Reversal of Mutant KRAS-Mediated Apoptosis Resistance by Concurrent Noxa/Bik Induction and Bcl-2/Bcl-xL Antagonism in Colon Cancer Cells

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Abstract

KRAS mutations are frequently detected in human colorectal cancer and contribute to de novo apoptosis resistance and ultimately therapeutic failure. To overcome KRAS-mediated apoptosis resistance, the irreversible proteasome inhibitor, carfilzomib, was evaluated and found to potently induce Noxa, which was dependent upon c-Myc, and Bik. Isogenic mutant versus wild-type KRAS carcinoma cells showed elevated Bcl-xL, confirmed by KRAS siRNA or ectopic expression. Upregulated Bcl-xL by mutant KRAS was mediated by ERK3 as indicated by ERK knockdown. Bcl-xL expression was regulated at the level of mRNA and protein as shown using actinomycin D and cycloheximide, respectively. Suppression of Bcl-xL by shRNA sensitized mutant KRAS cells to carfilzomib. Concurrent Bcl-xL antagonism by the BH3 mimic ABT-263 combined with carfilzomib synergistically enhanced apoptosis that was dependent on Bax or p53, and was attenuated by Noxa or Bik shRNA. In support of this strategy, ectopically expressed Noxa enhanced apoptosis by ABT-263. Carfilzomib-induced Noxa and Bik sequestered Mcl-1 and ABT-263 released Bik and Bak from Bcl-xL, suggesting a mechanism for drug synergy. These preclinical findings establish mutant KRAS-mediated Bcl-xL upregulation as a key mechanism of apoptosis resistance in KRAS-mutant colorectal cancer. Furthermore, antagonizing Bcl-xL enabled carfilzomib-induced Noxa and Bik to induce synergistic apoptosis that reversed KRAS-mediated resistance.

Implications: This novel study reveals a promising treatment strategy to overcome apoptosis resistance in KRAS-mutant colorectal cancer by concurrent upregulation of Noxa/Bik and antagonism of Bcl-xL. Mol Cancer Res; 13(4); 659–69. ©2014 AACR.

Introduction

Colorectal cancer is second only to lung cancer as a cause of cancer-related mortality in the United States (1). Although advances in colorectal cancer treatment have occurred, therapeutic options remain limited for approximately 50% of patients whose tumors carry activating mutations in the KRAS oncogene (exons 2, 3, 4; ref. 2). Mutant KRAS is associated with treatment resistance due, in part, to defective apoptotic signaling (3). KRAS mutations are known to interfere with apoptosis resistance to antibodies against the EGFR (4). To date, attempts to develop drugs that target mutant Ras proteins have been unsuccessful. Recent studies using large-scale RNA interference screens have identified cells expressing oncogenic KRAS to be vulnerable to proteasome inhibition (5). The ubiquitin–proteasome system is an important regulator of tumor cell growth, and proteasome inhibitors are attractive candidates for combination with other targeted agents. Increased proteasomal activity characterizes human cancer cells and is necessary to degrade ubiquitinated proteins via the 26S proteasome (consists of a 20S core particle and two regulatory 19S regulatory caps; ref. 6). Protein targets include those involved in apoptosis and cell-cycle regulation as well as in tumor progression (7). The proteasome inhibitor bortezomib was relatively ineffective against solid tumors in clinical trials (8), and limiting factors include the reversibility of proteasome activity that requires frequent and extended treatment for its effective suppression. In addition, defective apoptotic signaling may also limit efficacy. An irreversible proteasome inhibitor, carfilzomib, shows activity against bortezomib-resistant cells and is approved by the U.S. Food and Drug Administration for the treatment of patients with relapsed/refractory multiple myeloma and mantle cell lymphoma (9, 10).

