Enhanced inhibition of ERK signaling by a novel allosteric MEK inhibitor, CH5126766, that suppresses feedback reactivation of RAF activity.

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NOTE
Nobuya Ishii and Naoki Harada contributed equally to this work.
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CONFLICT OF INTEREST

Nobuya Ishii, Naoki Harada, Kazuhiro Ohara, Takaaki Miura, Hiroshi Sakamoto, Yutaka Matsuda, Yasushi Tomii, Yukako Tachibana-Kondo, Hitoshi Iikura, Toshihiro Aoki, Nobuo Shimma, Mikio Arisawa, Yuko Aoki are full-time employees of Chugai Pharmaceutical Co., Ltd. Hitoshi Iikura, Toshihiro Aoki, Yasushi Tomii, Naoki Harada, and Toshiyuki Sakai filed a patent (EP1982982) on CH5126766 and its derivatives. This study was funded by Chugai Pharmaceutical Co., Ltd.

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Tumors with mutant RAS are often dependent on ERK signaling for growth, however, MEK inhibitors have only marginal antitumor activity in these tumors. MEK inhibitors relieve ERK dependent feedback inhibition of RAF and cause induction of MEK phosphorylation. We have now identified a MEK inhibitor, CH5126766 (RO5126766), that has the unique property of inhibiting RAF kinase as well. CH5126766 binding causes MEK to adopt a conformation in which it cannot be phosphorylated by and released from RAF. This results in formation of a stable MEK/RAF complex and inhibition of RAF kinase. Consistent with this mechanism, this drug does not induce MEK phosphorylation. CH5126766 inhibits ERK signaling output more effectively than a standard MEK inhibitor that induces MEK phosphorylation and has potent antitumor activity as well. These results suggest that relief of RAF feedback limits pathway inhibition by standard MEK inhibitors. CH5126766 represents a new type of MEK inhibitor that causes MEK to become a dominant negative inhibitor of RAF and which, in doing so, may have enhanced therapeutic activity in ERK dependent tumors with mutant RAS.
INTRODUCTION

The RAS-RAF-MEK-ERK signaling pathway is activated in many human tumors, including those with BRAF, RAS and NF1 mutations and some with activated growth factor receptors. The pathway has been shown to play a role in driving proliferation, suppressing apoptosis and in mediating other aspects of the transformed phenotype and is thought to be necessary for the maintenance of the growth and viability of many tumors (1). This has led to efforts to develop inhibitors of components of this pathway as antitumor agents (2). Recently, inhibitors of the MEK and RAF kinases have met with some success in the treatment of melanomas with V600E or V600K BRAF mutations (3, 4, 5). RAF inhibitors only inhibit ERK signaling in cells with activating mutation of BRAF and activate ERK signaling in other cells (6, 7). They therefore have a wide therapeutic index and remarkable activity in melanoma patients with mutant BRAF, but clearly cannot be effective in tumors with mutant RAS due to paradoxical activation of RAF (7, 8, 9).

MEK inhibitors have significant activity in patients with mutant BRAF melanoma (3), and some activity in patients with RAS mutant tumors (10, 11, 12). However, the ability of MEK inhibitors to potently inhibit ERK signaling may be limited by their toxicity and by relief of ERK dependent-feedback inhibition of RAF, which causes induction of MEK phosphorylation (13).

Here, we describe a novel allosteric MEK inhibitor CH5126766 (RO5126766) that was generated by derivatization of a drug identified in a screen for compounds that induces P27Kip1 expression in tumor cells. CH5126766 inhibits MEK but also suppresses feedback induction of RAF-dependent MEK phosphorylation. In KRAS mutant tumor xenograft models, CH5126766 causes greater suppression of
ERK pathway output and antitumor activity compared to that elicited by a MEK inhibitor that induces
RAF-mediated MEK phosphorylation.
MATERIALS AND METHODS

Recombinant proteins and cell lines

For RAF biochemical enzyme assays, MEK1 K97R (C-terminally His$_6$ tagged full length MEK1 with K97R mutation, Millipore), B-RAF wt (N-terminally GST-His$_6$-thrombin cleavage site fused to BRAF 417-766, ProQinase), B-RAF V600E (N-terminally GST-His$_6$-thrombin cleavage site fused to BRAF 417-766 with a V600E mutation, ProQinase), Raf-1 (N-terminally GST-tagged Raf-1 306-end with mutations Y340D and Y341D, Millipore) were used. For MEK biochemical assays, MEK1 S218E/S222E (N-terminally His$_6$ fused full length MEK1 with S218E and S222E mutations) and MAP Kinase 2/Erk 2 (N-terminally His$_6$ fused full length full length mouse MAP Kinase 2/Erk2, Millipore) were used. For biophysical analysis, N-terminally His$_6$ tagged unphosphorylated full length wild-type MEK1 kinase (1-393) (MAP2K1 (MEK1) Recombinant Human Protein, P3093) and N-terminally GST-fused phosphorylated full length wild-type MEK1 kinase (1-393) (MAP2K1, 07-141) were purchased from Invitrogen and Carna Bioscience respectively.

N-terminally GST-fused BRAF kinase domain (433-726) (GST-BRAF), N-terminal GST-tagged CRAF kinase domain (306-648) Y340D/Y341D (GST-CRAF) and N-terminal GST-tagged BRAF kinase domain (433-726) with V600E mutation (GST-BRAF V600E) were purchased from Carna Bioscience (BRAF (09-112), RAF1 (09-125) and BRAF [V600E], respectively). All cell lines except for human leukemic monocyte lymphoma cell line U937 were obtained from the American Type Culture Collection (ATCC) and cultured under the conditions that are described on the ATCC website (http://www.atcc.org/). U937 is a kind gift from Dr. Y. Honma at Saitama Cancer Center Research Institute and was maintained in RPMI
1640 supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin.

