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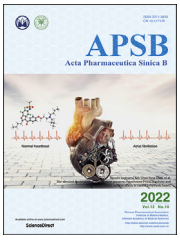

LETTER TO THE EDITOR

Discovery of novel heteroaryl alkynes for highly potent KIT^{D816V} cells inhibition to treat gastrointestinal stromal tumors

KEYWORDS

KIT^{D816V}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 apotosis[1]. Beyond surgical removal, targeted tyrosine kinase inhibitors (TKI) such as imatinib (**1**), sunitinib (**2**) and regorafenib (**3**) 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.

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[2]. Targeting KIT^{D816V} proved a formidable challenge for drug development and only a few multikinase inhibitors (**4**–**6,** Supporting Information Fig. S1) and newly approved drugs (**7**–**8,** Fig. S1) for drug resistant GISTs, show the potential to overcome KIT^{D816V} mutants. However, the clinical benefit of these medications is still limited due to their insufficient activity toward the KIT^{D816V} 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^{D816V} mutant, while minimizing the risk of potential toxicity by reducing the off-target effects. Many efforts (**9**–**12,** Fig. S1) have been made to achieve these goals, but so far none of them show highly potent and specific to KIT^{D816V} cells and sufficient *in vivo* anti-tumor efficacy in KIT^{D816V} xenograft models.

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

1. Structural optimizations and biological evaluation for potent KIT^{D816V} inhibitors

In our previous work[3], a series of *o*-aminopyridyl alkyne CSF-1R inhibitors (such as **13**, Fig. 1A) with type II binding modes were identified. On the other hand, according to the switch control concepts[4], type II inhibitors can also inhibit a variety of KIT mutants, even secondary mutations located in A-loop, such as D816V,

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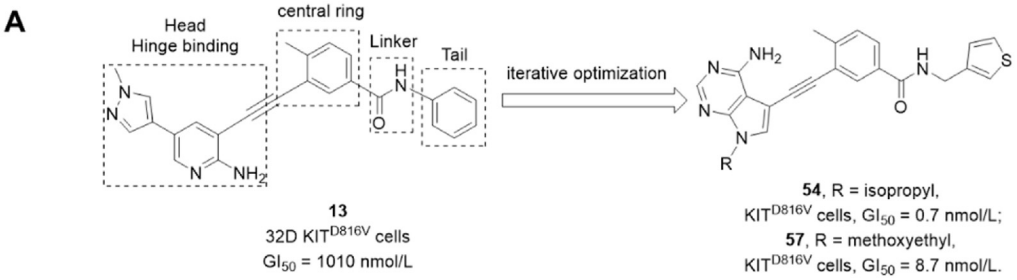

