

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

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Abstract

Purpose: Two clinical-stage anticancer drugs, the Bcl-2 inhibitor ABT-263, and the MDM2 inhibitor SAR405838 achieve complete tumor regression in animal models of leukemia but also induce acquired resistance. Elucidation of acquired resistance mechanisms and development of strategies to overcome the resistance are critical for their successful clinical development.

Experimental Design: We employed RS4;11 and MV4;11 cell lines, two acute leukemia models, to investigate acquired resistance mechanisms for both drugs *in vitro* and *in vivo* and evaluated several treatment regimens in xenograft mouse models to improve longer-term, complete tumor regression.

Results: Resistance to either SAR405838 or ABT-263 (or its analogue ABT-737) develops in acute leukemia models *in vitro* and *in vivo*. RS4;11 and MV4;11 tumors treated with SAR405838 acquire resistance to the drug by mutation of the *TP53* gene or

compromise of p53 protein function. RS4;11 tumors treated with either ABT-263 or ABT-737 acquire resistance primarily through downregulation of BAX but not BAK. When acute leukemia cells become highly resistant to the MDM2 inhibitor, they retain their sensitivity to the Bcl-2 inhibitors, or *vice versa*. Certain sequential or combination treatment of SAR405838 and ABT-263 can achieve longer-term tumor regression than treatment with either agent alone.

Conclusions: Our study provides new insights into the mechanisms of acquired resistance of Bcl-2 and MDM2 inhibitors in acute leukemia models and suggests that certain sequential or combination treatment of these two distinct classes of apoptosis-inducing agents should be tested as new treatment strategies for acute leukemia in the clinic. *Clin Cancer Res*; 21(11); 2558–68. ©2015 AACR.

Introduction

Acute lymphoblastic leukemia (ALL) and acute myelogenous leukemia (AML) remain incurable with conventional chemotherapy, and new therapeutic strategies are needed (1–3). Targeting key apoptosis regulators to promote apoptosis in tumor cells is being pursued as a promising cancer therapeutic strategy (4).

Bcl-2 family proteins are a class of master regulators of apoptosis (5–9). Bcl-2, the founding member of the family, is overexpressed in patients with leukemia and is an attractive therapeutic target (10, 11). Intense research efforts have yielded potent small-molecule inhibitors of Bcl-2 such as ABT-737 (12) and its orally active analogue ABT-263 (navitoclax; ref. 13). Both compounds have demonstrated impressive single-agent activity

against leukemia (12–14). Both compounds bind to Bcl-2, Bcl-xL, and Bcl-w with high affinities, but Bcl-2 appears to be their most critical target in leukemia cells *in vivo* (15). Accordingly, we refer to them as Bcl-2 inhibitors in this study.

The tumor suppressor p53 is another attractive therapeutic target for leukemia (16–21). In about 90% of leukemias, p53 retains its wild-type status, but its function is effectively inhibited by its endogenous cellular antagonist MDM2 (22–26). Small molecules designed to block the p53-MDM2 interaction (MDM2 inhibitors) activate the tumor suppressor function of wild-type p53 (27–30). Several highly potent MDM2 inhibitors, such as RG7112 (29, 31) and SAR405838 (32), are now in clinical trials for cancer treatment.

Although both ABT-263 (13) and SAR405838 (32) can achieve complete tumor regression in xenograft models of leukemia, tumors eventually regrow after termination of the treatment, suggesting the emergence of resistance to both classes of drugs. Such acquired resistance is 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 (34–39), no study of their *in vivo* acquired resistance mechanisms has been reported.

In this study, we have elucidated acquired resistance mechanisms for the Bcl-2 and MDM2 inhibitors *in vitro* and *in vivo* using the RS4;11 and the MV4;11 leukemia cell lines. The RS4;11 cell line was established from an ALL patient, whereas the MV4;11 cell line was established from a patient with AML. Both leukemia cell lines contain wild-type p53 and harbor a chromosomal t(4;11)

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Translational Relevance

Acquired resistance of tumor cells to anticancer drugs is a major cause of the failure of clinical cancer therapy. We have investigated the mechanisms of acquired resistance for two classes of novel anticancer drugs currently in clinical trials, ABT-263, a Bcl-2 inhibitor, and SAR405838, an MDM2 inhibitor. Although both drugs yield rapid and complete tumor regression in an acute leukemia xenograft model in mice, tumors regrow after cessation of treatment. Tumors that become highly resistant to SAR405838 retain sensitivity to ABT-263 and *vice versa*, and sequential treatment with ABT-263 first followed by SAR405838 or their combination was found to achieve longer-term tumor regression than either single agent. This suggests that sequential treatment or a combination of these two classes of novel apoptosis-inducing agents should be explored as a clinical strategy to overcome acquired drug resistance in acute leukemia patients.

translocation. Although the RS4;11 cell line harbors wild-type FLT3, the MV4;11 cell line harbors a *FLT3-ITD* mutation, a common (25%–30%) mutation associated with poor prognosis in AML patients (40–42). Both cell lines are sensitive to apoptosis induction by Bcl-2 and MDM2 inhibitors *in vitro* and are therefore excellent models to investigate the acquired resistance of leukemia cells to these two classes of apoptosis-inducing agents.

Our study has yielded new insights into the resistance mechanisms for both classes of drugs and resulted in novel therapeutic strategies.

Materials and Methods

Reagents and antibodies

SAR405838 was provided by Sanofi. ABT compounds were purchased from Selleck Chemicals. Rabbit antibodies for caspase-3, PARP, Mcl-1 (D35A5), Bcl-xL (54H6) and mouse antibody for caspase-7 were obtained from Cell Signaling Technology; rabbit antibodies for GAPDH and BAK (G-23) and mouse antibodies for BAX (6A7 and 6D149) and Bcl-2 were from Santa Cruz Biotechnology; 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 Pharmingen.

Cell culture, cell viability, and apoptosis assays

RS4;11 and MV4;11 cell lines were purchased from the American Type Culture Collection (ATCC), where authentication is performed by short tandem repeat analysis, and cultured as recommended for a maximum of 3 months. All *in vivo*-acquired resistant sublines were cultured for a maximum of 15 passages. Cell viability was evaluated by a Water Soluble Tetrazolium (WST) assay (Dojindo; ref. 43). Apoptosis was analyzed using Annexin V-FLUOS staining kit (Roche Applied Science). Differences in mean values of cell apoptosis among different groups were analyzed by two-way ANOVA using Prism, with a *P* value of <0.05 being considered significant.

Resistant cell lines

Both parental cell lines were treated with ABT-737 starting from 10 nmol/L for 72 hours. The cells were then rinsed, and the

remaining live cells were expanded in regular medium. This process was repeated with increased drug concentration till 10 μ mol/L, 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 μ mol/L. DMSO-treated cell lines were generated as controls.

shRNA interferences

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; ref. 35). The oligonucleotides were annealed and ligated into a self-inactivating lentiviral vector under the control of the H1 promoter (44). 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. Ninety-six hours after infection, the infected cells were sorted for GFP fluorescence.

p53 mutation analysis

Primers to amplify and sequence genomic DNA for exons 2 to 11 of human *TP53* were used according to Hauser and colleagues (45). Primers to amplify and sequence cDNA for exons 2 to 11 of human p53 were used according to Aziz and colleagues (36). Mutation surveyor (SoftGenetics LLC) 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, 5×10^6 tumor cells with 50% Matrigel were injected subcutaneously on the dorsal side of SCID mice. For efficacy experiments, tumor sizes and animal weights were measured 2 to 3 times per week with tumor volume (mm^3) = $(\text{length} \times \text{width}^2)/2$. Significance (*P*) was calculated by unpaired two-tailed *t* test using Prism, with *P* value of <0.05 being considered significant.

