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Discovery of a novel covalent CDK4/6 inhibitor based on palbociclib scaffold

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ABSTRACT

Cyclin-dependent kinases 4 and 6 (CDK4/6), which are involved in dynamic regulation of cell cycle, play an indispensable role in controlling the tumor growth. Here, based on the scaffold of palbociclib, we designed and synthesized a series of covalent CDK4/6 inhibitors that targeted amino acid Thr107. The optimized compound **C-13** exhibited potent *in vitro* anticancer activity against CDK4/6 with high selectivity over CDK4/6. Moreover, **C-13** showed significant tumor growth inhibition in MDA-MB-231 tumor xenograft model (TGI of 93.49% at dose of 40 mg/kg) without causing significant weight loss and toxicity during the treatment period.

1. Introduction

The cyclin-dependent protein kinases (CDKs) are serine/threonine kinases that belong to GMGC family (CDKs, mitogen-activated protein kinase, glycogen synthase kinase, and CDK-like kinases) [1]. They are classified as regulators of cell cycles (CDKs1-6, 11 and 14–18) and transcriptions (CDKs7-13, 19 and 20), which are activated at certain points of the cell cycle and play a significant role in cell cycle control [2,3]. The uncontrolled deregulation of the cell cycle is a hallmark of cancer. Cell cycle arrest in cancer could suppress the growth and metastasis of tumor. Hence, CDKs are regarded as promising targets for the treatment of cancers and some diseases [4]. Among the CDKs, cyclin-dependent kinases 4 and 6 (CDK4/6) are responsible for cell cycle entrance and hyperactivated in cancer cells [5]. CDK4/6 plays an important role in promoting the G1 to S cell cycle transition by phosphorylating the tumor suppressor retinoblastoma (Rb). Once Rb is phosphorylated, it loses its E2F inhibitory effect, allowing the transcription of E2F-regulated genes, and G1 to S phase progression [6,7]. In 1990s, due to the low selectivity toward CDKs, the first generation CDK inhibitors such as Seliciclib, Dinaciclib and Milciclib show severe toxicity (Fig. 1) [8]. Thereafter, a new generation of selective CDK4/6 inhibitors (Fig. 1), such as Palbociclib (PD0332991), Ribociclib (LEE011) and Abemaciclib (LY2835219) were approved by FDA with improved effectiveness and decreased adverse effects [9]. Among these agents, the CDK4/6 inhibitor palbociclib in 2015 was regarded as a landmark, which applied to a combination therapy for estrogen receptor-positive and HER2-negative advanced breast cancer, contributing to prolongation of progression-free-survival [10,11].

In recent years, with the rapid development of the structural biology and bioinformatics, the rational design of targeted covalent inhibitors (TCIs) attracts more and more attentions [12,13]. Over the past several decades, several TCIs have been approved by the Food and Drug Administration, such as Afatinib, Ibrutinib, while more are in clinical research or being used as proteomic tools for the drugs discovery [14–16]. Covalent ligand-target interactions exhibit significant pharmacological benefits, including improved

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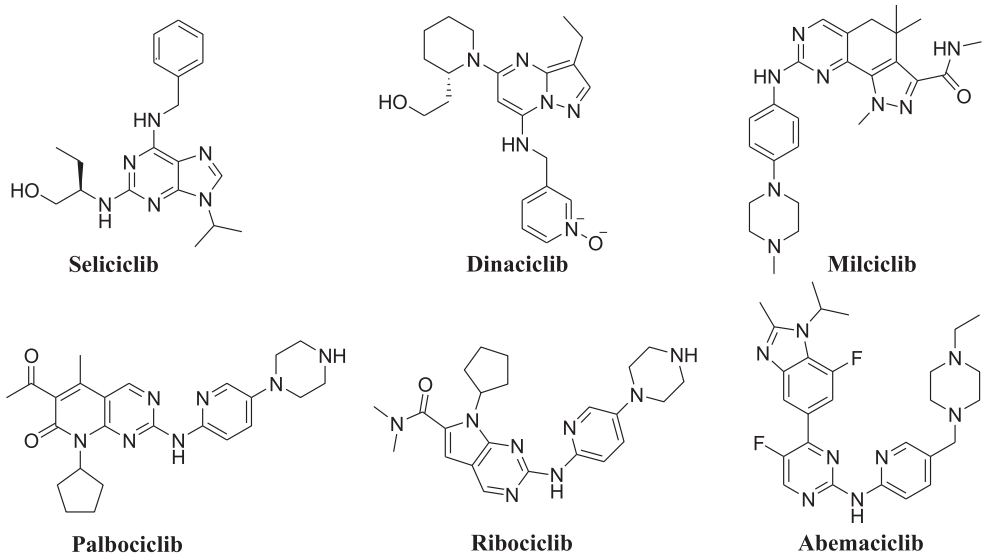


Fig. 1. Structures of several CDK inhibitors.

selectivity, potent cellular efficiency, less frequency and lower doses [17,18].

Through careful observation of the crystal structure of CDK6 in complex with palbociclib (PDB: 2EUF), Thr107, which located at the solvent accessible region of the binding pocket was found, which can be utilized as the nucleophile. Besides, we noted that the pyrido-[2,3-d]pyrimidin-7-ones ring of palbociclib was pivotal for specificity and activity. In addition, the piperazinylpyridine substituent of palbociclib is pointing out of the binding pocket toward the solvent region of the binding pocket. Replacing the piperazine group with some kinds of heterocyclic groups, including piperidine, 4-hydroxypiperidine, morpholine, and 3,5-dimethylmorpholine, has little effect on the binding affinity, demonstrating that addition of a substituent group in this position might only exert a limited influence on the inhibitor activity and specificity [19,20]. It should be noted that the distance between the Thr107 hydroxyl and piperazine nitrogen is 3.4−3.6 Å, which intrigue us to strategically place an electrophilic warhead with or without a linker on the position of piperazine group, which will make the inhibitor form the covalent bond with amino acid Thr107, which is much stronger than non-covalent interactions [21].

Subsequently, in order to discover and develop a potent and selective CDK4/6 inhibitor, we report the research of a series of novel covalent inhibitors based on the palbociclib scaffold that targeted key Thr107 of the CDK6. Finally, compound **C-13** was obtained with potent and highly selective CDK4/6 inhibition activity and excellent anticancer activity *in vivo*.

2. Result and discussion

2.1. Covalent molecular design

Based on the crystal structure of CDK6 in complex with palbociclib (2EUF) (Fig. 2), we noticed that Thr107, which located in the edge of the binding pocket can be utilized as a nucleophile for covalent bonding. It was observed that the NH group on piperazine of palbociclib, which is located at the solvent region, is an ideal modification site for attachment of warheads without blocking the crucial binding interaction. It should be noted that there is a distance of 3.4−3.6 Å between piperazine nitrogen and Thr107, due to the flexibility of the solvent accessible region, there might be a huge change of the distance, therefore a series of linkers with different length and composition was considered (Table 2).

As far as the electrophilic moieties were concerned, the research of Christian Jöst found that acryl- and chloroacetylamides/anilides exhibited astonishingly low off-target reactivity [22]. Acrylamide is a classical Michael-acceptor, targeting nucleophiles like general cysteine [23] or threonine [24] in some cases [22]. Besides, the chloroacetylamide group is a Michael-acceptor, which contributes to the covalent binding with an active site cysteine via nucleophilic substitution of the halogen in metazachlor [25]. Therefore, in this work three electrophilic moieties, acrylamide, α-halogen ketones, and cyanogroup were investigated (Table 1).

The NH group in piperazine of palbociclib is located at the solvent region, which is suitable for attachment of linkers without loss of vital binding interaction. To obtain selective and potent covalent inhibitors for CDK4/6, a series of palbociclib derivatives with three different electrophilic warheads and linkers were designed and synthesized.

2.2. Synthetic chemistry

In this work, 39 compounds were synthesized and characterized (Table 2). The designed CDK6-targeting covalent compounds required the corresponding palbociclib as the initial materials. All synthetic steps were presented in the following schemes.

Carboxylic acid derivatives of palbociclib were prepared in Scheme 1. They were synthesized by coupling palbociclib with *tert*-butyl bromoacetate or *tert*-butyl acrylate under alkaline condition, followed by acidolysis to get compound **PD-3** or **PD-5** (Scheme 1).

It was critical to construct the central structure for the synthesis of A series compound (Scheme 2). Firstly, a series of alkyl linker with different length and two hydrophilic alkyl ether chains were employed to react with acryloyl chloride, followed by N-Boc deprotection with CF_3CO_2H to give amines **4a**-**4d**, **7a**-**7b**. Then the target compounds **A-1** to **A-12** were obtained via acylation between **PD-3** or **PD-5** and intermediate amine in the presence of HATU as well as DIPEA.

In order to get the A series and B series compounds, intermediates **8a**-**8d**, **9a**-**9b**, **10a**-**10d** and **11a**-**11b** were synthesized by acylation using palbociclib and Mono-Boc protected diamines as materials. Subsequently, the Boc-protecting group of the

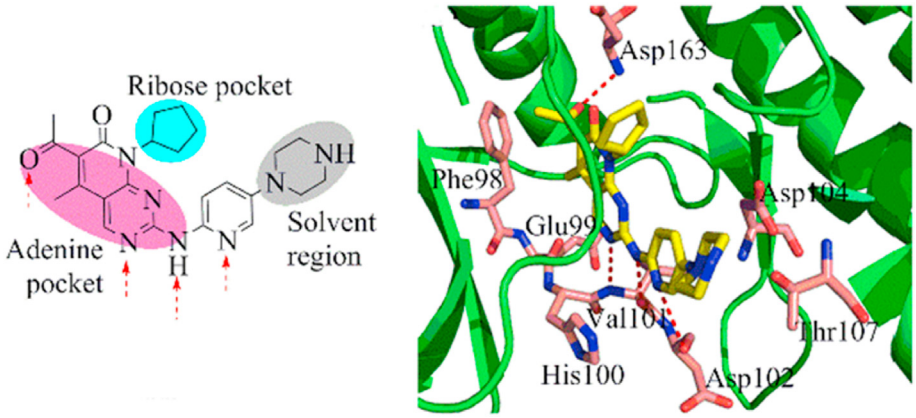
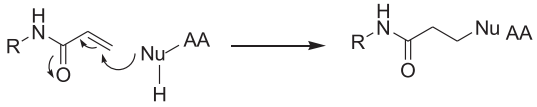
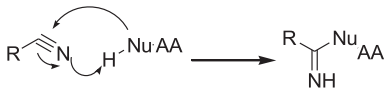
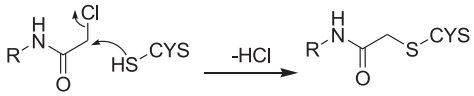


Fig. 2. Structure of Palbociclib and co-crystal of Palbociclib binding with CDK6 (2EUF).

Table 1
Three chosen electrophiles for covalent protein modification.

Electrophile	Targeted nucleophiles	Binding mode
	Cysteine Threonine	
	Serine Cysteine	
	Cysteine	

intermediate was removed under acid condition, which coupled bromoacetonitrile to afford compounds **B-1** to **B-12** (Scheme 3) or reacted with chloroacetyl chloride to generate compounds **C-1** to **C-12** (Scheme 4). In addition, palbociclib was reacted with acryloyl chloride or bromoacetonitrile to get the compounds **A-13** and **B-13** in one step process, respectively. The desired covalent compound **C-13** was produced through an acylation coupling of palbociclib with chloroacetyl chloride in the presence of triethylamine.

2.3. Antiproliferative activity in vitro

All the obtained compounds were assessed for anti-proliferative activity against a panel of cancer lines by MTT assay, including human breast cancer cell line (MCF-7, MDA-MB-231, MDA-MB-453) and human non-small cell lung cancer cell line (H1299), utilizing palbociclib as a reference drug. As Table 3 shown, the A series and B series compounds displayed a significant loss in cellular activity in comparison with the C series compounds, indicating that the electrophilic alpha-chloroacetamide group may be more suitable than the other two electrophilic warheads when targeted to Thr107 of CDK6. In MDA-MB-453 cell line, compounds **C-3**, **C-4**, **C-9** and **C-13** of the C series exhibited excellent anticancer potency compared with the positive control, and the IC_{50} values ranges from 1.75 to 3.55 μM. However, the compound **C-3**, **C-4** and **C-9** showed less anti-proliferation activity on other three cell lines than palbociclib, which might be associated with the linker length and membrane permeability. It should be noted that, without the linker, compound **C-13** exhibited the most potent anticancer activity with IC_{50} values against MDA-MB-231, MDA-MB-453 and H1299 at 1.21 μM, 1.75 μM and 2.49 μM, respectively, which is about 4-fold than palbociclib.

2.4. Binding mode analysis of C-13 with CDK6

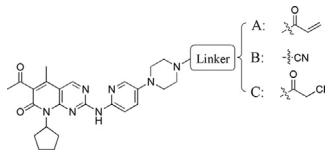
To interpret the activity of **C-13**, the potential binding mode of **C-13** with CDK6 was investigated by using AutoDock 4.0 [26]. As shown in Fig. 3, the original four hydrogen bonds between palbociclib group and CDK6 were maintained. Besides, it was observed that the terminal electrophilic chloroacetylamide group could approach Thr107 effectively, indicating that the CDK6 protein might form a stable covalent bond with the chloroacetylamide group of compound **C-13** without adding of extra linker group.

To primarily verify the covalent nature of compound **C-13**, the crucial terminal chloride was replaced by hydrogen or methyl group, e.g compound **D-1** and **D-2** were synthesized (Fig. 4). After MTT assay in four tumor cell lines, they almost had no anti-proliferative potency (>50 μM) against the breast cell lines, indicating that the chloride atom might form a covalent bond with Thr107 of CDK6 protein, which play a vital role in the improved anti-proliferative potency.

2.5. Plate clone formation assay and cellular toxicity of compound C-13

To further investigate the activity of compound **C-13**, plate clone formation assay was conducted, which was incubated with MDA-MB-231 cells for clone formation. From Fig. 5A, when treated with

Table 2
Chemical structures of designed CDK4/6 covalent inhibitors derived from palbociclib, three chosen electrophiles and different linkers used for synthesis..



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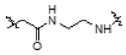
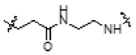
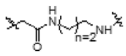
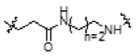
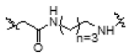
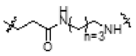
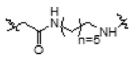
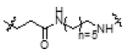
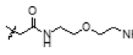
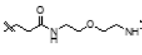
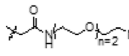
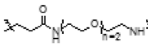
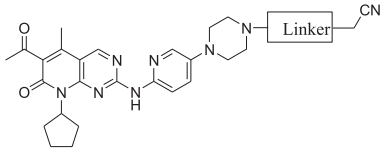
A-1		A-2	
A-3		A-4	
A-5		A-6	
A-7		A-8	
A-9		A-10	
A-11		A-12	
A-13	No linker		



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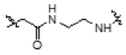
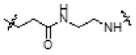
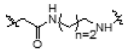
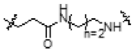
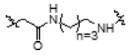
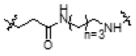
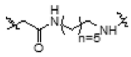
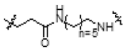
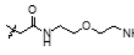
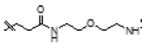
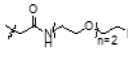
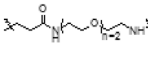
B-1		B-2	
B-3		B-4	
B-5		B-6	
B-7		B-8	
B-9		B-10	
B-11		B-12	
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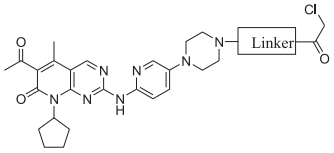


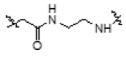
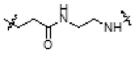
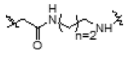
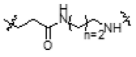
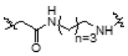
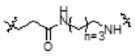
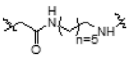
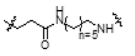
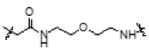
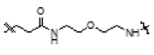
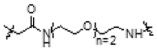
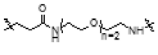
Compounds	Linkers	Compounds	Linkers
C-1		C-2	
C-3		C-4	

Table 2 (*continued*)

Compounds	Linkers	Compounds	Linkers
C-5		C-6	
C-7		C-8	
C-9		C-10	
C-11		C-12	
C-13	No linker		

compound **C-13** at 0.16 μM, the proliferation of MDA-MB-231 cells is evidently impaired in comparison with the untreated control group. Furthermore, at the concentration of 0.625 μM, nearly no clone formed.

As for the covalent inhibitor, it is very necessary to evaluate its toxicity. Hence, the cytotoxic effect assay associated with compound **C-13** against human normal liver cell line (LO2) was conducted. As can be seen in **Fig. 5B**, when compared with palbociclib, compound **C-13** presented an approximately 2-fold drop in toxicity toward LO2 cells (5 μM up to 20 μM), indicating its safety.

