Identification of the MEK1(F129L) Activating Mutation as a Potential Mechanism of Acquired Resistance to MEK Inhibition in Human Cancers Carrying the B-Raf V600E Mutation

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Abstract

Although targeting the Ras/Raf/MEK pathway remains a promising anticancer strategy, mitogen-activated protein/extracellular signal-regulated kinase (ERK) kinase (MEK) inhibitors in clinical development are likely to be limited in their ability to produce durable clinical responses due to the emergence of acquired drug resistance. To identify potential mechanisms of such resistance, we established MEK inhibitor–resistant clones of human HT-29 colon cancer cells (HT-29R cells) that harbor the B-RafV600E mutation. HT-29R cells were specifically resistant to MEK inhibition in vitro and in vivo, with drug-induced elevation of MEK/ERK and their downstream targets primarily accountable for drug resistance. We identified MEK1(F129L) mutation as a molecular mechanism responsible for MEK/ERK pathway activation. In an isogenic cell system that extended these findings into other cancer cell lines, the MEK1(F129L) mutant exhibited higher intrinsic kinase activity than wild-type MEK1 [MEK1(WT)], leading to potent activation of ERK and downstream targets. The MEK1(F129L) mutation also strengthened binding to c-Raf, suggesting an underlying mechanism of higher intrinsic kinase activity. Notably, the combined use of Raf and MEK inhibitors overcame the observed drug resistance and exhibited greater synergy in HT-29R cells than the drug-sensitive HT-29 parental cells. Overall, our findings suggested that mutations in MEK1 can lead to acquired resistance in patients treated with MEK inhibitors and that a combined inhibition of Raf and MEK may be potentially useful as a strategy to bypass or prevent drug resistance in the clinic. Cancer Res; 71(16); 5535–45. ©2011 AACR.

Introduction

Current cancer drug development is evolving toward treatment with molecularly targeted agents, driven by the discovery of genetic alterations in cancer and the signaling pathways they alter. In some patients with cancers driven by a “driver” oncogene, targeted therapies have shown remarkable improvements in clinical response and survival. Successful examples include imatinib in chronic myelogenous leukemia with BCR-ABL translocation (1, 2) and in gastrointestinal stromal tumors with activating c-KIT mutation (3), gefitinib and erlotinib in non–small-cell lung cancers (NSCLC) harboring an activating epidermal growth factor receptor (EGFR) mutation (4), and trastuzumab in breast cancer with Her-2 amplification (5). On the other hand, the rapid acquisition of drug resistance often limits the overall therapeutic impact. This highlights the need for greater understanding of intrinsic (de novo) or acquired resistance mechanisms of the targeted therapy. Oncogenic Ras and B-Raf mutations frequently occur in human tumors. Ras mutations are found in approximately 30% of human tumors (6). B-Raf mutations also occur in many cancers, with the highest incidence in melanoma (60%–70%; ref. 7). Ras or B-Raf mutations lead to aberrant activation of the Ras/Raf/MEK/ERK signal cascade that correlates with tumor progression and poor prognosis (8). The Ras/Raf/MEK pathway components have therefore become attractive targets for cancer therapy. Many new agents have entered human clinical trials targeting Raf or MEK [mitogen-activated protein/extracellular signal-regulated kinase (ERK) kinase], RG7204/PLX4032 (vemurafenib), an orally selective B-Raf inhibitor, exhibits impressive antitumor activity in B-RafV600E mutant metastatic melanomas (9, 10) and significantly improved patient survival (2011 ASCO). A companion diagnostic test to select B-RafV600E mutant tumors is necessary (11–14). Highly selective MEK
inhibitors (GSK1120212, AZD6244, GDC-0973/XL518, and RO4987655) evaluated in early clinical studies may provide benefit in a broader patient population carrying B-Raf wild-type (WT) or Ras mutant tumors. To date, the observed clinical response for MEK inhibitors as single agents has been encouraging but limited (15–17). This likely resulted from potent MEK inhibition in normal cells leading to a narrow therapeutic window. Limited durable efficacy of MEK inhibitors may also result from de novo or drug-resistant acquired resistance in cancers due to complicated signaling pathway cross-talk and compensatory feedback regulation. Thus, it is important to have a better understanding of the molecular mechanism underlying acquired resistance. In this study, we established the colorectal cancer cell (HT-29R) clones resistant to MEK inhibition by chronically exposing HT-29 cells to RO4927350, a highly selective MEK inhibitor (18). HT-29 parental cells harbor HT-29R clones resistant to MEK inhibition by chronically exposure.

