Characterization of a Novel Mitogen-Activated Protein Kinase Kinase 1/2 Inhibitor with a Unique Mechanism of Action for Cancer Therapy

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Abstract

The mitogen-activated protein kinase (MAPK) signal transduction pathway plays a central role in regulating tumor cell growth, survival, differentiation, and angiogenesis. The key components of the Ras/Raf/MEK/ERK signal module are frequently altered in human cancers. Targeting this pathway represents a promising anticancer strategy. Small molecule inhibitors targeting MEK1/2 have shown promise in the clinic; however, ultimate clinical proof-of-concept remains elusive. Here, we report a potent and highly selective non-ATP-competitive MEK1/2 inhibitor, RO4927350, with a novel chemical structure and unique mechanism of action. It selectively blocks the MAPK pathway signaling both in vitro and in vivo, which results in significant antitumor efficacy in a broad spectrum of tumor models. Compared with previously reported MEK inhibitors, RO4927350 inhibits not only ERK1/2 but also MEK1/2 phosphorylation. In cancer cells, high basal levels of phospho-MEK1/2 rather than phospho-ERK1/2 seem to correlate with greater sensitivity to RO4927350. Furthermore, RO4927350 prevents a feedback increase in MEK phosphorylation, which has been observed with other MEK inhibitors. We show that B-Raf rather than C-Raf plays a critical role in the feedback regulation. The unique MAPK signaling blockade mediated by RO4927350 in cancer may reduce the risk of developing drug resistance. Thus, RO4927350 represents a novel therapeutic modality in cancers with aberrant MAPK pathway activation. [Cancer Res 2009;69(5):1924–32]

Introduction

The mitogen-activated protein kinase (MAPK) pathway represents one of the best characterized signaling pathways involved in the development and progression of human cancers. This pathway, via the Ras/Raf/MEK/ERK signal cascade, is responsible for transmitting and amplifying mitogenic signals from the cell surface to the nucleus where activated transcription factors regulate gene expression and determine cell fate. The constitutive activation of this pathway is sufficient to induce cellular transformation. Deregulation of the MAPK pathway, due to aberrant receptor tyrosine kinase (RTK) activation and Ras and/or B-Raf mutations, is frequently found in human cancers and represents a major factor in determining abnormal cell growth (1). Ras oncogenic mutations occur in ~30% of all human cancers with the highest incidences in adenocarcinomas of the pancreas (90%), colon (45%), and lung (35%; ref. 2). Oncogenic mutations in B-Raf have been found in 66% of primary melanomas and less frequently in other tumors such as colon (12%), ovarian (30%), and papillary thyroid cancers (35–70%; refs. 3–5). Furthermore, aberrant activation of the MAPK pathway correlates with tumor progression and poor prognosis in patients with various cancers such as breast, colorectal, prostate, renal cell carcinoma, non–small cell lung cancer (NSCLC), and melanoma. Thus, key players of the MAPK pathway are attractive targets for the development of cancer therapeutic agents (1, 6, 7).

MEK1 and MEK2 (MEK1/2) are members of a large family of dual-specificity kinases (MEK1-7) that phosphorylate threonine and tyrosine residues of various MAPKs. Thus far, the only known substrates of MEK1/2 are ERK1/2. Although MEK1/2 mutations have not been found in human cancers, the constitutive expression of MEK1/2 is sufficient to induce transformation (8, 9). Targeting MEK1/2 with a small molecule inhibitor has the potential to prevent all upstream aberrant oncogenic signaling (RTK, Ras, and B-Raf). In the clinic, highly selective MEK inhibitors with related core chemical structures, namely PD0325901 (Pfizer; ref. 10), ARRY142886/AZD6244 (Array Biopharma/AstraZeneca; ref. 11), and XL518 (Exelixis/Genentech) have been evaluated for clinical proof-of-concept (POC) in phase I/II trials. PD0325901 and ARRY142886/AZD6244 have shown partial responses and stable diseases in some patients with pancreatic cancer, NSCLC, and malignant melanoma; however, ultimate clinical POC for these MEK inhibitors has yet to be shown.

In the current study, we report that RO4927350 is a highly selective and potent MEK inhibitor with novel structure and mechanism of action in cancer cells. RO4927350 exhibits significant antitumor activity in vitro and in vivo by selectively blocking the MAPK pathway signaling via a unique tandem inhibition of the phosphorylation of both ERK1/2 and MEK1/2. This unique mode of MAPK signal blockade may provide additional clinical benefit to ensure sufficient and durable pathway inhibition.