High throughput screening for compounds that induce p27\(^{\text{Kip1}}\) expression.

High throughput screening to identify compounds that induce p27\(^{\text{Kip1}}\) utilized a reporter gene assay with a human p27\(^{\text{Kip1}}\) gene promoter region. The reporter plasmid p27PF-Luc contained a DNA fragment comprising the XhoI site (-3568) to Smal site (-12) of the p27\(^{\text{Kip1}}\) gene promoter cloned upstream of the firefly luciferase reporter gene in pGVB21. This plasmid was transiently transfected into U937 cells by electroporation. The transfected cells were exposed to 10 \(\mu\)M of individual 230,000 compounds from Chugai's chemical library for 48 h, the luciferase activity was measured, and p27\(^{\text{Kip1}}\) protein induction was confirmed with p27\(^{\text{Kip1}}\) ELISA assay after 2 days incubation of tumor cell lines with compounds.

MEK and RAF kinase enzyme assays

The inhibitory activities against CRAF, BRAF or BRAF V600E enzymes were measured by quantification of phosphorylation of inactive K97R MEK1 (MEK1 (Millipore) by recombinant RAF proteins (BRAF: B-RAF wt (ProQinase), BRAF V600E: B-RAF V600E (ProQinase) or CRAF: Raf-1 (Millipore) with Europium-anti-MEK1/2 (pSer218/222) antibody (PerkinElmer) and SureLight allopheocyanine-anti-6his antibody (PerkinElmer) by measuring time-resolved fluorescence (TRF). Alternatively, the inhibitory activities against the RAF enzymes were measured by quantification of phosphorylation of a fluorescein labeled peptide corresponding to human MEK1 212-224 and human MEK2 217-229.
(5-FI-SGQLIDSMANSFV-NH₂, MEKtide) by using the IMAP fluorescence polarization (FP) Screening Express Kit (Molecular Devices).

Inhibition of MEK1 was evaluated by a coupled assay with active MEK1 (MEK1 S218E/S222E, ProQinase) and unactive dephosphorylated ERK2 (MAP Kinase 2/Erk 2, Millipore). The phosphorylation of a fluorescent labeled peptide substrate (FAM-Erktide, IPTTPITTTYFFK-5FAM-COOH) by ERK2 was quantified by using the IMAP FP Screening Express Kit (Molecular Devices).

Surface plasmon resonance (SPR)

All of the biosensor experiments were conducted on the Biacore 2000 or Biacore T100 systems (GE Healthcare) at 15°C with a flow rate of 30 µL/min. For direct binding experiments of test compounds to MEK, His₆-MEK1 was minimally biotinylated with sulfo-NHS-LC-LC biotin (Thermo Scientific), and then coupled to a streptavidin-coated sensor chip (GE Healthcare). Solutions of test compounds were injected over the surface for one minute or two minutes and then the flow was switched to a running buffer: Tris-based saline (50 mM tris(hydroxyethyl)carboxymethane-HCl, pH 7.6, 150 mM NaCl, 10 mM MgCl₂, 1 mM dithiothreitol (DTT), 0.01% P-20, and 1% dimethyl sulfoxide (DMSO)).

For direct binding assays of test compounds to RAF, N-terminal GST tagged BRAF or CRAF was captured on the surface of a CM5 sensor chip (GE Healthcare) by anti-GST polyclonal antibodies that were pre-immobilized on the chip according to the manufacturer’s instructions. Then, 10 µM of test compound solutions in 1% DMSO were injected over the prepared sensor chip. Phosphate-based saline
(10 mM phosphate, pH 7.4, 138 mM NaCl, 2.7 mM KCl, 10 mM MgCl2, 1 mM DTT, 0.01% P-20, and 1%
DMSO) was used as a running buffer.

The effects of CH5126766 and PD0325901 on the BRAF- or CRAF-MEK1 interactions were determined
using single-cycle kinetics due to the slow dissociation of His6-MEK1 from RAF. In these experiments,
His6-MEK1 solutions at concentrations of 0.0256, 0.064, 0.16, 0.4 and 1 μM, were injected sequentially in
order of increasing concentration over the sensor chip in the absence or presence of 3 μM of the test
compounds and then the dissociation constants of His6-MEK1 were calculated for the immobilized BRAF
or CRAF for each condition. In these experiments, 500 μM ATP was added to the running buffer.

The resulting sensorgrams were double-referenced, DMSO-calibrated, and fitted to determine kinetic
parameters by using SCRUBBER2 (BioLogics), BIAevaluation ver3.1, T100 evaluation ver2.0 or or T200
evaluation ver1.0 (GE Healthcare) software.

Co-immunoprecipitation of MEK1 and BRAF

The 293H cells transfected with 2 μg FLAG-tagged full length ARAF, BRAF, CRAF or BRAF V600E
plasmid DNA (8) or HCT116 cells were treated with 0.1% DMSO or MEK inhibitors (50 nM PD0325901 or
250 nM CH5126766 in 0.1% DMSO) for 1 h (293H transfected cells) or 2 hours (HCT116 cells). Cells
were lysed in 1% NP40 lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 10% glycerol and 1 mM EDTA)
supplemented with 2.5 mM sodium orthovanadate, 10 mM PMSF and protease inhibitor cocktail (EMD).
For immunoprecipitation of FLAG-tagged RAF proteins, agarose conjugated anti-FLAG antibody beads
(Invitrogen) were added to precipitate FLAG-tagged proteins. For immunoprecipitation of intact BRAF proteins, Dynabeads Protein G (Invitrogen) and anti-BRAF antibody mixed were incubated with the cell lysate for immunoprecipitation for 1 h at 4°C. Immunoprecipitations were washed three times in the lysis buffer and subjected to WB analysis.

