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

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Abstract

Oncogenic B-RAF, which drives cell transformation and proliferation, has been detected in approximate 50% of human malignant melanomas and 5-15% of colorectal cancers (CRC). Despite the remarkable clinical activities achieved by vemurafenib and dabrafenib in treating B-RAF\textsuperscript{V600E} metastatic melanoma, their clinical efficacy in B-RAF\textsuperscript{V600E} CRC is far less impressive. Prior studies suggested that feedback activation of EGFR and MAPK signalling upon B-RAF inhibition might contribute to the relative unresponsiveness of CRC to the first generation B-RAF 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 B-RAF\textsuperscript{V600E}-activated ERK phosphorylation and cell proliferation. It demonstrates selective cytotoxicity and preferentially inhibits proliferation of cancer cells harbouring B-RAF\textsuperscript{V600E} and EGFR mutation/amplification. In B-RAF\textsuperscript{V600E} CRC 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 B-RAF\textsuperscript{V600E} mutation. These findings support BGB-283 as a potent antitumor drug candidate with clinical potential for treating CRC harbouring B-RAF\textsuperscript{V600E} mutation.
Introduction

The mitogen-activated protein kinase (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). B-RAF, 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 \textit{B-RAF} gene in ~50% of human malignant melanomas and 15% of colorectal cancers (CRC) (6-11), with the V600E mutation accounting for at least 90% of oncogenic B-RAF mutations (6). This V600E mutation introduces a negative charge in the B-RAF kinase domain that mimics and bypasses the phosphorylation required for B-RAF activation, which is normally achieved through growth factor activated receptor tyrosine kinases. As a result, this “gain of function” of B-RAF gives rise to a constitutive MAPK signalling that promotes tumor progression, in which B-RAF$^{V600E}$ activates MEK1/2 in a RAS-independent manner (12-15).

Small molecules that selectively target mutant B-RAF exhibit good efficacy and yield impressive clinical responses in melanoma patients with the B-RAF$^{V600E}$ mutation. The RAF inhibitors that selectively inhibit BRAF$^{V600E}$ 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 B-RAF inhibitors in benefiting melanoma patients confirms B-RAF\textsuperscript{V600E} as a \textit{bona fide} oncogenic target and validates the utility of cancer therapies that target B-RAF and MAPK signalling (4, 5). However, these first generation B-RAF inhibitors still have limitations including development of cutaneous squamous cell carcinomas (cSCCs) and treatment-related keratoacanthomas (KAs) due to paradoxical activation of MAPK signalling. Additionally, they have inadequate clinical activity outside of melanoma with B-RAF\textsuperscript{V600E} mutation (16, 18-20). In particular, the clinical response among CRC patients with B-RAF\textsuperscript{V600E} mutations is much lower than that observed in melanoma patients. For example, vemurafenib was reported to have a mere 5% objective response rates in CRC patients with B-RAF\textsuperscript{V600E} 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 B-RAF\textsuperscript{V600E} melanoma and CRC to the first generation B-RAF inhibitors (22, 23). Combination of B-RAF and EGFR inhibitors was found to significantly enhance the efficacy in preclinical models (22, 23). Therefore, a second generation B-RAF inhibitor that can inhibit both BRAF V600E and EGFR driven RAF activation may have a therapeutic advantage in treating B-RAF mutated CRC.

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 cell lines harbouring B-RAF^{V600E} or EGFR mutations. BGB-283 is highly efficacious in B-RAF^{V600E} CRC xenograft models including HT29, Colo205, and two primary tumor xenografts harbouring BRAF^{V600E} mutation. In addition, BGB-283 shows compelling efficacy in WiDr xenograft model where EGFR reactivation was shown to be induced upon B-RAF inhibition (22, 23). Together, these data suggest that BGB-283 is a novel RAF kinase and EGFR inhibitor with therapeutic potential in targeting oncogenic B-RAF 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 (HNMR), liquid chromatography-mass spectrometry (LC-MS) and high performance liquid chromatography (HPLC). Reference compounds were purchased from the following sources: PLX4032 and dabrafenib, WuXi AppTec; Gefitinib, MedChem; Erlotinib, Beijing Ouhe Technology Co. Ltd.; Cetuximab (Merck KGaA, Germany), Beijing Cancer Hospital. Stock solutions of compounds were prepared in dimethyl sulfoxide.

