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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Rational design and development of novel NAE inhibitors for the treatment of pancreatic cancer

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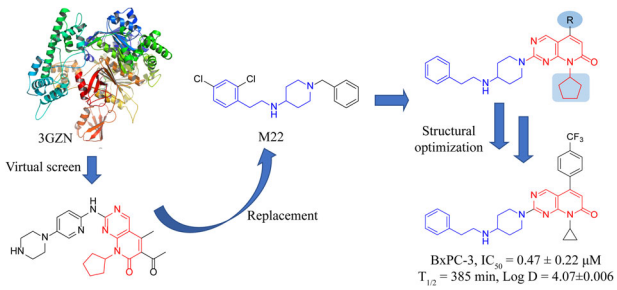
Abstract

Pancreatic cancer remains clinically challenging because of the lack of efficient targeted therapies and high aggressiveness. NEDD8 activating enzyme (NAE) plays a critical role in various cellular functions in cancers. Herein, we report the synthesis, optimization, and evaluation of a new series of pyrido[2,3-d]pyrimidin-7(8*H*)-one derivative as highly selective and efficacious NAE inhibitors, enabling rapid degradation of related substrates and potent inhibition of BxPC-3 cell proliferation. Moreover, western blot assays demonstrated that compound **51** could inhibit NAE activity, resulting in apoptosis in BxPC-3 cells. Furthermore, compound **51** induced cell apoptosis in vitro and inhibited tumor growth in BxPC-3 xenograft models. Our work established that **51** was a highly potent and efficacious NAE inhibitor, representing an effective strategy and great potential as a new targeted therapy for pancreatic cancer.

Graphical abstract



Keywords Virtual screening · NAE inhibitor · Pancreatic cancer · Structural optimization · Synthetic chemistry · Anti-tumor

Introduction

Pancreatic cancer is a highly malignant tumor of the digestive system, and its etiology and pathogenesis are still unclear. As reported, pancreatic cancer has the poorest prognosis of any common solid malignancy, with a 5-year overall survival rate of ~10%, lagging far behind the improved survival of other solid tumors [1]. Despite being relatively uncommon, pancreatic cancer is expected to become the second leading cause of cancer death by the end of the decade [2]. Disappointingly, pancreatic cancer exhibits resistance to many antineoplastic therapies due to rapid progression and low rates of pathologic complete response even with the most effective systemic agents and radiotherapy [3]. To significantly prolong the nonprogressive

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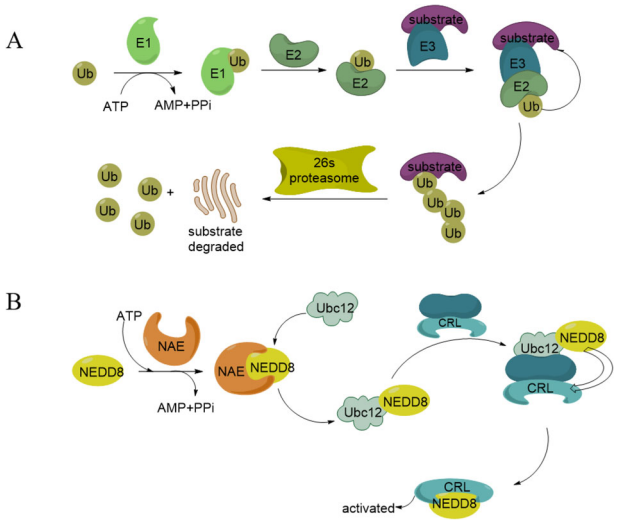


Fig. 1 **A** Summary of the UPS. **B** The NEDD8 conjugation pathway

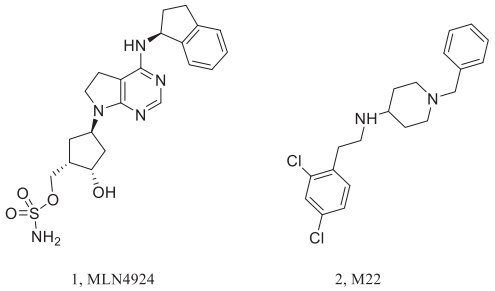


Fig. 2 Structure of MLN4924 and M22

survival of patients with pancreatic cancer, developing new anti-pancreatic cancer drugs is extremely important.

The ubiquitin-proteasome system (UPS) is a multi-component system responsible for protein degradation, which participates in the degradation of more than 80% of intracellular proteins [4–8]. Dysregulation of UPS can lead to abnormal activation or inhibition of signaling pathways, which may lead to tumors or other disease progressions [9]. The ubiquitinylation pathway is carried out by three distinct enzymatic steps. Initially, ubiquitin is activated by the ubiquitin-activating enzyme (E1) in an ATP-dependent reaction. The ubiquitinylation pathway is executed. Ubiquitin-activating enzyme E1 delivers activated ubiquitin molecules to ubiquitin-conjugating enzyme E2, and then E2-bound ubiquitin is linked to target proteins by ubiquitin ligase E3 (as shown in Fig. 1A). The UPS pathway has been intensively studied as a therapeutic target, although medications targeting the three enzymes (E1, E2, and E3) are still in the early stages of development [10]. Bortezomib [11], is the first FDA-approved proteasome inhibitor drug targeting the ubiquitin-proteasome pathway, but Bortezomib is not very effective due to its poor selectivity in substrate degradation [12].

In addition to the ubiquitin proteins, Ubiquitin-like proteins conjugation pathways, including NEDD8 and SUMO, have also been identified as critical players in the protein degradation process [13]. It covalently couples the ubiquitin-like small molecule NEDD8 to the substrate protein through a tertiary enzyme-linked reaction, thereby affecting the stability, conformation, and function of the substrate protein. (as shown in Fig. 1B). NEDD8 is initially activated by NAE in an ATP-dependent reaction and then transferred to the NEDD8 conjugating enzyme, Ubc12 (also known as UBE2M). Finally, with the coordinated action of Ubc12, NEDD8 is attached to cullin proteins, which are the central scaffold of cullin-RING ubiquitin E3 ligases (CRLs). Therefore, NAE mediates the degradation of proteins regulated by CRLs [10]. The substantial evidence indicated that the neddylation pathway is involved in a broad array of cellular functions and dysregulation of neddylation can lead to tumorigenesis [14–21]. Meanwhile, overactive neddylation has been reported in a variety of malignancies [21–24]. Hence, inhibiting NAE represents a potential approach to treating human cancers. MLN4924 [25] (shown in Fig. 2) is the first and only inhibitor of NAE that entered clinical trials to treat several types of cancer, especially hematologic malignancies [26–32]. However, clinical trials have shown that MLN4924 has a limited scope of application and general efficacy, which underlines the urgent need to develop novel NAE inhibitors [33, 34].

In our previous work, **M22** (as shown in Fig. 2) was identified as a novel reversible inhibitor of NAE through high-throughput virtual screening [35, 36]. As with A549 (lung cancer) cells, **M22** induced apoptosis in AGS (gastric cancer) cells in nude mice, inhibited tumor growth in multiple cancer cell lines with IC_{50} values in the nanomolar range, and showed low toxicity in a zebrafish model, which represents an ideal lead structure for the design of new antitumor agents. A schematic of the overall receptor-based virtual screening procedure according to our previous work is presented in Fig. 3 [35]. To screen and obtain the scaffold with drug-like properties, we downloaded 7941 molecules from the DrugBank Database and screened out 2259 marketed drug molecules through Prepare ligands in Discovery Studio 2016 (DS 2016). These small molecules were docked into the MLN4924 binding pocket (PDB ID: 3GZN) of NAE by the *CDOCKER* molecular docking module. The docking poses were scored by the scoring functions *CDOCKER_INTERATION_ENERGY, Ligscore, PLP*, and *Jain*. Finally, 124 compounds were divided into 10 categories by calculating the Tanimoto Coefficient (Tc) with ECFP_6. Finally, 22 compounds with diverse structures (as shown in Fig. S1) were obtained by manual selection. According to our experience in medicinal chemistry, the pyrido[2,3-*d*]pyrimidin-7(8*H*)-one structure was determined to replace the *N*-benzyl group in **M22** to obtain the general structural formula

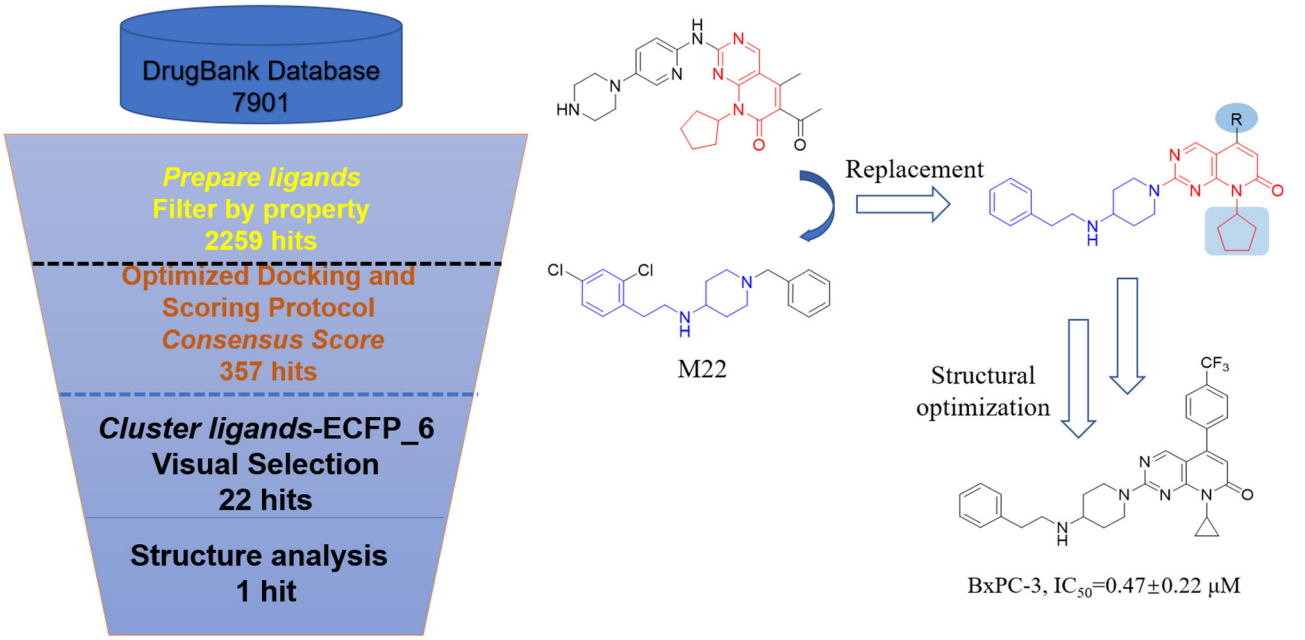


Fig. 3 The workflow of the virtual screening and the drug design of NAE inhibitors. 22 hits were filtered from DrugBank by docking scores and *Cluster Ligands*, then pyrido[2,3-*d*]pyrimidin-7(8*H*)-one structure was selceted to general structural formula and optimizated to our target compounds

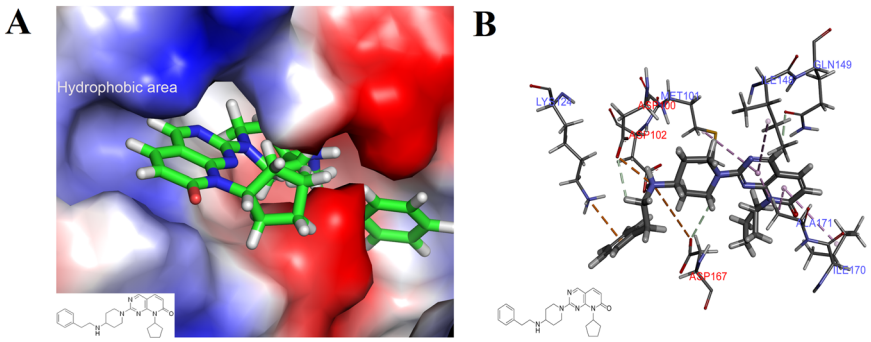


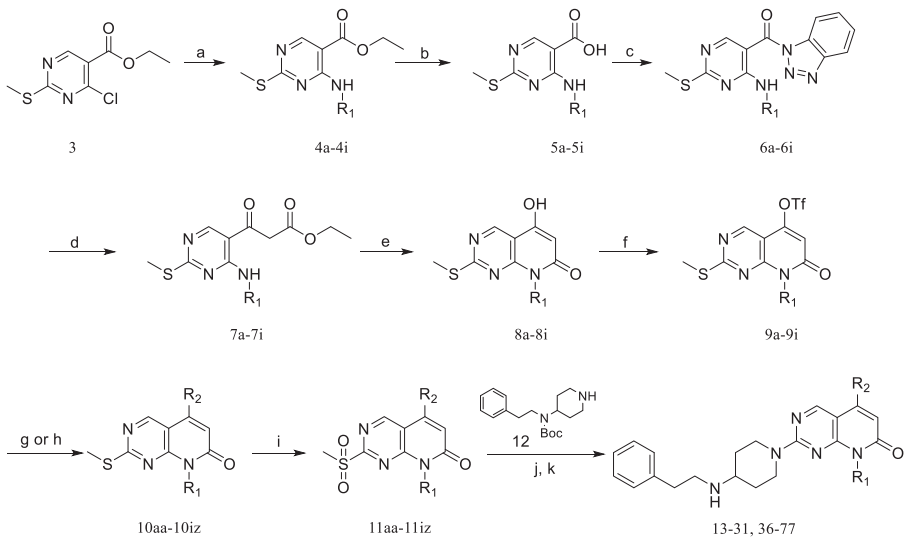
Fig. 4 **A** Docking diagram of structural formula and NAE. **B** Interaction between structural formula and NAE

of our target compounds. As shown in Fig. 4, the general formula could be accommodated in the ATP binding site of NAE. Predicted ligand-protein interaction showed that the pyrimidinone ring could form π-alkyl with Ile148, Ala171, Met101, and Ile170, and form a hydrogen bond with Asp102, Asp167, and Gln149. Compared with *N*-benzyl, the pyrimidinone ring can increase the interaction force, improving NAE inhibition. Then a series of structural optimizations were carried out based on the general structural formula. Almost 65 compounds exhibited suppression of BxPC-3 cell proliferation with IC_{50} values in the low dose range. Among those compounds, compound **51** had excellent potency against BxPC-3 cells ($IC_{50} = 0.47$ μM). In further investigation of the antitumor mechanism, compound **51** could directly inhibit NAE in the enzyme-based assay, induce apoptosis in BxPC-3 cells, and show antitumor efficacy in BxPC-3 xenograft mouse models. Moreover, compound **51** has low toxicity and a reasonable Log D value.

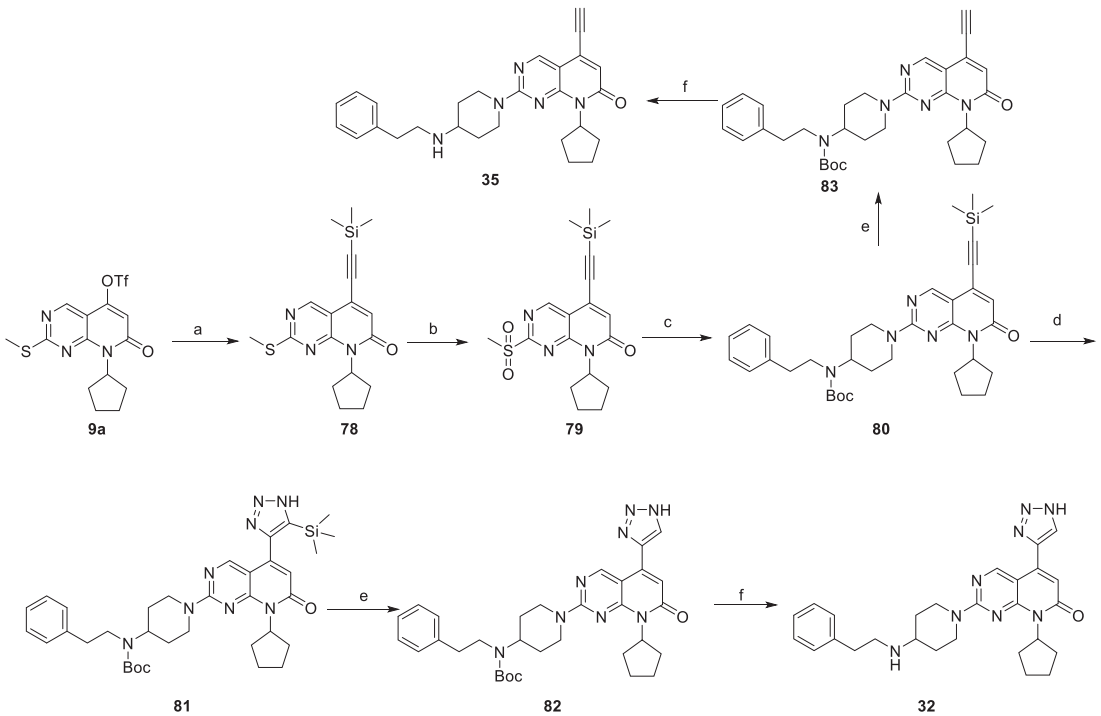
Results and discussion

Chemistry

The synthetic strategy used to prepare the target compounds **13-31**, **36-77** is outlined in Scheme 1 and the typical intermediate **12** was prepared as described formerly [36]. Starting from compound **3**, intermediates **4a**−**4i** were obtained by a nucleophilic substitution reaction with amines. Intermediates **5a-5i** were obtained by a simple hydrolysis reaction. Then **7a-7i** were obtained by activating of carboxyl group by condensation with benzotriazole, the substitution of tert-butyl malonate, and removal of the Boc group. Under the alkaline condition, **7a-7i** cyclized to form intermediates **8a-8i**, which reacted with trifluoromethane sulfonic anhydride to form the critical intermediate trifluoromethanesulfonate **9a–9i**. From **9a–9i**, intermediates **10aa–10iz** were prepared by the Suzuki coupling reaction



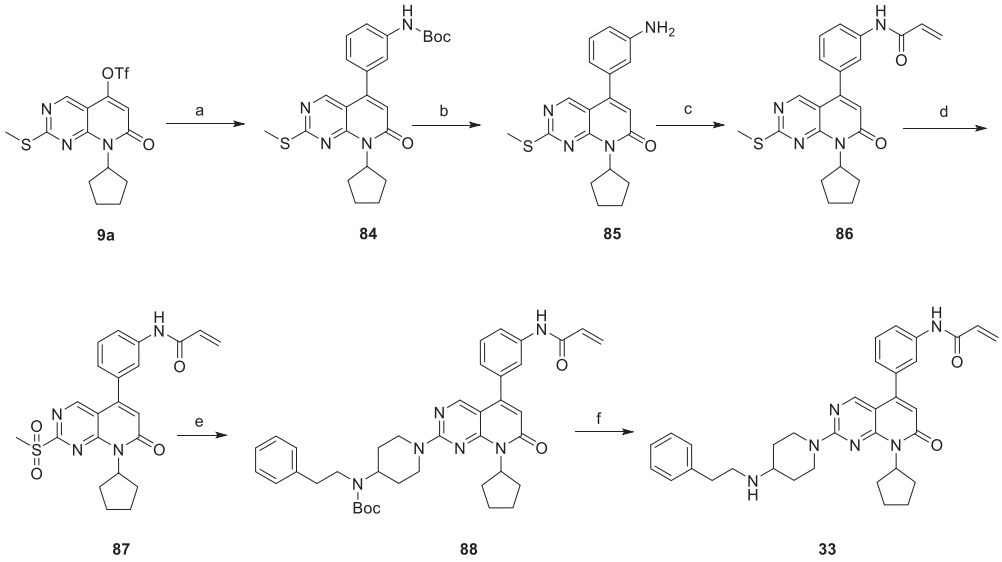
Scheme 1 Synthetic Routes of Compounds **13-31**, **36-77** [a]. [a]Reagents and conditions: **a** Amine compounds, Et_3N, THF, rt, 1 h, 60–91% yield; **b** 1 M NaOH, reflux, EtOH, 1 h, 75%-80% yield; **c** EDCI, HOBt, benzotriazole, DCM, Rt, 8 h, 57–74% yield; **d** (I) NaH, ethyl tert-butyl malonate, THF, 0 °C, 2 h, (II) TFA, DCM, rt, 64–75% yield over two steps; **e** DBU, EtOH, reflux, 2 h, 74–79% yield; **f** $(TfO)_2$, Et_3N, DCM, 0 °C, 1 h, 72–74% yield; **g** Arylboronic acid, $Pd(PPh_3)_4$, Cs_2CO_3, THF, reflux, 73%-79% yield; **h** Terminal alkynes, $Pd(PPh_3)_4$, CuI, Et_3N, THF, reflux, 69.2–73.4% yield; **i** m-CPBA, DCM, rt, 84–89% yield; **j** Intermediate 12, Toluene, reflux, 79–86% yield; **j** TFA, DCM, rt, 89–92% yield



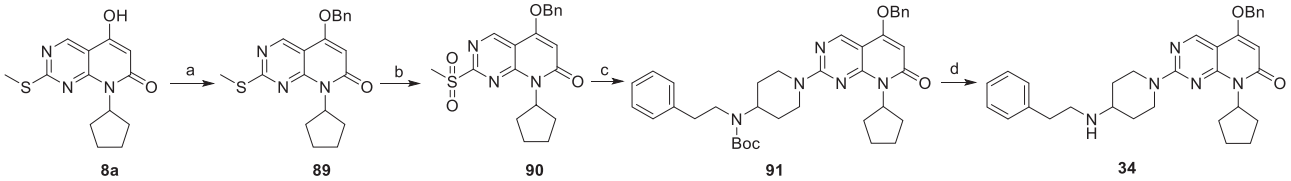
Scheme 2 Synthetic Routes of Compounds **32, 35** [a]. [a]Reagents and conditions: **a** Trimethylsilyl acetylene, $Pd(PPh_3)_4$, CuI, Et_3N, THF, reflux, 84% yield; **b** m-CPBA, DCM, rt, 85% yield; **c** Toluene, reflux, 87% yield; **d** NaN_3, DMSO, 100 °C, 3 h, 89% yield; **e** TBAF, THF, rt, 5 min; **f** TFA, DCM, rt, 89–92% yield over two steps

or Sonogashira coupling reaction. After oxidation and coupling with the previously synthesized compound **12**, target compounds **13–31**, **36–77** were obtained by removing the Boc group.

