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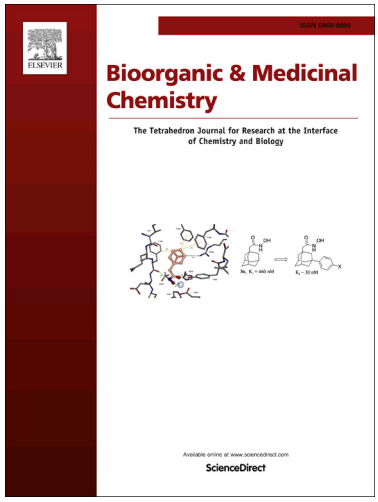

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Discovery of thieno[3,2-c]pyridin-4-amines as novel Bruton's tyrosine kinase (BTK) Inhibitors

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Footnotes

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Abstract: A novel series of BTK inhibitors bearing thieno[3,2-c]pyridin-4-amine framework as the core scaffold were designed, synthesized and well characterized. In this paper, twenty one compounds displayed variant inhibitory activities against BTK in vitro, and compound **14g** showed the most potent inhibitory activity agianst BTK enzyme, with the IC_{50} value of 12.8 nM. Moreover, compounds **14g** displayed relatively good kinase selectivity and was subsequently evaluated in vivo for profiling its PK properties. This work identified the thieno[3,2-c]pyridin-4-amine derivatives as novel BTK inhibitors and verified the value of thieno[3,2-c]pyridin-4-amine scaffold in drug design.

Keywords: BTK inhibitors, thieno[3,2-c]pyridin-4-amine, inhibitory activity, kinase selectivity.

1. Introduction

Bruton's tyrosine kinase (BTK) is a member of Tec family of cytoplasmic mammalian non-receptor tyrosine kinases. It is a crucial terminal kinase in the B cell receptor (BCR) signaling pathway and essential for the development and activation of B cells. It has been shown that aberrant activation of B cells plays a central role in the pathogenesis of B-cell lymphomas and various autoimmune diseases. Therefore, inhibition of BTK has become an attractive and potential therapeutic approach for the treatment of human diseases associated with B cells lymphoproliferative disorders and autoimmune disorders, such as hematological malignancies and rheumatoid arthritis (RA) [1-2].

During recent years, a number of research groups are eager to pursue small molecular BTK inhibitors, and many BTK-targeting agents have progressed to clinical stages. Among these, ibrutinib (**1**, PCI-32765, Imbruvica™) is the most successful BTK inhibitor, which was developed by Pharmacyclics and approved by US FDA for the treatment of mantle cell lymphoma (MCL) and chronic lymphocytic leukemia (CLL) in the past 2 years. And now, it is also ongoing in the clinical evaluation for other indications [3]. In addition, several BTK inhibitors including spebrutinib (**2**, CC-292, AVL-292), ONO-4059 (**3**) and ACP-196 are currently moved into the phase II clinical evaluation, and several molecules including MSC-2364447, PRN1008, BGB-3111 and HM-71224 are quickly advanced into phase I clinical trials, although most of their structures are still not disclosed [4-6]. Except that, there are some agents including CGI-1746, GDC-0834, RN-486 (**4**) reported in discovery stage [7-15]. Recently, we have also reported our medicinal chemistry work on the discovery of BTK inhibitors, which are structurally similar to RN-486, such as compounds **5** and **6** [16-17]. In general, the reported small molecular BTK inhibitors can be classified into two classes (irreversible inhibitors and reversible inhibitors) by their binding modes with the BTK catalytic domains. Irreversible BTK inhibitors contain a Michael addition receptor moiety in the structures which can form a covalent bind with the conserved cysteine-481 residue of BTK enzyme to achieve strong binding [1]. The agents including ibrutinib, spebrutinib and ONO-4059 belong to this class. It is believed that irreversible BTK inhibitors can achieve significant clinical benefit for treating hematological malignancies. For example,

ibrutinib shows significant progression free survival benefit and overall survival benefit in both MCL and CLL clinical trials. Nevertheless, it is thought reversible BTK inhibitors might be more appropriate for treating RA. RA is different from hematological malignancies, and is non-life-threatening disease. However, the irreversible inhibitors might give some unexpected off-target toxicity because of the potential of covalently binding to non-target proteins and achieving the drug–protein conjugate. Thus, the development of reversible BTK inhibitors would be more suitable to fulfill the treatment need for non-life-threatening RA disease [18-19]. Among the reported reversible BTK inhibitors, 5-phenylpyrazinone-based or 5-phenylpyridinone-based inhibitors, such as GDC-0834 and RN-486, demonstrated high BTK inhibitory potency and excellent selectivity. However, GDC-0834 was suspended in phase I because of poor pharmacokinetic properties, and RN486 was not any progressed in the past 3 years [4]. Thus, there is still eagerly need to develop new classes of reversible BTK inhibitors.

(Insert Figure 1 and title for Figure 1 here)

Ibrutinib is a selective irreversible BTK inhibitor, showing very high potency against BTK (IC_{50} = 0.5 nM). Ibrutinib can irreversibly bind to BTK because of the acrylamide moiety which can form a covalent bind with the conserved cysteine-481 residue of BTK. In fact, ibrutinib was structurally optimized from the precursor compound PCI-29732, which was reported to be another potent BTK inhibitor (IC_{50} = 8.2 nM). Due to lack of Michael addition receptor moiety, PCI-29732 is regard to non-covalently bind to BTK enzyme. PCI-29732 is chemically similar to compound B43. The publicly available crystal structure of B43 with BTK (PDB 3GEN) disclosed the binding mode of this type of reversible BTK inhibitors [20-21]. Generally, the backbone of 4-amino pyrrolopyrimidine occupies the ATP binding pocket and makes several important interactions with the hinge. The 4-amine directly forms two H-bonds with the gatekeeper Thr-474 and the backbone Glu-475. The N-3 nitrogen of the pyrimidine participates in an H-bond with Met-477, and the phenoxyphenyl group enters a hydrophobic pocket and forms a face-to-edge π-stacking interaction with Phe-540. These interactions above are critical for BTK activity. Bioisosteric replacement is a highly attractive method in the drug design and

discovery, and we have used this strategy to discover several novel chemical series in hedgehog inhibitors programs [22]. Thieno[3,2-c]pyridines are very valuable heterocyclic scaffolds which are abundantly described as central structural motifs in the design of kinase inhibitors [23]. Based on the deeply understanding of the binding mode of B43 with BTK enzyme and the principles of bioisosterism, we designed a novel series of BTK inhibitors containing thieno[3,2-c]pyridin-4-amine scaffold. In the new designed series, thieno[3,2-c]pyridin-4-amine group are expected to occupy the ATP binding pocket and keep the key hydrogen bonds, and the C3-phenoxyphenyl and its bioisosteric groups can form the π-stacking interaction. Meanwhile, we tried to introduce alkyl and aryl groups on the C7-position of thieno[3,2-c]pyridines to explore the SAR of the thieno[3,2-c]pyrid-4-amine scaffold. To the best of our knowledge, there is the first time to describe the SARs on this position. Herein, we report our recent effort on the synthesis and SARs of this series of BTK inhibitors.

(Insert Figure 2 and title for Figure 2 here)

2. Chemistry

As summarized in Table 1, the thieno[3,2-c]pyridin-4-amine derivatives (**12a–h, 14a-i, 19a-b and 24a-b**) were designed and synthesized. The synthetic routes for all target compounds are illustrated in Schemes **1**–**2**.

Compounds **12a–h** and **14a-i** were synthesized according to Scheme **1**. Commercially available 3-bromothieno[3,2-c]pyridin-4-amine **9** was treated with (4-phenoxyphenyl)boronic acid under palladium catalysis to give the Suzuki coupling product **10** in 96 % yield. Iodination of **10** using N-iodosuccinimide afforded the key intermediate **11** in 70 % yield. Then treating **11** with various boronic acid reagents under the Suzuki coupling condition provided the target compounds **12a-h** as well as the intermediate **13** in 35-90 % yields. Deprotection of **13** with trifluoroacetic acid provided the target compound **14a** in 92 % yield, which was subsequently treated with various halides under alkaline condition to produce target compound **14b-i** in 40-95 % yields.