B-RAF\textsuperscript{V600E} kinase domain/BGB-283 co-crystallization and structure determination

B-RAF\textsuperscript{V600E} (444-723) was expressed and purified using methods similar to those previously reported (22). To co-crystallize B-RAF\textsuperscript{V600E} with BGB-283, protein solution was incubated with BGB-283 at a ratio of 1:5 for 1 h, and mixed with reservoir solution (100 mM Bis Tris at pH 6.5, 23% PEG3350, and 200 mM MgCl\textsubscript{2}) at equal volume. Co-crystals grew by sitting drop vapour diffusion method at 4 °C. Diffraction data was collected from Synchrotron Radiation beam line (Shanghai Synchrotron Radiation Facility). Crystals belonged to the space group P212121 (a =49.395 Å, b=101.601 Å, c =109.786 Å) and contain two B-RAF\textsuperscript{V600E} molecule 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 (Table S1). Complex structure of B-RAF$_{\text{V600E}}$ kinase domain with BGB-283 was submitted to Protein Data Bank with a 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-resonance 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 60-120 minutes at room temperature (RT), ATP (final concentration at 100 μM) and kinase substrates were added to initiate the reaction. The reaction was stopped by an equal volume of stop/detection solution according to the manufacture’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 PHERArastar FS plate reader (BMG Labtech).

BGB-283 was screened for activity in a panel of 277 kinases at a fixed concentration of 10 μM by Life Technologies using their standard assays at Km concentration of ATP for perspective kinases. The IC$_{50}$ was then determined for kinases showing >80% inhibition at 10 μM BGB-283.
Cell culture

A375, Sk-Mel-28, HT29, Colo205, WiDr, Ba/F3, A431, HCC827, SW620, HCT116, and cell lines used in cell panel profiling were purchased from American Type Culture Collection (ATCC). Cell lines were tested and authenticated at ATCC prior to purchase using morphology, karyotyping, and PCR based approaches. All the cell lines were cultured in the designated medium supplemented with 10% fetal bovine serum (FBS, Thermo Scientific), 100 units/mL penicillin (Gibco), 0.1 mg/mL streptomycin (Gibco) and in a humidified 37°C environment with 5% CO₂. Cell lines were reinstated from frozen stocks laid down within three passages from the original cells purchased and passaged 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 min incubation. EGF stimulated cell growth was achieved by addition of EGF to DMEM or RPMI supplemented with 10% FBS.

Western blotting analysis

For in vitro studies, cells were harvested after 1 hour treatment at 37°C and lysed immediately as previously described (24). For in vivo 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 min 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 4-12% Bis-Tris protein gels (Life Technologies) and transferred to nitrocellulose membranes using iBlot™ Dry Blotting System (Life Technologies). Blots were blocked with TBSTM (50 mM Tris (pH 7.5), 150 mM NaCl, 0.1% Tween 20, and 5% non-fat milk) at RT for 1 h and probed with indicated antibodies diluted in TBSTM. The membranes were probed for phospho-proteins then stripped to probe for total proteins. For re-probing, the membranes were stripped in stripping buffer (25 mM glycine, pH 2.0, 1% SDS) for 30-60 min at RT, rinsed twice with TBST for 10 min 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-B-RAF (SC-5248), Santa Cruz Biotechnology; anti-C-RAF (610152), BD Biosciences; antibodies to MEK (9122, 9126), phospho-MEK1/2 (Ser217/221) (9154), ERK (4695), phospho-ERK1/2 (Thr202/Tyr204) (4370), EGFR (2646s), phospho-AKT (Ser473) (4060), AKT (4685), c-Myc (3058), DUSP6 (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 $3 \times 10^4$ per well of a 96-well plate and left to attach for 16 hours. Growth medium was then replaced with 100 μL of DMEM containing no serum. Cells were then treated with a 10-point titration of compound. After 1 hour of compound treatment, 50 μL of lysis buffer (Cisbio) were added to each well. Plates were then incubated at room temperature with shaking for 30 minutes. A total of 16 μL 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 μL of Eu$^{3+}$- or Tb$^{3+}$- cryptate (donor) labeled anti-ERK or anti-EGFR antibody (Cisbio) and 2 μL 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 (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 lysing, 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). EC₅₀ 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., P.R.China. 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 2.5-5×10⁶ cells in 200 μl PBS in the right front flank via a 26-gauge needle. When the average tumor size reached 110~200 mm³, animals were randomized to treatment groups (7-9 mice per group) and treated twice per day (BID) or once daily (QD) by oral gavage (p.o.) with vehicle alone or 2.5-30 mg/kg of BGB-283. As control, mice were treated with erlotinib (100 mg/kg QD), 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 prior to dosing.
For BCCO-002 and BCCO-028 primary human tumor xenografts (PDX), colorectal cancer samples were collected from Beijing Cancer Hospital after patient's informed consent and immediately transferred in DMEM culture medium contained 200 U/mL penicillin and 200 mg/mL streptomycin. Within 2-4 h of surgery, small fragments (3 mm×3 mm×3 mm) were subcutaneously engrafted into the scapular area or flank of anesthetized NOD/SCID mice. After 3 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 100-200 mm³, 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, BID) or dabrafenib (50 mg/kg, BID). A further group received intravenous cetuximab (40mg/kg every three 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 End Points**

