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RG7204 (PLX4032), a Selective BRAF^{V600E} Inhibitor, Displays Potent Antitumor Activity in Preclinical Melanoma Models

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Abstract

The BRAF^{V600E} mutation is common in several human cancers, especially melanoma. RG7204 (PLX4032) is a small-molecule inhibitor of BRAF^{V600E} kinase activity that is in phase II and phase III clinical testing. Here, we report a preclinical characterization of the antitumor activity of RG7204 using established *in vitro* and *in vivo* models of malignant melanoma. RG7204 potently inhibited proliferation and mitogen-activated protein/extracellular signal-regulated kinase (ERK) kinase and ERK phosphorylation in a panel of tumor cell lines, including melanoma cell lines expressing BRAF^{V600E} or other mutant BRAF proteins altered at codon 600. In contrast, RG7204 lacked activity in cell lines that express wild-type BRAF or non-V600 mutations. In several tumor xenograft models of BRAF^{V600E}-expressing melanoma, we found that RG7204 treatment caused partial or complete tumor regressions and improved animal survival, in a dose-dependent manner. There was no toxicity observed in any dose group in any of the *in vivo* models tested. Our findings offer evidence of the potent antitumor activity of RG7204 against melanomas harboring the mutant *BRAF^{V600E}* gene. *Cancer Res; 70(13); 5518–27. ©2010 AACR.*

Introduction

The Ras-Raf-MEK-ERK signaling pathway has been implicated in human oncogenesis (1–3). This pathway normally connects extracellular signals, such as growth factors and hormones, to the nucleus, leading to the expression of genes that regulate cell proliferation, differentiation, and survival (2). When a ligand binds to its receptor tyrosine kinase on the plasma membrane, it stimulates the activity of Ras. One major effector of Ras is the Raf family of serine/threonine kinases, which comprises A-Raf, BRAF, and C-Raf (4–7). Raf proteins signal through phosphorylation and activation of a downstream kinase, mitogen-activated protein (MAP)/extracellular signal-regulated kinase (ERK) kinase (MEK), which subsequently phosphorylates and activates ERK (8). The Ras-Raf-MEK-ERK pathway may be constitutively activated in human cancers through mutations in Ras or Raf (1–3).

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Mutations in BRAF have been found in ~8% of human cancers, including ~50% of melanomas, 30% to 70% of thyroid cancers, 30% of serous low-grade ovarian cancers, and 10% of colorectal cancers (2, 9). The majority of BRAF mutations detected in human cancer cell lines and primary tumors are substitutions of a single amino acid in the kinase domain (V600E; ref. 9). The V600E mutation results in constitutively active BRAF that is independent of activation by Ras (9).

Based on its association with human cancers, BRAF has been a target of small-molecule therapies to treat cancer (1, 3). Raf inhibitors, such as BAY 43-9006 (sorafenib), are not selective for BRAF, with activity against multiple kinase targets (10). Other small-molecule BRAF inhibitors include RAF265 (11), XL281 (12), AZ628 (13), GSK2118436 (14), and GDC-0879 (15).

RG7204 (formerly PLX4032) is a small-molecule inhibitor that inhibits BRAF^{V600E} with a IC_{50} of 30 nmol/L (data not shown). It is undergoing phase II and III clinical investigation. In a previous preclinical study, RG7204 had an antiproliferative effect in melanoma and thyroid cell lines, with a concomitant dose-dependent block of MEK1/2 phosphorylation (16). In addition, RG7204 induced apoptosis in a melanoma cell line (16).

A phase I dose-escalation study with RG7204 showed promising results, particularly in patients with metastatic melanoma (17, 18). Melanoma patients with the BRAF^{V600E} mutation were dosed with RG7204 formulated for increased bioavailability. Eleven of 16 patients treated with >240 mg bid had partial regression, and three others showed a lesser response that did not reach the Response Evaluation Criteria In Solid Tumor (RECIST) criteria for partial regression

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(17–19). In a phase I extension study using the maximum tolerable dose of 960 mg bid, of 27 evaluable patients with metastatic melanoma, 18 met the RECIST criteria for partial regression, and there was one complete regression in a patient with stage M1a disease (i.e., with cutaneous metastases; ref. 19). It should be noted that the majority of the melanoma patients in these studies had stage M1c disease, indicating a poor prognosis (17–20).

This report extends the previous findings with RG7204 to panels of melanoma cell lines and other tumor cell lines with and without the $BRAF^{V600E}$ mutation, further showing the selectivity of RG7204 for $BRAF^{V600E}$. We also investigated the effect of RG7204 on tumor regression and survival in $BRAF^{V600E}$-bearing melanoma tumor xenograft models using the same high-bioavailability microprecipitated bulk powder (MBP) formulation that is used in the clinical trials. The data generated from these preclinical *in vivo* efficacy studies with melanoma xenograft models have foreshadowed the clinical results observed in patients with metastatic melanoma.

Materials and Methods

Cell lines and reagents

The LOX IMVI (LOX) cells were provided by the Biological Testing Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute-Frederick Cancer Research and Development Center. HMVII cells were purchased from the European Collection of Cell Cultures, Health Protection Agency Culture Collections. MKN74 cells were purchased from the Japanese Collection of Research Bioresources, Health Science Research Resources Bank. WM239A, WM1341D, WM3152, and WM1789 cell lines were obtained from Dr. Meenhard Herlyn (The Wistar Institute, University of Pennsylvania, Philadelphia, PA). All other cell lines were purchased from the American Type Culture Collection. All cell lines were maintained in the designated medium (Supplementary Table S1) supplemented with the indicated concentration of heat-inactivated fetal bovine serum [Life Technologies/Bethesda Research Laboratories (BRL)] and 2 mmol/L L-glutamine (Life Technologies/BRL).

The following antibodies were from Cell Signaling Technology: anti–phospho-ERK1/2 (Thr202/Tyr204), anti–phospho-MEK1/2 (Ser217/221), and anti-MEK1/2. Anti-ERK1/2 antibody was from Millipore.

