A novel B-RAF inhibitor blocks interleukin-8 (IL-8) synthesis in human melanoma xenografts, revealing IL-8 as a potential pharmacodynamic biomarker

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Abstract

B-RAF mutations have been identified in the majority of melanoma and a large fraction of colorectal and papillary thyroid carcinoma. Drug discovery efforts targeting mutated B-RAF have yielded several interesting molecules, and currently, three compounds are undergoing clinical evaluation. Inhibition of B-RAF in animal models leads to a slowing of tumor growth and, in some cases, tumor reduction. Described within is a novel series of diaryl imidazoles with potent, single-digit nanomolar, anti-B-RAF activity. One compound from this series has been detailed here and has been shown to block B-RAF^{V600E}-dependent extracellular signal-regulated kinase 1/2 phosphorylation in SK-MEL-28 melanoma cells as well as soft agar colony formation and proliferation. Importantly, interleukin-8 (IL-8) was identified by quantitative real-time PCR and ELISA as a product of the elevated mitogen-activated protein kinase signaling in these cells. Plasma concentrations of IL-8 in mice bearing melanoma xenografts were significantly reduced following exposure to B-RAF inhibitors. Taken together, these data suggest that IL-8 could serve as a tractable clinical biomarker. [Mol Cancer Ther 2008;7(3):492–9]

Introduction

The Raf kinases are key mediators of growth factor signals that effect cell survival, development, and proliferation. Of the three known isoforms of Raf, B-RAF is the sole family member known to be an oncogene (1–3). Indeed, mutations resulting in B-RAF activation, specifically B-RAF^{V600E}, have been identified in several cancers including melanoma, colorectal carcinoma, and papillary thyroid carcinoma (4–6). Mutational studies and crystallography have shown that the V600E mutation destabilizes the inactive conformation of the enzyme and produces a constitutively active kinase with ~500-fold increase in activity (7). Cells expressing this form of mutated B-RAF have been shown to have very high basal extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation. As a result, these cells produce large quantities of growth factors and chemokines dependent on mitogen-activated protein kinase signaling. One of these target genes encodes interleukin-8 (IL-8), a proinflammatory chemokine and potent autocrine factor known to promote tumor cell growth and angiogenesis (8). At least two known pathways are responsible for production of IL-8. ERK1/2 is known to activate AP1 was well as induce its expression, thus up-regulating IL-8 (9–11). In addition, phosphoinositide-3-kinase activation signals to nuclear factor-κB, thus inducing IL-8 expression (12).

IL-8 has already received attention as a prognostic factor for melanoma patients (13). Thus, it has been shown that patients with elevated levels of basic fibroblast growth factor, angiogenin, vascular endothelial growth factor, and IL-8 have poor survival rates. Interestingly, IL-8 was not found to be an early marker for melanoma development as it was only elevated in stage II and III patients. However, tumor burden and IL-8 did serve as independent predictive factors of progression-free survival. Consequently, IL-8 has emerged as an important measure of not only a patient's tumor burden but also their potential for disease-free survival.

The mitogen-activated protein kinase pathway is a well-accepted target for cancer therapy. Importantly, a compound with Raf inhibitory activity, Bay 43-9006 (sorafenib), recently gained approval for the treatment of renal cell carcinoma (14, 15). In addition, MEK inhibitors have shown early promise (16–18). Currently, clinical evaluation of drugs requires reproducible and robust biomarkers. The programs mentioned above have used phospho-ERK levels in *ex vivo* phorbol 12-myristate 13-acetate–stimulated peripheral blood mononuclear cells from drug-treated patients as a pharmacodynamic biomarker (19). These approaches are valid for evaluating compound doses and resultant bioavailability but do not necessarily correlate to intratumor, target-dependent activity. Analysis of a surrogate tumor-dependent biomarker is an important measure of a compound's ability to penetrate tumor tissue and therefore helps to guide dosing and potentially predict efficacy. There are several cases where tumor-dependent biomarkers are routinely used to assess disease progression. For example, prostate-specific antigen is a well-known

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indicator of benign prostatic hyperplasia as well as prostate cancer and has been used clinically since at least 1989 (20). Serum levels of P-selectin, cytokeratins, and sErbB1 are also gaining attention as potential tumor biomarkers (21–23). Other markers, MART1 and gp100, to name but a few, are commonly analyzed but require invasive biopsies and immunohistochemistry (24), thus making them far less convenient.

Described within is a series of diaryl imidazoles with potent anti-B-RAF kinase activity. These compounds have been shown to inhibit ERK1/2 phosphorylation and IL-8 production in a variety of melanoma cell lines harboring the B-RAF$^{V600E/D}$ mutations. Importantly, compounds in this series reduce plasma and tumor IL-8 protein and mRNA levels when dosed in animals with established human melanoma xenograft tumors.

Materials and Methods

Kinase Assays

The RAF kinases were assayed at a concentration of 5 nmol/L in kinase reaction buffer [50 mmol/L HEPES (pH 7.4), 100 mmol/L NaCl, 10 mmol/L $MgCl_2$, Complete protease inhibitor cocktail (Roche Applied Science; used at a dilution of 1:200 when a tablet is dissolved in 1 mL water), 1 mmol/L NaF]. Briefly, compounds were diluted in 30% DMSO, 50 mmol/L HEPES and 10 μL were dispensed to reaction plates. Next, 50 μL of a buffer containing mouse MEK1-GST (100 nmol/L final) and [^{33}P]ATP (GE Healthcare; 10 μCi/mL final) were added to appropriate wells. Finally, 40 μL RAF kinase was added. Following 1-h incubation at room temperature, assays were stopped by the addition of 10 mmol/L ATP in 1% bovine serum albumin, 50 mmol/L EDTA. This mixture (80 μL) was transferred to 1% bovine serum albumin–blocked Flash Plates (Perkin-Elmer) coated previously overnight with a 1:2,500 dilution of anti-GST antibody (GE Healthcare). These plates were incubated for 2 h with shaking at room temperature. Plates were washed three times with PBS, 0.1% Triton X-100 and counted on a Top Count (Perkin-Elmer). IC_{50}s were determined by plotting percent of control against log M compound concentration and performing a nonlinear regression fit in GraphPad Prism (GraphPad Software).

