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The effectiveness of HS-72 variants in inhibition of heat shock protein 72

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THE EFFECTIVENESS OF HS-72 VARIANTS IN INHIBITION OF HEAT SHOCK PROTEIN 72

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ABSTRACT

Heat shock proteins (HSPs) play important roles in the process of maintaining proteostasis in a cell. HSP72, the inducible form of the HSP70 family, is expressed in response to stress on the cell or tissue, including those stresses caused by tumor growth. Increasing evidence suggests that HSP72 is necessary for a cancerous cell to survive under the stresses of a tumor microenvironment. This has naturally raised interest in identifying an inhibitor selective for HSP72. The Haystead Laboratory at Duke University identified such a small-molecule inhibitor, referred to as HS-72, and proposed the scaffold as an ideal starting point to develop a family of therapeutic agents targeting HSP72.

This work examines the potency and effectiveness of HS-72 and a number of its analogs developed by the Haystead Laboratory. These results suggest that HS-159 is a more effective inhibitor of HSP72 on a range of human tumor cell lines than HS-72. Further studies are needed to quantify how much more potent HS-159 is than HS-72 and potentially identify even more potent compounds.

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LIST OF ABBREVIATIONS

AAD70 ..... AIF-Derived Decoy for HSP70

Dox ..... doxycycline

EGF ..... Epidermal Growth Factor

FLECS ..... Fluorescence-Linked Enzyme Chemoproteomic Strategy

HSE ..... Heat Shock Element

HSF1 ..... Heat Shock Factor 1

HSP ..... Heat Shock Protein

HSP72 ..... Heat Shock Protein 72

HSR ..... Heat Shock Response

LD_{50} ..... Lethal Dose, 50% aka Median Lethal Dose

NBD ..... Nucleotide Binding Domain

OIS ..... Oncogene Induced Senescence

PES 2-phenylethynesulfonamide

INTRODUCTION

In recent years, the scientific community has grown very interested in the role of heat shock proteins (HSP) in cancer and their potential as therapeutic targets. The 72 kDa heat shock protein, HSP72, has drawn specific interest because of its inducible nature and overexpression in the cancer phenotype. This study seeks to characterize the anticancer activity of a series of small molecule inhibitors of HSP72 that were identified at Duke.

Heat Shock Proteins

Heat shock proteins were first discovered in 1962 and were originally defined in the context of their increased expression following exposure to heat, heavy metals, and oxidative stress (Lianos et al., 2015; Powers & Workman, 2007). They are grouped into families by size and function: HSP90, HSP70, HSP60, and small HSPs (Jego, Hazoumé, Seigneuric, & Garrido, 2013; Lianos et al., 2015; Powers & Workman, 2007). HSPs are highly evolutionarily conserved across species (Howe et al., 2014; Jego et al., 2013). Most HSPs regulate essential functions within cells. For example, they serve as molecular chaperones, protecting client proteins from proteolysis. They are involved in the assembly and maintenance of protein complexes, and intracellular protein trafficking and secretion. Heat shock proteins have also been shown to regulate transcription factors (Lianos et al., 2015).

Induction of HSPs is a key component of the heat shock or stress response (HSR), which is characterized by a series of biochemical and molecular events that protect cells from initial and repeat exposure to proteotoxic stress or injury (Powers & Workman, 2007). Indeed, HSPs are thought to be necessary for cell survival under stress

(Calderwood, Khaleque, Sawyer, & Ciocca, 2006). The cytoprotective effects include anti-apoptotic mechanisms by which HSPs bind to and inactivate key players in the apoptotic machinery (Jego et al., 2013).

The HSP70 family is of specific interest in cancer biology. This family is highly conserved and consists of HSPs between 66kDa-78kDa (Powers & Workman, 2007). There are eight mammalian HSPs in this family (Howe et al., 2014). All members have similar structures in their N-terminal ATPase and substrate-binding domains. The ~10kDa C-terminal domain is the most variable and contains the EEVD motif in most isoforms; this is the site where co-chaperones interact. HSP70 family members protect against programmed cell death at different points along the apoptotic pathway. HSP70 can prevent the loss of mitochondrial membrane potential upon exposure to apoptotic stimuli, blocking apoptogenic factor release (e.g. cytochrome c or AIF). Alternatively, HSP70 can act downstream of the mitochondria, directly inhibiting APAF-1 or AIF. HSP70 also potently inhibits caspase-independent death by modulating the pro-apoptotic JNK pathway (Powers & Workman, 2007).

The HSP70 family includes both the highly stress-inducible HSP72 (HSP70i, HSPA1, HSP70-1) and the constitutively expressed Hsc70 (HSP73, HSPA8). Under normal conditions, HSP70s are ATP-dependent, requiring ATP hydrolysis in the N-terminal NBD (nucleotide binding domain) (Howe et al., 2014; Jego et al., 2013). We are particularly interested in the actions of HSP72, which we will consider later.

Heat Shock Proteins and Cancer

Recent evidence shows that protein levels of HSPs are elevated in the cancer phenotype. This suggests that cancer cells are more dependent on HSPs, perhaps because of the hostile conditions created by deregulated oncogenes or the stress of the solid tumor microenvironment, which includes nutrient starvation, hypoxia, and acidosis (Powers & Workman, 2007). HSP levels are increased in most stages of tumor development and in the process of developing drug resistance. When non-malignant cell undergo oncogenic transformation, HSPs protect newly transformed cells from undergoing oncogene induced senescence (OIS), instead, enabling them to grow and survive (Calderwood et al., 2006). In addition, cytotoxic chemotherapies can induce HSP expression in tumor cells, which may play a role in the development of therapeutic resistance. Moreover, studies have shown that increased HSP levels in tumors are associated with poor clinical outcomes (Jego et al., 2013).

In mouse models, overexpression of HSP70 resulted in increased tumor growth, metastatic dissemination and resistance to treatment with chemotherapeutic agents. Conversely, depletion or inhibition of HSP70 resulted in decreased tumor size (Jego et al., 2013). Akerfelt et al. showed that mice lacking HSF1, the transcription factor that regulates HSP expression, have lower tumor incidence and better survival in a classical chemical skin carcinogenesis model as well as in a model with an oncogenic mutation of p53. They found similar results with human cancer cell lines with HSF1 depleted by RNAi strategies (Åkerfelt, Morimoto, & Sistonen, 2010).

HSP72

HSP72 is of particular interest as a target for therapeutic treatment. In unstressed non-malignant cells, HSP72 is expressed at low or undetectable levels. Upon exposure to proteotoxic stress, HSP72 protein expression increases (Howe et al., 2014; Volloch & Sherman, 1999). When the stress is removed, levels decrease to baseline as HSP72 is degraded, or aggregates into granules where it is then permanently inactivated (Volloch & Sherman, 1999). The inducing stress is not limited to heat shock, but can also include viral infection, transformation, and various forms of cancer (Howe et al., 2014).

High levels of HSP72 protein are expressed in tumors, and overexpression correlates with a highly aggressive phenotype including increased invasiveness and metastatic dissemination, chemo-resistance, and poor clinical outcome (Howe et al., 2014; Jego et al., 2013; Lianos et al., 2015; Volloch & Sherman, 1999; Yaglom, Gabai, & Sherman, 2007). HSP72 can protect cancer cells from stress by providing another route to survive in the inhospitable tumor microenvironment e.g. hypoxia, or in the presence of denatured protein aggregates caused by chemotherapy and radiation (Howe et al., 2014). Indeed, it seems that constitutively high HSP72 expression is essential for cancer cell survival. Down-regulating HSP72 has a significantly stronger effect in transformed cell lines than in primary cell lines, indicating that tumor cells seem to constitute a stressed phenotype and are more dependent on the cytoprotective effects of HSP72 (Jego et al., 2013). When HSP72 is depleted in tumorigenic and non-tumorigenic cell lines, the tumorigenic cells show significantly increased sensitivity to the killing effects of cytotoxic agents, whereas the non-tumorigenic lines are unaffected (Howe et al., 2014).

Volloch et al. sought to understand the mechanism underlying the association between HSP72 overexpression and oncogenic transformation. It was believed that oncogenic transformation induces HSP72 expression, but could the converse be true? They tested the question in mice and found that sustained elevated levels of HSP72 result in oncogenic transformation in immortalized cells. It is noteworthy to mention that aged mammalian cells show a decreased ability to express HSP72 in response to stress. Without this phenomena, as cells faced the stress of aging, HSP72 would be constitutively expressed and greatly increase the likelihood of cancer occurrence as people age (Volloch & Sherman, 1999).

HSP72 has a key role in cancer initiation and progression because it helps cells avoid the apoptosis that would otherwise be caused by tumor microenvironment stress and by anti-cancer drugs. HSP72 can inhibit apoptosis at many points, through both the intrinsic and extrinsic apoptotic pathways (Lianos et al., 2015). HSP72 inhibits events in mitochondrial-mediated apoptosis by blocking Bax translocation to the mitochondria, which in turn blocks cytochrome C release and prevents apoptosis. HSP72 also inhibits death receptors 4 and 5, preventing TNF-related ligand formation of the death-induced signaling complex that promotes apoptosis. HSP72 also mediates caspase dependent and independent apoptosis by binding Apaf-1 and thus blocking procaspase-9 recruitment to the apoptosome in the caspase-dependent pathway, and by JNK inhibition in the caspase-independent pathway (Calderwood et al., 2006; Howe et al., 2014). Cells that evade apoptosis through these mechanisms can still undergo necrosis. Moderate levels of

necrosis lead to inflammation and angiogenesis, which promotes tumor cell invasion and metastasis (Calderwood et al., 2006).

Cell proliferation can also be limited by terminal cell senescence. Targeted siRNA mediated depletion of HSP72 in newly transformed human epithelial cell lines has been shown to trigger cell senescence via p53-dependent and independent pathways as evidenced by increased senescence associated β –galactosidase activity (Calderwood et al., 2006; Lianos et al., 2015; Yaglom et al., 2007). Decreased HSP72 can also inactivate the cell cycle kinase, Cdc2, causing G2-M cell cycle arrest and cell senescence (Yaglom et al., 2007).

Challenges of Developing a Therapeutic Agent and Previous Attempts

While there is good rationale to develop an agent to inhibit HSP72 as a therapeutic treatment for cancer, the process has proved difficult. One of the most difficult challenges is that HSP72 shares 90% of its sequence with the constitutively active Hsc70, inhibition of which would be lethal. The only known phenotype of the HSP72 knockout mice is reduced sperm count in male mice. Other challenges to identifying an HSP72 inhibitor include its poorly defined physiological substrates and the fact that its nuclear binding pocket is always occupied by ATP or ADP, limiting small molecule accessibility. Past inhibitors have failed to discriminate between members of the HSP70 family or have performed poorly in vivo (Howe et al., 2014).

