Preclinical In vivo Evaluation of Efficacy, Pharmacokinetics, and Pharmacodynamics of a Novel MEK1/2 Kinase Inhibitor RO5068760 in Multiple Tumor Models

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Abstract
Targeting the Ras/Raf/mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) pathway represents a promising anticancer strategy. Recently, we have reported a novel class of potent and selective non–ATP-competitive MEK1/2 inhibitors with a unique structure and mechanism of action. RO5068760 is a representative of this class showing significant efficacy in a broad spectrum of tumors with aberrant mitogen-activated protein kinase pathway activation. To understand the relationship between systemic exposures and target (MEK1/2) inhibition as well as tumor growth inhibition, the current study presents a detailed in vivo characterization of efficacy, pharmacokinetics, and pharmacodynamics of RO5068760 in multiple xenograft tumor models. For inhibition of MEK1/2 as measured by the phosphorylated ERK levels, the estimated EC₅₀s in plasma were 1.36 μmol/L (880 ng/mL) and 3.35 μmol/L (2168 ng/mL) in LOX melanoma and HT-29 colorectal cancer models, respectively. A similar EC₅₀ (1.41 μmol/L or 915 ng/mL) was observed in monkey peripheral blood lymphocytes. To achieve tumor growth inhibition (≥90%), an average plasma drug concentration of 0.65 or 5.23 μmol/L was required in B-RafV600E or K-Ras mutant tumor models, respectively, which were remarkably similar to the IC₉₀ values (0.64 or 4.1 μmol/L) determined in vitro for cellular growth inhibition. With equivalent in vivo systemic exposures, RO5068760 showed superior efficacy in tumors harboring B-RafV600E mutation. The plasma concentration time profiles indicate that constant p-ERK suppression (>50%) may not be required for optimal efficacy, especially in highly responsive tumors. This study may facilitate future clinical trial design in using biochemical markers for early proof of mechanism and in selecting the right patients and optimal dose regimen. Mol Cancer Ther; 9(1); 134–44. ©2010 AACR.

Introduction
Aberrant activation of the Ras/Raf/mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) signal cascade correlates with tumor progression and poor prognosis in many cancer patients. Dysregulation of the Ras/Raf/MEK/ERK pathway, due to aberrant receptor tyrosine kinase activation and Ras or B-Raf mutation, is frequently found in human cancers and represents a major factor in determining abnormal cell growth (1). Ras oncogenic mutations occur in ∼30% of all human cancers with the highest incidence in adenocarcinomas of the pancreas (90%), colon (45%), and lung (35%; ref. 2). Recently, K-Ras mutation was found to confer resistance to epidermal growth factor receptor–targeted cancer therapy (3–5). Oncogenic mutations in B-Raf were also found in 66% of primary melanomas and less frequently in other tumors such as colon (12%), ovarian (30%), and papillary thyroid cancers (35–70%; refs. 6–8). MEK1/2 are serine/threonine kinases responsible for phosphorylating ERK1/2, which then translocate to the nucleus where they regulate the expression of genes associated with the key regulators of cellular proliferation and survival. Although MEK1/2 are rarely mutated in cancer, overexpression of MEK1/2 is sufficient to induce transformation. Thus, the MAPK pathway components have become attractive targets under extensive investigation for cancer therapeutic intervention (9–11).

Many agents targeting Ras, Raf, or MEK have entered human clinical trials; however, none have been approved as cancer therapeutics. For targeting Ras, the farnesyltransferase inhibitors have shown disappointing clinical
efficacy, which now can be explained by the fact that N-Ras and K-Ras have alternative processing pathways that substitute for farnesylation (12, 13). Sorafenib was developed as a Raf inhibitor, but was later found to be a multikinase inhibitor that exhibits antitumor efficacy mainly through its antiangiogenic activity against other targets such as KDR, Flt-3, etc. (9). Most recently, PLX4032, an orally active selective B-Raf inhibitor under codevelopment by Roche and Plexxikon, exhibited significant antitumor activity in V600E B-Raf mutant tumors (14). For targeting MEK, highly selective inhibitors [PD0325901 (Pfizer), AZD6244 (Array Biopharma/Anstromeca), XL518 (Exelixis/Genentech), and RDEA 119 (Ardea Biosciences)] have been evaluated in phase I/II studies. PD0325901 and AZD6244 have shown some signs of clinical activity in the patients with pancreatic cancer, non–small cell lung cancer (NSCLC) and malignant melanoma (15, 16). However, robust clinical proof of concept for an agent targeting the Ras/Raf/MEK pathway is still pending.