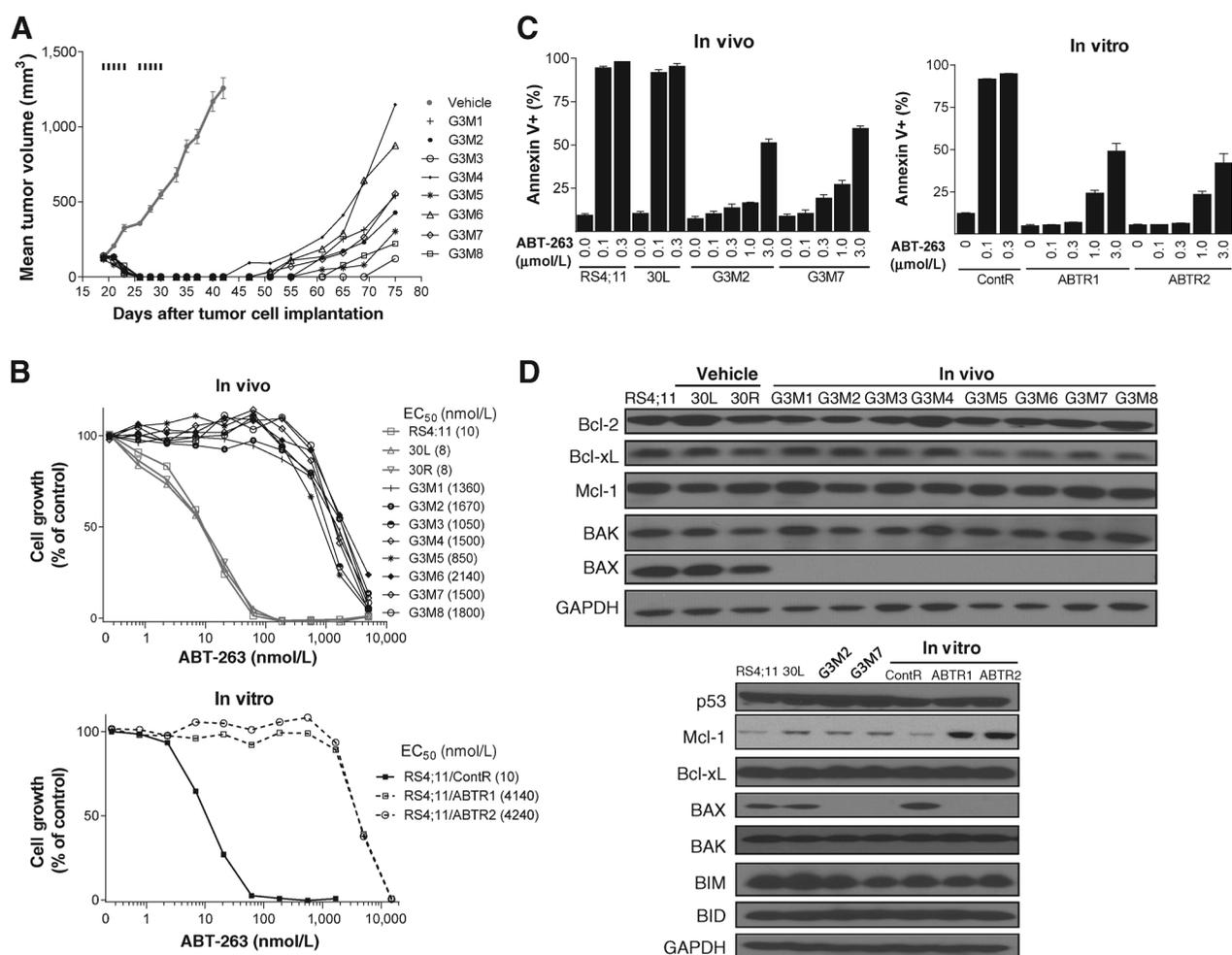
Results

Establishment and characterization of RS4;11 sublines resistant to the Bcl-2 inhibitors

We first employed the RS4;11 cell line to investigate the acquired resistance mechanisms to the Bcl-2 inhibitors.

Treatment of RS4;11 xenograft tumors in mice with ABT-737 yielded complete tumor regression (Fig. 1A), but all tumors regrew after termination of the treatment. We isolated RS4;11 tumor cells from the regrown tumors and established 8 sublines (G3M1–G3M8). Although the RS4;11 parental cell line and 2 representative sublines (30L and 30R) established from the vehicle-treated tumors are equally sensitive to both ABT-737 and ABT-263 in a cell growth assay, all 8 sublines obtained from the ABT-737-treated tumors become resistant to ABT-263 and ABT-737, with an average IC_{50} value >100-fold higher than the control cell lines (Fig. 1B and Supplementary Fig. S1A). ABT-737 and ABT-263 also become ineffective in induction of apoptosis in the G3M1–G3M8 sublines (Fig. 1C and Supplementary Fig. S1B).

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**Figure 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 or ABT-737 at 100 mg/kg, i.p. for 5 days per week for 2 weeks. Vehicle-treated tumors and ABT-737-treated, regrown tumors were harvested and cultured to generate sublines. B, sensitivity of *in vivo* and *in vitro* 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, and cell viability was determined by a Water Soluble Tetrazolium (WST) assay. C, sensitivity of *in vivo* and *in vitro* RS4;11 sublines to ABT-263 in an apoptosis assay. Parental RS4;11 cell line and sublines established from RS4;11 xenografts (30L, vehicle-treated; G3M2 and G3M7, SAR405838-treated) and RS4;11 sublines established *in vitro* (ContR, vehicle-treated; ABTR1 and ABTR2, SAR405838-treated) were treated with ABT-263 or vehicle control for 24 hours for apoptosis analysis by flow cytometry with Annexin V/Propidium iodide (PI) 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, 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.

Upregulation of the Mcl-1 protein was identified as the primary acquired resistance mechanism to ABT-737 (34, 35). Western blotting, however, showed that the resistant G3M1–G3M8 and the sensitive control cell lines have similar levels of Mcl-1 expression (Fig. 1D). Profiling of other Bcl-2 family members showed that compared with the control cell lines, BAX protein is profoundly downregulated in all the 8 resistant cell lines (Fig. 1D), but other Bcl-2 proteins, including BAK, have similar levels between resistant and sensitive cell lines (Fig. 1D and Supplementary Fig. S1C).

