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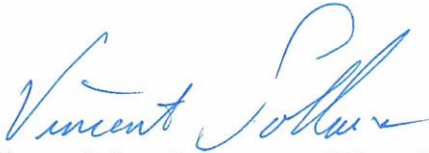
CRIPPLING RAPID EVOLUTION OF METASTASIS AND DRUG RESISTANCE IN A549 NON-SMALL CELL LUNG CANCER CELLS WITH THE CLINICALLY RELEVANT HSP90 INHIBITOR AUY922

A dissertation submitted to
the Graduate College of
Marshall University
In partial fulfillment of
the requirements for the degree of
Doctor of Philosophy
In
Biomedical Sciences
by
Nickolas Anthony Bacon
Approved by
Dr. Vincent Sollars, Committee Chairperson
Dr. Piyali Dasgupta
Dr. Emine Koc
Dr. Wei Li
Dr. William McCumbee

Marshall University
May 2021

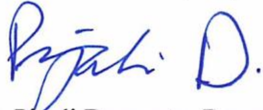
APPROVAL OF THESIS

We, the faculty supervising the work of Nickolas Anthony Bacon, affirm that the dissertation, *Crippling Rapid Evolution of Metastasis and Drug Resistance in A549 Non-Small Cell Lung Cancer Cells with the Clinically Relevant HSP90 Inhibitor AUY922*, meets the high academic standards for original scholarship and creative work established by the Biomedical Sciences program and the Graduate College of Marshall University. This work also conforms to the editorial standards of our discipline and the Graduate College of Marshall University. With our signatures, we approve the manuscript for publication.



February 25, 2021

Dr. Vincent Sollars, Department of Biomedical Sciences — Committee Chairperson — Date



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ABSTRACT

The ability for species to evolve new features in response to changing circumstances in order to survive and propagate is a ubiquitous observation on both the macroscopic and microscopic levels of living systems. It should be no surprise, then, that diseases such as cancer utilize their own forms of adaptation to perpetuate themselves when exposed to external threats. Indeed, concepts drawn from Darwinian evolution are now widely accepted to help explain certain aspects of carcinogenesis and malignant progression, the sum of which have come to be known as the theory of tumor evolution. Since metastasis and drug resistance are features that manifest toward the late stages in the disease after withstanding numerous selective pressures, cancer cells harboring these features can be viewed as the most evolutionarily fit. Just as many forms of life rely on common adaptive mechanisms to promote their survival during dramatic shifts in their environment, metastatic and drug resistant cancers may rely upon common cellular mechanisms to promote their survival when faced with untenable circumstances. We hypothesize that one of the oldest genes in the human genome, HSP90, functions as a link between metastatic and drug resistant behavior of cancer. We believe this occurs through HSP90's relationship in supporting the function of gene products that define the cancer hallmarks and clinical evidence suggesting HSP90 is important in progressing cancer into advanced stages. In the following chapters we discuss HSP90 and its role in orchestrating evolution of metastatic and drug resistant phenotypes. We use the clinically relevant HSP90 inhibitor, AUY922, to explore our assertions *in vitro* in the context of non-small cell lung cancer (NSCLC), which is prone to evolving metastatic and drug resistant phenotypes. We examine the implications for our findings, future directions, and new possibilities for utilizing HSP90 inhibitors to treat cancer.

CHAPTER 1: INTRODUCTION

Heat shock protein 90 (HSP90) is among some of the most primordial and well conserved genes in the human genome (Schlesinger, 1990). It has retained its function in all of eukarya, from yeast to humans, with homologs in numerous prokarya as well. Despite its age in the genomes of almost every organism on earth, it has only been in the last 60 years that we have been able to study it in any sort of detail. Early studies taught us that HSP90 is necessary in order to maintain viability in eukaryotic cells (Picard, 2002), indicating that evolution has placed a high priority in preserving its function over the millennia. However, the dependence eukaryotic cells have on HSP90 to maintain function has made it difficult to study, as genetic manipulation of HSP90 usually results in cells and organisms that are hypersensitive to heat stress (Picard, 2002). This has been a significant barrier to understanding what, if any, relevance HSP90 has to medicine. At the very minimum, these observations delineated HSP90's role in managing stressful stimuli, with further research describing HSP90 as essential in managing various forms of cellular stress other than heat stress (Courgeon, Maisonhaute, & Best-Belpomme, 1984; Heikkila, Schultz, Iatrou, & Gedamu, 1982; Michel & Starka, 1986; Yura, Tobe, Ito, & Osawa, 1984).

Since the 1970's, it has been possible to target HSP90 pharmacologically (DeBoer, Meulman, Wnuk, & Peterson, 1970), making it easier to interrogate its function without severely compromising biological systems. Decades of research manipulating HSP90 pharmacologically have produced some surprising revelations. One of the most interesting patterns of discovery has been that drugging HSP90 can generate unexpected, novel phenotypes at the single cell and whole organism levels (Lawag et al., 2017; Queitsch, Sangster, & Lindquist, 2002; Rohner et al., 2013; Sollars et al., 2003). Some of these phenotypes are somewhat disjointed and would have

little chance in helping the organism achieve success in a natural setting subject to the forces of Darwinian evolution. This suggests that HSP90 may be important in reinforcing phenotypes that will promote the fitness of organisms in their environment, especially under stressful conditions.

It is now widely accepted that the genetic, epigenetic, and phenotypic changes that cancers accrue can be explained by concepts pulled from Darwinian evolution, a theory known as tumor evolution (Casas-Selves & Degregori, 2011; Greaves, 2015; Lacina et al., 2019). The phenotypes malignant tumors evolve in the advanced stages of the disease can be viewed as the most fit since these cells survived late into the disease process. In the late stages of cancer, these phenotypes almost always result in metastatic and drug resistant behavior. If HSP90 is important in reinforcing fitness in species, then it is possible that HSP90 may be reinforcing fitness of cancers, in particular, fitness coupled to metastasis and drug resistance. Interestingly, HSP90 inhibitors have also demonstrated potent and specific anticancer effects in a variety of tumors.

In the following chapters, we discuss HSP90's role in the heat shock response, and posit in what way HSP90 is essential in perpetuating the evolution of metastatic and drug resistant phenotypes in cancer. We will support our claims with scientific evidence demonstrating pharmacological inhibition of HSP90 attenuates metastatic and drug resistant phenotypes *in vitro*. We will close by examining the implications of this evidence, future directions, and considering novel ways to use HSP90 inhibitors to improve cancer treatment outcomes.

The Heat Shock Response

Organisms are exposed to a plethora of environmental insults that threaten cellular homeostasis and rely on cellular programs that respond promptly to these stressors to maintain normal cell functions. One of the most ancient and evolutionarily conserved responses to stress found in all of eukarya on earth is the heat shock response (Ritossa, 1996; Schlesinger, 1990).

An increase in just a few degrees can cause proteins to denature, become entangled, and aggregate within a cell. This disruption of proteostasis signals a dramatic increase in the production of a class of proteins known as heat shock proteins (HSPs) (Lindquist, 1986). Members of this class include HSP100, HSP90, HSP70, HSP60, and HSP40. They function as molecular chaperones, physically interacting with and manipulating misfolded, unstable and damaged proteins in order to aid them in reaching a mature conformation, ultimately maintaining appropriate trafficking, signaling integrity, and degradation of proteins in the face of heat stress. Proteins that rely on HSPs to maintain their native conformation are referred to as clients, with some HSPs having very specific clientele while others, such as HSP90, chaperone hundreds of clients stretching across multiple, indispensable cellular processes. Further research revealed that other forms of stress, such as hypoxia, acidosis, and chemical damage also result in disruption of proteostasis and up regulation of HSPs (Courgeon et al., 1984; Heikkila et al., 1982; Michel & Starka, 1986; Yura et al., 1984). Since other forms of stress can up regulate HSPs, the heat shock response has also come to be known as the “stress” response. When the source of stress is removed, HSPs return to their basal state, but remain primed to increase when faced with stress in the future.

The wide range of gene products in essential cell processes engaged by HSPs during stressful stimuli codifies the heat shock response as an adaptive mechanism, functioning primarily to maintain smooth cellular function in the face of stress. This has obvious benefits in that all biological tissues are likely to experience stress at some point in time, so having access to mechanisms permitting the cell to adapt to stress is critical to maintaining tissue integrity. Unfortunately, the heat shock response can enable certain diseases such as cancer. Indeed, numerous studies have demonstrated elevated HSP expression in a variety of human cancers

(Calderwood, Khaleque, Sawyer, & Ciocca, 2006; Sherman & Multhoff, 2007). This should not be surprising as one of the overarching themes in cancer is the acquisition of mutations resulting in unstable gene products that require additional processing by HSPs in order to function. Furthermore, many of the stressful stimuli associated with induction of HSPs also occur in cancer. For example, the accelerated growth and proliferation of neoplastic tissue can promote the formation of lactic acidosis in the tumor microenvironment. Additionally, application of high dose chemotherapy to treat cancer can be expected to supply the chemical stress necessary to induce HSP expression (Tiligada, 2006). Of the HSPs implicated in driving oncogenic processes, HSP90 is considered unique as it has distinct characteristics that separate it from other HSPs. Additionally, HSP90 may function differently in neoplastic tissue relative to normal tissue.

HSP90

Vertebrates retain two isoforms of HSP90, HSP90α and HSP90β. HSP90α is considered the inducible isoform, and its expression increases when a cell is exposed to stressful stimuli. HSP90β operates in a constitutive fashion, and is widely expressed at basal conditions (Millson et al., 2007). HSP90α and HSP90β share 86% sequence homology (Langer & Fasold, 2001) and are thought to be derived from a gene duplication event 500 million years ago (Krone & Sass, 1994). HSP90 is almost exclusively found in the cytoplasm, although evidence is accumulating that it functions in the nucleus as well (Calderwood & Neckers, 2016), leaving open the possibility HSP90 directly influences transcriptional changes and epigenetic mechanisms, in addition to its classical chaperone function (Lawag et al., 2017; Sollars et al., 2003). It forms a

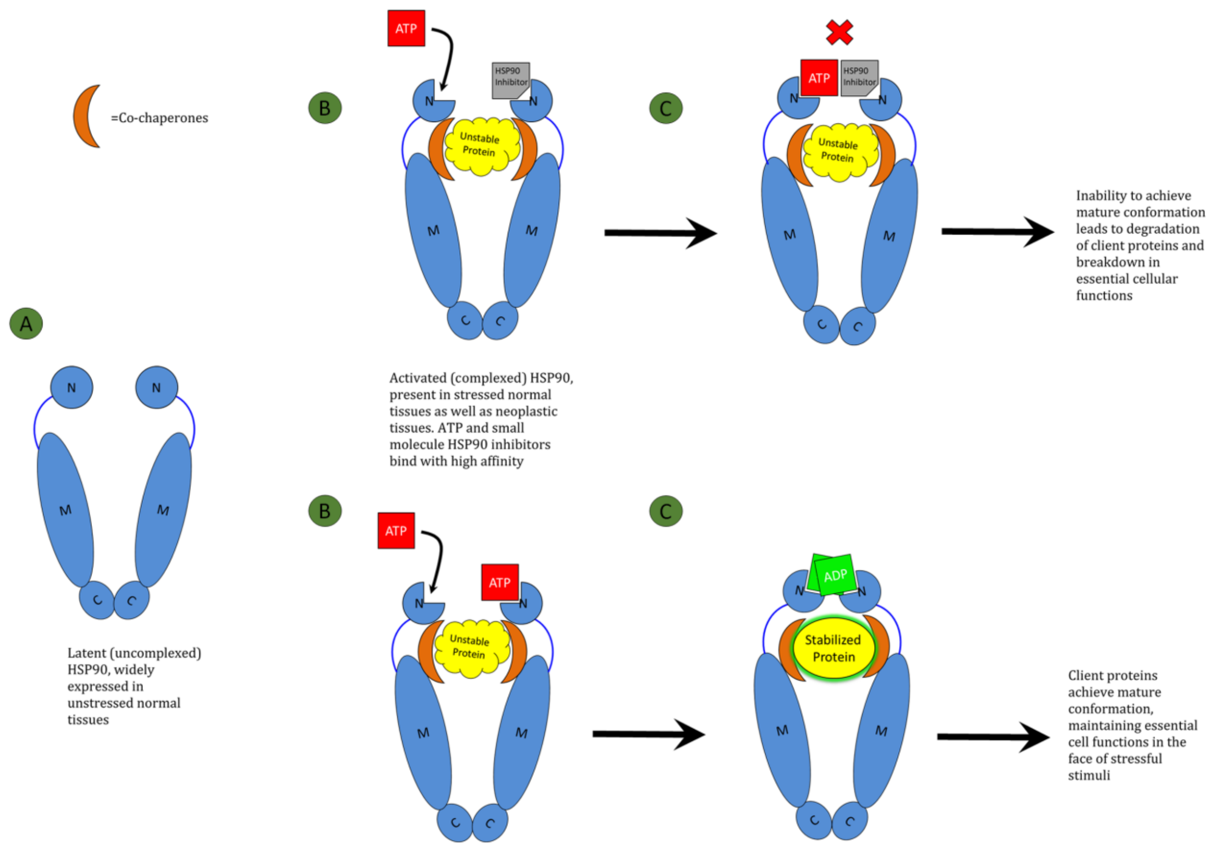

Figure 1. HSP90 Chaperone Function
(A) Illustration of the three functional domains of HSP90, N=N terminal domain, M=middle domain, C=C terminal domain. The N terminal domain containing the nucleotide binding pocket in latent HSP90 has low affinity for ATP or small molecule HSP90 inhibitors (blunt ends). The C terminal domain is necessary for dimerization. (B) Upon binding to co-chaperones that aid in presentation of the unstable protein client to the active site, HSP90 achieves an activated conformation with high affinity for small molecule HSP90 inhibitors (top) (indicated by notch in N-terminal domain) and for ATP (bottom). (C)(Top) In the presence of small molecule inhibitors, HSP90 cannot complete chaperone function and client protein cannot achieve mature conformation, leading to proteosomal degradation and breakdown of essential cell functions during stress. (Bottom) ATP hydrolysis drives "pincer" mechanism, which assists client proteins in successfully reaching a mature conformation under stress.

dimer at physiological temperatures and contains an N-terminal domain, a middle-domain and a C-terminal domain **(Figure 1A)**. HSP90 is ATP dependent and contains a nucleotide-binding

pocket belonging to the GHKL family in its N-terminal domain (Prodromou et al., 1997). This ATP binding pocket is distinct from other chaperones and kinases and is the target of small molecule HSP90 inhibitors **(Figure 1B).** The middle domain contains the binding site for clients and for co-chaperones that assist HSP90 in its chaperone function (Hawle et al., 2006). The C-terminal domain is necessary for HSP90 dimerization (Harris, Shiau, & Agard, 2004; Minami, Kimura, Kawasaki, Suzuki, & Yahara, 1994; Nemoto, Ohara-Nemoto, Ota, Takagi, & Yokoyama, 1995) and also binds certain clients. These three domains work in concert with co-chaperones to manipulate unstable proteins into a functional, active conformation **(Figure 1).** When HSP90 is bound to co-chaperones containing an unstable protein in need of achieving a mature conformation, it is referred to as "activated" HSP90 (Kamal & Burrows, 2004). This state is found in stressed tissues and cancer cells, and is the state in which HSP90 is most receptive to pharmacological inhibition. HSP90 not bound to clients and co-chaperones is known as "latent" HSP90 (Kamal & Burrows, 2004). The latent form of HSP90 is commonly found in normal, unstressed tissues.

Unlike other HSPs, HSP90 is essential in all eukaryotes and is present in large excess, representing 1-2% of a cell's total proteome in unstressed conditions (Borkovich, Farrelly, Finkelstein, Taulien, & Lindquist, 1989; Taipale et al., 2012; Taipale et al., 2014). Excess HSP90 expression in unstressed tissues is thought to provide a buffer for cells to quickly manage transient levels of incoming proteotoxic stress. HSP90 also engages its clients differently than its HSP cousins. Other HSPs interact with their clients transiently while many clients associated with HSP90, such as steroid hormone receptors, require continuous interaction in order to maintain a functional conformation (Pratt & Toft, 1997). Additionally, while HSP90 has hundreds of clients, they tend to focus around kinases, signal transducers, and transcription

factors that are key nodes to an assortment of cell signaling and developmental pathways (Taipale, Jarosz, & Lindquist, 2010; Taipale et al., 2014). Therefore, mechanisms that limit HSP90 function may have substantial effects on a cell's behavior and ability to manage exposure to stressful stimuli.