Recently, we have reported a new class of MEK inhibitors with novel chemical structure and mechanism of action (17). RO5068760 belongs to this class and exhibits a unique mode of MAPK signal blockade. Here, we describe an extensive preclinical investigation of efficacy, pharmacokinetics (PK), and pharmacodynamics (PD) of RO5068760 in nude mice bearing human xenografts. Our results provide a good understanding of the relevant systemic drug exposures that are required for RO5068760 to achieve significant target suppression as well as tumor growth inhibition (TGI). These results may provide guidance for future clinical trial design in the selection of sensitive patient population, the use of biochemical markers for early proof of mechanism, and the selection of an optimal dose and regimen.

Materials and Methods

Compounds

The test compound RO5068760, systematic name, ((25,3S)-2-[(R)-4-[2-(1R,2,3-dihydroxy-propoxy)-phenyl]-2,5-dioxo-imidazolidin-1-yl]-N-(2-fluoro-4-ido-phenyl)-3-phenylbutyramide, was synthesized according to the procedure described in patent application WO 2007096259. RO5068760 was formulated as a suspension in 2% Klucel LF with 0.1% Tween 80 (v/v) in water for oral administration in nude mice. RO5068760 was formulated as micronized suspensions or tablets for oral administration in monkey.

Cell Lines

Cell lines were purchased from American Type Culture Collection and were maintained in the designated medium supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies/Bethesda Research Laboratories) and 2 mmol/L L-glutamine (Life Technologies/Bethesda Research Laboratories). The LOX IMVI cells were provided by the Biological Testing Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute (Frederick, MD).

Western Blot Analysis and MTT Assay In vitro

Cells were seeded at appropriate density (70–75% confluent) 1 d before the compound treatment. Upon compound treatment at various concentrations, p-ERK, cyclin D1, and p27 levels were analyzed by Western blot analysis, and cell proliferation/growth was analyzed by MTT as described previously (17).

Pharmacokinetic Analysis

Plasma samples were collected from two tumor-bearing mice per time point at the end of efficacy studies at 1, 2, 4, 8, and 12 h postdose [twice daily dosing and at 1, 2, 4, 8, 24 h for once daily dosing]. Plasma samples were stabilized with acetic acid to inhibit conversion to the epimer. The mean plasma concentration time profile was generated based on composite data for both RO5068760 and its less active epimer RO5128368. In the HT-29 xenograft study, 1-h sample was not collected and data were only generated for RO5068760. Noncompartmental pharmacokinetic analysis was done on mean plasma data using Watson Lims Software. Area-under-the-curve (AUC) for the dosing interval was calculated using the linear trapezoidal rule. The concentration at 0 h was defined as 0 ng/mL. The maximum plasma concentration (Cmax) was taken directly from the plasma concentration time profile.

Bioanalytic Method

The concentrations of RO5068760 and its epimer, RO5128368, in mouse plasma or tumor were determined using a selective and reproducible bioanalytic method. Tumor samples were homogenized in a volume of PBS equivalent to thrice the tissue weight and processed similarly as with plasma samples. Isotopic internal standards were used for some of the studies. The analytes were extracted from plasma or tissue homogenates by protein precipitation. Reconstituted samples were analyzed by liquid chromatography tandem mass spectrometry in the positive mode. The lower limit of quantification for both analytes was 5.0 ng/mL in plasma and tumor homogenates.

Western Blot Analysis (PD Assay of Phosphorylated ERK Levels in Xenograft Tumors)

Xenograft tumors were harvested and flash frozen at the indicated time points and stored at −80°C. Protein was extracted by homogenization (Autogizer, TOMTEC) in the presence of 2 to 5 mL cell lysis buffer (Cell Signaling Technology) containing protease inhibitors (Roche Diagnostics). After incubation on ice for 30 min, the lysates were centrifuged at 14,000 rpm for 15 min to clear the insoluble fragments. The protein concentrations were determined and equal amounts of total protein were resolved on 4% to 12% NuPage gradient gel (Invitrogen) and blotted with the antibodies as indicated. The densitometric quantitation of specific bands was determined using Multi Gauge Software (Fujifilm). The total ERK1/2 values
served to normalize the phosphorylated ERK (p-ERK)1/2 signal values. After the initial subtraction of background signal, the p-ERK1/2 to total-ERK1/2 ratio was determined. This value for the control sample (vehicle treated) was set to 100% (or 0% inhibition) and the values for the compound dosed samples were expressed as percent control or percent inhibition relative to the control sample.

