BGB-283, a Novel RAF Kinase and EGFR Inhibitor, Displays Potent Antitumor Activity in BRAF-Mutated Colorectal Cancers

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Abstract

Oncogenic BRAF, which drives cell transformation and proliferation, has been detected in approximately 50% of human malignant melanomas and 5% to 15% of colorectal cancers. Despite the remarkable clinical activities achieved by vemurafenib and dabrafenib in treating BRAFV600E metastatic melanoma, their clinical efficacy in BRAFV600E colorectal cancer is far less impressive. Prior studies suggested that feedback activation of EGFR and MAPK signaling upon BRAF inhibition might contribute to the relative unresponsiveness of colorectal cancer to the first-generation BRAF inhibitors. Here, we report characterization of a dual RAF kinase/EGFR inhibitor, BGB-283, which is currently under clinical investigation. In vitro, BGB-283 potently inhibits BRAFV600E-activated ERK phosphorylation and cell proliferation. It demonstrates selective cytotoxicity and preferentially inhibits proliferation of cancer cells harboring BRAFV600E and EGFR mutation/amplification. In BRAFV600E colorectal cancer cell lines, BGB-283 effectively inhibits the reactivation of EGFR and EGFR-mediated cell proliferation. In vivo, BGB-283 treatment leads to dose-dependent tumor growth inhibition accompanied by partial and complete tumor regressions in both cell line-derived and primary human colorectal tumor xenografts bearing BRAFV600E mutation. These findings support BGB-283 as a potent antitumor drug candidate with clinical potential for treating colorectal cancer harboring BRAFV600E mutation. Mol Cancer Ther; 14(10); 2187–97. © 2015 AACR.

Introduction

The MAPK pathway plays an essential role in regulating cell proliferation and survival. Activation of the RAS–RAF–MEK–ERK kinase cascade by external stimuli transduces signals from the plasma membrane into the cell nucleus to control gene expression and determine cell fate (1). Aberrant activation of the MAPK signal transduction pathway is frequently found in different types of cancers, contributing to increased cell division, suppressed apoptosis, and enhanced cell motility and invasion (2, 3). BRAF, one of the three members of the RAF kinase family, has been identified as a target for cancer therapy (4, 5). A sequencing screen of 923 cancer samples detected mutations in the BRAF gene in approximately 50% of human malignant melanomas and 15% of colorectal cancers (6–11), with the V600E mutation accounting for at least 90% of oncogenic BRAF mutations (6). This V600E mutation introduces a negative charge in the BRAF kinase domain that mimics and bypasses the phosphorylation required for BRAF activation, which is normally achieved through growth factor–activated receptor tyrosine kinases. As a result, this "gain of function" of BRAF gives rise to a constitutive MAPK signaling that promotes tumor progression, in which BRAFV600E activates MEK1/2 in a RAS-independent manner (12–15).

Small molecules that selectively target mutant BRAF exhibit good efficacy and yield impressive clinical responses in melanoma patients with BRAFV600E mutation. The RAF inhibitors that selectively inhibit BRAFV600E tumors, vemurafenib (PLX4032) and dabrafenib (GSK 2118436), have generated objective response rates from 50% to 70%, respectively, in early clinical trials treating metastatic melanoma (5, 16, 17). The clinical experience with these BRAF inhibitors in benefitting melanoma patients confirms BRAFV600E as a bona fide oncogenic target and validates the utility of cancer therapies that target BRAF and MAPK signaling (4, 5). However, these first-generation BRAF inhibitors still have limitations, including development of cutaneous squamous cell carcinomas and treatment-related keratoacanthomas (KA) due to paradoxical activation of MAPK signaling. In addition, they have inadequate clinical activity outside of melanoma.
with BRAF<sup>V600E</sup> mutation (16, 18–20). In particular, the clinical response among colorectal cancer patients with BRAF<sup>V600E</sup> mutations is much lower than that observed in melanoma patients. For example, vemurafenib was reported to have a mere 5% objective response rates in colorectal cancer patients with BRAF<sup>V600E</sup> mutation (21). Recent studies suggest that increased EGFR activity through a feedback activation mechanism might account for the drastic difference in objective response rates between BRAF<sup>V600E</sup> melanoma and colorectal cancer to the first-generation BRAF inhibitors (22, 23). Combination of BRAF and EGFR inhibitors was found to significantly enhance the efficacy in preclinical models (22, 23). Therefore, a second-generation BRAF inhibitor that can inhibit both BRAF<sup>V600E</sup> and EGFR-driven RAF activation may have a therapeutic advantage in treating BRAF-mutated colorectal cancer.

