mTOR Inhibition Specifically Sensitizes Colorectal Cancers with KRAS or BRAF Mutations to BCL-2/BCL-XL Inhibition by Suppressing MCL-1

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ABSTRACT

Colorectal cancers harboring KRAS or BRAF mutations are refractory to current targeted therapies. Using data from a high-throughput drug screen, we have developed a novel therapeutic strategy that targets the apoptotic machinery using the BCL-2 family inhibitor ABT-263 (navitoclax) in combination with a TORC1/2 inhibitor, AZD8055. This combination leads to efficient apoptosis specifically in KRAS- and BRAF-mutant but not wild-type (WT) colorectal cancer cells. This specific susceptibility results from TORC1/2 inhibition leading to suppression of MCL-1 expression in mutant, but not WT, colorectal cancers, leading to abrogation of BIM/MCL-1 complexes. This combination strategy leads to tumor regressions in both KRAS-mutant colorectal cancer xenograft and genetically engineered mouse models of colorectal cancer, but not in the corresponding KRAS-WT colorectal cancer models. These data suggest that the combination of BCL-2/BCL-XL inhibitors with TORC1/2 inhibitors constitutes a promising targeted therapy strategy to treat these recalcitrant cancers.

SIGNIFICANCE: Effective targeted therapies directed against colorectal cancer with activating mutations in KRAS remain elusive. We have leveraged drug-screen data from a large panel of human colorectal cancers to uncover an effective, rational targeted therapy strategy that has preferential activity in colorectal cancers with KRAS or BRAF mutations. This combination may be developed for clinical testing. Cancer Discov; 4(1); 42–52. © 2013 AACR.

See related commentary by Russo et al., p. 19.
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INTRODUCTION

KRAS mutations are observed in approximately 30% to 45% of colorectal cancers (1–3). These mutations lead to potent activation of the MEK–ERK (MAP–ERK kinase–extracellular signal–regulated kinase) signaling pathway (4). Although therapies targeting EGFR receptor (EGFR) have some efficacy in colorectal cancers without KRAS mutations (1, 5–8), these therapies most likely fail because the MEK–ERK pathway is sustained by mutant KRAS in the presence of EGFR inhibitory antibodies. Direct inhibitors of mutant KRAS protein are not yet available; therefore, efforts are often focused on targets in signaling pathways whose inhibition alone or in combination may be effective for this subset of cancers (9–16). Indeed, multiple approaches, including the combination of phosphoinositide 3-kinase (PI3K) and MEK pathway inhibitors, are being examined in clinical trials. Mutant BRAF, which is directly downstream of KRAS, also leads to hyperactivation of the MEK–ERK pathway. BRAF mutations occur in roughly 5% to 15% of colorectal cancers (1–3, 17), and are generally mutually exclusive with KRAS mutations (1). In fact, a recent report highlighted gene expression similarities in these two genetically distinct mutant colorectal cancers, underscoring the overlap in signaling downstream from these mutant oncogenes (18). Single-agent BRAF inhibitors have been largely ineffective in BRAF-mutant colorectal cancers (19), despite activity in BRAF-mutant melanomas (20). However, some laboratory models of BRAF-mutant colorectal cancers are sensitive to the combination of BRAF and receptor tyrosine kinase inhibitors, particularly EGFR inhibitors, and this approach is currently under evaluation in the clinic (21, 22). Although some of these novel therapeutic strategies for KRAS- and BRAF-mutant colorectal cancers being explored in clinical trials will hopefully demonstrate some activity, it is very likely that clinical resistance will emerge, necessitating additional treatment strategies. Thus, there continues to be an urgent need to develop additional targeted therapies for KRAS-mutant as well as BRAF-mutant colorectal cancers.

We sought to uncover targeted therapy strategies that demonstrate specificity toward KRAS- or BRAF-mutant colorectal cancers compared with their wild-type (WT) counterparts. We leveraged the results from a high-throughput screen that assessed the sensitivity of over 600 cell lines to 130 drugs (23). Because the induction of both apoptosis and growth arrest is a hallmark of many successful targeted therapy approaches (24–26), we built upon the screen results and further mechanistic insights to establish a combination strategy producing these biologic effects.

