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Treatment Strategies in Acute Myelogenous Leukemia : Investigating HSP90 and P53 as Targets

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TREATMENT STRATEGIES IN ACUTE MYELOGENOUS LEUKEMIA: INVESTIGATING HSP90 AND P53 AS TARGETS

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Abstract

Acute myelogenous leukemia (AML) is the deadliest of the lymphatic and bone marrow cancers. Patients diagnosed with AML have a five year survival rate of 23.4%. AML is characterized by an accumulation of undifferentiated and functionless myeloid precursors in the bone marrow and blood. Heat shock protein 90 (Hsp90) inhibitors, such as 17-AAG, an analog of geldanamycin (GM), are currently undergoing phase I, and II and III clinical trials for various cancers with mixed results. I found distinct responses to 17-AAG treatment among the AML cell lines tested. I also discovered two mutant *TP53* alleles in Kasumi-3 cells, an AML cell line that was very sensitive to 17-AAG. I hypothesized that this high sensitivity was due to the degradation of mutant p53, a known Hsp90 binding protein. Although I was not able to show that this mutant p53 was acting in an oncogenic fashion, it still raises the possibility that tailoring 17-AAG treatment to patients with known oncogenic *TP53* alleles might prove to be very effective. Kasumi-3 cells will provide a valuable model to determine which biomarkers may indicate sensitivity to Hsp90 inhibition. In addition to studying the role of Hsp90 inhibition in cancer, I also explored the hypothesis that Hsp90 acts as a morphological capacitor for evolution in a mammalian model. EML cells were used as a model for hematopoiesis and treated with GM to inhibit Hsp90. It was postulated that this treatment would cause a trans-differentiation event or have an effect on differentiation. What was observed was an increase in GM treated cell survival when selective conditions were applied. This data suggests that GM treatment was giving the cells a selective advantage. Unfortunately, experimental results were very variable and I was unable to obtain consistent results upon numerous replicate experiments. The various diverse responses to Hsp90 inhibition presented in these studies may help explain the mixed results of 17-AAG in clinical trials.

Dedication

I would like to dedicate this work to my parents, my late father Eric W. Stephan died my first semester at Marshall and my mother Janice L. Stephan. It was through their guidance that I learned that to succeed working hard and perseverance is the key. They are responsible in a large part for who I am today and I appreciate them always being there for me.

Acknowledgments

First, I would sincerely like to thank my husband, Dr. Andrew Napper. It is through his support that I have been able to do this. He took care of things on the home front so that I could concentrate on my studies. In addition, he always listened to all my troubles when things went wrong and celebrated with me when things went right! I would also like to thank my children, Alex and Aaron, and my mom, Jan, for their big role as my personal cheerleaders. I would like to thank my lab mates present and past: Mindy Varney, Jasjeet Bhullar, Ayah Arafa, James Buchanan, Jonathan Lewis and last but certainly not least, Harsh Pratap. We were always there for each other and I doubt I could have done this without them!

I'd like to thank Dr. F. Heath Damron and Kari Wilson for the opportunity to collaborate and do some interesting work in the microbiology field. I really appreciate Dr. Pier Paolo Claudio's help. He played a big role in my p53 project. He gave me great advice and provided me with the antibodies that I desperately needed to test my hypotheses. He was also a sympathetic ear and was willing to help me in any way he could. Dr. Yulia Dementieva gave me great advice and assisted me with statistical analysis. I would like to give Dr. John Wilkinson special thanks for his help in trouble shooting westerns. He really helped me with some tough problems and his door was always open if I needed advice about anything. I'd also like to thank Adam Hall for helping me clean up my southern blot that took me forever to produce and Melissa Bonner who helped figure out how to electroporate my cells. Anne Silvis was also very helpful, being a sympathetic ear and a great supply of antibodies! I'd like to give special thanks to all my BMS friends over the years who have made this experience much more fun, especially Mindy and Heath. Because of them, I got to do some really fun and crazy things that I never thought I'd get into! My whole graduate experience has helped me grow as person, and they were a big part of that.

Lastly, I'd like to thank my advisor, Dr. Sollars for his guidance and patience. Thank you to my committee Dr. Green, Dr. Niles, Dr. Primerano, and Dr. Yu. The time they all spent at my

meetings and the advice they gave me helped me through some rough times and aided in my education. I sincerely appreciate the time and effort all five have put into me. Thank you all.

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List of symbols/nomenclature

7-AAD ----- 7-Aminoactinomycin D

17-AAG ----- 17-N-Allylamino-17-demethoxygeldanamycin

ABC ----- ATP-binding cassette

ADP ----- Adenine diphosphate

AML ----- Acute myelogenous leukemia

APL ----- Acute promyelocytic leukemia

ARF ----- Alternative reading frame gene product

ATM ----- Ataxia telangiectasia mutated

ATP ----- Adenine triphosphate

ATR ----- Ataxia telangiectasia and Rad3 related

ATRA ----- All-trans retinoic acid

BAX ----- Bcl-2–associated X protein

BCR ----- Breakpoint cluster region

CDC ----- Cell division cycle proteins

CLP ----- Common lymphoid progenitor

CM ----- Conditioned medium

CMP ----- Common myeloid progenitor

Cpt ----- Camptothecin

EMP ----- Erythroid megakaryoctye progenitor

EMEM ----- Eagle's minimum essential medium

EML ----- Erythrocyte myeloid lymphocyte

EPO ----- Erythropoietin

ER ----- Endoplasmic reticulum

EVI1 ----- Ectopic viral integration site-1

FITC ----- Fluorescein isothiocyanate

FLT3 ----- FMS like kinase

GADD45 ----- Growth Arrest and DNA Damage

GAPDH ----- Glyceraldehyde 3-phosphate dehydrogenase

G-CSF ----- Granulocyte colony stimulating factor

GM ----- Geldanamycin

GM-CSF----- Granulocyte macrophage colony stimulating factor

GMP----- Granulocyte macrophage progenitor

HIP----- Hsp0 interacting protein

HOP----- Hsp70-Hsp90 organizing protein

HSC----- Hematopoietic stem cell

Hsp----- Heat shock proteins

IC50----- Half maximal inhibitory concentration

IL-3----- Interleukin 3

IMDM----- Iscove's modified Dulbecco's medium

M-CSF----- Macrophage colony stimulating factor

MDM2----- Murine double minute

MDR1----- Multidrug resistant protein

PBS----- Phosphate buffered saline

P-gp----- P-glycoprotein

PUMA----- p53 upregulated modulator of apoptosis

PI3K----- Phosphatidylinositol 3-kinases

PML----- Promyelocytic leukemia gene

PRIMA-1----- p53 reactivation and induction of massive apoptosis

RARα----- Retinoic acid receptor α

RITA----- Reactivation of p53 and induction of tumor cell apoptosis

SCF----- Stem cell factor

SNP----- Single nucleotide polymorphism

SP-1----- Specific protein 1

TBS----- Tris buffered saline

TPO----- Thrombopoietin

TPR----- Tetratricopeptide repeat

Vp----- Verapamil

Chapter 1 : Introduction

Leukemia

About five hundred billion blood cells need to be replaced in the human body each day. The hematopoietic stem cell (HSC), which resides mostly in bone marrow, is the pluripotent precursor cell that gives rise to all the different cell types in the blood (**Figure 1.1**). It stays in a relatively quiescent state and retains self renewal capabilities. External signals, such as cytokines, initiate asymmetric division of the HSC resulting in either a common lymphoid progenitor (CLP) or a common myeloid progenitor (CMP). These progenitors are very different from the hematopoietic stem cell in that they can divide much more rapidly; however, they have a finite lifetime. The CLP cells divide and undergo successive stages of differentiation to create more committed progenitor cells of the lymphoid lineage until mature cells, including B and T cells, are produced. The CMP produces more committed myeloid progenitors that eventually mature into granulocytes, macrophages, megakaryoctyes and erythrocytes (Terskikh et al., 2003; Passague et al., 2003).

HSC maintenance is very complex and involves many factors. Probably the most important cytokines involved in HSC maintenance are thrombopoietin (TPO) and stem cell factor (SCF). The SCF receptor, KIT, and the TPO receptor, c-mpl, are both highly expressed on HSCs. TPO has been reported to suppress apoptosis in HSCs, which indicates its role in HSC maintenance may be to promote cell survival. Furthermore, mice engineered with mutations in KIT were reported to have 10- to 20- times less HSC than their wild-type counterparts (reviewed in Kent et al., 2007; Blank et al., 2007). Another study provided additional evidence of the importance of SCF in HSC maintenance by showing that injection of an anti-kit antibody into adult mice causes a significant decrease in the number of HSCs as well as progenitor cells in the bone marrow (Ogawa, 1991). This antibody binds to the KIT receptor and causes its inactivation. This study also highlights the importance of SCF in progenitor cell biology. SCF can act synergistically with other cytokines, such as interleukin-3 (IL-3), granulocyte monocyte colony stimulating factor (GM-CSF) and

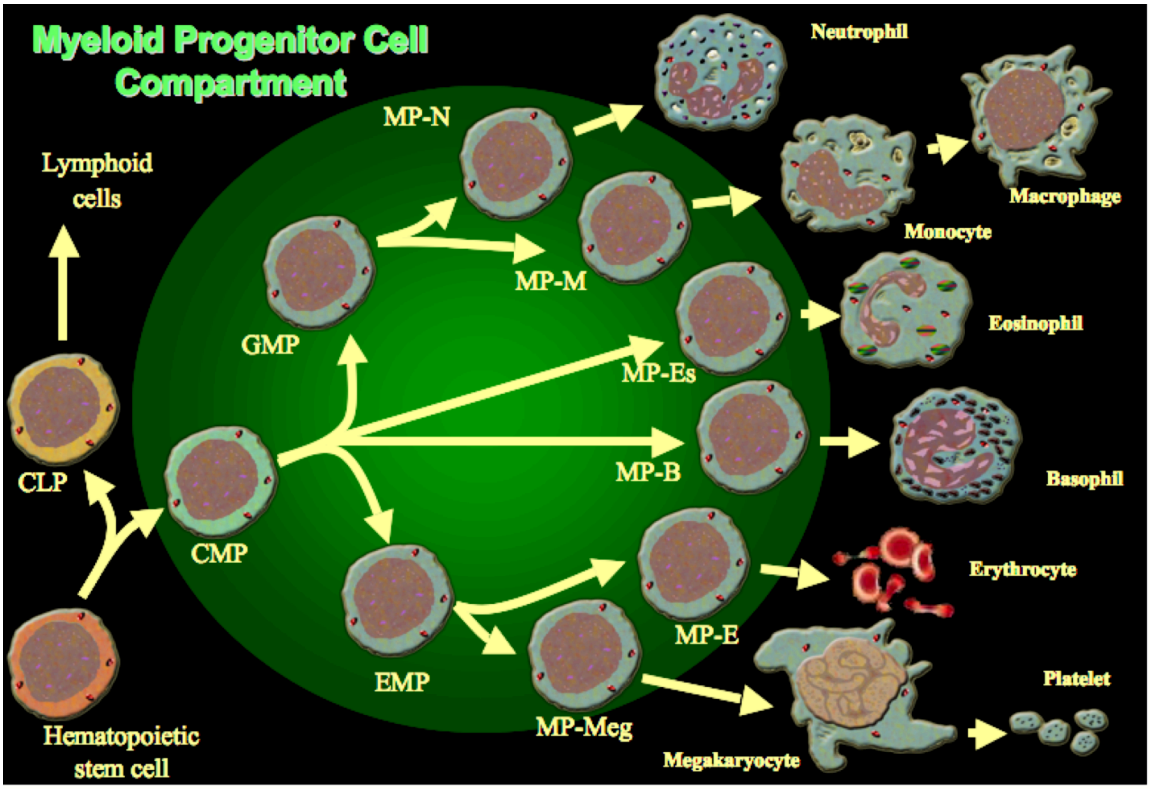

Figure 1.1. Proposed mechanism for hematopoiesis. The myeloid compartment is outlined in green. Asymmetric division of the HSC is initiated and can result in either a CLP or a CMP. The CLP cells divide and undergo successive stages of differentiation to create more committed progenitor cells of the lymphoid lineage until mature cells, including B and T cells and natural killer cells, are produced. The CMP produces more committed myeloid progenitors that eventually mature into neutrophils, basophils, macrophages, megakaryoctyes and erythrocytes. Abbreviations: hematopoietic stem cell (HSC), common myeloid progenitor cell (CMP), common lymphoid progenitor cell (CLP), erythroid and megakaryocyte progenitor cell (EMP), and granulocyte and monocyte progenitor cell (GMP). The committed progenitor cells are abbreviated as follows: neutrophil (MP-N), monocyte (MP-M), eosinophil (MP-Es), basophil (MP-B), erythrocyte (MP-E), and megakaryocyte (MP-Meg).

erythropoietin (EPO), to increase the number and size of myeloid progenitor colonies formed in semisolid media (Broudy, 1997).

The role of cytokines, as well as other factors, involved in hematopoiesis is very complex and there is a great amount of redundancy in their function (Metcalf, 1993). Many studies have elucidated their importance in myeloid progenitor proliferation, survival and differentiation (reviewed in Kaushansky, 2006). IL-3, SCF and TPO are important in the production of the erythrocyte megakaryocyte progenitors (EMP) from a CMP. The continued presence of either TPO or EPO dictates whether these progenitors progress to produce mature megakaryocytes or

erythrocytes, respectively. On the other hand, the presence of GM-CSF promotes the creation of the granulocyte macrophage progenitor (GMP) from a CMP. This committed progenitor requires macrophage colony stimulating factor (M-CSF) or granulocyte colony stimulating factor (G-CSF) to mature along the monocyte lineage or the granulocyte lineage respectively (**Figure 1.1**).

Understanding the pathways controlling hematopoiesis is essential for studying leukemia. It is unique from other cancers in that the uncontrolled accumulation of cancer cells circulate throughout the body, instead of forming a mass or a tumor. As a result, vascularization and metastasis are not necessary for this disease to progress. As the disease advances, more and more immature and functionless cells, called blast cells, accumulate in the bone marrow and peripheral blood. This inhibits the bone marrow from producing mature, functional white blood cells that are needed for the immune system and red blood cells, which are needed for delivery of oxygen to tissues. As a result, common symptoms for leukemia are anemia, fatigue, easy bruising (from lack of platelets), as well as symptoms of a suppressed immune system such as frequent infections.

Leukemia accounts for about one third of cancers in children 0–14 and is the 5th most common cause of cancer deaths overall. Those diagnosed with leukemia have an overall five year survival rate of 54%; however, leukemia causes more death than any other cancer in children and young adults under 20 years of age (Leukemia, Lymphoma, Myeloma, Facts 2009-2010, June 2009). Although overall survival rates have increased in recent years, these statistics clearly show that more treatment options are needed.

There are four major types of leukemia: acute and chronic lymphocytic leukemia and acute and chronic myelogenous leukemia. As the names suggest, they are categorized by which arm of the hematopoietic system the cancer cells originated from and the extent of blast cell accumulation. Lymphocytic leukemias are characterized by accumulation of lymphocytic precursor cells whereas the myeloid leukemias have an increased proliferation of myeloid precursors. The acute leukemias are characterized by a rapid increase of blast cells that interfere with normal hematopoietic function, while, the chronic leukemias progress more slowly. There

is a buildup of immature blast cells, but not as extensively as in the acute leukemias. Chronic leukemias often progress to the acute category, which is also called blast crisis. The acute leukemias, as the name implies, are the most severe and life threatening.

Acute myelogenous leukemia (AML) is the most deadly among the four major types. Patients diagnosed with AML have a five year survival rate of 23.4%. The median age at diagnosis for AML is 70 years; however, elderly patients in general only survive a few months after diagnosis. AML can be further sub-classified based on the characteristics of the main proliferating blast cell. The French-American-British classification is commonly used, which categorizes AML into eight different subtypes listed in **Table 1.1** (Bennet et al., 1976). These subtypes can be identified by examining the cells in the bone marrow and blood by either microscopy, cytogenetic methods, or through cell surface marker expression via flow cytometry. Because different subtypes have varying responses to therapy, identifying the subtype of AML can help tailor patient treatment options.

Chromosome alterations are very common in AML, which are detected in over 50% of cases (Mrozek et al., 2004). These alterations have been accepted as the strongest prognostic factor for survival and therapy response. Therefore, patients are generally placed into one of three large

FAB subtype	Description
M0	Undifferentiated
M1	Myeloblastic without differentiation
M2	Myeloblastic with maturation
M3	Promyelocytic
M4	Myelomonocytic
M5	Monocytic
M6	Erythroleukemia
M7	Megakaryoblastic

Table 1.1. French-American-British (FAB) classification of AML. Subtypes are based on the type of cell from which the leukemia developed and the degree of maturity. These help physicians in deciding treatment options for patients.

risk groups based on the cytogenetic makeup of their leukemia cells: favorable, intermediate or adverse (Grimwade et al., 1998). Treatment options are based on type of leukemia, age, and the

cytogenetic risk group of the patient. It should be noted that tailoring treatment using the cytogenetic risk groups does have limitations because the groups are based on common chromosomal abnormalities. Therefore, patients with uncommon chromosomal aberrations, such as p53 deletion, may not receive appropriate treatment because these aberrations are poorly understood in AML.

A diverse array of mutations has been implicated in AML progression. These mutations can be classed into two groups. Class I mutations are those that stimulate signal transduction pathways resulting in increased proliferation or survival of the cell. Examples include receptor tyrosine kinases such as, FMS like tyrosine kinase 3, FLT3 (Reilly, 2003), and KIT (Gari et al., 1999). KIT is the receptor for SCF and promotes HSC maintenance. FLT3 is also expressed in HSC and early progenitors and has been reported to facilitate proliferation in these cells (Kaushansky, 2006). These mutations usually result in constitutive activation in the absence of ligand binding resulting in unregulated growth.

Class II mutations are those that occur within transcription factors or components of the transcriptional machinery (classes of mutations reviewed in Renneville et al., 2008). The three classes of transcription factors that are most commonly affected are core binding factors (Example: AML1), retinoic acid receptor (RARα), and homeobox family members (example: HOXA9), which are all important in myeloid cell development. These mutations often result in a block in differentiation of the cells, a hallmark of AML. A classic example of this type of mutation is the chromosomal translocation t(15;17) which results in the fusion of the retinoic acid receptor alpha (RARα) gene with the promyelocytic leukemia (PML) gene. This mutation is found in 95% of acute promyelocytic leukemias (APL) and creates a chimeric protein product that blocks differentiation of granulocytes. Treatment with retinoic acid, the ligand that activates RARα, results in the differentiation of the immature precursor cells and has a high response rate in the treatment of patients with APL (Melnick et al., 1999; Tallman et al., 2002).

Interestingly, these fusion proteins can promote leukemogenesis, although with a latent period, when expressed in mouse models. This latent period suggests that another mutation is required before AML is induced. Consistent with the multi-hit model, one hypothesis is that a combination of class I and class II mutations is required in order for leukemogenesis to occur (Dash and Gilliland, 2001).

Kasumi-3 cells are a relatively new AML cell line with the classification of M0. They were isolated from a 57 year old Japanese patient, who unfortunately died 16 days after he was admitted to the hospital. These cells have a complex karyotype including a translocation between chromosome 3 and 7 [t(3;7)(q27;q22)], that results in aberrant expression of *ectopic viral integration site-1* (*EVI1*)(Asou et al., 1996). EVI1 is an early transcription factor and its expression has been correlated with poor prognosis in patients with AML. This transcription factor has recently been reported to be essential for HSC maintenance and proliferation and is hypothesized to participate in generation of leukemic stem cells (Goyama and Kurokawa, 2009). Since this cell line is relatively new, data is lacking regarding its molecular profile. In chapters 2 and 3, I report that these cells have two mutated *TP53* alleles and are sensitive to Hsp90 inhibition.

In contrast, HL-60, KG-1a and THP-1 cells have been available for more than three decades. They have been used as a model for hematopoiesis because they can be induced to differentiate by addition of various compounds. HL-60 cells were isolated from a patient with promyelocytic leukemia, and are classified as M3 (Gallager et al., 1979). KG-1a cells are a subclone of their parental cell line, KG-1. These cells were isolated from a patient with erythroleukemia that progressed into full blown AML (Koeffler et al., 1980). Both of these cell types also have a complex karyotype. THP-1 cells were isolated from a one-year old patient with monocytic leukemia and have the classification of M5 (Tsuchiya et al., 1980). They have frequently been used as a human macrophage model because they can be easily differentiated into monocytes by treating with phorbol esters. These four cell lines offer a range of different types of AML and provide a good model to study effects of chemical treatments for this disease.

Hsp90

Molecular characteristics of Hsp90

Chaperones are proteins that bind to other proteins and assist them in attaining a stable and active conformation. Some chaperones function by preventing the formation of deleterious aggregates that often occur in newly synthesized proteins, which allows for natural folding to take place. In addition, they can also contribute to folding the protein into their correct three-dimensional structures (Hartl, 1996). Stressful conditions such as irregular temperature changes, abnormal pH and nutrient deprivation can result in protein denaturation as well as production of new proteins to assist the cell's recovery. As a result, these conditions cause the induction of a special class of chaperones called heat shock proteins. These proteins help to refold denatured proteins and aid in the folding of newly synthesized proteins. Thus heat shock proteins play a key role in a cell's stress response by maximizing the number of active proteins, which in turn contributes to the cell's health and survival (Linquist and Craig, 1988).

Heat shock protein 90 (Hsp90) is one of these very important chaperones. Under normal conditions, Hsp90 constitutes 1–2% of soluble cellular protein. Many signaling molecules, such as kinases, hormone receptors and transcription factors have been identified as Hsp90 binding targets, which are termed client proteins. These client proteins depend directly on Hsp90 to reach their biologically active conformation (reviewed in Pearl et al., 2008). Hsp90 is an essential part of a large multi-protein complex that facilitates folding these proteins.

Hsp90 has five known isoforms that are a result of gene duplication events. Humans have two cytosolic isoforms, Hsp90α and Hsp90β. Hsp90α is the inducible isoform and Hsp90β is the isoform that is constitutively expressed. These two isoforms are 76% homologous (Moore et al., 1989) but are not completely interchangeable. Studies in yeast have demonstrated that expression of either isoform can rescue viability in cells that lack endogenous expression of Hsp90. However, there does seem to be a difference in client proteins between the two isoforms (Millson et al., 2007). Interestingly, this same study indicated that although yeasts expressing

Hsp90β were able to survive, they were highly sensitive to Hsp90 inhibition. Although studies are being conducted to understand the difference between these two isoforms, much is still not understood. Hsp90N (Grammatikakis et al., 2002) is another Hsp90 isoform that is mostly associated with the membrane and lacks the N-terminal domain of the protein. However, existence of this isoform in humans is now controversial (Zurawska et al., 2008).

The other two known isoforms are only present in higher eukaryotes; however, not much is known about their client proteins. GRP94 is associated with the endoplasmic reticulum (Ni and Lee, 2007) and is 50% homologous to the cytoplasmic isoforms (Gupta et all, 1995). Studies have shown GRP94 to be involved in the folding of components of the immune system (Nichitta et al., 2004; Melnick et al., 1992). It has also been implicated in the secretion of insulin-like growth factors (Wanderling et al., 2007). TRAP1 is a mitochondrial isoform (Song et al., 1995; Felts et al., 2000). The biological role of this isoform is very poorly understood. However, a recent study indicated that TRAP1 could play an important role in regulation of reactive oxygen species in the mitochondria (Im et al., 2007). Studies to discover the functions of these two Hsp90 isoforms are ongoing.

Hsp90 protein has four main domains (**Figure 1.2**) The N-terminal domain contains an ATP/ADP binding site that is very important for Hsp90 function. As will be explained later, this is a common site for binding of natural and synthetic inhibitors. Next, there is a small, highly charged linker region that is thought to provide the protein with more flexibility and can also facilitate client binding. This region is absent in TRAP1. The middle region, which is thought to be where protein clients bind, connects the linker to the C-terminal domain. Finally, the C-terminal domain contains the dimerization domain as well as another nucleotide binding domain capable of binding adenine as well as guanine nucleotides (Soti et al., 2003; Marcu et al., 2000). In the cytosolic forms of Hsp90, the C-terminal domain also contains a tetratricopeptide repeat (TPR) binding region, (composed of amino acids MEEVD). This is a site for some co-chaperones, such as HOP (Hsp70-Hsp90 organizing protein), to bind. In GRP94, this domain is replaced with

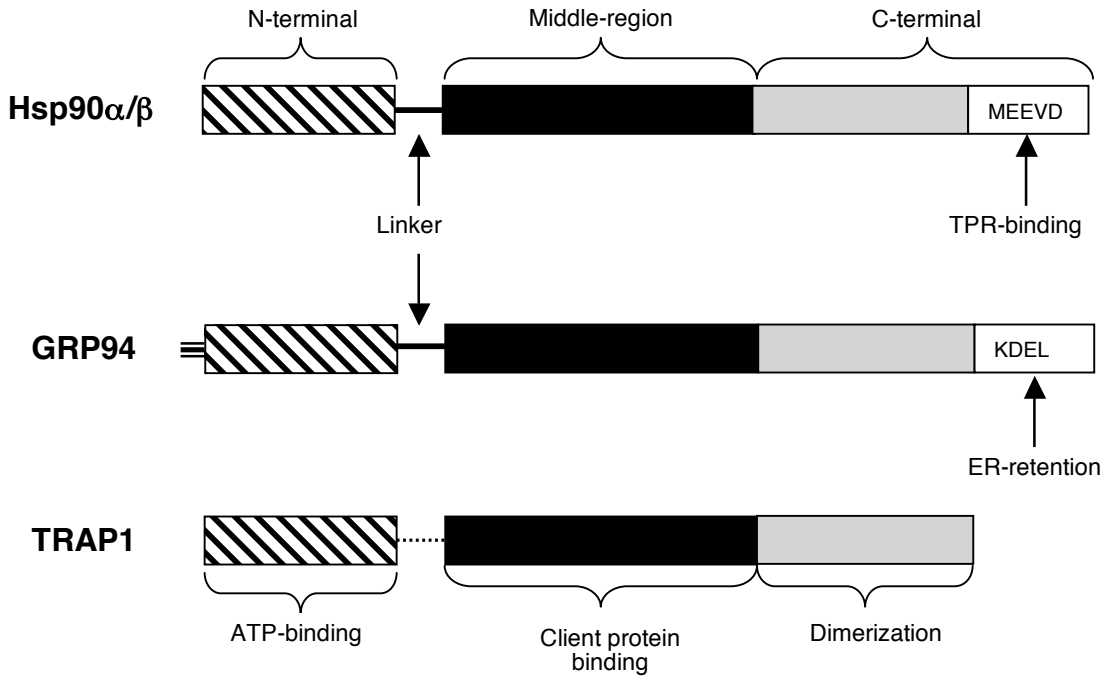

Figure 1.2. Structure of Hsp90 isoforms. Hsp90 protein structure has four main functional domains. The N-terminal domain holds an ATP binding domain. The middle region is thought to contain client protein binding sites. The linker domain is a positively charged region that links the N-terminal and the middle region of the protein. The C-terminal houses the dimerization domain necessary for Hsp90 to function. The TPR-binding site is where co-chaperones such as HOP can bind. Hsp90α/β are the cytosolic isoforms. GRP94 contains an ER-retention signal in place of the TPR-binding site and thus it functions mostly in the endoplasmic reticulum. It also has a short addition to the N-terminal region, the function of which is unknown. TRAP1 lacks the linker region and the very end of the C-terminal and is mostly associated with the mitochondria. All three isoforms contain the different functional domains marked on the bottom of the schematic and the three regional domains indicated in the top of the schematic.

an endoplasmic reticulum retention domain and is totally absent in TRAP1 (structure reviewed in Prodromou and Pearl, 2003). All three isoforms share the ATP-binding, client protein binding and dimerization domains

The protein folding pathway is not completely understood, but data support that the folding process takes place in a multi-protein complex with Hsp90 being a central player (**Figure 1.3**). Hsp90 forms a homodimer at its C-terminal end, resulting in a U-shaped structure that allows the two N-terminal domains to become available for protein interactions. Hsp70 forms a complex with new proteins to stabilize and deliver them to Hsp90. HIP (Hsp70 interacting

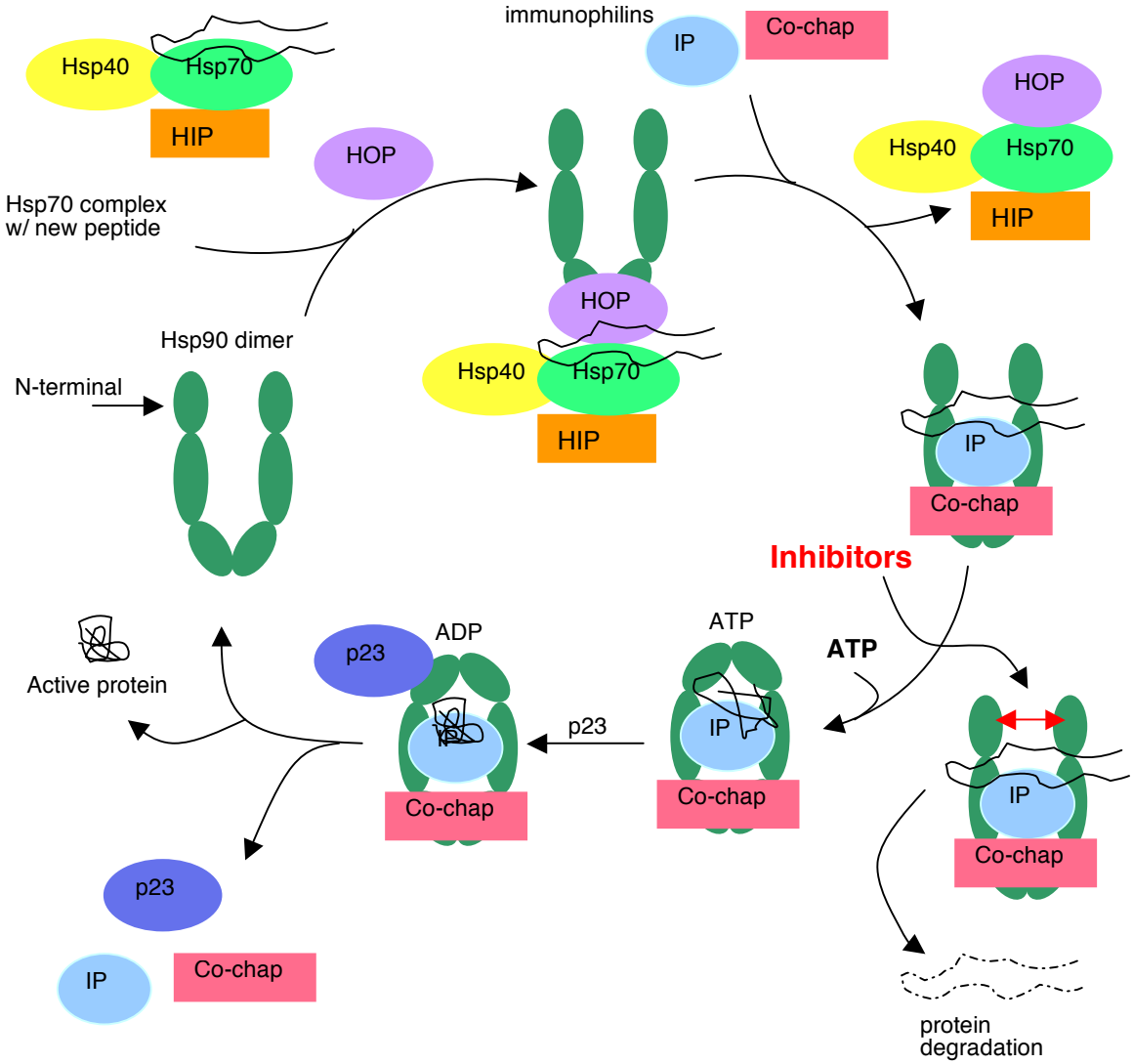

Figure 1.3. Proposed Hsp90 chaperone cycle. Hsp90 forms a homodimer at its C-terminal end. Hsp70 forms a complex with new proteins to stabilize and deliver them to Hsp90. HIP (Hsp70 interacting protein) and Hsp40 helps in stabilizing this complex. The Hsp70 complex binds to Hsp90 through HOP with its TRP domains that connect the two. The client protein is then loaded and the Hsp70 complex is released. Other co-chaperones and immunophilins, which are peptidyl-prolyl cis-trans isomerases, then bind to form the folding complex. ATP is then recruited which results in the dimerization of the N-terminal domains forming a "clamp" around the client protein. p23 acts to stabilize the clamp and facilitates ATP hydrolysis. This protein folding complex helps the client find its three-dimensional structure before it is ultimately released as a mature protein.

protein) and Hsp40 helps in stabilizing this complex. The Hsp70 complex binds to Hsp90 through HOP, which contains TRP domains that can bind both Hsp90 and Hsp70 and thus connect the two. The client protein is then loaded into Hsp90's middle domain and the Hsp70 complex is released. CDC37 is a known Hsp90 co-chaperone that has recently been found to

specifically aid in the loading of kinases into the complex and stays associated until folding is complete. At this point, other co-chaperones and immunophilins, which are peptidyl-prolyl cis-trans isomerases, bind to form the folding complex. ATP is then recruited to the N-terminal pocket of Hsp90 resulting in the dimerization of these domains and the formation of a "clamp" around the client protein (Chadli et al., 2000; Prodromou et al., 2000). p23 is then recruited and acts to stabilize the clamp and facilitate ATP hydrolysis. This protein folding complex helps the client find its three-dimensional structure before it is ultimately released as a mature protein. This releasing process is poorly understood, but p23 (Pratt et al., 2003) is hypothesized to facilitate the process (folding process reviewed in Buchner, 1999; Blagg and Kerr, 2006). Most Hsp90 inhibitors bind in the N-terminal ATP pocket thus preventing the clamp formation (**Figure 1.3**) and ultimately resulting in the degradation of the protein client.

