Targeting Inducible Heat Shock Protein 70 in Cancer and Dengue Virus Pathogenesis

with a Novel Small Molecule Inhibitor

by

Matthew Keller Howe

Department of Pharmacology and Cancer Biology
Duke University

Date:_______________________

Approved:

___________________________
Timothy Haystead, Supervisor

___________________________
Stacy Horner

___________________________
Donald McDonnell

___________________________
Dennis Thiele

Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor of Philosophy in the Department of
Pharmacology and Cancer Biology in the Graduate School
of Duke University

2015
ABSTRACT
Targeting Inducible Heat Shock Protein 70 in Cancer and Dengue Virus Pathogenesis
with a Novel Small Molecule Inhibitor

by
Matthew Keller Howe
Department of Pharmacology and Cancer Biology
Duke University

Date: ______________________
Approved:

_________________________
Timothy Haystead, Supervisor

_________________________
Stacy Horner

_________________________
Donald McDonnell

_________________________
Dennis Thiele

An abstract of a dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor of Philosophy in the Department of
Pharmacology and Cancer Biology in the Graduate School
of Duke University

2015
Abstract

Inducible Heat shock protein (Hsp70i) is a protein chaperone that is utilized during tumorigenesis and viral infections for efficient propagation. Overexpression of Hsp70i is observed in a wide spectrum of human tumors, and this overexpression correlates with metastasis, poor outcomes, and resistance to chemotherapy in patients. Hsp70i aids in cancer cell propagation through regulation of anti-apoptotic and cell survival pathways. Furthermore, Hsp70i is induced following infection for several viruses and aids viral propagation, in part through regulation of anti-apoptotic pathways as well as promoting the folding of newly synthesized proteins. Due to the parallel role of Hsp70i in both cancer and viral pathogenesis, identification of small-molecule inhibitors selective for Hsp70i could provide tools for the development of novel therapeutics and further elucidate the role of Hsp70i in both cancer and viral infections.

To date, few Hsp70 inhibitors have been identified and characterized, and their efficacy in clinical settings is unknown. Through the fluorescence-linked enzyme chemoproteomic strategy (FLECS) screen, an allosteric inhibitor selective for Hsp70i was identified, called HS-72. We show that HS-72 is highly selective for Hsp70i, over the broader purinome and other Hsp70 family members, in particular the closely related constitutively active Hsp70 family member, Hsc70. Additionally, HS-72 acts as an allosteric inhibitor to induce a conformational change and inhibit Hsp70i activity. HS-72 displays hallmarks of Hsp70i inhibition in vitro by promoting Hsp70i substrate protein degradation, protein aggregation, and selective growth inhibition of cancer cells. In wild type mice HS-72 is well tolerated and a limited PK study shows HS-72 is bioavailable. Furthermore, in a MMTV-neu breast cancer mouse model, HS-72 shows efficacy to
inhibit tumor growth and promote survival.

Due to the similar utilization of Hsp70i in cancer and viral pathogenesis, this suggests the potential for HS-72 as an antiviral agent. Dengue virus (DENV) is of great public health importance due to estimates of up to 400 million infections per year, coupled with the geographic distribution of the virus, which is now endemic in over 100 countries worldwide. There is also a pressing need for DENV interventions, owing to the lack of approved vaccines or antiviral therapies. DENV is reliant on host factors throughout the viral life cycle and Hsp70i has been implicated as a host factor in DENV pathogenesis. Additionally, the complete role of Hsp70i in DENV pathogenesis remains to be elucidated, highlighting a unique opportunity to use HS-72 as a tool to specifically probe Hsp70i function. In monocytes, Hsp70i is expressed at low levels preceding DENV infection, but Hsp70i expression is induced upon DENV infection. Furthermore, inducing Hsp70i expression prior to infection, correlates with an increase in DENV infection. Targeting Hsp70i with HS-72, results in a dose dependent reduction in DENV infected monocytes, while cell viability was maintained, through inhibiting the entry stage of the viral life cycle. Following infection, Hsp70i localizes to the cell surface and interacts with the DENV receptor complex to mediate viral entry. While, HS-72 treatment results in a disruption of the interaction of Hsp70i with the DENV receptor complex, yielding a reduction in infected cells.

Collectively this work further supports Hsp70i as an anticancer and anti-dengue virus target, and identifies HS-72, a chemical scaffold that is amenable to resynthesis and iteration, as an ideal starting point for a new generation of therapeutics targeting Hsp70i.
Dedication

I dedicate this work to my grandma, Betty Howe.
## Contents

Abstract ........................................................................................................................................ iv  
List of Tables ................................................................................................................................. xi  
List of Figures ............................................................................................................................... xii  
List of Abbreviations .................................................................................................................... xvii  
Acknowledgements ....................................................................................................................... xxii  

1. Introduction ............................................................................................................................... 1  
   1.1 Heat Shock Protein 70 .......................................................................................................... 1  
   1.2 Hsp70 as an Anticancer Target ......................................................................................... 4  
      1.2.1 Hsp90 as an Anticancer Target .................................................................................. 5  
   1.3 Hsp70 Inhibitors ................................................................................................................ 6  
   1.4 Molecular Chaperones in Viral Pathogenesis ...................................................................... 9  
      1.4.1 Hsp90 as an Antiviral Target ..................................................................................... 12  
      1.4.2 Hsp70 as an Antiviral Target .................................................................................... 16  
   1.5 Dengue Virus .................................................................................................................... 20  
   1.6 Conclusion .......................................................................................................................... 25  

2. Experimental Procedures .......................................................................................................... 29  
   2.1 Identification of an Allosteric Small Molecule Inhibitor Selective for the Inducible Form of Hsp70 .............................................................................................................. 29  
      2.1.1 Fluorescence linked enzyme chemoproteomic strategy (FLECS) Screen ................. 29  
      2.1.2 Endogenous Hsp70 Elution ....................................................................................... 30  
      2.1.3 Caspase 3/Caspase 7 Assay ...................................................................................... 30  
      2.1.4 Degradation Assay .................................................................................................... 31  
      2.1.5 Protein Purification .................................................................................................... 31
2.1.6 Thermofluor Assay ................................................................. 32
2.1.7 ATPase Assay ........................................................................ 32
2.1.8 Docking Studies ..................................................................... 33
2.1.9 Partial Proteolysis .................................................................. 33
2.1.10 Mass Spectrometry ................................................................. 33
2.1.11 Limited Proteolysis ................................................................. 34
2.1.12 Aggregation Assay ................................................................. 34
2.1.13 Cell Proliferation ................................................................. 34
2.1.14 HS-72 in vivo Studies .............................................................. 35
   2.1.14.1 Maximum Tolerated Dose (MTD) and Blood Workup ........ 35
   2.1.14.2 HS-72 Pharmacokinetics (PK) ........................................ 35
   2.1.14.3 Efficacy Studies ........................................................... 37
2.2 HS-72 Demonstrates Anti-Dengue Virus Activity, Validating Hsp70i as a Host Antiviral Target .......................................................... 37
   2.2.1 Dengue Virus Proteomic Profiling ........................................ 37
   2.2.2 Flow Cytometry .................................................................. 38
   2.2.3 Foci Forming Assay ............................................................. 39
   2.2.4 Time of Addition Assay ....................................................... 39
   2.2.5 Attachment and Entry Assay ................................................ 40
   2.2.6 Biotin Labeling of Surface Proteins ...................................... 40
   2.2.7 Proximity Ligation Assay (PLA) .......................................... 41
2.3 Cell Lines, Antibodies, and Virus Preparation ................................ 42
2.4 Western Blotting ....................................................................... 43
2.5 Compounds ............................................................................. 44
2.6 Statistical Analysis ........................................................................................................... 44

3. Identification of an Allosteric Small Molecule Inhibitor Selective for the Inducible Form of Hsp70 ................................................................. 46

3.1 Identification of the Hsp70i Inhibitor, HS-72 ................................................................. 46

3.1.1 Confirmation of GFP-Hsp70i binding to the ATP Resin. ........................................... 47

3.1.2 FLECS Screening Yields Highly Selective Hsp70i Interactors. .............................. 48

3.1.3 Identification of a Caspase-Activating Cell-Permeable Compound that Targets Hsp70i ....................................................................................... 52

3.2. Characterization of HS-72 .............................................................................................. 56

3.2.1 HS-72 Selectively Targets Hsp70i Over Other Members of the Hsp70 Superfamily. ........................................................................................................ 56

3.2.2 HS-72 is an Allosteric Inhibitor of Hsp70i. ................................................................. 59

3.2.3 Hsp70i C306D Mutation Perturbs HS-72 binding ....................................................... 64

3.2.4 HS-72 Allosteric Interaction Induces a Conformation Change in Hsp70i .............. 64

3.3. HS-72 Shows Hallmarks of Hsp70i Inhibition in vitro and Efficacy in a MMTV-neu Breast Cancer Mouse Model ......................................................... 69

3.3.1 HS-72 Induces Cellular Protein Aggregation ............................................................ 69

3.3.2 HS-72 Inhibits Cancer Cell Proliferation .................................................................. 70

3.3.3 HS-72 Acts Synergistically with HS-10 .................................................................... 72

3.4 HS-72 is Bioavailable and Shows Efficacy in a Spontaneous Mouse Mammary Tumor Model ...................................................................................... 74

3.4.1 MTD and Complete Blood Workup Shows HS-72 is not Toxic in Wild-Type Mice. ........................................................................................................ 74

3.4.2 Pharmacokinetic and Distribution Studies Show HS-72 is Bioavailable in Wild- Type Mice ............................................................................................... 76

3.4.3 HS-72 Displays Efficacy in MMTV-neu Mice ............................................................ 79

3.5 Conclusions ....................................................................................................................... 82
List of Tables

Table 1: Select Viruses that Utilize Hsp70 and Hsp90................................................................. 10
List of Figures

Figure 1: Hsp70 Structure. (A) Structure of Hsp70 (E. coli, DnaK) in the ATP-bound conformation (PDB 4B9Q) and (B) in the ADP-bound apo conformation (PDB 2KHO). ATP represented in (A) as ball-and-stick model. .............................................................................................................................. 3

Figure 2: Chemical Structures of Select Hsp70 Inhibitors .......................................................................................................................... 8

Figure 3: HealthMap of current reports of Dengue cases, as of 30 June 2015; also showing risk areas by country from Absent in blue to Present in red. Adapted from HealthMap.org/dengue, accessed 30 June 2015 ...................................................................................................................... 21

Figure 4: Schematic of DENV genome. ................................................................................................................................................... 24

Figure 5: Recombinant GFP-Hsp70i is readily captured on γ-phosphate linked ATP resin and is competitively released with ATP/ADP, consistent with nucleotide induced conformational changes. ................................................................................................................................. 48

Figure 6: Overview of FLECS protocol. ............................................................................................................................................... 49

Figure 7: (A) Screening of Hsp70 by FLECS identified 197 based on fluorescence readings. (B) 197 hits sorted by specificity for Hsp70i against the entire FLECS library. (C) Structure of HS-72 and location on the (A) Hsp70 screen array and the (B) FLECS library array. .............................................................................................................................................. 50

Figure 8: (A) Western blotting confirmed presence of Hsp70i in 60 hits. (B) 22 hits were confirmed by Western blot to elute endogenous Hsp70i. (A-B) Final lead, HS-72 is highlighted by box. ................................................................................................................................. 51

Figure 9: Structures of 22 Hits from FLECS. ............................................................................................................................................ 52

Figure 10: HS-72 shows cell permeability and hallmarks of Hsp70i inhibition through caspase-3/7 activation and substrate degradation assays. (A-C Mean ± SEM). ....................................................................................................................... 55

Figure 11: HS-72 selectively elutes Hsp70i from (A,C) HEK 293 cell lysate and (B) pig bladder tissue lysate bound to the ATP resin .................................................................................................................................................. 57

Figure 12: The HS-72 scaffold is highly selective for Hsp70i over the constitutively active Hsc70 and the wider purinome. ............................................................................................................................................... 59

Figure 13: R and S enantiomer elution of GFP-Hsp70i from ATP resin ........................................................................................................................ 60

Figure 14: Purification of (A) Hsp70i and (B) Hsc70. .............................................................................................................................. 60
Figure 15: (A) The Hsp90 inhibitor, HS-10, and (B) ATP/ADP increase the T_m of Hsp90 and Hsp70i, respectively. ............................................................................................................................ 61

Figure 16: (A) HS-72 decreases the T_m of Hsp70i, (C) which is amplified in the presence of ATP, (B, D) while HS-71 has no Effect on T_m. (E) HS-72 has no effect on the T_m of Hsp70i in the presence of ADP. (F) Detergents had no effect on the ability of HS-72 to destabilize Hsp70i. ............................................................................................................................ 62

Figure 17: HS-72 does not change the Tm of Hsc70 ± ATP.............................................. 63

Figure 18: HS-72 does not reduce Hsp70 ATPase activity in the presence or absence of co-chaperone, Hlj1. Hsp70 + DMSO and Hsp70 + Hlj1 + DMSO indicated by dashed lines. Hsp70 + HS-72 is in green, and Hsp70 + Hlj1 + HS-72 is in red. Hlj1 + DMSO is indicated by dashed lines with squares. ............................................................................................................................ 63

Figure 19: HS-72 does not change the T_m of Hsp70i C306D mutant ± ATP................. 64

Figure 20: Docking reveals two potential allosteric sites of HS-72 interaction with Hsp70i. ............................................................................................................................ 65

Figure 21: Native protein digestion studies supports HS-72 as an allosteric inhibitor of Hsp70i. Full length Hsp70i indicated with black arrow and fragments resulting from proteolysis indicated with blue arrows............................................................................................................................ 66

Figure 22: HS-72 does not bind Hsp70i in the same sites as (A-B) VER or (C-D) PES due to no additive or synergistic effects on the T_m of Hsp70i in Thermofluor. (Mean ± SEM). ............................................................................................................................ 68

Figure 23: HS-72 inhibits Hsp70i activity in a Huntington’s cell model. .................... 69

Figure 24: HS-72 inhibits Hsp70i activity across multiple tumorigenic cell lines. (Mean ± SEM). ............................................................................................................................ 71

Figure 25: HS-71 minimally inhibits cancer cell proliferation. (Mean ± SEM). ............ 72

Figure 26: Combination treatment with HS-72 and HS-10 induces degradation of HER2 and Akt more efficiently than either HS-72 or HS-10 alone in MCF7 and BT474 Cells. . 73

Figure 27: HS-72 and HS-10 show synergism inhibiting proliferation of (A) SKBr3 and (B) MFC7 cancer cell lines. (Mean ± SEM)............................................................................................................................. 74

Figure 28: (A) MTD and (B-D) blood workup show no toxicity from HS-72 in wild-type mice............................................................................................................................ 75

Figure 29: Structure of internal standard, HS-156, used for PK study analyzing HS-72 in plasma, liver, and kidney............................................................................................................................ 76
Figure 30: (A) Plasma, (B) kidney, and (C) liver samples plotted on a standard curve.

Figure 31: LC-MS analysis of (A) plasma, (B) kidney, and (C) liver following i.p. injection, 20mpk, at the indicated time points over 24 hours, show HS-72 has a high degree of bioavailability in wild-type mice. (Mean ± SEM).

Figure 32: (A) HS-72 alone and in (B) combination with HS-10, inhibits proliferation of a MMTV-neu derived cell line, NF639. (Mean ± SEM).

Figure 33: (A) HS-72 promotes reduction in tumor volume as well as an (B) Increased median survival time in MMTV-neu mice when compared to animals receiving no treatment. (A; Mean ± SEM).

Figure 34: Proteomic analysis reveals induction of a 70kDa protein following DENV infection. Lanes labeled “C” indicate uninfected control samples; lanes labeled “V” indicated DENV infected samples.

Figure 35: Induction of Hsp70i was confirmed following DENV infection, in U937+DC-SIGN cells. E protein serves as a positive control for infection, GAPDH serves as a loading control. Graph on right is quantification of Western Blot, using Image J software.

Figure 36: HS-10 treatment results in an increase in Hsp70i expression in U937+DC-SIGN cells. GAPDH serves as a loading control. The graph on the right shows quantification of the bands from the western blot, using Image J software.

Figure 37: U937+DC-SIGN cells were pretreated with HS-10, to induce Hsp70i expression prior to DENV infection, resulting in a dose dependent increase in infectivity as determined by (A) flow cytometry or (B) foci forming assay. (Mean ± SEM).

Figure 38: Total and surface Hsp70i expression was induced in infected U937+DC-SIGN cells and infected cells pretreated with HS-10. Graphs represent quantification of respective histograms. (Mean ± SEM).

Figure 39: Acute HS-10 treatment has no effect on DENV infection of U937+DC-SIGN cells.

Figure 40: Pretreatment with Hsp70i or DC-SIGN antibodies reduces DENV infection in U937+DC-SIGN cells. The nonspecific antibody serves as a negative control, while the DC-SIGN antibody serves as a positive control. (Mean ± SEM).

Figure 41: (A) HS-72 yields a dose dependent reduction in DENV infection as determined by flow cytometry. (B) Viability analysis corresponding to percent infection in (A) shows viability was maintained when HS-72 <90µM. (C) HS-72 reduced DENV infection in a dose dependent manner as determined by a decrease in foci forming units. (Mean ± SEM).
Figure 42: (A) HS-72 inhibits DENV infection at early stages of the viral life cycle as determined by percent infection in a time of addition assay. (B) Cell number, corresponding to percent infection in (A), is maintained throughout the time of addition assay between control and HS-72 treated groups, indicating no significant change in cell viability. (Mean ± SEM). ................................................................. 96

Figure 43: An attachment/entry assay shows that HS-72 is working to inhibit DENV infection at the entry stage of the viral life cycle, as determined by the fold change of viral RNA compared to GAPDH. (Mean ± SEM). ................................................................. 97

Figure 44: Hsp70i surface expression was determined, by flow cytometry, at the indicated time points post-infection, showing a significant increase 1 hour post-infection compared to uninfected cells. While, Hsp70i surface expression has significantly decreased by 4 and 24 hours post-infection. Graph represents quantification of respective histograms (Mean ± SEM). ................................................................. 99

Figure 45: Biotinylation of surface Hsp70i shows Hsp70i localizing to the cell surface following DENV infection. While in DENV infected cells treated with HS-72, Hsp70i is maintained on the cell surface until 24 hours post-infection. ................................................................. 100

Figure 46: Hsp70i surface expression was determined in HS-72 treated cells at the indicated time points post-infection by flow cytometry, showing a significant increase 1 hour, 4 hours, and 24 hours post-infection compared to uninfected cells. Graph represents quantification of respective histograms (Mean ± SEM). ................................................................. 101

Figure 47: Treating uninfected U937+DC-SIGN cells with HS-72 did not significantly change the surface expression of Hsp70i. The graph on the right represents quantification of the histogram (Mean ± SEM). ................................................................. 103

Figure 48: Hsp70i surface expression was determined in cells treated with inactivated DENV in the (A) absence and (B) presence of HS-72, which showed no change compared to uninfected cells. (C-D) Graphs represent quantification of histograms shown in (A) and (B), respectively (Mean ± SEM). ................................................................. 104

Figure 49: Inactivated DENV does not infect U937+DC-SIGN cells. ...................... 105

Figure 50: Hsp70i surface expression was increased, as determined by flow cytometry, in cells treated with HS-10, while HS-72 treatment had no significant effect at (A) 6 and (B) 24 hours. Combination treatment with HS-72 and HS-10 maintains Hsp70i on the cell surface 24 hours post-treatment compared to HS-10 treatment alone. Graphs represent quantification of respective histograms (Mean ± SEM). ................................................................. 105

Figure 51: PLA shows an in situ interaction of Hsp70i with the DENV E protein, indicated by distinct red punctae, which is disrupted by HS-72, as observed by a decrease in red punctae. Graph represents quantification of respective images from PLA (Mean ± SEM). Representative images shown with insets showing magnified portion of image.
designated by boxes. Hsp70i - E protein interaction represented by red punctae, DAPI shown in blue to represent cell nuclei, green staining represents cell membranes. ...... 107

Figure 52: Cells not treated with primary antibodies show minimal punctae, indicating minimal background fluorescence and highlighting the specificity of the PLA. DAPI shown in blue to represent cell nuclei, green staining represents cell membranes. ...... 108

Figure 53: PLA shows an in situ interaction of Hsp70i with DC-SIGN, indicated by distinct red punctae, which is disrupted by HS-72, as observed by a decrease in red punctae. Graph represents quantification of respective images from PLA (Mean ± SEM). Representative images shown with insets showing magnified portion of image designated by boxes. Hsp70i - DC-SIGN interaction represented by red punctae, DAPI shown in blue to represent cell nuclei, green staining represents cell membranes. ...... 109

Figure 54: Hsp70i localizes to the cell surface and interacts with the DENV receptor complex to aid in viral entry, while HS-72 treatment disrupts the interaction of Hsp70i with the DENV receptor complex, which reduces viral entry. 1) A DENV virion attaches to a host cell through binding a receptor such as DC-SIGN. 2) DENV binding causes a change in Hsp70i localization to the cell surface, where Hsp70i interacts with the DENV receptor complex. 3) Hsp70i continues to interact with the DENV receptor complex and aids in entry of DENV. 4) By late stages of the DENV lifecycle, localization of Hsp70i is primarily cytoplasmic. Once the DENV genome is released into the host cell, synthesis of viral proteins, and replication of viral RNA occurs, which is followed by packaging and export of mature DENV virions from the infected host cell. 5) In cells treated with HS-72, a DENV virion attaches to a host cell through binding a receptor such as DC-SIGN. 6) DENV binding causes a change in Hsp70i localization to the cell surface and Hsp70i interacts with the DENV receptor complex. 7) The interaction of Hsp70i with the DENV receptor complex is disrupted and Hsp70i is maintained on the cell surface. This could be due to the change in Hsp70i conformation induced by HS-72, which would inhibit Hsp70i in mediating entry of DENV. Ultimately, HS-72 treatment leads to a reduction in DENV infection. Schematic of HSP70 represented in blue, DC-SIGN represented in green, DENV virion represented in red, DENV genome represented in orange, and HS-72 represented by chemical structure in lower panel........................ 112
List of Abbreviations

