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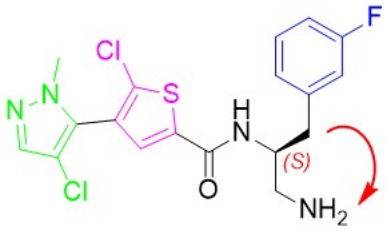
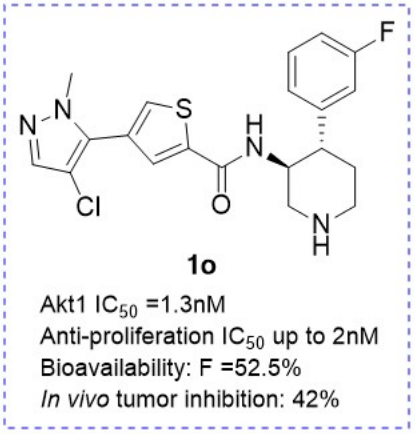


Afuresertib
GSK-2110183
K_i = 0.08 nM



1o
Akt1 IC_{50} =1.3nM
Anti-proliferation IC_{50} up to 2nM
Bioavailability: F =52.5%
In vivo tumor inhibition: 42%

Discovery of Pyrazole-thiophene Derivatives as Highly Potent, Orally Active Akt Inhibitors

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Abstract:

A series of pyrazole-thiophene derivatives exhibiting good Akt inhibitory activities were obtained on the basis of conformational restriction strategy, leading to the discovery of compound **1d** and **1o** which showed excellent *in vitro* antitumor effect against a variety of hematologic cancer cells and their potential of inducing apoptosis, blocking the cell cycles at *S* phase and significantly inhibiting the phosphorylation of downstream biomarkers of Akt kinase of cancer cells. Amongst, compound **1o** also exhibited good PK profiles and inhibited about 40% tumor growth in MM1S xenograft model. Compound **1o** might be a potential candidate for further development.

Keywords: pyrazole-thiophene derivatives; Akt inhibitor; antitumor

1. Introduction

Serine/threonine kinase Akt (also known as protein kinase B or PKB) has become a major focus of attention due to its essential role in diverse cellular processes.[1] As we known, PI3K/Akt signaling pathway is abnormal in cancer cells and plays a pivotal role in neoplastic transformation.[2, 3] Akt is considered to be a key mediator of the PI3K pathway, and the inhibition of Akt can decrease the tumor growth effectively.[4-9] So far, a number of Akt inhibitors have been under clinical evaluation (**Figure 1**), demonstrating its potential value for cancer therapy.

In particular, a phase III clinical trial is ongoing to investigate the effectiveness of GDC0068 in the treatment of triple-negative breast cancer (TNBC) (NCT03337724). Besides, AZD5363 in combination with paclitaxel for the treatment of TNBC also provide a positive outcome in a phase II clinical trial.[10] **GSK2141795** (GSK-795 or Uprosertib) and **GSK2110183** (GSK-183 or Afuresertib) are ATP-competitive Akt inhibitors developed by the GSK company, which are currently in clinical trials for the evaluation of its therapeutic effect against different types of cancer, such as melanoma, breast cancer, etc. Thus, it is fully demonstrated that the promising prospect of Akt inhibitors as anti-cancer agents.

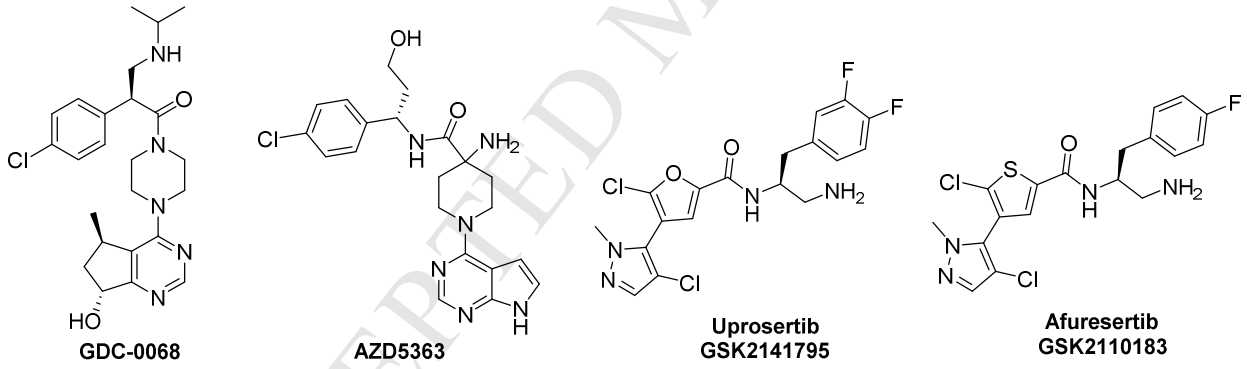


Figure 1. Chemical structures of representative Akt inhibitors.

Conformational restriction is a common methodology for drug design, which has been widely applied in drug discovery in a variety of diseases,[11, 12] including cancer and Alzheimer's disease, as well as in the development of antibacterial or antifungal agents. In our previous studies,[13-17] a series of 4,4-disubstituted or 3,4-disubstituted/3,4,6-trisubstituted (unpublished data) piperidine derivatives featuring more restricted conformation in contrast to GSK-795 were rationally designed and synthesized, exhibiting excellent Akt inhibitory activities and good *in vitro* or *in vivo* properties. Thus, we thought that conformational restriction strategy may also be suitable to be applied in GSK-183. Herein, as a continuing effort, compound A bearing (3S,4S) piperidine skeleton was

synthesized as shown in **Figure 2**. Since the synthetic method we used [18] gave trace (3S,4R)- or (3R,4S)-product, and we have demonstrated that (3S, 4S)-product was more favorable for binding with Akt1 than that of (3R,4R)-product (unpublished data), the (3S, 4S)-configuration was chosen for further study. Whereas, the Akt inhibitory activity of **A** decreased dramatically, exhibiting an IC_{50} value of 102 nM. Following research indicated that different link mode of thiophen ring can upregulate the Akt inhibitory potential of this series compound. Furthermore, the antitumor mechanism, PK profiles, *in vitro* or *in vivo* antitumor activities of the most promising compound **1d** and **1o** were studied.

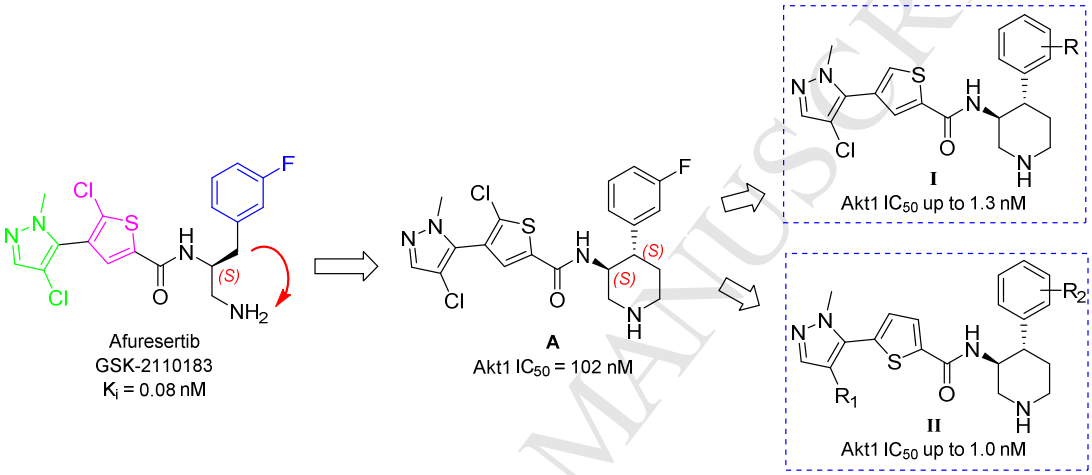
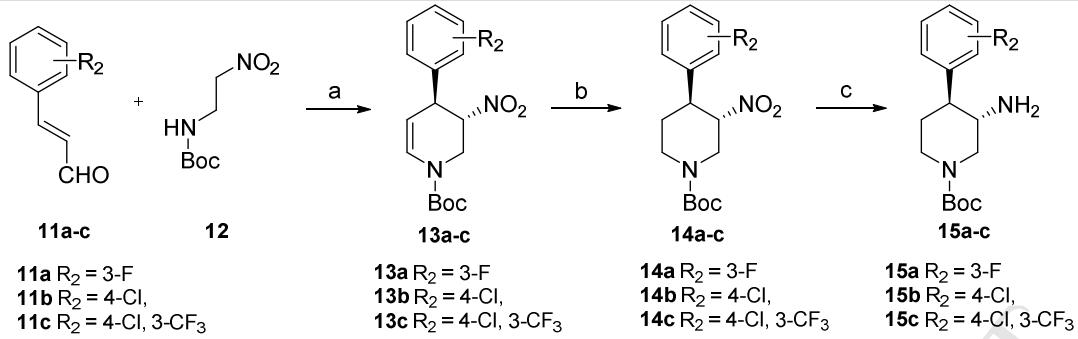


Figure 2. Design rationale based on the structure of GSK-183.

2. Results and discussion

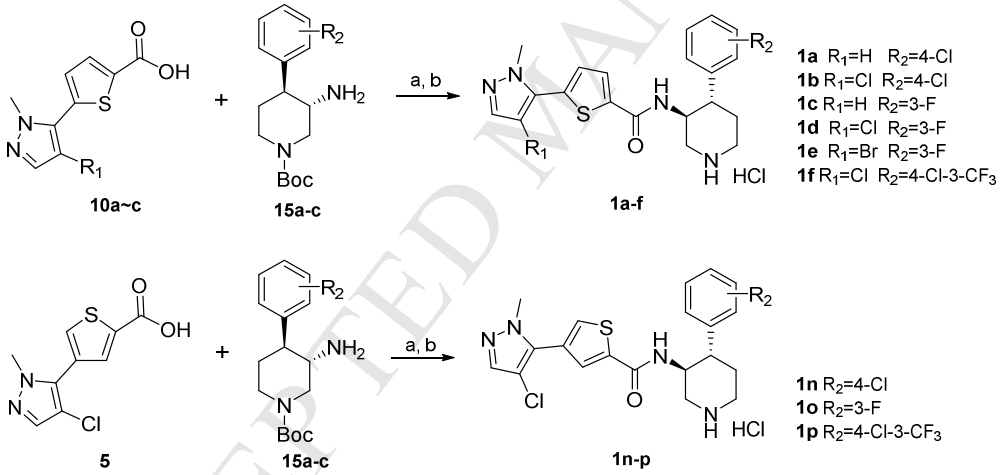
2.1 Chemistry

Synthesis of biaryl carboxylic acids and 3,4-disubstituted piperidines. The synthetic route for the preparation of the acid fragments was similar to the literature conditions (patent: WO2008098104, **Figure S1**). The synthesis of 3,4-disubstituted piperidine derivatives was shown in **Scheme 1**, compounds **11a-c** underwent S Jorgensen-Hayashi reagent catalyzed cyclization [18] with compound **12** to provide the (3S,4S) compound **13a-c**. Treatment of the compounds with Et_3SiH, and following in the presence of Fe/NH_4Cl provided 3-amino-4-aryl piperidine (**15a-c**).



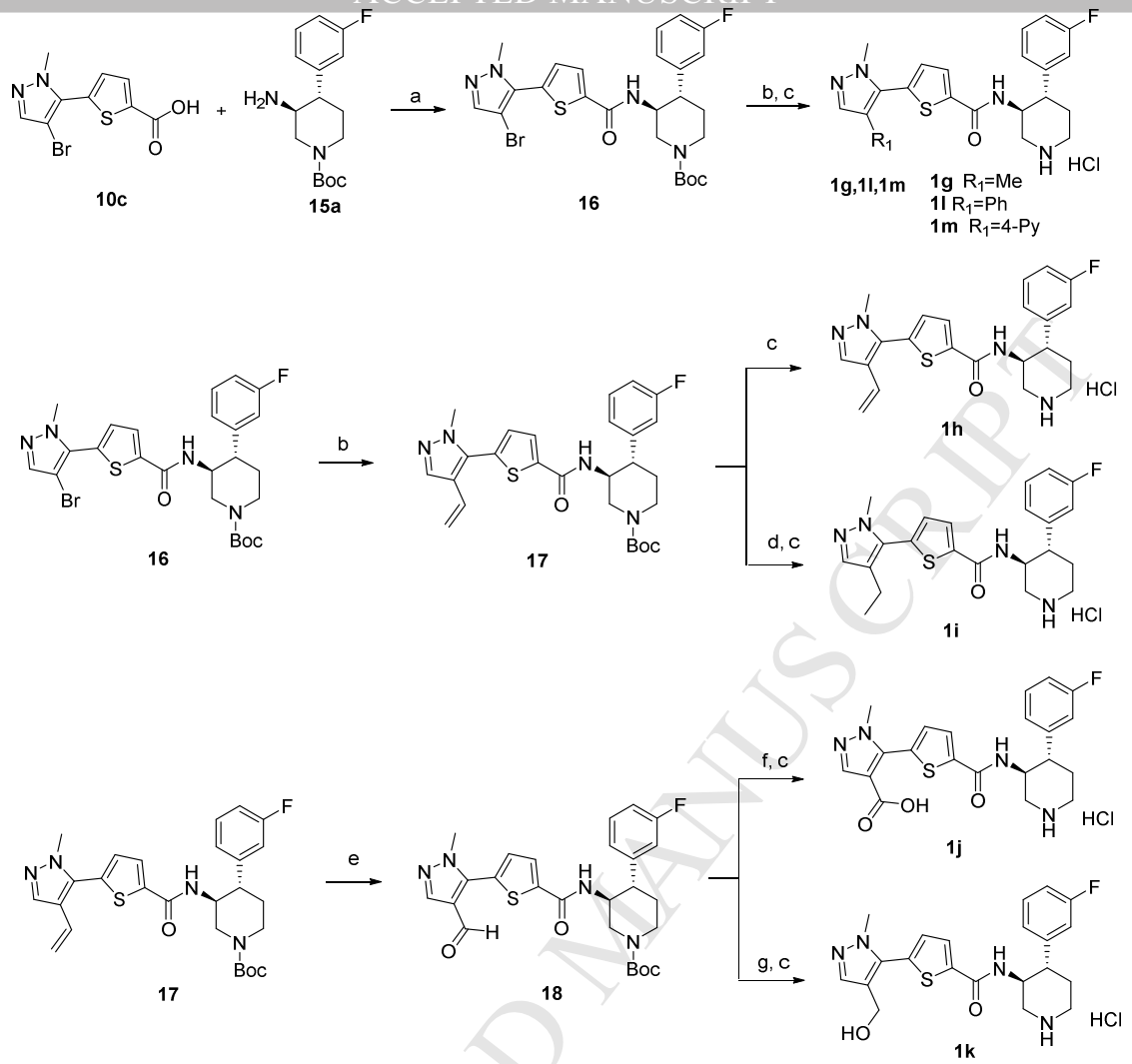
Scheme 1. (a) (S)-2-(diphenyl((trimethylsilyl)oxy)methyl)pyrrolidine, dichloromethane; ii. TFA, dichloromethane; (b) i. EtSiH, TFA; ii. Boc_2O, TEA, dichloromethane; (g) Fe, NH_4Cl, $EtOH/H_2O$.