(Insert Scheme 1 and legend for Scheme 1 here)

Compounds **19a–b** and **24a-b** were prepared according to Scheme **2**. Condensation of the commercially available boronobenzoic acid (**15a-b**) and aniline gave **16a-b**, which were subsequently reacted with 3-bromothieno[3,2-c]pyridin-4-amine **9** using Suzuki coupling protocols to generate **17a-b**. Treating **17a-b** with N-iodosuccinimide provided the iodides **18a-b**, followed by Suzuki coupling reaction to afford the target compounds **19a-b**. Likewise, the similar synthetic routes were employed to synthesize compounds **24a-b**. Starting from the commercially available 4-aminophenylboronic acid pinacol ester (**20**) and m-chlorobenzoyl chloride, the desired condensation product **21** was obtained, which was followed by Suzuki coupling reaction, iodination and again Suzuki coupling reaction, leading to the target compounds **24a-b**.

(Insert Scheme 2 and legend for Scheme 2 here)

3. Result and discussion

3.1. In vitro enzymatic assay of the new compounds against BTK kinase

The newly synthesized thieno[3,2-c]pyridin-4-amine derivatives (**12a–h, 14a-i, 19a-b and 24a-b**) were evaluated for their abilities to inhibit the BTK enzymatic activity. The vitro IC_{50} values are summarized in Table **1**.

(Insert Table 1 and title for Table 1 here)

Among the thieno[3,2-c]pyridin-4-amine series, when the six-membered ring aryl group were introduced at the C7-position, such as phenyl (**12a**), 4-pyridyl (**12b**), 4-morpholinyl substituted phenyl (**12c**), 4-phenoxy substituted phenyl (**12d**), and 6-methoxynaphthalenyl (**12e**), the resulting compounds almost lost inhibitory effect against BTK (IC_{50} > 1000 nM). However, the five-membered ring aryl substituted compounds **12f** and **14a** displayed weak potency against BTK. C7-furan-3-yl alogue **12f** showed an IC_{50} value of 767.5 nM, while C7-1H-pyrazol-4-yl alogue **14a** was weaker than **12f**, with an IC_{50} value of 1052 nM. The introduction of three-membered ring of cyclopropyl (**12g**) or linear alkyl of n-butyl (**12h**) in the C7-position resulted in BTK inhibitory activity lost (IC_{50} > 10000 nM, both). This initial SAR trend appeared that five-membered aryl substituted at C7-position was a preference. Subsequently, a survey of several substituents

at the N1-position of pyrazolyl was investigated. Interestingly, Compounds **14b** bearing N1-methyl group showed an IC_{50} value of 107.4 nM, which was 10 fold more potent than no-substituted pyrazolyl **14a**, while N1-methyl was changed into bulky alkyl groups such as ethyl (**14c**), n-butyl (**14d**), benzyl (**14e**) or methoxypropyl (**14f**) afforded reduced BTK inhibitory activity, compared to **14b**. However, unexpectedly, compound **14g** bearing a 2-(methylamino)-2-oxoethyl at N1-position of pyrazolyl exhibited a higher potency against BTK, with an IC_{50} value of 12.8 nM. Compared with these, 2-(methylamino)-2-oxoethyl (**14g**) produced increased activity likely due to the amide or the carbonyl group. An attempt of replacement of 2-(methylamino)-2-oxoethyl with dimethylaminosulfuryl (**14h**) and acetyl (**14i**) led to reduced potency, which appeared that the position of the amide or the carbonyl group was critical for BTK inhibitory activity. N-(2-(methylamino)-2-oxoethyl)-1H-pyrazol-1-yl moiety at the C7-position may provide an extra interaction with BTK residue, thereby showing the higher inhibitory activity. Based on the best two substituents at the C7-position, the C3-position modification was attempted to carry out. The replacement of the oxygen linker of C3-phenoxyphenyl with an amide afforded compounds **19a** and **19b**, as well as **24a** and **24b**. However, unfortunately, these compounds showed reduced inhibitory activities. This finding suggested that the π-electron properties of the subsutituents on the C3-position of thieno[3,2-c]pyrid-4-amine scaffold significantly affected the BTK inhibitory activity.

3.2. Kinase selectivity assay

From the BTK inhibition results above, compound **14b** and **14g** were picked out as the representative BTK inhibitors among the thieno[3,2-c]pyridin-4-amine series, and they were further evaluated for their inhibitory activities against 4 kinds of our in-house RTKs. The data were summarized in Table **2**. Compared to BTK enzyme, both compounds only showed moderate inhibitory activities against KDR kinase, and moreover, compound **14g** displayed 10-fold more selective against BTK than KDR. This result indicated that **14g** was a relatively selective BTK inhibitor, and deserving further identification against other kinase profiles.

(Insert Table 2 and title for Table 2 here)

3.3. In vivo pharmacokinetic (PK) profiles

Compound **14g** stood out as the most potent BTK inhibitor in this thieno[3,2-c]pyridin-4-amine series, with an IC_{50} value of 12.8 nM. Therefore, it was further investigated by profiling its pharmacokinetic (PK) properties in vivo. Table **3** illustrated the PK profiles of compound **14g** in SD rats, by iv (2.5 mg/kg) and po (10 mg/kg) administration. Following intravenous injection with 2.5 mg/kg in SD rats, **14g** showed a satisfactory exposure (AUC = 1459.1 hr*ng/mL) and volume of distribution (Vz = 900 mL/kg), but a short half-time ($T_{1/2}$ = 0.36 h). The short elimination phase half life was probably due to its large systemic clearance (Cl = 1716.4 mL/hr/kg). Subsequently, compound **14g** was further evaluated by oral administration of 10 mg/kg dose. It was found the drug was also quickly eliminated, with large systemic clearance of 8670.1 mL/hr/kg and short half time ($T_{1/2}$ = 1.01 h), although the area-under-curve (AUC = 1365.7 hr*ng/mL) and the oral bioavailability (F = 23.4 %) are acceptable in this dose. The PK properties of compound **14g** suggested that some groups in this structure maybe easily be metabolized and eliminated, thereby exhibiting a short half-life. Thus, in the next structural modification, efforts should be made for reducing the metabolic effect and optimizing the PK characteristics, meanwhile, increasing the in vitro biological activities.

(Insert Table 3 and title for Table 3 here)

3.4. Docking study

In an effort to elucidate the binding mode for this kind of thieno[3,2-c]pyridin-4-amines, We performed the molecular docking of compound **14g** with BTK enzyme (PDB code 3GEN). The CDOCKER program of the Discovery Studio 2.5 software package was used, and the binding model of compound **14g** with BTK enzyme was shown in Figure **3**. It was found that the entire structure of **14g** was favorably located in the BTK pocket. The 4-amino group of thieno[3,2-c]pyridine fragment of **14g** formed two hydrogen bonds with Thr-474 and Glu-475, respectively. Furthermore, the N atom in the 5-position of thieno[3,2-c]pyridine generated another hydrogen bonding interaction with the backbone of Met-477 in the BTK kinase hinge

region. These three hydrogen bonds were very critical for maintaining the BTK inhibitory activity. Meanwhile, the N' atom in the 2-position of pyrazolyl group substituted at C7-position of thieno[3,2-c]pyridin-4-amine formed a hydrogen bond with the water-36, which was similar to **B43** with BTK. Interestingly, it was noticeable that the carbonyl of 2-(methylamino)-2-oxoethyl group in 1-position of pyrazolyl could afford a hydrogen bond interaction with Cys-481, which might contribute to the higher BTK inhibition of compound **14g** in the series. Overall, the docking results afforded some understandings on the observed BTK inhibitory activities for the thieno[3,2-c]pyridin-4-amines.

(Insert Figure 3 and title for Figure 3 here)

4. Conclusion

In this paper, we have developed a novel series of BTK inhibitors bearing thieno[3,2-c]pyridin-4-amine framework as the core scaffold. Twenty one compounds were design, synthized, and evaluated. These compounds displayed variant inhibitory activities against BTK in vitro, and compound **14g** gave the highest potency with the IC_{50} value of 12.8 nM. Moreover, compounds **14g** displayed relatively good kinase selectivity. However, in vivo evaluation showed that compound **14g** had poor PK properties. Thus, further structural modification is deserved to be made for improving the PK characteristics, as well as increasing the in vitro biological activities.

