CEP-32496: A Novel Orally Active BRAF\textsuperscript{V600E} Inhibitor with Selective Cellular and In Vivo Antitumor Activity

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Abstract

Mutations in the BRAF gene have been identified in approximately 7% of cancers, including 60% to 70% of melanomas, 29% to 83% of papillary thyroid carcinomas, 4% to 16% colorectal cancers, and a lesser extent in serous ovarian and non–small cell lung cancers. The V600E mutation is found in the vast majority of cases and is an activating mutation, conferring transforming and immortalization potential to cells. CEP-32496 is a potent BRAF inhibitor in an \textit{in vitro} binding assay for mutated BRAF\textsuperscript{V600E} \((K_d \text{BRAF}^{V600E} = 14 \text{ nmol/L})\) and in a mitogen-activated protein (MAP)/extracellular signal–regulated (ER) kinase (MEK) phosphorylation (pMEK) inhibition assay in human melanoma (A375) and colorectal cancer (Colo-205) cell lines \((IC_{50} = 78 \text{ and } 60 \text{ nmol/L})\). \textit{In vitro}, CEP-32496 has multikinase binding activity at other cancer targets of interest; however, it exhibits selective cellular cytotoxicity for BRAF\textsuperscript{V600E} versus wild-type cells. CEP-32496 is orally bioavailable in multiple preclinical species (>95% in rats, dogs, and monkeys) and has single oral dose pharmacodynamic inhibition (10–55 mg/kg) of both pMEK and pERK in BRAF\textsuperscript{V600E} colon carcinoma xenografts in nude mice. Sustained tumor stasis and regressions are observed with oral administration (30–100 mg/kg twice daily) against BRAF\textsuperscript{V600E} melanoma and colon carcinoma xenografts, with no adverse effects. Little or no epithelial hyperplasia was observed in rodents and primates with prolonged oral administration and sustained exposure. CEP-32496 benchmarks favorably with respect to other kinase inhibitors, including RAF-265 (phase I), sorafenib, (approved), and vemurafenib (PLX4032/RG7204, approved). CEP-32496 represents a novel and pharmacologically active BRAF inhibitor with a favorable side effect profile currently in clinical development.

Introduction

Mutations in cellular signaling pathways that regulate cell proliferation and survival are often found in human cancers (1). Activating “driving” mutations that occur frequently or that are mutually exclusive within a pathway are strongly correlated with cellular dependency and uncontrolled growth, proliferation, and migration and represent favorable molecular targets for cancer therapy (2–5).

Activation of the mitogen-activated protein kinase (MAPK) pathway is associated with normal cell growth and proliferation in response to mitogens and trophins. Extracellular ligand binding to receptor tyrosine kinases (RTK) activates RAS, which recruits RAF to the cellular membrane where it is phosphorylated (6). The RAF family of serine/threonine kinases consists of 3 known human isoforms, A, B, and C (CRAF synonymous with RAF-1). These isoforms share a common architecture in 3 highly conserved regions but are distinct in their expression profiles and regulatory functions, although the BRAF isoform is thought to be the major signal transducer in the activation of the MAP/ERK (MEK)/extracellular signal–regulated kinase (ERK) pathway (5–8).

Mutations in the BRAF gene are among the most common identified in human cancers (1, 9–12). BRAF mutations have been identified in approximately 7% of all cancers evaluated, including 60% to 70% of melanomas, 29% to 83% of papillary thyroid carcinomas, 4% to 16% of colorectal cancers, 5% to 13% of serous ovarian cancers, 1% to 2% of non–small cell lung cancers, and more recently in hairy cell leukemia (4, 13–20). While
several mutated forms of BRAF have been identified, the V600E mutation is found in the vast majority of cases (~90%) and is a BRAF kinase–activating mutation (500-fold activity over wild-type). This mutant form confers transforming and immortalization potential to tumor cells, resulting in the hyperactivation of MEK/ERK signaling and enhanced tumor cell proliferation, aneuploidy, and survival (2, 21–23). Although BRAF<sub>V600E</sub> is oncogenic, emerging evidence indicates that this genotype contributes to the development of predominantly benign nevi that progress to a full malignant phenotype in the context of additional genetic events in human tumors (12). In contrast to the high incidence of BRAF mutations in human cancers, CRAF mutations are rare, presumably due to low basal kinase activity (24). Consequently, BRAF has received the most attention as a therapeutic target in oncology and clinical proof of concept has been established in melanoma with the BRAF inhibitors vemurafenib (PLX4032/RG7204), XL281, and GSK2118436 (25–27).

We describe herein CEP-32496 as a quinazoline BRAF inhibitor that potently inhibits MEK phosphorylation and cell proliferation in vitro in cells harboring the BRAF<sub>V600E</sub> mutation. CEP-32496 potently binds to wild-type BRAF <em>in vitro</em> but is selectively cytotoxic to cell lines harboring the BRAF<sub>V600E</sub> mutation versus BRAF wild-type cells. Furthermore, CEP-32496 shows favorable pharmaceutical properties and is orally bioavailable with sustained plasma and tissue exposures in all preclinical species examined. CEP-32496 is highly efficacious in mouse xenograft models using BRAF<sub>V600E</sub>–mutated human cell lines, with pharmacodynamic (PD) evidence of BRAF-mediated tumor growth inhibition (TGI) as shown by modulation of both MEK and ERK phosphorylation in tumor xenografts.

