Failure to Induce Apoptosis via BCL-2 Family Proteins Underlies Lack of Efficacy of Combined MEK and PI3K Inhibitors for KRAS-Mutant Lung Cancers

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Abstract
Although several groups have demonstrated that concomitant use of MEK and phosphoinositide 3-kinase (PI3K) inhibitors (MEKi/PI3Ki) can induce dramatic tumor regressions in mouse models of KRAS-mutant non–small cell lung cancer (NSCLC), ongoing clinical trials investigating this strategy have been underwhelming to date. While efficacy may be hampered by a narrow therapeutic index, the contribution of biologic heterogeneity in the response of KRAS-mutant NSCLCs to MEKi/PI3Ki has been largely unexplored. In this study, we find that most human KRAS-mutant NSCLC cell lines fail to undergo marked apoptosis in response to MEKi/PI3Ki, which is key for tumor responsiveness in vivo. This heterogeneity of apoptotic response occurs despite relatively uniform induction of growth arrest. Using a targeted short hairpin RNA screen of BCL-2 family members, we identify BIM, PUMA, and BCL-XL as key regulators of the apoptotic response induced by MEKi/PI3Ki, with decreased expression of BIM and PUMA relative to BCL-XL in cell lines with intrinsic resistance. In addition, by modeling adaptive resistance to MEKi/PI3Ki both in vitro and in vivo, we find that, upon the development of resistance, tumors have a diminished apoptotic response due to downregulation of BIM and PUMA. These results suggest that the inability to induce apoptosis may limit the effectiveness of MEKi/PI3Ki for KRAS-mutant NSCLCs by contributing to intrinsic and adaptive resistance to this therapy. Cancer Res; 74(11); 3146–56. ©2014 AACR.

Introduction
Advances in the understanding of genetic alterations in non–small cell lung cancer (NSCLC) have given rise to therapies that target specific oncogenic signaling pathways. For example, cancers that harbor activating EGFR receptor (EGFR) mutations or the EML4-ALK translocation are highly sensitive to the EGFR inhibitors erlotinib and gefitinib or the ALK/MET inhibitor crizotinib, respectively, with response rates of approximately 60% to 70% (1–3). However, no highly effective therapies have been developed for cancers harboring mutant KRAS, which account for 20% to 25% of NSCLCs as well as 35% to 45% of colon cancers and 80% to 95% of pancreatic cancers. Thus, there continues to be a great need for new therapeutic strategies for KRAS-mutant cancers.

Activating mutations in KRAS lead to impaired GTPase activity and constitutive activation of multiple signaling pathways that regulate growth and survival, including RAF/MEK/ERK, RalGDS, and in some instances, phosphoinositide 3-kinase (PI3K)/AKT (4). While attempts to directly target the mutant KRAS protein have so far proven unsuccessful, an alternative approach is to target these downstream signaling pathways in combination. Several groups have demonstrated that dual inhibition of MEK and PI3K leads to tumor regression in experimental models of KRAS-mutant cancers (5–8). This has spurred the rapid clinical development of this combination for KRAS-mutant cancers, and although responses have been noted, the emerging data have been underwhelming to date (9–12). Although the tolerability of this combination when administered daily remains questionable, it is also not clear what proportion of KRAS-mutant NSCLCs are even truly sensitive to combined MEKi/PI3Ki, and the molecular features that underlie sensitivity to this therapy have not been defined.

In cancers driven by receptor tyrosine kinases (RTK) such as EGFR and ALK, inhibition of the corresponding RTK leads to suppression of the MEK and PI3K pathways, resulting in cell-cycle arrest and apoptosis (13). Induction of apoptosis is critical for tumor regressions in vivo, and a diminished apoptotic response contributes to intrinsic resistance to EGFR inhibitors in EGFR-mutant NSCLCs (14, 15). These signaling pathways modulate the BCL-2 family of pro- and antiapoptotic...
proteins that regulate the mitochondrial apoptotic response. For instance, extracellular signal–regulated kinase (ERK)–mediated phosphorylation of the proapoptotic BCL-2 family member BIM leads to its proteasomal degradation (16), and suppression of ERK signaling results in increased BIM protein levels, which is key for response to tyrosine kinase inhibitors (TKI; refs. 13, 17–19). Furthermore, in some instances, targeted therapies may impact the expression of antiapoptotic mediators such as BCL-XL and MCL-1 by their effects on the NF-κB and TORC1, respectively (13, 20). Thus, altering the balance of pro- and antiapoptotic mediators by suppression of activated kinase signaling pathways is a critical component of effective targeted therapies.

