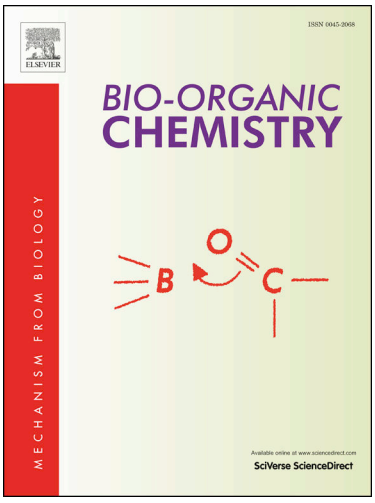
Journal Pre-proofs



Design, Synthesis, and Biological Evaluation of substituted 2-(thiophen-2-yl)-1,3,5-triazine derivatives as Potential dual PI3Kα/mTOR inhibitors

Binliang Zhang, Qian Zhang, Zhen Xiao, Xin Sun, Zunhua Yang, Qi Gu, Ziqin Liu, Ting Xie, Qingqing Jin, Pengwu Zheng, Shan Xu, Wufu Zhu

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Design, Synthesis, and Biological Evaluation of substituted 2-(thiophen-2-yl)-1,3,5-triazine derivatives as Potential dual PI3Kα/mTOR inhibitors

Binliang Zhang [a], Qian Zhang [a], Zhen Xiao [a], Xin Sun [a], Zunhua Yang [b], Qi Gu [a], Ziqin Liu [a], Ting Xie [a], Qingqing Jin [a], Pengwu Zheng [a], Shan Xu [a,*], Wufu Zhu [a,*]

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Abstract：

The phosphoinositide 3-kinase (PI3K) and mammalian target of rapamycin (mTOR) have been regarded as promising targets for the treatment of cancer. Herein, we synthesized a new series of substituted 2-(thiophen-2-yl)-1,3,5-triazine derivatives as novel PI3Kα/mTOR dual inhibitors for cancer therapy. All compounds were evaluated for the IC_{50} values against three cancer cell lines (A549, MCF-7 and Hela). Most of the target compounds exhibited moderate to excellent anti-tumor activities against these three tested cancer cell lines especially against A549 and Hela cancer cell lines. Among them, the most promising compound **13g** showed excellent anti-tumor potency for A549, MCF-7 and Hela cell lines with IC_{50} values of 0.20±0.05 μM, 1.25±0.11 μM and 1.03±0.24 μM, respectively. Notably, according to the result of enzymatic activity assay, compound **13g** was identified as a novel PI3Kα/mTOR dual inhibitor, which had an approximately 10-fold improvement in mTOR inhibition, compared to the class I PI3K inhibitor **1** (pictilisib, GDC-0941), with IC_{50} values of 525 nM to 48 nM. And western blot analysis indicated compound **13g** could efficiently suppress the phosphorylation of AKT at the dose of 0.1 μM, which further demonstrated compound **13g** had significant inhibitory effect on the PI3K/Akt/mTOR pathway. Furthermore, compound **13g** could stimulate A549 cells arrest at G0/G1 phase in a dose-dependent manner, and induced apoptosis at a low concentration.

Keywords: 2-(Thiophen-2-yl)-1,3,5-triazine; PI3Kα/mTOR inhibitors; Anti-tumor activity; Scaffold hopping

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1. Introduction

The phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT)/the mammalian target of rapamycin (mTOR) signaling pathway plays a critical role in a diverse set of cellular functions, including cell growth, proliferation, motility, differentiation, and survival, which has been identified as promising target for the treatment of cancer, providing validated therapeutic targets associated with malignancies [1-4]. At present, some PI3K inhibitor drugs had been approved by FDA, such as Alpelisib (BYL719) [5], Copanlisib (BAY 80-6946) [6], Idelalisib (GS-1101) [7] and so on.

Along this critical signaling pathway, a variety of kinase inhibitors were discovered, such as allosteric mTOR inhibitor, ATP-competitive mTOR inhibitor, pan-PI3K inhibitor, PI3K/mTOR dual inhibitor and a more selective inhibitor of the class I PI3K family. Among them, dual PI3K/mTOR inhibitors appear to increase efficacy and reduce the likelihood of inducing drug resistance as they target the pathway at two nodal points. In recent years, many PI3K/mTOR dual inhibitors were discovered including PI-103 [8], GSK2126458 [9], compoud **3j** [10], Apitolisib (GDC-0980) [11] and so on (**Figure 1**). Among them, GSK2126458 and Apitelisib (GDC-0980) are now in clinical phase I.

It has been reported that most of these PI3K inhibitors share similar structural and chemical features, i.e., a central six-membered heterocycle substituted by a morpholine with a hydrogen bond donor group [12] (**Figure 1**). Among them, **1** (pictilisib, GDC-0941) [13] was the first potent class I PI3K inhibitor developed by Genentech, which was served as a useful reference to explore novel PI3K inhibitors. Starting with **1** (pictilisib, GDC-0941), considerable structure−activity relationship (SAR) investigations have been focused on the substituents of the thienopyrimidine core, and some potential compounds were developed such as apitolisib (GDC-0980). However, these explorations are restricted to the replacement of the indazole with aryl or heteroaryl group, and modification of the thieno[3,2-*d*]pyrimidine core of **1** have barely been reported. It is worth mentioning that several series of PI3K inhibitors were designed by incorporating 1,3,5-triazine, a symmetrical structure to improve the solubility and potency, such as ZSTK474 [14] (**Figure 1**). Therefore, thieno[3,2-*d*]pyrimidine and 1,3,5-triazine had been proven to be an effectual scaffold with anti-tumor.

In this study, we applied scaffold hopping method to develop several series of 2-(thiophen-2-yl)-1,3,5-triazine derivatives as novel PI3Kα/mTOR dual inhibitors. As a result, three series of substituted 2-(thiophen-2-yl)-1,3,5-triazine derivatives (**11a-11g**, **12a-12g** and **13a-13g**) were designed and synthesized. In addition, the bioactivities against three cancer cell lines (human lung adenocarcinoma cells A549, human breast cancer cells MCF-7 and human cervical cancer cells Hela) and the normal human fetal lung fibroblast (WI-38) cell line were evaluated.

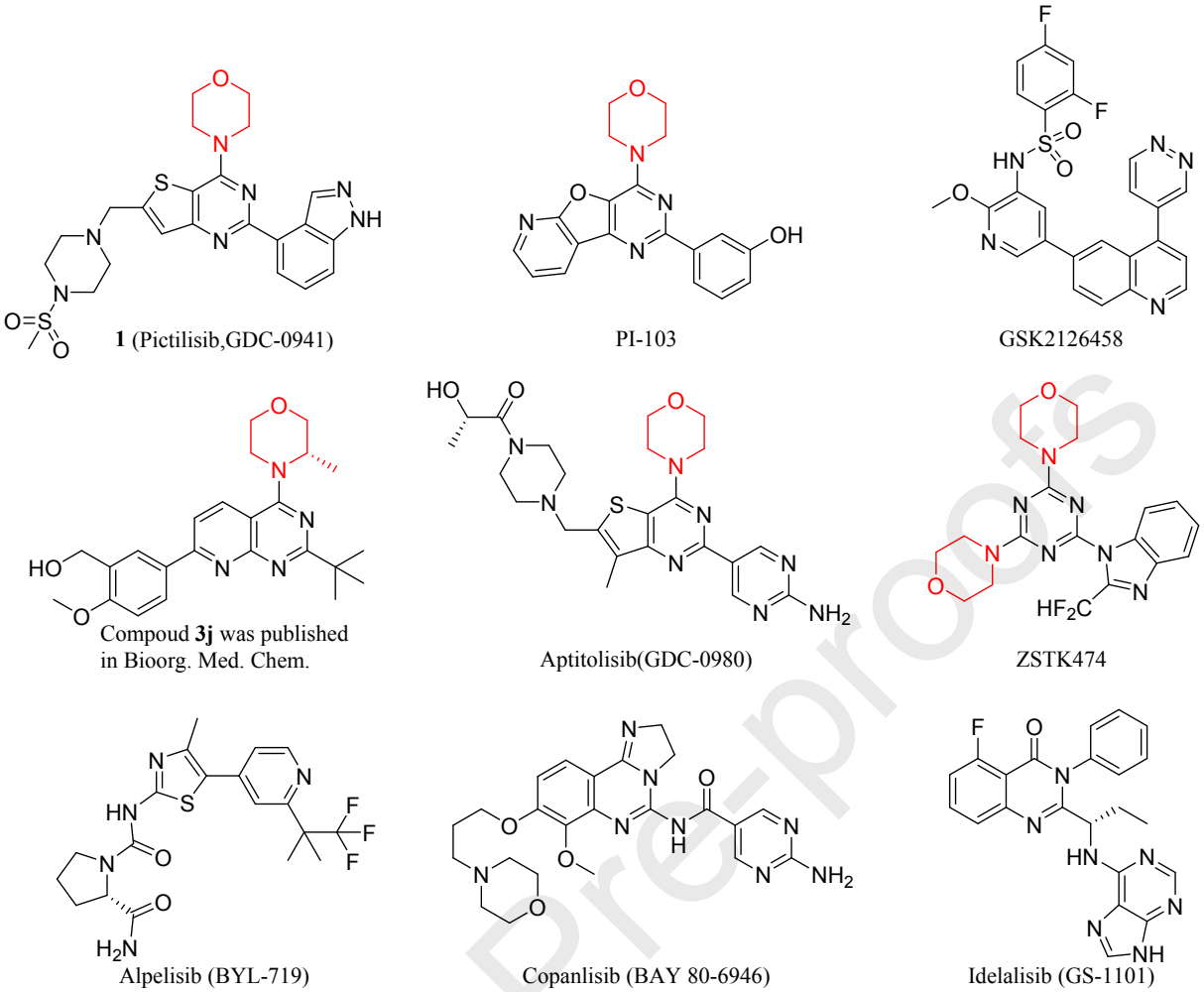


Figure 1. Examples of PI3K inhibitors.

2. Results and discussion

2.1 Design strategy

To guide our design, we docked GDC-0941 and ZSTK474 into PI3Kα protein (PDB code 4L23). As depicted in **Figure 2A**, the whole GDC-0941 skeleton embedded into hydrophobic pocket of PI3Kα kinase and closely combined with PI3Kα kinase. In this cocrystal structure, the morpholine oxygen of GDC-0941 formed a crucial hydrogen bond interaction with the hinge region of PI3Kα kinase through the amide of Val851, and the indazole moiety points toward the affinity pocket where two nitrogen atoms on the indazole make key interactions with Asp810 and Tyr836, respectively. In addition, the 4-methanesulfonylpiperazin-1-ylmethyl group extended out to solvent, but the sulfonyl oxygen atom could not form any specific hydrogen bond with the solvent front of PI3Kα kinase. As illustrated in **Figure 2B**, the orientation of the whole molecular skeleton of ZSTK474 into hydrophobic pocket of PI3Kα protein was basically the same as that of GDC-0941. ZSTK474 also combined well with PI3Kα kinase and formed two strong hydrogen bonds with Val851 and Lys802. According to the molecular simulation results and previous research of PI3K inhibitors, a variety of core can be

accommodated in PI3K kinase cavity, which further indicated that sufficient space was available within the ATP binding site allowing for the possibility of modifying the thieno[3,2-*d*]pyrimidine core of GDC-0941.

In this research, we investigated the possibility of scaffold hopping from the thieno[3,2-*d*]pyrimidine core of GDC-0941 to 2-(thiophen-2-yl)-1,3,5-triazine, which was the principal breakthrough and innovation of our research, as illustrated in **Figure 3**. Through this strategy, the spatial volume and length of 2-(thiophen-2-yl)-1,3,5-triazine could be further expanded to occupy the protein cavity. We suspected that substituted 2-(thiophen-2-yl)-1,3,5-triazine derivatives would better embed into the deep ATP pocket, occupying space adjacent to Asp810 side chain. A morpholine portion was introduced as the hinge region binding moiety, heterocyclic aromatic or substituted aryl group at the C-6 position of the 2-(thiophen-2-yl)-1,3,5-triazine core as the possible affinity element, which interacted with the deeper hydrophobic pocket. Compared with the thieno[3,2-*d*]pyrimidine core of GDC-0941, the conformation of the new core had changed, so our strategy was to introduce appropriate substituents on thiophene ring to explore potential interactions with the residue Gln859 nearby. Herein, we disclosed the preparation and biological evaluation of a series of substituted 2-(thiophen-2-yl)-1,3,5-triazine derivatives, which demonstrated potent inhibition of the PI3K/AKT/mTOR pathway, culminating in the discovery of **13g**.