Materials and Methods

Compounds. RO4927350, systematic name (2S,3S)-2-[(R)-4-[4-(2-hydroxy-ethoxy)-phenyl]-2,5-dioxo-imidazolidin-1-yl]-3-phenyl-N-[(4-propionyl-thiazol-2-yl)-butyramide, was synthesized according to the
procedure described in patent application (WO2006018188). MEK inhibitor U0126 and ERK inhibitor FR180204 (5-[2-phenylpyrazolo][1,5-a]pyridin-3-yl)-1H-pyrazolo[3,4-c]pyridazin-3-ylamine) were purchased from EMD Biosciences.

**Reagents for kinase assays.** Recombinant human C-Raf, recombinant human glutathione 3-transferase (GST)-free MEK1, and recombinant human GST-ERK2 were prepared as kinases. The FITC-labeled ERK substrate peptide is FITC-AC-Ac-Me-Leu-Thr-Pro-Leu-Seq-Pro-Gly-Pro-Phe-Ala-NH2. IMAP binding reagent (beads) and IMAP binding buffer 5× were purchased from Molecular Devices; Eu-labeled antiphosphotyrosine was purchased from Perkin-Elmer; Phycocyanin goat anti-GST (Type 1)-alloyphocyanin was purchased from Prozyme.

**In vitro MEK kinase (Raf/MEK/ERK cascade) IMAP and homogenous time-resolved fluorescence analyses.** IMAP reactions were carried out in 384-well plates containing serially diluted test compounds. Final reaction conditions were 50 μmol/L ATP, 1 mmol/L active C-Raf, 22.5 nmol/L inactive MEK, 90.5 nmol/L inactive ERK, 0.5 mmol/L FITC-labeled ERK substrate peptide, in the presence of 10 mmol/L HEPES (pH 7.0), 10 mmol/L MgCl2, 50 mmol/L NaCl, 100 μmol/L NaVO4, 1 mmol/L DTT, and 0.2 mg/ml bovine serum albumin (BSA). Reaction mixture (18 μl) was incubated for 1 h at 37°C and stopped by transferring 2 μl of reaction mixture to 30 μl of 1:100 IMAP beads buffer (Molecular Devices). After overnight incubation at room temperature, plates were read for fluorescence polarization (FP) on a LJI Acqreader. FP reading (in mP) was used to calculate reaction rate. All assays were semi-automated by a CybiWell workstation.

**Homogenous time-resolved fluorescence (HTRF) reaction** was carried out under various ATP conditions in 384-well plates containing serial dilution tested compounds. Final reaction conditions were 2.3 mmol/L active C-Raf, 2.3 mmol/L inactive MEK, 28.7 mmol/L inactive ERK, 3 mmol/L Eu-labeled antiphosphotyrosine, 30 mmol/L Phycocyanin goat anti-GST (Type 1)-alloyphocyanin, in the presence of 50 mmol/L HEPES (pH 7.0), 5 mmol/L MgCl2, 100 μmol/L NaVO4, 1 mmol/L DTT, and 0.2 mg/ml BSA. Compounds were incubated with C-Raf and MEK for 30 min at 37°C, then ERK was added for another 30-min incubation at 37°C. The kinase reaction was stopped by the addition of Eu/ACP in the presence of 20 mmol/L EDTA for an additional 1 h incubation at room temperature. Plates were read on an Envision reader. Compound IC₅₀ values were determined from interplate triplicate sets of data.

**Biacore binding assay.** GST cleaved human recombinant MEK was minimally biotinylated with Sulfo-NHS-LC-Biotin (Pierce) in a reagent to protein ratio of 0.31. Free biotin was removed with exhaustive dialysis after 20-h incubation at 4°C. Kinetic binding studies were performed on Biacore SS1. Biotinylated MEK was captured on a Biacore streptavidin sensor chip (Series S Sensor Chip SA) at a density of 5758 resonance units (RU). One RU corresponds to ~1 pg of protein/mm². Test compound was dissolved in 100% DMSO at 10 mmol/L and diluted in a 2-fold concentration series from 2 mmol/L to 1.95 mmol/L. Running buffer was 10 mmol/L HEPES (pH 7.4), 50 mmol/L NaCl, 1 mmol/L DTT, 10 mmol/L MgCl2, 0.005% P20, 50 μmol/L ATP, and 5% DMSO. Kinetic analysis was performed using Biacore BI/evaluation software using a simple model for 1:1 (Langmuir) binding.