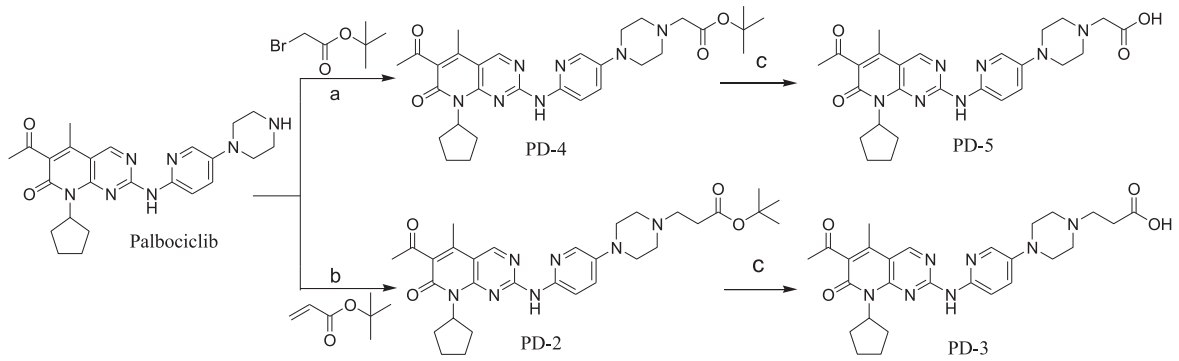
2.6. CDK4/6 kinase inhibition

Next, several C series compounds were chosen to evaluate the CDK4 and CDK6 kinase inhibition activities. As shown in Table 4, most compounds of this series exhibited potent inhibitory efficacy, the average %inhibition was >50%, which might be ascribed to the extra chloroacetylamide electrophilic warhead to palbociclib. Among these compounds, the compound **C-5** showed higher inhibition activity toward CDK4 (average %inhibition was 83.2%) and CDK6 (average %inhibition was 62.95%) with the concentration of 20 nM. However, the anticancer activity of **C-5** was noticeable weaker than **C-13**, which might result from poor cellular permeability and solubility. It should be noted that when the chloroacetylamide group was replaced with an acetamide or propenamide group, compounds **D-1** and **D-2** exhibited greatly compromised enzymatic potency, resulting in <50% inhibition rate toward CDK4 and CDK6 at a concentration of 20 nM, indicating the important role of chloroacetylamide group in forming of covalent bond with Thr107. Compound **C-13** shows slightly higher in IC_{50} of enzymatic inhibitory activities when compared with palbociclib (Table 5), due to the covalent bonding, **C-13** displayed 4-fold improved cellular potency in proliferation assay compared with palbociclib.

2.7. Cell-based inhibition of p-Rb and cell cycle arrest caused by compound C-13

CDK4/6 inhibitor is expected to induce cell cycle arrest by triggering accumulation of cancer cells at G1 in an Rb-dependent manner [27]. We compared the effects of compound **C-13** and palbociclib on the cell cycle profile of MDA-MB-231 cells through the flow cytometry analysis (Fig. 6). As expected, compound **C-13** and palbociclib induced a dose-dependent accumulation of these cells in the G1 phase. For instance, treatment with 1.5 μM of compound **C-13** led to around 20% increase in G1 cells compared to untreated cells.

To broadly assess the effect of compound **C-13** on the CDK4/6 and the phosphorylation of Rb (S780), Western blot assay on the



Scheme 1. Synthesis of **PD-2** and **PD-4**: a. 1.5 eq *tert*-butyl bromoacetate, 10.0 eq DIPEA, DCM, r.t, 4 h; b. 3.0 eq *tert*-butyl acrylate, 3.0 eq DBU, DCM, r.t, 5 h; c. DCM/TFA = 3:1, r.t, 5 h.

MDA-MB-231 cells was performed. As shown in Fig. 7, the compound **C-13** can blocked CDK4/6→Rb→E2F signaling pathway dose-dependently when incubated with MDA-MB-231 cells for 24 h. Notably, the treatment of MDA-MB-231 with compound **C-13** resulted in blockage of the Rb phosphorylation upon dose escalation, which is agreement with its cellular CDK4/6 targeted mechanism of action.

2.8. Compound C-13 induced apoptosis of MDA-MB-231 cells

In order to confirm the effect of compound **C-13** on apoptosis, the annexin V-FITC/propidium iodide staining assay on human breast tumor MDA-MB-231 cells was conducted. As shown in Fig. 8, the compound **C-13** is capable of inducing apoptosis in a dose-dependent manner. When the concentration ranges from 0.625 μM to 10 μM, MDA-MB-231 cells showed a pronounced increase in the percentage of early apoptotic stage from 4.24% to 68.48%. Apart from that, around 83% of treated cells entered apoptosis after 24 h treatment with compound **C-13** at the concentration of 10 μM. Based on the results, it seemed that the apoptosis was responsible for the observed anti-proliferative impact of compound **C-13**.

2.9. CDK kinases panel screening

High selectivity is an important factor in the design of safe and potent covalent inhibitor. So, compound **C-13** was further evaluated against a kinase panel of CDK families. Within a panel of enzymes, compound **C-13** exhibited absolute selectivity for CDK4/6 with little or no activity against other CDKs (Table 6), whose profile was similar with palbociclib.

2.10. In vivo antiproliferative activity of compound C-13

The anti-tumor activity *in vivo* of compound **C-13** was evaluated based on its covalent binding and ideal cellular activity. As illustrated in Fig. 9, after treatment by compound **C-13**, tumor volume and mass is significantly reduced in a dose-dependent manner. Tumor growth inhibitions (TGI) of 104.68%, 93.49% were observed in the MDA-MB-231 xenograft model at doses of 80 mg/kg and 40 mg/kg. Palbociclib at 40 mg/kg doses was 86.82%, slightly less active than compound **C-13** (93.49%, 40 mg/kg), demonstrating the advantage of compound **C-13** compared with palbociclib. Besides, compound **C-13** did not caused significant weight loss at high dose groups, suggesting its low toxicity.

3. Conclusions

In this study, we reported the design and synthesis of a novel series of CDK6-targeting covalent inhibitors based on the palbociclib scaffold. Three electrophilic warhead and a series of linker with different length and composition were evaluated. Among them, compound **C-13**, with the conjugation of palbociclib and chloroacetylamide electrophilic warhead, showed enhanced cytotoxic effects on human breast cancer cell line (MDA-MB-231 and MDA-MB-453) as well as human non-small cell lung cancer cell line (H1299) in comparison with palbociclib *in vitro*. In addition, **C-13** exhibited high selectivity over CDK4/6 in CDK kinases panel screening. Compound **C-13** also made impact significant inhibitory on phosphorylation of Rb by inducing G1 cell cycle arrest. In MDA-MB-231 xenograft model, compound **C-13** reduced tumor growth potently without causing significant weight loss and toxicity.

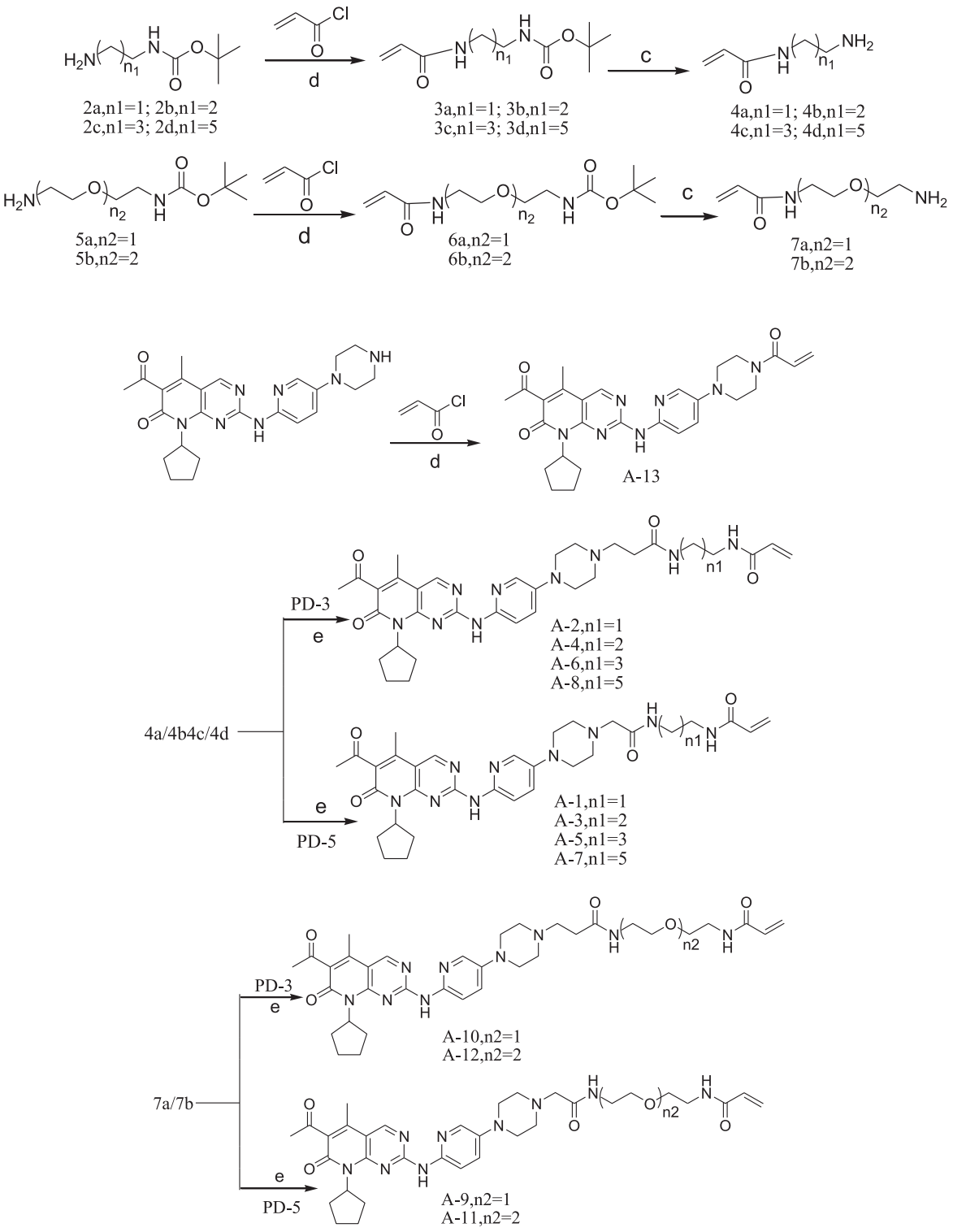
4. Experimental section

4.1. Chemistry

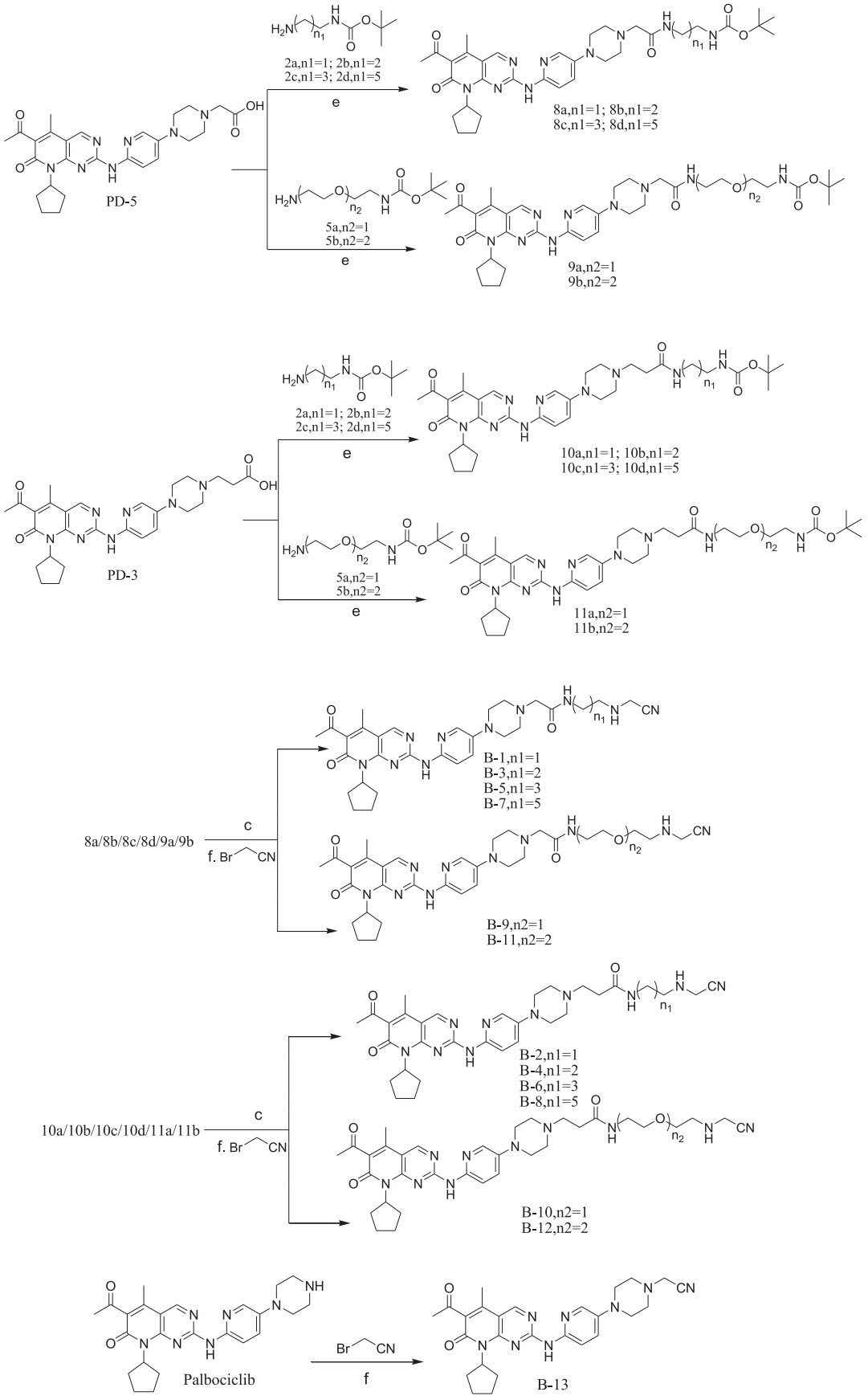
All reagents and solvents were obtained from commercial sources and used without further purification. Analytical thin chromatography (TLC) was performed on Yantai Shandong silica gel plates with QF254 fluorescence indicator, and spots were visualized by the UV night. Flash column chromatography was carried out using 200−400 mesh silica gel from Qingdao Haiyang. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded at 400 MHz on Bruker AMX 400 spectrometer, using residual deuterated solvent ($CDCl_3$, DMSO-d_6, CD_3OD) as standard reference. And chemical shifts are recored in ppm relative to the thtramethylsilane (TMS), and coupling constants (J) are noted in hertz. All the final compounds were examined with HPLC, and the purities of the biologically evaluated compounds were ≥95%.

4.1.1. tert-butyl3-(4-(6-(6-acetyl-8-cyclopentyl-5-methyl-7-oxo-7,8-dihydropyrido [2,3 -d]pyr-imidin-2-ylamino)pyridin-3-yl) piperazin-1-yl)propanoate (*PD-2*)

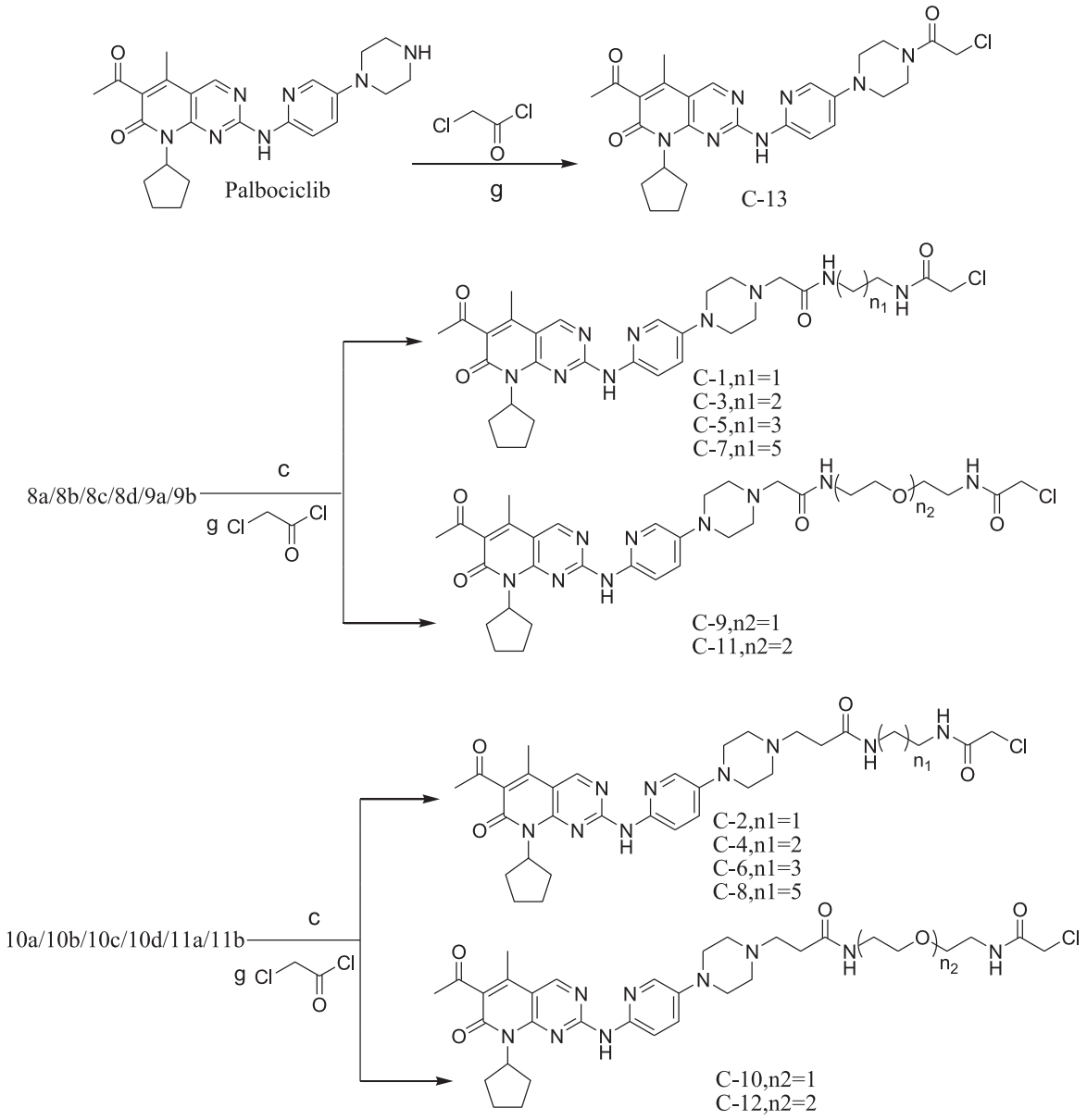
Tert-Butyl acrylate (0.045 mL, 0.312 mmol) and DBU (0.044 mL, 0.296 mmol) were added to a solution of Palbociclib (44.75 mg, 0.1 mmol) in DCM (15 mL), and the mixture was stirred at room temperature for 5 h. When TLC indicated full consumption of the starting material, the reaction was evaporated under reduced pressure. Then the reaction was diluted with EtOAc and washed twice with brine. The green layer was dried over anhydrous sodium sulfate, and concentrated in vacuo. The residue was purified by column chromatography to give **PD-2** as a green solid (54 mg, 94%).^{1}H NMR (400 MHz, $CDCl_3$) δ 8.87 (s, 1H), 8.16 (d, J = 9.1 Hz, 1H),



Scheme 2. Synthesis of the A series of compounds: d. 1.5 eq acryloyl chloride, 3.0 eq TEA, 0−25 °C, 4 h; e. 1.2 eq HATU, 3.0 eq DIPEA, DMF, r.t, overnight.