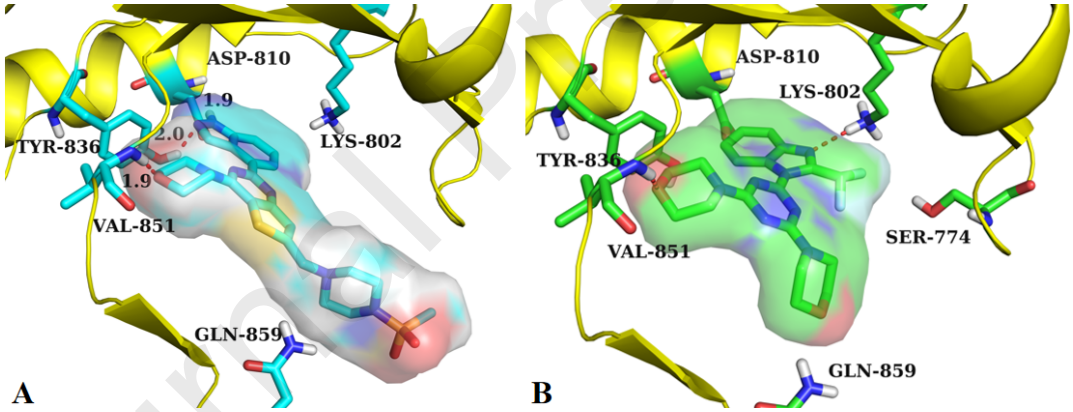


Figure 2. The docking result of GDC-0941 and ZSTK474 bound to PI3Kα protein (PDB code 4L23)

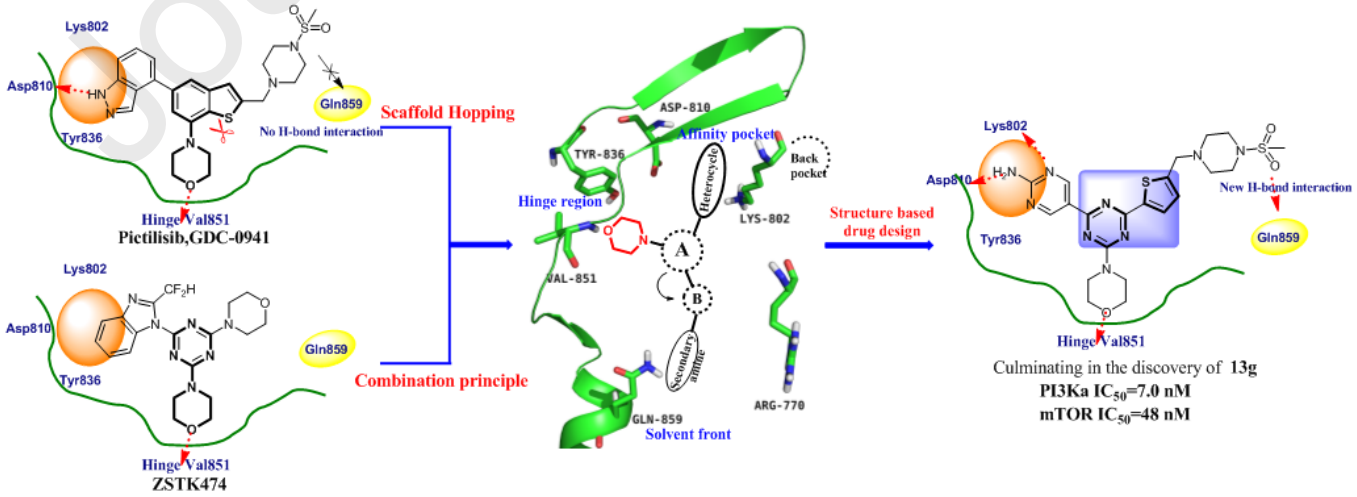
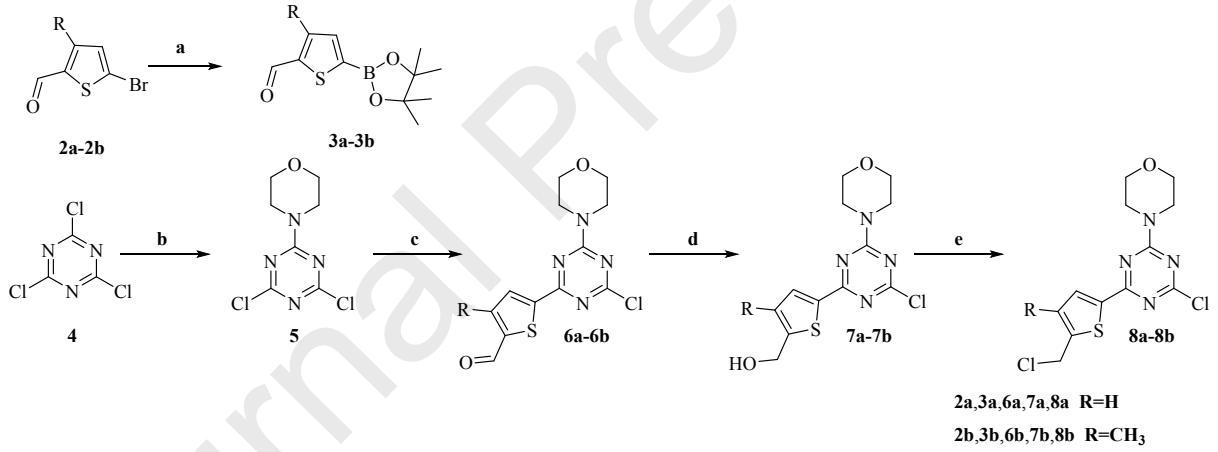


Figure 3. Design of series derivatives based on **1**.

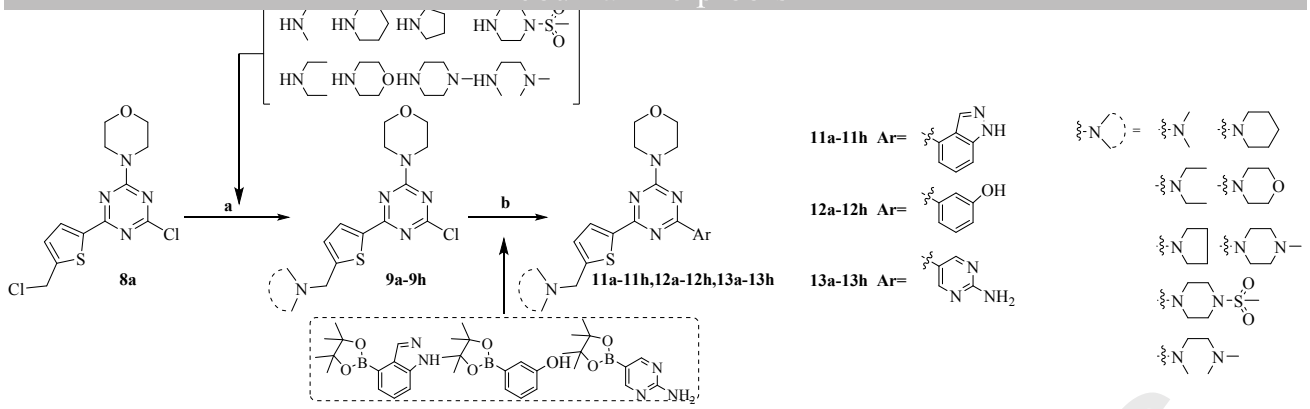
2.2. Chemistry

The synthetic route of the intermediate compounds **8a-8b** were shown in **Scheme 1**. Under the catalysis of bis(triphenylphosphine)palladium(II) dichloride, compounds **2a-2b** were reacted with bis(pinacolato)diboron through Suzuki coupling reaction to generate compounds **3a-3b**. In acetone solution, the nucleophilic reaction between cyanuric chloride and morpholine yielded compound **5**. Subsequently, coupling compound **5** with **3a-3b** *via* Suzuki reaction afforded corresponding target compounds **6a-6b**. Treatment of **6a-6b** with $NaBH_4$ in MeOH in ice bath provided **7a-7b** as a primary alcohol, which reacted with thionyl chloride to give compounds **8a-8b**. The synthetic route of the target compounds **11a-11h, 12a-12h, 13a-13h, 14a-14b, 15a-15b** and **16a-16b** were shown in **Schemes 2** and **3**. Compound **8a** and various secondary amines were reacted to give compounds **9a-9h** and then coupled with 1*H*-indazole-4-boronic acid pinacol ester, 3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenol and 2-aminopyrimidine-5-boronic acid pinacol ester to obtain compounds **11a-11h**, **12a-12h** and **13a-13h**. At the same way, compound **8b** substituted with 1-(methylsulfonyl) piperazine and morpholine to obtain compounds **10a-10b**. Finally, three kinds of aromatic nucleus were introduced to compounds **10a-10b** by Suzuki coupling reaction to obtain compounds **14a-14b**, **15a-15b** and **16a-16b**.



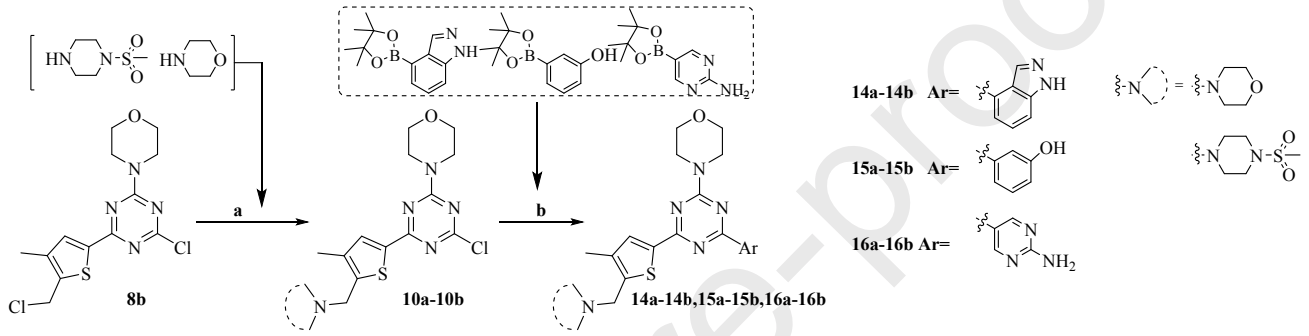
Scheme 1. Synthetic route of the compounds **8a-8b**.

Reagents and conditions: (a) Bis(pinacolato)diboron, KAc, $Pd(PPh_3)Cl_2$, 1,2-dimethoxyethane, 80 ºC, 6 h; (b) Morpholine, Acetone, 0 ºC, 1 h; (c) H_2O, K_2CO_3, $Pd(PPh_3)Cl_2$, 75 ºC, 12 h; (d) MeOH, $NaBH_4$, 0 ºC, 0.5 h; (e) CH_2Cl_2, $SOCl_2$, DMF, r.t., 2 h.



Scheme 2. Synthetic route of the compounds **11a-11h**, **12a-12h** and **13a-13h**.

Reagents and conditions: (a) K_2CO_3, Isopropyl alcohol, 55 ºC, 1.5 h; (b) H_2O, K_2CO_3, $Pd(PPh_3)Cl_2$, 130 ºC, 8 h.



Scheme 3. Synthetic route of the compounds **14a-14b**, **15a-15b** and **16a-16b**.

Reagents and conditions: (a) K_2CO_3, Isopropyl alcohol, 55 ºC, 1.5 h; (b) H_2O, K_2CO_3, $Pd(PPh_3)Cl_2$, 130 ºC, 8 h.

