Dissecting Therapeutic Resistance to ERK Inhibition

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Abstract

The MAPK pathway is a key driver of tumorigenesis, and mutations in the RAS and BRAF genes are among the most common in human cancers. Mutations in RAS occur in a variety of tumor types and are important in driving MAPK and PI3K pathway activation (1, 2). Mutations in BRAF occur in approximately 50% of metastatic melanomas, frequently at the V600E residue, making this the most common oncogenic event in this disease and the primary contributor to MEK and ERK activation.

Introduction

BRAF mutations in melanoma patients lead to constitutive activation of the downstream MAPK pathway. BRAF and MEK inhibitors have demonstrated remarkable antitumor responses in patients with mutant BRAF melanoma. This approach has been extended to patients with mutant V600E melanoma. However, initial tumor shrinkage is transient and the vast majority of patients develop resistance. We previously reported that SCH772984, an ERK 1/2 inhibitor, effectively suppressed MAPK pathway signaling and cell proliferation in BRAF, MEK, and concurrent BRAF/MEK inhibitor-resistant tumor models. ERK inhibitors are currently being evaluated in clinical trials and, in anticipation of the likelihood of clinical resistance, we sought to prospectively model acquired resistance to SCH772984. Our data show that long-term exposure of cells to SCH772984 leads to acquired resistance, attributable to a mutation of glycine to aspartic acid (G186E) in the DFG motif of ERK1. Structural and biophysical studies demonstrated specific defects in SCH772984 binding to mutant ERK. Taken together, these studies describe the interaction of SCH772984 with ERK and identify a novel mechanism of ERK inhibitor resistance through mutation of a single residue within the DFG motif. Mol Cancer Ther; 15(4); 548–59. ©2016 AACR.
We recently reported the discovery and characterization of a novel and specific ERK 1/2 inhibitor SCH772984 with a unique mechanism of action (23). SCH772984 not only inhibits ERK enzymatic activity effectively but also its phosphorylation by MEK. More recently, SCH772984 was shown to be a potent inhibitor of a majority of melanoma cell lines irrespective of their BRAF mutational status and their sensitivity to BRAF inhibitors (24). Although the clinical efficacy of ERK inhibitors has yet to be demonstrated, experience with small-molecule kinase inhibitors such as BRAF or MEK inhibitors suggests that even when high response rates are elicited, tumor cells still retain a strong propensity to develop resistance. Anticipating the development of resistance to ERK inhibitors in the clinic, we undertook a prospective study to investigate the mechanism(s) of resistance to SCH772984. We have also reported previously that SCH772984 demonstrated a more limited efficacy profile in RAS-mutant cell lines as compared with BRAF-mutant cell lines (23). The results presented in this article seek to extend those observations and to understand ERK inhibitor resistance specifically in the context of a KRAS-mutant cell line, not a BRAF-mutant cell line. This impetus to focus on a KRAS-mutant line was due to the lower sensitivity of ERK inhibitors in this context (although we acknowledge that the resistance profile generated in a BRAF-mutant cell line could utilize different mechanisms).

Materials and Methods

Total RNA extraction and cDNA synthesis

Parental or resistant HCT-116 cells untreated or treated with SCH772984 were collected and extraction of RNA was done using the RNeasy Mini Kit (Qiagen), as per the manufacturer's instructions. RNA samples were eluted in 50 μL of nuclease-free water. cDNA was generated from 2 μg of total RNA by using a murine MLV Reverse Transcriptase First-Strand cDNA Synthesis Kit (Applied Biosystems).

Genomic DNA sequencing

Primer extension sequencing was performed by GENEWIZ, Inc on an Applied Biosystems BigDye version 3.1. The reactions were subsequently run on Applied Biosystem’s 3730xl DNA Analyzer for sequencing of all coding exons in KRAS, HRAS, NRAS, MAP2K1 (MEK1), MAP2K2 (MEK2), MAPK1 (ERK2), MAPK3 (ERK1), or BRAF (coding exon 6 and 11-15).

Real-time PCR for MAPK gene expression

PCRs were performed and monitored using an ABI Prism 7900 Sequence Detection system (PerkinElmer, Applied Biosystems). The PCR master mix with AmpliTaq Gold DNA polymerase was used and cDNA samples were analyzed in duplicate. Primers and probes were used at concentrations of 100 and 125 nmol/L per reaction, respectively. After an initial denaturation step of 95°C for 10 minutes, the cDNA products were amplified with 40 PCR cycles (denaturation: 95°C for 15 seconds; extension: 60°C for 1 minute). For each sample, the Ct value was determined as the cycle number at which the fluorescence intensity reached 0.05; this value was chosen after confirming that all curves were in the exponential phase of amplification. Relative expression was calculated using the ΔΔCt method using the following equations: ΔΔCt (sample) = Ct (target) – Ct (reference); relative quantity = 2–ΔΔCt. Differential expression of genes was identified using significance analysis. For each cDNA sample, the Ct value of each target sequence was normalized to the reference genes (GAPDH, tubulin, and actin).

Protein overexpression in stable cell lines

Cells were infected with lentivirus produced from lentiORF constructs (pLOC vector) expressing either RFP, ERK1WT, ERK1G186D, or ERK2WT. Cells were selected in blastocidin (20 μg/mL) for 10 days before Western blotting or testing in proliferation assays.

Western blot analysis

Cell lysates for Western blotting were made using MPER lysis buffer (Thermo Scientific) with protease inhibitor cocktail (Roche). Protein samples (20 μg per well) were separated on 4% to 20% Tris-HCl gels and then transferred to a nitrocellulose membrane. Western blot analyses were probed with antibodies selective for the following proteins: p-ERK1/2 (T202/Y204, Cell Signaling Technologies, cat no. 4695), ERK1/2 (Cell Signaling Technology, cat no. 9107), pRSK (Millipore, cat no. 04-419), RSK (BD Biosciences, cat no. 610226), and actin (Cell Signaling Technology, cat no. 4967).

Cell Lines and treatments

Parental HCT-116 cell line was obtained from ATCC in June 2013 and authenticated using DNA fingerprinting short tandem repeat assays. The cell line was then tested for pathogen contamination. Parental HCT-116 cells were used within four passages before generating HCT-116–resistant cells. Both parental HCT-116 and HCT-116 SCH772984-resistant cells were again tested in February 2014 using fingerprinting short tandem repeat assays and sequenced. Cells were maintained in DMEM with 10% FBS with or without inhibitors. Cell proliferation experiments were performed in a 96-well plate format (six replicates), at a plating density of 4,000 cells per well. At 24 hours after cell seeding, cells were treated with serially diluted inhibitor (10 μmol/L–0.001 μmol/L). Cells were then quantified on day 5 using ViaLight Luminescence kit (Promega) following the manufacturer’s instructions. Data were then analyzed using the SoftMax pro plate reader/software. For resistant cell line generation, HCT-116 cells were grown in DMEM with 10% heat-inactivated FBS media in the presence of increasing concentrations of SCH772984 inhibitor (0.1–2 μmol/L) over approximately 8 months until resistant cells acquired growth properties similar to untreated parental HCT-116 cells.