5. Experimental

5.1 Chemistry

All chemical reagents were purchased from commercial vendors and used without further purification unless noted especially. The melting points for the compounds were performed on a Melt-Temp II apparatus and uncorrected. ^{1}H NMR spectra (400 MHz) and ^{13}C NMR (100 MHz) spectra data were recorded in $CDCl_3$ or DMSO-d_6 on a Bruker BioSpin AG (Ultrashield Plus AV 400) spectrometer. MS data were recorded at an Agilent-6120 quadrupole LC/MS (ESI) while HRMS were recorded at a Water Q-Tof micro mass spectrometer. The HPLC study for the compounds was verified using a

mixture of solvent (methanol/water or acetonitrile/water) at the flow rate of 2 mL/min and peak detection at 254 nm under UV. Column chromatography was carried out on silica gel (200–300 mesh) purchased from Qindao Ocean Chemical Company of China. Thin-layer chromatography (TLC) analyses were carried out on silica gel GF254. Ibrutinib was synthesized according to the reference literature.

3-(4-Phenoxyphenyl)thieno[3,2-c]pyridin-4-amine (10). A solution of 3-bromothieno[3,2-c]pyridin-4-amine (3 g, 13 mmol), $Pd(PPh_3)_4$ (1.5 g, 1.3 mmol) and (4-phenoxyphenyl)boronic acid (3.06 g, 14.3 mmol) in toluene (20 mL) was degassed with nitrogen for 5 min followed by addition of EtOH (4 mL), H_2O (2 mL) and Na_2CO_3 (3.5 g, 32.5 mmol) under continuous flow of nitrogen. The reaction mixture was stirred at 90 ^oC for 2 h. The reaction mixture was cooled, filtered through celite, diluted with water (45 mL), and extracted with (3 × 60 mL) ethyl acetate. The combined organic layers were dried over sodium sulfate and were concentrated in vacuo, the crude product was purified on a silica gel column using (20-80 % ethyl acetate/hexanes) as eluent to afford **10** (4.0 g, 96 %) as a white solid. MS m/z 319.1 $[M+H]^+$; ^1H NMR (400 MHz, DMSO-d_6) δ ppm 7.84-7.82 (d, *J*=5.60 Hz, 1H), 7.59 (s, 1H), 7.48-7.43 (m, 4H), 7.27-7.26 (d, *J*=5.60 Hz, 1H), 7.22-7.18 (t, *J*=14.4 Hz, 1H), 7.14-7.10 (t, *J*=15.6 Hz, 4H), 5.43 (brs, 2H).

7-Iodo-3-(4-phenoxyphenyl)thieno[3,2-c]pyridin-4-amine (11). A solution of 3-(4-phenoxyphenyl)thieno[3,2-c]pyridin-4-amine (2.5 g, 7.9 mmol) and N-Iodosuccinimide (2.14 g, 9.5 mmol) in DMF (10 mL) was stirred at r t for 2 h. After completion of reaction, the reaction mixture was diluted with the mixed ice and water, the precipitated solid was filtered off and the crude product was purified on a silica gel column using (20-80 % ethyl acetate/ petroleum ether) as eluent to afford **11** (2.4 g, 70.0 %) as a yellow solid. MS m/z 445.0 $[M+H]^+$; ^1H NMR (400 MHz, DMSO-d_6) δ ppm 8.02 (s, 1H), 7.57 (s, 1H), 7.48-7.42 (m, 4H), 7.22-7.18 (t, *J*=14.8 Hz, 1H), 7.14-7.10 (t, *J*=15.2 Hz, 4H), 5.61 (brs, 2H).

3-(4-Phenoxyphenyl)-7-phenylthieno[3,2-c]pyridin-4-amine (12a). A solution of 7-iodo-3-(4-phenoxyphenyl)thieno[3,2-c]pyridin-4-amine (100 mg, 0.225 mmol), $Pd(PPh_3)_4$ (26 mg, 0.0225 mmol) and phenylboronic acid (33 mg, 0.27 mmol) in Ethylene glycol dimethyl ether (10 mL) was degassed with nitrogen for 5 min followed

by addition of H_2O (2 mL) and Na_2CO_3 (72 mg, 0.675 mmol) under continuous flow of nitrogen. The reaction mixture was stirred at 80 ^oC for 3 h. The reaction mixture was cooled, filtered through celite, diluted with water (45 mL), and extracted with (3 × 20 mL) ethyl acetate. The combined organic layers were dried over sodium sulfate and were concentrated in vacuo, the crude product was purified on a silica gel column using (20-50 % ethyl acetate/ petroleum ether) as eluent to afford **12a** (71 mg, 80.1 %) as a white solid. Mp:172-173.4 ^oC; MS m/z 395.1 $[M+H]^+$; ^1H NMR (400 MHz, $CDCl_3$) δ ppm 7.99 (s, 1H), 7.66 (s, 1H), 7.52-7.39 (m, 9H) 7.20-7.18 (t, *J*=14.8 Hz, 1H), 7.13-7.09 (t, *J*=15.2 Hz, 4H), 4.86 (brs, 2H); HPLC 95.4 %; HRMS (ESI) m/z calcd for $C_{25}H_{18}N_2OS$ $[M+H]^+$. 395.1140, found 395.1218.

3-(4-Phenoxyphenyl)-7-(pyridin-4-yl)thieno[3,2-c]pyridin-4-amine (12b). 12b was synthesized by the general method described above compound **12a** (27 mg, 66.3 %). Mp: 230.2-231.8 ^oC; MS m/z 396.1 $[M+H]^+$; ^1H NMR (400 MHz, $CDCl_3$) δ ppm 8.72-8.71 (d, *J*=5.56 Hz, 2H), 8.04 (s, 1H), 7.63-7.62 (d, *J*=5.52 Hz, 2H), 7.45-7.39 (dd, *J*=8.44 Hz, *J*=7.64 Hz, 4H), 7.20-7.19 (t, *J*=4.44 Hz, 2H), 7.12-7.10 (d, *J*=8.56 Hz, 4H), 4.87 (brs, 2H); HPLC 99.4 %; HRMS(ESI) m/z calcd for $C_{24}H_{18}N_3OS$ $[M+H]^+$. 396.1092, found 396.1172.

7-(4-Morpholinophenyl)-3-(4-phenoxyphenyl)thieno[3,2-c]pyridin-4-amine (12c). 12c was synthesized by the general method described above compound **12a** (28 mg, 67.1 %). Mp: 207.7-209.4 ^oC; MS m/z 480.2 $[M+H]^+$; ^1H NMR (400 MHz, DMSO-d_6) δ ppm 7.85 (s, 1H), 7.62 (s, 1H), 7.53-7.43 (m, 7H), 7.22-7.21 (t, *J*=8.54 Hz, 1H), 7.15-7.07 (m, 5H), 5.45 (brs, 2H), 3.78-3.76 (t, *J*=8.00 Hz, 4H), 3.19-3.17 (t, *J*=8.00 Hz, 4H); HPLC 95.4 %; HRMS (ESI) m/z calcd for $C_{29}H_{25}N_3O_2S$ $[M+H]^+$. 480.1740, found 480.1756.

3,7-Bis(4-phenoxyphenyl)thieno[3,2-c]pyridin-4-amine (12d). 12d was synthesized by the general method described above compound **12a** (22 mg, 76.1 %). Mp: 185.5-187.3 ^oC; MS m/z 487.2 $[M+H]^+$; ^1H NMR (400 MHz, $CDCl_3$) δ ppm 7.92 (s, 1H), 7.63-7.61 (d, *J*=8.04 Hz, 2H), 7.46-7.43 (d, *J*=8.34 Hz, 2H), 7.42-7.36 (dd, *J*=8.00 Hz, *J*=8.00 Hz,4H), 7.20-7.17 (t, *J*=13.20 Hz, 2H), 7.15-7.10 (m, 8H); HPLC 95.4 %; HRMS (ESI) m/z calcd for $C_{31}H_{22}N_2O_2S$ $[M+H]^+$. 487.1478, found 487.1482.