Chemical syntheses of compounds **32** and **35** are depicted in Scheme 2. The key intermediate **9a** was coupled by Sonogashira reaction, and then intermediate **79** was oxidized and coupled with side chain **12** to obtain



Scheme 3 Synthetic Routes of Compounds **33** [a]. [a]Reagents and conditions: **a** 3-(N-Boc-amino) phenylboronic acid, $Pd(PPh_3)_4$, Na_2CO_3, THF, reflux, 73% yield; **b** TFA, DCM, rt, and 89% yield; **c** Acryloyl chloride, $NaHCO_3$, CH_3CN, rt; **d** m-CPBA, DCM, rt, 84% yield; **e** Intermediate **12**, toluene, reflux, 88% yield; **f** TFA, DCM, rt, 92% yield



Scheme 4 Synthetic Routes of Compounds **34** [a]. [a]Reagents and conditions: **a** Benzyl bromide, $NaHCO_3$, CH_3CN, reflux, 83% yield; **b** *m*-CPBA, DCM, rt, 76% yield; **c** Intermediate 12, toluene, reflux, 89% yield; **d** TFA, DCM, rt, 90% yield

intermediate **80**, which condensed with sodium azide to form intermediate **81**. Finally, the trimethylsilyl and Boc were removed to obtain the target compound **32**. Compound **35** was obtained by removing trimethylsilyl and the Boc group from intermediate **80**.

The synthetic route of compound **33** was described in Scheme 3. The key intermediate **9a** was coupled with 3-(N-Boc-amino) phenylboronic acid by Suzuki reaction to obtain compound **84**, followed by removal of the Boc protective group and condensed with acryloyl chloride to form intermediate **86**. Then the target compound **33** was obtained as described previously.

The syntheses of compound **34** were outlined in Scheme 4. The key intermediate **8a** was condensed with benzyl bromide and then the remaining synthesis steps were the same as Scheme 1.

Structure-based molecular design strategy

As shown in Fig. 4A, a hydrophobic pocket was found above the 5-position of pyrido[2,3-*d*]pyrimidin-7(8*H*)-one. Therefore, hydrophobic substituents were introduced to pyrido[2,3-*d*]pyrimidin-7(8*H*)-one to occupy the hydrophobic region. Besides these, biaryl structures are privileged structures in antitumor drugs due to their rotatable bonds, suitable stiffness, bulk and shape, and suitable for binding [36, 37]. Therefore, hydrophilic and hydrophobic aryl rings were introduced at the 5-positions of pyrido[2,3-*d*]pyrimidin-7(8*H*)-one (**3-35**, shown in Fig. 5 and Table 1). Biological evaluation results are summarized in Table 1 and Table S1. It was discovered that modifying the 5-positions of pyrido[2,3-*d*]pyrimidin-7(8*H*)-one with phenyl group (**13–29**) increased anti-proliferation activities against BxPC-3 (pancreatic cancer), HCT-116 (colorectal carcinoma), and A549 (lung cancer), comparing with **M22**. Cancer cells used in this study were neddylation upregulated [38, 39]. Compounds **30-41** also showed more potent anti-proliferation activities than **M22**. It was disclosed that when introducing substituents such as fluorine to the meta- or para-position of the benzene ring, the resulting compound **15** or **16** had increased inhibition against BxPC-3 with IC_{50} values of 1.45 μM and 0.96 μM, respectively. When an electron-withdrawing group such as $-CF_3$ or electron-donating group such as $-OCH_3$ was installed at the benzene ring, the cancer cells' inhibitory activities of compounds **19–22** were dramatically increased compared to

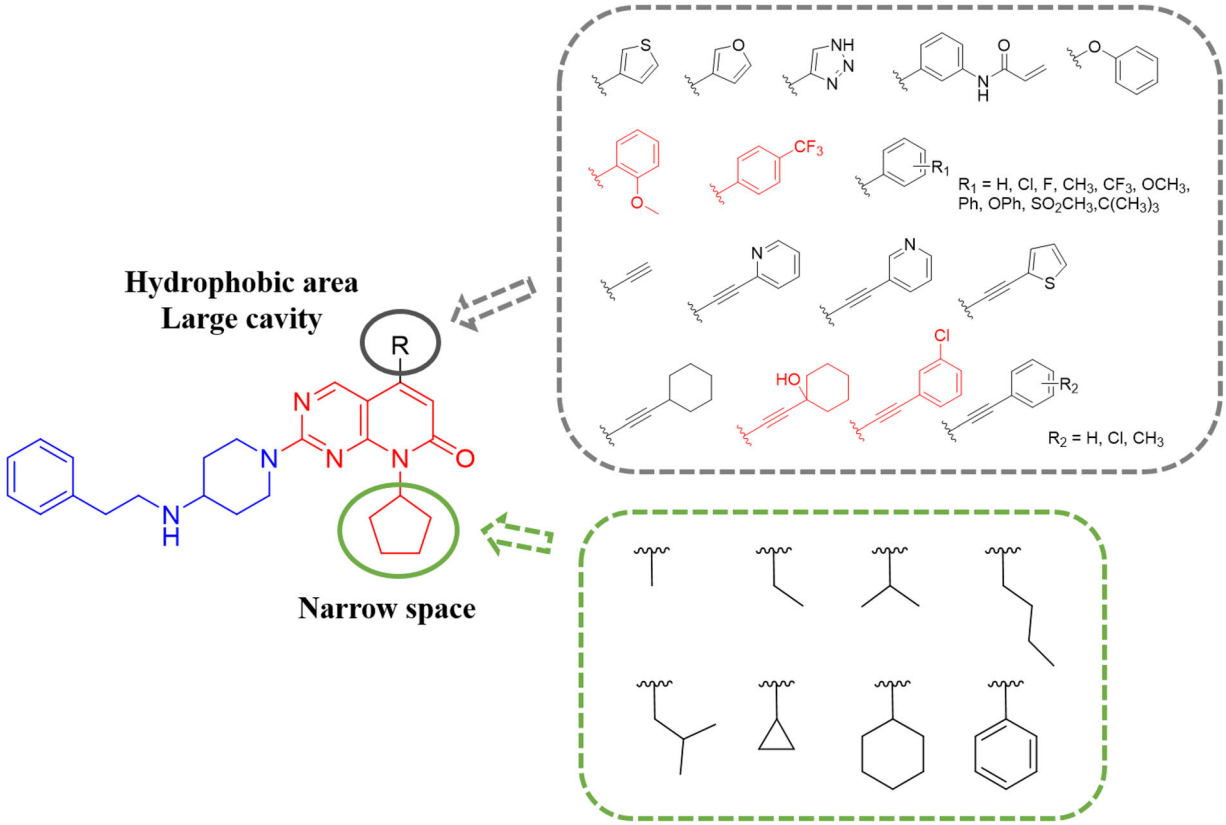


Fig. 5 SAR of designed NAE Inhibitors

M22. However, these optimized compounds have similar activities, it seems that antiproliferative activity depends on general structural formula and the aromatic group modification could not improve the activity a lot.

Because of its rigidity and hydrophobicity structure, the ethynyl group was introduced to pyrido[2,3-*d*]pyrimidin-7(8*H*)-one with rings in this study. Therefore, compounds **35-45** (shown in Table 1) were designed, synthesized, and tested. As shown in Tables 1 and S1, those 5-ethynyl substituted derivatives had increased antiproliferation activities, indicating that introducing ethynyl groups on the 5-position of pyrido[2,3-*d*]pyrimidin-7(8*H*)-one would also contribute to increased antiproliferation activity. Compounds **45** and **39** had the lowest IC_{50} value for inhibiting the proliferation of BxPC-3 and HCT-116 cells.

To obtain more effective antitumor drugs, the dominant group of the first round of structural modification was retained. The structural modification was conducted focusing on the 8-position of pyrido[2,3-*d*]pyrimidin-7(8*H*)-one. Through the strategy of group replacement, the 8-position alkane was changed. The length and volume of the base chain could change the position of the compound in the binding site, evaluate the binding effect of different groups, and design a series of new compounds (as shown in Fig. 5). We introduced linear and cyclic alkanes of different sizes to investigate the effect of volume on compound activity. As shown in Tables 2 and S2, compounds **51**, **59**, **67**, and **75** with cyclopropyl structure generally showed comparable activity to the positive drugs in BxPC-3 cell assays, and the volume is just right. In addition, cyclopropane has directionality, and the H of cyclopropane acts as a nonclassical hydrogen bond donor to form hydrogen bonds.

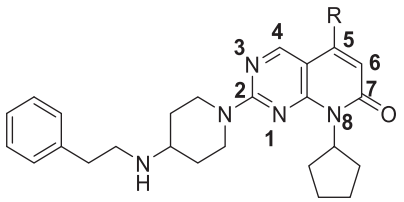
In vitro cytotoxicity of selected compounds

NAE is widely expressed in non-cancerous cells, but it is expressed at a low level. NAE inhibitors may have some effect on non-cancer cells at high concentrations. Based on cell proliferative assay results, **51**, **58**, **59**, **64**, **67**, and **72** were chosen to investigate their toxicity against normal cells L-02 (Human Liver Cells). On average, tested compounds exhibited a 3–10 times antiproliferative effect on BxPC-3 cells compared to normal cells, (shown in Fig. 6 and Table 3) suggesting that the proliferation inhibition on BxPC-3 cells was due to NAE inhibition rather than general toxicity.

Half-life and intrinsic clearance of 51, 59, 64, 72

During the early discovery phase of compounds, the human-derived liver microsomes in vitro incubation

Table 1 Compounds **13**–**45** and their anti-tumor Activity against BxPC-3



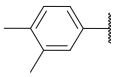
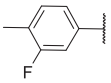
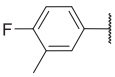
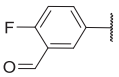
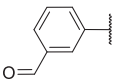
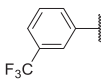
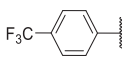
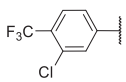
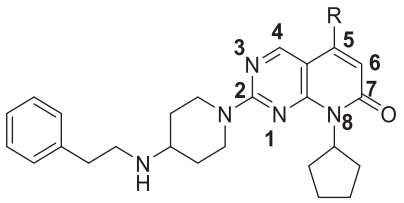
Compd.	R	Cellular IC_{50} (μM)[a] BxPC-3	Log D	-CDOCKER_ INTERACTION_ENERGY
13		2.87 ± 0.71	4.74 ± 0.19	52.39
14		1.56 ± 0.38	4.10 ± 0.01	42.62
15		1.45 ± 0.04	4.32 ± 0.008	--
16		0.96 ± 0.44	4.06 ± 0.007	40.31
17		2.22 ± 0.15	3.77 ± 0.004	39.71
18		0.90 ± 0.10	3.80 ± 0.01	26.06
19		1.92 ± 0.04	4.14 ± 0.007	48.66
20		1.30 ± 0.75	4.15 ± 0.013	37.42
21		0.99 ± 0.03	4.30 ± 0.021	39.50
22		1.78 ± 0.14	4.05 ± 0.002	48.78
23		1.27 ± 0.04	4.33 ± 0.009	36.36

Table 1 (continued)



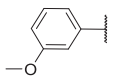
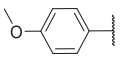
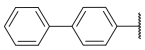
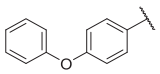
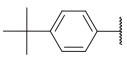
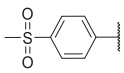
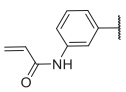
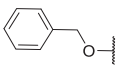
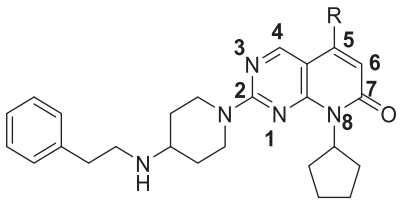
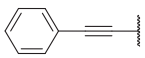
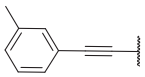
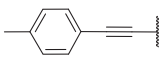
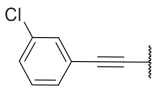
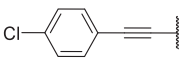
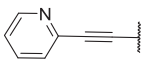
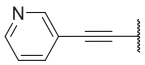
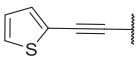
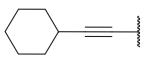
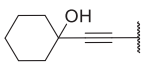
Compd.	R	Cellular IC_{50} (μM)[a] BxPC-3	Log D	-CDOCKER_ INTERACTION_ENERGY
24		1.81 ± 0.06	4.81 ± 0.009	40.03
25		1.37 ± 0.07	3.93 ± 0.002	33.77
26		2.54 ± 0.05	4.09 ± 0.001	43.21
27		2.89 ± 0.74	3.97 ± 0.004	47.74
28		1.95 ± 0.10	3.66 ± 0.068	42.07
29		2.64 ± 0.73	3.47 ± 0.006	43.75
30		2.35 ± 0.12	4.14 ± 0.046	54.88
31		1.41 ± 0.02	4.14 ± 0.005	56.19
32		2.87 ± 0.22	3.43 ± 0.01	57.73
33		2.42 ± 0.13	3.80 ± 0.006	32.21
34		1.50 ± 0.11	3.60 ± 0.003	32.48
35		1.83 ± 0.09	3.98 ± 0.002	34.79

Table 1 (continued)



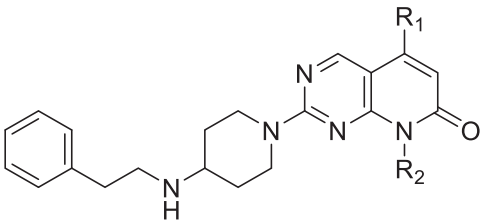
Compd.	R	Cellular IC_{50} (μM)[a] BxPC-3	Log D	-CDOCKER_ INTERACTION_ENERGY
36		1.39 ± 0.22	3.55 ± 0.002	34.45
37		1.79 ± 0.07	4.08 ± 0.003	35.56
38		2.09 ± 0.05	3.65 ± 0.003	32.33
39		1.76 ± 0.10	3.54 ± 0.01	51.26
40		2.14 ± 0.53	3.83 ± 0.10	49.39
41		1.90 ± 0.05	3.79 ± 0.004	44.20
42		3.07 ± 0.73	3.60 ± 0.019	41.58
43		2.37 ± 0.15	3.48 ± 0.001	46.47
44		1.24 ± 0.08	4.35 ± 0.023	47.31
45		0.69 ± 0.19	4.09 ± 0.001	31.52
M22		3.96 ± 0.79		48.66
MLN4924		0.25 ± 0.21		64.69

[a]IC50 values are expressed as the mean ± SD from three independent experiments

method were used to assess the risk of compounds being metabolized in the body. As usual, half-life ($T_{1/2}$) and intrinsic clearance (CL_{int}) were used to evaluate the druggability of compounds **51**, **59**, **64**, **and 72**. As shown in Table 4, **51** has a better half-life and inherent clearance rate than MLN4924.

51 was chosen for further validation because it had the lowest IC_{50} value against BxPC-3 cells and was moderately

Table 2 Compounds **46**–**77** and their anti-tumor activity against BxPC-3



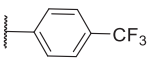
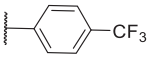
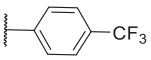
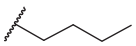
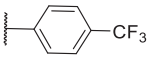
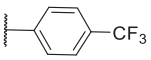
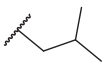
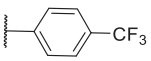
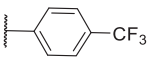
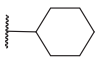
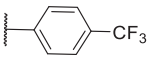
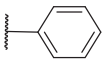
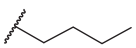
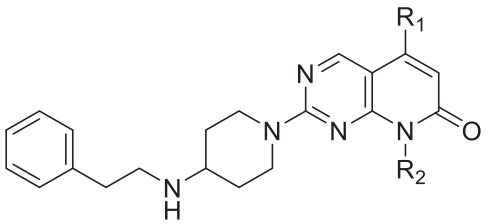
Compd.	R_1	R_2	Cellular IC_{50} (μM) BxPC-3	Log D	-CDOCKER_ INTERACTION _ENERGY
46			1.20 ± 0.04	4.08 ± 0.04	53.60
47			1.39 ± 0.16	5.60 ± 0.18	51.49
48			1.32 ± 0.22	4.17 ± 0.008	42.67
49			1.7 ± 0.07	4.34 ± 0.22	51.60
50			1.21 ± 0.07	3.85 ± 0.001	46.26
51			0.47 ± 0.22	4.07 ± 0.006	55.11
52			2.04 ± 0.05	3.98 ± 0.006	35.27
53			1.16 ± 0.12	3.66 ± 0.004	32.79
54			3.57 ± 0.04	3.92 ± 0.001	49.77
55			1.31 ± 0.17	4.41 ± 0.01	53.23
56			1.83 ± 0.03	3.88 ± 0.006	51.91

Table 2 (continued)



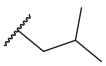
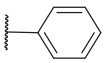
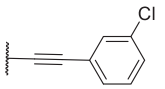
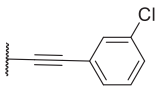
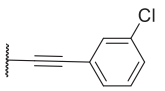
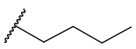
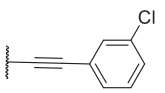
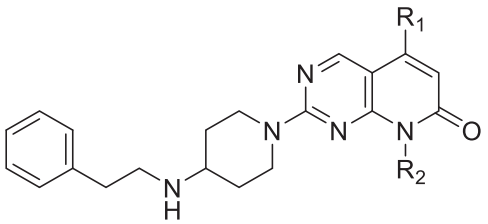
Compd.	R_1	R_2	Cellular IC_{50} (μM) BxPC-3	Log D	-CDOCKER_ INTERACTION _ENERGY
57			1.25 ± 0.15	4.27 ± 0.02	48.26
58			0.87 ± 0.39	3.61 ± 0.02	53.02
59			0.95 ± 0.25	2.95 ± 0.005	48.33
60			1.17 ± 0.15	3.62 ± 0.002	33.64
61			1.93 ± 0.14	3.39 ± 0.001	34.36
62			1.68 ± 0.15	2.97 ± 0.002	57.06
63			1.07 ± 0.11	2.98 ± 0.004	52.17
64			0.69 ± 0.09	3.87 ± 0.04	47.41
65			1.60 ± 0.04	3.96 ± 0.02	45.44

Table 2 (continued)



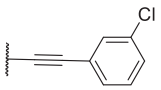
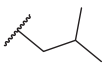
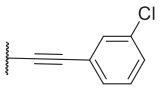
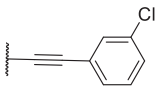
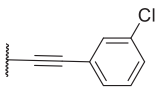
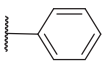
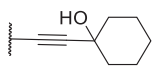
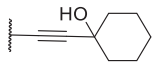
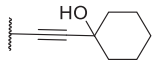
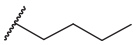
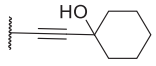
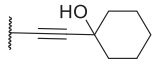
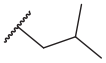
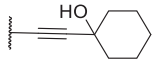
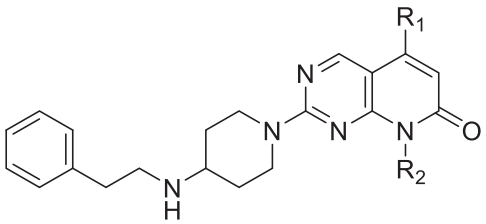
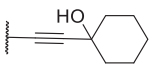
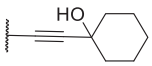
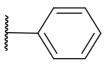
Compd.	R_1	R_2	Cellular IC_{50} (μM) BxPC-3	Log D	-CDOCKER_ INTERACTION _ENERGY
66			1.21 ± 0.07	3.79 ± 0.023	49.23
67			0.62 ± 0.13	3.60 ± 0.002	49.93
68			3.15 ± 0.11	3.48 ± 0.002	42.56
69			1.59 ± 0.83	3.72 ± 0.001	32.76
70			1.55 ± 0.07	3.85 ± 0.02	55.31
71			1.70 ± 0.06	3.47 ± 0.001	58.03
72			0.56 ± 0.07	3.78 ± 0.08	45.79
73			1.01 ± 0.09	5.23 ± 0.01	60.61
74			1.31 ± 0.05	4.08 ± 0.001	57.77
75			1.59 ± 0.18	3.95 ± 0.002	55.25

Table 2 (continued)



Compd.	R_1	R_2	Cellular IC_{50} (μM) BxPC-3	Log D	-CDOCKER_ INTERACTION _ENERGY
76			1.00 ± 0.08	5.07 ± 0.04	60.61
77			1.80 ± 0.06	3.66 ± 0.01	--
M22			3.96 ± 0.79		48.66
MLN4924			0.43 ± 0.39		64.69

[a]IC50 values are expressed as the mean ± SD from three independent experiments

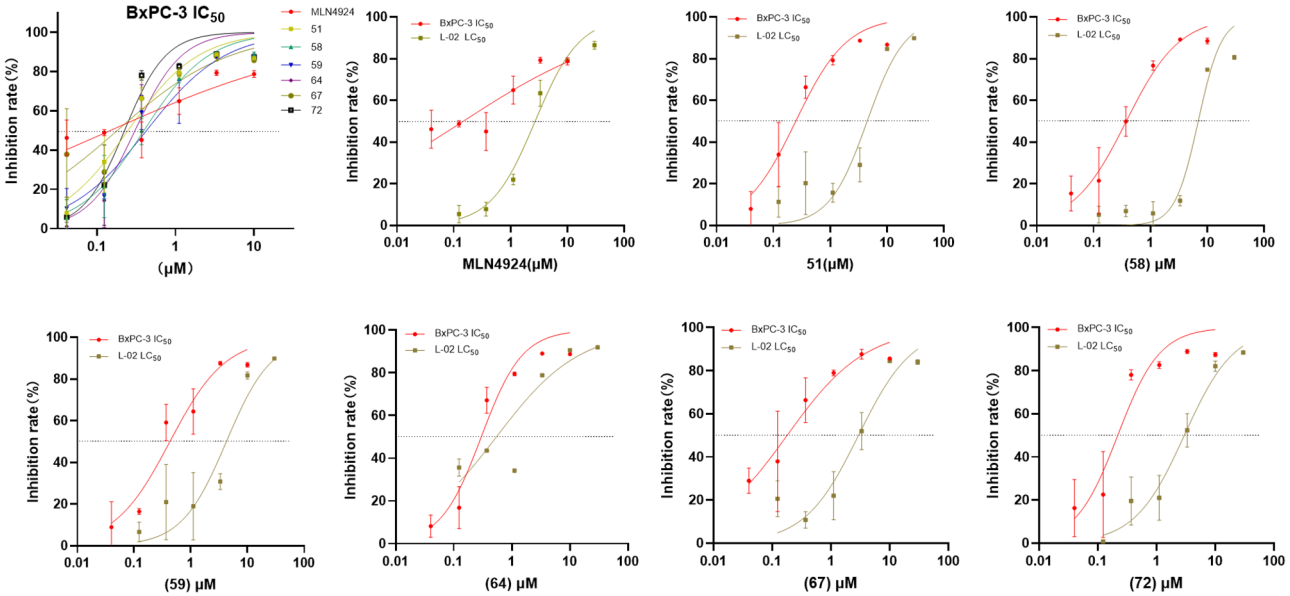
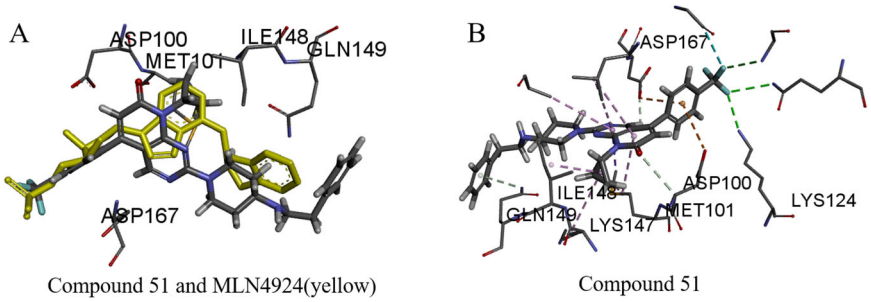


Fig. 6 The IC_{50} and LC_{50} dose–response curves of tested compounds

selective for normal cells and had a longer half-life. In addition, **51** is a relatively high binding force in molecular docking. Molecular docking (Fig. 3B) demonstrated that compound **51** had similar binding mode with the structural formula and formed three key hydrogen bonds with Asp100, Ile148 and Gln149 and a hydrogen bond with Asp167 which is similar to the co-crystal structure of MLN4924 (Fig. 7).