Synthesis of target compounds. The synthetic route for the preparation of the target compounds was shown in **Scheme 2**. Treatment of the acids with the amines in the presence of EDCI and HOBt provided the condensed intermediates. Then, deprotection with HCl in ethyl acetate yielded the target compounds **1a-f** and **1n-p**.



Scheme 2. (a) EDCI, HOBt, DIPEA; (b) EA, HCl.

Condensation of pyrazol-thiophene carboxylic acid **10c** and (3S, 4S)-N-Boc-3-amino-4-arylpiperidine **15a** afforded intermediate **16** which subsequently coupled with different boronic acids and de-Boc protected to give the title compound **1g**, **1l** and **1m**. In addition, the intermediate **16** can be coupled with an ethylene boronic anhydride pyridine complex to obtain an ethylene intermediate **17** which then deprotected to remove Boc to obtain compound **1h**, or reduced with H_2 to obtain compound **1i**. Compound **18** was obtained by oxidizing compound **17** with $OsO_4/NaIO_4$/NMO system. Then further oxidized and deprotected to get compound **1j**, or reduced and deprotected to get compound **1k**.



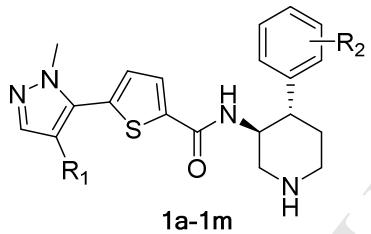
Scheme 3. (a) EDCI, HOBt, DIPEA; (b) substituted boronic acids or vinylboronic anhydride pyridine complex, K_2CO_3, $Pd_2(dba)_3$, dioxane; (c) EA, HCl; (d) H_2, Pd/C, MeOH; (e) OsO_4/$NaIO_4$, NMO, THF/H_2O; (f) $RuCl_3$, $NaIO_4$, CH_3CN/H_2O; (g) $NaBH_4$, THF/MeOH.

2.2 *In vitro* activity evaluation

We investigated the 2,5-substituted thiophene linkage at first. As shown in **Table 1**, replacement of the hydrogen of R_1 group with chlorin resulted in a 10-folds increasement of potency (**1a** vs **1b**, **1c** vs **1d**). Whereas, after introducing bromine to R_1 (compound **1e**), a loss of inhibitory activity was observed. The Akt inhibitory activity of compounds with 4-Cl or 3-F substituent on the phenyl of 4-piperidine performed better than that of compounds with 4-Cl-3-CF_3 substituent (**1b** or **1d** vs **1f**). Hence, 3-F substituent was chosen as the privileged group for further exploration. Other groups on 4-pyrazole (R_1) were also investigated, the IC_{50} value could still remain below 10 nM unless the size of the substituent was small such as methyl group (**1g**). Introducing other large groups such as vinyl (**1h**), ethyl (**1i**), carboxyl (**1j**), hydroxymethyl (**1k**), phenyl (**1l**) or 4-pyridyl (**1m**) would lead

to a dramatically loss of Akt1 inhibitory potency of compounds, indicating that the tolerance of this position was limited. Besides, proliferation inhibitory activities of compounds against OVCAR-8 and HCT116 cells were also evaluated. Most of the compounds exhibiting better Akt1 inhibitory potency performed better anti-proliferation activity against OVCAR-8 and HCT116 cells. Compounds with R_1 of acid or basic group, the anti-proliferation activity decreased dramatically such as compounds **1j** and **1m**, which IC_{50} values were > 20 μM.

Table 1. Akt1 enzymatic and cancer cell line activities for compounds **1a**-**1m**.



1a-1m

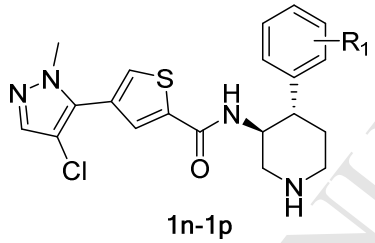
Compd.	R_1	R_2	Akt1 IC_{50} (nM)	IC_{50} (μM)[a]	
				OVCAR-8	HCT116
1a	H	4-Cl	10.2	1.39	8.87
1b	Cl	4-Cl	1.8	1.47	3.02
1c	H	3-F	10.6	1.59	1.37
1d	Cl	3-F	1.0	0.23	0.44
1e	Br	3-F	5.46	NT[b]	0.89
1f	Cl	4-Cl-3-CF_3	65.2	0.64	1.41
1g	Me	3-F	9.0	1.74	1.78
1h	vinyl	3-F	46.9	3.19	6.85
1i	Et	3-F	48.9	2.70	2.83
1j	carboxyl	3-F	101.0	>50	>50
1k	CH_2OH	3-F	62.2	7.17	7.42
1l	Ph	3-F	70.4	2.51	4.42
1m	4-Py	3-F	99.9	27.99	24.01
GSK2141795	-	-	9.6	0.54	1.75
GSK2110183	-	-	16.9	NT[b]	NT[b]

a. The IC_{50} values are an average of three independent determinations.

b. NT =Not Tested.

Furthermore, the compounds with 2,4-substituted thiophene linkage were also tested. As shown in **Table 2**, similar to the SAR of compounds with 2,5-substituted thiophene linkage, 3-F substituents (**1o**) showed better activity than those of 4-Cl or 4-Cl-3-CF_3 substituents (**1n** or **1p**), respectively. The anti-proliferation activity of compound **1o** was better than those of **1n** and **1p**, respectively. Amongst, compound **1d** and **1o** exhibited better Akt inhibitory or anti-proliferation activity than that of the positive **GSK2141795** and **GSK2110183.**

Table 2. Akt1 enzymatic and cancer cell line activities for compounds **1n**-**1p**.



1n-1p

Compd.	R_1	Akt1 IC_{50} (nM)	IC_{50} (μM)[a]	
			OVCAR-8	HCT116
1n	4-Cl	13.7	5.69	1.21
1o	3-F	1.26	NT[b]	0.95
1p	4-Cl-3-CF_3	35.3	0.58	1.85
GSK2141795	-	9.6	0.54	1.75
GSK2110183	-	16.9	NT[b]	NT[b]

a. The IC_{50} values are an average of three independent determinations.

b. NT =Not Tested.

2.3 *In vitro* anti-proliferation activity

The activity of compound **1d** or **1o** was measured by its ability to inhibit growth of a panel of 11 cell lines derived from hematologic tumors, by a standard proliferation assay. As shown in **Table 3**, compound **1o** showed better inhibitory potency against MM1S, RMPI8226, CEM-C1and MOLT-4 cell line than compound **1d**. Among the cell lines, MM1S, CEM-C1 and CCRF-CEM exhibited highly sensitive to compound **1o**, which IC_{50} values were < 10 nM. Whereas, both of the compounds performed the lowest sensitivity against K562 cell line. Moreover, compound **1d** and **1o** showed better anti-proliferation activity than that of **GSK2141795** against some cell lines such as

MM1S, RMPI8226, MV4-11 et al.

Table 3. hematologic cancer cell line activities for compounds **1d** and **1o**.

Cell lines	IC_{50} (nM)[a]		
	1d	**1o**	**GSK2141795**
MM1S	21	2	32
RMPI8226	263	49	538
CEM-C1	28	7	30
MOLT-4	50	14	66
MV4-11	135	441	635
HUT78	298	NT[b]	378
JVM-2	208	NT[b]	293
U937	78	188	101
CCRF-CEM	NT[b]	8	NT[b]
Mino	NT[b]	531	NT[b]
K562	> 3000	> 3000	>3000

a. The IC_{50} values are an average of three independent determinations.

b. NT =Not Tested.

2.4 Compound 1d and 1o induce apoptosis and cell cycles blockage of cancer cells

To determine whether the antiproliferative activity of **1d** and **1o** on colon cancer cell (HCT116) proliferation was accompanied by enhanced cancer cell apoptosis, the percentages of apoptotic cells were tested by conducting a flow cytometry assay. The results indicated that compound **1d** and **1o** both had an apoptosis-inducing effect in a dose-dependent manner, with 89.62 % and 24.58 % of apoptotic cells were detected at the concentration of 10 μM respectively (**Figure 3A** and **B**). Compound **1o** showed weaker apoptosis-inducing effect than **1d**. Besides, western blot analysis was further performed to test the Akt inhibitory ability of compound **1d** and **1o** in tumor cells. The results demonstrated that, upon treatment with the inhibitor **E22** *in vitro*, a dose-dependent significant reduction in the phosphorylation of GSK3β and PRAS40 was observed in HCT116 cells. Consistent with other ATP-competitive Akt kinase depressors,[19-21] both of **1d** and **1o** showed a concentration-dependent feedback increase in Akt phosphorylation (**Figure 3C**). In addition, it was

further demonstrated that compound **1d** and **1o** can also arrest cell cycle progression of HCT116 cells into the S phase staring from a concentration of 0.4 μM (**Figure 4A** and **B**).

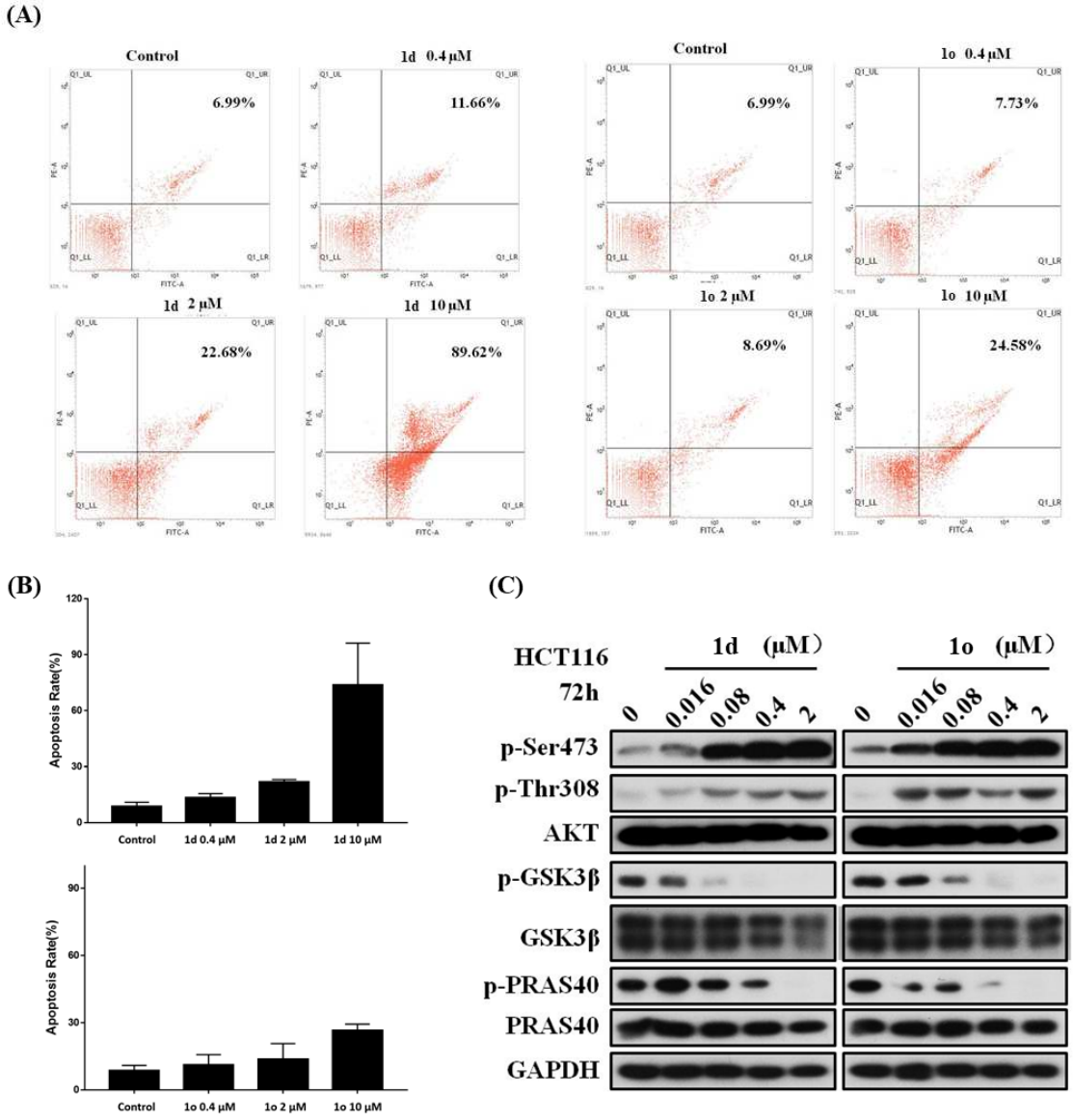


Figure 3. Compound **1d** and **1o** induce apoptosis *in vitro.* (A) Apoptosis was assessed upon treatment with the indicated concentrations (0.4-10 μM) by flow cytometry using the FL1-H channel (Annexin-V) and FL2-H channel (PI) of a Becton Dickinson FACS Calibur. Representative dot plots are shown, and the results are summarized in (B). (C) Western blot analysis of total AKT, p-AKT, PRAS40, p-PRAS40, GSK3β and p- GSK3β in the colon cancer cell line HCT116. Results are presented as the mean ± SD from three independent experiments.