**AMBIT kinase profiling assays.** Kinase-tagged T7 phage binding assays for 227 kinases were developed at Abbott Biosciences as described (12).** Antibodies and reagents.** Anti-p-ERK (T292/Y204), anti-p-MEK (Ser27/212), anti-MEK, anti-p-AKT (S473), anti-AKT, anti-p-stress-activated protein kinase (SAPK) (Ser-Jun-NH2 kinase (JNK; T83,Y85), anti-SAPK/JNK, anti-p-p38 (T180/Y182), anti-p38, –Mcl-1, anti-p21, anti-p-Elk (Ser383), anti-p-Mnk1 (Thr197/202), anti-p-ERK1/2 (Ser398). Anti-p-p90RSK(Ser380), anti-PARP, anti-Cyclin D1, and anti-CDK4 were from Cell Signaling Technology. Anti-ERK and anti-p27 were from Upstate. Anti-p-actin was from Sigma. Anti-ε-Myc was from EMD Biosciences. Anti-B-Raf was from Santa Cruz Biotechnology and anti-C-Raf was from BD Biosciences.

**Cell lines.** Cell lines were purchased from American Type Culture Collection and maintained in the designated medium supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies/Bethesda Research Laboratories) and 2 mmol/L L-glutamine (Life Technologies/Bethesda Research Laboratories). The LOX IMVI cells were provided by the Biological Testing Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, NCI-Frederick.

**Western blot analysis.** Cells were seeded at appropriate density (70–75% confluent) in 6-well plates 1 d before compound treatment. Upon compound treatment at various concentrations, the cells were harvested and lysed immediately with 1× cell lysis buffer (Cell Signaling Technology). Where indicated, cells were serum-starved for 16 h, treated with compounds for 1 h, and stimulated with 500 nmol/L phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) for 5 min at 37°C. Tumor tissues were harvested at the indicated time points and stored at −80°C. Protein was extracted by homogenization (Autogestor; TOMTEC) in the presence of 1× cell lysis buffer (Cell Signaling Technology) containing protease inhibitors (Roche Diagnostics). After incubation on ice for 30 min, the lysates were centrifuged at 14,000 rpm for 15 min to clear insoluble fragments. Equal amounts of total protein were resolved on 4% to 12% NuPAGE gradient gel (Invitrogen) and blotted with antibodies as indicated. The chemiluminescent signal was generated with enhanced chemiluminescence Plus and detected with Fujifilm LAS-3000 imager. The densitometric quantitation of specific bands was determined using Multi Gauge Software (Fujifilm).

**3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyldiazotrazolium bromide assays.** The seeds were seeded 1 d before compound treatment and exposed to compounds for 5 d. Cell respiration, as an indicator of cell viability, was measured by the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-diazotrazolium bromide (MTT) to formazan. The formation of MTT into formazan by viable cells was assessed by a microplate reader at 570 nm.

**Cell cycle analysis.** Subconfluent cells were treated with compound for 24 h, harvested, and then rinsed with PBS and fixed with 70% ethanol at −20°C. Cells were stained in a PI/RNase staining buffer (BD Biosciences) at room temperature for 15 min and analyzed for DNA content by flow cytometry. Cell cycle distribution and histograms were generated with FlowJo (Tree Star, Inc.) software.

**siRNA transfection.** HT-29 and DU 145 cells were transfected with 50 nmol/L nontargeting (NT), B-Raf, or C-Raf siRNAs (Dharmacon siGenome Smartpool) using Lipofectamine RNAi Max (Invitrogen). After 48 h, cells were treated with DMSO, RO4927350 (10 μmol/L), or U0126 (10 μmol/L) for 1 h and harvested. Cell lysates were resolved on 4% to 12% NuPAGE gradient gels (Invitrogen) and blotted with antibodies as indicated.

**In vivo xenograft tumor models.** Human cancer cells were implanted s.c. in the right flank of female nude mice (Charles River Laboratories). Once the xenograft tumors reached a mean tumor volume of ~100 to 200 mm³, mice were randomized to treatment groups (n = 10 per group) and received either vehicle or compound at the dose indicated. Tumor volume and mouse body weight were measured twice to thrice weekly. Efficacy data were graphically represented as the mean tumor volume ± SE. Tumor volume (in cubic millimeters) was calculated using the ellipsoid formula: \[ V = \frac{4}{3} \pi \left( \frac{D \times d^2}{2} \right) \] where \( D \) represents the large diameter of the tumor, and \( d \) represents the small diameter. Tumor volumes of treated groups were presented as percentages of tumor volumes of the control groups (%T/C), using the formula: \[ \frac{100 \times (T - T_0)}{C - C_0} \] where \( T \) represents mean total tumor volume of a treated group on a specific day during the experiment, \( T_0 \) represented mean tumor volume of the same treated group on the first day of treatment, \( C \) represented mean tumor volume of a control group on the specific day during the experiment, and \( C_0 \) represented mean tumor volume of the same treated group on the first day of treatment. Percent tumor growth inhibition was calculated using the formula 100-%T/C. Statistical analysis was determined by the rank sum test (SigmaStat, version 2.0, Jandel Scientific). Differences between groups were considered to be significant when the \( p \) value was ≤0.05.