In this study, we investigated the response of human KRAS-mutant NSCLCs to MEKi/PI3Ki. Using a collection of human KRAS-mutant NSCLC cell lines, we observed that the majority had impaired responsiveness to MEKi/PI3Ki, resulting from the differential ability of MEKi/PI3Ki to induce a robust apoptotic response. Furthermore, loss of the apoptotic response was associated with the development of resistance to MEKi/PI3Ki in vitro and in vivo. These results provide insight into the molecular mechanisms underlying sensitivity and resistance of KRAS-mutant NSCLCs to MEKi/PI3Ki therapy.

Materials and Methods

Cell lines and reagents

Human KRAS-mutant and EGFR-mutant NSCLC cell lines were obtained from the Center for Molecular Therapeutics at the Massachusetts General Hospital (MGH) Cancer Center (Boston, MA), which performs routine single-nucleotide polymorphism (SNP) and short tandem repeat authentication (21); cell lines were passaged for less than 6 months following receipt. A427-R- and DV-90-R–resistant cell lines were generated by exposing parental cell lines to 1 μmol/L AZD6244/GDC-0941 for 3 days followed by drug washout for 3 days. Cells were treated for 5 to 10 cycles, followed by maintenance in the continuous presence of drug. The N1, N2 (treatment-naive), and R1, R2, R3 (AZD6244/BEZ235-resistant) lines are tumor-derived cell lines from Kras p53L/L mouse strains harboring a conditional activating mutation (G12D) at the endogenous Kras locus and conditional Tp53 knockout were described previously (22). Tumor burden was quantified according to the formula $V = 0.52 \times L \times W^2$. Mice with established tumors were randomized to drug treatment groups: 25 mg/kg AZD6244 (0.5% methylcellulose/0.4% polysorbate), 100 mg/kg GDC-0941 (0.5% methylcellulose/0.2% Tween-80), 100 mg/kg ABT-263 (30% PEG400/60% Phosal 50 PG/10% ethanol), or combinations. Drug treatments were administered by oral gavage. Kras and Kras p53L/L mouse strains harboring a conditional activating mutation (G12D) were developed in approximately 2 weeks. Tumors were measured with electronic calipers, and the tumor volume was calculated according to the formula $V = 0.52 \times L \times W^2$. Visible tumors were injected subcutaneously into the flanks of athymic nude mice (6–8 weeks). Visible tumors developed in approximately 2 weeks. Tumors were measured with electronic calipers, and the tumor volume was calculated according to the formula $V = 0.52 \times L \times W^2$. Visible tumors were injected subcutaneously into the flanks of athymic nude mice (6–8 weeks).

Cell proliferation analysis

Cell lines were seeded 24 hours before addition of drug. Cells were treated with drugs for 72 hours or as indicated and proliferation determined by CellTiter-Glo assay (Promega). For time course experiments, multiple identical plates were seeded and at indicated time points were frozen at −80°C. All plates were then developed at the end of the experiment simultaneously.

Annexin/propidium iodide staining by flow cytometry

Cells were seeded at low density 24 hours before drug addition. After 72 hours, floating and adherent cells were collected and stained with propidium iodide (PI) and Cy5-Annexin V and analyzed by flow cytometry. Percentage of apoptotic cells was calculated by subtracting percentage of Annexin-positive cells in vehicle control from percentage of Annexin-positive cells with drug treatment.

Short hairpin RNA screen

Bacterial plKO shRNA clones (4–10 per gene) were obtained from the Molecular Phenotyping Laboratory at MGH. Lentiviral stocks were produced according to the RNAi Consortium protocol from the Broad Institute (Cambridge, MA). Protein knockdown efficiency was determined by Western blotting using A549 cells, and hairpins with the best knockdown were selected for use (Supplementary Table S2). Cell lines were infected with lentivirus in an arrayed 96-well format at a multiplicity of infection of 2–3. After 24 hours, the virus was removed and cells were cultured for 48 hours in normal media. Cells were then treated with 1 μmol/L AZD6244/GDC-0941 for 48 hours, and cell viability and caspase-3/7 activity were determined by ApoTox-Glo assay (Promega).

