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Enhanced Inhibition of ERK Signaling by a Novel Allosteric MEK Inhibitor, CH5126766, That Suppresses Feedback Reactivation of RAF Activity

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Abstract

Tumors with mutant RAS are often dependent on extracellular signal–regulated kinase (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 that, in doing so, may have enhanced therapeutic activity in ERK-dependent tumors with mutant RAS. *Cancer Res; 73(13); 4050–60. ©2013 AACR.*

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 (3–5). RAF inhibitors only inhibit extracellular signal–regulated kinase (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 patients with melanoma with mutant BRAF but clearly cannot be effective in tumors with mutant RAS due to paradoxical activation of RAF (7–9). MEK inhibitors have significant activity in patients with mutant BRAF melanoma (3) and some activity in patients with RAS-mutant tumors (10–12). 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 $p27^{Kip1}$ 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 with 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

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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), and 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 mitogen-activated protein (MAP) kinase 2/Erk 2 (N-terminally His_6-fused 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, Saitama, Japan, and was maintained in RPMI-1640 supplemented with 10% FBS and 1% penicillin/streptomycin.

High-throughput screening for compounds that induce $p27^{Kip1}$ expression

High-throughput screening to identify compounds that induce $p27^{Kip1}$ used a reporter gene assay with a human $p27^{Kip1}$ gene promoter region. The reporter plasmid p27PF-Luc contained a DNA fragment comprising the *Xho*I site (−3,568) to *Sma*I site (−12) of the $p27^{Kip1}$ 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 μmol/L of individual 230,000 compounds from Chugai's chemical library for 48 hours, the luciferase activity was measured, and $p27^{Kip1}$ protein induction was confirmed with $p27^{Kip1}$ ELISA assay after 2-day 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 allophycocyanine-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-Fl-SGQLIDSMANSFV-NH_2, 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, IPTTPITTTYFFFK-5FAM-COOH) by ERK2 was quantified by using the IMAP FP Screening Express Kit (Molecular Devices).

Surface plasmon resonance

All of the biosensor experiments were carried out 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, His_6-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 1 or 2 minutes and then the flow was switched to a running buffer: Tris-based saline [50 mmol/L tris(hyroxyethyl)carboxymethane-HCl, pH 7.6, 150 mmol/L NaCl, 10 mmol/L $MgCl_2$, 1 mmol/L 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 μmol/L of test compound solutions in 1% DMSO were injected over the prepared sensor chip. Phosphate-based saline (10 mmol/L phosphate, pH 7.4, 138 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L $MgCl_2$, 1 mmol/L 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 His_6-MEK1 from RAF. In these experiments, His_6-MEK1 solutions at concentrations of 0.0256, 0.064, 0.16, 0.4, and 1 μmol/L, were injected sequentially in order of increasing concentration over the sensor chip in the absence or presence of 3 μmol/L of the test compounds and then the dissociation constants of His_6-MEK1 were calculated for the immobilized BRAF or CRAF for each condition. In these experiments, 500 μmol/L 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 T200 evaluation ver1.0 (GE Healthcare) software.

Coimmunoprecipitation 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 nmol/L PD0325901 or 250 nmol/L CH5126766 in 0.1% DMSO) for 1 hour (293H-transfected cells) or 2 hours (HCT116 cells). Cells were lysed in 1% NP40 lysis buffer (50 mmol/L TRIS, pH 7.5, 150 nmol/L NaCl, 10% glycerol, and 1 mmol/L EDTA) supplemented with 2.5 mmol/L sodium orthovanadate, 10