Fluorescence-Activated Cell Sorting Analysis (Ex vivo PD Assay of p-ERK Levels in Peripheral Blood Mononuclear Cells)

The procedure for the detection of p-ERK in whole blood by flow cytometry was based on the procedure described by Chow et al. (18). Whole blood was drawn from monkeys before or after dosing at indicated time points using (K2)EDTA as an anticoagulant; the blood was stored at 4°C. Whole blood (0.1 mL) was stimulated with phorbol myristate acetate (PMA; Sigma-Aldrich) at a final concentration of 400 μM and incubated for 10 min at 37°C. Formaldehyde (Polysciences) was then added to a final concentration of 4% and the samples were held at room temperature for 10 min. RBC were lysed by the addition of a Trion X-100/PBS solution to a final concentration of 0.1% for 30 min at room temperature. The remaining cells are washed and collected by centrifugation and resuspended in methanol (50%). Following incubation with methanol, the cells were stained with fluorescent-conjugated phospho-p44/42 MAPK (T202/Y204) antibody (Alexa Fluor 488 conjugate). The lymphocyte population was identified and gated based on a dot plot of forward scatter versus side scatter. Within the gated population, the mean fluorescence intensity (MFI) of p-ERK (T202/Y204) was determined. The MFI value for the predose sample (control) was set to 100% (or 0% inhibition) and the values for the compound dosed samples were expressed as percent control or percent inhibition relative to the control sample.

In vivo Efficacy Studies in Xenograft Tumor Models

Human cancer cells were implanted s.c. in the right flank of female nude mice. When mean tumor volume reached approximately 100 to 200 mm³, mice were randomized to treatment groups (n = 10 per group) and received either vehicle or compound at the dose indicated. Tumor volume and mouse body weight were measured twice to thrice weekly. Efficacy data were graphically represented as the mean tumor volume ± SEM. Tumor volume (in cubic millimeters) was calculated as described previously (17). Statistical analysis was determined by Mann-Whitney rank-sum test, one-way ANOVA, and post hoc Bonferroni t test (SigmaStat, version 2.0). Differences between groups were considered significant when the probability value was ≤0.05. Differences between groups were considered biologically significant when percent TGI was ≥60%.

PK-PD Modeling

The PD values and the corresponding PK values generated from the same animals were used for EC50 determination by Xlfit using 4 Parameter Logistic Model or Sigmoidal Dose-Response Model (205) where y = A + [(B−A)/(1+(C/x)^D)]

Results

In vitro and In vivo Characteristics of MEK Inhibitor RO5068760

As described previously (17), we have identified a new class of MEK inhibitors that contains a novel substituted hydantoin ring system. After lead optimization, a highly potent and selective MEK1/2 inhibitor RO5068760 was identified (Table 1). In vitro, RO5068760 potently inhibits MEK1 kinase activity with an IC50 value of 0.025 ± 0.012 μM in a Raf/MEK/ERK cascade assay. It is a non–ATP-competitive inhibitor and lacks activity (IC50 > 10 μM) against any other kinase in a large panel of kinase assays tested (>200 kinases; Table 1; ref. 17). RO5068760 significantly inhibits MAPK pathway activity as evidenced by the dose-dependent inhibition of both ERK and MEK phosphorylation (p-ERK and p-MEK) in many human cancer cell lines. Representative IC50 values for p-ERK inhibition are shown in Table 1 as 0.022 μM (LOX melanoma), 0.012 μM (HT-29 colorectal cancer), 0.082 μM (H460 NSCLC, and 0.069 μM (MIA PaCa-2 pancreatic cancer). Many lines of evidence suggest that p-ERK inhibition alone is not sufficient to predict the antitumor activities of MEK inhibitors (17, 19). RO5068760 specifically inhibited cell growth in cancer cell lines with activating pathway gene mutations (B-Raf or K-Ras), and the representative IC50/IC90 values are 0.018/0.72 μM in LOX (B-RafV600E), 0.11/0.56 μM in HT-29 (B-RafV600E), 0.30/3.08 μM in H460 (K-RasG12C), and 1.25/5.11 μM in MIA PaCa-2 (K-RasQ61K) cancer cells. The B-RafV600E mutant lines are more sensitive compared with the Ras mutant lines with average IC50 values of 0.64 μM versus 4.1 μM (Table 1). In cancer cells sensitive to MEK inhibition, specific suppression of p-MEK and p-ERK by RO5068760 led to the downregulation of CDK4/cyclin D1 and upregulation of p27. Dysregulation of these well-known cell cycle regulators resulted in G1 cell cycle arrest and subsequently led to the apoptotic cell death in responsive cancer cells (Supplementary Fig. S1).

