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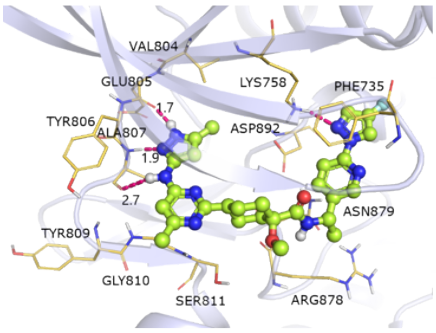

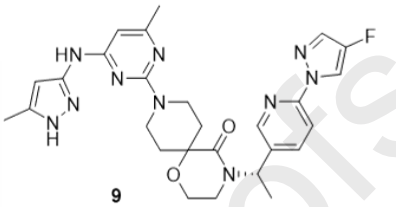

9

RET (wt) IC_{50} 1.29 nM
MDR1 A to B (ER) 4.3 cm/s (2.7)
Vd 1.5 L/kg

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Discovery and Optimization of Selective RET Inhibitors via Scaffold Hopping

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ABSTRACT

Aberrant alterations of rearranged during transfection (RET) have been identified as actionable drivers of multiple cancers, including thyroid carcinoma and lung cancer. Currently, several approved multikinase inhibitors such as vandetanib and cabozantinib demonstrate clinical activity in patients with RET-rearranged or RET-mutant cancers. However, the observed response rates are only modest and the 'off-target' toxicities resulted from the inhibition of other kinases is also a concern. Herein, we designed and synthesized a series of RET inhibitors based on the structure of selective RET inhibitor BLU-667 and investigated their biological activities. We identified compound **9** as a novel potent and selective RET inhibitor with improved drug-like properties. Compound **9** exhibits a selective inhibitory profile with an inhibitory concentration 50 (IC_{50}) of 1.29 nM for RET and 1.97 (RET V804M) or 0.99 (RET M918T) for mutant RETs. The proliferation of Ba/F3 cells transformed with NSCLC related KIF5B-RET fusion was effectively suppressed by compound **9** (IC_{50} = 19 nM). Additionally, compound **9** displayed less 'off-target' effects than BLU-667. In mouse xenograft models, compound **9** repressed tumor growth driven by KIF5B-RET-Ba/F3 cells in a dose-dependent manner. Based on its exceptional kinase selectivity, good potency and high exposure in tumor tissues, compound **9** represents a promising lead for the discovery of RET directed therapeutic agents and the study of RET-driven tumor biology.

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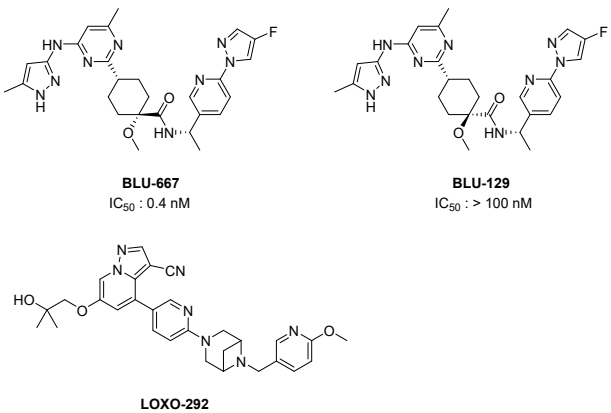
1. I

RET is a transmembrane receptor tyrosine kinase which is encoded by the proto-oncogene RET located on chromosome 10. [1] This kinase, which is crucial for the development and homeostasis of some tissues such as enteric nervous system and male germ-cell tissues,[2-6] is composed of an extracellular cadherin domain, cysteine-rich region, transmembrane domain, and an intracellular kinase domain.[7-8] Upon activation, RET dimerizes and auto phosphorylates its intracellular kinase domain.[5,9-11] Subsequently, the recruitment of signaling adaptors induces several signaling transduction cascades including MAPK, PI3K, PKA and PKC pathways which are involved in cell apoptosis and proliferation.[12-16] Two primary types of RET aberrations were identified to be oncogenic,[17,18] including chromosomal rearrangements and missense mutations. Rearrangements of the RET proto-oncogene with various upstream partner genes can produce hybrid proteins with ligand independent kinase activities.[19-21] Mutation of the RET can also result in the activation of its kinase activities and various point mutations have been reported to be involved in the pathogenesis of thyroid cancers and lung cancers.[22-26] Downstream signaling cascades might be enhanced by RET hyperactivation and thus leading to uncontrolled cell proliferation.

