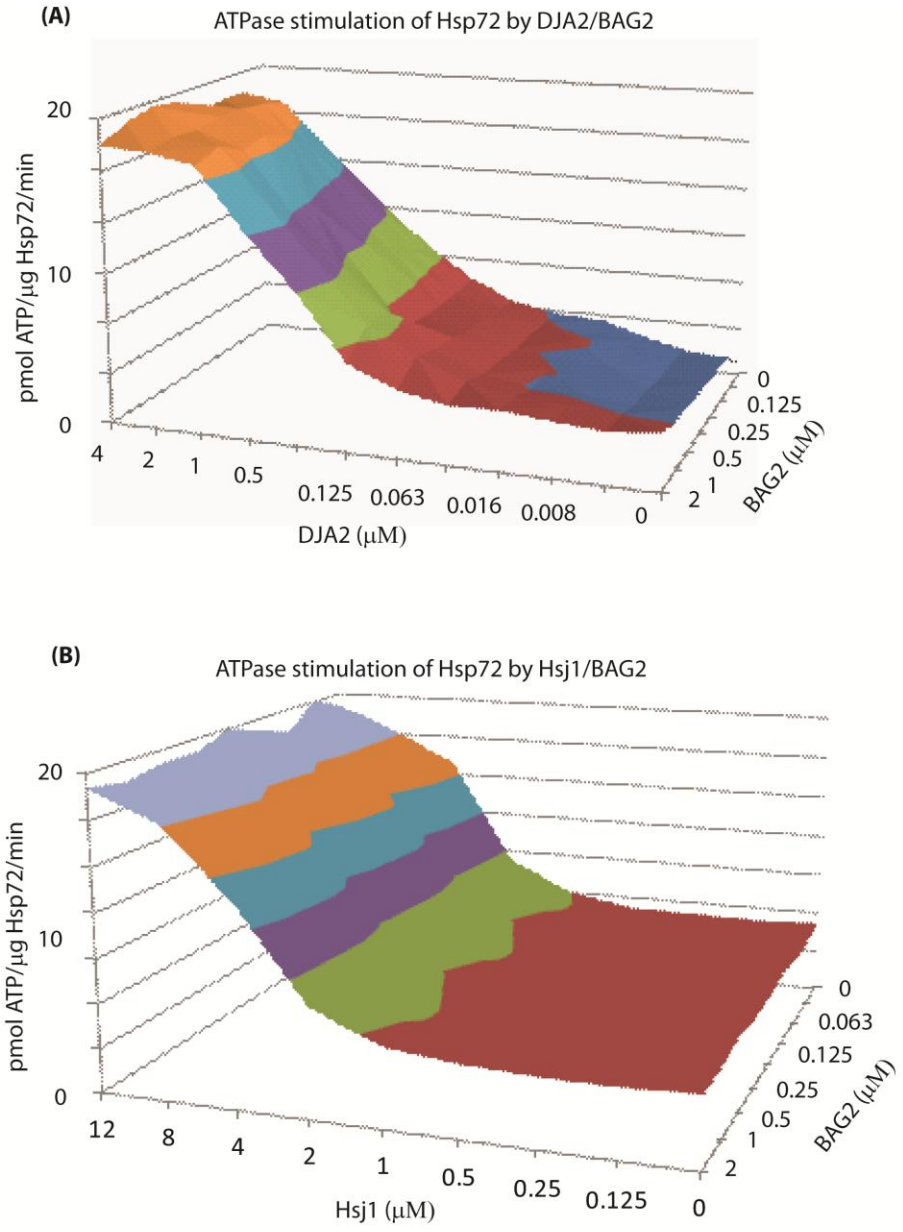

Figure 3.4 Stimulation of the ATPase activity of Hsp72 by different Hsp40s. (A) Stimulation profile of DJA2/BAG2. DJA1 also exhibited a similar profile. (B) Stimulation profile of Hsj1/BAG2. Experiments were repeated twice.

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The full description of this data is found in the Appendix, and the results in Figure 3.4 are representative of what we found. Specifically, we saw that the different J-proteins were able to stimulate the ATPase activities of both Hsp72 and Hsc70 (Figure 3.4 and Appendix 3.1), but that they had different "potency", as described by their half-maximal concentrations required for stimulation (Figure 3.4 and Table 3.1). DJA1 and DJA2 had similar K_m values of (~0.5μM) against both Hsp72 and Hsc70, while Hsj1 had a higher K_m value of (~4. 5μM). All of the human J proteins had the same maximum stimulation value (V_{max} ~18 pmol ATP/μg/min), suggesting that only the affinity of the different J proteins might be different. Surprisingly, none the NEFs had any stimulatory affect. This is surprising because GrpE stimulates 2-6 fold under these conditions. Thus, human Hsp70/NEF systems also differ from the prokaryotic. We note that these experiments do not rule out an effect in single turnover assays or under conditions of specific substrates or J combinations.

Table 3.1 ATPase activity of human co-chaperones with Hsp72 and Hsc70. NEFs (BAG1,BAG2,BAG3) did not have any stimulatory effect.

	Hsp72		Hsc70	
	V_{max} pmol ATP/μg/min	K_m μM	V_{max} pmol ATP/μg/min	K_m μM
DJA1	18.62 ± 1.28	0.48 ± 0.14	18.19 ± 2.14	0.46 ± 0.09
DJA2	17.93 ± 1.23	0.42 ± 0.19	16.39 ± 1.47	0.51 ± 0.4
Hsj1	17.52 ± 1.95	4.23 ± 0.9	18.32 ± 2.45	4.4 ± 0.34
	V_{max} (pmol ATP/μg/min)		K_m (μM)	
DnaK/DnaJ	9.8 ± 0.6		0.58 ± 0.11	
DnaK/GrpE	3.8 ± 0.7		0.021 ± 0.009	

Concentration of Hsp72/Hsc70/DnaK was 0.5uM. DJA1,DJA2 and DnaJ were varied from 0-4uM. HSj1 was varied from 0-12 uM. NEFs were varied from 0-2uM.

3.2.4 Only DJA2 can stimulate the *in vitro* refolding capacity of Hsc70/Hsp72

One of the functions of Hsp70s is to refold damaged proteins, and this activity can be measured *in vitro* by luciferase refolding assays. In the prokaryotic system, full refolding of denatured luciferase requires all three components and the process is absolutely dependent on DnaK and DnaJ [27]. We tested the 12 different human Hsp70 combinations and found that only DJA2 supported refolding in combination with either Hsc70 or Hsp72 (Figure 3.5 and Appendix 3.2). In a very interesting finding, none of the other co-chaperones was able to support refolding (e.g. see Figure 3.5B). Again, this result is in stark contrast to the prokaryotic system and it reiterates the functional complexity of the human chaperone system.

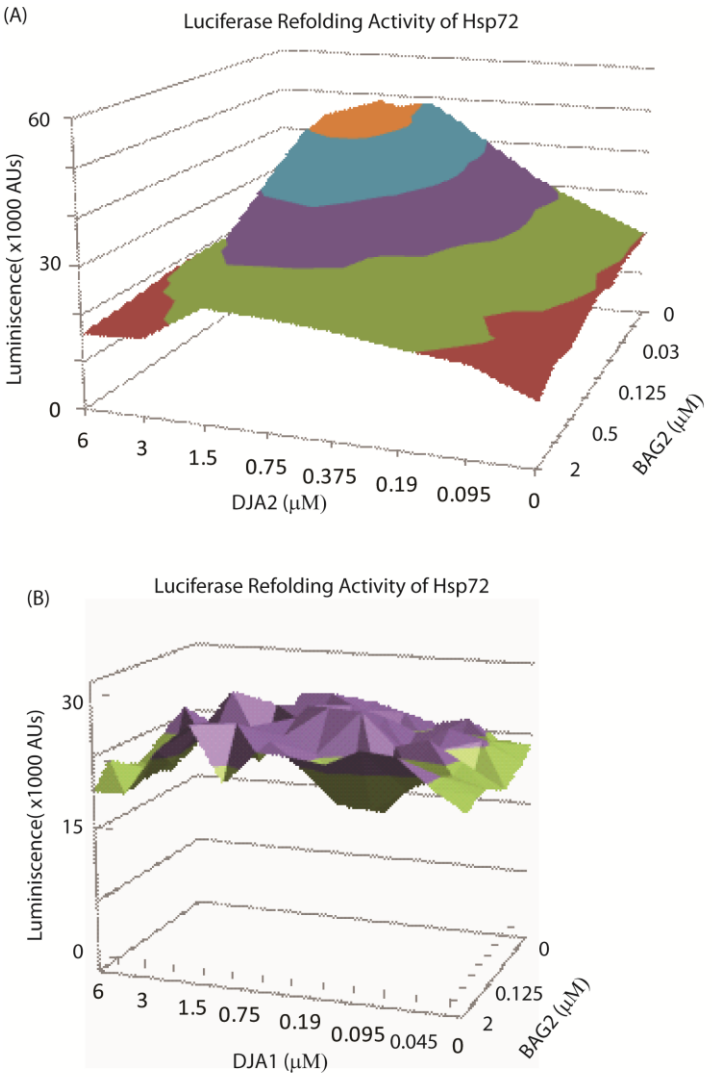
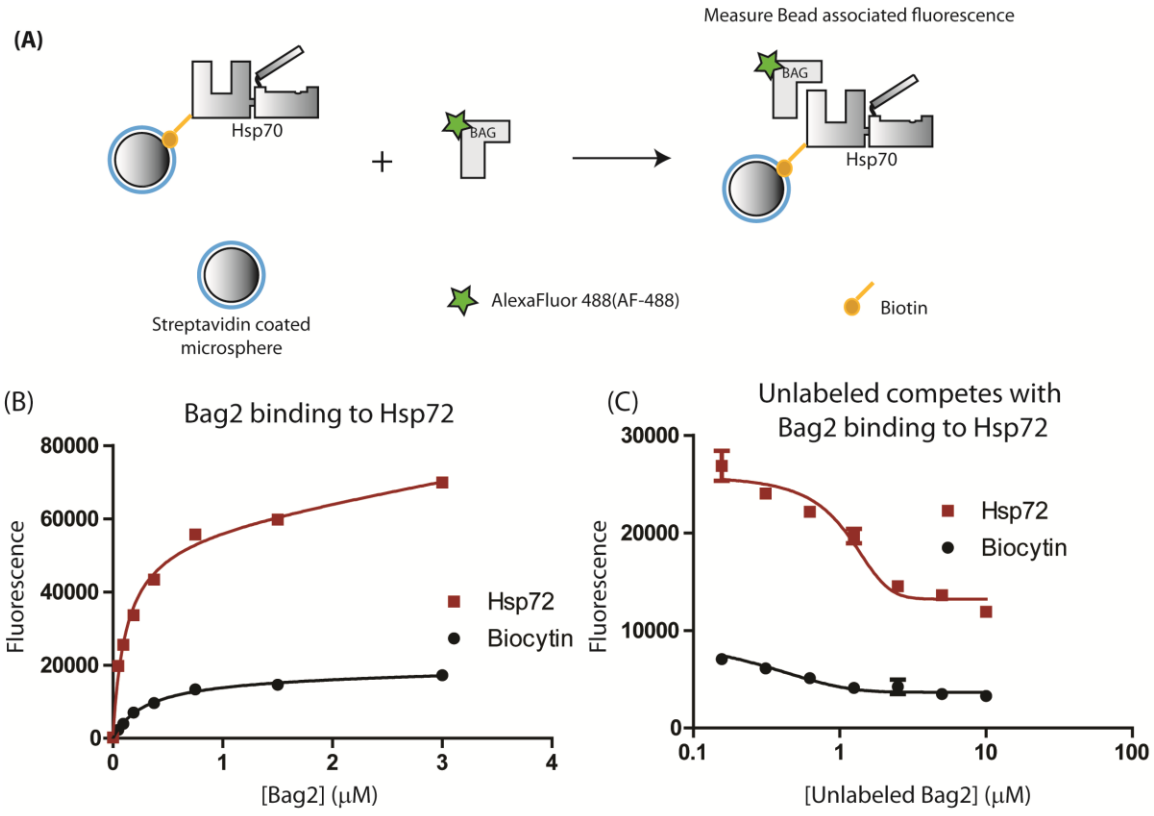


Figure 3.5 Luciferase refolding activity of Hsp72 as stimulated by co-chaperones. (A) DJA2 was functionally competent to fold denatured luciferase, while BAG2 displayed no effect in the assay. (B) DJA1 was unable to fold denatured luciferase. Data is the average of two experiments.

3.2.5 Human NEFs bind directly to human Hsp70s

It was somewhat surprising to us that the human BAG-type NEFs were not able to stimulate either ATP turnover or the refolding capacity of Hsp70s. Next, we wanted to determine whether or not these proteins bind to Hsp70s. If they didn't bind to Hsp70s, then they might be partially unfolded or otherwise damaged, which might explain why they didn't assist ATPase or refolding activity. Circular dichroism studies conformed that



	K_d	IC_{50}
Bag2	122 ± 3.6 nM	12.7 ± 1.6 μM
Bag3	39.1 ± 3.6 nM	424.7 ± 23.6 nM

Figure 3.6 Flow cytometry binding assay for BAGs and Hsp70s. (A) Schematic of the assay. Biotinylated Hsp70 is immobilized on streptavidin beads and incubated with AF-488 labeled BAG proteins. Bead associated fluorescence is measured. (B) Bag2 binds in a dose dependent manner to Hsp72. (C) Unlabeled Bag2 can compete with labeled protein in binding to Hsp72. Experiments were performedin duplicate

the BAGs appeared folded, but to further clarify this issue, we develop a flow cytometry-based binding assay to measure the binding affinities of the BAG1-3 proteins to Hsp70 (Figure 3.6).

Briefly, biotinylated Hsp70 is loaded onto streptavidin coated microspheres and incubated with BAG protein that had been labeled with a fluorophore (AlexaFluor488). Both the Hsp70 and BAG protein were labeled with amine reactive probes and the free probe was removed, as described in the Materials and Methods. We then measure the bead-associated fluorescence to determine apparent binding constants. Importantly, beads coated with biocytin served as the negative control to account for non-specific binding. In these studies, we observe a dose-dependent binding with both BAG2 (K_d 122 ± 3.6 nM) and BAG3 (K_d 39.1 ± 3.6 nM) (Figure 3.6B). To confirm that the observed binding event is specific we competed with unlabeled BAG (BAG2 IC_{50} = 12.7 ± 1.6μM, BAG3 IC_{50} = 423.7 ± 23.6 nM). These binding affinities have recently been verified by another graduate student, Jennifer Rauch, using isothermal calorimetry (ITC). In addition, Ms. Rauch found that BAG1 binds Hsp70 with an affinity of 212 ± 19.8nM (preliminary results). Thus, we conclude that the BAG proteins bind Hsp70, but they are not potent nucleotide exchange factors or stimulators of luciferase refolding *in vitro*. Rather, they may serve as scaffolding proteins to recruit Hsp70 to subcellular locations or into specific partnerships.

3.2.6 Gray Box screen against the human chaperone complex (Hsp72/DJA2/BAG2)

The biochemical characterization of the human Hsp70 systems (described above)

suggested that a screen against human Hsp70 systems may yield sufficient signal:noise for high throughput chemical screening. Next, we considered which specific complex we should use in the screen. Because DJA2 was active in the ATPase assays and it was the only human J protein with functional refolding activity, this co-chaperone seemed like a clear choice. Based on what we learned in the biochemical characterization studies and in Chapter 2, we used DJA2 near its K_m value (Appendix 3.3), to increase the odds of finding activators. The two human Hsp70s, Hsp72 and Hsc70, were very similar in all our assays, but we chose Hsp72 since it appears to be most important in tau biology (see Chapter 1 and [28]). Finally, we decided to include BAG2. The reasoning behind this last choice is that BAG2 is expressed in neuronal systems and it forms a tight complex with Hsp72 (as discussed above). Thus, although it doesn't directly contribute to ATP turnover, we wanted to provide a more "physiological" target. In other words, any compounds that bind to the BAG2-Hsp72 interface would need to compete with this contact *in vivo*, so we wanted to model this environment in the HTS campaign.

