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Design, synthesis and biological evaluation of novel 2,4-diaminopyrimidine derivatives as potent antitumor agents

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Abstract

For developing novel therapeutic agents with anticancer activities, two series of novel 2,4-diaminopyrimidine derivatives possessing triazolopiperazine or 1,4,8-triazaspiro[4.5]decan-3-one scaffolds were designed and synthesized. The preliminary investigation showed that some compounds displayed moderate to excellent potency against four tested cancer cell lines as compared with palbociclib and momelotinib. In particular, the most promising compounds **9k** and **13f** showed the most potent antitumor activities with IC_{50} values of 2.14/1.98 μM, 3.59/2.78 μM, 5.52/4.27 μM, and 3.69/4.01 μM against A549, HCT-116, PC-3 and MCF-7 cell lines, respectively. The structure-activity relationships (SARs) studies were conducted through the variation in the moiety of aromatic ring and the terminal aniline on pyrimidine core. Furthermore, the mechanism of their anticancer activity was clarified by further explorations in the bioactivity. The results showed that compound **9k** obviously inhibited proliferation of A549 cell lines, induced a great decrease in mitochondrial membrane potential leading to apoptosis of cancer cells, suppressed the migration of tumor cells and prolonged the A549 cells cycle distribution, represented the blockage at G2-M phase and accumulation at S phase.

Key words: Antiproliferative activity; Synthesis; aminopyrimidine derivatives; Structure-activity relationships

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1. Introduction

Cancer is one of the most serious diseases and the leading cause of morbidity and mortality[1]. There were an estimated 18.1 million new cases of cancer and 9.6 million deaths from cancer worldwide in 2018[2]. This casts great socioeconomic burdens. Cancer treatment and prevention has long been a crucial problem and a challenge to the medical profession as well. Thus, for several decades, researchers have been devoting themselves to finding effective clinical approaches for the treatment of cancer and searching for novel anticancer agents[3]. For these purposes, combination principle strategy has been widely used to develop new antineoplastic drugs that act synergistically on multiple targets by covalently fusing two or more active pharmacophores in a single-hybrid molecule with dual or multiple anticancer activitives[4].

More and more evidence indicated that the heterocyclic scaffold was a significant tool for finding new active substances with many potential applications[5-6]. Especially, aminopyrimidine and its bioisosteric structures have been recommended as a privileged structure, and an important class of chemotherapeutic drugs have emerged for the treatment of cancers, such as CDK4/6 inhibitor(palbociclib), JAK inbibitor(momelotinib), VEGFR inhibitor(pazopanib) and Aurora A inhibitor(alisertib), *et al*[7-9]. These characteristics demonstrated that the extent of ongoing interests toward new 2-aminopyrimidine derivatives and prompted us to develop this pharmacophore as novel potential bioactive molecules(Fig.1).

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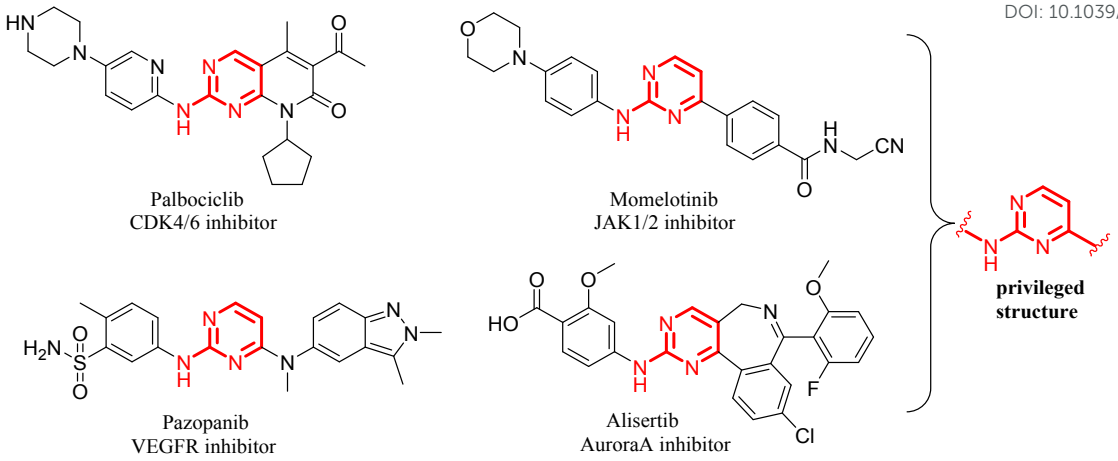

Fig. 1 Structures of representative kinase inhibitors containing aminopyrimidine nuclei

Triazolopiperazine derivatives had a variety of biological activities, such as hypoglycemic[10], anti-inflammatory[11], anti-virus[12],antitumor activity [13], and so on. In recent years, a series of antitumor compounds with triazolopiperazines moiety have been designed and synthesized. Among these derivatives, AZ0108 has been reported to demonstrate significant inhibitory activity against PARP. The co-crystal structure of AZ0108 in complex with PARP domains revealed that the 1,2,4-triazole framework played key roles in the interaction with PARP kinase due to their binding modes and the presence of hydrogen bonds. Thus, the development of such small molecules with triazolopiperazines framework, which can readily bind with various enzymes and receptors through hydrogen bonds, is a viable approach to develop new activity compounds. Spiropiperidines belongs to a relevant class of molecules for various GPCR target such as anti-inflammatory angents or δ-orpioid agonists, and could offer a useful structure for the discovery of new active compounds. To our knowledge, the spiropiperidines structure was rarely reported in the antitumor filed. For enriching the structural diversity, we also introduced the 1,4,8-triazaspiro[4.5]decan-3-one fragment to replace triazolopiperazines moiety as its good

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bioactivity as well as had the hydrogen bond donor and acceptor, which could have the interaction with the receptors[14]. (Fig.2).

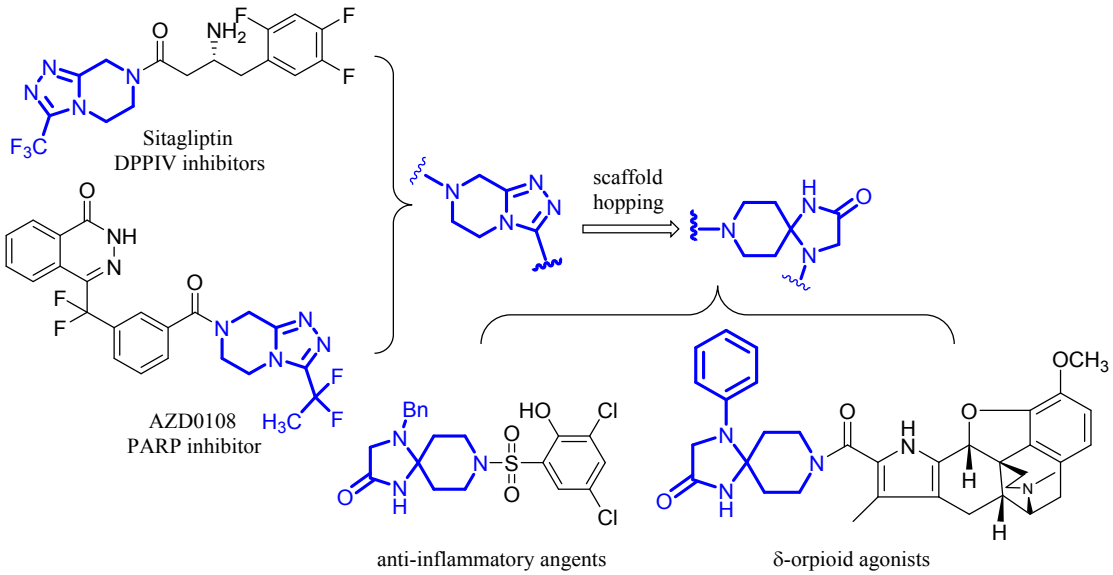

Fig. 2 Structures of representative triazolopiperazines derivatives and scaffold hopping to 1,4,8-triazaspiro[4.5]decan-3-ones framework

Judging from these observations, it was thought of interest to study the combination of 2-aminopyrimidine moiety and triazolopiperazine/1,4,8-triazaspiro[4.5]decan-3-one framework combination, which were expected to exhibit synergistic antitumor effect. Accordingly, two series of 2-aminopyrimidine derivatives have been designed and synthesized(Fig.3). Various substituents were introduced to the terminal phenyl ring with the purpose of exploring the influence of substituents on anticancer activity by regulating the electronic and steric effects. Further modifications were performed by introducing various aliphatic amino groups into the hydrophilic tail which exposing to the solvent [15-16].

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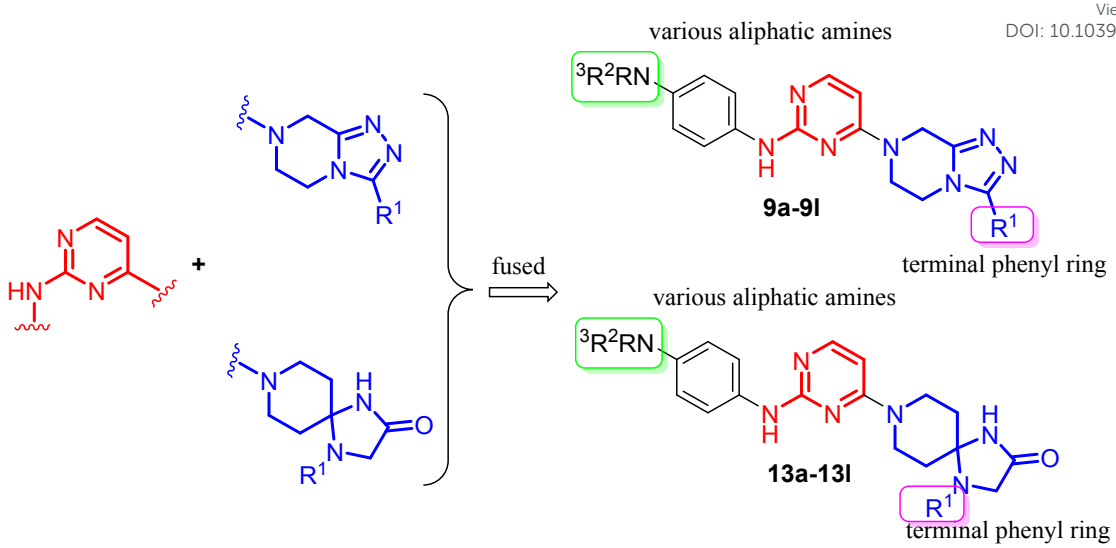

Fig. 3 Design strategy for the aminopyrimidine derivatives containing triazolopiperazine or 1,4,8-triazaspiro[4.5]decan-3-one scaffolds.