Proteasome inhibitors have been shown to induce proapoptotic BH3-only proteins (11), but have also been shown to interfere with the degradation of antiapoptotic Mcl-1 (12). In a prior study, we found that bortezomib can upregulate proapoptotic Noxa expression to increase apoptotic susceptibility in colorectal cancer cell lines (13). However, human cancers are commonly resistant to apoptosis due to overexpression of antiapoptotic Bcl-2 family proteins or alternatively, due to downregulation of proapoptotic BH3-only proteins (14). Furthermore, the mechanism of defective apoptosis in KRAS-mutant cells remains poorly defined. Small-molecule inhibitors have been developed that bind to the BH3 hydrophobic binding groove of Bcl-2, Bcl-xL, or also Mcl-1. These BH3 mimetics mimic the function of endogenous BH3-only proteins and therefore, possess the ability to tip the balance in
favor of promoting tumor cell apoptosis. ABT-263 is an orally bioavailable inhibitor of Bcl-2/Bcl-xl that promotes apoptosis and has shown antitumor activity both in vitro and in vivo (15, 16). This drug is under active clinical development in patients with hematologic malignancies and small cell lung cancer (17). ABT-263 does not antagonize Mcl-1 (18, 19) in contrast to obatoclax that is not currently in active clinical development.

In this study, we sought to elucidate the mechanism of apoptosis resistance in KRAS-mutant cells and evaluated a novel strategy for its circumvention. Specifically, we induced proapoptotic BH3-only proteins by proteasome inhibition and concurrently antagonized antiapoptotic Bcl-2/Bcl-xl proteins using a BH3 mimetic agent, which we found to interact synergistically to reverse KRAS-mediated apoptosis resistance.

Materials and Methods

Cell culture and drugs

Human colorectal cancer cell lines (HCT116 and SW620) had been obtained from the ATCC. Isogenic cell lines containing KRAS wild-type (HCT116, #152; DLD1, #197) or mutant (HCT116, #154; DLD1, #196) alleles, and HCT116 cells with Bax−/− or P53−/− were obtained from Dr. B. Vogelstein (Johns Hopkins University, Baltimore, MD). All cells were grown as monolayers in RPMI (Invitrogen) supplemented with 10% (v/v) FBS and 1% antibiotic/antimycotic (Invitrogen). HEK293T cells were grown in DMEM (Sigma) and supplemented as above. Cells were treated with carfilzomib (LC Laboratories) alone or combined with ABT-263 (Sellekchem). Carfilzomib and ABT-263 were prepared as 1 or 10 mmol/L stock solutions in DMSO, respectively, and stored at −20°C. Inhibitors of transcription, for example, actinomycin D, or translation, for example, cyclohexamide, were purchased from Sigma.

Lentiviral expression

The production of virus and the transduction of target cells were performed using a standard procedure, as previously described (20). The lentiviral shRNA expression vectors for c-myc and a nontargeting control vector were obtained from Sigma and Addgene, respectively. Noxa, Bcl-xl, Mcl-1, or Bak shRNA were generated as described previously (13, 21, 22). The targeting shRNA sets against Noxa, Bik, or Bcl-xl were purchased from Openbiosystems. The lentiviral shRNA expression construct was packaged in pseudotyped viral particles and transduced into target cells in Opti-MEM (Invitrogen) containing 8 μg/mL of polybrene (Sigma). Following incubation of the cells overnight at 37°C, media were removed and replaced with the original growth media. Puromycin (2–4 μg/mL; Sigma) was added at 48 hours after transduction and the puromycin-resistant pool of cells was obtained and used for subsequent experiments.

Transfection of siRNA

Cells were seeded 1 day before transfection at 30% to 50% confluence in growth medium without antibiotics. ERK1/2 siRNA (Cell Signaling Technology) or small pool KRAS siRNA (Dharmacon) were mixed with lipofectamine RNAiMax (Invitrogen) in OPTI-MEM medium, mixed gently and incubated to form a complex. The mixture was then added dropwise to cells to achieve an siRNA final concentration of 50 mmol/L. Cells were then incubated at 37°C and knockdown efficiency was determined 48 hours after transfection.

Competitive reverse transcription PCR

Total RNA was extracted from cells using the RNeasy Mini Kit (Qiagen) and RNA integrity was confirmed using an Agilent Bioanalyzer 2000. Competitive reverse transcription PCR (RT-PCR) was performed using a one-step RT-PCR kit (Qiagen) with mixing of Bcl-xl (forward: 5′-GATCCCCATTGAGCGAGCTTTAAG-CAAG-3′; reverse: 5′-CCCCATCCCCGGAAGATGCATTCATCCTAC-3′) and β-actin (forward: 5′-TCACCCACACTGCGCCATCTCAGA-3′; reverse: 5′-CAGCGGAAGCTCTGACTTGATGG-3′) primers at molar ratio of 1:1. Reverse transcription was coupled with PCR (+25 cycles) on a thermocycler (Applied Biosystems). PCR products were quantified on the Agilent Bioanalyzer 2000 using the DNA 12,000 kit.