**Materials and Methods**

**Chemical synthesis of CEP-32496**

CEP-32496 was synthesized at Ambit Biosciences Corporation (28, 29).

**In vitro binding**

Biochemical kinase competition binding assays were conducted using Ambit’s KinomeScan as described previously (ref. 30; www.kinomescan.com).

**Cell assays**

**Activity in mutated versus wild-type BRAF cell lines.** A375 (obtained on August 9, 2007), SK-MEL-28 (June 27, 2007), HT-144 (June 24, 2007), Colo-205 (September 29, 2004), HCT116 (July 1, 2009), Hs578T (November 24, 2008), LNCAp (August 8, 2007), DU145 (September 4, 2008), and PC-3 (March 6, 2009) cell lines were obtained from, and cultured in media recommended by, American Type Culture Collection (ATCC). Colo-679 (June 27, 2007) was obtained from, and cultured in media recommended by, DSMZ. Cell lines were authenticated at each cell bank before purchase by their standard short tandem repeat (STR) DNA typing methodology. Within 1 month of receipt, cultures were grown out for several passages and aliquots of each were frozen. For experimental use, each cell type was thawed and grown out for no more than 2 months before use in an experiment. For MEK phosphorylation and cell viability assays, cells were cultured in 0.5% serum overnight before addition of compounds (9-point curve). Cells were incubated with compounds for 2 hours before lysis for the pMEK endpoint. Total MEK and pMEK levels were determined from the lysates using colorimetric ELISA assays (Cell Signaling Technologies). EC<sub>50</sub> values were calculated from total MEK normalized dose–response curves. For cell proliferation and viability assays, equal cell numbers were plated and incubated with compounds for 72 hours followed by addition of CellTiter-Blue (Promega) to detect viable cells after 3 hours of incubation with reagent. This assay is based on the cellular metabolism of resazurin to resorufin. Nonviable cells lack metabolic capacity and do not generate a fluorescent signal. EC<sub>50</sub> values were determined from the fluorometric product. Briefly, dimethyl sulfoxide–treated cells were included as negative control and no cell wells were used to define the baseline fluorescence. EC<sub>50</sub> curves were fitted to the negative control (100%). In all experiments with CEP-32496, the highest concentrations of drug led to reduction of fluorescence to within 10% the baseline (no cells) indicative of a cytotoxic response. Experiments were carried out in duplicate and repeated at least once.

**CRAF IC<sub>50</sub>.** Inhibition was measured in HeLa cells, which express high levels of BRAF<sub>Wt</sub>, CRAF<sub>Wt</sub>, and RAS<sub>Wt</sub>, following stimulation with 12-O-tetradecanoylphorbol-13-acetate as described (31). HeLa cells were obtained from the ATCC on May 5, 2009, were authenticated before purchase using the STR DNA typing methodology noted above, and were used within 2 months of purchase. Both CRAF phosphorylation (serine 338) and downstream ERK phosphorylation (pp42/44) were measured in the presence of 0 to 3,000 nmol/L CEP-32496. To address the potential for transactivation of RAF complexes in tumor cells with BRAF<sub>Wt</sub> and CRAF<sub>Wt</sub>, but possessing a mutated RAS gene, Calu-6 non–small cell lung adenocarcinoma cells were evaluated for the effects of 0 to 30,000 nmol/L CEP-32496 on MEK phosphorylation.

**Non-RAF cellular kinase assays.** All tumor cell lines were purchased from the ATCC, MAP and mycoplasma–tested (BioReliance Corp.), and deemed contamination–free before <em>in vitro</em> and <em>in vivo</em> studies. Authentication of tumor lines was done as described above using STR DNA typing methodology. K562 human chronic myelogenous leukemia (for ABL and BCR-ABL; obtained on October 5, 2007), TT-1 human medullary thyroid carcinoma (for activated RET; obtained on September 7, 2008), BxPC-3 and ASPC-1 human pancreatic carcinoma (for EPHA2; obtained on July 20, 2004), and Jurkat human T-cell lymphoma (for LCK; obtained on March 12, 2007) cell lines were used for these cellular assays. Primary low-passage human umbilical vein endothelial cells (HUVEC)
for VEGFR1 and VEGFR2 were obtained (April 24, 2008) from Clonetics (Lonza Walkersville Inc.), similarly MAP and Mycoplasma-tested and authenticated as above. For all cell assays, cell lines were expanded within 1 month of receipt and aliquots were frozen. For experimental use, each cell type was thawed and grown out for no more than 3 to 5 passages (<4 months) before use in an experiment. Various tumor cell lines were incubated with the indicated concentrations of CEP-32496 for 2 hours in a serum-free media for EC50 determinations. EphA2, ASPC-1, and BxPC-3 cells were additionally stimulated with rmEphinA1/Fc at 1 μg/mL for 10 minutes before harvesting. For KDR/VEGFR2 and FLT-1/VEGFR1, HUVECs were grown overnight in a medium containing 0.5% FBS, followed by 2 hours of incubation with inhibitors in a serum-free media. HUVECs were stimulated with VEGF at 3 nmol/L and vanadate at 100 μmol/L for 10 minutes before harvesting. Cell extracts were prepared in a Triton-based lysis buffer (Cell Signaling #9803) supplemented with protease inhibitors (Roche Complete Mini), and expression of phosphorylated and total proteins was analyzed by Western blotting using specific antibodies according to manufacturers’ recommendations. Western blot analyses were scanned and quantified (GelPro), and the ratio of phospho/total was calculated for each sample and presented as a percentage of control. The following antibodies used in these studies: pABL (#2861), ABL (#2862), pRET (#3221), RET (#3220), pEphA2 (#3970), EphA2 (#3974), pVEGFR2 (#2478), and VEGFR2 (#2479) were obtained from Cell Signaling; pFLT-1/VEGFR2 (sc-101821) and FLT-1 (sc-316) from Santa Cruz Biotechnology; and pEPHA2 (#3970), pVEGFR2 (#2479), and VEGFR2 (#2479) were obtained from Cell Signaling; pFLT-1/VEGFR2 (sc-101821) and FLT-1 (sc-316) from Santa Cruz Biotechnology; and FLT-1/pVEGFR1 (AF4170) from R&D Systems. Mouse recombinant EphinA1/Fc (602-A1) and human recombinant VEGF165 (293-VE) were from R&D Systems.