Perhaps the most profound aspect of HSP90 is its influence on rapid acquisition of novel traits through its role as both a potentiator and capacitor of phenotypic variation, at both the single cell and whole organism levels. As a potentiator of phenotypic variation, HSP90 can use its chaperone function to properly fold and support the function of unstable signal transducers, influencing phenotypic switches that can occur from selective pressures downstream of these signal transducers. For example, calcineurin relies on HSP90 to function (Imai & Yahara, 2000; Kumar, Musiyenko, & Barik, 2005), and studies involving yeast showed calcineurin requires HSP90 in order to promote rapid acquisition of resistance to antifungal drugs (Cowen & Lindquist, 2005). As a capacitor of phenotypic variation, HSP90 chaperone activity can mask phenotypic variation associated with cryptic genetic alterations in a cell or organism until extreme stresses or small molecule inhibition of HSP90 overwhelm its chaperone function and reveals them (Lawag et al., 2017; Queitsch et al., 2002; Rohner et al., 2013; Sangster et al., 2007; Sollars et al., 2003). This is the setting in which phenotypes with apparent decreases in evolutionary fitness have been observed (Queitsch et al., 2002; Sangster et al., 2007; Sollars et al., 2003). In fact, reductions in fitness were directly observed through reduced number of seeds produced by *A. thaliana* plants with attenuated HSP90 function (Sangster et al., 2007). Taken together, one would expect HSP90's dual role as a potentiator and capacitor of novel phenotypes to have a measurable effect on the emergence of new phenotypes in a setting subject to the rules of Darwinian evolution, such as cancer.

Tumor Evolution, Intratumoral Heterogeneity, and HSP90

Applying the concept of Darwinian evolution to cancer is not a cutting edge idea (Nowell, 1976), but it was not until the last few decades that technological advances have permitted researchers to study cancer at the level of detail necessary to build a strong case that concepts pulled from Darwinian evolution can explain certain aspects of carcinogenesis (Casas-Selves & Degregori, 2011; Greaves, 2015; Lacina et al., 2019). On a fundamental level, cancers evolve over time because they fulfill the three conditions of Darwinian evolution: propagation of genetic material to the next generation; heterogeneous phenotypes existing in one biological system; and the existence of selective pressures that act on that heterogeneity so that the fittest survive.

Fulfillment of all three conditions is exemplified by some of the most basic observations of neoplastic behavior. Propagation of genetic material is derived from the understanding that cancer is a clonal disease. As each cancer cell divides, it passes on its genetic material to daughter cells, thus promulgating the neoplastic phenotype. Metastasis is an example of heterogeneity developing in a cancer. If a cancer started in one place, and then transplanted to a distant site, there was a point where the primary tumor became a heterogeneous population of cancer cells: some willing to migrate to a new tissue landscape, others content on their primary place of residence. Lastly, decades of clinical observations of remission followed by relapse of drug resistant cancers is evidence of tumors experiencing, adapting to, and overcoming selective pressures of therapy over time. The development of chemotherapeutic resistance actually encapsulates all three Darwinian conditions. After selective pressure of therapy is overcome, either through intrinsic or acquired resistance mechanisms, surviving cancer cells will again continue to proliferate, passing on to their progeny the genetic code that permitted resistance to

occur, which eventually manifests as multidrug resistant relapse (Gatenby & Brown, 2018). There are other examples of neoplastic behavior that fulfill Darwinian conditions, but metastasis and drug resistance are highlighted here because they are two consequences of tumor evolution and two major sources of treatment failure in cancer.

In order to maintain perpetual evolution, the most vital of the Darwinian conditions is the existence of heterogeneous populations. Without heterogeneity, the potential to change is substantially constrained, regardless of the ability to propagate genetic material or the presence of selective pressures. In terms of cancer, this is known as intratumoral heterogeneity (ITH), which for this discussion refers to the diversity of malignant phenotypes that exist within a tumor as a product of their various genetic and epigenetic alterations. Numerous sequencing studies of a range of temporal and spatially distinct tumor types reveal an astounding spectrum of cellular diversity in any individual patient's tumor (Bolli et al., 2014; de Bruin et al., 2014; Eckert et al., 2016; Uchi et al., 2016; Yates et al., 2015; Zhang et al., 2014). This pool of diversity provides the substrate for Darwinian processes to test and select the most advantageous malignant phenotypes to withstand the stresses of genetic alterations, microenvironmental pressures, energy demands, and cytotoxic therapy that cancer cells eventually experience. ITH has also been linked to development of metastasis and drug resistance (Bhang et al., 2015; Hallou, Jennings, & Kabla, 2017; Piotrowska et al., 2015; Wei et al., 2017), making the formation of heterogeneous tumor populations a central component of treatment failure.

The source of ITH in cancer is predominantly derived from cell intrinsic processes that define the disease. Cancer is typically described in terms of accumulating mutations in the genetic code that results in protein products that are nonfunctional, severely compromised, or

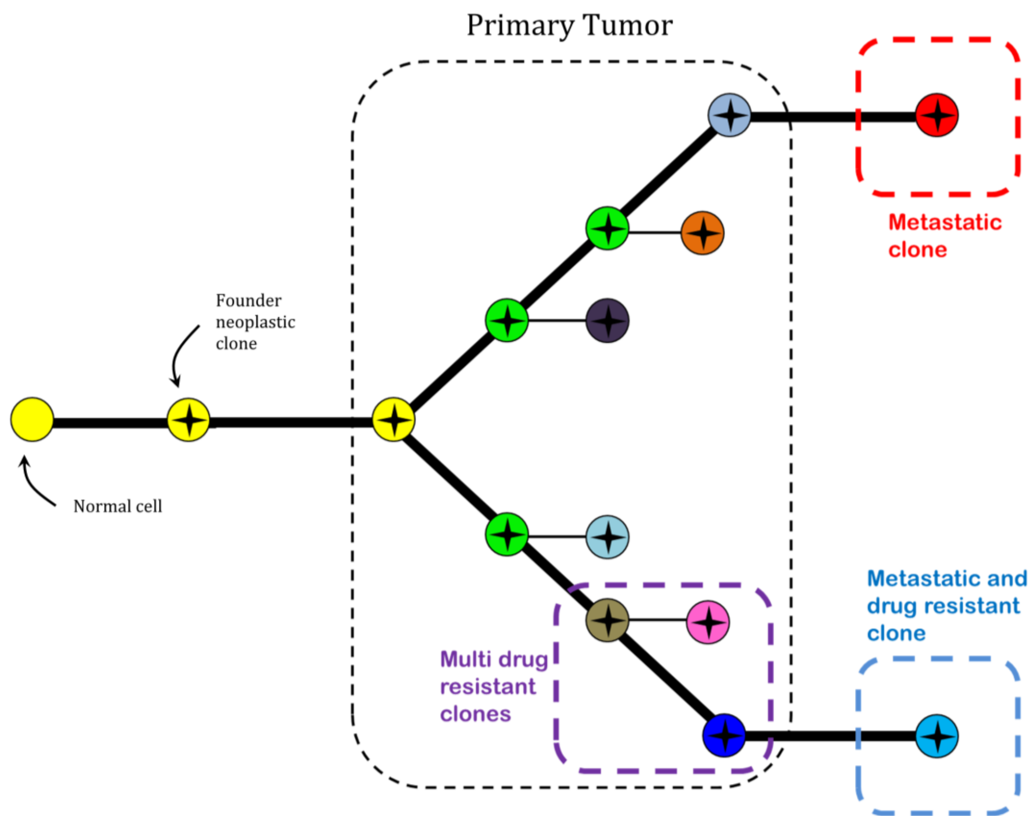

Figure 2. Illustration of the Consequences of Genetic Instability or Epigenetic Modifications on the Formation of Heterogeneous Populations in a Tumor. The four-pointed star represents the silencing of tumor suppressor genes that sets forth the cascade of genomic instability and accumulation of oncogenic mutations. As the tumor grows and clonal progeny accumulate mutations, they may acquire driver mutations (thick lines) that provide a fitness advantage and changes in their identity (change in color). Tumor cells may also acquire passenger mutations (thin lines) that change their identity, but offers no additional fitness advantage. Over time, tumor cells evolve metastatic and drug resistant features.

inappropriately activated. A necessary mutational event that initiates malignant transformation is the silencing of tumor suppressor genes. These genes function to guard the genome against accumulation of oncogenic mutations that promote abnormal cellular behavior. If a mutation occurs in a tumor suppressor gene that renders it nonfunctional, mutations can accumulate unchecked, ultimately permitting the formation of a neoplastic clone that grows, divides, and invades surrounding tissue beyond its normal capacity. As the newly transformed clone

proliferates into a small group of progeny that also lack tumor suppressor functions as acquired from their parent, new mutations are permitted to accumulate unrestricted with each cell division. This process is known as genomic instability and is a major source of genetic ITH in cancer (Burrell, McGranahan, Bartek, & Swanton, 2013). As incidental mutations amass with each round of the cell cycle, genotypes begin to diverge from one another, over time forming trunks and branches that resemble a phylogenetic tree **(Figure 2).**

Many mutations will be deleterious to neoplastic sub clones, resulting in their rapid removal from the tumor population. If a mutation occurs in a gene that imparts a fitness advantage to a cancer cell, this mutation will persist and be passed on to future cellular progeny. These mutations are known as driver mutations (Vogelstein et al., 2013), and in the context of branching evolution, represent the trunks and large branches of a cancer's phylogenetic tree **(Figure 2).** They may also acquire passenger mutations, which are supplementary mutations that occur alongside driver mutations that do not impart any particular selective advantage relative to surrounding cancer cells. Some evidence suggests passenger mutations may actually become detrimental if too many accumulate (McFarland, Korolev, Kryukov, Sunyaev, & Mirny, 2013; Vogelstein et al., 2013). Passenger mutations make up the smallest branches of the phylogenetic tree. Over time, many trunks and branches may form, generating an array of genotypes that result in distinct cellular identities and behaviors **(Figure 2)**. Computer models utilizing patient biopsy data and known driver mutation frequencies support the notion that a wide variety of distinct genotypes can be derived from a single malignant common ancestor over time (Waclaw et al., 2015), propelled primarily by genomic instability (Greaves & Maley, 2012).

Diversity in a tumor is not strictly limited to genetic variation. Epigenetic variation is also abundant in a tumor (Easwaran, Tsai, & Baylin, 2014). Epigenetics refers to changes in gene

expression without a change in the DNA sequence itself that can be propagated through mitosis or meiosis. This usually refers to mechanisms regulating chromatin structure such as DNA methylation or histone modifications. Changes in DNA methylation can operate similar to mutations in terms of how they influence gene expression, with hypermethylation of CpG islands leading to gene silencing, and hypomethylation of CpG islands leading to gene activation. For example, hypermethylation of tumor suppressor genes, such as pRB, can silence its expression and initiate the formation of a retinoblastoma tumor (Greger, Passarge, Hopping, Messmer, & Horsthemke, 1989). There are fewer examples of specific oncogene activation through hypomethylation, however global hypomethylation is a well-documented phenomena in cancer and may promote an even greater increase in genomic instability since looser chromatin is more prone to breakage and translocation events (Javadekar & Raghavan, 2015).

If an epigenetic modification commonly results in gene expression changes that confer a selective advantage, it is referred to as an epi-driver gene (Vogelstein et al., 2013). Epi-driver genes are analogous to driver mutations, forming the trunk and large branches of a cancer's phylogenetic tree. There are two important implications for epigenetic variation in a tumor. The first is that two genetically identical cancer cells may have different epigenetic signatures, which may lead them to behave differently. This adds a notable layer of depth to the notion of ITH. Second, epigenetic modifications are sensitive to the environment and may occur rapidly, much faster than it could take for driver mutations to become apparent. Rapid epigenetic changes are thought to be a major cause of acquired drug resistance in cancer (Flinders et al., 2016).

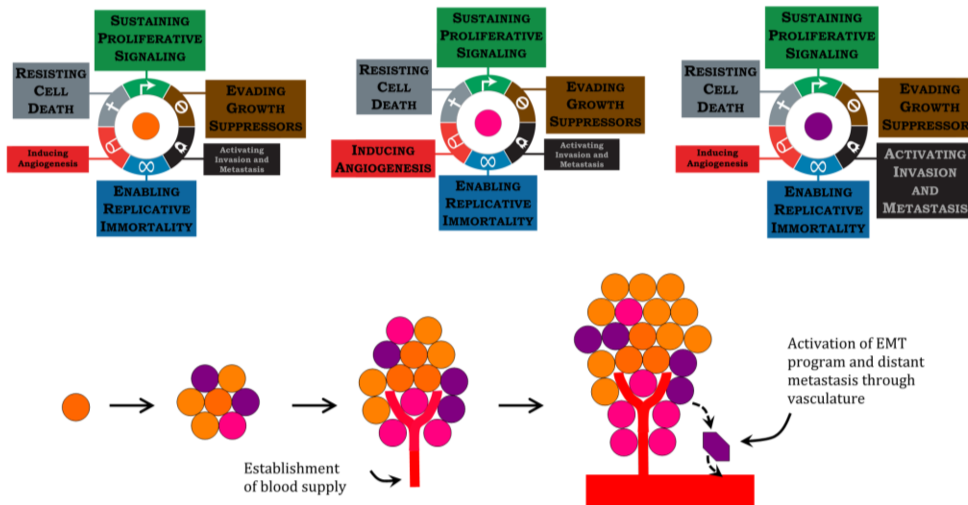

Figure 3. The Relationship Between Intratumoral Heterogeneity (ITH) and Tumor Evolution According to the Cancer Hallmarks.
Over time the tumor evolves into three basic phenotypes, each represented by different colors (orange, purple, pink). The size of the colored boxes represents the relative dependence the clone has on the corresponding cancer hallmark to maintain its phenotype. Founder mutations begin the first neoplastic clone (orange). As it divides over time, the progeny may accumulate mutations or epigenetic changes, and sub clones may form with new abilities. A sub clone may gain the ability to induce angiogenesis (pink) through secretion of pro-angiogenic factors like VEGF to establish a blood supply and provide the tumor with additional nutrients. As time progresses, further changes in gene expression may generate a metastatic sub clone through activation of cellular programs like EMT (purple) that permit invasion and metastatic spread to distant sites in the body. Adapted from Hanahan and Weinberg 2000.

Epigenetic modifications also drive transdifferentiation of cancer cells undergoing epithelial to mesenchymal transition (EMT), eventually resulting in induction of the metastatic cascade during cancer progression (Tam & Weinberg, 2013).

The concept of ITH makes it appear that cancers evolve new phenotypes in stochastic and unpredictable ways. However, after decades of research a pattern has taken shape. In 2000, Hanahan and Weinberg published their seminal work, “Hallmarks of Cancer”(Hanahan & Weinberg, 2000), in order to sum up and stratify the dizzying array of cellular behaviors that manifest in tumors. They would later update their analysis of the cancer hallmarks by adding four emerging hallmarks (Hanahan & Weinberg, 2011), but we will only refer to the original six

hallmarks as they have withstood the test of time. The hallmarks of cancer represent a pattern of discernible neoplastic behaviors driven by mutated gene products (Hanahan & Weinberg, 2000) and aberrant epigenetic modifications (Easwaran et al., 2014; Flavahan, Gaskell, & Bernstein, 2017). With this said, the cancer hallmarks can be referred to as a cooperative set of phenotypes selected for according to the theory of tumor evolution. Given what we also know about the assortment of phenotypes that can exist within a tumor, there are certainly distinct populations that depend on cancer hallmarks to varying degrees to maintain their survival during the course of disease progression **(Figure 3).**

Where does HSP90 fit into this discussion? One of the most interesting aspects of HSP90 is many of its clients are also gene products that define the cancer hallmarks. In fact, if one were to compare the list of verified HSP90 clients (Picard, 2019) to gene products that drive the cancer hallmarks (Moser, Lang, & Stoeltzing, 2009), they would discover considerable overlap. **(Figure 4)** This relationship has undergone intense investigation and numerous studies report that HSP90 is essential in promoting the cancer hallmarks, from the acquisition of immortal characters to the development of metastasis and drug resistance (Kim et al., 2008; Nolan, Franco, Hance, Hayward, & Isaacs, 2015; Whitesell et al., 2014). Since the hallmarks of cancer represent a heterogeneous group of features malignant neoplasms are bound to achieve over time, and HSP90 appears necessary to maintain them, then HSP90 may be promoting ITH by stabilizing mutated and unstable gene products that delineate the cancer hallmarks through its potentiator function as a chaperone. Moreover, the large pool of excess HSP90 also serves to steady

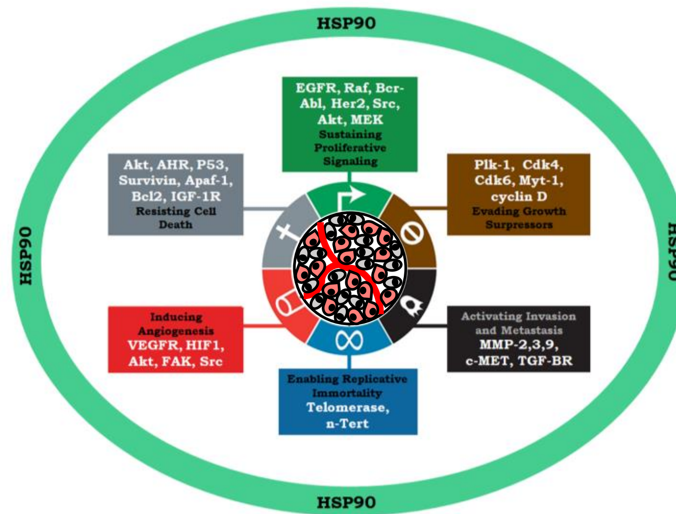

Figure 4. HSP90 and the Cancer Hallmarks
Depiction of the overlap of HSP90 clients with gene products within the hallmarks of cancer. Adapted from Hanahan and Weinberg 2000.

