

**Heat Shock Protein 70 (Hsp70) Acts as a Guardian Against  
Multiple Cell Death Pathways**

**by**

**Sharan Ram Srinivasan**

**A dissertation submitted in partial fulfillment  
of the requirements for the degree of  
Doctor of Philosophy  
(Chemical Biology)  
in The University of Michigan  
2014**

**Doctoral Committee:**

**Associate Professor Jason E. Gestwicki, Co-Chair  
Professor Duxin Sun, Co-Chair  
Professor Colin S. Duckett  
Assistant Professor Daniel R. Southworth**

## **Dedication**

To my mentors and teachers, past and present,  
For your wisdom, patience, and kindness.  
And to my family,  
For a constant stream of  
Loving support and encouragement.

## Acknowledgements

The work described herein would not be possible without the support and assistance from numerous people. I would like to begin by thanking my primary advisor, Jason Gestwicki. He has taught me so much, from the ability to properly ask scientific questions to the importance of developing an emotional bond to my work. He has let me forge my own path to the extent possible, learning from my mistakes along the way, and for that I am extremely grateful. I could not have asked for a more encouraging, patient, and insightful mentor. I would also like to thank the members of the Gestwicki Lab, both past and present. This work would not have been possible without the early efforts of Yoshinari Miyata, who made some of the earliest analogs of MKT-077. I would especially like to thank Xiaokai Li, who undertook this daunting project alongside me from the very beginning. He has truly helped this project become a success story of chemical biology. I would also like to thank Jennifer Rauch and Zapporah Young for their contributions to this work, particularly in studying the interactions between Hsp70 and co-chaperones. Thanks to Atta, Anne, Victoria, and Srikanth, who welcomed me into the lab with open arms. Even Tomoko, who told me my first experiment was bound to fail (it only half did). Thanks to Leah, for her revisions to my written work, critical questions, and stimulating discussions about literature and the future of science. More recently, Bryan Duniak and Laura Cesa have rejuvenated the lab with their personal expertise in physical and biological chemistries. I would also like to thank Andrea Thompson and Matt Smith, who took me on as their little brother and mentored me in

both the technical and mental approaches to my experiments. It has been an honor to work with such passionate, intelligent, and hard-working individuals.

I've also had the unique pleasure of working for not one, but two mentors during this thesis work. I can't express enough thanks to Colin Duckett, who understood my hesitation to move to California, and who gave me a desk and bench to complete my work. More importantly, he gave me the mentorship I needed to complete this dissertation. For that, I am extremely indebted. The members of the Duckett Lab have also provided valuable insight and criticism to my work and its implications outside the sphere of chaperones and chemical biology. I would like to thank Andy and Niall, for their enthusiasm towards my work and countless stimulating discussions. Thanks also to Melissa and Annie, for making me feel welcome in such a new environment.

Science is a team effort, and I've had the joy of working with some outstanding collaborators, including: Jooho Chung, Dr. Duxin Sun, and Dr. Erik Zuiderweg. Thank you for your time, patience, and expertise. I am grateful to the members of my committee for their insight and advice towards my training.

I would also like to thank the staff and faculty of the Program in Chemical Biology for providing me a home during my time as a PhD student. In particular, I would like to thank Prof. Anna Mapp, Laura Howe, and Traci Swan, for creating a welcoming environment. In addition to PCB, I would like to thank the staff of the MSTP for their constant assistance. Ellen, Laurie, and Hilkka have answered questions and solved countless issues, always with a smile. I would also like to express my appreciation to Ron Koenig for his direction and advice regarding my training as a physician scientist.

On a more personal note, I would like to thank those people who stimulated my passion for science at a young age, and those who nourished it along the way, encouraging me to seek a career as a scientist. Specifically, I would like to thank my Mrs. Gulati and Mrs. Razi for helping me uncover an aptitude for mathematics. Thanks to Mr. Tom Redig, my high school chemistry teacher, who showed me the wonders of the field. I would also like to thank Profs. SonBinh Nguyen and Erik Sontheimer at Northwestern University, who both invited me to work in their labs as an undergraduate, and taught me how to apply scientific principles from a textbook to research questions at the bench top.

Finally, I am grateful for my family, who has sacrificed so much to see me through this stage in my training. My sister and parents have been a constant source of encouragement and affection. They showed confidence in me even when I doubted myself. I thank my girlfriend, Heather, who put up with random scientific musings about apoptosis versus necroptosis. This support system has given me the ability to constantly reach higher and meet any challenge. Everything I've ever done or will do in the future is because of you, and for you.

## Preface

This dissertation is a compilation of both published and unpublished work towards the goal of understanding the roles of Heat Shock Protein 70 (Hsp70) in cancer. Chapter 1 is primarily based on a review that has been submitted to *Current Topics in Medicinal Chemistry*, entitled “Allosteric Inhibitors of Hsp70: Drugging the Second Chaperone of Tumorigenesis.” This manuscript summarizes the pro-survival roles played by molecular chaperones as well as the efforts to pharmaceutically target Hsp70. Chapter 2 is derived, in part, from a manuscript where we adapted the rhoadcyanine, MKT-077, as a selective Hsp70 inhibitor. Using structure-guided design, we developed more potent and metabolically stable derivatives for use as chemical probes and clinical candidates. This citation is “Analogues of the Allosteric Heat Shock Protein 70 (Hsp70) Inhibitor, MKT-077, As Anti-Cancer Agents” **2013 ACS Med. Chem. Lett.** 4(11): 1042-7. In Chapter 3, we use the most potent of the charged analogs, JG-98, to explore the roles of Hsp70 in cancer. The major discovery of this work was that Hsp70 acts at a hub of multiple cell death pathways, capable of directing both apoptotic and necroptotic cell deaths. This work is being prepared for submission to *Science*. In Chapter 4, we made significant progress towards identifying the oncogenic roles played by individual members of the Hsp70 family. To achieve this goal, we developed JG-13, a neutral derivative of MKT-077 that selectively affects cytosolic Hsp70s. By comparing the effects of JG-13 with JG-98, we were able to parse out the differences between

unique Hsp70 isoforms, and how they contribute to maintaining the cancer phenotype. A manuscript describing this work is in preparation. In Chapter 5, we discuss the implications of this work and present possible future directions.

## Table of Contents

<b>Dedication .....</b>	<b>ii</b>
<b>Acknowledgements .....</b>	<b>iii</b>
<b>Preface .....</b>	<b>vi</b>
<b>List of Figures .....</b>	<b>xi</b>
<b>List of Schemes .....</b>	<b>xiii</b>
<b>List of Appendices.....</b>	<b>xiv</b>
<b>Abstract .....</b>	<b>xv</b>
<b>Chapter</b>	
<b>1. Protein Quality Control in Cancer: Drugging the Molecular Chaperones</b>	
1.1 Abstract.....	1
1.2 Chaperones as Permissive Oncogenes.....	2
1.3 Hsp90: “The Cancer Chaperone”.....	2
1.4 Hsp70: The Second Chaperone of Tumorigenesis.....	5
1.5 Pharmacological Targeting of Hsp70.....	12
1.6 Combination Warfare .....	26
1.7 Conclusions and Future Directions .....	27
1.8 References.....	30

## **2. Development of a Tool to Probe Hsp70 Function: Synthetic Derivatives of the Allosteric Modulator, MKT-077**

2.1 Abstract .....	35
2.2 Introduction .....	36
2.3 Results and Discussion.....	38
2.4 Conclusion .....	56
2.5 Notes.....	58
2.6 Experimental procedures .....	59
2.7 Appendices .....	63
2.8 References.....	65

## **3. Hsp70 Acts as a General Guardian of Cell Survival By Inhibiting Multiple Cell Death Pathways**

3.1 Abstract .....	67
3.2 Introduction .....	68
3.3 Results .....	70
3.4 Discussion.....	83
3.5 Conclusion .....	88
3.6 Notes.....	89
3.7 Experimental Procedures.....	90
3.8 Appendices .....	92
3.9 References.....	94

<b>4. Deconvoluting the Roles of Distinct Members of the Hsp70 Family in Cancer</b>	
4.1 Abstract .....	97
4.2 Introduction .....	98
4.3 Results .....	100
4.4 Discussion.....	112
4.5 Conclusion .....	115
4.6 Notes.....	116
4.7 Experimental Procedures.....	117
4.8 Appendices .....	120
4.9 References.....	123
<b>5. Conclusions and future directions: Unraveling the Signaling Roles of Hsp70 in Cancer and Development of Hsp70 Inhibitors as Therapeutics</b>	
5.1 Abstract .....	125
5.2 Conclusions .....	126
5.3 Future Directions.....	133
5.4 Final thoughts .....	140
5.5 References.....	141

## List of Figures

### Figure

1.1	Architecture and Dynamics of heat shock protein 70 (Hsp70) .....	6
1.2	Hsp70 promotes cell survival and oncogenic proliferation through multiple pathways .....	10
1.3	Hsp70 chaperones substrates of multiple pathways important for cell survival .....	11
1.4	Binding sites for some known Hsp70 inhibitors.....	13
1.5	MKT-077 Allosterically Modulates Hsp70 Function.....	25
2.1	Characterization of YM-01 Stability and Metabolism .....	39
2.2	MKT-077 analogs show greatly improved metabolic stability .....	46
2.3	JG-98 Binds Hsp70 With Greater Affinity than MKT-077 .....	48
2.4	JG-98 binds to a conserved, allosteric site on an Hsp70 family member	50
2.5	MKT-077 Analogs Bind to the NBD of Hsp70 .....	51
2.6	Improved Anti-Cancer Activity of MKT-077 Analogs .....	53
2.7	JG-98 Perturbs Co-Chaperone Modulation of Hsp70 Function .....	55
3.1	JG-98 Induces Hsp90 Client Degradation and Induces Apoptosis .....	71
3.2	JG-98 is Synergistic with Inhibitors of PQC Machinery .....	74
3.3	Hsp70 Inhibition Elicits Rapid Cell Death.....	75
3.4	JG-98 Induces Cell Death Independent of Bcl-2 Status.....	77
3.5	Caspase Inhibition Switches Cell Death Morphology.....	79
3.6	JG-98 Induces Cell Death Through a Novel RIP1-Dependent Process...	81
3.7	Hsp90 Guards Against Multiple Cell Death Pathways by Engaging RIP1.....	87
4.1	JG-13 Displays Increased Toxicity Over JG-98 .....	102

4.2	JG-13 Traps Hsp70 in the ADP-Bound State .....	<b>105</b>
4.3	JG-13 Induces a Cell Death with a Unique Profile .....	<b>107</b>
4.4	JG-13 Differs from Charged MKT-077 Analogs in effects on Hsp90 Clients .....	<b>109</b>
4.5	Synergistic Activity of JG-13 with Other Inhibitors of PQC Machinery ...	<b>111</b>
5.1	Improvements to the MKT-077 Scaffold.....	<b>129</b>
5.2	MKT-077 Analogs Trigger Multiple Cell Death Pathways .....	<b>132</b>
5.3	Mass Spectrometry Tools to Reveal Hsp70-Dependent Proteome .....	<b>135</b>
5.4	MKT-077 Analogs Have Activity <i>in vivo</i> .....	<b>138</b>

## **List of Schemes**

### **Scheme**

2.1	Synthetic Route to MKT-077 and Analogues .....	<b>40</b>
4.1	Development of JG-13, a potent anti-cancer compound and Hsp70 inhibitor.....	<b>100</b>

## **List of Appendices**

### **Appendix**

2.1	Biotinylated MKT-077 Analogs Bind Hsc70 .....	<b>63</b>
2.2	MKT-077 Derivatives Exhibit Preferential Dependence on mtHsp70 .....	<b>64</b>
3.1	Flow Cytometry Indicates JG-98 Triggers an Apoptotic Cell Death .....	<b>92</b>
3.2	Inactivation of Both Apoptosis and Necroptosis is Required to Inactivate JG-98's Cytotoxic Effects .....	<b>93</b>
4.1	JG-13 Induces a Rapid Cell Death .....	<b>120</b>
4.2	JG-13 Mildly Affects Hsp90 Clients.....	<b>121</b>
4.3	JG-13 is At Least Partially Dependent on RIP1 Kinase Activity .....	<b>122</b>

## Abstract

**Co-Chair: Jason E. Gestwicki; Co-Chair: Duxin Sun**

The proper balance between protein synthesis, folding, and turnover is termed protein homeostasis, or proteostasis. Cancer cells are especially reliant on the activities of molecular chaperones, such as heat shock proteins 70 (Hsp70) and 90 (Hsp90), which are critical to protein quality control. Hsp70 and Hsp90 collaborate to buffer cancer cells against unstable oncogenic mutations, and these chaperones are thought to prevent apoptosis during rapid metabolism and cell division. While Hsp90's specific roles in cancer are relatively well described and inhibitors are being studied in multiple clinical trials, less is known about Hsp70.

In this thesis, we aimed to characterize Hsp70's roles in cancer signaling. Towards this goal, we developed the first selective and potent inhibitors of Hsp70, based on the rhodocyanine MKT-077. We found that these molecules, including JG-98 and JG-13, have potent cytotoxic activity in multiple cancer cell lines, with minimal effect on normal fibroblasts. Moreover, these compounds were highly synergistic with inhibitors of either Hsp90 or the proteasome. Using JG-98 as a chemical probe, we found that Hsp70 suppresses apoptotic cell death through a novel process dependent on RIP1 kinase. Further, under conditions in which apoptosis was inhibited, JG-98 triggered a strong necrotic phenotype, suggesting that Hsp70 helps regulate a key

cellular “decision” to proceed with either apoptosis or necrosis. Thus, Hsp70 shares some cellular roles in common with Hsp90, but also has important differences that might be exploited in the pursuit of anti-cancer agents with new mechanisms-of-action.

Hsp70 is actually a family with thirteen members in humans, and it is still unclear if these isoforms play distinct or overlapping roles in cancer. To better understand this question, we have made significant efforts towards designing small molecules that target cytosolic and mitochondrial Hsp70s specifically. These probes have the potential to further transform our understanding of Hsp70 as a cancer chaperone.

Taken together, these studies reveal that Hsp70 operates in a much more global cytoprotective fashion in cancer cells than previously suggested. This work also makes significant strides towards the development of improved chemotherapeutics targeting the protein quality control machinery.

## **Chapter 1**

### **Protein Quality Control in Cancer: Drugging the Molecular Chaperones**

#### **1.1 Abstract**

Cancer cells survive in the presence of a number of cytotoxic stresses that would normally result in cell death. To accomplish this feat, most cancers express elevated levels of molecular chaperones, especially heat shock protein 70 (Hsp70) and heat shock protein 90 (Hsp90). These chaperones stabilize numerous anti-apoptotic “clients”, including Raf-1, Akt-1 and Her2, providing resistance to cell death and protecting against radiation and chemotherapy. These pro-survival activities make Hsp70 and Hsp90 promising nodes for potential anti-cancer therapies. While inhibitors of Hsp90 are well known, progress in the development of Hsp70 inhibitors has proven more difficult. Hsp70 binds tightly to ATP through a highly conserved domain of the actin/hexokinase/Hsp70 superfamily, making it challenging to identify inhibitors that selectively compete with ATP for binding to the active site. Alternatively, some progress has been made on compounds that target important allosteric sites on the chaperone. In some cases, these allosteric pockets have been shown to control key protein-protein interactions between Hsp70 and its co-chaperones, providing multiple possible avenues for disrupting chaperone functions. In this chapter, we review the evidence linking Hsp70 to cancer signaling pathways and provide an update on recent progress in developing allosteric inhibitors.

## **1.2 Chaperones as Permissive Oncogenes**

Tumor cells typically express high levels of the molecular chaperones Hsp70 and Hsp90 (1-6). These chaperones are thought to be permissive of the cancer phenotype, in part, because they protect oncogenes from degradation. For example, many oncogenes contain destabilizing mutations or aberrant chromosomal translocations that would normally render the proteins prone to rapid turnover. However, unique to cancer cells, chaperones are found preferentially in complex with co-chaperones (7), which increases their activity and buffers against aberrant mutations. At the same time, chaperones also protect against the cytotoxicity that would normally occur in the presence of oxidative damage associated with rapid cell division (8, 9). Indeed, tumorigenesis is so dependent on the activity of chaperones, that cancer cells have been described as being “addicted” to these factors (10-12). Thus, Hsp90 and Hsp70, sitting at the intersection of multiple cell survival pathways, have emerged as potential hubs for anti-cancer drug development.

## **1.3 Hsp90, “The Cancer Chaperone”**

Hsp90 is an ATPase composed of an N-terminal domain that binds ATP, a middle domain that mediates homodimerization and a C-terminal domain (reviewed in (13)). All of these domains have been proposed to interact with clients. Hsp90 is active as a homodimer and recent structural insights have provided snapshots of Hsp90 in its various apo-, ATP- and ADP-bound states (14, 15). These structures have shown that nucleotide cycling in the N-terminal domains drive major conformational changes, including rotation of the two Hsp90 monomers from an open “V-state” to form a more

closed structure. These actions are thought to regulate the capture and release of Hsp90's clients. Clients are typically stabilized by their interaction with Hsp90, while release from the chaperone is often associated with degradation.

There are three major categories of Hsp90 inhibitors, which are divided into categories based on their binding sites on the chaperone. The N-terminal domain is the site of binding for geldanamycin, its analogs and many other synthetic inhibitors, such as PU-H71, STA-9090 and NVP-AUY922. These molecules compete with ATP (16-19), and binding of these compounds appears to stall nucleotide cycling, trap an "open" state of the dimer and favor client release. In turn, release of clients from Hsp90 seems to restore their normal vulnerability to rapid degradation. More recently, compounds related to novobiocin, such as KU-174, have been found to interact near the C-terminus of Hsp90 (20, 21). Interestingly, these compounds also destabilize Hsp90 clients, despite the fact that they bind a distinct domain and don't directly compete for binding to nucleotide. The mechanism of client degradation after binding novobiocin analogs is not yet clear, but it likely involves changes in allosteric communication or conformational shifts within Hsp90 that leads to reduced client interactions. Finally, some reported Hsp90 inhibitors impede interactions with co-chaperones (22-24). A variety of co-chaperones, including p23, Aha1, Hop and cdc37 associate with Hsp90, regulating its ATPase activity and helping to guide its selection of clients (25, 26). As previously mentioned, complexes between Hsp90 and its co-chaperones are enriched in cancer cells (7), signifying that the oncogenic roles of Hsp90 are linked to cooperation between these factors. Consistent with this idea, compounds that inhibit protein-protein interactions between Hsp90 and its co-chaperones disrupt Hsp90 functions. Similar to

the other categories of inhibitors, these compounds also destabilize Hsp90 clients. For example, analogs of sansalvamide A bind the middle domain of Hsp90 and interrupt binding of Hsp90 to IP6K2 (27), while analogs of gedunin appear to selectively inactivate the co-chaperone p23 (22). It is interesting that many different compounds, operating at multiple binding sites on Hsp90, all disrupt client stability. These results suggest that Hsp90 is a sensitive, dynamic “machine” and that interrupting any of its various activities (e.g. ATP cycling, co-chaperone interactions) reduces its ability to protect clients. As will be discussed below, similar patterns are emerging for the Hsp70 system, which seems to present parallel opportunities for inhibition.

