The Mutant KRAS Gene Up-regulates BCL-XL Protein via STAT3 to Confer Apoptosis Resistance That Is Reversed by BIM Protein Induction and BCL-XL Antagonism*

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Results: Mutant KRAS up-regulates BCL-XL via STAT3. A MEK inhibitor induces BIM by loss of phosphorylation (Ser69) and interacts synergistically with a BH3 mimetic.

Conclusion: STAT3-mediated BCL-XL inhibits apoptosis in mutant KRAS cells that is overcome by a MEK inhibitor and a BH3 mimetic.

Significance: STAT3-BCL-XL represents a key mechanism of apoptosis resistance by activated KRAS.

In colorectal cancers with oncogenic GTPase Kras (KRAS) mutations, inhibition of downstream MEK/ERK signaling has shown limited efficacy, in part because of failure to induce a robust apoptotic response. We studied the mechanism of apoptosis resistance in mutant KRAS cells and sought to enhance the efficacy of a KRAS-specific MEK/ERK inhibitor, GDC-0623. GDC-0623 was shown to potently up-regulate BIM expression to a greater extent versus other MEK inhibitors in isogenic KRAS HCT116 and mutant KRAS SW620 colon cancer cells. ERK silencing enhanced BIM up-regulation by GDC-0623 that was due to its loss of phosphorylation at Ser69, confirmed by a BIM-EL phosphorylation-defective mutant (S69G) that increased protein stability and blocked BIM induction. Despite BIM and BIK induction, the isogenic KRAS mutant versus wild-type cells remained resistant to GDC-0623-induced apoptosis, in part because of up-regulation of BCL-XL. KRAS knockdown by a doxycycline-inducible shRNA attenuated BCL-XL expression. BCL-XL knockdown sensitized KRAS mutant cells to GDC-0623-mediated apoptosis, as did the BH3 mimetic ABT-263. GDC-0623 plus ABT-263 induced a synergistic apoptosis by a mechanism that includes release of BIM from its sequesteration by BCL-XL. Furthermore, mutant KRAS activated p-STAT3 (Tyr705) in the absence of IL-6 secretion, and STAT3 knockdown reduced BCL-XL mRNA and protein expression. These data suggest that BCL-XL up-regulation by STAT3 contributes to mutant KRAS-mediated apoptosis resistance. Such resistance can be overcome by potent BIM induction and concurrent BCL-XL antagonism to enable a synergistic apoptotic response.

Oncogenic GTPase Kras (KRAS)5 mutations can be detected in ~50% of human colorectal cancers (CRCs) (1) and result in a protein with impaired GTPase activity that enables KRAS to remain activated (2). Mutated KRAS has been shown to contribute to de novo apoptosis resistance, failure of anticaner drug treatment, and a poor prognosis in colon cancer patients receiving adjuvant chemotherapy (3). To date, direct targeting of mutant KRAS has not been achieved, and there are no effective targeted agents for use in KRAS mutant CRCs. MEK is a serine/threonine kinase that lies downstream of both RAS and RAF in a canonical RAF/MEK/ERK pathway that regulates key cellular activities, including differentiation, proliferation, and survival (4). The downstream position of MEK in this cascade makes it an attractive therapeutic target for patients whose tumors carry upstream gain-of-function mutations. Studies of multiple allosteric inhibitors of MEK in KRAS mutant cancers demonstrate target inhibition (5) but have generally produced stable disease in early-phase clinical trials (6–9). In contrast to BRAF mutant melanomas, this limited efficacy indicates that different mechanisms of inhibition are required for optimal antitumor activity in each genotype. Structural and functional analyses indicate that the novel MEK inhibitor GDC-0623 can achieve superior efficacy in KRAS-driven tumors by forming a strong hydrogen bond interaction with Ser212 in MEK that is critical for blocking MEK feedback phosphorylation by wild-type RAF (10). On the basis of favorable preclinical data, GDC-0623 is currently being studied in a phase I clinical trial.