rapid and widespread changes in gene expression that can occur under the direction of epi-driver genes. Stated another way, HSP90 may play a fundamental role in supporting the emergence of various cancer hallmarks and thus, provides the medium by which ITH can manifest, ultimately setting the table for Darwinian processes to advance tumor evolution and subsequent disease progression **(Figure 5).**

The association between HSP90 in supporting ITH and propelling tumor evolution is further strengthened when one considers the clinical impact HSP90 expression has on a patient's cancer. Clinical evidence indicates that increased HSP90 expression is associated with various cancers and has been linked to a poor clinical prognosis (Flandrin et al., 2008; Pick et al., 2007; Thomas et al., 2005; Wu, Huang, Liu, & Liu, 2015; Zagouri et al., 2010). High HSP90 expression was associated with increased Her-2/neu and estrogen receptor expression, large tumors, high nuclear grade, and lymph node involvement in breast cancers (Pick et al., 2007), all

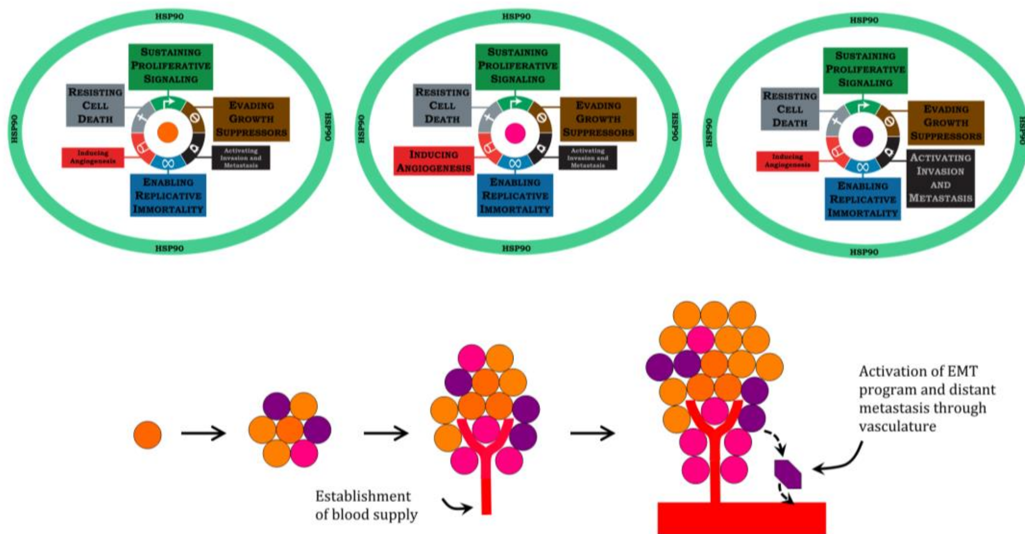

Figure 5. Unification of HSP90, ITH, and Tumor Evolution According to the Cancer Hallmarks
Illustration of HSP90 promoting ITH, through simultaneous support of neoplastic sub clones that depend on cancer hallmarks to varying degrees (orange, pink, and purple circles). The size of the colored boxes represents the relative dependence the clone has on the corresponding cancer hallmark to maintain its phenotype, with HSP90 encircling all six original cancer hallmarks indicating it is supporting the current state of each sub clones cancer hallmarks, but is also poised to support other cancer hallmarks when a cancer cell may require more dependence. Adapted from Hanahan and Weinberg 2000.

heterogeneous features that require significant changes in the primary tumor in order to occur. It is also important to note that both the estrogen and Her2/neu receptors are HSP90 clients. HSP90 over expression in gastric cancer patients correlated with increased depth of invasion, lymph node involvement, and a higher stage of disease progression (J. Wang, Cui, Zhang, Wu, & Tang, 2013), again a mix of features that necessitate changes within the primary tumor in order to manifest.

Interestingly, while HSP90 overexpression is strongly linked to poor prognoses in cancers, HSP90 itself is not mutated in cancer (Workman, Burrows, Neckers, & Rosen, 2007), suggesting that the native conformation of HSP90 provides a significant fitness advantage to evolving cancer cells. This fitness advantage may be inherent to HSP90's ability to support the

cancer hallmarks, which eventually generates the level of ITH necessary for cancers to evolve to advanced stages (Bhang et al., 2015; Gainor et al., 2016; Wei et al., 2017). Since HSP90 is not mutated in most cancers, this also makes it a lucrative target for sustained small molecule inhibition to potentially limit ITH and restrict tumor evolution.

However, there is one small problem with this assertion. As previously stated, research suggests that HSP90 can influence phenotypic diversity in two ways. It acts as a potentiator of phenotypic diversity by using its chaperone activity to support the function of unstable proteins that rely on HSP90 in order to transfer signals for phenotypic change. HSP90 can also act like a capacitor for phenotypic diversity by masking the effect of small changes in the genetic code on proteins through reinforcement of the wild type phenotype, also known as canalization (Waddington, 1959). As a capacitor, HSP90 stores cryptic phenotypic diversity and only reveals them when its chaperone function becomes overwhelmed, either through small molecule inhibition or various forms of extreme stress (Lawag et al., 2017; Rohner et al., 2013; Sollars et al., 2003). If inhibition of HSP90 can reveal additional phenotypic diversity, and thus contribute to ITH, how do we reconcile using HSP90 inhibitors to limit tumor heterogeneity and stall a cancer's evolution?

The answer may lie in studying some of the effects HSP90 inhibition has on whole organisms. When HSP90 is inhibited in the small flowering plant, *Arapidopsis thaliana*, multiple changes occurred in the plant including organ number defects, deformed leaves, and smaller quantities of seeds (Queitsch et al., 2002; Sangster et al., 2007). HSP90 inhibition or inactivation in *Drosophila melanogaster* produced a population of flies with limb like structures growing out of the eye that normally was not there (Sollars et al., 2003). In each of these cases, inhibition of HSP90 during the organisms' development generated new phenotypes that would not likely

promote the long-term success of that organism in its natural environment. In other words, they are less fit than their wild type counterparts. This decrease in fitness was directly postulated in the study of *A. Thaliana* (Sangster et al., 2007). The disordered eye phenotype in *D. melanogaster* is not likely to result in reproductive success of that fly in an uncontrolled, natural setting, since finding a mate and avoiding predators is certainly easier with vision completely intact. Indeed, it would be very rare to naturally find a fruit fly with an appendage growing out of its eye raiding that decomposing banana peel in your trash!

In sum, HSP90 inhibition may reveal phenotypic diversity, but this diversity generates evolutionary "dead ends" in an uncontrolled setting. Something similar may be happening in the case of cancers that undergo HSP90 inhibition. Therefore, targeting HSP90 may limit the evolution of cancers in two ways. First, by abrogating potentiation of the cancer hallmarks that ultimately manifests in ITH. Second, by discharging cryptic variation stored in HSP90's capacitor function, effectively revealing evolutionary "dead ends" that are incompatible with long-term tumor maintenance.

HSP90 Inhibitors

Targeting HSP90 with small molecule inhibitors to treat cancer has been the center of considerable debate for almost 50 years. The first HSP90 inhibitors discovered were geldanamycin and radicicol, two naturally occurring compounds that demonstrated anti-cancer activity *in vitro*. It was soon observed that they destroyed cancer by destabilizing the interaction between HSP90 and its clients that are also major oncogenes (Blagosklonny, Toretsky, Bohen, & Neckers, 1996; Grenert et al., 1997; Whitesell, Mimnaugh, De Costa, Myers, & Neckers, 1994). For example, oncogenes like v-SRC and the dominant negative form of p53, major drivers of carcinogenesis, rely on HSP90 to maintain oncogenic stability (Walerych et al., 2004; C. Wang

& Chen, 2003; Xu & Lindquist, 1993). If oncogenes lose interaction with HSP90, they are targeted for degradation by the proteasome, thus limiting their role in orchestrating neoplastic behavior. Moreover, the unusual conformation ATP adopts in the nucleotide-binding pocket of HSP90 is also achieved by geldanamycin and radicicol, making these compounds very selective in targeting HSP90 function. This is unlike other cancer drugs that target the ATP binding pocket of kinases, which share significant homology in the ATP binding pocket with other kinases, leading to off target effects.

These observations generated a flurry of research interest in HSP90 inhibitors to treat cancer. It also garnered a fair amount of skepticism, and for good reason. Since HSP90 is important in maintaining viability in normal cells, many believed that HSP90 inhibition would lead to overwhelming adverse effects, rendering them inappropriate for clinical application. At first, skeptics appeared to be correct as both geldanamycin and radicicol failed in clinical trials due to toxic effects and poor solubility. This propelled research to improve the solubility profile and pharmacodynamics of HSP90 inhibitors. Further research would reveal that HSP90 inhibitors have the highest affinity for tissues that have HSP90 in its "activated" form compared to tissues with HSP90 in a latent state (Kamal et al., 2003). It appears that cancer cells have much of its HSP90 in an active complex (Kamal et al., 2003), likely because it is busy maintaining mutated gene products of the cancer hallmarks. This makes HSP90 inhibitors both specific to their target through the unique GHKL binding site and specific to neoplastic tissue, a highly coveted property of any cancer therapy. Further studies have shown that certain HSP90 inhibitors can accumulate in neoplastic tissue relative to normal tissues (Eccles et al., 2008; Jensen et al., 2008), suggesting that HSP90 inhibitors could still be effective against cancers if given at low doses in the right frequency.

Research efforts and advances in medicinal chemistry have led to the development of over a dozen different HSP90 inhibitors to date. Improvements in the solubility profiles of these drugs have permitted many of them to progress through various phases of the clinical trial process. HSP90 inhibitors like 17-AAG, 17-DMAG, and AUY922 have all progressed past phase I trials; another HSP90 inhibitor, ganetespib (STA-9090), made it as far as phase III. None have been approved, but clinical trials are still active or recruiting for multiple cancers. What we have learned from clinical trials is that HSP90 inhibitors can deliver a specific cytotoxic effect to cancer cells, and that the best responses are achieved when HSP90 inhibitors are used in combination with other therapies (Bendell et al., 2015; Johnson et al., 2015; Kong et al., 2016) (Johnson et al., 2015; Modi et al., 2011).

Despite improvements in the pharmacology of HSP90 inhibitors and some measurable success in clinical trials, adverse effects and inconsistent tumor responses continue to hamstring their progression to an approved therapy for cancer. Concerns regarding parallel induction of the cancer's survival response, impairment of antitumor immune mechanisms, cardiac arrhythmias, and hepatotoxicity have emerged from clinical trials (Whitesell & Lindquist, 2005).

These trepidations may be due to the nature of the clinical trial process for cancer chemotherapies. When a chemotherapy enters phase I clinical trials, a small group of patients are enrolled, and the drug's efficacy is evaluated by titrating to the maximum tolerated dose (MTD) a patient can physiologically withstand and weighing the costs of adverse effects to the benefits of tumor response to therapy. If a proper balance can be achieved, the chemotherapy under investigation moves on to the next phase of the clinical trial. In the subsequent phases, the chemotherapy will be compared to current gold standard treatments and the cohort sizes will grow considerably, but the dose administered will continue to be given proximal to the MTD.

What is interesting in terms of HSP90 inhibitors is that a fair number of them pass through phase I, indicating a tolerable level can be achieved; but, they never get approved as a single agent for cancer therapy due to inconsistent tumor responses relative to the gold standard treatments. The source of inconsistency may be derived from the high doses demanded by the clinical trial process, causing global cellular stress and inadvertent induction of the heat shock response. As previously mentioned, the heat shock response is an adaptive mechanism, and its unintended activation hands the cancer a tool to promote evolution and survival, rather than limit it. Also, administering high doses of HSP90 inhibitors does not maximize the increased affinity HSP90 inhibitors have for neoplastic tissue.

If HSP90 inhibitors are specific to their target (Kamal et al., 2003) and can accumulate within neoplastic tissue *in vivo* (Eccles et al., 2008; Jensen et al., 2008), low dose administration at strategic frequencies could be a clinically viable approach to remove issues associated with inadvertent induction of the heat shock response. Furthermore, the ability for HSP90 inhibitors to shut down multiple cancer hallmarks simultaneously may dampen the formation of excessive ITH, which in turn slows the clock on tumor evolution and disease progression. This may place HSP90 inhibitors as a unique chemotherapy that can be used specifically to confront drug resistance and metastasis in cancer. This is already starting to be revealed in studies showing HSP90 inhibition to be useful in abrogating development of resistance to certain chemotherapies (Jacobson et al., 2016; Nagaraju et al., 2019; Whitesell et al., 2014) and deconstructing molecular pathways involved in epithelial to mesenchymal transition (EMT), migration and metastasis *in vitro,* and, in animal xenografts of different human cancer cell lines (Chong et al., 2019; Nagaraju et al., 2015).

Furthermore, evidence from clinical trials indicates that HSP90 inhibitors can be safely

paired with conventional therapies (Bendell et al., 2015; Eroglu et al., 2018; Johnson et al., 2015). Since HSP90 inhibitors can attenuate metastasis and drug resistance, it suggests that they may be especially useful to pair with anti-cancer agents that are very potent early in the treatment process, but are known to promote evolution of drug resistance and metastasis later, like paclitaxel (Datta et al., 2017) (Karagiannis et al., 2017; Volk-Draper et al., 2014).

In the following chapters, we examine the clinically relevant HSP90 inhibitor, AUY922, in its ability to curtail both metastatic and drug resistant phenotypes in cancer. We investigate these processes in the context of non-small cell lung cancer (NSCLC), which is often drug resistant and metastatic at the time of diagnosis or during the treatment process (Gabor et al., 2004; Popper, 2016; Shanker, Willcutts, Roth, & Ramesh, 2010; Sosa Iglesias, Giuranno, Dubois, Theys, & Vooijs, 2018). Our results indicate that equivalent doses of AUY922 below those that are currently recommended in clinical trials can restrict the acquisition of drug resistant and metastatic phenotypes *in vitro*. We then discuss repurposing small molecule HSP90 inhibitors as the first form of therapy designed to curb tumor evolution and disease progression, which may be used alongside conventional therapies to generate sustained tumor remission.

Chapter 2: Low Dose HSP90 Inhibition with AUY922 Blunts Rapid Evolution of Metastatic and Drug Resistant Phenotypes Induced by TGF-β and Paclitaxel in A549 cells

Abstract

Despite advances in cancer treatment, metastasis and drug resistance continue to contribute to treatment failure. Since these are features that often occur toward the late stages in the disease after withstanding numerous selective pressures, they may rely on a shared adaptive mechanism in order to persist. The heat shock response is one of the most well conserved adaptive responses to cellular stress found in nature. A major player in the heat shock response is HSP90, and some studies suggest it can facilitate the evolution of drug resistance and metastasis in cancer. Non-small cell lung cancers (NSCLCs) are strongly associated with metastasis and drug resistance either at the time of diagnosis or early in the treatment process. We explored the role of HSP90 in the evolution of metastatic and drug resistant features in NSCLC by treating A549 cells with AUY922, a clinically relevant HSP90 inhibitor, and inducing metastatic and drug resistant phenotypes via TGF-β and paclitaxel, respectively. We used flow cytometry to measure changes in E-Cadherin, a marker for epithelial to mesenchymal transition (EMT) and two ABC transporters associated with drug resistant lung cancers. We found that metastatic and efflux dependent drug resistant features negatively correlated with AUY922 treatment. We followed our results with functional assays relevant to metastasis and ABC transporters to confirm our results. Together our data indicates that HSP90 inhibition with AUY922 can limit the acquisition of metastatic and drug resistant phenotypes in A549 cells at equivalent, clinically appropriate doses.

Background

Lung cancer is the most commonly diagnosed cancer and leading cause of cancer mortality worldwide regardless of sex (Bray et al., 2018). 85% of lung cancers are non-small cell lung cancers (NSCLC) (Herbst, Morgensztern, & Boshoff, 2018), with metastasis and chemotherapeutic resistance common features of these cancers either at the time of diagnosis or manifesting during the treatment process (Gabor et al., 2004; Popper, 2016; Shanker et al., 2010; Sosa Iglesias et al., 2018). Therefore, discovering ways to mitigate metastasis and chemotherapeutic resistance in NSCLCs will be beneficial to a substantial number of cancer patients.

Metastasis is the spread of cancer cells from the primary tumor site to distant organs in the body where they grow and disrupt normal organ function (Fidler & Kripke, 2015). The step-wise process by which carcinomas undergo the complex changes in cell signaling and gene expression required to complete metastasis is referred to as epithelial-mesenchymal transition (EMT) (Kalluri & Weinberg, 2009). The initial stages of EMT in carcinomas begin with loss of cell-cell junctions. A major protein involved in maintaining cell-cell junctions that is lost during EMT is E-cadherin (Onder et al., 2008). Loss of E-cadherin facilitates dissolution of cell-cell junctions, permitting cancer cells to migrate from their primary site as they undergo further changes to invade local vasculature to travel to distant organs in the body. Numerous studies in human tumor samples have demonstrated that reduced E-cadherin expression is associated with dedifferentiation and lymphogeneous spread (Cheng, Nagabhushan, Pretlow, Amini, & Pretlow, 1996; Dorudi, Sheffield, Poulsom, Northover, & Hart, 1993; Oka et al., 1992; Schipper et al., 1991; Siitonen et al., 1996), including NSCLCs (Lee, Wu, Chen, & Chang, 2000; Lim, Jang, Kim, & Park, 2000; Sulzer, Leers, van Noord, Bollen, & Theunissen, 1998). This makes E-

cadherin a reliable and clinically relevant marker for *in vitro* study of metastasis in NSCLC cell lines.

