LETTER TO THE EDITOR

Discovery of novel heteroaryl alkynes for highly potent KIT<sup>D816V</sup> cells inhibition to treat gastrointestinal stromal tumors

**KEYWORDS**
KIT<sup>D816V</sup> cells; Mutation; Drug resistance; Gastrointestinal stromal tumors

To the Editor:

Gastrointestinal stromal tumors (GIST) is the most popular mesenchymal tumor in the gastrointestinal tract with approximately 80% of GIST harboring gain-of-function mutations at either the extracellular region (exon 9) or the juxtamembrane domain (JMD, exon 11) of KIT, resulting in uncontrolled proliferation and resistance to apoptosis<sup>1</sup>. Beyond surgical removal, targeted tyrosine kinase inhibitors (TKI) such as imatinib (<sup>1</sup>), sunitinib (<sup>2</sup>) and regorafenib (<sup>3</sup>) have been approved for the treatment of GIST by inhibiting malignant proliferation driven by KIT. However, these therapies are limited due to the acquisition of polyclonal secondary resistance mutations in ATP pocket (exons 13/14) and activation loop (A-loop, exons 17/18) of KIT.<sup>2</sup> D816V, one of the major mutant forms in A-loop, shifts the KIT activation equilibrium from an inactive to active state and renders it insensitive to the approved treatments<sup>2</sup>. Targeting KIT<sup>D816V</sup> posed a formidable challenge for drug development and only a few multikinase inhibitors (<sup>4</sup>–<sup>6</sup>, Supporting Information Fig. S1) and newly approved drugs (<sup>7</sup>–<sup>8</sup>, Fig. S1) for drug resistant GISTs, show the potential to overcome KIT<sup>D816V</sup> mutants. However, the clinical benefit of these medications is still limited due to their insufficient activity toward the KIT<sup>D816V</sup> mutant as well as unwanted side effects from low selectivity profiles over the wild-type KIT and other kinases. Beyond these approved medications for GIST, there is still an unmet clinical need for highly potent therapeutic agents to overcome drug resistant KIT<sup>D816V</sup> mutant, while minimizing the risk of potential toxicity by reducing the off-target effects. Many efforts (<sup>9</sup>–<sup>12</sup>, <sup>Fig. S1</sup>) have been made to achieve these goals, but so far none of them show highly potent and specific to KIT<sup>D816V</sup> cells and sufficient <i>in vivo</i> anti-tumor efficacy in KIT<sup>D816V</sup> xenograft models.

In this study, we report novel heteroaryl alkynyl compounds that are highly potent and specific against KIT<sup>D816V</sup> cells. The most promising compound, <sup>54</sup> displayed high potency against transformed 32D cell lines bearing KIT<sup>D816V</sup> mutant (GI<sub>50</sub> = 0.7 nmol/L) as well as varies of KIT mutations. Moreover, <sup>54</sup> demonstrated excellent selectivity profiles between KIT<sup>D816V</sup> mutant cells and a panel of cell lines, with no obvious toxicity for 32D normal cells (GI<sub>50</sub> > 1000 nmol/L) and high therapeutic windows of >491.7-fold change related to the KIT<sup>WT</sup> cells (NCI-H526, Mo7e and HMC-1 cells). Also, it showed much lower activities against EGFR<sup>WT</sup> amplified A431 cells and PDGFR-<i>a</i><sup>WT</sup> driven U118MG cells (GI<sub>50</sub> > 1000 nmol/L). In addition, <sup>54</sup> displayed acceptable PK properties and significant <i>in vivo</i> anti-tumor efficacy in 32D KIT<sup>D816V</sup> xenograft mice models with low toxicity. The high potency and specificity, together with favorable druggabilities of <sup>54</sup> indicates that this compound can serve as a promising candidate for treatment of GIST.

1. **Structural optimizations and biological evaluation for potent KIT<sup>D816V</sup> inhibitors**

In our previous work<sup>3</sup>, a series of o-aminopyridyl alkyne CSF-1R inhibitors (such as <sup>13</sup>, <sup>Fig. 1A</sup>) with type II binding modes were identified. On the other hand, according to the switch control concepts<sup>4</sup>, type II inhibitors can also inhibit a variety of KIT mutants, even secondary mutations located in A-loop, such as D816V,
The iterative optimization based on a CSF-1R inhibitor (13) led to the discovery of highly potent KITD816V inhibitor (49).

**Figure 1**  (A) The discovery of novel heteroaryl alkynes (54 and 57) as specific KITD816V inhibitors based on the previous CSF-1R inhibitor 13. (B) Blockage of the activation of KIT and the downstream STAT3 and ERK phosphorylation was analyzed by Western-blotting in 32D KITD816V cells after 4-h treatment with 54 and 57. (C) Antiproliferative effects of compounds 54, 57 and ripretinib against a variety of KIT/mutant-transformed 32D cells and cell lines with amplification of wild-type KIT, PDGFRα and EGFR. (D) PK properties were determined in male ICR (CD-1) mice (n = 3) using LC/MS/MS. (E)–(F) 54 and 57 suppressed the growth of 32D KITD816V xenografts in vivo and effects of these compounds on body weight in the 32D KITD816V xenografts models. Nude mice bearing 32D KITD816V xenografts were randomized into vehicle (n = 10) or treatment (n = 6) groups, and given treatment as indicated. Tumor volume and body weight were measured on the indicated days. *P < 0.05, **P < 0.001 versus vehicle group.
by switching back to its inactive state inducing by the binding of switch inhibition type II inhibitor. Thus, we hypothesized that our type II CSF-1R inhibitors might display inhibition effects against some KIT mutants due to the structural homology with CSF-1R and the precedent established by compounds like 1.

