Preclinical Development

Molecular Cancer Therapeutics

ERK Inhibition Overcomes Acquired Resistance to MEK Inhibitors

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Abstract

The RAS/RAF/MEK pathway is activated in more than 30% of human cancers, most commonly via mutation in the *K-ras* oncogene and also via mutations in BRAF. Several allosteric mitogen-activated protein/extracellular signal–regulated kinase (MEK) inhibitors, aimed at treating tumors with RAS/RAF pathway alterations, are in clinical development. However, acquired resistance to these inhibitors has been documented both in preclinical and clinical samples. To identify strategies to overcome this resistance, we have derived three independent MEK inhibitor–resistant cell lines. Resistance to allosteric MEK inhibitors in these cell lines was consistently linked to acquired mutations in the allosteric binding pocket of MEK. In one cell line, concurrent amplification of mutant K-ras was observed in conjunction with MEK allosteric pocket mutations. Clonal analysis showed that both resistance mechanisms occur in the same cell and contribute to enhanced resistance. Importantly, in all cases the MEK-resistant cell lines retained their addiction to the mitogen-activated protein kinase (MAPK) pathway, as evidenced by their sensitivity to a selective inhibitor of the ERK1/2 kinases. These data suggest that tumors with acquired MEK inhibitor resistance remain dependent on the MAPK pathway and are therefore sensitive to inhibitors that act downstream of the mutated MEK target. Importantly, we show that dual inhibition of MEK and ERK by small molecule inhibitors was synergistic and acted to both inhibit the emergence of resistance, as well as to overcome acquired resistance to MEK inhibitors. Therefore, our data provide a rationale for cotargeting multiple nodes within the MAPK signaling cascade in *K-ras* mutant tumors to maximize therapeutic benefit for patients. *Mol Cancer Ther; 11(5); 1143–54. ©2012 AACR.*

Introduction

The RAS/RAF/MEK pathway is widely activated in human cancers and thus has attracted significant interest as a therapeutic target for cancer (1). Efforts to target RAS directly have not been successful to date, but recent clinical trials with BRAF and mitogen-activated protein/extracellular signal–regulated kinase (MEK) inhibitors have suggested that targeting these downstream RAS effectors holds promise in the treatment of cancers harboring oncogenic alterations in the pathway (2). Although clinical responses and antitumor activity can be impressive, particularly for BRAF inhibitors in BRAF mutant melanoma, the majority of patients ultimately develop clinical resistance and progressive disease on these agents (2, 3). Preclinical studies have identified multiple mechanisms of acquired resistance to BRAF inhibitors, including switching between RAF isoforms (4), upregulation of RTK or NRAS signaling (5), and reactivation of mitogen-activated protein kinase (MAPK) signaling via COT activation (6) or a MEK kinase activating mutation (7). Similarly, preclinical studies have identified distinct mechanisms by which cells acquire resistance to MEK inhibition, including amplification of mutant BRAF (8), STAT3 upregulation (9), or mutations in the allosteric pocket of MEK that can directly block binding of inhibitors to the MEK kinase or lead to constitutive MEK kinase activity (10, 11). MEK mutations have also been described in tumor samples from patients treated with MEK (11) or BRAF inhibitors (7), showing clinical relevance.

In comparison with RAF and MEK inhibitors, the development of small molecule inhibitors against ERK1/2, the kinases that act directly downstream of MEK, has lagged behind. Nevertheless, examples of selective ERK1/2 inhibitors have been reported and are currently in preclinical development (12, 13). In this study, we set out to characterize the mechanisms of acquired resistance to MEK allosteric inhibitors in cancer cell lines harboring

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Note: Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

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doi: 10.1158/1535-7163.MCT-11-1010

oncogenic *K-ras* mutations and identify strategies to overcome this resistance, with a focus on cotargeting ERK and MEK. Our goal was to determine whether *K-ras* mutant, MEK-resistant cells maintained addiction to the MAPK pathway and sensitivity to inhibition of the pathway at the level of ERK1/2.

Our major finding is that MEK inhibitor–resistant cells consistently maintain their addiction to the MAPK pathway and their sensitivity to a selective inhibitor of ERK1/2. Combination treatment of naive *K-ras* mutant cells with MEK plus ERK inhibitors inhibited the outgrowth of resistant cells, whereas ERK inhibitor treatment of cells with acquired MEK inhibitor resistance cells effectively blocked their proliferation. These findings support the rationale for the clinical development of small molecule ERK1/2 inhibitors in conjunction with MEK inhibitors as a therapeutic strategy for patients with MAPK pathway–addicted (*K-ras* mutant and BRAF mutant) tumors.

Materials and Methods

Cell lines

MDA-MB-231, LoVo, HCT-116, H2122, and BL2122 cells were obtained from American Type Culture Collection (ATCC) and archived in the Genentech cell bank. COLO206F and COLO206F-AR cells were kindly provided by Ryan Corcoran and Jeffrey Engelman (Massachusetts General Hospital, Charlestown, MA). Starting parental cell lines for MDA-MB-231, LoVo, and HCT-116 were authenticated at Genetica, Inc., (www.genetica.com) using a Multiplex STR assay, whereas H2122 and BL2122 cells were assayed at early passage after acquisition from ATCC. All cell lines were maintained in RPMI 1640 or Dulbecco's Modified Eagle's Medium supplemented with 10% FBS (Sigma), nonessential amino acids, and 2 mmol/L L-glutamine.

Compounds and cell viability experiments

All studies in this article describing MEK and ERK inhibition have used the potent and selective MEK1/2 inhibitors PD0325901 (Pfizer; ref. 14) and G-573 (Genentech; ref. 15), as well as the ERK inhibitor (12, 13), to evaluate the effects of MEK and ERK inhibition. Selectivity of the ERK inhibitor for ERK1/2 across a panel of kinases, and for KRAS mutant versus matched normal cells, is provided in Supplementary Fig. S1. The phosphoinositide 3-kinase (PI3K) inhibitors and BRAF inhibitors used in this study were provided by the Genentech Medicinal Chemistry department and have been described previously (16–18). The structures of the ERK, MEK, PI3K, and BRAF inhibitors used are provided. All other compounds were purchased commercially. Cell viability and bromodeoxyuridine (BrdUrd) studies were conducted as described previously (19, 20). All *in vitro* cell viability experiments were repeated independently at least 3 times with similar results.

Resistant cell line selection

The breast cancer cell line MDA-MB-231 and colon cancer cell lines LoVo and HCT-116 were grown in increasing concentrations of the MEK inhibitor PD0325901 (Pfizer), until they grew normally in a concentration of 10 μmol/L for MDA-MB-231 and 5 μmol/L for LoVo and HCT-116. Resistant clones of HCT-116 MEK-R were isolated by fluorescence-activated cell sorting individual cells into 96-well plates.

Western blot analyses and transfections

For MEK and ERK inhibitor dose response blots, cells were plated at a density of 9×10^5 cells per well into 4-well plates and dosed the following day with 2-fold or 3-fold serial dilutions of ERKi, MEKi-1, MEKi-2, starting at 10 μmol/L for 24 hours. Details of antibodies and conditions are provided in the Supplementary Materials and Methods.