Since activating RET rearrangements and mutations have been identified to be oncogenic,[16,27] the development of RET inhibitors as targeted therapeutics to meet clinical needs has attracted much attentions.[17,18] In the past decade, several multikinase inhibitors with activities against RET have been evaluated in patients with tumors involving RET aberrations.[28-30] These multikinase inhibitors, including cabozantinib, vandetanib, sorafenib and others have documented IC_{50} ranging from <5 nM to 100 nM against full-length wild-type RET.[31-34] However, although moderate objective response rate has been reported, the drug-related adverse events cast a shadow over the application of multikinase inhibitors in the treatment of patients with RET associated tumors. The 'off-target' toxicities of these multikinase inhibitors were thought to result from inhibitions against a variety of kinases in addition to RET.[17] Considering that these drugs were initially developed as inhibitors of other kinases such as VEGFR2, EGFR and MET.[18] To improve the clinical efficacy and minimize 'off-target' toxicities of RET-directed targeted therapies, novel and potent inhibitors with highly selectivity against RET need to be developed. Two RET-specific inhibitors, BLU-667 and LOXO-292 have entered clinical trials and were approved recently (**Figure 1**).[35-39] According to a clinical trial in patients with advanced RET fusion malignancies, BLU-667 exhibited 95% and 83% objective response rate in NSCLC (non-small cell lung cancer) and PTC (papillary thyroid cancer), respectively.[37] However, BLU-667 also showed some side effects including anemia, white blood cell count decrease and neutropenia which was thought to result from the inhibition of JAK2 and FLT3.[40,41] Moreover, BLU-667 is a PGP substrate and the relatively high efflux ratio of BLU-667 (P_{app} A to B = 1.5 cm/s, efflux ratio = 20.6) might require higher dose to secure enough exposures in may cause more "on-target" side effects as RET is broadly expressed in normal cells.[42,43] Collectively, although BLU-667 shed lights on precision targeted therapy for RET-driven cancers, there is still room for improvement of drug-like properties.[35]

a

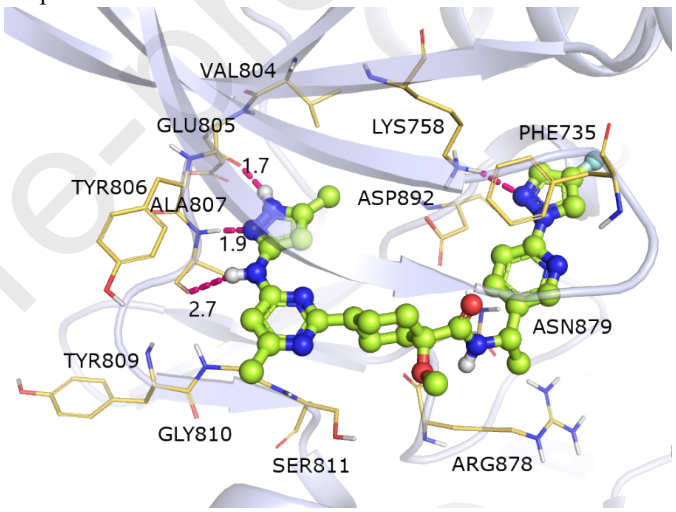


b

2. Result and discussion

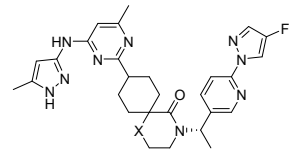
Figure 1. (a) Structure of BLU-667, BLU-129 and LOXO-292; (b) Docking result of BLU-667 with RET (2IVU was used as docking template)