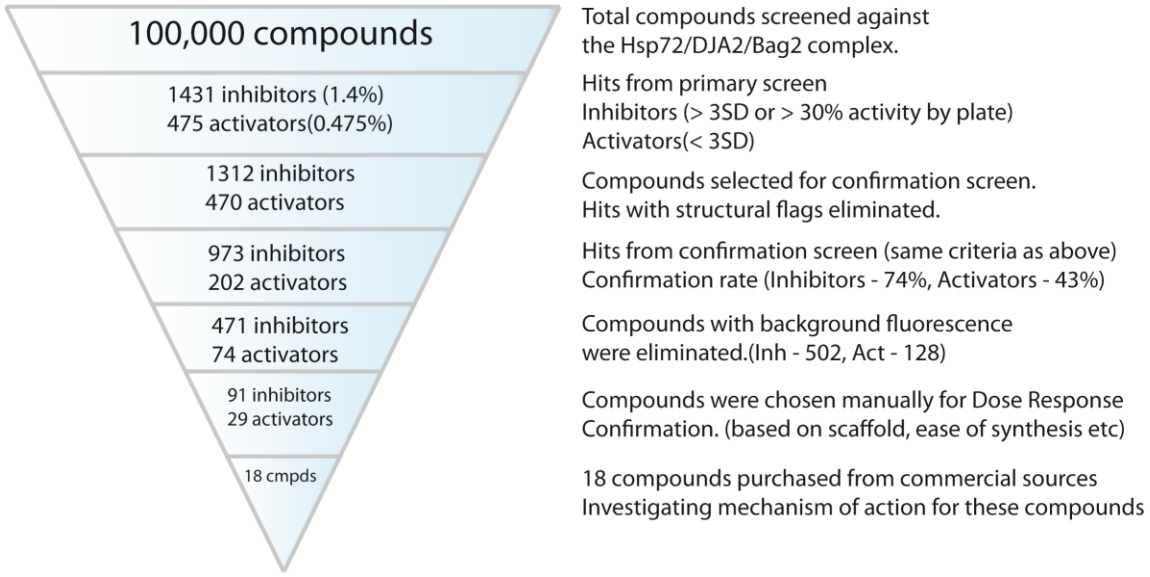


Figure3.7 HTS screening efforts against Hsp72/DJA2/Bag2 chaperone complex.

The primary HTS screen was performed with the ChemBridge 100K library, which is a structurally diverse chemical collection of drug-like molecules. The assay was carried out as described in Chapter 2. The protein concentrations were Hsp72 (0.5 μM), DJA2 (0.1 μM) BAG2 (0.25 μM) and ATP (1 mM). Test compounds were included at final concentrations of 40 μM. The positive control was all of the components except the Hsp72. The negative control was DMSO. From this screen, active compounds were selected based on two criteria: (a) samples that either increased or decreased the phosphate production by 30% or (b) samples that had signals more than 3 standard deviations (3SD) from the negative controls. Using these criteria, we identified 1312 inhibitors and 470 activators. To confirm these activities, hits were subjected to a confirmation screen in duplicate using the original library compound. This process reduced the inhibitors to 973/1312 and activators to 202/470. Next, because the assay was fluorescence-based, we tested the intrinsic fluorescence of the inhibitors and activators and removed any that had values greater than 110% of DMSO control. This filter reduced the actives to: 471/1312 inhibitors and 74/470 activators. The top 91 inhibitors and 29 activators were then selected for dose response curves (eight concentrations between 0 and 100 μM). From this analysis, we repurchased 18 compounds (8 activators and 10 inhibitors) based on their potency and the synthetic tractability of the scaffold. These re-purchased compounds were then retested in ATPase assays (activators - Appendix 3.4, inhibitors - Appendix 3.5). We were pleased to find that 5 of these compounds (all inhibitors) retained activity (Figure 3.8), which is a typical confirmation rate upon retesting of fresh compound (CCG, personnel communication).

Characterization of these inhibitors to identify their mechanism-of-inhibition and their binding sites is currently ongoing in the Gestwicki laboratory.

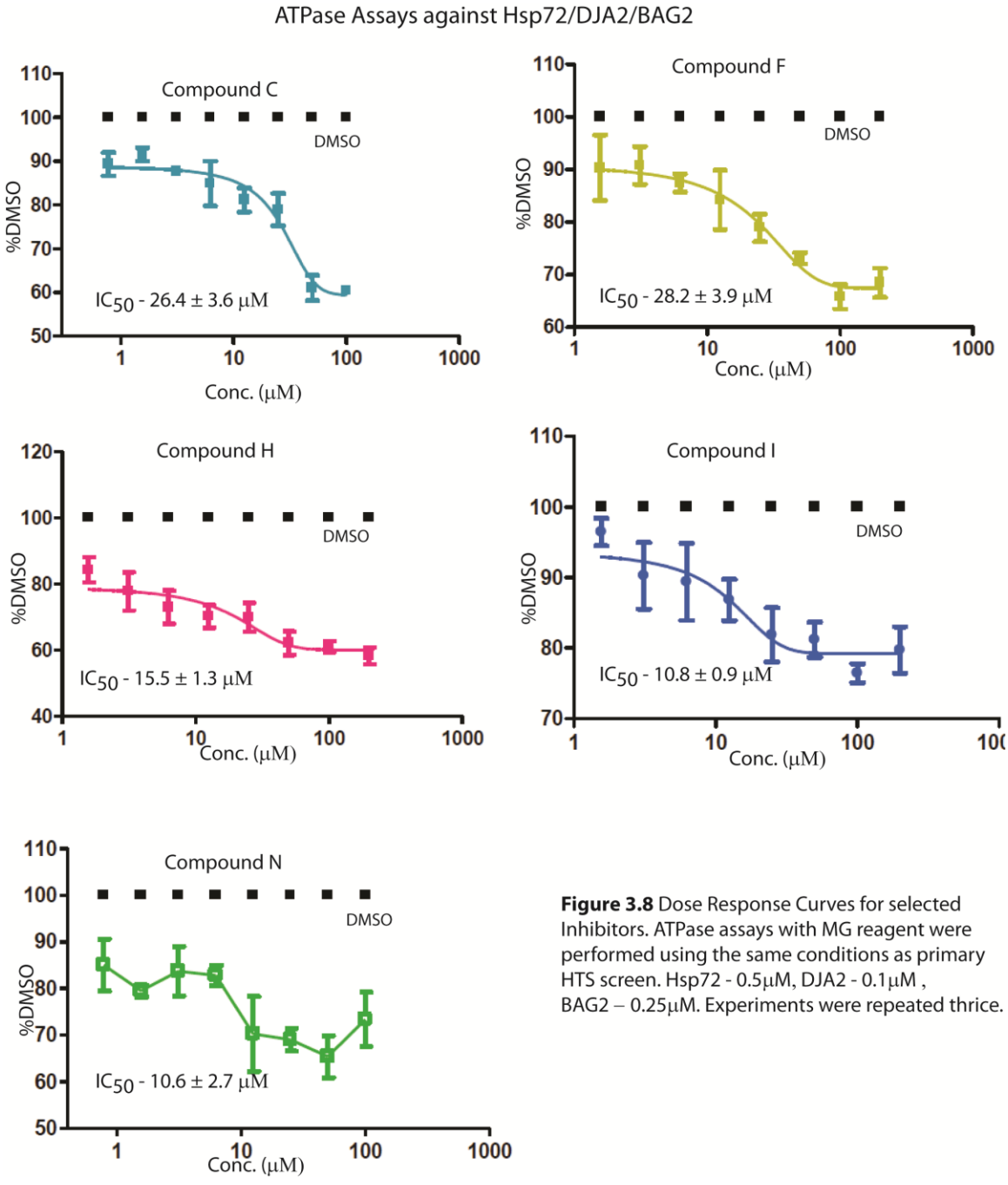


Figure 3.8 Dose Response Curves for selected Inhibitors. ATPase assays with MG reagent were performed using the same conditions as primary HTS screen. Hsp72 - 0.5μM, DJA2 - 0.1μM , BAG2 – 0.25μM. Experiments were repeated thrice.

3.2.7 Hits compounds exhibit selectivity against different chaperone complexes

As mentioned in Chapter 1, the human Hsp70 system is highly similar to the prokaryotic system. For example, DnaK is more than 50% identical and 75% similar to human Hsp72. Thus, we wanted to ascertain whether the five inhibitors identified above would retain activity against the prokaryotic DnaK-DnaJ-GrpE system. We were pleased to find that these compounds were also inhibitors of this system, although the IC_{50} values were not identical (Figure 3.9 and Table 3.2). More work is needed to understand the mechanisms and potential selectivity of these compounds, but this finding provides further support for the potential of this screening method and the potential of these active compounds.

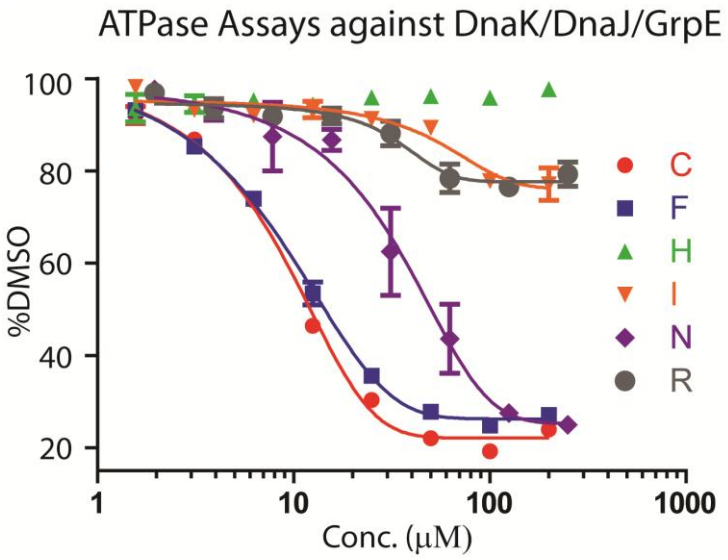


Figure 3.9 Inhibitors are selective against different complexes. H does not inhibit DnaK/DnaJ/GrpE (Pro.) complex while it inhibits the Hsp72/DJA2/BAG2 (Hum.) human complex. R does the inverse, it inhibits the Pro. complex but has no effect against the human complex. I,N are less potent against the Pro. complex than the human complex, while C,F are more potent agains the Pro. complex. ATPase assays were performed in triplicate using MG reagent. DnaK - 0.5μM, DnaJ - 0.5μM , GrpE – 0.5μM.

	DnaK/DnaJ/GrpE		Hsp72/DJA2/BAG2	
	IC_{50} (μM)	% inhibition	IC_{50} (μM)	% inhibition
Compound C	6.83 ± 0.14	22	26.4 ± 3.6	60
Compound F	7.471 ± 0.19	31	28.2 ± 3.9	68
Compound H	No effect	0	15.5 ± 1.3	60
Compound I	77.52 ± 4.95	78	10.8 ± 0.9	81
Compound N	44.77 ± 1.47	25	10.6 ± 2.7	70
Compound R	26.32 ± 2.45	80	No effect	0

Table 3.2 Compounds inhibit the bacterial and human chaperone complexes with different affinities. ATPase assays with MG reagent were performed using the same conditions as primary HTS screen. DnaK/Hsp72 - 0.5μM, DnaJ/DJA2 - 0.1μM , GrpE/BAG2– 0.25μM

3.3 Discussion

The major goals of the work in this Chapter were to characterize human Hsp70 chaperone systems using biochemical methods and use this information to implement a HTS experiment. These studies are inspired by the efforts in Chapter 2, in which we describe the concept of "gray box" screening, which is a new method designed to identify inhibitors of protein-protein interactions. The major challenge of this approach is that the multi-protein complex must be reconstituted and, thus, the biochemical activity of the co-factors must be defined. Towards that goal for the human Hsp70 system, we characterized the effects of co-chaperones on ATPase and luciferase refolding activity. During this process, we made a number of unanticipated findings that suggest substantial differences between the human and prokaryotic systems. For example, we found that human Hsp70s bind to model substrate with only a 4-fold weaker affinity, yet this

interaction does not stimulate ATPase activity. This result is in stark contrast to what is found in DnaK [29, 30]. These results suggest that there are substantial differences in the allostery in the human and prokaryotic systems. Recent findings from the Mokranjac group have provided some further support of this idea. Using a FRET assay, they found that DnaK is less flexible than human mitochondrial Hsp70 [31]. Thus, despite the high homology between these proteins at the sequence and structural level, there might be considerable differences in more subtle regulatory aspects, such as allostery and dynamics.

Another surprising finding is that different J proteins have different apparent affinities for Hsp70s. Specifically, we found that Hsj1 has a weaker potency than DJA1 and DJA2. This seems to occur despite the near identity of the conserved J domains (~88%), suggesting that regions outside of the J domain may be responsible for the differences. In fact, the G/F rich domain of DnaJ has been found to contribute to ATPase stimulation [10, 32] and this region is less conserved in DJA1, DJA2 and Hsj1 (~55%), providing a possible mechanism. Moreover, despite the ability of these J proteins to stimulate ATP turnover, only DJA2 was able to support refolding. Dogma in the chaperone field has suggested that ATP stimulation is required for luciferase folding, yet these results clearly demonstrate that these two activities are not directly linked. Work by other members of the Gestwicki group (using point mutants) has also supported this model [33].

Finally, we found that the human BAG proteins fail to stimulate steady state ATP turnover. Although this finding does not preclude the idea that BAG proteins might

stimulate in single turnover assays, they clearly show that human BAGs have a different function than prokaryotic GrpE in the ATPase cycle. This difference was not due to a failure to bind Hsp70, because we used flow cytometry and ITC to confirm this protein-protein interaction and found values similar to those reported in the literature [34]. We are currently pursuing structures of Hsp72 bound to BAG2 to help us understand these differences. Together, these findings have highlighted important and unexpected differences between the prokaryotic and human Hsp70 systems.

In the final part of this Chapter, we successfully carried out a HTS experiment using the Hsp72-BAG2-DJA2 complex as the target and identified small molecules that modulate the human chaperone system. These findings provide good support of our "gray box" screening concept. Moreover, these results have provided approximately 5 new chemical scaffolds with potential to be used as novel Hsp70 modulators. These chemical probes may be new tools for tuning the protein-protein interactions in the Hsp70 chaperone complexes.

Notes

Srikanth Patury and Jason Gestwicki designed the experiments. Tomoko Komiyama and Amanda Lewis helped with the protein purification and HTS experiments. Tomoko Komiyama, Robert Briski, Bryan Dunyak performed follow-up studies on the hit compounds. Jennifer Rauch aided in the BAG expression and binding assays. Tom McQuade and Steve Vander Roest helped run the chemical screen in the CCG.

3.4 Experimental Procedures

3.4.1 Protein purification

Hsp72, Hsc70, DJA2, BAG2, BAG3 were cloned into the pMCSG7 plasmid (Midwest Center for Structural Genomics, Bethesda, MD) by ligation independent cloning. For Hsp72, Hsc70 expressiont, 25 mL of overnight (37 °C) LB culture of Rosetta (DE3) culture was poured into 1 L of Terrific Broth. After 3 hours incubation at 37 °C, the culture was cooled down to 28 ^{o}C for 2 hours before overnight induction of expression with 200 μM IPTG overnight, and the cell pellet was stored at -80 °C until use. For DJA2, BAG2, BAG3, a single colony of Rosetta (DE3) culture was inoculated into 5 mL LB. After 6 hours of incubation at 37 °C, the 5 mL starter culture was poured into 1 L Terrific Broth and the larger culture was incubated at 37 °C until OD_{600} reached 0.4 - 0.6. Expression was induced by 500 μM IPTG under 18 °C for ~15 hours with shaking and the cell pellet was stored in -80 °C until use. All proteins were purified by standard Ni purification protocol using Ni-NTA His•Bind® Resin (Novagen, Darmstadt, Germany) as following. The cell pellet was resuspended in His-binding buffer (25 mM Tris, 600 mM KCl, 5 mM imidazole, pH 8.5) supplement with protease inhibitor cocktail (1 tablet per liter of original culture) and the bacterial cells were lysed by sonication (3.5 minutes, 30 sec on/off cycle, 63%). The cell debris was removed by centrifugation at 20,000 rpm for 45 mins. The supernatant was incubated with Ni-NTA resin for 2 hours at 4 °C. To remove the contaminated ATPase activity in the J-protein and NEF samples, the His-proein-bound Ni-NTA resin was washed extensively by 100 mL of His-binding buffer, 150 mL 25 mM Tris buffer (40 mM Imidazole, 300 mM KCl, 3% ethanol, pH 8.0), and

finally with 500 mL 25 mM Tris buffer (30 mM imidazole, 100 mM KCl, 3% ethanol, pH 8.0). The proteins were eluted by His-elution buffer (50 mM Tris, 300 mM Imidazole, 150 mM KCl, pH = 8.0) and the J-proteins were concentrated, exchanged into 25 mM HEPES buffer (150 mM KCl, 5 mM $MgCl_2$, pH 7.5) and stored at -80 °C until use. For the BAG proteins an additional purification step included the MonoQ column.

3.4.2 ATPase Assays

We followed the ATPase assay protocol developed by Lyra Chang, a graduate student in the lab[29]. Briefly, samples were prepared with the addition of Hsp70, Hsp40, NEFs to a total volume of 15 µl in each well. Next, 10 µl of 2.5 mM ATP was added to start the reaction. The final concentrations were: ATP (1 mM), Hsp70 (0.5 µM) unless otherwise noted. Intrinsic ATPase rate was measured with Hsp70 (0.6 µM) in the absence of co-chaperones or substrate. The final concentrations of Hsp70, Hsp40, NEF are reported for each experiment in the results section. For steady state conditions, samples were incubated at 37 °C for three hours, then 80 µl of malachite green reagent was added to each well, immediately followed by 10 µl of 32% (w/v) sodium citrate. Samples were mixed thoroughly and incubated at 37 °C for 15 minutes. Finally, OD_{620} was measured. All experiments were preformed in triplicate and the signal from non-specific ATP hydrolysis in controls lacking Hsp70 was subtracted. A phosphate standard curve (using potassium dibasic phosphate) was generated each day and used to convert the units to pmol P_i/µg DnaK/min.