Protein purification for crystallization

BL21 (DE3) cells were transformed with plasmids coding for rat wild-type or G186D mutant ERK2 protein. Unphosphorylated ERK2 proteins were expressed and purified on ice (4°C) as follows: cell pellets were resuspended in 5 mL/g of lysis buffer (0.05 mol/L sodium phosphate, pH 8.0, 0.3 mol/L NaCl, 10 mmol/L L-mercaptoethanol, 10,000 U/L benzozene, and 5 mL/L of Calbiochem Protease Inhibitor Cocktail Set III), homogenized using a Dounce Homogenizer, and then lysed with three passes through a Microfluidizer. The lysate was centrifuged at an rmax of 200,000 × g for 1 hour to remove cell debris. The supernatant was applied to a Qiagen Ni-NTA superflo agarose column (40 mL) pre-equilibrated with Buffer A (lysis buffer, pH 7.5, without benzozene and protease inhibitors). The column was then washed as follows: 1 column volume (CV) of Buffer A. 3 CV of Buffer B (Buffer A with 25 mmol/L imidazole), 2 CV of Buffer B containing 1 mol/L NaCl, and 2 CV of Buffer A with 45 mmol/L imidazole. The column was...
then eluted with Buffer A containing 250 mmol/L imidazole. The eluted pool was diazylized (three exchanges) against 4 L of MonoQ Buffer (25 mmol/L Tris-Cl, pH 7.8, 0.05 mol/L NaCl, 10% (v/v) glycerol, 1 mmol/L EDTA, and 5 mmol/L DTT). The diazylated Ni-NTA pool was applied to an HR 16/10 MonoQ column at 1 mL/minute. After loading, the column was washed with 1 CV (20 mL) of MonoQ Buffer and then eluted with a linear sodium chloride gradient (600 mL of MonoQ Buffer (0–0.5 mol/L NaCl). The first of two major protein peaks representing unphosphorylated ERK2 (eluted at ~0.15 mol/L NaCl) were pooled and concentrated to 15.8 mg/mL using spin concentrators (10,000 MWCO, Amicon).

Protein crystallization, data collection, and structure determination

Crystals of rat ERK2 [MAHs 1–358 or C167D mutant] were generated using the hanging drop method. Each drop consisted of 2 μL of approximately 15 to 20 mg/mL ERK2 protein (precomplexed with 1 mmol/L olomoucine) and 2 μL crystallization solution (50 mmol/L MES, pH 6.4, 2 M ammonium sulfate, 5% PEG 400, 1% glycerol, 0.5% DMSO, and 1 mmol/L TCEP). The 4-μL drop was seeded on a glass slide and inverted over a reservoir containing 0.7 mL of the same crystallization solution and incubated at 24°C. In 1 to 2 weeks, crystals grew to a sufficient size for use in compound-soaking experiments. A crystal was transferred into 300 μL of a soaking buffer (50 mmol/L MES, pH 6.4, 2 M ammonium sulfate, 5% PEG 400, 1% glycerol, 0.5% DMSO, and 1 mmol/L TCEP). The 4-μL drop was seeded on a glass slide and inverted over a reservoir containing 0.7 mL of the same crystallization solution and incubated at 4°C. In 1 to 2 weeks, crystals grew to a sufficient size for use in compound-soaking experiments. A crystal was transferred into a cryogenic buffer (100 mmol/L MES, pH 6.4, 1.8 M ammonium sulfate, 5% PEG 400, and 27.5% glycerol) for 15 seconds, then cooled into a loop and flash frozen in liquid nitrogen. Diffraction data were obtained at APS IMCA beam line using a MAR M165 CCD for wild-type ERK2 and a Dectris 6M for ERK2G167D. Data collection and refinement data were obtained at APS IMCA beam line using a MAR M165 CCD for wild-type ERK2 and a Dectris 6M for ERK2G167D. Data collection and refinement statistics are summarized in Table 1.

Purification of inactive ERK enzymes

DNA encoding human wild-type ERK1 enzyme (Genbank nucleotide reference sequence NM_002746) with eight additionality N-terminal Histidine residues (His10) was synthesized (Gene-wiz, Inc.) and cloned into bacterial expression vector pGEX6p-3 using the Sall and NotI sites. This construct, when translated, produced an intact recombinant ERK1 enzyme with two N-terminal affinity tags, GST and His10. ERK1C167D was cloned from the wild-type template using a QuikChange kit (Stratagene). Both constructs were transformed into E. coli and induced with 0.5 mmol/L IPTG at OD600 nm = ~0.8, and overexpression was allowed to proceed for approximately 16 hours. The induction temperature for WT ERK1 enzyme and ERK1C167D mutant was 25°C and 16°C, respectively. The inactive forms of WT and mutant ERK1 enzyme were each purified as follows: cells from a 10-L culture were resuspended with 400 mL of lysis buffer (300 mmol/L NaCl, 50 mmol/L Tris, pH 8, 10% glycerol, 0.1% Triton X100, 2 mmol/L DTT, 0.5 mmol/L EDTA, and Roche cocktail inhibitor tablets), and lysed using either a microfluidizer or sonicator. Cell lysates were clarified by ultracentrifugation and the cleared supernatant was batch bound (30 minutes at 4°C) to 15 mL of glutathione beads (GE, cat no. 17-5132-02) precalibrated with wash buffer (1 mmol/L NaCl, 5% glycerol, 25 mmol/L Tris, pH 7.5, 0.25 mmol/L EDTA, and 1 mmol/L DTT). The lyse-beads mixture was poured into a gravity column and unbound proteins were washed with approximately 500 mL of wash buffer or until no protein could be detected from the flow through with Bradford reagent. The target protein was eluted with elution buffer containing 10 mmol/L reduced glutathione (50 mmol/L HEPES, pH 7.3, 10 mmol/L reduced glutathione, 5% glycerol, 1 mmol/L DTT). Target protein was confirmed on SDS-PAGE prior to pooling. Pooled fractions were concentrated to approximately 5 mL (~1.5% of SEC bed volume) and fractionated over a HiLoad 26/60 Superdex 200 Size Exclusion Column (SEC buffer: 25 mmol/L Tris, pH 7.2, 0.26 mol/L NaCl, 10% glycerol, 1 mmol/L DTT, and 0.1 mmol/L EDTA). Fractions containing target protein were pooled and concentrated for storage. Final protein yield with >98% purity from a 10-L culture was approximately 20 mg. ERK2 wild-type and mutant were similarly generated.

