IDENTIFICATION AND CHARACTERIZATION OF NATURAL COMPOUND INHIBITORS OF THE HSP90 CHAPERONE COMPLEX

By

JASON LEE DAVENPORT

Bachelor of Science in Biochemistry
Oklahoma Christian University
Oklahoma City, OK
2006

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
DOCTOR OF PHILOSOPHY
July, 2013

IDENTIFICATION AND CHARACTERIZATION OF

NATURAL COMPOUND INHIBITORS OF THE

HSP90 CHAPERONE COMPLEX

Dissertation Approved:

Dr. Robert Matts

Dissertation Adviser

Dr. Andrew Mort

Dr. Junpeng Deng

Dr. Patricia Canaan

Dr. Jeff Hadwiger

ACKNOWLEDGEMENTS

For all of my successes, both professionally and personally, I owe a great deal of credit to a great many people. First, I need to thank Dr. Matts for taking me into his laboratory, and for guiding my growth and development as a student, and as a scientist. His knowledge, experience, patience, and confidence in his students has allowed me to develop and independent, methodical, and overall mature and productive approach to science. I also owe thanks to the current and past members of my advisory committee, Dr. Andrew Mort, Dr. Junpeng Deng, Dr. Patricia Canaan, Dr. Jeff Hadwiger, and Dr. Stacey Benson. Their direction, advice, and patience have been much appreciated, and have given me a diverse and challenging system of support carrying out my research and writing.

I also would like to acknowledge my parents, for whom my education has always been a top priority. It has been largely thanks to their support and encouragement that I have had the desire and ability to pursue this degree and the skills and opportunity that come with it. Similarly, I have had a number of great friends and family members who have supported me in so many ways, and have made my educational journey not only better, but highly enjoyable. Finally, I would like to give credit to the large number of teachers, mentors, friendly acquaintances, and people who have simply served as examples in my learning process leading up to this day.

Acknowledgements reflect the views of the author and are not endorsed by committee members or Oklahoma State University.

Name: JASON DAVENPORT

Date of Degree: MAY 2013

Title of Study: IDENTIFICATION AND CHARACTERIZATION OF NATURAL COMPOUND INHIBITORS OF THE HSP90 CHAPERONE COMPLEX

Major Field: BIOCHEMISTRY AND MOLECULAR BIOLOGY

The 90 kDa Heat Shock Protein (Hsp90) has emerged as a major therapeutic target for a number of diseases, the most notable of which is cancer. Accordingly, many groups are attempting to develop small molecules that modulate the activity of this chaperone and its associated co-chaperones. At the same time, many of these same diseases have traditionally been treated using substances derived from local plant species. Recognizing the intersection between these two medicinal strategies, our group has sought to find novel inhibitory compounds against the Hsp90 chaperone machine by employing high-throughput screens of natural compound libraries. In this work, we report the results of four such screens, showing that a number of potential Hsp90 inhibitors have already been used successfully in various medical traditions for the treatment of multiple diseases. Characterization of structure-activity relationships using a 1,4- nathoquinone scaffold that was common to several of our natural product hits, demonstrated that the luciferase renaturation assay that forms the basis of our screens is comparable to cell-based assays. Four compound hits, anthothecol, garcinol, rottlerin, and piperlongumine, were further characterized and demonstrated to inhibit the proliferation of human cancer cells, and the maturation of an Hsp90-dependent kinase. Additionally, one of the compounds, gambogic acid, was shown to disrupt inter-molecular interactions of the Hsp90 chaperone complex, and to interact with Hsp90 itself. These compounds and their derivatives represent potential novel therapeutics against cancer and other diseases in which Hsp90 plays a role.

TABLE OF CONTENTS

Chapter Page

LIST OF TABLES

Table Page

LIST OF FIGURES

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

For thousands of years, traditional medicine has relied, to a large degree, on substances available in the natural world. These have come mostly from various types of plants, but also from animals and other living things. In the modern era, beginning two centuries ago with the purification of morphine from poppy seed pods, many maladies have been treated with chemicals derived from traditional natural sources. Among these are psychotic conditions, microbial infections and their symptoms, heart conditions, and others have been treatable in this manner [1]. Many of the compounds from multiple ancient traditions are being studied, and their molecular targets being determined. In addition to the basic investigation into the compounds' activities, researchers are attempting to find compounds to be used against specific diseases, such as cancer [2].

To this end, many groups have turned their attention to the 90 kDa Heat Shock Protein (Hsp90) as a therapeutic target. The importance of this molecule is difficult to overstate. It is a nearly universally conserved protein that serves as a major molecular hub in most organisms. The chaperone plays a critical role in protein stability, signal transduction, protein localization, and others. Hsp90's significance in a medicinal context has been realized, as its central involvement in the underlying processes of cellular transformation has been observed [3].

Hsp90 homologs are found in an enormous variety of organisms, including plants, animals, and many microbial life forms. Except for archaea, the organisms of every kingdom contain at least one

Hsp90 gene [4]. HtpG is the homolog of Hsp90 found in E. coli, but it is not essential under non-stress conditions. Importantly, however, all studied eukaryotes require Hsp90 for viability [5]

In organisms from bacteria to humans, and including viral proteins, Hsp90 has been shown to interact with well over 300 proteins. These interactions were determined for the cytosolic form alone. [6] The interactors of the mitochondrial and endosplasmic reticulum isoforms of Hsp90 have not been comprehensively examined, although they will certainly increase the number.

The significance of Hsp90's interactors lies not only in their number, but in their functions. The majority of proteins that are dependent upon Hsp90 (client proteins) for their activity are involved in either signal transduction or localization of proteins. A large number of the chaperone's interacting proteins are receptors, kinases, or transcription factors.[6] Additionally, mitochondrial import receptors [7], assembly of the kinetochore complex [8], proteins involved in DNA packaging and modification, and other proteins are dependent on Hsp90 for their function, whether as clients, or simply as subunits of the same functional complex.

An additional, critical function that Hsp90 serves is the one for which it was named. Hsp90 belongs to the family of proteins called heat shock proteins, because in response to elevated temperatures, their expression is increased. In addition to heat, other stresses that result in the denaturing of proteins, such as heavy metals, reactive oxygen species, cold, and others, Hsp90 and the other heat shock proteins are up-regulated, and act by binding the exposed hydrophobic portions of unfolded proteins, and promoting their refolding, rather than aggregation [4, 9]. At least some cancer lines have been shown to constitutively express otherwise inducible heat shock proteins, as well as display

localization of these proteins to cellular and extracellular areas that they are not found in normal cells [10].

ATPase and chaperone cycle

Hsp90 has ATPase activity which drives its active chaperoning activity. The process of folding or refolding of a substrate protein involves the switching between several conformations of Hsp90 and its co-chaperones, accompanied by the binding and hydrolysis of ATP and release of ADP. This cycle and the specific combination of co-chaperones in the complex are intricately linked. Different co-chaperones have various effects on the ATPase activity of Hsp90. Some of them are known to increase the rate of ATP hydrolysis, while others are known to decrease it. Still others do not, in themselves, have an observable effect on the ATPase, but influence other properties of the chaperone cycle.

Hsp90, in conjunction with its co-chaperones, exists as a clamp-shaped dimer (Figure 1.1). The C-terminal domains mediate dimerization that was thought to be constitutive.[11-12] More recent evidence suggests that, while the C-termini are usually dimerized, the Hsp90 dimer does occasionally exist in a conformation where dimerization is mediated solely by the N-termini. It should be noted that this dynamic behavior was observed in fluorescently labeled, purified Hsp90, and its physiological relevance has not yet been established [13]. The current model of its ATPase cycle is shown below (Figure 2). Once the cycle is completed, it can begin again, presumably with either the same client molecule, or a different one. Differences in co-chaperone composition of the Hsp90 complex, and in the complex's function within a cell, are observed when the complex is biased toward specific points in the cycle. These changes hold significance for therapeutic intervention, which will be discussed later.

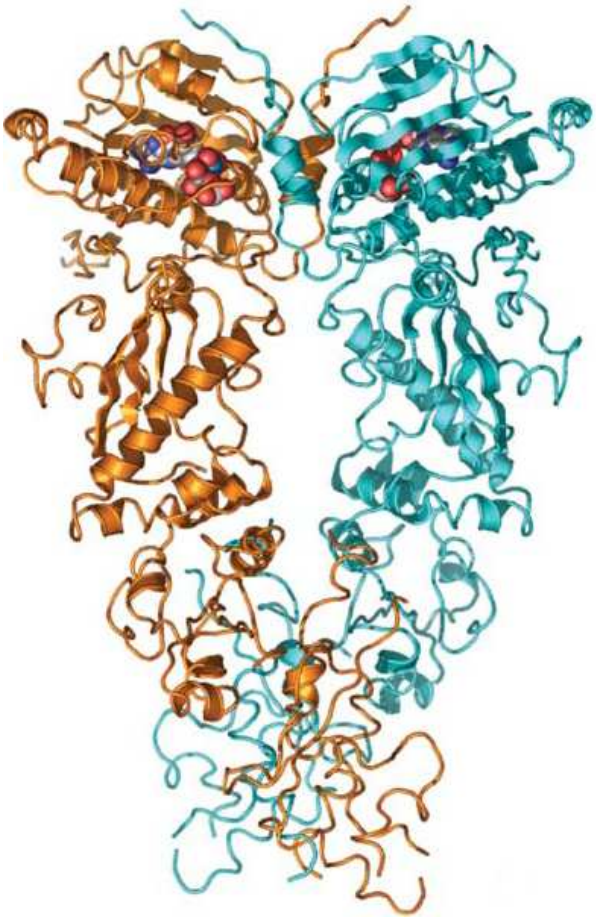


Figure 1.1. Full length crystal structure of yeast Hsp90 with non-hydrolyzable ATP analog bound to N-terminal ATP binding site. [14]

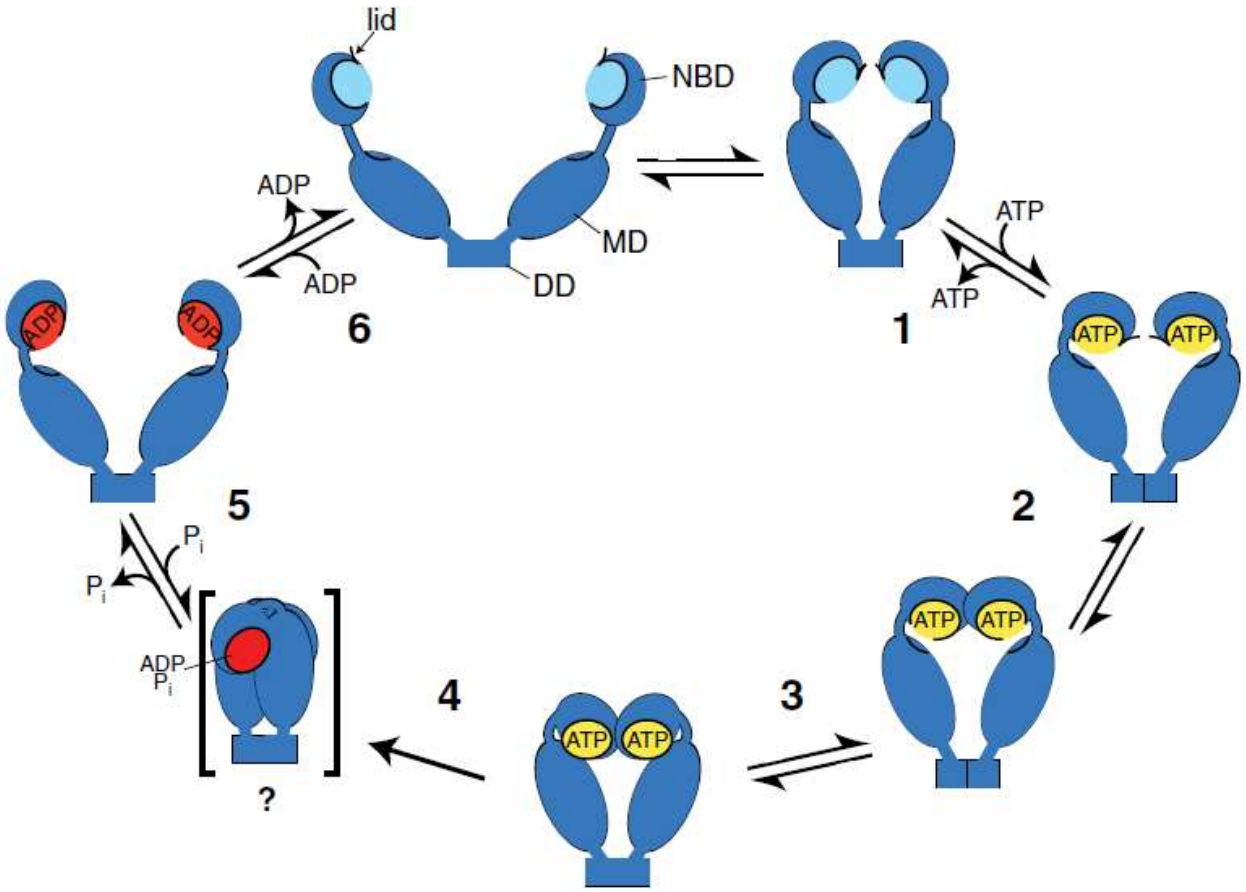


Figure 1.2. Current proposed model of the Hsp90 ATPase cycle.[15]

Hsp90's 'default' state, in humans, is an open form, with no nucleotide bound, and exists in an equilibrium of positions between steps 6 and 1. Following binding of ATP (Step 1), a helical lid segment closes over the ATP, resulting in N-terminal dimerization (Step 2) [16-18], although this likely results from a shift in equilibrium of closed versus open Hsp90 molecules, rather than an all-or-nothing phenomenon [19]. The closed position of the lid segment stabilizes this conformation (Step 3) and promotes ATP hydrolysis (Step 4) [20]. There is some evidence that, after hydrolysis of the ATP (Step 4), the two monomers engage in a highly compact, ADP-bound conformation, although this conformation has only been observed transiently.[21] After this, the clamp opens (Step 5), ADP is released (Step 6)

Co-chaperones

Hsp90 functions as part of a complex of proteins. Aside from the client, these additional proteins have been termed co-chaperones. In eukaryotes, over twenty co-chaperones have been identified. Some of these proteins have been shown to affect the rate of ATP hydrolysis by Hsp90. Others are, more often than not, found when certain types of substrate proteins are involved. The precise mechanisms, and to an extent, functions, of the co-chaperones in general have not yet been elucidated. A few of the co-chaperones are reviewed here briefly.

Of the proteins counted among co-chaperones of Hsp90, Hsp70 may be the one that is the most important in its roles outside of the Hsp90 complex. Hsp70 is essential for a large number of cellular functions where Hsp90 is not involved, or at least, is not involved directly. Hsp70 chaperones many nascent peptides to maturity and in conjunction with Hsp90, prevents aggregation under stress conditions and promotes disaggregation, and serves to target proteins for degradation by both the lysosomal and proteasomal machineries. It also plays varying roles in the regulation of apoptosis, in addition to other roles [22].

Hsp70 and the Heat Shock Cognate 70 (Hsc70) are often studied and discussed interchangeably. In fact, much of the investigation into these proteins is done using antibodies that do not distinguish between the two. Similar to Hsp90's isoforms, Hsc70 is constitutively expressed, while Hsp70 is induced upon protein stress. While the two proteins have differences in binding specificity and substrate affinity, for the purposes of their interactions with Hsp90, we will discuss them as a single protein. Hsp70 consists of an N-terminal ATP binding domain and a C-terminal substrate binding domain. Like Hsp90, the C-terminal domain contains an MEEVD motif, allowing it to interact with tetratricopeptide repeat (TPR) proteins.

Hop, or Hsp organizing protein, is primarily known to serve as an adaptor for the transfer of client proteins from Hsp70 to Hsp90. After binding Hsp40 and the client protein, Hsp70 then binds to Hop,

which then facilitates the transfer of the client to Hsp90. Hop's ability to link Hsp70 and Hsp90 is mediated by the presence of multiple TPR domains. These domains bind the conserved MEEVD motifs present in both the Hsp90 and Hsp70 proteins. Hop is also known to inhibit the binding of ATP by Hsp90, effectively reducing the rate at which Hsp90 hydrolyzes ATP, pausing the ATPase cycle before transfer of the client from Hsp70 [23].

The binding and transfer of a client protein by Hsp70 also requires the activity of Hsp40. Hsp40 is part of a family of proteins referred to as J domain proteins. They each contain a J domain, which consists of a four-helix motif, two of which are involved in a coiled-coil structure. The domain contains an invariant His-Pro-Asp tripeptide in one of its loops. These proteins are essential for most of Hsp70's functions, conferring binding specificity for given processes [24], and stimulating Hsp70 to hydrolyze ATP, converting the protein to its high affinity client-binding state.

P23, at 23 kDa is the smallest of the known Hsp90 co-chaperones. The protein consists of an eight-member, anti-parallel beta sandwich, with a highly conserved seven-residue segment in one of the loops, which has been shown to be involved in binding to Hsp90. P23 binds to,[25] and stabilizes [26] the closed, or 'late', conformation of Hsp90. This interaction is stabilized by the binding of ATP [18]. P23 is said to have passive chaperoning activity, in that it can bind to, and prevent the aggregation of, denatured proteins. This is unlikely to reveal anything about the mechanism of its function within the Hsp90 complex, as nascent proteins chaperoned by Hsp90 are in a near-native state.

In the Hsp90 complex, Cdc37 is predominantly involved with the chaperoning of kinases, for which its presence is essential [27]. In this context, according to the most recently proposed model, a dimer of phosphorylated Cdc37 molecules bind to a client kinase monomer. Upon dissociation of one of the Cdc37 monomers, the remaining Cdc37-kinase complex binds an Hsp90 dimer. After interaction with Protein Phosphatase 5 (PP5), another Hsp90 co-chaperone, and subsequent dephosphorylation of

Cdc37, the complex dissociates, resulting in a mature kinase, or a reiteration of the cycle. Additionally, Cdc37 inhibits the ATPase activity of Hsp90, but does so without interfering with ATP binding [28-29]. However, Cdc37 has functions that are independent of Hsp90, or only partially dependent on it. Cdc37 possesses passive chaperoning activity, which likely results at least partially from its ability to bind client proteins in the absence of Hsp90.

Another Hsp90 co-chaperone is the Activator of Hsp90 ATPase 1 (Aha1) which, as its name suggests, stimulates the ATPase activity of the chaperone. Like Hsp90, Aha1 is up-regulated under stress conditions [30]. The exact role of Aha1 is not understood, although its importance to client activation has been demonstrated. Increased expression of Aha1 results in increased activation of client proteins, while decreased expression results in decreased activation of client proteins and increased sensitivity to Hsp90 inhibition [31].

The co-chaperones discussed here are sufficient to chaperone many clients, including hormone receptors. Kinases, however, generally require the action of Cdc37. While the functions of none of the members of the complex are entirely understood, those of the other co-chaperones are understood even less so. Among them are Protein Phosphatase 5 (PP5), which is known to regulate the activity of Hsp90 and Cdc37; Cyp40, FKBP52, Sgt1, and several others. In some cases a specific function or set of functions of the co-chaperones is known. In others, the co-chaperone just seems to direct specificity of interaction between Hsp90 and individual proteins or groups of proteins. The various functions of the other co-chaperones continue to be pieced together.

Hsp90 exists as four known isoforms in mammals. Hsp90β, which is constitutively expressed, is localized to the cytoplasm along with the inducible Hsp90α. The 94 kDa Glucose-regulated Protein (Grp94) is localized to the endoplasmic reticulum, and the Tumor Necrosis Factor Receptor Associated Protein (TRAP-1) is the mitochondrial version.

Hsp90 and cancer

The role of Hsp90 in the development and progression of cancer is perhaps its most studied aspect. Specifically, a great deal of work has characterized the interaction between Hsp90 and the oncoproteins that it supports. In 2000, Hanahan and Weinberg proposed six hallmarks by which cancer could be defined. These were: resistance to cell death; sustained proliferative signaling; evasion of growth suppressors; activation of invasion and metastasis; enabling of replicative immortality; and induction of angiogenesis. More recently, the same researchers have proposed an additional four characteristics of cancer cells. Two of these are cellular hallmarks similar to the previous six: deregulation of cellular energetics; and evasion of immune destruction. Finally, two 'enabling characteristics' have also been described: genome instability and mutation; and tumor-promoting inflammation [32]. All of these characteristics are represented by the group of proteins for which Hsp90 is known to be essential for stability and activity. Additionally, the list of Hsp90 clients covers a large number of proteins that are not included in these functions [33].

The expression of Hsp90 and other chaperones is generally increased in transformed cells, and this altered expression is necessary for their survival and growth. Its isoforms are often altered in cancer cells. The mitochondrial isoform TRAP-1 is highly expressed in the mitochondria of a number of tumor types, while expression is much lower in normal tissues [34].

One consequence of continuous proliferation is shortened telomeres, which leads to cellular senescence. Cancer cells overcome this problem by having an active telomerase. Hsp90 and its co-chaperone, p23, are required for proper assembly and function of the telomerase complex [35]. In yeast, the Hsp90 homolog, Hsp82, was shown in vitro to 'switch' a DNA-binding complex containing Cdc13 and telomerase from a capping function to an extending function [36].

Cancer cells in metastatic tumors are often found to be hypoxic. This state, rather than being a barrier to cancer cell proliferation, may actually be an advantage. Hypoxia-inducible factor-1, which is up-regulated in response to a decreased oxygen level, is a transcription factor involved in the

expression of glucose transporters, glycolytic enzymes, and proteins required for angiogenesis. HIF-1α is an Hsp90-dependent protein. It has been shown to interact with Hsp90, and treatment with an Hsp90 inhibitor disrupts activation of HIF-1, even under hypoxic conditions [37].

HRI

The heme-regulated inhibitor (HRI) of eIF2α is one of the many kinases that are dependent on Hsp90 for its stability and function. The kinase phosphorylates the alpha subunit of eukaryotic translation initiation factor 2. This results in suppression of translation, specifically of globins in reticulocytes, and other cell types, and is of importance during conditions of stress that can negatively impact protein homeostasis [38]. Accordingly, in the presence of denatured protein, HRI phosphorylates eIF2α [39]. Additionally, inhibition of the proteasome also results in phosphorylation of eIF2α, exclusively by HRI [40].

The study of HRI has provided insight into the nature of the relationship between Hsp90 and some of its kinase clients, such as the interaction with Hsp90, not just for folding and activation, but as a sort of holding function until the kinase is needed. HRI interacts with Hsp90 during translation in rabbit reticulocyte lysate. During heme-deficiency, HRI undergoes auto-phosphorylation, as part of its maturation, and activates, and is released from the Hsp90 complex. Without maturation, HRI remains bound to Hsp90. Treatment of the lysate with the Hsp90 inhibitor geldanamycin blocks the activation of HRI, and the phosphorylation of HRI's substrate, eIF2α, and results in the dissociation of HRI from Hsp90 [41].

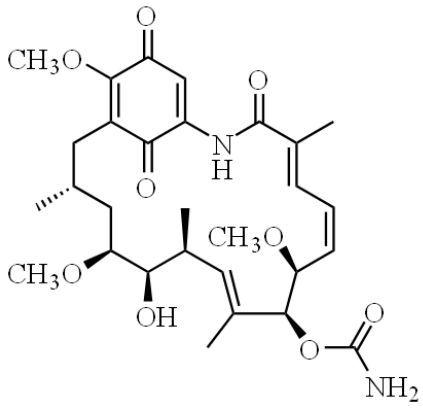
We've discussed here a few specific examples of the function of Hsp90 clients in the survival and growth of cancer cells. However, there are many more proteins involved in the process. Other examples of Hsp90 clients involved in cancer are Akt [42], which antagonizes apoptosis , matrix metalloproteinase 2 (MMP2) [43], which is involved in tissue invasion and metastasis, the MAP kinase Raf [44], which is involved in growth, proliferation, and apoptosis resistance.

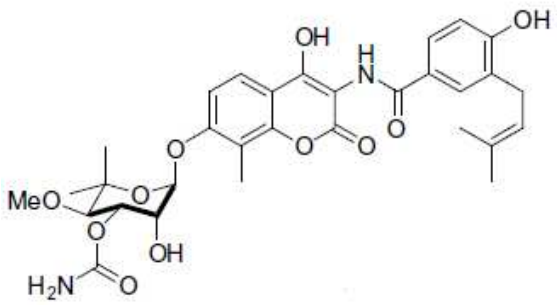
An example of the 'buffering' activity possessed by Hsp90 that is critical to the growth and survival of transformed cells is mutant p53. As already discussed, many of the proteins chaperoned by Hsp90 are those involved in proliferative and pro-survival signal transduction. P53, by contrast, has a generally antagonistic role regarding these pathways. Mutant forms of the p53 tumor suppressor protein are found in the majority of cancers. While normal p53 plays an important role in the prevention of tumor growth by inducing cell cycle arrest and apoptosis in the presence of DNA damage, the mutant form does not have such activity. The mutant form requires extended interaction with Hsp90, compared to the wildtype [45]. Additionally, the mutant form localizes with Hsp90 in the cytoplasm, rather than the nucleus [46]. Given that p53 acts as a tetramer, even a modest buildup of the mutant protein could impair its normal regulatory activity. P53 is an example of Hsp90 supporting the dysfunction of a molecule normally involved in the dampening of cell growth, rather than its acceleration, as is the case with many other Hsp90 clients. This further underscores the importance of Hsp90 to the survival and growth of cancer cells.

There are many more proteins involved in cancer and other diseases that depend on Hsp90 for their activity and stability. An updated list of Hsp90's interactors is maintained by the laboratory of Didier Picard and can be found here: http://www.picard.ch/downloads/Hsp90interactors.pdf.

Inhibitors

Since the discovery of the classical Hsp90 inhibitor, geldanamycin (Figure 1.3), many additional inhibitors have been synthesized or discovered. Several of these are derivatives of geldanamycin, such as 17-AAG, 17-DMAG, and Retaspimycin, among others. Other inhibitors, like geldanamycin, were originally identified as antibiotics, but were discovered to have anti-cancer properties, such as radicicol [47-48] and herbimycin, which is part of the geldanamycin family of compounds [49]. While these compounds bind to the N-terminal ATP binding site of Hsp90, another group of inhibitors, like novobiocin (Figure 1.3) and its analogs, bind to the C-terminus.





Geldanamycin Novobiocin

Figure 1.3. Strucures of the classical Hsp90 inhibitors geldanamycin and novobiocin.

As mentioned previously, most Hsp90 inhibitors bind the N-terminal ATP-binding site. While ATP-binding sites are found in a very large number of proteins, Hsp90 inhibitors are fairly specific, and bind mostly to Hsp90 and a small number of other proteins, including histidine kinases [50], the PhoQ sensor kinase [51], and Type II DNA Topoisomerase [52]. These proteins, and Hsp90 itself, belong to a superfamily known as the GHKL proteins. These proteins contain an unusual ATP-binding motif called the Bergerat fold. Whereas ATP-binding domains usually consist of four parallel beta sheets packed between two pairs of alpha helices, the Bergerat fold consists of an a layer of anti-parallel beta sheets facing a layer of three alpha helices [53]. ATP bound to Hsc70 assumes an open, linear conformation. In contrast, ATP bound Hsp90 is in a distinctly more contorted, compact form (Figure 1.4).

Cancer cells

The cytotoxic and anti-proliferative effects of Hsp90 inhibitors on cultured cancer cell lines provide some of the strongest rationale for investigating their potential as therapeutic agents. Hsp90 inhibitors have demonstrated a strong ability to slow or stop proliferation and to induce apoptosis in a number of cancer cell lines. Among these are leukemia and KB cells [54], lymphoblastic leukemia cell lines containing the Philadelphia chromosomal rearrangement [55], breast, colon, endometrial, and ovarian cancer [56], spinal neuroblastoma [57], bile duct cancer [58], and others.

Hsp90 inhibitors have also demonstrated effectiveness against cancer cells that have developed resistance to other cancer treatments, such as radiation therapy [59], proteasome inhibitors [60], and other anti-cancer pharmaceuticals, such as the mitotic inhibitor paclitaxel [61] and the tyrosine kinase inhibitor imatinib [62].

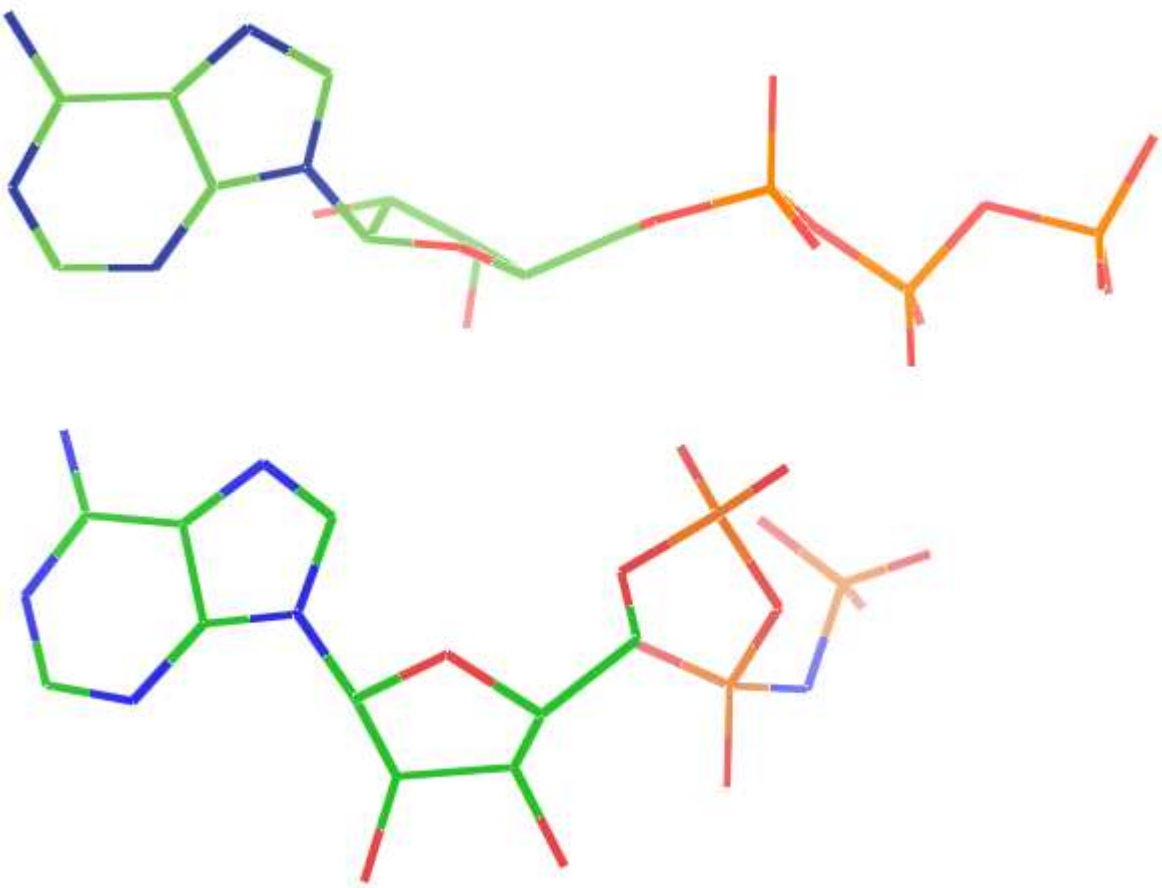


Figure 1.4. The conformation of ATP while bound to Hsc70 and the non-hydrolyzable ATP analog AMP-PNP while bound to Hsp90. [63]

Additionally, Hsp90 inhibitors often display a synergistic activity with other forms of treatment, greatly lowering the concentrations of both compounds necessary for anti-proliferative or cytotoxic activity [64].

In this study, we have used three cells lines to study the effects of putative Hsp90 inhibitors. These cell lines are MCF-7, HeLa, and SkBr3. MCF-7, a breast cancer cell line, was chosen because these cells overexpress the Hsp90-dependent estrogen receptor and androgen receptor. They do not, however, express the Hsp90-dependent ErbB2 protein. SkBr3 cells, another breast cancer cell line, overexpresses ErbB2, allowing a comparison between the effects of putative Hsp90 inhibitors on different Hsp90 client proteins, as well as between two cells types from the same tissue. HeLa cells are cervical cancer cells that are known to be resilient against multiple types of stress, and are good potential indicators of an Hsp90 inhibitor's effectiveness.

In vivo studies have been carried out to determine the efficacy of Hsp90 inhibitors against tumor growth. In a mouse model of prostate cancer, the mitochondrial Hsp90 isoform inhibitor gamitrinib blocked the development of prostate tumors, and prevented metastatsis [65].

Inhibitors of Hsp90 have several effects on the Hsp90 dimer itself, as well as its association with its co-chaperones and client proteins. Inhibitors of Hsp90 bind to different regions of the protein, although the majority of compounds with appreciable affinity seem to bind the N-terminal ATP site. Others bind to a putative C-terminal ATP-binding site. Different inhibitors also have different effects on the chaperone. Given the difficulties of crystallography, inhibitor discovery has outpaced the production of crystal structures of Hsp90 with bound inhibitors. However, other methods have allowed the elucidation of these effects. All studied inhibitors lock, or strongly bias, the protein into one specific part of its ATPase cycle. The conformational biases introduced by the inhibitors alter the proteolytic sensitivity of Hsp90, producing distinct cleavage patterns. Additionally, the repertoire of co-chaperones is conformation dependent. Some inhibitors, such as the well-established

geldanamycin and its derivatives, favor the more 'open' conformation, which is normally found when Hsp90 has no nucleotide bound. When treated with trypsin, geldanamycin-bound Hsp90 produces a gel pattern that is little, if any, changed from that of untreated Hsp90. This conformation, which is thought of as the intermediate portion of the ATP cycle, also features bound Hsp70 and the Hsp70-Hsp90 organizing protein (HOP). The molybdate ion has been used in Hsp90 studies to examine the late phase of the ATP cycle. When lysate or recombinant protein is treated with sodium molybdate, the interactions of the co-chaperones p23 and Cdc37, as well as FKBP52, with Hsp90 are stabilized. Proteolytic digestion results suggest that a portion of the C-terminus becomes structured as a result of the binding of molybdate. Novobiocin, another well-studied inhibitor, binds the C-terminus of Hsp90. The binding of this compound favors a conformation of Hsp90 that results in dissociation of the chaperone from nascent client protein. Additionally, this dissociation seems to take place at a point in the ATP cycle at which the 'late' complex has formed. This results in a complex consisting of the client and the co-chaperones p23 and PP5, but not the co-chaperone Cdc37 or Hsp90 itself [66].