Quercetin, a naturally occurring flavonoid, has been found to inhibit HSP expression by modulating HSF1 activity and thus inhibiting cancer cell growth both in vitro and in vivo. This inhibitor is selective to tumor cells compared with non-malignant,

cells; however, it is not specific as it has promiscuous inhibitory effects on many protein kinases. KNK437, a lactam, is another inhibitor that showed early promise. Both of these drugs, however, are not ideal for clinical use because of their low potency; they require high concentrations on the order of 100-500 μM to be effective. The specificity of the inhibitors has to be questioned at these high concentrations (Powers & Workman, 2007).

Jego et al. showed that ADD70 (AIF derived decoy for HSP70), an HSP70 inhibitor, reduced the size of established tumor xenografts and led to tumor growth delay in mice. ADD70 also sensitized the mice to cisplatin, a DNA alkylating agent used to treat a variety of solid tumors. Notably, ADD70 is specific to HSP72 but not constitutively active Hsc70. These effects were not observed in immunodeficient animals. The mechanism of ADD70 antitumor activity has been attributed to a CD8+ T-cell immune response (Jego et al., 2013; Powers & Workman, 2007). Recent investigation has also identified PES (2-phenylethynesulfonamide) as a small molecule inhibitor for HSP70. PES causes protein aggregation leading to autophagy or caspase-dependent apoptosis. Both ADD70 and PES target the peptide binding domain (Jego et al., 2013).

It is widely recognized that inhibition of HSP90 in vitro and in vivo results in upregulation of HSP72. This effect is demonstrated by studies using siRNA mediated depletion of HSP72, which results in increased sensitivity of leukemia cell lines to the antitumor activity of HSP90 small molecule inhibitors (Powers & Workman, 2007). 17AAG, an HSP90 inhibitor that showed clinical efficacy in Phase I and II trials, is selective for tumor cells but may also lead to resistance to cancer therapy because HSP90 is no longer able to buffer against altered phenotype cancer cells (Calderwood et al.,

2006). Naturally, it will be desirable to combine HSR modulators to evaluate the potential synergistic effect of HSP90 and HSP72 inhibitors together.

Identification of HS-72 and Its Analogs

In a recent publication from the Haystead Laboratory at Duke University, Howe et al. were able to identify an allosteric small molecule inhibitor that selectively binds to HSP72. They identified the compound by using fluorescence-linked enzyme chemoproteomic strategy (FLECS) on a chemical screen of their in-house library of 3,379 purine-like molecules. Notably this inhibitor is selective over Hsc70. HS-72 is an allosteric inhibitor thought to bind at the NBD of HSP72 (Figure 1). It is hypothesized that HS-72 binds HSP72 causing a conformational change that alters the surface exposure to trypsin, protecting certain sequences from trypsin degradation. In murine studies, HS-72 appears to be well-tolerated, bioavailable, and reduced tumor growth in the MMTV-neu breast cancer model (Howe et al., 2014).

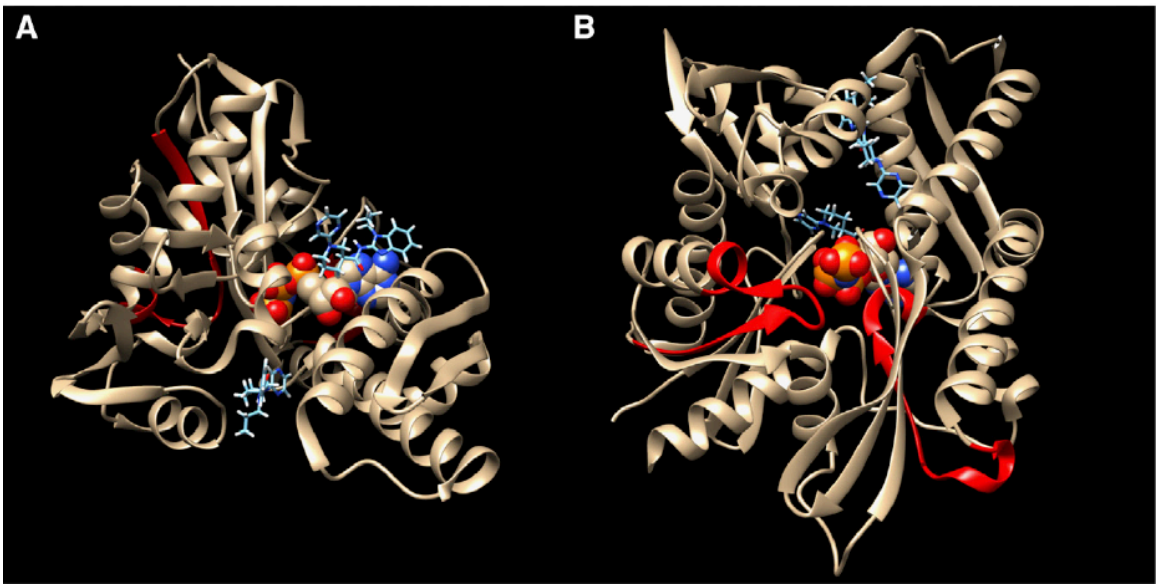


Figure 1: Molecular Modeling Supports HS-72 as an Allosteric Inhibitor of HSP72. These models show two different views of the HSP72 NBD and highlight two potential binding sites of HS-72 (Figure adapted from Howe et al., 2014).

For the above reasons, HS-72 is considered an ideal starting point for further chemical modification to improve potency. The initial isolate was racemic, so a separate R confirmation (now known as HS-71) and the more effective S confirmation (HS-72) were resynthesized (Howe et al., 2014). The Haystead Laboratory next developed nineteen analogs of HS-72 with structures shown in Figure 2. We sought to test which of these analogs exhibit increased potency compared with HS-72.

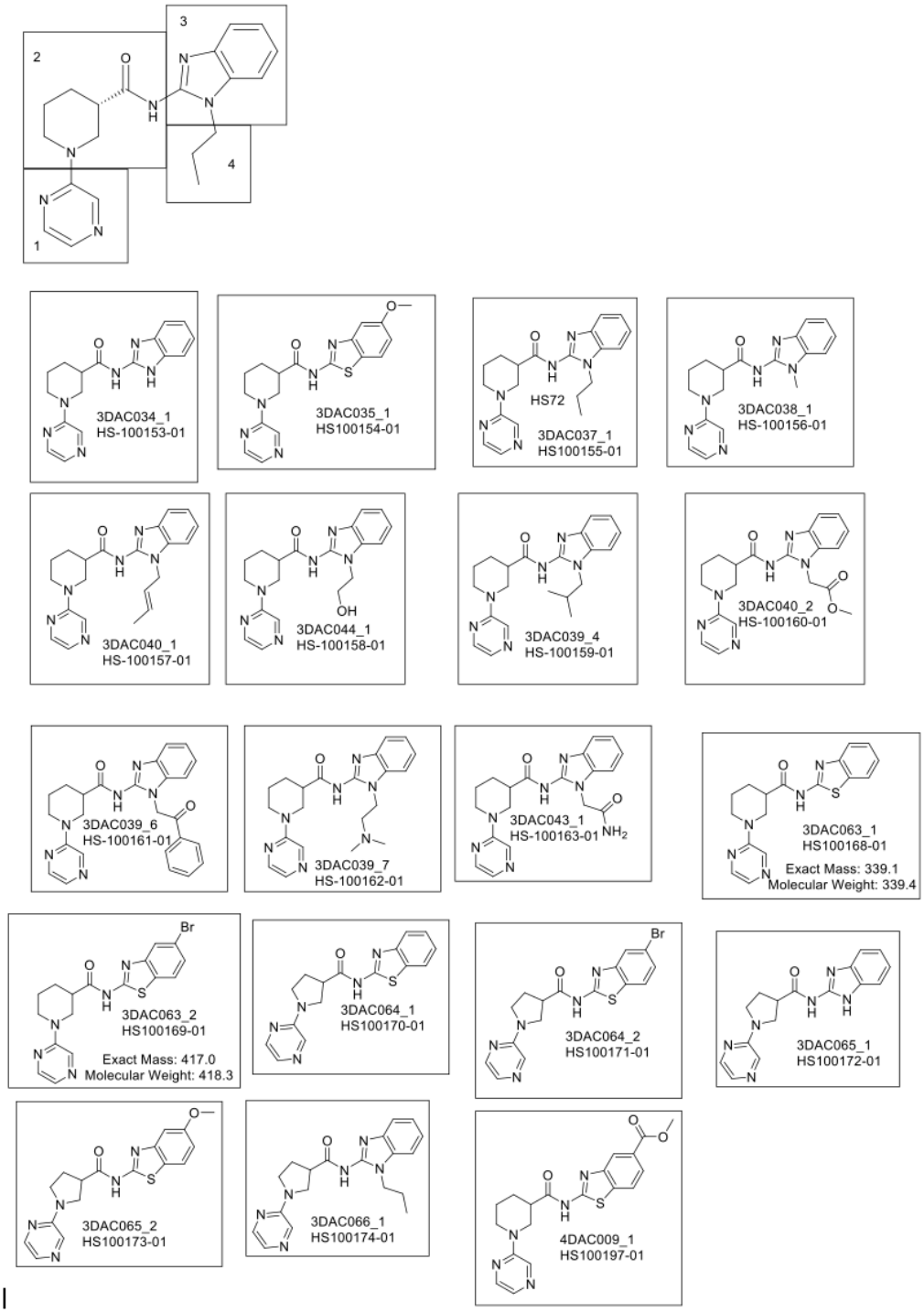


Figure 2: HS-72 Analog Structures. Chemical structures of the resynthesized analogs of HS-72 (Figure supplied by the Haystead Laboratory).

Specific Objectives

In order to identify an analog more potent than HS-72 we will:

(1) Assess the effect of each compound on cell viability at a range of concentrations using an ATP concentration luminescence assay.

(2) Evaluate the consistency of a promising compound(s) on the viability of different human tumor and non-malignant cell lines.

(3) Determine the effects of a more potent compound(s) on cell cycle kinetics and viability.

(4) Assess the effect of HSP72 inhibitors on oncogene-induced senescence.

We expect these studies will show:

(1) There is at least one analog that is more potent than HS-72 at inhibiting HSP72 and inducing cell death.

(2) A more potent HSP72 inhibitor exerts its effects by inhibiting anti-apoptotic pathways resulting in increased cell death and an increased proportion of cells in early apoptosis.

(3) A more potent HSP72 inhibitor will trigger cell senescence, which will eventually lead to apoptosis.

METHODS

Tissue Culture

The following human cell lines were used for these studies: (i) MCF10A, an immortalized, non-malignant breast epithelial cell line; (ii) MCF10A cells expressing the p110 oncogenic truncated form of HER2 under the control of doxycycline-inducible promoter; (iii) BT474 cells, a human invasive ductal breast carcinoma cell line; and (iv) T pancreatic tumor cells. These cell lines were maintained in log growth phase in tissue culture throughout the course of experiments. Transformed and untransformed MCF10A cells were each cultured separately with or without 0.25 μg/mL doxycycline.