mmol/L phenylmethylsulfonyl fluoride (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 hour at 4°C. Immunoprecipitations were washed 3 times in the lysis buffer and subjected to Western blot 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. A total of 5×10^6 (HCT116) or 1×10^7 (Calu-6 and COLO205) tumor cells per mouse were injected subcutaneously into the right flank of the 7- to 9-week-old mice. When tumor volume reached to 200 mm^3 (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: $TGI = [1 - (T - T_0)/(C - C_0)] \times 100$, where T and T_0 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 C_0 were the mean tumor volumes for the control group. The value of the 50% effective dose (ED_{50}) for each compound was calculated on the basis of the formula for the straight line that connected the 2 points for which y was equal to 50%. Each treatment group included 4 to 6 animals. Statistical analysis was conducted by using Dunnett 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 G_1 arrest in a human lung large cell carcinoma cell line NCI-H460 (KRAS Q61H) and 2 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 antiproliferative activity obtained in a panel of tumor cell lines exposed to different agents. Among the 21 antitumor agents tested, including CH5126766, antimetabolites (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 (camptothecin), 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 KINOME*scan* panel with 256 kinases (DiscoveRx) at 10 μmol/L, 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). Because the initial KINOME*scan* panel did not include MEK1 and MEK2, we separately confirmed the binding of CH5126766 to MEK1 and MEK2 with KINOME*scan* profiling. The K_d for MEK1 and MEK2 was 2.9 and 13 nmol/L, respectively (Supplementary Table S2).

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 hours, and the phosphorylation status of MEK and ERK were analyzed. As shown in Fig. 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 2 RAF inhibitors (GDC-0879 and PLX-4720), phosphorylation of MEK and ERK was 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