2. Chemistry

The general synthetic routes of the target compounds are shown in **Scheme 1-3**. The intermediate 2-hydrazinylpyrazine (**2**) was prepared from the commercially available 2-chloropyrazine (**1**) and hydrazine hydrate in good yield according to the known procedure[17]. Intermediate **2** was condensed with appropriate benzaldehydes in ethanol at room temperature to give the corresponding hydrazones which was not isolated in the next step and was treated by chloramine T trihydrate though the oxidative cyclisation cascade to afford the fused triazolopyrazine derivatives (**4**). The key intermediates (**5**) were obtained *via* the substitution reaction of intermediates **4** with 2,4-dichloropyrimidine. These above were mentioned in **Scheme 1**. Side chain anilines (**8**) were prepared in two steps as shown in **Scheme 2**. Aromatic nucleophilic substitution of fluorine in 4-fluoronitrobenzene by aliphatic amines were readily achieved in DMF at 120 °C. The resulting nitrobenzenes were reduced to anilines using Pd/C in EtOH and generally used without further purification. The synthetic route to compounds **9a-j** and

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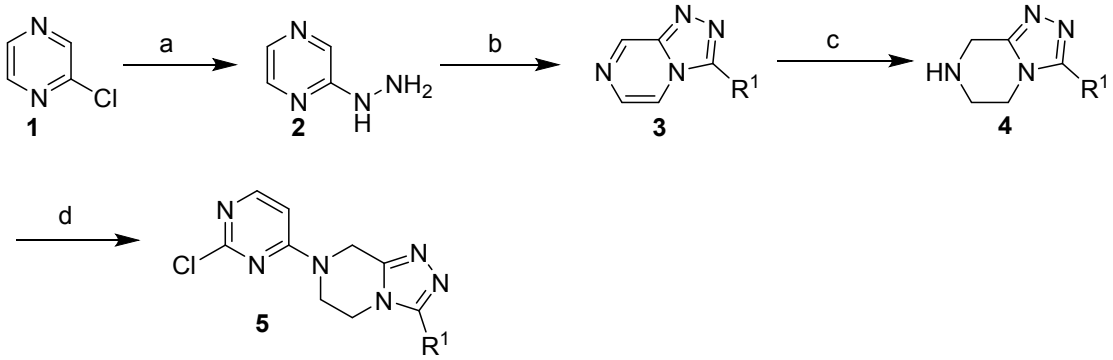
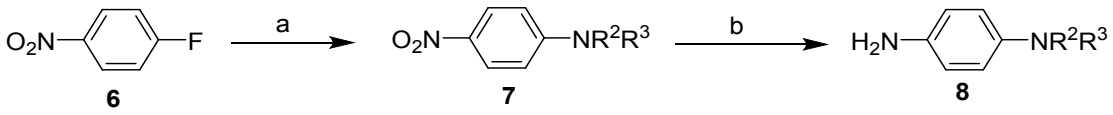
13a-j was outlined in **Scheme 3**. The intermediate (**11**) could easily obtain by following the 2,4-dichloropyrimidine in the presence of K_2CO_3 and the 4-position that was displaced by 4-piperidone[18]. Subsequently, amination of **5** and **11** by corresponding aryl amines (**8**) in the presence of *p*-toluenesulfonic acid furnished target compounds **9a-l** and **12**. Finally, target compounds **13a-j** were successfully afforded via the condensation of **12** with appropriate 2-(phenylamino)acetamides in 1,2-dichloroethane (DCE) at 90 °C with amounts of camphorsulfonic acid. Their spectral data were in full agreement with the proposed structures.



Scheme 1. General scheme for the synthesis of key intermediates **5**; Reagents and conditions: (a)80% hydrazine hydrate, 65 °C, 10 h; (b)i: appropriate aromatic aldehyde, EtOH, r.t. 1 h; ii: chloramine T trihydrate, r.t. ovevnight; (c) H_2, 10 % Pd/C, EtOH, r.t., 18 h; (d)2,4-dichloropyrimidine, TEA, DMF, r.t., 2 h;



Scheme 2. Preparation of the side chain anilines; Reagents and conditions: (a) Amines R^1R^2NH, K_2CO_3, DMF, 120 °C, 3 h; (b) H_2, 10 % Pd/C, EtOH, r.t., 5 h.

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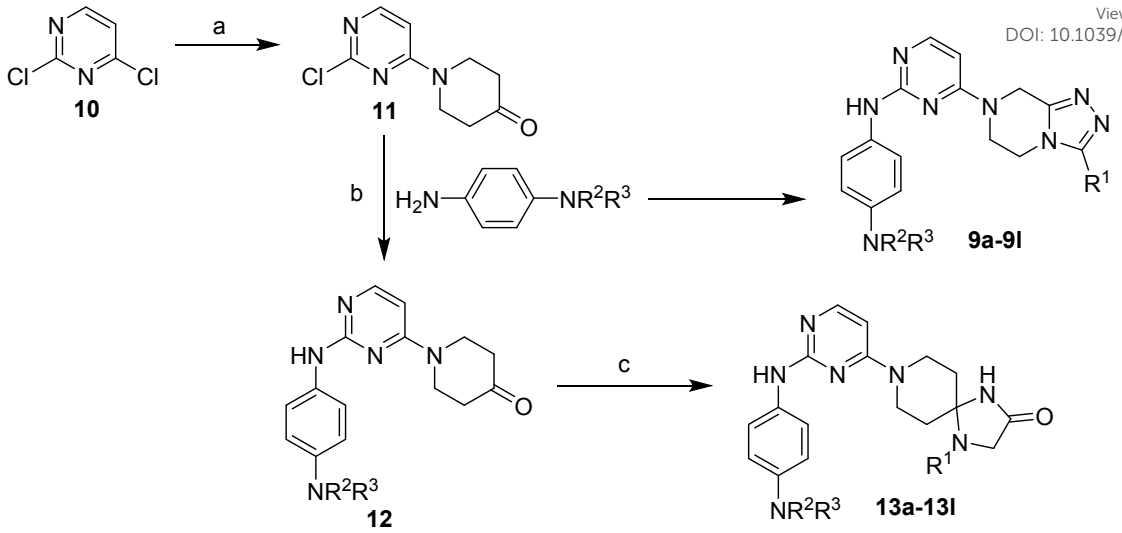

Scheme 3. General scheme for the synthesis of target compounds; Reagents and conditions: (a) 4-piperidone hydrate hydrochloride, K_2CO_3, MeCN, r.t. (b) *p*-Toluenesulfonic acid, isopropanol, 90 °C, 24 h; (c) appropriate 2-(phenylamino)acetamide , Camphorsulfonic acid, DCE, 90 °C, 24 h.

3.Results and discussion

3.1 In vitro antitumor activity and SARs study

The in *vitro* antitumor activities of the target compounds were evaluated in A549 (human lung cancer), HCT-116 (human colon cancer), PC-3 (human prostate cancer) cell lines and MCF-7 (human breast cancer) by the MTT assay. Using palbociclib and Momelotinib as the positive controls, the bioactivity data summarized in **Tables 1** that are presented as half-maximal inhibitory concentration (IC_{50}) values.

As illustrated in **Table 1**, most of the prepared compounds showed moderate-to-excellent potency against four tested cell lines in the μM range, which suggested that the combination of 2-aminopyrimidine moiety and triazolopiperazines/1,4,8-triazaspiro[4.5]decan-3-one framework exhibit potent synergistic antitumor effect. Our initial effort was to explore the cytotoxic activity of compounds with different substituents on the phenyl ring in order to understand the structure-activity relationship. A small set of compounds (**9a**-**9e**) with different aryl groups were synthesized and evaluated for their cytotoxic activity. Compound **9e** (R_1=3-Cl-4-F-Ph) exhibited good activity against A549 and HCT-116 cells with IC_{50} values of 4.95 and 10.76 μM. According

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to this result, the introduction of electron-withdrawing groups(EWGs) exhibited a positive effect on the cytotoxic activity on the A549 cells, such as compound **9b** (R_1 = 4-fluorophenyl IC_{50} = 7.99 μM), **9e** (R_1 = 3-chloro-4-fluorophenyl, IC_{50} = 4.95 μM), which are more potent than **9a** (R_1 = phenyl IC_{50} = 19.97 μM) and indicated monosubstitution of phenyl is more preferred. By contrast, electron-donating groups (EDGs) such as 4-methyl (**9c**) exhibited a negative effect. After the optimization of R_1 moiety, the modification of amino groups NR_2R_3 was carried out. Preliminary SARs indicated that the introduction of different amino groups at the C-2 position of pyrimidine moiety had a significant influence on activity, which suggested that the hydrophilic group contributed much to their potency. Replacement of the oxygen atom of morpholine group in **9e** by sulfur atom or carbon atom(**9h** and **9f**) led to the increased potency, indicating that the presence of carbon atom was a critical factor in anti-proliferative activities. Generally, five-membered pyrrolidine analogs were less active than corresponding six-membered inhibitors (**9f** *vs.* **9g**) despite of possessing fairly basic nitrogen atom, highlighting the importance of ring size. 2,6-Dimethypiperazinyl group, showed the most potent antitumor activities with the IC_{50} values of 2.14 μM, 3.59 μM, 5.52 μM, and 3.69 μM against A549, HCT-116, PC-3 and MCF-7 cell lines, respectively. In order to decrease the polarity of piperazine ring, *N*-methyl was embeded on the piperazine ring, and the results indicated that the hydrophilic *N*-methylpiperazinyl (**9j**) had negative impact on the activities. Besides, a loss in potency was observed once the piperazinyl group in **9f** was "opened" to dimethylamine (**9i**). Meanwhile, 4-methylpiperidine also showed negative impact on the activities. Obviously, all target compounds possessed selectivity for A549 and HCT-116 cancer cell lines, and had the makings of good candidate for lung and colorectal cancer.

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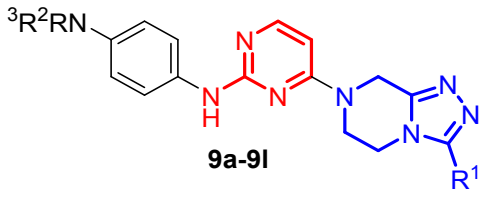
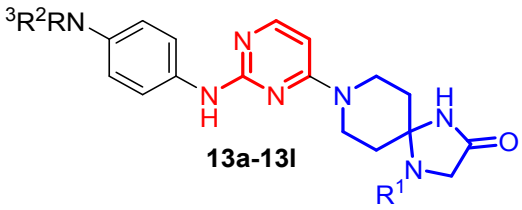
Further investigations were performed to investigate the influence of the 1,4,8-triazaspiro[4.5]decan-3-one moiety at the 4-position of pyrimidine core on the cytotoxic activity. To begin with, we discussed the cytotoxic activity of different substituents on the phenyl ring. 4-Fluorophenyl (**13c**) was selected as a positive group than other EWGs, such as 3-chloro-4-fluorophenyl (**13d**) and 4-chlorophenyl(**13e**). Compared with the EWGs, 4-methylphenyl (**13b**) as an EDG exhibited weaker activity. Replacement of the sulfur atom of thiomorpholine group (**13f**, IC_{50} = 2.78μM and 4.27μM against HCT-116 and PC-3) by oxygen atom (**13c**), caused the potency to be lowered by 1.7- and 2-fold, respectively. Five-membered pyrrolidine analogs were more active than corresponding six-membered inhibitors. Notably, the most promising compound **13f** displayed stronger potency than momelotinib and palbociclib in A549, HCT-116, PC-3 and cells with IC_{50} values of 1.98 μM, 2.78 μM and 4.27 μM, respectively. When the pyrrolidino group in **13g** was "opened" to diethylamine (**13l**), the potency decreased slightly. These encouraging results provided a valuable lead compounds **9k** and **13f** and highlighted the potential for further development.

Table 1. Structures and cytotoxicity of compounds (**9a-9l** and **13a-13l**).