RESULTS

Data obtained from our recently described high-throughput drug screen (23, 27) allowed us to compare the efficacy of drugs between KRAS- and BRAF-mutant human colorectal cancers versus WT human colorectal cancers. Included among the large number of compounds in the drug screen was ABT-263, a BCL-2/BCL-XL inhibitor (BH3 mimetic) that has demonstrated preclinical efficacy in some tumors (28, 29) and is under clinical evaluation as a single agent or in combination with chemotherapy (30, 31). In this study, we found that ABT-263 had similar activity in KRAS- and BRAF-mutant colorectal cancers compared with WT colorectal cancers (Fig. 1A). In contrast to ABT-263, a different BH3 mimetic, obatoclax, neutralizes another BCL-2 family member, MCL-1, in addition to BCL-2 and BCL-XL (32). Unlike ABT-263, obatoclax was more effective in both KRAS- and BRAF-mutant colorectal cancers than in WT colorectal cancers (Fig. 1B). The selectivity of obatoclax for mutant colorectal cancers was notable, as many common chemotherapies and experimental therapies did not discriminate between the mutant and WT colorectal cancers (Supplementary Fig. S1; P = NS for all comparisons). The differential sensitivity to obatoclax was not explained simply by expression levels of either MCL-1 or other BCL-2 family members (Supplementary Fig. S2A and S2B). Consistent with the increased sensitivity of mutant colorectal cancers to obatoclax, RNA interference (RNAi) knockdown of MCL1 sensitized mutant colorectal cancers, but not WT colorectal cancers, to ABT-263 (Fig. 1C and Supplementary Fig. S2C). In total, these findings suggest that, in comparison with their WT counterparts, mutant cells have a heightened sensitivity to combined inhibition of MCL-1, BCL-XL, and BCL-2.

These data indicated that targeting BCL-2, BCL-XL, and MCL-1 may be an effective therapeutic strategy in mutant colorectal cancers. However, obatoclax has significant toxicity, possibly off-target, limiting its clinical utility (33–35). Furthermore, many effective targeted therapies lead to both apoptosis and growth arrest, and inhibition of BCL-2 family members might be expected to induce apoptosis (29), but fail to strongly impair proliferation. Thus, we sought an alternative approach to both induce apoptosis and concomitantly block proliferation. Previous studies had demonstrated that efficient translation of MCL1 mRNA requires cap-dependent translation in some cancers (36–38). mTOR complex 1 (TORC1) inhibition, via loss of 4EBP1 (also referred to as EIF4EBP1) phosphorylation, results in a decrease of MCL-1 protein expression in these cancers (37–40). Consistent with those results, we found that treatment of KRAS- and BRAF-mutant colorectal cancers with the TORC1/2 inhibitor AZD8055 led to a decrease in MCL-1 expression (Fig. 1D and Supplementary Figs. S2D and S3). Interestingly, AZD8055 did not substantially suppress MCL-1 expression in KRAS- and BRAF-WT cells (Fig. 1E). Indeed, the regulation of MCL-1 expression by TORC1/2 seemed to be different between WT and mutant colorectal cancers, as AZD8055 effectively suppressed TORC1 and TORC2 in both genetic backgrounds as measured by its effect on phospho-4EBP, phospho-4EBP1, and phospho-AKT at residue 473 (Fig. 1D and E). Consistent with this finding, treatment with AZD8055 led to a G1 arrest in both WT and mutant colorectal cancer, and thus mutant and WT colorectal cancers had similar sensitivities to single-agent AZD8055 (Supplementary Fig. S4A and S4B). The suppression of MCL-1 protein expression by AZD8055 in mutant cells was not associated with a decrease in MCL1 mRNA levels (Supplementary Fig. S5A), consistent with an effect on translation of the MCL1 mRNA in these cancers. Furthermore, MCL1 mRNA levels only slightly increased in both mutant and WT cells following AZD8055 treatment, suggesting there is no differential feedback of MCL1 transcription contributing to differences in MCL-1 protein expression between the cancer types (Supplementary Fig. S5A). A time-course
**Figure 1.** KRAS- and BRAF-mutant (MT) colorectal cancers have increased sensitivity to obatoclax compared with their WT counterparts and also have MCL-1 expression under the regulation of TORC1/2. KRAS-mutant, BRAF-mutant, and WT colorectal cells were treated with increasing concentrations of (A) the BCL-2/XL inhibitor ABT-263 or (B) the BCL-2/XL/MCL-1 inhibitor obatoclax for 72 hours; cell number was determined, and IC$_{50}$ values were calculated (and converted to natural log, y-axis). Student t tests were performed between the two groups (KRAS- or BRAF-mutant vs. WT) and P values were determined (NS = not significant; n = 27). C, KRAS-mutant SW620 cells were transfected with 50 nmol/L scrambled (sc) or 50 nmol/L MCL1 siRNA for 24 hours. An aliquot of transfected cells was used to prepare lysates for Western blot analyses with the indicated antibodies (bottom) or reseeded and the next day treated for 48 hours with vehicle (no Rx) or 1 μmol/L ABT-263; percentage of apoptotic cells was quantified by fluorescence-activated cell sorting (FACS) analysis (n = 3). D, KRAS-mutant or (E) KRAS/BRAF-WT colorectal cell lines were treated for 16 hours with no drug (no Rx), 500 nmol/L AZD8055, 1 μmol/L ABT-263, or combination AZD8055/ABT-263, and proteins from lysates were subjected to Western blot analyses with the indicated antibodies. The numbers in the panels represent the amount of MCL-1 normalized to GAPDH. The detection of MCL-1 and GAPDH was performed on the same membrane. p, phosphorylated.
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Figure 2. Increased induction of apoptosis in KRAS- and BRAF-mutant (MT) colorectal cancers treated with combined ABT-263/AZD8055 compared with KRAS/BRAF-WT colorectal cancers. A, the indicated cell lines were treated with no drug, 500 nmol/L AZD8055, 1 μmol/L ABT-263, or combination AZD8055/ABT-263 for 72 hours. Percentage of apoptotic cells was quantified by fluorescence-activated cell sorting (FACS) analysis. Each bar graph represents the amount of apoptosis induced by treatment relative to no drug treatment. Error bars are the SD (n = 3). B and C, isogenic HCT-116 cells that were either KRAS mutant or WT were treated with no drug (no Rx), 500 nmol/L AZD8055, 1 μmol/L ABT-263, or the combination of ABT-263/AZD8055 (263/8055) for (B) 72 hours and the percentage of apoptotic cells was quantified by FACS, or (C) overnight and proteins from lysates were subjected to Western blot analyses with the indicated antibodies. For B, each bar graph represents the amount of apoptosis induced by treatment relative to no drug treatment. Error bars are the SD (n = 3).