17-AAG: 17-\(N\)-allylamino-17-demethoxygeldanamycin
17-DMAG: 17-Dimethylaminoethylamino-17-demethoxygeldanamycin
ADE: Antibody Dependent Enhancement
ADP: Adenosine Diphosphate
Aha1: activator of heat shock 90 kDa protein ATPase homolog 1
AMC: 7-Amino-4-methylcoumarin
Apaf-1: Apoptotic protease-activating factor-1
ATCC: American Type Culture Collection
ATP: Adenosine Triphosphate
Azt: Azidothymidine
Bax: Bcl2-associated X protein
Bcl-2: B-cell lymphoma-2
Biw: biweekly dosing
BSA: bovine serum albumin
CBC: Complete blood count
Cdc37: cell division cycle 37
Cdk9: Cyclin-dependent kinase 9
CHIKV: Chikungunya virus
CHIP: Carboxyl-Terminus of Hsp70 Interacting Protein
Cyp40: Cyclophilin 40
DAPI: 4',6-diamidino-2-phenylindole
DC-SIGN: dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DENV: Dengue virus
DHF: Dengue Hemorrhagic Fever
DISC: Death-Induced Signaling Complex
DMEM: Dulbecco’s Modified Eagle Medium
DMSO: Dimethyl sulfoxide
DSS: Dengue Shock Syndrome
DTT: Dithiothreitol
E1A: Early region 1A
E protein: Envelope Protein
EBV: Epstein-Barr virus
EDTA: Ethylenediaminetetraacetic acid
EGF: Epidermal Growth Factor
EIC: Extracted ion chromatograms
EMEM: Eagle’s Minimum Essential Media
FBS: Fetal Bovine Serum
FLECS: fluorescence linked enzyme chemoproteomic strategy
FLIP(S): FLICE inhibitory protein
GFP: Green Fluorescent Protein
Gran + Mono: Granuloctye + Monocyte count
GRP75//78/94: glucose regulated protein 75/78/94
HBV: Hepatitis B virus
Hct: hematocrit
HCV: Hepatitis C virus
Hgb: hemoglobin

Hip: Hsp70-interacting protein

HIV: human immunodeficiency virus

Hop: Hsp70-Hsp90 organizing protein

HPV: Human Papillomavirus

Hsc70: Heat Shock Cognate 70

Hsp70: Heat Shock Protein 70

Hsp70i: Inducible Heat Shock Protein 70

Hsp90: Heat Shock Protein 90

HSV: herpes simplex virus

HT: High-Throughput

httQ74-GFP: 74-glutamine repeats from exon 1 of human Huntington, fused to GFP

i.p.: Intraperitoneal

JEV: Japanese encephalitis virus

JNK: c-Jun N-terminal kinase

LCMS: Liquid chromatography–mass spectrometry

LYMF: lymphocytes

MALDI-MS: matrix-assisted laser desorption-ionization mass spectrometry

MEM: Minimum Essential Media

MMTV: Mouse Mamary Tumor Virus

MOI: multiplicity of infection

MP1U: Mouse Phase 1 Unit

MS: mass spectrometry
MTD: Maximum Tolerated Dose
NBD: nucleotide binding domain
NS5A/3: Non-structural protein 5A/3
ORF29p: VZV open reading frame 29 protein
PBS: phosphate-buffered saline
PBS-T: PBS with 0.01% Tween-20
PES: Pifithrin-µ
PFA: Paraformaldehyde
PK: Pharmacokinetics
PLA: Proximity Ligation Assay
pRB: Retinoblastoma protein
PTFE: Polytetrafluoroethylene
PVDF: polyvinylidene difluoride
Qd: daily dosing
RCA: rolling circle amplification
Rbc: red blood cell count
RFU: relative fluorescence units
SAR: structure-activity relationship
SBD: substrate binding domain
SV40: Simian virus
TBS: Tris-buffered saline
TBS-T: TBS with 0.01% Tween-20
TCA: Trichloroacetic acid
TIC: total ion chromatogram

TLC: thin-layer chromatography

TPR: tetratricopeptide repeat

TOF/TOF: time of flight mass spectrometry

VP1/2/3/4/7: Viral protein 1/2/3/4/7

VSV: Vesicular Stomatitis virus

VZV: Varicella zoster virus

Wbc: White blood cell count

WGA-488: wheat germ agglutinin conjugated to Alexa flour 488

w.w.: wet weight

YFV: Yellow Fever Virus
Acknowledgements

I would like to thank my wife, Bethany, for the endless support and encouragement she has provided throughout my time in graduate school. She has been the guinea pig for listening to my many presentations, which I think she could pretty much give at this point. She also allowed me to put her English degree to work, by editing my writing to make sure it is grammatically correct.

Thanks to my parents and siblings, George, Tina, Ashley, Cooper, and Kasey, and my new parents Donna and Harry, for all of their support. They are always there for me when times are tough, but more importantly when there was an excuse to celebrate. I want to especially thank my parents for their unwavering support and guidance, not just in graduate school, but also in life.

I would like to thank Tim for giving me the opportunity to undertake my graduate work in his lab. I thank him for the guidance he provided that allowed me to grow and develop as a scientist and in life. I am lucky and grateful to have Tim as a mentor during graduate school and I hope to be able to continue to learn from him in the future.

I would like to thank all the members of the Haystead lab, Aaron, Brittany, Dave, Dave, Jared, Khaldon, Lauren, Philip, and Yazan, I couldn’t have asked for a better group to work and interact with on a daily basis.

In addition to the contributions from the members of the Haystead lab, I would like to acknowledge all of the collaborators on these projects. The many collaborators include: the Thiele lab, Dennis Thiele, Alex Jaeger, and Eileen Molzberger; Neil Spector; the Garcia-Blanco lab, Mariano Garcia-Blanco and Brandt Levitt; from the
MP1U at UNC, David Darr, Jamie Jordan, and Lucas Hunter; and from the Brodsky lab at the University of Pittsburg, Jeff Brodsky and Theodore Gobillot.

I would like to thank the members of my thesis committee: Dr. Stacy Horner, Dr. Donald McDonnell, and Dr. Dennis Thiele. I am grateful for all of the advice and mentoring that you have provided in directing my research.

I would especially like to thank Dr. Donald McDonnell. He has been an invaluable source of advice and support for many years, which guided me on the path that I am on today. I am grateful to be able to talk with him about anything, from career advice to bike racing.
1. Introduction

Cancer and viral infections exploit various host proteins for efficient propagation, such as the Heat Shock Protein 70 (Hsp70) family of molecular chaperone proteins. Hsp70 is utilized in propagation of cancer and viral infections due to its role in various cellular processes, in particular through the regulation of anti-apoptotic pathways as well as through assisting in the proper folding of newly synthesized proteins, which will aid cells to survive the cellular stress associated with tumorigenesis and viral infections. In the Haystead laboratory, we are interested in using small molecules to target protein chaperones, such as Hsp70 as well as Hsp90, due to the parallel utilization of these proteins during tumorigenesis and viral infections. Interestingly, one of the first effective human immunodeficiency virus (HIV) antivirals, Azidothymidine (AZT), was identified as a potent anticancer agent, further highlighting the parallels between cancer and viral infections.

In this dissertation I will discuss the identification and development of a small molecule inhibitor that targets Hsp70i, which is used to further elucidate the role of Hsp70i in both cancer and viral pathogenesis, and supports Hsp70i as a therapeutic target.

1.1 Heat Shock Protein 70

Hsp70 is a protein chaperone that plays an essential role in maintaining cellular homeostasis. Hsp70 has many housekeeping functions, including the folding of nascent proteins, refolding of misfolded proteins, protein transport between cellular compartments, breakdown of unstable proteins, removal of protein complexes, and control of regulatory proteins (Daugaard et al., 2007).
Hsp70 is an evolutionary conserved protein that is found in species ranging from bacteria, and plants, to humans (Hunt and Morimoto, 1985). There are several Hsp70 family members, including the constitutively active and stress inducible isoforms. The inducible form of Hsp70 and the constitutively active form of Hsp70, are localized to the cytosol and nucleus, while the inducible form can also be found in lysosomes (Daugaard et al., 2007). There are also Hsp70 family members localized to the endoplasmic reticulum, GRP78, or to the mitochondria, Grp75 (Daugaard et al., 2007). The stress inducible Hsp70 (also called Hsp70i, Hsp72, Hsp70-1, HspA1A/HspA1B; herein will be designated Hsp70i) is present in low or undetectable levels in most unstressed normal cells and tissues; however, expression levels rapidly increase in response to stress conditions such as heat shock, viral infection or transformation. Deletion of its immediate paralog, the constitutively active heat shock protein cognate 70 (Hsc70) is developmentally lethal, whereas deletion of Hsp70i results in sterility of male mice, but no other overt phenotype (Dix et al., 1996). Hsp70i and Hsc70 are highly related, sharing 90% sequence identity, with most of the amino acid variability confined to the NBD. There is greater sequence divergence with respect to other Hsp70 family members (50-80% identity), especially within the NBD (Daugaard et al., 2007). The close sequence similarity between Hsp70i and Hsc70 has contributed to past difficulties in separating the two proteins' functions, both pharmacologically and with RNA interference approaches.

Structurally Hsp70 family members are similar, consisting of three functional domains, a nucleotide-binding domain (NBD) in the N-terminal region, a substrate-binding domain (SBD) in the C-terminal region, and a linker in the middle (Figure 1) (Howe et al., 2014). The SBD is comprised of a β sandwich, consisting of two twisted four-stranded β sheets, which form the pocket (Jiang et al., 2005; Mayer, 2010). The β
sandwich pocket is covered by a α-helix, composed of five helices, forming the lid. The chaperone activity of Hsp70 is a function of the C-terminus EEVD sequence in cooperation with other chaperones such as Hsp40, Hsp90, Hip, Hop, CHIP and Bag1 (Tavaria et al., 1996). Much of Hsp70 function and activity is regulated through interactions with co-chaperones. The J-domain co-chaperones, such as Hsp40, bind to the NBD of Hsp70 and assist in stimulating its ATPase activity (Hohfeld et al., 2001). The nucleotide exchange factor (NEF) co-chaperones, Bag-1, Hsp110, and Hip, catalyze the release of ADP to complete the ATP cycle (Hohfeld et al., 2001). The TPR domain co-chaperone Hop, is involved in the formation of Hsp70-Hsp90 complexes and assists in substrate transfer (Hohfeld et al., 2001). CHIP, another TPR domain co-chaperone, also mediates Hsp70-Hsp90 binding but may also ubiquitinate some Hsp70 substrate proteins through its ubiquitin ligase activity, which leads to protein degradation (Ramos, 2011).

Figure 1: Hsp70 Structure. (A) Structure of Hsp70 (E. coli, DnaK) in the ATP-bound conformation (PDB 4B9Q) and (B) in the ADP-bound apo conformation (PBD 2KHO). ATP represented in (A) as ball-and-stick model.
ATP hydrolysis is essential for Hsp70 function. ATP binding to Hsp70 occurs through the β and γ phosphates being submerged in the NBD and stabilized through interactions with the NBD subdomains and K⁺ and Mg²⁺ cations (Massey, 2010). Upon ATP hydrolysis, Hsp70 undergoes conformational changes, which are highlighted in Figure 1, that are regulated by nucleotide binding and the presence of substrate protein interactions (Evans et al., 2010). Recent nuclear magnetic resonance data has shown a high flexibility in the subdomains that make up the NBD, leading to an opening and closing of the nucleotide binding cleft, when nucleotide is released and bound respectively (Mayer and Bukau, 2005).

1.2 Hsp70 as an Anticancer Target

Current research has shown that there is a higher level of Hsp70i expression in malignant tumors, such as breast, prostate, and colon cancers, compared to non-malignant tissue (Shu and Huang, 2008). Hsp70i is up regulated in response to stress such as heat, hypoxia, and nutrient deprivation, stressors that are present in the tumor microenvironment (Shu and Huang, 2008). Furthermore, in general there is a correlation in heat shock protein overexpression and resistance to cancer therapeutics, highlighting the role of Hsp70i in promoting cancer cell survival under conditions that are otherwise lethal (Jego et al., 2013). The chaperone function of the heat shock proteins, assisting in the proper folding of proteins and the prevention of protein aggregates, is one method of providing cellular protection (Jego et al., 2013). Hsp70i regulates apoptotic pathways, both extrinsic and intrinsic apoptotic pathways (Goloudina et al., 2012; Jego et al., 2013). This occurs by preventing TNF-related apoptosis-inducing ligand formation of the death-induced signaling complex (DISC) through inhibition of death receptors 4 and 5,
as well as by inhibiting events in mitochondrial-mediated apoptosis (Guo et al., 2005b). Intrinsically, Hsp70i prevents cytochrome c release from the mitochondria through preventing Bax translocation (Yang et al., 2012). Hsp70i also prevents apoptosis through JNK inhibition as well as through binding to Apaf-1, which blocks recruitment of procaspase-9 to the apotosome (Beere, 2001; Park et al., 2001). Furthermore, Hsp70i activates Akt, which regulates signaling pathways involved in cell survival (Joly et al., 2010). Hsp70i also protects cancer cells from oncogenic stress induced by up-regulation of specific oncogenes such as HER2. Increased expression of Hsp70i correlates with resistance to chemotherapy and radiation and therefore poor clinical outcomes by providing cancer cells a route to survive and proliferate in the presence of noxious stimuli such as hypoxia or denatured protein aggregates. These data have led to the proposal that cancer cells are dependent on Hsp70i for survival (Goloudina et al., 2012). This hypothesis is supported by Hsp70i depletion studies in which tumor cell death and sensitivity to chemotherapeutic drugs were evident, while non-tumorigenic cell lines were unaffected by Hsp70i depletion (Nylandsted et al., 2002). Due to the evidence from these studies, much work has been done to develop inhibitors to target Hsp70i for potential cancer therapeutics.

1.2.1 Hsp90 as an Anticancer Target

Similar to what has been reported with Hsp70, there is an increased level of the protein chaperone, Heat Shock Protein 90 (Hsp90), expression in tumors, due to stressors in the tumor microenvironment, which promotes resistance to therapy and cellular survival (Barrott and Haystead, 2013). The chaperone function of Hsp90, to assist in the proper folding of proteins and the prevention of protein aggregates, also
aids in cancer cell survival (Jego et al., 2013). Hsp90 regulates extrinsic apoptotic pathways through inhibiting activation of the DISC through association with and transport of FLICE inhibitory protein to the DISC (Panner et al., 2007). Intrinsically, Hsp90 prevents cytochrome c release through forming a complex with Bcl-2 (Cohen-Saidon et al., 2006). Hsp90 binds to Apaf-1, preventing apoptosis through caspase inhibition (Pandey et al., 2000). Furthermore, Hsp90 regulates signaling pathways involved in cell survival through activating Akt (Joly et al., 2010).

Due to the role of Hsp90 in cancer pathogenesis, inhibitors targeting Hsp90 as anticancer therapeutics have been developed, and have had a great deal of success. Currently there are 17 Hsp90 inhibitors in clinical trials, from phase 1 to phase 3, for multiple types of cancer. The inhibitors are either derived from natural products such as 17-AAG and radicicol, or synthetic such as IPI-504, VER52296, PU-H71, and SNX-5422 (Barrott and Haystead, 2013; Fadden et al., 2010; Jego et al., 2013). Hsp90 inhibitors competitively bind the ATP binding site in the NBD, thereby preventing hydrolysis of ATP and Hsp90 function in vivo (Jego et al., 2013).

1.3 Hsp70 Inhibitors

While, several Hsp70 inhibitors have been developed, targeting Hsp70 with small molecule inhibitors has been faced with several challenges. First, there is close sequence identity between Hsp70i and Hsc70, making it difficult to discriminate between the family members. Additionally, the conformational state of Hsp70 may limit small molecule accessibility to the ATP binding site (Qi et al., 2013). When in the closed conformation there is little accessibility for NBD active-site accessibility by a small molecule. Furthermore, the polar interactions present in the NBD as well as the high
affinity for ADP, adds to the difficulty to target the ATP binding site (Massey, 2010). These complications have prevented current Hsp70 inhibitors from discriminating between Hsp70 family members and show little efficacy in vivo (Massey, 2010). Current Hsp70 inhibitors include NSC 630668-R/1, VER-155008, MKT-077, Pifithrin-μ, MAL3-101, and YK-5 (Figure 2) (Powers et al., 2010; Rodina et al., 2013). NSC 630668-R/1, inhibits ATPase activity but does not discriminate Hsp70i from Hsc70 (Fewell et al., 2001). VER-155008 shows broad specificity with other Heat Shock Protein family members, largely because it is a nucleotide derivative. It also contains two potentially labile, perhaps by design, benzyl groups (Massey, 2010). MKT-077 is a rhodacyanine dye that was identified to have anticancer activity, from a screen of compounds obtained from the Fuji Photo Film Co., Ltd. (Koya et al., 1996). MKT-077 binds the NBD of Hsp70 and interacts with the mitochondrial Hsp70 family member (Wadhwa et al., 2000). It was found that MKT-077 has selectivity for inhibiting proliferation in cancer cells and was tested in phase I clinical trials; however severe renal dysfunction was observed in patients, which halted additional trials (Britten et al., 2000; Propper et al., 1999). Pifithrin-μ (PES) has been shown to interact with the SBD of both Hsc70 and Hsp70i and disrupt client protein interaction in vitro (Leu et al., 2009). However, recent evidence suggests that the PES interaction with Hsp70 is through non-specific interactions (Schlecht et al., 2013). The molecule promotes caspase-dependent cell death only in tumor cells, suggesting some specificity to Hsp70i in vitro, although p53 binding has also been shown, which may explain its antitumor actions (Leu et al., 2009). Moreover, MKT-077 and PES have potential reactive groups that render them covalent modifiers, which may contribute to side effects in vivo. Due to these challenges, several recent efforts have focused on development of small molecule inhibitors of Hsp70i that target allosteric
sites, as opposed to the nucleotide binding site, to regulate its function (Assimon et al., 2013; Taldone et al., 2014). MAL3-101 is a second-generation derivative of NSC 630668-R/1 and has been shown to compromise co-chaperone-stimulated Hsp70 ATPase activity, suggesting it is an allosteric regulator, although the exact binding site of this molecule remains unknown (Braunstein et al., 2011). Like NSC 630668-R/1, MAL3-101 is quite large and has a number of labile ester groups. YK5 is an allosteric inhibitor of Hsp70, recently identified using modeling techniques, but this molecule does not discriminate between Hsp70i and Hsc70 (Rodina et al., 2013).

![Chemical Structures of Select Hsp70 Inhibitors](image)

Figure 2: Chemical Structures of Select Hsp70 Inhibitors
1.4 Molecular Chaperones in Viral Pathogenesis

Viruses are dependent on host factors for successful replication and propagation, due to the genome of many viruses being limited to a few proteins. Host factors that are utilized include protein chaperones, such as Hsp70 and Hsp90, and are used throughout the lifecycle of a variety of viruses’. Table 1 highlights a subset of the work showing the role of Hsp70 and Hsp90 in the life cycle of numerous viruses. The exploitation of Hsp70 and Hsp90 in viral propagation is similar to the roles of these proteins in cancer progression. Upon infection of a host cell a virus rapidly induces the production and synthesis of numerous viral proteins in a small timeframe. At this step in the viral lifecycle, Hsp70 and Hsp90 are utilized to ensure proper folding and function of the newly synthesized viral proteins. This utilization of heat shock proteins is highlighted by viral capsid proteins being assembled into an intricate structure, which yields a potential for the formation of aggregates (Cobbold et al., 2001). Proper folding and assembly of viral proteins illustrates the requirement for Hsp70 and Hsp90 in one step in the viral lifecycle. Furthermore, viruses need to rewire cellular signaling pathways to avoid host immune detection and to prevent apoptosis of a host cell to ensure production of mature virions. Hsp70 and Hsp90 are involved in the life cycle of many viruses including DNA viruses and positive and negative sense RNA viruses. The role of Hsp70 and Hsp90 in viral lifecycles is further demonstrated due to heat shock protein expression being induced following viral infection (Brenner and Wainberg, 1999). The role of Hsp70 and Hsp90 in a diverse set of viruses shows the potential for Hsp70 and Hsp90 as antiviral targets.
Table 1: Select Viruses that Utilize Hsp70 and Hsp90.