Competition Studies

An ELISA was developed to determine if Johnson & Johnson B-RAF inhibitors were ATP competitive. Briefly, kinase assays were set up as above with the following exceptions. Substrate solutions containing 2× mouse MEK were prepared with 2× cold ATP (200, 40, 8, 1.6, and 0.32 μmol/L). These substrate solutions were combined with dilution series of compounds before the addition of B-RAF. Assays were incubated for 15 min at room temperature and terminated by the addition of 10 μL 500 mmol/L EDTA. Assay mixtures were transferred to high binding ELISA plates (Corning) coated previously and blocked with anti-GST as above and incubated with shaking at room temperature for 2 h. Plates were washed three times as above and probed for 1 h with anti-phospho-MEK1/2 (Cell Signaling) at a 1:2,500 dilution in PBS with 1% bovine serum albumin and washed again. Finally, plates were incubated for 1 h with anti-rabbit horseradish peroxidase (Pierce) at 1:50,000 as above and washed. Plates were developed with TMB substrate (Chemicon) and stopped by the addition of 25 μL of 3 N HCl. Dose-response curves and competitive inhibition curves were produced with GraphPad Prism. K_i were determined from nonlinear regression analysis of data, where $K_{mapp} = K_m * (1 + I / K_i)$ and $Y = V_{max}$ $V_{max} * X / (K_{mapp} + X)$ (where X is concentration of ATP).

Protein Expression

B-RAF-6HIS, B-RAF^{V600E}-6HIS, C-RAF-6HIS, and mouse MEK-GST were produced with a baculovirus expression system (Invitrogen). The RAF proteins were purified on nickel resin (Qiagen) and mouse MEK was purified on glutathione-Sepharose (GE Healthcare) followed by gel filtration on an S200 (GE Healthcare) to exclude endogenous insect glutathione-*S*-transferase. Isolated proteins were frozen at -80°C in the presence of 20% glycerol.

Inhibition of ERK Phosphorylation in B-RAF$^{V600E/D}$ Cell Lines

Melanoma cell lines A101D, A2058, A375, SW-MEL-28, WM-266-4 (B-RAF^{V600D}), colon carcinoma RKO, and glioblastoma multiforme DBTRG-05MG were obtained from American Type Culture Collection and cultured according to the recommended conditions. Cells were plated at 10,000 per well in a 96-well tissue culture plate the day before analysis. Growth medium was replaced by medium containing a dilution series of compound (previously diluted to 100× final concentration in 100% DMSO) resulting in a final DMSO concentration of 1%. Cells were returned to the 37°C incubator for 1 h after which they were washed once with ice-cold PBS and then lysed and assayed according to the manufacturer's instructions for the phospho-ERK2 ELISA kit (R&D Systems). Briefly, cell lysates were incubated in anti-ERK2-coated plates for 1 h, with shaking, at room temperature. Anti-phospho-ERK2 antibody was then applied to the plates for 1 h followed by washing and an anti-goat horseradish peroxidase–conjugated antibody for 30 min. Plates were washed and then developed with TMB substrate (Chemicon). IC_{50} values were determined by plotting B/Bo versus log M and applying a one-site competition nonlinear regression fit in GraphPad Prism.

Soft Agar Colony Formation

Soft agar colony formation assays were done as follows. Bottom layers were prepared first: 1% DNA grade agar was brought to boil in a microwave and cooled to 40°C in a water bath. DMEM (2×) was warmed to 40°C in water bath and allowed to equilibrate for at least 30 min. Equal volumes of the two solutions were mixed to give 0.5% agar plus 1× DMEM. Two milliliters were added to each well of six-well tissue culture plates and allowed to set. For top agar: 0.6% agar was brought to boil in a microwave and cooled to 40°C in a water bath. Two 2× RPMI plus 10% fetal bovine serum, penicillin, streptomycin, and glutamine were warmed to the same temperature. SK-MEL-28 cells

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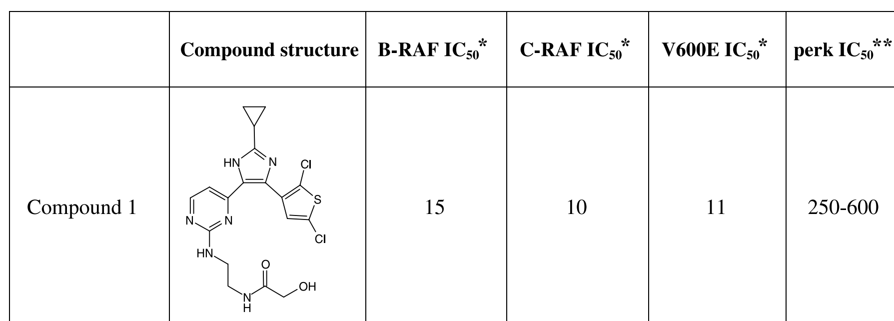

	Compound structure	B-RAF IC_{50}*	C-RAF IC_{50}*	V600E IC_{50}*	perk IC_{50}**
Compound 1		15	10	11	250-600

Figure 1. *In vitro* pharmacology of B-RAF inhibitor. Activity in nmol/L; *, *in vitro* kinase assays; **, SK-Mel-28 melanoma cell ELISA.

were counted and adjusted to 500,000/mL. Dilution series of compounds were prepared at 2× in 1 mL of 2× medium in 15 mL tubes and kept at 40°C. The following steps were done in rapid succession. To compound containing medium 2× agarose, 0.1 mL of cell suspension was added then quickly mixed and poured onto bottom agar layer.

Plates were incubated at 37°C in humidified incubator for 10 to 14 days adding 2 mL 1× medium after 1 week. Plates were stained with 1.0 mL of 0.005% crystal violet for >1 h, and colonies were counted in five high-power fields on a dissecting microscope.

