A Novel RAF Kinase Inhibitor with DFG-Out-Binding Mode: High Efficacy in BRAF-Mutant Tumor Xenograft Models in the Absence of Normal Tissue Hyperproliferation

Irene C. Waizenegger1, Anke Baum1, Steffen Steurer2, Heinz Stadtmüller2, Gerd Bader2, Otmar Schaaf3, Pilar Garin-Chesa1, Andreas Schlattl1, Norbert Schweifer3, Christian Haslinger1, Florian Colbatzky4, Sien Mousa4, Arno Kalkuhl4, Norbert Kraut1, and Günther R. Adolf1

Abstract

BI 882370 is a highly potent and selective RAF inhibitor that binds to the DFG-out (inactive) conformation of the BRAF kinase. The compound inhibited proliferation of human BRAF-mutant melanoma cells with 100× higher potency (1–10 nmol/L) than vemurafenib, whereas wild-type cells were not affected at 1,000 nmol/L. BI 882370 administered orally was efficacious in multiple mouse models of BRAF-mutant melanomas and colorectal carcinomas, and at 25 mg/kg twice daily showed superior efficacy compared with vemurafenib, dabrafenib, or trametinib (dosed to provide exposures reached in patients). To model drug resistance, A375 melanoma–bearing mice were initially treated with vemurafenib; all tumors responded with regression, but the majority subsequently resumed growth. Trametinib did not show any efficacy in this progressing population. BI 882370 induced tumor regression; however, resistance developed within 3 weeks. BI 882370 in combination with trametinib resulted in more pronounced regressions, and resistance was not observed during 5 weeks of second-line therapy. Importantly, mice treated with BI 882370 did not show any body weight loss or clinical signs of tolerability, and no pathologic changes were observed in several major organs investigated, including skin. Furthermore, a pilot study in rats (up to 60 mg/kg daily for 2 weeks) indicated lack of toxicity in terms of clinical chemistry, hematology, pathology, and toxicogenomics. Our results indicate the feasibility of developing novel compounds that provide an improved therapeutic window compared with first-generation BRAF inhibitors, resulting in more pronounced and long-lasting pathway suppression and thus improved efficacy. Mol Cancer Ther. 15(3); 354–65. ©2016 AACR.

Introduction

In multiple normal tissues, the MAPK signaling pathway transmits extracellular signals via tyrosine kinase receptors, RAS, RAF, MEK, and ERK proteins to regulate diverse cellular functions, including survival and proliferation. Hyperactivation of this pathway due to increased transducer expression (based on focal gene amplification) or higher intrinsic activity (due to gain-of-function point mutations, deletions, or chromosomal rearrangements) contributes to the pathogenesis of a wide range of solid tumors as well as hematologic malignancies, with mutations in RAS GTPases as the most frequent drivers of malignant proliferation. Genetic alterations of members of the RAF family of serine/threonine kinases are likewise involved in multiple types of cancers. Whereas mutations in ARAF and CRAF are very rare, BRAF mutations are found with widely varying frequency across multiple cancer types, ranging from about 5% in colorectal and non–small cell lung carcinoma (NSCLC) to 50% in malignant melanoma and close to 100% in hairy cell leukemia. Among a variety of BRAF alterations identified to date, point mutations affecting amino acid position 600 are by far the most frequent ones (V600E or V600K; melanoma, >90%); the oncogenic potential of these and the dependence of BRAF-mutant melanomas on the activity of the mutant protein was clearly demonstrated in a variety of in vivo and animal disease models. Subsequently, multiple drug discovery programs to synthesize potent and drug-like BRAF inhibitors were initiated, and the first compound, vemurafenib, was approved for treatment of melanoma patients in 2011, only nine years after the initial report on BRAF mutations in cancer (1, 2).

During clinical development of vemurafenib and, subsequently, of further BRAF inhibitors such as dabrafenib, two main issues limiting the utility of this class of compounds became apparent. First, treatment resulted in the progression of skin lesions...
with preexisting RAS mutations, in particular of squamous cell carcinomas and keratoacanthomas, in a subset of patients; mechanistically, these adverse events involve a paradoxical, inhibitor-induced activation of wild-type (WT) B/CRAF signaling, increased pathway output, and thus increased proliferation. Clinically, these skin malignancies are not of major concern, as the number of lesions per patient is usually small, and the tumors can be removed surgically (3). Initial concerns that BRAF inhibitors might also promote progression of other RAS-mutant cancers have not been substantiated to date, although a few individual cases have been reported (4). More importantly, the benefit provided by BRAF inhibitors to melanoma patients was found to be quite limited in duration. Following initial, often dramatic regression of lesions, secondary resistance develops in a majority of patients over the course of a few months of treatment. More recent clinical trials have shown that simultaneous treatment with a BRAF (dabrafenib) and a MEK inhibitor (trametinib) provides superior outcomes compared with single-agent therapy in terms of higher response rates and prolonged progression-free and overall survival, and the BRAF–MEK inhibitor combination is now the standard of care for BRAFV600–mutant melanoma (5, 6). Nevertheless, the improvement achieved by combination therapy appears modest, and a high medical need for effective treatment of relapsing patients remains. Recently, Girotti and colleagues reported on novel RAF inhibitors that showed efficacy in preclinical melanoma models resistant to the vemurafenib analogue PLX4720 (7). Interestingly, these compounds, like BI 882370, bind to the inactive conformation of BRAF; in addition to RAF kinases, however, they also inhibit SRC family kinases with similar potency. Although SRC inhibition may well contribute to efficacy, the therapeutic window that can be achieved by combined RAF/SRC inhibition remains to be determined.