Hsp90 binds to at least 1,000 proteins in the cell, including many pro-survival kinases and transcription factors (28-31). Many of the clients of Hsp90 have metastable domains, such as steroid-binding clefts, nucleotide-binding cassettes and latent protein-protein interaction motifs. Binding of Hsp90 to these client proteins is thought to protect them from degradation by “hiding” the metastable regions from the protein quality control systems (32, 33). Accordingly, a diagnostic feature of Hsp90 inhibitors is that they destabilize multiple clients, including Akt, Raf-1, Her2, and Bcr-Abl (28-31). Interestingly, Hsp90 inhibitors typically have little effect on normal cells, such as fibroblasts. It isn’t yet clear why some chaperone clients become so sensitive in cancer cells, but it seems plausible that the clients in non-cancerous cells may fold more effectively and be less dependent on Hsp90 function. Based on this logic, Hsp90 has been called the “cancer chaperone” (12, 34, 35).

Several Hsp90 inhibitors, mostly targeting the N-terminal domain, have entered clinical trials as anti-cancer therapies (35-38). While Hsp90 inhibitors exhibit potent

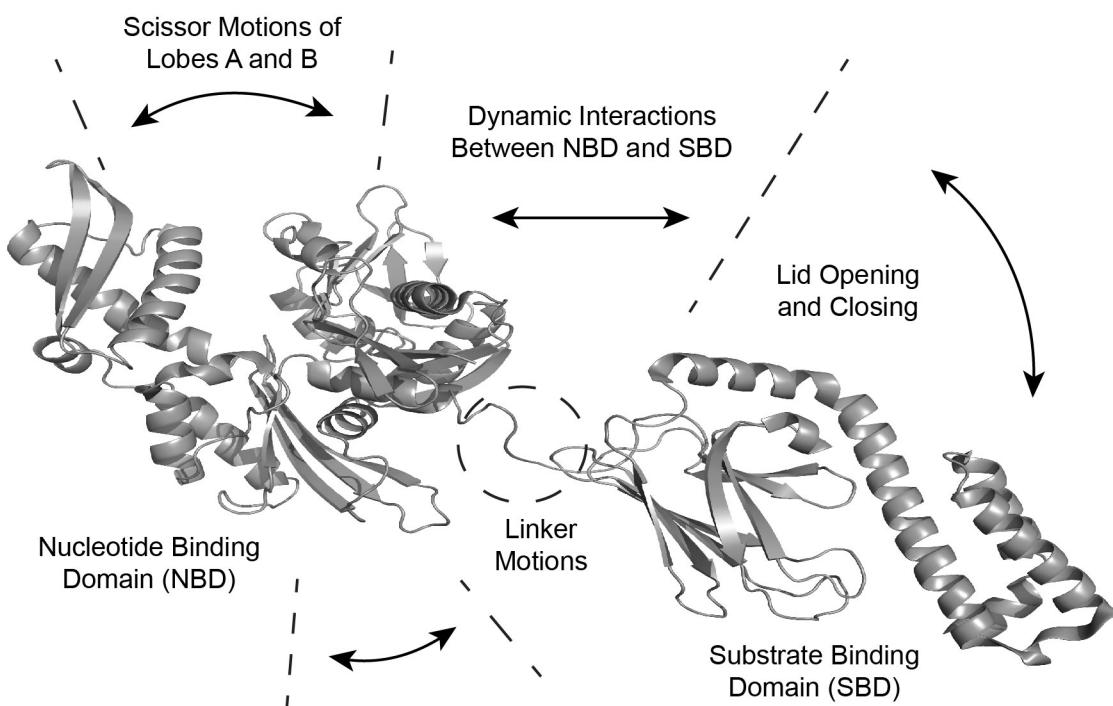
activity against a variety of cancer lines, including those already resistant to conventional therapeutics (39, 40), the results of clinical trials so far suggest that the efficacy of these compounds may be short lived. Specifically, tolerance to Hsp90 inhibitors occurs relatively rapidly, with eventual clinical relapse (41-44). Though the exact mechanism for this acquired resistance is unclear, one potential explanation is that Hsp70 may compensate for loss of Hsp90 function. Indeed, one biomarker for N-terminal Hsp90 inhibitors is that they activate a stress response, dramatically elevating the levels of Hsp70 and other chaperones (44-47). In turn, high levels of Hsp70 may be able to partially compensate for loss of Hsp90 function in cancer cells, suggesting that Hsp70 may be an additional facilitator of oncogenic activity.

#### **1.4 Hsp70: the Second Chaperone of Tumorigenesis**

Hsp70 consists of a 44kDa nucleotide-binding domain (NBD) and a 26kDa substrate-binding domain (SBD) (Figure 1.1) (48). The NBD includes a nucleotide-binding cassette of the Hsp70/actin/hexokinase superfamily that binds very tightly to ATP ( $K_d \sim 0.1$  to  $0.5$   $\mu\text{M}$ ) (49) and has intrinsic hydrolysis activity (50). The SBD is comprised of a  $\beta$ -sandwich subdomain and a  $\alpha$ -helical “lid”. Hydrophobic regions in client proteins bind in a groove of the  $\beta$ -sandwich that is “covered” by the lid (51). The NBD and SBD are connected by a short, hydrophobic linker that is important for inter-domain allostery (52, 53).

Detailed NMR and crystallography studies have shown that Hsp70 is a highly flexible protein that is regulated by multiple allosteric mechanisms (54). For example, the two lobes of the NBD collapse onto each other in response to hydrolysis of ATP

(55). This “scissor” motion helps communicate nucleotide status in the remainder of the protein, because the NBD is closely associated with the SBD in the ATP-bound state, while hydrolysis releases this inter-domain contact (56-58). A consequence of this motion is that the helical lid moves from an “open” configuration impinging on the NBD, to a “closed” state on the SBD (Figure 1.1). These coordinated motions occur over dozens of Ångstroms and involve rearrangements of nearly every subdomain.



**Figure 1.1 Architecture and dynamics of heat shock protein 70 (Hsp70).**  
Allosteric changes during ATP hydrolysis occur both within and between domains.  
PDB ID: 2KHO.

Allosteric motions in Hsp70 are further regulated by binding to co-chaperones. Hsp40s (or J-proteins) are co-chaperones that bind to Hsp70 via a conserved J domain. Binding of the J domain in a region between the NBD and SBD helps release their interdomain contacts, mobilizing the linker region, and promoting ATP hydrolysis (56). After hydrolysis, ADP is replaced with ATP by another co-chaperone class, nucleotide-exchanged factors (NEFs). NEFs, such as BAG1, make contacts with the two lobes of Hsp70's NBD and favor the "open" configuration (59). This allosteric motion allows ADP to be released and ATP to bind. Finally, tetratricopeptide repeat (TPR) domain proteins, such as Hsc70-organizing protein (HOP) and the C-terminal Hsc70-Interacting protein (CHIP), interact at the C-terminal EEVD motif of the SBD (60). The TPR co-chaperones appear to coordinate "hand-off" of clients to other pathways. For example, HOP binds to both Hsp70 and Hsp90 and it has been shown to facilitate transfer of clients between the chaperones, while CHIP is an E3 ligase that ubiquitinates substrates, thus targeting them for proteasomal degradation. Thus, these various families of co-chaperones (Hsp40s, NEFs and TPR proteins) bind Hsp70 and help regulate its cellular actions. Moreover, there are ~40 Hsp40 genes in human cells, in addition to dozens of NEFs and TPR-domain co-chaperones. It is thought that specific combinations of Hsp70s and individual members of the Hsp40 family may be partially dedicated to subsets of chaperone activities (61-63), a model supported by recent genetic studies (64, 65). Thus, the combinatorial assembly of co-chaperones onto Hsp70 is thought to generate a series of distinct complexes that may execute the specific actions of the chaperone.

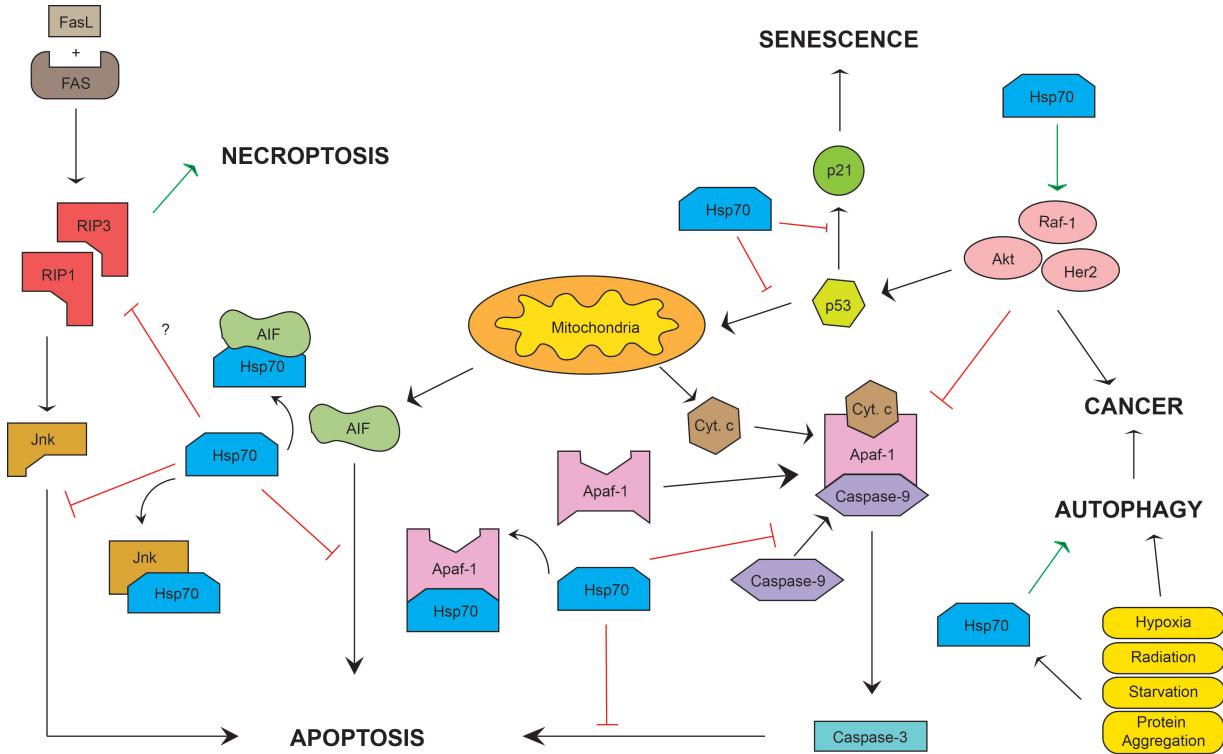
Hsp70 is thought to have a large number of clients. Indeed, studies using peptide arrays have suggested that the clients of Hsp70 include virtually any protein with exposed hydrophobic regions (66). NMR and crystallography studies have supported this idea by showing that Hsp70 makes most of its contacts with the amide backbone of client peptides (48, 67). This mode of binding would be predicted to allow binding to most, if not all, proteins (68). Thus, the theoretical clients of Hsp70 would appear to include the same pool governed by Hsp90, in addition to a wide range of other proteins. Indeed, several studies have shown that knockdown of Hsp70 leads to degradation of oncogenic Hsp90 clients, such as Akt and Her2 (69-74). However, one might initially presume that destabilization of all presumed Hsp70 clients might be broadly cytotoxic to all cells, not just cancer cells. However, experimental studies have shown that this is not the case, as will be discussed below. Accordingly, one of the major outstanding questions in the field is how Hsp70 clients are chosen, how these clients might be different in cancer cells, and how Hsp70 and its co-chaperones might cooperate in this process.

There are approximately 13 different Hsp70 genes in humans and members of the Hsp70 family are found in the cytosol, the nucleus and all of the organelles (75). The major cytosolic family members are Hsc70 (HSPA8), which is constitutively expressed, and Hsp72 (HSPA1), which is expressed in response to stress. Cancer cells appear to have elevated Hsp72 levels, consistent with a basal level of cellular stress. Other Hsp70 family members include Grp75 (HSPA9; mortalin), which is expressed in the mitochondria. Recently, Grp75 has also been found in the cytosol and proposed to play an important cytoplasmic role in cancer (76, 77). In the endoplasmic reticulum, Grp78

(HSPA5; BiP) plays roles in proteostasis of the secretion system (78), which is especially important in leukemia cells that have active secretion systems. Seminal studies in the Workman laboratory have shown that siRNA knockdowns of *both* Hsc70 and Hsp70 are required to arrest tumor cell growth (72). Likewise, Grp75 has been implicated in preventing apoptosis and senescence in MCF7, U2OS, and COS7 cells (79, 80). It isn't yet clear which Hsp70s might be the best targets for anti-cancer therapy. Moreover, discriminating between the family members with small molecules might be difficult, as they are highly conserved (~50% identical). However, differences in their subcellular localization or the identity of their co-chaperones might eventually be used to focus inhibitors on specific family members. This approach will be discussed in more detail in Chapter 4.

#### **1.4.1 Evidence Linking Hsp70 to Cancer**

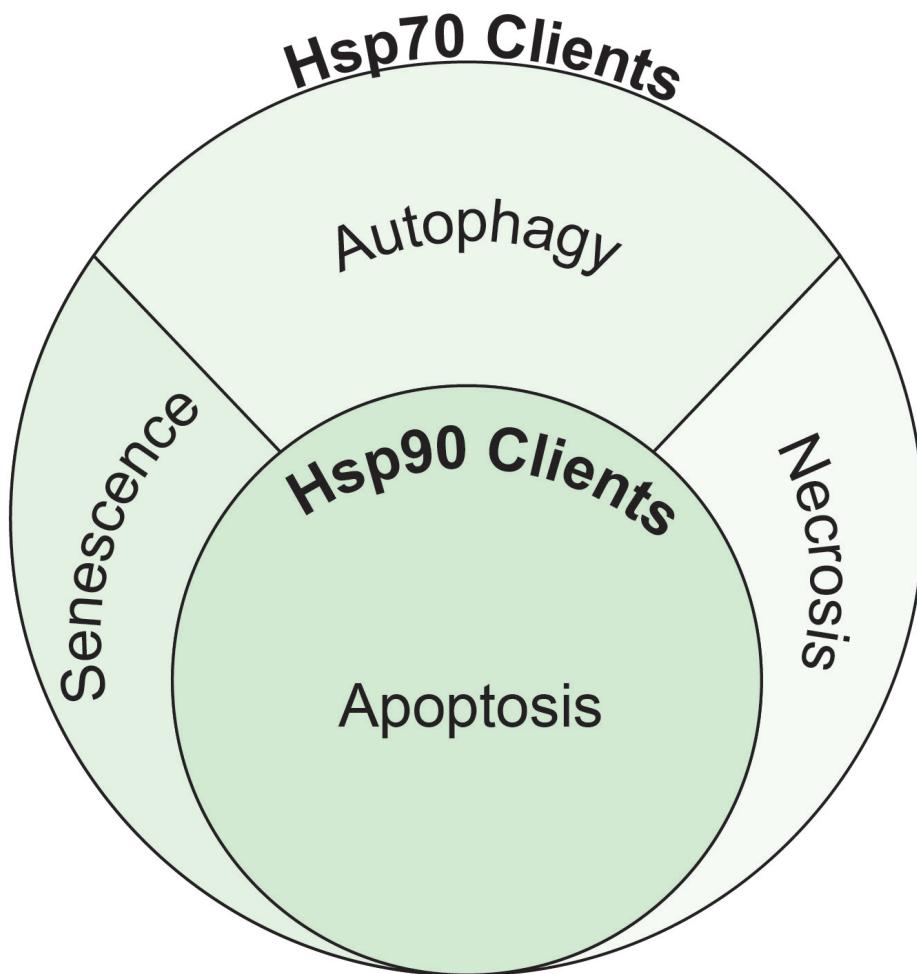
Hsp70 levels are constitutively elevated in both solid and liquid tumors when compared to normal or immortalized cells (81, 82). Further, its expression levels correlate with disease progression in multiple cancers (83) and Hsp70 expression directly predicts resistance to an array of chemotherapeutics, radiation therapies (3, 5, 6, 72, 84) and immune-mediated destruction of cancer cell (85, 86). These observations have driven interest in better understanding the roles of Hsp70 in cancer and exploring this chaperone as a potential drug target.



**Figure 1.2. Hsp70 promotes cell survival and oncogenic proliferation through multiple pathways.** Although Hsp70 has been shown to inhibit apoptosis by interacting with several key effectors of the programmed cell death pathway, its chaperone effects have also been noted to promote autophagy, inhibit senescence, and potentially influence necroptosis, or programmed necrosis.

The expression of Hsp70 in such a diverse set of tumors suggests that it may act more broadly towards oncogenic proliferation instead of modulating only a single pathway. Indeed, Hsp70 binds c-jun (JNK) (87), APAF-1 (88), and AIF (89), inhibiting the formation of the death-inducing signaling complex (90); it also prevents Bcl-2 translocation to the mitochondria (91, 92) and prevents caspase-9 recruitment to the apoptosome (88) (Figure 1.2). Thus, Hsp70 is thought to guard against both extrinsic and intrinsic death triggers, as well as caspase-dependent and -independent forms of apoptosis. Additionally, recent results suggest that Hsp70 inhibition might trigger necrosis (93), senescence (3, 6) and/or autophagic cell death (11). These observations support the notion that Hsp70 serves as a general hub of pro-survival activities and that

it might be a weak link in many cancers. In fact, a comparison between the pathways reported to be regulated by Hsp90 and those controlled by Hsp70 suggests that Hsp70 may have an even broader role in cell survival (Figure 1.3).

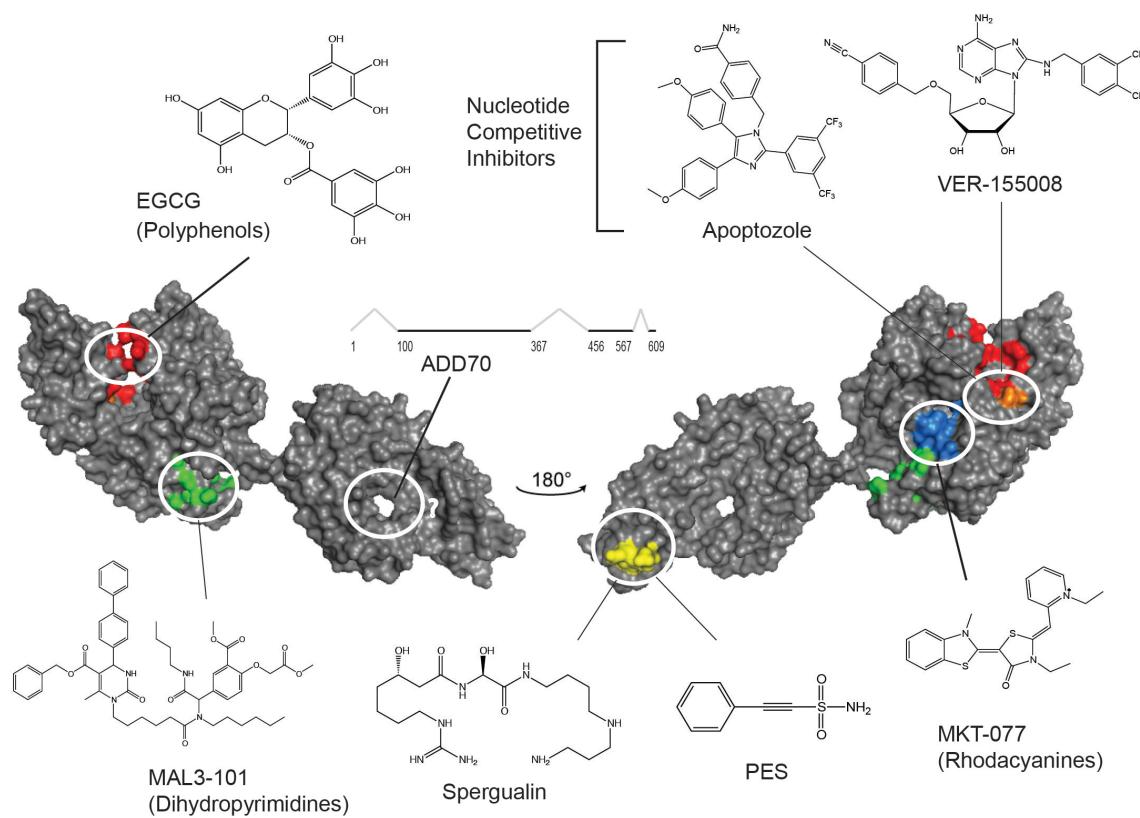


**Figure 1.3 Hsp70 chaperones substrates of multiple pathways important for cell survival.** The “Hsp70-ome” consists of proteins involved in senescence, autophagy, and necrosis. As indicated, the Hsp90-clientele that is so important to apoptosis defines only a subset of the “70-ome.”

