Precision Targeted Therapy with BLU-667 for RET-Driven Cancers

Vivek Subbiah¹, Justin F. Gainor², Rami Rahal³, Jason D. Brubaker³, Joseph L. Kim³, Michelle Maynard³, Wei Hu³, Qiongfang Cao³, Michael P. Sheets³, Douglas Wilson³, Kevin J. Wilson³, Lucian DiPietro³, Paul Fleming³, Michael Palmer³, Mimi I. Hu⁴, Lori Wirth², Marcia S. Brose⁵, Sai-Hong Ignatius Ou⁶, Matthew Taylor⁷, Elena Garralda⁸, Stephen Miller³, Beni Wolf³, Christoph Lengauer³, Timothy Guzi³, and Erica K. Evans³
The receptor tyrosine kinase rearranged during transfection (RET) is an onco-
genic driver activated in multiple cancers, including non–small cell lung cancer
(NSCLC), medullary thyroid cancer (MTC), and papillary thyroid cancer. No approved therapies have
been designed to target RET; treatment has been limited to multikinase inhibitors (MKI), which can
have significant off-target toxicities and limited efficacy. BLU-667 is a highly potent and selective RET
inhibitor designed to overcome these limitations. In vitro, BLU-667 demonstrated ≥10-fold increased
potency over approved MKIs against oncogenic RET variants and resistance mutants. In vivo, BLU-667
potently inhibited growth of NSCLC and thyroid cancer xenografts driven by various RET mutations
and fusions without inhibiting VEGFR2. In first-in-human testing, BLU-667 significantly inhibited RET
signaling and induced durable clinical responses in patients with RET-altered NSCLC and MTC without
notable off-target toxicity, providing clinical validation for selective RET targeting.

SIGNIFICANCE: Patients with RET-driven cancers derive limited benefit from available MKIs. BLU-
667 is a potent and selective RET inhibitor that induces tumor regression in cancer models with RET
mutations and fusions. BLU-667 attenuated RET signaling and produced durable clinical responses
in patients with RET-altered tumors, clinically validating selective RET targeting. Cancer Discov; 8(7):
836–49. ©2018 AACR.

See related commentary by Iams and Lovly, p. 797.

INTRODUCTION

Targeting oncogenic driver kinases with specifically tai-
lored inhibitors has transformed the management of a variety of
hematologic malignancies and solid tumors. First-gen-
eration kinase inhibitors directed against oncogenic driv-
ers such as BCR–ABL fusions (imatinib), EGFR mutations
(gefitinib and erlotinib), and ALK rearrangements (crizotinib)
have demonstrated vast improvements over cytotoxic che-
motherapy in chronic myelogenous leukemia and kinase-driven
non–small cell lung cancer (NSCLC), respectively (1–4). These
everly established a new treatment paradigm for the use of targeted kinase inhibitors to benefit genetically
defined patient populations. Despite the effectiveness of these approaches, however, acquired resistance to therapy
has emerged as a major clinical challenge, spurring efforts to develop more potent and selective next-generation kinase
inhibitors that also encompass activity against observed and predicted resistance mutations (5–9).

Rearranged during transfection (RET) is a receptor tyros-
ine kinase and bona fide oncogene that drives various cancers
(10–16). RET normally plays a critical role in kidney morpho-
genesis and embryonic development of the enteric nervous
system. However, oncogenic RET alterations promote ligand-
independent, constitutive RET kinase activation, which drives
tumorigenesis. To date, two major mechanisms of RET kinase
activation have been described: (i) RET point mutations and (ii)
RET gene rearrangements. RET missense mutations can occur
in extracellular cysteine residues (e.g., C620R and C634R/W),
which trigger aberrant receptor dimerization, or in the intra-
cellular kinase domain, which promotes ligand-independent
kinase activation (e.g., V804L/M, M918T; ref. 17). Activating
point mutations are most commonly found in medullary thy-
roid cancer (MTC); approximately 50% of sporadic MTCs har-
bor activating RET mutations, and nearly all cases of familial
MTC contain a germline activating RET mutation (17, 18).
Alternatively, RET activation can occur via gene rearrangement.
RET rearrangements create a fusion protein juxtaposing the
RET kinase domain and a dimerization domain of another pro-
tein, creating a constitutively activated dimer (19). RET fusions
are seen in 10% to 20% of papillary thyroid cancer (PTC), 1% to
2% of NSCLCs, and multiple other cancer subtypes, including
colorectal and breast cancers (10–18). Together, these data indi-
cate that dysregulated RET signaling can act as an important
driver across multiple tumor indications.

Although RET was one of the first kinase fusions cloned
from an epithelial tumor (20), patients with RET-driven
cancers have derived only modest benefit from RET-directed
strategies to date. It should be noted, however, that RET
therapies thus far have largely centered around multikinase
inhibitors (MKI) that have been repurposed to treat patients with RET alterations. For example, the MKIs cabozantinib and vandetanib were originally designed to target other kinases, such as VEGFR2, tyrosine–protein kinase MET, and EGFR, and they inhibit these targets more potently than RET (21, 22). Cabozantinib and vandetanib are both approved for the treatment of patients with metastatic or locally advanced MTC and have documented activity in patients with RET fusion–driven NSCLC, yet each agent has produced limited disease control and lower response rates compared with selective kinase inhibitors targeting other oncogenic drivers in NSCLC (23–26). In addition, the significant side-effect profiles of each can either limit use in certain patients or limit the dose that patients can tolerate. Furthermore, these agents are biochemically inactive against the RET V804L/M mutants. This gatekeeper position is associated with acquired resistance to tyrosine kinase inhibitors in other targeted therapy/kinase pairs, and mutations at this residue act as primary driver mutations in a subset of hereditary MTCs. Additional MKIs with ancillary RET activity, such as sunitinib, lenvatinib, regorafenib, and RXDX-105, have also been tested in RET-driven tumors with equally disappointing results (27). Together, these findings have led some investigators to question whether RET is a dominant oncogenic driver or whether currently available MKIs insufficiently inhibit RET in vivo due to narrow therapeutic indices.