In the course of a discovery program aiming for the identification of improved BRAF inhibitors, we noted that various compounds from the same chemical class displayed widely varying therapeutic windows in mice. Whereas some compounds, even though reasonably potent and selective BRAF inhibitors in in vitro experiments and efficacious in tumor xenograft models, induced hyperproliferation of various stratified and transitional epithelia in mice, in accordance with previous reports (8–10), others achieved similar efficacy in the absence of detectable histologic changes. Although some of these differences may be due to off-target effects, we reasoned that different modes of drug–target interaction may play a fundamental role in determining the therapeutic index and thus the extent and duration of tumor responses. We here present the chemical structure, target binding mode, and preclinical pharmacologic profile of a highly potent and selective RAF inhibitor that displays favorable characteristics in models of BRAF-mutant cancers.

Materials and Methods

Compounds

Compounds were synthesized and/or formulated according to published procedures: BI 882370 (I-142; ref. 11); afatinib (BIBW 2992; ref. 12); vemurafenib (example P-0956; refs. 13, 14); dabrafenib (example S8a; ref. 15); trametinib (example 4-I; ref. 16); and GDC-0879 (example 110; ref. 17). In addition, vemurafenib (Zelboraf), cetuximab (Erbitux), and irinotecan (Campto) were purchased from Roche, Merck, and Pfizer, respectively.

Structural research

A histidine-tagged mutant form of the human BRAF kinase domain (18) expressed in E. coli was purified by affinity chromatography on Ni Sepharose, ion exchange chromatography and gel filtration. Crystals were grown by the vapor diffusion method. Datasets were collected at 100K on the X60SA beamline at SLS and processed with XDS (19). Structures were solved by molecular replacement using a published BRAF structure and refined by iterative cycles of model building using COOT (20) and refinement by REFMAC (21). The structures were deposited at RCSB [accession codes 5CSX (BI 882370) and 5CSW (dabrafenib)]. All crystallographic work was done by Proteros Biostructures GmbH (more details are available in the Supplementary Materials and Methods).

Kinase assays

IC50 values were determined by Proteros Biostructures GmbH, following published methods (22).

Cell lines and cellular assays

Cell lines were cultured according to the manufacturer’s instruction and authenticated by short tandem repeat (STR) analysis at Boehringer Ingelheim on the dates indicated. A101 (obtained, October 9, 2006; STR, September 15, 2015), A375 (obtained, July 7, 2006; STR, April 14, 2012), C32 (obtained, January 1, 2007; STR, April 28, 2014), COLO 205 (obtained, February 21, 2003; STR, May 4, 2011), G-361 (obtained, October 16, 2006; STR, September 9, 2015), HCT-116 (obtained, June 17, 2011; STR, April 17, 2012), HT-29 (obtained, January 24, 2012; STR, February 28, 2013), LS411N (obtained, August 24, 2006; STR, June 16, 2015), NCI-H1395 (obtained, July 20, 2011; STR, March 15, 2012), NCI-H1666 (obtained, December 13, 2004; STR, September 15, 2015), NCI-H1755 (obtained, July 20, 2011; STR, March 15, 2012), and SK-MEL-28 (obtained, October 15, 2007; STR, December 11, 2014) were from the ATCC. CAL-12T (obtained, August 21, 2006; STR, September 15, 2015) were from the DSMZ, and HCC-364 (obtained, June 22, 2013; STR, August 12, 2013) were from University of Texas Southwestern Medical Center (Dallas, TX). BRO cell line was a kind gift from Prof. Piet Borst (Amsterdam Free University, Amsterdam, the Netherlands) in 1995; STR data for BRO cells are from September 9, 2014, no published reference STR available.

Proliferation assays. Cell lines were analyzed by alamarBlue (AbD Serotec), except HT-29 and COLO 205, which were tested by 3H-thymidine incorporation assay. In brief, cells were seeded into 96-well plates, and the next day, "time zero" plates were read, or serially diluted compounds were added to test plates (final DMSO concentration 1%; compounds were usually tested in duplicates). After 3 days of compound treatment, the antiproliferative effect was determined by measuring the metabolic activity of remaining cells using alamarBlue. GI50 or EC50 values were calculated by nonlinear regression curve fit (sigmoidal dose response with variable hill slope). To determine the capacity of cells to incorporate 3H-thymidine, 0.1 μCi/
well $^3$H-thymidine (Amersham TRK296) was added after 3 days of compound treatment. After additional 16 hours of incubation, plates were frozen (−20 °C) to lye cells. Cell lysates were prepared and harvested on MultiScreen Harvest Plates FC (Merck Millipore, #MAHFC1H60); 25 μL MicroScint/well was added to dried filter plates, and the plates were measured with a Wallac TriLux 1450 MicroBeta Counter. EC$_{50}$ values were calculated as described previously. In assays performed at Eurofins Panlabs, cells were incubated for 3 days, fixed, and stained with DAPI nuclear dye. Cell counts were determined by fluorescence imaging, and EC$_{50}$ values were determined by comparing cultures treated for 3 days and cultures fixed at the beginning of the incubation period.

**Phospho-ERK assay.** A375 (10,000 cells/well) or SK-MEL-28 (7,500 cells/well) were plated in 96-well flat bottom Costar plates (#3598), and the next day, cells were treated with compounds for 2 hours, fixed with 4% formaldehyde, permeabilized and washed several times with 0.1% Triton X-100/PBS (20 minutes), blocked with 5% nonfat dry milk in TBST, and incubated with anti-phospho-ERK1/2 (Sigma #M8159; 1:500) overnight. After several washing steps with 0.1% Tween-20/PBS, cells were stained with HRPO-coupled rabbit anti-mouse IgG (Dako, #P0161; 1:1,000; 1 hour), washed, and stained with TMB substrate (Bender MedSystems, #BMS406); the reaction was stopped after 5 to 30 minutes by 1 mol/L phosphoric acid. The plates were measured at 450 nm using a Wallac Victor 2. The EC$_{50}$ was calculated as described above.
To address the reversibility of phospho-ERK modulation cells were treated with compounds for 2 hours, washed 5 times with medium, and incubated further until they were processed as described above.