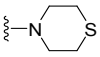
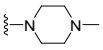
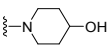


9a-9l



13a-13l

Compd.	R^1	NR^2R^3	IC_{50}(μM)			
			A549	HCT-11	PC-3	MCF-7
9a	-Ph		19.97	>30	>30	>30
9b	4-F-Ph		7.99	13.72	>30	>30

9c	4-Me-Ph		18.35	16.16	>30	15.48
9d	4-CF_3-Ph		16.27	12.95	>30	>30
9e	3-Cl-4-F-Ph		4.95	10.76	>30	12.08
9f	3-Cl-4-F-Ph		**2.99**	**3.50**	>30	12.62
9g	3-Cl-4-F-Ph		8.91	4.51	14.68	15.55
9h	3-Cl-4-F-Ph		5.17	9.71	14.21	13.90
9i	3-Cl-4-F-Ph		6.27	6.47	8.83	10.36
9j	3-Cl-4-F-Ph		20.60	>30	>30	>30
9k	3-Cl-4-F-Ph		**2.14**	**3.59**	**5.52**	**3.69**
9l	3-Cl-4-F-Ph		15.56	>30	>30	21.02
13a	-Ph		21.63	16.02	>30	>30
13b	4-Me-Ph		16.18	18.34	21.40	17.33
13c	4-F-Ph		4.26	4.87	8.42	6.65
13d	3-Cl-4-F-Ph		11.20	14.78	16.78	6.64
13e	4-Cl-Ph		17.70	11.79	>30	>30
13f	4-F-Ph		**1.98**	**2.78**	**4.27**	**4.01**
13g	4-F-Ph		**3.18**	**3.98**	**3.63**	**6.20**
13h	4-F-Ph		4.41	4.03	4.71	3.28
13i	4-F-Ph		3.00	7.01	4.56	6.78
13j	4-F-Ph		>30	15.20	29.90	19.49
13k	4-F-Ph		10.57	5.90	10.56	6.78
13l	4-F-Ph		4.34	3.62	5.93	5.40
Palbociclib			2.02	3.69	6.82	3.52
Momelotinib			1.56	3.25	4.21	3.30

3.2 Wound-healing assay.

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The effect of compound **9k** on migration of cancer cells was investigated by the wound healing assay for migration, which was an important feature of metastatic cancers. **Fig 4** and **5** indicated that concentrations markedly suppressed the wound healing in a concentration-dependent manner.

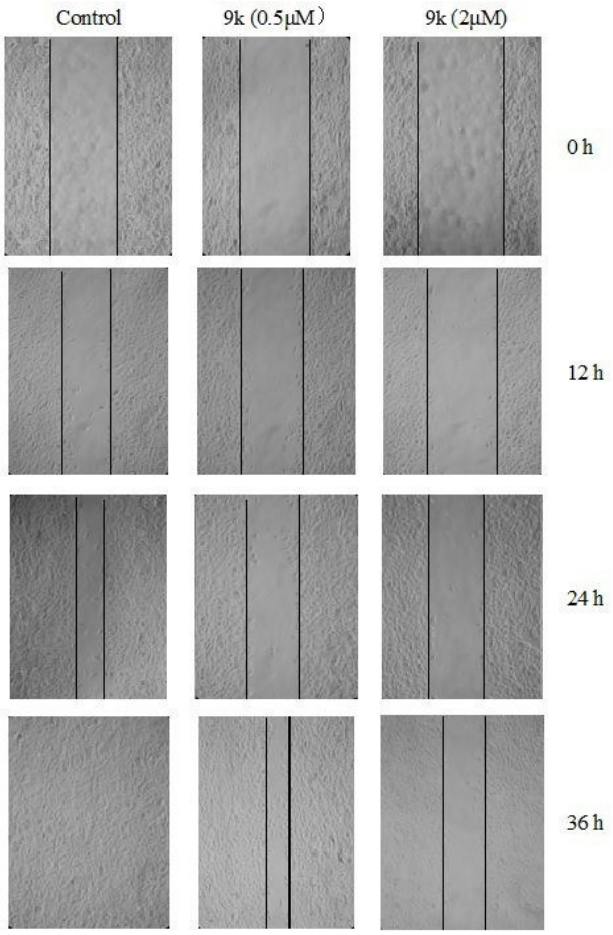

Fig 4 *In vitro* wound healing assay on A549 cells. Phase contrast images were obtained by the treatment of compound 9k at indicated concentrations for 0, 12, 24 and 36 h.

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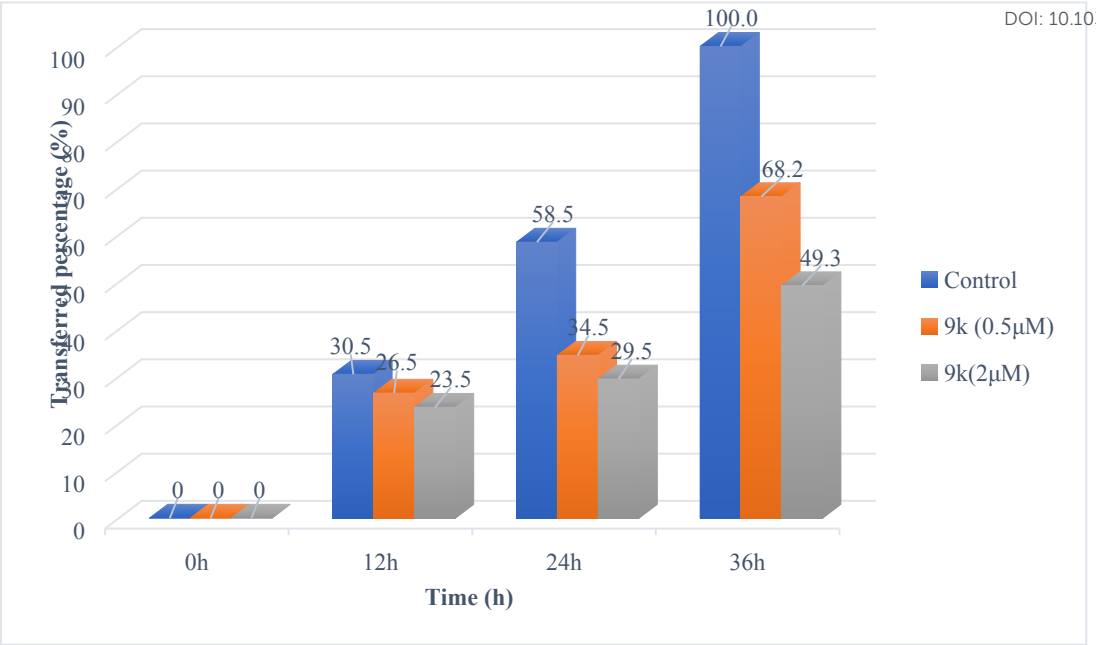

Fig. 5 *In vitro* wound healing on A549 cells of **9k**. Columnar graph represents the transferred percentage distribution at different concentrations and at different time

3.3 Cell apoptosis study

To investigate the molecular mechanisms of action preliminarily, cell apoptosis analysis of A549 cells treated with the optimal compounds **9k** and **13g** were performed using Annexin V/propidium iodide (PI) double staining. Annexin V was able to penetrate through intact membranes of live cells and colors DNA as green fluorescence, while PI was only taken up by apoptotic cells with damaged membranes coloring DNA as red fluorescence. As shown in **Fig. 6**, flow cytometric analysis data showed the percentage of Annexin V-positive cells was significantly increased after exposure to increasing dose of the optimal compounds, compared with medium control (4.3% *vs* 92.7% in **9k** and 4.3% *vs* 20.4% in **13g** at 10 μM). These results suggested that the optimal compounds can induce apoptosis in vitro and this is one of mechanisms by which the compounds exerted its antiproliferative activity.

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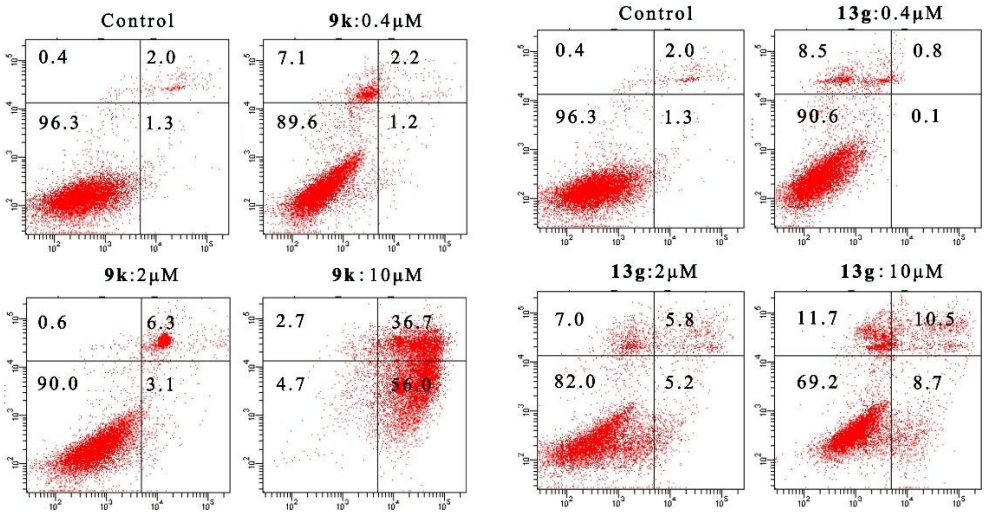

Fig. 6 Effect of compound 9k and 13g on cell apoptosis in A549 cells.

3.4 Cell cycle analysis

The cell cycle analysis was performed to investigate the prevention of proliferation in A549 cells with the potent compound **9k** and **13g**. After treatment of A549 cells with the test compounds for 24 h at indicated concentrations (**9k**: 0.08 μM and 2μM; **13g**:0.16 μM and 4 μM), the cells were fixed and stained with PI, the DNA content was analyzed by flow cytometry. The obtained results were compared with non-treated A549 cells, as control. As shown in **Fig. 7**, treatment of A549 cells with **9k** at 0.08, 2 μM concentrations increased the percentage of S-phase cells from 13.43% (as control group) to 26.78%, 30.24%, and G2-M phase from 2.35% (as control group) to 10.81%, 8.30% and with **13g** at 0.16, 4 μM concentrations increased the percentage of S-phase cells from 13.43% (as control group) to 25.63%, 28.02%, and G2-M phase from 2.35% (as control group) to 12.53%, 11.23%, respectively. These results confirmed that the test compounds significantly prolonged the A549 cells cycle distribution, represented the blockage at G2-M phase and accumulation at S phase.

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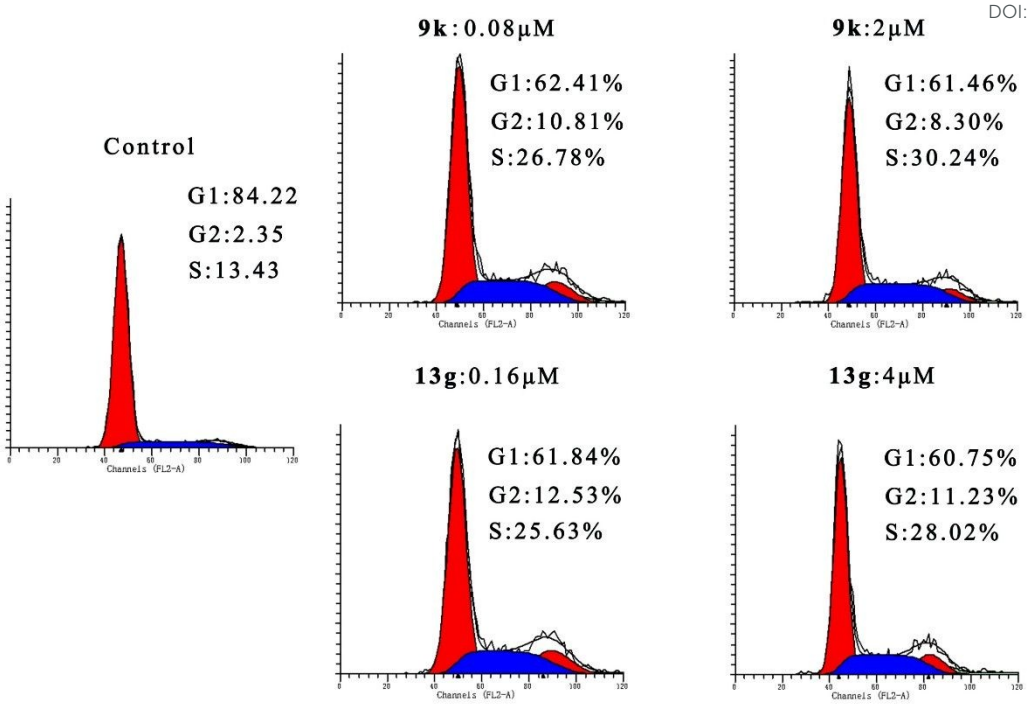

Fig. 7 Effect of compound **9k** and **13g** on the cell cycle distribution of A549 cells.