Degradation

Treatment of cells, and in some cases, cell-free systems, with an inhibitor of Hsp90, results in the degradation of Hsp90-dependent client proteins, such as EGFR and ErbB2 (Figure 5). Without the support of the Hsp90 chaperone complex, client proteins become unstable and are poly-ubiquitinated and degraded by the proteasome. The ubiquitination of the client protein is mediated by the E3 ligase, C-terminus of Hsp70 Interacting Protein (CHIP). This protein, as the name states, interacts with Hsp70 through a TPR domain, and, along with Hsp40, facilitates the degradation of proteins that are not successfully folded by the Hsp90 machinery, and CHIP's interaction with the client is increased upon Hsp90 inhibition. [67] While the exact mechanism is not yet fully understood, it is clear that Hsp90 plays a part in the degradation of its own client proteins, as its presence is necessary for efficient ubiquitination of the unfolded protein [68].

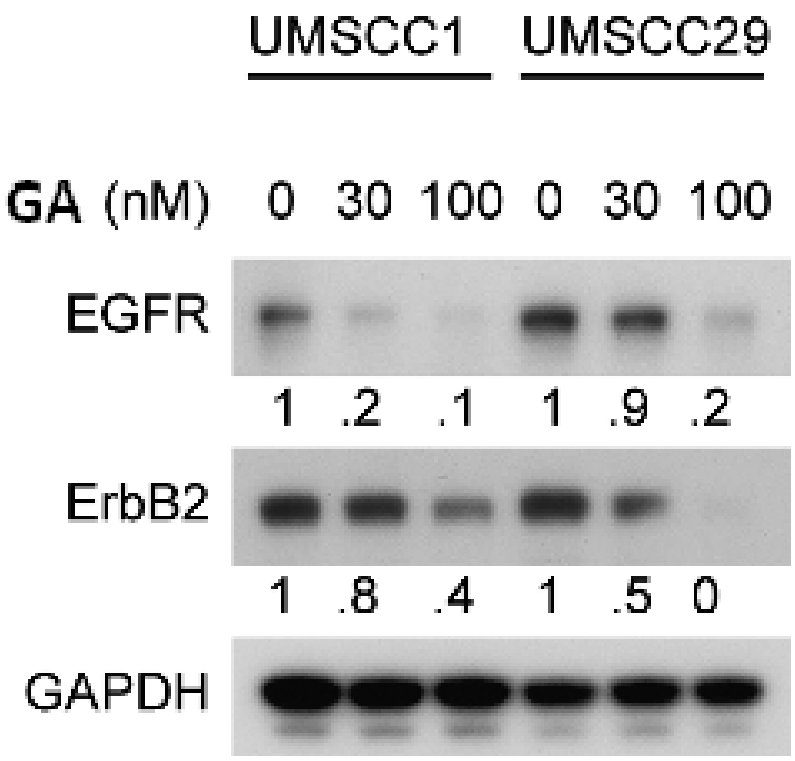


Figure 1.5. Treatment of two carcinoma cell lines with geldanamycin reduces the expression of the Hsp90 client proteins EGFR and ErbB2. [69]

Whatever the mechanism, this depletion of Hsp90-dependent proteins, specifically the signal transduction molecules that are essential for the survival and growth of cancer, is one of the properties that makes the chaperone a high-profile target for therapeutic intervention. With the destruction of these molecules, cancer cells are often unable to survive and proliferate, and populations of cancer cells have even been observed to revert to normal upon treatment with an Hsp90 inhibitor.

Given the importance of Hsp90 in cancer and other diseases, the search for small molecules that can modulate its activity is now an area of intense focus. In this study, we report some of the progress made by our group in this effort.

CHAPTER II

HIGH-THROUGHPUT SCREEN FOR INHIBITORS OF HSP90

AND FURTHER STUDY OF SELECTED HITS

Introduction

Since its emergence as a major molecular target for cancer therapeutics, Hsp90 has been the focus of a great deal of research. Basic research has seen the expansion of the group of proteins termed 'clients', which rely on Hsp90, not only for their activity and function, but for their stability. It has long been established that disruption of Hsp90 function results in reduced stability and degradation of these client proteins. It has also been firmly established that the majority of the proteins dependent on Hsp90 for their function are involved in signal transduction. Additionally, these proteins reside in diverse pathways with wide-ranging functions. Accordingly, inhibition of Hsp90 has equally wide-ranging effects. These effects have been observed most starkly in cancer cells, which depend more than normal cells on Hsp90-dependent signal transduction capabilities.

Owing to its importance to the viability of cancer cells, a great deal of effort has been dedicated to the discovery and characterization of inhibitors of Hsp90 and its chaperone complex. These studies have taken many forms, relied on many techniques, and have screened large numbers of compounds for their ability to modulate Hsp90 function.

One such study was undertaken by our lab. Our efforts to identify compounds that inhibit Hsp90 have focused on compounds found in nature. The well-studied inhibitors of Hsp90, geldanamycin, radicicol, and novobiocin, were all isolated from bacteria and fungi. In order to identify additional inhibitors, we have screened libraries consisting of compounds from various natural sources, such as trees, sea sponge, and others.

Our screen, and eventual characterization of hits, has made use of rabbit reticulocyte lysate. Reticulocyte lysates, the lysates of immature red blood cells, have been used for decades for in vitro translation studies, and are replete with Hsp90 and other molecular chaperones. As such, they are useful for studying the functions and interactions of these proteins.

In order to use the reticulocyte system to screen for inhibitors of the Hsp90 complex, we make use of the fact that Hsp90 is involved, not only in the folding and activation of nascent proteins belonging to its specific client set, but also, unfolded proteins in general. For our study, we used thermally denatured firefly luciferase. Incubation with chaperone-replete rabbit reticulocyte lysate will restore the activity of thermally denatured firefly luciferase. Additionally, while incubation at 37° C will result in a loss of luciferase activity, incubation at 37° C with reticulocyte lysate causes no change in activity. These observations indicate both active and passive chaperoning capabilities of rabbit reticulocyte lysate, with respect to luciferase. Immunoprecipitation of denatured luciferase from reticulocyte lysate, as well as treatment with Hsp90 inhibitors, indicate that Hsp90 and its co-chaperones are involved and essential for the refolding of thermally denatured luciferase in the lysate [70]. We make use of this fact for the screening of potential Hsp90 inhibitors. Thermally denatured luciferase is introduced into the reticulocyte lysate. After a one to three hour incubation at room temperature, a substantial increase in luciferase activity occurs, as observed by the addition of an assay buffer containing the natural substrate of luciferase, luciferin. However, when an Hsp90 inhibitor, such as

geldanamycin, is added to the lysate, little if any change in luciferase activity is observed. In this way, we can screen large numbers of compounds for potential activity against Hsp90 or its co-chaperones. Here we describe our system for the high-throughput screening of commercially available compounds for their ability to inhibit the Hsp90-dependent refolding of firefly luciferase, and the characterization of a select group of the resulting hits.

Materials and Methods

Rabbit reticulocyte lysate

Rabbit reticulocyte lysates were purchased from Green Hectares. The purchased lysate was prepared by lysing one volume of packed reticulocytes in two volumes of deionized water, followed by centrifugation for twenty minutes at 15,000 X g.

Denatured Luciferase

Recombinant luciferase from Promega was diluted to 0.5 mg/mL in buffer consisting of 25 mM Tricine-HCl (pH 7.8), 8 mM $MgSO_4$, 0.1 mM EDTA, and 10 mg/mL acetylated BSA. Next, the solution was adjusted to include 10% glycerol and 1% Triton X-100. Finally, the luciferase solution was heated to ~41°. Once the activity of the luciferase reached ~1% of its initial value, the mixture was placed on ice, or flash frozen in liquid nitrogen and placed at -80° for storage.

To prepare the denatured luciferase for use in re-folding assays, 125 uL of the 0.5 mg/mL mixture was added into a 10 mL mixture containing 80 mM Tris HCl, pH 7.7, 8 mM $Mg(OAc)_2$, 300 mM KCl, 12 mM ATP, and 20 mM creatine phosphate, and 0.8 mg/mL creatine phosphokinase.

Assay Buffer

The assay buffer, which contains the luciferase substrate luciferin, consisted of 75 mM Tricine-HCl, pH 7.8, 24 mM MgSO4, 300 uM EDTA, 2 mM DTT, 313 uM D-luciferin, 640 uM coenzyme A, 660 uM ATP, 150 mM KCl, 10% (v/v) Triton X-100, 20% (v/v) glycerol, and 3.5% DMSO.

Compounds

The compound libraries screened were from the following companies, and contained the respective number of compounds: TimTec – 240; Analyticon – 2728; Biofocus – 272; BioMol – 579.

Compounds were reconstituted in 100% DMSO. Stocks, at a concentration of 1 mg/mL, were diluted 30-fold into nano-pure water for the assays. The assay was performed in 96-well microplates. To each well was added 30 uL of the water/DMSO compound solution, 15 uL of the reticulocyte lysate preparation, and 15 uL of the luciferase reagent. The plates were agitated and then allowed to incubate at 25° C for one to three hours. After the incubation, 60 uL of assay buffer containing luciferin was added to each well. The plates were then placed on a Lumac microplate luminometer, and luminescence was measured in relative light units, with an integration time of 10 seconds. Compounds that inhibited luciferase refolding by approximately fifty percent were then titrated into a refolding reaction containing native luciferase, to control for direct inhibition of luciferase.

Results

A number of compounds were found to substantially inhibit the renaturation of luciferase in our lysate system. After control screening with native luciferase to eliminate false positives that were likely luciferase inhibitors, a number of compounds were selected for further study.

The compounds listed below in Tables 2.1 – 2.9 have been compiled because they have been determined to inhibit the Hsp90-dependent refolding of luciferase, without inhibiting luciferase itself. The compounds have been sorted largely by structural classification, although some do not fit well into any grouping with others.

Because of Hsp90's central role in signal transduction and protein shuttling, its inhibition has widespread and sometimes varied effects within a cell. Depending on the conditions, the physiological manifestations of this inhibition can also vary. For example, Hsp90 is highly involved in the inflammatory response, as several mediating proteins, such as IκB Kinase (IKK) [71] and nitric oxide synthase [72], are dependent on Hsp90 for their function. Accordingly, Hsp90 inhibitors result in the down-regulation of these proteins and display anti-inflammatory activity, which makes them prominent in traditional medicine. In the case of cancer cells, accelerated growth and cell division is maintained by Hsp90-dependent clients. Thus, treatment with Hsp90 inhibitors results in the slowing of cell growth, and potentially, death. Similarly, the effects of Hsp90's inhibition can be seen in other medically relevant ways, including activity against viruses, bacteria, fungi, and parasites, specifically the causative agent of malaria, *Plasmodium falciparum*.

The significance of this to our screen, in addition to being the primary reasons for our study, is that novel compounds not known to have any activity against Hsp90 or its co-chaperones can be implicated in this role by their being reported to have multiple, seeming unrelated, medically relevant biological activities. As will be shown below, some of the compounds identified in our screen have been specifically shown to inhibit the activities of proteins known to be dependent on Hsp90 for their function. These proteins include Akt, STAT-3, Her2 (ErbB2), Insulin-like Growth Factor Receptor (IGFR), Endothelial Growth Factor Receptor (EGFR), and others. Compounds reported to block the actions of these proteins, or their downstream signaling partners, such as NF-kB, are of special interest to our study. Also, Hsp90 is known to be required

for the activity of viral polymerases [73-74], so anti-viral activity is another mark of an Hsp90 inhibitor. Additionally, even though they haven't been identified as Hsp90 inhibitors, most of the compounds in our screen belong to compound families that contain known, or likely, Hsp90 inhibitors. Below, we give examples of the biological activities that make each class of compounds, or specific compounds, good candidates as Hsp90 inhibitors. References supporting the compounds' potential as Hsp90 inhibitors are included in the tables.

Sesquiterpene lactones

Some of the compound hits in our screen belong to the family of compounds known as sesquiterpene lactones. These compounds are characterized by a fifteen-carbon skeleton formed from the joining of three isoprene units. The other defining feature of the molecules is a lactone group. These compounds are found in many types of plants, and have long been used for various purposes in traditional medicine. Given their effectiveness in the treatment of a wide variety of ailments, and their observed action on multiple cellular functions and molecular targets, these compounds are promising candidates as Hsp90 inhibitors.

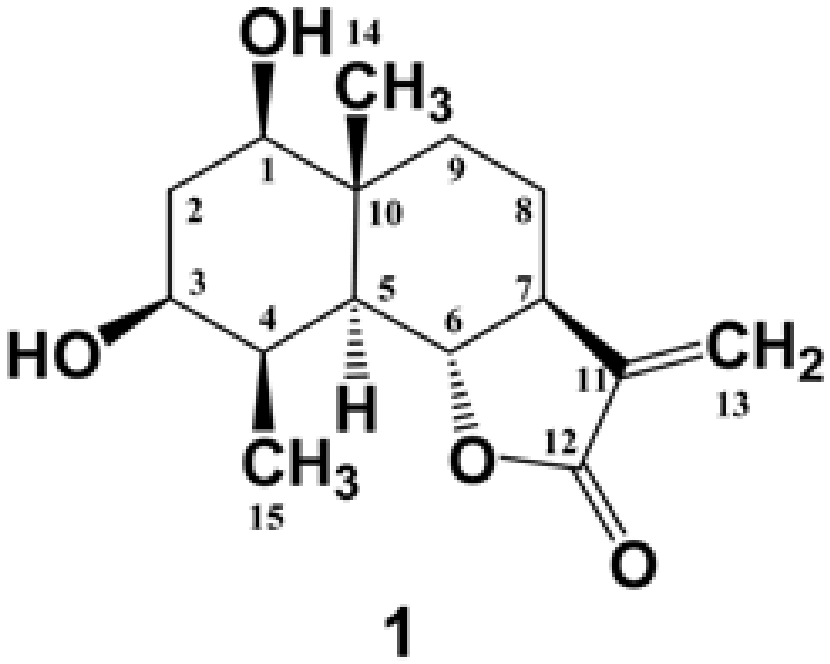
Sesquiterpene lactones have been grouped into seven general classes according to their structures. They are germacranolides, eudesmanolides, eremophilanolides, guaianolides, pseudoguaianolides, hypocretenolides, and iso-seco-tanapartholides. Compounds with reported biological activity come from all of the groups, although germacranolides, guaianolides, and pseudoguaianolides appear to be the most prominent [75].

Two sesquiterpene lactones, isodeoxyelephantopin, and its nearly identical analog, deoxyelephantopin, were shown to inhibit the proliferation of mouse fibroblast tumor cells. The two compounds also inhibited DNA replication in both proliferating lymphocytes and tumor ascites [76]. Another pair of compounds fitting into this family, costunolide and eremanthin, were extracted from the ornamental plant *Costus speciosus*, and displayed anti-fungal activity

similar to the standard anti-fungal, fluconazole, against two species of *Trichophyton*, and somewhat weaker activity against several other fungi [77].

As nitric oxide is a mediator of inflammation, a compound's effect on nitric oxide production is an indicator of its anti-inflammatory potential. This is especially relevant to our study, because nitric oxide synthase is a known Hsp90 client. A eudesmanolide (Figure 2.1) isolated from *Taraxacum mongolicum*, and assayed for its ability to inhibit nitric oxide production in RAW 264.7 mouse macrophages, displayed an IC_{50} of ~60 uM [78].

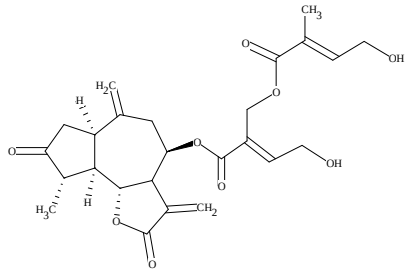
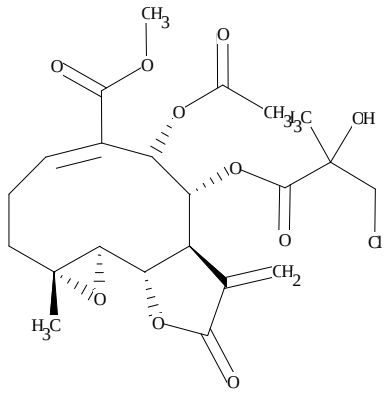
Hit #6, parthenolide, is from a different class of sesquiterpene lactones, but has a nearly identical structure. Additionally, it contains a methene group at the same location as the previously mentioned compound, a functional group that is the sole distinguishing feature from another, less active compound in the same study. Hit #4, MEGxm0_000041, contains a very similar moiety as its core, but also contains an esterified, un-saturated 8-carbon fatty acid molecule. Two compounds from the plant Eupatorium lindleyanum, eupalinolide A and eupalinolide B, are of the germacranolide sub-class. They induced the expression of several heat shock proteins, including Hsp70 and Hsp90, in mouse squamous cell carcinoma and melanoma cells. The compounds were also shown to activate HSF1 [79], a potential indicator of an Hsp90 inhibitor. Hit #2, also belongs to this class of compounds, and has some similarity in its attached moieties.

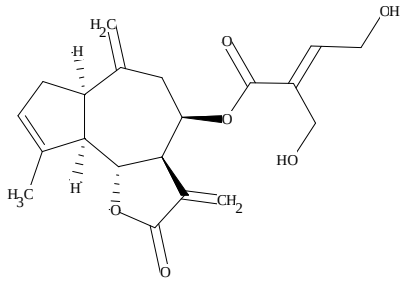
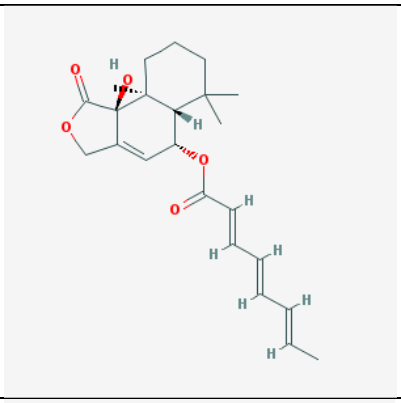
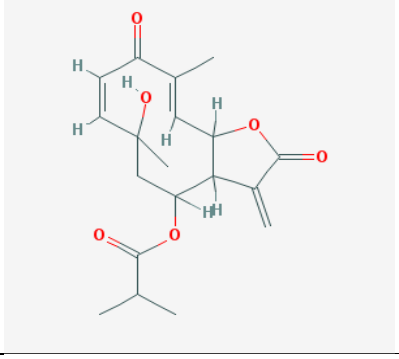
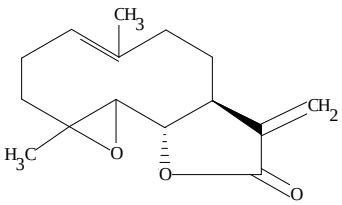


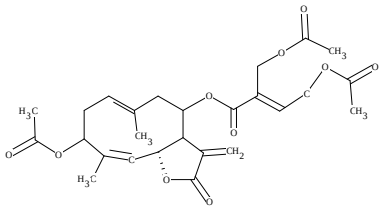
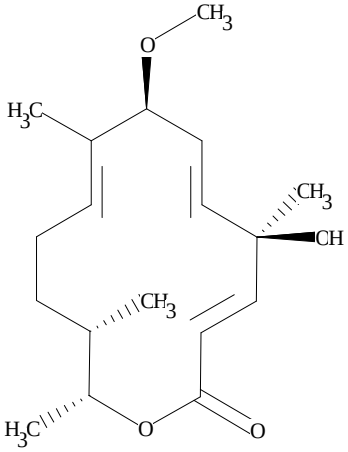
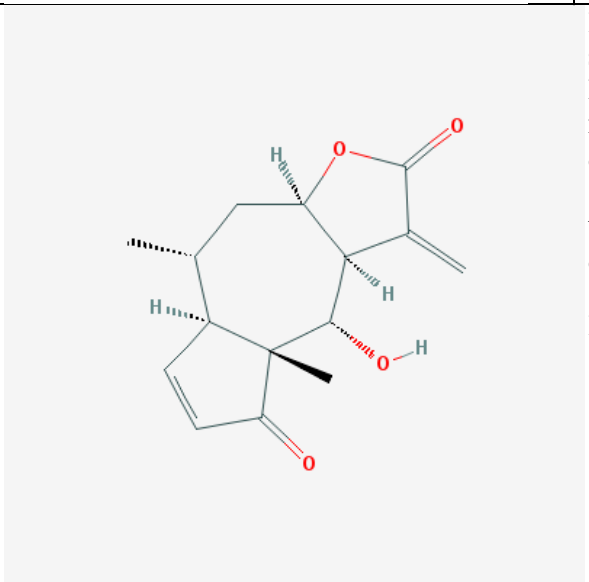
1

Figure 2.1. Structure of a Eudesmanolide from *Taraxacum mongolicum*

Table 2.1. Sesquiterpene lactone compounds

#	Name, or location within assay.	Structure	Properties
1	17-C3 Guaianolide		IC_{50} ~ 60 uM
2	29-F9 Germacranolide		IC_{50} ~ 40 uM

3	17-F4 Guaianolide		IC_{50} ~ 40uM
4	Eudesmanolide		Anti- L5178Y lymphoblastic tumor, PC12 neuroendocrine tumor, HeLa cervical cancer [80] IC_{50} ~ 60 uM
5	Germacranolide		Anti-malarial [81]
6	Parthenolide Germacranolide		IC_{50} > 100 uM Activity discussed below.

7	17-C4		IC_{50} ~ 30 uM
8	16-H2		IC_{50} ~ 10 uM
9	Helenalin		Induction of ROS and suppression of NF-kB; suppression of Bcl-2-mediated resistance to apoptosis [82]. Anti-leukemic [83]. Inhibition of human telomerase [84]. Induction of autophagy and cell cycle arrest [85]. IC_{50} ~ 80 uM

Compound 6, parthenolide, has been identified as an anti-tumor and anti-inflammatory agent, and is currently in clinical trials for acute myeloid leukemia, acute lymphoblastic leukemia, and other types of blood and lymph node cancers [75].

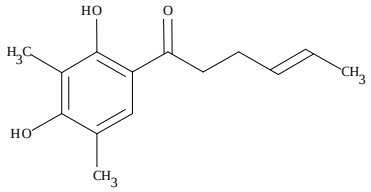
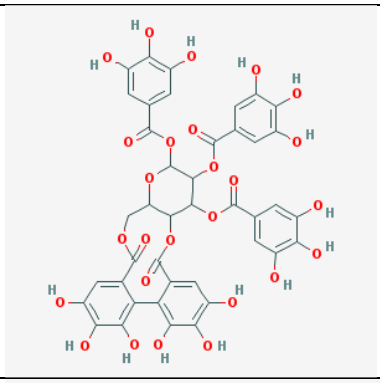
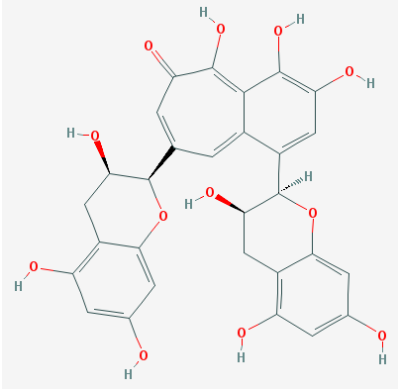
Parthenolide's anti-cancer and anti-inflammatory activities have been attributed to multiple mechanisms. It was shown to inhibit the activation of NFkB by IkB, even when the kinase was constitutively active [86]. It was also able to sensitize TRAIL-resistant cancer cells by inhibiting STAT3 activation [87].

Polyphenols

Polyphenols are defined as those compounds that contain phenolic moieties. Often, these substituents are poly-hydroxylated. The family of compounds is large, and contains multiple subtypes. Many of the hits from our screen fall into this classification, and fall across multiple subclasses.

Polyphenols are often described as anti-oxidants, and are observed to protect against ROS in vitro. The actual mechanisms behind these activities, however, have not been determined.

Table 2.2. Polyphenols and related compounds

#	Name or location within assay	Structure	Properties	
10	F2 2’3’-dihydrosorbicillin		IC_{50} > 100 uM	
11	Tellimagrandin II 151590 Polyphenol		Anti-HIV Suppressed tumor growth and increased lifespan in mouse sarcoma 180 [88]. IC_{50} ~ 70 uM	
12	Theaflavin		Anti-influenza and anti-inflammatory [89]. Anti-proliferative against leukemia cells via down-regulation of Akt; implicated as inhibitor of Hsp90 [90]. Inhibits NFkB and MAPK signaling [91].	

Flavonoids

Flavonoids are a large and diverse group of compounds within the class of polyphenolics. Flavonoids likely comprise around half of all identified polyphenolic compounds. Aside from their aromaticity, the molecules have no single unifying characteristic, except that they all contain two or more six-membered rings, as well as at least one oxygen atom, as an ether or a ketone. Many of the compounds contain additional keto- and –hydroxyl groups. This family of compounds exists in abundance in a number of substances used in traditional medicine. These substances have been attributed activities against allergy, inflammation, infection, tumors, diarrhea, and others. They've also been credited with wound healing and other beneficial properties. As ubiquitous as flavonoids are in plants, they are found in many foods. Examples are quercitin, EGCG, resveratrol, and others.

Several of the compound hits in our screen belong to the flavanoid family. Some of the compounds contain the typical bicyclic core with a benzene ring fused to a pyran or pyrone group, with a phenyl group attached in the flavan, isoflavan, or neoflavan configuration. These then contain additional phenyl or aliphatic groups of varying saturation and oxygen attachment. Additionally, some of the compounds fall into the subgroup of flavanoids known as chalcones. These compounds are metabolic precursors to the previously described flavonoids. The chalcones are characterized by two benzenes bridged by a 2-propen-1-one group.

Flavonoids are found throughout the plant kingdom. They have been used in various forms for treatment of multiple conditions for centuries. Many flavonoids have proven anti-microbial activity. Argentine folk medicine has made use of a plant containing the glycosylated flavonol, quercetagetin-7-arabinosyl-galactoside, for treatment of infectious diseases [92]. In another study, epigallocatechin gallate (EGCG), a type of flavonoid found abundantly in green tea, demonstrated strong anti-bacterial activity, resulting from damage to the lipid bilayer [93].

However, EGCG has also been demonstrated to bind to Hsp90 and to down-regulate the expression and activation of its clients [94].

In several studies over the course of twenty years, apigenin, a simple flavone, was assayed for its anti-bacterial activity, and was found to be active against over fifteen types of pathogenic bacteria, including *S. aureus*, MRSA, *E. coli*, *P. aeruginosa*, and *K. pneumonia* [95].

In addition to anti-microbial activity, these types of compounds demonstrate anti-cancer activities of varied sorts. Apigenin (Figure 2) has demonstrated strong *in vitro* anti-tumor and anti-angiogenic activity against human lung, prostate, and ovarian cancer cells. In each of these, the expression of VEGF and the Hsp90-dependent HIF-1alpha were suppressed [96-97].

Among our hits, four compounds, # 9, 10, 12, and 13, contain the same flavone core that apigenin has. Another two contain a flavonone core that is nearly identical, except for saturation in place of the C-2 double bond in flavones.

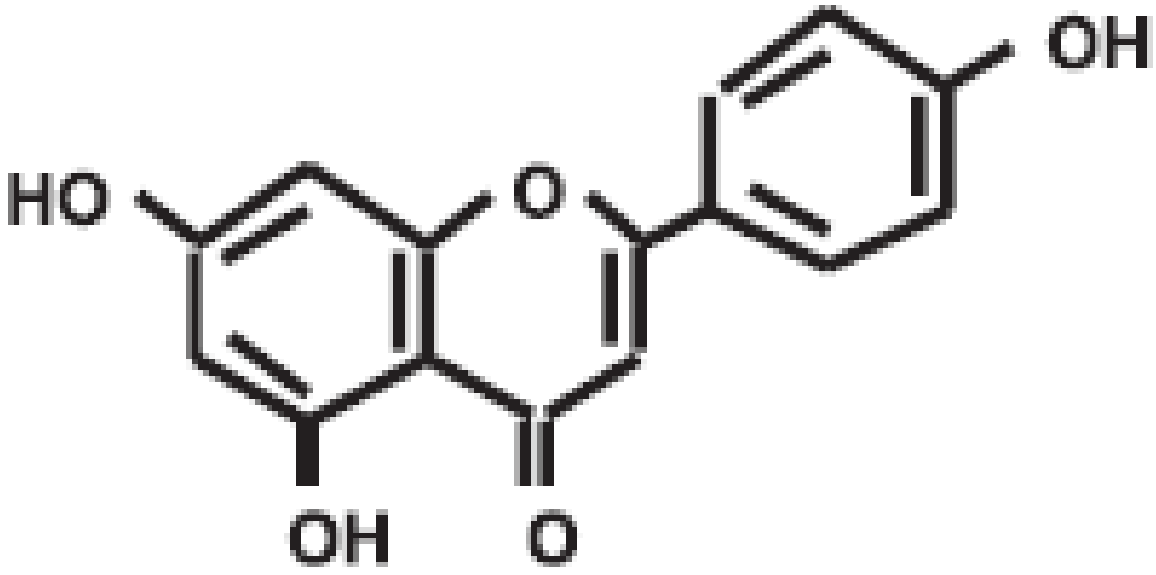
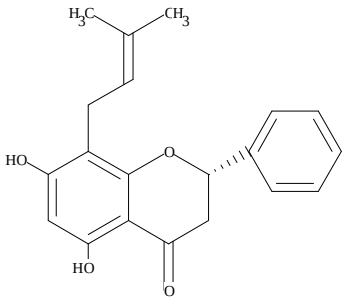
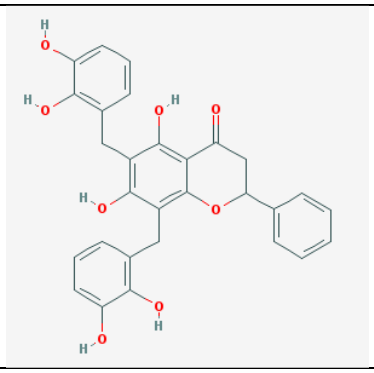
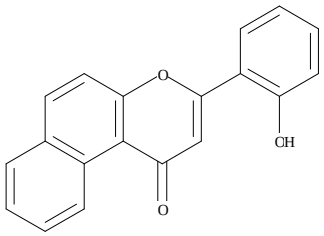
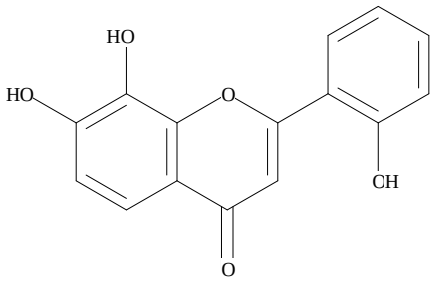
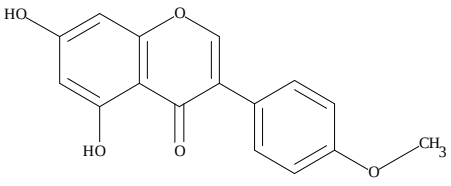
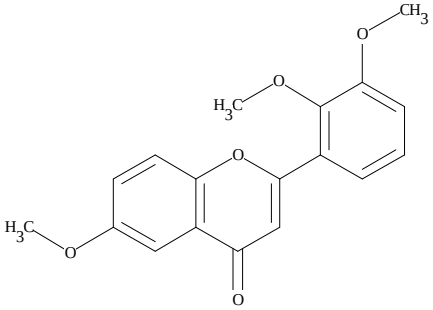
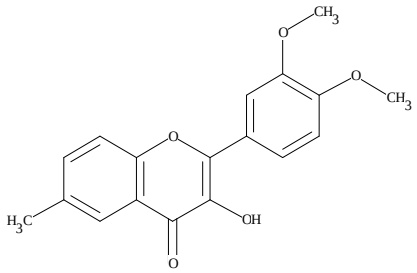
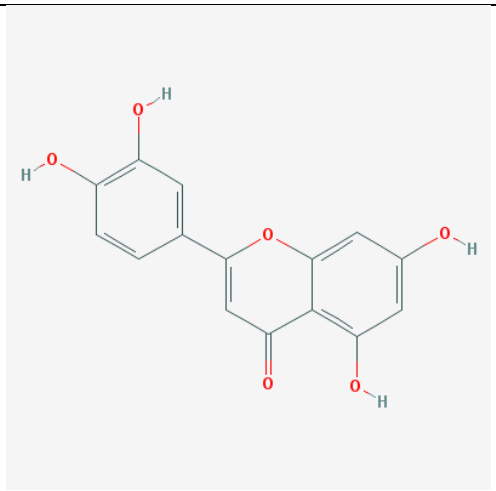


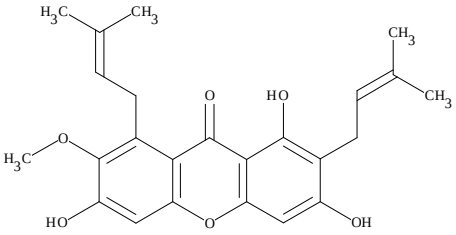
Figure 2.2. Structure of apigenin

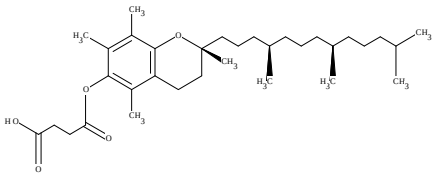
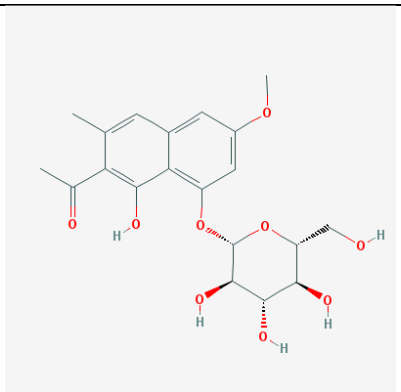
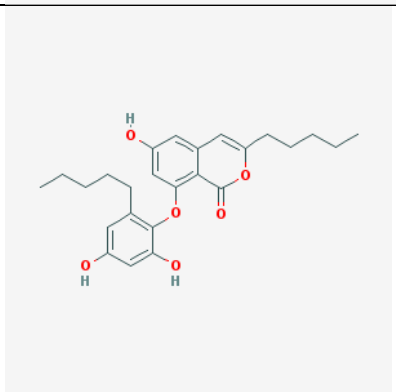
Table 2.3. Flavonoid compounds

#	Name or location within assay	Structure	Properties
13	(2S)-5,7-dihydroxy-8-(3-methylbut-2-enyl)-2-phenylchroman-4-one Flavanone		IC_{50} > 75 uM
14	MolPort-005-945-561 45360115 Flavonone		None reported IC_{50} ~ 70 uM
15	2'-hydroxy-b-naphthoflavone Flavone		IC_{50} ~ 350 uM Some inhibition of luciferase

16	7,8-dihydroxy-2-(2-hydroxyphenyl) chromen-4-one Flavone		IC_{50} ~ 10 uM Some inhibition of luciferase
17	Biochanin A 5,7-dihydroxy-3-(4-methoxyphenyl) chromen-4-one Isoflavone		Anti-inflammatory. Inhibits iNOS, p38 MAPK, and NFkB activation. Anti-proliferative and cyto-toxic activity against RAW 264.7 macrophages and HT-29 colon carcinoma cells. Less active against RAW 264.7. [98] IC_{50} > 90 uM
18	2',3',6-Trimethoxyflavone, 97% 2-(2,3-dimethoxyphenyl)-6-methoxychromen-4-one		IC_{50} > 90 uM

19	3',4'-Dimethoxy-3-hydroxy-6-methylflavone 2-(3,4-dimethoxyphenyl)-3-hydroxy-6-methylchromen-4-one		IC_{50} ~ 30 – 80 uM
20	Luteolin		Induction of unfolded protein response and apoptosis in neuroblastoma [99]. Inhibits LPS-activated, Akt-mediated activation of NFkB in macrophages, blocking production of TNFa [100]. Anti-tumor activity through EGFR pathway suppression in breast cancer cells [101]. Shown to inhibit Hsp90 [102].

