Investigation of Resistant Mechanisms of Apoptosis-Inducing Anticancer Agents

by

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Dedication

I dedicate my dissertation to my sister, Cicely Hoffman-Luca. She has been and always will be one of the most influential people in my life. Her tenacity and strength inspired me to reach my goal of obtaining my PhD. She has never left my side and I thank her immensely for her never ending support and love.

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Abstract

Cancer cells have acquired the ability to survive by up-regulating survival pathways or down-regulating cell death pathways that results in their resistance to apoptosis. Integral players involved in the activation or inhibition of apoptosis include the tumor suppressor p53 and the Bcl-2 family of proteins. In 50% of human cancers p53 is mutated resulting in inactivation of its tumor suppressor activity, however, in the remaining cancers p53 is functionally inhibited by its direct interaction with the MDM2 protein. This interaction has been extensively studied and has resulted in the discovery of small molecule MDM2-p53 interaction inhibitors like SAR405838. The Bcl-2 family of proteins, key regulators of apoptosis, has inspired a plethora of investigations aimed at targeting this family to activate apoptosis in tumor cells. Examples of these apoptosis-inducing agents are the Bcl-2/Bcl-xL inhibitors ABT-737 and ABT-263. Although these agents show promise in clinical trials for the treatment of cancer, it is fully expected that resistance to these drugs will develop. Here, I have investigated the acquired resistance mechanisms *in vitro* and *in vivo* to apoptosis-inducing small molecule anticancer agents in acute leukemia and osteosarcoma models.

Despite impressive initial antitumor activity, the tumor regression achieved by ABT-737/ABT-263 and SAR405838 in xenograft models of acute leukemia is transitory; tumors eventually regrew after treatments were terminated, suggesting emergence of resistance to both classes of drugs. Analysis of the regrown tumors initially treated with the inhibitors showed that

the acute leukemia cells acquire resistance to SAR405838 by mutation of the p53 gene or compromised p53 function, and to ABT-263 through down-regulation of pro-apoptotic Bcl-2 family member BAX. Combination and sequential treatment of SAR405838 and ABT-263 achieved longer term tumor regression without signs of toxicity than either agent alone in acute leukemia models.

In the osteosarcoma model, which harbors an amplified MDM2 gene and wild-type p53, only *in vitro* exposure to SAR405838 resulted in acquired resistance whereas *in vivo* exposure did not. Similar to the findings in the acute leukemia models, analysis of the SJSA-1 cells that developed resistance *in vitro* to SAR405838 showed that p53 is mutated in the DNA binding domain. In comparison, a number of cell lines were established by culturing tumors that regrew after SAR405838 treatment and, surprisingly, these sublines retained sensitivity to SAR405838. Analysis of these sublines showed that p53 either maintains its wild-type status or harbors a single heterozygous C176F mutation located in the DNA binding domain of p53. Computational modeling suggests that the p53 C176F mutant is still functional. Taken together, these data suggest that both *in vitro* and *in vivo* models of resistance should be employed in order to better identify resistance that may occur in a clinical setting.

Chapter 1

Introduction

The treatment of cancer has improved greatly over the last several decades, mainly due to the development of highly efficacious chemotherapeutics and targeted therapies. However, the ability of cancer cells to develop resistance to drug treatment remains a significant impediment to successful disease treatment. Numerous investigations have identified a myriad of mechanisms in which cancer cells can elude drug therapy, and it has become evident that resistance exists against every effective drug. The determination of the mechanisms of drug resistance can help to identify patients that may benefit the most from the drug and to develop rational treatment strategies to overcome resistance.

Drug resistance results when diseases become tolerant to pharmaceutical treatments. The concept was first recognized when bacteria became resistant to certain antibiotics and has now been identified in other diseases such as cancer. Several mechanisms of resistance have been identified such as drug inactivation, drug efflux, drug target alterations, DNA damage repair, cell death inhibition and microenvironment-mediated resistance[1-3]. For the purpose of this dissertation I will primarily focus on the mechanisms of resistance involving resistance of cancer cells to cell death induction by apoptosis inducing agents.

Cells have an inherent ability to self-destruct called programmed cell death or apoptosis. Defects in the apoptotic machinery can eventually lead to expansion of populations of cancerous cells. Resistance to apoptosis can also increase the tumor cells ability to escape analysis by the

immune system. Furthermore, defects in the apoptosis pathways can result in cancer cell's resistance to therapy. Thus, resistance to apoptosis has been termed one of the hallmarks of cancer and is an important clinical problem. This dissertation will focus on elucidating the mechanisms of resistance in cancer models, both *in vitro* and *in vivo*, that arise as a result of treatment with small molecule inhibitors designed to target defects in the apoptosis machinery. More specifically, I will focus on the mechanisms of resistance to inhibitors of the p53-MDM2 interaction and anti-apoptotic protein members of the B cell lymphoma 2 (Bcl-2) family. The results from these studies may lead to the development of rationale treatment strategies aimed at overcoming cancer drug resistance.

p53

Over 30 years ago, the p53 gene was first identified as an oncogene, but over ten years later was found to be a tumor suppressor[4,5]. The p53 gene contains 11 exons spanning 20 kilo bases in chromosome 17p. Both the loss of chromosome 17p and p53 inactivating mutations are common in cancers. The p53 gene was implicated in the rare inherited condition, Li-Fraumeni syndrome, which is associated with frequent occurrence of several types of cancer in affected families [6]. Furthermore, it was determined that the p53 protein does not function properly in most human cancers. In order to investigate the role of the p53 gene in mammalian development and tumorigenesis, mice with p53 deficiency were utilized. Observations showed that the mice deficient for p53 were prone to develop cancer[7]. In humans, approximately 50% of tumors harbor a mutated form of *TP53* (gene that encodes the p53 protein) and in the remaining 50% p53 can be altered in a myriad of ways[8,9]. A large body of accumulated studies has demonstrated that all cancer cells harbor either defective p53 or malfunctions in the p53 network.

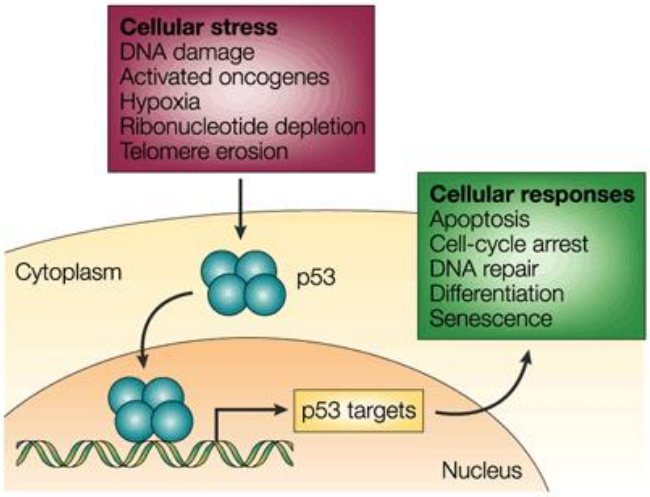

Figure 1.1 p53 signaling pathway

The tumor suppressor protein p53 is a multifunctional transcriptional factor that regulates cellular processes that affects DNA repair, senescence, angiogenesis, cell cycle, differentiation and apoptosis [5] (Figure 1.1[9]). In unstressed cells p53 exists at very low concentrations, however, under stress (DNA damage, activated oncogenes, etc) p53 protein accumulates in the cell. Upon accumulation the p53 protein will bind in its tetrameric form to the p53 response elements, which include a large and versatile group of responsive genes[10]. Thus, p53 acts as a transcription factor controlling these important cellular processes. Without p53, damaged and stressed cells continue to multiply and thus are likely to become cancerous. There are several mechanisms of inactivation of p53 that have been found in human cancer. These mechanisms include mutations in the p53 gene[8], increased degradation of p53 by the direct binding of the MDM2 oncoprotein[11,12], deletion of the carboxy terminal domain of p53, mutations in downstream regulators and mislocalization of p53 to the cytoplasm instead of the nucleus[5,13].

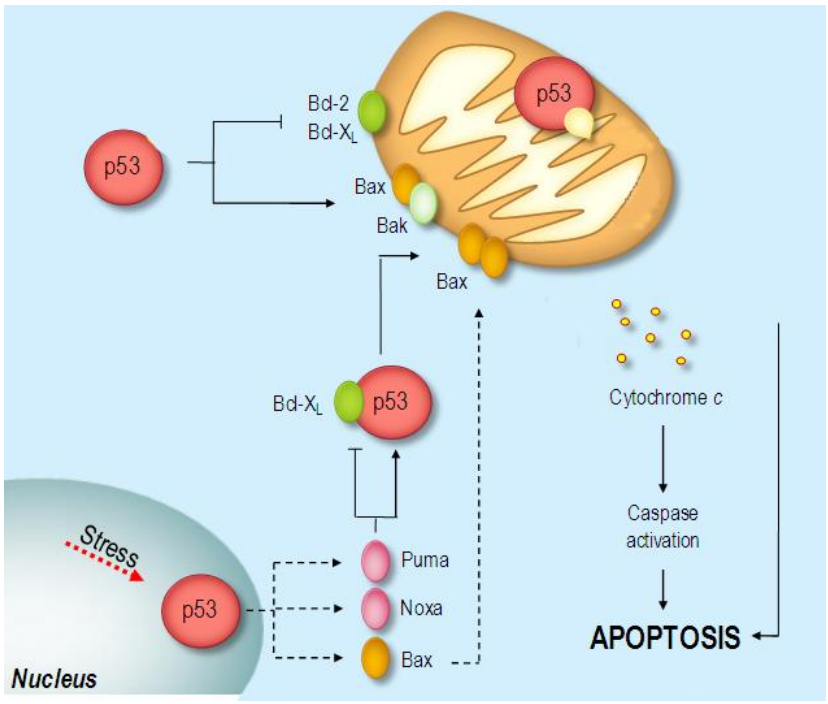

Figure 1.2 Cytosolic and mitochondrial p53 apoptotic pathways

One of the more prominent cellular responses to p53 is the activation of apoptosis. Apoptosis signals can employ two pathways: the extrinsic pathway, which is activated through cell surface receptors, or the intrinsic pathway (see section titled Bcl-2 Family for more details), which responds to stress signals[14]. The Bcl-2 family of proteins play a central role in the activation of the intrinsic apoptotic pathway (Figure 1.2[15]). Apoptosis is induced by p53 primarily through induction of the pro-apoptotic Bcl-2 family members, such as NOXA and p53 upregulated modulator of apoptosis (PUMA)[16,17]. The PUMA protein is able to bind to the mitochondrial anti-apoptotic proteins such as Bcl-2, Bcl-xL, Bcl-w, Mcl-1 and Bcl-2A1, whereas NOXA is only able to bind to Mcl-1 and Bcl-2A1[18]. Apoptosis is initiated once PUMA and/or NOXA inhibit the Bcl-2 anti-apoptotic family members[19]. Apoptosis can also be directly activated by translocation of the p53 protein to the mitochondria resulting in activation of the pro-apoptotic Bcl-2 family members, such as BAX and BID[20,21]. The mechanism of transcription-independent p53-mediated tumor suppression is another route in which p53 can

activate apoptosis. Complexation of the DNA-binding region of p53 with the anti-apoptotic proteins Bcl-2 and Bcl-xL frees their pro-apoptotic family members to activate apoptosis[22].

Initially, p53 was termed the 'guardian of the genome' due to its ability to mediate G1 arrest as a result of DNA damage[23]. Transient or irreversible p53-mediated cell cycle arrest involves the transcriptional activation of the cyclin-dependent kinase (CDK) inhibitor p21[24,25]. Enhanced levels of p21 inactivate the kinases responsible for driving cell cycle progression[26]. Analysis of cells from p21 deficient mice revealed that activation of p21 by p53 plays a major role in mediating the G_1 cell cycle arrest[27].

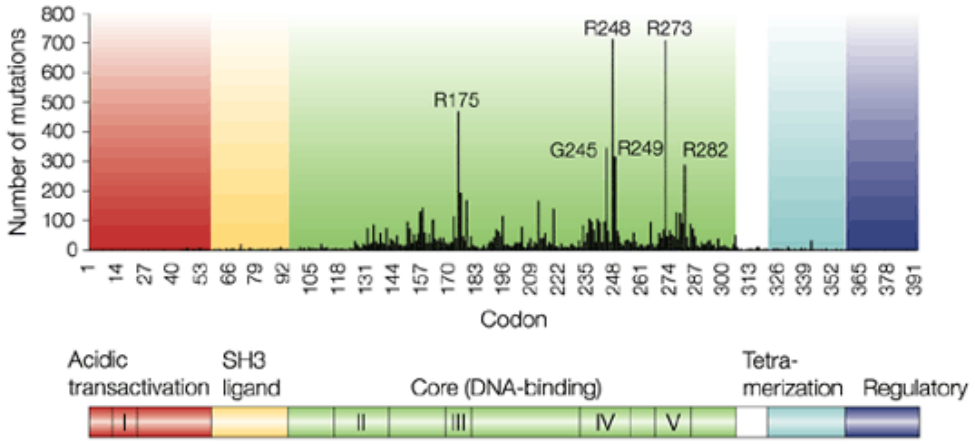

Figure 1.3 Frequency and distribution of TP53 mutations
The p53 protein comprises 393-residues that contains an NH-terminal transactivation domain, a proline-rich SH3 ligand, a core DNA-binding domain, a tetramerization domain and a carboxy-terminal regulatory domain[28]. A histogram of p53 missense mutations identifies the frequency of each mutation[29].

As mentioned previously, p53 function in cancers can be inactivated due to mutations within the TP53 gene. Mutations have been found in almost every region of the p53 protein, however, approximately 95% of the mutations identified occur in the sequence specific DNA binding domain (amino acids 100 to 300) (Figure 1.3[29]). Most frequently the tumor associated alterations in p53 result in missense mutations, which leads to the substitution of a single amino acid that can be stably expressed in the tumor cell[30]. Currently, the p53 field has identified six

key residues that are most commonly mutated and have been termed the "hot spot" mutations. These "hot spot" mutations − 175, 245, 248, 249, 273, and 282 − generally lead to a loss of function of the wild-type activity of p53 (Figure 1.3)[28]. In many tumor cells where p53 harbors a single amino acid substitution its ability to express p53 is retained, despite the mutation. These mutated p53 proteins are commonly more stable than wild-type p53 and are at higher concentrations in the tumor cells[13].

MDM2-p53 Interaction

In the remaining cancers that do not harbor mutated p53, p53 can be functionally inhibited by its primary cellular inhibitor, murine double minute 2 (MDM2). Initially the MDM2 oncogene was found overexpressed by amplification in a spontaneously transformed cell line (3T3DM)[31]. MDM2 contains a p53 binding domain at the N-terminus and a RING (really interesting new gene) domain at the C-terminus functioning as an E3 ubiquitin ligase, which targets both p53 and itself for degradation by the proteasome[32]. The first functional role attributed to MDM2 was its ability to inhibit p53 transcriptional activation[33]. A common feature of many tumors, specifically tumors with wild-type p53, is they exhibit amplification of the MDM2 gene or altered expression of the MDM2 protein[32]. These facts lead to the conclusion that the major oncogenic role of MDM2 is to block the transcriptional activity of p53.

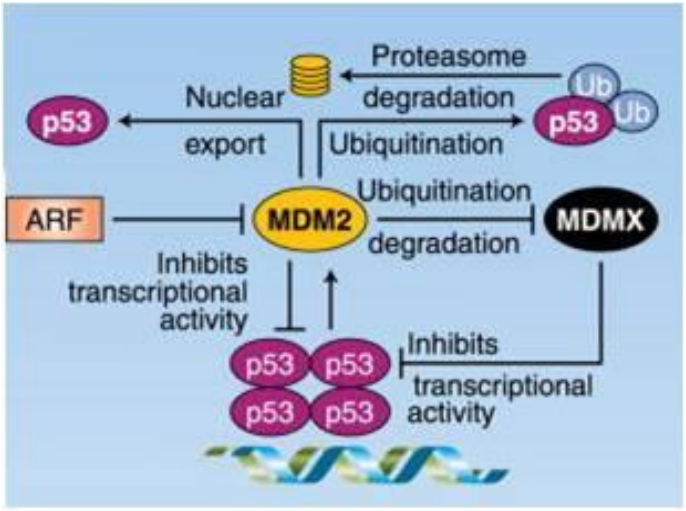

Figure 1.4 MDM2 and p53 auto-regulatory feedback loop

The MDM2-p53 interaction has been a highly investigated area in research and discovery of new cancer therapy. MDM2 and p53 form an auto-regulatory feedback loop in which the two proteins mutually control each other's cellular level (Figure 1.4[34]). When the level of MDM2 increases, it binds to and inactivates p53 by blocking its transactivation domain and by targeting the protein for ubiquitin dependent degradation by the proteasome[33]. MDM2 binding to p53 also induces the export of p53 out of the nucleus which inhibits p53's ability to access target genes[12]. The importance of MDM2 in the control of the activity of p53 was demonstrated when mice lacking MDM2 died as embryos, but additional deletion of TP53 rescued them from death[35]. MDMX, a homolog of MDM2, can also directly bind to the transactivation domain of p53 and inhibit the activity of p53, however, MDMX does not induce degradation of p53. The tumor suppressor ARF accumulates in the nucleus where it forms stable complexes with MDM2 and sequesters it into the nucleolus, thereby preventing MDM2 from inhibiting p53 activity[36].

MDM2 is clearly an important modulator of various pathways in the oncogenic process. The release of p53 from MDM2 leading to cell cycle arrest and apoptosis has emerged as an important interaction for the treatment and prevention of cancer. High resolution crystal

structures of the NH_2 terminus of MDM2 complexed with short p53 peptides provided necessary information about the interaction[37]. The structures show that the MDM2-p53 interaction involves a hydrophobic pocket in MDM2 with four key hydrophobic residues in p53, which include Phe19, Leu22, Trp23 and Leu26[34]. The identification of the well-defined pocket and key interactions involved in the binding of MDM2 to p53 provided the basis for the design of non-peptide, drug-like small molecule inhibitors of the MDM2-p53 interaction to reactivate p53.

The Nutlins were the first class of bona fide, potent, specific and orally bioavailable small molecule MDM2 inhibitors[38]. The Nutlins act by mimicking p53 and binding in the p53 binding pocket of MDM2, leading to the activation of cell cycle arrest, apoptosis and senescence in cancers with wild-type p53[39]. A derivative of Nutlin-3 with improved binding affinity to MDM2 and pharmacokinetics, RG7112, was advanced into clinical trials[40,41]. RG7112 was well tolerated in patients with liposarcoma, however, some patients experienced hematological toxicity[42]. RG7112 is currently in Phase I clinical trials as a single agent for patients with advanced solid tumors or leukemia and in combination in patients with AML (http://clinicaltrials.gov).

The MI compounds are a spiro-oxindole class of compounds designed by our laboratory. MI-219 binds to MDM2 with high affinity and specificity, particularly over MDMX[43]. Chemical modifications of MI-219 lead to the discovery of MI-77301 (SAR405838) which exhibits improved binding affinity to MDM2, cellular activity and pharmacokinetics[44]. The MDM2 inhibitors are able to induce both cell cycle arrest and cell death in tumor cells. Studies using Nutlin-3, RG7112, MI-219 and MI-77301 (SAR405838) show that these inhibitors selectively inhibit cell growth and induce cell cycle arrest in p53 wild-type tumor cell lines[38,40,43,44].

The effect of MDM2-p53 interaction inhibitors on normal tissue is of great importance when evaluating these inhibitors from a therapeutic standpoint. Preclinical studies in mice have

shown good tolerability of the Nutlin and the MI compounds with no sign of toxicity[38,43,44]. In normal cells, the MDM2 inhibitors activate p53 and induce cell cycle arrest but not cell death[38,43]. Radiation and chemotherapy treatment of mice induces profound apoptosis in small intestine crypts and the thymus, tissues that are highly susceptible to p53-induced apoptosis[43]. MI-219 treatment activates p53 with minimal p53 accumulation and does not result in apoptosis in normal tissues[43]. The exact mechanism for the lack of toxicity in normal tissues post MDM2 inhibitor treatment is yet to be determined.

Bcl-2 Family

Although p53 plays a prominent role in apoptosis induction, the Bcl-2 family are the master regulators of apoptosis at the mitochondria[14,45,46]. The proteins in the Bcl-2 family regulate apoptosis in response to physiological prompts and cytotoxic agents[45]. While normal cells have low expression levels of the antiapoptotic Bcl-2 and Bcl-xL proteins, these proteins are highly overexpressed in many different types of human tumors. Overexpression of Bcl-2 antiapoptotic proteins have been linked to poor prognosis in several types of cancer as well as clinical resistance to current cancer treatments[47]. Laboratory studies and clinical observations have established that overexpression of Bcl-2 or Bcl-xL causes cancer cells to become more resistant to chemotherapeutic agents, thus the development of agents that can inhibit the Bcl-2 family of proteins demonstrates promise in the treatment of cancer[48].

As mentioned above, apoptosis occurs through two distinct mechanisms, the extrinsic and intrinsic pathways. The extrinsic apoptotic pathway is initiated by ligation of the tumor necrosis factor family leading to downstream activation of caspase-8, which in turn activates effector caspases such as caspase-3[49]. The intrinsic pathway is regulated by the Bcl-2 family of proteins,

which controls whether a cell lives or commits to death through the mitochondrial apoptotic pathway[50]. The Bcl-2 family can be divided into three groups based on sequence homology and function: first, the proapoptotic Bcl-2 homology domain (BH3)-only proteins (such as BID, BIM, PUMA, NOXA, and BAD), second, the multi-domain proapoptotic proteins (BAX and BAK) and lastly, the antiapoptotic proteins (Bcl-2, Bcl-xL, Bcl-w, Mcl-1 and A1) (Figure 1.5)[51]. The initiation of apoptosis requires both proapoptotic proteins and the related BH3 protein interaction domain. Death signals result in the BH3-only proteins switching off survival function by inserting their BH3 domain into a hydrophobic pocket on their antiapoptotic relatives [52]. More simply, when the amount of proapoptotic proteins surmounts the binding ability of antiapoptotic proteins, apoptosis progresses (Figure 1.6).

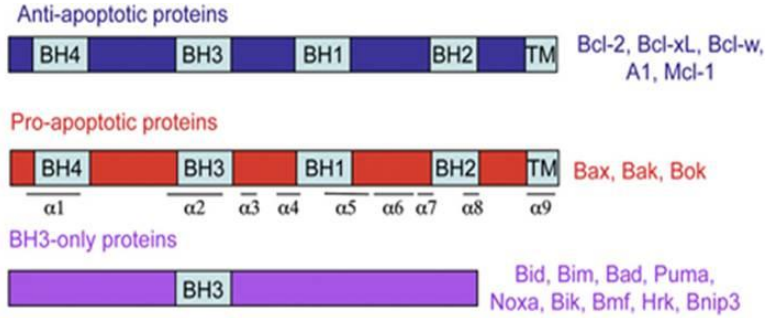

Figure 1.5 Structure of the Bcl-2 family of proteins
The Bcl-2 family of proteins is divided into three groups based on their Bcl-2 homology (BH) domains. Proapoptotic and antiapoptotic proteins have 4 BH domains and the BH3-only proteins, contain only the BH3 domain[53].

The BH3 domain and the presence of either BAX or BAK are necessary for the death function of the BH3-only proteins[54]. The targeted deletions of BAX or BAK produced mice with a relatively mild phenotype[55]. However, the deletion of both BAX and BAK resulted in mice with a large number of defects. The essential roles of BAX and BAK in apoptosis were further emphasized in double knockout mouse embryonic fibroblast cells where the cells were resistant to a myriad of apoptotic stimuli[56]. Once BAX and/or BAK are free from their anti-apoptotic

family members they homo-oligomerize and permeabilize the mitochondrian, a process termed mitochondrial outer membrane permeabilization (MOMP), releasing factors such as cytochrome c and SMAC promoting activation of the caspases resulting in cell death (Figure 1.6[2])[46,53].

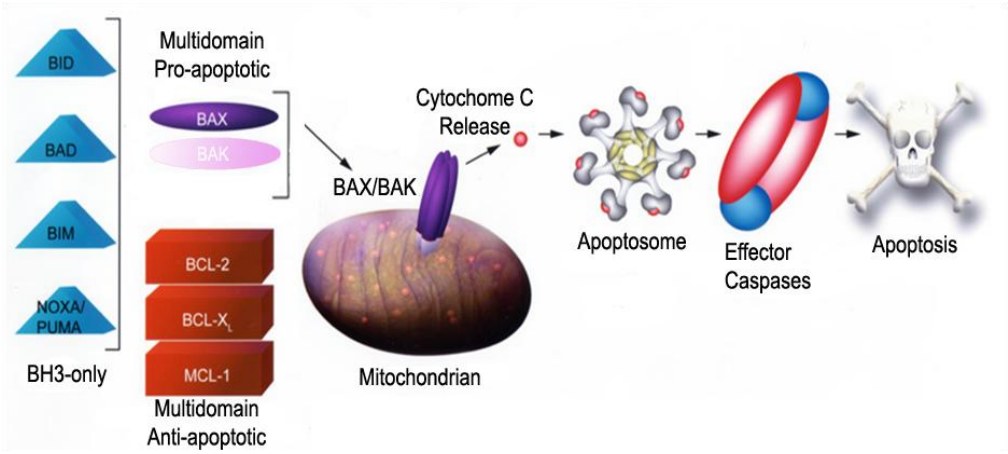

Figure 1.6 Bcl-2 protein family: key regulators of apoptosis

Over the last decade, BAX and BAK have been classified as homologs or functionally redundant. However, there has been more recent evidence suggesting that BAX and BAK play different roles in apoptosis in various cell types. In HCT116 human colon carcinoma cells, BAK is expendable for apoptosis induced by stimuli such as Bcl-2/Bcl-xL small molecule ABT-737, but not for fluorouracil-induced apoptosis[57]. In both a leukemia and lymphoma model, the knockout of BAX conferred resistance to ABT-737[58]. However, the loss of BAK or BAX has yet to be identified as a consequence of acquired drug resistance to apoptotic stimuli.

Evidence that further supports the potentially different roles of BAX and BAK are their different locations in the cell. Upon apoptotic stimuli, BAX is primarily cytosolic and translocates to the mitochondrial outer membrane where it oligomerizes and inserts[53]. Conversely, BAK is primarily located in the cytosol where it inserts into the mitochondrial outer membrane by a C-terminal transmembrane domain[59]. Controversy as to how BAX and BAK are activated leading to cell death is another area suggesting that these antiapoptotic proteins play

different roles in apoptosis. It remains to be determined whether the BH3-only protein PUMA (p53 up-regulated modulator of apoptosis) can promote ligand-induced activation of BAX. However, Gallenne *et al*. suggested that PUMA is competent to trigger BAX activity by itself[60]. In healthy cells, BAK associates with Mcl-1 and Bcl-xL, but not with other antiapoptotic members such as Bcl-2 and A1. Thus, BAK is held in check mainly by Mcl-1 and Bcl-xL and only induces apoptosis if freed from both[61].

The balance between the pro- and antiapoptotic proteins in a cancer cell plays a huge role in whether a cell will commit to death or not. For example, a cell may express a large amount of antiapoptotic proteins that are available to bind and sequester their proapoptotic family members and therefore be protected from extreme levels of pro-death signaling, such as signals that would result from chemotherapy treatment. Conversely, a cancer cell can have a very small amount of antiapoptotic proteins leaving it ill-equipped to defend itself against stress. The cells with high levels of antiapoptotic proteins are 'unprimed' for death, whereas cells with low levels of antiapoptotic proteins are 'primed' for death[62]. Assays can be employed to measure whether a cancer cell is primed for death and thus how readily it will undergo apoptosis[63]. The ability to profile cancer cells has allowed for the determination of how tumor cells will respond to cytotoxic chemotherapy[64]. Furthermore, profiling cancer cells has helped shed light on cancer cells that will readily undergo apoptosis when exposed to BH3 mimetics[65].

Targeting the Bcl-2 family of proteins has proven to be a promising approach to the treatment of cancers with over-expression of Bcl-2. The BH3 domain has been extensively studied including extensive crystallographic studies[52]. These studies provided the building blocks for the development of BH3 mimetics. BH3 mimetics are small molecules designed to mimic BH3 proteins by binding and sequestering Bcl-2 anti-apoptotic family members. Several

natural product derivatives and rationally designed compounds have been developed as BH3 mimetics, including ABT-737 and its oral analog ABT-263 (navitolax), ABT-199 and gossypol (AT-101)[66-69].

AT-101 (gossypol) is a natural product derived from cotton seed, stem and root that is an oral pan-Bcl-2 inhibitor[70]. AT-101 binds with submicromolar affinity to Bcl-2, Bcl-xL and Mcl-1[71]. The drug works by inducing apoptosis by acting as a BH3 mimetic and as a p53 independent activator of NOXA and PUMA[72,73]. The cytotoxicity of AT-101 may also be attributed to DNA cleavage and/or the generation of reactive oxygen species[73]. The phase I trials were promising; AT-101 was well tolerated in patients and demonstrated cytoreductive activities in several malignancies[69]. Unfortunately, phase II trials in prostate and lung cancers, both in single agent and combination treatment regimens failed to be clinically efficacious[69].

Highly potent small-molecule inhibitors of Bcl-2/Bcl-xL have been designed including, ABT-737 and ABT-263 from Abbott Laboratories[66,67]. ABT-737 and ABT-263 have similar binding profiles, both of which bind with high affinity (K_i < 1 nM) to Bcl-2, Bcl-xL and Bcl-w. Consistent with its chemical binding profile to these Bcl-2 proteins, ABT-737/ABT-263 effectively induces apoptosis in cancer cell lines with high levels of Bcl-2/Bcl-xL both *in vitro* and *in vivo*[67]. ABT-737/ABT-263 acts by binding to anti-apoptotic family members such as Bcl-2, Bcl-w and Bcl-xL, but does not bind members such as Mcl-1 and A1. The antagonization of these anti-apoptotic family members leads to the downstream activation of pro-apoptotic family members, such as BAX and BAK. The efficacy of ABT-737/263 in inducing apoptosis is cell specific[74]. In various *in vivo* models of small cell lung carcinomas (SCLCs), lymphoma, and myeloid leukemias, ABT-737/ABT-263 has been effective in causing tumor regression[67]. ABT-263 clinical trials have been and are currently being conducted in patients with chronic

lymphocytic leukemia (CLL)[75], lymphoma[76] and lung cancer[77]. Partial response was observed in patients with relapsed or refractory CLL, however, ABT-263 had minimal success as a single agent in treatment of lung cancer[77]. Thrombocytopenia was observed in the clinical trials as the dose-limiting toxicity, because ABT-263 targets Bcl-xL which is critical for platelet survival[78,79]. Luckily, the platelet decrease was transient and reversible[75-77,80].

Many studies have investigated Bcl-2/Bcl-xL inhibitors in combination with conventional chemotherapy agents and targeted therapies. Potent synergistic responses have been observed between ABT-737 and dexamethasone in multiple myeloma patient samples[81]. Concurrent use of the S-phase blocker, zoledronic acid, with ABT-737 induces synergistic cell death in cholangiocarcinoma cell lines[82]. Both ABT-737 and ABT-263 significantly enhance the activity of chemotherapy agents[83,84]. Also, ABT-263 has been shown to increase the efficacy of the kinesin-5 inhibitor, paclitaxel, and the MAP kinase (MEK) inhibitor, G-963[85,86]. Currently, several phase II clinical trials are investigating the combination of ABT-263 in lymphoid cancers and solid tumors (http://clinicaltrials.gov).

In order to resolve the dose-limiting toxicity of ABT-263, ABT-199 (navitoclax) was designed to selectively target Bcl-2. ABT-199 potently inhibits Bcl-2 function and inhibits growth of Bcl-2 dependent tumors *in vivo* and spares human platelets. In three patients with refractory CLL a single dose of ABT-199 resulted in tumor lysis within 24 hours[68]. However, on target tumor lysis syndrome (TLS) has been observed in a subset of patients treated with ABT-199 and thus dosing regiments must be tailored to mitigate this risk[87]. Despite the dose-limiting toxicity of ABT-199, the drug has been successful in phase I trials in patients with refractory or relapsed CLL or non-Hodgkin lymphoma[68]. Phase II clinical trials are ongoing to investigate combination treatment with ABT-199 (http://clinicaltrials.gov).

Numerous *in vitro* studies have investigated the role of the Bcl-2 family in resistance to Bcl-2 family inhibitors. Multiple studies performed with ABT-737/ABT-263 acquired resistant cell lines show increased levels of anti-apoptotic proteins Mcl-1 and Bfl-1/A1, which are not targeted by ABT-737[88-91]. Acute myeloid leukemia cell lines with high expression of Mcl-1 are resistant to ABT-737[88]. Investigations performed with lymphoma cell lines with acquired resistance to ABT-737 also observed an upregulation of Mcl-1 and Bfl-1/A1[89]. All of the *in vitro* studies have lead to similar findings: the upregulation of Mcl-1 confers resistance to Bcl-2/Bcl-xL inhibitors. As with any *in vitro* study there is always the same question: how does this correlate to whole living organisms? Is there a different phenotype for resistance to Bcl-2/Bcl-xL inhibitors *in vivo*?