To investigate the acquisition of resistance to the Bcl-2 inhibitors *in vitro*, the RS4;11 cells were exposed to gradually increasing concentrations of ABT-737. This yielded two sublines (ABTR1 and ABTR2), which exhibit >100-fold resistance

to ABT-263 compared with the parental RS4;11 cell line in a cell growth assay (Fig. 1B) and are also insensitive to apoptosis induction by ABT-263 (Fig. 1C). Western blotting showed that the BAX protein, but not BAK, is also greatly downregulated in ABTR1 and ABTR2 sublines when compared with the parental cell line. However, in contrast with the lack of Mcl-1 upregulation in all the 8 *in vivo*-resistant sublines, Mcl-1 is upregulated in the two *in vitro*-resistant sublines when compared with the control cell lines (Fig. 1D).

We analyzed the mRNA levels for both BAX and Mcl-1 in these resistant and control cell lines by qPCR. Our data showed that the mRNA levels of BAX are reduced by approximately 70% in each of these resistant sublines obtained from *in vitro* and *in vivo* ABT-737 treatment compared with the RS4;11 parental and vehicle

control-treated cell lines (Supplementary Fig. S1D). However, the Mcl-1 mRNA levels are very similar in these two resistant cell lines compared with the RS4;11 parental cell line (Supplementary Fig. S1D). Hence, although the reduced transcription of BAX clearly contributes to the reduction in BAX protein in these *in vitro*- and *in vivo*-resistant sublines, the increase of Mcl-1 protein in these two *in vitro*-resistant sublines is not due to a change in Mcl-1 transcription.

Establishment and characterization of RS4;11-resistant sublines to the MDM2 inhibitor

The MDM2 inhibitor SAR405838 inhibits cell growth with $IC_{50} = 140$ nmol/L and effectively induces apoptosis in the RS4;11 cell line (Fig. 2).

Treatment of the RS4;11 xenograft tumors in mice with SAR405838 induced rapid and complete tumor regression, which persisted for >30 days, but eventually the tumors returned (Fig. 2A). Harvesting and culturing all regrown tumors treated with SAR405838 established 7 sublines (G7M1-G7M7). A cell growth assay showed that although two sublines, G7M2 and G7M4, exhibited 5-fold greater resistance to SAR405838 compared with the parental and vehicle-treated cell lines, the other 5 sublines showed >35-fold resistance to SAR405838 (Fig. 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. 2C and Supplementary Fig. S2A).

We also established two RS4;11 sublines (MIR1 and MIR2) *in vitro* by gradually increasing the concentration of SAR405838 in

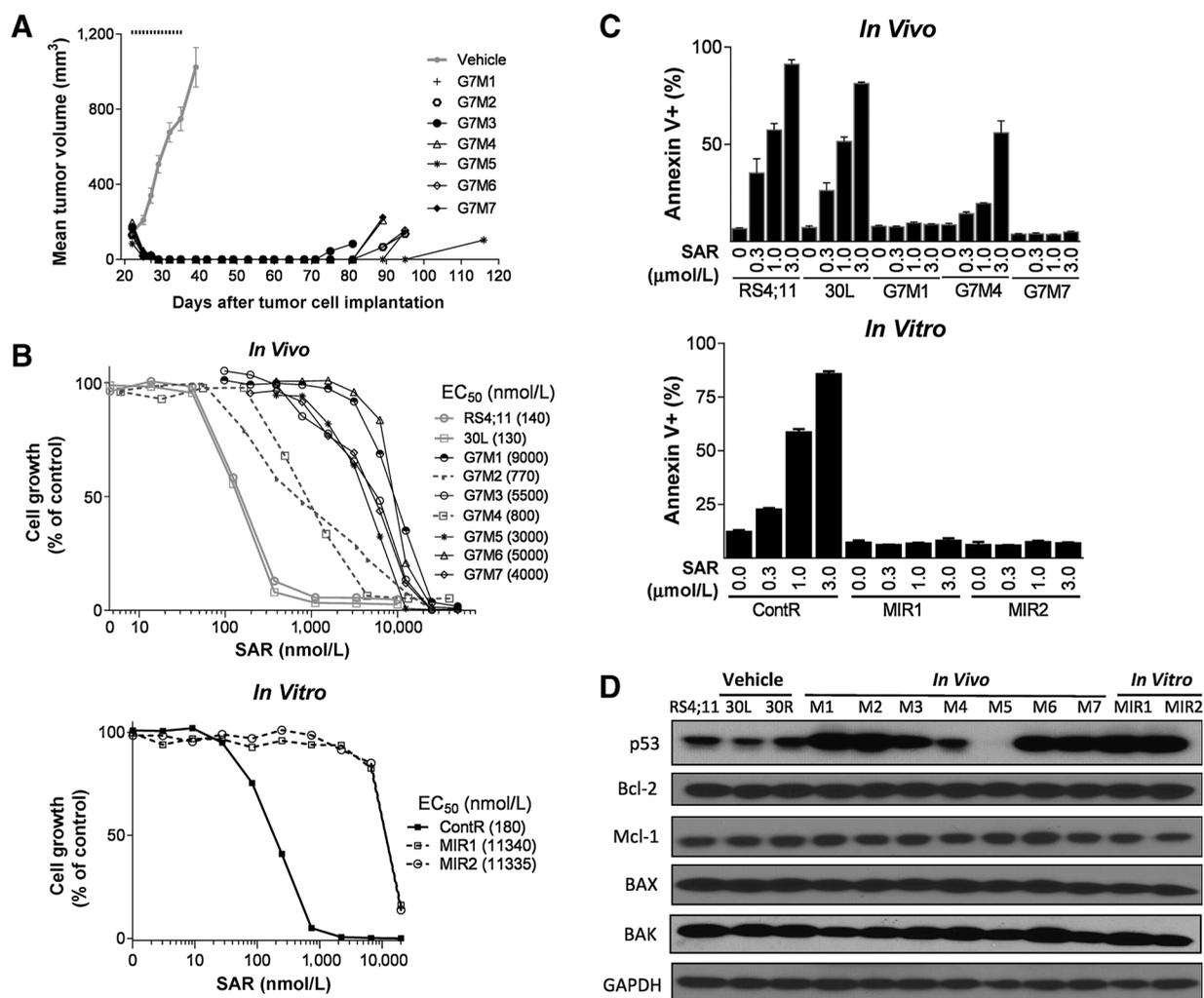


Figure 2.

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 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 mm³ and then cultured to establish sublines. B, sensitivity of *in vivo* and *in vitro* 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) and RS4;11 sublines established *in vitro* (ContR, vehicle-treated; MIR1 and MIR2, SAR405838-treated) were treated with SAR405838, and cell viability was determined by a WST assay. C, sensitivity of *in vivo* and *in vitro* RS4;11 sublines to SAR405838 in an apoptosis assay. RS4;11 parental and established sublines were treated with SAR405838 for 24 hours for apoptosis analysis by flow cytometry with Annexin V/PI double staining. D, 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.

the cell culture. Both the MIR1 and MIR2 sublines are >60-fold less sensitive to SAR405838 than the control cell lines in a cell growth assay (Fig. 2B), and their resistance was confirmed in an apoptosis assay (Fig. 2C).

Because the activity of SAR405838 depends upon wild-type p53 (32), we analyzed the *TP53* mutation by sequencing exons 2 to 11 in all the *in vitro* and *in vivo* sublines obtained with SAR405838 treatment. Both the MIR1 and MIR2 *in vitro* sublines and 5 of 7 of the *in vivo* sublines harbor *TP53* mutations (Supplementary Table S1).

