Discovery of a novel ERK inhibitor with activity in models of acquired resistance to BRAF and MEK inhibitors

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Abstract

The high frequency of activating RAS or BRAF mutations in cancer provides strong rationale for targeting the MAPK pathway. Selective BRAF and MEK inhibitors have shown clinical efficacy in melanoma patients. However, the majority of responses are transient and resistance is often associated with pathway reactivation of the ERK signaling pathway. Here we describe the identification and characterization of SCH772984, a novel and selective inhibitor of ERK1/2 which displays behaviors of both type I and type II kinase inhibitors. SCH772984 has nanomolar cellular potency on tumor cells with mutations in BRAF, NRAS, or KRAS and induces tumor regressions in xenograft models at tolerated doses. Importantly, SCH772984 effectively inhibited MAPK signaling and cell proliferation in BRAF or MEK inhibitor resistant models as well as in tumor cells resistant to concurrent treatment with BRAF and MEK inhibitors. These data support the clinical development of ERK inhibitors for tumors refractory to MAPK inhibitors.

Significance

BRAF and MEK inhibitors have activity in MAPK-dependent cancers with BRAF or RAS mutations. However, resistance is associated with pathway alterations resulting in pERK reactivation. Here we describe a novel ERK1/2 kinase inhibitor that has anti-tumor activity in naïve and MAPK inhibitor resistant cells containing BRAF or RAS mutations.
Introduction

Specific inhibitors of RAF and MEK kinases have been developed to target BRAF and RAS mutant tumors (1-6). In particular, the BRAF inhibitor vemurafenib (formerly PLX4032) has been FDA approved for the treatment of BRAF<sup>V600E</sup> mutant metastatic melanoma (7). Although these results are encouraging, durable responses are limited by acquired resistance occurring at a median time of approximately 6-7 months (2, 8). The combination of the BRAF inhibitor dabrafenib with the MEK inhibitor trametinib has demonstrated significant clinical benefit in BRAF mutant melanoma compared with BRAF or MEK inhibitor monotherapy (1). Interestingly, BRAF/MEK inhibitor combination treatment has been demonstrated to block pERK reactivation in melanoma cells (9, 10), a phenomenon which may possibly reduce the emergence of clinical resistance. Mechanisms of resistance to BRAF/MEK combination therapy are currently unknown but ERK represents an attractive downstream target to exploit. To this end, we developed and characterized SCH772984, a novel, specific inhibitor of ERK1/2 activity and demonstrate robust efficacy in RAS or BRAF mutant cancer cells. Moreover, SCH772984 was also effective in single-agent BRAF or MEK inhibitor resistance as well as in cells that became resistant to the combination of these agents.

Results

A selective ERK inhibitor was identified using an affinity-based mass spectroscopy high throughput platform (11). A library of approximately 5 million compounds was screened for binding to the unphosphorylated form of the ERK2 protein. Synthetic chemistry efforts improved enzymatic potency and selectivity, culminating in
the synthesis of the ATP competitive compound, SCH772984 (Figure 1A). SCH772984 potently inhibited ERK1 and ERK2 activity with IC$_{50}$ values of 4 nM and 1 nM, respectively (Figure 1B). SCH772984 is highly selective with only seven kinases out of 300 tested showing greater than 50% inhibition at a concentration of 1 μM (Supplementary Table S1).

Treatment of the $BRAF^{V600E}$ mutant human melanoma cell line LOXIMV1 (LOX) with SCH772984 resulted in a dose-dependent inhibition of phosphorylation of the ERK substrate p90 ribosomal S6 kinase (T359/S363 phospho-RSK) (Figure 1C). Unexpectedly, SCH772984 also inhibited phosphorylation of residues in the activation loop of ERK itself (T202/Y204 and T185/Y187 of ERK1 and ERK2, respectively), a modification catalyzed by the ERK-activating kinases, MEK1/2. This latter observation was unexpected given that SCH772984 did not directly inhibit MEK1, MEK2, BRAF, or CRAF enzyme activity (Figure 1B and Supplementary Table S1). To further rule out a direct interaction between SCH772984 and MEK, the binding of SCH772984 to MEK1 (or ERK2 as a positive control) was evaluated biophysically using a temperature dependent fluorescence (TdF) assay (Figure 1D and Supplementary Figure S1). As a control, the allosteric MEK inhibitor GSK1120212 specifically bound MEK1. Consistent with results from the kinase panel, SCH772984 bound both purified un-phosphorylated and phosphorylated ERK2 proteins but did not bind to purified MEK1.