MCF10A cells were cultured in 500mL DMEM/F12 base medium supplemented with 25mL heat inactivated horse serum, 100μL EGF (100 mg/mL), 250μL hydrocortisone (1mg/mL), 50μL choleraToxin (1mg/mL), 500μL insulin (10mg/mL) and 5.0mL pen/strep. BT474 cells were cultured in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum. DT cells were cultured in DMEM/M3:BaseF (Incell Corporation) base medium supplemented with 10% heat inactivated fetal bovine serum.

ATP Concentration Luminescence Assay

To identify the most promising HSP72 inhibitor, each of the analogs was tested at a range of concentrations in BT474 cells, and ATP concentrations were measured to determine the concentration of remaining viable cells. BT474 cells were plated at 3×10^3 cells per well on 96-well plates. Medium was replaced and individual compounds were added 24 hours later at the following concentrations (μM): 0, 4.5, 6, 9, 12, 18, 25, 38, 50,

and 75. After 72 hours, the medium was removed and Promega Luciferase Reagent was added, and after 10 minutes the plates were read. The luminescence was measured using a GENios Luminescence Microplate Reader.

This process was later repeated using HS-72, HS-157, HS-159, and HS-160 in BT474, DT, MCF10A p110 -Dox, and MCF10A p110 +Dox cell lines.

β -Galactosidase Activity for Senescence

Western blot analysis was used to test for senescence associated β-galactosidase activity. MCF10A, MCF10A p110 -Dox, and MF10A p110 +Dox cells were plated at 1.4 x 10^4 cells per well under the following treatment conditions for each compound: no compound, 40 μM 0.01% DMSO, 10 μM HS-72, 20 μM HS-72, 30 μM HS-72, 40 μM HS-72, 5 μM HS-159, 10 μM HS-159, 15 μM HS-159, 20 μM HS-159, 30 μM HS-159, and 40 μM HS-160. The cells were maintained for two weeks and then protein extracts were collected for Western blot analysis.

To collect for Western blot analysis, the cells were collected in trypsin, washed several times in PBS and centrifuged, and the pellet was re-suspended in RIPA lysis buffer. After another 10 minute centrifuge, the supernatant was collected and added to a loading buffer. The samples were then loaded onto Biorad Mini-PROTEAN TGX. Vertical gel electrophoresis was run with the Biorad Mini-PROTEAN tetra cell, and the gel was then transferred to a membrane with a Thermofisher iBlot Gel Transfer Device.

The membranes were blocked in a solution of 5% dry milk in PBS-T for an hour. The membranes were soaked in a solution of 1% dry milk in PBS-T with the following Santa Cruz primary antibodies overnight: α- β-galactosidase (Mouse) at 1:500, α-actin

(Mouse) at 1:2000, and α-HSP70 (Rat) at 1:2000. The next day, the membranes were washed in PBS-T for one hour, with fresh PBS-T every 15 minutes. Then they were soaked in a solution of 1% dry milk in PBS-T with the following Santa Cruz secondary antibodies for one hour: 800-labeled anti-mouse antibody at 1:10,000 and 670-labeled anti-rat antibody at 1:10,000. The membranes were once again washed with PBS-T for one hour with 15 minute PBS-T changes. They were then rinsed with water and scanned on the Odyssey Infrared Imaging System.

Flow Cytometry

BT474 cells were plated at 4.2 x 10^4 cells per well on a 12-well plate. After 24 hours, the medium was replaced and compounds were added. Three wells were treated with HS-72 at 15 μM, 40 μM, and 50 μM. Three wells were treated with HS-159 at 5 μM, 10 μM, and 20 μM. Three wells were treated with HS-160 at 50 μM each, and three wells were treated with no compound. The cells were grown in compound for 72 hours. At this point the cells were dissociated with trypsin and collected. The samples were sent to Duke University's Flow Cytometry Shared Resource facility for flow cytometric analysis with Annexin V and 7AAD markers to mark for early apoptosis and cell death, respectively.

RESULTS

ATP Concentration Luminescence Assay on Each Analog

A dose-response curve was plotted for each compound where the average luminescence at each concentration of compound, representative of cell viability, was compared to the no compound treatment control. The data was fit with a third degree polynomial line of best fit and the concentration at which 50% of cells are predicted to be dead, an approximated LD_{50} was determined for each compound. Those values are shown in Table 1. The original graphs for each compound are provided in Appendix A. HS-159 shows the lowest LD_{50} at 10.88 μM. HS-157 and HS-197 were less potent with LD_{50} values of 16.24 μM and 18.01 μM, respectively. The averaged LD_{50} of HS-72 across four independent experiments was 18.53 μM.

Table 1. LD_{50} Values of Compound Analogs
The concentration curves were fit with a third degree polynomial curve. The concentration at which the percent of luminescence compared to the non-compound control luminescence value was predicted to be 50% was calculated. If there were multiple plates for the same compound, those values were averaged to give the values below.
*The third degree polynomial fit does not have a solution for y=50%.

DRUG	LD-50 (uM)
HS-72	18.53
HS-197	18.01
HS-174	59.31
HS-173	107.77
HS-172	N/A*
HS-171	84.48
HS-170	57.35
HS-169	N/A*
HS-168	61.05
HS-163	76.83
HS-162	77.18
HS-161	25.27
HS-160	N/A*
HS-159	10.88
HS-158	N/A*
HS-157	16.24
HS-156	59.09
HS-155	31.41
HS-154	N/A*
HS-153	N/A*

HS-159 ATP Concentration Luminescence Assay on Different Cell Lines

A dose-response curve was plotted for each compound on each of the four cell lines where the average luminescence at each concentration, representing a measure of cell viability, was compared to the no compound treatment control. As shown in Figure 3,

the effect of HS-159 was consistent on DT and BT474 cell lines as well as the MCF10A p110 cell line with and without doxycycline.

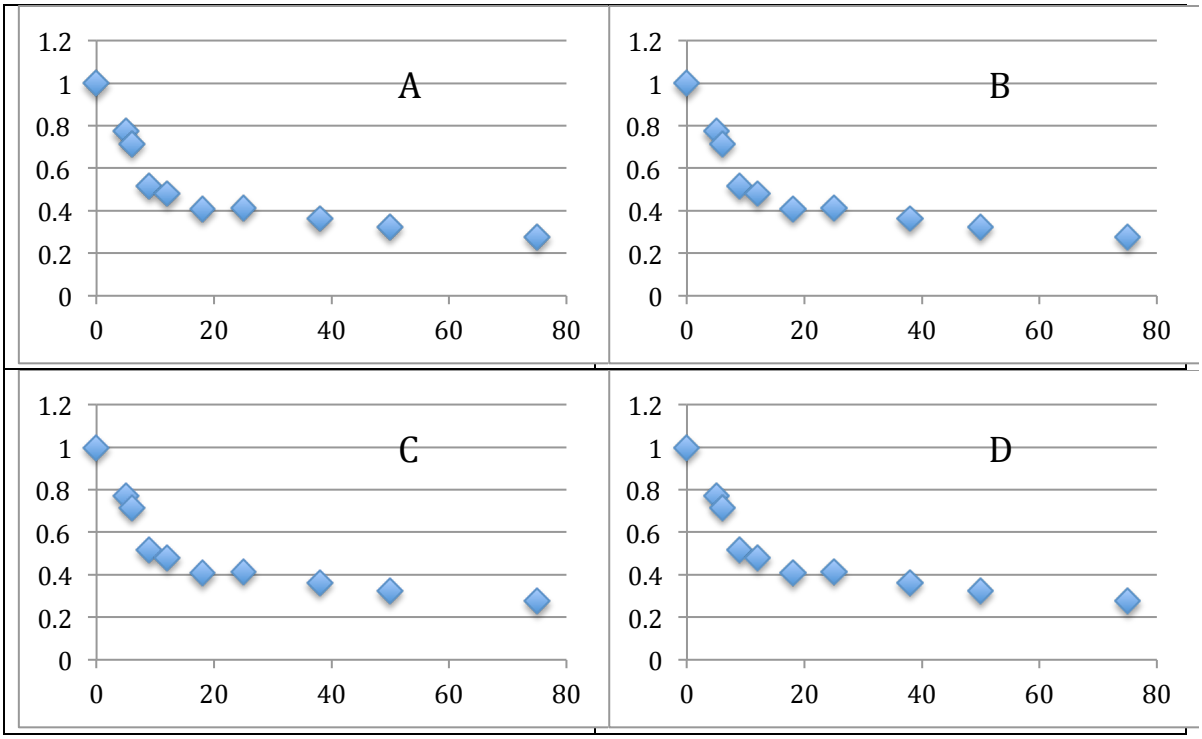


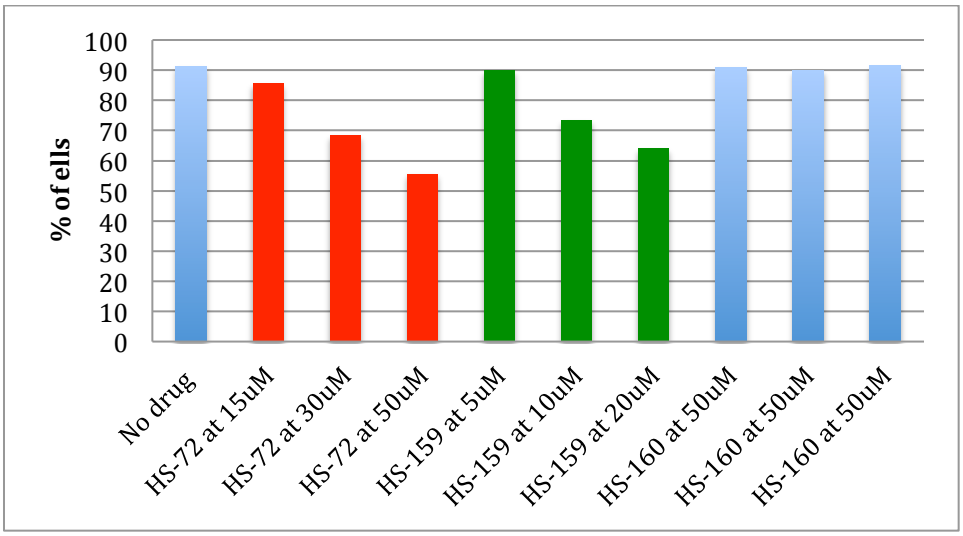
Figure 3: Luminescence Graphs for HS-159 on Different Cell Lines.
These graphs show the result of ATP luminescence measurements for the indicated cell lines treated with HS-159 for 72 hours. The y-axes represent % compared to the control, and the x-axes represent concentration in µM. (A) BT474 cells, (B) DT cells, (C) MCF10A p110 cells without doxycycline, (D) MCF10A p110 cells with 0.25 µg/mL doxycycline.

HS-159 Flow Cytometry

Treating cells with HS-160 showed no appreciable difference to the control group. Compared to controls, treatment with HS-72 and HS-159 resulted in an increased number and proportion of cells in early apoptosis. There was a direct correlation between concentration of compound and induction of apoptosis. In side by side comparison at low concentrations of compound, HS-159 increased the proportion of early apoptotic cells

compared with HS-72. Specifically, when cells were treated with HS-72 at 30 µM, flow cytometry showed 68.53% of cells were viable and 18.18% were undergoing early apoptosis. At only 20 µM, 64.17% of cells treated with HS-159 were viable and 21.4% were undergoing early apoptosis.