**Materials and Methods**

**Compounds**

RO4927350 was synthesized according to the procedure described in patent application (WO2006018188). The Raf inhibitor PLX4032 was synthesized in house. The MEK inhibitor RO4987655/CH4987655 was synthesized by Chugai Pharmaceuticals. The MEK inhibitor PD0325901, the Raf inhibitor PLX720, sorafenib, paclitaxel, doxorubicin, and irinotecan were purchased from American Chemicals Custom Corporation, ChemieTek, or Sigma Aldrich.

**Cell lines and antibodies**

The HT-29 cell line was purchased from American Type Culture Collection and maintained in the designated medium. Antibodies against Ras, phosphorylated (p)-ERK1/2 (Thr202/Tyr204), p-RAF1/2 (Ser217/221), MEK1/2, p-RSK (Ser380), RSK, p-MNK (Thr197/202), MNK, p-BAD (Ser112), p-p85/70 S6 kinase (Thr421/Ser424), p-S6 ribosomal protein (p-S6 RP; Ser235/236), p-S6 RP (Ser240/244), p-CREB (Ser133), DUSP-6, p-akt (Ser473), p-4E-BP (Thr37/46), p-4E-BP (Ser65), p-4E-BP (Ser240/244), p-CREB (Ser133), p-S6 ribosomal protein (p-S6 RP; Ser240/244), p-CREB (Ser133), DUSP-6, p-akt (Ser473), p-4E-BP (Thr37/46), p-4E-BP (Ser65), p-4E-BP (Thr70), p-mTOR (S2481), and p-mTOR (S2448) were purchased from Cell Signaling Technology. Anti-B-Raf, anti-c-Raf, anti-β-actin were purchased from Santa Cruz Biotechnology, BD Transduction Laboratories, and Sigma Aldrich, respectively. Anti-DUSP-10 was from Abnova. Anti-ERK was from Millipore.

**Generation of the MEK inhibitor–resistant clones (HT-29R)**

HT-29 cells were cultured in the presence of gradually increasing concentrations of RO4927350 up to 20 μM/L. After 10 months, HT-29R resistant clones were selected and maintained in the presence of 5 μM/L RO4927350. HT-29R control clones were selected by the same single-cell subclonal procedure in the absence of the drug.

**Sequencing of B-Raf, c-Raf, MEK1, and MEK2 DNA**

Genomic DNAs were isolated using the Qiagen DNA Blood & Tissue Kit. All 18 coding exons of B-Raf, 11 coding exons of MEK1, and c-Raf coding exons 10 to 14 were PCR-amplified with designed primers and bidirectionally sequenced by Genewiz DNA Sequencing Services using Sanger’s method. The coding exon 2, exon 3, exon 5, and exon 6 of MEK2 were sequenced internally using the following primer sets: CTTGAGGTCCTGAGGTCTGC (forward) and GCC-TGGAAGTCAATCAGAAATGC (reverse) for exon 2, TGGTGCTT-GACCATGTTGG (forward) and AAGAGATCCCTGGAAAGC (reverse) for exon 3, CAGCACTGTCTCGTCTCTGG (forward) and GAGAAGGGGAGGAGAACG (reverse) for exons 5 and 6.

**Western blot analysis and cell viability assays in vitro**

Western blotting and MTT assays were carried out as previously described (18). The CellTiter-Glo luminescent cell viability assays (Promega) were carried out according to the manufacturer’s instruction. For combination MTT assays, cells were simultaneously treated with various concentrations of 2 agents at a fixed ratio across a range of activities. Cell viability was evaluated after 5 days by the MTT assay. The isobologram and combination index (CI) were calculated according to the Chou and Talalay median effect principal, using CalcuSyn software version 2 (Biosoft). The computed isobologram is a graphical representation of the 2-agent interaction and is formed by plotting the individual drug doses required to achieve a single-agent effect on their respective x and y axes; a line connecting the 2 points is drawn. Combination data points that fall on, above, or below the line represent additive, antagonism, or synergy, respectively. The calculated CI values provide a qualitative measure of the degree of drug interaction in terms of additive effect (CI = 1), synergism (CI < 1), or antagonism (CI > 1) for a given endpoint of the effect measurement such as EC50 or EC90.