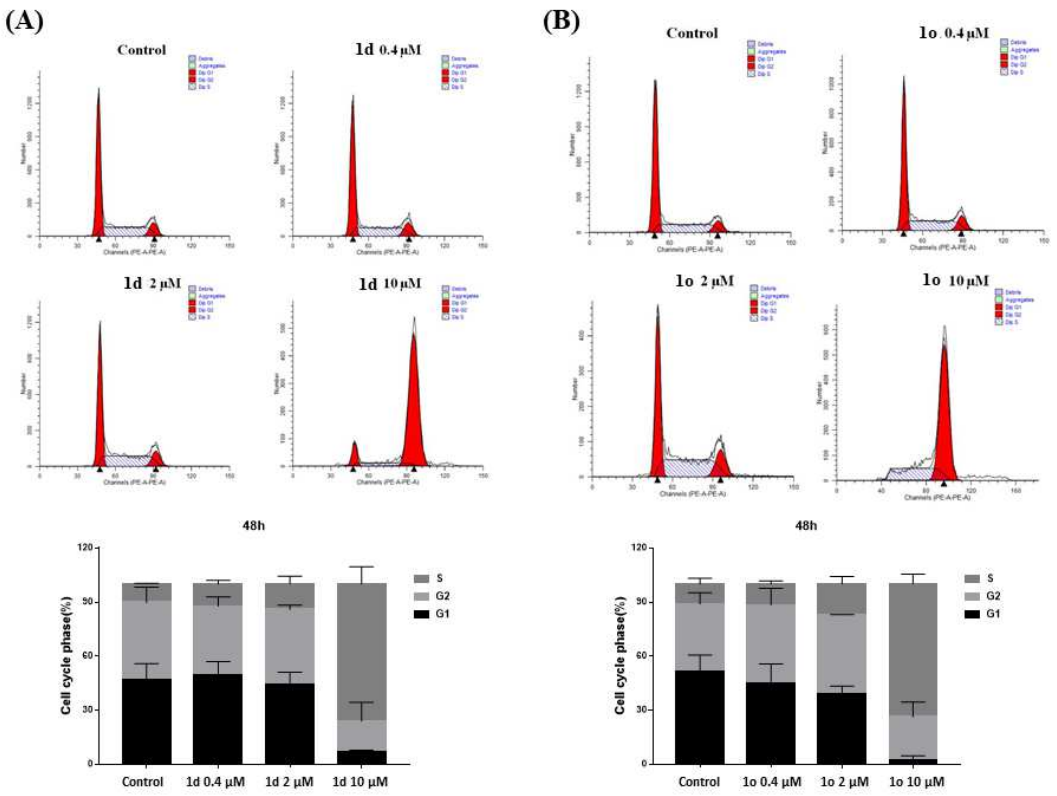


Figure 4. Compound **1d** (A) and **1o** (B) arrested cell cycle progression in HCT116 cells. Results are presented as the mean ± SD from three independent experiments.

2.5 Pharmacokinetic study

On the basis of Akt inhibitory and anti-cancer cell proliferation potency, compound **1o** was chosen to further test its pharmacokinetic properties. As shown in **Table 4**, after 10 mg/kg oral dosing (compound was dissolved in saline) in rats, a good absorption and exposure were observed, which $t_{1/2}$, C_{max} and AUC_{0-t} values were 2.75 h, 423 μg/L and 3886 μg /L·h respectively. The bioavailability of **1o** was 52.5 %, which was favorable for *in vivo* efficacy study. Furthermore, compound **1o** also exhibited moderate hERG inhibition (40 % @ 3 μM). The *in vitro* stability of **1o** was also evaluated in liver microsomes and plasma. The stability of compound **1o** was good in the plasma, but showed less stable in the liver microsomes of rat.

Table 4. Pharmacokinetic profile of compound **1o**

Parameters	1o	
	p.o. (10mg/kg)	i.v. (2mg/kg)

T_{max} (h)	2.75	0.083
$T_{1/2}$ (h)	4.74	3.52
C_{max} (μg/L)	423.0	1439.0
AUC_{0-t} (μg /L·h)	3886.0	1481.1
$AUC_{0-\infty}$(μg/L·h)	3940.5	1507.7
F(%)	52.5	
hERG inhibition rate (%, @ 3 μM)	40.0	
Liver microsome stability (%, remaining percent @1.5 h) [mouse, rat, dog, human]	82.7, 63.7, 81.3, 88.3	
Plasma stability (%, remaining percent @1.5 h) [mouse, human]	> 99, > 99	

2.6 *In vivo* xenograft tumor inhibition

The *in vivo* anti-tumor activity of compound **1o** was determined in the MM1S xenografts model in nude mice. After 14 days treatment with compound **1o** (15 or 30 mg/kg, p.o., q.d.), body weight loss of mice was observed (**Figure 5A**). In addition, both dosages showed remarkable tumor growth inhibition and tumor weight control (**Figure 5B**, **C** and **D**). The tumor growth inhibition rate of compound **1o** at the dose of 15 mg/kg was comparable to that of 30 mg/kg (40.70% vs. 42.07% inhibition respectively), and slightly better than that of **GSK2141795** (36.77% inhibition), demonstrating a suitable point for further development.

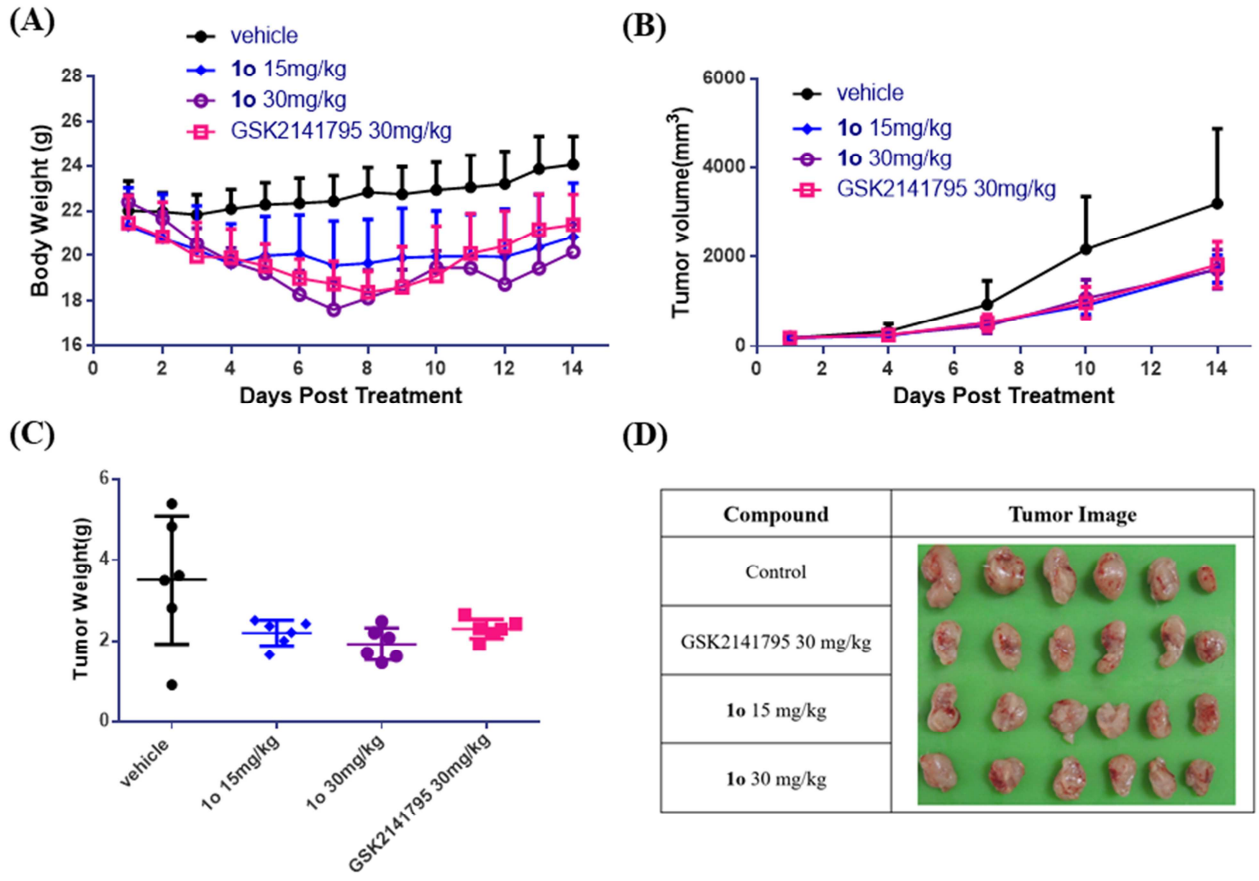


Figure 5. Compound **1o** develops an anti-tumor effect through inhibiting the kinase activity of Akt. (A, B, C and D) The mice transplanted with MM1S xenografts were randomly divided into four groups (N = 5 mice/group) and gave an injection of **1o** at 15 or 30 mg/kg and **GSK2141795** at 30 mg/kg or vehicle daily for a period of 14 days. The mice body weight (A), tumor volume (B), tumor weight (C) and tumor image (D) were recorded. Data represent the mean ± SD.

2.7 Molecular docking

In order to explore the possible binding mode of compound **1o** with Akt protein, computational docking and molecular dynamics were performed. First, the complex of 4GV1 (PDB code) [22] with **1o** was simulated for 20 ns. The RMSD value of the protein backbone was calculated in a trajectory from 0 to 20 ns. As shown in **Figure 6A**, after about 500 ps of simulation, the RMSD became stable, indicating that dynamic equilibrium was attained. The amino acid residues such as Val164, Met227, Ala230 and Thr291 contributed most for the binding between Akt protein and **1o** (**Figure 6B**). The hydrogen bond distance between **1o** and Asp292 indicated that the system reached equilibrium conditions (**Figure 6C**). Based on the relative stable conformation, **1o** was hydrogen bonded with the Akt residue Ala230 and Asp292. Besides, the 4-phenyl group inserted into the

hydrophobic pocket formed by the residues of Gly162, Val164, Lys179 and Leu181 (**Figure 6D**).