In order to further elucidate the mechanism of action of SCH772984, we compared its MAPK pathway inhibition to that of the previously described ATP competitive ERK inhibitor VTX-11e (12, 13). In the $BRAF^{V600E}$ mutant A375 melanoma cell line, both compounds inhibited the formation of phospho-RSK in a dose-dependent
manner (Figure 1E). Interestingly, and in contrast to the inhibition of phospho-ERK seen with SCH772984, phospho-ERK increased in cells treated with VTX-11e. VTX-11e-mediated reactivation of phospho-ERK is likely due to feedback activation as previously described (reviewed in (14)). Increased ERK phosphorylation was similarly observed following PLX4032 treatment in \textit{KRAS}-mutant cell lines (Supplementary Figure S2). This was abolished by treatment with SCH772984 confirming that SCH772984 prevents MEK-mediated ERK phosphorylation. Interestingly, while the inhibition of phospho-ERK and phospho-RSK by SCH772984 was sustained for 24 hours, by 36 hours the inhibition of phospho-ERK was lost while inhibition of phospho-RSK was maintained (Supplementary Figure S3). Further pathway analysis demonstrated that SCH772984 reduced pCRAF S289/S296/S301 phosphorylation (direct ERK phosphorylation sites that inhibit CRAF activity (15)) suggesting that rebound ERK phosphorylation is a result of CRAF upregulation. Taken together, we hypothesize that, in addition to ATP-competitive inhibition of ERK1/2 catalytic activity, SCH772984 induces or stabilizes a conformational state in ERK that prevents activation by MEK and this is sufficient to maintain ERK inhibition even in the presence of feedback signaling by CRAF.

The studies described above established SCH772984 as a potent inhibitor of ERK in \textit{BRAF} and \textit{KRAS} mutant cells. To determine the selectivity of SCH772984 in these contexts, we assessed in vitro anti-proliferative activity in a panel of 121 human tumor cell lines (Figure 2A, Supplementary Figure S4, and Supplementary Table S2). SCH772984 demonstrated EC50 values <500 nM (defined as responders) in approximately 88% and 49% of \textit{BRAF}-mutant (n=25) or \textit{RAS}-mutant (n=35) tumor lines, respectively (Figure 2A). Flow cytometric analysis of SCH772984-sensitive melanoma
cells revealed a G1 arrest as well as an increase in the sub-G1 fraction indicative of apoptosis (Supplementary Figure S5). Less than 20% of cells wildtype for both RAS and BRAF (n=61) were sensitive to SCH772984 (Figure 2A).

In vivo anti-tumor efficacy of SCH772984 was evaluated in BRAF or KRAS mutant xenograft models established from human melanoma or pancreatic carcinoma cell lines. Treatment of BRAF mutant LOX melanoma xenografts with SCH772984 (50 mg/kg b.i.d.) led to 98% tumor regression (Figure 2B). Dose-dependent anti-tumor activity was also observed in the KRAS mutant pancreatic MiaPaCa model with 36% regression at 50 mg/kg b.i.d (Figure 2C). Importantly, tumor regression was accompanied by robust inhibition of ERK phosphorylation in tumor tissue (Figure 2D). SCH772984 was well tolerated on this schedule as measured by morbidity, lethality or body weight loss (Figure 2E).

MAPK inhibitors are currently in development for BRAF and RAS mutant cancers, and the BRAF inhibitor vemurafenib (PLX4032) is approved for the treatment of BRAF-mutant metastatic melanoma. Despite impressive initial clinical responses to MAPK inhibitors, most patients relapse (reviewed in (16)). To determine whether SCH772984 was efficacious in models of BRAF- or MEK-inhibitor resistance, we generated BRAF-mutant melanoma or KRAS-mutant colorectal cancer cell lines resistant to the BRAF inhibitor PLX4032 or the MEK inhibitor GSK1120212, respectively. Cell lines were serially-passaged in the presence of escalating concentrations of compound over a period of 4-8 months. Cell line pools became resistant to high dose PLX4032 (10 µM) or GSK1120212 (1 µM) as evidenced by robust proliferation in the presence of inhibitor (data not shown). Acquired activating mutations in KRAS$^{G13D}$ or NRAS$^{G12C}$ were
identified in PLX4032-resistant *BRAF*-mutant A375 melanoma (Figure 3A-B) or PLX4032-resistant *BRAF*-mutant RKO colorectal (Supplementary Figure S6) lines, respectively. GSK1120212 resistance was associated with acquired mutations in *MEK1* (*MEK1*V211D mutation in *BRAF*-mutant RKO and *MEK1*G128D/L215P mutations in *KRAS*-mutant HCT116; Figures 3C), consistent with previously described alleles which confer AZD6244 resistance (17). Acquisition of drug resistance was accompanied by pathway reactivation as indicated by increased baseline phospho-ERK levels (Figure 3B) and increased transcription of *DUSP6*, *MYC*, *LIF* and *IL8*, well known MAPK target genes (Supplementary Figure S7). SCH772984 demonstrated potent growth inhibition in all of the resistant models described above with IC50s similar to those observed in parental lines (Figure 3 and Supplementary Figure S6). As expected, phospho-ERK, phospho-RSK and mRNA levels for *DUSP6*, *MYC*, *LIF* and *IL8* were all decreased following SCH772984 treatment (Figures 3B and Supplementary Figure S7).