**In vivo evaluation of the pharmacodynamic effect of RO4927350 in peripheral blood mononuclear cells in cynomolgus monkey.** RO4927350 was orally administered to cynomolgus monkey at 50 mg/kg and whole blood was collected pre-dose and at 2, 12, or 24 h postdose. The blood samples were treated with PMA (400 nmol/L) for 5 min at 37°C. Peripheral blood mononuclear cells (PBMC) were isolated using
Lymphocyte separation medium (MP Biomedicals) according to the manufacturer's instructions. Cells were lysed with 1× cell lysis buffer (Cell Signaling Technology). The relative p-ERK/ERK levels were analyzed by Western blot and quantified by Fuji imaging system.

Results

**RO4927350 is a highly selective and potent novel MEK inhibitor in vitro.** Using a Raf/MEK/ERK cascade assay in high-throughput screening, we identified a new class of potent MEK1/2 inhibitors that contain a substituted hydantoin ring. RO4927350 is a representative of this class. Biacore analysis showed its direct binding to unphosphorylated MEK with high binding affinity as evidenced by its fast rate of association and its slow rate of dissociation (Fig. 1A). RO4927350 potently inhibited MEK1 kinase activity with an IC<sub>50</sub> value of 23 nmol/L in a Raf/MEK/ERK cascade assay (Fig. 1B) and showed consistent MEK inhibition in the range of 2 to 250 μmol/L concentrations of ATP (Fig. 1C), which suggests that RO4927350 is a non–ATP-competitive inhibitor. Its high specificity against MEK was determined in various assays (Table 1). RO4927350 was active in C-Raf/MEK/ERK and MEK/ERK assays with a Ki of 0.053 and 0.184 μmol/L, respectively, but inactive against C-Raf (Ki >50 μmol/L) when the compound was tested in the Caliper C-Raf assay using a peptide as substrate (GQLIDS-MANSFVGR-NH<sub>2</sub>). The selectivity of RO4927350 was further shown in both internal (a panel of diverse kinases; Table 1) and external kinase assay panel (Ambit profiling of 227 kinases). RO4927350 at 10 μmol/L was inactive against all other kinases including C-Raf (Raf1) and B-Raf in Ambit profiling (data not shown). Thus, RO4927350 is a novel potent and selective MEK1/2 inhibitor.

**RO4927350 selectively inhibits both ERK and MEK phosphorylation and affects cell proliferation in cancer cells.** As ERK1/2 are the only known substrates of MEK1/2, ERK1/2 phosphorylation (p-ERK1/2) levels were used to determine the degree of MEK1/2 inhibition in human HT-29 colorectal and LOX melanoma cells. Both cell lines harbor B-Raf V600E mutations and have high basal levels of ERK and MEK phosphorylation.

Table 1. Selectivity testing of RO4927350 in various kinase assays

<table>
<thead>
<tr>
<th>Kinase (enzyme)</th>
<th>Assay format</th>
<th>Ki (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-RAF/MEK/ERK cascade</td>
<td>IMAP</td>
<td>0.053</td>
</tr>
<tr>
<td>C-RAF</td>
<td>Caliper technology</td>
<td>&gt;50</td>
</tr>
<tr>
<td>MEK/ERK cascade</td>
<td>Caliper technology</td>
<td>0.184</td>
</tr>
<tr>
<td>ERK</td>
<td>IMAP</td>
<td>&gt;10</td>
</tr>
<tr>
<td>Others (ABL, AKT1, AURORA, CDK2, CHK2, CK16, DYRK1A, EGFR, EphB3, FGFR1, IKK&lt;sub&gt;h&lt;/sub&gt;, IRAK4, KDR, LCK, MST2, NEK2, PDGFR, PIM1, PKD, ROCK2, SRC)</td>
<td>IMAP</td>
<td>&gt;10</td>
</tr>
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NOTE: Caliper assays were performed according to manufacturer's instructions. IMAP assays were performed as described in the Materials and Methods with changing enzyme concentrations and peptide substrates optimized for each testing enzyme.
RO4927350 significantly and dose-dependently suppressed basal levels of ERK phosphorylation (p-ERK; Fig. 2A). Interestingly, RO4927350 also significantly and dose-dependently inhibited basal levels of MEK phosphorylation (p-MEK; Fig. 2B). RO4927350 selectively inhibited Raf/MEK/ERK pathway activity in cells while having no effect on the activation status of other closely related intracellular signaling molecules such as p38 and AKT (Fig. 2B).