Cellular proliferation assays

Cellular proliferation was evaluated by 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; Sigma) assay. Briefly, cells were plated in 96-well microtiter plates at a density of 1,000 to 5,000 cells per well in a volume of 180 μL. For the assay, RG7204 was prepared at 10 times the final assay concentration in media containing 1% DMSO. Twenty-four hours after cell plating, 20 μL of the appropriate dilution were added to plates in duplicate. The plates were assayed for proliferation 6 days after the cells were plated according to the procedure originally described by Mosmann (21). Percent inhibition was calculated using the formula:

$$\text{Percent inhibition} = 100 - \left[\frac{\text{Mean absorbance of experimental wells}}{\text{Mean absorbance of control wells}}\right] \text{x } 100$$

The IC_{50} was determined from the regression of a plot of the logarithm of the concentration versus percent inhibition by XLfit (version 4.2; IDBS) using the Dose-Response One-Site Model (#205).

Western blot analysis

For sample preparation from cell lines, the cells were seeded at appropriate density (70–75% confluent) in six-well plates 1 day before compound treatment. Upon compound treatment at various drug concentrations for 2 hours at 37°C, the cells were harvested and lysed immediately. For sample preparation from tumor xenografts, tumors were harvested 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 lysis buffer. After incubation on ice for 20 to 30 minutes, the lysates were centrifuged at 14,000 rpm for 15 minutes. The protein concentrations of the lysates were determined.

Equal amounts of total protein for cell lysates and for tumor lysates were resolved on 4% to 12% NuPage gradient polyacrylamide gels (Invitrogen) and blotted with the indicated antibodies. The chemiluminescent signal was generated with Enhanced Chemiluminescence Plus Western Blotting Detection Reagents (Amersham Biosciences) and detected with a Fujifilm LAS-3000 imager. The densitometric quantitation of specific bands was determined using the Multi Gauge Software (Fujifilm).

Animals

Athymic nude mice (Crl:NU-Foxn1nu), ages 13 to 14 weeks, and weighing approximately 23 to 25 g, were purchased from Charles River Laboratories. The health of all animals was monitored daily by gross observation and analysis of blood samples of sentinel animals. All animal experiments were done in accordance with protocols approved by the Institutional Animal Care and Use Committees.

Tumor xenografts

For the LOX xenografts, 2×10^6 cells in 0.2 mL of PBS were injected s.c. into the right lateral flank. For the Colo829 and A375 xenografts, test animals were implanted s.c. on the right lateral flank on day 0 with 30- to 50-mg tumor fragments using a 12-gauge trocar needle.

Test agents for *in vivo* studies

RG7204, formulated as MBP, was suspended at the desired concentration as needed for each dose group in an aqueous vehicle containing 2% Klucel LF (Hydroxypropylcellulose; Aqualon) and adjusted to pH 4 with dilute HCl. Temozolomide (Schering-Plough) was purchased as 250-mg capsules. Capsules were opened and combined into one bulk supply. To prepare the stock dosing material, temozolomide was first

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dissolved in 100% DMSO followed by dilution with saline to form a final milky white suspension in 10% DMSO/90% saline (pH 3.4).

Efficacy and safety end points

Tumor volumes were calculated using the following ellipsoid formula: $[D \times (d^2)]/2$, in which D represents the large diameter of the tumor, and d represents the small diameter. Tumor volumes of treated groups are presented as percentages of tumor volumes of the control groups (%T/C) using the following formula: $100 \times [(T - T_0)/(C - C_0)]$, in which T represents mean tumor volume of a treated group on a specific day during the experiment, T_0 represents mean tumor volume of the same treated group on the first day of treatment, C represents mean tumor volume of a control group on the specific day during the experiment, and C_0 represents mean tumor volume of the same treated group on the first day of treatment. Percent tumor growth inhibition was calculated as $100 - \%T/C$, with >100% tumor growth inhibition representing regression. Survival was calculated using a predefined cutoff volume of 2,000 mm^3 as a surrogate for mortality. The percent increase in life span was calculated as follows:

$$100 \times \frac{[(\text{MDD-treated tumor-bearing animals}) - (\text{MDD control tumor-bearing animals})]}{\text{MDD control tumor-bearing animals}}$$

in which MDD represents median day of death. Each treatment group included 8 to 10 animals. Average percentage weight change was used as a surrogate end point for tolerability in all experiments. Toxicity was defined as ≥20% of mice showing ≥20% body weight loss and/or mortality. The health status of animals was also checked daily by veterinary staff, and tumor volume and weights were recorded two to three times a week.

Statistical methods

Statistical analysis consisted of Mann-Whitney Rank Sum Test, one-way ANOVA, and *post hoc* Bonferroni t test (SigmaStat, version 2.0; Jandel Scientific). Survival was analyzed by the Kaplan-Meier method. Treated groups were compared with the vehicle group, and survival comparisons between groups were analyzed by log-rank test (GraphPad Prism, version 4.3; GraphPad Software). Differences between groups were considered significant when the P value was ≤ 0.05.

Results

Cell line mutation status and RG7204 effects *in vitro*

Cellular proliferation. The effect of RG7204 on cellular proliferation was assessed using a panel of 32 tumor cell lines (Supplementary Table S1). BRAF, K-Ras, and N-Ras mutational status was recorded for each cell line.

In 17 melanoma cell lines, RG7204 was a potent inhibitor of proliferation in those expressing $BRAF^{V600E}$ but not $BRAF^{WT}$ (Fig. 1). RG7204 also potently inhibited proliferation of melanoma cell lines expressing other codon 600 BRAF mutations (V600D, V600K, and V600R; see Supplementary Table S1 and Fig. 1). In addition, proliferation of the WM1789 melanoma cell line expressing $BRAF^{K601E}$ was moderately inhibited by RG7204.