Chemotherapeutic resistance can manifest as a result of cell cycle alterations, insensitivity to apoptosis, altered drug metabolism, increased DNA damage repair, and increased drug efflux (Szakacs, Paterson, Ludwig, Booth-Genthe, & Gottesman, 2006). Increased drug efflux is a common mechanism cancer cells use to protect themselves from chemotherapeutic damage and is mediated by a family of transmembrane transporters known as ATP-binding cassette (ABC) transporters (Fletcher, Williams, Henderson, Norris, & Haber, 2016). There are 48 known ABC transporters, and three, ABCB1, ABCC1, and ABCG2, are most commonly found to drive efflux dependent drug resistance in human cancers (Sharom, 2008). This is presumably because these three transporters have considerable chemotherapeutic substrate overlap (Sharom, 2008). Two of these ABC transporters, ABCB1 and ABCC1, are commonly overexpressed in human NSCLC tumors (Nooter et al., 1996; Sugawara et al., 1995; Wright et al., 1998), and can play major roles in conferring multidrug resistance in NSCLCs (Berger et al., 2005; Oshika et al., 1998; Ota et al., 1995; Volm, Mattern, & Samsel, 1991). Despite these studies, there is some controversy as to whether ABC transporters play a significant role in drug resistant cancers, as studies employing ABC transporter inhibitors during cancer treatment failed to show clinical benefit (Binkhathlan & Lavasanifar, 2013). Recently, however, some experts in the field of ABC transporters are calling for a reevaluation of ABC transporters in cancer by pointing out inconsistencies and pitfalls in previous studies that dismissed the role of ABC transporters in multi drug resistant cancers (Robey et al., 2018). In any case, since these molecular pumps physically interact with common chemotherapies and can alter the pharmacokinetics and bioavailability of other drugs, their study should not be totally ignored.

It is understood that metastatic and drug resistant cancer cells within a tumor emerge as a consequence of the complex dynamics of tumor evolution (Foo & Michor, 2014; Gatenby & Brown, 2018; Turajlic & Swanton, 2016). Given the selective pressures of chemotherapy and the harsh tumor microenvironment to overcome, there may be a common adaptive mechanism that metastatic and drug resistant cancer cells share in order to persist in these stressful conditions and contribute to disease progression. An adaptive response to stress that is fundamental to all organisms is the heat shock response (Schlesinger, 1990). HSP90 is a major player in the heat shock response known to influence numerous essential cell processes. Previous studies have shown HSP90 to facilitate evolution of novel phenotypes (Jarosz & Lindquist, 2010; Queitsch et al., 2002; Rohner et al., 2013; Sollars et al., 2003), including drug resistant phenotypes (Cowen & Lindquist, 2005; Vincent, Lancaster, Scherz-Shouval, Whitesell, & Lindquist, 2013; Whitesell et al., 2014). HSP90 also enables the metastatic cascade, as pointed out in an exhaustive review (Tsutsumi, Beebe, & Neckers, 2009). Clinical evidence supports this hypothesis, as HSP90 overexpression in gastric and breast cancers correlates with high tumor grade, depth of invasion, and lymph node involvement (Pick et al., 2007; J. Wang et al., 2013), all indicators of strong induction of the metastatic cascade. Furthermore, increased HSP90 expression in various human cancers correlates with a poor prognosis (Flandrin et al., 2008; Pick et al., 2007; Thomas et al., 2005; J. Wang et al., 2013; Zagouri, Bournakis, Koutsoukos, & Papadimitriou, 2012).

Several lines of recent evidence indicate that pharmacological HSP90 inhibition can limit molecular pathways involved in EMT, migration and metastasis *in vitro* and in animal xenografts of different cancer cell lines (Chong et al., 2019; Nagaraju et al., 2015). Whether other clinically relevant HSP90 inhibitors can limit these processes in NSCLC cell lines has not been documented. Furthermore, certain HSP90 inhibitors have demonstrated synergistic anticancer

effects with conventional cytotoxic chemotherapies such as paclitaxel (Munster, Basso, Solit, Norton, & Rosen, 2001; Solit, Basso, Olshen, Scher, & Rosen, 2003) and doxorubicin (Munster et al., 2001). While the results of these studies attributed the synergistic effect to mechanisms related to cell signaling, paclitaxel is a known substrate for ABCB1 and doxorubicin is a known substrate for both ABCB1 and ABCC1 (Cole et al., 1992; Sharom, 2008). This leaves open the possibility that perhaps some of the synergistic effects of HSP90 inhibition may be due to intracellular accumulation of paclitaxel and doxorubicin as a result of altered ABC transporter expression. The theoretical basis for this partly exists due to the fact that ABCC1 is a verified HSP90 client (Picard, 2019), and HSP90 inhibition classically results in degradation of client proteins. However, no relationship between HSP90 inhibition and reduced expression of ABC transporters has been demonstrated in the context of multidrug resistant cancer to date.

In the present study we ask whether treatment with the clinically relevant HSP90 inhibitor, AUY922, given at the same dose and exposure time, could limit rapid acquisition of metastatic traits and ABC transporter driven drug resistance in A549 cells, a common NSCLC cell line. AUY922 is unique among HSP90 inhibitors as it is readily soluble in ethanol and can inhibit both the inducible (HSP90α) and constitutive (HSP90β) isoforms of HSP90 at low nanomolar concentrations (Eccles et al., 2008; Garon et al., 2013). We were guided by the work of others who used relatively low doses of HSP90 inhibition to abrogate emergence of drug resistant phenotypes in MCF-7 breast cancer cells (Whitesell et al., 2014). If successful, our work would further strengthen the case for other clinically relevant HSP90 inhibitors, and AUY922 in particular, to be deployed in the clinic at low doses as an adjunct to prevent acquisition of drug resistance and metastasis that can be associated with some cytotoxic chemotherapies (Karagiannis et al., 2017; Volk-Draper et al., 2014). Furthermore, successfully

utilizing smaller doses of chemotherapy can be expected to improve the quality of life for cancer patients undergoing chemotherapy.

Materials and Methods

Cell Culture

A549 cells were purchased from the European Collection of Authenticated Cell Cultures (ECACC)(Account #: 86012804; Lot: 165012) and cultured in RPMI-1640 media supplemented with 10% FBS and 25mM HEPES buffer. All experiments were performed between passages 5 and 20 from the original purchased vial.

Cell Viability Assay (XTT)

The viability of A549 cells under AUY922 and paclitaxel treatment was assessed in parallel using the XTT assay (Biotinium; catalog #: 30007) per manufacturer recommended protocol. Plates were read on Spectramaxi3x® microplate reader at wavelength 450nM. Each experiment was performed three times.

Western Blot

A549 cells were seeded in six-well plates at $4.0x10^5$ cells/well and allowed to attach overnight. The next day, medium was aspirated and replaced with 1.0mL fresh growth medium and treated with indicated drug. After 6 hours, medium was aspirated and the cells were harvested, quantified, and transferred to nitrocellulose membranes as described(Mahmood & Yang, 2012). Primary antibodies were used at a dilution of 1:1500 mouse anti-HSP70 (Enzo,ADI-SPA-810-D, clone: C92F-3A-5) and 1:10000 mouse anti-GAPDH (cat#:12345). Species-specific europium-conjugated secondary antibody (ScanLater goat anti mouse; part#: R7562; Molecular Devices®) was used. Afterward, membranes were washed with TBS-T and

analyzed via Spectramaxi3x® western blot cartridge (Molecular Devices®). Two independent experiments were performed in triplicate (n=6).

FOUR DAY EMT INDUCTION IMMUNOPHENOTYPING

A549 cells were seeded into six-well plates (50,000 cells/well) and allowed to attach overnight. Medium was aspirated and replaced with 2.0mL fresh growth medium and treated with drug for 48 hours. After, medium was aspirated from each well and replaced with 2.0mL of fresh growth medium. One plate from each treatment group was treated with 10ng/mL TGF-β (Peproptech, Cat#:100-21), while the others remained in untreated medium for an additional 48 hours. Afterward, cells were detached with trypsin for 15 minutes, placed into flow tubes, washed, stained for viability (Zombie-NIR, 77184, Biolegend), counted via typan blue, normalized to cell numbers, and blocked as previously described (Lawag et al., 2017). Samples were stained with the following fluorescently conjugated antibodies: Alexafluor®647 anti-human E-Cadherin (BD Biosciences, 56371, clone: 67A4), Alexafluor®488 anti-human ABCC1 (BioLegend, 370306, clone: QCRL-3), and Brilliant Violet ®421 ABCB1 (BD Biosciences, 566015, clone: UIC2) per sample for 30 minutes on ice protected from light. Samples were washed twice with 2.0mL cold FACS buffer, centrifuging at 300g for 5 minutes between washes. After second wash, samples were decanted and gently resuspended in residual buffer with a pipet tip to generate a homogenous single cell suspension and analyzed via flow cytometry. Three independent experiments were performed in triplicate for each treatment group (n=9).

WOUND HEALING ASSAY

A549 cells were seeded into six well plates (50,000 cells/well) and allowed to attach overnight. Cultures were treated with drug for 48 hours. After, cells were detached with trypsin, collected in separate 50mL tubes and spun at 300g for 5 minutes. Supernatant was decanted, and

pellets were resuspended in 1.0mL fresh growth medium. $3.0x10^5$ cells were collected from each tube and resuspended in 400µL of fresh growth medium. We empirically determined that this cell concentration would yield a confluent monolayer when seeded into 20µL drops and allowed to attach overnight (15,000 cells/drop). Three 20µL drops were seeded into the top row of three six well plates, one plate per treatment group (9 drops per treatment group in total) and allowed to attach overnight. Cells were then scratched with a 200µL pipet tip and 1.0mL of fresh growth medium was added to each well. Pictures and measurements were taken at time 0 hours and time 18 hours over the middle of the colonies by drawing a single straight line through the nuclei of cells on the left side of the migration front and dropping three perpendicular lines across the viewing field (one at the top, one through the middle and one at the bottom) to the migration front on the right. Migration index was calculated by dividing average starting gap width by the average gap width at 18 hours. Two independent experiments were performed (n=18 for each treatment group).

Trans-epithelial Electric Resistance (TER) Assay

A549 cells were seeded into 6 transwells (10,000 cells/well) and allowed to grow for 72 hours. At this time point, fresh medium was given, and three wells were treated with 20 nM of AUY922. TER measurements were taken at 24 hours within 5 minutes of removing from the incubator so as to eliminate the influence of temperature on our measurements. Final values were obtained by subtracting the resistance of the bathing solution and an empty support. Results are expressed as ohms per square centimeter ($\Omega \cdot cm^2$). To compare effects obtained in different monolayers on different days, we normalized data as described (Larre et al., 2010). Two independent experiments were performed in triplicate (n=6).

EIGHT DAY MDR INDUCTION IMMUNOPHENOTYPING

In these experiments we utilized what we learned from the up regulation of ABC transporters under paclitaxel treatment during the four day EMT induction experiments. We treated with AUY922 during the first 48 hours of the experiment as before, but instead of inducing changes in E-cadherin with TGF-β, we induced changes in ABC transporters with paclitaxel. We extended the experimental timeline from four days to eight days to provide cells pretreated with AUY922 an additional 2 days to expand in untreated media after their initial treatment during the first 48 hours of the experiment (expansion in untreated medium during days 2-4) and after induction of ABC transporters with paclitaxel during days 4-6 (expansion in untreated medium during days 6-8). A549 cells were collected from stock flask and thirty 20μL drops (8,000 cells/drop, $2.4x10^5$ cells/petri dish) were seeded into 100mm petri dishes and allowed to attach overnight. This method of seeding provided reproducible circular colonies over the course of the experiment that captures some of the three dimensional dynamics that exists within a tumor, with cells at the periphery having fewer cell neighbors and more access to space and nutrients compared to cells in the middle of the colony (a "pseudo-slice" of the tumor). The following day, 7.0 mL of fresh growth medium was added to each petri dish and cells were treated with AUY922. After 48 hours, treated medium was aspirated and replaced with 8.0mL of fresh untreated growth medium. After an additional 2 days, medium was aspirated and replaced with 10.0mL of fresh growth medium. At this time point some petri dishes were treated with 10nM of paclitaxel (MDR induction), while the others remained untreated (no MDR induction). Petri dishes were allowed to grow for an additional 2 days. On day 6, medium was aspirated from each petri dish and replaced with 10.0mL of fresh untreated growth medium and allowed to grow for an additional 2 days. On day 8, cells were harvested. The entire growth medium from

each petri dish was placed into separate 50mL tubes and petri dishes were washed once with 5.0mL PBS, which was subsequently aspirated. Cells were detached with trypsin/4mM EDTA for 20 minutes at 37°C, and placed into corresponding 50mL tubes with previously collected growth medium. Cells were washed, stained for viability, counted, normalized to cell numbers, and blocked as previously described (Lawag et al., 2017). Each sample was stained with fluorescently conjugated antibodies: Alexafluor®488 anti-human ABCC1 (BioLegend, 370306, clone:QCRL-3), and Brilliant Violet ®421 ABCB1 (BD Biosciences, 566015, clone:UIC2) per sample for 30 minutes on ice protected from light. Samples were washed twice with 2.0mL cold FACS buffer, decanted and centrifuged at 300g for 5 minutes between washes. After second wash, samples were decanted and pellets gently resuspended in residual cold FACS buffer with a pipet tip to generate a homogenous single cell suspension and analyzed via flow cytometry. At least two independent experiments were performed with at least two biological replicates per treatment (no MDR induction n=5, MDR induction n=8).

DOXORUBICIN EFFLUX ASSAY

A549 cells were seeded in thirty 20µL drops in 100mm petri dishes, and cultured as described in the eight-day experiment for MDR induction. On day eight, cells were harvested and $2.5x10^5$ cells were placed into flow tubes, washed with growth medium and centrifuged at 300g for 5 minutes. Supernatant was decanted, and pellets were gently resuspended in 500µL of warm growth medium treated with 10µM of doxorubicin. Cells were incubated at 37°C for 30 minutes to load with doxorubicin. Samples were washed in growth medium and centrifuged at 300g for 5 minutes. Supernatant was decanted, and pellets resuspended in 1.0mL of growth medium. Samples were incubated at 37°C with agitation for 2 hours. Afterward, samples were washed with 2.0mL of room temperature PBS followed by centrifugation at 300g for 5 minutes.

Supernatant was decanted, and pellets were resuspended in 200µL of Zombie-NIR viability reagent, which was applied per manufacturer's instructions. After the final wash and decant, cells were gently resuspended in residual cold FACS buffer with a pipet tip to generate a homogenous single cells suspension and analyzed via flow cytometry. Three independent experiments were performed in at least duplicate (n=8).

Four, Six and Eight Day Culture Growth

A549 cells were collected from stock flask, seeded in 20µL drops in 100mm petri dishes, and cultured as described in the eight-day experiment for both non-MDR induction and MDR induction groups. On day four and six, cells were washed and detached as described in the eight-day experiment. Instead of resuspending in 1.0mL of zombie reagent, cells were resuspended in 1.0mL of fresh growth medium, and a 10µL aliquot was taken from each sample to count via hemacytomter and 0.4% typan blue staining. For samples without MDR induction with paclitaxel, a single experiment was performed in triplicate for day four and day six (n=3 for each day). For samples with MDR induction with paclitaxel, two independent experiments in triplicate were performed on days four and six (n=6 for each day). Day eight counts were a combination of counts gathered on day eight from MDR induction immunophenotyping and doxorubicin efflux studies.

Flow Cytometric Analysis

All flow cytometric analysis were performed via Novocyte ® 3000 flow cytometer (ACEA Biosciences, Inc., San Diego, California). 50,000 single cell events were analyzed. All data analysis was performed using NovoExpress Software version 1.3.0 (ACEA Biosciences, Inc., San Diego, California). For all antibodies, we used fluorescence minus one (FMO) and corresponding isotype controls to determine positive staining background. All data analysis was

performed using NovoExpress Software version 1.3.0 (ACEA Biosciences, Inc., San Diego, California).

Statistical Analysis

All statistical analysis was performed using Prism8.0a software (GraphPad Software Inc., San Diego, California). Appropriate statistical tests were performed with corresponding significance values indicate in figure legends. For the E-cadherin flow cytometry studies and wound-healing assay, analysis was performed using one-way ANOVA with Tukey's test for multiple comparisons. For trans-epithelial resistance, eight-day flow cytometry studies without MDR induction, eight-day growth curves, and doxorubicin efflux studies, analysis was performed using Student's t test for unpaired data. For ABC transporter flow cytometry studies with/without EMT and with MDR induction, analysis was performed using one-way ANOVA with Dunnet's test for multiple comparisons.