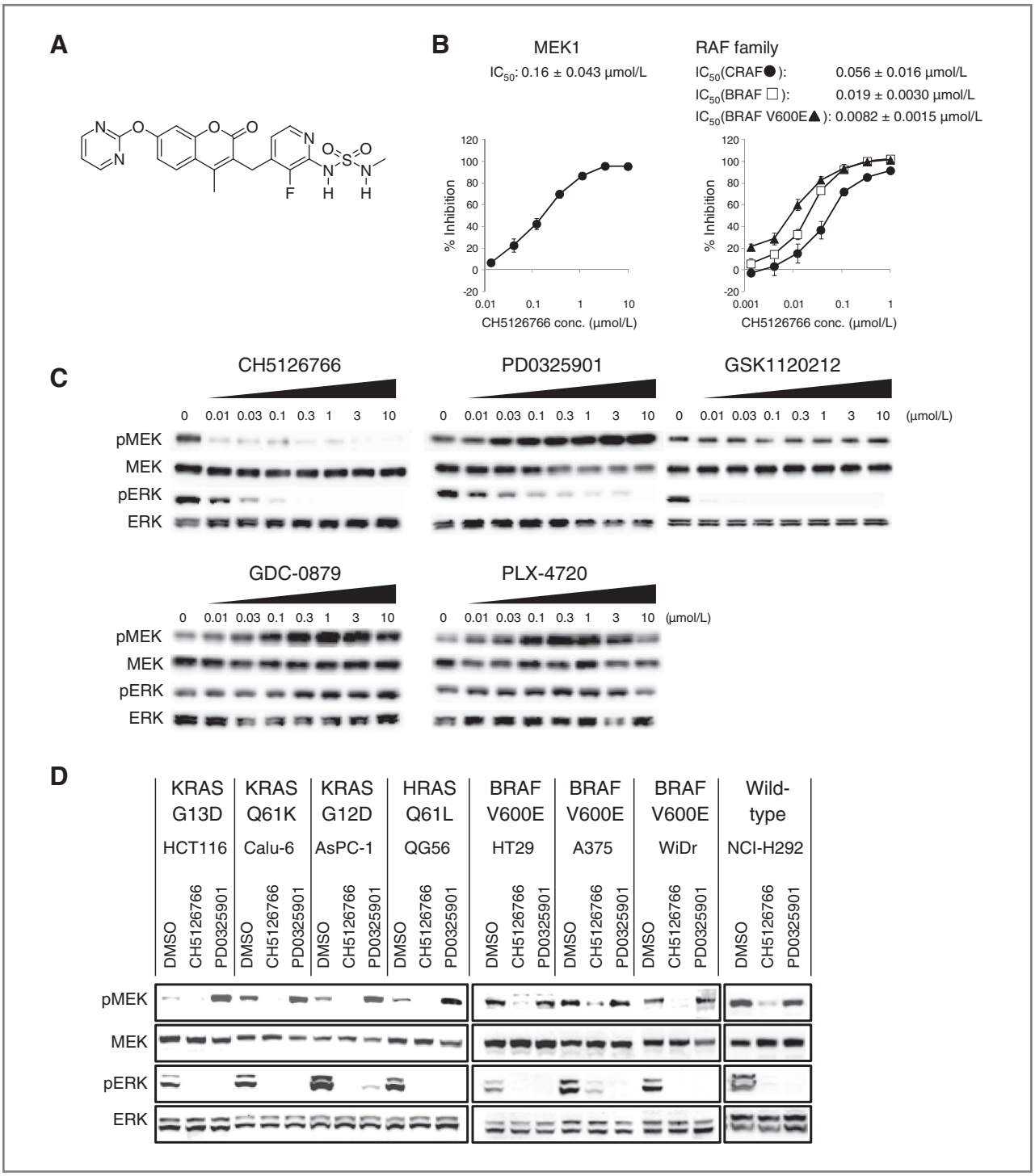

Figure 1. MEK and RAF inhibitory activities of CH5126766. A, chemical structure of CH5126766 (*N*-(3-fluoro-4-{[4-methyl-7-(2-pyrimidinyloxy)-2*H*-chromen-2-on-3-yl]methyl}-2-pyridyl)-*N*'-methylsulfamide). B, inhibitory activity of CH5126766 in cell-free MEK1 and RAF family enzyme assays. For MEK1 kinase, ERK2 activation by active MEK1 (S218E/S222E) was quantified by coupling with an ERK assay using FAM-Erktide as a substrate of ERK2 and then measuring its fluorescence polarization change. For RAF family kinases, phosphorylated His_6-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-His_6 antibody. IC_{50} values were expressed as a mean value ± SD. 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 hours were monitored with Western blotting. 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 μmol/L CH5126766 or 0.25 μmol/L PD0325901 for 2 hours. DMSO was used as the negative control.

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 2 kinases, RAF and MEK, one of which is required for the activation of the other.

We therefore used surface plasmon resonance (SPR) analyses to assess whether CH5126766 binds directly to MEK, BRAF, or CRAF. Our SPR analyses with immobilized His_6-MEK1 showed that both CH5126766 and PD0325901 bind to His_6-MEK1 (Fig. 2). In the presence of 50 μmol/L ATP, the binding signal of CH5126766 or PD0325901 to His_6-MEK1 was additive with that obtained with ATP alone. These data suggest that CH5126766 does not compete with ATP for binding to His_6-MEK but that instead it binds to another site in the protein. This is consistent with our data that the K_D of CH5126766 for His_6-MEK1 are similar in the presence or absence of 50 μmol/L ATP (16 and 6.1 nmol/L, respectively). Moreover, the binding signal obtained with His_6-MEK1 in the presence of 0.2 μmol/L CH5126766 and 0.2 μmol/L PD0325901 was not additive with that obtained with 0.2 μmol/L 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 CH5126766 for MEK1. This is consistent with the previous observations that this region is important for MEK1 binding of other allosteric MEK inhibitors (16).