Retroviral expression of mutant KRAS

The retroviral expression vector pBabe-KRAS (G12V) [Addgene] was packaged into pseudotyped retrovirus, as previously described (23). Retrovirus was then transduced into isogenic HCT116 cells containing only wild-type KRAS. Nontransduced cells were eliminated by puromycin selection.

Ectopic doxycycline-inducible expression of Noxa

A lentiviral inducible expression vector pTRIPZ was double digested by AgeI/EcoRI and ligated with the coding region of Noxa that was digested with the same restriction enzymes. Cloning of pTRIPZ-Noxa was then performed using standard techniques. Pseudotyped lentivirus was packaged as described above, except that second-generation helper plasmids, pMD2.G and PsPAX2 (Addgene; #12259 and #12260), were used.

Apoptosis assay and analysis of drug synergy

Apoptosis was analyzed by annexin V staining and quantified by flow cytometry, as previously described (22). Briefly, cells were incubated with drugs at prespecified time points and adherent cells were detached using trypsin that allowed their combination with floating cells. Cells were pelleted by centrifugation and washed three times with cold PBS. Cells were then incubated with annexin V conjugated with FITC (BD Biosciences), and these cell populations were then labeled with fluorescent dyes to enable their quantitation by flow cytometry.

To evaluate for an interaction between carfilzomib and ABT-263, cells were treated with carfilzomib, ABT-263, or their combination at a fixed ratio and apoptosis was quantitated by annexin V staining as described above. The means of triplicate experiments were used to compute the combination index (CI) per the method of Chou and Talalay (24) using Compusyn software (ComboSyn, Inc.). CI values <1 were consistent with drug synergy.

Immunoblotting and immunoprecipitation

Protein samples were prepared in a lysis buffer [5 mmol/L MgCl2, 137 mmol/L KCL, 1 mmol/L EDTA, 10 mmol/L EGTA, 1% CHAPS, 10 mmol/L HEPES (pH 7.5)] supplemented with a protease inhibitor cocktail (Sigma). Protein samples were normalized using a NanoDrop measurement method (Thermo Scientific). Cell lysates were incubated with primary antibodies for 3 to 6 hours at 4°C and immunocomplexes were then captured with magnetic beads conjugated with protein A/G (Pierce). After washing three times in lysis buffer, the immunoprecipitated proteins were eluted with 2× LDS sample buffer (Invitrogen), and then loaded onto a 14% SDS-PAGE gel for protein separation that was followed by an electrical transfer onto a polyvinylidene difluoride
(PVDF) membrane (Bio-Rad). Immunoblotting was performed using standard procedures as previously described (25). Primary antibodies used included those against Noxa (Calbiochem), Puma (Abcam), Mcl-1 (BD Pharmingen), Bcl-xL (Calbiochem), caspase-8 (BD Pharmingen), and tubulin (Sigma). All other antibodies were obtained from Cell Signaling Technology.

Statistical analysis
The values shown in Annexin V and RT-PCR experiments represent the mean ± SD for triplicate experiments. Statistical significance was determined using the Student t test. A P value < 0.05 was considered statistically significant.

Results
Mutant KRAS upregulates antiapoptotic Bcl-xL expression
To date, the mechanism of defective apoptotic signaling in KRAS-mutant colorectal cancer cells remains poorly understood. We studied the mechanism of KRAS-mediated apoptosis resistance using isogenic HCT116 and DLD1 colorectal cancer cell lines containing KRAS wild-type or mutant alleles where the other homologous copy had been somatically deleted by gene knock-out (26). The KRAS mutational status of these cell lines was verified by gene sequencing before usage (data not shown). Mutated KRAS was associated with constitutive activation of MEK and ERK shown by phosphorylation in both isogenic cell lines (Fig. 1A). Analysis of proapoptotic BH3-only proteins in mutant versus wild-type KRAS cells revealed similar expression of Noxa, Bik, and Puma expression. However, mutant versus wild-type KRAS cells showed upregulation of antiapoptotic Bcl-xL proteins without change in Bcl-2 or Mcl-1 (Fig. 1A). Bcl-xL upregulation by mutated KRAS was confirmed using ectopic mutant KRAS (G12V) or KRAS siRNA that were shown to increase or decrease Bcl-xL expression, respectively (Fig. 1B). A role for ERK in mediating Bcl-xL upregulation by mutant KRAS was suggested using ERK siRNA that attenuated Bcl-xL protein expression (Fig. 1C).