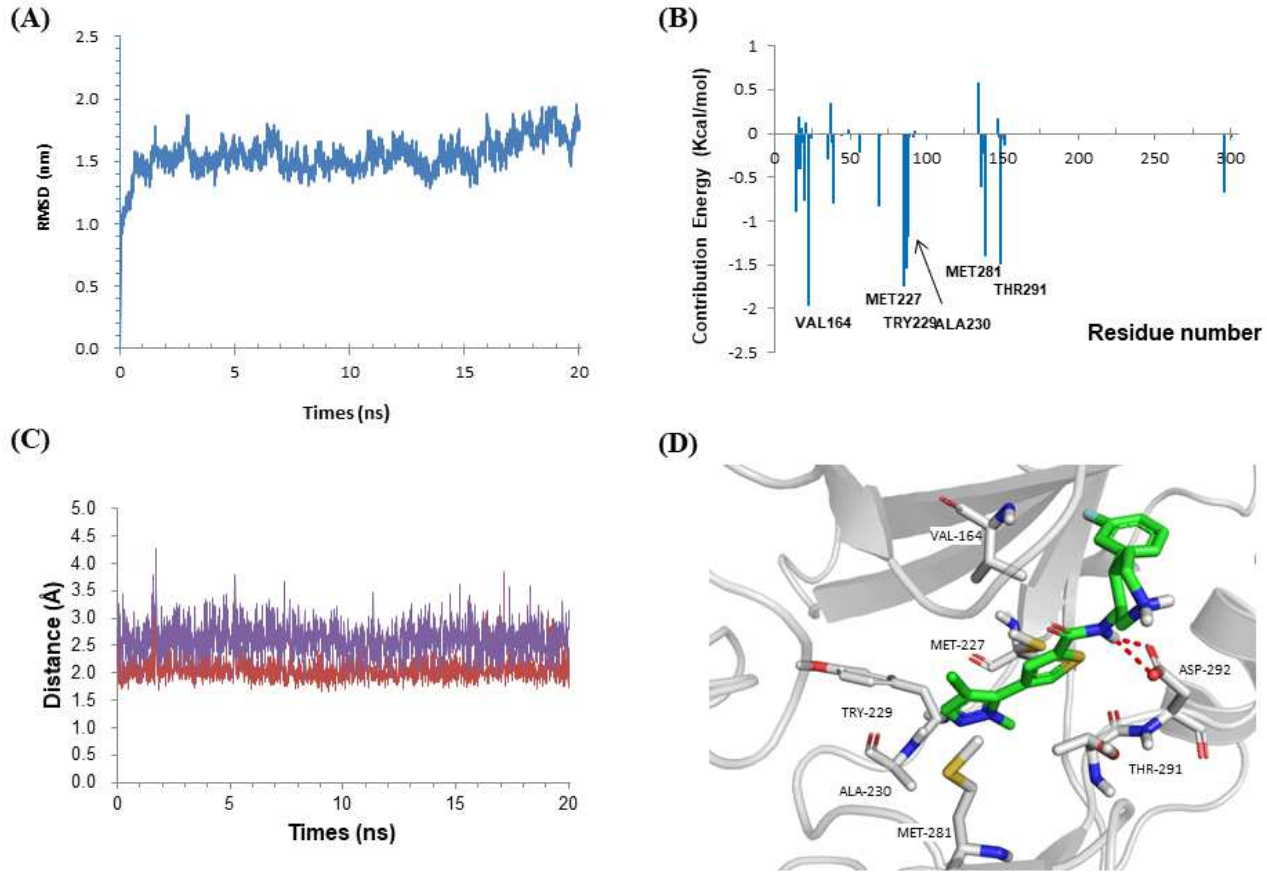


Figure 6. MD simulations and analysis of the interaction of compound **E22** with 4GV1. (A) RMSD of Akt backbone during the 20 ns simulation time; (B) Plot of the inhibitor-residue interaction spectrum of the two hydrogen bonds (purple and red) formed between the amide-NH of **1o** and the Asp-292 amino acid residue; (C) Distance plot of Asp292 with **1o**; (D) 3D plot of the binding pattern.

3. Conclusion

Based on the conformational restriction strategy, the activities of compounds with thiophene linkage were explored, leading to the discovery of compound **1d** and **1o**. Both of the compounds inhibited a variety of hematologic cancer cells and showed their potential of inducing apoptosis and blocking the cell cycles at S phase of cancer cells. Compound **1o** exhibited excellent PK profiles and *in vivo* tumor inhibition activity, demonstrating **1o** would be a promising candidate for further development.

4. Materials and Methods

4.1 Chemistry

^{1}H NMR and ^{13}C NMR spectra were recorded at 500 MHz using a Bruker AVANCE III spectrometer in $CDCl_3$, or DMSO-d6 solution, with tetramethylsilane (TMS) serving as internal standard. Chemical shift values (δ) were reported in ppm. Multiplicities are recorded by the following abbreviations: s, singlet; d, double; t, triplet; q, quartet; m, multiplet; J, coupling constant (Hz). High resolution mass spectrum (HRMS) was obtained from Agilent Technologies 6224 TOF LC/MS. The purities of compounds for biological testing were assessed by NMR and HPLC, and the purities were ≥95 %. The analytical HPLC was performed on a Agilent 1260 Infinity II (LC03) machine and a C18 reversed-phase column (Agilent Eclipse XDB-C18, 4.6*250 mm, 5 μm), with a flow rate of 1.0 mL/min, the detection by UV absorbance at a wavelength of 254 nm, the column temperature was 25 □, eluting with water (0.1% trifluoroacetic acid) as A phase and methanol as B phase (0 min, A phase: 90%, B phase: 10%; 8 min, A phase: 10%, B phase: 90%; 13 min, A phase: 10%, B phase: 90%; 15 min, A phase: 90%, B phase: 10%; 20 min, A phase: 90%, B phase: 10%). The melting point was recorded from Büchi M-560. The specific optical rotation was obtained from an Insmark IP-cligi 300 machine, with a temperature of 20 ^{o}C, the detection wavelength was 589 nm, the compound concentration was 0.1 g/100 mL and the length of the polarization tube was 100 mm. The optical data was an average of three independent determinations. Unless otherwise noted, reagents and solvents were obtained from commercial suppliers and without further purification.

General procedure A: (for the synthesis of compounds **3** and **9**) Tetrakistriphenylphosphane Pd (0) (3.45 g, 3 mmol) was added to a stirred suspension of 4-halogen substituted aromatic ester or aldehyde (30 mmol), 1-methyl-5-(4,4,5,5-tetramethyl-[1,3,2] dioxaborolan-2-yl)-1H-pyrazole (7.5 g, 36 mmol) and potassium phosphate (12g, 45 mmol) in dimethylformamide (100 mL) at 0 °C under nitrogen protection. The reaction mixture was heated at 100 °C for 10 h, then poured into H_2O (300 mL) and extracted with ethyl acetate (100 mL × 3). The combined organic layers were washed with saturated brine (200 mL × 3), dried over Na_2SO_4, and concentrated under vacuum to afford the crude product, then purified by column chromatography.

General procedure B: (for the synthesis of compounds **10a-c**) To a solution of biaryl ester (10 mmol) in dry THF/DMF (v/v, 10: 1) at 0 °C was added NCS or NBS (11 mmol) in one portion. The reaction mixture was stirred at r.t. for 5 h. After it was fully reacted, the mixture was poured into H_2O (50 mL) and extracted with ethyl acetate (30 mL × 3). The combined organic layers were washed with saturated brine (50 mL × 2), dried over Na_2SO_4 and concentrated under vacuum. The

residue was purified by column chromatography.

To a solution of the above intermediate products (8.8 mmol) in dry THF/EtOH (v/v, 4: 1) was slowly added aqueous NaOH (3 N, 10 mL, 30 mmol) and the mixture was stirred at r.t. for 5 h. After it was fully reacted, the solvent was removed under vacuum and the resulting crude mixture was dissolved in H_2O (30 mL) and acidified with 1 N hydrochloric acid until pH 2~3, and extracted with ethyl acetate (30 mL × 3). The combined organic layers were washed with saturated brine, dried over anhydrous Na_2SO_4, and then concentrated to dryness under vacuum to provide the white solid.

General procedure C: (for the synthesis of compounds **13a-c**) To a solution of compound **12** (1.5 mmol), (S)-2-(diphenyl((trimethylsilyl)oxy)methyl)pyrrolidine (33 mg, 0.1 mmol) and benzoicacid (25 mg, 0.2 mmol) in dry dichloromethane (2 mL) was slowly added substituted cinnamyl aldehyde **11a-c** (1 mmol) at 0 °C. After the addition was complete, the reaction was warmed up to room temperature and stirred overnight. The stirring was continued until aldehyde was consumed (monitored by TLC). The solution was diluted to 10 mL with dichloromethane and trifluoroacetic acid (148 μL, 2 mmol) was added dropwise, then the reaction mixture was stirred for another 5 h. The reaction was quenched by the dropping of 1N aqueous sodium bicarbonate solution (10 mL) and stirred for another 10 min. Water phase was extracted with ethyl acetate (10 mL × 3), and the combined organic layers were washed with saturated brine (20 mL × 2) dried over anhydrous sodium sulfate and concentrated. Purification by flash column chromatography afforded yellow solid.

General procedure D: (for the synthesis of compounds **14a-c**) To a solution of 3,4-disubstituted piperidine compound (5.4 mmol), triethylsilane (1.8 mL, 11 mmol) in dichloromethane (25 mL) was added dropwise trifluoroacetic acid (3.7 mL, 50 mmol) at 0 °C. After the addition was complete, the reaction was warmed up to room temperature and stirred overnight. Then the reaction was quenched by the dropping of saturated aqueous sodium bicarbonate (50 mL) and stirred for another 10 min. Water phase was extracted with dichloromethane (20 mL × 3), and the combined organic layers were washed with saturated brine (20 mL × 2) dried over anhydrous sodium sulfate and concentrated in vacuo to give an oil. To a suspension of the residue and triethylamine (1.4 mL, 10 mmol) in tetrahydrofuran (45 mL), di-*tert*-butyl dicarbonate (3.7 mL, 50 mmol) was added in batches at 0 °C. After the addition was complete, the reaction was warmed up to room temperature and stirred for 5 h. After solvent removal, the residue was dissolved in ethyl acetate (50 mL),

washed by 0.5N hydrochloric acid (20 mL × 2) and saturated brine (20 mL × 2) and dried over anhydrous sodium sulphate. After solvent removal, the residue was purified by column chromatography to afford product as a white solid.

General procedure E: (for the synthesis of compounds **15a-c**) To a suspension of 3,4-disubstituted piperidine compound (4.2 mmol) in ethyl alcohol - water (v/v, 3: 1, 40mL) was added iron powder (2.82 g, 50.4 mmol) and ammonium chloride (1.0 g, 16.8 mmol), then the mixture was refluxed for 5 h with mechanical stirring. After it is fully reacted, the mixture was filtered and filtrate was concentrated under vacuum. The residue was dissolved in ethyl acetate (50 mL), washed by saturated aqueous sodium bicarbonate (20 mL × 2) and saturated brine (20 mL × 2) and dried over anhydrous sodium sulphate. After solvent removal, the residue was dried in vacuum to give gray solid.

General procedure F: (for the synthesis of compounds **1a-f**, **1n-p** and **16**) To a suspension of the acid (0.2 mmol), EDCI (69 mg, 0.36 mmol), HOBT (49 mg, 0.36 mmol) and DIPEA (87 μL, 0.5 mmol) in dichloromethane was added the amine (58.8 mg, 0.2 mmol), then the mixture was reacted for 5 h at room temperature with mechanical stirring. After it was fully reacted, the mixture was concentrated under vacuum. The residue was dissolved in ethyl acetate (50 mL), washed by 1N HCl (20 mL × 2) and saturated brine (20 mL × 2) and dried over anhydrous sodium sulphate. After solvent removal, the residue was purified by column chromatography to afford the product.

To a solution of the above product in ethyl acetate was added HCl saturated ethyl acetate, stirred at room temperature for 6 h. After it was fully reacted, the mixture was filtered, the resulted solid was washed with diethyl ether. The solid was dried in vacuum to afford target product as white solid.

General procedure G: (for the synthesis of compounds **1g, 1l, 1m**) To a suspension of substituted boric acid (0.5 mmol) and K_2CO_3 (41 mg, 0.3 mmol) was added $Pd_2(dba)_3$ (10 mg, 0.01 mmol) and 10% tri-tert-butylphosphine pentane solution (60 μL, 0.03 mmol) in dioxane and H_2O (v/v, 5:1). The mixture was heated to 70 ^oC and stirred overnight. After the reaction was completed, the reaction solution was poured into hydrogen peroxide solution (10 mL), extracted with ethyl acetate (10 mL × 3), and the combined organic layers were washed with saturated brine (20 mL × 2) dried over anhydrous sodium sulfate and concentrated. Purification by flash column chromatography afforded the product.

To a solution of the above product in ethyl acetate was added HCl saturated ethyl acetate, stirred

at room temperature for 6 h. After it was fully reacted, the mixture was filtered, the resulted solid was washed with diethyl ether. The solid was dried in vacuum to afford target product as white solid.

After the SAR study, 16 compounds were obtained in total, the compound information of the intermediates can be found in the Supporting Information.

4-(1-Methyl-1H-pyrazol-5-yl)thiophene-2-carbaldehyde (3)

General procedure A, Yield: 81%; ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.98 (d, J = 1.1 Hz, 1H), 7.85 (d, J = 1.1 Hz, 1H), 7.77 (s, 1H), 7.53 (d, J = 1.9 Hz, 1H), 6.39 (d, J = 1.9 Hz, 1H), 3.97 (s, 3H). ESI-MS: m/z = 193 [M + H]+.

4-(4-Chloro-1-methyl-1H-pyrazol-5-yl)thiophene-2-carbaldehyde (4)

General procedure B, Yield: 90%; ^{1}H NMR (500 MHz, $CDCl_3$) δ 10.01 (d, J = 1.1 Hz, 1H), 7.92 (d, J = 1.1 Hz, 1H), 7.90 (s, 1H), 7.52 (s, 1H), 3.91 (s, 3H). ESI-MS: m/z =227 [M + H]+.

4-(4-Chloro-1-methyl-1H-pyrazol-5-yl)thiophene-2-carboxylic acid (5)

To a solution of compound **4** (2 mmol) in acetone(15 mL) was added $KMnO_4$ (0.38 g, 2.4 mmol) in batches at 0 ^{o}C. The mixture was stirred at r.t. for 3 h. After it was fully reacted, the mixture was filtered and the filtrate was concentrated under vacuum. The residue was purified by column chromatography. Yield: 85%; ^{1}H NMR (500 MHz, DMSO-*d6*) δ 13.35 (s, 1H), 8.20 (d, J = 1.5 Hz, 1H), 7.93 (d, J = 1.5 Hz, 1H), 7.64 (s, 1H), 3.84 (s, 3H). ESI-MS: m/z = 241 [M − H]−.

5-Bromothiophene-2-carboxylic acid (7)

To a solution of compound **6** (2 mmol) in acetone (15 mL) was added $KMnO_4$ (0.38 g, 2.4 mmol) in batches at 0 ^{o}C. The mixture was stirred at r.t. for 3h. After it was fully reacted, the mixture was filtrated and filtrate was concentrated under vacuum. The residue was purified by column chromatography. Yield: 85%; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.64 (d, *J* = 4.5 Hz, 1H), 7.11 (d, *J* = 4.5 Hz, 1H). ESI-MS: *m/z* = 205 $[M - H]^-$.