Tumor volumes were calculated using the formula: \( V = 0.5 \times (a \times b^2) \), 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 mm³ for at least three consecutive measurements. Tumor growth inhibition (TGI) was calculated using the following formula: % growth inhibition = $100 \times \frac{1 - (\text{treated } t - \text{treated } t_0)}{(\text{placebo } t - \text{placebo } t_0)}$, in which treated $t$ represents tumor volume at day $t$ in treated group, treated $t_0$ represents tumor volume of the same treated group on the first day of treatment, placebo $t$ represents placebo tumor volume day $t$ in control group, placebo $t_0$ 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

$$5-\left((1R,1aS,6bR)-1-(6-\text{(trifluoromethyl)}-\text{1H-benzo[d]imidazol-2-yl})-1a,6b\text{-dihydro-1H-cyclopropa[b]benzofuran-5-yl})\text{oxy})-3,4\text{-dihydro-1,8-naphthyridin-2(1H)-one}\right)$$

(Figure 1A), is a synthetic inhibitor designed to interact with the ATP-binding site of the $\text{B-RAF}^{\text{V600E}}$ (Figure 1B). The crystal structure of BGB-283/ $\text{B-RAF}^{\text{V600E}}$ kinase domain complex revealed that BGB-283 binds to the ATP-binding pocket of $\text{B-RAF}^{\text{V600E}}$ in an inactive conformation (Figure 1B, Table S1). BGB-283 binds to both protomers of $\text{B-RAF}^{\text{V600E}}$ with a DFG-out and $\alpha$-C helix-in conformation. In comparison, PLX4032 binds to only one of the protomers in DFG-out and $\alpha$-C helix-in conformation in its co-crystal structure with $\text{B-RAF}^{\text{V600E}}$ (PDB code: 3OG7) (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 enzimidazole and trifluoro groups locate in the pocket formed by $\alpha$-C helix and activation loop (Figure 1B). There are two hydrogen bonds formed between BGB-283 and the $\text{B-RAF}$ protein. One is between hydroxyl group of E501 on the $\alpha$-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 hinge region of $\text{B-RAF}^{\text{V600E}}$. 
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 B-RAF<sup>V600E</sup>, WT B-RAF, C-RAF Y340/341D, and WT A-RAF <em>in vitro</em> (Table 1A). In particular, BGB-283 potently inhibited the activity of the recombinant B-RAF<sup>V600E</sup> kinase domain with an IC<sub>50</sub> value of 23 ± 5 nM (Table 1A). The inhibition by BGB-283 was time-dependent and with a slow off-rate. The estimated t<sub>1/2</sub> of dissociation was measured to be much longer than 240 min (Figure S1A). BGB-283 was further shown to be a reversible inhibitor of the B-RAF kinase (Table S3). The selectivity of BGB-283 was evaluated by measuring the percentage of inhibition against 279 kinases at 10 μM, a concentration that is approximately 450 times higher than its IC<sub>50</sub> value against purified kinase domain of B-RAF<sup>V600E</sup>. Of the 279 kinases tested, 43 of them showed > 80% of inhibition by 10 μM BGB-283 (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 (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 IC<sub>50</sub>s of 29 ± 18 nM, and EGFR T790M/L858R mutant with IC<sub>50</sub>s of 495 ± 124 nM (Table 1A).
BGB-283 potently inhibits B-RAF\textsuperscript{V600E}-activated ERK phosphorylation and cell proliferation