7-(6-Methoxynaphthalen-2-yl)-3-(4-phenoxyphenyl)thieno[3,2-c]pyridin-4-amine (12e). 12e was synthesized by the general method described above compound **12a** (32 mg, 72.1 %). Mp: 205.1-207.2 °C; MS m/z 475.2 $[M+H]^+$; ^1H NMR (400 MHz, $CDCl_3$) δ ppm 8.06-8.05 (d, *J*=3.4 Hz, 2H), 7.87-7.85 (d, *J*=8.04 Hz, 1H), 7.82-7.80 (t, *J*=8.41 Hz, 1H), 7.77-7.75 (d, *J*=8.00 Hz, 1H), 7.47-7.45 (d, *J*=8.20 Hz, 2H), 7.42-7.39 (t, *J*=12.18 Hz, 2H), 7.21-7.16 (dd, *J*=6.04 Hz, *J*=4.84 Hz, 4H), 7.12-7.10 (dd, *J*=2.32 Hz, *J*=2.46 Hz, 4H), 4.75 (brs, 2H), 3.96 (s, 3H); HPLC 97.7 %; HRMS (ESI) m/z calcd for $C_{30}H_{22}N_2O_2S$ $[M+H]^+$. 485.1474, found 485.1485.

7-(Furan-2-yl)-3-(4-phenoxyphenyl)thieno[3,2-c]pyridin-4-amine (12f). 12f was synthesized by the general method described above compound **12a** (38 mg, 63.0 %). Mp: 163.4-165.1 °C; MS m/z 385.1 $[M+H]^+$; ^1H NMR (400 MHz, $CDCl_3$) δ ppm 8.05 (s, 1H), 7.89 (s, 1H), 7.56 (s, 1H), 7.44-7.38 (dd, *J*=8.04 Hz, *J*=8.46 Hz, 4H), 7.21-7.18 (t, *J*=12.04 Hz, 2H), 7.11-7.08 (dd, *J*=3.20 Hz, *J*=4.00 Hz, 4H), 6.83 (s, 1H), 4.74 (brs, 2H); HPLC 97.5 %; HRMS (ESI) m/z calcd for $C_{23}H_{16}N_2O_2S$ $[M+H]^+$. 385.1005, found 385.1018.

7-Cyclopropyl-3-(4-phenoxyphenyl)thieno[3,2-c]pyridin-4-amine (12g). 12g was synthesized by the general method described above compound **12a** (21 mg, 42.0 %). Mp: 180.5-182.4 °C; MS m/z 359.1 $[M+H]^+$; ^1H NMR (400 MHz, $CDCl_3$) δ ppm 7.69 (s, 1H), 7.40 (s, 4H), 7.15 (s, 2H), 7.09 (s, 4H), 4.66 (brs, 2H), 1.97 (s, 1H), 0.97 (s, 2H), 0.75 (s, 2H). HPLC 95.8 %; HRMS (ESI) m/z calcd for $C_{22}H_{18}N_2OS$ $[M+H]^+$. 359.1212, found 359.1211.

7-Butyl-3-(4-phenoxyphenyl)thieno[3,2-c]pyridin-4-amine (12h). 12h was synthesized by the general method described above compound **12a** (21 mg, 42.0 %). Mp: 147.5-148.6 °C; MS m/z 375.2 $[M+H]^+$; ^1H NMR (400 MHz, $CDCl_3$) δ ppm 7.72 (s, 1H), 7.43-7.37 (dd, *J*=8.04 Hz, *J*=7.82 Hz, 4H), 719-7.15 (t, *J*=15.60 Hz, 1H), 7.12 (s, 1H), 711-7.07 (t, *J*=15.80 Hz, 4H), 4.56(br s, 2H), 719-7.15 (t, *J*=15.60 Hz, 1H), 2.78-2.75(t, *J*=12.00 Hz, 2H), 1.77-1.70 (m, 2H), 1.45-1.38 (m, 2H), 0.98-0.95 (q, *J*=12.40 Hz, 3H); HPLC 95.4 %; HRMS (ESI) m/z calcd for $C_{23}H_{22}N_2OS$ $[M+H]^+$. 375.1526, found 375.1538.

Tert-butyl 4-(4-amino-3-(4-phenoxyphenyl)thieno[3,2-c]pyridin-7-yl)-1H-pyrazole

-1-carboxylate (13). A solution of **11** (150 mg, 0.337 mmol), 1-Boc-pyrazole-4-boronic acid pinacol ester (100 mg, 0.337 mmol) and $Pd(dppf)Cl_2 \cdot CH_2Cl_2$ (27 mg, 0.0337 mmol) in 1,4-dioxane (10 mL) was degassed with nitrogen for 5 min followed by addition Na_2CO_3 (107 mg, 2M in water) under continuous flow of nitrogen. The reaction mixture was stirred at 80 ^oC for 1 h, the catalyst was removed by filtration through Celite and the filtrate concentrated to a residue. The residue was used in the next reaction without further purification. MS m/z 485.1 $[M+H]^+$.

3-(4-Phenoxyphenyl)-7-(1H-pyrazol-4-yl)thieno[3,2-c]pyridin-4-amine (14a). A mixture of the crude product **13** (100 mg, 0.21 mmol) was stirred in CF_3COOH (3 mL) at r t for 2 h, after completion of reaction, the reaction mixture was diluted with the mixed ice and water, and then 1M NaOH solution was added to alkalify to pH 7-8, the precipitated solid was filtered, and concentrated under reduced pressure. The crude product was purified on a silica gel column using (5-10 % CH_3OH/ DCM) as eluent to afford **14a** (59 mg, 75 %) as a gray solid. Mp: 232.3-234.2 ^oC; MS m/z 385.1 $[M+H]^+$. ^1H NMR (400 MHz, DMSO-d_6) δ ppm 13.09 (brs, 1H), 8.15 (brs, 1H), 8.06 (s, 1H), 7.95 (br s, 1H), 7.54 (s, 1H), 7.48-7.43 (dd, *J*=7.20 Hz, *J*=8.20 Hz, 4H), 7.22-7.18 (t, *J*=6.80 Hz, 1H), 7.14-7.12 (dd, *J*=2.80 Hz, *J*=2.80 Hz, 4H), 5.42 (brs, 2H); HPLC 99.7 %; HRMS (ESI) m/z calcd for $C_{22}H_{16}N_4OS$ $[M+H]^+$.385.1117, found 385.1128.

7-(1-Methyl-1H-pyrazol-4-yl)-3-(4-phenoxyphenyl)thieno[3,2-c]pyridin-4-amine (14b). A suspension of **14a** (100 mg, 0.26 mmol), CH_3I (55 mg, 0.39 mmol) and K_2CO_3 (71.7 mg, 0.52 mmol) in DMF (5 mL) was stirred for 12 h at rt. After completion of reaction, the reaction mixture was diluted with the mixed ice and water, the precipitated solid was filtered off and the crude product was purified on a silica gel column using (20-50%) ethyl acetate/ petroleum ether) as eluent to afford **14b** (19mg, 42.0%) as a white solid. Mp: 201-203 ^oC; MS m/z 456.2 $[M+H]^+$; ^1H NMR (400 MHz, DMSO-d_6) δppm 8.17 (s, 1H), 8.07 (br s, 2H), 7.93 (s, 1H), 7.56 (s, 1H), 7.51-7.43 (dd, *J*=8.00 Hz, *J*=8.20 Hz, 4H), 7.22-7.19 (t, *J*=6.40 Hz, 1H), 7.14-7.12 (dd, *J*=4.40 Hz, *J*=4.00 Hz, 4H), 5.44 (brs, 2H), 4.86 (s, 2H), 2.65 (s, 1H); HPLC 98.6%; HRMS(ESI) m/z calcd for $C_{25}H_{21}N_5O_2S$ $[M+H]^+$. 456.1488, found 456.1501.

7-(1-Ethyl-1H-pyrazol-4-yl)-3-(4-phenoxyphenyl)thieno[3,2-c]pyridin-4-amine

(14c). 14c was synthesized by the general method described above compound **14b** (90 mg, 87.1 %) as a white solid. Mp: 180.5-182.0 ^{o}C; MS m/z 399.2 $[M+H]^+$. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 7.91-7.90 (d, *J*=6.20 Hz, 1H), 7.76 (s, 1H), 7.65 (s, 1H), 7.43-7.38 (dd, *J*=8.40 Hz, *J*=7.80 Hz, 4H), 7.21-7.15 (dd, *J*=6.00 Hz, *J*=7.60 Hz, 2H), 7.12-7.07 (dd, *J*=4.80 Hz, *J*=5.20 Hz, 4H), 5.20 (brs, 2H), 4.00 (s, 3H); HPLC 95.3 %; HRMS (ESI) m/z calcd for $C_{23}H_{18}N_4OS$ $[M+H]^+$.399.1274, found 399.1276.