In vivo, when administered orally, RO5068760 showed dose-dependent antitumor activity in corresponding human tumor xenografts with aberrant MAPK pathway activation. As shown in Table 1 and Fig. 1, in vivo antitumor activity of RO5068760 was evaluated using various oral doses (from 6.25–500 mg/kg) and schedules (once or twice daily, once weekly). The conventional once or twice daily dose schedule was designed to deliver continuous drug exposure in vivo. The exploratory once weekly schedule was chosen to determine whether a short pulse treatment with the highest Cmax level could be as effective as continuous dosing. We went with the highest concentration feasible for making an oral suspension that delivered the 500 mg/kg dose level. RO5068760 showed
statistically significant TGI (>65% TGI, P < 0.005) in all tumor-bearing mice treated with the drug twice daily or once daily compared with vehicle-treated control mice. The maximum tolerated dose was not identified with any given regimens and there were no gross signs of adverse effect to the treatment for 3 weeks (data not shown). In the LOX melanoma model harboring B-RafV600E mutation, animals treated at doses as low as 12.5 mg/kg twice daily exhibited partial (PR) or complete regression (CR). At a dose of 100 mg/kg twice daily, PR or CR was shown in four of four tumor models tested (P < 0.001). Substantial antitumor activity was observed in the LOX melanoma model even at 500 mg/kg once weekly with three PRs and five CRs compared with vehicle controls (P < 0.001). However, treatment with 500 mg/kg once weekly in H460 lung and MIA PaCa-2 pancreatic models harboring K-Ras mutations did not yield biologically significant TGI (48% and 42%, respectively). Given the same total dose, weekly dosing was less effective and twice daily dosing yielded better antitumor efficacy compared with once daily dosing in K-Ras mutant tumors, suggesting that Cmax is not driving efficacy and continued exposure to RO5068760 may provide better clinical benefit. These in vivo results are consistent with the cytostatic nature of a MEK inhibitor.

**The PK-PD Relationship of RO5068760 in Nude Mice Bearing Human Tumor Xenografts**

At the efficacious dose levels, the detailed PK-PD (p-ERK inhibition) relationship of RO5068760 was evaluated in the LOX melanoma and HT-29 colorectal

### Table 1. Highly selective novel MEK inhibitor RO5068760

**In vitro characteristics of RO5068760**

| Chemical structure: C28H27FIN3O6 | Molecular weight: 647.45 |

| **In vitro IC50 for MEK1 (μM)** | 0.025 ± 0.012 |
| **In vitro IC50 for other kinases* (μM)** | >10 |
| **In vitro IC50 for cellular p-ERK inhibition (μM)** | 0.012 (HT-29 colorectal) |
| | 0.082 (H460 NSCLC) |
| | 0.069 (MIA PaCa-2 pancreas) |
| **In vitro IC50/IC90 for cell growth inhibition (μmol/L)** | 0.018/0.72 (LOX) |
| | 0.11/0.56 (HT-29) |
| | 0.30/3.08 (H460) |
| | 1.25/5.11 (MIA PaCa-2) |

**In vivo antitumor efficacy of RO5068760**

<table>
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<th>Tumor type</th>
<th>Mutation</th>
<th>6.25 mg/kg twice daily</th>
<th>12.5 mg/kg twice daily</th>
<th>25 mg/kg twice daily</th>
<th>50 mg/kg twice daily</th>
<th>100 mg/kg twice daily</th>
<th>200 mg/kg twice daily</th>
<th>500 mg/kg once weekly</th>
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<tr>
<td></td>
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<td>(4/10 PR)</td>
<td>(10/10 CR)</td>
<td>(10/10 CR)</td>
<td>(10/10 CR)</td>
<td>(10/10 CR)</td>
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<td>(3/10 PR)</td>
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<tr>
<td>HT-29 (colorectal)</td>
<td>B-Raf (V600E)</td>
<td>65</td>
<td>71</td>
<td>84 (3/8 PR)</td>
<td>&gt;100 (4/8 PR)</td>
<td>95 (4/8 PR)</td>
<td>&gt;100 (4/8 PR)</td>
<td>&gt;100 (4/8 PR)</td>
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<tr>
<td>MIA PaCa-2 (pancreas)</td>
<td>KRAS (G12C)</td>
<td>64</td>
<td>83</td>
<td>90 (2/10 PR)</td>
<td>80</td>
<td>48%</td>
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<tr>
<td>H460 (NSCLC)</td>
<td>KRAS (Q61H)</td>
<td>64</td>
<td>83</td>
<td>90 (2/10 PR)</td>
<td>80</td>
<td>48%</td>
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Abbreviations: CR, complete regression; PR, partial regression.
*Kinase panels were described by Daouti, et al. (2009).
xenograft models. After a single oral dose of RO5068760 at 100 mg/kg in nude mice bearing human LOX melanoma xenografts, tumors were excised at 0, 1, 2, 4, 8, 12 hours postdosing, and p-ERK, total ERK, cyclin D1, and p27 levels were analyzed by Western blot (Fig. 2A). The PD effects were determined by the percentage of p-ERK/ERK inhibition in treated tumors compared with the vehicle-treated controls. The plasma samples were collected from the same test animals, and drug concentrations in both plasma and tumor tissues were measured. As shown in Table 2, PD effects (p-ERK inhibition) correlated well with drug levels in both plasma and tumor tissues. After fitting to a four-parameter logistic (sigmoidal dose-response) model using XLfit, the estimated EC50 values were 880 ng/mL (1.36 μmol/L) and 928 ng/g in plasma and xenograft tumors, respectively (Fig. 2A).

