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Article

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Design, Synthesis and Biological Evaluation of Dimorpholine Substituted Thienopyrimidines as Potential Class I PI3K/ mTOR Dual Inhibitors

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KEYWORDS : Class I PI3K/ mTOR Inhibitor; PI3K/AKT/mTOR pathway; Morpholine; Antitumor.

ABSTRACT: Dysfunctional signaling of the PI3K/AKT/mTOR pathway in cancer, and its crucial role in cell growth and survival have made it a much desired target for cancer therapeutics. A series of dimorpholine substituted thienopyrimidine derivatives had been prepared and evaluated *in vitro* and *in vivo*. Among them, compound **14o** was identified as a dual Class I PI3K and mTOR kinase inhibitor, which had an approximately 8-fold improvement in

mTOR inhibition relative to the class I PI3K inhibitor **1** (pictilisib, GDC-0941). Western blot analysis confirmed that **14o** could mechanistic modulation of cellular PI3K/AKT/mTOR pathway through inhibiting phosphorylation of both AKT and S6 in human cancer cell lines. In addition, **14o** demonstrated significant efficacy in SKOV-3 and U87MG tumor xenograft models without causing significant weight loss and toxicity.

Introduction

The PI3K/AKT/mTOR pathway is one of the most frequently dysregulated signaling cascades in human malignancies, and plays a central role in cell proliferation, survival, migration, and metabolism.[1]

There are eight mammalian PI3K enzymes, which are grouped into IA, 1B, II, and III according to their sequence, homology, and substrate preferences. Class IA PI3Ks are heterodimers that contain one of three PI3K catalytic subunits (p110α, p110β and p110δ) and a p85 regulatory subunits, whereas Class IB PI3Ks consist of a p110γ catalytic subunit and regulatory subunit p101 or p87.[2] Class I PI3Ks are responsible for the conversion of phosphatidylinositol 4,5-diphosphate (PIP2) to phosphatidylinositol 3,4,5-triphosphate (PIP3).[3] The generation of PIP3 leads to the downstream activation of serine-threonine kinase AKT, which integrates multiple signaling pathways to regulate cell growth and metabolism.[4] Moreover, the gene *PIK3CA* that encode the p110α subunit, is overexpressed or mutated in a wide variety of cancers including ovarian, colorectal, glioblastoma, and gastric cancers.[5-8] The phosphatase and tensin homologue gene (*PTEN*), which serves as a critical negative regulator of PI3K signaling by converting PIP3 back to PIP2, is among the most frequently lost or mutated tumour suppressor genes.[9] In addition, in cancer cells expressing normal *PI3K* and *PTEN* genes, other

lesions are present that can activate the PI3K signaling pathway (mutation and/or amplification of genes encoding RTKs, RAS and AKT).[1] Active PI3K signaling is probably an important mechanism of resistance to various targeted cancer therapies.[10]

The kinase mTOR, a member of the PIKK (phosphatidylinositol like kinase) family, has a high degree of active site similarity with PI3Ks. mTOR can be activated by AKT and lead to increased protein synthesis and cell growth.[11, 12] The relevancy between mTOR and cancers has been validated by several approved drugs such as everolimus and temsirolimus for the treatment of advanced renal cell carcinoma.[13, 14] Dual PI3K/mTOR inhibitors appear to offer an augment on better efficacy and less likelihood to induce drug resistance, as they target the pathway at two nodal points.[12, 15, 16] In recent years, many PI3K/mTOR dual inhibitors were discovered including PF-04691502[17], PI-103[18], voxtalisib (XL765)[19], apitolisib (GDC-0980)[20], dactolisib (BEZ235)[21] and so on (Figure 1).

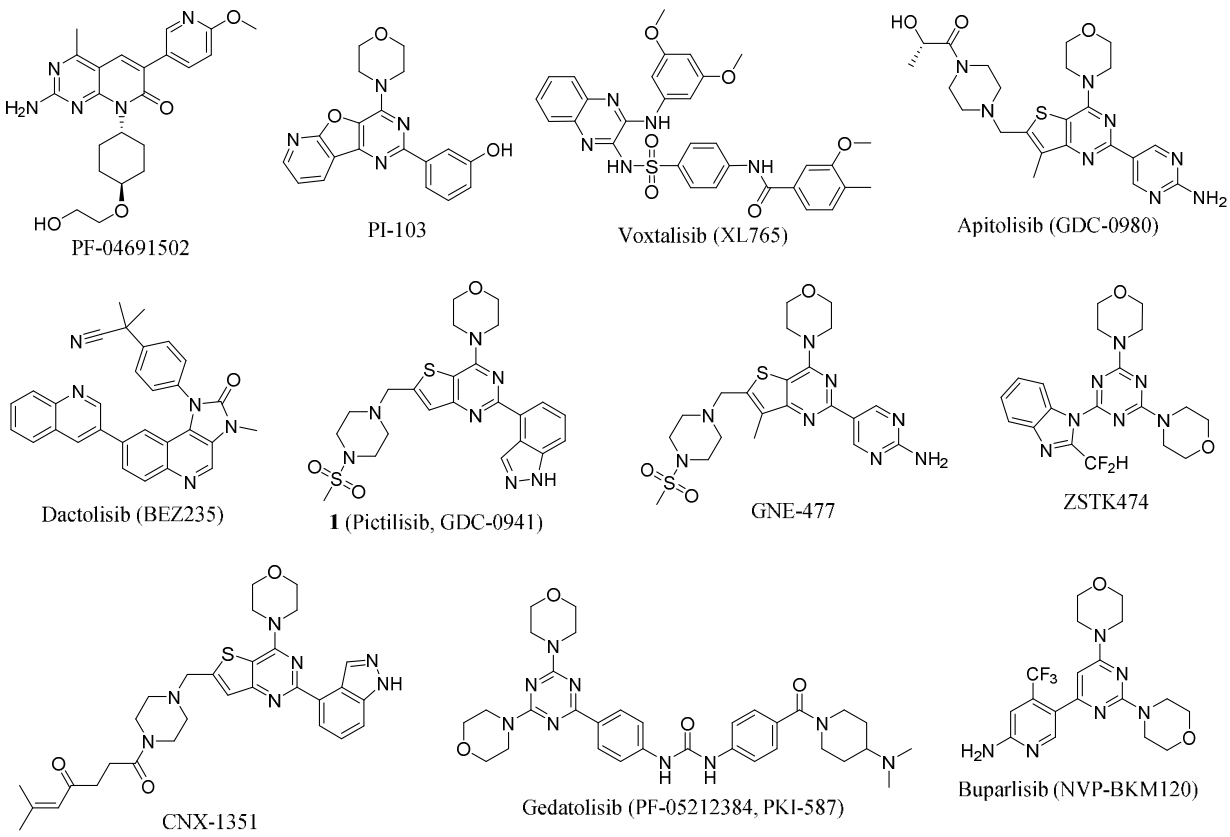


Figure 1. Examples of PI3K inhibitors

It has been reported that the morpholine ring is the most common feature of PI3K inhibitors (Figure 1), and the morpholine oxygen may form critical hydrogen bond with amino acid residues.[22]

The potent class I PI3K inhibitor **1** [23], currently in phase II clinical trials for the treatment of cancer, served as a useful template to explore new PI3K inhibitors. Starting with **1**, considerable structure−activity relationship (SAR) investigations have been focused on the substituents of thienopyrimidine core, and several advanced compounds were developed such as CNX-1351[24] and GNE-477[25]. However, for the C-2 position, these explorations are restricted to the replacement of the indazole with aryl or heteroaryl group, and the alkyl, cycloalkyl group or

saturated heterocycles substituents have barely been reported.[20, 25-28] It was conceivable that the potency as well as other drug-like properties could be further optimized through modification of **1** (Figure 2).

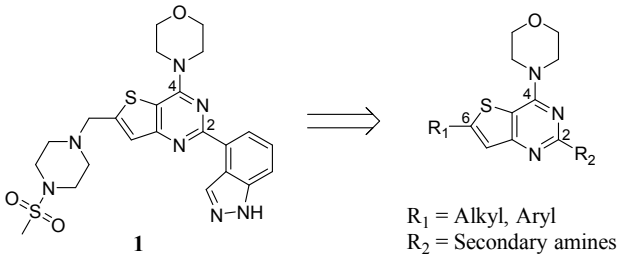


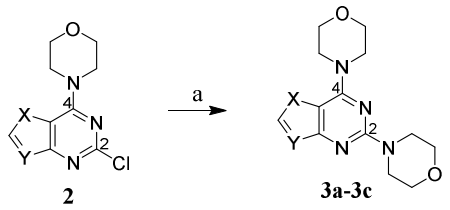
Figure 2. Design of series derivatives based on **1**.

It is worth mentioning that, several PI3K inhibitors were designed by incorporation of the second morpholine group into the triazine or pyrimidine core to improve the solubility or potency, such as ZSTK474[29], buparlisib (NVP-BKM120)[30] and gedatolisib (PF-05212384, PKI-587)[31] (Figure 1). Herein we disclose the preparation and biological evaluation of a series of dimorpholine substituted thienopyrimidine derivatives that demonstrate potent inhibition of PI3K/AKT/mTOR passway, culminating in the discovery of **14o**.

CHEMISTRY

As depicted in Scheme 1, the desired 2,4-dimorpholine substituted pyrimidine derivatives (**3a-3c**) were prepared from 2-chloro-4-morpholinopyrimidine derivatives[32-34] **2** via amination on the C-2 position with morpholine.

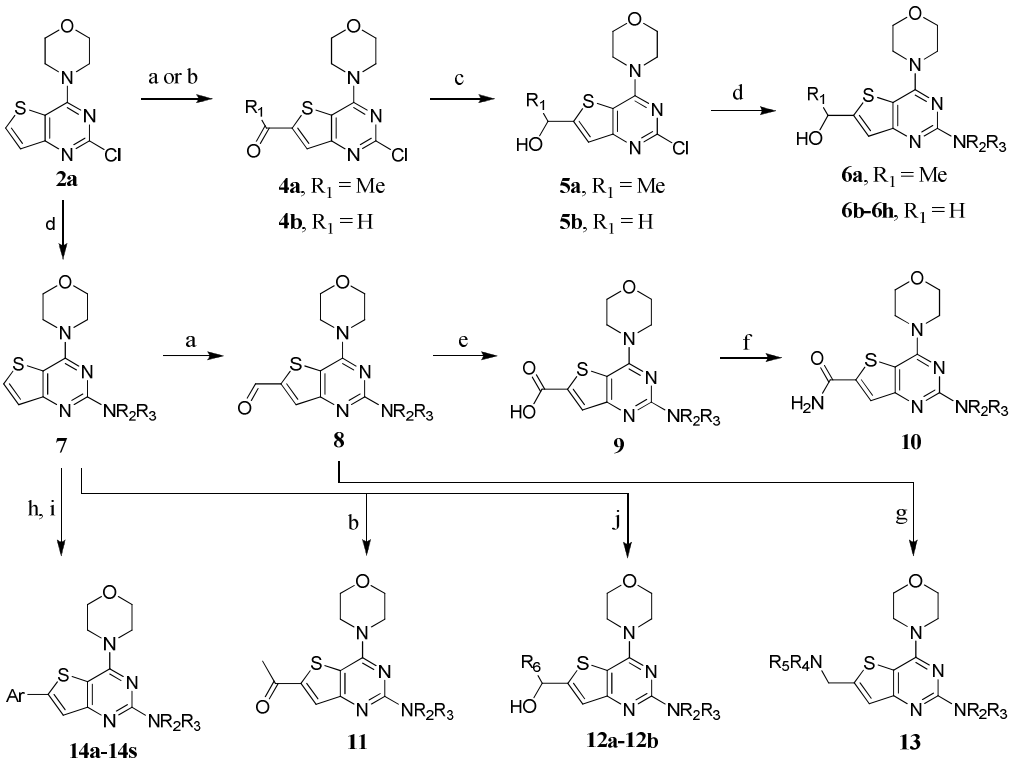
Scheme 1. Synthesis of Compounds 3a-3c



(a) Morpholine, TFA, n-Butanol, reflux.