Pharmacokinetics

Rodent pharmacokinetic (PK) studies were conducted in accordance with the Institute for Laboratory Animal Research Guide for the Care and Use of Laboratory Animals and the USDA Animal Welfare Act and Cephalon Inc. Institutional Animal Care and Use Committee guidelines. Sprague-Dawley rats (Charles River; 250–350 g) and athymic nude mice (Charles River; 20–25 g) were administered CEP-32496 intravenously or orally formulated in either Pharmatek#6 (Hot Rod Chemistry; Pharmatek, Inc.) or 22% to 50% 2-hydroxypropyl-β-cyclodextrin (HPBCD) or HPBCD at various doses (as free base equivalent), following a minimum of 2 days of acclimation to the vivarium. Blood samples were collected at 5 (intravenously only), 15, 30 minutes, 1, 2, 4, 6, and 24 hours into K3EDTA tubes for plasma isolation. Compound levels in plasma were quantitatively analyzed by LC-MS/MS (API 4000-Qtrap; Applied Biosystems) following protein precipitation with acetonitrile containing an internal standard, and pharmacokinetic parameters were calculated from the normalized liquid chromatography/tandem mass spectrometric (LC/MS-MS) peak areas using a noncompartmental model with WinNonlin version 5.2, using the linear trapezoidal estimation method.

Following overnight fasting, CEP-32496-HCl formulated in 22% HPBCD was administered to male cynomolgus monkeys (2.6–3.4 kg) and beagle dogs (10.3–11.9 kg) as the free base equivalent at oral doses of 3 and 10 mg/kg and intravenous dose of 1 mg/kg (n = 3 animals) in a cross-over design (≥7-day washout period). Blood samples were collected via femoral vein before dosing and at preselected time points through 24 hours postdose. Concentrations of CEP-32496 in plasma were determined by LC/MS-MS and noncompartmental analysis per rodent pharmacokinetics.

Microsomal stability

The stability of CEP-32496 was determined in rat, dog, cynomolgus monkey (In Vitro Technologies), and human (CellzDirect; 0.5 mg/mL) pooled liver microsomes as previously reported (29).

CYP450 inhibition and induction

The potential for CEP-32496 to inhibit 5 major cytochrome P450 (CYP450) isoenforms was assessed as previously described (29). The activity of CEP-32496 on CYP3A4 induction was measured using a cell-based reporter gene assay (Puracyp).

In vivo tumor pharmacodynamics and efficacy

For in vivo biochemical efficacy assays (tumor pharmacokinetic/pharmacodynamic studies), athymic nude mice bearing Colo-205 or A375 BRAFV600E subcutaneous tumors were treated with a single daily oral dose of vehicle (HPBCD) or inhibitor similar to the previously described protocol (32, 33). At appropriate time points over a 24-hour period, animals were sacrificed and plasma, tumors, and select tissues (lung and liver) were collected and lysates prepared. Total and phosphorylated MEK and ERK in tumors were measured by immunoblotting and ELISA methods. Compound levels in plasma, tumor, and tissue samples were analyzed and quantitated by LC/MS-MS.

For antitumor efficacy studies, established tumor-bearing athymic nude mice were treated orally with vehicle (HPBCD) or inhibitor following a twice daily dosing schedule for a period of 15 days. Mice were monitored for signs of morbidity (behavior and body weight loss) and tumors were measured 3 times per week. Immediately following the final dose, a subset of the animals was sacrificed and plasma, tumors, and select tissues were harvested and compound levels in the samples were measured by LC/MS-MS. The remaining animals were evaluated for a period of 7 to 10 days to assess any potential residual side effects of treatment.

As reference, all pharmacodynamic and efficacy studies included sorafenib and RAF-265 at the published maximally effective doses of 60 mg/kg orally once daily and 100 mg/kg orally every other day respectively.
**Results**

**In vitro binding**

CEP-32496 potently bound to the RAF family of kinases in vitro in the Ambit KinomeScan Assay ($K_d = 14–39$ nmol/L; Table 1). The kinase selectivity profile of CEP-32496 was evaluated by measuring binding constants for a panel of 400 kinases (356 unique nonmutant kinases). As shown in Fig. 2 (and Supplementary Data), CEP-32496 had modest kinase selectivity, binding to 30% of the 356 nonmutant kinases in the panel with a $K_d \leq 3$ µmol/L, generating an $S(3 \mumol/L)$ value of 0.3 (34).