Combination experiments. Combination data were analyzed in R version 2.12.1 (21) and results summarized graphically across the combinations tested. To assess inhibition under various drug combinations in excess of that predicted by Bliss additivity, smoothed estimates of the 2 drugs' single-agent curves were used to derive a predicted inhibition response surface, as in Borisy and colleagues (22). More details are provided in Supplementary Methods.

Three-dimensional cultures

For 3-dimensional (3-D) cultures, MDA-MB-231 parental and MEK-R cells were trypsinized from monolayer cultures and plated on top of commercially available laminin-rich matrix produced from Engelbreth-Holm-Swarm tumors (Matrigel; BD Biosciences) using a previously described protocol (20).

Sequencing parental and resistant cell lines

Exonic regions of MEK1, MEK2, and KRAS were amplified by nested PCR of genomic DNA and sequenced in both directions using Big Dye Terminator Kit reagents and an ABI PRISM 3730xl DNA analyzer (Applied Biosystems).

Gene expression microarray and copy number analyses

Gene expression analysis of breast cancer cell lines MDA-MB-231 parental and MEK-R lines was as described previously (20). Pathway relationships for genes differentially expressed between MDA-MB-231 parental and MEK-R cells was determined using Ingenuity software. Copy number analyses were carried out after correcting for GC-content bias following Diskin and colleagues (23) and then segmented using CBS (24). These analyses are described in more detail in the Supplementary Materials and Methods.

Results

Selection of MEK-resistant basal-like breast cancer cells

We initially focused our efforts on a *K-ras* mutant basal-like breast tumor model, representing an indication with poor prognosis and limited therapeutic options (25).

Breast cancer is a heterogeneous disease with well-defined molecular subtypes referred to as luminal, HER2 positive, and basal-like (26). Previous work has suggested that basal-like breast cancer models are dependent on RAS/RAF/MEK signaling and show a high degree of sensitivity to MEK inhibition relative to models representing the other subtypes (20, 27). We exposed *K-ras* mutant MDA-MB-231 basal-like breast cancer cells to increasing concentrations of the selective allosteric MEK inhibitor PD0325901 (ref. 28; referred to as MEKi-1) over a period of several months and gradually selected a pool of cells that could grow normally in the presence of 10 μmol/L MEKi-1. Analysis of cellular inhibition using an ATP-based cell viability assay showed a shift in IC_{50} for MEKi-1 from 0.3 μmol/L in the parental line to greater than 10 μmol/L in MEK-resistant (MEK-R) cells (Fig. 1A, top). The

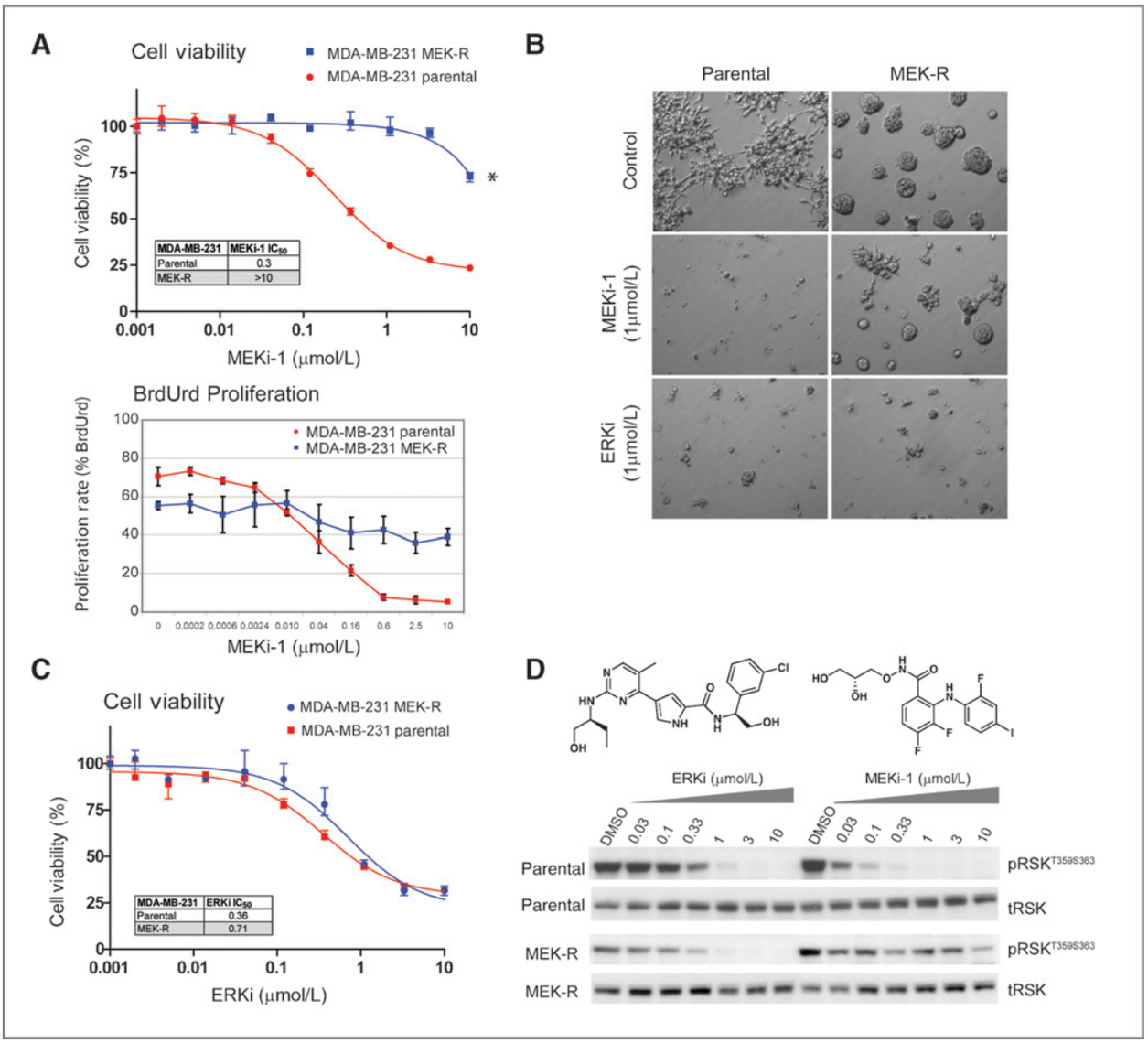

Figure 1. *K-ras* mutant MDA-MB-231 MEK-resistant (MEK-R) cells are insensitive to 2 independent MEK inhibitors but retain sensitivity to ERK inhibition for both cell viability and pharmacodynamic responses. A, top, MDA-MB-231 parental and MEK-resistant (MEK-R) cells were treated with a range of concentrations of MEKi-1 (PD0325901) or MEKi-2 (G-573) for 72 hours and cell viability was measured by ATP-based viability assays. Cells were plated in quadruplicate for each condition, and cell viability experiments were repeated independently at least 3 times with similar results. Bottom, parental and MEK-R cells were treated with MEKi-1for 72 hours and BrdUrd incorporation over a 5-hour labeling period was quantitated by high content imaging. Asterisks indicate that IC_{50} values are significantly different based on nonoverlapping 95% confidence intervals (CI) from nonlinear regression analysis. B, MDA-MB-231 parental and MEK-R cells were grown in 3-D culture in the presence or absence of 1 μmol/L MEKi-1 or ERKi and representatives from triplicate wells were photographed to assess cell morphology and response to MEK inhibition. C, effects of MEKi versus ERKi on cell viability measured by ATP-based viability assay in parental and MEK-R cells. D, MEK-R cells fail to show downregulation of the ERK target pRSK in response to MEK inhibitor treatment but do show downregulation of pRSK in response to ERK inhibitor treatment. Cells treated for 24 hours with a serial dilution of MEKi-1 and ERKi before Western blotting. Total RSK levels are shown as a loading control. Structures for MEKi-1 and ERKi are shown above panel D. DMSO, dimethyl sulfoxide.