Hsp90 and cancer

The aberrant activity of multiple signaling pathways is necessary for the progression of cancer. Interruption of just one of these abnormally activated pathways alone is frequently not effective for treatment. Therefore, the identification of new targets that are responsible for activation of multiple pathways has become important for the development of new cancer therapeutics. To date, Hsp90α/β has been found to interact with 70 kinases, 20 transcription factors (including steroid receptors) and 100 other proteins (see http://www.picard.ch). Through the folding machinery mentioned above, Hsp90 acts to stabilize these proteins and help them attain their biologically active form. Many of these client proteins are directly involved in signal transduction pathways that are associated with oncogenesis. In addition to normal protein clients, Hsp90 has been implicated in the maintenance of many mutated and chimeric proteins that result in transformation, such a p53 and BCR/ABL respectively (Blagosklonny et al., 1996; Nimmanapalli et al., 2001). Thus, Hsp90 has become an exciting new target for development of cancer therapeutics.

Inhibition of Hsp90 completely disrupts the folding process and targets client proteins for degradation. Inhibition can be accomplished through the binding of small molecules in the N-terminal ATP binding domain or in the C-terminal nucleotide binding domain. Studies investigating inhibitors that are designed to disrupt Hsp90 protein interactions with its binding partners in the multi-protein folding complex have also shown some success (inhibitors extensively reviewed in Yanyan et al., 2009).

Geldanamycin is a benzoquinone ansamycin antibiotic that binds in the N-terminal ATP binding pocket of Hsp90 and inhibits its ATP binding. This inhibition causes the degradation of Hsp90 client proteins and has been used as a tool to identify additional Hsp90 clients. Unfortunately, geldanamycin is poorly soluble in water and was found to be highly hepatotoxic, making it unsuitable for clinical trials. 17-allylamino-17-desmethoxygeldanamycin (17-AAG) was designed to improve the therapeutic index of geldanamycin by making it more water soluble. As a result, 17-AAG has been investigated in many different cancers and is currently the focus of numerous clinical trials (Usmani et al., 2009).

Many studies have investigated 17-AAG effects in leukemia cells alone or in combination with other chemotherapeutic agents. FLT3 is a frequent target for gain-of-function mutations in AML. Mutant FLT3 is an Hsp90 client and was shown to be degraded with Hsp90 inhibition (Minami et al., 2002; Yao et al., 2003). Molm13 and MV4-11 are leukemia cell lines that have activating FLT3 mutations. Treatment with 17-AAG and etoposide, a topoisomerase II inhibitor, was shown to have a synergistic effect on cell death in these two cell lines. This effect was not as pronounced in cell lines that were tested which had wild-type FLT3, which illustrates the importance of Hsp90 in stabilizing mutant proteins (Yao et al., 2007). Treatment of HL-60 and Jurkat leukemia cells sequentially with 17-AAG and then arsenic trioxide resulted in the same synergistic effect in cell death (Pelicano et al., 2006). 17-AAG was also shown to act in combination with cytarabine in HL-60 and ML1 leukemia cells to cause apoptosis (Messa et al., 2005).

ABL is a tyrosine kinase that is constitutively activated by the fusion with BCR found in most chronic myeloid leukemias. Gleevec is a tyrosine kinase inhibitor that has had great success in treating chronic myeloid leukemia patients with BCR/ABL positive cells. Nimmanapolli et al. developed Gleevec resistant K562 cells, which have endogenous BCR/ABL expression, and BCR/ABL expressing HL-60 cells (Nimmanapalli et al., 2002). This group was able to show that treatment of these resistant cells with 17-AAG induced apoptosis. In addition, patients that had failed Gleevec therapy had increased apoptosis of leukemia cells with 17-AAG treatment. These studies illustrate that targeting Hsp90 in AML with specific mutated client proteins, such as FLT3 may prove to be effective treatment.

A long term study examined the expression of heat shock proteins in 98 patients newly diagnosed with AML over a period of up to 4.5 years. These patients varied in their FAB subtype as well as their karyotypic profile. The expression of heat shock proteins in leukemic cells was much higher compared to expression in normal marrow. High expression of Hsp90 was correlated with high levels of multi-resistant protein expression. In addition, low Hsp90 expression was correlated with complete remission of the patients. The overall survival of patients was also significantly lower in those with high expression of Hsp90 (24.2 months versus 4.9 months) (Thomas et al., 2005).

A more recent study from the same group, confirmed that high Hsp90 expression is correlated with poor prognosis. They subsequently showed that higher percentages of Hsp90 positive cells were associated with PI3K/AKT activation and with BCL-2 positive cells. Patients who obtained complete remission also had significantly lower percentages of Hsp90 positive blast cells at diagnosis. Ex vivo studies demonstrated that samples with higher expression of Hsp90 had improved survival in growth factor-free medium. Additionally, samples that formed higher numbers of colonies in semi-solid medium had higher percentages of Hsp90 positive cells. Importantly, they also demonstrated that samples with a high percentage of Hsp90 positive cells were the most susceptible to 17-AAG treatment. The 17-AAG IC50 of samples with at least 50% Hsp90 positive cells was 1 μM, compared to an IC50 of almost 10 μM in those samples

with the lowest Hsp90 expression (Flanderin et al., 2008). In other words, those samples with high Hsp90 expression needed a dose 10 times *less* than those samples with low Hsp90 expression to kill of 50% of the cells. These two studies demonstrate the role of Hsp90, as well as other heat shock proteins, in the progression of AML.

Early phase I/II clinical trials of tanespimycin, or 17-AAG, have shown it to be well tolerated in patients with advanced cancers. The drug is administered intravenously and patient side effects include fatigue, thrombocytopenia, abdominal pain and some hapatotoxicity, all of which are reversible. Degradation of Hsp90 client proteins and up-regulation of Hsp70 was measured in tumor biopsies and peripheral blood monocytes to demonstrate that treatments were effectively inhibiting Hsp90 function. However, there was minimal disease response in these clinical trials suggesting that 17-AAG alone may not be an effective treatment strategy (reviewed in Reikvam et al., 2009; Usmani et al., 2009).

Data from clinical trials involving AML patients has been sparse. However, recent results from a phase I clinical study with alvespimycin, a new geldanamycin derivative, show that it is more water soluble than 17-AAG, more potent in inhibiting Hsp90, and has a longer half-life in the circulation. Data from this clinical trial demonstrated induction of Hsp70 expression and apoptosis in bone marrow blast cells (Lancet et al., 2010). These results suggest that more effective Hsp90 inhibitors may improve the efficacy of treatment in clinical trials.

The studies discussed above indicate that leukemia cells with high Hsp90 expression are more susceptible to 17-AAG treatment (Thomas et al., 2005; Flanderin et al., 2008). Furthermore, another study demonstrated that Hsp90 derived from tumor tissue is present mostly in the active folding complex. Since Hsp90 in normal tissue is mostly uncomplexed (Kamal et al., 2003), these results demonstrate that Hsp90 is highly active in cancerous cells. Hsp90 is known to stabilize a variety of signaling proteins that have random mutations and are required for cancer cell survival (Neckers, 2002). The over expression of these signaling proteins can also cause the up-regulation of Hsp90 seen in many cancers. As a result, cancer cells become more dependent on Hsp90, which makes them more sensitive to inhibitors than normal cells.

Hsp90 and phenotypic plasticity

Previous studies in *Drosophila* and *Arabidopsis* provided evidence that Hsp90 inhibition could cause the inheritance of unexpected morphological phenotypes. In *Drosophila* a reduction of Hsp90 function, either by pharmacological inhibition or mutant genotype, allowed phenotypic variation which affected many different adult features. The structures affected depended on the genetic background. Upon selection of these phenotypes, they became fixed independent of Hsp90 mutation and also became more penetrant. The authors stated that these observations were due to multiple genetic determinants that mapped to the second and third chromosome (Linquist et al., 1998). This group also showed that inhibition of Hsp90 in *Arabidopsis* resulted in the creation of atypical phenotypes (Queitsch et al., 2002), which demonstrates that the effect can be seen across species. It should be noted that the specific phenotypes seen were strain dependent, indicating that the mechanism could be genetically based.

However, another study in *Drosophila* provided evidence that Hsp90 inhibition could elicit phenotypic variation through epigenetic modulation. Epigenetics is defined as reversible, heritable changes in gene regulation that occur without a change in DNA sequence. These changes are brought about by histone modifications, such as acetylation, or by DNA methylation. Genetic or pharmacological inhibition of the Hsp90 homolog, Hsp83, had similar effects to heterozygous mutations in a variety chromatin remodeling genes. A transdifferentiation event, seen as a limb-like outgrowth in the eye, was observed in offspring whose parents were fed geldanamycin. The percentage of offspring with the eye phenotype increased upon selective breeding, in the absence of Hsp90 inhibition, suggesting an epigenetic mechanism. Further studies showed a reversal of the phenotype with histone deacetylase inhibitors, providing a link between Hsp90 and epigenetic modulation (Sollars et al., 2003).

These three important studies have led to the hypothesis that Hsp90 acts as a "capacitor" for morphological evolution. In other words, Hsp90 acts as a buffer to stabilize certain

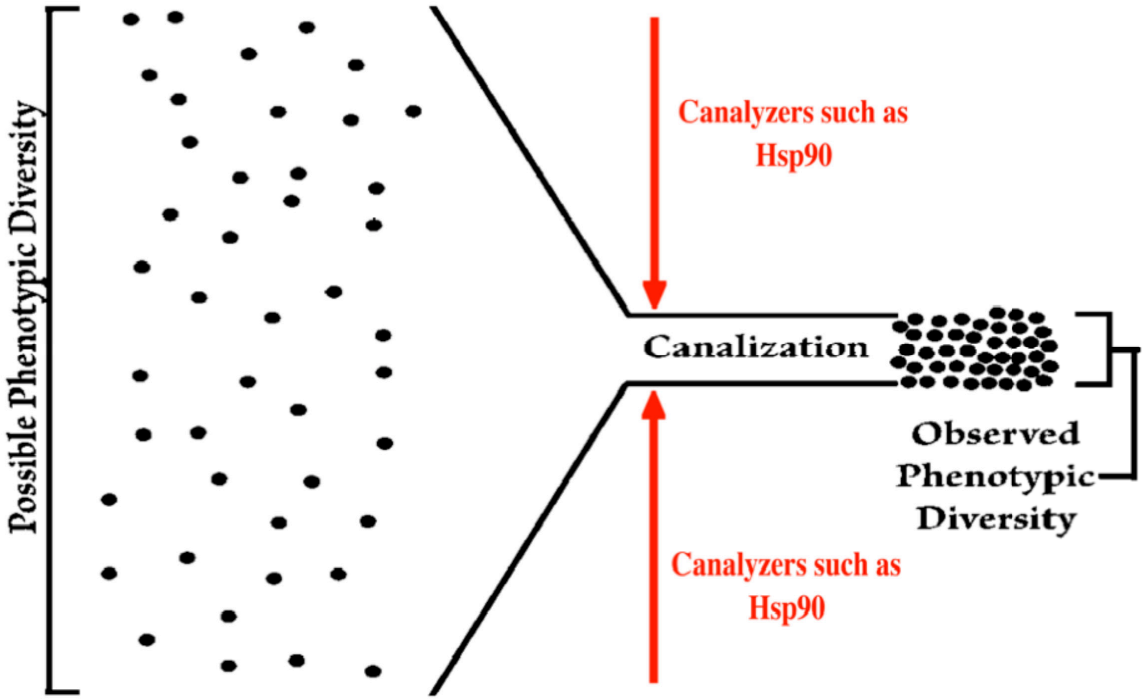

Figure 1.4. Canalization of phenotypic diversity. The potential expression patterns of genes from one organism to the next in a particular species are diverse enough to give a wide range of phenotypes that are not present in the population. This is theorized to be the result of canalization of these phenotypes during development. Meaning, the present morphology of the species is favored and variations in gene expression that would result in deviation from this model are compensated for by canalyzers (or capacitors) present in the system. However, if the canalyzer is knocked out or inhibited, the result is a release of varying phenotypes. This can allow for a greater adaptability of the species when selective pressures are applied or in the case of cancer progression a greater potential for full transformation.

phenotypes. Conrad Waddington first developed the theory of canalization in evolution (Waddington, 1942). He hypothesized that certain traits become fixed in a population so that even minor variations, through an unknown mechanism, still result in the same phenotypic outcome. Hsp90 fits very nicely into this theory (**Figure 1.4**). Non-deleterious mutations could go unnoticed due to the help of Hsp90 in folding and activation of proteins. Hsp90 function could be a possible mechanism for canalization, and has been called "Waddington's widget" for that reason (Ruden et al., 2003). Hsp90 could therefore act as a capacitor in that it "stores" phenotypic variation. In times of stress, Hsp90 activity would be reduced due to an increase in protein production and misfolded proteins placing a higher demand on its activity. Higher demand would cause a decrease in activity which could bring about the release of varying

phenotypes. Selection of phenotypes that are beneficial to the organism would become fixed in the population allowing for adaptation to occur.

While studies have demonstrated Hsp90's ability to act as a phenotypic capacitor for morphological evolution in *Drosophila* and *Arabidopsis*, data is lacking for this effect in mammalian systems. In studies that will be presented later, I have attempted to reproduce these results in a mammalian hematopoietic model, with mixed results. Even though my data were not reproducible, there is still the possibility that this phenomenon occurs in mammals. If this can be demonstrated, it would have a large impact on how Hsp90 inhibitors are used in cancer treatment. If Hsp90 acts as a phenotypic capacitor in humans, then its inhibition could make cancer cells more adaptable to their environment.

Hsp90 plays a role in many signal transduction pathways by its specific interaction with signaling proteins. Hsp90 has been proposed not only to participate in the folding process, but also to poise many of these signaling proteins in preparation for propagating these signals. In fact, Hsp90 has been shown to interact with over 10% of the yeast proteome, including chromatin remodeling complexes (Zhao et al., 2005). It would be reasonable to expect this interaction occurs in mammalian models since Hsp90 is so highly conserved in many different species. This provides further evidence that Hsp90 could be involved in epigenetic modulations. With Hsp90's diverse array of protein associations it is not surprising that perturbation in Hsp90 function could allow phenotypic variation.

p53

The discovery of p53

In 1979 investigators analyzed protein content of cells transformed by the SV40 virus by immunoprecipitation using sera from rodents with SV40-induced tumors. They found a novel 53 kD protein that bound to the SV40 T-antigen. Interestingly, this protein was also immunoprecipitated using the same sera from uninfected carcinoma cell lines, indicating this

protein was of cellular origin (Linzer and Levine, 1979). Later, it came to be known as p53 based upon its apparent molecular weight.

Determining the function of this protein has taken many investigators down a winding but remarkable road. At first it was thought to be a tumor antigen because animals immunized with tumor cells produced antibodies to p53 (DeLeo et al., 1979; Linzer and Levine, 1979). This finding initiated a race to isolate the cDNA in order to test the function of this protein in oncogenesis. Subsequent transfection studies indicated that p53 acts as an oncogene due to its apparent ability to cooperate with oncogenic RAS and immortalize cells (Eliyahu et al., 1984; Jenkins et al., 1984; Parada et al., 1984). At the same time these studies were ongoing researchers in Levine's group were performing the same experiment, but with a different p53 cDNA clone, but obtaining an opposite result. This prompted the two groups to exchange cDNA clones and perform the experiments again. Interestingly, they were able to reproduce each other's original results. However, comparison of the two cDNA clone's amino acid sequences held the key to the discrepancy. They were identical apart from one amino acid at position 135. Subsequent studies showed that wild-type p53 had an alanine at position 135 while the clone that acted as an oncogene had a valine at this amino acid position. This finding clearly demonstrated that one amino acid difference in p53 could make the difference between a protein that promotes oncogenesis and one that acts as a tumor suppressor (Hinds et al., 1989). These studies, along with others classified p53 as a tumor suppressor and documented the possible oncogenic properties of mutant p53.

p53 structure, regulation and function

In humans the p53 gene, *TP53*, is located on the short arm of chromosome 17. It is 20 kb in length and contains 11 exons and 10 introns. The first exon is non-coding, with the translational start site located in exon 2. The full length wild-type p53 cDNA is 1182 nucleotides long and encodes a 363 amino acid protein. Recently ten isoforms of p53 were identified (Bourdon et al., 2005; Rohaly et al., 2005). These are formed through alternative splicing in intron 9 and 2 and an

additional promoter located within intron 4. A combination of all these alternative splicing and promoter events results in the ten known isoforms to date (Bourdon, 2007).

It is apparent that these isoforms are expressed in normal tissue in a tissue specific manner. Two of these isoforms, Δ133p53 and Δ40p53, are known to act in a dominant negative manner to full length p53 due to the fact that they still have the oligomerization domain, but lack the transactivation domain (Curtois et al., 2002; Ghosh et al., 2004). Preliminary studies show the different p53 isoforms that are capable of DNA binding show a differential preference for p53-responsive promoters and can result in alternate p53 responses (Bourdon et al., 2005; Rohaly et al., 2005). This differential gene regulation has been proposed as a possible mechanism for the tissue specific actions of p53. Bone marrow or peripheral blood samples express seven isoforms (Bourdon et al., 2005; Anensen et al., 2006). This finding raises the possibility that protein levels of the different p53 isoforms could be important in leukemia. However, the functions of these isoforms and how they interact in the canonical p53 pathways are still poorly understood.

p53 consists of five main domains (**Figure 1.5**). The N-terminal region of the protein contains the transactivation domain and a proline rich domain. The core domain of the protein contains a large DNA binding domain, this region is also the target of 95% of the point mutations in p53 that is expressed in a large number of different types of cancer (Vousden and Lu, 2002). An oligomerization domain is located in the C-terminal region of the protein. This allows for two p53 molecules to dimerize and then bind to another dimer, ultimately forming a tetramer. The C-terminal region also contains a regulatory domain with several lysine residues that can either be ubiquitinated or acetylated. These post translational modifications have a direct effect on p53 stabilization and activation.

Under normal conditions, p53 has a very short half-life, on the order of 20 minutes. This is mostly due to its negative regulator MDM2, also known as HDM2 in humans. HDM2 is an E3 ubiquitin ligase that binds to the N-terminal transactivation domain of the protein and inhibits

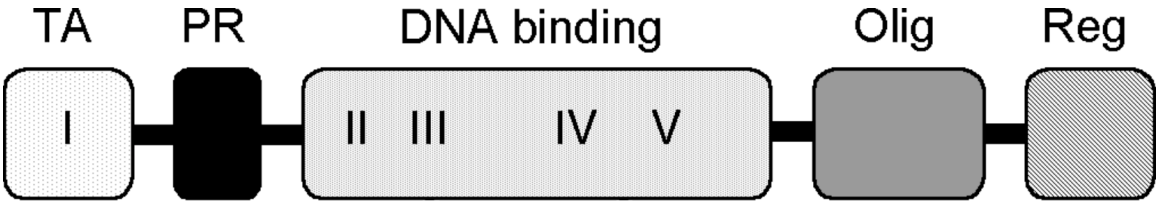

Figure 1.5. Schematic of p53 protein structure. p53 protein can be divided into five functional domains. The N-terminal region has the transactivation domain (TA) and the proline rich (PR) domain. The central region contains the DNA binding domain. The C-terminal holds the oligomerization domain (OD) and the regulatory domain (Reg). The Roman numerals indicate highly conserved regions of the *TP53* gene.

p53 function. It can also ubiquitinate p53 in the C-terminal region causing its subsequent degradation by the proteasome, resulting in low protein levels (Woods and Vousden, 2001; Michael and Oren, 2002). However, when a cell experiences stress such as DNA damage, hypoxia, ribonucleotide depletion, or deregulated oncogene expression, p53 rapidly accumulates in the cell. This is can result either from the covalent modification of the p53, such as acetylation and phosphorylation that activate p53 and inhibit HDM2 binding, or by modifying HDM2 and preventing its interaction with p53 (**Figure 1.6**).

In normal cells, p53 is constitutively expressed. Regulation of protein levels are accomplished through post-translational modifications as discussed above. This is a common control mechanism used to regulate stress proteins such as hypoxia-inducible factor-α. It allows cells to rapidly respond to stress by stabilizing instead of synthesizing proteins. Stabilization of p53 allows for the swift activation of pathways that prevent the propagation of cells that could cause cancer and other pathologies.

The role of p53 as a transcription factor has been intensely studied and is necessary for tumor suppressor activity. Once p53 is activated, it forms a tetramer that binds to specific sequences of DNA activating transcription of target genes (El-Deiry et al., 1992; Funk et al., 1992). Depending on tissue type and the extent of cellular damage, this can result in one of two outcomes. The cell can undergo cell cycle arrest, which occurs when p21, GADD45 and 14-3-3 protein expression is up-regulated. Cell cycle arrest buys the cell time to repair its DNA and thus

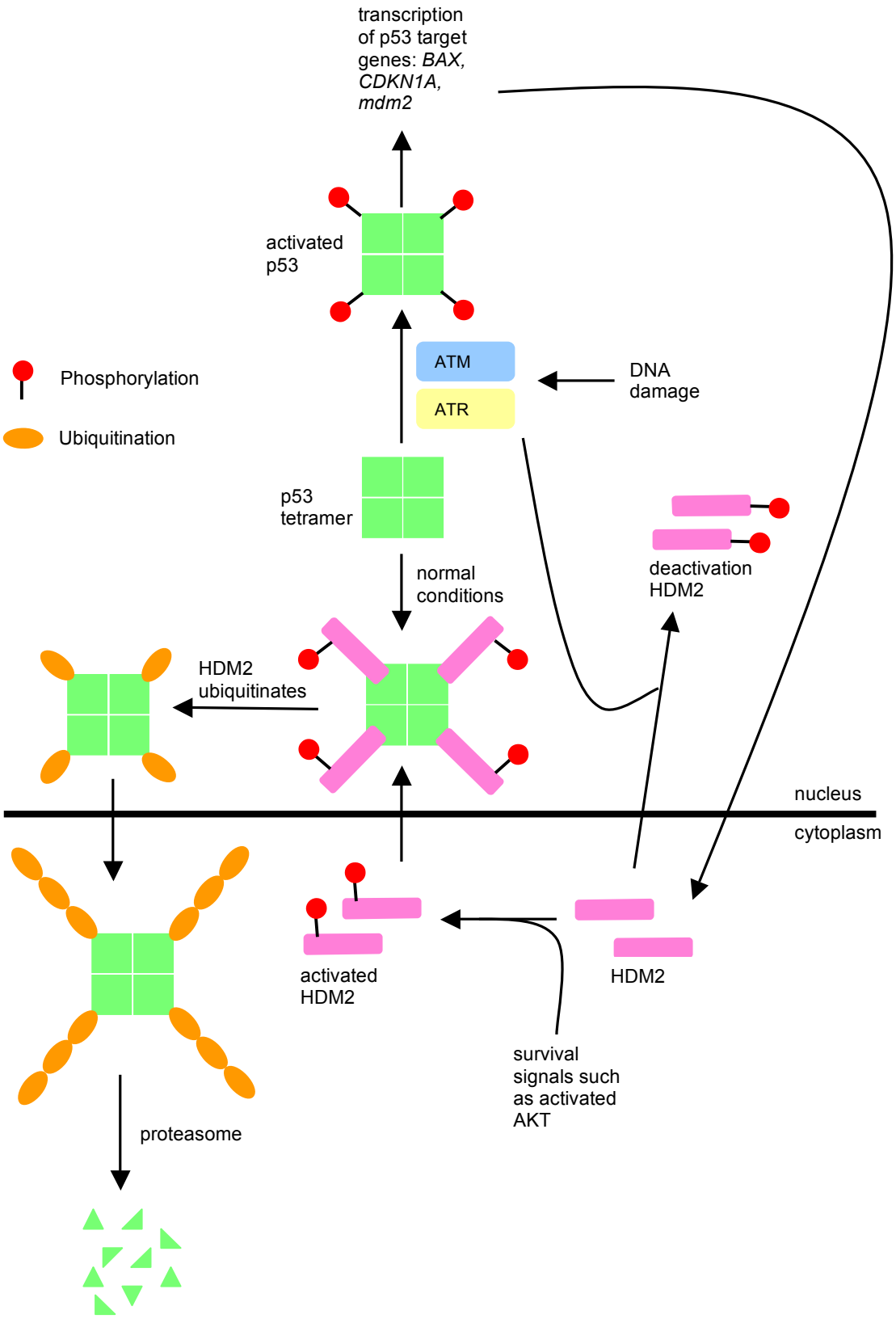
transcription of p53 target genes: *BAX, CDKN1A, mdm2*
activated p53
Phosphorylation
Ubiquitination
ATM
ATR
DNA damage
p53 tetramer
normal conditions
deactivation HDM2
HDM2 ubiquitinates
nucleus
cytoplasm
activated HDM2
HDM2
survival signals such as activated AKT
proteasome

Figure 1.6 (previous page). Control of p53 levels by various kinases. Under normal conditions HDM2 ubiquitinates p53, which causes its export from the nucleus followed by polyubiquitination and targeting to the proteasome where it is degraded. This keeps p53 levels quite low during homeostasis. However, when stress is applied, such as DNA damage, kinases such as ATM and ATR activate p53 via phosphorylation. This phosphorylation is thought to disrupt HDM2 binding to p53 and thus lengthening its half-life, which then allows it to activate transcription of target genes. ATM and ATR also phosphorylate HDM2 in an inhibitory manner to prevent its interaction with p53. Survival signals can activate kinases such as AKT, which can phosphorylate HDM2 causing its activation. This subsequently causes p53 to be degraded, which then prevents p53 from initiating an apoptosis response.

promotes survival. p53 can also activate pro-apoptotic genes such as *BAX*, *PUMA,* and *NOXA,* that forces the cell to undergo programmed cell death. By being a major switch to control these two pathways, p53 prevents the propagation of damaged cells that could contain mutations and become cancerous.

Indeed, p53 plays a central role in protecting the integrity of the genome, and thus preventing cellular transformation. The significance of this role is demonstrated by three key observations. One is that over 50% of all cancers have mutant p53, making it the most frequent mutated gene identified in human cancer. The second is Li-Fraumeni syndrome, which is a genetic disease often caused by a germline mutation in p53. Li-Fraumeni syndrome patients usually develop tumors by early adulthood with the loss of heterozygosity (Malkin et al., 1990). Finally, mice that are p53-null have normal development but develop cancer within 3-6 months of birth (Donehower et al., 1992; Jacks et al., 1994). These important functions of p53 have prompted scientists to call it the 'guardian of the genome' (Lane, 1992).