To develop potent and selective RET inhibitors, we started with the binding mode analysis of BLU-667 with RET (**Figure 1b**). The co-crystal structure 2IVU, a complex of RET with ZD6474/Vandetanib, was used as the modeling template.[44] Modeling result suggested that amino pyrazole moiety formed three hydrogen bonds with hinge residues Ala807 and Glu805. The hydrophobic hexane ring served as a bridge linking the pyrimidine ring and the pyridine ring. The fluoropyrazole group which is attached to the pyridine ring extended deep into a pocket close to Lys758, suggesting the existence of an additional hydrogen bond. Moreover, the pyridine and fluor pyrazole are in close contact with the Phe735 residue of the P-loop, indicating the presence of additional π-π interaction. The conformation of



the linker is important to position the pyridine-pyrazole moiety in the right direction, as the potency of compound BLU-129 decreased significantly (IC_{50} > 100 nM). Based on the structure of BLU-667 and the binding model analysis, we speculated that conformation restriction by connecting the methoxy group and amide to form a spiro skeleton might be a promising strategy to improve permeability and mitigate the liability as a PGP substrate along with the reduction of one hydrogen bond donor. Compound **1** was thus synthesized and the activity against RET (wild-type) and RET (V804M) as well as RET (M918T) was tested. It was found that compound **1** has an IC_{50} of 1.12 nM, 5.28 nM and 2.99 nM against RET (wild-type), V804M mutation and M918T mutation (**Table 1**) respectively. Compound **2** was synthesized by replacing the oxygen atom with a carbon atom and showed potencies against RET (wild-type) and V804M mutation comparable to those of

Tab



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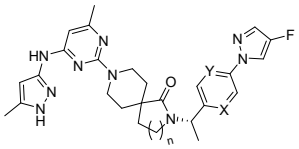
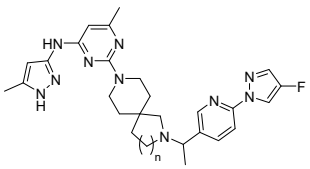
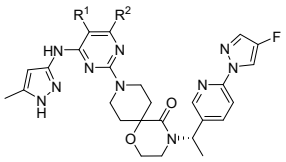
2: X = C



3: n = 1, X = C, Y = N
4: n = 2, X = C, Y = N
5: n = 2, X = N, Y = N
6: n = 2, X = CF, Y = C



7: n =1
8: n = 2



9: R^1 = H, R^2 = Me
10:R^1 = F, R^2 = H

Compound No.	IC_{50} (nM)		
	RET (wt)	RET (V804M)	RET (M918T)
BLU-667	0.78	1.93	0.70
1	1.12	5.28	2.99
2	0.90	1.06	3.09
3	1.58	3.42	ND[a]
4	0.36	0.71	1.14
5	0.68	1.62	0.61
6	1.39	1.95	2.42
7	73.87	133.60	ND[a]
8	92.99	128.70	ND[a]
9	1.29	1.97	0.90
10	19.10	13.50	95.50

[a]ND: not determined.

BLU-667. The activity of compound **2** against M918T mutation was four times lower than BLU-667 with an IC_{50} of 3.09 nM. Encouraged by those results, compound **3-6** were prepared to investigate the effect of the ring size on potencies. To simplify the synthetic route, the cyclohexane ring in **2** was substituted with a piperidine ring in **3-6**. Six-membered lactam ring was found to be superior to five membered lactam ring, since compound **4** was two times more potent than BLU-667 against wild-type RET and V804M mutations (**4** versus **3** and BLU-667). The influence of the ring size is consistent with the modeling result which reveals that the geometry of the inhibitor is crucial for favorable interaction with RET. Pyridine ring of **4** can be substituted with pyrazine ring affording compound **5** which was found to be almost the same potency as BLU-667. Introduction of a fluorine atom to the pyridine ring of **4** slightly decreased the activity by 2 to 3-fold, suggesting subtle electronic effect of the substituent (**5**, **6** versus **4**). Compound **7** and **8** was synthesized by removing the oxygen atom of the amide group of **3** and **4**. However, **7** and **8** were about 100-fold less potent than BLU-667, which indicated that amide might participate in additional hydrogen bond formation although the docking model of BLU-667 against RET did not suggest such interaction. In addition, we synthesized compound **9** to explore the substituent influence of the pyrimidine ring. Compound **9** showed an IC_{50} of 1.29 nM against wild-type RET, which is 2-fold less potent than BLU-667. However, an IC_{50} of 1.97 nM and 0.90 nM