mechanism of action of MEK inhibition in MDA-MB-231 cells has been previously shown to involve reduced proliferation rather than apoptosis (20), so we next examined effects of MEKi-1 on BrdUrd incorporation in parental and MEK-R cells. We observed that although MEK-R cells showed a slightly lower proliferation rate at baseline (57% vs. 71% BrdUrd incorporation over 5 hours), they showed only minimal reduction of BrdUrd incorporation in the presence of increasing MEKi-1 concentration compared with parental cells (Fig. 1A, bottom). To confirm that this resistance was not unique to MEKi-1, we verified that MEK-R cells are similarly resistant to a second allosteric MEK inhibitor (G-573, referred to as MEKi-2; ref. 15) in a cell viability assay (Supplementary Fig. S2). In addition, resistance was stable when cells were cultured for 3 months in the absence of drug and then reassayed for sensitivity to MEK inhibition, suggesting a stable genetic change (Supplementary Fig. S2). Differential effects between parental and MEK-R cells seemed specific to MEK inhibition, as both lines responded similarly when screened with a panel of other targeted and chemotherapeutic agents, including PI3 kinase inhibitors (18, 29), the HSP90 inhibitor geldanamycin, and chemotherapeutics such as paclitaxel and etoposide (Supplementary Fig. S2).

To assay resistance in a setting that may be more reflective of *in vivo* biology, we cultured both parental and MEK-R cells in a 3-D matrix of collagen and laminin (Matrigel) and assayed for sensitivity to MEK inhibition as well as for changes in morphology in the resistant cells (Fig. 1B). We found that growth of parental cells in 3-D culture was completely inhibited at a concentration of 1 μmol/L MEK inhibitor, whereas MEK-R cells were still able to grow and form colonies at this concentration. In addition, we found that MEK-R cells plated and grown in 3-D culture adopted a much more rounded morphology compared with the stellate morphology exhibited by parental cells. This rounded morphology is typically associated with a more luminal epithelial phenotype in cell lines, whereas the stellate morphology is associated with more invasive basal-like mesenchymal lines (30). Furthermore, microarray gene expression profiling of RNA from parental cells cultured in standard media and MEK-R cells cultured in 10 μmol/L MEKi-1 showed upregulation of genes involved in luminal epithelial cell fates and concomitant downregulation of mesenchymal cell fate regulators in MEK-R cells (Supplementary Fig. S3). These findings are consistent with recent studies showing that MAPK signaling can regulate the mesenchymal fate in basal-like breast cancer cells (31) and suggest that selection of MDA-MB-231 cells in the presence of a MEK inhibitor seems to have downregulated this pathway and resulted in mesenchymal-to-epithelial transition.

MEK-R KRAS mutant cells retain sensitivity to ERK inhibition

Because MEK-R cells maintain ERK and RSK activation in the presence of MEK inhibitor, we next tested whether they are still dependent on signaling through the RAS/MEK/ERK axis for survival and proliferation by treating with a selective and potent inhibitor of ERK1/2 (ERKi refs. 12, 13). Selectivity testing for activity of this inhibitor against a panel of 200 other kinases failed to identify any target other than ERK that was substantially inhibited (Supplementary Fig. S1A), and this compound was substantially more potent in KRAS mutant H2122 cells than in normal, immortalized BL2122 cells derived from the same patient (Supplementary Fig. S1B). We found that parental and MEK-R cells showed similar sensitivity to this selective ERK inhibitor in cell viability assays conducted in both 3-D culture and standard 2-D format, suggesting that ERK inhibition can overcome MEK inhibitor resistance (Fig. 1B and C, respectively). Furthermore, although MEK inhibition was unable to suppress levels of the ERK target sites on phospho- $RSK^{T359Ser363}$ in MEK-R cells, ERK inhibitors effectively suppressed $pRSK^{T359Ser363}$ levels with similar potency as in parental cells (Fig. 1D). These findings strongly suggested that KRAS mutant MEK-R cells retain overall dependence on RAS/MEK/ERK signaling despite their insensitivity to pathway inhibition at the level of MEK.

We expanded our analysis to additional tumor cell lines from other tumor cell lines harboring *K-ras* mutations. Because up to 40% of colorectal cancers harbor activating *K-ras* mutations (32), we used the colorectal lines HCT-116 and LoVo, both of which show strong *in vitro* sensitivity to 2 different allosteric MEK inhibitors (Fig. 2A). We were able to select derivatives of both lines that could grow normally in the presence of 5 μmol/L MEKi-1 and were also resistant to MEKi-2 (Fig. 2A). Similar to the MDA-MB-231 MEK-R cells, both HCT-116 and LoVo MEK-R cells seemed to retain dependence on RAS/MEK/ERK signaling as ERK inhibition was able to decrease cell proliferation and inhibit pRSK to a similar degree as was observed in parental cells (Fig. 2A and B). In addition, we examined the effects of ERK and MEK inhibitors on MAPK substrates more globally by immunoblotting lysates from treated HCT-116 parental and MEK-R cells with an antibody recognizing the canonical ERK phosphorylation motif PXtP (Fig. 2C). We found that although in parental cells both ERKi and MEKi could abolish ERK substrate phosphorylation, in contrast, in MEK-R cells, basal ERK substrate phosphorylation was higher and was only abolished by ERKi treatment. Thus, in all MEK-R cell lines analyzed, MEK resistance and pathway activation could be effectively overcome by ERK inhibition.

Molecular mechanisms of MEK resistance

The observation that all of the MEK-R lines identified in this study retained sensitivity to ERK inhibition suggested that the mechanism of resistance was acting at or above the level of MEK itself, as ERK is a direct downstream substrate of MEK. To identify such mechanisms of resistance, we first sequenced the coding regions of MEK1 and MEK2. Mutational analysis showed that MEK-R cells derived from MDA-MB-231 and HCT-116 cells harbor