Methyl 5-bromothiophene-2-carboxylate (8)

To a solution of compound 7 (5.2 g, 25 mmol) in 50 mL anhydrous MeOH was slowly added $SOCl_2$ (2.6 mL, 37.5 mmol) at 0 □, then 1 mL anhydrous DMF was added and refluxed for 3 hours. After the reaction was completed, the mixture was concentrated under vacuum. The residue was dissolved in EtOAc and washed with saturated $NaHCO_3$ solution (20 mL × 3), brine (20 mL × 3) and dried over Na_2SO_4 and concentrated. Purification by flash column chromatography afforded the white solid (5.0 g, 22.7 mmol). Yield: 91%; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.54 (d, *J* = 4.0 Hz, 1H), 7.07

(d, *J* = 4.0 Hz, 1H), 3.87 (s, 3H). ESI-MS: *m/z* = 221 $[M + H]^+$.

Methyl 5-(1-methyl-1H-pyrazol-5-yl)thiophene-2-carboxylate (9)

General procedure A, Yield: 73%; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.78 (d, *J* = 3.9 Hz, 1H), 7.49 (d, *J* = 1.8 Hz, 1H), 7.16 (d, *J* = 3.9 Hz, 1H), 6.47 (d, *J* = 1.8 Hz, 1H), 4.02 (s, 3H), 3.91 (s, 3H). ESI-MS: *m/z* = 223 $[M + H]^+$.

5-(1-Methyl-1H-pyrazol-5-yl)thiophene-2-carboxylic acid (10a)

General procedure B, Yield: 88%; ^{1}H NMR (500 MHz, DMSO-*d6*) δ 13.28 (s, 1H), 7.74 (d, *J* = 3.9 Hz, 1H), 7.47 (d, *J* = 2.0 Hz, 1H), 7.44 (d, *J* = 3.9 Hz, 1H), 6.61 (d, *J* = 2.0 Hz, 1H), 3.96 (s, 3H). ESI-MS: *m/z* = 207 $[M - H]^-$.

5-(4-Chloro-1-methyl-1H-pyrazol-5-yl)thiophene-2-carboxylic acid (10b)

General procedure B, Yield: 91%; ^{1}H NMR (500 MHz, DMSO-*d6*) δ 13.44 (s, 1H), 7.83 (d, *J* = 3.9 Hz, 1H), 7.72 (s, 1H), 7.52 (d, *J* = 3.9 Hz, 1H), 3.92 (s, 3H). ESI-MS: *m/z* = 241 $[M - H]^-$.

5-(4-Bromo-1-methyl-1H-pyrazol-5-yl)thiophene-2-carboxylic acid (10c)

General procedure B, Yield: 87%; ^{1}H NMR (500 MHz, DMSO-*d6*) δ 13.41 (s, 1H), 7.83 (d, *J* = 3.9 Hz, 1H), 7.71 (s, 1H), 7.50 (d, *J* = 3.9 Hz, 1H), 3.91 (s, 3H). ESI-MS: *m/z* = 285 $[M - H]^-$.

Tert-butyl (3S,4S)-4-(3-fluorophenyl)-3-nitro-3,4-dihydropyridine-1(2H)-carboxylate (13a)

General procedure C, yield: 47%; Specific optical rotation: -181.015^o; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.35 – 7.28 (m, 1H), 7.19 – 6.93 (m, 4H), 4.93 and 4.82 (d, *J* = 4.3 Hz, and d, *J* = 5.3 Hz, 1H, 1:1.3 ratio due to amide rotamers), 4.69 – 4.59 (m, 1H), 4.26 – 4.22 (m, 1H), 4.17 – 4.06 (m, 1H), 3.92 and 3.99 (d, *J* = 12.5 Hz, and d, *J* = 12.2 Hz, 1H, 1:1.3 ratio due to amide rotamers), 1.52 (s, 9H). ^{13}C NMR (125 MHz, $CDCl_3$, 1:1.3 ratio due to amide rotamers) δ 163.20 (d, *J* = 246.3 Hz), 151.71 and 151.57, 142.42 (d, *J* = 6.6 Hz), 130.73 (d, *J* = 8.7 Hz), 126.67 and 126.98, 123.85 (d, *J* = 2.5 Hz), 115.23, 115.07, 103.48 and 103.07, 84.73, 82.54, 42.62 and 42.56, 42.32 and 41.56, 28.31. ESI-MS: *m/z* = 345 $[M + Na]^+$.

Tert-butyl (3S,4S)-4-(4-chlorophenyl)-3-nitro-3,4-dihydropyridine-1(2H)-carboxylate (13b)

General procedure C, yield: 55%; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.32 (d, *J* = 8.4 Hz, 2H), 7.18 (d, *J* = 8.4 Hz, 2H), 7.14 and 6.99 (d, *J* = 7.5 Hz, and d, *J* = 7.6 Hz, 1H, 1:1.2 ratio due to amide rotamers), 4.91 and 4.81 (d, *J* = 6.5 Hz, and d, *J* = 6.0 Hz, 1H, 1:1.2 ratio due to amide rotamers), 4.64 – 4.56 (m, 1H), 4.23 – 4.19 (m, 1H), 4.09 – 3.91 (m, 2H), 1.52 (s, 9H). ESI-MS: *m/z* = 361 $[M + Na]^+$.

Tert-butyl

(3S,4S)-4-(4-chloro-3-(trifluoromethyl)phenyl)-3-nitro-3,4-dihydropyridine-1(2H)-carboxylate (13c)

General procedure C, yield: 72%; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.56 (d, *J* = 1.9 Hz, 1H), 7.49 (d, *J* = 8.3 Hz, 1H), 7.36 (dd, *J* = 8.3, 1.9 Hz, 1H), 7.18 and 7.03 (d, *J* = 7.9 Hz, and d, *J* = 7.8 Hz, 1H, 1:1.2 ratio due to amide rotamers), 4.89 and 4.78 (d, *J* = 6.0 Hz, and d, *J* = 6.0 Hz, 1H, 1:1.2 ratio due to amide rotamers), 4.66 – 4.58 (m, 1H), 4.29 – 4.26 (m, 1H), 4.11 – 3.97 (m, 2H), 1.53 (s, 9H). ESI-MS: *m/z* = 429 $[M + Na]^+$.

Tert-butyl (3S,4S)-4-(3-fluorophenyl)-3-nitropiperidine-1-carboxylate (14a)

General procedure D, yield: 85%; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.32 – 7.26 (m, 1H), 7.00 – 6.95 (m, 2H), 6.93 – 6.89 (m, 1H), 4.84 – 4.55 (m, 2H), 4.27 (m, 1H), 3.30 (td, *J* = 12.2, 4.1 Hz, 1H), 3.19 (m, 1H), 2.88 (m, 1H), 1.96 (d, *J* = 13.7 Hz, 1H), 1.79 – 1.73 (m, 1H), 1.50 (s, 9H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 163.13 (d, *J* = 246.3 Hz), 154.09, 141.62 (d, *J* = 7.5 Hz), 130.75 (d, *J* = 7.5 Hz), 123.03 (d, *J* = 2.5 Hz), 115.12 (d, *J* = 20.0 Hz), 114.40 (d, *J* = 21.3 Hz), 86.78, 81.29, 46.83, 44.35, 43.81, 31.80, 28.47.

Tert-butyl (3S,4S)-4-(4-chlorophenyl)-3-nitropiperidine-1-carboxylate(14b)

General procedure D, yield: 91%; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.29 (d, *J* = 8.4 Hz, 2H), 7.13 (d, *J* = 8.4 Hz, 2H), 4.84 – 4.57 (m, 2H), 4.28 – 4.25 (m, 1H), 3.34 – 3.08 (m, 2H), 2.89 – 2.87 (m, 1H), 1.93 (d, *J* = 13.8 Hz, 1H), 1.81 – 1.70 (m, 1H), 1.49 (s, 9H). ESI-MS: *m/z* = 325 $[M - CH_3]^+$.

Tert-butyl

(3S,4S)-4-(4-chloro-3-(trifluoromethyl)phenyl)-3-nitropiperidine-1-carboxylate(14c)

General procedure D, yield: 92%; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.51 (d, *J* = 2.1 Hz, 1H), 7.46 (d, *J* = 8.3 Hz, 1H), 7.32 (dd, *J* = 8.3, 2.1 Hz, 1H), 4.67 (dd, *J* = 10.6, 7.3 Hz, 1H), 4.31 – 4.28 (m, 1H), 3.78 – 3.71 (m, 1H), 3.38 – 3.31 (m, 1H), 3.29 – 3.07 (m, 1H), 2.90 – 2.87 (m, 1H), 1.95 (d, *J* = 13.5 Hz, 1H), 1.81 – 1.70 (m, 1H), 1.49 (s, 9H).

Tert-butyl (3S,4S)-3-amino-4-(3-fluorophenyl)piperidine-1-carboxylate(15a)

General procedure E, yield: 81%; ^{1}H NMR (500 MHz, DMSO-*d6*) δ 7.38 (td, *J* = 7.9, 6.3 Hz, 1H), 7.23 (dd, *J* = 10.3, 2.0 Hz, 1H), 7.17 (d, *J* = 7.7 Hz, 1H), 7.10 – 7.06 (m, 1H), 4.37 (d, *J* = 10.6 Hz, 1H), 4.04 – 3.94 (m, 1H), 3.18 (dd, *J* = 10.4, 6.5 Hz, 1H), 2.90 – 2.61 (m, 3H), 1.79 – 1.70 (m, 1H), 1.67 – 1.57 (m, 1H), 1.43 (s, 9H). ESI-MS: *m/z* = 295 $[M + H]^+$.

Tert-butyl (3S,4S)-3-amino-4-(4-chlorophenyl)piperidine-1-carboxylate(15b)

General procedure E, yield: 85%; ESI-MS: *m/z* = 311 $[M + H]^+$.

Tert-butyl

(3S,4S)-3-amino-4-(4-chloro-3-(trifluoromethyl)phenyl)piperidine-1-carboxylate(15c)

General procedure E, yield: 96%; ESI-MS: *m/z* = 379 $[M + H]^+$.

Tert-butyl

(3S,4S)-3-(5-(4-bromo-1-methyl-1H-pyrazol-5-yl)thiophene-2-carboxamido)-4-(3-fluorophenyl)piperidine-1-carboxylate (16)

General procedure F, Yield: 82%; 1H NMR (500 MHz, $CDCl_3$) δ 7.49 (s, 1H), 7.30 – 7.23 (m, 2H), 7.10 (d, J = 3.9 Hz, 1H), 7.04 (d, J = 7.8 Hz, 1H), 6.97 – 6.94 (m, 1H), 6.92 – 6.88 (m, 1H), 6.00 (br s, 1H), 4.55 – 4.48 (m, 1H), 4.31 – 4.11 (m, 2H), 3.86 (s, 3H), 2.94 – 2.69 (m, 3H), 1.99 – 1.90 (m, 1H), 1.80 – 1.71 (m, 1H), 1.49 (s, 9H). ESI-MS: m/z = 585 $[M + Na]^+$.

Tert-butyl

(3S,4S)-4-(3-fluorophenyl)-3-(5-(1-methyl-4-vinyl-1H-pyrazol-5-yl)thiophene-2-carboxamido) piperidine-1-carboxylate (17)

Tris(dibenzylideneacetone)dipalladium ($Pd_2(dba)_3$,10 mg, 0.01 mmol) and 10% tri-tert-butylphosphine pentane solution (60 μL, 0.03 mmol) were dissolved in 1 mL anhydrous DMF. After stirring at room temperature for 3 h, a mixed solution (5 mL) of dioxane and H_2O (v/v, 5:1) was added slowly under N_2 protection. To the above solution was added compound 16 (56.3 mg, 0.1 mmol), vinylboronic anhydride pyridine complex (120 mg, 0.5 mmol) and K_2CO_3 (41 mg, 0.3 mmol) at 0 □, and then heated to 70 □ for 12 h. After the reaction was completed, the mixture was poured into hydrogen peroxide (10 mL), and then extracted with ethyl acetate (10 mL × 3), the combined organic layers were washed with saturated brine (20 mL × 2), dried over anhydrous sodium sulfate and concentrated. Purification by flash column chromatography (petroleum ether: EtOAc = 3:1-1:1) afforded the product. Yield: 83%; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.69 (s, 1H), 7.33 – 7.27 (m, 2H), 7.06 (d, *J* = 8.0 Hz, 1H), 6.99 – 6.90 (m, 3H), 6.42 (dd, *J* = 17.6, 11.1 Hz, 1H), 5.68 (s, 1H), 5.51 (dd, *J* = 17.6, 1.2 Hz, 1H), 5.09 (dd, *J* = 11.1, 1.2 Hz, 1H), 4.53 (d, *J* = 9.7 Hz, 1H), 4.37 – 4.10 (m, 2H), 3.82 (s, 3H), 2.87 – 2.71 (m, 3H), 2.01 – 1.90 (m, 1H), 1.82 – 1.74 (m, 1H), 1.51 (s, 9H). ESI-MS: *m/z* = 533 $[M + Na]^+$.