Preparation of activated ERK enzymes

The active form of ERK enzyme used in this manuscript was generated through in vitro phosphorylation using a constitutively active MEK1 enzyme. Specifically, purified inactive ERK and active MEK1 were incubated at approximately 1:1 molar ratio at approximately 20 mmol/L complex concentration in the presence of 50 mmol/L HEPES, pH 7.3, 5% glycerol, 1 mmol/L DTT, 20 mmol/L MgCl2, and 4 mmol/L ATP for 2 hours at room temperature, followed by overnight incubation at 4°C. MEK1 was successfully removed from GST-ERK dimers using a Superdex 200 SEC column.

Activated ERK inhibition assays for IC50 determination

The IC50 of SCH772984 for the activated forms of ERK1 and ERK2, and their respective DFG single amino acid substitution

<table>
<thead>
<tr>
<th>Crystal</th>
<th>PDB code</th>
<th>WT rat-ERK2</th>
<th>G167D rat-ERK2</th>
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<tr>
<td>SCH772984</td>
<td>SHD4</td>
<td>SCH772984</td>
<td>SHD7</td>
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Table 1. Statistics for crystal data collection and refinement

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<th>Data collection</th>
<th>Crystal</th>
<th>PDB code</th>
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<td>Wavelength</td>
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<td>Space group</td>
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<td>P2,2,2</td>
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<tr>
<td>Unit cell parameters (Å, °)</td>
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<td>a = 71.5, b = 91.4, c = 63.2</td>
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<tr>
<td>Resolution range (Å)</td>
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<td>15.0-1.45</td>
</tr>
<tr>
<td>Multiplicity</td>
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<td>97.8 (94.7)</td>
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<tr>
<td>Unique reflections</td>
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<td>44,558</td>
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<tr>
<td>Rmerge (%)</td>
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<td>5.1 (6.0)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>97.8 (94.7)</td>
<td>92.7 (91.4)</td>
</tr>
<tr>
<td>R(merge) (%)</td>
<td>5.1 (6.0)</td>
<td>27.6 (3.7)</td>
</tr>
</tbody>
</table>

Refinement

| Rmerge/Refl (% | 17.6/19.9 | 18.1/20.7 |
| No. of protein atoms | 5,675 | 5,872 |
| No. of water | 275 | 274 |
| No. of heteroatoms | 390 | 377 |
| R.m.s.d bonds (Å) | 0.009 | 0.010 |
| R.m.s.d angles (°) | 1.0 | 1.0 |
mutants (ERK1-G186D and ERK2-G169D), were determined using a LANCE Ultra ULight assay (PerkinElmer). IC₅₀ values were determined from a 1:5-point 3-fold dilution titration range (wild-type ERK, 5 mmol/L–1.05 pmol/L; mutant ERK, 100 mmol/L–21 pmol/L) using the following procedure: compound was dispensed into each well of a black nonbinding surface 384-well microplate (Corning, cat no. 3820), followed by the addition of 7.5 μL of 1X kinase buffer (50 mmol/L HEPES, pH 7.3, 0.01% Brij 35, 10 mmol/L MgCl₂, and 1 mmol/L DTT) containing ATP and enzyme. After preincubation (30 minutes), each reaction was initiated by the addition of 2.5 μL 1X kinase buffer containing MBP-Ulight labeled peptide (PerkinElmer, cat no. TRF0109-M). The final reaction in each well (10 μL) contained 125 pmol/L ERK enzyme, 50 mmol/L MBP-Ulight peptide, and 45 μmol/L ATP and <1% DMSO. Phosphorylation reactions were incubated for 90 minutes (ERK1 and ERK1-G186D) or 60 minutes (ERK2 and ERK2-G169D). Reactions were immediately quenched by the addition of 10 μL of 1X quench/detection buffer (PerkinElmer, cat no. CR97-100) containing 4 nmol/L of anti-pY-MBP antibody (PerkinElmer, cat no. TRF0201-M). Following incubation with detection reagents (60 minutes), reaction plates were read on a PerkinElmer EnVision plate reader using a standard TR-FRET protocol. IC₅₀ values were determined by a 4-parameter robust fit of log₁₀ inhibitor concentrations versus TR-FRET ratio values using Prism (GraphPad Software, version 6.04).

Surface plasmon resonance (SPR) experiments were conducted using a Biacore T200 system (GE Healthcare). Recombinant GST-tagged ERK proteins were diluted to 0.5 pmol/L in 1× Biacore running buffer HBS-EP⁺ (GE, cat no. BR-1006-69) containing 3% DMSO and captured on the surface of a preactivated Series S CM5 Biacore chip (GE, cat no. BR-1005-30) using a GST Capture Kit (GE, cat no. BR-1002-23), according to the manufacturer’s instructions. Briefly, anti-GST antibody was immobilized onto the chip by amine coupling, followed by ethanolamine injection to block any remaining active groups. To condition the chip, cycles of GST injection, followed by a regeneration step, were utilized to block any high-affinity GST-binding sites. Final anti-GST antibody immobilization levels were typically approximately 7,500 RU. For kinetic experiments conducted in high-performance mode, surface regeneration was followed by a protein capture step between each inhibitor injection. Surface regeneration solution (10 mmol/L Glycine-HCl, pH 1.8) was injected twice over all four CM5 chip flow cells for 120 seconds at 100 μL/minute. Regeneration was followed by injection of protein (0.5 pmol/L) for 120 seconds at 5 μL/minute across the designated flow cell (2, 3, or 4), with injection of GST (same conditions) over flow cell 1 as a reference. Final ERK protein capture levels were approximately 1,250 ± 150 RU. To determine binding kinetics of the inhibitor to ERK proteins, SCH772984 was then passed over all four flow cells at 50 μL/minute for 180 seconds, followed by a dissociation period of 300 to 1,000 seconds. After the dissociation period, the chip was regenerated back to baseline as described above. This was repeated for each inhibitor injection over nine injections of a 2/3-fold dilution titration range starting between 25 nmol/L and 5,000 nmol/L that included a single replicate. Binding data were solvent corrected and double reference subtracted with a reference flow cell and blank injections to account for any bulk refractive index changes or any small amount of injection noise, baseline drift, and nonspecific binding. Quality control and evaluation were performed using the Biacore T200 Evaluation Software. The entire dataset was fit to either a 1:1 interaction model to give k₉₅, k₉₄, and Kᵥ values or a steady-state binding affinity model yielding Kᵥ. For inactive wild-type ERK1 (capture level ~1,250 ± 150 RU) and inactive wild-type ERK2 (performed in previous experiments with capture level ~3,500 RU), binding experiments were conducted in single-cycle kinetics mode. For inactive wild-type ERK1, inhibitor injections were performed at 50 μL/minute with a contact time of 420 seconds and a dissociation time of 2,400 seconds. Following five sequential injections of a 3-fold dilution titration range of SCH772984 starting at 50 nmol/L, surface regeneration was performed as described above. For inactive wild-type ERK2, inhibitor injections (5 sequential injections of a 3-fold dilution titration range of SCH772984 starting at 27 nmol/L) were performed at 30 μL/minute to accommodate a longer contact time of 600 seconds and a dissociation time of 3,600 seconds. Both datasets were fit to a 1:1 interaction model yielding k₉₅, k₉₄, and Kᵥ values. All assays were run at 25°C in 1× Biacore running buffer HBS-EP⁺ (GE, cat no. BR-1006-69) containing 3% DMSO.