Scheme 2 describes the general procedure for preparing thieno[3,2-*d*]pyrimidine derivatives. Lithiation of the common intermediate **2a** followed by acylation with DMF or DMAc provided **4a-4b.** Reduction of **4a-4b** by $NaBH_4$ and then nucleophilic substitution with appropriate amine yielded compounds **6a-6h.** Alternatively, the lithium anion of **7** could be trapped with aldehyde to give the secondary alcohol derivatives **12a** and **12b**. Ketone derivative **11** could be easily obtained through trapping of lithium anion of **7** with DMAc. Oxidization of aldehyde **8** with *m*-CPBA resulted in the carboxylic acid derivative **9**. And then, the corresponding primary amide **10** was obtained through amidation**.** Reductive amination of **8** using appropriate amine and sodium triacetoxyborohydride provided the compound **13**. Also in Scheme 2, the intermediate **7** could be converted to the arylated derivatives **14a-14s** through iodization and Suzuki coupling (For **14c**, Stille coupling was used).[25] It was worth noting that **14q** was prepared similarly to **14o** from the methyl substituted starting material.

Scheme 2. Synthesis of thienopyrimidine analogues



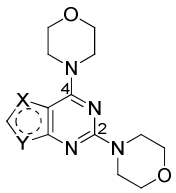
(a) n-BuLi, THF, -78 °C, DMF; (b) n-BuLi, THF, -78 °C, DMAc; (c) $NaBH_4$, MeOH; (d) HNR_1R_2, TFA, n-Butanol, reflux; (e) *m*-CPBA, DCM; (f) NH_4Cl, HOAt, HATU, DIPEA, DMF; (g) N-methanesulfonylpiperazine, 1,2-DCE, $HC(OCH_3)_3$, $Na(OAc)_3BH$; (h) n-BuLi, THF, -78 °C, I_2; (i) Suzuki coupling: boronic acid/ester, Na_2CO_3, H_2O, dioxane, $PdCl_2(dppf)_2$, 100 °C or Stille coupling with stannane; (j) n-BuLi, THF, -78 °C, Aldehyde.

RESULTS AND DISCUSSION

Kinase-Glo Plus luminescent assay and Lance Ultra Assay were used for evaluating the inhibitory activity of the compounds against PI3Kα and mTOR, respectively.

As presented in Table 1, the hit compound **3a** showed promising activity against PI3Kα isoform. Then replacement of the core structure of **3a** with purine (**3b**) or pyrrolo[2,3-d]pyrimidine[34] (**3c**) caused a decrease in activity, suggesting that thieno[3,2-d]pyrimidine analogues were worthy of further optimization, although poor mTOR inhibitory activities were observed.

Table 1. IC_{50} Values for Enzymatic Inhibition of PI3Kα and mTOR

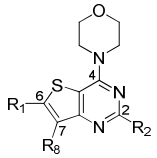


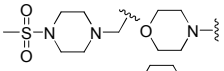
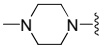
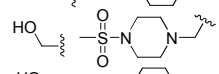
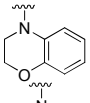
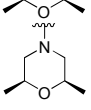
Compd	X	Y	IC50[a] (nM)	
			PI3Kα	mTOR
3a	S	CH	1875	>2000
3b	N	NH	>10000	>2000
3c	CH	NH	6184	>2000

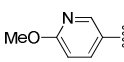
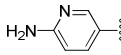
[a]IC_{50} values for enzymatic inhibition of PI3Kα and mTOR. The IC_{50} values are the mean of at least three experiments, with typical variations of less than 20%.

Subsequent efforts were focused on the substitutions of the C-6 position of thieno[3,2-*d*]pyrimidine ring due to the synthetic accessibility of this position (Table 2). Compared with compound **3a**, **6b** substituted with a methylol group in the C-6 position showed a dramatic increase in PI3Kα inhibitory activity. Similarly, the carboxylic acid derivative **9** showed comparable potency to **6b**. In contrast, ketone **11** and tertiary amine **13** derivatives displayed decreased inhibitory activities toward PI3Kα.

Table 2. In Vitro PI3Kα and mTOR Inhibitory Activities



Compd [a]	R_1	R_2	IC_{50} [b] (nM)	
			PI3Kα	mTOR
6a			122	632
6b			216	>2000
9			237	>2000
11			675	>2000
13			4817	>2000
6c			1034	>2000
6d			279	>2000
6e			249	603
6f			681	336
6g			216	463
6h			299	335
10			191	865
12a			239	>2000
12b			2050	>2000
14a			>10000	>2000
14b			>10000	225
14c			694	270
14d			281	612

Compd[a]	R_1	R_2	IC_{50} [b] (nM)	
			PI3Kα	mTOR
14e			>10000	>2000
14f			>10000	>2000
14g			165	70
14h			9468	>2000
14i			24	424
14j			>10000	>2000
14k			2203	109
14l			3562	222
14m			>10000	>2000
14n			4185	172
14o			15	16
14p			1242	>2000
14q[c]			197	73
14r			995	427
14s			4955	>2000
1			4.8	134

[a] Unless otherwise noted, R_8= H. [b] IC_{50} values for enzymatic inhibition of PI3Kα and mTOR. The IC_{50} values are the mean of triplicate measurements, with typical variations of less than 20%.[c] R_8= Methyl.

Next, we conducted an SAR study by replacing the C2-morpholine of **6b** with other isosteres. The piperazine derivatives **6c** and **6d** showed decreased potency when compared to **6b**. By contrast, the thiomorpholine analogue **6e** showed slightly decrease in PI3Kα inhibitory activity relative to **6b**, but an improved potency toward mTOR. Modifications to the C2-morpholine revealed that compound **6g** with *cis*-2,6-dimethyl substituent on morpholine ring showed improved inhibitory activity against mTOR and maintained PI3Kα inhibitory activity when compared to **6b** and **6e**. Next, we continued to modify the C-6 position of thieno[3,2-*d*]pyrimidine core, mainly because of the greatest degree of flexibility of this region in terms of SAR.

Upon introducing a methyl group to the α position of primary alcohol **6g**, **6a** displayed slightly higher activity against PI3Kα with an IC_{50} of 122 nM. However, other secondary alcohols **12a** and **12b** did not improve the inhibitory activity in comparison with **6g**. **10** with a primary amide group at C-6 position displayed slightly lower activities against PI3Kα and mTOR when compared to **6a**.

Meanwhile, we concentrated our efforts to modify the C-6 position of the thienopyrimidine core with aromatic and heteroaromatic rings. Phenyl substituted analogues **14a** and **14b** dramatically decreased PI3Kα inhibitory activity with IC_{50} values of over 10 μM. **14c**-**14e** were characterized by replacing the C-6 benzene of **14a** with pyridine. To our delight, *meta*-substituted pyridine **14d** showed promising inhibition activities toward PI3Kα and mTOR when compared with *ortho*- and *para*-substituted pyridine derivatives (**14c** and **14e**). 2-Aminopyridyl derivative **14g** exhibited 9-fold improvement in mTOR inhibition relative to **14d**. Further optimization of **14g** followed, **14i**

with aminopyrimidine group displayed stronger inhibitory activity on PI3Kα with IC_{50} value of 24 nM.

Five-membered heteroaromatic rings were introduced at C-6 position for further SAR investigations. The PI3Kα inhibition activities decreased dramatically when pyrrole, furan, indazole and thiophene (**14j**-**14n**) were installed. In contrast, upon introducing 5-pyrazole led to a more potent compound **14o**, which inhibits PI3Kα with a remarkable IC_{50} of 15 nM. It is worth mentioning that, **14o** displayed stronger inhibitory activity on mTOR with IC_{50} values of 16 nM in comparison to **1** with 134 nM. For PI3Kα enzyme potency, the *N*-methyl substituted analogue **14p** was about 80-fold less potent than the corresponding NH analogue **14o**. Substitution with a methyl group in the C-7 position, compound **14q** showed a potent reduction in activities on PI3Kα and mTOR relative to **14o**, possibly because of the methyl group induces a conformational change.[35] Replacement of the 5-pyrazolyl group with 4-pyrazolyl (**14r** and **14s**) resulted in a large reduction in potency. These results suggested that the pyrazole NH of **14o** forms an important hydrogen bonding interaction with the target protein.

The activities of **14i** and **14o** against the Class I PI3Ks and mTOR were presented in Table 3. Interestingly, **14i** also showed an IC_{50} value of 35 nM against γ-isozyme, which was 2-fold higher activity than **14o**. It was noteworthy that, compound **14o** was almost equipotent against PI3Kα, PI3Kδ and mTOR, while displaying modest levels of selectivity against PI3Kβ and PI3Kγ, making it a promising Class I PI3K/mTOR dual inhibitor.

Table 3. Activities of 14i and 14o against Class I PI3Ks and mTOR (IC_{50} Values[a] in nM)

Compd	PI3Kα	PI3Kβ	PI3Kγ	PI3Kδ	mTOR
14i	24	317	35	171	424
14o	15	175	75	10	16
1	4.8	33	46	3.3	134

[a]IC_{50} values are the mean of triplicate measurements.

Genetic aberrations of PI3K pathway frequently occur in various human cancers.[36, 37] Inhibition of cell proliferation was measured using the CCK-8 assay with the U87MG (*PTEN* null) human glioblastoma cell line, LNCaP (*PTEN* null) human prostate cancer cell line, PC-3 (*PTEN* null) human prostate cancer cell line and SKOV-3 (*PIK3CA* mutation) human ovarian cancer cell line[17, 38-40], and **1** was employed as positive control (Table 4). Compounds with IC_{50} values less than 500 nM on PI3Kα or mTOR were subjected to these cell lines. In general, these results correlated with their effects on kinases activities.

14o showed somewhat more potent than **1** in these cancer cell lines with submicromolar IC_{50}s, possibly due to the increased mTOR inhibition activity. Unexpectedly, **14i** performed well on kinase but exhibited weak cellular inhibition. Hence, **14o** was selected for further antitumor evaluation *in vitro* and *in vivo*.

Table 4. Antiproliferative Activities Various Cell Lines.

Compd	IC50[a] (μM)			
	PC-3	LNCaP	U87MG	SKOV-3
6a	1.38	2.59	3.29	1.22
6b	6.00	2.06	17.47	8.24
6d	14.99	6.16	>25	23.33
6e	2.26	4.2	>25	2.18
6f	2.67	1.82	21.2	2.89
6g	1.05	1.78	6.59	4.99
6h	1.32	2.63	>25	2.17
9	>25	23.55	2.44	>25
10	4.32	3.27	>25	7.11
12a	2.97	1.60	>25	9.12
14b	2.49	2.38	9.88	1.80
14c	2.75	4.43	4.45	0.99
14d	12.85	3.0	>25	15.68
14g	7.00	1.13	>25	2.90
14i	3.67	4.53	>25	10.02
14k	>25	2.64	12.80	5.91
14l	5.92	6.77	>25	21.77
14n	3.05	2.39	>25	18.88
14o	0.446	0.21	0.43	0.504
14q	1.74	1.10	8.76	0.83
14r	>25	4.05	>25	10.12
1	0.457	0.34	0.55	0.864

[a]IC_{50}, the mean value of triplicate measurements.