Compound 51 inhibited NAE activity in the enzyme-based assay

An enzymatic assay using an E2-mediated thioester product of Ubc12-NEDD8 was used to determine the level of NAE-mediated production in a cell-free system. NAE activity is reduced when the Ubc12-NEDD8 conjugation level is lower. Recombinant human NAE was incubated with



Compound 51 and MLN4924(yellow) Compound 51

Fig. 7 **A** Merging between top-ranked **51** pose and MLN4924 conformation (shown in yellow) from NAE crystal (PDB ID:3G ZN). **B** Docking modes of compounds **51** with NAE

Table 3 LC_{50} values of tested compounds (μM, $n = 3$)[a]

Compd.	LC_{50}(μM) L-02
51	4.90 ± 0.05
58	6.42 ± 0.06
59	6.08 ± 0.09
64	2.82 ± 0.10
67	3.11 ± 0.49
72	3.13 ± 0.85
MLN4924	2.11 ± 0.17

[a]LC_{50} indicates the compound concentration required to inhibit cell viability by 50%

Table 4 The metabolic stability of **51**, **59**, **64**, **72**

Compd.	$T_{1/2}$(min)	CL_{int}(mL*min^{-1}*mg^{-1})
51	385	0.0036
59	101.91	0.0136
64	54.14	0.0128
72	117.76	0.0067
MLN4924	238	0.0058

NEDD8, Ubc12, and ATP in the presence of compound **51** for 1 h. Then levels of Ubc12-NEDD8 thioester product were detected by western blot. The intensity of Ubc12-NEDD8 bands was reduced in a concentration-dependent manner. The formation of Ubc12-NEDD8 was inhibited at 3.33 M (as shown in Fig. 8), indicating that compound **51** was an effective NAE inhibitor.

Effects of 51 on NAE-associated signaling pathway

Compound 51 was tested for its ability to inhibit NAE activity in BxPC-3 cells by western blot in the following study. As shown in Fig. 9A, NAE activated NEDD8 and then transferred it to Ubc12, resulting in an accumulation of the Ubc12-NEDD8 complex in the control group. After treatment with **51** for 24 h, the NEDD8 combined Ubc12 was noticeably decreased concentration-dependently,

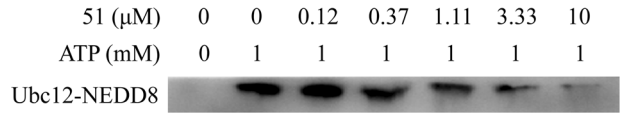


Fig. 8 Compound **51** inhibited Ubc12-NEDD8 formation in a dose-dependent manner in an enzyme assay

indicating that NAE activity was inhibited by **51**. Inhibition of NAE can lead to the inactivation of CRL, causing the accumulation of substrates such as p27, CDT1, and NRF2, resulting in cell cycle arrest and induction of apoptosis. BxPC-3 cells were treated with **51** for 24 h, p27, CDT1, and NRF2 began to accumulate, suggesting that **51** could stabilize CRL substrates by suppressing neddylation.

Apoptosis induced by 51 in BxPC-3 Cells

Flow cytometry was used to assess the effect of compound 51 on inducing apoptosis in BxPC-3 cells. The results showed that in **51**-treated BxPC-3 cells, apoptotic cells appeared in a dose-dependent pattern (Fig. 10). The apoptotic rates (Annexin V-EGFP) of BxPC-3 cells were increased to 82.87 percent of total cells (15 μM, 24 h) after treatment with **51**, whereas only a few cells were observed as apoptotic in the control group. BxPC-3 cells went through the process of transitioning from early to late apoptosis as the concentration of **51** increased. These findings suggested that compound **51** could trigger apoptosis in a concentration-dependent manner.

The occurrence of most tumors is accompanied by the abnormal expression of the tumor suppressor p53. When cells are damaged, the level of p53 in the cells will increase rapidly and induce cell apoptosis. In cell apoptosis, characteristic apoptotic bodies are formed, which are regulated by Bcl-2. When the cell is apoptotic, the expression of Bax becomes more substantial, and the expression of Bcl-2 becomes weaker. The ratio of Bax/Bcl-2 determines the direction of cell apoptosis. The ratio of Bax/Bcl-2 is upregulated to promote cell apoptosis, and the ratio of Bax/Bcl-2 is down-regulated to inhibit cell apoptosis. This experiment investigated the effect of compound **51** on intracellular p53, Caspase 3, Bax, and Bcl-2. After BxPC-3 cells were treated

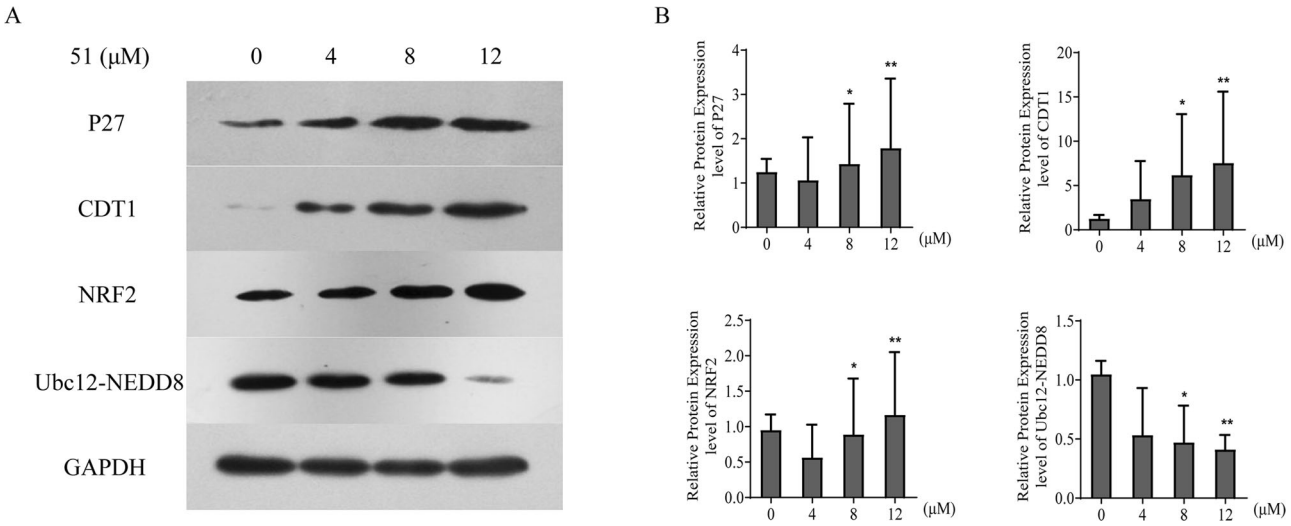


Fig. 9 **A** Compound **51** could inhibit neddylation and CRL substrate degradation in BxPC-3 cells. **B** The relative protein expression level of P27, CDT1, NRF2, Ubc12-NEDD8. Total cell lysates were subjected to Western blot analysis. GAPDH was used as a loading control. $^{**}P < 0.01$, $^{*}P < 0.05$

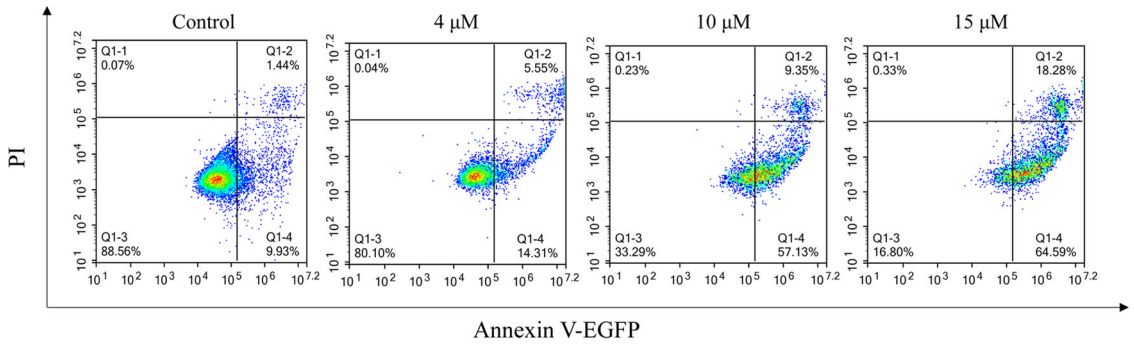


Fig. 10 Flow cytometric analysis of the apoptotic effect of **51** in BxPC-3 cells through Annexin-V-EGFP/PI staining assay

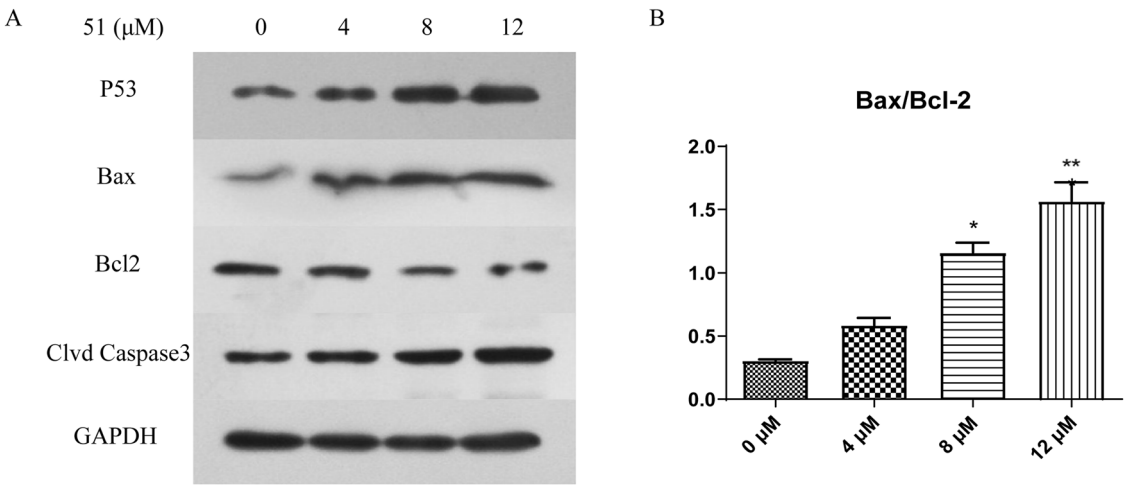


Fig. 11 **A** Expression of apoptosis protein induced by Compound **51** **B** Relative protein expression level of Bax/Bcl-2. $^{**}P < 0.01$, $^{*}P < 0.05$

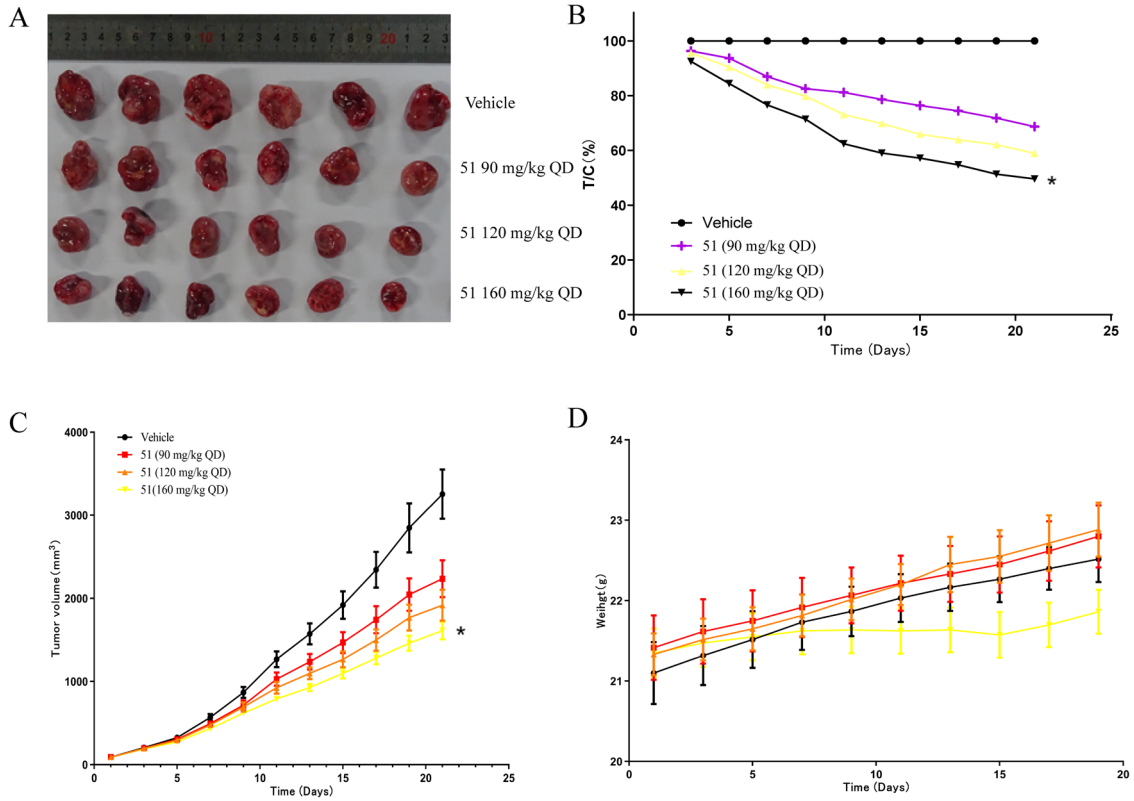


Fig. 12 In vivo activities of compound **51** in cell-derived xenografts. Nude mice bearing subcutaneous xenografts derived from BxPC-3 were injected subcutaneously daily with 90, 120, or 160 mg/kg compound **51**, respectively for 21 consecutive days. **A** Anatomical nude mice's tumor tissues untreated or treated with **51**. **B** Relative rate of tumor proliferation. **C** The tumor weight values were measured daily. **D** The growing curves of mice's body weight. Data are shown as mean ± SEM, $n = 6$; $^*P < 0.05$

with **51** for 24 h, the protein content was detected by Western blot. Results are shown in Fig. 11. As the dosage increased, p53 protein rose, the ratio of Bax/Bcl-2 increased, cell apoptosis increased, and apoptosis markers gradually accumulated.

Those results further supported that compound **51** could induce apoptosis in a concentration-dependent manner.

In vivo study of compound 51

We investigated the antitumor activity of the new NAE inhibitor **51** in vivo based on its promising in vitro potency. Animals were given subcutaneous injections of vehicle or compound **51** for 21 days. In our preliminary study, the maximum tolerated single dose for compound **51** was determined as 160 mg/kg. Therefore, compound **51** was given at 90 mg/kg, 120 mg/kg, or 160 mg/kg once daily (QD) consecutively. As shown in Fig. 12, **51**-treated mice showed significant suppression of tumor growth in a dose-dependent manner, compared to vehicle-treated mice. No significant change in body weight was observed at those doses, indicating that the treatments have no apparent toxicity in the xenograft models. Results show that compound **51** has a certain antitumor effect and has the potential to treat pancreatic cancer.

Conclusion

The NEDD8-activating enzyme (NAE), which regulates the degradation and turnover of cancer-related proteins by activating the cullin-RING E3 ubiquitin ligases, is an emerging target for cancer therapy. This research conducted a structural hopping strategy and synthesized a series of compounds by structural optimization. Candidates with potent anticancer activities were discovered during in vitro activity testing in three cancer cells, particularly in BxPC-3 cells. In a preliminary investigation, the best candidate compound **51** showed the least undesirable side effects and fulfilled lipophilicity after cytotoxicity testing. In the in vitro incubation experiment of liver microsomes, candidate compound **51** showed the best half-life. In addition, compound **51** induced a dose–response drop in the level of Ubc12-NEDD8, indicating that it was an NAE inhibitor. Further research revealed that compound **51** induced apoptosis in BxPC-3 cells and reduced tumor development. In general, compound **51** is a novel and highly effective NAE inhibitor for pancreatic cancer, worthy of further analysis.

Experimental

Chemistry general

All reactions were performed in glassware containing a Teflon-coated stir bar. Commercially available solvents and reagents were obtained from Adamas, Accela, Aikonchem, Sinopharm Chemical, Energy Chemical, Aladdin, Macklin, Meyer, Leyan, MCE, Bidepharm, Sigma-Aldrich, J&K, and TCI, and used without further purification. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker 300, 400, or 500 MHz NMR spectrometer. Chemical shifts (δ) were reported in ppm downfield from an internal tetramethylsilane standard. High-resolution mass spectrometry (HRMS) analysis was recorded on an HP1100 spectrometer. Flash column chromatography on silica gel (200−300 mesh) was used for the routine purification of reaction products. All reactions were monitored by thin-layer chromatography on silica gel plates (15 mm × 50 mm) and spots were visualized under UV light at 254 nm. HPLC analysis was conducted for all compounds on a Shimadzu LC system (Inertsil ODS-SP C18 column, 4.6 mm × 250 mm, 5 μM). Most compounds are >95% pure by HPLC analysis, compounds 28, 30, 36, 39, 44, 49, 53, and 62 are >93% pure by HPLC analysis.

Synthetic procedures

General method for the synthesis of 4a-4i

Raw material **3** (50 mmol), corresponding amine (75 mmol), and triethylamine (150 mmol) were added into a round bottom flask with 150 mL THF in turn, the mixture was stirred at 35 °C for 1 h. Excess solids were filtered out, and the filtrate was evaporated under reduced pressure to remove THF. Then the round bottom flask was reconstituted with 150 mL ethyl acetate, washed with saturated NH_4Cl solution (100 mL × 3), and then washed with saturated NaCl solution three times (100 mL × 3), anhydrous sulfuric acid. The organic phase was dried with sodium and filtered. The filtrate was rotated under reduced pressure to remove the solvent, 15 mL of toluene was added, and the solvent was removed under reduced pressure by rotary evaporation to obtain a yellow oil. The crude product was used for the next step without any purification.

General method for the synthesis of 5a–5i

The prepared product **4a–4i** (50 mmol) and 30 mL 1 mol L^{-1} sodium hydroxide solution were added into a 250 mL round bottom flask with 25 mL ethanol. The mixture was heated to reflux for 5 h. The ethanol was removed by rotary evaporation, the pH was adjusted to 3–4 with 1 mol L^{-1} hydrochloric acid, a large amount of white solid was precipitated, filtered by suction, the filter cake was washed with a small amount of water, *n*-hexane, and dried to obtain white solid. The crude product was used for the next step without any purification.

General method for the Synthesis of 6a–6i

The prepared product **5a–5i** (50 mmol), benzotriazole (100 mmol), EDCI (60 mmol), and HOBt (150 mmol) were added into a 250 mL round bottom flask with 150 mL dichloromethane. The mixture was stirred at 35 °C for 16 h, and the mixture gradually became clear. After the reaction was completed, silica gel was added to make sand and purified by column chromatography (PE:EA = 15:1) to obtain white crystals, the product was used for the next step.

General method for the synthesis of 7a-7i

NaH (75 mmol) was added into a 250 mL round-bottom flask with 100 mL anhydrous THF under an N_2 atmosphere. The mixture was stirred in an ice bath for 30 min. When the internal temperature dropped to 0–5 °C, propylene glycol Ethyl tert-butyl ester (75 mmol) was added and the mixture was stirred at 0 °C for 30 min. 70 mL of prepared product **6a–6i** (50 mmol) in THF was added into a round-bottom flask under an N_2 atmosphere, the mixture was stirred in an ice bath for 3 h. After the reaction was completed, 200 mL of saturated aqueous ammonium chloride solution was added, most of the THF was distilled under reduced pressure, and the mixture was extracted three times with EA (100 mL × 3), and washed with saturated aqueous NaCl (100 mL × 3), dried over sodium sulfate, filtered, and the solvent was evaporated under reduced pressure to obtain a crude product. the crude product was reconstituted with 50 mL of dichloromethane, stirred magnetically, and 25 mL of trifluoroacetic acid was added. An irritating gas was generated. After the reaction was completed, the mixture was evaporated under reduced pressure with 25 mL toluene and purified and separated by column chromatography (PE:EA = 10:1) to obtain yellow oil, the product was used for the next step.

General method for the synthesis of 8a-8i

The prepared products **7a–7i** (50 mmol) and DBU (100 mmol) were added into a 100 mL round bottom flask with 20 mL absolute ethanol. The mixture was heated to reflux for 2 h. The temperature was cooled down naturally, and most of the ethanol in the system was removed by rotary evaporation under reduced pressure. 1 mol L^{-1} hydrochloric acid was added to adjust the pH of the reaction system to 2–3. A large amount of white solid precipitated,

filtered, and filtered. The cake was washed with a small amount of water and dried by blowing to obtain a white solid, the product was used for the next step.

General method for the synthesis of 9a–9i

The prepared product **8a–8i** (50 mmol) was added into a 250 mL round bottom flask with 70 mL anhydrous dichloromethane, and triethylamine (75 mmol) was added. The mixture was cooled to 0 °C, and then trifluoromethane sulfonic anhydride (75 mmol) was added dropwise with a constant pressure dropping funnel under the atmosphere of N_2. The reaction system first turned wine red and then yellow. After the reaction was completed, the mixture was purified by column chromatography (PE:EA = 24:1) to obtain a white solid, the product was used for the next step.

General method for the synthesis of 10aa-10iz

The prepared product **9a-9i** (10 mmol), Cs_2CO_3 (20 mmol), $Pd(PPh_3)_4$ (0.5 mmol), and Phenylboronic acid compounds (15 mmol) were added into a 50 mL round bottom flask with 20 mL anhydrous THF. The mixture was heated to reflux for 1.5 h under an N_2 atmosphere. After the reaction was completed, the mixture was purified by column chromatography (PE:EA = 5:1) to obtain a solid product. Or the prepared products **9a-9i** (10 mmol), triethylamine (30 mmol), CuI (1 mmol), $Pd(PPh_3)_4$ (1 mmol), and alkynes compounds (15 mmol) were added into a 50 mL round bottom flask with 20 mL anhydrous THF. The mixture was heated to reflux for 1.5 h under an N_2 atmosphere. After the reaction was completed, the mixture was purified by column chromatography (PE:EA = 50:1) to obtain the solid product, the product was used for the next step.