**Immunoblot.** Lysates were prepared from cells treated for 2 or 24 h. Harvested cells were lysed in 20 mmol/L Tris pH 7.7, 100 mmol/L NaCl, 20 mmol/L β-glycerophosphate, 5 mmol/L MgCl2, 1 mmol/L NaF, 0.1% Triton X-100, 5% glycerol plus freshly added 1 mmol/L DTT, 0.1 μmol/L okadaic acid, 1 mmol/L sodium vanadate, and Complete Mini Protease Inhibitor Tablets (Roche, #11836170001). For SDS-PAGE, equal amounts of protein/lane were loaded. After gel transfer onto PVDF membrane, immunoblots were performed using the following antibodies: phospho-MEK1/2 (Cell Signaling Technology #9112; 1:1,000), MEK1 (BD Biosciences #610121; 1:10,000), phospho-ERK (Sigma #8159; 1:30,000), ERK1/2 (Upstate Biotechnology #06-182; 1:5,000), cyclin D1/2 (Upstate Biotechnology #05-362; 1:5,000), Kip1/p27 (BD Biosciences #610241; 1:5,000), and α-tubulin (Sigma #T6199; 1:4,000). Secondary antibodies were from DAKO (sc-2004 or #P0447). ECL detection was performed.

**Coimmunoprecipitation.** Cells were treated for 1 hour with various compounds prior to harvesting. NP40 lysates and CRAF immunoprecipitates were prepared according to ref. 23 using rabbit anti-CRAF (Santa Cruz Biotechnology, sc-227) and analyzed by immunoblot using mouse anti-BRAF (Santa Cruz Biotechnology, sc-5284).

**Animal studies.** All experiments were carried out under ethics committee–approved protocols and in compliance with federal guidelines for the humane treatment and care of laboratory animals.

**Compound formulations.** BI 882370 was dissolved in 0.5% Natrosol (pH 3, citric acid). Vemurafenib in amorphous form was stabilized by co precipitation with hydroxypropylmethyl cellulose acetate succinate (HPMC-AS). The polymer–vemurafenib powder (30% drug load) was suspended in 2% Klucel LF hydroxypropylcellulose (pH 3–3.5, HCl). For one of the experiments (Fig. 4), Zelboraf tablets were crushed and milled, and the resulting powder was suspended in 0.5% Natrosol (pH 3, HCl). Dabrafenib was suspended in 0.5% HPMC-AS and 0.02% Tween 80 (pH 8, NaOH). Trametinib was administered as a suspension in 1% Natrosol/5% DMSO.

**Mouse xenograft studies.** A375, COLO 205, or G-361 (5 × 106), or HT-29 (1.5 × 106) cells were injected subcutaneously into 7-week-old female BomTac:NMRI-Foxn1nu mice (Taconic). Mice were randomized when tumors were well established. All compounds were administered intragastrically (10 ml/kg). Tumor diameters were measured 3 times a week, and volumes were calculated according to the formula "tumor volume = length × diameter2 × π/6". Mice were inspected daily for clinical signs, and body weight was determined 3 times weekly. Median TGI values were calculated as follows:

\[ TGI = 100 \times \left(1 - \frac{(treated_{final \ day} - treated_{day1})}{(control_{final \ day} - control_{day1})}\right)\]

The P values obtained from the Mann–Whitney U test were adjusted using the Bonferroni–Holm correction. For graphical representation of tumor growth kinetics, data were plotted as average with SEM.

On the last day of experiments, blood samples were taken under isoflurane anesthesia to determine drug plasma concentrations. For pharmacokinetic/pharmacodynamic analyses, blood samples and tumors were either snap frozen to determine drug concentrations or formalin-fixed and paraffin-embedded for IHC evaluation.

**IHC.** Immunohistochemical analysis was carried out in formalin-fixed paraffin-embedded tumor samples from xenograft models. The indirect immunoperoxidase assay Envision System (Dako #4003) was used with 3,3'-diaminobenzidine solution (Sigma Aldrich, # D5905) as a substrate for the immunoreaction and the staining was performed according to the manufacturer’s instruction. Sections were counterstained with hematoxylin. The rabbit monoclonal antibody against phospho-ERK1/2 (Cell Signaling, #4376) and an antibody against Cyclin D1 (Biocare medical, #CP236A) were used to monitor pathway modulation under treatment.

For more details, see Supplementary Materials and Methods section.

Table 1. RAF isoform inhibition and kinase selectivity

<table>
<thead>
<tr>
<th>A. Binding mode</th>
<th>Vemurafenib</th>
<th>Dabrafenib</th>
<th>BI 882370</th>
</tr>
</thead>
<tbody>
<tr>
<td>DFG-in</td>
<td>DFG-in</td>
<td>DFG-out</td>
<td></td>
</tr>
<tr>
<td>Kinase IC50 (nmol/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BRAF</td>
<td>72</td>
<td>1</td>
<td>0.4</td>
</tr>
<tr>
<td>BRAF inhibitor</td>
<td>117</td>
<td>2</td>
<td>0.8</td>
</tr>
<tr>
<td>CRAF</td>
<td>33</td>
<td>0.7</td>
<td>0.6</td>
</tr>
<tr>
<td>Kinase IC50 (nmol/L)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BRAF inhibitor</td>
<td>240</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>BRAF</td>
<td>560</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>CRAF</td>
<td>220</td>
<td>16</td>
<td>3</td>
</tr>
</tbody>
</table>

**Selectivity (number of kinases inhibited by >50% at 1,000 nmol/L/total number of kinases)**

| 6/243 | 24/258 | 15/238 |

NOTE: The IC50 values and IC50 for BI 882370, vemurafenib, and dabrafenib were determined on BRAFV600E mutant, BRAF WT, and CRAF kinase. Selectivity was determined by testing the compounds on a large panel of kinases at a single concentration (1,000 nmol/L); inhibition by >50% compared with controls was scored as positive.