In cell lines from other tumor types (lung, gastric, breast, pancreatic, and skin), RG7204 had effect only on the breast

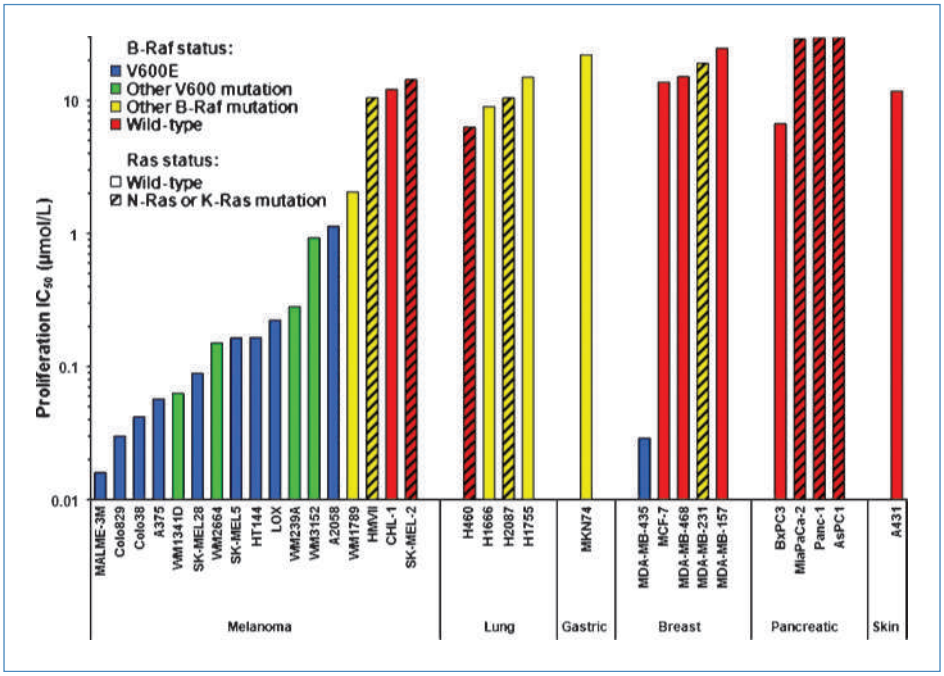

Figure 1. Inhibition of proliferation of tumor cell lines by RG7204 depends on BRAF mutational status. The inhibition of cellular proliferation by varying concentrations of RG7204 was determined by MTT assay. The IC_{50} for each cell line was determined by regression of the inhibition data using a one-site dose-response model. Cell lines are color coded according to tumor type and BRAF and Ras mutational status.

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cancer cell line MDA-MB-435, which expresses $BRAF^{V600E}$ and wild-type Ras (Supplementary Table S1 and Fig. 1). None of the other lung, gastric, breast, pancreatic, or skin cancer cell lines tested harbor the V600E or other V600 codon mutations, and RG7204 did not inhibit their proliferation, contrasting with the consistent antiproliferative effects in $BRAF^{V600E}$ mutant cell lines. IC_{50} values in most of these nonresponsive cell lines were >10 μmol/L (Supplementary Table S1; Fig. 1).

MEK and ERK phosphorylation. The effect of RG7204 on MEK and ERK phosphorylation was investigated in melanoma cell lines expressing $BRAF^{V600E}$, $BRAF^{V600D}$, $BRAF^{V600R}$, $BRAF^{G469V}$, or $BRAF^{WT}$. As shown in Fig. 2, RG7204 inhibited the phosphorylation of MEK and ERK in the two representative $BRAF^{V600E}$-expressing melanoma cell lines, Colo829 and LOX (Fig. 2A and B). RG7204 also inhibited MEK and ERK phosphorylation in the WM2664 and WM1341D cell lines, which expresses $BRAF^{V600D}$ and $BRAF^{V600R}$, respectively (Fig. 2C). Therefore, suppression of ERK and MEK phosphorylation by RG7204 correlates with its inhibition of cellular proliferation in melanoma cells harboring mutations at the V600 position.

The effect of RG7204 on MEK and ERK phosphorylation in melanoma cell lines that do not express codon 600 BRAF mutations was more variable (Fig. 2D). In HMVII cells, which express $BRAF^{G469V}$ and $N\text{-}Ras^{Q61K}$, RG7204 had little effect on the phosphorylation of MEK or ERK (Fig. 2D). CHL-1 cells express both $BRAF^{WT}$ and $N\text{-}Ras^{WT}$ and have low constitutive levels of MEK and ERK phosphorylation (Fig. 2D), and RG7204 induced MEK and ERK phosphorylation at high concentrations in CHL-1 cells (Fig. 2D). In SK-MEL-2 cells, which express $BRAF^{WT}$ and $N\text{-}Ras^{Q61R}$, RG7204 had a biphasic effect on MEK phosphorylation: phosphorylation was induced at low RG7204 concentrations and inhibited at higher concentrations (Fig. 2D). RG7204 had little effect on ERK phosphorylation in the SK-MEL-2 cell line (Fig. 2D). The mechanism underlying RG7204-induced MEK and ERK phosphorylation is under investigation.

Cell cycle and apoptosis. The effect of RG7204 treatment for 48 hours on the expression of cyclin D1 and cleaved poly (ADP-ribose) polymerase (PARP) was determined in three melanoma cell lines (Supplementary Fig. S1). In cell lines expressing $BRAF^{V600E}$, A375, and Colo829, cyclin D1 expression was decreased by RG7204 treatment, indicative of cell cycle arrest. RG7204 did not affect cyclin D1 expression in SK-MEL-2 cells expressing $BRAF^{WT}$ (Supplementary Fig. S1). RG7204 treatment was associated with increased cleavage of PARP, a marker of apoptosis, in A375 and Colo829 cells, but this effect was not observed in SK-MEL-2 cells (Supplementary Fig. S1).