The BH3-only proteins appear to be critical for the response to targeted therapies, including EGFR receptor (EGFR) and combined MEK/Pi3K inhibitors (11, 12). MEK/ERK inhibitors have

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5 The abbreviations used are: KRAS, GTPase Kras; CRC, colorectal cancer; CI, combination index.
been shown to induce the pro-apoptotic BH3-only BIM protein by suppression of ERK-mediated phosphorylation, which blocks pro- tease-mediated BIM degradation (13). BIM is the most potent BH3-only protein and can bind and neutralize all anti-apoptotic B cell lymphoma 2 (BCL-2) proteins, whereas NOXA, p53 upregulated modulator of apoptosis (PUMA), BID and BIK show greater selectivity (14). Therefore, up-regulation of BIM may offer the greatest potential to improve therapeutic efficacy. However, the induction of BIM proteins by MEK/ERK inhibition (15, 16) can result in BIM being bound and inhibited by anti-apoptotic BCL-2 and/or BCL-XL, which are frequently overexpressed in solid tumors (17). This antagonism may explain the modest apoptotic and largely cytostatic effect of MEK/ERK inhibitors in KRAS mutant tumor xenografts (18) as well as predominantly stable disease in patient studies (6, 7). Of note, STAT3 can regulate the transcription of oncogenic and inflammatory genes, including BCL-XL, Myc, cyclin D1, COX-2, and IL-1β (19). STAT3 is constitutively activated in colon cancers (20) and has been shown to mediate diverse cellular responses, including inflammation, cell proliferation, and apoptosis.

In this work, we demonstrate that MEK/ERK inhibition by GDC-0623 can up-regulate BIM expression because of its loss of phosphorylation at Ser69. Importantly, GDC-0623 was found to more potently up-regulate BIM compared with other MEK inhibitors, and, therefore, its ability to overcome mutant KRAS-mediated apoptosis resistance was examined. The ability of GDC-0623 to trigger apoptosis was limited by mutant KRAS-mediated overexpression of BCL-XL, which was found to be regulated by STAT3. BCL-XL antagonism by gene knockdown or the BH3 mimetic ABT-263 was shown to synergistically enhance apoptosis induction in combination with GDC-0623 in KRAS mutant cells. The mechanism of this effect was in part due to the release of BIM from its sequestration by BCL-XL, as shown using ABT-263. Dependence on BIM was confirmed by BIM knockdown, which abrogated the ability of GDC-0623 plus ABT-263 to trigger cell death. Together, these data suggest a novel strategy to circumvent apoptosis resistance in KRAS mutant CRC cells.

Experimental Procedures

Cell Culture and Drugs—The isogenic HCT116 human CRC cell line containing KRAS wild-type (no. 152) or mutant (no. 154) alleles was obtained from Dr. B. Vogelstein (Johns Hopkins University). The KRAS mutant SW620 cell line was obtained from the ATCC. HS683 (glioma), U373 (glioblastoma), and U87 (glioblastoma) cell lines (gifts from Dr. J. Sarkaria, Mayo Clinic) were used as controls. Authentication of cell lines was not performed within the previous 6 months. Cell lines are routinely tested for Mycoplasma contamination every 3 months with a MycoAlert mycoplasma detection set (Lonza, Allendale, NJ). All cells were grown as monolayers in RPMI medium (Invitrogen) supplemented with 10% (v/v) FBS and 1% antibiotic-antimycotic (Invitrogen), but HEK293T cells, which were utilized for pseudovirus production, were grown in DMEM (Sigma) and supplemented as above. Cells were treated with GDC-0623 (ActiveBiochem, Maplewood, NJ) alone or combined with ABT-263 (Sellekchem, Houston, TX) and with carfilzomb

where shown (LC Labs, Woburn, MA). GDC-0623 and ABT-263 were prepared as 1 mmol/liter and 10 mmol/liter stock solutions in DMSO, respectively, and stored at −20 °C.

Lentiviral shRNA Expression—Virus production using HEK293T cells and transduction of target cells were performed utilizing a standard procedure described previously (21). The non-targeting shRNA expression vector was obtained from Addgene (Cambridge, MA). BIM and BCL-XL shRNAs were generated as described previously (21, 22). For BIK, the targeting sequence was ACACCTAAAGGAGAACATAA. All other shRNA constructs were purchased from GE Dharmacon (Lafayette, CO). For transduction of lentiviral shRNA expression constructs (packaged as pseudotyped viral particles) into target cells, the growth medium of recipient cells was replaced with Opti-MEM (Invitrogen) containing 8 μg/ml Polybrene (Sigma) and appropriate amounts of lentivirus. The cells were incubated overnight at 37 °C, and the medium was replaced the following day. Puromycin (2–4 μg/ml, Sigma) was added 48 h post-transduction, and the puromycin-resistant pool of cells was used for subsequent experiments.