*BRAF* amplification, as well as expression of drug resistant *BRAF* splice-variants lacking N-terminal regulatory regions, have been described as clinical resistance mechanisms in PLX4032-relapsed melanoma patients while *MEK1*P124L was identified in a patient who relapsed on treatment with the MEK inhibitor AZD6244 (10, 17-19). Importantly, *BRAF* amplification has been demonstrated to mediate cross-resistance to MEK inhibitors (10) while *MEK* mutations mediate cross-resistance to BRAF inhibitors (17, 20). To test whether ERK blockade was efficacious in the context of these clinically observed resistance mechanisms, stable cell lines expressing each of these variants were engineered in a *BRAF*V600E A375 background. As shown in Figure 3D, over-expression of *KRAS*G13D (positive control), *BRAF*V600E (to simulate amplification) or *BRAF*V600EΔ2-8 all
conferred resistance to PLX4032 while over-expression of MEKI\textsuperscript{P124L} (residue proximal to N-terminal negative inhibitory domain) conferred resistance to GSK1120212. In addition, expression of MEKI\textsuperscript{F129L} (allosteric binding site mutant with enhanced activity (17, 21)) or MEKI\textsuperscript{DD} (constitutively active mutant with aspartic acid replacement of activating loop serine residues (17)) all mediated resistance to PLX4032 (Supplementary Figure S8). Treatment with SCH772984 was efficacious in all contexts described above, suggesting that cells which circumvent BRAF or MEK inhibitor therapy and reactivate ERK signaling remain dependent on ERK.

In a recent clinical study, the combination of a BRAF inhibitor with a MEK inhibitor doubled the progression free survival benefit relative to either monotherapy (1). As this combination could potentially become the standard-of-care for BRAF\textsuperscript{V600E} melanoma, we sought to develop melanoma models resistant to BRAF/MEK double blockade and evaluate their responsiveness to ERK inhibition. BRAF\textsuperscript{V600E} containing A101D melanoma cells were double-selected with PLX4032 and GSK1120212 until resistant cells proliferated at similar rates compared to parental (Figure 4A). BRAF and MEK inhibitor combination resistance led to strong reactivation of the MAPK pathway as measured by phospho-ERK and phospho-RSK, either in the presence or absence of both compounds (Figure 4B). SCH772984 effectively reduced phospho-ERK/phospho-RSK (Figure 4B) and inhibited proliferation in the dually-resistant A101D melanoma cells in the presence or absence of both BRAF/MEK inhibitors with IC50s similar to the parental line (Figures 4A, C). Similar results were obtained with BRAF/MEK inhibitor combination resistant BRAF\textsuperscript{-}mutant RKO colorectal line (Supplementary Figure S9).
MAPK pathway mutational analysis of parental and dual-resistant lines identified acquired \( NRAS^{G12C}, MEKI^{V21ID}, \) and \( MEKI^{L215P} \) in PLX4032-resistant RKO cells consistent with MAPK pathway reactivation (Supplementary Figure S9). Interestingly, dual-resistant A101D cells did not acquire mutations in \( BRAF, HRAS, NRAS, KRAS, MAP2K1 \) (MEK1), \( MAP2K2 \) (MEK2), \( MAPK1 \) (ERK2), or \( MAPK3 \) (ERK1) in all coding exons analyzed (see Materials and Methods). However, in addition to strong ERK reactivation (Figure 4) we also identified upregulation of \( BRAF, CRAF, PDGFRB, IGF1RB, \) and \( pAKT \) in this line (Supplementary Figure S10) consistent with previous reported resistance mechanisms for vemurafenib as well as cross-resistance mechanisms for MEK inhibitors. Taken together, these results suggest that ERK inhibitors such as SCH772984 could be beneficial for BRAF and MEK inhibitor refractory disease with ERK reactivation.