Efficacy of RG7204 in tumor xenograft models

RG7204 formulation and dosing rationale. Pharmacokinetic (PK) parameters were determined for RG7204 administered in two formulations, corn oil or MBP, in tumor-bearing nude mice (Supplementary Table S2). A nondose proportional relationship was observed with the corn oil formulation (Supplementary Table S2), resulting in suboptimal antitumor efficacy (data not shown). In contrast, dose-proportional increases in exposure were observed in animals treated with RG7204 in the MBP formulation (Supplementary Table S2), suggesting that increased drug exposure, which can be achieved with the MBP formulation, was required to achieve optimal *in vivo* results. Therefore, the MBP formulation, also used in the clinical trials, was used in the subsequent efficacy studies reported here.

Due to a lack of toxicity, a maximum tolerable dose for RG7204 was not reached in mice. A dose of 100 mg/kg bid was selected as the highest dose to be tested in efficacy studies.

RG7204 efficacy, PK, and PD in a LOX melanoma model. The effect of three doses of RG7204 (12.5, 25, and 75 mg/kg bid) on antitumor activity and survival was determined in mice bearing LOX tumor xenografts (Fig. 3). RG7204 significantly inhibited tumor growth and induced tumor regression for all three doses studied, with complete regression in 10 of 10 mice treated with 25 and 75 mg/kg bid, and five of nine mice treated with 12.5 mg/kg bid; four of nine mice treated with 12.5 mg/kg bid of RG7204 showed partial tumor regression (Supplementary Table S3; Fig. 3A). RG7204 significantly increased survival relative to vehicle in a dose-responsive manner (Supplementary Table S3; Fig. 3B).

Eight of 10 mice in the 75 mg/kg bid group were considered complete cures and died of natural causes (Supplementary Table S3). No gross signs of metastasis were observed at necropsy (data not shown). Even after complete regression, the tumors in the mice treated with the lower doses recurred (data not shown).

In addition, the relationship between RG7204 concentration (PK) and effect on MEK and ERK phosphorylation [pharmacodynamics (PD)] was investigated in the same LOX melanoma tumor xenograft model. After a single oral dose of RG7204 at 100 mg/kg in nude mice bearing human LOX melanoma xenografts, tumors were harvested at various time points post dosing, and pMEK, total MEK, pERK, and total ERK were analyzed by Western blot. The PD effects were determined by the percentage of pMEK/MEK and pERK/ERK inhibitions in RG7204-treated tumors compared with the vehicle-treated tumor samples. There seemed to be an association between plasma concentration and inhibition of MEK and ERK phosphorylation, with the highest plasma concentration (124 μmol/L at 2 h) corresponding to the highest mean percent inhibition of phosphorylation (70.5% for MEK and 52% for ERK; Supplementary Table S3; Fig. 4). In contrast, mean tumor concentrations of RG7204 increased slightly from 2 hours postdose (30.2 μmol/L) to peak at 8 hours postdose (37.8 μmol/L; Supplementary Table S3; Fig. 4). Both plasma and tumor concentrations of RG7204 decreased to ≤3 μmol/L at 24 hours postdose (Supplementary Table S3; Fig. 4). After peaking at 2 hours postdose, mean percent inhibition of MEK phosphorylation remained fairly steady out to 8 hours postdose (64.8%) and diminished to 26.0% at 24 hours postdose (Supplementary Table S3; Fig. 4). For ERK phosphorylation, mean percent inhibition showed a sharper decrease; at 24 hours postdose, no inhibition remained (Supplementary Table S3; Fig. 4). The degree of the pathway inhibition

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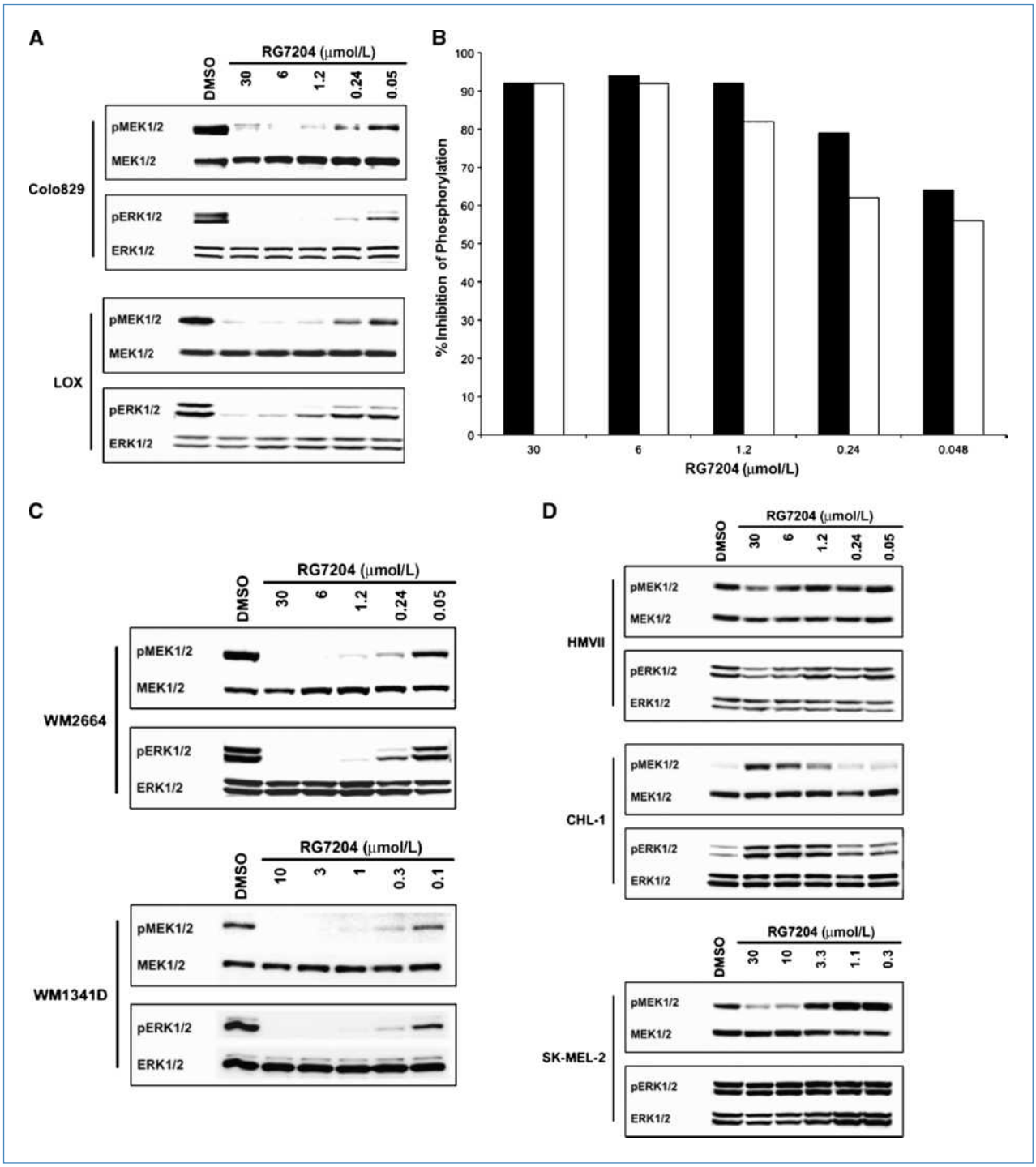