2.3. Biological evaluation

Three cancer cell lines A549, MCF-7 and Hela were selected for the anti-tumor activities of the target compounds (**11a-11h**, **12a-12h**, **13a-13h**, **14a-14b**, **15a-15b** and **16a-16b**) by 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay, using PI3K inhibitor GDC-0941 as positive control. Furthermore, the preferred target compounds (**11c**, **11e**, **11g**, **12c**, **12e**, **12g**, **13c**, **13e** and **13g**) were evaluated for the IC_{50} values against PI3Kα and mTOR kinases by the mobility shift assay, together with reference compounds PI-103 and GDC-0941, and according to the results of kinase inhibitory activity, the compounds **13e** and **13g** were further evaluated for other tyrosine kinases to test the enzyme-based selectivity. As shown in **Table 1** and **Table 2**, the results expressed as half-maximal inhibitory concentration (IC_{50}) values and the values were the average of three independent experiments. Moreover, in order to investigate the mode of cell proliferation inhibition by compound **13g**, cell cycle distribution analysis (**Figure 5**) and apoptosis analysis (**Figure 6**) were performed on A549 cells. In addition, AO single staining (**Figure 7**) and western blot (**Figure 8**) were carried out in this study.

As can be seen, most of compounds displayed moderate to excellent anti-tumor activities against three cancer cells lines (A549, MCF-7 and Hela) with potency from single-digit μM to nM range in **Tables 1** and **2**. In general, the target

compounds had more potent anti-tumor activities against both A549 and Hela cell lines than that of MCF-7 cell line. What's more, we could easily see that the third series of compounds **13a-13h** were much more active than the other series of compounds **11a-11h** and **12a-12h** against the three cancer cell lines, which suggested that introduction of 2-aminopyrimidine group to the compounds were preferable to the anti-tumor activity. We speculated that the nitrogen atom on 2-aminopyrimidine can provide appropriate hydrogen bond donor based on the above results, which may contribute to high binding affinity. Among these compounds, six of them were more potent than GDC-0941 against one or more cell lines. The most promising compound **13g** exhibited the best activity against A549, MCF-7 and Hela cell lines with the IC_{50} values of 0.20±0.05 μM, 1.25±0.11 μM and 1.03±0.24 μM, respectively, which were better than that of lead drug GDC-0941.

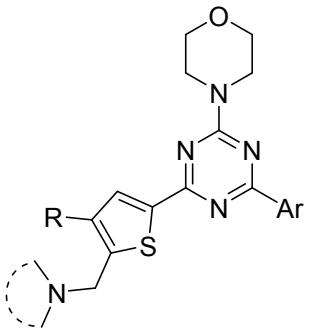
As showed in **Table 1**, it is worth mentioning that the substituents of the secondary amines had an important influence on anti-tumor activity. Compared with **11a** and **11b**, **12a** and **12b**, **13a** and **13b**, it was found that the compounds containing dimethylamine groups were much more active than compounds containing diethylamine groups against the three cancer cell lines. Similarly, the same trend was observed between pyrrolidine unit and piperidine unit. What's more, introduction of morpholine and 1-(methylsulfonyl)piperazine to the compounds (**11e** and **11g**, **12e** and **12g**, **13e** and **13g**) were more beneficial to the anti-tumor activity than that of other secondary amines. We speculated that morpholine and 1-(methylsulfonyl)piperazine groups were preferred toward the solvent front region of PI3Kα or mTOR, which can provide appropriate hydrogen bond donor to form hydrogen bonds with amino acid residues to enhance anti-tumor activity.

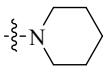
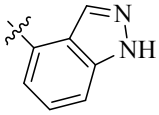
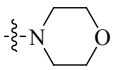
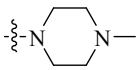
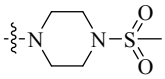
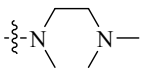
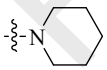
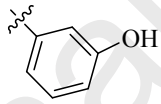
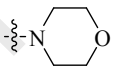
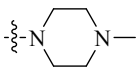
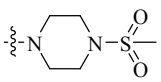
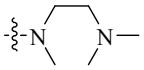
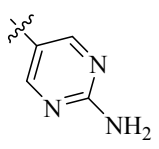
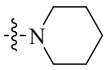
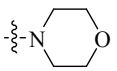
Inspired by Apitolisib (GDC-0980), the electron-donating group methyl was introduced into thiophene ring to change the electron cloud distribution of the compounds in order to improve the anti-tumor activity of the compounds. Therefore, a methyl group was introduced into the thiophene ring of compounds **11e**, **11g**, **12e**, **12g**, **13e** and **13g** with excellent anti-tumor activity. The results were shown in **Table 2**, these six compounds displayed moderate anticancer activity against three cancer cells lines (A549, MCF-7 and Hela). However, introducing a methyl group on thiophene ring resulted in decreasing anti-tumor activity, which was not as expected.

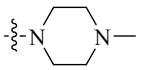
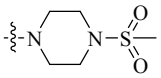
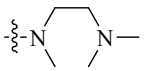
The most promising compound **13g** showed only a slight improvement in anti-tumor activity compared to the class I PI3K inhibitor GDC-0941. Therefore, their cytotoxic activity against normal cells were further compared. In this study, compounds **13e** and **13g** were selected for cytotoxic activity against WI-38 normal cell line *in vitro*. The results of the test were presented in **Table 3**, which shown that compound **13g** exhibited lower cytotoxic activity against WI-38 normal cell line compared to Class I PI3K inhibitor GDC-0941. Furthermore, the cytotoxic activity of compound **13e** on WI-38 normal cell line was similar to that of compound **13g**, and their IC_{50} values were greater than 20 μM. These data indicated that three cancer cells lines (A549, MCF-7 and Hela) were more sensitive than the normal cells.

Table 1

Structures and activity of target compounds **11a-11h**, **12a-12h** and **13a-13h**.



Compd.	R	Ar		IC_{50}[a] (μM)		
				A549	MCF-7	Hela
11a	H			5.58±0.74	8.21±0.91	6.38±0.80
11b	H			8.47±0.92	12.85±1.10	6.81±0.83
11c	H			4.69±0.67	5.71±0.75	4.44±0.64
11d	H			21.86±1.34	12.93±1.11	17.69±1.38
11e	H			**2.32±0.78**	3.68±0.69	3.62±0.55
11f	H			8.43±0.92	12.79±1.10	5.12±0.71
11g	H			**1.11±0.83**	**0.33±0.89**	3.98±0.69
11h	H			51.67±1.71	20.77±1.31	49.63±1.69
12a	H			13.26±1.02	6.46±0.81	5.13±0.71
12b	H			15.7±1.19	8.36±0.92	13.63±1.13
12c	H			8.83±0.94	4.15±0.61	3.60±0.55
12d	H			23.57±1.37	22.56±1.35	7.24±0.86
12e	H			**1.44±0.08**	**2.47±0.09**	**0.38±0.07**
12f	H			21.27±1.32	6.96±0.84	8.82±0.94
12g	H			**0.32±0.02**	3.50±0.38	**2.36±0.55**
12h	H			21.27±1.02	31.87±0.92	47.26±1.43
13a	H			4.84±0.16	5.21±0.28	4.73±0.19
13b	H			7.06±0.32	10.12±0.31	11.34±0.51
13c	H			**1.15±0.22**	**1.21±0.18**	**1.07±0.09**
13d	H			15.11±1.02	20.45±1.21	11.46±0.43
13e	H			**1.50±0.14**	**2.65±0.08**	**1.84±0.11**

13f	H			7.83±2.21	15.69±1.28	8.52±0.23
13g	H			**0.20±0.05**	**1.25±0.11**	**1.03±0.24**
13h	H			18.38±1.72	40.07±1.92	27.46±1.35
GDC-0941[b]	-	-	-	1.21±0.13	1.47±0.10	3.72±0.26

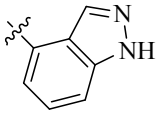
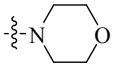
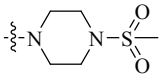
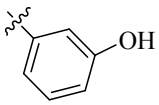
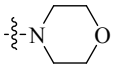
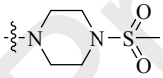
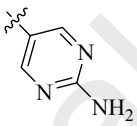
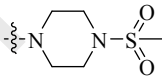
The bold values showed excellent anti-tumor activity (0.2-2.65 μM).

[a]The values are an average of three separate determinations.

[b]Used as the positive control.

Table 2

Structures and activity of target compounds **14a-14b**, **15a-15b** and **16a-16b**.

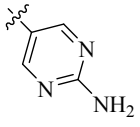
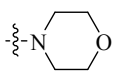
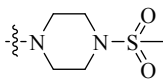
Compd.	R	Ar		IC_{50}[a] (μM)		
				A549	**MCF-7**	**Hela**
14a	CH_3			9.89±1.90	11.81±1.52	13.97±1.14
14b	CH_3			6.55±0.81	5.57±0.74	4.00±0.60
15a	CH_3			11.85±1.07	6.38±0.80	9.61±0.98
15b	CH_3			4.26±0.52	8.36±0.92	5.13±0.71
16a	CH_3			5.34±0.16	6.11±0.18	5.85±0.15
16b	CH_3			3.86±0.84	7.04±0.84	3.52±0.14
GDC-0941[b]	-	-	-	1.20±0.13	1.47±0.10	3.72±0.26

[a]The values are an average of three separate determinations.

[b]Used as the positive control.

Table 3

Cytotoxic activity of compounds **13e** and **13g** against WI-38 normal cell line *in Vitro*

Compd.	R	Ar		IC_{50} (μM)
				WI-38
13e	H			>20
13g	H			>20
GDC-0941[a]	-	-	-	8.4±1.93

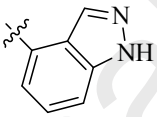
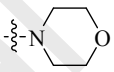
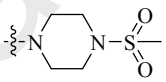
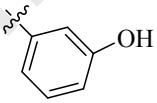
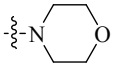
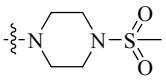
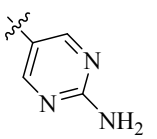
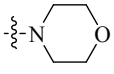
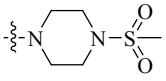
[a]Used as the positive control.

2.4. PI3Kα and mTOR enzymatic assays *in vitro*

Experiments against PI3Kα and mTOR kinases of nine selected compounds (**11c**, **11e**, **11g**, **12c**, **12e**, **12g**, **13c**, **13e** and **13g**) as well as the lead compounds GDC-0941 and PI-103 were carried out in this paper for further study. In **Table 4**, obviously, the overall activity against PI3Kα kinase of compounds **13e** and **13g** were equal to the reference compound GDC-0941, with the IC_{50} values of 9.5 nM and 7.0 nM against PI3Kα kinase. As can be seen, the overall activity against mTOR kinase of the selected compounds were better than that of the reference compound GDC-0941. Notably, compounds **13e** and **13g** showed excellent inhibitory activity to mTOR with the IC_{50} values of 69 nM and 48 nM, which had an approximately 7-fold to 10-fold improvement in mTOR inhibition, compared to the class I PI3K inhibitor GDC-0941. What's more, the overall activity against PI3Kα and mTOR kinases of compounds **13e** and **13g** were slightly less than the reference compound PI-103. In a word, the results prompted us that compounds **13e** and **13g** may become promising PI3Kα/mTOR dual inhibitors.

Table 4

PI3Kα and mTOR kinases inhibitory activity of compounds **11c**, **11e**, **11g**, **12c**, **12e**, **12g**, **13c**, **13e**, **13g**, GDC-0941 and PI-103.

Compd.	R	Ar		IC_{50} (nM)	
				PI3Ka	mTOR
11c	H			747±36.29	>1000
11e	H			731±40.65	485±19.21
11g	H			716±33.62	439±21.09
12c	H			395±24.32	864±56.74
12e	H			**194±13.91**	**183±15.87**
12g	H			**55±4.20**	**195±11.69**
13c	H			151±8.12	483±17.63
13e	H			**9.5±1.14**	**69±6.35**
13g	H			**7.0±0.99**	**48±2.47**
GDC-0941[a]	-	-	-	6.0±1.12	525±28.33
PI-103[a]	-	-	-	5.1±1.03	21±3.73

[a]Used as the positive control.