**Plasmid constructs and transfection**

Human full-length MEK1(WT) and mutant MEK1(F129L) expression vectors were cloned into vector pCMV6 with myc-DDK tag at its C-terminus (from Origene). The mutation of MEK1(F129L) was generated by mutagenesis changing C387 to A and was confirmed by sequencing. HEK293 cells were transfected with MEK1(WT) or MEK1(F129L) expression vector, using Lipofectamine 2000 (Invitrogen).

**Immunoprecipitation–Western blot analysis**

Cells were transfected with FLAG-tagged MEK1(WT) or MEK1(F129L) plasmids with or without B-RafV600E cotransfection. After 48 hours, cells were lysed and immunoprecipitation (IP) was carried out using anti-FLAG M2 affinity gel (Sigma). Immune complexes were analyzed by Western blotting. For in vitro kinase assays, the bound protein was eluted with FLAG peptides, and the FLAG-tagged proteins [MEK1(WT) or MEK1(F129L)] were analyzed by Western blotting and quantified by measuring band density against

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various concentrations of FLAG standard loaded on the same gel.

**In vitro kinase assays and compound IC$_{50}$ determination by IMAP assay**

MEK kinase activity was determined by immobilized metal ion affinity-based fluorescence polarization (IMAP) assay as previously described (18). In brief, kinase buffer containing 55 nmol/L MEK1(WT) or MEK1(F129L) with or without 3 nmol/L c-Raf, 90.5 nmol/L inactive ERK, 0.5 μmol/L fluorescein isothiocyanate–labeled ERK substrate peptide, and various concentrations of compound in the presence of 50 μmol/L ATP were added into the assay plate and incubated for 1 hour at 37°C. The plate was incubated at 37°C for 20 minutes for MEK activation in the presence of c-Raf. The percentage of activity and the IC$_{50}$ values of compounds were calculated.

**In vivo efficacy studies in xenograft tumor models**

In vivo testing was done as previously described (19). Briefly, HT-29 cancer cells were implanted s.c. in nude mice. Once the xenograft tumors reached a mean tumor volume of approximately 100 to 200 mm$^3$, mice were randomized to treatment groups (n = 10 per group) and received either vehicle or the compounds at the indicated dose. RO4927350 was formulated as a fine suspension containing 0.09% methyl paraben, 0.01% propyl paraben, 2.0% Klucel LF, and 0.1% polysorbate 80 in purified water and given orally twice daily for approximately 3 weeks. Irinotecan was provided in a stock sterile saline solution of 20 mg/mL, administered intraperitoneally (i.p.) every 4 days for approximately 3 weeks. Efficacy data were graphically represented as the mean tumor volume ± SEM. Tumor volume (in cubic millimeters) and statistical analysis were determined as previously described (19).

**Results**

**Establishment of colorectal cancer cell clones (HT-29R) with acquired resistance to the highly selective MEK inhibitor RO4927350 in vitro and in vivo**

The HT-29 colorectal cancer cell line was previously shown to harbor the B-Raf V600E mutation and is highly sensitive to MEK inhibition (18, 19). To explore potential mechanisms of drug-acquired resistance, we exposed HT-29 cells to gradually increasing concentrations of the allosteric MEK inhibitor RO4927350. After 10 months, surviving cells were subcloned to establish RO4927350-resistant cells (HT-29R), which were subsequently maintained in the presence of 5 μmol/L RO4927350. The control sensitive cells (HT-29S) were subcloned in the absence of the MEK inhibitor. HT-29S and HT-29R cells (5 clones from each category) were treated with various concentrations (0.37–10 μmol/L) of the MEK inhibitor RO4927350 for 24 to 120 hours, and representative results are shown in Fig. 1. RO4927350 inhibited HT-29S9 cell growth in a dose-dependent and time-dependent manner (Fig. 1A), whereas HT-29R1 cells showed significant resistance to RO4927350 (Fig. 1B).