Results

AUY922 and Paclitaxel have Similar Effects on A549 Cell Viability after 48 Hours Exposure

Before investigating metastatic and drug resistant properties of A549 cells, we had to evaluate their sensitivity to AUY922 and paclitaxel. Paclitaxel was used in many of our studies as it is currently used in combination with other chemotherapies in NSCLC and has been associated with development of metastasis (Karagiannis et al., 2017; Volk-Draper et al., 2014) and ABC transporter driven drug resistance (Datta et al., 2017). Intriguingly, A549 cells displayed almost identical viability curves to increasing doses of either drug at 48 hours of exposure **(Figure 6A).** Since we were most interested in evaluating how A549 cells change in

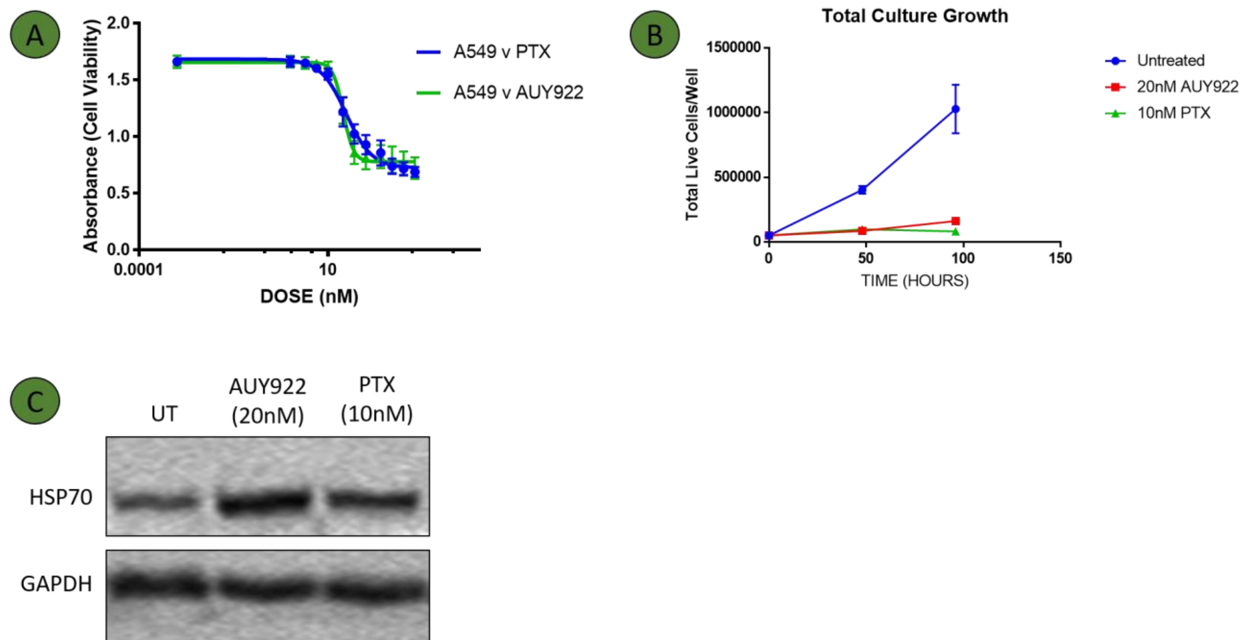

Figure 6. Chemosensitivy of A549 Cells to Paclitaxel and AUY922 and HSP70 Induction (A): Chemosensitivity of A549 cells to 48 hour exposure to increasing concentrations of Paclitaxel (blue) and AUY922 (green) ranging from 0.001-2,000nM. Viability was determined using the XTT assay. Each dose was performed in sextet per individual experiment. Data represents the mean ±95% CI of three independent experiments. EC50 A549 v PTX: 30.2 (95% CI 25.0-36.8nM); EC50 A549v AUY922: 25.4 (95% CI 22.3-28.5nM) (B) Growth curves of A549 cells after 48 hours of indicated drug followed by 48 hours of untreated medium. (C) Induction of HSP70 expression by HSP90 inhibition with AUY922 after only six hours of exposure. A representative blot of two independent experiments performed in triplicate is shown.

response to these drugs, not necessarily how they are killed, we wanted to refrain from using high doses of either drug in our experiments to avoid selecting for a particular metastatic or drug resistant phenotype in the A549 cell population. We decided we could achieve this by focusing on doses below the EC50, which in this case would be doses less than 25nM for AUY922 and less than 30nM for Paclitaxel **(Figure 6A).**

Since paclitaxel can exhibit additional growth inhibition beyond 48 hours **(Figure 6B),** we decided that 10nM treatment with paclitaxel would be appropriate to further ensure we do not select for any particular phenotype, as our planned experimental endpoints were to extend well beyond 48 hours. We chose 20nM of AUY922 as this dose was enough to demonstrate up

regulation of HSP70 **(Figure 6C)** and is below the EC50. Up regulation of HSP70 is considered a reliable indicator of successful pharmacological HSP90 inhibition (Whitesell et al., 2014). We used C_{max} values gathered from clinical trials (Doi et al., 2014; Kong et al., 2016; Seggewiss-Bernhardt et al., 2015; Sessa et al., 2013) to confirm that 20nM of AUY922 is well within a clinically relevant treatment range.

AUY922 Promotes E-cadherin Surface Expression in A549 Cells, Which Remains Durable after EMT Induction with TGF-b

Induction of EMT is a well-known method to study metastasis *in vitro* and is thought to drive metastatic changes *in vivo* (Tsai & Yang, 2013; Zhao et al., 2016). A hallmark of EMT is loss of E-cadherin and studies have demonstrated reduced E-cadherin expression in NSCLC tumors derived from clinical samples correlates with poor tumor differentiation and invasion of local structures including vasculature (Lee et al., 2000). Therefore, we reasoned that E-cadherin would be a suitable marker to evaluate changes related to metastatic progression that would translate to the clinic.

We exposed A549 cells to 20nM of AUY922 or 10nM of paclitaxel for 48 hours, followed with 48 hours in untreated media, and captured phenotypic changes via flow cytometry. Treatment with AUY922 promoted the E-cadherin positive fraction while paclitaxel reduced E-cadherin positive fraction, relative to the control **(Figure 7 A, B).** Additionally, there was a significant increase in median fluorescence intensity (MFI) in AUY922 treated group cultures relative to either paclitaxel treated cultures or control cultures, an indication that the positive fraction had an overall increase in E-cadherin expression per cell in the positive fraction **(Figure 7C).**

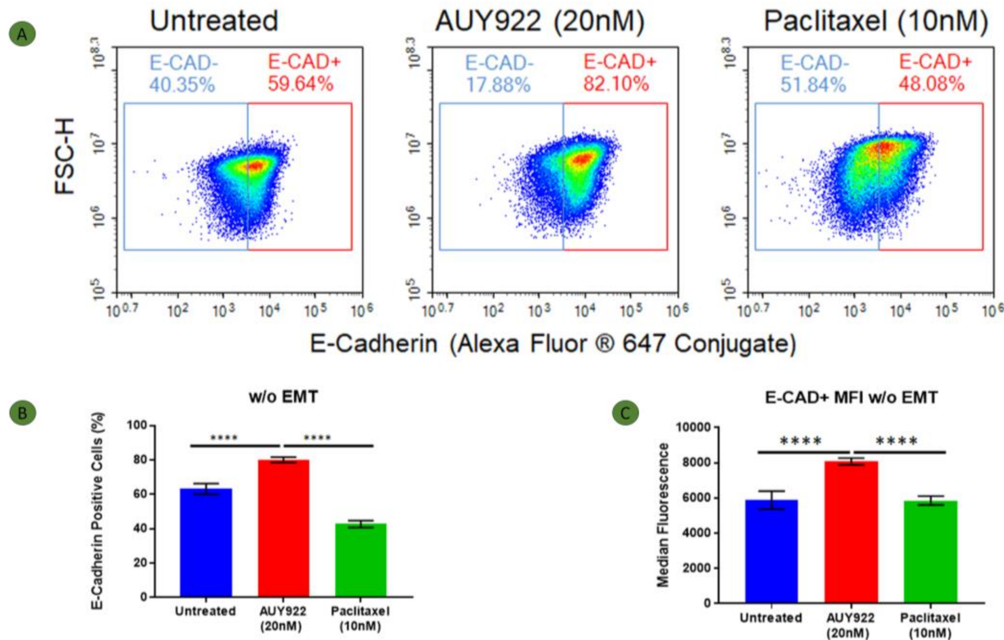

Figure 7. E-cadherin Immunophenotyping without EMT Induction
(A) Flow cytometric analysis of E-Cadherin expression after 48 hours of treatment followed by 48 hours in untreated medium (w/o EMT induction). (B) Quantification of the percentage of cells positive for E-Cadherin w/o EMT induction. Data is representative of the mean ±SEM of three independent experiments performed in triplicate (n=9). (C) Quantification of the median fluorescence intensity of the E-cadherin positive fraction. Data is representative of the mean ±SEM of three independent experiments performed in triplicate (n=9). ****P<0.0001.

To evaluate the durability of the increase in E-cadherin expression triggered by AUY922, we decided to follow the initial 48-hour treatment period with 48 hours in TGF-β treated media. TGF-β is a well-known and potent inducer of EMT in A549 cells (Kasai, Allen, Mason, Kamimura, & Zhang, 2005). While 20nM of AUY922 did not completely desensitize A549 cells to TGF-β driven EMT in these cultures, both the percent positive fraction and the median fluorescence were significantly higher than either the paclitaxel treated or control cultures **(Figure 8A-C).** Together, this data suggests that AUY922 generates changes in A549 cells that promote E-cadherin expression that remains durable even in the presence of strong drivers of the metastatic cascade such as TGF-β, relative to paclitaxel treatments or control cultures.

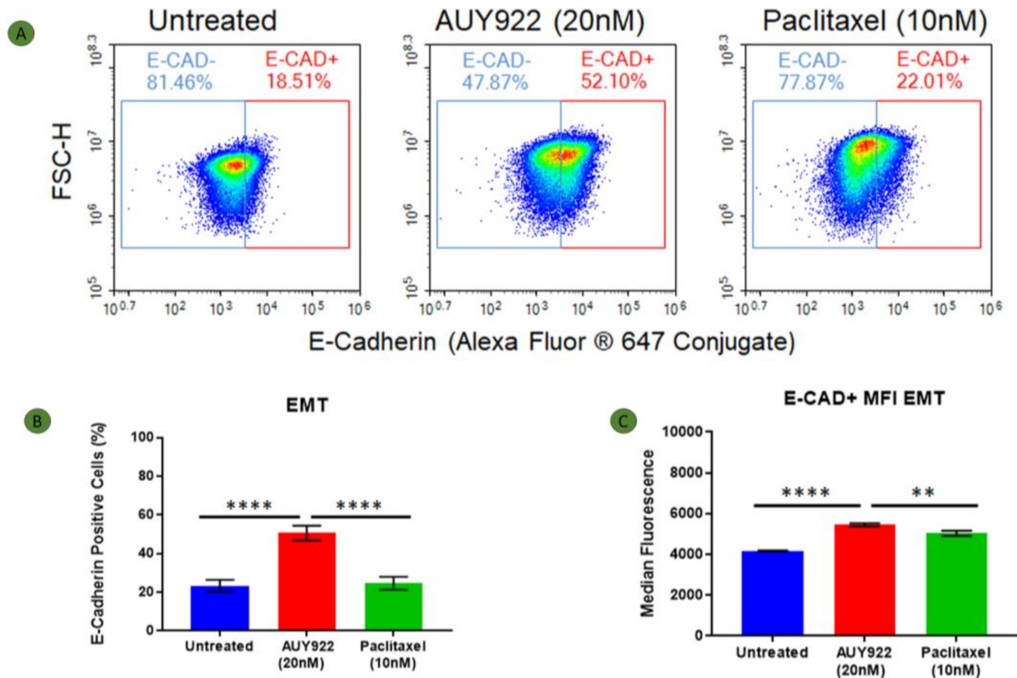

Figure 8. E-cadherin Immunophenotyping with EMT Induction
(A) Flow cytometric analysis of E-Cadherin expression after 48 hours of treatment followed by 48 hours EMT induction in medium supplemented with 10ng/mL of TGF-β. (B) Quantification of the percentage of cells positive for E-Cadherin with EMT induction. Data is representative of the mean ±SEM of three independent experiments performed in triplicate (n=9).
(C) Quantification of the median fluorescence intensity of the E-cadherin positive fraction with EMT induction. Data is representative of the mean ±SEM of three independent experiments performed in triplicate (n=9). **P<0.01; ****P<0.0001.

AUY922 LIMITS MOTILITY OF A549 CELLS

If E-cadherin loss is associated with increased invasion and metastasis, then it is reasonable that the increase in E-cadherin we observed with AUY922 treatment could influence the migratory capacity of A549 cells. To investigate this, we exposed A549 cells to 20nM of AUY922 and 10nM of paclitaxel for 48 hours and performed a wound-healing assay as described. As expected, AUY922 treated cells had drastically limited migration capacity compared to paclitaxel treated or control cultures **(Figure 9A, B).**

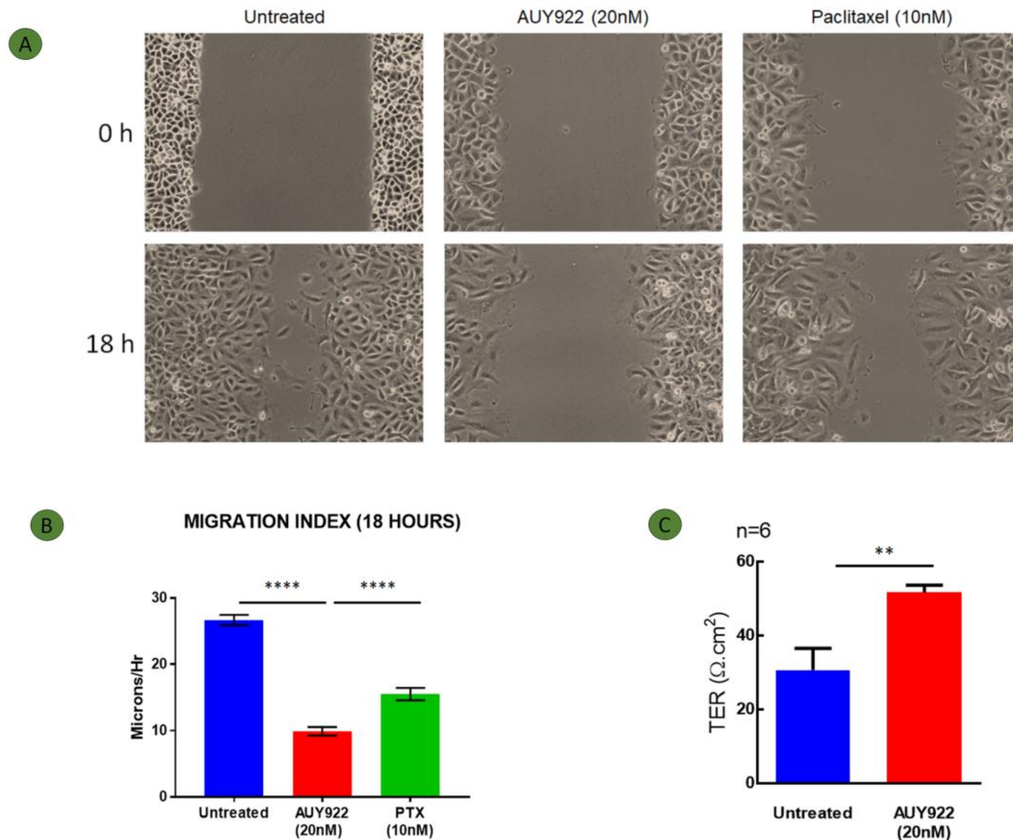

Figure 9: Effect of AUY922 Treatment on Migratory Capacity and Tight Junction Formation in A549 Cells.

(A) After 48 hours of drug exposure, cell numbers were normalized by reseeding in 20uL drops (15,000 cells/drop) and allowed to attach overnight. The circular colonies that formed were then scratched with 200uL pipette tip and pictures were taken at time 0 hours (top) and 18 hours (bottom). Two individual experiments were performed. Images are representative scratches from an individual colony. There were 9 total colonies per experiment per treatment group (n=18). (B) Quantification of mean migration index calculated by dividing starting gap width by the gap width at 18 hours. (C) Change in trans-epithelial electric resistance (TER) across confluent monolayers of A549 cells after 24 hours of 20nM AUY922 treatment. Two independent experiments were performed in triplicate (n=6); All data is representative of the mean ±SEM of two independent experiments performed as described**P<0.01, ****P<0.0001

AUY922 Promotes Tight Junction Formation in A549 Cells

The increase in E cadherin expression under AUY922 treatment prompted us to find additional details to identify a more definitive cause for the lack of migration in the scratch assay. Increased E-cadherin expression may indicate the promotion of tight junctions between cells (Mendonsa, Na, & Gumbiner, 2018). To investigate this, we plated A549 cells in transwell

plates and measured tight junction formation via trans-epithelia resistance (TER) in each of our previous treatment conditions. At 24 hours, there was a substantial TER increase in AUY922 treated cultures relative to control cultures **(Figure 9C).**

This observation helps explain the lack of migration of AUY922 treated A549 cells in the wound healing assays. By promoting tight junction formation, the increased intensity in cell-cell adhesion makes it less likely for the cells at the edge of the scratch to migrate into the gap. This places AUY922 as a potential cell-cell adhesion enhancer, which has benefits in the context of cancer metastasis as increases in cell-cell adhesion discourages cancer cells from migrating outside the primary tumor.