**Cellular kinase selectivity and cytotoxic selectivity for mutant BRAF**

To assess the ability of CEP-32496 to inhibit BRAF$^{V600E}$, a mechanistic assay was used that measured MEK phosphorylation, the direct downstream target of BRAF$^{V600E}$. When incubated with cell lines harboring the BRAF$^{V600E}$ mutation in culture medium containing low serum (0.5% FBS), CEP-32496 inhibited MEK phosphorylation in a concentration-dependent manner (IC$_{50}$ = 84 and 60 nmol/L; A375 and Colo-205 cells, respectively). To assess the potential cytotoxic selectivity against mutant versus wild-type BRAF tumor cells, a functional readout of cell viability was used. CEP-32496 was tested in a panel of BRAF mutant [A375, SK-MEL-28, Colo-679, HT-144 (skin), and Colo-205 (colon)] and wild-type [HCT116 (colon), Hs578T (breast), LNCaP, DU145, and PC-3 (prostate)] cell lines (Fig. 1A and Table 2). CEP-32496 showed a selective cytotoxicity profile (15- to 85-fold shift in cell viability) against tumor cell lines with the V600E mutation versus wild-type BRAF. The only wild-type cell line in which CEP-32496 had appreciable activity was HCT116 (IC$_{50}$ = 669 nmol/L), a colonic epithelial cell line with a known RAS mutation and wild-type p53, a combination previously shown to confer sensitivity to inhibition of the MAPK pathway (35). In agreement with recent reports evaluating other inhibitors of mutant BRAF (31, 36), CEP-32496 lacked appreciable cellular CRAF inhibition (IC$_{50}$ ≥ 3,000 nmol/L) and induced, in a concentration-related manner, the activation of MEK (Fig. 1B).

<table>
<thead>
<tr>
<th>Kinase target</th>
<th>Ambit Kinome Scan $K_d$, nmol/L</th>
<th>Millipore enzyme IC$_{50}$, nmol/L</th>
<th>Cellular IC$_{50}$, nmol/L, human cell line</th>
</tr>
</thead>
<tbody>
<tr>
<td>BRAF</td>
<td>36</td>
<td>na</td>
<td>2,736 (Hs578T)</td>
</tr>
<tr>
<td>BRAF$^{V600E}$</td>
<td>14</td>
<td>na</td>
<td>60 (Colo-205)</td>
</tr>
<tr>
<td>CRAF</td>
<td>39</td>
<td>146</td>
<td>≥3000 (HeLa)</td>
</tr>
<tr>
<td>ABL, BCR-ABL</td>
<td>2.8</td>
<td>6</td>
<td>39 (K562; ABL)</td>
</tr>
<tr>
<td>RET</td>
<td>1.5</td>
<td>7</td>
<td>21 (TT-1)</td>
</tr>
<tr>
<td>EPHA2</td>
<td>14</td>
<td>41</td>
<td>27 (BxPC-3)</td>
</tr>
<tr>
<td>KDR/VEGFR2</td>
<td>7.9</td>
<td>43</td>
<td>≥700 (HUVEC)</td>
</tr>
<tr>
<td>FLT-1/VEGFR1</td>
<td>14</td>
<td>1</td>
<td>≥1,000 (HUVEC)</td>
</tr>
<tr>
<td>LCK</td>
<td>2.2</td>
<td>15</td>
<td>694 (Jurkat)</td>
</tr>
<tr>
<td>CKIT</td>
<td>2.4</td>
<td>na</td>
<td>1,000 (A431)</td>
</tr>
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</table>

**NOTE:** CEP-32496 potently binds in vitro to both mutated (V600E) and WT BRAF, as well as to the related family member CRAF. While CEP-32496 is not a selective binder in vitro, selective kinase inhibition in cells was observed in a panel of cell lines tested. Abbreviation: na, not available.

![Figure 1](https://example.com/figure1.png)

Figure 1. A, EC$_{50}$ values in mutant versus wild-type BRAF cell lines. CEP-32496 is preferentially cytotoxic in cell lines harboring the V600E mutation versus WT BRAF (with the exception of the MAPK-dysregulated HCT116 cell line). B, Western blot analysis showing CEP-32496 concentration-dependent induction of pMEK in Calu-6 Cells, which harbor WT BRAF and CRAF but mutated KRAS (KRAS$^{G12V}$).
and ERK (data not shown) in tumor cells with wild-type BRAF and CRAF and mutated KRAS.

In vitro binding data suggested only modest kinase selectivity of CEP-32496, which was shifted to greater selectivity in enzyme assays, indicating conformational (or activation state) dependence, and to relative kinase selectivity in a battery of cellular biochemical assays, which are conducted under physiologic ATP concentrations. These observations are consistent with type 2 kinase inhibitors, which bind to the active, but not inactive, conformation of kinase targets (37). In a cellular panel of 6 non-RAF targets of interest to which CEP-32496 was bound with potency similar to BRAFV600E, CEP-32496 was largely inactive in inhibiting cellular phosphorylation (IC50 values = 1–2 μmol/L) against VEGFR1 and VEGFR2 in HUVECs. Similarly, CEP-32496 was largely inactive in inhibiting phosphorylation of LCK (IC50 = 0.8 μmol/L) in lymphoma cells, and KIT (IC50 = 1.4 μmol/L) in carcinoma cells, despite the in vitro binding profile in KinomeScan (Fig. 2). Potent target inhibition for ABL/BCR-ABL, RET, and EPHA2 was observed in specific tumor cell lines in which these kinases are known to play important roles (IC50 range = 27–70 nmol/L), confirming the in vitro kinome binding data (Table 1). These results suggest that CEP-32496 may have potential therapeutic application against additional oncogenic protein kinase-driven or -dependent cancers, specifically BCR-ABL- and RET-mediated malignancies.