Other Mechanisms of Cancer Drug Resistance

Drug resistance due to defects and/or inhibition of the apoptosis pathway has been discussed so far; however it is important to discuss other mechanisms of resistance. One of the more common mechanisms of resistance to a broad range of anticancer drugs is what is known as multidrug resistance. Multidrug resistance is simultaneous resistance to several structurally unrelated drugs that do not share a common mechanism of action. Different types of cellular multidrug resistance have been described; however the general mechanism involves expression of ATP-dependent efflux pumps[92]. The transporters known as ATP-binding cassette (ABC) transporters are able to efflux drugs out of the tumor cell or reduce drug uptake into the cell. The protein encoded by the multidrug resistant (MDR1) gene is P-glycoprotein (Pgp), which is one of the most thoroughly studied ABC proteins[93,94]. MDR1/P-gp (ABCB1) functions as an energy dependent plasma membrane efflux pump that actively transports drugs out of cells. Cancers

such as acute myeloid leukemia (AML), breast cancer and small-cell lung cancer show higher expression levels of MDR/P-gp after chemotherapy[95]. Not all multidrug resistant cells express MDR1/P-gp, which lead to the discovery of the multidrug resistant associated protein 1 (MRP1 or ABCC1)[96]. The discovery of MRP1 instigated the search for homologs, which lead to the discovery of eight additional members of the ABCC subfamily of transporters[97]. Overexpression of MRP1 in tumor cells confers resistance to drugs such as anthracyclines, vinca alkaloids, and methotrexate[98]. Studies in human cancer cell lines selected for mitoxantrone resistance resulted in the discovery of another member of the ABC transporters[99,100]. This transporter (ABCG2) is known as the breast cancer resistance protein (BCRP). Multidrug resistance remains a challenge to successful treatment of cancer.

Drug inactivation is yet another complex mechanism employed in living systems that results in drug resistance. Interactions between a drug and different proteins *in vivo* can modify, partially degrade, or complex the drug with other molecules or proteins leading to drug activation. Cancer cells can develop resistance by decreasing drug activation due to the fact that several cancer therapies must undergo metabolic activation in order to achieve clinical efficacy[101,102]. Examples of drug activation and inactivation include the cytochrome P450 (CYP) system, glutathione-*S*-transferase (GST) superfamily and uridine diphospho-glucuronosyltransferase (UGT) superfamily[1,103]. An example of where decreased drug activation results in resistance, is observed in the treatment of acute myeloid leukemia with cytarabine (AraC), a nucleoside drug[104]. Activation of AraC requires several phosphorylation events that convert the drug to AraC-triphosphate. Drug resistance to AraC results when down regulation or mutation in the metabolic pathway, specifically deoxycitidine kinase, develops[104,105].

For several years, DNA damaging agents were highly effective drugs for the treatment of cancer[106]. However, the repair of damaged DNA has been found to play a prominent role in anticancer drug resistance. The DNA damage response mechanism can reverse the drug induced damage resulting from treatment with drugs that directly or indirectly damage DNA. An example of this phenomenon has been observed in resistance that results from use of cisplatin, a platinum containing drug resulting in harmful DNA crosslinks leading to apoptosis[107]. Several resistance mechanisms to platinum containing drugs have been identified including inactivation by glutathione and other thiol containing proteins as well as increased DNA repair[108]. Nucleotide excision repair, a key player in the development of resistance to platinum-based drugs, is mainly responsible for repairing bulky DNA adducts such as those resulting from interaction with cisplatin[109]. Homologous recombination is another form of DNA repair associated with reversing platinum-based drug damage[109,110]. Taken together, the efficacy of platinum-based drugs depends on the cancer cells ability to evade activation of DNA damage response mechanisms.

Although genetic alterations in tumor cells play a key role in tumor development and resistance mechanisms, the microenvironment including the extracellular matrix and tumor vasculature hold an integral part in the progression of the disease and the development of resistance[3,111-114]. Examples of resistant mechanisms involving the tumor microenvironment include cell-adhesion mediated resistance and the secretion of soluble growth factors or cytokines[3,115]. Cell-adhesion mediated resistance has resulted in response to many therapeutics, including chemotherapy and targeted therapies[116,117]. Stromal derived hepatocyte growth factor (HGF) has been shown to result in resistance to BRAF inhibition in BRAF mutant melanoma cells by increased phosphorylation of the HGF receptor MET[118]. Patients with increased

expression of HGF exhibited reduced response to BRAF inhibitor treatment[118]. These studies further support that the interaction between a tumor cell and its microenvironment is important to determine mechanisms of resistance to drug therapies. Unfortunately, resistance mechanisms to cancer drugs are commonly investigated utilizing cell lines that develop resistance *in vitro* absent from environmental factors. Thus, *in vitro* development of drug resistance may not be an accurate representation of the resistance mechanisms that will arise in a clinical setting. Furthermore, drug exposure to cancer cells in *in vitro* conditions does not account for the pharmacokinetics involved in drug treatment in a whole animal[119,120]. Taken together, it is necessary to investigate mechanisms of resistance that develop *in vivo* in order to more accurately understand what will result in human patients.

Treatment Strategies

A major shift has begun in the treatment of cancer from a standardized regiment of chemotherapy drugs for specific cancers to one where the underlying molecular mechanism and oncogenic target of individual tumors are considered in order to best treat patients. The number and diversity of cancer drug resistance mechanisms highlights the need for rational treatment strategies aimed at avoiding and overcoming drug resistance. Combination and sequential use of therapies has been stated as the best treatment options to overcome cancer drug resistance[121-124]. Combination treatment strategies should be more effective than single agent treatment because targeting multiple pathways at once can lead to a synergistic cell death response and certain drugs may sensitize drug resistant cancer cells to other drugs[125-128]. For example, pretreatment with histone deacetylase and methylation inhibitors in cisplatin resistant ovarian cancer cells re-sensitized these cells to cell death by cisplatin[125]. Gefitinib, a BCRP inhibitor, reversed resistance

to anticancer agents in breast cancer cells by inhibiting drug efflux, subsequently the combination of gefitinib and irenotecan resulted in increased survival *in vivo* compared to single agent treatment[129]. In a study performed with p53 mutant pancreatic tumor cells the combination of AZD7762 (checkpoint kinase 1 inhibitor) and olaparib (PARP1 inhibitor) sensitized the cells to radiation treatment[123]. These studies and several others emphasize the potential benefit of combination and/or sequential treatment regimens for treating resistant or relapsed cancers.

Combination and sequential treatment strategies also address the fact that cancer cell populations are heterogeneous. Recent studies show that a fraction of cells within the heterogeneous population possess drug resistance prior to treatment[130-133]. For example, a study in acute myeloid leukemia (AML) identified two clones, one sensitive to drug treatment and the other resistant to drug treatment[130]. By definition, drug treatment of cancers results in the death of drug sensitive cancer cells, thus the drug resistant fraction of cancer cells can survive and expand resulting in the relapse of cancer. This possibility could exist in all forms of cancer. However, it is also possible for cancers to be less heterogeneous than others. In a clonal composition study of breast cancer both monogenomic and polygenomic tumors were identified[132]. The polygenomic tumors may contain different types of clonal subpopulations, all of which could have varying degrees of sensitivity or resistance to drug treatment.

Combination studies using MDM2-p53 interaction inhibitors and Bcl-2/Bcl-xL inhibitors has the added advantage of inducing p53 target genes leading to apoptosis and cell cycle arrest along with inhibition of highly expressed antiapoptotic Bcl-2 family members, Bcl-2 and Bcl-xL. Further inhibition of antiapoptotic Bcl-2 proteins can be achieved by successful inhibition of MDM2 resulting in activation of p53 and an increase in PUMA, which has been shown to antagonize Mcl-1[16]. Less frequent dosing and decreased drug dosage of the drugs could both

delay the onset of resistance and result in less toxicity in patients. In cancers where Bcl-2 is overexpressed and p53 wild-type status is retained, the targeted combination therapy of Bcl-2 and MDM2-p53 interaction inhibitors investigated in this dissertation constitute a mechanism-based therapy with considerable clinical potential. Overall, these studies suggest that combination and/or sequential treatment of cancers is an attractive treatment strategy and should be further investigated.

Aims

The treatment of cancer has improved greatly over the last several decades, mainly due to the development of highly efficacious chemotherapeutics and targeted therapies. However, the ability of cancer cells to develop resistance to drug treatment remains a significant impediment to successful cancer treatment. Numerous investigations have identified a myriad of mechanisms in which cancer cells can elude drug therapy, and it has become evident that resistance exists against every effective drug. The determination of the mechanisms of drug resistance can help elucidate valuable information about how to circumvent drug resistance in cancer cells. Targeting key apoptosis regulators such as the MDM2-p53 interaction and the commonly overexpressed Bcl-2 protein, is an exciting, new therapeutic strategy for reactivating apoptosis in cancer cells that have acquired resistance to cell death[45,134,135]. To date, non-peptide small molecule Bcl-2 inhibitors and MDM2-p53 interaction inhibitors have been developed and several such inhibitors have been advanced into clinical trials[75,136,137]. While such small-molecule drugs have shown promise in initial clinical trials, it is fully expected that cancer cells will acquire resistance to these novel anticancer agents[138].

The overall aims of my dissertation are as follows:

1. To investigate the mechanisms of resistance in cancer to MDM2-p53 interaction inhibitors and Bcl-2/Bcl-xL inhibitors both *in vitro* and *in vivo*.

2. To identify rationale treatment strategies aimed at overcoming and circumventing resistance in order to increase tumor-free survival.

3. To address differences in the development of resistance to MDM2-p53 interaction inhibitors and Bcl-2/Bcl-xL inhibitors both *in vitro* and *in vivo*.

References

1. Housman, G. *et al.* Drug resistance in cancer: an overview. *Cancers (Basel)* **6**, 1769-92 (2014).
2. DeVita, V.T., Hellman, S. & Rosenberg, S.A. *Cancer, principles & practice of oncology*, lxxv, 2898 p. (Lippincott Williams & Wilkins, Philadelphia, PA, 2005).
3. Ramos, P. & Bentires-Alj, M. Mechanism-based cancer therapy: resistance to therapy, therapy for resistance. *Oncogene* (2014).
4. Lane, D.P. & Crawford, L.V. T antigen is bound to a host protein in SV40-transformed cells. *Nature* **278**, 261-3 (1979).
5. Vogelstein, B., Lane, D. & Levine, A.J. Surfing the p53 network. *Nature* **408**, 307-10 (2000).
6. Malkin, D. *et al.* Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms. *Science* **250**, 1233-8 (1990).
7. Donehower, L.A. *et al.* Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature* **356**, 215-21 (1992).
8. Hainaut, P. & Hollstein, M. p53 and human cancer: the first ten thousand mutations. *Adv Cancer Res* **77**, 81-137 (2000).
9. Chene, P. Inhibiting the p53-MDM2 interaction: an important target for cancer therapy. *Nat Rev Cancer* **3**, 102-9 (2003).
10. Laptenko, O. & Prives, C. Transcriptional regulation by p53: one protein, many possibilities. *Cell Death Differ* **13**, 951-61 (2006).
11. Wu, X., Bayle, J.H., Olson, D. & Levine, A.J. The p53-mdm-2 autoregulatory feedback loop. *Genes Dev* **7**, 1126-32 (1993).
12. Freedman, D.A., Wu, L. & Levine, A.J. Functions of the MDM2 oncoprotein. *Cell Mol Life Sci* **55**, 96-107 (1999).
13. Vousden, K.H. & Lu, X. Live or let die: the cell's response to p53. *Nat Rev Cancer* **2**, 594-604 (2002).
14. Green, D.R. Apoptotic pathways: the roads to ruin. *Cell* **94**, 695-8 (1998).
15. Amaral, J.D., Xavier, J.M., Steer, C.J. & Rodrigues, C.M. The role of p53 in apoptosis. *Discov Med* **9**, 145-52 (2010).
16. Shibue, T. *et al.* Differential contribution of Puma and Noxa in dual regulation of p53-mediated apoptotic pathways. *EMBO J* **25**, 4952-62 (2006).
17. Jeffers, J.R. *et al.* Puma is an essential mediator of p53-dependent and -independent apoptotic pathways. *Cancer Cell* **4**, 321-8 (2003).
18. Chen, L. *et al.* Differential targeting of prosurvival Bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function. *Mol Cell* **17**, 393-403 (2005).
19. Oda, E. *et al.* Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. *Science* **288**, 1053-8 (2000).
20. Sax, J.K. *et al.* BID regulation by p53 contributes to chemosensitivity. *Nat Cell Biol* **4**, 842-9 (2002).
21. Miyashita, T. *et al.* Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression in vitro and in vivo. *Oncogene* **9**, 1799-805 (1994).
22. Mihara, M. *et al.* p53 has a direct apoptogenic role at the mitochondria. *Mol Cell* **11**, 577-90 (2003).
23. Lane, D.P. Cancer. p53, guardian of the genome. *Nature* **358**, 15-6 (1992).
24. el-Deiry, W.S. *et al.* WAF1, a potential mediator of p53 tumor suppression. *Cell* **75**, 817-25 (1993).
25. Levine, A.J. p53, the cellular gatekeeper for growth and division. *Cell* **88**, 323-31 (1997).

26. Bates, S. & Vousden, K.H. p53 in signaling checkpoint arrest or apoptosis. *Curr Opin Genet Dev* **6**, 12-8 (1996).
27. Sax, J.K. & El-Deiry, W.S. p53 downstream targets and chemosensitivity. *Cell Death Differ* **10**, 413-7 (2003).
28. Joerger, A.C. & Fersht, A.R. Structural biology of the tumor suppressor p53. *Annu Rev Biochem* **77**, 557-82 (2008).
29. Bullock, A.N. & Fersht, A.R. Rescuing the function of mutant p53. *Nat Rev Cancer* **1**, 68-76 (2001).
30. Brosh, R. & Rotter, V. When mutants gain new powers: news from the mutant p53 field. *Nat Rev Cancer* **9**, 701-13 (2009).
31. Fakharzadeh, S.S., Trusko, S.P. & George, D.L. Tumorigenic potential associated with enhanced expression of a gene that is amplified in a mouse tumor cell line. *EMBO J* **10**, 1565-9 (1991).
32. Wade, M., Li, Y.C. & Wahl, G.M. MDM2, MDMX and p53 in oncogenesis and cancer therapy. *Nat Rev Cancer* **13**, 83-96 (2013).
33. Momand, J., Zambetti, G.P., Olson, D.C., George, D. & Levine, A.J. The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. *Cell* **69**, 1237-45 (1992).
34. Shangary, S. & Wang, S. Targeting the MDM2-p53 interaction for cancer therapy. *Clin Cancer Res* **14**, 5318-24 (2008).
35. Montes de Oca Luna, R., Wagner, D.S. & Lozano, G. Rescue of early embryonic lethality in mdm2-deficient mice by deletion of p53. *Nature* **378**, 203-6 (1995).
36. Weber, J.D., Taylor, L.J., Roussel, M.F., Sherr, C.J. & Bar-Sagi, D. Nucleolar Arf sequesters Mdm2 and activates p53. *Nat Cell Biol* **1**, 20-6 (1999).
37. Kussie, P.H. *et al.* Structure of the MDM2 oncoprotein bound to the p53 tumor suppressor transactivation domain. *Science* **274**, 948-53 (1996).
38. Vassilev, L.T. *et al.* In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. *Science* **303**, 844-8 (2004).
39. Tovar, C. *et al.* Small-molecule MDM2 antagonists reveal aberrant p53 signaling in cancer: implications for therapy. *Proc Natl Acad Sci U S A* **103**, 1888-93 (2006).
40. Tovar, C. *et al.* MDM2 small-molecule antagonist RG7112 activates p53 signaling and regresses human tumors in preclinical cancer models. *Cancer Res* **73**, 2587-97 (2013).
41. Vu, B. *et al.* Discovery of RG7112: A Small-Molecule MDM2 Inhibitor in Clinical Development. *ACS Med Chem Lett* **4**, 466-9 (2013).
42. Ray-Coquard, I. *et al.* Effect of the MDM2 antagonist RG7112 on the P53 pathway in patients with MDM2-amplified, well-differentiated or dedifferentiated liposarcoma: an exploratory proof-of-mechanism study. *Lancet Oncol* **13**, 1133-40 (2012).
43. Shangary, S. *et al.* Temporal activation of p53 by a specific MDM2 inhibitor is selectively toxic to tumors and leads to complete tumor growth inhibition. *Proc Natl Acad Sci U S A* **105**, 3933-8 (2008).
44. Wang, S. *et al.* SAR405838: An optimized inhibitor of MDM2-p53 interaction that induces complete and durable tumor regression. *Cancer Res* (2014).
45. Danial, N.N. & Korsmeyer, S.J. Cell death: critical control points. *Cell* **116**, 205-19 (2004).
46. Green, D.R. & Kroemer, G. The pathophysiology of mitochondrial cell death. *Science* **305**, 626-9 (2004).
47. Chao, D.T. & Korsmeyer, S.J. BCL-2 family: regulators of cell death. *Annu Rev Immunol* **16**, 395-419 (1998).
48. Reed, J.C. Bcl-2 family proteins: strategies for overcoming chemoresistance in cancer. *Adv Pharmacol* **41**, 501-32 (1997).

49. Letai, A.G. Diagnosing and exploiting cancer's addiction to blocks in apoptosis. *Nat Rev Cancer* **8**, 121-32 (2008).
50. Cory, S. & Adams, J.M. The Bcl2 family: regulators of the cellular life-or-death switch. *Nat Rev Cancer* **2**, 647-56 (2002).
51. Gross, A., McDonnell, J.M. & Korsmeyer, S.J. BCL-2 family members and the mitochondria in apoptosis. *Genes Dev* **13**, 1899-911 (1999).
52. Cragg, M.S., Harris, C., Strasser, A. & Scott, C.L. Unleashing the power of inhibitors of oncogenic kinases through BH3 mimetics. *Nat Rev Cancer* **9**, 321-6 (2009).
53. Martinou, J.C. & Youle, R.J. Mitochondria in apoptosis: Bcl-2 family members and mitochondrial dynamics. *Dev Cell* **21**, 92-101 (2011).
54. Cheng, E.H. *et al.* BCL-2, BCL-X(L) sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis. *Mol Cell* **8**, 705-11 (2001).
55. Knudson, C.M., Tung, K.S., Tourtellotte, W.G., Brown, G.A. & Korsmeyer, S.J. Bax-deficient mice with lymphoid hyperplasia and male germ cell death. *Science* **270**, 96-9 (1995).
56. Wei, M.C. *et al.* Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. *Science* **292**, 727-30 (2001).
57. Wang, C. & Youle, R.J. Predominant requirement of Bax for apoptosis in HCT116 cells is determined by Mcl-1's inhibitory effect on Bak. *Oncogene* (2011).
58. Kojima, K. *et al.* Decreased sensitivity of 17p-deleted chronic lymphocytic leukemia cells to a small molecule BCL-2 antagonist ABT-737. *Cancer* (2011).
59. Cheng, E.H., Sheiko, T.V., Fisher, J.K., Craigen, W.J. & Korsmeyer, S.J. VDAC2 inhibits BAK activation and mitochondrial apoptosis. *Science* **301**, 513-7 (2003).
60. Gallenne, T. *et al.* Bax activation by the BH3-only protein Puma promotes cell dependence on antiapoptotic Bcl-2 family members. *J Cell Biol* **185**, 279-90 (2009).
61. Willis, S.N. *et al.* Proapoptotic Bak is sequestered by Mcl-1 and Bcl-xL, but not Bcl-2, until displaced by BH3-only proteins. *Genes Dev* **19**, 1294-305 (2005).
62. Certo, M. *et al.* Mitochondria primed by death signals determine cellular addiction to antiapoptotic BCL-2 family members. *Cancer Cell* **9**, 351-65 (2006).
63. Ni Chonghaile, T. *et al.* Pretreatment mitochondrial priming correlates with clinical response to cytotoxic chemotherapy. *Science* **334**, 1129-33 (2011).
64. Sarosiek, K.A., Ni Chonghaile, T. & Letai, A. Mitochondria: gatekeepers of response to chemotherapy. *Trends Cell Biol* **23**, 612-9 (2013).
65. Ni Chonghaile, T. & Letai, A. Mimicking the BH3 domain to kill cancer cells. *Oncogene* **27 Suppl 1**, S149-57 (2008).
66. Tse, C. *et al.* ABT-263: a potent and orally bioavailable Bcl-2 family inhibitor. *Cancer Res* **68**, 3421-8 (2008).
67. Oltersdorf, T. *et al.* An inhibitor of Bcl-2 family proteins induces regression of solid tumours. *Nature* **435**, 677-81 (2005).
68. Souers, A.J. *et al.* ABT-199, a potent and selective BCL-2 inhibitor, achieves antitumor activity while sparing platelets. *Nat Med* **19**, 202-8 (2013).
69. Liu, G. *et al.* An open-label, multicenter, phase I/II study of single-agent AT-101 in men with castrate-resistant prostate cancer. *Clin Cancer Res* **15**, 3172-6 (2009).
70. Wu, D. An overview of the clinical pharmacology and therapeutic potential of gossypol as a male contraceptive agent and in gynaecological disease. *Drugs* **38**, 333-41 (1989).
71. Kitada, S. *et al.* Discovery, characterization, and structure-activity relationships studies of proapoptotic polyphenols targeting B-cell lymphocyte/leukemia-2 proteins. *J Med Chem* **46**, 4259-64 (2003).

72. Meng, Y. *et al.* Natural BH3 mimetic (-)-gossypol chemosensitizes human prostate cancer via Bcl-xL inhibition accompanied by increase of Puma and Noxa. *Mol Cancer Ther* **7**, 2192-202 (2008).
73. Balakrishnan, K., Wierda, W.G., Keating, M.J. & Gandhi, V. Gossypol, a BH3 mimetic, induces apoptosis in chronic lymphocytic leukemia cells. *Blood* **112**, 1971-80 (2008).
74. Tahir, S.K. *et al.* Influence of Bcl-2 family members on the cellular response of small-cell lung cancer cell lines to ABT-737. *Cancer Res* **67**, 1176-83 (2007).
75. Roberts, A.W. *et al.* Substantial susceptibility of chronic lymphocytic leukemia to BCL2 inhibition: results of a phase I study of navitoclax in patients with relapsed or refractory disease. *J Clin Oncol* **30**, 488-96 (2012).
76. Wilson, W.H. *et al.* Navitoclax, a targeted high-affinity inhibitor of BCL-2, in lymphoid malignancies: a phase 1 dose-escalation study of safety, pharmacokinetics, pharmacodynamics, and antitumour activity. *Lancet Oncol* **11**, 1149-59 (2010).
77. Gandhi, L. *et al.* Phase I study of Navitoclax (ABT-263), a novel Bcl-2 family inhibitor, in patients with small-cell lung cancer and other solid tumors. *J Clin Oncol* **29**, 909-16 (2011).
78. Schoenwaelder, S.M. *et al.* Bcl-xL-inhibitory BH3 mimetics can induce a transient thrombocytopathy that undermines the hemostatic function of platelets. *Blood* **118**, 1663-74 (2011).
79. Zhang, H. *et al.* Bcl-2 family proteins are essential for platelet survival. *Cell Death Differ* **14**, 943-51 (2007).
80. Rudin, C.M. *et al.* Phase II study of single-agent navitoclax (ABT-263) and biomarker correlates in patients with relapsed small cell lung cancer. *Clin Cancer Res* **18**, 3163-9 (2012).
81. Trudel, S. *et al.* The Bcl-2 family protein inhibitor, ABT-737, has substantial antimyeloma activity and shows synergistic effect with dexamethasone and melphalan. *Clin Cancer Res* **13**, 621-9 (2007).
82. Romani, A.A. *et al.* The BH3-mimetic ABT-737 targets the apoptotic machinery in cholangiocarcinoma cell lines resulting in synergistic interactions with zoledronic acid. *Cancer Chemother Pharmacol* **67**, 557-67 (2011).
83. Kang, M.H. *et al.* Activity of vincristine, L-ASP, and dexamethasone against acute lymphoblastic leukemia is enhanced by the BH3-mimetic ABT-737 in vitro and in vivo. *Blood* **110**, 2057-66 (2007).
84. Ackler, S. *et al.* The Bcl-2 inhibitor ABT-263 enhances the response of multiple chemotherapeutic regimens in hematologic tumors in vivo. *Cancer Chemother Pharmacol* **66**, 869-80 (2010).
85. Shi, J., Zhou, Y., Huang, H.C. & Mitchison, T.J. Navitoclax (ABT-263) accelerates apoptosis during drug-induced mitotic arrest by antagonizing Bcl-xL. *Cancer Res* **71**, 4518-26 (2011).
86. Tan, N. *et al.* Bcl-2/Bcl-xL inhibition increases the efficacy of MEK inhibition alone and in combination with PI3 kinase inhibition in lung and pancreatic tumor models. *Mol Cancer Ther* **12**, 853-64 (2013).
87. Ng, S.Y. & Davids, M.S. Selective Bcl-2 inhibition to treat chronic lymphocytic leukemia and non-Hodgkin lymphoma. *Clin Adv Hematol Oncol* **12**, 224-9 (2014).
88. Konopleva, M. *et al.* Mechanisms of apoptosis sensitivity and resistance to the BH3 mimetic ABT-737 in acute myeloid leukemia. *Cancer Cell* **10**, 375-88 (2006).
89. Yecies, D., Carlson, N.E., Deng, J. & Letai, A. Acquired resistance to ABT-737 in lymphoma cells that up-regulate MCL-1 and BFL-1. *Blood* **115**, 3304-13 (2010).
90. Deng, J. *et al.* BH3 profiling identifies three distinct classes of apoptotic blocks to predict response to ABT-737 and conventional chemotherapeutic agents. *Cancer Cell* **12**, 171-85 (2007).
91. van Delft, M.F. *et al.* The BH3 mimetic ABT-737 targets selective Bcl-2 proteins and efficiently induces apoptosis via Bak/Bax if Mcl-1 is neutralized. *Cancer Cell* **10**, 389-99 (2006).

92. Gottesman, M.M., Fojo, T. & Bates, S.E. Multidrug resistance in cancer: role of ATP-dependent transporters. *Nat Rev Cancer* **2**, 48-58 (2002).
93. Ueda, K., Cardarelli, C., Gottesman, M.M. & Pastan, I. Expression of a full-length cDNA for the human "MDR1" gene confers resistance to colchicine, doxorubicin, and vinblastine. *Proc Natl Acad Sci U S A* **84**, 3004-8 (1987).
94. Fojo, A.T. *et al.* Expression of a multidrug-resistance gene in human tumors and tissues. *Proc Natl Acad Sci U S A* **84**, 265-9 (1987).
95. Shaffer, B.C. *et al.* Drug resistance: still a daunting challenge to the successful treatment of AML. *Drug Resist Updat* **15**, 62-9 (2012).
96. Cole, S.P. *et al.* Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. *Science* **258**, 1650-4 (1992).
97. Borst, P., Evers, R., Kool, M. & Wijnholds, J. A family of drug transporters: the multidrug resistance-associated proteins. *J Natl Cancer Inst* **92**, 1295-302 (2000).
98. Gillet, J.P., Efferth, T. & Remacle, J. Chemotherapy-induced resistance by ATP-binding cassette transporter genes. *Biochim Biophys Acta* **1775**, 237-62 (2007).
99. Miyake, K. *et al.* Molecular cloning of cDNAs which are highly overexpressed in mitoxantrone-resistant cells: demonstration of homology to ABC transport genes. *Cancer Res* **59**, 8-13 (1999).
100. Doyle, L.A. *et al.* A multidrug resistance transporter from human MCF-7 breast cancer cells. *Proc Natl Acad Sci U S A* **95**, 15665-70 (1998).
101. Rochat, B. Importance of influx and efflux systems and xenobiotic metabolizing enzymes in intratumoral disposition of anticancer agents. *Curr Cancer Drug Targets* **9**, 652-74 (2009).
102. Zahreddine, H. & Borden, K.L. Mechanisms and insights into drug resistance in cancer. *Front Pharmacol* **4**, 28 (2013).
103. Michael, M. & Doherty, M.M. Tumoral drug metabolism: overview and its implications for cancer therapy. *J Clin Oncol* **23**, 205-29 (2005).
104. Sampath, D. *et al.* Pharmacodynamics of cytarabine alone and in combination with 7-hydroxystaurosporine (UCN-01) in AML blasts in vitro and during a clinical trial. *Blood* **107**, 2517-24 (2006).
105. Cai, J. *et al.* Two distinct molecular mechanisms underlying cytarabine resistance in human leukemic cells. *Cancer Res* **68**, 2349-57 (2008).
106. Gurova, K. New hopes from old drugs: revisiting DNA-binding small molecules as anticancer agents. *Future Oncol* **5**, 1685-704 (2009).
107. Chu, G. Cellular responses to cisplatin. The roles of DNA-binding proteins and DNA repair. *J Biol Chem* **269**, 787-90 (1994).
108. Galluzzi, L. *et al.* Molecular mechanisms of cisplatin resistance. *Oncogene* **31**, 1869-83 (2012).
109. Bonanno, L., Favaretto, A. & Rosell, R. Platinum drugs and DNA repair mechanisms in lung cancer. *Anticancer Res* **34**, 493-501 (2014).
110. Olaussen, K.A. *et al.* DNA repair by ERCC1 in non-small-cell lung cancer and cisplatin-based adjuvant chemotherapy. *N Engl J Med* **355**, 983-91 (2006).
111. Meads, M.B., Gatenby, R.A. & Dalton, W.S. Environment-mediated drug resistance: a major contributor to minimal residual disease. *Nat Rev Cancer* **9**, 665-74 (2009).
112. Junttila, M.R. & de Sauvage, F.J. Influence of tumour micro-environment heterogeneity on therapeutic response. *Nature* **501**, 346-54 (2013).
113. Joyce, J.A. & Pollard, J.W. Microenvironmental regulation of metastasis. *Nat Rev Cancer* **9**, 239-52 (2009).
114. Teicher, B.A. *et al.* Tumor resistance to alkylating agents conferred by mechanisms operative only in vivo. *Science* **247**, 1457-61 (1990).

115. Wilson, T.R. *et al.* Widespread potential for growth-factor-driven resistance to anticancer kinase inhibitors. *Nature* **487**, 505-9 (2012).
116. Huang, C. *et al.* beta1 integrin mediates an alternative survival pathway in breast cancer cells resistant to lapatinib. *Breast Cancer Res* **13**, R84 (2011).
117. Muranen, T. *et al.* Inhibition of PI3K/mTOR leads to adaptive resistance in matrix-attached cancer cells. *Cancer Cell* **21**, 227-39 (2012).
118. Straussman, R. *et al.* Tumour micro-environment elicits innate resistance to RAF inhibitors through HGF secretion. *Nature* **487**, 500-4 (2012).
119. Goodman, L.S., Gilman, A., Brunton, L.L., Lazo, J.S. & Parker, K.L. *Goodman & Gilman's the pharmacological basis of therapeutics*, xxiii, 2021 p. (McGraw-Hill, New York, 2006).
120. Peterson, J.K. & Houghton, P.J. Integrating pharmacology and in vivo cancer models in preclinical and clinical drug development. *Eur J Cancer* **40**, 837-44 (2004).
121. Byler, S. *et al.* Genetic and epigenetic aspects of breast cancer progression and therapy. *Anticancer Res* **34**, 1071-7 (2014).
122. Sarkar, S., Goldgar, S., Byler, S., Rosenthal, S. & Heerboth, S. Demethylation and re-expression of epigenetically silenced tumor suppressor genes: sensitization of cancer cells by combination therapy. *Epigenomics* **5**, 87-94 (2013).
123. Vance, S. *et al.* Selective radiosensitization of p53 mutant pancreatic cancer cells by combined inhibition of Chk1 and PARP1. *Cell Cycle* **10**, 4321-9 (2011).
124. Shi, H., Kong, X., Ribas, A. & Lo, R.S. Combinatorial treatments that overcome PDGFRbeta-driven resistance of melanoma cells to V600EB-RAF inhibition. *Cancer Res* **71**, 5067-74 (2011).
125. Cacan, E., Ali, M.W., Boyd, N.H., Hooks, S.B. & Greer, S.F. Inhibition of HDAC1 and DNMT1 modulate RGS10 expression and decrease ovarian cancer chemoresistance. *PLoS One* **9**, e87455 (2014).
126. Juergens, R.A. *et al.* Combination epigenetic therapy has efficacy in patients with refractory advanced non-small cell lung cancer. *Cancer Discov* **1**, 598-607 (2011).
127. Sarkar, S. *et al.* Histone deacetylase inhibitors reverse CpG methylation by regulating DNMT1 through ERK signaling. *Anticancer Res* **31**, 2723-32 (2011).
128. Johannessen, C.M. *et al.* A melanocyte lineage program confers resistance to MAP kinase pathway inhibition. *Nature* **504**, 138-42 (2013).
129. Yanase, K. *et al.* Gefitinib reverses breast cancer resistance protein-mediated drug resistance. *Mol Cancer Ther* **3**, 1119-25 (2004).
130. Parkin, B. *et al.* Clonal evolution and devolution after chemotherapy in adult acute myelogenous leukemia. *Blood* **121**, 369-77 (2013).
131. Lapidot, T. *et al.* A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. *Nature* **367**, 645-8 (1994).
132. Navin, N. *et al.* Inferring tumor progression from genomic heterogeneity. *Genome Res* **20**, 68-80 (2010).
133. Campbell, P.J. *et al.* The patterns and dynamics of genomic instability in metastatic pancreatic cancer. *Nature* **467**, 1109-13 (2010).
134. Hanahan, D. & Weinberg, R.A. The hallmarks of cancer. *Cell* **100**, 57-70 (2000).
135. Campos, L. *et al.* High expression of bcl-2 protein in acute myeloid leukemia cells is associated with poor response to chemotherapy. *Blood* **81**, 3091-6 (1993).
136. Vassilev, L.T. p53 Activation by small molecules: application in oncology. *J Med Chem* **48**, 4491-9 (2005).
137. Saddler, C. *et al.* Comprehensive biomarker and genomic analysis identifies p53 status as the major determinant of response to MDM2 inhibitors in chronic lymphocytic leukemia. *Blood* **111**, 1584-93 (2008).