2.5. Enzymatic selectivity assays

In order to examine whether compounds **13e** and **13g** were selective PI3Kα/mTOR dual inhibitors, compounds **13e** and **13g** were evaluated against other tyrosine kinases. The obtained results were presented in **Figure 4**, which suggested that compound **13g** exhibited low inhibitory effects against EGFR (8.3%), c-Met (2.0%), VEGFR-2 (0.5%), and EGFR $^{T790M/L858R}$ (2.3%) at the concentration of 1 μM. However, compound **13g** inhibited mTOR and PI3Kα kinases by more than 93% at the dose of 1 μM, especially PI3Kα kinase, up to 100%. The selectivity of compound **13e** for kinases was similar to that of compound **13g**. These data demonstrated that compounds **13e** and **13g** were selective PI3Kα/mTOR dual inhibitors.

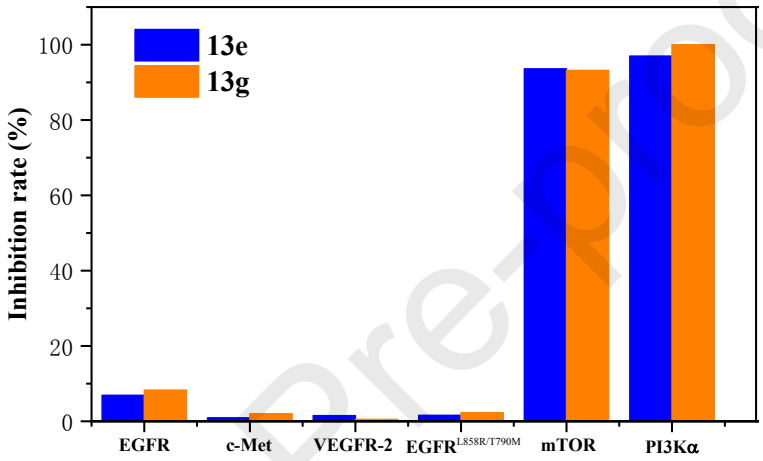


Figure 4. Inhibition of tyrosine kinases by compounds **13e** and **13g** at 1 μM

2.6. Effect on cell cycle progression.

To explore the way of cell proliferation inhibition by compound **13g**, cell cycle distribution analysis was carried out on A549 cells. The results were shown in **Figure 5**. As can be seen, compared with the control group, compound **13g** can induce cell cycle arrest of A549 cells at the G0/G1 checkpoint at low concentration, in a dose-dependent manner. As the concentration of compound **13g** increasing, compound **13g** significantly increased the G0/G1 phase population from 69.44% to 77.70%, while the S phase was decreased, while no significant change in G2/M phase was observed. What's more, at a concentration of 1.6 μM, compound **13g** induced cell cycle arrest of A549 cells at the G0/G1 checkpoint with 77.70%, which was similar to that of GDC-0941 (76.22%).

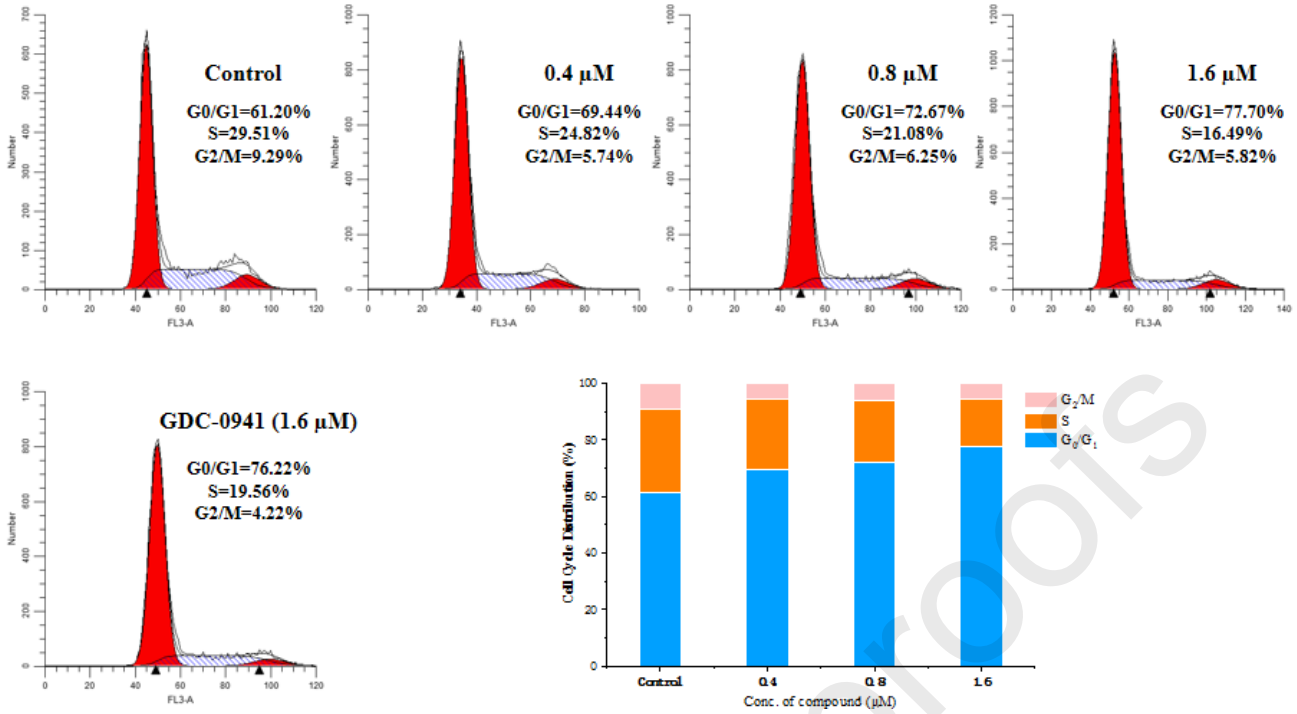


Figure 5. Cell cycle progression analyses of A549 cells treated with compound **13g** and GDC-0941 for 48 h**.**

2.7. Effect on apoptosis

To obtain more insight into the growth inhibition mechanism of tumor cells, the influence of compound **13g** on apoptosis was investigated. A549 cells were treated with 0.4 μM, 0.8 μM and 1.6 μM of compound **13g** for 48 h, respectively, and then the flow cytometric apoptosis experiments were carried out. The results of apoptosis analysis were illustrated in **Figure 6**, which showed that compound **13g** (the total apoptosis rate was 16.05% at 1.6 μM) could induce 12.51% increase of apoptotic cells, compared with the control group (the total apoptosis rate was 3.54%). As the concentration of compound **13g** increasing, the total apoptosis rate was also increased, from 7.18% to 16.05%, in a dose-dependent manner. These findings demonstrated that compound **13g** successfully induced apoptosis in a dose-dependent manner, and the effect was similar to that of GDC-0941.

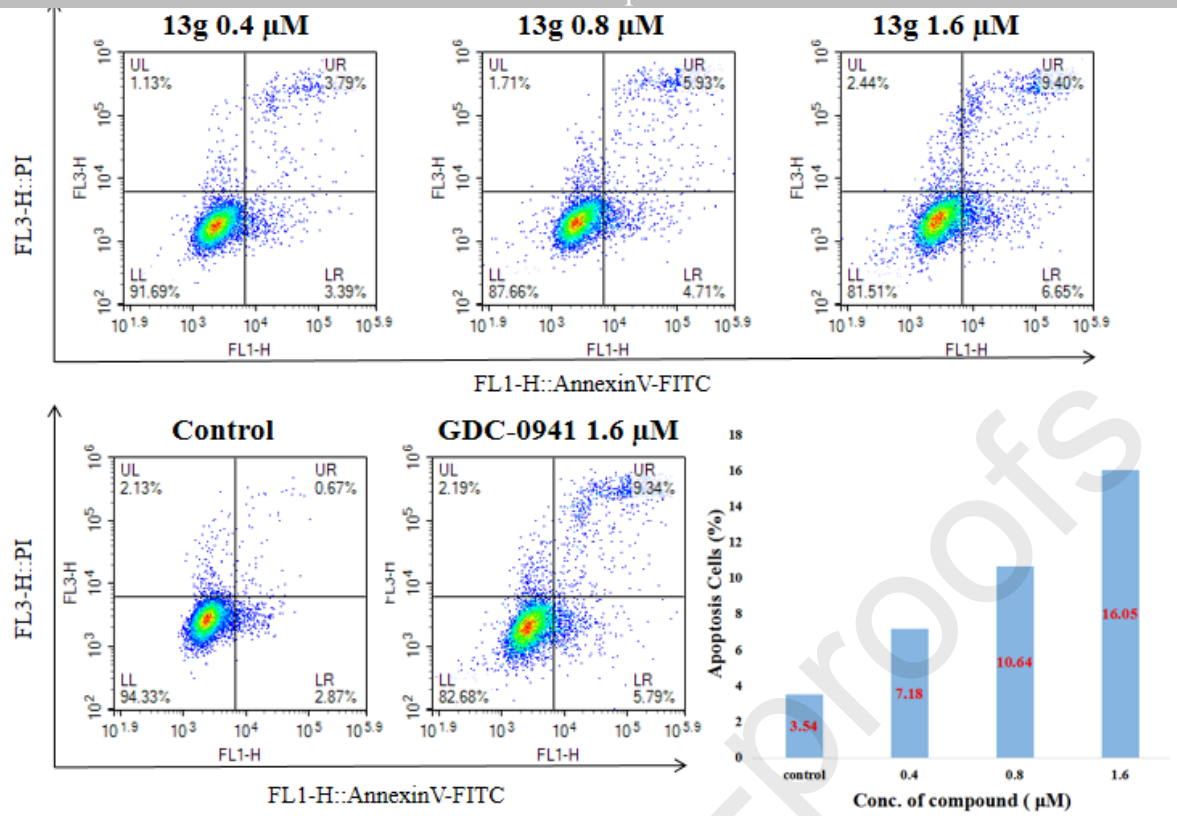


Figure 6. Apoptosis analyses of A549 cells treated with compound **13g** and GDC-0941 for 48 h**.**

2.8. Morphologic changes of A549 cells under inverted microscopy and fluorescence microscopy

In order to evaluate whether compound **13g** was able to induce apoptotic, the apoptotic experiments were performed to detect the effect of compound **13g** on A549 cells by acridine orange (AO) single staining.

As shown in **Figure 7**, the control cells (**Figure 7A**) were showing that the nucleus of A549 cells were stained with acridine orange (AO) from fluorescence microscopy, and shape of the cell was full, and the edge was clear and the refraction was great. However, when the cells were treated with 0.2 μM and 0.8 μM of compound **13g**, it can be seen in **Figures 7B** and **7C** that the A549 cells showed significant evidence of apoptosis mediated cell death, such as cell shrinkage. It indicated that compound **13g** could induce apoptosis of A549 cells, which were consistent with the former result of the flow cytometric apoptosis experiments.

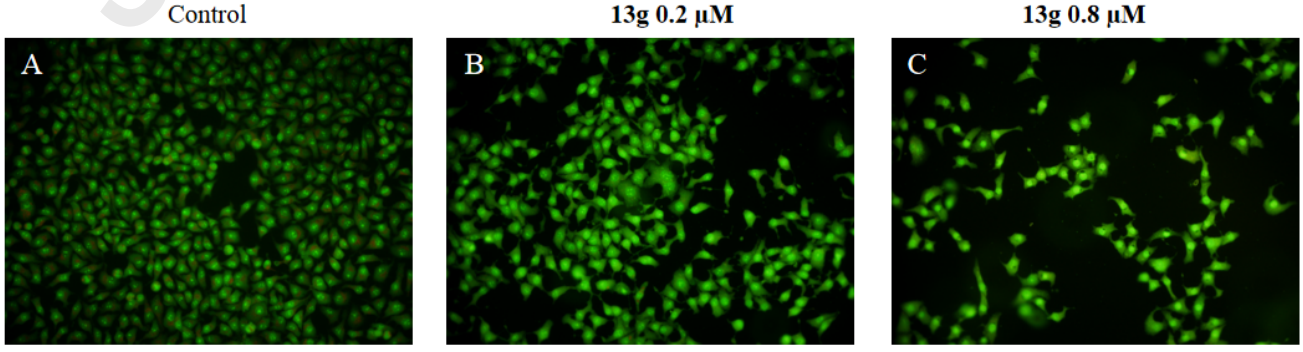


Figure 7. Morphologic changes of A549 cells under inverted microscopy and fluorescence microscopy.