The intracellular PI3K pathway inhibitory activities of **14o** were evaluated by Western blot analysis. We observed that **14o** could inhibit the PI3K/AKT/mTOR pathway inside the cancer cells, as indicated by the dose-dependent decreases in phosphorylation of AKT and its downstream target S6 in U87MG and SKOV-3 cell lines (Figure 3).

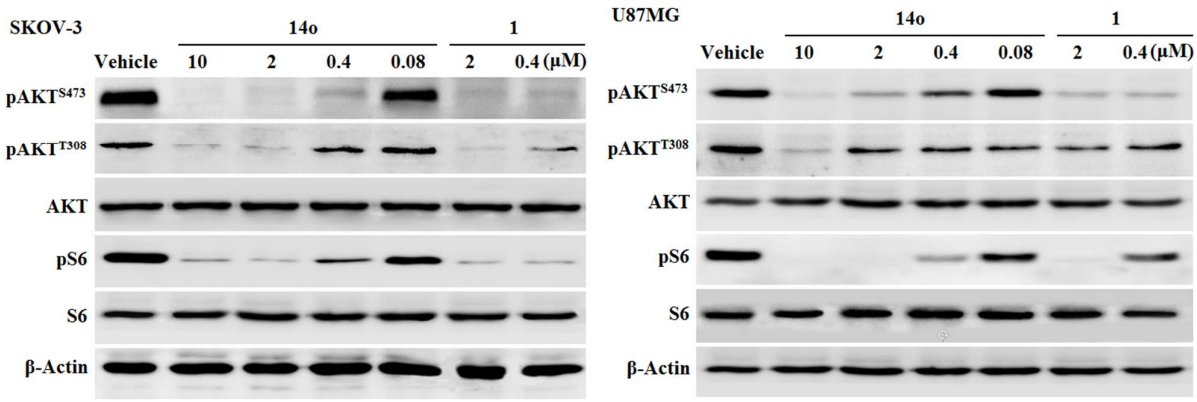


Figure 3. Effects of **14o** on pAKT, AKT, pS6 and S6.

To examine the possible binding modes of **14o**, docking analysis was performed using Autodock 4.0[41]. The protein crystal structure of PI3Kα (PDB 4L23)[42] and mTOR (PDB 4JT5)[43] was used, followed by molecular dynamics simulations to study the binding modes using the Amber 12 package[44].

Three hydrogen bonds interaction between **14o** and PI3Kα were observed. The oxygen of C4 morpholine formed a hydrogen bond with Lys802, while C2 dimethylmorpholine oxygen formed an additional hydrogen bond interaction with the hydroxy group of Ser773. The pyrazole moiety interacted with the hinge Val851 through a hydrogen bond and formed hydrophobic interactions with the side chains of Val850, Val851, Met922, Phe930 and Ile932 (Figure 4A). Docking modes of **14o** with mTOR showed that, *cis*-2,6-dimethylmorpholine has the hydrophobic contacts with Leu2185,

Trp2239 (hinge region) and Ile2237 (Figure 4B). More importantly, the pyrazole group formed two hydrogen bonds to the residues of Lys2187 and Glu2190, which may contributed to the improved mTOR inhibition activity.

The binding modes of **14o** suggested that the newly introduced *cis*-2,6-dimethylmorpholine and pyrazole moieties had hydrophobic and/or hydrogen-bond interactions with PI3Kα and mTOR binding sites, which were in accordance with the SAR study in Table 2.

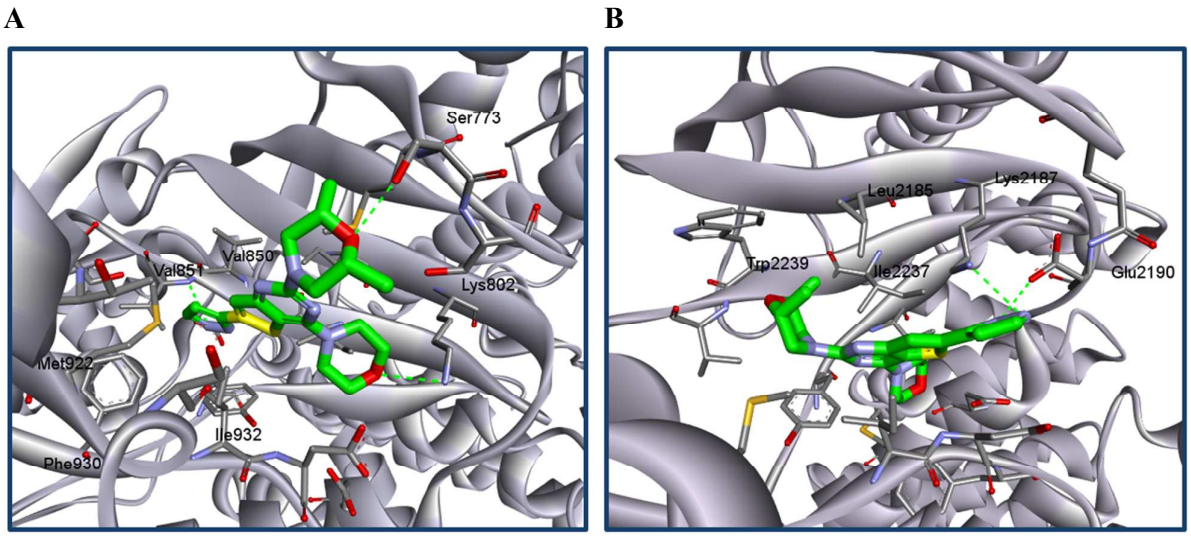
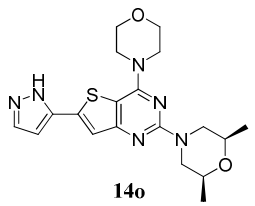


Figure 4. (A) Proposed binding mode of compound **14o** in PI3Kα model. (B) Docking modes for **14o** into protein crystal structure of mTOR.

The pharmacokinetic properties of **14o** were evaluated in Sprague-Dawley rats (Table 5). Moderate plasma clearance and volume of distribution were found after intravenous administration at 2 mg/kg. Acceptable oral bioavailability (41%) was achieved in this species.

Table 5. Pharmacokinetic Data for Compound 14o in Rat



14o

IV (2 mg/kg)[a]		PO (10 mg/kg)[b]			
Cl	V_{ss}	$T_{1/2}$	C_{max}	AUC	F
(mL/min/kg)	(L/kg)	h	(μM)	(μM·h)	%
29	4.4	1.5	1.4	5.2	41

[a]Male Sprague−Dawley rats were dosed intravenously with 2 mg/kg of **14o** prepared in 40% PEG400/ 5% DMSO/ 55% Saline. Compound **14o** was administered PO at the 10 mg/kg in 0.5% sodium carboxymethylcellulose with 0.2% Tween 80.

On the basis of the appealing biochemical and cellular activity and pharmacokinetic properties, the antitumor efficacy of **14o** was evaluated *in vivo*.

BALB/c nude mice bearing SKOV-3 and U87MG xenograft tumors were treated with **14o** by intragastric administration for 24 and 18 days, respectively. As shown in Figure 5A, the growth of xenograft tumors was significantly inhibited by **14o** in a dose-dependent manner. The tumor growth inhibition (TGI) of 89.6%, 75.9%, 48.5% were observed in the SKOV-3 model at the dose of 60, 30 and 15 mg/kg, respectively. For the U87MG model, administration of **14o** at doses of 40 and 20 mg/kg caused respective TGI of 81.2% and 69.2% ($p < 0.05$). In contrast, **1** as positive control showed less potent than **14o** in both SKOV-3 and U87MG models at the same dose. Moreover, **14o** did not cause significant weight loss and toxicity during the treatment period (See SI).

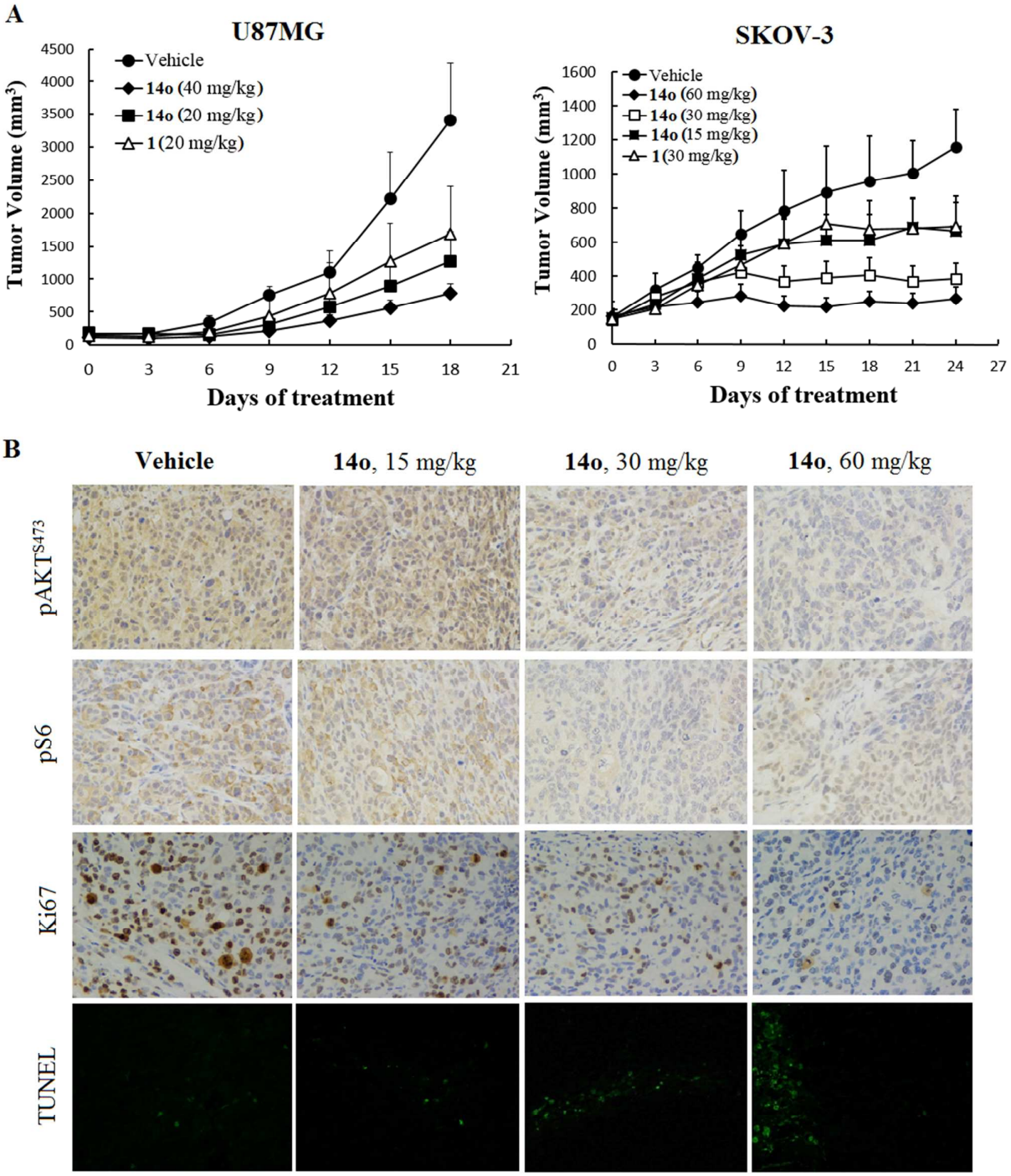


Figure 5. Pharmacodynamic profile of **14o** *in vivo*. (A) Growth inhibitory effect of **14o** on established U87MG and SKOV-3 xenografts in female BALB/c nude mice (N = 10 per group, mean ± SD). (B) Immunohistochemical staining of pAKT(S473), pS6, Ki67 and TUNEL in vehicle or **14o**-treated tumors of SKOV-3 xenograft model.