Furthermore, the PK-PD (p-ERK inhibition) relationship of RO5068760 was evaluated at two different efficacious dose levels (50 and 100 mg/kg) in nude mice bearing human HT-29 colorectal cancer xenograft tumors (Table 2). Tumors tissues were excised at 2, 4, 8, and 12 hours after single oral administration of RO5068760. p-ERK and total ERK levels were analyzed by Western blot. Similar to the LOX model, the PD (p-ERK inhibition) effect of RO5068760 and the corresponding plasma and tumor drug concentrations were determined and fitted to the four-parameter logistic model; the estimated EC50 values were 2168 ng/mL (3.35 μmol/L) and 1001 ng/g in plasma and xenograft tumors, respectively (Fig. 2B).

**The PK-PD Relationship of RO5068760 in Cynomolgus Monkey**

In clinical trials for an investigational drug, tumor tissue may not be always available. To establish a PK/PD relationship in an easily accessible surrogate tissue such as blood cells, PK-PD studies were done with RO5068760 in cynomolgus monkeys. After oral administration of a single dose of RO5068760, monkey whole blood and plasma were collected from animals predose and at 1, 3, 6, 12, and 24 hours postdosing. Drug concentrations...
were determined in plasma, and p-ERK inhibition was examined in whole blood by fluorescence-activated cell sorting after stimulation with PMA (Fig. 2C). In fluorescence-activated cell sorting analysis, lymphocytes, with their high nucleus/cytoplasm ratio, show different light scattering characteristics based on their size and absorbance (granularity). Thus, it became a common strategy to use electronic gates on the basis of forward scatter versus side scatter to focus the analysis on the lymphocyte population. Peripheral blood mononuclear cells have a low basal level of ERK phosphorylation. Upon PMA stimulation, p-ERK was significantly increased. After a single oral dose of RO5068760 in monkeys, significant suppression of PMA-stimulated ERK phosphorylation in peripheral blood mononuclear cells was achieved, which correlated well with the drug concentrations measured in the corresponding plasma samples. The PK/PD relationship was modeled based on a sigmoidal dose-response as shown in Fig. 2C; the EC₅₀ was estimated to be 915 ng/mL (1.41 μmol/L) in cynomolgous monkeys.

The Relationship between RO5068760 Systemic Exposures and Antitumor Efficacy in Nude Mice Bearing Human Xenograft Tumors

Orally active RO5068760 showed dose-dependent antitumor activity in a variety of human tumor xenografts (Table 1; Fig. 1). To further characterize the relationship between RO5068760 systemic exposures and antitumor efficacy, at the end of the efficacy studies, the plasma drug concentrations of RO5068760 were measured at various time points after the final dose and PK parameters including the AUC₀₋₁₂ (twice daily) or AUC₀₋₂₄ (once daily) were determined (Table 3). RO5068760 showed reasonably consistent drug exposures across different tumor models with the exception of the HT-29 model where plasma drug concentrations were lower compared with others at the same dose levels. After 3 to 4 weeks of treatment, the chronic drug exposures were equivalent to that of acute (single) doses in all models. At comparable systemic drug exposures [C_max, AUC, and average drug concentration (C_avg) in Table 3], RO5068760 showed superior in vivo efficacy in B-RafV600E xenografts (LOX and HT-29) compared with K-Ras mutant xenografts (H460 and MIA PaCa-2). As C_τrough levels were negligible regardless of dose levels (Supplementary Fig. S2) and C_max was not driving efficacy, the C_avg in plasma were calculated and plotted against the corresponding antitumor activity (%TGI). The estimated C_avg in plasma required for achieving tumor stasis (≥90% TGI) was 423 ng/mL (0.65 μmol/L) or 3389 ng/mL (5.23 μmol/L) in B-RafV600E or K-Ras mutant tumors, respectively (Fig. 3). Interestingly, these C_avg values for tumor stasis (≥90% TGI) are remarkably similar to the average IC₉₀ (0.64 or 4.1 μmol/L) determined in vitro for anti-proliferation in the B-RafV600E or K-Ras mutant cells, respectively (Table 1).