Discussion

Targeted drug therapy in biomarker selected patient populations is gaining broad acceptance as an effective way to treat cancer based on the inherent genetics of the tumor. Toward that end, SCH772984, a potent and selective ERK inhibitor, was developed for treating malignancies dependent on dysregulated MAPK signaling. Our data implicate ERK inhibition as a therapeutic option for the treatment of patients with \( BRAF, KRAS, \) or \( NRAS \) mutant tumors including patients who relapse on BRAF or MEK inhibitor therapy. SCH772984 possessed strong, preferential activity in a large panel of biomarker-positive (i.e. \( BRAF, KRAS, \) and \( NRAS \) mutant) cell lines \textit{in vitro} and also produced \textit{in vivo} tumor regressions in xenograft models. Like other MAPK inhibitors, SCH772984
demonstrated broader activity in a panel of BRAF-mutant cells than in KRAS- or NRAS-mutants. This was expected given the known importance of MAPK signaling downstream of activated BRAF. However, in the future it will be important to understand why some RAS mutant cells are naturally more resistant to SCH772984 as these mechanisms could limit its clinical utility.

It certain contexts, it has been demonstrated that RAF inhibitor efficacy can be limited by ERK reactivation that occurs within 24 hours (22). Since SCH772984 effectively inhibited both ERK enzymatic activity as well as its phosphorylation by MEK, we wondered whether SCH772984 might have the ability to block ERK phosphorylation in the context of negative feedback pathway activation. Like vemurafenib, SCH772984 induced rebound signaling kinetics. MAPK signaling was nearly completely eliminated through the initial 24 hours of compound treatment. Thereafter, a loss of ERK-mediated inhibitory CRAF phosphorylation (S289/S296/S301) (15) was observed as well as dramatic increases in pERK. Despite negative feedback activation up to and including pERK, SCH772984 maintained a signaling blockade between ERK and RSK. These results suggest that SCH772984 has a stronger ability to inhibit ERK catalytic activity than MEK phosphorylation of ERK (which was apparently overwhelmed in this setting). Furthermore, this durable inhibition of pRSK in the context of negative feedback, favorably distinguishes SCH772984 from BRAF inhibitors.

While tumors can take multiple paths to RAF inhibitor resistance, one common feature appears to be the reactivation of the ERK pathway. Because of its ability to inhibit at a distinct, downstream intervention point in the MAPK pathway, SCH772984 has the potential to overcome resistance induced by MEK or BRAF inhibitors. We
examined SCH772984 in various models of previously described, clinically relevant BRAF or MEK inhibitor resistance, including acquired RAS mutations, acquired MEK1 mutations, overexpression of BRAF\textsuperscript{V600E} or a splice-variant lacking an amino-terminal RAS-binding domain, as well as ectopic expression of various MEK1 mutants (including constitutively-active). In all scenarios tested, SCH772984 treatment produced IC\textsubscript{50} values in viability assays similar to what was observed in parental cells. One exception was the overexpression of either BRAF\textsuperscript{V600E} or a BRAF\textsuperscript{V600E} splice-variant lacking exons 2-8 (insensitive to vemurafenib inhibition due to constitutive dimerization) whereby we observed ~10 fold IC\textsubscript{50} shift toward cross-resistance. Currently, the mechanism responsible for this low-level cross-resistance is unknown and further study is necessary. However, when we attempted to hyper-activate ERK to high levels (i.e. overexpression of constitutively-active MEK1), we observed little-to-no cross-resistance. This suggests that the ERK node may represent a rate-limiting step within the MAPK pathway that is subject to less fluctuation with regard to biological signaling (and hence less potential for IC\textsubscript{50} shift during cross-resistance). For these reasons, targeting ERK may be more effective than targeting MEK especially in light of evidence suggesting limited clinical trametinib activity in BRAF inhibitor refractory melanoma (23).

Development of effective combination strategies is expected to reduce the incidence of clinical resistance that is common with single agent targeted therapies. The combination of BRAF and MEK inhibition in BRAF-mutant melanoma is a good example of the clinical benefit that can be obtained from this approach. Although the clinical mechanisms of resistance to these combinations are largely unknown, we expect that multiple mechanisms resulting in pathway reactivation, or perhaps a single downstream
mechanism, might be sufficient for tumor escape. In this study, we demonstrate pERK reactivation in two models of BRAF and MEK combination therapy resistance. SCH772984 was effective in blocking proliferation in both models. Interestingly, in one model the dual-resistant cells did not acquire mutations in the MAPK pathway but rather upregulated known RTK’s previously described to mediate PLX4032 resistance as well as cross-resistance to MEK inhibitors (i.e. PDGFRB) (24). Acquired RTK activation in dual-resistant A101D cells was accompanied by both pERK and pAKT activation. However, this cell line was still dependent on ERK signaling as demonstrated by its upregulation of BRAF and CRAF as well as its response to SCH772984. Taken together, our results suggest that clinical resistance to BRAF/MEK combination therapy will likely involve multiple mechanisms resulting in ERK pathway reactivation. Moreover, our data indicate that these mechanisms will likely be similar to those reported to mediate resistance to BRAF or MEK as single agents.