Metabolic stability and CYP450 liability profile

Following in vitro incubation of CEP-32496 with mouse, rat, dog, monkey, and human liver microsomes, the intrinsic clearance (Clint) was low and t1/2 values were above 60 minutes for all species tested. In vitro assays using pooled human liver microsomes indicated that CEP-32496 is a modest to poor inhibitor of CYP450, having IC50 values greater than 11 μmol/L for 4 of the 5 major human isoforms (CYP3A4, 2D6, 2C9, and 1A2) and only modest activity for CYP2C19 (IC50 = 3.4 μmol/L). The activity of CEP-32496 on CYP3A4 induction was measured using a cell-based reporter gene assay that showed no CYP3A4 induction (≤1-fold) at concentrations up to 30 μmol/L.

Preclinical pharmacokinetic profile

Single-dose pharmacokinetic studies in rats showed dose-related increases in plasma Cmax and area under curve (AUC) from 3 to 100 and 3 to 30 mg/kg, respectively (Fig. 3A and Table 3). By 100 mg/kg, the elimination phase over 0 to 24 hours was extended, indicating saturation of elimination, resulting in a slightly greater than dose proportional increase in AUC. Oral bioavailability (%F) ranged from 96% to 100% over the linear range. Plasma exposures at equivalent doses in mice were greater than in rats. The pharmacokinetic parameters in nonrodent species were similarly favorable, with CEP-32496 showing...
low clearance, high volume of distribution, and high F% in dogs and monkeys (Fig. 3B and Table 3). In vivo clearance in rat, dog, and monkey was low, in good correlation with in vitro data, leading to the prediction of low human clearance. In all species, the compound was well tolerated at all doses with no abnormal observations. Specifically, administration of CEP-32496 in dogs at 1 mg/kg intravenously and 3 and 10 mg/kg orally showed low clearance and a high volume of distribution, resulting in a long terminal half-life ($t_{1/2} = 12$ hours). Similarly, in monkeys, intravenous administration at 1 mg/kg resulted in a 4.9-hour half-life with low clearance and high volume of distribution, indicating extensive distribution to tissues. Following oral administration of CEP-32496 at 3 or 10 mg/kg, systemic exposure ($C_{max}$ and $AUC_{0-\infty}$) increased in a dose-related, but slightly greater than dose proportional manner, with a mean F% of approximately 100%.

**Evaluation of BRAF pathway target inhibition (pMEK and pERK) and in vivo efficacy**

**Tumor PK/PD.** The magnitude and duration of BRAF$^{V600E}$ inhibition in tumor lysates was assessed by the level of inhibition of downstream signaling pathways, assessed by normalized inhibition of pMEK and pERK as in vivo tumor pharmacodynamic endpoints. Inhibition of MEK phosphorylation in BRAF$^{V600E}$-positive subcutaneous Colo-205 tumor xenografts in nude mice was detected following administration of CEP-32496 (10, 30, and 55 mg/kg orally twice daily; Fig. 4A). CEP-32496 at 10 and 30 mg/kg resulted in significant ($P < 0.03$) inhibition of normalized pMEK in tumor lysates relative to vehicle controls at 2 and 6 hours but returned to baseline by 10 hours, whereas a 55 mg/kg dose resulted in a 75% to 57% ($P < 0.03$) inhibition of pMEK at 2 through 10-hour postadministration, with normalization to baseline by 24 hours.

Inhibition of normalized pERK was comparable, but more robust and sustained in magnitude than that observed for pMEK (Fig. 4B). A rebound to higher than control pMEK and pERK levels was reproducibly noted at later time points, when plasma and tumor drug levels were low. The rebound beyond baseline pERK levels at low plasma concentrations may be attributed to the abrogation of a negative feedback mechanism for regulation of pERK signaling and/or the result of off-target kinase modulation and cross-signaling in the MEK/ERK pathway (6, 8, 38).

In general, the magnitude and duration of normalized pMEK inhibition was consistent with plasma and particularly tumor concentrations over the dose range and time course of the pharmacodynamic assessment (Fig. 4C). RAF-265 administered at its optimally effective dose of 100 mg/kg orally every other day resulted in significant ($P < 0.03$) inhibition sustained over 2 to 24 hours (data not

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**Table 3. Summary of pharmacokinetic parameters for all species at a nominal dose**