3.5 In vitro enzyme assay

Based on the cellular assays, potent compounds were selected for further in *vitro* CDK4, JAK2, VEGFR2, PI3Kα and FLT3% inhibition at 1 μM. The results were summarized in **Table 2**. As shown in **Table 2**, all compounds poorly inhibited CDK4, VEGFR2 , PI3Kα and FLT3 kinases with % inhibition values ranging from 1% to 20%. However, most of compounds displayed more highly inhibition against JAK2 kinases with % inhibition values ranging from 42.9% to 72.3%. Even though it showed more selectivity against JAK2, the inhibitory activity of compound **9k** was still less than momelotinib. These results indicated that two series of novel compounds worth further studying as new potential anticancer agent for the treatment of human cancers.

Table 2 Some kinases activity of selected compounds and positive control

Compd.	Inhibition(%)				
	JAK2	CDK4	VEGFR2	PI3Kα	FLT3
9f	55.5	4.3	9.7	8.7	7.3

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9k	72.3	2.9	20.0	5.6	9.4
13f	69.1	3.1	8.4	2.5	3.2
13k	42.9	3.4	9.9	1.8	0.5
Momelotinib	88.7	-	-	-	-
Palbociclib	-	92.5	-	-	-

3.6 molecular docking

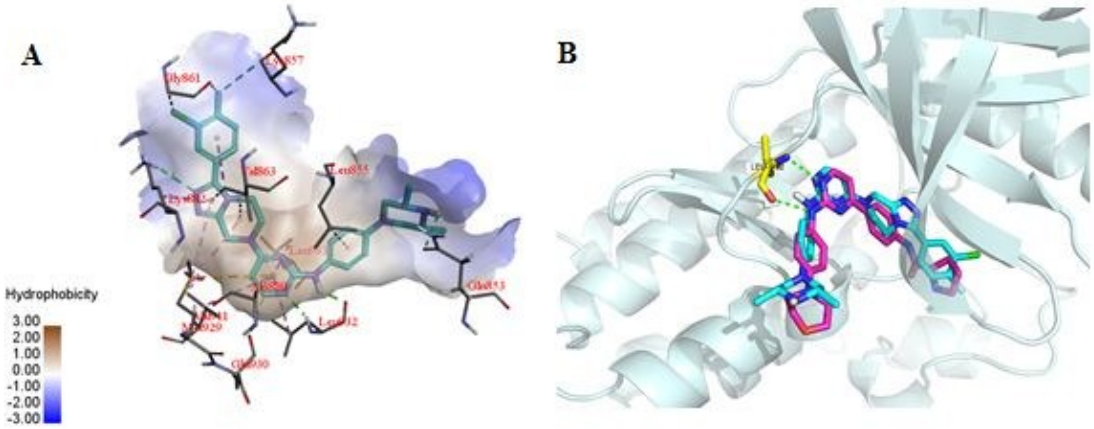

Fig. 8 A): The JAK2 active site in complex with compound 9k and its hydrophobic surface (brown: highly hydrophobic, blue: highly hydrophilic). The protein was displayed by secondary structure. Compounds were shown in colored sticks (cyan: carbon atom, blue: nitrogen atom, red: oxygen atom, white: hydrogen atom, brown: sulfur atom). B): 9k overlapping with XL019(pink sticks)

In order to better understand the binding mode, molecular docking models of **9k** were performed based upon the cocrystal structure of JAK2 with XL019 (PDB code: 4BBF), respectively. As shown in **Fig.8**, compound **9k** occupied the kinase domain in a similar way to XL019, forming two hydrogen bonds *via* 1-position nitrogen atom and 2-position NH of the pyrimidine nuclei with Leu 932 at the hinge area. In addition, 1-position NH of the 2,6-dimethypiperazinyl formed another hydrogen bond with Gln853 and the N atom of triazolopiperazine formed hydrogen bond with Lys882. In the meantime, the π-akyl interaction between triazolopiperazine and Val863, Met929 and Gly861 was also present.

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3.7 Physicochemical and ADME parameters

Furthermore, SwissADME (a free web tool to evaluate pharmacokinetics, drug likeness and medicinal chemistry friendliness of small molecules) was used to predict the physicochemical and ADME properties of the synthesized compounds.

As demonstrated in **Table 3**, the active compounds (**9k**、**13f** and **13g**) shown variable permeability based on gastrointestinal absorption (GI), according to the BOILED-Egg predictive model (Brain Or IntestinaL EstimateD permeation method). All predicted compounds showed high gastrointestinal absorption. With respect to oral bioavailability, it's expected 0.55 of probability of oral bioavailability score >10% in the rat for all compounds.

Table 3. Physicochemical properties and ADME properties of most active compounds.

Compd.	MW (g/mol) <500	H-bond acceptors <10	H-bond donors <5	Log P o/w <5	Violation Lipinski Rule of 5	[a]PSA (Å²) ≤140	Rotatable bonds <10	[b]BBB	[c]GI	[d]BS
9k	534.03	6	2	3.99	1	87.03	5	No	High	0.55
13f	519.64	4	2	3.61	1	101.93	5	No	High	0.55
13g	487.57	4	2	3.74	0	76.63	5	Yes	High	0.55

[a]PSA - Polar surface area. [b]BBB – blood-brain barrier. [c]GI – Gastrointestinal absorption. [d]BS – Bioavailability Score.

4. Conclusion

In current investigation, two series of novel 2,4-diaminopyrimidine derivatives with triazolopiperazine or 1,4,8-triazaspiro[4.5]decan-3-one framework, were firstly designed and synthesized. The structure-activity relationship (SAR) was discussed in details and the results demonstrated that the most promising compound **9k** and **13f** showed the most potent antitumor activities. Combined with the results of the molecular docking and enzymatic studies, the JAK2 was very likely to be one of the drug targets of the compound**s**. In summary, the SARs studies

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together with the pharmacological assays identified these series of compounds as promising anti-cancer agents, which will lead to the promising development of new drugs.

5. Experimental procedures

5.1 *Chemistry*

All melting points were acquired on a Mettler Melting Point MP70 apparatus (Mettler, Toledo, Switzerland) and uncorrected. Mass spectra (MS) were taken in ESI mode on Agilent 1100 LC-MS (Agilent, palo Alto, CA, USA). Reactions were monitored by thin-layer chromatography (TLC) on silica plates (F-254) and visualized under UV light.^{1}H NMR and ^{13}C NMR spectra were performed using Bruker spectrometers (Bruker Bioscience, respectively, Billerica, MA, USA) with TMS as an internal standard. Column chromatography was run on silica gel (200-300 mesh) from Qingdao Ocean Chemicals (Qingdao, Shandong, China). Unless otherwise noted, all materials were obtained from commercially available sources and used without further purification.

*5.1.1 General procedure for preparation of compounds (**9a**)*

*5.1.1.1 2-hydrazinylpyrazine (**2**)*

Under nitrogen atmosphere, 2-chloropyrazine (96 mL, 1073 mmol) was added dropwise to aqueous hydrazine (544 mL, 6009 mmol) at 65 °C over 1 h. After the addition, stirring was continued at 65 °C for 10 h. The mixture was cooled to room temperature and the yellow flakes were filtered off. The solid was washed with a small amount of water and dried under vacuum to afford pale yellow solid (60 g). m.p.: 111.7 – 113.3 °C.MS (ESI) *m/z*: 111.2 $[M+H]^+$

*5.1.1.2 3-phenyl -[1,2,4]triazolo[4,3-a]pyrazines (**3a**)*

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To a stirred suspension of **2** (5 g, 34.5 mmol) in ethanol (80 mL) was added the benzaldehyde (4.82g , 34.5 mmol) and this resulted mixturewas stirred at room temperature for 60 min. The completion of the reaction was confirmed by TLC. After disappearance of the starting aldehyde, chloramine T trihydrate (15.44g, 51.75 mmol) was added to the reaction mixture, which was stirred overnight, until consumption of the intermediate hydrazine **3** formed in the reaction. The mixture was diluted with addition of chilled water and the precipitate was filtered off, washed with water followed by cold ethanol and air-dried to give the corresponding 3-penyl-[1,2,4]triazolo[4,3-a]pyrazine **3a** as a yellow solid (6.2g). Yield: 70%; mp: 159.2–162.0 °C. MS (ESI) m/z (%): 197.98 [M+H]+. ^{1}H NMR (400MHz, DMSO-d6) δ 9.50 (s, 1 H), 8.64 (d, J = 5.0 Hz, 1 H), 7.99 – 7.96 (m, 3 H), 7.66 - 7.73 (m, 3 H).

*5.1.1.3 3-phenyl-5,6,7,8-tetrahydro-[1,2,4]triazolo[4,3-a]pyrazine(**4a**)*

3-Phenyl-[1,2,4]triazolo[4,3-a]pyrazine **3** (2.01 g) was hydrogenated under atmospheric hydrogen with 0.5g 10% Pd/C as a catalyst in ethanol (20 mL) at room temperature for 18 h. The reaction mixture was filtered through and concentrated. Purification by flash chromatography (silica gel, 10% methanol/dichloromethane) afforded 3-phenyl -5,6,7,8-tetrahydro-[1,2,4]triazolo[4,3-a]pyrazine (**4a**) as a white solid(1.5g). Yield: 75%; MS (ESI) *m/z*: 201.20[M+H]$^+$. ^{1}H NMR (400 MHz, DMSO) δ 7.94 – 7.64 (m, 2H), 7.59 – 7.44 (m, 3H), 4.13 (t, *J* = 5.5 Hz, 2H), 3.77 (s, 2H), 2.84 (t, *J* = 5.5 Hz, 2H)

*5.1.1.4 7-(2-chloropyrimidin-4-yl)-3-phenyl -5,6,7,8-tetrahydro-[1,2,4]triazolo[4,3-a]pyrazine(**5a**)*

2,4-dichloropyrimidine (0.82g, 5.5mmol) was dissolved in 10mL N,N-dimethylformamide (DMF), trimethylamine(0.75g, 7.5mmol) was added, followed by

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3-phenyl-5,6,7,8-tetrahydro-[1,2,4]triazolo[4,3-a]pyrazine **4a**(1g, 5.0mmol). The mixture was stirred at room temperature for 2 h and then poured into stirring ice-water. The resulting precipitate was filtered and dried to obtain compound **5a** as a white solid (0.96g). Yield: 62%; MS (ESI) *m/z*: 312.22 $[M+H]^+$. ^1H NMR (400 MHz, DMSO) δ 8.23 (d, *J* = 6.1 Hz, 1H), 7.81-7.75 (m, 2H), 7.59-7.53 (m, 2H), 7.10 (d, *J* = 6.2 Hz, 1H), 5.06 (s, 2H), 4.26 (t, *J* = 5.2 Hz, 2H), 4.13 (t, *J* = 5.3 Hz, 2H).