Inhibition of IL-8 Expression *In vitro*

Melanoma cell lines A101D, A2058, A375, SW-MEL-28, WM-266-4 (B-RAF^{V600D}), colon carcinoma RKO, and glioblastoma multiforme DBTRG-05MG were exposed to compound 1 as for the phospho-ERK assay (described above) with the exception that the exposure time was 4 h. Supernatants were tested for IL-8 levels in an ELISA (M1918; Research Diagnostics). RNA was isolated from cell lysates (RNeasy; Qiagen) and quantitated (RNA LabChip; Agilent). Quantitative real-time PCR was done with IL-8 and GAPDH TaqMan gene expression assays (IL-8, Hs00174103_m1; GAPDH, 4333764T; Applied Biosystems) on a 7500 Fast Real-Time System according to the manufacturer's specifications.

Inhibition of IL-8 Expression *In vivo*

WM-266-4 melanoma cells were resuspended in medium to 2 × 10^7/mL, mixed 1:1 with Matrigel (BD 354234), and left on ice. *nu/nu* (nude) mice were injected in the right rear flank with 100 μL cell suspension. Tumors were allowed to grow to 200 to 500 mm^3 before treatment. Animals were handled according to Institutional Animal Care and Use Committees protocol and regulations. Mice were sampled via retro-orbital bleed 18 h before dosing to obtain initial IL-8 levels. Before dosing, compound 1 was formulated at 6 mg/mL in a vehicle consisting of 5% pharmasolve and 30% SBE-cyclodextrin (CyDex). Mice received 100 μL (30 mg/kg) of compound or vehicle by s.c. injection. Plasma levels were maintained above 1 μmol/L over a 12-h period. At 9 and 12 h after the first dose, mice were euthanized via CO_2 asphyxiation and blood and tumor samples were taken. IL-8 levels in plasma were determined with an ELISA. For IL-8 mRNA studies, animals were treated as above with the following modifications. Mice received one or two doses of compound or vehicle at t_0 and $t_{2.5}$. Animals were euthanized as above at 2 or 4 h after the first dose. Tumors were placed immediately into liquid nitrogen. mRNA and cDNA were prepared according to standard procedures as above. IL-8 and GAPDH mRNA levels were quantitated with TaqMan RT-PCR (Applied Biosystems) as stated previously.

Results

Diaryl Imidazoles Are Potent Inhibitors of B-RAF Kinase Activity

An in-house medicinal chemistry effort was initiated to identify B-RAF inhibitors with desirable drug-like qualities, including selectivity over other kinases, potency, and manageable pharmacokinetic properties. One example from this effort, referred to here as ''compound 1,'' contains pyrimidine and dichloro thiophene as the two aryl substituents of the imidazole core, is highlighted herein. Compound 1, with an ethylenediamine side chain, had K_i values versus recombinant B-RAF, B-RAF^{V600E}, and C-RAF in the low nanomolar range (Fig. 1). Compounds from this series were also found to be ATP-competitive inhibitors, showing increased IC_{50} in the presence of increasing ATP concentrations (Fig. 2A). Compound 1, when docked in the ATP-binding pocket of the B-RAF crystal structure, is predicted to make donor-acceptor interactions with pyrimidine and aniline nitrogens and the protein backbone at Cys^{531} (Fig. 2B). When analyzed for selectivity, compound 1 was found to block five kinases at >90% inhibition at 1 μmol/L compound and 10 μmol/L ATP (Supplementary Table S1).[1] Furthermore, when screened in-house for isoform selectivity, these compounds showed equipotent inhibition of B-RAF^{V600E} and C-RAF (Fig. 1).

Intracellular ERK1/2 Phosphorylation Is Blocked in Cells Treated with Compound 1

As mentioned, B-RAF^{V600E} has ~500-fold greater kinase activity than wild-type B-RAF. Consequently, many cells lines expressing mutant B-RAF have elevated phospho-ERK1/2 levels that are readily detected by standard ELISA techniques. Thus, phospho-ERK levels have served as a valuable measure of the intracellular activity of B-RAF inhibitors. For our first studies, we chose to evaluate the

[1] Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

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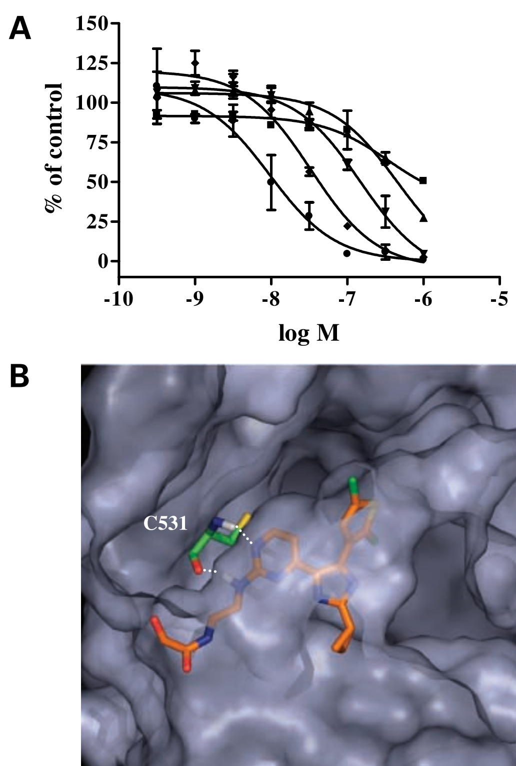

Figure 2. ATP competition and computer-aided docking of compound 1. Dose-dependent inhibition of B-RAF was evaluated in the presence of increasing amounts of ATP and compound 1 (**A**). The K_i of compound 1 was calculated to be 8 nmol/L. ATP concentrations were 100 (■), 20 (▲), 4 (▼), 0.8 (♦), and 0.16 (●) μmol/L. **B,** compound 1 docked in model of B-RAF crystal structure. *White lines,* donor-acceptor interactions with Cys^{531}.

effect of B-RAF inhibitors in several different tumor types in an effort to establish how broadly applicable these compounds would be at blocking ERK phosphorylation. Therefore, melanoma cell lines A101D, A2058, A375, SK-MEL-28, and WM-266-4, colon cancer line RKO, and glioblastoma multiforme line DBTRG-05MG, all harboring activating B-RAF mutations, were exposed to compound 1 for 1 h and then the level of phospho-ERK was determined by ELISA. Compound 1 had a very similar effect in all of the cells tested, with an average IC_{50} of 440 ± 122 nmol/L (Table 1). Because compound 1 showed no cellular selectivity, we focused our future work on the melanoma lines SK-MEL-28 and WM-266-4. Of these two, we chose to use SK-MEL-28 for routine screening of compounds *in vitro* and WM-266-4 for xenograft studies (as described below).