of AZD8055 treatment in mutant cells demonstrated clear suppression of MCL-1 protein expression within 2 hours of treatment (Supplementary Fig. S5B). In contrast, MCL-1 expression was not diminished in WT cells at any time point examined (Supplementary Fig. S5B).

Because TORC1/2 inhibition effectively suppressed MCL-1 expression in KRAS- or BRAF-mutant cells, we explored whether TORC1/2 inhibitors would combine with ABT-263 to induce apoptosis specifically in these genetic backgrounds (analogous to MCL1 siRNA sensitizing to ABT-263; Fig. 1C). It is notable that ABT-263 usually induces expression of MCL-1, and that the differential effects of AZD8055 on the expression of MCL-1 between mutant and WT cells were often more dramatic when AZD8055 was combined with ABT-263 (Fig. 1D and E and Supplementary Figs. S2D and S3). We assessed the induction of apoptosis across a panel of human mutant and WT colorectal cancer cell lines. In addition to human cancer cell lines, we also included ex vivo mouse cell lines derived from colorectal cancers induced in Kras-mutant (Kras/Apc/p53) and WT (Apc/p53) genetically engineered mouse models (GEMM) of colorectal cancer (42, 43). The combination of ABT-263 and AZD8055 induced substantial apoptosis in the mutant human and murine colorectal cancers (average apoptosis 59.4% ± 4.0%; Fig. 2A). In contrast, marked apoptosis was not observed in any of the WT colorectal cancer cell lines (average apoptosis 12.5% ± 2.6%; Fig. 2A). We also compared the effect of this combination on a pair of isogenic HCT-116 cell lines with and without KRAS mutations (44). We observed that the KRAS-mutant HCT-116 cells had approximately a 7-fold increase.

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Figure 3. TORC inhibition, but not MEK inhibition, downregulates MCL-1 and disrupts MCL-1:BIM complexes following ABT-263 therapy, leading to enhanced apoptosis. **A**, KRAS-mutant SW620 colorectal cells expressing doxycycline-inducible KRAS shRNA vectors were treated with or without 50 ng/mL doxycycline (DOX) for 72 hours, and lysates were subjected to Western blot analyses with the indicated antibodies. **B**, KRAS-mutant SW620 colorectal cells were treated for 16 hours with no drug (no Rx), 500 nmol/L AZD8055, or 1 μmol/L AZD6244, and lysates were subjected to Western blot analyses with the indicated antibodies. **C**, stable clones of SW620 cells expressing GFP alone or a GFP–IRES–MCL-1 were sorted by fluorescence-activated cell sorting (FACS) analysis by fluorescence intensity, and the populations of cells were separated. Western blot analyses of the resultant cell lines are shown in the bottom. Cells were treated for 72 hours with the ABT-263/AZD8055 combination therapy and apoptosis was determined by FACS. Each bar graph represents the amount of apoptosis induced by treatment relative to no drug treatment. Error bars are the SD (n = 3). **D**, SW620 parental cells underwent immunoprecipitation (IP) with antibodies targeting BIM (left), MCL-1 (middle), or immunoglobulin G (IgG; left and middle) following 6 hours of no drug (no Rx), 500 nmol/L AZD8055, 1 μmol/L ABT-263, combination therapy (263/8055), 1 μmol/L AZD6244, or combination of AZD6244 and ABT-263 (263/6244). Precipitates were analyzed by Western blot analyses with the indicated antibodies. Arrow indicates BCL-XL that migrates more slowly than the light chain. Whole-cell lysates were set aside before immunoprecipitation and were subjected to Western blot analyses with the indicated antibodies (right). **E**, the schematic displays how ABT-263 and AZD8055 combination therapy affect the balance of BCL-2 family proteins to induce apoptosis in KRAS-mutant cells. Mitochondria are depicted in green.