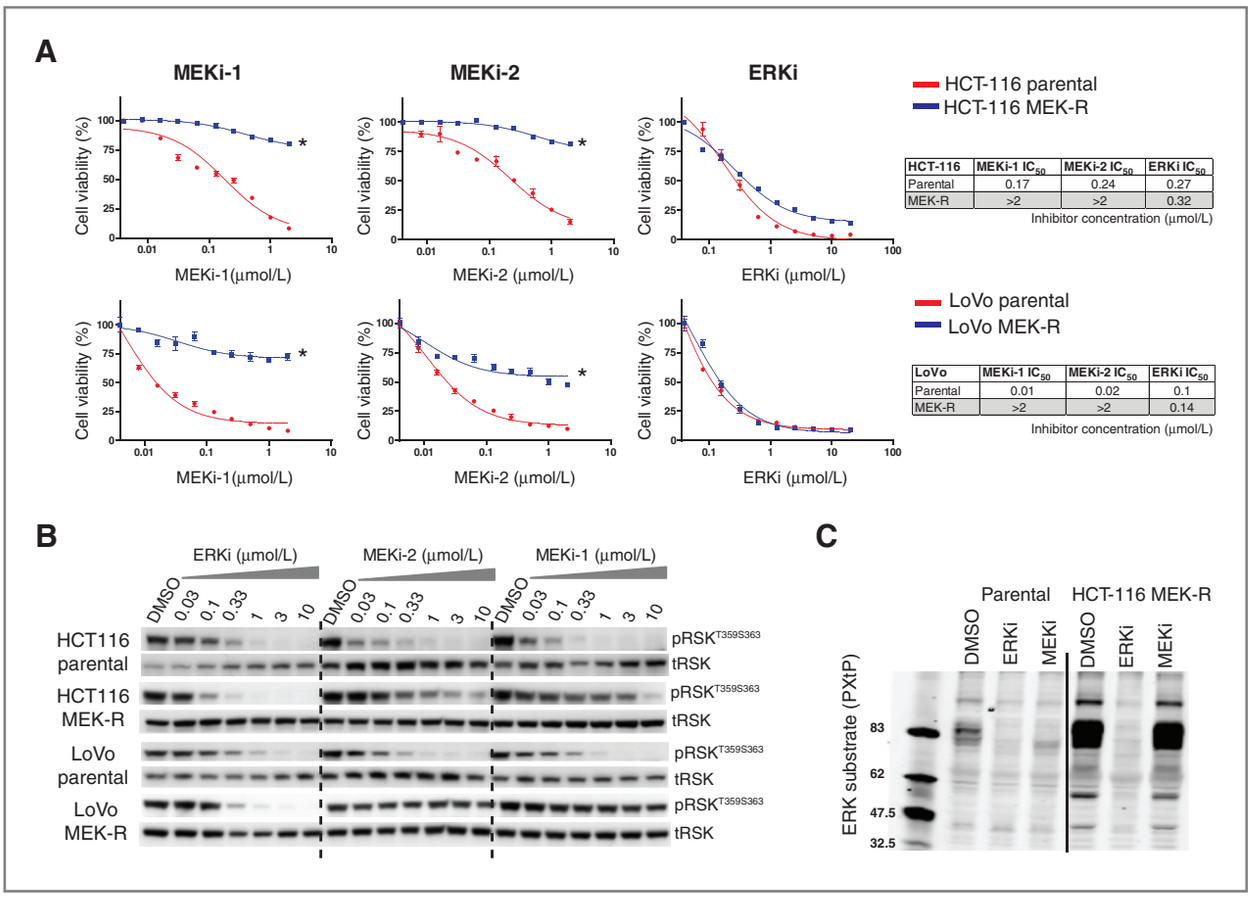

Figure 2. Two independent MEK-R *K-ras* mutant colon cell lines show cross-resistance to a second MEK inhibitor but retain sensitivity to ERK inhibition. A, cell viability in response to inhibitors was assessed by ATP-based cell viability assays. Asterisks indicate nonoverlapping 95% CIs for IC_{50} values. B, Western blot analysis indicating that MEK-R lines show pRSK downregulation in response to ERK inhibitors but not MEK inhibitors. Cells were treated for 24 hours with inhibitors. Total RSK levels are shown as a loading control. C, effects of MEK and ERK inhibition on global phosphorylation levels of the consensus ERK substrate motif PXtP in HCT-116 parental and MEK-R cells. DMSO, dimethyl sulfoxide.

mutations in MEK1 that are predicted to result in L115P and F129L substitutions, respectively, whereas those derived from LoVo cells harbor a mutation in MEK2 predicted to cause a V215E substitution (homologous to V211D mutations in MEK1; Table 1, Supplementary Fig. S4). These mutations are all heterozygous and occur within the allosteric binding pocket in which arylamine MEK inhibitors are known to bind (Fig. 3A). In addition, the identified mutations have all previously been shown to confer *in vitro* MEK inhibitor resistance in random mutagenesis experiments followed by stable transfection functional assays of MEK inhibitor resistance in BRAF mutant cells (11). The L115P and V211D mutations confer resistance by abrogating MEK inhibitor binding, while F129L also increases intrinsic kinase activity of MEK (10). All 3 of our cell lines retained the activating *K-ras* (G13D) allele. To assess whether the MEK mutations were present in a preexisting subpopulation or arose *de novo* in the presence of MEK inhibitor, we used digital PCR with a TaqMan genotyping assay for the L115P-causing mutation on DNA from parental MDA-MB-231 cells (Supplementary Fig. S5). We screened approximately 68,850 genome equivalents in this assay and did not detect any evidence of the L115P alteration. These data suggested that the L115P mutation is either not present, or is only present in a very small subpopulation, in the parental cell line. To show that these mutations can abrogate MEK

Table 1. Summary of MEK1, MEK2, and KRAS mutational status of 3 KRAS mutant cells lines and their MEK-R derivatives

Cell line	MEK1	MEK2	KRAS
MDA-MB-231 parental	WT	WT	G13D
MDA-MB-231 MEK-R	**L115P**	WT	G13D
HCT-116 parental	WT	WT	G13D
HCT-116 MEK-R	**F129L**	WT	G13D
LoVo parental	WT	WT	G13D
LoVo MEK-R	WT	V215E	G13D