**Figure 1.** HT-29R cells exhibited acquired resistance to MEK inhibitor RO4927350 in vitro (A and B) and in vivo (C and D). In vitro, HT-29S9 (A) and HT-29R1 (B) cells were treated with various concentrations of RO4927350 and cell viability was measured at various time points. In vivo, mice bearing xenograft tumors derived from HT-29S9 (C) and HT-29R1 (D) were treated with RO4927350 [orally twice a day (bidpo) at 100 mg/kg bid] and irinotecan [60 mg/kg i.p. every 4 days (q4d)].
To confirm that in vitro generated HT-29R clones remain resistant to MEK inhibition in vivo, we established xenograft tumors derived from sensitive and resistant clones and monitored their tumor growth kinetics (data not shown). We selected 2 representative clones with similar tumor growth kinetics (HT-29S9 and HT-29R1) and carried out in vivo efficacy studies using MEK inhibitor RO4927350 and irinotecan (Fig. IC and D). RO4927350, when administered orally at 100 mg/kg twice daily for 3 weeks, effectively inhibited HT-29S9 xenograft tumor growth resulting in statistically significant tumor growth inhibition (TGI; 85% TGI, P < 0.001) compared with vehicle-treated controls (Fig. IC). In contrast, HT-29R1 xenograft tumors treated with RO4927350, using the same treatment dose and schedule, resulted in only 36% TGI (P = 0.003; Fig. 1D). Irinotecan, when administered i.p. at 60 mg/kg every 4 days, resulted in equivalent statistically significant tumor growth inhibition (P < 0.001) in both models (88% TGI in HT-29S9 and 82% in HT-29R1; Fig. 1C and D). No body weight loss was observed in mice from any treatment group (data not shown).

**HT-29R clones are resistant to a wide range of Raf/MEK inhibitors**

To further examine whether HT-29R clones are cross-resistant to other cancer therapeutic agents, we treated HT-29S and HT-29R cells (2 representative clones from each category) with various agents including MEK inhibitors (RO4927350, RO4987655, and PD0325901), Raf inhibitors (PLX4720, RG7204, and sorafenib), paclitaxel, doxorubicin, and irinotecan. Cell proliferation was analyzed, and average IC50 and IC90 values of each agent were determined (Table 1). In comparison with HT-29S clones, HT-29R clones exhibited significantly reduced sensitivity to all MEK inhibitors (RO4927350, RO4987655, and PD0325901) with diversified chemical structures (15, 18, 20, 21), with increased average IC90 values ranging from 29- to 65-fold. HT-29R clones displayed 10- or 13-fold increased IC50 to the highly selective Raf inhibitors PLX4720 or RG7204, respectively. Interestingly, Raf inhibitors produced minimal change in IC90 (RG7204) or maximal effective concentration (PLX4720), suggesting that the HT-29R cells are cross-resistant to Raf inhibition at a lower concentration range, but the sensitivity can be restored at higher drug levels. However, we cannot completely rule out off-target effects at higher concentrations because PLX4720 and RG7204 are ATP-competitive Raf inhibitors. On the other hand, HT-29R and HT-29S clones showed equivalent sensitivity (less than 2-fold difference in both IC50 and IC90) to sorafenib, a nonselective Raf inhibitor, and other agents with different mechanisms of action (paclitaxel, doxorubicin, and irinotecan; Table 1). These results show that HT-29R clones are specifically resistant to MEK/Raf inhibition, suggesting that the acquired resistance mechanism is likely involved in pathway specific dysregulation.

**Acquired resistance to MEK inhibition is associated with an elevation of the Raf/MEK/ERK pathway activity**

To understand how the Raf/MEK/ERK pathway is dysregulated in HT-29R cells, we profiled sensitive and resistant clones to examine pathway component expression and activation. Representative results are shown in Fig. 2A. The protein expression levels of key components including Ras, c-Raf, MEK, and ERK were not significantly elevated (less than 1.5-fold) in resistant clones compared with sensitive clones. On the other hand, the MEK/ERK pathway activity was highly elevated in HT-29R clones compared with HT-29S clones as evidenced by the significant increase in the phosphorylation levels of MEK, ERK, and their downstream targets (RSK, MNK, BAD, CREB, S6 RP; Fig. 2A). Pathway activation was also observed in the in vivo xenograft tumors derived from a resistant clone (HT-29R1) showing increased levels of p-MEK, p-ERK, p-RSK, p-MNK, p-BAD, p-S6 RP, and DUSP6 and DUSP10 (known ERK downstream target genes) compared with the xenograft tumors derived from a sensitive clone (HT-29S9; Fig. 2B). All resistant clones tested showed significantly elevated MEK/ERK and downstream pathway activation (Supplementary Fig. S1). The elevation is likely occurring at or below the level of Raf within the Ras/Raf/MEK/ERK signaling cascade. When we carried out phospho-receptor tyrosine kinase (RTK) array analysis (R&D Systems) to simultaneously detect the relative tyrosine phosphorylation levels of 42 different RTKs in both HT-29S and HT-29R clones, none of these RTK phosphorylation levels were found elevated in HT-29R (data not shown). We also confirmed that neither basal nor stimulated levels of Ras activity were altered in HT-29R (data not shown). Moreover, the IP-kinase assays showed that B-Raf activity was not significantly different between HT-29R and HT-29S (data not shown). The c-Raf activity was barely detectable by IP-kinase assays, and phosphorylated c-Raf (Ser338) in all clones (R and S) was barely detectable.