Stimulation curves were evaluated by fitting the data using a hyperbolic fit with a non-zero intercept;

$$y = V_{max} * x / (K_m + x) + b$$

The non-linear fit was performed using GraphPad Prism version 4.0 for Windows (GraphPad Software San Diego, CA). The protocol for the HTS ATPase assay was taken from the work published by Yoshi Miyata, a graduate student in the Gestwicki lab[35].

3.4.3 Luciferase Refolding Assays

The luciferase refolding activity of DnaK WT and mutants were evaluated as described with minor changes (ref). Briefly, denatured firefly luciferase was prepared by beginning with a concentrated stock (8.2 μM) of luciferase with 6 M GuHCl in 25 mM HEPES buffer (50 mM potassium acetate, 5 mM DTT, pH 7.2). This stock was incubated at room temperature for one hour and then diluted to 0.2 μM with the same HEPES buffer without GuHCl. This preparation was used as the stock solution for final sample preparation. Enzyme mix (10 μl) containing Hsp70, Hsp40, NEF, denatured firefly luciferase in 39 mM HEPES (170 mM potassium acetate, 1.7 mM magnesium acetate, 3 mM DTT, 12 mM creatine phosphate, 50 U/ml creatine kinase, pH 7.6) was first added into each well of 96-well white plate and then 4 μl of 3.5 mM ATP, dissolved in water, was added to start the reaction. The final concentration of Hsp70 was 1 μM, denatured luciferase was 8 nM, and ATP was 1 mM unless otherwise noted. The concentration of Hsp40 and NEF are reported for each experiment in the results section. After one hour of incubation at 37 °C, equilibrium was reached and 14 μl of 0.5% or 2% (v/v) SteadyGlo reagent in 50 mM glycine buffer (30 mM $MgSO_4$, 10 mM ATP and 4 mM DTT, pH 7.8) was added into each well, and the luminescence was measured. For each experiment, the signal from a negative control containing everything but Hsp70 was subtracted. The rate of refolding

was obtained by converting the luminescence unit into luciferase concentration using standard curve, and analyzing by linear regression fit using GraphPad Prism 4.0 (for Windows).

3.4.4 Holdase Activity Assays

Native luciferase was diluted to 0.032 μM in 50 mM HEPES (10 mM $MgSO_4$, 300 mM KCl, 20 mM DTT, pH 7.5) and loaded into a 96-well PCR plate (Thermofisher) (5 μl/well). Next, 5 μl of 2 μM Hsp70 was added in triplicate into the wells containing luciferase. The reaction mixtures were heated to 39.5 °C for 8 minutes and the samples were transferred into a 96-well, opaque, white microtiter plate (Thermofisher). Next, 10 μl of 0.5 % v/v SteadyGlo reagent, as described in the previous section, was added to each well and the luminescence was measured.

3.4.5 Octet Red Binding Assays

Octet RED platform binding experiments were conducted at 25ºC using

Streptavidin coated biosensors (SA sensors, ForteBio, Cat no 18-0019) on the Octet

RED biosensor platform (ForteBio) in 25mM HEPES, 5mM $MgCl_2$, 10mM KCl, 0.3% Tween-20, 1mM ATP pH 7.5. Sensor tips were pre-wet for 15 min in buffer immediately prior to use, and the 96 well micro-plates were filled with 200μl of sample or buffer per well and agitated at 1000rpm. Biotinylated DnaK and Hsp72 (0.1mg/ml) were immobilized on SA sensors to saturation for 20 min (typical loading levels 2.5nm). Binding interaction of NRLLLTG was monitored over a range of concentrations (association phase for 5 min, dissociation phase for 5 min). Data was analyzed using both

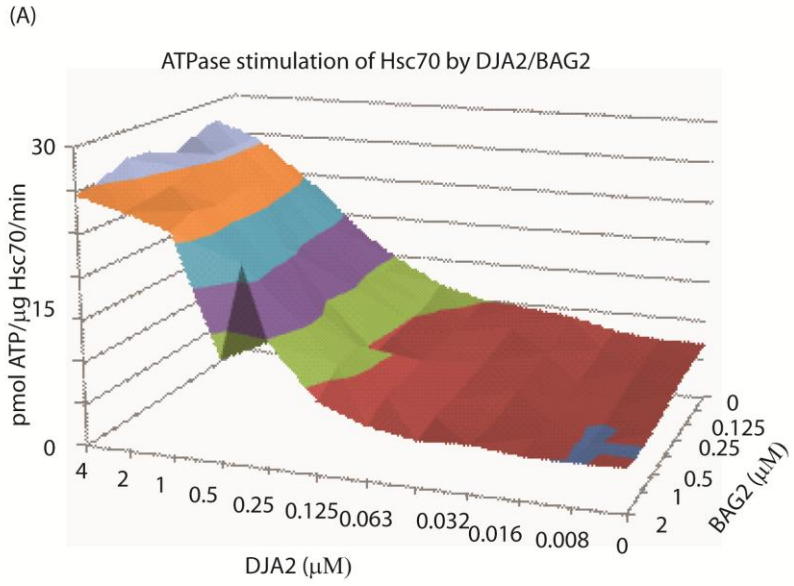
kinetic (1:1 binding, global fit) and steady state analysis and the binding constants were indistinguishable. Reference sensors were used to correct for background and baseline drift. Experiments were done in duplicate.

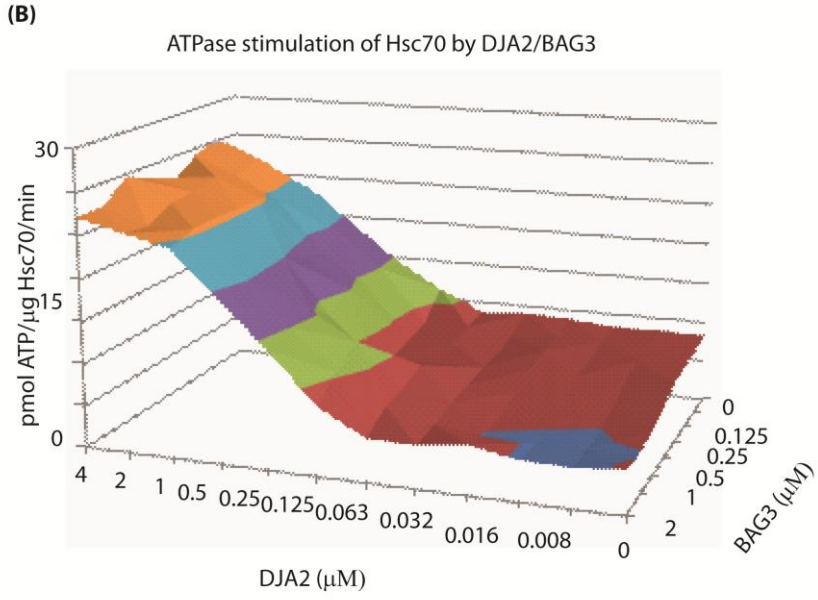
3.4.6 Flow Cytometry-based binding assays

Biotinylated Hsp72 was attached to polysterene streptavidin coated beads (Spherotech) and mixed with increasing concentrations of Alexa Fluor 488 labeled Bag protein in buffer A (25mM HEPES, 5mM $MgCl_2$, 10mM KCl, 0.3% Tween-20 pH 7.5). Binding was detected by measuring bead-associated fluorescence using an Accuri C6 Flow Cytometer. Competition experiments were performed in a similar manner with fluorophore labeled Bag protein (30nM) and titrating in either unlabeled Hsp72 or Bag. Beads capped with biocytin were used as a negative control for each experiment.

3.5 Appendices

Appendix 3.1





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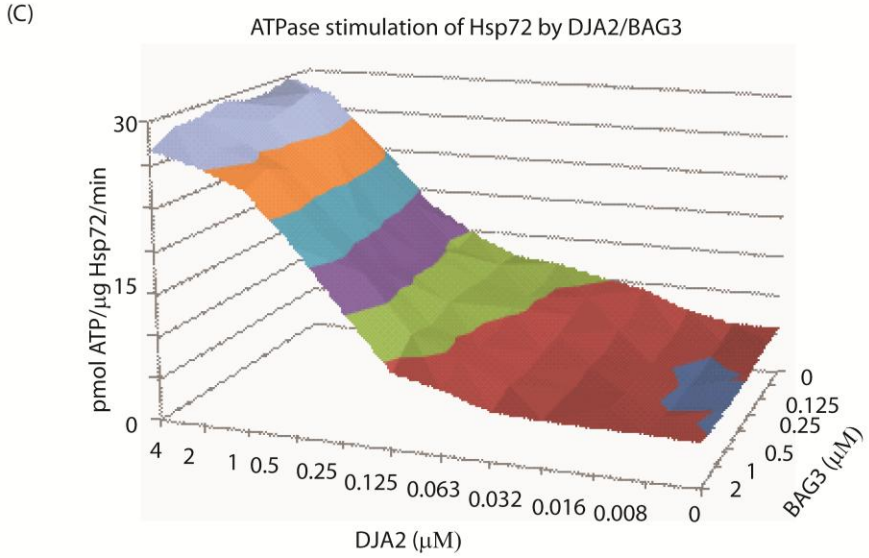
(C)



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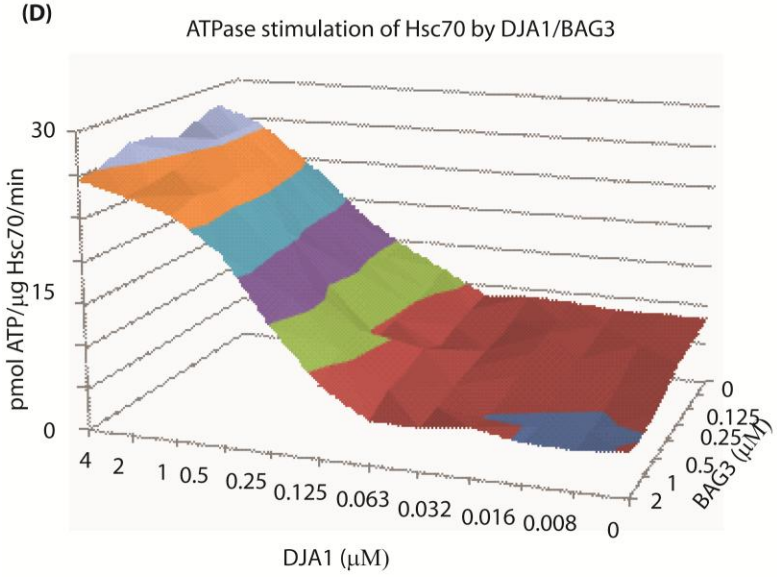

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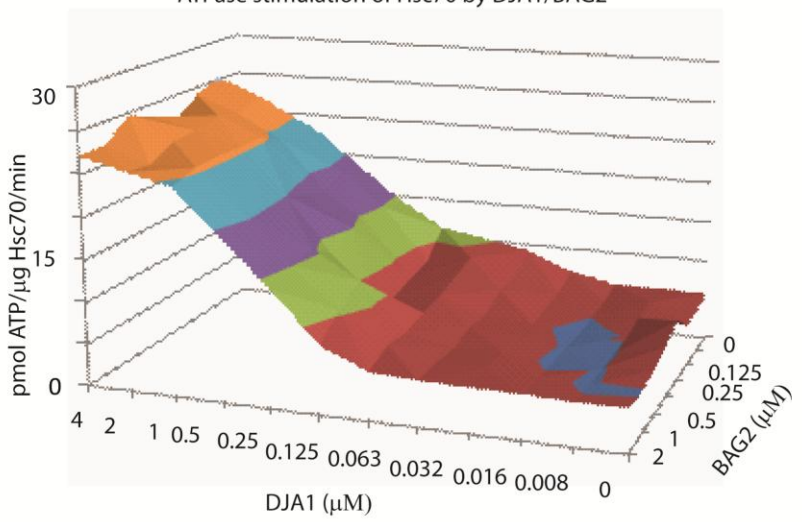
(E)



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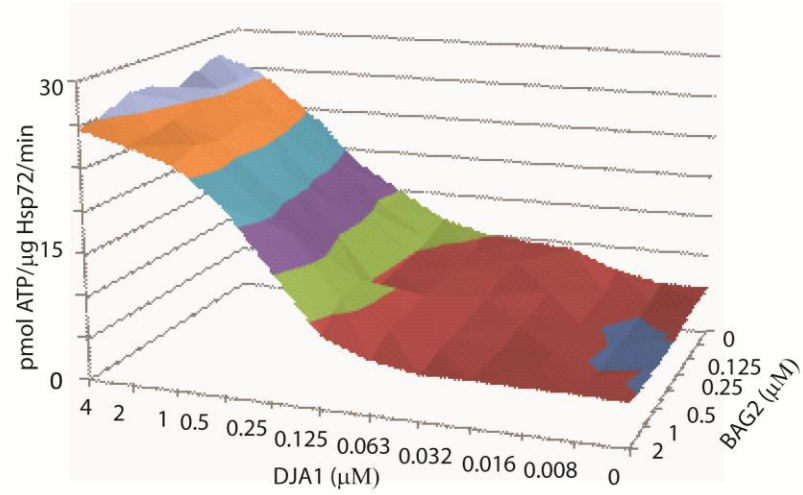

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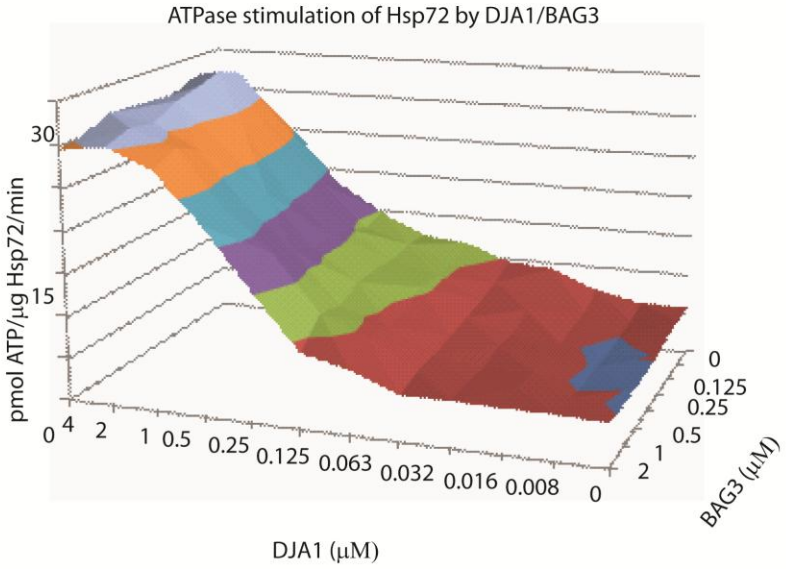
(G)



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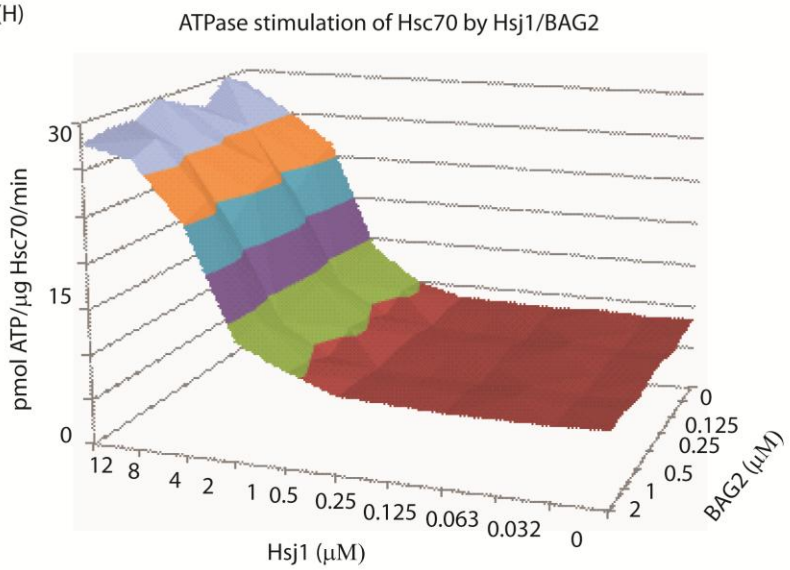