A



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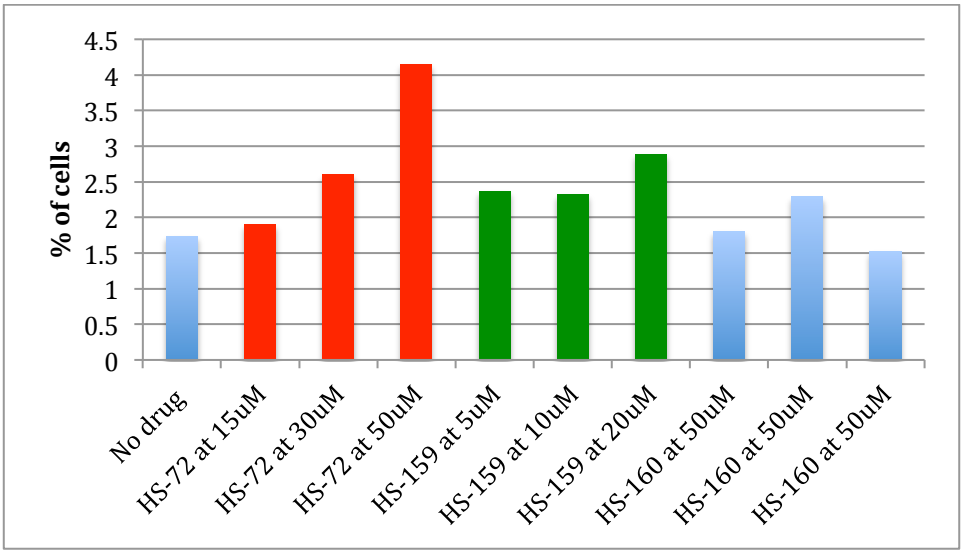


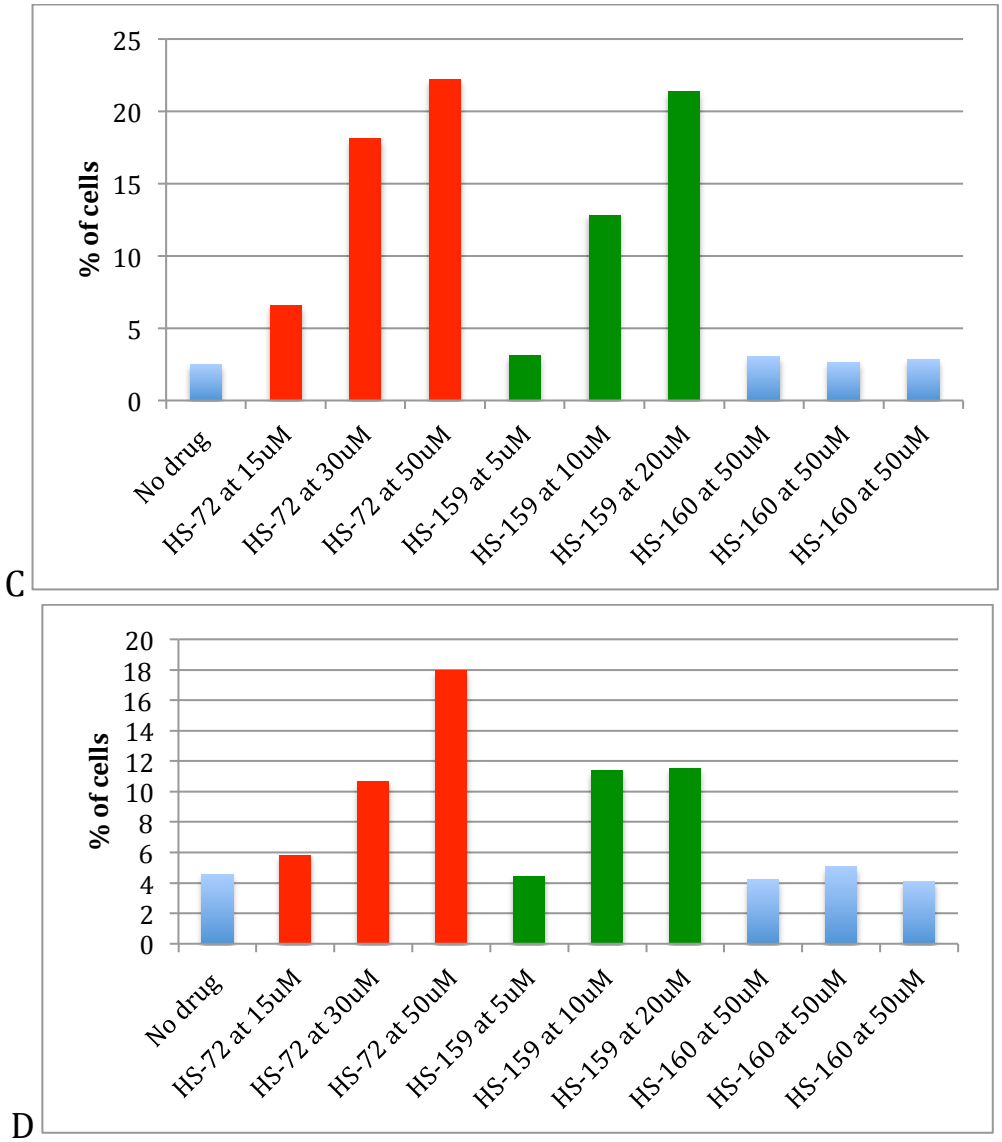


Figure 4: Flow Cytometry Results of HS-159 and HS-72 on BT474 cells.
The flow cytometry results on BT474 cells treated for three days with HS-72, HS-159 and control conditions of no drug and the negative control HS-160 are shown above. (A) 7AAD and Annexin V negative viable cells; (B) Annexin V positive/7AAD negative early apoptotic cells; (C) 7AAD positive/Annexin V negative dead cells; and (D) Annexin V and 7AAD positive apoptotic cells.

β –Galactosidase Staining

The results of staining for β –galactosidase activity were inconclusive. With cell densities clearly being variable after compound treatment for one or two weeks and no way to quantify the density of stained cells, we could not determine the effect that each drug treatment had on relative levels of senescence.

β –Galactosidase Protein Western Blot Analysis

The Western blots were analyzed for the strength of signal, with the strength of each signal normalized to the strength of the actin signal for each sample. When the β –galactosidase : actin ratio was normalized to the DMSO control for each cell condition, the experimental conditions, those samples treated with HS-72 and HS-159, showed a lower ratio of β –galactosidase, indicating lower relative levels of senescence than the control. This data is presented in Table 2 and Figure 5.

Table 2: Western Blot Analysis Normalization for MCF10A p110.
The tables show the intensity of signal for the antibody to each protein, the β–gal :actin normalization, and the normalization to the DMSO control. These results are from MCF10A-p110 cells treated with each compound for 2 weeks. Note that the concentration of HS-160 was 40 μM, HS-159 at 5 μM, and HS-72 at 10 μM. (A) Without doxycycline. (B) With 0.25 μg/mL doxycycline.

A	DMSO	HS-160	HS-159	HS-72
β –galactosidase	5100	68400	40900	36300
actin	323000	333000	460000	325000
β –galactosidase : actin	0.159	0.205	0.0889	0.112
normalized	1	1.288	0.558	0.701

B	DMSO	HS-160	HS-159	HS-72
β –galactosidase	90500	97600	79000	49300
actin	243000	314000	309000	226000
β –galactosidase : actin	0.372	0.311	0.256	0.218
normalized	1	0.835	0.686	0.586

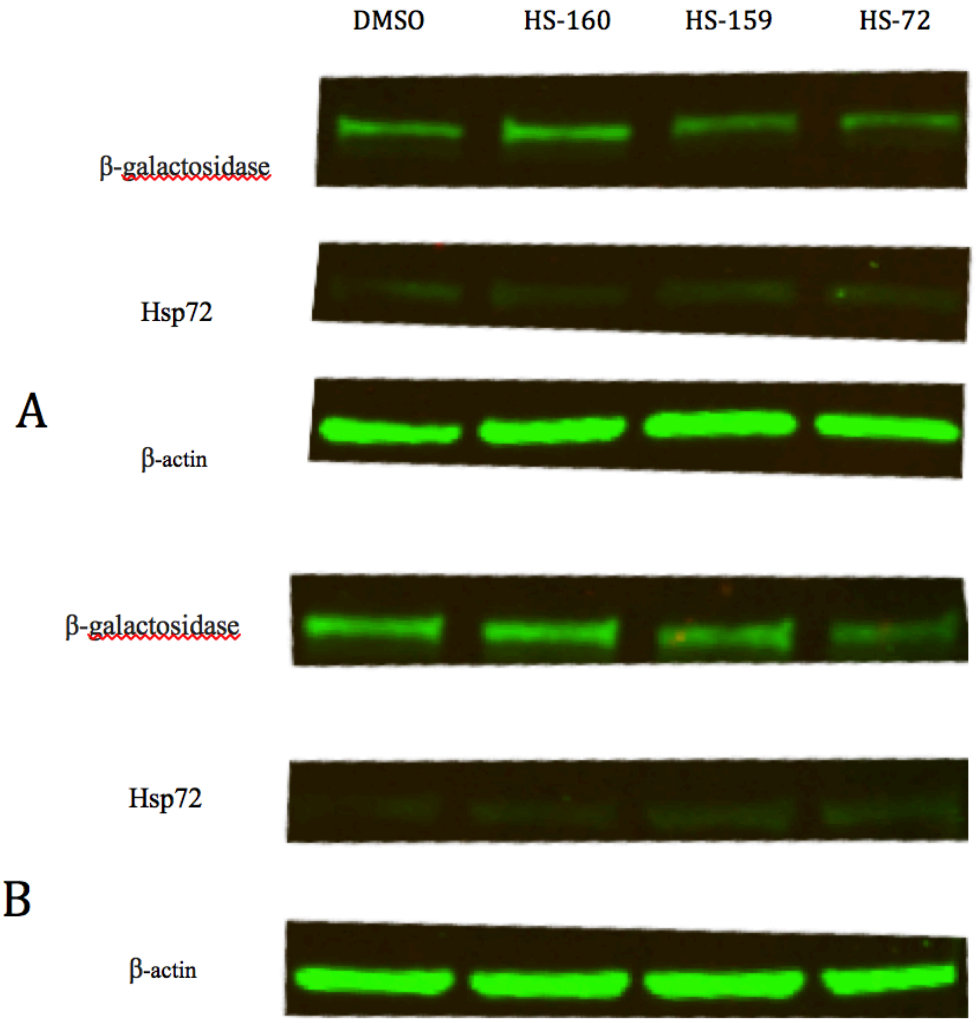


Figure 5: Western Blot Analysis in MCF10A-p110 cells.
MCF10A-p110 after 2 weeks of treatment with the indicated compounds. Note that HS-160 was administered at 40 µM, HS-159 at 5 µM, and HS-72 at 10 µM. (A) Without doxycycline. (B) With 0.25 µg/mL doxycycline.