**Table 1. HT-29R cells are specifically resistant to Raf/MEK inhibition**

<table>
<thead>
<tr>
<th>Compound</th>
<th>MTT IC50 (mM)</th>
<th>HT-29S IC50 (mM)</th>
<th>HT-29R IC50 (mM)</th>
<th>Ratio R/S</th>
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detectable, which is consistent with previous findings that c-Raf activity is very low in B-RafV600E cancer lines (22). These results show that acquired resistance to MEK inhibition is associated with a significant elevation of MEK/ERK pathway activity without alteration in upstream component (Ras/Raf) expression (Fig. 2A) or Ras GTPase and Raf kinase activities (data not shown).

An activating mutation MEK1(F129L) was identified in HT-29R cells
To understand the underlying mechanism for pathway elevation in HT-29R cells, we profiled HT-29R versus HT-29S to identify potential genetic alterations. We carried out full exome sequencing of B-Raf, c-Raf, MEK1, and MEK2 in representative sensitive and resistant clones (5 clones from each category). No additional B-Raf mutations were found and no c-Raf and MEK2 mutations were detected (data not shown) in HT-29R cells. Interestingly, we identified a single-nucleotide change (C387A) resulting in amino acid substitution F129L (Fig. 3A) within the MEK1 allosteric drug-binding pocket (23, 24) in 2 clones (R1 and R9). To examine the potential impact of mutant MEK1(F129L) on MEK inhibition, R1 and R9 cells were treated with various concentrations of RO4927350, the p-ERK levels were examined by Western blotting, and EC50 values were determined. As shown in Fig. 3B, the elevated p-ERK in MEK1(F129L)-expressing cells was suppressed by RO4927350 but higher concentrations were required (Fig. 4B). These results suggest that the MEK1(F129L) mutation may be an activating mutation increasing downstream signaling requiring higher drug concentrations for inhibition.

MEK1(F129L) exhibits higher intrinsic kinase activity, binds c-Raf more strongly compared with MEK1(WT), and confers resistance to MEK inhibition
To validate MEK1(F129L) as an activating mutation, we directly measured kinase activity of the purified MEK1(F129L) proteins in a cell-free system. The FLAG-tagged MEK1(WT) and MEK1(F129L) proteins were purified from HEK293 transfectants by using an anti-FLAG M2 affinity gel. Total levels of purified FLAG-tagged MEK1(WT) and MEK1(F129L) were
comparable, but p-MEK levels were significantly higher in the F129L preparation (Fig. 4C, top). Enzyme activities of MEK1 (WT) and MEK1(F129L) were measured in vitro in a cell-free system by quantitating the phosphorylation of ERK peptide by the IMAP assay. Furthermore, we examined the dose–response effect of the MEK inhibitor (RO4987655) on MEK1 (WT) or MEK1(F129L) with or without the addition of c-Raf (Fig. 4C, bottom). In the absence of c-Raf, MEK1(WT) showed negligible basal enzyme activity whereas MEK1(F129L) displayed higher basal enzyme activity than MEK1(WT) correlating with observed higher p-MEK levels within MEK1(F129L) (Fig. 4C, top). Basal activity of MEK1(F129L) can be suppressed by a MEK inhibitor with only a minor increase (~2-fold) in IC50 value as compared with MEK1(WT). In the presence of c-Raf, MEK1(WT) and MEK1(F129L) showed equivalent enzyme activities; however, MEK1(F129L) was more resistant to MEK inhibition than MEK1(WT), with approximately 7-fold-increased IC50 value observed. These results show that the MEK1(F129L) mutation possesses higher intrinsic kinase activity leading to resistance to MEK inhibition.