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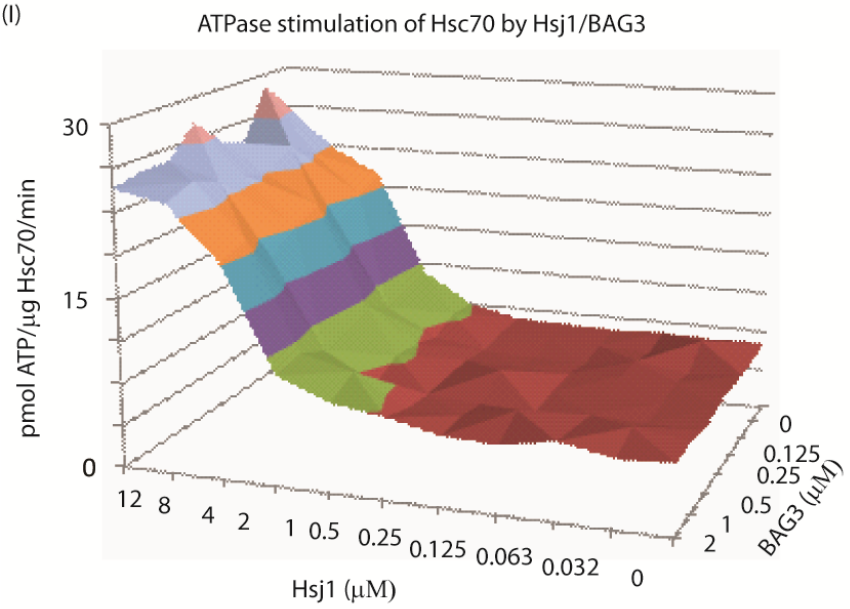
(I)



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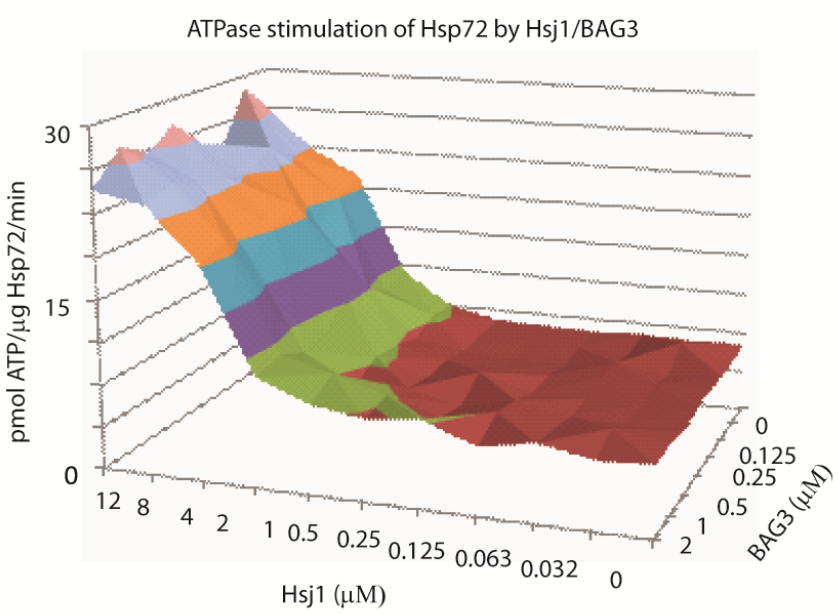
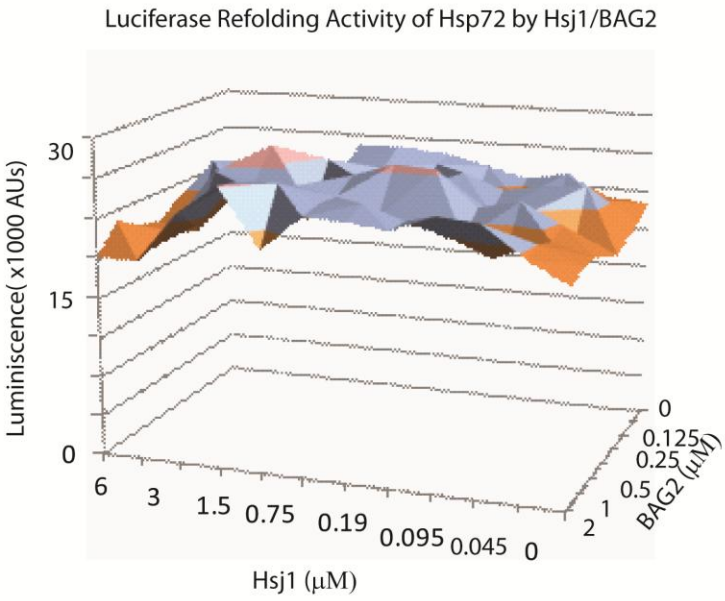


Appendix 3.2

(A)



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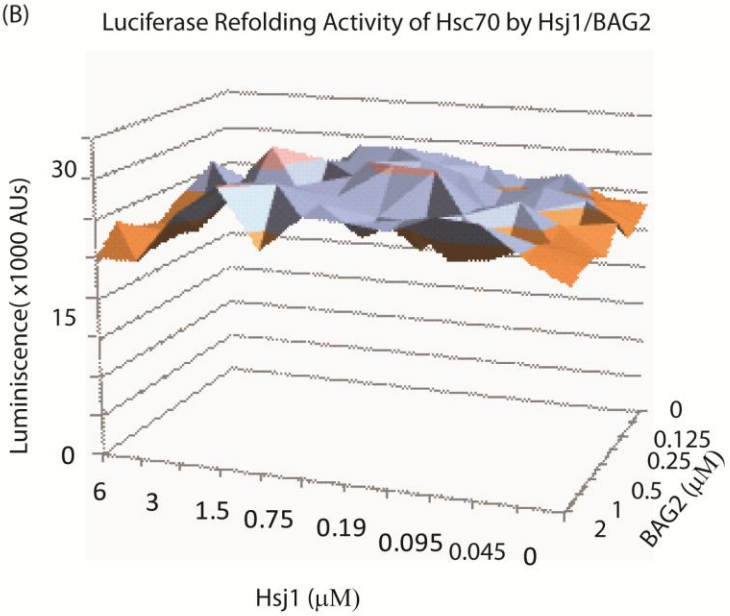

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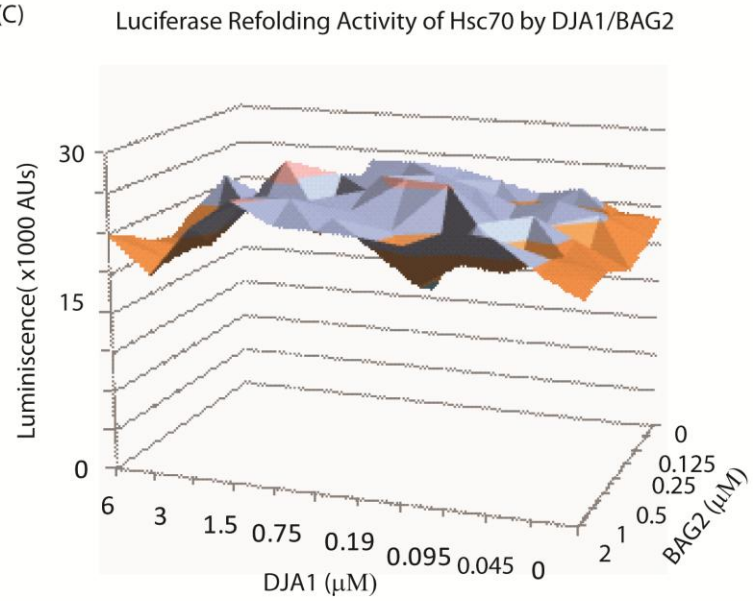

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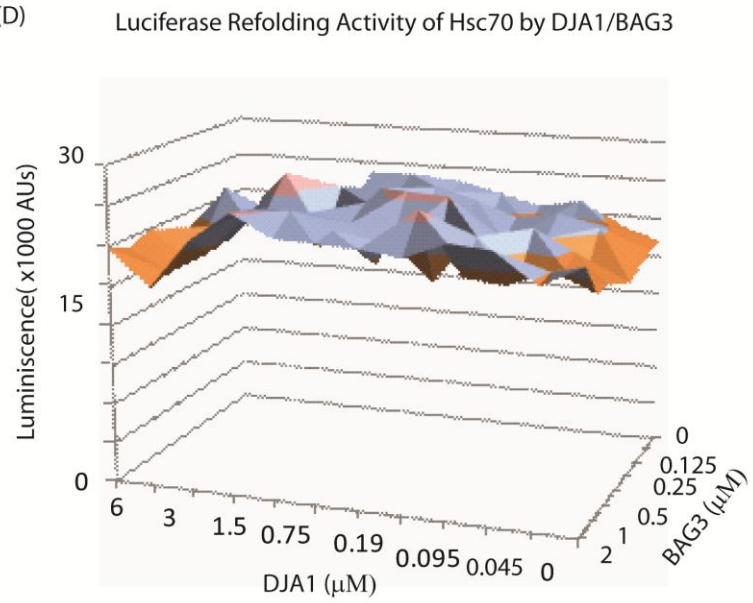

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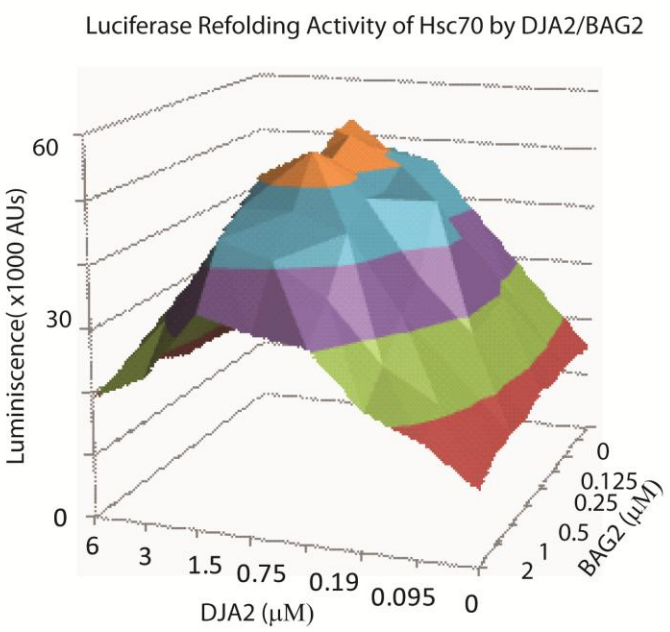

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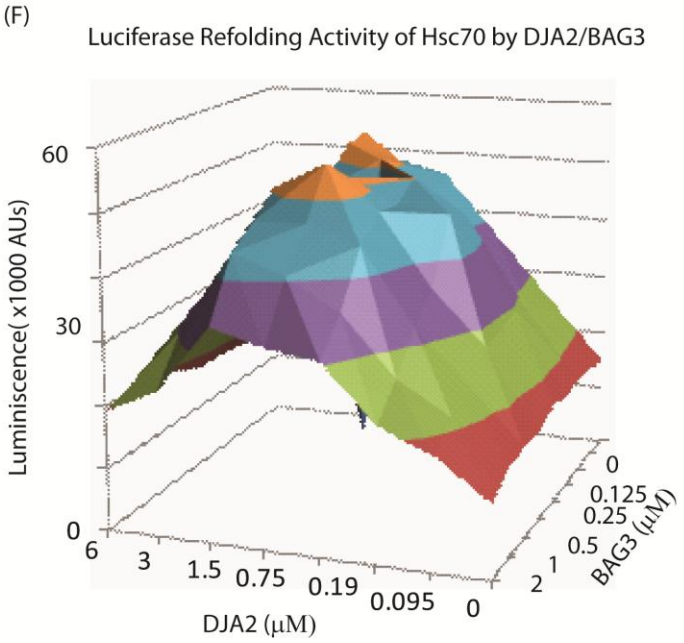

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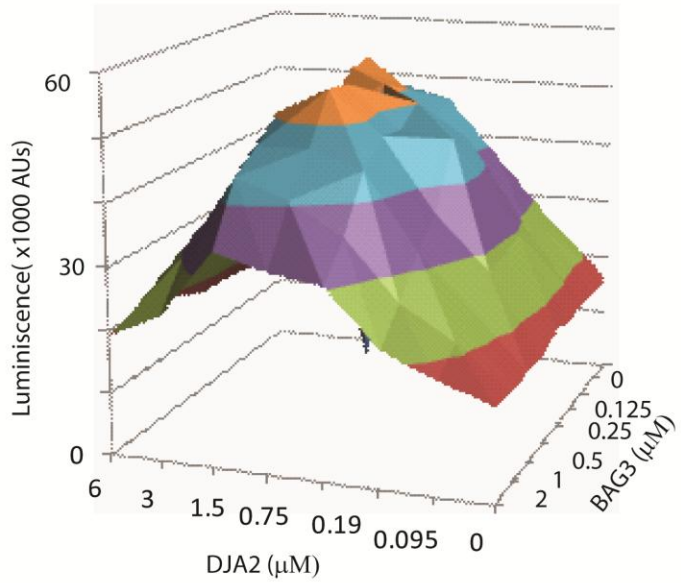
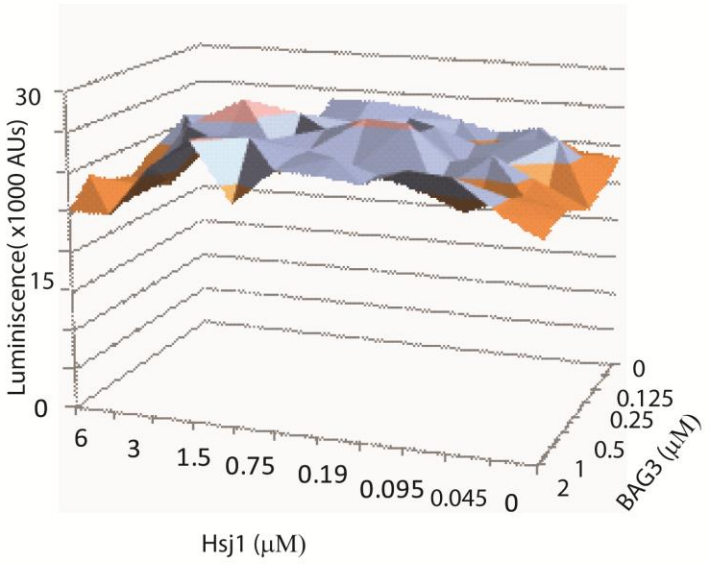
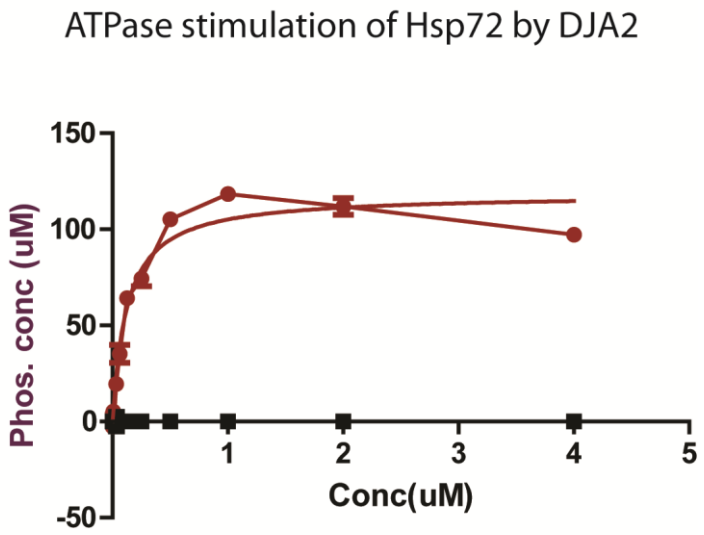
(G) Luciferase Refolding Activity of Hsp72 by DJA2/BAG3