DISCUSSION

This study confirms the results of the Howe et al. study showing that HS-72 is an inhibitor of HSP72. It also demonstrates that HS-159 is a more potent inhibitor of HSP72 with a measured LD_{50} of 10.88μM compared to 18.53μM. HS-157 and HS-197 proved to be effective inhibitors, but not significantly more potent than HS-72. We also showed that other analogs of HS-72 that were tested are either ineffective or less potent than HS-72. These include HS-153, HS-154, HS-155, HS-156, HS-158, HS-160, HS-161, HS-162, HS-163, HS-168, HS-169, HS-170, HS-171, HS-172, HS-173, and HS-174.

Preliminary tests of HS-159 on various cell lines suggest that HS-159 is an effective and potent inhibitor on a range of cell lines and has clinical potential in breast and pancreatic cancers, as well as potentially other tumor types. Flow cytometry results showed that HS-159, to a greater degree than HS-72, caused early apoptosis. This confirms our hypothesis that HS-159 inhibition of HSP72 diminishes the anti-apoptotic effect of HSP72.

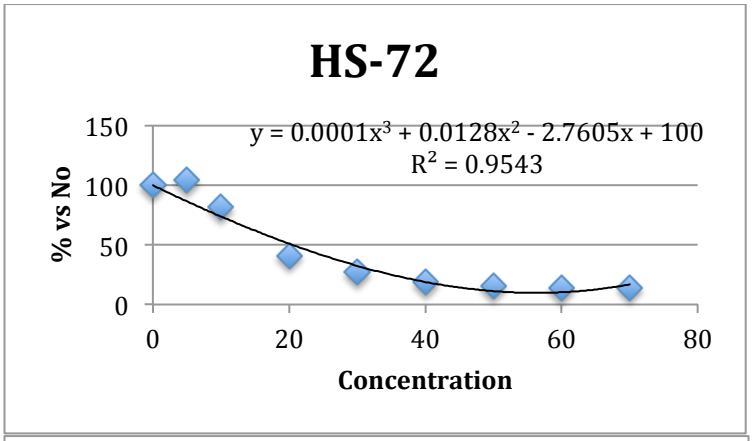
Our initial hypothesis was that HS-72 and HS-159 inhibit HSP72, which in turn increases cell senescence in response to oncogenic transformation, in this case the p110 oncogenic form of HER2. However, Western blot protein analysis of cells treated with these compounds does not reveal an increase in senescence as measured by β – galactosidase activity. We collected protein extracts for Western blot analysis after one and two weeks of treatment with HS-72 and HS-159. In light of these results, it is possible that HS-72 and HS-159 exert their antitumor effects through a pathway involving increased cell senescence, but by the time the cells were collected, they had

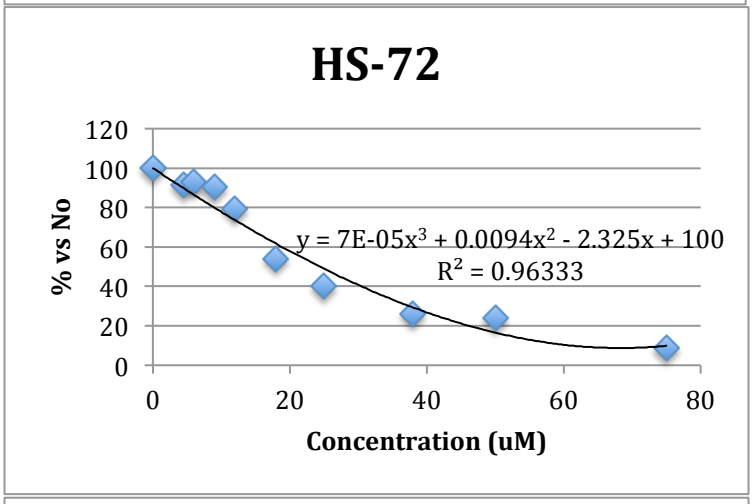
died. Further studies with Western blot analysis at earlier time periods are necessary to test this theory.

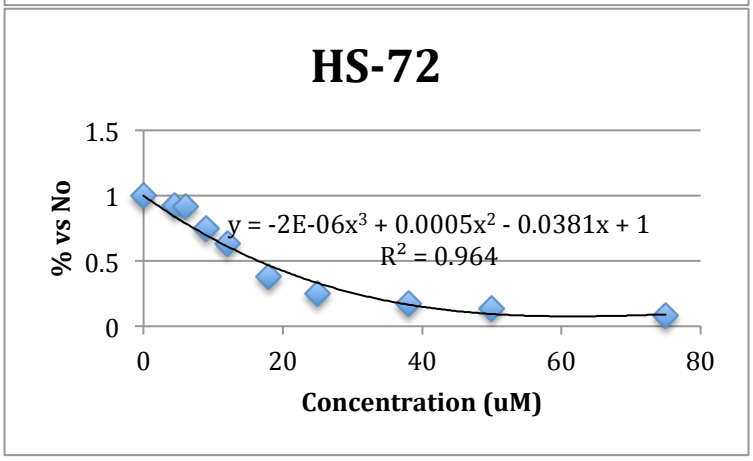
While the discovery of a more potent inhibitor than HS-72 is promising, it is noteworthy to mention that this study represents a very small sample size and HS-159 is less than twice as potent as HS-72. Ideally, another inhibitor can be developed or identified that is an order of magnitude more potent than HS-72. The modification from HS-72 to HS-159 involves increased branching of the side chain (Figure 2, see designation 4). Thus, further modification or addition of methyl groups to this side chain shows promise as a starting point for development of a more effective compound.

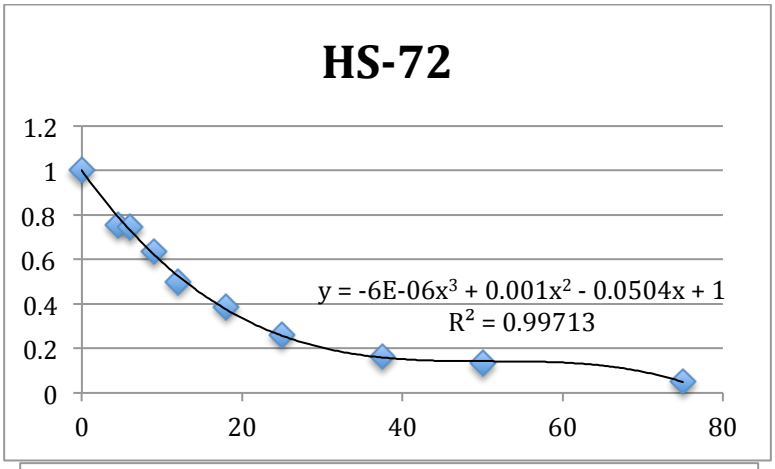
Further studies should also examine the effects of combining an HSP72 inhibitor such as HS-159 with an HSP90 inhibitor. We suggest that these two inhibitors may act synergistically and will have the greatest clinical potential when combined.

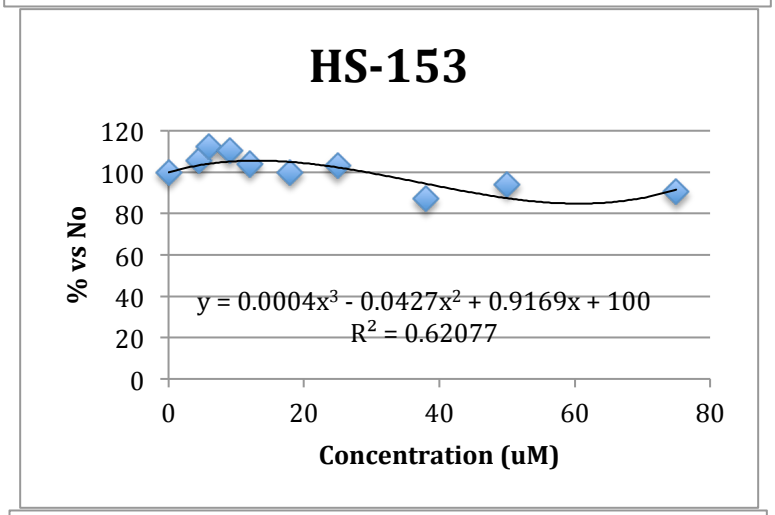
APPENDIX A

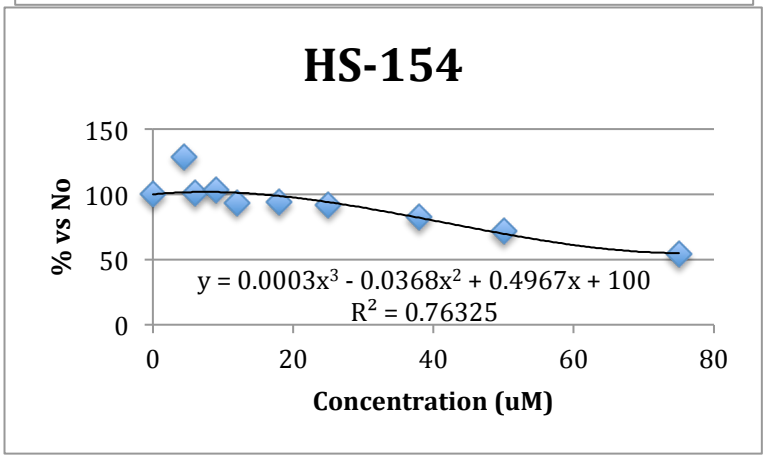

HS-72
y = 0.0001x³ + 0.0128x² - 2.7605x + 100
R² = 0.9543
% vs No
150
100
50
0
0
20
40
60
80
Concentration

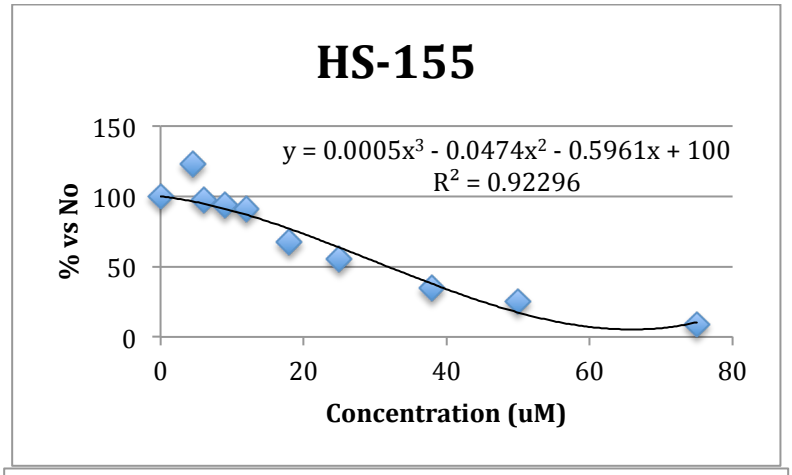

HS-72
y = 7E-05x³ + 0.0094x² - 2.325x + 100
R² = 0.96333
% vs No
120
100
80
60
40
20
0
0
20
40
60
80
Concentration (uM)