ved, indicating higher selectivity of compound **9** toward RET mutations. The activity of compound **10** dropped dramatically and was 10-fold less potent than compound **9**.

Next, we evaluated the cellular anti-proliferation activities, microsomal metabolic stabilities and permeabilities of highly potent compound **2**, **4**, **5** and **9** (**Table 2**). To our delight, compound **2** showed higher permeability than BLU-667 and the microsomal metabolic stability maintained. Although compound **4** and **5** were more potent toward RET than compound **2**, the high clearance rate in mouse microsomes associated with compound **4** and **5** limited their further developments. The microsomal metabolic stability of **9** was acceptable and it has higher permeability and lower efflux ratio. The higher permeability may be attributed to the decrease of amide NH group, which serves as a hydrogen bond donor.

Table 2. Cell anti-proliferation activity, microsomal metabolic stability and permeability of selected RET inhibitors

Compound No.	Cell assay (nM) [a]	MMS Clint (liver) (mL/min/kg) (H/M)	P_{app} A to B (cm/s), (efflux ratio)
BLU-667	17.0	<8.6/<38.0	1.5, (20.6)
2	ND[b]	<8.6/<38.0	3.5, (7.5)
4	14.0	36.4/155	1.2, (7.4)
5	19.0	31.3/136	2.1, (1.9)
9	19.0	24.1/79.0	4.3, (2.7)

[a]cell activity was test against KIF5B-RET Ba/F3 cell lines. [b]ND: not determined.

Considering that compound **9** had higher activity against RET mutations relative to wild-type RET and better permeability, **9** was further evaluated in mouse PK study (**Table 3**). Compound **9** was found to be equally stable with BLU-667 (Cl = 6.6 mL/kg/min versus Cl = 5.0 mL/kg/min) and had a larger tissue distribution than BLU-667 (V_d = 1.5 L/kg versus V_d = 0.7 L/kg). With a bioavailability of 60%, compound **9** showed an AUC of 27723 nM * h in plasma, which was about three fifth that of BLU-667.

Table 3. Mouse PK of compound **9**

Properties	BLU-667	**9**
Cl (mL/kg/min)	5.0	6.6
Vd (L/kg)	0.7	1.5
AUC (nM * h)[a]	46062	27723
F%	77.2	59.7

[a]AUC (p.o. in 10 mg/kg)

Moreover, Compound **9** was profiled against a panel of kinase to evaluate the selectivity profile (See Supporting Information), and exhibited similar selectivity with BLU-667. It is worth mentioning that the activity of **9** against JAK2 and FLT3 were 2 and 5-fold less potent than BLU-667 (**Table 4**), respectively, potentially suggesting lower 'off-target' toxicity in the future development of **9**.