Table 2. RAF isoform inhibition and kinase selectivity

<table>
<thead>
<tr>
<th>B. Kinase selectivity of BI 882370</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kinase IC50 (nmol/L)</td>
</tr>
<tr>
<td>Kinase</td>
</tr>
<tr>
<td>--------</td>
</tr>
<tr>
<td>CSFIR</td>
</tr>
<tr>
<td>LCK</td>
</tr>
<tr>
<td>SMRS</td>
</tr>
<tr>
<td>FGR</td>
</tr>
<tr>
<td>PTK6</td>
</tr>
<tr>
<td>YES1</td>
</tr>
<tr>
<td>RET</td>
</tr>
<tr>
<td>KIT</td>
</tr>
<tr>
<td>SRC</td>
</tr>
<tr>
<td>BTK</td>
</tr>
<tr>
<td>SRC</td>
</tr>
<tr>
<td>LYN B</td>
</tr>
<tr>
<td>LYN A</td>
</tr>
<tr>
<td>FRK</td>
</tr>
<tr>
<td>POGFR A</td>
</tr>
</tbody>
</table>

NOTE: IC50 values for all kinases that scored positive in the primary screen of BI 882370.
NOTE: EC50 values were determined on BRAF-mutant and WT melanoma cell lines (A101D, A375, SK-MEL-28, G-361, and BRO) and colorectal cancer cell lines (COLO 205, HT-29, LS411N, and HCT-116) using cell-based ELISA technology for the detection of p-ERK and alamarBlue, DAPI-labelled nuclei (C), or [3H]-thymidine incorporation tests (D) for determining antiproliferative activity.

Table 2. Pharmacodynamic biomarker modulation and antiproliferative effect on cancer cell lines

<table>
<thead>
<tr>
<th>Condition</th>
<th>Vemurafenib</th>
<th>Dabrafenib</th>
<th>BI 882370</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO (nmol/L)</td>
<td>p-ERK (A375)</td>
<td>59</td>
<td>5</td>
</tr>
<tr>
<td>p-ERK (SK-MEL-28)</td>
<td>71</td>
<td>5</td>
<td>0.7</td>
</tr>
<tr>
<td>Proliferation EC50 (nmol/L)</td>
<td>A375V600E</td>
<td>110</td>
<td>4</td>
</tr>
<tr>
<td>SK-MEL-28V600E</td>
<td>264</td>
<td>7</td>
<td>1</td>
</tr>
<tr>
<td>G-361V600E</td>
<td>1,272</td>
<td>39</td>
<td>6</td>
</tr>
<tr>
<td>A101DV600E</td>
<td>216</td>
<td>Not done</td>
<td>2.6</td>
</tr>
<tr>
<td>A375V600E</td>
<td>83</td>
<td>Not done</td>
<td>1.4</td>
</tr>
<tr>
<td>BRO-mutant</td>
<td>&gt;20,000</td>
<td>7,581</td>
<td>5,960</td>
</tr>
<tr>
<td>COLO 205V600E</td>
<td>227</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>HT-29V600E</td>
<td>196</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>LS411NV600E</td>
<td>177</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>HCT-116WT</td>
<td>&gt;20,000</td>
<td>&gt;20,000</td>
<td>5,768</td>
</tr>
</tbody>
</table>

NOTE: EC50 values were determined on BRAF-mutant and WT melanoma (A101D, A375, SK-MEL-28, G-361, and BRO) and colorectal cancer cell lines (COLO 205, HT-29, LS411N, and HCT-116) using cell-based ELISA technology for the detection of p-ERK and alamarBlue, DAPI-labelled nuclei (C), or [3H]-thymidine incorporation tests (D) for determining antiproliferative activity.

Results

Chemical structure and binding mode

BI 882370 (Fig. 1) is a compound resulting from an extensive lead optimization program to identify highly potent and selective RAF inhibitors. The target-binding mode was investigated by X-ray crystallography using a modified form of the BRAF kinase domain with improved solubility (18). The structure of the approved RAF inhibitor dabrafenib in complex with the same protein was solved with improved solubility (18). The structure of the approved RAF inhibitor dabrafenib in complex with the same protein was solved with improved solubility (18). The structure of the approved RAF inhibitor dabrafenib in complex with the same protein was solved with improved solubility (18). The structure of the approved RAF inhibitor dabrafenib in complex with the same protein was solved with improved solubility (18).

BI 882370 potently inhibited the oncogenic BRAFV600E-mutant as well as the WT BRAF and CRAF kinases, with similar IC50 values achieved (Fig. 1 and Supplementary Fig. S1; Supplementary Tables S1 and S2).

Dabrafenib was found to bind to the hinge region in the cleft between the N- and C-lobes of the kinase, i.e., to the ATP binding site, in the "DFG-in" (active) conformation of the enzyme. The compound shows a classical 2-point hinge-binding interaction via its aminopyridyl moiety: the sulfonamide oxygen forms H-bonds to the backbone NH of Phe595 and the side chain of Lys594. The nitrogen atom of the sulfonamide moiety is within H-bonding distance (3.3 Å) to the main chain NH of Asp594. This suggests that the nitrogen is in a deprotonated state, stabilizing the DFG-in conformation of the kinase.