Figure 2. Specific inhibition of MAPK pathway signals and cell proliferation by RO4927350 in cancer cells. A, dose-dependent inhibition of ERK phosphorylation by RO4927350 in HT-29 colorectal and LOX melanoma cancer cells. Cells were treated with various concentrations of RO4927350 for 1 h and cell lysates were analyzed by Western blot analysis using antibodies against phosphorylated ERK1/2 (p-ERK1/2); the same blots were then analyzed with antibodies against total ERK1/2. B, RO4927350 selectively inhibits the phosphorylation of both ERK and MEK, but not AKT and p38, in HT-29 colorectal and LOX melanoma cancer cells. Cells were treated with various concentrations of RO4927350 for 1 h and cell lysates were analyzed by Western blot analysis using various antibodies against phospho-proteins as indicated, then the same blots were probed with antibodies against respective total proteins. C, relatively high basal levels of p-MEK/MEK, but not p-ERK/ERK, seemed to be sensitive to RO4927350 treatment in cancer cells. Western blot analysis of basal p-MEK/MEK and p-ERK/MEK levels in various cancer cells. β-Actin as the loading control (top). The charts (bottom) show the relative basal levels of p-MEK/MEK and p-ERK/ERK (X axis) plotted against the IC50 of RO4927350 in the respective WT B-Raf cancer cells with or without Ras mutation (Y axis). The p-MEK/MEK and p-ERK/ERK levels were determined by quantification of the Western blot bands.

RO4927350 was active in suppressing ERK1/2 phosphorylation in many human tumor cell lines with IC50 values ranging from 0.056 to 3.51 μmol/L (Supplementary Table S1). To examine the subsequent cellular consequences of p-ERK suppression, the antiproliferative effect of RO4927350 was examined by MTT assay. RO4927350 potently inhibited cell proliferation in many cell lines with activating pathway gene mutations (B-Raf or Ras) with IC50...
values between 0.29 and 5.2 μmol/L. As previously reported with other MEK inhibitors, tumor cell lines containing the B-Raf V600E mutation were highly sensitive to RO4927350 treatment (Supplementary Table S1; ref. 13). In addition, the results in Supplementary Table S1 indicate that p-ERK suppression alone is not sufficient to predict the antiproliferative activity of MEK inhibitor RO4927350 in cancer cells. To identify additional factors determining the sensitivity of cancer cell lines responding to RO4927350, we profiled both basal p-ERK and p-MEK levels by Western blot (Fig. 2C). B-Raf mutant tumor cell lines were found to have relatively high baseline levels of p-MEK1/2. In B-Raf wild-type (WT) lines, especially in cells with WT Ras and B-Raf, high basal levels of p-MEK, but not p-ERK, seemed to be another predictive factor for tumor cell responsiveness to RO4927350 treatment (such as in BxPC-3 and 22Rv1; Fig. 2C). Comparison of the IC50 values in a paired normal fibroblast Malme-3 (>30 μmol/L) versus malignant melanoma Malme-3M (0.15 μmol/L) cell lines showed that the melanoma tumor cell with B-RafV600E was more susceptible to RO4927350 treatment than its normal counterpart. These results suggest that MEK inhibitor RO4927350 is highly active in cancer cells with aberrant MAPK pathway signaling, which is likely reflected by the phosphorylation levels of MEK1/2 rather than ERK1/2.

**Mechanism of action of MEK inhibitor RO4927350 in cancer cells.** To understand the underlying mechanism of action of RO4927350 in affecting cancer cell proliferation, we analyzed its effect on the cell cycle in tumor cells that were either sensitive or resistant to drug treatment. In responsive HT-29 colorectal cancer cells, specific inhibition of MEK1/2 and ERK1/2 phosphorylation by RO4927350 (Fig. 2B) led to G1 phase cell cycle arrest (Fig. 3A). Correspondingly, treatment with RO4927350 resulted in the down-regulation of CDK4, cyclin D1, and the up-regulation of p21 and p27 (Fig. 3B), key cell cycle regulators for G1-S phase progression. In contrast, in resistant DU 145 prostate cancer cells, RO4927350 potently suppressed ERK1/2 phosphorylation with an IC50 of 0.270 μmol/L (Supplementary Table S1) leading to the up-regulation of p21 and p27 but minimal down-regulation of CDK4 and cyclin D1 (Fig. 3B) and negligible G1 phase of cell cycle arrest (Fig. 3A). Similar results were observed in other sensitive and resistant cancer cells (LOX melanoma and MCF7 breast cancer cells; Fig. 3C). Furthermore, RO4927350 was shown to down-regulate cell cycle regulators c-Myc and Mcl-1, ultimately inducing apoptotic cell death as evident by PARP cleavage in sensitive LOX but not resistant MCF7 cells.

