ABSTRACT
BH3 mimic drugs, which inhibit prosurvival BCL2 family proteins, have limited single-agent activity in solid tumor models. The potential of BH3 mimetics for these cancers may depend on their ability to potentiate the apoptotic response to chemotherapy and targeted therapies. Using a novel class of potent and selective MCL1 inhibitors, we demonstrate that concurrent MEK + MCL1 inhibition induces apoptosis and tumor regression in KRAS-mutant non–small cell lung cancer (NSCLC) models, which respond poorly to MEK inhibition alone. Susceptibility to BH3 mimetics that target either MCL1 or BCL-xL was determined by the differential binding of proapoptotic BCL2 proteins to MCL1 or BCL-xL, respectively. The efficacy of dual MEK + MCL1 blockade was augmented by prior transient exposure to BCL-xL inhibitors, which promotes the binding of proapoptotic BCL2 proteins to MCL1. This suggests a novel strategy for integrating BH3 mimetics that target different BCL2 family proteins for KRAS-mutant NSCLC.

SIGNIFICANCE: Defining the molecular basis for MCL1 versus BCL-xL dependency will be essential for effective prioritization of BH3 mimic combination therapies in the clinic. We discover a novel strategy for integrating BCL-xL and MCL1 inhibitors to drive and subsequently exploit apoptotic dependencies of KRAS-mutant NSCLCs treated with MEK inhibitors. Cancer Discov; 8(12); 1598–613. © 2018 AACR.

See related commentary by Leber et al., p. 1511.
INTRODUCTION

KRAS, a small GTPase that activates MAPK signaling, is one of the most frequently mutated driver oncogenes (1). KRAS mutations, which are largely localized to residues G12, G13, and Q61, decrease either intrinsic and/or GAP-mediated hydrolysis, causing constitutive activation of MAPK and other downstream signaling pathways (2). Although effective molecular targeted therapies that inhibit oncogenic mutant kinases in the RAS–MAPK pathway have been developed (e.g., EGFR inhibitors for EGFR-mutant NSCLC, BRAF inhibitors for BRAF-mutant melanoma), there currently are no approved targeted therapies for KRAS-mutant cancers. KRAS mutations are found in 20% to 25% of patients with non–small cell lung cancer (NSCLC) and predict lack of response to EGFR inhibitors (3). Attempts to target downstream MAPK signaling with inhibitors of MEK1/2 have yielded disappointing results (4, 5), and strategies that simultaneously target multiple signaling pathways have been limited by toxicity (6, 7). Most recently, a novel class of KRAS inhibitors that covalently bind to the G12C mutant has been described (8, 9), although these have yet to be tested in the clinic. Thus, there remains an urgent need for new therapeutic strategies that can target KRAS-mutant cancers.

Several studies have shown that suppression of MAPK signaling, either by depletion of mutant KRAS or by pharmacologic inhibition of downstream MEK1/2, is insufficient to induce apoptosis in a significant number of KRAS-mutant cell lines (10–12). Therapeutic strategies that cotarget kinase signaling pathways and apoptotic regulators may increase apoptosis and convert cytostatic responses into tumor regressions (13). Activated kinase signaling pathways such as MAPK (RAS/RAF/MEK/ERK) and PI3K/AKT converge on the BCL2 protein family, which regulates the mitochondrial or intrinsic apoptotic response (14). In cells with MAPK activation, ERK phosphorylation suppresses the proapoptotic BH3 protein BIM by targeting it for degradation (15, 16). MEK inhibition causes BIM to accumulate (16); however, BIM can be neutralized by prosurvival BCL2 family members such as BCL-xL or MCL1. Combining MEK inhibitors with the BH3 mimetic navitoclax (ABT-263), which prevents the binding of BIM to BCL2 and BCL-xL, led to greater apoptosis and tumor...
regression in KRAS experimental models compared with MEK inhibitors alone (11), and a clinical trial evaluating this combination is currently ongoing (NCT02079740, www.clinicaltrials.gov). To date, these approaches have been limited to targeting BCL2 and BCL-xL due to the lack of selective and potent inhibitors that target other members of the BCL2 family.

MCL1 is frequently amplified in lung cancers (17), and the development of potent and selective MCL1 inhibitors has long been of interest. Recently, a novel MCL1 inhibitor, S63845, with in vitro activity was reported (18). Significant activity was observed in leukemia, myeloma, and lymphoma models, and several different MCL1 inhibitors are currently in clinical development for these malignancies (NCT02992483, NCT02979366, and NCT02675452; www.clinicaltrials.gov). Single-agent activity of S63845 was limited in solid tumor models including NSCLC and breast cancers; however, combining S63845 with relevant kinase inhibitors led to decreased cell viability of BRAF-, EGFR-, and HER2-addicted cell lines in vitro, providing proof of principle that MCL1 inhibition, similar to BCL-xL inhibition, may potentiate the response to kinase inhibitor–targeted therapies. However, due to the lack of studies that directly compare analogous combination strategies that target either MCL1 or BCL-xL, the optimal pairing of kinase inhibitors with BH3 mimetics that target different BCL2 family proteins in specific subsets of cancer remains undefined.

Here, we assessed the activity of a novel class of potent and selective spiromacrocyclic MCL1 inhibitors in combination with MEK inhibition in KRAS-mutant NSCLC models and compared this to the parallel strategy of MEK + BCL-xL inhibition. Distinct but overlapping subsets of KRAS-mutant NSCLC models were more sensitive to MEK + MCL1 versus MEK + BCL-xL inhibition, which was determined by the binding interactions between specific BCL2 family proteins. By altering the cellular localization and interactions between BCL2 family proteins with transient exposure to BCL-xL inhibitors, KRAS-mutant NSCLC cells could be induced into an MCL1-dependent state with increased sensitivity to MEK + MCL1 inhibition. These results provide a rationale for the clinical evaluation of MEK + MCL1 inhibitors in KRAS-mutant NSCLC and suggest a broader strategy for integrating MCL1 and BCL-xL inhibitors to maximize efficacy of kinase inhibitor–targeted therapies.

RESULTS

Combination Drug Screen Identifies Synergistic Activity between AM-8621 and MAPK Pathway Inhibitors in Cell Lines with MAPK PathwayActivation

Recently developed potent and selective MCL1 inhibitors have demonstrated limited single-agent activity against solid tumor malignancies (18, 19), suggesting that drug combinations may be required to maximize the potential clinical benefit of this class of inhibitors for these cancers. In order to determine agents that synergistically enhance the activity of MCL1 inhibition, 187 compounds targeting a diverse array of mechanisms (Supplementary Table S1) were screened in pairwise combinations with a novel potent and selective MCL1 inhibitor, AM-8621, against a panel of 15 diverse solid tumor cancer cell lines that had a range of sensitivities to AM-8621 (Supplementary Fig. S1A–S1D). AM-8621 is the prototypical member of a novel class of spiromacrocyclic MCL1 inhibitors, which also includes the tool compound AM-4907 and the clinical compound AMG 176, which exhibit potent and selective MCL1 inhibition in vitro and in vivo (19). Synergistic drug combinations were determined using a Loewe excess additivity model (20). A number of potential clinically relevant synergistic combinations were identified, including conventional cytotoxic chemotherapeutic agents, HSP90 inhibitors, and BH3 mimetics targeting BCL-xL/BCL2 (Fig. 1A; Supplementary Fig. S1E).