Here, we describe BLU-667, a next-generation small-molecule RET inhibitor specifically designed for highly potent and selective targeting of oncogenic RET alterations, including the most prevalent RET fusions (e.g., KIF5B–RET and CCDC6–RET) and RET-activating mutations (e.g., C634W, M918T, and V804L/M). BLU-667 demonstrated increased RET potency and selectivity relative to MKIs both in vitro and in vivo models of RET-driven thyroid, lung, and colorectal cancers. In early clinical testing, BLU-667 attenuated RET signaling and induced durable responses in patients with RET-altered NSCLC and MTC without notable off-target toxicity, establishing initial proof of principle for highly selective RET targeting in RET-driven malignancies.

RESULTS

BLU-667 Potently and Selectively Inhibits RET

A library of over 10,000 agnostically designed kinase inhibitors with greater than 60 chemical scaffolds and annotated versus the human kinome was interrogated to identify compounds with inhibitory activity against wild-type (WT) RET and oncogenic RET variants (M918T, V804L, and V804M), while maintaining selectivity against other human kinases. Iterative medicinal chemistry optimization was performed with these initial compounds to improve RET potency, selectivity, and pharmaceutical properties, leading to the generation of BLU-667 (Fig. 1A). In biochemical assays, BLU-667 inhibited the kinase activity of WT RET with subnanomolar potency (IC50 0.4 nmol/L; Table 1). Compared with the MKIs cabozantinib, vandetanib, and RXDX-105 (IC50 11, 4, and 3 nmol/L, respectively), BLU-667 was 8- to 28-fold more potent against WT RET (Fig. 1B; Table 1). Moreover, BLU-667 demonstrated potent activity (IC50 0.4 nmol/L) against common oncogenic RET alterations, including RETM918T, an
activating mutation found in MTC, as well as the CCDC6-RET fusion observed in PTC and NSCLC (17, 28).

A common therapeutic vulnerability of kinase inhibitors in the clinic is the development of on-target resistance mutations, particularly at the gatekeeper position, that stericly hinder inhibitor binding. These mutations have been observed in chronic myeloid leukemia (BCR-ABL T315I), EGFR-mutant NSCLC (EGFR T790M), and ALK-rearranged NSCLC (ALK L1196M; ref. 6). Interestingly, RET gatekeeper mutations at the V804 residue (V804L and V804M) can occur as germline mutations in patients with MTC (29) and have also been shown to confer resistance to MKIs in vitro (29). Cabozantinib and vandetanib were poor inhibitors of these mutants with approximately 100- to 10,000-fold higher IC50s than against WT RET (Fig. 1B; Table 1). By contrast, BLU-667 was designed to inhibit these resistance mutations and demonstrated the same subnanomolar potency as observed against WT RET (IC50 of 0.3 and 0.4 nmol/L for RET V804L and RET V804M, respectively; Fig. 1B; Table 1).

Currently available MKIs with RET activity (e.g., cabozantinib and vandetanib) are characterized by activity against multiple kinase targets, most notably the receptor tyrosine kinase VEGFR2, which regulates angiogenesis and permeability of the vascular endothelium (30). Pharmacologic inhibition of VEGFR2 activity can result in clinically relevant dose-limiting toxicities such as hypertension, thrombosis, and hemorrhage. We therefore designed BLU-667 to have enhanced activity and selectivity for RET versus VEGFR2. Whereas cabozantinib, vandetanib, and RXDX-105 displayed little to no selectivity for RET over VEGFR2, BLU-667 was 88-fold more potent against RET (Table 1). To confirm the high selectivity of BLU-667 for RET across human kinases, we screened a panel of 371 kinases for inhibition by BLU-667 at 300 nmol/L. To refine our understanding of the selectivity that BLU-667 has for RET, we determined the IC50 for kinases inhibited greater than 50% (Supplementary Table S3). Cellular screening confirmed BLU-667’s specificity for RET over VEGFR2 (Supplementary Table S1). Thus, BLU-667 is more potent on RET than the MKIs being studied in a cellular setting. Ba/F3 cells were engineered to express a KIF5B-RET fusion and treated with BLU-667, RXDX-105, cabozantinib, or vandetanib. BLU-667 inhibited RET autophosphorylation with a cellular IC50 of 5 nmol/L, at least 10 times more potently than cabozantinib, vandetanib, and RXDX-105 (IC50s of 61.9, 833, and 128.6 nmol/L, respectively; Fig. 1D).