**Efficacy experiments in mouse xenograft models**

All *in vivo* studies were approved by the Chugai Institutional Animal Care and Use Committee. Female BALB-nu/nu mice (CAnN.Cg-Foxn1nu/CrlCrlj nu/nu) were obtained from Charles River Laboratories Japan and maintained under pathogen-free conditions. These mice were given access to standard mouse chow and water *ad libitum*. 5 × 10⁶ (HCT116) or 1 × 10⁷ (Calu-6 and COLO205) tumor cells/mouse were injected subcutaneously into the right flank of the 7–9-week-old mice. When tumor volume reached to 200 mm³ (day 0), the mice were randomized, and vehicle (5% DMSO and 10% 2-hydroxypropyl-β-cyclodextrin (HPCD) solution in distilled water), CH5126766 or PD0325901 was administered orally once a day at the indicated doses. For the drug administration, CH5126766 was dissolved in distilled water containing 5% DMSO and 10% HPCD and PD0325901 was dissolved in distilled water containing 5% ethanol and 5% Cremophor EL. Drugs were administrated at the maximum tolerated dose (MTD). The MTD was defined as the highest dose that produced less than 20% weight loss and no mortality. Tumor growth inhibition (TGI) was calculated using the following formula: 

\[ TGI = \left[ 1 - \frac{(T - T_0)}{(C - C_0)} \right] \times 100 \]

where T and T₀ were the mean tumor volumes on a specific experimental day.
and on the first day of treatment, respectively, for the experimental groups and likewise, where C and C0 were the mean tumor volumes for the control group. The value of the 50% effective dose (ED$_{50}$) for each compound was calculated based on the formula for the straight line that connected the two points for which y was equal to 50%. Each treatment group included 4 to 6 animals. Statistical analysis was performed by using Dunnett’s test or Wilcoxon test with SAS version 8.02 (SAS Institute). The criterion for statistical significance was $p < 0.05$. 


RESULTS

Discovery of CH5126766 and its inhibition of ERK signaling

CH5126766 (RO5126766; Fig. 1A) was obtained by iterative rounds of derivatization of a hit compound from a cell-based high-throughput screen for compounds that induce expression of the cell cycle inhibitor p27^Kip1 in tumor cells. CH5126766 induced p27^Kip1 protein expression and caused G1 arrest in a human lung large cell carcinoma cell line NCI-H460 (KRAS Q61H) and two colorectal adenocarcinoma cell lines, HT29 (BRAF V600E) and HCT116 (KRAS G13D) (Supplementary Fig. S1A and S1B). We attempted to determine the target of the drug by using the COMPARE drug screening algorithm (14), which analyzes the pattern of anti-proliferative activity obtained in a panel of tumor cell lines exposed to different agents. Among the 21 antitumor agents tested, including CH5126766, anti-metabolites (5-fluorouracil, gemcitabine and methotrexate), a DNA damaging agent (cisplatin), a DNA intercalator (doxorubicin), an alkylating agent (mytomycin C) and inhibitors of MEK (CI-1040, PD0325901 and AZD6244), EGFR (erlotinib), Bcr-Abl (imatinib and dasatinib), multiple kinases (sorafenib and sunitinib), HSP90 (geldanamycin), proteasome (bortezomib), mTOR (rapamycin), PI3K (LY294002), topoisomerase I (camptotecin) and microtubules (paclitaxel), only MEK inhibitors were associated with a pattern of inhibition similar to that obtained with CH5126766 (correlation coefficients greater than 0.8, Supplementary Table S1).

MEK is a component of the cascade of kinases responsible for the activation of ERK signaling.

To determine whether the effects of the drug were due to inhibition of MEK or RAF, we assessed its
effects on their catalytic activity in cell-free kinase assays (Fig. 1B). CH5126766 effectively inhibited
activation of ERK2 protein by MEK1 and the phosphorylation of MEK1 protein by the RAF family of
enzymes. Thus, the drug inhibited the activity of both RAF kinase and MEK kinase in the in vitro
biochemical assays. In a KINOMEscan panel with 256 kinases (DiscoveRx) at 10 μM, CH5126766 was
suggested to bind to CRAF (82% inhibition of an ATP analog binding) and BRAF (89% inhibition of an ATP
analog binding), but not to 254 other kinases (Supplementary Table S2). Since the initial KINOMEscan
panel did not include MEK1 and MEK2, we separately confirmed the binding of CH5126766 to MEK1 and
MEK2 with KINOMEscan profiling. The Kd for MEK1 and MEK2 was 2.9 and 13 nM, respectively
(Supplementary Table S2).

In order to examine whether the drug also inhibits these targets in cells, we investigated the
effect of CH5126766 on phosphorylation of MEK and ERK in tumor cells. HCT116 KRAS mutant
colorectal cancer cells were treated with CH5126766 for 2 h, and the phosphorylation status of MEK and
ERK were analyzed. As shown in Figure 1C, CH5126766 reduced the levels of phospho-MEK and
phospho-ERK to undetectable levels, whereas other MEK inhibitors PD0325901 and GSK1120212 only
inhibited ERK phosphorylation. When HCT116 cells were treated with either of two RAF inhibitors
(GDC-0879 and PLX-4720), phosphorylation of MEK and ERK were induced as previously reported in
response to paradoxical activation of RAF kinase (Fig. 1C). Thus, the effects of CH5126766 on ERK
signaling in HCT116 were different from those of both MEK inhibitors and RAF inhibitors.