General method for the synthesis of 11aa-11iz

The prepared product **10aa-10iz** (1 mmol) and m-CPBA (3 mmol) were added into a 50 mL round-bottom flask with 20 mL dichloromethane. The mixture was stirred at room temperature for 1 h. 5 mL saturated Na_2SO_3 aqueous solution was added to quench the reaction, the mixture was washed with saturated Na_2CO_3 aqueous solution (10 mL × 3) and saturated NaCl aqueous solution (10 mL × 3), and dried with anhydrous sodium sulfate. The mixture was filtered and evaporated under reduced pressure to obtain oil, the product was used for the next step.

8-Cyclopropyl-2-(4-(phenethylamino)piperidin-1-yl)-5-(4-(trifluoromethyl)phenyl)pyrido[2,3-d]pyrimidin-7(8H)-one (51) The prepared oil **11gi** (5 mmol) and compound **12** were added into a 25 mL round bottom flask with 15 mL toluene. The mixture was heated to reflux for 2 h under N_2 atmosphere. After the reaction was completed, the mixture was cooled to room temperature, and the mixture was purified by column chromatography (PE:EA = 30:1) to obtain solid product. Then white solid (0.5 mmol) and 4 mL of trifluoroacetic acid were added into a 25 mL round bottom flask with 20 mL of dichloromethane, stirred magnetically. The mixture was stirred at room temperature for 1 h. After the reaction was completed, 10 mL of toluene was added, and the solvent was distilled off under reduced pressure. An oily substance was obtained, which was slurried by adding 20 mL of ether, filtered, the filter cake was washed with ether, and dried to obtain 173 mg of white solid powder, 98% yield, 98.8% HPLC purity. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.73 (s, 2H), 8.25 (s, 1H), 7.55 (d, J = 4.9 Hz, 2H), 7.51 (d, J = 4.8 Hz, 2H), 7.29–7.16 (m, 4H), 6.28 (s, 1H), 5.02 (d, J = 8.1 Hz, 2H), 3.31 (s, 1H), 3.20 (s, 2H), 3.05-2.96 (m, 4H), 2.83–2.79 (m, 1H), 2.20 (d, J = 6.9 Hz, 2H), 1.84 (s, 1H), 1.80–1.73 (m, 2H), 1.19 (q, J = 12.7 Hz, 2H), 0.89 (d, J = 4.3 Hz, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 164.1, 159.9, 157.7, 157.2, 147.4, 138.9, 136.0, 131.3 (q, J_{CF} = 32.6 Hz), 129.0, 128.9, 128.5, 127.3, 125.8, 125.7 (q, J_{CF} = 10.8 Hz), 122.0, 118.5, 117.6, 114.6, 104.9, 55.8, 46.1, 42.3, 32.5, 28.2, 25.1, 9.4. HRMS (ESI) (m/z): calculated for $C_{30}H_{31}F_3N_5O$ $[M + H]^+$ 534.2404; found, 534.2481.

8-Cyclopentyl-2-(4-(phenethylamino)piperidin-1-yl)-5-phenylpyrido[2,3-d]pyrimidin-7(8H)-one (13) The title compound was prepared from **3** according to the preparation of **51**. 158 mg, 18% yield, 99.4% HPLC Purity. ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.75 (s, 2H), 8.37 (s, 1H), 7.46 (t, J = 5.0 Hz, 3H), 7.39 (t, J = 7.5 Hz, 2H), 7.27 (q, J = 14.5 Hz, 2H), 7.22 (t, J = 14.0 Hz, 1H), 7.15 (d, J = 7.0 Hz, 2H), 6.30 (s, 1H), 5.84 (t, J = 18.0 Hz, 1H), 4.93 (d, J = 13.5 Hz, 2H), 3.31 (s, 1H), 3.20 (s, 2H), 3.04–2.96 (m, 4H), 2.40–2.35 (m, 2H), 2.18 (d, J = 11.5 Hz, 2H), 1.98 (s, 2H), 1.82 (t, J = 9.0 Hz, 2H), 1.80–1.64 (m, 4H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 163.6, 159.7, 157.9, 156.5, 148.6, 136.1, 135.3, 129.1, 129.0, 128.7, 128.6, 128.5, 127.3, 117.6, 105.6, 55.9, 53.5, 46.1, 42.4, 32.5, 28.2, 27.9, 25.6. HRMS (ESI) (m/z): calculated for $C_{31}H_{35}N_5O$ $[M + H]^+$ 494.2842; found, 494.2923.

8-Cyclopentyl-5-(3,4-dimethylphenyl)-2-(4-(phenethylamino)piperidin-1-yl)pyrido[2,3-d]pyrimidin-7(8H)-one (14) The title compound was prepared from **3** according to the preparation of **51**. 56 mg, 22% yield, 97.8% HPLC purity. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.75 (s, 1H), 8.44 (s, 2H), 7.31–7.23 (m, 4H), 7.22–7.11 (m, 4H), 6.29 (s, 1H), 5.96–5.80 (m, 1H), 4.95 (d, J = 13.5 Hz, 2H), 3.31 (s, 1H), 3.18 (s, 2H), 3.06–2.92 (m, 4H), 2.37 (d, J = 10.8 Hz, 1H), 2.31 (d, J = 5.7 Hz, 6H), 2.18 (d, J = 10.8 Hz, 2H), 1.96 (d, J = 4.8 Hz, 2H), 1.87 (m, 3H), 1.81–1.63 (m, 6H). ^{13}C

NMR (125 MHz, $CDCl_3$) δ 163.7, 159.6, 158.0, 156.5, 148.7, 137.8, 137.1, 136.1, 132.8, 129.9, 129.8, 129.0, 128.5, 127.3, 126.1, 117.4, 105.7, 55.9, 53.4, 46.1, 42.4, 32.5, 28.2, 27.9, 25.6, 19.8, 19.6. HRMS (ESI) (m/z): calculated for $C_{33}H_{39}N_5O$ $[M + H]^+$ 522.3155; found, 522.3229.

8-Cyclopentyl-5-(3-fluoro-4-methylphenyl)-2-(4-(phenethylamino)piperidin-1-yl)pyrido[2,3-d]pyrimidin-7(8H)-one (15) The title compound was prepared from **3** according to the preparation of **51**. 156 mg, 30% yield, 94.8% HPLC purity. ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.75 (s, 2H), 8.38 (s, 1H), 7.25 (t, $J = 19.5$ Hz, 3H), 7.21 (d, $J = 6.5$ Hz, 1H), 7.15 (d, $J = 7.0$ Hz, 2H), 7.06 (t, $J = 17.0$ Hz, 2H), 6.27 (s, 1H), 5.83 (t, $J = 18.0$ Hz, 1H), 4.94 (d, $J = 12.0$ Hz, 2H), 3.31 (s, 1H), 3.19 (s, 2H), 3.04-2.94 (m, 4H), 3.34 (s, 5H), 2.19 (d, $J = 11.5$ Hz, 2H), 1.98 (s, 2H), 1.81-1.75 (m, 4H), 1.64 (d, $J = 3.5$ Hz, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 163.4, 162.2 (d, $J_{CF} = 245.0$ Hz), 159.7, 157.7, 156.5, 147.3, 136.1, 134.6 (d, $J_{CF} = 7.5$ Hz), 131.9, 131.8, 128.9, 128.5, 127.3, 126.1 (d, $J_{CF} = 15.0$ Hz), 124.1, 117.6, 115.4, 115.3, 105.3, 55.8, 53.5, 46.1, 42.4, 32.5, 28.2, 27.8, 25.6, 14.4, 14.4. HRMS (ESI) (m/z): calculated for $C_{32}H_{36}FN_5O$ $[M + H]^+$ 526.2904; found, 526.2979.

8-Cyclopentyl-5-(4-fluoro-3-methylphenyl)-2-(4-(phenethylamino)piperidin-1-yl)pyrido[2,3-d]pyrimidin-7(8H)-one (16) The title compound was prepared from **7** according to the preparation of **51**. 230 mg, 44% yield, 99.8% HPLC purity. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.73 (s, 2H), 8.36 (s, 1H), 7.80 (d, $J = 8.0$ Hz, 1H), 7.30–7.06 (m, 8H), 6.26 (s, 1H), 5.88–5.76 (m, 1H), 4.92 (d, $J = 14.1$ Hz, 2H), 3.30 (s, 1H), 3.20 (s, 2H), 3.05–2.92 (m, 4H), 2.37 (s, 2H), 2.32 (s, 3H), 2.17 (d, $J = 11.7$ Hz, 2H), 1.98 (s, 2H), 1.80–1.63 (m, 6H). ^{13}C NMR (125 MHz, DMSO-d_6 + $CDCl_3$) δ 162.6, 161.0 (d, $J_{CF} = 246.3$ Hz), 159.1, 157.1, 155.8, 147.1, 136.2, 136.1, 131.2, 131.1, 130.4 (d, $J_{CF} = 3.8$ Hz), 128.2, 128.1, 127.2, 127.1 126.4, 124.8 (d, $J_{CF} = 17.5$ Hz), 116.6, 114.9, 114.7, 104.5, 54.7, 52.7, 51.8, 45.4, 45.1, 41.8, 41.3, 31.7, 31.7, 27.5, 27.2, 25.0, 24.6, 14.0, 13.9. HRMS (ESI) (m/z): calculated for $C_{32}H_{36}FN_5O$ $[M + H]^+$ 526.2904; found, 522. 526.2989.

5-(8-Cyclopentyl-7-oxo-2-(4-(phenethylamino)piperidin-1-yl)-7,8-dihydropyrido[2,3-d]pyrimidin-5-yl)-2-fluorobenzaldehyde (17) The title compound was prepared from **3** according to the preparation of **51**. 91 mg, 19% yield, 99.8% HPLC purity. ^{1}H NMR (500 MHz, $CDCl_3$) δ 10.04 (s, 1H), 9.74 (s, 2H), 8.24 (s, 1H), 7.91 (q, $J = 8.6$ Hz, 1H), 7.65–7.62 (m, 1H), 7.34–7.15 (m, 6H), 6.27 (s, 1H), 5.86-5.79 (m, 1H), 4.93 (d, $J = 13.2$ Hz, 2H), 3.31 (s, 1H), 3.20 (s, 2H), 3.04–2.95 (m, 4H), 2.38–2.33 (m, 2H), 2.19 (d, $J = 11.8$ Hz, 2H), 1.98 (t, $J = 13.8$ Hz, 2H), 1.83–1.72 (m, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 186.3 (q, $J_{CF} = 5.9$ Hz), 166.5, 163.1, 158.1 (d, $J_{CF} = 240.9$ Hz), 157.2, 146.1, 136.3, 136.2, 136.0, 132.2, 129.0, 128.8, 128.5, 127.3, 124.4 (d, $J_{CF} = 8.9$ Hz), 118.2, 117.6, 117.3, 105.1, 55.8, 53.6, 46.1, 42.4, 32.5, 28.2, 27.8, 25.6. HRMS (ESI) (m/z): calculated for $C_{32}H_{34}FN_5O_2$ $[M + H]^+$ 540.2697; found, 540.2729.

3-(8-Cyclopentyl-7-oxo-2-(4-(phenethylamino)piperidin-1-yl)-7,8-dihydropyrido[2,3-d]pyrimidin-5-yl)benzaldehyde (18) The title compound was prepared from **3** according to the preparation of **51**. 105 mg, 20% yield, 97.6% HPLC purity. ^{1}H NMR (300 MHz, $CDCl_3$) δ 10.07 (s, 1H), 9.74 (s, 2H), 8.28 (s, 1H), 8.00 (q, $J = 7.5$ Hz, 1H), 7.90 (s, 1H), 7.68 (t, $J = 12.6$ Hz, 2H), 7.30–7.14 (m, 5H), 6.31 (s, 1H), 5.84 (t, $J = 17.7$ Hz, 1H), 4.93 (d, $J = 12.9$ Hz, 2H), 3.31 (s, 1H), 3.19 (s, 2H), 3.05-2.93 (m, 4H), 2.35 (t, $J = 15.0$ Hz, 2H), 2.18 (d, $J = 11.6$ Hz, 2H), 1.98 (s, 2H), 1.85–1.63 (m, 6H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 191.4, 163.2, 162.1, 161.8, 159.8, 157.4, 156.6, 147.1, 136.8, 136.3, 136.0, 134.3, 130.2, 129.6, 128.9, 128.5, 127.3, 118.1, 105.2, 55.8, 53.6, 46.1, 42.4, 32.4, 28.1, 27.8, 25.6. HRMS (ESI) (m/z): calculated for $C_{32}H_{35}N_5O_2$ $[M + H]^+$ 522.2791; found, 522.2867.

8-Cyclopentyl-2-(4-(phenethylamino)piperidin-1-yl)-5-(2-(trifluoromethyl)phenyl)pyrido[2,3-d]pyrimidin-7(8H)-one (19) The title compound was prepared from **3** according to the preparation of **51**. 184 mg, 33% yield, 99.4% HPLC purity. ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.74 (s, 2H), 8.26 (s, 1H), 7.73 (d, $J = 7.5$ Hz, 1H), 7.66–7.57 (m, 3H), 7.29–7.21 (m, 4H), 7.15 (d, $J = 7.5$ Hz, 2H), 6.30 (s, 1H), 5.84 (t, $J = 18.0$ Hz, 1H), 4.93 (d, $J = 13.5$ Hz, 2H), 3.30 (s, 1H), 3.19 (s, 2H), 3.04-2.95 (m, 4H), 2.39-2.34 (m, 2H), 2.19 (d, $J = 12.0$ Hz, 2H), 1.83-1.80 (m, 4H), 1.77–1.64 (m, 4H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 163.0, 159.7, 157.7, 155.8, 145.7, 136.1, 133.5, 131.7, 130.8, 129.1, 129.0, 128.9, 128.8, 128.6, 128.5, 127.3, 126.5, 126.4, 124.7, 122.5, 118.9, 116.5 (q, $J_{CF} = 290.0$ Hz), 115.37, 113.04, 106.6, 55.8, 53.6, 46.1, 42.3, 32.4, 28.1, 27.9, 25.6. HRMS (ESI) (m/z): calculated for $C_{32}H_{34}F_3N_5O$ $[M + H]^+$ 562.2715; found, 562.2796.

8-Cyclopentyl-2-(4-(phenethylamino)piperidin-1-yl)-5-(3-(trifluoromethyl)phenyl)pyrido[2,3-d]pyrimidin-7(8H)-one (20) The title compound was prepared from **3** according to the preparation of **51**. 150 mg, 27% yield, 99.1% HPLC purity. ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.75 (s, 2H), 8.27 (s, 1H), 7.74 (d, $J = 8.0$ Hz, 2H), 7.51 (d, $J = 8.0$ Hz, 2H), 7.28–7.21 (m, 3H), 7.15 (d, $J = 7.5$ Hz, 2H), 6.30 (s, 1H), 5.87–5.80 (m, 1H), 4.94 (d, $J = 13.0$ Hz, 2H), 3.32 (s, 1H), 3.20 (s, 2H), 3.04–2.95 (m, 4H), 2.38 (t, $J = 11.0$ Hz, 2H), 2.19 (d, $J = 11.0$ Hz, 2H), 1.99 (s, 2H), 1.84–1.73 (m, 4H),

1.64 (d, $J = 5.0$ Hz, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 163.2, 159.8, 157.4, 156.5, 147.0, 138.9, 136.0, 131.7, 131.4 (q, $J_{CF} = 32.5$ Hz), 129.1, 128.9, 128.5, 127.3, 127.1, 125.8 (d, $J_{CF} = 68.7$ Hz), 124.9, 122.8, 118.0, 117.7, 115.4, 105.1, 55.8, 53.6, 46.3, 42.4, 32.4, 28.1, 27.8, 25.6. HRMS (ESI) (m/z): calculated for $C_{32}H_{34}F_3N_5O$ $[M + H]^+$ 562.2715; found, 562.2796.

8-Cyclopentyl-2-(4-(phenethylamino)piperidin-1-yl)-5-(4-(trifluoromethyl)phenyl)pyrido[2,3-d]pyrimidin-7(8H)-one (21) The title compound was prepared from **3** according to the preparation of **51**. 150 mg, 27% yield, 99.1% HPLC purity. ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.75 (s, 2H), 8.27 (s, 1H), 7.74 (d, $J = 8.0$ Hz, 2H), 7.51 (d, $J = 8.0$ Hz, 2H), 7.28–7.21 (m, 3H), 7.15 (d, $J = 7.5$ Hz, 2H), 6.30 (s, 1H), 5.87–5.80 (m, 1H), 4.94 (d, $J = 13.0$ Hz, 2H), 3.32 (s, 1H), 3.20 (s, 2H), 3.04–2.95 (m, 4H), 2.38 (t, $J = 11.0$ Hz, 2H), 2.19 (d, $J = 11.0$ Hz, 2H), 1.99 (s, 2H), 1.84–1.73 (m, 4H), 1.64 (d, $J = 5.0$ Hz, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 163.2, 159.8, 157.4, 156.5, 147.0, 138.9, 136.0, 131.3 (d, $J_{CF} = 32.5$ Hz), 129.1, 129.0, 128.5, 127.3, 127.1, 125.8, 125.8, 124.9, 122.8, 118.0, 117.7, 115.4, 105.1, 55.8, 53.6, 46.1, 42.4, 32.4, 28.1, 27.8, 25.6. HRMS (ESI) (m/z): calculated for $C_{32}H_{34}F_3N_5O$ $[M + H]^+$ 562.2715; found, 562.2796.

5-(3-Chloro-4-(trifluoromethyl)phenyl)-8-cyclopentyl-2-(4-(phenethylamino)piperidin-1-yl)pyrido[2,3-d]pyrimidin-7(8H)-one (22) The title compound was prepared from **3** according to the preparation of **51**. 158 mg, 27% yield, 99.7% HPLC purity. ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.72 (s, 2H), 8.24 (s, 1H), 7.80 (d, $J = 8.0$ Hz, 1H), 7.55 (s, 1H), 7.38 (d, $J = 8.0$ Hz, 1H), 7.29–7.21 (m, 2H), 7.15 (d, $J = 7.0$ Hz, 2H), 6.28 (s, 1H), 5.86–5.79 (m, 1H), 4.94 (d, $J = 13.0$ Hz, 2H), 3.32 (s, 1H), 3.19 (d, $J = 8.5$ Hz, 2H), 3.04–2.95 (m, 4H), 2.36 (s, 2H), 2.19 (d, $J = 11.5$ Hz, 2H), 1.99 (s, 2H), 1.84–1.72 (m, 6H), 1.65 (d, $J = 4.5$ Hz, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 163.0, 159.8, 157.1, 156.5, 145.5, 140.3, 136.0, 133.0, 131.4, 129.1, 128.9, 128.9, 128.5, 128.1, 128.0, 128.0, 127.3, 126.9, 125.8, 123.7, 121.5, 118.2, 104.7, 55.8, 53.7, 46.1, 42.4, 32.4, 28.1, 27.8, 25.6. HRMS (ESI) (m/z): calculated for $C_{32}H_{33}ClF_3N_5O$ $[M + H]^+$ 596.2326; found, 596.2404.

8-Cyclopentyl-5-(2-methoxyphenyl)-2-(4-(phenethylamino)piperidin-1-yl)pyrido[2,3-d]pyrimidin-7(8H)-one (23) The title compound was prepared from **3** according to the preparation of **51**. 135 mg, 11% yield, 98.9% HPLC purity. ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.74 (s, 2H), 8.08 (s, 1H), 7.42 (t, $J = 14.5$ Hz, 1H), 7.27 (t, $J = 14.5$ Hz, 2H), 7.21 (t, $J = 15.5$ Hz, 2H), 7.15 (d, $J = 7.5$ Hz, 2H), 7.05 (t, $J = 14.5$ Hz, 1H), 6.98 (d, $J = 8.0$ Hz, 2H), 6.27 (s, 1H), 5.87–5.80 (m, 1H), 4.93 (d, $J = 13.5$ Hz, 2H), 3.74 (s, 3H), 3.30 (s, 1H), 3.20 (s, 2H), 3.03 (t, $J = 16.5$ Hz, 2H), 2.95 (t, $J = 21.0$ Hz, 2H), 2.39 (d, $J = 2.5$ Hz, 2H), 2.18 (d, $J = 11.0$ Hz, 2H), 1.97 (s, 2H), 1.83–1.74 (m, 4H), 1.63 (d, $J = 5.0$ Hz, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 163.9, 159.6, 158.4, 156.4, 155.9, 146.3, 136.1, 130.6, 130.5, 128.9, 128.5, 127.3, 124.2, 121.0, 118.7, 111.0, 105.7, 55.9, 55.5, 53.4, 46.1, 42.4, 32.4, 28.2, 27.9, 25.6. HRMS (ESI) (m/z): calculated for $C_{32}H_{37}N_5O_2$ $[M + H]^+$ 524.2947; found, 524.3030.

8-Cyclopentyl-5-(3-methoxyphenyl)-2-(4-(phenethylamino)piperidin-1-yl)pyrido[2,3-d]pyrimidin-7(8H)-one (24) The title compound was prepared from **3** according to the preparation of **51**. 187 mg, 36% yield, 99.8% HPLC purity. ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.75 (s, 2H), 8.41 (s, 1H), 7.37 (t, $J = 16.0$ Hz, 1H), 7.28–7.23 (m, 3H), 7.20 (d, $J = 20.0$ Hz, 2H), 7.15 (d, $J = 7.0$ Hz, 2H), 7.00–6.91 (m, 1H), 6.31 (s, 1H), 5.87–5.80 (m, 1H), 4.94 (d, $J = 13.0$ Hz, 2H), 3.83 (s, 3H), 3.31 (s, 1H), 3.20 (s, 2H), 3.04–2.94 (m, 4H), 2.38 (t, $J = 26.5$ Hz, 2H), 2.18 (d, $J = 11.5$ Hz, 2H), 1.98 (s, 2H), 1.83–1.72 (m, 4H), 1.64 (d, $J = 5.0$ Hz, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 163.6, 159.8, 159.7, 157.9, 156.5, 148.5, 136.6, 136.1, 129.8, 129.0, 128.5, 127.3, 121.0, 117.5, 114.8, 114.2, 105.5, 55.9, 55.3, 53.5, 46.1, 42.4, 32.4, 28.2, 27.8, 25.6. HRMS (ESI) (m/z): calculated for $C_{32}H_{37}N_5O_2$ $[M + H]^+$ 524.2947; found, 524.3026.