138. Gottesman, M.M. Mechanisms of cancer drug resistance. *Annu Rev Med* **53**, 615-27 (2002).

Chapter 2

Elucidation of Acquired Resistance to Bcl-2 and MDM2 Inhibitors in Acute Leukemia In Vitro and In Vivo

Abstract

Acquired resistance of tumors to anticancer therapies is a major clinical concern. We have investigated the acquired resistance mechanisms *in vitro* and *in vivo* in acute leukemia models for two novel anticancer drugs in clinical development, the MDM2-p53 interaction inhibitor SAR405838 and the dual Bcl-2/Bcl-xL inhibitor ABT-263. Both drugs are highly effective in induction of apoptosis in the ALL RS4;11 cell line *in vitro* and in the xenograft tissue and achieve complete RS4;11 xenograft tumor regression in mice. However, resistance develops for both drugs *in vitro* and *in vivo*. Analysis of the regrown tumors showed that the RS4;11 tumor cells acquire resistance to SAR405838 by mutation of p53 gene or compromised p53 function and to ABT-263 through down-regulation of pro-apoptotic Bcl-2 family member BAX. *In vitro* and *in vivo*, RS4;11 cells that become resistance to SAR405838 retain sensitivity to ABT-263 and conversely, RS4;11 cells that develop resistance to ABT-263 also retain sensitivity to SAR405838. Sequential treatment of RS4;11 xenografts with ABT-263 followed by SAR405838 led to a longer tumor free survival than either single agent. Combination of

SAR405838 and ABT-263 achieved longer term tumor regression without signs of toxicity than either agent alone. Our study provides new insights into the resistant mechanisms for Bcl-2/Bcl-xL and MDM2 inhibitors in acute leukemia and suggests that sequential or combination treatment of these two distinct classes of apoptosis-inducing agents should be explored as a new treatment strategy for acute leukemia with the goal of overcoming acquired drug resistance and improving long-term clinical outcome for patients.

Introduction

Leukemias such as acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML), remain incurable with conventional chemotherapy and new therapeutic strategies are urgently needed[1,2]. Since resistance to apoptosis is a hallmark of cancers, including leukemia, targeting key apoptosis regulators with the goal of overcoming apoptosis resistance in tumor cells has been pursued as a promising cancer therapeutic approach[3].

Bcl-2 family proteins are a class of master regulators of apoptosis[4-8]. The original member of the family, Bcl-2 is overexpressed in patients with leukemia and is an attractive therapeutic target[9,10]. Intense research efforts have yielded potent small-molecule Bcl-2 inhibitors such as ABT-737 [11] and its orally active analogue ABT-263 (navitoclax)[12]. Both ABT-737 and ABT-263 bind to Bcl-2, Bcl-xL and Bcl-w with very high affinities, but it has been shown that Bcl-2, but not Bcl-xL or Bcl-w, is the most critical target for these compounds in leukemia cells *in vivo*[13]. In this study we refer to these compounds as Bcl-2 inhibitors. In both preclinical and clinical trials, these ABT compounds have shown impressive activity as single agents against leukemia[11,12,14].

The tumor suppressor p53 represents another attractive therapeutic target for leukemia[15-20]. In about 90% of leukemias, p53 retains its wild-type status, but its function is effectively inhibited by its endogenous cellular antagonist MDM2[21-25]. As a consequence, MDM2 inhibitors, small molecules designed to block the p53-MDM2 protein-protein interaction can activate wild-type p53[26-29]. Nutlin-3 is the first potent, specific, *bona fide* MDM2 inhibitor [29] and its analogue, RG7112, has been advanced into clinical development[30]. In preclinical studies, RG7112 is capable of inducing tumor regression in xenograft models of leukemia in mice[31] and is being tested in clinical trials in leukemia. In addition to RG7112, six other highly potent MDM2 inhibitors, including SAR405838 designed in our laboratory, are now in clinical trials for cancer treatment[32].

Despite impressive initial antitumor activity, the complete tumor regression achieved by ABT-737/ABT-263[12] and RG7112[31] in xenograft models of leukemia was transitory; tumors eventually regrew after the treatments were terminated, suggesting emergence of resistance to both classes of drugs. Indeed, acquired resistance is, in general, a major cause of cancer drug failure in clinical trials [33]. Although resistance mechanisms for Bcl-2 and MDM2 inhibitors have been investigated in cell culture models[34-38], no study has been performed to investigate the acquired resistance mechanisms *in vivo*. In the present study, we have elucidated resistant mechanisms for both classes of apoptosis-inducing drugs *in vitro* and *in vivo* using the RS4;11 acute lymphoblastic leukemia cell line and the MV4;11 acute myeloid leukemia cell line.

To investigate resistance mechanisms for Bcl-2 inhibitors, we have employed ABT-737 and ABT-263, which bind to Bcl-2, Bcl-xL and Bcl-w with high affinities and show high specificity over Mcl-1 and A1 proteins. ABT-737 and ABT-263 have the same mechanism of action in apoptosis induction in tumor cells[5,11,12] and the orally active ABT-263 is currently in

Phase I/II clinical trials for cancer treatment. To investigate resistant mechanisms for MDM2 inhibitors, we employed SAR405838. SAR405838 binds to MDM2 with a K_i value of 0.88 nM, has a very high specificity over other proteins, and is capable of inducing complete tumor regression in multiple models. SAR405838 has progressed into Phase I clinical trials for cancer treatment.

In this study, we have elucidated acquired resistance mechanisms for these two classes of apoptosis-inducing drugs *in vitro* and *in vivo* using the RS4;11 ALL and the MV4;11 AML cell lines. This has yielded insights into the resistance mechanisms for both classes of drugs and has led to the development of new therapeutic strategies which deliver longer tumor-free survival in mice.

Materials and Methods

Reagents and antibodies. SAR405838 was synthesized using the same method[39]. ABT-263 and ABT-737 were purchased from Selleck Chemicals (Houston, TX). Rabbit antibodies for caspase-3, PARP, Mcl-1 (D35A5), Bcl-xL (54H6) and mouse antibody for caspase-7 were obtained from Cell Signaling Technology (Danvers, MA); rabbit antibodies for GAPDH and BAK (G-23) and mouse antibodies for BAX (6A7 and 6D149) and Bcl-2 were from Santa Cruz Biotechnology (Dallas, TX); mouse antibody p53 (Ab-6) and MDM2 (Ab-1) and rabbit PUMA (Ab-1) were from Calbiochem (Millipore). Mouse antibody for p21 was from BD Pharminogen (San Jose, CA).

Cell Culture, cell viability, and apoptosis assays. RS4;11 and MV4;11 cell lines were purchased from American Type Culture Collection and cultured as recommended. Cell viability

was evaluated by a WST-8 assay (Dojindo)[40]. Apoptosis was analyzed using Annexin V-FLUOS staining kit (Roche Applied Science, Indianapolis, IN). Differences in mean values of cell apoptosis among different groups were analyzed by 2-way ANOVA using Prism, with a *P* value of <0.05 being considered significant.

Resistant Cell Lines. RS4;11 and MV4;11 parental cell lines were treated with ABT-737 starting from 10 nM for 72 hrs. The cells were then rinsed and the remaining live cells were expanded in normal medium. This process was repeated with increased drug concentration till 10 μM and surviving cells were utilized for subsequent experiments. An identical protocol was utilized to obtain *in vitro* sublines resistant to SAR405838, with the exception of the final drug concentration being 20 μM. DMSO treated cell lines were generated for use as control.

Stable short hairpin interfering RNA constructs. Short 19-bp hairpins for generating RNA interference: BAX (nucleotides 239-257, Genbank NM138761), BAK (nucleotides 535-553, Genbank NM001188) and p53 (nucleotides 611-629 Genbank NM000546)[35]. The oligonucleotides were annealed and ligated into a self-inactivating lentiviral vector under the control of the H1 promoter [41]. The vector also carried the GFP reporter gene under control of the human ubiquitin-C promoter to monitor infection efficiency. A scrambled shRNA construct was utilized as a control[35]. Lentiviral shRNA virus-containing supernatant, generated by the University of Michigan Vector Core, was used to infect RS4;11 and MV4;11 cells. At 96 h post infection the cells were sorted for GFP fluorescence.

p53 Mutation analysis. Primers to amplify and sequence genomic DNA for exons 2 to 11 of human p53 were used according to Hauser *et al.*[2]. Primers to amplify and sequence cDNA for exons 2 to 11 of human p53 were used according to Aziz *et al.*[36]. Mutation surveyor (SoftGenetics LLC) software was used to compare experimental sequences against Refseq GenBank as well as by visual inspection of sequence tracings.

***In vivo* xenograft studies.** To develop xenograft tumors, 5 x 10^6 tumor cells with 50% Matrigel were injected subcutaneously on the dorsal side of SCID mice. All groups had 6-12 mice unless otherwise stated. Tumor sizes and animal weights were measured 2-3 times per week with tumor volume (mm^3) = (length x $width^2$)/2. Significance (*P*) was calculated by unpaired two-tailed *t* test using Prism. *P* value of < 0.05 being considered significant.

Synergy studies. Synergism, additive effects and antagonism was assessed using the Chou-Talalay[42] method and Calcusyn software (Biosoft, Ferguson, MO). The effect on cell growth was expressed as a fraction of cells that were killed by the single agent treatment compared to the combination treatment versus untreated cells utilizing the WST-8 assay at 4 days. The effect on apoptosis was determined using flow cytometry in order to identify the percentage of Annexin V-positive cells in the single agent treatment compared to the combination treatment versus control treated cells at 24 h for RS4;11 cells and 48 h for MV4;11 cells.

Results

Establishment and characterization of RS4;11 sublines resistant in vitro and in vivo to ABT-263/ABT-737

The acute lymphoblastic leukemia RS4;11 cell line has a t(4;11) chromosomal rearrangement and has been shown *in vitro* and *in vivo* to be sensitive to ABT-737/ABT-263[12,43].

Treatment of RS4;11 xenograft tumors in mice with ABT-737 results in complete tumor regression (Fig. 2.1A), but all tumors regrow shortly after termination of the treatment. We isolated RS4;11 tumor cells from the regrown tumors and established 8 sublines (G3M1-G3M8). While the RS4;11 parental cell line and 2 representative sublines (30L and 30R) established from the vehicle- treated tumors are highly sensitive in a cell growth assay to both ABT-737 and ABT-263, all 8 sublines obtained from the ABT-737 treated tumors have increased resistance to ABT-263 and ABT-737, with an average IC_{50} value >100-fold higher than the control cell lines (Fig. 2.1B and SI Fig. S2.1B). Apoptosis analysis by flow cytometry confirmed that these sublines (G3M1-G3M8) are indeed highly resistant to ABT-737 and ABT-263 (Fig. 2.1C and SI Fig. S2.1C).

In previous studies, upregulation of the Mcl-1 protein was shown to be a primary mechanism for acquired resistance of tumor cells to ABT-737[34,35]. Western blotting, however, showed that the highly resistant sublines (G3M1-G3M8) and the sensitive control cell lines have similar levels of Mcl-1 expression (Fig. 2.1G). Profiling of other Bcl-2 family members showed that compared to the control cell lines, BAX protein, but not its homolog BAK, is consistently and profoundly down-regulated in all the 8 resistant cell lines (Fig. 2.1F). However, in comparison to the control cell lines, other Bcl-2 protein levels are not significantly different in these resistant sublines (G3M1-G3M8) (Fig. 2.1F and SI Fig. S2.1A).

To investigate the acquisition of resistance to the Bcl-2 inhibitors of the RS4;11 cell line *in vitro*, the RS4;11 cells were exposed to gradually increasing concentrations (10 nM gradually increasing to 10 μM) of ABT-737. This yielded two sublines (ABTR1 and ABTR2) which, in a cell growth assay exhibit >100-fold resistance to ABT-263 compared to the parental RS4;11 cell line (Fig. 2.1D). This resistance of the ABTR1 and ABTR2 sublines to ABT-263 was further confirmed in an apoptosis assay (Fig. 2.1E). Western blotting showed that the BAX protein, but not BAK, is profoundly down-regulated in ABTR1 and ABTR2 sublines when compared to the parental cell line, similar to what was observed in the 8 *in vivo* cell lines (Fig. 2.1G). However, in contrast to the lack of Mcl-1 upregulation in all the 8 *in vivo* sublines, Mcl-1 is clearly upregulated in the ABTR sublines when compared to the control cell lines (Fig. 2.1G).

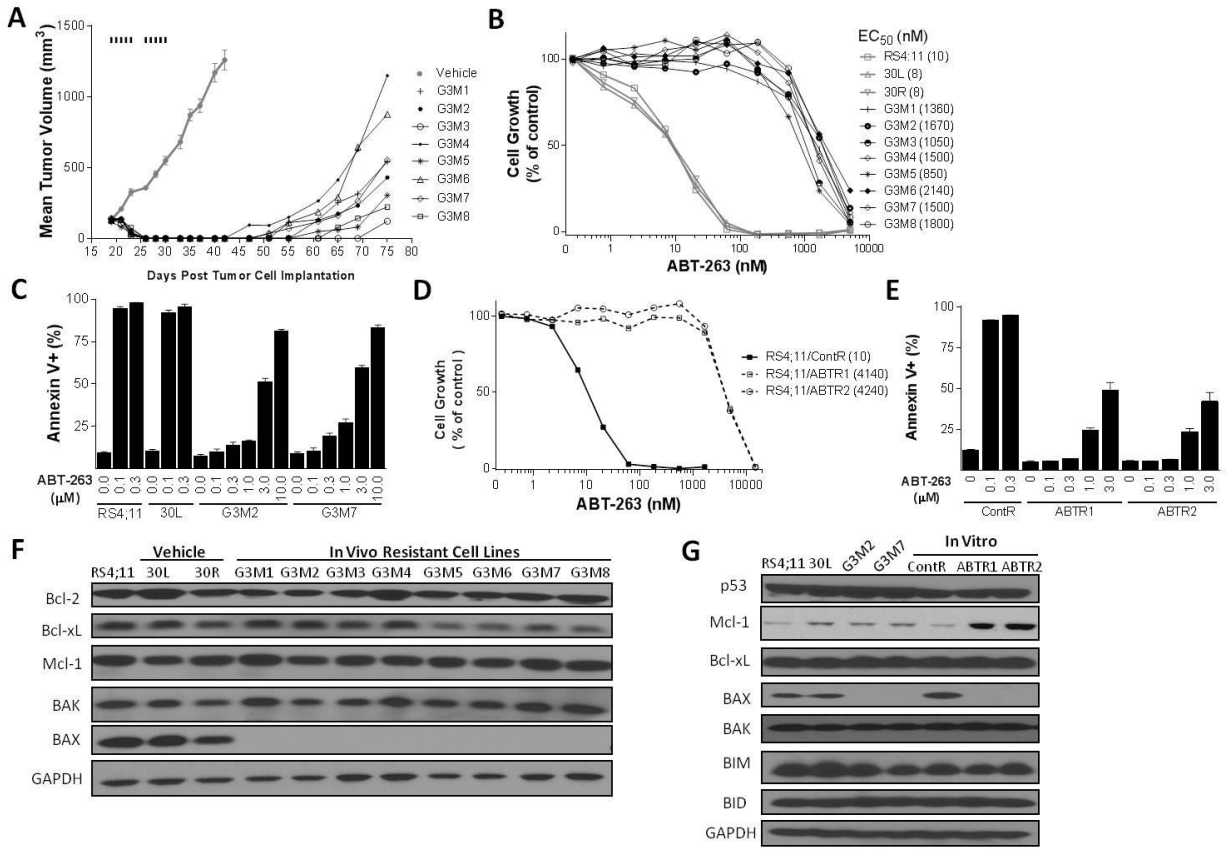

Figure 2.1 Establishment and characterization of *in vitro* and *in vivo* RS4;11 sublines resistant to ABT-263/ABT-737.

A, Establishment of *in vivo* RS4;11 sublines. RS4;11 xenograft tumors were treated with vehicle (grey) or ABT-737 at 100 mg/kg, i.p. for 5 days per week for 2 weeks (black). Vehicle treated tumors and ABT-737-treated, regrown tumors were harvested and cultured to generate sublines. B, Sensitivity of *in vivo* RS4;11 sublines to ABT-263 in a cell growth assay. Parental, two representative vehicle-treated sublines (30L and 30R) and 8 sublines (G3M1-G3M8) generated from ABT-treated tumors were treated with ABT-263 or vehicle control for 4 days for cell viability analysis using a WST assay. C, Sensitivity of *in vivo* RS4;11 sublines to ABT-263 in an apoptosis assay. Parental RS4;11 cell line and one representative subline from vehicle-treated tumors, two representative sublines from ABT-treated tumors (G3M2 and G3M7) were treated with ABT-263 or vehicle control for 24 h for apoptosis analysis by flow cytometry with Annexin V/P.I. double staining. Data (mean ± SD) are from triplicates, including both early (Annexin V-positive/PI-negative) and late (Annexin V-positive/PI-positive) apoptotic cells. D, Sensitivity of *in vitro* RS4;11 sublines to ABT-263 in a cell growth assay using the same protocol as in (B). E, Sensitivity of *in vitro* RS4;11 sublines to ABT-263 in an apoptosis assay using the same protocol as in (C). F and G, Immunoblotting of Bcl-2 family proteins for *in vitro* and *in vivo* RS4;11 resistant sublines and control lines. GAPDH was used as the loading control.

Establishment and characterization of *in vitro* and *in vivo* RS4;11 resistant sublines to the MDM2 inhibitor SAR405838

The MDM2 inhibitor SAR405838 effectively inhibits cell growth with IC_{50} = 140 nM (Fig. 2.2B) and induces apoptosis in the RS4;11 cell line, which has wild-type p53 status (Fig. 2.2C).

In vivo, treatment of the RS4;11 xenograft tumors in mice with SAR405838 induces rapid and complete tumor regression, which persists for >30 days, but eventually the tumors return (Fig. 2.2A). Harvesting and culturing all tumors regrown after treatment with SAR405838 established 7 sublines (G7M1-G7M7). A cell growth assay showed that while two sublines, G7M2 and G7M4, exhibit 5-fold greater resistance to SAR405838 compared with the parental and vehicle treated cell lines, the other 5 sublines show an average of >35-fold increased resistance to SAR405838 (Fig. 2.2B). The moderate resistance to SAR405838 in the G7M4 subline and more significant resistance in the G7M1 and G7M7 sublines were confirmed in an apoptosis assay (Fig. 2.2C and SI Fig. S2.2A).

We also established two RS4;11 sublines (MIR1 and MIR2) *in vitro* by gradually increasing the concentration of SAR405838 in the cell culture. Both the MIR1 and MIR2 sublines exhibit >60-fold greater resistance to SAR405838 in a cell growth assay compared with the control cell lines (Fig. 2.2D) and their profound resistance was confirmed in an apoptosis assay (Fig. 2.2E).

The activity of SAR405838 depends upon wild-type p53[32], and consequently we analyzed the p53 mutation by sequencing exons 2-11 in all the *in vitro* and *in vivo* sublines obtained after the SAR405838 treatment (SI Table S2.1). Both the MIR1 and MIR2 *in vitro* sublines and 5 out of 7 of the *in vivo* sublines were found to harbor p53 mutation(s).

Immunoblotting showed that, compared to the control cell lines, basal p53 protein expression is increased in all the sublines harboring p53 mutation(s) (G7M1-3, 6-7 and MIR1-2) (Fig. 2.2F). In contrast to the loss of BAX expression in the resistant sublines obtained from the ABT-737/ABT-263 treatment, similar levels of BAX protein were found in all the sublines obtained from treatment with SAR405838 and in the parental cell line (Fig. 2.2F).

To investigate if any of these sublines obtained from treatment with SAR405838 still retained functional p53, we examined induction by SAR405838 treatment of two p53-regulated proteins, p21 and MDM2, and also accumulation of p53 protein and PARP cleavage in these sublines (SI Fig. S2.2B and C). No significant induction of p21 protein, PARP cleavage or p53 accumulation is observed in the sublines harboring p53 mutation (G7M1, G7M3 and G7M7) when treated with SAR405838, indicative of the absence of p53 activation (SI Fig. S2.2B). In the G7M4 subline lacking p53 mutation, SAR405838 induces a dose-dependent increase of p21 and p53 proteins and cleavage of PARP, indicative of p53 activation and apoptosis induction, but with a reduced potency as compared to that in the parental cell line (SI Fig. S2C). In G7M5,

which has no expression of p53 at the basal level (Fig. 2.2F), SAR405838 fails to induce p53 and p21 upregulation and PARP cleavage, indicative of non-functional p53 (SI Fig. S2.2C).

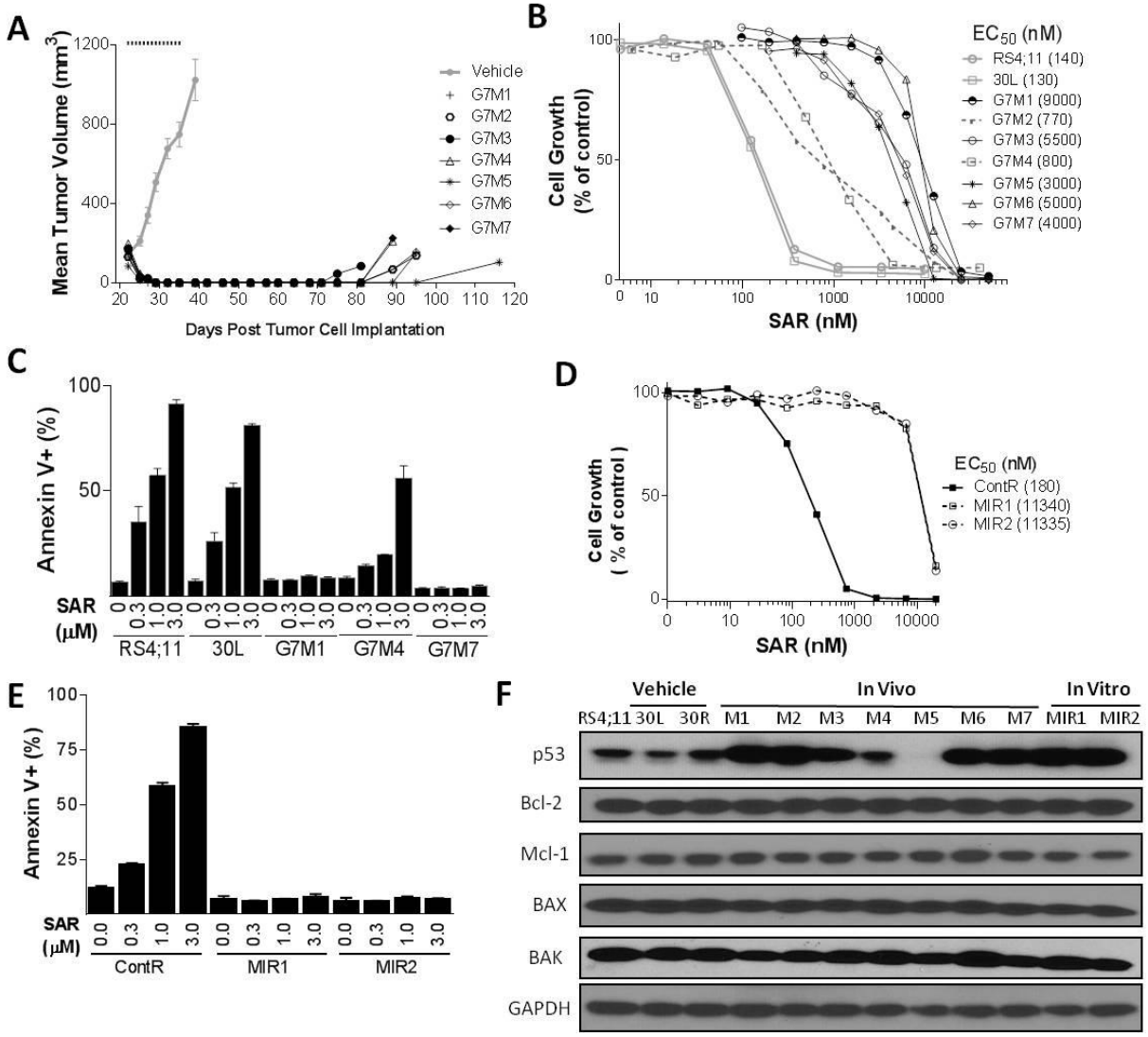

Figure 2.2 Establishment and characterization of RS4;11 sublines obtained from *in vitro* and *in vivo* treatment with SAR405838.

A, Establishment of *in vivo* RS4;11 sublines. RS4;11 xenograft tumors were treated with vehicle (grey) or 200 mg/kg of SAR405838 orally for 21 days and SAR405838-treated tumors (G7M1-G7M7) were harvested when the regressed tumors regrew to approximately 100 mm^3 and then cultured to establish sublines. B, Sensitivity of *in vivo* RS4;11 sublines to SAR405838 in a cell growth assay. Parental RS4;11 cell line and sublines established from RS4;11 xenografts (30L, vehicle-treated; G7M1-G7M7, SAR405838-treated) were treated with SAR405838 for 4 days for cell viability analysis in a WST assay. C, Sensitivity of *in vivo* RS4;11 sublines to SAR405838 in an apoptosis assay. Parental RS4;11 cell line and sublines established from representative RS4;11 xenografts were treated with SAR405838 for 24 h for apoptosis analysis by flow cytometry with Annexin V/P.I. double staining. D, Sensitivity of *in vitro* RS4;11 sublines to

SAR405838 in a cell growth assay using the same protocol as in (B). E, Sensitivity of *in vitro* RS4;11 sublines to SAR405838 in an apoptosis assay using the same protocol as in (C). F, Immunoblotting of representative Bcl-2 family proteins and p53 for *in vitro* and *in vivo* RS4;11 sublines and control lines, with GAPDH as the loading control.

Taken together, these data show that treatment of the RS4;11 cell line *in vitro* and *in vivo* by SAR405838 yielded SAR405838-resistant sublines, which have either inactive, mutated p53 or p53 with compromised function.

RS4;11 sublines resistant to Bcl-2 inhibitors retain their sensitivity to MDM2 inhibitor

Exons 2-11 were sequenced for each of the RS4;11 *in vitro* and *in vivo* sublines resistant to ABT compounds. No p53 mutation could be detected, suggesting that these sublines retain wild-type p53 status and sensitivity to MDM2 inhibitors.

SAR405838 effectively inhibits cell growth in the ABTR1 and ABTR2 *in vitro* sublines (Fig. 2.3A) and induces dose-dependent apoptosis in the ABTR1 and ABTR2 sublines, albeit with modestly reduced potency compared to that in the control RS4;11 cell line (Fig. 2.3B).

In the 8 *in vivo* sublines (G3M1-G3M8) that developed profound resistance to ABT compounds, SAR405838 is still very effective in inhibition of cell growth (Fig. 2.3C). Apoptosis induction by SAR405838 is attenuated by a factor of only 2-3 in the G3M1 and G3M6 sublines compared to the RS4;11 parental and vehicle-treated cell lines (Fig. 2.3D). In the RS4;11 parental, and the G3M1 and G3M6 sublines, SAR405838 effectively and dose-dependently induces both activation of p53 and apoptosis, as shown by upregulation of p53, p21, MDM2 and PUMA proteins, and cleavage of caspase-3, caspase-7 and PARP (Fig. 2.3E).

Hence, when the RS4;11 cells develop profound acquired resistance to Bcl-2 inhibitors ABT-737 and ABT-263, they are still sensitive to the MDM2 inhibitor SAR405838, and lack cross-resistance.

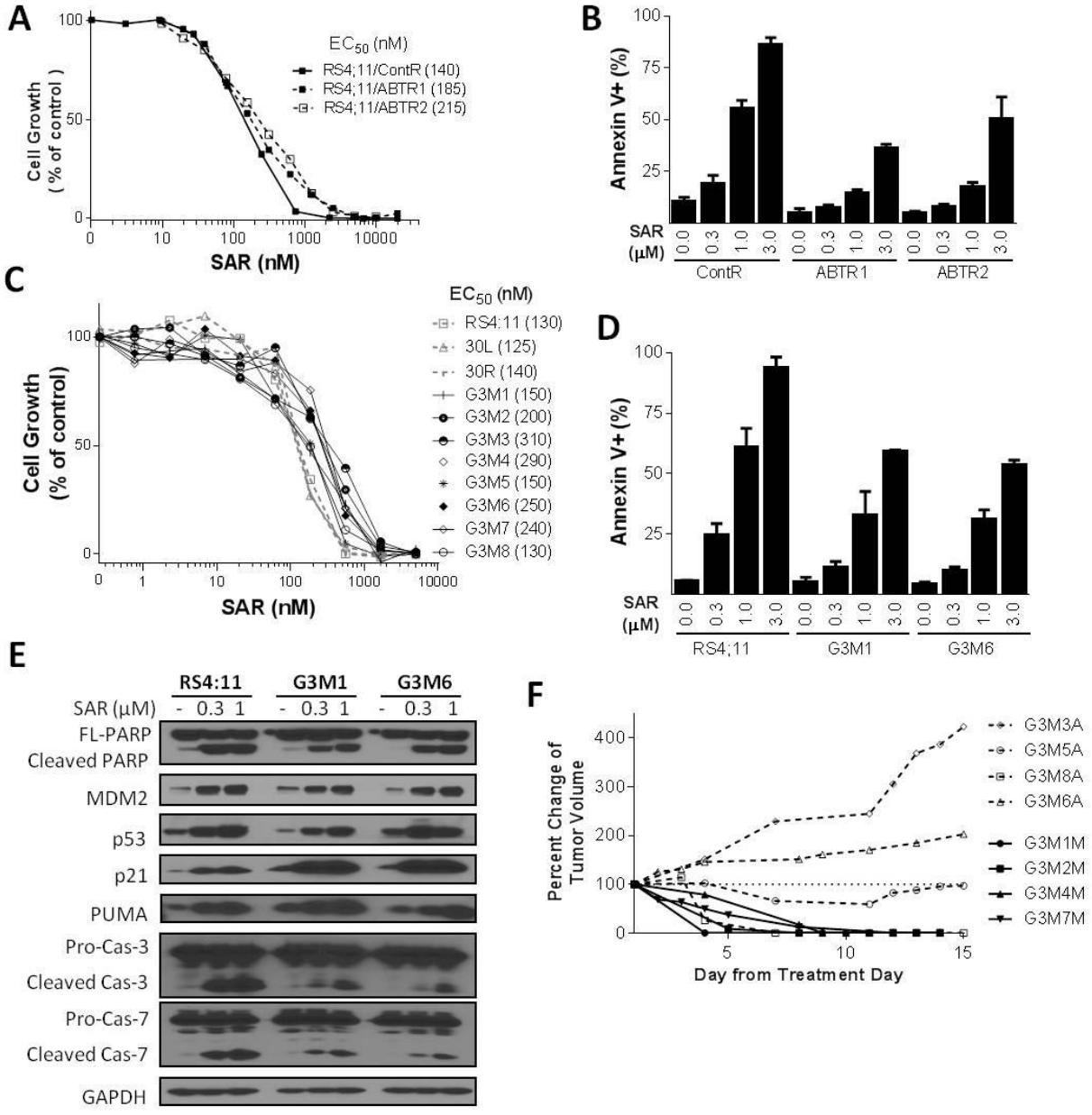

Figure 2.3 Evaluation of SAR405838 in RS4;11 sublines obtained from in vitro or in vivo treatment with ABT-737.

A and C, RS4;11 parental and sublines were treated for 4 days with SAR405838 for cell viability analysis by a WST assay. B and D, RS4;11 parental and representative sublines were treated

with SAR405838 for 24 h for apoptosis analysis by flow cytometry with Annexin V/P.I. double staining. E, RS4;11 parental and two representative sublines were treated with SAR405838 for 24 h for immunoblotting of p53 and p53-regulated proteins and biochemical markers of apoptosis. F, Regrown RS4;11 xenografts treated with ABT-263 for daily, 21 days at 100 mg/kg *via* oral gavage were retreated with either SAR405838 at 100 mg/kg (solid lines) or ABT-263 (dashed lines) at 150 mg/kg, both *via* oral gavage, daily for 14 days. Data are presented as percent change of the tumor volume for each mouse.

RS4;11 sublines resistant to SAR405838 retain sensitivity to ABT-263

We next evaluated ABT-263 for its effectiveness in the RS4;11 sublines obtained by the treatment with SAR405838. In cell growth and apoptosis assays, both *in vitro* (MIR1 and MIR2) and *in vivo* (G7M1-G7M7) sublines show sensitivity to ABT-263 similar to that of the RS4;11 control cell lines (Fig. 2.4A-D). Based upon western blot analysis of cleavage of caspase-3, caspase-7 and PARP (Fig. 2.4E), ABT-263 is equally effective in induction of apoptosis in representative sublines such as G7M1, G7M4 and G7M7 (Fig. 4D). Hence, while p53 mutation or compromised p53 function greatly diminishes the activity of the MDM2 inhibitor SAR405838, it has no effect on the activity of the Bcl-2 inhibitor ABT-263.