In MEK1(F129L)-expressing cells, levels of total c-Raf and phosphorylated c-Raf (S338) are not changed as compared with MEK1(WT) (Fig. 4D and data not shown). To further understand the underlying molecular mechanisms responsible for increased activity of MEK1(F129L), we analyzed MEK1 and Raf complex formation in cells. When comparable levels of WT or F129L MEK1 were expressed in HEK293 cells with or without B-RafV600E coexpression (Fig. 4D), MEK1(F129L) significantly activated ERK phosphorylation, although somewhat less than observed with B-RafV600E (Fig. 4D, top). MEK1 complexes were immunoprecipitated with anti-FLAG antibody, and Raf/MEK levels were examined by Western blotting. Increased levels of c-Raf were immunoprecipitated with MEK1(F129L) compared with MEK1(WT) regardless of B-RafV600E coexpression. The immunoprecipitated B-Raf was comparable between MEK1(WT) and MEK1(F129L) (Fig. 4D, bottom). These results suggest that the increased intrinsic kinase activity of MEK1(F129L) may be due to its stronger binding affinity to c-Raf that leads to a sustained MEK activation.

To confirm these findings in cancer cell lines natively harboring the B-RafV600E mutation, MEK1(WT) and MEK1(F129L) were expressed in A375 melanoma cells. Comparable levels of MEK1(WT) and MEK1(F129L) were expressed (detected by anti-FLAG, anti-MEK1, and anti-p-MEK1/2 antibodies, Fig. 5A, bottom). The MEK1(F129L) specifically induced ERK phosphorylation (Fig. 5A, top), bound more c-Raf but not B-Raf (Fig. 5B), and displayed significant resistance to MEK inhibition, as evidenced by a 10-fold increase in the IC50 (Fig. 5C and D). Similar results were observed in LOX melanoma and MDA-MB-435 cancer cells carrying the B-RafV600E mutation (data not shown).

Combined inhibition of MEK and B-Raf overcomes resistance exhibiting greater synergy in HT-29R cells

To determine whether the combination of Raf and MEK inhibition could overcome MEK1(F129L)-conferred resistance, we carried out combination studies using the MEK inhibitor RO4987655 and the Raf inhibitor RG7204 in HT-29S9 and HT-29R1 cells. Cell viability was measured in the presence of each agent alone or in combination. The results were analyzed by CalcuSyn software and displayed as simulated isobolograms.
For both the sensitive and resistant cell lines, the combination IC50 decreased on the lower left of the diagonal, indicating synergism between the 2 agents. Consistently, the calculated CI values were less than 1 representing a synergistic outcome (Fig. 6A). It is worth noting that lower CI values were observed across effective levels (IC50, IC75, and IC90) in HT-29R1 cells than in HT-29S9 cells, suggesting a greater synergy of MEK and Raf inhibition in the resistant clone. Representative results in Fig. 6B clearly show greater synergistic effects of MEK and Raf inhibition in HT-29R (1 and 9) clones than in HT-29S (1 and 9) clones. Greater synergistic effects were also observed in HT-29R than in HT-29S treated with alternative

(Fig. 6A). For both the sensitive and resistant cell lines, the combination IC50 decreased on the lower left of the diagonal, indicating synergism between the 2 agents. Consistently, the calculated CI values were less than 1 representing a synergistic outcome (Fig. 6A). It is worth noting that lower CI values were observed across effective levels (IC50, IC75, and IC90) in HT-29R1 cells than in HT-29S9 cells, suggesting a greater synergy of MEK and Raf inhibition in the resistant clone. Representative results in Fig. 6B clearly show greater synergistic effects of MEK and Raf inhibition in HT-29R (1 and 9) clones than in HT-29S (1 and 9) clones. Greater synergistic effects were also observed in HT-29R than in HT-29S treated with alternative
MEK and Raf inhibitors (RO4927350 and PLX4720, data not shown). Correspondingly, the combination treatment with MEK and Raf inhibitors (at concentrations resulting in >90% growth inhibition) more effectively suppressed p-MEK, p-ERK, and downstream signal cyclin D1 in both sensitive and resistant cells (Fig. 6C). Together, our results indicate that the combination of Raf and MEK inhibition could be a potential therapeutic strategy not only to enhance clinical efficacy but also to bypass or prevent drug-acquired resistance in B-RafV600E patients treated with MEK or Raf inhibitors.