BLU-667 Inhibits RET Signaling and Proliferation in RET-Driven Cancer Cell Lines

Constitutively activated RET leads to autophosphorylation of the kinase, recruitment of adapter proteins (e.g., SHC), and downstream activation of the MAPK pathway (31). To assess BLU-667 in a cellular setting, Ba/F3 cells were engineered to express a KIF5B-RET fusion and treated with BLU-667, RXDX-105, cabozantinib, or vandetanib. BLU-667 inhibited RET signaling in a panel of RET-driven cell lines: LC2/ad (CCDC6-RET, NSCLC), MZ-CRC-1 (RET M918T, MTC), and TT (RET M918T, MTC). BLU-667 inhibited phosphorylation of RET, SHC, and ERK1/2 at concentrations as low as 0.4 nmol/L in these cell lines (Fig. 2A–C). To confirm MAPK pathway suppression, we measured the impact of BLU-667 on expression of DUSP6 and SPSR4, two genes transcriptionally regulated by ERK1/2 (32, 33). In all cell lines tested, regardless of lineage or type of RET alteration, BLU-667 decreased the abundance of these transcripts in a dose-dependent manner but did not affect the expression of GSK3B, a component of the parallel PI3K–AKT pathway (Fig. 2D–F). Collectively, these data indicate that BLU-667 specifically abrogates RET signaling in RET-altered cancers from diverse lineages. Importantly, RET pathway inhibition with BLU-667 also more potently inhibited proliferation of RET-altered cell lines relative to MKIs (Supplementary Table S2).

We next assessed the activity of BLU-667 against RET V804L and V804M gatekeeper mutations which confer resistance to cabozantinib and vandetanib. In unbiased N-ethyl-N-nitosourea (ENU) mutagenesis screens in KIF5B-RET Ba/F3 cells, we found amino acid substitution at the V804 residue more potently inhibited proliferation of RET V804L and V804M, respectively (Fig. 1B; Table 1).

Table 1. Biochemical potency of BLU-667 and MKIs against activated RET mutants and fusion variants

<table>
<thead>
<tr>
<th>Compound</th>
<th>WT RET</th>
<th>RET V804L</th>
<th>RET V804M</th>
<th>RET M918T</th>
<th>CCDC6-RET</th>
<th>VEGFR2</th>
</tr>
</thead>
<tbody>
<tr>
<td>BLU-667</td>
<td>0.4</td>
<td>0.3</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>35</td>
</tr>
<tr>
<td>Cabozantinib</td>
<td>11</td>
<td>45</td>
<td>162</td>
<td>8</td>
<td>34</td>
<td>2</td>
</tr>
<tr>
<td>Vandetanib</td>
<td>4</td>
<td>3,597</td>
<td>726</td>
<td>7</td>
<td>20</td>
<td>4</td>
</tr>
<tr>
<td>RXDX-105</td>
<td>3</td>
<td>188</td>
<td>102</td>
<td>4</td>
<td>7</td>
<td>17</td>
</tr>
</tbody>
</table>

Currently available MKIs with RET activity (e.g., cabozantinib and vandetanib) are characterized by activity against multiple kinase targets, most notably the receptor tyrosine kinase VEGFR2, which regulates angiogenesis and permeability of the vascular endothelium (30). Pharmacologic inhibition of VEGFR2 activity can result in clinically relevant dose-limiting toxicities such as hypertension, thrombosis, and hemorrhage. We therefore designed BLU-667 to have enhanced activity and selectivity for RET versus VEGFR2. Whereas cabozantinib, vandetanib, and RXDX-105 displayed little to no selectivity for RET over VEGFR2, BLU-667 was 88-fold more potent against RET (Table 1). To confirm the high selectivity of BLU-667 for RET across human kinases, we screened a panel of 371 kinases for inhibition by BLU-667 at 300 nmol/L. To refine our understanding of the selectivity that BLU-667 has for RET, we determined the IC50 for kinases inhibited greater than 50% (Supplementary Table S3). Cellular screening confirmed BLU-667’s specificity for RET over VEGFR2 (Supplementary Table S1). Thus, BLU-667 is more potent on RET than the MKIs being studied in RET-driven malignancies and highly selective across human kinases.
Figure 2. RET signaling pathways are inhibited by BLU-667. A–C, Immunoblots of whole cell lysates from (A) LC2/ad NSCLC cells, (B) MZ-CRC-1 MTC cells, or (C) TT MTC cells treated with BLU-667, cabozantinib, or vandetanib at indicated concentrations (nmol/L) for 90 minutes. Phospho- and total RET, SHC, and ERK1/2 were interrogated. In LC2/ad cells, pERK is represented by the top two bands of the triplet.

D–F, Relative transcript expression of RET pathway targets DUSP6, SPRY4, and GSK3β 7 hours after treatment of (D) LC2/ad, (E) MZ-CRC-1 cells, or (F) TT MTC cells with BLU-667 or cabozantinib. Data are the mean ± SD. *, P < 0.05; **, P < 0.01; *** P < 0.001, two-sided Student t test. SD, standard deviation.
BLU-667 Inhibits RET Alterations in Cancer

**Figure 3.** Antitumor activity of BLU-667 across RET-driven solid tumor models in vivo. Antitumor activity of BLU-667 compared with cabozantinib and waterfalk plot showing the best response as a percentage change in tumor volume taken on the last day of treatment in KIF5B–RET Ba/F3 allografts (A, B), KIF5B–RET V804L Ba/F3 allografts (C, D), and RET C634W TT xenografts (E, F). Nine mice were included in each treatment group. Data are the mean ± SEM. **P < 0.01, ***P < 0.001, one-way ANOVA with a Dunnett multiple comparisons test (RET C634W TT xenografts) or repeated measures two-way ANOVA (KIF5B–RET and KIF5B–RET V804L Ba/F3 allografts). b.i.d., twice daily; q.d., once daily.