Tert-butyl

(3S,4S)-4-(3-fluorophenyl)-3-(5-(4-formyl-1-methyl-1H-pyrazol-5-yl)thiophene-2-carboxamido)piperidine-1-carboxylate(18)

Compound **17** (102 mg, 0.2 mmol) was dissolved in a mixture of 10 mL THF and H_2O (v/v, 3:1) on

the ice bath. Osmium tetroxide (5 mg) and N-methylmorpholine oxide (47 mg, 0.4 mmol) were added, and then stirred at room temperature overnight. $NaIO_4$ (107 mg, 0.5 mmol) was added to the reaction and stirred for 3 hours at room temperature. After the reaction was completed, the reaction liquid was poured into the saturated $Na_2S_2O_3$ solution (20 mL). Extracted with ethyl acetate (10 mL × 3), the combined organic layers were washed with saturated brine (20 mL × 2), dried over anhydrous sodium sulfate and concentrated. Purification by flash column chromatography afforded the white solids (94 mg, 0.184 mmol). Yield: 92%; ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.72 (s, 1H), 8.02 (s, 1H), 7.32 – 7.27 (m, 2H), 7.18 (d, J = 3.8 Hz, 1H), 7.05 (d, J = 7.2 Hz, 1H), 7.00 – 6.91 (m, 2H), 5.76 (br s, 1H), 4.57 – 4.50 (m, 1H), 4.34 – 4.14 (m, 2H), 3.89 (s, 3H), 2.94 – 2.71 (m, 3H), 1.98 – 1.91 (m, 1H), 1.85 – 1.75 (m, 1H), 1.51 (s, 9H). ESI-MS: *m/z* = 535 $[M + Na]^+$.

N-((3S,4S)-4-(4-chlorophenyl)piperidin-3-yl)-5-(1-methyl-1H-pyrazol-5-yl)thiophene-2-carboxamide hydrochloride (1a)

General procedure F, yield: 72%; Retention time: 9.373 min, purity: 96.62 %; ^{1}H NMR (500 MHz, DMSO-*d6*) δ 9.55 (d, *J* = 9.3 Hz, 1H), 9.46 (d, *J* = 10.5 Hz, 1H), 9.04 (d, *J* = 8.9 Hz, 1H), 7.89 (d, *J* = 3.9 Hz, 1H), 7.45 (d, *J* = 1.9 Hz, 1H), 7.37 – 7.29 (m, 5H), 6.53 (d, *J* = 1.9 Hz, 1H), 4.54 – 4.45 (m, 1H), 3.92 (s, 3H), 3.36 (dd, *J* = 9.2, 4.7 Hz, 2H), 3.23 – 3.18 (m, 1H), 2.99 – 2.92 (m, 2H), 2.11 – 1.99 (m, 1H), 1.97 – 1.92 (m, 1H). ^{13}C NMR (125 MHz, DMSO-*d6*) δ 160.14, 140.86, 139.43, 138.13, 135.36, 134.94, 131.27, 129.43, 129.27, 128.33, 127.46, 106.85, 47.39, 46.41, 43.93, 43.25, 38.08, 29.31. ESI-MS: *m/z* = 401 $[M + H]^+$. HRMS (ESI) (m/z): calcd for $C_{20}H_{22}ClN_4OS$ $[M + H]^+$ 401.1197, found 401.1212.

5-(4-Chloro-1-methyl-1H-pyrazol-5-yl)-N-((3S,4S)-4-(4-chlorophenyl)piperidin-3-yl)thiophene-2-carboxamide hydrochloride (1b)

General procedure F, yield: 80%; ^{1}H NMR (500 MHz, DMSO-*d6*) δ 9.45 (d, *J* = 9.5 Hz, 1H), 9.36 (d, *J* = 10.3 Hz, 1H), 9.04 (d, *J* = 8.9 Hz, 1H), 7.92 (d, *J* = 3.9 Hz, 1H), 7.66 (s, 1H), 7.40 (d, *J* = 3.9 Hz, 1H), 7.36 – 7.28 (m, 5H), 4.57 – 4.49 (m, 1H), 3.86 (s, 3H), 3.36 (dd, *J* = 14.0, 7.0 Hz, 2H), 3.21 (td, *J* = 11.7, 4.0 Hz, 1H), 2.97 (q, *J* = 10.9 Hz, 2H), 2.09 – 1.94 (m, 2H). ^{13}C NMR (125 MHz, DMSO-*d6*) δ 160.03, 141.13, 140.76, 136.67, 132.09, 131.27, 130.97, 129.97, 129.35, 128.75, 128.31, 108.49, 47.37, 46.35, 43.85, 43.24, 38.96, 29.37. ESI-MS: *m/z* = 435 $[M + H]^+$. HRMS (ESI) (m/z): calcd for $C_{20}H_{21}Cl_2N_4OS$ $[M + H]^+$ 435.0808, found 435.0828.

N-((3S,4S)-4-(3-fluorophenyl)piperidin-3-yl)-5-(1-methyl-1H-pyrazol-5-yl)thiophene-2-carboxamide hydrochlorid (1c)

General procedure F, yield: 54%; Retention time: 9.191 min, purity: 96.23 %; Melting point: 206~208 ^{o}C; ^{1}H NMR (500 MHz, MeOD) δ 8.02 (d, *J* = 2.6 Hz, 1H), 7.74 (d, *J* = 4.0 Hz, 1H), 7.46 (d, *J* = 3.9 Hz, 1H), 7.29 (dd, *J* = 14.0, 7.9 Hz, 1H), 7.19 (d, *J* = 7.8 Hz, 1H), 7.14 (dd, *J* = 10.0, 1.9 Hz, 1H), 6.92 (td, *J* = 8.5, 2.3 Hz, 1H), 6.82 (d, *J* = 2.6 Hz, 1H), 4.65 (td, *J* = 11.6, 4.3 Hz, 1H), 4.10 (s, 3H), 3.64 – 3.61 (m, 1H), 3.56 (d, *J* = 12.7 Hz, 1H), 3.30 – 3.18 (m, 3H), 2.22 – 2.08 (m, 2H). ^{13}C NMR (125 MHz, MeOD) δ 164.22 (d, *J* = 243.8 Hz), 162.60, 144.90 (d, *J* = 6.3 Hz), 142.59, 140.68, 136.93, 133.53, 131.40 (d, *J* = 7.5 Hz), 131.19, 130.46, 124.71 (d, *J* = 2.5 Hz), 115.47 (d, *J* = 21.3 Hz), 115.03 (d, *J* = 21.3 Hz), 109.01, 49.38, 47.92, 46.29, 45.07, 38.06, 31.12. ESI-MS: *m/z* = 385 $[M + H]^{+}$.

5-(4-Chloro-1-methyl-1H-pyrazol-5-yl)-N-((3S,4S)-4-(3-fluorophenyl)piperidin-3-yl)thiophene -2-carboxamide hydrochloride (1d)

General procedure F, yield: 78%; Melting point: 200~202 ^{o}C; ^{1}H NMR (500 MHz, MeOD) δ 7.62 (d, *J* = 3.8 Hz, 1H), 7.52 (s, 1H), 7.35 – 7.28 (m, 2H), 7.16 (d, *J* = 7.8 Hz, 1H), 7.10 (d, *J* = 10.1 Hz, 1H), 6.95 (td, *J* = 8.5, 2.2 Hz, 1H), 4.58 (td, *J* = 11.6, 4.2 Hz, 1H), 3.87 (s, 3H), 3.65 – 3.59 (m, 1H), 3.54 (d, *J* = 12.7 Hz, 1H), 3.20 (t, *J* = 13.0 Hz, 2H), 3.13 (t, *J* = 12.0 Hz, 1H), 2.20 (d, *J* = 12.4 Hz, 1H), 2.12 – 2.02 (m, 1H). ^{13}C NMR (125 MHz, MeOD) δ 164.35 (d, *J* = 247.5 Hz), 163.15, 144.73 (d, *J* = 6.3 Hz), 141.50, 138.11, 133.94, 133.57, 131.51 (d, *J* = 7.5 Hz), 131.00, 129.97, 124.56, 115.41 (d, *J* = 22.5 Hz), 115.19 (d, *J* = 21.3 Hz), 111.29, 49.41, 48.06, 46.42, 45.17, 39.19, 31.33. ESI-MS: *m/z* = 419 $[M + H]^{+}$. HRMS (ESI) (*m/z*): calcd for $C_{20}H_{21}ClFN_4OS$ $[M + H]^{+}$ 419.1109, found 419.1113.

5-(4-Bromo-1-methyl-1H-pyrazol-5-yl)-N-((3S,4S)-4-(3-fluorophenyl)piperidin-3-yl)thiophene-2-carboxamide hydrochlorid (1e)

General procedure F, yield: 89%; Retention time: 9.860 min, purity: 95.58 %; Melting point: 189~192 ^{o}C; ^{1}H NMR (500 MHz, MeOD) δ 7.66 (d, *J* = 3.9 Hz, 1H), 7.54 (s, 1H), 7.33 – 7.28 (m, 1H), 7.26 (d, *J* = 3.9 Hz, 1H), 7.18 (d, *J* = 7.7 Hz, 1H), 7.12 (d, *J* = 9.9 Hz, 1H), 6.94 (td, *J* = 8.5, 2.2 Hz, 1H), 4.61 (td, *J* = 11.5, 4.1 Hz, 1H), 3.85 (s, 3H), 3.65 – 3.59 (m, 1H), 3.55 (d, *J* = 12.3 Hz, 1H), 3.28 – 3.12 (m, 3H), 2.20 (d, *J* = 13.7 Hz, 1H), 2.15 – 2.03 (m, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 164.30 (d, *J* = 243.8 Hz), 163.06, 144.80 (d, *J* = 6.3 Hz), 141.70, 140.36, 135.61, 133.95, 131.47 (d, *J* = 7.5 Hz), 131.39, 129.95, 124.61, 115.43 (d, *J* = 21.3 Hz), 115.13 (d, *J* = 21.3 Hz), 96.03, 49.34, 48.01, 46.33, 45.14, 39.12, 31.27. ESI-MS: *m/z* = 463 $[M + H]^{+}$. HRMS (ESI) (*m/z*): calcd for $C_{20}H_{21}BrFN_4OS$ $[M + H]^{+}$ 463.0603, found 463.0649.

5-(4-Chloro-1-methyl-1H-pyrazol-5-yl)-N-((3S,4S)-4-(4-chloro-3-(trifluoromethyl)phenyl)piperidin-3-yl)thiophene-2-carboxamide hydrochlorid (1f)

General procedure F, yield: 77%; Retention time: 10.630 min, purity: 95.89 %; ^{1}H NMR (500 MHz, DMSO-*d6*) δ 9.45 (d, *J* = 9.0 Hz, 1H), 9.26 (d, *J* = 9.8 Hz, 1H), 9.12 (d, *J* = 8.9 Hz, 1H), 7.92 (d, *J* = 3.6 Hz, 1H), 7.79 (s, 1H), 7.70 – 7.63 (m, 2H), 7.59 (d, *J* = 8.1 Hz, 1H), 7.42 (d, *J* = 3.7 Hz, 1H), 4.58 – 4.48 (m, 1H), 3.86 (s, 3H), 3.42 – 3.27 (m, 3H), 3.02 – 2.91 (m, 2H), 2.15 – 1.97 (m, 2H). ^{13}C NMR (125 MHz, DMSO-*d6*) δ 160.09, 141.63, 140.83, 136.60, 132.76, 132.00, 131.61, 130.98, 129.86, 128.86, 128.70, 127.31 (q, *J* = 5.1 Hz), 126.24 (q, *J* = 30.1 Hz), 122.74 (q, *J* = 271.4 Hz), 108.44, 47.28, 46.25, 43.72, 43.10, 38.83, 28.68. ESI-MS: *m/z* = 503 $[M + H]^+$. HRMS (ESI) (*m/z*): calcd for $C_{21}H_{20}Cl_2F_3N_4OS$ $[M + H]^+$ 503.0687, found 503.0711.

5-(1,4-Dimethyl-1H-pyrazol-5-yl)-N-((3S,4S)-4-(3-fluorophenyl)piperidin-3-yl)thiophene-2-carboxamide hydrochloride (1g)

General procedure G yield: 51%; ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.73 (s, 1H), 7.68 (d, *J* = 3.8 Hz, 1H), 7.38 – 7.25 (m, 2H), 7.17 (d, *J* = 7.4 Hz, 1H), 7.11 (d, *J* = 8.8 Hz, 1H), 6.96 (dd, *J* = 11.5, 5.3 Hz, 1H), 4.63 – 4.55 (m, 1H), 3.90 (s, 3H), 3.68 – 3.50 (m, 3H), 3.25 – 3.10 (m, 2H), 2.20 (d, *J* = 14.0 Hz, 1H), 2.11 (s, 3H), 2.09 – 2.02 (m, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 164.33 (d, *J* = 242.5 Hz), 162.92, 144.79 (d, *J* = 7.5 Hz), 142.37, 137.55, 137.39, 133.64, 131.84, 131.52 (d, *J* = 8.8 Hz), 130.20, 124.61, 118.99, 115.44 (d, *J* = 21.3 Hz), 115.16 (d, *J* = 21.3 Hz), 49.28, 48.02, 46.40, 45.13, 37.71, 31.33, 9.38. ESI-MS: *m/z* = 399 $[M + H]^+$.