Table 4. Kinases selectivity profile

Kinase	IC_{50} (nM)[a]	
	BLU-667	**9**

JAK2	2.0	4.4
FLT3	6.5	30.8

[a]IC_{50} was determined using radiolabeled assay

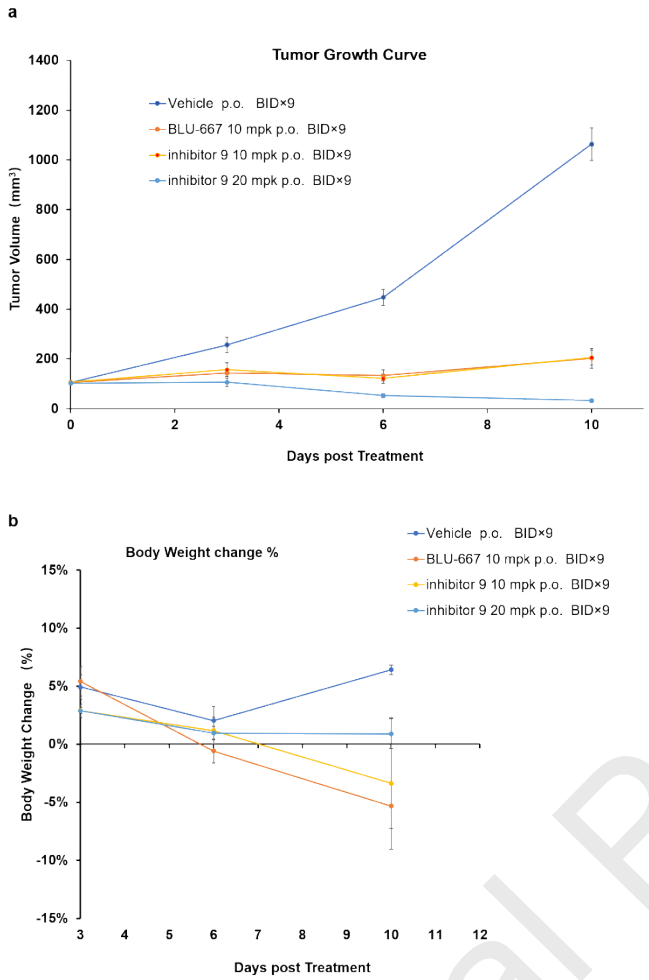


Figure 2. *In vivo* anticancer effect of BLU-667 and **9** in KIF5B-RET Ba/F3 cell line derived xenograft model. (a) Tumor volume changes after treatment of BLU-667 and compound **9**. Values represent the average ± SEM (n = 6 per group). Tumor volume = V_t (measurement of the tumor volume) – V_0 (initial tumor volume). (b) Bodyweight changes of each group (%).

To further assess the effectiveness of compound **9** in suppressing tumor growth, CDX model was developed by transplanting KIF5B-RET Ba/F3 cells subcutaneously in immunodeficient nude/nude mouse. After treating with BLU-667 and compound **9** for 10 days, the tumor growth was remarkably suppressed comparison to that of vehicle group. Compound **9** administered 10 mg/kg (PO QD) produced comparable efficacy with BLU-667 at the same dose and the efficacy of **9** was better at 20 mg/kg dosage with nearly complete tumor regression (**Figure 2a**). The body weights in all four groups were also examined and all study group animals were well tolerated (**Figure 2b**). Moreover, after 10 days treatment, exposure of compound **9** at 8.5 h after administration was determined to be 3935 nM in plasma and 8100 nM/kg in tumor tissue at 10 mg/kg dosage. In contrast to the higher tumor tissue exposure of compound **9**, BLU-667 was found to has higher plasma exposure (6315 nM in plasma and 2522 nmol/kg in tumor at 10 mg/kg dosage) (See Supporting Information), which was consistent with the higher volume distribution of **9** comparable to BLU-667 in mice. Combining the lower potency of

ssue exposure, it's reasonable to speculate that the hematopoietic side effects associated with JAK2 inhibition and high plasma exposure might be alleviated in the future.

In summary, we synthesized a series of selective RET inhibitors based on the structure of BLU-667. The introduction of a spirocyclic scaffold on BLU-667 resulted in conformation restriction and cLogP decrease, which was speculated to be a promising strategy to improve potency and permeability and reduce PGP efflux. To our delight, *in vitro* and *in vivo* studies indicate that compound **9** exhibits higher permeability, tissue distribution, tumor tissue exposure and better kinases selectivity against JAK2 and FLT3, suggesting compound **9** is a promising candidate in RET targeted therapy.

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Declaration of interests

The authors declare no competing financial interest.

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