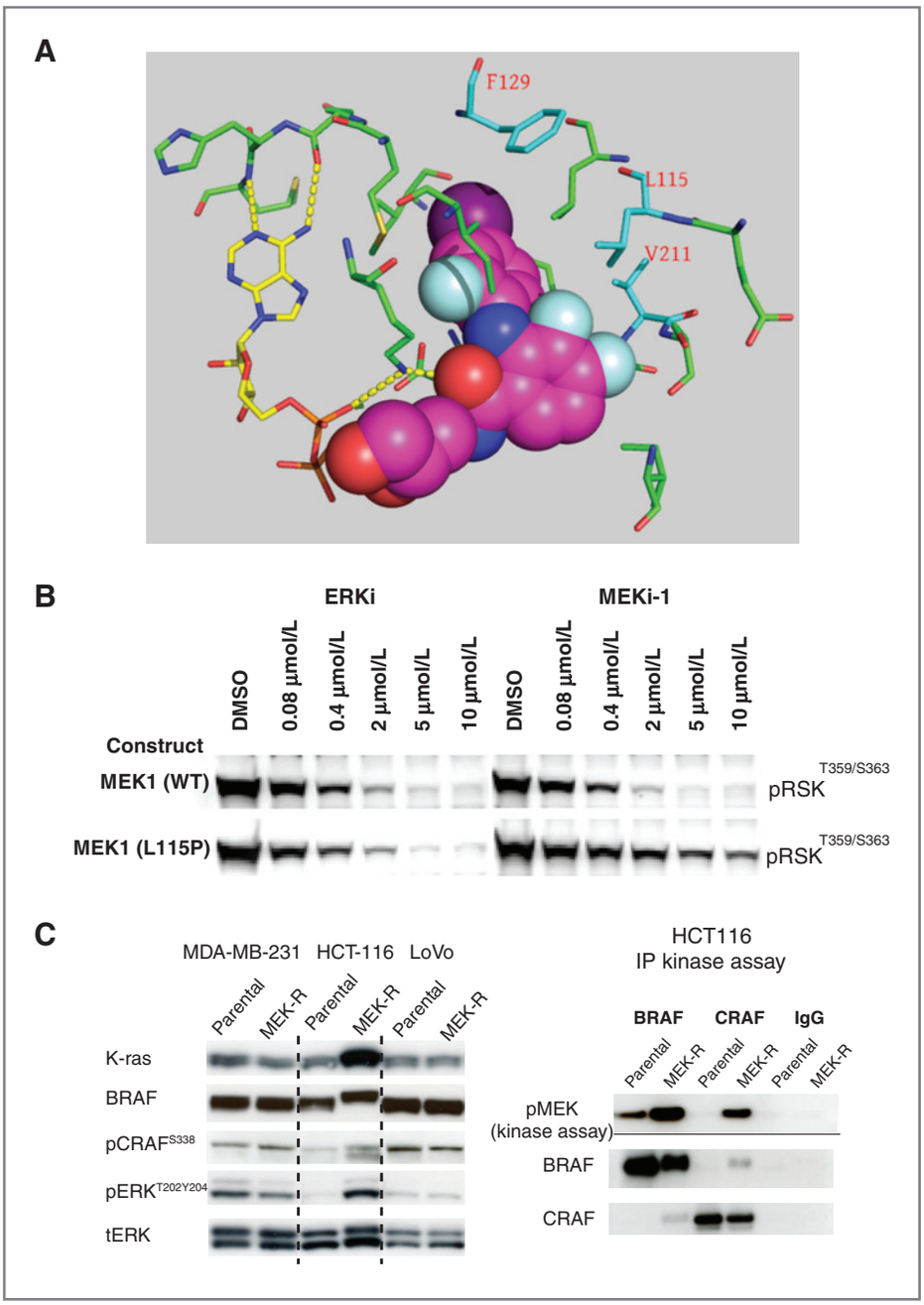

Figure 3. MEK-R cell lines harbor allosteric inhibitor binding site mutations in either MEK1 or MEK2 that block MEK inhibitor but do not affect ERK inhibitor pharmacodynamic responses. A, X-ray structure (PDB code: 3EQG) depicting positions of mutations within the allosteric binding pocket of MEK for arylamine class of inhibitors, critical for binding. B, effect of transient transfection of MEK1 wild-type or L115P mutant form on ERK versus MEK inhibitor–mediated downregulation of pRSK levels in HCT-116 cells. Ectopic expression of the L115P mutation abrogates pharmacodynamic response to a MEK inhibitor. The transfection efficiency in this experiment was 68% based on expression of FLAG-tagged MEK (L115P), as described in the Supplementary Materials and Methods. C, left, Western blot analysis comparing basal levels and phosphorylation of key Ras/RAF/MAPK expression components in parental versus MEK-R cells. Right, immunoprecipitation/Western blot assays to compare levels of CRAF and BRAF kinase activity in parental and MEK-R cells. DMSO, dimethyl sulfoxide.

inhibitor responsiveness but have no effect on ERK inhibitor sensitivity in the context of the KRAS mutant cell lines used in this study, we introduced the L115P mutation into MEK1 by site-directed mutagenesis and transfected HCT-116 parental cells with this construct or a wild-type version of MEK1. We found that expression of MEK1 (L115P) effectively blocked MEK inhibitor–mediated downregulation of pRSK in transfected cells but did not affect ERK inhibitor–mediated downregulation (Fig. 3B). These data suggested that although MEK-R cells may be escaping from tumor growth inhibition due to the failure of MEK inhibitors to bind and inhibit mutant MEK, they retain the ability to respond to ERK inhibition.

We next sought to investigate the upregulation of ERK substrate phosphorylation observed in HCT-116 cells (Fig. 2C) by assaying for levels of MAPK signaling pathway components in all 3 MEK-R–resistant lines. Our Western blot data showed overexpression of *K-Ras* along with induction of phosphorylated CRAF and phosphorylated ERK, selectively in the HCT-116 MEK-R cells (Fig. 3C, left panel) and not in the MDA-MB-231 or LoVo MEK-R cells. HCT-116 MEK-R cells also displayed increased

BRAF and CRAF activity levels as shown by an immunoprecipitation/kinase assay (Fig. 3C). These results, along with the ERK substrate results previously described, suggested that the pathway is hyperactivated in HCT-116 cells, and that additional mechanisms of resistance might be involved in this tumor model.

To identify additional mechanisms of resistance, we assessed global copy number data from comparative genomic hybridization (CGH) arrays for differences between parental and resistant cell lines. In the case of MDA-MB-231 and LoVo cells, this analysis did not reveal any substantial differences in copy number for BRAF, MEK1/2, ERK1/2, or *K-ras* or any other loci. In contrast, HCT-116 MEK-R cells showed high-level amplification, specifically at the *K-ras* locus, but not other loci, compared with parental cells (Fig. 4A). Quantitation of copy number by quantitative genomic PCR indicated that the HCT-116 MEK-R cells carry 23 copies of *K-ras* (Supplementary Fig. S6A), and sequence analysis suggested selective amplification of the mutant *K-ras* allele, because a wild-type allele peak is not detectable in the sequencing trace (Supplementary Fig. S6B). *K-ras* amplification has previously been reported to underlie acquired MEK resistance in derivatives of this cell line selected for resistance to the MEK inhibitor AZD-6244 (33). Because the HCT-116 MEK-R pool showed evidence of 2 distinct mechanisms of resistance, a MEK1 (F129L) mutation and KRAS amplification, we next asked whether these alterations were occurring in the same cells or in different cells within the pool population, perhaps representing distinct clonal lineages. We cloned individual cells in 96-well plates, waited until the population expanded, then sequenced MEK1 and determined KRAS copy number via qPCR assay. This analysis revealed surprising heterogeneity (Supplementary Fig. S7, Fig. 4), with the majority of cells