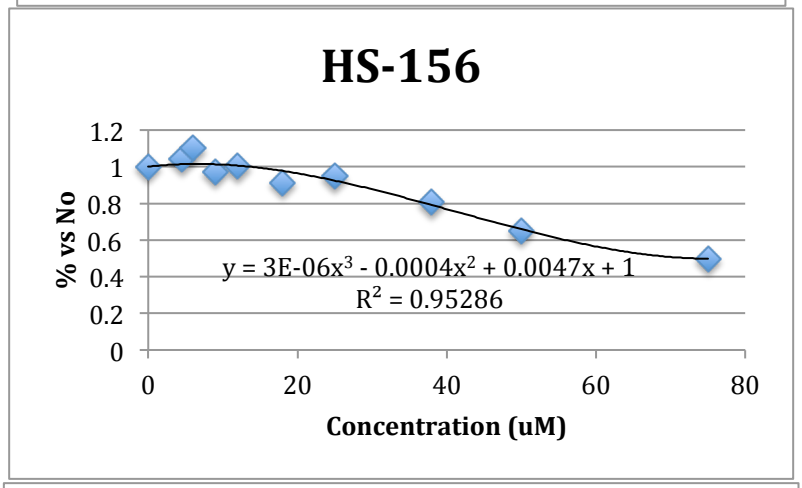

HS-72
y = -2E-06x³ + 0.0005x² - 0.0381x + 1
R² = 0.964
% vs No
1.5
1
0.5
0
0
20
40
60
80
Concentration (uM)

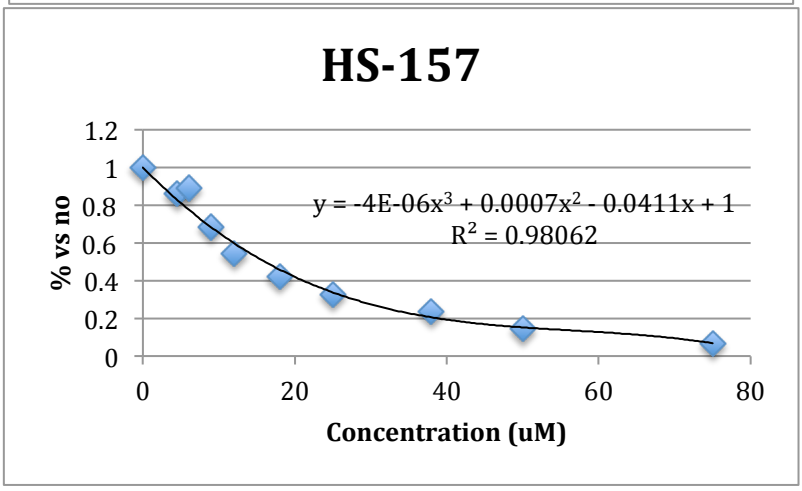

HS-72
1.2
1
0.8
0.6
0.4
0.2
0
0
20
40
60
80
y = -6E-06x³ + 0.001x² - 0.0504x + 1
R² = 0.99713

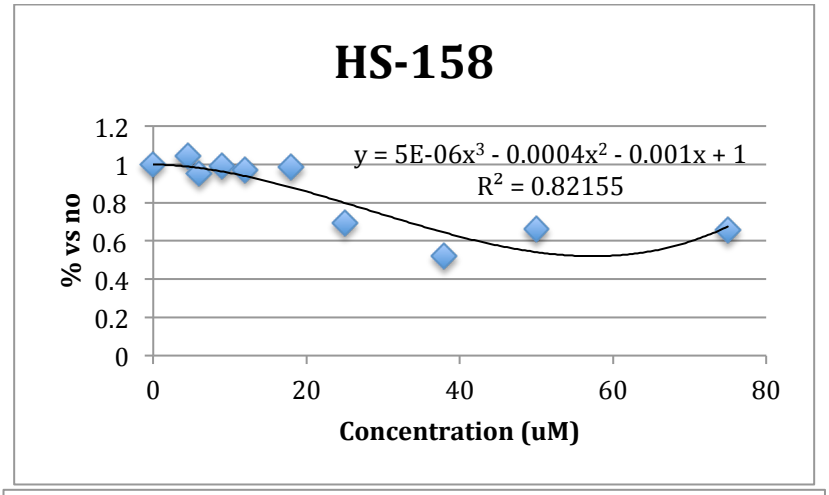

HS-153
120
100
80
60
40
20
0
% vs No
0
20
40
60
80
Concentration (uM)
y = 0.0004x³ - 0.0427x² + 0.9169x + 100
R² = 0.62077

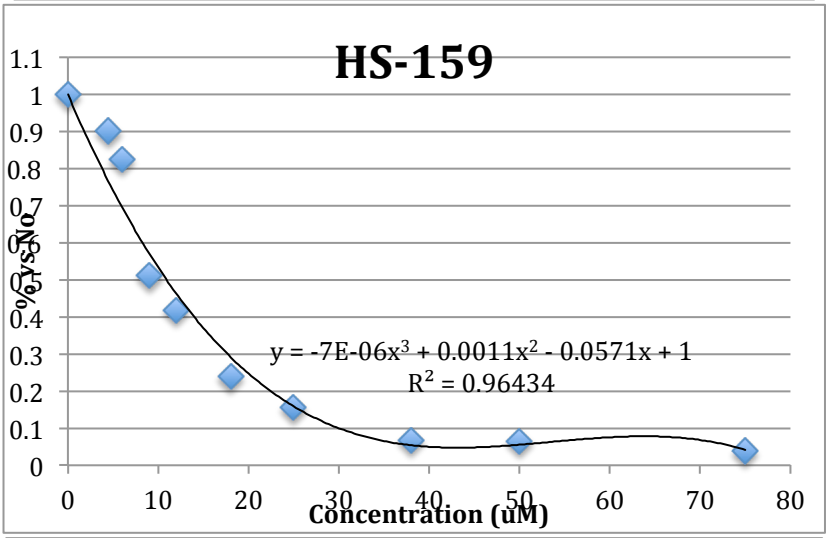

HS-154
150
100
50
0
% vs No
0
20
40
60
80
Concentration (uM)
y = 0.0003x³ - 0.0368x² + 0.4967x + 100
R² = 0.76325

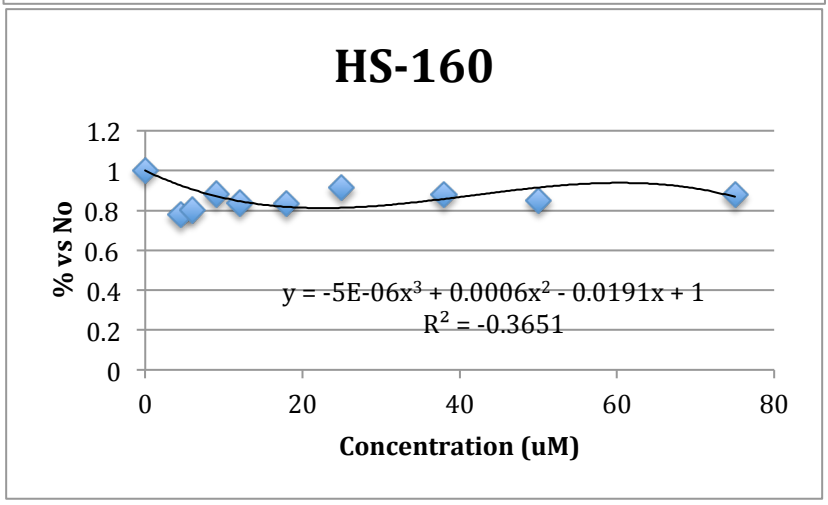

HS-155
y = 0.0005x³ - 0.0474x² - 0.5961x + 100
R² = 0.92296
% vs No
150
100
50
0
0
20
40
60
80
Concentration (uM)

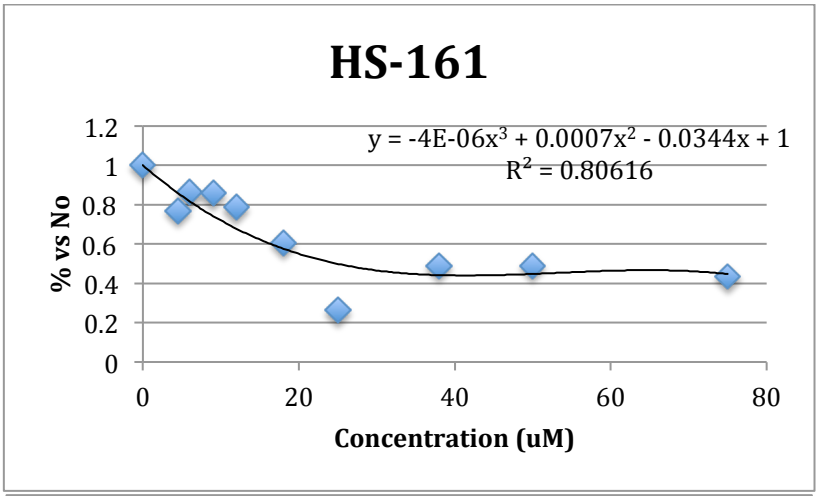

HS-156
y = 3E-06x³ - 0.0004x² + 0.0047x + 1
R² = 0.95286
% vs No
1.2
1
0.8
0.6
0.4
0.2
0
0
20
40
60
80
Concentration (uM)

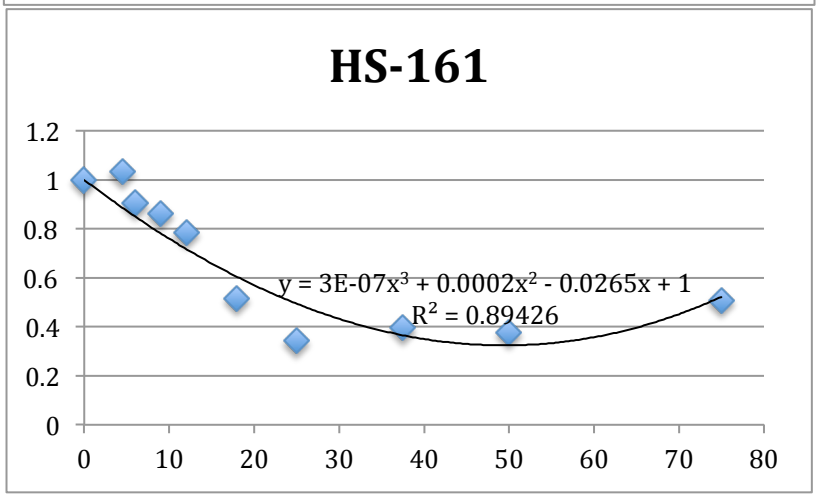

HS-157
y = -4E-06x³ + 0.0007x² - 0.0411x + 1
R² = 0.98062
% vs no
1.2
1
0.8
0.6
0.4
0.2
0
0
20
40
60
80
Concentration (uM)