<table>
<thead>
<tr>
<th>Virus Family</th>
<th>Virus</th>
<th>Hsp70 role</th>
<th>Hsp90 role</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flaviviridae</td>
<td>DENV</td>
<td>Virus binding. Induction following infection.</td>
<td>Virus binding.</td>
<td>(Reyes-Del Valle et al., 2005); (Padwad et al., 2010)</td>
</tr>
<tr>
<td></td>
<td>HCV</td>
<td>Folding of capsid and NS5A proteins.</td>
<td>Maturation and preservation of NS3. NS5A association and replication complex formation.</td>
<td>(Choukhi et al., 1998); (Gonzalez et al., 2009); (Ujino et al., 2009); (Okamoto et al., 2006)</td>
</tr>
<tr>
<td></td>
<td>JEV</td>
<td>Colocalizes with replication complex and with NS3 and NS5 proteins.</td>
<td></td>
<td>(Ye et al., 2013)</td>
</tr>
<tr>
<td></td>
<td>YFV</td>
<td>Induced late in infection</td>
<td></td>
<td>(Lefeuvre et al., 2006)</td>
</tr>
<tr>
<td>Hepadnaviridae</td>
<td>HBV</td>
<td>Proper folding and assembly of L and envelope proteins.</td>
<td>Reverse transcriptase function.</td>
<td>(Cho et al., 2003); (Hu et al., 2004)</td>
</tr>
<tr>
<td>Herpesviridae</td>
<td>HSV</td>
<td>Mediate binding of initiator protein with origin of replication.</td>
<td>Proper folding and localization of HSV polymerase.</td>
<td>(Tanguy Le Gac and Boehmer, 2002); (Burch and Weller, 2005)</td>
</tr>
<tr>
<td></td>
<td>EBV</td>
<td>Virus dependent induction. Interaction with nuclear antigen-LP.</td>
<td>Virus dependent induction. Cell survival.</td>
<td>(Cheung and Dosch, 1993); (Kitay and Rowe, 1996); (Jeon et al., 2007)</td>
</tr>
<tr>
<td></td>
<td>VZV</td>
<td>Mediate replication through colocalization in replication complexes with viral DNA binding protein ORF29p.</td>
<td>Mediate replication through colocalization in replication complexes with viral DNA binding protein ORF29p.</td>
<td>(Kyratsous and Silverstein, 2007)</td>
</tr>
<tr>
<td>Orthomyxoviridae</td>
<td>Influenza</td>
<td>Component of Localization of</td>
<td></td>
<td>(Sagara and)</td>
</tr>
<tr>
<td>Family</td>
<td>Virus</td>
<td>Process</td>
<td>Protein/Region</td>
<td>References</td>
</tr>
<tr>
<td>-----------------</td>
<td>-------</td>
<td>--------------------------------------------------------------------------</td>
<td>--------------------------------------------------------------------------------</td>
<td>----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Papillomavirus</td>
<td>HPV</td>
<td>Increased expression following infection. Mediate genome replication. Virion assembly and disassembly.</td>
<td>polymerase subunits to nucleus.</td>
<td>Kawai, 1992; (Manzoor et al., 2014); (Naito et al., 2007)</td>
</tr>
<tr>
<td>Picornaviridae</td>
<td>Poliovirus</td>
<td>Forms complex with capsid P1 protein</td>
<td>Folding and maturation of capsid protein.</td>
<td>(Liao et al., 2005); (Lin et al., 2002); (Chromy et al., 2003)</td>
</tr>
<tr>
<td>Polyomaviridae</td>
<td>SV40</td>
<td>Induction following infection. Proper folding, localization, and assembly of capsid.</td>
<td>Induction following infection. Proper folding of large T antigen.</td>
<td>(Khandjian and Turler, 1983); (Chromy et al., 2003); (Miyata and Yahara, 2000)</td>
</tr>
<tr>
<td>Poxviridae</td>
<td>Vaccinia Virus</td>
<td>Induced following infection. Folding of viral proteins.</td>
<td>Folding of core protein.</td>
<td>(Sedger et al., 1996); (Jindal and Young, 1992); (Hung et al., 2002)</td>
</tr>
<tr>
<td>Reoviridae</td>
<td>Reovirus</td>
<td>Folding of sigma1 viral protein.</td>
<td>Folding of sigma1 viral protein.</td>
<td>(Leone et al., 1996); (Gilmore et al., 1998)</td>
</tr>
<tr>
<td>Reoviridae</td>
<td>Rotavirus</td>
<td>Part of receptor complex mediating viral binding.</td>
<td>Proper folding of nonstructural protein 3, leading to efficient viral replication.</td>
<td>(Dutta et al., 2011; Guerrero et al., 2002)</td>
</tr>
<tr>
<td>Retroviridae</td>
<td>HIV</td>
<td>Increased expression following infection. HIV transcription.</td>
<td>HIV transcription. Reactivation from latency.</td>
<td>(Wainberg et al., 1997); (O'Keeffe et al., 2000); (Anderson et al., 2014)</td>
</tr>
<tr>
<td>Rhabdoviridae</td>
<td>VSV</td>
<td>Component of virion. Folding of viral G</td>
<td>Stabilization of large subunit of VSV</td>
<td>(Sagara and Kawai, 1992); (de Silva et al., 1996)</td>
</tr>
<tr>
<td>Virus Family</td>
<td>Virus</td>
<td>Function</td>
<td>Interaction</td>
<td>Reference</td>
</tr>
<tr>
<td>-------------</td>
<td>-------</td>
<td>----------</td>
<td>-------------</td>
<td>-----------</td>
</tr>
<tr>
<td>Togaviridae</td>
<td>CHIKV</td>
<td>Part of receptor complex mediating virus binding and entry.</td>
<td>Interaction with viral proteins nsP3 and nsP4, mediating viral replication.</td>
<td>(Paingankar and Arankalle, 2014); (Rathore et al., 2014)</td>
</tr>
<tr>
<td>Measles virus</td>
<td></td>
<td>Replication, proper folding of N protein.</td>
<td></td>
<td>(Zhang et al., 2002); (Connor et al., 2007); (Paingankar and Arankalle, 2014); (Rathore et al., 2014)</td>
</tr>
</tbody>
</table>

### 1.4.1 Hsp90 as an Antiviral Target

Despite the number of Hsp90 inhibitors that have been developed and the previous studies showing that Hsp90 is involved in viral propagation, only a few small molecule Hsp90 inhibitors have been tested for antiviral activity. One Hsp90 inhibitor that has been shown to have antiviral activity is the natural product geldanamycin, which is a first generation Hsp90 inhibitor. Given that there is an observed antiviral effect with geldanamycin, this shows the possibility for recently developed less toxic synthetic small molecule Hsp90 inhibitors, such as PU-H71, SNX-5422, and ganetespib, to be used as antivirals (Barrott and Haystead, 2013; Jego et al., 2013). The current research using geldanamycin has further elucidated the role of Hsp90 in viral pathogenesis, has shown that Hsp90 is a *bona fide* antiviral target, and indicates the potential for Hsp90 inhibitors to be used as antiviral drugs. Geldanamycin has been used extensively as an anticancer agent and entered phase I clinical trials, which were subsequently suspended due to toxicity concerns (Supko et al., 1995). Recent evidence highlighting a role for Hsp90 in the lifecycle of many viruses has shown the potential for Hsp90 as an antiviral target and therefore the use of Hsp90 inhibitors as antiviral agents.
Several structural and nonstructural viral proteins from DNA and RNA viruses are dependent on Hsp90 for proper folding, activity, and maturation, and are therefore classified as clients of Hsp90. The HBV viral polymerase is a client of Hsp90 and Hsp90 activity is required for the polymerase function (Hu et al., 2004). This is supported by treatment with geldanamycin, leading to an inhibition in reverse transcription of pregenomic RNA into DNA and incorporation of pregenomic RNA into nucleocapsids from decreased polymerase activity. Proper folding and localization of the rotavirus nonstructural protein 3 (NSP3) is dependent on Hsp90 (Dutta et al., 2011). Treatment with 17-AAG or 17-DMAG, modified analogs of geldanamycin, results in reduced viral replication through loss of NSP3 nuclear localization (Dutta et al., 2011). The HCV viral proteins NS3 and NS5A are clients of Hsp90 and require Hsp90 for proper function (Okamoto et al., 2006; Ujino et al., 2009). 17-AAG or geldanamycin treatment leads to a decrease in HSV NS3 or NS5A protein levels, respectively, and subsequently a decrease in viral replication (Okamoto et al., 2006; Ujino et al., 2009). Hsp90 stabilizes the large subunit of the VSV polymerase (L protein), and treatment of infected cells with geldanamycin or radicicol leads to degradation of the L protein and a reduction in viral replication (Connor et al., 2007). Another nonstructural protein that is an Hsp90 client is the HSV polymerase, UL30, which is properly localized and stabilized by Hsp90 activity (Burch and Weller, 2005). Hsp90 inhibition, with geldanamycin, leads to mislocalized and degraded UL30 and therefore reduced viral replication and titer (Burch and Weller, 2005). The influenza A polymerase subunits PB1 and PB2 associate with and require Hsp90 activity for proper function, which is supported by geldanamycin and 17-AAG treatment leading to degradation in PB1 and PB2 and therefore a reduction in viral titer (Chase et al., 2008).
Hsp90 along with Hsp70, forms a complex that regulates the proper localization of the VZV DNA binding protein, ORF29p (Kyratsous and Silverstein, 2007). Disruption of this complex leads to improper localization and decreased VZV replication (Kyratsous and Silverstein, 2007). Another viral protein that is regulated by both Hsp90 and Hsp70 is the SV40 large T antigen (LT). Hsp90 has been shown to interact with the SV40 LT, and treatment with geldanamycin leads to a reduction in protein levels of LT in COS7 cells (Miyata and Yahara, 2000).

The structural proteins of several viruses are regulated by Hsp90 activity. In vaccinia virus, Hsp90 associates with its client, viral core protein 4a, and also aids in the replication of the virus (Hung et al., 2002). Geldanamycin treatment delays vaccinia virus replication and leads to a reduction in viral titer (Hung et al., 2002). Another client that Hsp90 properly folds, while in the immature conformation, is the reovirus protein sigma1. Geldanamycin treatment inhibits the formation of the mature sigma1 complex (Gilmore et al., 1998). The viral capsid protein of the Picornaviridae family of viruses, including poliovirus, rhinovirus, and coxsakievirus, is also a client of Hsp90. The mature conformation of the capsid protein is formed following cleavage of a precursor protein, and interaction with Hsp90 is required for successful cleavage of the precursor protein (Geller et al., 2007). Furthermore, treatment with geldanamycin or 17-AAG inhibits poliovirus, rhinovirus, and coxsakievirus replication (Geller et al., 2007). This occurs because the picornavirus capsid protein, P1, relies on Hsp90 for proper folding (Geller et al., 2007).

Hsp90 mediates HIV replication through formation of a complex, not through interaction with viral client proteins. Hsp90 along with Cdc37 form a complex with Cdk9/cyclin T1, which allows for interaction of RNA polymerase II with HIV protein Tat,
leading to viral transcription (O'Keeffe et al., 2000). Hsp90 inhibition with geldanamycin leads to disrupting the Cdk9/cyclin T1 complex and ultimately perturbing viral transcription (O'Keeffe et al., 2000). As mentioned previously, Hsp90 is involved in regulated intrinsic and extrinsic apoptotic pathways. Hsp90 is utilized by cancer cells to evade apoptosis when faced with cellular stresses, such as hypoxia or anticancer treatment (Jego et al., 2013). Following infection, viruses will utilize Hsp90 to prevent apoptosis (Geller et al., 2012). Furthermore, treatment with geldanamycin and its analogue 17-AAG have been shown to lead to cell death in NK/T-cell lymphoma cells infected with Epstein-Barr virus (Jeon et al., 2007). In human cytomegalovirus infected lung fibroblasts, geldanamycin treatment resulted in inhibited protein synthesis and viral titer, because Hsp90 inhibition led to inactivation of the Akt signaling pathway, which is critical for human cytomegalovirus propagation (Basha et al., 2005).

Furthermore, the Haystead lab has shown antiviral indications with the clinical candidate Hsp90 inhibitor, SNX-5422. Studies have shown that SNX-5422 potently inhibits HIV replication in CD4+ T cells, acting at several stages of the virus life cycle including viral trafficking, entry and integration (Unpublished Results). Additionally, a recent collaboration with the Vasudevan lab at Duke-NUS, showed that SNX-5422 and a close structural analog, called HS-10, potently inhibits CHIKV infection in both in vitro and in vivo models (Rathore et al., 2014). Novel interactions between CHIKV non-structural protein 3 and non-structural protein 4 with Hsp90 were observed in pull down experiments, and silencing of Hsp90 transcripts with siRNA were shown to disrupt CHIKV replication in cultured cells (Rathore et al., 2014). Finally, in a CHIKV mouse model, SNX-5422 dramatically reduced viral titers and reduced inflammation in severe infection as well as the associated musculopathy (Rathore et al., 2014).
1.4.2 Hsp70 as an Antiviral Target

As previously discussed, Hsp70 inhibitors have been developed for anticancer applications, while there has been little work done with the current inhibitors for use as antivirals. As Table 1 highlights, Hsp70 has far reaching roles in viral pathogenesis, which indicates that Hsp70 inhibitors could have potent antiviral activity. It has been observed in several viruses that there is an increase in Hsp70 expression following infection. In Epstein-Barr virus, Hsp70 mRNA is induced and subsequently Hsp70 protein levels following infection (Cheung and Dosch, 1993). Hsp70 gene expression is specifically induced following adenovirus, HSV, and SV40 infection, while expression of other stress-associated and heat shock protein genes are not induced (Mayer, 2005). There is also an observed increase in Hsp70 expression at the early stages of HIV infection, which leads to an increase in Hsp70 present on the cell membrane (Brenner and Wainberg, 1999). The presence of Hsp70 on the cell membrane may indicate a role in mediating binding and internalization of the virions. The initial increase in Hsp70 expression is reduced during viral replication, but is increased once again at the late stages of the viral lifecycle that correspond with virion release (Brenner and Wainberg, 1999). Additionally, Hsp70 is involved in folding, assembly, and localization of the HIV envelope protein and associates with the HIV p24 viral protein (Earl et al., 1991). Furthermore, Hsp70 is integrated into infectious HIV virions that are exported from cells, which correlates with increased Hsp70 expression late in the viral lifecycle (Brenner and Wainberg, 1999).

Hsp70 mediates virus binding as part of the receptor complex for multiple viruses. As previously mentioned, Hsp70 is found on the cell surface following HIV infection and possibly mediating viral binding and entry. Hsp70 has been identified as a
part of the receptor complex for rotavirus binding, and anti-Hsp70 antibodies inhibit rotavirus infection (Guerrero et al., 2002). The viral proteins VP4 and VP7, which are located on the surface of the virion, interact with Hsp70 during viral binding (Guerrero et al., 2002). The Hsp70 family member, GRP78, has been identified as part of the receptor complex, along with integrin α,β3, for coxsackievirus A9 and, GRP78 is essential both for coxsackievirus A9 binding and internalization into host cells (Triantafilou et al., 2002).

In addition to mediating viral entry, Hsp70 is involved in the viral life cycle in post-internalization steps. Hsp70 associates with the viral capsid protein of adenovirus serotype 2 after the virus has been internalized into cells (Niewiarowska et al., 1992). There is also a colocalization of Hsp70 with adenovirus particles in the nucleus of infected cells (Niewiarowska et al., 1992). This colocalization, between Hsp70 and the hexon viral protein, regulates viral DNA import into the nucleus (Saphire et al., 2000). Hsp70 is also involved in the replication steps of viral life cycles. Binding of the E1 helicase of human papillomavirus to the origin of replication is increased by interaction with Hsp70 and is dependent on the ATPase activity of Hsp70 (Liu et al., 1998). Hsp70 mediates replication of HSV-1 through association with the UL9 helicase protein. Association of UL9 with Hsp70 increases affinity of UL9 binding to the HSV-1 origin of replication and facilitates viral replication (Tanguy Le Gac and Boehmer, 2002). In addition to aiding viral binding and replication, Hsp70 also mediates viral protein folding. The HCV envelope proteins E1 and E2 assemble into a heterodimer, and aggregation can occur during this assembly (Choukhi et al., 1998). GRP78 has been shown to interact with E1 and E2 to prevent the aggregation of these proteins (Choukhi et al., 1998). Association with Hsp70 mediates the proper folding of the polyomavirus capsid
proteins, VP1, VP2, and VP3 (Cripe et al., 1995). This association occurs when Hsp70 colocalizes with VP1, VP2, and VP3 in the nucleus following polyomavirus infection (Cripe et al., 1995). Nascent chains of the reovirus attachment protein, sigma1, associate with and are folded properly by Hsp70 (Leone et al., 1996). Hsp70 as well as GRP78 associate with the large L envelope protein of HBV, which is a transmembrane protein, with cytosolic and ER domains (Lambert and Prange, 2003). The association with Hsp70 and GRP78 maintains proper folding of the L protein as well as a transmembrane orientation (Lambert and Prange, 2003).

Cellular transformation that occurs following viral infection is aided and regulated by Hsp70 activity. This transformation involves the virus reactivating the cell cycle in quiescent cells. The involvement of Hsp70 in this process has been shown in the SV40 virus (Mayer, 2005). The transforming activity of SV40 is through the viral large and small T antigen, TAg (Mayer, 2005). SV40 TAg contains a J domain, which allows for interaction with Hsp70. During viral transformation and to initiate replication, SV40 TAg associates with pRB, p107, p103, and the E2F transcription factors in a manner dependent on interaction with Hsp70 and Hsp70 ATPase activity (Mayer, 2005). This initiates viral replication and reinitiates the cell cycle. The activity of the HPV E1A viral protein is similar to that of SV40 TAg and E1A interacts directly with Hsp70 during cellular transformation (Mayer, 2005). The role of Hsp70 in cellular transformation during viral infection directly parallels its involvement in the transformation of cancer cells (Ciocca et al., 2013).

In addition to cellular transformation, Hsp70 aids in the survival of cells following viral infection. As mentioned previously, Hsp70 regulates extrinsic and intrinsic apoptotic pathways, which is also utilized by cancer cells faced with extracellular and intracellular
stresses (Jego et al., 2013). The induction of Hsp70 that is observed upon infection with numerous viruses may have multiple functions, including serving as a mechanism to escape apoptosis. This is essential for viral propagation, and viruses are known to inhibit many apoptotic pathways, from inhibiting caspase activation, cytochrome c release, and activation of the death receptors (Benedict et al., 2002). Hsp70 can prevent apoptosis in cancer cells through binding p53 (Zylicz et al., 2001). The same interaction with p53 has been observed with SV40 TAg, a viral protein previously mentioned that interacts with Hsp70 (Benedict et al., 2002). This interaction could provide viruses a Hsp70 mediated mechanism for evading apoptosis following infection.

As mentioned previously, the SV40 TAg contains a J domain in its N-terminal domain, which allows for interactions with Hsp70 (Mayer, 2005). Along with SV40 the poxvirus, Molluscum contagiosum virus consists of protein with a J domain (Moratilla et al., 1997). It has been shown that TAg is involved throughout the viral lifecycle and mutations that disrupt the J domain interacting with Hsp70 inhibit viral propagation (DeCaprio, 1999). This further highlights Hsp70 being required by viruses for efficient replication and propagation, since these viral proteins contain J domains, which stimulate Hsp70 ATPase activity.

Hsp70 has also been identified as a host factor that mediates dengue virus (DENV) infection, however the complete role of Hsp70i in the DENV life cycle remains to be elucidated. Previous studies have shown that siRNA knockdown of Hsp70 results in decreased DENV RNA copy numbers in supernatants and decreased intracellular DENV load (Padwad et al., 2010). Furthermore, Hsp70 was identified through affinity chromatography to be part of the DENV receptor complex, however it is not fully elucidated which member of the Hsp70 family member is part of the complex (Reyes-Del
Valle et al., 2005). Hsp70 family members as a part of the DENV receptor complex is supported by previous studies showing a reduction in DENV infected human and mosquito cells when pretreated with Hsp70 antibodies (Reyes-Del Valle et al., 2005; Vega-Almeida et al., 2013). Additionally, it has been shown that there is an increase in the number of DENV infected cells following heat shock (Chavez-Salinas et al., 2008). The increase in infection correlates with an increase in Hsp70 present on the cell surface, but the different Hsp70 family members are not discriminated (Chavez-Salinas et al., 2008). Therefore, the precise role of the inducible Hsp70 family member remains to be fully elucidated. Due to a lack of approved antiviral treatments or vaccines for DENV, understanding the role of DENV host proteins and development of inhibitors could translate into much needed DENV interventions.

1.5 Dengue Virus

DENV is the leading cause of arthropod transmitted disease in humans, consisting of four distinct serotypes (DENV 1-4), which are transmitted to humans through mosquito vectors, primarily Aedes aegypti and A. albopictus (Guzman et al., 2010; Kyle and Harris, 2008; Wilder-Smith et al., 2010). In 1970, DENV epidemics had only been reported in nine countries, but by 2009, DENV was reported to be endemic in over 100 countries worldwide, primarily in tropical and sub-tropical regions (Guzman et al., 2010). Figure 4 shows the current cases of DENV, as of 30 June 2015, as well as the country-associated risk for transmission, further highlighting the global burden of the disease (Adapted from HealthMap.org/dengue, accessed 30 June 2015). While DENV is not endemic to the continental United States, the virus is endemic in the United States territory Puerto Rico, and travel associated cases are frequently reported in the
continental United States. However, there are increasing reports of incidence of locally acquired cases, primarily in south Florida, but also in regions along the Texas-Mexico border and Hawai‘i, further illustrating the geographical spread of DENV into the United States (Franco et al., 2010). An estimated three billion people worldwide are at risk for infection and recent reports estimate that the number of infections each year could be close to 400 million (Bhatt et al., 2013; Guzman et al., 2010). Due to the expanding geographical distribution of DENV and the frequency of epidemics, the World Health Organization has classified DENV as a major international public health concern (Wilder-Smith et al., 2010).

Figure 3: HealthMap of current reports of Dengue cases, as of 30 June 2015; also showing risk areas by country from Absent in blue to Present in red. Adapted from HealthMap.org/dengue, accessed 30 June 2015.

Infection can manifest as the self-limited febrile illness known as dengue fever, or the potentially fatal dengue hemorrhagic fever (DHF) and dengue shock syndrome.
(DSS) (Back and Lundkvist, 2013). DHF results in bleeding, low levels of blood platelets, and blood plasma leakage. DSS is more severe than DHF and occurs when blood pressure drops to dangerously low levels. DENV infection results in at least 500,000 annual hospitalizations, while DHF and DSS are the leading cause of hospitalization and death of children in eight countries (Coller and Clements, 2011; Whitehead et al., 2007). Infection with one serotype elicits immunity to that serotype, but not to the others. However, secondary infection of another DENV serotype frequently results in DHF, by a process called antibody dependent enhancement (ADE) (Coller and Clements, 2011). This is believed to be due to non-neutralizing cross-reactive antibodies binding to virus particles and causing increased uptake of the virus during secondary infection. In addition to DENV effects on human health, there is also a significant impact on the economies of developing countries where the virus is endemic. In endemic countries, the burden of DENV is approximately 1,300 disability-adjusted life-years per million people, which is similar to other diseases in these regions, such as tuberculosis (Gubler and Meltzer, 1999).