21	MANGOSTIN 1,7-bis(3-methylbut-2-enyl)-3,6,8-trihydroxy-2-methoxyxanthen-9-one		Xanthanoid - Similar to gambogic acid Induces cell cycle arrest and apoptosis in colon [103] and prostate cancer cells [104]. Blocks activation of MAP and Akt pathways [105]. IC_{50} ~ 60 uM	

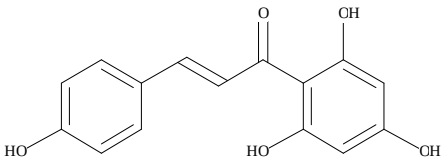
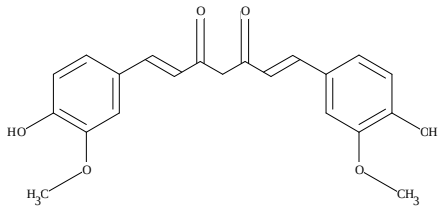
22	(+)-α-Tocopherol acid succinate		IC_{50} ~ 50 uM	
23	CHEMBL109330		Possible anti-oxidant activity [106]	
24	MolPort-001-742-269 38356110		None reported IC_{50} ~ 65 uM	

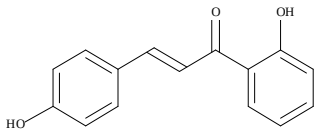
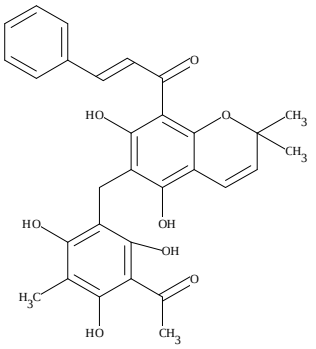
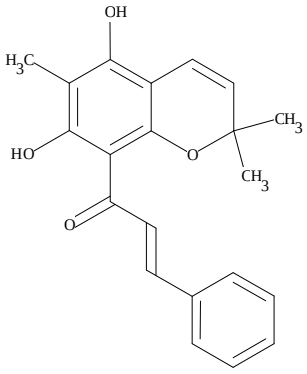
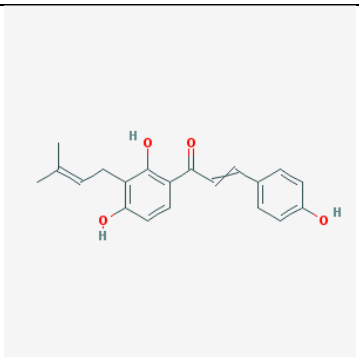
Chalcones

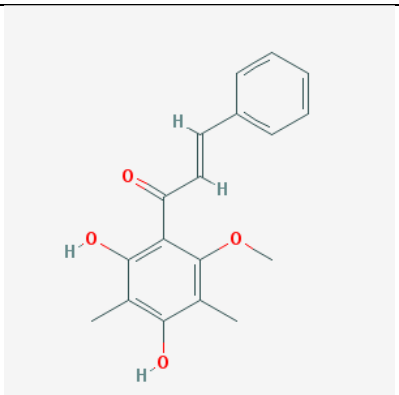
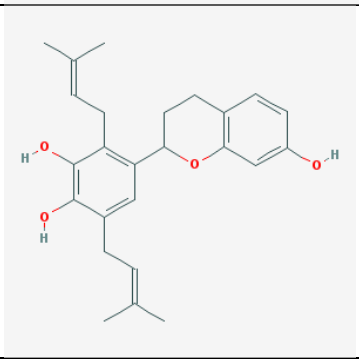
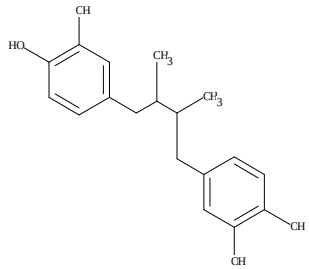
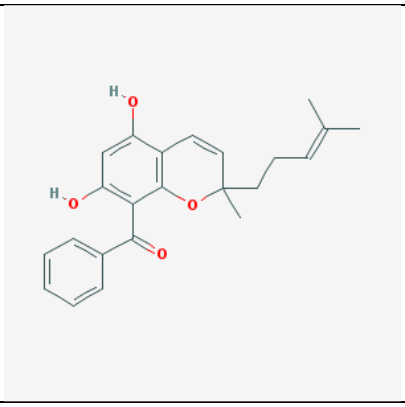
Chalcones are a structurally distinct subclass of flavonoids to which several of our hits belong. Chalcones have many of the same biochemical characteristics as the other flavonoids. As with

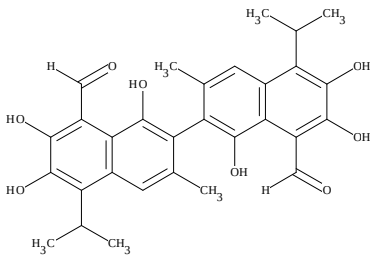
flavonoids generally, chalcones have anti-fungal[107], anti-inflammatory[108], anti-tumorogenic [109], anti-HIV, and anti-plasmodial activities [110], among others. Significantly, a number of proteins were identified as targets for a group of chalcones. Several of these proteins are known to be dependent on Hsp90. Included among them were Akt, NF-kB, mTOR, STAT3, HIF-1α, iNOS, and others([111]). Also, although it comprises only a fraction of its mass, a chalcone unit is present in the putative Hsp90 inhibitor, rottlerin.

Table 2.4. Chalcone compounds

#	Name or location within assay	Structure	Properties
25	Phloretin [60-82-2]		Induction of apoptosis in breast cancer cells via p53 induction and Bcl-xl degradation [112] IC_{50} ~ 35 – 90 uM
26	Curcumin [458-37-7] (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) Similar to chalcone		Multiple effects. Anti-oxidant, anti-inflammatory, suppression of NF-kB activation, anti-proliferative to several cancers [113]. Reported Hsp90 inhibitor [114]. IC_{50} ~ 70 uM

27	2',4-Dihydroxychalcone (2E)-1-(2-hydroxyphenyl)-3-(4-hydroxyphenyl)prop-2-en-1-one Chalcone		IC_{50} ~ 120 uM
28	Rotterlin Contains chalcone moiety.		Likely Hsp90 inhibitor. See Chapter V IC_{50} ~ 20 – 50 uM
29	18-E6 Contains chalcone moiety Similar to Rottlerin and catechin.		IC_{50} ~ 60 uM
30	AC1L4WK5 193568		IC_{50} ~ 50 uM

31	2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone 10424762		Anti-bacterial; anti-fungal [115-116] IC_{50} ~ 70 uM
32	AC1MR5D9 3512639 Polyphenol Flavan-3-ol		None reported Related compound – Catechin (CID 9064) – Tumor Hsp90 inhibition, et al… Emory University Molecular Libraries Screening Center – HTS for Tumor Hsp90 Inhibitors – PubChem ID IC_{50} ~ 60 uM
33	Nordihydroguaiaretic acid		Phase II study for effect on prostate cancer. Increased doubling time of PSA. Thought to inhibit IGF1R and HER2. Effectiveness less than pre-determined threshold, probably largely because of liver metabolism resulting in decreased bioavailability [117]. IC_{50} ~ 35 – 80 uM
34	CID 16070714 Similar to catechin		IC_{50} ~ 50 uM

35	Gossypol		Anti-oxidant; broad anti-cancer activity. Contraceptive activity. Anti-viral. Anti-protozoan. Anti-microbial[118] IC_{50} ~ 50 uM

Alkaloids

Alkaloids are broadly defined as containing basic nitrogen atoms in their ring structures. As this definition includes a large number of possible compounds, the family is more practically broken down into smaller divisions. Regardless, the compounds within the family that have demonstrated biological activities are themselves diverse, with no single structure or group of structures being a requisite for these activities. For the classification purposes of our screen, we will regard alkaloids as compounds that contain cyclic nitrogen atoms. However, alkaloid compounds containing another relevant group, such as a quinone, will be placed in that group. Like the other compound groups represented in our screen, alkaloids have demonstrated a wide range of medically relevant bioactivities. Included are anti-tumor, anti-hypertensive, anti-depressant, anti-microbial, anti-inflammatory, and other activities [119]. A well-known example of a medicinal alkaloid is quinine (Figure 2.3), isolated from the tropical medicinal plant *Cinchona succirubra*, which has been used to treat malaria for hundreds of years [81].

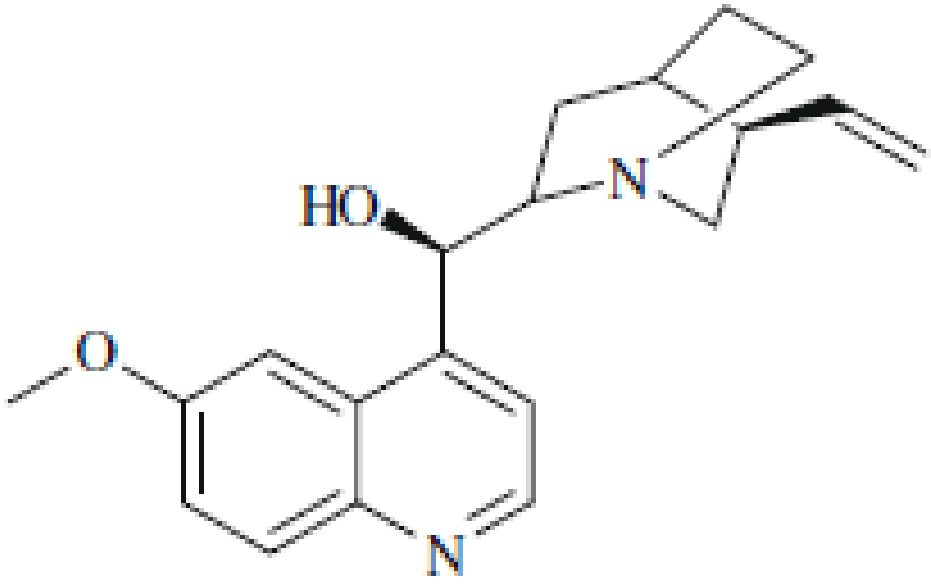
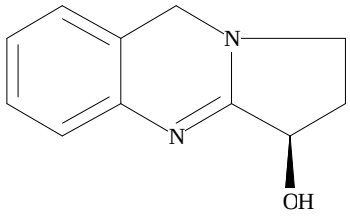
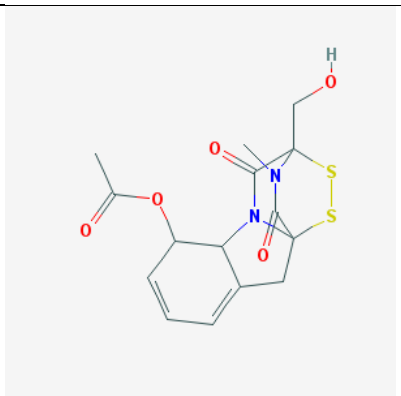
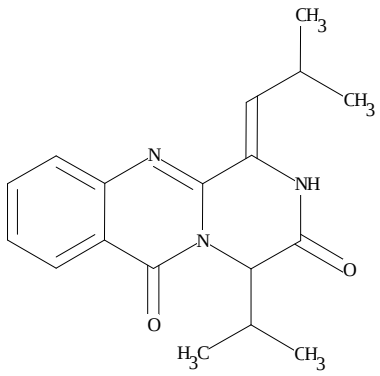
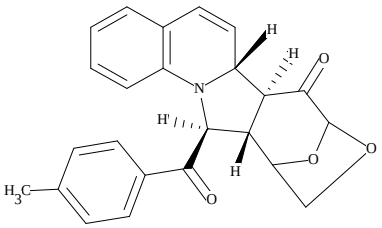
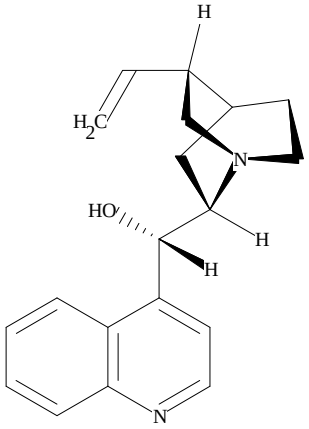
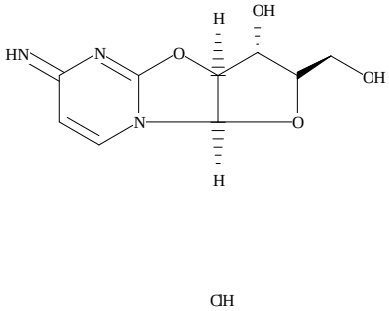
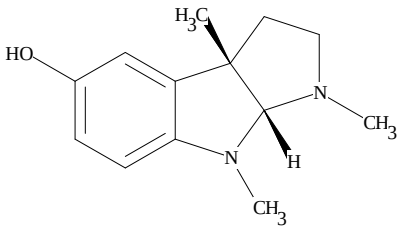
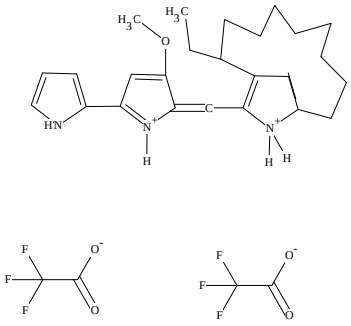
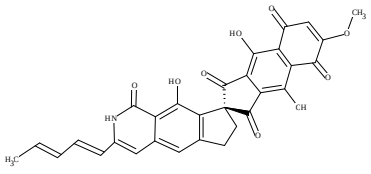


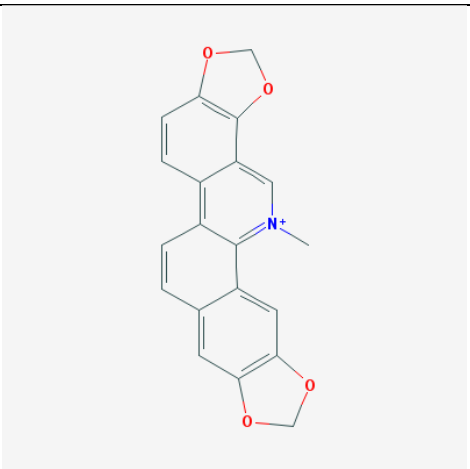
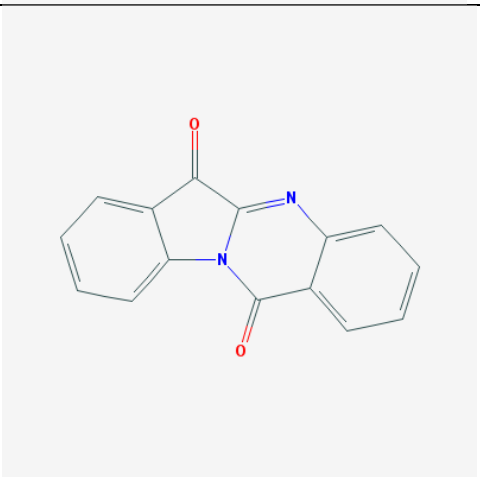
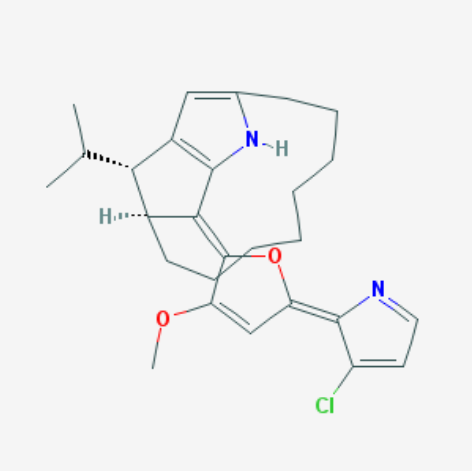
Figure 2.3. Structure of quinine

Table 2.5. Alkaloid compounds

#	Name or location within assay	Structure	Properties
36	Peganine 72610		Immuno-modulation. Anti- mycobacterial; leishmania Prostaglandin potentiation [120-124] IC_{50} ~ 130 uM
37	MEGxm0_000173 21127802		None reported Closely related compound, Gliotoxin (CID 6223) cited for multiple activities. Inhibitor of NF-κB, farnesyl transferase; anti-mycobacterial; anti-Hepatitis C; induction of apoptosis and necrosis [125-130] IC_{50} ~ 15 uM
38	19-A7		IC_{50} ~ 60 uM

39	(2S,3S,14S,13R)-3-[(4-methylphenyl)carbonyl]-17,19-dioxa-4-azapentacyclo[14.2.1.0<2,14>.0<4,13>.0<5,10>]nonadeca-5(10),6,8,11-tetraen-15-one		IC_{50} ~ 45 uM
40	(+)-Cinchonine ((5R)-5-vinylquinuclidin-2-yl)(1S)-4-quinolylmethan-1-ol		IC_{50} ~ 30 uM
41	(3aS,2R,3R,9aR)-2-(hydroxymethyl)-6-imino-9-hydro-2H,3H-oxolano[2,3-d]pyrimidi no[2,1-b]1,3-oxazolidin-3-ol, chloride		IC_{50} ~ 50 uM

42	(-)-Eseroline, Fumarate salt (3aS,8aR)-1,8,3a-trimethylpyrrolidino[2,3-b]indolin-5-ol		IC_{50} ~ 50 – 90 uM
43	metacycloprodigiosin, Streptorubin		Cyto-toxic activity against human lung, liver and blood cancer cells, and mouse lymphoma cells [131]. IC_{50} ~ 10 uM
46	Fredericamycin		IC_{50} ~ 3 uM

48	Sanguinarine		Induces cell cycle arrest and apoptosis in human cervical cancer cells [132]. IC_{50} ~ 70 uM
49	Tryptanthrin		Induced apoptosis in human leukemia cells [133]. Inhibition of prostaglandin E2 and nitric oxide in mouse macrophages [134]. IC_{50} ~ 15 uM
50	Roseophilin		Stereoisomer 1 or 2

Aporphines

Aporphines comprise a subset of alkaloid compounds characterized by an aromatic, tetracyclic skeleton, containing three benzene moieties, and a fourth cycle containing the alkaloid nitrogen. Many derivatives exist, several of which appeared in our screen. Most of the compounds in this family, that are in our screen, are highly similar, with some being very nearly identical, differing only by the presence or location of a hydroxyl, methoxy, or keto group. Some, however, differ a little more, perhaps containing an additional ring, or existing as a covalent dimer of two normal aporphine molecules.

While there is little known about most of the aporphine compounds in our screen, some appear to have no previous designation, there are other highly similar compounds that have previously been isolated and studied. One such compound, glaucine, shows a host of activities *in vitro*, including relaxation of bronchia and inhibition of its contraction, reduction in superoxide generation in stimulated polymorphonuclear leukocytes and eosinophils, reduction of elastase release, leukotriene production, and intracellular Ca^{2+} in PMN's, platelet aggregation, and eosinophil peroxidase release. These effects of the compound make it a likely bronchiodilator and anti-inflammatory. [135]

Aporphines have also demonstrated *in vitro* anti-viral activity. A number of the compounds inhibit polio-virus infection of cultured mammalian cells by 50% at a range of low micromolar concentrations. [136-137] Additionally, the aporphines dicentrine, glaucine, corydine, and apomorphine, which are analogs of the aporphines in our study, demonstrated anti-proliferative activity against mouse leukemia, melanoma, bladder cancer, and colon cancer cells [138].

As discussed earlier, Hsp90 contains a distinct ATP-binding domain, the specificity of which is being exploited in the search for inhibitors. This domain, called the Bergerat fold, is shared by a few other protein families, including the gyrase type of topoisomerase II. Some aporphines are

inhibitors of topoisomerase II, increasing the likelihood that highly similar compounds from our screen are inhibitors of Hsp90. One such inhibitor is liriodenine (Figure 4). This compound has shown activity against human cancer cells [139], gram-positive bacteria, yeast, and fungi [140-141]. Additionally, liriodenine inhibits the activity of topoisomerase II *in vitro*.[142]

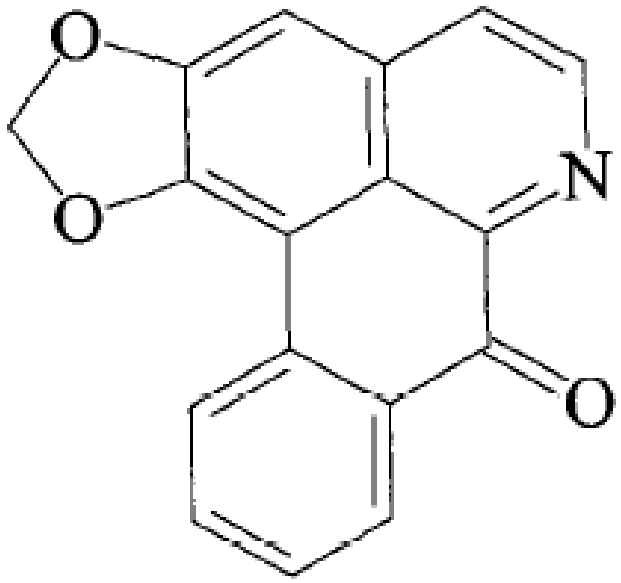
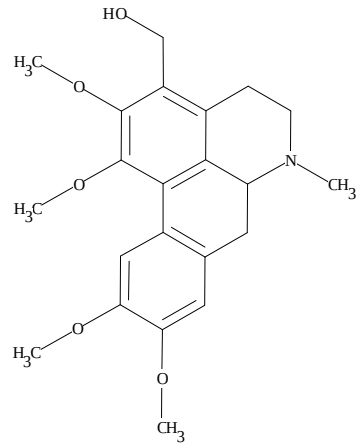
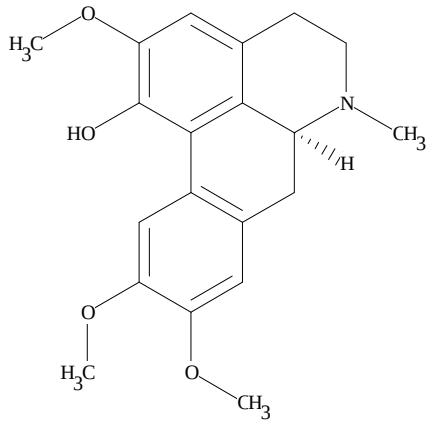
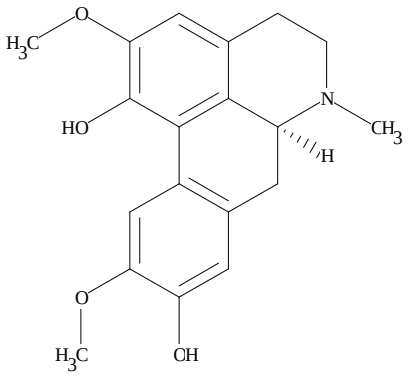
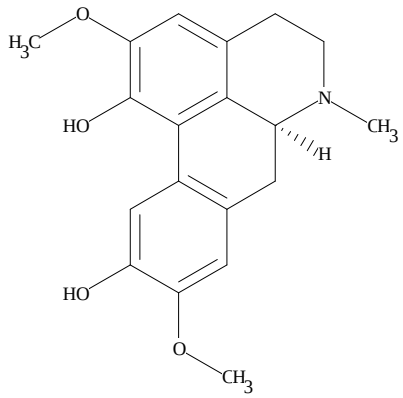
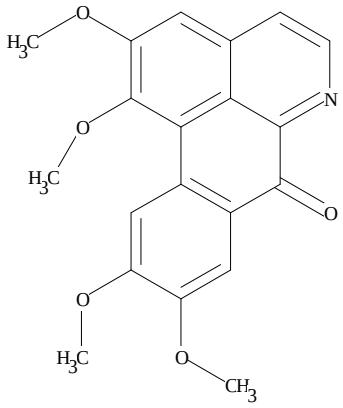
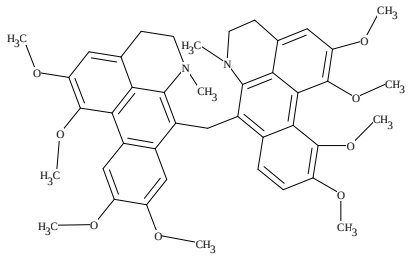
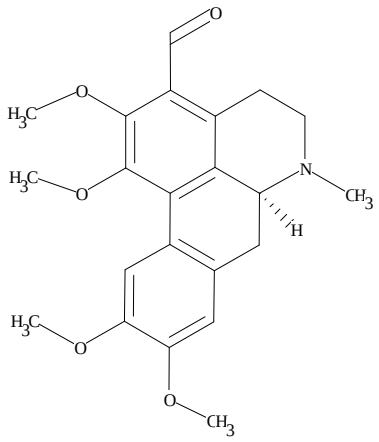
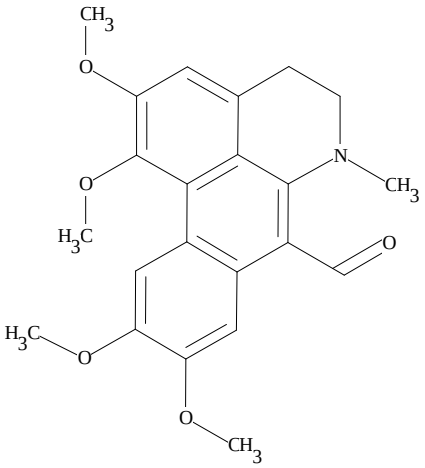
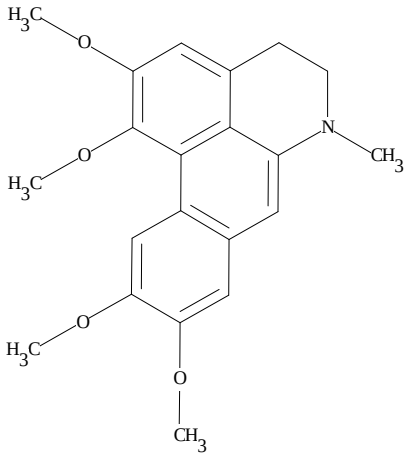


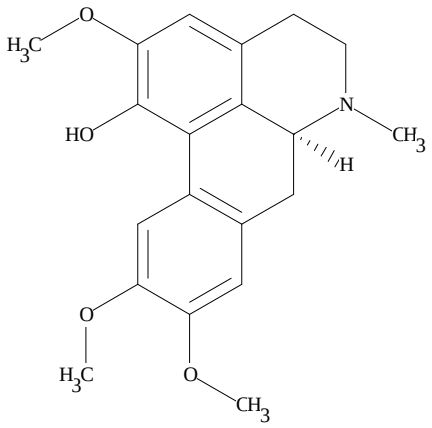
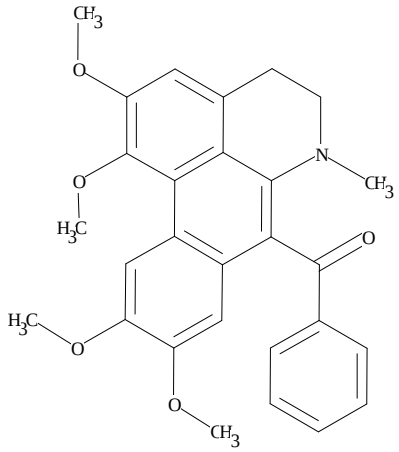
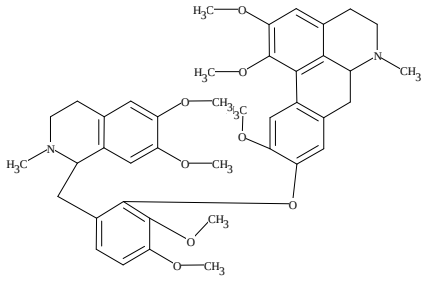
Figure 2.4. Structure of liriodenine

Table 2.6. Aporphine compounds

#	Name	Structure	Properties
51			IC_{50} ~ 80 uM
52	Thaliporphine, thalicmidine		IC_{50} ~ 9 uM
53	Isoboldine		IC_{50} ~ 30 – 80 uM

54	Bracteoline		IC_{50} ~ 80 uM
55	7-Oxoglaucine		IC_{50} ~ 80 uM
56			IC_{50} ~ 100 – 400 nM

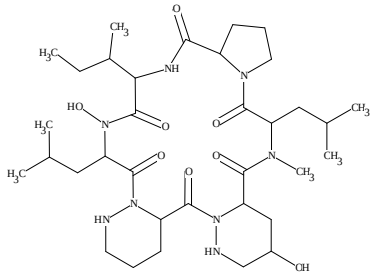
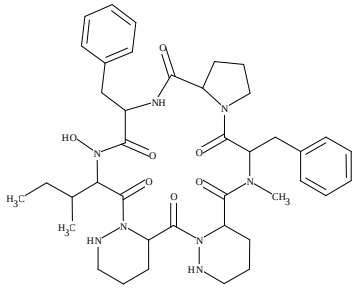
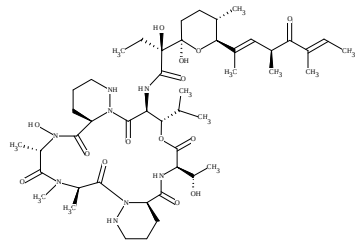
57			IC_{50} ~ 8 – 20 uM
58	Product of chemical transformation of Dehydroglaucine		IC_{50} ~ 1 – 3 uM
59	Dehydroglaucine		IC_{50} ~ 900 nM

60	Thaliporphine, thalicmidine		
61	Product of chemical transformation of Dehydroglaucine		IC_{50} ~ 7 – 20 uM
62			Similar to groups of anti-malarial [81] and anti-inflammatory compounds [119]. Compounds share 3-ring structure. IC_{50} > 30 uM

Cyclic peptides

The laboratory of Shelli McAlpine has investigated the anti-cancer and Hsp90-inhibiting activities of a number of naturally sourced macro-cyclic peptides. These molecules and their derivatives have demonstrated relatively potent activity against a number of cancers [143]. The rationale behind the pursuit of these particular molecules is that similar compounds have been identified as antibiotics and anti-fungals, as well as having anti-cancer activity. The polypeptide nature of these compounds may confer on them the ability to mimic hydrophobic portions of client proteins. The three compounds in our screen all contain moieties that could mimic hydrophobic amino acids.