N-((3S,4S)-4-(3-fluorophenyl)piperidin-3-yl)-5-(1-methyl-4-vinyl-1H-pyrazol-5-yl)thiophene-2-carboxamide hydrochlorid (1h)

To a solution of compound **17** in ethyl acetate was added HCl saturated ethyl acetate, stirred at room temperature for 6 h. After it was fully reacted, the mixture was filtered, the resulted solid was washed with diethyl ether. The solid was dried in vacuum to afford target product as white solid. Yield: 89%; Melting point: 238~240 ^{o}C; ^{1}H NMR (500 MHz, $CDCl_3$, 3:2 ratio due to atropisomers) δ 7.93 and 7.85 (s, 1H), 7.70 (s, 1H), 7.35 – 7.06 (m, 4H), 6.94 (t, *J* = 8.0 Hz, 1H), 6.43 (dd, *J* = 17.5, 11.1 Hz, 1H), 5.59 (d, *J* = 17.5 Hz, 1H), 5.16 (d, *J* = 11.1 Hz, 1H), 4.67 – 4.55 (m, 1H), 3.86 and 3.83 (s, 3H), 3.67 – 3.51 (m, 2H), 3.29 – 3.15 (m, 3H), 2.23 – 2.04 (m, 2H). ESI-MS: *m/z* = 411 $[M + H]^+$. HRMS (ESI) (*m/z*): calcd for $C_{22}H_{24}FN_4OS$ $[M + H]^+$ 411.1655, found 411.1654.

5-(4-Ethyl-1-methyl-1H-pyrazol-5-yl)-N-((3S,4S)-4-(3-fluorophenyl)piperidin-3-yl)thiophene-2-carboxamide hydrochlorid (1i)

To a solution of compound 17 (51 mg, 0.1 mmol) in 3 mL MeOH was added 10% Pd/C (5 mg) at 0 □. The atmosphere of the reaction system was replaced by hydrogen three times and reacted overnight. After the reaction was completed, Pd/C was removed by filtrated, and the filtrate was concentrated to afford gray solid. To a solution of the above solid in ethyl acetate was added HCl saturated ethyl acetate, stirred at room temperature for 6 h. After it was fully reacted, the mixture was filtered, the resulted solid was washed with diethyl ether. The solid was dried in vacuum to afford target product as white solid. Yield: 72%; Retention time: 9.876 min, purity: 95.64 %; Melting point: 226~228 ^{o}C; ^{1}H NMR (500 MHz, MeOD) δ 7.90 (s, 1H), 7.74 (d, *J* = 3.8 Hz, 1H), 7.34 – 7.27 (m, 2H), 7.19 (d, *J* = 7.7 Hz, 1H), 7.13 (d, *J* = 10.0 Hz, 1H), 6.94 (td, *J* = 8.5, 2.2 Hz, 1H), 4.62 (td, *J* = 11.6, 4.3 Hz, 1H), 3.91 (s, 3H), 3.64 – 3.60 (m, 1H), 3.55 (d, *J* = 12.6 Hz, 1H), 3.30 – 3.15 (m, 3H), 2.52 (q, *J* = 7.5 Hz, 2H), 2.23 – 2.05 (m, 2H), 1.15 (t, *J* = 7.5 Hz, 3H). ^{13}C NMR (125 MHz, MeOD) δ 164.28 (d, *J* = 242.5 Hz), 162.78, 144.86 (d, *J* = 6.3 Hz), 142.94, 137.35, 135.84, 132.75, 132.37, 131.46 (d, *J* = 7.5 Hz), 130.26, 126.08, 124.65 (d, *J* = 2.5 Hz), 115.48 (d, *J* = 21.3 Hz), 115.10 (d, *J* = 21.3 Hz), 49.38, 47.96, 46.32, 45.11, 37.47, 31.24, 18.27, 15.07. ESI-MS: *m/z* = 413 $[M + H]^+$. HRMS (ESI) (*m/z*): calcd for $C_{22}H_{26}FN_4OS$ $[M + H]^+$ 413.1811, found 413.1838.

5-(5-(((3S,4S)-4-(3-fluorophenyl)piperidin-3-yl)carbamoyl)thiophen-2-yl)-1-methyl-1H-pyrazole-4-carboxylic acid hydrochloride (1j)

To a 10 mL mixture of CH_3CN and hydrogen peroxide (v/v, 1:1) was added compound **18** (102 mg, 0.2 mmol), ruthenium trichloride (4 mg, catalytic capacity) and $NaIO_4$ (214 mg, 1 mmol) at 0 □, and then reacted overnight at room temperature. After the reaction was completed, the black insoluble substances were removed by filtration, and the filtrate was extracted with ethyl acetate (10 mL × 3), the combined organic layers were washed with saturated brine (20 mL × 2), dried over anhydrous sodium sulfate and concentrated. Purification by flash column chromatography (DCM: MeOH = 30:1-10:1) afforded the N-Boc protective products as white solid; ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.01 (s, 1H), 7.31 – 7.23 (m, 2H), 7.09 – 7.01 (m, 2H), 6.98 (d, J = 9.8 Hz, 1H), 6.93 – 6.88 (m, 1H), 6.13 (brs, 1H), 4.52 – 4.35 (m, 1H), 4.26 – 4.13 (m, 2H), 3.79 (s, 3H), 2.99 – 2.75 (m, 3H), 2.02 – 1.91 (m, 1H), 1.86 – 1.74 (m, 1H), 1.49 (s, 9H). ESI-MS: m/z = 567 $[M + K]^+$.

To a solution of the above solid in ethyl acetate was added HCl saturated ethyl acetate, stirred at room temperature for 6 h. After it was fully reacted, the mixture was filtered, the resulted solid was washed with diethyl ether. The solid was dried in vacuum to afford target product as white solid.

Yield: 55%; Melting point: 181~183 ^{o}C; Specific optical rotation: -40.687^{o}; ^{1}H NMR (500 MHz, MeOD) δ 7.94 (s, 1H), 7.61 (d, *J* = 3.1 Hz, 1H), 7.37 – 7.28 (m, 1H), 7.22 (d, *J* = 3.2 Hz, 1H), 7.19 – 7.07 (m, 2H), 6.96 (t, *J* = 7.6 Hz, 1H), 4.65 – 4.55 (m, 1H), 3.75 (s, 3H), 3.61 (d, *J* = 7.0 Hz, 1H), 3.53 (d, *J* = 11.0 Hz, 1H), 3.26 – 3.07 (m, 3H), 2.20 – 2.02 (m, 2H). ^{13}C NMR (125 MHz, MeOD) δ 165.45, 163.32 (d, *J* = 242.5 Hz), 163.19, 144.80 (d, *J* = 7.5 Hz), 142.35, 142.23, 139.29, 134.30, 132.43, 131.53 (d, *J* = 7.5 Hz), 129.56, 124.63, 115.59, 115.40 (d, *J* = 21.3 Hz), 115.16 (d, *J* = 21.3 Hz), 49.27, 48.07, 46.35, 45.22, 37.99, 31.41. ESI-MS: *m/z* = 429 $[M + H]^{+}$. HRMS (ESI) (*m/z*): calcd for $C_{21}H_{22}FN_4O_3S$ $[M + H]^{+}$ 429.1397, found 429.1380.

N-((3S,4S)-4-(3-fluorophenyl)piperidin-3-yl)-5-(4-(hydroxymethyl)-1-methyl-1H-pyrazol-5-yl)thiophene-2-carboxamide hydrochloride (1k)

Compound 18 (94 mg, 0.184 mmol) was dissolved in a 5 mL mixture of THF and MeOH (v/v, 10:1). Then $NaBH_4$ (11 mg, 0.3 mmol) was added slowly to the mixture at 0 □, and reacted at room temperature for 2 h. After the reaction was completed, the mixture was cooled to 0 □ and quenched with 10% NH_4Cl solution (5 mL). The mixture was extracted with ethyl acetate (10 mL × 3), the combined organic layers were washed with saturated brine (20 mL × 2), dried over anhydrous sodium sulfate and concentrated. Purification by flash column chromatography afforded the foam solid. Yield: 88%;^{1}H NMR (500 MHz, MeOD, 1:9 ratio due to atropisomers) δ 7.84 and 7.78 (s, 1H), 7.67 (d, *J* = 3.9 Hz, 1H), 7.35 – 7.27 (m, 2H), 7.17 (d, *J* = 7.7 Hz, 1H), 7.11 (d, *J* = 10.0 Hz, 1H), 6.96 (dd, *J* = 11.8, 5.1 Hz, 1H), 4.59 (td, *J* = 11.6, 4.3 Hz, 1H), 4.45 and 4.27 (s, 2H), 3.92 and 3.92 (s, 3H), 3.64 – 3.60 (m, 1H), 3.54 (d, *J* = 12.6 Hz, 1H), 3.26 – 3.10 (m, 3H), 2.20 (d, *J* = 12.9 Hz, 1H), 2.11 – 2.04 (m, 1H). ^{13}C NMR (125 MHz, MeOD) δ 164.33 (d, *J* = 242.5 Hz), 163.02, 144.80 (d, *J* = 7.5 Hz), 142.39, 139.18, 137.67, 133.74, 131.89, 131.51 (d, *J* = 8.8 Hz), 130.12, 124.60 (d, *J* = 2.5 Hz), 120.09, 115.44 (d, *J* = 21.3 Hz), 115.16 (d, *J* = 21.3 Hz), 65.19, 49.28, 48.01, 46.39, 45.13, 37.92, 31.33. ESI-MS: *m/z* = 415 $[M + H]^{+}$.

N-((3S,4S)-4-(3-fluorophenyl)piperidin-3-yl)-5-(1-methyl-4-phenyl-1H-pyrazol-5-yl)thiophene-2-carboxamide hydrochloride (1l)

General procedure G yield: 76%; Melting point: 190~193 ^{o}C; ^{1}H NMR (500 MHz, MeOD) δ 7.90 (s, 1H), 7.67 (d, *J* = 3.2 Hz, 1H), 7.31 – 7.06 (m, 9H), 6.94 (t, *J* = 7.5 Hz, 1H), 4.63 – 4.53 (m, 1H), 3.83 (s, 3H), 3.58 – 3.44 (m, 2H), 3.25 – 3.07 (m, 3H), 2.22 – 2.03 (m, 2H). ^{13}C NMR (125 MHz, MeOD) δ 164.27 (d, *J* = 243.8 Hz), 162.91, 144.81 (d, *J* = 7.5 Hz), 142.51, 137.36, 134.75, 134.66, 132.48, 132.41, 131.46 (d, *J* = 7.5 Hz), 130.31, 129.68, 128.89, 128.36, 124.89, 124.59, 115.46 (d, *J*

= 22.5 Hz), 115.09 (d, *J* = 21.3 Hz), 49.34, 47.96, 46.27, 45.11, 37.59, 31.20. ESI-MS: *m/z* = 461 $[M + H]^+$. HRMS (ESI) (*m/z*): calcd for $C_{26}H_{26}FN_4OS$ $[M + H]^+$ 461.1811, found 461.1846.

N-((3S,4S)-4-(3-fluorophenyl)piperidin-3-yl)-5-(1-methyl-4-(pyridin-4-yl)-1H-pyrazol-5-yl)thiophene-2-carboxamide hydrochloride (1m)

General procedure G yield: 46%;^{1}H NMR (500 MHz, MeOD) δ 8.59 (d, *J* = 6.7 Hz, 2H), 8.27 (s, 1H), 7.83 (d, *J* = 6.7 Hz, 2H), 7.78 (d, *J* = 3.8 Hz, 1H), 7.36 – 7.31 (m, 2H), 7.19 (d, *J* = 7.7 Hz, 1H), 7.12 (d, *J* = 9.9 Hz, 1H), 6.98 (td, *J* = 8.4, 2.1 Hz, 1H), 4.59 (dt, *J* = 11.6, 5.8 Hz, 1H), 3.81 (s, 3H), 3.61 – 3.57 (m, 1H), 3.56 – 3.51 (m, 1H), 3.29 – 3.10 (m, 3H), 2.21 – 2.16 (m, 1H), 2.11 – 2.04 (m, 1H). ^{13}C NMR (125 MHz, MeOD) δ 164.33 (d, *J* = 243.8 Hz), 162.69, 152.06, 145.04, 144.89 (d, *J* = 7.5 Hz), 144.08, 142.37, 140.41, 136.89, 133.48, 133.24, 131.57 (d, *J* = 8.8 Hz), 130.84, 127.15, 124.62, 124.28, 118.99, 115.50 (d, *J* = 21.3 Hz), 115.18 (d, *J* = 21.3 Hz), 49.40, 47.95, 46.26, 45.10, 37.94, 31.37. ESI-MS: *m/z* = 462 $[M + H]^+$. HRMS (ESI) (*m/z*): calcd for $C_{25}H_{25}FN_5OS$ $[M + H]^+$ 462.1764, found 462.1765.

4-(4-Chloro-1-methyl-1H-pyrazol-5-yl)-N-((3S,4S)-4-(4-chlorophenyl)piperidin-3-yl)thiophene-2-carboxamide (2R,3R)-2,3-dihydroxysuccinate (1n)

General procedure F, yield: 47%; ^{1}H NMR (400 MHz, DMSO-*d6*) δ 8.76 (d, *J* = 7.8 Hz, 1H), 8.03 (s, 1H), 7.88 (s, 1H), 7.62 (s, 1H), 7.39 – 7.25 (m, 4H), 4.55 – 4.39 (m, 1H), 4.17 (s, 2H), 3.81 (s, 3H), 3.46 – 3.31 (m, 2H), 3.08 – 2.87 (m, 3H), 2.06 – 1.91 (m, 2H). ^{13}C NMR (100 MHz, DMSO-*d6*) δ 174.03, 160.10, 140.80, 139.95, 136.39, 133.96, 131.55, 131.28, 129.35, 128.45, 128.33, 127.33, 107.34, 72.11, 47.66, 46.55, 44.20, 43.21, 38.60, 29.72. HRMS (ESI) (*m/z*): calcd for $C_{20}H_{21}Cl_2N_4OS$ $[M + H]^+$ 435.0813, found 435.0737.