Currently there are no approved therapies or vaccines for DENV (Bhatt et al., 2013). The primary method for controlling the spread of the virus is through management of the vector population, which has failed in endemic countries. This is due to the ability of A. aegypti to live in crowded urban environments as well as a lack of resources for battling the mosquito vector in endemic countries, which ultimately leads to unimpeded transmission of DENV (Morrison et al., 2008). While there are currently no approved vaccines, several candidates are currently in various stages of clinical development, which include a chimeric live attenuated, DNA, inactivated, infectious clone live attenuated, live attenuated, virus-like particles, and virus vector vaccines.
Recent results from a phase III clinical trial suggest a vaccine could be approved in the near future (Thomas, 2015; Villar et al., 2015). However, the biggest challenge faced in vaccine development is to ensure protection across all four DENV serotypes, which is further complicated by lack of a robust animal model to replicate the pathogenesis of severe dengue disease in humans (Guzman and Harris, 2014; Slifka, 2014; Thisyakorn and Thisyakorn, 2014; Thomas, 2014). If a vaccine fails to efficiently protect against one of the serotypes there is an increased possibility of severe dengue disease due to ADE. In addition to protecting against all four serotypes, the vaccine must elicit long-lasting protection, be affordable, and easily transported to vaccination sites (Guzman and Harris, 2014). The lack of vector control, which when coupled with the difficulties to develop a vaccine that protects against all four serotypes, stresses the need to develop novel and effective DENV therapeutics (Coller and Clements, 2011; Morrison et al., 2008).

DENV is a member of the genus *Flavivirus* with a genome of approximately 11kB that encodes for 3 structural proteins, the Capsid (C), premembrane/membrane (prM), and envelope (E), and 7 non-structural proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5 (Figure 5) (Ross, 2010). The 3 structural proteins are required for assembly into virus particles. NS3 is a serine protease and ATP-dependent helicase and NS5 is a methyltransferase and RNA-dependent RNA polymerase. NS2B is a cofactor for the NS3 protease and NS4B plays a role in inhibiting the interferon response (Ross, 2010). NS1 and NS4A are both involved in the viral replication process, but the exact role of these proteins remain to be elucidated (Miller
et al., 2007; Muller and Young, 2013). NS2A is involved in viral replication as well as the maturation of DENV virions (Xie et al., 2013).

![Diagram of DENV genome]

Figure 4: Schematic of DENV genome.

Upon being bit by an infected mosquito, DENV virions will bind to cell surface receptors in the newly infected human. One such attachment factor is dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) (Tassaneetrithep et al., 2003). While no putative DENV receptor has been identified, multiple receptors and attachment factors have been discovered (Cruz-Oliveira et al., 2015). Therefore it is suggested that there is a DENV receptor complex as opposed to a single DENV receptor, which is further supported by the ability of DENV to infect both mammalian and insect cells (Cruz-Oliveira et al., 2015). Following internalization, the viral RNA is released into the host cell cytoplasm. Initial translation of the viral RNA results in a single polyprotein, which is cleaved by viral and host proteases to yield the 10 DENV proteins (Back and Lundkvist, 2013). Replication of the viral RNA then ensues, with the newly synthesized RNA packaged into immature virions. An immature virion is trafficked through the trans-Golgi network, resulting in conformational changes in the membrane protein rendering the virion mature and infectious, which is finally exported from the host cell (Back and Lundkvist, 2013).

Due to the relatively small genome, host factors are utilized for efficient replication and propagation of DENV, and recent studies have aimed to identify potential
Targeting host proteins for antiviral therapeutics provides an advantage over targeting viral proteins because host proteins are not as susceptible to mutations that result in the development of drug resistant strains, which can occur when targeting viral proteins (Noble et al., 2010). As previously mentioned, one such host factor that has recently been identified that is involved in DENV pathogenesis, but precise role remains to be elucidated, is the Hsp70 family of proteins, including Hsp70i and Hsc70. Development of inhibitors targeting Hsp70i and better understanding the role of Hsp70i in DENV pathogenesis, could lead to important therapeutic interventions for reducing the global burden of DENV.

1.6 Conclusion

There are several lines of evidence, which were previously discussed, suggesting that Hsp70 is a viable target for anticancer therapy and more recently as a viable antiviral target for a wide variety of viral infections. However, further development of Hsp70 inhibitors is warranted to see a clinical impact from targeting Hsp70 for anticancer and antiviral treatments. Hsp90 has been established as an anticancer target and previous studies suggest Hsp90 as a viable antiviral target as well. As previously mentioned, Hsp90 inhibitors have been extensively developed, indicating a potential clinical impact in the near future from targeting Hsp90 for antiviral therapy. Furthermore, in the case of Hsp90, there is a two-decade clinical history with inhibitors targeting the protein for multiple indications in cancer (Neckers and Workman, 2012). Over 38 clinical trials for 17 Hsp90 inhibitors have been conducted in the US alone, from phase 1 to phase 3, and many experts in the field expect FDA approval for one or more of the
synthetic Hsp90 inhibitors in the very near future. From our discussion therefore, it would seem that there might be much to gain by testing one or more Hsp90 inhibitors that are efficacious in cancer as antiviral drugs against viral infections such as HIV. This may lead to an immediate clinical impact, since the human toxicities and pharmacological properties of most clinically relevant Hsp90 inhibitors are very well understood. It is also likely that the therapeutic window to achieve antiviral activity in vivo would be considerably lower than used to treat tumors. Typically, most cancer trials involve dosing patients are close to the maximum tolerated dose. One would imagine when used as an antiviral, an Hsp90 inhibitor would be used at lower dose over an acute period to clear an infection. As previously mentioned, one of the first effective antivirals, AZT, for HIV came from a cancer program (Broder, 2010). Additionally, in the case of AZT, dosing levels for antiviral activity were considerably lower than used to achieve efficacy for cancer (Chow et al., 2009).

A major advantage of targeting Hsp70 and Hsp90 as opposed to viral proteins is the issue of drug resistance. Although many successful antiviral drugs have been developed, they are all subject to development of drug resistance because of a high mutation rate that is seen in certain viruses, such as RNA viruses (Domingo and Holland, 1997). These mutations can lead to changes in viral proteins and render antiviral drugs ineffective (Pillay and Zambon, 1998). To offset drug resistance, combination therapies have been developed in which two or more virally encoded proteins are targeted simultaneously. This has been very successful in managing long term HIV treatment, although is not infallible, and even the most compliant of patients can develop resistance (Pennings, 2013). For these reasons, many groups have aimed to identify host factors that would be viable alternatives for antiviral therapy (Lou et al.,
2014; Tayyari and Hegele, 2012; Zeisel et al., 2013). Hsp70 and Hsp90, acting in their cellular chaperone capacity as well as involvement in regulating a wide range of cellular pathways, may make them ideal host antiviral targets. The fact that neither protein would be under the direct genetic control of any invading virus renders them essentially immune from resistance arising through a single point mutation, as typically occurs with most antivirals targeting virally encoded proteins (Richman, 2006). Indeed, there is some evidence that viruses may never develop resistance to Hsp90 inhibitors. For example, poliovirus, rhinovirus, and coxsackievirus do not develop resistance to Hsp90 inhibitors in vitro and poliovirus does not develop resistance in vivo (Geller et al., 2007).

Furthermore, there has been no reported resistance to Hsp90 inhibitors that lack the chemical backbone that is found in 17-AAG, 17-DMAG, and IPI-504, when used as anticancer drugs (Trepel et al., 2010). Indeed, with respect to drug resistance, there are many parallels between cancer and viral infections where Hsp90 is concerned, and this is also likely to also be true with Hsp70. In cancer, cellular Hsp90 and Hsp70 are often exploited by oncogenes, which use its chaperone functions to stabilize the oncogenically expressed proteins (Barrott and Haystead, 2013; Jego et al., 2013). If one inhibits Hsp90 or Hsp70 function, the expressed oncogene becomes unstable and the cancer cells cease to grow (Neckers and Workman, 2012; Powers et al., 2008). Drug pressure in this case is therefore not directed at the oncogene itself, but at the heat shock proteins. Similarly, in the case of Hsp90 and Hsp70 acting as antiviral targets, drug pressure would be outside the immediate genetic control of the virus. Under these circumstances, more than a single point mutation would therefore be required to promote resistance to an Hsp90 or Hsp70 inhibitor. Therefore, the host cell may have to involve a new drug
transporter or adapt an existing metabolic enzyme to effect drug resistance when placed under selective pressure with an Hsp90 or Hsp70 inhibitor.

The Haystead laboratory is certainly not alone in thinking about Hsp70 and Hsp90 as antiviral targets. The development of Hsp70 and Hsp90 as antiviral targets and their inhibitors as antiviral drugs was the focus of a meeting at the NIH in 2013 titled “Protein Homeostasis and Viral Infection: From Mechanisms to Therapeutics”. The meeting highlighted current work targeting both Hsp70 and Hsp90 in a variety of viruses, including HIV, poliovirus, DENV, and EBV. Much of the research thus far has been with geldanamycin and the second-generation derivatives, 17-AAG, and 17-DMAG. These compounds have been shown to elicit antiviral activity, which indicates that the potent and selective synthetic Hsp90 inhibitors will also yield antiviral activity. As discussed, unlike their natural product counterparts, many synthetic Hsp90 inhibitors have shown to be well tolerated in humans, with some close to FDA approval for certain cancers. While Hsp70 inhibitors have not been as extensively developed as Hsp90 inhibitors, current research showing the parallel role of Hsp70 in oncogenesis and in the life cycle of many viruses’, highlights the potential for these inhibitors as well. Therefore, we predict that in the near future Hsp70 inhibitors could have an immediate and dual impact in the clinic as an antiviral and anticancer therapeutics.
2. Experimental Procedures

2.1 Identification of an Allosteric Small Molecule Inhibitor Selective for the Inducible Form of Hsp70

2.1.1 Fluorescence linked enzyme chemoproteomic strategy (FLECS) Screen

A pEGFP-tagged Hsp70i (plasmid 15215, Addgene, Cambridge, MA) was used in the FLECS assay and was originally cloned by Evan Eisenburg (Zeng et al., 2004). ATP used in the assay was purchased from Sigma (St. Louis, MO) and a 200 mM stock was prepared with low salt buffer (150 mM NaCl, 25 mM Tris, pH 7.5, 60 mM MgCl2). The γ-phosphate ATP sepharose was synthesized as previously described and stored in low salt buffer (Carlson et al., 2013). FuGENE 6 transfection reagent (Roche, Mannheim, Germany) was used for transfection of GFP-Hsp70i into HEK 293T cells, following the manufacturer protocol. The transfection ensued for 48 hours, upon which time the cells were harvested and lysed in cell lysis buffer (150 mM NaCl, 50 mM Tris, pH 7.5, 1% Triton X-100, 1 mM EDTA (Ethylenediaminetetraacetic acid), 1 mM DTT (Dithiothreitol), and 1 tablet Complete Mini protease inhibitor (Roche)). Cell lysates were stored at -80°C until further use. Upon binding the resin lysates were washed 3x with high stringency wash buffer (1 M NaCl, 25 mM Tris, pH 7.5, 60 mM MgCl2, 1 mM DTT) and 3x with low stringency wash buffer (150 mM NaCl, 25 mM Tris, pH 7.5, 60 mM MgCl2, 1 mM DTT). Next the lysates were transferred to 0.2 µm PVDF (polyvinylidene difluoride) filter 96-well plate (Corning, Corning, NY) sitting on top of a black flat-bottomed 96-well catch plate (Corning). The plates were spun down using an Eppendorf Centrifuge 5810 (Hamburg, Germany) at 2000 rpm for 2 min.
2.1.2 Endogenous Hsp70 Elution

Pig bladder tissue was used for eluting endogenous Hsp70 from the γ-phosphate ATP sepharose resin. Tissue was homogenized in liquid nitrogen and stored at -80°C until further use. Tissue mass was measured (grams) and then 2.5 X volume (mL) of tissue lysis buffer (50 mM HEPES, 60 mM MgCl2, 60 mM KCl, 1 mM DTT) was added and homogenized on a laboratory blender. A total of 25 grams of homogenized tissue was typically used for each experiment. Subsequent to lysis, the tissue was centrifuged at 35,000 RPM in a Beckman (Brea, CA) Type 45 Ti rotor for 45 minutes at 4°C, and then filtered over silica wool. The filtered supernatant was then added to the γ-phosphate ATP sepharose resin and washed as previously described in the FLECS screen. Compounds were used to elute Hsp70 and the elutions were then separated by SDS-PAGE and analyzed by Western blot or the gels were visualized by silver stain.

2.1.3 Caspase 3/Caspase 7 Assay

The Amplite™ Fluorimetric Caspase3/7 assay kit (AAT Bioquest, Sunnyvale, CA) was used per the manufacturers instructions. Briefly, a fluorometric indicator, Ac-DEVD-7-Amino-4-methylcoumarin (AMC), was used to determine caspase activity. Cleavage of AMC by caspases resulted in a fluorescent signal that can be assessed at 440-460nm with an excitation of 340-350nm. Cells were seeded at 60,000 cells/well in a 96 well plate and treated with compound for the indicated period of time. Diluted caspase 3 and caspase 7 assay solution was added to each well and incubated at room temperature for 2 hours protected from light, upon which time fluorescence was measured on the Victor X2 plate reader (Perkin Elmer, Waltham, MA).
2.1.4 Degradation Assay

The specified cells were seeded overnight and then treated with the indicated concentration of compound for 24 hours. The cells were then harvested and subjected to analysis by Western blot.

2.1.5 Protein Purification

Jason Gestwicki (University of California, San Francisco) generously provided plasmids for human HSPA1A (Hsp70i), HSPA8 (Hsc70), and HSPA1A C306D mutant. Rosetta competent cells were transformed with plasmids and single colonies were picked from streaked LB/ampicillin/chloramphenicol plates. Cultures were grown at 37°C for 4-6 hours, were cooled to 15°C and expression was induced overnight with 200µM isopropyl 1-thio-β-D-galactopyranoside. Cells were pelleted and resuspended in Ni-lysis buffer (50mM Na2HPO4, 300mM NaCl, 10mM Imidazole, 0.05% Tween-20) supplemented with Complete Mini protease inhibitor tablets (Roche, Mannheim, Germany) and 1M DTT, and then sonicated. The cells were again pelleted and the supernatant was incubated with cOmplete His-Tag purification resin (Roche, Mannheim, Germany) for 5 hours at 4°C. The resin was washed with Ni-wash buffer (50mM Na2HPO4, 300mM NaCl, 20mM Imidazole, 0.05% Tween-20) and eluted with Ni-elution buffer (50mM Na2HPO4, 300mM NaCl, 250mM Imidazole, 0.05% Tween-20). The elution was then incubated with tobacco etch virus protease overnight at 4°C. The following day the elution was incubated with the γ-phosphate ATP sepharose resin for 30 minutes at 4°C. Next the resin was washed with ATP-wash buffer (50mMTris-HCl, 60mM MgCl2, 60mM KCl, 10mM Citrate) supplemented with 1mM DTT and then ATP-wash buffer supplemented with 2mM ATP was added to the resin to elute the protein. The final
elutions were concentrated in Amicon Ultra-15 Centrifugal Filter Units (EMD Millipore, Billerica, MA) and buffer exchanged into 25mM HEPES, 5mM MgCl$_2$, 10mM KCl (pH 7.5) and stored at -80°C until use.

2.1.6 Thermofluor Assay

SYPRO orange (Molecular Probes, Eugene, OR) was diluted 1:1000 in 25mM HEPES, 5mM MgCl$_2$, and 10mM KCl (pH 7.5), and purified Hsp70i, Hsc70, Hsp70i C306D, or Hsp90 was then added to a final dilution of 0.04 mg/ml. Where indicated 0.001% or 0.01% Triton X-100 (Sigma) was also added. The indicated compound or Dimethyl sulfoxide (DMSO) was then added at the specified concentration and each sample was added as 5 replicates to wells of a 384 well plate (BioRad, Hercules, CA). A melt curve protocol (25°C to 90°C, increasing 0.5°C and a plate reading every 30 seconds) was run on a CFX384 Touch™ Real-Time PCR Detection System (BioRad).

To determine the midpoint of the protein unfolding transition or $T_m$, GraphPad Prisim4 (La Jolla, CA) was used to normalize the melt curve and to calculate the first derivate of the melt curve, with the steepest point of the slope being the $T_m$.

2.1.7 ATPase Assay

Single turnover assays were performed as previously described (Fewell et al., 2004). Briefly, Hsp70 was incubated with $^{32}$P-ATP and cold ATP in single turnover buffer (1M KCl, 250mM HEPES, 110mM MgOAc) for 30 minutes on ice. $^{32}$P-ATP-Hsp70 complex was purified from a Nick Column-Sephadex G-50 (Amersham, Piscataway, NJ). Glycerol was added and the complexes were stored at -80°C. To determine ATP hydrolysis, a sample was thawed and added to single turnover buffer containing Hlj1 and compound added after 60 seconds. At the specified time points an aliquot of the reaction
is removed, added to stop solution (2M LiCl, 4M formic acid, 36mM ATP) and spotted on a thin-layer chromatography (TLC) plate. The percentage of ATP hydrolyzed to ADP and \( P_i \) was then calculated.

### 2.1.8 Docking Studies

HS-72 was docked into the crystal structure of the Hsp70i nucleotide-binding domain (NDB) bound to AMP-pnp (PDB: 2E8A) using the SwissDock program. The returned clusters were distributed between two binding sites. Chimera was used to visualize the putative binding sites of HS-72 on the Hsp70i NBD.

### 2.1.9 Partial Proteolysis

Hsp70i (8µg for SDS-PAGE analysis, 2µg for mass spec analysis) was incubated with 1mM ATP, 1mM ADP, 100µM HS72, 100µM HS72 + 1mM ATP, or 100µM HS72 + 1mM ADP for 30 min. at room temperature. Hsp70i was digested by adding 0.1µg of trypsin (Promega, Madison, WI) per 2µg of protein and was quenched by addition of 25% trifluoroacetic acid for mass spec analysis or addition of 5x SDS loading buffer and boiling for SDS-PAGE analysis at 2 hours, or 24 hours. Gels were visualized by silver stain.

### 2.1.10 Mass Spectrometry

Tryptic peptides were subjected to matrix-assisted laser desorption-ionization mass spectrometry (MALDI-MS) on an ABSCIEX TOF/TOF (time of flight) 5800 mass spectrometer. Positive mode time of flight was used to identify peptides, and individual peptides were sequenced by MS/MS. All sequence and peptide fingerprint data was searched using the UniProt database.
2.1.11 Limited Proteolysis

Limited proteolysis was performed as previously described (Seguin et al., 2012). Briefly, 4µg of purified Hsp70i was incubated with HS-72, DMSO, and/or the indicated nucleotide for 20 minutes on ice. 1.8ng of Proteinase K was added and incubated at 37°C for 5 minutes. The reaction was quenched with 100% Trichloroacetic acid (TCA) and incubated for 10 minutes on ice. Reactions were centrifuged for 10 minutes at 13,000 rpm at 4°C and the supernatants were removed. The pellets were resuspended in TCA sample buffer (80 mM Tris HCl pH 8, 8 mM EDTA, 120 mM DTT, 3.5% SDS, 0.29% glycerol, 0.08% Tris base, 0.01% bromophenol blue), separated by SDS-PAGE, and visualized by silver stain.

2.1.12 Aggregation Assay

The PC12 rat neuronal cell line, which expresses Huntington exon 1 containing 74 glutamine repeats, is fused to GFP (httQ-GFP), and is under the control of a doxycycline promoter, was used. Cells were treated with HS-72 for 18 hours prior to a doxycycline addition for 48 hours. The soluble and pellet fraction were then separated by centrifugation at 14,000 rpm for 15 minutes and both fractions were assayed for httQ-GFP by solubilizing with SDS followed by western blotting with antibodies against GFP.

2.1.13 Cell Proliferation

Cell proliferation was determined using a Hoechst stain (Sigma) to quantify DNA. 5,000 cells of the designated cell line were plated in 96 well plates and treated the next day, designated as time point 0, with the indicated concentration of the specified compound maintained for the duration of the assay. At the indicated time points the media was removed and plate frozen at -80°C. Double distilled H₂O was then added and
the plates were incubated at 37°C for 1 hour. After 1 hour the plates were frozen at -80°C. After freezing, the plates were thawed and Hoechst stain was diluted 1:1000 in TNE buffer (10mM Tris, 2M NaCl, 1mM Na<sub>2</sub>EDTA). The final fluorescence was measured on the Victor X2 plate reader at 355/460 nm, and cell proliferation was determined with the formula: % Cell Proliferation = 100 x (Sample fluorescence – background fluorescence) / (Control fluorescence – background fluorescence).

2.1.14 HS-72 in vivo Studies

2.1.14.1 Maximum Tolerated Dose (MTD) and Blood Workup

The 5 cohorts consisted of 3 mice each given HS-72 BiW and administered Intraperitoneal (i.p.) in DMSO at 1, 5, 10, 20 and 30mpk using female FVBs aged to 10 weeks. Body Mass was measured weekly and the mice were monitored for signs of toxicity as per Mouse Phase 1 Unit (MP1U) standard protocol. For blood analysis, 4 mice were injected i.p. with HS-72 on day 1 and 4, with blood drawn on day 5. 4 mice receiving no treatment were used as controls.