## **1.5 Pharmacological Targeting of Hsp70**

Despite its roles in governing the oncoproteome, no Hsp70 inhibitors are currently in clinical trials. However, several groups are working towards developing Hsp70-targeting molecules, and some of their efforts will be discussed here.

One emerging theme from this work is that the known Hsp70 inhibitors bind multiple sites that are dispersed across the protein surface (Figure 1.4). These sites are found in both the SBD and the NBD and the compounds include those that are competitive for ATP binding and those that bind a variety of allosteric sites. To organize this discussion, the various compounds will be categorized by their reported binding site and whether they are orthosteric or allosteric inhibitors.



**Figure 1.4 Binding sites for some known Hsp70 inhibitors.** The approximate binding sites of Hsp70-targeted molecules and representative chemical structures are shown. The allosteric sites on Hsp70 are distributed across its surface, but are clustered at sites of protein-protein interactions.

### **1.5.1 Compounds that bind in the SBD of Hsp70**

#### **1.5.1.1 PES (or PFTμ)**

Originally discovered out of a screen for molecules that activate p53-mediated apoptosis, 2-phenylethylnesulfonamide (PES or PFTμ) was found to interact with the SBD of Hsp70 (Figure 1.4) (11). A biotinylated version of PES binds Hsp70 in cell lysates, and specifically to the lid region of the SBD (aa 573-616), as measured by mass spectrometry. Further mutagenesis and docking studies suggest that the compound may bind in a helical bundle within the SBD's lid (94). This region of Hsp70 has relatively low conservation amongst Hsp70 family members and PES has been shown to bind Hsp72, but not Grp75/Mortalin or Grp78/BiP.

Analysis of PES-treated cell lysates showed that the compound disrupts binding of Hsp70 to p53, suggesting that some of the contacts between the chaperone and its clients may be disrupted by PES. Moreover, PES appears to block the interactions of Hsp70 with some of its co-chaperones, including CHIP, Hsp40 (DjB1), and Bag-1M. The structural basis of this effect on clients and co-chaperones is not yet clear. One possibility is that the compound disrupts the lid architecture, which might interrupt binding to some clients and TPR-domain co-chaperones, such as CHIP, which bind to the C-terminal EEVD motif. However, it is less clear why binding in the lid region would impact Hsp40 or BAG domain interactions, which are thought to occur far from that site (56, 59). It is possible that binding may allosterically regulate these distal protein-protein contacts. Another idea is that PES globally destabilizes Hsp70's structural architecture, thereby disrupting its protein-protein interactions with all three classes of co-chaperones

and its clients.

PES-treated cells exhibit striking vacuolization of the cytosol, suggesting that the compound may impair lysosomal activity (11, 95). Further, PES elevates the levels of LC3-II in multiple cell lines, signifying increased autophagic flux. This activity appears to be particularly toxic to acute myeloid leukemia (AML) and acute lymphoblastoid leukemia (ALL) cells, suggesting that PES might be promising in the treatment of acute leukemias (96).

Based on the results with PES, a recent study explored synthetic analogs. PES is a simple, “fragment-like” molecule (Figure 1.4) with a high ligand efficiency (LE), which makes it an attractive scaffold for further elaboration. Interestingly, the derivation studies showed that the amide could be replaced with a pyrrolidine without a loss of activity, but that reducing the acetylene was not tolerated. From those studies, a derivative containing a chloro group at the meta-position of the benzyl moiety (2-(3-chlorophenyl)ethynesulfonamide; PES-Cl) was identified (94). This compound was more potent than PES in cell viability studies and appeared to share the other hallmarks of PES activity in cells (e.g. vacuolization, LC3-II accumulation). However, only a handful of PES analogs have been tested and detailed structure-activity relationships (SAR) have not emerged. Further, a recent manuscript calls into question whether PES acts through the Hsp70 system (97).

### 1.5.1.2 ADD70

Hsp70 counters apoptotic death, in part, by interacting with apoptosis-inducing factor (AIF) and preventing its translocation from the mitochondria to the nucleus (98). Schmitt *et al.* used deletion mutants of AIF to map the precise region of AIF that binds Hsp70. They used this information to build a cytosolic AIF-mimetic, named ADD70, which competes with endogenous AIF for binding to Hsp70. Although the mimetic itself is not cytotoxic, ADD70 does sensitize cancer cells to staurosporine, vinblastine, and other therapeutics. Based on analogy with other Hsp70 clients, one might assume that AIF binds in the SBD of Hsp70s (Figure 1.4), however, this hypothesis has not been tested. Further, membrane-permeable, synthetic analogs haven't yet been developed.

The lack of toxicity in response to ADD70 is interesting because most of the other molecules that bind Hsp70 are cytotoxic as stand-alone agents (see below). Thus, ADD70 may bind to the chaperone in a unique way, interrupting only select chaperone functions. Another possibility is that the region of ADD70 that binds Hsp70 is a hub for other protein-protein interactions. If this is the case, then the activity of ADD70 may be a function of multiple pathways, including Hsp70. Further mechanistic work will be required to address these issues.

One interesting clue to the function of ADD70 comes from studies on its treatment of immunocompetent mice. Although ADD70 was not inherently cytotoxic in immunodeficient mouse models, it decreased tumor growth in immunocompetent mice. Histological examinations confirmed infiltration of CD8+ cytotoxic T-cells in tumor cells expressing ADD70-GFP (98). Thus, ADD70 appears to enhance immunogenicity through an unknown mechanism.

### 1.5.1.3 Spergualin

The polyamine natural product, spergualin, was originally identified as a potent anti-bacterial agent with broad spectrum activity (99). Subsequent worked showed that this molecule also has anti-cancer and immunosuppressive activities (100-102). Synthetic efforts have removed some of the metabolic liabilities of spergualin, including the hydroxyl at carbon 15, which have greatly improved the physical properties of the chemical series (103, 104). The most advanced of the spergualin analogs, 15-deoxy-spergualin (15-DSG) is clinically approved in Japan for the treatment of acute allograft rejection, making this molecule the lone Hsp70 inhibitor approved for human use (105, 106).

15-DSG was found to be selective for Hsp70 in cells, using mass spectrometry and pull down studies (107, 108). Further, this compound was shown to bind to the C-terminus of Hsp70s and chemically crosslink to the EEVD motif. As discussed above, this region of the SBD is interesting because it serves as a site for interactions with TPR domain co-chaperones. Thus, it is possible that spergualin analogs may disrupt co-chaperone interactions, although this has not been shown. Further, there is not a crystal structure of 15-DSG bound to Hsp70, so the exact binding site is uncertain and SAR studies have not benefitted from structural knowledge.

Early SAR studies were focused on three key regions of 15-DSG, the central  $\alpha$ -hydroxyglycine core, the guanidylated fatty chain and the spermidine-derived polyamine. Nishizawa and co-workers replaced the central  $\alpha$ -hydroxyglycine with various  $\alpha$ - or  $\omega$ -amino acids and the anti-tumor activities of these analogs were evaluated against L1210 (IMC) mouse leukemia cells (103). From the resulting SAR,

the authors concluded that the central  $\alpha$ -hydroxyglycine could be replaced with either glycine or an L-serine residue. The authors also altered the length and flexibility of the guanidinoheptanoic acid region to study its role in anti-tumor activity. It was discovered that a 4-guandino-phenylbutyric acid shared similar anti-tumor activity with 15-DSG, suggesting that significant bulk could be tolerated in that region. Umeda and co-workers subsequently studied modifications of the spermidine moiety (109). The SAR concluded that both the primary and secondary amino groups were important for anti-tumor activities. Together, these studies have shown a relatively narrow SAR around 15-DSG.

Based on its success as an immunosuppressant, 15-DSG was explored as an anti-cancer agent in a Phase I clinical trial (80 and 2792 mg/m<sup>2</sup>/day) against refractory solid tumors in 56 patients (110). However, the average plasma concentration was only 0.07 to 7  $\mu$ g/mL, largely because of rapid clearance (terminal half-life of 1.9 hr). These concentrations are below the anti-tumor activity of the compounds in many cancer cell lines, suggesting that more potent and stable analogs are needed. Dose-limiting toxicity was reversible hypotension, which occurred in five of six patients with the highest dosage, but it isn't clear whether this toxicity was on- or off-target.

The mechanism by which spergualin analogs activate cell death is not yet clear. Also, additional medicinal chemistry efforts will likely be required to improve the potency and stability of these molecules. However, the relative safety of 15-DSG in humans makes this scaffold an attractive option for further mechanistic studies.

## **1.5.2 Compounds that bind in the NBD of Hsp70**

### **1.5.2.1. ATP/ADP Competitive Inhibitors**

Hsp70 binds ATP with an affinity that is nearly 1000-fold tighter than binding of nucleotide to Hsp90 (49). As such, it is more difficult for compounds to compete with ATP in Hsp70's nucleotide-binding pocket. These challenges have been nicely summarized by Massey, who compared the hit rates from fragment screens performed against multiple nucleotide-binding targets, including Hsp70 and Hsp90. This analysis showed that Hsp70 had a hit rate of only 0.4%, while Hsp90 had a hit rate of 4.4%. High hit rates are typically associated with better “druggability” and they have been shown to be a predictor of future success in fragment elaboration campaigns. Thus, Massey concluded that the ATP-binding site of Hsp70 is an unusually poor drug target, having one of the worst hit rates yet described. Further, the ATP-binding cassette in Hsp70 is highly conserved amongst several abundant proteins, such as actin and hexokinase. Thus, it is predicted to be difficult to selectively inhibit Hsp70 by this approach.

Despite these issues, a number of competitive inhibitors have been reported to bind in the ATP-binding cassette of Hsp70s (Figure 1.4) and these have been found to have anti-tumor activity. Before discussions of allosteric inhibitors, it may be illuminating to examine these efforts in more detail.

#### **1.5.2.1.1 Apoptozole**

Apoptozole was discovered in a screen of imidazoles that trigger apoptosis (111). A resin-linked version of apoptozole was used to show that the compound binds to the NBDs of both Hsc70 and Hsp72, and a fluorescent version of the molecule co-localized with anti-Hsp70 antibodies in cells. Using an analog of apoptozole with an installed electrophile, the binding site of the compound was further mapped *in vitro* to a region near the nucleotide-binding pocket (112). Using this distance constraint, computational modeling results suggested that the compound might compete with nucleotide. Consistent with this model, apoptozole mildly inhibits the ATPase activity of Hsp70 (~60% reduction in ATPase rate at 200 µM). However, verification of this binding site by formal competition studies and/or structural studies has not been reported.

#### **1.5.2.1.2 VER-155008**

In a rational approach, Williamson, Massey and co-workers developed a collection of adenosine analogs and screened them in a fluorescence polarization (FP) assay for binding to Hsp70. This process yielded multiple analogs with IC<sub>50</sub> values in the low micromolar range. Co-crystallization confirmed that two of these molecules bind in the ATP-binding cleft. From this starting point, analogs were developed and the most potent of these compounds, VER-155008, had an IC<sub>50</sub> value of 0.5 µM. This compound also had promising anti-proliferative activity in HCT116 cells, with an EC<sub>50</sub> of 5 µM, and it destabilized some of the expected client proteins, including Raf1 and Her2 (113). Subsequent work showed that VER-155008 induced both caspase-dependent and - independent apoptosis in colon carcinoma cells (70). These studies have demonstrated

that inhibitors can be developed starting from structural knowledge of the Hsp70 nucleotide-binding site.

In a recent study, the same team has reported the development of new inhibitors of Grp78, the ER-resident Hsp70 family member. Starting with the BAG1-Hsc70 crystal structure, they designed adenosine-mimetics that are selective for Grp78 over Hsc70 (114). Co-crystal structures suggest that polar groups appended to the ribose ring of VER-155008 disfavor binding to Grp78, which has an isoleucine residue rather than a polar threonine in the key amino acid 37. Consistent with this idea, VER-155008, which has a hydrophobic cyano-benzyl group in that position, was not selective for any specific Hsp70 family member; in fact, its anti-proliferative activity likely comes from pan-inhibition of Hsp70s.

The next step in the development of these compounds is to understand whether they selectively bind to Hsp70 in cells, and if any differences in activity are observed in cancer cells compared to healthy ones. Further, it will be interesting to understand why competitive binding leads to client degradation. Is the inability of Hsp70 to actively cycle ATP/ADP linked to client turnover? Does the compound trap a specific state of Hsp70 that is linked to the degradation pathways? In the toolbox of available Hsp70 inhibitors, active-site competitors will likely serve an important role.

### **1.5.2.2. Allosteric Modulators**

One promising way to inhibit Hsp70's activity in cancer cells may be to target molecules to the chaperone's many allosteric sites (115, 116). This approach might be expected to avoid some of the problems associated with active-site inhibitors, including selectively targeting an ATP-binding fold that is highly conserved amongst many proteins. Another potential advantage of targeting allosteric sites is that Hsp70 cooperates with a large number of important co-chaperones. Many allosteric sites in Hsp70 are located at sites of binding to co-chaperones (see Figure 1.1). Thus, molecules that bind allosteric sites might have profound effects on recruitment of co-chaperones and might be more specific to cancer cells, where chaperones exist predominately in super complexes.

#### **1.5.2.2.1 MAL3-101**

Members of the Hsp40 family of co-chaperones are critical regulators of Hsp70 (56, 59, 60). Using a screen for modulators of Hsp70's ATPase activity, Fewell *et al.* discovered MAL3-101, a dihydropyrimidine that inhibits Hsp40-enhanced ATPase activity without affecting Hsp70's endogenous functions (117). Subsequent work showed that analogs of MAL3-101 bind at the interface of the Hsp70-Hsp40 complex and regulate formation of this heterodimer (118) (Figure 1.4). The binding site of the dihydropyrimidines is well conserved and members of this chemical series have activity against multiple human, yeast and prokaryotic Hsp70s (119-121).

The ER resident Hsp70, BiP, collaborates with a series of Hsp40s to coordinate quality control in this organelle (122-124). Because BIP-Hsp40 pairs regulate secretion

of properly folded immunoglobulins (78), MAL3-101 was tested in a multiple myeloma model and it was found to exhibit anti-proliferative activity both *in vitro* and *in vivo*. Moreover, MAL3-101 was synergistic with the Hsp90 inhibitor, 17-AAG, and the proteasome inhibitor, MG-132 (125). These results suggest that inhibition of the Hsp70-Hsp40 pair is a viable way to interrupt chaperone functions and induce apoptosis in cancer cells. This finding is important because the dihydropyrimidines have little activity against isolated Hsp70, so their anti-proliferative activity likely originates from activity against the Hsp70-Hsp40 complex rather than the chaperone alone. The next steps in the development of this chemical series are to improve the affinity and potency of the compounds.

#### **1.5.2.2.2 MKT-077**

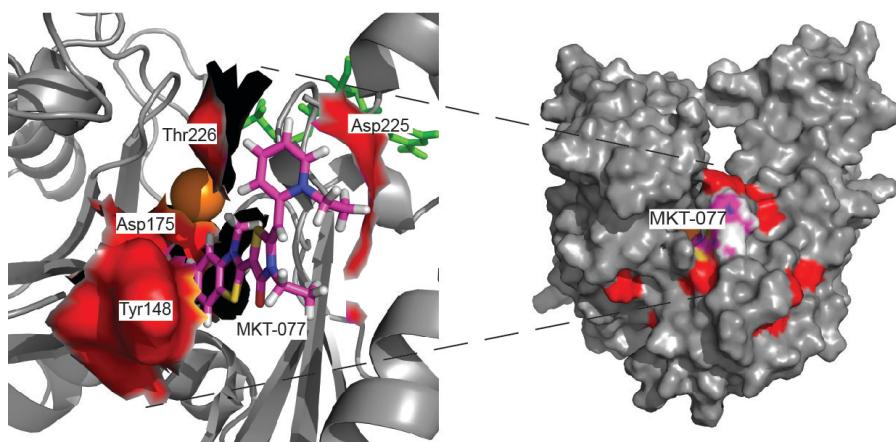
MKT-077 was first synthesized as rhodacyanine dye by Fuji Film and it was subsequently shown to have potent activity against multiple cancer cell lines (126-128). Interestingly, this compound had little toxicity against either fibroblasts or immortalized epithelial lines. This discrimination led to the testing of MKT-077 in Phase 1 clinical trials (129). The trial was halted due to renal magnesium wasting; however, enthusiasm for this chemical series remains strong and it represents one of the few Hsp70 inhibitors to advance to clinical trials.

MKT-077 exerts its anti-cancer activity by binding to Hsp70 (80). Specifically, NMR and docking studies have shown that the compound binds to a conserved allosteric pocket at the junction between subdomains IA and IIA of the NBD (130) (Figure 1.5.A). Although this pocket is adjacent to the ATP-binding cleft, MKT-077

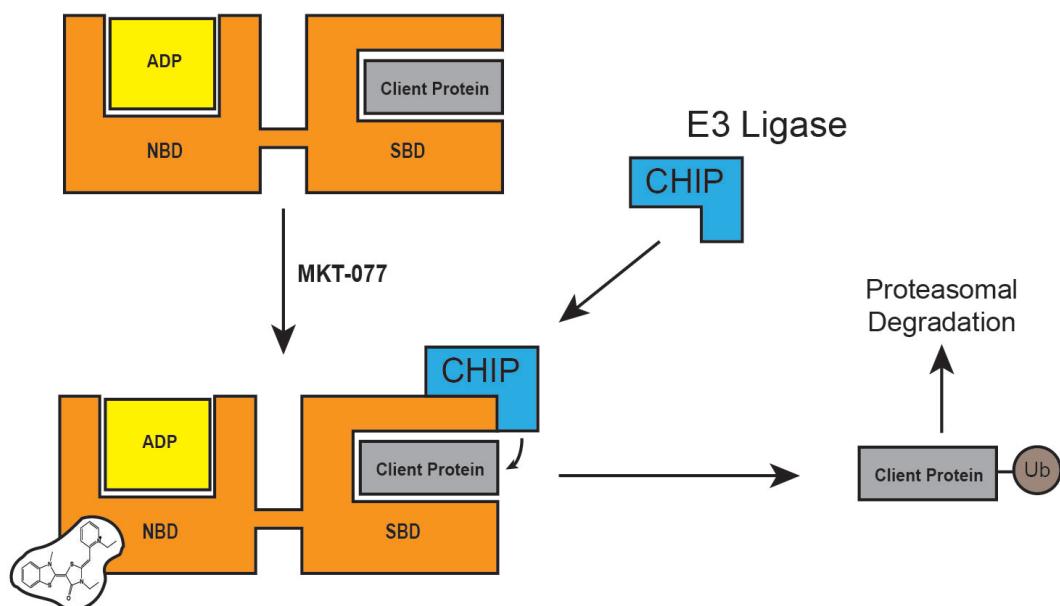
analogs do not compete for binding to nucleotide. Rather, binding of MKT-077 appears to trap the chaperone in the ADP conformation (79, 130), limiting client release (131) (Figure 1.5.B). Consistent with this idea, MKT-077 stabilizes binding of Hsp70 to model substrates *in vitro* (132, 133). While tighter binding of Hsp90 to clients might be expected to be protective, tighter binding of Hsp70 appears to destabilize Hsp70 clients, such as Akt (134). One possible mechanism for this activity is that prolonged dwell time of Hsp70 on clients may recruit effectors of the ubiquitin-proteasome system (135). Consistent with this idea, work in the nuclear hormone receptor system has suggested that Hsp70 is linked to client degradation, while Hsp90 is generally protective (136).