The effects of CH5126766 on ERK signaling were further assessed in a panel of tumor cells with or
as a function of genotype (RAS/RAF wt, BRAF V600E mutant, mutant RAS) and compared with those obtained with PD0325901. In tumors with RAS mutation, CH5126766 effectively inhibited both MEK and ERK phosphorylation (Fig. 1D). This differs from the effects of PD0325901, which inhibited ERK phosphorylation and induced MEK phosphorylation in these cells (Fig. 1D). CH5126766 also inhibited MEK and ERK phosphorylation in mutant BRAF tumors as well as WT RAS/BRAF cells whereas the MEK inhibitor PD0325901 inhibited ERK phosphorylation in these cells and had no apparent effect on MEK phosphorylation (Fig. 1D). Thus, the effects of CH5126766 on ERK signaling in tumor cells and in cell free kinase assays differ from those obtained with other inhibitors of RAF or MEK and are consistent with finding that it inhibits both kinases in vitro assays.

Mechanism of inhibition of MEK and ERK phosphorylation

It seemed unlikely, however, that the effects of CH5126766 are due to its selective and independent binding to two kinases, RAF and MEK, one of which is required for the activation of the other. We therefore utilized surface plasmon resonance (SPR) analyses to assess whether CH5126766 binds directly to MEK, BRAF or CRAF. Our SPR analyses with immobilized His\textsubscript{6}-MEK1 showed that both CH5126766 and PD0325901 bind to His\textsubscript{6}-MEK1 (Fig. 2). In the presence of 50 µM ATP, the binding signal of CH5126766 or PD0325901 to His\textsubscript{6}-MEK1 was additive with that obtained with ATP alone. These data suggest that CH5126766 does not compete with ATP for binding to His\textsubscript{6}-MEK, but that instead it binds to another site in the protein. This is consistent with our data that the $K_\text{D}$s of CH5126766 for His\textsubscript{6}-MEK1 are
similar in the presence or absence of 50 µM ATP (16 nM and 6.1 nM, respectively). Moreover, the binding
signal obtained with His6-MEK1 in the presence of 0.2 µM CH5126766 and 0.2 µM PD0325901 was not
additive with that obtained with 0.2 µM PD0325901 alone in our SPR analysis (Fig. 2). Thus, we conclude
that CH5126766 binds to the known site of binding (15) of the allosteric inhibitors MEK inhibitors, such as
PD0325901. In our biochemical MEK kinase assay, CH5126766 did not inhibit the kinase activity of
phosphorylated GST-MEK1 on S218 and S222 (Supplementary Fig. S3). This suggests that
phosphorylation of S218 and S222 is critical for the affinity of CH5126777 for MEK1. This is consistent
with the previous observations that this region is important for MEK1 binding of other allosteric MEK
inhibitors (16).

By contrast, SPR analyses revealed no evidence for binding of CH5126766 to immobilized
GST-BRAF or GST-CRAF (Fig. 3A). These data suggest that the inhibition of His6-MEK1 K97R
phosphorylation by CH5126766 (Fig. 1B) is not due to a direct interaction of the drug with RAF kinases.
Indeed, when a peptide substrate for RAF (MEKtide, corresponding to human MEK1 212-224 and human
MEK2 217-229 (SGQLIDSMANSFV-NH2)) was substituted for the MEK1 protein as the substrate in an in
vitro assay of CRAF kinase activity, CH5126766 did not affect the peptide phosphorylation
(Supplementary Fig. S2). Given these data, we concluded our initial evidence that CH5126766 bound to
RAF proteins in the KINOMEscan profiling (Supplementary Table S2) was an artifact of the extremely
high concentration (10 µM) of drug employed in this assay compared to the much lower concentrations of
drug required to inhibit MEK phosphorylation in cells (~0.01 µM, Fig. 1C).
These results suggested the hypothesis that CH5126766 inhibits RAF kinase by binding to MEK1. To test this idea, we used SPR to determine whether the drug alters the interaction between RAF and MEK. CH5126766 decreased the dissociation rate of His6-MEK1 from GST-BRAF (Fig. 3B and Supplementary Table S4). We observed similar effects when we substituted GST-CRAF for GST-BRAF. In contrast, PD0325901 had no effect on the kinetics of dissociation of this protein complex. Moreover, co-immunoprecipitation analysis with lysates of 293H cells engineered to express FLAG-tagged RAF proteins showed that CH5126766 causes MEK1/2 to interact with all three FLAG-tagged RAF proteins, including FLAG-tagged BRAF V600E, whereas PD0325901 causes MEK1/2 to interact with FLAG-tagged wild type ARAF and CRAF, but not FLAG-tagged wild type BRAF and mutant BRAF (Fig. 3C). Whereas considerable MEK2 was immunoprecipitated with each of the FLAG-RAF from lysates of cells treated with CH5126766, the relative amount of MEK1 pulled down with FLAG- wild type BRAF was much greater than that pulled down with FLAG-CRAF or FLAG-BRAF V600E. Whether this reflects differences in affinity of MEK1 and MEK2 to the different RAF proteins has not yet been addressed. Similar results were obtained when we used KRAS mutated HCT116 cells and immunoprecipitated endogenous BRAF. In the cells treated with CH5126766, MEK and CRAF were co-immunoprecipitated with BRAF, whereas in the DMSO or PD0325901 treated cells, only CRAF was co-immunoprecipitated with BRAF (Fig. 3D). The results suggest that CH5126766-bound MEK binds to and inhibits RAF kinase activity. To assess this possibility, the ability of CRAF to phosphorylate the MEKtide in an in vitro kinase assay was evaluated in the presence or absence of unphosphorylated His6-MEK1 proteins and MEK or RAF inhibitors (Fig. 3E).
The pan-RAF inhibitor PLX4720 inhibited CRAF activity whether or not His6-MEK1 was present and PD0325901 did not inhibit the phosphorylation of the MEKtide by CRAF kinase in either circumstance. In contrast, CH5126766 inhibited CRAF activity in a His6-MEK1-dependent manner (Fig. 3E). Moreover, the inhibition of CRAF activity by CH5126766 was dependent on the concentration of His6-MEK1. These results suggest that binding of CH5126766 to MEK causes MEK to bind to and inhibit RAF and inhibit its kinase activity. Taken together, the data suggest a model in which the drug binds to MEK and causes it to adopt a conformation in which it binds to RAF, but cannot be phosphorylated by and released from RAF. As a consequence, CH5126766 causes MEK to become a dominant negative inhibitor of RAF.