How exactly p53 makes the choice between apoptosis and cell cycle arrest has been extensively studied, but is still not completely understood. One hypothesis is that the decision between the life and death of a cell actually does not reside with p53, but with the intracellular and extracellular signaling events present with p53 activation. These signals can come from the cell-cell and cell-matrix interactions, or simply by activation of pro-survival signaling pathways such as AKT (Li, 2002). Survival signals present at the time of p53 activation could make a cell more prone to cell cycle arrest. It has also been proposed that specific covalent modifications to p53 can dictate which promoters it binds and activates. p53 protein binding partners can also

dictate promoter specificity. It seems abundantly clear that p53 acts as an important element in a complex network of signals and protein interactions that ultimately decides cell fate. p53 may be central in this network, but downstream effects of p53 activation can be largely dependent on availability and activation of other proteins (extensively reviewed in Oren, 2003).

When a cell is experiencing certain types of stress, e.g. DNA damage, p53 protein needs to be stabilized so that its accumulation can stimulate target pathways. When DNA damage occurs, the protein kinases ATM and ATR become activated and phosphorylate p53 in the N-terminal region (**Figure 1.6**). This disrupts HDM2 association with p53 and prevents its subsequent ubiquitination (Bannin et al., 1998; Canman et al., 1998). ATM can also phosphorylate HDM2 in such a way that causes it to be functionally inactive towards p53 (Maya et al., 2001). These actions increase p53 protein levels in the cell and increase its activity. The outcome of p53 action depends in a large part on the extent of DNA damage that has occurred.

HDM2 is frequently a target in cancer because of its role in p53 regulation. Hyper-proliferation induced by oncogenes, such as *β-catenin*, can induce p53 activity. $p14^{ARF}$, an alternative reading frame gene product of the *INK4a* locus (Sherr, 1998), is a known tumor suppressor that is frequently disabled in many cancers. ARF binds to HDM2 directly and blocks its ability to inhibit p53 function (Sherr et al., 2001). Deregulation of *β-catenin* activity has been shown to induce ARF protein expression (Damalas et al., 2001), which ultimately leads to p53 activation and cell cycle arrest. Activated AKT can also act directly on HDM2 in an opposite manner. Survival signals activate AKT, which allows it to phosphorylate HDM2 on different residues from those mentioned above to cause its relocation to the nucleus (**Figure 1.6**). There it can bind and ubiquitinate p53 causing its export from the nucleus and degradation (Mayo et al., 2001; Ogawara et al., 2002). These studies show that HDM2 can be phosphorylated on different residues by other pathways to increase or decrease its activity, which ultimately affect p53 function.

Mutant p53 in cancer

There are three mechanisms through which mutant p53 is thought to contribute to tumor development: (1) loss of wild-type p53 function; (2) dominant-negative inhibition of wild-type function; and (3) gain-of-function mutations. Loss of the wild-type tumor suppressor abilities of p53 would critically compromise the ability of a cell to respond to many different types of stress. As discussed previously, loss-of-function can be acquired by deleterious mutation or deletion of either *TP53* itself or one of the regulators of p53 activity. This would be especially advantageous to a precancerous cell. Without p53, genomic alterations would go unnoticed and provide the cell with a means to acquire new characteristics that could aid in tumor progression. The cell would also lack a means to detect uncontrolled proliferation, which is one of the hallmarks of cancer.

Of the 50% of mutant p53 genes in cancer, 90% are missense mutations that give rise to a full length stable protein that shows an increase in accumulation compared to wild-type. This distinguishes p53 from other tumor suppressor genes in which inactivation often results in truncated proteins or complete lack of protein expression. The majority of the missense mutations are in the DNA binding domain, disrupting sequence-specific DNA binding. At the same time, the oligomerization domain would remain intact allowing the mutant form to hetero-oligomerize with wild-type p53 (if wild-type is present). Since tetramer formation is necessary for DNA binding, it is conceivable that hetero-oligomerization would inhibit wild-type p53 from binding to DNA and activating transcription. This would result in a classic dominant-negative phenotype, whereby the mutant form dominantly inhibits the wild-type form and results in the same effect as what is seen in p53-null cells. Several studies have clearly shown this phenomenon to be present. For example, mice with endogenous wild-type p53 and engineered to express a missense p53 mutant in trans have a higher rate of developing tumors (Lavigueur et al., 1989; Harvey et al., 1995).

The very high frequency of missense mutations of p53 in cancer resulting in a stable protein suggests that those cancer cells with mutant p53 obtain a selective advantage during oncogenesis. This selective advantage indicates that mutant p53 could have other functions in the cell rather than just resulting in loss-of-function (reviewed in Kim and Deppert, 2004; Strano et al., 2007). Studies have been undertaken that test this hypothesis by ectopically expressing various p53 mutants in a cells without endogenous p53. Expression of mutant p53 in cells that lack endogenous p53 resulted in an increase in tumorigenicity (Shaulsky et al., 1991; Pohl et al., 1988; Dittmer et al., 1993). Many other studies have demonstrated that mutant p53 can induce the expression of genes important in tumor cell progression such as *MDR1*, *EGFR* and *c-Myc* (Chin et al., 1992; Deb et al., 1994; Frazier et al., 1998). More recently microarray analysis has shown that mutant p53 can alter global gene expression in different cell lines (O'Farrel et al., 2004). These studies make the case for mutant *TP53* acting as a transcription factor that has a different set of target genes to that of wild-type.

The possibility of mutant p53 acting as a transcription factor begs the question: can missense mutant p53 proteins still bind to DNA? This question has remained under vigorous debate. Despite numerous efforts of comparing promoter regions of mutant p53 target genes, no consensus sequence has been found to date. However, chromatin immunoprecipitation (ChIP) assays have demonstrated that mutant p53 proteins do indeed physically associate with the promoters whose expression they regulate (Zalcenstein et al., 2003). This demonstrates that mutant p53 proteins can target promoters in a specific manner, but that no sequence homology between promoters has been discovered.

Two mechanisms have been postulated to explain how mutant p53 can associate with its respective target promoter. One is that the mutant protein can bind to DNA based on the specific DNA structure. This hypothesis is supported by the findings that wild-type p53 can interact with DNA in a sequence specific AND non-specific manner. As discussed earlier, the sequence specific mode is facilitated by the DNA binding domain of the protein. However, the non-specific manner in which p53 can bind DNA is strictly dependent on DNA topography

(reviewed in Kim and Deppert, 2003) and is accomplished through the C-terminal domain. In fact, the C-terminus was found to be required for efficient binding to chromatin in vitro (Epinosa and Emerson, 2001). Wild-type p53 can bind with high affinity to double-stranded and single stranded DNA, secondary DNA structures and bulges in DNA caused by mismatched bases. As one can imagine, this method of binding DNA could facilitate the DNA repair function of p53.

This evidence suggests that DNA binding and transactivation of promoters by wild-type p53 may result from the sequence specific and non-specific interactions making the process much more complex than initially proposed (Yakovleva et al., 2002). These interactions of the C-terminal domain of p53 with DNA structure could allow mutant proteins, with inactive sequence specific DNA binding domains, to bind DNA and possibly facilitate transcription. This is further supported by a study demonstrating that mutant p53 was able to bind non-linear DNA structures, such as stem-loop and four-way junction structures, with high affinity, but unable to bind the linear counterpart of the same sequence (Goehler et al., 2005).

Another possible mechanism for the association of mutant p53 with various target promoters is its interaction with other sequence specific transcription factors. One example is specific protein 1 (SP1) (Gualberto and Baldwin, 1995; Chicas et al., 2000), a transcription factor that participates in the regulation of genes involved in proliferation, resistance to apoptosis and angiogenesis. (Black et al., 2001). Interestingly, wild-type p53 inhibits SP1 dependent gene expression, but mutant p53 acts synergistically to amplify transcriptional activation of SP1 (Bargonetti et al., 1997). Similar effects are seen with ETS-1, a proto-oncogene that regulates expression of angiogenic and extracellular matrix remodeling genes (Sampath et al., 2001). Expression of these types of proteins is typically associated with tumors of a more invasive phenotype and thus can be indicative of poor prognosis.

p53 and leukemia

About 10% of myeloid and lymphoid leukemias have mutated p53 at the time of diagnosis (Mitani et al., 2007). However, p53 inactivation is often more commonly associated with

progression of hematological malignancies, such as the progression of CML to blast crisis (Sander et al., 1993; Nakai et al., 1994). As discussed previously, chromosomal aberrations is a common occurrence in AML. In fact, loss of 17p is often associated with a complex abnormal karyotype, and is correlated with poor outcome (Haferlach et al., 2008). A recent study indicated that a single *TP53* deletion was a strong independent negative prognostic factor for disease free survival. This was also true of patients without the presence of other chromosomal abnormalities (Seifert et al., 2009). Another study showed that patients with abnormal chromosome 17 had significantly higher drug resistance and shorter overall survival (Nahi et al., 2008). These studies show that although *TP53* mutations in AML are uncommon, inactivating *TP53* can have a devastating outcome.

A very early case study revealed that a *TP53* mutation became dominant in the peripheral blood cells of chronic myeloid leukemia patient after progression to blast crisis. After successful treatment and return of the patient's condition to chronic phase, the mutant *TP53* clones could not be detected by sequencing, indicating that the *TP53* wild-type clones were predominant. This case study suggests *TP53* alteration can play a role in the acceleration from chronic phase to blast crisis in chronic myeloid leukemia (Foti et al., 1991).

Interestingly, a differential p53 isoform expression pattern was detected in peripheral blood cells before and after chemotherapy in AML patients (Anensen et al., 2006). Two isoforms, Δp53 and p53α (full length), were prevalent before the induction of chemotherapy. After two hours of the start of chemotherapy, p53α was up-regulated along with common p53 targets such as p21 and BAX. This prompted the authors to suggest that it is the alpha isoform that contributes the most in the p53 response of AML cells to stress.

Since *TP53* is mutated in only about 10% of myeloid and lymphoid leukemias, clinical drug studies have been designed to enhance the activity of wild-type p53. Nutlin and MI-63 both bind to HDM2 and inhibit its interaction with p53, resulting in an increased amount and activity of this protein. RITA (reactivation of p53 and induction of tumor cell apoptosis) is a furanic compound that binds to the N-terminal domain of p53 and causes a conformational change that reduces

HDM2 interaction with p53. There are some compounds available that can also reactivate mutant forms of p53. One such compound is PRIMA-1 (p53 reactivation and induction of massive apoptosis) that restores wild-type conformation to p53 through a mechanism that is not entirely understood. PRIMA-1 activation induces apoptosis without toxic effects to normal cells. CP-31398 is thought to stabilize the DNA binding domain and rescue DNA binding activity of p53 mutants. MIRA-1 rescues the transcriptional transactivation of p53 mutants by aiding the mutant protein in folding to a more native conformation (reviewed in Saha et al., 2010).

In studies that will be presented in Chapter 3, an AML cell line Kasumi-3 was found to possess two mutant alleles of *TP53*. We demonstrated that this cell line was very sensitive to Hsp90 inhibition by 17-AAG and that the p53 protein present in the cells was degraded with treatment. Therefore, we investigated whether the p53 present in these cells had oncogenic function.

Interaction of mutant p53 with the genome of cancer cells is very complex and may involve more than one mechanism of action. A further complication is the thousands of different *TP53* mutations reported in various types of cancer (Petitjean et al., 2007) and not all mutant p53 proteins have the same activity. Some mutant p53 proteins may act in a dominant negative manner rendering wild-type p53 useless, thus promoting tumor progression. Some mutants may act as transcriptional activators either by binding the DNA directly or through binding to protein complexes that can either abrogate or potentiate gene expression. The recent discovery of different p53 isoforms adds yet another layer of complexity to studying the role of p53.

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Chapter 2 : 17-N-Allylamino-17-demethoxygeldanamycin induces a diverse response in human AML cells

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Abstract

The goal of this study was to ascertain the specific effects of 17-N-Allylamino-17-demethoxygeldanamycin (17-AAG) treatment in human acute myelogenous leukemia (AML). Four human leukemia cell lines were treated with varying doses of 17-AAG followed by analysis of toxicity, apoptosis, proliferation, and cell cycle. Treatment with 17-AAG caused the cells to accumulate in the G_2/M phase. However, the effect was variable among the four AML cell lines. The expression of p21, p53 and P-glycoprotein (MDR1) activity were among the possible mechanisms responsible for the differential cell line response to 17-AAG. Exploiting these differences may allow for more effective combinatory treatments in patients with AML.

Introduction

Hsp90 is a constitutively expressed protein that makes up 1-2% of cellular protein (Csremely et al., 1998). It is a member of the chaperone protein family, whose function includes protein folding and quality control. In times of stress, Hsp90 expression is up-regulated to assist the cell's recovery by stabilizing misfolded proteins. Under normal conditions, Hsp90 is required for the activation of many signaling proteins including protein kinases and transcription factors (Pearl et al., 2008). In cancer, it acts to stabilize a variety of mutated and over-expressed signaling proteins that are required for cancer cell survival (Neckers, 2002). As a result, cancer cells become more dependent on Hsp90, which makes them more sensitive to inhibitors of this protein than normal cells. Thus, Hsp90 has become an exciting new target in chemotherapy for cancer.

Geldanamycin is an ansamycin antibiotic that inhibits Hsp90 by competitively binding in the ATP binding pocket located in the N-terminal domain of the protein. Although it showed promise as an anticancer agent, it was eventually determined that the drug was highly hepatotoxic. An analogue of geldanamycin, 17-AAG, was developed in order to improve the therapeutic index. These drugs ultimately cause the proteasomal degradation of Hsp90 client proteins by inhibiting the ATPase activity necessary for Hsp90 to function as a chaperone (Workman, 2004; Usmani et al., 2009). Because Hsp90 clients include many signaling proteins, inhibitors such as 17-AAG can have an impact on multiple signaling pathways making them desirable therapeutic agents. (extensively reviewed in Reikvam et al., 2009)

Recently, a phase I clinical trial investigating alvespimycin treatment, another geldanamycin derivative, in AML showed complete remission in 3 out of 17 patients and 1 patient achieved a 50% reduction in bone marrow blasts (Lancet et al., 2010). These results demonstrate that Hsp90 inhibition can produce clinically relevant effects; however, there is a need to determine the mechanisms responsible for the positive responses in order to improve response rate for AML patients. Consequently, the goal of this study was to ascertain the cellular and molecular changes that occur with 17-AAG treatment in human AML. To that end, the human leukemia cell

lines HL-60, KG-1a, THP-1 and Kasumi-3 cells, representing a variety of AML subtypes, were studied. Apoptosis, proliferation, cell cycle, and differentiation studies were performed after exposure of these cell lines to 17-AAG for various periods of time. Our data indicate that there was a diverse response among these AML cell types to 17-AAG treatment. These findings suggest that tailoring treatment on an individual basis may prove to be more effective in treating AML with 17-AAG.

Materials and Methods

Materials

p21 (clone CP36, CP74) and GAPDH (clone 6C5) antibodies were purchased from Millipore (Temecula, CA). Secondary antibody used with p21 was purchased from Abcam (Cambridge, MA). CDC2 and CDC25c (clones pstaire & H-6) antibodies were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). p53 antibody (clone DO-1) was a kind gift from Dr. Pier Palo Claudio. Clone DO-1 recognizes amino acids 21-25 in the transactivating domain of the protein, and in our hands resulted in a single band. This epitope makes it specific for the full length isoforms α, β, γ and the truncated isoform Δp53 (Bourdon, 2007; Anensen et al., 2006). Rabbit secondary antibody was purchased from Cell Signaling (Boston, MA), and mouse secondary was purchased from Amersham Biosciences. 17-AAG was purchased from A. G. Scientific, (San Diego, CA). Rh123 and Verapamil were purchased from Sigma (St. Louis, MO)

Cell Culture

All cell lines were purchased from American Type Culture Collection (Manassas, VA) and grown in the recommended culture medium and incubated at 37 ºC with 5% CO_2.

Apoptosis Studies

Cells were seeded at $2x10^5$ cells/mL and treated with vehicle, 2 or 3 μM of 17-AAG. After 48 hours, cells were labeled with pacific blue conjugated annexin V (Molecular Probes, Eugene OR)

and 7-aminoactinomycin D (BD Pharmingen, San Jose, CA) according to the Annexin V product sheet. Fluorescence was then measured by flow cytometry on a BD FACSAria flow cytometer. Data was analyzed using Flowjo 8.8.6 (Mac version).

Proliferation Studies

Cell Trace CSFE Proliferation Kit was purchased from Molecular Probes (Eugene, OR). The basic protocol was followed as given on the product information sheet to load the cells with dye. The cells were then seeded at $2x10^5$ cells/mL and were treated with vehicle, 2 or 3 μM 17-AAG. HL-60 cells and THP-1 cells were treated for 48 hours and Kasumi-3 cells were treated for 72 hours before collection. KG-1a cells were treated for 48 hours, counted and reseeded at $2x10^5$ cells/mL in fresh treated medium. 48 hours later (96 hours total) the cells were collected. All cells were fixed in 2% paraformaldehyde at time of collection for at least 18 hours before measuring fluorescence by flow cytometry. Median fluorescent values were determined using Flowjo 8.8.6 (Mac version).

Cell cycle

Cells were seeded at $2x10^5$ cells/mL and treated with vehicle, 0.25, 0.50, 1.0, 2.0 or 3.0 μM of 17-AAG. After 48 hours, HL-60, THP-1 and Kasumi-3 cells were collected and fixed in 70% ethanol. KG-1a cells were counted and resuspended at $2x10^5$ cells/mL in fresh treated medium. After an additional 48 hours, the cells were also fixed in 70% ethanol. DNA staining was performed by addition of 50 μg/mL of propidium iodide and 250 μg/mL of RNAse A (both purchased from Sigma, St. Louis, MO) in PBS and incubated at 37 ºC for 30 minutes. Cells were then placed on ice and fluorescence was measured by flow cytometry. Subsequent data was analyzed using ModFit LT for MacIntel.

Western Blotting

Protein was extracted by resuspension of the cells in RIPA lysis buffer [25mM Tris pH 7.4, 1% Triton X100, 1% SDS, 1% Na deoxycholate, 150mM NaCl + Halt protease inhibitor

(Thermoscientific, Rockford, IL)]. Protein was quantified using BCA Protein Assay Kit (Pierce, Rockford, IL). 50 μg of protein was loaded onto a 10% polyacrylamide gel and subjected to electrophoresis. Protein was then transferred to a nitrocellulose membrane and blocked with 5% milk in 0.1% Tween in tris-buffered saline (TBST). Antibodies were incubated over night at 4 °C in blocking solution (except for CDC25c, which was incubated in ½ block) at 1:1000 dilution. Washes and secondary incubations (1:3000 dilution) were performed at room temperature. Membranes were stripped by gently rocking in medium stripping buffer (200 mM glycine, 0.1% SDS, & 1% Tween, pH 2.2) for five minutes two times at room temperature. The membrane was then washed by rocking in TBST four times for five minutes each. The immunoreactivity bands were visualized using chemiluminescence (ECL) detection reagents or Super Signal West Pico (Thermoscientific, Rockford, IL). The bands on the autoradiograms were quantified using Quantity One 4.5.2 software program from Bio-Rad by densitometry.

Rh123 efflux assay

Rh123 efflux assay was performed as previously described (Petriz and Garcia-Lopez, 1997). Briefly, cells were seeded at 2 x 10^5 cells/mL. Rh123 was added at a final concentration of 200 ng/mL for Rh123 positive samples. Verapamil, at a final concentration of 50 μM, was added to the appropriate cultures. Cells were incubated at 37 °C for one hour. The cells were then washed once with PBS and resuspended in fresh medium with or without Verapamil. After 90 minutes incubation the cells were collected by centrifugation and resuspended in FACS buffer for analysis by flow cytometry as described above.

Verapamil combined with 17-AAG treatments

Cell cycle profile and apoptosis assays were performed as described above with the following treatments: 2 x 10^5 cells/mL were seeded into a six well plate and treated with vehicle, 50 μM Verapamil, 3 μM 17-AAG or both. After 24 hours a portion of the culture was harvested and fixed as described above for cell cycle analysis. Cells were analyzed for apoptosis after 48 hours of treatment.

Statistical analysis

All statistical analysis was performed using SigmaStat, Systat Software, Inc. Analysis of variance was performed on all data sets where normality and equal variance assumptions were met. Student's t-test was performed with Tukey's correction for multiple comparisons. Significance was assigned for all data with $p<0.05$. Cell cycle data sets were analyzed using Dunnett's method, comparing each group to the control. For all other non-normal data, ANOVA on Ranks was performed. Pairwise testing was performed using Dunn's method for multiple comparisons.

Results

Human leukemia cells have varying susceptibilities to 17-AAG treatment

A 17-AAG toxicity assay was performed on the four AML cell lines. Cells were seeded at $2x10^5$ cells/mL and incubated with varying concentrations of 17-AAG ranging from 2 μM to 15 μM for 48 hours. At this time, erythrosin B dye exclusion counts were performed. The results illustrate that there are distinct differences in the susceptibility of these cell lines to 17-AAG treatment (**Figure 2.1A**). KG-1a cells are still able to proliferate as demonstrated by an increase in cell number at lower treatment levels. In fact, these cells did not attain an apparent static state until they were treated with 8 μM of 17-AAG. On the other hand, Kasumi-3 cells exhibit a much higher susceptibility to apoptosis than the other cell lines, as can be seen by the low cell counts even at the lowest concentrations of treatment. These cells also continued to decline in cell number with greater doses of 17-AAG, unlike the other three cell lines which were able to sustain a constant cell concentration at higher doses.

To determine if 17-AAG was inducing apoptosis, annexin V studies were performed. It should be noted that in phase I clinical trials, the circulating concentration of 17-AAG in peripheral blood was less than 3.5 μM (Grem et al., 2005). Because of this, the rest of the experiments performed in this study centered on the physiologically relevant concentrations of 2

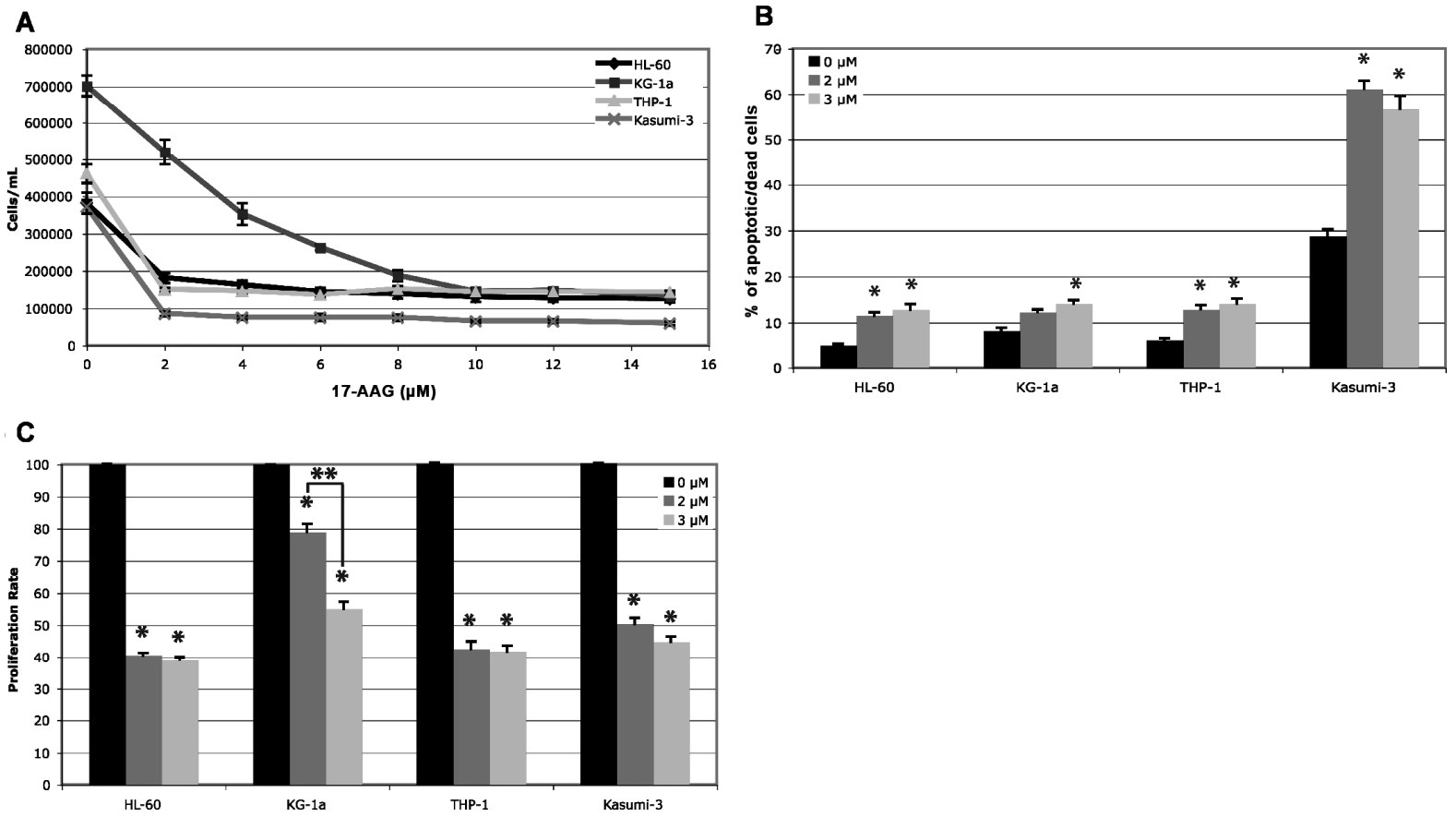

Figure 2.1. 17-AAG treatment inhibits proliferation in human leukemia cells and causes apoptosis. A) Cells were seeded at a density of $2x10^5$ cells/mL at indicated 17-AAG concentrations. Cell counts were performed after 48 hours. **B)** Cells were treated with 0, 2 or 3 μM of 17-AAG for 48 hours. Apoptosis was assessed using annexin V and 7-aminoactinomycin D. Graphs show percentage of apoptotic and dead cells combined at the various concentrations. **C)** Cells were loaded with CSFE dye and treated with 17-AAG as outlined in the materials and methods. Median fluorescence was measured by flow cytometry. Proliferation rate was assessed by calculating the median fluorescence percentage of untreated values to treated values. All results depicted here are representative of three separate experiments performed in triplicate (mean + S.E.M.). (*) designates significance of $p<0.05$ when data is compared to the vehicle control, (**) designates significance of $p<0.05$ when 3 μM treatment is compared to the 2 μM treated cells.

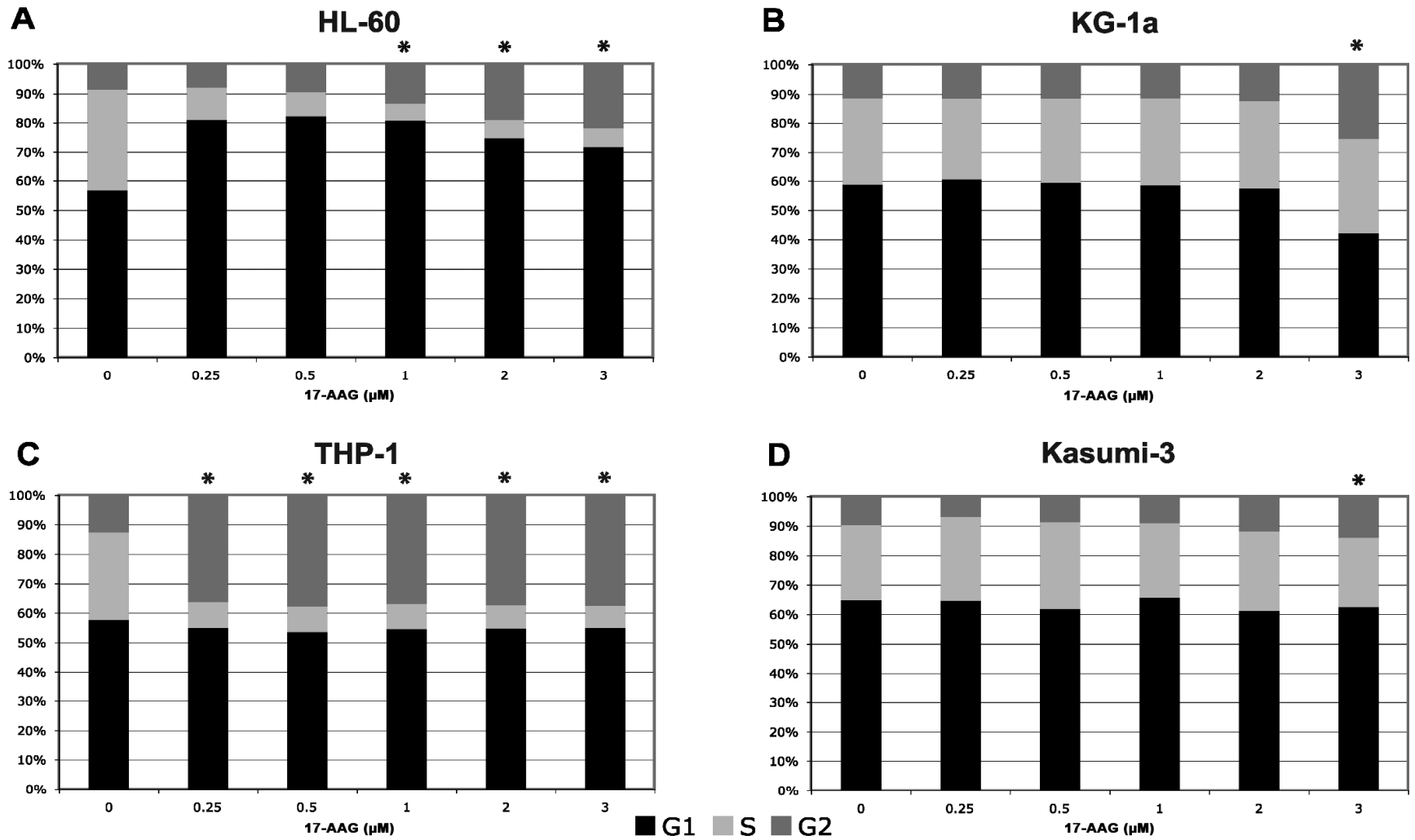

Figure 2.2. 17-AAG treatment causes arrest in G_2/M phase of the cell cycle. Cells were treated as outlined in the materials and methods with the indicated concentrations of 17-AAG. Cell cycle was measured by flow cytometry. Graphs illustrate percentage of cells in each phase: **A**) represents HL-60, **B)** KG-1a, **C)** THP-1 and **D)** Kasumi-3 cells. Results depicted here are the mean values representative of three separate experiments performed in triplicate. (*) indicates $p<0.05$ as compared to the vehicle control.

and 3 μM. The cells were seeded as described above and treated with 0, 2 and 3 μM of 17-AAG. After 48 hours the cells were collected and labeled with annexin V and 7-aminoactinomycin D as a dead cell counter stain. HL-60, KG-1a and THP-1 cells showed significant apoptosis and/or cell death; however, there was only about a 5% increase compared to vehicle control (**Figure 2.1B**). On the other hand, treatment induced a greater percentage of cellular apoptosis and death with short-term treatment in Kasumi-3 cells (**Figure 2.1B**). These data indicate that Kasumi-3 cells are much more vulnerable to short term 17-AAG treatment than the other cell lines.