The mechanism by which MKT-077 and its analogs suppress growth of cancer cells is not yet clear. MKT-077 and its analogs appear to trigger apoptosis, as judged by microscopy and their effects on apoptotic clients (80, 137). However, Hsp70 has been implicated in senescence (3, 6, 79), cell cycle arrest (138, 139), and even necrosis (93). The exact mechanisms, their relative importance and the molecular details of the pathways have not been identified. In Chapters 2 - 4, I describe efforts towards developing more potent MKT-077 analogs and using them as probes to clarify these issues for the first time. The MKT-077 scaffold was chosen for this effort because it has the best-characterized binding site on Hsp70s and it has the most compelling selectivity in cells, compared to other Hsp70 inhibitors. Thus, we considered it the most promising scaffold for building the first chemical probes for this chaperone.

### A. MKT-077 Binds an Allosteric Pocket on Hsp70



### B. MKT-077 Inhibits Hsp70-Bag1 Interaction and Stabilizes ADP Conformation



**Figure 1.5 MKT-077 Allosterically Modulates Hsp70 Function.** (A). Left: MKT-077, shown in magenta, binds to an allosteric pocket on the NBD of Hsp70. Its pocket is guarded by charged residues while the interior remains significantly more hydrophobic. Right: Despite interacting close to the ATP-binding site, MKT-077 causes allosteric perturbations several angstroms away, as detected by NMR. Figure adapted from Rousaki et al. (B). MKT-077 stabilizes the ADP conformation, trapping the client protein in a high-affinity state for Hsp70, thereby recruiting members of the ubiquitin-proteasome system.

## 1.6 Combination Warfare

Most current chemotherapeutic regimens contain some permutation of vinblastine, cisplatin, methotrexate, prednisone, adriamycin, and any of several other cytotoxic drugs. Cocktails of these active agents are used to maximize therapeutic efficacy, while reducing toxicity, side effects and resistance. In this context, it is worth discussing how Hsp70 inhibitors, such as the ones reviewed here, might be used in rational combination with other compounds.

As described earlier, Hsp90 acts as a central hub in cancer cells, directing the fates of hundreds of signaling effectors (12, 34, 35). Thus, combining Hsp90 inhibitors with inhibitors of other pathways would be expected to be synergistic. Indeed, combining Hsp90 inhibitors with conventional chemotherapeutics or radiation therapy creates powerful regimens (140-142). Further, Hsp90 inhibitors have been shown to restore sensitivity in cell lines previously resistant to drugs such as tamoxifen, doxorubicin, or trastuzumab (40, 143, 144). Similarly, the idea of combining an Hsp90 inhibitor with a drug targeting Hsp70 has been suggested by many groups (10, 73). Hsp70's induction upon HSF1 release makes it an ideal target to complement Hsp90-mediated therapy. In fact, synergy has been later observed between Hsp90 inhibitors and either VER-155008 or MAL3-101 (70, 125, 145).

Another interesting combination approach may be to combine Hsp70 inhibitors with different mechanisms-of-action. As discussed above, targeting different sites on Hsp70 produces non-redundant outcomes. For example, ADD70 sensitizes cells while PES, VER-155008, and MKT-077 are actively cytotoxic. Thus combinations of these compounds might be expected to be synergistic. Further, compounds such as MAL3-

101 that target the Hsp70-Hsp40 complex might be synergistic with compounds that target other regions. In this way, the Hsp70 complex itself might provide a rich source of novel combinations. In Chapters 3 and 4, I explore these ideas using more potent, selective, and stable derivatives of MKT-077.

## 1.7 Conclusions and Future Directions

Cancer cells must cope with the stress of rapid division and high mutational rates, ultimately becoming “addicted” to chaperones. Thus, proteins within the protein quality control system, such as molecular chaperones, have become desirable drug targets. Inhibition of these systems is thought to disrupt multiple anti-death pathways at once (12, 146, 147), providing a powerful disruption of central signaling hubs.

In this backdrop, genetic studies have strongly implicated Hsp70 as a particularly good anti-cancer target, either as a stand-alone target or as a target that might impact resistance to other chemotherapeutics. (41-43). However, the suitability of Hsp70 as a drug target remains somewhat of an enigma. Part of the difficulty of “inhibiting” this system is that it isn’t yet clear what inhibition means or whether all types of inhibition will yield the same outcomes. As mentioned above, one goal might be to stabilize Hsp70 interactions with clients, potentially by targeting allostery in the chaperone. Hsp70 undergoes a complex series of dynamic conformational changes and engages in multiple protein-protein interactions with co-chaperones and client proteins. Further, it has broad cellular activity in protein folding, disaggregation, the assembly of multi-protein complexes, subcellular trafficking, protein turnover, antigen presentation and transcription (148-151). Current models suggest that these varied chaperone activities

arise from unique combinations of Hsp70 family members and their co-chaperones. Thus, the desired Hsp70 “target” may actually be a specific set of multiple proteins. This idea is illustrated by MAL3-101, which inhibits the Hsp70-Hsp40 combination (117, 118).

As this field develops, it will be interesting to know whether all types of inhibitors cause destabilization of oncogenic clients, as is the case for Hsp90 inhibitors. However, current data with compounds such as ADD70 suggest that Hsp70 may be a different type of target. We propose that each type of Hsp70 “inhibitor” might have unique capabilities, depending on what types of allosteric programs it interacts with. Thus, the suite of Hsp70 inhibitors is likely to include numerous molecules with non-redundant mechanisms of action.

To this end, emerging data suggests that Hsp70 may not be acting purely to oppose apoptosis, but more generally as a pro-survival factor, acting as a “back-up” to Hsp90 in maintaining the oncogenic proteome. Thus, the combination of Hsp70 inhibitors with molecules targeting Hsp90, the proteasome, and other protein quality control pathways promises to be a powerful approach towards safer and more effective chemotherapeutic regimens.

In this dissertation, several of these key questions are addressed. In Chapter 2, I describe the development of more potent and stable Hsp70 inhibitors, improving on the MKT-077 scaffold. In Chapters 3 and 4, I use these tool compounds to reveal the roles Hsp70 plays in maintaining the cancer phenotype. Finally, in Chapter 5, I discuss future studies to explore Hsp70 as a hub of cancer cell signaling, and the impact of adding these inhibitors to chemotherapeutic regimens.

## **Notes**

This work has been, in part, submitted as an invited review to Current Topics in Medicinal Chemistry entitled “Allosteric Inhibitors of Hsp70: Drugging the Second Chaperone of Tumorigenesis.” Sharan R Srinivasan, Xiaokai Li, and Jason E. Gestwicki contributed intellectually to this review.

## 1.8 References

1. M. Ferrarini, S. Heltai, M. R. Zocchi, C. Rugarli, *Int J Cancer* **51**, 613 (Jun 19, 1992).
2. T. M. Gress *et al.*, *Cancer Res* **54**, 547 (Jan 15, 1994).
3. J. A. Yaglom, V. L. Gabai, M. Y. Sherman, *Cancer Res* **67**, 2373 (Mar 1, 2007).
4. M. Yano, Z. Naito, S. Tanaka, G. Asano, *Jpn J Cancer Res* **87**, 908 (Sep, 1996).
5. V. L. Gabai, K. R. Budagova, M. Y. Sherman, *Oncogene* **24**, 3328 (May 5, 2005).
6. V. L. Gabai, J. A. Yaglom, T. Waldman, M. Y. Sherman, *Mol Cell Biol* **29**, 559 (Jan, 2009).
7. A. Kamal *et al.*, *Nature* **425**, 407 (Sep 25, 2003).
8. C. Jolly, R. I. Morimoto, *J Natl Cancer Inst* **92**, 1564 (Oct 4, 2000).
9. J. Luo, N. L. Solimini, S. J. Elledge, *Cell* **136**, 823 (Mar 6, 2009).
10. A. R. Goloudina, O. N. Demidov, C. Garrido, *Cancer Lett* **325**, 117 (Dec 28, 2012).
11. J. I. Leu, J. Pimkina, A. Frank, M. E. Murphy, D. L. George, *Mol Cell* **36**, 15 (Oct 9, 2009).
12. J. Trepel, M. Mollapour, G. Giaccone, L. Neckers, *Nat Rev Cancer* **10**, 537 (Aug, 2010).
13. L. H. Pearl, C. Prodromou, *Annu Rev Biochem* **75**, 271 (2006).
14. K. A. Krukenberg, T. O. Street, L. A. Lavery, D. A. Agard, *Q Rev Biophys* **44**, 229 (May, 2011).
15. D. R. Southworth, D. A. Agard, *Mol Cell* **32**, 631 (Dec 5, 2008).
16. J. R. Porter, C. C. Fritz, K. M. Depew, *Curr Opin Chem Biol* **14**, 412 (Jun, 2010).
17. T. W. Schulte *et al.*, *Cell Stress Chaperones* **3**, 100 (Jun, 1998).
18. T. W. Schulte, L. M. Neckers, *Cancer Chemother Pharmacol* **42**, 273 (1998).
19. L. Whitesell, E. G. Mimnaugh, B. De Costa, C. E. Myers, L. M. Neckers, *Proc Natl Acad Sci U S A* **91**, 8324 (Aug 30, 1994).
20. A. Donnelly, B. S. Blagg, *Curr Med Chem* **15**, 2702 (2008).
21. J. D. Eskew *et al.*, *BMC Cancer* **11**, 468 (2011).
22. C. A. Patwardhan *et al.*, *J Biol Chem* **288**, 7313 (Mar 8, 2013).
23. G. Pimienta, K. M. Herbert, L. Regan, *Mol Pharm* **8**, 2252 (Dec 5, 2011).
24. T. Zhang *et al.*, *Mol Cancer Ther* **7**, 162 (Jan, 2008).
25. A. Zuehlke, J. L. Johnson, *Biopolymers* **93**, 211 (Mar, 2010).
26. J. Li, J. Soroka, J. Buchner, *Biochim Biophys Acta* **1823**, 624 (Mar, 2012).
27. R. C. Vasko *et al.*, *ACS Med Chem Lett* **1**, 4 (Jan 1, 2010).
28. V. C. da Silva, C. H. Ramos, *J Proteomics* **75**, 2790 (Jun 6, 2012).
29. R. S. Samant, P. A. Clarke, P. Workman, *Cell Cycle* **11**, 1301 (Apr 1, 2012).
30. M. Taipale, D. F. Jarosz, S. Lindquist, *Nat Rev Mol Cell Biol* **11**, 515 (Jul, 2010).
31. M. Taipale *et al.*, *Cell* **150**, 987 (Aug 31, 2012).
32. P. Connell *et al.*, *Nat Cell Biol* **3**, 93 (Jan, 2001).
33. W. Xu *et al.*, *Proc Natl Acad Sci U S A* **99**, 12847 (Oct 1, 2002).
34. L. H. Pearl, C. Prodromou, P. Workman, *Biochem J* **410**, 439 (Mar 15, 2008).
35. L. Whitesell, S. L. Lindquist, *Nat Rev Cancer* **5**, 761 (Oct, 2005).
36. N. I. o. Health, N. C. Institute. (Bethesda, 2013), vol. 2013.
37. K. Helmbrecht, E. Zeise, L. Rensing, *Cell Prolif* **33**, 341 (Dec, 2000).

38. A. L. Joly, G. Wettstein, G. Mignot, F. Ghiringhelli, C. Garrido, *J Innate Immun* **2**, 238 (2010).
39. K. Jhaveri, S. Modi, *Adv Pharmacol* **65**, 471 (2012).
40. X. Lu, L. Xiao, L. Wang, D. M. Ruden, *Biochem Pharmacol* **83**, 995 (Apr 15, 2012).
41. N. Gaspar et al., *Cancer Res* **69**, 1966 (Mar 1, 2009).
42. S. Kummar et al., *Eur J Cancer* **46**, 340 (Jan, 2010).
43. J. E. Lancet et al., *Leukemia* **24**, 699 (Apr, 2010).
44. S. Pacey et al., *Clin Cancer Res* **17**, 1561 (Mar 15, 2011).
45. J. Madrigal-Matute et al., *Cardiovasc Res* **86**, 330 (May 1, 2010).
46. R. Rao et al., *Cancer Biol Ther* **8**, 1273 (Jul, 2009).
47. J. Zou, Y. Guo, T. Guettouche, D. F. Smith, R. Voellmy, *Cell* **94**, 471 (Aug 21, 1998).
48. E. B. Bertelsen, L. Chang, J. E. Gestwicki, E. R. Zuiderweg, *Proc Natl Acad Sci U S A* **106**, 8471 (May 26, 2009).
49. A. J. Massey, *J Med Chem* **53**, 7280 (Oct 28, 2010).
50. P. Bork, C. Sander, A. Valencia, *Proc Natl Acad Sci U S A* **89**, 7290 (Aug 15, 1992).
51. H. Wang et al., *Biochemistry* **37**, 7929 (Jun 2, 1998).
52. R. G. Smock et al., *Mol Syst Biol* **6**, 414 (Sep 21, 2010).
53. J. F. Swain et al., *Mol Cell* **26**, 27 (Apr 13, 2007).
54. E. R. Zuiderweg et al., *Top Curr Chem* **328**, 99 (2013).
55. Y. Zhang, E. R. Zuiderweg, *Proc Natl Acad Sci U S A* **101**, 10272 (Jul 13, 2004).
56. A. Ahmad et al., *Proc Natl Acad Sci U S A* **108**, 18966 (Nov 22, 2011).
57. M. P. Mayer et al., *Nat Struct Biol* **7**, 586 (Jul, 2000).
58. M. Vogel, M. P. Mayer, B. Bukau, *J Biol Chem* **281**, 38705 (Dec 15, 2006).
59. H. Sondermann et al., *Science* **291**, 1553 (Feb 23, 2001).
60. F. H. Liu, S. J. Wu, S. M. Hu, C. D. Hsiao, C. Wang, *J Biol Chem* **274**, 34425 (Nov 26, 1999).
61. G. C. Meacham et al., *EMBO J* **18**, 1492 (Mar 15, 1999).
62. J. S. Liu et al., *J Biol Chem* **273**, 30704 (Nov 13, 1998).
63. A. J. Caplan, D. M. Cyr, M. G. Douglas, *Cell* **71**, 1143 (Dec 24, 1992).
64. A. T. Gillies, R. Taylor, J. E. Gestwicki, *Mol Biosyst* **8**, 2901 (Nov, 2012).
65. H. H. Kampinga, E. A. Craig, *Nat Rev Mol Cell Biol* **11**, 579 (Aug, 2010).
66. S. Rudiger, L. Germeroth, J. Schneider-Mergener, B. Bukau, *EMBO J* **16**, 1501 (Apr 1, 1997).
67. X. Zhu et al., *Science* **272**, 1606 (Jun 14, 1996).
68. S. R. Srinivasan, A. T. Gillies, L. Chang, A. D. Thompson, J. E. Gestwicki, *Mol Biosyst* **8**, 2323 (Sep, 2012).
69. J. Koren, 3rd et al., *J Biol Chem* **285**, 2498 (Jan 22, 2010).
70. A. J. Massey et al., *Cancer Chemother Pharmacol* **66**, 535 (Aug, 2010).
71. L. Meng, C. Hunt, J. A. Yaglom, V. L. Gabai, M. Y. Sherman, *Oncogene* **30**, 2836 (Jun 23, 2011).
72. M. V. Powers, P. A. Clarke, P. Workman, *Cancer Cell* **14**, 250 (Sep 9, 2008).
73. M. V. Powers et al., *Cell Cycle* **9**, 1542 (Apr 15, 2010).
74. D. Walerych et al., *Oncogene* **28**, 4284 (Dec 3, 2009).

75. M. Jaattela, *Ann Med* **31**, 261 (Aug, 1999).
76. Q. Ran *et al.*, *Biochem Biophys Res Commun* **275**, 174 (Aug 18, 2000).
77. W. J. Lu *et al.*, *Cell Death Differ* **18**, 1046 (Jun, 2011).
78. M. R. Knittler, S. Dirks, I. G. Haas, *Proc Natl Acad Sci U S A* **92**, 1764 (Feb 28, 1995).
79. C. C. Deocaris *et al.*, *Cancer Lett* **252**, 259 (Jul 18, 2007).
80. R. Wadhwa *et al.*, *Cancer Res* **60**, 6818 (Dec 15, 2000).
81. J. Nylandsted, K. Brand, M. Jaattela, *Ann N Y Acad Sci* **926**, 122 (2000).
82. M. Rohde *et al.*, *Genes Dev* **19**, 570 (Mar 1, 2005).
83. K. Nanbu *et al.*, *Cancer Detect Prev* **22**, 549 (1998).
84. D. R. Ciocca, S. K. Calderwood, *Cell Stress Chaperones* **10**, 86 (Summer, 2005).
85. M. Jaattela, D. Wissing, *J Exp Med* **177**, 231 (Jan 1, 1993).
86. F. Chalmin *et al.*, *J Clin Invest* **120**, 457 (Feb, 2010).
87. H. S. Park, J. S. Lee, S. H. Huh, J. S. Seo, E. J. Choi, *EMBO J* **20**, 446 (Feb 1, 2001).
88. A. Saleh, S. M. Srinivasula, L. Balkir, P. D. Robbins, E. S. Alnemri, *Nat Cell Biol* **2**, 476 (Aug, 2000).
89. L. Ravagnan *et al.*, *Nat Cell Biol* **3**, 839 (Sep, 2001).
90. F. Guo *et al.*, *Blood* **105**, 1246 (Feb 1, 2005).
91. T. Gotoh, K. Terada, S. Oyadomari, M. Mori, *Cell Death Differ* **11**, 390 (Apr, 2004).
92. A. R. Stankiewicz, G. Lachapelle, C. P. Foo, S. M. Radicioni, D. D. Mosser, *J Biol Chem* **280**, 38729 (Nov 18, 2005).
93. A. E. Kabakov, V. L. Gabai, *Exp Cell Res* **217**, 15 (Mar, 1995).
94. G. M. Balaburski *et al.*, *Mol Cancer Res* **11**, 219 (Mar, 2013).
95. J. I. Leu, J. Pimkina, P. Pandey, M. E. Murphy, D. L. George, *Mol Cancer Res* **9**, 936 (Jul, 2011).
96. M. Kaiser *et al.*, *Blood Cancer J* **1**, e28 (Jul, 2011).
97. R. Schlecht *et al.*, *PLoS One* **8**, e78443 (2013).
98. E. Schmitt *et al.*, *Cancer Res* **66**, 4191 (Apr 15, 2006).
99. H. Umezawa *et al.*, *J Antibiot (Tokyo)* **34**, 1622 (Dec, 1981).
100. K. Nishikawa *et al.*, *J. Antibiot.* **39**, 1461 (1986).
101. K. Nemoto *et al.*, *J. Antibiot.* **40**, 1193 (1987).
102. K. Nemoto *et al.*, *J. Antibiot.* **40**, 1448 (1987).
103. R. Nishizawa *et al.*, *J. Antibiot.* **41**, 1629 (1988).
104. L. Lebreton, J. Annat, P. Derrepas, P. Dutartre, P. Renaut, *J. Med. Chem.* **42**, 277 (1999/01/01, 1999).
105. N. R. Krieger, S. Emre, *Pediatr. Transplant.* **8**, 594 (2004).
106. D. B. Kaufman *et al.*, *Transplant. Rev.* **10**, 160 (1996).
107. S. G. Nadler *et al.*, *Biochem. Biophys. Res. Commun.* **253**, 176 (Dec 9, 1998).
108. S. G. Nadler, M. A. Tepper, B. Schacter, C. E. Mazzucco, *Science* **258**, 484 (Oct 16, 1992).
109. Y. Umeda *et al.*, *J. Antibiot.* **40**, 1303 (1987).
110. K. A. Havlin *et al.*, *Anti-Cancer Drugs* **6**, 229 (1995).
111. D. R. Williams, S. K. Ko, S. Park, M. R. Lee, I. Shin, *Angew Chem Int Ed Engl* **47**, 7466 (2008).