Figure 2. Effect of RG7204 on MEK and ERK phosphorylation in melanoma cell lines. In all panels, cells were treated with the indicated concentration of RG7204 for 2 h before lysis. Western blot analysis was performed with antibodies specific for phospho-MEK1/2 (pMEK1/2), total MEK1/2, phospho-ERK1/2 (pERK1/2), and total ERK1/2. A, RG7204 inhibited phosphorylation of MEK and ERK in Colo829 and LOX melanoma cell lines bearing $BRAF^{V600E}$. B, the effect of RG7204 on MEK and ERK phosphorylation in LOX cells was quantitated. Total MEK1/2 and ERK1/2 values served to normalize pMEK1/2 and pERK1/2 values to correct for differences in protein loading. After the initial subtraction of the background signal, the ratios of pMEK1/2 to total MEK1/2 and of pERK1/2 to total ERK1/2 were determined. The value for the control sample (DMSO) was set to 100% (or 0% inhibition), and the values for the compound-treated samples were expressed as inhibition relative to the control sample. C, RG7204 inhibited phosphorylation of MEK and ERK in melanoma cell lines bearing other V600 BRAF mutations: WM2664 ($BRAF^{V600D}$) and WM1341D ($BRAF^{V600R}$). D, RG7204 had varying effects on phosphorylation of MEK and ERK in wild-type BRAF-bearing melanoma cell lines, CHL-1 ($BRAF^{WT}$) and SK-MEL2 ($BRAF^{WT}$), and a noncodon 600–mutated BRAF-bearing melanoma cell line, HMVII ($BRAF^{G469V}$).

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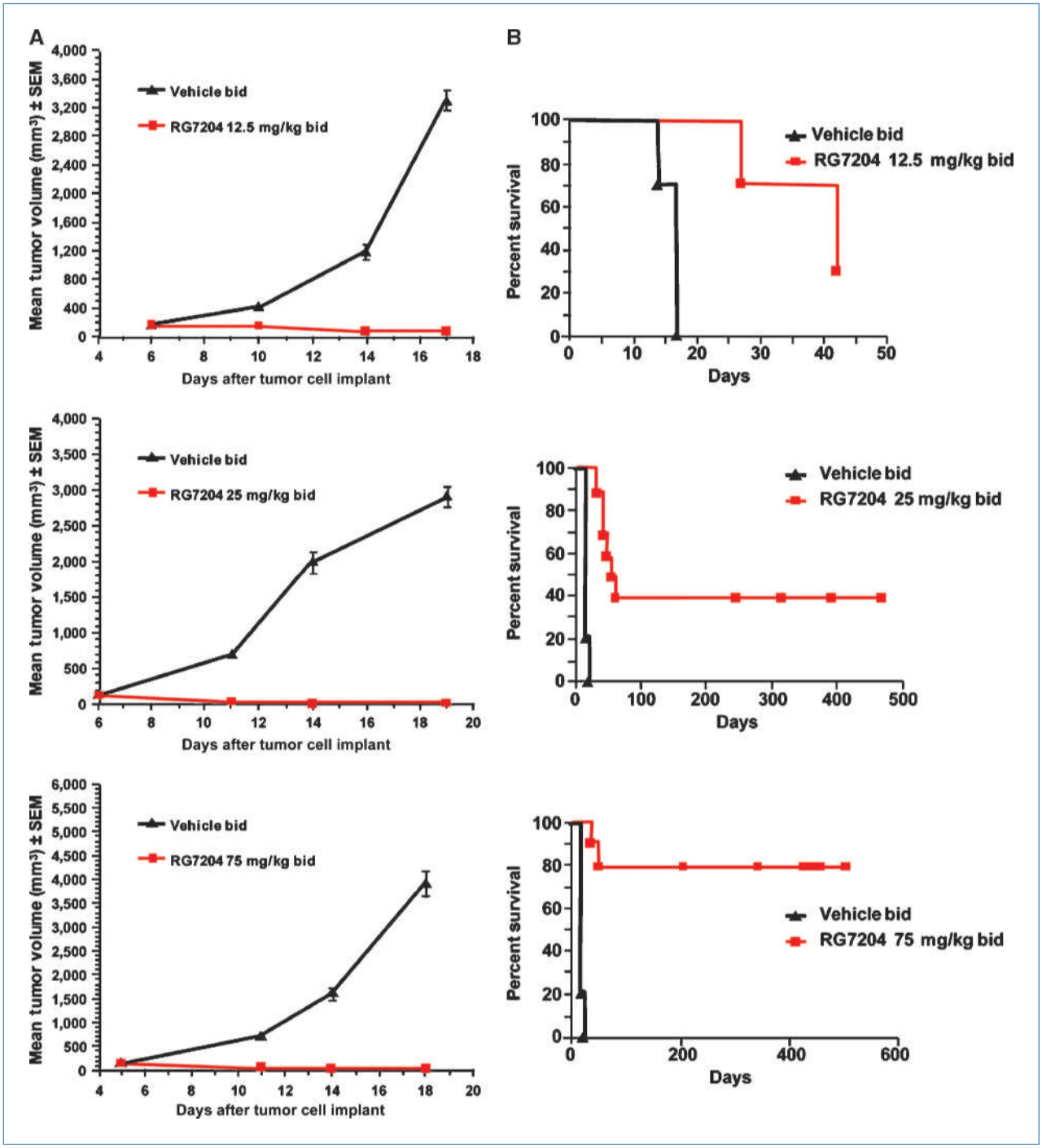