Immunoblotting showed that, compared with the control cell lines, basal p53 protein expression is increased in all the sublines harboring *TP53* mutations (G7M1-3, 6-7 and MIR1-2; Fig. 2D). qPCR analysis showed that p53 mRNA levels are not significantly changed in G7M1, G7M2, MIR1, and MIR2 sublines with a mutated p53 compared with the parental RS4;11 cell line but much lower in the G7M5 subline with a low level of p53 protein (Supplementary Fig. S2B). In contrast with 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. 2D).

To investigate if any of these sublines obtained from SAR405838 treatment still retains functional p53, we examined p21, a p53-regulated protein, accumulation of p53 protein and PARP cleavage in these sublines treated with SAR405838 (Supplementary Fig. S2C and S2D). 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 with that in the parental cell line (Supplementary Fig. S2C). In G7M5, which has no expression of p53 at the basal level (Fig. 2D), SAR405838 fails to induce p53 and p21 upregulation

and PARP cleavage (Supplementary Fig. S2C). 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 (Supplementary Fig. S2D).

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

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

Exons 2 to 11 were sequenced in all the RS4;11-resistant sublines to ABT compounds. No *TP53* mutation could be detected, suggesting that these sublines may contain wild-type p53 and could be still sensitive to MDM2 inhibitors.

Indeed, SAR405838 potently inhibits cell growth and induces dose-dependent apoptosis in the ABTR1 and ABTR2 sublines (Fig. 3), albeit with modestly reduced potency compared with that in the control RS4;11 cell line.

In the 8 *in vivo* sublines (G3M1–G3M8) resistant to ABT compounds, SAR405838 is still effective in inhibition of cell growth (Fig. 3A). Apoptosis induction by SAR405838 is attenuated by only 2 to 3 times in the G3M1 and G3M6 sublines compared with the RS4;11 parental and vehicle-treated cell lines (Fig. 3B). In the RS4;11 parental, and the G3M1 and G3M6 sublines, SAR405838 dose-dependently induces activation of p53 and apoptosis, as shown by upregulation of p53, p21, MDM2, and PUMA proteins, and cleavage of caspase-3/-7 and PARP (Fig. 3C).

Hence, when the RS4;11 cells acquire profound resistance to the Bcl-2 inhibitors, the cells are still sensitive to the MDM2 inhibitor, thus lacking cross-resistance.

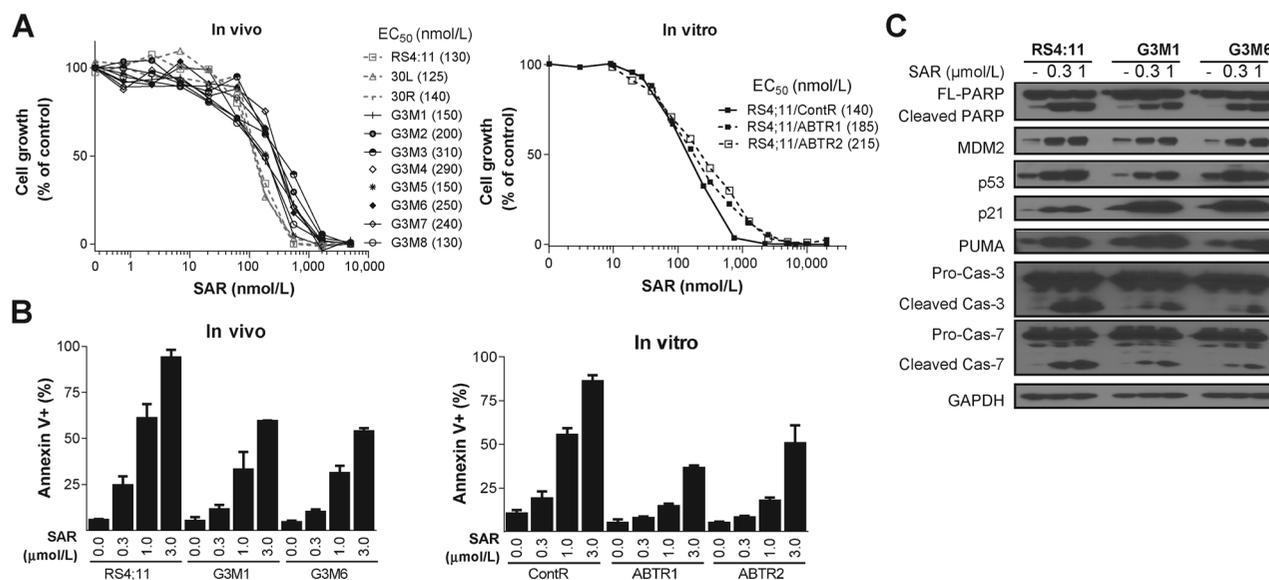
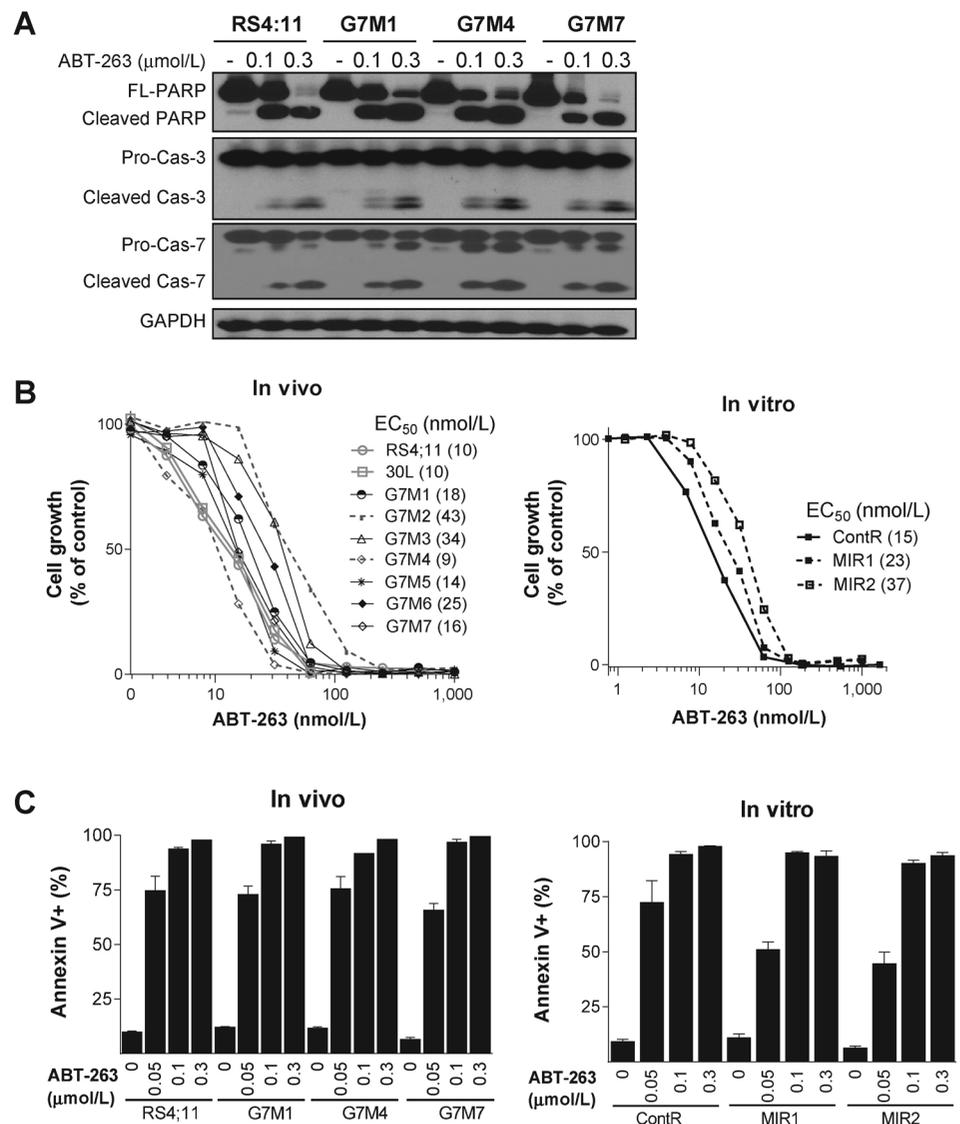