The effect of BGB-283 on oncogenic B-RAF-mediated ERK phosphorylation was investigated in A375, a B-RAF\textsuperscript{V600E}-driven melanoma cell line. Consistent with data from biochemical characterization, BGB-283 potently inhibited ERK phosphorylation (Figure 2A). The potency of BGB-283 in-inhibiting B-RAF\textsuperscript{V600E}-driven ERK phosphorylation was further confirmed by a quantitative TR-FRET assay in four different cell lines harbouring B-RAF\textsuperscript{V600E}, with IC\textsubscript{50} values ranging from 32 nM to 153 nM (Table 1B). Previously, it was reported that PLX4032 treatment of SW620 and HCT116 cell lines expressing B-RAF\textsuperscript{WT} 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 (Figure 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 EGFR E746-A750 deletion. BGB-283 inhibited the EGF induced EGFR autophosphorylation on Tyr1068 in A431 cells in a dose-dependent manner (Figure 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 (Figure 2C and S1B). It has been reported that B-RAF inhibition could induce feedback activation of EGFR thus contributing to the insensitivity of B-RAF\textsuperscript{V600E} colon cancer cells to PLX4032 (22, 23). To evaluate the feedback activation of EGFR upon B-RAF inhibition with BGB-283 or PLX4032 treatment, two B-RAF\textsuperscript{V600E} colon cancer cell lines (HT-29 and WiDr) were treated by the compounds and measured for EGFR and ERK phosphorylation levels. PLX4032 treatment resulted in an up-regulation 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 signalling as represented by c-Myc expression in both cell lines after 24 h treatment. Addition of an EGFR inhibitor, erlotinib, abrogated the EGFR reactivation induced by PLX4032 and led to better inhibition of MAPK signalling after 24 h. In comparison, BGB-283 alone reduced EGFR phosphorylation, and inhibited ERK phosphorylation and c-Myc expression more effectively than PLX4032 (Figure 2D). These findings suggest that the anti-EGFR activity of BGB-283 could suppress the feedback activation of EGFR signalling upon BRAF inhibition (Figure 2D).

Ba/F3 cells normally grow in an IL-3-dependent manner (29), but their growth can be rendered IL-3 independent by over-expression of EGFR Exon19 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 Exon19 deletion or EGFR L858R mutants,
suggesting that BGB-283 could inhibit EGFR-driven cell proliferation (Table 2A, Figure S1C). Interestingly, BGB-283 also showed moderate inhibitory effect on EGFR Exon19δ/T790M mutant that is insensitive to gefitinib (Table 2A, Figure S1C). This result is consistent with the observation from biochemical studies where BGB-283 showed inhibition to T790M containing EGFR mutant. There were no significant difference in sensitivity to BRAF inhibitors between B-RAFV600E melanoma cells (A375) and B-RAFV600E colon cancer cells (HT29 and WiDr) in both short term and long term proliferation assay (Table 2B). However, in the presence of the EGFR ligand EGF, both HT29 and WiDr became insensitive to PLX4032 at concentration up to 10 μM. 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 EGF induced cell proliferation in B-RAFV600E 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 mg/kg and 30 mg/kg BID or QD effectively inhibited tumor growth (Figure 2E and 2F). 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 tumor growth inhibition effect at 100 mg/kg QD in HCC827 xenograft model (Figure 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 B-RAF\textsuperscript{V600E} or harbouring 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 three days. The mutational status of B-RAF, H-RAS, K-RAS and N-RAS was noted for each cell line (Table S2, Figure 3). BGB-283 selectively inhibited the growth of cancer cell lines expressing B-RAF\textsuperscript{V600E} but not B-RAF\textsuperscript{WT}, similar to PLX4032 (Figure 3A and 3B). The EC\textsubscript{50} values in the majority of nonresponsive cell lines were >10 μM (Figure 3A and Table S2). In addition, BGB-283 was found to inhibit proliferation of the HCC827 lung cancer cell line with EGFR Exon19 deletion, ZR-75-30 breast cancer cell line with Her2 amplification, and NCI-H322M lung cancer cell line with EGFR overexpression (Figure 3A).