BLU-667 inhibits RET phosphorylation and RET downstream signaling in vitro and in vivo, with potent antitumor activity in preclinical and clinical studies (34). To demonstrate that RET pathway inhibition correlated with antitumor activity in vivo, RET pathway inhibition was assessed in tumor lysates harvested after BLU-667 administration. BLU-667 decreased pRET and pSHC in a dose-dependent manner and maintained inhibition of both targets throughout the 12-hour dosing period at the efficacious dose of 30 mg/kg twice daily (Fig. 4B; Supplementary Fig. S3). In the KIF5B–RET NSCLC PDX, inhibition of RET phosphorylation led to a sustained decrease in expression of the MAPK target genes DUSP6 and SPRY4 (Fig. 4C). By contrast, 60 mg/kg once-daily cabozantinib displayed only transient suppression of DUSP6 and SPRY4 expression, indicating incomplete suppression of RET pathway signaling and raising the possibility that some of the antitumor activity of cabozantinib may be mediated through non-RET inhibitory mechanisms.

Increases in circulating VEGFA and decreases in soluble VEGFR2 (sVEGFR2) are two biomarkers for VEGFR2 inhibition in preclinical and clinical studies (34). To demonstrate BLU-667 specificity for RET over VEGFR2 in vivo, we measured plasma levels of these biomarkers in the KIF5B–RET PDX tumor-bearing mice treated with cabozantinib or BLU-667.
from the *in vitro* experiment shown in Fig. 4A. Consistent with its potent VEGFR2 biochemical activity, cabozantinib significantly increased the level of circulating VEGFA (*P* < 0.001) and decreased sVEGFR2 compared with vehicle treatment (*P* < 0.01; Fig. 4D; Supplementary Fig. S4). By contrast, BLU-667 did not significantly alter the levels of circulating VEGFA or sVEGFR2 at any dose tested. Together, these data indicate that BLU-667 potently and selectively inhibited RET without modulating VEGFR2 *in vitro*, confirming that selective RET inhibition is sufficient for antitumor activity in these preclinical tumor models.

**BLU-667 Exhibits Clinical Activity in Early Studies**

To investigate the clinical impact of BLU-667 on RET-driven malignancies, a phase I, first-in-human, dose-escalation study of BLU-667 in patients with advanced, unresectable NSCLC, thyroid cancer, or other solid tumors was initiated (NCT03037385). Both tyrosine kinase inhibitor (TKI)–naïve and TKI-refractory patients with any number of lines of therapy were eligible for enrollment in the study.

A 27-year-old patient with sporadic MTC harboring multiple RET mutations (L629P, D631_R635DELINS, and V637R) was among the first patients to enroll in the first-in-human BLU-667 trial. The patient was TKI-naïve prior to the start of BLU-667 treatment, with highly invasive disease that required emergent tracheostomy and extensive surgery, including total thyroidectomy, central neck dissection, bilateral levels 1 through 4 neck dissection, total thymectomy, and median sternotomy. The postoperative course was complicated by chylothorax. Multidisciplinary medical consensus was against radiotherapy to the neck, and restaging scans showed left paratracheal disease with
tracheal and esophageal invasion as well as metastatic disease to the lungs and liver. The two FDA-approved multikinase drugs for MTC (vandetanib and cabozantinib) were not considered appropriate for this patient given the associated risk of VEGFR-related toxicities that can include impaired wound healing and increase the risk of fistula formation and hemorrhage (35, 36). Therefore, the patient was enrolled on the BLU-667 clinical trial and began treatment at the second dose level (60 mg, once daily). Remarkably, after 28 days of BLU-667 therapy, there was a >90% reduction in the serum tumor marker calcitonin (Fig. 5A). After 8 weeks, target lesions were reduced by 19%. After successive dose escalations of BLU-667 to 200 mg once daily, the patient achieved partial response with >30% tumor reduction per RECIST version 1.1 (Fig. 5B). This patient subsequently escalated to 300 mg once daily BLU-667 and achieved a confirmed partial response (47% maximal reduction) at 10 months. Overall, carcinoembryonic antigen (CEA) levels decreased by 57% over this period. Improved health status with BLU-667 treatment allowed for removal of the patient’s tracheostomy tube and a return to baseline body weight after several kilograms of weight loss prior to treatment. BLU-667 has been well tolerated throughout 11 months of continuous treatment with the only drug-related adverse event being transient grade 1 decrease in white blood cells, which resolved without drug interruption or dose modification. This patient remains progression free and on therapy for more than 11 months.

Substantial antitumor activity was also observed in a second patient with MTC. In this case, a 56-year-old with sporadic RETM918T-mutant disease, who had responded and then progressed on vandetanib, initiated therapy with BLU-667, 300 mg once daily. Early signals of clinical activity emerged...
within the first few weeks of BLU-667 treatment: Serum calcitonin decreased >90% and CEA decreased by 75% after 28 days (Fig. 5C). RET<sub>M918T</sub>-circulating tumor DNA (ctDNA) decreased by 47% after 28 days and was not detectable after 56 days. Paired tumor biopsies collected pretreatment and 28 days after treatment demonstrated a 93% reduction in DUSP6 and an 86% reduction in SPRY4 mRNA expression, confirming RET-pathway inhibition within the tumor (Supplementary Fig. S5). Importantly, these indications of activity were confirmed by radiographic response (~35%) per RECIST 1.1 after 8 weeks (Fig. 5D). The patient tolerated BLU-667 treatment well without dose interruption; drug-related adverse events were grade 1 nausea and hyperphosphatemia. The patient continues on therapy more than 8 months with a confirmed partial response (maximum 47% reduction).