We describe herein BGB-283, a novel fused tricyclic benzoimidazole compound that inhibits both BRAF and EGFR. BGB-283 potently inhibits RAF family kinases and EGFR activities in biochemical assays. It demonstrates selective cytotoxicity to colorectal cancer cell lines harboring BRAF<sup>V600E</sup> or EGFR mutations. BGB-283 is highly efficacious in BRAF<sup>V600E</sup> colorectal cancer xenograft models, including HT29, Colo205, and two primary tumor xenografts harboring BRAF<sup>V600E</sup> mutation. In addition, BGB-283 shows compelling efficacy in a WiDr xenograft model where EGFR reactivation was shown to be induced upon BRAF inhibition (22, 23). Together, these data suggest that BGB-283 is a novel RAF kinase and EGFR inhibitor with therapeutic potential in targeting oncogenic BRAF in human colorectal carcinoma.

Materials and Methods

Material

BGB-283 used in this study exceeded a purity of 99% as measured by proton nuclear magnetic resonance, liquid chromatography–mass spectrometry and high-performance liquid chromatography. Reference compounds were purchased from the following sources: PLX4032 and dabrafenib, WuXi AppTec; gefitinib, MenCha, erlotinib, Beijing Ouhu Technology Co., Ltd.; cetuximab (Merck KGaA), Beijing Cancer Hospital (Beijing, P.R. China). Stock solutions of compounds were prepared in DMSO.

BRAF<sup>V600E</sup> kinase domain/BGB-283 co-crystallization and structure determination

BRAF<sup>V600E</sup> (444–723) was expressed and purified using methods similar to those previously reported (22). To co-crystallize BRAF<sup>V600E</sup> with BGB-283, protein solution was incubated with BGB-283 at a ratio of 1:5 for 1 hour, and mixed with reservoir solution (100 mmol/L Bis Tris at pH 6.5, 23% PEG3350, and 200 mmol/L MgCl<sub>2</sub>) at equal volume. Co-crystals grew by sitting drop vapor diffusion method at 4°C. Diffraction data were collected from the Synchrotron Radiation beam line (Shanghai Synchrotron Radiation Facility). Crystals belonged to the space group P2<sub>1</sub>2<sub>1</sub>2<sub>1</sub> (a = 49.395 Å, b = 101.601 Å, c = 109.786 Å) and contain two BRAF<sup>V600E</sup> molecules in an asymmetric unit. Diffraction images were processed and scaled with HKL2000. Phase was solved using software MOLREP by molecular replacement method with previously published structure. The resultant model was subsequently refined in PHENIX using rigid-body refinement and maximum likelihood method (Supplementary Table S1). Complex structure of the BRAF<sup>V600E</sup> kinase domain with BGB-283 was submitted to the Protein Data Bank (PDB) with the PDB ID code 4R5Y.

In vitro kinase assay

Compounds were tested for inhibition of RAF and WT EGFR kinase activity in assays based on time-resolved fluorescence energy transfer (TR-FRET) methodology. MEK1 (K97R) was used as a substrate for RAF kinases and a biotinylated peptide substrate was used for EGFR (61TK0BLC, CisBio Bioassays). The kinase was incubated with a serial dilution of compounds for 60 to 120 minutes at room temperature (RT), ATP (final concentration at 100 μmol/L) and kinase substrates were added to initiate the reaction. The reaction was stopped by an equal volume of stop/detection solution according to the manufacturer’s instruction (CisBio Bioassays). Plates were sealed and incubated at RT for 2 hours, and the TR-FRET signals (ratio of fluorescence emission at 665 nm over emission at 620 nm with excitation at 337 nm wavelength) were recorded on a PHERAstar FS plate reader (BMG Labtech).

BGB-283 was screened for activity in a panel of 277 kinases at a fixed concentration of 10 μmol/L by Life Technologies using their standard assays at Km concentration of ATP for perspective kinases. The IC<sub>50</sub> was then determined for kinases showing >80% inhibition at 10 μmol/L BGB-283.

Cell culture

A375, Sk-Me1-28, HT29, Colo205, WiDr, Ba/F3, A431, HCC827, SW620, HCT116, and cell lines used in cell panel profiling were purchased from ATCC. Cell lines were tested and authenticated at ATCC before purchase using morphology, karyotyping, and PCR-based approaches. All the cell lines were cultured in the designated medium supplemented with 10% FBS (Thermo Scientific), 100 U/mL penicillin (Gibco), 0.1 mg/mL streptomycin (Gibco), and in a humidified 37°C environment with 5% CO<sub>2</sub>. Cell lines were reconstituted from frozen stocks laid down within three passages from the original cells purchased and passed no more than 30 times. Culturing condition for cell panel profiling is listed in Supplementary Table S2. Stimulation of EGFR phosphorylation in A431 cells was achieved by addition of EGF to serum-free DMEM with 10-minute incubation. EGF-stimulated cell growth was achieved by addition of EGF to DMEM or RPMI supplemented with 10% FBS.