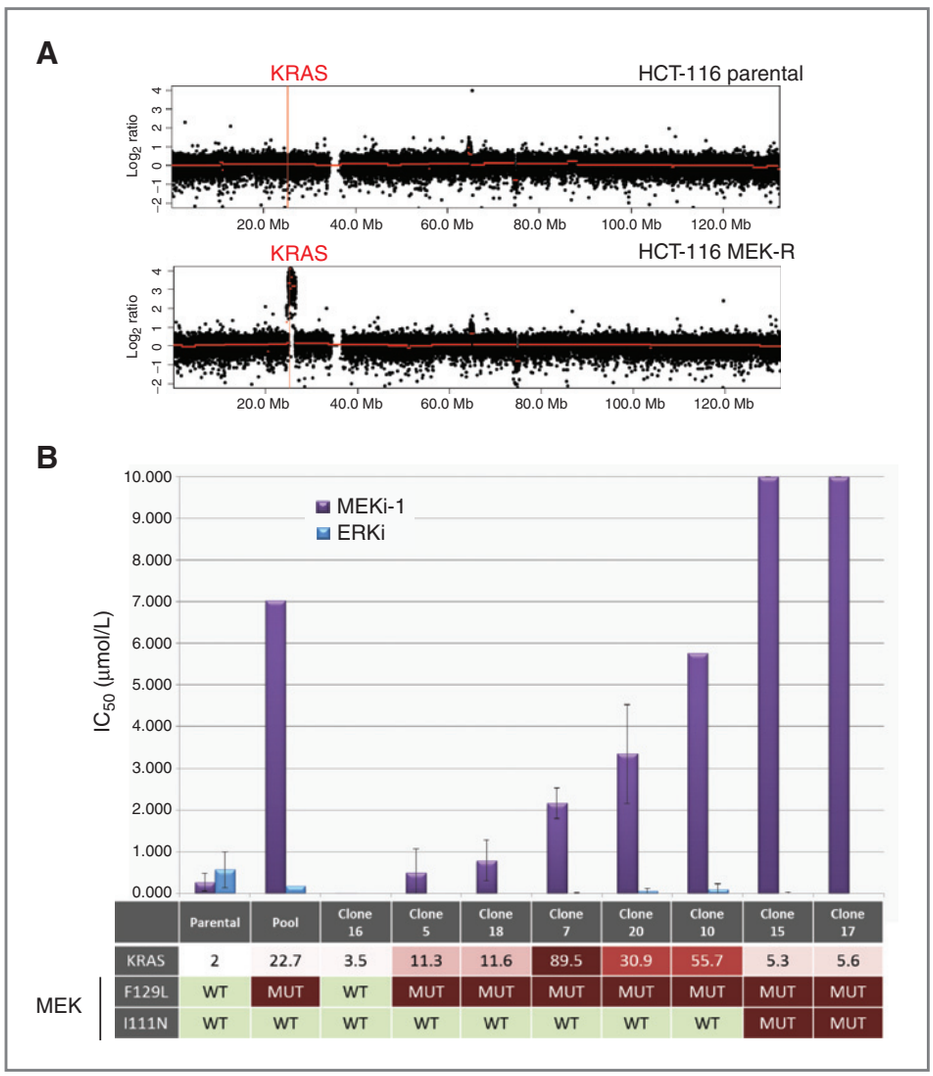

Figure 4. HCT-116 MEK-R cells harbor multiple resistance mechanisms. A, CGH measurement of DNA copy number along the length of chromosome 12 shows focal *K-ras* amplification in HCT-116 MEK-R cells (below) but not in the HCT-116 parental line (above). Black dots represent the values for each probe and the red lines represent the mean copy number value for contiguous chromosomal segments. B, individual clones derived from the MEK-R pool were analyzed for MEK1 mutations and level of KRAS amplification, then tested for sensitivity to MEK and ERK inhibition. Y axis shows IC_{50} for MEK or ERK inhibitors, and genotypes for each clone are illustrated in the chart. Resistant clones contain at least 2 candidate resistance mechanisms.

having both the F129L mutation and varying degrees of KRAS amplification. One clone was wild-type for MEK1 with very low levels of KRAS amplification, similar to parental cells. Surprisingly, we also identified 2 clones with low levels of KRAS amplification that harbored both F129L as well as a second MEK1 mutation predicted to cause an I111N substitution. This alteration has also been previously described to block MEK inhibitor binding (11). We next screened a representative set of clones for sensitivity to MEKi-1 and ERKi (Fig. 4B) and found that the MEK1 F129L/I111N double mutant cells showed the greatest resistance to MEK inhibitors, whereas clones with KRAS amplification and a concurrent F129L mutation showed a correlation between greater resistance to MEK inhibitors and high KRAS copy number above a certain threshold (approximately 30 copies). This pattern of mutations and resistance suggests that the F129L MEK1 mutation was selected for early on, and during subsequent selection, different subsets of clones acquired additional genetic events that increased their resistance, either high-level K-Ras amplification or an additional I111N MEK1 mutation, to more effectively block MEK inhibitor binding. It is notable that all of the clones retained strong sensitivity to the ERK inhibitor, suggesting that downstream inhibition at the level of ERK1/2 can overcome multiple mechanisms of resistance in the same cell (Fig. 4B).

Amplification of BRAF has also been shown to underlie resistance to MEK and RAF inhibitors in BRAF mutant tumors, in which RAF plus MEK inhibitor combinations are currently being tested in clinical trials with promising results (8, 33). Because ERK inhibitors remained effective in *K-ras* mutant amplified cells, we next sought to determine whether they would remain effective in MEK inhibitor resistant, BRAF mutant amplified cells. A previous report showed that BRAF mutant colorectal cancer COLO206F cells with acquired resistance to the AZD-6244 MEK inhibitor (COLO206F-AR) displayed 10 to 15 copies of mutant BRAF (8). We treated COLO206F parental and COLO206F-AR MEK-resistant cells with the 2 MEK inhibitors, the ERK inhibitor and the BRAF inhibitor PLX4720 (34). We found that, in contrast to MEK and BRAF inhibitors, the ERK inhibitor again largely maintained its ability to block proliferation and pathway suppression at the level of pRSK in the BRAF-amplified COLO206F-AR cells, with only an approximately 4-fold shift in sensitivity to ERKi relative to parental cells compared with a 125-