AUY922 Treatment does not Induce Surface Expression of ABCB1 or ABCC1 Compared to Paclitaxel Treatment

During the EMT induction experiments where we exposed A549 cells to 48 hours of AUY922 or paclitaxel and followed with 48 hours of untreated medium or TGF-β treated medium to measure E-cadherin expression, we also probed expression of ABCB1 and ABCC1. These two ABC transporters are also commonly known as P-glycoprotein (P-gp) and multidrug resistance protein 1 (MRP1), respectively. In agreement with others (Synold, Dussault, & Forman, 2001; Vesel et al., 2017), paclitaxel treatment in A549 cells promoted a slight increase in ABCB1 positive fraction relative to the control cultures at the end of the four day period, with enhanced ABCB1 positive fraction under induction of EMT with TGF-β in the final 48-hour time period **(Figure 10A-C)**. Paclitaxel treated cultures also had a similar increase in ABCC1

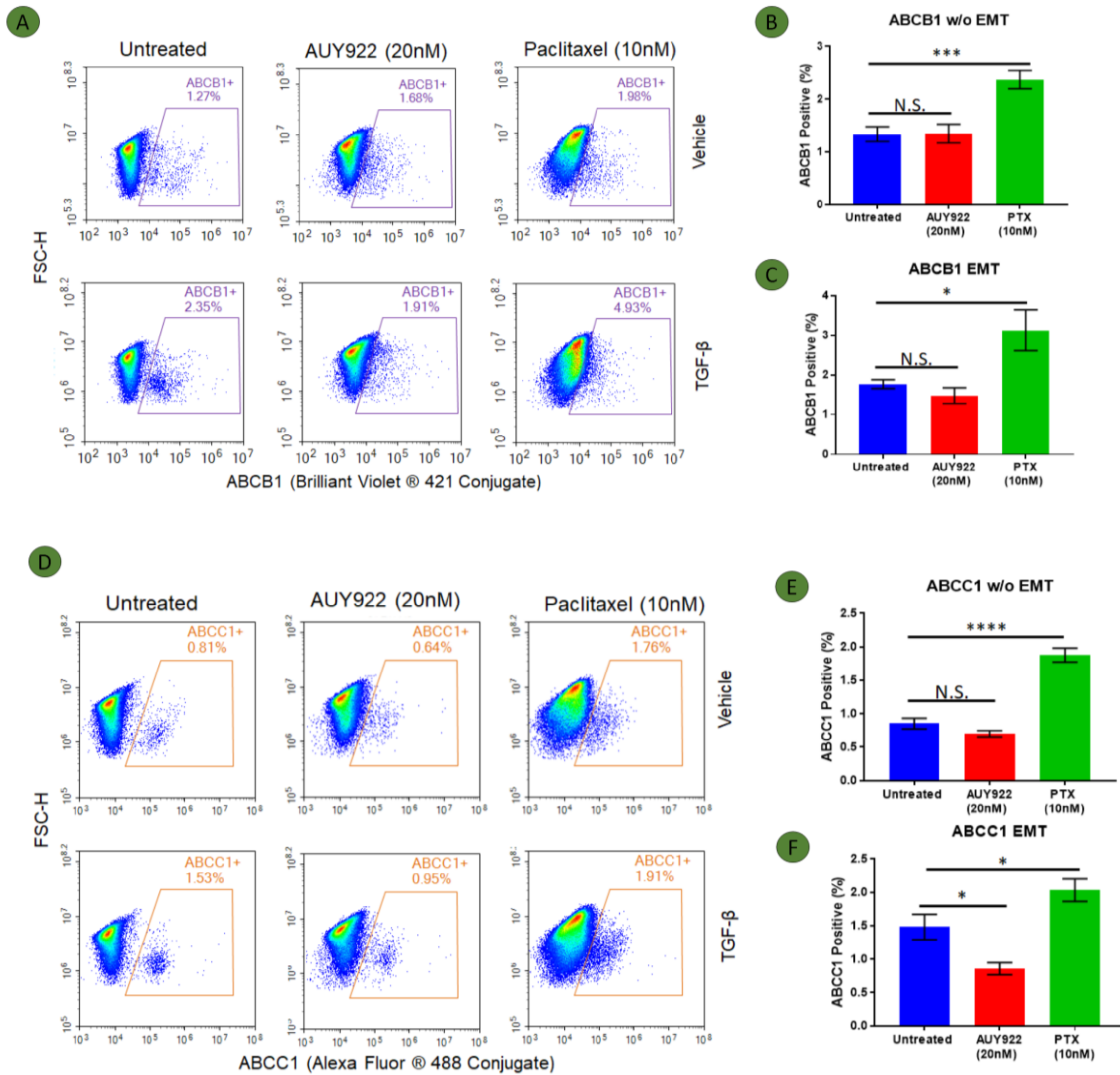

Figure 10: Drug Transporter Expression in EMT Induction Experiments.
(A) ABCB1 expression without EMT induction (Top) and with EMT induction with TGF-β (Bottom) of indicated drug treatments. (B) Quantification of the percentage of cells positive for ABCB1 without EMT induction. Data is representative of the mean ±SEM of three independent experiments performed in triplicate (n=9). (C) Quantification of the percentage of cells positive for ABCB1 and EMT induction. Data is representative of the mean ±SEM of three independent experiments performed in triplicate (n=9). (D) ABCC1 expression without EMT induction (Top) and with EMT induction with TGF-β (Bottom) of indicated treatments. (E) Quantification of three independent experiments performed in triplicate (n=9). (F) Quantification of percentage of cells positive for ABCC1 with EMT induction. Data is representative of the mean ±SEM of three independent experiments performed in triplicate (n=9). *P<0.05, ***P<0.001, ****P<0.0001.

positive fraction relative to the control with or without TGF-β in the final 48 hours before capture **(Figure 10D-F).**

In contrast, there was no increase in ABCB1 or ABCC1 relative to the control in AUY922 treated cultures regardless of TGF-β treatment (**Figure 10**). While the changes in AUY922 cultures were of little statistical significance compared to control cultures, considered as a whole there was a general downward trend, especially in AUY922 treated cultures exposed to TGF-β in the final 48 hours before capture **(Figure 10C, F).** This was interesting to us since A549 cultures treated with AUY922 received a chemical stimulus at twice the molar dose of paclitaxel cultures, yet remained insensitive to induction to part of the xenobiotic defense response for which ABC transporters evolved to carry out (Dean & Annilo, 2005). With this information, we formed a new strategy to maximize the resolution of our putative observations of the reduced positive fraction of ABCB1 and ABCC1 under AUY922 treatment. From here forward, loss or gain of ABCB1 and ABCC1 will be referred to as the MDR phenotype.

SINGLE TREATMENT WITH AUY922 REDUCES MDR PHENOTYPE AND DESENSITIZES A549 CELLS TO INDUCTION OF MDR PHENOTYPE VIA PACLITAXEL TREATMENT

In order to maximize the resolution of our observations of putative reduction in ABC transporter expression with AUY922 treatment, we altered the protocol as described in the methods. This experimental timeline mimics a scenario in which a cancer patient may receive multiple chemotherapies in cycles on alternating days, adding a level of clinical relevance to this experimental strategy.

Our adjustments to the experimental protocol proved to be successful. A549 cells treated with 20nM AUY922 for 48 hours followed by fresh media for the remaining 6 days

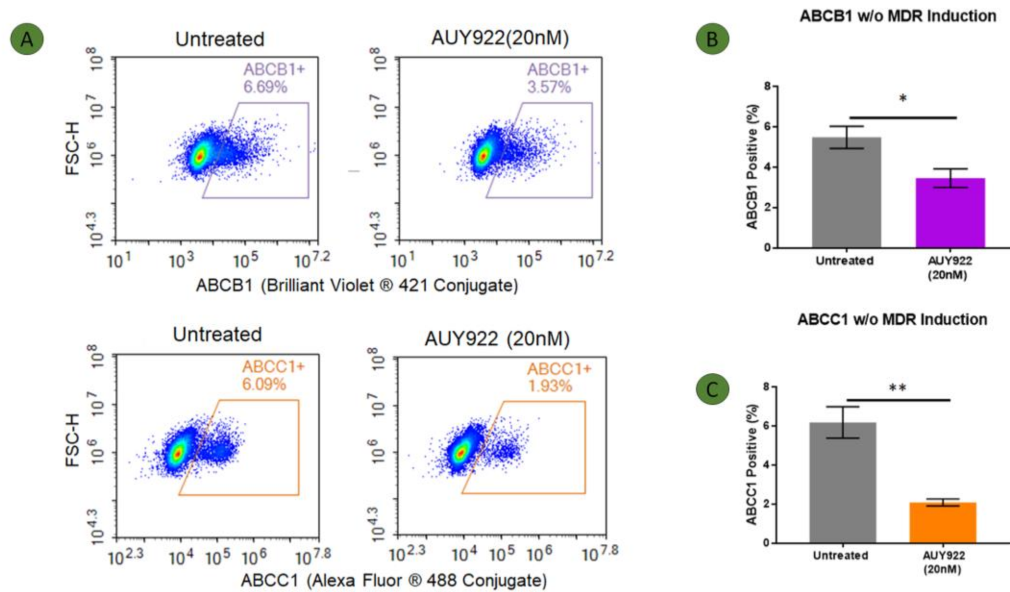

Figure 11: Flow Cytometric Analysis of ABC Transporters Captured at Day 8 after Indicated Drug Treatment.
(A) (Top) ABCB1 (Bottom) ABCC1 expression in A549 cells after a single treatment with 20nM AUY922. (B) Quantification of the percentage of cells positive for ABCB1 captured at day 8 (C) Quantification of the percentage of cells positive for ABCC1 captured at day 8. All data is representative of the mean ±SEM of two independent experiments performed at least in duplicate (n=5). *P<0.05, **P<0.01.

demonstrated reduced positive fraction of the MDR phenotype **(Figure 11A-C)** relative to control cultures that received fresh untreated media every 48 hours for 8 days **(Figure 11A-C)**.

Additionally, cells pretreated with 20nM AUY922 for the first 48 hours appeared to be insensitive to induction of the MDR phenotype with 10nM paclitaxel on days 4-6 compared to control cultures that only received 10nM of paclitaxel on days 4-6 and showed dramatic induction of the MDR phenotype **(Figure 12A-D).** The observation of reduced induction of the MDR phenotype under AUY922 treatment appeared to be dose dependent, as pretreatment with 10nM of AUY922 also demonstrated a proportional decrease in the MDR phenotype **(Figure 12A-D)**. It appears that just as AUY922 treatment desensitized A549 cells to acquisition of a metastatic phenotype through TGF-β driven EMT, they also appear insensitive in attaining the

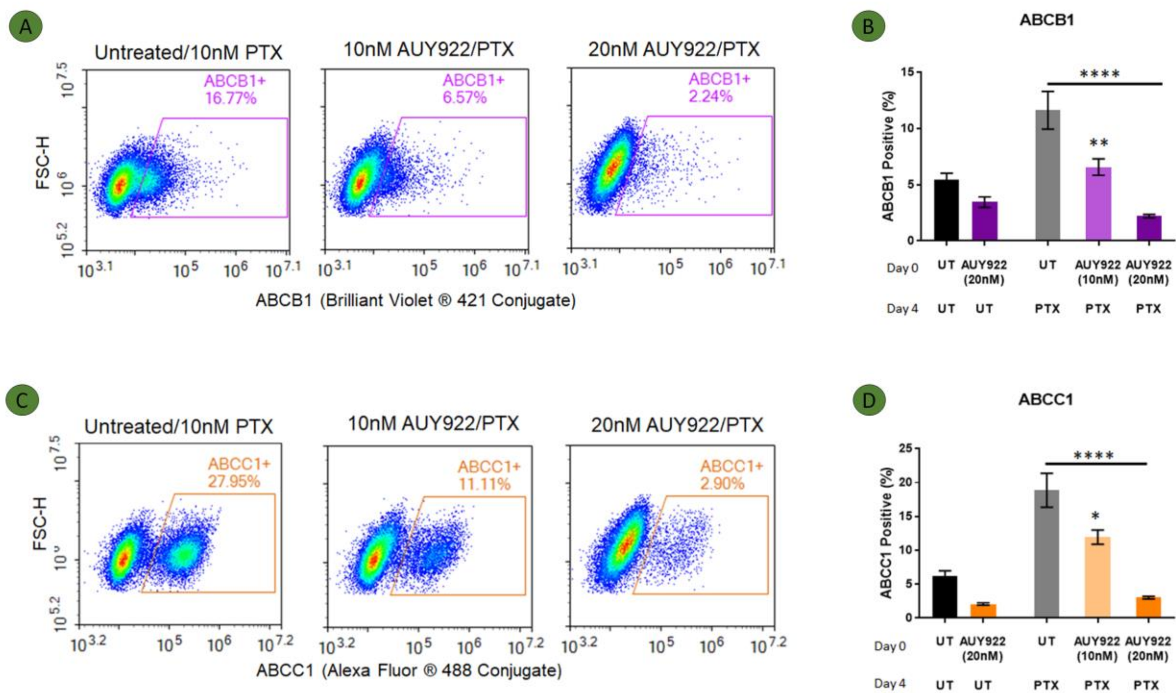

Figure 12: Flow Cytometric Analysis of ABC Transporter Expression Captured at day 8 for Cultures Treated with 10nM Paclitaxel to Induce MDR.
(A) ABCB1 expression in A549 cells after a single treatment of AUY922 followed by MDR induction with 10nM Paclitaxel (PTX) as described. (B) Quantification of the percentage of cells positive for ABCB1 captured at 192 hours. (C)ABCC1 expression in A549 cells after a single treatment of AUY922 followed by MDR induction with 10nM Paclitaxel (PTX) as described. (D) Quantification of the percentage of cells positive for ABCC1 captured at 192 hours. PTX=10nM Paclitaxel. Text before the slash indicates treatment conditions during the first 48 hours of the experiment. Text after the slash indicates treatment conditions from days 4 to 6. All data is representative of the mean ±SEM of three independent experiments performed at least in duplicate (n=8). *P<0.05, **P<0.01, ****P<0.0001

MDR phenotype. Furthermore, MDR induction with paclitaxel generated a robust double positive ABCB1 and ABCC1 population, which was not present in cultures that did not receive MDR induction with paclitaxel (data not shown). This was also where the greatest reduction in the MDR phenotype as a result of AUY922 treatment appeared to occur **(Figure 13).** This is a significant finding because ABCB1 and ABCC1 have considerable substrate overlap in

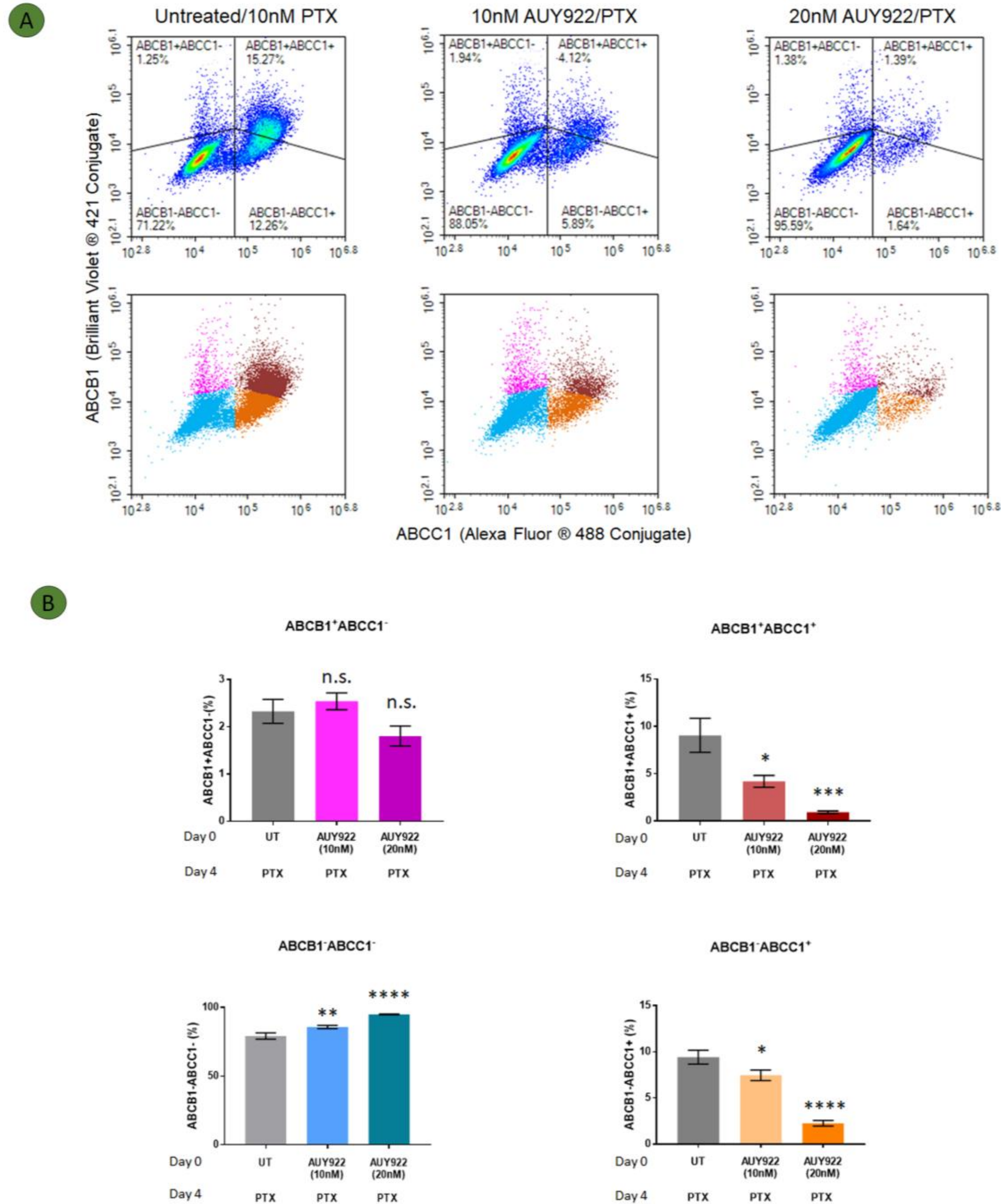

Figure 13. Flow Cytometric Analysis Captured at Day 8 of All Four Populations of A549 Cells after MDR Onduction on Day 4 with 10nM Paclitaxel (PTX)