To determine whether Bcl-xL is transcriptionally regulated by mutant KRAS, we analyzed Bcl-xL mRNA expression by competitive RT-PCR using β-actin as an internal control. Bcl-xL transcripts were shown to be upregulated approximately 1.4-fold in mutant versus wild-type HCT116 KRAS cells (Fig. 1D). Cells were then treated with the transcription inhibitor, actinomycin D, which was shown to suppress both Bcl-xL mRNA transcripts (Fig. 1E) and protein expression (Fig. 1F) in a time-dependent manner. Inhibition of protein translation using cyclohexamide was also shown to attenuate Bcl-xL expression (Fig. 1F), suggesting that mutated KRAS can regulate Bcl-xL by both transcriptional and posttranscriptional mechanisms.

Carfilzomib induces expression of proapoptotic Noxa and Bik proteins
Recent data suggested that KRAS-mutant tumor cells are vulnerable to proteasome inhibition (5). We determined whether carfilzomib can induce proapoptotic BH3-only proteins that may regulate apoptotic susceptibility in colorectal cancer cells. We found that carfilzomib can potently induce expression of Noxa and Bik proteins in a dose-dependent manner in both mutant and wild-type KRAS HCT116 cells (Fig. 2A). Potent induction of these BH3-only proteins was also observed in KRAS-mutant SW620 cells that was dosage and time-dependent (Fig. 2A–C). Carfilzomib also induced expression of c-myc and antiapoptotic Mcl-1 (Fig 2A–D), which have been shown to undergo degradation by the proteasome (27). Mcl-1 is a short-lived protein (half-life of ~1 hour; ref. 28) whose induction by carfilzomib is consistent with inhibition of proteasomal activity. Noxa induction by bortezomib has been shown to be c-myc-dependent (27). Accordingly, we suppressed c-myc using shRNA and found that c-myc shRNA can attenuate carfilzomib-induced Noxa (Fig. 2D). Together, these data suggest that Noxa induction by proteasome inhibitors is mediated by c-myc.

ABT-263 and carfilzomib interact synergistically to overcome Bcl-xL-mediated apoptosis resistance in KRAS-mutant cells
Given that carfilzomib potently induced Noxa and Bik expression in KRAS-mutant and wild-type tumor cells, we determined whether induction of these proteins was sufficient to overcome apoptosis resistance in KRAS-mutant cells. However, we found that KRAS-mutant versus wild-type isogenic HCT116 cells showed less carfilzomib-induced apoptosis shown by an annexin V labeling (Fig 3A) and reduced caspase-8, -9, and -3 cleavage (Fig. 3C). Isogenic DLD1 cells with mutant KRAS were similarly more resistant to carfilzomib as compared with their wild-type counterpart (data not shown). Given the finding of Bcl-xL upregulation in KRAS-mutant cells, we determined whether inhibition of Bcl-2/Bcl-xL by ABT-263 can reverse the observed resistance to carfilzomib-induced apoptosis in KRAS-mutant cells. Treatment with the combination of carfilzomib plus ABT-263 significantly enhanced apoptosis as compared with either drug alone in both KRAS-mutant and wild-type HCT116 cells (Fig. 3A and C). Similarly, the drug combination markedly enhanced apoptosis compared with carfilzomib or ABT-263 alone in KRAS-mutant SW620 cells (Fig. 3B and D). Enhanced caspase activation in cells treated with the drug combination versus single agents was associated with reduced Bcl-xL and Mcl-1 expression (Fig. 3C), both of which can be cleaved by activated caspase-3 (29, 30). These data indicate that ABT-263 can restore sensitivity to carfilzomib in mutant KRAS HCT116 and SW620 cell lines.