In 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 His_6-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-NH_2)] 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 KINOME*scan*

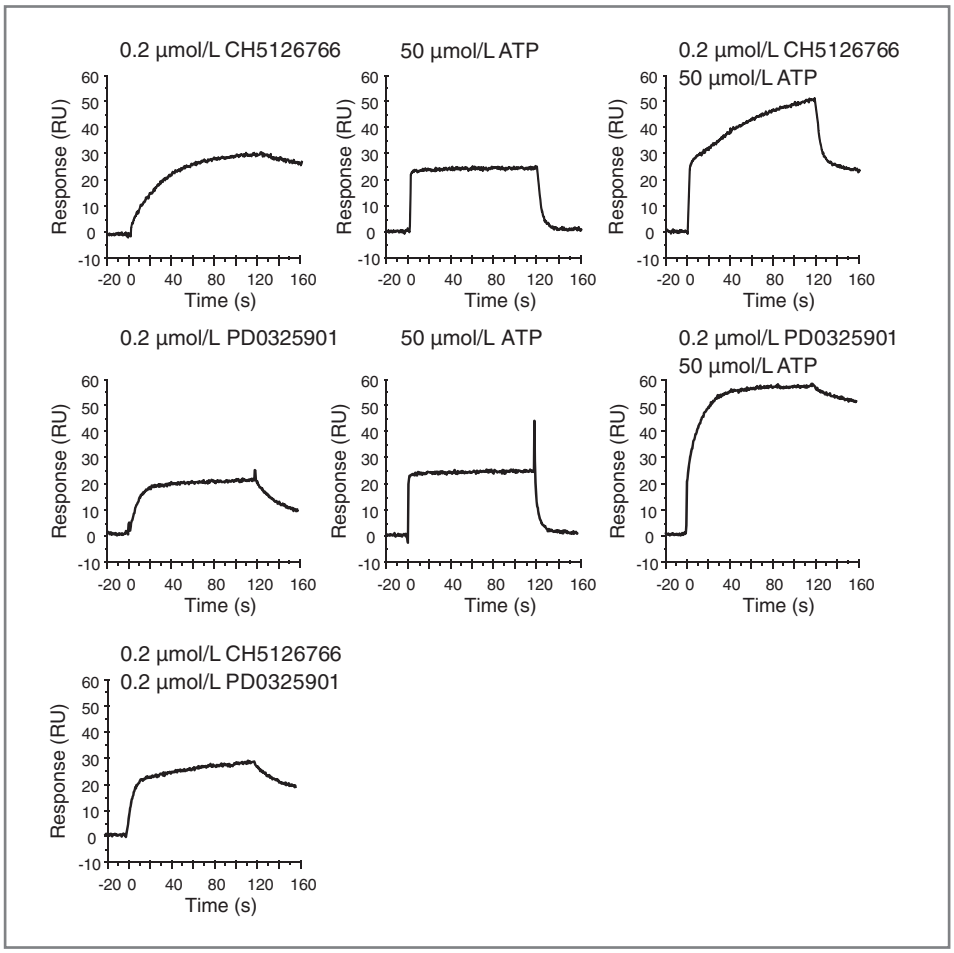

Figure 2. Inhibition mode of MEK by CH5126766. SPR sensorgram of the interaction between CH5126766 and immobilized His_6-MEK1 in the presence or absence of 50 μmol/L ATP or 0.2 μmol/L PD0325901. Test solutions were injected over the sensor chip during the period from time point 0 to 120 seconds.