**A novel mechanism of action of RO4927350 that differentiates it from other MEK inhibitors.** The dual inhibition of MEK and ERK phosphorylation is thus far unique to RO4927350 and its analogues, clearly differentiated this class of molecules from other MEK inhibitors that inhibit ERK but not MEK phosphorylation (14, 15). Previous studies have shown a negative feedback mechanism exists in cells that precisely controls hyperactive p-ERK phosphorylation.

Figure 3. Mechanism of actions of MEK inhibitor RO4927350 in cancer cells. A, RO4927350 induces G1 cell cycle arrest only in responsive cancer cells. HT-29 and DU 145 cancer cells were treated with DMSO or RO4927350 (5 μmol/L) for 24 h and cell cycle profiles were examined by fluorescence-activated cell sorting analysis. The values for cell cycle distribution were derived using FlowJo software. B, RO4927350 affects the expressions of genes that are critical to G1-S phase transition in cell cycle. HT-29 and DU 145 cells were treated with RO4927350 for 16 or 24 h, and the cell lysates were analyzed by Western blot using antibodies as indicated. C, RO4927350 induces G1 cell cycle arrest and subsequent apoptotic cell death only in responsive cancer cells. Western blot analysis of cell lysates from LOX or MCF7 cells with or without RO4927350 treatment.
signaling via direct regulation of Raf-1 (C-Raf). Raf-1 activity is sustained in cells treated with U0126, a non-ATP competitive MEK inhibitor (16). Because treatment with RO4927350 results in decreased ERK and MEK phosphorylation in cancer cells (Fig. 2B), it was interesting to examine whether RO4927350 differs from U0126 in this regard. As the results in Fig. 4A show, basal levels of p-MEK are high in HT-29 (B-RafV600E) cells and low in DU 145 (WT Ras/B-Raf) cells. RO4927350 significantly suppressed the basal phosphorylation levels of MEK and ERK in both cell lines, whereas U0126 only weakly inhibited MEK phosphorylation in HT-29 cells and resulted in a dose-dependent feedback increase in p-MEK levels in DU 145 cells. ERK inhibitor (FR180204), as a control, had minimal effect on MEK or ERK phosphorylation. It is worth noting that other MEK inhibitors (PD0325901, AZD6244) currently in clinical development have shown a similar induction of feedback increase in p-MEK levels in WT B-Raf cells (14, 15).

We next compared the effects of RO4927350 and U0126 on mitogen-stimulated MAPK pathway activation in HT-29 and DU 145 cells. When cells were stimulated with PMA (Fig. 4B), the MAPK pathway signaling components were highly activated as shown by the elevated levels of p-MEK, p-ERK, p-RSK, p-ELK, and p-MNK compared with untreated controls (Fig. 4B). RO4927350 suppressed PMA-induced activation of MAPK pathway components including MEK in both cell lines. U0126 only suppressed components of the MAPK pathway downstream of MEK. As expected, ERK inhibitor (FR180204) only inhibited protein activation downstream of ERK in both cell lines.

Previous studies have suggested that Raf-1 (C-Raf) might be the target of negative feedback inhibition after ERK hyperactivation (16). We specifically knocked down B-Raf or C-Raf in HT-29 and DU 145 cells to determine whether the depletion of either Raf could influence U0126-induced feedback activation of MEK. When cells were transfected with control or Raf siRNAs, a significant depletion of B-Raf or C-Raf by the respective siRNAs was shown in the HT-29 and DU 145 cells (Fig. 4C, two top panels). The basal levels of p-MEK and p-ERK were reduced by B-Raf but not C-Raf knockdown.
The B-Raf knockdown resulted in a decrease in basal p-MEK and p-ERK levels in both cell lines regardless of B-Raf mutational status (lanes 2 and 11), although the effect on p-ERK was higher in the B-RafV600E mutant line (HT-29), which is consistent with the current understanding that oncogenic B-RafV600E mutation predominantly drives aberrant MAPK pathway activation in tumor cells bearing this mutation. Knockdown of C-Raf (lanes 3 and 12); however, either had no effect (in HT-29 cells) or led to an increase in basal level of p-MEK (in DU 145). When siRNA-transfected HT-29 cells were treated with RO4927350, significant suppression of both MEK and ERK phosphorylation was observed regardless of Raf knockdown (lanes 4–6). U0126, on the other hand, induced modest inhibition of MEK phosphorylation, which was further enhanced by the knockdown of B-Raf but not C-Raf (lanes 7–9). When siRNA-transfected DU 145 cells were treated with RO4927350, ERK phosphorylation was significantly suppressed with no feedback activation of MEK (lanes 13–15). U0126 induced a feedback increase in MEK phosphorylation (lane 16) similar to the cells depleted of C-Raf (lane 12), and this effect was partially relieved by B-Raf (lane 17) but not C-Raf knockdown (lane 18). These data suggest that MEK phosphorylation levels in tumor cells are mainly driven by B-Raf rather than C-Raf activity. The differential mechanism of inhibition of MEK phosphorylation by RO4927350 in tumor cells prevents a feedback increase of MEK that is observed with other MEK inhibitors. The novel mode of action of RO4927350 has a similar effect as siRNA-mediated B-Raf depletion in tumor cells. U0126, on the other hand, seems to mimic the effects observed in tumor cells after siRNA-mediated C-Raf depletion.