in apoptosis following combination therapy compared with the KRAS WT HCT-116 cells (Fig. 2B). Of note, MCL-1 levels decreased significantly more following AZD8055 (and the AZD8055/ABT-263 combination) treatment in mutant cells compared with the WT cells (Fig. 2C; 10-fold vs. two-fold, respectively). In total, these findings support the notion that mutant colorectal cancers may be particularly more sensitive to this combination than their WT counterparts.

These findings prompted us to further examine the regulation of MCL-1 in KRAS-mutant cancers and the mechanism by which its inhibition synergized with ABT-263 to promote apoptosis. Knockdown of KRAS using inducible short hairpin RNA (shRNA) was sufficient to suppress 4EBP1 phosphorylation, and, accordingly, MCL-1 in KRAS-mutant SW620 cells (Fig. 3A). However, MEK inhibition alone did not suppress phosphorylation of 4EBP1, and, accordingly, it did not decrease expression.
of MCL-1 (Fig. 3B). This finding is consistent with a report suggesting that other KRAS-regulated pathways, in addition to MEK–ERK, contribute to phosphorylation of 4EBP1 (45). In contrast, KRAS knockdown did not decrease MCL-1 levels in KRAS-WT CW-2 cells (Supplementary Fig. S3C).

We further interrogated the molecular mechanisms by which the combination of TORC1/2 and BCL-2/XL inhibitors led to apoptosis in the mutant cancers. To determine whether the induction of apoptosis by the AZD8055/ABT-263 combination was indeed dependent on the suppression of MCL-1 expression, we expressed exogenous MCL-1 via lentiviral gene delivery. As expected, the exogenous expression of MCL-1 suppressed the induction of apoptosis (Fig. 3C and Supplementary Fig. S6A). To understand how the drug combination affected the protein complexes of the BCL-2 family, we performed immunoprecipitations in the absence and presence of inhibitors. Previous studies have shown that BIM plays a critical role in BH3 mimetic (including ABT-263)-induced apoptosis (46, 47). Analysis of immunoprecipitated BIM and MCL-1 complexes revealed that ABT-263 treatment abrogated the interaction between BIM and BCL-XL. However, ABT-263 treatment led to a proportional increase in the association between MCL-1 and BIM (Fig. 3D). These findings suggest that the BIM released from BCL-2 and BCL-XL in response to ABT-263 was bound to MCL-1, preventing apoptosis. Adding AZD8055 to ABT-263 resulted in loss of BIM/MCL-1 complexes (Fig. 3D) corresponding to decreases in MCL-1 expression in the whole-cell extracts (Fig. 3D). AZD8055 treatment also led to reduced complexes between MCL-1 and BAK due to loss of MCL-1 (Fig. 3D, middle), likely contributing to the amount of apoptosis induced by the combination. In KRAS-WT cells, analysis of immunoprecipitated BIM complexes revealed that, similar to the KRAS-mutant cells, ABT-263 treatment blocked the interaction between BIM and BCL-XL, and that treatment led to a proportional increase in the association between MCL-1 and BIM. However, in contrast to the mutant cells, AZD8055 treatment did not reduce BIM/MCL-1 complexes (Supplementary Fig. S6B), consistent with the lack of reduction in MCL-1 following AZD8055 treatment in the total cell lysates (Supplementary Fig. S6B) and the lack of efficacy of the combination (Fig. 2A). A model depicting the impact of the combination on the BCL-2 family is shown in Fig. 3E. Of note, this mechanism of apoptosis is distinct from the recently described combination of ABT-263 and MEK inhibitor (16), AZD6244. Unlike AZD8055, the MEK inhibitor does not decrease levels of MCL-1, but rather increases levels of BIM (Fig. 3D).