Discussion

Acquired drug resistance remains the major challenge to targeted cancer therapy that leads to patient relapse with limited survival benefit (25). Therefore, it is important to identify mechanisms underlying acquired resistance. By continuously exposing cancer cells to a highly selective anticancer agent in vitro over a period of time, the majority of cells are eliminated and only drug-resistant clones are expanded. This is an experimental strategy that has been successfully used to identify MET amplification as a mechanism of acquired resistance to EGFR kinase inhibitor in NSCLC (26), and c-Raf overexpression as a potential mechanism of acquired resistance to B-Raf inhibitor in melanoma (27). In this study, we established a cell model system (HT-29R) resistant to MEK inhibition. Moreover, we identified a MEK1(F129L) mutation located within the allosteric drug binding pocket of MEK that exhibits elevated intrinsic kinase activity leading to a significant elevation of Raf/MEK/ERK pathway signaling conferring resistance to MEK inhibition. We further showed that this mutation stabilizes the MEK/c-Raf complex formation, a possible mechanism underlying the elevated MEK1(F129L) activity. Combination inhibition of Raf and MEK could overcome the resistance exhibiting greater synergy in the resistant cells. This study suggests that the MEK1 mutation could be a potential drug-acquired resistance mechanism emerging in MEK inhibitor–treated cancer patients.

Somatic mutations in MEK1 and MEK2 have thus far not been reported in human cancers except a heterozygous missense substitution MEK1(D67N) that was identified in an ovarian cancer cell line (28). Germline mutations, including F535S and Y130C MEK1 and F57C MEK2, are the first naturally occurring MEK mutations identified in patients with cardiofaciocutaneous (CFC) syndrome (29). These germline mutations remain sensitive to MEK and Raf inhibition (30). By carrying out a random mutagenesis screen, Emery and colleagues reported that many artificial MEK1 mutations situated within the allosteric drug binding pocket or α-helix C showed robust resistance to MEK inhibition (31). In addition, they showed that other MEK mutations located within the N-terminal negative regulatory helix (A), similar to the germline mutations reported in CFC, also confer resistance to MEK inhibition. One such mutation, MEK1(P124L), was identified as a highly selective MEK inhibitor, RO4927350. HT-29 cells harboring the B-Raf/V600E mutation are originally sensitive to MEK inhibition (18, 19). By comparative profiling of sensitive and resistant clones, we reveal a significant elevation of MEK/ERK and downstream signaling consistent with the acquired resistance to MEK inhibition. Moreover, we identified a MEK1(F129L) mutation located within the allosteric binding pocket that exhibits elevated intrinsic kinase activity leading to a significant elevation of Raf/MEK/ERK pathway signaling conferring resistance to MEK inhibition. We further showed that this mutation stabilizes the MEK/c-Raf complex formation, a possible mechanism underlying the elevated MEK1(F129L) activity. Combination inhibition of Raf and MEK could overcome the resistance exhibiting greater synergy in the resistant cells. This study suggests that the MEK1 mutation could be a potential drug-acquired resistance mechanism emerging in MEK inhibitor–treated cancer patients.
in a resistant metastatic focus that emerged in a melanoma patient treated with the MEK inhibitor AZD6244. We provide experimental evidence to show that the MEK1(F129L) mutation within the allosteric binding pocket occurs after long-term treatment with a MEK inhibitor in B-RafV600E colorectal cancer cells, leading to increased intrinsic MEK1 activation and resistance to MEK inhibition. We further show that the MEK1(F129L) mutation changed the binding affinity to c-Raf, which could be the underlying mechanism of increased MEK1 (F129L) activity. To our knowledge, this is the first report of altered Raf/MEK complex formation leading to a functional impact on the pathway activity. It is intriguing to find phenylalanine (F)-to-leucine (L) substitution on MEK1, a fairly conserved mutation from a structural point of view with minimal physical property changes in size and charge, significantly enhances its intrinsic kinase activity and activates downstream signals. While this manuscript was under review, a new study reported that the MEK1(C121S) mutation was

Figure 6. Combination of MEK and Raf inhibitor showed greater synergy in HT-29R cells than in HT-29S. Cells were treated with MEK and Raf inhibitors (RO4987655 and RG7204) and cell viability was evaluated as described in Materials and Methods. A, the isobologram and CI were determined. B, greater synergy was shown with the representative data points. C, Western blot analysis of cellular proteins in cells treated with RO4987655 and RG7204 as indicated, wherein combination treatment resulted in 92% and 95% growth inhibition in HT-29S and HT-29R cells, respectively.
found in a patient with melanoma who developed resistance to the Raf inhibitor PLX4032 after an initial dramatic response (32).