Table 2.7. Cyclic peptide compounds

#	Name or location within assay	Structure	Properties
44	P12		IC_{50} ~ 10 uM
45	Cyclopeptide L-156373		IC_{50} ~ 10 uM
47	Antibiotic A83586C M14		Anti-proliferative activity against human colon, lung, breast, and bone cancer cells [144]. IC_{50} ~ 10 uM

Quinones

Quinones represent an extremely large and diverse family of compounds. Essentially, the only thing that differentiates a quinone from any other class of compounds is the presence of two keto-groups on an aromatic ring. As such, an enormous array of combinations of functional and structural groups that incorporate this characteristic is possible. For the purposes of our screen, we have grouped the quinones together, not because of any great similarity in their structure, but the potential commonality of their chemical properties; specifically, that they are usually redox-active, making them promising compounds with which to treat cancer. The production of reactive oxygen species resulting from exposure to quinone-containing compounds is a process that is potentially destructive to any cell. Cancer cells, however, are often less resilient to chemical insults like these. It should also be noted that some Hsp90 inhibitors contain quinone moieties. It has not been established if the redox potential of these compounds contributes to the anti-cancer efficacy of these compounds by increasing their ability to inhibit Hsp90, increasing the stress on the cells in addition to that caused by Hsp90 inhibition, or some combination of the two. It should be noted, however, that reduction of 17-DMAG and 17-AAG to their hydroquinone forms increased their cytotoxicity to cancer cells [145]

Some well-established Hsp90 inhibitors, such as geldanamycin (Figure 5) and its derivatives, contain quinone moieties. Consequently, we have observed reticulocyte lysate treated with these inhibitors to have a distinct dark red color, attributable to met-hemoglobin formation resulting from the oxidative activity from these compounds. Similarly, geldanamycin has been shown to generate reactive oxygen species in vitro and in cell culture [146]. Structural studies demonstrating direct binding of these compounds to the ATP-binding site in the N-terminus of Hsp90 implies that the redox activity of these compounds is not highly relevant to their biological activity, although this has not been clearly determined. Accordingly, the antibiotic, rifamycin,

which contains a reduced quinone moiety within an overall similar anthroquinone ansamycin molecule, potentially has the same type of activity as geldanamycin.

Within our screen, eight hits were quinones. They are characterized by two basic core structures, with varying additions in the form of cyclic structures and functional groups.

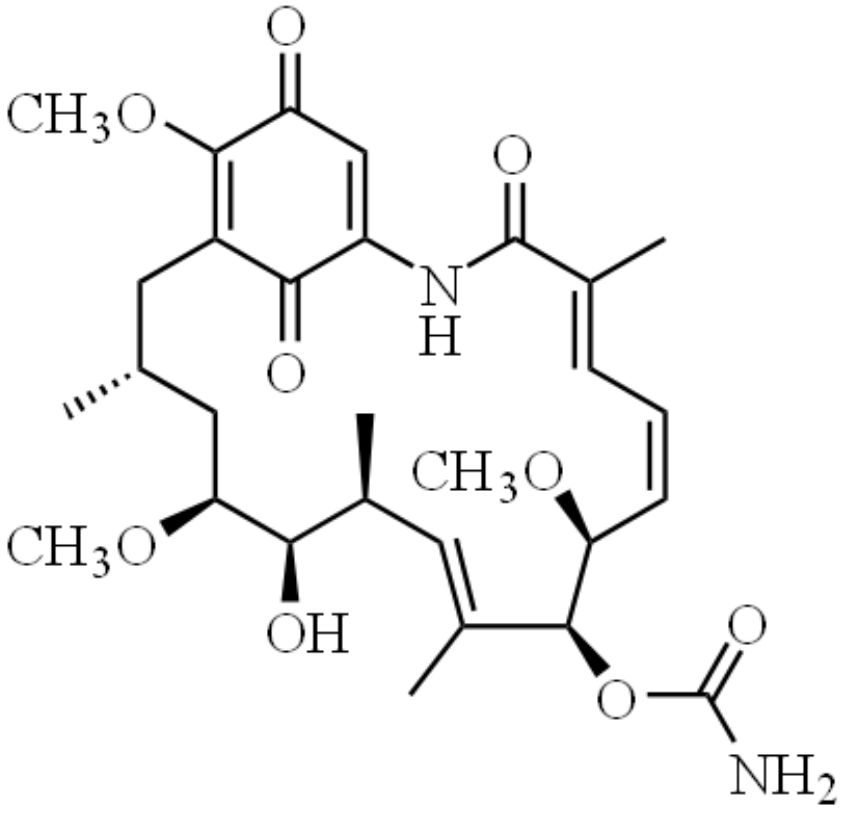
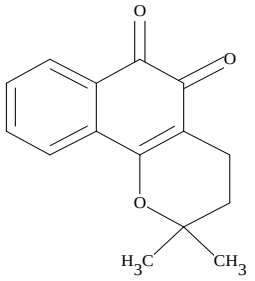
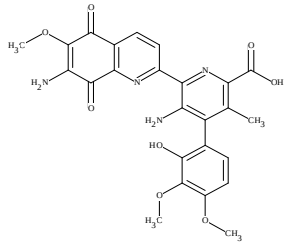
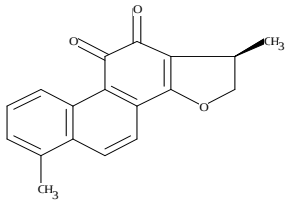
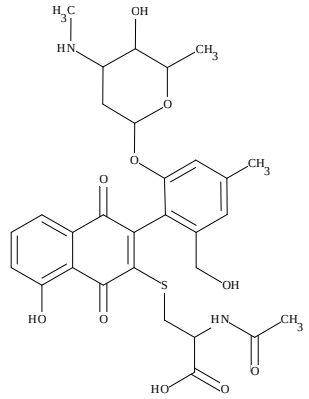
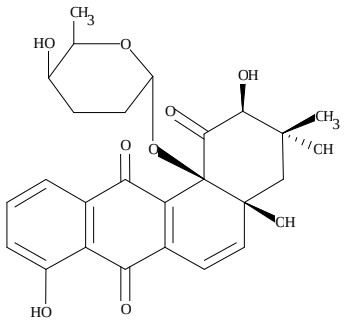
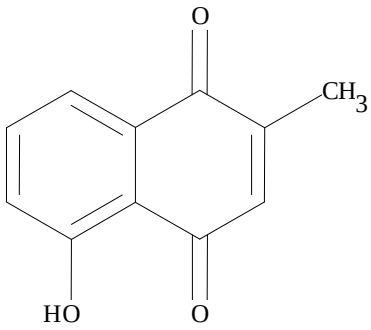
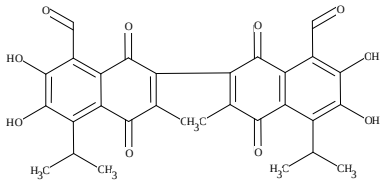
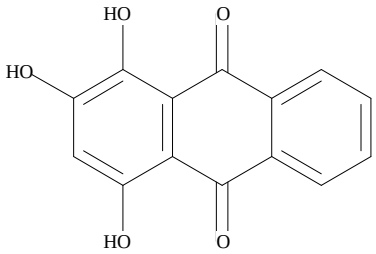
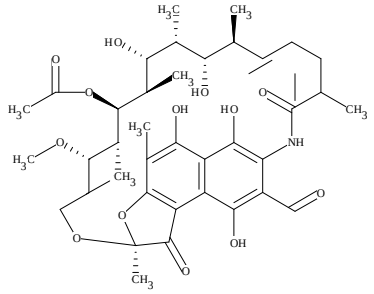


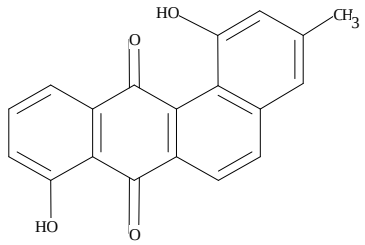
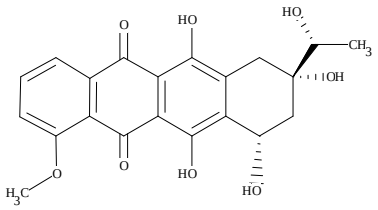
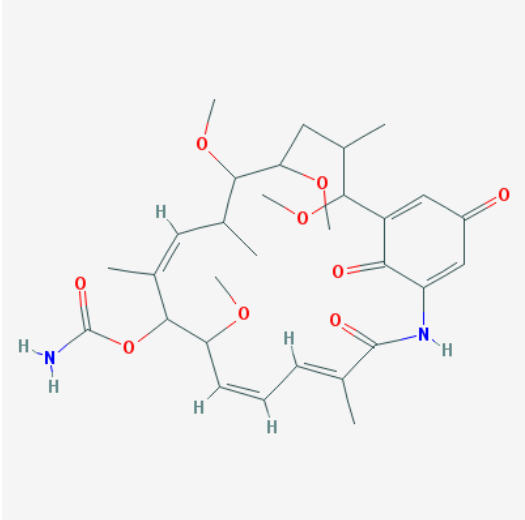
Figure 2.5. Structure of geldanamycin

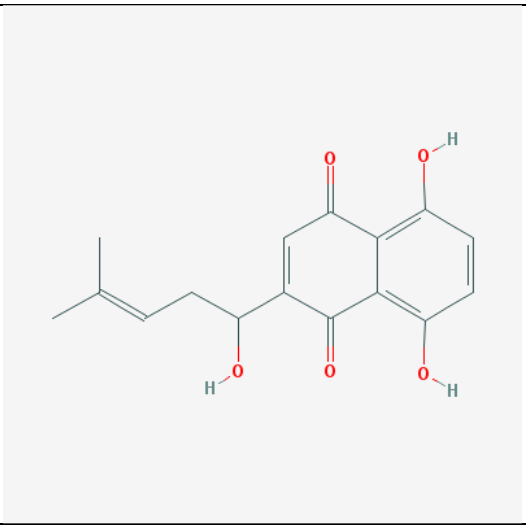
Table 2.8. Quinone compounds

#	Name or location within assay	Structure	Properties
63	B-Lapachone 3885		Anti- HL-60 leukemia; U87 glioblastoma [147-153] IC_{50} ~ 20 uM
64	Streptonigrin 5351165		Disruption of NF-κB activation. Anti-bacterial, anti-fungal, anti-viral, anti-glioma [154-159] IC_{50} ~15 uM
65	Dihydrotanshinone 11425923		Inhibition of Hypoxia-Inducible Factor 1 activation; Vitamin D receptor Anti- AGS gastric cancer cells; hepatocellular carcinomas [160-162] IC_{50} ~ 80 uM

66	2- A5		IC_{50} ~ 10 uM
67	30-D10		IC_{50} ~ 2 uM
68	Plumbagin		Plumbagin has been widely studied for its anti-cancer activity. It's been shown to be effective against a variety of cancers, including pancreatic, ovarian, skin, lung, blood, and others. It also has anti-inflammatory activity [163]. Additionally, molecules reported to be targets of plumbagin are the endothelial growth factor receptor (EGFR), STAT-3 [164], and AKT, among others [163]. IC_{50} < 55 nM

69	7-[8-formyl-6,7-dihydroxy-3-methyl-5-(methylethyl)-1,4-dioxo(2-naphthyl)]-2,3-dihydroxy-6-methyl-4-(methylethyl)-5,8-dioxonaphthalene carbaldehyde		IC_{50} ~ 6 uM
70	1,2,4-trihydroxyanthracene-9,10-dione		IC_{50} ~ 40 – 100 uM
71	3-formyl Rifamycin SV		IC_{50} ~ 15 – 35 uM

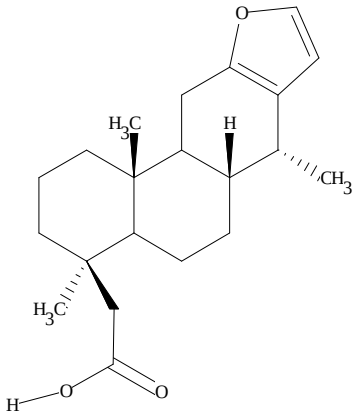
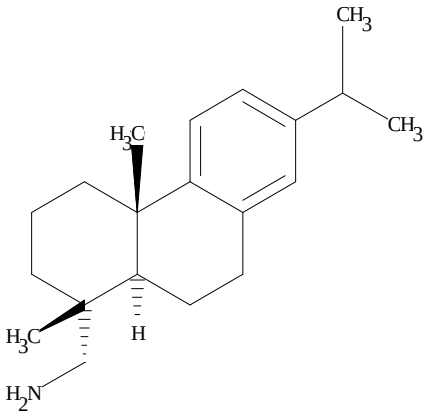
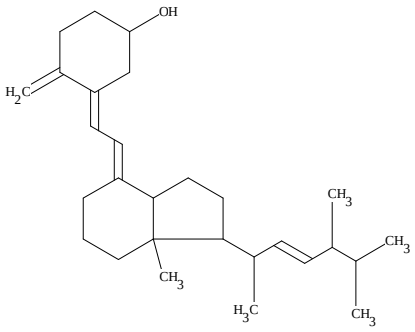
72	Tetrangulol G2		IC_{50} ~ 20 uM
73	Dihydrodaunomycinone Leukaemomycinone-D B-112 B4		IC_{50} > 60 uM
74	Herbimycin		Established inhibitor of Hsp90 [165]. Possesses antibiotic and antitumor activities [166-167]. Belongs to benzoquinone ansamycin family, of which geldanamycin is a member. IC_{50} ~ 40 uM

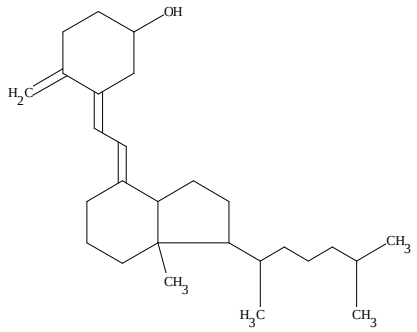
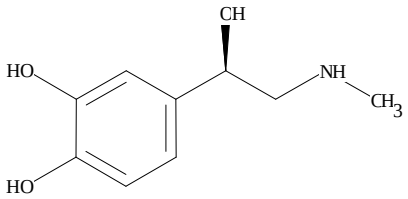
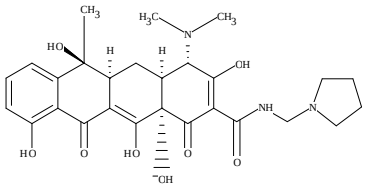
75	Shikonin		Anti-inflammatory, anti-tumor, wide-ranging activities reported [168] IC_{50} ~ 75 uM

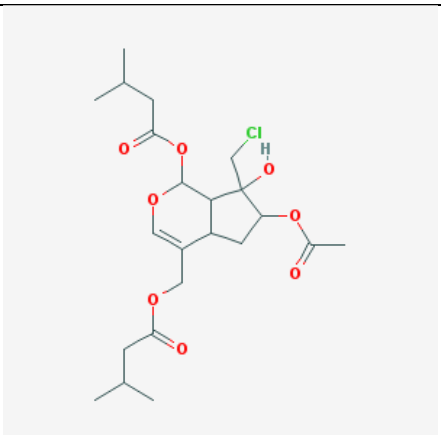
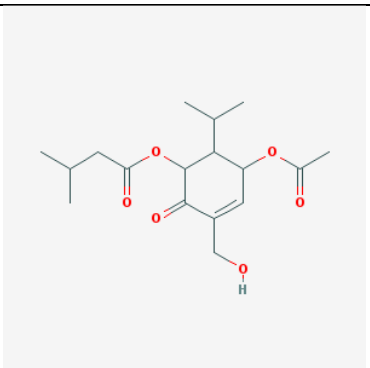
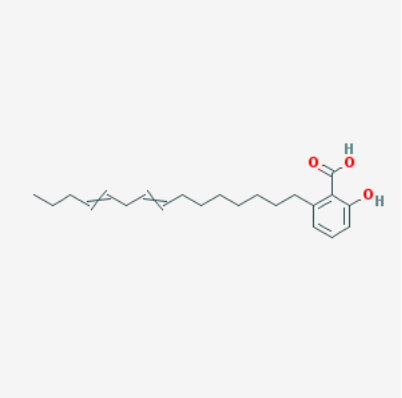
Other Compounds

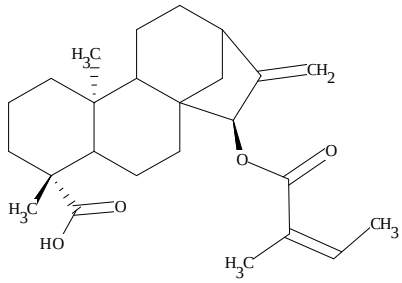
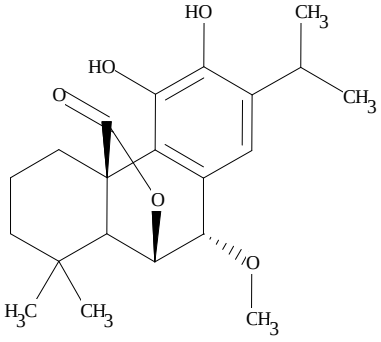
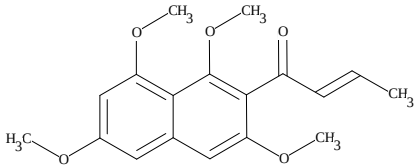
The compounds in this section do not fit well into any of the previously described families. Some of them are known biochemicals, such as Vitamin D2 and L-adrenaline, which haven't been implicated as Hsp90 inhibitors, although Vitamin D2 has demonstrated anti-cancer activity [169]. Others are more exotic and little, if anything is known about them.

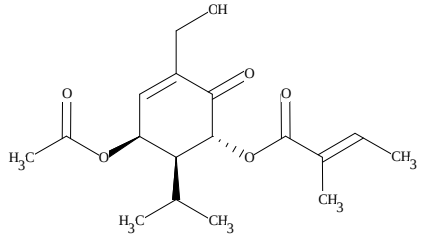
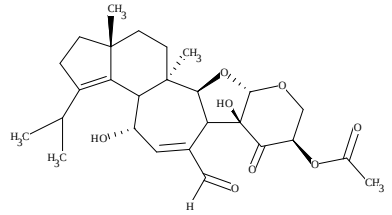
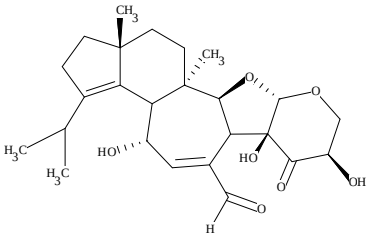
Table 2.9. Other compounds

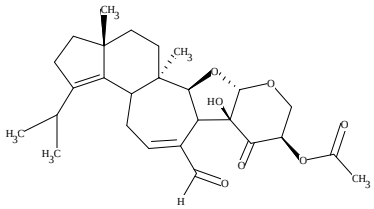
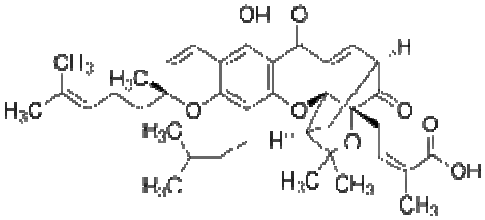
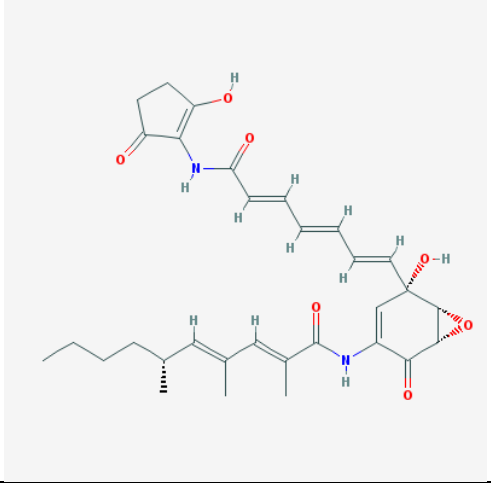
#	Name or location within assay	Structure	Properties
76	2-((2R,6R,10R,11R)-2,6,11-trimethyl-15-oxatetracyclo[8.7.0.0<2,7>.0<12,16>]heptadeca-12(16),13-dien-6-yl)acetic acid		IC_{50} > 75 uM
77	(+)-Dehydroabietylamine or Leelamine [(4aS,1R,10aR)-1,4a-dimethyl-7-(methylethyl)-1,2,3,4,9,10,10a,4a-octahydrophenanthryl]methylamine		IC_{50} ~ 30 – 75 uM
78	Vitamin D2 Hormone		IC_{50} ~ 60 uM Demonstrated modulatory effects on cancer and autoimmune disorders [170]

79	3-{2-[7-(1,5-dimethylhexyl)-6-methylbicyclo[4.3.0]non-2-ylidene] Hormone		IC_{50} ~ 60 uM
80	L-Adrenaline, 98+% Hormone		IC_{50} ~ 55 uM
81	Rolitetracycline		IC_{50} ~ 20 uM

82	MolPort-005-945-572 13455459		None reported Closely related compound, CHEMBL560620 (CID 12821893) – Anti- A549, HCT8, Bel7402, and PC3M cancer lines [80] IC_{50} ~ 50 uM
83	45360154	No structure	No reports IC_{50} ~ 50 uM
84	MolPort-005-944-833 45359640		None reported Closely related compound, MEGxp0_001860 (CID 15699875) – Anti- BJeLR transformed fibroblasts Inhibitor of STK33, MITF ROS induction in cancer cells Broad institute Screen - Run ID's 2007-01-W01-02-03; 2013-01-A01-03-01; 2021-01-A01-05-04; 2044-01_Activator_SinglePoint_HTS_Activity IC_{50} ~ 50 uM
85	CID 53984538		IC_{50} ~ 10 uM

86	17-A4		IC_{50} ~ 40 uM
87	18-G11		IC_{50} ~ 30 uM
88	28-D6		IC_{50} ~ 5 uM

89	28-E3		IC_{50} ~ 15 uM
90	H15 Striatal-B		Inhibition of cell growth of multiple cancer lines including non-small cell lung, melanoma, colon, leukemia, and others. – National Cancer Institute Cell Line Growth Inhibition Assay IC_{50} ~ 2 uM
91	G13 Striatal C		Only information for Striatal B. Similar activity is likely.

92	G15 Striatal A		Only information for Striatal B. Similar activity is likely.
93	Gambogic Acid		Demonstrated to inhibit Hsp90 [171-172]. IC_{50} ~ 2 uM
94	Manumycin A		Known as a farnesyl transferase inhibitor, but has been shown to induce ROS [173-174] production, down-regulate Akt and MEK [174], STAT3, and Telomerase[173], and induces autophagy in pancreatic cancer cells [175] and apoptosis in multiple lines [173-174].

Further Characterization of Putative Inhibitors

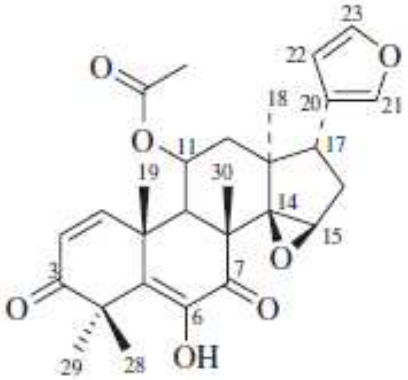
Based on results from our high-throughput screen and a search of the literature for compounds that display broad biological activities, we have identified four compounds as potential Hsp90 inhibitors: anthothecol, garcinol, rottlerin, and piperlongumine.

Anthothecol (Figure 6) is a limonoid compound isolated from the tree *Khaya anthotheca*, and possesses low micromolar activity against the growth of *Plasmodium falciparum* in erythrocytes [176], which is a hallmark of Hsp90 inhibitors. The compound's structure is also very similar to

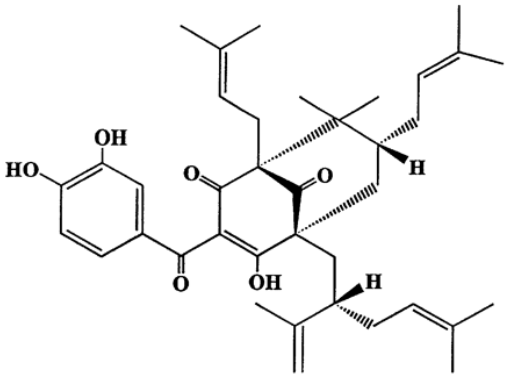
that of the Hsp90 inhibitor gedunin [177]. Anthothecol was identified as a potential Hsp90 inhibitor in a high throughput screen of a Microsource natural product library (Matts, R.L., unpublished data).

Garcinol (Figure 2.6) was chosen for further study, because it has demonstrated the ability to induce apoptosis in a number of cancers, including breast, colon, kidney, prostate, leukemia [178], pancreatic[179], and others. Additionally, garcinol can inhibit angiogenesis through down-regulation of Prostaglandin E2, VEGF, and IL-8 [179]. Garcinol has anti-oxidant and anti-inflammatory properties, as it inhibits the production of ROS and nitric oxide [180].

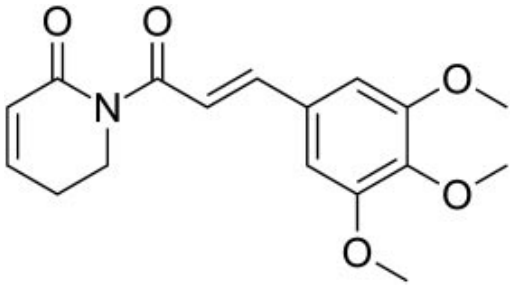
Piperlongumine (Figure 2.6) has been shown to suppress Platelet-derived Growth Factor (PDGF) signaling [181]. Piperlongumine also inhibits the proliferation of prostate cancer cells and causes depletion of the androgen receptor, a well-known Hsp90 client protein [182].



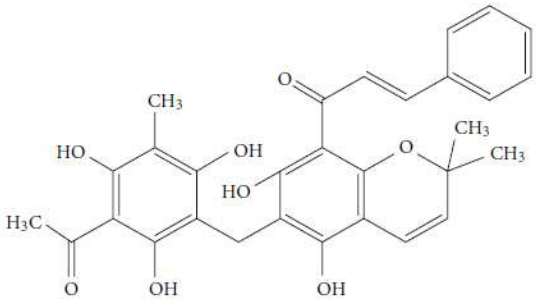
Anthothecol



Garcinol



Piperlongumine



Rottlerin

Figure 2.6. Structures of anthothecol, garcinol, rottlerin, and piperlongumine

Of the four compounds chosen for additional study, rottlerin (Figure 2.6) is easily the best known. It has been used in traditional medicine, and has demonstrated many physiologically significant biological activities. Rottlerin is isolated from the tropical tree *Mallotus Philippinensis*. Rottlerin displays cytotoxicity against a number of cancer types, including lung, breast, lymphocytic leukemia, and multiple myelomas. This activity has largely been attributed to inhibition of PKCδ [183], although the drug has been demonstrated to act independently of this protein [184]. An array of human malignant tumor cells was treated with rottlerin, and all lines were found to undergo apoptosis mediated by Death Receptor 5 (DR5) [185]. Rottlerin has also been reported to inhibit the kinases PRAK, MAPKAP-2, Akt, and CaMK [186].

In our study, we provide evidence that further implicates these compounds as inhibitors of the Hsp90 complex. We show that, in addition to inhibiting the proliferation of cancer cells, the compounds also inhibit the Hsp90-dependent folding of thermally denatured luciferase in a dose-dependent manner, and block the Hsp90-dependent maturation of the HRI kinase.

Inhibition of Hsp90-mediated refolding of denatured luciferase

Anthothecol, garcinol, rottlerin, and piperlongumine all display the ability to inhibit the refolding of luciferase at micromolar concentrations in reticulocyte lysate (Figure 2.7), implicating them as inhibitors of Hsp90. Anthothecol and rottlerin were identified as potential Hsp90 inhibitors by their ability to inhibit refolding of thermally denatured luciferase in screens of natural compound libraries.

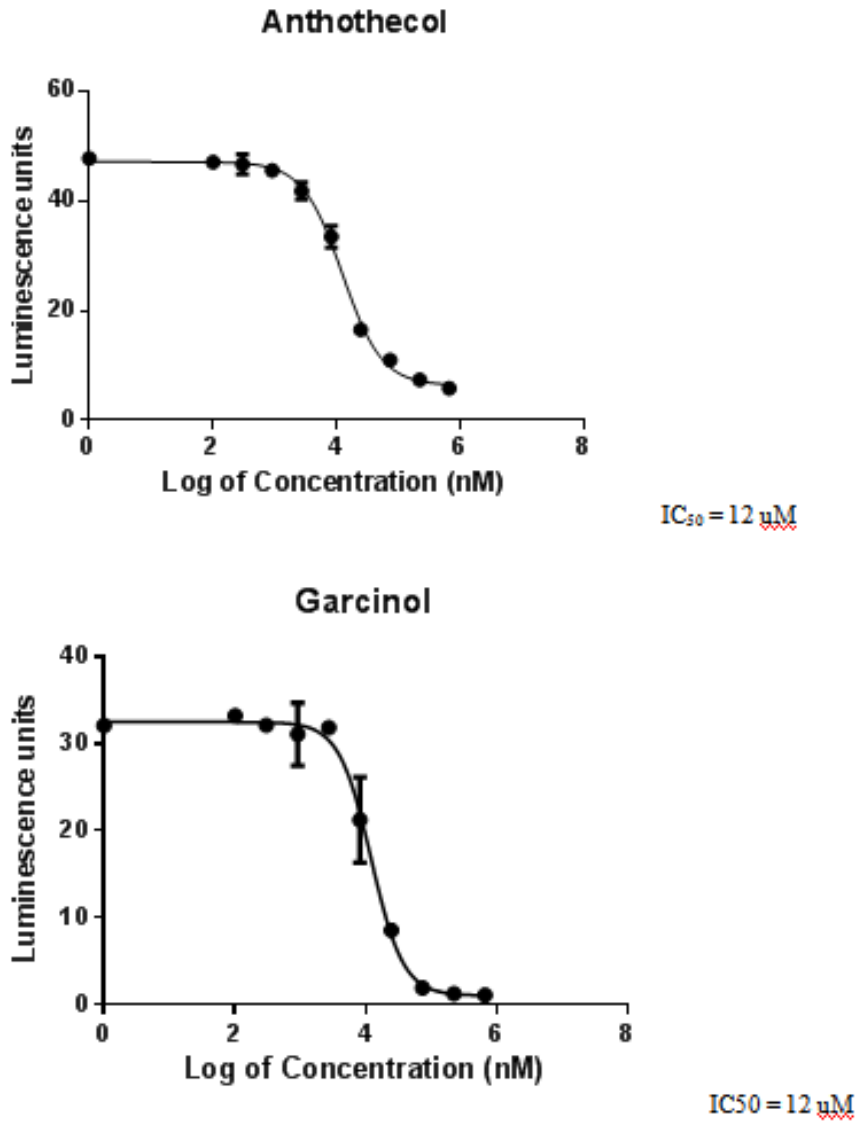
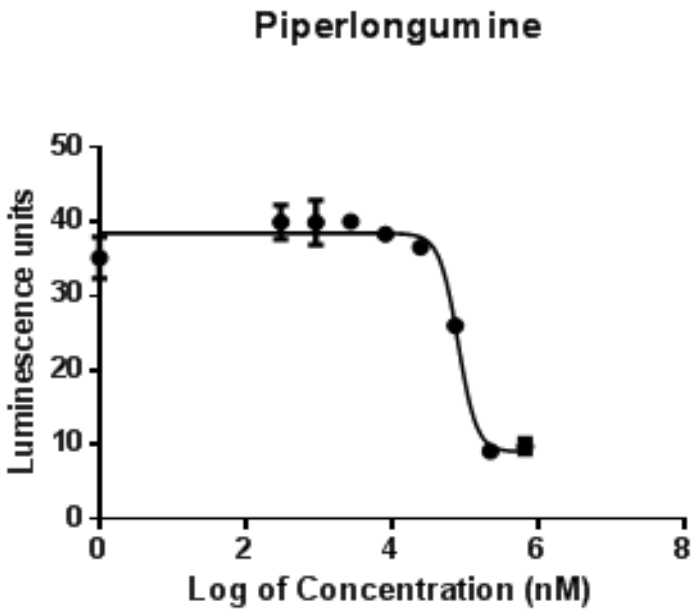
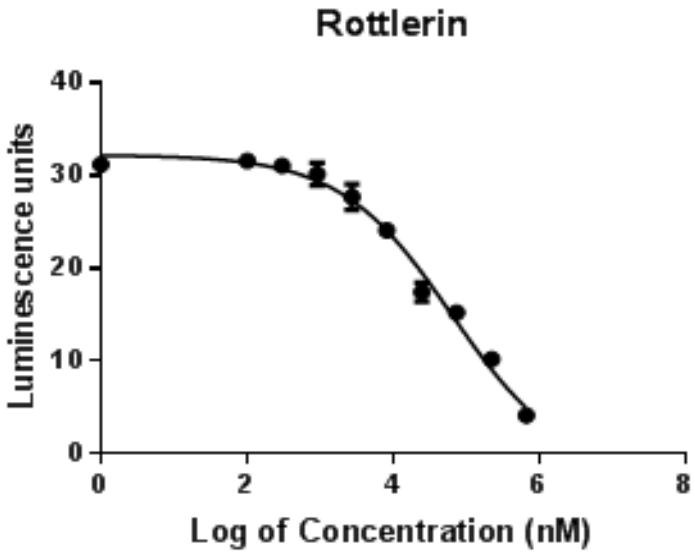


Figure 2.7. Effect of anthothecol, garcinol, rottlerin, and piperlongumine on the refolding of denatured luciferase in reticulocyte lysate.
Each compound was titrated into wells containing denatured luciferase and reticulocyte lysate. After a two hours incubation period, assay buffer containing luciferin was added, and relative luminescence was measured. The Y-axis is relative luminescence, and the X-axis is the concentration of drug in nM.