2.9. Western blot assay *in vitro*

To further determine whether the target compounds would inhibit the activation of both PI3K/Akt/mTOR signalings in cancer cell, western blot assay was performed to test the expression of relative proteins. As shown in **Figure 8**, a dose-dependent inhibition of the phosphorylation of AKT was found after the treatment of compound **13g**. Compound **13g** could efficiently suppress the phosphorylation of AKT at the dose of 0.1 μM. These results were in accordance with the results of enzymatic assays, which further demonstrated compound **13g** had significant inhibitory effect on the PI3K/Akt/mTOR pathway. Furthermore, western blot analysis indicated that compound **13g** significantly reduced the protein expression of p-GSK-3β in A549 cells, in a dose-dependent, but total GSK-3β protein level remained unchanged.

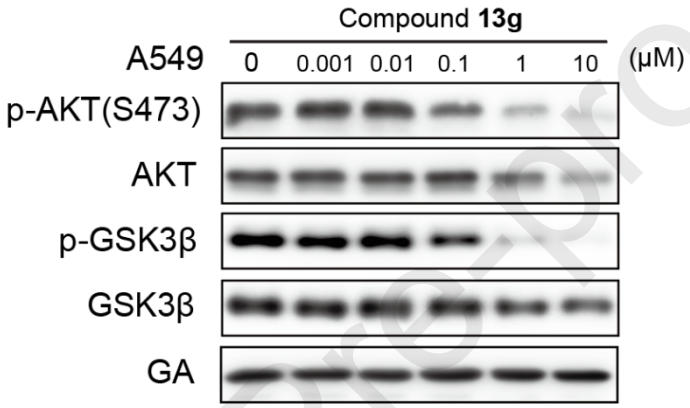


Figure 8. Effects of compound **13g** on the phosphorylation level of AKT in A549 cells after 24 h exposure.

2.10. Molecular docking study

In order to explore the binding modes of target compounds with the active site of PI3Kα (PDB code: 4L23) and mTOR (PDB code: 4JT6), molecular docking simulation studies were performed by AutoDock 4.2 software (The Scripps Research Institute, USA) and the docking results were processed and modified in PyMOL 1.8.x software (https://pymol.org). According to the above test results, the representative compound **13g** as ligand examples was selected, as the best PI3Kα/mTOR inhibitor in this study. As shown in **Figure 9**, the binding mode of **13g** to PI3Kα was very similar to that of GDC-0941, which was responsible for similar potency against PI3Kα. The orientation of the whole molecular skeleton of compound **13g** into hydrophobic pocket of PI3Kα protein was basically the same as that of GDC-0941 and the morpholine formed a key hydrogen bond with Val851 (Val2240 on mTOR) in the hinge region with the hydrogen bond lengths of 1.9 Å (1.8 Å, mTOR). The amino group at the 2-aminopyrimidine engaged in another hydrogen bond with Asp810 and Lys802 (Tyr2225 and Asp2195 on mTOR) with the hydrogen bond lengths of 2.5 Å and 2.6 Å, respectively, which was the reason why the three series of compounds **13a-13h** were much more active than the other series of compounds **11a-11h** and **12a-12h** against the three cancer cell lines. Furthermore, 1-(methylsulfonyl)piperazine group of compound **13g** was preferred

toward the solvent front region of PI3Kα and an additional hydrogen bond was formed between the oxygen of 1-(methylsulfonyl)piperazine group and the residue GLN859 with the hydrogen bond lengths of 1.8 Å, which verifies our conjecture above. In general, these results of the molecular docking study showed that 2-(thiophen-2-yl)-1,3,5-triazine derivatives bearing 1-(methylsulfonyl)piperazine group and 2-aminopyrimidine group could act synergistically to interact with the active binding site of PI3Kα and mTOR, which claimed that compound **13g** may be a potential PI3K/mTOR dual inhibitors. Additionally, based on the results of structure-activity relationships analysis and molecular docking study, more potential PI3K/mTOR dual inhibitors may allow to be designed rationally.

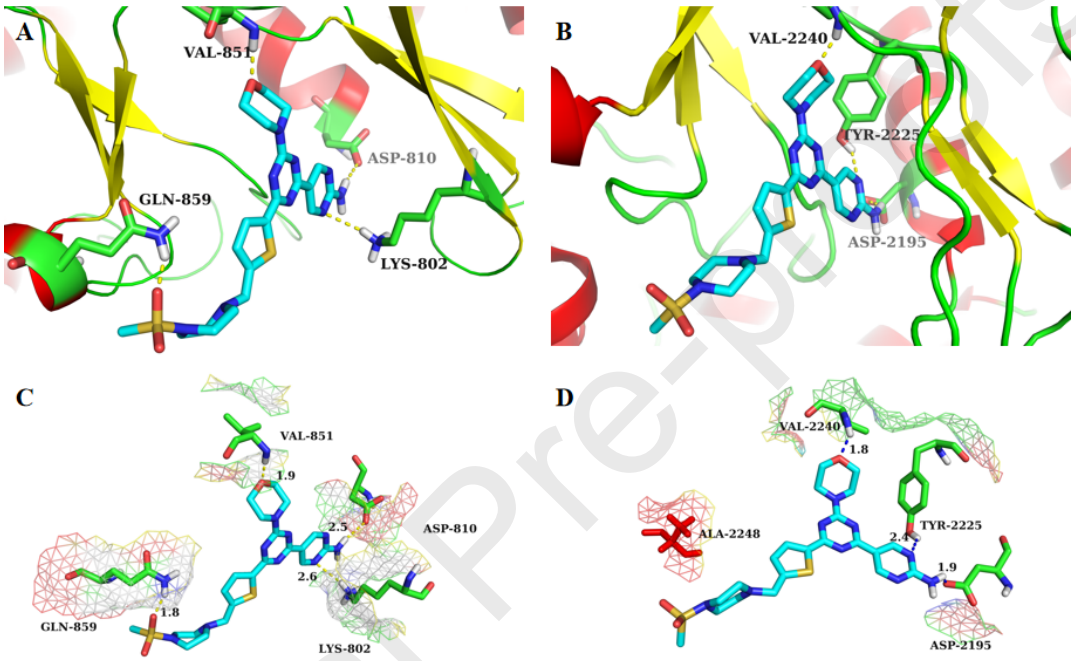


Figure 9. Docking mode of compound **13g**.
(A) (C) The binding model of representative compound **13g** bound to PI3Kα (PDB code: 4L23).
(B) (D) The binding model of representative compound **13g** bound to mTOR (PDB code: 4JT6).

3. Conclusions

In summary, based on the class I PI3K inhibitor GDC-0941, a new series of substituted 2-(thiophen-2-yl)-1,3,5-triazine derivatives as potent PI3Kα/mTOR inhibitors were developed and evaluated them for the IC_{50} values against three cancer cell lines (A549, MCF-7 and Hela). Most of the compounds showed moderate to excellent anti-tumor activity against the different cancer cells. Notably, compound **13g** showed the best activity against A549, MCF-7 and Hela cancer cell lines with IC_{50} values of 0.20±0.05 μM, 1.25±0.11 μM and 1.03±0.24 μM. According to the result of enzymatic activity assay and western blot, compound **13g** was identified as novel PI3Kα/mTOR dual inhibitors, which had an approximately 10-fold improvement in mTOR inhibition relative to the class I PI3K inhibitor Pictilisib (GDC-0941). Moreover, compound **13g** could stimulate A549 cells arrest at G0/G1 phase and induced apoptosis at a low concentration. By far, the existing data indicated that compound **13g** may become a potential PI3Kα/mTOR dual inhibitor for oncology indications.

4. Experimental section

4.1. Chemistry

Unless otherwise required, all reagents were obtained from commercial analytical grade and were used without further purification. All melting points were obtained on a Büchi Melting Point B-540 apparatus (Büchi Labortechnik, Flawil, Switzerland) and were uncorrected. TLC analysis was carried out on silica gel plates GF254 (Qindao Haiyang Chemical, China) with fluorescent indicator 254 nm. Column chromatography was run on silica gel (200-300 mesh) from Qingdao Ocean Chemicals (Qingdao, Shandong, China). Mass spectrometry (MS) was performed on Waters High Resolution Quadrupole Time of Flight Tandem Mass Spectrometry (QTOF). The purity of the compound was determined by Agilent 1260 liquid chromatograph fitted with an Inertex-C18 column. ^{1}HNMR and ^{13}CNMR spectra were recorded on Bruker ARX-400, 400 MHz spectrometers (Bruker Bioscience, Billerica, MA, USA) with tetramethylsilane (TMS) as an internal standard.

4.2. *Preparation of 5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)thiophene-2-carbaldehyde (**3a**) and 3-methyl-5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)thiophene-2-carbaldehyde (**3b**)*

Compound **3a** and **3b** were synthesized according to the procedures in previous research [15], and yields are 76.9% and 68.3%, respectively.

4.3. *Preparation of 4-(4,6-dichloro-1,3,5-triazin-2-yl)morpholine (**5**)*

To a stirred solution of cyanuric chloride (6.5 g, 35 mmol) in acetone (80 mL), a solution of morpholine (2.0 g, 23 mmol) in acetone (20 mL) was added dropwise at 0 ºC. The resulting mixture was quenched with water, filtered, washed with water and dried to get compound **5** (4.3 g, 79.6%).

4.4. *Preparation of 5-(4-chloro-6-morpholino-1,3,5-triazin-2-yl)thiophene-2-carbaldehyde (**6a**)*

Compound **3a** (3.0 g, 12.6 mmol) was combined with bis(triphenylphosphine)palladium(II)chloride (0.21 g) and compound **5** (3.0 g, 12.7 mmol) in 1,2-dimethoxyethane (100 mL) and 25 mL of 1 M potassium carbonate in water. The reaction mixture was heated to 75 ºC for 8 h and monitored by TLC. After cooling to room temperature, the reaction was quenched with saturated aqueous NaCl and then extracted with DCM. The combined organic layer was dried over anhydrous Na_2SO_4 and concentrated in reduced pressure to obtain the crude product. The crude produc was purified through a column chromatography on silica with petroleum ether/ethyl acetate as eluent to obtain **6a** as yellow solid in 75.9% yield.

4.5. *Preparation of 5-(4-chloro-6-morpholino-1,3,5-triazin-2-yl)-3-methylthiophene-2-carbaldehyde (**6b**)*

The synthetic method of compound **6b** was the same to compound **6a**. Compound **6b** was obtained as yellow solid in 81.7% yield.

4.6. *Preparation of (5-(4-chloro-6-morpholino-1,3,5-triazin-2-yl)thiophen-2-yl)methanol (**7a**)*

To a solution of compound **6a** (1 g, 3.2 mmol) in methanol (10 mL) was added sodium borohydride (0.4 g, 10.5 mmol) in small portions at 0 and the reaction was stirred for 1 h. The solvent was evaporated and the residue partitioned between ethyl acetate (20 mL) and 10% ammonium chloride solution (10 mL). The organic layer was washed with water (10 mL), dried over sodium sulfate and evaporated to obtain compound (0.9 g, 90.0%) as yellow solid.

4.7. *Preparation of (5-(4-chloro-6-morpholino-1,3,5-triazin-2-yl)-3-methylthiophen-2-yl)methanol (**7b**)*

The synthetic method of compound **7b** was the same to compound **7a**. Compound **7b** was obtained as yellow solid in 90.0% yield.

4.8. *Preparation of 4-(4-chloro-6-(5-(chloromethyl)thiophen-2-yl)-1,3,5-triazin-2-yl)morpholine (**8a**)*

To a stirred solution of compound **7a** (2.0 g, 6.3 mmol) in CH_2C1_2 (50 mL), thionyl chloride (2.3 mL, 19.3 mmol) and a few drops of DMF were added. The mixture was stirred at room temperature for 4 hours and monitored by TLC. After completion, the reaction was quenched with saturated aqueous NaCl and then extracted with DCM. The combined organic layer was dried over anhydrous Na_2SO_4, filtered and concentrated to get yellow solid in 96.4% yield, and then compound **8a** directly used for further reaction.