112. H. J. Cho *et al.*, *J Am Chem Soc* **133**, 20267 (Dec 21, 2011).
113. D. S. Williamson *et al.*, *J Med Chem* **52**, 1510 (Mar 26, 2009).
114. A. T. Macias *et al.*, *J Med Chem* **54**, 4034 (Jun 23, 2011).
115. C. G. Evans, L. Chang, J. E. Gestwicki, *J Med Chem* **53**, 4585 (Jun 24, 2010).
116. V. A. Assimon, A. T. Gillies, J. N. Rauch, J. E. Gestwicki, *Curr Pharm Des* **19**, 404 (2013).
117. S. W. Fewell *et al.*, *J Biol Chem* **279**, 51131 (Dec 3, 2004).
118. S. Wisen *et al.*, *ACS Chem Biol* **5**, 611 (Jun 18, 2010).
119. U. K. Jinwal *et al.*, *J Neurosci* **29**, 12079 (Sep 30, 2009).
120. S. Wisen, J. Androsavich, C. G. Evans, L. Chang, J. E. Gestwicki, *Bioorg Med Chem Lett* **18**, 60 (Jan 1, 2008).
121. L. Chang *et al.*, *Anal Biochem* **372**, 167 (Jan 15, 2008).
122. G. Schlenstedt, S. Harris, B. Risse, R. Lill, P. A. Silver, *J Cell Biol* **129**, 979 (May, 1995).
123. S. Nishikawa, T. Endo, *J Biol Chem* **272**, 12889 (May 16, 1997).
124. I. Sadler *et al.*, *J Cell Biol* **109**, 2665 (Dec, 1989).
125. M. J. Braunstein *et al.*, *J Oncol* **2011**, 232037 (2011).
126. Y. Chiba *et al.*, *Anticancer Res* **18**, 1047 (Mar-Apr, 1998).
127. Y. Chiba *et al.*, *J Surg Oncol* **69**, 105 (Oct, 1998).
128. K. Koya *et al.*, *Cancer Res* **56**, 538 (Feb 1, 1996).
129. C. D. Britten *et al.*, *Clin Cancer Res* **6**, 42 (Jan, 2000).
130. A. Rousaki *et al.*, *J Mol Biol* **411**, 614 (Aug 19, 2011).
131. J. Abisambra *et al.*, *Biol Psychiatry*, (Apr 19, 2013).
132. A. M. Wang *et al.*, *Nat Chem Biol* **9**, 112 (Feb, 2013).
133. Y. Miyata *et al.*, *ACS Chem Neurosci*, (Mar 20, 2013).
134. J. Koren, 3rd *et al.*, *PLoS One* **7**, e35566 (2012).
135. Y. Miyata, J. Koren, J. Kiray, C. A. Dickey, J. E. Gestwicki, *Future Med Chem* **3**, 1523 (Sep, 2011).
136. W. B. Pratt, Y. Morishima, H. M. Peng, Y. Osawa, *Exp Biol Med (Maywood)* **235**, 278 (Mar, 2010).
137. X. Li, S. R. Srinivasan, Z. T. Young, D. Sun, J. E. Gestwicki, *ACS Med Chem Lett*, (2013).
138. J. Karlseder *et al.*, *Biochem Biophys Res Commun* **220**, 153 (Mar 7, 1996).
139. S. Iordanskiy *et al.*, *J Virol* **78**, 9697 (Sep, 2004).
140. A. V. McNamara, M. Barclay, A. J. Watson, J. R. Jenkins, *Biochem Pharmacol* **83**, 355 (Feb 1, 2012).
141. L. Stingl *et al.*, *Br J Cancer* **102**, 1578 (May 25, 2010).
142. Z. A. Wainberg *et al.*, *Mol Cancer Ther* **12**, 509 (Apr, 2013).
143. M. Tatokoro *et al.*, *Int J Cancer* **131**, 987 (Aug 15, 2012).
144. H. Zhang *et al.*, *Int J Cancer* **126**, 1226 (Mar 1, 2010).
145. E. L. Davenport *et al.*, *Leukemia* **24**, 1804 (Oct, 2010).
146. L. J. Crawford, B. Walker, A. E. Irvine, *J Cell Commun Signal* **5**, 101 (Jun, 2011).
147. R. Mathew, V. Karantza-Wadsworth, E. White, *Nat Rev Cancer* **7**, 961 (Dec, 2007).
148. B. Bercovich *et al.*, *J Biol Chem* **272**, 9002 (Apr 4, 1997).
149. J. Frydman, *Annu Rev Biochem* **70**, 603 (2001).

150. F. U. Hartl, A. Bracher, M. Hayer-Hartl, *Nature* **475**, 324 (Jul 21, 2011).
151. N. Kettern, C. Rogon, A. Limmer, H. Schild, J. Hohfeld, *PLoS One* **6**, e16398 (2011).

## Chapter 2

### Development of a Tool to Probe Hsp70 Function: Synthetic Derivatives of the Allosteric Modulator, MKT-077.

#### 2.1 Abstract

The rhodacyanine, MKT-077, has anti-proliferative activity against cancer cell lines through its ability to inhibit members of the heat shock protein 70 (Hsp70) family of molecular chaperones. However, MKT-077 is rapidly metabolized ( $t_{1/2} < 5$  min) and is only moderately efficacious ( $EC_{50} \sim 2$   $\mu$ M), which limits its use as either a chemical probe or potential therapeutic. In this chapter, we report the design, synthesis and characterization of MKT-077 analogs. This work resulted in compound **30** (JG-98), a derivative with an improved microsomal half-life ( $t_{1/2} = 37$  min) and increased potency against the breast cancer cell lines MDA-MB-231 and MCF-7 ( $EC_{50}$  values of  $0.4 \pm 0.03$   $\mu$ M and  $0.7 \pm 0.2$ , respectively). Additionally, JG-98 bound Hsc70 *in vitro* with an affinity over 60 times that of MKT-077 and NMR titration experiments confirmed that JG-98 binds to the conserved allosteric site on Hsp70. These studies advance MKT-077 analogs as the first chemical probes for studying Hsp70's roles in cancer (described in Chapters 3 and 4).

## **2.2 Introduction**

### **2.2.1 Targeting Hsp70 in Cancer**

As outlined in Chapter 1, heat shock protein 70 (Hsp70) is an ATP-dependent molecular chaperone that plays essential roles in protein homeostasis (1, 2). There are thirteen members of the Hsp70 family in mammals (3), including the constitutively expressed, cytosolic heat shock cognate 70 (Hsc70, HSPA8), the stress-inducible, cytoplasmic heat shock protein 70 (Hsp72, HSPA1) and the mitochondrial Hsp70 (mtHsp70, mot-2, HSPA9). These chaperones bind and stabilize a wide array of proteins, including many kinases and transcription factors involved in pro-survival signaling (2, 4, 5). Accordingly, high levels of Hsp70 activity are associated with immortalized cells and poor prognosis in multiple types of cancer, including breast cancer, endometrial cancer and cervical cancer (6). Many cancer cells appear to become “addicted” to these high chaperone levels and activity, as dual silencing of Hsc70 and Hsp72 leads to cell death only in tumor cells, but not normal fibroblasts (7). For these reasons, Hsp70 has emerged as a potential target for anti-cancer agents (1). Inhibition of Hsp70s might be especially powerful in combination with other chemotherapies because the levels of Hsp72 are dramatically increased after exposure to other therapies, such as Hsp90 inhibitors (8), proteasome inhibitors (9), and radiation (10). However, small molecules targeting Hsp70 have lagged in development, in part, due to its high affinity for ATP (11), making inhibitors against this pocket lackluster. As such, we have decided to pursue an alternative strategy.

## **2.2.2 MKT-077: Allosteric Inhibitor of Hsp70**

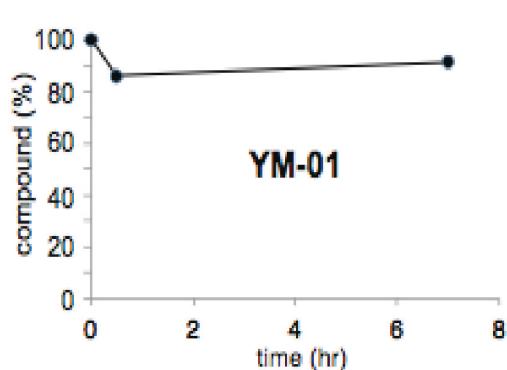
MKT-077 is a cationic rhodacyanine that was originally developed as a dye and later found to have promising anti-proliferative activity against numerous cancer cell lines, including those derived from bladder carcinomas, colon carcinomas, breast carcinomas, melanomas, and pancreatic carcinomas (12-14). This activity led MKT-077 to be advanced to Phase I clinical trials against solid tumors. However, it was abandoned due to renal toxicity, specifically magnesium wasting (15). More recently, Wadhwa and colleagues revisited the scaffold and used a biotinylated version of MKT-077 to show that this compound derives its anti-cancer activity by binding to members of the Hsp70 family, including Hsc70 and mtHsp70 (14, 16, 17). Subsequent work showed that a portion of the anti-proliferative activity of MKT-077 involved release of the tumor suppressor, p53, from mtHsp70 (14). Recently, we used NMR to discover that MKT-077 binds Hsc70 at an allosteric site within the nucleotide-binding domain (NBD) (18). Interestingly, this site is conserved in the major Hsp70 family members, including Hsp72 and mtHsp70, and it appears to be important in the chaperone's ATP hydrolysis cycle because an analog of MKT-077, YM-01, disrupts nucleotide turnover (16, 18). Treatment with YM-01 leads to degradation of the chaperone "client" Akt1 in tamoxifen-resistant MCF7 breast cancer cells (19), suggesting that binding to this allosteric site also disrupts the cellular functions of Hsp70s, at least in cancer cells. However, MKT-077 and YM-01 are not toxic to fibroblasts or normal epithelial cells (20). Thus, MKT-077 only appears to be toxic to the cells that are "addicted" to elevated levels and activity of chaperones, reminiscent of the mechanisms that are commonly invoked for Hsp90 and proteasome inhibitors (21, 22). Unfortunately, MKT-077's rapid metabolism

has limited its use, making it difficult to employ as a tool compound in cells or animals. Here we describe efforts to improve upon MKT-077 to develop a tool compound to probe Hsp70's oncogenic activity.

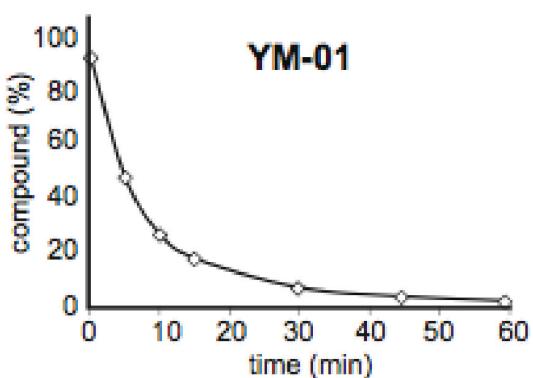
### **2.3 Results and Discussion**

To better understand MKT-077 metabolism, our group first incubated a close analog, YM-01, with mouse liver microsomes and confirmed that it is susceptible to rapid metabolism, with a lifetime of only  $4.4 \pm 1.0$  min (23). LC-MS/MS analysis of the treated YM-01 revealed that the benzothiazole and pyridinium rings were the major sites of P<sub>450</sub> oxidation (24) (Figure 2.1).

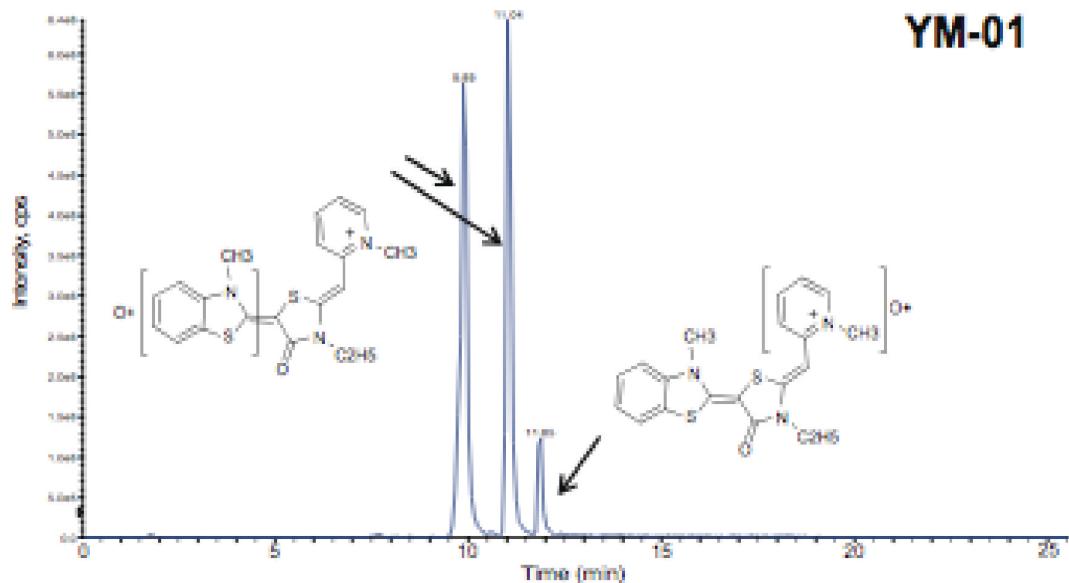
### A. YM-01 is stable in water



### B. YM-01 is Rapidly Metabolized



### C. LC-MS Analysis of YM-01 Metabolites Reveals Vulnerable Sites



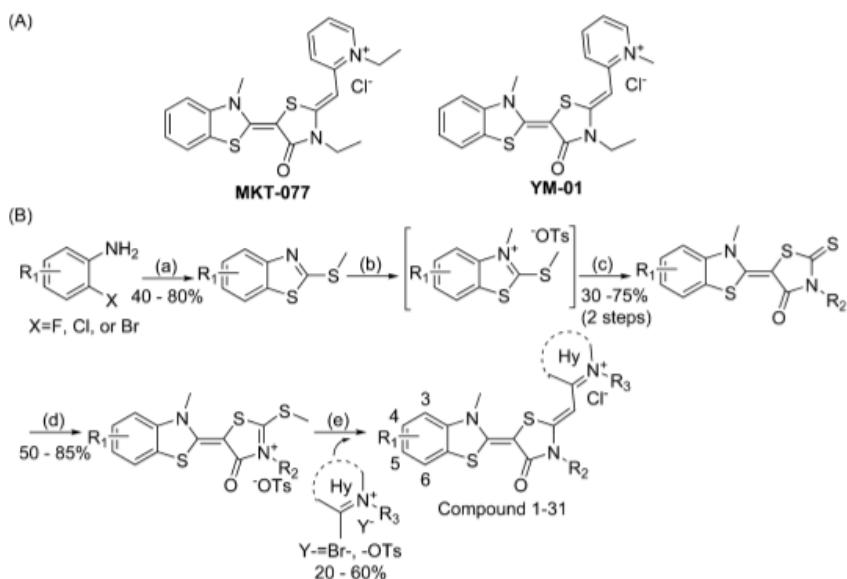
**Figure 2.1. Characterization of YM-01 Stability and Metabolism.**

(A) YM-01 is relatively stable in water, determined by LC-MS. (B) YM-01 is rapidly degraded in liver microsomes. (C) Metabolite analysis by LC-MS/MS reveals preferred sites for CYP450 oxidation. Figure adapted from Miyata et al (ACS Chem Neurosci. 2013).

### 2.3.1 General Synthetic Approach

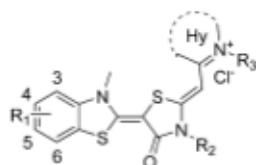
Guided by those studies, a postdoctoral fellow in the group, Xiaokai Li, used a previously reported synthetic route (Scheme 2.1) (16) to generate MKT-077 analogs that are designed to remove these metabolic liabilities. Briefly, the syntheses started with cyclization of substituted anilines with potassium ethyl xanthate, followed by methylation of the cyclized products by iodomethane under mild basic conditions. The resulting benzothiazoles were activated by methyl *p*-toluenesulfonate, and coupled with a series of *N*-substituted rhodanines. These products were methylated by methyl *p*-toluenesulfonate, followed by another coupling with an activated heterocycle. The products were dissolved in methanol and passed through a chloride exchange column to generate the final compounds **1-31** in overall yields between 20% and 40% (Table 2.1).

**Scheme 1. Synthetic Route to MKT-077 and Analogues<sup>a</sup>**



<sup>a</sup>(A) Chemical structures of MKT-077 and YM-01. (B) General synthetic route to analogues. (a) 1. Potassium ethyl xanthate, DMF, 4 h, 125 °C; 2. methyl iodide, triethyl- amine, ethanol, 1 h, 80 °C; (b) methyl *p*-toluenesulfonate, anisole, 125 °C; (c) *N*-substituted rhodanine, triethylamine, acetonitrile, 4 h, 25 °C; (d) methyl *p*-toluenesulfonate, DMF, 3 h, 135 °C; (e) 1. triethylamine, acetonitrile, 3 h, 80 °C, 2. Cl-ion exchange column.