Due to the resistance of KG-1a cells to treatment, they were exposed to 17-AAG for 96 hours with a re-treatment after 48 hours in order to obtain the similar results as the other cell lines. All the other cell lines were treated for 48 hours unless otherwise noted.

Dye dilution assays were performed next to confirm that treatment slowed proliferation of the cells. Kasumi-3 cells doubling time is quite long, ranging from 50-60 hours. Therefore, in this particular study, proliferation rate for this cell line was assessed after 72 hours to give the cells ample time to double. The proliferation rate of HL-60, THP-1 and Kasumi-3 cells was decreased significantly by up to 60% with 2 and 3 μM treatment (**Figure 2.1C**). KG-1a cells exhibit a significant dose dependent reduction in proliferation rate, 20% for 2 μM and 45% for 3 μM. These data indicate that even with more stringent treatment, KG-1a cells were still less responsive to 17-AAG treatment when compared to the other cell lines.

17-AAG treatment causes G_2/M arrest

The cells were treated with a range of 17-AAG concentrations and cell cycle was assessed using flow cytometry. HL-60 cells arrest in G_1 at the lower concentrations of 17-AAG, but high concentrations result in a 2.0 to 2.5-fold increase in the percentage of cells in the G_2/M phase compared to the vehicle control (**Figure 2.2A**). Not surprisingly, KG-1a cells show a 2-fold increase in G_2/M only with the highest concentration of 17-AAG (**Figure 2.2B**). KG-1a cells' lack of response in the lower concentrations of treatment provides further evidence of their resistant phenotype. THP-1 cells however, arrested at the lowest concentrations tested, with a 3-

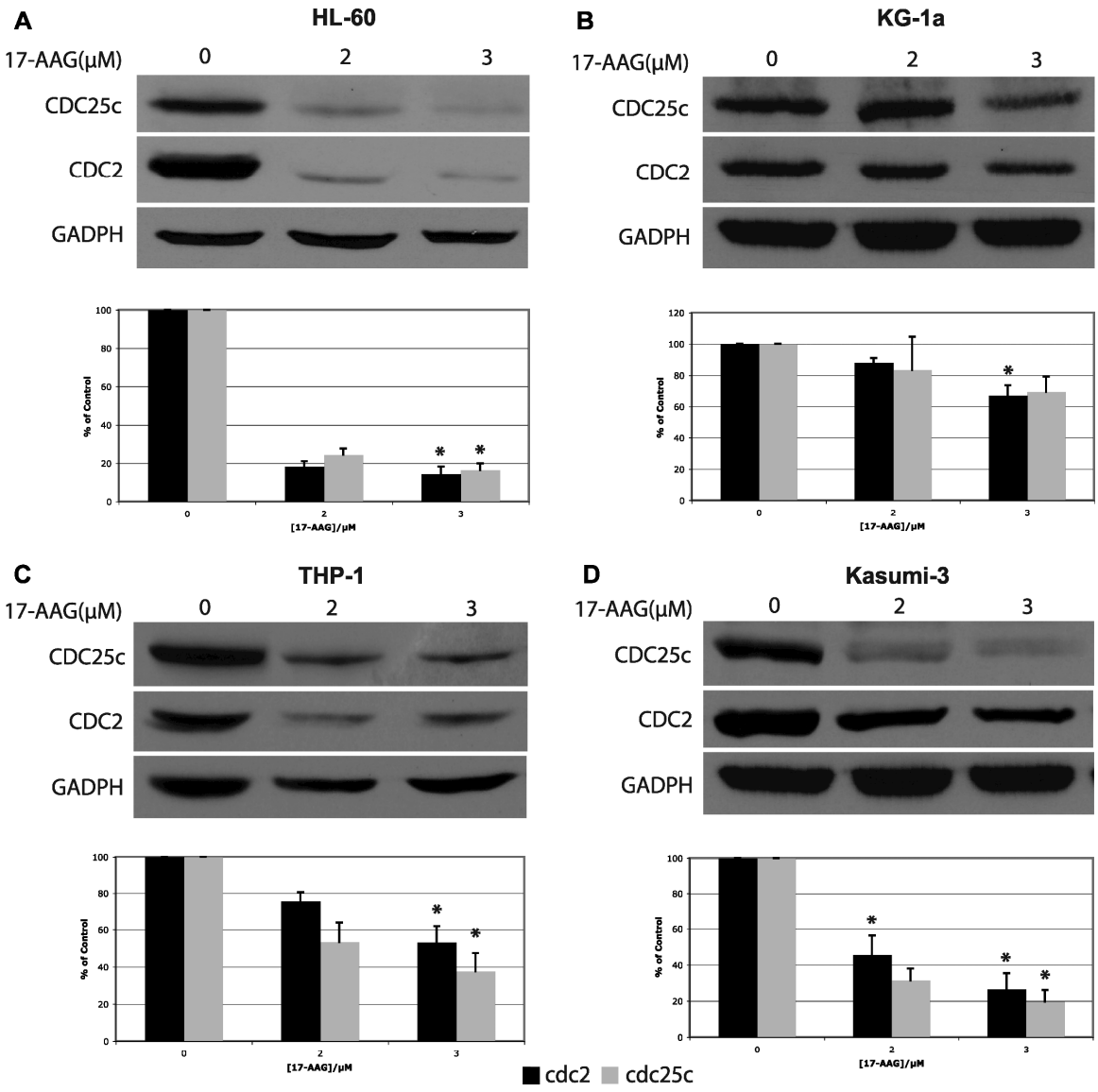

Figure 2.3. CDC2 and CDC25c levels are diminished with 17-AAG treatment. Cells were treated with 0, 2 and 3 μM 17-AAG as outlined in the materials and methods. Total cell lysates were subjected to SDS PAGE analysis on a 10% polyacrylamide gel and blotted onto a nitrocellulose membrane. Each panel shows a representative western blot after probing with CDC2 and CDC25c antibodies: **A)** represents HL-60, **B)** KG-1a, **C)** THP-1 and **D)** Kasumi-3. Graphs represent densitometry from 3-4 experiments (mean + S.E.M.). (*) indicates $p<0.05$ as compared to the vehicle control.

fold increase in G_2/M phase cells. This occurred with no concurrent change in G_1 phase cells (**Figure 2.2C**). Kasumi-3 cells accumulated to approximately 14% in G_2/M phase with the highest concentration of 17-AAG, however this was still very low compared to the other cell lines (**Figure 2.2D**). This result was surprising given that this cell line was the most susceptible to treatment. This suggests an alternate mechanism of cell death for Kasumi-3 cells and illustrates the complexity of the cell lines' responses to 17-AAG treatment.

17-AAG depletes CDC25c and CDC2 cell cycle protein levels

CDC25c and CDC2 were recently described as Hsp90 client proteins and subsequently down regulated with 17-AAG treatment in glioblastoma and lung cancer (Senju et al., 2006; Garcia-Morales et al., 2007). These two proteins are very important regulators of G_2/M progression (Stark and Taylor, 2006). In all of the AML cell lines, both proteins were diminished with Hsp90 inhibition (**Figure 2.3A**, **B**, **C** and **D**). According to the densitometry results, CDC2 and CDC25c proteins were degraded significantly in THP-1, Kasumi-3, and HL-60 cells treatment. However, only CDC2 was degraded to a significant extent in KG-1a cells at the highest concentration tested. It is interesting that the two proteins are degraded to a greater degree in HL-60 cells considering THP-1 cells undergo cell cycle arrest much more readily. This is suggestive of an additional mechanism facilitating G_2/M arrest in THP-1 cells.

p21 is up-regulated with 17-AAG treatment in THP-1 cells

p21 is an important protein that is up-regulated in times of stress by p53 (el-Deiry et al., 1994; Ohnishi et al., 1996). Recently, p21 has been shown to be up-regulated independently of p53 in chronic lymphocytic leukemias tested with geldanamycin (Lin et al., 2008). Expression of p21 promotes cell cycle arrest and differentiation and has been shown to disrupt CDC25c interactions with CDC2 (Abbas and Dutta, 2009). To determine whether p21 correlated with the observed G_2/M arrest, protein levels were measured by western blot in the four cell lines. Protein levels of p21 were persistently elevated in treated THP-1 cells, but was not induced in the

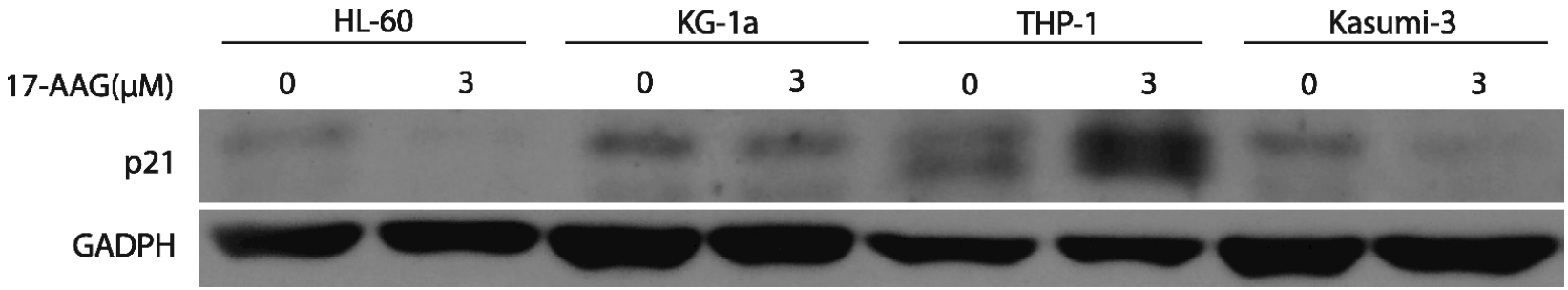

Figure 2.4. THP-1 cells up-regulate p21 with 17-AAG treatment. Cells were treated with 17-AAG as outlined in the material and methods. Total cell lysates were western blotted and probed with p21 antibody. The blot shown is representative of three blots in which all blots exhibits the same effect.

other cell lines (**Figure 2.4**). Up-regulation of p21 provides a possible mechanism for the high propensity of THP-1 cells to arrest in the G_2/M phase of the cell cycle with 17-AAG treatment.

p53 is significantly down-regulated with 17-AAG treatment in Kasumi-3 cells

Wild-type p53 is known to help facilitate apoptosis in response to chemotherapeutic drugs (Lotem and Sachs, 1993; Lowe et al., 1993). Therefore, it was hypothesized that Kasumi-3 cells' high sensitivity to 17-AAG treatment was due to wild type p53 expression. Western blotting was performed to assess p53 expression in all of the cell lines. The antibody used in this study recognizes an epitope in the N-terminal region of p53 (21-25 aa), which makes it is specific for the full length isoforms α, β, γ and the truncated isoform Δp53 (Anensen et al., 2006; Bourdon, 2007). A recent study revealed that AML patients have higher levels of Δp53 compared to αp53 in their leukemic cells. However, after receiving chemotherapy, there was a fast up-regulation of αp53 protein levels and p53 target genes (Anensen et al., 2006). This indicates the importance of the αp53 isoform in the chemotherapeutic response of AML patients. Indeed, p53 protein was highly expressed in Kasumi-3 cells as compared to the p53 over-expressing cell line Wi38 (**Figure 2.5A**). The other cell lines did not express detectable levels of p53 as previously reported (Kastan et al., 1991; Sugimoto et al., 1992; Akashi et al., 1999), and only a single band was detected in Kasumi-3 cell lysate.

Western blotting also revealed that p53 levels were significantly reduced with Hsp90 inhibition in Kasumi-3 cells (**Figure 2.5B**). This is consistent with mutant p53 being a known Hsp90 client that is degraded upon Hsp90 inhibition (Blagosklonny et al., 1996; Sepehrnia et al., 1996; Whitesell et al., 1998; Nagata, et al., 1999). This evidence suggests that Kasumi-3 cells harbor mutant p53 and not wild type. Sequencing of the p53 cDNA revealed that Kasumi-3 cells do indeed express mutant forms of p53 (see Chapter 3). Studies investigating these mutations are currently underway. Mutations and deletions of p53 occur in over 50% of all cancers (Hainaut and Hollstein, 2000) and have been shown to protect cancer cells from apoptosis

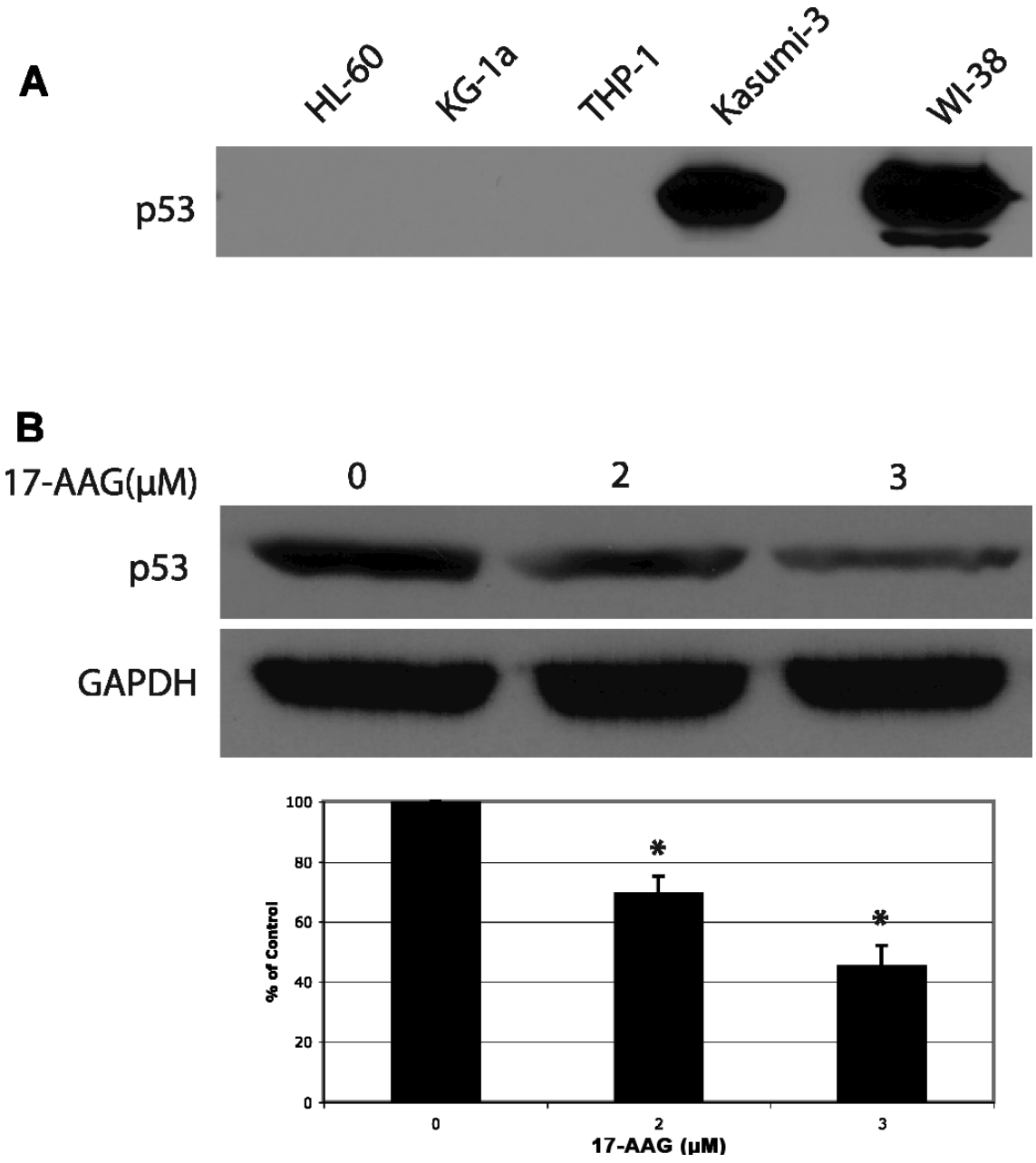

Figure 2.5. p53 is significantly down-regulated with 17-AAG treatment in Kasumi-3 cells. A) untreated cellular protein was blotted and probed with p53 antibody. WI-38 cells transfected with a p53 over-expression vector was used as a positive control. **B**) Kasumi-3 cells were treated for 48 hours with 0, 2 and 3 μM 17-AAG. p53 levels were measured by western blot. A representative blot is shown. Densitometry from four independent trials is graphed in panel under blot (mean + S.E.M.). (*) indicates $p<0.05$ as compared to the vehicle control.

(Cadwell and Zambetti, 2001; Dittmer et al., 1993; Kim and Deppert, 2004). It is conceivable that degradation of mutant p53 could be a possible mechanism for Kasumi-3 cells' high sensitivity to 17-AAG treatment.

KG-1a cells have P-glycoprotein (P-gp) activity facilitating their resistance to 17-AAG treatment

P-gp is an ATP-binding cassette (ABC) transporter that allows for efficient efflux of substrates from cells. It has been well characterized as a mechanism in multi-drug resistance (MDR), due to its inherent ability to clear chemotherapeutic agents from cells before they can inflict harm. KG-1a cells are known to have active P-gp (Bailly et al., 1995). In order to determine if this was the mechanism causing KG-1a cells' resistant phenotype, the efflux capacity of the different cell lines were measured. KG-1a cells have P-gp activity, as illustrated by decreased fluorescence in non-inhibited cells (**Figure 2.6A,** black histogram). This effect was ablated when 50 μM of verapamil (Vp), a specific P-gp inhibitor, was added to the cell culture (**Figure 2.6A**, shaded histogram). THP-1 and HL-60 cells however, were not able to efflux Rh123 to a significant degree (**Figure 2.6B** and **C**, black histograms). Results shown are representative of three independent experiments. KG-1a cells were then treated with a combination of 17-AAG and verapamil to determine whether inhibition of P-gp would result in a more susceptible phenotype. Cells were seeded as described above and treated with vehicle, 50 μM verapamil, 3 μM 17-AAG or a combination of both reagents. The cell cycle profile of each treatment group was measured after 24 hours. Treatment with 17-AAG and verapamil alone did result in a slight increase in the number of G_1 phase cells. This effect is not surprising since verapamil has been previously shown to inhibit growth of primary AML cells in vitro (Bruserud et al., 1995; Bruserud et al., 1993). However, this effect is ablated when the cells are treated with both reagents (**Figure 2.6D**). In fact, a 2-fold increase in the number of cells in G_2/M phase of the cell cycle was observed, which is more indicative of the phenotype displayed by the other cell lines in this study. There was also a significant increase in dead and apoptotic cells after 48 hours of 17-AAG

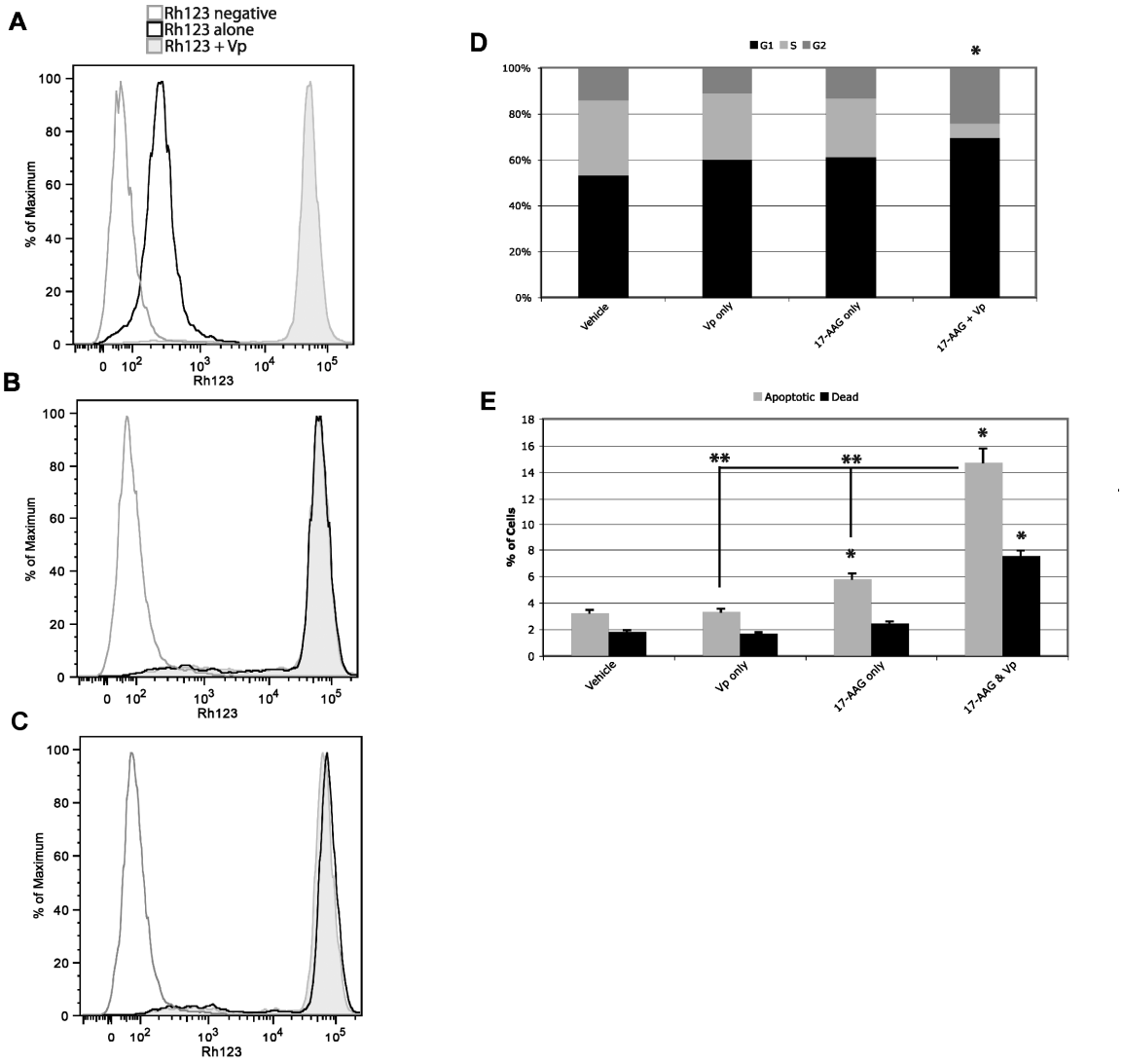

Figure 2.6. KG-1a cells possess P-gp activity. Cells were loaded with Rh123 dye in the presence or absence of 50 μM verapamil (Vp) for an hour. Dye efflux was measured by flow cytometry. Each panel shows a representative graph of fluorescence from three independent trials. **A)** KG-1a, **B)** HL-60, and **C)** THP-1. In panel **D**) KG-1a cells were treated with 50 μM of Vp alone, 3 μM of 17-AAG or a combination of both. After 24 hours, cell cycle was measured by flow cytometry. Results depicted are the means from three independent trials each performed in triplicate. **E)** Cells from the same cultures were incubated an additional 24 hours. At this time, they were labeled with annexin V and 7-aminoactinomycin D. Graph shows percentage of cells undergoing apoptosis and cellular death. Data is from three independent trials (mean + S.E.M.). (*) indicates $p<0.05$ as compared to the vehicle control (**) indicates $p<0.05$ as compared to single treatment.

treatment (**Figure 2.6E**). Not only was the increase significant when compared to vehicle control, it was also significant when compared to cells treated with Vp or 17-AAG alone. These results illustrate that P-gp activity is clearly playing a role in KG-1a cells resistance to 17-AAG treatment.

Discussion

Our data indicate that the response of AML cell lines to 17-AAG treatment is diverse and that Hsp90 operates to stabilize AML cancer cells by varied mechanisms. Previous studies have shown that 17-AAG sensitivity is variable among primary AML cells treated in vitro. This effect was correlated to Hsp90 expression, which was also found to be heterogeneous among the samples tested (Flandrin et al., 2008). Another study confirmed this observation showing that heat shock protein expression is highly variable among AML patients (Thomas et al., 2005). Both studies reported that high expression of heat shock proteins was associated with lower complete remission rates, illustrating the importance of heat shock proteins in AML. Our results add to the current known data by offering further mechanisms to explain the diversity of sensitivities of AML cells.

We verified a previous report (Flandrin et al., 2008) that 17-AAG treatment inhibits AML cell growth. Further analysis revealed the cells undergo G_2/M arrest, although this response was certainly not equivalent. We observed a 5 – 25% increase of cells within the G_2/M phase upon 17-AAG treatment among the cell lines tested. KG-1a cells required a more aggressive treatment regimen to achieve cell cycle arrest. THP-1 cells exhibited the highest susceptibility to 17-AAG treatment with a high percentage of cells accumulating in the G_2/M phase at the lowest concentration tested. HL-60 cells initially arrested in G_1 at 0.25 – 1.0 μM; however, they began accumulating in G_2/M at 2 – 3 μM of 17-AAG. G_1 arrest has been observed in malignant pleural mesothelioma and in Jurkat cells (Okamoto et al., 2008; Shelton et al., 2009) with 17-AAG treatment. The exact mechanism responsible for this shift of cell cycle compartment with increasing concentrations of 17-AAG is not known. Kasumi-3 cells also show a small, but

significant, accumulation in G_2/M phase when treated with 3 μM 17-AAG. However, these cells display a much higher level of apoptosis with 48 hours treatment as compared to the other cell lines (Fig. 1B). This indicates that there is an alternate mechanism that is directing them to undergo apoptosis instead of cell cycle arrest.

p21 is an important effector in cell cycle arrest. Therefore, p21 expression was measured in the cell lines to determine whether its induction correlated with cell cycle arrest. We found that 17-AAG only up-regulated p21 in THP-1 cells. This result correlates with THP-1 cells being the most sensitive to 17-AAG induced G_2/M arrest. It is tempting to speculate that p21 expression is the driving force behind this effect; however, more studies are needed to confirm this hypothesis.

Our data suggest that targeting mutant p53 with 17-AAG may prove to be an effective therapy in AML patients having this mutation. Evidence presented in this study indicates Kasumi-3 cells harbor mutant p53. Some forms of mutant p53 have been shown to protect cancer cells against apoptosis and enhance their tumorigenic potential (Cadwell and Zambetti, 2001). Loss of mutant p53, as seen with 17-AAG treatment (**Figure 2.5B**), could possibly alleviate this protection from apoptosis causing the cells to die instead of undergo cell cycle arrest. Additional studies, such as siRNA knock down of p53, are needed to support this hypothesis. Mutant p53 could be an important biomarker for 17-AAG treatment in AML as well as other cancers.

A previous study published by Ryningen et. al. (Ryningen et al., 2006) illustrated that low BCL-2 versus BAX ratios were associated with high in vitro apoptosis of primary AML cells. They also found that low Hsp70 levels were related to low vitality of the cells. It is possible that upon added stress of Hsp90 inhibition, Kasumi-3 cells may succumb to treatment due to an unfavorable ratio of these proteins. Further studies need to be performed to determine the mechanism for Kasumi-3 cells' high sensitivity to Hsp90 inhibition.

Overall our study shows that a possible reason for the lackluster response of 17-AAG in clinical trials is due to the heterogeneous nature of this cancer. Among the cell lines in this

study, a diverse response was elicited by 17-AAG treatment. Three different possible mechanisms for this diversity were observed. Clinical trials are ongoing investigating 17-AAG as a single agent, as well as in combination with other chemotherapeutic agents. Specific biomarkers, such as mutant p53 or P-gp, might aid in targeting patients that will benefit from 17-AAG treatment either alone or in combination with another drug. Future clinical studies are needed to determine whether mutant p53 or P-glycoprotein can predict patient response to 17-aag treatment.

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Chapter 3 : Kasumi-3 cells possess two different mutant *TP53* alleles

Abstract

p53 is a well known tumor suppressor protein. 90% of the mutations in its gene that are present in cancer result in production of a stable protein. Most of these mutations arise in the DNA binding domain and lead to an accumulation of the protein. We report here that the acute myelogenous leukemia (AML) cell line, Kasumi-3, is heterozygous for two point mutations and homozygous for the 72R polymorphism within the p53 gene. The p53 protein present in these cells is non-functional and no gene amplification was detected. Knockdown studies of p53 revealed that the mutant p53 is not involved in cell survival or P-glycoprotein expression in this cell line. However, Kasumi-3 cells can still serve as an important tool to study the effects of mutant p53 in various forms of cancer.

Introduction

p53 has been well established as a transcription factor that has tumor suppressor activities. Under normal conditions, it has a short half-life due to its rapid degradation. Upon cellular stresses, such as heat shock and DNA damage, a rapid accumulation of the protein occurs. This accumulation results in the expression of various genes that are involved in DNA damage control, including *CDKN1A,* which encodes p21 that induces cell cycle arrest, *BAX* an inducer of apoptosis, and *mdm2,* which encodes HDM2 that is a negative regulator of p53. This creates a tightly regulated pathway that can quickly be activated and then deactivated when conditions return to normal (reviewed in Oren, 2003). Expression of these genes and other p53 targets are key in regulating cellular processes that prevent transformation.

In order to circumvent its tumor suppressor activities, *TP53* is mutated in over 50% of all cancers. Interestingly, over 90% of these mutations are missense mutations in the DNA binding region but result in the expression of a stable full length protein (Vousden and Lu, 2003). The high incidence of these mutant p53 proteins in cancer has led to the hypothesis that they offer cancer cells a survival advantage. In fact, oncogenic activities have been reported with some mutant p53 proteins (reviewed in Kim and Deppert, 2004).