(A) (Top) Representative flow plots demonstrating strong induction of ABCB1+ABCC1+ (double positive) population that decreases under AUY922 treatment in a dose dependent manner. Text before the slash indicates conditions from hours 0-48, text after the slash indicates conditions from hours 96-144. (A)(Bottom) Same view, but with colors designating the four populations to give a sense of the heterogeneity within the cultures. (B) Quantification of the percentage of cells in all four populations captured on day 8 according to the colors in (A). Data is representative of the mean ±SEM of three independent experiments performed at least in duplicate (n=8). *P<0.05, **P<0.01, ****P<0.0001

chemotherapies commonly used to treat lung cancer (Sharom, 2008), and experimental evidence suggests expression of multiple ABC transporters are required for cancers to achieve multidrug resistance (Bartholomae et al., 2016; Marzac et al., 2011) (Robey et al., 2018). This means that HSP90 inhibition with AUY922 has the potential to limit the formation of lung cancer cells that can generate the greatest efflux of chemotherapies across their cell membrane.

Loss of MDR Phenotype from AUY922 Treatment has a Functional Consequence that Impacts A549 Cell Survival

Our results indicating that treatment with AUY922 desensitizes A549 cells to induction of the MDR phenotype with paclitaxel suggest that these cells are more sensitive to cell death via paclitaxel treatment than controls treated with paclitaxel alone on day 4. However, given that ABC transporters are only one of several methods in which cancer cells can generate multi drug resistance, and the wide range of signaling pathways that may be altered by HSP90 inhibition, it is possible that we could be reducing one mechanism of multidrug resistance with AUY922 while reinforcing another. To investigate if AUY922 altered the viability of A549 cells over the 8-day period, we began tracking the culture growth at important time points in the experiment, in particular days 4, 6 and 8. A549 cells that received fresh medium every 2 days for 8 days showed classical unimpeded exponential growth, while A549 cells that received 20nM of AUY922 for the first two days followed by fresh medium every two days for the next 6 days showed a significant slowing of culture growth at each time point throughout the experiment, but an overall positive slope to the growth curve **(Figure 14A).**

In the treatment groups that received 10nM of paclitaxel on day 4 followed by fresh medium on day 6, more complex changes in culture growth were observed**.** A549 cells that

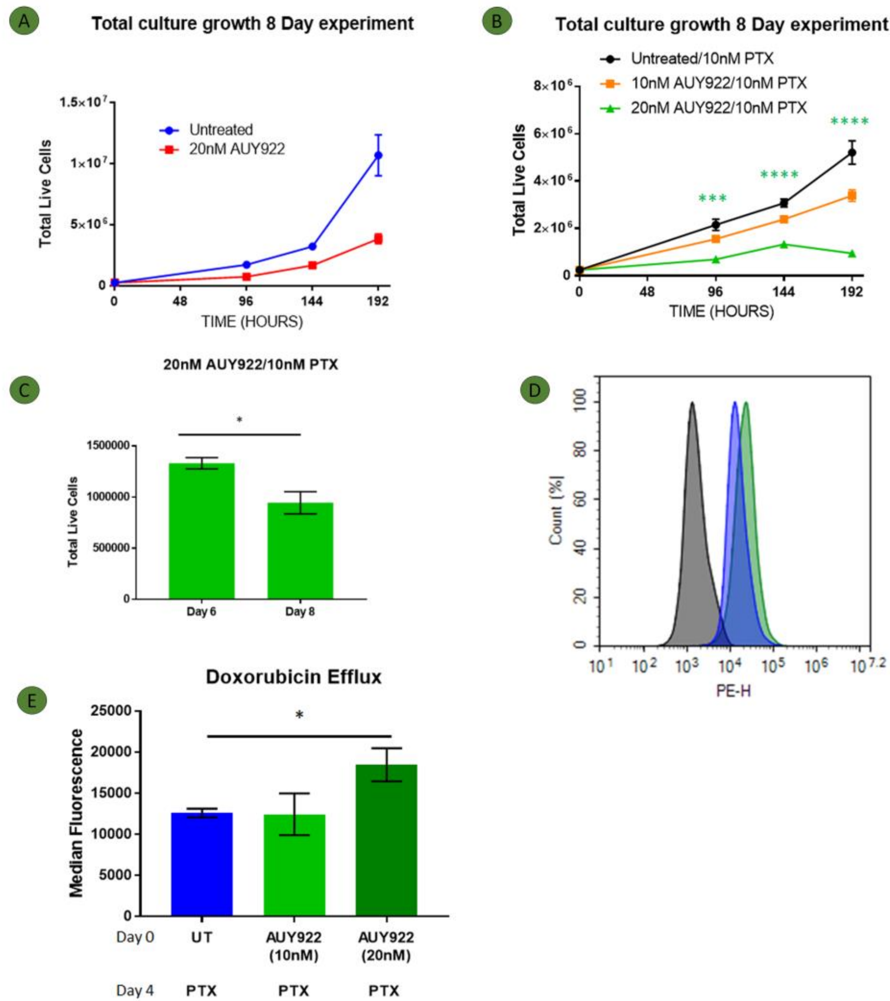

Figure 14: Growth Curve during 8 Day ABC Transporter Experiments and Doxorubicin Efflux Assay

(A) Growth curve of A549 cultures during the 8 day experiment after a single 48 hour 20nM treatment of AUY922. (B) Growth curve of A549 cultures exposed to 10nM of paclitaxel to induce MDR as described after 48 hour pretreatment with indicated doses of AUY922 (n=6). (C) Comparison of viable cells at hours 144 and hours 192 in cultures pretreated with 20nM AUY922 followed by MDR induction, indicating significant difference in viability. (D) Doxorubicin efflux assay representative flow cytometry histogram median fluorescence intensity of the total population of A549 cultures treated with 10nM of paclitaxel on day 4 (blue) and A549 cells pretreated with 20nM of AUY922 before receiving 10nM of paclitaxel on day 4 (green). Black curve represents negative control. (E) Doxorubicin efflux assay quantification of the median fluorescence intensity of doxorubicin accumulation of the total cell population treated as indicated (n=8). In (B) and (C), text before the slash indicates conditions from hours 0-48, text after the slash indicates conditions from hours 96-144. All data is representative of the mean ±SEM of three independent experiments performed at least in duplicate. *P<0.05, **P<0.01, ***P<0.001, ***P<0.001, ****P<0.0001

remained untreated until they received 10nM of paclitaxel on day 4 showed a slowing of growth from days 4 to 6, but on days 6 to 8 demonstrated accelerated growth **(Figure 14B black line).** In contrast, A549 cells treated with 10nM and 20nM AUY922 showed a similar slowing of culture growth from days 4 to 6, but a dose dependent decrease in the slope of the growth curve from days 6 to 8 **(Figure 14B orange and green lines)**. In the case of 20nM of AUY922 treatment during the first two days, there was a negative slope to the growth curve from days 6-8.

These results may be explained by our previous findings of reduced MDR phenotype in A549 cells pretreated with AUY922 captured on day 8 via flow cytometry **(Figures 5 and 6)**. When A549 cells treated with AUY922 throughout the first 2 days of the experiment are unable to access the MDR phenotype during paclitaxel treatment during days 4-6, they cannot efflux paclitaxel when they receive fresh medium on days 6-8, thus promoting A549 cell death and a decrease in the slope of the growth curve during this time **(Figure 14B, C).**

This analysis only explains the role of ABCB1 in paclitaxel efflux, since ABCC1 effluxes paclitaxel poorly (Borst, Evers, Kool, & Wijnholds, 2000). To account for both ABCB1 and ABCC1 in the MDR phenotype and the results of the growth curve in cultures that received paclitaxel on day four, we decided to perform an efflux assay using doxorubicin as a fluorescent substrate. Doxorubicin is a substrate for both ABCB1 and ABCC1 (Sharom, 2008) and has intrinsic fluorescent properties that make it ideal to study drug efflux via flow cytometry. This method in using doxorubicin to study efflux activity of ABC transporters has been used by other investigators (Chen et al., 2015; Punia, Raina, Agarwal, & Singh, 2017) and we developed our assay in accordance with these studies. We performed the eight-day experiment just as before, but instead of immunophenotyping for ABCB1and ABCC1, we loaded the cells with

doxorubicin and provided a washout period to allow doxorubicin efflux, before analysis via flow cytometry. If our observation of the reduced MDR phenotype in AUY922 treated A549 cells is valid, these cultures should accumulate doxorubicin to a greater extent than A549 cells that received paclitaxel alone. Indeed, this was our result **(Figure 14D),** with significant change occurring in A549 cells that received 20nM of AUY922 **(Figure 14E).** Our efflux assay did not indicate a significant change between cultures that only received MDR induction with paclitaxel on day four compared to cultures treated with 10nM AUY922 before MDR induction with paclitaxel, even though there was a significant decrease in the MDR phenotype in these cells. This may be due to a threshold effect, where accumulation of drug in the A549 cell population does not occur unless transporter activity is reduced to a threshold level.

Together, the growth curve analysis and the efflux assay serves to support the results of the decreased MDR phenotype in AUY922 treated cells captured via flow cytometry during the eight-day experiment. The growth curve analysis also shows that we are not engaging other drug resistance mechanisms that are sufficient to restore cell survival as a product of AUY922 treatment. Furthermore, the resultant reduction in ABCB1 and ABCC1 positive fraction in the culture is sufficient to generate a functional consequence that leads to intracellular accumulation of chemotherapy, promoting cell death.

Discussion

Previous studies have implicated HSP90 in facilitating cellular mechanisms that drive metastasis and drug resistance in several cancer types, and that pharmacological HSP90 inhibition can deconstruct these mechanisms (Chong et al., 2019; Nagaraju et al., 2015; Whitesell et al., 2014). However, these studies only evaluated drug resistance and metastasis separately. Here we demonstrate that HSP90 inhibition with AUY922 can limit both metastatic

and drug resistant features in A549 NSCLC cells at the same clinically relevant dose. Moreover, these changes can be maintained even in the presence of strong inducers for metastatic and drug resistant phenotypes over a relatively prolonged time frame.

Our findings support the work of others demonstrating HSP90 inhibition abrogates EMT in other cancers (Chong et al., 2019; Nagaraju et al., 2015). However, this is the first documentation of these observations in A549 NSCLC cells with AUY922. The increased E-cadherin expression in our flow cytometry studies combined with our wound healing assays and TER measurements indicates that AUY922 treatment enhances cell-cell adhesion. This is pertinent to NSCLC, which is often locally advanced at the time of diagnosis (Gabor et al., 2004; Popper, 2016). Immediate intervention with low dose AUY922 may be a useful treatment strategy in preventing further progression of the metastatic cascade in these cancers.

By carefully studying the flow cytometry plots during the four day EMT induction experiments, we also have evidence that HS90 inhibition with AUY922 simplifies the heterogeneity in the cultures relative to untreated or paclitaxel treated cultures in terms of E-cadherin positive and E-cadherin negative cells. In cultures that did not receive EMT induction, after a single treatment with AUY922, the culture shifts to mostly E-cadherin positive, whereas the paclitaxel cultures and the control have more balanced percentages of E-cadherin positive and E-cadherin negative populations. Under paclitaxel treatment, the heterogeneity is even more dramatic as the flow plots reveal what appear to be two distinct populations beginning to diverge from one another in the culture, whereas the control culture maintains a robust positive and negative population that is grouped close together. During EMT induction with TGF- β, AUY922 cultures do not transition as far as the two cultures that retained balanced positive and negative E-cadherin populations. Even on this relatively short time scale, these findings support

the hypothesis that HSP90 inhibition can reduce ITH such that it is less prone to acquiring metastatic properties.

To the best of our knowledge, we are the first to document the relationship between HSP90 inhibition with AUY922 in A549 cells and reduced cell surface expression of ABCB1 and ABCC1 with any HSP90 inhibitor in any cancer cell line. A single treatment with AUY922 is enough to maintain a significant reduction in both ABCB1 and ABCC1 for up to 8 days. Moreover, pretreatment with AUY922 suppressed cell surface expression of ABCB1 and ABCC1, even when stimulated to induce ABC transporter expression with paclitaxel. We were able to demonstrate a functional consequence of these findings by tracking cell growth over the eight-day experimental timeline and through efflux studies using doxorubicin, which is a substrate for both ABCB1 and ABCC1, as a fluorescent drug accumulation marker. Our findings and experimental design are clinically relevant since both ABCB1 and ABCC1 are thought to play a significant role in drug resistant NSCLCs (Berger et al., 2005; Oshika et al., 1998; Ota et al., 1995; Volm et al., 1991), and paclitaxel is still used to treat NSCLC. Additionally, ABC transporters, in particular ABCB1, can contribute to drug resistance in other cancers (Goldstein et al., 1989; Sharom, 2008), potentially making our findings applicable to a wide variety of cancers.

Here too, we see how AUY922 treatment simplifies the heterogeneity in the A549 culture populations when the MDR phenotype is induced. In control cultures that received MDR induction with paclitaxel, four populations of cells emerged in terms of ABCB1 and ABCC1 surface expression. On the other hand, AUY922 treatment reduced heterogeneity in the cultures in a dose dependent manner, such that the cultures became increasingly double negative for ABCB1 and ABCC1 expression. This is further support for the hypothesis that HSP90 inhibition

can reduce heterogeneity in cancer cell populations such that they are less likely to become drug resistant.

When it was discovered that ABC transporters drive multidrug resistance in cancer, numerous pharmacological inhibitors were developed to limit ABC transporter driven multidrug resistance (Robey et al., 2018). However, clinical evaluations of these inhibitors failed to demonstrate any benefit. This was later determined to be because most of these inhibitors only targeted a single ABC transporter at a time, and it is now suspected that expression of multiple ABC transporters is necessary to confer multidrug resistance in cancer (Robey et al., 2018). Furthermore, these drugs were not well tolerated in combination with chemotherapy (Robey et al., 2018). Our flow cytometry experiments suggest that it is possible to get around some of these problems by using AUY922 to remove cell surface expression of two major ABC transporters thought to confer multidrug resistance in a variety of cancers, including NSCLC. If ABCB1 and ABCC1 are not expressed on the cell surface, they cannot be expected to play a role in efflux dependent multi drug resistance.

The down regulation of ABCB1 and ABCC1 on the surface of A549 cells treated with AUY922 may also explain a relatively common side effect of this drug observed in clinical trials. A frequently reported adverse effect of AUY922 is various visual disturbances, and these occur most often in treatment cohorts receiving the highest dose of AUY922 (Bendell et al., 2015; Doi et al., 2014; Felip et al., 2018; Johnson et al., 2015; Kong et al., 2016; Seggewiss-Bernhardt et al., 2015; Sessa et al., 2013). Others have postulated that this might be related to a certain sensitivity of the retina to AUY922 (Kong et al., 2016; Zhou et al., 2013), however these investigators did not provide any direct cellular mechanism relevant to retinal physiology to explain this sensitivity. The blood retinal barrier (BRB) is a privileged site in the body. As such

it contains high expression of ABC transporters including ABCB1 and ABCC1 (Chapy et al., 2016) that not only protect the retina from xenobiotic insult, but also govern multiple biological gradients important for normal function. Application of high doses of AUY922 in clinical trials may be down regulating ABC transporter expression in the retina and altering its ability to properly maintain certain gradients, leading to changes in vision (Bendell et al., 2015; Piotrowska et al., 2018; Sessa et al., 2013). Even though our work is in a different tissue type, our results support this hypothesis when considering that almost all tissues in the human body utilize ABC transporters to some degree. Changes in vision are not likely due to apoptosis, as clinical trials report visual changes are reversible once AUY922 treatment is removed. However, this can only be definitively confirmed by studies evaluating ABC transporters in retinal tissue treated with AUY922.