**Table 1. Summary of the Antiproliferative Activities and Microsomal Stabilities of MKT-077 and Its Derivatives<sup>a</sup>**



compd	R1	R2	R3	Hy	MDA-MB-231 EC <sub>50</sub> (μM)	MCF-7 EC <sub>50</sub> (μM)	MEF <sup>b</sup> EC <sub>50</sub> (μM)	microsome stability t <sub>1/2</sub> (min)
MKT-077	H	ethyl	ethyl	2-pyridyl	1.4 ± 0.2	2.2 ± 0.2	>50	<5
YM-01	H	ethyl	methyl	2-pyridyl	2.0 ± 0.2	5.2 ± 0.8	>50	<5
1	3-F	ethyl	methyl	2-pyridyl	4.0 ± 0.4	2.2 ± 0.4	>50	15
2	4-F	ethyl	methyl	2-pyridyl	7.7 ± 0.6	18 ± 4.4	>50	23
3	5-F	ethyl	methyl	2-pyridyl	5.6 ± 0.4	6.2 ± 0.8	>50	7.7
4	6-F	ethyl	methyl	2-pyridyl	6.2 ± 1.0	8.5 ± 3.0	>50	16
5	3-F	ethyl	ethyl	2-pyridyl	3.8 ± 0.2	1.0 ± 0.3	>30	NT <sup>c</sup>
6	4-F	ethyl	ethyl	2-pyridyl	13 ± 0.7	2.4 ± 0.6	>30	NT
7	5-F	ethyl	ethyl	2-pyridyl	8.3 ± 1.1	0.9 ± 0.3	>30	NT
8	6-F	ethyl	ethyl	2-pyridyl	2.8 ± 0.2	0.8 ± 0.1	27 ± 3.0	NT
9	3-Cl	ethyl	methyl	2-pyridyl	4.4 ± 0.6	7.8 ± 1.8	>50	NT
10	4-Cl	ethyl	methyl	2-pyridyl	4.6 ± 0.4	28 ± 4.3	>50	NT
11	5-Cl	ethyl	methyl	2-pyridyl	8.3 ± 0.7	9.5 ± 3.1	>50	8.2
12	6-Cl	ethyl	methyl	2-pyridyl	4.1 ± 0.5	5.2 ± 1.0	>50	NT
13	4-OMe	ethyl	methyl	2-pyridyl	2.8 ± 0.4	14 ± 2.6	>50	NT
14	5-OMe	ethyl	methyl	2-pyridyl	4.0 ± 0.4	11 ± 2.2	>50	10
15	4-CF <sub>3</sub>	ethyl	methyl	2-pyridyl	21 ± 4.5	>30	>50	NT
16	5-CF <sub>3</sub>	ethyl	methyl	2-pyridyl	19 ± 2.9	>30	>50	NT
17	H	allyl	methyl	2-pyridyl	3.6 ± 0.4	8.4 ± 2.4	>50	NT
18	H	benzyl	methyl	2-pyridyl	1.8 ± 0.3	2.9 ± 0.6	>50	NT
19	H	ethyl	propyl	2-pyridyl	1.6 ± 0.2	2.8 ± 0.8	>50	NT
20	H	ethyl	benzyl	2-pyridyl	1.0 ± 0.2	1.5 ± 0.2	>50	NT
21	H	ethyl	2-hydroxyethyl	2-pyridyl	24 ± 3.3	6.9 ± 2.1	>50	NT
22	H	ethyl	methyl	4-pyridyl	1.7 ± 0.1	19 ± 4.9	>50	NT
23	H	ethyl	methyl	2-pyrimidinyl	5.8 ± 0.8	9.1 ± 1.6	>50	NT
24	H	ethyl	methyl	2-thiazolyl	5.1 ± 0.3	0.7 ± 0.1	>50	NT
25	H	ethyl	ethyl	2-thiazolyl	2.2 ± 0.2	0.8 ± 0.1	>30	NT
26	H	ethyl	benzyl	2-thiazolyl	0.5 ± 0.1	0.6 ± 0.04	6.8 ± 0.4	NT
27 (JG-83)	3-F	ethyl	benzyl	2-thiazolyl	0.4 ± 0.03	1.0 ± 0.2	8.3 ± 0.7	NT
28 (JG-84)	3-Cl	ethyl	benzyl	2-thiazolyl	0.4 ± 0.02	0.8 ± 0.2	7.0 ± 0.6	8.8
29	4-Cl	ethyl	benzyl	2-thiazolyl	0.6 ± 0.1	0.8 ± 0.2	>30	NT
30 (JG-98)	5-Cl	ethyl	benzyl	2-thiazolyl	0.4 ± 0.03	0.7 ± 0.2	24 ± 1.3	37
31	6-Cl	ethyl	benzyl	2-thiazolyl	0.5 ± 0.1	1.0 ± 0.8	>30	NT

<sup>a</sup>Antiproliferative activity was measured using MTT assays. Results are the average of experiments performed in triplicate, and error is SEM. For stability studies, compounds (1 μM) were incubated with mouse liver microsomes and degradation monitored by LC-MS (see the Supporting Information). <sup>b</sup>From C57BL/6 mice. <sup>c</sup>Not tested or poorly soluble.

### 2.3.2 Substitutions on the Benzothiazole Ring Improve Metabolic Stability

To find an analog of MKT-077 with better potential as a chemical probe for studying Hsp70's roles in cancer, MKT-077, YM-01 and compounds **1-31** were evaluated for anti-proliferative activity in MDA-MB-231 and MCF7 breast cancer cells using MTT assays (25). In addition, their activity against normal mouse embryonic fibroblasts (MEFs) was also measured. We would expect that a useful MKT-077 analog would be less toxic to untransformed cells. Similarly, the stability of the compounds in the presence of mouse liver microsomes was recorded to identify analogs more stable than MKT-077.

Using these tests, MKT-077 had EC<sub>50</sub> values of 1.4 ± 0.2 µM and 2.2 ± 0.2 µM against the breast cancer cells (Table 2.1). YM-01, which differs from MKT-077 only in the methyl substitution in the R<sub>3</sub> position, was only slightly weaker (EC<sub>50</sub> values of 2.0 ± 0.2 and 5.2 ± 0.8 µM). Consistent with the literature, neither molecule was toxic to MEFs (EC<sub>50</sub> > 50 µM) and both had short half-lives in microsomes (t<sub>1/2</sub> < 5 min). Guided by the earlier metabolic studies (Figure 2.1), we found introducing a fluorine in the 3, 4, 5 or 6 positions of the benzothiazole (R<sub>1</sub>) of YM-01 (compounds **1-4**) significantly improved stability as measured by liver microsomes (t<sub>1/2</sub> = 15, 23, 7.7 and 16 min, respectively). However, these substitutions also reduced potency (EC<sub>50</sub> values between 2.2 and 18 µM). When the same fluorinations were introduced in the context of the ethyl modification at the R<sub>3</sub> position (compound **5-8**), potency was only slightly improved (EC<sub>50</sub> between 0.8 and 13 µM). Replacing fluorines for chlorines in the YM-01 scaffold (compounds **9-12**) did not improve potency and, in fact, further increased the EC<sub>50</sub> values to between 4.1 and 28 µM. Similarly, placing trifluoromethyl or methoxy groups

at positions 4' and 5' (compounds **13-16**) decreased activity to between 3 and more than 30  $\mu\text{M}$  (Table 2.1). Together, these results suggested that electrophilic substitutions on the benzothiazole protected against metabolism, but only smaller moieties were tolerated. Larger groups reduced anti-proliferative activity, likely due to steric constraints imposed on the benzothiazole ring by the binding pocket of Hsp70 (see below).

### 2.3.3 Hydrophobic Functionalization of the Pyridinium Ring Improves Potency

In an attempt to increase the potency of this series, we made substitutions at the central rhodanine ring ( $R_2$ ) and the *N*-substitutions of the pyridinium ( $R_3$ ). Replacing the  $R_2$  ethyl group of MKT-077 with an allyl or benzyl group (compounds **17** and **18**) did not substantially affect activity against either cell line. Likewise, replacing the ethyl moiety of the pyridinium ( $R_3$ ) with a propyl group (compound **19**) did not significantly improve potency ( $\text{EC}_{50}$  values  $2.8 \pm 0.8$  and  $1.6 \pm 0.2 \mu\text{M}$  against MCF7 and MDA-MB-231 cells, respectively). However, increasing the size of this substituent to a benzyl group (compound **20**) improved activity by approximately 2-fold ( $\text{EC}_{50}$  values of  $1.5 \pm 0.2$  and  $1.0 \pm 0.2 \mu\text{M}$ ). The more hydrophilic 2-hydroxyethyl (compound **21**) decreased potency ( $\text{EC}_{50}$  values  $6.9 \pm 2.1$  and  $24 \pm 3.3 \mu\text{M}$ ), suggesting that hydrophobic groups were favored in this position.

### **2.3.4 Thiazolium Ring is More Potent than Pyridinium**

Next, we made modifications to the pyridinium heterocycle (Hy). Changing the position of the heteroatom in the pyridinium from the 2 to 4 position (compound **22**) did not significantly influence anti-proliferative activity in MDA-MB-231 cells ( $EC_{50}$   $1.7 \pm 0.1 \mu\text{M}$ ), while the activity was reduced in MCF-7 cells ( $EC_{50}$   $19 \pm 4.9 \mu\text{M}$ ). Similarly, adding another nitrogen to the ring (compound **23**) reduced potency by approximately 2-fold, while replacing the pyridinium with a thiazolium (compound **24**) improved the potency by 3-fold in MCF-7 cells ( $EC_{50}$   $0.7 \pm 0.1 \mu\text{M}$ ) and slightly reduced activity in MDA-MB-231 cells ( $EC_{50}$   $5.1 \pm 0.3 \mu\text{M}$ ). To test whether the improvement in activity by the 2-thiazolyl group at  $R_3$  would be robust, we also generated analogs with this modification in the context of ethyl and benzyl substitutions at  $R_3$  (compounds **25** and **26**, respectively). These compounds followed the structure-activity trends of the earlier molecules, with the cytotoxicity of compound **26** improved by 2-fold in MDA-MB-231 cells and 4-fold in MCF7 cells, with  $EC_{50}$  of  $0.5 \pm 0.1$  and  $0.6 \pm 0.04$  respectively.

### **2.3.5 JG-98, an Improved MKT-077 Analog that Incorporates the Initial Structure-Activity Relationships**

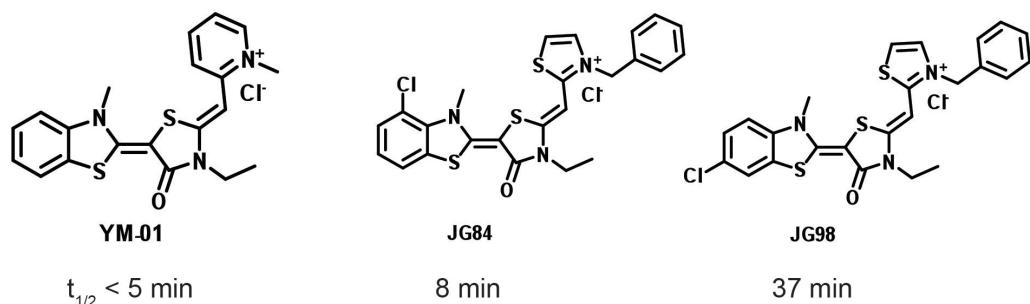
Together, these results suggested that a modest increase in anti-proliferative potency might be gained by switching the pyridinium for a thiazolium, and that appending a benzyl moiety to this ring might further promote activity. Combining these features with halogen replacements on the benzothiazole ring resulted in compounds **27-31**. As halogenation of the benzothiazole ring at the 5' position had proven most effective, compound **30** (JG-98) was taken forward for further studies.

### **2.3.5.1 JG-98 Has Improved Metabolic Stability *in vitro* and *in vivo***

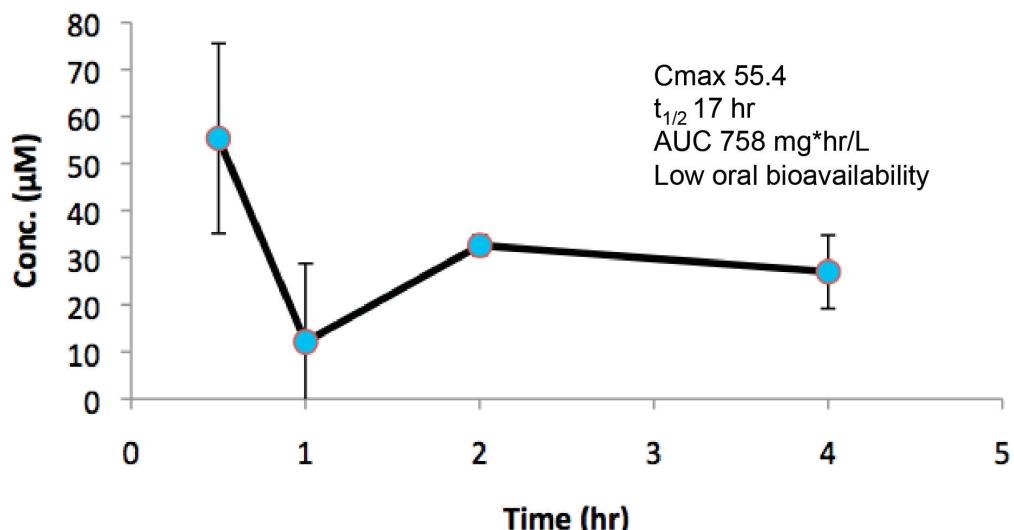
To confirm that combining the above-described structural changes led to an improved oxidative stability, we analyzed compound metabolism in liver microsomes. We found that JG-98 had a lifetime of 37 min, which is at least 7-fold better than MKT-077 or YM-01 (Figure 2.2.A). JG-84 was not significantly more stable than YM-01 ( $t_{1/2} = 8$  min), further confirming that halogenation of the 5' position on the benzothiazole ring was important.

In collaboration with Duxin Sun (University of Michigan), we also examined JG-98's stability *in vivo* by performing intraperitoneal injections of CD-1 mice and measuring drug levels in the blood stream (Figure 2.2.B). Despite a poor oral bioavailability, JG-98 displayed favorable pharmacokinetics *in vivo* ( $t_{1/2} = 17$  hrs), warranting further investigation.

### A. Metabolic stabilities of MKT-077 Analogs



### B. In vivo stability of JG-98 dosed i.p. in CD-1 mice.

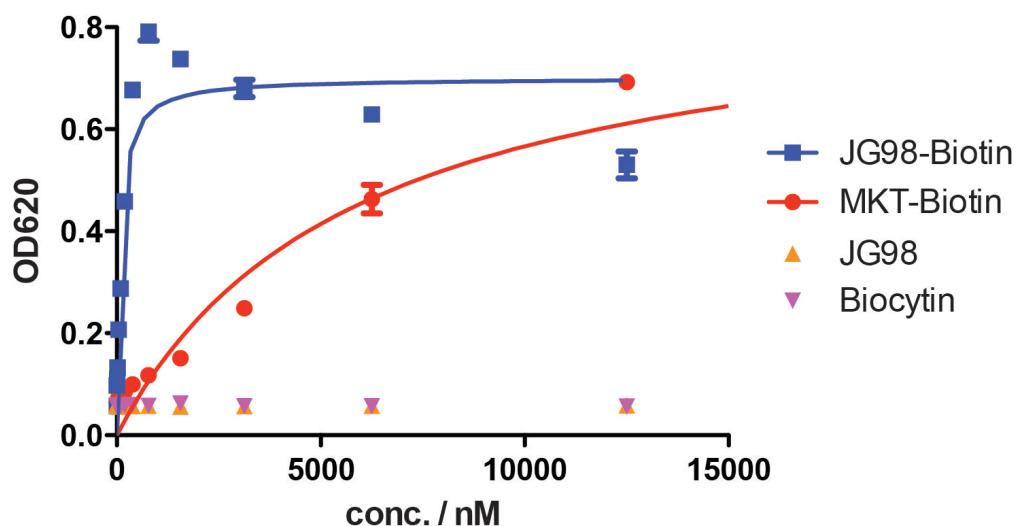


**Figure 2.2. MKT-077 Analogs show greatly improved metabolic stability.**  
**(A)** Half-life comparison of YM-01 with newer analogs. Half-lives were determined by liver microsome studies. **(B)** Plasma levels of JG-98 measured after intraperitoneal (i.p.) dosing of CD-1 mice. Figure adapted from Li, X. et al (ACS Med Chem Lett., 2013).

### **2.3.5.2 JG-98 Interacts with Hsp70**

#### **2.3.5.2.1 JG-98 Binds Tightly to Hsc70 *in vitro***

Next, we wanted to explore whether JG-98 retained binding to Hsc70 *in vitro*. To test this idea, we synthesized a biotinylated version of JG-98, using a modification of the previous synthetic route (Appendix 2.1.A). Using a previously reported ELISA procedure (16), the affinity ( $K_D$ ) of JG98-biotin for purified, human Hsc70 was calculated to be  $87 \pm 16$  nM (Figure 2.3), which was ~60 times improved over the affinity of Hsc70 for a biotinylated version of MKT-077 ( $K_D = 5.8 \pm 0.77$   $\mu\text{M}$ ). Thus, the chemical modifications leading to JG-98 not only improve its metabolic stability, but also dramatically increase its affinity for binding to Hsp70 *in vitro*.



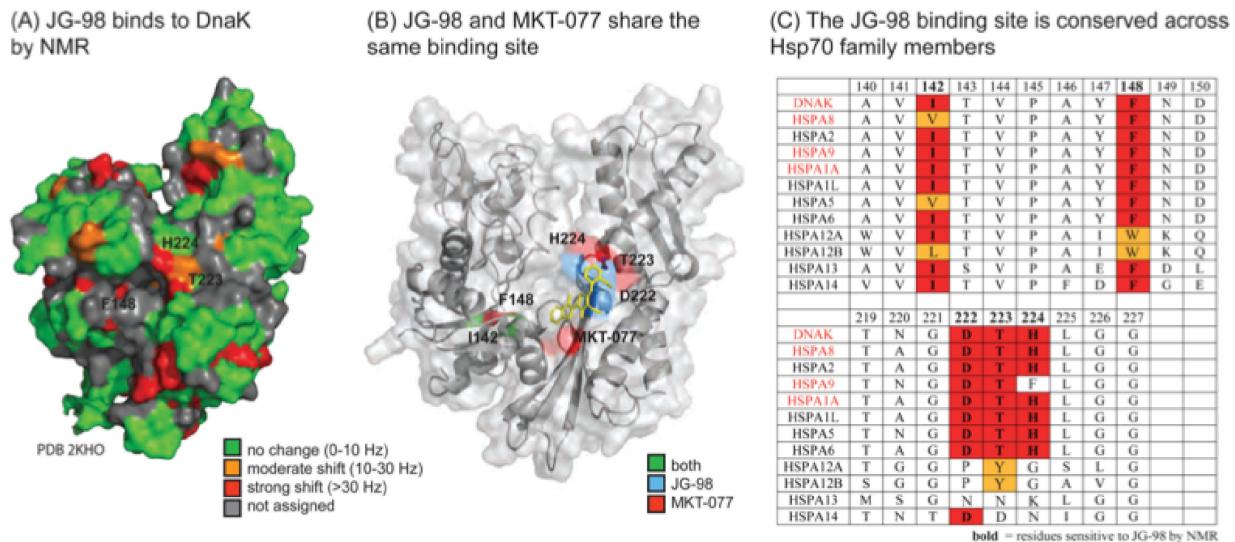
	MKT-Biotin	JG98-Biotin
One site -- Specific binding		
Best-fit values		
Bmax	0.8954	0.7004
Kd	5810	87.01
Std. Error		
Bmax	0.03747	0.02457
Kd	771.2	15.56
95% Confidence Intervals		
Bmax	0.8199 to 0.9709	0.6508 to 0.7501
Kd	4256 to 7363	55.57 to 118.4
Goodness of Fit		
Degrees of Freedom	46	40
R2	0.9522	0.9010

**Figure 2.3. JG-98 Binds Hsp70 with Greater Affinity than MKT-077.** Affinities were measured in an ELISA. Hsp70 was immobilized and incubated with biotinylated versions of MKT-077 or JG-98. Binding events were detected with streptavidin-HRP followed by TMB substrate.