Transfection of siRNA—Cells were seeded 1 day before transfection at 30–50% confluence in growth medium without antibiotics. STAT3 siRNA (Cell Signaling Technology, Danvers, MA) and Lipofectamine RNAiMax (Invitrogen) were diluted in OPTI-MEM medium, mixed gently, and incubated to allow complex formation. The cells were then transfected by adding the RNAi-Lipofectamine complex dropwise to medium to achieve a siRNA concentration of 50 nmol/liter. Cells were then incubated at 37 °C, and knockdown efficiency was determined 48 h post-transfection.

ELISA—Determination of the IL-6 concentration in the media was performed by ELISA on the basis of a quantitative sandwich immunoassay technique according to the manufacturer (Biolegend, San Diego, CA). The IL-6 ELISA was linear between 0–500 pg/ml. Isogenic KRAS mutant and wild-type HCT116 and DLD1 colon cancer cell lines were plated at 0.5 × 10^6/ml and incubated for 96 h with and without LPS stimulation (10 μg/ml). HMC-1 mastocytoma cells were used as a positive control with and without stimulation (phorbol 12-myristate 13-acetate (PMA) + ionomycin) for 72 h. The ELISA reaction was measured using a spectrophotometer.

Competitive RT-PCR—Total RNA was extracted from cells using the RNA Easy mini kit (Qiagen, Germantown, MD), and RNA integrity was evaluated using an Agilent Bioanalyzer 2000. Competitive RT-PCR was performed with a one-step RT-PCR kit (Qiagen) using the following primer sets containing an equimolar ratio of BIM (forward, 5'-GCCGCCACCTGGCAGC-3'; reverse, 5'-ACACAGGGGATCTTCTCATAA-3') or IL-6 (forward, 5'-ATGAACTCCTTCTCCAAAGGGC-3'; reverse, 5'-GAAGGCCCTCAGGCTGACTG-3') against β-actin (forward, 5'-TCACCACACTTGCCACCTGAG-3'; reverse, 5'-CAGCCGAAACCGTCATTGCAATGG-3'). Reverse transcription was coupled with PCR (25 cycles) on a thermocycler (Applied Biosystems, Grand Island, NY). PCR products were quantified on the Agilent Bioanalyzer 2000 using the DNA 12,000 kit. In brief, samples were loaded onto DNA microchips, and the DNA fragments were then separated by capillary electrophoresis. The target DNA sizes and relative
quantities were calculated on the basis of DNA ladders and an internal marker, respectively. The associated software then generates agarose gel–like images.

**Lentiviral Expression of Inducible KRAS shRNA**—The pTRIPZ lentiviral vector containing an inducible shRNA (Dharmacon) was packaged into pseudotyped lentivirus with helper plasmids pMD2.G (Addgene, catalog no. 12259) and PsPAX2 (Addgene, catalog no. 12260). Target cells were transduced with concentrated lentivirus and selected with puromycin. Knockdown efficiency was evaluated by immunoblotting after induction of KRAS shRNA by doxycycline (2–10 ng/ml).

**Site-directed Mutagenesis and Doxycycline-inducible Ectopic Expression**—Wild-type BIM or KRAS (G12D) cDNA was purchased from Origene (Rockville, MD) and Addgene, respectively. The ORF was subcloned into pTRIPZ, whereby red fluorescence protein and shRNA in the original vector were removed by restriction enzyme digestion. Compatible cohesive ends were utilized to facilitate ligation that avoided cleavage of existing sites in the BIM ORF. BIM S69G or S69E mutants were generated by overlapping PCR using primers containing the desired mutations, which were then subcloned into pTRIPZ. Induction was achieved by doxycycline (2–10 ng/ml).

**Apoptosis Assay and Calculation of a Combination Index (CI)**—Apoptosis was analyzed by annexin V+ staining and quantified by flow cytometry as described previously (23). Briefly, cells were incubated with the study drugs at prespecified time points. Trypsin was added to detach adherent cells that were then combined with floating cells. Cells were pelleted by centrifugation, and the pellet was washed three times in cold PBS. Cells were incubated with annexin V conjugated with FITC (BD Biosciences). The labeled cell populations were quantitated by flow cytometry. The means of triplicate experiments were used to compute the CI using CompuSyn software (ComboSyn Inc., Paramus, NJ). CI < 1 indicated drug synergy.