Western blotting analysis

For <i>in vitro</i> studies, cells were harvested after 1 hour treatment at 37°C and lysed immediately as previously described (24). For <i>in vivo</i> studies, tumors were harvested at the indicated time points, snap-frozen in liquid nitrogen, and stored at –80°C. Tumors were homogenized in 500 μL lysis buffer in MP homogenization unit (Fast prep-24; MP bio, Cat. # 6004.2) and lysates were then centrifuged at 13,000 rpm for 10 minutes at 4°C to remove insoluble debris. The protein concentration of lysates was determined using the Pierce BCA protein assay kit (Thermo Scientific). Proteins were separated by 10% SDS-PAGE gel or NuPAGE Novex 4% to 12% Bis-Tris protein gels (Life Technologies) and transferred to nitrocellulose membranes using iBlot Dry Blotting System (Life Technologies). Blots were blocked with TBSTM [50 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 0.1% Tween 20, and 5% non-fat milk] at RT for 1 hour and probed with indicated antibodies diluted in TBSTM. The membranes were probed for phospho-proteins and then stripped to probe for total proteins. For reprobing, the membranes were stripped in stripping buffer (25 mmol/L glycine, pH 2.0, 1% SDS) for 30 to 60 minutes at RT, rinsed twice with TBST for 10 minutes, and probed for other...
proteins. Antigen–antibody complexes were visualized using the chemiluminescent substrate (Millipore) and detected with Image Quant LAS4000 mini digital imaging system (GE Healthcare).

Antibodies used were obtained commercially from the following sources: anti-BRAF (SC-5248), Santa Cruz Biotechnology; anti-C-Raf (610152), BD Biosciences; antibodies to MEK (9122, 9126), phospho-MEK1/2 (Ser217/211) (9154), ERK (4695), phospho-ERK1/2 (Thr202/Tyr204) (4370), EGFR (2646s), phospho-AKT (Ser473; 4060), AKT (4685), DLSp6 (5605), phospho-EGFR (Tyr1068; 3777), GAPDH (2118s) and anti-rabbit IgG horseradish peroxidase (HRP)-linked secondary antibody, Cell Signaling Technology; anti-mouse IgG HRP-linked secondary antibody, Sigma-Aldrich.

Cell-based phospho-ERK and phospho-EGFR detection assay
Cellular phospho-ERK and phospho-EGFR were measured using a TR-FRET–based method. Cells were seeded at 3 x 10^4 per well of a 96-well plate and left to attach for 16 hours. Growth medium was then replaced with 100 mL of DMEM containing no serum. Cells were then treated with a 10-point titration of compound. After 1 hour of compound treatment, cells were then incubated at room temperature with shaking for 30 minutes. A total of 16 mL of cell lysate from each well of a 96-well plate was transferred to a 384-well small volume white plate. Lysate from each well was incubated with 2 mL of Eu^{3+} or Tb^{3+}-cryptate (donor) labeled anti-ERK or anti-EGFR antibody (Cisbio) and 2 mL of D2 (acceptor) labeled anti-phospho-ERK or anti-phospho-EGFR antibody (Cisbio) for 2 hours at room temperature. FRET signals were measured using a PHERAstar FS reader (BMG Labtech).

Proliferation assay
The growth-inhibitory activity of compounds in a panel of melanoma, colon, breast, and lung cancer cells was determined using CellTiter-Glo luminescent cell viability assay (Promega). The number of cells seeded per well of a 96-well plate was optimized for each cell line to ensure logarithmic growth over the 3 days treatment period (Supplementary Table S2). Cells were left to attach for 16 hours and then treated with a 10-point dilution series in duplicate. Following a 3-day exposure to the compound, a volume of CellTiter-Glo reagent equal to the volume of cell culture medium present in each well was added. Mixture was mixed on an orbital shaker for 2 minutes to allow cell lysis, followed by 10 minutes incubation at room temperature to allow development and stabilization of luminescent signal. Luminescent signal was measured using PHERAstar FS reader (BMG Labtech). EC_{50} values for cell viability were determined with GraphPad Prism software.

Tumor xenografts experiments
Female NOD/SCID and BALB/c nude mice, ages 4 to 6 weeks, and weighing approximately 18 g, were purchased from Beijing HFK Bioscience Co., Ltd. All procedures involving animals were conducted in accordance with the Institutional Animal Care and Use Committee (IACUC) of BeiGene. For HCC827, A431, HT29, Colo205, and WiDr xenografts, each mouse was injected subcutaneously with 2.5 to 5 x 10^6 cells in 200 mL PBS in the right front flank via a 26-gauge needle. When the average tumor size reached 110 to 200 mm^3, animals were randomized to treatment groups (7–9 mice per group) and treated twice per day (b.i.d.) or once daily (q.d.) by oral gavage (p.o.) with vehicle alone or 2.5 to 30 mg/kg of BGB-283. As control, mice were treated with erlotinib (100 mg/kg q.d) or cetuximab (40 mg/kg twice weekly). BGB-283 and erlotinib were formulated at the desired concentration as a homogenous suspension in 0.5% (w/v) methylcellulose in purified water. Cetuximab was formulated by diluting the injection solution with saline before dosing.