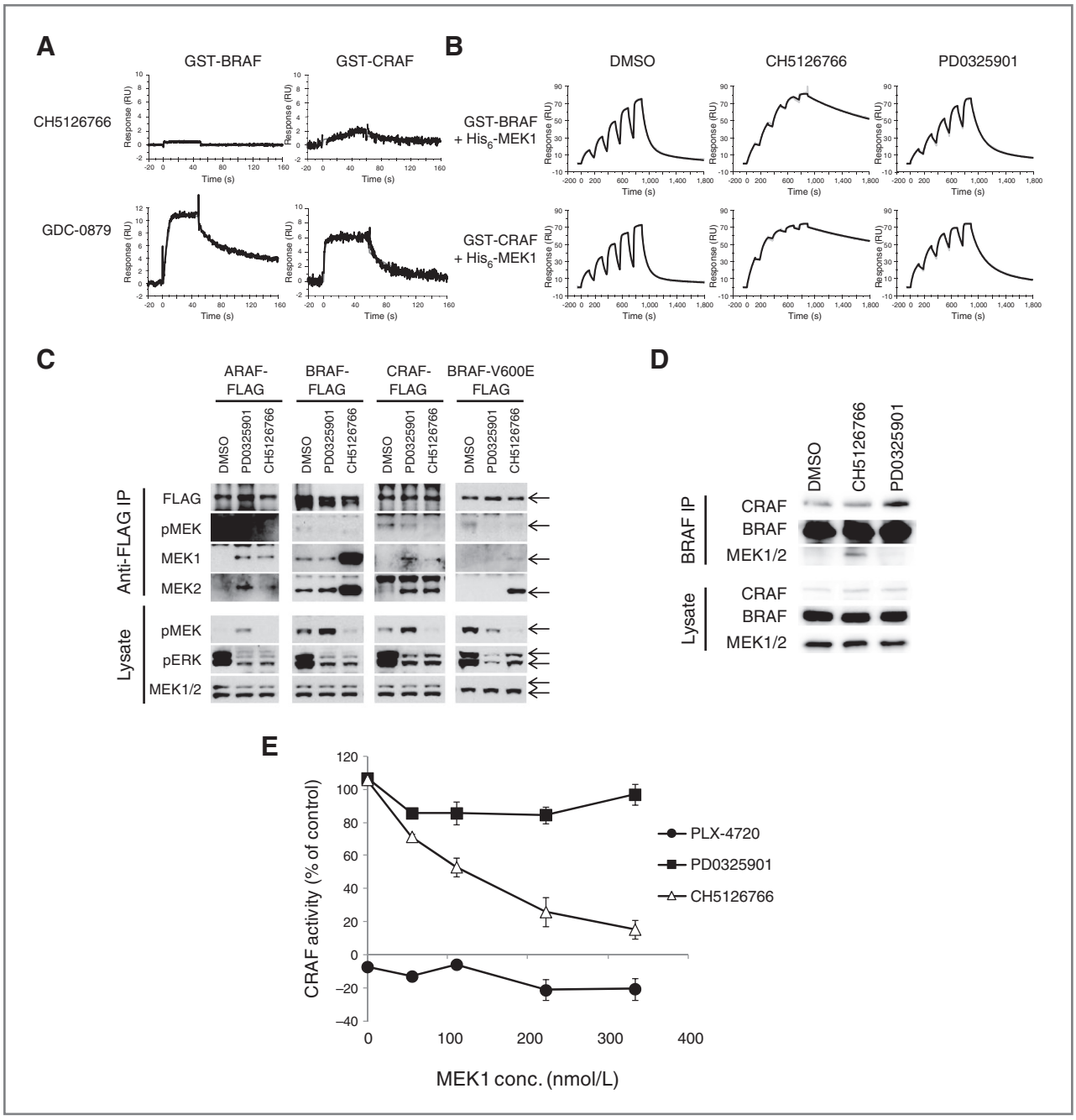

Figure 3. Inhibition mode of RAF kinases by CH5126766. A, SPR analyses between immobilized GST-BRAF or GST-CRAF and CH5126766. The binding activity of immobilized GST-BRAF and GST-CRAF was confirmed with an ATP-competitive pan-RAF inhibitor GDC-0879. The theoretical curves obtained by the global fitting of the sensorgrams are also shown (thin lines). B, SPR sensorgrams of the interaction between His_6-MEK1 and immobilized GST-BRAF or GST-CRAF in the presence of 500 μmol/L ATP, 500 μmol/L ATP plus 3 μmol/L CH5126766, or 500 μmol/L ATP plus 3 μmol/L 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 5 different concentrations of MEK1 were injected sequentially in order of increasing concentrations. Further details of these experiments are described in Materials and 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 nmol/L PD0325901 or 250 nmol/L CH5126766 or 0.1% DMSO for 1 hour. FLAG-RAF family proteins were immunoprecipitated with anti-FLAG antibody, and coimmunoprecipitated 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 nmol/L PD0325901 or 250 nmol/L CH5126766 or 0.1% DMSO for 2 hours. An anti-BRAF antibody was used for the coimmunoprecipitation 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 μmol/L CH5126766, PLX-4720 or PD0325901 with various concentrations of His_6-MEK1.