4-(4-Chloro-1-methyl-1H-pyrazol-5-yl)-N-((3S,4S)-4-(3-fluorophenyl)piperidin-3-yl)thiophene-2-carboxamide hydrochloride (1o)

General procedure F, yield: 69%; Retention time: 9.735 min, purity: 96.10 %; Melting point: 189~192 ^{o}C; Specific optical rotation: -88.742^o; ^{1}H NMR (500 MHz, MeOD) δ 7.89 (d, *J* = 1.0 Hz, 1H), 7.79 (s, 1H), 7.52 (s, 1H), 7.32 – 7.27 (m, 1H), 7.17 (d, *J* = 7.7 Hz, 1H), 7.11 (d, *J* = 10.0 Hz, 1H), 6.97 – 6.92 (m, 1H), 4.59 (td, *J* = 11.5, 4.1 Hz, 1H), 3.83 (s, 3H), 3.64 – 3.59 (m, 1H), 3.57 – 3.50 (m, 1H), 3.26 – 3.13 (m, 3H), 2.22 – 2.03 (m, 2H). ^{13}C NMR (125 MHz, MeOD) δ 164.31 (d, *J* = 243.8 Hz), 163.06, 144.76 (d, *J* = 7.5 Hz), 140.44, 137.78, 136.00, 133.02, 131.48 (d, *J* = 7.5 Hz), 130.08, 129.10, 124.62 (d, *J* = 2.5 Hz), 115.45 (d, *J* = 21.3 Hz), 115.15 (d, *J* = 21.3 Hz), 110.12, 49.46, 48.05, 46.39, 45.14, 38.86, 31.18. ESI-MS: *m/z* = 419 $[M + H]^+$. HRMS (ESI) (*m/z*): calcd

for $C_{20}H_{21}ClFN_4OS$ $[M + H]^+$ 419.1109, found 419.1115.

4-(4-Chloro-1-methyl-1H-pyrazol-5-yl)-N-((3S,4S)-4-(4-chloro-3-(trifluoromethyl)phenyl)piperidin-3-yl)thiophene-2-carboxamide hydrochloride (1p)

General procedure F, yield: 49%; Retention time: 10.568 min, purity: 95.69 %; ^{1}H NMR (500 MHz, DMSO-*d6*) δ 9.34 (s, 1H), 9.20 (s, 1H), 9.09 (d, *J* = 8.9 Hz, 1H), 8.04 (d, *J* = 1.2 Hz, 1H), 7.79 (d, *J* = 1.5 Hz, 1H), 7.70 – 7.63 (m, 3H), 7.50 (d, *J* = 8.3 Hz, 1H), 4.54 – 4.46 (m, 1H), 3.82 (s, 3H), 3.31 – 3.27 (m, 2H), 3.02 – 2.93 (m, 2H), 2.72 (q, *J* = 11.1 Hz, 1H), 2.14 – 1.94 (m, 2H). ^{13}C NMR (125 MHz, DMSO-*d6*) δ 168.81, 160.10, 141.74, 139.71, 136.36, 133.95, 132.86, 131.64, 128.92, 128.67, 127.51 (q, *J* = 4.6 Hz), 127.28, 126.24 (q, *J* = 32.5 Hz), 122.88 (q, *J* = 271.2 Hz), 107.36, 47.43, 46.38, 44.05, 42.95, 38.59, 29.06. ESI-MS: *m/z* = 503 $[M + H]^+$. HRMS (ESI) (*m/z*): calcd for $C_{21}H_{20}Cl_2F_3N_4OS$ $[M + H]^+$ 503.0687, found 503.0680.

4.2 Akt1 inhibitory activity assay

The Akt1 inhibitory activities were determined using the homogeneous time-resolved fluorescence (HTRF) KinEASE-STK S1 kit (Cat.#62ST2PEC, Cisbio) according to the manufacturer's instructions. Akt1 kinase was expressed and purified from Escherichia coli system in-house. In short, the kinase reactions were carried out in a 384 ProxiPlate with 10 µL reaction volume per well containing 0.25 µg/mL Akt1, 1 µM peptide substrate, test compound and 20 µM ATP in assay buffer (50 mM HEPES pH 7.5, 0.01% BRIJ-35, 5 mM $MgCl_2$, 1 mM EGTA). After incubation for 1 h at r.t., the reaction was stopped by the addition of 5µL Sa-XL665 and 5 µL STK Antibody-Eu(K) in EDTA. The plate was sealed and incubated for 1 h at r.t., and the resulting TR-FRET signal was measured on Envision-PerkinElmer. The fluorescence emission was measured at 615 nm (cryptate) and 665 nm (XL665). An Emission Ratio was calculated (665/615) for each well and the Percent inhibitions were expressed as follows: Percent inhibition = (max-sample Ratio)/ (max-min)*100 ("Min" means the Ratio of no enzyme control and "max" means the Ratio of DMSO control). Compounds were initially tested at a fixed concentration (1 µg/mL), and those displaying more than 50% inhibition were further tested for dose–response IC_{50} values.

4.3 Cell culture

Human ovarian carcinoma cells (OVCAR8) and human colon cancer cells (HCT116) were obtained from the Cell Bank of the China Science Academy, Shanghai and were cultured in DMEM, F-12K or RPMI-1640 medium (Gibco Laboratories), supplemented with 10% fetal bovine serum (Gibco Laboratories), 105 U/L penicillin, and 100 mg/L streptomycin at 37 °C in an atmosphere containing

5% CO2. Cells were grown to 70% to 80% confluency in dishes or cell culture plates and treated under various conditions as indicated.

4.4 Anti-proliferative assay

OVCAR8 and HCT116 cells were treated with the compounds at various doses for 72h, and cell proliferation was tested by a sulforhodamine B (SRB) protein assay (Sigma, S1402). Cells (5000/well) were incubated with 10% trichloroacetic acid (TCA) for 1 h (4 °C) and then stained with SRB for 20 min. The SRB was washed away with 1% glacial acetic acid, and 100 μ l of 1% Tris-base was added to each well. The optical density (OD) was determined at 515 nm by a Multiskan Spectrum plate reader (Thermo Electron Corporation, Marietta, OH, USA).

4.5 Western blot assay

Whole protein extracts from cultured cells or tissues were prepared and subjected to western blotting. Protein samples were size-fractionated by 10% SDS-PAGE, analysed by immunoblotting and visualized by enhanced chemiluminescence (ECL, Amersham Biosciences, Castle Hill, Australia) and then exposed on X-ray film. Proteins in the lysates were equalized and then analyzed by specific antibodies. The primary antibodies were listed as follows: AKT (CST, 9272S), pAKT-Thr308 (Santa, sc-16646), pAKT-Ser473 (Santa, sc-7985), PRAS40 (CST, 2691P), p-PRAS40 (CST, 13175P), GSK-3β (Santa, sc-7291), p-GSK-3β (CST, 8566S), β-Actin (Santa, SC-1615). Appropriate secondary antibodies and ECL were used to visualize the protein signaling.

4.6 Flow cytometry

HCT116 cells were seeded in 6-well dish plates at 1×10^5 cells/well and exposed to different concentrations of compounds. After 72 h, the cells were harvested and stained with Annexin-V/PI Solution using the FITC Annexin V Apoptosis Detection Kit I (BD Bioscience, 556547) for 10–15 min and resuspended in binding buffer. Then the fluorescence emission at 530 nm and 585 nm using 488 nm excitation were measured by flow cytometry (BD FACSCalibur).

4.7 hERG inhibition assay

Currents were recorded from HEK-293 cells, using the whole-cell patch-clamp technique. The cells were transferred to a perfusion chamber and the perfusion was performed with extracellular fluid. The extracellular fluid (mM): K Aspartate, 130; $MgCl_2$, 5; EGTA, 5; HEPES, 10; Tris-ATP, 4; pH 7.2. Electrodes were pulled using a dual-stage glass micropipette puller (Narishige PC-10, Japan). Current traces of hERG channels were elicited by applying a pulse from -80 mV to +40 mV for 4 s followed by a step to -40 mV for 2 s. The procedure was repeated every 20 seconds. After the

maximum current was stabilized, the tested compounds (3 μM) were perfused. The inhibition rate was calculated when the current was stable. All data represent at least 3 independent experiments (n = 3).

4.8 Liver microsome stability assay

1 mg/mL microsome solution (purchased from Ruide Research Institute for Liver Diseases (Shanghai) Co. Ltd) was mixed with 20 mL of 50 mM NADPH (Aladdin) solution to prepare a microsome-NADPH solution. 500 μL of the microsome-NADPH solution was pre-warmed at 37 ^{o}C for 5 minutes. 5 μL of a 100 μg/mL test article solution was then added to initiate the reaction. The incubation mixture was kept at 37 ^{o}C and 100 μL aliquots were taken at 0, 15, and 45 minutes. In each aliquot, the reaction was quenched using 400 μL of methanol containing 1 μg/mL internal standard compound (from the in-house database). After quenching, the mixtures were vortexed and centrifuged. The supernatant was transferred and 10 μL was injected into an API4000 + LC/MS system. The peak area ratio of a test article versus the internal standard was used in the calculation of the rate of disappearance of a test article.

4.9 Plasma stability assay

A 500 μL aliquot of rat plasma or human plasma (purchased from Shanghai Yuduo Biotechnology Company) was added to 5 μL of a test article solution at 100 μg/mL to give a final solution. The mixture was incubated at 37 ^{o}C with gentle agitation. An aliquot of 100 μL of the reaction mixture was taken at 0, 15, and 45 minutes and quenched using 400 μL of methanol containing 1 μg/mL internal standard compound (from the in-house database). After quenching, the mixture was vortexed and centrifuged and 10 μL of the resulting solution was injected into an API4000 + LC/MS system. The percentage remaining of the test article at the incubation times of 15- and 45-minutes relative to that at 0 minutes was calculated using the peak area ratio of the test article versus the internal standard.

4.10 Pharmacokinetic studies

This study was performed in strict accordance with the Laboratory Animal Management Regulations (State Scientific and Technological Commission Publication No. 8-27 Rev. 2017) and was approved by Zhejiang University Laboratory Animal Center (Hangzhou, China). SD rats (purchased from Zhejiang Academy of Medical Sciences) were administered compound in saline by oral gavage or intravenous injection. Venous blood (100 μL) samples were collected at 0, 0.167, 0.5, 1, 2, 3, 4, 8, 24 and 48 h for oral gavage or 0, 0.083, 0.25, 0.5, 1, 2, 4, 8, 24 and 48 h for intravenous

injection. Plasma was separated from whole blood by centrifugation and stored at -20 ^{o}C until analysis. Compound levels were determined using a Waters Xevo TQ LC-MS/MS system. The C_{max}, T_{max}, $t_{1/2}$ and AUC were evaluated using Analyst 1.5.1.

4.11 Xenotransplantation

BALB/c (nu/nu) mice (National Rodent Laboratory Animal Resource, Shanghai), 6–8 weeks of age, were used for all experiments. When MM1S xenograft tumors reached 100 mm^3, mice were randomly divided into four groups. Compounds were dissolved in the solvent: DMSO:PEG400:H_2O=1:20:79. The dosing group received **1o** (15 or 30 mg/kg) and **GSK2141795** (30 mg/kg) orally once daily for 14 days, and the mice in control group were orally administered the solvent for 14 days.

Statements of Ethical Approval: The animal study was conducted in full compliance with the Guide for Care and Use of Laboratory Animals and approved by the Shanghai Model Organisms Center, -Inc..

4.12 Molecular docking and dynamic simulation

The molecular docking was performed using Ligand Docking implanted in Maestro. Parameters were maintained at the default configuration. The docked structure of compound **1o** complexed within the active pocket of 4GV1 was used as the initial structures for molecular dynamic calculations using Discovery Studio 2.5. A CHARMm force field was applied to the complex and the resulting system was subjected to double-fold minimization (10,000 cycles of steepest descent minimization and 10,000 cycles of conjugate gradient minimization). The system was heated from 50 K to 300 K over a period of 4 ps and subsequently equilibrated for 500 ps. Starting from the last frame of the equilibration, a production simulation was performed for 20000 ps using the NPT simulation (The isothermic-isobaric ensemble simulation) ensemble under a constant temperature of 300 K and pressure of 1 atm. Other parameters of MD simulation were maintained at the default Discovery Studio configuration.

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Author Contributions

W.Z. and J.C. designed and synthesized the compounds, analyzed the data and drafted the manuscript, Y.W synthesized the compounds, L.X. and X.H. performed the biological experiments, G.C. performed the docking calculations, Y.Z., J.L., X.D. and J.L. conceived the study and helped to analyze the data.

‡ W.Z. and J.C. contributed equally to this work.

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Declaration of Interests

The authors declare no competing financial interest.

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Highlights:

- Compound **1d** and **1o** exhibiting good Akt inhibitory activities were obtained on the basis of conformational restriction strategy.
- Compound **1d** and **1o** showed excellent *in vitro* antitumor effect against a variety of hematologic cancer cells, and significantly induced apoptosis, blocked the cell cycles at *S* phase and inhibited the phosphorylation of downstream biomarkers of Akt kinase of cancer cells.
- Compound **1o** exhibited good PK profiles and a 40% tumor growth inhibition in MM1S xenograft tumor model.