Mouse treatment studies

All mouse studies were conducted through Institutional Animal Care and Use Committee–approved animal protocols in accordance with institutional guidelines. For xenograft studies, cell line suspensions were prepared in i:10 Matrigel, and 5 × 10^6 cells were injected subcutaneously into the flanks of athymic nude mice (6–8 weeks). Visible tumors developed in approximately 2 weeks. Tumors were measured with electronic calipers, and the tumor volume was calculated according to the formula $V = 0.52 \times L \times W^2$. Visible tumors were injected subcutaneously into the flanks of athymic nude mice (6–8 weeks). Visible tumors were injected subcutaneously into the flanks of athymic nude mice (6–8 weeks).

Data and statistical analysis

Data were analyzed using GraphPad Prism software (GraphPad Software). Western blot band quantitation was performed using SynGene Gene Tools Software. All correlation calculations were carried out using the Spearman correlation test. Comparisons between groups (e.g., experimental vs. control) were made using paired or unpaired t tests as appropriate. P < 0.05 was considered to be statistically significant and is indicated by asterisks unless otherwise noted.
Results

KRAS-mutant NSCLC cell lines have highly variable apoptotic response to MEKi/PI3Ki

Our earlier studies suggested that the responses to MEKi/PI3Ki in genetically engineered mouse KRAS-mutant NSCLC models can be variable, depending on the presence of concurrent secondary mutations (5, 24). For example, in these studies, lung adenocarcinomas driven by mutant KRAS in the absence of accompanying mutations had dramatic regressions (75% reduction in tumor burden) to combined treatment with the MEK inhibitor AZD6244 (selumetinib) and the dual PI3K/mTOR inhibitor NVP-BEZ235, whereas tumors with concurrent Lkb1 deletion had minimal responses (20% reduction in tumor volume). We also examined the response of Kras-mutant tumors with secondary Trp53 deletion (22) to treatment with AZD6244/BEZ235 and observed a transient response of intermediate magnitude (60% reduction in tumor volume) that was followed by rapid development of drug resistance after 4 weeks (Supplementary Fig. S1). Similar responses were observed with AZD6244 in combination with the pan-PI3K inhibitors GDC-0941 or BKM120. These data indicate that the presence of secondary mutations can diminish the response of KRAS-mutant NSCLCs to combined MEKi/PI3Ki therapy and suggest that significant heterogeneity of responsiveness may potentially exist among KRAS-mutant lung cancers in the clinic.

Human KRAS-mutant NSCLCs typically occur in the setting of tobacco smoke exposure and exhibit a high mutational burden (25). In contrast to the relative genetic simplicity of mouse models, cell lines derived from human tumors harbor multiple concurrent mutations or deletions in genes regulating growth and survival pathways (Supplementary Table S1). To further model the response of human KRAS-mutant NSCLCs to MEKi/PI3Ki, we assessed the effects of AZD6244 in combination with GDC-0941 on cell proliferation, cell-cycle progression, and apoptosis using a panel of 20 human KRAS-mutant NSCLC cell lines. MEKi/PI3Ki induced G1 arrest and reduced cell proliferation by 50% to 90% in all cell lines (Supplementary Fig. S2). Maximal inhibition of cell proliferation occurred with dual pathway inhibition (Supplementary Fig. S3). Treatment with AZD6244 alone increased AKT phosphorylation in several cell lines, underscoring the potential benefit of dual pathway inhibition to suppress activation of feedback signaling loops. Of note, the concentration of inhibitors chosen for use in subsequent studies was the lowest concentration that achieved >90% pathway inhibition.

Unexpectedly, we observed that the apoptotic response to MEKi/PI3Ki was highly variable despite the relatively uniform inhibition of cell-cycle progression (Fig. 1A; Supplementary Fig. S4A and S4B). Only a minority of cell lines exhibited a robust apoptotic response similar to that of the EGFR-mutant HCC827 NSCLC cell line treated with the EGFR inhibitor gefitinib (13, 26). Comparison of the effects of MEKi/PI3Ki on cell proliferation between low- and high-apoptosis cell lines revealed that cell lines with a minimal apoptotic response exhibited a net positive proliferative response in the presence of MEKi/PI3Ki, albeit reduced relative to vehicle-treated cells (Fig. 1B; Supplementary Fig. S3D). In contrast, cell lines with a high apoptotic response had a net cytotoxic response to MEKi/PI3Ki, similar to HCC827 cells treated with gefitinib. Inhibiting apoptosis with the pan-caspase inhibitor QVD-Oph converted the cytotoxic response of A427 cells to a net proliferative response (Fig. 1C; Supplementary Fig. S4C), demonstrating the important contribution of apoptosis in addition to growth arrest for the in vitro effectiveness of MEKi/PI3Ki. Across the entire cell line panel, a cytotoxic response to MEKi/PI3Ki coincided with a high degree of both cell-cycle arrest and apoptosis and occurred only in a minority of cell lines (Fig. 1D). Furthermore, TP53 or LKB1 mutational status did not correlate apoptotic responsiveness to MEKi/PI3Ki (Supplementary Fig. S5), although it is worth noting that the within each of these subgroups, cell lines harbored varied additional mutations (Supplementary Table S1).