Given the unusual activation profile of KIT that the A-loop is independent on the presence of phosphate and the type II inhibition in general is difficult to reproduce in biochemical assays, the screening was carried out on 32D cell lines transformed with KITD816V mutant. The result showed that compound 13 displayed potential to (GI50 = 1010 nmol/L) inhibit the proliferation of these drug-resistant GIST cells. Subsequently, an iterative medicinal chemistry optimization focused on the “linker”, “tail”, “central ring” and “hinge binder” was carried out to yield heteroaryl alkenes (represented by 54 and 57) as highly potent inhibitors against KITD816V cells (Fig. 1A).

2. Further biological assays of compounds 54 and 57 in vitro

The blockage effects of 54 and 57 on the KIT signaling pathways was confirmed in 32D KITD816V cell lines (Fig. 1B). It was found that these compounds displayed potent inhibition effects against the phosphorylation of KITD816V and the downstream STAT3 and ERK phosphorylation in KITD816V cells, which were more potent than that of ripretinib.

Compounds 54 and 57 was selected for further investigation in a panel of 32D cell lines with broad-spectrum mutants, which are transformed by a variety of clinically important KIT mutants as shown in Fig. 1C. The results showed that these compounds displayed strong potency against these KIT/mutants. Compound 54 showed a low nMolar and even picomolar GI50 value against variants of V559D (GI50 = 0.09 nmol/L), V559D/V654A (GI50 = 1.1 nmol/L), and V559D/N822K (GI50 = 0.8 nmol/L), which was 16.7, 18.8 and 2.2 times more potent than that of ripretinib, respectively. Moreover, the therapeutic windows for wild-type KIT related to these KIT/mutants were determined in ripretinib, respectively. Moreover, the therapeutic windows for wild-type KIT related to these KIT/mutants were determined in ripretinib, respectively.

The results showed that these compounds exhibited considerable inhibitory effects toward the KIT wt amplified cells (Mo7e cells, GI50 = 0.7 nmol/L) and a variety of clinically important KIT mutants including both the primary mutations and secondary mutations. Also, this compound showed superior selectivity profiles over the 32D parental cells, and those cell lines driven by KIT wt, EGFR wt and PDGFR wt with a TGI (tumor growth inhibition) value of 45% and 71%, respectively. As shown in Fig. 1E, compound 57 inhibited the growth of 32D KITD816V xenografts with a TGI (260.1 nmol/L) and short t1/2 (1.34 h). Based on these PK properties, both compounds were administered twice a day (BID) via oral administration to maintain consistent plasma concentrations over a longer period of time and suppress the growth of cancer cells in vivo. Meanwhile, the dosage of 57 was increased appropriately given its relatively lower drug exposure as compared to the 54.

3. The in vivo PK and anti-tumor efficacy evaluations of compounds 54 and 57

The preliminary pharmacokinetic properties of 54 and 57 were evaluated in mice (Fig. 1D). After a single dose administration of these compounds (i.e., 2 mg/kg; p.o., 10 mg/kg), the key PK parameters of these compounds were determined. The results displayed that 54 exhibited the moderate bioavailability (29.9%) and drug exposure (AUClast = 1278 h·ng/mL) as well as the relatively short t1/2 (2.29 h) using 10 mpk oral administration. Whereas compound 57 displayed poor oral PK properties with low drug exposure (AUClast = 233 h·ng/mL) and short t1/2 (1.34 h). Based on these PK properties, both compounds were administered twice a day (BID) via oral administration to maintain consistent plasma concentrations over a longer period of time and suppress the growth of cancer cells in vivo. Meanwhile, the dosage of 57 was increased appropriately given its relatively lower drug exposure as compared to the 54.

In conclusion, starting from our previously reported type II inhibitors of CSF-1R with o-aminopyridinyl alkyne scaffold, an iterative medicinal chemistry optimization was carried out to improve the anti-KITD816V activity, selectivity, and drug-like characteristics, leading to the discovery of the most promising compound 54, with a novel heteroaryl alkyne scaffold. In vitro, 54 potently inhibited the KITD816V variant cells (GI50 = 0.7 nmol/L) and a variety of clinically important KIT mutants including both the primary mutations and secondary mutations. Also, this compound showed superior selectivity profiles over the 32D parental cells, and those cell lines driven by KITwt, EGFRwt and PDGFRwt which could increase its therapeutic benefits for the GIT treatment driven by KIT/mutants and minimize the potential risk of myelosuppression, and other toxicities related to the physiological function of KIT signaling pathways. Further biological assays of compounds 54 and 57 in vivo exhibited acceptable druggabilities and excellent inhibitory efficacy in 32D KITD816V xenograft mouse models, which is resistant to ripretinib. To conclude, this study identifies compound 54 as a promising therapeutic agent for GIST with the potential to overcome clinical resistance induced by KITD816V.
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Author contributions

Zhicheng Xie, Yihao Guo, Taiwen Chen, Xin Li and Yu Zhang carried out the synthetic experiments. Lin Li, Mi Zhang, Yongpeng Li and Xi Zhu carried out the biology experiments. Youhong Hu and Liguang Lou supervised the project. Zhicheng Xie, Lin Li, Youhong Hu and Liguang Lou wrote and finalized the manuscript.

Conflicts of interest

The authors have no conflicts of interest to declare.

Appendix A. Supporting information

Supporting data to this article can be found online at https://doi.org/10.1016/j.apsb.2022.07.015.

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