5.1.1.5

N-*(4-morpholinophenyl)-4-(3-phenyl-5,6-dihydro-[1,2,4]triazolo[4,3-*a*]pyrazin-7(8*H*)-yl)pyrimidin-2-amine(**9a**)*

To a stirred solution of compound **5a** (1 mmol) in 8 mL isopropanol was added 4-morpholinoaniline **8** (1.1 mmol) and *p*-toluenesulfonic acid (1.1 mmol). The mixture was heated to 90 °C for 24 h. The reaction mixture was quenched by saturated Na_2CO_3 aqueous solution, and then was extracted with DCM and the organic phase was washed with water, dried over anhydrous Na_2SO_4. The combined organic layer was concentrated under reduced pressure and was further purified by flash column chromatography using dichloromethane/methanol as eluent to afford product **9a** as a white solid.

Yield: 32%; m.p.: 225.4 – 227.5 °C; HRMS (ESI-Q-TOF, m/z) : 455.2301 $[M+H]^+$; ^1H NMR (400 MHz, DMSO-d_6) δ 8.96 (s, 1H), 8.04 (d, *J* = 5.9 Hz, 1H), 7.90 – 7.72 (m, 3H), 7.66 – 7.46 (m, 5H), 6.88 (d, *J* = 9.0 Hz, 2H), 6.43 (d, *J* = 6.0 Hz, 1H), 5.04 (s, 2H), 4.24 (t, *J* = 5.2 Hz, 2H), 4.07 (t, *J* = 5.3 Hz, 2H), 3.78 – 3.68 (m, 4H), 3.12 – 2.98 (m, 4H). ^{13}C NMR (101 MHz, DMSO) δ 162.2, 160.0, 157.6, 152.8, 149.3, 146.2, 133.8, 130.1, 129.3, 128.3, 127.3, 120.5, 116.0, 95.3,

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66.6, 49.8, 43.5, 41.5, 41.1; Anal. Calcd for $C_{25}H_{26}N_8O$: C, 66.06; H, 5.77; N, 24.65; Found: C, 66.07; H, 5.74; N, 24.62.

Compounds **9b-9l** were synthesized by the general procedure of compound **9a** with corresponding raw materials.

4-(3-(4-fluorophenyl)-5,6-dihydro-[1,2,4]triazolo[4,3-a]pyrazin-7(8H)-yl)-N-(4-morpholinopheny l)pyrimidin-2-amine (**9b**)

Yield: 37%; m.p.: 283.0 – 285.5 °C; HRMS (ESI-Q-TOF, m/z): 473.2215 $[M+H]^+$;^{1}H NMR (400 MHz, DMSO-d_6) δ 8.96 (s, 1H), 8.04 (d, J = 5.9 Hz, 1H), 7.86 – 7.79 (m, 2H), 7.56 (d, J = 8.9 Hz, 2H), 7.40 (t, J = 8.8 Hz, 2H), 6.92 – 6.86 (m, 2H), 6.43 (d, J = 6.0 Hz, 1H), 5.04 (s, 2H), 4.23 (t, J = 5.0 Hz, 2H), 4.7 (t, J = 5.1 Hz, 2H), 3.78 – 3.68 (m, 4H), 3.08 – 2.96 (m, 4H).^{13}C NMR (101 MHz, DMSO) δ 164.5, 162.2, 160.0, 157.6, 152.1, 149.3, 146.2, 133.8, 130.8, 123.9, 120.5, 116.4, 116.08, 95.3, 66.6, 49.8, 43.4, 41.5, 41.0. Anal. Calcd for $C_{25}H_{25}FN_8O$: C, 63.55; H, 5.33; N, 23.71; Found: C, 63.52; H, 5.35; N, 23.73.

N-(4-morpholinophenyl)-4-(3-(*p*-tolyl)-5,6-dihydro-[1,2,4]triazolo[4,3-*a*]pyrazin-7(8*H*)-yl)pyrimi din-2-amine(**9c**)

Yield: 34%; m.p.: 270.5 – 273.5 °C; HRMS (ESI-Q-TOF, m/z): 469.2468 $[M+H]^+$;^{1}H NMR (400 MHz, DMSO-d_6) δ 8.95 (s, 1H), 8.04 (d, J = 5.9 Hz, 1H), 7.66 (d, J = 8.1 Hz, 2H), 7.57 (d, J = 9.0 Hz, 2H), 7.36 (d, J = 8.0 Hz, 2H), 6.88 (d, J = 9.0 Hz, 2H), 6.42 (d, J = 6.0 Hz, 1H), 5.03 (s, 2H), 4.22 (t, J = 5.1 Hz, 2H), 4.06 (t, J = 5.3 Hz, 2H), 3.78 – 3.68 (m, 4H), 3.08 – 2.96 (m, 4H), 2.38 (s, 3H). ^{13}C NMR (101 MHz, DMSO) δ 162.2, 160.0, 157.6, 152.8, 149.1, 146.2, 139.8, 133.8, 129.9, 128.2, 124.5, 120.5, 116.0, 95.3, 66.6, 49.8, 43.4, 41.5, 41.1, 21.3. Anal. Calcd for $C_{26}H_{28}N_8O$: C, 66.65; H, 6.02; N, 23.91; Found: C, 66.67; H, 6.04; N, 23.96.

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N-(4-morpholinophenyl)-4-(3-(4-(trifluoromethyl)phenyl)-5,6-dihydro-[1,2,4]triazolo[4,3-a]pyrazin-7(8H)-yl)pyrimidin-2-amine (**9d**)

Yield: 35%; m.p.: 272.4 – 275.2 °C; HRMS (ESI-Q-TOF, m/z): 523.2187 $[M+H]^+$;^{1}H NMR (400 MHz, DMSO-d_6) δ 8.96 (s, 1H), 8.05-8.01 (m, 3H), 7.92 (d, J = 8.3 Hz, 2H), 7.56 (d, J = 9.0 Hz, 2H), 6.89 (d, J = 9.0 Hz, 2H), 6.44 (d, J = 6.0 Hz, 1H), 5.07 (s, 2H), 4.30 (t, J = 5.2 Hz, 2H), 4.09 (t, J = 5.4 Hz, 2H), 3.78 – 3.68 (m, 4H), 3.08 – 2.96 (m, 4H). ^{13}C NMR (101 MHz, DMSO) δ 162.2, 160.0, 157.7, 151.8, 149.9, 146.3, 133.8, 131.3, 130.3, 130.1, 129.1, 126.3, 125.8, 123.1, 120.6, 116.0, 95.3, 66.6, 49.8, 43.6, 41.4, 41.0. Anal. Calcd for $C_{26}H_{25}F_3N_8O$: C, 59.76; H, 4.82; N, 21.44; Found: C, 59.73; H, 4.81; N, 21.46.

4-(3-(3-chloro-4-fluorophenyl)-5,6-dihydro-[1,2,4]triazolo[4,3-a]pyrazin-7(8H)-yl)-N-(4-morpholinophenyl)pyrimidin-2-amine (**9e**)

Yield: 38%; m.p.: 253.4 – 255.2 °C; HRMS (ESI-Q-TOF, m/z):507.1828$[M+H]^+$; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.97 (s, 1H), 8.04 (d, J = 6.0 Hz, 1H), 8.00 (dd, J = 7.1, 2.1 Hz, 1H), 7.82-7.77 (m, 1H), 7.63-7.55 (m, 3H), 6.88 (d, J = 9.0 Hz, 2H), 6.44 (d, J = 6.0 Hz, 1H), 5.06 (s, 2H), 4.26 (t, J = 5.1 Hz, 2H), 4.06 (t, J = 5.3 Hz, 2H), 3.78 – 3.68 (m, 4H), 3.08 – 2.96 (m, 4H). ^{13}C NMR (101 MHz, DMSO) δ 162.2, 160.0, 157.6, 157.2, 151.1, 149.6, 146.2, 133.8, 130.4, 129.4, 125.2, 120.5, 118.1, 116.0, 95.3, 66.6, 49.8, 43.3, 41.4, 41.0. Anal. Calcd for $C_{25}H_{24}ClFN_8O$: C, 59.23; H, 4.77; N, 22.10; Found: C, 59.20; H, 4.78; N, 22.13.

4-(3-(3-chloro-4-fluorophenyl)-5,6-dihydro-[1,2,4]triazolo[4,3-a]pyrazin-7(8H)-yl)-N-(4-(piperidin-1-yl)phenyl)pyrimidin-2-amine (**9f**)

Yield: 30%; m.p.: 293.4 – 295.7°C; HRMS (ESI-Q-TOF, m/z): 505.2028$[M+H]^+$; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.94 (s, 1H), 8.08 – 7.97 (m, 2H), 7.82-7.78(m, 1H), 7.62 (t, J = 8.9 Hz, 1H),

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7.52 (d, *J* = 9.0 Hz, 2H), 6.86 (d, *J* = 9.0 Hz, 2H), 6.43 (d, *J* = 6.0 Hz, 1H), 5.06 (s, 2H), 4.25 (t, *J* = 5.3 Hz, 2H), 4.06 (t, *J* = 4.9 Hz, 2H), 3.06 – 2.99 (m, 4H), 1.67-1.59 (m, 4H), 1.53-1.46 (m, 2H). ^{13}C NMR (101 MHz, DMSO) δ 162.2, 160.0, 158.4, 157.6, 151.1, 149.6, 147.2, 133.3, 130.4, 129.4, 125.2, 120.7, 120.6, 118.1, 116.9, 95.3, 51.0, 43.3, 41.4, 41.0, 25.9, 24.3. Anal. Calcd for $C_{26}H_{26}ClFN_8$: C, 61.84; H, 5.19; N, 22.19; Found: C, 61.88; H, 5.16; N, 22.20.

4-(3-(3-chloro-4-fluorophenyl)-5,6-dihydro-[1,2,4]triazolo[4,3-*a*]pyrazin-7(8*H*)-yl)-*N*-(4-(pyrrolidin-1-yl)phenyl)pyrimidin-2-amine (**9g**)

Yield: 30%; m.p.: 264.4 – 266.7°C; HRMS (ESI-Q-TOF, m/z): 491.1880[M+H]$^+$; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.77 (s, 1H), 8.00 (d, *J* = 5.5 Hz, 2H), 7.82-7.77 (m, 1H), 7.61 (t, *J* = 8.9 Hz, 1H), 7.45 (d, *J* = 8.7 Hz, 2H), 6.49 (d, *J* = 8.7 Hz, 2H), 6.38 (d, *J* = 5.9 Hz, 1H), 5.04 (s, 2H), 4.24 (t, *J* = 5.3 Hz, 2H), 4.05 (t, *J* = 5.1 Hz, 2H), 3.24-3.14(d, *J* = 6.3 Hz, 4H), 1.99-1.89 (q, *J* = 4.8, 3.3 Hz, 4H). ^{13}C NMR (101 MHz, DMSO) δ 162.2, 160.2, 158.4, 157.7, 151.1, 149.6, 143.9, 130.4, 130.1, 129.5, 125.2, 121.6, 120.5, 118.1, 111.9, 94.8, 48.0, 43.3, 41.3, 40.9, 25.3. Anal. Calcd for $C_{25}H_{24}ClFN_8$: C, 61.16; H, 4.93; N, 22.82; Found: C, 61.18; H, 4.97; N, 21.82.