<table>
<thead>
<tr>
<th>Species</th>
<th>Route</th>
<th>Dose, mg/kg</th>
<th>$C_l$, mL/min/kg</th>
<th>$V_d$, L/kg</th>
<th>$C_{max}$, µM/L</th>
<th>$T_{max}$, h</th>
<th>$AUC_{0-\infty}$, µM•h/L</th>
<th>$t_{1/2}$, h</th>
<th>F%</th>
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<tbody>
<tr>
<td>Mouse</td>
<td>oral</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>394</td>
<td>na</td>
<td></td>
</tr>
<tr>
<td>Rat</td>
<td>i.v.</td>
<td>1</td>
<td>4.17</td>
<td>2.8</td>
<td>6.97</td>
<td>2.0</td>
<td>75.1</td>
<td>10</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>oral</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>7.96</td>
<td>7.8</td>
<td></td>
</tr>
<tr>
<td>Dog</td>
<td>i.v.</td>
<td>1</td>
<td>5.00</td>
<td>4.5</td>
<td>5.21</td>
<td>2.0</td>
<td>63.6</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td></td>
<td>oral</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>7.20</td>
<td>9.8</td>
<td>100</td>
</tr>
<tr>
<td>Monkey</td>
<td>i.v.</td>
<td>1</td>
<td>0.40</td>
<td>2.5</td>
<td>4.28</td>
<td>4.0</td>
<td>92.2</td>
<td>4.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>oral</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4.70</td>
<td>13</td>
<td>100</td>
</tr>
</tbody>
</table>

Abbreviations: na, not available.
shown). The duration of inhibition of pMEK was consistent with sustained and elevated tumor concentrations over this time course and with the published pharmacologic profile of RAF-265 (39).

**Antitumor efficacy**

The oral antitumor efficacy and tolerability of CEP-32496 was evaluated in mouse subcutaneous human Colo-205 and HT-29 colon carcinoma and A375 melanoma xenograft models containing BRAFV600E (17, 39). Oral doses were administered twice daily, unless otherwise noted.

In established Colo-205 tumor xenografts, CEP-32496 was without effect at 10 mg/kg, whereas 30 and 100 mg/kg resulted in tumor stasis and a 40% and 80%, respectively, incidence of partial tumor regressions (Fig. 5A and Table 4).

**Figure 4.** In vivo tumor pharmacodynamic inhibition of mutant BRAF–mediated downstream signaling as measured by normalized MEK (A) and ERK (B) phosphorylation in Colo-205 tumors from mice over a 24-hour period following single oral administration of CEP-32496 at 10, 30, and 55 mg/kg, and corresponding plasma and tumor levels (C). Values shown are mean ± SEM from 2 studies, n = 3 tumor-bearing mice per time point per study. Veh, vehicle.

**Figure 5.** Antitumor efficacy profile of CEP-32496 in BRAF mutant tumor xenografts. CEP-32496 was administered orally to mice bearing established human tumor subcutaneous xenografts with the BRAFV600E genotype (Colo-205 colorectal carcinoma and A375 melanoma). A, dose response (left) for Colo-205 TGI and incidence of tumor regressions (right). B, dose response (left) for A375 efficacy with minimal antitumor activity at 10 mg/kg, and TGI and incidence of tumor regressions (right) at doses of 30 and 100 mg/kg was observed. No mortality or significant body weight loss was observed in either model.
All doses were well tolerated with no mortality and minimal body weight loss (<5% relative to vehicle controls). Minimum plasma exposures of CEP-32496 associated with antitumor efficacy in the Colo-205 tumor model were 4 to 6 μmol/L (at Cmax) at the minimum efficacious dose of 30 mg/kg. Following cessation of treatment, Colo-205 tumors exhibited slow regrowth followed by a 3- to 4-day plateau and subsequent resumption in tumor growth at a rate comparable with controls (data not shown). Confirmation of the antitumor efficacy (TGI and regressions) of CEP-32496 in BRAF-mutated colon tumors was shown to a comparable degree in established HT-29 colon carcinoma xenografts. Administration of CEP-32496 at 30 mg/kg once and twice daily for 20 days resulted in 50% and 64% TGI relative to controls and a 10% and 20% incidence of partial responses, respectively. Furthermore, CEP-32496 administration at 100 mg/kg once and twice daily resulted in 72% and 91% TGI relative to controls and a 50% and 90% incidence of partial responses, respectively (data not shown).

In A375 melanoma human xenografts, the minimum effective dose of 30 mg/kg resulted in a pronounced (P < 0.05) TGI beginning at day 10 of dosing with essentially tumor stasis (P < 0.0001) at day 15 (termination of study) and a 40% incidence of partial responses (Fig. 5B and Table 4). All dosing regimens were well tolerated with no mortality and minimal body weight loss (<5% relative to vehicle-matched controls). Minimum plasma exposures of CEP-32496 associated with antitumor efficacy in the A375 tumor model were similar to those observed in Colo-205 tumors (4–6 μmol/L at Cmax) at the minimum efficacious oral dose of 30 mg/kg. Similar to the profile observed for Colo-205 tumor xenografts, on cessation of treatment, a 7- to 10-day lag period was followed by resumption of tumor growth at a rate comparable with controls, indicating that sustained administration and exposure of CEP-32496 was associated with antitumor efficacy in the both the Colo-205 and A375 melanoma models.

CEP-32496 was compared in vivo to sorafenib and RAF-265 at the published maximally effective doses of 60 mg/kg orally once daily and 100 mg/kg orally every other day, respectively. Administration of CEP-32496 in the Colo-205 and A375 tumor models resulted in pharmacodynamics and antitumor effect superior to that of sorafenib and comparable with RAF-265, but with an improved tolerability profile to the latter compound, based upon the changes in body weight observed in these studies (Table 4).