Figure 3. RG7204 inhibits tumor growth and prolongs survival in a $BRAF^{V600E}$-bearing LOX melanoma xenograft model. Groups of mice were treated with RG7204 administered bid at doses of 12.5, 25, and 75 mg/kg for 11 to 13 d starting on day 5 to 6 after implantation. A, efficacy data are plotted as mean tumor volume (in mm^3) ± SEM. B, survival data are plotted as percent of animals surviving in each group using a predefined cutoff volume of 2,000 mm^3 as a surrogate for survival.

represented by pMEK and pERK inhibition (70.5% for MEK and 52% for ERK) seemed low when compared with the antitumor effect (regressions). This may be due to the fact that the PD readout was measured by Western blot of the xenograft tumors, which also contain stromal tissue and infiltrating cells that do not have MAP kinase signaling affected by treatment. Immunohistochemical staining of pMEK and pERK has been used to analyze clinical samples of patients

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treated with RG7204 and shows good correlation between pERK inhibition and clinical response (20).

RG7204 efficacy in a Colo829 melanoma model. The efficacy of RG7204 was compared with that of temozolomide in mice bearing Colo829 tumor xenografts (Fig. 5A and B). Pilot tolerability studies determined the maximum tolerable dose of temozolomide to be 100 mg/kg once daily for 5 days (data not shown). RG7204 at 100 mg/kg bid for 21 days showed greatly improved antitumor activity compared both with vehicle ($P = 0.001$) and with temozolomide at 100 mg/kg once daily for 5 days ($P < 0.001$) at the end of the study on day 38 after the tumor cell implant (Fig. 5A). There was complete tumor regression in all 10 mice treated with RG7204 by the end of the study. In contrast, there was spontaneous partial regression in one animal treated with temozolomide and no complete regression in this group. There was no significant difference in antitumor activity between temozolomide and vehicle at the end of the study ($P = 0.740$).

Survival in the mice treated with RG7204 was significantly better than in those treated with vehicle ($P = 0.0008$) or with temozolomide ($P = 0.0004$; Fig. 5B). Compared with animals in the vehicle group, RG7204-treated mice showed a 61% increased life span. Temozolomide did not significantly improve life span compared with vehicle (13%; $P = 0.2986$). There was no observed toxicity for RG7204 or temozolomide in the Colo829 tumor xenograft study.

RG7204 efficacy in an A375 melanoma model. The A375 melanoma xenograft model was used to evaluate the effect of RG7204 on tumor growth inhibition and overall survival. In addition, this model was used to investigate the effect of reducing the dose of RG7204, after starting at a high dose, on antitumor activity and survival. This study design mimics the clinical situation in which a patient's dose may need to be reduced, and may provide insight into the effect of dose reduction in the clinical setting. Mice were given oral doses of RG7204 at 75 or 25 mg/kg bid for 11 days, or they were given doses of 75 mg/kg bid for 7 days followed by 25 mg/kg bid for 4 days. RG7204 had statistically equivalent tumor growth inhibition in the animals given 75 mg/kg bid and those given 75 mg/kg bid followed by 25 mg/kg bid ($P > 0.05$), with complete regression of the tumors in 10 of 10 mice in those treatment groups (Fig. 5C). Unlike the LOX melanoma model, some of the regressed tumors from this study as well as the study with the Colo829 model did recur after treatment was stopped, even at the highest doses, although they were significantly delayed compared with the vehicle control (data not shown). These mice were not retreated; and therefore, it is unknown whether tumors had developed resistance to RG7204. Studies into the mechanisms of acquired resistance to RG7204 treatment are ongoing.

The 75 mg/kg group and the group given 75 mg/kg followed by 25 mg/kg also had equivalent improvements in survival ($P = 0.1368$), with prolonged survival compared with the vehicle control of 227% and 131%, respectively ($P < 0.0001$, for both; Fig. 5D). The group given 25 mg/kg bid had a 31% increased life span compared with the vehicle group ($P = 0.0098$), which was significantly lower than that seen in either the 75 mg/kg group or the 75 mg/kg followed by 25 mg/kg group ($P = 0.0019$ and $P = 0.0175$, respectively). Analysis of the tumor growth inhibition associated with the two dosing regimens did not reveal an effect of dose reduction from 75 to 25 mg/kg over a 4-day interval. Although not statistically significant, there may be a trend that suggests that mice receiving the dose reduction had less survival benefit. This