Scheme 3. Synthesis of the B series of compounds: f. 3.0 eq bromoacetonitrile, 3.0 eq K_2CO_3, 1.5 eq KI, DMF, 80 °C, overnight.



Scheme 4. Synthesis of the C series of compounds: Conditions: g. 2.0 eq chloroacetyl chloride, 3.0 eq TEA, THF, 0°C-r.t, 5 h.

8.08 (d, J = 2.7 Hz, 1H), 7.32 (dd, J = 9.1, 2.9 Hz, 1H), 3.27–3.17 (m, 4H), 2.74 (t, J = 7.3 Hz, 2H), 2.70–2.61 (m, 4H), 2.55 (s, 3H), 2.46 (t, J = 7.3 Hz, 2H), 2.42–2.31 (m, 5H), 2.10–2.02 (m, 2H), 1.92–1.87 (m, 2H), 1.45 (s, 9H), 1.26 (s, 3H).

*4.1.2. tert-butyl 2-(4-(6-(6-acetyl-8-cyclopentyl-5-methyl-7-oxo-7,8-dihydropyrido[2,3-d]pyrimidin-2-ylamino)pyridin-3-yl) piperazin-1-yl)acetate (**PD-4**)*

Tert-Butyl bromoacetate (0.054 mL, 0.375 mmol) and DIPEA (0.412 mL, 0.296 mmol) were added to a solution of Palbociclib (111.88 mg, 0.25 mmol) in DCM (30 mL), and the mixture was stirred at room temperature for 5 h. When TLC indicated full consumption of the starting material, the reaction was evaporated under reduced pressure. Then the reaction was extracted with EtOAc and washed twice with brine. The green layer was dried over anhydrous Na_2SO_4, and concentrated in vacuo. The material was purified by column chromatography to afford the compound **PD-4** as a green solid after concentaration (115 mg, 82%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.89 (s, 1H), 8.15 (d, J = 9.2 Hz, 1H), 8.11 (d, J = 2.8 Hz, 1H), 7.33 (dd, J = 9.1, 3.0 Hz, 1H), 3.30–3.23 (m, 4H), 3.20 (s, 2H), 2.84–2.74 (m, 4H), 2.55 (s, 3H), 2.42–2.31 (m, 5H), 2.09–2.02 (m, 2H), 1.92–1.86 (m, 2H), 1.48 (s, 9H), 1.26 (s, 3H).

*4.1.3. tert-butyl 2-acrylamidoethylcarbamate (**3a**)*

TEA (0.209 mL, 1.5 mmol) was added to a solution of compound **2a** (80.06 mg, 0.5 mmol) in DCM (15 mL), and stirred at 0 °C for 5 min. Acryloyl chloride (0.06 mL, 0.75 mmol) was added dropwise under a nitrogen atmosphere at 0 °C, the mixture was stirred at room temperature for 4 h. The reaction was monitored by TLC. After completion of the reaction, it was evaporated under reduced pressure. Water (20 mL) was added and the mixture was extracted with EtOAc (20 mL × 3) and organic layer washed twice with brine, dried over anhydrous Na_2SO_4. The organic layer was concentrated in vacuo. The crude products **3a** were purified on silica gel column chromatography to give compound **3a** (90 mg, 84%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.26 (d, J = 17.0 Hz, 1H), 6.11 (dd, J = 17.1,

Table 3
Antiproliferative activities of compounds against various cell lines *in vitro*.

Compound	IC50 (μM)			
	H1299	MDA-MB-231	MDA-MB-453	MCF-7
A-1	>20	18.67 ± 1.56	>20	>20
A-2	>20	>20	>20	>20
A-3	>20	18.03 ± 0.47	>20	>20
A-4	>20	18.29 ± 1.34	>20	>20
A-5	>20	>20	>20	>20
A-6	>20	>20	>20	>20
A-7	>20	14.85 ± 0.37	>20	>20
A-8	>20	>20	>20	>20
A-9	>20	>20	15.43 ± 0.78	>20
A-10	>20	>20	5.25 ± 0.12	>20
A-11	>20	>20	10.25 ± 0.91	>20
A-12	>20	>20	8.19 ± 0.56	>20
A-13	8.50 ± 0.38	>20	6.96 ± 0.48	>20
B-1	>20	>20	9.89 ± 0.74	>20
B-2	>20	>20	>20	>20
B-3	19.20 ± 1.22	>20	15.46 ± 0.98	>20
B-4	>20	>20	>20	>20
B-5	>20	>20	>20	>20
B-6	8.42 ± 0.49	12.50 ± 0.93	12.82 ± 0.71	>20
B-7	>20	>20	>20	>20
B-8	>20	>20	>20	>20
B-9	>20	>20	>20	>20
B-10	>20	>20	>20	>20
B-11	>20	>20	>20	>20
B-12	>20	>20	>20	>20
B-13	>20	17.86 ± 1.48	>20	>20
C-1	>20	10.23 ± 0.89	7.60 ± 0.38	2.39 ± 0.12
C-2	>20	>20	>20	>20
C-3	7.41 ± 0.31	7.80 ± 0.55	1.92 ± 0.11	5.22 ± 0.41
C-4	7.41 ± 0.43	9.67 ± 0.68	1.96 ± 0.13	5.48 ± 0.24
C-5	>20	8.32 ± 0.44	8.93 ± 0.36	12.4 ± 1.03
C-6	>20	>20	7.01 ± 0.43	16.6 ± 1.21
C-7	8.88 ± 0.45	9.89 ± 0.56	5.11 ± 0.32	14.5 ± 1.01
C-8	>20	>20	>20	>20
C-9	16.08 ± 1.23	>20	3.55 ± 0.21	4.25 ± 0.12
C-10	>20	>20	6.11 ± 0.33	11.5 ± 0.75
C-11	>20	>20	>20	>20
C-12	>20	8.92 ± 0.54	9.20 ± 0.49	15.8 ± 1.09
C-13	**2.49 ± 0.15**	**1.21 ± 0.10**	**1.75 ± 0.14**	**0.38 ± 0.02**
D-1	>50	>50	>50	>50
D-2	>50	142.5 ± 2.34	71.46 ± 3.42	25.67 ± 1.58
Palbociclib	8.92 ± 1.82	4.72 ± 0.36	6.93 ± 0.53	0.34 ± 0.03

10.2 Hz, 1H), 5.63 (dd, J = 10.2, 1.3 Hz, 1H), 3.44 (t, J = 5.5 Hz, 2H), 3.31 (t, J = 5.5 Hz, 2H), 1.43 (s, 9H).

*4.1.4. tert-butyl 3-acrylamidopropylcarbamate (**3b**)*

Compound **3b** (87 mg, 76%) was synthesized with similar procedures as **3a**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.30–6.11 (m, 2H), 5.62 (dd, J = 9.9, 1.9 Hz, 1H), 3.36 (t, J = 6.4 Hz, 2H), 3.17 (t, J = 6.1 Hz, 2H), 1.71–1.62 (m, 2H), 1.44 (s, 9H).

*4.1.5. tert-butyl 4-acrylamidobutylcarbamate (**3c**)*

Compound **3c** (98 mg, 81%) was synthesized with similar procedures as **3a**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.31–6.11 (m, 2H), 5.61 (dd, J = 10.0, 1.8 Hz, 1H), 3.34 (t, J = 6.5 Hz, 2H), 3.13 (t, J = 6.1 Hz, 2H), 1.59–1.48 (m, 4H), 1.43 (s, 9H).

*4.1.6. tert-butyl 6-acrylamidohexylcarbamate (**3d**)*

Compound **3d** (108 mg, 80%) was synthesized with similar procedures as **3a**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.29–6.11 (m, 2H), 5.60 (dd, J = 10.1, 1.5 Hz, 1H), 3.31 (t, J = 6.8 Hz, 2H), 3.10 (t, J = 6.2 Hz, 2H), 1.56–1.42 (m, 13H), 1.34 (s, 4H).

*4.1.7. tert-butyl 2-(2-acrylamidoethoxy)ethylcarbamate (**6a**)*

Compound **6a** (79 mg, 61%) was synthesized with similar procedures as **3a**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.30–6.16 (m, 2H), 5.64 (dd, J = 10.1, 1.6 Hz, 1H), 3.60–3.48 (m, 6H), 3.31 (m, 2H), 1.45 (s, 9H).

*4.1.8. tert-butyl 2-(2-(2-acrylamidoethoxy)ethoxy)ethylcarbamate (**6b**)*

Compound **6b** (105 mg, 70%) was synthesized with similar procedures as **3a**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.35–6.09 (m, 2H), 5.62 (dd, J = 10.1 Hz, 1.4 Hz, 1H), 3.65–3.52 (m, 10H), 3.33 (t, J = 13.1 Hz, 2H), 1.45 (s, 9H).

*4.1.9. tert-butyl2op-(2-(4-(6-(6-acetyl-8-cyclopentyl-5-methyl-7-oxo-7,8-dihydryrido [2,3-d]pyrimidin-2-ylamino)pyridin-3-yl) piperazin-1-yl)acetamido)ethylcarbamate (**8a**)*

To a solution of compound **PD-5** (50 mg, 0.099 mmol) in DMF (15 mL) was added compound **2a** (19 mg, 0.119 mmol), HATU (45 mg, 0.119 mmol), DIPEA (0.048 mL, 0.297 mmol) and the resulting solution was stirred at room temperature overnight. Then water (30 mL) was added and the mixture was extracted with ethyl acetate (20 mL × 3), washed with brine (30 mL × 3). The green organic layer was dried over anhydrous Na_2SO_4, concentrated under reduced pressure to intermediate product. The crude material was purified by flash column chromatography to give compound **8a** (9 mg, 14%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.85 (s, 1H), 8.18 (d, J = 9.1 Hz, 1H), 8.05 (d, J = 2.8 Hz, 1H), 7.33 (dd, J = 9.1, 2.9 Hz, 1H), 4.04 (s, 2H), 3.50 (s, 4H), 3.28–3.19 (m, 4H), 3.12 (s, 2H), 2.77–2.68 (m, 4H), 2.55 (s, 3H), 2.41–2.30 (m, 5H), 2.10–2.03 (m, 2H), 1.92–1.86 (m, 2H), 1.26 (s, 3H).

*4.1.10. tert-butyl 3-(2-(4-(6-(6-acetyl-8-cyclopentyl-5-methyl-7-oxo-7,8-dihydropyrido [2,3-d]pyrimidin-2-ylamino)pyridin-3-yl) piperazin-1-yl)acetamido)propylcarbamate (**8b**)*

Compound **8b** (25 mg, 38%) was synthesized with similar procedures as **8a**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.87 (s, 1H), 8.17 (d, J = 9.1 Hz, 1H), 8.07 (d, J = 2.8 Hz, 1H), 7.33 (dd, J = 9.1, 2.9 Hz, 1H), 3.35 (t, J = 6.5 Hz, 2H), 3.30–3.23 (m, 4H), 3.18 (t, 2H), 3.11 (s, 2H), 2.79–2.70 (m, 4H), 2.55 (s, 3H), 2.41–2.30 (m, 5H), 2.11–2.02 (m, 2H), 1.89–1.87 (m, 2H), 1.69–1.66 (m, 2H), 1.44 (s, 9H), 1.25 (s, 3H).

4.1.11. tert-butyl 4-(2-(4-(6-(6-acetyl-8-cyclopentyl-5-methyl-7-oxo-7,8-dihydropyrido

***[2,3-d]pyrimidin-2-ylamino)pyridin-3-yl)piperazin-1-yl)acetamido)butylcarbamate (8c)*.**

Compound **8c** (18 mg, 27%) was synthesized with similar procedures as **8a**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.92 (s, 1H), 8.17 (d, J = 9.1 Hz, 1H), 8.08 (d, J = 2.8 Hz, 1H), 7.32 (dd, J = 9.1, 2.9 Hz, 1H), 3.33 (t, J = 6.1 Hz, 2H), 3.25–3.21 (m, 4H), 3.16–3.09 (m, 4H), 2.76–2.70 (m, 4H), 2.55 (s, 3H), 2.40–2.33 (m, 5H), 2.10–2.05 (m, 2H), 1.93–1.85 (m, 2H), 1.59–1.52 (m, 4H), 1.43 (s, 9H), 1.26 (s, 3H).

*4.1.12. tert-butyl 6-(2-(4-(6-(6-acetyl-8-cyclopentyl-5-methyl-7-oxo-7,8-dihydropyrido [2,3-d]pyrimidin-2-ylamino)pyridin-3-yl) piperazin-1-yl)acetamido)hexylcarbamate (**8d**)*

Compound **8d** (35 mg, 50%) was synthesized with similar procedures as **8a**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.90 (s, 1H), 8.17 (d, J = 9.1 Hz, 1H), 8.08 (d, J = 2.8 Hz, 1H), 7.34 (dd, J = 9.1, 2.9 Hz, 1H), 3.34–3.14 (m, 8H), 3.10 (s, 2H), 2.79–2.70 (m, 4H), 2.55 (s, 3H), 2.41–2.31 (m, 5H), 2.10–2.04 (m, 2H), 1.93–1.85 (m, 2H), 1.73–1.66 (m, 2H), 1.56–1.51 (m, 2H), 1.43 (s, 9H), 1.37–1.34 (m, 4H), 1.27 (s, 3H).

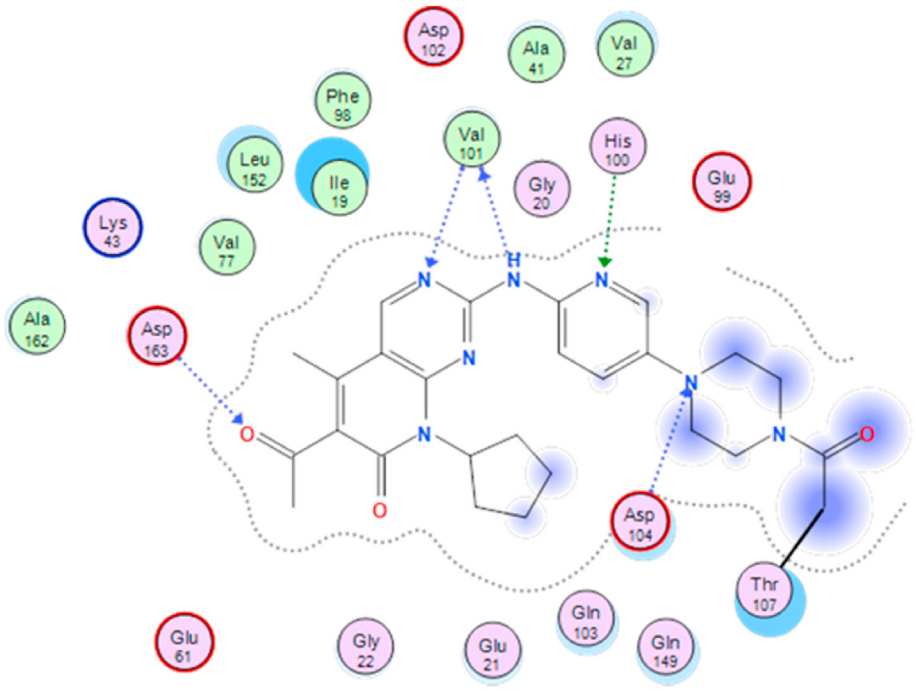


Fig. 3. Predicted binding mode of compound **C-13** with CDK6.

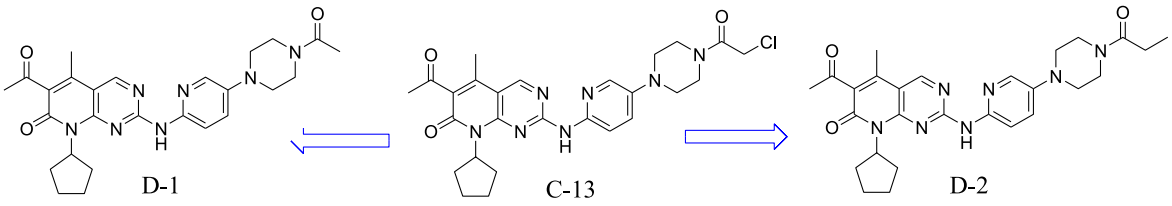


Fig. 4. Chemical structures of compounds **D-1** and **D-2**.