(H) Luciferase Refolding Activity of Hsp72 by Hsj1/BAG3

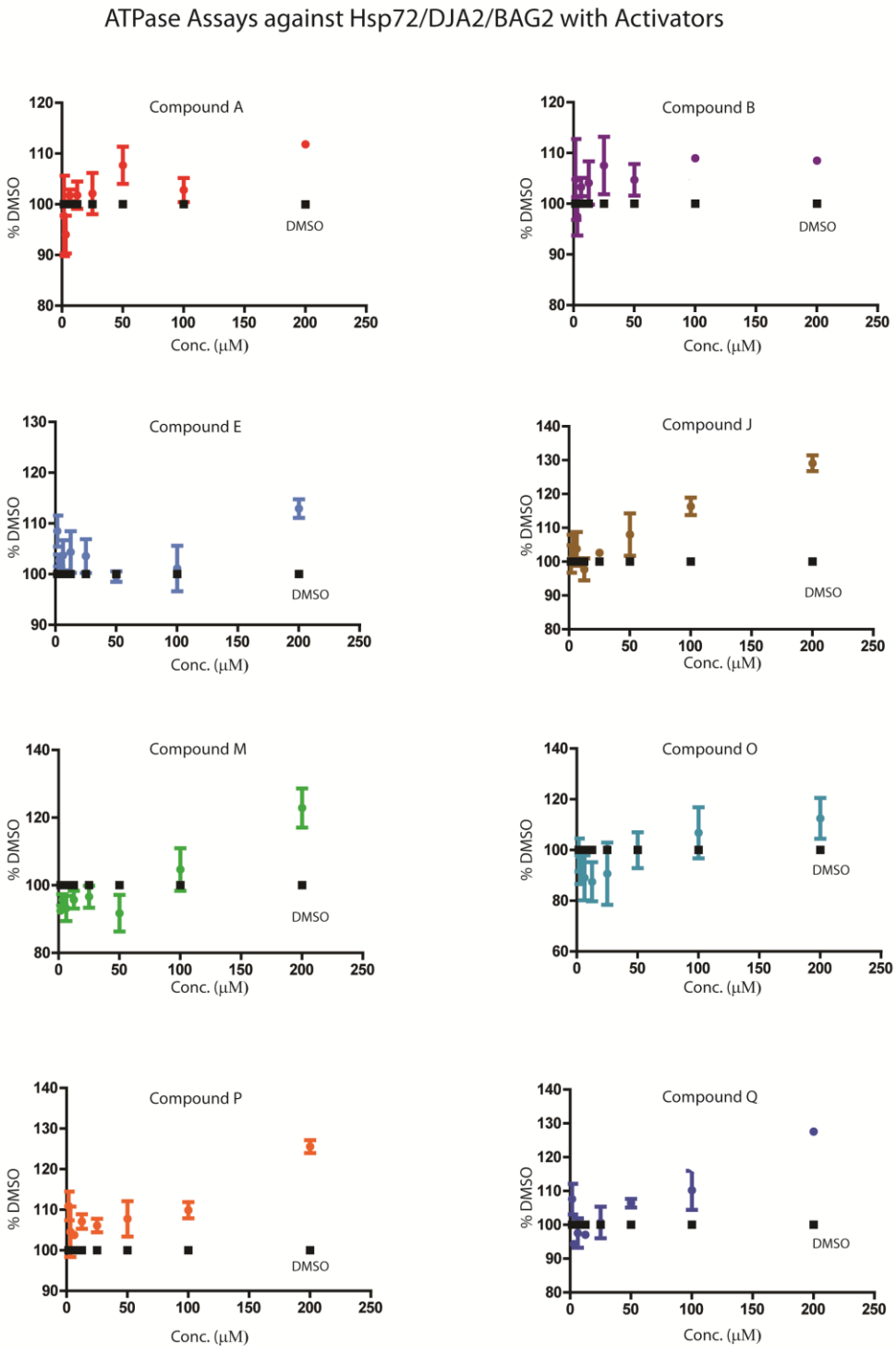


Appendix 3.3



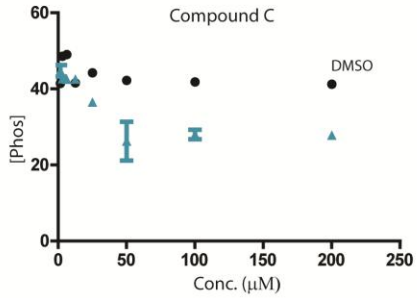
Michaelis-Menten	
Best-fit values	
VMAX	118.3
KM	0.1251

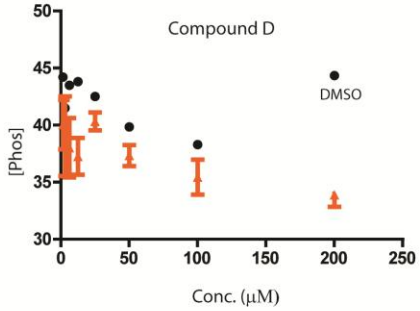
Appendix 3.4 Dose Response Curves for repurchased Activators. ATPase assays with MG reagent were performed using the same conditions as primary HTS screen. Hsp72 - 0.5μM, DJA2 - 0.1μM , BAG2– 0.25μM

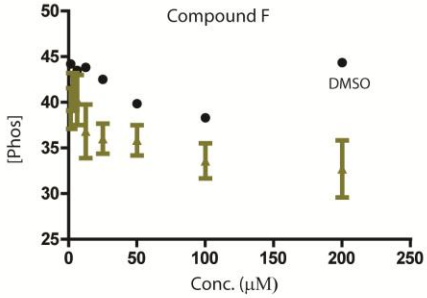


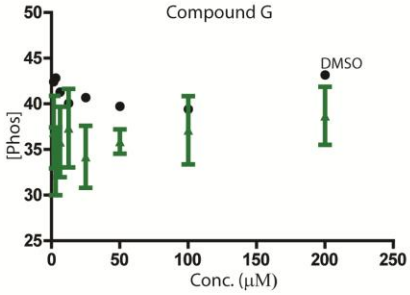
Appendix 3.5 Dose Response Curves for repurchased Inhibitors. ATPase assays with MG reagent were performed using the same conditions as primary HTS screen. Hsp72 - 0.5μM, DJA2 - 0.1μM , BAG2– 0.25μM

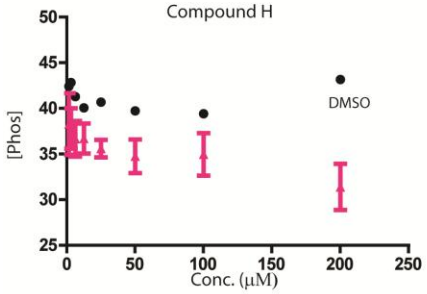
ATPase Assays against Hsp72/DJA2/BAG2 with Inhibitors

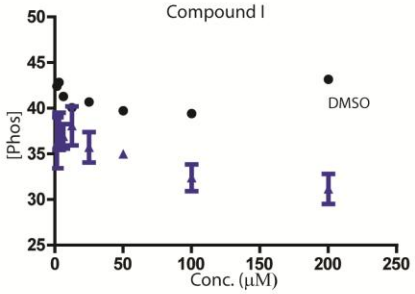


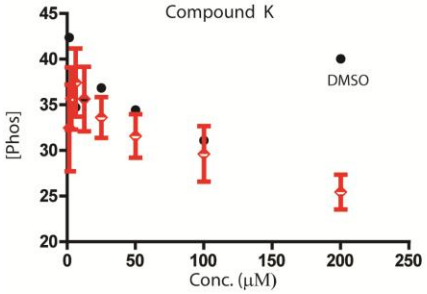


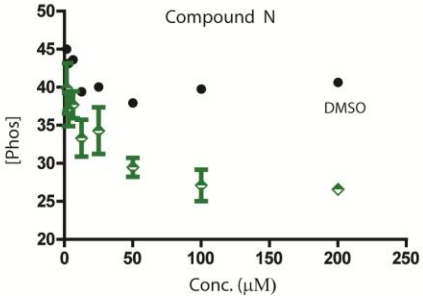






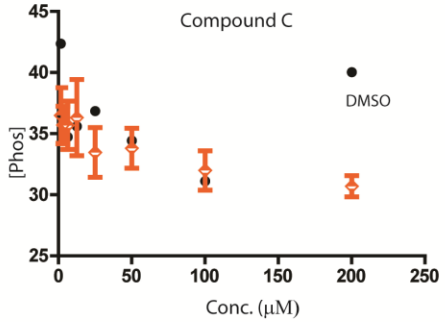


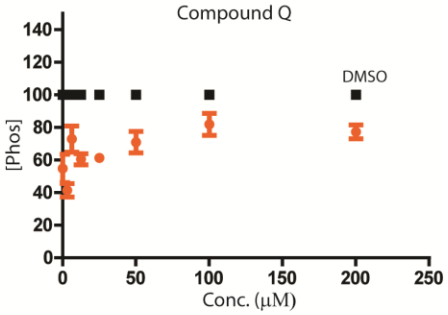


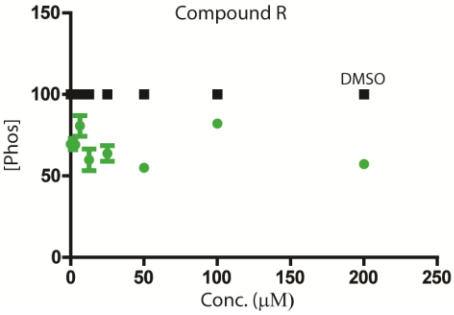


Appendix 3.5 continued.

ATPase Assays against Hsp72/DJA2/BAG2 with Inhibitors







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Chapter 4

Biophysical characterization of the Hsp70-CHIP chaperone complex

4.1 Abstract

One of the major goals of this thesis work is to characterize the biochemical properties of multi-protein chaperone complexes. As described in Chapters 2 and 3, these insights have permitted the discovery of new chemical inhibitors via "gray box" screening. In this Chapter, we focus on understanding the interaction between human Hsp70 and tetratricopeptide (TPR) domain co-chaperones, one of the least well-described co-chaperone interactions. As discussed in Chapter 1, the Hsp70/Hsp90 molecular chaperone machinery plays a central role in protein quality control in the cell by assisting in the folding and/or degradation of protein substrates. Folding is coupled to cycles of ATP hydrolysis and is regulated by a cohort of Hsp70- and Hsp90-bound co-chaperones. However, not every attempt to fold is successful, and the chaperone machinery can also direct misfolded proteins to the degradation pathways, including the ubiquitin-dependent proteasome system (UPS). A complex between Hsp70 and the E3 ubiquitin ligase CHIP (carboxyl terminus of Hsc70-interacting protein) appears to be particularly important in promoting the UPS-mediated degradation of a number of substrates. Based on pull-down assays, CHIP appears to bind to both Hsp70 and Hsp90 through its tetratricopeptide

(TPR) domain, but less is known about the affinity of this interaction and whether other regions of the proteins might contribute. To better understand this process, we have measured the affinity of CHIP binding and to the chaperones and mapped the key domains using truncation. These studies confirmed that much of the free energy of binding originates with CHIP's TPR domain binding to the C termini of Hsp70 and Hsp90, but additional contacts in the substrate-binding domain (SBD) of Hsp70 also make significant contributions. We also found that none of these CHIP-Hsp70 interactions impacted the steady state ATP turnover rate of Hsp70. Finally, we used *in vitro* ubiquitination assays to demonstrate that the TPR and U-box domains of CHIP are necessary for chaperone ubiquitination. Together, these studies suggest that interaction between the TPR domain of CHIP with molecular chaperones, while not sufficient to fully describe the affinity, is critically important in facilitation substrate ubiquitiylation, possibly by fine-tuning the relative position of substrate and CHIP in the Hsp70 complex. Together, these results are expected to significantly assist efforts to discover and develop small molecules that inhibit the Hsp70-CHIP complex.

4.1.1 Hsp70 and Hsp90 chaperones triage misfolded proteins

As discussed in Chapter 1, the major chaperones Hsp70 and Hsp90 act together to regulate the function, trafficking and turnover of multiple protein substrates [1] Both chaperones bind client proteins in an ATP-dependent manner and their activities are regulated by a variety of co-chaperones [2, 3] When the Hsp70/Hps90 chaperones encounter a misfolded protein, they are thought to first attempt to rescue the protein via the refolding pathway and , at the same time, sequester the misfolded species to prevent

toxic interactions[4]. However, Hsp70 and Hsp90 have also been linked to the degradation of substrates via the proteasome and autophagy pathways [5]. Comparatively less is known about the mechanisms of chaperone-assisted degradation.

4.1.2 CHIP links chaperones to the ubiquitin-dependent degradation pathway

Proteins destined for degradation by the ubiquitin-dependent proteasome system (UPS) are labeled with a polyubiquitin chain and sent to the 26S proteasome [6]. Briefly, ubiquitylation of substrates is mediated by a series of three factors, namely activating (E1), conjugating (E2) and ligase (E3) enzymes [7]. There is considerable evidence that Hsp70/Hsp90 chaperones promote degradation of substrates such as glucocorticoid receptors (GR)[8] and cystic fibrosis transmembrane conductance regulator (CFTR) [9] by directly recruiting E3 ligases. The two E3 ligases known to interact with Hsp70/Hsp90 are Parkin and CHIP [10, 11]. CHIP appears to be particularly important and it is a 35 k-Da protein that was first identified in a yeast two hybrid screen for proteins containing tetratricopeptide (TPR) domains [12]. For some proteins, such as the GR, CHIP appears to be the sole dedicated E3 responsible for turnover, whereas other substrates, such as neuronal nitric oxide synthase (nNOS), can be degraded by multiple E3 ligases [13]. CHIP doesn't appear to act by itself but, rather, its activity is Hsp70- and Hsp90-dependent [14, 15]. CHIP binds to both Hsp70 and Hsp90 at their C-terminal 'EEVD' motifs and this interaction seems to require CHIP's TPR domain [12, 16]. In turn, CHIP binds to the UbcH5 family of E2-conjugating enzymes through a carboxyl terminal U-box domain[17]. Through formation of this multi-protein complex, CHIP has been shown to ubiquitinate Hsp70/Hsp90 substrates, diverting them to the proteasome [18].

4.1.3 To fold or to degrade: TPR co-chaperones determine function

In addition to CHIP, the TPR domain is a chaperone-binding motif found in several co-chaperones. The TPR domain is characterized by a tandem arrangement of three 34 amino acid motif that forms an antiparallel α-helical hairpin. Most proteins that have TPR domains also have additional domains with other activities and, thus, these co-chaperones are thought to recruit unique capabilities to the Hsp70 complex. For example, HOP has three domains (TPR1, TPR2A, and TPR2B) with three TPR motifs each [19]. HOP preferably binds to the ADP-bound form of Hsp70 via TPR1 and TPR2B, while TPR2A specifically binds to Hsp90 [20]. In this way, Hop bridges Hsp70 and Hsp90, assists substrate transfer between these chaperones and is believed to promote substrate folding. Thus, although both HOP and CHIP bind via TPR domains, the outcomes of these interactions are diametrically opposed: HOP favors folding, while CHIP favors degradation. Based on these observations and many others, it is thought that competition between TPR co-chaperones might drive combinatorial assembly of chaperone complexes with specific functions.

4.1.4 The molecular mechanism of the Hsp70/Hsp90/CHIP triage decisions is not known

Despite the importance of the TPR-chaperone interaction, surprisingly little is known about the biochemistry of these protein complexes. Most of what is known comes from overexpression and pulldown experiments, such as glutathione-S-transferase (GST) fusion protein assays [12, 18, 21]. Based on these studies, the TPR domain and an adjacent charged region of CHIP appear to be necessary for its interaction with Hsp70,

while the carboxy terminal domain (CTD) of Hsc70, including the EEVD motif, are required on the other side [18]. However, biochemical studies on the Hop-Hsp70 interaction suggest that binding between Hsp70 and Hop is not solely dependent on TPR-EEVD interactions [19]. Further, a recent structure of the Hsp70-Hop-Hsp90 complex also suggested the potential importance of contacts outside the EEVD [22]. Thus, our hypothesis is that the CHIP interactions with Hsp70/Hsp90 may involve important, and previously under-studied, contacts outside the EEVD-TPR contact. To clarify these important questions, our goal was to biophysically characterize the binding determinants between CHIP and Hsp70.

4.2 Results

4.2.1 Design of CHIP and Hsp70 truncations

Qualitative "domain mapping" analysis between CHIP and Hsp70 that has been performed by Ballinger et al [12], in which they generated a series of truncations in both CHIP and Hsp70 (Figure 4.1). Accordingly, we designed a similar set of constructs including, a TPR domain deletion (ΔTPR) and two C-terminal truncations 1-142 and 1-197 (CHIP-142 and CHIP-197, respectively). We also deleted the first 25 amino acids, which have been shown to be important in auto-ubiquitylation of CHIP (CHIP Δ25). On the Hsp70 side of the protein-protein contact, we deleted the EEVD motif (Hsp70-ΔEEVD) and also used the prokaryotic Hsp70, which naturally lacks the EEVD. Next, we also made a truncation (Hsp70-555) in which the entire C-terminal "lid" domain (CTD) is deleted. Finally, the nucleotide binding domain (NBD) of Hsp70 has been

shown to be dispensable for binding to CHIP, so we also generated this construct (Hsp70-NBD).

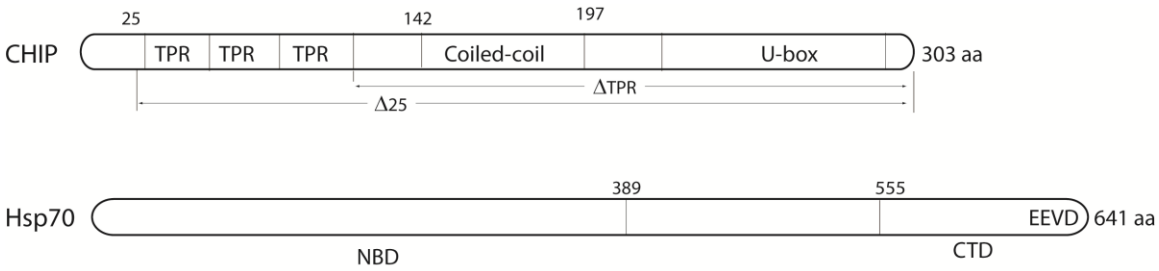

Figure 4.1 Design of the CHIP and Hsp70 fragments. CHIP has 3 TPR domains in amino terminus and the functional U-box in the C-terminus. The N-terminal 25 residues are important for its auto-ubiquitylation. Hsp70 has a Nucleotide Binding Domain(NBD)(1-389). The C-terminal Carboxy Terminal Domain(CTD) has been shown to be involved in the interaction with CHIP.