To further confirm that **14o** blocked the PI3K and mTOR activity *in vivo*, immunohistochemical (IHC) analyses of SKOV-3 tumors collected at the end of the dosing period were carried out. As shown in Figure 5B, **14o** caused a dose-dependent decrease of $pAKT^{S473}$ and pS6 *in vivo*. In addition, dose-dependent decreases in staining for Ki67 were observed, which confirmed a pronounced decrease in tumor cell proliferation. Furthermore, increased apoptosis judged by TUNEL staining was observed in SKOV-3 tumors.

CONCLUSIONS

Structural modifications of **1** led to the discovery of **14o**, which had been shown to be a potent Class I PI3K and mTOR kinase dual inhibitor. Compound **14o** showed a strong inhibitory effect on the growth of U87MG and SKOV-3 xenografts in nude mice. IHC analyses of **14o**-treated SKOV-3 tumors demonstrated a dose-dependent decrease of $pAKT^{S473}$ and pS6, confirming that **14o** blocked the PI3K/AKT/mTOR passway *in vivo*. By far, the existing data indicated that **14o** was a promising Class I PI3K/mTOR dual inhibitor for oncology indications. The antitumor properties and other biological activities of **14o** *in vivo* are presently being investigated.

EXPERIMENTAL SECTION

All solvents and reagents obtained from commercial sources were used without further purification. Flash column chromatography was performed using silica gel from Qingdao Haiyang. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AMX 400 spectrometer and were calibrated using TMS or residual deuterated solvent as an internal reference ($CDCl_3$: 7.26 ppm for ^{1}H NMR and 77.16 ppm for ^{13}C NMR; d_6-DMSO: 2.50 ppm for ^{1}H NMR and 39.52 ppm for ^{13}C NMR). The purity of final

compounds (>95%) were determined by high-performance liquid chromatography (HPLC) analysis performed on Waters 2695 Series chromatographs. HRMS spectra were recorded on a Shimadzu LCMS-IT-TOF.

General Procedure A for the Synthesis of **3a-3c**

To a solution of compound **2** (1 mmol) in 1-butanol (2 mL) was added morpholine (174 mg, 2 mmol), followed by trifluoroacetic acid (342 mg, 3 mmol). The solution was stirred at 125 °C overnight. The reaction solution was concentrated and then EtOAc (10 mL) was added. The resulting mixture was washed with saturated $NaHCO_3$ (10 mL) and brine (10 mL). The organic phase was then dried (Na_2SO_4), filtered, and concentrated under reduced pressure. Pure product was obtained after flash column chromatography.

4,4'-(Thieno[3,2-d]pyrimidine-2,4-diyl)dimorpholine (**3a**) was obtained from 4-(2-chlorothieno[3,2-d]pyrimidin-4-yl)morpholine (**2a**) and morpholine following the *General Procedure A*. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.60 (d, J = 5.5 Hz, 1H), 7.17 (d, J = 5.5 Hz, 1H), 3.93 – 3.87 (m, 4H), 3.86 – 3.75 (m, 12H); ^{13}C NMR (101 MHz, $CDCl_3$) δ: 164.03, 160.48, 158.76, 131.55, 124.39, 105.78, 67.13, 66.91, 46.43, 45.03. HRMS (DART-TOF) calculated for $C_{14}H_{19}N_4O_2S$ $[M+H]^+$ m/z 307.1223, found 307.1199.

4,4'-(9H-Purine-2,6-diyl)dimorpholine (**3b**) was obtained from 4-(2-chloro-9H-purin-6-yl)morpholine (**2b**) and morpholine following the *General Procedure A*. ^{1}H NMR (400 MHz, d_6-DMSO) δ: 12.43 (s, 1H), 7.78 (s, 1H), 4.33 – 3.94 (m, 4H), 3.72 – 3.56 (m, 12H); ^{13}C NMR (101 MHz, d_6-DMSO) δ: 158.16, 153.64, 153.16, 135.73, 113.38, 66.22, 66.10, 44.76. HRMS (DART-TOF) calculated for $C_{13}H_{19}N_6O_2$ $[M+H]^+$ m/z 291.1564, found 291.1551.

4,4'-(7H-Pyrrolo[2,3-d]pyrimidine-2,4-diyl)dimorpholine **(3c)** was obtained from 4-(2-chloro-7H-pyrrolo[2,3-d]pyrimidin-4-yl)morpholine (**2c**) and morpholine following the *General Procedure A*. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 9.69 (s, 1H), 6.75 (dd, *J* = 3.5, 2.3 Hz, 1H), 6.35 (dd, *J* = 3.5, 2.0 Hz, 1H), 3.93 – 3.87 (m, 4H), 3.86 – 3.81 (m, 4H), 3.81 – 3.76 (m, 4H), 3.76 – 3.69 (m, 4H); ^{13}C NMR (101 MHz, $CDCl_3$) δ: 158.83, 157.67, 155.01, 117.78, 101.69, 96.58, 67.13, 66.96, 46.00, 45.31. HRMS (DART-TOF) calculated for $C_{14}H_{20}N_5O_2$ $[M+H]^+$ m/z 290.1612, found 290.1664.

General Procedure B for the Synthesis of **6a-6h**

To a suspension of 4-(2-chlorothieno[3,2-d]pyrimidin-4-yl)morpholine (**2a**, 1.28 g, 5 mmol) in dry tetrahydrofuran (20 mL) at -78 °C was added a 2.5 M solution of n-butyllithium in hexanes (2.4 mL, 1.2 equiv, 6 mmol). After stirring for 1 h, dry N,N-dimethylformamide (548 mg, 1.5 equiv, 7.5 mmol, for **4b**) or N,N-Dimethylacetamide (653 mg, 1.5 equiv, 7.5 mmol, for **4a**) was added. The reaction mixture was stirred for 1 h at -78 °C and then warmed slowly to room temperature and stirred for another 2 h. The reaction mixture was poured onto ice/water and extracted with EtOAc. The organic phase was then dried (Na_2SO_4), filtered, and concentrated under reduced pressure. **4a or 4b** was obtained after flash column chromatography.

A solution of **4a** (596 mg, 2 mmol) or **4b** (567 mg, 2 mmol) in MeOH (10 mL) at 0 °C was treated with sodium borohydride (757 mg, 20 mmol). The solution was allowed to warm to room temperature and stirred for 30 min. The reaction mixture was quenched with cold water and extracted with ethyl acetate. The organic layer dried (Na_2SO_4), filtered, and concentrated under reduced pressure to give the crude product **5a** or **5b**. The reduced product **5a** or **5b** (0.5 mmol) was dissolved in 1-butanol (2 mL), and appropriate amine (1 mmol) was added, followed by trifluoroacetic acid

(170 mg, 1.5 mmol). The mixture was stirred at 125 °C overnight and monitored by TLC. The reaction solution was concentrated and then EtOAc (10 mL) was added. The resulting mixture was washed with saturated $NaHCO_3$ (10 mL) and brine (10 mL). The organic phase was then dried (Na_2SO_4), filtered, and concentrated under reduced pressure. Pure product **6a**-**6h** was obtained after flash column chromatography.

1-(2-(cis-2,6-Dimethylmorpholino)-4-morpholinothieno[3,2-d]pyrimidin-6-yl)ethanol (**6a**) was obtained from **5a** and cis-2,6-dimethylmorpholine following the *General Procedure B*. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 6.97 (d, *J* = 0.7 Hz, 1H), 5.11 (q, *J* = 6.2 Hz, 1H), 4.48 (dd, *J* = 12.7, 1.1 Hz, 2H), 3.90 – 3.77 (m, 8H), 3.71 – 3.61 (m, 2H), 2.56 (dd, *J* = 13.0, 10.7 Hz, 2H), 1.59 (d, *J* = 6.4 Hz, 3H), 1.26 (d, *J* = 6.2 Hz, 6H); ^{13}C NMR (101 MHz, $CDCl_3$) δ: 163.78, 160.07, 158.54, 156.42, 119.76, 104.88, 71.94, 66.91, 66.70, 50.11, 46.40, 25.17, 19.11. HRMS (DART-TOF) calculated for $C_{18}H_{27}N_4O_3S$ $[M+H]^+$ m/z 379.1798, found 379.1777.

(2,4-Dimorpholinothieno[3,2-d]pyrimidin-6-yl)methanol (**6b**) was obtained from **5b** and morpholine following the *General Procedure B*. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.01 (s, 1H), 4.87 (s, 2H), 3.90 – 3.85 (m, 4H), 3.84 – 3.80 (m, 4H), 3.79 – 3.72 (m, 8H); ^{13}C NMR (101 MHz, $CDCl_3$) δ: 163.70, 160.37, 158.47, 150.80, 121.31, 105.60, 67.10, 66.90, 60.74, 46.42, 45.00. HRMS (DART-TOF) calculated for $C_{15}H_{21}N_4O_3S$ $[M+H]^+$ m/z 337.1329, found 337.1287.

(2-(4-Methylpiperazin-1-yl)-4-morpholinothieno[3,2-d]pyrimidin-6-yl)methanol (**6c**) was obtained from **5b** and 1-methylpiperazine following the *General Procedure B*. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 6.99 (s, 1H), 4.85 (s, 2H), 3.83 (dd, *J* = 14.0, 4.3 Hz, 12H), 2.97 (br s, 1H), 2.51 (t, *J* = 4.4 Hz, 4H), 2.35 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ: 163.77, 160.22, 158.45, 151.10, 121.16, 105.33, 66.90,

60.60, 55.12, 46.40, 46.26, 44.21. HRMS (DART-TOF) calculated for $C_{16}H_{24}N_5O_2S$ $[M+H]^+$ m/z 350.1645, found 350.1528.

(2-(4-(Methylsulfonyl)piperazin-1-yl)-4-morpholinothieno[3,2-d]pyrimidin-6-yl)methanol (**6d**) was obtained from **5b** and 1-(methylsulfonyl)piperazine following the *General Procedure B*. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 6.98 (s, 1H), 5.76 (t, J = 5.7 Hz, 1H), 4.72 (d, J = 5.7 Hz, 2H), 3.94 – 3.76 (m, 8H), 3.77 – 3.64 (m, 4H), 3.15 (dd, J = 6.1, 2.7 Hz, 4H), 2.87 (s, 3H); ^{13}C NMR (101 MHz, d_6-DMSO) δ: 163.42, 159.12, 157.81, 153.99, 119.39, 104.03, 65.96, 58.75, 45.84, 45.25, 43.41, 33.70. HRMS (DART-TOF) calculated for $C_{16}H_{24}N_5O_4S_2$ $[M+H]^+$ m/z 414.1264, found 414.1278.

(4-Morpholino-2-thiomorpholinothieno[3,2-d]pyrimidin-6-yl)methanol (**6e**) was obtained from **5b** and thiomorpholine following the *General Procedure B*. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 6.99 (s, 1H), 4.86 (s, 2H), 4.22 – 4.04 (m, 4H), 3.91 – 3.72 (m, 8H), 2.73 – 2.56 (m, 4H); ^{13}C NMR (101 MHz, $CDCl_3$) δ: 163.89, 159.69, 158.58, 150.66, 121.38, 105.13, 66.89, 60.76, 46.95, 46.44, 27.02. HRMS (DART-TOF) calculated for $C_{15}H_{21}N_4O_2S_2$ $[M+H]^+$ m/z 353.1100, found 352.1021.