Beyond MTC, important antitumor activity was also observed in RET-altered NSCLC. A 37-year-old patient with metastatic NSCLC, who had progressed on cisplatin, pemetrexed, and bevacizumab, had tumor tissue test positive for a RET fusion via FISH analysis. The patient initiated treatment with 200 mg once daily BLU-667, and ctDNA analysis at baseline revealed a canonical KIF5B–RET fusion and co-occurring TP53 mutation. Tumor reduction (~25%) was noted at first radiographic assessment after 8 weeks of treatment and correlated with a concomitant decline in KIF5B–RET and TP53 ctDNA levels (Fig. 6A). The patient achieved a partial response on the second radiographic assessment after 16 weeks (Fig. 6B) and continues on treatment (more than 10 months) with a confirmed partial response. As observed in the patients with MTC described here, BLU-667 has been well tolerated, with all drug-related adverse events being grade 1 and including constipation (resolved), dry skin, rash, and leukopenia.

Consistent with the clinical activity of BLU-667 in TKI-resistant MTC, we also observed a rapid clinical response in a patient with TKI-refractory KIF5B–RET NSCLC. This

Figure 6. Partial response achieved with BLU-667 in a metastatic KIF5B–RET NSCLC tumor. A, Tumor and KIF5B–RET and TP53 ctDNA reduction over the course of treatment with 200 mg once daily BLU-667 demonstrates rapid response to BLU-667. B, CT scan illustrates tumor (circled) at baseline (top) and after 32 weeks of BLU-667 treatment (bottom). C, Tumor reduction over 4 cycles of treatment with 300 mg BLU-667. ctDNA not available for this patient. D, CT scan of the patient’s tumor (circled) at baseline (top) and after 16 weeks of BLU-667 treatment (bottom). C, cycle number; D, day.
72-year-old former smoker with locally advanced NSCLC had received concurrent chemoradiation with cisplatin and pemetrexed, was then treated with carboplatin and nab-paclitaxel, and eventually progressed. Next-generation sequencing of the tumor tissue revealed a KIF5B–RET fusion, and the patient was enrolled on a clinical trial testing a combination regimen of vandetanib and everolimus (NCT01582191). The patient achieved a partial response, but restaging scans performed after 11 cycles showed progressive disease, which was associated with clinical symptoms of increasing dyspnea and worsening performance status. The patient was then enrolled on the phase I trial of BLU-667. After 16 weeks of BLU-667 (300 mg once daily), the patient had a partial response with 34% reduction of tumor volume (Fig. 6C and D) and improvement of dyspnea and performance status. BLU-667 has been well tolerated throughout treatment, and the patient has not experienced drug-related adverse events. This patient continues on treatment more than 18 weeks. This early clinical experience with BLU-667 demonstrates that this selective RET inhibitor can achieve response per RECIST criteria in both TKI-naïve and TKI-refractory tumors driven by KIF5B–RET, the most prevalent RET fusion found in NSCLC, and suggests the promise of this targeted therapy for this currently underserved patient population.

**DISCUSSION**

Targeted kinase inhibitors have produced significant improvements in the outcomes of patients across a broad range of kinase-driven cancers. This approach has been particularly successful in NSCLC, where several dysregulated tyrosine kinases such as EGFR, ALK, and ROS1 have been identified and selectively targeted, transforming a once-poor diagnosis into one with several highly effective treatment options (1, 3, 4, 37). By analogy, RET-rearranged NSCLC may represent a similar opportunity, as lung tumors with oncogenic RET activation typically lack other known driver mutations. Yet, despite a seemingly strong scientific rationale and precedence for precision therapy in kinase-mutated lung cancers, patients with RET-driven NSCLC, as well as other RET-driven tumors, have few good therapeutic options at present. The impact of MKIs with ancillary RET activity has been disappointing in this patient population, likely because of an inability to sufficiently inhibit oncogenic signaling driven by activating RET aberrations at maximally tolerated doses. Moreover, very much like ALK- and ROS1-rearranged NSCLC, patients with RET-rearranged NSCLC are characterized by a higher percentage of never-smokers than the general NSCLC population (28). As such, these tumors are likely to have a lower mutation burden and a decreased likelihood of significant benefit from immunotherapies (38–40), further underscoring the need for potent, tailored RET inhibitors.

MTC is a rare malignancy for which vandetanib and cabo-zantinib were approved in 2011 and 2012, respectively. However, the multikinase target profiles of these agents make their mechanisms of action unclear and their off-target safety profiles complex. In nonhereditary MTC with an estimated RET mutation rate of 50%, and in hereditary MTC that is almost always associated with a germline RET mutation, selective RET inhibition could be highly impactful and provide an additional option for patients who have progressed on, or have contraindications for, MKI therapy.

BLU-667 was designed as a potent and highly selective inhibitor of activating RET alterations found in NSCLC, thyroid cancer, and other solid tumors. BLU-667 was equally potent across various RET fusions and mutants, including CCDC6–RET, KIF5B–RET, and mutations at the gatekeeper residue: V804L, V804M, and the newly identified V804E amino acid substitution. The equipotent activity of BLU-667 across all oncogenic RET variants further differentiates it from MKIs with RET-inhibitory activity. For example, preliminary clinical trial results with RXDX-105 demonstrated no objective responses in patients with KIF5B–RET NSCLC; clinical activity was observed only among CCDC6–RET- or NCOA4–RET-driven tumors (41). This lack of response to RXDX-105 in patients with KIF5B–RET NSCLC has raised the important question of whether KIF5B–RET truly drives these tumors or whether KIF5B–RET-driven tumors have a different biology, less dependent on RET kinase activity and thus less sensitive to RET inhibition. Given the results reported here with BLU-667, this explanation of reduced RET dependence in KIF5B–RET tumors seems unlikely and is further refuted by the recent report of a KIF5B–RET NSCLC partial response per RECIST criteria observed with the RET inhibitor LOXO-292 (42). BLU-667 inhibited KIF5B–RET autophosphorylation over 20-fold more potently than RXDX-105 in vitro and provides a sound rationale for the preferential activity seen with BLU-667.