To prioritize clinically promising MCL1 inhibitor combinations, we considered the spectrum of oncogenic driver mutations present in the cell lines. One of the most notable genotype-associated synergies was between AM-8621 and drugs that inhibit the MAPK pathway (MEK and BRAF inhibitors) in cell lines with oncogenic MAPK pathway activation (Fig. 1B). Six of 15 cell lines showed statistically significant synergy with a majority of the MAPK pathway inhibitors (MEK and BRAF) included in the screen, and eight cell lines showed statistically significant synergy with a majority of MEK inhibitors. Of these eight cell lines, five harbored mutations known to cause MAPK pathway activation (A427, H23: KRAS; H1395, G-361: BRAF; H1437: MEK1). Additionally, two of the three other cell lines that did not harbor MAPK-activating mutations have previously been demonstrated to have functional MAPK pathway activation (HCC70: triple-negative breast cancer with activated RAS gene signature, ref. 21; G402: malignant rhabdoid tumor with hyperactivation of FGFR1, ref. 22). We next confirmed synergy between AM-8621 and PD032591 (MEK inhibitor) and vemurafenib (BRAF inhibitor), as well as the clinically relevant MEK inhibitor trametinib and BRAF inhibitor dabrafenib, in KRAS- and BRAF-mutant cells by testing the heterologous (A × B) or corresponding self-crosses (A × A, B × B) for all possible dose combinations in a 10 × 10 complete combination matrix. Strong synergy was again observed between AM-8621 and the MEK inhibitors PD0325901 and trametinib in A427 and H23 cells (KRAS-mutant NSCLC) and G-361 cells (BRAF-mutant melanoma), synergy was not observed in the KRAS wild-type H661 (NSCLC) or DMS-273 (small-cell lung cancer) cell lines (Fig. 1C). Synergy was also observed between AM-8621 and the BRAF inhibitors dabrafenib and vemurafenib in G-361 cells (Fig. 1C). No synergy was observed with the corresponding self-crosses. These results suggest that combining MEK + MCL1 inhibitors may be a promising combination strategy specifically for cancers with MAPK pathway activation.

KRAS-Mutant NSCLCs Are Sensitive to Combination MEK + MCL1 or BCL-xL Inhibitors

Recent studies have demonstrated that combining MEK inhibitors with BH3 mimetics targeting BCL-xL/BCL2 (e.g., navitoclax or ABT-263) synergistically induces apoptosis in KRAS-mutant cancer models (11, 12). Based on the results of the combination drug screen, we hypothesized that a parallel strategy targeting both MEK and MCL1 might be effective against KRAS-mutant cancers, particularly those that do not respond to MEK + BCL-xL inhibition. To assess the relative dependency of KRAS-mutant cell lines on MCL1 and BCL-xL, we first tested whether AM-8621, navitoclax, or venetoclax
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Notably, significant activity of the AM-8621 + navitoclax combination was observed in cell lines that were completely insensitive to AM-8621 alone (e.g., A549 and LU-99A). This heterogeneity was reflected in the degree of caspase-3/7 activation and apoptosis induced by MEK inhibitor (MEKi) + BH3 mimetic combinations (Supplementary Fig. S2F; Fig. 2C) and overall decrease in cell viability (Fig. 2D). The apoptotic effect of AM-8621 in combination with trametinib was recapitulated by siRNA-mediated MCL1 knockdown (Supplementary Fig. S2H), validating an on-target mechanism of MCL1 inhibition by AM-8621. We also confirmed that the effects of navitoclax were due primarily to inhibition of BCL-xL, as similar results were obtained using the BCL-xL–selective inhibitor A1331852 (23) but not the BCL2-selective inhibitor venetoclax (Supplementary Fig. S2H). These data demonstrate that inhibition of MCL1 or BCL-xL potentiates the effects of MEK inhibition in distinct but overlapping subsets of KRAS-mutant NSCLC.

Sensitivity to MCL1 and BCL-xL Inhibition Is Determined by Specific BCL2 Family Protein Interactions

Given the potential heterogeneity of response of KRAS-mutant NSCLCs to MEK inhibitor + BH3 mimetic combinations, a more complete understanding of the underlying (ABT-199, a selective BCL2 inhibitor) alone could decrease cell viability. Whereas most KRAS-mutant colorectal cancer cell lines were more sensitive to navitoclax, subsets of KRAS-mutant NSCLC cell lines were partially sensitive to either AM-8621 or navitoclax, both, or neither (Supplementary Fig. S2A–S2C). Very little activity of venetoclax was observed in either NSCLC or colorectal cancer cell lines.

We next sought to determine the degree of heterogeneity in the response of KRAS-mutant NSCLC cells to combined MEK + MCL1 inhibition. Strong synergy was observed with the combination of AM-8621 and trametinib in KRAS-mutant NSCLC cell lines with partial sensitivity to AM-8621 alone (e.g., H23 and H2030), and the combination of navitoclax and trametinib was synergistic in cell lines with partial sensitivity to navitoclax alone (e.g., H1734 and H1944; Fig. 2A and Supplementary Fig. S2D). Across the entire KRAS-mutant NSCLC and colorectal cancer cell line panels, navitoclax strongly enhanced the inhibitory effect of trametinib in almost all colorectal cell lines (Supplementary Fig. S2E), whereas the response of KRAS-mutant NSCLC cell lines was heterogeneous, with subsets of cell lines exhibiting enhancement with AM-8621, navitoclax, both, or neither (Fig. 2B).

Notably, significant activity of the AM-8621 + trametinib combination was observed in cell lines that were completely insensitive to AM-8621 alone (e.g., A549 and LU-99A). This heterogeneity was reflected in the degree of caspase-3/7 activation and apoptosis induced by MEK inhibitor (MEKi) + BH3 mimetic combinations (Supplementary Fig. S2F; Fig. 2C) and overall decrease in cell viability (Fig. 2D). The apoptotic effect of AM-8621 in combination with trametinib was recapitulated by siRNA-mediated MCL1 knockdown (Supplementary Fig. S2H), validating an on-target mechanism of MCL1 inhibition by AM-8621. We also confirmed that the effects of navitoclax were due primarily to inhibition of BCL-xL, as similar results were obtained using the BCL-xL–selective inhibitor A1331852 (23) but not the BCL2-selective inhibitor venetoclax (Supplementary Fig. S2H). These data demonstrate that inhibition of MCL1 or BCL-xL potentiates the effects of MEK inhibition in distinct but overlapping subsets of KRAS-mutant NSCLC.

Sensitivity to MCL1 and BCL-xL Inhibition Is Determined by Specific BCL2 Family Protein Interactions

Given the potential heterogeneity of response of KRAS-mutant NSCLCs to MEK inhibitor + BH3 mimetic combinations, a more complete understanding of the underlying...
KRAS-mutant NSCLC cell lines exhibit a heterogeneous pattern of synergy between MEK inhibitors and BH3 mimetics. A, Cell lines were treated with trametinib (TRAM) in combination with AM-8621 or navitoclax (nav) in a 8 × 6 dose matrix, and synergy was calculated according to the Loewe excess additivity model. B, Cell lines were treated with increasing concentrations of trametinib alone or in the presence of 1 μmol/L AM-8621 or navitoclax for 72 hours, and viability was determined. The enhancement of growth inhibition by the addition of AM-8621 or navitoclax to trametinib was determined by normalizing the viability after combination treatment to trametinib alone for each dose point (0% = no effect; 100% = complete cell killing). The bottom plot compares the relative enhancement achieved by the addition of AM-8621 (red) or navitoclax (blue) to trametinib for the entire KRAS-NSCLC cell line panel. C, Combining trametinib (100 nmol/L) with AM-8621 (1 μmol/L) or navitoclax (1 μmol/L) leads to increased apoptosis as determined by annexin V staining. D, Effect of combining trametinib (100 nmol/L) with AM-8621 (1 μmol/L) or navitoclax (1 μmol/L) on cell viability. Cells were treated for 72 hours and stained with crystal violet.