(2-(2H-Benzo[b][1,4]oxazin-4(3H)-yl)-4-morpholinothieno[3,2-d]pyrimidin-6-yl)methanol (**6f**) was obtained from **5b** and 3,4-dihydro-2H-benzo[b][1,4]oxazine following the *General Procedure B*. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.96 (dd, J = 8.2, 1.0 Hz, 1H), 7.06 (s, 1H), 6.99 – 6.81 (m, 3H), 4.85 (s, 2H), 4.30 (t, J = 4.2 Hz, 2H), 4.25 (t, J = 4.2 Hz, 2H), 3.94 – 3.72 (m, 8H), 2.74 (br s, 1H); ^{13}C NMR (101 MHz, $CDCl_3$) δ: 163.29, 158.18, 157.72, 151.29, 146.30, 128.24, 124.11, 123.45, 121.34, 119.53, 117.03, 107.01, 66.88, 66.08, 60.63, 46.44, 42.75. HRMS (DART-TOF) calculated for $C_{19}H_{21}N_4O_3S$ $[M+H]^+$ m/z 385.1329, found 385.1283.

(2-(cis-2,6-Dimethylmorpholino)-4-morpholinothieno[3,2-d]pyrimidin-6-yl)methanol (**6g**) was obtained from **5b** and cis-2,6-dimethylmorpholine following the *General Procedure B*. ^{1}H NMR (400 MHz, d_6-DMSO) δ: 6.96 (s, 1H), 5.77 (t, *J* = 5.2 Hz, 1H), 4.71 (d, *J* = 4.2 Hz, 2H), 4.44 (dd, *J* = 12.9, 1.5 Hz, 2H), 3.82 – 3.77 (m, 4H), 3.75 – 3.70 (m, 4H), 3.59 – 3.48 (m, 2H), 2.43 (dd, *J* = 13.0, 10.7 Hz, 2H), 1.15 (d, *J* = 6.2 Hz, 6H); ^{13}C NMR (101 MHz, d_6-DMSO) δ: 163.43, 159.27, 157.79, 153.79, 119.41, 103.81, 71.03, 65.98, 58.75, 49.52, 45.82, 18.82. HRMS (DART-TOF) calculated for $C_{17}H_{25}N_4O_3S$ $[M+H]^+$ m/z 365.1642, found 365.1642.

(R)-(2-(3-Methylmorpholino)-4-morpholinothieno[3,2-d]pyrimidin-6-yl)methanol (**6h**) was obtained from **5b** and (R)-3-methylmorpholine following the *General Procedure B*. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 6.97 (s, 1H), 4.82 (s, 2H), 4.68 – 4.60 (m, 1H), 4.23 (dd, *J* = 13.4, 1.9 Hz, 1H), 3.96 (dd, *J* = 11.2, 3.2 Hz, 1H), 3.91 – 3.78 (m, 8H), 3.76 – 3.68 (m, 2H), 3.55 (td, *J* = 12.0, 2.9 Hz, 1H), 3.25 (td, *J* = 13.0, 3.6 Hz, 1H), 2.82 (s, 1H), 1.26 (d, *J* = 6.7 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ: 163.66, 159.67, 158.44, 151.05, 121.07, 105.24, 71.47, 67.32, 66.84, 60.48, 47.14, 46.36, 39.49, 13.56. HRMS (DART-TOF) calculated for $C_{16}H_{23}N_4O_3S$ $[M+H]^+$ m/z 351.1485, found 351.1354.

2,4-Dimorpholinothieno[3,2-d]pyrimidine-6-carboxylic acid (**9**)

To a suspension of 4,4'-(thieno[3,2-d]pyrimidine-2,4-diyl)dimorpholine (**3a**, 1.54 g, 5 mmol) in dry tetrahydrofuran (20 mL) at -78 °C was added a 2.5 M solution of n-butyllithium in hexanes (2.4 mL, 1.2 equiv, 6 mmol). After stirring for 1 h, dry N,N-dimethylformamide (548 mg, 1.5 equiv, 7.5 mmol) was added. The reaction mixture was stirred for 1 h at -78 °C and then warmed slowly to room temperature and stirred for another 2 h. The reaction mixture was poured onto ice/water and extracted with EtOAc. The organic phase was then dried (Na_2SO_4), filtered, and concentrated under

reduced pressure. 2,4-dimorpholinothieno[3,2-d]pyrimidine-6-carbaldehyde (**8a**) was obtained after flash column chromatography. Compound **8a** (334 mg, 1mmol) was suspended in dichloromethane (5 mL) followed the addition of *m*-CPBA (75%, 230 mg, 1.33 mmol). The resulting mixture was heated to reflux for 24 h. Upon completion, the mixture was cooled to room temperature and concentrated. Product 9 was obtained after flash column chromatography (207 mg, 59% yield). ^{1}H NMR (400 MHz, d_6-DMSO) δ: 7.21 (s, 1H), 3.84 – 3.77 (m, 4H), 3.77 – 3.70 (m, 4H), 3.67 – 3.59 (m, 8H); ^{13}C NMR (101 MHz, d_6-DMSO) δ: 164.21, 163.24, 159.48, 158.17, 152.73, 123.22, 106.94, 66.14, 66.00, 45.91, 44.50. HRMS (DART-TOF) calculated for $C_{15}H_{19}N_4O_4S$ $[M+H]^+$ m/z 351.1122, found 351.1091.

2-(cis-2,6-Dimethylmorpholino)-4-morpholinothieno[3,2-d]pyrimidine-6-carboxamide **(10)**

2-(cis-2,6-dimethylmorpholino)-4-morpholinothieno[3,2-d]pyrimidine-6-carboxylic acid **(9b)** was prepared similarly to **9.** To a mixture of **9b** (189 mg, 0.5 mmol), 1-hydroxy-7-azabenzotriazole (HOAt, 13.6 mg, 0.1 mmol), O-(7-azabenzotriazol-1-yl)-(N,N,N',N'-tetramethyluronium hexafluorophosphate (HATU, 285 mg, 0.75 mmol), and N,N-diisopropylethylamine (258 mg, 2 mmol) in DMF (1.5 mL) was added ammonium chloride (80 mg, 1.5 mmol). The reaction mixture was stirred overnight at room temperature. The mixture was diluted with EtOAc, washed with saturated aqueous $NaHCO_3$ and brine. The organic phase was then dried (Na_2SO_4), filtered, and concentrated under reduced pressure. The residue was purified by silica gel chromatography to afford **10** (134 mg, 71% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.49 (s, 1H), 6.18 (br s, 2H), 4.50 (dd, J = 12.9, 1.4 Hz, 2H), 3.94 – 3.80 (m, 8H), 3.66 (dqd, J = 12.5, 6.2, 2.4 Hz, 2H), 2.58 (dd, J = 13.1, 10.7 Hz, 2H), 1.27 (d, J = 6.2 Hz, 6H); ^{13}C NMR (101 MHz, $CDCl_3$) δ: 163.76, 162.91, 160.15,

158.71, 142.25, 124.89, 108.60, 71.90, 66.81, 49.98, 46.48, 19.10. HRMS (DART-TOF) calculated for $C_{17}H_{24}N_5O_3S$ $[M+H]^+$ m/z 378.1594, found 378.1591.

1-(2,4-Dimorpholinothieno[3,2-d]pyrimidin-6-yl)ethanone (**11**)

To a suspension of 4,4'-(thieno[3,2-d]pyrimidine-2,4-diyl)dimorpholine (**3a**, 153 mg, 0.5 mmol) in dry tetrahydrofuran (2 mL) at -78 °C was added a 2.5 M solution of n-butyllithium in hexanes (0.24 mL, 1.2 equiv, 0.6 mmol). After stirring for 1 h, dry N,N-Dimethylacetamide (65 mg, 1.5 equiv, 0.75 mmol) was added. The reaction mixture was stirred for 1 h at -78 °C and then warmed slowly to room temperature and stirred for another 2 h. The reaction mixture was poured onto ice/water and extracted with EtOAc. The organic phase was then dried (Na_2SO_4), filtered, and concentrated under reduced pressure. **11** was obtained after flash column chromatography (106 mg, 61% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.70 (s, 1H), 3.94 – 3.88 (m, 4H), 3.85 – 3.76 (m, 12H), 2.64 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ: 192.50, 162.96, 160.47, 158.88, 146.97, 129.04, 110.24, 67.02, 66.78, 46.50, 44.84, 27.01. HRMS (DART-TOF) calculated for $C_{16}H_{21}N_4O_3S$ $[M+H]^+$ m/z 349.1329, found 349.1310.

General Procedure C for the Synthesis of **12a-12b**

cis-2,6-Dimethyl-4-(4-morpholinothieno[3,2-d]pyrimidin-2-yl)morpholine (**7a**) was prepared similarly to **3a**. To a suspension of cis-2,6-dimethyl-4-(4-morpholinothieno[3,2-d]pyrimidin-2-yl)morpholine (**7a**, 0**.**17 g, 0.5 mmol) in dry tetrahydrofuran (2 mL) at -78 °C was added a 2.5 M solution of n-butyllithium in hexanes (0.24 mL, 1.2 equiv, 0.6 mmol). After stirring for 1 h, dry aldehyde (1.2 equiv, 0.6 mmol) was added. The reaction mixture was stirred for 1 h at -78 °C and then warmed slowly to room temperature and stirred for another 2 h. The reaction mixture was

poured onto ice/water and extracted with EtOAc. The organic phase was then dried (Na_2SO_4), filtered, and concentrated under reduced pressure. Compound **12** was obtained after flash column chromatography.

1-(2-(cis-2,6-Dimethylmorpholino)-4-morpholinothieno[3,2-d]pyrimidin-6-yl)propan-1-ol (**12a**) was obtained from **7a** and propionaldehyde following the *General Procedure C*. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 6.92 (s, 1H), 4.79 (t, *J* = 6.4 Hz, 1H), 4.47 (d, *J* = 12.5 Hz, 2H), 3.90 – 3.77 (m, 8H), 3.71 – 3.60 (m, 2H), 3.14 (br s, 1H), 2.55 (dd, *J* = 12.9, 10.8 Hz, 2H), 1.91 – 1.75 (m, 2H), 1.26 (d, *J* = 6.2 Hz, 6H), 0.96 (t, *J* = 7.4 Hz, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ: 163.46, 159.89, 158.42, 155.49 120.12, 104.84, 71.90, 71.77, 66.84, 50.07, 46.33, 31.93, 19.06, 9.89. HRMS (DART-TOF) calculated for $C_{19}H_{29}N_4O_3S$ $[M+H]^+$ m/z 393.1955, found 393.1900.

1-(2-(cis-2,6-Dimethylmorpholino)-4-morpholinothieno[3,2-d]pyrimidin-6-yl)-2-phenylethanol **(12b)** was obtained from **7a** and phenylacetaldehyde following the *General Procedure C*. ^{1}H NMR (400 MHz, $CDCl_3$) δ: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.34 – 7.21 (m, 5H), 6.99 (s, 1H), 5.16 (dd, *J* = 8.3, 4.6 Hz, 1H), 4.49 (d, *J* = 11.6 Hz, 2H), 3.91 – 3.79 (m, 8H), 3.71 – 3.60 (m, 2H), 3.13 (ddd, *J* = 22.2, 13.7, 6.6 Hz, 2H), 2.56 (dd, *J* = 13.0, 10.7 Hz, 2H), 2.18 (br s, 1H), 1.26 (d, *J* = 6.2 Hz, 6H); ^{13}C NMR (101 MHz, $CDCl_3$) δ: 158.53, 154.24, 136.89, 129.63, 128.86, 127.20, 120.37, 104.96, 71.95, 71.58, 66.92, 50.10, 46.44, 45.74, 19.12. HRMS (DART-TOF) calculated for $C_{24}H_{31}N_4O_3S$ $[M+H]^+$ m/z 455.2111, found 455.2086.