We evaluated the interaction between carfilzomib and ABT-263 using a median dose effect method with calculation of a CI using a fixed dose ratio (24). The effect of the drug combination on apoptosis was found to be synergistic, that is, CI values < 1.0, in both KRAS-mutant and wild-type HCT116 cells (Fig. 3A) and in KRAS-mutant SW620 cells. Of note, the CI value was significantly higher in KRAS-mutant compared with wild-type HCT116 cells (P = 0.04). These data indicate that carfilzomib alone is insufficient to overcome mutated KRAS-mediated apoptosis resistance and that concurrent Bcl-xL antagonism is needed to achieve substantial apoptosis in KRAS-mutant colorectal cancer cells.

Suppression of Noxa using two independent shRNAs was shown to significantly attenuate carfilzomib-induced cleavage of caspase-8, -9, and -3 (compared with control shRNA) in KRAS-mutant SW620 cells (Fig. 4A). To demonstrate that Noxa induction underlies the ability of carfilzomib to enhance ABT-263–induced apoptosis, we ectopically expressed Noxa using a doxycycline-inducible system. Doxycycline-induced Noxa was shown to significantly augment ABT-263–induced caspase-3 cleavage (Fig. 4B), indicating that Noxa induction is a key effector of carfilzomib-induced apoptosis. Knockdown of Bik by shRNA was also shown to reduce caspase cleavage by carfilzomib combined with ABT-263 (Fig. 4C). In contrast to Noxa or Bik knockdown, suppression of Bcl-xL by shRNA was shown to sensitize
Figure 1. Mutant KRAS upregulates antiapoptotic Bcl-xL expression. A, isogenic KRAS-mutant (mt) versus wild-type (wt) HCT116 or DLD1 cells show activation of downstream effectors MEK and ERK and overexpression of antiapoptotic Bcl-xL (left) shown by immunoblotting. B, ectopic expression of mutant KRAS by a retrovirus or KRAS knockdown by siRNA was performed in isogenic KRAS HCT116 cells. Protein expression of KRAS or Bcl-xL was determined by immunoblotting. Tubulin was used as a control for protein loading. C, HCT116 cells were incubated with control or ERK siRNA and the effect on Bcl-xL expression was determined by immunoblotting. D, a competitive RT-PCR assay was performed to quantitate Bcl-xL transcripts using β-actin (ACTB) as an internal control (top). The ratio of Bcl-xL to ACTB transcripts was then plotted in wt versus mt KRAS cells (bottom). *, P < 0.05. E and F, inhibition of Bcl-xL transcription by actinomycin D (ACT-D) or cyclohexamide (CHX) reduced Bcl-xL protein expression in a time-dependent manner. HCT116 cells were treated with actinomycin D or cyclohexamide for indicated times. Total RNA and whole-cell protein lysates were prepared to detect Bcl-xL transcripts (E) and proteins (F) using competitive RT-PCR and immunoblotting.
KRAS-mutant HCT116 and SW620 cells to carfilzomib-induced apoptosis as evidenced by caspase-8, -9, and -3 cleavage (Fig. 5A). Although suppression of Bcl-xL was shown to synergistically enhance carfilzomib-induced apoptosis, suppression of Mcl-1 using shRNA modestly enhanced apoptosis induced by carfilzomib (Fig. 5A, B, and D). Consistent with lack of ability of ABT-263 to inhibit Mcl-1 (19), suppression of Mcl-1 markedly augmented ABT-263–induced apoptosis (Fig. 5D). Together, these data indicate that induction of Noxa/Bik and antagonism of Bcl-xL collectively contribute to the synergistic interaction between carfilzomib and ABT-263 in KRAS-mutant cells.

Noxa and Bik sequester Mcl-1, whereas ABT-263 releases Bik and Bak from Bcl-xL to promote apoptosis.