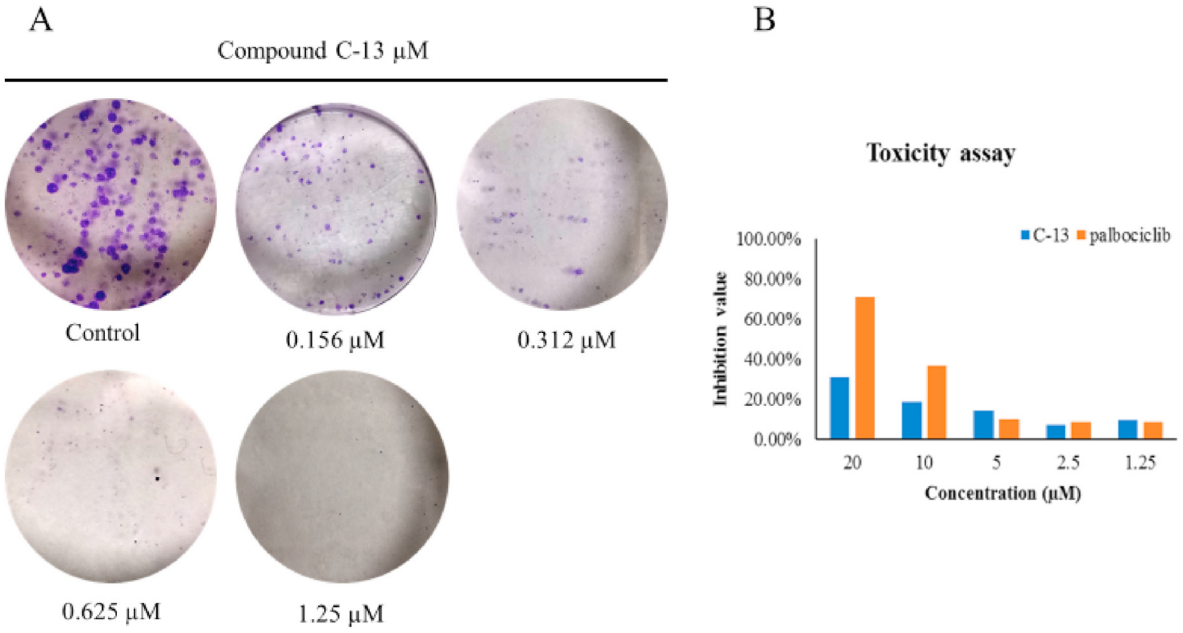


Fig. 5. (A) Colony-formation assays. MDA-MB-231 cells were treated with diverse concentration of **C-13**, untreated cells were used as the control. (B) Toxicity of **C-13** against LO2.

Table 4
The average %inhibition of compounds against CDK4 and CDK6 at the concentration of 20 nM and 200 nM *in vitro*.

Compound	CDK6 (Average % inhibition)		CDK4 (Average % inhibition)	
	200 nM	20 nM	200 nM	20 nM
C-1	93.42	60.77	104.20	81.60
C-3	91.74	56.98	100.70	92.10
C-4	93.09	65.47	96.40	79.00
C-5	94.81	62.95	104.00	83.20
C-6	91.95	52.11	100.40	89.20
C-7	90.90	45.68	98.20	71.30
C-9	92.71	61.40	101.60	76.70
C-10	94.39	60.47	103.20	91.20
C-13	93.76	61.69	95.30	81.60
D-1	87.62	43.20	100.40	68.50
D-2	91.36	42.95	103.00	69.80
Palbociclib	98.17	70.94	104.70	94.10

Table 5
The enzymatic inhibitory activities of **C-13** toward CDK4 and CDK6 *in vitro*.

	Compound C-13 (nM)	Palbociclib (nM)
CDK6 (Cyclin D3)	14 ± 1.01	10.27 ± 0.63
CDK4 (Cyclin D3)	6.1 ± 0.32	3.44 ± 0.16

*4.1.13. tert-butyl2-(2-(2-(2-(4-(6-(6-acetyl-8-cyclopentyl-5-methyl-7-oxo-7,8-dihydrop yrido [2,3-d]pyrimidin-2-ylamino) pyridin-3-yl)piperazin-1-yl)acetamido)ethoxy)etho xy) ethylcarbamate (**9b**)*

Compound **9b** (20 mg, 29%) was synthesized with similar procedures as **8a**.^{1}H NMR (400 MHz, $CDCl_3$) δ 8.87 (s, 1H), 8.17 (d, J = 9.7 Hz, 1H), 8.07 (d, J = 2.8 Hz, 1H), 7.34 (dd, J = 9.1, 2.9 Hz, 1H), 3.75–3.56 (m, 8H), 3.53 (t, J = 5.2 Hz, 4H), 3.26–3.20 (m, 4H), 3.12 (s, 2H), 2.55 (s, 3H), 2.41–2.33 (m, 5H), 2.10–2.04 (m, 2H), 1.93–1.86 (m, 2H), 1.44 (s, 9H), 1.26 (s, 3H).

*4.1.14. tert-butyl 2-(3-(4-(6-(6-acetyl-8-cyclopentyl-5-methyl-7-oxo-7,8-dihydropyrido [2,3-d]pyrimidin-2-ylamino)pyridin-3-yl) piperazin-1-yl)propanamido)ethylcarbamate (**10a**)*

To a solution of compound **PD-3** (50 mg, 0.0963 mmol) in DMF (15 mL) was added compound **2a** (19 mg, 0.116 mmol), HATU (44.1 mg, 0.115 mmol), DIPEA (0.048 mL, 0.289 mmol) and the resulting solution was stirred at room temperature overnight. Then water (30 mL) was added and the mixture was extracted with ethyl acetate (20 mL × 3), washed with brine (30 mL × 3). The green organic layer was dried over anhydrous Na_2SO_4, concentrated under reduced pressure to intermediate product. The crude material was purified by flash column chromatography to give compound **10a** (23 mg, 36%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.82 (s, 1H), 8.18 (d, J = 9.1 Hz, 1H), 8.05 (d, J = 2.6 Hz, 1H), 7.33 (dd, J = 9.1, 2.8 Hz, 1H), 3.41–3.34 (m, 2H), 3.30–3.21 (m, 6H), 2.78–2.62 (m, 6H), 2.55 (s, 3H), 2.44 (t, J = 6.1 Hz, 2H), 2.40–2.30 (m, 5H), 2.11–2.03 (m, 2H), 1.93–1.85 (m, 2H), 1.43 (s, 9H), 1.25 (s, 3H).

*4.1.15. tert-butyl 3-(3-(4-(6-(6-acetyl-8-cyclopentyl-5-methyl-7-oxo-7,8-dihydropyrido [2,3-d]pyrimidin-2-ylamino)pyridin-3-yl) piperazin-yl)propanamido)propylcarbamate (**10b**)*

Compound **10b** (18 mg, 28%) was synthesized with similar procedures as **10a**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.87 (s, 1H), 8.17 (d, J = 9.1 Hz, 1H), 8.08 (d, J = 2.8 Hz, 1H), 7.33 (dd, J = 9.1, 2.9 Hz, 1H), 3.30 (t, J = 6.1 Hz, 2H), 3.26–3.21 (m, 4H), 3.17 (t, J = 6.1 Hz, 2H), 2.78–2.66 (m, 6H), 2.55 (s, 3H), 2.45 (t, J = 6.3 Hz, 2H), 2.39–2.30 (m, 5H), 2.11–2.03 (m, 2H), 1.91–1.87 (m, 2H), 1.72–1.66 (m, 2H), 1.42 (s, 9H), 1.26 (s, 3H).

*4.1.16. tert-butyl4-(3-(4-(6-(6-acetyl-8-cyclopentyl-5-methyl-7-oxo-7,8-dihydropyrido [2,3-d]pyrimidin-2-ylamino)pyridin-3-yl) piperazin-1-yl)propanamido)butylcarbamate (**10c**)*

Compound **10c** (36 mg, 54%) was synthesized with similar procedures as **10a**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.87 (s, 1H), 8.17 (d, J = 8.6 Hz, 1H), 8.08 (d, J = 2.8 Hz, 1H), 7.34 (dd, J = 9.1, 3.0 Hz, 1H), 3.29–3.19 (m, 6H), 3.15–3.10 (m, 2H), 2.77–2.67 (m, 6H), 2.55 (s, 3H), 2.44 (t, J = 6.1 Hz, 2H), 2.40–2.30 (m, 5H), 2.13–1.98 (m, 4H), 1.93–1.85 (m, 2H), 1.72–1.67 (m, 2H), 1.42 (s, 9H), 1.26 (s, 3H).

*4.1.17. tert-butyl2-(2-(2-(3-(4-(6-(6-acetyl-8-cyclopentyl-5-methyl-7-oxo-7,8-dihydropyrido[2,3-d]pyri-midin-2-ylamino) pyridin-3-yl)piperazin-1-yl)propanamido)ethoxy) ethoxy) ethylcarbamate (**11b**)*

Compound **11b** (43 mg, 64%) was synthesized with similar procedures as **10a**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.87 (s, 1H), 8.19 (d, J = 9.1 Hz, 1H), 8.08 (d, J = 2.8 Hz, 1H), 7.34 (dd, J = 9.1, 2.9 Hz, 1H), 3.62–3.55 (m, 6H), 3.53–3.43 (m, 4H), 3.31–3.20 (m, 6H), 2.79–2.67 (m, 6H), 2.55 (s, 3H), 2.45 (t, J = 6.3 Hz, 2H), 2.42–2.33 (m, 5H), 2.10–2.03 (m, 2H), 1.92–1.84 (m, 2H), 1.44 (s, 9H), 1.25 (s, 3H).

*4.1.18. N-(2-(2-(4-(6-(6-acetyl-8-cyclopentyl-5-methyl-7-oxo-7,8-dihydropyrido[2,3-d] pyrimidin-2-ylamino)pyridin-3-yl)piperazin-1-yl)acetamido)ethyl)acrylamide (**A-1**)*

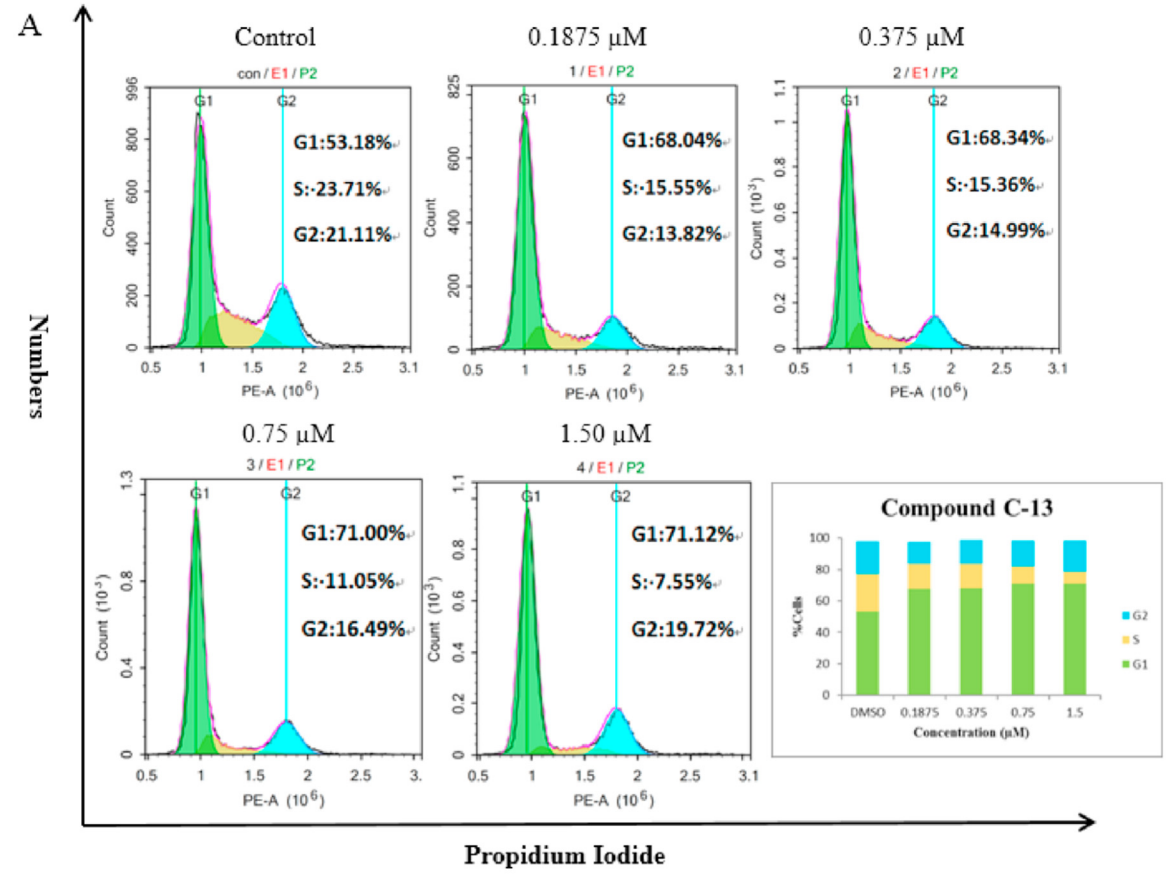
To a solution of compound **3a** (50 mg, 0.233 mmol) in DCM (3 mL) was added TFA (1 mL) and the resulting solution was stirred for 3 h at room temperature. The mixture was evaporated under reduced pressure, which directly dissolved into DMF (10 mL). To the DMF solution was added **PD-5** (98 mg, 0.194 mmol), HATU (89 mg, 0.233 mmol), DIPEA (261 mg, 1.94 mmol). The reaction was stirred at room temperature overnight. Then water (30 mL) was added and the mixture was extracted with ethyl acetate (20 mL × 3), washed with brine (30 mL × 3). The green organic layer was dried over anhydrous Na_2SO_4, concentrated under reduced pressure to crude product. The crude material was purified by flash column chromatography to give **A-1** as a green solid (16 mg, 14%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.89 (s, 1H), 8.17 (d, J = 9.1 Hz, 1H), 8.07 (d, J = 2.7 Hz, 1H), 7.32 (dd, J = 9.1, 2.9 Hz, 1H), 6.26 (dd, J = 17.0, 1.5 Hz, 1H), 6.12 (dd, J = 17.0, 10.2 Hz, 1H), 5.64 (dd, J = 10.2, 1.5 Hz, 1H), 3.51 (s, 4H), 3.29–3.19 (m, 4H), 3.09 (s, 2H), 2.78–2.67 (m, 4H), 2.55 (s, 3H), 2.43–2.30 (m, 5H), 2.11–2.03 (m, 2H), 1.93–1.85 (m, 2H), 1.26 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 202.70, 171.53, 166.26, 161.43, 158.13, 157.32, 155.55, 145.17, 143.33, 141.86, 136.56, 130.84, 130.64, 126.51, 125.97, 113.58, 107.60, 61.43, 54.13, 53.33, 49.48, 40.29, 39.15, 31.54, 28.07, 25.74, 13.95. HRMS (DART-TOF) calculated for $C_{31}H_{39}N_9O_4$ $[M + H]^+$ *m/z* 602.3199, found 602.3196.

*4.1.19. N-(2-(3-(4-(6-(6-acetyl-8-cyclopentyl-5-methyl-7-oxo-7,8-dihydropyrido[2,3-d]pyrimidin-2-ylamino)pyridin-3-yl)piperazin-1-yl)propanamido)ethyl)acrylamide (**A-2**)*

A-2 (21 mg, 24%) was synthesized with similar procedures as **A-1.** ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.79 (s, 1H), 8.18 (d, J = 8.8 Hz, 1H), 8.02 (d, J = 2.9 Hz, 1H), 7.33 (dd, J = 9.1, 2.9 Hz, 1H), 6.23 (dd, J = 17.0, 1.4 Hz, 1H), 6.09 (dd, J = 16.9, 10.1 Hz, 1H), 5.61 (dd, J = 10.1, 1.4 Hz,1H), 3.48 (t, J = 10.4 Hz, 4H), 3.21 (t, J = 4.3 Hz, 4H), 2.81–2.61 (m, 6H), 2.55 (s, 3H), 2.44 (t, J = 5.9 Hz, 2H), 2.40–2.26 (m, 5H), 2.10–2.03 (m, 2H), 1.92–1.85 (m, 2H), 1.26 (s, 3H). HRMS (DART-TOF) calculated for $C_{32}H_{41}N_9O_4$ $[M + Na]^+$ *m/z* 638.3174, found 638.3172.