Results

Acquired resistance to small-molecule kinase inhibitors often occurs after prolonged treatment or exposure to drug (26). We have previously published on the discovery of a novel ERK1/2 inhibitor SCH772984 (23). To model the acquisition of resistance to this ERK inhibitor, we used the KRAS-mutant HCT-116 colorectal cell line (termed HCT-116R), which is sensitive to SCH772984 (23). To generate drug resistance, cells were serially passaged in the presence of increasing concentrations of SCH772984 over a period of 4 months. Although the vast majority of cells were rapidly killed in the presence of this inhibitor, cell line pools eventually emerged (termed HCT-116R pool) that were resistant to a high dose of drug as evidenced by robust proliferation in the presence of SCH772984 (2 μmol/L). Proliferation dose–response curves showed that the resistant cell pool was unaffected by up to 10 μmol/L SCH772984 (Fig. 1A). Several single-cell clones were isolated from the resistant pool and cell lines established and cultured in the presence of 2 μmol/L of SCH772984. One resistant clonal cell line (termed HCT-116R) was selected for the series of studies described below to characterize the mechanism of resistance. Proliferation assays showed that HCT-116R was not sensitive to high concentrations of SCH772984 (Fig. 1B). IncuCyte analysis confirmed that proliferation rates of HCT116R cells were comparable with parental cells (HCT116; Fig. 1C and D). Proliferation of HCT116R cells was not significantly affected by the presence of high concentrations of SCH772984. When grown in the absence of SCH772984 for a period of 8 weeks, HCT-116R cells maintained their SCH772984-resistant phenotype, indicating a stable mechanism of resistance (data not shown).

Next, we investigated whether acquisition of drug resistance was accompanied by MAPK pathway reactivation. SCH772984 not only inhibits the intrinsic kinase activity of ERK but also blocks MEK-mediated ERK phosphorylation (23). Phospho-ERK, which is abolished upon compound treatment of HCT-116 cells, is reestablished in resistant cells to levels similar to untreated parental cells. Phospho-RSK, a downstream substrate of ERK, was reduced in parental cells upon SCH772984 treatment. Baseline levels of phospho-RSK can be clearly seen in the resistant cells...
although the levels are lower than that seen with parental cells; importantly, these levels are unresponsive to addition of SCH772984 (Fig. 2A). To compare downstream pathway activity between parental and resistant cells in the presence of SCH772984, we assessed the transcriptional output from a select panel of genes that have been well documented as MAPK-responsive genes in BRAF-mutant melanoma (Fig. 2B). Although the overall output was lower in the resistant cells, we observed clear differences between the parental and resistant cell lines in response to SCH772984 treatment. In each case, although the transcriptional readout in the parental cell line decreased strongly upon drug treatment, the same readout in the resistant line showed little to no change. These data suggest that MAPK downstream transcriptional activity in the resistant line is no longer sensitive to ERK inhibition. To distinguish between compound-specific and pathway-specific resistance, we tested the effect of a previously described ATP-competitive ERK inhibitor and tool compound, VTX-11e. As shown in Fig. 2C, sensitivity of both parental and resistant HCT-116 cells to VTX-11e is essentially similar (IC_{50} of 0.45 µmol/L and 2 µmol/L, respectively). We also evaluated whether the resistant cells still retain sensitivity to inhibition of MEK, the direct upstream kinase of ERK. To this end, we compared the effect of the MEK inhibitor GSK1120212 on parental and resistant cells. As shown in Fig. 2D, GSK1120212 is equipotent at inhibiting pERK in both cell lines (IC_{50} of 5.8 nmol/L in parental cells vs. 1.4 nmol/L in resistant cells). Taken together, these data are consistent with the reactivation of the MAPK pathway in SCH772984-resistant cells, albeit at a lower overall level compared with parental cells; this reactivation, while insensitive to SCH772984, can be abrogated by a MEK inhibitor.

In an effort to identify the mechanism(s) of acquired resistance to SCH772984, the coding regions of RAS, RAF, MEK1, MEK2, ERK1, and ERK2 from 15 single-cell–derived clones including HCT-116\(^{\text{R}}\) were sequenced. Sequence analysis showed that in every clone sequenced, SCH772984 resistance is associated with an acquired mutation in ERK1 in the DFG activation segment with the substitution of glycine\(^{186}\) to aspartic acid. Sequencing traces (with the mutant allelic frequencies high-lighted) for the clone chosen for our studies (HCT-116\(^{\text{R}}\)) are shown in the Supplementary Fig. S1. Sequencing of parental HCT-116 cells did not detect cells carrying ERK1\(^{G186D}\) mutation preexisting in the population. Of note, we also observed ERK2\(^{G180D}\) mutation in cell lines that were made resistant to the ERK inhibitor MK88933 (data not shown). MK88933, which is in the same structural class as SCH772984, is in phase I clinical trials.

To confirm that the \(G186D\) mutation did indeed confer resistance, we overexpressed ERK1\(^{G186D}\) mutant, or wild-type ERK1 or ERK2 proteins, in HCT116\(^{\text{R}}\) cells. Treatment with SCH772984 did not significantly inhibit proliferation (Fig. 3A) or phospho-ERK / phospho-RSK levels (Fig. 3B) in ERK1\(^{G186D}\)–expressing cells, when compared with cells overexpressing wild-type ERK1 or ERK2. These data demonstrate that the presence of mutant ERK1\(^{G186D}\) is sufficient to confer resistance to SCH772984. Given the similarity in the activities of ERK1 and ERK2, and the conserved nature of the DFG motif residues (Fig. 3C), we predict that the analogous mutation in ERK2 might function similarly.