For BCCO-002 and BCCO-028 primary human tumor xenografts (PDX), colorectal cancer samples were collected from Beijing Cancer Hospital (Beijing, P.R. China) after patient's informed consent and immediately transferred in DMEM culture medium contained 200 U/mL penicillin and 200 mg/mL streptomycin. Within 2 to 4 hours of surgery, small fragments (3 mm x 3 mm x 3 mm) were subcutaneously engrafted into the scapular area or flank of anesthetized NOD/SCID mice. After three successful passages on NOD/SCID, tumors were subsequently passaged in BALB/c nude mice. Efficacy studies were conducted within six passages of the patient tumors. When the average tumor size reaches 100 to 200 mm^3, animals were randomized to treatment groups (8 mice per group) and treated orally with vehicle (0.5% MC) alone, BGB-283 (5–10 mg/kg, b.i.d.) or dabrafenib (50 mg/kg, b.i.d.). A further group received intravenous cetuximab (40 mg/kg every 3 days). BGB-283 was formulated as described above. Dabrafenib was formulated in 10% DMSO + 90% HP-β-CD (Hydroxypropyl-β-cyclodextrin)/PBS.

In both cell line and primary tumor xenograft studies, individual body weights and tumor volumes were determined twice weekly, with mice being monitored daily for clinical signs of toxicity during the study.

Efficacy endpoints
Tumor volumes were calculated using the formula: V = 0.5 x (a x b^2), in which a and b are the long and short diameters of the tumor, respectively. Partial regression (PR) was defined as tumor volume smaller than 50% of the starting tumor volume on the first day of dosing for at least three consecutive measurements. Complete regression (CR) was defined as tumor volume less than 14 mm^3 for at least three consecutive measurements. Tumor growth inhibition (TGI) was calculated using the following formula: % growth inhibition = 100 x [1 – (treated t0 – placebo t0)]/ (placebo t0 – placebo t0)], in which treated t represents tumor volume at day t in the treated group, treated t0 represents tumor volume of the same treated group on the first day of treatment, placebo t represents placebo tumor volume at day t in the control group, placebo t0 represents tumor volume of the same group on the first day of treatment. Statistical analysis was conducted using the Student t test. P < 0.05 was considered statistically significant.

Results
BGB-283 is an inhibitor of RAF family kinases and EGFR
BGB-283, with the chemical formula 5-(((1R,1aS,6bR)-1-(6-( trifluoromethyl)-1H-benzo[d]imidazol-2-yl)-1a,6b-dihydro-1H-cycloprop[b]benzofuran-5-yl)oxy)-3,4-dihydro-1,8-naphthyridin-2(1H)-one (Fig. 1A), is a synthetic inhibitor designed to interact with the ATP-binding site of the BRAF^V600E (Fig. 1B). The crystal structure of the BGB-283/BRAF^V600E kinase domain complex revealed that BGB-283 binds to the ATP-binding pocket of BRAF^V600E in an inactive conformation (Fig. 1B and Supplementary Table S1). BGB-283 binds to both protomers of BRAF^V600E and BRAF^V600E.
BRAFV600E with a DFG-out and α-C helix-in conformation. In comparison, PLX4032 binds to only one of the protomers in DFG-out and α-C helix-in conformation in its co-crystal structure with BRAFV600E (PDB code: 3OG7; ref. 25). The pyridine lactam group of BGB-283 interacts with the hinge region, while the fused tricycle group binds between the N-terminal lobe domain and activation loop. The benzimidazole and trifluoro groups locate in the pocket formed by α-C helix and activation loop (Fig. 1B). There are two hydrogen bonds formed between BGB-283 and the BRAF protein. One is between hydroxyl group of E501 on the α-C helix and the nitrogen atom from the benzimidazole. The other is between the nitrogen atom on the pyridine lactam group and the carbonyl of C532 from the hinge region of BRAFV600E.

The biochemical potency of BGB-283 in inhibiting RAF kinases was measured under steady-state conditions using GST-MEK1 (K97R) as a substrate. BGB-283 was shown to be a pan-RAF inhibitor of the kinase domains of BRAFV600E, WT BRAF, C-RAF Y340/341D, and WT A-RAF in vitro (Table 1A). In particular, BRAFV600E potently inhibited the activity of the recombinant BRAFV600E kinase domain with an IC50 value of 23 ± 5 nmol/L (Table 1A). The inhibition by BGB-283 was time dependent and with a slow off-rate. The estimated t1/2 of dissociation was measured to be much longer than 240 minutes (Supplementary Fig. S1A). BGB-283 was further shown to be a reversible inhibitor of the BRAF kinase (Supplementary Table S3). The selectivity of BGB-283 was evaluated by measuring the percentage of inhibition against 279 kinases at 10 μmol/L, a concentration that is approximately 450 times higher than its IC50 value against the purified kinase domain of BRAFV600E. Of the 279 kinases tested, 43 of them showed >80% of inhibition by 10 μmol/L BGB-283 (Supplementary Table S4). Further profiling of BGB-283 against these 43 kinases revealed its inhibition of several other kinases, including EGFR, DDR1, DDR2, EPHA3, FLT3, VEGFR2, and ZAK (Supplementary Table S5). The biochemical inhibition of EGFR by BGB-283 was further characterized in the TR-FRET assay. BGB-283 was shown to inhibit the kinase activity of EGFR with IC50 of 29 ± 18 nmol/L, and EGFR T790M/L858R mutant with IC50 of 495 ± 124 nmol/L (Table 1A).