Small-Molecule Inhibitors of B-RAF^{V600E} Block Cell Proliferation and Colony Formation

Recent studies with interfering RNA techniques have validated the role of B-RAF^{V600E} in cell proliferation, survival, and invasion (25). Here, small-molecule inhibitors are shown to possess similar properties. Thus, to evaluate the effect on anchorage-independent growth and cell proliferation, we tested compound 1 in a traditional soft colony agar formation assay as well as thymidine uptake proliferation assays. First, SK-MEL-28 were plated on agar in the presence of a range of compound doses and allowed to grow for 14 days. Compound 1 blocked colony formation with an apparent IC_{50} of ~3 to 5 μmol/L (Fig. 3A). In addition, compound 1 had an IC_{50} of 2 μmol/L for inhibition of SK-MEL-28 proliferation over a 72-h period (Fig. 3B) with no appreciable loss in cell viability.

IL-8 Expression Is Dependent on B-RAF Activity in Melanoma Cells with V600E/D Mutation

IL-8 expression is typically low to undetectable in resting cells. On exposure to proinflammatory or mitogenic signals, IL-8 levels rise dramatically (26). One key observation is that cells harboring the B-RAF$^{V600E/D}$ mutation mimic a stimulated phenotype as they have high endogenous levels of phospho-ERK and constitutively produce IL-8 (27). These apparently ideal characteristics led us to evaluate IL-8 as a potential pharmacodynamic biomarker. Thus, we analyzed the effect of compound 1 on IL-8 production in SK-MEL-28, the same cells investigated for compound 1 effect on ERK phosphorylation. Cells were treated with compound 1 for 4 h and supernatants were analyzed for IL-8 levels. Interestingly, the colon cancer lines RKO, HT29, and Colo205 did not produce IL-8 at detectable

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Table 1. Effect of compound 1 on ERK phosphorylation and IL-8 production in various cancer cell lines and relative levels of phospho-ERK and IL-8

Cell line	Phospho-ERK IC_{50}*	IL-8 IC_{50}*	Phospho-ERK†,‡	IL-8 ng/mL‡
WM-266-4§	405	109	798	0.728
SK-Mel-28§	328	109	1109	0.203
A101D§	574	860	1055	1.080
A2058§	631	278	665	1.664
A375§	320	889	539	0.188
DBTRG-05MG‖	364	267	689	0.378
RKO¶	457	NA	415	0

*Activity in nmol/L.
†Relative levels based on absorbance units.
‡Determined from vehicle controls for each cell line.
§Melanoma.
‖Neuroblastoma.
¶Colorectal cancer.

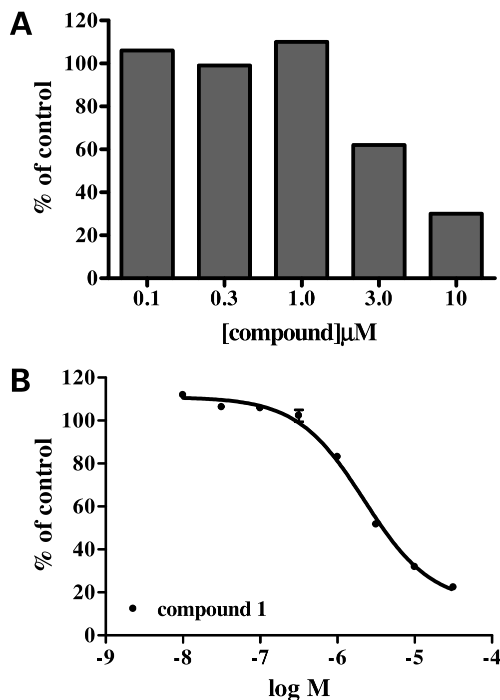

Figure 3. Effect of compound 1 on proliferation and soft colony formation. SK-MEL-28 cells were analyzed for their ability to form colonies in soft agar in the presence or absence of compound 1. **A,** colony numbers were determined by counting five high-power fields and plotted versus compound concentration. **B,** compound 1 was evaluated for its ability to block cell proliferation. SK-MEL-28 cells were treated with compound 1 in the presence of [^{14}C]thymidine over a 72-h period. Inhibition of proliferation was based on amount of [^{14}C]thymidine incorporated in compound 1 – treated cells compared with vehicle-treated cells.

levels, whereas the melanoma lines secreted variable amounts of IL-8 from 200 to 1,700 pg/mL (Table 1). Furthermore, there was no apparent correlation between phospho-ERK levels and amount of IL-8 produced. When evaluated for its potency in these cells, compound 1 showed dose-dependent inhibition of IL-8 production with IC_{50}s ranging from 109 to 889 nmol/L (Table 1). We sought to elucidate the mechanism of this reduction in IL-8 levels by analyzing IL-8 mRNA in compound 1–treated cells using quantitative real-time PCR. To simplify this experiment, we selected one cell line, SK-MEL-28, and exposed the cells to 10 μmol/L compound 1 for up to 6 h. As seen in Fig. 4, compound 1 dramatically reduced IL-8 message levels with the maximum effect being a 23-fold reduction observed at the 4-h time point. These results indicate that compound 1 blocked IL-8 synthesis at the transcriptional level, further elucidating the role of oncogenic B-RAF in regulating autocrine factors.