To investigate the role of apoptosis in the response to MEKi/PI3Ki in vivo, we established xenograft tumors in mice from KRAS-mutant cell lines with high (A427) or low (SW1573) apoptotic response. Before implantation, these cells were modified to express Gaussia luciferase, which is secreted into the blood allowing for precise quantitation of tumor cell viability (ref. 27 and Supplementary Fig. S6). Combination treatment of A427 xenografts with AZD6244/GDC-0941 resulted in decreased blood luciferase signal, indicating decreased tumor cell viability as well as increased cleaved caspase-3 immunostaining (Fig. 1E and F). In contrast, combination treatment of SW1573 xenografts failed to activate caspase-3 and did not reduce blood luciferase signal despite comparable inhibition of both p-ERK and p-AKT (Supplementary Fig. S7). Taken together, these results suggest that failure to induce apoptosis may limit the efficacy of combined MEK and PI3K inhibition for KRAS-mutant NSCLCs in vitro and in vivo.

Variable apoptotic response is due to differential activation of mitochondrial apoptotic pathway

Tyrosine kinase signaling pathways modulate the mitochondrial pathway of apoptosis, which is regulated by the BCL-2 family proteins. MEKi/PI3Ki effectively suppressed the respective signaling pathways in cells with both high and low levels of apoptosis (Supplementary Figs. S3C and S8A) but only resulted in activation of BAX and cleavage of caspase-3 and PARP in cells undergoing apoptosis (Supplementary Fig. S8). To further assess whether induction of apoptosis results from variability in the downstream inhibition of MEK and PI3K pathways, we examined transcriptional outputs following MEKi/PI3Ki treatment. mRNA levels of the MEK/ERK pathway targets DUSP6, SPRY4, and EGR-1 were dramatically reduced in all cell lines, regardless of degree of apoptotic response (Supplementary Fig. S9). Similarly, mRNA levels of the FOXO3a transcriptional targets HER3 and TRAIL were induced in low- and high-apoptosis cell lines, consistent with comparable inhibition of PI3K/AKT. We also assessed whether differential activation of another RAS effector pathway, RalGDS, which has been shown to play a role in RAS-induced tumorigenesis (28, 29), might contribute to the differential apoptotic response. However, pull-down assays of GTP-bound RAL-A, a small GTPase activated by RalGDS, revealed no clear differences in RAL-A activation in response to MEKi/PI3Ki between high- and low-apoptosis cell lines (Supplementary Fig. S10). In fact, a modest increase in GTP-bound RAL-A was observed...
following MEKi/PI3Ki in all three low-apoptosis cell lines and two of three high-apoptosis cell lines, possibly due to suppression of negative feedback loops. In addition, there was no clear difference in baseline RAL-A activation between low- and high-apoptosis cell lines. This suggests that the differential apoptotic response induced by MEKi/PI3Ki is not simply explained by variable inhibition of RAS effector pathways but may result from differential ability of the MEK and PI3K pathways to modulate the BCL-2 family of apoptotic regulatory proteins.

The apoptotic response to MEKi/PI3Ki in KRAS-mutant NSCLC cell lines does not correlate with baseline mitochondrial apoptotic priming

Recently, the concept of mitochondrial priming has been proposed to explain the relative sensitivity of cancers to chemotherapeutic agents (30). In this paradigm, priming is a function of the proximity of a cell to the apoptotic threshold, determined by the collective expression of pro- versus anti-apoptotic BCL-2 family proteins. The extent of priming can be determined experimentally by BH3 profiling, which measures mitochondrial depolarization in response to exogenously added BH3 peptides that mimic the proapoptotic activity of BH3-only proteins, including BIM, BID, and PUMA (31, 32). To investigate whether the apoptotic response to MEKi/PI3Ki among KRAS-mutant NSCLC cell lines is a function of mitochondrial priming, we performed BH3 profiling on five high- and five low-apoptosis cells. However, no correlation between baseline mitochondrial priming and apoptotic response to MEKi/PI3Ki was observed (Supplementary Fig. S11). This suggests that sensitivity of KRAS-
mutant NSCLCs to MEK/PI3Ki may be more dependent on dynamic changes in specific BCL-2 family proteins than overall baseline proximity to the apoptotic threshold, in contrast to what has been found for the less selective conventional cytotoxic chemotherapies (30).

