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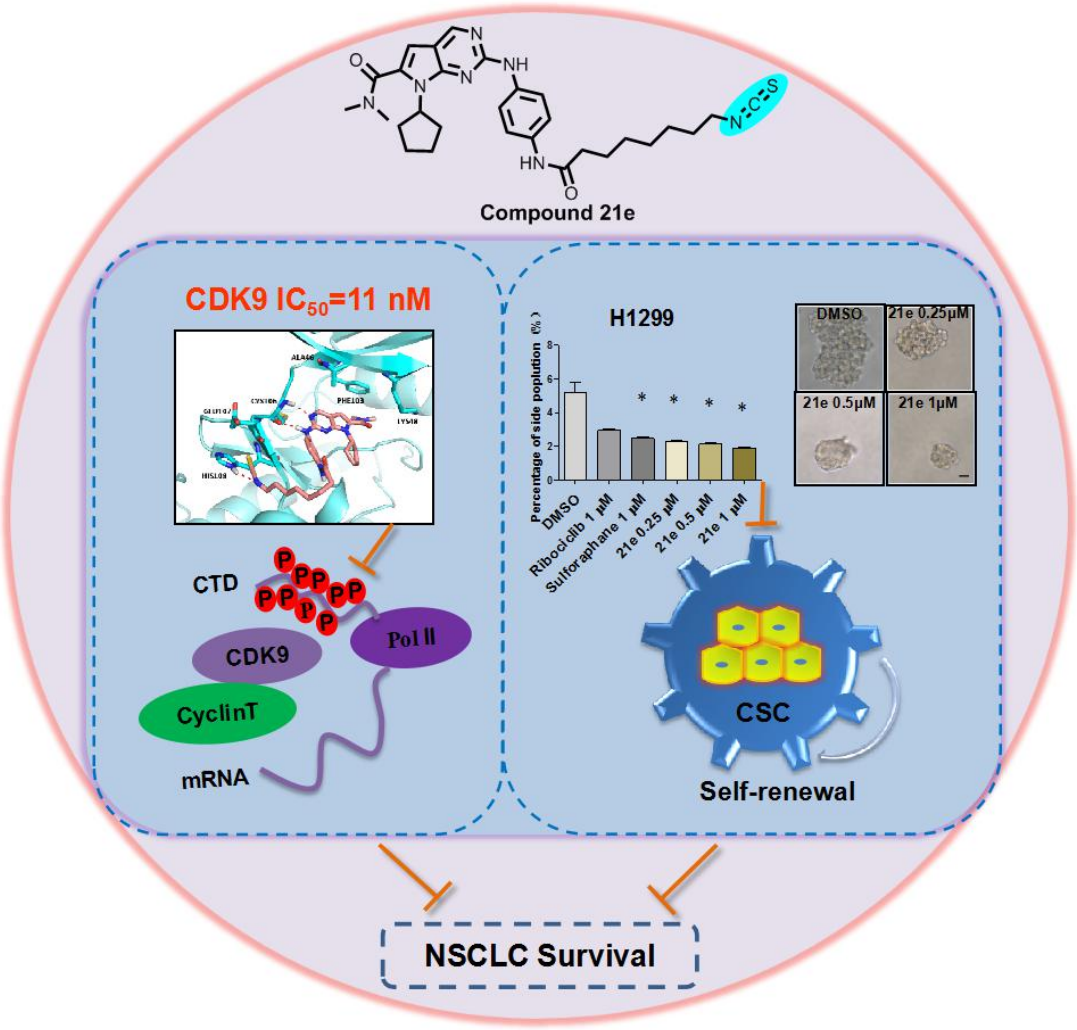

A selective, potent and oral CDK9 inhibitor, **21e** (IC_{50} = 11 nM) with stemness suppression properties of NSCLC was discovered. In H1299 xenograft mouse model, **21e** at 20 mg/kg led to significant tumor regression without obvious toxicity.

Novel Cyclin-dependent Kinase 9 (CDK9) Inhibitor with Suppression of Cancer Stemness Activity against Non-Small-Cell Lung Cancer

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ABSTRACT

A series of novel, highly potent, selective CDK9 inhibitors with cancer stem cells (CSCs) inhibition activity were designed and synthesized for non-small-cell lung cancer (NSCLC) therapy. Structure-activity relationship analysis based on enzymatic and cellular activities led to the discovery of a promising inhibitor **21e**. **21e** potently inhibited CDK9 with IC_{50} value of 11 nM and suppressed the stemness properties of NSCLC effectively. It could decrease the stemness phenotypes of NSCLC cells, including tumor sphere formation, side-population and stemness markers abundance. **21e** displayed good selectivity over the CDK family kinases and kinase profiling assay against 381 kinases. In addition, **21e** inhibited cell proliferation, colony-formation, and cell cycle progression and induced apoptosis in NSCLC. In H1299 xenograft mouse model, a once-daily dose of compound **21e** at 20 mg/kg significantly suppressed the tumor growth without obvious toxicity. Studies of mechanisms of action indicated that **21e** efficiently inhibited CDK9 signaling pathway and stemness both *in vitro* and *in vivo*. Collectively, **21e** as a novel CDK9 inhibitor with CSCs inhibition properties could be a promising agent for the treatment of NSCLC.