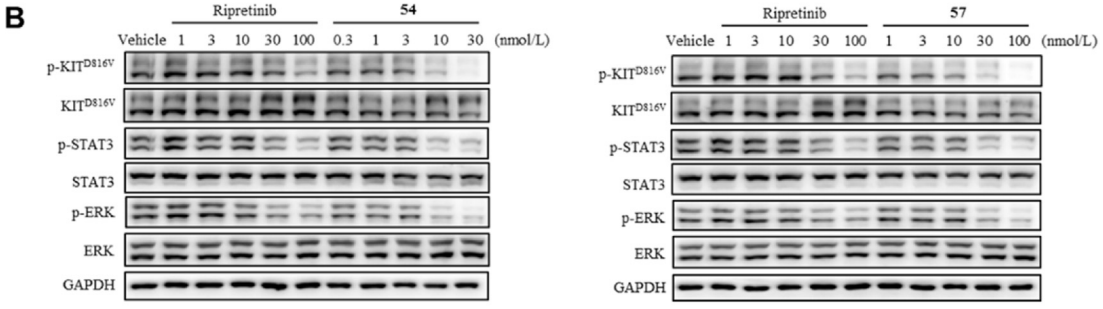

C

No.	32D KIT/mutant cell lines (GI$_{50}$, nmo/L)					cell lines with amplified wild-type kinases (GI$_{50}$, nmol/L)				
						KIT^{wt}			PDGFRα^{wt}	EGFR^{wt}
	D816V	V559D	V559D/ V654A	V559D/ Y823D	V559D/ N822K	NCI-H526	Mo7e	HMC-1	U118MG	A431
54	0.7±0.1	0.1±0.03	1.1±0.4	30.4±1.0	0.8±0.6	>1000	344.2±59.4	>1000	>1000	>1000
57	8.7±2.8	0.2±0.06	16.4±4.1	84.5±18.6	10.5±6.5	>1000	>1000	>1000	>1000	>1000
Ripretinib	12.6±0.4	1.5±0.7	20.7±7.5	2.8±0.7	1.8±0.6	>1000	260.0±52.5	260.1±128.8	268.9±5.5	>1000

D

Compd.	Dose	$t_{1/2}$ (h)	T_{max} (h)	C_{max} (ng/mL)	AUC$_{last}$ (h·ng/mL)	CL$_{_obs}$ (mL/min/kg)	MRT$_{INF_obs}$ (h)	$Vss_{\_obs}$ (mL/kg)	F (%)
54	*p.o.* 10 mpk	2.29	0.42	579	1278	-	3.40	-	29.9
	i.v. 2 mpk	1.17	-	-	855	39.9	0.81	1908	
57	*p.o.* 10 mpk	1.34	0.25	308	233	-	0.49	-	8.8
	i.v. 2 mpk	0.36	-	-	529	63.3	0.31	1189	

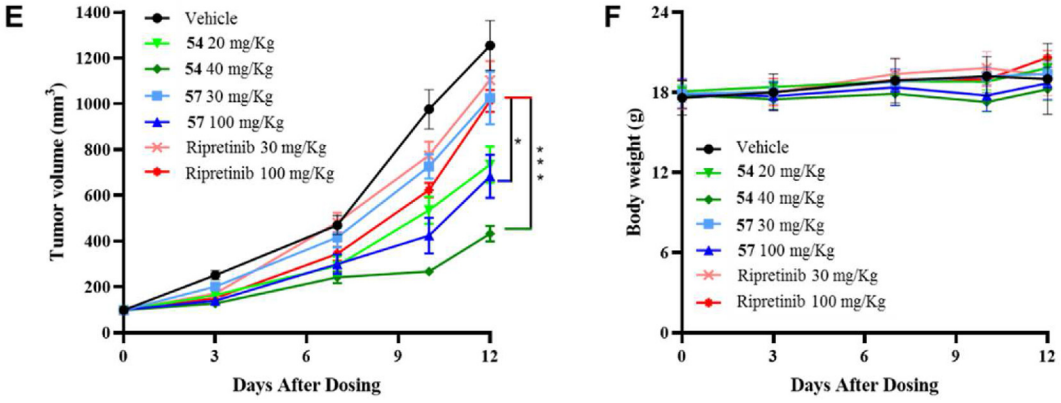

Figure 1 (A) The discovery of novel heteroaryl alkynes (**54** and **57**) as specific KIT^{D816V} inhibitors based on the previous CSF-1R inhibitor **13**. (B) Blockage of the activation of KIT and the downstream STAT3 and ERK phosphorylation was analyzed by Western-blotting in 32D KIT^{D816V} cells 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 KIT^{D816V} xenografts tumor *in vivo* and effects of these compounds on body weight in the 32D KIT^{D816V} xenografts models. Nude mice bearing 32D KIT^{D816V} 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[5] 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 KIT^{D816V} mutant. The result showed that compound **13** displayed potential to (GI_{50} = 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 alkynes (represented by **54** and **57**) as highly potent inhibitors against KIT^{D816V} 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 KIT^{D816V} cell lines (Fig. 1B). It was found that these compounds showed potent inhibition effects against the phosphorylation of KIT^{D816V} and the downstream STAT3 and ERK phosphorylation in KIT^{D816V} 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 nomolar and even picomolar GI_{50} value against mutants of V559D (GI_{50} = 0.09 nmol/L), V559D/V654A (GI_{50} = 1.1 nmol/L), and V559D/N822K (GI_{50} = 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 those cells with amplified wild-type KIT, including NCI-H526, Mo7e and HMC-1 cells. Interestingly, compared to the KIT/mutants cell lines, **54** and **57** displayed much lower potency to these cell lines, indicating the enhanced therapeutic outcome for GIST treatment driven by KIT/mutants and minimized the potential risk of myelosuppression, and other toxicities related to the physiological function of KIT signaling pathways. Also, further investigation of selectivity profiles showed that these compounds displayed relatively low activities against a panel of cell lines dependent on other kinases, including $EGFR^{wt}$, $PDGFR\alpha^{wt}$ with favorable therapeutic windows of more than 1428.6-fold comparing to the KIT^{D81V} mutant cells. Whereas, ripretinib exhibited considerable inhibitory effects toward the KIT^{wt} amplified cells (Mo7e cells, GI_{50} = 260.6 nmol/L; HMC-1 cells, GI_{50} = 260.1 nmol/L) and the $PDGFR\alpha^{wt}$ amplified cells (U118MG cells, GI_{50} = 268.9 nmol/L), showing relatively low therapeutic windows to the KIT/mutants.