8-Cyclopentyl-5-(4-methoxyphenyl)-2-(4-(phenethylamino)piperidin-1-yl)pyrido[2,3-d]pyrimidin-7(8H)-one (25) The title compound was prepared from **3** according to the preparation of **51**. 155 mg, 30% yield, 99.7% HPLC purity. ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.76 (s, 2H), 8.43 (s, 1H), 7.32 (t, $J = 8.5$ Hz, 2H), 7.27 (t, $J = 14.0$ Hz, 2H), 7.22 (t, $J = 14.0$ Hz, 1H), 7.15 (d, $J = 7.0$ Hz, 2H), 6.98 (d, $J = 8.5$ Hz, 2H), 6.27 (s, 1H), 5.85–5.81 (m, 1H), 4.94 (d, $J = 13.0$ Hz, 2H), 3.86 (s, 3H), 3.31 (s, 1H), 3.20 (s, 2H), 3.03–2.94 (m, 4H), 2.37 (s, 2H), 2.19 (d, $J = 11.5$ Hz, 2H), 2.19 (d, $J = 11.5$ Hz, 2H), 1.97 (s, 2H), 1.77 (t, $J = 30.0$ Hz, 4H), 1.63 (d, $J = 4.0$ Hz, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 163.7, 160.4, 159.6, 157.9, 156.5, 148.3, 136.1, 130.0, 128.9, 128.5, 127.5, 127.3, 117.2, 114.2, 105.7, 55.9, 55.4, 53.4, 46.1, 42.4, 32.4, 28.2, 27.8, 25.6. HRMS (ESI) (m/z): calculated for $C_{32}H_{37}N_5O_2$ $[M + H]^+$ 524.2947; found, 524.3016.

5-([1,1′-Biphenyl]-4-yl)-8-cyclopentyl-2-(4-(phenethylamino)piperidin-1-yl)pyrido[2,3-d]pyrimidin-7(8H)-one (26) The title compound was prepared from **3** according to the preparation of **51**. 137 mg, 28% yield, 99.7% HPLC purity. ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.76 (s, 2H), 8.45 (s, 1H), 7.69–7.62 (m, 4H), 7.47 (t, $J = 15.5$ Hz, 4H), 7.39 (t, $J = 17.5$ Hz,1H), 7.27 (d, $J = 7.5$ Hz, 1H), 7.26–7.15 (m, 5H), 6.35 (s, 1H), 5.87–5.82 (m, 4H), 2.39 (d, $J = 2.5$ Hz,

2H), 1.99 (s, 2H), 1.84–1.65 (m, 4H), 1.20 (t, $J = 14.0$ Hz, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 163.6, 159.7, 157.9, 156.6, 148.2, 142.1, 140.2, 136.1, 134.1, 129.1, 129.0, 128.9, 128.5, 127.8, 127.4, 127.3, 127.1, 117.6, 105.2, 55.9, 53.5, 46.1, 42.4, 32.5, 28.2, 27.9, 25.6. HRMS (ESI) (m/z): calculated for $C_{37}H_{39}N_5O$ $[M + H]^+$ 570.3155; found, 570.3241.

8-Cyclopentyl-2-(4-(phenethylamino)piperidin-1-yl)-5-(4-phenoxyphenyl)pyrido[2,3-d]pyrimidin-7(8H)-one (27) The title compound was prepared from **3** according to the preparation of **51**. 127 mg, 23% yield, 99.7% HPLC purity. ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.76 (s, 2H), 8.42 (s, 1H), 7.39–7.34 (m, 4H), 7.27 (t, $J = 15.0$ Hz, 3H), 7.23–7.15 (m, 3H), 7.07 (d, $J = 7.0$ Hz, 4H), 6.29 (s, 1H), 5.83 (t, $J = 18.0$ Hz, 1H), 4.94 (d, $J = 13.0$ Hz, 2H), 3.31 (s, 1H), 3.19 (s, 2H), 3.04–2.94 (m, 4H), 2.37 (s, 2H), 2.19 (d, $J = 11.0$ Hz, 2H), 1.98 (s, 2H), 1.82–1.72 (m, 4H), 1.63 (d, $J = 4.5$ Hz, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 163.6, 159.7, 158.6, 157.8, 156.6, 148.0, 136.1, 130.2, 129.9, 129.8, 129.0, 127.3, 124.0, 119.6, 118.5, 117.5, 105.6, 55.9, 53.5, 46.1, 42.4, 32.5, 28.2, 27.9, 25.6. HRMS (ESI) (m/z): calculated for $C_{37}H_{39}N_5O_2$ $[M + H]^+$ 586.3104; found, 586.3188.

5-(4-(Tert-butyl)phenyl)-8-cyclopentyl-2-(4-(phenethylamino)piperidin-1-yl)pyrido[2,3-d]pyrimidin-7(8H)-one (28) The title compound was prepared from **3** according to the preparation of **51**. 140 mg, 26% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.75 (s, 2H), 8.43 (s, 1H), 7.47 (d, $J = 8.1$ Hz, 2H), 7.34–7.22 (m, 5H), 7.15 (d, $J = 6.9$ Hz, 2H), 6.31 (s, 1H), 5.84 (t, $J = 17.7$ Hz, 1H), 4.93 (d, $J = 13.5$ Hz, 2H), 3.31 (s, 1H), 3.19 (s, 2H), 3.05–2.92 (m, 4H), 2.37 (d, $J = 3.0$ Hz, 2H), 2.18 (d, $J = 9.9$ Hz, 2H), 1.97 (s, 2H), 1.77 (t, $J = 8.4$ Hz, 4H), 1.65 (t, $J = 10.8$ Hz, 2H), 1.37 (s, 9H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 163.7, 159.6, 158.0, 156.5, 152.4, 148.7, 136.1, 132.3, 128.9, 128.5, 128.4, 127.3, 125.7, 117.4, 105.7, 55.9, 53.5, 46.1, 42.4, 34.8, 32.5, 31.3, 28.2, 27.9, 25.6. HRMS (ESI) (m/z): calculated for $C_{35}H_{43}N_5O$ $[M + H]^+$ 550.3468; found, 550.3543.

8-Cyclopentyl-5-(4-(methylsulfonyl)phenyl)-2-(4-(phenethylamino)piperidin-1-yl)pyrido[2,3-d]pyrimidin-7(8H)-one (29) The title compound was prepared from **3** according to the preparation of **51**. 93 mg, 17% yield, 98.3% HPLC purity. ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.72 (s, 2H), 8.23 (s, 1H), 8.06 (d, $J = 8.0$ Hz, 2H), 7.59 (d, $J = 8.5$ Hz, 2H), 7.29–7.22 (m, 3H), 7.15 (d, $J = 7.0$ Hz, 2H), 6.30 (s, 1H), 5.83 (t, $J = 18.0$ Hz, 1H), 4.94 (d, $J = 13.5$ Hz, 2H), 3.31 (s, 1H), 3.20 (d, $J = 24.5$ Hz, 2H), 3.05 (s, 3H), 3.03–2.95 (m, 4H), 2.36 (t, $J = 27.5$ Hz, 2H), 2.19 (d, $J = 11.0$ Hz, 2H), 1.98 (s, 2H), 1.84–1.72 (m, 6H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 163.1, 159.8, 157.3, 156.5, 146.5, 141.3, 140.9, 136.0, 129.7, 129.0, 128.5, 127.9, 127.4, 118.1, 104.9, 55.7, 53.7, 46.1, 44.5, 42.4, 32.4, 28.1, 27.8, 25.6. HRMS (ESI) (m/z): calculated for $C_{32}H_{37}N_5O_2$ $[M + H]^+$ 572.2617; found, 572.2692.

8-Cyclopentyl-5-(4-methoxyphenyl)-2-(4-(phenethylamino)piperidin-1-yl)pyrido[2,3-d]pyrimidin-7(8H)-one (30) The title compound was prepared from **3** according to the preparation of **51**. 165 mg, 40% yield. ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.76 (s, 2H), 8.64 (s, 1H), 7.68 (s, 1H), 7.55 (t, $J = 3.0$ Hz, 1H), 7.29–7.21 (m, 3H), 7.16 (d, $J = 7.0$ Hz, 2H), 6.60 (d, $J = 0.5$ Hz, 1H), 6.35 (s, 1H), 5.80 (t, $J = 18.0$ Hz, 1H), 4.94 (d, $J = 13.5$ Hz, 2H), 3.32 (s, 1H), 3.20 (s, 2H), 3.05–2.95 (m, 4H), 2.37 (s, 3H), 2.35 (t, $J = 11.5$ Hz, 2H), 2.31 (d, $J = 7.5$ Hz, 2H), 2.20 (d, $J = 11.5$ Hz, 2H), 1.98–1.93 (m, 4H), 1.79 (q, $J = 15.0$ Hz, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 163.6, 159.7, 157.2, 156.5, 143.9, 141.4, 1391, 128.9, 128.5, 127.3, 120.4, 116.8, 110.7, 105.2, 55.8, 53.5, 46.1, 42.4, 32.5, 28.2, 27.8, 25.6. HRMS (ESI) (m/z): calculated for $C_{29}H_{33}N_5O_2$ $[M + H]^+$ 484.2634; found, 484.2790.

8-Cyclopentyl-2-(4-(phenethylamino)piperidin-1-yl)-5-(thiophen-3-yl)pyrido[2,3-d]pyrimidin-7(8H)-one (31) The title compound was prepared from **3** according to the preparation of **51**. 160 mg, 26% yield, 99.6% HPLC purity. ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.76 (s, 2H), 8.55 (s, 1H), 7.68 (s, 1H), 7.46–7.44 (m, 2H), 7.29–7.20 (m, 4H), 7.17 (t, $J = 20.5$ Hz, 2H), 6.36 (s, 1H), 5.82 (t, $J = 17.0$ Hz, 1H), 4.94 (d, $J = 14.0$ Hz, 2H), 3.31 (s, 1H), 3.20 (s, 2H), 3.04–2.94 (m, 4H), 2.38–2.33 (m, 2H), 2.19 (d, $J = 11.5$ Hz, 2H), 1.97 (d, $J = 8.0$ Hz, 2H), 1.81–1.74 (m, 6H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 163.6, 159.7, 157.6, 156.4, 143.2, 136.0, 135.8, 128.9, 128.5, 127.9, 127.3, 126.7, 125.2, 117.2, 105.5, 55.8, 53.5, 46.1, 42.4, 32.4, 28.1, 27.8, 25.6. HRMS (ESI) (m/z): calculated for $C_{29}H_{33}N_5OS$ $[M + H]^+$ 500.2406; found, 500.2476.

8-Cyclopentyl-2-(methylthio)-5-((trimethylsilyl)ethynyl)pyrido[2,3-d]pyrimidin-7(8H)-one (78) Compound **9a** (0.5 g, 1.22 mmol), Et_3N (0.37 g, 3.06 mmol), CuI (0.019 g, 0.122 mmol), $Pd(PPh_3)_4$ (0.071 g, 0.061 mmol), trimethylsilylacetylene (0.3 g, 3.05 mmol) were added to a 25 mL round-bottomed flask with 10 mL THF. The mixture was heated to reflux for 6 h under the atmosphere of N_2. After the reaction was completed, the resulting mixture was purified and separated by column chromatography (PE:EA = 30:1) to obtain 0.47 g of a yellow solid product.

8-Cyclopentyl-2-(methylsulfonyl)-5-((trimethylsilyl)ethynyl)pyrido[2,3-d]pyrimidin-7(8H)-one (79) Compound **78** (0.47 g, 1.32 mmol) and *m*-CPBA (0.71 g, 3.95 mmol) was

added into a 50 mL round bottom flask with 25 mL DCM. The reaction was carried out at room temperature for 2 h. After the reaction was completed, 10 mL of saturated sodium sulfite was added for quenching. The resulting mixture was washed with saturated sodium carbonate (15 mL × 3) and saturated brine (15 mL × 3). The separated organic layer was dried over anhydrous sodium sulfate, filtered, and distilled under a reduced pressure to obtain 0.61 g yellow solid.

Tert-butyl (1-(8-cyclopentyl-7-oxo-5-((trimethylsilyl)ethynyl)-7,8-dihydropyrido[2,3-d]pyrimidin-2-yl)piperidine-4-yl)(phenethyl)carbamate (80) Compound **79** (0.61 g, 1.57 mmol), compound **12** (0.95 g, 3.13 mmol), and 10 mL toluene were added into a 25 mL round bottom flask. The reaction system was heated to reflux for 3 h under an atmosphere of N2. After the reaction was completed, the mixture was cooled to room temperature and subjected to column chromatography (PE:EA = 32:1) to obtain 0.45 g of white solid. 47% yield.

Tert-butyl (1-(8-cyclopentyl-7-oxo-5-(5-(trimethylsilyl)-1H-1,2,3-triazole-4-yl)-7,8-dihydropyrido[2,3-d]pyrimidin-2-yl) piperidine-4-yl)(phenethyl)carbamate (81) Compound **80** (0.22 g, 0.36 mmol) and 10 mL toluene were added into a 25 mL round bottom flask. After dissolution, NaN_3 (0.05 g, 0.72 mmol) was added into bottom. The reaction system was heated to 100 °C for 3 h under an atmosphere of N_2. After the reaction was completed, the resulting mixture was added to 70 mL ethyl acetate and washed with water (30 mL × 3), and the separated organic layer was dried over anhydrous sodium sulfate, filtered, and distilled under a reduced pressure to provide 0.2 g product.

Tert-butyl (1-(8-cyclopentyl-7-oxo-5-(1H-1,2,3-triazol-4-yl)-7,8-dihydropyrido[2,3-d]pyrimidin-2-yl)piperidin-4-yl)(phenethyl)carbamate (82) Compound **81** (0.22 g, 0.3 mmol) and 10 mL THF were added into a 25 mL round bottom flask. After dissolution, 0.49 mL 1 M TBAF tetrahydrofuran solution was added. The mixture was stirred under atmosphere of N_2 for 10 min, and distilled under a reduced pressure to obtain 0.2 g product.

8-Cyclopentyl-2-(4-(phenethylamino)piperidin-1-yl)-5-(1H-1,2,3-triazol-4-yl)pyrido[2,3-d]pyrimidin-7(8H)-one (32) Compound **82** (0.2 g, 0.34 mmol) and 20 mL of dichloromethane were added to a 25 mL round bottom flask. After dissolution, trifluoroacetic acid (4 mL, 54.4 mmol) was added, and the mixture was stirred at room temperature for 1 h. After the reaction was completed, 5 mL of toluene was added into and distilled under reduced pressure. Then 10 mL ether was added into stir for 16 h. The resulting residue was filtered, and the filter cake was wash with ether, dried to obtain 22 mg pale yellow solid product, 2% yield, 98.8% HPLC purity. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.69 (s, 1H), 7.99 (s, 1H), 7.31 (t, $J = 15.0$ Hz, 2H), 7.22 (q, $J = 16.5$ Hz, 3H), 6.66 (s, 1H), 5.92–5.84 (m, 1H), 4.75 (d, $J = 12.5$ Hz, 2H), 3.18 (t, $J = 23.5$ Hz, 1H), 3.04 (t, $J = 14.0$ Hz, 2H), 2.93–2.87 (m, 3H), 2.41 (d, $J = 8.5$ Hz, 2H), 2.03 (s, 4H), 1.85 (d, $J = 8.5$ Hz, 2H), 1.69 (d, $J = 5.0$ Hz, 2H), 1.37 (q, $J = 30.5$ Hz, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 191.4, 163.3, 159.7, 157.4, 1566, 147.12, 136.8, 136.0, 134.3, 130.2, 129.7, 129.0, 128.5, 127.35, 118.0, 105.2, 55.8, 53.6, 46.1, 42.4, 32.5, 28.2, 27.9, 25.6. HRMS (ESI) (m/z): calculated for $C_{33}H_{41}N_5O_2$ $[M + H]^+$ 485.2699; found, 485.2780.

5-(3-Aminophenyl)-8-cyclopentyl-2-(methylthio)pyrido[2,3-d]pyrimidin-7(8H)-one (85) Compound **9a** (0.45 g, 1.10 mmol), Na_2CO_3 (0.23 g, 2.12 mmol), $Pd(PPh_3)_4$ (0.102 g, 0.088 mmol) and 3-Boc-aminophenyl boronic acid (0.39 g, 1.65 mmol) were added into a 50 mL round-bottomed flask with 20 mL anhydrous THF. The reaction system was heated to reflux for 2 h under the atmosphere of N_2. After the reaction was completed, the resulting residue was subjected to column chromatography (PE:EA = 5:1) to obtain a 0.45 g oily substance. Then 5 mL of dichloromethane and trifluoroacetic acid (4 mL, 54.4 mmol) was added to the round bottom flask. The mixture was stirred at room temperature for 1 h. After the reaction was completed, 5 mL toluene was added and the mixture was distilled under a reduced pressure to obtain a 0.48 g product.

N-(3-(8-cyclopentyl-2-(methylthio)-7-oxo-7,8-dihydropyrido[2,3-d]pyrimidin-5-yl)phenyl)acrylamide (86) Compound **85** (0.48 g, 1.03 mmol) and $NaHCO_3$ (0.26 g, 3.08 mmol) were added into a 50 mL round bottom flask with 20 mL MeCN, then acryloyl chloride (0.14 g, 1.54 mmol) was slowly added dropwise under ice bath. The mixture was stirred at room temperature for 30 min. After the completion of the reaction, the mixture was quenched by adding 50 mL of water, extracted with ethyl acetate (30 mL × 3), The separated organic layer was dried over anhydrous sodium sulfate, filtered, and distilled under a reduced pressure. The resulting residue was subjected to column chromatography (PE:EA = 6:1) to obtain 0.24 g oily substance. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.60 (s, 1H), 7.74 (d, $J = 5.6$ Hz, 2H), 7.69 (s, 1H), 7.47 (t, $J = 15.8$ Hz, 1H), 7.14 (d, $J = 7.6$ Hz, 1H), 6.48 (d, $J = 12.9$ Hz, 1H), 6.42 (s, 1H), 6.30–6.24 (m, 1H), 6.04–5.92 (m, 1H), 5.78 (d, $J = 10.1$ Hz, 1H), 2.62 (s, 3H), 2.40 (q, $J = 19.2$ Hz, 2H), 2.09 (t, $J = 7.2$ Hz, 2H), 1.93 (t, $J = 9.2$ Hz, 2H), 1.72 (t, $J = 11.2$ Hz, 2H). MS (ESI) (m/z): calculated for $C_{22}H_{22}N_4O_2S$ $[M + H]^+$ 407.15; found, 407.16.

N-(3-(8-cyclopentyl-7-oxo-2-(4-(phenethylamino)piperidin-1-yl)-7,8-dihydropyrido[2,3-d]pyrimidin-5-yl)phenyl)acrylamide (33) The title compound was prepared according to the preparation of **51**. 93 mg, 7% yield, 97.3% HPLC purity. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.48 (s, 2H), 8.57 (s, 1H), 8.24 (s, 1H), 7.69 (s, 1H), 7.43 (t, $J = 15.9$ Hz, 1H), 7.26–7.16 (m, 5H), 7.07 (d, $J = 7.5$ Hz, 1H), 6.55–6.45 (m, 2H), 6.32 (s, 1H), 5.75 (d, $J = 10.8$ Hz, 1H),5.65 (t, $J = 17.9$ Hz, 1H), 4.93 (s, 2H), 3.56 (s, 1H), 3.26 (s, 2H), 3.12-3.09 (t, $J = 15.0$ Hz, 2H), 2.98 (t, $J = 25.6$ Hz, 2H), 2.25 (s, 2H), 1.97 (s, 2H), 1.81 (s, 1H), 1.73 (s, 2H), 1.54 (d, $J = 10.4$ Hz, 2H), 1.46 (s, 2H), 1.36 (s, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 191.4, 163.3, 159.7, 157.4, 156.6, 147.1, 136.8, 136.3, 136.0, 134.3, 130.2, 129.7, 129.0, 128.5, 127.3, 118.0, 105.2, 55.8, 53.6, 46.1, 42.4, 32.4, 28.2, 27.9, 25.6. HRMS (ESI) (m/z): calculated for $C_{34}H_{38}N_6O_2$ $[M + H]^+$ 563.3056; found, 563.3146.

5-(Benzyloxy)-8-cyclopentyl-2-(methylthio)pyrido[2,3-d]pyrimidin-7(8H)-one (89) Compound **8a** (1 g, 3.6 mmol) K_2CO_3 (1 g, 7.2 mmol), and benzyl bromide (1.23 g, 7.2 mmol) were added into a 50 mL round-bottom flask with 10 mL acetonitrile. The mixture was heated to reflux. After the reaction was completed, the mixture was filtered, cooled, and crystallized, filtered to obtain a 0.3 g product, the filtrate was purified by column chromatography (PE:EA = 10: 1) to obtain 0.33 g of white solid, a total of 0.63 g of product was obtained. 47.6% yield.

5-(Benzyloxy)-8-cyclopentyl-2-(4-(phenethylamino)piperidin-1-yl)pyrido[2,3-d]pyrimidin-7(8H)-one (34) The title compound was prepared according to the preparation of **51**. 52 mg, 15% yield, 98.9% HPLC purity. ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.86 (s, 2H), 8.76 (s, 1H), 8.66 (d, $J = 4.5$ Hz, 1H), 7.83 (t, $J = 14.0$ Hz, 1H), 7.68 (d, $J = 7.5$ Hz, 1H), 7.42–7.35 (m, 5H), 7.28–7.16 (m, 5H), 5.80 (s, 1H), 5.70–5.63 (m, 1H), 5.14 (s, 2H), 4.92 (d, $J = 12.0$ Hz, 2H), 3.34 (s, 1H), 3.21 (s, 2H), 3.04 (t, $J = 16.0$ Hz, 2H), 2.95 (t, $J = 25.0$ Hz, 2H), 2.18 (d, $J = 10.0$ Hz, 4H), 1.87 (s, 2H), 1.71–1.65 (m, 4H), 1.57 (d, $J = 4.5$ Hz, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 165.3, 161.2, 160.3, 155.8, 154.9, 136.2, 135.2, 128.9, 128.7, 128.6, 128.5, 127.5, 127.3, 101.6, 95.0, 70.5, 55.8, 53.1, 46.1, 42.5, 32.5, 28.1, 27.9, 25.5. HRMS (ESI) (m/z): calculated for $C_{32}H_{37}N_5O_2$ $[M + H]^+$ 524.2947; found, 524.3027.

8-Cyclopentyl-5-ethynyl-2-(4-(phenethylamine)piperidine-1-yl)pyrido[2,3-d]pyrimidin-7(8H)-one (35) Compound **78** (0.19 g, 0.31 mmol) and 0.50 mL 1 M TBAF were added to a 25 mL round bottom flask with 10 mL THF. The resulting mixture was stirred for 5 min under an atmosphere of N2. The resulting residue was subjected to column chromatography (PE:EA = 9:1) to obtain 0.17 g of oil. 20 mL dichloromethane and trifluoroacetic acid (4 mL, 54.4 mmol) was added to the round bottom flask and stirred at room temperature for 1 h. After the reaction was completed, 5 mL toluene was added and distilled under reduced pressure. Then 10 mL ether was added to stir for 16 h. The resulting residue was filtered, and the filter cake was washed with ether and dried to obtain 70 mg of white solid powder, 23.8% yield, 92.9% HPLC purity.