The combined action of MKIs on RET and VEGFR2 has been postulated by some to contribute positively to antitumor activity, especially in MTC. However, we have shown that BLU-667 was able to achieve high levels of RET inhibition and antitumor activity both preclinically and clinically without concomitant VEGFR2 inhibition, suggesting it is not required for effective treatment of RET-altered tumors and better avoided considering the associated toxicities. The closest BLU-667 kinase off-target identified in biochemical screening was JAK1, with IC50 shifted 20-fold relative to RET. Yet evidence of clinically significant JAK inhibition (reduced reticulocytes, red blood cells, neutrophils/monocytes) has not appeared among the four patients described above, suggesting the preferential activity of BLU-667 for RET versus JAK was preserved in vivo. Given this, it is unlikely that JAK inhibitory activity plays a part in the BLU-667 antitumor activity, unless only low levels of JAK inhibition are required. Future studies will illuminate the full spectrum of BLU-667 activity, but all data to date support the view that RET inhibition is sufficient for antitumor activity and that high levels of sustained target inhibition with a selective kinase inhibitor can deliver meaningful clinical responses with potentially fewer off-target side effects, making long-term drug administration more tolerable for patients.

It has been proposed that the inclusion of a pharmacodynamic assessment of molecularly targeted therapies in clinical trials can streamline the drug-development process (43, 44). Pharmacodynamic biomarkers have been successfully utilized for the clinical development of kinase inhibitors, including imatinib and gefitinib (44–46). In this study, BLU-667 dose-dependently inhibited RET and SHC activation, which...
mirrored the inhibition of DUSP6 and SPRY4 transcription across RET-driven preclinical models, indicating that these transcripts can serve as biomarkers for RET inhibitory activity. The translational capability of these markers was established in this study in which MTC tumor shrinkage induced by BLU-667 treatment was associated with efficient inhibition of DUSP6 and SPRY4 expression within the tumor tissue. To our knowledge, this represents the first confirmation of RET target engagement by a small-molecule inhibitor, multitargeted or selective, within the clinical setting and may be used in this and future studies to more precisely define the optimal dose and schedule required for effective RET inhibition.

The specificity and potency of BLU-667 for RET, its preliminary antitumor activity in patients with RET-altered MTC or NSCLC, and its seemingly favorable clinical safety profile in these early studies suggest that BLU-667 has potential to improve outcomes in patients with RET-driven cancers while limiting off-target adverse effects. Importantly, selective RET inhibition by BLU-667 was sufficient for antitumor activity, in patients with KIF5B–RET NSCLC that were TKI naïve or TKI refractory and in a patient with vandetanib-resistant MTC. By offering a highly selective medicine specifically tailored for the oncogenic driver, we hope BLU-667 will enable patients with RET-driven tumors to benefit from the recent advances in genomic profiling that have revolutionized treatment options for patients with kinase-driven diseases.

**METHODS**

**BLU-667 Synthesis**

The synthesis of BLU-667 is described in WO 2017/079140; example 5, compound 130 (47). Details can be found at https://patentscope.wipo.int/search/en/detail.jsf?docid=WO2017079140.

**In Vitro Biochemical Activity Assay**

Recombinant RET enzyme and enzyme variants (V804L, V804M, M918T, and CDC6-RET) were purchased from ProQinase GmbH, and recombinant VEGFR2 enzyme was purchased from Carna Bio USA, all containing N-terminal Glutathione S-transferase tags. Cabozantinib, vandetanib, and RDX1-105 were purchased from Selleck Chemicals. Kinase/peptide substrate pairs, serially diluted compounds, and cofactors required were prepared in a reaction buffer at their respective Michaelis constant (Km) for ATP and incubated for 120 minutes. The reactions were stopped by the addition of an EDTA-containing buffer and proportional enzymatic activities were measured using the Perkin Elmer electrophoretic mobility shift platform (EZReader, Perkin Elmer). Potency was calculated from EZReader data with 1% DMSO and 10 μmol/L ATP to generate biochemical IC50 for each compound.

**Human Kinase Selectivity**

BLU-667 was screened at 300 nmol/L for inhibitory activity across a panel of 371 kinases (Reaction Biology Corporation). The 23 kinases inhibited >50% at 300 nmol/L were selected for full 10 point concentration–response curves with BLU-667 (1 μmol/L maximum concentration) at 200 μmol/L ATP to generate biochemical IC50 (Reaction Biology Corp) using 3P-ATP (10 mCi/mL) to initiate the reaction, followed by the detection of kinase activity by a filter-binding method.