apoptotic dependencies that determine drug sensitivity will be critical for selecting the most effective combination. Profiling the response to AM-8621 across a large cancer cell line panel demonstrated an inverse correlation between AM-8621 sensitivity and BCL-xL expression (19), in agreement with a recent study that assessed the sensitivity of 563845 in a more limited set of hematologic cancer cell lines (18). Comparison of MCL1 and BCL-xL expression levels in NSCLC and colorectal cancer cell lines in the Cancer Cell Line Encyclopedia revealed that as a group, colorectal cancer cell lines exhibited higher BCL-xL and lower MCL1 expression than NSCLC cell lines (Supplementary Fig. S3A), consistent with our observation that KRAS-mutant colorectal cancer cell lines were more sensitive to BCL-xL inhibition (Supplementary Fig. S2). However, when examining individual cell lines within our panel of KRAS-mutant NSCLC cell lines, we did not observe an obvious correlation between combination drug sensitivity and expression of MCL1 or BCL-xL mRNA, or ratios of BIM to MCL1 or BCL-xL (Supplementary Fig. S3B). We also did not observe subsets of KRAS-mutant NSCLC cell lines with inverse coexpression of BCL-xL and MCL1 (e.g., MCL1\textsuperscript{hi}/BCL-xL\textsuperscript{lo} or MCL1\textsuperscript{lo}/BCL-xL\textsuperscript{hi}) that might obviously predict differential drug sensitivity. In general, NSCLC cell lines with greater MCL1 tended to also have greater BCL-xL expression. A similar pattern was observed in KRAS-mutant NSCLC tumors (Supplementary Fig. S3C and S3D). Therefore, baseline expression levels of BCL-xL or MCL1 may not be sufficiently robust to predict sensitivity to MEK + MCL1 or BCL-xL inhibition for individual cell lines or tumors.

To determine the molecular basis for the differential sensitivity to MEK + MCL1 versus BCL-xL inhibition, we examined the impact of drug treatment on apoptotic signaling. As expected, inhibition of phospho-ERK by trametinib led to accumulation of the BIM protein (Supplementary Fig. S3E). Treatment with AM-8621 or navitoclax interrupted BIM:MCL1 and BIM:BCL-xL interactions, respectively (Supplementary Fig. S3F), with a commensurate increase in BIM binding to the uninhibited prosurvival partner, consistent with the ability of prosurvival BCL2 family proteins to buffer BIM liberated by BH3 mimetics. To compare the effects of BIM induction on apoptotic priming in H2030 and H1734, we performed BH3 profiling (24) before and after treatment with trametinib. Trametinib treatment increased overall
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Figure 3. Mitochondrial BCL2 family interactions determine sensitivity profile to BH3 mimetics. A, The change in apoptotic priming after treatment with 100 nmol/L trametinib for 16 hours was determined by BH3 profiling, which assesses mitochondria depolarization (MOMP) after treatment with BH3 peptides. Total apoptotic priming is determined by BID peptide, whereas MS1 and HRK peptides are specific for MCL1 and BCL-xL dependency, respectively. Data are the mean and standard error of four independent experiments. B, Direct binding interactions between BCL2 family proteins. MCL1 and BCL-xL were immunoprecipitated, and interacting proteins were determined by western blotting (Ig, immunoglobulin control; In, total cell lysate input; IP, immunoprecipitated fraction; S, supernatant). Right plot summarizes binding of proapoptotic BCL2 family proteins to MCL1 versus BCL-xL. C, Subcellular localization of BCL2 family proteins after 24-hour drug treatment. D, Effect of drug treatment on binding interactions between BCL2 family proteins. Cells were treated with 100 nmol/L trametinib (tram), 1 μmol/L AM-8621 (AM), and/or 1 μmol/L navitoclax (nav) as indicated for 24 hours (M, MCL1 immunoprecipitation; B, BCL-xL immunoprecipitation; In, Input; sm, MCL1 supernatant; sb, BCL-xL supernatant).

apoptotic priming (BID peptide) in both cell lines, indicating that proapoptotic signaling was induced by the treatment (Fig. 3A). Notably, in H2030 cells, this corresponded to increased MCL1 dependence, whereas H1734 cells became more BCL-xL dependent, in agreement with the combination treatment data (Fig. 2).

We next examined whether the differences in apoptotic dependency result from differential binding of BIM and other proapoptotic BCL2 proteins to MCL1 or BCL-xL. At baseline, MCL1 was localized primarily to the mitochondria in most cell lines, whereas BCL-xL was variably distributed between the mitochondria and cytosol (Supplementary Fig. S3G). BIM was exclusively localized to the mitochondrial fraction, indicating that the critical BIM:MCL1 and BIM:BCL-xL interactions occur at the mitochondria. To directly compare the amount of BIM bound to MCL1 relative to BCL-xL, we performed immunoprecipitation of MCL1 and BCL-xL and assessed for binding of proapoptotic BCL2 family members.
In A427, H23, and H2030 cells, which are MCL1 dependent, BIM was preferentially bound to MCL1 at baseline (Fig. 3B). In contrast, BIM was bound to BCL-xL as well as MCL1 in BCL-xL–dependent H1944 and H1734 cells. Additionally, we observed that BAK was bound to MCL1 in H23 and H2030 cells, whereas BAX was bound to BCL-xL in H1944 and H1734 cells. Treatment of H2030 cells with trametinib led to additional accumulation of BIM at the mitochondria (Fig. 3C) and increased binding of BIM to MCL1, explaining the increase in MCL1 priming revealed by BH3 profiling (Fig. 3D). Treatment with combined trametinib + AM-8621 prevented the accumulation of BIM on MCL1 and led to a decrease in mitochondrial BIM, suggesting that other prosurvival BCL2 family proteins such as BCL-xL are insufficient to neutralize and stabilize BIM once it is liberated from MCL1. In H1734 cells, trametinib treatment led to BIM accumulation on BCL-xL as well as MCL1; cotreatment with ABT263 + trametinib displaced both BIM and BAX from BCL-xL (Fig. 3D). Thus, the sensitivity of H2030 and H1734 cells to combined MEK + BH3 mimetics appears to be determined by a primary dependency on MCL1 or BCL-xL, respectively, to neutralize multiple proapoptotic BCL2 family members. Together, these results point to the complexity and diversity of interactions between BCL2 family proteins that determine sensitivity to MEKi + BH3 mimic combinations.

Combination MEK + MCL1 Inhibition Leads to KRAS-Mutant NSCLC Tumor Regression

To evaluate the in vivo activity of MEK + MCL1 inhibition, we generated H2030 and A549 subcutaneous xenograft tumors in immunocompromised mice. Consistent with prior studies of KRAS-mutant xenograft tumors (25, 26), treatment with trametinib suppressed phospho-ERK signaling and led to modest tumor growth inhibition in both models (Fig. 4A and B; Supplementary Fig. S4A and S4B). To investigate the effects of MCL1 inhibition in vivo, we used AM-4907, a structural analogue of AM-8621 with improved oral bioavailability (Supplementary Fig. S1A; ref. 19). AM-4907 modestly slowed the growth of H2030 xenograft tumors, but had little activity against A549 xenograft tumors, largely mirroring the response of the respective cell lines to AM-8621 (Supplementary Fig. S2B). Combination treatment with trametinib and AM-4907 led to activation of cleaved caspase-3 (Fig. 4B) and caused regression of both A549 and H2030 xenograft tumors (Fig. 4A). Similar results were also observed in A427 xenograft tumors (Supplementary Fig. S4C). The differential sensitivity of H2030 xenograft tumors to BH3 mimic combinations was consistent with the in vitro drug sensitivity, with the combination of trametinib + navitoclax showing little activity beyond that of trametinib alone (Supplementary Fig. S4D).