8-Cyclopentyl-2-(4-(phenethylamino)piperidin-1-yl)-5-(phenylethynyl)pyrido[2,3-d]pyrimidin-7(8H)-one (36) The title compound was prepared according to the preparation of **51**. 20 mg, 48% yield, 94.2% HPLC purity. ^{1}H NMR (300 MHz, DMSO-d_6) δ 9.93 (s, 1H), 8.70 (s, 2H), 7.75 (q, $J = 9.3$ Hz, 2H), 7.53–7.46 (m, 3H), 7.38–7.25 (m, 5H), 6.52 (s, 1H), 5.82–5.76 (m, 1H), 4.79 (d, $J = 12.0$ Hz, 2H), 3.49 (s, 1H), 3.42–3.34 (m, 3H), 3.24 (s, 2H), 3.10 (t, $J = 24.6$ Hz, 2H), 2.94 (t, $J = 16.5$ Hz, 2H), 2.28 (s, 2H), 2.19 (d, $J = 10.8$ Hz, 2H), 1.95 (s, 2H), 1.81 (t, $J = 9.3$ Hz, 2H), 1.67 (t, $J = 10.2$ Hz, 2H), 1.51 (d, $J = 9.0$ Hz, 2H), 1.10 (t, $J = 14.1$ Hz, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 163.3, 160.1, 157.7, 156.0, 136.0, 132.0, 129.9, 129.7, 129.0, 128.6, 128.5, 127.3, 121.6, 120.9, 105.4, 99.3, 82.4, 55.9, 53.5, 46.2, 42.4. HRMS (ESI) (m/z): calculated for $C_{33}H_{35}N_5O$ $[M + H]^+$ 517.2842; found, 518.2921.

8-Cyclopentyl-2-(4-(phenethylamino)piperidin-1-yl)-5-(m-tolylethynyl)pyrido[2,3-d]pyrimidin-7(8H)-one (37) The title compound was prepared according to the preparation of **51**. 108 mg, 21% yield, 99.7% HPLC purity. ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.76 (s, 2H), 8.85 (s, 1H), 7.39 (t, $J = 16.0$ Hz, 2H), 7.28 (t, $J = 19.0$ Hz, 5H), 7.23–7.16 (m, 2H), 6.51 (s, 1H), 5.81–5.74 (m, 1H), 4.96 (d, $J = 12.5$ Hz, 2H), 3.31 (s, 1H), 3.20 (s, 2H), 3.03–2.95 (m, 4H), 2.37 (s, 3H), 2.32 (d, $J = 9.0$ Hz, 2H), 2.20 (d, $J = 11.5$ Hz, 2H), 1.97 (s, 2H), 1.77 (d, $J = 11.5$ Hz, 4H), 1.63 (d, $J = 4.5$ Hz, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 163.3, 160.1, 157.8, 156.0, 138.4, 136.0, 132.6, 130.6, 130.0, 129.1, 129.0, 128.5, 128.5, 127.4, 121.4, 120.9, 105.4, 99.6, 82.0, 55.9, 53.4, 46.2, 42.4, 32.5, 28.2, 27.9, 25.6, 21.2. HRMS (ESI) (m/z): calculated for $C_{34}H_{37}N_5O$ $[M + H]^+$ 532.2998; found, 532.3082.

8-Cyclopentyl-2-(4-(phenethylamino)piperidin-1-yl)-5-(p-tolylethynyl)pyrido[2,3-d]pyrimidin-7(8H)-one (38) The title compound was prepared according to the preparation of **51**. 159 mg, 31% yield, 99.2% HPLC purity. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.73 (s, 2H), 8.84 (s, 1H), 7.45 (d, $J = 7.9$ Hz, 2H), 7.31–7.15 (m, 7H), 6.51 (s, 1H), 5.80–5.74 (m, 1H), 4.94 (d, $J = 14.1$ Hz, 2H), 3.31 (s, 1H), 3.18 (d, $J = 6.8$ Hz, 2H), 3.06–2.94 (m, 4H), 2.38 (s, 3H), 2.30 (d, $J = 7.5$ Hz, 2H), 2.19 (d, $J = 11.3$ Hz, 2H), 1.97 (s, 2H),

1.82–1.62 (m, 6H). ^{13}C NMR (125 MHz, DMSO-d_6 + $CDCl_3$) δ 162.4, 159.5, 157.1, 155.4, 139.6, 136.2, 136.1, 131.4, 129.5, 128.9, 128.2, 128.1, 126.5, 119.8, 119.6, 117.8, 117.5, 115.2, 104.4, 99.1, 81.3, 54.7, 52.7, 51.9, 45.4, 45.2, 41.9, 41.4, 40.0, 39.8, 39.7, 39.5, 39.3, 39.2, 39.0, 31.8, 31.7, 27.6, 27.3, 25.0, 24.6, 21.0. HRMS (ESI) (m/z): calculated for $C_{34}H_{37}N_5O$ $[M + H]^+$ 532.2998; found, 532.3097.

5-((3-Chlorophenyl)ethynyl)-8-cyclopentyl-2-(4-(phenethylamino)piperidin-1-yl)pyrido[2,3-d]pyrimidin-7(8H)-one (39) The title compound was prepared according to the preparation of **51**. 118 mg, 22% yield, 93.8% HPLC purity. ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.76 (s, 2H), 8.80 (s, 1H), 7.56 (s, 1H), 7.43 (t, $J = 30.0$ Hz 1H), 7.34 (d, $J = 23.0$ Hz, 1H), 7.31 (t, $J = 8.5$ Hz, 1H), 7.29 (t, $J = 15.0$ Hz, 1H), 7.25 (t, $J = 15.0$ Hz, 4H), 7.19 (t, $J = 15.0$ Hz, 2H), 6.52 (s, 1H), 5.77 (q, $J = 25.0$ Hz 1H), 4.95 (d, $J = 12.5$ Hz, 2H), 3.31 (s, 1H), 3.20 (s, 2H), 3.03–2.96 (m, 4H), 2.31 (t, $J = 10.5$ Hz, 2H), 2.20 (d, $J = 11.0$ Hz, 2H), 1.97 (s, 2H), 1.77 (q, $J = 30.0$ Hz, 4H), 1.63 (d, $J = 4.5$ Hz, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 163.1, 160.1, 157.6, 156.1, 136.0, 134.5, 131.8, 130.1, 129.9, 129.8, 129.3, 129.0, 128.5, 127.4, 123.3, 121.3, 105.2, 97.4, 83.6, 77.2, 55.8, 53.5, 46.2, 42.4, 32.5, 28.2, 27.9, 25.6. HRMS (ESI) (m/z): calculated for $C_{32}H_{37}N_5O_2$ $[M + H]^+$ 552.2452; found, 552.2529.

5-((4-Chlorophenyl)ethynyl)-8-cyclopentyl-2-(4-(phenethylamino)piperidin-1-yl)pyrido[2,3-d]pyrimidin-7(8H)-one (40) The title compound was prepared according to the preparation of **51**. 100 mg, 15% yield, 99.4% HPLC purity. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.76 (s, 2H), 8.80 (s, 1H), 7.49 (d, $J = 8.4$ Hz, 2H), 7.36 (d, $J = 8.7$ Hz, 2H), 7.29 (t, $J = 15.0$ Hz, 3H), 7.23–7.15 (m, 2H), 6.52 (s, 1H), 5.83–5.74 (m, 1H), 4.94 (d, $J = 12.9$ Hz, 2H), 3.31 (s, 1H), 3.19 (s, 2H), 3.06–2.94 (m, 4H), 2.29 (t, $J = 7.8$ Hz, 2H), 2.20 (d, $J = 11.4$ Hz, 2H), 1.97 (s, 2H), 1.82–1.70 (m, 4H), 1.64 (t, $J = 10.2$ Hz, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 163.1, 160.1, 157.6, 156.0, 136.0, 135.9, 133.2, 129.5, 129.0, 129.0, 128.5, 127.3, 121.1, 120.0, 105.2, 97.9, 83.3, 55.8, 53.5, 46.2, 42.4, 32.5, 28.2, 27.9, 25.6. HRMS (ESI) (m/z): calculated for $C_{33}H_{34}ClN_5O$ $[M + H]^+$ 552.2452; found, 552.2529.

8-Cyclopentyl-2-(4-(phenethylamino)piperidin-1-yl)-5-(pyridin-2-ylethynyl)pyrido[2,3-d]pyrimidin-7(8H)-one (41) The title compound was prepared according to the preparation of **51**. 76 mg, 12% yield, 97.6% HPLC purity. ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.68 (s, 2H), 8.85 (s, 1H), 8.80 (s, 1H), 8.66 (d, $J = 3.5$ Hz, 2H), 7.97 (d, $J = 8.0$ Hz, 1H), 7.44 (q, $J = 12.5$ Hz, 1H), 7.31–7.22 (m, 3H), 7.16 (t, $J = 15.5$ Hz, 2H), 6.57 (s, 1H), 5.80–5.76 (t, $J = 18.0$ Hz, 1H), 4.96 (d, $J = 12.0$ Hz, 2H), 3.32 (s, 1H), 3.22–3.15 (m, 2H), 3.05–2.97 (m, 4H), 2.35–2.31 (m, 2H), 2.20 (d, $J = 11.5$ Hz, 2H), 1.98 (s, 2H), 1.82–1.73 (m, 6H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 163.0, 160.1, 157.5, 156.1, 151.1, 148.3, 140.3, 136.0, 129.0, 128.9, 128.5, 127.4, 123.9, 121.7, 105.0, 94.4, 86.2, 55.9, 54.6, 53.6, 46.3, 46.2, 43.8, 42.4, 36.0, 32.5, 28.2, 27.9, 25.7. HRMS (ESI) (m/z): calculated for $C_{32}H_{34}N_6O$ $[M + H]^+$ 519.2794; found, 519.2863.

8-Cyclopentyl-2-(4-(phenethylamino)piperidin-1-yl)-5-(pyridin-3-ylethynyl)pyrido[2,3-d]pyrimidin-7(8H)-one (42) The title compound was prepared according to the preparation of **51**. 56 mg, 8.8% yield, 99.1% HPLC purity. ^{1}H NMR (500 MHz, DMSO-d_6 + $CDCl_3$) δ 9.57 (s, 2H), 8.89 (s, 1H), 8.66 (d, $J = 4.5$ Hz, 1H), 7.83 (t, $J = 14.0$ Hz, 1H), 7.68 (d, $J = 7.5$ Hz, 1H), 7.41 (q, $J = 12.0$ Hz, 1H), 7.32 (t, $J = 15.0$ Hz, 2H), 7.25 (d, $J = 7.5$ Hz, 3H), 6.52 (s, 1H), 5.82 (t, $J = 15.0$ Hz, 1H), 4.94 (s, 2H), 3.41 (s, 1H), 3.20 (s, 2H), 3.04 (d, $J = 9.0$ Hz, 4H), 2.32 (d, $J = 8.0$ Hz, 2H), 2.22 (d, $J = 11.5$ Hz, 2H), 2.01 (s, 2H), 1.85–1.84 (t, $J = 8.0$ Hz, 2H), 1.69 (d, $J = 10.5$ Hz, 4H). ^{13}C NMR (125 MHz, DMSO-d_6 + $CDCl_3$) δ 162.0, 159.6, 157.1, 155.4, 149.8, 141.1, 136.3, 136.1, 128.3, 128.2, 128.1, 127.5, 126.4, 123.7, 120.6, 104.1, 97.1, 80.7, 54.5, 52.7, 45.1, 41.8, 40.0, 39.8, 39.7, 39.5, 39.3, 39.2, 39.0, 31.7, 27.52, 27.2, 25.0. HRMS (ESI) (m/z): calculated for $C_{32}H_{34}N_6O$ $[M + H]^+$ 519.2794; found, 519.2864.

8-Cyclopentyl-2-(4-(phenethylamino)piperidin-1-yl)-5-(thiophen-2-ylethynyl)pyrido[2,3-d]pyrimidin-7(8H)-one (43) The title compound was prepared according to the preparation of **51**. 167 mg, 26% yield, 98.9% HPLC purity. ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.73 (s, 2H), 8.79 (s, 1H), 7.40 (d, $J = 5.9$ Hz, 2H) 7.30–7.21 (m, 3H), 7.16 (d, $J = 7.2$ Hz, 2H), 7.06 (d, $J = 3.7$ Hz, 1H), 6.50 (s, 1H), 5.79–5.75 (t, $J = 20.0$ Hz, 1H), 4.95 (d, $J = 12.3$ Hz, 2H), 3.31 (s, 1H), 3.20 (s, 2H), 3.05–2.95 (m, 4H), 2.31 (s, 2H), 2.20 (d, $J = 11.3$ Hz, 2H), 1.97 (s, 2H), 1.79 (s, 4H), 1.63 (d, $J = 4.1$ Hz, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 162.4, 159.4, 156.9, 155.3, 136.1, 133.4, 129.0, 128.9, 128.1, 128.1, 128.0, 127.0, 126.4, 120.5, 119.3, 104.1, 91.9, 85.6, 54.7, 52.7, 51.8, 45.4, 45.2, 41.8, 41.4, 39.7, 39.5, 39.4, 39.2, 39.0, 31.70, 31.6, 27.5, 27.2, 25.0, 24.5. HRMS (ESI) (m/z): calculated for $C_{31}H_{33}N_5OS$ $[M + H]^+$ 524.2406; found, 524.2505.

5-(Cyclohexylethynyl)-8-cyclopentyl-2-(4-(phenethylamino)piperidin-1-yl)pyrido[2,3-d]pyrimidin-7(8H)-one (44) The title compound was prepared according to the preparation of **51**. 128 mg, 20% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.75 (s, 2H), 8.73 (s, 1H), 7.31–7.15 (m, 5H), 6.39 (s, 1H), 5.75 (t, $J = 17.7$ Hz, 1H), 4.93 (d, $J = 13.5$ Hz, 2H), 3.30 (s, 1H),

3.19 (s, 2H), 3.05–2.92 (m, 4H), 2.72–2.66 (m, 1H), 2.26 (q, $J = 25.2$ Hz, 4H), 1.93 (t, $J = 13.5$ Hz, 4H), 1.76 (t, $J = 8.7$ Hz, 6H), 1.60 (t, $J = 17.4$ Hz, 5H), 1.39 (d, $J = 6.9$ Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 136.0, 130.7, 128.9, 128.5, 127.3, 120.7, 105.9, 105.6, 74.1, 55.9, 53.3, 46.1, 42.4, 32.5, 32.2, 29.9, 28.2, 27.9, 25.7, 25.6, 24.7. HRMS (ESI) (m/z): calculated for $C_{33}H_{41}N_5O$ $[M + H]^+$ 524.3311; found, 524.3388.

8-Cyclopentyl-5-((1-hydroxycyclohexyl)ethynyl)-2-(4-(phenethylamino)piperidin-1-yl)pyrido[2,3-d]pyrimidin-7(8H)-one (45) The title compound was prepared according to the preparation of **51**. 140 mg, 21% yield, 99.3% HPLC purity. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.74 (s, 2H), 8.66 (s, 1H), 7.29 (q, $J = 17.1$ Hz, 3H), 7.20 (q, $J = 21.9$ Hz, 2H), 6.44 (s, 1H), 5.74 (t, $J = 17.7$ Hz, 1H), 4.86 (d, $J = 12.6$ Hz, 2H), 3.30 (s, 1H), 3.20 (s, 2H), 3.01 (q, $J = 7.2$ Hz, 4H), 2.62 (s, 1H), 3.34–2.19 (m, 4H), 2.06–1.97 (m, 4H), 1.79–1.54 (m, 14H), 1.32 (d, $J = 10.5$ Hz, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 163.4, 156.0, 157.6, 155.8, 136.0, 129.8, 128.9, 128.5, 127.3, 121.3, 105.4, 104.1, 76.7, 76.6, 68.9, 56.0, 53.5, 46.4, 42.3, 39.6, 32.5, 28.2, 27.9, 25.6, 25.1, 23.3. HRMS (ESI) (m/z): calculated for $C_{33}H_{41}N_5O_2$ $[M + H]^+$ 540.3260; found, 540.3338.

8-Methyl-2-(4-(phenethylamino)piperidin-1-yl)-5-(4-(trifluoromethyl)phenyl)pyrido[2,3-d]pyrimidine-7(8H)-one (46) The title compound was prepared with methylamine hydrochloride as the starting material. The synthesis method is similar to **51**. White solid, 115 mg, 8% yield, 99.0% HPLC purity. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.74 (s, 2H), 8.28 (s, 1H), 7.75 (d, $J = 4.8$ Hz, 2H), 7.53 (d, $J = 4.8$ Hz, 2H), 7.29–7.15 (m, 5H), 6.35 (s, 1H), 5.00 (d, $J = 8.2$ Hz, 2H), 3.61 (s, 1H), 3.31 (s, 1H), 3.20 (s, 2H), 3.04–2.95 (m, 5H), 2.20 (d, $J = 6.9$ Hz, 2H), 1.87 (s, 1H), 1.79–1.72 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 163.1, 160.1, 157.3, 156.2, 147.5, 138.9, 136.0, 131.4 (q, $J_{CF} = 32.6$ Hz), 129.1, 128.9, 128.5, 127.3, 125.8, 125.8, 125.6, 116.8, 104.6, 55.8, 46.2, 42.2, 32.5, 28.2, 27.6. HRMS (ESI) (m/z): calculated for $C_{28}H_{28}F_3N_5O$ $[M + H]^+$ 508.2246; found, 508.2339.

8-Ethyl-2-(4-(phenethylamino)piperidin-1-yl)-5-(4-(trifluoromethyl)phenyl)pyrido[2,3-d]pyrimidine-7(8H)-one (47) The title compound was prepared with ethylamine aqueous solution as the starting material. The synthesis method is similar to **51**. White solid, 119 mg, 7.8% yield, 98.9% HPLC purity. ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.72 (s, 1H), 8.41 (s, 1H), 7.46 (s, 1H), 7.38 (s, 1H), 7.27–7.34 (m, 1H), 7.25 (s, 2H), 7.23 (d, $J = 7.5$ Hz, 1H), 7.16 (t, $J = 7.7$ Hz, 3H), 5.84–5.92 (m, 1H), 4.97 (d, $J = 14.0$ Hz, 2H), 3.30 (s, 1H), 3.20 (s, 2H), 3.04 (t, $J = 8.2$ Hz, 2H), 2.97 (t, $J = 12.2$ Hz, 2H), 2.37 (q, $J = 6.7$ Hz, 2H), 2.29 (d, $J = 10.0$ Hz, 6H), 2.21 (d, $J = 11.5$ Hz, 2H), 1.99 (t, $J = 6.5$ Hz, 2H), 1.76–1.86 (m, 4H), 1.64 (d, $J = 5.5$ Hz, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 162.6, 160.2, 157.38, 155.7, 147.4, 139.1, 136.1, 131.5 (q, $J_{CF} = 19.6$ Hz), 129.1, 129.0, 128.5, 127.4, 125.8, 125.8, 122.8, 117.3, 104.8, 55.8, 46.2, 42.3, 36.0, 32.5, 28.2, 12.7. HRMS (ESI) (m/z): calculated for $C_{33}H_{39}N_5O$ $[M + H]^+$ 522.3163; found, 522.3236.

8-Butyl-2-(4-(phenethylamino)piperidin-1-yl)-5-(4-(trifluoromethyl)phenyl)pyrido[2,3-d]pyrimidine-7(8H)-one (48) The title compound was prepared with *N*-butylamine as the starting material. The synthesis method is similar to **51**. White solid, 162 mg, 11% yield, 98.1% HPLC purity. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.72 (s, 2H), 8.28 (s, 1H), 7.75 (d, $J = 4.8$ Hz, 2H), 7.74 (d, $J = 4.8$ Hz, 2H), 7.29–7.21 (m, 3H), 7.16 (d, $J = 4.3$ Hz, 2H), 6.34 (s, 1H), 4.97 (d, $J = 8.1$ Hz, 2H), 4.31 (t, $J = 8.97$ Hz, 3H), 3.31 (s, 1H), 1.79–1.66 (m, 4H), 1.39 (q, $J = 13.4$ Hz, 2H), 0.95 (t, $J = 4.4$ Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 164.0, 159.5, 158.3, 156.4, 155.6, 146.4, 136.1, 130.6, 130.5, 129.0, 128.5, 127.3, 124.2, 121.0, 118.6, 111.0, 105.7, 55.9, 55.5, 46.1, 45.3, 42.4, 32.44, 28.2, 19.4. HRMS (ESI) (m/z): calculated for $C_{31}H_{34}F_3N_5O$ $[M + H]^+$ 550.2715; found, 550.2791.

8-Isopropyl-2-(4-(phenethylamino)piperidin-1-yl)-5-(4-(trifluoromethyl)phenyl)pyrido[2,3-d]pyrimidine-7(8H)-ketone (49) The title compound was prepared with isopropylamine as the starting material. The synthesis method is similar to **51**. White solid, 138 mg, 9% yield, 94.6% HPLC purity. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.72 (s, 2H), 8.27 (s,1H), 7.74 (d, $J = 8.4$ Hz, 2H), 7.52 (d, $J = 8.1$ Hz, 2H), 7.30–7.15 (m, 6H), 6.30 (s, 1H), 5.77–5.68 (s, 1H), 4.96 (d, $J = 13.8$ Hz, 2H), 3.31–3.17 (m, 3H), 3.06–2.94 (m, 4H), 2.21 (d, $J = 11.4$ Hz, 2H), 1.82–1.73 (m, 5H), 1.58 (d, $J = 6.9$ Hz, 6H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 163.2, 159.7, 157.4, 156.2, 147.1, 138.9, 136.2, 129.1, 129.0, 128.5, 127.4, 125.8, 125.8, 118.0, 105.1, 55.8, 46.1, 45.6, 42.4, 32.5, 28.2, 19.4. HRMS (ESI) (m/z): calculated for $C_{30}H_{32}F_3N_5O$ $[M + H]^+$ 536.2559; found, 536.2645.

8-Isobutyl-2-(4-(phenethylamino)piperidin-1-yl)-5-(4-(trifluoromethyl)phenyl)pyrido[2,3-d]pyrimidine-7(8H)-one (50) The title compound was prepared with isobutylamine as the starting material. The synthesis method is similar to **51**. White solid, 136 mg, 9% yield, 98.1% HPLC purity. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.73 (s, 2H), 8.29 (s,1H), 7.75 (d, $J = 8.1$ Hz, 2H), 7.54 (d, $J = 8.0$ Hz, 2H), 7.3–7.23 (m,5H), 7.16 (d, $J = 6.7$ Hz, 2H), 6.35 (s, 1H), 4.97 (d, $J = 13.5$ Hz, 2H), 4.17 (d, $J = 7.2$ Hz, 2H), 3.49–2.93 (m, 7H), 1.76 (q, $J = 33.5$ Hz, 2H), 0.93 (t, $J = 6.7$ Hz, 6H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 163.1, 162.4, 162.2, 161.9,

160.0, 157.4, 156.3, 147.4, 139.0, 131.4 (q, $J_{CF} = 32.5$ Hz), 129.1, 129.0, 128.5, 127.4, 127.1, 125.8, 125.8, 125.8, 124.9, 122.8, 120.6, 117.7, 111.3, 105.4, 104.6, 55.8, 47.7, 46.2, 42.3, 32.5, 28.2, 27.3, 20.4. HRMS (ESI) (m/z): calculated for $C_{31}H_{34}F_3N_5O$ $[M + H]^+$ 550.2715; found, 550.2799.