profiling (Supplementary Table S2) was an artifact of the extremely high concentration (10 μmol/L) of drug used in this assay compared with the much lower concentrations of drug required to inhibit MEK phosphorylation in cells (~0.01 μmol/L, 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 His_6-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, coimmunoprecipitation analysis with lysates of 293H cells engineered to express FLAG-tagged RAF proteins showed that CH5126766 causes MEK1/2 to interact with all 3 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 coimmunoprecipitated with BRAF, whereas in the DMSO- or PD0325901-treated cells, only CRAF was coimmunoprecipitated 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 His_6-MEK1 proteins and MEK or RAF inhibitors (Fig. 3E). The pan-RAF inhibitor PLX4720 inhibited CRAF activity whether or not His_6-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 His_6-MEK1–dependent manner (Fig. 3E). Moreover, the inhibition of CRAF activity by CH5126766 was dependent on the concentration of His_6-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, 10–12). 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 MTD in the HCT116 model (1.5 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 hours from the first drug administration (Fig. 4B). Moreover, in HCT116 models, the ED_{50} for CH5126766 and PD0325901 were 0.056 and 0.80 mg/kg, respectively (Supplementary Fig. S3). Therefore, the doses used for this experiment were 26.8- and 31.3-fold higher doses than the 50% effective doses, respectively. As shown in Fig. 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 2 drugs, the tumors in Fig. 4A were resected 4 hours after the first dose or 4 hours 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 4 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 hours 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 Fig. 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 that resistance of these