To evaluate the efficacy of MEK + MCL1 inhibition in addition to clinically relevant in vivo models, we generated primary patient-derived xenograft (PDX) mouse tumor models from KRAS-mutant adenocarcinomas (Supplementary Table S2) that represented a variety of KRAS point mutations as well as co-occurring mutations. Mice bearing PDX tumors were treated with trametinib or the combination of trametinib + AM-4907 or trametinib + AMG 176 (the clinical analogue) for 2 weeks and evaluated for tumor regression. Whereas tumor regression was observed with single-agent trametinib in only 1 of 8 models (MGH6024, <30%), combination treatment led to average tumor regression in 6 of 8 models, with four achieving greater than 30% average tumor volume reduction (Fig. 4C; Supplementary Fig. S4E). Additionally, for 6 of 7 evaluable models, the combination of trametinib + AM-4907 had statistically greater antitumor activity than trametinib alone. In contrast, the combination of trametinib + navitoclax showed minimal activity beyond trametinib alone in 4 of 4 models tested (Supplementary Fig. S4E and S4F). Consistent with these results, coimmunoprecipitation experiments confirmed that BIM was preferentially bound to MCL1 in MGH1070 and MGH1089-1 tumors (Supplementary Fig. S4G). Together, these results demonstrate in vivo efficacy of combined inhibition of MEK and MCL1 and suggest that this may be a promising therapeutic approach for treatment of KRAS-mutant NSCLC in the clinic.

Prior BCL-xL Inhibition Increases MCL1 Dependence and Increases Sensitivity to MCL1 Inhibitors

The development of selective MCL1 inhibitors with potent in vivo activity now provides the opportunity to rationally design therapies that can specifically target each of the major prosurvival BCL2 family proteins that protect cells during tyrosine kinase inhibitor treatment (14). However, our results suggest the selection of the most appropriate BH3 mimic may be hampered by the lack of clinically feasible biomarkers (e.g., gene mutations, RNA, or protein expression levels) that can robustly predict whether tumors are MCL1 or BCL-xL dependent. To circumvent this problem, we investigated whether intermittent treatment with alternating MCL1 and BCL-xL inhibitors might represent a practical strategy to maximize treatment efficacy without the need to prospectively determine apoptotic dependencies. We treated H2030 cells with AM-8621 or navitoclax in combination with trametinib for 4 days, followed by drug washout for 3 days (for detailed treatment schemas, see Supplementary Table S3). After washout,
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A

% Change in tumor volume

Time (days)

P < 0.05

Control

AM-4907

Trametinib

AM-4907 + trametinib

B

H&E

Phospho-ERK

Cleaved caspase-3

Control

Trametinib

AM-4907

Trametinib + AM-4907

C

% Change in tumor volume

Trametinib

Trametinib + MCL1i

MGH6024

MGH1112

MGH1091

MGH1089

MGH1088

MGH1070

MGH1065

MGH1040

P < 0.05

P < 0.01

N.S.
cells were rechallenged with either the same or opposite drug combination, for up to three rounds of treatment. Unexpectedly, we observed that after exposure to navitoclax + trametinib, cells became exquisitely sensitive to AM-8621 + trametinib (Supplementary Fig. S5A). To determine whether “pretreating” cells with navitoclax might sensitize to MCL1 inhibition, we selected cell lines with varied MCL1 dependencies and treated them sequentially with navitoclax, drug washout, and finally AM-8621 + trametinib. Although navitoclax pretreatment had little impact on the sensitivity to trametinib alone, cell lines became more sensitive to the AM-8621 + trametinib combination, as indicated by the degree of separation (ΔAUC) between the trametinib-only and trametinib + AM-8621 dose–response curves (Fig. 5A–C). Conversely, pretreatment with AM-8621 did not sensitize cells to combined navitoclax + trametinib (Fig. 5B and C). The increased sensitivity was observed after a range of navitoclax pretreatment and drug washout periods (Supplementary Fig. S5B) and corresponded with increased apoptosis (Fig. S5D).

We next examined whether navitoclax pretreatment causes KRAS-mutant NSCLC cells to become sensitive to AM-8621 alone. Whereas H2030 cells, which exhibit baseline sensitivity to MCL1 inhibition, became more sensitive to AM-8621, cell lines with little or no baseline MCL1 dependence (A549, H1944, and H1734) did not become sensitive to single-agent AM-8621 (Supplementary Fig. SSC and SSD). Thus, in the majority of cells, an additional apoptotic stimulus such as BIM induction is still required to trigger an apoptotic response, suggesting that sequential application of BCL-xL followed by MCL1 inhibitors might selectively sensitize KRAS-mutant NSCLC cells to MCL1 inhibition. To test this, we compared the response of KRAS-mutant NSCLC lines with non–KRAS-mutant lung adenocarcinoma, squamous cell carcinoma, and noncancer cell lines. Navitoclax pretreatment increased the sensitivity of the majority of KRAS-mutant NSCLC cells to AM-8621 + trametinib (Fig. 5E), with the exception of KRAS-mutant NSCLC cells that were completely insensitive to both MEK + MCL1 and MEK + BCL-xL inhibition at baseline (H460 and H1606). Of note, cell lines that were sensitive to MEK + BCL-xL inhibition but not MEK + MCL1 inhibition at baseline (H1734 and H441) became sensitive to MEK + MCL1 inhibition after navitoclax pretreatment. We did not observe sensitization of non–KRAS-mutant cells including KRAS wild-type lung cancer cells and cancer-associated fibroblast cells, consistent with the notion that this approach selectively targets KRAS-mutant cells that have MAPK-mediated suppression of BIM. Finally, to verify that the effect of navitoclax pretreatment was mediated by BCL-xL, we tested the BCL-xL–selective inhibitor A1331852 and observed even more pronounced sensitization in KRAS-mutant NSCLC cells, but not in non–KRAS-mutant cell lines (Fig. 5E). Overall, these results suggest that sequential application of BH3 mimetics can be used to selectively sensitize KRAS-mutant NSCLC cells to MEK inhibition.

To determine the mechanism of increased MEK + MCL1 inhibitor sensitivity of KRAS-mutant cells after transient BCL-xL inhibitor treatment, we first examined whether pretreatment with navitoclax induced changes in the expression of BCL2 family genes. After both navitoclax treatment and washout, we did not observe any consistent changes in the RNA transcript levels in H2030, H2009, H1734, and H1944 cells that could explain the sensitization (Supplementary Fig. S5E). We next examined the binding interactions and subcellular localization of BCL2 family proteins. In H2030 cells, in which the majority of BIM is already bound to MCL1 at baseline, navitoclax or A1331852 pretreatment caused a further modest increase in the relative amount of BIM bound to MCL1 which persisted even after drug washout (Supplementary Fig. S5F). An even more pronounced increase in BIM bound to MCL1 was observed in H2009, H1944, and H1734, which exhibit less MCL1 dependence at baseline but become sensitive to MEK + MCL1 inhibition after navitoclax or A1331852 pretreatment (Fig. S5F). In contrast, there was little or no increase in BIM bound to MCL1 after navitoclax or A1331852 pretreatment in cells that did not sensitize (H358 and H460). Conversely, there was no increase in BIM bound to BCL-xL after pretreatment with AM-8621 (Supplementary Fig. S5G), consistent with the lack of sensitization to BCL-xL inhibition with the reciprocal pretreatment strategy (Fig. 5B and C). We also observed additional changes in the subcellular localization of BCL2 family proteins, with an increase in MCL1 and BIM and/or decrease in BCL-xL protein at the mitochondria (Supplementary Fig. S5H and SSI), all consistent with increased dependence on MCL1.