7-(1-Butyl-1H-pyrazol-4-yl)-3-(4-phenoxyphenyl)thieno[3,2-c]pyridin-4-amine (14d). 14d was synthesized by the general method described above compound **14b** (38.5 mg, 83.0 %). Mp: 160-162 ^{o}C; MS m/z 413.2 $[M+H]^+$; ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 8.02 (s, 1H), 7.90 (s, 1H), 7.78 (s, 1H), 7.45-7.38 (dd, *J*=8.40 Hz, *J*=8.00 Hz, 4H), 7.18-7.16 (t, *J*=8.20 Hz, 2H), 7.11-7.08 (dd, *J*=4.20 Hz, *J*=4.40 Hz, 4H), 4.68 (brs, 2H), 4.28-4.27 (q, *J*=4.00 Hz, 2H), 1.60-1.56 (t, *J*=2.40 Hz, 3H); HPLC 99.0 %; HRMS (ESI) m/z calcd for $C_{24}H_{20}N_4OS$ $[M+H]^+$.413.1430, found 413.1432.

7-(1-Benzyl-1H-pyrazol-4-yl)-3-(4-phenoxyphenyl)thieno[3,2-c]pyridin-4-amine (14e). 14e was synthesized by the general method described above compound **14b** (18.3 mg, 57.2 %). Mp: 165-167 ^{o}C; MS m/z 441.2 $[M+H]^+$; ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 8.00 (s, 1H), 7.89 (s, 1H), 7.76 (s, 1H), 7.44-7.38 (dd, *J*=8.40 Hz, *J*=8.00 Hz, 4H), 7.20-7.18 (t, *J*=8.20 Hz, 2H), 7.12-7.08 (dd, *J*=4.20 Hz, *J*=4.40 Hz, 4H), 4.87 (brs, 2H), 4.23-4.219(t, *J*=14.0 Hz, 2H), 1.93 (m, 2H), 1.41 (m, 2H), 1.00-0.97 (t, *J*=9.20 Hz, 3H); HPLC 95.5 %; HRMS (ESI) m/z calcd for $C_{26}H_{24}N_4OS$ $[M+H]^+$.441.1670, found 441.1673.

7-(1-(3-Methoxypropyl)-1H-pyrazol-4-yl)-3-(4-phenoxyphenyl)thieno[3,2-c]pyridin-4-amine (14f). 14f was synthesized by the general method described above compound **14b** (21 mg, 86.5 %). Mp: 182-183.5 ^{o}C; MS m/z 475.2 $[M+H]^+$; ^{1}H NMR (400 MHz, $CDCl_3$) δppm 8.02 (s, 1H), 7.95 (s, 1H), 7.77 (s, 1H), 7.44-7.30(m, 9H), 7.19-7.15(m, 2H), 7.11-7.08 (dd, *J*=4.20 Hz, *J*=4.40 Hz, 4H), 5.41 (s, 2H), 4.67 (s, 2H); HPLC 95.6 %; HRMS (ESI) m/z calcd for $C_{29}H_{22}N_4OS$ $[M+H]^+$. 475.1587, found 475.1588.

2-(4-(4-Amino-3-(4-phenoxyphenyl)thieno[3,2-c]pyridin-7-yl)-1H-pyrazol-1-yl)-N-methylacetamide (14g). 14g was synthesized by the general method described above

compound **14b** (23 mg, 65.2 %). Mp: 145-147 °C; MS m/z 457.2 $[M+H]^+$; ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.17 (s, 1H), 8.05 (s, 1H), 7.91 (s, 1H), 7.56 (s, 1H), 7.50-7.43 (dd, *J*=8.80 Hz, *J*=8.40 Hz, 4H), 7.22-7.19 (t, *J*=12.4 Hz, 1H), 7.15-7.11 (dd, *J*=4.40 Hz, *J*=4.20 Hz, 4H), 4.86 (brs, 2H), 4.33 (t, 2H), 3.40 (t, 2H), 3.39 (s, 3H), 2.20 (t, 2H); HPLC 96.6 %; HRMS (ESI) m/z calcd for $C_{26}H_{24}N_4O_2S$ $[M+H]^+$. 457.1692, found 457.1700.

4-(4-Amino-3-(4-phenoxyphenyl)thieno[3,2-c]pyridin-7-yl)-N,N-dimethyl-1H-pyrazole-1-sulfonamide (14h). 14h was synthesized by the general method described above compound **14b** (18 mg, 88.5 %). Mp: 170-172 °C; MS m/z 492.2 $[M+H]^+$; ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.61 (s, 1H), 8.43 (s, 1H), 8.22 (s, 1H), 7.63 (s, 1H), 7.51-7.43 (dd, *J*=8.80 Hz, *J*=8.40 Hz, 4H), 7.23-7.19 (t, *J*=12.4 Hz, 1H), 7.15-7.13 (dd, *J*=4.40 Hz, *J*=4.20 Hz, 4H), 5.81 (brs, 2H), 2.93 (s, 6H); HPLC 98.5 %; HRMS (ESI) m/z calcd for $C_{24}H_{21}N_5O_3S_2$ $[M+H]^+$. 492.1158, found 492.1172.

1-(4-(4-Amino-3-(4-phenoxyphenyl)thieno[3,2-c]pyridin-7-yl)-1H-pyrazol-1-yl)ethanone (14i). 14i was synthesized by the general method described above compound **14b** (25 mg, 62.5 %). Mp: 169-171 °C; MS m/z 427.2 $[M+H]^+$; ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 8.70 (s, 1H), 8.48 (s, 1H), 8.28 (s, 1H), 7.61 (s, 1H), 7.52-7.44 (dd, *J*=8.40 Hz, *J*=8.40 Hz, 4H), 7.24-7.20 (t, *J*=12.4 Hz, 1H), 7.16-7.13 (dd, *J*=4.40 Hz, *J*=4.20 Hz, 4H), 5.66 (brs, 2H), 2.71 (s, 3H); HPLC 98.5 %; HRMS (ESI) m/z calcd for $C_{24}H_{18}N_4O_2S$ $[M+H]^+$. 427.1223, found 427.1225.

(3-(Phenylcarbamoyl)phenyl)boronic acid (16a). A suspension of 3-boronobenzoic acid (1.0 g, 5.9 mmol), aniline (0.65g, 7 mmol), Et_3N (1.29 g, 12 mmol) and PyBop (3.6 g, 7mmol) in DMF(10 mL) was stirred for 12 h at r t. After completion of reaction, the solution was diluted with H_2O (15 mL), and then the product was extracted three times with EtOAc (50 mL). The combined organic layer was dried over Na_2SO_4, and the solvent was removed in vacuo, the crude product was purified on a silica gel column using (1-5 %) CH_3OH/ DCM as eluent to afford **16a** (1.47 g, 75 %) as a white solid. MS m/z 241.2 $[M+H]^+$.

(4-(Phenylcarbamoyl)phenyl)boronic acid (16b). 16b was synthesized by the general method described above compound **16a** (1.53 g, 40.8 %). MS m/z 241.2 $[M+H]^+$.

3-(4-Aminothieno[3,2-c]pyridin-3-yl)-N-phenylbenzamide (17a). **17a** was synthesized by the general method described above compound **12a** (0.89 g, 78.3 %). MS m/z 345.2 $[M+H]^+$.

4-(4-Aminothieno[3,2-c]pyridin-3-yl)-N-phenylbenzamide (17b). **17b** was synthesized by the general method described above compound **12a** (1.21 g, 83.1 %). MS m/z 345.2 $[M+H]^+$.

3-(4-Amino-7-iodothieno[3,2-c]pyridin-3-yl)-N-phenylbenzamide (18a). **18a** was synthesized by the general method described above compound **11** (0.41 g, 47.1 %). MS m/z 470.2 $[M+H]^+$.

4-(4-Amino-7-iodothieno[3,2-c]pyridin-3-yl)-N-phenylbenzamide (18b). **18b** was synthesized by the general method described above compound **11** (0.56 g, 50.3 %). MS m/z 470.2 $[M+H]^+$. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 10.41 (s, 1H), 8.10-8.08 (d, *J* = 4.80 Hz, 2H), 8.06 (s, 1H), 7.82-7.80 (d, *J* = 8.00 Hz, 2H), 7.69 (s, 1H), 7.65-7.63 (d, *J* = 8.00 Hz, 2H), 7.39-7.35 (t, *J* = 16.00 Hz, 2H), 7.14-7.12 (d, *J* = 8.00 Hz, 1H), 5.62 (brs, 2H).