**Cell Culture**

The TT MTC cell line was purchased from the ATCC (2014) and grown in 20% FBS Ultraculture with Glutamax (ThermoFisher Scientific). LC2/ad was obtained from Riken (2014) and grown in 1:1 Ham’s F12/RPMI medium supplemented with 15% FBS, 1 × penicillin-streptomycin, and 25 mmol/L l-arginine. For 4T1 tumors (Hepes) and HEK293 cells (Hepes). MZ-CRC-1 and TPC-1 cell lines were obtained in 2014 from the University of Colorado Denver. MZ-CRC-1 cells were grown in bMEM with 10% FBS; TPC-1 cells were grown in RPMI medium with 10% FBS. Parental Ba/F3 cells were purchased from Leibnitz Institute DSMZ German Collection of Microorganisms and Cell Culture (2013) and grown in RPMI medium with 10% FBS, 1 × penicillin-streptomycin, and 5 ng/mL murine interleukin-3 (MIL). Ba/F3 KIF5B–RET cell lines were grown in RPMI medium with 10% FBS, 1 × penicillin-streptomycin, and 1 ng/mL dosycycline. Cell lines were authenticated by periodic short tandem repeat (STR) profiling (PowerPlex 16HS by Promega, testing performed at Genentech) and confirmed to be Mycoplasma free (PCR-based detection by DDC Medical and Genentech). TT cells were last authenticated by STR in May 2015, LC2/ad in August 2016, MZ-CRC-1 in March 2017, Ba/F3 cells were not authenticated. Cell lines were last tested for Mycoplasma in August 2016 (LC2/ad, TT) and August 2017 (MZ-CRC-1, Ba/F3). All cell lines were thawed and passed at least 3 times before initiating experiments. Cells were discarded after 18 passages.

**Generation of KIF5B–RET Ba/F3 Cells**

The DNA encoding the amino acid sequence of human KIF5B–RET variant 1 was placed in a lentivirus vector under a doxycycline-inducible promoter to maximize expression with a carboxyl-terminal FLAG epitope to facilitate immunodetection of the fusion by anti-FLAG antibodies. Lentiviral-mediated gene transduction was used to express KIF5B–RET in Ba/F3 cells; KIF5B–RET-dependent cells were selected by IL2 withdrawal and confirmed to express the KIF5B–RET fusion protein by immunoblot analysis.

**Proliferation Assays**

KIF5B–RET Ba/F3 cells were exposed to compound concentrations ranging from 25 μmol/L to 95.4 μmol/L for 48 hours, and proliferation was assessed with Cell Titer Glo (Promega). TT, MZ-CRC-1, TPC-1, or LC2/ad cells were exposed to compound for 4 days and proliferation was measured by BrdU incorporation (Abcam). Lentiviral-mediated gene transduction was used to express KIF5B–RET in Ba/F3 cells; KIF5B–RET-dependent cells were selected by IL3 withdrawal and confirmed to express the KIF5B–RET fusion protein by immunoblot analysis.

**ENU Mutagenesis Assays**

To generate Ba/F3 cells carrying V804 substitutions, WT KIF5B–RET Ba/F3 cells were mutagenized overnight with ENU and plated in 96-well plates for a period of 2 weeks in the presence of 6 concentrations of MKIs (ponatinib, regorafenib, cabozantinib, or vandetanib). The concentrations chosen ranged from 20 to 640 μmol/L for the proliferation IC50 for each compound: 125 nmol/L to 4 μmol/L, 20 to 640 nmol/L for ponatinib, and 250 nmol/L to 8 μmol/L of vandetanib. Genomic DNA was isolated from resistant clones, and Sanger sequencing was used to identify those that harbored substitutions.

**Immunoblot**

Cells were treated with the indicated compound concentrations for 90 minutes. For all cell lines except LC2/ad, cell pellets were lyzed in Phospho-Safe lysis buffer (EMD Millipore). To lyse the LC2/ad cells, 1 × NuPAGE lithium dodecyl sulfate (LDS) sample buffer (Thermo Fisher Scientific) with 5% mercaptoethanol preheated to 95°C was...
added to each well. Total cell lysates were resolved by SDS-PAGE and transferred onto nitrocellulose membranes (Bio-Rad). Primary antibodies included phospho-RET (Y1062, sc-20252-R; Santa Cruz Biotechnology), total RET (#3323; Cell Signaling Technology), phospho-SHC (Y239/Y240, #2434; Cell Signaling Technology), phospho-ERK1/2 (T202/Y204, #4370; Cell Signaling Technology), total ERK1/2 (#9107; Cell Signaling Technology), total SHC antibody (06-203; EMD Millipore), and total Beta-Actin (a53280; Abcam). Secondary antibodies (IRDye 800-conjugated rabbit IgG and IRDye 700-conjugated mouse IgG) were from Rockland Immunochemicals Inc. Immunoblots were imaged using the Odyssey fluorescent imaging system (LI-COR Biosciences).

**AlphaLISA Phospho-RET Assay**

Cell lines were exposed to BLU-667 or reference compound for 90 minutes with AlphaLISA Lysis Buffer (Perkin Elmer) supplemented with protease/phosphatase inhibitors. Autophosphorylated RET was detected with antibody against phospho-RET Y1062 (sc-20252-R, Santa Cruz Biotechnology). Anti-FLAG donor beads (Perkin Elmer) or anti-Rabbit acceptor beads (Perkin Elmer) were added to cell lysates and agitated for 1 hour each. An Evasion Multilabel Reader (PerkinElmer) was used to detect the AlphaLISA signal.

**DUSP6 and SPRY4 Expression Analysis**

Cells were treated with the indicated compounds for 7 hours before lysis with Buffer RLT (QIAGEN) containing 1% β-mercaptoethanol. Total RNA was isolated using the RNeasy Plus Mini Kit (QIAGEN) according to the manufacturer’s instructions. First-strand cDNA was synthesized using the SuperScript VILO Master Mix (Thermo Fisher Scientific) according to the manufacturer’s instructions. Real-time qPCR was run on the Viia 7 Real Time PCR System (Thermo Fisher Scientific). For qRT-PCR, the expression of the reference gene glucuronidase beta (GUSB) was used to normalize expression of the target genes DUSP6, SPRY4, and glycogen synthase kinase 3 beta (GSK3B). Duplicate qRT-PCR reactions were analyzed for each sample, and QuantStudio Real-Time PCR software (Life Technologies) normalized the average expression of DUSP6, SPRY4, or GSK3B to the average expression of the reference gene GUSB in each sample.