2.1.14.2 HS-72 Pharmacokinetics (PK)

Wild-type mice were injected i.p. with HS-72 and sacrificed 5 minutes, 1 hour, 4 hours, 8 hours, and 24 hours post injection. Untreated animals were included as control and called 0 minutes. Each time point consisted of 3 animals. At the indicated time points liver, kidney, and blood was harvested from each animal. Whole blood was centrifuged and only the plasma was retained. Whole liver and kidneys were frozen and stored at -80°C until processing. Before quantifying HS-72 in the plasma and tissue a standard curve was made using HS-72 and HS-156, a close structural analogue to HS-72 as the internal standard. HS-72 and HS-156 were diluted in water and were further
diluted 1:4 in acetonitrile. The resulting solution was then filtered through a 0.2µm Polytetrafluoroethylene (PTFE) membrane (VWR, Radnor, PA). LCMS analyses were performed on an Agilent 2100 LC system (Santa Clara, CA) connected to an Agilent 6311 Ion Trap Liquid chromatography–mass spectrometry (LCMS). Samples were injected (15 µL injection volume) onto an Agilent Eclipse Plus C18 column (4.6 mm i.d. x 150 mm, 5 µm particle size) at room temperature. Mobile phases A (0.2% formic acid in water) and B (0.2% formic acid in acetonitrile) were mixed to form a gradient of 5%-100% B over 9 minutes. MS analysis was performed in positive ionization mode (Nebulizer 50 psi, Dry Gas 12.0 L/min, Dry Temperature 350 °C, Capillary 31 nA, Ramp Range 4.5-1.5 kV). The detection mass target was 350 m/z and the scan range was 100-1000 m/z. Data was analyzed on Bruker Data Analysis software for 6300 Series Ion Trap v.4.0 sp2. Extracted ion chromatograms (EIC) were generated for target masses [M+H]⁺, m/z 337 and [M+H]⁺, m/z 365 and peak areas were determined using standard integration protocol or manual integration. The total ion chromatogram (TIC), UV chromatogram, and mass spectra (MS) were also obtained for each run. The area under the curve from the EIC for HS-72 [M+H]⁺, m/z 365 and for HS-156 [M+H]⁺, m/z 337 was determined. The EIC area ratio for HS-72 compared to HS-156 was calculated and this ratio was used to plot a standard curve based on the known concentrations of HS-72.

The plasma samples were processed by diluting 1:4 in acetonitrile, and filtered through a 0.2µm PTFE membrane. 15µL of the solution was run through an Eclipse Plus C18 column and analyzed by an Agilent Ion Trap 6130 LC-MS. The resulting EIC ratios from HS-72 compared to HS-156 were then determined, which was used to calculate the concentration of HS-72 in the diluted sample. The concentration in solution of HS-72 was adjusted for the 1:4 dilution that occurs during sample preparation. The final
concentration of HS-72 in plasma was calculated per mL of plasma. The liver and kidney samples were weighed and homogenized in tissue lysis buffer. The resulting homogenate was then processed and analyzed in the same manner as the plasma samples. The resulting EIC ratios from HS-72 compared to HS-156 were then determined, which was used to calculate the concentration of HS-72 in the diluted sample. The concentration in solution of HS-72 was adjusted for the 1:4 dilution that occurs during sample preparation. Final concentration of HS-72 in the kidney and liver was calculated per gram of tissue using the weight of each tissue measured before sample processing. The information for the raw files for all samples is located in Appendix D.

2.1.14.3 Efficacy Studies

Mouse Mammary Tumor Virus (MMTV)-neu mice, a HER2 overexpression breast cancer mouse model in which HER2 is under the transcriptional control of the mouse mammary tumor virus promoter/enhancer, were treated with the indicated doses and dosing schedule (Taneja et al., 2009). All doses were delivered through i.p. injections using DMSO and their tumors were calipered once weekly. The mice were culled upon reaching tumor burden or if they expressed signs of toxicity as per MP1U standard protocol.

2.2 HS-72 Demonstrates Anti-Dengue Virus Activity, Validating Hsp70i as a Host Antiviral Target.

2.2.1 Dengue Virus Proteomic Profiling

Huh7 cells were infected with DENV at a multiplicity of infection (MOI) of 10. 2 hours before cell harvest, cell media was spiked with 440µCi of $[^{35}S]$-methionine and
[35S]-cysteine (Easy tag EXPRESS®; Perkin Elmer, Waltham, MA). Cells were lysed in lysis buffer (0.1% Triton; 150mM NaCl; 60mM MgCl₂; 25mM Tris-HCl, pH 7.5; 1mM DTT, 1µM Microcystin (Cayman Chemical, Ann Arbor, MI), and 1x protease inhibitor tablet (Roche, Mannheim, Germany)), clarified by centrifugation, and incubated with a γ-phosphate ATP-affinity resin. The resin was washed 3x with low salt wash buffer (50mM Tris, pH 7.5; 150mM NaCl; and 60mM Mg Cl₂) and bound proteins were removed from the resin by boiling in 5x SDS running buffer. Samples were subjected to SDS-PAGE and analyzed by silver stain. The gel was then dried and analyzed by autoradiogram for seven days. Indicated bands were excised and identified by MALDI-MS on an ABSCIEX (Framingham, MA) TOF/TOF 5800 mass spectrometer.

2.2.2 Flow Cytometry

U937+DC-SIGN cells were plated at 3 x 10⁵ cells/well and treated with compounds or antibody where indicated. The duration of antibody pretreatment with Hsp70i, DC-SIGN, or a nonspecific antibody was 2 hours prior to addition of DENV. Cells were then infected with DENV at a MOI = 1, where indicated. To inactivate DENV, an equivalent of a MOI = 1 of DENV was heated at 55°C for 30 minutes prior to addition to cells, where indicated. After a 1 hour incubation, virus media was removed and compound containing media was added. For antibody pretreatment, media only was added following virus incubation. Compounds were maintained for duration of experiments. At the indicated time points post-infection, cells were incubated in Fixable Viability Dye eFluor® 780 (eBioscience, San Diego, CA) following the manufacture protocol. Cells were washed 2x in PBS + 0.5% bovine serum albumin (BSA) and fixed in 2% Paraformaldehyde (PFA). Where indicated, cells were permeablized with ice-cold methanol. Cells were incubated in primary antibody followed by the matching Alexa
Flour® 488 (Invitrogen, Carlsbad, CA) secondary antibody. Samples were collected using the BD Facs Canto II (BD bioscience, Franklin Lakes, NJ). Data was analyzed using FlowJo version 10 software (FlowJo, LLC, Ashland, OR). Non-specific isotype control antibody (Thermo Scientific, Rockford, IL) was used as a control.

2.2.3 Foci Forming Assay

Virus culture supernatant from U937+DC-SIGN cells treated with the indicated compounds was added to Vero cells in 10-fold serial dilutions. Vero cells were incubated for 1 hour with virus media. After this incubation, 200μL of 1:1 tragacanth gum/2x Eagle’s Minimum Essential Media (EMEM) overlay with 2% FBS was added to each well and incubated for 4 days. The overlay solution was removed and cells were fixed with 4% PFA in PBS, permeabilized in 0.5% Triton X-100, and blocked in PBS + 0.1% tween and 1% normal donkey serum (Millipore; Temecula, CA). Viral foci were identified using the DENV E protein antibody 4G2 followed incubation with Alexa Fluor® 488 secondary antibody. Foci were visualized using an AMG EVOS® FL Imaging System (Life Technologies, Carlsbad, CA).

2.2.4 Time of Addition Assay

U937+DC-SIGN cells were plated at 3 x 10⁵ cells/well. At the indicated time points, cells were treated with HS-72 (75μM). At time point zero, cells were then infected with DENV at a MOI = 1. After a 1 hour incubation, virus media was removed. HS-72 was maintained from the time of treatment for duration of the assay. Cells were fixed in 4% PFA, permeabilized in PBS + 0.5% Triton X-100, and blocked in PBS + 1% normal donkey serum. Infected cells were identified using the DENV E protein antibody 4G2 followed incubation with Alexa Fluor® 488 secondary antibody. 4′,6-diamidino-2-
phenylindole (DAPI) was used to designate cells. Cells were imaged using the Cellomics Array Scan VTI system (Thermo Scientific). Percent infection and cell number were determined using vHCS Scan software version 5 (Thermo Scientific).

2.2.5 Attachment and Entry Assay

U937+DC-SIGN cells were plated at 3 x 10^5 cells/well and treated with HS-72 (75µM) for 1 hour, cells were then cooled to 4°C, and DENV was added at a MOI = 1. After 1 hour, RNA was extracted from cells, which corresponds to the attachment stage. Separately, cells were warmed to 37°C for 1 hour, and RNA were extracted, which corresponds to the entry stage of the life cycle. According to the manufactures instructions, RNA was extracted using the Arum RNA kit (BioRad) and cDNA was generated using the iScript cDNA synthesis kit (BioRad). cDNA along with primers and SYBR® Green Supermix (Biorad) were added according to the manufactures instructions for qPCR. qPCR was run at an initial 95°C for 5 minutes, then 40 cycles of 95°C for 15 seconds and 60°C for 30 seconds, on a CFX384 Touch Real-Time PCR Detection System (BioRad). The fold change of viral RNA vs. GAPDH, which served as the control, was determined. Primers are as follows: DENV-FWD 5’-AATATGCTGAAACGCGAGAGA-3’, DENV-REV 5’-GGGATTGTTAGGAAACGAAGG-3’, GAPDH-FWD 5’-GAGTCAACGGATTTGGCTCGT-3’, and GAPDH-REV 5’-TTGATTTGGAGGGATCTCG-3’.

2.2.6 Biotin Labeling of Surface Proteins

U937+DC-SIGN cells were plated at 3 x 10^5 cells/well and treated with HS-72 (75µM) where indicated. Cells were then infected with DENV at a MOI = 1, where indicated. At the indicated time point post-infection, cells were put on ice in a 4°C cold
room to maintain proteins on the cell surface. Modification of a previously published protocol was used for biotin labeling (Gabriel et al., 2009). Briefly, cells were washed 3x with PBS\(^{2+}\) (PBS + 1.5mM MgCl\(_2\), 0.2mM Ca Cl\(_2\)) and incubated with Sulfo-NHS-SS-biotin (Thermo Scientific) 2x for 15 minutes. Free biotin was quenched by incubating 2x for 15 minutes in PBS\(^{2+}\) supplemented with 100mM glycine. Cells were lysed in RPIA buffer (10mM Tris, pH 7.4; 150mM NaCl; 1 mM EDTA; 0.1% SDS; 1% Triton; 1% sodium deoxycholate) supplemented with 1x protease inhibitor tablet (Roche). Protein concentration was determined and 50µg of protein is incubated with Avidin sepharose resin. The resin was washed with RIPA buffer, incubated with 2x Laemmli sample buffer (BioRad) to cleave the NHS-SS-biotin disulfide bond. The eluted proteins yielded the membrane bound fraction, while the cellular fraction was protein that remained unbound after incubation with the avidin-sepharose resin, were subjected to SDS-PAGE and western blot analysis.

### 2.2.7 Proximity Ligation Assay (PLA)

U937+DC-SIGN cells were plated at 3 x 10\(^5\) cells/well and treated with HS-72 (75µM) where indicated. Cells were then infected with DENV at a MOI = 1, where indicated, attached to poly-lysine treated cover slips (VWR, Radnor, PA), and fixed in 4% PFA. Cells were washed in PBS, incubated in wheat germ agglutinin conjugated to Alexa flour 488 (WGA-488; Life Technologies) to stain cell membranes, blocked in 5% normal goat serum (Abcam; Cambridge, MA), and incubated with primary antibodies. Proximity Ligation Assay (PLA) reagents were used according to the manufactures instructions (Sigma). Briefly, PLA probes were added to samples, followed by the addition of ligation solution to hybridize and link the probes in a circle. The ligated circle serves as the template for rolling circle amplification (RCA) through addition of
polymerase along with fluorescently labeled oligonucleotides, which will hybridize with
the RCA product and yield a fluorescent signal. The cover slips were then mounted with
mounting medium containing DAPI to slides and imaged using a Zeiss Axio Imager
widefield fluorescence microscope. To quantify images: the percentage of cells with red
punctae was quantified by counting cell number as well as the number of cells with red
punctae for 5 separate fields of view.

2.3 Cell Lines, Antibodies, and Virus Preparation

HEK-293T (ATCC® ACS-4500™), MCF7 (ATCC® HTB-22™), HeLa (ATCC®
CCL-2™), HepG2 (ATCC® HB-8065™), T47D (ATCC® CRL-2865™) and NF639
(ATCC® CRL-3090™) cell lines were obtained from American Type Culture Collection
(ATCC) and were grown in Dulbecco’s Modified Eagle Medium (DMEM) supplemented
with 10% Fetal Bovine Serum (FBS). African green monkey Vero cells (ATCC CCL-81)
and Aedes albopictus C6/36 cells (ATCC CRL-1660) were obtained from the ATCC.
Vero cells were maintained in DMEM media supplemented with 10% FBS and 1x
penicillin/streptomycin. C6/36 cells were maintained in Minimum Essential Media (MEM)
supplemented with 10% FBS, 1% HEPES, 1% sodium pyruvate, 1% non-essential
amino acids, and 1x penicillin/streptomycin. BT474, SkBr3, LNCaP, and RWPE1 cell
lines were provided by Donald McDonnell (Duke University) and were grown in RPMI-
1640 medium supplemented with 10% FBS and non-essential amino acids. MCF-10A
cell line was provided by Donald McDonnell and were grown in DMEM/F12 medium
supplemented with 5% horse serum, 0.02% Epidermal Growth Factor (EGF), 0.05%
Hydrocortisone, 0.01% Cholera Toxin, 0.1% Insulin, and 1% penicillin/streptomycin.
PC12 cell line expressing httQ74-GFP was provided by Dennis Thiele (Duke University)
and were grown in DMEM supplemented with 5% FBS, 10% horse serum, 100ug/ml G418, 75ug/ml Hygromycin B, and 100U/ml penicillin/streptomycin plus supplements (Neef et al., 2010). U937+DC-SIGN cells were provided by Aravinda de Silva (University of North Carolina at Chapel Hill) and were grown in RPMI-1640 media supplemented with 10% heat inactivated FBS, 1% L-glutamine, 50µM beta-mercaptoethanol, 1% non-essential amino acids, and 1x penicillin/streptomycin. Huh7 cells were provided by Mariano Garcia-Blanco (University of Texas Medical Branch) and were grown in DMEM media supplemented with 10% FBS and 1x penicillin/streptomycin. All cell lines were maintained at 37°C in an atmosphere of 5% CO2, except for C6/36, which was maintained at 28°C and 5% CO2.

GFP, Her2, Akt, Hsp70i, Hsc70, Grp78, Grp75, Hsp90, DC-SIGN, and GAPDH antibodies were purchased from Cell Signaling Technology (Danvers, MA). The 4G2 mouse monoclonal antibody for DENV Envelope (E) protein generously provided by Mariano Garcia-Blanco (University of Texas Medical Branch). Dengue Virus 2-NGC (DENV) stocks were prepared by incubating 500µL of DENV in 4.5mL serum free media on confluent C6/36 cells. After 1 hour incubation, virus media was replaced with complete growth media supplemented with 2% FBS and 5mM HEPES. Cells were incubated for 72 hours and cell media was collected and centrifuged at 4,000 rpm for five minutes. The supernatants were saved and stored at -80°C. Virus titer was quantified by foci forming assay.

2.4 Western Blotting
SDS-PAGE was carried out using the Criterion™ Cell system using pre-casted 4-20% or 4-15% Criterion™ Tris-HCl gels (BioRad, Hercules, CA). For Western blotting gels were
run at 200V for 1 hour using the PowerPac basic power supply (BioRad, Hercules, CA). Next, gels were transferred to nitrocellulose for blotting at 100V for 1 hour (Fisher Scientific, Waltham, MA). Nitrocellulose membranes were blocked with 5% dry non-fat milk in phosphate-buffered saline (PBS) with 0.01% Tween-20 (PBS-T) or with 5% dry non-fat milk in Tris-buffered saline (TBS) with 0.01% Tween-20 (TBS-T) for 1 hour at room temperature. Membranes were incubated with primary antibodies overnight at 4°C. The next day membranes were washed 3X in PBS-T or TBS-T, based on manufacture recommendations, incubated for 1 hour at room temperature with secondary antibodies, and further washed 3X in PBS-T or TBS-T. ECL Plus Western blotting reagent (Pierce Biotechnology, Rockford, IL) was used to detect antibodies. Where indicated, bands were quantified using ImageJ software (Schneider et al., 2012).

2.5 Compounds

HS-72 was synthesized in house and described in Appendix A (Howe et al., 2014). HS-72 resin was synthesized in house and described in Appendix B (Howe et al., 2014). HS-10 was synthesized in house as previously described (Hughes et al., 2012). VER-15508, pifithrin-µ (PES), and Ribavirin were purchased from Sigma and resuspended in DMSO. ATP and ADP were purchased from Sigma and resuspended in low salt buffer (150 mM NaCl, 25 mM Tris, pH 7.5, 60 mM MgCl2).

2.6 Statistical Analysis

All statistical analysis performed using GraphPad Prisim4 (La Jolla, CA). Significance determined, by t test unless otherwise specified, as p < 0.05. Where indicated, * p<0.05; *** p<0.001; compared to control. Thermofluor data was analyzed using a one-way ANOVA with a Newman-Keuls posttest, mean ± SEM. All proliferation
data was analyzed using a two-way ANOVA with a Bonferroni posttest, mean ± SEM. Tumor growth analysis was analyzed using a two-way ANOVA with a Bonferroni posttest, comparing the HS-72 treated animals and control animals at each time point, mean ± SEM. Tumor growth was also analyzed by linear regression to compare the slope of the lines between the HS-72 treated animals and control animals.
3. Identification of an Allosteric Small Molecule Inhibitor Selective for the Inducible Form of Hsp70.

3.1 Identification of the Hsp70i Inhibitor, HS-72.

As previously described, Hsp70i is utilized by cancer and viral infections for efficient propagation, due to Hsp70i regulating similar pathways involved in the progression of both disease states. Current inhibitors are not able to discriminate the different Hsp70 family members, in particular Hsp70i and Hsc70. From a drug discovery perspective, Hsp70i presents a number of challenges, not the least of which being its close sequence identity with Hsc70. Specific, physiological substrates of Hsp70i are poorly defined and high throughput assays based on chaperone or trafficking activities are limited (Kang et al., 2008). As previously described, when Hsp70i is in the closed conformation, there is limited ability for small molecule inhibitors to target the nucleotide-binding site. In cells, Hsp70s may be reminiscent of small G proteins in which the nucleotide-binding pocket is always occupied, undergoing GTP/GDP exchange upon activation, again limiting small molecule accessibility. In the case of Hsp70i, the protein has high affinity for ADP, which is likely exchanged with ATP through allosteric regulation (Powers et al., 2010). Crystallographic and nuclear magnetic resonance studies have also shown that co-chaperone interactions induce altered conformational states (Evans et al., 2010). Because of these many complications, most Hsp70 inhibitors have either failed to discriminate between various Hsp70 family members or perform poorly in vivo (Massey, 2010).

To identify novel Hsp70 inhibitors that discriminate Hsp70i from Hsc70 and other family members, we used a fluorescence linked enzyme chemoproteomic strategy.
(FLECS) to screen an in house library of 3379 purine like molecules (Carlson et al., 2013).

### 3.1.1 Confirmation of GFP-Hsp70i binding to the ATP Resin.

For screening of Hsp70i inhibitors by FLECS, GFP-Hsp70i was expressed in HEK293 cells, extracts prepared, incubated directly with γ-phosphate-linked ATP-Sepharose and then eluted with ATP or ADP (Figure 5). These studies demonstrated that the GFP-fusion protein has a fully functional nucleotide-binding pocket and that binding occurs in a reversible manner. Based on the crystal structure of the Hsp70i homologue DnaK with bound ATP, showing limited solvent accessibility, recovery of the fusion protein on γ-linked ATP resin was at first surprising (Figure 1A). This is because the γ-phosphate oxygen on the immobilized ATP is tethered to polyethylene glycol (PEG), which is expected to sterically hinder Hsp70i binding. We therefore propose that the fusion protein is recovered through binding Hsp70i in the apo or ADP-bound form, which is subsequently driven into the ATP-bound conformation when exposed to the γ-linked ATP resin (Figure 1B). Once bound, the protein is retained because of an inability to hydrolyze the PEG-linked phosphate. The dramatic differences in elution between ATP compared to ADP shown in Figure 1A are consistent with this hypothesis. Once bound, exposure to low µM [Mg²⁺ATP] enables the protein to turn over and be released. In contrast, mM [ADP] was required to compete the bound fusion protein from the immobilized nucleotide. These findings suggested an opportunity to use the FLECS approach to identify selective inhibitors of Hsp70i that either act competitively at the ATP binding site or allosterically to regulate nucleotide binding.
Recombinant GFP-Hsp70i is readily captured on γ-phosphate linked ATP resin and is competitively released with ATP/ADP, consistent with nucleotide induced conformational changes.

3.1.2 FLECS Screening Yields Highly Selective Hsp70i Interactors.

Assembly of our focused library, consisting of 3379 purine like compounds, was described previously (Carlson et al., 2013). Figure 6 describes FLECS, illustrating how individual compounds are screened in parallel against the ATP medium charged with GFP-Hsp70i. Briefly, GFP-Hsp70i was expressed in HEK 293T cells and crude cell lysate was then added to the ATP resin (Figure 6A). Following several wash steps the bound GFP-Hsp70i was plated along with the compounds from the library in 96-well filter plates with ATP serving as a positive control, or with buffer to serve as a negative control (Figure 6B-C). The lysates were eluted from the filter plate onto a catch plate by centrifugation and the fluorescence of the eluates was measured (Figure 6D-E). Those compounds that disrupted the Hsp70i-ATP association resulted in an increased fluorescence signal over the buffer only samples.
The primary screen identified 197 hits from the library, which were sorted based on fluorescence readings and illustrated in Figure 7A, with dark red indicating strongly fluorescent compounds and light yellow indicates readings near or at background. The 197 were also sorted by their specificity towards GFP-Hsp70i over other purinome members that had also been screened against the same chemical library by FLECS, which is illustrated in Figure 7B, showing compounds that are active in multiple assays are gray-blue to dark red, while compounds that are active only in a single screen are light blue, highlighting the specificity of FLECS. The compounds that were active in multiple assays were removed from consideration.
Next, the presence of GFP-Hsp70i in the eluates from the 197 primary hits was determined by Western blot. This reduced the collection to 60 compounds and also eliminated auto fluorescent false positive molecules (Figure 8A). Next we tested the ability of the 60 compounds for elution of native Hsp70i from the ATP resin using pig bladder extracts, a rich source of native Hsp70i (Figure 8B). This reduced the final collection to 22 diverse structures (0.65% of the library), showing selectivity towards both recombinant human and native mammalian Hsp70i (Figure 9).