Administration of Compound 1 to Nude Mice Bearing Human Melanoma Xenografts Results in Reduction of Plasma IL-8 and Tumor IL-8 mRNA

Clinical studies have shown that certain melanoma patients have elevated levels of IL-8 in their blood, which has served as a surrogate marker for tumor burden (13). In addition, the *in vitro* studies described herein show that IL-8 levels can be dramatically reduced in SK-MEL-28 cells treated with small-molecule B-RAF inhibitors. Taken together, these data suggest that IL-8 may serve as a predictive pharmacodynamic biomarker. Thus, an initial xenograft study was done to determine if mice harboring human melanoma xenografts also had elevated IL-8 in their blood and whether this correlated with tumor volume. For these experiments, the melanoma line WM-266-4 harboring the V600D mutation (equivalent in B-RAF kinase activity to V600E; ref. 7) was chosen because xenograft tumors from this line grew more consistently in size and rate (data not shown) and produced more IL-8 than SK-MEL-28, with levels after 4 h in culture of 730 versus 200 pg/mL for SK-MEL-28 (see Table 1). Furthermore, compound 1 blocked IL-8 production in WM-266-4 and SK-MEL-28 with the same IC_{50} (Table 1). Hence, in this pilot xenograft study, nude mice were inoculated with either 1×10^6 or 5×10^6 WM-266-4 cells to identify optimal tumor growth conditions and evaluate IL-8 levels. Tumors were measured every 2 to 3 days and mice were bled over the course of the study to track IL-8 levels along with tumor growth (Fig. 5A and *B*). IL-8 plasma levels reached a maximum of 13 pg/mL in the mice that received 5×10^6 cells and 8 pg/mL in the 1×10^6 group. IL-8 plasma levels were well correlated with tumor size over the course of the study (Fig. 5C; $r^2 = 0.61$; $P < 0.0001$). Importantly, mice that were not inoculated with tumor cells had no detectable IL-8 in their plasma. Also, because the ELISA used for detection was highly specific for IL-8, it was concluded that only tumor-derived IL-8 was measured in these studies and not the endogenous mouse homologue, KC. Due to these findings, that IL-8 was readily detectable in mice with human melanoma tumors, experiments were done to evaluate IL-8 as a pharmacodynamic readout during compound treatment. Thus, mice with WM-266-4 xenograft tumors at an average volume of 500 mm^3 received s.c. administration of compound 1 at 30 mg/kg over a 12-h period such that plasma drug levels would be >1 μmol/L (12 h was chosen due to the long half-life of plasma IL-8 as deduced from ref. 28). Further, s.c. administration was the preferred route

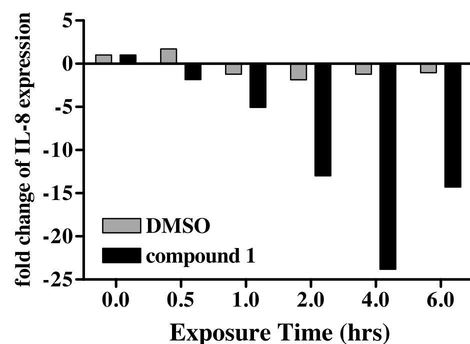

Figure 4. Analysis of compound 1 effect on IL-8 mRNA synthesis in SK-MEL-28 melanoma cells. SK-MEL-28 melanoma cells were treated with either DMSO (*gray columns*) or 10 μmol/L compound 1 (*black columns*) for up to 6 h. Quantitative real-time PCR was done to determine effect of compound on IL-8 mRNA levels.

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of dosing as compound 1 had poor oral bioavailability. Blood and tumors were taken at 9 and 12 h after the first dose. Analysis of plasma samples revealed that treatment with compound 1 reduced IL-8 by 40% at 9 h ($P = 0.05$) and 84% at 12 h ($P = 0.02$; Fig. 6A). In support of these findings, tumor tissue was tested for drug levels at the end of the study and found to be, on the average, 3 µmol/L or >10-fold the IC_{50} required for inhibiting ERK phosphorylation and IL-8 production in WM-266-4 melanoma cells *in vitro*. Taken together, these data suggest that compound 1 is able to penetrate tumor stroma and block B-RAF activity leading to the abolishment of ERK-dependent gene transcription. To substantiate this concept as well as shorten these studies, experiments were done to investigate the effect of s.c. compound administration on IL-8 mRNA levels in tumors. A shorter time course was chosen for these studies as *in vitro* experiments showed that a maximal effect on mRNA was observed 4 h after compound administration with a potential loss of effect at 6 h, whereas IL-8 clearance from plasma was modeled to take at least 6 to 9 h to reach baseline. In addition, the ultimate goal of these experiments was to establish a routine pharmacodynamic screen and dosing over a 12-h period was considered to be less desirable. Thus, mice received either one dose of compound 1 at 30 mg/kg at t_0 or two doses of compound 1 at 30 mg/kg, one at t_0 and the second at $t_{2.5}$, tumors were then harvested at 2 or 4 h after the first dose. TaqMan quantitative real-time PCR analysis of tumor cDNA revealed a 2-fold reduction in IL-8 mRNA for both dose groups, which was significant ($P = 0.048$) when compared with vehicle controls (Fig. 6B). Taken together, these results suggest that serum IL-8 protein and tumor IL-8 mRNA could serve as surrogate biomarkers for compound efficacy in xenograft pharmacokinetic/pharmacodynamic models, which may translate to clinical utility.

Discussion

Mitogen-activated protein kinase inhibitors have been under development for the treatment of a variety of diseases for nearly 15 years. Recent advances in understanding the pathophysiologic role of dysregulated RAF signaling has led to the validation of this pathway in mediating disease and development of therapies for a variety of cancers. Indeed, since the identification of B-RAF mutations in a majority of melanomas in 2002 (1), several series of novel inhibitors have been produced and are heading towards (29, 30), or already in, the clinic. Described here is an example from our own B-RAF drug discovery program that has further elucidated the effect of inhibiting mutant B-RAF with *in vitro* and *in vivo* melanoma models.