To understand why the effects of navitoclax persist despite drug washout, we quantified the amount of intracellular navitoclax after drug washout. In both H2030 and H1734 cells, approximately 50% of intracellular navitoclax was retained 24 hours after washout, and this remained stable for up to 96 hours (Supplementary Fig. S5J). In contrast, AM-8621 was depleted from cells immediately upon removal of drug from the culture media (Supplementary Fig. S5K), again consistent with its inability to sensitize cells to BCL-xL inhibition. All together, these results suggest that transient exposure of KRAS-mutant NSCLC cells to BCL-xL inhibitors leads to sustained loading of BIM on MCL1, increasing MCL1 dependence and thus poising cells to more readily undergo apoptosis upon subsequent treatment with combination MEK + MCL1 inhibitors. Indeed, navitoclax pretreated H2030 and H1734 cells underwent rapid mitochondrial depolarization and cytochrome C release after treatment with trametinib + AM-8621 compared with vehicle-pretreated cells (Fig. 5G; Supplementary Fig. S5L).

Pretreatment with BCL-xL Inhibitors May Permit Alternative MEK + MCL1 Inhibitor Dosing Strategies

The use of MEK inhibitors in the clinic is associated with significant toxicity in many patients (27), and this may limit the promise of MEK-based combination therapies. Alternative drug administration schedules such as intermittent dosing have the potential to minimize toxicity, but may come with the cost of decreased efficacy. We hypothesized that increasing the MCL1 inhibitor sensitivity of KRAS-mutant NSCLC cells by pretreatment with BCL-xL inhibitors might offset decreased activity of intermittent MEK + MCL1 inhibition. To test this, we monitored cell viability of KRAS-mutant NSCLC cell lines during treatment with continuous or intermittent trametinib + AM-8621 (3 days on/4 days off), after either vehicle or navitoclax pretreatment. We also assessed patient-derived cell lines that corresponded to PDX models used for the in vivo studies (independently generated...
Combination MEK + MCL1 Inhibitors for KRAS-Mutant NSCLC

...regressions upon treatment with trametinib... MCL1 inhibition... H2030 xenografts that were pretreated with navitoclax or A1331852 experienced deeper and more rapid tumor regressions upon treatment with trametinib + +... for KRAS-Mutant NSCLCs with BCL-xL inhibitors increases sensitivity to combined MEK + MCL1 inhibition... BCL-xL inhibition... MCL1 inhibition... Figure 5. Transient BCL-xL inhibition alters BCL2 family interactions and increases sensitivity to MCL1 inhibition. A, Navitoclax (Nav) pretreatment increases sensitivity to combination MEK + MCL1 inhibition. Cells were pretreated with navitoclax for 48 hours followed by drug washout for 48 hours and then treated with trametinib (Tram) or AM-8621 (AM) for 72 hours (blue boxes, navitoclax; red boxes, AMG-8621; open boxes, vehicle; line, drug washout period). For detailed treatment schemas, see Supplementary Table S3. B, Navitoclax pretreatment increases sensitivity to combined MEK + MCL1 inhibition; however, the reciprocal strategy of AM-8621 pretreatment does not increase sensitivity to MEK + BCL-xL inhibition. AUC corresponds to the shaded area between trametinib-only (black) and trametinib + BH3 mimetic (colored) curves and is a measure of the sensitization effect. C, Comparison of the increase in sensitization observed with the reciprocal pretreatment strategies. Data are replotted from B, and each dot represents the increase in MCL1 sensitization after pretreatment with navitoclax (red) or BCL-xL sensitization after pretreatment with AM-8621 (blue) for each cell line. D, Cells were pretreated with 1 μmol/L navitoclax for 48 hours followed by drug washout for 48 hours and then treated with 100 nmol/L trametinib, 1 μmol/L navitoclax, 1 μmol/L AM-8621, or combination for 72 hours. Apoptosis was determined by propidium iodide (PI)/annexin staining. E, Navitoclax or A1331852 pretreatment sensitizes KRAS-mutant NSCLC cells that exhibit baseline sensitivity to either MEK + MCL1 or MEK + BCL-xL inhibition, but not KRAS-mutant NSCLC cells without baseline sensitivity or non-KRAS-mutant cells. F, Navitoclax or A1331852 pretreatment leads to increased BIM bound to MCL1 in cells that exhibit sensitization (H2009, H1944, and H1734), but not in cells that do not sensitze (H358 and H460). G, Navitoclax pretreated cells undergo rapid mitochondrial depolarization and release of cytochrome C upon treatment with trametinib + AM-8621.

from the clinical tumor samples, except for MGH1070x, which was derived from the established mouse PDX tumor. Intermittent trametinib + AM-8621 was slightly less effective than continuous treatment (P = 0.055) for reducing cell viability (Fig. 6A). However, when cells were pretreated with navitoclax, intermittent trametinib + AM-8621 led to dramatically reduced cell viability in all cell lines. Finally, we tested whether BCL-xL inhibitor pretreatment sensitizes KRAS-mutant xenograft tumors to MEK + MCL1 inhibition in vivo. H2030 xenografts that were pretreated with navitoclax or A1331852 experienced deeper and more rapid tumor regressions upon treatment with trametinib + AM-4907 or AMG 176 combinations compared with vehicle-pretreated tumors (Fig. 6B). Thus, transient treatment of KRAS-mutant NSCLCs with BCL-xL inhibitors increases sensitivity to combined MEK + MCL1 inhibition in vitro and in vivo and may enable intermittent dosing strategies with preservation of antitumor efficacy.

**DISCUSSION**

The development of MCL1 inhibitors with potent and selective in vivo activity represents a major advance in our ability to individually target the three major prosurvival BCL2...
family proteins that regulate the apoptotic response: MCL1, BCL2, and BCL-XL. Although BH3 mimetics that target these three proteins have demonstrated activity against various hematologic malignancies (18, 19, 28), emerging data suggest that solid tumor malignancies such as NSCLC and breast cancer will be largely refractory to these drugs when used alone. For these cancers, the promise of BH3 mimetics lies in their ability to potentiate the apoptotic response of other therapeutic modalities such as targeted therapies or cytotoxic chemotherapy.

A number of studies have demonstrated that the BCL2/BCL-xL inhibitors ABT-737 and ABT-263 (navitoclax) can synergize with targeted therapies such as EGFR inhibitors in NSCLC (29–31) and MEK inhibitors in BRAF melanoma (32) and KRAS-mutant cancers (11). Similarly, two recent studies demonstrated that the novel MCL1 inhibitor S63845 synergizes with targeted therapies and chemotherapy in breast cancer, melanoma, and lung cancer models (18, 33). Our combinatorial drug screen similarly identified strong synergistic associations between the novel spiromacrocyclic MCL1 inhibitor AM-8621 and a number of clinically relevant compounds that may warrant further investigation. In this study, we focused on synergy between AM-8621 and MEK inhibitors in KRAS-mutant lung cancers because of the clear association of an oncogenic driver mutation and the lack of effective targeted therapy options for these patients. In a subset of KRAS-mutant NSCLC cell lines, combining the MEK inhibitor trametinib with AM-8621 led to robust activation of apoptosis and potent suppression of cell viability. These results reinforce the notion that therapies that target oncogenic dependencies and induce proapoptotic proteins (e.g., induction of BIM upon MEK inhibition) drive dependency on BCL-XL or MCL1 that can be therapeutically exploited with BH3 mimetics. Combining trametinib with the orally bioavailable analogue AM-4907 or the clinical compound AMG 176 induced tumor regressions in KRAS-mutant NSCLC models in vivo, providing a strong rationale for the clinical investigation of this combination for KRAS-mutant NSCLC.