Figure 2.7. Continued



$IC_{50} = 80\ \mu M$



$IC_{50} = 63\ \mu M$

Inhibition of HRI maturation

The heme-regulated inhibitor of eIF2α is an Hsp90 client kinase which, upon folding by Hsp90, will activate, or mature, by autophosphorylation when heme is deficient. This activation is dependent on functional Hsp90, and can be detected as a slight band shift when separated on a gel (Figure 2.8). Similar to the known Hsp90 inhibitor, geldanamycin, GA, anthothecol, garcinol, rottlerin, and piperlongumine inhibited the maturation of HRI, as seen by the absence of the low mobility form of HRI.

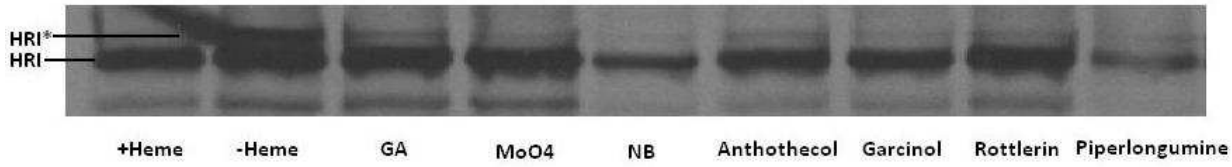


Figure 2.8. Effect of compounds on HRI's Hsp90-dependent maturation
HRI was translated in the presence of ^{35}S-labeled methionine and transferred to heme-deficient lysate for maturation. Translated protein was separated by SDS PAGE, transferred to PVDF membrane, and visualized by X-ray film exposure. The phosphorylated active form of the kinase is indicated with an asterisk. Lanes were treated as follows: heme, no heme, 20 uM geldanamycin (GA), 20 mM sodium molybdate, 20 mM novobiocin, and 100 uM each of anthothecol, garcinol, rottlerin, and piperlongumine.

Compounds Inhibit Proliferation of Human Cancer Cells

We tested the anti-proliferative activity of the four compounds on two human breast cancer cell lines (Figure 2.9). We used an MTS assay, in which living cells reduce the reagent to a chromophore detectable at a specific wavelength. Possibly resulting from their specific absorption wavelengths, wells treated with anthothecol and garcinol did not yield usable data, although previous unpublished observations indicate that an concentration range of 2 – 5 uM of these drugs results in the detachment of approximately half of these cells from their growing surface in cell culture. MCF-7 and SkBr3 both exhibited a dose-dependent decrease in cell viability after treatment with rottlerin and piperlongumine. For MCF-7 cells, both drugs cause a 50% reduction in growth at the 5 – 10 uM range (Figure 2.9, A and B). For SkBr3 cells, there was a decrease in signal intensity even in the vehicle control, possibly resulting from chemical changes in the medium. This complicates our ability to determine an IC_{50} for proliferation, however, there is still a dose-dependent downward trend (Figure 2.9, C and D).

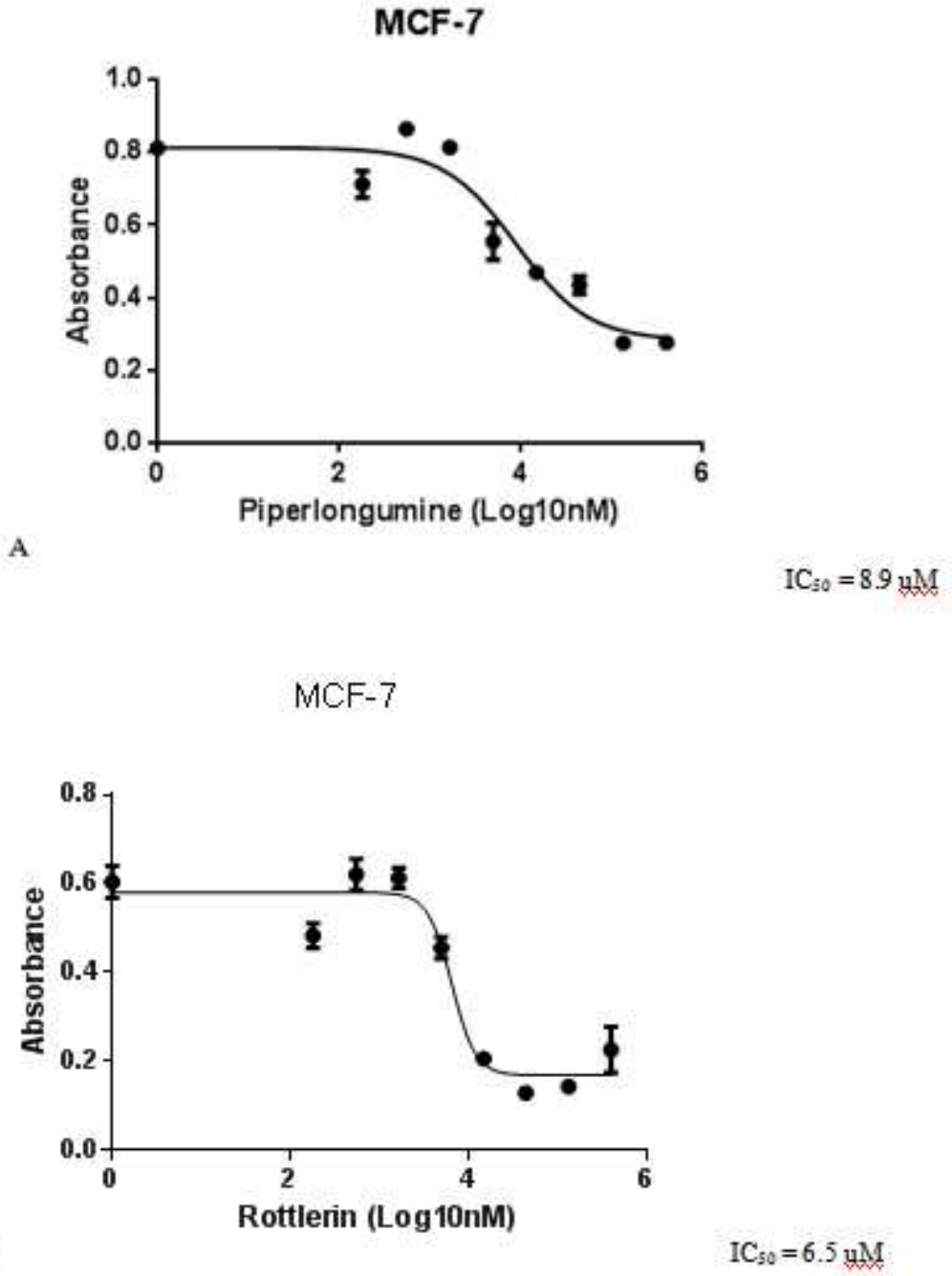
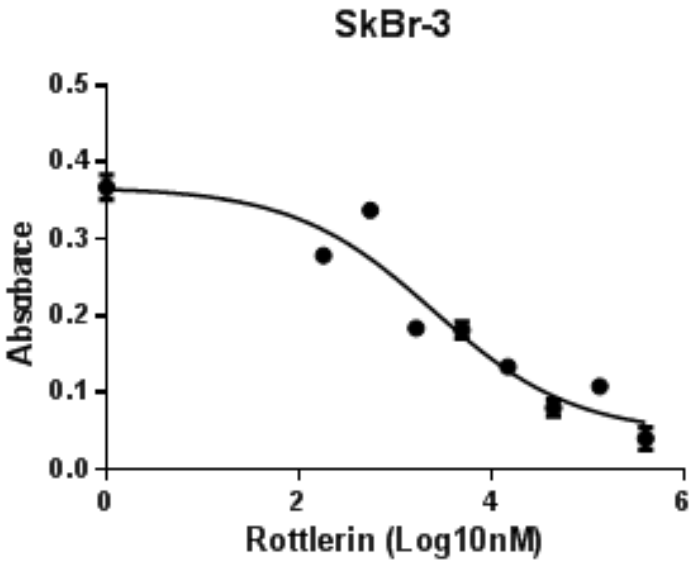


Figure 2.9. Effects of rottlerin and piperlongumine on the proliferation of MCF-7 and SkBr3 human breast cancer cells.

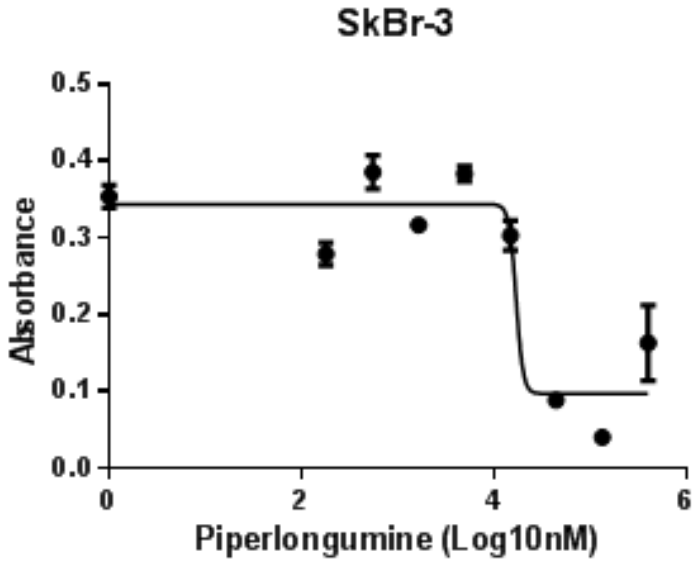
MCF-7 and SkBr3 cell lines were treated in culture with rottlerin (B and C) and piperlongumine (A and D), and DMSO as a control. Proliferation was assessed at 24 hr using an MTS assay. Proliferation is defined as the colorimetric intensity difference between wells treated with DMSO

and wells treated with the compounds. The Y-axis is the relative colorimetric intensity; The X-axis is the concentration of drugs in nM.

Figure 2.9. - Continued



C $IC_{50} = 2.5\ \mu M$



D $IC_{50} = 17\ \mu M$

Conclusion

The compounds identified in our screen are diverse. The compounds with perhaps the greatest potential as Hsp90 inhibitors are those that have already been identified as having specific activities, but against multiple targets. Often, reports by separate laboratories will demonstrate activity of a compound against a certain kinase or pathway, while showing that related proteins or pathways are unaffected. When multiple targets with such effects are demonstrated, it is a strong indicator that the compound in question may be an Hsp90 inhibitor. Hsp90 is essential for the function of kinases, receptors, and other proteins from varied and wide-ranging pathways in the cell, but often plays no part in the function of closely related proteins. This scattered, yet specific, involvement of Hsp90 often precludes the detection of Hsp90's importance by groups studying a narrow portion of the proteomic landscape. Accompanying the effects of these compounds are usually anti-proliferative cyto-toxic activities against cancer cells. Compounds that demonstrate these somewhat pleiotropic effects are among the first to be considered for additional study.

Another tell-tale sign of an Hsp90 inhibitor among screen hits is anti-microbial activity. The earliest Hsp90 inhibitors, such as radicicol, geldanamycin, and novobiocin, were identified as antibiotics before their activity against Hsp90 was elucidated.

Some compounds closely resemble compounds that are known intercalating agents. While these compounds could theoretically have some value as anti-cancer and anti-microbial drugs, we generally choose to bypass them, given the potential off-target toxicity.

Another characteristic of compounds that potentially limit their usefulness is that some of them are redox active. While this fact may or may not affect their activity as Hsp90 inhibitors, the compound can potentially induce an oxidative stress on the cell, which is a potential problem in itself. The results presented here implicate the compounds anthothecol, garcinol, rottlerin, and

piperlongumine as inhibitors of the Hsp90 chaperone complex. Further study of the compounds will be necessary to confirm their status as inhibitors. This will include assessment of their ability to reduce expression of Hsp90-dependent client proteins in cancer cells, and for anthothecol and garcinol, measurement of their anti-proliferative activity. Additionally, physical interaction between the compounds and Hsp90 will need to be determined. Furthermore, we will determine whether or not the compounds bind Hsp90, and we will try to determine the mechanism of binding; specifically, if they bind to the N-terminus or the C-terminus, and where in each domain such binding occurs.

CHAPTER III

COMPARISON OF CELL-BASED AND LUCIFERASE-BASED ASSAYS OF THE ACTIVITIES OF HSP90 INHIBITORS THAT CONTAIN THE 1,4-NAPHTHOQUINONE SCAFFOLD

Introduction

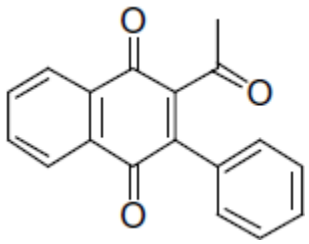
While some inhibitors of the Hsp90 complex are currently in clinical trials, the search for new molecules in this class continues. In collaboration with the laboratory of Dr. Brian Blagg at the University of Kansas, our laboratory carried out a high throughput screen of small molecules for their ability to inhibit the Hsp90-dependent refolding of thermally denatured firefly luciferase. Among the hits from this screen were three compounds (HTS1, HTS2, HTS3) bearing similar core structures. Each compound contained a bicyclic quinone moiety. Two of the compounds are naphthoquinones, while the other contains a furazan ring in place of the fused benzene. These compounds induced the degradation of the Hsp90 client protein Her2, as determined by ELISA, at concentrations from 1 to 3 uM. Finally, the compounds demonstrated anti-proliferative activity against MCF-7 and SkBr3 human breast cancer lines in the nM to low uM range (Table 3.1).

In addition to the cell-based assays performed by Dr. Blagg's group on the derivative compounds, we performed a titrated luciferase refolding assay on them. We wanted to determine how well the compounds' potency correlated between the two types of assay. This holds significance for the compounds as potential therapeutic agents, as solubility problems not

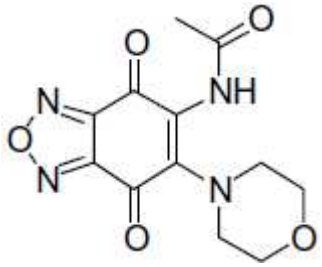
detected in cell-based assays, yet apparent in luciferase refolding assays, can negatively affect the bio-availability of a potential drug. Additionally, while living cells may have ways to metabolize or remove certain compounds, masking their molecular activity within the cell, the luciferase assay is more likely to provide evidence of their interaction with Hsp90. Here we describe the synthesis and cell-based activity of a set of putative Hsp90 inhibitors, and compare them to their observed activity against the Hsp90-dependent refolding of firefly luciferase.

Table 3.1. Hits from KU high-throughput screen.

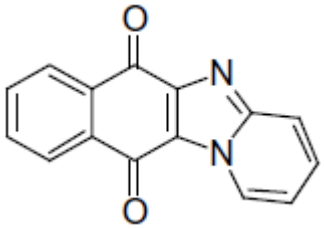
Compound	Luciferase Assay IC_{50} (uM)	Her2 ELISA IC_{50} (uM)	Anti-proliferation IC_{50} (uM)	
			MCF-7	SkBr3
HTS1	0.25	3.3 ± 1	0.21 ± .02	0.82 ± .02
HTS2	0.38	1.2 ± 0.2	19 ± 4	2.9 ± 0.5
HTS3	0.02	1.2 ± 0.3	0.83 ± .13	0.83 ± 0.23



HTS 1



HTS 2



HTS 3

Materials and Methods

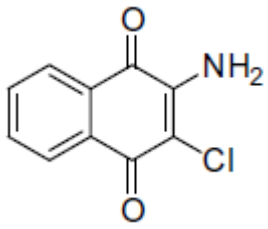
Screen for Hsp90 inhibitory activity

The ability of the synthesized compounds to inhibit the Hsp90-dependent folding of denatured luciferase was assayed as described in Chapter 2.

Synthesis of 1,4 Naphthoquinone derivatives to determine structure-activity relationships

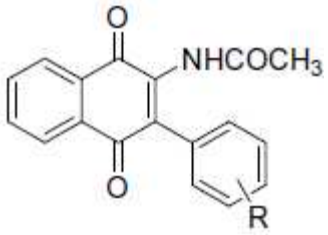
Because of their potent inhibition of Hsp90-dependent luciferase refolding, and their activity against cell proliferation and expression of an Hsp90 client protein, the core structure of these compounds was chosen as a scaffold on which to build potential inhibitors of Hsp90, and to explore the structural characteristics that confer optimal inhibitory activity against the chaperone.

The scaffold used for all of the compounds in the study was 2-amino-3-chloro-1,4-naphthoquinone (1). From this beginning structure, three families of compounds were created, as well as an additional pair of compounds.



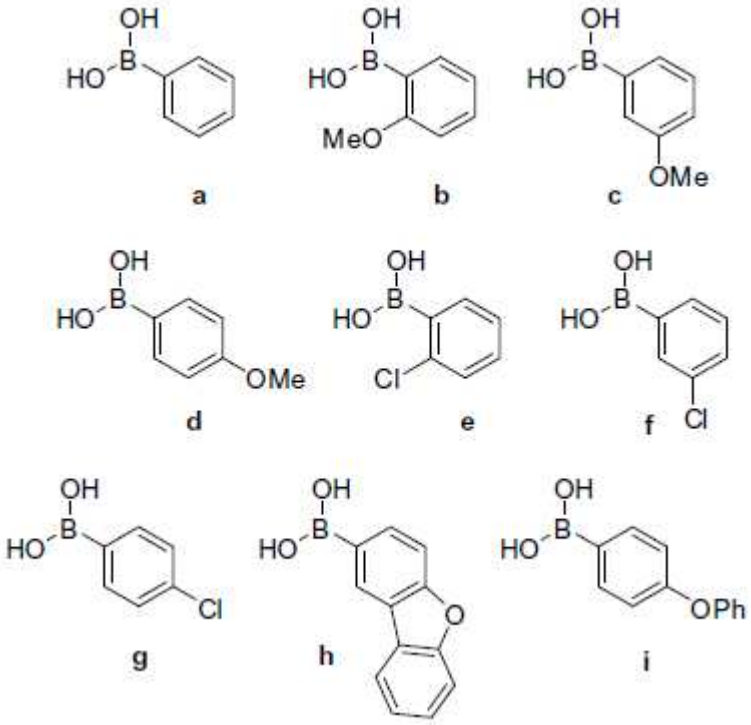
1

To synthesize the first two compound families, the amino group at the 2-position was replaced with an acetamide group for the first family (3), or a benzamide group for the second family (4). For both families, the chlorine at the 3-position was then replaced with an assortment of substituted aryl groups (R).



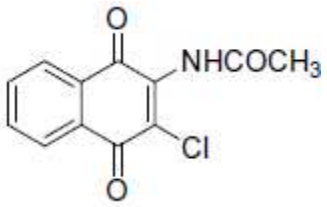
3 **4**

Scaffolds for compound groups 3 and 4.

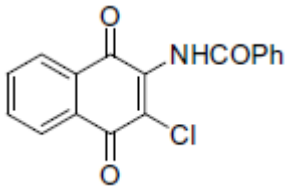


Aryl groups included in compound groups 3 and 4.

For comparison of the contributions of each of the added aryl groups, compared to the core structure, compounds (5) and (6) were created. These two molecules were identical to the (3) and (4) core structures, respectively, except that they retained the 3-position chlorine.

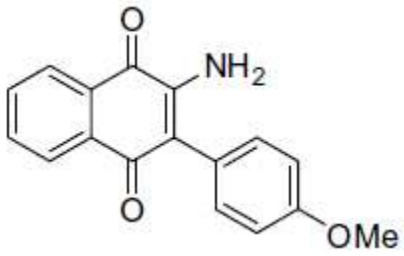


Compound 5

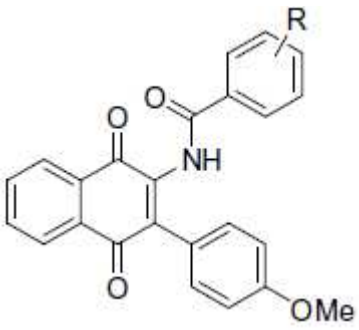


Compound 6

One of the compounds from this family (2d) was then used as the scaffold for the third family of compounds. This third family incorporates the aryl groups a-i shown below. (13). In place of the 2-position amino group, a variety of aryl groups (R) attached through an amide link were added, as well as one cyclohexane addition.

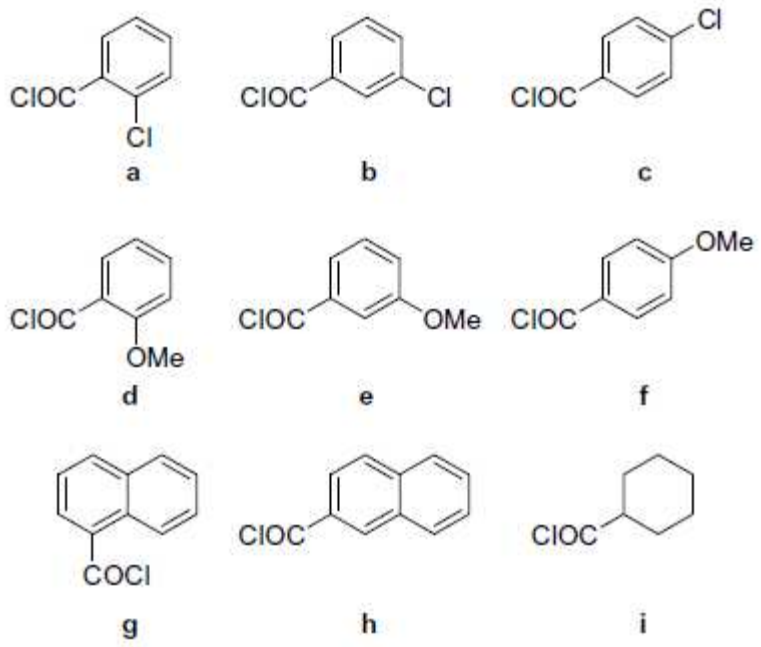


Compound 2d



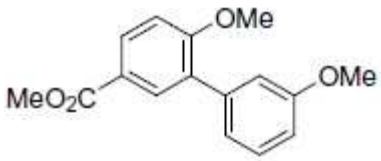
13

Scaffold for compound group 13.

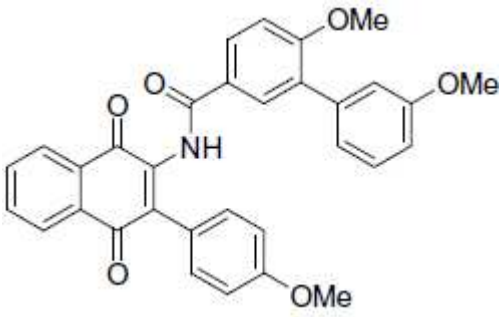


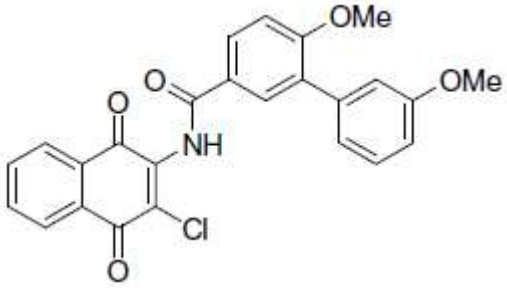
Aryl groups used in compound group 13

To create the final two compounds, a biaryl group that was shown to confer greater potency to a novobiocin derivative than novobiocin itself [187] was attached to compounds 1 and 2d, to form compounds 11 and 12.



Biaryl moiety used in compounds 11 and 12.





Compound 11 **Compound 12**

Results and Discussion

Generally, the compounds' ability to inhibit the refolding of denatured luciferase correlated fairly well with their anti-proliferative and Her2 degradation-inducing activities (Table 3.2). There were, however, exceptions. Compound 4b, a benzamide with a methoxy-phenyl group at the 3-position, had low uM IC_{50} (1.9 – 3.0) activity in all three cell-based assays. Its IC_{50} for the refolding of luciferase, however, was more than 10-fold higher, 39 uM. Compound 13f, a benzamide that contains two methoxy phenyl groups, demonstrated a similar, although slightly smaller disparity. This compound also had a low uM IC_{50} (1.5 – 6.4) in the cell-based assays, but had an IC_{50} of 24 uM in the luciferase assay. Although there are many possible explanations for these results, the simplest is solubility. Some of the compounds being tested precipitate in the aqueous solution of the reticulocyte lysate, whereas in the cell-based assays, the cell membranes offer a different solvent environment in which the solubilities of the compounds, or at least their uptake and delivery to the target molecules, could be greater.

Other possibilities for the difference in performance of one compound between different assays still warrant consideration.

The Hsp90 protein complex that does the refolding work in reticulocyte lysate does not contain cdc37. This co-chaperone is often, though not always, involved in chaperoning kinases. Inhibition of cdc37, or disruption of its interaction with Hsp90, is one mechanism by which an inhibitor could act, which would result in less inhibition of luciferase refolding, if any at all. If cdc37 is in fact the selective factor, then this represents a way to screen for inhibitors that are more specific to cdc37, which might have their own medicinal applications for other ailments in which kinase clients of Hsp90 play a particularly important role.

Additionally, the reticulocyte lysate is quasi-physiological, as opposed to the cells used in the other assays. Structures, pathways, and other systems that are intact in a living cell, but not in the lysate, could be more or less dependent on the Hsp90 complex's function than pure denatured luficerase. Similarly, cellular metabolism could cause a chemical change in the compounds that does not take place in the lysate.

Table 3.2. Activities of synthesized compounds

Compound	Her2 ELISA IC_{50} (uM)	Anti-proliferation IC_{50} (uM)		Luciferase Renaturation IC_{50} (uM)
		MCF-7	SkBr3	
HTS1	3.3 ± 1	.21 ± .02	0.82 ± .02	0.25
3b	2.5 ± 1.4	2.2 ± 0.7	1.7 ± 0.3	1.6 ± 0.5
3g	5.3 ± 2.0	1.4 ± 0.2	1.8 ± 0.4	0.2 ± .03
4b	3.0 ± 1.4	2.2 ± 0.1	1.9 ± 0.7	39 ± 16
4g	1.8 ± 0.5	2.6 ± 0.1	8.5 ± 0.6	1.4 ± 0.3
5	6.6 ± 0.9	1.7 ± 0.1	0.6 ± 0.003	5.0 ± 1.5
11	>100	>100	>100	86 ± 22
12	1.7 ± .04	3.0 ± 0.4	1.7 ± 0.5	1.6 ± 0.4
13e	44.2 ± 4.0	1.8 ± 0.1	6.4 ± 0.2	2.5 ± 1.6
13f	2.4 ± .08	1.5 ± 0.3	6.4 ± 0.1	24.0 ± 15
13i	2.5 ± 1.2	1.9 ± 0.6	4.8 ± 1.3	1.4 ± 0.3

Compounds 11 and 12 differ only by the presence of a para-methoxy phenyl group on 11 in place of the chlorine on 12. This structural difference resulted in a large disparity in the activities of the two compounds. Compound 12 was one of the more potent inhibitors, with an IC_{50} value across all four assays ranging from about 1.5 to 3 uM. Compound 11, however, had IC_{50}'s above 100 uM in the cell-based assays, and 86 uM for luciferase renaturation. An ortho-methoxy phenyl group is also present in compounds 3b and 4b, both of which have lower activities in the luciferase assay than their analogs, 3g and 4g, which contain a para-chloro phenyl group. This pattern could implicate this region of the molecule as one which, when bound to its target, is somewhat near an area of the protein that is sensitive to large aliphatic groups, but more accommodating to smaller or more negatively charged ones.

As mentioned previously, compound 13f had a higher IC_{50} than some of its closely related compounds, at least when used in the luciferase assay. For this group of compounds, the phenyl group attachment is at the 2 position versus the 3 position. Judging from the lower, and varied, activities of the compounds with this family with larger group, or aliphatic groups, it would appear that this region of the molecule interacts with a portion of its target that is somewhat specific for particular sizes and charges in the structure. For instance, 13e and 13f differ only by the position of the methoxy group on the phenyl ring (meta and para, respectively). This difference in position, however, appears to confer a nearly twenty-fold decrease in the IC_{50} of 13f.

The purpose of this study was to create new inhibitors of the Hsp90 chaperone complex, as well as to probe the structures for characteristics leading to increased affinity for the protein. Most of the synthesized derivatives of the original screen hits had activities in the low uM range as determined by cell-based assays and luciferase renaturation assay. Exceptions to this were generally molecules with large, bicyclic additions, specifically those that increased the width of the molecule, likely reflecting steric clashing against the perimeter of the binding site. Also, the increase in the bulk of a molecule likely has a negative effect on its solubility. Additionally,

some of the better performers also contained a chloride addition, probably reflecting a better fit for smaller and/or more negatively charged moieties. Future work will likely involve the fine-tuning and modeling of these structural features and their interactions with the Hsp90 molecule.

CHAPTER IV

GAMBOGIC ACID, A NATURAL PRODUCT INHIBITOR OF HSP90

A high-throughput screening of natural product libraries identified gambogic acid, a component of the exudates of several *Garcinia* species, as a potential Hsp90 inhibitor, in addition to the known Hsp90 inhibitor celastrol. Subsequent testing established that gambogic acid inhibited cell proliferation, brought about the degradation of Hsp90 client proteins in cultured cells, and induced the expression of Hsp70 and Hsp90, which are hallmarks of Hsp90 inhibition. Gambogic acid also disrupted the interaction of Hsp90, Hsp70 and Cdc37 with and blocked the maturation of an Hsp90-dependent client (the heme-regulated eIF2□ kinase) *in vitro.* Surface plasmon resonance spectroscopy indicated that gambogic acid bound to the N-terminal domain of Hsp90 with a low micromolar K_d, in a manner that was not competitive with the Hsp90 inhibitor geldanamycin. Molecular docking experiments support the posit that gambogic acid binds Hsp90 a site distinct from Hsp90's ATP binding pocket. The data firmly established gambogic acid as a novel Hsp90 inhibitor and provides evidence of a new site that can be targeted for the development of improved Hsp90 inhibitors.

Introduction

The 90-kDa heat shock protein (Hsp90) is the core component of an oligomeric chaperone machine whose function is required for the viability of all eukaryotic cells. Hsp90 functions with a cohort of co-chaperones to facilitate the folding, activation and stabilization of numerous client proteins, many of which function in regulating signal transduction pathways [14, 188-189]. Among the plethora of Hsp90-dependent clients are proteins that function in pathways that represent all six hallmarks of cancer [190-196]. Thus, inhibition of Hsp90 function simultaneously incapacitates multiple client proteins, providing a combinatorial attack on cellular oncogenic processes. Consequently, Hsp90 has emerged as an exciting new target for the development of anti-tumor agents.

Natural product inhibitors of Hsp90 have been discovered that target binding sites in Hsp90's N- and C-terminal domains. Geldanamycin and radicicol, which are produced by the soil actinomycetes species *Streptomyces hygroscopicus* and the mycoparasitic fungus *Humicola fuscoatra*, respectively, bind to the ATP binding pocket in Hsp90's N-terminal domain [14, 188-189], while novobiocin, which is produced by *Streptomyces spheroids*, inhibits Hsp90 function by binding to Hsp90's C-terminal domain [66, 197-198]. Other natural products with well known anti-tumor and/ or chemopreventative properties, but poorly characterized mechanisms of action have been discovered to exhibit inhibitory activity toward Hsp90: epigallocatechin gallate (EGCG) [55, 94, 199-200], the well-know anti-oxidant found in green tea; gedunin, a tetranortriterpenoid isolated from the Indian neem tree [177, 201-202]; celastrol, a quinone methide triterpene, that is a pharmacologically active compound present in Thunder God Vine root extracts [201, 203]; and the rotenoid deguelin [204-205].

Derivatives of geldanamycin and other compounds that target the ATP-binding pocket in Hsp90's N-terminal domain have entered more than 20 clinical trials for the treatment of cancer

[195, 206-209]. Clinical complications have arisen in phase II trials of several Hsp90 inhibitors, with incidences of hepato-, cardio-, and ocular toxicity having significantly dampened enthusiasm for clinical use of Hsp90 inhibition [195]. Consequently, there is an ongoing search for Hsp90 inhibitors with superior chemotherapeutic properties for the treatment of cancers.

To this end, we have screened natural product libraries for compounds that inhibit Hsp90-dependent refolding of thermally denatured firefly luciferase. We presumed that natural products would represent a fertile territory for the identification of new Hsp90-inhibitors, as it is reasonable to expect that evolutionary pressures would give plants that produce secondary metabolites which inhibit Hsp90 a competitive advantage, because such compounds would inhibit the growth and development of insect pests and other pathogens. Celastrol, a known Hsp90 inhibitor [201, 203], and gambogic acid, a component of the exudates of several *Garcinia* species that has been used medicinally for centuries in southeast Asia, were identified as inhibitors of luciferase refolding in screens of natural product libraries from Microsource and Biomol.