**PUMA, BIM, and BCL-XL mediate the apoptotic response of KRAS-mutant NSCLC cell lines to MEK/PI3Ki**

To determine the direct mediators of MEK/PI3Ki-induced apoptosis, we performed a targeted short hairpin RNA (shRNA) screen of the BCL-2 family and assessed the impact on MEK/PI3Ki-induced activation of caspase-3/7 in 5 high-apoptosis KRAS-mutant NSCLC cell lines (A427, DV-90, H2009, H1792, and A549). Each hairpin was verified to knockdown expression of the target gene at the protein level (Supplementary Table S2). In aggregate, knockdown of proapoptotic BH3-only proteins PUMA and BIM and the effector protein BAX led to reduced caspase-3/7 activation (Fig. 2A). To confirm these results, we established cell lines with stable knockdown of BIM, PUMA and BAX and assessed the apoptotic response to MEK/PI3Ki. Confirming the screen results, depletion of PUMA, BIM, or BAX in A427 and A549 cells reduced caspase-3/7 activation in response to MEK/PI3Ki compared with shGFP-infected control cells (Fig. 2B) and decreased the percentage of apoptotic cells (Fig. 2C). Similar protection from apoptosis was observed after transfection with unrelated siRNAs targeting BIM, PUMA, and BAX, reducing the likelihood that this effect is due to nonspecific or off-target effects of the hairpins (Supplementary Fig. S12). This indicates that BIM and PUMA play a role in mediating the apoptotic response to MEK/PI3Ki in KRAS-mutant NSCLC cells.

As shown above, MEK/PI3Ki failed to induce substantial apoptosis in many of the KRAS-mutant NSCLC cell lines. To investigate whether antiapoptotic BCL-2 family proteins play a role in mediating intrinsic resistance to MEK/PI3Ki, we performed shRNA-mediated knockdown of BCL-XL, MCL-1, BCL-2, BCL-W, and BFL-1/A1 in 10 low-apoptosis KRAS-mutant NSCLC cell lines. Knockdown of BCL-XL and MCL-1 alone resulted in increased basal apoptosis and potentiated the apoptotic response to MEK/PI3Ki (Fig. 2D). Notably,
knockdown of BCL-2 and other prosurvival BCL-2 family members failed to do so. Because gene knockdown of BCL-XL and MCL-1 led to substantially decreased viability and complicated the selection of cell lines with stable knockdown, we employed a pharmacologic strategy to confirm the hypothesis that antiapoptotic BCL-2 family proteins such as BCL-XL contribute to resistance to MEKi/P3Ki. In the majority of cell lines, the dual BCL-XL/BCL-2 inhibitor ABT-263 (navitoclax) stimulated a moderate degree of apoptosis, whereas a robust apoptotic response was observed after MEKi/P3Ki treatment in the presence of ABT-263 (Fig. 2E). Consistent with the shRNA results, this effect of ABT-263 was specific to inhibition of BCL-XL, as ABT-199, a selective BCL-2 inhibitor (33), showed no effect (Supplementary Fig. S13). Given the lack of a selective MCL-1 inhibitor, we were unable to similarly test the effect of MCL-1 inhibition on sensitivity to MEKi/P3Ki. Nevertheless these results demonstrate that prosurvival BCL-2 family proteins such as BCL-XL protect some KRAS-mutant NSCLC cells from MEKi/P3Ki-induced apoptosis.

We next examined the effect of MEKi/P3Ki on the expression of BCL-2 family proteins. Both PUMA and BIM protein accumulated after MEKi/P3Ki treatment in both low- and high-apoptosis cells, whereas BCL-XL and MCL-1 levels were largely unchanged in the majority of cell lines (Supplementary Fig. S14A). The absolute expression levels of any of these BCL-2 family members alone (BIM, PUMA, BCL-XL, or MCL-1) at baseline or after drug treatment did not correlate with apoptotic sensitivity. However, because apoptosis is determined by the relative balance of pro- and antiapoptotic BCL-2 family proteins, we also analyzed the collective expression levels of BIM and PUMA versus BCL-XL and MCL-1. Importantly, both at baseline and after drug treatment, the expression of BIM and PUMA protein levels relative to BCL-XL correlated highly with apoptotic response (Fig. 2F), whereas accounting for the expression of MCL-1 did not improve the correlation (Supplementary Fig. S14B).