Figure 3.

Evaluation of SAR405838 in RS4;11 xenografts or sublines treated with Bcl-2 inhibitors. A, RS4;11 parental and sublines previously exposed to ABT-737 *in vivo* (G3M1–G3M8) and *in vitro* (ABTR1 and ABTR2) were treated for 4 days with SAR405838, and cell viability was determined by a WST assay. B, RS4;11 parental and representative sublines were treated with SAR405838 for 24 hours for apoptosis analysis by flow cytometry with Annexin V/PI double staining. C, RS4;11 parental and two representative sublines (G3M1 and G3M6) were treated with SAR405838 for 24 hours for immunoblotting of p53 and p53-regulated proteins and biochemical markers of apoptosis.

**Figure 4.**

Evaluation of ABT-263 in RS4:11 xenografts or sublines treated with SAR405838. A, representative RS4:11 sublines were treated with ABT-263 for 24 hours, and biochemical markers of apoptosis were analyzed by immunoblotting. B, RS4:11 parental and sublines were treated with ABT-263 for 4 days, and cell viability was determined by a WST assay. C, cells were treated with ABT-263 for 24 hours for apoptosis analysis by flow cytometry with Annexin V/PI double staining.

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 with SAR405838 treatment. In cell growth and apoptosis assays, both *in vitro* (MIR1 and MIR2) and *in vivo* (G7M1–G7M7) sublines show similar sensitivity to ABT-263, as compared with the RS4:11 parental cell line (Fig. 4). Based upon cleavage of caspase-3, caspase-7, and PARP (Fig. 4A), ABT-263 is equally effective in inhibiting cell growth and induction of apoptosis in representative sublines such as G7M1, G7M4, and G7M7 and the parental cell line (Fig. 4B and C). Hence, although p53 mutation or compromised p53 function greatly diminishes the activity of the MDM2 inhibitor, it has no effect on the activity of the Bcl-2 inhibitor.

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

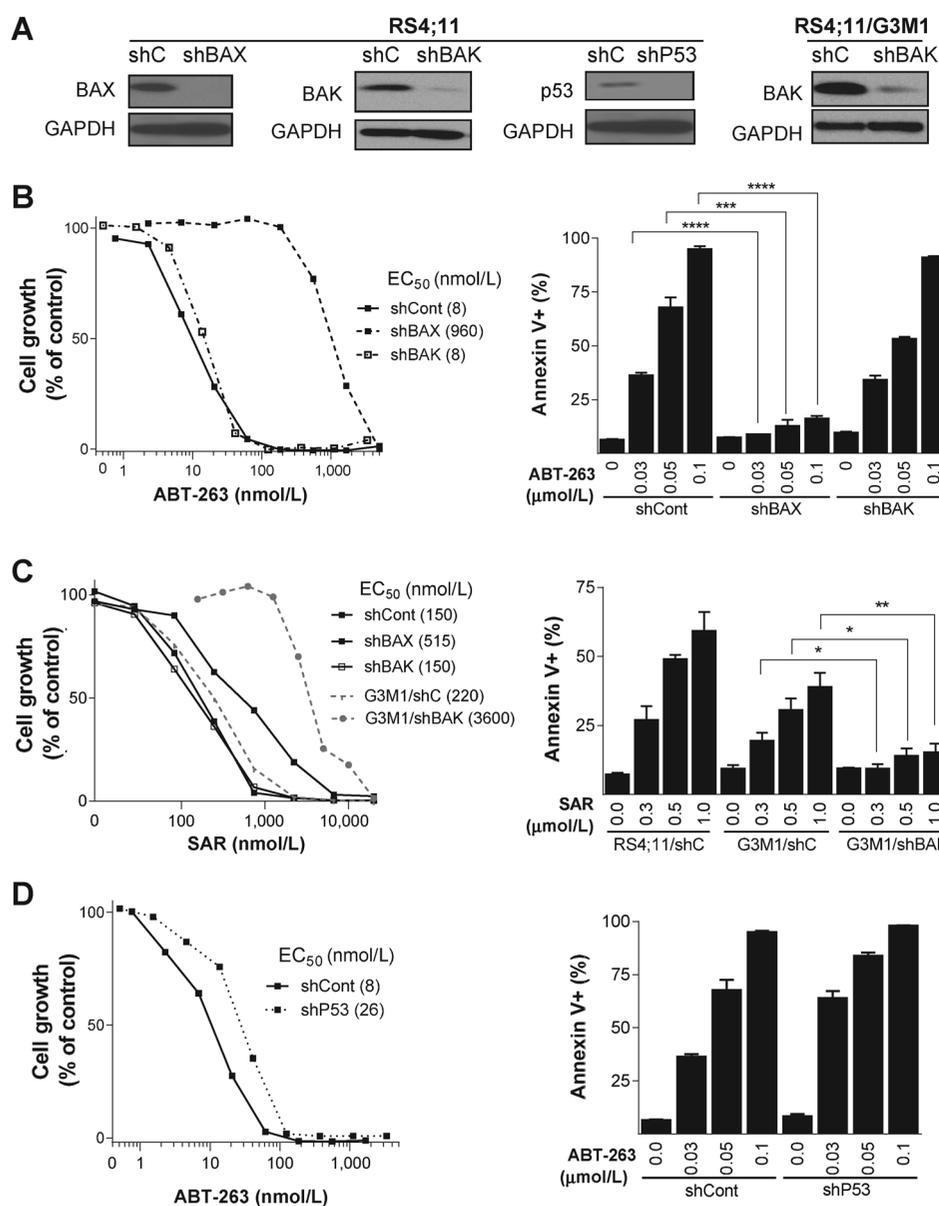
When the RS4:11 cell line was treated with ABT-737 *in vitro* and *in vivo*, BAX, but not BAK, is profoundly downregulated (Fig. 1D),

and the cells become resistant to ABT-737 and ABT-263 but retain their sensitivity to SAR405838 (Fig. 3). This suggested that BAX and BAK play different roles in the activity of these two different classes of drugs, which was further investigated.