We recently reported that Kasumi-3 cells, an AML cell line, are especially sensitive to Heat shock protein 90 (Hsp90) inhibition and provided evidence to support the hypothesis that these cells express mutant p53 (Napper and Sollars, in press). We report here that Kasumi-3 cells are homozygous for the P72R polymorphism and heterozygous for two separate point mutations of the *TP53* gene.

Materials and Methods

Materials

p21 (clone CP36, CP74) and GADPH (clone 6C5) antibodies were purchased from Millipore (Temecula, CA). Secondary antibody used with p21 was purchased from Abcam (Cambridge,

MA). BAX (clone 2D2) antibody was purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). p53 and HDM2 antibodies (clone DO-1 and A10, respectively) were a kind gift from Dr. Pier Palo Claudio. Rabbit secondary antibody was purchased from Cell Signaling (Boston, MA) and mouse secondary was purchased from Amersham Biosciences. Camptothecin was purchased from Sigma (St. Louis, MO). Digoxygenin labeling kit (cat# 11 585 614 910) was purchased from Roche. EcoRI, NcoI, HindIII, and StuI were purchased from Promega (Madison, WI). The siRNA for p53 (Cat# sc-29435) and control (Cat# sc-37007) were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The P-glycoprotein antibody, isotype control and secondary antibody used in this study were obtained from BD (cat # 557001, 55740 and 550826 respectively).

Cell culture

Kasumi-3 and A549 cell lines were purchased from the American Type Culture Collection (Manassas, VA) and grown in the recommended culture medium and incubated at 37 ºC with 5% CO_2.

Sequencing

p53 cDNAs were obtained by isolating RNA from Kasumi-3 cells using the Master Pure kit from Epicenter (Madison, WI). The cDNA was prepared using the Advantage RT-for-PCR kit from Clontech (Mountain View, CA). The p53 cDNAS were PCR amplified using primers described previously (Liu and Bodmer, 2006). The resulting PCR products were cloned using an Invitrogen TOPO TA Cloning kit. Clones containing the plasmid were selected by plating on LB plates containing 40 μg/mL of kanamycin. Plasmids were harvested from 3 mL an overnight liquid culture using the Zyppy Plasmid Prep kit from Zymo Research (Orange, CA) and sequenced using M13 primers. The 1.8 kb insert necessitated sequencing from an internal primer (5'-gtgaaatattctccatccagtg-3') as well as the external M13 primers. Four of the 1.8 kb PCR products and 16 of the 1.5 kb PCR products were sequenced from the recombinant plasmids by the Genomics Core Facility at Marshall University School of Medicine.

Genomic sequencing was accomplished by first isolating genomic DNA from Kasumi-3 cells using the GenElute Mammalian DNA Prep Kit from Sigma (St. Louis, MO). Subsequently, the genomic regions containing the three polymorphisms found by sequencing the cDNAs were PCR amplified followed by gel purification. These PCR products were then sequenced on an ABI 3130 Genetic Analyzer in the MU Genomics Core Facility. The primers used for sequencing and PCR amplification are as follows. For the 782+1G>A mutation primers were described previously for exon 7 (Liu and Bodmer, 2006); for the P72R polymorphism and the 484A>T mutation the primers were described previously to amplify exon 4 and 5 respectively (Kandel et al., 2000). Three PCR products of each region were sequenced.

p53 transcriptional activity assay

Kasumi-3 were seeded at $3x10^5$ cells/mL and treated with DMSO or 300 nM camptothecin. A549 cells were grown to 50-70% confluence before treatment. Cells were harvested 24 hours after treatment and protein was extracted by resuspension of the cells in RIPA lysis buffer [25mM Tris pH 7.4, 1% triton X100, 1% SDS, 1% Na Deoxycholate, 150mM NaCl + Halt protease inhibitor (Thermoscientific, Rockford, IL). Protein (20 μg) was loaded from each sample and probed with p21, HDM2, BAX, p53 and GAPDH antibodies.

Southern blotting

For probe preparation, template DNA was obtained by amplification of one of the plasmids containing the large insert and subsequently purified using Qiaquick PCR Purification Kit from Qiagen (Valencia, CA). This PCR product (1 μg) was used in the probe labeling reaction following the protocol provided in the Digoxygenin labeling kit. DNA was extracted from each cell line using GenElute Mammalian DNA Prep Kits from Sigma (St. Louis, MO). DNA (12 μg) was digested with 5u/μg of EcoRI, HindIII, StuI and NcoI for 3 hours at 37 ºC in a total volume of 200 μL. The DNA was then precipitated and equal amounts (8-10 μg) were loaded onto a 0.7%, 7 mm thick agarose gel. DNA fragments were separated by electrophoresis at 2.5 v/cm for about 1 hour, when the bromophenol blue was about ⅔ of the way down the gel. All gel treatments

were performed with gentle rocking at room temperature. The gel was first depurinated with 0.25 M HCl for 15 min. The gel was then treated with denaturation solution (1.5 M NaCl, 0.5 M NaOH) 2x for 20 minutes each. This was followed by treatment with neutralization solution (1.5 M NaCl, 0.5 M TrisCl, pH 7.0) 2x for 20 minutes each. The DNA was then transferred to a nitrocellulose membrane with 20x SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0) using a vacuum blotter (model 785) from BioRad at 6 mmHg for 90 minutes. DNA fragments were crosslinked to the membrane with a UV crosslinker (Fisher FB-UVXL-1000); membranes were rinsed once in 2x SSC and allowed to air dry. The filter was then pre-hybridized using the hybridization buffer from the kit. Hybridization was performed over night at 37ºC with gentle rocking and 25 ng/mL of probe. The membrane was then washed 2x at room temperature with 0.1% SDS, 2x SSC for 5 minutes each followed by two more 15 minute washes at 63ºC with 0.1% SDS, 0.5x SSC in a UVP Hybridization Oven (model HB-100). Detection of the digoxygenin labeled probe was based on Digoxygenin labeling kit's protocol. For the best result, the membrane was exposed to film for 20 minutes after the initial 1 hour of increasing signal was achieved.

p53 knockdown studies

The day before transfection, Kasumi-3 cells were seeded at 3-4 x 10^5 cells/mL in fresh medium. Electroporation was done by pipetting 800 nM of siRNA, control or p53, in a cuvette. 100 μL of media without serum containing 2 x 10^6 cells was added to the cuvette. The cells were gently mixed to ensure even distribution of siRNA. The cuvette was then electroporated using a mammalian electroporator (Elector Square Porator model ECM830 from BTX Genetronics) with 2 pulses of 250 volts for 13 ms each. The cells were then plated in 3 mL of full media in a 6 well plate. Protein knockdown and other assays were performed 72 hours later. Apoptosis and efflux assays were performed as described previously (Chapter 2 materials and methods). P-glycoprotein expression was measured by first blocking Fc receptors with human IgG for 20 minutes. The antibody was then added in FACS buffer (3% BSA, 0.02% sodium azide, 1mM EDTA in PBS) at 0.2ug/10^6 cells for 30 minutes 4ºC. The cells were washed once and secondary

conjugated to APC was added to a total volume of 100 μL. The cells were incubated again for 30 minutes at 4ºC, then washed once and subjected to analysis on a BD FACSAria.

Statistical analysis

Statistical analysis was performed using SigmaStat, Systat Software, Inc. Student's t-test was performed and significance was assigned for all data with $p<0.05$.

Results

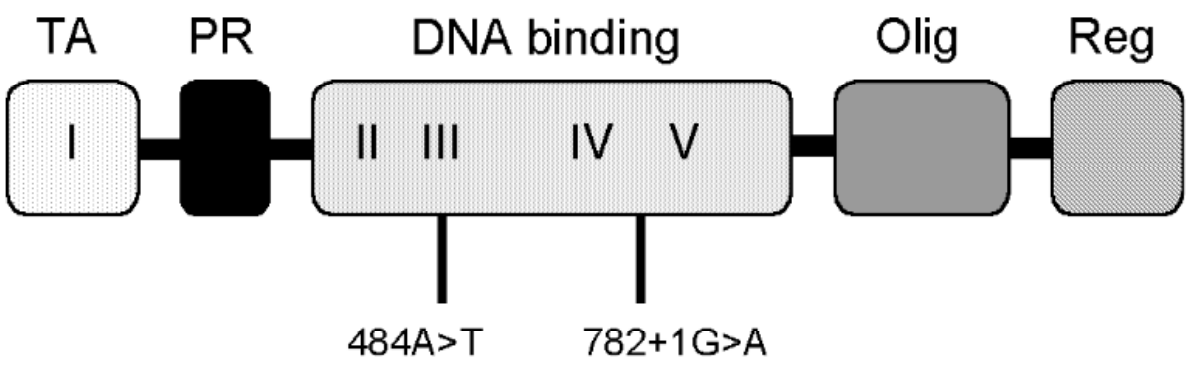
Kasumi-3 cells are heterozygous for two mutant *TP53* alleles

p53 cDNAS from Kasumi-3 cells were prepared by PCR amplification of the entire open reading frame. Surprisingly, two products were obtained, the expected 1.5 kb product and a larger 1.8 kb product. Sequencing of several clones of each of the products revealed that both products contain a single nucleotide polymorphism, 215C>G (**Figure 3.1B** and **C**). Translation of these mRNAs results in the amino acid change, P72R, which turns out to be a common polymorphism.

The smaller product also contains a point mutation, 484A>T (**Figure 3.1B**). This results in a missense mutation in the DNA binding region of the protein with the amino acid substitution I162F (**Figure 3.1A**). The larger product also contains a point mutation in the intron 7 donor site, 782+1G>A (Figure 3.1C), which results in retention of intron 7 in the mature messenger RNA. Translation of this mRNA would produce a truncated form of p53 due to a stop codon located within the intronic sequence (**Figure 3.1C**). Therefore, protein structures after exon 7 would be absent, including the oligomerization domain and the regulatory domain (**Figure 3.1A**).

This data led to the hypothesis that Kasumi-3 cells are homozygous for the P72R polymorphism and heterozygous for the two point mutations. In order to confirm this, the three regions of genomic DNA containing the polymorphisms were PCR-amplified and sequenced.

A



B

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   1 ATGGAGGAGC CGCAGTCAGA TCCTAGCGTC GAGCCCCCTC TGAGTCAGGA AACATTTTCA GACCTATGGA AACTACTTCC TGAAAACAAC GTTCTGTCCC
 101 CCTTGCCGTC CCAAGCAATG GATGATTTGA TGCTGTCCCC GGACGATATT GAACAATGGT TCACTGAAGA CCCAGGTCCA GATGAAGCTC CCAGAATGCC
 201 AGAGGCTGCT CCCCGCGTGG CCCCTGCACC AGCAGCTCCT ACACCGGCGG CCCCTGCACC AGCCCCCTCC TGGCCCCTGT CATCTTCTGT CCCTTCCCAG
 301 AAAACCTACC AGGGCAGCTA CGGTTTCCGT CTGGGCTTCT TGCATTCTGG GACAGCCAAG TCTGTGACTT GCACGTACTC CCCTGCCCTC AACAAGATGT
 401 TTTGCCAACT GGCCAAGACC TGCCCTGTGC AGCTGTGGGT TGATTCCACA CCCCCGCCCG GCACCCGCGT CCGCGCCATG GCCATCTACA AGCAGTCACA
 501 GCACATGACG GAGGTTGTGA GGCGCTGCCC CCACCATGAG CGCTGCTCAG ATAGCGATGG TCTGGCCCCT CCTCAGCATC TTATCCGAGT GGAAGGAAAT
 601 TTGCGTGTGG AGTATTTGGA TGACAGAAAC ACTTTTCGAC ATAGTGTGGT GGTGCCCTAT GAGCCGCCTG AGGTTGGCTC TGACTGTACC ACCATCCACT
 701 ACAACTACAT GTGTAACAGT TCCTGCATGG GCGGCATGAA CCGGAGGCCC ATCCTCACCA TCATCACACT GGAAGACTCC AGTGGTAATC TACTGGGACG
 801 GAACAGCTTT GAGGTGCGTG TTTGTGCCTG TCCTGGGAGA GACCGGCGCA CAGAGGAAGA GAATCTCCGC AAGAAAGGGG AGCCTCACCA CGAGCTGCCC
 901 CCAGCGAGCA CTAAGCGAGC ACTGCCCAAC AACACCAGCT CCTCTCCCCA GCCAAAGAAG AAACCACTGG ATGGAGAATA TTTCACCCTT CAGATCCGTG
1001 GGCGTGAGCG CTTCGAGATG TTCCGAGAGC TGAATGAGGC CTTGGAACTC AAGGATGCCC AGGCTGGGAA GGAGCCAGGG GGGAGCAGGG CTCACTCCAG
1101 CCACCTGAAG TCCAAAAAGG GTCAGTCTAC CTCCCGCCAT AAAAAACTCA TGTTCAAGAC AGAAGGGCCT GACTCAGACT GA
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C

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   1 ATGGAGGAGC CGCAGTCAGA TCCTAGCGTC GAGCCCCCTC TGAGTCAGGA AACATTTTCA GACCTATGGA AACTACTTCC TGAAAACAAC GTTCTGTCCC
 101 CCTTGCCGTC CCAAGCAATG GATGATTTGA TGCTGTCCCC GGACGATATT GAACAATGGT TCACTGAAGA CCCAGGTCCA GATGAAGCTC CCAGAATGCC
 201 AGAGGCTGCT CCCCGCGTGG CCCCTGCACC AGCAGCTCCT ACACCGGCGG CCCCTGCACC AGCCCCCTCC TGGCCCCTGT CATCTTCTGT CCCTTCCCAG
 301 AAAACCTACC AGGGCAGCTA CGGTTTCCGT CTGGGCTTCT TGCATTCTGG GACAGCCAAG TCTGTGACTT GCACGTACTC CCCTGCCCTC AACAAGATGT
 401 TTTGCCAACT GGCCAAGACC TGCCCTGTGC AGCTGTGGGT TGATTCCACA CCCCCGCCCG GCACCCGCGT CCGCGCCATG GCCATCTACA AGCAGTCACA
 501 GCACATGACG GAGGTTGTGA GGCGCTGCCC CCACCATGAG CGCTGCTCAG ATAGCGATGG TCTGGCCCCT CCTCAGCATC TTATCCGAGT GGAAGGAAAT
 601 TTGCGTGTGG AGTATTTGGA TGACAGAAAC ACTTTTCGAC ATAGTGTGGT GGTGCCCTAT GAGCCGCCTG AGGTTGGCTC TGACTGTACC ACCATCCACT
 701 ACAACTACAT GTGTAACAGT TCCTGCATGG GCGGCATGAA CCGGAGGCCC ATCCTCACCA TCATCACACT GGAAGACTCC AGATCAGGAG CCACTTGCCA
 801 CCCTGCCACAG TGGCCTGCTG TGCCCCAGCC TCTGCTTGCC TCTGACCCCT GGGCCCACCT CTTACCGATT TCTTCCACAC TACTACCCAT CCACCTCTCA
 901 TCACATCCCC GGCGGGGAAT CCCCTTACTG CTCCCACTCA GTTTCTCTTT CTCTGGCTTT GGGACCTCTT AACCTGTGGC TTCTCCCCCA CCTACCTGGA
1001 GCTGGAGCTT AGGCTCCCAG AAGGACAAGG GTGGTTGGGA GTAGATGGAG CCTGGTTTTT TAAATGGGAC AGGTAGGACC TGATTTCCTT ACTGCCTCTT
1101 GCTTTCTCTT TCCTATCCTG AGTAGTGGTA ATCTACTGGG ACGGAACAGC TTTGAGGTGC GTGTTTGTGC CTGTCCTGGG AGAGACCGGC GCACAGAGGA
1201 AGAGAATCTC CGCAAGAAAG GGGAGCCTCA CCACGAGCTG CCCCCAGGGA GCACTAAGCG AGCACTGCCC AACAACACCA GCTCCTCTCC CCAGCCAAAG
1301 AAGAAACCAC TGGATGGAGA ATATTTCACC CTTCAGATCC GTGGGCGTGA GCGCTTCGAG ATGTTCCGAG AGCTGAATGA GGCCTTGGAA CTCAAGGATG
1401 CCCAGGCTGG GAAGGAGCCA GGGGGGAGCA GGGCTCACTC CAGCCACCTG AAGTCCAAAA AGGGTCAGTC TACCTCCCGC CATAAAAAAC TCATGTTCAA
1501 GACAGAAGGG CCTGACTCAG ACTGA
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Figure 3.1. Kasumi-3 cells have two mutant *TP53* alleles. A) Schematic of p53 protein structure can be divided into five functional domains. The N-terminal region has the transactivation domain (TA) and the proline rich (PR) domain. The central region contains the DNA binding domain. The C-terminal holds the oligomerization domain (OD) and the regulatory domain (Reg). The Roman numerals indicate highly conserved regions of the *TP53* gene. Placement of the two point mutations in reference to the protein domains is indicated. Both mutations are in the DNA binding region. **B)** The 484A>T point mutation and the 215C>G polymorphism are indicated (highlighted in yellow) within the cDNA sequence. The base pair changes results in the amino acid change P72R and I162F upon translation. **C)** The second mutation, 782+1G>A, is in the intron 7 donor site (highlighted in yellow), which entraps intron 7 (highlighted in green) in the mature messenger RNA. A stop codon (highlighted in red) is encountered within the intronic region which would result in a truncated protein lacking the C-terminal domain. This cDNA also contains the 215C>G polymorphism (highlighted in yellow).

Figure 3.2 shows a representative example, out of three, of each sequencing result with the base in question highlighted with an orange rectangle. A single black peak, representing G, is seen at base 215 on the DNA sequencing trace when exon 4 was amplified (**Figure 3.2A**). This would be expected if the cells were homozygous for the P72R polymorphism. When exon 5 was sequenced, two peaks are observed at base 484, a green (indicating an A) and a red peak (indicating a T), which can be seen on the DNA sequence trace (**Figure 3.2B**). Exon 7 and the flanking regions were also amplified and the sequencing trace shows a black and a green peak at base 782+1 (**Figure 3.2C**), the intron donor site of intron 7. These data support the hypothesis that Kasumi-3 cells are heterozygous for two mutant *TP53* alleles, both of which also contain the P72R polymorphism.

Kasumi-3 cells lack p53 transcriptional activity

Next, we sought to verify the transcriptional activity of these p53 mutant proteins in Kasumi-3 and A549 cells. To that end, we treated the cells with 300 nM of camptothecin (Cpt), a topoisomerase inhibitor, to induce DNA damage. A549 cells, which have wild type p53, were used as a positive control to show our treatments could elicit a p53 response. After a 24 hour exposure to Cpt, regulation of p53 target proteins p21, BAX, and HDM2 was measured by western blotting. Results confirmed that p21 and BAX were significantly up-regulated in A549 cells with Cpt treatment, a result not observed in Kasumi-3 cells (**Figure 3.3A** & **D**). Also, upon

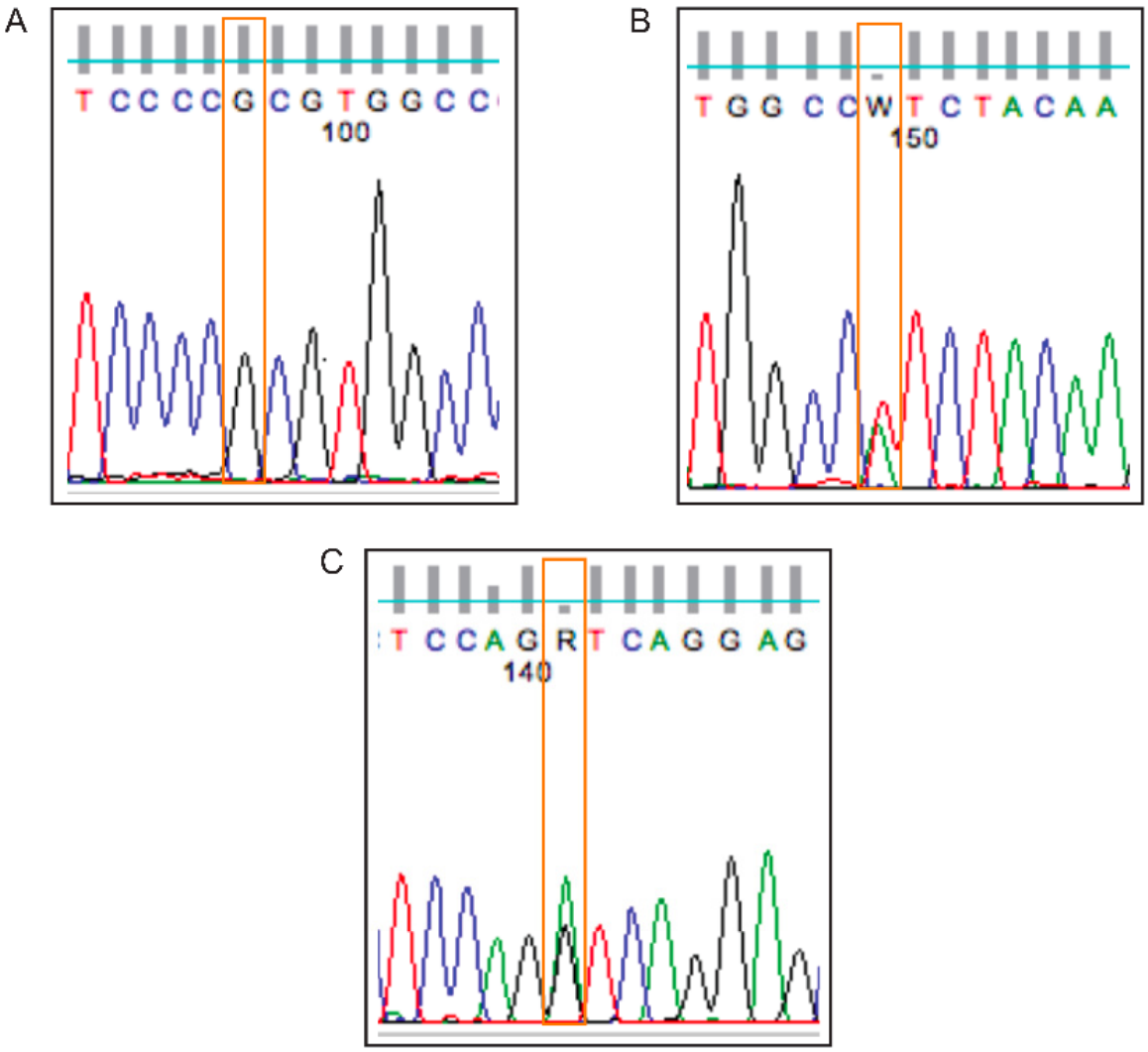

Figure 3.2. DNA sequencing traces of the regions of genomic DNA with the point differences found in the cDNAs. Panel **A)** represents the genomic region containing the P72R polymorphism**, B)** the 484A>T region and **C)** the 782+1G>A region. Results show that A) is a homozygote and B) and C) are heterozygous for the representative point mutations.

DNA damage, p53 is rapidly stabilized and thus an increase in protein level is generally seen. We found that p53 up-regulation occurred in A549 cells but not in Kasumi-3 cells (**Figure 3.3A** & **B**). HDM2 was not significantly up-regulated in either cell line(**Figure 3.3A** & **C**). These results indicate that the p53 present in Kasumi-3 cells lacks wild type function in response to DNA damage.

TP53 is not amplified in Kasumi-3 cells

The presence of two separate mutations in *TP53* could indicate the presence of multiple copies of the gene throughout the genome. We therefore performed a Southern Blot to determine whether *TP53* was amplified in Kasumi-3 cells. Kasumi-3 and A549 cell DNA were isolated and subjected to restriction enzyme digestion with four different enzymes: EcoRI, NcoI, StuI and HindIII. The DNA samples were separated on a 0.7% agarose gel and blotted onto a positively charged nitrocellulose membrane. It was subsequently probed with a digoxygenin labeled probe produced from PCR amplification of the large p53 cDNA insert. We anticipated that if TP53 gene was amplified in Kasumi-3 cells, either an increase in band intensity or a different pattern of bands from the wild type DNA, A549 cells, would be observed. As can be seen in **Figure 3.4** neither of these results were obtained. This indicates that *TP53* is most likely present as a single locus with two alleles, both of which are mutated.

Transfection of p53 siRNA can cause protein knockdown in Kasumi-3 cells

Ongoing studies indicate that some mutant p53 proteins can actually act to contribute to cancer progression by inducing the expression of genes that can facilitate transformation (Kim and Deppert, 2004). We have presented here evidence that Kasumi-3 cells express mutant p53 protein. We previously reported that Kasumi-3 cells are sensitive to the Hsp90 inhibitor, 17-AAG, and that this treatment coincides with a reduction in p53 levels (Napper and Sollars, in press). This data led us to hypothesize that the mutant p53 protein in Kasumi-3 cells is acting in an oncogenic capacity and that because of the aberrant transcriptional program, the cells become dependent on it for survival. Consequently, when mutant p53 levels are degraded by

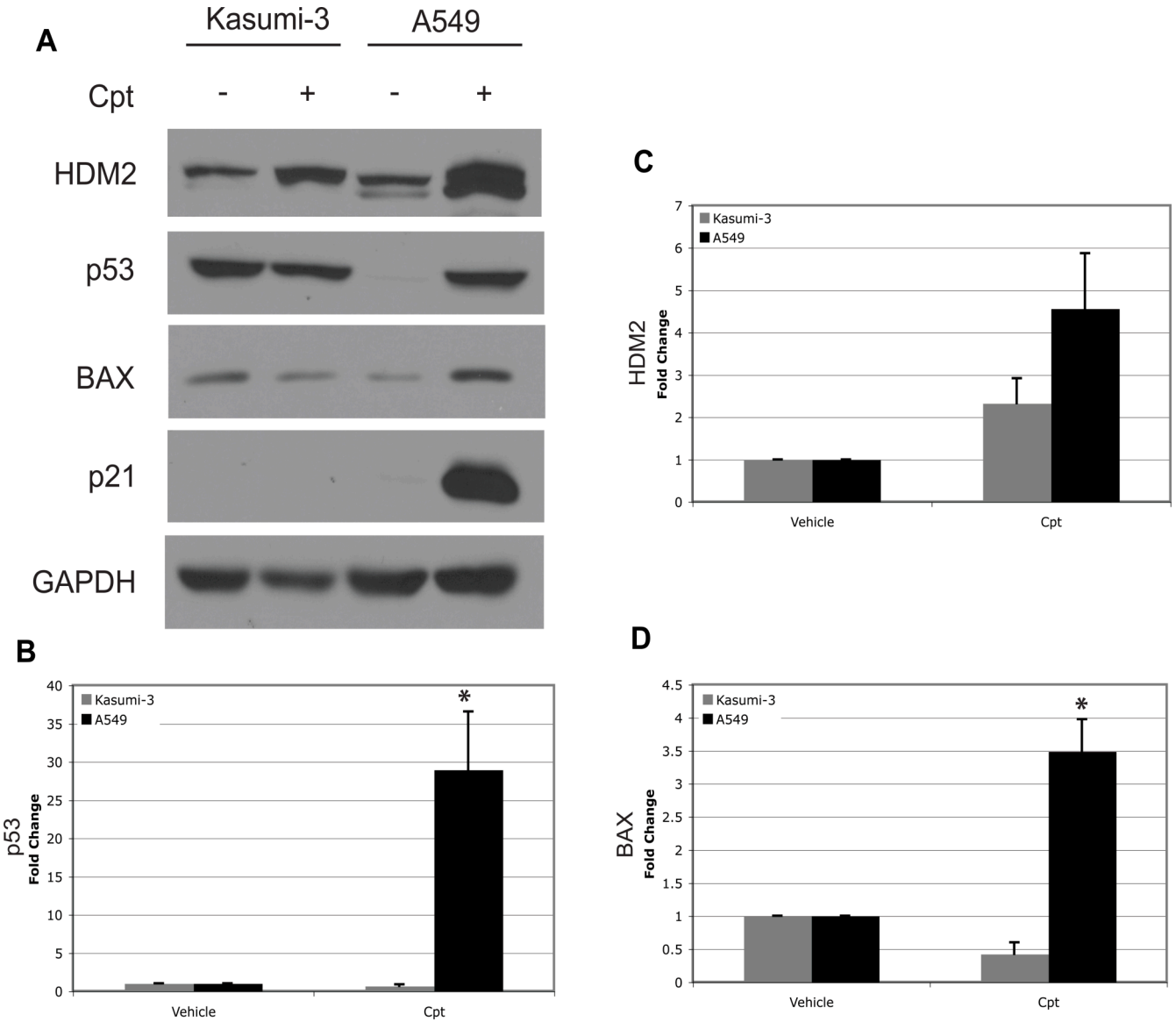

Figure 3.3. Kasumi-3 p53 protein does not have wild type function with DNA damage. DNA damage was induced in Kasumi-3 cells and A549 cells by treatment with 300 nM of camptothecin (Cpt) for 24 hours. **A)** After treatment cell lysates were harvested and subjected to western blotting and three p53 target proteins were probed. The blot is representative of three experiments. Kasumi-3 cells are deficient in wild type p53 activity as can be seen by lack of up-regulation of p53 target genes. A549 cells were used as a positive control and do exhibit wild type p53 activity. Graphs below and to the right represent densitometry from 3 experiments (mean + S.E.M.) as compared to the vehicle treated cells for **B)** p53, **C)** HDM2, and **D)** BAX. (*) indicates $p<0.05$ as compared to the vehicle control.