4,4'-(6-((4-(Methylsulfonyl)piperazin-1-yl)methyl)thieno[3,2-d]pyrimidine-2,4-diyl)dimorpholine **(13)**. A mixture of **8a** (167 mg, 0.5 mmol), 1-methanesulfonyl-piperazine hydrochloride (120 mg, 1.2 equiv), and trimethylorthoformate (159 mg, 3 equiv) was stirred in 1,2-dichloroethane (2 mL) for 6 h

at room temperature. To this mixture was added sodium triacetoxyborohydride (265 mg, 2.5 equiv), and the reaction mixture was stirred for 24 h at room temperature and then quenched with brine, extracted with dichloromethane, dried over $MgSO_4$, and the solvent removed in vacuo. The crude product was purified by chromatography (104 mg, 43%). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.02 (s, 1H), 3.93 – 3.68 (m, 18H), 3.34 – 3.22 (m, 4H), 2.79 (s, 3H), 2.68 – 2.59 (m, 4H); ^{13}C NMR (101 MHz, $CDCl_3$) δ: 160.39, 158.43, 148.07, 123.27, 105.86, 67.09, 66.90, 57.49, 52.40, 46.40, 45.94, 44.97, 34.54. HRMS (DART-TOF) calculated for $C_{20}H_{31}N_6O_4S_2$ $[M+H]^+$ m/z 483.1843, found 483.1769.

General Procedure D for the Synthesis of **14a**-**14s** (**14c** and **14q** were not included)

To a stirred solution of **7a** (1.67 g, 5 mmol) in dry THF (20 mL) was added a 2.5 M solution of n-butyllithium in hexanes (3 mL, 1.5 equiv, 7.5 mmol) at -78 °C over a period of 10 minutes, stirred for 2 h at -40 °C followed by addition of iodine (1.9 g, 7.5 mmol) in THF (5 mL) at -78 °C. The mixture was warmed slowly to room temperature and stirred for 8 h. After the completion of reaction (monitored by TLC), the reaction was quenched with saturated ammonium chloride and extracted with EtOAc (2 x 50 mL). The organic layer was washed with sodium thiosulphate solution, dried over anhydrous Na_2SO_4 and concentrated in vacuo. The crude product was purified by chromatography to afford iodine substituted compound **15** (1.7 g, 75%)

Under N_2 atmosphere, a mixture of iodine substituted compound **15** (230 mg, 0.5 mmol), boronic acid/ester (1.2 equiv), Na_2CO_3 (106 mg, 2 equiv), $PdCl_2(dppf)_2$ (18 mg, 5 mol%), 1,4-dioxane (1.5 ml), and water (0.5 mL) was heated to 100 °C for 16 h, the reaction mixture was cooled, diluted with

ethyl acetate, washed with brine, dried over Na_2SO_4, and the solvent was removed in vacuo. Purification on silica yielded the desired compound **14a-14s** (**14c** and **14q** are not included).

cis-2,6-Dimethyl-4-(4-morpholino-6-phenylthieno[3,2-d]pyrimidin-2-yl)morpholine (**14a**) was obtained from **15** and phenylboronic acid following the *General Procedure D*. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.74 – 7.63 (m, 2H), 7.48 – 7.32 (m, 4H), 4.53 (dd, *J* = 12.9, 1.4 Hz, 2H), 3.94 – 3.82 (m, 8H), 3.69 (dqd, *J* = 12.3, 6.1, 2.4 Hz, 2H), 2.59 (dd, *J* = 13.0, 10.7 Hz, 2H), 1.28 (d, *J* = 6.2 Hz, 6H); ^{13}C NMR (101 MHz, $CDCl_3$) δ: 164.48, 160.18, 158.46, 149.18, 133.50, 129.27, 129.18, 126.37, 119.90, 105.37, 71.94, 66.92, 50.08, 46.47, 19.12. HRMS (DART-TOF) calculated for $C_{22}H_{27}N_4O_2S$ $[M+H]^+$ m/z 411.1849, found 411.1860.

2-(2-(cis-2,6-Dimethylmorpholino)-4-morpholinothieno[3,2-d]pyrimidin-6-yl)phenol (**14b**) was obtained from **15** and 2-hydroxyphenylboronic acid following the *General Procedure D*. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.76 (s, 1H), 7.52 (dd, *J* = 8.0, 1.3 Hz, 1H), 7.23 (td, *J* = 8.2, 1.4 Hz, 1H), 6.97 – 6.91 (m, 2H), 4.50 (d, *J* = 12.2 Hz, 2H), 3.96 – 3.82 (m, 8H), 3.74 – 3.64 (m, 2H), 2.61 (dd, *J* = 12.8, 10.8 Hz, 2H), 1.24 (d, *J* = 6.2 Hz, 6H); ^{13}C NMR (101 MHz, $CDCl_3$) δ: 159.91, 158.25, 154.17, 145.75, 130.46, 129.36, 122.78, 120.63, 120.50, 117.02, 105.54, 71.99, 66.94, 50.43, 46.52, 19.09. HRMS (DART-TOF) calculated for $C_{22}H_{27}N_4O_3S$ $[M+H]^+$ m/z 427.1798, found 427.1806.

cis-2,6-Dimethyl-4-(4-morpholino-6-(pyridin-2-yl)thieno[3,2-d]pyrimidin-2-yl)morpholine (**14c**) Under N_2 atmosphere, a mixture of **15** (230 mg, 0.5 mmol), 2-(tributylstannyl)pyridine (368 mg, 2 equiv), $PdCl_2(PPh_3)_2$ (18 mg, 5 mol%), in dry DMF was heated to 100 °C for 2 days, the reaction mixture was cooled, diluted with ethyl acetate, washed saturated aqueous $NaHCO_3$ solution and brine respectively, dried over Na_2SO_4, and the solvent was removed in vacuo. Purification on silica

yielded the desired compound **14c** (109 mg, 53%). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.62 (d, *J* = 4.7 Hz, 1H), 7.82 – 7.72 (m, 2H), 7.61 (s, 1H), 7.29 – 7.23 (m, 1H), 4.53 (d, *J* = 12.8 Hz, 2H), 3.97 – 3.93 (m, 4H), 3.87 – 3.82 (m, 4H), 3.74 – 3.64 (m, 2H), 2.59 (dd, *J* = 12.8, 10.9 Hz, 2H), 1.28 (d, *J* = 6.2 Hz, 6H); ^{13}C NMR (101 MHz, $CDCl_3$) δ: 164.16, 160.11, 158.65, 151.73, 149.77, 149.30, 137.04, 123.60, 120.85, 119.87, 107.08, 71.91, 66.90, 50.04, 46.48, 19.10. HRMS (DART-TOF) calculated for $C_{21}H_{26}N_5O_2S$ $[M+H]^+$ m/z 412.1802, found 412.1763.

cis-2,6-Dimethyl-4-(4-morpholino-6-(pyridin-3-yl)thieno[3,2-d]pyrimidin-2-yl)morpholine (**14d**) was obtained from **15** and 3-pyridineboronic acid pinacol ester following the *General Procedure D*. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.95 (d, *J* = 1.5 Hz, 1H), 8.61 (d, *J* = 3.8 Hz, 1H), 7.94 (d, *J* = 8.0 Hz, 1H), 7.44 (s, 1H), 7.37 (dd, *J* = 7.8, 4.9 Hz, 1H), 4.53 (d, *J* = 12.2 Hz, 2H), 3.95 – 3.84 (m, 8H), 3.74 – 3.64 (m, 2H), 2.60 (dd, *J* = 12.8, 10.9 Hz, 2H), 1.28 (d, *J* = 6.2 Hz, 6H); ^{13}C NMR (101 MHz, $CDCl_3$) δ: 164.25, 160.19, 158.45, 150.11, 147.35, 145.09, 133.43, 129.61, 123.88, 121.12, 105.67, 71.91, 66.87, 50.01, 46.45, 19.10. HRMS (DART-TOF) calculated for $C_{21}H_{26}N_5O_2S$ $[M+H]^+$ m/z 412.1802, found 412.1764.

cis-2,6-Dimethyl-4-(4-morpholino-6-(pyridin-4-yl)thieno[3,2-d]pyrimidin-2-yl)morpholine (**14e**) was obtained from **15** and pyridine-4-boronic acid following the *General Procedure D*. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.66 (dd, *J* = 4.6, 1.6 Hz, 2H), 7.55 (s, 1H), 7.53 (dd, *J* = 4.5, 1.6 Hz, 2H), 4.53 (dd, *J* = 13.1, 1.6 Hz, 2H), 3.94 – 3.89 (m, 4H), 3.89 – 3.84 (m, 4H), 3.73 – 3.64 (m, 2H), 2.60 (dd, *J* = 13.2, 10.7 Hz, 2H), 1.28 (d, *J* = 6.3 Hz, 6H); ^{13}C NMR (101 MHz, $CDCl_3$) δ: 163.99, 160.17, 158.48, 150.69, 145.44, 140.65, 122.24, 120.26, 106.12, 71.88, 66.82, 49.97, 46.43, 19.08. HRMS (DART-TOF) calculated for $C_{21}H_{26}N_5O_2S$ $[M+H]^+$ m/z 412.1802, found 412.1764.

cis-4-(6-(6-Methoxypyridin-3-yl)-4-morpholinothieno[3,2-d]pyrimidin-2-yl)-2,6-dimethylmorpholine (**14f**) was obtained from **15** and 2-methoxy-5-pyridineboronic acid following the *General Procedure D*. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.50 (d, *J* = 2.3 Hz, 1H), 7.83 (dd, *J* = 8.6, 2.5 Hz, 1H), 7.30 (s, 1H), 6.81 (d, *J* = 8.7 Hz, 1H), 4.52 (dd, *J* = 13.1, 1.6 Hz, 2H), 3.98 (s, 3H), 3.92 – 3.83 (m, 8H), 3.69 (dqd, *J* = 12.5, 6.2, 2.4 Hz, 2H), 2.59 (dd, *J* = 13.1, 10.7 Hz, 2H), 1.28 (d, *J* = 6.2 Hz, 6H); ^{13}C NMR (101 MHz, $CDCl_3$) δ: 164.74, 164.49, 160.22, 158.42, 145.81, 144.68, 136.59, 123.16, 119.51, 111.42, 104.99, 71.95, 66.92, 53.92, 50.07, 46.48, 19.13. HRMS (DART-TOF) calculated for $C_{22}H_{28}N_5O_3S$ $[M+H]^+$ m/z 442.1907, found 442.1869.

5-(2-(cis-2,6-Dimethylmorpholino)-4-morpholinothieno[3,2-d]pyrimidin-6-yl)pyridin-2-amine (**14g**) was obtained from **15** and 2-aminopyridine-5-boronic acid pinacol ester following the *General Procedure D*. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.41 (d, *J* = 2.0 Hz, 1H), 7.70 (dd, *J* = 8.6, 2.3 Hz, 1H), 7.25 (s, 1H), 6.54 (d, *J* = 8.6 Hz, 1H), 4.80 (s, 2H), 4.52 (dd, *J* = 13.0, 1.6 Hz, 2H), 3.92 – 3.83 (m, 8H), 3.73 – 3.63 (m, 2H), 2.58 (dd, *J* = 13.1, 10.7 Hz, 2H), 1.27 (d, *J* = 6.2 Hz, 6H); ^{13}C NMR (101 MHz, $CDCl_3$) δ: 164.43, 160.07, 158.86, 158.31, 146.64, 146.01, 135.72, 120.15, 118.20, 108.62, 104.41, 71.92, 66.89, 50.06, 46.43, 19.09. HRMS (DART-TOF) calculated for $C_{21}H_{27}N_6O_2S$ $[M+H]^+$ m/z 427.1911, found 427.1902.

cis-2,6-Dimethyl-4-(4-morpholino-6-(pyrimidin-5-yl)thieno[3,2-d]pyrimidin-2-yl)morpholine (**14h**) was obtained from **15** and 5-pyrimidinylboronic acid following the *General Procedure D*. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 9.21 (s, 1H), 9.02 (s, 2H), 7.48 (d, *J* = 2.0 Hz, 1H), 4.53 (dd, *J* = 12.9, 1.1 Hz, 2H), 4.01 – 3.83 (m, 8H), 3.76 – 3.60 (m, 2H), 2.60 (dd, *J* = 12.9, 10.8 Hz, 2H), 1.28 (d, *J* = 6.2 Hz, 6H); ^{13}C NMR (101 MHz, $CDCl_3$) δ: 164.07, 160.21, 158.61, 158.43, 153.90, 140.82, 128.00,

122.16, 105.95, 71.87, 66.81, 49.95, 46.42, 19.08. HRMS (DART-TOF) calculated for $C_{20}H_{25}N_6O_2S$ $[M+H]^+$ m/z 413.1754, found 413.1719.