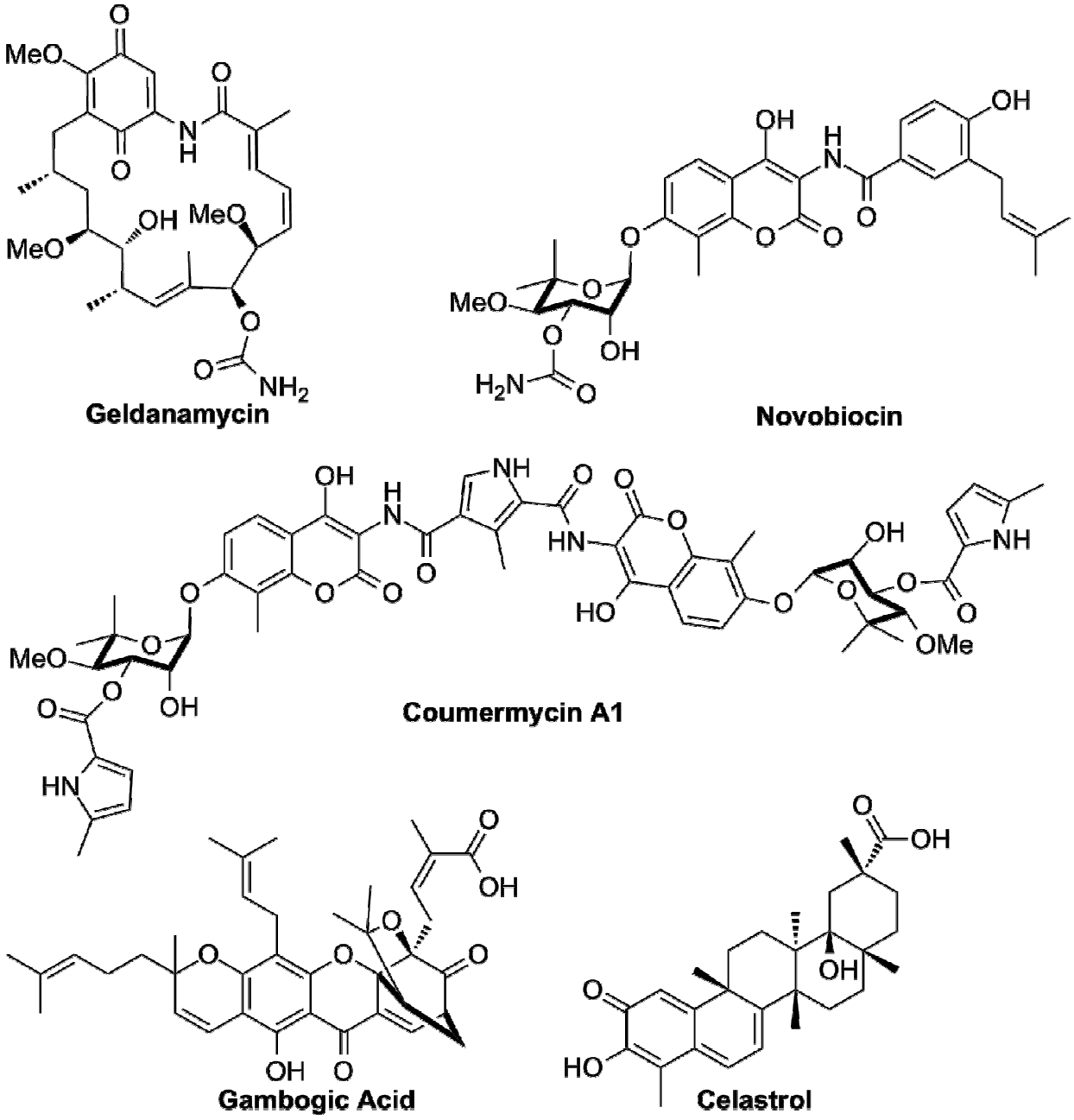


Figure 4.1. Structures of the Hsp90 inhibitors geldanamycin, novobiocin, coumermycin A1, celastrol, and gambogic acid.

Gambogic acid, like Hsp90 inhibitors, has anti-tumor [210-214], anti-angiogenic [212, 215-216], and anti-metastatic [217-218] activities (reviewed in [219-221]), but a poorly characterized mechanism of action. In addition, like Hsp90 inhibitors [193, 222], gambogic acid has been observed to be selectively cytotoxic to cancer versus normal cells [214, 223]. While gambogic acid has been reported to induce apoptosis in cancer cells by binding to the transferrin receptor [224-225], the cytotoxic activity of gambogic acid has also been found to have a transferrin receptor-independent component [226]. Here we present the characterization of gambogic acid's Hsp90 inhibitory activity, and compare its mechanism of action to other Hsp90 inhibitors.

Materials and Methods

Screen for inhibitors of Hsp90-dependent Luciferase Refolding

Natural product libraries from Microsource and Biomol were screened as previously described for inhibitors of Hsp90-dependent refolding of thermally denatured firefly luciferase [227]. Positive hits were then screen against native luciferase to eliminate false-positive that were direct inhibitors of luciferase [70, 228]. Subsequently, celastrol (CalBiochem) and gambogic acid (Biomol) were titrated into reticulocyte lysate containing thermally denatured luciferase to determine the concentration of drug required to inhibit luciferase refolding by 50% (IC_{50}) compared to the DMSO control as previously described [70, 228]. Reactions were carried out in triplicate at room temperature in 96-well microtiter plates, and experiments were repeated at least twice, with relative light unit (RLU) production measured using a L_{Max}II (Molecular Devices) microplate reader and a 10 sec integration time [70, 228].

Effect of gambogic acid on cell proliferation

MCF-7 and HeLa cells were grown in Gibco Modified Essential Medium, supplemented with non-essential amino acids, 2 mM glutamine, and 10% fetal bovine serum. SkBr3 cells were maintained in McCoy's 5A media (Iwakata & Grace modification, Cellgro) with L-glutamine supplemented with streptomycin (500 μg/ml), penicillin (100 units/ml), and 10% fetal bovine serum. Cells were grown to confluence in a humidified atmosphere (37 °C, 5% CO_2). Cells were seeded at 2000 cells per well in a clear 96-well plate, media volume was brought to 100 □l, and the cells were allowed to attach overnight. The next day, varying concentrations of compound or 1% DMSO vehicle control were added to the wells. Cells were then incubated at 37 ^{o}C for 72 hours. Cell viability was determined using the Promega Cell Titer 96 Aqueous One Solution Cell Proliferation Assay, which makes use of a soluble tetrazolium compound that is converted into a chromophore by living cells. After incubation with compounds, 20 □L of the assay substrate solution were added to the wells, and the plate was incubated at 37 ^{o}C for an additional hour. Absorbance at 490 nm was then read on a Molecular Devices Versamax plate reader, and values were expressed as percent of cells incubated in DMSO alone.

Effect of Gambogic acid on the interaction of Hsp90 and its co-chaperones with the heme-regulated eIF2α kinase (HRI)

His-tagged kinase-dead HRI/K199R was synthesized by coupled transcription-translation (TnT) in reticulocyte lysate in the presence of [^{35}S]methionine [66, 229-230]. After 20 min of synthesis, compounds or an equivalent volume of DMSO or water were added at the concentrations indicated in the figure legend and synthesis was continued for an additional 10 min. Subsequently, aliquots were taken for immunoadsorption of His-tagged HRI with anti-His-tag antibodies bound to anti-mouse-IgG agarose resin [66, 229-230]. Agarose resins containing bound anti-His-tag antibody were washed four times with 10 mM PIPES (pH = 7.2) plus 100 mM

NaCl and analyzed by SDS-PAGE and Western blotting for co-adsorbed Hsp90, Hsp70 and Cdc37 as previously described [66, 229-230]. Lysate lacking plasmid encoding His-tagged HRI was used as the control for non-specific binding.

Effect of Gambogic acid on Hsp90-dependent HRI maturation/ activation

[^{35}S]-labeled his-tagged wild type HRI or K199R mutant was generated by TnT in reticulocyte lysate as described above. After 30 min, samples were diluted into 7 volumes of hemin-supplemented or heme-deficient lysate containing DMSO, water or drugs at the same concentration present in the TnT lysate and incubated for an additional 45 min [66, 229-230]. The His-tagged HRI was adsorbed from samples by the addition of NTA-Ni resin on ice for 1 h. Resins were washed, eluted by boiling in SDS-sample buffer, and samples were analyzed by SDS-PAGE, and autoradiography after electrotransfer to PVDF membrane to detect a shift in HRI's electrophoretic mobility that is dependent upon its Hsp90-dependent maturtion/activation [66, 229-230].

Gambogic acid-induced depletion of Hsp90-dependent proteins from cultured MCF7 and SKBr3 breast cancer and HeLa cells

MCF-7, SkBr3 and Hela cells were grown to confluence as described above, seeded in culture dishes ($1x10^6$/dish; brand) and allowed to attach overnight. Gambogic acid was added at the concentrations indicated in the figure and the cells were incubated for an additional 36 h. Cells were harvested and analyzed for Hsp90 client protein degradation (Her2, Raf-1, and Akt) and heat shock protein induction (Hsp90 and Hsp70) as described previously [231]. For comparison, cell were incubated with DMSO (1%) or geldanamycin 500 nM), and extracts were Western blotted for actin as a loading control.

Surface plasmon resonance spectroscopy of gambogic acid binding to Hsp90

Insect Sf9 cells overexpressing human Hsp90α were cultured and harvested by the Baculovirus/Monoclonal Antibody Core facility at Baylor College of Medicine. Hsp90 was extracted and purified (>98% pure) as described previously [17, 232], but without the initial DEAE-cellulose chromatography step. Bacterial *E. coli* DE-3 Star cells carrying plasmids for the expression of either the N-terminal (Hsp90NT: amino acids 1-241 with a C-terminal -GELRSGC tail) or C-terminal (Hsp90CT: amino acids 531-732) domains of Hsp90 were cultured in LB media and induced with IPTG. Recombinant Hsp90NT and Hsp90CT were purified using NiNTA column. Following elution of from the NiNTA column the N-terminal His-tag was cleaved using TEV protease (Invitrogen), followed by size-exclusion chromatography on Superdex 200. The Hsp90 containing fractions were pooled, concentrated and dialyzed against 10 mM Hepes (pH 7.5) containing150 mM NaCl and 10% glycerol, stored at -80°C until use. Prior to the use of the Hsp90NT or Hsp90CT were reacted according to the manufactures recommended protocol with the EZ-Link® Maleimide-PEG2-Biotin (Thermo Scientific) then buffer exchanged into fresh 10 mM Hepes (pH 7.5) containing150 mM NaCl to eliminated free biotin entities.

The surface of a SSO1 CO_2H SPR sensor chip mounted in a SensiQ SPR instrument (ICX Nomadics) was activated by treatment with N-3-dimethylaminopropyl-N'-ethylcarbodiimide hydrochloride and N-hydroxysuccinimide for preferential cross-linking of full length Hsp90's N-terminus to the surface. For immobilization of Hsp90, 250 µl of Hsp90 (6.2 mg/ml) in 10 mM Hepes buffer (pH 7.4) containing 150 mM NaCl was injected at a flowrate of 10 µl/min, resulting in 2000 response units of protein captured on the experimental surface of the chip. Then, 1 M ethanolamine (pH 8) was used to quench the remaining activated groups, and the surface washed with buffer containing 10 mM PIPES pH 7.4, 300 mM NaCl, and 2% DMSO. The surface of a SSO3 BioCap SPR sensor chip was mounted in a SensiQ SPR instrument (ICX Nomadics) and

either the biotinylated Hsp90NT (7.0 mg/ml) or Hsp90CT (6.8 mg/ml) was discrete injected over the experimental channel at a flow rate of 10 μl/min, resulting in an capture of Hsp90NT at 1400 response units and Hsp90CT at 1250 response units of protein on the experimental surface of the chip. The chips were then washed with assay buffer prior to experimental analysis.

Gambogic acid was diluted in assay buffer containing 10 mM PIPES pH 7.4, 300 mM NaCl, and 2% DMSO and injected over the surface of each derivatized chip at a flow rate of 15 μl/min at 25°C at the indicated concentrations. Additionally, for competition studies gambogic acid was diluted in assay buffer containing 20 μM geldanamycin. All measurements were done in triplicate. SPR binding curves were analyzed using QDAT software (ICX Nomadics) to calculate the k_a, k_d and K_D.

Docking studies of geldanamycin and gambogic acid with the N-terminal domain of Hsp90

In silico docking of geldanamycin and gambogic acid with the 3D coordinates of the X-ray crystal structures of the N-terminal domain of HSP90 with bound geldanamycin and in the open conformation, PDB IDs 1YET and 1YES respectively, was accomplished using the Autodock program [233] downloaded from the Molecular Graphic Laboratory of the Scripps Research Institute. The AutoDock program was chosen because it uses a genetic algorithm to generate the poses of the ligand inside a known or predicted binding site utilizing the Lamarckian version of genetic algorithm where the changes in conformations adopted by molecules after *in situ* optimization are used as subsequent poses for the offspring.

In our docking experiments, all waters were removed form the 3D X-ray coordinate while Gasteiger charges were placed on the X-ray structures of the N-terminal domain of HSP90 along with geldanamycin and gambogic acid using tools from the Autodock suite. A grid box centered on the N-terminal HSP90 domain with definitions of 126x126x126 points and 0.4 Å spacing was

chosen for ligand docking experiments. The docking parameters consisted of setting the population size to 300, the number of generations set to 27,000, the number of evaluation set to 20,000,000 while the number of docking runs was set to 50 with a cutoff of 1 Å for the root mean square tolerance for the grouping of each docking run.

While the binding mode of geldanamycin with Hsp90 has been determined through x-ray crystallography [234], the binding mode of gambogic acid with Hsp90 has yet to be determined through either NMR or x-ray crystallography. The docking of geldanamycin to the apo X-ray structure of Hsp90NT, PDB ID 1YET, was used as a control to test and validate our docking parameters. As expected, Geldanamycin docked to the binding site identified in the crystal structure with an average binding energy of -9.65 kcal/mol and a 1 Å average root mean square deviation from the reference structure, Figure 4.6.

Results and Discussion

Identification of gambogic acid as a putative Hsp90 inhibitor from a high-thoughput screen of natural product libraries

Screening of natural product libraries purchased from Microsource and Biomol for compounds that inhibited Hsp90-dependent refolding of luciferase identified gambogic acid as a potential Hsp90-inhibitor, along with the known Hsp90 inhibitor, celastrol, among other compounds. Neither celastrol or gambogic acid had any direct effect on the activity of native luciferase. Upon titration of various concentrations of the drugs into the refolding assay (Fig. 4.2A), celastrol and gambogic acid were found to inhibit luciferase refolding by 50% (IC_{50}) at a concentration of 20 and 2 uM, respectively.

Effect of gambogic acid on cancer cell proliferation

Gambogic acid has been demonstrated in numerous studies to inhibit the proliferation of a variety of cancer cell lines [211-213, 225, 235-243] (reviewed in [219-221]). To determine whether gambogic acid's anti-proliferative activity could be correlated with its Hsp90-inhibitory activity, we examined the effect of varying concentrations of gambogic acid on the growth/ viability of HeLa cells, and MCF7 and SK-Br3 breast cancer cell lines. Gambogic acid inhibited the proliferation of HeLa, MCF7 and SK-Br3 cells in a concentration dependent manner (Fig. 4.2B). Growth of the HeLa, MCF7 and SK-Br3 cells were inhibited by 50% by treatment with 1.5, 2.0 and 0.8 □M gambogic acid, respectively. The highest concentrations of gambogic acid were cytotoxic as evidenced by detachment a significant number of cells from the surface of the culture flasks. Thus, gambogic acid's IC_{50} for inhibition of cell proliferation correlated well with its IC_{50} for the inhibition of luciferase refolding.

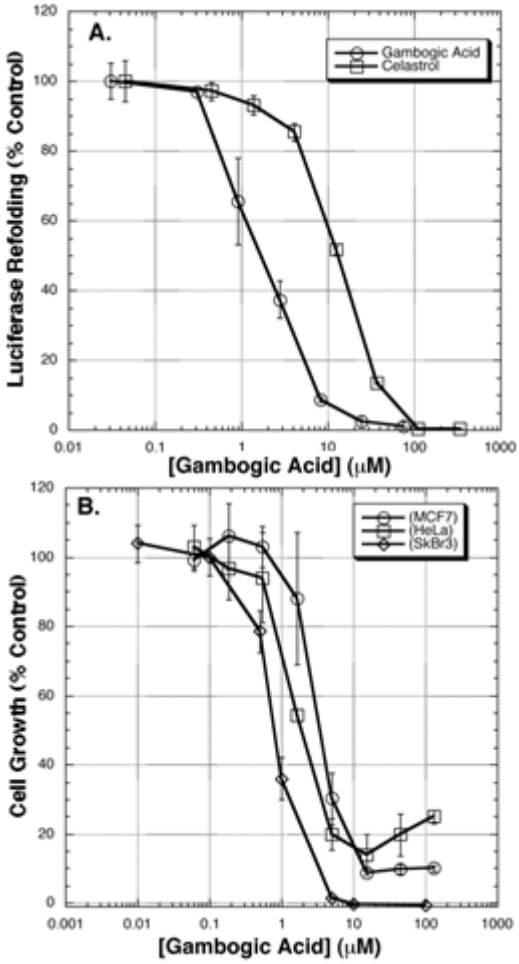


Figure 4.2. Effect of gambogic acid and celastrol on Hsp90-dependent luciferase refolding in reticulocyte lysate (A), and effect of gambogic acid on cell proliferation of HeLa cells, and MCF7 and SkBr3 breast cancer cells. Experiments were carried out as described under "Experimental Procedures". Data for SkBr3 and HeLa cells generated by Laura Peterson.

Gambogic acid-induced depletion of Hsp90-dependent proteins

Treatment of cultured cells with known Hsp90 inhibitors deplete the cells of Hsp90-dependent proteins in a time and concentration dependent manner. To further characterize gambogic acid as a potential Hsp90 inhibitor, MCF7 and Sk-Br3 cells were treated with varying concentration of gambogic acid for 36 hr, and equivalent amounts of protein from cell extracts were Western blotted for Hsp70 and Hsp90, and the Hsp90-dependent proteins Her2, Akt, and Raf-1, using actin as a loading control and geldanamycin as a positive control for Hsp90-inhibition. Gambogic acid was observed to deplete MCF7 and Sk-Br3 cells of Her2, Akt and Raf-1 in a concentration dependent fashion (Fig. 4.3), that correlated well with the IC_{50} value for gambogic acid-induced inhibition of the proliferation of these cell lines. In addition, gambogic acid induced Hsp90 and Hsp70 expression, another hallmark of Hsp90-inhibition. Gambogic acid had a similar effect on the levels of Her2, Raf-1 and Akt in HeLa cells (not shown). These results further support the hypothesis that the anti-proliferative effect of gambogic acid on cancer cell growth is mediated, at least in part, by its ability to inhibit Hsp90.

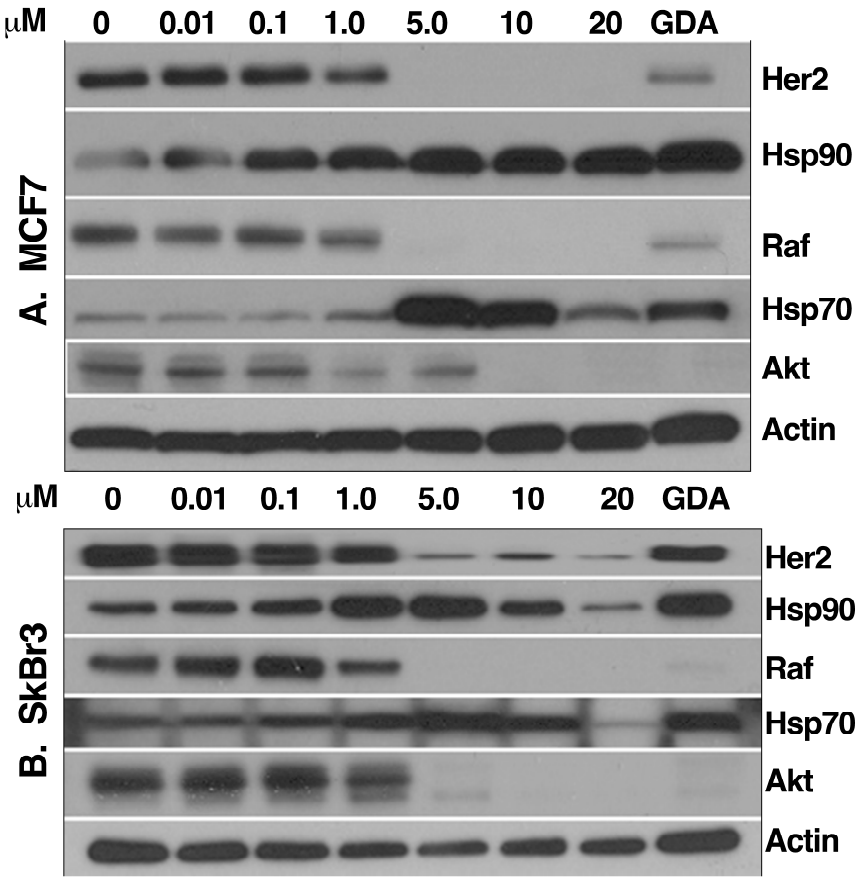


Figure 4.3. Gambogic acid-induced degradation of Hsp90 client proteins. Gambogic acid was incubated with (A) MCF7 and (B) SkBr3 breast cancer cells at concentrations, (μM) indicated in the figure. was evaluated for its ability to downregulate several client proteins as described in the methods section. Geldanamycin (500 nM) and DMSO were used as positive and negative controls, resepctively. Cell extracts were prepared and equivalent amounts of protein were Western blotted for the indicated proteins as described under "Experimental Procedures". Data generated by Laura Peterson.

Effect of gambogic acid on the association of Hsp90 chaperone components with HRI and HRI's Hsp90-dependent maturation

HRI is a heme-regulated eIF2α kinase that requires Hsp90, Hsp70 and Cdc37 for its maturation and activation under heme-deficient conditions [41, 229, 244]. To further characterize the mechanism of action of gambogic acid we compared the effects of gambogic acid on the binding of Hsp90 chaperone components to HRI, to the effects of geldanamycin and celastrol, which bind to Hsp90's N-terminal domain, and novobiocin and coumermycin A1, which inhibit Hsp90 by binding to its C-terminal domain. The non-activatable K199R mutant of HRI was used in these experiments as it is unable to mature and interacts constitutively with Hsp90. In these experiments, the immunadsorbed samples were washed with low ionic strength buffer, as it distinguishes between the mechanism of action of geladanmycin and celastrol, which bind to different sites in Hsp90's N-terminal domain. As shown in Figure 4.4A (lane 3), geldanamycin causes the accumulation of Hsp90 in complexes with client proteins containing intermediate components of the Hsp90 reaction cycle, reproducibly increasing the amount of Hsp70 co-adsorbed with HRI/K199R compared to the DMSO control. When immunoadsorptions from geldanamycin treated lysate are washed with low ionic strength buffers, Hsp90 remains bound to kinase clients (here HRI/K199R), but the binding of Cdc37 is disrupted [245]. In the presence of molybdate, which stabilizes "late" Hsp90 complexes, the binding of Hsp90 and Cdc37 to HRI/K199R was enhanced, and the binding of Hsp70 was unaffected (lane 4). In contrast, under similar condition celastrol disrupts the binding of Hsp90, Hsp70 and Cdc37 to HRI (Fig. 4.4A, lane 5). As previously reported, novobiocin also disrupted the interaction of Hsp90, Hsp70 and Cdc37 with HRI/K199R, as did the bivalent novobiocin related compound coumermycin A1 (Fig. 4.4A, lanes 6 & 7). Gambogic acid similarly blocked the binding of Hsp90, Hsp70 and Cdc37 (lane 8) to HRI/K199R indicating that it affected the interaction of Hsp90 chaperone components in a manner distinct from geldanamycin.

Subsequently, we examined the effects of gambogic acid on HRI activation upon incubation in heme-deficient lysate. Hsp90-dependent maturation and activation of HRI is accompanied by a change in its electrophoretic mobility to a more slowly migrating species (Fig. 4.4B). After 40 min of incubation of HRI in heme-deficient lysate, approximately half of the [^{35}S]HRI had a slower electrophoretic mobility upon SDS-PAGE analysis compared to HRI incubated in heme-replete lysate (lane 2 vs 1). Gambogic acid inhibited the maturation/ activation of HRI in heme-deficient lysate to an extent similar to the known Hsp90 inhibitors geldanamycin, celastrol, and molybdate. The C-terminal Hsp90 inhibitors novobiocin and coumermycin A1 also inhibited the maruration/ activation of HRI, but it was also apparent that these compounds reduced the quantity of [^{35}S]HRI that was present in the samples.

To determine whether the decreased recovery of [^{35}S]HRI was due to less [^{35}S]HRI being present, aliquots were taken from the samples prior to the beginning of the maturational incubation. Autoradiography of aliquots taken from samples prior to their further incubation for 45 min indicated that an equivalent amount of [^{35}S]HRI was present in each sample (Fig. 4.4C, 0 min: upper panel). Incubation of [^{35}S]HRI in the presence of novobiocin or coumermycin A1 resulted in a greater than 90% loss of the [^{35}S]HRI (Fig. 4.4C, lanes 6 & 7). However, significantly less [^{35}S]HRI was lost upon incubation of samples in the presence of geldanamycin, celastrol, or gambogic acid compared to the DMSO control. A similar effect of novobiocin and coumermycin A1 has been observed on stability of Akt generated by TnT in reticulocyte lysate (not shown). Thus, the absence of an effect of gambogic acid on HRI stability distinguishes its mechanism of action from that of C-terminal inhibitors. In addition, the effect of Hsp90 inhibition on the stability of nascent kinases in reticulocyte lysate appears to be a property that further distinguishes inhibitors of Hsp90 that bind to its C-terminal domain from inhibitors the bind to its N-terminal domain.

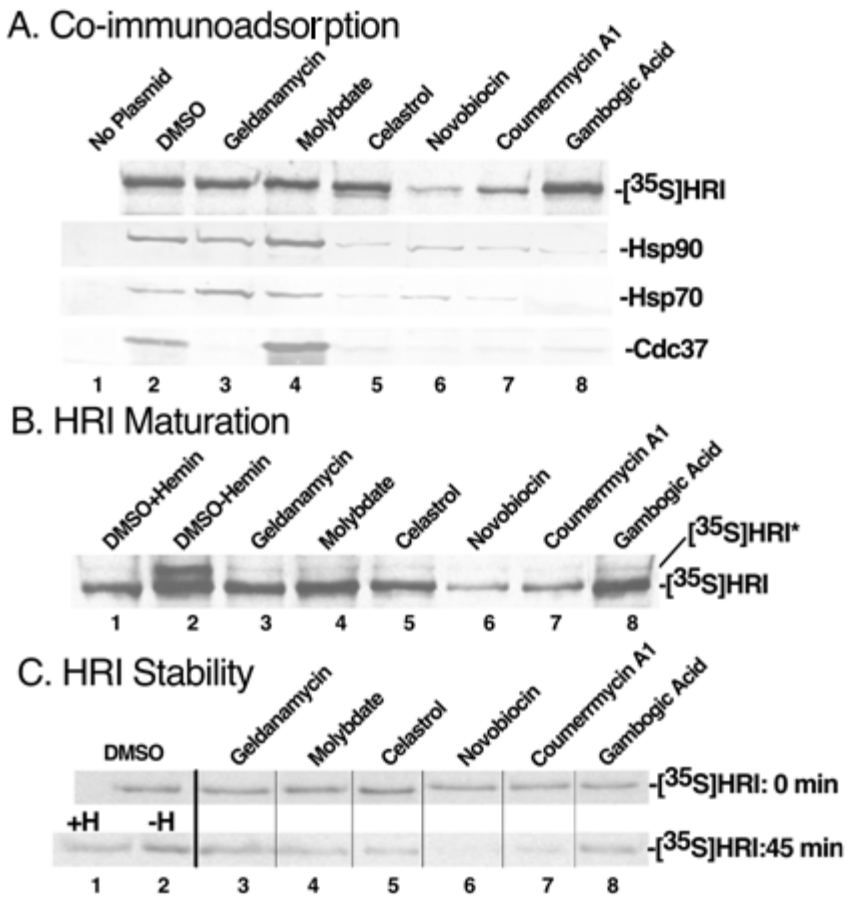


Figure 4.4. Effect of Hsp90 inhibitors on the interaction of Hsp90 and its co-chaperones with HRI (A), on HRI's Hsp90-dependent maturation (B), and on HRI stability (C). **A.** [^{35}S]His-tagged HRI/K199R was synthesized by TnT in reticulocyte lysate as described under "Materials and Methods". After 10 min, DMSO (4% v/v, lanes 1 & 2), geldanamycin (80 uM, lane 3), sodium molybdate (20 mM, lane 4), celastrol (100 uM, lane 5), novobiocin (4.0 mM, lane 6), coumermycin A1 (400 uM, lane 7), or gambogic acid (50 uM, lane 8) were added followed by an additional 40 min of incubation. [^{35}S]His-tagged HRI/K199R was then immunoadsorbed with anti-His antibodies and samples were analyzed for co-adsorbing Hsp90, Hsp70, and Cdc37 by SDS-PAGE and Western blotting. Lane 1: TnT lysate containing no plasmid as the control for non-specific binding. Top panel: autoradiogram of immunoadsorbed [^{35}S]HRI/K199R. **B.** [^{35}S]His-tagged HRI was synthesized in reticulocyte lysate as described under "Materials and Methods". After 20 min, DMSO (4% v/v, lanes 1, 2 & 3), geldanamycin (80 uM, lane 4), sodium molybdate (20 mM, lane 5), celastrol (100 uM, lane 6), novobiocin (4.0 mM, lane 7), coumermycin A1 (400 uM, lane 8), or gambogic acid (50 uM, lane 9) were added followed by an additional 10 of incubation. An aliquot of the TnT lysate was then transferred to hemin supplemented (20 uM, lane 2) or heme-deficient (lanes 1 & 3-12) lysate containing and equivalent concentration of each addition, followed by an additional 45 min of incubation. The samples were then analyzed for HRI maturation by SDS-PAGE and autoradiography as described under " Materials and Methods". Lane 1: TnT lysate containing no plasmid. [^{35}S]HRI*: mature, active HRI. C. [^{35}S]His-tagged HRI was synthesized in reticulocyte lysate and treated with DMSO or Hsp90-inhibitors as described above. Aliquots of each reaction were taken prior to (upper panel, 0 min) and 45 min after (lower panel, 45 min) dilution into and incubation in heme-

supplemented (lane1) or heme-deficient (lanes 2-8).

Surface plasmon renonance (SPR) analysis of the binding of gambogic acid to Hsp90's N-terminal domain

To determine whether gambogic acid was indeed interacting with Hsp90's N-terminal domain, we cloned the N-terminal domain of Hsp90α (Hsp90NT; amino acids 1-241) with a 7 amino acid extension at its C-terminus containing a C-terminal Cys residue. Recombinant Hsp90NT was biotinylated and immobilized onto a neutravidin sensor chip for analysis of gambogic acid binding by surface plasmon resonance spectroscopy (SPR). Full length Hsp90 and the C-terminal domain of Hsp90 (Hsp90CT, amino acids 531-732) were immobilized on sensor chips which were used as positive and negative controls, respectively. Gambogic acid was observed to bind specifically to full length Hsp90 (Fig. 5A) and Hsp90NT (Fig. 5B), but not to Hsp90CT (not shown). Kinetic analysis of the binding and dissociation kinetics indicated that gambogic acid bound to both Hsp90 and Hsp90NT with similar association constants (k_a), dissociation constants (k_d) and calculated Kd (9.8 versus 7.6 uM, respectively: Table 1). Gambogic acid was also observed to bind Hsp90NT in the presence of 20 uM geldanamycin in the analyte buffer, with the geldanmycin having no significant effect on the measured k_a and k_d or the calculated K_d (Table 1). Thus, the data indicate, that gambogic acid binds to the N-terminal domain of Hsp90, and like celastrol [201], it binds to a site distinct from the ATP binding pocket. In addition, the K_d for gambogic acid binding to Hsp90 correlated well with the IC_{50}s for gambogic acid-induced inhibition of other Hsp90-dependent processes, further supporting Hsp90 as one of gambogic acid's physiological targets.

Table 4.1. Constants for the binding of Gambogic Acid to Hsp90

Protein	k_a (M^{-1} S^{-1})	k_d (S^{-1})	K_D (μM)
Full length Hsp90	1.16(8)e^3	0.0113(4)	9.8(2)
Hsp90NT	1.47(6)e^3	0.01122(7)	7.6(3)
Hsp90NT (+20 □M GA)	1.62(8)e^3	0.0114(7)	7.0(4)
Hsp90CT	No Binding		

Figure 4.5. SPR analysis of the interaction of gambogic acid with (A) full length Hsp90 and (B) the N-terminal domain of Hsp90. A. Injection of 1.0, 10, 25 and 50 uM gambogic acid over a SPR chip containing bound full length Hsp90. B. Injection of 0.5, 5, 15 and 25 uM gambogic acid over a SPR chip containing bound Hsp90NT. Black line: sensorgram of binding and dissociation; dotted gray line: curve fit. Data generated by Jacob Manjarrez.

Virtual docking of gambogic acid to Hsp90NT

We approached the problem of identifying where gambogic acid putatively binds to the HSP90 N-terminal domain by using the "blind" docking method [246] in which the entire HSP90 N-terminal domain not just the geldanamycin binding site was used to search for the lowest possible binding energy for gambogic acid. The apo- and geldanamycin-bound Hsp90NT crystal structure, PDB ID 1YET, along with the open or unbound crystal structure of the Hsp90NT, PDB ID 1YES, were used for docking of gambogic acid to determine if it would compete for the geldanamycin binding site or bind elsewhere. These two structures share a 0.25Å RMSD of the Cα backbone spanning the entire 221 residues of the crystal structures with the majority of conformational changes occurring in residues 105-112 [234]. In all three docking experiments, Gambogic acid preferentially bound to the hydrophobic cleft created by α-helix 9 (H9) and β-sheet strand 8 (S8), residues 200-222. The calculated average binding affinities for Gambogic acid for this region were -9.44, -9.50 and -9.37 kcal/mol for the apo, geldanamycin-bound and open structures, respectively. Docking results predict aliphatic hydrophobic interactions would occur between the gambogic acid substituent arms and the aliphatic side chains of H9, while the xanthenone moiety would lie on the Cα backbone of S8 making potential pi interaction with the carbonyl and amide bonds. Additionally, a salt bridge is formed between K208 and a gambogic acid carboxyl group, and K284 forms a hydrogen bond with another gambogic acid carboxyl group. These interactions stabilize the binding of gambogic acid to Hsp90 (Fig. 4.6). The regions of H9 and S8 undergo minor conformational changes with a 0.15Å RMSD of the Cα residues between residues 200-222.