RAF inhibitors have been available as tool compounds since the development of GW5074, ZM336372, and Bay 43-9006 (sorafenib) nearly a decade ago. These compounds show a variety of activities in kinase and cell-based assays. For example, ZM336372 exhibits potent inhibition of B-RAF kinase activity, with an IC_{50} of 100 nmol/L, but, interestingly, induces rapid and strong activation of mitogen-activated protein kinase signaling independent of pathway agonism (31). Bay 43-9006, also a potent B-RAF inhibitor (~20 nmol/L), has mixed allosteric and ATP competitive binding modes. This compound is ~500-fold less potent in blocking ERK phosphorylation in cells that express B-RAF^{V600E}. This decrease in activity could be influenced by the fact that Bay 43-9006 binds preferentially to the inactive conformation of RAF kinases, a thermodynamically unfavored state for B-RAF^{V600E} (7). Described here is a purely ATP competitive compound that shows submicromolar kinase and cellular activity. Although a crystal structure of this compound bound to B-RAF is not available, docking of compound 1 suggests that a

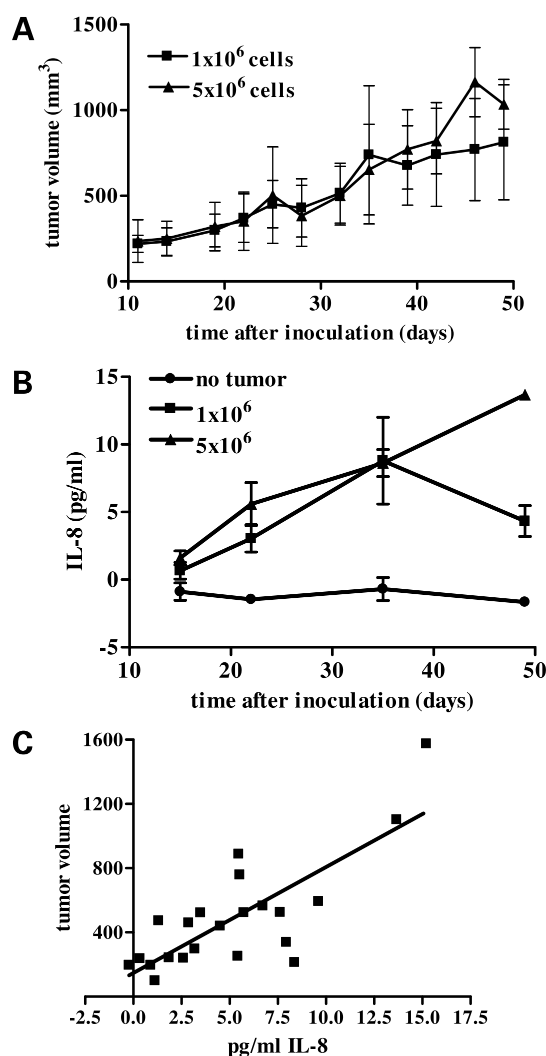

Figure 5. Evaluation of IL-8 plasma levels in nude mice bearing WM-266-4 human melanoma xenografts. Nude mice were injected in the right rear flank with either 1×10^6 (■) or 5×10^6 (▲) WM-266-4; tumor volumes were measured every 2 to 3 d (**A**) and plasma samples were taken throughout the course of the study to determine IL-8 levels [**B**; no cells (●), 1×10^6 (■), or 5×10^6 (▲)]. **C,** correlation between IL-8 plasma levels and tumor volumes at the end of the 6-wk study.

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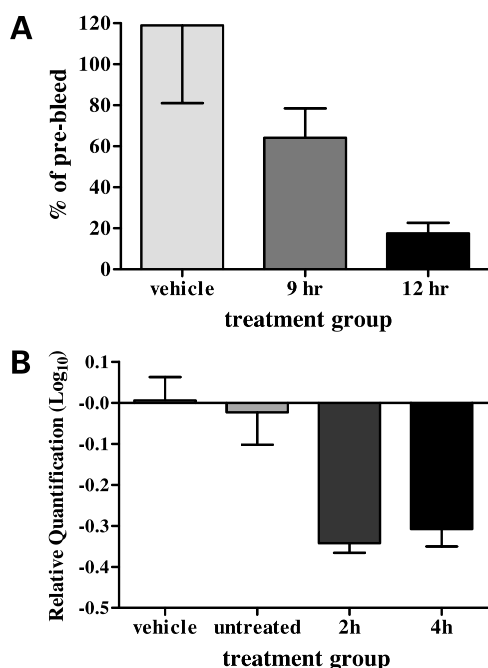

Figure 6. Evaluation of compound 1 effect on IL-8 production and mRNA synthesis in mice bearing established WM-266-4 melanoma xenografts. Nude mice bearing 500 mm^3 (average volume) WM-266-4 melanoma xenograft tumors received s.c. doses of either vehicle (*light gray column*) or compound 1 at 30 mg/kg over a 9-h (*dark gray column*) or 12-h (*black column*) period. Plasma samples were prepared at the end of the study from which IL-8 levels were determined. **B,** nude mice, as in **A**, were left untreated (*light gray column*) or received either one s.c. dose of compound 1 at 30 mg/kg (2 h; *dark gray column*), two doses 2.5 h apart (4 h; *black column*) or vehicle (*white column*). Tumors were subsequently processed for TaqMan RT-PCR analysis of IL-8 mRNA as in Materials and Methods.

pyrimidine nitrogen and the aniline nitrogen perform donor-acceptor interactions with the protein backbone at Cys^{531} (Fig. 2B). Further, the thiophene ring is favored to occupy a hydrophobic pocket adjacent to the ATP-binding site, whereas the ethylene diamine side chain points toward the solvent exposed space. This binding mode is similar to that found with other diaryl imidazole based kinase inhibitors, such as SB203580 bound to p38α (32).