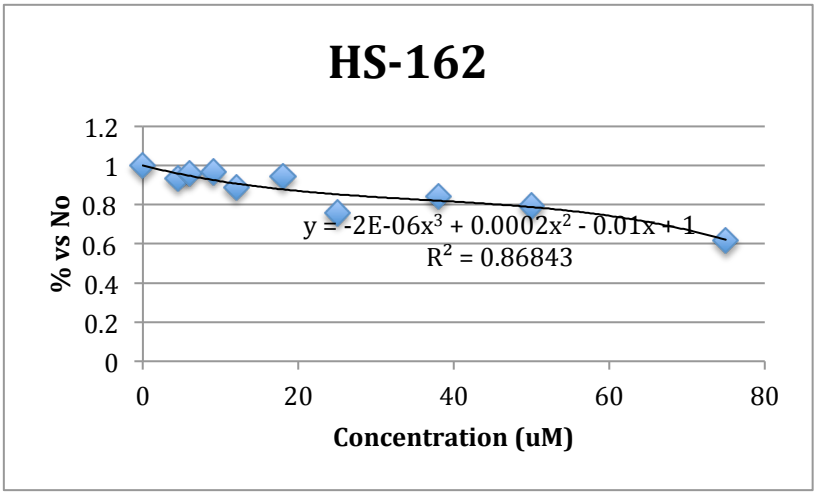

HS-158
y = 5E-06x³ - 0.0004x² - 0.001x + 1
R² = 0.82155
% vs no
Concentration (uM)

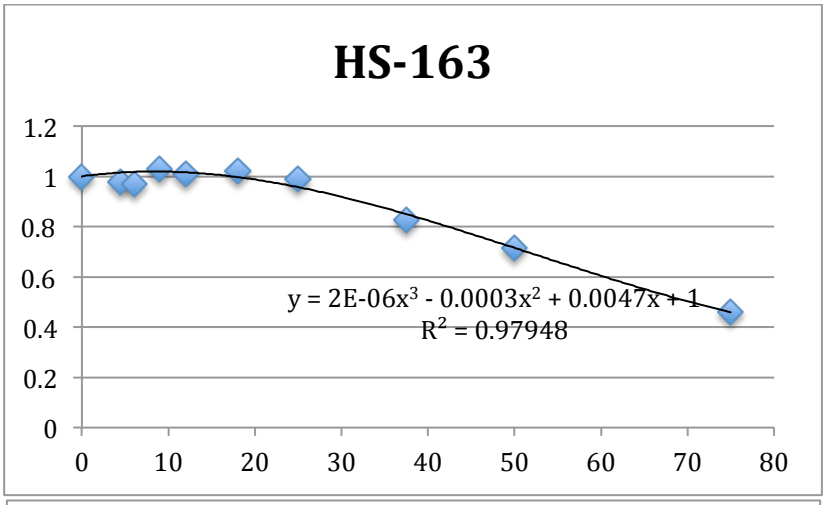

HS-159
y = -7E-06x³ + 0.0011x² - 0.0571x + 1
R² = 0.96434
% vs No
Concentration (uM)

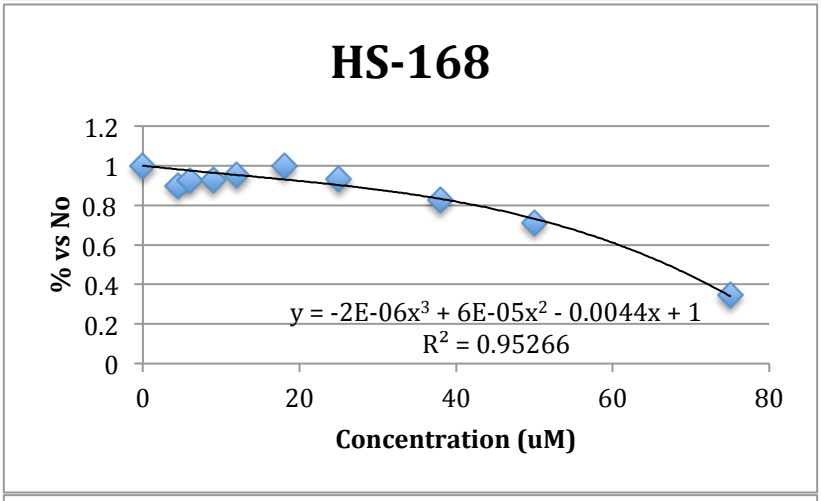

HS-160
y = -5E-06x³ + 0.0006x² - 0.0191x + 1
R² = -0.3651
% vs No
Concentration (uM)

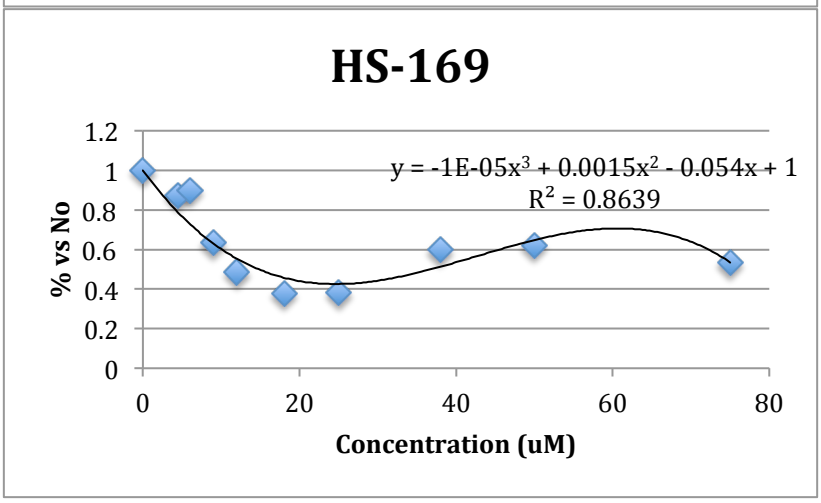

HS-161
y = -4E-06x³ + 0.0007x² - 0.0344x + 1
R² = 0.80616
% vs No
Concentration (uM)
0
0.2
0.4
0.6
0.8
1
1.2
0
20
40
60
80

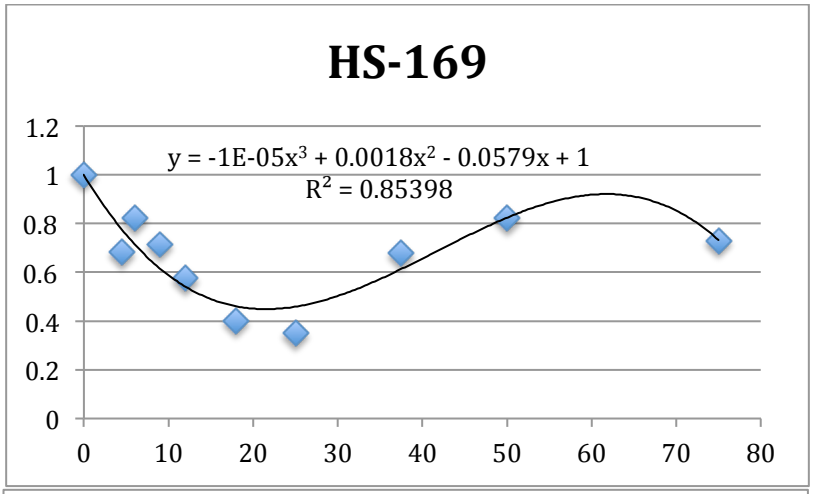

HS-161
y = 3E-07x³ + 0.0002x² - 0.0265x + 1
R² = 0.89426
0
0.2
0.4
0.6
0.8
1
1.2
0
10
20
30
40
50
60
70
80

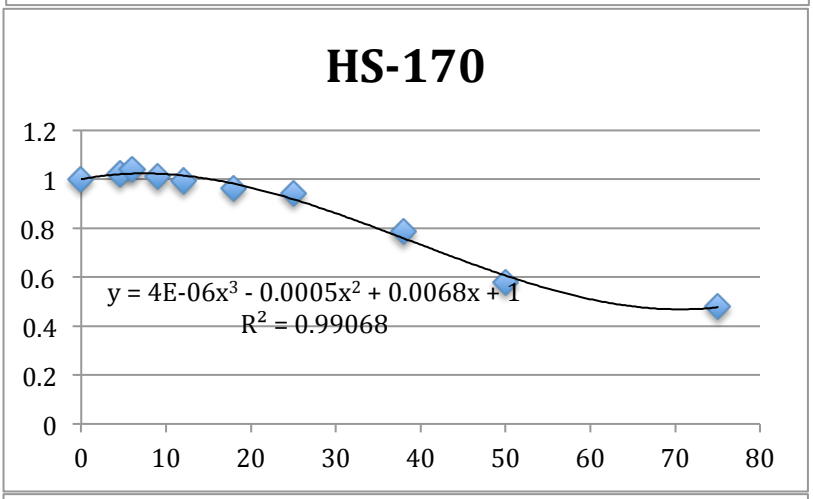

HS-162
y = -2E-06x³ + 0.0002x² - 0.01x + 1
R² = 0.86843
% vs No
Concentration (uM)
0
0.2
0.4
0.6
0.8
1
1.2
0
20
40
60
80

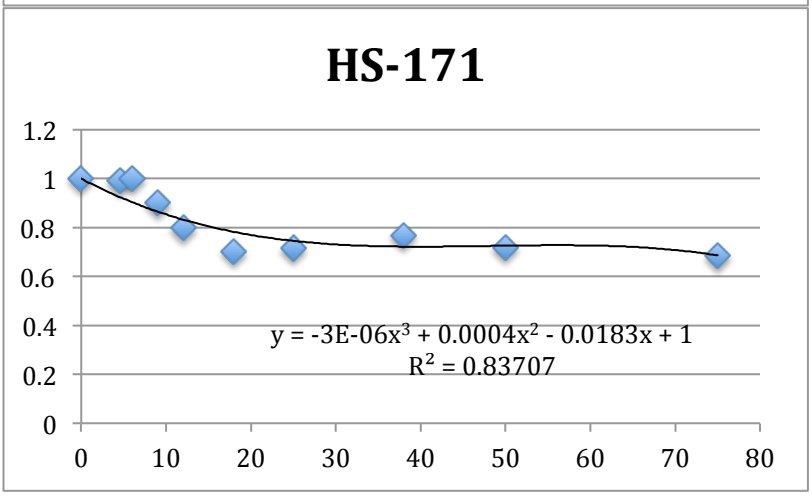

HS-163
1.2
1
0.8
0.6
0.4
0.2
0
0
10
20
30
40
50
60
70
80
y = 2E-06x³ - 0.0003x² + 0.0047x + 1
R² = 0.97948