BRAFV600E potently inhibits BRAFV600E-activated ERK phosphorylation and cell proliferation

The effect of BGB-283 on oncogenic BRAF-mediated ERK phosphorylation was investigated in A375, a BRAFV600E-driven melanoma cell line. Consistent with data from biochemical characterization, BGB-283 potently inhibited BRAF phosphorylation (Fig. 2A). The potency of BGB-283 in inhibiting BRAFV600E-driven

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**Table 1. In vitro kinase and cellular activity of BGB-283**

**A. Biochemical activity of BGB-283 against RAF family and EGFR kinases.**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>IC50 (nmol/L; mean ± SD)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>BRAFV600E kinase domain (aa416-766)</td>
<td>23 ± 5</td>
<td>7</td>
</tr>
<tr>
<td>WT BRAF kinase domain (aa416-766)</td>
<td>32 ± 8</td>
<td>3</td>
</tr>
<tr>
<td>C-RAF Y340/341D kinase domain (aa306-648)</td>
<td>7.0 ± 2.3</td>
<td>6</td>
</tr>
<tr>
<td>WT A-RAF kinase domain (aa282-609)</td>
<td>5.6</td>
<td>1</td>
</tr>
<tr>
<td>EGFR kinase domain (aa669-1210)</td>
<td>29 ± 18</td>
<td>10</td>
</tr>
<tr>
<td>EGFR 7979M/L858R (aa669-1210)</td>
<td>495 ± 124</td>
<td>2</td>
</tr>
</tbody>
</table>

**B. Inhibition of ERK or EGFR phosphorylation by BGB-283 in different cancer cell lines.**

<table>
<thead>
<tr>
<th>Phosphorylated kinase</th>
<th>Cell lines</th>
<th>BGB-283 IC50 (nmol/L; mean ± SD)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>pERK</td>
<td>A375</td>
<td>64 ± 31</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Sk-Mel-28</td>
<td>95 ± 58</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>HT29</td>
<td>50 ± 18</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Colo205</td>
<td>92 ± 57</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>A431</td>
<td>385 ± 40</td>
<td>7</td>
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<tr>
<td></td>
<td>HCC827</td>
<td>195 ± 102</td>
<td>2</td>
</tr>
</tbody>
</table>

**Figure 1.**

BGB-283, a compound designed for inhibiting oncogenic BRAF. A, chemical structure of BGB-283. B, the crystal structure of BGB-283 bound to BRAFV600E. Dashed lines are hydrogen bonds.

**Table 1A.** Biochemical activity of BGB-283 against RAF family and EGFR kinases.

<table>
<thead>
<tr>
<th>Enzyme</th>
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<tbody>
<tr>
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<tr>
<td>WT BRAF kinase domain (aa416-766)</td>
<td>32 ± 8</td>
<td>3</td>
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<tr>
<td>C-RAF Y340/341D kinase domain (aa306-648)</td>
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</tr>
<tr>
<td>WT A-RAF kinase domain (aa282-609)</td>
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<td>EGFR 7979M/L858R (aa669-1210)</td>
<td>495 ± 124</td>
<td>2</td>
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</tbody>
</table>
Figure 2.

BGB-283 potently inhibited ERK phosphorylation and EGFR activity. A, immunoblot analyses for BRAF, phospho-ERK1/2, ERK1/2, and GAPDH in A375 cell lysates prepared 1 hour after treatment with indicated concentrations of PLX4032 or BGB-283. B, immunoblot analyses for BRAF, phospho-ERK1/2, ERK1/2, and GAPDH in HCT116 and SW620 cell lysates prepared 1 hour after being treated with indicated concentrations of PLX4032 and BGB-283. C, immunoblot analyses for EGFR, phospho-Tyr1068-EGFR, phospho-ERK1/2, ERK1/2, and GAPDH in A431 (EGFR overexpression) cell lysates prepared 2 hours after 20 ng/mL EGF stimulation and treatment with indicated concentrations of PLX4032, BGB-283, or gefitinib. D, immunoblot analyses for phospho-Tyr1068-EGFR, EGFR, phospho-Thr202/Tyr204-ERK1/2, ERK1/2, c-Myc, and GAPDH in WiDr and HT29 cell lysates prepared 3 and 24 hours after treatment with indicated compound diluted in RPMI-1640 with 5% FBS. HCC827 (EGFR E746-A750 deletion; E) tumor cells (5 × 10⁶) or A431 (EGFR overexpression; F) tumor cells (5 × 10⁶) were implanted subcutaneously in female BALB/c nude mice. When the tumors reached a mean volume of approximately 100 to 180 mm³ in size, mice were randomly allocated into groups and treated as indicated. Data are presented as average tumor volume ± SEM of 7 animals in each group.
ERK phosphorylation was further confirmed by a quantitative TR-FRET assay in four different cell lines harboring BRAF\textsuperscript{V600E}, with IC\textsubscript{50} values ranging from 32 to 153 nmol/L (Table 1B). Previously, it was reported that PLX4032 treatment of SW620 and HCT116 cell lines expressing BRAF\textsuperscript{V600E} resulted in a paradoxical induction of ERK1/2 phosphorylation (26–28). Unlike PLX4032, BGB-283 induced much less ERK activation in both of the two cell lines (Fig. 2B).