Although sustained drug exposure or target inhibition may be optimal for efficacy as monotherapy, safety concerns raised during the clinical investigation of other MEK inhibitors prompted us to ask what was the minimal duration and magnitude of target (p-ERK) inhibition required for efficacy. First, the mean plasma concentration time profiles of RO5068760 (Supplementary Fig. S2) at doses resulting in a significant TGI (>90% TGI) in xenograft models were assessed for the time above the mean EC₉₀ (1.36 + 3.35 = 2.36 μmol/L or 1525 ng/mL) of p-ERK suppression in tumors (Fig. 2A and B). In the LOX model dosed with 12.5 mg/kg twice daily, which achieved CR/PR, the plasma concentrations of RO5068760 remained just below the EC₉₀ (1525 ng/mL), whereas in the other tumor models dosed at 100 mg/kg twice daily, which resulted in tumor stasis, plasma levels of RO5068760 were sustained above EC₉₀ (1525 ng/mL) for 5 to 7 hours (HT-29, MIA PaCa-2, and H460) within a 12-hour dosing interval. In a second assessment, the maximal plasma levels were compared with the EC₉₀ of p-ERK inhibition (∼9,000 ng/mL). In the MIA PaCa-2 and H460 xenograft models, C_max levels in plasma reached or exceeded the EC₉₀ of p-ERK suppression, whereas in the LOX and HT-29 models, maximal plasma levels required for a similar degree of efficacy were significantly lower (Table 3). This suggests that K-Ras mutant tumors require a higher degree and longer duration of p-ERK suppression for optimal efficacy compared with B-Raf mutant tumors. In addition, in all models at efficacious dose levels, C_τrough was minimal, indicating that constant p-ERK suppression (>50%) may not be required for efficacy, especially in the most responsive tumor populations.

Discussion

The Ras/Raf/MEK/ERK pathway is aberrantly activated in many human cancers. Ultimate clinical proof of concept in cancer therapy for a selective Ras/Raf/MEK/ERK pathway inhibitor remains elusive. The most advanced investigational MEK inhibitors (PD0325901 and AZD6244) share similar chemical core structures and biological properties. Both agents have shown some clinical benefits including partial response and stable disease, but have also revealed potential safety issues that were not known to be mechanism or chemo-type related (15, 16). We have identified a new class of MEK inhibitors with a novel structure and a unique mechanism of action that may offer differential clinical benefit in cancer patients (17). Many challenges remain in the development of MEK inhibitors: (a) the selection of the most responsive patient population; (b) the use of biochemical markers for early proof of mechanism of action and to define an optimal dose regimen for target inhibition; (c) the use of surrogate markers to predict early response; and (d) the selection of optimal combination partners and regimens for rational combination therapy. The current study presents a detailed in vivo characterization of efficacy, PK, and PD of RO5068760 in multiple human tumor xenograft models. The relationships between RO5068760 systemic exposures and the target (ERK phosphorylation) inhibition as well as TGI were evaluated. The plasma EC₉₀...
required for p-ERK suppression and the average plasma EC₉₀ for tumor stasis were determined. The minimal duration and magnitude of p-ERK inhibition required for optimal efficacy were estimated. These results may provide guidance for future clinical trial design in the selection of a responsive patient population, the use of biochemical markers for early proof of mechanism, and the selection of an optimal dose regimen.

For the inhibition of ERK phosphorylation (p-ERK) in LOX melanoma and HT-29 colorectal cancer xenografts, the estimated EC₅₀ in plasma were 1.36 μmol/L (880 ng/mL) and 3.35 μmol/L (2168 ng/mL), respectively, and the estimated EC₅₀ in tumor tissue were 928 ng/g and 1001 ng/g, respectively (Fig. 2A and B). The difference in plasma EC₅₀ values between the two tumor models could be the result of analytic variability due to the limited actual data available around the predicted EC₅₀ values. The tumor concentration time profile generally follows the plasma concentration time profile. Only at the 12 hours postdose did tumor drug concentration exceed plasma concentration, indicating a slightly slower elimination from the tumor. Assuming that distribution of the drug is similar between the xenograft and the patient tumors, the preclinical results support that plasma concentration in the clinic could be used for an initial estimation of drug concentration and target inhibition