Our docking results are consistent with gambogic acid being a non-competitive inhibitor of the Hsp90 N-terminal domain. The region of H9 and S8 of the HSP90 N-terminal domain undergoes little change between the geldanamycin-bound structure and the open conformation structure with a 0.15Å RMSD of the Cα backbone of residues 200-222.

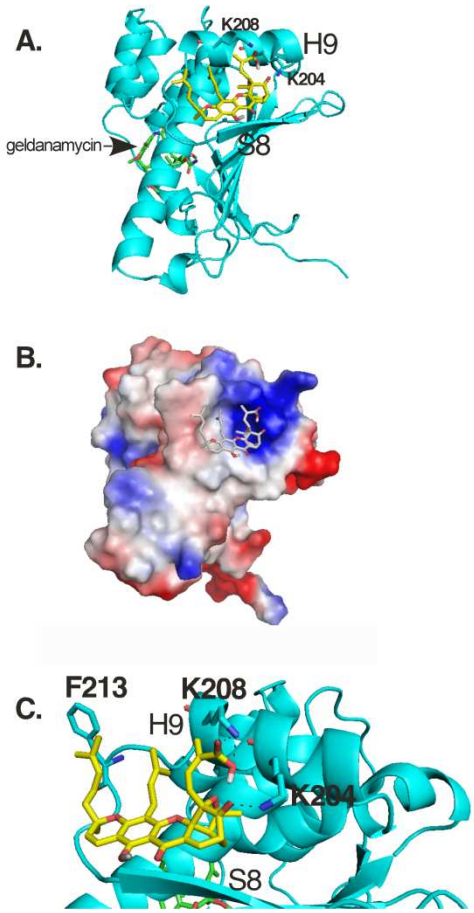


Figure 4.6. Models of gambogic acid docked to geldanamycin-bound Hsp90NT. A. Ribbon diagram of Hsp90NT with the carbons of **1** and geldanamycin shown in yellow and green, respectively. B. Electrostatic surface potential of Hsp90NT shown in the same orientation as in A. C. Close-up showing the salt bridge and H-bond formed between K208 and K204 with gambogic acid.

In this study we present data characterizing the effects of two inhibitors of the Hsp90 chaperone complex on the complex itself, as well as on cancer cells. Gambogic acid has demonstrated biological activities relevant to the treatment of cancer and other diseases. These activities were mediated by its effect on known Hsp90 client proteins, including, but not limited to, induction of cell cycle arrest and inhibition of migration in vascular smooth muscle cells by blocking the activity of the platelet derived growth factor receptor and the G protein rac1 [247], suppression of STAT3 activation and induction of apoptosis in multiple myeloma cells [248], inhibition of TNFα-induced prostate cancer cell invasion via blockade of the Akt pathway [249], and induction of mutant p53 degradation in breast cancer cells [250]. Despite the large number of reports on gambogic acid's activities, it has only recently been identified as an inhibitor of Hsp90. We have demonstrated its cytotoxicity to cancer cells, its ability to inhibit the Hsp90-mediated refolding of thermally denatured luciferase, its direct binding to Hsp90, and its effects on the interaction of Hsp90 with its co-chaperones and a nascent client protein. In the study of gambogic acid's effects on the Hsp90 complex in the chaperoning of HRI, we also examined the activity of another relatively new Hsp90 inhibitor, celastrol. This compound, which has been studied in an Hsp90 context more thoroughly, had similar effects on the chaperone-co-chaperone-kinase complex to those of gambogic acid. Both compounds abrogated the interaction of HRI with Hsp90, as well as the co-chaperones Hsp70 and Cdc37. In this way, the compounds act in a manner similar to that of the C-terminal inhibitors novobiocin and coumermycin A1. Unlike these compounds, however, celastrol and gambogic acid did not cause the degradation of the client protein after a 45 minute incubation. It might be the case that celastrol and gambogic acid have identical binding mechanism, causing the same changes in the Hsp90 complex. However, additional biophysical studies with celastrol will be needed to make a determination.

CHAPTER V

CONCLUSION AND FUTURE DIRECTIONS

Summary

In this work, we have demonstrated two over-arching concepts. First, we have shown that natural products are rich sources of bioactive compounds, many of which are likely inhibitors of the Hsp90 chaperone complex. Second, we have demonstrated the utility of a luciferase-based assay for high-throughput screening to identify such compounds, and for approximating their affinity for the Hsp90 complex.

Our high-throughput screen consisted of four natural compound libraries from commercially available sources. The total number of compounds screened was approximately 3800. The number of hits, defined as those compounds that inhibited luciferase refolding by 50%, was 95. This represents a 2.5% hit rate for the compounds. Other high-throughput screens are being carried out in the search for Hsp90 inhibitors. Not only are the methods used varied, but the libraries, as well. Some of the screens make use of chemical libraries that contain many synthetic compounds. In one study, 21,000 molecules targeted as ATP analogs were screened for their ability to bind Hsp82, the yeast homolog of Hsp90. This screen yielded two hits, a <.01% rate. [251]. Another screen, which was somewhat more successful in identifying potential Hsp90 inhibitors, used a collection of compounds that consisted of natural compounds, various drugs already in use, and known pharmacologically active compounds. This screen produced 46

compounds out of 4000 screened, a >1% rate. Additionally, they were able to determine specificity for binding of the *Plasmodium falciparum* Hsp90, versus human Hsp90, which narrowed the hits to three [252]. Our lab has previously carried out a high-throughput screen of 20,000 compound chemical library at the University of Kansas High Throughput Screening Laboratory, using the luciferase refolding assay to identify hits as we have in this study. A hit rate of 0.6 % (120 compounds) was observed.

In our own high-throughput screen, there were a number of compounds that have no designation other than a catalog number. These compounds have not been characterized or studied in any way, other than the elucidation of their structure. This strengthens the case for further investigation into compounds from natural sources, as they could represent a large, untapped reservoir of potential therapeutics. There are numerous plants in traditional medicinal use all over the world whose extracts have been shown to have medicinal value, and whose chemical compositions have not been determined. More relevant to our study, many of these specifically have anti-cancer activity. A few examples: *Lindera obtusiloba* from China and Korea, inhibits proliferation and induces apoptosis in hepatocellular carcinoma [253]. *Trametes robiniophila* from China inhibits the proliferation of umbilical vein endothelial cells and mammary tumor cells [254]. *Equisetum hyemale*, found everywhere but Australia and New Zealand, was cytotoxic to mouse leukemia cells [255]. Ethanol extract of *Hedyotis diffusa* from China induced cell cycle arrest in human colon carcinoma cells [256]. A traditional Chinese formula containing glossy privet fruit and the herbs *Carapax trionycis* and *Polygonum cuspidatum* was shown to induce apoptosis in human hepatocarcinoma cells [257]. A study of Mayan botanical literature found ten species that displayed some cytotoxic activity to at least one human cancer cell line each. The most potent extract was the root bark from *Aeschynomene fascicularis*. Others included *Bonellia macrocarpa*, *Casearia corymbosa*, and *Alvaradoa amorphoides* [258]. The flavonoid-containing fraction from *Saraca asoca*, used in traditional Indian medicine, applied topically, was effective

in reducing chemically induced tumor formation [259]. Certain extracts of the seeds of *Glinus lotoides*, used in Asia and Africa, showed apoptotic cytotoxicity against multiple human carcinoma cell lines [260].

These examples are only a small fraction of the plants and other natural sources from which medical traditions throughout the world derive therapeutics. Many of these have only been characterized by simple solvent extractions. Some have not been fractionated at all.

Taken together, the results of our screen, the results of other screens using different types of compound libraries, and the ongoing work being done with natural products all support our hypothesis that natural compound sources are more likely to yield Hsp90 inhibitors than typical chemical sources, and point to these natural sources as viable targets for the continued pursuit of improved inhibitors of Hsp90 that are likely to yield large numbers of candidate compounds.

This template is best used for directly typing in your content. However, you can paste text into the document, but use caution as pasting can produce varying results.

REFERENCES

1. Wachtel-Galor, S. and I.F.F. Benzie, *Herbal Medicine: An Introduction to Its History, Usage, Regulation, Current Trends, and Research Needs.* 2011.
2. Ichikawa, H., et al., *Anticancer drugs designed by mother nature: ancient drugs but modern targets.* Curr Pharm Des, 2007. **13**(33): p. 3400-16.
3. Whitesell, L. and S.L. Lindquist, *HSP90 and the chaperoning of cancer.* Nat Rev Cancer, 2005. **5**(10): p. 761-72.
4. Chen, B., D. Zhong, and A. Monteiro, *Comparative genomics and evolution of the HSP90 family of genes across all kingdoms of organisms.* BMC Genomics, 2006. **7**: p. 156.
5. Taipale, M., D.F. Jarosz, and S. Lindquist, *HSP90 at the hub of protein homeostasis: emerging mechanistic insights.* Nat Rev Mol Cell Biol, 2010. **11**(7): p. 515-28.
6. Picard, D., *Hsp90 Interactors.* 2012.
7. Fan, A.C., et al., *Interaction between the human mitochondrial import receptors Tom20 and Tom70 in vitro suggests a chaperone displacement mechanism.* J Biol Chem, 2011. **286**(37): p. 32208-19.
8. Bansal, P.K., R. Abdulle, and K. Kitagawa, *Sgt1 associates with Hsp90: an initial step of assembly of the core kinetochore complex.* Mol Cell Biol, 2004. **24**(18): p. 8069-79.
9. Morimoto, R.I., et al., *The heat-shock response: regulation and function of heat-shock proteins and molecular chaperones.* Essays Biochem, 1997. **32**: p. 17-29.
10. Ferrarini, M., et al., *Unusual expression and localization of heat-shock proteins in human tumor cells.* Int J Cancer, 1992. **51**(4): p. 613-9.
11. Harris, S.F., A.K. Shiau, and D.A. Agard, *The crystal structure of the carboxy-terminal dimerization domain of htpG, the Escherichia coli Hsp90, reveals a potential substrate binding site.* Structure, 2004. **12**(6): p. 1087-97.
12. Minami, Y., et al., *The carboxy-terminal region of mammalian HSP90 is required for its dimerization and function in vivo.* Mol Cell Biol, 1994. **14**(2): p. 1459-64.
13. Ratzke, C., et al., *Dynamics of heat shock protein 90 C-terminal dimerization is an important part of its conformational cycle.* Proc Natl Acad Sci U S A, 2010. **107**(37): p. 16101-6.
14. Pearl, L.H. and C. Prodromou, *Structure and mechanism of the hsp90 molecular chaperone machinery.* Annu Rev Biochem, 2006. **75**: p. 271-94.
15. Mayer, M.P., C. Prodromou, and J. Frydman, *The Hsp90 mosaic: a picture emerges.* Nat Struct Mol Biol, 2009. **16**(1): p. 2-6.
16. Csermely, P., et al., *ATP induces a conformational change of the 90-kDa heat shock protein (hsp90).* J Biol Chem, 1993. **268**(3): p. 1901-7.
17. Grenert, J.P., et al., *The amino-terminal domain of heat shock protein 90 (hsp90) that binds geldanamycin is an ATP/ADP switch domain that regulates hsp90 conformation.* J Biol Chem, 1997. **272**(38): p. 23843-50.
18. Sullivan, W., et al., *Nucleotides and two functional states of hsp90.* J Biol Chem, 1997. **272**(12): p. 8007-12.

1. Wachtel-Galor, S. and I.F.F. Benzie, *Herbal Medicine: An Introduction to Its History, Usage, Regulation, Current Trends, and Research Needs.* 2011.
2. Ichikawa, H., et al., *Anticancer drugs designed by mother nature: ancient drugs but modern targets.* Curr Pharm Des, 2007. **13**(33): p. 3400-16.
3. Whitesell, L. and S.L. Lindquist, *HSP90 and the chaperoning of cancer.* Nat Rev Cancer, 2005. **5**(10): p. 761-72.

4. Chen, B., D. Zhong, and A. Monteiro, *Comparative genomics and evolution of the HSP90 family of genes across all kingdoms of organisms.* BMC Genomics, 2006. **7**: p. 156.
5. Taipale, M., D.F. Jarosz, and S. Lindquist, *HSP90 at the hub of protein homeostasis: emerging mechanistic insights.* Nat Rev Mol Cell Biol, 2010. **11**(7): p. 515-28.
6. Picard, D., *Hsp90 Interactors.* 2012.
7. Fan, A.C., et al., *Interaction between the human mitochondrial import receptors Tom20 and Tom70 in vitro suggests a chaperone displacement mechanism.* J Biol Chem, 2011. **286**(37): p. 32208-19.
8. Bansal, P.K., R. Abdulle, and K. Kitagawa, *Sgt1 associates with Hsp90: an initial step of assembly of the core kinetochore complex.* Mol Cell Biol, 2004. **24**(18): p. 8069-79.
9. Morimoto, R.I., et al., *The heat-shock response: regulation and function of heat-shock proteins and molecular chaperones.* Essays Biochem, 1997. **32**: p. 17-29.
10. Ferrarini, M., et al., *Unusual expression and localization of heat-shock proteins in human tumor cells.* Int J Cancer, 1992. **51**(4): p. 613-9.
11. Harris, S.F., A.K. Shiau, and D.A. Agard, *The crystal structure of the carboxy-terminal dimerization domain of htpG, the Escherichia coli Hsp90, reveals a potential substrate binding site.* Structure, 2004. **12**(6): p. 1087-97.
12. Minami, Y., et al., *The carboxy-terminal region of mammalian HSP90 is required for its dimerization and function in vivo.* Mol Cell Biol, 1994. **14**(2): p. 1459-64.
13. Ratzke, C., et al., *Dynamics of heat shock protein 90 C-terminal dimerization is an important part of its conformational cycle.* Proc Natl Acad Sci U S A, 2010. **107**(37): p. 16101-6.
14. Pearl, L.H. and C. Prodromou, *Structure and mechanism of the hsp90 molecular chaperone machinery.* Annu Rev Biochem, 2006. **75**: p. 271-94.
15. Mayer, M.P., C. Prodromou, and J. Frydman, *The Hsp90 mosaic: a picture emerges.* Nat Struct Mol Biol, 2009. **16**(1): p. 2-6.
16. Csermely, P., et al., *ATP induces a conformational change of the 90-kDa heat shock protein (hsp90).* J Biol Chem, 1993. **268**(3): p. 1901-7.
17. Grenert, J.P., et al., *The amino-terminal domain of heat shock protein 90 (hsp90) that binds geldanamycin is an ATP/ADP switch domain that regulates hsp90 conformation.* J Biol Chem, 1997. **272**(38): p. 23843-50.
18. Sullivan, W., et al., *Nucleotides and two functional states of hsp90.* J Biol Chem, 1997. **272**(12): p. 8007-12.
19. Southworth, D.R. and D.A. Agard, *Species-dependent ensembles of conserved conformational states define the Hsp90 chaperone ATPase cycle.* Mol Cell, 2008. **32**(5): p. 631-40.
20. Prodromou, C., et al., *The ATPase cycle of Hsp90 drives a molecular 'clamp' via transient dimerization of the N-terminal domains.* EMBO J, 2000. **19**(16): p. 4383-92.
21. Shiau, A.K., et al., *Structural Analysis of E. coli hsp90 reveals dramatic nucleotide-dependent conformational rearrangements.* Cell, 2006. **127**(2): p. 329-40.
22. Liu, T., C.K. Daniels, and S. Cao, *Comprehensive review on the HSC70 functions, interactions with related molecules and involvement in clinical diseases and therapeutic potential.* Pharmacol Ther, 2012. **136**(3): p. 354-74.
23. Prodromou, C., et al., *Regulation of Hsp90 ATPase activity by tetratricopeptide repeat (TPR)-domain co-chaperones.* EMBO J, 1999. **18**(3): p. 754-62.
24. Kampinga, H.H. and E.A. Craig, *The HSP70 chaperone machinery: J proteins as drivers of functional specificity.* Nat Rev Mol Cell Biol, 2010. **11**(8): p. 579-92.
25. Grenert, J.P., B.D. Johnson, and D.O. Toft, *The importance of ATP binding and hydrolysis by hsp90 in formation and function of protein heterocomplexes.* J Biol Chem, 1999. **274**(25): p. 17525-33.

26. Johnson, J.L. and D.O. Toft, *Binding of p23 and hsp90 during assembly with the progesterone receptor.* Mol Endocrinol, 1995. **9**(6): p. 670-8.
27. MacLean, M. and D. Picard, *Cdc37 goes beyond Hsp90 and kinases.* Cell Stress Chaperones, 2003. **8**(2): p. 114-9.
28. Siligardi, G., et al., *Regulation of Hsp90 ATPase activity by the co-chaperone Cdc37p/p50cdc37.* J Biol Chem, 2002. **277**(23): p. 20151-9.
29. Roe, S.M., et al., *The Mechanism of Hsp90 regulation by the protein kinase-specific cochaperone p50(cdc37).* Cell, 2004. **116**(1): p. 87-98.
30. Panaretou, B., et al., *Activation of the ATPase activity of hsp90 by the stress-regulated cochaperone aha1.* Mol Cell, 2002. **10**(6): p. 1307-18.
31. Holmes, J.L., et al., *Silencing of HSP90 cochaperone AHA1 expression decreases client protein activation and increases cellular sensitivity to the HSP90 inhibitor 17-allylamino-17-demethoxygeldanamycin.* Cancer Res, 2008. **68**(4): p. 1188-97.
32. Hanahan, D. and R.A. Weinberg, *Hallmarks of cancer: the next generation.* Cell, 2011. **144**(5): p. 646-74.
33. da Silva, V.C. and C.H. Ramos, *The network interaction of the human cytosolic 90kDa heat shock protein Hsp90: A target for cancer therapeutics.* J Proteomics, 2012. **75**(10): p. 2790-802.
34. Kang, B.H., et al., *Regulation of tumor cell mitochondrial homeostasis by an organelle-specific Hsp90 chaperone network.* Cell, 2007. **131**(2): p. 257-70.
35. Holt, S.E., et al., *Functional requirement of p23 and Hsp90 in telomerase complexes.* Genes Dev, 1999. **13**(7): p. 817-26.
36. DeZwaan, D.C., et al., *The Hsp82 molecular chaperone promotes a switch between unextendable and extendable telomere states.* Nat Struct Mol Biol, 2009. **16**(7): p. 711-6.
37. Minet, E., et al., *Hypoxia-induced activation of HIF-1: role of HIF-1alpha-Hsp90 interaction.* FEBS Lett, 1999. **460**(2): p. 251-6.
38. Chen, J.J. and I.M. London, *Regulation of protein synthesis by heme-regulated eIF-2 alpha kinase.* Trends Biochem Sci, 1995. **20**(3): p. 105-8.
39. Matts, R.L., R. Hurst, and Z. Xu, *Denatured proteins inhibit translation in hemin-supplemented rabbit reticulocyte lysate by inducing the activation of the heme-regulated eIF-2 alpha kinase.* Biochemistry, 1993. **32**(29): p. 7323-8.
40. Yerlikaya, A., S.R. Kimball, and B.A. Stanley, *Phosphorylation of eIF2alpha in response to 26S proteasome inhibition is mediated by the haem-regulated inhibitor (HRI) kinase.* Biochem J, 2008. **412**(3): p. 579-88.
41. Uma, S., et al., *Hsp90 is obligatory for the heme-regulated eIF-2alpha kinase to acquire and maintain an activable conformation.* J Biol Chem, 1997. **272**(17): p. 11648-56.
42. Basso, A.D., et al., *Akt forms an intracellular complex with heat shock protein 90 (Hsp90) and Cdc37 and is destabilized by inhibitors of Hsp90 function.* J Biol Chem, 2002. **277**(42): p. 39858-66.
43. Eustace, B.K., et al., *Functional proteomic screens reveal an essential extracellular role for hsp90 alpha in cancer cell invasiveness.* Nat Cell Biol, 2004. **6**(6): p. 507-14.
44. Wartmann, M. and R.J. Davis, *The native structure of the activated Raf protein kinase is a membrane-bound multi-subunit complex.* J Biol Chem, 1994. **269**(9): p. 6695-701.
45. Blagosklonny, M.V., et al., *Mutant conformation of p53 translated in vitro or in vivo requires functional HSP90.* Proc Natl Acad Sci U S A, 1996. **93**(16): p. 8379-83.
46. Sepehrnia, B., et al., *Heat shock protein 84 forms a complex with mutant p53 protein predominantly within a cytoplasmic compartment of the cell.* J Biol Chem, 1996. **271**(25): p. 15084-90.
47. Delmotte, P. and J. Delmotte-Plaque, *A new antifungal substance of fungal origin.* Nature, 1953. **171**(4347): p. 344.

48. Kwon, H.J., et al., *Potent and specific inhibition of p60v-src protein kinase both in vivo and in vitro by radicicol.* Cancer Res, 1992. **52**(24): p. 6926-30.
49. Furusaki, A., et al., *Herbimycin A: an ansamycin antibiotic; X-ray crystal structure.* J Antibiot (Tokyo), 1980. **33**(7): p. 781-2.
50. Besant, P.G., et al., *Inhibition of branched-chain alpha-keto acid dehydrogenase kinase and Sln1 yeast histidine kinase by the antifungal antibiotic radicicol.* Mol Pharmacol, 2002. **62**(2): p. 289-96.
51. Guarnieri, M.T., et al., *The Hsp90 inhibitor radicicol interacts with the ATP-binding pocket of bacterial sensor kinase PhoQ.* J Mol Biol, 2008. **379**(1): p. 82-93.
52. Gellert, M., et al., *Novobiocin and coumermycin inhibit DNA supercoiling catalyzed by DNA gyrase.* Proc Natl Acad Sci U S A, 1976. **73**(12): p. 4474-8.
53. Dutta, R. and M. Inouye, *GHKL, an emergent ATPase/kinase superfamily.* Trends Biochem Sci, 2000. **25**(1): p. 24-8.
54. DeBoer, C., et al., *Geldanamycin, a new antibiotic.* J Antibiot (Tokyo), 1970. **23**(9): p. 442-7.
55. Tong, W.G., et al., *The synthetic heat shock protein 90 (Hsp90) inhibitor EC141 induces degradation of Bcr-Abl p190 protein and apoptosis of Ph-positive acute lymphoblastic leukemia cells.* Invest New Drugs, 2011. **29**(6): p. 1206-12.
56. Radanyi, C., et al., *Antiproliferative and apoptotic activities of tosylcyclonovobiocic acids as potent heat shock protein 90 inhibitors in human cancer cells.* Cancer Lett, 2009. **274**(1): p. 88-94.
57. Whitesell, L., et al., *Benzoquinonoid ansamycins possess selective tumoricidal activity unrelated to src kinase inhibition.* Cancer Res, 1992. **52**(7): p. 1721-8.
58. Zhang, J., et al., *The heat shock protein 90 inhibitor 17-AAG suppresses growth and induces apoptosis in human cholangiocarcinoma cells.* Clin Exp Med, 2012.
59. Camphausen, K. and P.J. Tofilon, *Inhibition of Hsp90: a multitarget approach to radiosensitization.* Clin Cancer Res, 2007. **13**(15 Pt 1): p. 4326-30.
60. Roue, G., et al., *The Hsp90 inhibitor IPI-504 overcomes bortezomib resistance in mantle cell lymphoma in vitro and in vivo by down-regulation of the prosurvival ER chaperone BiP/Grp78.* Blood, 2011. **117**(4): p. 1270-9.
61. Sain, N., et al., *Potentiation of paclitaxel activity by the HSP90 inhibitor 17-allylamino-17-demethoxygeldanamycin in human ovarian carcinoma cell lines with high levels of activated AKT.* Mol Cancer Ther, 2006. **5**(5): p. 1197-208.
62. Smyth, T., et al., *The HSP90 Inhibitor, AT13387, Is Effective against Imatinib-Sensitive and -Resistant Gastrointestinal Stromal Tumor Models.* Mol Cancer Ther, 2012. **11**(8): p. 1799-808.
63. Prodromou, C., et al., *Identification and structural characterization of the ATP/ADP-binding site in the Hsp90 molecular chaperone.* Cell, 1997. **90**(1): p. 65-75.
64. Jia, W., et al., *Synergistic antileukemic interactions between 17-AAG and UCN-01 involve interruption of RAF/MEK- and AKT-related pathways.* Blood, 2003. **102**(5): p. 1824-32.
65. Kang, B.H., et al., *Targeted inhibition of mitochondrial Hsp90 suppresses localised and metastatic prostate cancer growth in a genetic mouse model of disease.* Br J Cancer, 2011. **104**(4): p. 629-34.
66. Yun, B.-G., et al., *Novobiocin induces a distinct conformation of Hsp90 and alters Hsp90-cochaperone-client interactions.* Biochemistry, 2004. **43**: p. 8217-29.
67. Faresse, N., et al., *Mineralocorticoid receptor degradation is promoted by Hsp90 inhibition and the ubiquitin-protein ligase CHIP.* Am J Physiol Renal Physiol, 2010. **299**(6): p. F1462-72.
68. Murata, S., et al., *CHIP is a chaperone-dependent E3 ligase that ubiquitylates unfolded protein.* EMBO Rep, 2001. **2**(12): p. 1133-8.

69. Ahsan, A., et al., *Wild-type EGFR is stabilized by direct interaction with HSP90 in cancer cells and tumors.* Neoplasia, 2012. **14**(8): p. 670-7.
70. Thulasiraman, V. and R.L. Matts, *Effect of geldanamycin on the kinetics of chaperone-mediated renaturation of firefly luciferase in rabbit reticulocyte lysate.* Biochemistry, 1996. **35**: p. 13443-13450.
71. Chen, G., P. Cao, and D.V. Goeddel, *TNF-induced recruitment and activation of the IKK complex require Cdc37 and Hsp90.* Mol Cell, 2002. **9**(2): p. 401-10.
72. Garcia-Cardena, G., et al., *Dynamic activation of endothelial nitric oxide synthase by Hsp90.* Nature, 1998. **392**(6678): p. 821-4.
73. Burch, A.D. and S.K. Weller, *Herpes simplex virus type 1 DNA polymerase requires the mammalian chaperone hsp90 for proper localization to the nucleus.* J Virol, 2005. **79**(16): p. 10740-9.
74. Kampmueller, K.M. and D.J. Miller, *The cellular chaperone heat shock protein 90 facilitates Flock House virus RNA replication in Drosophila cells.* J Virol, 2005. **79**(11): p. 6827-37.
75. Ghantous, A., et al., *What made sesquiterpene lactones reach cancer clinical trials?* Drug Discov Today, 2010. **15**(15-16): p. 668-78.
76. Geetha, B.S., et al., *Sesquiterpene lactones isolated from Elephantopus scaber L. inhibits human lymphocyte proliferation and the growth of tumour cell lines and induces apoptosis in vitro.* J Biomed Biotechnol, 2012. **2012**: p. 721285.
77. Duraipandiyan, V., et al., *Antimicrobial activity of sesquiterpene lactones isolated from traditional medicinal plant, Costus speciosus (Koen ex.Retz.) Sm.* BMC Complement Altern Med, 2012. **12**: p. 13.
78. Kim, Y.H., et al., *Eudesmanolides from Taraxacum mongolicum and their inhibitory effects on the production of nitric oxide.* Arch Pharm Res, 2011. **34**(1): p. 37-41.
79. Yamashita, Y., et al., *Purification and characterization of HSP-inducers from Eupatorium lindleyanum.* Biochem Pharmacol, 2012. **83**(7): p. 909-22.
80. Lin, S., et al., *Acylated iridoids with cytotoxicity from Valeriana jatamansi.* J Nat Prod, 2009. **72**(4): p. 650-5.
81. Kaur, K., et al., *Antimalarials from nature.* Bioorg Med Chem, 2009. **17**(9): p. 3229-56.
82. Hoffmann, R., et al., *Helenalin bypasses Bcl-2-mediated cell death resistance by inhibiting NF-kappaB and promoting reactive oxygen species generation.* Biochem Pharmacol, 2011. **82**(5): p. 453-63.
83. Pettit, G.R. and G.M. Cragg, *Antineoplastic agents. 32. The pseudoguaianolide helenalin.* Experientia, 1973. **29**(7): p. 781.
84. Huang, P.R., Y.M. Yeh, and T.C. Wang, *Potent inhibition of human telomerase by helenalin.* Cancer Lett, 2005. **227**(2): p. 169-74.
85. Lim, C.B., et al., *NF-kappaB p65 repression by the sesquiterpene lactone, Helenalin, contributes to the induction of autophagy cell death.* BMC Complement Altern Med, 2012. **12**: p. 93.
86. Kwok, B.H., et al., *The anti-inflammatory natural product parthenolide from the medicinal herb Feverfew directly binds to and inhibits IkappaB kinase.* Chem Biol, 2001. **8**(8): p. 759-66.
87. Carlisi, D., et al., *Parthenolide sensitizes hepatocellular carcinoma cells to TRAIL by inducing the expression of death receptors through inhibition of STAT3 activation.* J Cell Physiol, 2011. **226**(6): p. 1632-41.
88. Nakashima H, M.T., Yamamoto N, Sakagami H, Tanuma S, Hatano T, Yoshida T, Okuda T, *Inhibition of Human Immunodeficiency Viral Replication by Tannins and Related Compounds.* Antiviral Research, 1992. **18**(1): p. 91-103.
89. Zu, M., et al., *In vitro anti-influenza virus and anti-inflammatory activities of theaflavin derivatives.* Antiviral Res, 2012. **94**(3): p. 217-24.

90. Halder, B., S. Das Gupta, and A. Gomes, *Black tea polyphenols induce human leukemic cell cycle arrest by inhibiting Akt signaling: possible involvement of Hsp90, Wnt/beta-catenin signaling and FOXO1.* FEBS J, 2012. **279**(16): p. 2876-91.
91. Kim, S. and Y.E. Joo, *Theaflavin Inhibits LPS-Induced IL-6, MCP-1, and ICAM-1 Expression in Bone Marrow-Derived Macrophages Through the Blockade of NF-kappaB and MAPK Signaling Pathways.* Chonnam Med J, 2011. **47**(2): p. 104-10.
92. Tereschuk, M.L., et al., *Antimicrobial activity of flavonoids from leaves of Tagetes minuta.* J Ethnopharmacol, 1997. **56**(3): p. 227-32.
93. Ikigai, H., et al., *Bactericidal catechins damage the lipid bilayer.* Biochim Biophys Acta, 1993. **1147**(1): p. 132-6.
94. Yin, Z., E.C. Henry, and T.A. Gasiewicz, *(-)-Epigallocatechin-3-gallate is a novel Hsp90 inhibitor.* Biochemistry, 2009. **48**(2): p. 336-45.
95. Cushnie, T.P. and A.J. Lamb, *Antimicrobial activity of flavonoids.* Int J Antimicrob Agents, 2005. **26**(5): p. 343-56.
96. Liu, L.Z., et al., *Apigenin inhibits expression of vascular endothelial growth factor and angiogenesis in human lung cancer cells: implication of chemoprevention of lung cancer.* Mol Pharmacol, 2005. **68**(3): p. 635-43.
97. Fang, J., et al., *Apigenin inhibits tumor angiogenesis through decreasing HIF-1alpha and VEGF expression.* Carcinogenesis, 2007. **28**(4): p. 858-64.
98. Kole, L., et al., *Biochanin-A, an isoflavon, showed anti-proliferative and anti-inflammatory activities through the inhibition of iNOS expression, p38-MAPK and ATF-2 phosphorylation and blocking NFkappaB nuclear translocation.* Eur J Pharmacol, 2011. **653**(1-3): p. 8-15.
99. Choi, A.Y., et al., *Luteolin induces apoptosis through endoplasmic reticulum stress and mitochondrial dysfunction in Neuro-2a mouse neuroblastoma cells.* Eur J Pharmacol, 2011. **668**(1-2): p. 115-26.
100. Xagorari, A., et al., *Luteolin inhibits an endotoxin-stimulated phosphorylation cascade and proinflammatory cytokine production in macrophages.* J Pharmacol Exp Ther, 2001. **296**(1): p. 181-7.
101. Lee, E.J., S.Y. Oh, and M.K. Sung, *Luteolin exerts anti-tumor activity through the suppression of epidermal growth factor receptor-mediated pathway in MDA-MB-231 ER-negative breast cancer cells.* Food Chem Toxicol, 2012. **50**(11): p. 4136-43.
102. Fu, J., et al., *Luteolin Induces Carcinoma Cell Apoptosis through Binding Hsp90 to Suppress Constitutive Activation of STAT3.* PLoS One, 2012. **7**(11): p. e49194.
103. Watanapokasin, R., et al., *Effects of alpha-mangostin on apoptosis induction of human colon cancer.* World J Gastroenterol, 2011. **17**(16): p. 2086-95.
104. Johnson, J.J., et al., *alpha-Mangostin, a xanthone from mangosteen fruit, promotes cell cycle arrest in prostate cancer and decreases xenograft tumor growth.* Carcinogenesis, 2012. **33**(2): p. 413-9.
105. Akao, Y., et al., *Anti-cancer effects of xanthones from pericarps of mangosteen.* Int J Mol Sci, 2008. **9**(3): p. 355-70.
106. Krenn, L., et al., *Sulfemodin 8-O-beta-D-glucoside, a new sulfated anthraquinone glycoside, and antioxidant phenolic compounds from Rheum emodi.* J Nat Prod, 2003. **66**(8): p. 1107-9.
107. Opletalova, V. and D. Sedivy, *[Chalcones and their heterocyclic analogs as potential antifungal chemotherapeutic agents].* Ceska Slov Farm, 1999. **48**(6): p. 252-5.
108. Herencia, F., et al., *Novel anti-inflammatory chalcone derivatives inhibit the induction of nitric oxide synthase and cyclooxygenase-2 in mouse peritoneal macrophages.* FEBS Lett, 1999. **453**(1-2): p. 129-34.