In contrast to dabrafenib, BI 882370 binds to the ATP binding site of the kinase positioned in the "DFG-out" conformation, considered to represent the enzymatically inactive state of the protein. N20 of the pyrimidyl moiety forms a classical H-bond to the hinge region; a nonclassical H-bond via the acidified hydrogen of C21 contributes to binding. The two sulfonamide oxygen atoms form H-bonds to the side chain of Lys601 and the backbone NH of Asp594, respectively. This binding mode stabilizes the DFG-out conformation by placing Phe595 of the DFG motif just below the pyrrolopyrimidine moiety of BI 882370, enabling an aromatic T-stacking interaction that may contribute to the high potency of this inhibitor.

Potency and selectivity in biochemical assays

In enzymatic assays using a cascade RAF–MEK–ERK setup, BI 882370 potently inhibited the oncogenic BRAFV600E-mutant as well as the WT BRAF and CRAF kinases, with similar IC50 values.
A Novel RAF Inhibitor with DFG-Out–Binding Mode

(0.4, 0.8, and 0.6 nmol/L, respectively; Table 1A). Reporter displacement assays resulted in $K_D$ values of 4, 6, and 3 nmol/L, respectively. Dabrafenib showed similar potency in enzymatic as well as competitive binding assays, whereas vemurafenib was about 100-fold less potent.

The selectivity of BI 882370 was determined at a concentration of 1,000 nmol/L on a panel of 253 kinases. The activity of 15 enzymes was inhibited by 50% or more; IC$_{50}$ values were determined for all of them (Table 1B). The most sensitive kinase CSF1R was inhibited with an IC$_{50}$ value of 39 nmol/L, i.e., with about 100-fold lower potency compared with BRAF$^{V600E}$ inhibition.

**Pathway inhibition and antiproliferative activity**

High potency of BI 882370 was observed in cellular assays. In human A375 melanoma cells carrying a homozygous BRAF$^{V600E}$ mutation, treatment for 2 hours resulted in a reduction of phospho-MEK1/2 and phospho-ERK1/2 signals as seen in immunoblots, with EC$_{50}$ values estimated as 0.3 nmol/L and complete suppression observed at 3 nmol/L (Fig. 2A). Treatment for 24

**Figure 3.** First-line therapy of human melanoma xenografts in nude mice. A, mice bearing A375 tumors (BRAF$^{V600E}$ homozygous) were treated with BI 882370 at 25 mg/kg twice daily (bid), with vemurafenib at 120 mg/kg once daily (qd), or with dabrafenib at 60 mg/kg once daily. Adjusted $P$ values: BI 882370 vs. vemurafenib, $P = 0.0002$ and vs. dabrafenib, $P = 0.0002$. B, mice bearing G-361 tumors (BRAF$^{V600V/E}$ heterozygous) were treated with BI 882370 at 12.5 or 25 mg/kg twice daily or with vemurafenib at 120 mg/kg once daily. Adjusted $P$ values: BI 882370 25 mg/kg twice daily vs. vemurafenib, $P = 0.0002$; BI 882370 12.5 mg/kg twice daily vs. vemurafenib, $P = 0.0070$). C, mice bearing G-361 tumors (BRAF$^{V600V/E}$ heterozygous) were treated with BI 882370 at 25 mg/kg twice daily, vemurafenib at 120 mg/kg once daily, dabrafenib at 50 mg/kg once daily, trametinib at 0.25 mg/kg twice daily, or a combination of trametinib with either BI 882370 or dabrafenib. The groups receiving BI 882370 and the combination groups were treated for 41 days and thereafter observed until day 60 without further treatment. On day 23, adjusted $P$ values were BI 882370 vs. vemurafenib, $P = 0.0018$, vs. trametinib, $P = 0.0018$, and vs. dabrafenib, $P = 0.0018$. [Further details and statistics are provided, including adjusted $P$ values and comparisons between treatments.]
Mice with tumor volumes between 200–500 mm³ used for second-line treatment

Waizenegger et al.

Published OnlineFirst February 25, 2016; DOI: 10.1158/1535-7163.MCT-15-0617
of BI 882370 and trametinib. Second-line treatment with vemurafenib (120 mg/kg once daily), trametinib [0.25 mg/kg twice daily (bid)], BI 882370 (25 mg/kg twice daily) or a combination (Table 2). BI 882370 showed EC$_{50}$ values of 0.5 and 0.7 nmol/L in A375 and SK-MEL-28 (BRAFV600E) melanoma cells, respectively. Dabrafenib was 5- to 10-fold less active, in spite of similar potency in biochemical tests (see above); vemurafenib was about 100-fold less potent than BI 882370.

Compound-induced RAF heterodimerization in BRAF WT cells is considered to be the initiating event for paradoxical pathway activation, resulting in hyperproliferation of normal as well as Ras-mutant cells (23–25). For BI 882370,coprecitation assays did not provide evidence of heterodimer formation in the NRAS-mutant melanoma cell line BRO at pharmacologically relevant concentrations; even at high concentrations (1–10 µmol/L), dimer formation was barely detectable (Fig. 2C). In contrast, the tool compound GDC-0879 (25) clearly induced formation of both CRAF/ARAF as well as CRAF/BRAF dimers. Nevertheless, BI 882370 induced phosphorylation of MEK1/2 and enhanced phosphorylation of ERK1/2 in BRO cells at concentrations between 3 and 300 nmol/L; importantly, however, expression of cyclins D1/D2 or Kip1/p27 was not affected (Fig. 2B).