BGB-283 exhibits anti-tumor activity in mouse xenograft models of CRC

The \textit{in vivo} efficacy of BGB-283 was assessed in subcutaneous xenograft models derived from HT29 and Colo205 CRC cell lines harbouring the B-RAF\textsuperscript{V600E} mutation. It was previously reported that vemurafenib had limited efficacy against HT-29 xenograft and combination with EGFR inhibitor improved its anti-tumor activity (22, 32). BGB-283 significantly inhibited tumor growth of HT29 xenograft (P<0.001) at 5 mg/kg BID, 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 \((P>0.05, \text{BGB-283} + \text{cetuximab vs. BGB-283})\) (Figure 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 (Figure 4B). More significantly, partial regression was observed at 10 mg/kg (1/7 mice). At 30 mg/kg BGB-283, regressions were observed in 3/7 mice (2 PR and 1 CR) (Table S6).

The tumor inhibitory activity of BGB-283 was further evaluated in human tumor tissue derived primary CRC xenograft models. A total of twenty-three patient-derived CRC models were established \textit{in vivo} and two of them, BCCO-002 and BCCO-028, were identified to harbour B-RAF\textsuperscript{V600E} mutation. Both of these patient-derived CRC models were sensitive to treatment with BGB-283 (Figures 4C and 4D); >100\% TGI was observed on day 24 following oral treatment with BGB-283 (10 mg/kg, BID) (Figure 4C and 4D, Table S6). For BCCO-002, partial regressions were observed in 2/8 (25\%) mice treated with BGB-283 (10 mg/kg BID). Addition of cetuximab did not further enhance the anti-tumor activity of BGB-283 \((P>0.05, \text{BGB-283} + \text{cetuximab vs. BGB-283})\) against BCCO-002, which is consistent with the results observed in the HT29 xenograft model (Figure 4A and 4C). BCCO-028 appeared to be more sensitive to treatment with BGB-283; partial regressions were observed in 3/8 (38\%) mice treated with BGB-283 (5 mg/kg BID). Increasing the BGB-283 to 10 mg/kg, resulted in regressions in 7/8 (88\%) mice (5 partial and 2 complete regressions). In contrast, dabrafenib (50 mg/kg BID)
treatment was less effective against BCCO-028 with an observed 86% TGI, and no tumor regression (Figure 4D, Table S6). It should be noted that for dabrafenib at 50 mg/kg BID, its exposure in mouse is already 2-3 fold higher than the exposure it has achieved in patients at 150 mg BID dosing. Treatment with BGB-283 at doses up to 30 mg/kg had no significant effect on body weight in any of the tumor models tested (Figure S2).

**BGB-283 inhibits phosphorylation of both ERK1/2 and EGFR and displays potent anti-tumor activity in WiDr tumor xenografts**

BGB-283 was further evaluated against WiDr tumor xenografts, a B-RAF<sup>V600E</sup> CRC model where strong feedback activation of EGFR was reported upon B-RAF inhibition in two independent studies (23). In both reports, it was shown that B-RAF<sup>V600E</sup>-selective inhibitors vemurafemib and its close analog PLX-4720 were inactive as single agent in WiDr xenografts, and their anti-tumor 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 WiDr xenograft model as single agent. In this study, BGB-283 was orally administrated to testing mice at 5 and 10 mg/kg twice daily (Figure 4E). 95% TGI was observed at lowest dosage of 5 mg/kg and >100% TGI plus partial regression in 4/8 (50%) mice was achieved at dosage of 10 mg/kg (Table S6). In order to determine whether the tumor suppression was correlated to effective inhibition of EGFR and MAPK signalling, phospho-EGFR (pEGFR), phospho-MEK (pMEK), and phospho-ERK (pERK) and its downstream DUSP6 levels in tumor
lysate were examined by western blot 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 (Figure 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 MAPK pathway. Its ability to inhibit EGFR may contribute to its potent anti-tumor activity in this WiDr xenograft model.
Discussion

In this report, we describe the activity of BGB-283, a second generation B-RAF 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, B-RAF, C-RAF and B-RAF$^{V600E}$. 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 anti-proliferation activity. BGB-283 potently inhibited the serum induced cell proliferation of B-RAF$^{V600E}$ mutant cancer cell lines, with IC$_{50}$s ranging from 137 nM to 580 nM. It showed little or no inhibitory activity in cell lines lack of B-RAF$^{V600E}$ mutation, with the exception for HCC827 lung cancer cell line (EGFR E746-A750 deletion), ZR-75-30 (Her2 amplification), and 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 anti-proliferative activities in the tested cancer cells. Despite the different kinase selectivity profile between BGB-283 and vemurafenib, both agents displayed noticeable selectivity towards cancer cells harbouring B-RAF$^{V600E}$ in cell viability assay (Figure 3A and B).