*4.1.20. N-(3-(2-(4-(6-(6-acetyl-8-cyclopentyl-5-methyl-7-oxo-7,8-dihydropyrido[2,3-d] pyrimidin-2-ylamino)pyridin-3-yl)piperazin-1-yl)acetamido)propyl)acrylamide (**A-3**)*

A-3 (9.8 mg, 11.3%) was synthesized with similar procedures as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0.375 μM
0.75 μM
1.50 μM
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S:·23.71%
G2:21.11%
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S:·15.55%
G2:13.82%
G1:68.34%
S:·15.36%
G2:14.99%
G1:71.00%
S:·11.05%
G2:16.49%
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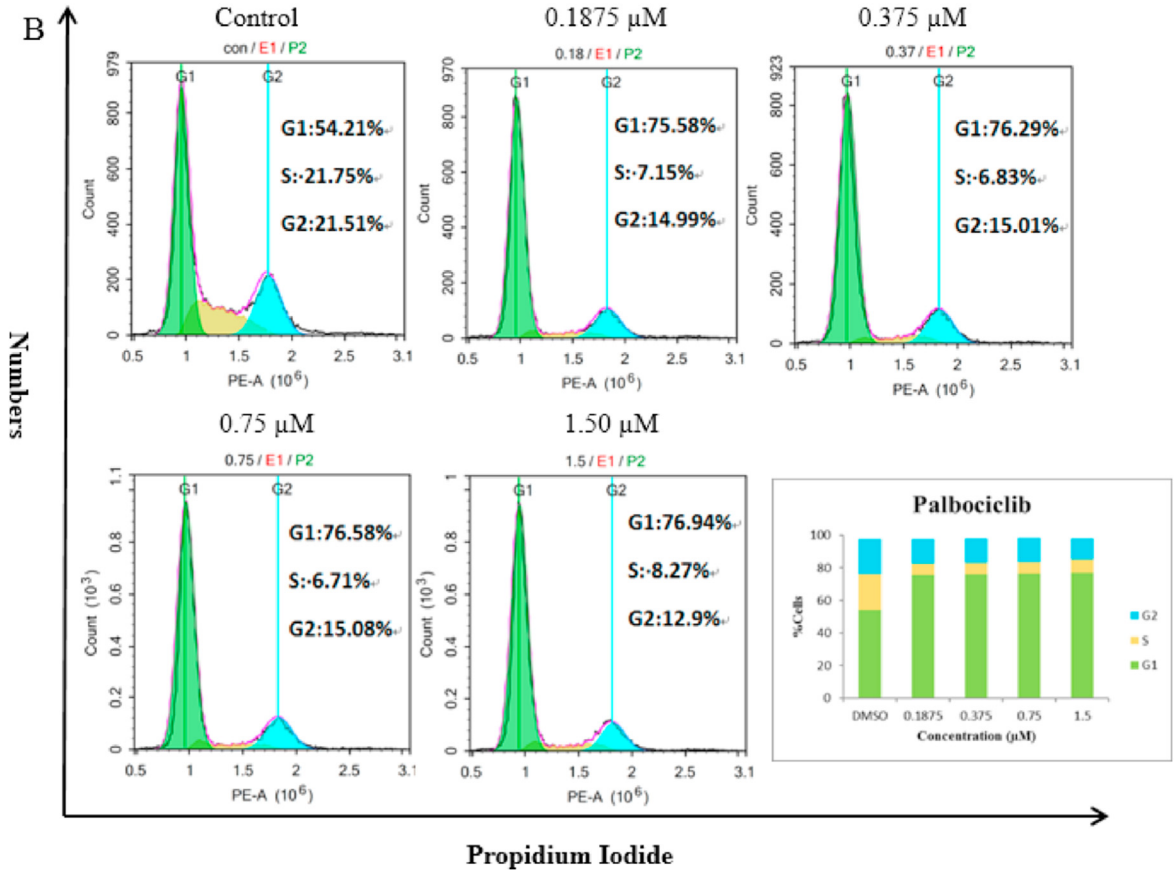
Compound C-13
%Cells
Concentration (μM)
DMSO
Numbers
Propidium Iodide


B
Control
0.1875 μM
0.375 μM
0.75 μM
1.50 μM
G1:54.21%
S:·21.75%
G2:21.51%
G1:75.58%
S:·7.15%
G2:14.99%
G1:76.29%
S:·6.83%
G2:15.01%
G1:76.58%
S:·6.71%
G2:15.08%
G1:76.94%
S:·8.27%
G2:12.9%
Palbociclib
%Cells
Concentration (μM)
DMSO
Numbers
Propidium Iodide

A-1. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.89 (s, 1H), 8.17 (d, J = 9.1 Hz, 1H), 8.07 (d, J = 2.7 Hz, 1H), 7.33 (dd, J = 9.1, 2.9 Hz, 1H), 6.27 (dd, J = 17.0, 1.4 Hz, 1H), 6.14 (dd, J = 17.0, 10.2 Hz, 1H), 5.63 (dd, J = 10.2, 1.4 Hz, 1H), 3.46–3.31 (m, 4H), 3.26–3.21 (m, 4H), 3.12 (s, 2H), 2.80–2.71 (m, 4H), 2.55 (s, 3H), 2.38 (s, 5H), 2.12–2.02 (m, 2H), 1.94–1.88 (m, 2H), 1.73–1.69 (m, 2H), 1.26 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 202.66, 170.85, 165.93, 161.42, 158.11, 157.30, 155.55, 145.16, 143.32, 141.83, 136.57, 131.14, 130.67, 126.12, 125.95, 113.57, 107.63, 61.53, 54.12, 53.41, 49.49, 35.89, 35.68, 31.54, 29.84, 28.07, 25.74, 13.95. HRMS (DART-TOF) calculated for $C_{32}H_{41}N_9O_4$ $[M + Na]^+$ *m/z* 638.3174, found 638.3175.

*4.1.21. N-(6-(4-(6-(6-acetyl-8-cyclopentyl-5-methyl-7-oxo-7,8-dihydropyrido[2,3-d] pyrimidin-2-ylamino)pyridin-3-yl)piperazin-1-yl)-4-oxohexyl)acrylamide (**A-4**)*

A-4 (22 mg, 20%) was synthesized with similar procedures as **A-1.** ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.82 (s, 1H), 8.18 (d, J = 9.0 Hz, 1H), 8.05 (d, J = 2.3 Hz, 1H), 7.34 (dd, J = 9.1, 2.6 Hz, 1H), 6.27 (dd, J = 16.9, 1.3 Hz, 1H), 6.13 (dd, J = 17.0, 10.2 Hz, 1H), 5.63 (dd, J = 10.1, 1.3 Hz, 1H), 3.41–3.28 (m, 4H), 3.27–3.16 (m, 4H), 2.79–2.61 (m, 6H), 2.55 (s, 3H), 2.46 (t, J = 6.1 Hz, 2H), 2.40–2.29 (m, 5H), 2.11–2.01 (m, 2H), 1.93–1.85 (m, 2H), 1.71–1.69 (m, 2H), 1.26 (s, 3H). HRMS (DART-TOF) calculated for $C_{33}H_{43}N_9O_4$ $[M + H]^+$ *m/z* 630.3512, found 630.3509.

*4.1.22. N-(4-(2-(4-(6-(6-acetyl-8-cyclopentyl-5-methyl-7-oxo-7,8-dihydropyrido[2,3-d] pyrimidin-2-ylamino)pyridin-3-yl)piperazin-1-yl)acetamido)butyl)acrylamide (**A-5**)*

A-5 (28 mg, 25%) was synthesized with similar procedures as **A-1.** ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.91 (s, 1H), 8.17 (d, J = 9.1 Hz, 1H), 8.07 (d, J = 2.7 Hz, 1H), 7.33 (dd, J = 9.1, 2.8 Hz, 1H), 6.27 (dd, J = 17.0, 1.5 Hz, 1H), 6.13 (dd, J = 17.0, 10.2 Hz, 1H), 5.62 (dd, J = 10.2, 1.5 Hz, 1H), 3.40–3.31 (m, 4H), 3.28–3.19 (m, 4H), 3.11 (s, 2H), 2.80–2.70 (m, 4H), 2.55 (s, 3H), 2.41–2.31 (m, 5H), 2.10–2.04 (m, 2H), 1.93–1.85 (m, 2H), 1.64–1.55 (m, 4H), 1.26 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 202.69, 170.15, 165.75, 161.42, 158.11, 157.32, 155.55, 145.19, 143.27, 141.86, 136.50, 130.96, 130.64, 126.22, 125.96, 113.57, 107.59, 61.55, 53.35, 49.48, 39.18, 38.51, 31.54, 28.07, 27.48, 26.57, 25.74, 13.95. HRMS (DART-TOF) calculated for $C_{33}H_{43}N_9O_4$ $[M + H]^+$ *m/z* 630.3512, found 630.3517.

*4.1.23. N-(4-(3-(4-(6-(6-acetyl-8-cyclopentyl-5-methyl-7-oxo-7,8-dihydropyrido[2,3-d] pyrimidin-2-ylamino)pyridin-3-yl)piperazin-1-yl)propanamido)butyl)acrylamide (**A-6**)*

A-6 (15 mg, 15%) was synthesized with similar procedures as **A-1.** ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.80 (s, 1H), 8.19 (d, J = 9.0 Hz, 1H), 8.04 (d, J = 2.5 Hz, 1H), 7.34 (dd, J = 9.1, 2.9 Hz, 1H), 6.25 (dd, J = 17.0, 1.3 Hz, 1H), 6.09 (dd, J = 17.0, 10.2 Hz, 1H), 5.61 (dd, J = 10.2, 1.3 Hz, 1H), 3.50–3.12 (m, 8H), 2.80–2.74 (m, 4H), 2.55 (s, 3H), 2.48 (t, J = 5.8 Hz, 2H), 2.41–2.22 (m, 5H), 2.11–2.04 (m, 2H), 1.91–1.85 (m, 2H), 1.63–1.53 (m, 4H), 1.26 (s, 3H). HRMS (DART-TOF) calculated for $C_{34}H_{45}N_9O_4$ $[M + H]^+$ *m/z* 644.3669, found 666.3667.

*4.1.24. N-(6-(2-(4-(6-(6-acetyl-8-cyclopentyl-5-methyl-7-oxo-7,8-dihydropyrido[2,3-d] pyrimidin-2-ylamino)pyridin-3-yl)piperazin-1-yl)acetamido)hexyl)acrylamide (**A-7**)*

A-7 (34 mg, 34%) was synthesized with similar procedures as **A-1.** ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.90 (s, 1H), 8.17 (d, J = 8.8 Hz, 1H), 8.08 (d, J = 2.8 Hz, 1H), 7.34 (dd, J = 9.1, 2.9 Hz, 1H), 6.27 (dd, J = 17.0, 1.5 Hz, 1H), 6.12 (dd, J = 17.0, 10.2 Hz, 1H), 5.61 (dd, J = 10.2, 1.5 Hz, 1H), 3.37–3.28 (m, 4H), 3.26–3.18 (m, 4H), 3.10 (s, 2H), 2.79–2.70 (m, 4H), 2.55 (s, 3H), 2.40–2.30 (m, 5H), 2.16–2.00 (m, 4H), 1.92–1.85 (m, 2H), 1.73–1.66 (m, 2H), 1.43–1.33 (m, 4H), 1.26 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 202.69, 169.85, 165.65, 161.42, 158.12, 157.31, 155.56, 145.21, 143.29, 141.85, 136.53, 131.03, 130.66, 126.10, 125.99, 113.60, 107.62, 61.60, 53.35, 49.54, 39.19, 38.61, 31.53, 29.62, 29.34, 28.06, 26.19, 26.15, 25.74, 13.95. HRMS (DART-TOF) calculated for $C_{35}H_{47}N_9O_4$ $[M + H]^+$ *m/z* 658.3825, found 658.3821.

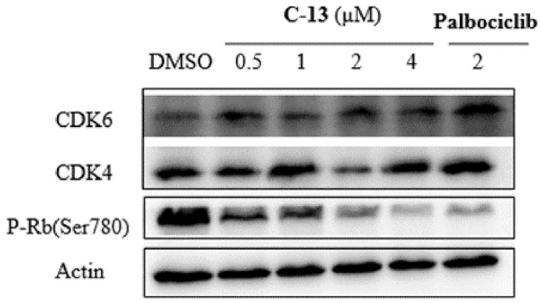


Fig. 7. Compound **C-13** inhibited the phosphorylation of Rb at Ser 780 in a dose-dependent manner in MDA-MB-231 cell lines.

*4.1.25. N-(6-(3-(4-(6-(6-acetyl-8-cyclopentyl-5-methyl-7-oxo-7,8-dihydropyrido[2,3-d]pyrimidin-2-ylamino)pyridin-3-yl)piperazin-1-yl)propanamido)hexyl)acrylamide (**A-8**)*

A-8 (16 mg, 13%) was synthesized with similar procedures as **A-1.** ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.82 (s, 1H), 8.18 (d, J = 9.0 Hz, 1H), 8.05 (d, J = 2.3 Hz, 1H), 7.34 (dd, J = 9.1, 2.6 Hz, 1H), 6.27 (dd, J = 16.9 Hz, 1.4 Hz, 1H), 6.13 (dd, J = 17.0, 10.2 Hz, 1H), 5.63 (dd, J = 10.1 Hz, 1.3 Hz, 1H), 3.40–3.30 (m, 4H), 3.27–3.17 (m, 4H), 2.81–2.66 (m, 6H), 2.55 (s, 3H), 2.46 (t, J = 6.1 Hz, 2H), 2.39–2.30 (m, 5H), 2.11–2.03 (m, 2H), 1.92–1.85 (m, 2H), 1.73–1.65 (m, 8H), 1.26 (s, 3H). HRMS (DART-TOF) calculated for $C_{36}H_{49}N_9O_4$ $[M + H]^+$ *m/z* 672.3982, found 672.3984.

*4.1.26. N-(2-(2-(2-(4-(6-(6-acetyl-8-cyclopentyl-5-methyl-7-oxo-7,8-dihydropyrido [2,3-d]pyrimidin-2-ylamino)pyridin-3-yl) piperazin-1-yl)acetamido)ethoxy)ethyl)acryl amide (**A-9**)*

A-9 (10 mg, 8%) was synthesized with similar procedures as **A-1.** ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.84 (s, 1H), 8.18 (d, J = 9.0 Hz, 1H), 8.05 (d, J = 2.7 Hz, 1H), 7.33 (dd, J = 9.1, 2.9 Hz, 1H), 6.27 (dd, J = 16.7 Hz, 1.6 Hz, 1H), 6.16 (dd, J = 17.0, 10.1 Hz, 1H), 5.61 (dd, J = 10.1, 1.6 Hz, 1H), 3.62–3.56 (m, 4H), 3.55–3.49 (m, 4H), 3.26–3.20 (m, 4H), 3.13 (s, 2H), 2.77–2.72 (m, 4H), 2.55 (s, 3H), 2.40–2.33 (m, 5H), 2.11–2.05 (m, 2H), 1.91–1.87 (m, 2H), 1.26 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 202.63, 166.20, 165.64, 161.41, 158.06, 157.22, 155.69, 155.54, 145.21, 142.57, 141.75, 136.53, 130.80, 126.49, 126.10, 113.61, 107.83, 70.02, 69.41, 61.58, 53.29, 49.53, 39.41, 38.58, 31.53, 28.09, 25.77, 13.96. HRMS (DART-TOF) calculated for $C_{34}H_{45}N_9O_5$ $[M + H]^+$ *m/z* 646.3461, found 646.3459.

*4.1.27. N-(2-(2-(3-(4-(6-(6-acetyl-8-cyclopentyl-5-methyl-7-oxo-7,8-dihydropyrido[2,3-d]pyrimidin-2-ylamino)pyridin-3-yl) piperazin-1-yl)propanamido)ethoxy)ethyl) acrylamide (**A-10**)*

A-10 (19 mg, 18%) was synthesized with similar procedures as **A-1.** ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.87 (s, 1H), 8.18 (d, J = 9.1 Hz, 1H), 8.07 (d, J = 2.8 Hz, 1H), 7.33 (dd, J = 9.1, 2.9 Hz, 1H), 6.29 (dd, J = 17.0, 1.6 Hz, 1H), 6.15 (dd, J = 17.0, 10.1 Hz, 1H), 5.62 (dd, J = 10.1, 1.5 Hz,

Fig. 6. MDA-MB-231 cell lines exhibited an obvious G1 arrest and a decrease of S phase after incubation with different doses of compound **C-13** (A) or palbociclib (B) for 12 h, the cell arrest progression was monitored by flow cytometery. Flow cytometry analysis of PI-stained cells with genomic DNA content. All the relative data to untreated cells and for comparison of the impact of indicated concentration on G1 phase.

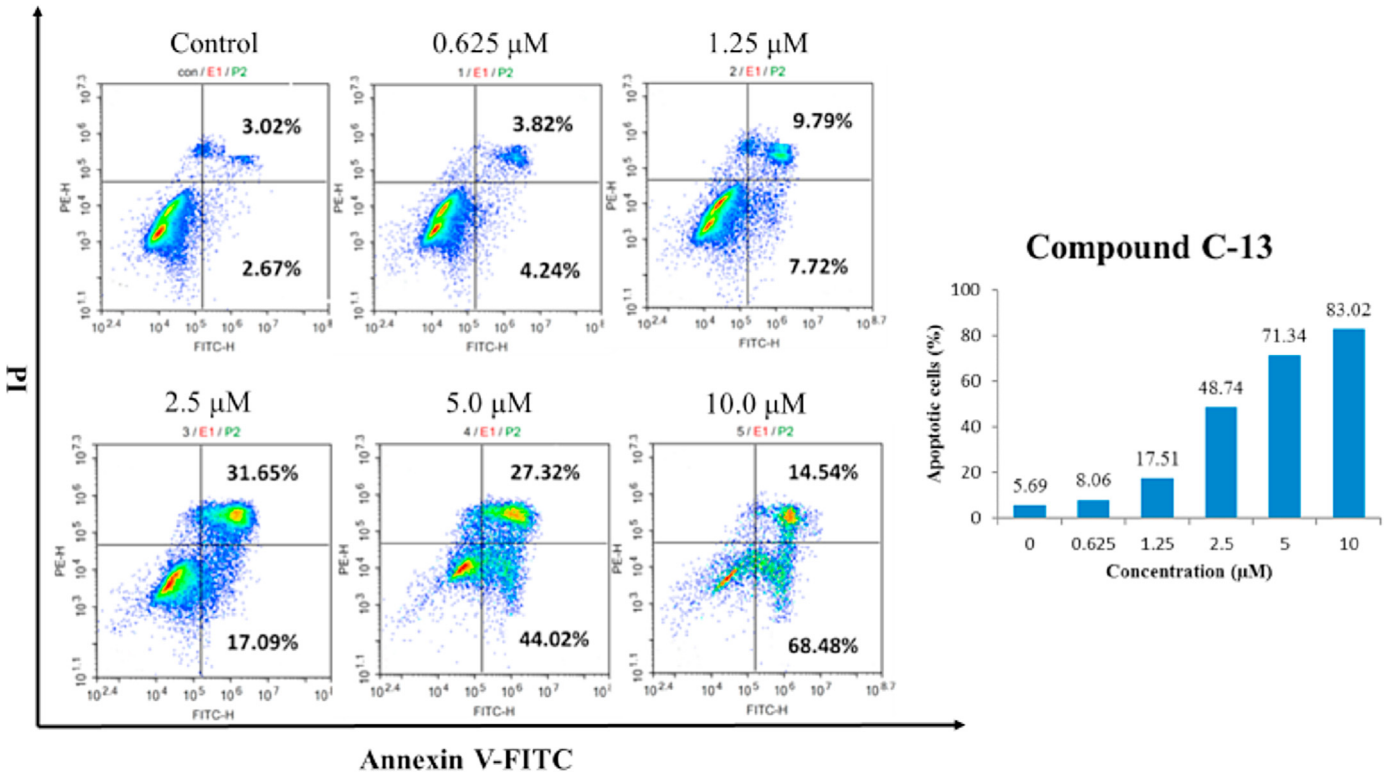


Fig. 8. Percentage of apoptotic cells treatment with different concentration of compound **C-13** for 24 h. The right picture showed that the percentage of cells suffered from apoptosis included the sum of early apoptotic cell and late apoptotic cell percentage. Untreated cells were regarded as control.