4.2.2 Development of a Surface Plasmon Resonance (SPR) binding assay to measure the CHIP-Hsp70 binding affinities

To measure the binding affinities, we developed an assay using Surface Plasmon Resonance (SPR). This platform employed a streptavidin (SA) Biacore chip in which we immobilized biotinylated Hsp70 or

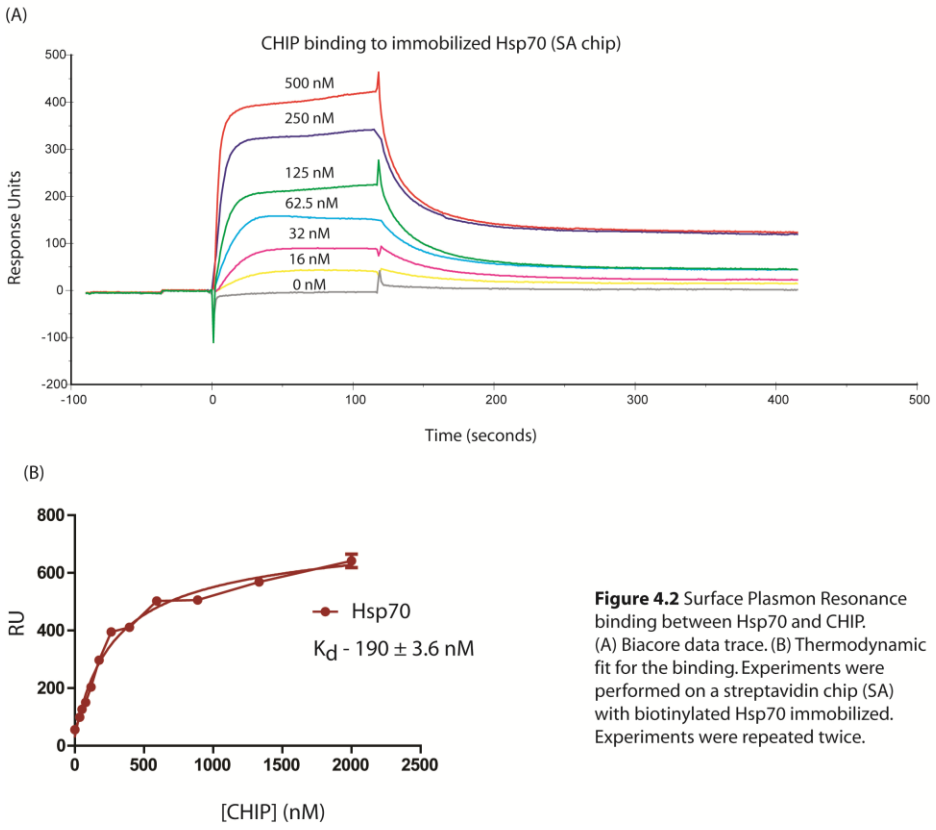

Figure 4.2 Surface Plasmon Resonance binding between Hsp70 and CHIP. (A) Biacore data trace. (B) Thermodynamic fit for the binding. Experiments were performed on a streptavidin chip (SA) with biotinylated Hsp70 immobilized. Experiments were repeated twice.

the various truncations. For each protein, we were able to achieve immobilization densities of ~1000 response units (RUs). We then evaluated the binding of recombinant CHIP (and truncations) by injecting several different concentrations (ranging from 0 to 500 nM) at a flow rate of 20 µL/min. Binding was determined by subtracting the signal from a control lane in which DnaK was immobilized (although binding to this lane was insignificant). Binding affinities were then determined using both kinetic and thermodynamic analyses yielding an apparent K_D of 190 ± 3.6 nM (Figure 4.2). To confirm that binding was specific, we verified that free Hsp70 competes with CHIP binding to the surface. This experiment provided an IC_{50} of 716 ± 90 nM at two different constant concentrations of CHIP (100 nM and 250 nM), suggesting that the interaction is specific.

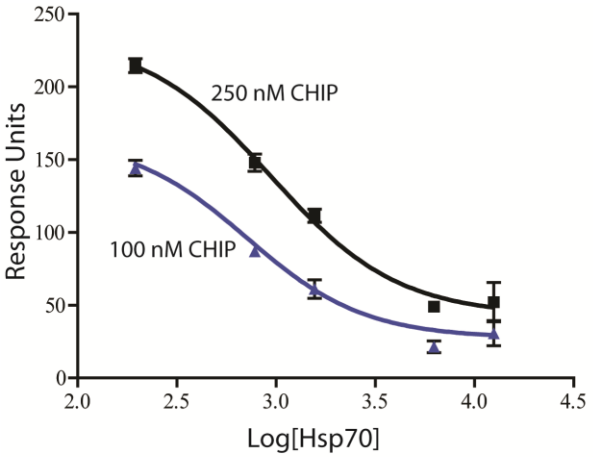

Figure 4.3 Competition of CHIP binding to immobilized Hsp70 by free Hsp70. Hsp70 was able to compete with CHIP in a dose dependent manner with a K_i of 716 ± 90 nM.

Using this SPR binding assay, next we measured the binding affinities of the different truncations (Table 4.1). In general, our quantitative findings agreed with the previous qualitative domain mapping analysis [12]. For example, the TPR domain of CHIP appeared to be important for the interaction, because deleting it resulted in a 3-fold loss in affinity. The coiled-coil domain between residues (142-197) of CHIP also appeared to contribute (Table 4.1). This is an interesting result because this domain is not conserved

in other TPR proteins. Thus, it may contribute to specificity in binding CHIP to Hsp70. When we examined the Hsp70 truncations, we found that domains in Hsp70 other than the EEVD region also contribute to the binding interactions. Specifically, we found that deletion of the EEVD region resulted in only a loss of 2-fold in affinity (Table 4.1). Moreover, the Hsp70-555 construct was still able to weakly bind to CHIP, indicating that residues in the substrate-binding domain (SBD) also participate. Together, these finding greatly enhance our knowledge of the contacts between Hsp70 and CHIP.

Table 4.1 Binding affinities (K_d values in nM) between Hsp70 and CHIP and various truncations, measured using Surface Plasmon Resonance(SPR). Representative raw traces are in Appendix 4.1

Construct	CHIP	CHIP1-142	CHIP1-197	CHIP Δ25	CHIP ΔTPR
Hsp70	190 ± 3.6	247 ± 36	220 ± 23	201 ± 34	601 ± 54
Hsp70 ΔEEVD	430 ± 36	520 ± 46	490 ± 42	440 ± 33	990 ± 65
Hsp70 1-555	835 ± 44	> 5 μM	920 ± 56	940 ± 74	> 5 μM
Hsp70 NBD	> 5 μM	> 5 μM	> 5 μM	> 5 μM	> 5 μM

4.2.3 CHIP does not display nucleotide dependence in binding to Hsp70

Since nucleotide binding and hydrolysis result in conformational changes in Hsp70, we wanted to measure the binding affinities for CHIP under different nucleotide states. Nucleotide dependence has already been shown for other TPR domain containing proteins, such as Hop, which binds Hsp70-ADP 2-fold better than Hsp70-ATP [20]. However, in our SPR studies we found that CHIP did not have a preference for Hsp70's nucleotide state. Both affinities were determined to be approximately 240 ± 13 nM. The functional significance of this finding is not yet clear, but work in the Gestwikci laboratory by another graduate student is exploring this issue.

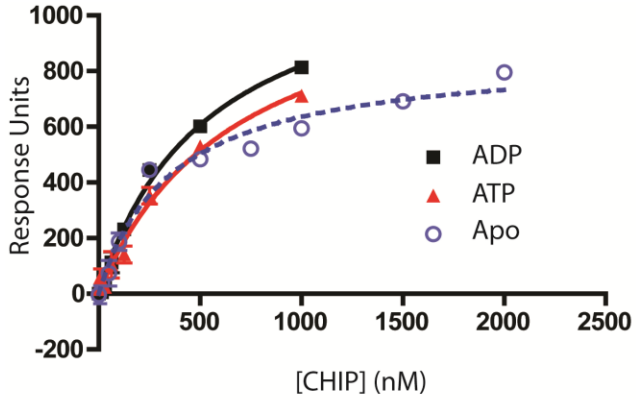

Figure 4.4 Nucleotide dependence of CHIP binding to Hsp70. Binding was performed with ATP, ADP or no nucleotide. There was no significant difference in the binding constants. The nucleotide was injected along with CHIP in the running buffer. Experiments were repeated twice.

4.2.4 CHIP binds to Hsp90 with slightly weaker affinity

CHIP has been shown to bind both Hsp90 and Hsp70, guiding triage decisions in protein quality control [18]. Traditionally, Hsp90 bound complexes are associated with pro-folding activity, while Hsp70 bound substrates are more prone to degradation. Thus, we wanted to measure if there was any difference in the binding affinities between CHIP and Hsp70/Hsp90. Briefly we immobilized biotinylated Hsp90 (1950 RUs) on the SA Biacore chip surface and then injected CHIP at a flow rate 20 µL/min.

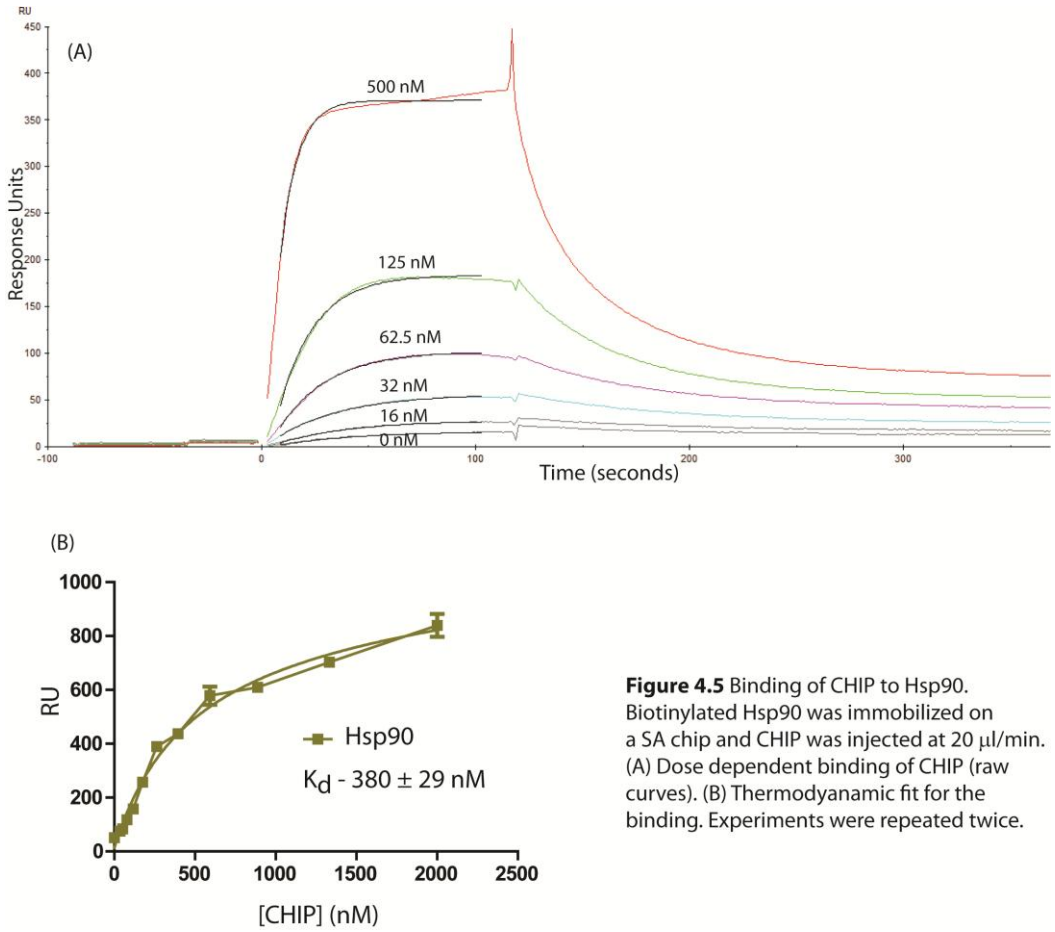

Figure 4.5 Binding of CHIP to Hsp90. Biotinylated Hsp90 was immobilized on a SA chip and CHIP was injected at 20 μl/min. (A) Dose dependent binding of CHIP (raw curves). (B) Thermodyanamic fit for the binding. Experiments were repeated twice.

In this platform, we found that CHIP bound Hsp90 with only a mildly weaker affinity (K_d = 380 ± 29 nM). Thus, CHIP binds both Hsp70 and Hsp90 with similar affinity. Again, the functional significance of this finding isn't yet clear, but it does suggest that other factors besides the binary protein-protein interaction affinity contribute to the ability of the Hsp70-CHIP complex to more strongly favor degradation.

4.2.5 FRET assays confirm binding between Hsp70/CHIP

Because any affinity measurement platform can be prone to artifacts, we wanted to develop a secondary assay to confirm the binding interactions. Specifically, we wanted to

use a solution phase, homogeneous platform to complement the surface-based SPR technique. Towards that goal, we used a Forster Energy Resonance Transfer (FRET)-type assay (Figure 4.6A). Briefly, Hsp70 was labeled with the fluorescent donor AlexaFluor488 (AF-488), which has an emission wavelength of 525 nm. Then, CHIP was labeled with Black Hole Quencher10 (BHQ-10), a strong FRET acceptor that absorbs at 507 nm. This system allows sensitive measurement of binding by an apparent quench in AlexaFluor488 fluorescence. Labeling of the two partners via lysine residues proceeded smoothly, with average incorporations of ~ 2-3 per protein. As predicted from the SPR studies, we observed a dose-dependent quench with increasing concentrations of labeled CHIP (Figure 4.6B). Interestingly, the observed affinity was stronger than that predicted by SPR (K_d – 55 ± 5.2 nM), suggesting that the surface may partially inhibit binding or that the labeling reactions strengthened the apparent affinity.

Next, we measured the ability of the various truncations to compete with the Hsp70-CHIP interaction. Briefly we incubated a constant concentration of AF-488 labeled Hsp70 (50 nM) and BHQ-10 labeled CHIP (50 nM) with unlabeled mutants for 1 hour and then monitored quenching. The relative results matched well with our SPR data, in that the TPR domain was found to be important for binding (Figure 4.6C). The CHIP-197 truncation was also able to compete, again showing that the coiled-coil domain contributes to the interaction. Similarly the Hsp70 truncations were also able to compete with the interaction (Figure 4.6D), consistent with the SPR results. Interestingly, synthetic EEVD peptide was not able to compete this interaction (data not shown), further suggesting that the EEVD-TPR contact, while a contributor to the interaction, is

not sufficient to describe the entire affinity.

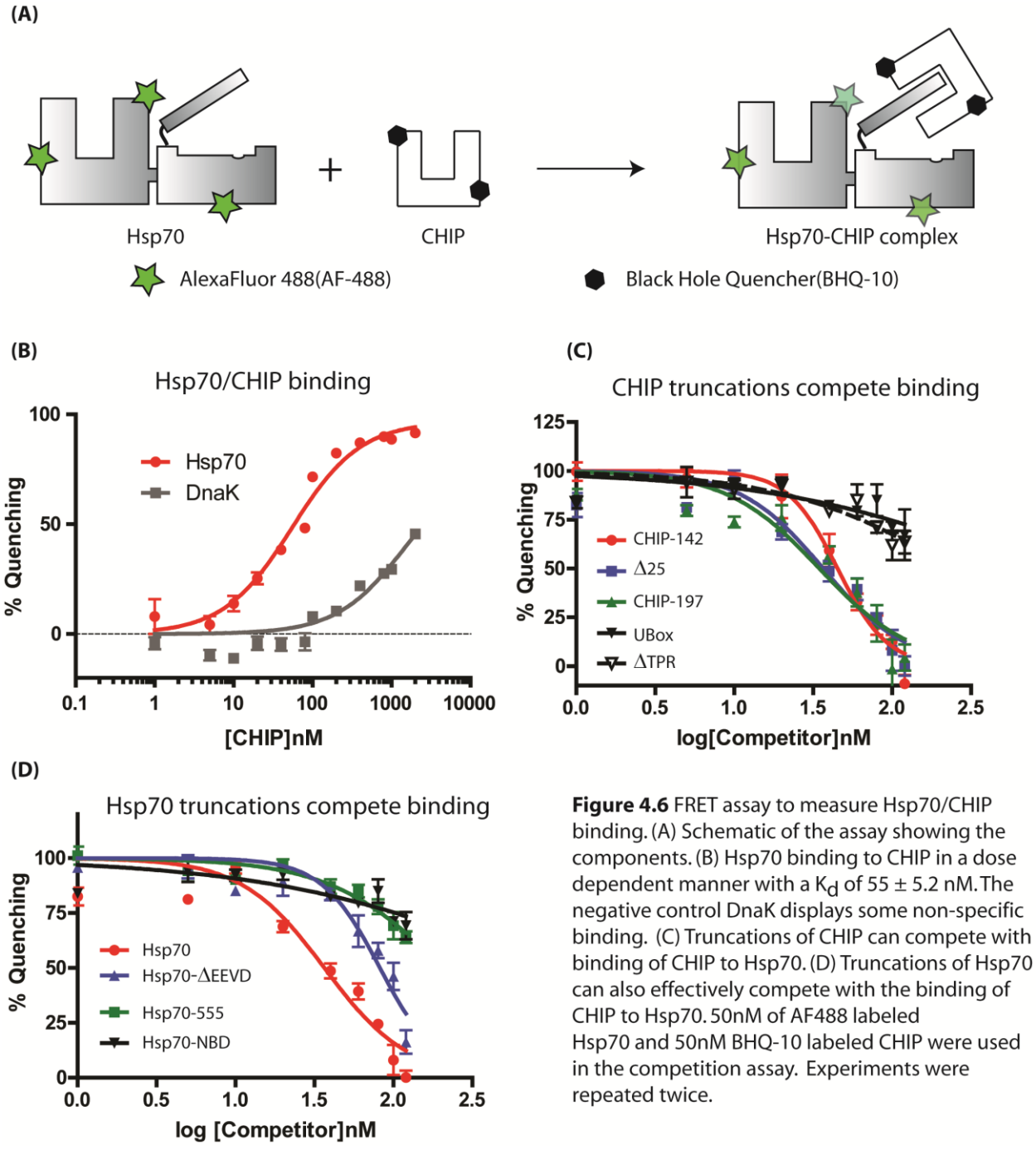

Figure 4.6 FRET assay to measure Hsp70/CHIP binding. (A) Schematic of the assay showing the components. (B) Hsp70 binding to CHIP in a dose dependent manner with a K_d of 55 ± 5.2 nM. The negative control DnaK displays some non-specific binding. (C) Truncations of CHIP can compete with binding of CHIP to Hsp70. (D) Truncations of Hsp70 can also effectively compete with the binding of CHIP to Hsp70. 50nM of AF488 labeled Hsp70 and 50nM BHQ-10 labeled CHIP were used in the competition assay. Experiments were repeated twice.