In spite of the remarkable responses to vemurafenib and dabrafenib in melanoma, the clinical response of other B-RAF$^{V600E}$ cancers to the first generation of B-RAF inhibitors is much less impressive (7, 17, 20, 21). The reported response of B-RAF$^{V600E}$ CRC 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 B-RAF inhibitors. This report demonstrates that BGB-283 is a *bona fide* EGFR inhibitor and displays good EGFR inhibitory activity in *in vitro* and *in vivo* experiments. In WiDr CRC cells, BGB-283 was shown to be able to inhibit the feedback activation of EGFR signalling and achieves sustained inhibition of pERK. This sustained inhibition of pERK translates into remarkable anti-tumor activity *in vivo*. Notably, BGB-283 single agent treatment at 10 mg/kg BID led to 50% partial regression in WiDr colorectal adenocarcinoma xenografts. In comparison, both PLX4720+cetuximab and vemurafenib+erlotinib combinations seemed to have achieved mostly tumor growth inhibition but not tumor regression in WiDr xenograft models (22, 23).

B-RAF^{V600E} mutation is reported to occur in 5-15% of CRC patients. Among the 23 CRC primary tumor xenograft models established in this study, two of them were found to have the B-RAF^{V600E} 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 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
CRC for B-RAF$^{V600E}$-selective inhibitors. A number of clinical trials that combines B-RAF inhibitors with EGFR small molecule inhibitors or monoclonal antibodies are currently on-going (see www.clinicaltrials.gov). The preclinical results reported in this study warrant evaluation of BGB-283 as a single agent in B-RAF$^{V600E}$ mutated CRC patients.
Acknowledgments

We are grateful to Dr. Zhan Yao and Dr. Neal Rosen for critical review of the manuscript. The grant supports for this study are Beijing Municipal Science and Technology Commission and National Science and Technology Major Project (No. 2013ZX09102005).
Reference


Tables

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 (nM) (mean ± SD)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-RAFV₆₀₀E kinase domain (aa416-766)</td>
<td>23 ± 5</td>
<td>7</td>
</tr>
<tr>
<td>WT B-RAF 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 T790M/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. BGB-283 potently inhibited BRAFV₆₀₀E–driven ERK phosphorylation in A375, SK-Mel-28, HT29, and Colo205 cells. BGB-283 also inhibited EGFR autophosphorylation in A431 and HCC827 cells, where EGFR is activated by overexpression or E746-A750 deletion.

<table>
<thead>
<tr>
<th>Phosphorylated kinase</th>
<th>Cell lines</th>
<th>BGB-283 IC₅₀ (nM) (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>pEGFR</td>
<td>A431</td>
<td>385 ± 40</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>HCC827</td>
<td>195 ± 102</td>
<td>2</td>
</tr>
</tbody>
</table>
Table 2. Inhibition of EGFR driven cell proliferation by BGB-283.

(A) BGB-283 inhibited EGFR-driven cell growth in Ba/F3 cells expressing different EGFR mutants.

<table>
<thead>
<tr>
<th></th>
<th>BGB-283 Mean EC&lt;sub&gt;50&lt;/sub&gt; ± SD (nM)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ba/F3</td>
<td>&gt;10000</td>
<td>1</td>
</tr>
<tr>
<td>Ba/F3 EGFR Exon19δ</td>
<td>188 ± 152</td>
<td>2</td>
</tr>
<tr>
<td>Ba/F3 EGFR L858R</td>
<td>224 ± 136</td>
<td>2</td>
</tr>
<tr>
<td>Ba/F3 EGFR Exon19δ/T790M</td>
<td>613 ± 40</td>
<td>2</td>
</tr>
</tbody>
</table>