Next, we asked whether our findings in mutant colorectal cancers extended to another type of KRAS-mutant cancer, KRAS-mutant non–small cell lung cancers (NSCLC). In contrast to KRAS-mutant colorectal cancer, AZD8055 failed to markedly downregulate MCL-1 in KRAS-mutant NSCLC cell lines (Supplementary Fig. S7A), and, accordingly, it failed to substantially increase the amount of apoptosis induced by ABT-263 in the NSCLC cell lines (Supplementary Fig. S7B). However, NSCLC cells were highly sensitive to ABT-263 treatment following MCL1 knockdown (Supplementary Fig. S7C), suggesting that KRAS-mutant cancers in general may be sensitive to simultaneous BCL-2, BCL-XL, and MCL-1 inhibition, but that TORC1 regulation of MCL-1 may be different between KRAS-mutant colorectal cancer and KRAS-mutant NSCLC. In support of this hypothesis, AZD8055 treatment led to a modest increase in the level of MCL1 transcript in KRAS-mutant NSCLCs (Supplementary Fig. S7D), similar to KRAS-mutant colorectal cancers and KRAS WT colorectal cancers (Supplementary Fig. S5A). Furthermore, KRAS knockdown in KRAS-mutant H2009 and H358 cells failed to significantly downregulate MCL-1 expression (Supplementary Fig. S7E) in contrast to KRAS knockdown in KRAS-mutant SW620 cells (Fig. 3A). Of note, we did not detect any differences between the stability of MCL-1 protein between KRAS-mutant colorectal cancer and KRAS-mutant NSCLC cell lines, as determined by Western blotting of MCL-1 following a short-term time-course of treatment with the protein translational inhibitor cycloheximide (Supplementary Fig. S8). Altogether, these data suggest that MCL-1 may be an important survival signal for KRAS-mutant cancers in general; however, the regulation of MCL-1 expression by TORC1/2 is not equivalent in KRAS-mutant NSCLCs.

Because the ABT-263/AZD8055 combination in KRAS-mutant colorectal cancers induces both apoptosis (Fig. 2A) and growth arrest (Supplementary Fig. S4A), we evaluated this therapeutic strategy in vivo against established KRAS-mutant colorectal cancers. In an SW620 KRAS-mutant colorectal cancer xenograft tumor model, combination treatment resulted in marked tumor growth impairment, and even some regressions (Fig. 4A, left). AZD8055 downregulated MCL-1 and there was a marked induction of cell death following combination therapy in vivo (Fig. 4B and Supplementary Fig. S9A). notably, SW620 tumors have been resistant to several other combination approaches in clinical development, highlighting the potential of the current approach (Supplementary Fig. S9B). As expected, the combination of AZD8055/ABT-263 failed to significantly prevent tumor growth or suppress MCL-1 expression in a KRAS-WT xenograft tumor model (Fig. 4A, right and B). Thus, this combination demonstrated more impressive activity against the KRAS-mutant colorectal cancer in vivo, consistent with the in vitro data.

We next tested this combination in GEMMs of Kras-mutant and Kras-WT colorectal cancer. These GEMMs are valuable preclinical models. In contrast to xenograft cancer models, the GEMMs develop in the physiologically relevant microenvironment of the colon and do not undergo growth selection in vitro before in vivo testing (42). Furthermore, the ability to monitor tumor growth and/or regressions using optical colonoscopy allows us to follow the dynamic tumor responses to treatment in real time, making this model particularly valuable and effective for evaluating experimental therapeutics. We examined the efficacy of the combination regimen on mice with colonic tumors that were Apc-mutant (null; ref. 48) with or without Kras mutation (42). The growth or regressions of individual tumors were monitored weekly by serial colonoscopies (42). The Kras-mutant colorectal cancer model treated with combination therapy exhibited shrinkage of all treated tumors (Fig. 4C and D). In contrast, the combination failed to induce tumor regressions in Kras-WT GEMMs (Fig. 4C and D), despite effective inhibition of TORC1/2 signaling in vivo (Fig. 4E). Therefore, GEMMs of colorectal cancer reflect our findings in human colorectal cancer cell lines assessed in vitro and as...
Figure 4. The combination of AZD8055/ABT-263 has in vivo efficacy in KRAS-mutant colorectal cancer. A, cohorts of KRAS-mutant SW620 xenograft tumors (left) were treated with no drug (control), AZD8055 (16 mg/kg/qd), ABT-263 (80 mg/kg/qd), or the combination (16 mg/kg/qd AZD8055, 80 mg/kg/qd ABT-263); qd, every day. Average tumor sizes for each treatment group are plotted. Cohorts of KRAS-WT KM12 xenograft tumors (right) were treated with no drug (control) or AZD8055/ABT-263 combination, and average tumor sizes are plotted. B, approximately 3 hours following the final drug treatment, mice were euthanized and tumors were harvested. Protein lysates were prepared from SW620 (left) and KM12 (right) tumors and were subjected to Western blot analyses with the indicated antibodies. C, representative images of tumors taken from serial colonoscopies of KRAS-mutant and KRAS-WT GEMMs as treated in A. Arrows depict each tumor. Tumor size was calculated by determining the percentage occlusion of the lumen as described in Methods. D, colorectal cancers in KRAS-mutant and KRAS-WT GEMMs were treated as in A for 28 days, and changes in tumor volume for each mouse are shown by a waterfall plot. E, lysates from KRAS-WT GEMM tumors were harvested approximately 3 hours following the last drug treatment of the experiment, and protein lysates were subjected to Western blotting analyses with the indicated antibodies. Each number represents a different tumor.
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**DISCUSSION**