All resistant clones tested showed significant MEK/ERK pathway elevation. It will be interesting to identify alternative molecular mechanisms in HT-29R clones without MEK1 mutation. Thus far, we were unable to identify any changes of RTK, Ras, and Raf activities in HT-29R clones without MEK1 mutation. Negative regulators of the MEK/ERK pathway such as various phosphatases would be the best candidates for further investigation. It is well known that many levels of cross-talk occur between the MEK/ERK and the phosphoinositide 3-kinase (PI3K)/AKT pathways. We analyzed phosphorylation levels of key PI3K pathway components (AKT, 4E-BP, mTOR) and found that phosphorylation of mTOR (S2448, S2481) was significantly elevated in HT-29R cells (Supplementary Fig. S2). mTOR is autophosphorylated at Ser2448 and phosphorylated at Ser2448 via both PI3K/AKT and/or p70 S6 kinase signaling pathways (33, 34). Interestingly, p-mTOR (S2448) is significantly higher in HT-29R (1 and 9) carrying the MEK1(F129L) mutation. We showed that MEK1(F129L) induced p70 S6 kinase activity (Fig. 4). It has been reported that p70 S6 kinase is a major effector of mTOR phosphorylation at Ser2448 (34). These results suggest that MEK1(F129L) induces p70 S6 kinase activation leading to the elevation of p-mTOR (S2448). Elevated mTOR phosphorylation in HT-29R cells is an interesting molecular mechanism for further investigation of the MEK and mTOR pathway cross-talk.

The Raf inhibitor PLX4032/PLX7204 exhibits impressive antitumor activity in B-RafV600E mutant metastatic melanomas with a remarkable response rate. On the other hand, antitumor efficacy is less promising in colorectal cancer patients bearing the same B-Raf mutation (35), which serves as a reminder of the complexity of cancer; nonetheless, RG7204 may offer hope for colorectal cancer patients as a reminder of the complexity of cancer; nonetheless, RG7204 may offer hope for colorectal cancer patients as part of combination therapy. Combination of Raf and MEK inhibition may be a promising therapeutic strategy (32). Here, we show that the MEK inhibitor–resistant lines are cross-resistant to the highly selective Raf inhibitors PLX4720/PLX4032 but not sorafenib, a nonselective c-Raf inhibitor. This is not unexpected, as sorafenib is characterized as a multikinase inhibitor and its antitumor activity is mainly driven by targeting KDR, Flt-3, etc., to suppress angiogenesis. Inhibition of c-Raf by sorafenib is not sufficient to suppress B-RafV600E cancer cell growth. Our study further implies that combination targeting of Raf and MEK not only broadens the patient population but also provides benefit in preventing or bypassing single drug-acquired resistance.

PI3K/PTEN pathway mutation in tumors significantly affects their sensitivity to MEK or Raf inhibitors. Combination targeting of Raf/MEK and PI3K/akt/mTOR pathways has been reported in many preclinical models (36, 37). For a comparative analysis, we carried out combination studies of MEK and PI3K inhibitors by using RO4927350 and PI-103 in HT-29S and HT-29R cells, in which a synergistic effect was observed in both sensitive and resistant clones, unlike the combination of Raf and MEK inhibitors, comparable CI values were seen (data not shown) consistent with the lack of elevation of p-AKT and p-4E-BP1 levels in HT-29R cells compared with HT-29S cells (Supplementary Fig. S2).

Our data support the need to monitor MEK1 mutation in the clinic during MEK inhibitor treatment, as these may indicate that drug-acquired resistance is occurring in the tumor. RO4987655 is in early clinical development (21). We examined MEK1 exon sequences in 4 archived predose tumors from melanoma and colorectal cancer patients, and as expected, there was no mutation found (data not shown). In the future, it will be interesting to validate our preclinical observations by examining whether MEK1 mutation can be found in patients relapsing following treatment with MEK inhibitors. Combination therapy targeting Raf and MEK may be applied accordingly.

Disclosure of Potential Conflicts of Interest

N. Rosen is a consultant, and other authors are employees of Hoffmann-La Roche Inc.

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References

MEK Inhibitor Acquired Resistance Mechanism


37. She QB, Halliovic E, Ye Q, Zhen W, Shirasawa S, Sasazuki T, et al. 4E-BP1 is a key effector of the oncogenic activation of the AKT and ERK signaling pathways that integrates their function in tumors. Cancer Cell 2010;18:39–51.
Identification of the MEK1(F129L) Activating Mutation as a Potential Mechanism of Acquired Resistance to MEK Inhibition in Human Cancers Carrying the B-RafV600E Mutation

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