We previously reported that baseline mRNA expression levels of BIM could predict response to TKI treatment in EGFR-mutant NSCLCs (14). Because the relative fold accumulation of BIM protein after TKI treatment is similar among high- and low-apoptosis cancers, pretreatment BIM mRNA levels effectively serve as a surrogate for on-treatment BIM levels. In an analogous manner, we observed that the difference in (BIM + PUMA)/BCL-XL ratios between high- and low-apoptosis cells was similar whether measured at baseline or after MEKi/P3Ki treatment (2.3- and 2.2-fold, respectively; Supplementary Fig. S14C). Therefore, we examined whether pretreatment mRNA levels of BIM and PUMA versus BCL-XL correlated with response to MEKi/P3Ki. Across the entire panel of KRAS-mutant NSCLC cell lines, the pretreatment ratio of BIM and PUMA relative to BCL-XL significantly correlated with apoptotic response to AZD6464/GDC-0941 (Supplementary Fig. S14D). Taken together, these results indicate that the apoptotic response of KRAS-mutant NSCLCs to MEKi/P3Ki is regulated by BIM, PUMA, and BCL-XL and suggest that differences in the relative expression of these proteins may underlie differential apoptotic sensitivity.

Restoration of apoptotic response sensitizes KRAS-mutant NSCLCs to MEKi/P3Ki

To establish whether decreased expression of proapoptotic relative to antiapoptotic BCL-2 proteins underlies insensitivity to MEKi/P3Ki, we tested whether altering the balance of BIM and BCL-XL reversed the resistance of KRAS-mutant NSCLC cell lines to MEKi/P3Ki. First, we inducibly expressed BIM in low-apoptosis H2030 and SW1573 cells. Ectopic expression of BIM restored the apoptotic response to MEKi/P3Ki (Fig. 3A; Supplementary Fig. S15) and resulted in a net cytotoxic response (Fig. 3B). Second, we assessed the effects of BCL-XL inhibition on cell proliferation. Treatment of cells with ABT-263, which restores the apoptotic response to MEKi/P3Ki (Fig. 2E), likewise led to a cytotoxic response in the majority of insensitive cell lines (Fig. 3C). In total, restoration of apoptosis either by increased expression of BIM or inhibition of BCL-XL correlated highly with conversion from a cytostatic to cytotoxic response to MEKi/P3Ki across the panel of insensitive cell lines (Fig. 3D). Finally, we examined whether BCL-XL inhibition would translate to improved efficacy of MEKi/P3Ki against insensitive KRAS-mutant NSCLCs in vivo. Consistent with the in vitro results, addition of ABT-263 to MEKi/P3Ki led to regression of SW1573 xenograft tumors (Fig. 3E; Supplementary Fig. S16). Altogether, these results demonstrate that the inability for MEKi/P3Ki to modulate BCL-2 family proteins sufficient to induce an apoptotic response contributes to intrinsic resistance of KRAS-mutant NSCLCs to MEKi/P3Ki in vitro and in vivo.

Rapid adaptation of KRAS-mutant NSCLCs to MEKi/P3Ki involves loss of apoptotic response

The clinical efficacy of targeted therapies is limited by the emergence of drug resistance (34). When studying the response of high-apoptosis KRAS-mutant NSCLC cell lines to MEKi/P3Ki, we observed that cells that survived the initial exposure to drug were less sensitive on repeated drug exposure (Supplementary Fig. S17A). This suggests that even when cells are initially sensitive to MEKi/P3Ki, efficacy might be limited by rapid adaptation to drug. To investigate whether suppression of apoptosis might underlie this adaptive response, we cultured A427 and DV-90 cells in the presence of AZD6244/GDC-0941 for 3 days followed by drug washout for 3 days (Supplementary Fig. S17B). Although initial drug treatment caused a robust apoptotic response, after 5 to 10 intermittent drug treatments, the resulting resistant A427-R and DV-90-R cells no longer exhibited a cytotoxic response and were able to survive in the continuous presence of MEKi/P3Ki (Fig. 4A). MEKi/P3Ki suppressed pERK, pAKT, and pS6 as well as cell-cycle progression in both resistant and parental cells (Fig. 4B and C; Supplementary Fig. S17C), indicating that resistance was not due to reactivation of these signaling pathways. In contrast, the resistant cells had a greatly diminished apoptotic response, after 7 days of continuous drug exposure (Fig. 4C). In these cells, the apoptosis induced by MEKi/P3Ki was blocked by the addition of BIM (Fig. 4D), suggesting that resistance to MEKi/P3Ki involves loss of the apoptotic response.