Given that ERK is directly downstream of BRAF and MEK, targeting the MAPK pathway at the level of ERK might offer unique advantages in a variety of MAPK resistance settings. Moreover, ERK directly signals to over 100 known cellular proteins that are important for tumor cell proliferation, survival, and apoptosis (i.e. pRsk, Myc, Bim, etc.). It will be important to define the optimal point of intervention in this critical signaling pathway in order to aid the development of effective therapeutics for BRAF and RAS mutant cancers. SCH772984 and related compounds provide potent and selective tools to help address this question either alone or in combination with other MAPK inhibitors.
Materials and Methods

Cell lines and treatments. For resistant cell line creation, cells were grown in Dulbecco’s modified Eagle medium (DMEM) with 10% heat-inactivated FBS media and increasing concentration of inhibitor (PLX4032 0.1-10 \( \mu M \); GSK1120212 0.01-1 \( \mu M \)) over ~4-8 months until resistant cells acquired growth properties similar to naïve parentals (at their top drug concentrations). For combination resistance, cells were incubated as above but with alternative dose escalation until a top-concentration was acquired (PLX4032 10 \( \mu M \) and GSK1120212 1 \( \mu M \)). The identities of all cell lines used for these studies were confirmed using short tandem repeat (STR) DNA analysis at ATCC. Stocks and dilutions of PLX4032, GSK1120212, and SCH772984 were made in DMSO solvent. Cell proliferation experiments were performed in a 96-well format (six replicates), and cells were plated at 4,000/well density. At 24 h after cell seeding, cells were treated with DMSO or 9 point IC50 dilution (0.001-10 \( \mu M \)) at 1% DMSO final for all concentrations. Viability was assayed on 5 days after dosing using ViaLight luminescence kit (Lonza) following the manufacturer’s recommendations (N=6, mean ± standard error). For cell line panel viability assay, cells were treated with SCH772984 for 4 days and assayed by CellTiterGlo luminescent cell viability assay (Promega). For Incucyte analysis, cells were plated as above in 96-well plates and image-based cell confluence data collected every 2 hours during live growth. For engineered resistant lines, cells were infected with lentivirus produced from lentiORF constructs (pLOC vector) expressing either red fluorescent protein (RFP), \( KRAS^{G13D} \), \( BRAF^{V600E} \), truncated \( BRAF^{V600E} \) lacking exons 2-8 (\( \Delta2-8 \)), \( MEK1^{P124L} \), \( MEK1^{F129L} \), or constitutively-active
MEK<sup>DD</sup> (S218D+S222D). Cells were blasticidin selected (20 µg/ml) and used for ViaLight assays as described above.

**Genomic DNA sequencing.** Primer extension sequencing was performed by GENEWIZ, Inc (South Plainfield, NJ) using Applied Biosystems BigDye version 3.1. The reactions were then 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).

**Total RNA extraction and cDNA synthesis.** Treated cells were collected with lysis buffer and extraction of RNA was performed using the RNAesy mini Kit (Ambion) as per manufacturer's directions. RNA samples were eluted in 50 µl of nuclease free water. cDNA was generated from 2 µg of RNA by using murine MLV reverse transcriptase first-strand cDNA synthesis kit (Applied Biosystems).

**Real-time PCR for MAPK gene expression.** PCR reactions were performed and monitored using an ABI Prism 7900 Sequence Detection system (Applied Biosystems). The PCR master mix was based on AmpliTaq Gold DNA polymerase; cDNA samples were analyzed in duplicate. Primers and probes were used at concentrations of 100 and 125 nM per reaction, respectively. After an initial denaturation step of 95°C for 10 min, the cDNA products were amplified with 40 PCR cycles (denaturation: 95°C for 15 s; extension: 60°C for 1 min). For each sample, the C<sub>t</sub> value was determined as the cycle number at which the fluorescence intensity reached 0.05; this value was chosen after
confirming that in this range all curves were in the exponential phase of amplification. Relative expression is calculated using the delta-Ct method using the following equations: \( \Delta Ct \text{ (Sample)} = Ct \text{ (Target)} - Ct \text{ (Reference)}; \) relative quantity = \( 2^{-\Delta Ct} \).