Keywords: CDK9, CSCs, Inhibitor, NSCLC

1. Introduction

Lung cancer is one of the most common malignant tumors characterized by high incidence and mortality as well as poor prognosis.[1] More than 85% of lung cancers are identified as non-small-cell lung cancer (NSCLC). Currently, conventional chemotherapy is the systemic therapy and prognosis remains poor. Resistance to chemotherapy, relapse in patients has always remained a challenging issue in NSCLC treatment. This resistance is attributed to a small population of cancer cells known as cancer stem cells (CSC).[2, 3] CSCs are with the key properties of self-renew and potential differentiation, which may play a pivotal role in carcinogenesis, growth, metastasis and drug resistance of NSCLC.[4-6] Traditional therapies, targeting rapidly proliferating cell populations, may kill the bulk of the tumor, yet are inefficient at targeting the CSCs. Elimination of CSCs is essential to improve the treatment of cancer.

Compared with chemotherapy, targeted therapies have the advantage of maximizing efficacy and reducing toxicity. Innovative adaptive study design combining of targeted therapy with CSCs inhibition may be used to expedite effective drug development. Preclinical studies have identified several potential targets, and cyclin-dependent kinase 9 (CDK9) is one of the targets that have recently caused increasing interest.[7-10] Inhibition of CDK9 selectively reduces many proteins up-regulated in many cancers, leading to the inhibition of proliferation and the promotion of apoptosis.[11-15] Recent studies have shown that blocking CDK9

activity provides an appealing therapeutic approach to curb lung cancer metastasis.[16]

CDK9 forms a functional complex with regulatory subunit cyclin T or cyclin K to control transcription progression.[17] This CDK9/cyclin T complex named positive transcription elongation factor b (P-TEFb) is a general transcription factor promoting the RNA synthesis in genetic programmes for cell growth and differentiation.[10, 18] As a component of the P-TEFb, CDK9 phosphorylates the C-terminal domain (CTD) of the largest subunit of the most RNA polymerase II (pol II) to stimulate transcription elongation of most protein coding genes.[19, 20] Phosphorylation of RNA pol II is regarded as a marker in preclinical pharmacodynamic research, which is much more involved in a variety of human pathological conditions, such as cancer.[8, 10] During the past decade, attempts have been made toward the exploitation of CDK9 inhibitor for anticancer potential. CDK9 shows high level of sequence conservation with other CDK family members, thus the design of selective CDK9 inhibitor is challenging. The first CDK9 inhibitor involved in clinical trial was flavopiridol with multiple CDK inhibition. Flavopiridol inhibits CDKs that govern cell cycle progression and phosphorylate the COOH-terminal domain of RNA polymerase II.[12, 21, 22] The antitumor activity of flavopiridol in chronic lymphocytic leukemia appears to be the CDK9-mediated downregulation of transcription of antiapoptotic proteins. While there are also several pan-CDK inhibitors in clinical studies,[23-25] potent and selective CDK9 inhibitors have only recently emerged.[26-28] These studies highlight

the need for the development and validation of CDK9 small molecule inhibitors.

Simultaneous inhibition CDK9 and tumor stem cell could be a promising strategy to enhance the antitumor activity. In an effort to discover CDK9 inhibitors with high potency of CSCs inhibition against NSCLC, we recently performed a rational drug design. In our study, we identified a series of novel compounds targeting CDK9 against human NSCLC. The compound **21e** exhibits excellent inhibition against CDK9 with IC_{50} value of 11 nM and inhibits stem cell properties of NSCLC effectively. The main purpose of this study is to perform a comprehensive evaluation to this compound, including *in vitro* and *in vivo* mechanism of action. Based on these finding, compound **21e** provides a potential treatment for the NSCLC.