5-(2-(cis-2,6-Dimethylmorpholino)-4-morpholinothieno[3,2-d]pyrimidin-6-yl)pyrimidin-2-amine (**14i**) was obtained from **15** and 2-aminopyrimidine-5-boronic acid pinacol ester following the *General Procedure D*. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 8.59 (s, 2H), 7.27 (s, 1H), 5.33 (s, 2H), 4.52 (dd, *J* = 13.0, 1.6 Hz, 2H), 3.92 – 3.83 (m, 8H), 3.72 – 3.63 (m, 2H), 2.59 (dd, *J* = 13.1, 10.7 Hz, 2H), 1.27 (d, *J* = 6.2 Hz, 6H); ^{13}C NMR (101 MHz, $CDCl_3$) δ: 164.44, 162.92, 160.22, 158.40, 155.97, 143.04, 119.04, 118.65, 104.63, 71.95, 66.91, 50.06, 46.47, 19.13. HRMS (DART-TOF) calculated for $C_{20}H_{26}N_7O_2S$ $[M+H]^+$ m/z 428.1863, found 428.1847.

cis-4-(6-(1H-Indazol-4-yl)-4-morpholinothieno[3,2-d]pyrimidin-2-yl)-2,6-dimethylmorpholine (**14j**) was obtained from **15** and 4-(tetramethyl-1,3,2-dioxaborolan-2-yl)-1H-indazole following the *General Procedure D*. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 10.75 (br s, 1H), 8.51 (s, 1H), 7.59 (s, 1H), 7.54 (d, *J* = 7.8 Hz, 1H), 7.49 – 7.40 (m, 2H), 4.56 (dd, *J* = 12.8, 1.2 Hz, 2H), 3.98 – 3.85 (m, 8H), 3.71 (dqd, *J* = 12.5, 6.1, 2.3 Hz, 2H), 2.62 (dd, *J* = 13.0, 10.7 Hz, 2H), 1.29 (d, *J* = 6.2 Hz, 6H); ^{13}C NMR (101 MHz, $CDCl_3$) δ: 158.52, 147.21, 140.81, 134.50, 127.09, 122.20, 121.05, 120.63, 110.73, 105.75, 72.00, 66.95, 50.15, 46.55, 19.14. HRMS (DART-TOF) calculated for $C_{23}H_{27}N_6O_2S$ $[M+H]^+$ m/z 451.1911, found 451.1756.

cis-4-(6-(Furan-2-yl)-4-morpholinothieno[3,2-d]pyrimidin-2-yl)-2,6-dimethylmorpholine (**14k**) was obtained from **15** and 2-furanboronic acid following the *General Procedure D*. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.49 (s, 1H), 7.29 (s, 1H), 6.71 (d, *J* = 3.2 Hz, 1H), 6.54 – 6.45 (m, 1H), 4.52 (d, *J* = 12.7 Hz, 2H), 3.93 – 3.80 (m, 8H), 3.74 – 3.61 (m, 2H), 2.58 (dd, *J* = 12.7, 10.9 Hz, 2H), 1.27 (d, *J* = 6.2

Hz, 6H); ^{13}C NMR (101 MHz, $CDCl_3$) δ: 164.23, 160.29, 158.59, 148.69, 143.33, 138.10, 118.52, 112.25, 108.23, 104.42, 71.96, 66.93, 50.09, 46.49, 19.14. HRMS (DART-TOF) calculated for $C_{20}H_{25}N_4O_3S$ $[M+H]^+$ m/z 401.1642, found 401.1605.

cis-4-(6-(Furan-3-yl)-4-morpholinothieno[3,2-d]pyrimidin-2-yl)-2,6-dimethylmorpholine (**14l**) was obtained from **15** and 3-furanboronic acid following the *General Procedure D*. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.78 (s, 1H), 7.49 (t, *J* = 1.6 Hz, 1H), 7.16 (s, 1H), 6.67 (dd, *J* = 1.7, 0.7 Hz, 1H), 4.51 (dd, *J* = 13.0, 1.6 Hz, 2H), 3.92 – 3.80 (m, 8H), 3.73 – 3.62 (m, 2H), 2.58 (dd, *J* = 13.1, 10.7 Hz, 2H), 1.27 (d, *J* = 6.2 Hz, 6H); ^{13}C NMR (101 MHz, $CDCl_3$) δ: 164.33, 160.22, 158.42, 144.24, 140.21, 139.67, 120.34, 119.93, 109.19, 104.20, 71.95, 66.93, 50.09, 46.44, 19.13. HRMS (DART-TOF) calculated for $C_{20}H_{25}N_4O_3S$ $[M+H]^+$ m/z 401.1642, found 401.1606.

cis-2,6-Dimethyl-4-(4-morpholino-6-(thiophen-3-yl)thieno[3,2-d]pyrimidin-2-yl)morpholine (**14m**) was obtained from **15** and 3-thiopheneboronic acid following the *General Procedure D*. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.56 (dd, *J* = 2.8, 1.3 Hz, 1H), 7.42 – 7.35 (m, 2H), 7.27 (s, 1H), 4.52 (dd, *J* = 13.0, 1.6 Hz, 2H), 3.96 – 3.79 (m, 8H), 3.73 – 3.64 (m, 2H), 2.58 (dd, *J* = 13.1, 10.7 Hz, 2H), 1.27 (d, *J* = 6.2 Hz, 6H); ^{13}C NMR (101 MHz, $CDCl_3$) δ: 164.43, 160.24, 158.48, 143.84, 134.99, 127.05, 126.08, 122.19, 119.79, 104.61, 71.96, 66.95, 50.10, 46.48, 19.14; HRMS (DART-TOF) calculated for $C_{20}H_{25}N_4O_2S_2$ $[M+H]^+$ m/z 417.1413, found 417.1291.

cis-2,6-Dimethyl-4-(4-morpholino-6-(1H-pyrrol-2-yl)thieno[3,2-d]pyrimidin-2-yl)morpholine (**14n**) was obtained from **15** and N-Boc-2-pyrroleboronic acid following the *General Procedure D*. ^{1}H NMR (400 MHz, d_6-DMSO) δ: 11.65 (s, 1H), 7.22 (s, 1H), 6.99 (dd, *J* = 3.8, 2.5 Hz, 1H), 6.54 (t, *J* = 3.5 Hz, 1H), 6.16 (dd, *J* = 5.8, 2.4 Hz, 1H), 4.44 (d, *J* = 11.4 Hz, 2H), 3.82 – 3.68 (m, 8H), 3.60 –

3.50 (m, 2H), 2.45 (dd, *J* = 12.9, 10.7 Hz, 2H), 1.16 (d, *J* = 6.2 Hz, 6H); ^{13}C NMR (101 MHz, d_6-DMSO) δ: 157.65, 124.82, 121.45, 115.09, 109.67, 108.49, 101.76, 71.03, 65.97, 49.52, 45.82, 18.81. HRMS (DART-TOF) calculated for $C_{20}H_{26}N_5O_2S$ $[M+H]^+$ m/z 400.1802, found 400.1752.

cis-2,6-Dimethyl-4-(4-morpholino-6-(1H-pyrazol-5-yl)thieno[3,2-d]pyrimidin-2-yl)morpholine (**14o**) was obtained from **15** and 1-(tetrahydropyran-2-yl)-1H-pyrazole-5-boronic acid pinacol ester following the *General Procedure D* and followed by deprotection (EtOH /conc. HCl = 10:1, 25 °C, 1h) . ^{1}H NMR (400 MHz, d_6-DMSO) δ: 13.14 (s, 1H), 7.86 (dd, *J* = 2.1, 1.6 Hz, 1H), 7.47 (s, 1H), 6.85 (t, *J* = 2.0 Hz, 1H), 4.46 (dd, *J* = 12.8, 1.5 Hz, 2H), 3.85 – 3.71 (m, 8H), 3.60 – 3.50 (m, 2H), 2.45 (dd, *J* = 12.9, 10.7 Hz, 2H), 1.16 (d, *J* = 6.2 Hz, 6H); ^{13}C NMR (101 MHz, d_6-DMSO) δ: 163.75, 159.38, 157.93, 144.84, 142.57, 130.48, 119.33, 103.38, 103.16, 71.02, 65.98, 49.53, 45.87, 18.82. HRMS (DART-TOF) calculated for $C_{19}H_{25}N_6O_2S$ $[M+H]^+$ m/z 401.1754, found 401.1713.

cis-2,6-Dimethyl-4-(6-(1-methyl-1H-pyrazol-5-yl)-4-morpholinothieno[3,2-d]pyrimidin-2-yl)morpholine (**14p**) was obtained from **15** and 1-methyl-1H-pyrazole-5-boronic acid pinacol ester following the *General Procedure D*. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.51 (d, *J* = 2.0 Hz, 1H), 7.23 (s, 1H), 6.50 (d, *J* = 2.0 Hz, 1H), 4.52 (dd, *J* = 13.1, 1.8 Hz, 2H), 4.06 (s, 3H), 3.92 – 3.81 (m, 8H), 3.68 (dqd, *J* = 12.5, 6.2, 2.4 Hz, 2H), 2.59 (dd, *J* = 13.1, 10.7 Hz, 2H), 1.28 (d, *J* = 6.2 Hz, 6H); ^{13}C NMR (101 MHz, $CDCl_3$) δ: 163.96, 160.29, 158.45, 138.86, 136.63, 122.88, 107.52, 105.62, 71.94, 66.88, 50.05, 46.47, 38.50, 19.12. HRMS (DART-TOF) calculated for $C_{20}H_{27}N_6O_2S$ $[M+H]^+$ m/z 415.1911, found 415.1877.

cis-2,6-Dimethyl-4-(7-methyl-4-morpholino-6-(1H-pyrazol-5-yl)thieno[3,2-d]pyrimidin-2-yl)morpholine (**14q**) **14q** was prepared similarly to **14o** from the methyl substituted starting material.