Inhibition of ERK signaling and tumor growth by CH5126766 in KRAS tumors in vivo.

MEK inhibitors have recently been shown to have antitumor activity in patients with mutant BRAF melanoma, and modest activity in patients with melanomas with mutant NRAS tumors (3, 10, 11, 12). It is possible that the feedback reactivation of RAF in response to MEK inhibition reduces the antitumor effects of these drugs by limiting their inhibition of ERK signaling. If this is the case, MEK inhibitors like CH5126766 that prevent induction of MEK phosphorylation by activated RAF could have enhanced antitumor activity in RAS tumors. To examine this possibility, we compared the antitumor effects of CH5126766 with those elicited by PD0325901 in an HCT116 (G13D KRAS) mouse xenograft model. In this experiment, we administrated CH5126766 or PD0325901 at their maximum tolerated dose (MTD) in the HCT16 model (1.5 mg/kg and 25 mg/kg, respectively). These doses inhibited pERK and
ERK signaling output at similar degrees in the tumors from the drug-treated mice at 4 h from the first drug administration (Fig. 4B). Moreover, in HCT116 models, the 50% effective dose of tumor growth inhibition (ED50) for CH5126766 and PD0325901 were 0.056 mg/kg and 0.80 mg/kg, respectively (Supplementary Fig. S3). Therefore, the doses used for this experiment were 26.8-fold and 31.3-fold higher doses than the 50% effective doses, respectively. As shown in Figure 4A, daily oral administration of either drug caused significant tumor regression of each these tumors. However, whereas inhibition of tumor growth was maintained for the entire 28-day treatment period in CH5126766-treated mice, tumor models receiving PD0325901 became refractory after 10 days of treatment. We observed similar differences between CH5126766 and PD0325901 in Calu-6, an anaplastic lung carcinoma model with Q61K mutant KRAS, but not in COLO205, a colorectal adenocarcinoma model with V600E mutant BRAF (Fig. 5A).

To assess whether differences in the extent of ERK pathway inhibition could account for the difference in efficacy of the two drugs, the tumors in Figure 4A were resected 4 h after the first dose or 4 h after the last of 34 days of daily dosing of the drug. In the PD0325901-treated tumors, MEK was highly phosphorylated (Fig. 4B). Although pMEK was induced by PD0325901 treatment, this was not associated with an increased ERK phosphorylation, which was undetectable in tumors treated with either drug even after multiple doses. It has been recently shown that pERK is a less quantitative measure of pathway activation than expression of ERK dependent genes (17). Whereas the levels of protein expression of four such genes, MKP2, MKP3, SPRY2 and SPRY4, were undetectable in tumors treated with CH5126766, substantial levels of SPRY2 and 4 and MKP3 were detected in the PD0325901-treated tumors after
multiple dosing. These differences were not observed 4 h after the initial dose of these inhibitors. Similar

effects were observed in CH5126766-treated Calu-6 tumors with Q61K KRAS, whereas no downstream
activation of ERK signaling was noted in PD0325901-treated COLO205 tumors with V600E BRAF even
after multiple dosing (Fig. 5B). The results suggest that CH5126766 is a more effective inhibitor of ERK
signaling output than PD0325901 in chronically treated tumors with RAS mutations.

We also examined whether the refractory tumors from the PD0325901-treated mice would
respond to CH5126766. The tumors that became refractory to PD0325901 were treated daily with either
PD0325901 or CH5126766. As shown in Figure 4C, the growth of the refractory tumors ceased in the
CH5126766-treated group but continued to increase in the PD0325901-treated group. These data
suggest the resistance of these tumors is secondary to the modest rebound in ERK signaling noted in
PD0325901-treated tumors and that the more prolonged growth inhibition with CH5126766 is due to more
effective inhibition of the pathway.
DISCUSSION

The elevation of ERK output in tumors with RAS or BRAF mutation and the sensitivity of such tumors to inhibitors of components of the pathway suggest that this may be a useful therapeutic strategy. RAF inhibitors selectively inhibit ERK activation in tumor cells with certain BRAF mutants that function as monomers. In other tumors, RAF inhibitors bind to and transactivate RAF dimers and ERK signaling (8). Thus, RAF inhibitors only effectively suppress ERK signaling in tumors in which the target is mutated, so they have a wider therapeutic index than MEK inhibitors. These drugs have remarkable clinical activity in melanomas with V600E BRAF mutation but they cannot be used to treat tumors in which the pathway is driven by other oncoproteins. Of note, antitumor activity in tumors with mutant BRAF requires very substantial (greater than 80%) inhibition of ERK output (18).