### 2.3.5.2.2 NMR Spectroscopy Reveals Binding Site of JG-98

Our group had previously found that MKT-077 binds to an allosteric site on Hsc70, which is not overlapping with the ATP-binding cleft (26). To determine if JG-98 shared this binding site, it was titrated into an aqueous solution of <sup>15</sup>N-labeled DnaK nucleotide binding domain (DnaK<sub>1-388</sub>) and its interactions with the protein monitored by NMR. We used DnaK, a prokaryotic ortholog of Hsp70 (27), in these studies because of its good behavior in solution and the availability of a large number of NMR resonance assignments (27). Titrating JG-98 (200 μM) into the NBD of <sup>1</sup>H-<sup>15</sup>N DnaK<sub>1-388</sub> (160 μM) showed strong chemical shifts (ΔHz > 30) in the TROSY-HSQC experiment. Strong shifts appeared in a deep, hydrophobic pocket formed by Ile142, Phe148 and other residues (Figure 2.4.A) and additional shifts were seen in nearby surface residues: Gly222, Asp223, and Thr224. A similar region was previously implicated in the binding of MKT-077 to Hsc70 (18) and an overlay of the two sets of compound-sensitive residues suggested that MKT-077 and JG-98 interact with the chaperone at the same site (Figure 2.4.B).

The major sites perturbed by JG-98 (residues I142, F148, D222, T223, H224) are highly conserved amongst prokaryotic and eukaryotic Hsp70s. To this point, Dr. Xiaokai Li has recently shown that JG-98 has nearly equal affinity for *E. coli* DnaK and human Hsc70 (personal conversation). These perturbed residues are also nearly invariant in the major human family members, such as Hsc70 (HSPA8), Hsp72 (HSPA1A) and mtHsp70 (HSPA9) (Figure 2.4.C). Thus, compounds of this class will likely have affinity for nearly all of the family members, as previously suggested (18).

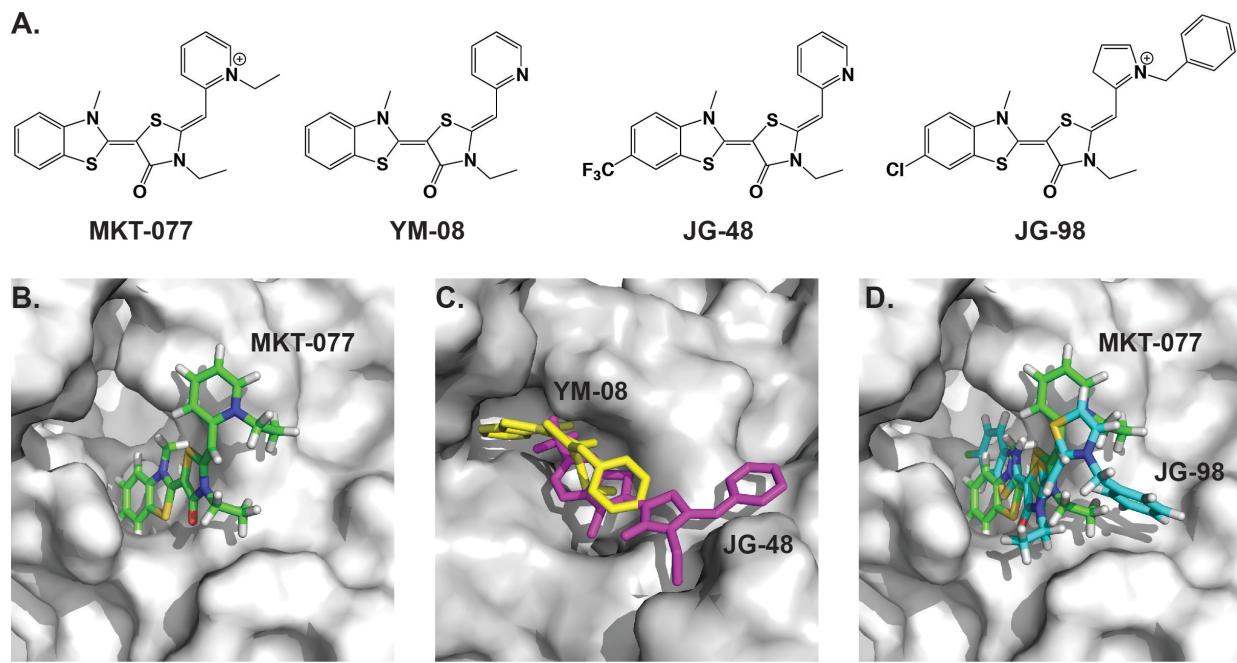


**Figure 2.4. JG-98 binds to a conserved, allosteric site on an Hsp70 family member.** (A) Titration of JG-98 into <sup>15</sup>N DnaK1–338 revealed chemical shifts, with a strong cluster in a deep pocket near F148. (B) JG-98 is predicted to bind the same allosteric pocket that accommodates MKT-077. The docked configuration of MKT-077 is shown (see text). (C) Alignment of DnaK and human Hsp70 family members, highlighting the cluster of residues sensitive to treatment with JG-98 (bold). Invariant residues are shown in red, conserved residues in orange, and nonconserved residues in white. Note that there is also high conservation outside the sensitive residues.

### 2.3.5.2.3 Docking of MKT-077 Analogs Unveils Structure-Activity Relationship

Despite interacting with Hsp70 at a similar site, JG-98 displays significantly increased binding affinity over MKT-077. To understand this, we used molecular modeling and docking to examine the conformational orientation of MKT-077 and select analogs (Figure 2.5). All analogs appear to bind to Hsp70 in a “benzothiazole-in” position. However, larger groups, like the trifluoromethyl moiety on JG-48, appear to prevent proper interaction of compound with the pocket (Figure 2.5.C). This is consistent with the decreased activity shown for these compounds (Table 2.1). Reduction to a smaller halogen functionalization of the benzothiazole ring allowed JG-98 to fit into the conserved pocket, though with a slightly offset orientation compared to MKT-077. The additional benzyl group off the thiazole ring also allowed for increased

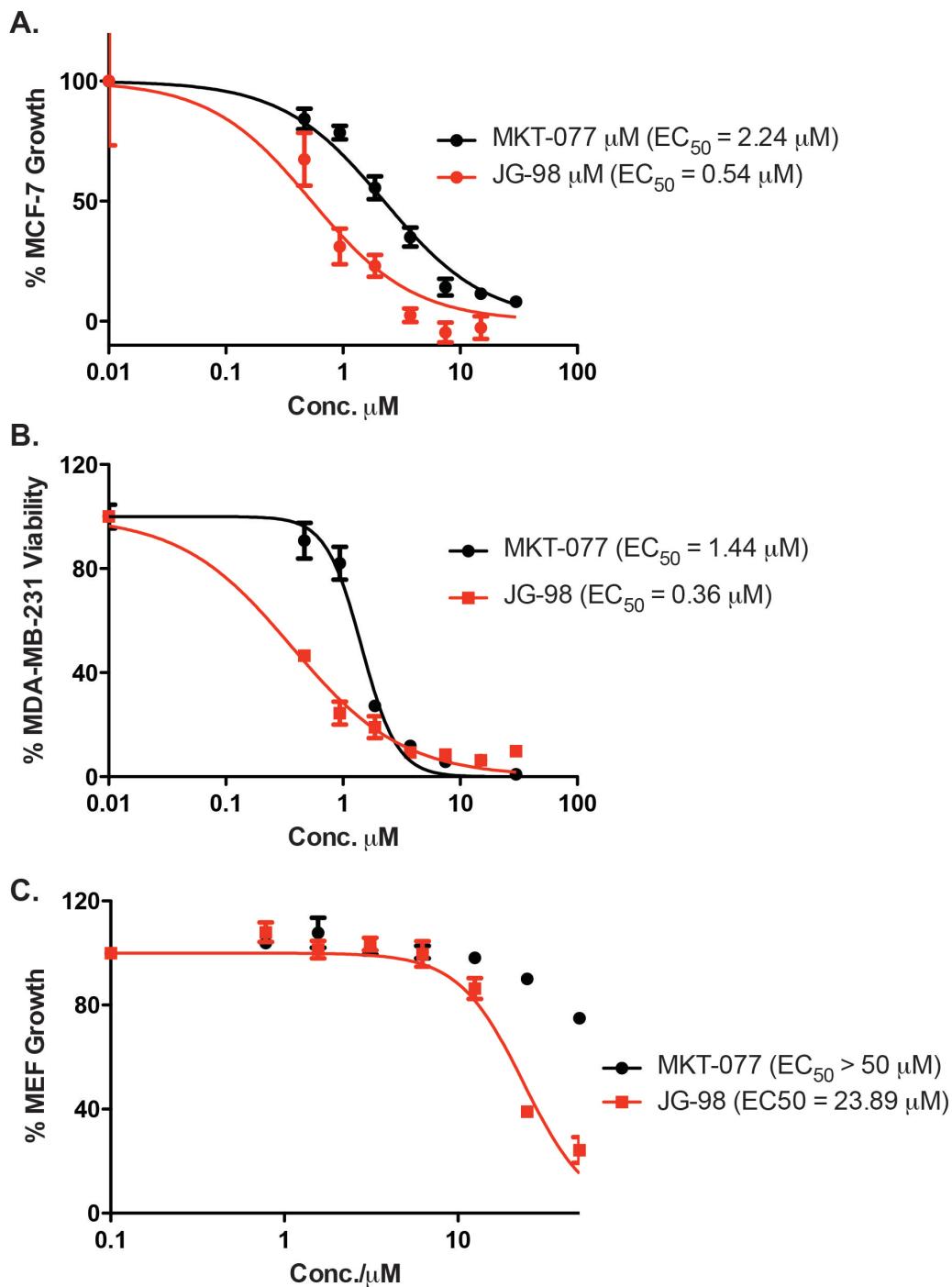
hydrophobic contact with the chaperone surface, contributing to the increased binding affinity (Figure 2.5.D).



**Figure 2.5 MKT-077 Analogs Bind to the NBD of Hsp70. (A)** Structures of MKT-077 and select analogs. **(B)** Docking structure of MKT-077. **(C)** Docking structures of YM-08 and JG-48. The trifluorocarbon group appears to prevent JG-48 from fully inserting into the desired pocket. **(D)** JG-98 docks to the same pocket as MKT-077, but with a slightly different conformation. The benzyl moiety also adds additional hydrophobic contacts, contributing to the increased affinity. Docking was performed using AutoDock4 and UCSFDock.

### **2.3.5.3 JG-98 Exhibits Improved Cytotoxicity Against Breast Cancer Cells**

To determine whether JG-98's improved metabolic stability and affinity for binding Hsp70 *in vitro* resulted in increased cytotoxicity, we treated MCF-7 and MDA-MB-231 cells and examined viability by the MTT assay. JG-98 exhibited significantly improved cytotoxicity against both cell lines ( $EC_{50} = 0.7 \pm 0.2 \mu\text{M}$  and  $0.4 \pm 0.03 \mu\text{M}$  respectively) (Figures 2.6.A and B). JG-98 had increased activity against MEFs compared to MKT-077, but the selectivity for cancer cells was still estimated to be between 20- and 50-fold, well within the therapeutic index range for most chemotherapeutic agents (Figure 2.6.C).



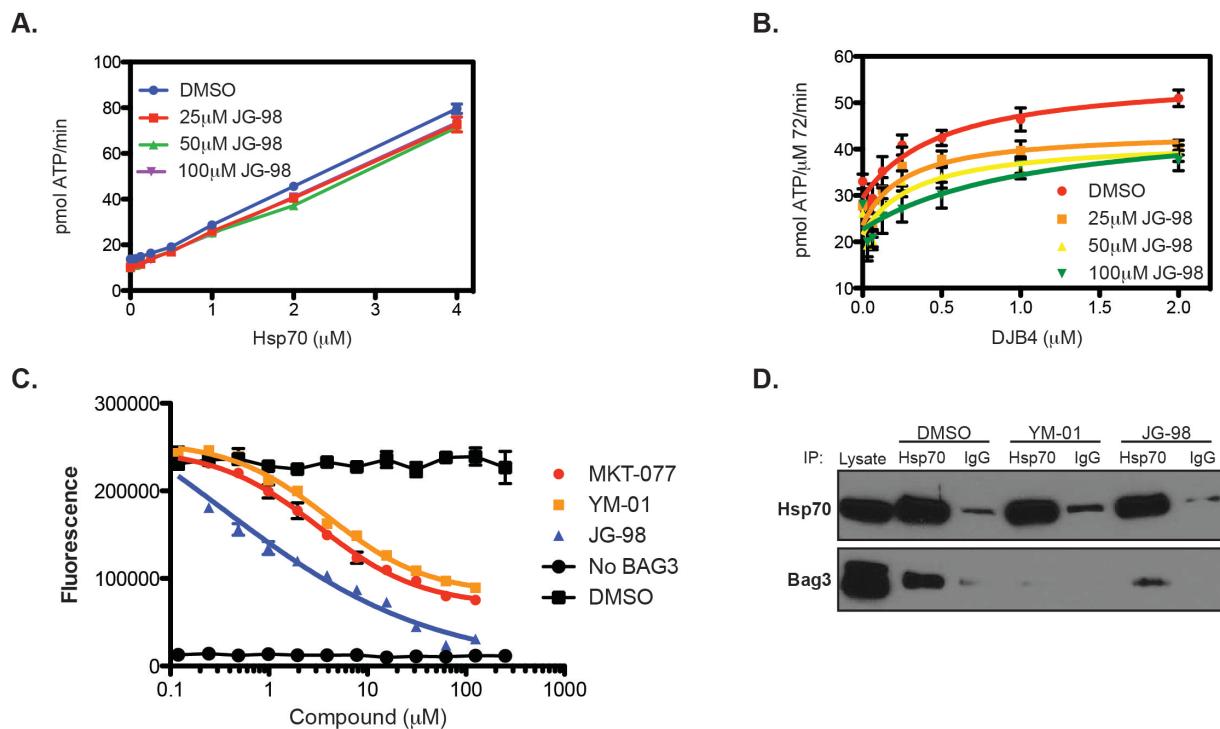
**Figure 2.6 Improved Anti-Cancer Activity of MKT-077 Analogs.**  
 JG-98 displays improved potency over MKT-077 in both MCF-7 (A) and MDA-MB-231 (B) cells. (C) JG-98 is slightly more toxic to MEFs, but retains a good therapeutic index (>20).

### **2.3.6 JG-98 Disrupts Hsp70's Cooperation with Co-chaperones**

Hsp70 is a multi-functional chaperone required for many aspects of a protein's life-cycle, including folding, trafficking, and degradation (28-31). To reduce its promiscuity and "fine-tune" its activity, Hsp70 is modulated by a class of proteins known as co-chaperones. These combinatorial complexes are thought to dictate the effect Hsp70 exerts on its client substrate (32-36). Further, these complexes are enriched in cancer cells to accommodate the need for increased proteostasis regulation (37), and are a potential target for selective drugging of Hsp70 activity in cancer cells.

To examine whether JG-98 inhibited Hsp70 by disrupting these interactions, we analyzed ATPase rate, a functional read-out of Hsp70 activity. We found that JG-98 had no effect on Hsp70's intrinsic activity (Figure 2.7.A), but significantly inhibited stimulation of Hsp70 ATPase rate by J-proteins, a class of Hsp70 co-chaperones (Figure 2.7.B).

MKT-077 analogs stall ATP cycling in Hsp70 by stabilizing the ADP-bound state (26, 38), leading Hsp70 to bind tightly to its peptide substrates. Another student in the Gestwicki Lab, Jenny Rauch, has recently shown that the ADP conformation has a decreased affinity for nucleotide exchange factor (NEF) co-chaperones (39). Accordingly, we find that JG-98, which traps Hsp70 in an ADP-conformation, reduces the interaction between Hsp70 and Bag3, a NEF co-chaperone, by both flow cytometry and co-immunoprecipitation (Figures 2.7.C and D).



**Figure 2.7 JG-98 Perturbs Co-Chaperone Modulation of Hsp70 Function. (A)** JG-98 doesn't affect the intrinsic ATPase activity of Hsp70. **(B)** J-protein stimulation of Hsp70 ATPase is decreased by incubation with JG-98. **(C)** MKT-077 analogs inhibit the association of Hsp70 with Bag3 co-chaperone. Protein interaction was measured in a flow cytometry platform. **(D)** JG-98 disrupts Hsp70-Bag3 binding in HeLaC3 cells. Hsp70 was immunoprecipitated and membranes were blotted for Bag3 association.

The ADP conformation of Hsp70 has a high affinity for both substrate as well as another co-chaperone, CHIP (C-terminal Hsp Interacting Protein), an E3 Ubiquitin Ligase. This increased Hsp70-CHIP interaction is thought to facilitate the degradation of Hsp70 client proteins via the proteasome (40, 41). This mechanism for MKT-077 derivatives such as JG-98, in comparison to the ATP-competitive compounds (42, 43), leads to a highly effective and selective disruption of Hsp70's oncogenic activities with limited toxicity.

## 2.4 Conclusions

MKT-077 had previously shown promise as an allosteric inhibitor of Hsp70. However, metabolic instability and relatively modest potency limited its use. In this Chapter, a library of MKT-077 derivatives was synthesized to identify a more stable and potent analog. Compared to either MKT-077 or YM-01, the most promising compounds, such as **30** (JG-98) had improved potencies against breast cancer cells and extended lifetimes in liver microsome studies. Further, JG-98 bound to a conserved, allosteric site on Hsp70 with tight affinity. Dr. Xiaokai Li is using knowledge of the binding site and structure-activity relationships (SAR) in continuing efforts to generate more potent MKT-077 analogs. Recent compounds from that effort have EC<sub>50</sub> values of ~70 nM (Li, X., unpublished work), suggesting that the allosteric pocket is amenable to tight binding, potent compounds.

Mechanistic studies suggest that JG-98 “traps” the ADP-bound state, decreases affinity for NEFs and interrupts the ATPase cycle. The resultant ADP conformation has a high affinity for substrate, furthering the idea that Hsp70’s domains can be modulated using allosteric perturbations. Zapporah Young, another student in the Gestwicki lab, has shown that MKT-077 derivatives not only increase Hsp70’s affinity for binding substrate in an *in vitro* ELISA platform, but also diminish the refolding capacity (Young, Z. et al, unpublished work).

Although inhibition of a central member of the protein-folding machinery might appear an overly toxic strategy, seminal work has shown that knockdown of Hsp70 isoforms is toxic to cancer cells only (44). Further, Hsp72<sup>-/-</sup> mice are viable, though sterile (45). Lastly, our lab in conjunction with the Dickey lab at USF, has shown that

MKT-077 derivatives are toxic only to cancer cells (19). Thus, drugging Hsp70 family members as anti-cancer therapies is a validated strategy, although uncertainty remains regarding the specific isoforms that should be targeted.

While it is largely thought that Hsp72, the stress-inducible isoform, is the key target, previous studies in the Workman lab have shown that knockdown of both Hsc70 and Hsp72 is crucial to cancer cell death (44). Our own work shows that MKT-077 derivatives act on both Hsc70 and Hsp72 *in vitro* and *in cellulo* (Figures 2.3, 2.7 and Appendix 2.1). Given the conservation of the binding site for these compounds, it is likely that activity extends to other Hsp70 isoforms as well. Indeed, Wadhwa et al. showed that MKT-077 bound both Hsc70 and mtHsp70 (Grp75). To corroborate this, in collaboration with the Weissman Laboratory at UCSF, using a genomic shRNA screen (46), we have shown that our new derivatives exhibit a preferential dependence on mtHsp70 (Appendix 2.2), though its actions on cytosolic isoforms remain clear.