<table>
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<tr>
<th>Tumor model</th>
<th>Oral dose level</th>
<th>Time postdose</th>
<th>% p-ERK inhibition</th>
<th>Plasma drug concentration (ng/mL)</th>
<th>Tumor drug concentration (ng/g)</th>
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<td>41</td>
<td>1,260</td>
<td>906</td>
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<td></td>
<td>12</td>
<td>1,560</td>
<td>995</td>
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<td></td>
<td></td>
<td>12 h</td>
<td>0</td>
<td>66</td>
<td>608</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>11</td>
<td>61</td>
<td>575</td>
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<td></td>
<td></td>
<td></td>
<td>60</td>
<td>115</td>
<td>188</td>
</tr>
<tr>
<td></td>
<td>100 mg/kg</td>
<td>2 h</td>
<td>77</td>
<td>9,700</td>
<td>2,090</td>
</tr>
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<td></td>
<td></td>
<td>76</td>
<td>9,360</td>
<td>2,860</td>
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<td></td>
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<td>4 h</td>
<td>79</td>
<td>6,030</td>
<td>3,080</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>83</td>
<td>6,090</td>
<td>945</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8 h</td>
<td>0</td>
<td>741</td>
<td>293</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>70</td>
<td>3,900</td>
<td>1,260</td>
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<tr>
<td></td>
<td></td>
<td>12 h</td>
<td>18</td>
<td>18</td>
<td>321</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0</td>
<td>195</td>
<td>505</td>
</tr>
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</table>

Figure 2. The in vivo PK-PD relationship of RO5068760. Nude mice bearing LOX melanoma (A) and HT-29 colorectal (B) xenograft tumors were treated with RO5068760 as indicated, and the xenograft tumors were harvested at various time points postdose. The p-ERK/ERK, cyclin D1, and p27 levels in the tumor lysates were examined by Western blot analysis. The tumor lysates were derived from two to three mice for each time point. Percentage of p-ERK inhibition was quantified and plotted against the respective drug concentrations in plasma derived from the same animal. The PK-PD relationship of RO5068760 in tumor-bearing mice was modeled and EC₅₀ was determined. C, cynomolgus monkey was orally dosed with RO5068760 at 40 mg/kg and whole blood was collected at 0, 2, 12, or 24 h postdose. The blood samples were treated with PMA (400 nmol/L) for 10 min, and p-ERK levels (MFI) in gated lymphocytes were analyzed by fluorescence-activated cell sorting. The p-ERK levels were quantified by MFI and plotted against respective plasma drug concentrations measured from the same animals. The PK-PD relationship in monkey was modeled and EC₅₀ was determined.
Furthermore, using peripheral blood lymphocytes in monkey as a surrogate tissue, a similar EC_{50} (1.41 μmol/L) was observed for the inhibition of PMA-stimulated ERK phosphorylation (Fig. 2C) compared with xenografts. Because blood cells are easily accessible in human subjects, these data can be generated early in man for proof of mechanism of action and a corresponding EC_{50} can be readily determined. The clinical and preclinical data can be compared to assess the degree and duration of drug action in man. With the challenges mentioned above for developing MEK inhibitors in clinic, the current studies provide guidance for future clinical trial design in the use of a biochemical marker for early proof of mechanism of action and for optimal dose regimen selection.

For achieving significant TGI (≥90% TGI), an estimated average plasma concentration of 0.65 or 5.23 μmol/L was

---

**Table 3.** The relationship between RO5068760 drug concentrations and TGI in multiple xenograft tumor models

<table>
<thead>
<tr>
<th>Tumor model</th>
<th>Dose* (mg/kg)</th>
<th>C_{max} (ng/mL)</th>
<th>AUC_{0-12} (ng*h/mL)</th>
<th>C_{ave} (ng/mL)</th>
<th>% TGI</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOX (B-RafV600E) melanoma</td>
<td>6.25</td>
<td>750</td>
<td>3,970</td>
<td>331</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>12.5</td>
<td>1,170</td>
<td>4,660</td>
<td>388</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>3,790</td>
<td>10,000</td>
<td>833</td>
<td>&gt;100  (10/10 CR)</td>
</tr>
<tr>
<td>HT-29 (B-RafV600E) colorectal</td>
<td>25</td>
<td>911</td>
<td>3,779</td>
<td>315</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>1,300</td>
<td>7,306</td>
<td>609</td>
<td>75</td>
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<tr>
<td></td>
<td>100</td>
<td>4,070</td>
<td>18,354</td>
<td>1,530</td>
<td>95 (4/10 PR)</td>
</tr>
<tr>
<td>H460 (K-RasQ61H) NSCLC</td>
<td>25</td>
<td>2,750</td>
<td>8,800</td>
<td>733</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>8,080</td>
<td>23,300</td>
<td>1,942</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>16,900</td>
<td>62,900</td>
<td>5,242</td>
<td>90 (2/10 PR)</td>
</tr>
<tr>
<td></td>
<td>200 once daily</td>
<td>20,700</td>
<td>122,000</td>
<td>5,083</td>
<td>80</td>
</tr>
<tr>
<td>MIA PaCa-2 (K-RasG12C) pancreas</td>
<td>25</td>
<td>3,960</td>
<td>12,700</td>
<td>1,058</td>
<td>71</td>
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<tr>
<td></td>
<td>50</td>
<td>5,240</td>
<td>18,800</td>
<td>1,567</td>
<td>84</td>
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<td>9,650</td>
<td>32,700</td>
<td>2,725</td>
<td>&gt;100 (4/8 PR)</td>
</tr>
<tr>
<td></td>
<td>200 once daily</td>
<td>15,500</td>
<td>66,200</td>
<td>2,758</td>
<td>97 (3/8 PR)</td>
</tr>
</tbody>
</table>