**Animal Studies**

All procedures relating to animal handling, care, and treatment were performed according to the guidelines approved by the Institutional Animal Care and Use Committee of the contract research organizations performing the study, following the guidance of the Association for Assessment and Accreditation of Laboratory Animal Care. Allograft and xenograft models using cell lines were performed at Shanghai ChemPartner, and BALB/c nude mice were inoculated subcutaneously into the right flank with 1×106 KIF5B–RET V804L Ba/F3 cells, 5×106 KIF5B–RET Ba/F3 cells, 5×106 KIF5B–RET V804L Ba/F3 cells, or 1×107 TT cells. Mice were divided into groups of 9, and treatments began when tumors were between 217 and 238 mm³. For PDX tumor models, CTG-0838 and colorectal cancer PDX tumors, respectively. Mice were divided into groups of 9, and treatments began when tumors were between 217 and 238 mm³. For PDX tumor models, CTG-0838 and colorectal cancer PDX tumors, respectively. For all comparisons, P < 0.05 was considered statistically significant.

**Disclosure of Potential Conflicts of Interest**

V. Subbiah reports receiving commercial research grants from Blueprint Medicines, Fujifilm, BERG, PharmaMar, D3, Pfizer, Luxo Oncology, MultiVir, Amgen, AbbVie, Roche/Genentech, Novartis, Incyte, Idera Pharmaceuticals, Bayer, GlaxoSmithKline, NanoCarrier, Vegenics, and Northwest Biotherapeutics. J.F. Gainor is a consultant/advisory board member for Bristol-Myers Squibb, Takeda, Novartis, Amgen, Pfizer, Genentech/Roche, Luxo, Merck, Incyte, Array BioPharma, and Theravance. R. Rahal has ownership interest in BluePrint Medicines. J.D. Brubaker has ownership interest in Blueprint Medicines. J.L. Kim has ownership interest in BluePrint Medicines. W. Hu has ownership interest in Blueprint Medicines. C. Hao has ownership interest in BluePrint Medicines. M.P. Sheets has ownership interest in BluePrint Medicines. D. Wilson has ownership interest in BluePrint Medicines. K.J. Wilson has ownership interest in BluePrint Medicines. L. DiPietro has ownership interest in BluePrint Medicines. M.I. Hu reports receiving commercial research support from Sanofi Genzyme. L. Wirth is a consultant/advisory board member for Eisai, Blueprint, and Luxo. M.S. Brose is a consultant/advisory board member for Blueprint Medicines. M. Taylor has received honoraria.
from the speakers bureaus of Bristol-Myers Squibb and Eisai Inc. and is a consultant/advisory board member for Lexo Oncology, Novartis, Blueprint Medicines, and Roche/Genentech. E. Garralda is a consultant/advisory board member for Roche and NeoMed Therapeutics. B. Wolf has ownership interest (including patents) in Blueprint Medicines. C. Lengauer has ownership interest in Blueprint Medicines. T. Guzi has ownership interest in Blueprint Medicines. E.K. Evans has ownership interest in Blueprint Medicines. No potential conflicts of interest were disclosed by the other authors.

**Authors’ Contributions**


Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): V. Subbiah, J.F. Gainor, M. Maynard, W. Hu, Q. Cao, M. Palmer, M.I. Hu, L. Wirth, M.S. Brose, S.-H. Ignatius Ou, M. Taylor, E. Garralda, B. Wolf, E.K. Evans


Administrative, technical, or material support (i.e., reporting or data management, writing and editing): E. Garralda, S. Miller, B. Wolf, C. Lengauer, T. Guzi, E.K. Evans


Other (compound design and synthesis): D. Wilson, L. DiPietro

Acknowledgments

We thank our colleagues at Blueprint Medicines for the thoughtful discussions and constructive feedback throughout the BLU-667 program and for the critical review of the manuscript. We also thank the scientists at Shanghai ChemPartner, CrownBio, and Champions Oncology for their excellent in vivo study support and execution, and Allison Cherry, PhD, of Team9 Science for medical writing support. Finally, we thank the patients and their families and the doctors and nurses for their participation in the ongoing phase I clinical trial with BLU-667. This work was funded by Blueprint Medicines.

Received March 30, 2018; revised April 9, 2018; accepted April 11, 2018; published first April 15, 2018.

**REFERENCES**


848 | CANCER DISCOVERY | JULY 2018 www.aacrjournals.org
Precision Targeted Therapy with BLU-667 for RET-Driven Cancers

Vivek Subbiah, Justin F. Gainor, Rami Rahal, et al.

Cancer Discov 2018;8:836-849. Published OnlineFirst April 15, 2018.

Updated version
Access the most recent version of this article at:
doi:10.1158/2159-8290.CD-18-0338

Supplementary Material
Access the most recent supplemental material at:
http://cancerdiscovery.aacrjournals.org/content/suppl/2018/04/12/2159-8290.CD-18-0338.DC1

Cited articles
This article cites 44 articles, 9 of which you can access for free at:
http://cancerdiscovery.aacrjournals.org/content/8/7/836.full#ref-list-1

Citing articles
This article has been cited by 23 HighWire-hosted articles. Access the articles at:
http://cancerdiscovery.aacrjournals.org/content/8/7/836.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, use this link
http://cancerdiscovery.aacrjournals.org/content/8/7/836.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.