4.9. *Preparation of 4-(4-chloro-6-(5-(chloromethyl)-4-methylthiophen-2-yl)-1,3,5-triazin-2-yl)morpholine (**8b**)*

The synthetic method of compound **8b** was the same to compound 8a. Compound **8b** was obtained as yellow solid in 95.7% yield.

4.10. *Preparation of 1-(5-(4-chloro-6-morpholino-1,3,5-triazin-2-yl)thiophen-2-yl)-N,N-dimethylmethanamine (**9a**)*

To a solution of compound **8a** (2.0 g, 0.012 mol), Dimethylamine (0.54 g, 0.012 mol) and K_2CO_3 (2.48 g, 0.018 mol) in isopropyl alcohol (20 mL) were added. The reaction mixture was heated to 55 ºC for 4 h and monitored by TLC. After completion, the reaction was quenched with saturated aqueous NaCl and then extracted with DCM. The combined organic layer was dried over anhydrous Na_2SO_4, filtered and concentrated to get yellow solid in 92.9% yield, and then compound **9a** directly used for further reaction.

4.11. *Preparation of compounds **9b-9h***

The synthetic method of compounds **9b-9h** was the same to compound **9a**. Compounds **9b-9h** as yellow solid in 80.0-95.6% yield.

4.12. *Preparation of 4-(4-chloro-6-(4-methyl-5-(morpholinomethyl)thiophen-2-yl)-1,3,5-triazin-2-yl)morpholine (**10a**)*

To a solution of compound **8b** (2.0 g, 0.012 mol), morpholine (0.54 g, 0.012 mol) and K_2CO_3 (2.48 g, 0.018 mol) in isopropyl alcohol (20 mL) were added. The reaction mixture was heated to 55 ºC for 4 h and monitored by TLC. After

completion, the reaction was quenched with saturated aqueous NaCl and then extracted with DCM. The combined organic layer was dried over anhydrous Na_2SO_4, filtered and concentrated to get yellow solid in 93.7% yield.

4.13. *Preparation of 4-(4-chloro-6-(4-methyl-5-((4-(methylsulfonyl)piperazin-1-yl)methyl)thiophen-2-yl)-1,3,5-triazin-2 -yl)morpholine (**10b**)*

The synthetic method of compound **10b** was the same to compound **10a**. Compound **10b** was obtained as yellow solid in 96.1% yield.

4.14. *General procedure for the preparation of compounds **11a-11h***

Compounds **9a-9h** was combined with bis(triphenylphosphine)palladium(II)chloride (0.21 g) and 1*H*-indazole- 4-boronic acid pinacol ester (1.68 g) in 1,2-dimethoxyethane (20 ml) and 15 mL of 1 M potassium carbonate in water. The reaction mixture was heated to 130 ºC for 4 h and monitored by TLC. After cooling to room temperature, the reaction was quenched with saturated aqueous NaCl and then extracted with DCM. The combined organic layer was dried over anhydrous Na_2SO_4 and concentrated in reduced pressure to obtain the crude product. The crude produc was purified through a column chromatography on silica with dichloromethane/methanol as eluent to produce **11a-11h** as yellow solid.

4.14.1. *1-(5-(4-(1H-indazol-4-yl)-6-morpholino-1,3,5-triazin-2-yl)thiophen-2-yl)-N,N-dimethylmethanamine (**11a**)*

Yellow solid in 40.7% yield. M.P.: 106.9-107.8 ºC. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.07 (s, 1H), 8.44 (d, J = 7.3 Hz, 1H), 8.06 (d, J = 3.6 Hz, 1H), 7.69 (d, J = 8.2 Hz, 1H), 7.54-7.52 (m, 1H), 7.03 (s, 1H), 4.08 (d, J = 15.9 Hz, 4H), 3.84 (d, J = 2.1 Hz, 5H), 3.74 (s, 2H), 2.37 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.04, 168.86, 168.59, 142.00, 138.93, 136.85, 128.40, 125.54 (2, C), 122.26 (2, C), 121.85, 118.65, 118.23, 66.31 (2, C), 56.62, 45.38 (2, C), 44.13 (2, C). TOF MS ES + (m/z): $(M + H)^+$, calcd for $C_{21}H_{23}N_7OS$: 422.1763, found, 422.1752.

4.14.2. *N-((5-(4-(1H-indazol-4-yl)-6-morpholino-1,3,5-triazin-2-yl)thiophen-2-yl)methyl)-N-ethylethanamine (**11b**)*

Yellow solid in 50.5% yield. M.P.: 115.9-117.1 ºC. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.06 (s, 1H), 8.43 (d, J = 7.2 Hz, 1H), 8.05 (d, J = 3.3 Hz, 1H), 7.69 (d, J = 7.9 Hz, 1H), 7.50 (t, J = 7.7 Hz, 1H), 7.02 (s, 1H), 4.06 (s, 4H), 3.90 (s, 2H), 3.83 (s, 4H), 2.65 (dd, J = 14.0, 6.9 Hz, 4H), 1.13 (t, J = 6.9 Hz, 6H). TOF MS ES + (m/z): $(M + H)^+$, calcd for $C_{23}H_{27}N_7OS$: 450.2076, found, 450.2060.

4.14.3. *4-(4-(1H-indazol-4-yl)-6-(5-(pyrrolidin-1-ylmethyl)thiophen-2-yl)-1,3,5-triazin-2-yl)morpholine (**11c**)*

Yellow solid in 30.9% yield. M.P.: 128.5-131.3 ºC. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.03 (s, 1H), 8.42 (s, 1H), 8.03 (s, 1H), 7.72 (s, 1H), 7.52 (s, 1H), 7.30 (s, 1H), 4.20 (s, 2H), 4.06 (s, 4H), 3.84 (s, 4H), 3.04 (s, 4H), 1.99 (s, 4H). TOF MS ES + (m/z): $(M + H)^+$, calcd for $C_{23}H_{25}N_7OS$: 448.1920, found, 448.1919.

4.14.4. *4-(4-(1H-indazol-4-yl)-6-(5-(piperidin-1-ylmethyl)thiophen-2-yl)-1,3,5-triazin-2-yl)morpholine (**11d**)*

Yellow solid in 61.2% yield. M.P.: 162.1-163.4 ºC. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.06 (s, 1H), 8.44 (s, 1H), 8.06 (s, 1H), 7.71 (s, 1H), 7.52 (s, 1H), 7.10 (s, 1H), 4.08 (s, 4H), 3.85 (s, 6H), 2.61 (s, 4H), 1.71 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.43, 166.62, 164.14, 140.25 (2, C), 136.16, 129.63, 126.42 (2, C), 125.67 (2, C), 122.59, 121.12, 112.73, 66.07 (2, C), 56.86, 53.18 (2, C), 43.16 (2, C), 28.95 (2, C), 24.59. TOF MS ES + (m/z): $(M + H)^+$, calcd for $C_{24}H_{27}N_7OS$: 462.2076, found, 462.2070.

4.14.5. *4-(4-(1H-indazol-4-yl)-6-(5-(morpholinomethyl)thiophen-2-yl)-1,3,5-triazin-2-yl)morpholine (**11e**)*

Light yellow solid in 45.6% yield. M.P.: 110.6-120.3 ºC. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.09 (s, 1H), 8.45 (s, 1H), 8.05 (s, 1H), 7.71 (s, 1H), 7.54 (s, 1H), 7.05 (s, 1H), 4.08 (s, 4H), 3.82 (d, J = 20.0 Hz, 12H), 2.59 (s, 4H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.45, 165.70, 164.30, 140.44, 139.41, 137.58, 130.33, 126.35 (2, C), 125.93 (2, C), 123.24, 121.79, 120.22, 66.80 (2, C), 60.45 (2, C), 58.46, 52.21 (2, C), 43.83 (2, C). TOF MS ES + (m/z): $(M + H)^+$, calcd for $C_{23}H_{25}N_7O_2S$: 464.1869, found, 464.1870.

4.14.6. *4-(4-(1H-indazol-4-yl)-6-(5-((4-methylpiperazin-1-yl)methyl)thiophen-2-yl)-1,3,5-triazin-2-yl)morpholine (**11f**)*

Light yellow solid in 59.3% yield. M.P.: 127.6-128.3 ºC. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.07 (s, 1H), 8.44 (d, J = 7.3 Hz, 1H), 8.04 (d, J = 3.7 Hz, 1H), 7.68 (d, J = 8.2 Hz, 1H), 7.51 (t, J = 7.8 Hz, 1H), 7.00 (d, J = 3.6 Hz, 1H), 4.07 (s, 4H), 3.85 (d, J = 4.5 Hz, 4H), 3.77 (s, 2H), 2.57 (s, 8H), 2.33 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.14, 166.45, 163.84, 140.91, 139.97, 135.83, 129.23, 126.13 (2, C), 125.34 (2, C), 123.34, 122.26, 120.84, 65.80 (2, C), 56.60, 53.88 (2, C), 51.70 (2, C), 44.70 (2, C), 42.86. TOF MS ES + (m/z): $(M + H)^+$, calcd for $C_{24}H_{28}N_8OS$: 477.2185, found, 477.2180.

4.14.7. *4-(4-(1H-indazol-4-yl)-6-(5-((4-(methylsulfonyl)piperazin-1-yl)methyl)thiophen-2-yl)-1,3,5-triazin-2-yl)morpholine (**11g**)*

Yellow solid in 63.2% yield. M.P.: 124.8-125.5 ºC. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.08 (s, 1H), 8.44 (d, J = 7.4 Hz, 1H), 8.03 (d, J = 3.5 Hz, 1H), 7.69 (d, J = 8.0 Hz, 1H), 7.52 (dd, J = 8.2, 4.6 Hz, 1H), 7.00 (s, 1H), 4.07 (s, 4H), 3.84-3.80 (m, 6H), 3.29 (s, 4H), 2.79 (s, 3H), 2.66 (s, 4H). TOF MS ES + (m/z): $(M + H)^+$, calcd for $C_{24}H_{28}N_8O_3S2$: 541.1804, found, 541.1808.

4.14.8. *N^1-((5-(4-(1H-indazol-4-yl)-6-morpholino-1,3,5-triazin-2-yl)thiophen-2-yl)methyl)-N^1,N^2,N^2-trimethylethane-1, 2-diamine (**11h**)*

Yellow solid in 48.7% yield. M.P.: 111.3-113.2 ºC. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.04 (s, 1H), 8.41 (s, 1H), 7.99 (s, 1H), 7.72 (s, 1H), 7.49 (s, 1H), 6.99 (s, 1H), 4.04 (s, 4H), 3.83 (s, 8H), 2.88 (d, J = 33.5 Hz, 4H), 2.66 (s, 6H), 2.34 (s, 3H). TOF MS ES + (m/z): $(M + H)^+$, calcd for $C_{24}H_{30}N_8OS$: 479.2342, found, 479.2351.

4.15. *General procedure for the synthesis of compounds **12a-12h** and **13a-13h***

The synthetic method of compounds **12a-12h** and **13a-13h** were similar to compounds **11a-11h**. The difference was that we needed to replace 1*H*-indazole-4-boronic acid pinacol ester with 3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan- 2-yl)phenol and 2-aminopyrimidine-5-boronic acid pinacol ester and the rest of the synthesis conditions were the same.

4.15.1. *3-(4-(5-((dimethylamino)methyl)thiophen-2-yl)-6-morpholino-1,3,5-triazin-2-yl)phenol (**12a**)*

Light yellow solid in 72.5% yield. M.P.: 133.5-134.7 ºC. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.14–7.89 (m, 3H), 7.36 (d, J = 6.9 Hz, 1H), 7.03 (d, J = 18.2 Hz, 2H), 4.00 (s, 4H), 3.84 (d, J = 18.8 Hz, 6H), 2.42 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) 170.18, 166.71, 164.22, 156.02, 141.83, 137.50, 129.60, 129.03, 127.39 (2, C), 119.99, 118.81, 114.92, 66.28 (2, C), 57.55, 43.98 (2, C), 43.17 (2, C). TOF MS ES + (m/z): $(M + H)^+$, calcd for $C_{20}H_{23}N_5O_2S$: 398.1651, found, 398.1652.