BGB-283 inhibits cellular EGFR activity and blocks cell proliferation and tumor growth driven by EGFR

The ability of BGB-283 in-inhibiting EGFR activity in cells was examined by measuring EGFR phosphorylation in A431 and HCC827 cells, where EGFR is activated by overexpression or EGF E746-A750 deletion. BGB-283 inhibited the EGFR-induced EGFR autophosphorylation on Tyr1068 in A431 cells in a dose-dependent manner (Fig. 2C and Table 1B). Gefitinib, a known EGFR inhibitor, also potently inhibited the same phosphorylation site. In comparison, no significant inhibition of EGFR autophosphorylation was observed in A431 and HCC827 cells treated with PLX4032 (Fig. 2C and Supplementary Fig. S1B). It has been reported that BRAF inhibition could induce feedback activation of EGFR thus contributing to the insensitivity of BRAF\textsuperscript{V600E} colon cancer cells to PLX4032 (22, 23). To evaluate the feedback activation of EGFR upon BRAF inhibition with BGB-283 or PLX4032 treatment, two BRAF\textsuperscript{V600E} colon cancer cell lines (HT29 and WiDr) were treated by the compounds and measured for EGFR and ERK phosphorylation levels. PLX4032 treatment resulted in an upregulation of phospho-EGFR on Tyr1068, and strongly increased downstream phospho-ERK in WiDr cells and partially reduced phospho-ERK levels in HT29 cells. Consequently, PLX4032 resulted in an insufficient inhibition of ERK-downstream signaling as represented by c-Myc expression in both cell lines after 24-hour treatment. Addition of an EGFR inhibitor, erlotinib, abrogated the EGFR reactivation induced by PLX4032 and led to better inhibition of MAPK signaling after 24 hours. In comparison, BGB-283 alone reduced EGFR phosphorylation, and inhibited ERK phosphorylation and c-Myc expression more effectively than PLX4032 (Fig. 2D). These findings suggest that the anti-EGFR activity of BGB-283 could suppress the feedback activation of EGFR signaling upon BRAF inhibition (Fig. 2D).

Ba/F3 cells normally grow in an IL3-dependent manner (29), but their growth can be rendered IL3 independent by overexpression of EGFR exon 19 deletion or EGFR L858R mutant (30, 31). In accordance with its EGFR inhibition effect, BGB-283 was found to block the proliferation of Ba/F3 cells that express EGFR exon 19 deletion or EGFR L858R mutants, suggesting that BGB-283 could inhibit EGFR-driven cell proliferation (Table 2A and Supplementary Fig. S1C). Interestingly, BGB-283 also showed moderate inhibitory effect on EGFR Exon19/790M mutant that is insensitive to gefitinib (Table 2A and Supplementary Fig. S1C). This result is consistent with the observation from biochemical studies where BGB-283 showed inhibition to 790M containing EGFR mutant. There were no significant differences in sensitivity to BRAF inhibitors between BRAF\textsuperscript{V600E} melanoma cells (A375) and BRAF\textsuperscript{V600E} colon cancer cells (HT29 and WiDr) in both short-term and long-term proliferation assay (Table 2B). However, in the present of the EGFR ligand EGF, both HT29 and WiDr became insensitive to PLX4032 at concentration up to 10 μmol/L. In contrast, BGB-283 retained inhibitory activity against HT29 and WiDr cells albeit with reduced potency (Table 2B). Together, these data demonstrate that BGB-283 inhibits EGFR in cells and can inhibit EGFR-induced cell proliferation in BRAF\textsuperscript{V600E} colon cancer cell lines.

The in vivo activity of BGB-283 in suppressing EGFR-mediated tumor growth was examined in HCC827 lung carcinoma and A431 epidermoid carcinoma xenografts. Consistent with in vitro study results, BGB-283 treatment at 10 and 30 mg/kg b.i.d. or qd effectively inhibited tumor growth (Fig. 2E and F). In correspondence with different anti-EGFR activities between the two cell lines (Table 1B), BGB-283 induced tumor regression in HCC827 but not in A431 xenograft. As a control, erlotinib, a potent EGFR inhibitor, achieved similar TGI effect at 100 mg/kg qd in the HCC827 xenograft model (Fig. 2E). In summary, these findings suggested that BGB-283 is a bona fide EGFR inhibitor both in vitro and in vivo.