Differentially-expressed genes were identified using significance analysis. For each cDNA sample, the \( C_t \) value of each target sequence was normalized to the reference genes (GAPDH, tubulin, and actin). Primer sets were obtained from Applied Biosystems for the following genes: \textit{DUSP6} (Hs00737962), \textit{MYC} (Hs00905030), \textit{LIF} (Hs00171455), \textit{IL8} (Hs00174103), \textit{GAPDH} (Hs02758991), \textit{ACTIN} (Hs00357333), and \textit{TUBULIN} (Hs00733770).

**Protein detection.** Cell lysates for western blotting were made in MPER (Thermo-scientific) lysis buffer with protease inhibitor cocktail (Roche). Protein (20 ug) was separated on 4-20\% Tris-HCL gel then transferred onto nitrocellulose membrane. Western blots were probed with antibodies against phospho-ERK1/2 T202/Y204 (Cell Signaling Technologies; CST #4695), ERK1/2 Cat# 9107 (CST #4695), phospho-RSK T359/S363 (Millipore #04-419), RSK (BD bioscience #610226), phospho-MEK1/2 S217/S221 (CST #9154), MEK1/2 (CST #9126), phospho-AKT S473 (CST #9271), AKT (CST #9272), phospho-BRAF S445 (CST #2696), BRAF (CST #9433), phospho-CRAF S289/S296/S301 (CST #9431), CRAF (CST #9432), PDGFRB (CST #4664), IGF1RB (CST 3027), GAPDH (Millipore #CB1001), and Actin (CST #4967).

**ERK2 IMAP enzymatic assay.** SCH772984 was tested in 8 point dilution curves in duplicate against purified ERK2 or ERK1. The enzyme was added to the reaction plate
and incubated with the compound before adding a solution of substrate peptide and ATP. 14µl of diluted enzyme (0.3ng active ERK2 per reaction) was added to each well of a 384-well plate. The plates were gently shaken to mix the reagents and incubated for 45 minutes at room temperature. The reaction was stopped with 60µl of IMAP Binding Solution (1:2200 dilutions of IMAP beads in 1X Binding Buffer). The plates were incubated at room temperature for an additional 0.5 hours to allow complete binding of phosphopeptides to the IMAP beads. Plates were read on the L JL Analyst.

**Cell cycle analysis by fluorescence-activated cell sorting.** Cell cycle analysis was performed by propidium iodide (PI) staining. LOX cells were collected after trypsinization, washed in PBS and centrifuged for 2 min at 1500xg. The cells were resuspended and fixed in 70% ethanol for at least 30 minutes on ice or overnight at -20°C. The cell pellets were washed with PBS and resuspended in 0.5 ml of propidium iodide (50 µg/ml)/0.1% Triton X-100 solution containing RNase and incubated at room temperature in the dark for 30 minutes. The PI stained cells were analyzed on a Becton Dickinson FACS Calibur flow cytometer Becton Dickinson, San Jose, CA, USA). The cell cycle profile was determined using both CellQuest (Becton Dickinson) and ModFit 3.0 (Verity Software, Topsham, ME, USA). CellQuest was used to acquire data and also determine the percentage of cells in the sub-G1 population. ModFit 3.0 software was used to determine the percentage of cells in G1, S and G2.

**Xenograft tumor growth assay.** Nude mice were injected subcutaneously with specific cell lines, grown to approximately 100 mm³ and randomized to treatment groups (10
mice per group) and treated intraperitoneal (i.p.) with either SCH772984 or vehicle according to the dosing schedule indicated in the figure legends. Tumor length (L), width (W), and height (H) were measured during and after the treatment periods by a caliper twice weekly on each mouse and then used to calculate tumor volume using the formula \((L \times W \times H)/2\). Animal body weights were measured on the same days twice weekly. Data was expressed as mean ± SEM. Upon completion of the experiment, vehicle- and SCH772984 treated tumor biopsies were processed for western blot analysis.

**Kinase panel screening.** The kinase inhibitory profile of SCH772984 was evaluated over a 310-kinase panel at Invitrogen.

**TdF Assay.** The unfolding of rat ERK2, rat phosphorylated ERK2 and human MEK1 proteins were performed at a final concentration of 0.5 μM each in the TdF assay buffer (10mM HEPES pH 7.4, 150mM NaCl, 5mM MgCl2, 1mM DTT) containing a final concentration of 5x Sypro Orange reporter dye. The samples contained 10, 5 or 2.5 μM compound at a final DMSO concentration of 2%. Samples were run at 5 μL in a white 384-well real time PCR plate (Roche Applied Science). The plates were sealed with a clear sealing film (Roche Applied Science) and assayed in the LightCycler 480-II (Roche Applied Science). The temperature was ramped from 23°C to 99°C in 15 minutes. The fluorescent intensity was collected at 15 data points per degree with excitation at 465 nm and emission at 580 nm.
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References


Supplemental information is included as a separate file.