To explore dose schedule, mice bearing established Colo-205 colon carcinoma xenografts were administered 30 mg/kg CEP-32496 once or twice daily continuously, 3 times weekly, or twice weekly, over a 25-day dosing period. As shown in Fig. 6 and Table 5, CEP-32496 showed schedule-dependent antitumor efficacy, with continuous twice and once daily administration optimal for both significant TGI and incidence of tumor regressions, whereas intermittent administration 3 times weekly (Monday, Wednesday, Friday once or twice daily) or

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**Table 4. Summary of in vivo efficacy data for CEP-32496 versus reference compounds, RAF-265 (CHIR-265), and sorafenib, in Colo-205 and A375 xenograft models, indicating a favorable profile for CEP-32496**

<table>
<thead>
<tr>
<th>Compound</th>
<th>A375 dose, mg/kg</th>
<th>Colo-205 dose, mg/kg</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEP-32496</td>
<td>30</td>
<td>60</td>
<td>No toxicity or PD, TS and 60% TGI and 60% PR, 80% PR, Transient TS and 30% PR, PR (MED)</td>
</tr>
<tr>
<td>Sorafenib</td>
<td>60</td>
<td>100</td>
<td>No toxicity or PD, 80% TGI and 60% PR, Transient TS and 30% PR, PR (MED)</td>
</tr>
<tr>
<td>RAF-265</td>
<td>30</td>
<td>60</td>
<td>No toxicity or PD, 80% TGI and 60% PR, Transient TS and 30% PR, PR (MED)</td>
</tr>
<tr>
<td>CHIR-265</td>
<td>30</td>
<td>60</td>
<td>No toxicity or PD, 80% TGI and 60% PR, Transient TS and 30% PR, PR (MED)</td>
</tr>
</tbody>
</table>

Abbreviations: BW, body weight; CR, complete regression; MED, maximally efficacious dose; PD, positive PD result; PR, partial regression; TS, tumor stasis.
twice weekly (Tuesday, Friday once or twice daily) showed significant TGI but no tumor regressions. These data support the hypothesis that sustained modulation of BRAF signaling in vivo is required for optimal therapeutic efficacy.

Discussion

CEP-32496 showed potent target activity in in vitro binding and cellular assays for mutated BRAFV600E and was modestly selective against other kinases \([S_{0.5}(\mu\text{mol/L})] = 0.3\) in competitive binding assays. However, CEP-32496 was not active at a cellular level against wild-type BRAF and half of the non-RAF targets tested in a panel of cell lines, showing a narrower profile of activity in cellular kinase assays than in in vitro binding assays. Nonetheless, the in vitro selectivity profile provides a roadmap for further exploration of potential targets, as well as potential off-target or undesirable pharmacology. Potent cellular activity at BCR-ABL, RET, and EPHA2 kinases is potentially desirable for clinical application in diseases including chronic myelogenous leukemia, thyroid malignancies, and pancreatic cancer (40). Several genetic mutations in thyroid cancer occur in the MAPK pathway, via activation of RET, RAS, or BRAF (16). Therefore, the inclusion of RET as a target should enlarge the population of patients with thyroid cancer who may benefit from CEP-32496 because these mutations, while typically mutually exclusive, are common in thyroid malignancies and common to the MAPK pathway (16). Conversely, the lack of inhibition at a cellular level for VEGFR1 and VEGFR2 is potentially a benefit with respect to the hypertension side effect profile that is associated with inhibition of these targets (41).

In the preclinical studies, CEP-32496 benchmarked favorably against known or purported BRAF inhibitors RAF-265 and sorafenib across a range of biochemical, cellular, metabolic, pharmacokinetic, and in vitro and in vivo pharmacologic parameters, particularly, in the demonstration of significant antitumor efficacy manifested by

Table 5. Summary of in vivo efficacy data for continuous versus intermittent administration shows a schedule-dependent dose response for optimal TGI and incidence of tumor regressions

<table>
<thead>
<tr>
<th>30 mg/kg dose schedule</th>
<th>% TGI</th>
<th>% Regressions</th>
<th>% Morbidity/mortality</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Twice daily</td>
<td>Tumor stasis</td>
<td>20</td>
<td>0</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Once daily</td>
<td>73</td>
<td>20</td>
<td>0</td>
<td>0.0005</td>
</tr>
<tr>
<td>M, W, F twice daily</td>
<td>67</td>
<td>0</td>
<td>0</td>
<td>0.001</td>
</tr>
<tr>
<td>M, W, F once daily</td>
<td>33</td>
<td>0</td>
<td>0</td>
<td>NS</td>
</tr>
<tr>
<td>T, F twice daily</td>
<td>58</td>
<td>0</td>
<td>0</td>
<td>0.02</td>
</tr>
<tr>
<td>T, F once daily</td>
<td>65</td>
<td>0</td>
<td>0</td>
<td>0.03</td>
</tr>
</tbody>
</table>

NOTE: \(P\) value versus vehicle-treated mice.
Abbreviation: NS, not significant.
partial and complete regressions in BRAFV600E-mutated melanoma and colorectal carcinomas with a favorable tolerability profile upon repeat oral administration. Direct experimental benchmarking against vemurafenib was not possible, as the preclinical profile of vemurafenib has only recently been published (42, 43). However, comparative assessments with the published preclinical profile of vemurafenib suggests comparability of CEP-32496 and vemurafenib with respect to a number of important parameters in each agent's preclinical profile, including in vitro enzyme and cellular potency against BRAF, degree of BRAFV600E-selective tumor cytotoxicity in vitro, tumor pharmacodynamic modulation of BRAF signalling (pMEK inhibition), the magnitude of antitumor efficacy (TGI, stasis, and regressions), and tolerability in BRAFV600E-containing tumor xenografts with once and twice daily oral administration (42).