To gain a better understanding of the mechanistic consequence of the mutation, the half maximal inhibitory concentration (IC\(_{50}\)) of SCH772984 for the active forms of both wild-type ERK1 and ERK1\(^{G186D}\) proteins were measured using an \textit{in vitro} TR-FRET

**Figure 1.**
Activity of SCH772984 in SCH772984-resistant HCT-116 cancer cell line pool (A) as well as single cell clone (B) demonstrates that both are resistant to SCH772984 at high concentrations. Viability was determined in 4-day CellTiterGlo assays. The y-axis plots percent viability versus SCH772984 concentration on the x-axis. IncuCyte analysis of real-time growth confluence (imaged every 2 hours) of HCT-116\(^{\text{P}}\) (C) and HCT-116\(^{\text{R}}\) cells (D).
A, SCH772984 potently inhibits in naïve HCT-116 cells but failed to inhibit phospho-ERK and phospho-RSK in resistant cells. B, SCH772984 effectively inhibits MAPK-dependent pathway gene expression in naïve but not in SCH772984-resistant HCT-116 cells. Naïve (HCT-116⁺) or HCT-116⁻ cells were either untreated or treated with vehicle (1% DMSO), SCH772984 (1 or 2 μmol/L) for 4, 16, or 24 hours. Expression of the MAPK-regulated genes was assessed by quantitative TaqMan PCR. Compound-treated samples from parental and resistant cells were normalized to their respective DMSO controls. C, comparison of VTX-11e on HCT-116⁺ and HCT-116⁻ cells (top, proliferation; bottom, target engagement). D, comparison of GSK1120212 on HCT-116⁺ and HCT-116⁻ cells (top, proliferation; bottom, target engagement).
kinase assay. As both enzymes exhibited similar $K_{D}$ (and maximal velocity, $V_{\text{max}}$) values for ATP (see Supplementary Fig. S2), the ATP concentration for wild-type and mutant enzyme was kept at $K_{D}$ (45 μmol/L). Three independent experiments were conducted in parallel for wild-type ERK1 and ERK1G186D proteins. IC_{50} values of 0.04 and 37 nmol/L respectively, were observed (Fig. 4A; Table 2, shaded region) indicating a 1,000-fold reduction in the ability of SCH772984 to inhibit mutant ERK1.

To further investigate the difference in inhibition demonstrated for mutant enzyme by SCH772984, we conducted binding studies using surface plasmon resonance (Biacore T200) in high-performance mode. In all cases, where saturation was attained, the maximal binding signal observed was >90% of the theoretical maximum level within error (see Supplementary Text S1). This indicated that all of the protein was properly folded. Inhibitor binding to the active forms of both wild-type ERK1 (Fig. 4B) and mutant ERK1G186D (Supplementary Fig. S3) was tested across 9-point inhibitor titration ranges of 25 to 0.2 nmol/L (2-fold dilution) and 5,000 to 0.76 nmol/L (3-fold dilution), respectively. A single replicate was incorporated to demonstrate signal reproducibility. The data for active-type ERK1 fit satisfactorily ($\chi^2 = 0.03, U$ value = 1) to a 1:1 binding model (Fig. 4B, black lines). For active mutant ERK1G186D, however, inhibitor binding and release were too rapid to resolve values for the individual on and off rate constants ($k_{on} > 2 \text{ s}^{-1}$, based on the instrument detection limit). Therefore, these data were fitted ($\chi^2 = 0.08$) to a steady-state binding model (solid line, Supplementary Fig. S3), yielding a $K_{D}$ value. Resultant fitted values for all relevant binding parameters for active wild-type ERK1 and mutant ERK1G186D are shown in Table 2 (left region). The equilibrium binding constants ($K_{D}$) determined for both active proteins were approximately 10-fold weaker than the corresponding IC_{50} values determined by TR-FRET. However, the large reduction in binding affinity (~1,000 fold) previously observed with the mutant was similarly observed in this analysis, a shift primarily attributable to differences in the off-rates.

Binding of SCH772984 to both inactive wild-type ERK1 and inactive mutant ERK1G186D was also analyzed using surface plasmon resonance (Biacore T200) in single cycle kinetics and high-performance modes, respectively (Fig 4C and D). For inactive wild-type ERK1, results from the sequential injections at 5 different inhibitor concentrations (50–0.62 nmol/L, 3-fold dilution) were fitted satisfactorily ($\chi^2 = 0.02, U$ value = 1) to a 1:1 binding model. For inactive mutant ERK1G186D, results from a 9-point inhibitor titration, incorporating a single replicate, from 1,666 to 0.76 nmol/L (3-fold dilution) were also fit satisfactorily ($\chi^2 = 0.02, U$ value = 1) to a 1:1 binding model. Values for the fitted parameters are shown in Table 2 (right region). Significantly, the $K_{D}$ values for SCH772984 with both the inactive wild-type ERK1 and inactive mutant ERK1G186D differ by approximately 200-fold, primarily expressed in a difference in the off-rates, with weaker binding to the mutant protein as observed for the active protein.

Given the significant sequence homology between ERK1 and ERK2 (Fig. 3C), we sought to extend these observations to ERK2. IC_{50} of SCH772984 for the active forms of both wild-type ERK2 and ERK2G169D proteins was determined using an in vitro TR-FRET kinase assay as described above for ERK1. The results are shown in Supplementary Fig. S4A and summarized in Supplementary Table S1 (shaded region). An identical set of surface plasmon resonance experiments was also repeated for both active and inactive wild-type ERK2 and mutant ERK2G169D (see Supplementary Information, Supplementary Table S1, and Supplementary Figs. S4 and S5). In general, the results were very similar to those observed for ERK1 (Table 2), with some minor differences (2- to 4-fold). Somewhat tighter binding of SCH772984 was observed with active mutant ERK2G169D (7.5-fold difference) than with active mutant ERK1G186D, but the overall trends remained predominantly the same. It may be worth noting that for both ERK1 and ERK2, $K_{D}$ values determined by Biacore analysis for the inactive wild-type and mutant proteins (Table 2 and Supplementary Table S1, right region) were similar to the IC_{50} values determined for the active wild-type and mutant proteins by TR-FRET analysis (Table 2 and Supplementary Table S1, shaded region). Taken together, these data also suggest that ERK2G169D would be expected to function similarly to ERK1G186D in the context of resistance and that the significant increase in SCH772984 off-rates (upon mutation) is primarily responsible. Finally, recent studies on ERK2 with

Figure 3.
A, overexpression of ERK1G186D mutant makes HCT-116 cells resistant to SCH772984. B, SCH772984 treatment of cells overexpressing ERK1G186D does not affect ERK or RSK phosphorylation levels. C, sequence alignment of human and rat ERK1 and 2 proteins showing conservation of the DFG motif in ERK1 and 2 and across species.
SCH772984, using a binding kinetics approach, also exhibited slow off-kinetics for this compound (27).