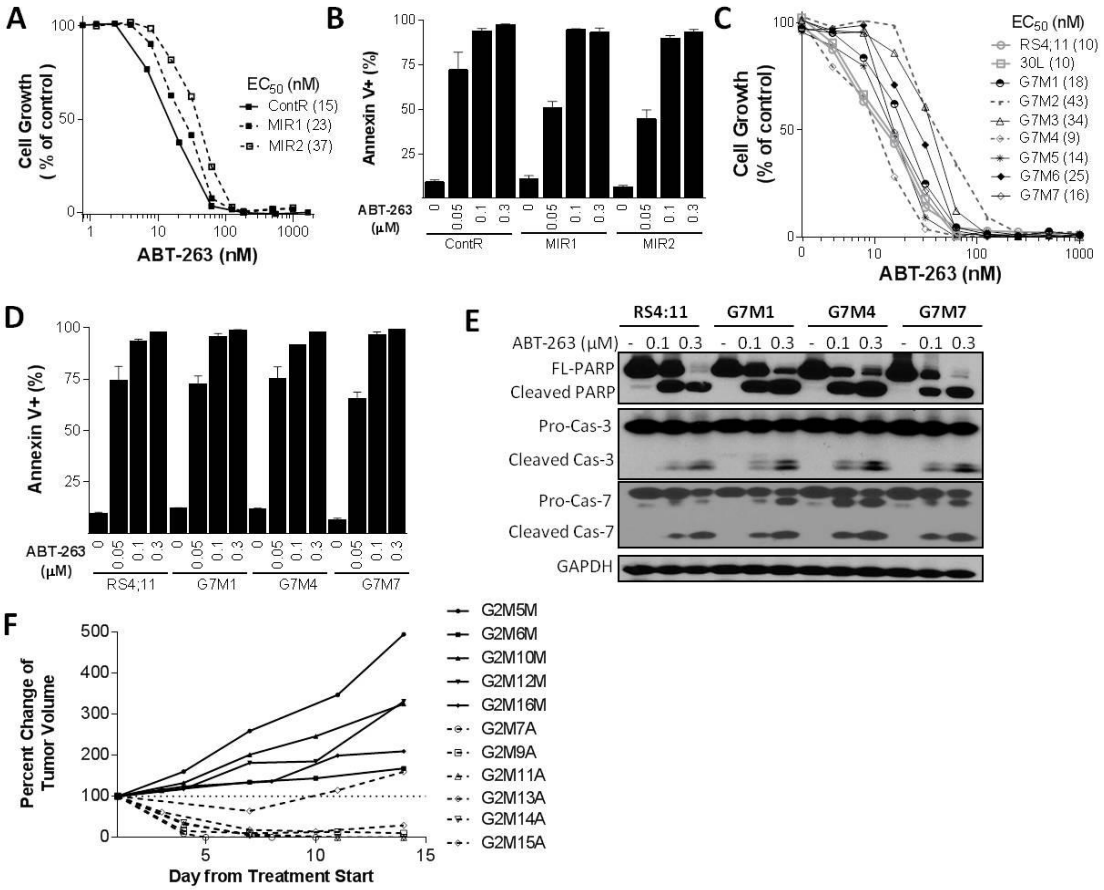

Figure 2.4 Evaluation of ABT-263 in RS4;11 sublines obtained from *in vitro* or *in vivo* treatment with SAR405838.

A and C, Cells were treated with ABT-263 for 4 days and cell viability was determined by a WST assay. B and D, Cells were treated with ABT-263 for 24 h for apoptosis analysis by flow cytometry with Annexin V/P.I. double staining. E, Representative RS4;11 sublines were treated with ABT-263 for 24 h and biochemical markers of apoptosis were analyzed by immunoblotting. F, Antitumor activity of ABT-263 and SAR405838 in regrown tumors initially treated with SAR405838. When regressed tumors initially treated with SAR405838 regrew to 100-200 mm^3, they were treated with either ABT-263 at 100 mg/kg (dashed lines), or SAR405838 (solid lines) at 200 mg/kg for 14 days. Data are presented as percent change of the tumor volume for each mouse.

Regrown tumors resistant to the initial drug are responsive to the second class of drug in mice

Although RS4;11 cells acquire resistance to Bcl-2 inhibitors ABT-263/ABT-737, they retain their sensitivity to the MDM2 inhibitor SAR405838 *in vitro* (Fig. 2.3A-E). Conversely,

when the RS4;11 cells acquire resistance to SAR405838, they remain sensitive to ABT-263 *in vitro* (Fig. 2.4A-E). These data suggest that although the RS4;11 tumors acquire resistance to one class of apoptosis-inducing agent, they may still be susceptible *in vivo* to a second class of apoptosis-inducing agents. We directly examined this possibility in mice bearing the RS4;11 xenograft tumors.

When RS4;11 xenograft tumors are treated with ABT-263, all tumors rapidly become undetectable (SI Fig. S2.3A) but regrow shortly after the treatment is ended. When the regrown tumors reached an average volume of 200 mm^3, they were randomized into two groups and treated with either the maximum tolerated dose of ABT-263 (150 mg/kg) or 100 mg/kg of SAR405838 (Fig. 2.3F and SI Fig. S2.3C). While one of the 4 tumors treated with ABT-263 regressed, the other three tumors showed no regression (Fig. 2.3F), in contrast to the rapid tumor regression observed following the initial treatment with ABT-263 at 100 mg/kg. In comparison, all 4 regrown tumors treated with SAR405838 experienced rapid and complete regression (Fig. 2.3F).

Similarly, treatment with SAR405838 at 100 mg/kg results in rapid and complete regression of RS4;11 tumors in mice (SI Fig. S2.4A), but all tumors regrow after termination of the treatment. When the regrown tumors reached an average volume of 200 mm^3, they were randomized into two groups and treated with either ABT-263 at 100 mg/kg or SAR405838 at 200 mg/kg (Fig. 2.4F), levels at which there was no sign of toxicity (SI Fig. S2.4C). All tumors treated with SAR405838 at 200 mg/kg continued to grow, but 5 out of 6 tumors treated with ABT-263 rapidly regressed (Fig. 2.4F). All cell lines isolated from tumors treated with ABT-263 at any point in either treatment protocol showed loss of BAX protein expression, while the expression of the BAK protein was not altered (SI Fig. S2.3B and S2.4B).

Taken together, these *in vivo* data clearly showed that when regrown xenograft tumors become resistant to the initial class of drug, they are still very responsive to the second class of drug, and lack cross-resistance.

Different roles of BAX in the activity of Bcl-2 and MDM2 inhibitors

When the parental RS4;11 cell line was treated with ABT-737/ABT-263 *in vitro* and *in vivo*, BAX, but not BAK is significantly down-regulated (Fig. 2.1F-G). Although becoming highly resistant to ABT compounds, the cells retain their sensitivity to SAR405838 (Fig. 2.3). This suggests that BAX and BAK may play different roles in the activity of these two different classes of apoptosis-inducing drugs. Consequently, we further investigated the role of BAX and BAK in cell growth inhibition and apoptosis induction assays for these two classes of drugs.

Efficient knock-down of BAX expression using lenti-virus shRNA in the RS4;11 parental cell line renders the cells >100-times less sensitive to ABT-263 in a cell growth assay and unresponsive to apoptosis induction by ABT-263 (Fig. 2.5A-C). However, efficient shRNA knock-down of BAK expression in the RS4;11 parental cell line has little or no effect on the activity of ABT-263 in both cell growth and apoptosis assays (Fig. 2.5A-C). For SAR405838, efficient knock-down of BAX expression in the RS4;11 parental cell line results in only a ~3-fold reduction in sensitivity (Fig. 2.5D) and efficient knock-down of BAK expression has no effect on the activity of SAR405838 (Fig. 2.5D).

Since BAX was profoundly down-regulated in all the RS4;11 sublines obtained from *in vitro* and *in vivo* treatment of ABT-737/ABT-263 (Fig. 2.1, SI Fig. S2.3B, S2.4B), we knocked down BAK in one of the sublines (G3M1) (Fig. 2.5A) and evaluated the sensitivity of the cells to SAR405838. Efficient knock-down of BAK in the G3M1 subline results in a >15-fold increase

in the IC_{50} value for SAR405838 in the cell growth assay and significantly reduces the apoptotic response to SAR405838 (Fig. 2.5D and E).

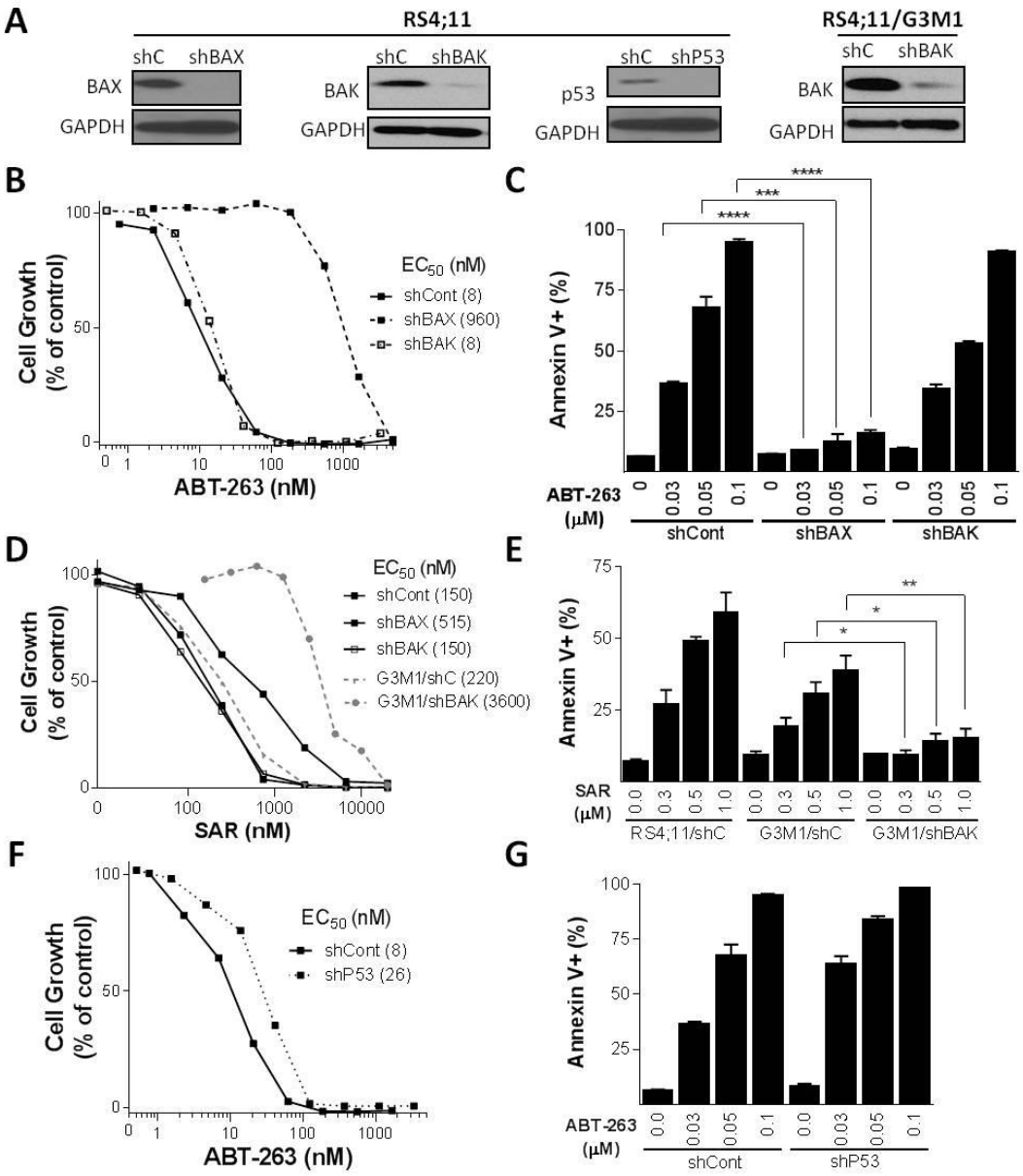

Figure 2.5 Investigation of the role of BAK, BAX and p53 in the activity of ABT-263 and SAR405838 in the RS4;11 cell line.

A, Immunoblots illustrating the efficiency of the shRNA-lentiviral approach in RS4;11 and RS4;11/G3M1 cells. B and C, BAX or BAK was knocked down by shRNA in the RS4;11 parental cell line and cells were treated with ABT-263 for 4 days for cell viability analysis by a WST assay (B), or treated with ABT-263 for 24 h for apoptosis analysis by flow cytometry with

Annexin V/P.I. double staining (C) (***, P <0.001; ****, P <0.0001; t test). D, BAK or BAX was efficiently knocked down by shRNA in the RS4;11 parental cell line or BAK was knocked down in G3M1 subline with a very low level of BAX. Cells were treated with SAR405838 for 4 days for cell viability analysis using a WST assay. E, Apoptosis analysis by flow cytometry with Annexin V/P.I. double staining. RS4;11/shControl, G3M1/shControl and G3M1/shBAK cells were treated with SAR405838 for 24 h (*, P <0.05; **, P <0.01; t test). F and G, p53 was knocked down by shRNA in the RS4;11 parental cell line and the cells were treated with ABT-263 for 4 days for cell viability analysis in a WST assay (F), or for 24 h for apoptosis analysis by flow cytometry with Annexin V/P.I. double staining (G).

These data show that while BAX, but not BAK, plays a dominant role in mediating the activity of Bcl-2 inhibitor ABT-263 in the RS4;11 cell line, the presence of either BAX or BAK is sufficient for effective apoptosis induction by the MDM2 inhibitor SAR405838. Absence of both BAX and BAK, however, renders the cells resistant to SAR405838.

p53 has a minimal role in the activity of Bcl-2 inhibitors

ABT-263 is very effective in RS4;11 sublines harboring mutated p53, suggesting that p53 has a minimal role in the activity of Bcl-2 inhibitors. To further examine this, we stably knocked down p53 in the RS4;11 parental cell line using lenti-virus shRNA (Fig. 2.5A). While efficient knock-down of p53 dramatically reduces the activity of SAR405838[32], it has only a modest effect on the activity of ABT-263 (Fig. 2.5F and G) and we concluded that p53 has a minimal role in the activity of Bcl-2 inhibitors in the RS4;11 cell line.

Lack of cross-resistance for MDM2 and Bcl-2 inhibitors in the MV4;11 acute leukemia cell line

We next extended our studies to the MV4;11 acute leukemia cell line, which also harbors a t4;11 chromosomal translocation.

First, we generated resistant sublines to SAR405838 and ABT-263. The MV.ABT-R cell line, generated by treating the MV4;11 cell line with ABT-263 *in vitro*, is >18-fold less sensitive

to ABT-263 than the control cell lines, but retains its sensitivity to SAR405838 (SI Fig. S2.5). Interestingly, western blot analysis reveals that while the MV.ABT-R subline shows no decrease in expression of BAX, it has increased expression of Mcl-1 protein compared to controls (SI Fig. S2.5C). However, stable knock-down of BAX expression in parental MV4;11 cells drastically attenuates cell growth inhibition and apoptosis induction by ABT-263 compared to the control cell line, but remains sensitive to SAR405838 (SI Fig. S2.6). In comparison, efficient knock-down of BAK expression in the parental MV4;11 cells has little to no effect on the activity of ABT-263 or SAR405838 (SI Fig. S2.6).

Similarly, the MV.MI-R cell line, generated by treating the MV4;11 cell line with SAR405838 *in vitro*, becomes >20-times less sensitive to SAR405838 than the control cell line in a cell growth assay but has the same sensitivity as the parental cell line to ABT-263 (SI Fig. S2.5). Sequencing exons 2-11 revealed that p53 has undergone a homozygous mutation, R248W, in the MV.MI-R cell line. To further test the role of p53 on the activity of ABT-263 and SAR405838, we stably knocked down p53 in the MV4;11 cell line (SI Fig. S2.7A). While efficient knock-down of p53 greatly reduces the activity of SAR405838, it has no effect on the activity of ABT-263 (SI Fig. S2.7B-D).

We next evaluated both SAR405838 and ABT-263 in mice bearing MV4;11 xenograft tumors. Although ABT-263 is fairly potent *in vitro*, it only inhibits tumor growth modestly *in vivo* at 100 mg/kg (SI Fig. 2.8A). In comparison, SAR405838 at 100 mg/kg induces rapid and complete, but transient tumor regression in 100% of mice bearing MV4;11 xenograft tumors (SI Fig. S2.8A). When regressed tumors treated with SAR405838 regrew, they were harvested and cultured to establish 6 sublines (G3M1-G3M6). Two representative tumors treated with vehicle were also harvested and cultured yielding two sublines (G1M3 and G1M7). A cell growth assay

showed that while two sublines (G3M2 and G3M5) exhibit moderate resistance to SAR405838 compared with the parental and vehicle treated cell lines, the other 4 sublines (G3M1, G3M3, G3M4 and G3M6) are highly resistant to SAR405838 (SI Fig. S2.8B). SAR405838 becomes ineffective in activation of apoptosis and p53 in these highly resistant sublines (SI Fig. S2.8C and D). Sequencing exons 2-11 showed that p53 has a homozygous mutation, R248W, in all of the highly resistant SAR405838 sublines. In comparison, p53 has a heterozygous mutation at R248W in the moderately resistant sublines. MV4;11 *in vivo* sublines resistant to SAR405838 are still sensitive to ABT-263 in both cell growth and apoptosis assays and exhibit little or no resistance, compared to control cell lines (SI Fig. S2.9).

Sequential and Combination treatment strategies improve 100% tumor-free survival

Although both Bcl-2 and MDM2 inhibitors used as single agents achieve rapid and complete regression of the RS4;11 xenograft tumors, tumors regrow shortly after cessation of the treatment. To determine the synergistic effects on cell growth and induction of apoptosis we combined ABT-263 and SAR405838 using a fixed-ratio experimental design. Our data showed that the combination of ABT-263 and SAR405838 in the RS4;11 and MV4;11 cell lines is highly synergistic based upon the combination index (CI) values (50) (SI Tables S2.2 and S2.3). Based upon the encouraging *in vitro* combination data, we investigated strategies with the goal of improving tumor-free survival of mice in the RS4;11 tumor xenograft model.

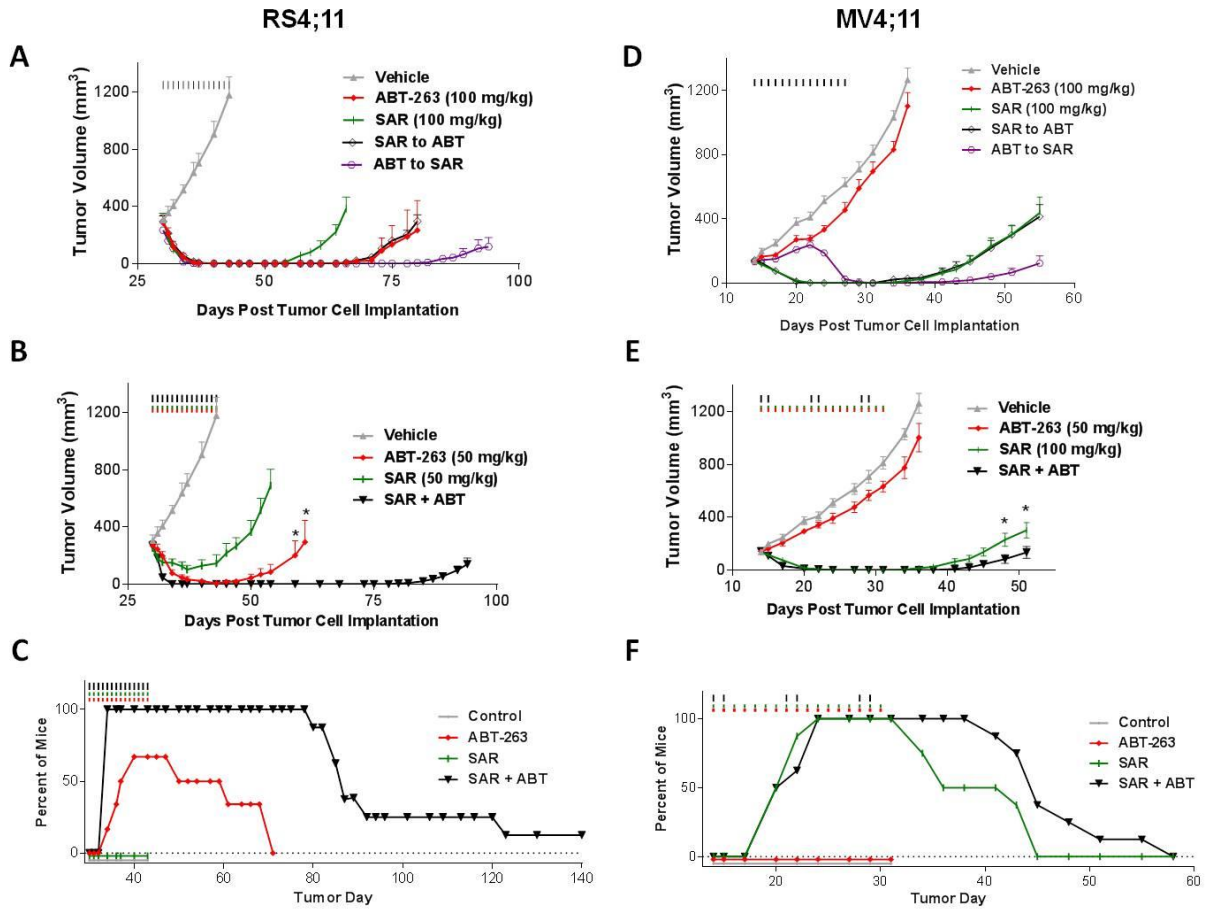

Figure 2.6 Antitumor activity of ABT-263 and SAR405838 alone, sequential treatment of the two drugs and their combination in the RS4;11 and MV4;11 xenografts models.
A, Antitumor activity of single agents and sequential treatment in the RS4;11 xenograft model. Mice were treated via oral gavage with vehicle, ABT-263 or SAR405838 for 14 days, or with one drug for 7 days, immediately followed with the other drug for another 7 days. B, Antitumor activity of ABT-263, SAR405838 and their combination in the RS4;11 xenograft model. When tumors grew to approximately 300 mm^3, mice were treated with vehicle, ABT-263, SAR405838 or their combination at 50 mg/kg for 14 days *via* oral gavage. C, Percentage of tumor-free survival of mice in the RS4;11 efficacy experiment shown in (B). D, Antitumor activity of single agents and sequential treatment in MV4;11 xenograft model. Mice were treated via oral gavage with vehicle, ABT-263 or SAR405838 for 18 days, or with one drug for 9 days, immediately followed with the other drug for another 9 days. E, Antitumor activity of ABT-263, SAR405838 and their combination in the MV4;11 xenograft model. When tumors grew to approximately 200 mm^3, mice were treated with vehicle, ABT-263, SAR405838 or their combination at 50 mg/kg of ABT-263 and 100 mg/kg of SAR405838 twice weekly for 18 days *via* oral gavage. F, Percentage of tumor-free survival of mice in the RS4;11 efficacy experiment shown in (E).

We tested two sequential treatment strategies *in vivo*: (1) ABT-263 daily for one week first followed by SAR405838 daily for another week or (2) SAR405838 daily for one week first

followed by ABT-263 daily for another week (Fig. 2.6A). Both strategies showed no signs of toxicity (SI Fig. S2.10A). The 100% tumor-free survival time was 12 days in the SAR405838 single-agent, daily treatment for 2 weeks and 24 days in the ABT-263 single-agent, daily treatment for 2 weeks. In comparison, the 100% tumor-free survival time was 41 days for the sequential treatment scheme of ABT-263 first for one week, followed by SAR405838 for another week, and 24 days for the reverse sequential treatment scheme (Fig. 2.6A). Hence, the sequential treatment with, first ABT-263, followed by SAR405838 is the most effective of the four treatment schemes in achieving tumor-free survival in 100% of mice.

Using a fixed-ratio experimental design, we assessed whether the combination of the Bcl-2/Bcl-xL inhibitor (ABT-263) and MDM2-p53 interaction inhibitor (SAR405838) would work in tandem to elicit a synergistic response in acute leukemia. Interaction studies between ABT-263 and SAR405838 showed synergistic effects on cell growth (SI Table S2.2) and induction of apoptosis (SI Table S2.3) in both RS4;11 and MV4;11 cells. The combination index (CI) values for cell growth and apoptosis were under 1.0 for both cell lines, suggesting a synergistic effect between ABT-263 and SAR405838.

We also tested whether the combination of ABT-263 and SAR405838 results in a longer tumor free survival than single agents. The MTD for the combination was determined to be 50 mg/kg for each agent in RS4;11 tumor-bearing mice. SAR405838 as a single agent at 50 mg/kg does not yield complete tumor regression in any animal, but ABT-263 as a single agent at 50 mg/kg achieves tumor regression in 4 out of 6 mice. The combination results in complete tumor regression in 100% of mice for a period of 44 days (Fig. 2.6B and C) with no sign of toxicity (SI Fig. S2.10B). Therefore, the combination of these two drugs at 50 mg/kg each is effective in

achieving and maintaining complete tumor regression in 100% of mice for a sustained period of time.

Strategies to improve tumor-free survival in mice bearing MV4;11 tumors

We next evaluated strategies with the goal of improving tumor-free survival in mice bearing MV4;11 tumors.

Although ABT-263 shows minimal *in vivo* anticancer activity when used as a single agent, either sequential treatment protocol (ABT-263 followed by SAR405838, or the reverse) results in tumor regression. Interestingly, the sequential treatment protocol, ABT-263 first then SAR405838, yields longer tumor regression than the reverse treatment protocol (Fig. 2.6D). The rapid regression upon treatment with SAR405838 after ABT-263 suggests that MV4;11 tumors treated with ABT-263 alone fail to develop resistance to SAR405838 *in vivo*. The combination of ABT-263 and SAR405838 both at 100 mg/kg is toxic in MV4;11 tumor-bearing mice, but the combination of ABT-263 at 50 mg/kg and SAR405838 at 100 mg/kg is well tolerated (SI Fig. S2.10C). While SAR405838 at 100 mg/kg for 18 days achieves complete tumor regression in 100% of mice for an average of 7 days, the combination of ABT-263 at 50 mg/kg and SAR405838 at 100 mg/kg with a less frequent dosing-schedule (twice a week for 3 weeks) achieves and maintains complete tumor regression in 100% of mice for an average of 14 days (Fig. 2.6E and F).

Discussion

Although a number of recently developed targeted anticancer drugs are highly effective in achieving complete response in the clinic, such responses are typically short-lived and patients

relapse quickly[1,44,45]. Therefore, it is critical to identify mechanism(s) of acquired resistance and to develop rational strategies to combat such resistance with the goal of improving the outcome of patients with the disease. In the present study, we elucidated the mechanisms of acquired resistance of two classes of apoptosis-inducing agents, the Bcl-2 inhibitors ABT-737/ABT-263 and the MDM2 inhibitor SAR405838 in two acute leukemia models *in vitro* and *in vivo*.

ABT-737 and ABT-263 are highly effective in inducing apoptosis in the RS4;11 cell line *in vitro*, achieving complete regression of RS4;11 xenograft tumors in mice, but both *in vitro* and *in vivo* treatments lead to the development of sublines that have profound acquired resistance to this class of drugs. BAX, but not BAK, is consistently down-regulated in each of these resistant sublines obtained from *in vitro* and *in vivo* treatments, suggesting a critical role for BAX (Fig. 2.1F, 2.1G, SI Fig. S2.3 and S2.4). Indeed, knock-down of BAX, but not BAK, in the parental RS4;11 cell line dramatically reduces the sensitivity of the tumor cells to ABT-737/ABT-263, confirming that BAX, but not BAK, is a key mediator for apoptosis induction by these Bcl-2 inhibitors (Fig. 2.5). Interestingly, while Mcl-1 is not upregulated in those resistant sublines obtained from *in vivo* treatment of RS4;11 tumors in mice with ABT-737, it is greatly increased in those resistant sublines obtained from *in vitro* treatment of ABT-737. One critical difference is that in *in vitro* treatment, the drug is present at a constant concentration and poses a continuous high threat to the survival of tumor cells. For *in vivo* treatment, the pressure from the drug for tumor cell survival fluctuates, as a result of the pharmacokinetic effect. Therefore, while both *in vitro* and *in vivo* treatments of the RS4;11 cell line with these Bcl-2 inhibitors result in acquired resistance, the precise mechanisms whereby resistance is acquired are subtly different. While Mcl-1 upregulation has been shown to be a primary mechanism of resistance to ABT-737/ABT-

263, our study shows that loss of BAX, but not upregulation of Mcl-1, is the primary resistant mechanism when the RS4;11 tumors are treated with ABT-737 in animals[34,35].

Similarly, *in vitro* and *in vivo* treatments of the RS4;11 cell line with the MDM2 inhibitor SAR405838 also result in acquired resistance. Our analyses showed that each of these resistant sublines harbors either an inactivating mutated p53 gene for its transcriptional activity or contains p53 with compromised transcriptional activity. As a result, in these resistant sublines with defective p53, SAR405838 is either completely ineffective or much less effective in activation of p53.

It is of great interest that all RS4;11 sublines obtained from *in vitro* and *in vivo* treatments with either ABT-737 or ABT-263, while becoming highly resistant to ABT compounds, retain their sensitivity to the MDM2 inhibitor SAR405838 *in vitro* (Fig. 2.3). Significantly, when regressed RS4;11 tumors treated with ABT-263 at 100 mg/kg regrow, they become much less responsive to even a higher dose of ABT-263 (150 mg/kg, the MTD) but undergo rapid tumor regression when treated with the MDM2 inhibitor SAR405838 (Fig. 2.3F). Similarly, ABT-263 is still very effective against RS4;11 resistant sublines generated with both *in vitro* and *in vivo* treatment of SAR405838 in both cell growth inhibition and apoptosis assays (Fig. 2.4). Furthermore, when RS4;11 tumors initially treated with SAR405838 regrow, they become unresponsive to the second round of SAR405838 treatment but undergo rapid tumor regression when treated with ABT-263 (Fig. 2.4F). Hence, our results show that because RS4;11 tumor cells develop different acquired resistant mechanisms to MDM2 and Bcl-2 inhibitors, tumors cells that become highly resistant to one class of drug retain their sensitivity to the second class of drug *in vitro*. Importantly, regrown tumors that are unresponsive to the initial drug in mice still undergo rapid tumor regression when treated with the second class of drug.

The lack of cross-resistance of the RS4;11 acute leukemia cells to Bcl-2 and MDM2 inhibitors suggests that p53 and BAX play different roles in the activities of these two classes of apoptosis-inducing agents. Efficient knock-down of p53 in the parental RS4;11 cell line significantly diminishes the activity of SAR405838 but has a minimal effect on the activity of ABT-263 (Fig. 2.5). Conversely, efficient knock-down of BAX has a profound effect in reducing the potency of ABT-263, but only has a modest effect on the activity of SAR405838. Interestingly, knock-down of BAK has no effect on the activity for both SAR405838 and ABT-263. However, down-regulation of BAK in a RS4;11 subline, which already has an undetectable level of BAX, results in resistance to apoptosis induction by SAR405838. These results suggest that presence of either BAK or BAX is sufficient for induction of apoptosis by MDM2 inhibitors (Fig. 2.5).

Since both ABT-263 and SAR405838 can completely regress the RS4;11 tumors in mice and all the cell lines cultured from relapsed tumors lack cross-resistance to these two classes of drugs, we tested two sequential and one combination treatment strategies for their effectiveness in achieving tumor-free survival. Sequential treatment with ABT-263 100 mg/kg daily for a week, followed by SAR405838 100 mg/kg daily for another week, can achieve 71% longer tumor-free survival in 100% of mice (41 days) than ABT-263 as a single agent daily for 2 weeks at the same dose (24 days) and 240% longer than SAR405838 used as a single agent daily for 2 weeks at the same dose (12 days) (Fig. 2.6A). The alternative sequential treatment is only equally effective, as compared to ABT-263 as a single agent daily for 2 weeks in achieving tumor-free survival in 100% of mice (Fig. 2.6A). Combination of ABT-263 and SAR405838 at their maximum tolerated dose (50 mg/kg for both) achieves complete tumor regression in 100% of the mice for 44 days in the RS4;11 xenograft model (Fig. 2.6B and C), much longer than both

drugs as single agents at 100 mg/kg. Both single agent treatments at 50 mg/kg fail to achieve tumor regression in 100% of mice.

These sequential and combination strategies using ABT-263 and SAR405838 were further evaluated in the MV4;11 xenograft model. In this model, ABT-263 is effective in inhibition of tumor growth but fails to achieve tumor regression, whereas SAR405838 at 100 mg/kg for 18 days achieves complete tumor regression but tumors regrow shortly after the treatment ceases (Fig. 2.6). Consistent with the data obtained from the RS4;11 model, treatment with ABT-263 first daily for 9 days, followed by treatment with SAR405838 daily for 9 days, leads to longer tumor-free survival for mice than treatment with SAR405838 as a single-agent for 18 days (Fig. 2.6D). Similar to the data obtained in the RS4;11 model, the combination of ABT-263 and SAR405838 both at 100 mg/kg is toxic to mice bearing MV4;11 tumors. However, combination of ABT-263 at 50 mg/kg and SAR405838 at 100 mg/kg twice weekly for 3 weeks is well tolerated and yields prolonged tumor regression compared to SAR405838 at 100 mg/kg for 18 days (Fig. 2.6E and F).

In summary, our study has shown that acute leukemia cells develop acquired resistance *in vitro* and *in vivo* when treated with either of two classes of apoptosis inducing agents, Bcl-2 inhibitors or MDM2 inhibitors. The mechanisms of acquisition of resistance for these two different classes of drugs are however completely different. Understanding of the acquired resistant mechanisms led us to develop both sequential and combination treatment strategies with these two different classes of apoptosis-inducing agents, which can effectively improve the tumor-free survival of mice over the single-agent treatments, which should be explored in clinical trials for the treatment of acute leukemia.