**Immunoprecipitation and Immunoblotting**—Protein samples were prepared in lysis buffer (5 mmol/liter MgCl₂, 137 mmol/liter KCl, 1 mmol/liter EDTA, 1 mmol/liter EGTA, 1% CHAPS, and 10 mmol/liter HEPES (pH 7.5)) supplemented with a protease inhibitor cocktail (Sigma). The protein concentration of the samples was measured using the nanodrop method (Thermo Scientific, Franklin, MA). Cell lysates were incubated with primary antibodies for 3 h at 4 °C. Immunocomplexes were captured using magnetic beads conjugated with protein A/G (Pierce) and then washed three times in lysis buffer. Immunoprecipitated proteins were eluted with 2× LDS sample buffer (Invitrogen) and loaded onto a 14% SDS-PAGE gel for separation, followed by an electrical transfer onto PVDF membranes (Bio-Rad). Immunoblotting was then performed as described previously (21). Primary antibodies included those against BCL-XL (Calbiochem), caspase-8 (BD Biosciences), and tubulin (Sigma). All other antibodies were purchased from Cell Signaling Technology.

**Statistical Analysis**—The values shown in the Annexin V and RT-PCR experiments represent the mean ± S.D. for triplicate experiments. Statistical significance was determined using Student’s t test in R programming language (24). p < 0.05 was considered statistically significant, by which the null hypothesis is rejected. A 95% confidence interval was also calculated to confirm significance.

**Results**

The MEK Inhibitor GDC-0623 Up-regulates BIM via Its Loss of Phosphorylation at Ser⁶⁹.—Given the key role of pro-apoptotic BIM in governing the apoptotic response to targeted therapies (11, 12), we examined the ability of BIM induction by MEK/ERK inhibition to enhance apoptotic susceptibility and to overcome mutant KRAS-mediated apoptosis resistance in CRC cells. Using isogenic HCT116 colon cancer cell lines containing either a mutant (no. 154) or wild-type (no. 152) KRAS allele in which the other copy was somatically deleted by homologous recombination (25), we evaluated the novel mutant KRAS-selective MEK/ERK inhibitor GDC-0623 (10). We found that GDC-0623 can inhibit ERK phosphorylation in association with potent induction of BIM and, to a lesser extent, BIK proteins in isogenic KRAS mutant and wild type HCT116 cells in a dose- and time-dependent manner (Fig. 1, A and B). Of the three major BIM isoforms (BIM(EL), BIM(L), and BIM(S)) that are generated by alternative splicing, Bim(EL) is the most abundant and contains a unique sequence that has been reported to be the target of phosphorylation by several MAP kinases (26). BIM and BIK induction by GDC-0623 was also observed in KRAS mutant SW620 cells (Fig. 1C). Compared with the MEK inhibitors selumetinib (AZD6244) or the mutant BRAF-specific MEK inhibitor cobimetinib (GDC-0973) (10), GDC-0623 induced BIM to a much greater extent than did the other drugs (Fig. 1D). ERK1/2 siRNA partially suppressed p-ERK, and the addition of GDC-0623 resulted in BIM up-regulation (Fig. 1E), suggesting that GDC-0623 can induce BIM via MEK/ERK inhibition. We then determined whether an increase in BIM gene transcription contributes to the up-regulation of BIM proteins. Although cells treated with GDC-0623 showed potent up-regulation of BIM proteins, no significant change in BIM mRNA levels were detected (Fig. 2A, left panel). The data suggest that activation of the transcription factor FOXO3a can mediate BIM expression by selumetinib (27). However, up-regulation of BIM proteins by GDC-0623 in CRC cells was unaffected by suppression of FOXO3a using shRNA (Fig. 2A, right panel), suggesting that up-regulation of BIM proteins by GDC-0623 may occur via an increase in BIM protein stability that has been shown to be regulated by its loss of phosphorylation at Ser⁶⁹ (28). We found that GDC-0623 can inhibit BIM-EL phosphorylation on Ser⁶⁹, leading to BIM accumulation. Specifically, ERK activation by PMA treatment induced BIM-EL phosphorylation, as shown by a mobility shift (Fig. 2B) and recognition with a p-BIM(Ser⁶⁹) antibody (Fig. 2C), which were abrogated rapidly by GDC-0623 treatment in both HCT116 and SW620 cell lines (Fig. 2, B and C).