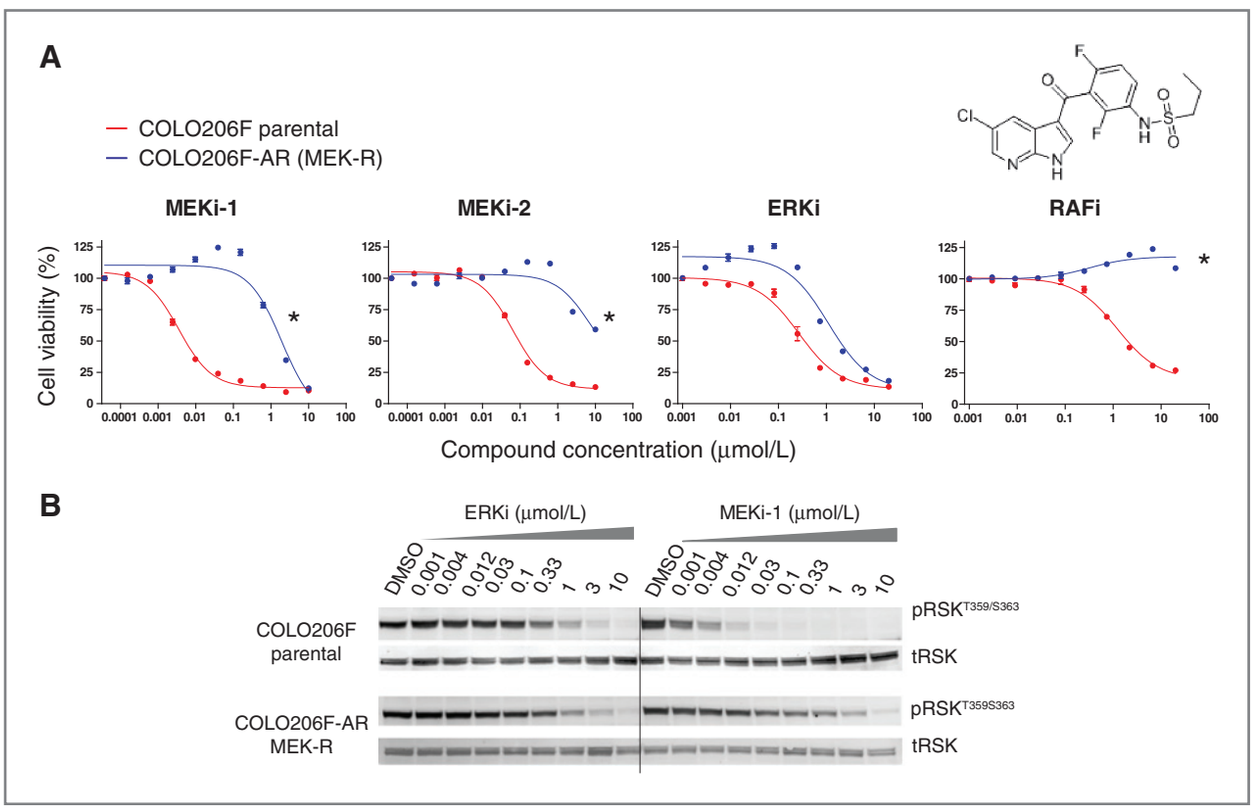

Figure 5. MEKi- and BRAFi-resistant COLO206F-AR cells with BRAF amplification maintain their sensitivity and response to ERK inhibition. A, cell viability of COLO206F parental and COLO206F-AR MEK-resistant cells after treatment with MEKi-1, MEKi-2, ERKi, and BRAFi (PLX4720), as determined via an ATP-based viability assay. Asterisks indicate nonoverlapping 95% CIs for IC_{50} values. B, measurement of pharmacodynamic response at the level of pRSK after a 24-hour treatment with serial dilutions of compounds shows potency shift for the MEK but not the ERK inhibitor. DMSO, dimethyl sulfoxide.

Table 2. Cellular IC_{50} as well as fold difference between the parental and MEK-R line

Cell line	IC_{50} MEKi-1 (μmol/L)	IC_{50} MEKi-2 (μmol/L)	IC_{50} ERKi (μmol/L)
COLO206F parental	0.01	0.08	0.3
COLO206F-AR MEK-R	1.5	10	1.25
IC_{50} shift MEK-R/parental	150×	125×	4.17×

to 150-fold shift in sensitivity to the 2 MEK inhibitors (Fig. 5 and Table 2). These data further supported the conclusion that mechanisms resulting in activation of the MAPK pathway at several nodes (K-Ras, B-Raf, and MEK) can all be overcome by downstream blockade of the MAPK pathway at the level of ERK1/2.

Combination strategies to overcome acquired MEK inhibitor resistance

An important question in cancer management is whether rational combinations of therapy given at the onset of therapy can delay or prevent resistance, or whether such combinations can overcome acquired resistance. To address the first question, we carried out short-term selection of MDA-MB-231 cells in the presence of MEKi alone, ERKi alone, or the combination of MEKi and ERKi. After 9 days of culture, we found significantly fewer resistant cells surviving in the presence of the combination versus either inhibitor alone (Fig. 6A). To address the second question, we treated the MEK-R cell lines described in this study with a combination of ERKi

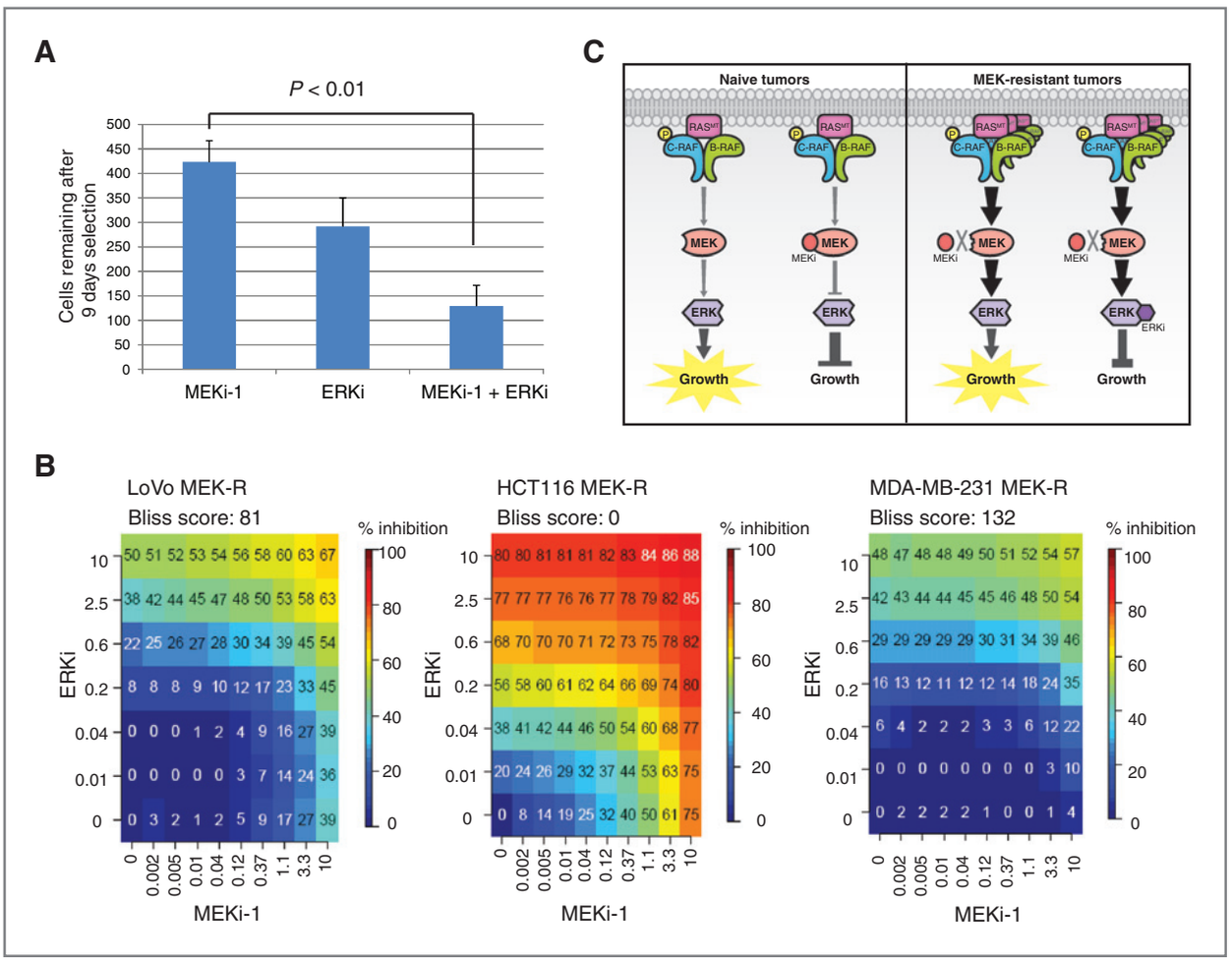

Figure 6. Dual inhibition of MEK and ERK abrogates *de novo* resistance and has synergistic effects on acquired resistance. A, MDA-MB-231 cells were treated for 9 days with the indicated inhibitors as single agents or in combination at a concentration of 2.5X IC_{50} with media changed every 3 days. Surviving cells were quantitated at the end of the experiment. B, MEK-R cells treated with MEK/ERK inhibitors as single agents and in combination across a range of concentrations. Percent inhibition is shown for each well and color coded based on the scale provided, with blue indicating no inhibition and red indicating 100% inhibition of cell viability. BLISS scores more than 0 indicate greater than additive effects. C, dual MEK and ERK inhibition is a strategy to more effectively shut down MAPK pathway flux, both in the context of naive MAPK-driven tumor cells (left) as well as in the context of acquired resistance to MEK inhibitors, by MEK mutation and/or upstream oncogene amplification (Mutant *K-ras* or BRAF).