To simulate transient compound exposure in vivo, the time course of pathway modulation after short-term in vitro treatment was analyzed using the A375 model. Cells were treated for 2 hours, washed extensively, incubated in medium without inhibitors, and harvested 1, 2, 3, 4, 6, or 24 hours thereafter to determine ERK phosphorylation by cell-based ELISA. Treatment with BI 882370 resulted in long-lasting suppression, with EC$_{50}$ values 24 hours after washout still in the range of 10 to 20 nmol/L. In contrast, ERK phosphorylation in cells pretreated with vemurafenib returned to control values within 1 hour of drug removal, even when the pretreatment concentration was as high as 10 µmol/L. In cells pretreated with dabrafenib, pathway suppression was observed for up to 4 hours after washout (EC$_{50}$ 5–15 nmol/L), whereas phospho-ERK levels returned to baseline after 6 hours [pretreatment concentrations up to 1,400 or 10,000 nmol/L (vemurafenib); Supplementary Fig. S2]. In an extended panel of BRAF-mutant human melanoma and colorectal cancer cell lines, BI 882370 showed EC$_{50}$ values in the range of 1 to 10 nmol/L (Table 2); in contrast, proliferation of BRAF WT cells was only inhibited at concentrations >1,000 nmol/L. In accordance with data on pathway inhibition presented above, dabrafenib was about 5-fold and vemurafenib about 100-fold less potent than BI 882370. In additional experiments to determine the drug sensitivity of BRAF-mutant NSCLC, the BRAFV600E cell line HCC-364 responded with an EC$_{50}$ of 7 nmol/L, whereas none of the cell lines with BRAFV600E or BRAFV600E mutation were sensitive (EC$_{50}$ > 1,000 nmol/L; NCI-H1395, NCI-H1666, NCI-H1755, and CAL-127).

### Efficacy in melanoma models

In initial dose-finding and scheduling experiments, we determined that BI 882370 showed higher efficacy when oral doses were given twice daily rather than once daily (same total daily dose; Supplementary Table S3, COLO 205 model). Treatment of mice bearing established A375 melanomas (50–100 mm$^3$) with BI 882370 at doses of 6.25 or 12.5 mg/kg twice daily resulted in high efficacy; on day 18, when the control group had to be sacrificed for ethical reasons (median tumor volume, 1,000 mm$^3$), the median TGI was higher than 100%, and partial regressions were observed in subsets of tumors. At 25 mg/kg twice daily, all tumors showed either partial (3/7) or complete (4/7) regression. For comparison, vemurafenib (120 mg/kg once daily) and dabrafenib (60 mg/kg once daily) resulted in TGI values of 89% (one partial regression) and 97% (2 partial regressions), respectively (Fig. 3A; Supplementary Table S3; see below for pharmacokinetic considerations). BI 882370 was well tolerated at all dose levels, as judged by body weight gain and clinical signs. 25 mg/kg administered twice daily was therefore used as the standard treatment dose/schedule for subsequent efficacy studies; an MTD has not been defined. Pharmacokinetic experiments indicated that under these conditions, drug exposure (AUC$_{0–24h}$) at the end of a treatment period of 2 to 3 weeks ranged from 12,000 to 25,000 nmol·h/L.

In additional experiments in the A375 model, we compared compound concentrations in tumors and plasma after a single oral dose of BI 882370 (50 mg/kg), dabrafenib (75 mg/kg), or vemurafenib (120 mg/kg). For BI 882370, tumor concentrations were higher than the plasma concentration at each time point investigated, in contrast to dabrafenib or vemurafenib (Supplementary Fig. S3). Following treatment with BI 882370, the phospho-ERK signal in tumor cells as determined by IHC was strongly or partially suppressed for at least 16 hours in spite of undetectable drug plasma concentrations at 16 hours (Supplementary Fig. S4).

G-361 melanoma cells with heterozygous BRAFV600E mutation are less sensitive to BRAF inhibitors than A375 cells in vitro (Table 2) and when grown as xenograft tumors may thus serve as a more discriminative pharmacologic model. In the first experiment (Fig. 3B, Supplementary Table S3), BI 882370 at 12.5 mg/kg twice daily resulted in a median TGI of 98% with 2 of 7 tumors showing partial regression; at 25 mg/kg twice daily, all tumors regressed. Vemurafenib, dosed at 120 mg/kg daily to achieve exposures that correspond to those observed in patients (AUC$_{0–24h}$ approximately 1,700,000 nmol·h/L for 960 mg twice daily; ref. 26), was less efficacious; after initial stabilization, tumors resumed growth after around 8 days of treatment. In an independent experiment (Fig. 3C, Supplementary Table S3), vemurafenib again showed only moderate efficacy. Similarly, dabrafenib at 50 mg/kg once daily (AUC$_{0–24h}$, 23,000 nmol·h/L) compared with AUC$_{0–24h}$, 8,400 nmol·h/L in patients upon repeat dosing with 150 mg twice daily; ref. 27) had only moderate impact on tumor growth.
The MEK inhibitor trametinib at the MTD was only moderately efficacious in delaying tumor growth (0.25 mg/kg twice daily; AUC_{0–24h} = 520 nmol·h/L, compared with 600 nmol·h/L upon repeat dosing with 2 mg once daily in patients; ref. 28). A combination of dabrafenib and trametinib showed markedly improved efficacy, stabilizing the tumor volume throughout the 42-day treatment period (1/7 tumors in regression); when treatment was discontinued, however, tumors started to regrow within a few days. BI 882370 dosed at 25 mg/kg twice daily showed high efficacy as a single agent, as all tumors partially regressed under treatment; upon discontinuation, tumor regrowth was markedly delayed. Interestingly, the combination of BI 882370 with trametinib did not further improve efficacy; in particular, no complete regression was observed.

Additional experiments were performed using the heterozygous BRAF^{V600E/K} A101D melanoma model. Both, BI 882370 (25 mg/kg twice daily) and trametinib (0.25 mg/kg twice daily) were highly efficacious, achieving partial regression of all tumors (n = 7/group); vemurafenib (120 mg/kg once daily) was less efficacious (TGI = 80%, no regressions; Supplementary Table S3).