8-Cyclohexyl-2-(4-(phenethylamino)piperidin-1-yl)-5-(4-(trifluoromethyl)phenyl)pyrido[2,3-d]pyrimidine-7(8H)-one (52) The title compound was prepared with cyclohexylamine as the starting material. The synthesis method is similar to **51**. White solid, 192 mg, 13% yield, 99.1% HPLC purity. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.73 (s, 1H), 8.25 (s, 1H), 7.73 (d, $J = 8.1$ Hz, 2H), 7.51 (t, $J = 8.0$ Hz, 2H), 7.28 (t, $J = 11.9$ Hz, 4H), 7.18 (q, $J = 22.5$ Hz, 2H), 6.30 (s, 1H), 5.32 (t, $J = 24.6$ Hz, 1H), 4.95 (d, $J = 13.4$ Hz, 1H), 3.25 (d, $J = 38.7$ Hz, 3H), 3.00 (q, $J = 31$ Hz, 4H), 2.66 (q, $J = 33.8$ Hz, 2H), 2.21 (d, $J = 11.6$ Hz, 2H), 1.87–1.66 (m, 8H), 1.38 (q, $J = 34.3$ Hz, 2H), 1.22 (t, $J = 23.2$ Hz, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 163.4, 159.6, 157.4, 156.4, 147.1, 138.9, 136.0, 131.2, 129.1, 129.0, 128.5, 127.4, 125.8, 125.8, 122.8, 105.1, 55.8, 46.1, 42.5, 32.4, 28.6, 28.1, 26.6, 25.8. HRMS (ESI) (m/z): calculated for $C_{33}H_{36}F_3N_5O$ $[M + H]^+$ 576.2872; found, 576.2937.

2-(4-(Phenethylamino)piperidin-1-yl)-8-phenyl-5-(4-(trifluoromethyl)phenyl)pyrido[2,3-d]pyrimidine-7(8H)-one (53) The title compound was prepared with aniline as the starting material. The synthesis method is similar to **51**. White solid, 138 mg, 9% yield, 94.1% HPLC purity. ^{1} H NMR (300 MHz, $CDCl_3$) δ 9.56 (s, 2H), 8.31 (s, 1H), 7.78 (d, $J = 8.0$ Hz, 2H), 7.59 (d, $J = 8.0$ Hz, 2H), 7.46–7.20 (m, 9H), 7.12 (d, $J = 6.9$ Hz, 2H), 4.52 (s, 2H), 3.11 (s, 3H), 2.95 (d, $J = 15.6$ Hz, 2H), 2.71 (s, 1H), 1.99 (d, $J = 9.5$ Hz, 2H), 1.55 (d, $J = 9.8$ Hz, 2H). ^{13}C NMR (125 MHz, $CDCl_3$ + DMSO-d_6) δ 162.0, 159.1, 156.6, 156.3, 147.7, 138.3, 136.0, 135.4, 130.4 (t, $J_{CF} = 32.5$ Hz), 128.4, 128.3, 128.0, 127.9, 127.6, 126.3, 125.1, 125.1, 124.3, 122.1, 116.3, 103.5, 55.5, 45.0, 41.2, 39.9, 39.7, 39.5, 39.4, 39.2, 39.0, 38.9, 31.6, 27.3. HRMS (ESI) (m/z): calculated for $C_{33}H_{30}F_3N_5O$ $[M + H]^+$ 570.2402; found, 570.2487.

5-(2-Methoxyphenyl)-8-methyl-2-(4-(phenethylamino)piperidin-1-yl)pyrido[2,3-d]pyrimidine-7(8H)-one (54) The title compound was prepared with methylamine hydrochloride as the starting material. The synthesis method is similar to **51**. White solid, 210 mg, 14% yield, 99.7% HPLC purity. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.73 (s, 2H), 8.09 (s, 1H), 7.43 (t, $J = 9.1$ Hz, 1H), 7.28–7.15 (m, 4H), 7.05 (t, $J = 8.8$ Hz, 1H), 6.99 (d, $J = 5.0$ Hz, 1H), 6.33 (s, 1H), 4.99 (d, $J = 8.1$ Hz, 2H), 3.73 (s, 3H), 3.60 (s, 3H), 3.29 (s, 1H), 3.19 (s, 2H), 3.04 (t, $J = 9.7$ Hz, 2H), 2.95 (t, $J = 15.0$ Hz, 2H), 2.19 (d, $J = 6.7$ Hz, 2H), 1.76 (d, $J = 6.0$ Hz, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 163.8, 162.4, 161.9, 159.9, 158.2, 156.3, 155.5, 146.7, 136.1, 130.6, 130.4, 128.8, 128.4, 127.2, 124.2, 120.9, 118.5, 117.3, 114.6, 111.0, 105.2, 55.8, 55.4, 46.1, 42.2, 32.4, 28.2, 27.4. HRMS (ESI) (m/z): calculated for $C_{28}H_{31}N_5O_2$ $[M + H]^+$ 470.2478; found, 470.2558.

8-Ethyl-5-(2-methoxyphenyl)-2-(4-(phenethylamino)piperidin-1-yl)pyrido[2,3-d]pyrimidine-7(8H)-one (55) The title compound was prepared with ethylamine aqueous solution as the starting material. The synthesis method is similar to **51**. White solid, 148 mg, 10% yield, 98.8% HPLC purity. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.75 (s, 2H), 8.10 (s, 1H), 7.44 (t, $J = 15.4$ Hz, 1H), 7.30–7.15 (m, 4H), 7.07–6.98 (m, 3H), 6.32 (s, 1H), 4.98 (d, $J = 13.6$ Hz, 2H), 4.35 (q, $J = 20.7$ Hz, 2H), 3.74 (s, 3H), 3.29 (s, 1H), 3.19 (s, 1H), 3.05–2.91 (m, 4H), 2.19 (d, $J = 11.9$ Hz, 2H), 1.76 (q, $J = 10.4$ Hz, 2H), 1.27 (t, $J = 13.9$ Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 163.3, 162.4, 162.1, 160.0, 158.3, 156.3, 155.0, 146.7, 136.1, 130.6, 130.5, 128.9, 128.5, 127.3, 124.3, 120.9, 117.8, 115.4, 111.0, 105.3, 55.9, 55.5, 46.1, 42.2, 35.8, 32.4, 28.2, 12.8. HRMS (ESI) (m/z): calculated for $C_{29}H_{33}N_5O_2$ $[M + H]^+$ 484.2634; found, 484.2714.

8-Butyl-5-(2-methoxyphenyl)-2-(4-(phenethylamino)piperidin-1-yl)pyrido[2,3-d]pyrimidine-7(8H)-one (56) The title compound was prepared with *N*-butylamine as the starting material. The synthesis method is similar to **51**. White solid, 130 mg, 9% yield, 98.4% HPLC purity. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.73 (s, 2H), 8.09 (s, 1H), 7.43 (t, $J = 9.4$ Hz, 1H), 7.28–7.15 (m, 4H), 7.05 (t, $J = 8.9$ Hz, 2H), 7.00 (s, 1H), 6.98 (s, 1H), 6.31 (s, 1H), 4.97 (d, $J = 8.2$ Hz, 2H), 4.30 (t, $J = 9.0$ Hz, 2H), 3.74 (s, 3H), 3.29 (s, 1H), 3.19 (s, 1H), 3.05-2.92 (m, 4H), 2.18 (d, $J = 7.0$ Hz, 2H), 2.06 (s, 1H), 1.76–1.67 (m, 4H), 1.39 (q, $J = 13.9$ Hz, 2H), 1.21 (t, $J = 8.4$ Hz, 3H).^{13}C NMR (75 MHz, $CDCl_3$) δ 163.4, 162.5, 159.9, 158.2, 156.3, 155.1, 146.6, 136.1, 130.6, 130.5, 128.9, 128.4, 127.2, 124.2, 120.9, 117.7, 111.0, 105.2, 55.9, 55.4, 46.1, 42.2, 40.52, 32.41, 29.7, 28.1, 20.4, 13.8. HRMS (ESI) (m/z): calculated for $C_{31}H_{37}N_5O_2$ $[M + H]^+$ 512.2947; found, 512.3026.

8-isopropyl-5-(2-methoxyphenyl)-2-(4-(phenethylamino) piperidin-1-yl)pyrido[2,3-d]pyrimidine-7(8H)-one (57) The title compound was prepared with isopropylamine as the starting material. The synthesis method is similar to **51**. White solid, 185 mg, 13% yield, 98.8% HPLC purity. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.73 (s, 2H), 8.08 (s, 1H), 7.43 (t, $J = 5.2$ Hz, 1H), 7.41–6.98 (m, 8H), 6.27 (s, 1H), 5.73 (s, 1H), 4.95 (d, $J = 8.2$ Hz, 2H), 3.74 (s, 3H), 3.29 (s, H), 3.19 (s, 2H), 3.04–2.92 (m, 4H), 1.79-1.72 (m, 2H), 1.58 (d, $J = 4.2$ Hz, 6H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 162.6,

159.3, 158.0, 156.0, 155.1, 146.2, 137.0, 130.8, 130.1, 128.6, 128.5, 126.7, 123.3, 120.9, 117.3, 111.6, 104.3, 55.5, 54.2, 44.7, 44.4, 42.1, 40.3, 40.1, 39.8, 39.5, 39.2, 38.9, 38.7, 31.7, 27.6, 19.2. HRMS (ESI) (m/z): calculated for $C_{30}H_{35}N_5O_2$ $[M + H]^+$ 498.2791; found, 498.2867.

8-Isobutyl-5-(2-methoxyphenyl)-2-(4-(phenethylamino) piperidin-1-yl)pyrido[2,3-d]pyrimidine-7(8H)-one (58) The title compound was prepared with isobutylamine as the starting material. The synthesis method is similar to **51**. White solid, 155 mg, 10% yield, 96.4% HPLC purity. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.74 (s, 2H), 8.09 (s, 1H), 7.43 (t, $J = 14.1$ Hz, 1H), 7.30–6.98 (m, 11H), 6.32 (s, 1H), 4.96 (d, $J = 13.7$ Hz, 2H), 4.16 (d, $J = 7.1$ Hz, 2H), 3.74 (s, 3H), 3.29–2.90 (m, 7H), 2.32–2.07 (m, 3H), 1.80–1.73 (m, 2H), 0.93 (q, $J = 6.7$ Hz, 6H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 163.8, 159.8, 158.3, 156.4, 155.6, 146.6, 136.1, 130.6, 130.5, 128.9, 128.5, 127.3, 124.3, 121.0, 117.9, 111.0, 105.2, 55.9, 55.5, 47.6, 46.1, 42.2, 32.4, 28.2, 27.3, 20.5. HRMS (ESI) (m/z): calculated for 498.2867 $[M + H]^+$ 512.2947; found, 512.3022.

8-Cyclopropyl-5-(2-methoxyphenyl)-2-(4-(phenethylamino) piperidin-1-yl)pyrido[2,3-d]pyrimidine-7(8H)-one (59) The title compound was prepared with cyclopropyl as the starting material. The synthesis method is similar to **51**. White solid, 182 mg, 12% yield, 98.6%. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.73 (s, 2H), 8.06 (s, 1H), 7.44 (t, $J = 5.2$ Hz, 1H), 7.29–7.15 (m, 6H), 7.05–6.98 (m, 2H), 6.26 (s, 1H), 5.02 (d, $J = 8.0$ Hz, 2H), 3.74 (s, 3H), 3.30 (s, 1H), 3.19 (3 s, 2H), 3.03 (t, $J = 10.1$ Hz, 2H), 2.96 (t, $J = 14.8$ Hz, 2H), 2.79 (s, 1H), 2.19 (d, $J = 7.0$ Hz, 2H), 1.96 (s, 1H), 1.76 (d, $J = 6.3$ Hz, 2H), 1.18 (d, $J = 4.2$ Hz, 2H), 0.88 (d, $J = 2.1$ Hz, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 164.7, 162.4, 162.0, 159.7, 158.1, 157.0, 156.3, 146.7, 136.1, 130.6, 130.4, 128.9, 128.5, 127.2, 124.2, 121.0, 118.1, 110.9, 105.4, 55.9, 55.4, 46.1, 42.2, 32.4, 28.2, 24.9, 9.3. HRMS (ESI) (m/z): calculated for $C_{30}H_{33}N_5O_2$ $[M + H]^+$ 496.2634; found, 496.2724.

8-Cyclohexyl-5-(2-methoxyphenyl)-2-(4-(phenethylamino) piperidin-1-yl)pyrido[2,3-d]pyrimidine-7(8H)-one (60) The title compound was prepared with cyclohexylamine as the starting material. The synthesis method is similar to **51**. White solid, 165 mg, 11% yield, 99.4% HPLC purity. ^{1} H NMR (300 MHz, $CDCl_3$) δ 9.73 (s, 1H), 8.07 (s, 1H), 7.44 (t, $J = 9.0$ Hz, 1H), 7.45 (t, $J = 8.0$ Hz, 2H), 7.3–7.06 (m, 7H), 7.04–6.97 (m, 2H), 6.28 (s, 1H), 5.32 (t, $J = 24.6$ Hz, 1H), 4.94 (d, $J = 13.2$ Hz, 2H), 3.74 (s, 1H), 3.30–3.18 (m, 3H), 3.06–2.92 (m, 4H), 2.69 (d, $J = 12.0$ Hz, 2H), 2.20 (d, $J = 11.1$ Hz 2H), 1.86–1.66 (m, 5H), 1.41–1.26 (m, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 162.5, 159.2, 158.0, 155.9, 155.2, 146.2, 137.0, 130.8, 130.1, 128.6, 128.5, 126.7, 123.3, 120.8, 111. 6, 104.3, 55.4, 54.2, 44.7, 42.1, 40.0, 39.8, 39.7, 39.5, 39.3, 39.2, 39.0, 31.7, 28.2, 27.6, 26.1, 25.3. HRMS (ESI) (m/z): calculated for $C_{33}H_{39}N_5O_2$ $[M + H]^+$ 538.3104; found, 538.3190.

5-(2-Methoxyphenyl)-8-phenyl-2-(4-(3-phenylpropyl)piperidin-1-yl)pyrido[2,3-d]pyrimidine-7(8H)-one (61) The title compound was prepared with aniline as the starting material. The synthesis method is similar to **51**. White solid, 85 mg, 6% yield, 96.4% HPLC purity. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.57 (s, 2H), 8.14 (s, 1H), 7.49-7.01 (m, 17 H), 6.42 (s, 1H), 3.78 (s, 3H), 2.97 (d, $J = 9.6$ Hz, 2H), 2.68 (t, $J = 18.9$ Hz, 2H), 1.99 (d, $J = 9.9$ Hz, 2H), 1.75 (s, 4H), 1.54 (d, $J = 8.4$ Hz, 2H).^{13}C NMR (75 MHz, DMSO-d_6) δ 162.2, 159.3, 157.8, 156.0, 156.0, 147.2, 137.0, 136.2, 130.9, 130.2, 128.8, 128.7, 128.6, 128.5, 127.9, 126.7, 123.4, 120.9, 117.0, 111.7, 103.9, 55.5, 54.0, 44.6, 41.5, 40.3, 40.1, 39.8, 39.5, 39.2, 38.9, 38.7, 31.6, 27.4. HRMS (ESI) (m/z): calculated for $C_{33}H_{33}N_5O_2$ $[M + H]^+$ 532.2634; found, 532.2712.

5-((3-chlorophenyl)ethynyl)-8-methyl-2-(4-(phenethylamino)piperidin-1-yl)pyrido[2,3-d]pyrimidine-7(8H)-one (62) The title compound was prepared with methylamine hydrochloride as the starting material. The synthesis method is similar to **51**. Yellow solid, 97 mg, 7% yield, 94.7% HPLC purity. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.75 (s, 2H), 8.79 (s, 1H), 7.57 (s, 1H), 7.46 (d, $J = 3.3$ Hz, 1H), 7.43 (d, $J = 14.8$ Hz, 1H), 7.39–7.22 (m, 4H), 7.17 (d, $J = 4.4$ Hz, 2H), 6.56 (s, 1H), 5.01 (d, $J = 7.8$ Hz, 2H), 3.56 (s, 3H), 3.31 (s, 1H), 3.20 (s, 2H), 3.06–2.96 (m, 4H), 2.22 (d, $J = 7.0$ Hz, 2H), 1.95 (s, 1H), 1.81–1.75 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 162.9, 160.3, 157.4, 155.6, 136.1, 134.5, 131.8, 130.1, 123.0, 129.8, 129.6, 128.9, 128.5, 127.3, 123.2, 120.0, 104.8, 97.5, 83.4, 55.8, 46.2, 42.3, 32.5, 28.2, 27.5. HRMS (ESI) (m/z): calculated for $C_{29}H_{28}ClN_5O$ $[M + H]^+$ 498.1982; found, 498.2053.

5-((3-chlorophenyl)ethynyl)-8-ethyl-2-(4-(phenethylamino) piperidin-1-yl)pyrido[2,3-d]pyrimidine-7(8H)-one (63) The title compound was prepared with ethylamine aqueous solution as the starting material. The synthesis method is similar to **51**. Yellow solid, 203 mg, 14% yield, 97.4% HPLC purity. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.83 (s, 1H), 7.83 (s, 1H), 7.65 (d, $J = 7.2$ Hz, 1H), 7.56–7.18 (m, 8H), 6.44 (s, 1H), 4.55 (d, $J = 9.0$ Hz, 2H), 4.20 (d, $J = 6.9$ Hz, 2H), 3.22–3.15 (m, 8H), 2.65 (dd, $J = 6.3$ Hz, $J = 6.6$ Hz, 6H), 1.91 (d, $J = 10.8$ Hz, 2H), 1.27–1.16 (m, 6H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 161.2, 160.0, 157.3, 154.6, 140.2, 133.4, 131.5, 130.6, 130.5, 130.0, 128.9, 128.5, 128.1, 125.7, 122.7, 118.7, 103.2, 96.8, 83.5, 53.8, 47.7, 42.3, 40.3, 40.1, 39.8, 39.5, 39.2, 38.9, 38.7, 36.0, 35.0, 31.5, 12.5. HRMS (ESI) (m/

z): calculated for $C_{30}H_{30}ClN_5O$ $[M + H]^+$ 512.2139; found, 512.2216.

8-Butyl-5-((3-chlorophenyl)ethynyl)-2-(4-(phenethylamino) piperidin-1-yl)pyrido[2,3-d]pyrimidine-7(8H)-one (64) The title compound was prepared with *N*-butylamine as the starting material. The synthesis method is similar to **51**. Yellow solid, 144 mg, 10% yield, 96.3% HPLC purity. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.76 (s, 2H), 8.80 (s, 1H), 7.56 (s, 1H), 7.46 (d, $J = 4.6$ Hz, 1H), 7.40–7.12 (m, 8H), 6.56 (s, 1H), 4.99 (d, $J = 7.4$ Hz, 2H), 4.26 (t, $J = 8.9$ Hz, 2H), 3.31 (s, 1H), 3.20 (s, 2H), 3.14-2.96 (m, 4H), 2.21 (d, $J = 6.8$ Hz, 2H), 1.78 (q, $J = 19.3$ Hz, 2H), 1.68–1.62 (m, 2H), 1.38 (q, $J = 13.3$ Hz, 2H), 0.98 (t, $J = 4.4$ Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 162.6, 162.5, 162.0, 160.3, 157.4, 155.3, 136.0, 134.5, 131.8, 130.8, 130.1, 129.9, 129.8, 130.7, 129.9, 129.8, 129.6, 129.3, 128.9, 128.5, 127.3, 123.2, 120.3, 118.5, 114.6, 104.8, 97.4, 83.4, 55.8, 46.1, 42.2, 40.5, 32.4, 29.6, 28.1, 20.3, 13.7. HRMS (ESI) (m/z): calculated for $C_{32}H_{34}ClN_5O$ $[M + H]^+$ 539.2452; found, 540.2525.

5-((3-chlorophenyl)ethynyl)-8-isopropyl-2-(4-(phenethylamino)piperidin-1-yl)pyrido[2,3-d]pyrimidine-7(8H)-one (65) The title compound was prepared with isopropylamine as the starting material. The synthesis method is similar to **51**. Yellow solid, 88 mg, 6% yield, 96.7% HPLC purity. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.76 (s, 2H), 8.79 (s, 1H), 7.56 (s, 1H), 7.45 (d, $J = 4.5$ Hz, 1H), 7.39 (d, $J = 4.8$ Hz, 1H), 7.33–7.16 (m, 8H), 6.51 (s, 1H), 5.65 (q, $J = 12.3$ Hz, 1H), 4.98 (d, $J = 7.5$ Hz, 2H), 3.47 (q, $J = 12.6$ Hz, 1H), 3.33 (s, 1H), 3.20 (d, $J = 4.5$ Hz, 2H), 3.05–2.97 (m, 4H), 2.22 (d, $J = 7.2$ Hz, 2H), 1.81–1.74 (m, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 163.1, 162.4, 162.1, 156.0, 157.6, 155.7, 136.0, 134.5, 131.8, 130.1, 129.9, 129.8, 129.4, 128.9, 128.5, 127.3, 123.2, 121.2, 105.1, 97.3, 83.3, 65.8, 55.8, 46.1, 45.5, 42.4, 32.4, 28.1, 19.3, 15.2. HRMS (ESI) (m/z): calculated for $C_{31}H_{32}ClN_5O_2$ $[M + H]^+$ 526.2295; found, 526.2383.

5-((3-chlorophenyl)ethynyl)-8-isobutyl-2-(4-(phenethylamino)piperidin-1-yl)pyrido[2,3-d]pyrimidine-7(8H)-one (66) The title compound was prepared with isobutylamine as the starting material. The synthesis method is similar to **51**. Yellow solid, 110 mg, 8% yield, 96.3% HPLC purity. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.89 (s, 2H), 7.88 (s, 1H), 7.68 (d, $J = 6.9$ Hz, 1H), 7.58 (d, $J = 7.2$ Hz, 1H), 7.51 (d, $J = 8.4$ Hz, 3H), 7.27–7.18 (m, 6H), 6.49 (s, 1H), 4.52 (s, 3H), 4.04 (d, $J = 6.0$ Hz, 3H), 3.25–3.18 (m, 7H), 2.79 (t, $J = 30.3$ Hz, 6H), 2.51 (s, 1H), 2.17 (t, $J = 12.0$ Hz, 2H), 1.89 (d, $J = 10.5$ Hz, 3H), 1.23 (d, $J = 8.1$ Hz, 3H), 0.87 (d, $J = 5.7$ Hz, 8H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 161.8, 159.9, 157.5, 155.3, 133.4, 131.6, 130.7, 130.6, 130.1, 129.0, 128.5, 128.1, 125.7, 122.7, 118.8, 103.2, 96.9, 83.5, 53.8, 47.9, 46.7, 42.3, 39.8, 39.5, 39.2, 38.9, 38.7, 36.1, 31.7, 26.7, 20.1. HRMS (ESI) (m/z): calculated for $C_{32}H_{34}ClN_5O_2$ $[M + H]^+$ 540.2452; found, 540.2544.