In order to move beyond proof-of-concept studies and define specific strategies for effectively deploying BH3 mimetics in the clinic, a more precise understanding of the specific functional apoptotic dependencies of different cancer subsets is needed. Now that potent and selective inhibitors that target MCL1, BCL2, and BCL-XL are available, it is possible to directly compare parallel approaches targeting each of these proteins. In this study, comparing the response of KRAS-mutant NSCLC and colorectal cancer cell lines with dual MEK + MCL1 or BCL-XL blockade yielded several important insights. First, we found that colorectal cancer cell lines are more uniformly BCL-XL-dependent than NSCLC cell lines. This finding is consistent with the observation that colorectal cancer cell lines as a group have higher BCL-XL and lower MCL1 expression levels, which would be expected to result in increased BCL-XL dependency. Whether this results from differences in co-occurring mutations or the influence of cell lineage remains to be determined; however, it suggests that tissue of origin may play an influential role in determining apoptotic dependencies independent of an oncogenic driver.
Second, our results suggest that the specific apoptotic dependencies of NSCLCs may be heterogeneous, with different KRAS-mutant NSCLC cell lines being dependent on MCL1, BCL-xl, both, or neither. Developing reliable and robust biomarkers that can predict these apoptotic dependencies and guide selection of BH3 mimetics will be crucial to guiding patient selection in the clinic. Many studies have demonstrated an inverse correlation between the sensitivity of BH3 mimetics and the expression level of the non-targeted prosurvival BCL2 family protein. For instance, high expression levels of MCL1 correlate with decreased sensitivity to ABT-737/navitoclax (34–36); conversely, sensitivity to S63845 and AM-8621 inversely correlates with BCL-xl expression levels (18, 19, 33). However, it is not clear if these correlations are sufficiently robust to enable MCL1 and BCL-xl expression levels to be used to predict drug sensitivity of individual tumors within cancer subclasses. For our KRAS-mutant NSCLC cell line panel, we found that expression levels of BCL2 family members were insufficient for predicting response to combination MEK + MCL1 versus MEK + BCL-xl inhibition. Coimmunoprecipitation experiments revealed notable differences in the binding interactions between proapoptotic BCL2 proteins and MCL1 or BCL-xl in cell lines that had similar levels of MCL1 and BCL-xl expression, and this corresponded to sensitivity to MCL1 or BCL-xl inhibition. This indicates that gene or protein expression levels alone incompletely describe the functional apoptotic dependencies that determine response to BH3 mimetics. BH3 profiling has recently emerged as a promising method for determining the apoptotic priming state of cancer cells (24, 37, 38), and in this study it differentiated between cells that were sensitive to trametinib + AM-8621 versus trametinib + navitoclax. It remains to be seen whether BH3 profiling can be effectively applied to solid-tumor malignancies for which routine diagnostic core biopsy tissue samples are scant.

Given these challenges, therapeutic strategies that incorporate both BCL-xl and MCL1 inhibitors might obviate the need to define specific apoptotic dependencies. In our combination drug screen, the combination of AM-8621 with ABT-737 or navitoclax was universally potent, with marked synergy observed against every cell line, suggesting that simultaneous administration might be associated with unacceptable toxicity. This motivated us to explore intercalating intermittent AM-8621 or navitoclax in combination with trametinib, a strategy that might also minimize the thrombocytopenia associated with navitoclax. Unexpectedly, we observed that transient exposure to BCL-xl inhibitors caused cells to become more sensitive to trametinib + AM-8621, including cells that exhibited no sensitivity to MCL1 inhibition at baseline. On a molecular level, pretreatment with navitoclax or A1331852 caused displacement of BIM from BCL-xl, retrotranslocation of BCL-xl from the mitochondria to the cytosol and, in some cases, an increase in mitochondrial MCL1. The net result of these changes was an increase in the amount of BIM bound to MCL1 at the mitochondria, positing the cell for rapid mitochondrial depolarization and release of cytchrome C upon treatment with trametinib + AM-8621. In contrast, cell lines that did not sensitize after navitoclax or A1331852 pretreatment exhibited very little increase in BIM bound to MCL1. The fact that the changes in BCL2 family protein interactions persist even after drug washout is likely facilitated by the long intracellular half-life of navitoclax. Although our studies did not directly determine whether navitoclax remains engaged in the BCL-xl binding pocket after washout, we did not observe restoration of BIM:BCL-xl interactions in H1734 cells after washout (Supplementary Fig. SF5). Interestingly, BCL-xl retrotranslocation from mitochondria to cytosol has been previously reported to protect cells from apoptosis by helping to recycle mitochondrial BAX back to its inactive cytoplasmic pool, and disruption of this process by ABT-737 increases both BCL-xl retrotranslocation and mitochondrial BAX localization (39). In our models, we did not observe any changes in the cytosolic–mitochondrial distribution of BAX after navitoclax pretreatment, suggesting that this mechanism does not account for the observed apoptotic sensitization.

Although this study focused specifically on KRAS-mutant NSCLC, the strategy of alternating BH3 mimetics that selectively target MCL1 and BCL-xl may be relevant to other oncogene-addicted cancers in which BH3-only proteins, such as BIM, can be induced by targeted therapies (40). It is noteworthy that sensitization after BCL-xl inhibitor pretreatment occurred only in cell lines that exhibited at least partial sensitivity to either MEK + MCL1 or MEK + BCL-xl inhibition. KRAS-mutant NSCLC cells that were completely insensitive to both trametinib + BH3 mimetic combinations did not become more sensitive MEK + MCL1 inhibitors after exposure to BCL-xl inhibitors, nor did KRAS wild-type lung cancer cells or stromal fibroblasts, which are not driven by oncogenic MAPK pathway activation. This suggests that matching the targeted therapy to oncogenic driver remains a critical determinant of selectivity, with BH3 mimetics acting as a potentiator of the apoptotic response. Thus, these results provide a rationale for further investigation of intercalated and intermittent dosing strategies that may maximize the efficacy of BH3 mimic-targeted therapy combinations in diverse cancer types.

**METHODS**

Cell Lines, Antibodies, and Reagents

KRAS-mutant NSCLC and colorectal cancer cell lines were obtained from the Center for Molecular Therapeutics at the Massachusetts General Hospital (MGH) Cancer Center, which performs routine SNP and short tandem repeat (STR) authentication. Additionally, cell lines underwent STR validation at the initiation of the project (Biosynthesis, Inc.). Cell lines were routinely tested for Mycoplasma during experimental use. Cell lines were maintained in RPMI supplemented with 5% FBS except A427, SW1573, H2009, H1573, GSPx, LS174T, LS1034, LoVo, SW1116, SW837, and T84 cells, which were maintained in DMEM/F12 supplemented with 5% FBS. Patient-derived NSCLC and cancer-associated fibroblast cell lines were established in our laboratory from surgical resections, core-needle biopsies, or pleural effusion samples as previously described (41), with the exception of the MGH1070x cell line, which was derived from a primary mouse PDX model. All patients signed informed consent to participate in a Dana-Farber/Harvard Cancer Center Institutional Review Board-approved protocol, giving permission for research to be performed on their samples. Clinically observed KRAS mutations (determined by MGH SNaPshot NGS genotyping panel) were verified in established cell lines. Established patient-derived cell lines were maintained in RPMI + 10% FBS. For cell culture and *in vivo* studies, trametinib, navitoclax, and AM-8621 were sensitive to trametinib versus trametinib + navitoclax. It remains to be seen whether BH3 profiling can be effectively applied to solid-tumor malignancies for which routine diagnostic core biopsy tissue samples are scant.
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(ABT-263), venetoclax (ABT-199), and A1331852 were purchased from Selleckchem. AM-8621, AM-4907, and AMG 176 were provided by Amgen. For cell culture studies, drugs were dissolved in DMSO to a final concentration of 10 mmol/L and stored at −20°C unless otherwise specified. 1 μmol/L concentration was used for in vitro cell culture experiments. For western blotting, the following antibodies were used: MCL1, BCL-xL, BIM, BAK, PUMA, Cytochrome C, COX-IV, HDAC1, β-tubulin, pERK1/2 T202/204, ERK1/2, and β-actin (Cell Signaling Technology); NOXA and BAX (Santa Cruz Biotechnology).