Table 6
Inhibitory activities of compound **C-13** against CDK family.

Protein kinase	IC_{50} (nM)
CDK1/CyCB1	>10000
CDK2/CycA2	7601
CDK4/CycD3	6.1 ± 0.32
CDK6/CycD3	14 ± 1.01
CDK7/CycH/MAT1	>3333
CDK9	1834

1H), 3.64–3.53 (m, 4H), 3.52–3.40 (m, 4H), 3.26–3.16 (m, 4H), 2.81–2.63 (m, 6H), 2.55 (s, 3H), 2.46 (t, J = 6.2 Hz, 2H), 2.41–2.30 (m, 5H), 1.93–1.85 (m, 2H), 1.74–1.64 (m, 2H), 1.26 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 202.70, 172.63, 165.76, 161.42, 158.12, 157.28, 155.56, 145.21, 143.28, 141.82, 136.41, 130.79, 130.70, 126.45, 125.94, 113.66, 107.67, 70.06, 69.40, 52.40, 49.49, 39.33, 38.75, 32.58, 31.53, 28.08, 25.75, 13.96. HRMS (DART-TOF) calculated for $C_{34}H_{45}N_9O_5$ $[M + Na]^+$ *m/z* 682.3436, found 682.3432.

*4.1.28. N-(2-(2-(2-(2-(4-(6-(6-acetyl-8-cyclopentyl-5-methyl-7-oxo-7,8-dihydropyrido [2,3-d]pyrimidin-2-ylamino)pyridin-3-yl) piperazin-1yl)acetamido)ethoxy)ethoxy)ethyl) acrylamide (**A-11**)*

A-11 (18 mg, 18%) was synthesized with similar procedures as **A-1.** ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.86 (s, 1H), 8.18 (d, J = 9.1 Hz, 1H), 8.06 (d, J = 2.8 Hz, 1H), 7.34 (dd, J = 9.1, 2.9 Hz, 1H), 6.28 (dd, J = 17.0, 1.5 Hz, 1H), 6.14 (dd, J = 17.0, 10.2 Hz, 1H), 5.62 (dd, J = 10.2, 1.5 Hz, 1H), 3.68–3.48 (m, 12H), 3.28–3.18 (m, 4H), 3.11 (s, 2H), 2.80–2.68 (m, 4H), 2.55 (s, 3H), 2.43–2.31 (m, 5H), 2.08–2.04 (m, 2H), 1.93–1.84 (m, 2H), 1.26 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 202.66, 170.12, 165.61, 161.41, 158.10, 157.27, 155.55, 145.21, 143.31, 141.80, 136.46, 130.85, 130.74, 126.41, 126.07, 113.67, 107.73, 70.35, 70.16, 69.92, 69.84, 61.57, 53.26, 49.55, 39.24, 38.72, 31.53, 28.08, 25.75, 13.96. HRMS (DART-TOF) calculated for $C_{35}H_{47}N_9O_6$ $[M + H]^+$ *m/z*

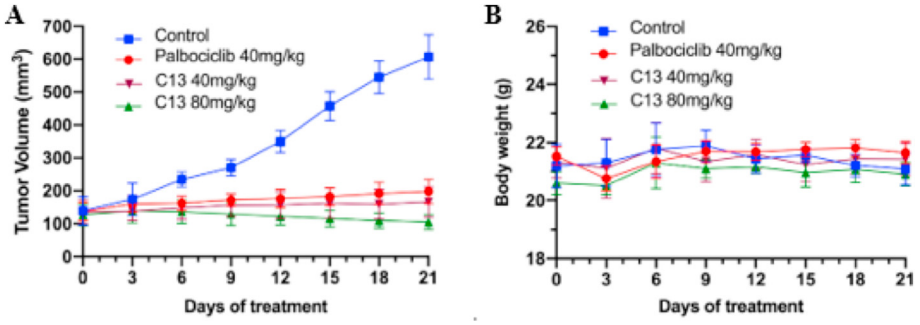


Fig. 9. Anticancer activity of compound **C-13** *in vivo*. (A) Growth inhibitory effect of compound **C-13** on established MDA-MB-231 xenografts in BALB/c nude mice (N = 3 per group); (B) Body weight of the mice during the treatment period.

690.3723, found 690.3716.

*4.1.29. N-(2-(2-(2-(3-(4-(6-(6-acetyl-8-cyclopentyl-5-methyl-7-oxo-7,8-dihydropyrido[2,3-d]pyrimidin-2-ylamino)pyridin-3-yl) piperazin-1-yl)propanamido)ethoxy)ethoxy) ethyl)acrylamide (**A-12**)*

A-12 (12 mg, 12%) was synthesized with similar procedures as **A-1.** ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.85 (s, 1H), 8.17 (d, J = 8.3 Hz, 1H), 8.06 (d, J = 3.6 Hz, 1H), 7.34 (dd, J = 9.1, 2.9 Hz, 1H), 6.28 (dd, J = 17.0, 1.5 Hz, 1H), 6.14 (dd, J = 17.0, 10.2 Hz, 1H), 5.62 (dd, J = 10.2, 1.5 Hz, 1H), 3.71−3.35 (m, 12H), 3.28−3.17 (m, 4H), 2.81−2.65 (m, 6H), 2.55 (s, 3H), 2.47−2.42 (m, 2H), 2.40−2.30 (m, 5H), 2.09−2.05 (m, 2H), 1.92−1.84 (m, 2H), 1.26 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 202.64, 172.36, 165.65, 161.41, 158.08, 157.24, 155.55, 145.16, 143.36, 141.77, 136.38, 130.85, 126.44, 126.11, 113.68, 107.81, 100.00, 70.29, 70.10, 69.81, 52.37, 49.48, 39.24, 38.92, 32.50, 31.53, 28.09, 25.77, 13.96. HRMS (DART-TOF) calculated for $C_{36}H_{49}N_9O_6$ $[M + H]^+$ *m/z* 704.3880, found 704.3882.

*4.1.30. 6-acetyl-2-(5-(4-acryloylpiperazin-1-yl)pyridin-2-ylamino)-8-cyclopentyl-5-me thylpyrido[2,3-d]pyrimidin-7(8H)-one (**A-13**)*

A-13 (18 mg, 80%) was synthesized with similar procedures as **A-1.** ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.90 (s, 1H), 8.21 (d, J = 9.1 Hz, 1H), 8.10 (d, J = 2.7 Hz, 1H), 7.35 (dd, J = 9.1, 2.9 Hz, 1H), 6.59 (dd, J = 17.0, 10.5 Hz, 1H), 6.35 (dd, J = 16.8, 1.8 Hz, 1H), 5.76 (dd, J = 10.5, 1.8 Hz, 1H), 3.92−3.74 (m, 4H), 3.26−3.15 (m, 4H), 2.54 (s, 3H), 2.43−2.30 (m, 5H), 2.11−2.03 (m, 2H), 1.93−1.85 (m, 2H), 1.26 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 202.63, 165.46, 161.40, 158.11, 157.29, 155.57, 145.82, 143.14, 141.80, 137.30, 130.79, 128.44, 127.17, 126.65, 113.59, 107.72, 54.15, 50.19, 49.75, 31.53, 28.07, 25.75, 13.97. HRMS (DART-TOF) calculated for $C_{27}H_{31}N_7O_3$ $[M + H]^+$ *m/z* 502.2562, found 502.2565.

*4.1.31. 2-(4-(6-(6-acetyl-8-cyclopentyl-5-methyl-7-oxo-7,8-dihydropyrido[2,3-d]pyri midin-2-ylamino)pyridin-3-yl)piperazin-1-yl)-N-(2-(cyanomethylamino)ethyl)acetami de (**B-1**)*

To a solution of compound **8a** (20 mg, 0.031 mmol) in DCM (3 mL) was added TFA (1 mL) and the solution was stirred for 3 h at room temperature. The mixture was evaporated under reduced pressure, which directly dissolved into DMF (10 mL). To the DMF solution was added bromoacetonitrile (0.007 mL, 0.0927 mmol), potassium carbonate (12.88 mg, 0.0927 mmol), potassium iodide (7.7 mg, 0.047 mmol). The resulting reaction was stirred at 80 °C overnight. The reaction was cooled to room temperature, quenched with water(30 mL), and extracted with ethyl acetate (20 mL × 3), washed with brine (30 mL × 3). The green organic layer was dried over anhydrous Na_2SO_4, concentrated under reduced pressure to crude product. The crude material was purified by flash column chromatography to give **B-1** as a green solid (9 mg, 50%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.80 (s, 1H), 8.18 (d, J = 9.0 Hz, 1H), 8.03 (d, J = 2.8 Hz, 1H), 7.33 (dd, J = 9.1, 2.9 Hz, 1H), 3.61 (s, 2H), 3.47 (t, J = 5.9 Hz, 2H), 3.28−3.17 (m, 4H), 3.12 (s, 2H), 2.92 (t, J = 5.8 Hz, 2H), 2.80−2.69 (m, 4H), 2.55 (s, 3H), 2.43−2.28 (m, 5H), 2.10−2.02 (m, 2H), 1.93−1.85 (m, 2H), 1.26 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 202.64, 170.45, 161.42, 158.06, 157.22, 155.54, 145.14, 143.34, 141.76, 136.57, 130.80, 126.04, 117.64, 113.59, 107.81, 61.52, 54.05, 53.34, 49.60, 48.56, 38.08, 37.08, 31.53, 28.09, 25.77, 13.96. HRMS (DART-TOF) calculated for $C_{30}H_{38}N_{10}O_3$ $[M + H]^+$ *m/z* 587.3202, found 587.3196.

*4.1.32. 3-(4-(6-(6-acetyl-8-cyclopentyl-5-methyl-7-oxo-7,8-dihydropyrido[2,3-d]pyri midin-2-ylamino)pyridin-3-yl)piperazin-1-yl)-N-(2-(cyanomethylamino)ethyl)propan amide (**B-2**)*

B-2 (18 mg, 36%) was synthesized with similar procedures as **B-1.** ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.82 (s, 1H), 8.19 (d, J = 9.1 Hz, 1H), 8.04 (d, J = 2.9 Hz, 1H), 7.34 (dd, J = 9.1, 3.0 Hz, 1H), 3.59 (s, 2H), 3.42 (t, J = 5.7 Hz, 2H), 3.27−3.20 (m, 4H), 2.88 (t, J = 5.7 Hz, 2H), 2.76−2.68 (m, 6H), 2.55 (s, 3H), 2.47 (t, J = 6.1 Hz, 2H), 2.39−2.33 (m, 5H), 2.08−2.03 (m, 2H), 1.89−1.85 (m, 2H), 1.26 (s, 3H). ^{13}C NMR (101 MHz, DMSO) δ 202.91, 171.64, 161.23, 159.03, 158.72, 155.24, 144.73, 143.92, 142.56, 135.83, 129.68, 125.15, 119.55, 115.59, 107.03, 54.49, 53.39, 52.70, 48.78, 48.08, 38.51, 36.91, 33.65, 31.77, 28.02, 25.57, 14.09. HRMS (DART-TOF) calculated for $C_{31}H_{40}N_{10}O_3$ $[M + H]^+$ *m/z* 601.3359, found 601.3362.

*4.1.33. 2-(4-(6-(6-acetyl-8-cyclopentyl-5-methyl-7-oxo-7,8-dihydropyrido[2,3-d]pyri midin-2-ylamino)pyridin-3-yl)piperazin-1-yl)-N-(3-(cyanomethylamino)propyl)aceta mide (**B-3**)*

B-3 (19 mg, 49%) was synthesized with similar procedures as **B-1.** ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.88 (s, 1H), 8.17 (d, J = 9.1 Hz, 1H), 8.06 (d, J = 2.8 Hz, 1H), 7.33 (dd, J = 9.0, 3.0 Hz, 1H), 3.60 (s, 2H), 3.42 (t, J = 6.5 Hz, 2H), 3.29−3.18 (m, 4H), 3.12 (s, 2H), 2.81−2.68 (m, 6H), 2.55 (s, 3H), 2.42−2.30 (m, 5H), 2.11−2.03 (m, 2H), 1.89−1.85 (m, 2H), 1.78−1.73 (m, 2H), 1.26 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 202.69, 170.24, 161.42, 158.11, 157.32, 155.55, 145.19, 143.27, 141.85, 136.48, 130.65, 125.94, 117.66, 113.57, 107.61, 61.51, 54.13, 53.38, 49.54, 46.04, 37.43, 36.52, 31.55, 29.36, 28.07, 25.74, 13.95. HRMS (DART-TOF) calculated for $C_{31}H_{40}N_{10}O_3$ $[M + H]^+$ *m/z* 601.3359, found 601.3352.

*4.1.34. 3-(4-(6-(6-acetyl-8-cyclopentyl-5-methyl-7-oxo-7,8-dihydropyrido[2,3-d]pyri midin-2-ylamino)pyridin-3-yl)piperazin-1-yl)-N-(3-(cyanomethylamino)propyl)propa namide (**B-4**)*

B-4 (8 mg, 25%) was synthesized with similar procedures as **B-1.** ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.81 (s, 1H), 8.19 (d, J = 9.0 Hz, 1H), 8.05 (d, J = 2.6 Hz, 1H), 7.34 (dd, J = 9.0, 2.9 Hz, 1H), 3.58 (s, 2H), 3.46−3.01 (m, 8H), 2.81−2.70 (m, 6H), 2.55 (s, 3H), 2.49−2.45 (m, 2H), 2.39−2.30 (m, 5H), 2.09−2.03 (m, 2H), 1.89−1.85 (m, 2H), 1.73−1.69 (m, 2H), 1.26 (s, 3H). ^{13}C NMR (101 MHz, DMSO) δ 202.92, 171.44, 161.24, 159.04, 158.72, 155.23, 144.73, 143.92, 142.56, 135.81, 129.68, 125.13, 119.59, 115.61, 107.03, 54.53, 53.38, 52.71, 48.80, 46.13, 37.16, 36.85, 33.72, 31.77, 29.50, 28.02, 25.57, 14.09. HRMS (DART-TOF) calculated for $C_{32}H_{42}N_{10}O_3$ $[M + H]^+$ *m/z* 615.3515, found 615.3509.

*4.1.35. 2-(4-(6-(6-acetyl-8-cyclopentyl-5-methyl-7-oxo-7,8-dihydropyrido[2,3-d]pyri midin-2-ylamino)pyridin-3-yl)piperazin-1-yl)-N-(4-(cyanomethylamino)butyl)acetami de (**B-5**)*

B-5 (10 mg, 44%) was synthesized with similar procedures as **B-1.** ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.85 (s, 1H), 8.18 (d, J = 9.1 Hz, 1H), 8.05 (d, J = 4.7 Hz, 1H), 7.33 (dd, J = 9.1, 3.0 Hz, 1H), 3.62 (s, 2H), 3.32 (t, J = 9.1 Hz, 2H), 3.27−3.16 (m, 4H), 3.10 (s, 2H), 2.90−2.60 (m, 6H), 2.55 (s, 3H), 2.42−2.29 (m, 5H), 2.10−2.02 (m, 2H), 1.92−1.84 (m, 2H), 1.64−1.50 (m, 4H), 1.26 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 202.65, 169.85, 161.42, 158.06, 157.21, 155.54, 145.15, 143.33, 141.75, 136.55, 130.81, 126.07, 117.76, 113.60, 107.83, 61.60, 54.05, 53.38, 49.60, 48.39, 38.68, 37.33, 31.53, 28.09, 27.45, 26.79, 25.77, 13.96. HRMS (DART-TOF) calculated for $C_{32}H_{42}N_{10}O_3$ $[M + H]^+$ *m/z* 615.3515, found 615.3520.

*4.1.36. 3-(4-(6-(6-acetyl-8-cyclopentyl-5-methyl-7-oxo-7,8-dihydropyrido[2,3-d]pyri midin-2-ylamino)pyridin-3-yl)piperazin-1-yl)-N-(4-(2-cyanoacetamido)butyl)propan amide (**B-6**)*

B-6 (11 mg, 34%) was synthesized with similar procedures as **B-1.** ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.85 (s, 1H), 8.19 (d, J = 9.1 Hz, 1H), 8.07 (d, J = 2.8 Hz, 1H), 7.35 (dd, J = 9.1, 2.9 Hz, 1H), 3.58 (s, 2H), 3.31−3.20 (m, 6H), 2.80−2.67 (m, 8H), 2.55 (s, 3H), 2.44 (t, J = 6.1 Hz, 2H), 2.40−2.30 (m, 5H), 2.10−2.03 (m, 2H), 1.92−1.85 (m, 2H), 1.59−1.50 (m, 4H), 1.26 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$)

δ 202.66, 172.15, 161.42, 158.09, 157.25, 155.55, 145.24, 143.26, 141.78, 136.52, 130.77, 126.00, 117.79, 113.62, 107.76, 54.07, 54.01, 52.41, 49.56, 48.40, 38.75, 37.31, 32.31, 31.53, 28.09, 27.27, 26.90, 25.77, 13.96. HRMS (DART-TOF) calculated for $C_{34}H_{44}N_{10}O_4$ [M + Na]$^+$ *m/z* 679.3439, found 679.3431.

*4.1.37. 2-(4-(6-(6-acetyl-8-cyclopentyl-5-methyl-7-oxo-7,8-dihydropyrido[2,3-d]pyrimidin-2-ylamino)pyridin-3-yl)piperazin-1-yl)-N-(6-(cyanomethylamino)hexyl)acetamide (**B-7**)*

B-7 (7 mg, 22%) was synthesized with similar procedures as **B-1.** ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.83 (s, 1H), 8.19 (d, *J* = 9.1 Hz, 1H), 8.04 (d, *J* = 2.9 Hz, 1H), 7.34 (dd, *J* = 9.1, 2.9 Hz, 1H), 3.59 (s, 2H), 3.30−3.21 (m, 6H), 3.11 (s, 2H), 2.77−2.71 (m, 6H), 2.55 (s, 3H), 2.39−2.33 (m, 5H), 2.10−2.03 (m, 2H), 1.92−1.86 (m, 2H), 1.58−1.50 (m, 4H), 1.42−1.34 (m, 4H), 1.26 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 202.63, 169.67, 161.42, 158.05, 157.20, 155.55, 145.14, 143.33, 141.75, 136.47, 130.83, 126.11, 117.83, 113.63, 107.84, 61.59, 54.05, 53.35, 49.58, 48.66, 38.80, 37.37, 31.53, 29.71, 29.31, 28.09, 26.63, 25.77, 13.96. HRMS (DART-TOF) calculated for $C_{34}H_{46}N_{10}O_3$ [M + H]$^+$ *m/z* 643.3828 found 643.3820.