Advanced colorectal cancers with KRAS or BRAF mutations require new therapeutic options. Here, we show that combining AZD8055 with ABT-263 induces robust apoptosis in KRAS- and BRAF-mutant human colorectal cancer cell lines. Furthermore, this combination produced tumor regressions in KRAS-mutant colorectal cancers in vivo both in human xenografts and in GEMMs, increasing the likelihood that this combination may have efficacy in clinical trials of KRAS-mutant colorectal cancers. Although our initial observation was that KRAS- and BRAF-mutant colorectal cancers had enhanced sensitivity to single-agent obatoclax compared with their WT counterparts, we believe that this combination is likely to be preferable to obatoclax for two main reasons: (i) BH3 mimetics alone induce apoptosis but do not seem to induce growth arrest, and (ii) clinical trial data suggest that the dose-limiting toxicities of obatoclax may preclude sufficient target inhibition, in contrast to ABT-263 (30, 34, 35). Furthermore, we are optimistic that this combination may have an attractive therapeutic index. Doses of AZD8055 as low as approximately 17 nmol/L effectively reduced MCL-1 levels and sensitized the cells to ABT-263 (Supplementary Fig. S10A and S10B). Even when we decreased the concentrations of AZD8055 in vivo from 16 to 2 mg/kg, significant tumor inhibition in the SW620 model was still achieved (Supplementary Fig. S10C).

Recently, there have been a number of studies that have sought effective approaches to treat KRAS-mutant cancers. These therapies include MEK inhibition in combination with BCL-2/XL inhibition (16), STK33 inhibition (13), TAK1 inhibition (14), MEK inhibition in combination with PI3K inhibition (11), GATA2 inhibition (15, 49), CDC6 inhibition (49), APC/C inhibition (50), and PLK1 inhibition (50). At this point, it is difficult to predict which approaches will ultimately emerge as effective therapies in the clinic, and potential therapeutic indices for each of these therapies have yet to be established. This will likely have a large impact on determining which approaches have the greatest clinical value. In addition, there may be different subsets of KRAS-mutant cancers that are susceptible to a particular combination. As an example, the combination of a MEK inhibitor with a BCL-2/XL inhibitor demonstrated efficacy across a large swath of KRAS-mutant cancers, including KRAS-mutant lung cancers, but tended to be less effective in KRAS-mutant cancers with mesenchymal features (16). In contrast, the combination of AZD8055 with a BCL-2/XL inhibitor seemed less active against KRAS-mutant lung cancers, but demonstrated potency against all KRAS-mutant colorectal cancers examined.

The current study offers some unique components that may facilitate drug development. Most published studies used RNAi approaches to identify potential therapeutic strategies, and thus further drug development efforts will be necessary to achieve clinical translation. The current study differs in that we used data from a high-throughput drug screen involving clinically relevant inhibitors to identify a treatment strategy specific for KRAS-mutant colorectal cancer. Although our current approach restricts the number of targets being explored (compared with comprehensive RNAi screens), it is rather straightforward to examine the efficacy of this drug combination in the clinic relatively quickly.

The combination developed in this study for KRAS-mutant colorectal cancers is less active in KRAS-WT colorectal cancers. The activity of this combination centers on the sensitization of these cancers to ABT-263 by suppression of MCL-1 expression upon treatment with TORC1/2 inhibitors. In contrast to the KRAS-mutant colorectal cancers, the WT colorectal cancers were not sensitized to ABT-263 by downregulation of MCL-1, nor did they have MCL-1 expression under the regulation of TORC. Although further studies will be required to determine the activity of this combination in other subsets of cancer, we speculate that this combination may be effective for cancers that have MCL-1 expression under strict regulation of the TORC1 pathway and are sensitive to simultaneous BCL-2/BCL-XL/MCL-1 disruption.

Overall, we believe that the combination of TORC inhibitors with BCL-2/XL inhibitors is mechanistically designed to induce apoptosis and growth arrest in the subset of colorectal cancers with KRAS or BRAF mutations and warrants further investigation as a potential clinical treatment of these recalcitrant cancers.

**METHODS**

**Cell Lines**

All the cell lines used in this study were provided by the Center for Molecular Therapeutics (CMT) at Massachusetts General Hospital (Boston, MA), with the exception of the GEMM cell lines (42) and the HCT-116 isogenic cell lines (44). The cell lines from CMT routinely undergo cell-line authentication testing by single-nucleotide polymorphism and short-tandem repeat analysis. These cell lines have been acquired over the past 5 years. Limited genotyping of human colorectal cancer cell lines used in the drug screen is shown in Supplementary Table S1. The human colorectal cancer cell lines used in the drug screen is shown in Supplementary Table S1. The human colorectal cancer cell lines used in Western blotting, apoptosis assays, and cell-cycle assays were grown in Dulbecco’s Modified Eagle Medium with 10% FBS in the presence of 1 μg/mL penicillin and streptomycin, except for the BRAF-mutant COLO-205 and LS-411N cell lines, which were grown in RPMI with 10% FBS. The KRAS-mutant lung cancer cell lines (H2009, H460, H2030, and H358) were grown in RPMI with 10% FBS.