Acquired resistance and second-line treatment

In the experiments described above, we had observed that A375 xenografts were sensitive to vemurafenib but rapidly acquired resistance. To model second-line therapy, we treated a large cohort of animals bearing A375 melanomas with vemurafenib (120 mg/kg once daily, 100% regressions). Tumors in control mice exhibited a poorly differentiated phenotype in contrast to well-differentiated adenocarcinomas observed after treatment with BI 882370 (data not shown).

Previous studies have demonstrated that partial resistance to BRAF inhibition in BRAF-mutant colorectal cancer is mediated by EGFR signaling (35). We have confirmed that the EGFR-specific antibody cetuximab as well as the ErbB family kinase inhibitor afatinib, although not significantly efficacious as single agents, synergize with BI 882370, achieving tumor growth control and even partial regressions in 5 and 6 of 7 mice per group, respectively (Supplementary Fig. S6B; Supplementary Table S3). Similarly, trametinib showed only moderate efficacy as a single agent (TGI = 82%, no regression) but markedly improved the efficacy of BI 882370 (TGI = 100%, 2/7 regressions). Finally, treatment of the animals with a combination of BI 882370, trametinib, and cetuximab resulted in regression of all tumors (7/7); however, complete regressions were not observed even with this triple drug combination (Supplementary Table S3; this table also includes additional results of other combination therapy regimens).

Efficacy in colorectal cancer models

In the BRAF^{V600E/K}-mutant COLO 205 model, previously reported to be sensitive to BRAF inhibition, treatment with vemurafenib at 120 mg/kg once daily for 2 weeks resulted in partial regression of 2 of 7 tumors (median TGI = 100%; Supplementary Table S3). BI 882370 at 25 mg/kg twice daily induced shrinkage of all tumors; 4 of these regressed completely. From this experiment, multiple organs and tissues of 5 animals were preserved and examined histologically; no evidence for BI 882370-induced hyperplasia or any other pathologic alterations was seen in skin, heart, lung, bladder, stomach, liver, kidney, and bladder (Supplementary Table S5). In an independent COLO 205 experiment, BI 882370 showed high efficacy at doses down to 6.25 mg/kg twice daily (Supplementary Fig. S5).

The HT-29 model (BRAF^{V600E/K}) was reported to be only moderately sensitive to BRAF inhibition (34). We confirmed this observation using vemurafenib at 120 mg/kg once daily, achieving a TGI of 60%. BI 882370 at 12.5 mg/kg twice daily resulted in 73% TGI; a minor improvement was noted when the dose was increased to 25 mg/kg twice daily (TGI = 85%). Further doubling of the dose to 50 mg/kg twice daily resulted in an increase of exposure from 25,000 to 62,000 nmol·h/L, but no increase in efficacy (TGI = 88%). Of note, BI 882370 was well tolerated even at this high dose, administered over a 3-week period (Supplementary Fig. S6A). Upon histopathologic examination (haematoxylin/eosin staining), tumors in control mice exhibited a poorly differentiated phenotype in contrast to well-differentiated adenocarcinomas observed after treatment with BI 882370 (data not shown).

Exploratory evaluation of safety and tolerability in rats

As noted above, a first study evaluating multiple organs of tumor-bearing mice treated for 2 weeks with BI 882370 at a highly efficacious dose level had not provided any evidence of pertinent pathologic changes. As a next step, an exploratory toxicology study was performed in male rats (Supplementary Table S6). Doses of 10, 30, and 60 mg/kg were administered once daily for...
2 weeks (5 animals per group). Pharmacokinetic analysis at the end of the study showed that the exposures achieved in the high-dose group exceeded those required for high efficacy in xenograft models (60 mg/kg; 43,000 nmol.h/L). Clinical chemistry and hematology investigations (days 3 and 14), histopathologic examination as well as toxicogenomic analysis of liver (days 1 and 14) and skin (day 14) did not reveal any toxicologically relevant changes.