*4.1.38. 3-(4-(6-(6-acetyl-8-cyclopentyl-5-methyl-7-oxo-7,8-dihydropyrido[2,3-d]pyri midin-2-ylamino)pyridin-3-yl)piperazin-1-yl)-N-(6-(cyanomethylamino)hexyl)propan amide (**B-8**)*

B-8 (11 mg, 32%) was synthesized with similar procedures as **B-1.** ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.82 (s, 1H), 8.19 (d, *J* = 9.1 Hz, 1H), 8.06 (d, *J* = 2.8 Hz, 1H), 7.35 (dd, *J* = 9.1, 3.0 Hz, 1H), 3.56 (s, 2H), 3.28−3.20 (m, 6H), 2.76−2.66 (m, 8H), 2.55 (s, 3H), 2.46−2.43 (m, 2H), 2.39−2.30 (m, 5H), 2.11−2.03 (m, 2H), 1.92−1.85 (m, 2H), 1.71−1.67 (m, 2H), 1.53−1.45 (m, 4H), 1.37−1.35 (m, 2H), 1.26 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 202.64, 172.08, 161.41, 158.07, 157.22, 155.55, 145.20, 143.29, 141.75, 136.41, 130.82, 126.03, 117.84, 113.64, 107.84, 54.03, 52.38, 49.58, 48.67, 38.90, 37.35, 32.29, 31.53, 29.56, 29.40, 28.10, 26.82, 26.69, 25.77, 13.96. HRMS (DART-TOF) calculated for $C_{35}H_{48}N_{10}O_3$ [M + H]$^+$ *m/z* 657.3985, found 657.3983.

*4.1.39. 2-(4-(6-(6-acetyl-8-cyclopentyl-5-methyl-7-oxo-7,8-dihydropyrido[2,3-d]pyrimidin-2-ylamino)pyridin-3-yl)piperazin-1-yl)-N-(2-(2-(cyanomethylamino)ethoxy)ethyl)acetamide (**B-9**)*

B-9 (9 mg, 26%) was synthesized with similar procedures as **B-1.** ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.83 (s, 1H), 8.18 (d, *J* = 9.1 Hz, 1H), 8.05 (d, *J* = 2.8 Hz, 1H), 7.35 (dd, *J* = 9.1, 2.9 Hz, 1H), 3.70−3.55 (m, 6H), 3.54−3.48 (m, 2H), 3.29−3.16 (m, 4H), 3.12 (s, 2H), 2.93−2.90 (m, 2H), 2.80−2.68 (m, 4H), 2.55 (s, 3H), 2.42−2.30 (m, 5H), 2.10−2.01 (m, 2H), 1.91−1.84 (m, 2H), 1.26 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 202.66, 170.00, 161.43, 158.06, 157.22, 155.54, 146.51, 143.37, 141.77, 136.59, 130.79, 126.10, 117.72, 113.61, 107.80, 69.97, 69.88, 61.57, 54.05, 53.32, 49.63, 48.25, 38.71, 37.46, 31.53, 29.69, 28.09, 25.77, 13.96. HRMS (DART-TOF) calculated for $C_{32}H_{42}N_{10}O_4$ [M + H]$^+$ *m/z* 631.3464, found 631.3466.

*4.1.40. 3-(4-(6-(6-acetyl-8-cyclopentyl-5-methyl-7-oxo-7,8-dihydropyrido[2,3-d]pyrimidin-2-ylamino)pyridin-3-yl)piperazin-1-yl)-N-(2-(2-(cyanomethylamino)ethoxy)ethyl)propanamide (**B-10**)*

B-10 (7 mg, 19%) was synthesized with similar procedures as **B-1.** ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.82 (s, 1H), 8.19 (d, *J* = 8.3 Hz, 1H), 8.04 (d, *J* = 2.9 Hz, 1H), 7.33 (dd, *J* = 9.1, 2.9 Hz, 1H), 3.68−3.42 (m, 10H), 3.28−3.20 (m, 4H), 2.78−2.69 (m, 6H), 2.55 (s, 3H), 2.49−2.45 (m, 2H), 2.40−2.32 (m, 5H), 2.09−2.04 (m, 2H), 1.90−1.85 (m, 2H), 1.26 (s, 3H). ^{13}C NMR (101 MHz, DMSO) δ 202.91, 171.66, 161.24, 159.03, 158.72, 156.72, 155.24, 144.74, 143.90, 142.56, 135.81, 129.69, 125.12, 115.60, 107.03, 69.38, 68.06, 54.42, 53.38, 52.69, 48.78, 38.81, 33.54, 31.77, 28.02, 25.57, 14.08. HRMS (DART-TOF) calculated for $C_{33}H_{44}N_{10}O_4$ [M + Na]$^+$ *m/z* 667.3439, found 667.3435.

*4.1.41. 2-(4-(6-(6-acetyl-8-cyclopentyl-5-methyl-7-oxo-7,8-dihydropyrido[2,3-d]pyrimidin-2-ylamino)pyridin-3-yl)piperazin-1-yl)-N-(2-(2-(2-(cyanomethylamino)ethoxy) ethoxy)ethyl) acetamide (**B-11**)*

B-11 (13 mg, 35%) was synthesized with similar procedures as **B-1.** ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.83 (s, 1H), 8.18 (d, *J* = 9.1 Hz, 1H), 8.04 (d, *J* = 2.8 Hz, 1H), 7.34 (dd, *J* = 9.1, 3.0 Hz, 1H), 3.64−3.57 (m, 10H), 3.53−3.49 (m, 2H), 3.25−3.19 (m, 4H), 3.11 (s, 2H), 2.90−2.86 (m, 2H), 2.76−2.70 (m, 4H), 2.55 (s, 3H), 2.39−2.33 (m, 5H), 2.10−2.03 (m, 2H), 1.90−1.86 (m, 2H), 1.26 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 202.65, 169.98, 161.42, 158.06, 157.21, 155.54, 145.08, 143.39, 141.75, 136.43, 130.81, 126.07, 117.81, 113.65, 107.84, 70.36, 70.20, 70.15, 69.98, 61.57, 54.04, 53.25, 49.56, 48.09, 38.72, 37.41, 31.53, 28.10, 25.77, 13.96. HRMS (DART-TOF) calculated for $C_{34}H_{46}N_{10}O_5$ [M + H]$^+$ *m/z* 675.3727, found 675.3723.

*4.1.42. 3-(4-(6-(6-acetyl-8-cyclopentyl-5-methyl-7-oxo-7,8-dihydropyrido[2,3-d]pyri midin-2-ylamino)pyridin-3-yl)piperazin-1-yl)-N-(2-(2-(2-(cyanomethylamino)ethoxy) ethoxy)ethyl) propanamide (**B-12**)*

B-12 (14 mg, 40%) was synthesized with similar procedures as **B-1.** ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.84 (s, 1H), 8.19 (d, *J* = 9.1 Hz, 1H), 8.06 (d, *J* = 2.8 Hz, 1H), 7.34 (dd, *J* = 9.1, 2.9 Hz, 1H), 3.68−3.41 (m, 14H), 3.25−3.20 (m, 4H), 2.76−2.69 (m, 6H), 2.55 (s, 3H), 2.47−2.44 (m, 2H), 2.40−2.33 (m, 5H), 2.09−2.04 (m, 2H), 1.92−1.85 (m, 2H), 1.26 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 202.68, 172.37, 161.41, 158.09, 157.51, 157.26, 155.55, 145.21, 143.35, 141.79, 136.32, 130.77, 126.14, 113.69, 107.76, 70.38, 70.12, 70.05, 68.70, 54.07, 53.83, 52.35, 49.49, 39.67, 38.80, 32.51, 31.53, 28.08, 25.75, 13.95. HRMS (DART-TOF) calculated for $C_{35}H_{48}N_{10}O_5$ [M + Na]$^+$ *m/z* 711.3701, found 711.3698.

*4.1.43. 2-(4-(6-(6-acetyl-8-cyclopentyl-5-methyl-7-oxo-7,8-dihydropyrido[2,3-d]pyri midin-2-ylamino)pyridin-3-yl)piperazin-1-yl)acetonitrile (**B-13**)*

B-13 (11 mg, 40%) was synthesized with similar procedures as **B-1.** ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.82 (s, 1H), 8.19 (d, *J* = 9.1 Hz, 1H), 8.04 (d, *J* = 2.8 Hz, 1H), 7.34 (dd, *J* = 9.1, 2.9 Hz, 1H), 3.60 (s, 2H), 3.30−3.20 (m, 4H), 2.83−2.78 (m, 4H), 2.55 (s, 3H), 2.41−2.30 (m, 5H), 2.11−2.03 (m, 2H), 1.92−1.86 (m, 2H), 1.26 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 202.63, 161.42, 158.06, 157.20, 155.55, 145.25, 143.25, 141.74, 136.71, 130.84, 126.35, 114.42, 113.64, 107.86, 54.07, 51.59, 49.23, 46.00, 31.53, 28.10, 25.77, 13.96. HRMS (DART-TOF) calculated for $C_{26}H_{30}N_8O_2$ [M + H]$^+$ *m/z* 487.2566, found 487.2566.

*4.1.44. 2-(4-(6-(6-acetyl-8-cyclopentyl-5-methyl-7-oxo-7,8-dihydropyrido[2,3-d]pyrimid-in-2-ylamino)pyridin-3-yl)piperazin-1-yl)-N-(2-(2-chloroacetamido)ethyl) aceta m-ide (**C-1**)*

TFA (1 mL) was added to a solution of compound **8a** (20 mg, 0.031 mmol) in DCM (3 mL), and stirred for 3 h at room temperature. The mixture was evaporated under reduced pressure, which directly dissolved into THF (15 mL). The THF solution was added triethylamine (0.013 mL, 0.093 mmol) which stirred at 0 °C for 5min. Chloroacetyl chloride (0.006 mL, 0.062 mmol) was added dropwise at 0 °C under N_2, the mixture was stirred at room temperature for 5 h. The reaction was monitored by TLC. After completion of the reaction, it was evaporated under reduced pressure. Water (20 mL) was added and the mixture was extracted with EtOAc (20 mL × 3) and organic layer washed twice with brine, dried over anhydrous Na_2SO_4. The green organic layer was concentrated in vacuo. The crude product was purified by flash column chromatography to give **C-1** as a green solid (9 mg, 46%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.85 (s, 1H), 8.18 (d, *J* = 9.1 Hz, 1H), 8.05 (d, *J* = 2.8 Hz, 1H), 7.33 (dd, *J* = 9.1, 2.9 Hz, 1H), 4.04 (s, 2H), 3.56−3.44 (m, 4H), 3.28−3.19 (m, 4H), 3.12 (s, 2H), 2.77−2.68 (m, 4H), 2.55 (s,

3H), 2.41–2.30 (m, 5H), 2.10–2.03 (m, 2H), 1.92–1.86 (m, 2H), 1.26 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 202.63, 171.21, 166.81, 161.42, 158.07, 157.23, 155.55, 145.18, 143.31, 141.76, 136.60, 130.80, 126.10, 113.62, 107.80, 61.40, 54.07, 53.39, 49.57, 42.54, 40.60, 38.79, 31.53, 28.09, 25.76, 13.96. HRMS (DART-TOF) calculated for $C_{30}H_{38}ClN_9O_4$ $[M + H]^+$ *m/z* 624.2809, found 624.2802.

*4.1.45. 3-(4-(6-(6-acetyl-8-cyclopentyl-5-methyl-7-oxo-7,8-dihydropyrido[2,3-d]pyrimid-in-2-ylamino)pyridin-3-yl)piperazin-1-yl)-N-(2-(2-chloroacetamido)ethyl)prop an-amide (**C-2**)*

C-2 (8 mg, 35%) was synthesized with similar procedures as **C-1**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.73 (s, 1H), 8.11 (d, *J* = 9.0 Hz, 1H), 7.96 (d, *J* = 2.8 Hz, 1H), 7.26 (dd, *J* = 9.1 Hz, 2.9 Hz, 1H), 3.98–3.95 (m, 2H), 3.40–3.35 (m, 4H), 3.19–3.15 (m, 4H), 2.72–2.65 (m, 6H), 2.48–2.47 (s, 3H), 2.45–2.42 (m, 2H), 2.31–2.28 (m, 5H), 2.03–1.98 (m, 2H), 1.83–1.77 (m, 2H), 1.26 (s, 3H). HRMS (DART-TOF) calculated for $C_{31}H_{40}ClN_9O_4$ $[M + H]^+$ *m/z* 638.2966, found 638.2961.

*4.1.46. 2-(4-(6-(6-acetyl-8-cyclopentyl-5-methyl-7-oxo-7,8-dihydropyrido[2,3-d]pyrimid-in-2-ylamino)pyridin-3-yl)piperazin-1-yl)-N-(3-(2-chloroacetamido)propyl)acet-amide (**C-3**)*

C-3 (15 mg, 61%) was synthesized with similar procedures as **C-1**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.80 (s, 1H), 8.15 (d, *J* = 9.2 Hz, 1H), 8.02 (d, *J* = 2.7 Hz, 1H), 7.34 (d, *J* = 9.2 Hz, 2.7 Hz, 1H), 4.06 (s, 2H), 3.41–3.32 (m, 4H), 3.29–3.20 (m, 4H), 3.13 (s, 2H), 2.80–2.71 (m, 4H), 2.58–2.52 (s, 3H), 2.41–2.31 (m, 5H), 2.10–2.03 (m, 2H), 1.91–1.85 (m, 2H), 1.74–1.66 (m, 4H), 1.26 (s, 3H). HRMS (DART-TOF) calculated for $C_{31}H_{40}ClN_9O_4$ $[M + H]^+$ *m/z* 638.2966, found 638.2967.

*4.1.47. 3-(4-(6-(6-acetyl-8-cyclopentyl-5-methyl-7-oxo-7,8-dihydropyrido[2,3-d]pyrimi-din-2-ylamino)pyridin-3-yl)piperazin-1-yl)-N-(3-(2-chloroacetamido)propyl) propanamide (**C-4**)*

C-4 (10 mg, 36%) was synthesized with similar procedures as **C-1**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.84 (s, 1H), 8.18 (d, *J* = 9.1 Hz, 1H), 8.06 (d, *J* = 2.8 Hz, 1H), 7.33 (dd, *J* = 9.1 Hz, 2.7 Hz, 1H), 4.04 (s, 2H), 3.38–3.30 (m, 4H), 3.24–3.20 (m, 4H), 2.76–2.70 (m, 6H), 2.56–2.54 (s, 3H), 2.49–2.46 (m, 2H), 2.39–2.33 (m, 5H), 2.09–2.03 (m, 2H), 1.90–1.86 (m, 2H), 1.70–1.67 (m, 2H), 1.26 (s, 3H). ^{13}C NMR (101 MHz, DMSO) δ 202.92, 171.58, 166.31, 161.23, 159.03, 158.72, 155.23, 144.72, 143.91, 142.56, 135.81, 129.68, 125.14, 115.59, 107.03, 54.52, 53.38, 52.71, 48.77, 43.13, 37.22, 36.54, 33.72, 31.77, 29.48, 28.03, 25.57, 14.08. HRMS (DART-TOF) calculated for $C_{32}H_{42}ClN_9O_4$ $[M + H]^+$ *m/z* 652.3122, found 652.3120.

*4.1.48. 2-(4-(6-(6-acetyl-8-cyclopentyl-5-methyl-7-oxo-7,8-dihydropyrido[2,3-d]pyrimid-in-2-ylamino)pyridin-3-yl)piperazin-1-yl)-N-(4-(2-chloroacetamido)butyl)aceta-mide (**C-5**)*

C-5 (9 mg, 23%) was synthesized with similar procedures as **C-1**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.86 (s, 1H), 8.18 (d, *J* = 9.1 Hz, 1H), 8.06 (d, *J* = 2.8 Hz, 1H), 7.34 (dd, *J* = 9.1, 2.9 Hz, 1H), 4.04 (s, 2H), 3.39–3.32 (m, 4H), 3.26–3.19 (m, 4H), 3.10 (s, 2H), 2.77–2.70 (m, 4H), 2.57–2.52 (s, 3H), 2.41–2.32 (m, 4H), 2.10–2.03 (m, 1H), 1.92–1.85 (m, 1H), 1.64–1.56 (m, 4H), 1.26 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 202.64, 169.96, 166.00, 161.42, 158.08, 157.24, 155.55, 145.18, 143.32, 141.78, 136.55, 130.78, 126.05, 113.60, 107.77, 61.59, 54.08, 53.38, 49.58, 42.68, 39.49, 38.47, 31.53, 28.08, 27.28, 26.77, 25.76, 13.96. HRMS (DART-TOF) calculated for $C_{32}H_{42}ClN_9O_4$ $[M + H]^+$ *m/z* 652.3122, found 652.3115.

*4.1.49. 3-(4-(6-(6-acetyl-8-cyclopentyl-5-methyl-7-oxo-7,8-dihydropyrido[2,3-d]pyrimid-in-2-ylamino)pyridin-3-yl)piperazin-1-yl)-N-(4-(2-chloroacetamido)butyl)propa-namide (**C-6**)*

C-6 (10 mg, 42%) was synthesized with similar procedures as **C-1**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.81 (s, 1H), 8.19 (d, *J* = 9.1 Hz, 1H), 8.04 (d, *J* = 2.8 Hz, 1H), 7.35 (dd, *J* = 9.1, 2.9 Hz, 1H), 4.00 (s, 2H), 3.37–3.26 (m, 4H), 3.25–3.18 (m, 4H), 2.77–2.67 (m, 6H), 2.55 (s, 3H), 2.47–2.44 (m, 2H), 2.39–2.31 (m, 5H), 2.11–2.04 (m, 2H), 1.91–1.86 (m, 2H), 1.77–1.73 (m, 4H), 1.26 (s, 3H). HRMS (DART-TOF) calculated for $C_{33}H_{44}ClN_9O_4$ $[M + H]^+$ *m/z* 666.3279, found 666.3286.