3-(4-Amino-7-(1-(2-(methylamino)-2-oxoethyl)-1H-pyrazol-4-yl)thieno[3,2-c]pyridin-3-yl)-N-phenylbenzamide (19a). **19a** was synthesized by the general method described above compound **13** (21 mg, 45.1 %). Mp: 171-173 ^{o}C; MS m/z 483.2 $[M+H]^+$; ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 10.35 (s, 1H), 8.21 (s, 1H), 8.13-8.08 (m, 4H), 7.97 (s, 1H), 7.79-7.77 (d, *J* = 8.00 Hz, 2H), 7.75-7.68 (q, 3H), 7.38-7.34 (d, *J* = 16.00 Hz, 2H), 7.13-7.11 (d, *J* = 8.00 Hz, 1H), 5.41 (s, 2H), 4.88 (s, 2H), 2.65 (s, 3H). HPLC 96.1 %; HRMS (ESI) m/z calcd for $C_{26}H_{22}N_6O_2S$ $[M+H]^+$. 483.1597, found 483.1601.

4-(4-Amino-7-(1-(2-(methylamino)-2-oxoethyl)-1H-pyrazol-4-yl)thieno[3,2-c]pyridin-3-yl)-N-phenylbenzamide (19b). **19b** was synthesized by the general method described above compound **13** (27 mg, 49.8 %). Mp: 178-180 ^{o}C; MS m/z 483.2 $[M+H]^+$; ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 10.42 (s, 1H), 8.21 (s, 1H), 8.13-8.10 (m, 4H), 7.97 (s, 1H), 7.83-7.81 (d, *J* = 8.00 Hz, 2H), 7.68-7.66 (d, 3H), 7.40-7.36 (d, *J* = 16.00 Hz, 2H), 7.14-7.12 (d, *J* = 8.00 Hz, 1H), 5.47 (s, 2H), 4.88 (s, 2H), 2.65 (s, 3H). HPLC 98.2%; HRMS (ESI) m/z calcd for $C_{26}H_{22}N_6O_2S$ $[M+H]^+$. 483.1598, found 483.1603.

3-Chloro-N-(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)benzamide

(21). 3-chlorobenzoyl chloride (175 mg, 1 mmol) in anhydrous THF (2 mL) was added dropwise to a solution of 4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)aniline (219 mg, 1 mmol) and Et_3N (152 g, 1.5 mmol) in 10 mL anhydrous THF of cooled to 0°C, and the reaction mixture was stirred at 0 °C for 1 h and at room temperature for 2 h. The solution was diluted with H_2O (5 mL), and then the product was extracted three times with EtOAc (20 mL). The combined organic layer was dried over Na_2SO_4, and the solvent was removed in vacuo, the residue was used in the next reaction without further purification. MS m/z 357.2 $[M+H]^+$.

N-(4-(4-Aminothieno[3,2-c]pyridin-3-yl)phenyl)-3-chlorobenzamide (22). **22** was synthesized by the general method described above compound **10** (151 mg, 53.7 %). MS m/z 379.1 $[M+H]^+$.

N-(4-(4-Amino-7-iodothieno[3,2-c]pyridin-3-yl)phenyl)-3-chlorobenzamide (23). **13** was synthesized by the general method described above compound 3 (102 mg, 43.2 %). MS m/z 505.1 $[M+H]^+$.

N-(4-(4-Amino-7-(1-methyl-1H-pyrazol-4-yl)thieno[3,2-c]pyridin-3-yl)phenyl)-3-chlorobenzamide (24a). **24a** was synthesized by the general method described above compound **12a** (30 mg, 38.5 %). Mp: 221-223 °C; MS m/z 460.1 $[M+H]^+$; ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 8.01 (s, 1H), 7.96 (s, 1H), 7.89-7.88 (m, 2H), 7.78-7.75 (t, *J* = 12.40 Hz, 4H), 7.57-7.44 (m, 4H), 4.73(s, 2H), 4.01 (s, 3H). HPLC 99.0 %; HRMS (ESI) m/z calcd for $C_{24}H_{18}ClN_5OS$ $[M+H]^+$. 460.0993, found 460.1002.

N-(4-(4-Amino-7-(1-(2-(methylamino)-2-oxoethyl)-1H-pyrazol-4-yl)thieno[3,2-c]pyridin-3-yl)phenyl)-3-chlorobenzamide (24b). **24b** was synthesized by the general method described above compound **12a** (38 mg, 35.8 %). Mp: 220-222 °C; MS m/z 517.1 $[M+H]^+$; ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 8.01-8.00 (m, 3H), 7.96-7.86 (m, 2H), 7.80-7.88 (d, *J* = 12.40 Hz, 3H), 7.55-7.47 (m, 4H), 7.21 (s, 1H), 6.45 (s, 1H), 5.01 (s, 2H), 4.90 (s, 2H), 2.85 (s, 3H). HPLC 99.0 %; HRMS (ESI) m/z calcd for $C_{26}H_{21}ClN_6O_2S$ $[M+H]^+$. 517.1208, found 517.1225.

5.2 BTK enzymatic assay

The HTRF kinase assay (components supplied as kit by Cisbio) was chosen for BTK

enzyme assays. It uses time resolved fluorescence resonance energy transfer (TR-FRET) to detect production of a phosphorylated substrate. A peptide substrate is labeled with a biotin that can bind to XL665 labeled streptavidin, and the anti-phosphoresidue antibody is labeled with Eu^{+}. Upon phosphorylation of the substrate, the antibody binds to phosphorylated substrate that enables TR-FRET detection in homogenous assay format. All the reagents used for the BTK kinase assays including their resources are BTK kinase (Invitrogen), HTRF kinEASE-TK kit (Cisbio Bioassays), ATP(Sigma), DTT(Sunshine), $MgCl_2$ and $MnCl_2$ (Sigma). The assay buffer was composed of 50 mM HEPES (pH 7.0), 5 mM $MgCl_2$, 5mM DTT, 0.1 % NaN3, 0.1 % BSA and 0.1 mM orthovanadate. The HTRF assays were preformed according to the manual in the kit. All reagents were dispensed into each well plate according to the orders as follow: (1) BTK enzyme: 0.5ng/μl, 4 μl; (2) Each compound as well as control: 0.008-50 mM; (3) Reagent: 22.4 μM ATP and 0.15 μM substrate, 2 μl; (4) Inculation: Ambient, at 25℃, 5 min; (5) Reagent: antibody and XL-665, 8 μl. Then following 1 hour incubation at room temperature fluorescence was measured on the PHERAStar FS microplate reader (BMG Lab Technologies). Signal was expressed in terms of HTRF ratio (fluorescence intensity at 665 nm/fluorescence intensity at 620 nm).

5.3 ELISA-based kinase selectivity assay.

In vitro kinase inhibition assays were carried out as described elsewhere. Briefly, 96-well plates were pre-coated with 0.2 mg/mL poly (Glu-Tyr, 4:1) (Sigma) overnight at 37°C. 0.05 mL aliquot of 0.01 mmol/L ATP diluted in kinases and reaction medium were added. Test compounds at various concentrations diluted in 0.01mL of 1 % DMSO (V/V) were added. The reaction mixtures were incubated for 60 min at 37°C. The wells were washed with PBS containing 0.1 % T-PBS for three times. The 0.1 mL Phosphorylated tyrosine substrate was added. The kinase reaction was incubated for for 60 min at 37°C, and then the wells were washed three times and then anti-mouse IgG (ZSGB-BIO; ZB-2305; 0.1 mL /well) coupled with horseradish peroxidase (HRP) was added and incubated for another 30 min. The TMB reaction was quenched by addition of 0.05mL of 2 M H_2SO_4. The optical density was measured at 450 nm by an ELISA reader. The IC_{50}

values were calculated for test compounds by using a regression analysis of the concentration/inhibition data.