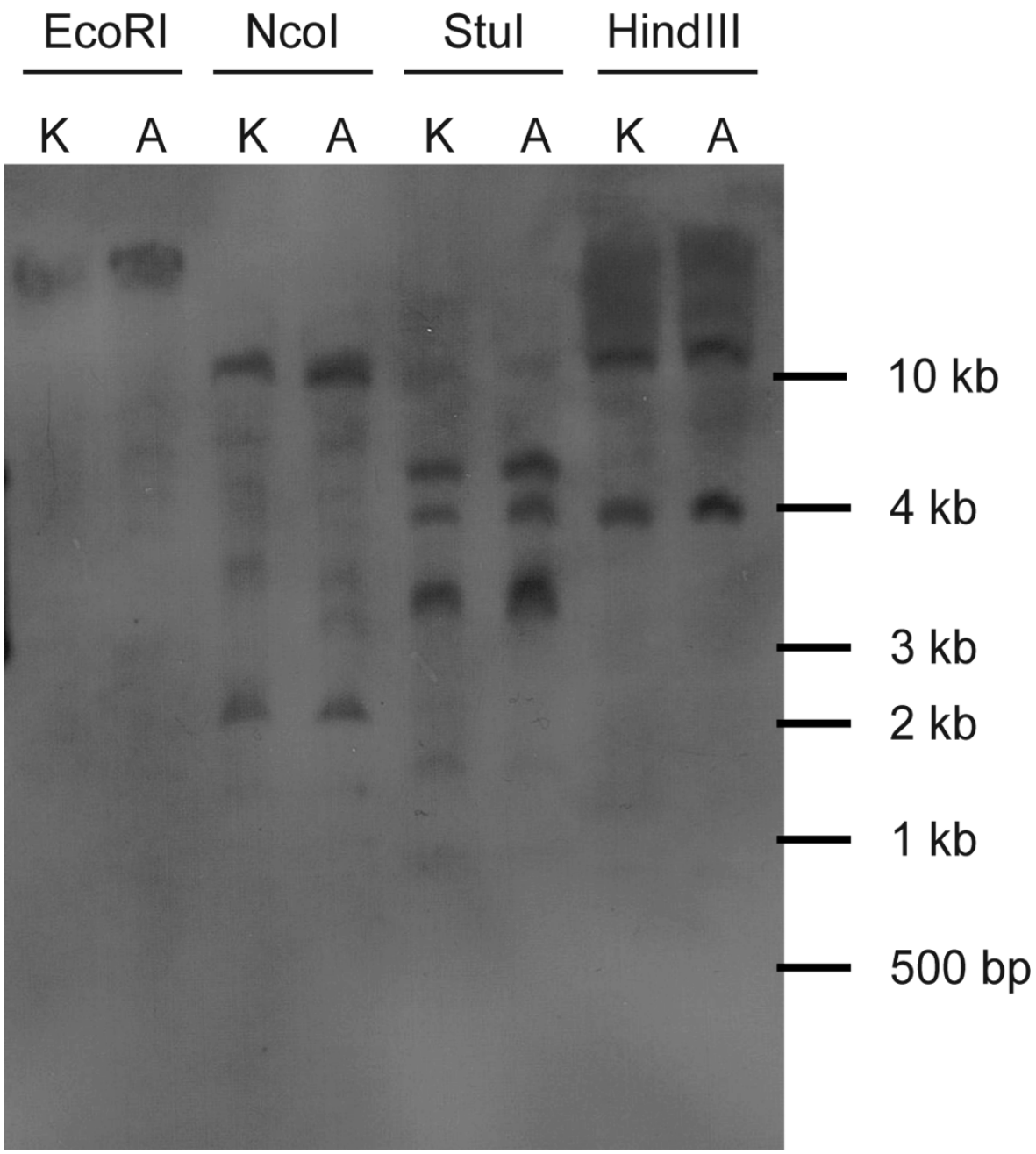

Figure 3.4. Kasumi-3 cells do not have amplified *TP53* gene. A southern blot was performed to verify that Kasumi-3 cells only have two copies of the *TP53* gene. A549 cells were once again used as a control. DNA was harvested from both cell lines and subjected to EcoRI, HindIII, StuI and NcoI digestion for 3 hours at 37ºC. Equal amounts (8-10 μg) were loaded and ran on a 0.7%, 7 mm thick agarose gel. The DNA was subsequently blotted and probed for *TP53*. The pattern of the bands for Kasumi-3 cells (marked K) and A549 cells (marked A) are identical which indicated that most likely Kasumi-3 cells do not have multiple copies of the p53 gene.

Hsp90 inhibition, the cells undergo apoptosis rather than cell cycle arrest, as seen with the other 17-AAG treated cell lines.

In order to test this hypothesis, we needed to reduce p53 levels in the absence of 17-AAG. Thus, we tried p53 siRNA transfection. Kasumi-3 cells are an undifferentiated form of AML (Asou et al., 1996) and as a result have a long doubling time of about 60 hours. Being that they are of hematopoietic origin, they are also a suspension cell line. All of these factors contribute to the difficultly in transfection by traditional means. Therefore, we had to resort to electroporation in order to attain efficient transfection. To assess transfection efficiency 2 x 10^6 cells were electroporated in the presence of 600 and 800 nM of control FITC labeled siRNA or 800 nM of non-labeled control siRNA. The cells were then plated in full media for at least 6 hours before analyzed by flow cytometry. High transfection efficiency (~88%) was achieved as indicated by the full histogram shift of the labeled siRNA transfected cells (green and blue) from the unlabeled siRNA transfected control (red) (**Figure 3.5A**).

In order to verify that p53 was being knocked-down at the protein level, cells were transfected with 800 nM of either non-specific control siRNA or p53 siRNA and whole cell lysates were harvested after 72 hours for analysis of protein expression by western blotting. **Figure 3.5B** shows that these transfection parameters do indeed result in a decrease of p53 protein levels. The blot shown is representative of three blots. 50-55% knockdown was attained in every experiment with 800 nM of siRNA. It should be noted that in our previous study, Hsp90 inhibition resulted in about a 50% knockdown of p53 (**Figure 2.5B**). Therefore, this amount of knockdown should be sufficient to test our hypothesis.

Mutant p53 knockdown does not affect cell viability or reduce P-glycoprotein activity

Kasumi-3 cells were transfected with 800 nM of siRNA, and 72 hours later, apoptosis levels were measured by annexin V labeling with 7-AAD as the dead cell stain. There was no difference in apoptosis levels between cells treated with p53 siRNA and control siRNA (**Figure 3.6A**). The

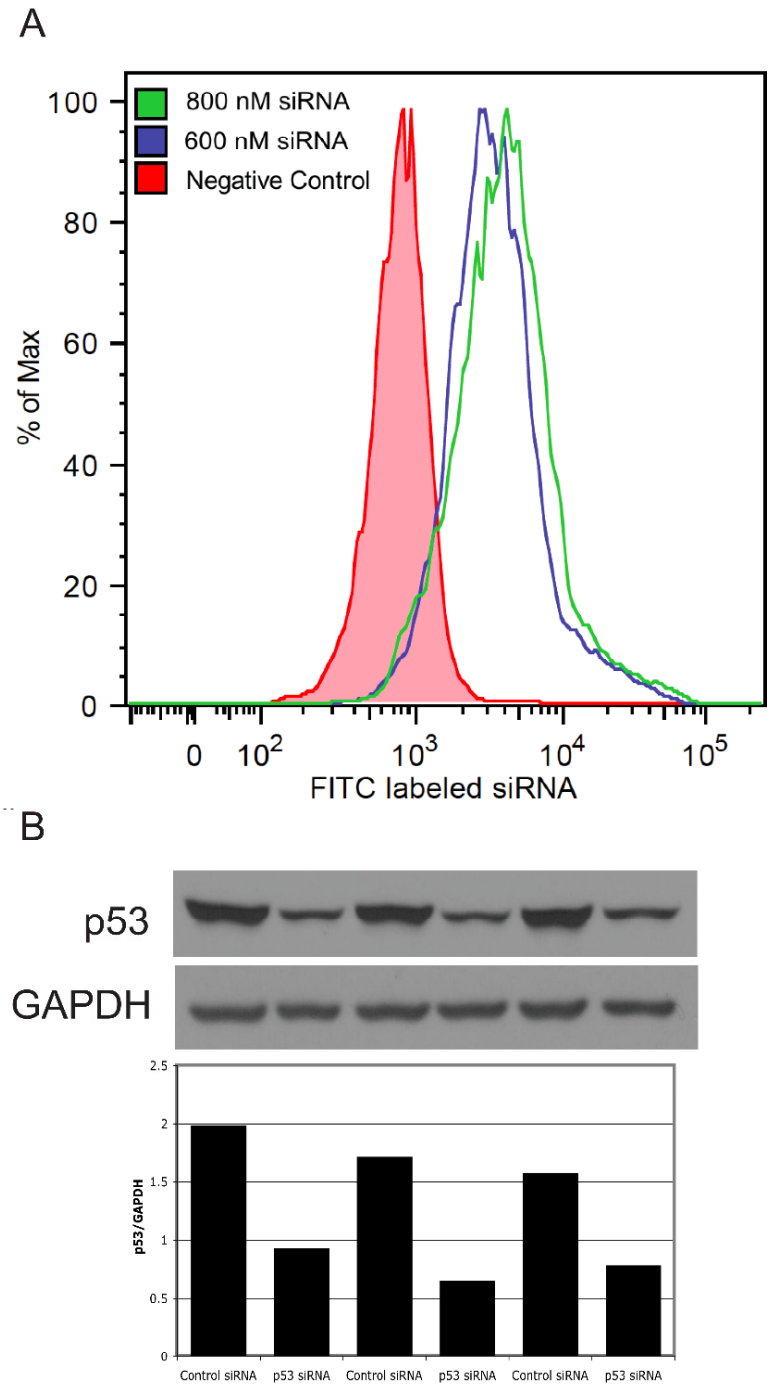

Figure 3.5. Kasumi-3 cells can be efficiently transfected with siRNA. A) Kasumi-3 cells were transfected with 600 and 800 nM of FITC tagged siRNA and analyzed by flow cytometry. Results clearly show that there is a definite histogram shift of the tagged siRNA compared to the non-tagged control siRNA. **B)** Western blotting was performed to verify that transfection of siRNA effectively knocked-down protein levels. Cells were transfected with 800 nM of p53 siRNA or non-specific control. After 72 hours p53 levels were assayed. Blot and panel underneath showing densitometry, indicates p53 protein and was knocked down by 55% (representative of three).

cells normally have a population of cells, about 30%, undergoing apoptosis at any given time (**Figure 2.2A**). However, the amount of apoptosis seen in these experiments indicates that the cells are undergoing apoptosis at an even higher rate, which is probably due to the harsh nature of electroporation in general.

Because electroporation can cause a large amount of cell death, without any contribution from the siRNA, any effects of mutant p53 knockdown could be masked. Therefore, another downstream target of mutant p53 was measured. P-glycoprotein (P-gp) is known to be up-regulated in the presence of mutant p53 (Chin et al., 1992). As described in chapter 2, P-gp is an ATP-binding cassette (ABC) transporter that pumps substrates out of cells. In our studies, we have discovered that P-gp activity facilitates KG-1a cells' resistance to 17-AAG treatment (**Figure 2.4**). We have also observed that Kasumi-3 cells have P-gp activity (**Figure 3.6C**); however, they are much more sensitive to 17-AAG treatment than KG-1a cells. This data led us to hypothesize that the mutant p53 in Kasumi-3 cells could be facilitating the expression of P-gp. This finding would fit well into the data reported in Chapter 2. Hsp90 inhibition down-regulates mutant p53 levels in the cells, which consequently causes the down-regulation of P-gp. This would make the cells much more sensitive to 17-AAG treatment, as was seen when P-gp activity was inhibited by verapamil in KG-1a cells (**Figure 2.6**). Thus, Hsp90 inhibition in this cell line could have a compound effect on their susceptibility to 17-AAG.

To test this hypothesis, the cells were once again transfected with 800 nM of either p53 or control siRNA and allowed to rest for 72 hours. After this time, the levels of P-gp were measured directly with antibody labeling followed by flow cytometry. There was no significant difference in P-gp expression between the different treatments (**Figure 3.6B**). Efflux assays were also performed on these same cells as a secondary assay to confirm the antibody labeling results. As can be seen in Figure 3.6C there was no difference in efflux capacity between the control siRNA treated cells (blue histogram) and the p53 siRNA treated cells (green histogram). In fact, both treatment groups were able to pump out the dye quite readily. Verapamil, a P-gp inhibitor, was

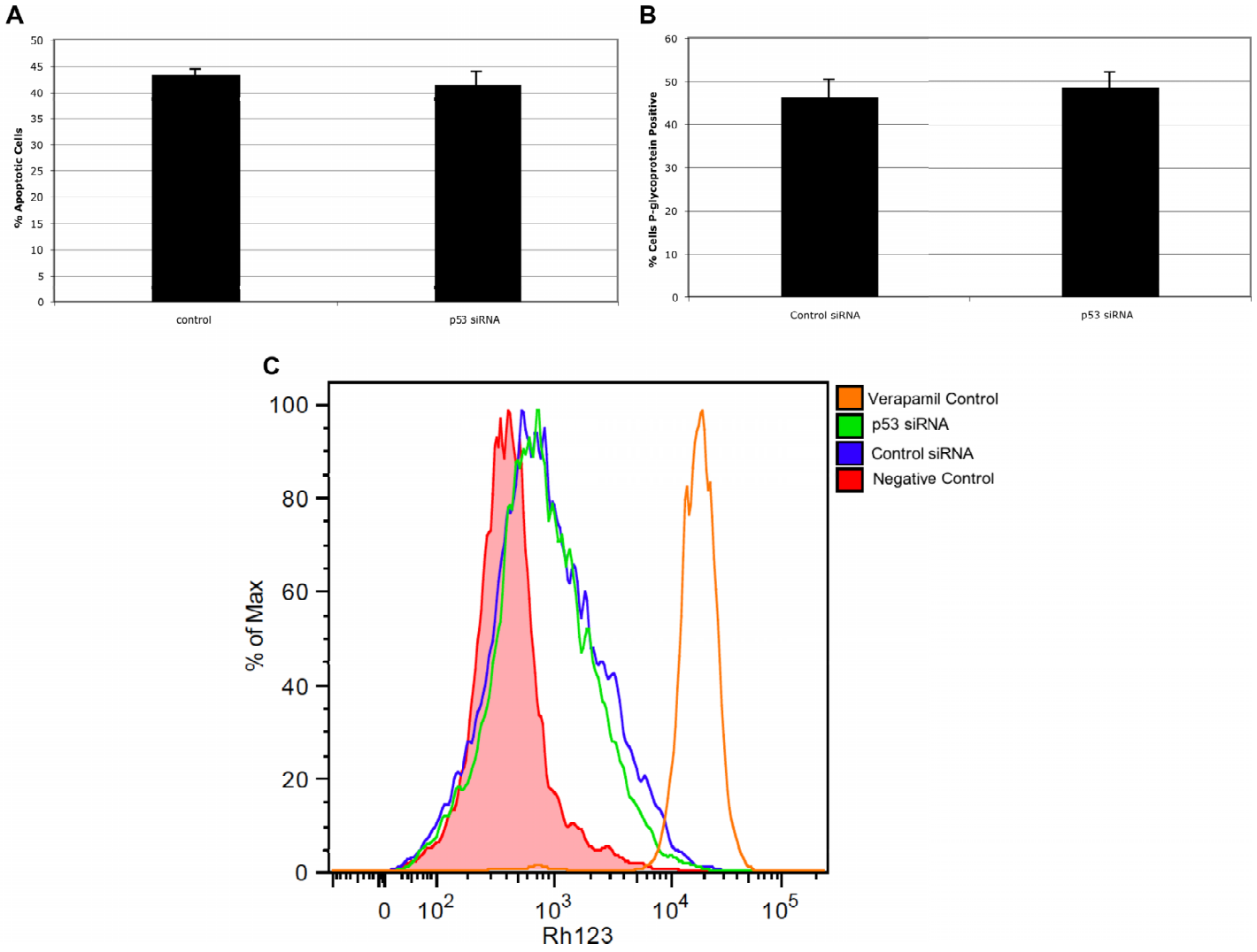
A
% Apoptotic Cells
50
45
40
35
30
25
20
15
10
5
0
control
p53 siRNA
B
% Cells P-glycoprotein Positive
60
50
40
30
20
10
0
Control siRNA
p53 siRNA
C
% of Max
100
80
60
40
20
0
0
10^2
10^3
10^4
10^5
Rh123
Verapamil Control
p53 siRNA
Control siRNA
Negative Control

Figure 3.6. Mutant p53 does not play a role in Kasumi-3 survival. A) Kasumi-3 cells were transfected with p53 siRNA or non-specific control and apoptosis was assayed after 72 hours by annexin V labeling with 7-AAD as a dead cell stain. There was no significant difference in the two treatment groups. Cells were transfected with p53 siRNA or non-specific control and P-glycoprotein levels and activity was measured. Panel **B)** shows that there is no significant difference in P-glycoprotein expression with p53 knockdown as assessed by flow cytometry. Panel **C)** shows that there is no difference in efflux capacity of the cells with p53 knockdown (green and blue histograms). As a positive control, cells were treated with 50 μM of verapamil, a P-glycoprotein inhibitor, during loading and rest period of the cells to show efficient dye loading (orange histogram).

used as a positive control to show the cells loaded the dye efficiently (orange histogram). Unfortunately, these data do not support our hypothesis that the mutant p53 in Kasumi-3 cells is acting in an oncogenic capacity. However, more studies are needed to completely rule out this possibility.

Discussion

We have identified two mutant *TP53* alleles in the Kasumi-3 cell line. The large cDNA product contains an uncommon point mutation that traps intron 7 within the mature p53 mRNA. Inclusion of intron 7 results in a truncated protein, due to the stop codon located in the intronic region. This means that even though most of the DNA binding domain would remain intact, the oligomerization domain, which is very important for p53 to function, would be absent. The p53 proteins have to tetramerize in order to bind to DNA and activate genes in a sequence specific manner (El-Deiry et al., 1992; Funk et al., 1992). Therefore, this mutant would be predicted to be non-functional and not able to act in a dominant negative manner, due to the lack of the oligomerization domain.

Whether this protein is stable is not clear. The presence of only a single 53 kD protein band in Western blot analysis suggests that it is rapidly degraded. Further evidence was provided by a previous study which analyzed p53 mutations in lung cancer (Takahashi et al., 1990). In this study, one tumor sample had a mutation in the intron 7 acceptor site causing it to be trapped in the p53 cDNA. This sample also lacked the presence of a normal allele. Immunoprecipitation assays with this particular sample were unsuccessful at pulling down any p53 protein when performed with antibodies recognizing the C-terminal and the N-terminal region.

Immunohistochemistry experiments confirmed the lack of p53 protein. This evidence collectively suggests that the protein product of this cDNA, with intron 7 trapping, would not be stable.

The single 484A>T mutation in the smaller cDNA product results in a substitution of a small side chain amino acid for a large side chain amino acid in the DNA binding domain. This would be foreseen to impose steric hindrance upon the protein structure. In fact, transactivation assays of over 2400 different mutant p53 proteins performed in yeast demonstrated this mutant to be non-functional (Kato et al., 2003). Nevertheless, we have confirmed through DNA damage assays that the p53 protein present in Kasumi-3 cells does not function as wild-type (**Figure 3.3)**.

An extensive search of the IARC database (Petitjean et al., 2007) revealed that both of these mutations have been detected in various types of cancer, such as lung and liver cancers. This is the first incidence of these mutations detected in leukemia. The P72R polymorphism is in the proline rich domain of p53 (see **Figure 3.1A**). It has an average frequency of heterozygotes of about 50% in populations analyzed (http://www-p53.iarc.fr/PolymorphismView.asp). Interestingly, this SNP becomes more prevalent as populations get closer to the equator (Olivier et al., 2010). Because it is so common, it has been extensively analyzed for a link to cancer; however, results from these studies have been inconsistent (Storey et al., 1998; Klug et al., 2001; Schmidt et al., 2007; Matakidou et al., 2003).

We hypothesized that the mutant p53 in the Kasumi-3 cells was acting in an oncogenic capacity. However, a knockdown of 50% of p53 levels with siRNA was not sufficient to induce apoptosis above the non-specific control. There was also no measured difference in P-gp levels and activity with p53 knockdown (**Figure 3.6**). These results do not support our hypothesis. Although, there is a possibility that the mutant p53 present in these cells may be facilitating other oncogenic functions of these cells that we have not measured.

In theory, the I162F mutant allele (**Figure 3.1B**), with the intact oligomerization domain, could exert a dominant negative effect. Therefore, this single mutation event would mimic a p53-null phenotype, which could stimulate quick progression of cancer. However, further

studies in which this mutant p53 protein is expressed in a wild type background, are needed to support this proposal. A recent study indicated that a single p53 deletion was a strong independent negative prognostic factor for disease free survival in AML (Seifert et al., 2009). This demonstrates that although p53 mutations in AML are uncommon, the effect of inactivating p53 can have a devastating outcome. This clinically relevant observation makes Kasumi-3 cells a valuable tool to study mutant p53 function in AML.

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Chapter 4 : EML cells exhibit a developmental plasticity when pre-treated with Hsp90 inhibitor geldanamycin

Abstract

New treatment strategies for AML are needed to increase patient survival. Heat shock protein 90 (Hsp90) inhibitors such as 17-AAG, an analog of geldanamycin (GM), are currently in phase I and II clinical trials for various leukemias and other cancers. Previous studies in *Drosophila* have shown that inhibition of Hsp90 by GM produced a trans-differentiation event in which eye tissue developed into a limb-like outgrowth. The outgrowth persisted after several generations in the absence of GM, indicating the occurrence of a heritable epigenetic event. Our current studies suggest that Hsp90 plays a similar role in EML cells, a mammalian hematopoietic stem cell line. By treating these cells with GM prior to differentiation, we have seen a dose dependent survival of the cells after selective conditions are imposed. Unfortunately our results were highly variable. However, our data could suggest that Hsp90 inhibition is causing an epigenetic modulation which results in cells that are more adaptable. Epigenetics has become increasingly studied as a mechanism enabling cancer progression by allowing precancerous cells to become more adaptable to their environment. Studying the role of Hsp90 in myeloid cell differentiation through epigenetic mechanisms will provide insights into normal blood development as well as possible treatment strategies for AML.

Introduction

A diverse set of important signaling proteins depends on Hsp90 for activation, including transcription factors and protein kinases. The inactivation of Hsp90 can therefore affect many systems in the cell (Pearl et al., 2008). It was shown that *Drosophila* bearing a mutant Hsp83 gene created offspring with phenotypic variations in many adult structures (Rutherford et. al., 1998) Because of this, the authors hypothesized that Hsp90 stores genetic morphological potential for later release or acts as a genetic capacitor.

A later study in *Drosophila* supported these findings that genetic or pharmacological inhibition of Hsp83 created phenotypic variations. However, this study provided evidence that this was based on an epigenetic mechanism (Sollars et. al., 2003). A trans-differentiation event, seen as a limb-like outgrowth in the eye, was observed in offspring whose parents were fed the Hsp90 inhibitor geldanamycin (GM), which was also seen in flies with heterozygous mutations in a chromatin remodeling gene. The percentage of offspring with the eye phenotype increased upon selective breeding in the absence of Hsp90 inhibition, which is suggestive of an epigenetic mechanism. Further studies showing a reversal of the phenotype with chromatin remodeling inhibitors provided a link between Hsp90 and epigenetic modulation (Sollars et. al., 2003).

In times of stress, Hsp90 is up-regulated to assist the cell in survival. Hsp90 becomes overwhelmed refolding and cleaning up misfolded proteins, which detracts from its normal function of activating signaling molecules. This is significant in evolutionary terms, because in times of stress, Hsp90 dependent pathways become perturbed which can generate more diverse phenotypes. If these phenotypes are a better fit to the environment, they can be selected for and become fixed in the population.

The studies detailed here were designed to test whether this phenomenon is conserved in mammalian model systems. As discussed previously, along the pathway from HSC to mature cell, different cell surface markers appear and disappear (Akashi et al., 2000; Terskikh et al., 2003). This provides a convenient method to track the development of a cell. Therefore, hematopoiesis provides a good model to study differentiation events brought on by Hsp90 inhibition.

EML cells are a hematopoietic stem cell line that was developed by inserting a dominant negative retinoic acid receptor (RAR) in hematopoietic stem/progenitor cells isolated from the bone marrow of BDF_1 mice (Tsai et al., 1994). The BDF_1 strain is produced by a C57BL/6 cross with DBA/2. By blocking retinoic acid receptor function, the cells can be cultured in normal cell culture conditions in the presence of stem cell factor (SCF) and retain their stem cell features. They can be induced to differentiate into any of the mature cells of the blood by various treatments, which makes them an ideal model for studying hematopoiesis.

It is our hypothesis that Hsp90 is an epigenetic modulator that can play a role in myeloid cell differentiation. To test this hypothesis, we treated EML cells prior to differentiation with GM. First, the differentiation protocol was confirmed by treating EML cells as prescribed followed by observation of morphological changes by microscopy and measurement of cell surface marker expression by flow cytometry. Toxicity assays were then performed by counting the cells after treatment with various concentrations of GM. In order to assay for specific epigenetic events, the cells were treated with GM 48 hours prior to differentiation. Cell surface marker expression and cell counts were performed at various points throughout the protocol to assess the effects of GM treatment in hematopoiesis.

Materials and Methods

Materials

Geldanamycin (cat. G3381), retinoic acid (cat. R2625) and Wright/Giemsa stain were purchased from Sigma. Antibodies and Fc block were all purchased from BD scientific lineage panel (cat. 5599710), Sca-1 (cat. 557403), CD117 (cat. 553355), CD127 (cat. 555288), Fc Block (cat. 553142), and streptavidin-APC (CAT. 554067). F4/80 was purchased from Caltag (cat. MF48004). GM-CSF was purchased from Stem Cell Technologies (Vancouver, BC).

Cell culture

EML.C1 and BHK cells were obtained from Dr. Schickwann Tsai at the University of Utah. EML cells were cultured in growth medium, which is base medium (IMDM supplemented with 20% heat inactivated horse serum) and 10% BHK conditioned medium, which contains SCF. The cells were incubated at 37 ºC and 5% CO_2 and maintained between 5 x 10^4 and 5 x 10^5 cells/mL by addition of media every 2-3 days. Cells were not passed more than 6-8 weeks for these experiments. Due to the synergistic effect of antibiotics with GM, the cells were cultured without antibiotics.

BHK cells were cultured in EMEM supplemented with 10% FBS at 37 ºC and 5% CO_2. They were sub-cultured when 80% confluence was reached, about every 3 days. To make conditioned media containing SCF, T-175 flasks were seeded and cells were grown to 80% confluence. Old media was discarded and 50 mL of fresh media was added to the flasks. After 48 hours, the media was collected and another 60 mL of media was added to the flasks. After another 48 the media was collected and the cells were discarded. All of the media were pooled and filtered through a 0.22 μm filter to sterilize, aliquoted and stored at -20 ºC.

WEHI-3 cells were obtained from ATCC (catalog # TIB-68). The cells were cultured in IMDM supplemented with 10% FBS at 37 ºC and 5% CO_2. They were maintained between 2 x 10^5 and 2 x 10^6 cells/mL by addition of media every 2-3 days. To obtain conditioned media containing IL-3, the cells were seeded at 3 x 10^5 cells/mL and cultured in fresh media for three days. The cultures were then centrifuged to collect the media and the cells were discarded. The media were filtered through a 0.22 μm filter to sterilize, aliquoted and stored at -20 ºC.

Differentiating EML cells

EML cells were resuspended at 1 x 10^5 cells/mL in base medium supplemented with 10 μM ATRA, 15% IL-3 and 10% BHK media, unless otherwise noted. After three days, the cells were counted, washed twice with PBS and resuspended at 1 x 10^6 cells/mL in base medium containing 10 ng/mL GM-CSF.

GM treatments

Cells were seeded at 2 x 10^5 cells/mL in growth medium. Cells were treated with 0, 15 and 20 nM GM with an equalization of vehicle among the treatments. After 24 hours, the cells were washed and reseeded at 2 x 10^5 cells/mL in growth medium for a rest period. After an additional 24-48 hours the cells were differentiated as described above.

Microscopy

Cells were differentiated for the first three days as described above. After this initial priming, the cells were washed and reseeded at 5 x 10^5 cells/mL in base medium containing 50 ng/mL of GM-CSF over cover slips. Cells on the cover slips were fed again by removing spent media and replacing with fresh media on days 7, 10 and 14. Before photographing, the cover slips were washed twice with PBS and fixed for 30 sec in methanol and allowed to dry. They were then dipped in Wright/Giemsa stain for 1 minute and then immediately washed with water. The cover slips were mounted using crystal mounting medium. Pictures were taken on days 10, 11, 13 and 19 using an Olympus BX51 epifluorescent microscope under 1000x magnification equipped with an Olympus DP70 Camera and associated software (DP Controller and DP manager), version 1.2.1.108.

Flow cytometry

Fc receptors were blocked by incubating the cells with 1 μg of Fc block per 1 x 10^6 cells for at least 20 minutes. After the Fc receptors were blocked, 1 x 10^5 cells were incubated with appropriate amount of antibody in 100 μL of FACS buffer (3% BSA, 0.02% sodium azide, 1mM EDTA in PBS) at 4 ºC for 30 minutes. Each sample was washed with 2 mL of FACS buffer. The appropriate amount of streptavidin conjugated to APC was then added for a total volume of 100 μL and incubated at 4 ºC for 30 minutes. The cells were washed again with 2 mL of FACS buffer. A total of 1 x 10^4 events were recorded on a BDFACSAria. Data were analyzed using FlowJo 8.8.6 software. Dmax subtraction method was used to calculate the percentage of positive cells.

Figure 4.1. Schematic of EML differentiation. EML cells can be induced to differentiate into granulocytes and macrophages first by treatment with all trans-retinoic acid (ATRA), interleukin-3 (IL-3), and stem cell factor (SCF) for three days. At this time, the cells are washed thoroughly and resuspended in medium containing granulocyte macrophage colony stimulating factor (GM-CSF), which further allows the cells to mature into macrophages and neutrophils within 5-10 days.