Supplemental Information

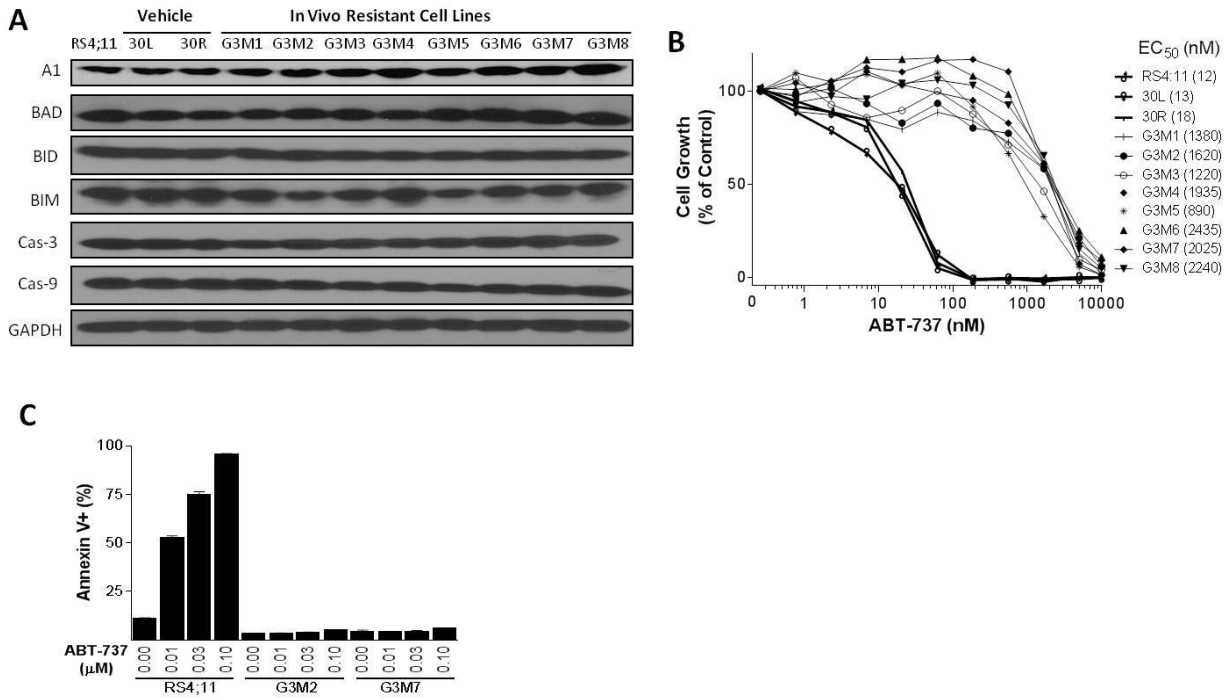

Figure S2.1 Characterization and sensitivity of ABT-737 resistant RS4;11 sublines isolated *in vivo.*

A, Immunoblotting for Bcl-2 family members and GAPDH in RS4;11 parental, vehicle (30L and 30R) and *in vivo* isolated sublines (G3M1-G3M8). B, Cells treated with ABT-737 for 4 days and cell viability was determined by WST assay. C, Cells were treated with ABT-737 for 24 h for apoptosis analysis by flow cytometry with Annexin V/P.I. double staining.

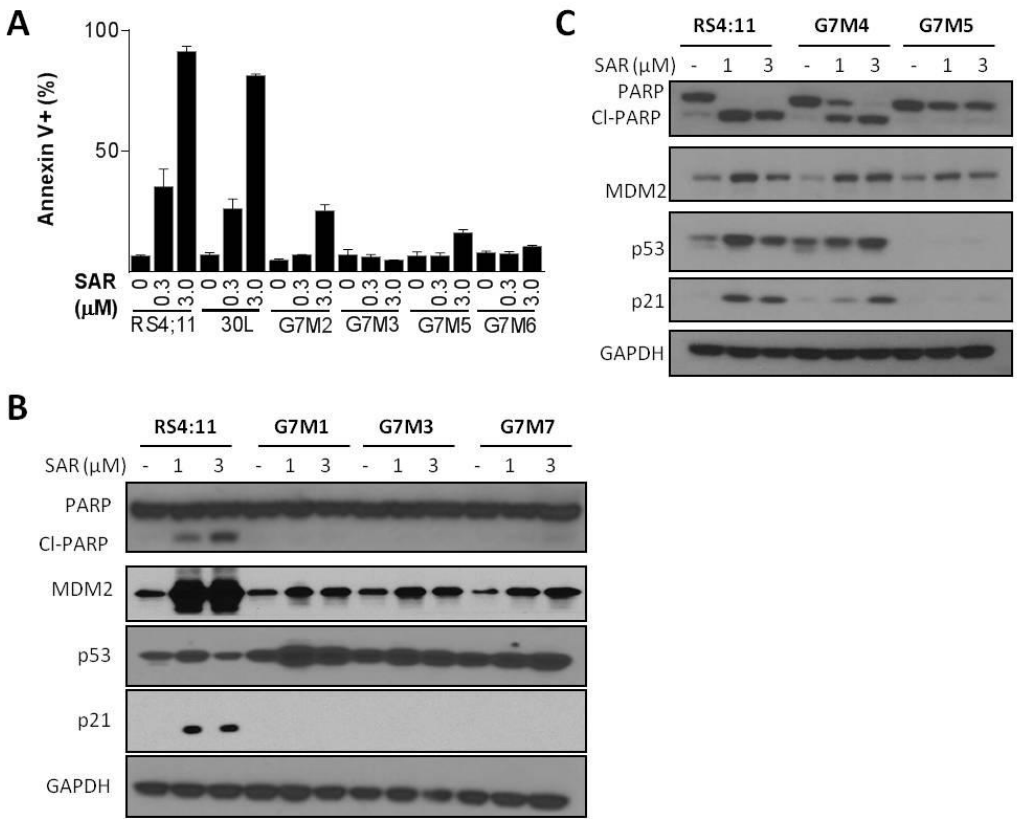

Figure S2.2 Variable Resistance to SAR405838 in the *in vivo* isolated RS4;11 sublines.
A, Cells were treated with SAR for 24 h for apoptosis analysis by flow cytometry with Annexin V/P.I. double staining. B and C, Immunoblotting showing the effect of SAR405838 after 24 h on apoptotic regulators as a function of dose.

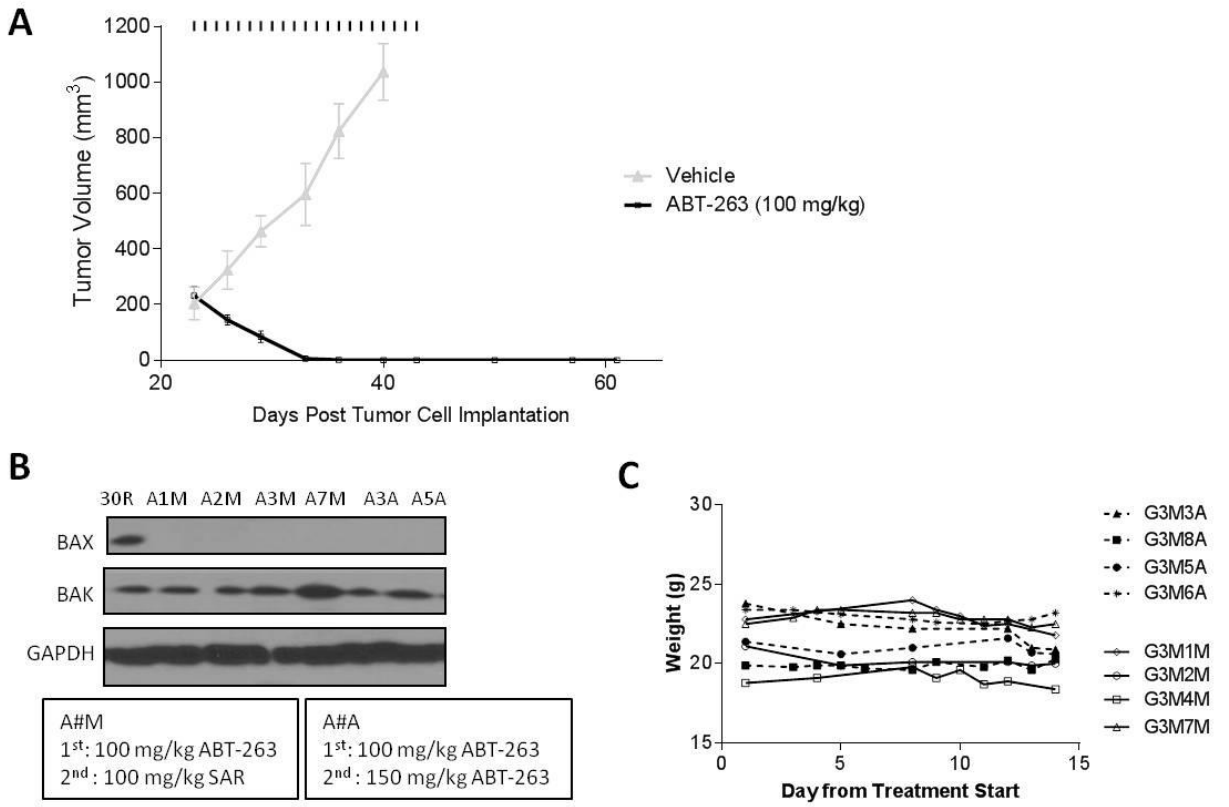

Figure S2.3 Treatment with ABT-263 followed by second treatment with ABT-263 or SAR405838.

A, Antitumor activity of RS4;11 xenografts treated with ABT-263 for 21 days at 100 mg/kg orally. B, Immunoblotting of BAX and BAK in sublines isolated from RS4;11 xenografts treated with ABT-263 first followed by a second treatment with either ABT-263 or SAR405838. C, Body weight change of mice in Fig. 2.3F.

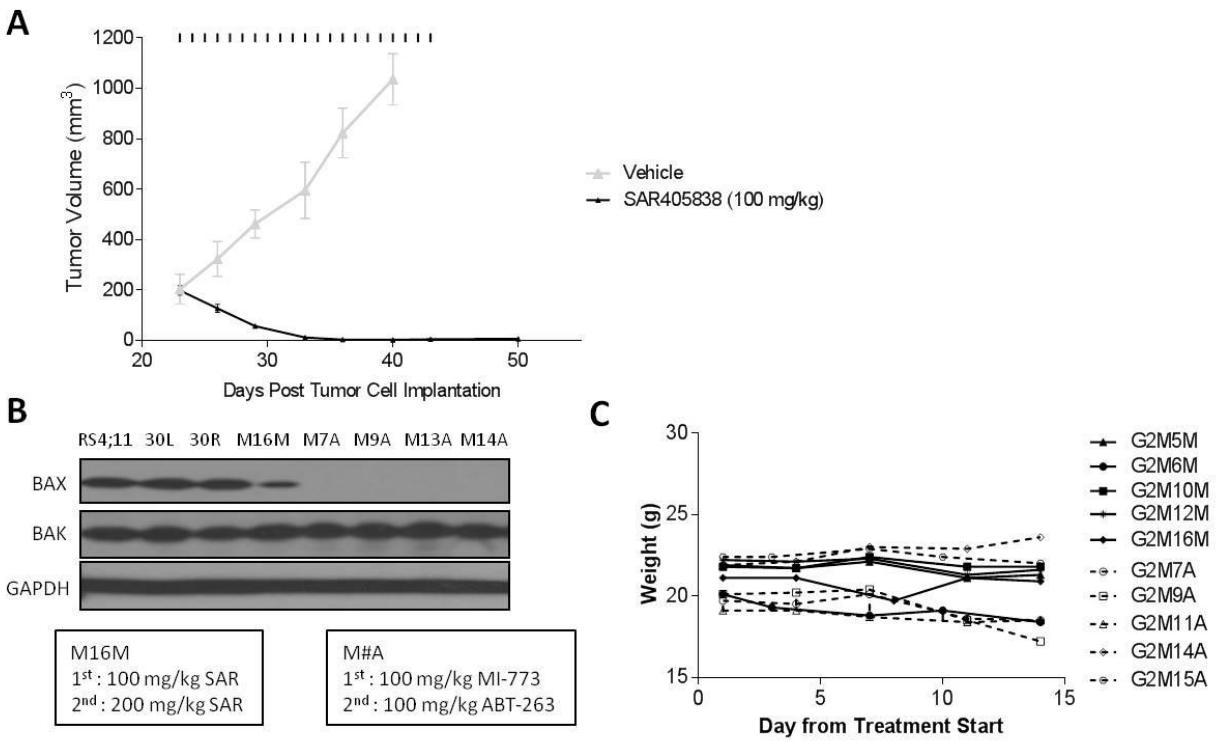

Figure S2.4 Treatment with SAR405838 followed by second treatment with ABT-263 or SAR405838.

A, Antitumor activity of RS4;11 xenografts treated with SAR405838 for 21 days at 100 mg/kg orally. B, Immunoblotting of BAX and BAK in sublines isolated from RS4;11 xenografts treated with SAR405838 first followed by a second treatment with either ABT-263 or SAR405838. C, Body weight change of mice in Fig. 2.4F.

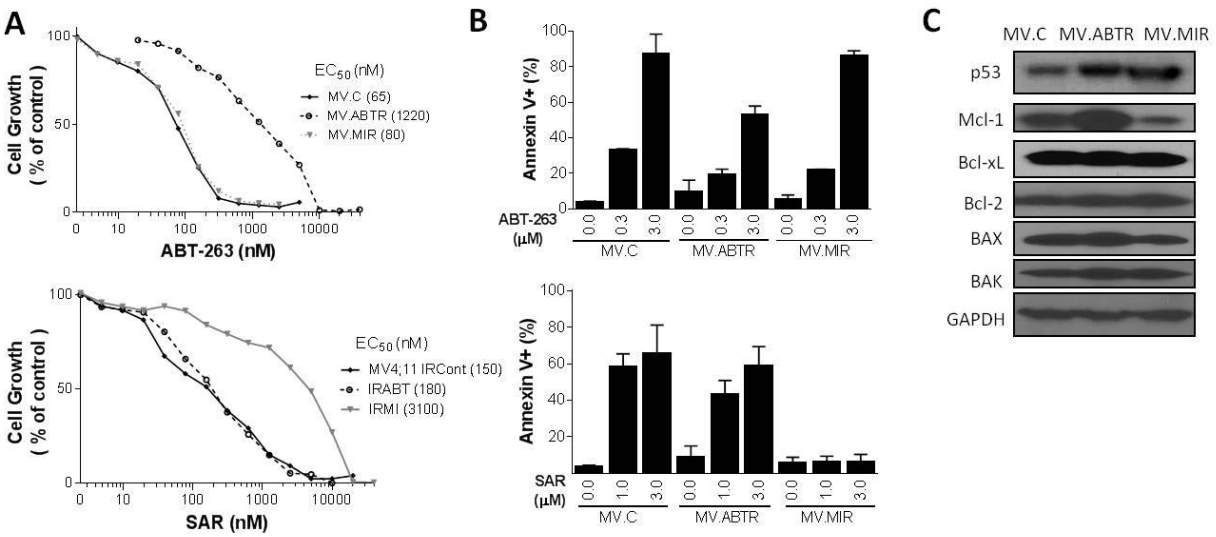

Figure S2.5 Characterization of resistance and sensitivity of ABT-263 and SAR405838 in MV4;11 *in vitro* model.

A, Cells were treated for 4 days with ABT-263 or SAR and cell viability was determined by WST assay. B, Resistant cells isolated from *in vitro* exposure to drug were treated with ABT-263 or SAR for 48 h for apoptosis analysis by flow cytometry with Annexin V/P.I. double staining. C, Immunoblotting for p53, Bcl-2 family members and GAPDH in MV4;11 resistant cells isolated from *in vitro* exposure to drug.

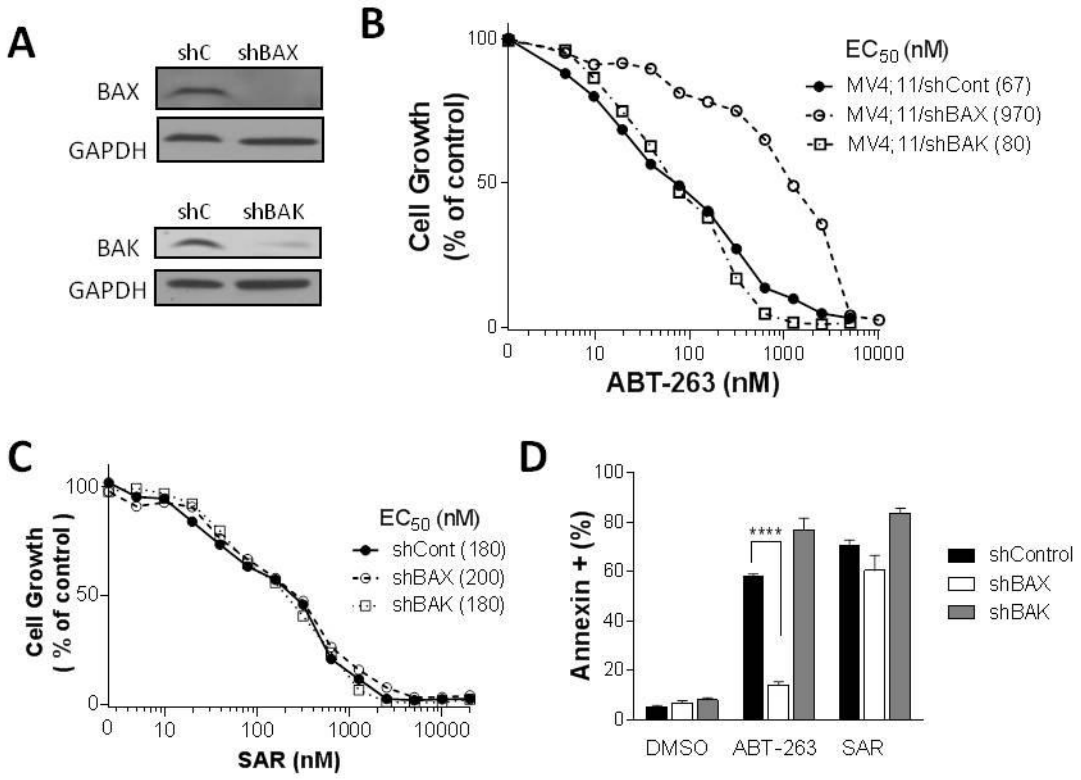

Figure S2.6 Suppression of BAX or BAK in MV4;11 cells.
A, Immunoblots showing efficiency of lentiviral knockdown of BAX and BAK. B, Cells treated with ABT-263 for 4 days and cell viability was determined by WST assay. C, cells treated with SAR for 4 days and cell viability was determined by WST assay. D, cells treated with 500 nM ABT-263 or 5 μM SAR for 48 h for apoptosis analysis by flow cytometry with Annexin V/P.I. double staining. Asterisks indicate significant difference ($P < 0.0001$).

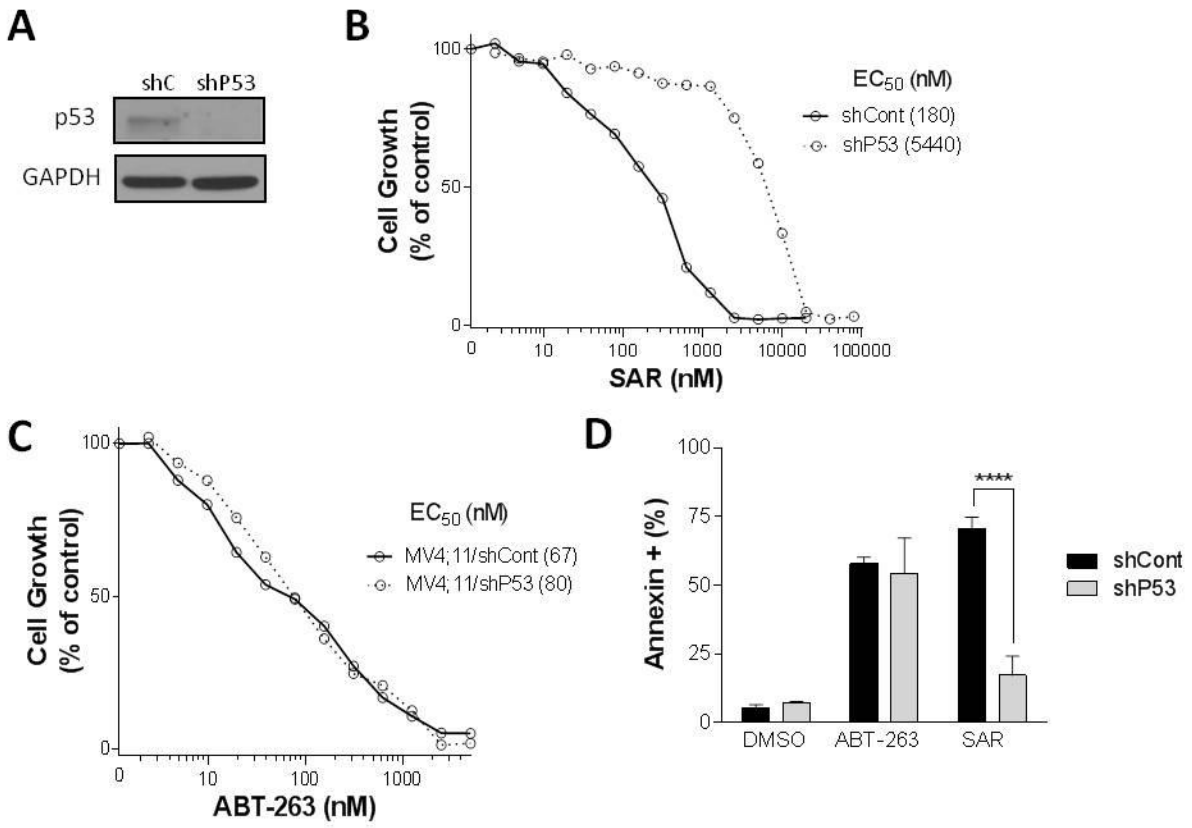

Figure S2.7 Suppression of p53 in MV4;11 confers resistance to SAR405838, but remains sensitive to ABT-263.

A, Immunoblot showing efficiency of lentiviral knockdown of p53. B and C, Cells treated with SAR or ABT-263 for 4 days and cell viability was determined by WST assay. D, Cells treated with 500 nM ABT-263 or 5 μM SAR for 48 h for apoptosis analysis by flow cytometry with Annexin V/P.I. double staining. Asterisks indicate significant difference ($P < 0.0001$).

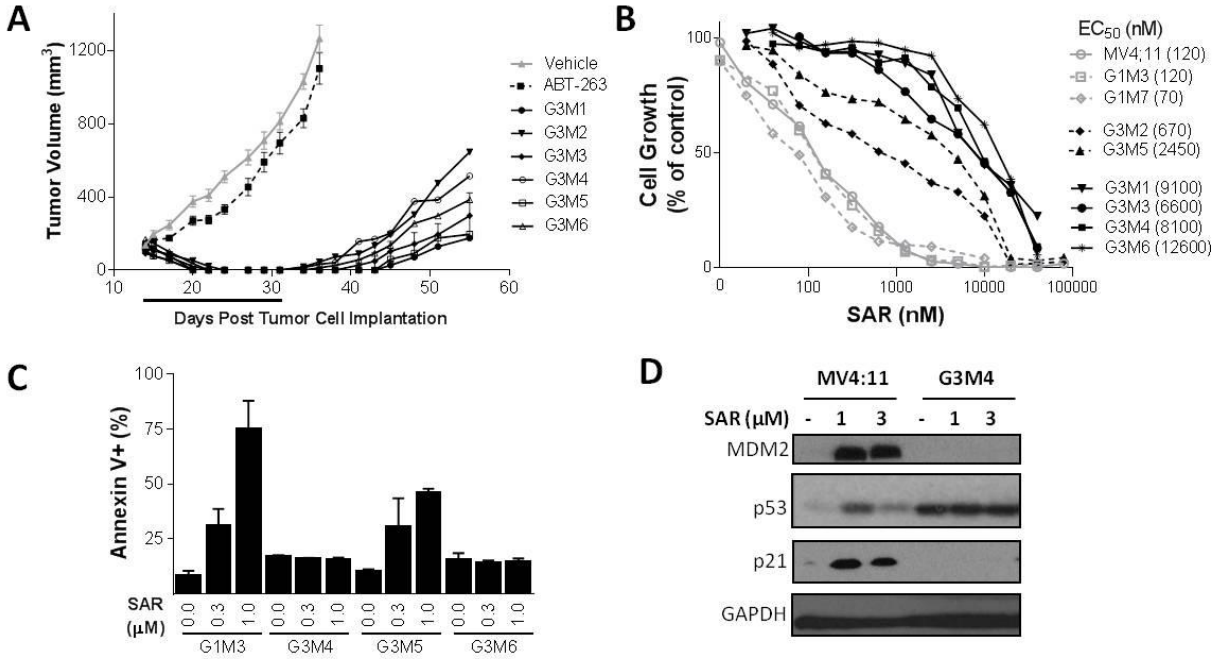

Figure S2.8 Characterization of MDM2-p53 inhibitor resistant MV4;11 sublines isolated *in vivo*.

A, MV4;11 xenograft tumors were treated with vehicle (*grey*), 100 mg/kg ABT-263 orally (*dashed line*) or 100 mg/kg of SAR orally (*black*, G3M1-G3M6) for 18 days. B, Tumor cells (G3M1-G3M6) isolated from MV4;11 xenografts were treated with SAR for 4 days and cell viability was evaluated by WST assay. C, Resistant cells isolated from *in vitro* exposure to drug were treated with ABT-263 or SAR for 48 h for apoptosis analysis by flow cytometry with Annexin V/P.I. double staining. D, Immunoblotting showing the effect of SAR after 24 h on apoptotic regulators as a function of dose.

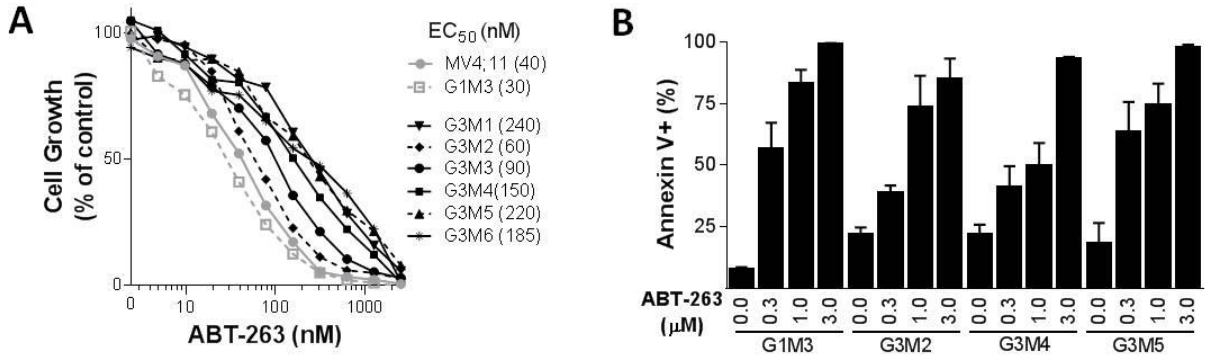

Figure S2.9 Activity of Bcl-2/Bcl-xL inhibitor in SAR405838 inhibitor resistant MV4;11 sublines isolated *in vivo*.

A, Tumor cells (G3M1-G3M6) isolated from MV4;11 xenografts were treated with SAR for 4 days and cell viability was evaluated by WST assay. B, Resistant cells isolated from *in vitro*

exposure to drug were treated with ABT-263 for 48 h for apoptosis analysis by flow cytometry with Annexin V/P.I. double staining.

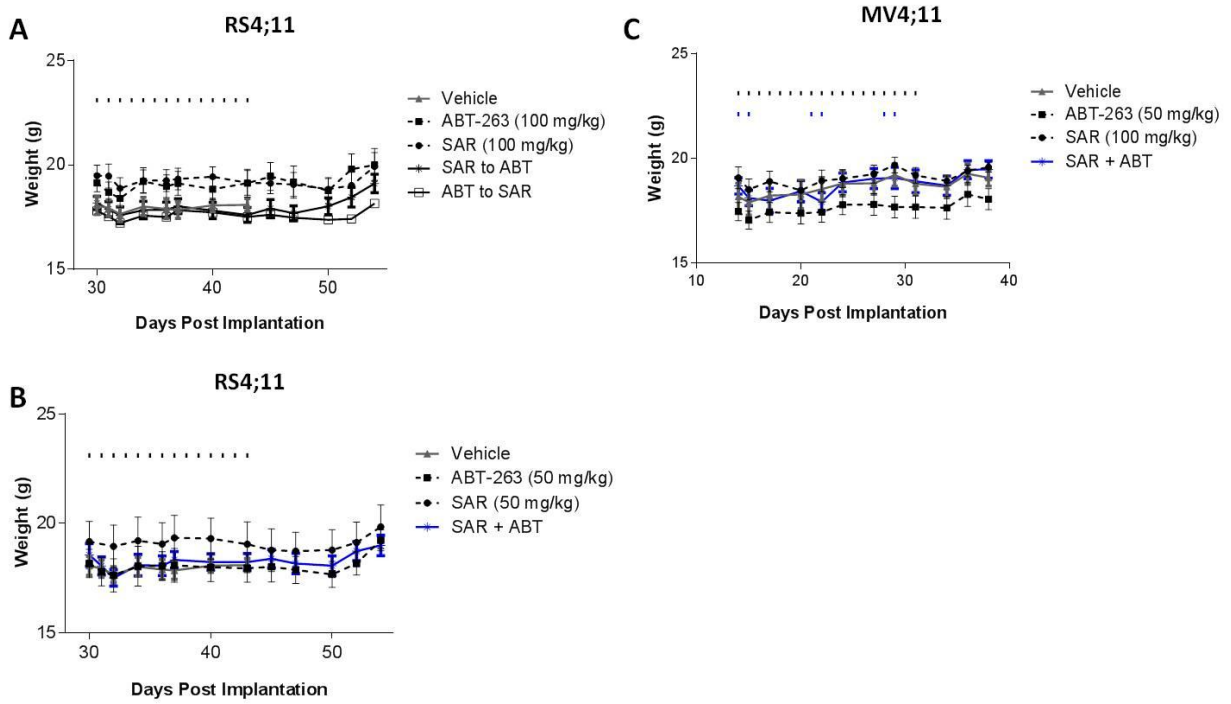

Figure S2.10 Body weight change of mice.
Body weight change in Fig. 2.6A (A), Fig. 2.6B (B) and Fig. 2.6E (C). Data shown are mean ± SEM for 6-8 mice.

Table S2.1 p53 sequencing results in RS4;11 sublines

Sample	Mutation
RS4;11	None
30L	None
30R	None
G3M1-G3M8	None
G7M1	Y220 Y/H
G7M2	I255 I/N
G7M3	S215S/G
G7M4	None
G7M5	None
G7M6	S215G
G7M7	Y236C
ContR	None
MIR1	S215 S/G
MIR2	Y236 Y/H, R249 R/G
ABTR1	None
ABTR2	None

Table S2.2 Combination index values for inhibitory effects of ABT-263 and SAR405838 on cell growth at a 1:1 ratio on cell growth analyzed by WST assay after 4 days.

	At ED_{50}	At ED_{75}
RS4;11	0.46	0.71
MV4;11	0.87	0.68

Table S2.3 Combination index values for apoptotic effects of ABT-263 and SAR405838.
Combination index values for apoptotic effects of ABT-263 and SAR405838 in the presence of increasing doses of ABT-263 and SAR405838 or combinations of the two agents at a 1:10 ratio for RS4;11 and a 1:1 ratio for MV4;11. Apoptosis was measured by Annexin V/P.I. double staining.