and MEKi. Combination effects were assessed in cell viability assays conducted across a matrixed array of concentrations of each compound. This approach allows visualization of percent inhibition at each combination of concentrations, as well as calculation of measures of synergy such as the Bliss independence model (35). Under Bliss independence, the fractional inhibition from a combination of 2 agents at specific concentrations is predicted as the sum of the inhibitions of each agent applied alone at the specific concentrations minus the product of those single-agent inhibitions (36). Deviations from additive effects in each well can be summed across the plate to give an overall Bliss score, with positive Bliss scores indicating greater than additive effects. We found that the MEKi + ERKi combination showed enhanced inhibition of cell growth compared with single-agent treatment in all 3 MEK-R cell lines (Fig. 6B). The effect was additive in HCT-116 MEK-R cells and synergistic (Bliss score > 0) in MDA-MB-231 and LoVo MEK-R cells. Therefore, the MEK–ERK inhibitor combination offers the potential of enhanced tumor growth inhibition and delayed resistance in treatment-naive patients, as well as enhanced inhibition in the setting of acquired MEK inhibitor resistance.

Discussion

Somatic activation of the Ras/RAF/MEK/ERK pathway is a major driving event that underlies the initiation and continued expression of the malignant phenotype in neoplastic cells. Pharmacologic inhibition of the pathway with selective inhibitors of MEK or BRAF potently suppresses growth of cancer cell lines harboring oncogenic activation of the pathway, such as those with *K-ras* and *BRAF* mutations (37). A number of agents targeting RAF and MEK have entered the clinic. BRAF inhibitors in particular have shown impressive clinical responses in patients with $BRAF^{V600E}$ mutant melanoma. For instance, treatment of metastatic melanoma patients with the selective inhibitor RG7204 has resulted in partial or complete tumor responses in nearly 80% of patients and extended progression-free survival from 2 months to at least 7 months (38). However, almost all patients eventually develop resistance and progressive worsening of disease, driven by the reactivation of the MAPK pathway in a $BRAF^{V600E}$–independent manner (3). As such, there is a pressing need for therapeutic strategies that can prevent the onset of resistance or overcome diverse resistance mechanisms once they have arisen.

Our observations, derived from multiple models, suggest that *K-ras* mutant tumors with acquired MEK inhibitor resistance often remain addicted to the MAPK pathway, as opposed to activating alternative redundant pathways. This is consistent with a recent report showing that in *K-Ras* mutant colorectal lines, PI3K pathway activity is driven by receptor tyrosine kinase rather than mutant *K-Ras* signaling (39). Furthermore, we show for the first time that selective ERK inhibitors can overcome this resistance and thus that ERK inhibition may constitute a therapeutic option for treating patients who have progressed on MEK inhibitor therapy and show reactivation of MAPK signaling. Previous studies linking allosteric site MEK point mutations to MEK inhibitor resistance used site-directed mutagenesis followed by overexpression of mutants and focused on a $BRAF^{V600E}$ background (11). Our study validates these mutations as a resistance mechanism by showing that they can arise spontaneously during long-term selection of *K-ras* mutant cells in the presence of MEK inhibitors. Together, these results suggest that mutational alteration of the target may be a common mechanism of resistance to MEK inhibitors in the clinic and provide a strong rationale for testing MEK mutation status, in parallel with other described resistance mechanisms, upon patient relapse.

Importantly, we show that MEK inhibitor–resistant cells can exhibit multiple concurrent resistance mechanisms in distinct clones within the same HCT-116 MEK-resistant tumor pool, namely the presence of 2 distinct MEK allosteric point mutations in a subset of clones and a MEK1 allosteric point mutation concurrently with high-level KRAS amplification in another subset. This is in contrast to a previous report suggesting that resistance in this model could be explained solely by KRAS amplification (33), suggesting that perhaps diverse mechanisms of resistance can arise under different experimental conditions. Importantly, treatment with an ERK inhibitor could still effectively inhibit HCT-116 MEK-R cells despite the presence of both upstream alterations. Why ERK inhibitors remain effective in the context of upstream oncogene amplification, although MEK inhibitors become less potent, is not entirely clear. We hypothesize that this phenomenon may be related to the fact that activation of MEK by these upstream oncogenes may change the steady-state conformation of MEK in a way that limits the accessibility of the allosteric binding pocket to MEK inhibitors (40). The ERK inhibitor described here is ATP competitive and may be less sensitive to altered conformation dynamics of activated ERK in the context of upstream oncogene amplification.

Clinical inhibition of MEK and BRAF is at an exciting crossroads with several examples of agents that have meaningful antitumor activity and survival benefit. Acquired resistance to these agents is a pressing issue, and we propose that ERK inhibitors can be valuable tools in combating BRAF and MEK inhibitor resistance. Our study is the first to provide preclinical evidence that administering combinations of MEK and ERK inhibitors in naive cells can prevent or delay the emergence of acquired resistance (Fig. 6C), as previously observed in the context of MEK/RAF inhibitor combinations (8, 11). Furthermore, we found that MEK/ERK inhibitor combinations can also be used to overcome acquired resistance, suggesting a treatment paradigm for patients who have progressed on single-agent MEK or BRAF inhibitor therapy. Additive or overlapping toxicity due

to vertical inhibition of 2 pathway components may be a concern with such an approach, though we note that the clinical combination of BRAF and MEK inhibitors has thus far been clinically well tolerated (41), boding well for MEK/ERK clinical combinations. Overall, these data further highlight the benefits of targeting multiple nodes within the MAPK pathway for achieving lasting responses in MAPK-addicted tumors and underscore the need to understand which of the various resistance mechanisms are at work in a given patient. Such efforts will require sustained commitment to the collection of serial biopsies at the time of disease progression, to identify the most appropriate therapy upon relapse.

Disclosure of Potential Conflicts of Interest

All authors are employees of Genentech, Inc. J. Moffat has ownership interest (including patents) in Roche.

Acknowledgments

The authors thank Dan Burdick, Danette Dudley, and Jake Schwarz for help with synthesis of the ERK inhibitor; Ryan Corcoran and Jeffrey Engelman for supplying the COLO206F and COLO206F-AR cells, Scott Sproul for technical assistance, and Jeff Settleman for comments on the manuscript.

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Received December 13, 2011; revised February 21, 2012; accepted February 23, 2012; published OnlineFirst March 8, 2012.

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