Figure 7: (A) Screening of Hsp70 by FLECS identified 197 based on fluorescence readings. (B) 197 hits sorted by specificity for Hsp70i against the entire FLECS library. (C) Structure of HS-72 and location on the (A) Hsp70 screen array and the (B) FLECS library array.
Figure 8: (A) Western blotting confirmed presence of Hsp70i in 60 hits. (B) 22 hits were confirmed by Western blot to elute endogenous Hsp70i. (A-B) Final lead, HS-72 is highlighted by box.
3.1.3 Identification of a Caspase-Activating Cell-Permeable Compound that Targets Hsp70i.

To further narrow the number of compounds, we first tested the 22 hits for their ability to activate caspase-3 and caspase-7 in various cancer cells, which is a hallmark of Hsp70i inhibition (Beere, 2001). Of the compounds tested, HS-72 ((S)-N-(1-propyl-1H-benzo[d]imidazol-2-yl)-1-(pyrazin-2-yl)piperidine-3-carboxamide) was most robust, inducing caspase activation in a dose dependent manner (Figure 10A). Other compounds were either less potent in this assay, or were cell impermeable and were not
pursued herein. The structure of the lead hit, HS-72, is shown in Figure 7C, while the
synthesis procedure for HS-72 is described in Appendix A. Furthermore, caspase
activation by HS-72 was reproducible across several cancer cell lines in a dose
dependent manner, at 6 hours (Figure 10B) and 24 hours (Figure 10C). As a second test
we examined the effect of HS-72 on the expression of Akt and HER2, two known client
proteins of Hsp70i, in BT474 breast cancer cells. Figure 10D shows dose dependent
reduction in Akt and HER2 with HS-72 treatment.
Figure 10: HS-72 shows cell permeability and hallmarks of Hsp70i inhibition through caspase-3/7 activation and substrate degradation assays. (A-C Mean ± SEM).
3.2. Characterization of HS-72.

The selective nature of HS-72 for the inducible Hsp70 family member is shown through Hsp70i elution from the ATP affinity resin along with Hsp70i binding an affinity resin created with the HS-72 scaffold. Using the thermofluor assay coupled with molecular modeling and native protein digestion studies, we highlight the allosteric interaction of HS-72 with Hsp70i.

3.2.1 HS-72 Selectively Targets Hsp70i Over Other Members of the Hsp70 Superfamily.

To test the selectivity of the HS-72 scaffold against the broader purinome, HEK 293T cell extracts or pig bladder tissue lysates were applied to the ATP resin and eluted with HS-72, as described for FLECS. The eluates were characterized by SDS-PAGE, silver stain, and MS analysis. Silver stain analysis for both HEK 293T cells and pig bladder lysates, confirmed elution of native Hsp70i and showed only a few non-specifically eluted proteins, indicating that HS-72 has a high degree of specificity within the wider purinome (Figure 11A-B). To more thoroughly determine the specificity of HS-72 for Hsp70i, the HEK 293T eluates were also analyzed for other Hsp70 family members Hsc70, Grp78, and Grp75, as well as Hsp90 by Western blot. This showed selective elution of Hsp70i by HS-72, with ATP serving as a positive control, showing elution of Hsp70 family members and Hsp90 (Figure 11C).
Next, an affinity resin using the HS-72 scaffold was synthesized in house. The synthesis procedure for the HS-72 affinity resin is described in Appendix B. HEK 293T cell lysate was applied to the resin and subjected to several washes, as described for FLECS. To confirm selectivity of the HS-72 affinity resin for Hsp70i, free HS-72 was
incubated with lysate to inhibit Hsp70i binding to the affinity resin. The linker on the resin was cleaved using sodium dithionite and the samples were subjected to SDS-PAGE and analyzed by Western blot, silver stain, and mass spectrometry (MS). When probing for Hsp70i by Western blot, the HS-72 affinity resin binds Hsp70i and free HS-72 is able to block this interaction (Figure 12A). Importantly, when probing for the closely related Hsp70 family member, Hsc70, the HS-72 affinity resin does not pull down Hsc70 (Figure 12B). This indicates that the HS-72 scaffold is highly selective for the inducible Hsp70 over the constitutively active Hsc70. Furthermore, silver stain and MS analysis reveals the HS-72 affinity resin pulls down Hsp70i, and this association is blocked with free HS-72, with very few non-specific interactions (Figure 12C). While Hsp90 is also pulled down, as shown in Figure 12C, this is a non-specific interaction with the media itself because the association is not blocked by free HS-72. These studies identified HS-72, a small molecule that can selectively discriminate Hsp70i from other members of the Hsp70 superfamily.
3.2.2 HS-72 is an Allosteric Inhibitor of Hsp70i.

Because the initial isolate of HS-72 was a racemic mixture, the molecule was resynthesized in its R and S enantiomeric forms. Figure 13 shows the S enantiomer more effectively elutes GFP-Hsp70i from ATP resin than the R enantiomer (referred to herein as HS-71). To characterize the S and R enantiomers in more detail we tested their effects on the thermal stability of purified Hsp70i and Hsc70 (Figure 14).
Figure 13: R and S enantiomer elution of GFP-Hsp70i from ATP resin.

Figure 14: Purification of (A) Hsp70i and (B) Hsc70.

The Thermoflour assay is used to show direct binding of a small molecule as measured by a change in melting temperature ($T_m$), (Cummings et al., 2006). In general, ATP competitive inhibitors impart a large degree of thermal stability to purine binding proteins because of the number of potential contacts within the nucleotide-binding pocket (Cummings et al., 2006). For example, Figure 15A shows that HS-10, an inhibitor
of Hsp90, increases the $T_m$ of this chaperone from 50°C to 60.5°C. Similarly, incubation of purified Hsp70i with ATP or ADP increased the $T_m$ by 4-5°C (Figure 15B).

![Graph A](image1.png)

**Figure 15:** (A) The Hsp90 inhibitor, HS-10, and (B) ATP/ADP increase the $T_m$ of Hsp90 and Hsp70i, respectively.

However, when we repeated the study with HS-72, the $T_m$ of Hsp70i decreased in a dose dependent manner by 0.1°C and 0.5°C, at 10µM and 100µM respectively (Figure 16A). Conversely, HS-71 had no effect on $T_m$ of Hsp70i, indicating that any effects observed with HS-72 cannot be explained by artifacts in the Thermofluor assay, such as non-specific ionic interactions or hydrophobic binding or fluorophore quenching (Figure 16B). The effect of HS-72 on thermal stability was more obvious when the experiment was repeated in the presence of ATP, with a decrease in $T_m$ of 0.5°C and 1°C at 10µM and 100µM respectively, while HS-71 had no effect on $T_m$ (Figure 16C-D). Significantly, HS-72 had no destabilizing effect on the ADP bound form (Figure 16E). Moreover, detergents had no effect on the ability of HS-72 to destabilize Hsp70i, eliminating the possibility of non-specific protein aggregation (Figure 16F).
Figure 16: (A) HS-72 decreases the $T_m$ of Hsp70i, (C) which is amplified in the presence of ATP, (B, D) while HS-71 has no Effect on $T_m$. (E) HS-72 has no effect on the $T_m$ of Hsp70i in the presence of ADP. (F) Detergents had no effect on the ability of HS-72 to destabilize Hsp70i.

Furthermore, when HS-72 was tested with purified Hsc70 in the Thermofluor assay, the compound failed to trigger a significant shift in Hsc70 $T_m$ in the presence or absence of ATP (Figure 17A-B). This further supports the selective nature of HS-72 for Hsp70i. Taken together these data suggest that although HS-72 is directly binding and selective for Hsp70i, its site(s) of interaction are unlikely to be in the ATP binding pocket.
This hypothesis is consistent with data showing that HS-72 does not directly inhibit ATP hydrolysis in single turnover assays with Hsp70i (Figure 18). Based on our Thermofluor data the most likely mechanism of HS-72 destabilization is via allosteric binding, which reduces the protein’s affinity for ATP. To explain HS-72’s action in this context, we hypothesize that upon binding to the ATP bound state the molecule induces large conformational changes, breaking a number of internal stabilizing contacts between the NBD and C terminal domain. This mechanism of action is also reminiscent of a small molecule allosteric inhibitor that decreases the $T_m$ of RGS4 (Blazer et al., 2010).

Figure 18: HS-72 does not reduce Hsp70 ATPase activity in the presence or absence of co-chaperone, Hlj1. Hsp70 + DMSO and Hsp70 + Hlj1 + DMSO indicated
Hsp70 + HS-72 is in green, and Hsp70 + Hlj1 + HS-72 is in red. Hlj1 + DMSO is indicated by dashed lines with squares.

3.2.3 Hsp70i C306D Mutation Perturbs HS-72 binding.

Miyata et al., using site directed mutagenesis, showed that Hsp70i C306 is a potential allosteric regulatory site within the NBD (Miyata et al., 2012). Interestingly, C306 is not conserved amongst other Hsp70 family members, including Hsc70. Consistent with this earlier work, Figure 19A-B shows that the $T_m$ of the Hsp70i C306D mutant was insensitive to HS-72, either in the presence or absence of ATP. The lack of effect of HS-72 on the thermal stability of Hsp70i C306D suggests that the molecule interacts either directly with C306 or that mutation of this residue results in a conformational change that renders the HS-72 binding site inaccessible.

Figure 19: HS-72 does not change the $T_m$ of Hsp70i C306D mutant ± ATP.

3.2.4 HS-72 Allosteric Interaction Induces a Conformation Change in Hsp70i.

In an attempt to gain some insight how HS-72 might be interacting with Hsp70i, we conducted a docking study of HS-72 with the crystal structure of the human NBD of Hsp70i containing AMP-pnp (PDB: 2E8A) using the SwissDock program (Grosdidier et al., 2011). Docking revealed 37 clusters, which were distributed between two binding
sites on either side of the bound ATP analogue, with the two stick structures representing HS-72 and AMP-pnp is shown as a space-filling molecule in the center, further supporting an allosteric mechanism of action (Figure 20A-B).

**Figure 20: Docking reveals two potential allosteric sites of HS-72 interaction with Hsp70i.**

Along with the docking studies we used partial proteolysis to identify potential sites of interactions. Partial proteolysis, visualized through silver stain, reveals a profound difference in the proteolytic pattern of Hsp70i in the presence of HS-72, which indicates that HS-72 induces a conformational change in Hsp70i over several time points, ranging from 2 hours to 24 hours, in the presence of trypsin (Figure 21A-B) to 15 minutes in the presence of Proteinase K (Figure 21C). Furthermore, MS analysis of the proteolytic pattern revealed specific residues that are protected from trypsin digestion upon inhibitor binding. Specifically, after 24 hours, peptides 141-155, 326-342, and 518-533, were all present in the samples treated with HS-72, but absent in the samples lacking HS-72 (Figure 21B and Appendix C). Furthermore, the residues that are
protected from trypsin digestion are highlighted in red in Figure 20A-B. We propose that the conformational change induced by HS-72 results in sequences 141-155, 326-342, and 518-533 to be protected from digestion. Collectively, these studies yield some insight as to the potential mode of HS-72 interaction with Hsp70i. The molecular docking studies reveal two putative binding sites that are distinct from the sequences that were protected from trypsin digestion. Therefore it is likely that HS-72 is inducing a conformational change in Hsp70i that alters surface exposure to trypsin and therefore protects the identified sequences from trypsin digestion.

Figure 21: Native protein digestion studies supports HS-72 as an allosteric inhibitor of Hsp70i. Full length Hsp70i indicated with black arrow and fragments resulting from proteolysis indicated with blue arrows.
To further investigate HS-72 sites of interaction, HS-72 was tested in combination with VER-15508 (VER) or pifithrin-µ (PES) by Thermofluor. There was an increase in Hsp70i $T_m$ with VER, consistent with previous work by Massey et al. showing binding of this compound in the active site of the NBD (Massey, 2010). When testing HS-72 and VER in combination there was no observed synergistic or additive effect on Hsp70i $T_m$ in the absence or presence of ATP, indicating that these molecules do not target Hsp70i at the same sites (Figure 22A-B). This further supports an allosteric binding site of HS-72, since VER is known to bind the active site in the NBD. When testing PES alone there was no dose dependent effect on Hsp70i $T_m$ (Figure 22C). Furthermore there was no synergistic or additive effect when testing HS-72 and PES in combination, indicating different binding sites on Hsp70i (Figure 22C-D). This indicates that HS-72 is not targeting the Hsp70i SBD, the putative primary site of PES binding (Leu et al., 2009).
Figure 22: HS-72 does not bind Hsp70i in the same sites as (A-B) VER or (C-D) PES due to no additive or synergistic effects on the T_m of Hsp70i in Thermofluor. (Mean ± SEM).
3.3. HS-72 Shows Hallmarks of Hsp70i Inhibition in vitro and Efficacy in a MMTV-neu Breast Cancer Mouse Model.

3.3.1 HS-72 Induces Cellular Protein Aggregation.

A hallmark of Hsp70i inhibition in cells is induction of protein aggregation, which was assayed using a cell culture model of Huntington’s disease. In this model, the PC12 rat neuronal cell line contains 74-glutamine repeats from exon 1 of human Huntington, fused to GFP (httQ74-GFP) (Wyttenbach et al., 2001). The httQ74-GFP is expressed stably and is inducible through a doxycycline-regulated promoter. We found an induction in protein aggregates in the presence of HS-72 compared with untreated controls, shown by an increase in the insoluble associated pellet fraction (Figure 23A). The pellet fraction is designated “P”, while the soluble fraction is designated “S”. Quantification of these bands shows a 50% increase in the insoluble associated pellet fraction in the HS-72 treated samples compared to untreated controls (Figure 23B).

Figure 23: HS-72 inhibits Hsp70i activity in a Huntington’s cell model.
3.3.2 HS-72 Inhibits Cancer Cell Proliferation.

Upregulation of Hsp70i has been implicated in tumorigenicity in breast and prostate cancers (Goloudina et al., 2012; Shu and Huang, 2008). To determine if HS-72 discriminates between various cell lines we carried out proliferation assays. Figure 24A-F shows that the inhibitor has potent anti-proliferative activity against the tumorigenic breast and prostate lines while the non-tumorigenic lines continued to proliferate in the presence of HS-72. There was a significant inhibition (p<0.001) of proliferation in all tumorigenic cell lines tested (Figure 24A-D). In contrast, the non-tumorigenic MCF10A cells continue to grow at all concentrations, while the non-tumorigenic RWPE1 cells were only inhibited at the highest concentration tested (Figure 24E-F). HS-71 treatment results in no effect on proliferation at 24 hours, which is a less potent effect when compared to HS-72, consistent with biochemical studies (Figure 25A-B). In SkBr3 cells there is an inhibition in proliferation at 48 and 72 hours at 25µM and 50µM (Figure 25A). In MCF7 cells there is inhibition at 48 hours at 25µM and 50µM, while at 72 hours significant inhibition is proliferation is observed in 50µM alone (Figure 25B).
Figure 24: HS-72 inhibits Hsp70i activity across multiple tumorigenic cell lines. (Mean ± SEM).
Figure 25: HS-71 minimally inhibits cancer cell proliferation. (Mean ± SEM).

3.3.3 HS-72 Acts Synergistically with HS-10.

To test if HS-72 acts synergistically with Hsp90 inhibitors we tested the effect of the Hsp90 inhibitor HS-10 in combination with HS-72 on the degradation of HER2 and Akt, which are classified as substrates or clients of Hsp70i and Hsp90, respectively. In the presence of HS-72, there is degradation of HER2 and Akt that is consistent with previous results (Figure 10 and 26). HS-10 alone also induced degradation of HER2 and Akt, as well as increased Hsp70 protein levels as expected, due to the negative regulatory role that Hsp90 has on Heat Shock Transcription Factor 1 (Figure 26). In combination of HS-72 and HS-10, the levels of HER2 were completely abolished and there was significant Akt degradation (Figure 26).
Next we determined the effect of the inhibitor combination on SkBr3 and MCF7 cell proliferation. Increasing amounts of HS-72 in addition to the HS-10 treatment resulted in an additive effect, potently inhibiting the proliferation of both cell lines more so than HS-72 or HS-10 alone (Figure 27A-B). These results add to the growing evidence that Hsp90 and Hsp70i inhibitor combinations are likely to have great therapeutic utility in the clinic (Guo et al., 2005a; Powers et al., 2009).
3.4 HS-72 is Bioavailable and Shows Efficacy in a Spontaneous Mouse Mammary Tumor Model.

3.4.1 MTD and Complete Blood Workup Shows HS-72 is not Toxic in Wild-Type Mice.

Prior to testing the efficacy of HS-72 in vivo, we performed a preliminary experiment to assess dose dependent effects in wild type mice. Healthy wild-type mice were dose escalated biweekly for 35 days and no adverse events, reduction in body weight, or altered behavior were observed up to 30 mg/kg (mpk) (Figure 28A). Additionally, a complete blood workup, which includes a complete blood count (CBC) analysis, a liver test, and a kidney test, was done following HS-72 treatment in wild-type mice. CBC analysis, which analyzes wbc (White blood cell count), LYMF (lymphocytes), rbc (red blood cell count), Gran + Mono (Granulocyte + Monocyte count), hct (hematocrit), and hgb (hemoglobin), following HS-72 treatment shows no adverse effect of HS-72 treatment (Figure 28B). A liver test analyzing ast (aspartate transaminase), alt (alanine transaminase), alb (albumin), and alkp (alkaline phosphatase), following HS-72
treatment also shows no adverse effects (Figure 28C). Finally, a kidney test analyzing 
bun (blood urea nitrogen), cl (chloride), na (sodium), k (potassium), and crea 
(creatinine), following HS-72 treatment also shows no adverse effects (Figure 28D).

These data indicate that HS-72 is not toxic to wild-type mice.

Figure 28: (A) MTD and (B-D) blood workup show no toxicity from HS-72 in 
 wild-type mice.
3.4.2 Pharmacokinetic and Distribution Studies Show HS-72 is Bioavailable in Wild-Type Mice.

A limited pharmacokinetic (PK) study was also performed using wild-type mice, analyzing and quantifying HS-72 in the plasma, liver, and kidney. Each sample was spiked with an internal standard, HS-156, which is a close structural analogue to HS-72, and analyzed by LC-MS (Figure 29).

![Figure 29: Structure of internal standard, HS-156, used for PK study analyzing HS-72 in plasma, liver, and kidney.](image)

The ratio of the extracted ion chromatogram (EIC) comparing HS-72 to HS-156 was plotted on a standard curve to quantify [HS-72] present in plasma, kidney, and liver samples (Figure 30A-C; access information for raw data files described in Appendix D). Concentration in solution for all samples adjusted for a 1:4 dilution factor that was used when processing the samples. Final concentration of HS-72 in plasma was calculated per mL of plasma (Figure 30A). Final concentration of HS-72 in the kidney and liver was calculated per gram of tissue using the weight of each tissue measured before sample processing (Figure 30B-C).
Figure 30: (A) Plasma, (B) kidney, and (C) liver samples plotted on a standard curve.

From the calculations using the standard curve, Figure 31A indicates that HS-72 is exponentially cleared from the plasma ($T_{1/2} \text{ elimination} = 0.4 \pm 0.1 \text{ hr; n=3; SEM}$) reaching $0.07 \pm 0.03 \text{ mmol/ml (n=3; SEM)}$ by 5 minutes post-intraperitoneal (i.p.) injection, clearing to $0.002 \pm 0.0002 \text{ mmol/ml (n=3; SEM)}$ by 8 hours, with only trace amounts detectable (<10 nmol/ml) by 24 hours. In kidney, HS-72 reached $0.43 \pm 0.08 \text{ mmol/gram tissue (wet weight (w.w.); n=3; SEM)}$ at 5 minutes and by 24 hours was retained at $0.02 \pm 0.004 \text{ mmol/g (w.w.; n=3; SEM)}$ (Figure 31B). In contrast, in liver HS-72 uptake peaked by 8 hours at $2.26 \pm 0.50 \text{ mmol/g (w.w.; n=3; SEM)}$ and was slowly cleared to $0.51 \pm 0.11 \text{ mmol/g (w.w.; n=3; SEM)}$ by 24 hours (Figure 31C). These findings show plasma HS-72 present at significant levels for at least 8 hours post i.p. and that HS-72 has a high degree of tissue bioavailability. In all 3 compartments, the parent MS ion of 365.2 Da was detected intact, with no evidence of rapid metabolism. The finding that HS-72 is absorbed to high [µM] levels following i.p. injection at 20mpk, particularly in liver, as well as kidney without adverse event, suggests the molecule is well tolerated in vivo.
Figure 31: LC-MS analysis of (A) plasma, (B) kidney, and (C) liver following i.p. injection, 20mpk, at the indicated time points over 24 hours, show HS-72 has a high degree of bioavailability in wild-type mice. (Mean ± SEM).

3.4.3 HS-72 Displays Efficacy in MMTV-neu Mice.

The apparent safety and bioavailability of HS-72 allowed us to test the efficacy of HS-72 to reduce tumor growth in the MMTV-neu breast cancer model. In this model, HER2 is overexpressed under the transcriptional control of the mouse mammary tumor virus (MMTV) promoter/enhancer, leading to spontaneous development of mammary
tumors (Taneja et al., 2009). To confirm that HS-72 would have efficacy in the MMTV-neu mouse model, HS-72 was tested in the NF639 cell line, which is derived from the mammary tumor of a MMTV-neu mouse. HS-72 was shown to potently inhibit proliferation of NF639 cells at 25µM and 50µM at 24, 48, and 72 hours, which is in a manner similar to previously tested cancer cell lines (Figure 32A). Furthermore, a synergistic effect on cell proliferation was observed when testing HS-72 and HS-10 in combination, thus highlighting the potential for combination therapy in the MMTV-neu mouse model (Figure 32B).