BGB-283 inhibits proliferation of tumor cells expressing BRAF\textsuperscript{V600E} or harboring an EGFR mutation

In order to test the selectivity of BGB-283 in suppressing cell proliferation, a panel of 107 human tumor cell lines was exposed to BGB-283 in the presence of serum and examined for viability after 3 days. The mutational status of BRAF, H-RAS, K-RAS, and N-RAS was noted for each cell line (Fig. 3 and Supplementary Table S2). BGB-283 selectively inhibited the growth of cancer cell lines expressing BRAF\textsuperscript{V600E} but not BRAF\textsuperscript{WT}, similar to PLX4032 (Fig. 3A and B). The EC\textsubscript{50} values in the majority of nonresponsive...
cell lines were >10 μmol/L (Fig. 3A and Supplementary Table S2). In addition, BGB-283 was found to inhibit proliferation of the HCC827 lung cancer cell line with EGFR exon 19 deletion, the ZR-75-30 breast cancer cell line with HER2 amplification, and the NCI-H322M lung cancer cell line with EGFR overexpression (Fig. 3A).

Figure 3. BGB-283 selectively inhibited proliferation of cancer cells harboring BRAFV600E and EGFR mutations. Antiproliferative effect of BGB-283 (A) and PLX4032 (B) following a 3D exposure across a panel of human cancer cell lines determined by the CellTiter-Glo assay.
BGB-283 exhibits antitumor activity in mouse xenograft models of colorectal cancer

The in vivo efficacy of BGB-283 was assessed in subcutaneous xenograft models derived from HT29 and Colo205 colorectal cancer cell lines harboring the BRAFV600E mutation. It was previously reported that vemurafenib had limited efficacy against HT-29 xenograft and combination with EGFR inhibitor improved its antitumor activity (22, 32). BGB-283 significantly inhibited tumor growth of HT29 xenograft (P < 0.001) at 5 mg/kg b.i.d., which was well tolerated by animals. Addition of cetuximab, an EGFR-targeting monoclonal antibody, did not further enhance the therapeutic effect of BGB-283 in this xenograft model (P > 0.05; BGB-283 + cetuximab vs. BGB-283; Fig. 4A), suggesting that BGB-283 alone might be sufficient in blocking the feedback activation of EGFR. Against Colo205 xenograft, BGB-283 produced dose-dependent tumor inhibition from 3 to 30 mg/kg (Fig. 4B). More significantly, partial regression was observed at 10 mg/kg (1/7 mice). At 30 mg/kg BGB-283, regressions were observed in 3 of 7 mice (2 PR and 1 CR; Supplementary Table S6).

The tumor inhibitory activity of BGB-283 was further evaluated in human tumor tissue derived primary colorectal xenograft models. A total of 23 patient-derived colorectal cancer models were established in vivo and two of them, BCCO-002 and BCCO-028, were identified to harbor BRAFV600E mutation. Both of these patient-derived colorectal cancer models were sensitive to treatment with BGB-283 (Fig. 4C and D); >100% TGI was observed on day 24 following oral treatment with BGB-283 (10 mg/kg, b.i.d.; Fig. 4C and D and Supplementary Table S6). For BCCO-002, partial regressions were observed in 2 of 8 (25%) mice treated with BGB-283 (10 mg/kg b.i.d.). Addition of cetuximab did not further enhance the antitumor activity of BGB-283 (P > 0.05, BGB-283 + cetuximab vs. BGB-283) against BCCO-002, which is consistent with the results observed in the HT29 xenograft model (Fig. 4A and C). BCCO-028 appeared to be more sensitive to treatment with BGB-283; partial regressions were observed in 3 of 8 (38%) mice treated with BGB-283 (5 mg/kg b.i.d.). Increasing the BGB-283 to 10 mg/kg, resulted in regressions in 7 of 8 (88%) mice (5 partial and 2 complete regressions). In contrast, dabrafenib (50 mg/kg b.i.d.) treatment was less effective against BCCO-028 with an observed 86% TGI, and no tumor regression (Fig. 4D Supplementary Table S6). It should be noted that for dabrafenib at 50 mg/kg b.i.d., its exposure in mouse is already 2- to 3-fold higher than the exposure it has achieved in human tumors. Despite the remarkable responses to vemurafenib and dabrafenib in melanoma, the clinical response of other BRAFV600E cancers to the first generation of BRAF inhibitors is much less impressive (7, 17, 20, 21). The reported response of BRAFV600E colorectal cancer to vemurafenib is merely 5% (21). Two independent studies suggested that EGFR feedback activation could be one of the main mechanisms of the observed resistance to first-generation BRAF inhibitors. This article demonstrates that the MAPK pathway is already 2- to 3-fold higher than the exposure it has achieved in mouse, and its close analogue PLX-4720 were inactive as single agent in WiDr xenografts, and their antitumor activities were markedly enhanced when combined with erlotinib or cetuximab (22, 23). In contrast, BGB-283 induced clear dose-dependent inhibition of tumor growth in the WiDr xenograft model as a single agent. In this study, BGB-283 was orally administrated to testing mice at 5 and 10 mg/kg twice daily (Fig. 4E). Ninety-five percent TGI was observed at lowest dosage of 5 mg/kg and >100% TGI plus partial regression in 4 of 8 (50%) mice was achieved at dosage of 10 mg/kg (Supplementary Table S6).