*4.1.50. 2-(4-(6-(6-acetyl-8-cyclopentyl-5-methyl-7-oxo-7,8-dihydropyrido[2,3-d]pyrimid-in-2-ylamino)pyridin-3-yl)piperazin-1-yl)-N-(6-(2-chloroacetamido)hexyl)aceta-mide (**C-7**)*

C-7 (9 mg, 23%) was synthesized with similar procedures as **C-1**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.84 (s, 1H), 8.17 (d, *J* = 9.1 Hz, 1H), 8.05 (d, *J* = 2.7 Hz, 1H), 7.34 (dd, *J* = 9.1, 2.9 Hz, 1H), 4.04 (s, 2H), 3.34–3.27 (m, 4H), 3.26–3.20 (m, 4H), 3.11 (s, 2H), 2.80–2.70 (m, 4H), 2.55 (s, 3H), 2.40–2.30 (m, 5H), 2.10–2.05 (m, 2H), 2.03–1.96 (m, 4H), 1.72–1.66 (m, 2H), 1.57–1.54 (m, 2H), 1.38–1.35 (m, 2H), 1.26 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 202.63, 169.54, 165.87, 161.40, 158.06, 157.23, 155.54, 145.17, 143.29, 141.76, 136.51, 130.77, 126.10, 113.63, 107.77, 61.55, 54.05, 53.32, 49.52, 42.70, 39.62, 38.76, 31.52, 29.63, 29.22, 28.08, 26.38, 26.28, 25.75, 13.95. HRMS (DART-TOF) calculated for $C_{34}H_{46}ClN_9O_4$ $[M + H]^+$ *m/z* 680.3435, found 680.3429.

*4.1.51. 3-(4-(6-(6-acetyl-8-cyclopentyl-5-methyl-7-oxo-7,8-dihydropyrido[2,3-d]pyrimid-in-2-ylamino)pyridin-3-yl)piperazin-1-yl)-N-(6-(2-chloroacetamido)hexyl)propa-namide (**C-8**)*

C-8 (4 mg, 17%) was synthesized with similar procedures as **C-1**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.82 (s, 1H), 8.19 (d, *J* = 9.1 Hz, 1H), 8.04 (d, *J* = 2.6 Hz, 1H), 7.35 (dd, *J* = 9.0, 2.8 Hz, 1H), 4.03 (s, 2H), 3.33–3.15 (m, 8H), 2.81–2.63 (m, 6H), 2.55 (s, 3H), 2.46–2.44 (m, 2H), 2.40–2.30 (m, 5H), 2.10–2.03 (m, 2H), 1.92–1.86 (m, 2H), 1.56–1.46 (m, 4H), 1.40–1.32 (m, 4H), 1.26 (s, 3H). HRMS (DART-TOF) calculated for $C_{35}H_{48}ClN_9O_4$ $[M + H]^+$ *m/z* 694.3592, found 694.3588.

*4.1.52. 2-(4-(6-(6-acetyl-8-cyclopentyl-5-methyl-7-oxo-7,8-dihydropyrido[2,3-d]pyrimid-in-2-ylamino)pyridin-3-yl)piperazin-1-yl)-N-(2-(2-(2-chloroacetamido)ethoxy) ethyl)acetamide (**C-9**)*

C-9 (15 mg, 61%) was synthesized with similar procedures as **C-1**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.85 (s, 1H), 8.18 (d, *J* = 9.1 Hz, 1H), 8.05 (d, *J* = 2.8 Hz, 1H), 7.33 (dd, *J* = 9.1, 3.0 Hz, 1H), 4.05 (s, 2H), 3.62–3.57 (m, 4H), 3.54–3.48 (m, 4H), 3.26–3.20 (m, 4H), 3.13 (s, 2H), 2.77–2.71 (m, 4H), 2.55 (s, 3H), 2.40–2.32 (m, 5H), 2.10–2.04 (m, 2H), 1.91–1.86 (m, 2H), 1.26 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 202.65, 170.14, 166.00, 161.42, 158.07, 157.23, 155.55, 145.18, 143.29, 141.78, 136.51, 130.78, 126.04, 113.62, 107.78, 70.01, 69.16, 61.60, 54.06, 53.33, 49.57, 42.67, 39.65, 38.68, 31.53, 28.09, 25.76, 13.96. HRMS (DART-TOF) calculated for $C_{32}H_{42}ClN_9O_5$ $[M + H]^+$ *m/z* 668.3071, found 668.3068.

*4.1.53. 3-(4-(6-(6-acetyl-8-cyclopentyl-5-methyl-7-oxo-7,8-dihydropyrido[2,3-d]pyrimid-in-2-ylamino)pyridin-3-yl)piperazin-1-yl)-N-(2-(2-(2-chloroacetamido)ethoxy) ethyl)propanamide (**C-10**)*

C-10 (18 mg, 65%) was synthesized with similar procedures as **C-1**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.85 (s, 1H), 8.19 (d, *J* = 9.1 Hz, 1H), 8.06 (d, *J* = 2.8 Hz, 1H), 7.34 (dd, *J* = 9.1, 2.9 Hz, 1H), 4.03 (s, 2H), 3.59–3.53 (m, 4H), 3.49–3.43 (m, 4H), 3.25–3.18 (m, 4H), 2.77–2.67 (m, 6H), 2.55 (s, 3H), 2.48–2.45 (m, 2H), 2.40–2.33 (m, 5H), 2.09–2.03 (m, 2H), 1.91–1.86 (m, 2H), 1.26 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 202.67, 172.36, 166.06, 161.42, 158.08, 157.24, 155.55, 145.17, 143.30, 141.78, 136.41, 130.77, 125.95, 113.63, 107.77, 70.03, 69.10, 54.06, 53.88, 52.41, 49.51, 42.66, 39.58, 38.82, 32.55, 31.53, 28.09, 25.77, 13.96. HRMS (DART-TOF) calculated for $C_{33}H_{44}ClN_9O_5$ $[M + H]^+$ *m/z* 682.3228, found 682.3222.

*4.1.54. 2-(4-(6-(6-acetyl-8-cyclopentyl-5-methyl-7-oxo-7,8-dihydropyrido[2,3-d]pyrimid-in-2-ylamino)pyridin-3-yl)piperazin-1-yl)-N-(2-(2-(2-(2-chloroacetamido)ethoxy) ethoxy)ethyl) acetamide (**C-11**)*

C-11 (12 mg, 49%) was synthesized with similar procedures as **C-1**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.82 (s, 1H), 8.17 (d, J = 9.0 Hz, 1H), 8.03 (d, J = 2.7 Hz, 1H), 7.34 (dd, J = 9.1, 2.9 Hz, 1H), 4.05 (s, 2H), 3.65−3.55 (m, 8H), 3.54−3.46 (m, 4H), 3.25−3.19 (m, 4H), 3.11 (s, 2H), 2.77−2.71 (m, 4H), 2.55 (s, 3H), 2.39−2.32 (m, 5H), 2.09−2.04 (m, 2H), 1.91−1.86 (m, 2H), 1.26 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 202.65, 169.91, 166.04, 161.41, 158.05, 157.21, 155.54, 145.14, 143.32, 141.75, 136.32, 130.82, 126.17, 113.69, 100.00, 70.41, 70.20, 70.00, 69.45, 61.53, 53.24, 49.53, 42.66, 39.52, 38.74, 31.52, 29.69, 28.09, 25.77, 13.96. HRMS (DART-TOF) calculated for $C_{34}H_{46}ClN_9O_6$ $[M + H]^+$ *m/z* 712.3334, found 712.3332.

*4.1.55. 3-(4-(6-(6-acetyl-8-cyclopentyl-5-methyl-7-oxo-7,8-dihydropyrido[2,3-d]pyrimid-in-2-ylamino)pyridin-3-yl)piperazin-1-yl)-N-(2-(2-(2-(2-chloroacetamido)ethoxy) ethoxy)ethyl) propanamide (**C-12**)*

C-12 (13 mg, 31%) was synthesized with similar procedures as **C-1**. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.84 (s, 1H), 8.18 (d, J = 9.1 Hz, 1H), 8.06 (d, J = 2.8 Hz, 1H), 7.34 (dd, J = 9.1, 2.9 Hz, 1H), 4.06 (s, 2H), 3.63−3.55 (m, 6H), 3.54−3.50 (m, 2H), 3.49−3.40 (m, 4H), 3.28−3.17 (m, 4H), 2.78−2.65 (m, 6H), 2.55 (s, 3H), 2.46−2.43 (m, 2H), 2.40−2.29 (m, 5H), 2.11−2.04 (m, 2H), 1.91−1.87 (m, 2H), 1.26 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 202.65, 172.22, 166.06, 161.42, 158.08, 157.24, 155.54, 145.14, 143.37, 141.78, 136.35, 130.78, 126.01, 113.65, 107.79, 70.34, 70.13, 69.39, 54.04, 53.88, 52.38, 49.47, 42.68, 39.51, 38.90, 32.57, 31.53, 28.09, 25.77, 13.96. HRMS (DART-TOF) calculated for $C_{35}H_{48}ClN_9O_6$ $[M + H]^+$ *m/z* 726.3490, found 726.3495.

*4.1.56. 6-acetyl-2-(5-(4-(2-chloroacetyl)piperazin-1-yl)pyridin-2-ylamino)-8-cyclopentyl-5-methylpyrido[2,3-d]pyrimidin-7(8H)-one (**C-13**)*

Triethylamine (0.03 mL, 0.2 mmol) was added to a solution of palbociclib (30 mg, 0.067 mmol) in DCM (15 mL), and stirred at 0 °C for 5min. Chloroacetyl chloride (0.015 mL, 0.134 mmol) was added dropwise at 0 °C under N_2, the mixture was stirred at room temperature for 5 h. The reaction was monitored by TLC. After completion of the reaction, it was evaporated under reduced pressure. Water (20 mL) was added and the mixture was extracted with EtOAc (20 mL × 3) and organic layer washed twice with brine, dried over anhydrous Na_2SO_4. The green organic layer was concentrated in vacuo. The crude product was purified by flash column chromatography to give **C-13** as a green solid (27 mg, 75%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.92 (s, 1H), 8.22 (d, J = 9.0 Hz, 1H), 8.11 (d, J = 2.6 Hz, 1H), 7.35 (dd, J = 9.1, 2.8 Hz, 1H), 4.14 (s, 2H), 3.85−3.72 (m, 4H), 3.27−3.17 (m, 4H), 2.55 (s, 3H), 2.42−2.33 (m, 5H), 2.12−2.03 (m, 2H), 1.93−1.85 (m, 2H), 1.26 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 202.61, 165.20, 161.39, 158.04, 157.21, 155.55, 145.89, 143.04, 141.72, 137.42, 130.92, 126.88, 113.59, 107.90, 54.10, 50.04, 49.68, 46.16, 41.98, 40.73, 31.52, 28.10, 25.77, 13.97. HRMS (DART-TOF) calculated for $C_{26}H_{30}ClN_7O_3$ $[M + Na]^+$ *m/z* 546.1991, found 546.1990.

4.2. Bioassays

4.2.1. Cellular proliferation assays

Human cancer lines MDA-MB-231, MDA-MB-453, MCF-7 and H1299 were obtained from the American Type Culture Collection (ATCC) and cultured at 37 °C with 5% CO_2 in DMEM, supplemented with 10% FBS and 1% Penicillin-Streptomycin (PS). Cells were seeded in 96-well tissue culture plates at 3−5 × 10^3 cells per well for 24 h. Compounds were dissolved in the same media, which multiple diluted and added in each well. After 3 days, MTT was added and cells were incubated for an additional 2 h. The absorbance values (OD) of the 96-well tissue culture plates was read at 450 nm operating a Spectra MAX M5 microplate spectrophotometer. Cell viability result was calculated by GraphPad Prism 6.0 software. The assay was repeated three times.

4.2.2. Molecular docking

To predict the potential binding mode of **C-13** with CDK4 and CDK6, docking studies was implemented by using AutoDock 4.0. The crystal structure of CDK6 was obtained from the Protein Data Bank (PDB ID: 2EUF), which converted from a PDB file to a PDBQT file with AutoDock Tools. All of the docking parameters were set to default.

4.2.3. Colony formation assay

MDA-MB-231 was seeded in a six-well plate with a concentration of 1000 cells per well and cultured at 37 °C with 5% CO_2 in incubator for 24 h. The target compound C-13 with different concentrations (0、0.1563、0.3125、0.625、1.25、2.5 μM) were added in each well and incubated for two weeks. After cells had grown for 14 days, it shown colony and was checked. Cells were fixed and stained with 100% methanol and 0.1% crystal violet for 25 min. At last, Colonies were reserved in 4 °C, that photographed and analyzed.

4.2.4. Cytotoxicity assessment

Human normal liver cell line (LO2) was seeded in 96-well plate (3000 cells/well) to assess cytotoxicity. LO2 cells were incubated 72 h in the presence of targeted compound **C-13**. Then MTT was added and cells were incubated for an additional 2 h. Subsequently, the absorbance values (OD) of the 96-well plates was measured using a Spectra MAX M5 microplate spectrophotometer. IC_{50} values were obtained by GraphPad Prism 6.0 software.

4.2.5. In vitro CDK kinase inhibition assay

The kinase assay was completed by Sundia. In short, the kinase inhibitory research of CDK4 (Cyclin D1) and CDK6 (Cyclin D3) were conducted for the optimized compounds as well as the control compound palbociclib, which evaluated by mobility shift assay (25 μM ATP for CDK4 and 20 μM ATP for CDK6). The kinase activity was determined through measuring the percentage of conversion by Caliper EZ Reader. IC_{50} values and curves fit were gained by GraphPad Prism 5.

4.2.6. Cell cycle analysis

MDA-MB-231 was seeded at 1 × 10^6 cells per well in 6-well plates and incubated 24 h at 37 °C with 5% CO_2. Targeted compound and palbociclib were dissolved in the same media, which diluted to diverse concentration and added in each well. After 12 h, the cells were harvested, which transferred to FACS tubes and centrifuged for 5 min. Cell pellets were collected, re-suspended in phosphate-buffered saline (PBS) and centrifuged for another 5 min. The upper layer PBS was dislodged and cell pellets were fixed with 70% ethanol at 4 °C for 1 h, centrifuged for 5 min and washed three times with PBS. The supernatant was removed and Propidium iodide (PI) cell cycle solution in PBS (50 μg/mL propidium iodide, 0.1 mg/mL RNase A) was added at 37 °C for 0.5 h. Finally, DNA content was quantified with a flow cytometer (BD Bioscience).

4.2.7. Western blot analysis

MDA-MB-231 cells were plated in 6-well plates, which incubated with various concentration of compound **C-13** for 24 h at 37 °C. When the cells were harvested, it was lysed with RIPA buffer,

protease inhibitors and benzonase. Then equal amount of protein were separated by employing SDS-PAGE before quantitating protein, and transferred onto PVDF membranes. The membranes were disposed by 5% BSA in PBS buffer with 0.1% PBST, then incubated with original antibodies for 24 h at 4 °C. Subsequently, it was washed with 0.1% PBST, and the incubation of mouse/rabbit secondary antibody was performed for 1 h. The primary antibodies CDK4 (NO. 12790), CDK6 (NO. 13331) and p-Rb S780 (NO. 8180) were purchased from Cell Signaling.

4.2.8. Annexin V/propidium iodide (PI) staining assay

MDA-MB-231 cells were seeded at 5×10^4 cells per well in a 6-well plate, which incubated 24 h at 37 °C with 5% CO_2. Cancer cells were treated with the increasing concentration (0.625 μM, 1.25 μM, 2.5 μM, 5 μM, 10 μM) of the compound **C-13** for 24 h, and the control cells were harvested by trypsinization. Then the harvested cells were washed with PBS and incubated with Annexin V-FITC solution (25 ng/mL) and Propidium Iodide staining solution (25 μg/mL; BD Biosciences) at 37 °C for 0.5 h. It was ultimately recorded and analyzed by the Gallios Flow cytometer (Beckman Coulter, Brea, CA, USA).

4.2.9. In vivo xenograft assay

Female BALB/c nude mice were purchased (Beijing HFK bioscience Co. Itd., Beijing, China), which housed in a pathogen free room with a 12 h light/dark schedule at 25 °C. MDA-MB-231 cells were harvested during the exponential-growth phase and washed twice with serum-free medium. Then MDA-MB-231 cells at a density of 1×10^7 were implanted subcutaneously into the mice (6–7 weeks old). When the tumor volume reached 100 mm^3, the mice were divided randomly (3 mice for each group) at this point in time. In the MDA-MB-231 model, the mice were dosed orally with compound **C-13** (40, 80 mg/kg/d, dissolved in 10% DMSO/10% PEG300/50 mM sodium lactate solution), palbociclib (40 mg/kg/d, dissolved in 10% DMSO/10% PEG300/50 mM sodium lactate solution), vehicle (10% DMSO/10% PEG300/50 mM sodium lactate solution) for 21 days. The sizes of the tumors and body weight were measured every three days. The tumor volume was determined by vernier caliper, which calculated as follows: tumor volume $= 0.5 \times a \times b^2$ (a represents long diameter; b represents short diameter). The percentage of tumor growth inhibition (TGI) was calculated as $100 \times$ {1-[(treated Final day − treated Initial day)/(control Final day − control Initial day)]}. All experimental technology and animal handling procedures have been approved by the Institutional Animal Care and Laboratory Animal Management Committee of Sichuan University.

Declaration of competing interest

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.

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Appendix A. Supplementary data

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