The compound described above was profiled in several standard oncology related assays. For example, compound 1 was tested for its ability to inhibit SK-MEL-28 proliferation and colony formation (Fig. 3A and *B*). The results suggest that inhibition of B-RAF activity in cancer cells could lead to prevention of proliferation, metastasis, and invasion. Indeed, studies with small interfering RNA have shown that suppression of $B\text{-}RAF^{V600E}$ expression in melanoma cells can block tumor growth and invasion and, in some cases, cause tumor shrinkage (25, 33, 34). Furthermore, recent evidence suggests that pharmacologic inhibition of $B\text{-}RAF^{V600E}$ in melanoma xenografts has a similar effect (35). To a certain degree, these results could be explained by the ability of RAF pathway inhibitors to reduce the expression of integrins on the cell surface, thereby reducing their metastatic potential (26). It is also known that mitogen-activated protein kinase activity can regulate integrin expression as well as actin polymerization (36). Therefore, disrupting the anchorage-independent growth and invasiveness of melanoma cells are important properties of B-RAF inhibitors. These results are all related to the finding that B-RAF inhibition led to potent blockade of ERK1/2 phosphorylation (Table 1).

The pharmacokinetic properties of the diaryl imidazoles described here have prevented their evaluation in conventional xenograft studies. Therefore, a program was initiated to elucidate a viable pharmacokinetic/pharmacodynamic biomarker for activity of our compounds in mice bearing melanoma xenograft tumors. Preliminary DNA chip studies with compounds similar to compound 1 (data not shown) and literature review (8, 37) led us to IL-8 as a potential surrogate marker for antitumor activity. Indeed, it was found that IL-8 was produced at high levels by several melanoma cell lines. IL-8 was also readily detectable in plasma samples from mice bearing melanoma xenografts and IL-8 levels increased with tumor burden (Fig. 5B). Furthermore, when SK-MEL-28 cells were treated with compound 1 *in vitro*, IL-8 mRNA synthesis was dramatically reduced (Fig. 4). Importantly, it was found that the IC_{50} for inhibition of ERK1/2 phosphorylation was similar to that for inhibiting IL-8 production, further suggesting the regulatory role of B-RAF in autocrine growth factor production. Subsequently, mice were dosed with compound 1 over a 4- or 12-h period to ensure a minimum level of 1 μmol/L inhibitor in the plasma. As shown in Fig. 6, significant reduction in tumor and IL-8 in plasma at 12 h (Fig. 6A) and IL-8 mRNA synthesis at 2 and 4 h (Fig. 6B) was observed. Taken together, these data strongly suggest that this compound not only penetrated tumor tissue but also potently inhibited intracellular $B\text{-}RAF^{V600E}$ activity. As mentioned above, it has been shown that the concentration of IL-8 in plasma of melanoma patients can be representative of tumor burden (13). Thus, tracking IL-8 plasma levels would be an important measure in melanoma clinical trials where B-RAF inhibitors are employed. Further investigations should be carried out to characterize the effect of B-RAF activating mutations on IL-8 overexpression by other known $B\text{-}RAF^{V600E}$-containing tumors, such as colorectal and papillary thyroid carcinoma. In our experiments, only melanoma cells produced appreciable amounts of IL-8 (Table 1), which appeared to be independent of phospho-ERK levels.

In summary, we have disclosed a novel series of potent diaryl imidazoles that inhibit $B\text{-}RAF^{V600E}$ *in vitro* and *in vivo*. These compounds should be useful for probing the mechanisms of B-RAF-dependent transformation and tumor biology. Furthermore, *in vivo* studies have revealed IL-8 plasma levels to be an important measure of a compound's ability to penetrate the tumor stroma and inhibit the B-RAF pathway. Finally, IL-8 could be an important noninvasive and quantitative pharmacodynamic biomarker for use in clinical trials where patients harbor tumors bearing B-RAF-activating mutations.

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References

1. Davies H, Bignell GR, Cox C, et al. Mutations of the BRAF gene in human cancer. Nature 2002;417:949 – 54.

2. Garnett MJ, Marais R. Guilty as charged: B-RAF is a human oncogene. Cancer Cell 2004;6:313 – 9.

3. Sharma A, Trivedi NR, Zimmerman MA, Tuveson DA, Smith CD, Robertson GP. Mutant V599EB-Raf regulates growth and vascular development of malignant melanoma tumors. Cancer Res 2005;65: 2412 – 21.

4. Chan TL, Zhao W, Leung SY, Yuen ST. BRAF and KRAS mutations in colorectal hyperplastic polyps and serrated adenomas. Cancer Res 2003; 63:4878 – 81.

5. Wang L, Cunningham JM, Winters JL, et al. BRAF mutations in colon cancer are not likely attributable to defective DNA mismatch repair. Cancer Res 2003;63:5209 – 12.

6. Deng G, Bell I, Crawley S, Gum J, et al. BRAF mutation is frequently present in sporadic colorectal cancer with methylated hMLH1, but not in hereditary nonpolyposis colorectal cancer. Clin Cancer Res 2004;10: 191 – 5.

7. Wan PT, Garnett MJ, Roe SM, et al. Mechanism of activation of the RAF-ERK signaling pathway by oncogenic mutations of B-RAF. Cell 2004; 116:855 – 67.

8. Sparmann A, Bar-Sagi D. Ras-induced interleukin-8 expression plays a critical role in tumor growth and angiogenesis. Cancer Cell 2004;6: 447 – 58.

9. Jones SA, Moser B, Thelen MA Comparison of post-receptor signal transduction events in Jurkat cells transfected with either IL-8R1 or IL-8R2. Chemokine mediated activation of p42/p44 MAP-kinase (ERK-2). FEBS Lett 1995;364:211 – 4.

10. Thompson HL, Marshall CJ, Saklatvala J. Characterization of two different forms of mitogen-activated protein kinase kinase induced in polymorphonuclear leukocytes following stimulation by *N*-formylmethionyl-leucyl-phenylalanine or granulocyte-macrophage colony-stimulating factor. J Biol Chem 1994;269:9486 – 92.