As opposed to RAF inhibitors, MEK inhibitors inhibit ERK signaling in all normal and tumor cells. They can therefore be used to treat RAS tumors. However, these drugs have only marginal therapeutic effects in patients with these tumors (10, 11, 12). RAS has been shown to activate more than ten effectors in model systems, of which three-RAF, PI3K, RAL-GDS-have been most strongly shown to mediate important aspects of transformation (19). It is possible that inhibition of RAF/MEK/ERK signaling alone is insufficient to markedly affect transformation. It has been shown, however, that the development of KRAS mutant induced lung cancer in genetically engineered mice requires c-RAF (20). Since the dosage of MEK inhibitor is limited by toxicity, it is also possible that not enough can be administered to inhibit the pathway enough to significantly affect tumor growth.
Relief of ERK dependent feedback inhibition of signaling may prevent maximal inhibition of pathway output by MEK inhibitors. Activation of ERK in cells is limited by ERK-dependent feedback inhibition of multiple upstream targets in the pathway and by ERK-dependent expression of the ERK phosphatases (MKPs) and SPRY family of proteins (21, 22, 23, 24, 25). In particular, activated ERK phosphorylates and inhibits CRAF kinase. Inhibition of ERK signaling by MEK inhibitors relieves ERK-dependent feedback inhibition of CRAF and induces MEK phosphorylation in most cells (26, 27). It is conceivable that phosphorylation of these sites (MEK S218 and S222) by RAF could reduce inhibition of MEK activity by the drug and reduce maximal inhibition of ERK (28), but this has not been demonstrated. This idea is consistent with our observations that feedback reactivation of RAF by conventional allosteric MEK inhibitors is more pronounced in cells with activated RAS in cells with mutant BRAF mutants or wild type RAS and BRAF (Fig. 1D) (17).

We now report the generation of a novel allosteric MEK inhibitor, CH5126766, that does not cause induction of MEK phosphorylation, despite its potent inhibition of ERK phosphorylation. This compound and PD0325901 compete for binding to the same site on MEK1 (Fig. 2) to which they bind with similar affinity (K_Ds for CH5126766 and PD0325901: 4.5 nM and 19 nM, respectively, Supplementary Table S3). Both inhibitors block ERK phosphorylation and signaling in cells. However, as opposed to PD0325901 and other MEK inhibitors, CH5126766 inhibits, rather than induces MEK phosphorylation (Fig. 1C &1D), a result that suggests that it directly inhibits RAF activity. This turns out to be the case, but only when full length MEK is present in the RAF kinase assay. Furthermore, the drug does not directly
bind to RAF (Fig. 3A).

The effects of the drug are explained by the demonstration that it causes MEK to bind to RAF. In this complex, the drug-bound MEK is not phosphorylated and its dissociation rate from RAF is much reduced compared to that of unbound MEK or MEK bound to PD0325901 (Fig. 3B, 3C & 3D). The dissociation of MEK protein from immobilized RAF protein was retarded by CH5126766: $k_{off}$ value was changed with a two order magnitude difference from $1.48 \times 10^{-2}$ to $2.76 \times 10^{-4}$ (BRAF-MEK1) and from $1.58 \times 10^{-2}$ to $1.62 \times 10^{-4}$ (CRAF-MEK1) by adding CH5126766. But it had no effect on the association of MEK with RAF: $k_{on}$ value was almost unchanged from $2.25 \times 10^{5}$ to $1.2 \times 10^{5}$ (BRAF-MEK1) and from $3.46 \times 10^{5}$ to $2.39 \times 10^{5}$ (CRAF-MEK1) by adding CH5126766 (Supplementary Table S4). Thus, MEK bound to CH5126766 is a dominant negative inhibitor of RAF. This is consistent with a model in which the binding of the drug to MEK causes the latter to adopt a conformation in which it cannot be phosphorylated by RAF.

CH5126766 preferentially binds to non-phosphorylated MEK1 rather than phosphorylated MEK1 in our cell free MEK kinase assay (Supplementary Fig. S3). Our current data strongly suggests that CH5126766 only binds to the unphosphorylated enzyme, inhibits it and prevents its phosphorylation. It does not inhibit the phosphorylated enzyme well, but rather traps the unphosphorylated enzyme so it cannot be phosphorylated. This is why the affinity for the unphosphorylated enzyme is high (KINOMEscan and SPR assays) and the drug is a potent inhibitor of MEK phosphorylation (RAF kinase in cells) but a less potent inhibitor of phosphorylated MEK in a kinase assay (Fig. 1B). Since the co-immunoprecipitated
MEK proteins with RAF proteins were not phosphorylated (Fig. 3C), it was suggested that phosphorylation of MEK causes its dissociation from RAF. From these lines of evidence, we speculate that the drug binds selectively to the non-phosphorylated form of MEK, locks it into the unphosphorylated conformation, and stabilizes MEK-RAF complex. The RAF/MEK drug bound complex is inactive and stable, thus the drug suppresses the feedback induction of MEK phosphorylation that occurs after ERK pathway inhibition in tumors exposed to other MEK inhibitors (Fig. 1C & 1D).

The cellular effects of CH5126766 thus appear to be those of a combined MEK and RAF inhibitor. Despite inhibition of RAF by CH5126766, it does not induce paradoxical activation of RAF kinases in cells in which active RAF is a dimer because it does not bind to RAF directly.