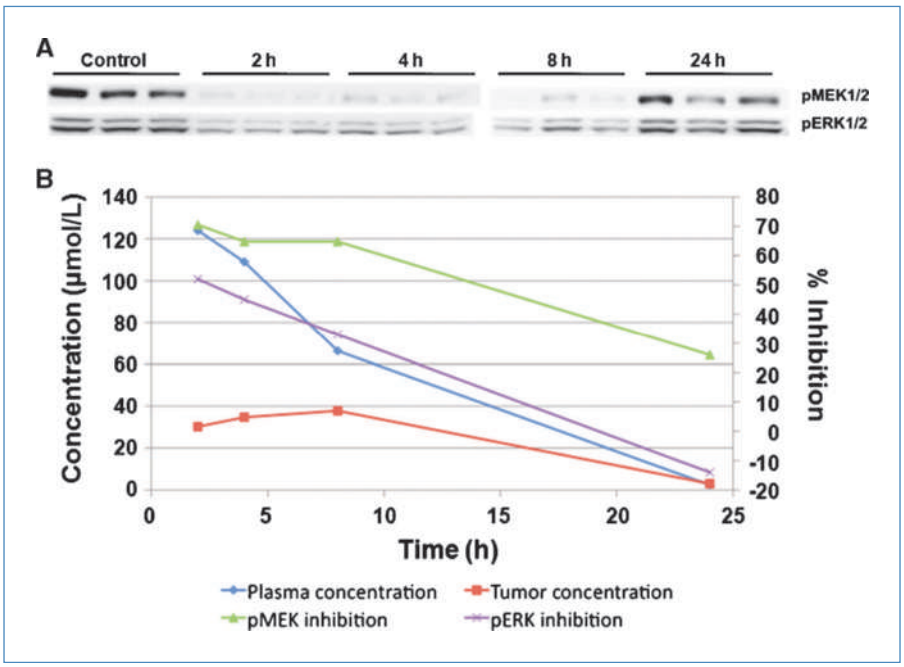

Figure 4. PD and PK relationship of RG7204 in the LOX melanoma xenograft model. Plasma and tumor samples were obtained at 2, 4, 8, and 24 h postdose for PD and PK analysis from mice administered a single dose of 100 mg/kg RG7204 orally when tumors were ~300 mm^3. A, tumor samples were subjected to Western blot analysis with antibodies specific for pMEK1/2 and pERK1/2. B, mean PD and PK parameters were calculated from three animals analyzed at each time point and plotted as a function of time postdose. RG7204 plasma and tumor concentrations are plotted on the left *Y*-axis. Inhibitions of MEK1/2 and ERK1/2 phosphorylation are plotted on the right *Y*-axis.

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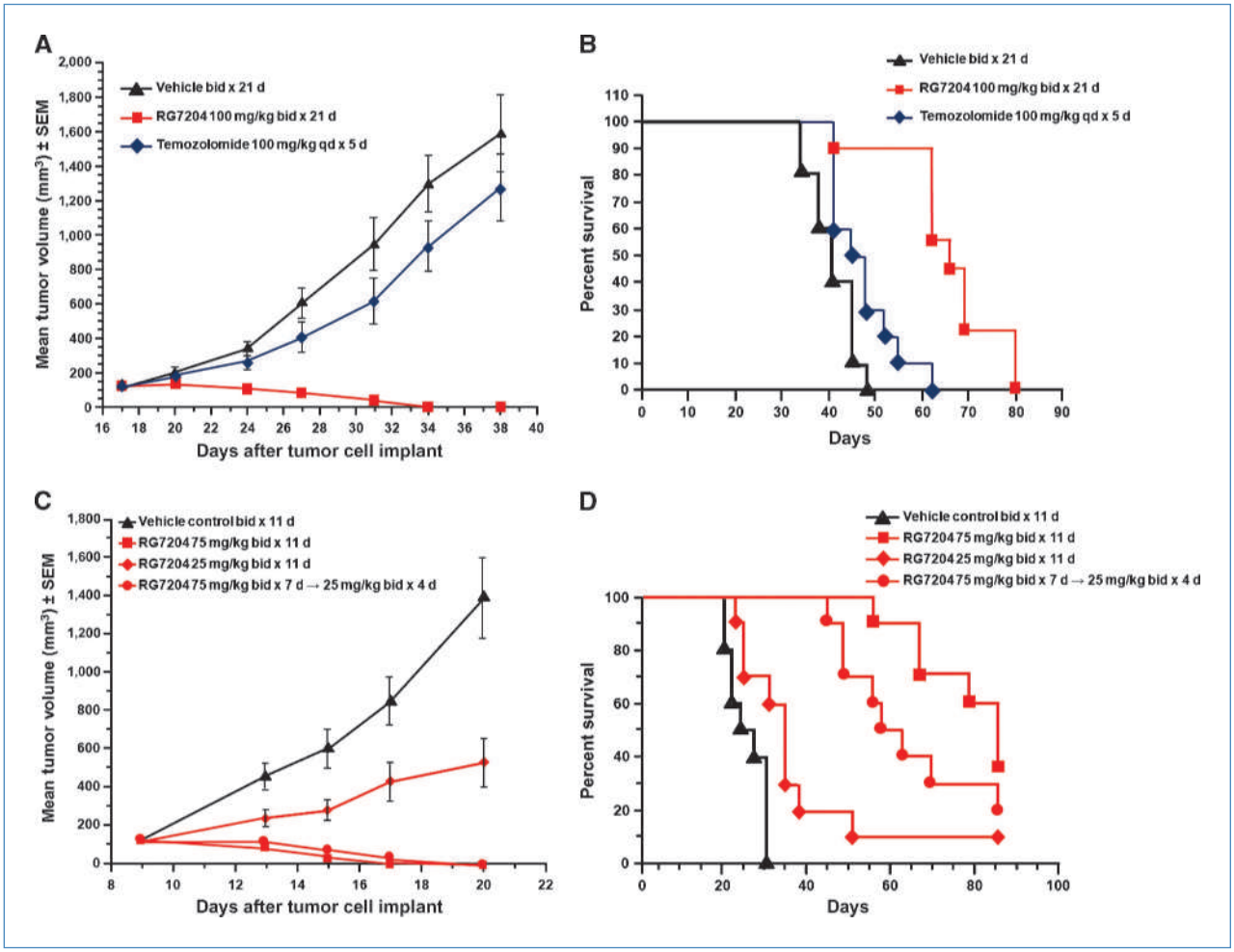

Figure 5. RG7204 inhibits tumor growth and prolongs survival in two $BRAF^{V600E}$-bearing melanoma xenograft models. Efficacy data are plotted as mean tumor volume (in mm^3) ± SEM. Survival data are plotted as percent of animals surviving in each group using a predefined cutoff volume of 2,000 mm^3 as a surrogate for survival. A and B, mice implanted with Colo829 xenografts were treated starting 17 d after implantation. Animals were treated with vehicle or RG7204 at a dose of 100 mg/kg bid for 21 d or with temozolomide at a dose of 100 mg/kg once daily for 5 d qd. C and D, mice implanted with A375 xenografts were treated starting 9 d after implantation. Mice were given oral doses of RG7204 at 75 or 25 mg/kg bid for 11 d or they were given doses of 75 mg/kg bid for 7 d followed by 25 mg/kg bid for 4 d.

may suggest that in the clinical setting, maintenance of the highest tolerated dose may provide the greatest benefit. Further work is needed to define temporal and exposure-effect relationships associated with dose reduction or different dose regimens.