*All doses were given twice daily other than those designated otherwise.

†AUC_{0-24} were presented as the dose schedules were once daily.

---

Figure 3. The estimated C_{ave} in plasma required for achieving tumor stasis (≥90% TGI) in multiple tumor models. Human cancer cells were implanted s.c. in nude mice. After palpable tumors were established, the tumor-bearing mice were dosed with RO5068760 as indicated by oral gavage (10 mice per group). The mean tumor volume in each dosing group were measured. The plasma drug concentrations of RO5068760 were measured at various time points after the final dose, and the C_{ave} in plasma were calculated and plotted against corresponding antitumor activity (% TGI; Table 3). The estimated C_{ave} in plasma required for achieving tumor stasis (≥90% TGI) was determined.
required for RO5068760 in B-RafV600E or KRas mutant
tumor models, respectively, which were remarkably
similar to the in vitro IC50 values (0.64 or 4.1 μmol/L)
for growth inhibition in these cells (Table 1; Fig. 3).
A more detailed analysis of the plasma concentration
time profiles (Supplementary Fig. S2) indicate that con-
stant p-ERK suppression (>50%) may not be necessary
for MEK inhibitors to achieve efficacy as monotherapy,
which is especially relevant for better management of
toxicities in normal cells. In addition, this provides flex-
ibility in dose regimen selection when used in combi-
nation with other cancer agents. Many data have sug-
gested that tumors harboring B-RafV600E mutation
are extremely sensitive to the MEK inhibition (19–22).
In line with these results, RO5068760 shows superior
in vitro efficacy in the B-RafV600E mutant xenografts
compared with the KRas mutant xenografts with equiva-
lent systemic exposures. In the B-Raf mutant xen-
ografts, the effective drug concentration required for
tumor regression were significantly lower than that in
the KRas mutant xenografts (Table 3). To assess the
duration and magnitude of p-ERK inhibition required
for optimal efficacy (>90% TGI), we found that in the
LOX model dosed with 12.5 mg/kg twice daily that
achieved CR/PR, the plasma concentrations remained
just below the EC50 (1,525 ng/mL) of p-ERK inhibition,
whereas in the other tumor models dosed at 100 mg/kg
twice daily that resulted in tumor stasis, plasma levels
of RO5068760 were sustained above EC50 (1525 ng/mL)
for 5 hours (HT-29) to 7 hours (MIA PaCa-2 and H460)
within a 12-hour dosing interval (Supplementary Fig. S2).
In the B-RafV600E tumors, the maximal degree of p-ERK
inhibition required for optimal efficacy (>90% TGI)
ranged from 40% to 80%, whereas in the KRas mutant
tumors ≥80% suppression was needed. These results
suggest that the KRas mutant tumors require a higher
degree and longer duration of p-ERK suppression for
optimal efficacy compared with B-Raf mutant tumors.
Our study provides guidance for testing MEK inhibitors
in the clinic and in the selected patient population using
an optimal biological dose for achieving efficacy and
reducing toxicity.

It is worth noting that there are several caveats associ-
ated with the preclinical PK-PD study described here:
first, the assumptions for monotherapy may not be di-
rectly applicable for combination therapy, which, how-
ever, is the most likely clinical treatment strategy for MEK
inhibitors considering the extensive cross-talk and feed-
back regulation between oncogenic signaling pathways.
In addition, the growth kinetics of preclinical xenografts
are different from that of human patient tumors, thus the
PK-PD (efficacy) relationship in preclinical models may
not be directly translatable. Lastly, human DMPK and
safety properties may differ from preclinical animals
(mice and monkey), which further complicate the inter-
pretation of preclinical data. Despite these limitations,
the current PK-PD study with the novel MEK inhibitor
RO5068760 may help to guide the clinical studies
through improved understanding of the relationship be-
tween drug concentrations of RO5068760 and the target
inhibition as well as the TGI in selected tumors highly
sensitive to the MEK inhibition.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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