We further examined the mechanism underlying synergy between carfilzomib and ABT-263. Caspase activation by carfilzomib alone or combined with ABT-263 was dependent upon Bax (Fig. 6A) and to a lesser extent on p53 (Fig. 6A) or Bak (Fig. 6B), as shown using gene knockout (Bax, p53) or knockdown (Bak) HCT116 cells. Importantly, carfilzomib-induced Noxa and Bik induction was unchanged in Bax or p53 knockout cells (Fig. 6A) in contrast to Puma that was attenuated in a p53-dependent manner (Fig. 6A). Furthermore, the
Figure 3. Mutant KRAS confers resistance to carfilzomib-induced apoptosis that can be reversed by ABT-263 resulting in synergistic drug interaction. A and B, isogenic KRAS HCT116 cells (A) or KRAS-mutant SW620 cells (B) were incubated with carfilzomib alone or combined with ABT-263 for 24 hours at the indicated doses. Apoptosis was quantified by annexin V+ staining using flow cytometry. Mean values of triplicate experiments are shown; bars represent SD. Drug synergy was evaluated using both drugs at a fixed ratio to determine the CI (see Materials and Methods). A CI <1 indicates a synergistic interaction. In HCT116 cells, significantly higher CI values were found in mutant versus wild-type cells (P = 0.04). C and D, analysis of caspase cleavage and antiapoptotic Bcl-xL and Mcl-1 expression by immunoblotting in isogenic HCT116 cells (C) or in KRAS mt SW620 cells (D) treated with carfilzomib alone or combined with ABT-263.
addition of ABT-263 to carfilzomib enhanced caspase-9 and -3 cleavage and augmented a Bax conformational change (Fig. 6C) that is consistent with engagement of mitochondrial apoptosis.

We studied interactions between proapoptotic and antiapoptotic Bcl-2 family proteins by immunoprecipitation. We found that carfilzomib-induced Noxa and Bik can each bind to and sequester Mcl-1 in cells treated with carfilzomib alone or combined with ABT-263 for 24 hours at indicated doses. Caspase cleavage was then analyzed by immunoblotting. A second Noxa shRNA was used to confirm its effect on drug-induced caspase-3 cleavage. B, mutant KRAS HCT116 or DLD1 cells were transduced with a lentiviral doxycycline (DOX)-inducible Noxa ectopic expression construct. Cells were treated with DOX in the presence or absence of ABT-263 and Noxa expression and caspase-3 cleavage were then analyzed. C, KRAS-mutant SW620 cells containing stable expression of Bik using two constructs or control shRNA were incubated with carfilzomib alone or combined with ABT-263 for 24 hours at indicated doses. Caspase cleavage was then analyzed.

Figure 4.
Knockdown of Noxa or Bik attenuates apoptosis induced by carfilzomib ± ABT-263. A, KRAS-mutant SW620 cells containing stable expression of Noxa (A) or control shRNA were incubated with carfilzomib alone or combined with ABT-263 for 24 hours at indicated doses. Caspase cleavage was then analyzed by immunoblotting. A second Noxa shRNA was used to confirm its effect on drug-induced caspase-3 cleavage. B, mutant KRAS HCT116 or DLD1 cells were transduced with a lentiviral doxycycline (DOX)-inducible Noxa ectopic expression construct. Cells were treated with DOX in the presence or absence of ABT-263 and Noxa expression and caspase-3 cleavage were then analyzed. C, KRAS-mutant SW620 cells containing stable expression of Bik using two constructs or control shRNA were incubated with carfilzomib alone or combined with ABT-263 for 24 hours at indicated doses. Caspase cleavage was then analyzed.
Discussion

Oncogenic KRAS mutations contribute to apoptosis resistance and treatment failure. We observed the novel finding of significant upregulation of antiapoptotic Bcl-xL expression in KRAS-mutant compared with wild-type cells that was regulated by ERK downstream of KRAS. Upregulation of Bcl-xL was confirmed using ectopic expression of mutant KRAS that increased Bcl-xL while siRNA knockdown of KRAS attenuated its expression. Regulation of mutant KRAS-mediated Bcl-xL upregulation occurred by both transcriptional and posttranscriptional mechanisms, indicating de novo synthesis of mRNA and its translation into protein. In an effort to reverse KRAS-mediated apoptosis resistance, we evaluated the irreversible proteasome inhibitor, carfilzomib, which induced Noxa and Bik expression. In a prior study, we found that bortezomib can similarly induce Noxa expression in colorectal cancer cells (13). Induction of Noxa by carfilzomib was dependent upon the level of oncogenic c-myc and a similar dependence was shown for bortezomib, whereby conserved myc-binding sites were identified in the Noxa promoter (27).