Discussion

Selective targeting of oncogenic mutations such as $BRAF^{V600E}$ allows anticancer therapies to be directed toward cancer cells rather than normal cells. RG7204, an inhibitor of $BRAF^{V600E}$, showed potent antitumor activity, causing dose-dependent complete and partial tumor regressions in melanoma xenograft models. This effect was accompanied by an improvement in survival and, in some cases, complete cures. In our prior experience with the aggressive LOX melanoma model, this phenomenon of complete cures rarely occurs with monotherapy. The aggressive nature of the LOX model is characterized by an exceptionally rapid growth rate, where in general, the tumor volume observed is >3,000 mm^3 at day 14 postimplantation. It is important to note that in this model, even after complete regression, tumors eventually recurred in animals in the lower dose group. This observation may suggest use of the maximal tolerated dose in the clinical setting for the best clinical outcome.

The substantial biochemical selectivity of RG7204 translates to minimal off-target activity and consequently overall minimal toxicity. Because of the very low toxicity of RG7204, a maximum tolerated dose was not achieved in the mice. Therefore, 75 or 100 mg/kg bid were used as optimal feasible doses for nude mice, as these doses allowed for workable suspensions for oral administration twice daily. An optimized amorphous formulation developed for clinical studies with much improved bioavailability enabled the achievement of

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substantial compound exposures in the mouse studies. As mentioned above, these doses resulted in complete cures in most of the treated mice. Nonetheless, PD studies suggested that at these doses, pathway inhibition in the tumor was incomplete. It is therefore likely that higher doses leading to greater pathway inhibition would be even more efficacious, further supporting the idea of using the highest tolerable dose in clinical studies.

In both cell lines and tumor xenograft models, potent antitumor activity of RG7204 was only observed when the $BRAF^{V600E}$ mutation was present. Inhibition of downstream signaling by RG7204 was also shown. Phosphorylation of MEK and ERK was inhibited with RG7204 treatment in cell lines and in tumor tissues bearing $BRAF^{V600E}$, indicating that the molecular mechanism of RG7204 is to block the downstream signaling resulting from constitutively active $BRAF^{V600E}$. RG7204 may also be effective in treating cancers other than melanoma, as shown by its antiproliferative activity in colorectal cancer and other tumor cell lines with the $BRAF^{V600E}$ mutation and by its RG7204 antitumor activity in $BRAF^{V600E}$-bearing colorectal cancer xenograft models.[6] However, it seems that the specific molecular effect of RG7204 may differ in different cell types. A previous study in a thyroid cancer cell line showed inhibition of cell growth but very little induction of apoptosis (16). In melanoma, acquisition of a BRAF mutation is likely a very early event, as even benign nevi harbor the mutation (22). In other tumor types, BRAF mutations may occur at other stages of tumorigenesis. It is likely that the response of different types of tumors to BRAF inhibition may correlate to the relative role of BRAF mutations in the context of coexistent alternative signaling pathways that are driving tumor development and maintenance in a given cancer.

The effects of RG7204 translated well from biochemical selectivity, to pathway inhibition in cells, to antitumor efficacy *in vivo*. Sensitivity to RG7204 correlated with activation loop mutations in BRAF, including codon 600 and 601 mutations. This is consistent with the binding mode that targets the form of the kinase that is stabilized by these mutations (23). It also should be noted that RG7204 showed no toxicity in any tumor xenograft model tested.

In conclusion, RG7204 has shown potent antitumor activity in preclinical melanoma models in which mutant $BRAF^{V600E}$ is expressed. Based on the exposures necessary for *in vivo* tumor stasis and regression, benchmarks of exposure in human studies were estimated, enabling critical decisions about dose escalations. These promising preclinical results have been corroborated by exciting phase I clinical trial data, including observations of tumor regression, particularly in metastatic melanoma patients (17–20).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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[6] Unpublished data.

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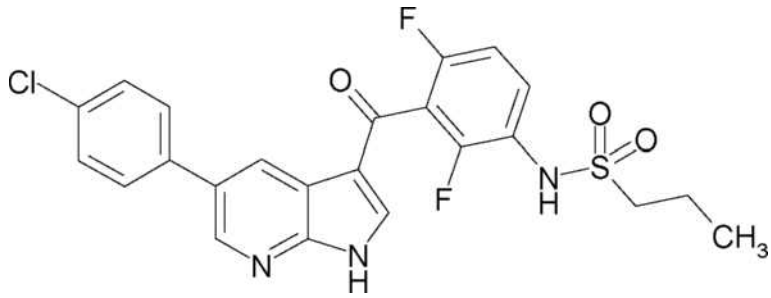
Cancer Research

Correction: RG7204 (PLX4032), a Selective $BRAF^{V600E}$ Inhibitor, Displays Potent Antitumor Activity in Preclinical Melanoma Models

In this article (Cancer Res 2010;70:5518–27), which was published in the July 1, 2010 issue of *Cancer Research* (1), the chemical structure of the compound described was not provided in the article. The structure is provided below.



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1. Yang H, Higgins B, Kolinsky K, et al. RG7204 (PLX4032), a selective $BRAF^{V600E}$ inhibitor, displays potent antitumor activity in preclinical melanoma models. Cancer Res 2010;70:5518–27.

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