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To investigate whether suppression of apoptosis might contribute to rapid adaptation to MEKi/PI3Ki in vivo, we used the Kras p53L/L lung adenocarcinoma model. These tumors initially respond to MEKi/PI3Ki but quickly develop resistance and progress after 4 weeks (Supplementary Fig. S1). We generated cell lines from Kras p53L/L tumors (R1, R2, R3) after progression on treatment with AZD6244/BEZ235, as well as from treatment-naïve Kras p53L/L tumors (N1, N2; Fig. 5A; Supplementary Fig. S18A and S18B). Cell lines from resistant tumors maintained resistance to MEKi/PI3Ki in vitro, with loss of the cytotoxic response exhibited by cell lines derived from treatment-naïve tumors (Fig. 5B). Importantly, resistant cell lines exhibited a significantly blunted apoptotic response to treatment with AZD6244/GDC-0941 (Fig. 5C; Supplementary Fig. S19). Notably, the pretreatment ratio of BIM relative to BCL-XL correlated with apoptotic response and was diminished in cells from resistant tumors (Fig. 5E). Finally, we examined whether pharmacologic inhibition of BCL-XL with ABT-263 could resensitize resistant tumor cell lines to MEKi/PI3Ki. Treatment of resistant Kras p53L/L cells with AZD6244/GDC0941 in the presence of 100 nmol/L of ABT-263 led to equivalent apoptosis as treatment-naïve cells with MEKi/PI3Ki alone (Fig. 5G). Altogether, these data indicate that a diminished apoptotic response associated with loss of expression of proapoptotic BCL-2 family proteins may result in rapid adaptation of KRAS-mutant NSCLCs to MEKi/PI3Ki in vitro and in vivo.

Discussion

There is increasing evidence that differences in the apoptotic response among cancers with the same primary oncogenic driver may result in variable clinical responses to targeted therapies (14, 15). In this study, we find that differences in the apoptotic response contribute to variable in vitro and in vivo...
Responsiveness of KRAS-mutant NSCLC experimental models to combined MEK and PI3K inhibition, a therapeutic combination currently being investigated in the clinic. Although emerging data from clinical studies indicate significant activity in KRAS-mutant ovarian cancers and some lung cancers, the activity in KRAS-mutant NSCLC in general has been underwhelming (9, 12, 35). While clinical efficacy may be limited by issues related to dosing and toxicity, our data may explain why the effectiveness of MEKi/PI3Ki may be limited, even when full suppression of the signaling pathways can be achieved. Because a large subset of KRAS-mutant lung cancers may be relatively insensitive MEKi/PI3Ki even with optimal inhibition, the findings in this study may become even more salient as innovative therapeutic dosing strategies are developed.

The extent to which secondary mutations affect response to targeted therapies is incompletely understood. Initial studies demonstrating impressive activity of MEKi/PI3Ki used genetically engineered mouse models of KRAS-mutant NSCLCs with no additional mutations (5). Subsequent studies using tumors with concomitant deletion of Trp53 or Lkb1 have shown less impressive responses of these mouse models to targeted therapies, including MEKi/PI3Ki (23, 24). However, there remain limited data on the impact of secondary mutations on clinical response to targeted therapies in patients. This may be especially relevant to KRAS-mutant lung cancers, which are associated with a history of cigarette smoking and exhibit increased mutational burden (25). In this study, we modeled the heterogeneity of human NSCLCs by employing a large panel of KRAS-mutant NSCLC cell lines that harbor numerous and diverse secondary mutations in key growth and survival pathways (Supplementary Table S1). Using these models, MEKi/PI3Ki demonstrated much less impressive activity than the original mouse studies with Kras mutations alone, more akin to what is being observed in the clinic. Despite induction of cell-cycle arrest across the panel of cell lines, MEKi/PI3Ki was largely ineffective at provoking sufficient apoptosis to cause a cytotoxic response in the majority of cell lines. In contrast to the genetically engineered mouse models, we did not observe statistically significant correlations between apoptotic response and TP53 or LKB1 mutational status. The reasons for this are unclear; however, it is possible that additional mutations present in the cell lines modify the responses to MEKi/PI3Ki. It is worth noting that all but one (A427) LKB1-mutant cell line exhibited low levels of apoptosis, consistent with the lack of response to MEKi/PI3Ki observed in the Kras Lkb1 mice (24). However, these studies point to the potential