To support BIM-EL phosphorylation as a mechanism of its stability, we generated a doxycycline-inducible system that enables ectopic expression of wild-type BIM or expression of mutant phospho-mimic (S69E) or phospho-deficient (S69G) BIM protein. The S69G BIM mutant showed a different mobility compared with the wild type and the S69E mutant (Fig. 2D, top panel). Furthermore, both wild-type BIM and the phospho-mimic BIM mutant (S69E) showed a PMA-induced mobility.
shift that was recognized by the p-BIM antibody. In contrast, the phospho-deficient BIM mutant (S69G) was relatively unresponsive to PMA treatment (Fig. 2D, top panel).

Mutation of BIM at S69G was shown to attenuate BIM degradation compared with the wild type or the S69E BIM mutant (Fig. 2D, center panel), consistent with the ability of the phospho-deficient BIM mutant to stabilize BIM proteins. Furthermore, treatment with GDC-0623 failed to increase the protein level of phospho-deficient BIM mutants (Fig. 2D, bottom panel). Together, these data suggest that loss of phosphorylation of BIM Ser69 is a critical event that leads to up-regulation of BIM via an increase in BIM protein stability.

Mutant KRAS Up-regulates BCL-XL and Confers Apoptosis Resistance to GDC-0623—In a recent study, we detected selective up-regulation of the anti-apoptotic BCL-XL protein in isogenic KRAS mutant versus wild-type HCT116 and DLD1 colon cancer cell lines (29). To confirm the up-regulation of BCL-XL by mutant KRAS, we generated stable KRAS mutant HCT116 cells that contain an inducible KRAS shRNA under the control of a doxycycline-responsive promoter. In this system, inducible knockdown of mutant KRAS was shown to suppress BCL-XL protein expression (Fig. 3A). Treatment of the HCT116 and SW620 cell lines with GDC-0623 at clinically relevant doses had no effect on BCL-XL expression (Fig. 3B). The effect of KRAS mutation status on the sensitivity to MEK inhibitors was then determined. GDC-0623 was shown to induce significantly less apoptosis in KRAS mutant compared with wild-type HCT116 cells, as shown by caspase cleavage and annexin V labeling (Fig. 3, C and D). Resistance remained despite an increase in the dose and duration of GDC-0623 treatment (Fig. 3D). This result is consistent with in vivo data in KRAS mutant tumor xenografts where MEK inhibitors induced only modest

**FIGURE 1.** The KRAS-specific MEK inhibitor GDC-0623 potently up-regulates the pro-apoptotic BH3-only protein BIM. A—C, dose- and time-dependent effects of GDC-0623 treatment on expression of BIM, BIK, p-ERK, and ERK in isogenic HCT116 cells (A and B, with WT or mutant (mt) KRAS alleles) or KRAS mutant SW620 cells (C). Tubulin served as a control for protein loading. D, comparison of the effect of MEK inhibitors on expression levels of p-ERK and BIM in KRAS mutant HCT116 cells. DMSO, dimethyl sulfoxide. E, effect of ERK siRNA on BIM expression in KRAS mutant HCT116 cells treated in the presence or absence of GDC-0623 (1 h).
apoptosis in the absence of significant tumor regression (18). A potential explanation is that solid tumors frequently express anti-apoptotic BCL-2/BCL-XL proteins that may disable pro-apoptotic BIM and BIK induced by MEK inhibition.

The BH3 Mimetic ABT-263 Synergistically Enhances GDC-0623-induced Apoptosis in KRAS Mutant Colon Cancer Cell Lines—On the basis of the finding that mutant KRAS up-regulates BCL-XL and shows resistance to GDC-0623, we hypothesized that antagonism of BCL-XL can release the potential of BIM and BIK induction to elicit robust apoptosis. In support of this, we found that suppression of BCL-XL using a lentiviral shRNA can potently enhance GDC-0623-induced caspase cleavage in both cell lines (Fig. 4A). This effect was confirmed with a second shRNA with a different BCL-XL targeting sequence (Fig. 4A). Antagonism of BCL-XL/BCL-2 function utilizing ABT-263 was shown to markedly enhance GDC-0623-