Given the high cellular potency and therapeutic windows of **54**, it was selected for further evaluation of kinome selectivity against a panel of 253 kinases (Supporting Information Table S6). Unfortunately, the molecular data did not match the cellular data. Compound **54** showed low inhibitory potency against KIT^{D816V} while displayed strong potency against KIT^{wt} and other KIT mutations (including D816H, V560G and V654A) at 1 μmol/L. We reasoned that **54** serving as a switch inhibitor, the inhibitory potency against KIT^{D816V} was closely dependent on the kinase conformation change, which was restricted in this molecular assay utilizing the non-natural KIT^{D816V} protein[5]. Further cellular thermal shift assay (CETSA) was carried out to confirm the on-target binding of compounds **54** and **57** (Supporting Information Fig. S2).

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.v., 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 (AUC_{last} = 1278 h·ng/mL) as well as the relatively short $t_{1/2}$ (2.29 h) using 10 mpk oral administration. Whereas compound **57** displayed poor oral PK properties with low drug exposure (AUC_{last} = 233 h·ng/mL) and short $t_{1/2}$ (1.34 h). Based on these PK properties, both compounds were administrated 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**.

The *in vivo* antitumor activity of compounds **54** and **57** was further investigated in 32D KIT^{D816V} xenograft mice models. Each group of mice were repeatedly administrated vehicle or compounds **54**, **57** and ripretinib twice a day *via* intragastric administration for 12 continuous days. As shown in Fig. 1E, compound **54** (20, 40 mg/kg, i.g., BID × 12) dose-dependently inhibited the growth of 32D KIT^{D816V} xenografts with a TGI (tumor growth inhibition) value of 45% and 71%, respectively. Compound **57** (30, 100 mg/kg, i.g., BID × 12) suppressed the growth of 32D KIT^{D816V} xenografts by 50% at high dose group (100 mg/kg). However, ripretinib (30, 100 mg/kg, i.g., BID × 12) showed no apparent efficacy in the 32D KIT^{D816V} xenograft mice models. In addition, no mortality or obvious body weight loss was observed during the whole period of treatment (Fig. 1F). Collectively, these results indicated that compounds **54** and **57** displayed significant *in vivo* anti-tumor efficacy in 32D KIT^{D816V} xenografts, which was much better than that of ripretinib.

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-KIT^{D816V} activity, selectivity, and drug-like characteristics, leading to the discovery of the most promising compound **54**, with a novel heteroaryl alkynyl scaffold. *In vitro*, **54** potently inhibited the KIT^{D816V} mutant cells (GI_{50} = 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 $PDGFR^{wt}$ which could increase its therapeutic benefits for the GIST treatment driven by KIT/mutants and minimize potential off-target toxicity related to the physiological function of these kinases signaling pathways. *In vivo*, **54** exhibited acceptable druggabilities and excellent inhibitory efficacy in 32D KIT^{D816V} xenograft mice model, 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 KIT^{D816V}.

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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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