Together, these studies advance the rhodacyanines as potential anti-cancer agents and improve their utility as chemical probes for studying Hsp70's roles in cancer. This is an important advance for my thesis work, because it provides the first well-characterized probe of Hsp70. Accordingly, JG-98 and a neutral derivative of MKT-077 will be used in Chapters 3 and 4 to better understand the roles of Hsp70 family members in cancer signaling.

## 2.5 Notes

This work has been, in part, published in *ACS Medicinal Chemistry Letters*. Xiaokai Li performed chemical synthesis of all compounds, in addition to screening of compounds in MDA-MB-231 cells, and *in vitro* binding studies by ELISA. Sharan R. Srinivasan performed screening of compounds in MCF-7 and MDA-MB-231 cells and docking studies along with Bryan Dunityak. Jamie Connarn helped with pharmacokinetic studies. Atta Ahmad performed NMR titration experiments. Zapporah T. Young and Adam M. Kabza also performed experiments. Genomic shRNA screens were completed by Martin Kampmann and Luke Gilbert in collaboration with the Weissman Lab at UCSF. Xiaokai Li, Sharan R. Srinivasan, and Jason E. Gestwicki helped in the preparation of the manuscript.

## **2.6 Experimental Procedures**

### **2.6.1 Chemistry**

Details of all chemical procedures, including synthesis and characterization of all compounds have been described in full in *Li et al* (2013) (47).

### **2.6.2 NMR Titration**

DnaK's nucleotide-binding domain (DnaK<sub>NBD</sub>; residues 1-388) was expressed in BL21 cells using M9 media enriched with <sup>15</sup>N. The cells were induced at an OD<sub>600</sub> = 0.5 and grown overnight at 26 °C. Cells were harvested at 12,227 x g, lysed with sonication (Fischer scientific model 500) and the lysate centrifuged at 27,216 x g. The supernatant was loaded on a pre-equilibrated FFQ (DEAE type anion exchanger) column. Fractions of eluent were identified for protein by OD280 and SDS-PAGE. Fraction containing DnaK<sub>NBD</sub> were pooled, dialyzed and loaded on a pre-equilibrated ATP-agarose column. The column was washed with buffer (25 mM TrisHCl, 10 mM KCl, pH 7.0) and eluted with 5 mM ADP in buffer. The concentration of the pooled protein fractions was determined by the BCA assay.

NMR samples of 160 µM <sup>15</sup>N labeled DnaK<sub>NBD</sub> (1-388) in 25 mM TrisHCl, 10 mM KCl, 8 mM MgCl<sub>2</sub>, 10 mM PO<sub>4</sub><sup>2-</sup>, 0.015% NaN<sub>3</sub>, 5% D<sub>2</sub>O and pH 7.0 was divided into two 300 µL samples. TROSY-HSQC spectra were then recorded for 3 hrs on a 800 MHz NMR magnet. The samples were treated with 6 µL of either DMSO alone or JG-98 (200 µM). TROSY-HSQC data was processed in NMR PIPES and converted to SPARKY format. Spectra were manually analyzed in SPARKY. Residues that shift in

the JG-98-treated sample, but not the DMSO-treated sample were mapped on the crystal structure of DnaK<sub>NBD</sub> (PDB ID: 2KHO) in PyMOL.

### **2.6.3 Cell Culture**

MCF-7 cells were cultured in DMEM with 10% fetal bovine serum (Invitrogen) and 1% penicillin-streptomycin (Invitrogen). MDA-MB-231 cells were cultured in DMEM with 10% FBS, 1% pen-strep, and 1% non-essential amino acids. All cells were maintained at 37 °C with 5% CO<sub>2</sub> in a humidified atmosphere.

### **2.6.4 Cell Survival Assay**

Cell viability was determined using a methyl thiazoyl tetrazolium (MTT) colorimetric assay (ATCC, catalog number 30-1010K) with the following modifications. Briefly, cells ( $5 \times 10^3$ ) were plated into 96-well assay plates in 0.1 ml media and allowed to attach overnight. Cells were then treated with compound at various concentrations in 0.2 mL media. After the 72-hour incubation period, cells were washed in PBS (3 x 200 µL), and 10 µL MTT reagent was added with 100 µL fresh media. Cells were then incubated for 4 hr in a humidified chamber at 37 °C with 5% CO<sub>2</sub>. Insoluble formazan crystals were solubilized by addition of 0.1 mL detergent solution (4 hr at room temp., dark). Resulting colored solutions were then quantified at an absorbance of 570 nm.

### **2.6.5 Microsomal Stability Assay**

LC-MS/MS parameters were optimized for each compound (see below). The enzyme β-NADPH (16.6 mg/mL) and master mix; 0.1 M phosphate buffer with 3.3 mM

$\text{MgCl}_2$ , mice liver microsomes (final concentration 500  $\mu\text{g/mL}$ ), and compound of interest (final concentration 1  $\mu\text{M}$ ) were incubated for 3 minutes at 37 °C. To begin the metabolic reaction, 20  $\mu\text{L}$  of  $\beta$ -NADPH was added to the master mix. The reaction was incubated at 37 °C for the duration of the experiment. An aliquot of 40  $\mu\text{L}$  was taken from the reaction and added to 120  $\mu\text{L}$  of internal standard (100 nM) in acetonitrile at time points 0, 5, 10, 15, 30, and 60 minutes. Samples were centrifuged at 14,000 rpm for 15 minutes to extract compound. After samples were analyzed by LC-MS/MS, peak areas for the compound and internal standard were plotted again the time to determine the half-life. Verapamil was used as a positive control.

The separation of compounds and internal standard was performed using an Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA) and Zobax SB-C18 column (2.1 × 50 mm, 3.5  $\mu\text{m}$ ) (Agilent Technologies). The compounds were eluted with a gradient as followed; 0-0.5 min solvent A 90% and solvent B 10%, .51-6 min solvent A 10% and solvent B 90%, and 6.1- 10min solvent A 90% and solvent B 10%. Solvent A contained 0.1% (v/v) FA in water and solvent B contained 0.1% (v/v) FA in acetonitrile. Injections of 5  $\mu\text{L}$  of samples were used in the HPLC, the elution ran for 10 minutes at a flow of 200  $\mu\text{L}/\text{min}$ . The MS detection used a QTRAP 3200 (Applied Biosystems/MDS Sciex, Foster City, CA) in the MRM mode. The temperature settings were 500 °C and the curtain gas, gas 1, and gas 2 were 30, 40, and 50 PSIG respectively. The ions produced were detected as following; JG-98 channel m/z 498 → m/z 407. The internal standard channel was m/z 400 → m/z 161.

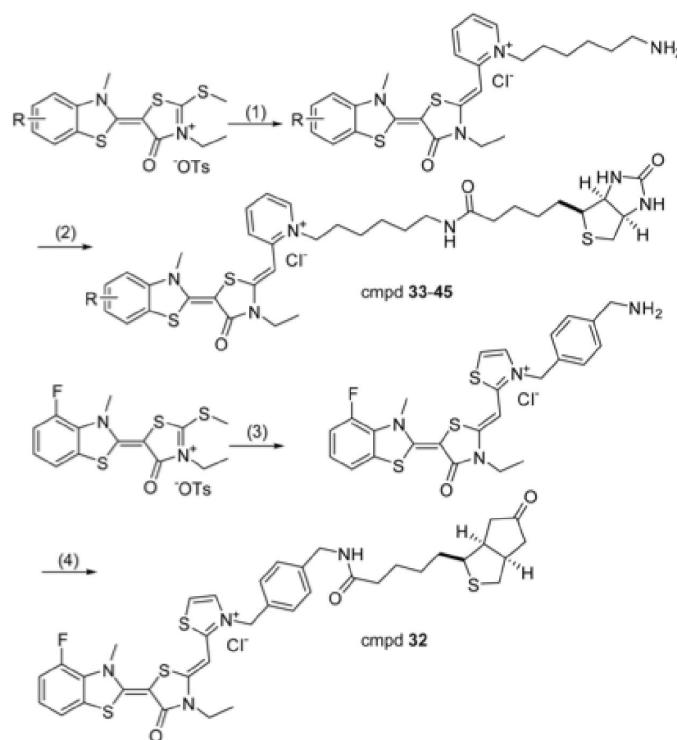
## **2.6.6 ELISA**

A sample of Hsc70 (50 µL; 0.06 mg/mL) was immobilized in 96-well plates in MES buffer (50 mM 2-(N-morpholino)ethanesulfonic acid, pH 5.2) at 37 °C overnight. Wells were washed three times with 150 µL TBS-T buffer (25 mM Tris, 140 mM NaCl, 2.7 mM KCl, 0.01% Tween-20, pH 7.4), then biotin labeled compound (final concentration 1 µM; 25 µL) was added and the mixture was incubated at room temperature for 3 hrs. The wells were washed again with 150 µL TBS-T three times prior to blocking with 100 µL 3% bovine serum albumin (BSA) in TBS-T for 5 min at room temperature. After the removal of the blocking solution, 50 µL of HRP-streptavidin (Pierce Biotechnology, 1:50000 TBS-T dilution) solution was added and the plates were incubated at room temperature for 1 hr. The HRP-streptavidin solution was removed and wells were washed three times with TBS-T. TMS substrate (Cell Signaling Technology 100 µL) was added and incubated at room temperature until a visible blue color was observed (~20 min). 1 M HCl stop solution was then added and the absorbance at 450 nm was recorded on a SpectraMax M5 multimode plate reader (Molecular Devices).

## 2.7 Appendices

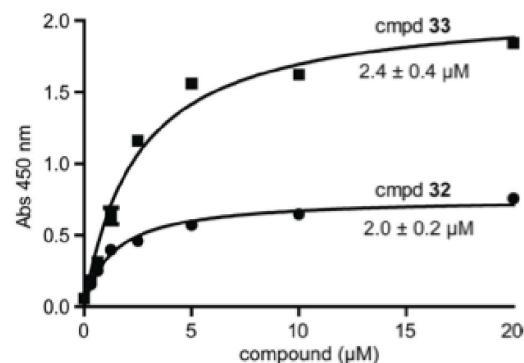
### 2.7.1 Biotinylated MKT-077 Analogs Bind Hsc70

(A) Synthesis of biotinylated MKT-077 analogues



- (1) a. 1-(6-((tert-butoxycarbonyl)amino)hexyl)-2-methylpyridin-1-ium bromide, triethylamine;
- b. Cl ion exchange column; c. trifluoroacetic acid
- (2) NHS-Biotin, triethylamine
- (3) a. 3-(4-((tert-butoxycarbonyl)amino)methyl)benzyl)-2-methylthiazol-3-ium bromide, triethylamine; b. Cl ion exchange column; c. trifluoroacetic acid
- (4) NHS-Biotin, triethylamine

(B) YM01-biotin and JG83-biotin have similar affinity for Hsc70

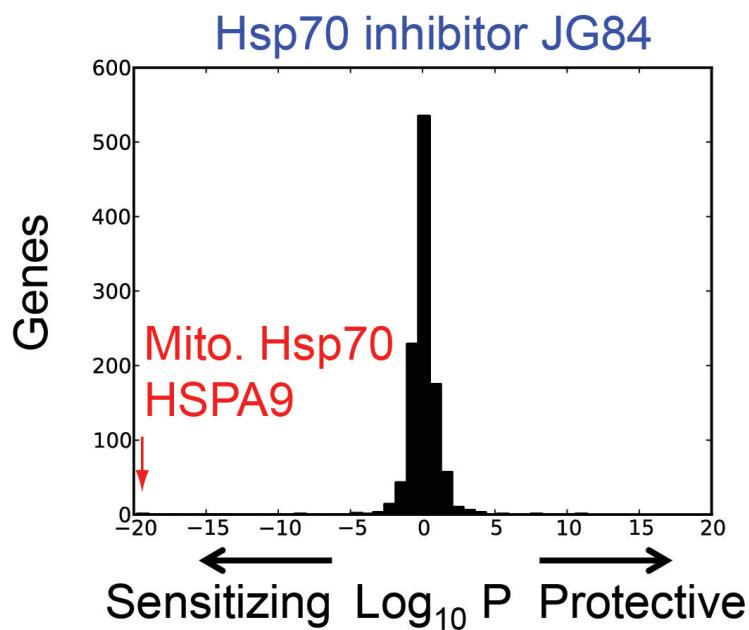


(C) Biotinylated analogues bind Hsc70

compound	R	K <sub>d</sub> / μM
33 (YM01-biotin)	H	2.0 ± 0.2
34	3-F	10.9 ± 4.4
35	4-F	2.3 ± 0.5
36	5-F	1.5 ± 0.2
37	6-F	1.9 ± 0.4
38	3-Cl	7.7 ± 1.0
39	4-Cl	4.0 ± 0.5
40	5-Cl	1.7 ± 0.3
41	6-Cl	2.6 ± 0.6
42	4-OMe	0.9 ± 0.1
43	4-CF <sub>3</sub>	0.4 ± 0.1
44	5-OMe	5.4 ± 0.7
45	5-CF <sub>3</sub>	1.0 ± 0.2

**Appendix 2.1. Biotinylated MKT-077 analogues bind Hsc70.** (A) Synthetic route to biotinylated MKT-077 analogues. (B) Biotinylated versions of YM-01 (33) and JG-83 (32) bind human Hsc70 with a similar affinity, as judged by ELISA. Results are the average of at least three experiments performed in duplicate. The error is SEM. (C) Binding affinities (KD) of compounds 33–45 to Hsc70 by ELISA. See the Supporting Information for details.

## 2.7.2 MKT-077 Derivatives Exhibit Preferential Dependence on mtHsp70



**Appendix 2.2 MKT-077 Derivatives Depend on mtHsp70 for Cytotoxic Effects.** A library of over 2,000 genes were targeted by shRNAs using lentiviral constructs, with multiple oligos per gene. A sublethal dose of JG-84 was added to look for sensitization to compound. MtHsp70, or HSPA9, appeared to most sensitize cells to JG-84, confirming target identification.

## 2.8 References

1. C. G. Evans, L. Chang, J. E. Gestwicki, *J. Med. Chem.* **53**, 4585 (Jun 24, 2010).
2. A. R. Goloudina, O. N. Demidov, C. Garrido, *Cancer Lett.* **325**, 117 (Dec, 2012).
3. H. H. Kampinga, E. A. Craig, *Nat. Rev. Mol. Cell Biol.* **11**, 579 (Aug, 2010).
4. M. Sherman, V. Gabai, C. O'Callaghan, J. Yaglom, *FEBS Lett.* **581**, 3711 (Jul 31, 2007).
5. V. L. Gabai, K. R. Budagova, M. Y. Sherman, *Oncogene* **24**, 3328 (May 5, 2005).
6. D. R. Ciocca, S. K. Calderwood, *Cell Stress Chaperones* **10**, 86 (Summer, 2005).
7. M. V. Powers, P. A. Clarke, P. Workman, *Cancer Cell* **14**, 250 (Sep 9, 2008).
8. F. Guo *et al.*, *Cancer Res.* **65**, 10536 (Nov 15, 2005).
9. L. Grossin *et al.*, *Biorheology* **41**, 521 (2004).
10. S. Sadekova, S. Lehnert, T. Y. K. Chow, *Int. J. Radiat. Biol.* **72**, 653 (Dec, 1997).
11. A. J. Massey, *J Med Chem* **53**, 7280 (Oct 28, 2010).
12. Y. Chiba *et al.*, *Anticancer Res.* **18**, 1047 (Mar-Apr, 1998).
13. K. Koya *et al.*, *Cancer Res.* **56**, 538 (Feb 1, 1996).
14. R. Wadhwa *et al.*, *Cancer Res.* **60**, 6818 (Dec 15, 2000).
15. C. D. Britten *et al.*, *Clin Cancer Res* **6**, 42 (Jan, 2000).
16. A. M. Wang *et al.*, *Nat. Chem. Biol.* **9**, 112 (Feb, 2013).
17. A. Tikoo *et al.*, *Cancer J.* **6**, 162 (May-Jun, 2000).
18. A. Rousaki *et al.*, *J. Mol. Biol.* **411**, 614 (Aug 19, 2011).
19. J. Koren, III *et al.*, *PLoS ONE* **7**, e35566 (2012).
20. R. Wadhwa *et al.*, *Cancer Res.* **62**, 4434 (Aug 1, 2002).
21. C. Didelot *et al.*, *Curr. Med. Chem.* **14**, 2839 (2007, 2007).
22. A. S. Duerfeldt, B. S. J. Blagg, *Bioorg. Med. Chem. Lett.* **20**, 4983 (Sep 1, 2010).
23. N. Tatsuta *et al.*, *Cancer Chemother. Pharmacol.* **43**, 295 (Apr, 1999).
24. Y. Miyata *et al.*, *ACS Chem. Neurosci.*, (2013).
25. T. Mosmann, *J. Immunol. Methods* **65**, 55 (1983, 1983).
26. A. Rousaki *et al.*, *J Mol Biol* **411**, 614 (Aug 19, 2011).
27. E. B. Bertelsen, L. Chang, J. E. Gestwicki, E. R. P. Zuiderweg, *Proc. Natl. Acad. Sci. U. S. A.* **106**, 8471 (May 26, 2009).
28. B. Bercovich *et al.*, *J Biol Chem* **272**, 9002 (Apr 4, 1997).
29. J. Frydman, *Annu Rev Biochem* **70**, 603 (2001).
30. F. U. Hartl, A. Bracher, M. Hayer-Hartl, *Nature* **475**, 324 (Jul 21, 2011).
31. N. Kettern, C. Rogon, A. Limmer, H. Schild, J. Hohfeld, *PLoS One* **6**, e16398 (2011).
32. A. J. Caplan, D. M. Cyr, M. G. Douglas, *Cell* **71**, 1143 (Dec 24, 1992).
33. A. T. Gillies, R. Taylor, J. E. Gestwicki, *Mol Biosyst* **8**, 2901 (Nov, 2012).
34. H. H. Kampinga, E. A. Craig, *Nat Rev Mol Cell Biol* **11**, 579 (Aug, 2010).
35. J. S. Liu *et al.*, *J Biol Chem* **273**, 30704 (Nov 13, 1998).
36. G. C. Meacham *et al.*, *EMBO J* **18**, 1492 (Mar 15, 1999).
37. A. Kamal *et al.*, *Nature* **425**, 407 (Sep 25, 2003).
38. A. M. Wang *et al.*, *Nat Chem Biol* **9**, 112 (Feb, 2013).
39. J. N. Rauch, J. E. Gestwicki, *J Biol Chem* **289**, 1402 (Jan 17, 2014).
40. P. Connell *et al.*, *Nat Cell Biol* **3**, 93 (Jan, 2001).

41. S. B. Qian, H. McDonough, F. Boellmann, D. M. Cyr, C. Patterson, *Nature* **440**, 551 (Mar 23, 2006).
42. A. J. Massey *et al.*, *Cancer Chemother Pharmacol* **66**, 535 (Aug, 2010).
43. D. S. Williamson *et al.*, *J Med Chem* **52**, 1510 (Mar 26, 2009).
44. M. V. Powers, P. A. Clarke, P. Workman, *Cancer Cell* **14**, 250 (Sep 9, 2008).
45. L. Huang, N. F. Mivechi, D. Moskophidis, *Mol Cell Biol* **21**, 8575 (Dec, 2001).
46. M. Kampmann, M. C. Bassik, J. S. Weissman, *Proc Natl Acad Sci U S A* **110**, E2317 (Jun 18, 2013).
47. X. Li, S. R. Srinivasan, Z. T. Young, D. Sun, J. E. Gestwicki, *ACS Med Chem Lett*, (2013).