Figure 32: (A) HS-72 alone and in (B) combination with HS-10, inhibits proliferation of a MMTV-neu derived cell line, NF639. (Mean ± SEM).

Tumor bearing MMTV-neu mice were treated i.p. with HS-72 at 20mpk on a biweekly (BiW) schedule for 21 days. At 21 days there is a significant reduction (p<0.05) in tumor volume in the HS-72 treated mice compared to untreated mice (Figure 33A). A linear regression analysis comparing the slopes of the HS-72 tumor volume vs. no treatment tumor volume is trending towards significance (p=0.08) (Figure 33A).

Furthermore, median survival of animals increased by 6 days in mice treated with HS-72
20mpk BiW, and by 13 days in animals treated with HS-72 20mpk qd (daily dosing), when compared to animals receiving no treatment (Figure 33B). One set of control animals was used for multiple treatments, yielding more control animals than treated animals for the efficacy studies. Collectively, these studies show that the HS-72 scaffold has no overt toxicities, exhibits tissue and tumor bioavailability, and demonstrates efficacy in a spontaneous mouse mammary tumor model even under conservative biweekly dosing conditions. Our findings indicate that further development of the HS-72 scaffold is warranted to improve its potency and drug like properties.

Figure 33: (A) HS-72 promotes reduction in tumor volume as well as an (B) increased median survival time in MMTV-neu mice when compared to animals receiving no treatment. (A; Mean ± SEM).
3.5 Conclusions

In this chapter we highlight the work leading to identification of a chemical scaffold, HS-72 ((S)-N-(1-propyl-1H-benzo[d]imidazol-2-yl)-1-(pyrazin-2-yl)piperidine-3-carboxamide), that exhibits specificity towards the inducible form of Hsp70, Hsp70i. Various biochemical approaches demonstrated the selectivity of HS-72 towards Hsp70i over other Hsp70 family members, in particular Hsc70. This includes selective elution from the γ-phosphate linked ATP resin, creation of a highly selective HS-72 affinity resin, a selective thermo destabilizing effect in the presence of ATP compared with Hsc70 and Hsp90, altered protease digestion patterns in the presence and absence of the inhibitor, and sensitivity to the S enantiomer over the R form HS-71. Our studies also show that HS-72 has a distinct mechanism of action in vitro by acting as an allosteric inhibitor of ATP binding, a feature that certainly underlies its ability to discriminate Hsp70i from other Hsp70 family members. This mode of action may also explain HS-72 selectivity against the broader purinome, since the molecule is not directly targeting the ATP binding pocket, which is also suggested by our molecular docking studies.

In cells, HS-72 bears all the hallmarks associated with inhibition of Hsp70i. At the molecular level this includes loss of HER2 and Akt expression in breast tumor cells and formation of protein aggregates in a cellular based model of Huntington’s disease. The inhibitor is also synergistic in combination with Hsp90 inhibitors, as determined by monitoring HER2 and Akt expression. In proliferation assays, HS-72 shows specificity towards more aggressive breast and prostate tumor cell lines, consistent with the specific role of Hsp70i in mediating metastatic progression in vivo. Importantly, HS-72 is well tolerated and bioavailable in mice with no evidence of overt toxicity at high doses. Collectively these data suggest that HS-72 is an attractive starting point for a medicinal
chemistry campaign to improve the potency and pharmacological properties of the molecule. With this longer-term aim in mind we tested the efficacy of HS-72 in the MMTV model, a murine model of spontaneous breast cancer in humans (Taneja et al., 2009). On a conservative biweekly administration cycle, HS-72 demonstrated significant inhibition of tumor growth with evidence of improved survival. Further, our PK study showed that plasma [HS-72] are maintained at >20 µM for at least 8 hours, levels that reflect its potency against various tumor cell lines in vitro.

It is important to highlight that the HS-72 molecule was a raw hit from the FLECS screen with no structure-activity relationship (SAR) done to improve the compound. From a medicinal chemistry perspective the HS-72 scaffold is attractive and we have shown that it is highly amenable to resynthesis; we have synthesized a HS-72 affinity resin and analogs can readily be created to drive future SAR studies. The central piperidine carboxamide scaffold that makes up the core of HS-72 is structurally distinct from all other described Hsp70i inhibitors. As previously highlighted, the prior inhibitors identified to target Hsp70s include NSC 630668-R/1, VER-155008, MAL3-101, MKT-077, PES, and YK5 (Powers et al., 2010; Rodina et al., 2013). There is considerable structural diversity amongst these inhibitors and generally the NBD domain has been favored for inhibitor development (Powers et al., 2010). However, the polar interactions present in the nucleotide binding pocket and its affinity for ATP have contributed to difficulties in selective inhibitor discovery (Massey, 2010). The full-length crystal structure of the nucleotide bound form shows that the nucleotide is completely enclosed, making the accessibility of small inhibitors difficult (Figure 1). Importantly, none of the approaches adopted thus far have been able to target specific Hsp70 family members, especially Hsp70i from Hsc70.
The FLECS assay employed in defining HS-72 is a simple quantitative binding assay that offers an alternate approach to defining inhibitors to enzymes/proteins that often seem intractable to conventional high-throughput (HT) screens. Because of its chaperoning and trafficking functions in vivo, Hsp70i is a good example of such intractability. To date few HT screens have been designed that enable Hsp70i to be screened based on its chaperone function or ATPase activity (Kang et al., 2008; Rowlands et al., 2010; Wisen and Gestwicki, 2008). Although Hsp70i possess ATPase activity, following turnover, ADP remains bound to the enzyme (Mayer and Bukau, 2005). This means that competitive inhibitors acting at the ATP binding site must bind with a very high affinity to displace the bound nucleotide. In this respect, the Hsp70s are reminiscent of small G proteins, which have nM affinity for both GTP and GDP and are very difficult to drug directly. We propose that Hsp70i is recovered on our ATP media as a result of the ADP-bound or apo form being forced into and retained in the active state when exposed to the immobilized nucleotide. This enabled discovery of HS-72, which reduces the affinity of Hsp70i for the immobilized ligand. We are currently exploring the possibility of screening libraries containing molecules that selectively block interactions with this media, rather than displacing the bound protein through competitive or allosteric means. Finally, the FLECS approach enabled our ability to establish HS-72’s selectivity, both against the Hsp70 family and the wider purinome. In our view, when targeting purine-utilizing enzymes, the intrinsic selectivity of one’s lead scaffold towards the enzyme of interest is a greater priority than initial potency. This limits off-target liabilities that can be very difficult to remove during lead optimization studies. For this reason, our prioritization of HS-72 over other Hsp70i hits in our screen was driven by the intrinsic nature of the scaffold towards the protein over several other classes of purinome.
members screened against the same library (Figure 7). The finding that HS-72 is well tolerated in mice, reaching 2.26 mmol/g (w.w.) in liver without modification, is perhaps a testament to this prioritization strategy.
4. HS-72 Demonstrates Anti-Dengue Virus Activity, Validating Hsp70i as a Host Antiviral Target.

An estimated three billion people are at risk of DENV infection worldwide and there are currently no approved therapeutic interventions for DENV infection. Due to the relatively small size of the DENV genome, DENV is reliant on host factors throughout the viral life cycle. Targeting host proteins for antiviral therapeutics provides an advantage over targeting viral proteins because host proteins are not as susceptible to mutations that result in the development of drug resistant strains, which can occur when targeting viral proteins (Noble et al., 2010). As previously described, one such host factor that has recently been identified that is involved in DENV pathogenesis is the Hsp70 family of proteins, including the Hsp70i and Hsc70 (Chavez-Salinas et al., 2008; Padwad et al., 2010; Reyes-Del Valle et al., 2005). Previous studies have shown that siRNA knockdown of Hsp70 results in decreased DENV RNA copy numbers in supernatants and decreased intracellular DENV load (Padwad et al., 2010). Furthermore, Hsp70 was identified through affinity chromatography to be part of the DENV receptor complex, however it is not fully elucidated which member of the Hsp70 family member is part of the complex (Reyes-Del Valle et al., 2005). Hsp70 family members as a part of the DENV receptor complex is supported by previous studies showing a reduction in DENV infected human and mosquito cells when pretreated with Hsp70 antibodies (Reyes-Del Valle et al., 2005; Vega-Almeida et al., 2013). Additionally, it has been shown that there is an increase in the number of DENV infected cells following heat shock (Chavez-Salinas et al., 2008). The increase in infection correlates with an increase in Hsp70 present on the cell surface, but the different Hsp70 family members are not discriminated (Chavez-Salinas et al., 2008). Therefore, the precise role of the inducible
Hsp70 family member remains to be fully elucidated. Using HS-72 as a tool to specifically target Hsp70i, allows for elucidating the role of the protein in DENV pathogenesis. Furthermore, previous studies implicating Hsp70i as a host antiviral target indicated HS-72 as a potential anti-DENV agent.

**4.1 Proteomic Analysis Shows Hsp70i is a DENV Host Factor.**

**4.1.1 Identification of Purine Binding Proteins Induced by DENV Infection.**

Previously, the Haystead laboratory has employed nucleotide affinity resins to capture and enrich *en masse* purine utilizing proteins (the purinome) from tumor cell and tissue extracts, with the goal of defining novel drug targets as well as to screen small molecule libraries for selective inhibitors, as highlighted for HS-72 in the previous chapter (Barrott et al., 2013; Carlson et al., 2013; Howe et al., 2014). Purine-binding proteins, by virtue of their nucleotide binding pockets represent approximately 50% of the estimated druggable purinome and include a diverse range of enzyme classes e.g. protein kinases, non-protein kinases, heat shock proteins, metabolic enzymes, DNA and RNA binding proteins (Haystead, 2006). Herein we employed the previously described affinity resin based on ATP immobilized through its γ-phosphate to define host purine utilizing proteins that may be induced or activated in response to DENV infection. Huh7 cells were treated with $[^{35}\text{S}]$ methionine/cysteine and virus (MOI = 10) and infection followed over 24 hours. At the indicated time points the cells were stopped and the purinome isolated by passing the radio-labeled cellular homogenates over the ATP resin in parallel. Bound proteins were then characterized by SDS-PAGE, autoradiography and MS analysis. Figure 34 shows marked increases in the translation of four ATP binding proteins in response to DENV-infection by 12-24 h post-infection at 105 kDa, 70 kDa, 65
kDa and 8 kDa. Three were identified by MS as virally encoded proteins NS5, NS3, and prM (Figure 34). Both NS5, and NS3 were expected to bind ATP via their respective polymerase, NTPase or helicase domains, however, the reasons for recovery of prM is unknown. All other proteins detected in the autoradiogram were unaffected by the infection, and identified by MS as host ATP binding proteins. We suspected that the 70 kDa protein may be Hsp70i, based on previous reports by others describing induction of the protein following DENV infection (Brenner and Wainberg, 1999; Cheung and Dosch, 1993; Lefeuvre et al., 2006; Mayer, 2005). Western blot analysis with antibodies to Hsp70i confirmed this conclusion (Figure 35).

![Image of protein analysis](image)

**Figure 34:** Proteomic analysis reveals induction of a 70kDa protein following DENV infection. Lanes labeled “C” indicate uninfected control samples; lanes labeled “V” indicated DENV infected samples.
**Figure 35:** Induction of Hsp70i was confirmed following DENV infection, in U937+DC-SIGN cells. E protein serves as a positive control for infection, GAPDH serves as a loading control. Graph on right is quantification of Western Blot, using Image J software.

**4.1.2 Hsp70i Mediates DENV Infection.**

To determine if the induction of Hsp70i was the result of a general cellular stress in response to DENV infection or likely to be facilitated by the virus itself, we induced expression of the protein in uninfected cells using the Hsp90 inhibitor, HS10 (Hughes et al., 2012). Induction of Hsp70i expression is a signature response to all Hsp90 inhibitors targeting the ATP binding domain of the protein (Fadden et al., 2010; Guo et al., 2005a; Powers and Workman, 2007). Hsp90 exists in a complex with heat shock transcription factor 1 (HSF-1) in cells and upon Hsp90 inhibition, the complex is disassociated, which allows HSF-1 to translocate to the nucleus (Powers et al., 2009). After trimerization, HSF-1 binds to heat shock elements on heat shock gene promoters, which elicits an increase in Hsp70i transcription (Powers et al., 2009). The induction of Hsp70i by Hsp90 inhibition is thought to be compensatory response to maintain cell viability (Guo et al., 2005a; Powers et al., 2009). Figure 36 shows treating uninfected cells with HS-10 for 6 hours results in a dose dependent increase in Hsp70i expression.
Figure 36: HS-10 treatment results in an increase in Hsp70i expression in U937+DC-SIGN cells. GAPDH serves as a loading control. The graph on the right shows quantification of the bands from the western blot, using Image J software.

When we infected the Hsp70i induced cells with DENV we observed a correlation of infectivity with Hsp70i expression compared with non-HS10 treated cells (Figure 36 and 37A-B). We also observed by flow cytometry an increase in surface Hsp70i expression, in both infected cells and infected cells pretreated with HS-10 (Figure 38). When HS-10 was administered without preincubation prior to addition of DENV, there is no effect on the percent of infected cells from non-treated cells (Figure 39). This finding is consistent with a requirement for the induction of Hsp70i expression, following Hsp90 inhibition, leading to increased infectivity. This experiment also showed that acute Hsp90 inhibition does not have acute antiviral activity against DENV in culture. Furthermore, pretreatment of U937+DC-SIGN cells with Hsp70i antibody reduces DENV infection, which is consistent with previous studies in human and mosquito cells (Figure 40) (Reyes-Del Valle et al., 2005; Vega-Almeida et al., 2013).
Figure 37: U937+DC-SIGN cells were pretreated with HS-10, to induce Hsp70i expression prior to DENV infection, resulting in a dose dependent increase in infectivity as determined by (A) flow cytometry or (B) foci forming assay. (Mean ± SEM).

Figure 38: Total and surface Hsp70i expression was induced in infected U937+DC-SIGN cells and infected cells pretreated with HS-10. Graphs represent quantification of respective histograms. (Mean ± SEM).
4.2 The Hsp70i Inhibitor, HS-72, Reduces DENV Infection.

Given the effects of Hsp70i induction on increasing infectivity, we tested the antiviral activity of the HS-72 DENV. As described in the previous chapter, HS-72 is a
recently identified highly specific allosteric small molecule inhibitor targeting Hsp70i (Howe et al., 2014). Additionally, the molecule discriminates Hsp70i from the closely related homolog, Hsc70, and exhibits bioavailability and efficacy in cell and animal models of human breast cancer (Howe et al., 2014). The human monocytic cell line, U937+DC-SIGN, was treated with HS-72 or Ribavirin for 1 hour prior to addition of DENV. At 24 hours post-infection, the cells were analyzed by flow cytometry for infection and viability (Figure 41A-B). Figure 41A-B shows treatment with HS-72 results in a dose dependent reduction in DENV infected cells with no significant increase in cell viability at <90µM. These data indicate a targeted reduction in infection through Hsp70i inhibition as opposed to non-discriminant cell toxicity from HS-72 treatment. The potency of HS-72 is comparable to Ribavirin, which was included as a positive control due to the established antiviral activity that has been previously reported against DENV in vitro (Takhampunya et al., 2006). In foci forming assays, using the supernatants from U937+DC-SIGN cells to infect Vero cells, HS-72 shows a similar dose dependent decrease in DENV infection, with an EC$_{50}$ of 22.8µM (Figure 41C).
Figure 41: (A) HS-72 yields a dose dependent reduction in DENV infection as determined by flow cytometry. (B) Viability analysis corresponding to percent infection in (A) shows viability was maintained when HS-72 <90µM. (C) HS-72 reduced DENV infection in a dose dependent manner as determined by a decrease in foci forming units. (Mean ± SEM).

4.2.1 HS-72 Inhibits DENV Entry into Host Cells

To determine the stage of the DENV life cycle inhibited by HS-72, U937+DC-SIGN cells were treated with the inhibitor 1 hour before addition of DENV, at the same time as DENV addition, and then 1 hour, 4 hours, 6 hours, and 12 hours post infection. At 24 hours after addition of DENV, percent infection and cell number were determined (Figure 42A-B). These studies showed that HS-72 was most effective when added prior to infection, or at the 0 or 60 minute time points, but ineffective when added 4 hours, 6 hours, or 12 hours post DENV infection (Figure 42A). These data indicate HS-72 is most likely working to block the DENV life cycle at early phases. Importantly, as observed earlier, no significant change in cell number between control and HS-72 treated cells was noted throughout the study, indicating cell viability was maintained (Figure 42B).
Figure 42: (A) HS-72 inhibits DENV infection at early stages of the viral life cycle as determined by percent infection in a time of addition assay. (B) Cell number, corresponding to percent infection in (A), is maintained throughout the time of addition assay between control and HS-72 treated groups, indicating no significant change in cell viability. (Mean ± SEM).
To discriminate the effect of HS-72 on the early stages of the DENV life cycle, an attachment and entry assay in U937+DC-SIGN cells was performed. Cells were treated with HS-72 and cooled to 4°C. To evaluate the effects of HS-72 on viral attachment, DENV was added to chilled cells for 1 hour in the presence and absence of HS-72. Separately, cells were transferred from 4°C to 37°C and incubated for an additional hour to allow for viral entry, plus and minus HS-72. Cells were then processed and analyzed by qPCR to quantify viral RNA. These studies showed that there is a significant decrease in viral RNA at the entry stage of the viral life cycle in cells treated with HS-72 (Figure 43). Collectively, these data indicate that HS-72 is working to inhibit infection by perturbing the entry stage of the DENV life cycle. Furthermore, this indicates that Hsp70i plays a role in mediating DENV entry into monocytes.

Figure 43: An attachment/entry assay shows that HS-72 is working to inhibit DENV infection at the entry stage of the viral life cycle, as determined by the fold change of viral RNA compared to GAPDH. (Mean ± SEM).
4.3 Hsp70i Localizes to the Cell surface Following DENV Infection.

Based on the finding that HS-72 works to disrupt entry of DENV into monocytes, we examined expression of Hsp70i on the cell surface. As discussed earlier, we had noted that DENV infection alone not only induced Hsp70i expression in U937+DC-SIGN cells, but also promoted expression of the protein on the plasma membrane (Figure 36 and 38). To further examine this observation, U937+DC-SIGN cells were infected with DENV and analyzed at time points that correlate with pre- and post-entry stages of the DENV life cycle by flow cytometry. Figure 44 shows there is a significant increase in cell surface Hsp70i 1 hour post-infection, compared to uninfected cells. However, by 4 and 24 hours post-infection, cell surface Hsp70i has decreased significantly compared to the 1 hour time point (Figure 44). Biotin labeling of Hsp70i through its surface cysteine residues on the cell surface further illustrates this change in localization. Isolating the membrane and cytoplasmic fraction of Hsp70i, following biotinylation of the membrane fraction, shows the increase in surface Hsp70i 1 hour post-infection, while there is a decrease in cytoplasmic Hsp70i compared to uninfected cells (Figure 45). By 24 hours post-infection, Hsp70i on the cell surface has decreased and there is a commensurate increase in cytoplasmic Hsp70i (Figure 45). This data indicates a change in Hsp70i localization following DENV infection to mediate virus entry into U937+DC-SIGN cells.
Figure 44: Hsp70i surface expression was determined, by flow cytometry, at the indicated time points post-infection, showing a significant increase 1 hour post-infection compared to uninfected cells. While, Hsp70i surface expression has significantly decreased by 4 and 24 hours post-infection. Graph represents quantification of respective histograms (Mean ± SEM).
Figure 45: Biotinylation of surface Hsp70i shows Hsp70i localizing to the cell surface following DENV infection. While in DENV infected cells treated with HS-72, Hsp70i is maintained on the cell surface until 24 hours post-infection.

Next, Hsp70i surface expression was examined in DENV infected U937-DC+SIGN cells treated with HS-72, at the same time points as previously described. There is also an observed increase in cell surface Hsp70i expression 1 hour post-infection in HS-72 treated cells compared to uninfected cells (Figure 46). However, by 4 hours and 24 hours post-infection, Hsp70i is continually maintained on the cell surface of HS-72 treated cells (Figure 46). This change in Hsp70i localization is further illustrated through biotinylation of the membrane fraction of Hsp70i in infected cells treated with HS-72, showing Hsp70i maintained on the cell surface by 24 hours post-infection (Figure 45). This indicates that inhibiting Hsp70i ATPase activity and inducing a conformational change with HS-72 maintains Hsp70i on the cell surface following DENV infection.
Figure 46: Hsp70i surface expression was determined in HS-72 treated cells at the indicated time points post-infection by flow cytometry, showing a significant increase 1 hour, 4 hours, and 24 hours post-infection compared to uninfected cells. Graph represents quantification of respective histograms (Mean ± SEM).