FIGURE 2. BIM up-regulation by GDC-0623 is due to increased protein stability mediated by loss of phosphorylation at Ser69. A, induction of BIM by GDC-0623 is independent of BIM transcription (left panel), and regulation by FOXO3A is shown using FOXO3A shRNA (right panel). Data were generated by competitive RT-PCR with β-actin (ACTB) serving as an internal loading control. DNA bands were separated and quantified using an Agilent Bioanalyzer 2100 whose associated software generates an agarose gel-like image (see “Experimental Procedures”). B, treatment with PMA induced BIM phosphorylation, indicated by a mobility shift to a higher molecular weight that was abrogated by GDC-0623 in isogenic KRAS mutant HCT116 (left panel) or SW620 (right panel) cells. DMSO, dimethyl sulfoxide. C, BIM phosphorylation was detected using a p-BIM (Ser69) antibody in both cell lines in the presence or absence of carfilzomib (50 nM), used to prevent protein proteasomal degradation. D, a lentiviral doxycycline (DOX)-inducible system was utilized to ectopically express BIM wild-type, S69G (phospho-deficient), or S69E (phospho-mimic) in KRAS mutant HCT116 cells. Both wild-type and S69E BIM showed a PMA-induced mobility shift that was recognized by the p-BIM antibody. In contrast, S69G BIM was relatively unresponsive to PMA treatment (top panel). Expression of BIM was induced with doxycycline (24 h) and then detected at the indicated time after doxycycline removal (center panel). In these cells, the effect of GDC-0623 treatment on the expression of BIM and p-ERK was determined (bottom panel).
induced apoptosis, as shown by annexin V labeling (Fig. 4 B) and caspase cleavage (Fig. 4 C). The combination of GDC-0623 and ABT-263 produced a 2-fold increase in apoptosis compared with either drug alone in both cell lines (Fig. 4 B). Calculation of the CI revealed a synergistic interaction between GDC-0623 and ABT-263, indicated by CI < 1.

Stable lentiviral shRNA knockdown of BIM or BIK markedly attenuated apoptosis induced by GDC-0623 plus ABT-263 (Fig. 5, A–C). To exclude nonspecific off-target effects, a second lentiviral shRNA construct was utilized for each gene that similarly reduced apoptosis induction (Fig. 4, A and C). Examination of the protein-protein interaction between BIM and BCL-XL or MCL-1 by immunoprecipitation revealed that binding of BIM to BCL-XL was abrogated by ABT-263 treatment (Fig. 5 D), consistent with the ability of ABT-263 to antagonize BCL-XL. These results demonstrate that BIM/BIK induction and concurrent BCL-XL antagonism enable robust apoptosis in CRC cells.

**STAT3 Regulates BCL-XL Induction in KRAS Mutant Cells—** We examined the role of STAT3 in the regulation of BCL-XL by mutant KRAS. Ectopic expression of mutant KRAS using a doxycycline-inducible system or isogenic KRAS mutant versus wild-type KRAS cells both showed activation of STAT3, as indicated by its phosphorylation at Tyr705 (Fig. 6 A). Because IL-6 is a known regulator of STAT3, we treated cells with exogenous IL-6, which was shown to up-regulate p-STAT3 and BCL-XL expression in wild-type KRAS cells. In mutant KRAS cells, IL-6 treatment increased p-STAT3 expression, but BCL-XL expression was not altered (Fig. 6 A). Mutant KRAS versus wild-type KRAS cells or cells with ectopic expression of mutant KRAS did not enhance IL-6 mRNA expression, as shown by competitive PCR (Fig. 6 B). We were unable to detect IL-6 secretion in the supernatant of wild-type or mutant KRAS isogenic HCT116 cells in the presence or absence of LPS stimulation (data not shown), although efficient secretion and detection of IL-6 in positive control cells can be achieved. Despite these findings using exogenous IL-6, suppression of STAT3 by shRNA or siRNA attenuated BCL-XL mRNA and protein expression (Fig. 6 C). It has been shown that oncogenic KRAS-driven cancers require TBK1, which regulates BCL-XL expression (30) and cell survival involving autocrine IL-6, CCL5, and STAT3 signaling (31). In KRAS mutant HCT116 cells, however, we found that...
Inhibition of BCL-XL synergistically enhances GDC-0623-induced apoptosis.