(B) BGB-283 inhibited EGF-induced cell proliferation in B-RAF<sup>V600E</sup> colon cancer cell lines.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>PLX4032 Mean EC&lt;sub&gt;50&lt;/sub&gt; ± SD (nM)</th>
<th>BGB-283 Mean EC&lt;sub&gt;50&lt;/sub&gt; ± SD (nM)</th>
<th>PLX4032 Mean EC&lt;sub&gt;50&lt;/sub&gt; ± SD (nM)</th>
<th>BGB-283 Mean EC&lt;sub&gt;50&lt;/sub&gt; ± SD (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No additional EGF</td>
<td>Plus 2.5 ng/mL EGF</td>
<td>No additional EGF</td>
<td>Plus 2.5 ng/mL EGF</td>
</tr>
<tr>
<td>A375</td>
<td>191 ± 16 (n=7)</td>
<td>200 ± 79 (n=5)</td>
<td>129 ± 61 (n=3)</td>
<td>150 ± 97 (n=3)</td>
</tr>
<tr>
<td>HT29</td>
<td>293 ± 103 (n=5)</td>
<td>270 ± 66 (n=5)</td>
<td>&gt;10,000 (n=2)</td>
<td>1,830 ± 927 (n=2)</td>
</tr>
<tr>
<td>WiDr</td>
<td>367 ± 132 (n=4)</td>
<td>305 ± 132 (n=4)</td>
<td>~10,000 (n=2)</td>
<td>1,890 ± 668 (n=2)</td>
</tr>
</tbody>
</table>
**Figure Legends**

**Figure 1. BGB-283: a compound designed for inhibiting oncogenic B-RAF.**

(A) Chemical structure of BGB-283. (B) The crystal structure of BGB-283 bound to B-RAF<sup>V600E</sup>. Dashed lines were hydrogen bonds.

**Figure 2. BGB-283 potently inhibited ERK phosphorylation and EGFR activity.**

(A) Immunoblots for B-RAF, phospho-ERK1/2, ERK1/2 and GAPDH in A375 cell lysates prepared 1 h after treatment with indicated concentrations of PLX4032 or BGB-283. (B) Immunoblots for B-RAF, phospho-ERK1/2, ERK1/2 and GAPDH in HCT116 and SW620 cell lysates prepared 1 h after being treated with indicated concentrations of PLX4032 and BGB-283. (C) Immunoblots for EGFR, phospho-Tyr1068-EGFR, phospho-ERK1/2, ERK1/2, and GAPDH in A431 (EGFR overexpression) cell lysates prepared 2 h after 20 ng/mL EGF stimulation and treatment with indicated concentrations of PLX4032, BGB-283, or Gefitinib. (D) Immunoblots 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 h after treatment with indicated compound diluted in RMPI-1640 with 5% FBS. (E) HCC827 (EGFR E746-A750 deletion) tumor cells (5x10<sup>6</sup>) or (F) A431 (EGFR overexpression) tumor cells (5x10<sup>6</sup>) were implanted subcutaneously in female BALB/c nude mice. When the tumors reached a mean volume of approximately 100-180 mm<sup>3</sup> in size, mice were randomly allocated into groups and treated as
indicated. Data are presented as average tumor volume ± standard error of the mean (SEM) of 7 animals in each group.

Figure 3. BGB-283 selectively inhibited proliferation of cancer cells harbouring B-RAF^{V600E} and EGFR mutations. Antiproliferative effect of (A) BGB-283 and (B) PLX4032 following a 3-d exposure across a panel of human cancer cell lines determined by cell titer-glo assay.

Figure 4. BGB-283 inhibited tumor growth in both cell-line derived and primary human colorectal cancer xenografts models harbouring B-RAF^{V600E} mutation. (A) HT29 tumor cells (3x10^6), (B) Colo205 tumor cells (2.5x10^6), or (E) WiDr tumor cells (5x10^6) were implanted subcutaneously in female BALB/c nude or NOD/SCID mice. When the tumors reached certain volume in size, mice were randomly allocated and treated as indicated. Data are presented as average tumor volume ± SEM in each group. (C) Primary colon cancer BCCO-002 or (D) BCCO-028 tumor fragments (P6; 3mm×3mm×3mm) were implanted subcutaneously in female BALB/c nude mice. When the tumors reached to certain volume in size, mice were randomly allocated and treated as indicated. Data are presented as average tumor volume ± SEM in each group. (F) Immunoblots for EGFR, phospho-EGFR, pAKT, AKT, pMEK, MEK, ERK1/2, phospho-ERK1/2, DUSP6 and GAPDH in WiDr xenograft tumor lysates. Tumor lysates were prepared 4 hours after the first dose the fifth dose on day 3 (BID ×3) with indicated
concentrations of BGB-283. Lysates from two representative mice each group were equally
mixed based on total protein concentrations and loaded for SDS-PAGE.
Figure 2
Figure 3
Molecular Cancer Therapeutics

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

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