Efficient shRNA knockdown of BAX expression 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. 5A and B). However, efficient shRNA knockdown 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. 5B). For SAR405838, efficient knockdown of BAX expression in the RS4:11 parental cell line results in only an approximately 3-fold reduction in sensitivity, and efficient knockdown of BAK expression has no effect on the activity of SAR405838 (Fig. 5C).

Because BAX was downregulated in all the RS4:11 sublines obtained from *in vitro* and *in vivo* treatment of the Bcl-2 inhibitors (Fig. 1; Supplementary Fig. S3A and S3B), we knocked down BAK in one of the sublines (G3M1; Fig. 5A) and evaluated the

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**Figure 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, 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 or treated with ABT-263 for 24 hours for apoptosis analysis by flow cytometry with Annexin V/PI double staining (***, $P < 0.001$; ****, $P < 0.0001$; t test). C, 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. Apoptosis analysis by flow cytometry with Annexin V/PI double staining. RS4;11/shControl, G3M1/shControl, and G3M1/shBAK cells were treated with SAR405838 for 24 hours (*, $P < 0.05$; **, $P < 0.01$; t test). D, 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 or for 24 hours for apoptosis analysis by flow cytometry with Annexin V/PI double staining.

sensitivity of the cells to SAR405838. Efficient knockdown of BAK in the G3M1 subline results in a >15-fold increase in the IC₅₀ value for SAR405838 in the cell growth assay and greatly reduces the apoptotic response to SAR405838 (Fig. 5C).

These data show that although BAX, but not BAK, plays a dominant role for the activity of ABT-263 in the RS4;11 cell line, the presence of either BAX or BAK is sufficient for effective apoptosis induction by SAR405838.

A minimal role of p53 in the activity of ABT-263

ABT-263 is effective in RS4;11 sublines harboring mutated TP53, 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 lentivirus shRNA (Fig. 5A). Although efficient knockdown of p53 dramatically reduces the activity of SAR405838 (32), it has only a modest effect on the

activity of ABT-263 (Fig. 5D). Hence, p53 has a minimal role in the activity of ABT-263 in the RS4;11 cell line.

RS4;11 xenograft tumors lack cross-resistance *in vivo* to Bcl-2 and MDM2 inhibitors

When RS4;11 cells acquire resistance to the Bcl-2 inhibitors, they retain their sensitivity to the MDM2 inhibitor *in vitro* (Fig. 3). Conversely, when the RS4;11 cells acquire resistance to SAR405838, they remain sensitive to ABT-263 *in vitro* (Fig. 4). These data suggest that when the RS4;11 tumors acquire resistance to one class of apoptosis-inducing agent, they may still be susceptible *in vivo* to the second class of apoptosis-inducing agent. We directly examined this possibility in mice.

When RS4;11 xenograft tumors were treated with ABT-263, all tumors rapidly became undetectable (Supplementary Fig. S3C), but regrew after the treatment was ended. When the regrown

tumors reached an average volume of 200 mm³, they were randomized into two groups and treated with the MTD of ABT-263 (150 mg/kg) or SAR405838 at 100 mg/kg (Fig. 6A and Supplementary Fig. S3C). Although one of the 4 tumors treated with ABT-263 regressed, the other three tumors had no regression. In comparison, all 4 regrown tumors treated with SAR405838 completely regressed (Fig. 6A).

Similarly, the RS4;11 tumors regressed rapidly when treated with SAR405838 at 100 mg/kg in mice (Supplementary Fig. S3D), but all tumors regrew after termination of the treatment. When the regrown tumors reached an average volume of 200 mm³, they were randomized into two groups and treated with either ABT-263 at 100 mg/kg or SAR405838 at 200 mg/kg (Fig. 6B and Supplementary Fig. S3D). All tumors treated with SAR405838 at

200 mg/kg continued to grow, but 5 of 6 tumors treated with ABT-263 regressed (Fig. 6B).

Our data show that when regrown RS4;11 xenograft tumors become resistant to the initial class of drug, they are still very responsive to the second class of drug, thus lacking cross-resistance.

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

Using the RS4;11 cell line, we showed that when the cells develop profound acquired resistance to one class of apoptosis-inducing agents, they retain their sensitivity to the other class of apoptosis-inducing agents. We next extended our studies both *in vitro* and *in vivo* to the MV4;11 cell line.