Figure 4. Loss of apoptotic response underlies adaptive resistance to MEKi/PI3Ki in vitro. A, A427 and A427-R cells were treated with 1 μmol/L AZD6244/GDC-0941 and cell proliferation measured. B, A427 and A427-R cells were treated with 1 μmol/L AZD6244/GDC-0941 for 24 hours. Bottom, quantitation of protein expression from Western blots. Data are mean and error of three independent experiments. C, cells were treated with 1 μmol/L AZD6244/GDC-0941 for 24 hours, stained with PI, and cell-cycle populations analyzed by flow cytometry. D, cells were treated with 1 μmol/L AZD6244/GDC-0941 and apoptosis determined by Annexin staining. Data, mean and SE of five to seven independent experiments.
limitation of genetically engineered mouse models for predicting response to novel therapeutic combinations.

While our study adds to the growing evidence for the importance of the apoptotic response for effective targeted therapies, it also suggests that loss of the apoptotic response secondary to downregulation of proapoptotic mediators may contribute to adaptive or acquired resistance to targeted therapies. This may be especially relevant to therapies that inhibit downstream effector signaling pathways such as MEK and PI3K rather than an RTK (e.g., mutant EGFR) because resistance to RTK inhibitors can be achieved by mutations in the receptor (e.g., EGFR T790M) or activation of alternate pathways (i.e., bypass tracks) that lead to reactivation of downstream signaling nodes such as MEK and PI3K (26, 36, 37). In contrast, KRAS-mutant NSCLC cell lines with acquired resistance to MEKi/PI3Ki still exhibited suppression of MEK/ERK and PI3K/AKT signaling and cell-cycle arrest upon drug treatment but this failed to translate into an apoptotic response. Thus, strategies to overcome either intrinsic or adaptive resistance may need to focus specifically on restoration of the apoptotic response such as use of epigenetic regulators to increase transcription of proapoptotic proteins or direct targeting of antiapoptotic BCL-2 family members (15, 38). As proof of concept, we demonstrate that restoration of BIM expression or use of ABT-263 can resensitize resistant KRAS-mutant human and mouse cancer cells, respectively, to MEKi/PI3Ki.

In summary, we feel that this study has important implications for the use of combined MEK and PI3K inhibitors and other targeted therapies for the treatment of KRAS-mutant NSCLCs. While MEKi/PI3Ki may be effective for some KRAS-mutant cancers, it is unlikely to be universally effective given the heterogeneity observed among human KRAS-mutant lung cancers. Future investigations of novel therapies for KRAS-mutant NSCLC should account for this heterogeneity to maximize potential for clinical success. In addition, the development of biomarkers beyond the primary oncogenic driver will likely be crucial for predicting which patients will respond to specific therapies. For example, apoptotic biomarkers combined with standard

Figure 5. Loss of apoptotic response underlies acquired resistance to MEKi/PI3Ki in vivo. A, cell lines established from resistant Kras p53f/f tumors after progression during MEKi/PI3Ki treatment. MRI images of R1 tumor nodule (arrow) at baseline, after treatment with AZD6244/BEZ235 (response, −72% change from baseline), and on-treatment progression (resistant, +200% change from nadir). B, cell lines derived from treatment-naive (N1) and resistant (R1) tumor nodules were treated with 1 μmol/L AZD6244/GDC-0941 or 1 μmol/L AZD6244/BEZ235. C, cell lines from resistant Kras p53f/f tumors (R1, R2, R3) and treatment-naive tumors (N1, N2) were treated with 1 μmol/L AZD6244/GDC-0941 and apoptosis determined by Annexin staining. Data, mean and SE of three to four independent experiments. D, BIM mRNA expression levels were determined by quantitative reverse transcription PCR. Data, mean and error of three independent experiments. E, correlation of BIM/BCL-XL ratios with apoptotic response for resistant (gray) and naïve (black) tumor-derived cell lines. F, cell lines were treated with AZD6244/GDC-0941 with or without ABT-263 and apoptosis determined. Data, mean and error of triplicate samples.
genotyping may be useful in predicting response to targeted therapies (14). Finally, given the importance of the apoptotic response, combination therapies that directly target BCL-2 family proteins may be a useful component of therapeutic strategies for KRAS-mutant cancers (39).

Disclosure of Potential Conflicts of Interest
A. Letai is a consultant/advisory board member of AbbVie. J.A. Engelman has received a commercial research grant from Novartis, AstraZeneca and is a consultant/advisory board member of Novartis, GSK, Genentech, and AstraZeneca. No potential conflicts of interest were disclosed by the other authors.

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References
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