5.4 Pharmacokinetic Profiles of compound 14b in SD rats

Compound **14g** were administered to 3 male SD rats (weight ranging from 180 g to 240 g) at doses of 2.5 mg/kg for iv administration as well as doses of 10 mg/kg for po administration for another 3 male SD rats. The dosing volume was 5 mL/kg. After administration, blood samples were collected at the point including 2 min, 5 min, 15 min, 30 min, 1 h, 2 h, 4 h, 6 h, 8 h, 10 h, and 24 h for analyses, the collected blood samples were centrifuged at 4000 rpm for 5 min at 4 ^{o}C, and then analyzed after protein precipitation. LC/MS/MS analysis of compound **14g** was performed under optimized conditions to obtain the best sensitivity and selectivity of the analyte in selected reaction monitoring mode (SRM) containing an internal standard. Plasma concentration-time data were measured by a noncompartmental approach using the software WinNonlin Enterprise, version 5.2 (Pharsight Co.,Mountain View, CA).

5.5 Molecular docking

The molecular docking procedure was referred to CDOCKER protocol within Discovery Studio 2.5. The protein-ligand complex crystal structure of **B43** bound to Btk (PDB code 3GEN) was chosen as the template. The initial 3D conformation of compound **14g** was optimized in the ChemBio3D Ultra using MM2 energy minimization method. The whole Btk enzyme was typed with CHARMm force field and the hydrogen atoms were added. The water molecules were deleted except those occupying at the ligand pocket. Binding site was defined as a sphere. Compound **14g** was docked in the defined active site. A set of 10 starting random conformations was generated. The top scoring pose of compound **14g** was retained and selected for analyzing.

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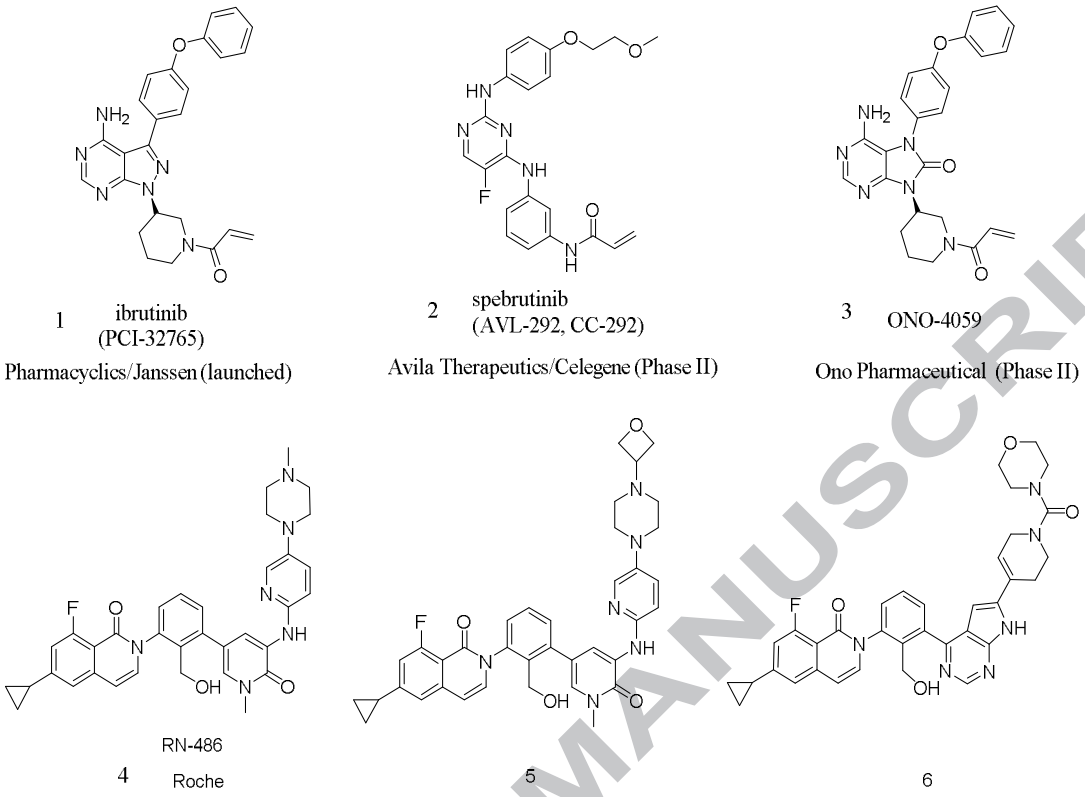
1



2

3 Figure 1. Representative structures of BTK inhibitors.

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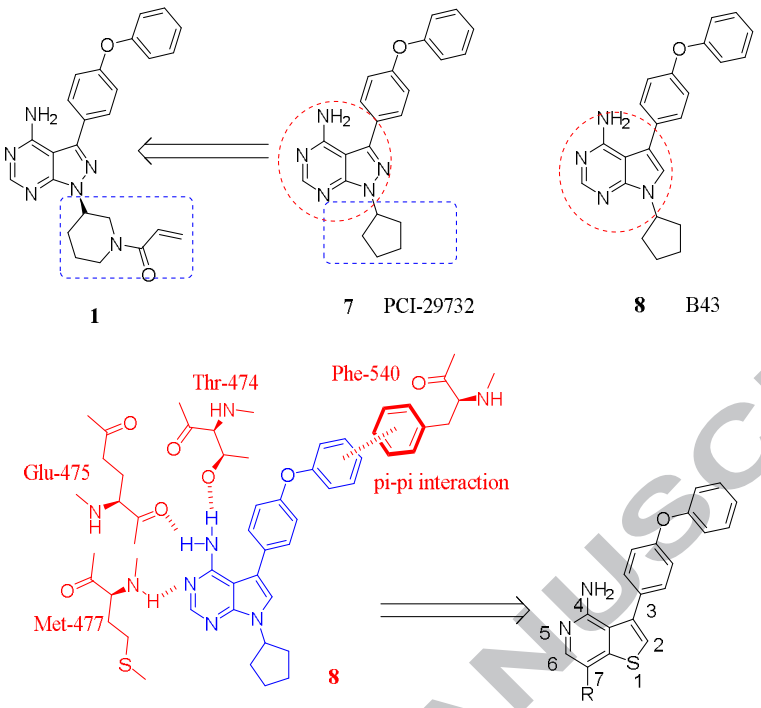