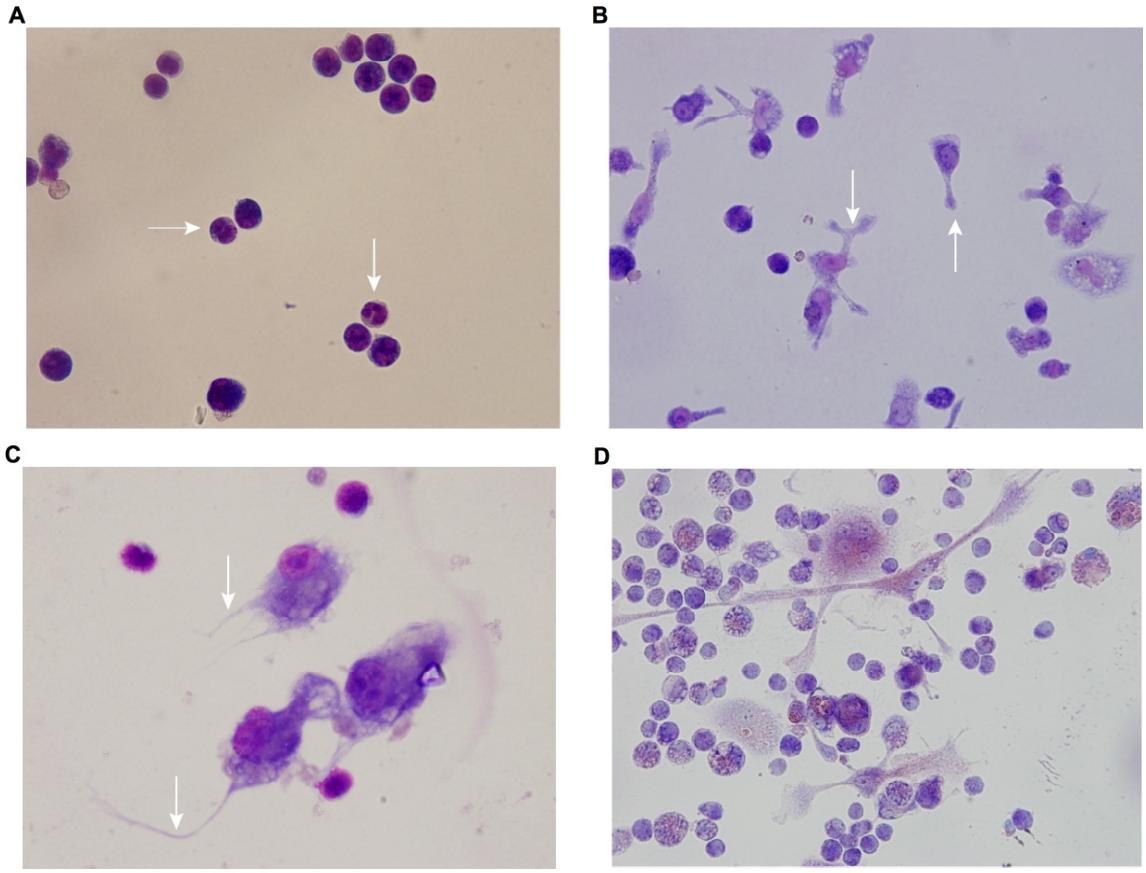


Figure 4.2. EML cells change in morphology when differentiation is induced. EML cells were microscopically examined at various stages to chart their morphological changes during differentiation. **A)** After 10 days of differentiation, the cells transitioned from suspension to more adherent cells and readily attached to cover slips. White arrow point out neutrophilic type cells as can be seen by their lobular nuclei. **B)** After 11 days of treatment, white arrows point out cells that began developing small projections. This is more indicative of macrophage development. **C)** and **D)** On day 14 and 19 of differentiation respectively, some of the cells became spread out and increased projections were observed (white arrows). However, there a majority of cells were still round with more monocytic morphological features.

Results

EML cells can be differentiated specifically along the macrophage/granulocytic lineage

We were particularly interested in the granulocyte/macrophage lineage. EML cells can be induced to differentiate down this pathway by treatment with all trans-retinoic acid (ATRA), interleukin-3 (IL-3), and stem cell factor SCF. Conditioned medium (CM) containing the cytokines SCF (BHK CM) and IL-3 (WEHI CM) was used to grow and differentiate the cells. Under normal

conditions, addition of 10% BHK CM to the culture medium was used to grow the cells. For the first experiments outlined here, differentiation was achieved by addition of 10% BHK CM, 15% WEHI CM and 10 μM ATRA for three days. At this time, the cells were washed and resuspended in medium containing granulocyte macrophage colony stimulating factor (GM-CSF), which induces differentiation into macrophages and neutrophils within 5-10 days (**Figure 4.1**).

In order to confirm differentiation, the cells were microscopically examined at various stages to chart their morphological changes. After 10 days of differentiation, the cells transitioned from suspension to more adherent cells and readily attached to cover slips. Neutrophilic type cells began appearing, as can be seen by their lobular nuclei (**Figure 4.2A**). After 11 days of treatment, cells began developing small projections that are more indicative of macrophage development (**Figure 4.2B**). On day 14 and 19 of differentiation, some of the cells became spread out and increased projections were observed. However, there a majority of cells were still round with more monocytic morphological features (**Figure 4.2C & D**).

Cell surface markers were also measured to track EML cell differentiation. **Table 4.1** lists the markers used in this study and the cell types that they target. The expression of these markers

Marker	Cell Type
CD117	Hematopoietic stem/progenitor cells
Sca-1	Early hematopoietic stem cells
CD11b	Macrophage/granulocytes
F4/80	Macrophages
CD127	Early lymphocytes
CD45	All leukocytes
Ly6G	Granulocytes
CD3ε	Lymphocytes
Ter119	Erythrocytes

Table 4.1. Markers used to determine stages of EML cell differentiation. The cell surface markers in the left column and the cell types which they identify are in the right hand column.

was measured everyday over a period of seven days via flow cytometry. **Figure 4.3A** illustrates that the treatments used in this study were successful in inducing differentiation following the

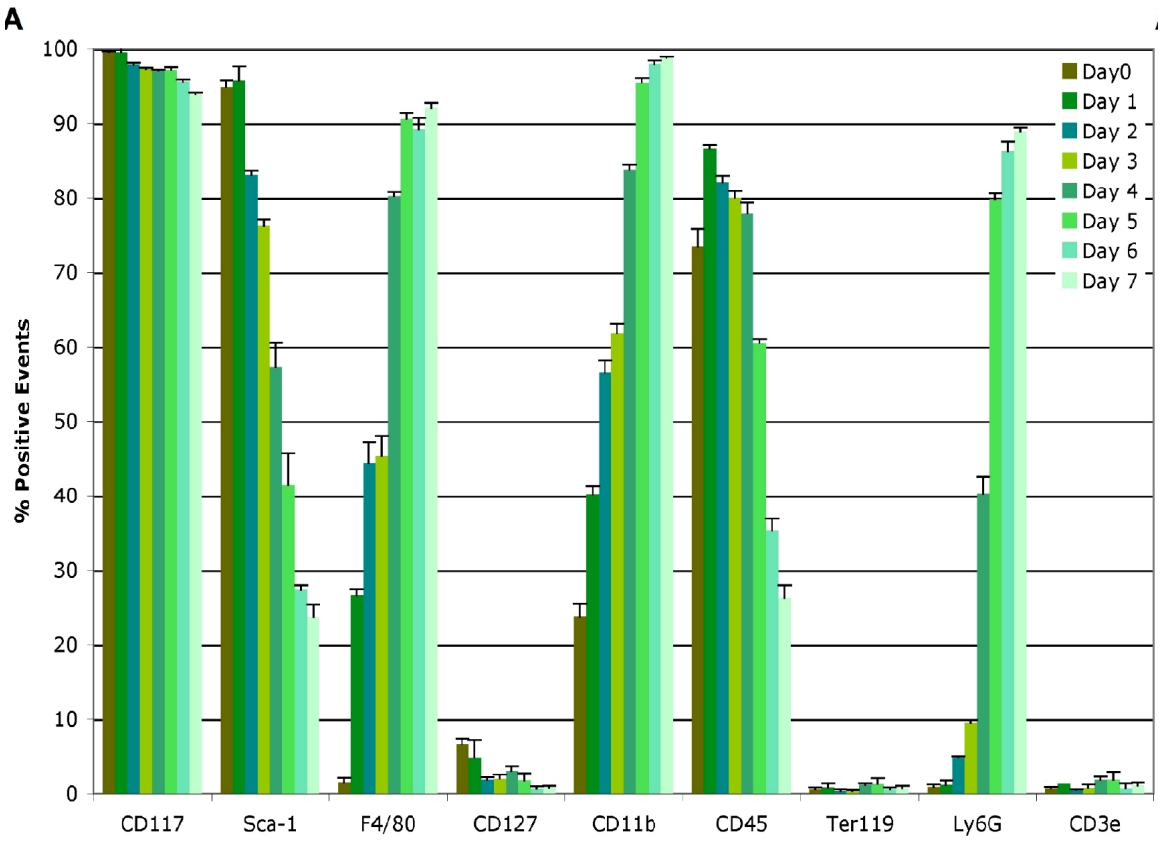
macrophage/granulocyte lineage. As expected, cells positive for Sca-1 decreased considerably. The median fluorescent values of CD117 also decreased dramatically with differentiation; however, this change was not reflected in the percentage positive cells due to the persistently high expression of this marker above background (**Figure 4.3B**). CD11b, Ly6G and F4/80 increased to 90% over the seven day period, showing that the cells were maturing into granulocyte/macrophage cells. On the other hand, CD127, Ter119 and CD3e remained at baseline levels, indicating that the cells were not differentiating toward the erythrocytic or lymphocytic lineage. All these data taken together illustrate that the protocol used in this study differentiates EML cells along the macrophage/granulocyte lineage.

EML cells exhibit a restriction point

During the extensive differentiation treatment, EML cells undergo rapid proliferation for the first three days as illustrated in **Figure 4.4**. It is during this time that we believe the cells are primed for further development. On day three, the cells were washed and then treated with media supplemented with GM-CSF to promote macrophage/granulocyte differentiation. During this media switch, a restriction point is observed, in that only those cells that have differentiated suitably are able to survive. Therefore, massive cell death of up to 80% is observed between days three and four of differentiation. After this initial step, the progenitors that survive are able to rapidly divide and differentiate.

High doses of GM is toxic to EML cells

In order to determine proper dosing of EML cells with GM, a toxicity assay was performed. Cells were seeded at 2×10^5 cells/mL and treated with varying doses of GM. Doses above 25 nM caused death among the cells as noted by a 50% decrease in cell number (**Figure 4.5**). In testing our hypothesis, it was necessary to treat the cells with GM at high enough doses to inhibit Hsp90, but not cause significant toxicity. Doses of 15 and 20 nM were chosen because the cells could still proliferate at 15 nM and at 20 nM the cell number remained at the seeded cell concentration.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30
20
10
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Day 2
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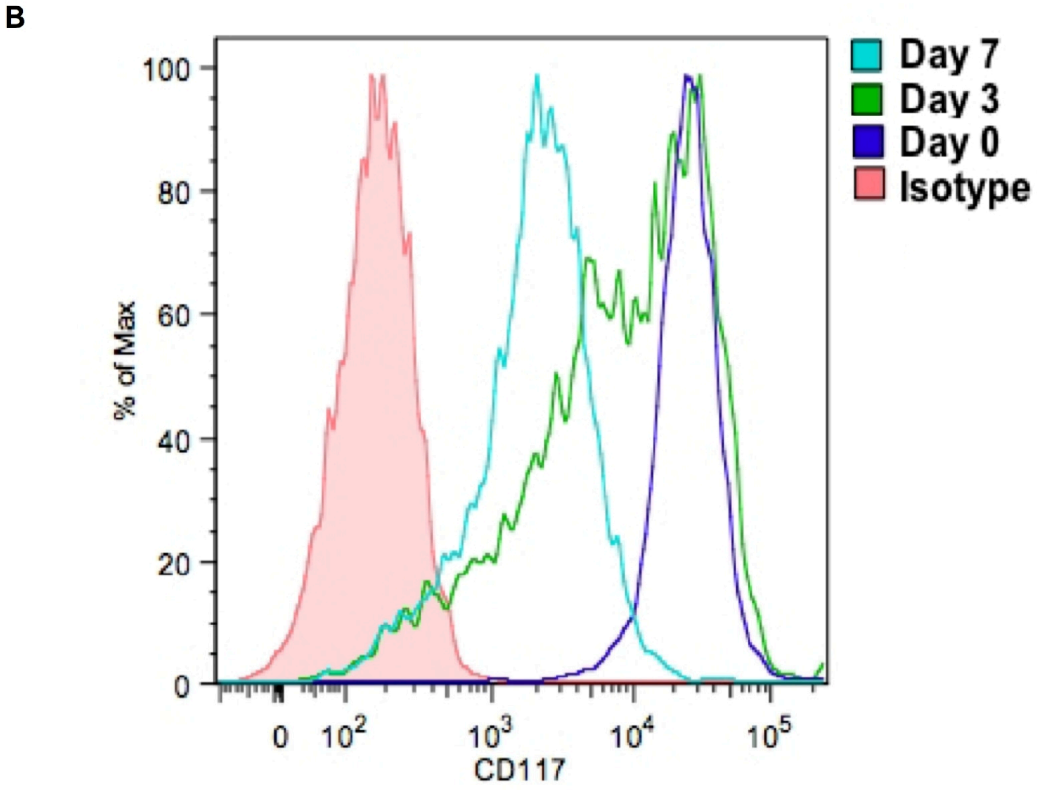
CD127
CD11b
CD45
Ter119
Ly6G
CD3e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Day 0
Isotype
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10^3
10^4
10^5
CD117

Figure 4.3. Marker profile of EML cells during differentiation. A) A total of nine cell surface markers were used to follow the differentiation progress of EML cells to a macrophage/granulocyte lineage over 7 days. CD117 and Sca-1 are stem cell markers. F4/80, CD11b and Ly6G are granulocyte/macrophage markers. CD127 and CD3e are lymphocyte markers. Ter119 is an erythrocyte marker and CD45 is a leukocyte marker. As anticipated, the number of cells expressing the stem cell markers decrease. The macrophage and granulocyte markers increase dramatically over the seven day period. This indicates that the protocol is functioning to differentiate the cells as expected. This study also provides a baseline of cell surface marker expression to compare against vehicle and GM treated cells. Results depicted are from three independent experiments performed in triplicate (mean + S.E.M.). **B)** The expression of CD117 does in decrease when the cells are differentiated as can be seen by the drop in median fluorescence over time. Histogram is representative of three experiments.

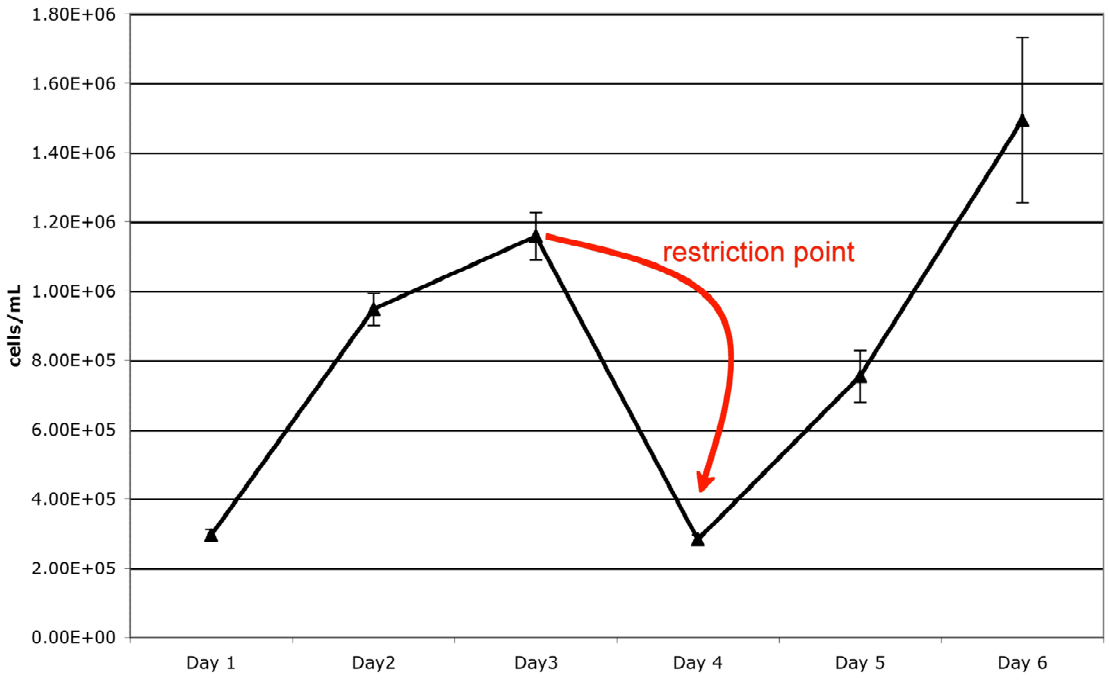


Figure 4.4. Cell counts during EML cell differentiation. The first stage of differentiating EML cells consists of incubating the cells with interleukin-3, stem cell factor, and all trans retinoic acid for three days. During this time cell numbers increase dramatically. After this initial priming, the media is switched to that containing granulocyte macrophage colony stimulating factor. In the next 24 hours an average of 80% of the cells die. This generates a selection window whereby only cells properly primed for differentiation survive. The cells that do survive continue to proliferate and develop into mature macrophages/neutrophils. Results depicted are from three independent experiments performed in triplicate (mean + S.E.M.).

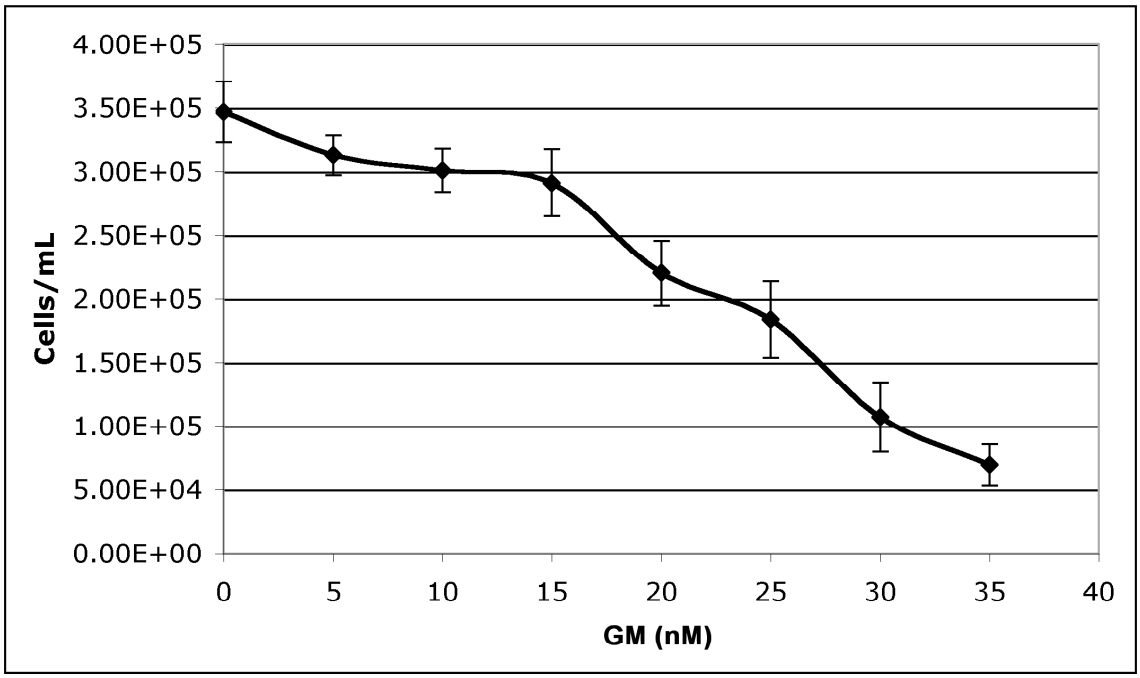
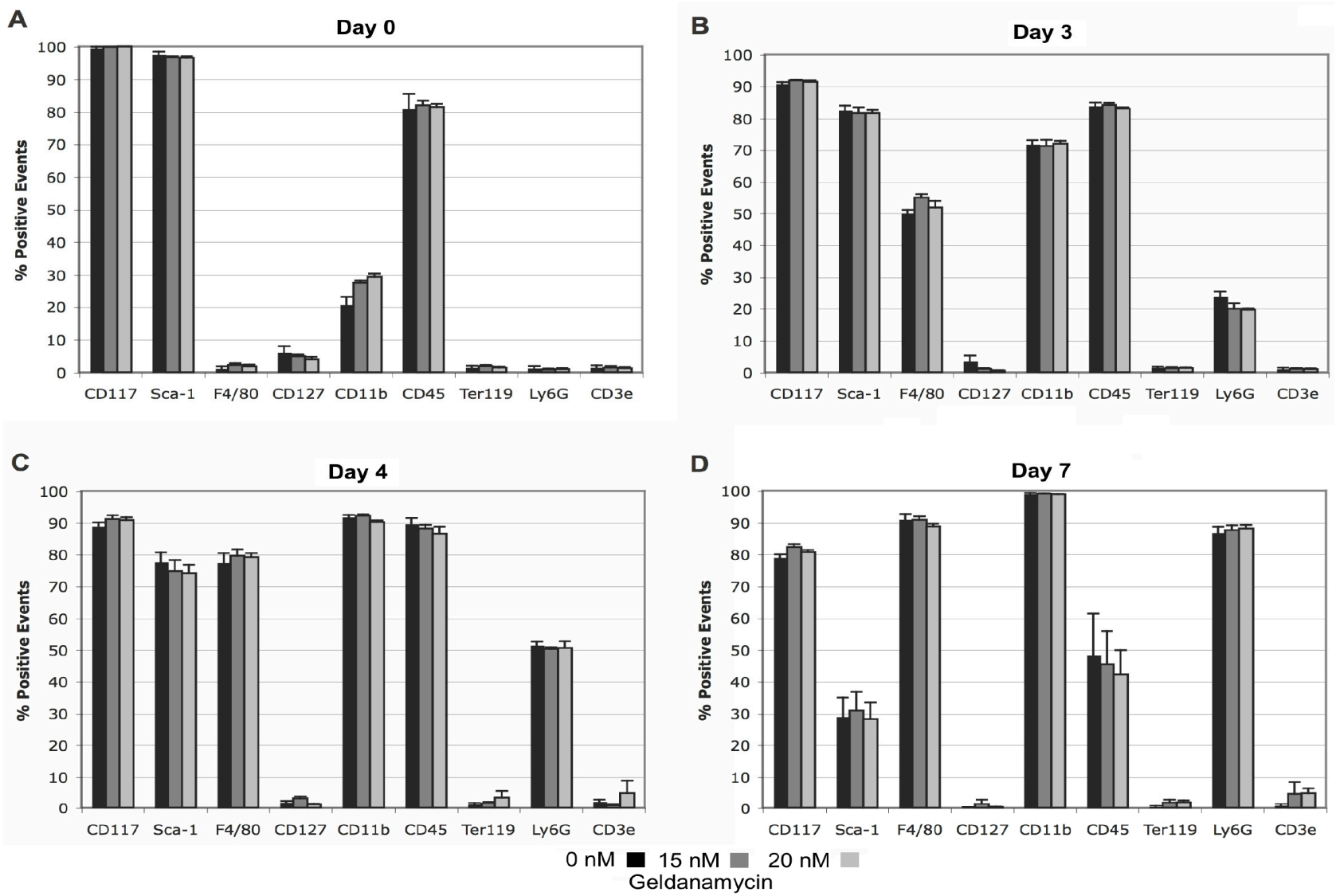


Figure 4.5. Toxicity of geldanamycin on EML cells. Geldanamycin is an HSP90 inhibitor. As a preliminary experiment to test the toxicity of this compound, a kill curve was constructed. 200,000 cells were incubated with varying concentrations of GM. After 24 hours, cell counts were performed using a hemocytometer. Results depicted are from three independent experiments performed in triplicate (mean + S.E.M.).

GM pre-treatment does not change EML cells' differentiation profile

The goal of this study was to ascertain the epigenetic affects of Hsp90 inhibition on hematopoiesis. Therefore, EML cells were pretreated with GM before differentiation was induced. The cells were treated with vehicle, 15 or 20 nM of GM for 24 hours followed by a 48 hour rest period. Given the short half-life of the GM/Hsp90 complex (Kamal et al., 2003), the brief inactivation of Hsp90 followed by a rest period will allow for the specific assay of epigenetic events. The cells were then differentiated as described above. Flow cytometry was used to measure cell surface marker expression before differentiation was induced (day 0), as well as on day 3, day 4 and day 6 of differentiation (**Figure 4.6A**, **B**, **C** and **D,** respectively). CD11b was significantly up-regulated on day 0 with GM pretreatment; however, this effect was ablated after differentiation was induced. There were no other differences in marker expression with GM pre-treatment.


A
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B
Day 3
C
Day 4
D
Day 7
% Positive Events
CD117
Sca-1
F4/80
CD127
CD11b
CD45
Ter119
Ly6G
CD3e
0 nM
15 nM
20 nM
Geldanamycin

Figure 4.6. Cell surface marker expression during differentiation does not vary significantly with GM treatment. Cell surface markers of EML cells were examined with GM treatment over six days. Based on the toxicity assay, the cells were treated with 0, 15nM, and 20nM of GM for 24 hours. The cells were then allowed to rest for 48 hours before they were differentiated. Cell surface markers where evaluated on **A)** day 0, **B)** day 3, **C)** day 4, and **D)** day 6 by flow cytometry. Surprisingly, there was no significant difference in the markers, except for one. On day 0 CD11b, which is a macrophage marker, was notably increased with GM treatment. However, this effect was lost during the differentiation process. Results depicted are from three independent experiments performed in duplicate (mean + S.E.M.).

Cells treated with GM have an increased adaptability through the restriction point

On day three of the differentiation protocol, the culture media was switched from that containing IL-3, SCF and ATRA to media containing just GM-CSF. Only those cells that are suitably primed for the media switch survive as seen in **Figure 4.4.** This environment provides an efficient way to assay increased adaptability of the cells. There was no significant difference in cell counts on day three of differentiation between the treatment groups (**Figure 4.7A**). However, a dose dependent increase in the survival of cells through this media switch was observed with GM pre-treatment (**Figure 4.7B**). These cell counts were performed on the same cultures as those in which the cell surface marker expression was measured in **Figure 4.6**.

The increased adaptability through the restriction point was not reproducible

Further studies, such as microarray analysis and extended dose response curves, were attempted in order to further our understanding of the exciting effect described above. Unfortunately, as **Figure 4.7C** illustrates, the same adaptability was not seen when these experiments were performed. Therefore, we hypothesized that a 48 rest period might be too long, making the epigenetic effects unstable. Subsequently, we tried resting the cells for only 24 hours. At first this seemed to work, as a significant increase in survival was observed through the restriction point with treatment (**Figure 4.7D**). However, this effect was short lived and still seemed to be unstable (**Figure 4.7E**).

In next set of experiments, we tried various methods to stabilize the phenotype. The cells were treated three times over a 30 hour time period. Because this was harsh to the cells, they

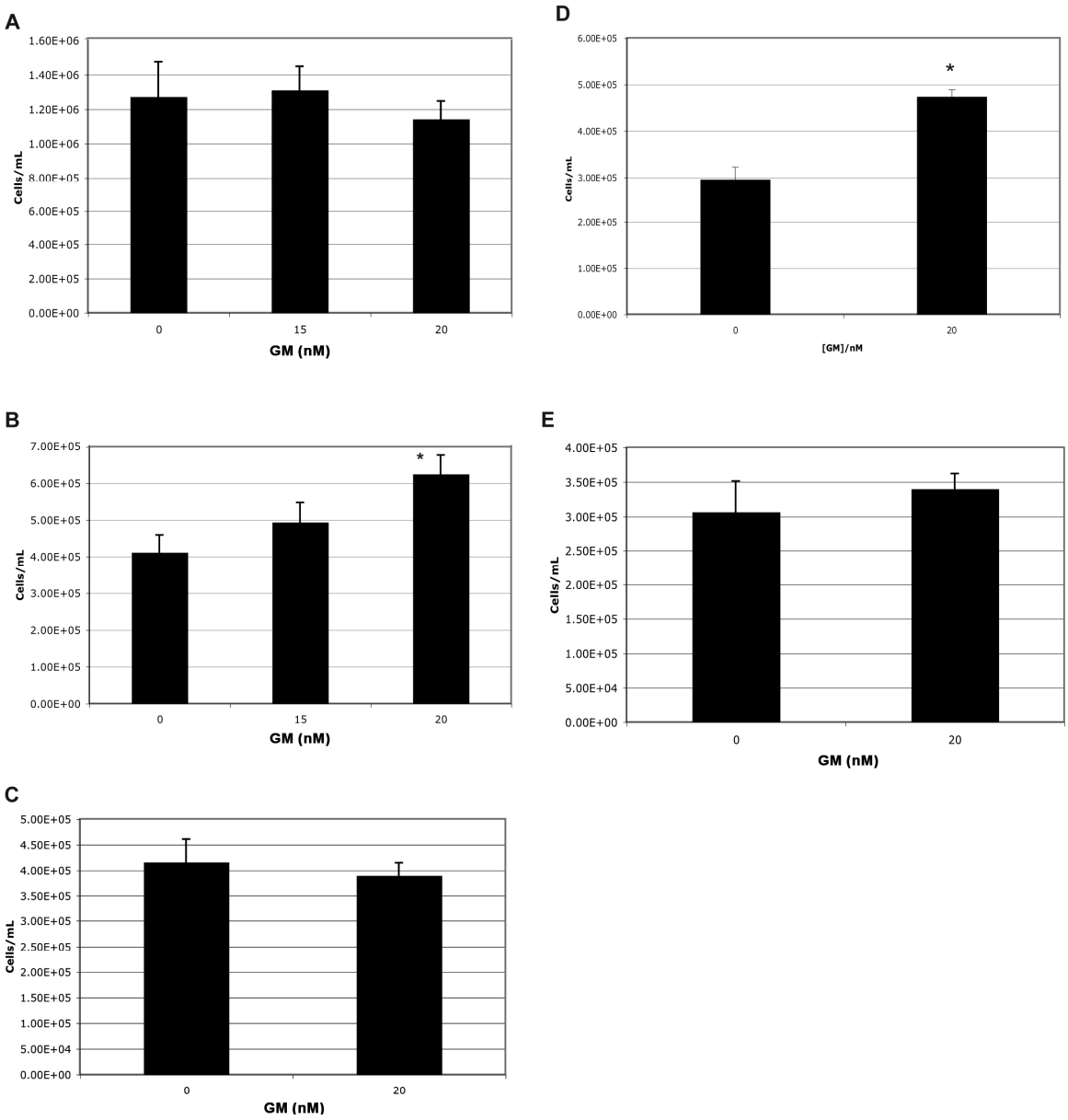

Figure 4.7. Geldanamycin offer cells a greater phenotypic plasticity that is unreliable. Cells were treated for 24 hours with GM prior to differentiation, with a 48 hour rest period. Cell counts in panels A) and B) were performed together alongside the marker study in Figure 4.6. **A)** There was no significant difference in cell counts performed on day 3 of differentiation. **B)** On day 4, a significant increase in cell survival was seen through the selection window that is dosage dependent. This indicates that inhibition of Hsp90 could be offering greater phenotypic diversity in the cells allowing them to respond to GM-CSF. Subsequent experiments were performed in the same manner after the above data was collected. **C)** Cell counts on day 4 were no longer significant when treated with the above conditions. 24 hours of GM treatment followed by 24 hours rest period before differentiation was then employed to try and induce the phenotype. **D)** and **E)** are two independent experiments that show that the results were still unreliable. Results depicted in A), B) and C) are from three independent experiments performed in triplicate (mean + S.E.M.). D) and E) are from one experiment performed in triplicate (mean + S.E.M.) under the same exact conditions.