	At ED_{50}	At ED_{75}
RS4;11	0.29	0.11
MV4;11	0.34	0.25

References

1. Pui, C.H. & Evans, W.E. Treatment of acute lymphoblastic leukemia. *N Engl J Med* **354**, 166-78 (2006).
2. Hauser, U. *et al.* Reliable detection of p53 aberrations in squamous cell carcinomas of the head and neck requires transcript analysis of the entire coding region. *Head Neck* **24**, 868-73 (2002).
3. Hanahan, D. & Weinberg, R.A. The hallmarks of cancer. *Cell* **100**, 57-70 (2000).
4. Danial, N.N. & Korsmeyer, S.J. Cell death: critical control points. *Cell* **116**, 205-19 (2004).
5. Cory, S. & Adams, J.M. The Bcl2 family: regulators of the cellular life-or-death switch. *Nat Rev Cancer* **2**, 647-56 (2002).
6. Martinou, J.C. & Youle, R.J. Mitochondria in apoptosis: Bcl-2 family members and mitochondrial dynamics. *Dev Cell* **21**, 92-101 (2011).
7. Chao, D.T. & Korsmeyer, S.J. BCL-2 family: regulators of cell death. *Annu Rev Immunol* **16**, 395-419 (1998).
8. Gross, A., McDonnell, J.M. & Korsmeyer, S.J. BCL-2 family members and the mitochondria in apoptosis. *Genes Dev* **13**, 1899-911 (1999).
9. Campos, L. *et al.* High expression of bcl-2 protein in acute myeloid leukemia cells is associated with poor response to chemotherapy. *Blood* **81**, 3091-6 (1993).
10. Robinson, B.W. *et al.* Abundant anti-apoptotic BCL-2 is a molecular target in leukaemias with t(4;11) translocation. *Br J Haematol* **141**, 827-39 (2008).
11. Oltersdorf, T. *et al.* An inhibitor of Bcl-2 family proteins induces regression of solid tumours. *Nature* **435**, 677-81 (2005).
12. Tse, C. *et al.* ABT-263: a potent and orally bioavailable Bcl-2 family inhibitor. *Cancer Res* **68**, 3421-8 (2008).
13. Merino, D. *et al.* Bcl-2, Bcl-x(L), and Bcl-w are not equivalent targets of ABT-737 and navitoclax (ABT-263) in lymphoid and leukemic cells. *Blood* **119**, 5807-16 (2012).
14. Roberts, A.W. *et al.* Substantial susceptibility of chronic lymphocytic leukemia to BCL2 inhibition: results of a phase I study of navitoclax in patients with relapsed or refractory disease. *J Clin Oncol* **30**, 488-96 (2012).
15. Vassilev, L.T. p53 Activation by small molecules: application in oncology. *J Med Chem* **48**, 4491-9 (2005).
16. Vogelstein, B., Lane, D. & Levine, A.J. Surfing the p53 network. *Nature* **408**, 307-10 (2000).
17. Levine, A.J. p53, the cellular gatekeeper for growth and division. *Cell* **88**, 323-31 (1997).
18. el-Deiry, W.S. Regulation of p53 downstream genes. *Semin Cancer Biol* **8**, 345-57 (1998).
19. Vousden, K.H. & Lu, X. Live or let die: the cell's response to p53. *Nat Rev Cancer* **2**, 594-604 (2002).
20. Fridman, J.S. & Lowe, S.W. Control of apoptosis by p53. *Oncogene* **22**, 9030-40 (2003).
21. Mitani, N., Niwa, Y. & Okamoto, Y. Surveyor nuclease-based detection of p53 gene mutations in haematological malignancy. *Ann Clin Biochem* **44**, 557-9 (2007).
22. Freedman, D.A., Wu, L. & Levine, A.J. Functions of the MDM2 oncoprotein. *Cell Mol Life Sci* **55**, 96-107 (1999).
23. Wu, X., Bayle, J.H., Olson, D. & Levine, A.J. The p53-mdm-2 autoregulatory feedback loop. *Genes Dev* **7**, 1126-32 (1993).

24. Momand, J., Wu, H.H. & Dasgupta, G. MDM2--master regulator of the p53 tumor suppressor protein. *Gene* **242**, 15-29 (2000).
25. Bond, G.L., Hu, W. & Levine, A.J. MDM2 is a central node in the p53 pathway: 12 years and counting. *Curr Cancer Drug Targets* **5**, 3-8 (2005).
26. Shangary, S. & Wang, S. Targeting the MDM2-p53 interaction for cancer therapy. *Clin Cancer Res* **14**, 5318-24 (2008).
27. Shangary, S. *et al.* Temporal activation of p53 by a specific MDM2 inhibitor is selectively toxic to tumors and leads to complete tumor growth inhibition. *Proc Natl Acad Sci U S A* **105**, 3933-8 (2008).
28. Vassilev, L.T. MDM2 inhibitors for cancer therapy. *Trends Mol Med* **13**, 23-31 (2007).
29. Vassilev, L.T. *et al.* In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. *Science* **303**, 844-8 (2004).
30. Tovar, C. *et al.* MDM2 small-molecule antagonist RG7112 activates p53 signaling and regresses human tumors in preclinical cancer models. *Cancer Res* **73**, 2587-97 (2013).
31. Carol, H. *et al.* Initial testing of the MDM2 inhibitor RG7112 by the Pediatric Preclinical Testing Program. *Pediatr Blood Cancer* **60**, 633-641 (Pediatr Blood Cancer. 2013).
32. Wang, S. *et al.* SAR405838: An optimized inhibitor of MDM2-p53 interaction that induces complete and durable tumor regression. *Cancer Res* (2014).
33. Gottesman, M.M. Mechanisms of cancer drug resistance. *Annu Rev Med* **53**, 615-27 (2002).
34. Yecies, D., Carlson, N.E., Deng, J. & Letai, A. Acquired resistance to ABT-737 in lymphoma cells that up-regulate MCL-1 and BFL-1. *Blood* **115**, 3304-13 (2010).
35. Konopleva, M. *et al.* Mechanisms of apoptosis sensitivity and resistance to the BH3 mimetic ABT-737 in acute myeloid leukemia. *Cancer Cell* **10**, 375-88 (2006).
36. Aziz, M.H., Shen, H. & Maki, C.G. Acquisition of p53 mutations in response to the non-genotoxic p53 activator Nutlin-3. *Oncogene* **30**, 4678-86 (2011).
37. Michaelis, M. *et al.* Adaptation of cancer cells from different entities to the MDM2 inhibitor nutlin-3 results in the emergence of p53-mutated multi-drug-resistant cancer cells. *Cell Death Dis* **2**, e243 (2011).
38. Kojima, K. *et al.* Decreased sensitivity of 17p-deleted chronic lymphocytic leukemia cells to a small molecule BCL-2 antagonist ABT-737. *Cancer* **118**, 1023-31 (2012).
39. Zhao, Y. *et al.* A potent small-molecule inhibitor of the MDM2-p53 interaction (MI-888) achieved complete and durable tumor regression in mice. *J Med Chem* **56**, 5553-61 (2013).
40. Lu, J. *et al.* SM-164: a novel, bivalent Smac mimetic that induces apoptosis and tumor regression by concurrent removal of the blockade of cIAP-1/2 and XIAP. *Cancer Res* **68**, 9384-93 (2008).
41. Lois, C., Hong, E.J., Pease, S., Brown, E.J. & Baltimore, D. Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. *Science* **295**, 868-872 (2002).
42. Chou, T.C. & Talalay, P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv Enzyme Regul* **22**, 27-55 (1984).
43. Kang, M.H. *et al.* Activity of vincristine, L-ASP, and dexamethasone against acute lymphoblastic leukemia is enhanced by the BH3-mimetic ABT-737 in vitro and in vivo. *Blood* **110**, 2057-66 (2007).
44. Rudin, C.M. *et al.* Phase II study of single-agent navitoclax (ABT-263) and biomarker correlates in patients with relapsed small cell lung cancer. *Clin Cancer Res* **18**, 3163-9 (2012).
45. Zauli, G., Voltan, R., Tisato, V. & Secchiero, P. State of the art of the therapeutic perspective of sorafenib against hematological malignancies. *Curr Med Chem* **19**, 4875-84 (2012).

Chapter 3

Critical difference in development of acquired resistance to MDM2 inhibitor SAR405838 *in vitro* and *in vivo*

Abstract

SAR405838 is a potent and specific MDM2 inhibitor currently being evaluated in Phase I clinical trials for the treatment of human cancer. In the present study, we investigated the development of acquired resistance to SAR405838 treatment *in vitro* and the lack of resistance *in vivo* using the SJSA-1 osteosarcoma cell line, which harbors amplified *MDM2* gene and wild-type p53. *In vitro* treatment of the SJSA-1 cells with SAR405838 resulted in acquired resistance to the drug. Analysis of these resistant cells showed that p53 is mutated in the DNA binding domain resulting in resistance to SAR405838 *in vitro*. Treatment of mice bearing the parental SJSA-1 xenograft tumors with SAR405838 led to rapid tumor regression, but tumors eventually returned after treatment cessation. A number of cell lines were established by culturing the regrown tumors and, surprisingly, these sublines showed minimal loss of sensitivity to SAR405838. Consistently, analysis of these sublines showed that p53 retains its wild-type status, with the exception of one subline, that harbored a single heterozygous mutation C176F located in the DNA binding domain. Computational modeling suggested that the p53 C176F mutant is still functional. Interestingly, cell lines established from xenografts of a representative subline

lacking the p53 C176F mutation treated with a second round of SAR405838 *in vivo* were still responsive to SAR405838, albeit with reduced sensitivity. Xenografts of a representative subline harboring the p53 C176F mutation treated with a second round of SAR405838 *in vivo* resulted in complete tumor regression. Our study showed that the SJSA-1 tumor cells acquired profound resistance to MDM2 inhibitor SAR405838 *in vitro* and developed inactivating p53 mutations in its DNA binding domain. Conversely, *in vivo* SJSA-1 tumor cells retained sensitivity to the MDM2 inhibitor after multiple treatment rounds of SAR405838. A deep sequencing study indicates that SAR405838 induces p53 mutations not initially present in the parental cell population. Our study suggests that tumor cells respond differently to drug treatment under *in vitro* and *in vivo* environments. Furthermore, these data suggest that both *in vitro* and *in vivo* models of resistance should be investigated in order to better identify resistance that will occur in a clinical setting.

Introduction

The tumor suppressor p53 is a regulator of cell cycle, apoptosis, DNA repair and senescence[1-4]. Approximately 50% of cancers maintain p53 wild-type status, but its function is tightly controlled by its primary cellular inhibitor, the MDM2 oncoprotein[5-8]. MDM2 acts by blocking the transactivation domain of p53, resulting in inhibition of p53 function[9]. p53 can activate MDM2 expression and upregulate MDM2 protein, which represses p53 by three mechanisms: 1) MDM2 binds to p53 and blocks its ability to activate transcription; 2) MDM2 ubiquitinates p53 and promotes its degradation; and 3) MDM2 is involved in the nuclear export of p53, thus rendering p53 inaccessible to its target DNAs[6,10]. These three inhibitory mechanisms of p53 by MDM2 are mediated by their direct protein-protein interaction. Due to the crucial role

of p53, targeting the MDM2-p53 interaction using non-peptide, cell permeable small-molecule inhibitors is being pursued as a new and novel therapeutic strategy for the treatment of human cancers retaining wild-type p53[9,11,12]. Recently, small-molecule inhibitors have been designed to block the MDM2-p53 interaction to activate p53. Seven highly potent MDM2 inhibitors, including RG7112[13,14] and SAR405838[15], also known as MI-77301, designed in our laboratory, are now in clinical trials for cancer treatment.

One critical question in the development and use of MDM2 inhibitors for cancer treatment is their potential to select for or acquire p53 mutations. It is well known that p53 wild-type cancer cells respond better to cancer therapies than p53 mutated cancer cells[16-18]. Current cancer treatments, such as radiation and chemotherapy agents that damage DNA can result in the acquisition of p53 mutations resulting in secondary cancers[19,20]. Initially it was hypothesized that activation of p53 through a non-genotoxic method, for example the MDM2 inhibitors, would avoid selection or acquisition of p53 mutated cells. However, this hypothesis was disproven in studies utilizing Nutlin-3, a small molecule MDM2 antagonist[21-23]. These studies exposed osteosarcoma, neuroblastoma, rhabdomyosarcoma and melanoma cell lines to Nutlin-3 for extended periods of time *in vitro*, which resulted in highly resistant sublines[21,24]. Several varying p53 mutations were identified in the resistant sublines. The authors state that because different p53 mutations were detected in separate experiments it can be argued that these mutated cells arose during the Nutlin treatment course and were not already present in the parental cell population. Although these studies suggest that p53 mutated cells are not being selected for under drug pressure, no studies have analyzed the presence of p53 mutated cells in the bulk parental population of cells before exposure to drug.

Despite impressive initial antitumor activity, the complete tumor regression in xenograft models of p53 wild-type cancers achieved by Nutlin and RG7112[25] is transitory; tumors eventually regrew after the treatments were terminated, suggesting emergence of resistance. Such acquired resistance is in general, a major cause of cancer drug failure in clinical trials[26]. To date, MDM2 inhibitors mechanisms of acquired drug resistance have only been studied in sublines that have developed resistance after long term exposure to drug *in vitro*[21,24], no studies have investigated acquired resistance developed *in vivo* to MDM2 inhibitors. Because a drug may induce different levels or mechanisms of resistance *in vitro* and *in vivo* environments[27-29], we have investigated the development of resistance to MDM2 inhibitor SAR405838 both *in vitro* and *in vivo* in the MDM2 amplified osteosarcoma (SJSA-1) cell line.

Our study has elucidated critical differences in the development of resistance *in vitro* versus *in vivo* resulting in exposure to MDM2 inhibitors and has shed light on the critical question of whether resistance to MDM2 inhibitors results from acquisition or selection of p53 mutated cells. Furthermore, we have identified that several rounds of SAR405838 treatment *in vivo* does not result in highly resistant cancer cells.

Materials and Methods

Reagents and antibodies. SAR405838 was synthesized using a method similar to that for MI-888[30]. The following primary antibodies were used: MDM2 (SMP-14, sc-965) and GAPDH (sc-5778) from Santa Cruz Biotechnology, p53 (DO-1, OP43) from Millipore and p21 (12D1) from Cell Signaling.

Cell Culture, cell viability, and apoptosis assays. SJSA-1 cell lines were purchased from American Type Culture Collection (ATCC) and cultured as recommended. Cell viability was evaluated by a WST-8 assay (Dojindo[31]). Apoptosis was analyzed using Annexin V-FLUOS staining kit (Roche Applied Science, Indianapolis, IN).

Stable short hairpin interfering RNA constructs. Published papers were used as guides to generate short 19-bp hairpins for RNA interference: p53 (nucleotides 611-629 Genbank NM000546)[32]. The oligonucleotides were annealed and ligated into a self-inactivating lentiviral vector under the control of the H1 promoter[33]. The vector used was also designed to carry the GFP reporter gene under control of the human ubiquitin-C promoter to monitor infection efficiency. A scrambled shRNA construct was also utilized as a control[32]. Lentiviral shRNA virus-containing supernatant was generated by the University of Michigan Vector Core. Virus-containing supernatant was used to infect SJSA-1 cells. At 96 h post-infection, the cells were sorted for GFP fluorescence and used for all subsequent experiments.

p53 Mutation Analysis. The p53 status was determined by sequence analysis. Total RNA was extracted with RNeasy Mini Kit from Qiagen Inc (Valencia, CA). Complimentary DNA (cDNA) was prepared by reverse transcription using SuperScript III First-Strand Synthesis SyperMix system from Invitrogen Corporation (Carlsbad, CA) following the manufacturer's protocol. Primers to amplify and sequence cDNA for exons 2 to 11 of human p53 were used from Aziz *et al.*[21]. The amplified p53 cDNA was sequenced by the University of Michigan Sequencing Core. Mutation surveyor (SoftGenetics LLC) software was used to compare

experimental sequences against Refseq GenBank as well as by visual inspection of sequence tracings.

***In vivo* xenograft studies.** To develop xenograft tumors, 5 x 10^6 tumor cells with 50% Matrigel were injected subcutaneously on the dorsal side of SCID mice. All groups had 6-8 mice. Tumor sizes and animal weights were measured 2-3 times per week with tumor volume (mm^3) = (length x $width^2$)/2. Tumor growth inhibition was calculated by the formula: 100% x (mean volume of controls - mean volume of treated)/ mean volume of controls at end of treatment.

Statistical Analysis. Differences in mean values of cell apoptosis among different groups were analyzed by 2-way ANOVA. For *in vivo* studies, significance (*P*) was calculated by Students *t* test, with a *P* value of less than 0.05 being considered significant. All statistical tests were 2-sided, and all statistical analysis was carried out using GraphPad Prism 6.

PacBio Experiment.

Amplification of TP53 was performed with PCR primers (see p53 Mutation Analysis). This amplicon was deeply sequenced on the Pacific Biosciences SMRT sequencer (PacBio). From the one PacBio flowcell 61,438 reads were generated, 43,445 mapped to the TP53 mRNA consensus sequence. Reads were examined for minor variant detection, using the RS_Minor_Variant module from PacBio.

Computational Modeling. The crystal structure of tetrameric p53 and the DNA of the BAX response element with the PDB entry: 4HEJ [34] was used in the computational modeling. In

preparation of the simulations, we have used the MOE program (Chemical Computing Group, Montreal, Quebec, Canada) to determine the protonation state of ionizable groups on p53 under the standard physiological condition. The MOE program was also used to perform the mutation of C176F in p53. In the C176F modeling, we selected the Phe rotamer that is not in van der Waal clashes with neighboring atoms. PMEMD from Amber (version 12)[35] was used for molecular dynamics simulations. The Amber 99SB force field parameters[36] were used for the amino acids. The force field parameters for the zinc ion were from Lu et. al[37].

To prepare the topology and coordinate files, counter ions were added to neutralize the charges in p53/DNA first and the system is then placed in a 13Å octahedral box of water (the TIP3P[38] water model). A minimization procedure of 1-1000 steps using conjugated gradient followed by 2000 steps steepest decent was first carried out. Then, a 500 ps constant volume and constant temperature (NVT) simulation was performed to raise the temperature of the system to 298K while constraining backbone atoms with a 5 kcal/mol/Å^2 force constant with reference to the crystal structure. A second 200 ps constant pressure and constant temperature (NPT) simulation at 298 K was performed while constraining backbone atoms with a 2 kcal/mol/Å^2 force constant with reference to the crystal structure. The system is then ready for the 6 ns production run in the isobaric isothermal (NPT, T = 298K and P = 1 atm) ensemble. A harmonic constraint with a 2 kcal/mol/Å^2 force constant was applied to the backbone atoms of the DNA double helix to ensure its integrity during the simulations. The SHAKE[39] algorithm was used to fix bonds involving hydrogen. The PME method[40] was used and the non-bonded cutoff distance was set at 12Å. The time step was 2 fs, and neighboring pairs list was updated every 20 steps. All the graphics were prepared using the PyMol program.

Results

Establishment and characterization of *in vitro* SJSA-1 sublines resistant to SAR405838

The MDM2 inhibitor SAR405838 effectively inhibits cell growth with IC_{50} = 120 nM in the SJSA-1 cell line, which has amplified MDM2 and wild-type p53 status (Fig. 3.1A).

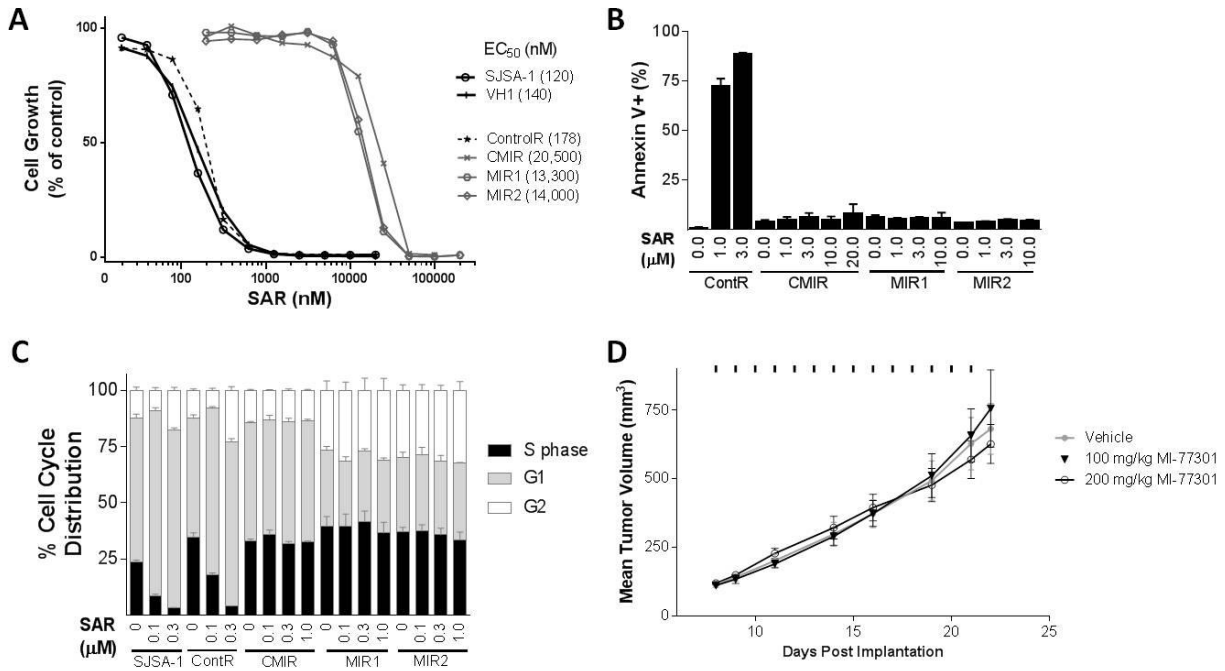

Figure 3.1 Characterization of MDM2-p53 interaction inhibitor resistant SJSA-1 sublines isolated *in vitro*

A, SJSA-1 cells developed resistance to MDM2 inhibitor *in vitro.* Cells were treated for 4 days with SAR405838 and cell viability was determined by WST assay. B, Cells were treated with SAR405838 for 48 h for apoptosis analysis with Annexin V/P.I. double staining. Data (mean ± SD) are from triplicates, including both early (Annexin V-positive/PI-negative) and late (Annexin V-positive/PI-positive) apoptotic cells. C, Cells were treated with SAR405838 for 24 h for cell cycle analysis by flow cytometry with P.I. staining. D, SCID mice bearing MIR2 tumors were treated with vehicle, 100 mg/kg or 200 mg/kg of SAR405838 by oral gavage for 14 days.

To investigate the acquired resistance of the SJSA-1 cell line *in vitro* to the MDM2 inhibitor, the SJSA-1 cells were exposed to SAR405838 *in vitro* by two different protocols (SI Fig. S3.1). We established an SJSA-1 subline (CMIR) by exposing SJSA-1 cells to 1 μM of SAR405838 for 72 hrs. The cells were then rinsed to remove SAR405838 and the remaining

cells were expanded in normal medium. This process was repeated 8 times and surviving cells were utilized for subsequent experiments (SI Fig. S3.1A). Conversely, an alternative protocol was used to establish SJSA-1 sublines (MIR1 and MIR2) by gradually increasing concentrations of SAR405838 (500 nM up to 20 μM) (SI Fig. S1B)[41]. These three SJSA-1 sublines exhibit >100-fold resistance to SAR405838 in a cell growth assay compared to the parental and control SJSA-1 cell lines (Fig. 3.1A). Resistance to SAR405838 was further confirmed by loss of apoptosis induction and cell cycle arrest (Fig. 3.1B and C).

Since the activity of MDM2 inhibitors is known to depend upon wild-type p53, we analyzed p53 mutation by sequencing exons 2-11 in the *in vitro* sublines obtained by SAR405838 treatment. All four SJSA-1 *in vitro* sublines and were found to harbor p53 mutation(s) in the DNA binding domain (SI Table S3.1).

To further asses the resistance of the *in vitro* SJSA-1 sublines, we investigated the efficacy of SAR405838 in xenografts of MIR2, which harbors a R273C mutation in p53. MIR2 xenografts treated with SAR405838 at both 100 mg/kg and 200 mg/kg daily for two weeks did not respond to treatment with no difference in tumor volume compared to the vehicle control group (Fig. 3.1D and SI Fig. S3.2A). Tumors were harvested after treatment cessation and analyzed for p53 mutations. All harvested tumor cell lines harbored the initially present heterozygous R273C mutation (Table S3.1B).

Taken together, these data show that treatment of the SJSA-1 cell line *in vitro* by SAR405838 yielded SAR405838-resistant sublines with p53 mutation(s) that are resistant to both *in vitro* and *in vivo* exposure to SAR405838.

Establishment and characterization of *in vivo* SJSA-1 sublines treated with one round of SAR405838

In vivo treatment of the SJSA-1 xenograft tumors in mice with 100 mg/kg of SAR405838 for two weeks induced rapid and complete tumor regression, which persisted for >40 days, but eventually the tumors returned (Fig. 3.2A and SI Fig. S3.2B). We isolated SJSA-1 tumor cells from the regrown tumors and established 5 sublines (7.2-7.4, 7.6 & 7.7). While the SJSA-1 parental cell line and the vehicle treated tumor subline (VH1) are highly sensitive in a cell growth assay to SAR405838, all the 5 sublines obtained from the SAR405838 treated tumors have IC_{50} values exhibiting a very subtle decrease in sensitivity with a 2 to 3-fold greater resistance than the control cell lines (Fig. 3.2B). Apoptosis and cell cycle analysis by flow cytometry confirmed that sublines remain sensitive to SAR405838, albeit with reduced sensitivity (Fig. 3.2C). We analyzed the p53 mutation status of the *in vivo* sublines obtained by SAR405838 treatment. Analysis showed that four out of the five sublines retained p53 wild-type status, with the exception of one subline (7.2), that harbored a single heterozygous mutation C176F (Table S3.2).

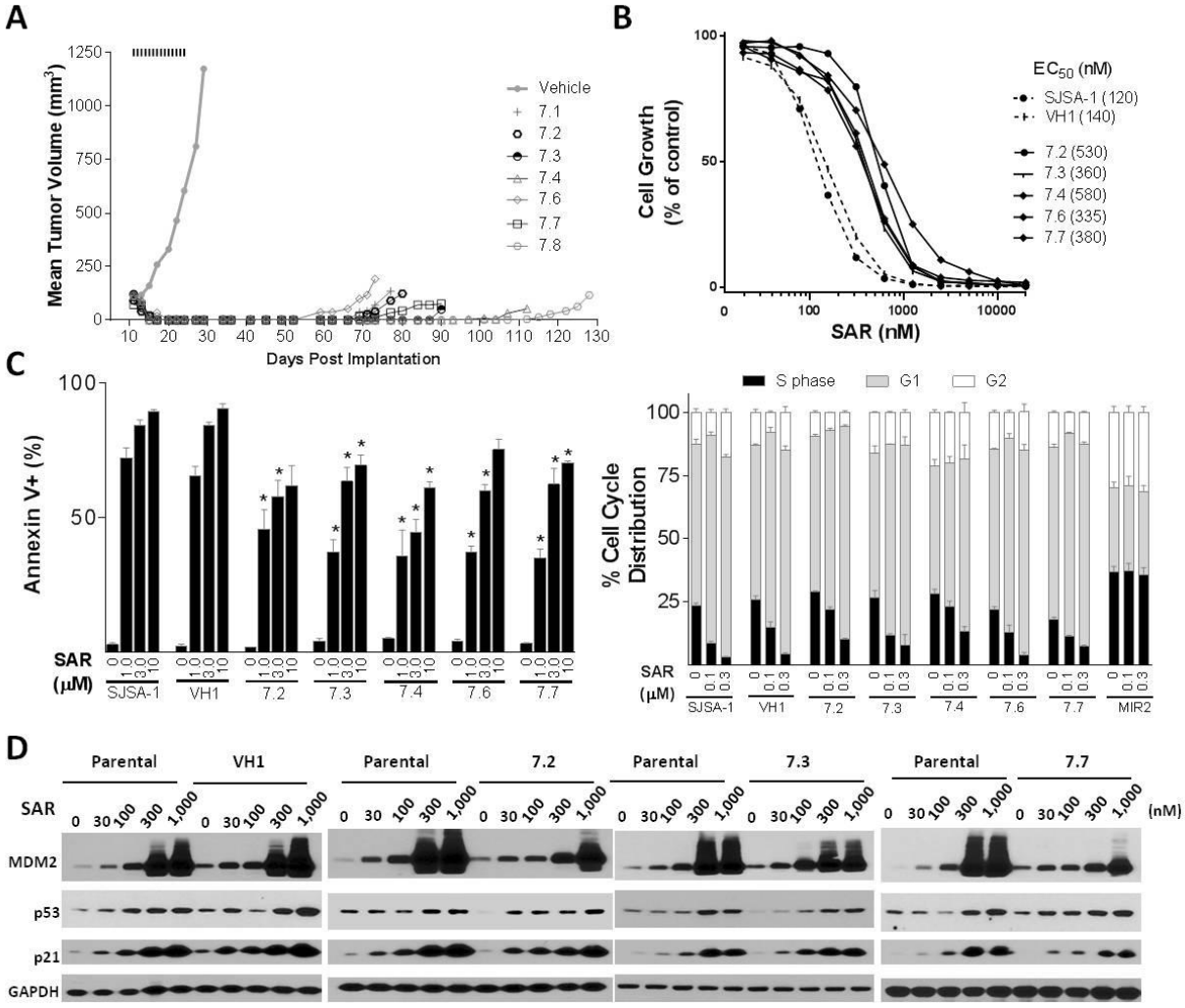

Figure 3.2 Characterization of MDM2 inhibitor *in vivo* treated SJSA-1 sublines isolated after a single round of SAR405838 treatment

A, SJSA-1 xenograft tumors were treated with vehicle or 100 mg/kg of SAR405838 orally for 14 days. B, Tumor cells (7.2-7.4, 7.6 and 7.7) isolated from SJSA-1 xenografts and vehicle treated xenograft (VH1) were treated with SAR405838 for 4 days and cell viability was evaluated by a WST assay. C, Examination of apoptosis induction and cell cycle arrest by flow cytometry. Cells were treated with SAR405838 for 48 h for apoptosis analysis by flow cytometry with Annexin V/P.I. double staining. The ttest was used to compare *in vivo* sublines to parental SJSA-1 cells. *, $P < 0.05$. Cells were treated with SAR405838 for 24 h for cell cycle analysis by flow cytometry with P.I. staining. D, Western blot analysis of p53, MDM2, p21 and GAPDH protein level in SJSA-1 parental, vehicle (VH1) and *in vivo* (7.2, 7.3 & 7.7) isolated sublines after 24 h treatment with SAR405838 at increasing concentrations.

To investigate if the *in vivo* sublines obtained from treatment with SAR405838 still retained functional p53, we examined induction of p21 and MDM2 and accumulation of p53

protein by SAR405838 treatment. In all the sublines, including the subline which harbored a p53 mutation, SAR405838 induces a dose-dependent increase of MDM2, p21 and p53 proteins, indicating p53 is still functional (Fig. 3.2D).

These data show that SJSA-1 cells treated *in vivo* with SAR405838 do not develop resistance to SAR405838 after one round of treatment.

Establishment and characterization of *in vivo* 7.6/SJSA-1 sublines treated with multiple rounds of SAR405838

In an attempt to isolate SJSA-1 cells resistant to SAR405838 after *in vivo* treatment, we treated the 7.6 subline (wild-type p53) xenografts with two more rounds of SAR405838 treatment (Fig. 3.3A). Mice harboring xenografts at approximately 100 mm^3 were treated with 100 mg/kg of SAR405838 for two weeks and tumors regressed during treatment. Tumors regrew after treatment cessation to approximately 100 mm^3 and were treated with 200 mg/kg for 8 days and tumors regressed with no sign of toxicity (SI Fig. S3.2C). Treated tumors were harvested and cultured resulting in 7 sublines (G2M1, G2M2, G2M4 - G2M8) along with two representative sublines established from the vehicle treated tumors (G1M2 and G1M6).

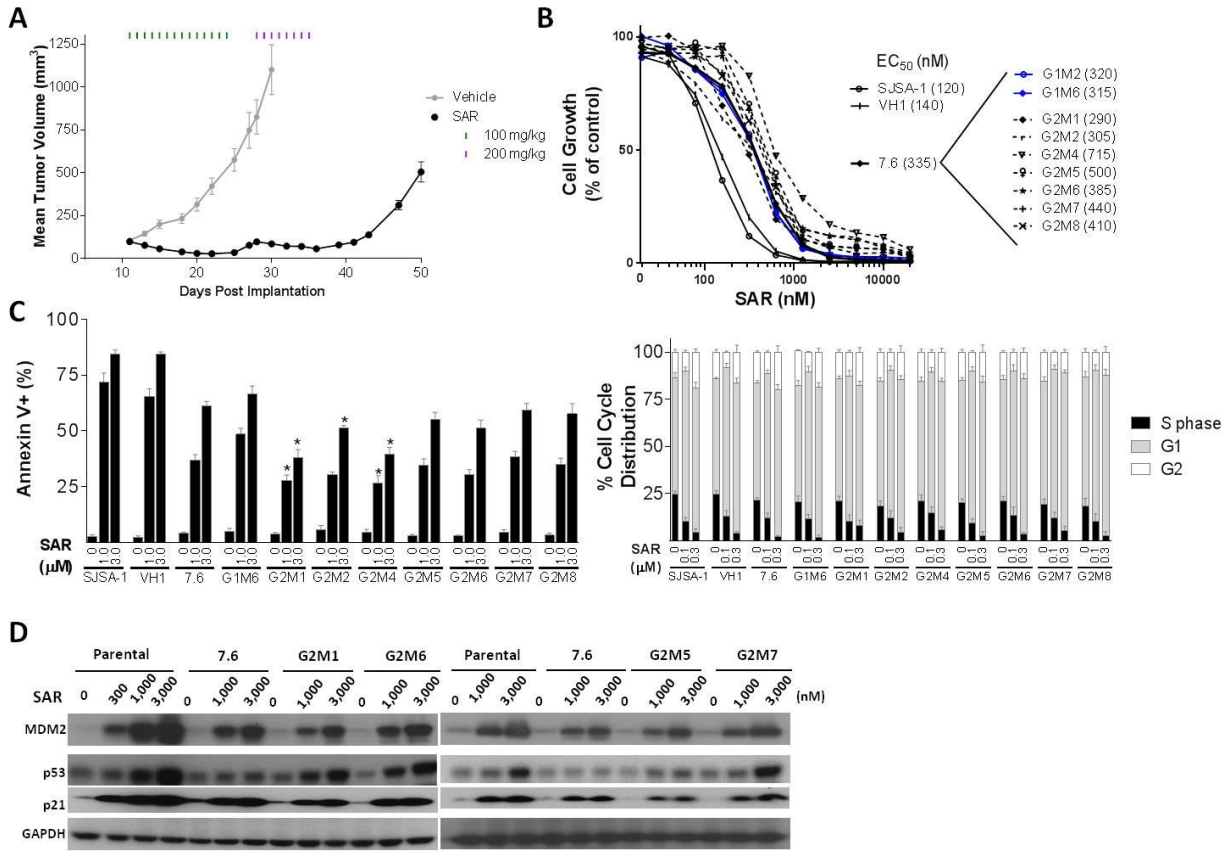

Figure 3.3 Establishment and characterization of *in vivo* 7.6/SJSA-1 sublines treated with multiple rounds of SAR405838

A, 7.6/SJSA-1 xenograft tumors were treated with vehicle or 100 mg/kg of SAR405838 orally for 14 days followed by 200 mg/kg SAR405838 for 8 days. B, Tumor cells isolated from 7.6/SJSA-1 xenografts and vehicle treated xenografts (G1M2 and G1M6) were treated with SAR405838 for 4 days and cell viability was evaluated by a WST assay. C, Cells were treated with SAR405838 for 48 h for apoptosis analysis by flow cytometry with Annexin V/P.I. double staining. The ttest was used to compare sublines (G2M1, G2M2, G2M4-G2M8) to 7.6 subline. *, $P < 0.05$. Cells were treated with SAR405838 for 24 h for cell cycle analysis by flow cytometry with P.I. staining. D, Western blot analysis of p53, MDM2, p21 and GAPDH protein level after 24 h treatment with SAR405838 at increasing concentrations.