A, effect of BCL-XL shRNA knockdown versus control shRNA on GDC-0623-induced caspase cleavage (CL) in KRAS mutant HCT116 or SW680 cells. FL, full-length. B and C, KRAS mutant (mut) cell lines were treated with GDC-0623, the BH3 mimetic ABT-263, or their combination at the indicated doses and times. Apoptosis induction was then determined by annexin V staining (B) and caspase cleavage (C).

FIGURE 4. Inhibition of BCL-XL synergistically enhances GDC-0623-induced apoptosis.
suppression of TBK1 by shRNA did not attenuate BCL-XL protein levels (Fig. 6D). This finding was confirmed using siRNA in KRAS mutant HCT116 and SW620 cells, where TBK1 suppression did not reduce STAT3 phosphorylation at Tyr705, nor did it attenuate BCL-XL expression (Fig. 6D). These data suggest that STAT3 mediates BCL-XL expression in an IL-6- or TBK1-independent manner.

Discussion

Mutant KRAS confers apoptosis resistance, which may represent an important mechanism of therapeutic failure in human cancers. Existing MEK inhibitors lack specificity against oncogenic KRAS versus BRAF mutations that converge on the MAPK signaling pathway. We studied the novel MEK inhibitor GDC-0623, which has been shown to have superior efficacy against KRAS compared with BRAF mutant tumors (10). We found that GDC-0623 treatment can potently up-regulate BIM and to a greater extent than observed for other MEK inhibitors that have undergone clinical evaluation (32, 33). ERK silencing in GDC-0623-treated cells was shown to enhance BIM induction. Importantly, BIM is the most potent pro-apoptotic BH3-only protein given its ability to neutralize all anti-apoptotic BCL-2 family proteins (14). In addition to BIM, GDC-0623 was also shown to induce BIK expression to further stimulate apoptosis. Mechanistically, MEK/ERK inhibition by GDC-0623 induced loss of BIM phosphorylation on Ser69, which has been shown to block its proteasomal degradation (28). We confirmed this finding using a BIM-EL phosphorylation-defective mutant (S69G) that increased BIM stability and blocked the ability of GDC-0623 to induce BIM. Although GDC-0623 up-regulated BIM protein expression, it did not alter BIM gene transcription. In another report, the transcription factor FOXO3a has been shown to mediate BIM up-regulation by the MEK inhibitor selumetinib (27). However, BIM up-regulation by GDC-0623 occurred independently of FOXO3a, as shown in FOXO3a knockdown cells. These data suggest that the mechanism of BIM protein up-regulation by GDC-0623 is post-translational and due to its loss of phosphorylation.

Despite BIM and BIK induction, isogenic KRAS mutant versus wild-type CRC cells remained resistant to GDC-0623-induced apoptosis. This resistance was in part due to up-regulation of BCL-XL, indicating that MEK/ERK inhibition and related BIM/BIK induction are insufficient to overcome mutant KRAS-mediated apoptosis resistance. These data may underlie the limited efficacy of MEK inhibitors against KRAS mutant tumors even when complete suppression of this signaling pathway is achieved. Although the mechanisms by which KRAS activation can suppress apoptosis are understood poorly, we found that mutant KRAS can up-regulate BCL-XL to confer

FIGURE 5. BIM regulates apoptosis induction by the combination of GDC-0623 and ABT-263 via a mechanism involving the release of BIM from BCL-XL protein. A–C, KRAS mutant HCT116 (A) or SW620 (B and C) cells with stable shRNA knockdown of BIM (A and B) or BIK (C) versus control shRNA were utilized. Cells were treated with GDC-0623, ABT-263, or a combination of both, and caspase cleavage (CL) was determined. FL, full-length. D, KRAS mutant HCT116 cells were treated with GDC-0623, ABT-263, or both in combination. Immunoprecipitation (IP) was then performed in whole cell lysates (WCL) using an anti-BIM antibody. The association of BIM with its binding partners BCL-XL or MCL-1 was determined in the coprecipitated protein complex by immunoblotting.