Since CH5126766 suppresses induction of MEK phosphorylation, it can be used to determine whether feedback reactivation of RAF reduces ERK inhibition by inhibitors such as PD0325901. Indeed, CH5126766 effectively inhibited ERK phosphorylation in vivo in RAS mutant xenografts and was a more potent inhibitor of ERK output and tumor growth than PD0325901 (Fig. 4 & Fig. 5). These data suggest but do not prove that preventing induction of pMEK accounts for the greater efficacy of this drug.

GSK1120212 (trametinib, JTP-74057), another MEK inhibitor, has significant therapeutic efficacy in patients with melanomas with BRAF V600E or V600K mutation (3) as well as in some RAS tumors (10). GSK1120212 was identified from a screen similar to the one from which CH5126766 was obtained, in this case, picking compounds that induced p15 expression (29, 30). This agent binds to the same site in MEK proteins as PD0325901 with higher affinity to MEK1 than PD0325901 (30) and induces phosphorylation of...
S222 but not S218 MEK1. In KRAS mutated cells, however, phosphorylation of MEK S222 was increased although phosphorylation of S218 MEK1 was prevented (16). It is possible that the significant clinical activity of this drug is due in part to partial suppression of feedback reactivation of ERK. As a class, such MEK inhibitors may offer the chance for enhanced pathway output inhibition and antitumor activity without necessarily increasing toxicity.

In the phase I clinical investigation of CH5126766, two partial responses in BRAF V600E melanoma and in one Q61K NRAS mutated melanoma patient was reported (12). However, the long plasma half-life of CH5126766 in human (60 h), and its continuous daily dosing were associated with a severe rash (12). Further progress in the use of the drugs to potently inhibit ERK signaling in tumors will require identification of the optimal doses and administration schedules in order to maximize inhibition of signaling and antitumor activity without unacceptable toxicity.

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REFERENCES


**FIGURE LEGENDS**

**Figure 1** MEK and RAF inhibitory activities of CH5126766. (A) Chemical structure of CH5126766 (N-(3-fluoro-4-[(4-methyl-7-(2-pyrimidinyloxy)-2H-chromen-2-on-3-yl)methyl]-2-pyridyl)-N'-methylsulfamide). (B) Inhibitory activity of CH5126766 in cell-free MEK1 and RAF family enzyme assays. For MEK1 kinase, ERK2 activation by active MEK1 (S218E/S222E) was quantified by coupling with an ERK assay using FAM-Erk tide as a substrate of ERK2, and then measuring its fluorescence polarization change. For RAF family kinases, phosphorylated His6-MEK1 by RAF family enzymes was quantified by time-resolved fluorescence resonance energy transfer (TR-FRET) between europium-labeled anti-pMEK and allophycocyanin (APC)-labeled anti-His6 antibody. IC50 values were expressed as a mean value ± standard deviation. (C) Inhibition of cellular pMEK and pERK in HCT116 cells by MEK inhibitors (CH5126766, PD0325901 and GSK1120212) or RAF inhibitors (GDC-0879 and PLX-4720). Levels of pMEK and pERK after treating HCT116 cells with various concentrations of the inhibitors for 2 h were monitored with western blotting. Dimethyl sulfoxide (DMSO) was used as the negative control. (D) Phosphorylation status of MEK and ERK in cell lines with KRAS G12D, KRAS G13D, KRAS Q61K, HRAS Q61K, or BRAF V600E mutations or wild-type RAS/RAF treated with 1 μM CH5126766 or 0.25 μM PD0325901 for 2 h. DMSO was used as the negative control.
Figure 2  Inhibition mode of MEK by CH5126766. SPR sensorgram of the interaction between CH5126766 and immobilized His<sub>6</sub>-MEK1 in the presence or absence of 50 µM ATP or 0.2 µM PD0325901. Test solutions were injected over the sensor chip during the period from time point 0 to 120 sec.

Figure 3  Inhibition mode of RAF kinases by CH5126766. (A) SPR analyses between immobilized GST-BRAF or GST-CRAF and CH5126766. The binding activity of immobilized GST-BRAF and GST-CRAF was confirmed with an ATP competitive pan-RAF inhibitor GDC-0879. The theoretical curves obtained by the global fitting of the sensorgrams, are also shown (thin lines). (B) SPR sensorgrams of the interaction between His<sub>6</sub>-MEK1 and immobilized GST-BRAF or GST-CRAF in the presence of 500 µM ATP, 500 µM ATP plus 3 µM CH5126766 or 500 µM ATP plus 3 µM PD0325901. The theoretical curves obtained by the global fitting of the sensorgrams, are also shown (thin lines). Single-cycle kinetics method was used for the analysis, where five different concentrations of MEK1 were injected sequentially in order of increasing concentrations. Further details of these experiments are described in Materials & Methods. (C) Interaction of MEK and RAF family proteins in the CH5126766-treated cells. 293H cells transfected with FLAG-tagged ARAF, BRAF CRAF and BRAF V600E were treated with 50 nM PD0325901 or 250 nM CH5126766, or 0.1% DMSO for 1 h. FLAG-RAF family proteins were immunoprecipitated with anti-FLAG antibody, and co-immunoprecipitated MEK1 and MEK2 were detected by western blotting. Arrows indicate specific signals in the analysis judging from their molecular weights. (D) Interaction of MEK, BRAF and CRAF in the CH5126766-treated tumor cells.
KRAS mutated HCT116 cells were treated with 50 nM PD0325901 or 250 nM CH5126766, or 0.1% DMSO for 2 h. An anti-BRAF antibody was used for the co-immunoprecipitation experiment with in the cell lysate from the drug treated HCT116 cells. MEK1/2, BRAF and CRAF proteins in the immunoprecipitated fraction were detected by western blotting. (E) MEK1-dependent inhibition of CRAF catalytic activity by CH5126766. A fluorescence polarization assay with a fluorescent-peptide substrate of RAF and GST-CRAF was conducted in the presence of 10 μM CH5126766, PLX-4720 or PD0325901 with various concentrations of His6 MEK1.