Surprisingly, established cell lines treated in total with three rounds of SAR405838 only exhibited IC_{50} values with >2 to 3-fold greater resistance than the control cell lines and no difference in sensitivity to the 7.6 subline and its vehicle controls (Fig. 3.3B). Analysis of apoptosis by flow cytometry confirmed that sublines (G2M5 - G2M8) were not significantly different in the level of induction by SAR405838 exposure compared to 7.6 subline and its

vehicle controls (Fig. 3.3C). Furthermore, SAR405838 was still capable of inducing cell cycle arrest in all sublines treated *in vivo* with three rounds of SAR405838 (Fig. 3.3C).

We analyzed the p53 mutation status of these sublines obtained from xenografts of 7.6/SJSA-1 (p53 wild-type status) treated with three rounds of SAR405838. Analysis showed that all 7 sublines harbored a single heterozygous mutation C176F (Table S3.3). In order to determine if these sublines retained functional p53, we examined induction of p21 and MDM2 and accumulation of p53 protein by SAR405838 treatment. In representative sublines SAR405838 induces a dose-dependent increase of MDM2, p21 and p53 proteins (Fig. 3.3D).

These data confirm that SJSA-1 cells that have undergone multiple rounds of SAR405838 treatment remain sensitive to treatment despite the development of a single heterozygous mutation C176F.

Establishment and characterization of *in vivo* 7.2/SJSA-1 sublines treated with SAR405838

Next, we attempted again to isolate SJSA-1 cells resistant to SAR405838 by treating the 7.2 subline (C176F) xenografts with another round of 100 mg/kg or an increased dose at 200 mg/kg of SAR405838 (Fig. 3.4A). Treatment with both doses of SAR405838 resulted in tumor regression with no observed toxicity (SI Fig. S3.2D), where the higher dose resulted in 5 out of 8 mice with undetectable tumors by treatment end. Harvesting and culturing of the 200 mg/kg SAR405838 group resulted in the establishment of 5 sublines (G3M2, G3M3, G3M5 - G3M7). Similar to the 7.6 subline xenograft sublines, the 7.2 sublines exhibited IC_{50} values with >4-fold greater resistance than the control cell lines and no difference in sensitivity to the 7.2 subline and its vehicle control (G1M4) (Fig. 3.4B). Analysis of apoptosis by flow cytometry confirmed that all sublines, other than G3M3, were not significantly different in the level of induction by SAR405838 exposure compared to 7.2 subline and its vehicle control (Fig. 3.4C). SAR405838

was still capable of inducing cell cycle arrest in all sublines treated *in vivo* with three rounds of SAR405838 (Fig. 3.4C).

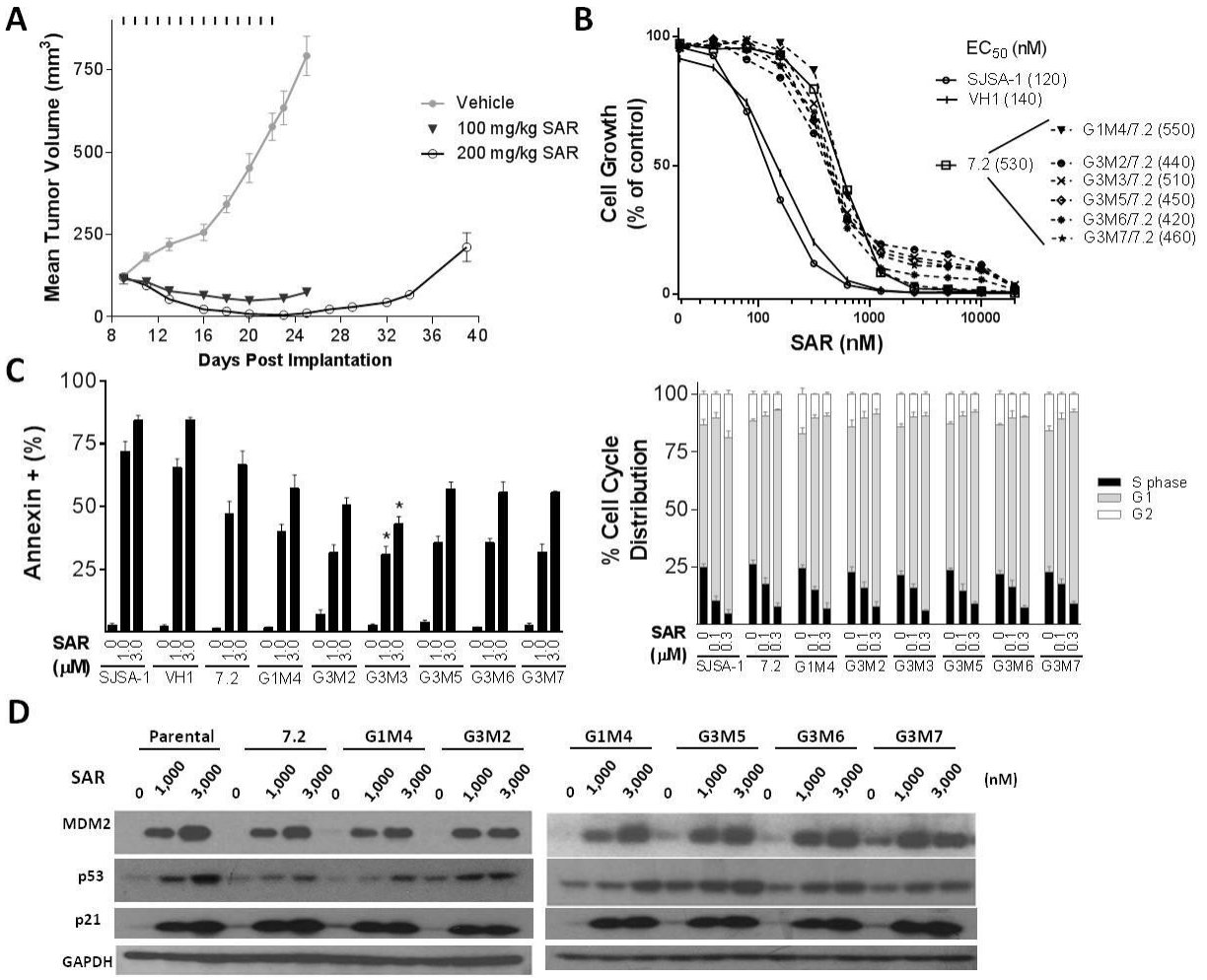

Figure 3.4 Establishment and characterization of *in vivo* 7.2/SJSA-1 sublines treated with SAR405838

A, 7.2/SJSA-1 xenograft tumors were treated with vehicle, 100 mg/kg or 200 mg/kg of SAR405838 orally for 14 day. B, Tumor cells isolated from 7.2/SJSA-1 xenografts and vehicle treated xenografts (G1M4) were treated with SAR405838 for 4 days and cell viability was evaluated by a WST assay. C, Cells were treated with SAR405838 for 48 h for apoptosis analysis by flow cytometry with Annexin V/P.I. double staining. The ttest was used to compare sublines (G3M2, G3M3, G3M5-G3M7) to 7.2 subline. *, $P < 0.05$. Cells were treated with SAR405838 for 24 h for cell cycle analysis by flow cytometry with P.I. staining. D, Western blot analysis of p53, MDM2, p21 and GAPDH protein level in SJSA-1 parental, 7.2/SJSA-1 subline (7.2), vehicle control treated 7.2/SJSA-1 sublines (G1M4) and 200 mg/kg SAR405838 treated sublines (G3M2, G3M5, G3M6, G3M7) after 24 h treatment with SAR405838 at increasing concentrations.

We analyzed the p53 mutation status of these sublines obtained from xenografts of 7.2/SJSA-1 (C176F) treated with 200 mg/kg of SAR405838. Analysis showed that all 5 sublines maintained the single heterozygous mutation C176F (Table S3.4). In order to determine if these sublines retained functional p53, we examined induction of p21 and MDM2 and accumulation of p53 protein by SAR405838 treatment. In representative sublines SAR405838 induces a dose-dependent increase of MDM2, p21 and p53 proteins (Fig. 3.4D).

These data confirm that SJSA-1 xenografts of 7.2 (C176F) remain sensitive to treatment with SAR405838.

MDM2 inhibitor *in vivo* treated sublines are sensitive to SAR405838 compared to the highly resistant *in vitro* resistant sublines

In order to examine the difference in the level of resistance of the *in vivo* and *in vitro* exposed cells to SAR405838 we employed apoptosis, cell cycle and western blot assays. In a cell growth inhibition assay SJSA-1 cells exposed to SAR405838 *in vitro* exhibit >100-fold greater resistance than the control cell lines versus the SJSA-1 cells exposed to SAR405838 *in vivo* which exhibit at most 6-fold greater resistance than the control lines (Fig. 3.5A). Analysis of apoptosis by flow clearly shows that induction of apoptosis is completely abolished in the SJSA-1 cells exposed to SAR405838 *in vitro* harboring a R273C mutation in p53 compared to strong apoptosis induction in SJSA-1 cells exposed SAR405838 *in vivo* harboring either wild type or a C176F mutation in p53 (Fig. 3.5B). SAR405838 exposure does not result in cell cycle arrest in sublines treated *in vitro* with SAR405838, whereas sublines treated with SAR405838 *in vivo* still induce cell cycle arrest in a dose dependent manner upon exposure to SAR405838 (Fig. 3.5B).

To further investigate the differences in acquired resistance of *in vitro* versus *in vivo* exposure to SAR405838 we analyzed the activation of the p53 pathway using western blot. Several representative SJSA-1 sublines treated *in vivo* with SAR405838 retained functional p53 status, represented by induction of MDM2, p53 and p21 in a dose dependent manner upon exposure to SAR405838, albeit with slightly reduced potency (Fig. 3.2D, 3.3D, 3.4D and 3.5C). Conversely, no significant induction of MDM2 or p21 protein was observed in the sublines, which developed resistance *in vitro* to SAR405838 (Fig. 3.5C). An induction of p53 protein was observed in both MIR1 and MIR2, however it is important to note that the untreated control had a higher p53 level compared to the parental line. The increased protein level of p53 in the untreated MIR1 and MIR2 sublines is consistent with the likely increased stability of the mutant p53[3].

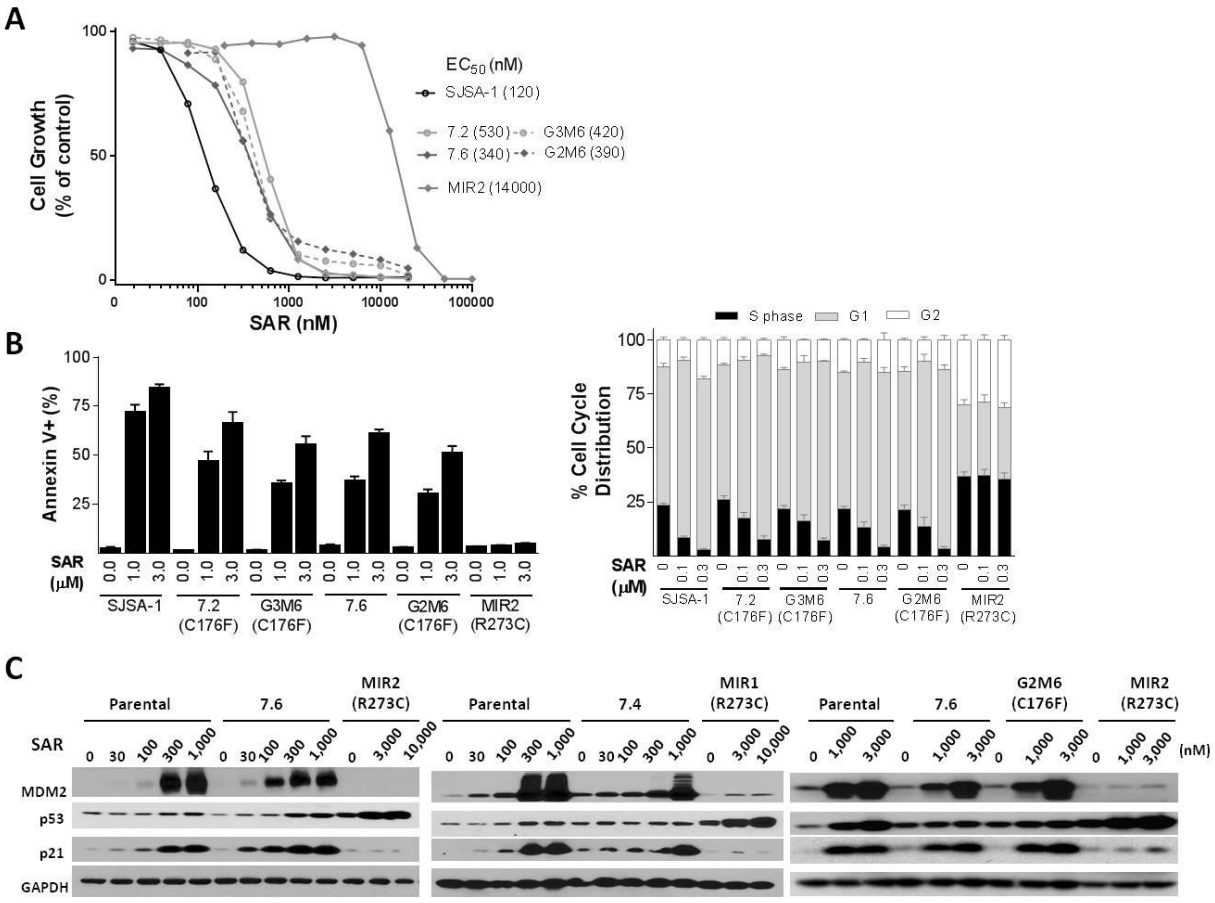

Figure 3.5 MDM2 inhibitor *in vivo* treated sublines (7.2, 7.6, G3M6 and G2M6) are sensitive to SAR405838 compared to the highly resistant *in vitro* resistant subline (MIR2)
A, SJSA-1 and representative sublines were treated with SAR405838 for 4 days and cell viability was evaluated by a WST assay. B, Cells were treated with SAR405838 for 48 h for apoptosis analysis by flow cytometry with Annexin V/P.I. double staining. Cells were treated with SAR405838 for 24 h for cell cycle analysis by flow cytometry with P.I. staining. C, Western blot analysis of p53, MDM2, p21 and GAPDH protein level in SJSA-1 parental, representative in vivo treated sublines and in vitro treated sublines (MIR1 and MIR2) after 24 h treatment with SAR405838 at increasing concentrations.

These data confirm that the level of resistance to SAR405838 in SJSA-1 cells exposed to SAR405838 *in vitro* versus *in vivo* is drastically different.

Deep sequencing of p53 in the SJSA-1 parental cell population

In order to determine whether a small population of p53 mutated cells were present in the SJSA-1 cell line we performed deep sequencing using the PacBio RS platform[42,43]. Primers were used to amplify the DNA binding domain of TP53 and amplicons were deeply sequenced on the Pacific Biosciences SMRT sequencer (PacBio). A distinct feature of this sequencing technique is that a single DNA amplicon can be sequenced multiple times by a DNA polymerase in a single run allowing for determination of a circular consensus (CCS)[42]. We limited the analysis reads to those that contained a minimum of 5 subreads (passes around the circular amplicon molecule) resulting in a 0.01% error rate; although individual consensus sequences can still be near 1% error[44]. This limitation resulted in 16,861 reads with an average of 11.3 passes and quality of insert equal to 0.993. We examined these reads for minor variant detection and found that there were no mutations in the DNA binding domain of the TP53 gene.

Next, we examined the PacBio data by a different method in order to determine if there are single mutations that occur at any one position when analyzing reads that contained a minimum of 10 subreads. This limitation resulted in an overall coverage of over 6,200 reads per position with a mean of 0.25% error across all positions with a standard deviation of 0.39%. We identified 19 positions in the nucleotide sequence with a predicted variation at a single nucleotide position with over 1% (1.02 - 4.11%) (SI Fig. S3.3). Of these 19 positions none of the mutations matched with the mutations found by Sanger sequencing in the acquired resistant sublines. This leads us to hypothesize that the 19 positions determined to have mutations at the low percentage of 1.02 to 4.11% are most likely due to PacBio error. However, overall these data suggest that if p53 mutant cells are present in the parental SJSA-1 cells and are selected for under drug pressure, it is at less than 1% of the population of cells.

Computational modeling of the interaction between the C176F p53 mutation and DNA

Our data clearly show that SAR405383 can effectively induce p53 activation in all SJSA-1 sublines containing a single heterozygous C176F p53 mutation. In the crystal structures of wild-type p53 in complex with DNA[45,46], C176, together with H179, C238, C242 residues, coordinates with a zinc ion and appears to be critical in maintaining the structural integrity of p53. Therefore, it was surprising that p53 with a heterozygous C176F mutation is functionally active as a transcriptional factor and we performed computational simulations to shed light on the structure and function for this heterozygous C176F p53 mutation.

p53 functions in a tetrameric unit in which two p53 monomers form a dimer to interact with DNA and two p53 dimers loosely interact to form a tetramer. The interaction interface between two p53 monomers are mediated *via* a hydrophobic interaction between 2-fold symmetrical positions of P177 and M243 from each monomer and the salt-bridge interaction between R181 of one p53 (denoted as p53A) and E180 of the second p53 (denoted p53B)[46] as shown in Fig. 3.6. In a 6 ns molecular dynamics (MD) simulation of the wild-type p53 tetramer complexed with DNA, we found the interaction interface is well-maintained. The salt-bridge interaction between R181 (p53A) and E180 (p53B) and R181 (p53B) and E180 (p53A) maintained distances between CZ of R181 and CD of E180 at 4.20 ± 0.68, 4.8 ± 0.77, 4.07 ± 0.20 and 4.17 ± 0.32 Å from MD simulation trajectories whereas they are 4.6, 4.8, 4.9, 5.0 Å in the crystal structure.

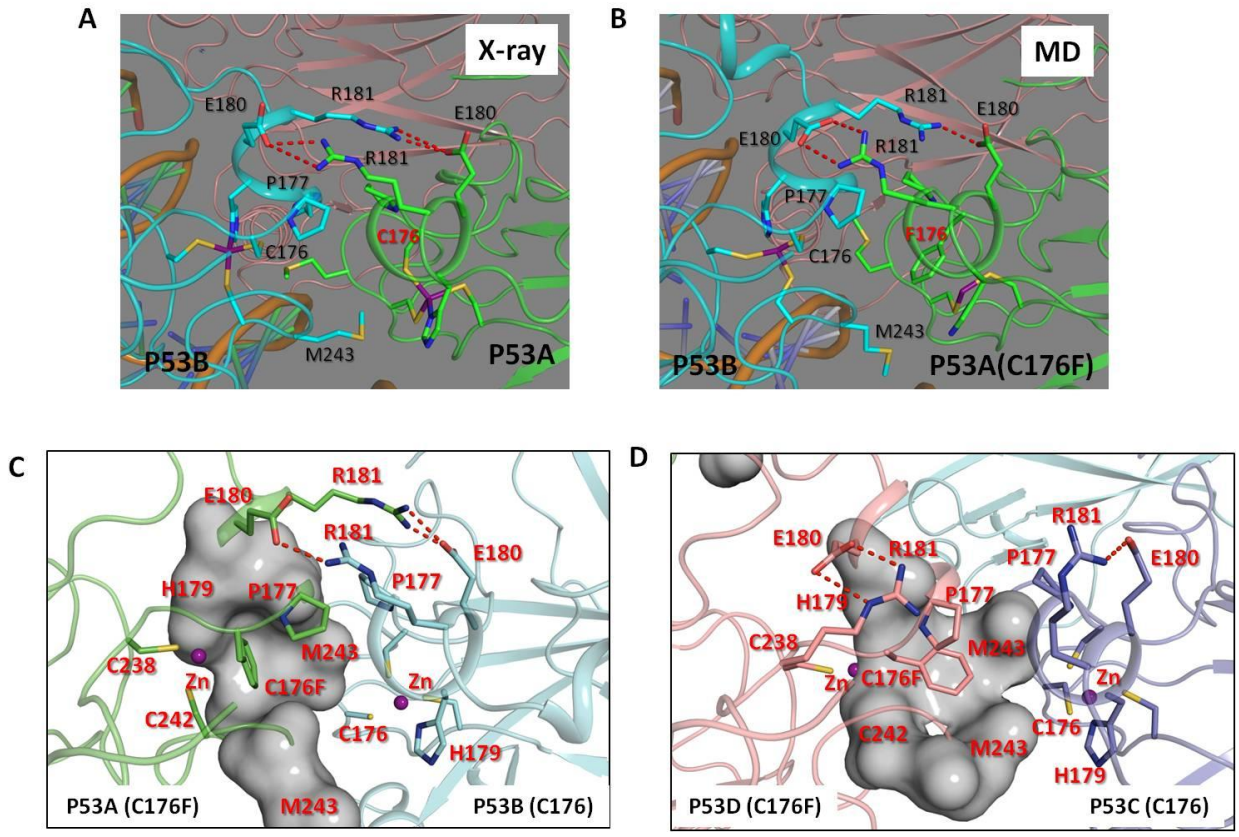

Figure 3.6 Modeling of the C176F mutation in p53 reveals the mutants' ability to bind DNA.
A, The loop-sheet-helix interaction interface between two p53 revealed by the crystal structure (PDB entry: 4HJE). B, The same interaction interface between one p53 wild-type and one (C176F) mutants in the final snapshot from the 6 ns MD simulation. Green and blue colored protein correspond to one p53 in a dimer. The backbone of DNA are shown in the orange color. The zinc ion is colored in purple. Hydrogen bonds are depicted in red dash lines. C and D, Two types of interaction between the p53 wild-type and p53 (C176F mutant) found from the final snapshot of the MD simulation corresponding to FCCF p53 tetrameric arrangement.

Due to the fact that the C176F p53 mutation is heterozygous, each p53 tetramer consists of two p53 wild-type molecules and two C176F p53 mutants and consequently, there are a total of six possible structural models for a p53 tetramer. In four of these six models, a wild-type p53 monomer molecule and a C176F p53 mutant monomer form a dimer which interacts with DNA. In two other models, two p53 wild-type monomers form a dimer and two C176F p53 mutant monomers form another dimer to interact with DNA. We denote these arrangements as FCCF, FCFC, CFCF, CFFC, FFCC, and CCFF, with C representing the wild-type p53 (C176) monomer

and F the C176F mutated p53 monomer (SI Table S3.5 and Fig. S3.4). We used the motional fluctuations of the p53 backbone atoms in these models and in the crystal structure to assess their structural stability. The FFCC model appears to be the least stable but the other five models have similar backbone motional fluctuations, when compared to the crystal structure. The FCCF tetramer appears to be the most stable. In each of the dimers, we also calculated the salt-bridge interaction distances between R181 and E180, an indicator of the structural stability of the dimer. The most stable FCCF tetramer also has a stronger R181 - E180 interaction than the other five models but this interaction is slightly weaker than in the wild-type p53 tetramer (SI Fig. S3.4).

Since the mutation of C176F results in a loss of zinc coordination, we analyzed the model structures of the FCCF tetramer from the MD simulations and compared them to those in the wild-type p53 tetramer to identify the additional stabilizing interactions. We found that C176F mutation allows the mutant to gain additional hydrophobic interactions with the M243 residue in a different p53 monomer and enhances cation-aromatic interactions with the zinc ion (Fig. 3.6C and D). These simulations therefore suggest that the p53 FCCF tetramer maintains its structural stability and its strong interactions with the DNA through those newly identified interactions and in this way, compensates partially for the loss of the zinc coordination with the C176 residue.

Discussion

SAR405838 inhibits the interaction between p53 and its inhibitor MDM2 and activates p53 responses in cancer cells. To date, studies investigating the acquisition of p53 mutations in cancer cells after *in vitro* exposure to MDM2 inhibitors has fostered the idea that targeting p53 will undoubtedly result in the development of deleterious p53 mutations[21,24]. In this study, we utilized the SJSA-1 cell line to establish *in vitro* and *in vivo* sublines that have been exposed to

the MDM2 inhibitor SAR405838. We identified that *in vitro* resistance sublines are highly resistant to SAR405838 due to p53 mutations that inhibit p53 activation whereas sublines established from xenografts treated with SAR405838 retain sensitivity to the inhibitor despite a uniformly identified single p53 mutation.

SAR405838 is highly effective in inducing apoptosis in the SJSA-1 cell lines *in vitro*, achieving complete regression of SJSA-1 xenograft tumors in mice, but only *in vitro* treatment leads to the development of sublines that have profound acquired resistance to the MDM2 inhibitor. The *in vitro* developed resistant cell lines were deemed highly resistant both in *in vitro* assays as well as in an *in vivo* study, where mice showed no response to the SAR405838 at either 100 or 200 mg/kg oral daily dosing for two weeks (Fig. 3.1D). Surprisingly, cell lines established from tumors treated with 100 mg/kg oral daily dosing for two weeks only resulted in a slight decrease in sensitivity to SAR405838 (Fig. 3.2). Sensitivity to SAR405838 of these established cell lines (7.2-7.4, 7.6 and 7.7) was confirmed *in vivo*. Our data show that in the 7.2/SJSA-1 and 7.6/SJSA-1 xenograft models, treatment with SAR405838 at 100 mg/kg daily for two weeks results in partial tumor regression for most tumors with over 100% tumor growth inhibition compared to vehicle controls (Fig. 3.3A and 3.4A). Furthermore, treatment of the 7.2/SJSA-1 xenograft model at 200 mg/kg daily for two weeks results in rapid and complete tumor regression in over 60% of mice with the remaining tumors reaching partial tumor regression (Fig. 3.4A).

We next sought to elucidate whether the difference in the level of resistance to SAR405838 in *in vitro* versus *in vivo* exposure to drug is dependent on the mutation status of p53. Whether a mutant is functional or not depends on the nature of the structural changes, which will affect the selectivity for various response elements such as p53 binding to DNA[47,48].

Initially all p53 mutants were considered to have equivalent affects on the function of p53, however, it is now known that different mutants can alter p53 function in vastly different ways[49]. Currently, there are two categories in which p53 mutants fall: the first termed as 'contact mutations' affect amino acids that contact DNA thus preventing wild-type transcriptional activity and the second termed as 'structural mutants' that disrupt the three dimensional structure of the protein[46,47]. We have identified that the SJSA-1 cancer cells exposed to MDM2 inhibitor SAR405838 by two different *in vitro* exposure protocols uniformly results in the acquisition of p53 mutant resistant sublines (Fig. 3.1). The SJSA-1 *in vitro* acquired SAR405838 resistant sublines harbor missense mutations located in the DNA binding domain of p53. All of the p53 mutations identified in this study have previously been identified in patient tumors (http://www-p53.iarc.fr/). The "hot spot" R273C mutation identified in both MIR1 and MIR2 sublines and the C277F mutation identified in CMIR are both common contact mutations[46]. R273 plays a crucial role in docking p53 to the DNA backbone and C277 directly contacts DNA bases[48]. Mutations leading to a change in the amino acid at these positions can dramatically reduce the DNA binding affinity[49,50]. Our data supports that these mutants ability to bind to DNA is reduced or lost because the *in vitro* resistant sublines are unable to activate p53 target proteins, apoptosis or cell cycle arrest (Fig. 3.1 and Fig. 3.5).

In drastic contrast to the *in vitro* acquired resistant sublines, established sublines from SJSA-1 xenografts that underwent single or multiple rounds of *in vivo* exposure to SAR405838 either maintain p53 wild-type status or acquire the C176F mutation in p53. Wild-type p53 amino acids C176, H179, C238 and C242 coordinate to a zinc ion to fold correctly[46]. Our data shows, unlike previously investigated Zn-binding-site mutants such as "hot spot" mutants R175C and R175H, the heterozygous C176F mutation does not result in loss of function of p53.

Surprisingly, although C176F no longer coordinates to the zinc ion, the gain of hydrophobic interaction between C176F and M243 and neighboring hydrophobic side chain atoms compensates the loss. Taken together, the computational modeling of the heterozygous C176F mutant, *in vitro* and *in vivo* assays with the SJSA-1 sublines harboring the heterozygous C176F mutation lead us to conclude that p53 remains functional and responsive to SAR405838.

The emergence of p53 mutated cells under SAR405838 exposure raises the questions as to where these cells originate from. SAR405838 treatment may result in the expansion of p53 mutated cell fractions already present in the parental SJSA-1 cell line. In two previous reports, nutlin-3 treatment of parental cell populations resulted in the p53 mutated sublines[21,24]. Their data may not be sufficient to conclude that nutlin-3 induces p53 mutations versus selects for p53 mutant cells already present in the parental population. However, our deep sequencing data, in corroboration with our resistant studies, supports these previous studies by showing that no p53 mutated cells are present at over 1% of the bulk population of SJSA-1 cells.

In conclusion, our results show that SAR405838 treatment of SJSA-1 xenografts does not result in resistance to the MDM2 inhibitor. Surprisingly, the SJSA-1 sublines established from several rounds of *in vivo* treatment with SAR405838 harbor a single heterozygous C176F mutation in p53. Computational modeling of the C176F mutation shows that this mutated p53 form is still able to bind DNA, which is also evident in *in vitro* assays showing that SAR405838 is still able to activate p53 target proteins, apoptosis and cell cycle arrest. Conversely, SAR405838 exposure *in vitro* to SJSA-1 cells results in p53 mutated sublines that are highly resistant to SAR405838. One critical difference is that in *in vitro* treatment, the drug is present at a constant concentration and poses a continuous high threat to the survival of tumor cells. For *in vivo* treatment, the pressure from the drug for tumor cell survival fluctuates, as a result of the

pharmacokinetic effect. While development of p53 mutations has been shown here and in other studies to be a primary mechanism of resistance *in vitro* to MDM2 inhibitors[21,24], our study shows that robust resistance does not develop *in vivo* to SAR405838 in the MDM2 amplified osteosarcoma (SJSA-1) cell line. Evidence presented in this study leads to the conclusion that *in vitro* exposure to the MDM2 inhibitor SAR405838 does not reflect what will take place in a clinical setting. The *in vivo* development of sublines treated with SAR405838 may be a much more accurate example of what will occur in the clinic, which suggests that patients can undergo several rounds of SAR405838 treatment with little to no development of resistance.