To visualize the disruption of ERK binding to SCH772984, we attempted to generate X-ray crystal structures of SCH772984 (Fig. 5A) bound to either wild-type or mutant rat ERK1 or ERK2 proteins. ERK2 bound to SCH772984 yielded crystals of good quality and high resolution. The structures of rat wild-type and ERK2G167D-mutant proteins (corresponding to human ERK1G186D and ERK2G169D; see Fig. 3C) bound to SCH772984 were determined at a resolution of 1.45 Å and 1.70 Å, respectively. Given the high degree of homology between ERK1 and ERK2 (and the observation that the binding mode of SCH772984 is conserved in human ERK1 and ERK2; ref. 28), as well as the results obtained from the surface plasmon resonance studies, we believe the observations from these studies are applicable to ERK1 as well. Rat ERK2 residue numbering is used in the description of the data.

Figure 5B is a surface representation overlay of the structures of apo ERK2 and ERK2 bound to SCH772984. The apo ERK2 structure shows the aromatic rings of Tyr34 and Tyr62 (both in magenta) stacked against each other to form a π–π interaction. Binding of SCH772984 induces a profound shift in the conformation of the glycine-rich loop particularly at Tyr34. The piperazine phenyl pyrimidine portion of SCH772984 induces a movement of Tyr34 (peach) moving it into the ATP site to occupy a pocket created by an approximately 5 Å movement of the glycine-rich loop. This causes the aromatic ring of Tyr34 to pack against the pyrrolidine ring of SCH772984. A small shift in position is also seen with Tyr62 (green ring). These changes would likely be incompatible with ATP binding.

The structures of apo ERK2 and ERK2 in complex with ATP have previously been published (29, 30). The ATP-competitive nature of SCH772984 is evident by its complete occupancy of the ATP-binding site in ERK2 (Fig. 5C). The indazole moiety of SCH772984 hydrogen bonds to the backbone carbonyl and amide nitrogen of hinge residues Asp104 and Met106, mimicking the adenine ring of ATP. The secondary amide linker and pyrrolidine form an extensive hydrogen bond network including the catalytic Lys52 and occupy the area where the triphosphate moiety of ATP binds. The ribose-binding region appears unoccupied by SCH772984. We had previously made the interesting observation that binding of SCH772984 to ERK prevents its phosphorylation by MEK, perhaps due to changes in conformation induced by the compound (23). The structure of ERK2 bound to SCH772984 now reveals the nature of this mechanism of action. Binding of SCH772984 produces a >1-Å movement at the beginning of the C-helix, which is transmitted to the C-terminal tail (Fig. 5D). These movements in the N-terminal domain likely prevent MEK from recognizing ERK2 as a substrate for phosphorylation. This is...
consistent with multiple interaction surfaces on ERK required for effective recognition and phosphorylation by MEK (29). In addition, the presence of SCH772984 in the newly formed binding site along the C-helix could lock ERK2 in a conformation that would not allow the N-terminal domain to generate motions required for phosphorylation upon binding of MEK.

When the binding mode of SCH772984 was compared with an ATP-competitive ERK inhibitor from a different structural class, VTX-11e (Fig. 5F), we observed that two thirds of VTX-11e overlap closely with the pyridine, indazole, and pyrrolidinone portions of SCH772984, whereas the chlorophenyl group of VTX-11e tucks underneath the glycine-rich loop and does not overlay SCH772984. Importantly, Tyr<sup>14</sup> of ERK2 occupies completely different sites when bound to each of the two compounds. The profound distortion of the glycine-rich loop and flipping of Tyr<sup>14</sup> into the ATP site that are induced by SCH772984, do not occur with VTX-11e, which does not extend as far toward the nC-helix of ERK. Thus, the π-π stacking interaction of Tyr<sup>14</sup> and Tyr<sup>65</sup> of ERK is not significantly disrupted upon VTX-11e binding. Another consequence of these different binding modes is that the distance between the CO<sub>a</sub> of G<sup>167</sup> and the nearest nonhydrogen atom of VTX-11e is nearly twice the distance between the CO<sub>a</sub> and the piperazine ring of SCH772984 (7.1 Å vs. 3.2 Å).

The structure of rat ERK<sub>2<sup>G167D</sup></sub>-mutant complex with SCH772984 shows a relatively conserved binding mode compared with wild-type (Fig. 5E). The pyridine, indazole, and pyrrolidinone portions of SCH772984 overlap almost perfectly with what is observed with wild-type ERK2. Significant changes start at the piperazine ring, which moves about 1 Å to avoid a steric clash with the additional atoms from Asp<sup>167</sup> of mutant ERK2. This produces an upward shift of the entire right side of SCH772984 as well as a 4.5-degree counterclockwise rotation about the pyrroline. As a consequence, there is a 1.5 Å movement of the terminal pyrimidine of SCH772984 altering the π-π stacking with the Tyr<sup>21</sup> aromatic ring. Interestingly, Asp<sup>167</sup> of mutant ERK2 forms a new salt bridge with Arg<sup>65</sup>. It is worth noting that although the shifts in the interaction of SCH772984 with mutant ERK2 appear minor, these changes are accompanied by a significant decrease in binding affinity (attributable to a rapid off-rate) observed in the surface plasmon resonance studies and by reactivation of the pathway.

**Discussion**

Acquisition of resistance upon prolonged drug treatment has been acknowledged as a major limitation of therapy with kinase inhibitors (26). Development of therapeutic options that overcome resistance to significantly impact the outcome of these patients depends on understanding the mechanistic basis for the resistance. We have previously shown that, SCH772984, a novel and selective ERK inhibitor, effectively inhibited cell proliferation and ERK signaling in BRAF or MEK inhibitor-resistant models (23) and ERK inhibition could therefore be an effective therapy option for relapsed patients who had reactivated the ERK pathway. In the current study, we consider the possibility that patients treated with an ERK inhibitor would eventually develop resistance. Given that ERK is the final signaling node in the MAPK pathway, understanding the biologic mechanism of ERK resistance should enable the design of the next generation of more effective therapies for patients who become refractory to an ERK inhibitor.