**Drug Screen**

The drug screen has previously been described (23), as has the website for publicly accessible drug-sensitivity data (ref. 27; www.cancerRxgene.org). Data analysis was performed using datasets available in early 2013, and these data are recorded in Supplementary Table S1.

**Antibodies and Reagents**

The following antibodies were used for Western blot analyses: phospho-AKT (473; cat #9271), phospho-ERK (cat #4370), phospho-4EBP1 (cat #2855), phospho-ERK (cat #9102), total RPS6 (cat #2401), total AKT (cat #2056), and BCL-XL (cat #2762) from Cell Signaling Technology. MCL-1 (S-19) antibody was from Santa Cruz Biotechnology. Antibody to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was purchased from Chemicon. Annexin-Cyt5 was from Biosource International. Propidium iodide was from Sigma-Aldrich. Opti-MEM was from Invitrogen. Protein A sepharose beads were from Amersham (GE Healthcare).
Western Blotting

For Western blotting of cellular lysates, cells were resuspended in lysis buffer (20 mM/L Tris, 150 mM/L NaCl, 1% Nonidet P-40, 0.1 mM EDTA, and protease and phosphatase inhibitors), incubated on ice for 10 minutes, and centrifuged at 14,000 rpm for 10 minutes. For whole-tumor lysates, these were prepared by homogenization with a TH tissue homogenizer (Omni International) in tissue lysis buffer (25 mM/L Tris–HCl (pH 7.6), 150 mM/L NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 1 mM/L dithiothreitol (DTT), and protease and phosphatase inhibitors), incubated for 10 minutes on ice, vigorously shaken for 30 minutes at 4°C at 1,400 rpm in a thermomixer, and centrifuged at 14,000 rpm for 10 minutes at 4°C. Protein concentration was determined by BCA Protein Assay (Pierce). Proteins were resolved using the NuPAGE Novex Midi Gel system on 4% to 12% Bis–Tris gels (Invitrogen), transferred to nylon membranes, and probed with the antibodies listed above. Representative blots are shown from several experiments. Chemiluminescence was detected with the Syngene G:Box camera (Synoptics). All measurements were performed in the linear range without saturation and were normalized to GAPDH or actin loading control.

Apoptosis

Cells were seeded at roughly 30% to 40% confluency and were treated with the indicated drug treatments, with no-treatment controls in parallel. Treatments were given in triplicate where indicated. Apoptosis experiments were performed as previously described (24) and analyzed on a BD LSR III (Becton Dickinson). The number of cells in quadrants II and IV (Annexin positive) were counted as apoptotic.

Cell Cycle

Cells were seeded at roughly 30% to 40% confluency and were treated overnight with the indicated treatments in triplicate, with no-treatment controls in parallel, and the cells were prepared for cell cycle analysis as previously described (24) and analyzed on a BD LSR III (Becton Dickinson).

siRNA Experiments

For the siRNA experiments, MCL1 and scrambled control oligos (Dharmacon smart pool) were used at a concentration of 50 nM/L and transfected with HiPerFect reagent (Qiagen). Briefly, cells were plated to achieve next-day confluency of roughly 60%. HiPerFect (75 μL) was added to 750 μL of Opti-MEM in a 1.5 mL Eppendorf tube. In parallel, 10 μM/L of siRNA was added to 750 μL of Opti-MEM in a separate 1.5 mL Eppendorf tube. After 10 minutes, the tubes were combined and mixed gently. Following 10 more minutes, the HiPerFect–siRNA mix was then drop-wise added to cells and gently shaken for 5 minutes. Twenty-four hours later, the cells were either resedeed in 6-well plates and treated with the appropriate drug the following day or lysed for Western blot analysis to determine the efficiency of the knockdown.

shRNA Experiments

For the shRNA experiments, cells were transduced with tetracycline-inducible shKRAS vectors as previously described (9). Following selection in 2 μg/mL puromycin, cells were grown in Tet-approved PBS and knockdown was performed in the presence of 50 ng/mL of doxycycline for 72 hours.

Protein Complex Immunoprecipitation

Following indicated drug treatments, cells were lysed in the same buffer as used for Western blotting. Of note, 20 μL of protein A sepharose beads were added to 250 to 500 μg of protein, and 5 μg of either MCL-1 antibody (S-19; Santa Cruz Biotechnology) or BIM antibody (cat# 2819; Cell Signaling Technology) was added to the lysates. Following overnight incubation with motion, the supernatant was collected and the cell pellets were washed three times with PBS.