RO4927350 exhibits significant in vivo antitumor efficacy that correlates with its inhibition of MAPK signaling. The antitumor activity of RO4927350 was investigated in the LOX human melanoma xenograft model in nude mice. LOX melanoma cells contain the most common activating B-Raf mutation (V600E). When RO4927350 was administered orally to LOX tumor–bearing mice twice daily with doses from 12.5 to 200 mg/kg for 11 days, dose-dependent antitumor activity was observed, with partial or complete tumor regressions elicited at doses above 50 mg/kg (Fig. 5A). In the same LOX tumor model, after a single oral dose of 100 mg/kg RO4927350, tumors were excised at various time points postdosing and tumor lysates were analyzed for phospho-ERK1/2 (p-ERK1/2), phospho-MEK1/2 (p-MEK1/2), and total ERK and MEK levels. Significant p-ERK1/2 and p-MEK1/2 inhibition in tumors was observed up to 8 hours postdosing from mice treated with the compound compared with the untreated (UT) or vehicle-treated controls (Fig. 5B). Plasma samples were collected from the same animals and the drug concentration in plasma was measured. Pharmacodynamic (PD) effects correlated well with drug levels in plasma (Fig. 5B). These results show that the antitumor activity of RO4927350 correlated well with specific MAPK pathway/target suppression in vivo.

Investigation of the breadth of in vivo antitumor activity of RO4927350 in multiple xenograft tumor models revealed anti-tumor activity that correlated well with in vitro studies. RO4927350 was orally administered twice daily at doses of 50, 100, or 200 mg/kg in tumor bearing mice for 3 to 4 weeks, and produced statistically significant and dose-dependent tumor growth inhibition in a variety of human tumor xenograft models including HT-29 colorectal, MDA-MB-435 breast, MIA PaCa-2 pancreas, and Calu-6 NSCLC (Supplementary Table S2). Partial tumor regressions were observed in HT-29, MIA PaCa-2 and MDA-MB-435 tumor models (Supplementary Table S2). RO4927350 was well-tolerated by the mice without significant body weight loss.

In vivo evaluation of the PK-PD relationship of RO4927350 in cynomolgus monkey. To further evaluate the pharmacologic properties of RO4927350 and establish the PD assay in surrogate tissue such as blood cells, which might help define the optimal dose-effect relationship in a clinical setting, we investigated RO4927350 in a nonrodent species. After a single oral dose of RO4927350 at 50 mg/kg in cynomolgus monkey, whole blood was...
collected at various time points: predose, 2, 12, and 24 hours postdose. The PD effects in PBMCs were examined by measuring PMA-stimulated p-ERK levels using Western blot analysis. The results in Fig. 6 show that RO4927350 significantly suppressed the PMA-stimulated p-ERK increase after a single oral dose.

**Discussion**

The Ras/Raf/MEK/ERK pathway has been extensively studied in cancer biology and its relevance for tumorigenesis has been well-established. Despite many efforts to identify a cancer therapeutic agent specifically targeting this pathway (e.g., Ras, Raf, or MEK inhibitors), none has yet been approved with the exception of the multitkiase inhibitor Sorafenib, which inhibits Raf, but exhibits antitumor efficacy mainly via its antiangiogenesis activity against other targets such as KDR, Flt-3, etc. (17, 18). Thus, ultimate clinical POC with a selective Ras/Raf/MEK/ERK pathway inhibitor for cancer therapy remains elusive. Recently, several MEK inhibitors have entered phase I/II clinical evaluation including PD0325901, AZD6244, and U0126; refs. 14, 15). Similar feedback activation has been observed in tumor cells treated with mammalian target of rapamycin (mTOR) inhibitors that induce a feedback activation of p-AKT. This feedback regulation has been suggested as a potential mechanism for resistance against mTOR inhibitor therapy (21, 22). Thus, the mechanistic differentiation of RO4927350 may provide additional clinical benefit such as a reduced risk of developing drug resistance compared with existing MEK inhibitors. Although ERK1/2 are the only known substrates of MEK1/2, we can not exclude the possibility that MEK1/2 may have other unidentified substrates that are also relevant for tumor cell growth/survival. MEK1/2 fibroblasts were defective in fibronectin-mediated cell migration, despite the fact that ERK1/2 activation was normal, indicating that MEK1 might have additional roles other than activation of ERK (23). RO4927350 might provide ERK-independent, but MEK1/2-dependent, antitumor activity. Moreover, the novel structure of RO4927350 has the potential to avoid chemotype-related liabilities compared with other MEK inhibitors with a similar core structure.