Author contributions

Figure legends

**Figure 1.** Biochemical and cell-based activity of SCH772984. (A) Chemical structure of SCH772984. (B) Effects of SCH772984 on kinase activity of ERK1, ERK2, and MEK1. (C) SCH772984 inhibits ERK & RSK phosphorylation. LOX *BRAF^V600E* melanoma cells were treated with increasing concentrations of SCH772984 and lysates were subjected to Western blot analysis using antibodies against phosphorylated ERK1/2 (pERK), phosphorylated p90 ribosomal S6 kinase (pRSK), total ERK (tERK), or total RSK (tRSK). (D) TdF binding activity of SCH772984, VTX-11e, or GSK1120212 to recombinant ERK2 and MEK1 enzymes. (E) Target engagement on pERK/pRSK for SCH772984 versus VTX-11e in *BRAF*-mutant A375 cells treated for 4 hours.

**Figure 2.** SCH772984 is efficacious in *BRAF* or *RAS* mutant tumor cells. (A) In vitro activity of SCH772984 in a panel of human tumor cell lines (summary of primary data from Supplementary Figure S3). (B) In vivo activity of SCH772984 in human LOX *BRAF*-mutant melanoma xenograft model. Female nude mice bearing human LOX *BRAF^V600E* tumors > 100 mm³ in size were dosed twice daily with vehicle or SCH772984 (12.5, 25, or 50 mg/kg) for 14 days (n=10 per group). Dosing occurred from day 1 to day 14. Tumor volume was measured and growth curves were derived from the mean tumor volume in each dose. The percent change in volume is shown after 14 days. Tumor regressions were observed at all doses (17% at 12.5 mg/kg, 84% at 25 mg/kg and 98% at 50 mg/kg). (C) In vivo activity of SCH772984 in human MiaPaCa *KRAS*-mutant pancreatic xenograft model. Mice were dosed twice daily with vehicle or with 25 mg/kg of SCH772984 or 50 mg/kg. Tumor regressions were observed at both doses (9% tumor
regression at 25 mg/kg and 36% tumor regression at 50 mg/kg dose). (D) Target engagement of SCH772984 on phospho-ERK in LOX melanoma xenograft tumors. On day 14 after the last dose (6 hours) of 12.5 mg/kg of SCH772984 (lanes 4-5) or vehicle (lanes 1-3) LOX xenograft tumors were harvested (3 mice per group) and homogenates from excised tumors were probed for phospho-ERK1/2 and total ERK1/2 levels by western blots. (E) Body weights of female nude mice with SCH772984 relative to control animals (in MiaPaCa xenograft). No drug related lethality was observed at all doses.

**Figure 3.** SCH772984 is efficacious in tumor cell lines refractory to either BRAF or MEK inhibitors. (A) SCH772984 is equally potent in naïve or PLX4032-resistant, **BRAF**-mutant A375 melanoma cells (A375BR). (B) SCH772984 potently inhibits phospho-ERK and phospho-RSK in naïve and PLX4032-resistant **BRAF**-mutant A375 melanoma cells. (C) SCH772984 is equally potent in naïve or GSK1120212-resistant, **BRAF**-mutant RKO colorectal cancer cells (RKOMR) or **KRAS**-mutant HCT116 colorectal cancer cells (HCT116MR). (D) SCH772984 is efficacious in BRAF mutant melanoma cells engineered to express various clinically-relevant BRAF or MEK inhibitor resistance mechanisms. The ectopic expression of **KRAS**<sup>G13D</sup> (as positive control), **BRAF**<sup>Y600E</sup>, or **BRAF**<sup>Y600EΔ8</sup>, but not red fluorescence protein (RFP as negative control), induces resistance to PLX4032. Ectopic expression of **MEK1**<sup>P124L</sup> induces resistance to GSK1120212. In all cases, little to no cross-resistance was observed with SCH772984. Cells were treated for 5 days followed by ViaLight assay. Overexpression of each was
confirmed by western blot (data not shown). All data is depicted as mean ± standard error (N=6).