4-(3-(3-chloro-4-fluorophenyl)-5,6-dihydro-[1,2,4]triazolo[4,3-a]pyrazin-7(8H)-yl)-N-(4-thiomorpholinophenyl)pyrimidin-2-amine (**9h**)

Yield: 38%; m.p.: 240.4 – 242.3°C; HRMS (ESI-Q-TOF, m/z): 523.1601[M+H]$^+$; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.99 (s, 1H), 8.09 – 7.92 (m, 2H), 7.83-7.77 (m, 1H), 7.60 (m, 3H), 6.88 (d, *J* = 9.0 Hz, 2H), 6.45 (d, *J* = 6.0 Hz,1H), 5.06 (s, 2H), 4.26 (t, *J* = 5.3 Hz, 2H), 4.07 (d, *J* = 5.3 Hz, 2H), 3.41 – 3.36 (m, 4H), 2.74 – 2.66 (m, 4H). ^{13}C NMR (101 MHz, DMSO) δ 162.2, 160.0, 158.4, 157.6, 151.1, 149.6, 146.4, 133.8, 130.4, 129.5, 125.2, 120.6, 118.1, 117.9, 117.7, 95.4, 52.6, 43.3, 41.4,

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41.0, 26.6. Anal. Calcd for $C_{25}H_{24}ClFN_8S$: C, 57.41; H, 4.63; N, 21.42; Found: C, 47.42; H, 4.66; N, 21.40.

N^1-(4-(3-(3-chloro-4-fluorophenyl)-5,6-dihydro-[1,2,4]triazolo[4,3-*a*]pyrazin-7(8*H*)-yl)pyrimidin-2-yl)-N^4,N^4-dimethylbenzene-1,4-diamine (**9i**)

Yield: 43%; m.p.: 260.8 – 263.3°C; HRMS (ESI-Q-TOF, m/z): 465.1628$[M+H]^+$; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.87 (s, 1H), 8.08 – 7.97 (m, 2H), 7.83-7.77 (m, 1H), 7.62 (t, *J* = 9.0 Hz, 1H), 7.49 (d, *J* = 9.1 Hz, 2H), 6.70 (d, *J* = 9.1 Hz, 2H), 6.41 (d, *J* = 6.0 Hz, 1H), 5.05 (s, 2H), 4.25 (t, *J* = 5.3 Hz, 2H), 4.06 (t, *J* = 5.3 Hz, 2H), 2.83 (s, 6H). ^{13}C NMR (101 MHz, DMSO) δ 162.2, 160.1, 158.4, 157.7, 151.1, 149.6, 146.4, 131.3, 130.5, 129.4, 125.2, 121.1, 120.5, 117.9, 113.44, 95.06, 43.3, 41.4, 41.2, 41.0. Anal. Calcd for $C_{23}H_{22}N_8$: C, 59.42; H, 4.77; N, 24.10; Found: C, 59.44; H, 4.74; N, 24.08.

4-(3-(3-chloro-4-fluorophenyl)-5,6-dihydro-[1,2,4]triazolo[4,3-*a*]pyrazin-7(8*H*)-yl)-*N*-(4-(4-methylpiperazin-1-yl)phenyl)pyrimidin-2-amine (**9j**)

Yield: 39.8%; m.p.: 229.4– 232.2°C; HRMS (ESI-Q-TOF, m/z): 520.2028 $[M+H]^+$; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.96 (s, 1H), 8.07 – 7.97 (m, 2H), 7.83-7.77 (m, 1H), 7.61-7.52 (m, 3H), 6.89 (d, *J* = 8.9 Hz, 2H), 6.44 (d, *J* = 6.0 Hz, 1H), 5.06 (s, 2H), 4.25 (t, *J* = 5.1 Hz, 2H), 4.07 (d, *J* = 5.3 Hz, 2H), 3.15-3.06 (m, 4H), 2.70 – 2.58 (m, 4H), 2.34 (s, 3H). ^{13}C NMR (151 MHz, DMSO) δ 162.2, 160.0, 158.4, 157.6, 151.1, 149.6, 146.1, 133.6, 130.5, 129.5, 125.3, 120.7, 120.6, 117.9, 116.4, 95.3, 54.9, 49.1, 45.8, 43.3, 41.4, 41.0, 40.4. Anal. Calcd for $C_{26}H_{27}ClFN_9$: C, 60.05; H, 5.23; N, 24.24; Found: C, 60.01; H, 5.19; N, 24.28.

4-(3-(3-chloro-4-fluorophenyl)-5,6-dihydro-[1,2,4]triazolo[4,3-*a*]pyrazin-7(8*H*)-yl)-*N*-(4-(3,5-dimethylpiperazin-1-yl)phenyl)pyrimidin-2-amine (**9k**)

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Yield: 30%; m.p.: 174.2– 178.1°C; HRMS (ESI-Q-TOF, m/z): 534.2296[M+H]$^+$;^{1}H NMR (400 MHz, DMSO-d_6) δ 8.96 (s, 1H), 8.06 – 7.97 (m, 2H), 7.80 (ddd, J = 8.7, 4.7, 2.2 Hz, 1H), 7.62 (t, J = 8.9 Hz, 1H), 7.53 (d, J = 8.7 Hz, 2H), 6.86 (d, J = 8.7 Hz, 2H), 6.43 (d, J = 6.0 Hz, 1H), 5.07 (s, 2H), 4.26 (t, J = 5.3 Hz, 2H), 4.07 (d, J = 5.2 Hz, 2H), 3.45-3.39 (m, 2H), 2.87 (m, 2H), 2.07 (t, J = 10.6 Hz, 2H), 1.03 (d, J = 6.2 Hz, 6H). ^{13}C NMR (101 MHz, DMSO) δ 162.2, 160.0, 158.4, 157.7, 151.1, 149.6, 146.4, 133.2, 130.5, 129.5, 125.2, 120.7, 120.6, 118.1, 116.3, 95.2, 56.4, 50.68, 43.37, 41.4, 41.0, 19.7. Anal. Calcd for $C_{27}H_{29}ClFN_9$: C, 60.73; H, 5.47; N, 23.61; Found: C, 60.75; H, 5.44; N, 23.58.

4-(3-(3-chloro-4-fluorophenyl)-5,6-dihydro-[1,2,4]triazolo[4,3-*a*]pyrazin-7(8*H*)-yl)-*N*-(4-(4-methylpiperidin-1-yl)phenyl)pyrimidin-2-amine (**9l**)

Yield: 28%; m.p.: 292.6– 298.1°C; HRMS (ESI-Q-TOF, m/z): 519.2192[M+H]$^+$;^{1}H NMR (400 MHz, DMSO-d_6) δ 8.92 (s, 1H), 8.08 – 7.96 (m, 2H), 7.83-7.77 (m, 1H), 7.62 (t, J = 9.0 Hz, 2H), 7.52 (d, J = 8.4 Hz, 1H), 6.86 (d, J = 8.9 Hz, 2H), 6.43 (d, J = 6.0 Hz, 1H), 5.05 (s, 2H), 4.25 (t, J = 5.3 Hz, 2H), 4.06 (t, J = 5.3 Hz, 2H), 3.53 (d, J = 11.0 Hz, 2H), 2.58 (d, J = 12.3 Hz, 2H), 1.69 (d, J = 13.0 Hz, 2H), 1.51-1.41 (m, 1H), 1.31-1.19 (m, 2H), 0.94 (d, J = 6.4 Hz, 3H). ^{13}C NMR (151 MHz, DMSO) δ 162.2, 160.0, 158.4, 157.6, 151.1, 149.6, 146.9, 130.7, 130.5, 129.4, 125.3, 120.7, 120.6, 118.1, 116.9, 95.3, 50.3, 43.3, 41.4, 41.0, 34.2, 30.5, 22.2. Anal. Calcd for $C_{27}H_{28}ClFN_8$: C, 62.48; H, 5.44; N, 21.59; Found: C, 62.50; H, 5.46; N, 22.53.

5.1.2 General Procedure for Preparation of (7)

Substituted amine (42 mmol) was added to a mixture of 4-fluoronitrobenzene (35 mmol) and K_2CO_3 (70 mmol) in 40 mL DMF. The reaction mixture was stirred at 120 °C and followed by TLC. After completion of the reaction, the mixture was poured into stirring ice-water. The

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resulting precipitate was filtered and dried to obtain compounds **7** as a yellow solid, Yield: 79.1-90.5%.

*5.1.3 General Procedure for Preparation of (**8**) c*

The substituted nitro compound **7** (15 mmol) was treated with 10% Pd-carbon (5% w/w). The reaction was subjected to hydrogenation under hydrogen gas at room temperature and the reaction was monitored by TLC. After completion of the reaction, the mixture was filtered through a Celite bed and concentrated in a vacuum to afford product **8**. Yield: 72.4-88.6%.

*5.1.4 General Procedure for Preparation of (**13a**)*

*4.1.4.1 1-(2-chloropyrimidin-4-yl)piperidin-4-one (**11**)*

A mixture of 4-piperidone hydrate hydrochloride (21.7g, 140mmol), 2,4-dichloropyrimidine (20g, 134.2mmol) and potassium carbonate (46.3g, 335.5mmol) in 200mL acetonitrile was stirred for 8 h at room temperature, after completion of the reaction, The reaction mixture was filtered through and concentrated. Purification by flash chromatography afforded **11** as a coulurless solid(20.6g). Yield: 73%. MS (ESI) *m/z*: 212.15 $[M+H]^+$, ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.13 (d, J = 6.2 Hz, 1H), 6.92 (d, J = 6.2 Hz, 1H), 4.01-3.80 (m, 4H), 2.53-2.48 (m, 4H).

*5.1.4.2 1-(2-((4-morpholinophenyl)amino)pyrimidin-4-yl)piperidin-4-one (**12a**)*

To a stirred solution of compound **11** (0.5g, 2.4 mmol) in 10 mL isopropanol was added 4-morpholinoaniline **8** (0.5g, 2.8 mmol) and *p*-toluenesulfonic acid (0.49g, 2.8 mmol). The mixture was heated to 90 °C for 10 h. The reaction mixture was quenched by saturated Na_2CO_3 aqueous solution, and then was extracted with DCM and the organic phase was washed with water, dried over anhydrous Na_2SO_4. The combined organic layer was concentrated under reduced

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pressure and was further purified by flash column chromatography using dichloromethane/methanol as eluent to afford product **12a** as a white solid.

Yield: 59%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.88 (s, 1H), 7.98 (d, J = 6.0 Hz, 1H), 7.58 (d, J = 8.9 Hz, 2H), 6.86 (d, J = 8.9 Hz, 2H), 6.30 (d, J = 6.0 Hz, 1H), 3.91 (t, J = 6.1 Hz, 4H), 3.73 (t, J = 4.7 Hz, 4H), 3.01 (t, J = 4.7 Hz, 4H), 2.45 (t, J = 6.1 Hz, 4H).

5.1.4.3

8-(2-((4-morpholinophenyl)amino)pyrimidin-4-yl)-4-phenyl-1,4,8-triazaspiro[4.5]decan-2-one

(13a)

A solution of **12a** (0.35g, 1.0 mmol), 2-(phenylamino)acetamide (0.14g, 1.2mmol) and camphorsulfonic acid (0.28g, 1.2mmol) in 5mL DCE was heated to 90 °C with stirring for 24 h. The solvent was then removed by a rotatory evaporator, the residue was dissolved in chloroform, washed with sodium hydrogen carbonate solution and water, and dried over anhydrous magnesium sulfate. The combined organic layer was concentrated under reduced pressure and was further purified by flash column chromatography using dichloromethane/methanol as eluent to afford product **13a** as a brown solid.