In order to determine whether the tumor suppression was correlated to effective inhibition of EGFR and MAPK signaling, phospho-EGFR (pEGFR), phospho-MEK (pMEK), and phospho-ERK (pERK) and its downstream DUSP6 levels in tumor lysate were examined by Western blot analysis at various dose levels of BGB-283. BGB-283 did not induce EGFR feedback activation as reported for vemurafenib. In addition, BGB-283 potently inhibited pEGFR after either the first or the fifth dose at both dosages. Correspondingly, BGB-283 potently inhibited MEK and ERK phosphorylation and DUSP6 expression in vivo when dosed repeatedly (Fig. 4F). There is no detectable difference on AKT phosphorylation. In sum, these findings showed that BGB-283, which inhibits both RAF family kinases and EGFR, could have sustained inhibition of the MAPK pathway. Its ability to inhibit EGFR may contribute to its potent antitumor activity in this WiDr xenograft model.

Discussion

In this article, we describe the activity of BGB-283, a second-generation BRAF inhibitor, with potential for the treatment of cancers with aberrations in the MAPK pathway. BGB-283 showed potent and reversible inhibitory activities against RAF family kinases, including wild-type A-RAF, BRAF, C-RAF, and BRAFV600E. In addition, BGB-283 also potently inhibited EGFR at both the biochemical and cellular level. BGB-283 demonstrated remarkable selectivity in a panel of 107 cancer cell lines for antiproliferative activity. BGB-283 potently inhibited the serum-induced cell proliferation of BRAFV600E-mutant cancer cell lines, with IC50 values ranging from 137 nmol/L to 580 nmol/L. It showed little or no inhibitory activity in cell lines lacking BRAFV600E mutation, with the exception of the HCC827 lung cancer cell line (EGFR E746-A750 deletion), ZR-75-30 (HER2 amplification), and the NCI-H322M lung cancer cell line (EGFR overexpression). These results suggested that RAF kinase and EGFR-inhibitory activities of BGB-283 contributed the most to its antiproliferative activities in the tested cancer cells. Despite the different kinase selectivity profile between BGB-283 and vemurafenib, both agents displayed noticeable selectivity toward cancer cells harboring BRAFV600E in a cell viability assay (Fig. 3A and B). Despite the remarkable responses to vemurafenib and dabrafenib in melanoma, the clinical response of other BRAFV600E cancers to the first generation of BRAF inhibitors is much less impressive (7, 17, 20, 21). The reported response of BRAFV600E colorectal cancer to vemurafenib is merely 5% (21). Two independent studies suggested that EGFR feedback activation could be one of the main mechanisms of the observed resistance to first-generation BRAF inhibitors. This article demonstrates that BGB-283 is a bona fide EGFR inhibitor and displays good EGFR inhibitory activity in vitro and in vivo experiments. In WiDr colorectal cancer cells, BGB-283 was shown to be able to inhibit the feedback activation of EGFR signaling and achieves sustained inhibition of pERK. This sustained inhibition of pERK translates into remarkable antitumor activity in vivo. Notably, BGB-283 single-agent treatment at 10 mg/kg b.i.d. led to 50% partial regression in WiDr colorectal adenocarcinoma xenografts. In comparison, both PLX4720+cetuximab and
Vemurafenib + erlotinib combinations seemed to have achieved mostly TGI but not tumor regression in WiDr xenograft models (22, 23). BRAF<sup>V600E</sup> mutation is reported to occur in 5% to 15% of colorectal cancer patients. Among the 23 colorectal cancer primary tumor xenograft models established in this study, two of them were found to have the BRAF<sup>V600E</sup> mutation. BGB-283 demonstrated good efficacy in both models with the objective response rate ranging from 25% to 100%. We are carrying out more comprehensive characterizations of these models and trying to better understand their biology.
to better understand the MAPK and EGFR pathways in these two primary tumor xenograft models. Currently, phase I clinical trials are in progress to test the safety, tolerability, pharmacokinetics, and pharmacodynamic activity of BGB-283 in human. To our knowledge, BGB-283 is the only small-molecule inhibitor in the clinic that simultaneously targets RAF kinases and EGFR. There have been strong interests from the community to test the hypothesis that EGFR feedback activation leads to lack of responses in colorectal cancer for BRAF^{V600E}-selective inhibitors. A number of clinical trials that combines BRAF inhibitors with EGFR small-molecule inhibitors or monoclonal antibodies are currently under way (see www.clinicaltrials.gov). The preclinical results reported in this study warrant evaluation of BGB-283 as a single agent in BRAF^{V600E}-mutated colorectal cancer patients.

Disclosure of Potential Conflicts of Interest

G. Zhang and D. Sutton have ownership interest in BeiGene (Beijing) Co., Ltd. C. Zhou reports receiving commercial research grant from and has ownership interest in BeiGene (Beijing) Co., Ltd. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions


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Authors’ Contributions


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References


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