Our data suggest that a mutation in the highly conserved DFG (Asp-Phe-Gly) motif, a central structural unit in kinases, may be an effective way for cells to acquire resistance to ERK inhibition. The acquired ERK<sub>G167D</sub> mutation was accompanied by pathway reactivation and proliferation in the presence of SCH772984. pERK levels that are abolished by compound treatment in parental cells are restored in resistant cells to levels observed in untreated parental cells. Overexpression of ERK<sub>1G167D</sub> in the parental cells is sufficient to mediate the same effect, indicating that mutation of a single amino acid residue is sufficient to confer resistance. These data support the hypothesis that ERK<sub>G167D</sub> is a binding-deficient mutant and not an "activated" mutant. It is also important to note that this is a heterozygous mutation and thus complete resistance is not expected with one wild-type allele still present in the cells. Therefore, it is likely that there is some degree of pathway restoration with the ERK mutant, though not complete. In addition, it is likely that the acquisition of resistance comes at a cost to the ERK enzyme (resulting in a "reduced fitness" phenotype), which is consistent with our observations that basal level of cellular ERK catalytic activity (as measured by pRSK and other downstream genes) is reduced in the resistant cells compared with parental.

How does a mutation of the terminal glycine to aspartate in the DFG motif confer insensitivity to SCH772984? For the sake of consistency with previously published work (29, 30), rat ERK2 amino acid numbering is used in the discussion below. The evolutionarily conserved DFG motif is found at the base of the activation loop and its orientation in kinases ("DFG in" or "DFG out") is associated with an "active" or "inactive" ATP-binding site conformation. In the "DFG in" conformation of ERK, the aspartate side chain of the DFG motif faces into the active site to facilitate catalysis. The neighboring phenylalanine occupies a hydrophobic pocket adjacent to the ATP-binding site. Finally, the glycine holds the active site open by interaction with the C-helix. In particular, interaction of G<sup>167</sup> with specific residues in the C-helix such as Arg<sup>65</sup> (which points toward the active site) and Gly<sup>14</sup>, further promotes the open conformation (31). Thus, the DFG motif plays a crucial role in maintenance of the conformational accessibility of the active site of ERK. SCH772984 binds the active form of the ATP-binding site of ERK, where all the conserved residues are in the proper orientation for catalysis. When Gly<sup>167</sup> is mutated, the aspartate side chains face the active site creating a steric clash that might contribute to instability of SCH773984.

**Table 2.** Summary of TR-FRET assay (IC<sub>50</sub> shaded region) and Biacore data (unshaded region) for SCH772984 binding to both active/inactive wild-type ERK<sub>1</sub> and active/inactive ERK<sub>G167D</sub> mutant, showing weaker binding for ERK<sub>G167D</sub>

<table>
<thead>
<tr>
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<th>Active</th>
<th>Inactive</th>
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<td><strong>ERK WT</strong></td>
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<tr>
<td>K&lt;sub&gt;i&lt;/sub&gt; (nmol/L)</td>
<td>0.04 ± 0.02</td>
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<tr>
<td>K&lt;sub&gt;b&lt;/sub&gt; (nmol/L)</td>
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<td>6.04 ± 0.01</td>
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<tr>
<td>K&lt;sub&gt;m&lt;/sub&gt; (M)</td>
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<td>10&lt;sup&gt;-6&lt;/sup&gt;</td>
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<td><strong>ERK G167D</strong></td>
<td>37.1 ± 14</td>
<td>590*</td>
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<tr>
<td>K&lt;sub&gt;i&lt;/sub&gt; (nmol/L)</td>
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<td>1.16 ± 0.001</td>
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<tr>
<td>K&lt;sub&gt;b&lt;/sub&gt; (nmol/L)</td>
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<td>100</td>
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<tr>
<td>t&lt;sub&gt;1/2&lt;/sub&gt; (min)</td>
<td>14</td>
<td>590*</td>
</tr>
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*See Supplementary Fig. S3.

**Upper limit estimate based on detection limit of Biacore instrument.**
Figure 5.
Structure determination of wild-type and ERK2<sup>G167D</sup> bound to SCH772984. A, chemical structure of SCH772984. B, comparison of the structures of rat ERK2 apo and ERK2 bound to SCH772984. In the apo structure Tyr<sup>34</sup> of the glycine-rich loop (shown in magenta color) is stacked against Tyr<sup>62</sup> (shown as a green aromatic ring). Compound binding causes Tyr<sup>34</sup> of the glycine-rich loop (shown in peach) to flip away from Try<sup>62</sup> and instead pack against the pyrrolidine moiety of SCH772984. The N- and C-terminal lobes are depicted in green and purple surfaces, respectively. MEK phosphorylation sites are highlighted in black, the DFG motif in red, and the hinge including the gatekeeper residue in yellow. SCH772984 is shown as orange sticks. C, overlay of SCH772984 and ATP bound to rat ERK2 protein. Structure of SCH772984 bound to ERK2 confirms the ATP-competitive nature of the compound. The indazole of SCH772984 overlaps the adenosine ring of ATP and the amide linker and pyrrolidine occupy the area bound by the triphosphate moiety of ATP. SCH772984 and ATP are shown as white and yellow sticks, respectively. ERK2 protein is depicted as a yellow ribbon. D, ribbon diagram of wild-type ERK2 bound to SCH772984 (peach color) overlaid with apo ERK2 (magenta) showing movement produced in the N-terminal domain could prevent MEK recognition/binding of ERK2 leading to loss of phosphorylation. ERK2 bound to SCH772984 is shown as peach ribbons and Apo ERK2 is shown as magenta ribbons. E, overlay of the structures of wild-type rat ERK2 (as green and peach colored ribbon) and mutant rat ERK2<sup>G167D</sup> (shown as yellow ribbon) proteins bound to SCH772984 showing a steric clash of the piperazine ring of SCH772984 with the additional atoms of Asp<sup>167</sup> of mutant ERK2. SCH772984 bound to wild-type ERK2 and ERK2<sup>G167D</sup> are shown as orange and yellow sticks respectively. F, overlay of the structures of SCH772984 (orange) and VTX-11e (green) bound to ERK2 showing that the profound distortion of the glycine-rich loop and flipping of Tyr<sup>34</sup> into the ATP site are uniquely induced by SCH772984.
binding to the active site. In contrast, in the case of VTX-11e, we speculate that although the aspartate side chain of mutant ERK forces a shift of SCH772984 leading to destabilization of its binding, the greater distance observed with VTX-11e (Fig. 5F) may preclude any impact of the aspartic acid substitution on its binding to ERK. This may be the basis for our experimental observation (Fig 2C) where proliferation of both parental and resistant cells was similarly sensitive to VTX-11e. We therefore speculate that ERK inhibitors structurally and mechanistically distinct from SCH772984 may be efficacious in cells resistance to it.