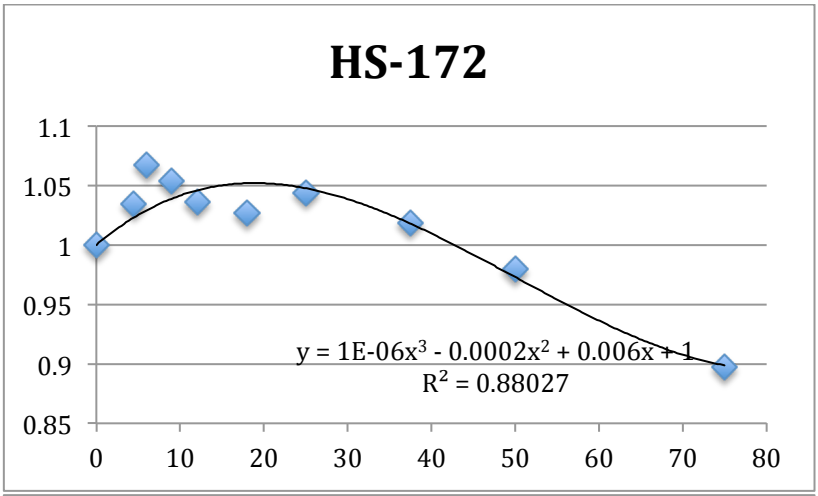

HS-168
% vs No
1.2
1
0.8
0.6
0.4
0.2
0
0
20
40
60
80
Concentration (uM)
y = -2E-06x³ + 6E-05x² - 0.0044x + 1
R² = 0.95266

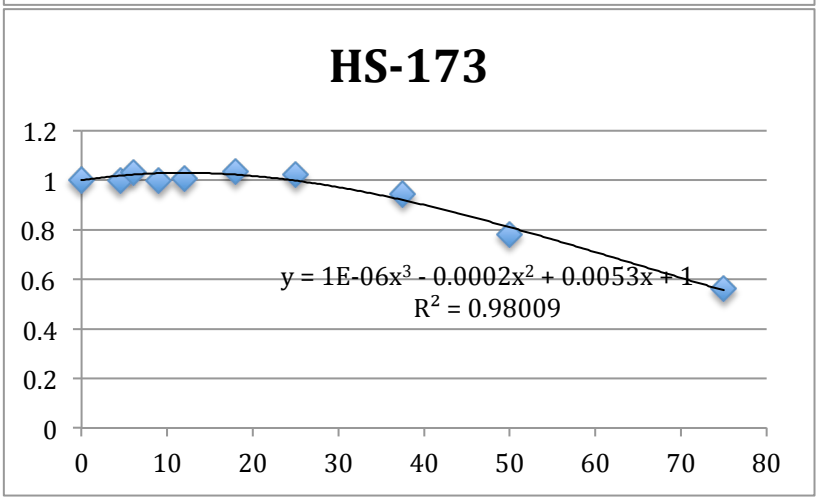

HS-169
% vs No
1.2
1
0.8
0.6
0.4
0.2
0
0
20
40
60
80
Concentration (uM)
y = -1E-05x³ + 0.0015x² - 0.054x + 1
R² = 0.8639

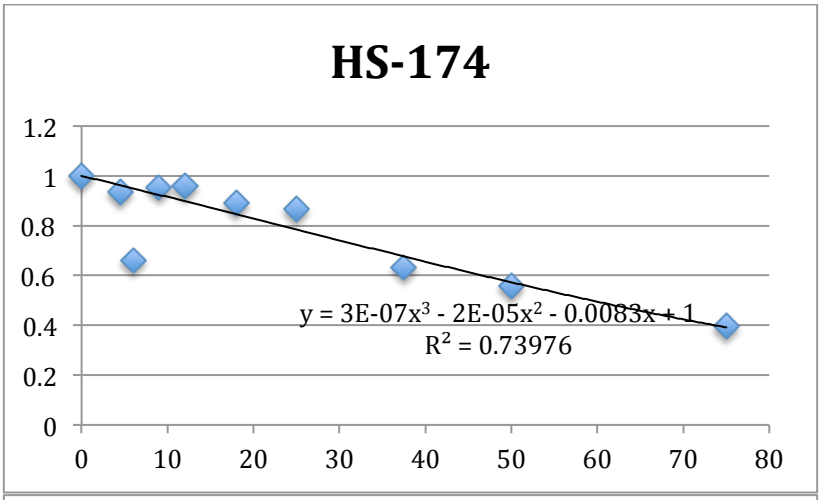

HS-169
y = -1E-05x³ + 0.0018x² - 0.0579x + 1
R² = 0.85398
1.2
1
0.8
0.6
0.4
0.2
0
0
10
20
30
40
50
60
70
80

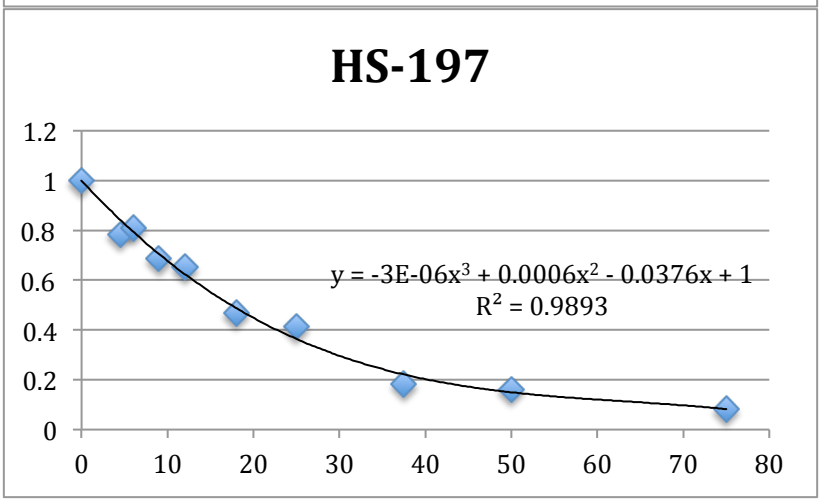

HS-170
y = 4E-06x³ - 0.0005x² + 0.0068x + 1
R² = 0.99068
1.2
1
0.8
0.6
0.4
0.2
0
0
10
20
30
40
50
60
70
80


HS-171
y = -3E-06x³ + 0.0004x² - 0.0183x + 1
R² = 0.83707
1.2
1
0.8
0.6
0.4
0.2
0
0
10
20
30
40
50
60
70
80


HS-172
1.1
1.05
1
0.95
0.9
0.85
0
10
20
30
40
50
60
70
80
y = 1E-06x³ - 0.0002x² + 0.006x + 1
R² = 0.88027


HS-173
1.2
1
0.8
0.6
0.4
0.2
0
0
10
20
30
40
50
60
70
80
y = 1E-06x³ - 0.0002x² + 0.0053x + 1
R² = 0.98009


HS-174
1.2
1
0.8
0.6
0.4
0.2
0
0
10
20
30
40
50
60
70
80
y = 3E-07x³ - 2E-05x² - 0.0083x + 1
R² = 0.73976


HS-197
1.2
1
0.8
0.6
0.4
0.2
0
0
10
20
30
40
50
60
70
80
y = -3E-06x³ + 0.0006x² - 0.0376x + 1
R² = 0.9893

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Education:

9/14 – present	**Boston University School of Medicine**, Boston, MA M.S. Degree in Medical Sciences [expected 05/16]
7/11 – 05/14	**Duke University**, Durham, NC B.S. in Mathematics
1/12 – 5/12	**Bucknell University**, Lewisburg, PA
8/10 – 4/11	**University of Pittsburgh**, Pittsburgh, PA

Research:

6/15 – 8/15	Study Coordinator for Open-Label Placebo Trial Served as CaRES intern, leading a team of two Research Specialists at UAB Comprehensive Cancer Center in a pilot clinical trial. Evaluated the feasibility and effects of using open-labeled administration of placebo pills on objective and psycho-social outcomes among cancer survivors seeking relief from cancer-related fatigue.
1/14 – 5/14	Mathematical Modeling Capstone Researched the outbreak of SARS and its epidemiology. Constructed and analyzed a mathematical model of various control methods on Mathematica. Presented our results to the mathematics department and WHO.
9/13 – 12/13	Biochemistry Research Summary Researched and reviewed a model of gastric bypass on mice and the biochemistry principles involved for an Introduction to Biochemistry course.
9/13 – 12/13	Intro to Human Development Research Thesis Researched and presented an experiment proposal on the cognitive development of individuals with Duchenne Muscular Dystrophy.

1/11 – 4/11 University of Pittsburgh Freshmen Engineering Conference Paper
Researched and presented an integrated approach to tissue engineering via mesenchymal stem cells, scaffolds, and bioreactors.

Employment:

Appalachia Service Project
Appalachia Service Project is a non-profit organization seeking to eradicate substandard housing within the Central Appalachia region.

5/12 – 8/12 Served as Volunteer Coordinator in Avery County, NC in 2012. Worked on a team of four to manage 500+ teenage and adult volunteers; manage an $80,000+ budget; select, plan for, and manage 17 construction projects through seven weeks of volunteer-administered repairs; engage with community leaders and community contacts. Worked unconventional hours to very tight deadlines.

5/14 – 8/14 Served as Staff Liaison assisting about 10 staff teams across TN, KY, VA, WV, and NC and liaising between the staffs and the non-profit's administration.

Leadership and Activities:

9/11 – 5/14 Duke Division I Women's Lacrosse Team
Participated in 30+ hours per week practicing, training, competing, and reviewing film. Represented Duke Athletics by supervising and hosting prospective student-athletes.

7/11 – 5/14 Oncology Recreation Therapy Volunteer, Duke Hospital
250+ total hours providing patients with interactive psychosocial therapy in order to develop, regain, or maintain the patients' capacity for full living. Led the Living History Program, which involved developing relationship with patients and writing a powerful one-page story about his or her life in order to strengthen connections between the patient and the care delivery team.

3/13 – 5/14 Duke Partnership for Service President
Managed an executive board of 14 that supported and coordinated the 70+ student service groups at Duke, connected individual students to those groups and other service initiatives, led events and campaigns to inspire the campus to service. Collaborated with school administration and various partners across Duke and Durham. Oversaw a budget of $80,000.

8/13 – 5/14 Transfer Advisory Council Chair

Serve as council Chair in 2013-2014 academic year managing 8 student counselors and coordinating all orientation events for the 40 transfer and exchange students matriculating to Duke. Maintained a community for the students throughout the year with occasional events to immerse them in student life at Duke. Developed personal relationships with 5 of the new students and facilitated their integration into Duke student life.

5/13 – 6/13 Coach for College Coach

Taught Physics and Life Skills and Coached Soccer to 150 Vietnamese 7th and 8th grade students in impoverished rural Vietnam. Worked as a team of 8 American college student athletes and 8 Vietnamese college students to plan, revise, and implement lesson plans in Physics, Math, Biological Sciences, English, Soccer, Basketball, Volleyball, and Baseball. Integrated the lessons to help the students to develop excitement for and proficiency in science, leadership, language, and life skills using sports as a means to applied learning.

Languages

Professional proficiency in Spanish.