4.15.2. *3-(4-(5-((diethylamino)methyl)thiophen-2-yl)-6-morpholino-1,3,5-triazin-2-yl)phenol (**12b**)*

Light yellow solid in 57.2% yield. M.P.: 116.4-117.7 ºC. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.87-7.85 (m, 3H), 7.17 (d, J = 8.0 Hz, 1H), 6.83 (d, J = 12.6 Hz, 2H), 3.82 (s, 6H), 3.64 (s, 4H), 2.51 (d, J = 6.2 Hz, 4H), 1.01–0.98 (m, 6H). TOF MS ES + (m/z): $(M + H)^+$, calcd for $C_{22}H_{27}N_5O_2S$: 426.1964 , found, 426.1958.

4.15.3. *3-(4-morpholino-6-(5-(pyrrolidin-1-ylmethyl)thiophen-2-yl)-1,3,5-triazin-2-yl)phenol (**12c**)*

Light yellow solid in 56.8% yield. M.P.: 134.5-135.7 ºC. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.02 (d, J = 7.7 Hz, 1H), 7.91 (d, J = 10.9 Hz, 2H), 7.33 (d, J = 7.4 Hz, 1H), 7.02 (d, J = 13.6 Hz, 2H), 3.98 (s, 6H), 3.80 (s, 4H), 2.76 (s, 4H), 1.87 (s, 4H). TOF MS ES + (m/z): $(M + H)^+$, calcd for $C_{22}H_{25}N_5O_2S$: 424.1807, found, 424.1808.

4.15.4. *3-(4-morpholino-6-(5-(piperidin-1-ylmethyl)thiophen-2-yl)-1,3,5-triazin-2-yl)phenol (**12d**)*

Light yellow solid in 54.7% yield. M.P.: 145.5-146.3 ºC. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.01 (d, J = 7.6 Hz, 1H), 7.93 (d, J = 3.4 Hz, 2H), 7.35 (t, J = 7.7 Hz, 1H), 7.05 (d, J = 7.1 Hz, 1H), 6.97 (s, 1H), 3.97 (d, J = 20.2 Hz, 6H), 3.82 (s, 4H), 2.66 (s, 4H), 1.70 (s, 4H), 1.45 (s, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.20, 166.67, 164.20, 156.31, 141.83, 137.33, 129.60, 129.08, 128.06 (2, C), 119.66, 119.05, 114.87, 66.31 (2, C), 57.02, 52.97 (2, C), 43.15 (2, C), 24.93 (2, C), 23.32. TOF MS ES + (m/z): $(M + H)^+$, calcd for $C_{23}H_{27}N_5O_2S$: 438.1964, found, 438.1958.

4.15.5. *3-(4-morpholino-6-(5-(morpholinomethyl)thiophen-2-yl)-1,3,5-triazin-2-yl)phenol (**12e**)*

Light yellow solid in 65.7% yield. M.P.: 120.5-122.8 ºC. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.16 (d, J = 7.8 Hz, 1H), 8.08 (d, J = 3.5 Hz, 2H), 7.46-7.43 (m, 1H), 7.14-7.11 (m, 2H), 4.12 (s, 4H), 3.90 (s, 10H), 2.70 (s, 4H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.58, 168.57, 164.73, 157.37, 141.27, 138.20, 130.73, 130.20, 129.59 (2, C), 120.96, 115.26, 117.61, 66.92 (2, C), 66.81 (2, C), 58.50, 53.40 (2, C), 43.69 (2, C). TOF MS ES + (m/z): $(M + H)^+$, calcd for $C_{22}H_{25}N_5O_3S$: 440.1756, found, 440.1761.

4.15.6. *3-(4-(5-((4-methylpiperazin-1-yl)methyl)thiophen-2-yl)-6-morpholino-1,3,5-triazin-2-yl)phenol (**12f**)*

Light yellow solid in 63.8% yield. M.P.: 220.5-221.3 ºC. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.98 (s, 1H), 7.89 (d, J = 8.8 Hz, 2H), 7.34 (s, 1H), 7.09 (s, 1H), 7.00 (s, 1H), 3.94 (s, 4H), 3.73 (s, 6H), 2.41 (d, J = 39.0 Hz, 8H), 2.17 (s, 3H). TOF MS ES + (m/z): $(M + H)^+$, calcd for $C_{23}H_{28}N_6O_2S$: 453.2073, found, 453.2075.

4.15.7. *3-(4-(5-((4-(methylsulfonyl)piperazin-1-yl)methyl)thiophen-2-yl)-6-morpholino-1,3,5-triazin-2-yl)phenol* ***(12g)***

Light yellow solid in 70.8% yield. M.P.: 156.3-157.7 ºC. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.04 (d, J = 19.5 Hz, 1H), 7.89 (d, J = 7.6 Hz, 2H), 7.34 (t, J = 7.7 Hz, 1H), 7.14 (s, 1H), 7.00 (d, J = 7.9 H z, 1H), 3.93 (d, J = 13.3 Hz, 4H), 3.73 (s, 6H), 3.47 (d, 4H), 3.15 (s, 3H), 2.90 (d, 4H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 171.61, 168.23, 165.68, 159.05, 141.94, 138.74, 131.91, 131.06, 128.67 (2, C), 120.84, 120.64, 116.40, 67.44 (2, C), 58.28, 56.17 (2, C), 53.93 (4, C), 44.92. TOF MS ES + (m/z): $(M + H)^+$, calcd for $C_{23}H_{28}N_6O_4S_2$: 517.1692, found, 517.1690.

4.15.8. *3-(4-(5-(((2-(dimethylamino)ethyl)(methyl)amino)methyl)thiophen-2-yl)-6-morpholino-1,3,5-triazin-2-yl)phenol* ***(12h)***

Yellow solid in 26.8% yield. M.P.: 121.9-213.4 ºC. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.10-7.91 (m, 3H), 7.36 (d, J = 6.9 Hz, 1H), 7.03 (d, J = 18.2 Hz, 2H), 3.92 (s, 6H), 3.71 (s, 4H), 2.75 (s, 4H), 2.67 (s, 6H), 2.35 (s, 3H). TOF MS ES + (m/z): $(M + H)^+$, calcd for $C_{23}H_{30}N_6O_2S$: 445.2229, found, 445.2215.

4.15.9. *5-(4-(5-((dimethylamino)methyl)thiophen-2-yl)-6-morpholino-1,3,5-triazin-2-yl)pyrimidin-2-amine* ***(13a)***

Yellow solid in 47.8% yield. M.P.: 115.3-116.9 ºC. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.31 (s, 2H), 7.99 (s, 1H), 7.03 (s, 1H), 5.48 (s, 2H), 4.00 (s, 4H), 3.81 (s, 4H), 3.76 (s, 2H), 2.38 (s, 6H). TOF MS ES + (m/z): $(M + H)^+$, calcd for $C_{18}H_{22}N_8OS$: 399.1716, found, 399.1719.

4.15.10. *5-(4-(5-((diethylamino)methyl)thiophen-2-yl)-6-morpholino-1,3,5-triazin-2-yl)pyrimidin-2-amine* ***(13b)***

Yellow solid in 36.6% yield. M.P.: 127.5-128.8 ºC. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.33 (s, 2H), 8.01 (s, 1H), 6.99 (s, 1H), 5.49 (s, 2H), 4.02 (s, 4H), 3.85 (d, J = 18.3 Hz, 6H), 2.64 (s, 4H), 1.71 (s, 6H). TOF MS ES + (m/z): $(M + H)^+$, calcd for $C_{20}H_{26}N_8OS$: 427.2029, found, 427.2035.

4.15.11. *5-(4-morpholino-6-(5-(pyrrolidin-1-ylmethyl)thiophen-2-yl)-1,3,5-triazin-2-yl)pyrimidin-2-amine* ***(13c)***

Yellow solid in 66.7% yield. M.P.: 166.3-167.5 ºC. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.29 (s, 2H), 7.97 (s, 1H), 6.97 (s, 1H), 5.57 (s, 2H), 3.99 (s, 4H), 3.83 (d, J = 22.4 Hz, 6H), 2.60 (s, 4H), 1.25 (s, 4H).^{13}C NMR (100 MHz, $CDCl_3$) δ 168.30, 167.26, 164.33, 159.45, 149.86 (2, C), 141.13 (2, C), 130.36, 126.28, 120.62, 66.77 (2, C), 55.18, 54.01 (2, C), 43.69 (2, C), 23.60 (2, C). TOF MS ES + (m/z): $(M + H)^+$, calcd for $C_{20}H_{24}N_8OS$:425.1872, found, 425.1866.

4.15.12. *5-(4-morpholino-6-(5-(piperidin-1-ylmethyl)thiophen-2-yl)-1,3,5-triazin-2-yl)pyrimidin-2-amine* ***(13d)***

Light yellow solid in 48.9% yield. M.P.: 250.8-251.5 ºC. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.32 (s, 2H), 7.99 (s, 1H),

6.98 (s, 1H), 5.42 (s, 2H), 4.01 (s, 4H), 3.82 (s, 4H), 3.76 (s, 2H), 2.50 (s, 4H), 1.27 (s, 6H). TOF MS ES + (m/z): $(M + H)^+$, calcd for, $C_{21}H_{26}N_8OS$: 439.2029, found, 439.2019.

4.15.13. *5-(4-morpholino-6-(5-(morpholinomethyl)thiophen-2-yl)-1,3,5-triazin-2-yl)pyrimidin-2-amine (**13e**)*

Light yellow solid in 35.9% yield. M.P.: 270.2-271.4 ºC. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.19 (s, 2H), 8.00 (s, 1H), 7.00 (s, 1H), 5.38 (s, 2H), 3.83 (d, J = 45.6 Hz, 14H), 1.27 (s, 4H). TOF MS ES + (m/z): $(M + H)^+$, calcd for $C_{20}H_{24}N_8O_2S$: 441.1821, found, 441.1813.

4.15.14. *5-(4-(5-((4-methylpiperazin-1-yl)methyl)thiophen-2-yl)-6-morpholino-1,3,5-triazin-2-yl)pyrimidin-2-amine (**13f**)*

Yellow solid in 63.2% yield. M.P.: 210.3-211.9 ºC. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.30 (s, 2H), 7.97 (d, J = 3.6 Hz, 1H), 6.98 (s, 1H), 5.62 (s, 2H), 4.00 (s, 4H), 3.84-3.74 (m, 6H), 2.59 (s, 8H), 2.35 (s, 3H). TOF MS ES + (m/z): $(M + H)^+$, calcd for $C_{21}H_{27}N_9OS$: 454.2138, found, 454.2142.

4.15.15. *5-(4-(5-((4-(methylsulfonyl)piperazin-1-yl)methyl)thiophen-2-yl)-6-morpholino-1,3,5-triazin-2-yl)pyrimidin-2-amine (**13g**)*

Yellow solid in 30.7% yield. M.P.: 261.1-262.4 ºC. ^{1}H NMR (400 MHz, CDCl3) δ 9.32 (s, 2H), 7.96 (s, 1H), 6.89 (s, 1H),5.48 (s, 2H), 4.03-4.00 (m, 4H), 3.84-3.82 (m, 4H), 3.73 (s, 2H), 3.31 (s, 4H), 2.83 (s, 3H), 2.68 (s, 4H). ^{13}C NMR (100MHz, $CDCl_3$) δ 168.40 (2, C), 165.49, 160.64, 149.79 (2, C), 142.76 (2, C), 131.59, 128.19, 121.72, 67.96 (2, C), 58.76, 56.08 (2, C), 53.78 (2, C), 46.84 (2, C), 44.89. TOF MS ES + (m/z): $(M + H)^+$, calcd for $C_{21}H_{27}N_9O_3S_2$: 518.1757, found, 518.1761.