Combination Drug Screen

**Viability Assay for Combination Screen.** The AM-8621 combination screen was performed at Zalicus. In brief, cells were seeded in growth media in 1.536-well tissue culture–treated plates at optimized seeding densities to ensure log phase growth throughout the compound treatment duration. Cells were incubated at 37°C and 5% CO2 for 24 hours before treatment. At the time of treatment, a set of assay plates (which do not receive treatment) was collected, and ATP levels were measured via ATPlite viability assay (PerkinElmer). These Tzero (T0) plates were read using ultrar sensitive luminescence on Envision Plate Readers. Treated assay plates were incubated with compound for 72 hours (see “Combination Screen Design” below). After 72 hours, plates were developed for endpoint analysis using ATPlite. All data points were collected via automated processes, quality controlled, and analyzed using Chalice software (Zalicus). Assay plates were accepted if they passed the following quality control standards: relative luciferase values were consistent throughout the entire experiment, Z-factor scores were greater than 0.6, and untreated/vehicle controls behaved consistently on the plate.

Growth inhibition (GI) values were calculated as a measure of cell viability. The cell viability of vehicle is measured at the time of dosing (T0) and after 72 hours (T72). A GI reading of 0% represents complete growth inhibition—cells treated with compound and T72 vehicle signals are equivalent. A GI of 100% represents complete growth inhibition—cells treated with compound and T0 vehicle signals are equivalent. A GI of 200% represents complete death of all cells in the culture well. GI was calculated by applying the following test equation:

\[
T < V_0 : 100 \times \left(1 - \frac{T - V_0}{V_0}\right)
\]

where \(T\) is the signal measure for a test article or combination, \(V\) is the vehicle-treated control measure, and \(V_0\) is the vehicle control measure at time zero. This formula is derived from the GI calculation used in the National Cancer Institute's NCI-60 high-throughput screen.

Combination Screen Design. Combination analysis data were collected in a 7 × 7 checkerboard optimized matrix for 15 cell lines screened in the 1.536-well format. One hundred eighty-seven enhancer compounds were combined with AM-8621 across the 15 cell line panel. In addition, 40 compounds were combined in self-cross analysis for each cell line. The starting concentration and fold dilution for AM-8621 and enhancer compounds were optimized to best fit the individual single-agent dose–response curves.

Synergy Score Analysis. To measure combination effects in excess of Loewe additivity, a scalar measure was used to characterize the strength of synergistic interaction termed the "synergy score." The synergy score is calculated as:

\[
\text{Synergy score} = \log f_x \log f_y \sum \max(0, I_{\text{dual}} - I_{\text{single}})
\]

The fractional inhibition for each component agent and combination point in the matrix is calculated relative to the median of all vehicle-treated control wells. The synergy score equation integrates the experimentally observed activity volume at each point in the matrix in excess of a model surface numerically derived from the activity of the component agents using the Loewe model for additivity. Additional terms in the synergy score equation (above) are used to normalize for various dilution factors used for individual agents and to allow for comparison of synergy scores across an entire experiment. The inclusion of positive inhibition gating or an Idatta multiplier removes noise near the zero-effect level and biases results for synergistic interactions that occur at high activity levels.

**Self-Cross–Based Combination Screen Analysis.** To objectively establish hit criteria for the AM-8621 combination screen, 40 compounds from the combination library were selected to be self-crossed across the 15 cell line panel as a means to empirically determine a baseline additive, non synergistic response. The identity of the 40 self-cross compounds was determined by selecting compounds with a variety of maximum response values and single-agent dose–response steepness. Those drug combinations, which yielded effect levels that statistically superseded those baseline additivity values, were considered synergistic.

The synergy score measure was used for the self-cross analysis. Synergy scores of self-crosses are expected to be additive by definition and therefore maintain a synergy score of zero. However, although some self-cross synergy scores are near zero, many are greater, suggesting that experimental noise or nonoptimal curve fitting of the single-agent dose responses is contributing to the slight perturbations in the score. Given the potential differences in cell line sensitivity to AM-8621 combination activities, we used a cell line–centric strategy for the self-cross–based combination screen analysis, focusing on self-cross behavior in individual cell lines versus global review of the cell line panel activity. Combinations where the synergy score was greater than the mean self-cross plus two standard deviations (2σ) were considered candidate synergies at the 95% confidence levels. A total of 960 hits were identified at 2σ above the mean (32% hit rate).

**Confirmatory High-Resolution Synergy Experiments.** For confirmatory synergy studies reported in Fig. 1C, similar methodologies to those described for the AM-8621 combination screen were used with the following exceptions: (i) studies were performed in 384-well plates and (ii) combinations and self-cross experiments were performed in 10 × 10 complete optimized matrices. All experiments were performed in duplicate.

**Cell Proliferation and Viability Assays**

For cell proliferation dose–response assays, cell lines were seeded into 96-well plates 24 hours before the addition of drug. Cell proliferation was determined by CellTiter-Glo assay (Promega) 72 hours after adding drug according to the manufacturer’s protocol. For short-term time-course experiments, plates were drugged in identical fashion at indicated time points; all plates in an experiment were developed with CellTiter-Glo simultaneously. Cell viability studies were performed using crystal violet staining. Cells were fixed with glutaraldehyde, washed with water, and stained with 0.1% crystal violet for 30 minutes. After imaging, staining was quantified by using 10% acetic acid to extract the stain and absorbance read at 590 nm. Long-term cell viability studies were performed using RealTime-Glo assay (Promega) according to the manufacturer’s protocol. Cell viability was assessed at the indicated time points, and fresh media were replaced immediately afterward. For navitoclax pretreatment studies, we observed similar sensitization over a range of pretreatment and washout time periods (48–72 hours); for consistency,
48-hour pretreatment/48-hour washout was used unless otherwise indicated.

**PI/Annexin Apoptosis Assay**

Cells were seeded in triplicate at low density 24 hours prior to drug addition. Forty-eight hours after adding drug, caspase cleavage was quantified using ApoTox-Glo Triplex Assay (Promega). Relative caspase cleavage was determined as follows: (luminescence)/[400Ex/515Em (viabilities)].

**Caspase 3/7 Apoptosis Assay**

Cells were seeded in triplicate at low density 24 hours prior to drug addition. Forty-eight hours after adding drug, caspase cleavage was quantified using ApoTox-Glo Triplex Assay (Promega). Relative caspase cleavage was determined as follows: (luminescence)/[400Ex/515Em (viabilities)].

**Subcellular Fractionation Assay**

Cells were incubated with drug for designated time periods. Following incubations, whole-cell, cytosolic, mitochondrial, and nuclear fractions were sequentially isolated using a cell fractionation kit (Abcam; catalog number 109719) according to the manufacturer’s protocol, with minor modifications. Purity of fractions was determined by western blot analysis using specific nuclear, cytosolic, and mitochondrial markers.

**Immunoprecipitation Assay**

Immunoblotting was performed per antibody manufacturer’s specifications. Cells were lysed using Trit Lysis Buffer and Protease inhibitor cocktail (Meso Scale Diagnostics) and immunoprecipitated overnight at 4°C with Protein A/G Agarose beads (Thermo Fisher) and the following antibodies: anti-MCL1 (BD Pharmingen), anti-BCL-xL (EMD Millipore), and anti-mouse IgG1 isotype control (Cell Signaling Technology). The immunoprecipitate, the supernatant, and a sample of the initial whole-cell lysate for each condition were then analyzed by western blot.

**siRNA Knockdown Studies**

Cells were plated in Opti-MEM media (Invitrogen), transfected with 50 nmol/L siRNAs specific for MCL1 (Thermo Fisher, 4392420; Qiagen, S10218120S) using HiPerfect. Twenty-four hours after transfection, whole-cell, cytosolic, mitochondrial, and nuclear fractions were collected 48 hours after transfection. For caspase 3/7 activity assays, drugs were added starting 48 hours after transfection.