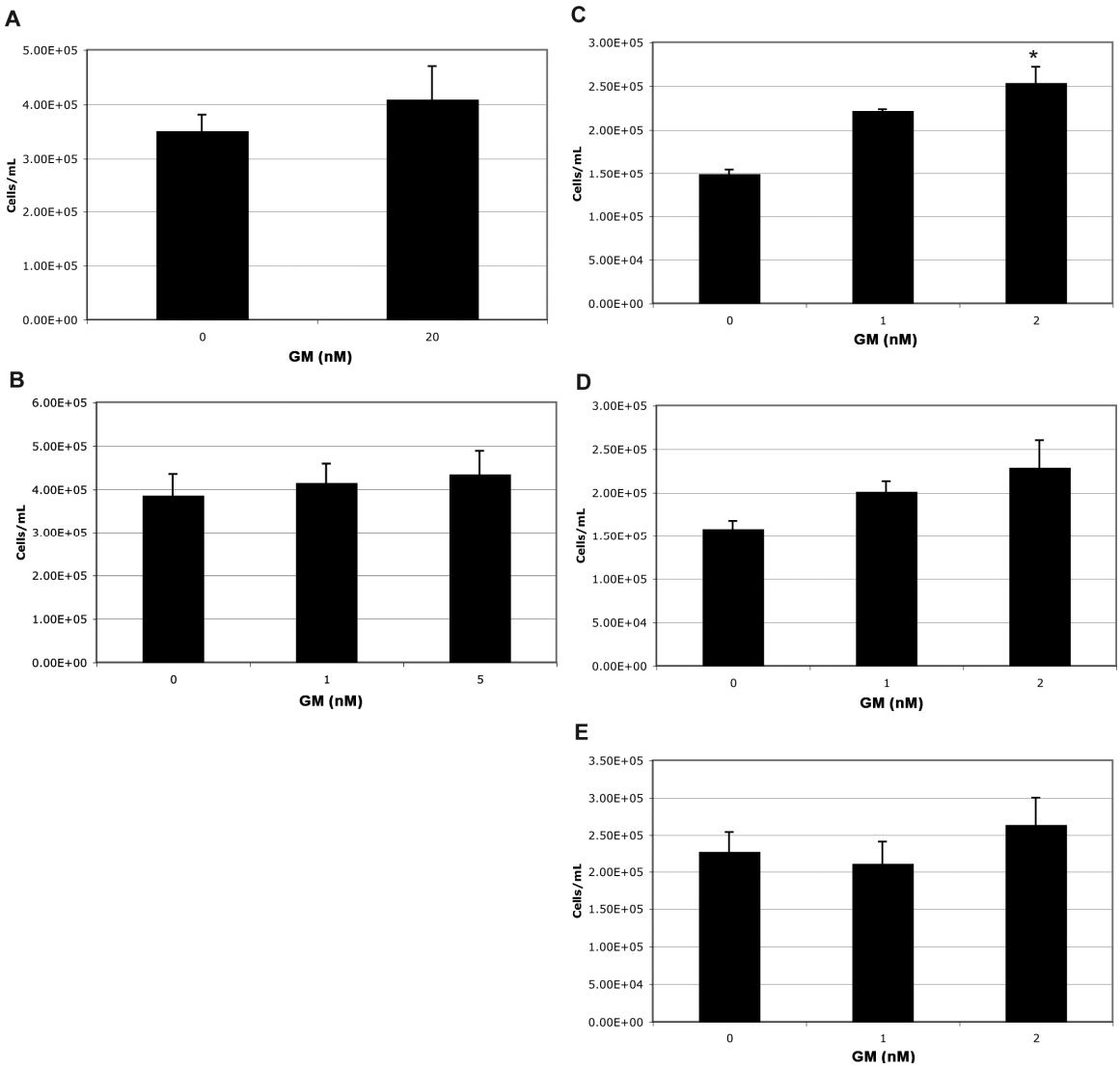

Figure 4.8. Efforts to try and rescue the plastic phenotype previously observed were unsuccessful. Different methods were employed to try and rescue the phenotype observed in Figure 4.7B. **A)** Cells were treated with GM three times over a 30 hour period and allowed to rest for 48 hours before differentiation was induced. No significant difference was seen in the cell counts on day 4. **B)** Cells were treated three times over a 72 hour period with low doses of GM before resting for 24 hours. Differentiation was then induced and cell counts on day 4 show a dose dependent increase in cell survival, but this was not significant. The next method that was tried was to pre-treat the cells with low doses of GM for 24 hours and then continue with treatment during differentiation. Panels **C), D)** and **E)** represent day 4 cell counts from one experiment performed in this manner. By separating the experiments it became clear how the data was variable. Results depicted in A), B) are from three independent experiments performed in triplicate (mean + S.E.M.). C), D), and E) are from one experiment performed in triplicate (mean + S.E.M.) under the same exact conditions. (*) indicates $p<0.05$ as compared to the vehicle control.

were allowed to rest for 48 hours before inducing differentiation. This method was not successful in bringing back the phenotype (**Figure 4.8A**). The next method we tried was treating at low a dose for a long period of time, 72 hours, and then resting the cells for 24 hours before differentiating them. This method brought back a similar trend, with a higher survival of the treated cells, but it was not significant (**Figure 4.8B**). One of the last techniques we tried was treating throughout differentiation. The cells were pre-treated with GM for 24 hours before differentiation was induced and then treated every day with fresh GM. At first it seemed like this would work to rescue the phenotype. A significant increase in survival through the restriction point was seen with treatment (**Figure 4.8C**). However, once again, this was short lived (**Figure 4.8D & E**). We also tried serum starvation to synchronize cells before treatment and varying the concentrations of IL-3, SCF and ATRA to try and stabilize the phenotype. We also tried treating for 48 hours, treating 4 times over a period of a day and a half at various concentrations of GM. Treating with 17-AAG was also tried as well. Again mixed results were obtained as seen in Figure 4.7 and 4.8, as none of these methods were successful in stabilizing the desired result.

Discussion

About 40% of the time we observed an increase in EML cell survival when selective conditions were applied. This increased plasticity observed in EML cells during differentiation with GM treatment could be explained in one of three ways: (1) the cells are more proliferative with GM treatment; (2) GM causes the cells to differentiate more quickly; (3) inhibition of Hsp90 causes the cells to become more plastic, which would make them more adaptable and thus enhance their survival through selective conditions. Cell counts on the day previous to the media switch were not significantly different between treatment groups (**Figure 4.7A**). This indicates the treated cells were not more proliferative. The marker studies revealed that pre-treating with GM did not change the differentiation profile of the cells (**Figure 4.3A**), so an acceleration in differentiation was not occurring. Therefore, we hypothesize that Hsp90 inhibition could be causing the cells to become more plastic. The inhibition of Hsp90 could

release a phenotype, or range of phenotypes, that allows the cells to be more adaptive to the selective environment of the media switch. Furthermore, this release occurs six days before the effect is observed, which would be suggestive of an epigenetic mechanism. If the results from this study were reproducible, it would corroborate previous data that Hsp90 acts an epigenetic morphological capacitor in a mammalian model.

This would be significant in cancer treatment. Precancerous cells must overcome a series of steps before they become full blown cancer (Hanahan and Weinberg, 2000). Survival within the host creates a Darwinian sort of environment whereby only those cancer cells that are able to adapt survive. Cancer cells are more susceptible to Hsp90 inhibition than normal cells, but Hsp90 inhibition could allow cancer cells to be more adaptable in certain types of cancer. Understanding how Hsp90 inhibition affects cell development will lead to a better understanding of treatment mechanisms.

The increased survival through the media switch experiments were repeated three times consistently. Unfortunately, when further experiments were performed to expand our understanding of this exciting result, reliability issues developed. Various methods were tried in order to stabilize the phenotype. Regrettably, none of these were successful. An unpredictable nature in the data seemed to develop as **Figure 4.7D** & **E** and **Figure 4.8C**, **D**, & **E** illustrate. The rest of the experiments performed with these cells were plagued with variable results.

We also tried varying the percentage of conditioned medium used during treatment of the cells and during differentiation. Again, unstable results were obtained. Sometimes the amount of conditioned medium seemed to affect the results, but upon repeating with the same lot of conditioned medium these effects were not seen. This made it difficult to implicate a causal role for the amount of SCF and IL-3 in the media. It should also be noted that using conditioned media could have contributed to the inconsistencies experienced with these studies. Although variability was seen within the same lot of CM, there could have been other factors present in the conditioned medium that may have had an effect on our system.

Two recent studies have demonstrated that EML cells exist in subpopulations with differing characteristics. One study sorted EML cells based on CD34 expression (Ye et al., 2005), which is a marker of a sub-fraction of HSCs and progenitors (Osawa et al., 1996). The authors found that there were equal fractions of CD34+ and CD34- among the lineage negative EML cells. These two sub-fractions were quite different in their response to cytokines. The CD34- population was unable to proliferate in the presence of SCF, even though they expressed high levels of SCF receptor. Interestingly, the CD34+ population was able to proliferate with SCF stimulation *and* reconstitute the original heterogeneous population of cells. The CD34- population was responsive to IL-3 alone, while the CD34+ population was not. This indicates that these two populations of cells behave very differently and the CD34+ population may represent a more primitive progenitor (Ye et al., 2005).

In a related study, other authors noted the broad range of expression of Sca-1 in EML cells via flow cytometry, and hypothesized that this was due to heterogeneity in the cell population. They sorted EML cells based on Sca-1 expression into high, mid and low populations. As was seen with CD34 expression, within hours the Sca-1 expression broadened for each sorted population regenerating the same presort expression pattern. They also examined whether these populations had different differentiation potentials. The Sca-1^{lo} population favored erythroid development and the Sca-1^{hi} populations favored granulocyte/macrophage development (Chang et al., 2008).

These studies demonstrate that there seems to be an inherent heterogeneity of EML cells, which could impact the results seen in our studies. This is corroborated by the findings that stem cells are heterogeneous by nature (Orkin and Zon, 2002; Roeder and Radtke, 2009). This could be due to the innate properties of the stem cells or the exposure to a variety of small disturbances from their surrounding environment. It has been hypothesized that these influences could cause "transcriptional noise", or small, random variations in transcriptional activity that can account for this heterogeneity (Chang et al., 2008).

Our results imply that the enhanced survival through the restriction point with GM treatment is a very complex phenotype, which may require specific conditions be met to observe the desired outcome. Given this, as well as reports on the heterogeneity of stem cells, it is not surprising that problems with reliability were encountered. It should be noted that this does not necessarily mean that Hsp90 is not involved in EML cell plasticity during differentiation. We believe there were unknown factors present that we could not control for that may have had deleterious effects on this complex phenotype.

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Chapter 5 : Discussion and Conclusions

AML is among the deadliest of the lymphatic and bone marrow cancers with a five year survival rate of 23.4%. Therefore, new treatment strategies are needed in order to improve patient survival. Heat shock protein 90 (Hsp90) is a molecular chaperone that serves a critical function in many different signaling cascades by ensuring proper protein structure, activity, proteolytic turnover, and localization. Because of this, it has been proposed that Hsp90 serves as a buffer to stabilize over expressed and mutated proteins helping cancer cells survive in the harsh environment of the host. Therefore, Hsp90 has become a target to cancer therapy. AML is characterized by an accumulation of undifferentiated and functionless myeloid precursors in the bone marrow and blood. Studying how Hsp90 impacts myeloid cell differentiation and AML cells specifically will provide insight into possible treatment strategies for AML. Therefore, the aim of our studies was to determine the specific effects of Hsp90 inhibition in hematopoiesis and AML. Mutant p53 is an Hsp90 client protein. Wild-type p53 is a tumor suppressor protein that is muted in 50% of total cancers and studies have shown that it can have oncogenic properties. Studies are needed to determine the role, if any, of mutant p53 in AML. Targeting mutant p53 might provide new treatment opportunities.

AML studies

We were able to show that Hsp90 inhibition, by treatment with 17-AAG, in leukemic cell lines caused cell cycle arrest in the G_2/M phase of the cell cycle. Previously, these findings were also observed in lung and glioblastoma cell lines (Garcia-Morales et al., 2007; Senju et al., 2006). However, the diverse reactions to 17-AAG treatment that we observed were not reported in these studies and correlates well with the fact that AML is a heterogeneous disease. Our data support previous reports that the block in G_2/M phase was due to the down-regulation of CDC2 and CDC25c, two important G_2/M checkpoint proteins that are known to be Hsp90 clients. Our

publication broadens the knowledge on how 17-AAG treatment can act specifically in AML and may shed light on the lack-luster response of patients to 17-AAG in clinical trials.

Studies have shown that Hsp70 and Hsp90 expression levels in cells can be indicative of their response to Hsp90 inhibition (Thomas et al., 2005; Flanderin et al., 2008). An additional experiment that would have been beneficial to my research would be to measure the expression of these two proteins in the cells before treatment. We may have been able to correlate their expression with 17-AAG susceptibility. Attempts were made to measure Hsp90 protein expression between the cell lines; however, difficulties with protein transfer during western blotting prevented accurate protein measurement.

Kasumi-3 studies

Mutations in the *TP53* gene occur more frequently than any other mutations found in cancer cells. These point mutations often result in the expression of a stable full length protein, which suggests that mutant p53 could be providing some sort of survival advantage to the cancer (Kim and Deppert, 2004). In fact, there is evidence that suggests that mutant forms of p53 can act in an oncogenic capacity and have been shown to activate transcription of genes that are important in oncogenesis. However, the mechanisms involved in these activities are poorly understood.

Even though mutant p53 is present in only about 10% of leukemias, a recent study indicated that a single p53 deletion was a strong independent negative prognostic factor for disease free survival in AML (Seifert et al., 2009). This demonstrates that although p53 mutations in AML are uncommon, the effect of inactivating p53 can have a devastating outcome on patient survival. In our studies, we found that p53 was mutated in the leukemia cell line Kasumi-3. It is interesting that this cell line harbors two separate mutant alleles. One mutant allele results in of intron 7 being trapped in the mature messenger RNA. This produces a truncated protein that is most likely not stable. The other is a point mutation in the DNA binding domain, which is the most common site for point mutations in the p53 gene. This allele probably codes for the up-regulated protein product seen in our experiments. The characterization of the mutations in p53

in Kasumi-3 cells provides a valuable tool to study p53 oncogenic function in general as well as in AML specifically.

This cell line was also very sensitive to Hsp90 inhibition, in that significant apoptosis was observed after just 48 hours of 17-AAG treatment. Hsp90 inhibition also caused significant down-regulation of mutant p53. These two observations led us to hypothesize that perhaps the cells were dependent on mutant p53 in some fashion and that this dependency was the mechanism behind the high sensitivity of this cell line.

This hypothesis however, was not validated. The mutant p53 was knocked down independently of Hsp90 inhibition. In order to mimic the 17-AAG studies, measurement of apoptosis was used as the endpoint of this experiment. Unfortunately, Kasumi-3 cells were difficult to transfect and as a result electroporation was used as a last resort for sufficient transfection efficiently. A 50% knockdown of p53 was achieved, but there was no difference in the apoptosis between the control and p53 siRNA treated cells. Regrettably, even though apoptosis was measured 72 hours after electroporation, apoptosis levels were around 50% in both treatment groups. This high rate of apoptosis could be masking the specific survival effects of mutant p53 protein knockdown in these cells.

Subsequently, we measured P-glycoprotein activity, which is a gene known to be up-regulated by mutant p53 (Chin et al., 1992). P-glycoprotein is an ABC transporter that is often expressed in drug resistant cases of AML and other cancers. It contributes to resistance through its ability to efficiently efflux drugs from cells. The thought behind this experiment was that perhaps there was a compound effect of 17-AAG down-regulating P-glycoprotein, through mutant p53 degradation, which would cause the cells to be more sensitive to 17-AAG. Unfortunately, there was no difference in P-glycoprotein activity with p53 knock down.

We were only able to study these two effects, apoptosis and P-glycoprotein expression, of mutant p53 knockdown due to time and monetary constraints. It is feasible that the methods we used to measure this effect were flawed, or that the mutant p53 could be affecting the cells in a different capacity than the two effects we studied. Therefore, it is still possible that the mutant

p53 in Kasumi-3 cells is acting in an oncogenic capacity. Perhaps looking at gene expression at the protein level of some other known mutant p53 targets would have provided evidence to support our hypothesis.

Kasumi-3 cells are under the AML classification of M0, which means they are an undifferentiated subtype. Stem cells are known to possess efflux pumping capabilities and in fact, they are sometimes isolated based on this ability. It is reasonable that Kasumi-3 cells, being undifferentiated, could express P-glycoprotein as a result of their immature precursor origins. P-glycoprotein and other drug resistant phenotypes are also common in AML. Perhaps, P-glycoprotein expression is accomplished through a completely separate mechanism in this cell line.

In clinical trials, there has been more success in treatments using 17-AAG and other Hsp90 inhibitors in combination with other drugs. Our results in Chapter 2, studying the effect of Hsp90 inhibition in several cell lines, agree with this result. If the mutant p53 in Kasumi-3 cells is acting in an oncogenic capacity, it is very possible that the compound effect of Hsp90 inhibition and mutant p53 degradation could be responsible for the sensitivity of these cells to 17-AAG. During the course of our studies, we were only able to knockdown the mutant p53 by about 50% either with 17-AAG or siRNA alone. It would be interesting to see if there was a synergistic effect of using p53 siRNA and 17-AAG. It would also be interesting to produce an inducible shRNA Kasumi-3 stable cell line to study the effects of mutant p53 knockdown. This would avoid the electroporation/apoptosis issue and allow p53 knockdown to be studied in conjunction with other drugs.

Another appealing experiment would be to take this particular p53 mutant and express it in a different p53-null cell line, such as HL-60. This would allow the direct assessment of phenotypes this mutant is capable of producing, if any. On a similar note, if this mutant p53 served in only a dominant-negative capacity to promote leukemogenesis in the original patient, it would be exciting to express it in a wild type p53 expressing AML cell line. Dominant-negative activity would be straightforward to measure. In our studies, these cells arrested growth with

treatment of camptothecin, which was most likely due to up-regulation of p21 in a p53 dependent manner. If this mutant p53 is acting as a dominant-negative this effect would be ablated in cells expressing both the mutant and the endogenous p53 protein.

It is remarkable that Kasumi-3 cells are so sensitive to Hsp90 inhibition given that they possess P-glycoprotein activity. KG-1a cells are very resistant to 17-AAG treatment and we were able to show that this was, at least in part, due to P-glycoprotein activity. This indicates that there must be some special mechanism(s) or protein client(s) responsible for Kasumi-3 cells' sensitivity. Discovering the reason of this susceptibility may uncover an important therapeutic target to use in conjunction with Hsp90 inhibition.

Kasumi-3 cells aberrantly express the EVI1 oncogene that has been implicated in leukemogenesis (Asou et al., 1996; Goyama and Kurokawa, 2009). Therefore, my next hypothesis would be that EVI1 is an Hsp90 client protein. This would be readily tested by measuring protein levels in the cells with and without 17-AAG treatment. Degradation of EVI1 would provide evidence that it is a client, but immunoprecipitation assays are needed for confirmation.

Kasumi-3 cells' dependence on EVI1 could be tested with EVI1 siRNA. However, as discussed earlier, this could have the same pitfalls as investigating mutant p53 in the same role. If EVI1 is an Hsp90 client, it would support the hypothesis that Hsp90 inhibition in this cell line has the compound effect that was hoped for when 17-AAG was introduced in clinical trials. One thing is certain, elucidating why Kasumi-3 cells are so susceptible to 17-AAG treatment would add much to our understanding of how Hsp90 contributes to cancer progression as well as targeting specific AML and other cancer patients who would benefit the most from this treatment.

EML studies

Because of the Darwinian nature in which cancer develops, any treatments that might impact the adaptability of cancer are important to understand. Therefore, the idea that Hsp90 can act as a genetic and epigenetic capacitor in *Drosophila* should be explored in a mammalian

model for this reason. Earlier studies showed in *Drosophila* that with parental Hsp90 inhibition, offspring were born with limb-like outgrowths in the eye. When selective conditions were imposed, this outgrowth continued over several generations without Hsp90 inhibition, indicating a heritable epigenetic mechanism (Sollars et al., 2003). We tried to replicate these findings in a mammalian hematopoietic stem cell model using EML cells. The cells were pretreated with geldanamycin before inducing differentiation. Initially, we were hoping for a trans-differentiation event in which the cells spontaneously differentiated, but we did not observe this change. We then looked for a trans-differentiation event during the normal differentiation protocol but this was also not observed. Perhaps our inability to detect these events is because the differentiation signals brought on by the cytokine stimulation are so strong that it would overcome any epigenetic modulation that could have taken place from Hsp90 inhibition.

In the *Drosophila* study, the eye tissue was sensitized by ectopic expression of Krüpple (Sollars et al., 2003), which is a gap gene important in the development of several thoracic and abdominal segments in the embryo. Ectopic expression of this developmental transcription factor caused the cells in the eye tissue to not have a clear developmental fate. When Hsp90 was inhibited, it caused these cells to trans-differentiate into appendages. One thing to consider in our EML studies is that there was no sensitizing agent present in our system. Perhaps if we had employed a sensitizer, such as expression of AML1, we may have also seen a trans-differentiation event.

We observed that with Hsp90 inhibition, cells that were primed to differentiate seemed to be more adaptable during the regular differentiation protocol. This is illustrated by their ability to survive better through a restriction point, which normally caused around 80% cell death. This finding was exciting because this adaptability had not previously been seen in a mammalian model, and the pretreatment of the cells suggested that it was caused by epigenetic change. Unfortunately, we encountered problems with replicating the experiments.

I have suggested that the non-reproducible results could be due to the inherent heterogeneity present in the cells, which has been documented in previous studies. One study sorted populations of CD34+ and CD34- EML cells. Interestingly, when comparing expression of Sca-1, an early stem cell marker in mice, and CD34, cells that were CD34+ were also Sca-1^{hi} and cells that were CD34- were Sca-1^{lo}. This suggests that cells that the CD34+ are more stem cell like than their CD34- counterparts (Ye et al., 2005).

These two populations also differed in their response to IL-3 and SCF. An essential experiment in this case would be to sort the two different populations of cells and immediately treat with geldanamycin and follow through with the experiment protocol. It is conceivable that perturbations in these populations could be partially responsible for the lack of reproducibility. The disturbances in the populations could be brought about through the conditioned medium used or other unknown factors in their environment. By sorting the two populations, these possible problems could be circumvented.

Another study observed Sca-1 expression to be highly variable in EML cells, and we have confirmed this finding in our studies (Chang et al., 2008). They went on to show that cells with differing levels of Sca-1 expression had different developmental tendencies. They also showed that when EML cells were sorted based on Sca-1 expression and subsequently cultured, the original population with broad Sca-1 expression was soon reproduced. Sorting EML cells based on Sca-1 expression followed by geldanamycin treatment would make a nice additional study to go along with those mentioned above. Perhaps these differing populations show different responses to geldanamycin treatment and enriching for one population over another would provide a more robust response.

In our studies with EML cells, we saw a significant increase in CD11b expression with Hsp90 inhibition without induction of differentiation. EML cells were produced by transfection with a dominant-negative retinoic acid receptor which blocks differentiation down the monocyte/macrophage lineage. Perhaps this block in differentiation obstructed the trans-differentiation event that could have occurred with treatment of geldanamycin alone. EML cells

are a continuously passaged cell line and there could be artifacts inherent in the cell line that could introduce confounding factors.

In light of our study of 17-AAG in the leukemia cell lines, further questions about geldanamycin treatment in EML cells has arisen. Treatment of EML cells with geldanamycin resulted in related cellar toxicity. At the doses that we used in our EML study, the cells were still able to proliferate or remained at a constant cell number. It would have been a good idea to do a cell cycle analysis study to determine if the cells were arresting in G_2/M phase as was seen in the leukemic cells. This data would have shown that G_2/M arrest was consistent in 'normal' cells.

Besides just focusing on the survival advantage of treated cells, it would have been interesting to look at the levels of acetylated histones after geldanamycin treatment. In the previous study, supplementing the flies' diet with histone deacetylase inhibitors lowered the frequency of outgrowths in the eye. This piece of evidence provided a molecular link to epigenetic modulation behind the phenotype. It would have been interesting if geldanamycin treatment in EML cells modulated histone acetylation, or even methylation and phosphorylation. An examination of the histone code may have proven to be more informative.

It is certainly possible that Hsp90 inhibition in EML cells was having an effect on gene expression without causing a measurable change in phenotype by the assays that we employed. These effects could have been ablated upon cytokine stimulation when differentiation was induced. Another procedure that would have provided additional information would have been a microarray analysis on EML cells with and without Hsp90 inhibition. This would have given us some idea of what genes were affected and would have allowed us to make a more informed hypotheses. Financial considerations did not allow use of this option.

Looking at how Hsp90 inhibition affects hematopoiesis in a whole organism using colony formation and flow cytometry assays of bone marrow could have also been very informative. Mice would have been a great model for this study given the experience our lab has with measuring stem and progenitor cells in bone marrow. Cells from the bone marrow can be cultured in semi-solid medium, containing different stimulatory cytokines. Every progenitor cell

present will form a colony after six days. The morphology of the cells within the colony can be assessed to determine what type of progenitor originally seeded that colony. The number of colonies present can be extrapolated back to the original seeding density of the culture to determine the frequency of progenitors present in the bone marrow. Flow cytometry can also be used to measure stem and progenitor cells by taking advantage of specific cell surface markers, which are well characterized in mice, present on each cell type. Treating the mice with 17-AAG followed by a complete bone marrow study would have broadened our understanding of the potential role that Hsp90 plays in hematopoiesis. These experiments could have also provided evidence to support the theory that Hsp90 acts as an evolutionary capacitor in mammalian models. However, financial and time constraints did not allow these experiments to be pursued.

Evidence of Hsp90 acting as an evolutionary capacitor in a mammalian model is still lacking, even though the first study in *Drosophila* was published over a decade ago (Rutherford et al., 1998). It would have been a great contribution to our understanding of Hsp90, as well as knowledge about evolution, if we would have obtained consistent results in the EML cell study. Such results would have contributed to our understanding of how Hsp90 inhibitors may work in cancer. If Hsp90 inhibition does indeed cause an increase in adaptability in hematopoietic cells, this could contribute to cancer progression in AML patients as well as other cancers.

Our model of choice may not have been the most efficient one with which to test our hypothesis. Embryonic stem cells might have provided another model system to use; however, we were not equipped to culture these types of cells. Perhaps in the future the confounding factors that plagued these experiments will be revealed and proper protocols implemented to control for them.

Hsp90 inhibitors were first proposed to be used in clinical trials because it is responsible for the folding and activation of many important signal transduction proteins, some of which are involved in cancer progression. Given that cancer is a multi-step process, it was thought that through Hsp90 inhibition, many pathways could be targeted with just one drug. Additionally, recent data show that cancer cells are dependent on Hsp90 and it is frequently up-regulated in

cancer. It is also present in active, complexed forms when compared to normal cells (Kamal et al., 2003). This dependence makes cancer cells more sensitive to Hsp90 inhibition than normal cells. Based on this knowledge, it would be feasible that Hsp90 inhibition would kill cancer cells without difficulty. However, Hsp90 inhibitors in clinical trials have not had the response that was hoped for as a single agent (Reikvam et al., 2009; Usmani et al., 2009).

Why aren't Hsp90 inhibitors more effective? One reason could be the dosing. Due to the Hepatotoxicity of available inhibitors, a high enough dose to impart damage to cancer cells cannot be achieved without unreasonable toxic side effects. However, in some studies, effective Hsp90 inhibition was attained as measured by down-regulation of client proteins and up-regulation of Hsp70. To try and combat these issues, the search for novel Hsp90 inhibitors that are more potent and less toxic is still ongoing.

We were able to show that between four human AML cell lines, the responses to 17-AAG were quite diverse. It is possible that the results of the clinical trials are due to diverse sensitivity of tumors to 17-AAG. Hsp90 inhibition has been shown in *Drosophila* and *Arabidopsis* to promote phenotypic diversity among progeny. An alternative hypothesis is that by inhibiting Hsp90 in human cancer, instead of eradicating the tumor, inhibition may allow the cancer cells to become more adaptable to their environment. Support for this hypothesis can be found in the existing literature and implied from our EML studies.

However, it should be noted that Hsp90 is often up-regulated in cancer due to the stressful conditions in which a cancer develops as well as the dependency of cancer on Hsp90 to stabilize mutated and misfolded proteins. Therefore, cancer is highly dependent on Hsp90 for its very survival, which makes the relationship of Hsp90 in cancer cells much more complex than in normal cells. This means that it is possible that the effects of Hsp90 inhibition in normal cells may not be the same as in cancer cells. Regardless, it is interesting to speculate on the reasons for the meager response of Hsp90 inhibition in clinical trials.

In summary, the studies presented here add to our understanding of how Hsp90 inhibition specifically acts in AML. We observed distinct responses to 17-AAG among the cell lines tested.

This may explain the lackluster results of this inhibitor in clinical trials. This study also led to the discovery of two alleles of mutant *TP53* in Kasumi-3 cells, an AML cell line that was very sensitive to 17-AAG. We hypothesized that the reason for this sensitivity was due to the degradation of mutant p53, a known Hsp90 client. Although we were not able to show that this mutant p53 was acting in an oncogenic fashion, it still raises the possibility that tailoring 17-AAG treatment to patients with known oncogenic *TP53* alleles might prove to be very effective. Unfortunately, we were not able to determine why Kasumi-3 cells were so sensitive to 17-AAG. However, they do provide a valuable model with which to determine which biomarkers may dictate sensitivity to Hsp90 inhibition. In addition, Kasumi-3 cells will make a good model to study mutant p53 function in cancer, as well as AML specifically.

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