Xenograft Studies

KrasG12D GEMM Homozygous mutant ApC and heterozygous mutant KrasG12D mice were generated as previously described (42).

Adenoviral Infection of Colon Epithelium

Tumors were induced in the colons of ApC-/-; KrasG12D/+ mice by adenosine-expressing cre recombinase and followed by optical colonoscopy as previously described (42).

In vivo Treatments

For the human xenografts, KM-12 (n = 5 for treatment group) or SW620 cells (n = 3 to 5 for each cohort) were injected (5 × 10⁶ cells/mouse) into the rear right flanks of athymic nude mice (Charles River Laboratories). When tumors reached approximately 100 to 200 mm³, treatments began. All drugs were administered directly to the stomach daily by oral gavage, unless otherwise indicated in the figure legend describing the data. For xenograft experiments, tumors were measured by electronic caliper two to three times a week, in two dimensions (length and width), and with the formula \( v = l \times w^2 \times \pi /6 \), where \( v \) = tumor volume, \( l \) = length, and \( w \) = width. For GEMMs, tumor size was determined by the Tumor Size Index as calculated by (tumor area/colon lumen area) × 100 (%; ref. 42).

All animal experiments were performed in accordance with Massachusetts General Hospital Animal Care and Use Committee or Tufts Institutional Animal Care and Use Committee.

Histopathology and Immunohistochemistry

Tissue was fixed in 10% paraformaldehyde overnight, embedded in paraffin, and sectioned at 4 μm. Slides were dewaxed by microwave heating in antigen retrieval solution (SAB; Vector Laboratories), treated for 10 minutes with hydrogen peroxide, incubated for 30 minutes in 1% sodium deoxycholate, 0.1% SDS, 1 mM/L dithiothreitol (DTT), and protease and phosphatase inhibitors, incubated for 10 minutes on ice, vigorously shaken for 30 minutes at 4°C at 1,400 rpm in a thermomixer, and centrifuged at 14,000 rpm for 10 minutes at 4°C. Protein concentration was determined by BCA Protein Assay (Pierce). Proteins were resolved using the NuPAGE Novex Midi Gel system on 4% to 12% Bis–Tris gels (Invitrogen), transferred to nylon membranes, and probed with the antibodies listed above. Representative blots are shown from several experiments. Chemiluminescence was detected with the Syngene G:Box camera (Synoptics). All measurements were performed in the linear range without saturation and were normalized to GAPDH or actin loading control.

Statistic Considerations

IC₅₀ values for cell lines were calculated as previously described in the drug screens (23). The statistical tests used in this study were Student t test (two-sided) and Wilcoxon rank-sum tests performed using GraphPad Prism Software or R version 2.14.1. Differences were considered statistically different if \( P < 0.05 \). The average amount of apoptosis between all the mutant groups and all the WT groups, as described in the text, is ±SEM.

Disclosure of Potential Conflicts of Interest

R.K. Jain has received commercial research grants from Dyax, MedImmune, and Roche, has ownership interest (including patents) in Xtuit Pharmaceuticals and Enlight, is a consultant/advisory board member of Nextcell, Zynrenta, Enlight (SAB), and SynDevRx (SAB), is on the Board of Directors of XTuit Pharmaceuticals, and is on the Board of Trustees of H&Q Healthcare Investors and H&Q Life Sciences Investors. J.A. Engelman has received commercial research grants from AstraZeneca and Novartis and is a consultant/advisory board member of Novartis, AstraZeneca, and Sanofi-Aventis. No potential conflicts of interest were disclosed by the other authors.

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Conflict-of-interest statement: The authors declare that they have no conflicts of interest.

Published OnlineFirst October 25, 2013; DOI: 10.1158/2159-8290.CD-13-0315

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**Grant Support**

This work was supported by DF/HCC Gastrointestinal Cancer SPORE P50 CA127003 (to J.A. Engelman), grants from the NIH: R01CA140594 (to J.A. Engelman), R01CA137008 (to J.A. Engelman), 1U54HG006097-01 (to C.H. Benes), a grant from the Wellcome Trust (086357; to C.H. Benes), awards from the Burroughs Wellcome Fund and the Howard Hughes Medical Institute (to M.N. Rivera), and an American Cancer Society Postdoctoral Fellowship (to A.C. Faber).

Received June 26, 2013; revised October 17, 2013; accepted October 24, 2013; published OnlineFirst October 25, 2013.

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**American Association for Cancer Research**

**JANUARY 2014**

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Cancer Discovery 2014;4:42-52. Published OnlineFirst October 25, 2013.

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