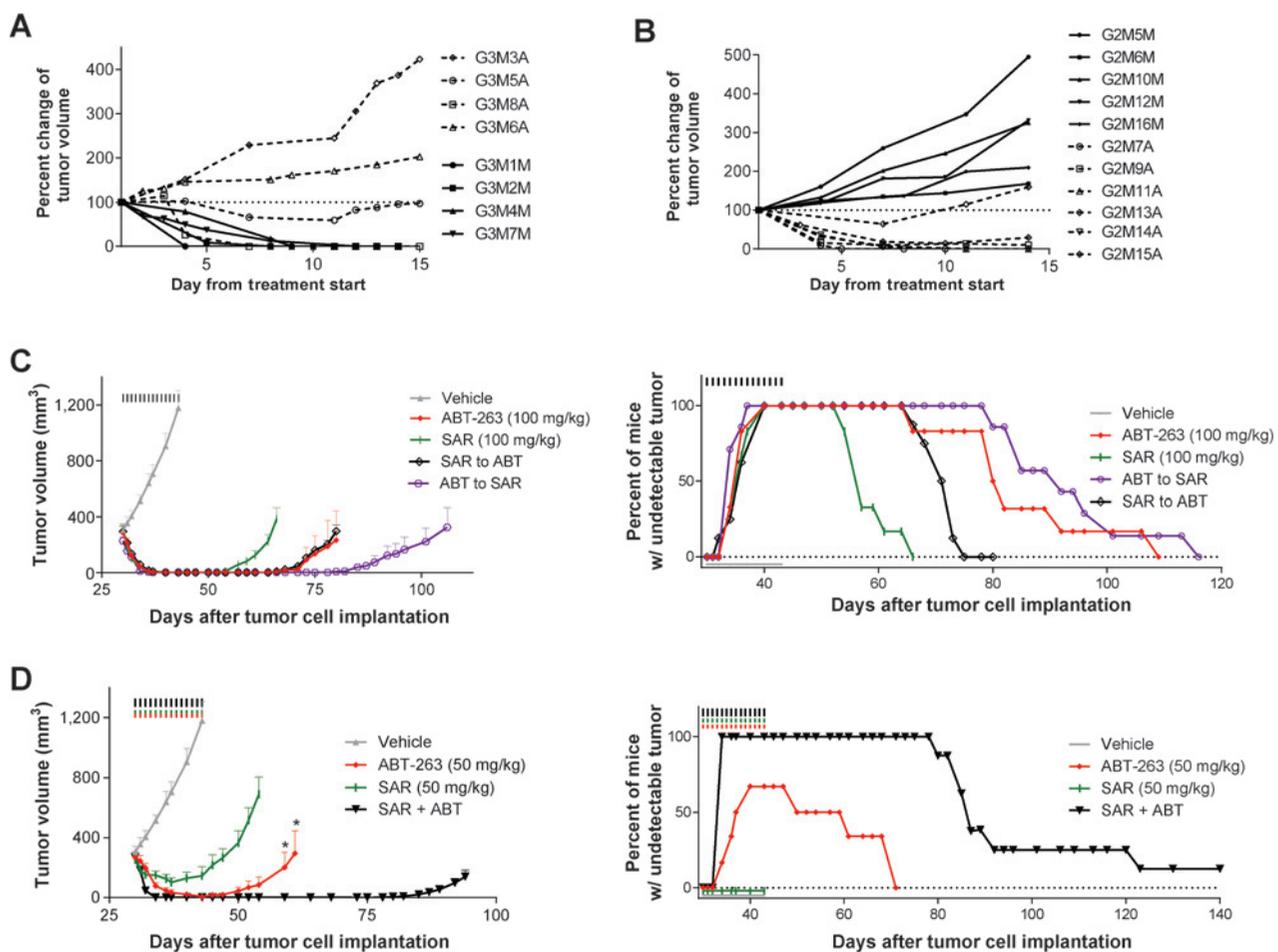


Figure 6.

Antitumor activity of ABT-263 and SAR405838 alone, sequential treatment of the two drugs, and their combination in the RS4;11 xenograft model. A, antitumor activity of ABT-263 and SAR405838 in regrown tumors initially treated with ABT-263. When regressed tumors initially treated with ABT-263 daily for 21 days at 100 mg/kg *via* oral gavage (Supplementary Fig. S3A) regrew to 100 to 200 mm³, they 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. B, antitumor activity of ABT-263 and SAR405838 in regrown tumors initially treated with SAR405838. When regressed tumors initially treated with SAR405838 for 21 days at 100 mg/kg (Supplementary Fig. S3D) regrew to 100 to 200 mm³, they were retreated 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. C, antitumor activity of single agents and sequential treatment. 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 an additional 7 days. Percentage of tumor-free survival of mice in the RS4;11 efficacy experiment shown on the left. D, antitumor activity of ABT-263, SAR405838, and their combination. When tumors grew to approximately 300 mm³, mice were treated with vehicle, ABT-263, SAR405838, or their combination at 50 mg/kg for 14 days *via* oral gavage (*, $P < 0.05$; *t* test for comparison of ABT-263 single-agent treatment at 50 mg/kg to the combination treatment). Percentage of tumor-free survival of mice in the RS4;11 efficacy experiment shown on the left.

The MV.ABT-R cell line was generated by treating the MV4;11 cell line with ABT-263 *in vitro*. MV.ABT-R is >18-fold less sensitive to ABT-263 than the parental cell line, but retains its sensitivity to SAR405838 (Supplementary Fig. S4A and S4B). Western blotting revealed that the MV.ABT-R subline shows no decrease in expression of BAX but has increased expression of Mcl-1 protein compared with the control lines (Supplementary Fig. S4C). Nevertheless, stable knockdown of BAX expression in the parental MV4;11 cell line also drastically attenuates cell growth inhibition and apoptosis induction by ABT-263 compared with the control cell line, but the cell line remains sensitive to SAR405838 (Supplementary Fig. S5). In contrast, efficient knockdown of BAK expression in the MV4;11 cell line has little or no effect on the activity of ABT-263 or SAR405838 (Supplementary Fig. S5).

We generated the MV.MI-R cell line by treating the MV4;11 cell line with SAR405838 *in vitro*. MV.MI-R is >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 (Supplementary Fig. S4). Sequencing of exons 2 to 11 reveals that p53 has 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 (Supplementary Fig. S6A), which greatly reduces the activity of SAR405838 but has no effect on the activity of ABT-263 (Supplementary Fig. S6B–S6D).

We then evaluated both SAR405838 and ABT-263 in mice bearing MV4;11 xenograft tumors. Although ABT-263 is quite potent *in vitro* (IC₅₀ = ~50 nmol/L in a cell growth assay), this drug at 100 mg/kg only modestly inhibits growth of MV4;11 xenograft tumors (Supplementary Fig. S7A). In comparison, SAR405838 at 100 mg/kg induces complete but transient tumor regression of all MV4;11 xenograft tumors (Supplementary Fig. S7A). 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). Cell growth assay showed that although two sublines (G3M2 and G3M5) exhibit moderate resistance to SAR405838 compared with the parental and vehicle-treated cell lines, the other 4 sublines are highly resistant to SAR405838 (Supplementary Fig. S7B). SAR405838 becomes ineffective in activation of p53 and induction of apoptosis in these resistant sublines (Supplementary Fig. S7C and S7D). Sequencing exons 2 to 11 showed that p53 has an R248W homozygous mutation in all of the highly resistant SAR405838 sublines but harbors an R248W heterozygous mutation in those moderately resistant sublines (G3M2 and G3M5). 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 drug resistance compared with the control cell lines (Supplementary Fig. S8).

Sequential strategies improve tumor-free survival

Although both Bcl-2 and MDM2 inhibitors as single agents yield complete regression of the RS4;11 xenograft tumors, tumors regrow shortly after cessation of their respective treatment. We investigated strategies to improve tumor-free survival of mice in the RS4;11 xenograft model.

Because these two classes of drugs lack cross-resistance, we tested two sequential treatment strategies *in vivo*: (i) ABT-263 daily for 1 week followed by SAR405838 daily for a second week or (ii) treatments in reverse order (Fig. 6C). Both strategies showed no

signs of toxicity (Supplementary Fig. S9A). The 100% tumor-free survival time of mice was 12 days when treated with SAR405838 alone for 2 weeks, and was 24 days when treated with ABT-263 alone for 2 weeks. In comparison, the 100% tumor-free survival time was 41 days with ABT-263 first for 1 week, followed by SAR405838 for another week, and 24 days for the reverse scheme (Fig. 6C). Hence, sequential treatment with ABT-263 first, followed by SAR405838, is the most effective of the four treatment schemes in achieving tumor-free survival in 100% of mice.

Combination strategies improve tumor-free survival

We investigated the synergistic effect combining ABT-263 and SAR405838 on cell growth and induction of apoptosis using a fixed-ratio design. Our data showed that the combination of these two drugs is highly synergistic based upon the combination index (CI) values (46) in the RS4;11 and MV4;11 cell lines (Supplementary Tables S2 and S3).

We next investigated if the combination of ABT-263 and SAR405838 can further improve the tumor-free survival of mice in the RS4;11 xenograft model. The MTD in RS4;11 tumor-bearing mice of the combination was determined to be 50 mg/kg for each agent. SAR405838 as a single agent at 50 mg/kg fails to yield complete tumor regression in any animal, but ABT-263 as a single agent at 50 mg/kg achieves tumor regression in 4 of 6 mice. The combination of the two drugs both at 50 mg/kg, however, results in complete tumor regression in 100% of mice for a period of 44 days (Fig. 6D) with no sign of toxicity (Supplementary Fig. S9B). Therefore, the combination of these two drugs at 50 mg/kg each is much more effective than either drug as a single agent at 100 mg/kg in achieving complete tumor regression in 100% of mice.