Figure 2. Design of the thieno[3,2-c]pyrid-4-amines as BTK inhibitors

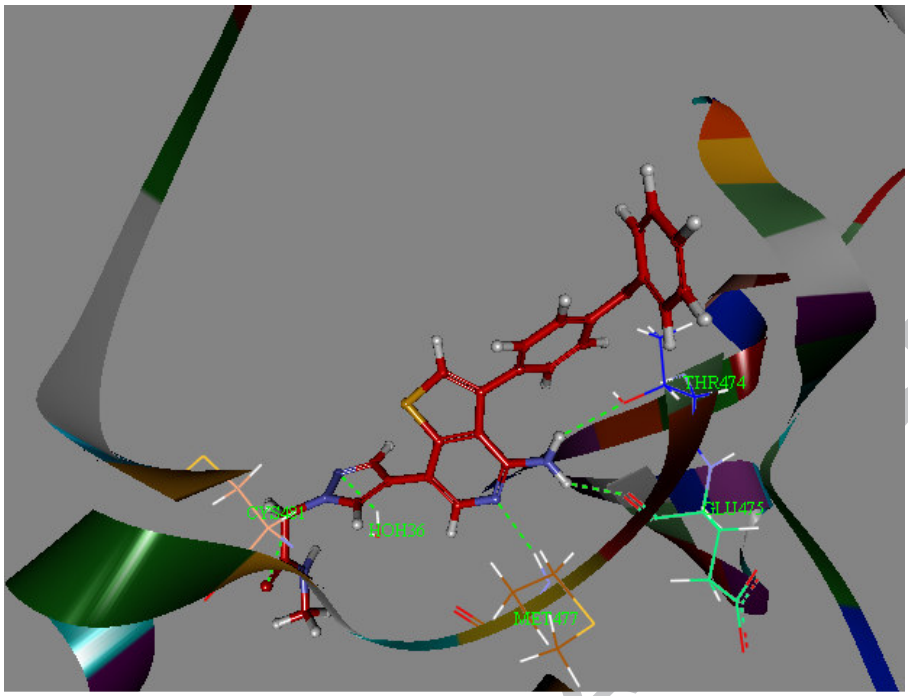
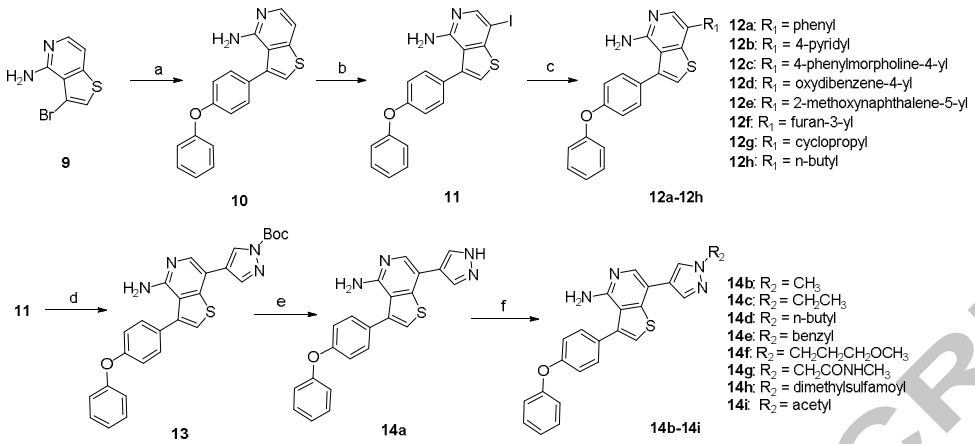
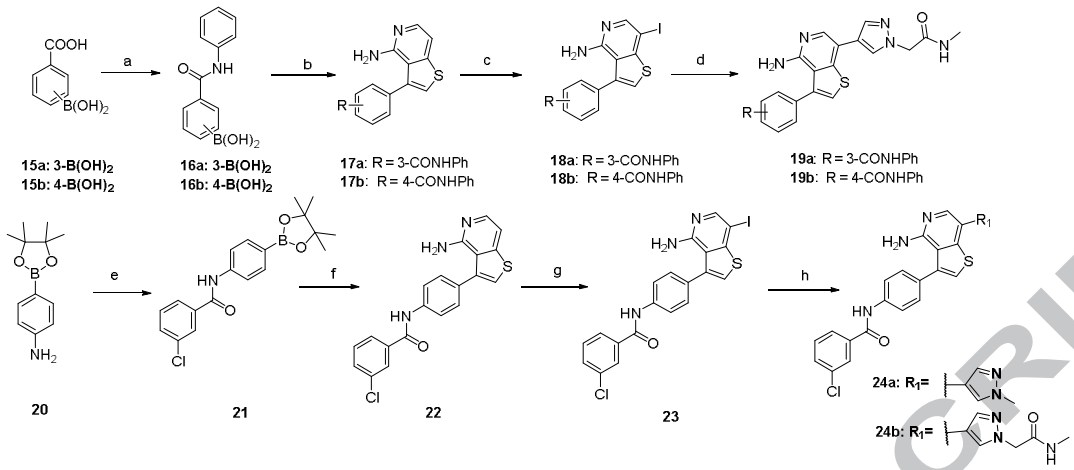


Figure 3. Docking mode of compound **14g** with BTK enzyme (PDB code: 3GEN). Selected residues Thr-474, Glu-475, Met-477, H_2O-36 and Cys-481 are shown. Hydrogen bonds are shown as green dashed lines.

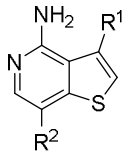


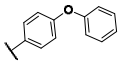
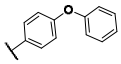
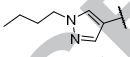
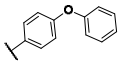
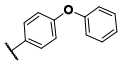
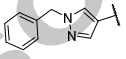
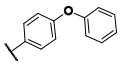
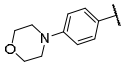
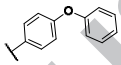
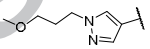
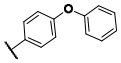
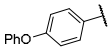
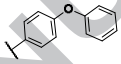
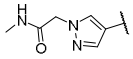
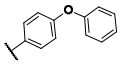
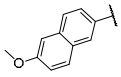
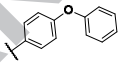
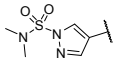
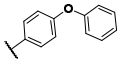
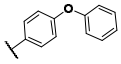
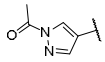
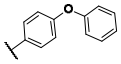
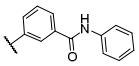
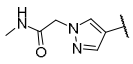
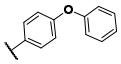
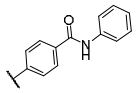
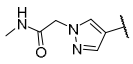
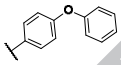
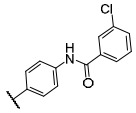
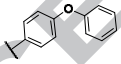
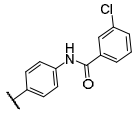
Scheme 1. Reagents and conditions: (a) $Pd(PPh_3)_4$, Na_2CO_3, EtOH, H_2O, toluene, 90 ^oC, 3 h, 96 %; (b) NIS, DMF, r t, 1 h, 70 %; (c) $R_1B(OH)_2$, $Pd(PPh_3)_4$, Na_2CO_3, DME, H_2O, 80 ^oC, 2-3 h, 35-90 %; (d) 1-Bocpyrazole-4-boronic acid pinacol ester, $Pd(dppf)Cl_2CH_2Cl_2$, Na_2CO_3, 1,4-dioxane, 100 ^oC,1-2 h, 55 %; (e) CF_3COOH, DCM, rt, 12 h, 92 %; (f) R^2X, K_2CO_3, DMF, 25 ^oC, 4-12 h, 40-95 %.



Scheme 2. Reagents and conditions: (a) aniline, PyBop, Et_3N, DMF, r t, 12 h; (b) compound **9**, $Pd(PPh_3)_4$, Na_2CO_3, DME, H_2O, 80 °C, 2-3 h; (c) NIS, DMF, r t, 1h, 50-55 %; (d) $Pd(dppf)Cl_2 \cdot CH_2Cl_2$, Na_2CO_3, 1,4-dioxane, 100 °C, 2 h; (e) m-Chlorobenzoyl chloride, Et_3N, DCM, 0-25 °C, 2 h, 85%; (f) $Pd(PPh_3)_4$, Na_2CO_3, DME, H_2O, 80 °C, 2-3 h, 80.2 %; (g) NIS, DMF, rt, 2 h, 57 %; (h) 1-Methylpyrazole-4-boronic acid pinacol ester for **24a** , N-methyl-2-(4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1H-pyrazol-1-yl)acetamide for **24b**, $Pd(dppf)Cl_2 \cdot CH_2Cl_2$, Na_2CO_3, 1,4-ioxane, 100 °C, 2 h.

Table 1. In vitro BTK enzymatic inhibition for thieno[3,2-c]pyrid-4-amine derivatives



Compds	R^1	R^2	Btk IC_{50}[nM] [a]	Compds	R^1	R^2	Btk IC_{50}[nM] [a]
12a			>10000	**14d**			387.1
12b			1954	**14e**			699.2
12c			2295	**14f**			279.7
12d			>10000	**14g**			12.8
12e			>10000	**14h**			527.6
12f			767.5	**14i**			454.2
12g			>10000	**19a**			291.5
12h			>10000	**19b**			137
14a			1052	**24a**			420.9
14b			107.4	**24b**			408.7
14c			176.2	**ibrutinib**	—	—	4.0

[a] Values are means of three experiments

Table 2. Kinase selectivity profiles of compounds **14b** and **14g**

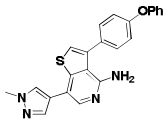
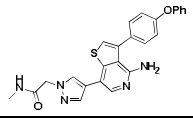
Compds	structures	IC_{50} (nM)				
		BTK	JAK2	JAK3	KDR	c-Met
14b		107.4	>10000	>10000	343	>10000
14g		12.8	>10000	>10000	284	>10000

Table 3. PK properties study for compound **14g** in SD rats [a]

compds	Dose (mg/kg)	C_{max} (ng/mL)	AUC_{0-t} (hr*ng/mL)	Vz (mL/kg)	Cl (mL/h/kg)	$MRT_{(0-t)}$ (h)	$T_{1/2}$ (h)	T_{max} (h)	F (%)
14g	2.5 (iv)[a]	2547.3	1459.1	900.0	1716.4	0.43	0.36	0.11	
	10 (po)[b]	855.5	1365.7	13320.6	8670.1	1.48	1.01	0.67	23.4

[a] Compound **14g** was formulated using 5 % DMA + 10 % solutol.

[b] Compound **14g** was formulated using 0.5 % HMPC + 0.5 % Twen-80.

Graphical Abstract

Discovery of thieno[3,2-c]pyridin-4-amines as novel Bruton's tyrosine kinase (BTK) Inhibitors

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