**Figure 4** Antitumor activity of CH5126766 in mouse xenograft tumor models. No animals died or more than 10% of their body weight gain and loss from baseline in these experiments. (A) Comparison of the efficacies of CH5126766 and PD0325901 in the HCT116 xenograft tumor model. CH5126766 or PD0325901 was administered orally every day for 4 weeks (*p = 0.0070, Wilcoxon test). (B) Phosphorylation states of signaling molecules in the HCT116 xenograft tumors resected from mice at 4 h from the first and final administration of the experiment shown in (A). (C) Efficacy of CH5126766 on the PD0325901-refractory tumors in the HCT116 xenograft tumor model. PD0325901 was administered orally every day for 25 days. Then, CH5126766 or PD0325901 was administered orally every day until the end of the study (*p = 0.0079, Wilcoxon test).
Figure 5  Effect of CH5126766 and PD0325901 in the Calu-6 (NSCLC, KRAS Q61K) and COLO205 (CRC, BRAF V600E) xenograft models. No animals died or more than 10% of their body weight changes from baseline in these experiments. (A) Antitumor efficacy of CH5126766 and PD0325901. CH5126766 or PD0325901 was orally administered every day for 11 days (*p < 0.0001, Dunnett’s test). (B) Phosphorylation status of signaling molecules in the Calu-6 and COLO205 xenograft tumors. The tumors were resected from mice at 4 h from the first and final administration of the experiments shown in Figure 5A.
Figure 1

A

B

RAF family
IC_{50} (CRAF): 0.056 ± 0.016 μM
IC_{50} (BRAF): 0.019 ± 0.0030 μM
IC_{50} (BRAF V600E): 0.0082 ± 0.0015 μM

C

D

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<th>KRAS</th>
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pMEK | MEK | pERK | ERK

| CH5126766 | 0 | 0.01 | 0.03 | 0.1 | 0.3 | 1 | 3 | 10 | 0 | 0.01 | 0.03 | 0.1 | 0.3 | 1 | 3 | 10 |
|-----------|---|------|------|-----|----|---|---|---|---|---|------|------|-----|----|---|---|---|
| DMSO      |   |      |      |     |    |   |   |   |   |   |      |      |     |    |   |   |   |
| CH5126766 |   |      |      |     |    |   |   |   |   |   |      |      |     |    |   |   |   |
| PD0325901 |   |      |      |     |    |   |   |   |   |   |      |      |     |    |   |   |   |
| GSK1120212|   |      |      |     |    |   |   |   |   |   |      |      |     |    |   |   |   |
| GDC-0879  |   |      |      |     |    |   |   |   |   |   |      |      |     |    |   |   |   |
| PLX-4720  |   |      |      |     |    |   |   |   |   |   |      |      |     |    |   |   |   |

MEK1 IC_{50}: 0.16 ± 0.043 μM

% Inhibition

% Inhibition

MEK

ERK

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Figure 2
Figure 3

A

GST-BRAF

GST-CRAF

CH5126766

DMSO

PD0325901

GDC-0879

GST-CRAF

+ His<sub>6</sub>-MEK1

GDC-0879

GST-BRAF

+ His<sub>6</sub>-MEK1

B

DMSO

CH5126766

PD0325901

C

ARAF-FLAG

BRAF-FLAG

CRAF-FLAG

BRAF-V600E-FLAG

DMSO

PD0325901

DMSO

PD0325901

DMSO

PD0325901

DMSO

PD0325901

DMSO

PD0325901

CRAF-BRAF

MEK1/2

Lyase

Anti-FLAG IP

MEK1

MEK2

pMEK

pERK

E

CRAF activity (% of control)

MEK1 conc. (nM)

PLX-4720

PD0325901

CH5126766
Figure 4

A

![Graph A showing tumor volume (mm$^3$) over post-implantation days](image)

B

**After single administration**

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**After 34 QD administrations**

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C

![Graph C showing tumor volume (mm$^3$) over post-implantation days](image)
Figure 5

A

Calu-6 (KRAS, NSCLC)

CH5126766

PD0325901

Tumor volume (mm$^3$)

Post implantation days

Vehicle

CH5126766 1.5 mg/kg, QD

PD0325901 25 mg/kg, QD

COLO205 (BRAF, CRC)

Tumor volume (mm$^3$)

Post implantation days

Vehicle

CH5126766 1.5 mg/kg, QD

PD0325901 25 mg/kg, QD

B

After single administration

After 11 QD administrations

Calu-6 (KRAS, NSCLC)

COLO205 (BRAF, CRC)

pMEK

MEK

pERK

ERK

SPRY2

SPRY4

MKP2

MKP3

tubulin

pMEK

MEK

pERK

ERK

SPRY2

SPRY4

MKP2

MKP3

tubulin

pMEK

MEK

pERK

ERK

SPRY2

SPRY4

MKP2

MKP3

tubulin

pMEK

MEK

pERK

ERK

SPRY2

SPRY4

MKP2

MKP3

tubulin
Enhanced inhibition of ERK signaling by a novel allosteric MEK inhibitor, CH5126766, that suppresses feedback reactivation of RAF activity


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