4.2.6 FRET assay to monitor Hsp90-CHIP binding shows similar results to SPR

We also wanted to confirm the Hsp90 binding affinities to CHIP in the FRET assay. This experiment was particularly important because a recent publication [23] reported that Hsp90 binds with a stronger affinity to CHIP than Hsp70. However, that study used ubiquitination assays, so we wanted to additionally measure the direct binding affinity. In this assay, similar to what we found with SPR, Hsp90 bound with a slightly weaker affinity than Hsp70 (81 ± 4.4nM) (Figure 4.7A), Next, we performed the binding of the truncations, which revealed that the required domains for binding of CHIP and Hsp90 parallel those needed for Hsp70 interactions. Specifically, the TPR domain is most important, with come contribution by the charged region (Figure 4.7B).

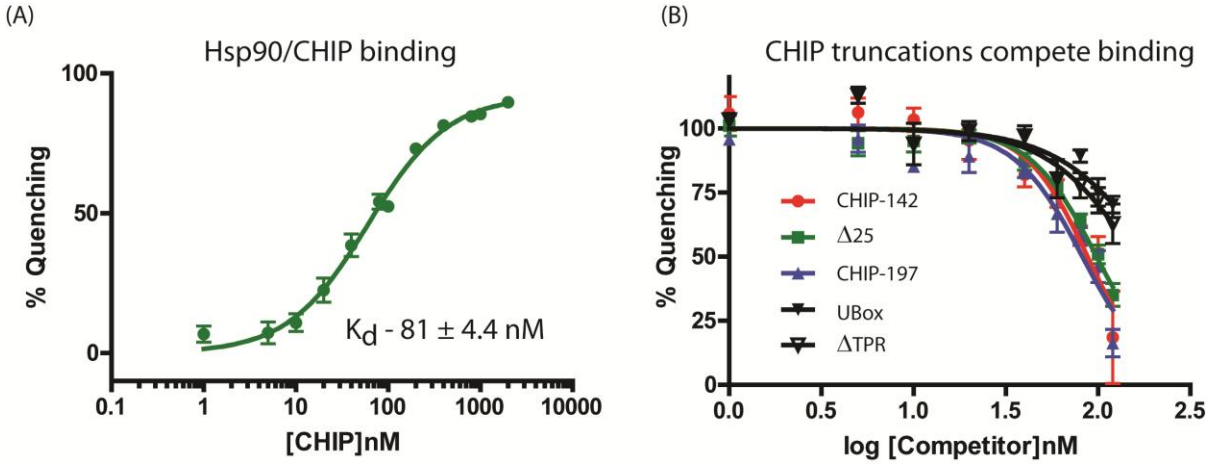

Figure 4.7 FRET assay to monitor Hsp90-CHIP binding. (A) BHQ-10 labeled CHIP binds in a dose-dependent manner to AF-488 labeled Hsp90. (B) This interaction can be competed with various truncations of CHIP. In the competition experiments, AF-488 labeled Hsp90 was kept constant at 50nM and BHQ-10 labeled CHIP was at 50nM. Experiments were repeated twice.

4.2.7 TPR domain is required for the ubiquitination of Hsp70

CHIP ubiquitinates Hsp70 in cells and this activity promote turnover of the chaperone[24]. Based on our biochemical studies, we wanted to assess which domains

were necessary for this functional activity of CHIP. Thus, we collaborated with the Paulson laboratory (Dept. of Neurology) to establish *in vitro* ubiquitination assays. Briefly CHIP and Hsp70 were incubated with a mixture of components required for the *in vitro* reconstitution of the ubiquitin cycle [6]: ubiquitin (Ub), E1 and E2 enzymes and ATP. CHIP_mediated conjugation of ubiquitin to Hsp70 was then measured (Figure 4.8). Using the series of CHIP truncations, we found that the TPR domain is absolutely required for the functional activity of CHIP. In addition, the truncations CHIP-142, CHIP-197 and CHIP Δ25 all lacked activity. Thus, multiple regions of CHIP are required for functional activity in the Hsp70-CHIP complex.

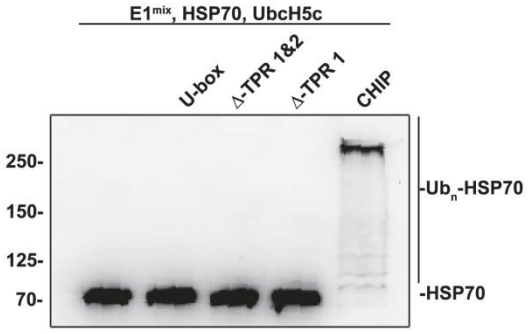

Figure 4.8 *In vitro* ubiquitation assay. TPR domain is required for the ubiquitination of Hsp70. UbcH5c is an E2 conjugating enzyme. The reaction was carried out at 37°C for 1hour.

4.3 Discussion

4.3.1 The TPR domain in CHIP and the EEVD motif in Hsp70 play important roles in binding but other regions also contribute

CHIP along with chaperones Hsp70 and Hsp90, plays a central role in the triage decisions [3]. In this Chapter, we sought to characterize, for the first time, the affinities between CHIP and the chaperones to determine which motifs contributed to binding. Using SPR and a FRET assay, we found that the TPR domain in CHIP is important for

binding to both Hsp70 and Hsp70, with the coiled-coil region also playing a role. On the other side, the EEVD motif of Hsp70 was also found to be involved in binding, but not required: the Hsp70-ΔEEVD truncation had only a 2-fold weaker affinity for CHIP. Further, the Hsp70-555 truncation retained some binding affinity, suggesting that regions in the carboxy terminal domain (CTD), interact with CHIP. This result parallels studies on the Hop-Hsp70 interaction [25].

4.3.2 Nucleotide state does not influence CHIP binding to Hsp70

Hop has been shown to preferentially bind Hsp70 in an ADP bound state [20]. Thus, we wanted to explore whether CHIP also shared this discriminatory ability. Unexpectedly, we found that the affinity of CHIP-HSp70 binding was nucleotide independent. Thus, we propose that the HOP-Hsp70 contact surface may be more extensive than the Hsp70-CHIP surface, allowing communication of the nucleotide-specific conformation between Hop and Hsp70, but not CHIP and Hsp70. More work is required to understand this difference and learn its potential functional significance in protein quality control.

4.3.3 Implications of this work on the development of small molecule inhibitors

A major theme of this thesis is that careful, biochemical and biophysical characterization of chaperone complexes can be important in the subsequent discovery of new chemical inhibitors, especially using “gray box” screening. Thus, a goal of this Chapter was to better understand and measure the binding of CHIP to Hsp70 and Hsp90, to enable potential chemical studies. Recently, Chris Evans and Matt Smith in the Gestwicki laboratory identified a class of natural products, based on spergualin, that inhibit the

Hsp70-CHIP interaction [26] (and unpublished work). Satisfyingly, the biophysical characterization of the Hsp70-CHIP interaction described in this Chapter has been useful in the rational synthesis of more potent and selective spergualin derivatives. I expect that future efforts will continue to identify novel inhibitors of TPR interactions that can be used to both (a) better understand the chaperone-TPR interactions and (b) probe the importance of this interaction in cellular and animal models of disease.

Notes:

Srikanth Patury and Jason Gestwicki designed the experiments. The ubiquitylation assays were performed by Matthew Scaglione and the FRET studies were performed, in part, by Matthew Smith. Andrea Thompson helped with SPR studies. Matthew Scaglione and Srikanth Patury made the CHIP and Hsp70 truncations. Srikanth Patury performed the bulk of the SPR studies, some FRET studies and the analyses.

4.4 Experimental Procedures

4.4.1 Protein purification

All Hsp70 truncations were cloned into the pMCSG7 plasmid (Midwest Center for Structural Genomics, Bethesda, MD) by ligation independent cloning and transformed into Rosetta (DE3) cells for expression, as previously described in Chapter 3. To express Hsp70 truncations, 25 mL of overnight (37 °C) LB culture of Rosetta (DE3) culture was poured into 1 L of Terrific Broth. After 3 hours incubation at 37 °C, the culture was cooled down to 28 ^{o}C for 2 hours before overnight induction of expression with 200 µM IPTG overnight, and the cell pellet was stored at -80 °C until use. The WT and Hsp70 truncations were purified using same procedures as described except the addition of the EDTA-free protease inhibitor cocktail (Roche) (1/4 tablet for each sample) in the lysis buffer and the omitting of final Ni-NTA resin clean-up step for Hsp70 truncations. All CHIP truncations were expressed in *E. coli* (BL21 DE3 LysS) and purified on glutathione sepharose resin (GE Healthcare)[27, 28]. All GST tagged proteins were cleaved with precission protease and purified prior to use. UbcH5c was purified as previously described [29].

4.4.2 Surface Plasmon Resonance experiments

A streptavidin(SA) chip (GE Healthcare) was docked and equilibrated with HBS buffer (10 mM HEPES, 150 mM NaCl, pH 7.4 containing 0.005% Tween-20) overnight at 5 uL/min. All experiments were performed at 25 °C. Proteins were biotinylated using Sulpho-NHS-LC-Biotin (Invitrogen, catalog B-6353) following the protocol provided by

Invitrogen(MP00143). Hsp70's were immobilized in MES buffer (20mM MES, 150 mM NaCl, pH 5.5, 0.01% Tween-20). Average immobilization responses for the various Hsp70 truncations were approximately 1200 RUs. After immobilization of all biotinylated proteins, the chip was equilibrated for at least 4 hours at 5 µL/min. Varying concentrations of CHIP and its truncations(10 nM – 2 µM) were prepared in HBS buffer and 40 µL were injected at 20 µL/min. For competition experiments, CHIP (100nM) and unlabeled Hsp70 (50 nM – 10 µM) were combined in HBS immediately prior to injection. For both direct and competition binding, the binding surface was regenerated with Regen buffer (HBS buffer, 100mM NaOH, 100mM $MgCl_2$) with a pulse injection of 20 µl until the baseline response was reached. Data was fit thermodynamically by using the change in RU value 5 seconds prior to the end of the association phase. The concentration dependent change in immobilization was then fit by non-linear regression analysis using GraphPad Prism software, from which K_d or K_i values were determined.

4.4.3 FRET assays

Protein samples were labeled and prepared as previously described[30]. Briefly, amine reactive Alex488 (Invitrogen) was used to label Hsp70 and Hsp90 by mixing the dye and protein at a 10:1 molar ratio in bicarbonate buffer (100mM $NaHCO_3$ [pH 8] containing 150mM NaCl). CHIP was similarly labeled using BHQ-10 (Biosearch Technologies) at a molar ratio of 50:1, dye: protein. Excess labels were removed by passage through Zeba desalting columns (Thermo Scientific) equilibrated with hepes-buffered saline. Using the extinction coefficients supplied by the manufacturer, label concentrations were determined for each protein sample and used to calculate the amount of molar

incorporation. MIR values for Hsp70, Hsp90, and CHIP were 3.2, 2.4, and 14.1 respectively. All quenching assays were performed at the indicated protein concentrations in 10mM Hepes buffer (ph 7.4) containing 150mM NaCl, and 0.05% Tween-20. Equilibrium binding measurements were taken in a 384-well microplate format using a SpectraMax M5 microplate reader (Molecular Devices). Each sample was excited at 480nm and total fluorescence was read at 525nm with a 515nm cut-off. The average fluorescence intensity of 15 reads was recorded for each sample.

4.4.4 Ubiquitination assays

Ubiquitination was typically performed for 1 hr at 37°C in 10 µl mixtures containing buffer A (50 mM Tris pH7.5, 50 mM KCl, 0.2 mM DTT), Ubmix (2.5 mM ATP, 5 mM MgCl2, 50 nM Ube1 (E1 enzyme), and 100 µM ubiquitin), 1 µM indicated E2, 1 µM CHIP, prepared as previously described [31]. Reactions were stopped by addition of SDS-Laemmli buffer and boiling, followed by separation of proteins by SDSPAGE and visualization by Western blotting with appropriate antibodies. Mouse mono-clonal antibody to Hsp70 (Assay Designs) was used to visualize ubiquitin transfer.

4.5 Appendices

Appendix 4.1

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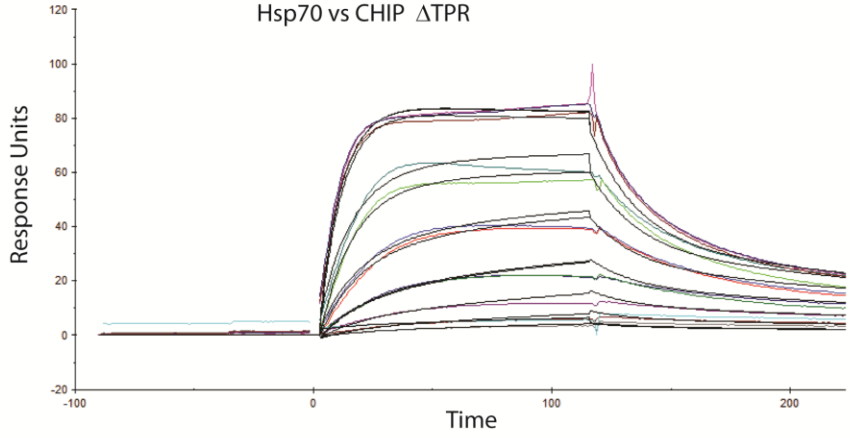

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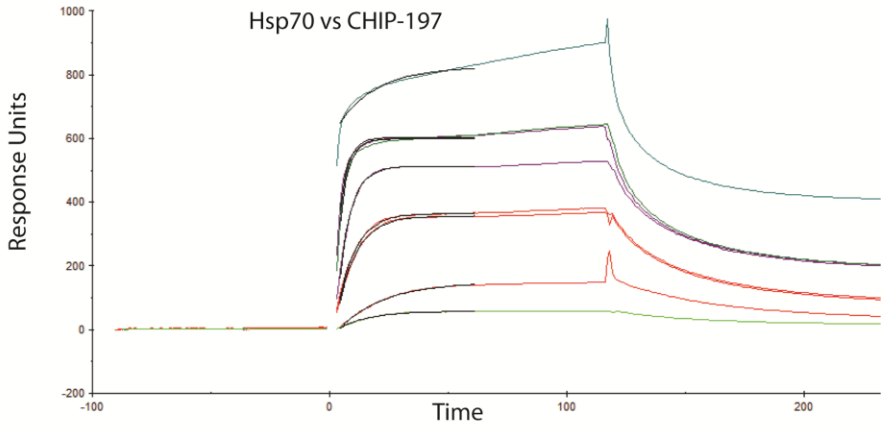

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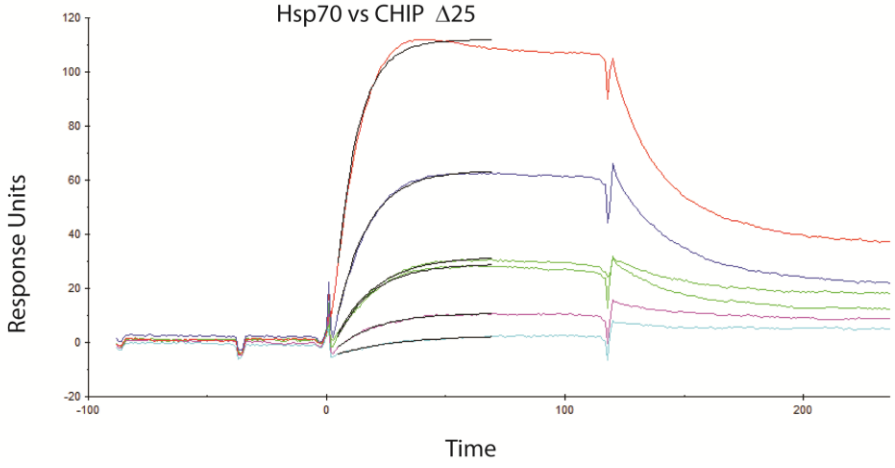

Appendix 4.1(continued..)