The in vitro cytotoxicity profile and in vivo pharmacodynamic and antitumor efficacy data in BRAFV600E-mutated tumor cell lines and human melanoma and colon carcinoma xenografts, respectively, with CEP-32496 suggest that continuous inhibition of BRAF signaling is required for optimal efficacy and that continuous administration in the clinic will likely be required. Therefore, the favorable pharmacokinetic profile of CEP-32496 in rodents, dogs, and monkeys (low clearance, high tissue distribution, and high oral bioavailability) is important for increasing the likelihood of clinical success. High exposure and bioavailability of CEP-32496 in preclinical species are expected to translate to favorable absorption in humans, and the in vitro/in vivo correlation of clearance predicts that clearance in humans will be low. In mice, the free concentration in plasma of CEP-32496, accounting for 99% murine plasma protein binding, exceeds the cellular IC₅₀ by about 4-fold for approximately 12 hours following a single oral dose at 100 mg/kg. Furthermore, dose-normalized exposure in other species is even greater, at about 8-fold for rats, dogs, and monkeys. Coupled with a high volume of distribution in preclinical species (≥2.5 L/kg) and good tumor exposure in nude mouse xenografts (50–190 μg h/mL), CEP-32496 is predicted to translate to a suitable human pharmacokinetic and tissue distribution profile.

Recent success in the clinic with the selective BRAFV600E inhibitors vemurafenib, XL281, and GSK2118436 has provided strong proof of concept for BRAF inhibition for the treatment of BRAF-mutated melanoma and other tumors in which BRAF is frequently mutated (25–27, 44). Clinical studies with vemurafenib in patients with metastatic melanoma with BRAFV600E have resulted in more than 50% response rates and improved progression-free survival (25, 42, 44). Improved overall survival was confirmed in a phase III trial, with response rates of more than 50% and overall survival rates at 6 months of 84% versus 64%, for vemurafenib versus dacarbazine (control), respectively (45). The remarkable clinical activity observed with vemurafenib in patients with malignant melanoma has provided further impetus for the development of novel and competitive therapeutic agents targeting BRAF in select tumor populations.

The potential on-target side effect profile of BRAF inhibitors in development has been a subject of considerable discussion (25, 44, 46). In clinical studies with vemurafenib in patients with BRAFV600E melanoma, a consistent adverse event was incidence of drug-related squamous cell carcinomas and benign keratocanthomas in 23% to 26% of successfully treated patients, a clinical presentation that was managed without treatment interruption (25, 42). Carnahan and colleagues (46) reported similar RAF inhibitor–related hyperplasia in multiple epithelial tissues and nonepithelial cells (heart and sciatic nerve) of rats and dogs. These observations suggest that pharmacologic inhibition of BRAF may result in homeostatic changes in selected epithelia, consistent with clinical observations of frequent hyperkeratosis, skin hyperplasia, and squamous cell carcinomas in patients treated with BRAF inhibitors. The precise mechanism(s) for this apparent on-target activity of BRAF inhibitors is unclear. Several recent reports suggest that upon exposure of noncancerous wild-type BRAF cells to selective BRAF inhibitors, the normal negative feedback regulation of the MAPK pathway at the level of ARAF and/or CRAF is inactivated, resulting in enhanced and sustained MAPK signaling. Alternatively, inhibition of BRAF in normal cells may stabilize or promote BRAF and CRAF heterodimers or CRAF homodimers, driving MAPK activation, and potentially, tissue hyperplasia (35, 36, 47). In a preclinical mouse model, activation in the presence of oncogenic, but not wild-type, KRAS resulted in induction of melanomas (47). These studies appear to reveal a potential unintended consequence of BRAF inhibition, particularly, in the presence of oncogenic KRAS mutations in patients.

In this regard, despite the BRAF-inhibitory profile and pronounced antitumor efficacy achieved in BRAFV600E-mutated tumor cell lines and human tumor xenografts with CEP-32496, this histologic phenotype of extensive tissue hyperplasia has not been observed in either pharmacologic or toxicologic studies in mice, rats, and cynomolgous monkeys upon repeat oral administration (15–28 days twice daily), despite dose-related toxicokinetic exposures (Cₘₐₓ) in rats and primates of 70 to 100 μg/mL (data not shown). The precise mechanism(s) for these observations, in contrast to that of several known BRAF inhibitors, including vemurafenib, is unclear and remains to be to be assessed in the clinic with CEP-32496.

The biochemical and cellular activity, pharmacokinetic and pharmacologic profile, favorable tolerability profile, including absence of pronounced hyperplasia and hypertrophy with repeat oral administration, and expanded activity profile against several additional therapeutically relevant kinase targets (BCR-ABL, RET, and EPHA2) will make CEP-32496 an important addition to the arsenal of novel therapies under clinical
development for the treatment of BRAF-dependent malignancies.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interests were disclosed.

Authors' Contributions

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J. James, M.F. Gardner, M.W. Holladay

Study supervision: J. James, B. Ruggeri, R.C. Armstrong, M. Williams, S.S. Bhagwat

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References
23. Cui Y, Quadagno TM. BRAF(V600E) signaling deregulates the mitotic spindle checkpoint through stabilizing Mps1 levels in melanoma cells. Oncogene 2008;27:3122–33.


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