4.15.16. *N^1-((5-(4-(2-aminopyrimidin-5-yl)-6-morpholino-1,3,5-triazin-2-yl)thiophen-2-yl)methyl)-N^1,N^2,N^2-trimethyl-thane-1,2-diamine (**13h**)*

Yellow solid in 49.8% yield . M.P.: 121.3-122.8 ºC. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.31 (s, 2H), 7.99 (d, J = 3.4 Hz, 1H), 7.00 (d, J = 3.2 Hz, 1H), 5.63 (s, 2H), 4.01 (s, 4H), 3.83 (s, 6H), 2.71 (s, 4H), 2.45 (s, 6H), 2.36 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 167.22, 166.20, 163.35, 158.45, 147.58 (2, C), 140.55 (2, C), 129.40, 126.09, 119.56, 65.77 (2, C), 56.20, 55.80, 53.03, 44.20 (2, C), 42.72 (2, C), 41.34. TOF MS ES+ (m/z): $(M + H)^+$, calcd for $C_{21}H_{29}N_9OS$: 456.2294, found, 456.2284.

4.16. *General procedure for the synthesis of compounds **14a-14b**, **15a-15b** and **16a-16b***

The synthetic method of compounds **14a-14b**, **15a-15b** and **16a-16b** were similar to compounds **11a-11h**. The difference was that we needed to replace compounds **8a** with compounds **8b** and and the rest of the synthesis conditions were the same.

4.16.1. *4-(4-(1H-indazol-4-yl)-6-(4-methyl-5-((4-(methylsulfonyl)piperazin-1-yl)methyl)thiophen-2-yl)-1,3,5-triazin-2-*

*yl)morpholine **(14a)***

Light yellow solid in 64.1% yield. M.P.: 132.4-133.5 ºC. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.10 (s, 1H), 8.47 (s, 1H), 7.95 (s, 1H), 7.70 (s, 1H), 7.54 (s, 1H), 4.09 (s, 6H), 3.86 (s, 4H),3.31 (s, 4H), 2.83 (s, 3H), 2.69 (s, 4H), 2.32 (s, 3H). TOF MS ES + (m/z): $(M + H)^+$, calcd for $C_{24}H_{27}N_7O_2S$: 478.2025, found, 478.2028.

4.16.2. *4-(4-(1H-indazol-4-yl)-6-(4-methyl-5-(morpholinomethyl)thiophen-2-yl)-1,3,5-triazin-2-yl)morpholine **(14b)***

Yellow solid in 43.8% yield. M.P.: 198.9-199.7 ºC. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.09 (s, 1H), 8.44 (s, 1H), 7.94 (d, J = 14.2 Hz, 1H), 7.73 (s, 1H), 7.53 (s, 1H), 4.09 (s, 4H), 3.81 (d, J = 36.2 Hz, 8H), 3.68 (s, 2H), 2.57 (s, 4H), 2.28 (s, 3H). TOF MS ES + (m/z): $(M + H)^+$, calcd for $C_{25}H_{30}N_8O_3S_2$: 555.1961, found, 555.1974.

4.16.3. *3-(4-(4-methyl-5-((4-(methylsulfonyl)piperazin-1-yl)methyl)thiophen-2-yl)-6-morpholino-1,3,5-triazin-2-yl)phenol **(15a)***

Light yellow solid in 53.4% yield. M.P.: 125.4-127.1 ºC. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.08 (s, 1H), 7.98 (s, 1H), 7.87 (s, 1H), 7.35 (s, 1H), 7.02 (s, 1H), 4.03 (s, 4H), 3.81 (s, 4H), 3.72 (s, 2H), 3.30 (s, 4H), 2.79 (s, 3H), 2.67 (s, 4H), 2.23 (s, 3H). TOF MS ES + (m/z): $(M + H)^+$, calcd for $C_{23}H_{27}N_5O_3S$: 454.1913, found, 454.1902.

5.16.4. *3-(4-(4-methyl-5-(morpholinomethyl)thiophen-2-yl)-6-morpholino-1,3,5-triazin-2-yl)phenol **(15b)***

Light yellow solid in 46.3% yield. M.P.: 232.7-233.5 ºC. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.01 (s, 2H), 7.89 (s, 1H), 7.35 (s, 1H), 7.04 (s, 1H), 4.02 (s, 8H), 3.81 (s, 4H), 2.30 (d, J = 29.0 Hz, 4H), 1.25 (s, 3H). TOF MS ES + (m/z): $(M + H)^+$, calcd for $C_{24}H_{30}N_6O_4S_2$: 531.1848, found, 531.1853.

4.16.5. *5-(4-(4-methyl-5-((4-(methylsulfonyl)piperazin-1-yl)methyl)thiophen-2-yl)-6-morpholino-1,3,5-triazin-2-yl)pyrimidin-2-amine **(16a)***

Light yellow solid in 60.1% yield. M.P.: 266.1-268.2 ºC. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.32 (s, 2H), 7.86 (s, 1H), 5.48 (s, 2H), 4.02-3.99 (m, 4H), 3.83-3.80 (m, 4H), 3.71 (s, 2H), 3.35 (s, 4H), 2.79 (s, 3H), 2.70 (s, 4H), 2.25 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.18, 167.14, 164.31, 159.53, 141.73 (2, C), 139.60, 136.15 (2, C), 133.47, 120.58, 66.83 (2, C), 55.20, 52.40 (2, C), 46.02 (4, C), 43.71, 14.10. TOF MS ES + (m/z): $(M + H)^+$, calcd for $C_{21}H_{26}N_8O_2S$: 455.1978, found, 455.1970.

4.16.6. *5-(4-(4-methyl-5-((4-(methylsulfonyl)piperazin-1-yl)methyl)thiophen-2-yl)-6-morpholino-1,3,5-triazin-2-yl)pyrimidin-2-amine **(16b)***

Light yellow solid in 39.1% yield. M.P.: 279.4-280.6 ºC. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.32 (s, 2H), 7.86 (s, 1H), 5.49 (s, 2H), 4.02 (s, 4H), 3.81 (d, J = 25.0 Hz, 8H), 3.67 (s, 2H), 2.57 (s, 4H), 2.26 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 168.16, 167.21, 164.28, 159.45, 142.02 (2, C), 139.30, 136.00 (2, C), 133.39, 120.73, 67.02 (2, C), 66.77 (2, C), 55.90,

53.64 (2, C), 43.69 (2, C), 14.00. TOF MS ES + (m/z): $(M + H)^+$, calcd for $C_{22}H_{29}N_9O_3S_2$: 532.1913, found, 532.1901.

4.17. Anti-tumor assay

The anti-tumor activities of target compounds (**11a-11h**, **12a-12h**, **13a-13h**, **14a-14b**, **15a-15b** and **16a-16b**) were evaluated with A549, MCF-7 and Hela cell lines by the standard MTT assay *in vitro*, with GDC-0941 as positive control. Specific operations according to our previous research [16].

4.18. Kinase selectivity assay

The target compounds (**11c**, **11e**, **11g**, **12c**, **12e**, **12g**, **13c**, **13e** and **13g**) are tested for their activity against PI3Kα and mTOR through the mobility shift assay, with GDC-0941 and PI-103 as positive controls. Specific operations according to our previous research [17].

4.19. Cell cycle progression assay

A549 cells were seeded in 6-well plates at a density of 1×10^6 cells and grown for 24h at 37 ºC in 5% CO_2, and then treated with GDC-0941 and compound **13g** for 48 h. Specific operations according to our previous research [18].

4.20. Cell apoptosis assay

A549 cells were seeded in a 6-well plates at 1×10^5 cells per well and incubated for 24 h, and treated with compound **13g** for 48 h. Specific operations according to our previous research [19].

4.21. Western blot analysis

A549 cells were treated with compound **13g** at different concentrations of 0.001 μM, 0.01 μM, 0.1 μM, 1 μM and 10 μM for 24 h, and then the protein of A549 cells were extracted by RIPA lysis buffer. The concentration of the cell lysates were measured by BCA protein Assay (California, USA). The Equal amount of protein was subjected to 10% SDS polyacrylamide gel electrophoresis and then transferred to PVDF membrane. Next, the PVDF membranes were incubated with various protein of target proteins (p-AKT (S473), AKT, p-GSK-3β, GSK-3β, GAPDH). Finally, exposure development was carried out with film in a dark room. The density of the bands were analyzed by Image Lab software.

4.22. Acridine orange (AO) single staining

The cancer cell apoptotic of target compound **13g** was evaluated with A549 cells by acridine orange single staining. Specific operations according to our previous research [20].

4.23. Molecular docking study

The co-crystal structure of PI3Kα in complex with PI-103 (PDB ID code: 4L23) and the co-crystal structure of mTOR in complex with PI-103 (PDB ID code: 4JT6) were chosen as the template to generate the docking modes. For the preparation of ligands, the 3D structures were generated and their energy minimizations were performed by AutoDock 4.2 software (The Scripps Research Institute, USA). The whole PI3Kα and mTOR enzymes were defined as a receptor and the site sphere were selected based on the PI-103 binding location of PI3Kα and mTOR, after that the molecule PI-103 was removed and **13g** was placed during the molecular docking procedure. Types of interactions of the docked PI3Kα and mTOR with ligand were analyzed and then the docking conformations were selected and saved based on calculated energy. All the docking results were processed and modified in PyMOL 1.8.x software (https: // pymol. org).

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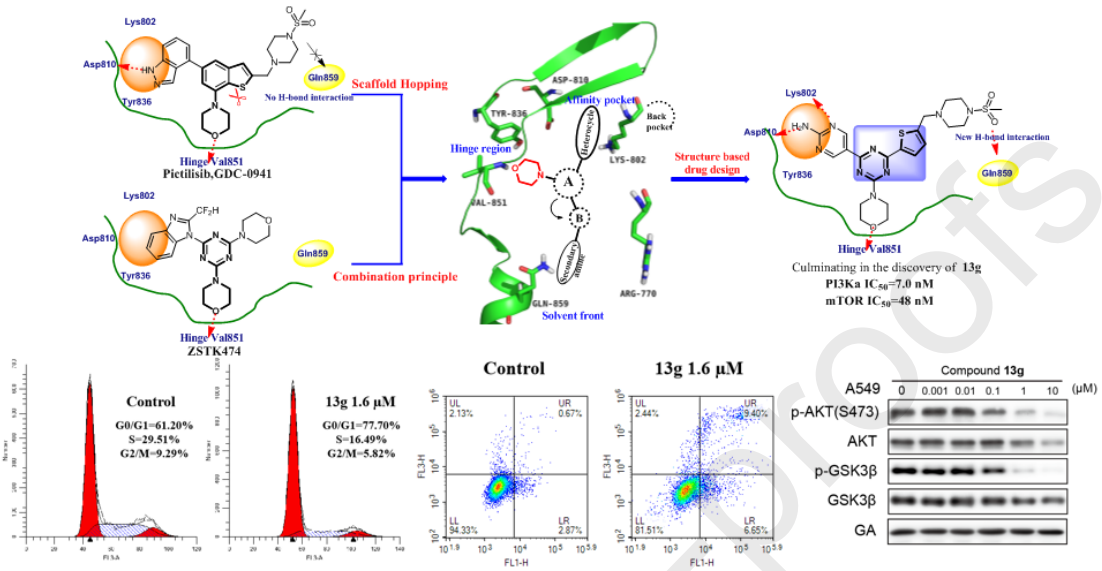
Graphical abstract



The preparation and biological evaluation of a series of substituted 2-(thiophen-2-yl)-1,3,5-triazine derivatives were disclosed, which demonstrated potent inhibition of the PI3K/AKT/mTOR pathway, culminating in the discovery of **13g**.

Research Highlights

- A new series of substituted 2-(thiophen-2-yl)-1,3,5-triazine derivatives as potent PI3Kα/mTOR dual inhibitors were discovered and developed.
- Most of the synthesized compounds showed moderate to significant antitumor activity.
- Compound **13g** showed excellent inhibitory activity to PI3Kα and mTOR kinases with the IC_{50} values of 7.0 nM and 48 nM, respectively.
- Compound **13g** had an approximately 10-fold improvement in mTOR inhibition (from 525 nM to 48 nM) relative to GDC-0941.
- Compound **13g** could stimulate A549 cells arrest at G0/G1 phase, in a dose-dependent manner, and induced apoptosis at a low concentration.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

none