**BH3 Profiling Assay**

BH3 profiling was performed as previously described (38). Briefly, cells were treated with trametinib at 100 nmol/L for 16 hours, and JC1-based BH3 profiling was performed on untreated versus trametinib-treated cells. Change in apoptotic priming was defined as the difference in the extent of mitochondrial depolarization induced by BID, MCL1, and HRK peptides in treated versus untreated cells.

**Navitoclax Washout Studies**

H2030 and H1734 cells were seeded in 10-cm dishes at a density of 5 × 105 cells/dish in 10 mL growth media containing 5% FBS. Plates were incubated overnight at 37°C and 5% CO2 to allow cells to adhere to the bottom of the plates. Cells were then treated with 1 μmol/L navitoclax or AM-8621 for 72 hours, followed by washout and replacement with 10 mL of compound-free growth media. At indicated time points post washout, media were aspirated from plates followed by two washes with 5 mL PBS. Plates were then stored at −80°C. To prepare for LC/MS analysis, plates were thawed, and 5 mL of acetonitrile was added to each plate followed by incubation at room temperature with shaking for 20 minutes. Samples were then spun down to collect supernatant, which was dried down and reconstituted in equal parts acetonitrile and water followed by analysis on an API 5500 mass spectrophotometer.

**Mouse Xenograft Assays and Drug Treatment**

All mouse studies were conducted through Institutional Animal Care and Use Committee–approved animal protocols in accordance with institutional guidelines. KRAS-mutant NSCLC PDX models were generated from surgical resections, core-needle biopsies, or pleural effusion samples by subcutaneous implantation into NSG mice (Jackson Labs). Subcutaneous tumors were serially passaged twice to fully establish each model. Clinically observed KRAS mutations were verified in each established model. For drug studies, PDX tumors were directly implanted subcutaneously into NSG or athymic nude (NE/Nu) mice and allowed to grow to 250 to 400 mm³. For H2030 and A549 xenograft studies, cell line suspensions were prepared in 1:1 matrigel/PBS, and 5 × 106 cells were injected unilaterally into the subcutaneous space on the flanks of athymic nude (Nu/Nu) mice and allowed to grow to approximately 350 mm³. Tumors were measured with electronic calipers, and the tumor volume was calculated according to the formula V = 0.52 × L × W². Mice with established tumors were randomized to drug treatment groups using covariate-adaptive randomization to minimize differences in baseline tumor volumes. Tumor volumes at the start of treatment (mean ± SD); A549 control 37 ± 28 mm³, A549 trametinib 382 ± 8 mm³, A549 AM-4907 374 ± 22 mm³, A549 trametinib + AM-4907 382 ± 11 mm³; H2030 control 315 ± 42 mm³, H2030 trametinib 318 ± 32 mm³, H2030 AM-4907 318 ± 60 mm³, H2030 trametinib + AM-4907 ± 33 mm³; H2030 navitoclax 227 ± 55 mm³, H2030 trametinib + navitoclax 283 ± 66 mm³; A427 control 201 ± 20 mm³, A427 trametinib 318 ± 16 mm³, A427 AM-4907 312 ± 17 mm³, A427 trametinib + AM-4907 305 ± 19 mm³. Trimetinib was dissolved in 0.5% HPMC/0.2% Tween 80 (pH 8.0) and administered by oral gavage daily at 3 mg/kg, 6 days per week. AM-4907 and AMG 176 were dissolved in 25% hydroxypropyl-beta-cycloextrin (pH8.0) and administered by oral gavage daily at 100 mg/kg and 50 mg/kg, respectively. Navitoclax was dissolved in 60% Phosal 50/30% polyethylene glycol (PEG) 400/10% ethanol and administered by oral gavage daily at 100 mg/kg. A13318752 was dissolved in 60% Phosal 50/27.5% PEG 400/10% ethanol/2.5% DMSO and administered by oral gavage at 25 mg/kg twice daily.

**IHC**

Histology was performed by HistoWiz Inc. (histowiz.com) using standard operating procedures and fully automated workflow. Formalin-fixed paraffin-embedded KRAS-mutant NSCLC xenograft tumors were sectioned at 4 μm. IHC was performed on a Bond Rx autostainer (Leica Biosystems) with enzyme treatment (1:1,000) using standard protocols. Antibodies used were phospho-ERK (1:100; #4307, Cell Signaling Technology) and cleaved caspase-3 (1:300; #9661, Cell Signaling Technology). Bond Polymer Refine Detection (Leica Biosystems) was used according to the manufacturer’s protocol. Sections were then counterstained with hematoxylin, dehydrated, and film covered using a Tissue-Tek Prisma and Coverslipper (Sakura).

**Confocal Fluorescence Microscopy**

Cells were seeded at low density on a Lab-Tek Chamber Slide System 48 hours prior to drug treatment. Cells were treated with 1 μmol/L A13318752 or vehicle for 48 hours, washed with PBS, and then cultured for 48 hours in media without drug. Cells were incubated with MitoTracker Red CMXRos (Thermo Fisher) for 30 minutes, fixed with 1% paraformaldehyde, and permeabilized with 0.2% Triton X-100. Cells were then blocked with 5% NGS/5% BSA for 1 hour and incubated with 1:500 anti-BCL-xL rabbit mAb (54H6, Cell Signaling Technology). Confocal Fluorescence Microscopy was used to evaluate the effect of treatment on mitochondrial morphology.
Technology) overnight at 4°C. Cells were washed with 0.05% Tween 20 and then incubated with 1:500 anti-rabbit IgG Alexa Fluor 488 conjugate (Cell Signaling Technology) for 1 h at room temperature. Finally, cells were stained with 1:1,000 DAPI for 10 minutes. Slides were imaged the next day on a Zeiss LSM 710 inverted laser scanning confocal microscope (excitation/emission: 495/519 nm for BCL-xL and 578/608 nm for MitoTracker) at 63× magnification.

Data and Statistical Analysis

Data were analyzed using GraphPad Prism software (GraphPad Software). Unless otherwise specified, data displayed are mean and standard error. Pair-wise comparisons between groups (e.g., experimental vs. control) were made using paired or unpaired t tests as appropriate. For xenograft tumor measurements, individual time points were compared using multiple t tests with Holm–Sidak correction for multiple comparisons. Unless otherwise indicated, P values below 0.05 were considered to be statistically significant.

Disclosure of Potential Conflicts of Interest

S. Caenepeel is a senior scientist at Amgen Inc. and has ownership interest (including stock, patents, etc.) in the same. L.V. Sequist is a consultant/advisory board member for AstraZeneca, Genentech, Blueprint Medicines, Pfizer, Novartis, Merrimack, and Boehringer Ingelheim. Z. Piotrowska is a consultant/advisory board member for AstraZeneca, Novartis, Takeda/Ariad, Guardant Health, and AbbVie. J.F. Gainor is a consultant/advisory board member for Novartis, Bristol-Myers Squibb, Incyte, Takeda, Pfizer, Roche, Amgen, Agios, Joune, Oncorusrus, and Regeneron. S.P. Brown has ownership interest (including stock, patents, etc.) in Amgen. A. Coxon has ownership interest (including stock, patents, etc.) in Amgen. P.E. Hughes has ownership interest (including stock, patents, etc.) in Amgen Inc. A.N. Hata reports receiving commercial research grants from Amgen, Novartis, and Relay Therapeutics. No potential conflicts of interest were disclosed by the other authors.

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Exploiting MCL1 Dependency with Combination MEK + MCL1 Inhibitors Leads to Induction of Apoptosis and Tumor Regression in KRAS-Mutant Non–Small Cell Lung Cancer

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