Supplemental Information

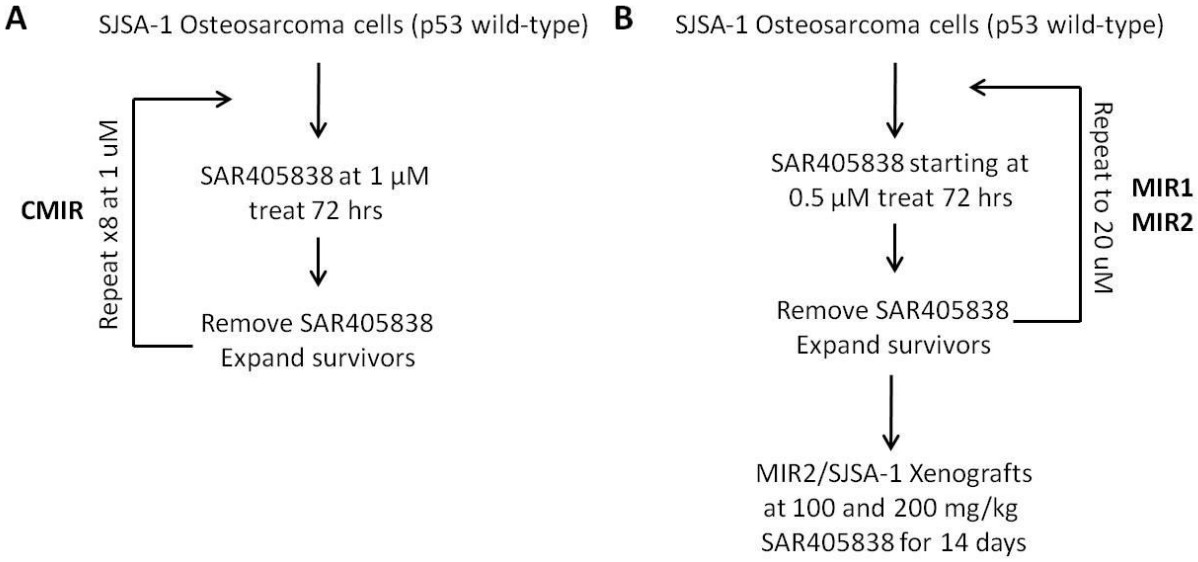

Figure S3.1 SAR405838 exposure and treatment protocols for CMIR, MIR1 and MIR2 resistant sublines

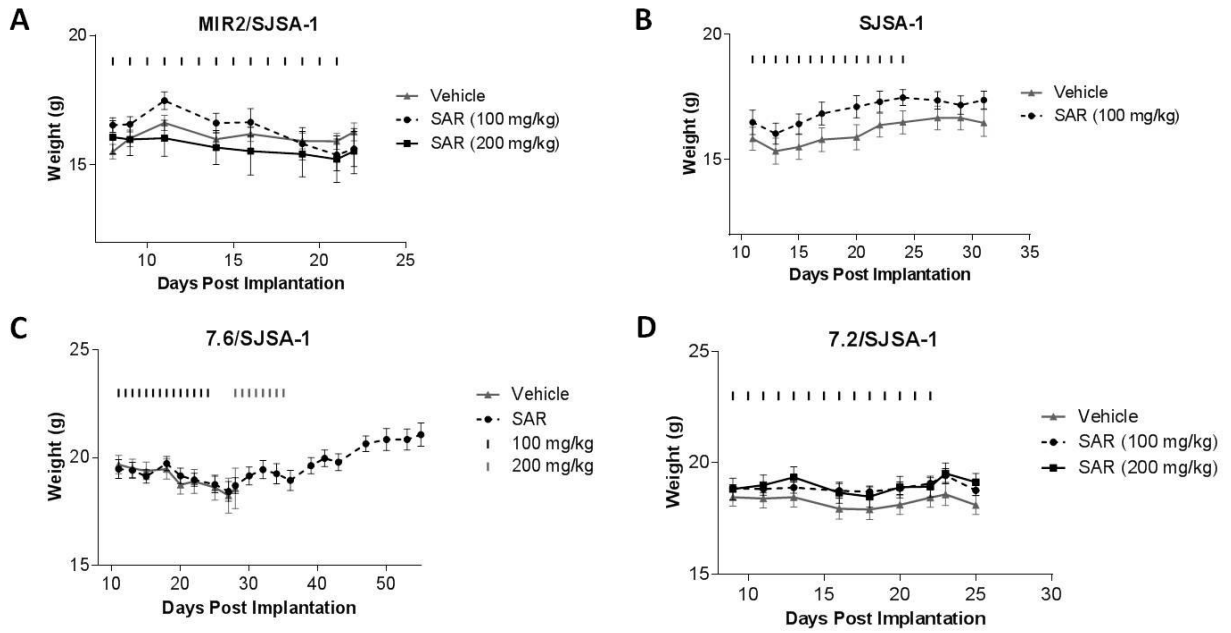

Figure S3.2 Body weight change of mice
Body weight change in mice in Fig. 3.1D (A), Fig. 3.2A (B), Fig. 3.3A (C) and Fig. 3.4A (D) . Data shown are mean ± SEM for 6-8 mice.

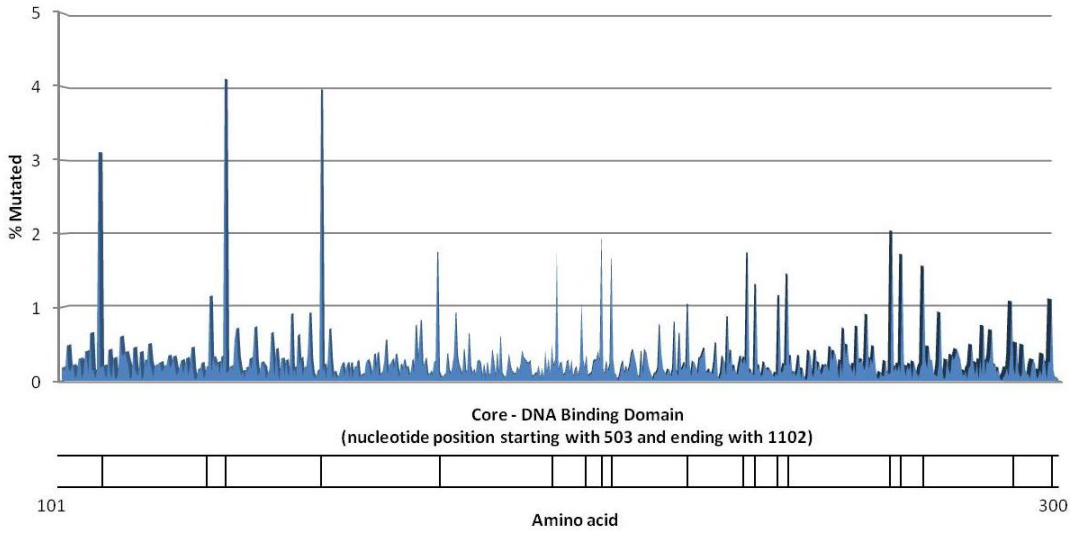

Figure S3.3 SJSA-1 parental cell population PacBio sequencing data.
Data represented as a graph of the percent a mutation was detected at each individual nucleotide position of the DNA binding domain of p53. The nucleotide positions correspond to amino acids 101 to 300. Vertical lines signify the location of the 19 positions with over 1% nucleotide variation.

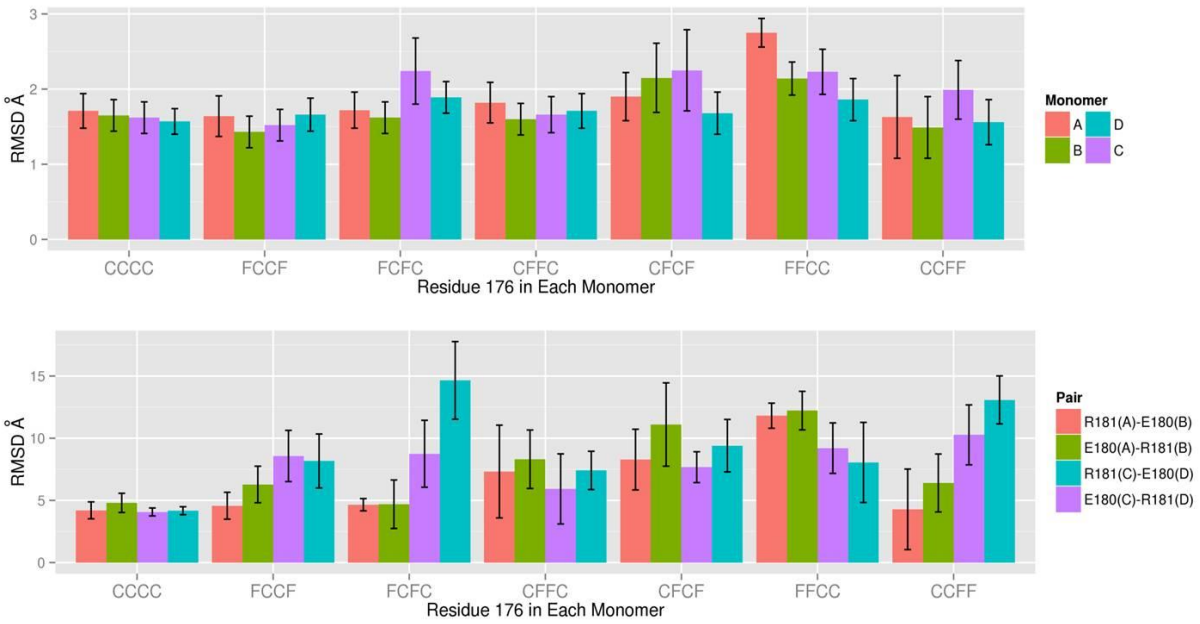

Figure S3.4 The root-mean-square deviations of the p53 backbone atoms from the wild-type crystal structures in four different tetrameric arrangements from the 6 ns MD simulations.

The distances between R181 (Cz) and E180(Cd) in the pair of dimeric p53 proteins calculated from the MD simulations. The standard deviations were shown in error bars. The arrangement of monomer A, B, C and D were shown in the legend.

Table S3.1 p53 sequencing results of SJSA-1 sublines established by *in vitro* exposure to SAR405838

A, p53 sequencing of parental SJSA-1, vehicle treated SJSA-1 sublines and *in vitro* resistant SJSA-1 sublines. B, p53 sequencing of *in vitro* resistant MIR2 sublines established after *in vivo* treatment with vehicle (G1M3) or 200 mg/kg/day of SAR405838 for two weeks (G3M1, G3M3 and G3M6).

A

Cell Line (EC_{50} SAR405838)	p53
SJSA-1 (120)	None
ContR (178)	None
MIR1 (13,300)	Heterozygous R273C
MIR2 (14,000)	Heterozygous R273C
CMIR (20,500)	Heterozygous L130R C277F

B

Cell Line	p53
MIR2	Heterozygous R273C
G1M3 (Vehicle treated)	
G3M1 (200 mg/kg SAR405838)	
G3M3 (200 mg/kg SAR405838)	
G3M6 (200 mg/kg SAR405838)	

Table S3.2 p53 sequencing results of SJSA-1 sublines established after *in vivo* treatment with 100 mg/kg/day of SAR405838 for two weeks

Cell Line (EC_{50} SAR405838)	p53
SJSA-1 (120)	None
VH1 (140)	None
7.2 (530)	Heterozygous C176F
7.3 (360)	None
7.4 (580)	None
7.6 (335)	None
7.7 (380)	None

Table S3.3 p53 sequencing results of 7.6/SJSA-1 sublines (no mutation) established after three rounds of *in vivo* SAR405838 treatment

Cell Line (EC_{50} SAR405838)	p53
SJSA-1 (120)	None
VH1 (140)	None
7.6 (335)	None
G1M2 (320)	None
G1M6 (315)	None
G2M1 (290)	Heterozygous C176F
G2M2 (305)	
G2M4 (715)	
G2M5 (500)	
G2M6 (385)	
G2M7 (440)	
G2M8 (410)	

Table S3.4 p53 sequencing results of 7.2/SJSA-1 subline (mutation C176F) established after *in vivo* treatment with 200 mg/kg/day of SAR405838 orally for two weeks

Cell Line (EC_{50} SAR405838)	p53
SJSA-1 (120)	None
VH1 (140)	None
7.2 (530)	Heterozygous C176F
G1M4 (550)	
G3M2 (440)	
G3M3 (510)	
G3M5 (450)	
G3M6 (420)	
G3M7 (460)	

Table S3.5 Root-mean square deviations of the wild-type and mutant p53 backbone atoms from the crystal structure and the distances between R181(Cz) and E180(Cd) in each dimeric p53 protein determined from the MD simulations.

RMSD(Å)	Monomer A	Monomer B	Monomer C	Monomer D
CCCC	1.71±0.23	1.65±0.21	1.62±0.21	1.57±0.17
FCCF	1.64±0.27	1.43±0.21	1.52±0.21	1.66±0.22
FCFC	1.72±0.19	1.62±0.22	2.24±0.30	1.89±0.28
FFCC	2.75±0.55	2.14±0.41	2.23±0.39	1.86±0.30
CCFF	1.63±0.24	1.49±0.21	1.99±0.44	1.56±0.21

Distance(Å)	R181(1)-E180(2)	E180(1)-R181(2)	R181(3)-E180(4)	E180(3)-R181(4)
CCCC	4.20±0.68	4.80±0.77	4.07±0.32	4.17±0.32
FCCF	4.57±1.08	6.28±1.47	8.57±2.06	8.17±2.17
FCFC	4.65±1.01	4.69±1.55	8.75±2.03	14.65±3.22
FFCC	11.81±3.24	12.22±2.33	9.20±2.41	8.05±1.93
CCFF	4.28±0.49	6.40±1.95	10.27±2.69	13.08±3.12

References

1. Teodoro, J.G., Evans, S.K. & Green, M.R. Inhibition of tumor angiogenesis by p53: a new role for the guardian of the genome. *J Mol Med (Berl)* **85**, 1175-86 (2007).
2. Levine, A.J. p53, the cellular gatekeeper for growth and division. *Cell* **88**, 323-31 (1997).
3. Vousden, K.H. & Lu, X. Live or let die: the cell's response to p53. *Nat Rev Cancer* **2**, 594-604 (2002).
4. Vogelstein, B., Lane, D. & Levine, A.J. Surfing the p53 network. *Nature* **408**, 307-10 (2000).
5. Hainaut, P. & Hollstein, M. p53 and human cancer: the first ten thousand mutations. *Adv Cancer Res* **77**, 81-137 (2000).
6. Freedman, D.A., Wu, L. & Levine, A.J. Functions of the MDM2 oncoprotein. *Cell Mol Life Sci* **55**, 96-107 (1999).

7. Fakharzadeh, S.S., Rosenblum-Vos, L., Murphy, M., Hoffman, E.K. & George, D.L. Structure and organization of amplified DNA on double minutes containing the mdm2 oncogene. *Genomics* **15**, 283-90 (1993).
8. Hollstein, M., Sidransky, D., Vogelstein, B. & Harris, C.C. p53 mutations in human cancers. *Science* **253**, 49-53 (1991).
9. Shangary, S. & Wang, S. Targeting the MDM2-p53 interaction for cancer therapy. *Clin Cancer Res* **14**, 5318-24 (2008).
10. Momand, J., Zambetti, G.P., Olson, D.C., George, D. & Levine, A.J. The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. *Cell* **69**, 1237-45 (1992).
11. Shangary, S. *et al.* Temporal activation of p53 by a specific MDM2 inhibitor is selectively toxic to tumors and leads to complete tumor growth inhibition. *Proc Natl Acad Sci U S A* **105**, 3933-8 (2008).
12. Vassilev, L.T. MDM2 inhibitors for cancer therapy. *Trends Mol Med* **13**, 23-31 (2007).
13. Vassilev, L.T. *et al.* In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. *Science* **303**, 844-8 (2004).
14. Tovar, C. *et al.* MDM2 small-molecule antagonist RG7112 activates p53 signaling and regresses human tumors in preclinical cancer models. *Cancer Res* **73**, 2587-97 (2013).
15. Wang, S. *et al.* SAR405838: An optimized inhibitor of MDM2-p53 interaction that induces complete and durable tumor regression. *Cancer Res* (2014).
16. Hainaut, P. & Wiman, K. *25 years of p53 research*, xi, 446 p. (Springer, Dordrecht, 2005).
17. Long, J. *et al.* Multiple distinct molecular mechanisms influence sensitivity and resistance to MDM2 inhibitors in adult acute myelogenous leukemia. *Blood* **116**, 71-80 (2010).
18. Saha, M.N., Micallef, J., Qiu, L. & Chang, H. Pharmacological activation of the p53 pathway in haematological malignancies. *J Clin Pathol* **63**, 204-9 (2010).
19. Christiansen, D.H., Andersen, M.K. & Pedersen-Bjergaard, J. Mutations with loss of heterozygosity of p53 are common in therapy-related myelodysplasia and acute myeloid leukemia after exposure to alkylating agents and significantly associated with deletion or loss of 5q, a complex karyotype, and a poor prognosis. *J Clin Oncol* **19**, 1405-13 (2001).
20. Leonard, D.G. *et al.* p53 mutations in leukemia and myelodysplastic syndrome after ovarian cancer. *Clin Cancer Res* **8**, 973-85 (2002).
21. Aziz, M.H., Shen, H. & Maki, C.G. Acquisition of p53 mutations in response to the non-genotoxic p53 activator Nutlin-3. *Oncogene* **30**, 4678-86 (2011).
22. Verma, R., Rigatti, M.J., Belinsky, G.S., Godman, C.A. & Giardina, C. DNA damage response to the Mdm2 inhibitor nutlin-3. *Biochem Pharmacol* **79**, 565-74 (2010).
23. Valentine, J.M., Kumar, S. & Moumen, A. A p53-independent role for the MDM2 antagonist Nutlin-3 in DNA damage response initiation. *BMC Cancer* **11**, 79 (2011).
24. Michaelis, M. *et al.* Adaptation of cancer cells from different entities to the MDM2 inhibitor nutlin-3 results in the emergence of p53-mutated multi-drug-resistant cancer cells. *Cell Death Dis* **2**, e243 (2011).
25. Carol, H. *et al.* Initial testing of the MDM2 inhibitor RG7112 by the Pediatric Preclinical Testing Program. *Pediatr Blood Cancer* **60**, 633-641 (Pediatr Blood Cancer. 2013).
26. Gottesman, M.M. Mechanisms of cancer drug resistance. *Annu Rev Med* **53**, 615-27 (2002).
27. Tredan, O., Galmarini, C.M., Patel, K. & Tannock, I.F. Drug resistance and the solid tumor microenvironment. *J Natl Cancer Inst* **99**, 1441-54 (2007).
28. Teicher, B.A. *et al.* Tumor resistance to alkylating agents conferred by mechanisms operative only in vivo. *Science* **247**, 1457-61 (1990).

29. Kerbel, R.S., St Croix, B., Florenes, V.A. & Rak, J. Induction and reversal of cell adhesion-dependent multicellular drug resistance in solid breast tumors. *Hum Cell* **9**, 257-64 (1996).
30. Zhao, Y. *et al.* A potent small-molecule inhibitor of the MDM2-p53 interaction (MI-888) achieved complete and durable tumor regression in mice. *J Med Chem* **56**, 5553-61 (2013).
31. Lu, J. *et al.* SM-164: a novel, bivalent Smac mimetic that induces apoptosis and tumor regression by concurrent removal of the blockade of cIAP-1/2 and XIAP. *Cancer Res* **68**, 9384-93 (2008).
32. Konopleva, M. *et al.* Mechanisms of apoptosis sensitivity and resistance to the BH3 mimetic ABT-737 in acute myeloid leukemia. *Cancer Cell* **10**, 375-88 (2006).
33. Lois, C., Hong, E.J., Pease, S., Brown, E.J. & Baltimore, D. Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. *Science* **295**, 868-872 (2002).
34. Chen, Y. *et al.* Structure of p53 binding to the BAX response element reveals DNA unwinding and compression to accommodate base-pair insertion. *Nucleic Acids Res* **41**, 8368-76 (2013).
35. Case, D.A. *et al.* AMBER 12. (University of California, San Francisco, 2012).
36. Wang, J., Cieplak, P. & Peter A. Kollman. How well does a restrained electrostatic potential (RESP) model perform in calculating conformational energies of organic and biological molecules? *Journal of Computational Chemistry* **21**, 1049-1074 (2000).
37. Lu, Q., Tan, Y.H. & Luo, R. Molecular dynamics simulations of p53 DNA-binding domain. *J Phys Chem B* **111**, 11538-45 (2007).
38. Jorgensen, W.L., Chandrasekhar, J., Madura, J.D., Impey, R.W. & Klein, M.L. Comparison of simple potential functions for simulating liquid water. *The Journal of Chemical Physics* **79**, 926-935 (1983).
39. Ryckaert, J.-P., Ciccotti, G. & Berendsen, H.J.C. Numerical integration of the cartesian equations of motion of a system with constraints: molecular dynamics of n-alkanes. *Journal of Computational Physics* **23**, 327-341 (1977).
40. Darden, T., York, D. & Pedersen, L. Particle Mesh Ewald - an N.Log(N) Method for Ewald Sums in Large Systems. *Journal of Chemical Physics* **98**, 10089-10092 (1993).
41. Yang, L.Y. & Trujillo, J.M. Biological characterization of multidrug-resistant human colon carcinoma sublines induced/selected by two methods. *Cancer Res* **50**, 3218-25 (1990).
42. Eid, J. *et al.* Real-time DNA sequencing from single polymerase molecules. *Science* **323**, 133-8 (2009).
43. Quail, M.A. *et al.* A tale of three next generation sequencing platforms: comparison of Ion Torrent, Pacific Biosciences and Illumina MiSeq sequencers. *BMC Genomics* **13**, 341 (2012).
44. Jiao, X. *et al.* A Benchmark Study on Error Assessment and Quality Control of CCS Reads Derived from the PacBio RS. *J Data Mining Genomics Proteomics* **4**(2013).
45. Cho, Y., Gorina, S., Jeffrey, P.D. & Pavletich, N.P. Crystal structure of a p53 tumor suppressor-DNA complex: understanding tumorigenic mutations. *Science* **265**, 346-55 (1994).
46. Joerger, A.C. & Fersht, A.R. Structural biology of the tumor suppressor p53. *Annu Rev Biochem* **77**, 557-82 (2008).
47. Brosh, R. & Rotter, V. When mutants gain new powers: news from the mutant p53 field. *Nat Rev Cancer* **9**, 701-13 (2009).
48. Kitayner, M. *et al.* Structural basis of DNA recognition by p53 tetramers. *Mol Cell* **22**, 741-53 (2006).
49. Muller, P.A. & Vousden, K.H. Mutant p53 in cancer: new functions and therapeutic opportunities. *Cancer Cell* **25**, 304-17 (2014).
50. Bullock, A.N. & Fersht, A.R. Rescuing the function of mutant p53. *Nat Rev Cancer* **1**, 68-76 (2001).

Chapter 4

Conclusion

The major findings of this dissertation are centralized around the goal of elucidation and development of more effective ways to treat cancer. I have investigated the mechanisms of resistance in cancer to both MDM2-p53 interaction inhibitors and Bcl-2/Bcl-xL inhibitors both *in vitro* and *in vivo*. I identified rational treatment strategies aimed at overcoming and circumventing resistance to these small molecule inhibitors. Lastly, I addressed the differences that arise in the development of resistance to these inhibitors *in vitro* versus *in vivo*.

Acquired resistance of tumor cells to anticancer drugs is a major cause of cancer therapy failure in the clinic. Cancer associated mutations in p53 are often found in the p53 DNA binding domain resulting in the inability of p53 to bind DNA and activate transcription[1]. In both acute leukemia models and in the osteosarcoma model (SJSA-1) of *in vitro* and *in vivo* acquired resistance to SAR405838, p53 mutations in the DNA binding domain were identified. Our analyses showed that each of the acute leukemia resistant sublines either harbor an inactivating mutated p53 gene or contain p53 with compromised transcriptional activity. The SJSA-1 *in vitro* acquired SAR405838 resistant sublines harbor deleterious missense mutations located in the DNA binding domain of p53. As a result, these resistant sublines are not sensitive to SAR405838 *in vitro* or *in vivo*. Overall, these data show that cancer cells that develop resistance to SAR405838 lack functional p53.

We have investigated the acquired resistance mechanisms *in vitro* and *in vivo* to the Bcl-2/Bcl-xL inhibitor ABT-737, as well as its orally active analogue ABT-263/navitoclax, which is currently in clinical trials for the treatment of several cancers including leukemia (http://clinicaltrial.gov). In both xenografts of relapsed acute lymphoblastic leukemia (ALL) model (RS4;11) and pediatric acute myeloid leukemia (AML) model (MV4;11), ABT-263 induced rapid and complete tumor regression, but tumors regrew shortly after treatment cessation. In our ALL model of acquired resistance, we found decreased expression of BAX both *in vitro* and *in vivo* as a repercussion of exposure to Bcl-2/Bcl-xL inhibitors. Both the RS4;11 and MV4;11 models retain sensitivity to ABT-737/ABT-263 when BAK is down-regulated, but become highly resistant when BAX is down-regulated. These data emphasize the different roles of the pro-apoptotic BAX and BAX proteins in tumor cell's response to Bcl-2/Bcl-xL inhibition. Furthermore, I have identified a new mechanism of acquired resistance to Bcl-2/Bcl-xL inhibitors, which differ from the previously identified mechanisms of resistance.

We have increased the depth of understanding resistant mechanisms to Bcl-2/Bcl-xL inhibitors and MDM2-p53 interaction inhibitors, paving the way to develop novel approaches to treating patients with reoccurring disease. Combination and sequential studies presented in this dissertation show that targeting both Bcl-2/Bcl-xL and MDM2 can achieve longer tumor free survival. Furthermore, I have investigated treatment strategies aimed at overcoming resistance to both Bcl-2/Bcl-xL and MDM2-p53 interactions inhibitors.

We define a targeted strategy to overcome ABT-263/navitoclax resistance in the intrinsically resistant MV4;11 model and in the RS4;11 model where resistance is driven by loss of Bcl-2 family member, BAX. We employed MDM2-p53 interaction inhibitor, SAR405838, *in vitro* and *in vivo* to re-activate apoptosis and cell growth inhibition. The apoptotic response of

p53 stimulation is typically mediated by its transcriptional upregulation of proapoptotic Bcl-2 family members, such as PUMA and/or BAX[2-4], thus the hypothesis is that the employment of SAR405838 will induce the reactivation of the Bcl-2 pathway leading to apoptosis[5]. Our data fully supports this hypothesis shown both by activation of PUMA and key apoptotic markers in the RS4;11 sublines resistant to ABT-737/ABT-263. Importantly, in the RS4;11 model SAR405838 achieves rapid and complete tumor regression in xenografts resistant to the Bcl-2/Bcl-xL inhibitor ABT-263. In the MV4;11 model initial treatment with ABT-263 does not result in tumor regression, however, immediate treatment with SAR405838 results in complete tumor regression. Taken together, these data show that MDM2-p53 interaction inhibitors can resensitize cancer cells resistant to Bcl-2/Bcl-xL inhibitors.

Excitingly, we have identified a strategy to activate apoptosis in highly resistant acute leukemia sublines independent of p53 status utilizing a Bcl-2/Bcl-xL inhibitor. ABT-263 is effective *in vitro* at activating apoptosis and cell growth inhibition in MDM2 inhibitor resistant RS4;11 and MV4;11 sublines. Furthermore, when RS4;11 tumors initially treated with SAR405838 regrow, they become unresponsive to the second round of SAR405838 treatment but undergo rapid tumor regression when treated with ABT-263. This suggests that the strategy of treating dysfunctional or mutated p53 cancers with Bcl-2/Bcl-xL inhibitors should be explored in the clinic.

Combination using MDM2-p53 interaction inhibitors and Bcl-2/Bcl-xL inhibitors has the added advantage of inducing p53 target genes leading to apoptosis and cell cycle arrest along with inhibition of highly expressed antiapoptotic Bcl-2 family members, Bcl-2 and Bcl-xL. Further inhibition of antiapoptotic Bcl-2 proteins can be achieved by successful inhibition of MDM2 resulting in activation of p53 and an increase in PUMA, which has been shown to

antagonize Mcl-1[4]. The ABT-263/SAR405383 combination in two acute leukemia xenograft models showed rapid and complete tumor regression with extended tumor free survival compared to single agent controls. Noteworthy is the MV4;11 model was only treated twice weekly with ABT-263/SAR405838 combination. Less frequent dosing of the drugs could both delay the onset of resistance and result in less toxicity in patients. Since Bcl-2 is overexpressed in acute leukemia and 80% of leukemia's retain p53 wild-type status, the targeted combination therapy proposed here would constitute a mechanism-based therapy with considerable clinical potential.

In order to elucidate the mechanisms of drug resistance, most studies have employed cell lines that have been exposed to drug *in vitro* and acquired resistance due to repeated and/or continuous exposure to drug[6-8]. Fewer investigations have utilized tumor cell lines, which developed resistance *in vivo*[9]. Based on findings presented in this dissertation, there are obvious differences in the mechanism of drug resistance and in the development of resistance when tumor cells are exposed to drug *in vitro* versus *in vivo*. One critical difference is that in *in vitro* treatment, the drug is present at a constant concentration and poses a continuous high threat to the survival of tumor cells. For *in vivo* treatment, the pressure from the drug for tumor cell survival fluctuates, as a result of the pharmacokinetic effect[9].

Differences were identified in the acute leukemia models when exposed to Bcl-2/Bcl-xL inhibitors *in vitro* versus *in vivo*. ABT-737/ABT-263 is highly effective in inducing apoptosis in the RS4;11 model *in vitro* and achieving complete regression of RS4;11 xenograft tumors in mice, but both *in vitro* and *in vivo* treatments lead to the development of sublines that have profound acquired resistance to this class of drugs. The *in vivo* exposure of RS4;11 xenografts to ABT-737/ABT-263 resulted in loss of expression of BAX, but not BAK. In contrast, the *in vitro*

exposure of RS4;11 cells to the Bcl-2/Bcl-xL inhibitor resulted in the loss of BAX expression as well as increased expression of the Mcl-1 protein. The loss of BAX protein expression in the Bcl-2/Bcl-xL inhibitor resistant cell lines is a finding in contrast to most studies published thus far in the literature, where increased expression of Mcl-1 or Bfl-1/A1 confers resistance to Bcl-2/Bcl-xL inhibitors[10-13]. The *in vitro* exposure of the MV4;11 model to ABT-263 also resulted in increased protein expression of Mcl-1. Although MV4;11 tumor cells were sensitive to ABT-263 *in vitro*, treatment *in vivo* illustrated MV4;11 cells are intrinsically resistant to the Bcl-2/Bcl-xL inhibitor, further emphasizing that sensitivity and resistance to drugs must be evaluated *in vivo*.

SAR405838 is highly effective in inducing apoptosis in the SJSA-1 cell lines *in vitro* and achieves complete regression of SJSA-1 xenograft tumors in mice, but only *in vitro* treatment leads to the development of sublines that have profound acquired resistance to the MDM2 inhibitor. The SJSA-1 *in vitro* acquired SAR405838 resistant sublines harbor deleterious missense mutations located in the DNA binding domain of p53. Our data corroborates that these p53 mutants ability to bind DNA is reduced because the *in vitro* resistant sublines are unable to activate p53 target proteins, induce apoptosis or inhibit cell cycle. In drastic contrast to the *in vitro* acquired resistant sublines, established sublines from SJSA-1 xenografts that underwent single or multiple rounds of *in vivo* exposure to SAR405838 either maintain p53 wild-type status or acquire the heterozygous C176F mutation in p53. Our data show that the C176F mutation does not result in loss of function of p53. Our modeling study suggests that although the C176F p53 mutant no longer coordinates to the zinc ion, the gain of hydrophobic interaction between C176F and M243 and neighboring hydrophobic side chain atoms partially compensates the loss[14]. Taken together, these data lead us to conclude that the p53 C176F mutated sublines retain functional p53 and are responsive to SAR405838 treatment. Our results show that SAR405838

treatment of SJSA-1 xenografts does not result in resistance to the MDM2 inhibitor. Evidence presented in this study leads to the conclusion that *in vitro* exposure to the MDM2 inhibitor SAR405838 may not reflect what will take place in a clinical setting. Furthermore, these data suggest that both *in vitro* and *in vivo* models of resistance should be investigated in order to better identify resistance that will occur in a clinical setting.

In summary, I provided substantial evidence to support the hypothesis that resistance to small molecule Bcl-2/Bcl-xL and MDM2-p53 interaction inhibitors can arise and that understanding the mechanisms of resistance to these agents allows for the development of rational treatment strategies. Furthermore, I have elucidated differences in development of resistance *in vitro* versus *in vivo* to Bcl-2/Bcl-xL and MDM2-p53 interaction inhibitors. These studies contribute to the continuous effort to treat cancer effectively with small molecule apoptosis-inducing agents.

References

1. Hollstein, M., Sidransky, D., Vogelstein, B. & Harris, C.C. p53 mutations in human cancers. *Science* **253**, 49-53 (1991).
2. Miyashita, T. & Reed, J.C. Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. *Cell* **80**, 293-9 (1995).
3. Sax, J.K. & El-Deiry, W.S. p53 downstream targets and chemosensitivity. *Cell Death Differ* **10**, 413-7 (2003).
4. Shibue, T. *et al.* Differential contribution of Puma and Noxa in dual regulation of p53-mediated apoptotic pathways. *EMBO J* **25**, 4952-62 (2006).
5. Geng, Y. *et al.* Cytoplasmic p53 and activated Bax regulate p53-dependent, transcription-independent neural precursor cell apoptosis. *J Histochem Cytochem* **58**, 265-75 (2010).
6. Yan, X.D., Li, M., Yuan, Y., Mao, N. & Pan, L.Y. Biological comparison of ovarian cancer resistant cell lines to cisplatin and Taxol by two different administrations. *Oncol Rep* **17**, 1163-9 (2007).
7. Twentyman, P.R., Fox, N.E., Wright, K.A. & Bleehen, N.M. Derivation and preliminary characterisation of adriamycin resistant lines of human lung cancer cells. *Br J Cancer* **53**, 529-37 (1986).
8. Yang, L.Y. & Trujillo, J.M. Biological characterization of multidrug-resistant human colon carcinoma sublines induced/selected by two methods. *Cancer Res* **50**, 3218-25 (1990).
9. Teicher, B.A. *et al.* Tumor resistance to alkylating agents conferred by mechanisms operative only in vivo. *Science* **247**, 1457-61 (1990).
10. Konopleva, M. *et al.* Mechanisms of apoptosis sensitivity and resistance to the BH3 mimetic ABT-737 in acute myeloid leukemia. *Cancer Cell* **10**, 375-88 (2006).
11. Yecies, D., Carlson, N.E., Deng, J. & Letai, A. Acquired resistance to ABT-737 in lymphoma cells that up-regulate MCL-1 and BFL-1. *Blood* **115**, 3304-13 (2010).
12. Deng, J. *et al.* BH3 profiling identifies three distinct classes of apoptotic blocks to predict response to ABT-737 and conventional chemotherapeutic agents. *Cancer Cell* **12**, 171-85 (2007).
13. van Delft, M.F. *et al.* The BH3 mimetic ABT-737 targets selective Bcl-2 proteins and efficiently induces apoptosis via Bak/Bax if Mcl-1 is neutralized. *Cancer Cell* **10**, 389-99 (2006).
14. Joerger, A.C. & Fersht, A.R. Structural biology of the tumor suppressor p53. *Annu Rev Biochem* **77**, 557-82 (2008).