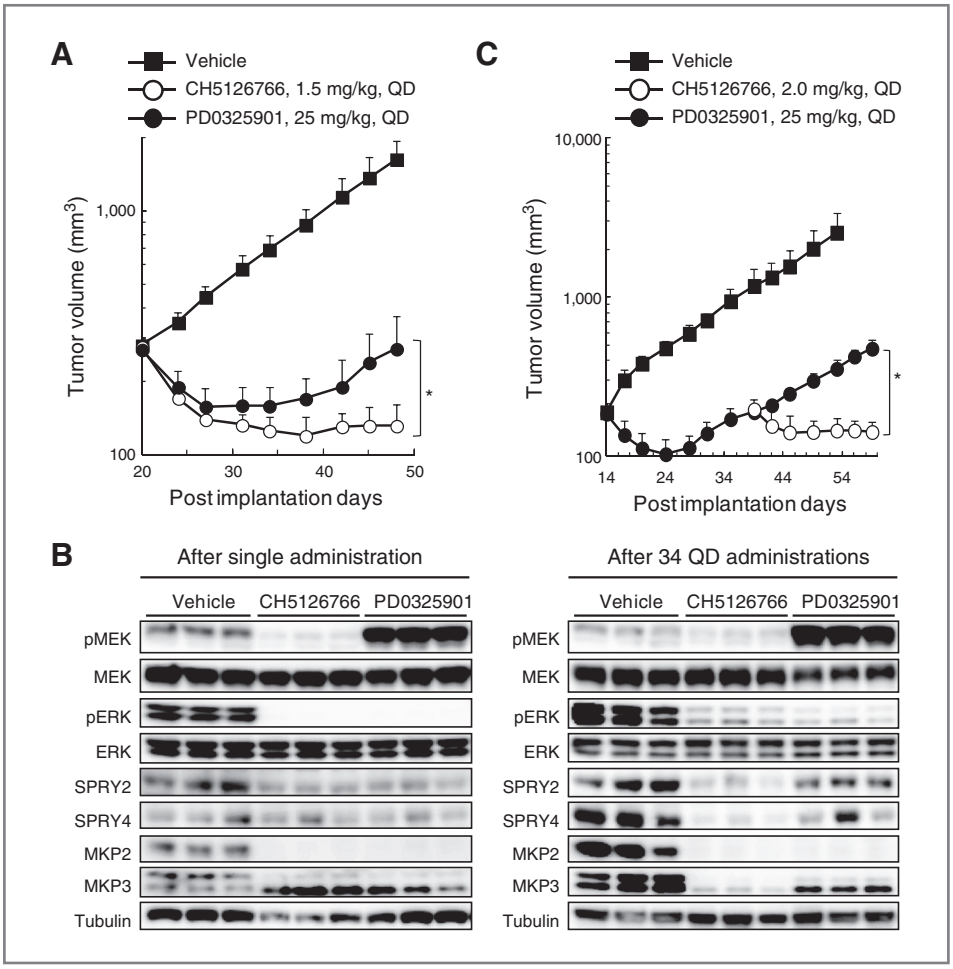

Figure 4. Antitumor activity of CH5126766 in mouse xenograft tumor models. No animals died or had more than 10% of their body weight gain and loss from baseline in these experiments. A, comparison of the efficacies of CH5126766 and PD0325901 in the HCT116 xenograft tumor model. CH5126766 or PD0325901 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 hours 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).

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 (>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 (10–12). RAS has been shown to activate more than 10 effectors in model systems, of which 3—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). Because 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 (21–25). 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