**Figure 4.** SCH772984 is efficacious in BRAF and MEK combination resistance in *BRAF*-mutant A101D melanoma cells. (A) Incucyte analysis of real time growth confluence (imaged every 2 hours) of parental and A101D BR + MR cells demonstrates similar growth rates despite the presence of high concentrations of PLX4032 (10 μM) and GSK1120212 (1 μM). Treatment with SCH772984 (either alone or in combination with BRAF + MEK inhibitor) is efficacious in both naïve and resistant lines. (B) A101D BR + MR cells acquire ERK pathway reactivation which is inhibited by SCH772984 (both in the presence and absence of the BRAF/MEK inhibitor combination during the experiment). (C) SCH772984 treatment IC50's on 5-day ViaLight viability assays for A101D parental and A101D BR + MR cells (both in the presence or absence of BRAF/MEK combination during initial plating; 10 μM PLX4032 + 1 μM GSK1120212). Data is derived from 10-point dose-titrations as described in Materials and Methods with all data is depicted as mean ± standard error (N=6).
**Figure 1**

**A**

![Chemical structure](image)

**B**

<table>
<thead>
<tr>
<th>Kinase</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
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<tbody>
<tr>
<td>ERK2</td>
<td>0.001</td>
</tr>
<tr>
<td>ERK1</td>
<td>0.004</td>
</tr>
<tr>
<td>MEK1</td>
<td>&gt;10</td>
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**C**

![Umbral](image)

**D**

<table>
<thead>
<tr>
<th>Compound</th>
<th>ERK (K&lt;sub&gt;D&lt;/sub&gt; (nM))</th>
<th>TM</th>
<th>pERK (K&lt;sub&gt;D&lt;/sub&gt; (nM))</th>
<th>TM</th>
<th>MEK (K&lt;sub&gt;D&lt;/sub&gt; (nM))</th>
<th>TM</th>
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<tbody>
<tr>
<td>GSK1120212</td>
<td>No binding</td>
<td>TM</td>
<td>No binding</td>
<td>0.23 ± 0.32</td>
<td>7 ± 2.7</td>
<td>8.0 ± 0.51</td>
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<tr>
<td>SCH772984</td>
<td>0.24 ± 0.4</td>
<td>10.3 ± 0.30</td>
<td>0.19 ± 0.12</td>
<td>9.8 ± 0.23</td>
<td>No binding</td>
<td>-0.17 ± 0.41</td>
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<tr>
<td>VTX-11e</td>
<td>5.4 ± 2.2</td>
<td>6.8 ± 1.3</td>
<td>0.11 ± 0.62</td>
<td>10.3 ± 1.3</td>
<td>No binding</td>
<td>-0.1 ± 0.3</td>
</tr>
</tbody>
</table>

**E**

![Western blot](image)
Figure 2

A

RAS genotype

% responders (EC50 < 500 nM)

wildtype  N = 61
KRAS mutant  N = 28
NRAS mutant  N = 7
BRAF mutant  N = 25

B

LOX (BRAF mutant melanoma)

Days of Treatment

Tumor Volume (mm$^3$)

Vehicle

SCH 772984 (12.5 mpk, i.p., bid)

SCH 772984 (25 mpk, i.p., bid)

SCH 772984 (50 mpk, i.p., bid)

C

MiaPaca (KRAS mutant pancreatic)

Days of Study

Tumor Volume (mm$^3$)

Vehicle (i.p., bid)

SCH 772984 (25 mpk, i.p., bid)

SCH 772984 (50 mpk, i.p., bid)

D

LOX (BRAF mutant melanoma)

Vehicle SCH772984 (12.5 mg/kg)

1 2 3 1 2 3

pERK ERK

E

MiaPaca (KRAS mutant pancreatic)

Days of Study

Body Weight (g)

Vehicle (i.p., bid)

SCH 772984 (25 mpk, i.p., bid)

SCH 772984 (50 mpk, i.p., bid)
Figure 3

A. Parental and BRAFi-resistant BRAF-mutant melanoma

B. BRAF-mutant melanoma target engagement (A375)

C. Parental and MEKi-resistant BRAF-mutant colorectal

D. Parental and MEKi-resistant KRAS-mutant colorectal
Figure 4

BRAFi + MEKi combination resistance in BRAF-mutant melanoma

A101D parental (Incucyte)

A101D parental (IncuCyte)

A101D BR+MR (IncuCyte)

B

IC50 for SCH772984 in 5-day viability assay (ViaLight)

<table>
<thead>
<tr>
<th>Tumor line</th>
<th>SCH772984 IC50 (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A101D parental</td>
<td>73</td>
</tr>
<tr>
<td>A101D BR+MR (with PLX4032+GSK1120212)</td>
<td>89</td>
</tr>
<tr>
<td>A101D BR+MR (without PLX4032+GSK1120212)</td>
<td>55</td>
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</table>
Erick J Morris, Sharda Jha, Clifford R Restino, et al.

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