Our previous results (23) implied that SCH772984 binds and inhibits ERK by a unique mechanism. Not only does SCH772984 inhibit ERK catalytic activity, but also prevents MEK phosphorylation of ERK. Our structural and biophysical data now demonstrate that the binding site of SCH772984 completely overlaps with the binding site for ATP (Fig. 5C); moreover, SCH772984 binds ERK2 with higher affinity than ATP (0.5 nmol/L and 45 μmol/L, respectively). Thus, once bound, SCH772984 can effectively prevent ATP access to the catalytic site, and by extension, substrate phosphorylation.

With regards to preventing phosphorylation by MEK, we had previously hypothesized that binding by SCH772984 induced or stabilized a conformational state in ERK that was not compatible with MEK phosphorylation. The structure of rat ERK2 bound to SCH772984 shows that although the compound shifts the π-π stacking interaction of Tyr34 and Tyr201 and forces a shift in the position of Tyr34, these changes do not appear to substantially shift the position of the residues Tyr34 and Tyr201 which are phosphorylated by MEK. However, compound binding does produce significant movements in the N-terminal domain via shifting of the positions of the C-helix and C-terminal tail (Fig. 5D). The C-helix is primarily involved in binding ATP and as SCH772984 predominantly occupies the same space as ATP, it pushes against the C-helix. This shift is in turn transmitted to the C-terminal tail. Wilsbacher and colleagues have previously demonstrated that the integrity of a fairly large surface area of the N-terminal structural domain needs to be maintained for a productive interaction between MEK and ERK (31). In particular, the sites of contact between MEK and ERK include residues from the C-helix, C-terminal tail, and the phosphorylation lip. Loss of conformational integrity of the N-terminal domain could destabilize MEK binding and/or its recognition of ERK.

Mutation of the glycine residue in the DFG loop in the resistance setting ensures that SCH772984 does not occupy the ATP site for any significant length of time. The Kᵦ values for SCH772984 with active human wild-type ERK1 and mutant ERK1<sup>G118D</sup> differ by approximately 1,000-fold. In addition, a significant difference in the off-rates was measured with weaker binding (rapid release) to the mutant protein (Fig. 4; Table 2). The acquired mutation thereby alleviates the perturbations to the N-terminal domain induced by SCH772984 binding. Therefore, MEK in SCH772984-resistant cells is able to bind and effectively phosphorylate ERK, which then phosphorylates RSK (Fig. 2A, 3A), resulting in the restoration of downstream transcriptional output (Fig. 2B). As a consequence, SCH772984-resistant cells retain sensitivity to a MEK inhibitor (Fig. 2D) raising the interesting possibility that treatment with a MEK inhibitor could be a therapeutic option for patients who develop resistance to this class of ERK inhibitors.

Collectively, these data support the hypothesis that ERK<sup>G118D</sup> is a binding-deficient mutant and not an "activated" mutant.

Interestingly, we did not identify any mutations in the "gatekeeper" residue in our SCH772984-resistant lines. A number of drug-resistant kinase mutants with altered gatekeeper residues have been identified in multiple kinases that have been effectively targeted clinically: BCR-ABL (T<sup>315I</sup>), EGFRT<sup>299M</sup>, KIT (T<sup>670I</sup>), and ALK (L<sup>1198M</sup>). Mutation of the gatekeeper residue in rat ERK2 (ERK2<sup>L1198S</sup>) to glycine or alanine has been shown to increase autoactivation by enhancing autophosphorylation of the Thr<sup>183</sup> and Tyr<sup>185</sup> in the activation loop (32). Exploring this further, we found that mutation of human ERK2 glutamine<sup>102S</sup> to glutamic acid (Q<sup>102S</sup>) was sufficient to mediate resistance in the presence of an ERK inhibitor through mechanisms that are not fully defined (data not shown). Thus, although no gatekeeper mutations were found in the current study, it is possible that such mutations may arise in patients upon sustained ERK inhibition. It is also worth noting that residue Tyr<sup>34</sup> plays a key role in accommodating SCH772984 in the active site of ERK. In order for the long piperazine–phenyl–pyrimidinyl tricyclic ring of SCH772984 to bind ERK, the side-chain of Tyr<sup>34</sup> flips under the glycine-rich loop toward the adenine-binding site, thus opening up a new side pocket. In this new conformation, Tyr<sup>34</sup> is positioned directly over the pyrrolidine ring of SCH772984, making a favorable hydrophobic interaction. One could speculate that a mutation of Tyr<sup>34</sup> to a residue less amenable to this drastic movement, may contribute to SCH772984 resistance in patients. Indeed, as Chaikuad and colleagues (28) recently showed, mutation of Tyr<sup>34</sup> to glutamic acid or alanine reduced the binding affinity of ERK to SCH772984.

ERK inhibitors are currently undergoing clinical trials in patients with activated or reactivated MAPK signaling. Acquired resistance to these agents may emerge in the clinic. This study is the first to provide preclinical evidence that a single amino acid mutation in the ERK DFG motif can lead to ERK inhibitor resistance. If this mechanism of resistance proves to be clinically relevant, our data suggest that a second-generation ERK inhibitor with a structurally distinct scaffold may be efficacious in this context (Fig. 2C). In addition, the compelling clinical proof-of-concept of BRAF/MEK inhibitor combination efficacy, combination studies with MEK/ERK or BRAF/ERK inhibitors may provide an alternative paradigm of treatment. Indeed, Wong and colleagues (24) recently provided preliminary evidence that chronic exposure of tumor cells in culture to a combination of SCH772984 and the BRAF inhibitor vemurafenib increased the time required to develop acquire resistance. It will therefore be important to further understand how resistance to targeting single nodes in the MAPK pathway compares with these combinations. In particular, selection pressures for mutations in MEK or ERK in the context of concurrent BRAF inhibition will be important to model, to enable administration of therapies most likely to provide maximal patient benefit.

Disclosure of Potential Conflicts of Interest

D. McMasters has ownership interest in Merck & Co., Inc. No potential conflicts of interest were disclosed by the other authors.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Jha, E.J. Morris, A. Hruza, M.S. Mansueto, G.K. Schroeder, J. Arbanas, D. McMasters, C.R. Restaino, A. Cooper, L. Jayaraman, A.A. Samatar

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Jha, E.J. Morris, M.S. Mansueto, S. Black, A. Mannarino, L. Jayaraman, A.A. Samatar

Study supervision: S. Jha, E.J. Morris, L. Jayaraman, A.A. Samatar

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