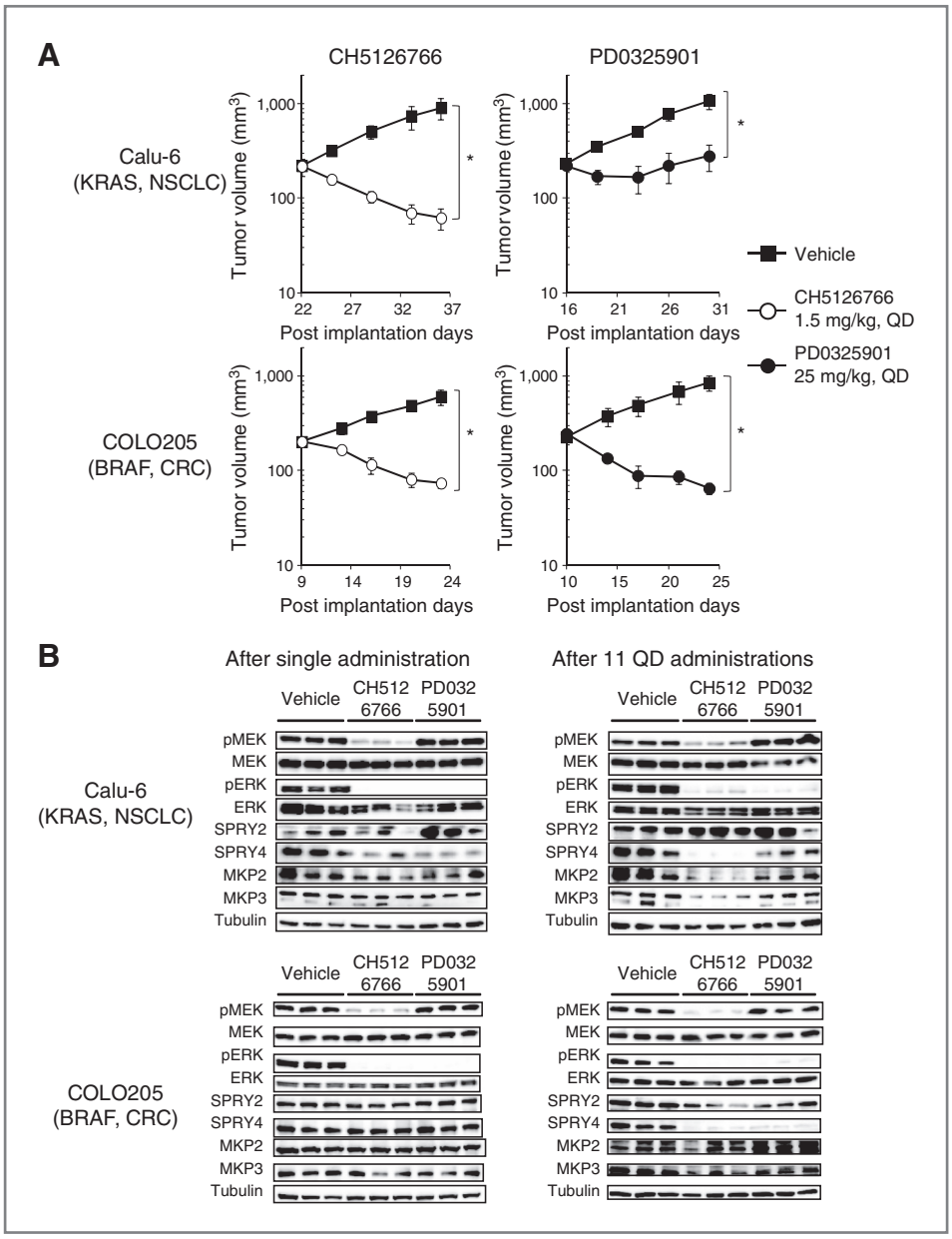

Figure 5. Effect of CH5126766 and PD0325901 in the Calu-6 (NSCLC, KRAS Q61K) and COLO205 (CRC, BRAF V600E) xenograft models. No animals died or had more than 10% of their body weight changes from baseline in these experiments. A, antitumor efficacy of CH5126766 and PD0325901. CH5126766 or PD0325901 was orally administered every day for 11 days (*, $P < 0.0001$, Dunnett test). B, phosphorylation status of signaling molecules in the Calu-6 and COLO205 xenograft tumors. The tumors were resected from mice at 4 hours from the first and final administration of the experiments shown in A.

maximal inhibition of ERK (28) but this has not been shown. 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; ref. 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_D for CH5126766 and PD0325901: 4.5 and 19 nmol/L, 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 and D), 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 with that of unbound MEK or MEK bound to PD0325901 (Fig. 3B–D). The dissociation of MEK protein from immobilized RAF protein was retarded by CH5126766: k_{off} value was changed with a 2-order magnitude difference from 1.48×10^{-2} to 2.76×10^{-4} (BRAF-

MEK1) and from 1.58×10^{-2} to 1.62×10^{-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 2.25×10^5 to 1.2×10^5 (BRAF-MEK1) and from 3.46×10^5 to 2.39×10^5 (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 nonphosphorylated 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 (KINOME*scan* 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). Because the coimmunoprecipitated 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 nonphosphorylated 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 and D). 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.

Because 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 and 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, 2 partial responses in BRAF V600E melanoma and in one Q61K NRAS-mutated patient with melanoma were reported (12). However, the long plasma half-life of CH5126766 in human (60 hours), 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 to maximize inhibition of signaling and antitumor activity without unacceptable toxicity.

Disclosure of Potential Conflicts of Interest

Y. Matsuda is employed (other than primary affiliation; e.g., consulting) and has ownership interest (including patents) in Chugai Pharmaceutical. N. Rosen has a commercial research grant from Bayer, Chugai, AstraZeneca, and Merck and is a consultant/advisory board member of Chugai Pharmaceutical and Millenium/Takeda and AstraZeneca. No potential conflicts of interest were disclosed by the other authors.

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