5-((3-chlorophenyl)ethynyl)-8-cyclopropyl-2-(4-(phenethylamino)piperidin-1-yl)pyrido[2,3-d]pyrimidine-7(8H)-one (67) The title compound was prepared with cyclopropyl as the starting material. The synthesis method is similar to **51**. Yellow solid, 77 mg, 5% yield, 98.3% HPLC purity. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.74 (s, 2H), 8.77 (s, 1H), 7.56 (d, $J = 1.0$ Hz, 1H), 7.55–7.16 (m, 9H), 6.51 (s, 1H), 5.04 (d, $J = 8.5$ Hz, 2H), 3.32 (s, 1H), 3.20 (s, 2H), 3.06–2.97 (m, 4H), 2.79–2.76 (m, 1H), 2.21 (d, $J = 6.5$ Hz, 2H), 1.81–1.74 (m, 4H), 1.21–1.15 (m, 2H), 0.85 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 164.0, 160.2, 157.3, 136.0, 134.5, 131.8, 130.1, 130.0, 129.8, 129.7, 129.0, 128.5, 127.3, 123.2, 120.8, 104.8, 97.6, 83.3, 55.9, 46.2, 42.3, 32.5, 28.2, 25.0, 9.3. HRMS (ESI) (m/z): calculated for $C_{31}H_{30}ClN_5O$ $[M + H]^+$ 524.2139; found, 524.2219.

5-((3-chlorophenyl)ethynyl)-8-cyclohexyl-2-(4-(phenethylamino)piperidin-1-yl)pyrido[2,3-d]pyrimidine-7(8H)-one (68) The title compound was prepared with cyclohexylamine as the starting material. The synthesis method is similar to **51**. Yellow solid, 200 mg, 14% yield, 98.3% HPLC purity. ^{1} H NMR (300 MHz, $CDCl_3$) δ 9.73 (s, 2H), 8.78 (s, 1H), 7.56 (d, $J = 3.3$ Hz, 2H), 7.47–7.16 (m, 7 H), 6.53 (s, 1H), 5.25 (t, $J = 24.3$ Hz, 2H), 4.97 (d, $J = 12.9$ Hz, 2H), 3.32(s, 1H), 3.21 (d, $J = 8.4$ Hz, 2H), 3.06–2.97 (m, 4H), 2.63 (q, $J = 34.5$ Hz, 2H), 2.23 (d, $J = 10.2$ Hz, 2H), 1.85–1.62 (m, 10H), 1.35 (t, $J = 12.6$ Hz, 2H), 1.21 (t, $J = 14.1$ Hz, 1H). ^{13}C NMR (125 MHz, $CDCl_3 + CD_3OD$) δ 163.6, 157.6, 155.7, 136.0, 131.7, 130.0, 129.9, 129.8, 128.8, 128.4, 127.2, 105.2, 97.6, 83.0, 55.4, 52.0, 49.6, 49.3, 49.0, 48.7, 48.4, 45.9, 45.7, 42.4, 41.9, 32.2, 28.5, 28.0, 26.4, 25.6, 24.7. HRMS (ESI) (m/z): calculated for $C_{34}H_{36}ClN_5O$ $[M + H]^+$ 566.2608; found, 566.2886.

5-((3-chlorophenyl)ethynyl)-2-(4-(phenethylamino)piperidin-1-yl)-8-phenylpyrido[2,3-d]pyrimidine-7(8H)-one (69) The title compound was prepared with aniline as the starting material. The synthesis method is similar to **51**. Yellow solid, 200 mg, 14% yield, 98.3% HPLC purity. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.89 (s, 1H), 7.79 (s, 1H), 7.65 (d, $J = 7.2$ Hz, 1H), 7.53–7.41 (m, 6H), 7.25–7.16 (m, 8H), 6.54 (s, 1H), 4.53 (s, 1H), 3.87 (s, 1H), 3.42–3.39 (m, 6H), 2.78 (d, $J = 6.6$ Hz, 4H), 2.71–2.52 (m, 4H), 1.76 (s, 3H), 1.12–1.10 (m, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 162.6, 161.6, 159.7, 157.1, 156.1, 140.0, 135.8, 133.5, 131.4, 130.4, 130.2, 129.8, 128.5, 128.4, 128.2, 127.9, 127.7, 125.5, 122.6, 119.1, 103.2, 97.0, 83.4, 53.6, 47.6, 41.9, 40.3, 40.1, 39.8, 39.5, 39.2, 39.0, 38.7, 36.0, 31.5. HRMS

(ESI) (m/z): calculated for $C_{34}H_{30}ClN_5O$ $[M + H]^+$ 560.2139; found, 560.2222.

5-((1-hydroxycyclohexyl)ethynyl)-8-methyl-2-(4-(phenethylamino)piperidin-1-yl)pyrido[2,3-d]pyrimidine-7(8H)-one (70) The title compound was prepared with methylamine hydrochloride as the starting material. The synthesis method is similar to **51**. White solid, 280 mg, 19% yield, 99.0% HPLC purity. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.73 (s, 2H), 8.69 (s, 1H), 7.31–7.17 (m, 5H), 6.47 (s, 1H), 4.93 (d, $J = 7.3$ Hz, 2H), 3.57 (s, 3H), 3.30 (s, 1H), 3.22 (s, 2H), 3.06–2.97 (m, 6H), 2.22 (d, $J = 6.8$ Hz, 3H), 2.13–2.04 (m, 6H), 1.80–1.71 (m, 4H), 1.61–1.54 (m, 2H), 1.33 (d, $J = 5.7$ Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3 + CD_3OD$) δ 163.5, 156.0, 157.5, 155.1, 135.7, 130.6, 128.5, 128.1, 126.9, 118.6, 104.8, 104.3, 76.3, 68.9, 68.1, 55.2, 48.7, 48.5, 48.3, 48.2, 48.0, 47.8, 47.6, 45.5, 41.9, 39.0, 31.9, 27.8, 27.0, 24.6, 22.9. HRMS (ESI) (m/z): calculated for $C_{29}H_{35}N_5O_2$ $[M + H]^+$ 486.2791; found, 486.2887.

8-Ethyl-5-((1-hydroxycyclohexyl)ethynyl)-2-(4-(phenethylamino)piperidin-1-yl)pyrido[2,3-d]pyrimidine-7(8H)-one (71) The title compound was prepared with *N*-butylamine as the starting material. The synthesis method is similar to **51**. White solid, 136 mg, 9% yield, 98.8% HPLC purity. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.73 (s, 2H), 8.67 (s, 1H), 7.30–7.16 (m, 5H), 6.46 (s, 1H), 4.92 (d, $J = 7.4$ Hz, 2H), 4.28 (q, $J = 12.3$ Hz, 2H), 3.31 (s, 1H), 3.21 (s, 2H), 3.05–2.96 (m, 5H), 2.21 (d, $J = 6.9$ Hz, 2H), 2.05 (d, $J = 7.4$ Hz, 2H), 1.78–1.71 (m, 6H), 1.60–1.53 (m,3H), 1.33–1.30 (m, 1H), 1.24 (t, $J = 4.2$ Hz, 3H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 161.3, 160.1, 157.2, 154.7, 137.0, 129.6, 128.6, 128.5, 126.7, 118.6, 105.5, 104.1, 75.8, 67.2, 54.1, 44.7, 41.9, 38.9, 35.1, 31.7, 27.63, 24.7, 22.6, 12.5. HRMS (ESI) (m/z): calculated for $C_{30}H_{37}N_5O_2$ $[M + H]^+$ 499.2947; found, 500.3027.

8-Butyl-5-((1-hydroxycyclohexyl)ethynyl)-2-(4-(phenethylamino)piperidin-1-yl)pyrido[2,3-d]pyrimidine-7 (8H)-one (72) The title compound was prepared with *N*-butylamine as the starting material. The synthesis method is similar to **51**. Yellow solid, 75 mg, 5% yield, 96.1% HPLC purity. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.74 (s, 2H), 8.69 (s, 1H), 7.30–7.16 (m, 5H), 6.47 (s, 1H), 4.92 (d, $J = 8.3$ Hz, 2H), 4.25 (t, $J = 8.9$ Hz, 2H), 3.30 (s, 1H), 3.21 (s, 2H), 3.05–2.98 (m, 4H), 2.54 (s, 1H), 2.20 (d, $J = 7.3$ Hz, 2H), 2.05 (d, $J = 7.6$ Hz, 2H), 1.78–1.56 (m, 12H), 1.38–1.34 (m, 3H), 0.95 (t, $J = 4.4$ Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 161.5, 160.2, 157.2, 154.9, 137.1, 129.6, 128.6, 128.5, 126.7, 118.5, 105.5, 104.0, 75.9, 67.2, 54.1, 44.7, 42.0, 31.7, 29.1, 27.6, 24.7, 22.7, 19.7, 13.6. HRMS (ESI) (m/z): calculated for $C_{32}H_{41}N_5O_2$ $[M + H]^+$ 528.3260; found, 528.3345.

5-((1-Hydroxycyclohexyl)ethynyl)-8-isopropyl-2-(4-(phenethylamino)piperidin-1-yl)pyrido[2,3-d]pyrimidine-7(8H)-one (73) The title compound was prepared with isopropylamine as the starting material. The synthesis method is similar to **51**. White solid, 88 mg, 6% yield, 96.4% HPLC purity. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.73 (s, 2H), 8.66 (s, 1H), 7.30–7.16 (m, 5H), 6.44 (s, 1H), 5.66–5.61 (m, 1H), 4.91 (t, $J = 7.8$ Hz, 2H), 3.31 (s, 1H), 3.21 (d, $J = 4.7$ Hz, 2H), 3.05–2.95 (m, 4H), 2.21 (d, $J = 6.8$ Hz, 2H), 2.04 (d, $J = 8.2$ Hz, 2H), 1.79–1.69 (m, 6H), 1.60–1.33 (m, 10H), 1.32 (d, $J = 4.3$ Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 163.5, 162.4, 159.8, 157.6, 155.4, 136.0, 123.0, 128.9, 128.5, 127.3, 121.0, 105.4, 104.1, 68.8, 55.9, 46.3, 45.5, 42.3, 39.6, 32.4, 28.1, 25.0, 23.2, 19.3. HRMS (ESI) (m/z): calculated for $C_{31}H_{39}N_5O_2$ $[M + H]^+$ 514.3104; found, 514.3195.

5-((1-Hydroxycyclohexyl)ethynyl)-8-isobutyl-2-(4-(phenethylamino)piperidin-1-yl)pyrido[2,3-d]pyrimidine-7(8H)-ketone (74) The title compound was prepared with isobutylamine as the starting material. The synthesis method is similar to **51**. White solid, 93 mg, 6% yield, 97.1% HPLC purity. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.73 (s, 2H), 8.71 (s, 1H), 7.30–7.16 (m, 5H), 6.48 (s, 1H), 4.93 (d, $J = 7.6$ Hz, 2H), 4.11 (d, $J = 4.3$ Hz, 2H), 3.30 (s, 1H), 3.20 (s, 2H), 3.04–2.96 (m, 4H), 2.69 (s, 2H), 2.19 (d, $J = 12.4$ Hz, 3H), 2.05 (d, $J = 7.1$ Hz, 2H), 1.79–1.70 (m, 6H), 1.61-1.54 (m, 3H), 1.33 (d, $J = 12.8$ Hz, 1H), 0.89 (s, 1H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 163.3, 162.5, 162.0, 160.1, 157.6, 155.5, 136.0, 130.1, 128.9, 128.5, 127.3, 120.2, 105.0, 104.1, 76.6, 68.9, 55.9, 47.5, 46.3, 42.1, 39.6, 32.4, 28.2, 27.2, 25.1, 23.3, 20.3. HRMS (ESI) (m/z): calculated for $C_{32}H_{41}N_5O_2$ $[M + H]^+$ 527.3260; found, 528.3345.

5-((1-Hydroxycyclohexyl)ethynyl)-8-cyclopropyl-2-(4-(phenethylamino)piperidin-1-yl)pyrido[2,3-d]pyrimidine-7(8H)-ketone (75) The title compound was prepared with cyclopropyl as the starting material. The synthesis method is similar to **51**. White solid, 107 mg, 7% yield, 95.0% HPLC purity. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.72 (s, 2H), 8.67 (s, 1H), 7.32–7.16 (m, 5H), 6.40 (s, 1H), 4.97 (d, $J = 16.2$ Hz, 2H), 3.32–3.22 (m, 3H), 3.01 (q, $J = 33.6$ Hz, 4H), 2.85–2.73 (m, 1H), 2.43 (s, 1H), 2.22 (d, $J = 40.8$ Hz, 2H), 2.04 (t, $J = 15.9$ Hz, 2H), 1.80–1.61 (m, 6H), 1.56 (q, $J = 29.1$ Hz, 3H), 1.35 (d, $J = 10.8$ Hz, 2H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 162.8, 159.9, 156.9, 136.9, 129.4, 128.6, 128.6, 126.8, 119.3, 105.6, 104.3, 75.9, 67.2, 54.3, 44.7, 42.0, 31.8, 27.7, 24.7, 22.7, 9.0. HRMS (ESI) (m/z): calculated for $C_{31}H_{37}N_5O_2$ $[M + H]^+$ 512.2947; found, 512.3033.

8-Cyclohexyl-5-((1-hydroxycyclohexyl)ethynyl)-2-(4-(phenethylamino)piperidin-1-yl)pyrido[2,3-d]pyrimidine-7(8H)-one (76) The title compound was prepared with cyclohexylamine as the starting material. The synthesis method is

similar to **51**. White solid, 78 mg, 5% yield, 98.1% HPLC purity. ^{1}H NMR (300 MHz, DMSO-d_6) δ 8.74 (s,1H), 7.31–7.16 (m, 5H), 6.28 (s, 1H), 5.70 (s, 1H), 5.14 (s, 1H), 4.54 (d, $J = 10.5$ Hz, 2H), 3.32–3.19 (m, 3H), 2.86–2.60 (m, 6H), 2.50 (s, 2H), 1.91–1.82 (m, 7H), 1.67–1.46 (m, 12H), 1.42–1.19 (m, 8H). ^{13}C NMR (125 MHz, $CDCl_3$ + CD_3OD) δ 158.7, 156.5, 136.9, 131.4, 129.7, 129.3, 128.1, 78.3, 69.3, 56.3, 50.0, 49.8, 49.7, 49.5, 49.3, 49.2, 49.0, 46.6, 43.3, 40.2, 33.1, 29.4, 28.9, 27.2, 25.8, 24.0. HRMS (ESI) (m/z): calculated for $C_{34}H_{43}N_5O_2$ $[M + H]^+$ 554.3417; found, 554.3497.

5-((1-hydroxycyclohexyl)ethynyl)-2-(4-(phenethylamino) piperidin-1-yl)-8-phenylpyrido[2,3-d]pyrimidine-7(8H)-one (77) The title compound was prepared with aniline as the starting material. The synthesis method is similar to **51**. White solid, 77 mg, 5% yield, 97.1% HPLC purity. ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.58 (s, 2H), 8.74 (s, 1H), 7.48–7.35 (m, 7H), 7.31–7.12 (m, 5H), 6.57 (s, 1H), 4.55 (s, 2H), 3.12 (s, 3H), 2.98–2.93 (m, 3H), 2.75 (s, 2H), 2.06–2.00 (m, 9H), 1.81–1.57 (m, 11H), 1.34–1.20 (m, 1H). ^{13}C NMR (125 MHz, $CDCl_3 + CD_3OD$) δ 163.4, 156.0, 157.7, 156.3, 136.0, 135.5, 131.7, 128.9, 128.8, 128.4, 128.3, 127.2, 119.7, 105.0, 105.0, 68.5, 55.3, 49.6, 49.3, 49.0, 48.8, 48.5, 48.2, 45.7, 41.9, 39.3, 32.1, 27.8, 24.9, 23.2. HRMS (ESI) (m/z): calculated for $C_{34}H_{37}N_5O_2$, $[M + H]^+$ 548.2947; found, 548.3033.

Tested compounds

All synthesized target compounds were characterized by ^{1}H NMR, ^{13}C NMR, HRMS, IR, and HPLC. The purity of all tested compounds was all over 90%. (Supplementary Figs. S2–S326).

Cytotoxicity assay

The cytotoxicity assay was completed using the MTT (sharp) assay. Human cell line L-02 was seeded in 96-well plates at a density of 3000 cells/well in a 100 μL culture medium. The cells were allowed to settle and attach for 12 h. Then the cell line was exposed to different concentrations of tested compounds in serial dilutions in a 150 μL medium and incubated for 72 h. Then 15 μL sterile MTT (5 mg/mL, dissolved in PBS) was added to each well and incubated with cells for 4 h at 37 °C. Purple formazan product was resuspended in DMSO (150 μL/well) and measured in a microplate reader at 490 nm wavelength. The experiment was repeated three times with three wells for each concentration of the tested agents. LC_{50} values were calculated from the inhibition curves by normalized nonlinear regression analysis using GraphPad Prism 8 software.

Enzyme-based NAE activity assay

The NEDD8 Conjugation Initiation Kit (K-800, R&D) was performed in the enzyme-based assay according to the instructions of the manufacturer. 2 μL NAE (2.5 μM), 2 μL NEDD8 (250 μM), 2 μL Ubc12 (50 μM), 2 μL reaction buffer and 10 μL water solution of **51** (0 μM, 0.12 μM, 0.37 μM, 1.11 μM, 3.33 μM, 10 μM) were added into 100 μL reaction tubes in order. Reaction solutions were incubated at 37 °C for 10 min. The reaction was initiated by the addition of 2 μL Mg^{2+}-ATP solution (10 mM), and the mixture was incubated at 37 °C for 60 min. In the negative control group, 2 μL ddH_2O was added instead of ATP solution. The reaction was quenched by the addition of 2 μL EDTA (1 M), and protein samples were electrophoresed under non-reducing conditions on a 12 % SDS-PAGE gel. The Ubc12-NEDD8 levels were determined by western blot analysis.

Cell-based NAE activity assay

To validate whether **51** could inhibit NAE in BxPC-3 cells, BxPC-3 cells which were treated with **51** under 0, 4, 8, 12 μM for 24 h were lysed and protein extracts were analyzed by western blot. The NEDD8 combined Ubc12 was noticeably decreased in a concentration-dependent manner, indicating that NAE activity was inhibited and that neddylation was suppressed by **51**.

Western blot analysis

Protein samples were transferred to a PVDF membrane. The membrane was blocked with 5% (w/v) milk for 1 h at room temperature and probed with primary antibody diluted (1:1000, v/v) in 5% (w/v) milk overnight at 4 °C. The membrane was washed with TBS/0.1% (v/v) Tween 20 (TBST) and incubated with horseradish peroxide-conjugated secondary antibody diluted (1:4000, v/v) in 5% (w/v) milk for 1.5 h at room temperature. Protein bands were detected using SignalFire™ ECL Reagent (6883, Cell Signaling Technology, CST). In this assay anti-Ubc12 antibody (Abcam) was used to measure Ubc12-NEDD8, Densitometry analysis of the Western blot was conducted by using Image J. All experiment was repeated two times.

Metabolic stability assay of liver microsomes

This assay was performed using the Human liver microsome mixed metabolic stability kit (0111013, PHASE) according to the instructions of the manufacturer. 10 μL NADPH regeneration system A solution (20×), 2 μL NADPH regeneration system B solution (100×), 1 μL tested compound (20 mM), 182 μL 0.1 M PBS buffer were added into 1 mL reaction

tubes in order, reaction solutions were incubated at 37 °C for 5 min. The reaction was initiated by the addition of 5 μL liver particles (20 mg/mL). 35 μL reaction solution was taken out each time and 165 μL of pre-cooled acetonitrile was added into the incubation system to stop the reaction at the set incubation time point of 0, 15, 30, 60, 90 min. After the sample was vortexed for 5 minutes, centrifuged at 12,000 rpm at 4 °C for 10 min at 12,000 rpm, and the supernatant was taken and injected with HPLC to detect the concentration of the drug. The concentration of the compound to be tested at the 0 min incubation time point is regarded as 100%, and other incubation time points are converted into the percentage remaining amount, and the natural logarithm of the percentage remaining amount at each time point is linear regression to the incubation time, and the slope is calculated **k**.

$$T_{1/2} = \frac{-0.693}{k}, CL_{int} = \frac{0.693}{T1/2(min)} * \frac{\text{Incubation system (mL)}}{\text{Liver microsomes(mg)}}$$

Apoptosis assay

Apoptosis was evaluated using Annexin V-EGFP Apoptosis Detection Kit (KGA102, KeyGEN BioTECH) following the manufacturer's instructions. BxPC-3 cells were seeded at 3×10^5 cells per well in six-well plates and allowed to attach in 12 h. Cells were treated with different concentrations of **51** (0 μM, 4 μM, 10 μM and 15 μM) for 12 h. Afterward, the cells were collected and washed twice with ice-cold PBS, and then resuspended in 500 μL 1× binding buffer, followed by the addition of 5 μL Annexin V-EGFP staining solution and 5 μL PI staining solution. After incubation at room temperature in the dark for 15 min, stained cells were analyzed immediately by flow cytometry (ACEA NovoCyte). In each sample, 20,000 cells were examined, and fluorescence was measured at an excitation wavelength of 488 nm through FL-1 (530 nm) and FL-3 (630 nm) filters.

In vivo anticancer activity experiments

BxPC-3 cells were established by inoculating 5×10^6 cells in nude mice, respectively. The efficacy study was initiated when tumors had reached ~90 mm^3. Tumor-bearing mice were randomly assigned to control and treatment groups and received the corresponding treatments as indicated. Tumor measurements were performed twice a week. All procedures for animal studies were approved by the Institutional Animal Care and Use Committee of the Jiangsu Institute of Materia Medica.

Log D measurement

Log D values of compounds were measured using the shake-flask method. Each compound was dissolved in 1 mL PBS (PBS was saturated with 1-octanol), and then an equal volume of 1-octanol (1-octanol was saturated with PBS) was added. The tube was shaken for 24 h at 37 °C to allow the compound to partition into each phase. After being centrifugated at 5000 rpm, a compound in each phase was determined by HPLC (Inertsil ODS-SP C18 column, 4.6 × 250 mm, 5 μm, SHIMADZU) at 254 nm. All the experiments were carried out in triplicate. The partition coefficient was calculated using the equation below.

$$\text{Log D} = \text{Log}\frac{AUC_{octanol}}{AUC_{PBS}}$$

Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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