Despite recent evidence that KRAS-mutant cancer cells have increased proteasomal activity (33) and display vulnerability to proteasome inhibition (5), we found increased resistance to carfilzomib in KRAS-mutant versus wild-type cells, indicating that induction of Noxa and Bik were insufficient to reverse apoptosis resistance. Therefore, we determined whether targeting Bcl-xL can overcome apoptosis resistance in these cells and used a BH3 mimetic drug. The addition of ABT-263 to low concentrations of carfilzomib was shown to potently enhance...
apoptosis and this interaction was highly synergistic in both isogenic HCT116 and in SW620 KRAS-mutant cells. Although drug synergy was observed in both wild-type and mutant KRAS cells, the CI in KRAS-mutant cells were significantly exceeded that in wild-type cells consistent with Bcl-xL upregulation in KRAS-mutant cells. Although synergy between a proteasome
inhibitor and a BH3 mimetic has been reported (34), the effect of this combination in relationship to KRAS status has not been studied previously. To explore the mechanism of synergy, we examined potential alterations in protein–protein interactions by the drug combination. ABT-263 was shown to displace Bak and Bik from their binding with Bcl-xl. Carfilzomib-induced proapoptotic effectors Noxa and Bik were shown to bind antiapoptotic Mcl-1. Upregulation of Mcl-1 by carfilzomib is a consequence of inhibiting its proteasome-mediated degradation (28). Importantly, Mcl-1 upregulation by carfilzomib can contribute to apoptosis resistance and ABT-263 does not inhibit Mcl-1. However, our data indicate that Mcl-1 is disabled by carfilzomib-induced Noxa and Bik as the drug combination induced a Bak conformational change (35) to a greater extent than did drug alone indicating mitoxantrone-mediated apoptosis. Drug-induced apoptosis was dependent upon Bak and its upstream regulator p53 (36), and to a lesser extent on Bak that may be related to its incomplete suppression or to a greater dependence upon Bak in our cells (37). In response to proteasome inhibition, conflicting data exist for the role of p53 in regulating apoptosis (38, 39). Induction of Noxa or Bik by carfilzomib was unaffected by p53 knockdown; however, we observed a p53-dependent induction of the BH3-only protein Puma that is consistent with its known regulation by p53 (40).

Carfilzomib and ABT-263 were shown to interact synergistically to overcome mutant KRAS-mediated apoptosis resistance in colorectal cancer cell lines. The mechanism underlying the observed synergistic interaction between the drug combinations involves modulation of Bcl-2 family protein–protein interactions that include dissociation of Bak and Bik from Bcl-xl by ABT-263 and the ability of Noxa and Bik induction by carfilzomib to sequester and disable Mcl-1. Although these effects contribute to the enhanced lethality of the drug combination, other as yet undefined mechanisms may also be contributory. In this regard, we recently demonstrated that bortezomib combined with ABT-263 can accumulate the ubiquitin binding protein and autophagy substrate p62/sequestosome 1 that can mediate caspase-8 aggregation/activation and subsequent apoptosis (23). The potential for in vivo efficacy of the carfilzomib and ABT-263 combination is supported by a recent study of this drug combined with BH3 mimetic obatoclax (also targets Mcl-1) where coadministration reduced tumor growth and increased survival of mice inoculated with germinal center lymphoma cells (41). Furthermore, this regimen exhibited minimal increases in toxicity toward normal cells in intact animals (41).

In conclusion, our findings demonstrate that Bcl-xl upregulation is an important mechanism of apoptosis resistance in mutant KRAS cells. Concurrent induction of proapoptotic Noxa/Bik and antagonism of Bcl-xl interaction synergistically to overcome KRAS-mediated apoptosis resistance. These findings warrant evaluation in an in vivo model and if confirmed, suggest a promising therapeutic strategy to overcome apoptosis resistance in KRAS-mutant colorectal cancer cells.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: K. Okamoto, S. Huang, F.A. Sinicrope
Development of methodology: K. Okamoto, S. Huang
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Zaanan, H. Kawakami, F.A. Sinicrope
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K. Okamoto, A. Zaanan, H. Kawakami, S. Huang, F.A. Sinicrope
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Huang, F.A. Sinicrope
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