^{1}H NMR (400 MHz, d_6-DMSO) δ: 13.22 (s, 1H), 7.91 (dd, *J* = 2.2, 1.6 Hz, 1H), 6.70 (t, *J* = 2.1 Hz, 1H), 4.53 (d, *J* = 11.4 Hz, 2H), 3.84 – 3.71 (m,8H), 3.62 – 3.52 (m, 2H), 2.47 (dd, *J* = 12.8, 10.6 Hz, 2H), 2.45 (s, 3H), 1.17 (d, *J* = 6.2 Hz, 6H); ^{13}C NMR (101 MHz, d_6-DMSO) δ: 163.19, 159.14, 158.01, 144.68, 136.42, 130.17, 127.07, 104.10, 102.88, 71.07, 66.00, 49.51, 45.90, 18.86, 12.12. HRMS (DART-TOF) calculated for $C_{20}H_{27}N_6O_2S$ $[M+H]^+$ m/z 415.1911, found 415.1866.

cis-2,6-Dimethyl-4-(4-morpholino-6-(1H-pyrazol-4-yl)thieno[3,2-d]pyrimidin-2-yl)morpholine (**14r**) was obtained from **15** and pyrazole-4-boronic acid pinacol ester following the *General Procedure D*. ^{1}H NMR (400 MHz, d_6-DMSO) δ: 13.22 (br s, 1H), 8.28 (s, 1H), 7.96 (s, 1H), 7.31 (s, 1H), 4.45 (dd, *J* = 12.9, 1.6 Hz, 2H), 3.85 – 3.69 (m, 8H), 3.55 (dqd, *J* = 12.3, 6.1, 2.3 Hz, 2H), 2.45 (dd, *J* = 13.0, 10.7 Hz, 2H), 1.15 (d, *J* = 6.2 Hz, 6H); ^{13}C NMR (101 MHz, d_6-DMSO) δ: 159.34, 157.69, 141.29, 117.72, 114.56, 102.34, 71.02, 65.97, 49.53, 45.80, 18.82. HRMS (DART-TOF) calculated for $C_{19}H_{25}N_6O_2S$ $[M+H]^+$ m/z 401.1754, found 401.1592.

cis-2,6-Dimethyl-4-(6-(1-methyl-1H-pyrazol-4-yl)-4-morpholinothieno[3,2-d]pyrimidin-2-yl)morpholine (**14s**) was obtained from **15** and 1-methyl-4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1H-pyrazole following the *General Procedure D*. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.74 (s, 1H), 7.64 (s, 1H), 7.11 (s, 1H), 4.51 (dd, *J* = 13.0, 1.6 Hz, 2H), 3.95 (s, 3H), 3.90 – 3.82 (m, 8H), 3.72 – 3.63 (m, 2H), 2.57 (dd, *J* = 13.1, 10.7 Hz, 2H), 1.27 (d, *J* = 6.2 Hz, 6H); ^{13}C NMR (101 MHz, $CDCl_3$) δ: 164.58, 160.22, 158.37, 140.60, 137.41, 127.83, 118.49, 116.56, 103.81, 71.95, 66.93, 50.10, 46.44, 39.38, 19.13. HRMS (DART-TOF) calculated for $C_{20}H_{27}N_6O_2S$ $[M+H]^+$ m/z 415.1911, found 415.1908.

PI3K Inhibition Assays.

The PI3K activity assay was conducted by Shanghai ChemPartner Co., Ltd (China).

Briefly, the compound, PI3K enzyme (PI3Kα from Invitrogen, PIK3Cβ from Millipore, PIK3Cγ from Invitrogen, PIK3Cδ from Millipore), PIP2 (Life) substrate, and ATP (25 μM, Sigma) were diluted in kinase buffer to the indicated concentrations. The assay plate was covered and incubated at room temperature (PI3Kα, PI3Kβ and PI3Kγ for 1 hour, PI3Kδ for 2 hours). Then, the Kinase-Glo reagent (Promega) was added to PI3Kα plate to stop the reaction and shaken for 15 min. For PI3Kδ, PI3Kβ and PI3Kγ inhibition assay, ADP-Glo reagent (Promega) was added and shaken slowly for 40 min, followed by addition of kinase detection reagent, shaken for 1 min and equilibrated for 60 min. The data was collected on FlexStation and presented in Excel. The curves were fitted by Graphpad Prism 5.0.

mTOR Inhibition Assays.

The mTOR inhibitory activity was evaluated by monitoring phosphorylation of mTOR's substrate 4EBP1, which was accomplished by Shanghai ChemPartner Co., Ltd (China). In short, the compound, mTOR (Millipore), ULight-4E-BP1 peptide substrate (PE), and ATP (13 μM, Sigma) were diluted in kinase buffer to the indicated concentrations. The assay plate was covered and incubated at room temperature for 30 min. Then, the kinase quench buffer (8 mM EDTA) and Eu-anti-phospho-4E-BP1 antibody (2 nM) were added. The mixtures were then incubated for 1h at room temperature. The data was collected on PerkinElmer EnVision Reader.

Pharmacokinetic Studies

Male SD rats (200-220 g, N = 3 per group) were dosed intravenously with 2 mg/kg of **14o** prepared in 40% PEG400/ 5% DMSO/ 55% Saline, and orally with 10 mg/kg in 0.5% sodium

carboxymethylcellulose/0.2% Tween 80. Blood samples were taken at 0 (prior to dosing), 0.25, 0.5, 1, 2, 4, 6, 12 and 24 h following oral dosing and at 0 (prior to dosing), 0.083, 0.25, 0.5, 1, 2, 4, 8 and 24 h following intravenous dosing. The samples were collected from jugular vein and stored in ice (0−4°C). Plasma was obtained from the blood samples by centrifugation (8000 rpm for 6 min at 2-8 °C) and stored at −80 °C. All samples of the compound were determined by LC−MS/MS (Shimadzu; API 4000). The assay lower limit of quantitation (LLOQ) was 1 ng/mL for **14o** in plasma. The pharmacokinetic parameters were analyzed by noncompartmental methods using WinNonlin 5.2. (Accomplished by Shanghai Medicilon Inc.)

Cell Viability Assay

The human cancer cell lines used were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured at 37℃ with 5% CO_2 in RPMI 1640 or DMEM, supplemented with 10% (v/v) fetal bovine serum (Gibco) and 1% (v/v) penicillin-streptomycin (HyClone). Cells in logarithmic phase were seeded in a 96-well plate at 2-5×10^3 cells per well for 24 hours (37℃, 5% CO_2). And then, an equal volume of medium containing various concentrations of test compounds was added to each well. After 72 h, CCK-8 reagent was added, and the cells were incubated for an additional 1-4 hours. The absorbance values (OD) of the 96-well plate was measured at 450 nm using SpectraMAX M5 microplate spectrophotometer (Molecular Devices). The IC_{50} values were means of at least three independent experiments and calculated by GraphPad Prism5 software.

Western blot analysis

Cells were treated with **14o** and **1** at the indicated concentrations for 24h at 37°C, then the cells were harvested, washed in ice-cold PBS, and lysed with RIPA buffer, protease inhibitors, phosphatase cocktails A and B, and PMSF (1 mM). Protein concentration was determined by the BCA Protein Assay Kit (beyotime#p0012s). The samples were subjected to SDS-PAGE and then transferred onto PVDF membranes (Millpore). The membranes were incubated overnight at 4°C with the primary antibody in 5% BSA/TBST buffer with gently shaking, then washed with 1×TBS/T 3 times, followed by incubation for 1 hour with a 1/5000 dilution of secondary HRP antibody in 5% nonfat milk/TBST. Primary antibodies used were: anti-AKT (1/1000 dilution, CST#4685s), anti-pAKTser473 (1/1000 dilution, CST#4058s), anti-pAKTthr308 (1/1000 dilution, CST#13038s), anti-S6RP (1/1000 dilution, CST#2217s), anti-pS6RP Ser235/236 (1/1000 dilution, CST#4858p) and anti-β-actin (1/5000 dilution, Zen BioScience#200068-8F10), and the target blots were detected with chemiluminescence system.

***In Vivo* Xenograft Studies.**

The female BALB/c nude mice were purchased (Beijing HFK Bioscience Co. ltd., Beijing, China). SKOV-3 and U87MG cells were harvested during the exponential-growth phase, washed twice with serum-free medium. Mice (6-7 weeks old and weighed 18-22 g) were injected subcutaneously with 5×10^6 SKOV-3 or U87MG cells, which were suspended in 0.1 mL of serum and antibiotic free growth medium. The tumors were allowed to grow to 150-200 mm^3, at which point the mice were divided randomly (10 mice for each group). In the SKOV-3 model, the mice were dosed orally with **14o** (15, 30, 60 mg/kg/d, suspended in 0.5% CMC-Na with 0.2% Tween-80), vehicle (0.5% CMC-Na with 0.2% Tween-80) and **1** (positive control, 30 mg/kg/d, suspended in 0.5% CMC-Na with 0.2%

Tween-80). In the U87MG model, the mice were dosed orally with **14o** (20, 40 mg/kg/d, suspended in 0.5% methylcellulose with 0.2% Tween-80), vehicle (0.5% methylcellulose with 0.2% Tween-80) and **1** (positive control, 20 mg/kg/d, suspended in 0.5% methylcellulose with 0.2% Tween-80). The body weight and tumor volume were measured every 3 days. The tumor volume was determined with Vernier calipers and calculated as follows: tumor volume = $a \times b^2/2$ (a, long diameter; b, short diameter). Percentage of tumor growth inhibition (TGI) was calculated as $100 \times \{1-[(Treated_{Final\ day}-Treated_{Initial\ day})/(Control_{Final\ day}-Control_{Initial\ day})]\}$. All animal experiments have been approved by Institutional Animal Care and Treatment Committee of Sichuan University in China (IACUC number: 20100318).

Immunohistochemical analysis

At the end of the dosing period, SKOV-3 tumors were collected. The tumors were fixed with formalin and embedded in paraffin. Sections were cut at 4-8 μm in thickness for histological and immunohistochemical analysis. The cell proliferation was detected by immunostaining with the Ki67 antibody using DAB chromogenic reagent kit (DAKO#k5007). Apoptosis was determined by transferase-mediated dUTP nick-end labeling (TUNEL) staining using an insitu cell death detection kit (Roche#TUN11684817). AKT^{Ser473} and $S6RP^{Ser235/236}$ phosphorylation were also detected by immunohistochemical analysis. Finally, images were captured with an Olympus digital camera attached to a light microscope.

Supporting Information.

The Supporting Information is available free of charge on the ACS Publications website at DOI:

Figure S1 describing the weight of mice during the treatment period; Scheme S1 listing the synthetic routes of compounds **10** and **12-14**; ^{1}H and ^{13}C NMR spectra of all final compounds.

Molecular formula strings (CSV)

Accession Codes

PDB codes 4L23 and 4JT5 were used for study of the binding modes of **14o** with PI3Kα and mTOR respectively. Authors will release the atomic coordinates and experimental data upon article publication.

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ABBREVIATIONS

PI3K, Phosphatidylinositol 3-Kinase; mTOR, Mammalian Target of Rapamycin; CCK-8, Cell Counting Kit-8; AKT, protein kinase B (PKB); RTK, receptor tyrosine kinase; THF, tetrahydrofuran; *m*-CPBA, 3-Chloroperoxybenzoic acid; TFA, trifluoroacetic acid; DMAc, N,N-Dimethylacetamide; DMF, N,N-Dimethylformamide.

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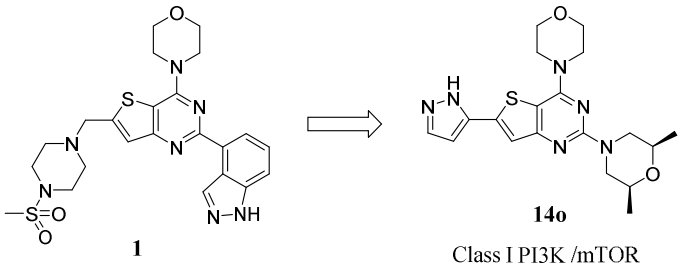

1
Class I PI3K Inhibitor

14o
Class I PI3K /mTOR
Dual Kinase Inhibitor

Structural modifications of Class I PI3K inhibitor **1** led to the discovery of **14o**, which had been shown to be a potent Class I PI3K/mTOR dual inhibitor.