**Discussion**

Approval of BRAF inhibitors for use in patients with BRAFV600E-mutant melanomas has provided an important improvement in treatment outcomes over the alkylating agent dacarbazine, the previous standard of melanoma therapy. In many patients, these targeted agents achieve rapid disease control, with response rates of approximately 50%. However, complete responses are rarely observed, and moreover, median progression-free survival is less than one year even in patients treated with a RAF/MEK inhibitor combination (6), with biomarker studies indicating that the MAPK pathway is active in progressing tumors in spite of continuous treatment. Dose escalation, an obvious measure to intensify pathway suppression, is not an option due to tolerability limitations. In recent years, a large number of pre-clinical studies as well as analyses of patient-derived tumor tissues have attempted to shed light on the mechanisms of resistance. Surprisingly, secondary point mutations in the mutant protein, a frequent cause of resistance to other kinase inhibitors, were not observed; instead, an unexpectedly broad spectrum of genetic alterations leading to reactivation of the MAPK pathway has been uncovered. More recently, it was shown that treatment of melanoma cells with BRAF inhibitors induces secretion of soluble factors that enhance the survival of drug-sensitive cells and accelerate the expansion of drug-resistant clones (36). Finally, experiments in mouse models have revealed a role of the tumor stroma in drug resistance, as melanoma-associated fibroblasts are activated by BRAF inhibitors to promote matrix remodeling, which in turn results in elevated integrin signaling in malignant cells and increased ERK activity independent of BRAF (37). In view of this plethora of potential resistance mechanisms, it appears questionable whether any single-targeted agent, or in fact any combination of targeted agents, may be able to overcome resistance or at least delay relapse to a clinically meaningful extent beyond that achieved by the approved dabrafenib and trametinib combination. We reasoned that further improvement of clinical outcomes may be realized by designing a novel RAF inhibitor with improved drug properties, such as high potency and selectivity to minimize off-target activity, favorable effects on tumor conformation with respect to undesired B/CRAF dimer formation, and long residence time on the target as well as favorable tissue distribution, all converging on an improved therapeutic window that enables stronger suppression of target activity for extended periods of time. The results we present here indicate that this strategy may be feasible. On the basis of preliminary experiments, we selected BI 882370 for in-depth profiling, a compound that binds to BRAF (and presumably also to A/RAF) in the inactive conformation termed "DFG-out" with reference to the position of the activation loop of the kinase. DFG-out binders, or type II inhibitors, in distinction to type I inhibitors that bind to the "DFG-in" active conformation, are considered to potentially achieve a higher degree of potency and selectivity and likely to possess greater cellular potency and slower dissociation rates than their type I counterparts (38). BI 882370 indeed showed higher cellular potency than approved BRAF inhibitors, high selectivity in a large kinase panel, long duration of action, and favorable tissue distribution with consequent long-term suppression of target activity in melanoma xenografts. In several xenograft models of BRAF-mutant cancers, BI 882370 showed superior efficacy in comparison with established BRAF and MEK inhibitors dosed to achieve exposures observed in patients. Of particular interest are the results of our attempt to model second-line treatment of A375 BRAFV600E melanomas after the failure of first-line vemurafenib. In the majority of vemurafenib-treated animals, we noted the characteristic pattern of initial regression and subsequent progression observed in patients. RNA sequencing of resistant tumors showed no evidence of point mutations, BRAF overexpression, or truncation, whereas the expression of several genes was consistently up or downregulated. These results indicate that epigenetic mechanisms are underlying drug resistance in this model, in accordance with recent findings in a subset of clinical melanoma samples (39). Progressing tumors were insensitive to treatment with a MEK inhibitor, again reflecting the clinical situation (40). In contrast, treatment of progressing tumors with BI 882370 resulted in tumor regression; however, resistance developed within about 2 to 3 weeks. Treatment with BI 882370 in combination with trametinib resulted in more pronounced regression, and no regrowth was observed within 5 weeks.

BI 882370 at doses of 25 mg/kg twice daily was well tolerated by mice for periods of at least several weeks; in one experiment, dose escalation to 50 mg/kg twice daily with concomitant higher exposures was likewise well tolerated. A MTD has not been defined. As a first step towards a more refined evaluation of safety and tolerability of BI 882370, tumor-bearing mice treated for 2 weeks were submitted to exploratory examination of multiple organs; neither epithelial hyperplasia nor any other pathologic changes were observed. In addition, exploratory toxicologic examination of male rats treated with doses up to 60 mg/kg once daily for 2 weeks, resulting in exposures in excess of those providing superior efficacy in xenograft models, did not result in any adverse clinical observations or any relevant findings in terms of clinical chemistry, hematology, pathology, or toxicogenomics.

Taken together, our results indicate that it may indeed be possible to develop second-generation RAF inhibitors that exhibit a superior therapeutic window compared with approved compounds. BI 882370, or agents with similar mode of target interaction and physicochemical properties, may provide higher response rates and longer duration of response and may be superior combination partners for MEK inhibitors as well as for novel agents suggested by recent preclinical studies, including inhibitors of RAF (37), of the PI3K pathway (41) as well as for BRAF-mutant colon carcinomas, with inhibitors of the EGFR (35).

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

Conception and design: I.C. Waizenegger, O. Schaaf, P. Garin-Chesa, F. Colbatzky, S. Mousa, N. Kraut, G.R. Adolf

Development of methodology: I.C. Waizenegger, O. Schaaf, P. Garin-Chesa, N. Schweifer, F. Colbatzky

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): I.C. Waizenegger, O. Schaaf, P. Garin-Chesa, F. Colbatzky, S. Mousa, A. Kalkuhl
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): I.C. Waizenegger, A. Baum, S. Steurer, H. Stadtmüller, G. Bader, O. Schaaf, P. Garin-Chesa, A. Schlattl, S. Mousa, N. Schweifer, C. Haslinger.

Writing, review, and/or revision of the manuscript: G. Bader, P. Garin-Chesa, A. Schlattl, F. Colbatzky, S. Mousa, N. Schweifer.

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): G. Bader, P. Garin-Chesa, A. Schlattl, F. Colbatzky, S. Mousa, N. Schweifer, C. Haslinger, Franziska Popp, Cristina Puri, Nina Rodi, Regina Ruzicka, Christian Salamon, Michaela Streicher, Alexander Wlasch, and Susanne Wollner-Gaido for their dedicated, excellent contributions to experiments described in this article and Thomas Bogeneder for discussion on medical perspectives.

Grant Support

This work was financially supported by Boehringer Ingelheim.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Acknowledgments

The authors acknowledge the contributions of the entire BRI BIA research and development team. In particular, the authors thank Horst Ahorn, Rosa Baumanngartner, Karin Bichler, Karin Bosch, Ralph Knoll, Karen Köhler, Franziska Bogenrieder for discussion on medical perspectives.

References


12. Himmelsbach F, Langkopf E, Blech S, Jung B, Baum ESF, inventors;


A Novel RAF Kinase Inhibitor with DFG-Out–Binding Mode: High Efficacy in BRAF-Mutant Tumor Xenograft Models in the Absence of Normal Tissue Hyperproliferation

Irene C. Waizenegger, Anke Baum, Steffen Steurer, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-15-0617

Supplementary Material
Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2016/02/26/1535-7163.MCT-15-0617.DC1

Cited articles
This article cites 33 articles, 6 of which you can access for free at:
http://mct.aacrjournals.org/content/15/3/354.full#ref-list-1

Citing articles
This article has been cited by 1 HighWire-hosted articles. Access the articles at:
http://mct.aacrjournals.org/content/15/3/354.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.