11. Na YJ, Jeon YJ, Suh JH, Kang JS, Yang KH, Kim HM. Suppression of IL-8 gene expression by radicicol is mediated through the inhibition of ERK1/2 and p38 signaling and negative regulation of NF-κB and AP-1. Int Immunopharmacol 2001;1:1877 – 87.

12. Sizemore N, Leung S, Stark GR. Activation of phosphatidylinositol 3-kinase in response to interleukin-1 leads to phosphorylation and activation of the NF-κB p65/RelA subunit. Mol Cell Biol 1999;19:4798 – 805.

13. Ugurel S, Rappl G, Tilgen W, Reinhold U. Increased serum concentration of angiogenic factors in malignant melanoma patients correlates with tumor progression and survival. J Clin Oncol 2001;19:577 – 83.

14. Ahmad T, Eisen T. Kinase inhibition with BAY 43-9006 in renal cell carcinoma. Clin Cancer Res 2004;10:6388 – 92S.

15. Lyons JF, Wilhelm S, Hibner B, Bollag G. Discovery of a novel Raf kinase inhibitor. Endocr Relat Cancer 2001;8:219 – 25.

16. Solit DB, Garraway LA, Pratilas CA, et al. BRAF mutation predicts sensitivity to MEK inhibition. Nature 2006;439:358 – 62.

17. Sebolt-Leopold JS, Herrera R. Targeting the mitogen-activated protein kinase cascade to treat cancer. Nat Rev Cancer 2004;4:937 – 47.

18. Wallace EM, Lyssikatos J, Blake JF, et al. Potent and selective mitogen-activated protein kinase kinase (MEK) 1,2 inhibitors. 1. 4-(4-Bromo-2-fluorophenylamino)-1-methylpyridin-2(1H)-ones. J Med Chem 2006;49:441 – 4.

19. Chow S, Patel H, Hedley DW. Measurement of MAPK activation by flow cytometry using phospho-specific antibodies to MEK and ERK: potential for pharmacodynamic monitoring of signal transduction inhibitors. Cytometry 2001;46:72 – 8.

20. Stamey TA, Kabalin JN, Ferrari M, Yang N. Prostate specific antigen in the diagnosis and treatment of adenocarcinoma of the prostate. IV. Anti-androgen treated patients. J Urol 1989;141:1088 – 90.

21. Coskun U, Sancak B, Sen I, et al. Serum P-selectin, soluble vascular cell adhesion molecule-I (s-VCAM-I) and soluble intercellular adhesion molecule-I (s-ICAM-I) levels in bladder carcinoma patients with different stages. Int Immunopharmacol 2006;6:672 – 7.

22. Baron AT, Lafky JM, Boardman CH, et al. Serum sErbB1 and epidermal growth factor levels as tumor biomarkers in women with stage III or IV epithelial ovarian cancer. Cancer Epidemiol Biomarkers Prev 1999; 8:129 – 37.

23. Barak V, Goike H, Panaretakis KW, Einarsson R. Clinical utility of cytokeratins as tumor markers. Clin Biochem 2004;37:529 – 40.

24. Fetsch PA, Filie AC, Steinberg SM, Abati A. Melanoma antigen expression in serial fine-needle aspiration samples in patients with metastatic malignant melanoma participating in immunotherapy clinical trials. Cancer Cytopathology 2001;93:409 – 14.

25. Hoeflich KP, Gray DC, Eby MT, et al. Oncogenic BRAF is required for tumor growth and maintenance in melanoma models. Cancer Res 2006; 66:999 – 1006.

26. Baggiolini M, Clark-Lewis I. Interleukin-8, a chemotactic and inflammatory cytokine. FEBS Lett 1992;307:97 – 101.

27. Gallagher PG, Bao Y, Prorock A, et al. Gene expression profiling reveals cross-talk between melanoma and fibroblasts: implications for host-tumor interactions in metastasis. Cancer Res 2005;65:4134 – 46.

28. Orlikowsky TW, Neunhoeffer F, Goelz R, et al. Evaluation of IL-8-concentrations in plasma and lysed EDTA-blood in healthy neonates and those with suspected early onset bacterial infection. Pediatr Res 2004;56: 804 – 9.

29. Takle AK, Brown MJ, Davies S, et al. The identification of potent and selective imidazole-based inhibitors of B-Raf kinase. Bioorg Med Chem Lett 2006;16:378 – 81.

30. King AJ, Patrick DR, Batorsky RS, et al. Demonstration of a genetic therapeutic index for tumors expressing oncogenic BRAF by the kinase inhibitor SB-590885. Cancer Res 2006;66:11100 – 5.

31. Hall-Jackson CA, Eyers PA, Cohen P, et al. Paradoxical activation of Raf by a novel Raf inhibitor. Chem Biol 1999;6:559 – 68.

32. Lee JC, Laydon JT, McDonnell PC, et al. A protein kinase involved in the regulation of inflammatory cytokine biosynthesis. Nature 1994;372: 739 – 46.

33. Woods D, Cherwinski H, Venetsanakos E, et al. Induction of β3-integrin gene expression by sustained activation of the Ras-regulated Raf-MEK-extracellular signal-regulated kinase signaling pathway. Mol Cell Biol 2001;21:3192 – 205.

34. Sumimoto H, Miyagishi M, Miyoshi H, et al. Inhibition of growth and invasive ability of melanoma by inactivation of mutated BRAF with lentivirus-mediated RNA interference. Oncogene 2004;23:6031 – 9.

35. Tsai J, Zhang J, Bremer R, Artis R, Hirth P, Bollag G. Development of a novel inhibitor of oncogenic b-raf. AACR Meet Abstr 2006;571a.

36. Pritchard CA, Hayes L, Wojnowski L, Zimmer A, Marais RM, Norman J C. B-Raf acts via the ROCKII/LIMK/cofilin pathway to maintain actin stress fibers in fibroblasts. Mol Cell Biol 2004;24:5937 – 52.

37. Leslie MC, Bar-Eli M. Regulation of gene expression in melanoma: new approaches for treatment. J Cell Biochem 2005;94:25 – 38.

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