Overall, the recent evidence that some treatment approaches may incidentally enable the metastatic cascade (Kajiyama et al., 2007; Karagiannis et al., 2017; Volk-Draper et al., 2014) and the persistence of drug resistant relapse after current treatment methods(Garraway & Janne, 2012) demands new, rational, treatment strategies that can curtail the emergence of metastatic and drug resistant cancer phenotypes simultaneously. The work presented in this chapter points to AUY922 as a possible drug candidate to accomplish this in NSCLC. We demonstrate that a single treatment with AUY922 at a relatively low, clinically appropriate dose can reduce phenotypic changes associated with both metastasis and efflux dependent drug resistance in A549 cells. These changes can be maintained even when potent inducers of metastatic and drug resistant phenotypes is applied. Additionally, we used AUY922 treatments in combination with drugs that are currently used to treat NSCLC, making a case that low dose pretreatment with AUY922 may prevent undesirable changes related to metastasis and drug resistance that has

been associated with conventional cytotoxic therapy like paclitaxel (Datta et al., 2017; Kajiyama et al., 2007; Karagiannis et al., 2017; Volk-Draper et al., 2014). This is reasonable for a clinical setting since other clinical trials using AUY922 at much higher doses have shown it to be well tolerated. Though some studies have shown AUY922 to be less well tolerated when paired with some conventional therapies (Seggewiss-Bernhardt et al., 2015), since we are arguing that lower doses should be used, tolerability can be expected to improve. Finally, we show circumstantial evidence that HSP90 inhibition can simplify phenotypes in culture such that they are less prone to providing heterogeneity for Darwinian processes to act on and drive tumor progression.

CHAPTER 3: CONCLUSIONS

Reflection

The exact role HSP90 plays in nature has been the center of considerable debate ever since drugs were first developed to target HSP90's activity decades ago. Targeting HSP90 in cancer came with heavy skepticism considering it is necessary for proper function of normal cells. These concerns have largely been put to rest as advances in the field have demonstrated that HSP90 inhibitors are specific to their target and accumulate in neoplastic tissue. These two highly desirable traits that are not easily found in other cancer drugs certainly suggests that HSP90 inhibitors should find their way into the cancer treatment process one way or another.

Our objective was to unite HSP90 inhibition in reducing the evolution of both metastatic and drug resistant features at a single relatively low dose. This is in contrast to other studies that investigated HSP90 inhibitors in deconstructing either drug resistance or metastasis, but not both simultaneously (Whitesell et al., 2014) (Chong et al., 2019; Nagaraju et al., 2015). We also wanted to study a drug resistance mechanism that could be widely applied to many cancers, such as ABC transporter driven drug resistance, which is distinct from previous studies (Whitesell et al., 2014).

Overall, we were successful in achieving our objective. We found that HSP90 inhibition with AUY922 at a single low dose could pointedly restrict metastatic and ABC transporter driven drug resistance features in A549 cells. This has strong implications for treatment of NSCLCs since these cancers are known to rapidly develop metastasis and efflux dependent drug resistance (Berger et al., 2005; Gabor et al., 2004; Oshika et al., 1998; Ota et al., 1995; Shanker et al., 2010; Sosa Iglesias et al., 2018; Volm et al., 1991). Furthermore, the ability to use one drug at low doses to curtail both of these features may increase the durability of treatment while

also improving the quality of life for cancer patients during treatment.

Study Weaknesses

While we were effective at demonstrating HSP90 inhibition with AUY922 restricted development of metastatic and drug resistant phenotypes, our work has some weaknesses. First, we only demonstrated our findings in a single lung cancer cell line. We did some preliminary studies in H596 cells, another lung cancer cell line, but we could not get the same results. This may be because H596 cells were relatively insensitive to HSP90 inhibition with AUY922, with an EC50>100nM according to our measurements. This suggests these cells are not as dependent on HSP90 as A549 cells are for viability. This may make the changes we document here in terms of cell adhesion and ABC transporters with AUY922 treatment exclusive to cancers that have a greater dependence on HSP90 to maintain cellular functions. In terms of cancer treatment, this may mean that in order to maximize the success of AUY922 in the clinic, patients should be stratified according to the dependence their cancers have on HSP90 in maintaining cancer hallmarks. Taking biopsies at different tumor regions and assessing the level of oncogene interaction with HSP90 via a co-immunoprecipitation assay optimized for the clinic could accomplish this. The more oncogenes that are associated with HSP90, the more likely a patient may benefit from HSP90 inhibitor therapy.

Another putative weakness is that this work lacks reproduction in an *in vivo* setting. We say putative here because as pointed out by others (Robey et al., 2018), one major confounding factor in delineating the relationship between multi drug resistant cancers and ABC transporters was that normal tissues and infiltrating immune cells that make up the tumor stroma also express ABC transporters, making it difficult to accurately assess changes in ABC transporter expression specifically occurring in neoplastic tissue. Therefore, it was reasonable to begin studying how

HSP90 inhibitors like AUY922 alter ABC transporters *in vitro* since this had never been studied before. However, the assertions made in this piece are significantly weaker without reproducing our results *in vivo*.

Contributions to the Field and Future Directions

Despite its weaknesses, this work has made some significant contributions to the field of medicine and cancer biology. Since our approach was relatively straightforward using very basic cell culture techniques, numerous future studies can be developed from these findings. Moreover, A549 cells are well studied and accessible to most research universities across the country, adding to the utility of the work presented here.

As previously stated, manipulating ABC transporters in multidrug resistant cancers has been attempted in the past with small molecule inhibition, but has come up short in delivering clinical efficacy (Robey et al., 2018). Here we show that it is possible to simply remove ABC transporters from the cell surface with AUY922, neutralizing their ability to efflux drugs from A549 NSCLC cells. Other cancer types prone to ABC transporter driven drug resistance should be studied for down regulation in ABC transporters when exposed to low doses of AUY922. These would include colorectal, pancreatic, liver, and breast cancers to name a few (Begicevic & Falasca, 2017). Additionally, other HSP90 inhibitors should be explored for this effect as well. HSP90 inhibitors like AUY922 that do not require dissolution in DMSO should be the primary focus since DMSO can induce ABC transporter expression (Nishimura, Ueda, & Naito, 2003).

The influence AUY922 has on ABC transporters in A549 cells shown in this work should be investigated in more detail to develop strategies to apply this effect in normal tissues. The ability to manipulate ABC transporter expression in normal tissues is relevant to medicine since it may improve bioavailability and tissue penetration of pharmacological therapies. For example,

a reason some drugs are not orally bioavailable is due to ABC transporter expression in the gut lining that pumps drugs back into the lumen before they can reach the basolateral membrane and cross into the bloodstream, significantly reducing the therapeutic effect of some drugs (Dietrich, Geier, & Oude Elferink, 2003). Furthermore, the blood brain barrier of the central nervous system is covered in ABC transporters that serve to protect it from xenobiotic insult, but becomes a significant obstacle for physicians attempting to apply pharmacological therapy to the central nervous system (Begley, 2004). It is unlikely that normal tissue will behave in the same way as neoplastic tissue when exposed to AUY922 because neoplastic tissue contains more activated HSP90. But if a mechanism can be identified in A549 cells that drives the reduced MDR phenotype when exposed to AUY922, that mechanism can be explored in detail in normal tissue and potentially exploited to down regulate ABC transporters to aid therapies in reaching their target tissue.

Finally, this work is distinct from other cancer research in that it successfully demonstrates the possibility of diminishing metastasis and drug resistance together, at the same dose, with a single drug. This is a significant discovery since the cellular mechanisms driving metastasis and drug resistance we show here are not known to operate through shared cellular mechanisms. Here we show evidence they are linked by HSP90. A549 cell xenograft studies in mice should be carried out immediately to evaluate whether the changes in metastatic and drug resistant features we observed with AUY922 *in vitro* are carried over *in vivo.* AUY922 should be administered to mice in low doses paired with paclitaxel or other chemotherapies in a clinically achievable treatment schedule. Mice should be followed for several months to evaluate progression of their tumors. Other HSP90 inhibitors that have shown success in low doses, such as ganetespib, should also be evaluated in parallel. If successful, this makes a strong case for

designing human clinical trials using HSP90 inhibitors in low doses to simultaneously restrict metastasis and drug resistance from developing in a patient's tumor.

Final Remarks

Clinical trials have demonstrated that AUY922 and other HSP90 inhibitors are more potent in combination with other conventional chemotherapies (Bendell et al., 2015; Johnson et al., 2015; Kong et al., 2016; Modi et al., 2011) than by themselves (Doi et al., 2014; Piotrowska et al., 2018; Seggewiss-Bernhardt et al., 2015). This is uncannily similar to the the antibiotic, clavulanic acid. Clavulanic acid is a weak antibiotic on its own (Brumfitt & Hamilton-Miller, 1984), but when combined with beta-lactam antibiotics, significantly potentiates their action through inhibition of microbial derived lactamases. Lactamase activity is an adaptive mechanism microbes developed to fight beta lactam producing fungi and is the main source of treatment failure of beta lactam antibiotics (Sykes, 2010). This was a significant problem until clavulanic acid was discovered, and it essentially revived a whole class of antibiotics that were rapidly becoming obsolete. Now, clavulanic acid is routinely used in combination with beta lactam antibiotics to successfully treat microbial infections resistant to beta lactam monotherapy.

The heat shock response, which is considered an adaptive response to cellular stress (Schlesinger, 1990), is known to play a significant role in cancer progression (Calderwood & Gong, 2016). Furthermore, a major chaperone protein integral to the heat shock response, HSP90, appears to support the evolution of metastatic and drug resistant cancers, which are responsible for the bulk of treatment failure. This is further strengthened when considering that HSP90 has multiple protein clients in each of the original six hallmarks of cancer (Hanahan & Weinberg, 2000) **(Figure 4)** including the hallmarks of "resistance to apoptosis" and "activation of invasion and metastasis". The results presented here demonstrate that AUY922, a potent

HSP90 inhibitor, can limit some of the phenotypic changes associated with metastatic and drug resistant cancers simultaneously. To date, there is not a class of drugs specifically used to suppress the evolution of metastatic and drug resistant phenotypes in cancer like there have been for the evolution of drug resistant microbial infections in the case of clavulanic acid. Could AUY922, and other HSP90 inhibitors, be operating like clavulanic acid by targeting an adaptive mechanism that many cancers appear to rely on to evolve metastatic and drug resistant phenotypes, both of which are responsible for treatment failure? Our results in addition to others certainly support that notion. Perhaps it is time to begin taking a closer look at using HSP90 inhibitors to shape the cancer, rather than kill it, to improve cancer treatment outcomes.

It is also possible that HSP90 inhibition in cancer has less to do with directly hamstringing HSP90's adaptive function and more to do with reducing ITH and revealing evolutionary "dead ends" stored in HSP90's capacitor function. Since HSP90 chaperones clients in every core cancer hallmark, limiting its function significantly reduces a cancer's ability to lean on any individual cancer hallmark to maintain survival since their gene products would all be simultaneously compromised **(Figure 15)**. This may force the emergence of phenotypes that are far less aggressive and may actually represent evolutionary "dead ends". In terms of the natural course of cancer, this refers to phenotypes that are less metastatic and drug resistant. We have some evidence for that here in this work. Another possible evolutionary "dead end" could be

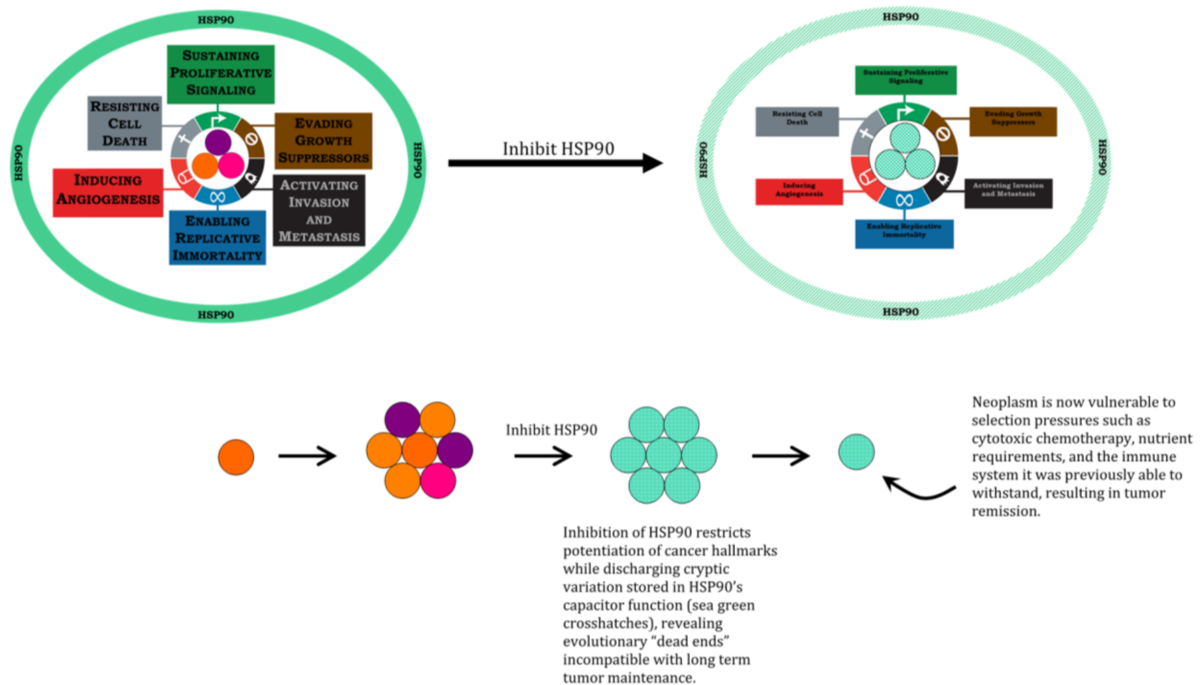

Figure 15: Hypothetical Illustration of HSP90 Inhibition Crippling Tumor Evolution
As in previous illustrations, HSP90 is shown orchestrating the cancer hallmarks in order to promote evolution of three tumor phenotypes (orange, pink purple), with enlarged colored boxes to denote the collective level of dependence the tumor has on the cancer hallmarks (LEFT). HSP90 inhibition shuts down the cancer hallmarks simultaneously, denoted by the shrunken colored boxes, bringing the tumor to a new state that is less able to evolve new neoplastic characteristics (sea green crosshatches). This may reveal evolutionary "dead ends" hidden in the cancer's genome, making the neoplasm more vulnerable to selective pressures it previously evolved to thwart, such as the immune system and chemotherapy.

resensitization of cancer cells to the immune system. There is already some evidence for this forming in studies demonstrating that ganetespib can sensitize cancer cells to T-cell mediated anti-tumor responses (Mbofung et al., 2017), though they do not discuss their findings in terms of tumor evolution. Revealing these evolutionary "dead ends" could fundamentally alter the trajectory of tumor evolution, perhaps even resulting in sustained tumor remission.

Given the complex nature of the mechanisms that drive metastasis and drug resistance in cancer, HSP90 inhibitors are unlikely to be a silver bullet. If we reframe cancer as a moving target that strives to evolve metastatic and drug resistant features over time according to the

theory of tumor evolution, we may discover alternative ways to prevent these features from manifesting in the first place. Therefore, more work should be dedicated to identifying cellular mechanisms and microenvironmental factors that drive both metastasis and drug resistance from the earliest stages of carcinogenesis to the final stages of advanced disease. If possible, we should develop drugs against single targets that are essential in driving both metastasis and drug resistance so as to leave room for combination therapy with cytotoxic agents to control tumor growth. Some investigators have already started working on this idea (Cao et al., 2018). This may not turn out to be the most effective way to kill the cancer outright, but if we can prevent drug resistance and metastasis from developing, there is a good chance we can control the disease and extend the lives of cancer patients. Even more, we may learn that approaching cancer treatment in this way may not require the levels of cytotoxic therapy currently recommended for cancer treatment, thus significantly improving the quality of life for patients during the treatment process.

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APPENDIX A: APPROVAL LETTER

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Office of Research Integrity

February 1, 2021

Nickolas Anthony Bacon
1015 West 5th Street, Rear
Huntington, WV 25701

Dear Mr. Bacon:

This letter is in response to the submitted dissertation abstract entitled *"Crippling Rapid Evolution of Metastasis and Drug Resistance in A549 Non-Small Cell Lung Cancer Cells with the Clinically Relevant HSP90 Inhibitor AUY922."* After assessing the abstract it has been deemed not to be human subject research and therefore exempt from oversight of the Marshall University Institutional Review Board (IRB). The Code of Federal Regulations (45CFR46) has set forth the criteria utilized in making this determination. Since the study does not involve human subjects as defined in DHHS regulation 45 CFR §46.102(e) it is not considered human subject research. If there are any changes to the abstract you provided then you would need to resubmit that information to the Office of Research Integrity for review and determination.

I appreciate your willingness to submit the abstract for determination. Please feel free to contact the Office of Research Integrity if you have any questions regarding future protocols that may require IRB review.

Sincerely,

Bruce F. Day, ThD, CIP
Director

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