Importantly, HS-72 alone does not change Hsp70i surface expression in uninfected U937-DC+SIGN cells, indicating the change in Hsp70i surface expression is mediated by infection and not by HS-72 treatment alone (Figure 47). Furthermore, U937+DC-SIGN cells treated with inactivated DENV, which was heated at 55°C for 30 minutes prior to treatment, show no change in cell surface Hsp70i compared to no virus at the indicated time points, in cells treated with or without HS-72 (Figure 48).
Inactivation of DENV results in no infection of U937+DC-SIGN cells (Figure 49). This indicates that successful binding and infection of U937+DC-SIGN cells mediates the change in Hsp70i localization. Additionally, treatment with HS-10 results in an increase in Hsp70i surface expression, which was previously described (Figure 38). Treatment with HS-10 in combination with HS-72 results in an increase in surface Hsp70i similar to HS-10 alone at 6 hours post-treatment, while Hsp70i is maintained on the cell surface 24 hours post-treatment with the combination treatment compared to HS-10 alone (Figure 50). In support of previous results, HS-72 alone does not significantly change Hsp70i surface expression (Figure 47 and 50). This data indicates that HS-72 maintains Hsp70i on the cell surface when cells are infected with DENV, as well as when cells are subjected to the stress of Hsp90 inhibition. Collectively, this data indicates upon DENV binding and subsequent infection of U937+DC-SIGN cells there is a change in Hsp70i localization to the cell surface. Inhibition of Hsp70i ATPase activity and a conformation change induce by HS-72, maintains Hsp70i on the cell surface in infected cells, but blocks viral entry, suggesting that fully functional Hsp70i is required for entry of DENV into host cells.
Figure 47: Treating uninfected U937+DC-SIGN cells with HS-72 did not significantly change the surface expression of Hsp70i. The graph on the right represents quantification of the histogram (Mean ± SEM).
Figure 48: Hsp70i surface expression was determined in cells treated with inactivated DENV in the (A) absence and (B) presence of HS-72, which showed no change compared to uninfected cells. (C-D) Graphs represent quantification of histograms shown in (A) and (B), respectively (Mean ± SEM).
Figure 49: Inactivated DENV does not infect U937+DC-SIGN cells.

Figure 50: Hsp70i surface expression was increased, as determined by flow cytometry, in cells treated with HS-10, while HS-72 treatment had no significant effect at (A) 6 and (B) 24 hours. Combination treatment with HS-72 and HS-10 maintains Hsp70i on the cell surface 24 hours post-treatment compared to HS-10 treatment alone. Graphs represent quantification of respective histograms (Mean ± SEM).
4.4 Hsp70i Interacts with the DENV Receptor Complex, which is Disrupted by HS-72.

To further elucidate how Hsp70i is mediating DENV entry an interaction between Hsp70i and the DENV receptor complex was investigated through proximity ligation assay (PLA). This assay allows for visualization of in situ interactions in cells by fluorescence microscopy (Koos et al., 2014). In PLA, primary antibodies were added to cells, followed by addition of PLA probes, which are conjugated with complementary oligonucleotides. Next the probes were hybridized to form a closed circle, which serves as the template for rolling circle amplification (RCA). Addition of fluorescently labeled oligonucleotides, which hybridized the RCA product, yields a fluorescent signal. This signal is visualized by fluorescence microscopy, representing an in situ interaction between the proteins of interest. Figure 51 shows distinct red punctae were observed, indicating an in situ interaction between Hsp70i and the DENV E protein at 1 and 4 hours post-infection. HS-72 treatment reduced the number of puncta, suggesting a disruption of the interaction at 4 hours post-infection (Figure 51). Uninfected cells or cells not treated with primary antibody show very little background fluorescence or red punctae, indicating the specificity of PLA (Figure 51 and 52).
Figure 51: PLA shows an *in situ* interaction of Hsp70i with the DENV E protein, indicated by distinct red punctae, which is disrupted by HS-72, as observed by a decrease in red punctae. Graph represents quantification of respective images from PLA (Mean ± SEM). Representative images shown with
accessory proteins showing magnified portion of image designated by boxes. Hsp70i - E protein interaction represented by red punctae, DAPI shown in blue to represent cell nuclei, green staining represents cell membranes.

Figure 52: Cells not treated with primary antibodies show minimal punctae, indicating minimal background fluorescence and highlighting the specificity of the PLA. DAPI shown in blue to represent cell nuclei, green staining represents cell membranes.

An *in situ* interaction of Hsp70i with the DC-SIGN receptor was also observed by PLA (Figure 53). DC-SIGN has been previously shown to be part of the DENV receptor complex that mediates DENV binding to host cells (Tassaneetrithep et al., 2003). PLA shows Hsp70i interacting with DC-SIGN at 1 and 4 hours post-infection, while HS-72 also disrupts this interaction 4 hours post-infection (Figure 53). An interaction is also observed in uninfected cells, because Hsp70i and DC-SIGN are host proteins (Figure 53). Collectively this data shows an in cell interaction of Hsp70i with the DENV E protein and Hsp70i with DC-SIGN, suggesting an interaction of Hsp70i with the DENV receptor complex during DENV binding and entry. HS-72 treatment results in disruption of Hsp70i associating with the DENV receptor complex to perturb entry of DENV into host cells, which ultimately results in a reduction of infected cells.
Figure 53: PLA shows an in situ interaction of Hsp70i with DC-SIGN, indicated by distinct red punctae, which is disrupted by HS-72, as observed by a decrease in red punctae. Graph represents quantification of respective images from PLA (Mean ± SEM). Representative images shown with insets showing
4.5 Conclusions

Because of the limited size of most viral genomes, the concept that viruses require components of the host cellular machinery to replicate is not surprising. However, defining which host factors are essential for viral replication using genetic, gene silencing or molecular biological approaches can often be problematic because these techniques often compromise cellular integrity, promote compensatory adaptations or simply trigger programmed cell death by activating apoptotic pathways. The outcome of such studies therefore becomes ambiguous, since any antiviral effect could be the result of a dying host cell. Small molecule inhibitors can greatly enable the role of a host protein to be better defined because they can be added at any stage of the life cycle, acutely or chronically, across cell lines and even species while maintaining cellular architecture. Small molecules generally inhibit biological activity rather than removing or mutating the targeted protein, which leads to unwanted compensatory effects. Ultimately, such studies can lead directly to the development of a new antiviral agent. Importantly host targets are not under the direct genetic control of the virus itself and therefore are not prone to early development of resistance under selective drug pressure. We suggest that our studies herein with HS-72, strongly indicate an essential role for Hsp70i in the DENV infection cycle and validate the protein as an antiviral host target. Data from our studies, highlighted in this chapter, that support this hypothesis can be summarized as follows: 1) proteomic studies show Hsp70i is induced and trafficked to the plasma membrane in response to DENV infection of the human liver cell line Huh7 and in a monocyte line; 2) pharmacological induction of Hsp70i expression with an
Hsp90 inhibitor potently promotes DENV infectivity; 3) HS-72, a highly selective allosteric inhibitor of Hsp70i exhibits anti-DENV activity in a monocyte cell line; 4) HS-72 blocks DENV entry by inhibiting Hsp70i at the plasma membrane; 5) cell imaging using the proximity ligation assay (PLA) show co-localization between Hsp70i, DENV E protein, and DC SIGN in monocytes, and these interactions are disrupted by HS-72. Collectively these findings suggest a model in which DENV, perhaps through interactions with DC-SIGN, triggers induced expression and trafficking of Hsp70i to the plasma membrane followed by binding to the virally encoded E protein. Presumably this triggers ATP turnover or a conformational change in Hsp70i that facilitates reinternalization along with the viral particle to promote infection. Inhibition of Hsp70i by HS-72 prevents Hsp70i reinternalization and disrupts Hsp70i interacting with the DENV receptor complex, therefore blocking viral entry (Figure 54). To test this hypothesis further detailed studies between Hsp70i, DENV E protein and DC SIGN receptor are certainly warranted. However, if this mechanism is correct, Hsp70i could be the second example of a druggable host protein that functions as a cell surface receptor for a virus that is necessary for viral entry. The anti-HIV drug Maraviroc functions to target the CCR5 protein and its interactions with CD4, to block HIV invasion of CD4+ T cells (Dorr et al., 2005).
Figure 54: Hsp70i localizes to the cell surface and interacts with the DENV receptor complex to aid in viral entry, while HS-72 treatment disrupts the interaction of Hsp70i with the DENV receptor complex, which reduces viral entry. 

1) A DENV virion attaches to a host cell through binding a receptor such as DC-SIGN. 
2) DENV binding causes a change in Hsp70i localization to the cell surface, where Hsp70i interacts with the DENV receptor complex. 
3) Hsp70i continues to interact with the DENV receptor complex and aids in entry of DENV. 
4) By late stages of the DENV lifecycle, localization of Hsp70i is primarily cytoplasmic. Once the DENV genome is released into the host cell, synthesis of viral proteins, and replication of viral RNA occurs, which is followed by packaging and export of mature DENV virions from the infected host cell. 
5) In cells treated with HS-72, a DENV virion attaches to a host cell through binding a receptor such as DC-SIGN. 
6) DENV binding causes a change in Hsp70i localization to the cell surface and Hsp70i interacts with the DENV receptor complex. 
7) The interaction of Hsp70i with the DENV receptor complex is disrupted and Hsp70i is maintained on the cell surface. This could be due to the change in Hsp70i conformation induced by HS-72, which would inhibit Hsp70i in mediating entry of DENV. Ultimately, HS-72
treatment leads to a reduction in DENV infection. Schematic of HSP70 represented in blue, DC-SIGN represented in green, DENV virion represented in red, DENV genome represented in orange, and HS-72 represented by chemical structure in lower panel.

As we have previously discussed, there is great interest in Hsp70i as a drug target for the treatment of various cancers (Jego et al., 2013; Powers et al., 2010). Hsp70i belongs to a group of closely related heat shock proteins, that share close sequence identity with the protein, in particular the non-inducible housekeeping protein, Hsc70. The Hsp70 proteins are thought to function in cellular protein homeostasis and are required for normal protein folding and stability. These activities often require ATPase activity and typically, like many heat shock proteins, the Hsp70s all possess ATP binding domains. This feature makes such proteins highly druggable and in cancer much attention has been focused in recent years on developing specific inhibitors for the heat shock proteins. In cancer, inhibition of Hsp90 or Hsp70 results in inhibition of cellular proliferation and metastasis (Powers et al., 2008, 2009). This response is associated with down regulation of the expression of various oncogenic client proteins such as HER2 and Akt (Powers et al., 2008, 2009). Indeed, the list of signal transduction proteins associated with both Hsp90 and Hsp70 is extensive (Powers et al., 2009). Our data described throughout, highlight several interesting parallels between oncogenic transformation and viral infection and their connections with the heat shock proteins. Cancer cells are often described as being addicted to heat shock proteins to maintain cellular proliferation (Jego et al., 2013). Heat shock proteins such as Hsp90 and Hsp70 are not oncogenes themselves, but are induced and sequestered by oncogenic proteins, promoting their proper folding and stability. Inhibiting Hsp90 or Hsp70 ATPase activity blocks this function, generally putting the cell into dormancy. For these reasons heat shock protein inhibitors are highly synergistic with antibodies or drugs that target the
oncogene itself (Leow et al., 2009; Wainberg et al., 2013). Therefore, the sequestration of Hsp70i by DENV to promote viral entry is analogous to oncogene sequestration of the protein and other Hsps. It is likely therefore, that inhibitors like HS-72, would be highly synergistic with any inhibitors targeting viral encoded nonstructural proteins or antibodies targeting viral surface proteins such as E protein or prM.

Our immediate goal is to improve the potency of HS-72 as an anti-DENV agent. In the previous chapter we discussed recent work in which we demonstrated that HS-72 has excellent pharmacokinetic and distribution properties in mice accumulating in tissues to [mM], with $t_{1/2}$ elimination times of $>24$ hours (Howe et al., 2014). The compound is well tolerated in mice and exhibited efficacy against the MMTV model of human breast cancer. Clearly the HS-72 scaffold has excellent drug like characteristics and should be tested in animal models of DENV infection across serotypes, either as a mono therapy or in combination with NS5 inhibitors (Lim et al., 2013; Schul et al., 2007). Given the synergistic effects often observed with heat shock inhibitors with targeted chemotherapeutics in cancer studies, HS-72 may show synergism with NS5 inhibitors that have failed to progress clinically due to dose limiting toxicities (Lai et al., 2014; Lim et al., 2013; McConnell and McAlpine, 2013). If HS-72 proves itself useful as an antiviral agent, one can draw another parallel with between cancer biology and antiviral development. As previously mentioned, the first clinically relevant antiretroviral drug AZT was originally derived from a cancer drug discovery program (Broder, 2010). In the case of AZT, the drug had dose limiting toxicities in cancer that limited its usefulness (Broder, 2010). However, its potency towards the HIV encoded reverse transcriptase greatly expanded its therapeutic window, especially when finally used in combination with protease inhibitors (Broder, 2010).
Appendix A

Synthesis of HS-72 ((S)-3): as described and performed by Dr. Phillip Hughes and
Dr. David Carlson.

(S)-1-(pyrazin-2-yl)piperidine-3-carboxylic acid (S)-1. (S)-(−)-3-
Piperidincarboxylic acid (250 mg, 1.94 mmol) and chloropyrazine (441 mg, 3.87 mmol)
were heated together with Hunig’s base (500 mg, 3.87 mmol) and ethanol (300 µL) at
120 °C for 16 h. TLC (4/1/1 : nBuOH/AcOH/H2O) showed a new product and only a
trace of starting material. The reaction mixture was concentrated, dissolved in DMSO
and purified by prep HPLC (5 to 100% methanol w/0.2% formic acid, 20 mL/m, Agilent
C-18, 21.1 x 25 cm) to give product (S)-1 (291 mg, 72%) as a white powder. MS (ESI)
[M+H]+ m/z = 208.0.

(S)-N-(1H-benzo[d]imidazol-2-yl)-1-(pyrazin-2-yl)piperidine-3-carboxamide (S)-2.
Compound (S)-1 (147 mg, 709 mmol) and 2-aminobenzimidazole (189 mg, 1.42 mmol)
were mixed with EDC (204 mg, 1.06 mmol), HOBT (96 mg, 0.71 mmol) and DMAP (9
mg, 71 mmol) and Hunig’s base (183 mg, 247 mL, 1.4 mmol) and dissolved in DMF (2 mL). TLC (9/1 : CH₂Cl₂/MeOH) showed the slow formation of product and loss of starting material. After 2 h, the reaction mixture was concentrated to remove DMF and chromatographed (gradient CH₂Cl₂ to 9/1 : CH₂Cl₂/MeOH). The product was triturated overnight in ethyl acetate/ hexanes to give (S)-2 (77.8 mg, 34%) as a white solid. MS (ESI) [M+H]+ m/z = 323.2.

(S)-N-(1-propyl-1H-benzo[d]imidazol-2-yl)-1-(pyrazin-2-yl)piperidine-3-carboxamide (S)-3. Amide (S)-2 (40 mg, 124 umol) was dissolved in DMSO (300 ul) and treated with sodium t-butoxide (124 uL of 2M solution) followed by 1-bromopropane (18.3 mg, 149 µmol) and stirred at RT. After 1 h, TLC (9/1 : CH₂Cl₂/MeOH) showed a new product and a little starting material. The sample was purified by prep HPLC (5 to 100% methanol w/0.2% formic acid, 20 mL/m, Agilent C-18, 21.1 x 25 cm) and recrystallized from ethyl acetate/heptanes to give (S)-3 (24 mg, 53%) as a white powder. (S)-3 was identical to commercial racemic 3 by TLC and LC/MS. MS (ESI) [M+H]+ m/z = 365.3.

HS-71, (R)-N-(1-propyl-1H-benzo[d]imidazol-2-yl)-1-(pyrazin-2-yl)piperidine-3-carboxamide, (R)-3, was prepared in an analogous manner from (R)-(--)3-piperidinocarboxylic acid.
Appendix B

Synthesis of HS-72 affinity resin ((S)-9): as described and performed by Dr. Phillip Hughes.

(S)-N-(1-(5-bromopentyl)-1H-benzo[d]imidazol-2-yl)-1-(pyrazin-2-yl)piperidine-3-carboxamide (S)-4. Amide (S)-2 (20 mg, 62 mmol) was dissolved in DMSO (300 ml) and treated with sodium t-butoxide (62 mL of 2 M solution) followed by 1,5-dibromopentane (17 mg, 74 mmol) and stirred RT. After about 1 h, TLC (9/1 : CH₂Cl₂/MeOH) and LC/MS showed a new product (m/z = 471.6). The product was purified by prep HPLC (5 to 100% methanol w/0.2% formic acid, 20 mL/m, Agilent C-18, 21.1 x 25 cm) to give (S)-4 as a yellow solid. The product was then used for the next reaction.
(S)-N-(1-(1-amino-4,7,10,13,16-pentaoxa-20-azapentacosan-25-yl)-1H-benzo[d]imidazol-2-yl)-1-(pyrazin-2-yl)piperidine-3-carboxamide (S)-5. Bromide (S)-4 (29 mg, 62 mmol) was dissolved in ethanol (2 mL) and treated with 1,19-diamino-4,7,10,13,16-pentaoxanonadecane (60 mg, 195 mmol) followed by DMSO (100 mL) and CH$_2$Cl$_2$ (1 mL) and stirred at RT for 3 days. The reaction mixture was concentrated and injected onto the prep HPLC (5 to 100% methanol, 20 mL/min, Agilent C-18, 21.1 x 25 cm) to give purified (S)-5 (12.9 mg, 30% from (S)-2) as a glass. LC/MS gives m/z = 669.4 [M + H]$^+$.  

**Cleavable linker activation**

Activated cleavable linker 7. The cleavable linker acid (Hughes et al., 2012) 6 (541 mg, 1.4 mmol), EDC (540 mg, 2.8 mmol) and N-hydroxysuccinimide (243 mg, 2.1
mmol) and a chip of DMAP were slurried in dichloromethane (10 mL) and DMF (1.5 mL). After 16 hour, TLC (70% ethyl acetate in hexanes) showed complete reaction. The reaction mixture was added to a column and chromatographed (silica gel, 18 x 3.5 cm, CH$_2$Cl$_2$ (200 mL), 25% EtOAc in CH$_2$Cl$_2$ (600 mL). The active fractions were combined and concentrated then triturated with hexanes ethyl acetate and filtered off to give the 7 (353 mg, 52%) as an orange powder.

Amine (S)-8. Amine (S)-5 (12.9 mg, 18.5 mmol) was dissolved 9/1: DCM/MeOH (1 mL) and treated with solid activated cleavable linker 7 (25 mg, 52 mmol), followed by Hunig’s base (10 mL). After stirring overnight, TLC showed mostly one product and LC/MS showed a big peak in the TIC with the right mass. UV showed nothing at 254 nm as usual. The sample was concentrated to an oil and chromatographed on silica gel eluting with a CH$_2$Cl$_2$ to 4/0.9/0.1 : CH$_2$Cl$_2$/MeOH/NH$_3$ gradient. The product was concentrated to give an orange glass. The sample was then dissolved in methylene chloride (1 mL) and TFA (1 mL). After 1 h, TLC (4/0.9/0.1 : CH$_2$Cl$_2$/MeOH/NH$_3$) showed a new product. The mixture was concentrated, diluted with ethanol and concentrated
again and then purified by HPLC (5 to 100% methanol, 20 mL/m, Agilent C-18, 21.1 x 25 cm) to give amine (S)-8 (5.5 mg, 31% overall) as an orange glass. LC/MS showed a single peak with m/z = 966.6 [M + H]+.

Buffers and solutions
Swelling solution 1 mM HCl
Coupling buffer 0.1 M NaHCO₃, 0.5 M NaCl, pH = 8.3
Capping solution 1 M ethanolamine
Low buffer 0.1 M AcOH/NaAcOH, 0.5 M NaCl pH = 4
High Buffer 0.1 M TRIS-HCl, 0.5 M NaCl pH = 8
Storage Buffer 0.1M KH₂PO₄, pH = 7.4 w/ 200 mg NaN₃/L

Roughly following GE Healthcare Instructions 71-7086-00 AFA.

Affinity resin (S)-9. In a 30 mL column, CNBr-activated Sepharose™ 4B (2 g) was swelled in 1 mM HCl (20 ml) and then washed with 1 mM HCl (400 mL). The resin
was washed with coupling buffer (20 mL) and then slurried with coupling buffer (10 mL). The mixture was then treated with amine (S)-8 (5.5 mg) in ethanol (1 mL) and tumbled at RT for 16 h. The resin was then drained (no color eluted) and washed with coupling buffer (5 x 10 mL), diluted with more coupling buffer (~10 mL) and treated with capping solution (200 mL) and rotated for 2 h. The solution was drained and the resin (S)-9 washed with 3 rounds of high buffer/low buffer (20 mL ea.) and finally washed with water (20 mL) and transferred in storage buffer (10 mL) to a 40 mL EPA vial and stored at 4°C.
Appendix C

Mass spectra of residues protected from trypsin digestion in the presence of HS-72, which were identified in partial proteolysis analysis and shown in Figure 21. Residues 326-342 shown in A, residues 141-155, shown in B, and residues 518-533 shown in C. Spectra for Hsp70i alone shown in blue and spectra for Hsp70i plus HS-72 shown in pink. The arrows designate the indicated residues, which highlight differences in the spectra.
Residues 326-342, protected from trypsin digestion indicated by arrow. Hsp70i spectra without HS-72 in blue. Hsp70i spectra with HS-72 in pink.
Residues 141-155, protected from trypsin digestion indicated by arrow. Hsp70i spectra without HS-72 in blue. Hsp70i spectra with HS-72 in pink.
C. Residues 518-533, protected from trypsin digestion indicated by arrow. Hsp70i spectra without HS-72 in blue. Hsp70i spectra with HS-72 in pink.
Appendix D

HS-72 Pharmacokinetic Raw Data Files

The raw files for all samples can be found in Supplemental Information 2, located on figshare and can be found here: http://dx.doi.org/10.6084/m9.figshare.1209606 with the TIC, EIC, UV chromatogram, and MS on the pages titled “Display Report – All Windows Selected Analysis”. The raw values for the EIC area under the curve for HS-72 and HS-156 are located on the pages titled “Compound Mass Spectrum List Report – MS”.
References


Muller, D.A., and Young, P.R. (2013). The flavivirus NS1 protein: molecular and structural biology, immunology, role in pathogenesis and application as a diagnostic biomarker. Antiviral research 98, 192-208.


140
Biography

Place and Date of Birth

Columbus, Ohio
25 April 1987

Education


Publications


Honors and Awards

Duke Scholars in Molecular Medicine; Infectious Disease Program. 2014 - 2015
Duke Graduate School Conference Travel Award; New Orleans, LA. 2014.
Fitzgerald Scholar Poster Award. 2013.