Similar to other MEK inhibitors, mechanistic studies suggest that RO4927350 regulates the downstream gene expression of Cyclin D1, CDK4, p21, and p27 and induces G1 phase cell cycle arrest in a subset of tumor cells. B-RafV600E mutant cells are particularly sensitive to RO4927350 treatment. Moreover, we show that RO4927350 exhibits differential antiproliferative effects in tumor cells that correlates with the difference in regulation of downstream gene expression in these cells. Only in responsive cells, RO4927350 regulates the expression of key apoptotic regulators c-Myc and Mcl-1, resulting in apoptosis. High basal levels of MEK rather than ERK phosphorylation in tumor cells seem to correlate with better responsiveness to RO4927350, especially in tumor cells carrying WT Ras/B-Raf genes. Interestingly, using siRNA-mediated knockdown of B-Raf or C-Raf, we identified a close correlation of p-MEK1/2 levels with B-Raf but not C-Raf activity because the p-MEK levels can be reduced by depletion of B-Raf but not C-Raf in cells (Fig. 4C). The inhibition of p-MEK levels was observed in cells treated with either RO4927350 or B-Raf siRNA, but not with other MEK inhibitors, which suggests that RO4927350 might exhibit antitumor effects in a similar manner as B-Raf knockdown in tumor cells. U0126 seems more closely related to C-Raf knockdown in its mode of action and leads to an increased level of p-MEK in WT B-Raf tumor cells, similar to cells depleted of C-Raf. Our results support the previously reported findings that B-Raf is the more potent activator of MEK in many cells and tissues, whereas C-Raf expression is dispensable for the transformed phenotype in human melanoma cells and may not always represent a predominant effector of MAPK signaling in human cells (24). Consistently, it has been shown that in the presence of similar levels of p-ERK, p-MEK levels are high in B-RafV600E mutant tumor cells but low in B-Raf WT tumor cells. The p-MEK levels are rapidly increased upon MEK inhibitor (PD0325901 and AZD6244) treatment of WT B-Raf but not mutant B-Raf cancer cells (14, 15). The feedback regulation was suggested to occur at/above the level of MEK. B-RafV600E can evade the feedback by up-regulation of the downstream effect proteins such

![Figure 6](https://example.com/figure6.png)

**Figure 6.** Inhibition of p-ERK in PBMC after a single oral dose of RO4927350 in monkey. Cynomolgus monkey was orally given with RO4927350 at 50 mg/kg and whole blood was collected at 0, 2, 12, or 24 h postdose. The blood samples were treated with PMA (400 nM/L) for 5 min, and PBMCs were purified and p-ERK levels in PBMCs were analyzed by Western blot (top). Bottom, the relative p-ERK/ERK levels were quantified.
as DUSPs, Sprouty etc. (14, 15). High basal levels of p-MEK occasionally observed in WT tumor cells may suggest an altered feedback regulation of the pathway in these cells. Interestingly, the WT tumor cells with high basal levels of p-MEK seem to be sensitive to RO4927350 treatment (Fig. 2C). Besides B-RafV600E mutation, identification of additional factors that can evade the feedback regulation of the MAPK pathway in tumors may provide new markers for prediction of response to MEK inhibitor treatment.

To show a clinical utility for MEK inhibitors, key questions still remain unanswered such as in which cancer patient population, what regimens, and what coterapeutic partners may be most effective. RO4927350 inhibits not only basal levels but also mitogen-stimulated increases in MEK1/2 phosphorylation (Figs. 3 and 4). This suggests that RO4927350 may prevent exogenous stimuli-induced MAPK pathway activation. Chemotherapeutic agent–induced MAPK pathway has been suggested to play a role in drug resistance (25). Thus, the combination of RO4927350 with other cancer therapeutic agents may be of benefit in a clinical setting. RO4927350 has shown antitumor activity as a single agent (Supplementary Table S2; Fig. 5). Combination with standard therapeutic agents has also shown additive or synergistic effects (data not shown). Collectively, our studies suggest the potential of RO4927350 and its analogues as targeted cancer therapeutic agents for tumors with aberrant MAPK pathway activation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

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