Discussion

Although molecularly targeted anticancer drugs can achieve complete clinical response, such responses are typically short-lived and patients relapse quickly (2, 47, 48). It is therefore critical to identify mechanism(s) of acquired resistance and to develop strategies to combat such resistance. 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 effectively induce apoptosis in the RS4;11 cell line *in vitro* and achieve complete regression of RS4;11 xenograft tumors in mice. Both *in vitro* and *in vivo* treatments, however, lead to the development of sublines that possess profound acquired resistance to this class of drugs. BAX, but not BAK, is consistently downregulated in each of these resistant sublines obtained from *in vitro* and *in vivo* treatments with the Bcl-2 inhibitors (Fig. 1D; Supplementary Fig. S3A and S3B), suggesting a critical role for BAX. Indeed, knockdown of BAX, but not BAK, in the parental RS4;11 cell line dramatically reduces the sensitivity of the tumor cells to ABT-263, confirming that BAX is a key mediator for apoptosis induction by ABT-263 (Fig. 5). Interestingly, although Mcl-1 is not upregulated in the resistant sublines obtained from *in vivo* treatment of RS4;11 tumors in mice with ABT-737, it is increased in the resistant sublines obtained from *in vitro* treatment of ABT-737 (Fig. 1D). Although Mcl-1 upregulation has been shown to be a primary mechanism of resistance to ABT-737 based upon *in vitro* data (34, 35), our study shows that loss of BAX, rather than upregulation of Mcl-1, is the primary

acquired resistance mechanism when the RS4;11 tumors are treated with ABT-737/ABT-263 in animals.

For the MDM2 inhibitor SAR405838, *in vitro* and *in vivo* treatments of the RS4;11 cell line with the drug also result in acquired resistance. Each of these resistant sublines either harbors a non-functional mutated p53 gene for its transcriptional activity or contains p53 with compromised transcriptional activity. Consequently, SAR405838 becomes either much less effective or completely ineffective in activation of p53 in these resistant cell lines (Fig. 2 and Supplementary Fig. S2).

When all RS4;11 sublines obtained from *in vitro* and *in vivo* treatments with either ABT-737 or ABT-263 become highly resistant to ABT compounds, they retain their sensitivity *in vitro* to SAR405838 (Fig. 3). When regressed RS4;11 tumors treated with ABT-263 regrow, these relapsed tumors become much less responsive to even a higher dose of ABT-263, but undergo rapid tumor regression when treated with SAR405838 (Fig. 6A). Conversely, ABT-263 is still very effective against RS4;11-resistant sublines generated by both *in vitro* and *in vivo* treatment of SAR405838 (Fig. 4). Further, when RS4;11 tumors initially treated with SAR405838 regrow, these relapsed tumors become unresponsive to the second round of SAR405838 treatment but undergo rapid tumor regression when treated with ABT-263 (Fig. 6B).

ABT-199 is a new, highly potent and selective Bcl-2 inhibitor currently in phase III clinical development for the treatment of acute leukemia (49). We evaluated ABT-199 in the RS4;11 parental cell line and sublines obtained with either ABT-737 or SAR405838 treatment (Supplementary Fig. S10 and S11). Similar to ABT-737 and ABT-263, ABT-199 is highly effective not only in the RS4;11 parental cell line, but also in sublines obtained from either *in vitro* or *in vivo* treatment with SAR405838. ABT-199 is ineffective in all sublines obtained from *in vitro* or *in vivo* treatment with ABT-737. Because ABT-199 is a selective Bcl-2 inhibitor, our data show that Bcl-2 is the primary cellular target for ABT-737 and ABT-263 in the RS4;11 cell line.

The lack of cross-resistance of the RS4;11 cell line 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. Indeed, knockdown of p53 in the parental RS4;11 cell line dramatically diminishes the activity of SAR405838 but has a minimal effect on the activity of ABT-263 (Fig. 5). In contrast, knockdown of BAX profoundly reduces the potency of ABT-263 but only has a modest effect on the activity of SAR405838.

The lack of cross-resistance for these two classes of apoptosis-inducing agents observed in the RS4;11 model was extended into the MV4;11 model both *in vitro* and *in vivo*. The MV4;11 cells develop profound acquired resistance *in vitro* upon treatment with either ABT-263 or SAR405838. When the MV4;11 cells develop acquired resistance to the one class of drug, they retain their sensitivity to the second class of drug. In mice, treatment of the MV4;11 xenografts with SAR405838, but not ABT-263, leads to complete tumor regression, but the regressed tumors regrow after the treatment is terminated (Supplementary Fig. S7). Sublines established from the regressed MV4;11 tumors treated with SAR405838 become highly resistant to SAR405838 but retain their sensitivity to ABT-263 *in vitro*. Mechanistically, MV4;11 cells develop profound acquired resistance to SAR405838 by a homozygous mutation of R248W of p53. Because ABT-263 failed to induce complete tumor regression of the MV4;11 tumors *in vivo*, we could not establish *in vivo*-resistant MV4;11 sublines to ABT-263. However, treatment of the MV4;11 cell line *in vitro* with ABT-

263 resulted in acquired resistance. Interestingly, upregulation of Mcl-1 protein, but not decrease of BAX, is observed in the two *in vitro*-resistant sublines.

Because 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 daily for 2 weeks at 100 mg/kg (24 days) and 240% longer than SAR405838 daily for 2 weeks at 100 mg/kg (12 days; Fig. 6C). However, the alternative sequential treatment is only equally effective, as compared with ABT-263 daily for 2 weeks in achieving tumor-free survival in 100% of mice (Fig. 6C). Combination of ABT-263 and SAR405838 at 50 mg/kg for both drugs achieves complete tumor regression in 100% of the mice for 44 days in the RS4;11 xenograft model (Fig. 6D), 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.

In summary, our study shows that acute leukemia cells develop acquired resistance *in vitro* and *in vivo* when treated with either a Bcl-2 inhibitor or an MDM2 inhibitor. The mechanisms of acquisition of resistance for these two classes of apoptosis-inducing agents are, however, completely different. New insights into the mechanisms of acquired resistance led us to develop both sequential and combination treatment strategies with these two classes of apoptosis-inducing agents to effectively improve the tumor-free survival of mice over the single-agent treatments, which should be evaluated for the treatment of acute leukemia in the clinic.

Disclosure of Potential Conflicts of Interest

S. Wang reports receiving research grants from Sanofi and has ownership interest (including patents) in Ascenta Therapeutics. Y. Zhao reports receiving royalties from the University of Michigan Ann Arbor from a patent on the MDM2 inhibitor, which is owned by the University and licensed to Sanofi and Ascenta Therapeutics. W. Sun reports receiving royalties from the University of Michigan from serial patents on spiroindole-type inhibitors, which are owned by the University and licensed to Sanofi. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

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Development of methodology: C.G. Hoffman-Luca, D. Ziazadeh
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C.G. Hoffman-Luca, D. Ziazadeh
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C.G. Hoffman-Luca, D. Ziazadeh, S. Wang
Writing, review, and/or revision of the manuscript: C.G. Hoffman-Luca, D. Ziazadeh, L. Debussche, S. Wang
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): D. Ziazadeh, D. McEachern, Y. Zhao, W. Sun
Study supervision: L. Debussche, S. Wang

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

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