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apoptosis resistance to MEK inhibition. Using a doxycycline-inducible system, suppression of mutant KRAS was shown to attenuate BCL-XL expression, consistent with our prior findings that ectopic expression of mutant KRAS can increase BCL-XL and that BCL-XL is increased in isogenic KRAS mutant versus wild type HCT116 and DLD1 CRC cells (29). The clinical relevance of this finding has been shown in human CRCs, where the level of BCL-XL protein expression by immunohistochemistry has been shown to be higher in KRAS mutant compared with wild-type tumors (34). We evaluated the potential for BCL-2/BCL-XL antagonism to increase apoptotic susceptibility, as evaluated with MEK inhibition in other tumor
types (35–37). Knockdown of BCL-XL by shRNA was shown to significantly enhance GDC-0623-induced apoptosis in KRAS mutant cells. Furthermore, the BH3 mimetic ABT-263 synergistically enhanced GDC-0623-induced apoptosis in KRAS mutant HCT116 and SW620 cell lines. The mechanism of synergy resulted from concurrent BIM/BIK up-regulation and antagonism of BCL-XL, which enabled a robust apoptotic response, as shown by BIM or BIK shRNA and the ability of ABT-263 to displace BIM from its sequestration by BCL-XL. These results provide important insights that include the following: up-regulation of BCL-XL is a novel mechanism of KRAS-mediated resistance in CRC cells, GDC-0623-induced induction of BIM is mediated by its loss of phosphorylation at Ser69, and GDC-0623 and ABT-263 interact synergistically to promote apoptosis in KRAS mutant CRC cells. Taken together, these data suggest that BCL-XL up-regulation is an important contributor to clinical resistance to MEK inhibitors in CRCs with KRAS mutations (38). Furthermore, our data suggest that the antitumor efficacy of targeted therapies that induce pro-apoptotic factors but produce only cytostatic effects in vivo can be enhanced by disabling anti-apoptotic proteins.

Elucidation of the mechanism by which mutant KRAS can confer apoptosis resistance is critical to the development of strategies to overcome such resistance. We have shown that mutant KRAS can up-regulate BCL-XL proteins, and that BCL-XL mRNA expression was also increased in KRAS mutant CRC cells (29). Here we demonstrate the novel finding that STAT3 knockdown can attenuate BCL-XL mRNA and protein expression, indicating that BCL-XL is transcriptionally regulated by STAT3. Regulation of BCL-XL by STAT3 was independent of the IκB kinase TBK1, as shown by knockdown of TBK1, which failed to reduce BCL-XL expression. This result is in contrast to findings in lung cancer cells (30) that suggest that this effect may depend upon tumor type. In support of the ability of STAT3 to regulate KRAS-mediated BCL-XL expression, we demonstrate that mutant KRAS can activate STAT3 by its phosphorylation at Tyr205 using both an isogenic system and doxycycline-inducible system. p-STAT3 Tyr205 is known to be critical for its transcriptional activity (39). IL-6 is a known inducer of p-STAT3. However, isogenic HCT116 colon cancer cells did not express detectable IL-6 at the level of mRNA or secreted protein, as reported previously (40), suggesting that STAT3 activation occurred independent of IL-6 to regulate Bcl-XL. Because STAT3 can be activated by cytokines, including IL-6 in the tumor microenvironment, potential paracrine effects are likely to occur in vivo. Other factors are known to contribute to BCL-XL expression (41), and STAT3 can also be regulated by multiple signaling pathways, including G protein-coupled receptor and Toll-like receptor 4 (42, 43). Of note, unphosphorylated STAT3 represents the bulk of total STAT3 and has been described to be oncongenic, in part, by modulating p-STAT3 gene targets with pro-oncogenic or pro-survival effects (c-Myc, c-fos, and BCL-XL as well as genes such as RANTES, cdc2, cyclin B1, IL-6, IL-8, Met, and M-RAS) through its cooperative binding with NF-κB to specific κB elements and/or other novel mechanisms (44).

In conclusion, we demonstrate a novel mechanism of KRAS-mediated apoptosis resistance because of STAT3-mediated up-regulation of BCL-XL, and the mechanism underlying potent BIM induction by GDC-0623 resulting from its loss of phosphorylation at Ser69. Concurrent induction of BIM and antagonism of BCL-XL were shown to interact synergistically to overcome apoptosis resistance in KRAS mutant CRC cells, suggesting an important therapeutic strategy. Currently, GDC-0623 is undergoing evaluation in a phase I clinical trial, and further study of its combination with a BH3 mimetic agent appears warranted in tumor types bearing KRAS mutations.

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