109. Makita, H., et al., *Chemoprevention of 4-nitroquinoline 1-oxide-induced rat oral carcinogenesis by the dietary flavonoids chalcone, 2-hydroxychalcone, and quercetin.* Cancer Res, 1996. **56**(21): p. 4904-9.
110. Casano, G., et al., *Anti-HIV and antiplasmodial activity of original flavonoid derivatives.* Bioorg Med Chem, 2010. **18**(16): p. 6012-23.
111. Yadav, V.R., et al., *The role of chalcones in suppression of NF-kappaB-mediated inflammation and cancer.* Int Immunopharmacol, 2011. **11**(3): p. 295-309.
112. Kim, M.S., et al., *Phloretin induces apoptosis in H-Ras MCF10A human breast tumor cells through the activation of p53 via JNK and p38 mitogen-activated protein kinase signaling.* Ann N Y Acad Sci, 2009. **1171**: p. 479-83.
113. Esatbeyoglu, T., et al., *Curcumin--from molecule to biological function.* Angew Chem Int Ed Engl, 2012. **51**(22): p. 5308-32.
114. Giommarelli, C., et al., *The enhancement of antiproliferative and proapoptotic activity of HDAC inhibitors by curcumin is mediated by Hsp90 inhibition.* Cell Mol Life Sci, 2010. **67**(6): p. 995-1004.
115. Belofsky, G., et al., *Phenolic metabolites of Dalea versicolor that enhance antibiotic activity against model pathogenic bacteria.* J Nat Prod, 2004. **67**(3): p. 481-4.
116. Gafner, S., et al., *Antifungal and antibacterial chalcones from Myrica serrata.* Planta Med, 1996. **62**(1): p. 67-9.
117. Friedlander, T.W., et al., *A phase II study of insulin-like growth factor receptor inhibition with nordihydroguaiaretic acid in men with non-metastatic hormone-sensitive prostate cancer.* Oncol Rep, 2012. **27**(1): p. 3-9.
118. Wang, X., et al., *Gossypol--a polyphenolic compound from cotton plant.* Adv Food Nutr Res, 2009. **58**: p. 215-63.
119. Souto, A.L., et al., *Anti-inflammatory activity of alkaloids: an update from 2000 to 2010.* Molecules, 2011. **16**(10): p. 8515-34.
120. Gibbs, B.F., *Differential modulation of IgE-dependent activation of human basophils by ambroxol and related secretolytic analogues.* Int J Immunopathol Pharmacol, 2009. **22**(4): p. 919-27.
121. Ignacimuthu, S. and N. Shanmugam, *Antimycobacterial activity of two natural alkaloids, vasicine acetate and 2-acetyl benzylamine, isolated from Indian shrub Adhatoda vasica Ness. leaves.* J Biosci, 2010. **35**(4): p. 565-70.
122. Khaliq, T., et al., *Peganine hydrochloride dihydrate an orally active antileishmanial agent.* Bioorg Med Chem Lett, 2009. **19**(9): p. 2585-6.
123. Lal, R. and P.L. Sharma, *Potentiation of prostaglandin evoked contractions of isolated rat uterus by vasicine hydrochloride.* Indian J Med Res, 1981. **73**: p. 641-8.
124. Misra, P., et al., *Antileishmanial activity mediated by apoptosis and structure-based target study of peganine hydrochloride dihydrate: an approach for rational drug design.* J Antimicrob Chemother, 2008. **62**(5): p. 998-1002.
125. Anselmi, K., et al., *Gliotoxin causes apoptosis and necrosis of rat Kupffer cells in vitro and in vivo in the absence of oxidative stress: exacerbation by caspase and serine protease inhibition.* J Hepatol, 2007. **47**(1): p. 103-13.
126. Dymock, B.W., P.S. Jones, and F.X. Wilson, *Novel approaches to the treatment of hepatitis C virus infection.* Antivir Chem Chemother, 2000. **11**(2): p. 79-96.
127. Murdock, K.C., *Antiviral agents. Chemical modifications of a disulfide antibiotic, acetylaranotin.* J Med Chem, 1974. **17**(8): p. 827-35.
128. Nicholas, G.M., et al., *Inhibition and kinetics of mycobacterium tuberculosis and mycobacterium smegmatis mycothiol-S-conjugate amidase by natural product inhibitors.* Bioorg Med Chem, 2003. **11**(4): p. 601-8.

129. Saha, B. and D. Nandi, *Farnesyltransferase inhibitors reduce Ras activation and ameliorate acetaminophen-induced liver injury in mice.* Hepatology, 2009. **50**(5): p. 1547-57.
130. Trebec-Reynolds, D.P., et al., *VEGF-A expression in osteoclasts is regulated by NF-kappaB induction of HIF-1alpha.* J Cell Biochem, 2010. **110**(2): p. 343-51.
131. Liu, R., et al., *Potent in vitro anticancer activity of metacycloprodigiosin and undecylprodigiosin from a sponge-derived actinomycete Saccharopolyspora sp. nov.* Arch Pharm Res, 2005. **28**(12): p. 1341-4.
132. Xu, J.Y., et al., *Sanguinarine inhibits growth of human cervical cancer cells through the induction of apoptosis.* Oncol Rep, 2012. **28**(6): p. 2264-70.
133. Miao, S., et al., *Proliferation-attenuating and apoptosis-inducing effects of tryptanthrin on human chronic myeloid leukemia k562 cell line in vitro.* Int J Mol Sci, 2011. **12**(6): p. 3831-45.
134. Ishihara, T., et al., *Tryptanthrin inhibits nitric oxide and prostaglandin E(2) synthesis by murine macrophages.* Eur J Pharmacol, 2000. **407**(1-2): p. 197-204.
135. Cortijo, J., et al., *Bronchodilator and anti-inflammatory activities of glaucine: In vitro studies in human airway smooth muscle and polymorphonuclear leukocytes.* Br J Pharmacol, 1999. **127**(7): p. 1641-51.
136. Boustie, J., et al., *Antipoliovirus structure-activity relationships of some aporphine alkaloids.* J Nat Prod, 1998. **61**(4): p. 480-4.
137. Spasova, M., et al., *Cinnamoyl- and hydroxycinnamoyl amides of glaucine and their antioxidative and antiviral activities.* Bioorg Med Chem, 2008. **16**(15): p. 7457-61.
138. Kondo, Y., et al., *Suppression of tumor cell growth and mitogen response by aporphine alkaloids, dicentrine, glaucine, corydine, and apomorphine.* J Pharmacobiodyn, 1990. **13**(7): p. 426-31.
139. Dong, X.P., et al., *Kmeriol and other aromatic constituents of Kmeria duperreana.* Pharm Res, 1989. **6**(7): p. 637-40.
140. Clark, A.M., et al., *In vivo efficacy of antifungal oxoaporphine alkaloids in experimental disseminated candidiasis.* Pharm Res, 1987. **4**(6): p. 495-8.
141. Hufford, C.D., et al., *Two antimicrobial alkaloids from heartwood of Liriodendron tulipifera L.* J Pharm Sci, 1975. **64**(5): p. 789-92.
142. Woo, S.H., et al., *Inhibition of topoisomerase II by liriodenine.* Biochem Pharmacol, 1997. **54**(4): p. 467-73.
143. Sellers, R.P., et al., *Design and synthesis of Hsp90 inhibitors: exploring the SAR of Sansalvamide A derivatives.* Bioorg Med Chem, 2010. **18**(18): p. 6822-56.
144. Hale, K.J., et al., *Synthesis of A83586C analogs with potent anticancer and beta-catenin/ TCF4/osteopontin inhibitory effects and insights into how A83586C modulates E2Fs and pRb.* Org Lett, 2009. **11**(3): p. 737-40.
145. Guo, W., et al., *The bioreduction of a series of benzoquinone ansamycins by NAD(P)H:quinone oxidoreductase 1 to more potent heat shock protein 90 inhibitors, the hydroquinone ansamycins.* Mol Pharmacol, 2006. **70**(4): p. 1194-203.
146. Dikalov, S., U. Landmesser, and D.G. Harrison, *Geldanamycin leads to superoxide formation by enzymatic and non-enzymatic redox cycling. Implications for studies of Hsp90 and endothelial cell nitric-oxide synthase.* J Biol Chem, 2002. **277**(28): p. 25480-5.
147. Boothman, D.A., S. Greer, and A.B. Pardee, *Potentiation of halogenated pyrimidine radiosensitizers in human carcinoma cells by beta-lapachone (3,4-dihydro-2,2-dimethyl-2H-naphtho[1,2-b]pyran- 5,6-dione), a novel DNA repair inhibitor.* Cancer Res, 1987. **47**(20): p. 5361-6.

148. Cavalcanti, B.C., et al., *Preclinical genotoxicology of nor-beta-lapachone in human cultured lymphocytes and Chinese hamster lung fibroblasts.* Chem Res Toxicol, 2011. **24**(9): p. 1560-74.
149. Duvoix, A., et al., *Effect of chemopreventive agents on glutathione S-transferase P1-1 gene expression mechanisms via activating protein 1 and nuclear factor kappaB inhibition.* Biochem Pharmacol, 2004. **68**(6): p. 1101-11.
150. Guiraud, P., et al., *Comparison of antibacterial and antifungal activities of lapachol and beta-lapachone.* Planta Med, 1994. **60**(4): p. 373-4.
151. Li, C.J., et al., *Three inhibitors of type 1 human immunodeficiency virus long terminal repeat-directed gene expression and virus replication.* Proc Natl Acad Sci U S A, 1993. **90**(5): p. 1839-42.
152. Park, E.J., K.S. Choi, and T.K. Kwon, *beta-Lapachone-induced reactive oxygen species (ROS) generation mediates autophagic cell death in glioma U87 MG cells.* Chem Biol Interact, 2011. **189**(1-2): p. 37-44.
153. Sacau, E.P., et al., *Inhibitory effects of lapachol derivatives on epstein-barr virus activation.* Bioorg Med Chem, 2003. **11**(4): p. 483-8.
154. Beall, H.D., et al., *Role of NAD(P)H:quinone oxidoreductase (DT-diaphorase) in cytotoxicity and induction of DNA damage by streptonigrin.* Biochem Pharmacol, 1996. **51**(5): p. 645-52.
155. Gu, B. and L.M. DeAngelis, *Enhanced cytotoxicity of bioreductive antitumor agents with dimethyl fumarate in human glioblastoma cells.* Anticancer Drugs, 2005. **16**(2): p. 167-74.
156. Okada, H., et al., *Biological properties of streptonigrin derivatives. II. Inhibition of reverse transcriptase activity.* J Antibiot (Tokyo), 1986. **39**(2): p. 306-8.
157. Ross, D., et al., *Bioactivation of quinones by DT-diaphorase, molecular, biochemical, and chemical studies.* Oncol Res, 1994. **6**(10-11): p. 493-500.
158. Take, Y., et al., *Comparative study on biological activities of heterocyclic quinones and streptonigrin.* J Antibiot (Tokyo), 1987. **40**(5): p. 679-84.
159. Wilson, J.B., et al., *The Chinese hamster FANCG/XRCC9 mutant NM3 fails to express the monoubiquitinated form of the FANCD2 protein, is hypersensitive to a range of DNA damaging agents and exhibits a normal level of spontaneous sister chromatid exchange.* Carcinogenesis, 2001. **22**(12): p. 1939-46.
160. Dat, N.T., et al., *Abietane diterpenes from Salvia miltiorrhiza inhibit the activation of hypoxia-inducible factor-1.* J Nat Prod, 2007. **70**(7): p. 1093-7.
161. Lee, W.Y., et al., *Cytotoxic effects of tanshinones from Salvia miltiorrhiza on doxorubicin-resistant human liver cancer cells.* J Nat Prod, 2010. **73**(5): p. 854-9.
162. Zhang, H., et al., *BsmI polymorphisms in vitamin D receptor gene are associated with diabetic nephropathy in type 2 diabetes in the Han Chinese population.* Gene, 2012. **495**(2): p. 183-8.
163. Padhye, S., et al., *Perspectives on medicinal properties of plumbagin and its analogs.* Med Res Rev, 2010.
164. Hafeez, B.B., et al., *Plumbagin, a plant derived natural agent inhibits the growth of pancreatic cancer cells in in vitro and in vivo via targeting EGFR, Stat3 and NF-kappaB signaling pathways.* Int J Cancer, 2012. **131**(9): p. 2175-86.
165. Sakagami, M., P. Morrison, and W.J. Welch, *Benzoquinoid ansamycins (herbimycin A and geldanamycin) interfere with the maturation of growth factor receptor tyrosine kinases.* Cell Stress Chaperones, 1999. **4**(1): p. 19-28.
166. Uehara, Y., et al., *Screening of agents which convert 'transformed morphology' of Rous sarcoma virus-infected rat kidney cells to 'normal morphology': identification of an active agent as herbimycin and its inhibition of intracellular src kinase.* Jpn J Cancer Res, 1985. **76**(8): p. 672-5.

167. Omura, S., et al., *Herbimycin, a new antibiotic produced by a strain of Streptomyces.* J Antibiot (Tokyo), 1979. **32**(4): p. 255-61.
168. Chen, X., et al., *Cellular pharmacology studies of shikonin derivatives.* Phytother Res, 2002. **16**(3): p. 199-209.
169. Wigington, D.P., S.A. Strugnell, and J.C. Knutson, *Pamidronate and 1,24(S)-dihydroxyvitamin D2 synergistically inhibit the growth of myeloma, breast and prostate cancer cells.* Anticancer Res, 2005. **25**(3B): p. 1909-17.
170. Wimalawansa, S.J., *Vitamin D in the new millennium.* Curr Osteoporos Rep, 2012. **10**(1): p. 4-15.
171. Zhang, L., et al., *Gambogic acid inhibits Hsp90 and deregulates TNF-alpha/NF-kappaB in HeLa cells.* Biochem Biophys Res Commun, 2010. **403**(3-4): p. 282-7.
172. Davenport, J., et al., *Gambogic acid, a natural product inhibitor of Hsp90.* J Nat Prod, 2011. **74**(5): p. 1085-92.
173. Dixit, D., et al., *Manumycin inhibits STAT3, telomerase activity, and growth of glioma cells by elevating intracellular reactive oxygen species generation.* Free Radic Biol Med, 2009. **47**(4): p. 364-74.
174. Sears, K.T., H. Daino, and G.B. Carey, *Reactive oxygen species-dependent destruction of MEK and Akt in Manumycin stimulated death of lymphoid tumor and myeloma cell lines.* Int J Cancer, 2008. **122**(7): p. 1496-505.
175. Pan, J., et al., *Autophagy induced by farnesyltransferase inhibitors in cancer cells.* Cancer Biol Ther, 2008. **7**(10): p. 1679-84.
176. Lee, S.E., et al., *Antimalarial activity of anthothecol derived from Khaya anthotheca (Meliaceae).* Phytomedicine, 2008. **15**(6-7): p. 533-5.
177. Brandt, G.E., et al., *Gedunin, a novel hsp90 inhibitor: semisynthesis of derivatives and preliminary structure-activity relationships.* J Med Chem, 2008. **51**(20): p. 6495-502.
178. Prasad, S., et al., *Garcinol potentiates TRAIL-induced apoptosis through modulation of death receptors and antiapoptotic proteins.* Mol Cancer Ther, 2010. **9**(4): p. 856-68.
179. Parasramka, M.A. and S.V. Gupta, *Garcinol inhibits cell proliferation and promotes apoptosis in pancreatic adenocarcinoma cells.* Nutr Cancer, 2011. **63**(3): p. 456-65.
180. Tanaka, T., et al., *Prevention of colonic aberrant crypt foci by dietary feeding of garcinol in male F344 rats.* Carcinogenesis, 2000. **21**(6): p. 1183-9.
181. Son, D.J., et al., *Piperlongumine inhibits atherosclerotic plaque formation and vascular smooth muscle cell proliferation by suppressing PDGF receptor signaling.* Biochem Biophys Res Commun, 2012. **427**(2): p. 349-54.
182. Golovine, K.V., et al., *Piperlongumine induces rapid depletion of the androgen receptor in human prostate cancer cells.* Prostate, 2012.
183. Maioli, E., C. Torricelli, and G. Valacchi, *Rottlerin and cancer: novel evidence and mechanisms.* ScientificWorldJournal, 2012. **2012**: p. 350826.
184. Singh, B.N., et al., *Rottlerin induces autophagy which leads to apoptotic cell death through inhibition of PI3K/Akt/mTOR pathway in human pancreatic cancer stem cells.* Biochem Pharmacol, 2012. **84**(9): p. 1154-63.
185. Lim, J.H., et al., *Rottlerin induces apoptosis via death receptor 5 (DR5) upregulation through CHOP-dependent and PKC delta-independent mechanism in human malignant tumor cells.* Carcinogenesis, 2009. **30**(5): p. 729-36.
186. Bain, J., et al., *The selectivity of protein kinase inhibitors: a further update.* Biochem J, 2007. **408**(3): p. 297-315.
187. Burlison, J.A., et al., *Development of novobiocin analogues that manifest anti-proliferative activity against several cancer cell lines.* J Org Chem, 2008. **73**(6): p. 2130-7.
188. Pearl, L.H., C. Prodromou, and P. Workman, *The Hsp90 molecular chaperone: an open and shut case for treatment.* Biochem J, 2008. **410**(3): p. 439-53.

189. Pratt, W.B. and D.O. Toft, *Regulation of signaling protein function and trafficking by the hsp90/hsp70-based chaperone machinery.* Exp Biol Med (Maywood), 2003. **228**(2): p. 111-33.
190. Trepel, J., et al., *Targeting the dynamic HSP90 complex in cancer.* Nat Rev Cancer, 2010. **10**(8): p. 537-49.
191. Neckers, L., *Heat shock protein 90: the cancer chaperone.* J Biosci, 2007. **32**(3): p. 517-30.
192. Workman, P., *Overview: translating Hsp90 biology into Hsp90 drugs.* Curr Cancer Drug Targets, 2003. **3**(5): p. 297-300.
193. Workman, P., *Altered states: selectively drugging the Hsp90 cancer chaperone.* Trends Mol Med, 2004. **10**(2): p. 47-51.
194. Hanahan, D. and R.A. Weinberg, *The hallmarks of cancer.* Cell, 2000. **100**(1): p. 57-70.
195. Holzbeierlein, J.M., A. Windsperger, and G. Vielhauer, *Hsp90: a drug target?* Curr Oncol Rep, 2010. **12**(2): p. 95-101.
196. Bishop, S.C., J.A. Burlison, and B.S. Blagg, *Hsp90: a novel target for the disruption of multiple signaling cascades.* Curr Cancer Drug Targets, 2007. **7**(4): p. 369-88.
197. Donnelly, A. and B.S. Blagg, *Novobiocin and additional inhibitors of the Hsp90 C-terminal nucleotide-binding pocket.* Curr Med Chem, 2008. **15**(26): p. 2702-17.
198. Marcu, M.G., et al., *The heat shock protein 90 antagonist novobiocin interacts with a previously unrecognized ATP-binding domain in the carboxyl terminus of the chaperone.* J Biol Chem, 2000. **275**(47): p. 37181-6.
199. Li, Y., et al., *(-)-Epigallocatechin-3-gallate inhibits Hsp90 function by impairing Hsp90 association with cochaperones in pancreatic cancer cell line Mia Paca-2.* Mol Pharm, 2009. **6**(4): p. 1152-9.
200. Palermo, C.M., C.A. Westlake, and T.A. Gasiewicz, *Epigallocatechin gallate inhibits aryl hydrocarbon receptor gene transcription through an indirect mechanism involving binding to a 90 kDa heat shock protein.* Biochemistry, 2005. **44**(13): p. 5041-52.
201. Hieronymus, H., et al., *Gene expression signature-based chemical genomic prediction identifies a novel class of HSP90 pathway modulators.* Cancer Cell, 2006. **10**(4): p. 321-30.
202. Lamb, J., et al., *The Connectivity Map: using gene-expression signatures to connect small molecules, genes, and disease.* Science, 2006. **313**(5795): p. 1929-35.
203. Zhang, T., et al., *A novel Hsp90 inhibitor to disrupt Hsp90/Cdc37 complex against pancreatic cancer cells.* Mol Cancer Ther, 2008. **7**(1): p. 162-70.
204. Kim, W.Y., et al., *Targeting heat shock protein 90 overrides the resistance of lung cancer cells by blocking radiation-induced stabilization of hypoxia-inducible factor-1alpha.* Cancer Res, 2009. **69**(4): p. 1624-32.
205. Oh, S.H., et al., *Structural basis for depletion of heat shock protein 90 client proteins by deguelin.* J Natl Cancer Inst, 2007. **99**(12): p. 949-61.
206. Biamonte, M.A., et al., *Heat shock protein 90: inhibitors in clinical trials.* J Med Chem, 2010. **53**(1): p. 3-17.
207. Koga, F., K. Kihara, and L. Neckers, *Inhibition of cancer invasion and metastasis by targeting the molecular chaperone heat-shock protein 90.* Anticancer Res, 2009. **29**(3): p. 797-807.
208. Porter, J.R., C.C. Fritz, and K.M. Depew, *Discovery and development of Hsp90 inhibitors: a promising pathway for cancer therapy.* Curr Opin Chem Biol, 2010. **14**(3): p. 412-20.
209. Kim, Y.S., et al., *Update on Hsp90 inhibitors in clinical trial.* Curr Top Med Chem, 2009. **9**(15): p. 1479-92.
210. Guo, Q.L., et al., *General gambogic acids inhibited growth of human hepatoma SMMC-7721 cells in vitro and in nude mice.* Acta Pharmacol Sin, 2004. **25**(6): p. 769-74.

211. Liu, W., et al., *Anticancer effect and apoptosis induction of gambogic acid in human gastric cancer line BGC-823.* World J Gastroenterol, 2005. **11**(24): p. 3655-9.
212. Qiang, L., et al., *Inhibition of glioblastoma growth and angiogenesis by gambogic acid: an in vitro and in vivo study.* Biochem Pharmacol, 2008. **75**(5): p. 1083-92.
213. Wu, Z.Q., et al., *Gambogic acid inhibits proliferation of human lung carcinoma SPC-A1 cells in vivo and in vitro and represses telomerase activity and telomerase reverse transcriptase mRNA expression in the cells.* Biol Pharm Bull, 2004. **27**(11): p. 1769-74.
214. Yang, Y., et al., *Differential apoptotic induction of gambogic acid, a novel anticancer natural product, on hepatoma cells and normal hepatocytes.* Cancer Lett, 2007. **256**(2): p. 259-66.
215. Lu, N., et al., *Gambogic acid inhibits angiogenesis through suppressing vascular endothelial growth factor-induced tyrosine phosphorylation of KDR/Flk-1.* Cancer Lett, 2007. **258**(1): p. 80-9.
216. Yi, T., et al., *Gambogic acid inhibits angiogenesis and prostate tumor growth by suppressing vascular endothelial growth factor receptor 2 signaling.* Cancer Res, 2008. **68**(6): p. 1843-50.
217. Qi, Q., et al., *Anti-invasive effect of gambogic acid in MDA-MB-231 human breast carcinoma cells.* Biochem Cell Biol, 2008. **86**(5): p. 386-95.
218. Zhao, J., et al., *Inhibition of alpha(4) integrin mediated adhesion was involved in the reduction of B16-F10 melanoma cells lung colonization in C57BL/6 mice treated with gambogic acid.* Eur J Pharmacol, 2008. **589**(1-3): p. 127-31.
219. Aggarwal, B.B., et al., *Potential of spice-derived phytochemicals for cancer prevention.* Planta Med, 2008. **74**(13): p. 1560-9.
220. Gupta, S.C., et al., *Regulation of survival, proliferation, invasion, angiogenesis, and metastasis of tumor cells through modulation of inflammatory pathways by nutraceuticals.* Cancer Metastasis Rev, 2010. **29**(3): p. 405-34.
221. Han, Q.B. and H.X. Xu, *Caged Garcinia xanthones: development since 1937.* Curr Med Chem, 2009. **16**(28): p. 3775-96.
222. Kamal, A., et al., *A high-affinity conformation of Hsp90 confers tumour selectivity on Hsp90 inhibitors.* Nature, 2003. **425**(6956): p. 407-10.
223. Zhao, L., et al., *Gambogic acid induces apoptosis and regulates expressions of Bax and Bcl-2 protein in human gastric carcinoma MGC-803 cells.* Biol Pharm Bull, 2004. **27**(7): p. 998-1003.
224. Kasibhatla, S., et al., *A role for transferrin receptor in triggering apoptosis when targeted with gambogic acid.* Proc Natl Acad Sci U S A, 2005. **102**(34): p. 12095-100.
225. Pandey, M.K., et al., *Gambogic acid, a novel ligand for transferrin receptor, potentiates TNF-induced apoptosis through modulation of the nuclear factor-kappaB signaling pathway.* Blood, 2007. **110**(10): p. 3517-25.
226. Ortiz-Sanchez, E., et al., *Enhanced cytotoxicity of an anti-transferrin receptor IgG3-avidin fusion protein in combination with gambogic acid against human malignant hematopoietic cells: functional relevance of iron, the receptor, and reactive oxygen species.* Leukemia, 2009. **23**(1): p. 59-70.
227. Galam, L., et al., *High-throughput assay for the identification of Hsp90 inhibitors based on Hsp90-dependent refolding of firefly luciferase.* Bioorg Med Chem, 2007. **15**(5): p. 1939-46.
228. Thulasiraman, V. and R.L. Matts, *Luciferase renaturation assays of chaperones and chaperone antagonists*, in *Methods in Molecular Biology: Bioluminescent Protocols*, R. LaRossa, Editor. 1997, Humana Press, Inc: Totowa, NJ. p. Chapt. 11.
229. Shao, J., et al., *Hsp90 Regulates p50^{cdc37} Function during the Biogenesis of the Active Conformation of the Heme-regulated eIF2a Kinase.* J. Biol. Chem., 2001. **276**: p. 206-214.

230. Shao, J., et al., *Functional dissection of cdc37: characterization of domain structure and amino acid residues critical for protein kinase binding.* Biochemistry, 2003. **42**(43): p. 12577-88.
231. Yu, X.M., et al., *Hsp90 inhibitors identified from a library of novobiocin analogues.* J Am Chem Soc, 2005. **127**(37): p. 12778-9.
232. Owen, B.A., et al., *Regulation of heat shock protein 90 (HSP90) ATPase activity by sequences in the carboxyl terminus.* J Biol Chem, 2002. **277**: p. 7086-91.
233. Morris, G.M., Goodsell, D.S., Halliday, R.S., Huey, R., Hart, W.E., Belew, R.K., Olson, A.J., *Automated Docking Using a Lamarckian Genetic Algorithm and an Empirical Binding Free Energy Function.* J. Comput. Chem., 1998. **19**(14): p. 1639-1662.
234. Stebbins, C.E., et al., *Crystal structure of an Hsp90-geldanamycin complex: targeting of a protein chaperone by an antitumor agent.* Cell, 1997. **89**(2): p. 239-50.
235. Hahnvajanawong, C., et al., *Apoptotic activity of caged xanthones from Garcinia hanburyi in cholangiocarcinoma cell lines.* World J Gastroenterol, 2010. **16**(18): p. 2235-43.
236. Wang, T., et al., *Gambogic acid, a potent inhibitor of survivin, reverses docetaxel resistance in gastric cancer cells.* Cancer Lett, 2008. **262**(2): p. 214-22.
237. Guo, Q.L., et al., *Inhibition of human telomerase reverse transcriptase gene expression by gambogic acid in human hepatoma SMMC-7721 cells.* Life Sci, 2006. **78**(11): p. 1238-45.
238. Yu, J., et al., *Repression of telomerase reverse transcriptase mRNA and hTERT promoter by gambogic acid in human gastric carcinoma cells.* Cancer Chemother Pharmacol, 2006. **58**(4): p. 434-43.
239. Zhang, H.Z., et al., *Discovery, characterization and SAR of gambogic acid as a potent apoptosis inducer by a HTS assay.* Bioorg Med Chem, 2004. **12**(2): p. 309-17.
240. Gu, H., et al., *Gambogic acid reduced bcl-2 expression via p53 in human breast MCF-7 cancer cells.* J Cancer Res Clin Oncol, 2009. **135**(12): p. 1777-82.
241. Xu, X., et al., *Gambogic acid induces apoptosis by regulating the expression of Bax and Bcl-2 and enhancing caspase-3 activity in human malignant melanoma A375 cells.* Int J Dermatol, 2009. **48**(2): p. 186-92.
242. Shu, W., et al., *Involvement of regulations of nucleophosmin and nucleoporins in gambogic acid-induced apoptosis in Jurkat cells.* Basic Clin Pharmacol Toxicol, 2008. **103**(6): p. 530-7.
243. Wang, Y., et al., *Gambogic acid induces death inducer-obliterator 1-mediated apoptosis in Jurkat T cells.* Acta Pharmacol Sin, 2008. **29**(3): p. 349-54.
244. Uma, S., V. Thulasiraman, and R.L. Matts, *Dual role for Hsc70 in the biogenesis and regulation of the heme-regulated kinase of the a subunit of eukaryotic translation initiation factor 2.* Mol. Cell. Biol., 1999. **19**: p. 5861-5871.
245. Yun, B.G., et al., *Novobiocin induces a distinct conformation of Hsp90 and alters Hsp90-cochaperone-client interactions.* Biochemistry, 2004. **43**(25): p. 8217-29.
246. Hetenyi, C. and D. van der Spoel, *Efficient docking of peptides to proteins without prior knowledge of the binding site.* Protein Sci, 2002. **11**(7): p. 1729-37.
247. Liu, Y., et al., *Gambogic acid induces G0/G1 cell cycle arrest and cell migration inhibition via suppressing PDGF receptor beta tyrosine phosphorylation and Rac1 activity in rat aortic smooth muscle cells.* J Atheroscler Thromb, 2010. **17**(9): p. 901-13.
248. Prasad, S., et al., *Gambogic acid inhibits STAT3 phosphorylation through activation of protein tyrosine phosphatase SHP-1: potential role in proliferation and apoptosis.* Cancer Prev Res (Phila), 2011. **4**(7): p. 1084-94.
249. Lu, L., et al., *Gambogic acid inhibits TNF-alpha-induced invasion of human prostate cancer PC3 cells in vitro through PI3K/Akt and NF-kappaB signaling pathways.* Acta Pharmacol Sin, 2012. **33**(4): p. 531-41.

250. Wang, J., et al., *Gambogic acid-induced degradation of mutant p53 is mediated by proteasome and related to CHIP.* J Cell Biochem, 2011. **112**(2): p. 509-19.
251. Vallee, F., et al., *Tricyclic series of heat shock protein 90 (Hsp90) inhibitors part I: discovery of tricyclic imidazo[4,5-c]pyridines as potent inhibitors of the Hsp90 molecular chaperone.* J Med Chem, 2011. **54**(20): p. 7206-19.
252. Shahinas, D., et al., *A repurposing strategy identifies novel synergistic inhibitors of Plasmodium falciparum heat shock protein 90.* J Med Chem, 2010. **53**(9): p. 3552-7.
253. Freise, C., et al., *A hepatoprotective Lindera obtusiloba extract suppresses growth and attenuates insulin like growth factor-1 receptor signaling and NF-kappaB activity in human liver cancer cell lines.* BMC Complement Altern Med, 2011. **11**: p. 39.
254. Wang, X., et al., *Anti-angiogenic and antitumor activities of Huaier aqueous extract.* Oncol Rep, 2012. **28**(4): p. 1167-75.
255. Li, H., et al., *Cell cycle arrest and cell apoptosis induced by Equisetum hyemale extract in murine leukemia L1210 cells.* J Ethnopharmacol, 2012. **144**(2): p. 322-7.
256. Lin, M., et al., *Hedyotis diffusa Willd extract inhibits HT-29 cell proliferation via cell cycle arrest.* Exp Ther Med, 2012. **4**(2): p. 307-310.
257. Hu, B., et al., *Liver Yin deficiency tonifying herbal extract induces apoptosis and cell senescence in Bel-7402 human hepatocarcinoma cells.* Exp Ther Med, 2012. **3**(1): p. 80-86.
258. Caamal-Fuentes, E., et al., *Screening of plants used in Mayan traditional medicine to treat cancer-like symptoms.* J Ethnopharmacol, 2011. **135**(3): p. 719-24.
259. Cibin, T.R., D.G. Devi, and A. Abraham, *Chemoprevention of two-stage skin cancer in vivo by Saraca asoca.* Integr Cancer Ther, 2012. **11**(3): p. 279-86.
260. Mengesha, A.E. and B.B. Youan, *Anticancer activity and nutritional value of extracts of the seed of Glinus lotoides.* J Nutr Sci Vitaminol (Tokyo), 2010. **56**(5): p. 311-8.

VITA

Jason Lee Davenport

Candidate for the Degree of

Doctor of Philosophy

Thesis: IDENTIFICATION AND CHARACTERIZATION OF NATURAL COMPOUND INHIBITORS OF THE HSP90 CHAPERONE COMPLEX

Major Field: Biochemistry and Molecular Biology

Biographical:

Education:

Completed the requirements for the Doctor of Philosophy in Biochemistry and Molecular Biology at Oklahoma State University, Stillwater, Oklahoma in May 2013.

Completed the requirements for the Bachelor of Science in Biochemistry at Oklahoma Christian University, Oklahoma City, Oklahoma in 2006.

Experience:

Research assistant at Oklahoma State University from August 2006 to July 2012. Research intern at Hyalose, L.L.C. from May to August 2005.