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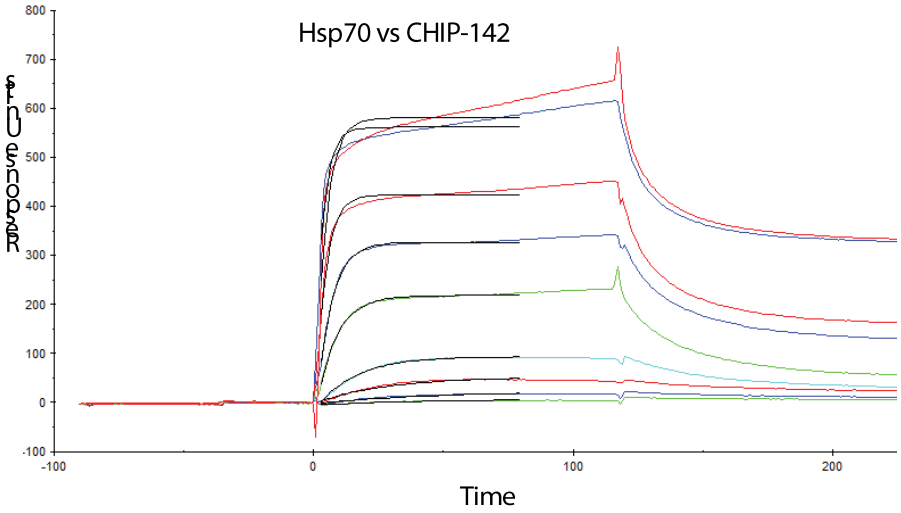

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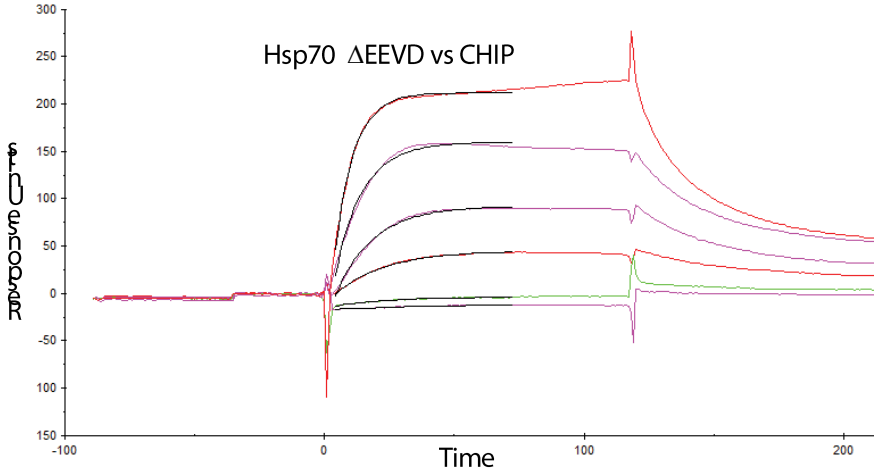

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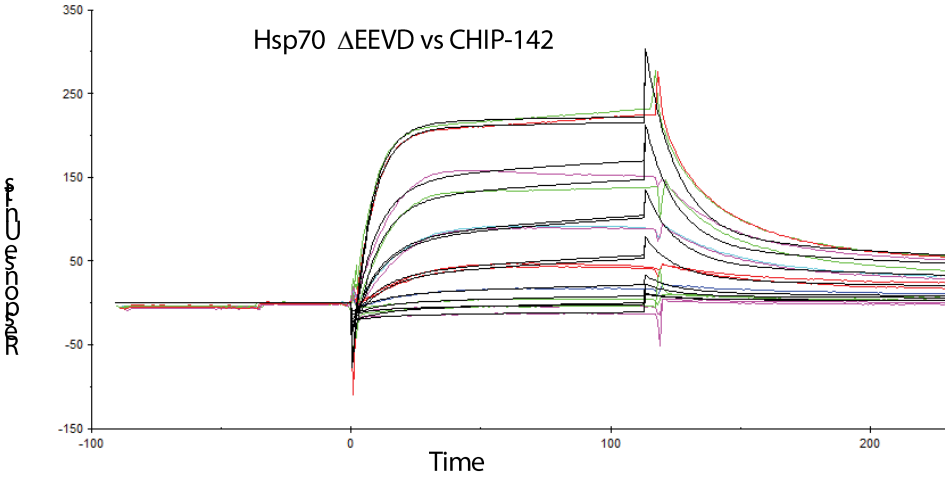

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Chapter 5

Conclusions and Future Directions

5.1 Conclusions

The heat shock protein 70 (Hsp70) family of chaperones is central to proteostasis decisions in the cell. Importantly, Hsp70s do not function alone; rather, they are assisted by a variety of co-chaperones as part of distinct complexes. This thesis focused on understanding how these complexes are formed, the biochemical features that differentiate these complexes and the identification of chemical probes that selectively perturb these complexes. The ultimate goal of this work is to better understand the logic of protein quality control in the cell.

When I first started working in the lab in 2006, there were only a few small molecules known to modulate the function of Hsp70s and these were often of dubious affinity and selectivity [1]. One of the reasons for this gap was that no HTS methods had been developed, severely limiting our capacity to identify potential inhibitors. Thus, from 2007-2010, Lyra Chang, Sussane Wisen, Yoshi Miyata and I in the Gestwicki laboratory spearheaded efforts to develop the first high throughput screens for Hsp70s. Together, we developed the idea that the best way to find inhibitors of Hsp70 would be to target the critical co-chaperone regions. Thus, we developed the concept of ‘gray box’ screening (a

term first coined by Lyra Chang). One of my major contributions to this process was the first comprehensive analysis of the biochemical and biophysical properties of the DnaK and human Hsp70 complexes. We realized relatively early on that knowledge of the turnover rates and optimal ratios between Hsp70:co-chaperone would be critical to a good screen. Thus, I focused my efforts during the past five years at characterizing these systems to enable informed HTS efforts. Most recently, I worked with Tomoko Komiyama to carry out a screen of 100K compounds (described in Chapter 3).

In addition, we found that it was important to deconvolute the target and mechanism of compounds after 'gray box' screening. While not as daunting as 'black box' screens, there are multiple potential targets in a screen against a reconstituted multi-protein complex, leading to greater urgency in understanding the mechanisms of the active compounds. My specific role in these efforts focused on the biophysical characterization of compounds, such as 115-7c, that were specific modulators of DnaK-DnaJ. In my opinion, the most fascinating finding was that I uncovered an unexpected allosteric mechanisms of action for 115-7c at the native protein-protein interface of DnaK and DnaJ. This interaction mimics the functional aspects of the natural co-chaperone DnaJ, despite the small size of the compound. This result could have far reaching implications in the study of inhibitors of protein-protein interactions.

In Chapter 3 and 4, we extended our gray-box screening strategy to human Hsp70 chaperone complexes. The first part of this process was originally intended to be a routine biochemical characterization of the turnover rates and affinity constants. However, both projects turned out to be much more interesting. Prior to these studies, the literature often assumed that, because of high sequence homology, the prokaryotic and

eukaryotic Hsp70 systems would behave similarly. Moreover, because the DnaK system had been best characterized, most experts in the field would assume that human Hsp70s would have similar properties. To be fair, we shared this notion. However, my studies strongly suggested that this assumption is false – the human Hsp70 system has many significant and important differences. For example, I found that not all J-proteins are functionally equivalent in our *in vitro* assays and that the NEFs are unable to stimulate the ATPase and refolding activities of human Hsp70s. We performed a HTS screen against one functionally competent chaperone complex Hsp72/DJA2/BAG2 and discovered a few promising lead hit compounds and efforts to characterize them are currently underway.

When the chaperone machinery fails in attempts to fold substrate proteins, it actively targets them to various degradation pathways. Hsp70 in complex with the E3 ubiquitin ligase CHIP is central to promoting the ubiquitin dependent proteosomal degradation of proteins. We characterized the binding affinity for this complex and, for the first time, mapped the key domains that are involved in this interaction *in vitro*. We found that the TPR domain of CHIP is important, but not absolutely required, in the interaction with molecular chaperones and that it is essential for the substrate ubiquitylation function of CHIP.

Together, my thesis projects are the first steps in understanding the molecular interactions involved in the assembly and functional outcomes of the Hsp70 chaperone complexes. The chemical probes identified will hopefully be useful in understanding the complicated biology of the Hsp70 family of proteins.

5.2 Future directions

5.2.1 Utilizing small molecules to identify chaperone complexes that interact with disease relevant substrates

As mentioned earlier, the combinatorial assembly of co-chaperones with Hsp70 leads to distinct outcomes, such as folding and degradation. To understand the role of Hsp70 as a triage machine, we need to understand the chaperone complexes that are associated with these outcomes. Further, we need to better understand which complexes are associated with specific disease-related substrates, such as tau [2-4]. One approach towards that end goal would be an unbiased proteomic approach in which we force the fate of a substrate using chemical probes and therefore enrich for a particular complex. For example, our "gray box" screens have identified Hsp70 inhibitors, such as 115-7c and methylene blue (MB), that dramatically and rapidly alter the fate of tau in HeLa cells, primary neurons and mouse brain slices [5, 6]. Andrea Thompson, a graduate student in the Gestwicki laboratory, is using mass spectrometry (MS) and co-immunoprecipitation of tau from HeLa cells to identify the chaperones that interact with tau after treatment with MB (Figure 5.1). Specifically, she treated cells with a combination of MB and a proteosomal inhibitor, bortezamib, to enrich for the complex of tau (with its associated chaperones) that is fated for degradation. The members of these complexes (e.g. the "play list") might serve as good candidates for future 'gray-box' screening efforts.

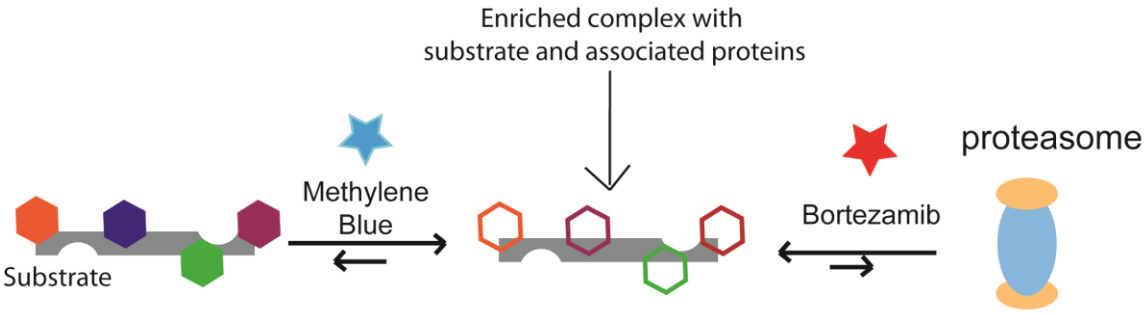

Figure 5.1 Utilizing chemical probes to identify chaperone complexes associated with substrates. Chaperone substrates such as Tau, polyQ associated proteins etc., interact with various chaperone complexes during their life cycle in the cell. We can use small molecules such as MethyleneBlue(MB), Bortezamib (proteasomal inhibitor) to enrich for intermediate complexes and conduct Mass Spectrometry(MS) analysis to identify proteins associated with a particular substrate.

5.2.2 Using chemical probes to dissect mechanistic details

In our collaboration with the Dickey group, we have shown that Hsp70 is central to the processing of tau in cells and animals [5]. However, it isn't yet clear how Hsp70 can distinguish between different tau variants, such as phosphorylated forms. As discussed in Chapter 1, J proteins are the co-chaperones that recruit Hsp70s into specific substrate interactions. Thus, one important future goal will be to identify the J protein that distinguishes "normal" from "abnormal" tau. Once J proteins that specifically stabilize abnormal tau are identified, we could develop inhibitors of this interaction in an attempt to destabilize tau. Although the work described in this thesis focused on the Hsp70-J protein interaction, future studies should also consider the J protein-substrate contact.

5.2.3 Development of biophysical techniques to monitor ternary complex formation

Techniques like SPR and ITC are very useful for determining the biophysical interactions between two binding partners. However, in the case of Hsp70, biology may be a product

of multiple co-chaperones and many protein-protein interactions. Thus, it will be useful to develop a technique to monitor the simultaneous assembly (and disassembly) of multi-protein complexes.

High Speed Capillary Electrophoresis (CE) with laser-induced fluorescence anisotropy (LIFA) detection is a promising solution to this problem. Briefly, one component (e.g. tau) could be labeled with a fluorophore and its interactions with partners, such as Hsp70, would result in the formation of a new peak (Figure 5.2). Likewise, additional partners (with unique labels) could be added to form larger and more heterogeneous complexes. This approach has been used to study interactions between SH2 domains and its substrate peptides [7, 8]. These experiments would help us understand how the various co-chaperones assemble into complexes.

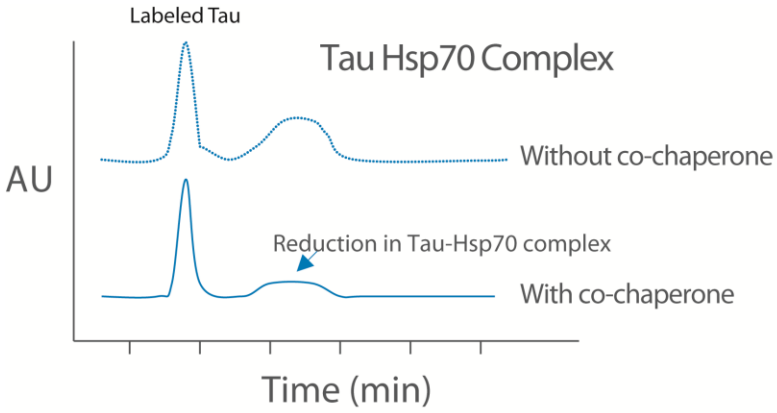

Figure 5.2 Capillary Electrophoresis technique to monitor Hsp70-Tau interaction

5.2.4 Potential multiplexed screens targeting the Hsp70 co-chaperone complexes

In Chapter 3, we developed a flow cytometry-based binding platform where we could monitor the binding interaction between the BAG proteins and Hsp70. Because human BAG proteins do not seem to impact ATP turnover (see Chapter 3), this platform might

be used to find inhibitors. This is an important goal because the BAG proteins have other domains that determine, in part, the functional specificity of Hsp70 complexes.

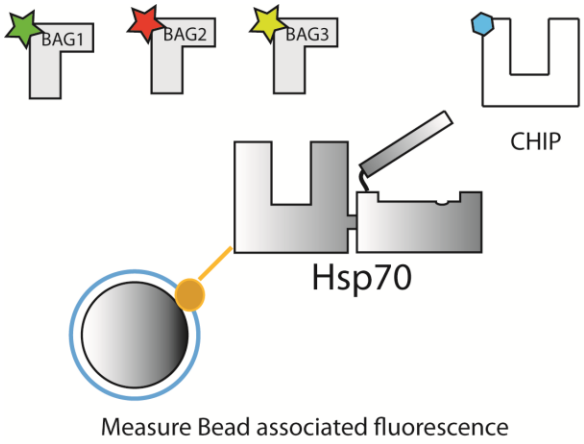

Figure 5.3 Multiplexed Flow assay where we can meaure upto 4 wavelenghts at a time. We can deconvolute any hit compounds after the screen to determine which binding partner the compound affects.

As envisioned, we would label the BAG proteins (BAG1-3) and CHIP with different fluorophores and perform the HTS screen against all the four components at the same time (Figure 5.3). We could then deconvolute the hit compounds after the primary screen, to determine their potential binding partner. This system may allow for the identification of chemical scaffolds targeting multiple Hsp70 interactions in a single, multiplexed primary screen.

5.3 Final Thoughts:

We have now successfully discovered and developed the first generation of small molecule probes that can modulate the function and activity of Hsp70. The current suite of compounds display only modest affinity and are more suitable as chemical probes rather than therapeutics. Since, Hsp70 operates as part of a combinatorial multi-protein complex with multiple protein-protein interfaces and allosteric sites, the chemical tools that are currently available to us will prove very useful to probe the complex the biology of Hsp70.

5.4 References

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