Yield: 23%. m.p.: 242.3 – 244.1 °C; HRMS (ESI-Q-TOF, m/z): 486.2614 $[M+H]^+$; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.61 (s, 1H), 8.86 (s, 1H), 7.95 (d, J = 6.1 Hz, 1H), 7.54 (d, J = 8.9 Hz, 2H), 7.17 (t, J = 7.9 Hz, 2H), 6.88-6.68 (m, 5H), 6.29 (d, J = 6.1 Hz, 1H), 4.59-4.20 (m, 2H), 3.86 (s, 2H), 3.77 – 3.66 (m, 4H), 3.20 (t, J = 13.2 Hz,2H), 3.06 – 2.97 (m, 4H), 2.48-2.42(m, 2H), 1.62 (d, J = 13.0 Hz, 2H). ^{13}C NMR (101 MHz, DMSO) δ 169.9, 162.0, 160.0, 159.4, 156.1, 146.3, 143.8, 133.6, 129.5, 120.6, 118.6, 116.0, 95.0, 77.3, 66.6, 51.1, 49.7, 34.1. Anal. Calcd for $C_{27}H_{31}N_7O_2$: C, 66.78; H, 6.44; N, 20.19; Found: C, 66.80; H, 6.42; N, 20.15.

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Compounds **13b-13l** were synthesized by the general procedure of compound **13a** with corresponding raw materials.

8-(2-((4-morpholinophenyl)amino)pyrimidin-4-yl)-4-(*p*-tolyl)-1,4,8-triazaspiro[4.5]decan-2-one (**13b**)

Yield: 20%. m.p.: 248.4 – 250.2 °C; HRMS (ESI-Q-TOF, m/z): 500.2771 $[M+H]^+$; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.53 (s, 1H), 8.81 (s, 1H), 7.94 (d, J = 6.0 Hz, 1H), 7.54 (d, J = 9.0 Hz, 2H), 6.99 (d, J = 8.4 Hz, 2H), 6.84 (d, J = 9.1 Hz, 2H), 6.72 (d, J = 8.6 Hz, 2H), 6.25 (d, J = 6.1 Hz, 1H), 4.63-4.25 (m, 2H) 3.82 (s, 2H), 3.76 – 3.66 (m, 4H), 3.16 (t, J = 12.7 Hz, 2H), 3.03 – 2.95 (m, 4H), 2.28 (td, J = 13.1, 5.0 Hz, 2H), 2.16 (s, 3H), 1.61 (d, J = 13.0 Hz, 2H). ^{13}C NMR (101 MHz, DMSO) δ 170.4, 161.9, 158.9, 155.2, 146.4, 141.5, 133.3, 129.9, 129.6, 128.5, 120.8, 117.7, 116.0, 114.6, 95.0, 77.3, 66.6, 55.3, 51.4, 49.7, 34.4, 20.4. Anal. Calcd for $C_{28}H_{33}N_7O_2$: C, 67.31; H, 6.66; N, 19.62; Found: C, 67.33; H, 6.62; N, 19.59.

4-(4-fluorophenyl)-8-(2-((4-morpholinophenyl)amino)pyrimidin-4-yl)-1,4,8-triazaspiro[4.5]decan-2-one (**13c**)

Yield: 19%. m.p.: 259.7 – 262.7 °C; HRMS (ESI-Q-TOF, m/z): 504.2520 $[M+H]^+$; ^{1} H NMR (400 MHz, DMSO-d_6) δ 9.54 (s, 1H), 8.81 (s, 1H), 7.92 (d, J = 6.0 Hz, 1H), 7.53 (d, J = 8.9 Hz, 2H), 7.04 (t, J = 8.8 Hz, 2H), 6.93 – 6.80 (m, 4H), 6.25 (d, J = 6.0 Hz, 1H), 4.60-4.19 (m, 2H), 3.85 (s, 2H), 3.75-3.68 (m, 4H), 3.16 (t, J = 13.2 Hz, 2H), 3.02-2.97 (m, 4H), 2.22 (td, J = 13.2, 5.0 Hz, 2H), 1.65 (d, J = 13.0 Hz, 2H). ^{13}C NMR (101 MHz, DMSO) δ 170.3, 161.9, 159.9, 156.9, 156.6, 146.1, 140.5, 134.0, 120.3, 119.3, 116.0, 115.8, 94.9, 77.5, 66.6, 51.7, 49.8, 34.4. Anal. Calcd for $C_{27}H_{30}FN_7O_2$: C, 64.40; H, 6.00; N, 19.47; Found: C, 64.39; H, 6.02; N, 19.50.

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4-(3-chloro-4-fluorophenyl)-8-(2-((4-morpholinophenyl)amino)pyrimidin-4-yl)-1,4,8-triazaspiro[4.5]decan-2-one (**13d**)

Yield: 19%. m.p.: 256.6 – 259.6 °C; HRMS (ESI-Q-TOF, m/z): 538.2131$[M+H]^+$; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.63 (s, 1H), 8.83 (s, 1H), 8.05 – 7.84 (m, 1H), 7.66 – 7.39 (m, 2H), 7.23 (dt, J = 10.6, 5.2 Hz, 1H), 6.89-6.74(m, 4H), 6.41 – 6.22 (m, 1H), 4.60-4.22 (m, 2H), 3.88 (s, 2H), 3.81 – 3.62 (m, 4H), 3.18 (t, J = 11.5 Hz, 2H), 3.00 (d, J = 5.2 Hz, 4H), 2.36 (d, J = 13.7 Hz, 2H), 1.64 (d, J = 13.0 Hz, 2H). ^{13}C NMR (101 MHz, DMSO) δ 169.6, 162.1, 159.97, 154.6, 152.3, 149.9, 146.1, 141.2, 133.9, 120.4, 119.9, 117.5, 117.3, 116.5, 116.0, 95.0, 77.5, 66.6, 51.4, 49.8, 33.9. Anal. Calcd for $C_{27}H_{29}ClFN_7O_2$: C, 60.28; H, 5.43; N, 18.22; Found: C, 60.25; H, 5.45; N, 18.21.

4-(4-chlorophenyl)-8-(2-((4-morpholinophenyl)amino)pyrimidin-4-yl)-1,4,8-triazaspiro[4.5]decan-2-one (**13e**)

Yield: 19%. m.p.: 211.0 – 213.6 °C; HRMS (ESI-Q-TOF, m/z):520.2224$[M+H]^+$; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.66 (s, 1H), 8.83 (s, 1H), 7.95 (d, J = 6.0 Hz, 1H), 7.55 (d, J = 9.0 Hz, 2H), 7.20 (d, J = 9.0 Hz, 2H), 6.85 (d, J = 9.1 Hz, 2H), 6.75 (d, J = 9.1 Hz, 2H), 6.27 (d, J = 6.1 Hz, 1H), 4.69-4.22 (m, 2H), 3.86 (s, 2H), 3.75-3.67 (m , 4H), 3.19 (t, J = 13.1 Hz, 2H), 3.06 – 2.90 (m, 4H), 2.44 (td, J = 13.1, 5.0 Hz, 2H), 1.63 (d, J = 12.9 Hz, 2H). ^{13}C NMR (101 MHz, DMSO) δ 169.6, 162.1, 160.1, 157.2, 146.0, 142.7, 134.1, 129.1, 122.2, 120.3, 117.1, 116.1, 94.9, 77.5, 66.6, 55.3, 51.1, 49.8, 34.0. Anal. Calcd for $C_{27}H_{30}ClN_7O_2$: C, 62.36; H, 5.82; N, 18.85; Found: C, 62.35; H, 5.85; N, 18.82.

4-(4-fluorophenyl)-8-(2-((4-thiomorpholinophenyl)amino)pyrimidin-4-yl)-1,4,8-triazaspiro[4.5]decan-2-one (**13f**)

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Yield: 21%. m.p.: 252.0 – 254.2 °C; HRMS (ESI-Q-TOF, m/z):520.2291[M+H]$^+$; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.56 (s, 1H), 8.81 (s, 1H), 7.93 (d, J = 5.9 Hz, 1H), 7.54 (d, J = 8.9 Hz, 2H), 7.04 (t, J = 8.8 Hz, 2H), 6.86 (m, 4H), 6.24 (d, J = 6.1 Hz, 1H), 4.74-4.22 (m, 2H), 3.85 (s, 2H), 3.16 (t, J = 13.3 Hz, 2H), 3.33-3.31 (m, 4H), 2.74 – 2.60 (m, 4H), 2.22 (td, J = 13.1, 5.1 Hz, 2H), 1.65 (d, J = 13.0 Hz, 2H).^{13}C NMR (101 MHz, DMSO) δ 170.3, 161.9, 160.0, 157.1, 156.7, 146.1, 140.5, 134.1, 120.3, 119.5, 117.8, 116.0, 94.9, 77.5, 52.6, 51.7, 34.4, 26.6. Anal. Calcd for $C_{27}H_{30}FN_7OS$: C, 62.41; H, 5.82; N, 18.87; Found: C, 62.38; H, 5.85; N, 18.89.

4-(4-fluorophenyl)-8-(2-((4-(pyrrolidin-1-yl)phenyl)amino)pyrimidin-4-yl)-1,4,8-triazaspiro[4.5]decan-2-one (**13g**)

Yield: 22%. m.p.: 232.4 – 235.6 °C; HRMS (ESI-Q-TOF, m/z): 488.2570[M+H]$^+$; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.54 (s, 1H), 8.59 (s, 1H), 7.90 (d, J = 5.9 Hz, 1H), 7.44 (d, J = 8.6 Hz, 2H), 7.04 (t, J = 8.8 Hz, 2H), 6.86 (dd, J = 9.2, 4.4 Hz, 2H), 6.46 (d, J = 8.8 Hz, 2H), 6.19 (d, J = 6.0 Hz, 1H), 4.60-4.21 (m, 2H), 3.85 (s, 2H), 3.24-3.05 (m, 6H), 2.21 (td, J = 13.1, 5.1 Hz, 2H), 1.99 – 1.82 (m, 4H), 1.64 (d, J = 12.8 Hz, 2H). ^{13}C NMR (101 MHz, DMSO) δ 170.3, 162.0, 160.3, 157.8, 157.2, 155.5, 143.6, 140.5, 130.4, 121.3, 119.4, 115.8, 111.9, 94.3, 77.5, 51.7, 48.0, 34.4, 25.3. Anal. Calcd for $C_{27}H_{30}FN_7O$: C, 66.51; H, 6.20; N, 20.11; Found: C, 66.55; H, 6.18; N, 20.11.

4-(4-fluorophenyl)-8-(2-((4-(4-methylpiperidin-1-yl)phenyl)amino)pyrimidin-4-yl)-1,4,8-triazaspiro[4.5]decan-2-one (**13h**)

Yield: 17%. m.p.: 222.4 – 225.1 °C; HRMS (ESI-Q-TOF, m/z):516.2883[M+H]$^+$; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.55 (s, 1H), 8.75 (s, 1H), 7.92 (d, J = 6.0 Hz, 1H), 7.50 (d, J = 8.9 Hz, 2H), 7.04 (t, J = 8.8 Hz, 2H), 6.90 – 6.70 (m, 4H), 6.23 (d, J = 6.1 Hz, 1H), 4.62-4.25 (m, 2H), 3.85 (s,

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2H), 3.58 – 3.44 (m, 2H), 3.23 – 3.07 (m, 2H), 2.55 (dd, *J* = 12.2, 2.5 Hz, 2H), 2.21 (td, *J* = 13.1, 5.0 Hz, 2H), 1.74 – 1.57 (m, 4H), 1.49-1.37 (m, 1H), 1.23 (qd, *J* = 11.8, 3.6 Hz, 2H), 0.93 (d, *J* = 6.5 Hz, 3H). ^{13}C NMR (101 MHz, DMSO) δ 170.3, 162.0, 160.1, 157.8, 157.1, 155.5, 146.6, 140.55, 133.5, 130.0, 120.3, 119.3, 116.9, 115.8, 94.7, 77.5, 51.7, 50.3, 34.2, 30.6, 22.2. Anal. Calcd for $C_{29}H_{34}FN_7O$: C, 67.55; H, 6.65; N, 19.02; Found: C, 67.56; H, 6.61; N, 19.05.

4-(4-fluorophenyl)-8-(2-((4-(piperidin-1-yl)phenyl)amino)pyrimidin-4-yl)-1,4,8-triazaspiro[4.5]decan-2-one (**13i**)

m.p.: 206.7 – 208.1 °C; HRMS (ESI-Q-TOF, m/z): 502.2728[M+H]$^+$; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.57 (s, 1H), 8.78 (s, 1H), 7.92 (d, *J* = 6.0 Hz, 1H), 7.51 (d, *J* = 9.0 Hz, 2H), 7.05 (t, *J* = 8.8 Hz, 2H), 6.95 – 6.73 (m, 4H), 6.23 (d, *J* = 6.1 Hz, 1H), 4.65-4.23 (s, 2H), 3.85 (s, 2H), 3.15 (t, *J* = 13.1 Hz, 2H), 2.99 (t, *J* = 5.4 Hz, 4H), 2.22 (td, *J* = 13.1, 5.1 Hz, 2H), 1.70 – 1.40 (m, 8H). ^{13}C NMR (101 MHz, DMSO) δ 170.3, 162.0, 160.1, 157.8, 157.1, 155.5, 146.9, 140.5, 133.6, 120.3, 119.3, 116.9, 115.8, 94.8, 77.5, 51.7, 51.1, 34.4, 25.9, 24.3. Anal. Calcd for $C_{28}H_{32}FN_7O$: C, 67.05; H, 6.43; N, 19.55; Found: C, 67.00; H, 6.47; N, 19.58.

4-(4-fluorophenyl)-8-(2-((4-(4-hydroxypiperidin-1-yl)phenyl)amino)pyrimidin-4-yl)-1,4,8-triazaspiro[4.5]decan-2-one (**13j**)

Yield: 23%. m.p.: 250.6 – 252.6 °C; HRMS (ESI-Q-TOF, m/z): 518.2134[M+H]$^+$; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.55 (s, 1H), 8.75 (s, 1H), 7.92 (d, *J* = 6.0 Hz, 1H), 7.50 (d, *J* = 8.7 Hz, 2H), 7.04 (t, *J* = 8.8 Hz, 2H), 6.92 – 6.80 (m, 4H), 6.23 (d, *J* = 6.0 Hz, 1H), 4.64 (s, 1H), 4.63-4.21 (m, 2H), 3.85 (s, 2H), 3.61-3.53 (m, 1H), 3.42-3.35(m, 2H), 3.15 (t, *J* = 13.1 Hz, 2H), 2.71 (ddd, *J* = 12.7, 10.3, 2.9 Hz, 2H), 2.21 (td, *J* = 13.2, 5.1 Hz, 2H), 1.84-1.76 (m, 2H), 1.65 (d, *J* = 12.8 Hz, 2H), 1.53-1.42 (m, 2H). ^{13}C NMR (151 MHz, DMSO) δ 170.2, 161.9, 160.0, 157.4, 157.1, 146.1,

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140.4, 133.4, 120.2, 119.3, 116.7, 115.8, 94.6, 77.4, 66.4, 51.6, 47.9, 34.4. Anal. Calcd for $C_{28}H_{32}FN_7O_2$: C, 64.97; H, 6.23; N, 18.94; Found: C, 64.95; H, 6.26; N, 18.96.

8-(2-((4-(dimethylamino)phenyl)amino)pyrimidin-4-yl)-4-(4-fluorophenyl)-1,4,8-triazaspiro[4.5]decan-2-one(**13k**)

Yield: 24%. m.p.: 197.5 – 199.9 °C; HRMS (ESI-Q-TOF, m/z):462.2414$[M+H]^+$; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.56 (s, 1H), 8.69 (s, 1H), 7.91 (d, J = 6.0 Hz, 1H), 7.48 (d, J = 9.0 Hz, 2H), 7.05 (t, J = 8.8 Hz, 2H), 6.86 (dd, J = 9.1, 4.6 Hz, 2H), 6.67 (d, J = 9.0 Hz, 2H), 6.21 (d, J = 6.1 Hz, 1H), 4.460-4.23 (m, 2H), 3.85 (s, 2H), 3.15 (t, J = 13.1 Hz, 2H), 2.81 (s, 6H), 2.21 (td, J = 13.1, 5.0 Hz, 2H), 1.65 (d, J = 13.0 Hz, 2H). ^{13}C NMR (101 MHz, DMSO) δ 170.3, 162.0, 160.2, 157.2, 156.6, 146.1, 140.5, 131.6, 120.8, 119.4, 115.8, 113.4, 94.5, 77.5, 51.7, 41.2, 34.4. Anal. Calcd for $C_{25}H_{28}FN_7O$: C, 65.06; H, 6.12; N, 21.24; Found: C, 65.08; H, 6.18; N, 20.21.

8-(2-((4-(diethylamino)phenyl)amino)pyrimidin-4-yl)-4-(4-fluorophenyl)-1,4,8-triazaspiro[4.5]decan-2-one (**13l**)

Yield: 20%. m.p.: 209.4 – 211.6 °C; HRMS (ESI-Q-TOF, m/z): 490.2725$[M+H]^+$; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.53 (s, 1H), 8.60 (s, 1H), 7.90 (d, J = 5.9 Hz, 1H), 7.43 (d, J = 8.8 Hz, 2H), 7.04 (t, J = 8.8 Hz, 2H), 6.89 – 6.84 (m, 2H), 6.60 (d, J = 8.8 Hz, 2H), 6.19 (d, J = 6.0 Hz, 1H), 4.58-4.29 (m, 2H), 3.85 (s, 2H), 3.25 (q, J = 7.0 Hz, 4H), 3.14 (t, J = 13.2 Hz, 2H), 2.21 (td, J = 13.1, 5.0 Hz, 2H), 1.65 (d, J = 13.0 Hz, 2H), 1.04 (t, J = 7.0 Hz, 6H). ^{13}C NMR (151 MHz, DMSO) δ 170.3, 161.9, 160.0, 156.7, 156.6, 143.1, 140.4, 130.4, 121.3, 119.2, 115.8, 112.9, 94.3, 77.4, 51.6, 44.3, 34.3, 12.8. Anal. Calcd for $C_{27}H_{32}FN_7O$: C, 66.24; H, 6.59; N, 20.03; Found: C, 66.25; H, 6.58; N, 20.01.

5.2.1 *Anti-proliferative assay*

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MTT assay was carried out on A549, PC-3, HCT116, MCF-7 cells to evaluate anti-proliferative activity of the target compounds. The cancer cell lines were cultured in minimum essential medium (MEM) supplement with 10% foetal bovine serum (FBS) with 10% fetal bovine serum in 96-well plates in 5% CO_2 at 37 °C for 24 h, then treated with the test compounds at various concentrations in 100 μL medium. After incubation for another 96 h, methyl thiazolyltetrazolium bromide (MTT) solution (50 μL of 2 mg/mL) was added to each well and incubated for additional 4 h. The medium was removed, the generated formazan in individual wells was dissolved in DMSO (100 μL) and measured at 570 nm using a 96-well plate reader. The inhibition data was calculated as the ratio of cell number in the treated group to that control group. The results expressed as IC_{50}, calculated using the Bacus Laboratories Incorporated Slide Scanner (Bliss) software.

5.2.2 *In vitro enzymatic assay*

The *in vitro* enzymatic assay of the compounds were evaluated by homogeneous time-resolved fluorescence(HTFR) assay. The kinase base buffer was consist of 50 mM HEPES (pH 7.5), 0.01% Triton X-100, 10 mM $MgCl_2$, and 2 mM DTT. The stop buffer contained a mixture of 100 mM HEPES (pH 7.5), 0.015% Brij-35, 0.2% Coating Reagent and 50 mM EDTA.

Initially, the tested compounds were diluted to 50-fold of the desired highest concentration in reaction by 100% DMSO. The tested compound dilution (100 μL) was transfered into a well in 96-well plate. Then, the controls were formed by adding 100 μL of 100% DMSO to two empty wells, which was marked as source plate. The intermediate plate was prepared by transferring 10 μL of compound from source plate to a new 96-well plate. In the intermediate plate, additional 90 μL of kinase buffer was added to each well. The intermediate plate was swayed for 10 min. Then,

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transferring 5 μL of each well from the 96-well intermediate plate to a 384-well plate in duplicates as the assay plate. In the each well of 384-well assay plate, the prepared enzyme solution (appropriate kinase in kinase base buffer) was added. The plate was then incubated at room temperature for 10 min. After that, the addition 10 μL of prepared peptide solution (FAM-labeled peptide and ATP in kinase base buffer) was added. The sample was incubated at 28 °C for 1 h, then 25 μL of stop buffer was added. The conversion data was copied from Caliper program, and the values were converted to inhibition values. Percent inhibition = (max - conversion)/ (max - min) × 100.

5.2.3 Cell migration assay

The A549 cells were trypsinized and added to a 6-well plate (5×10^4/well), then, allowed to grow to 100% confluence. A linear wound was made by a pipet tips across the confluent cell layer of each well. A549 cells were washed twice with PBS, and then incubated with culture medium containing DMSO or different concentrations of compound **9k**. After treatment for 12, 24 and 36 h, the images were taken by fluorescence microscope (Olympus, Tokyo, Japan).

5.2.4 Morphology assays of apoptotic cells

Apoptosis was measured by flow cytometry using Annexin V/propidium iodide (PI) double staining. A549 cells were added to a final concentration of 1×106/mL in a 6-well plate, and the plate was incubated for 24 h. Cells were treated with various concentrations of compounds. After being cultured for 48 h, control cells and treated cells were harvested and washed with PBS, and then resuspended in 100μL 1×binding buffer incubated in the mixture of 5μL Annexin V-FTIC and 5μL PI for 10 min at room temperature in dark place. The cells were resuspended in 400μL 1×binding buffer just before flow cytometric analysis.

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5.2.5 cell cycle assays.

Cell cycling was measured by propidium iodide (PI) staining. A549 cells were added to a final c ncentration of 1×106/ml in a 6-well plate, and the plate was incubated for 24 h. Cells were treated with of various concentrations of compounds. After being cultured for 24 h, control cells and treated cells were harvested and washed with PBS, then fixed with 70% ethanol and stored at 4℃ over night. Then, the ethanol-suspended cells were centrifuged, washed with PBS and stained with propidium iodide (PI) for 30 min at room temperature. followed by measuring of cell fluorescence.

Conflicts of interest

There are no conflicts to declare.

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Two series of 2-aminopyrimidine derivatives possessing triazolopiperazine or 1,4,8-triazaspiro[4.5]decan-3-one scaffolds were designed, synthesized and evaluated for their biological activity.