2. Results and discussion

2.1. CDK9 inhibitors with CSCs inhibition activity design strategy.

On basis of our recent studies,[29, 30] combining the pyrrolo-[2,3-*d*] pyrimidines-2-amine pharmacophore of ribociclib with other hydrophobic functional group has been successful to enhance the CDK9 inhibition activity obviously. These results support the view that the pyrrolo-[2,3-*d*] pyrimidines-2-amine scaffold might be optimized to yield a useful CDK9 inhibitor. In order to achieve this goal, we have performed docking studies to promote CDK9 specific inhibition. We docked ribociclib in CDK9 and CDK6, as ribociclib is a CDK4/6 inhibitor applied in clinic (**Figure 1A and 1B**). It shows that aminopyrimidine forms two key hydrogen bonds

to the backbone residue VAL101 and CYS106 in the hinge region of CDK6 and CDK9 respectively. By contrast, much bigger hydrophobic pocket formed obviously by HIS108 and GLY112 adjacent to hinge region exits in CDK9. Meanwhile, the two residues were substituted to residue GLN103 and THR107 at the same position in CDK6 (**Figure 1C**). It means that the hydrophobic pocket in CDK9 could accommodate more flexible and bigger fragment resulting in potent selectivity against CDK6, which is consistent to our previous studies. Therefore, decision of the flexible and bigger hydrophobic moiety appears to be particularly important in sustain the scaffold of the aminopyrimidine to play multiple biological function.

Sulforaphane (SFN) is a well-known natural compound within the isothiocyanate group and abrogates tumorigenesis and metastatic progression by targeting CSCs. [31-37] As reported, SFN potentiates the anticancer effects of gemcitabine, doxorubicin, cisplatin, or 5-flurouracil on cancer cell line while also increasing cytotoxicity of CSCs.[38-40] The combination of SFN with bafilomycin A1, as an inhibitor of autophagy, enhances apoptotic effect in breast cancer.[41] Combination therapies that SFN with other therapeutic agents favor a potential treatment modality for cancer, which provide a promising strategy of drug design. [42, 43] Isothiocyanate group with CSCs inhibition function as the pharmacophore of SFN molecule, was chosen as the hydrophobic substituent to improve inhibitory and selectivity against CDK9. [7] Thus, merged molecules, pyrrolo-[2,3-*d*] pyrimidines-2-amine from ribociclib as CDK9 pharmacophore and isothiocyanate from SFN as targeting CSCs

pharmacophore, were designed. The substitute group on the NH at the C2-position of the pyrimidine was replaced by a tail substituent containing isothiocyanate, linker and a substituted aromatic ring (**Figure 1D**). The diversity of the tail substituents was performed to improve the inhibitory activity against CDK9 and CSCs.

2.2. Chemistry.

Structural optimization was focused on the pyrimidine, Ar and linker regions (**Figure 1**). The synthetic routes were carried out as shown in **Scheme 1-3**. The synthesis of the final compounds **9a**~**9e** and **10aa**~**10de** were depicted in **Scheme 1**. Briefly, the commercially obtained **1** or **2a**~**2d** reacted with various mono-Boc-diamine with different carbon lengths (**3a**~**3e**) to obtain the intermediates **4a**~**4e** and **5aa**~**5de** with free aromatic amine, which then reacted with commercially available **6** *via* Buchwald–Hartwig coupling catalyzed by palladium acetate to afford the key intermediates **7a**~**7e** and **8aa**~**8de**. After deprotection by trifluoroacetic acid, final compounds **9a**~**9e** and **10aa**~**10de** were readily prepared within carbon disulfide and *N*,*N*'-dicylohexylcarbodiimide. In **Scheme 2**, aromatic diamine (**11a**~**11c** or **12**) condensed with Boc-NH-$(CH_2)_n$-COOH (**13a**~**13e**) to afford intermediates **14a**~**14g** and **15**. Then final compounds **20**, **21a**~**21g** and **22** were generated through three steps using synthetic strategy in the similar manner of **Scheme 1**. As shown in **Scheme 3**, *p*-nitroaniline analogs containing 2- or 3- substituent (**23a**~**23c**) were treated with Boc anhydride, and then were reduced to give the corresponding primary amine (**24a**~**24c)**. After that, **24a**~**24c** underwent Buchwald–Hartwig coupling reaction and deprotection

to produce **26a**~**26c**. The condensation reaction between the key start material **13e** and amine analogs (including ribociclib, **26a**~**26c** and **27**) yielded intermediates **28**, **29a**~**29c** and **30**. After removal of the *N*-Boc protecting group, the final compounds **31**, **32a**~**32c**, **33**, **34** were prepared with carbon disulfide.

2.3. Enzyme inhibitory activity and SAR.

The results of the enzymatic-inhibition assays are listed in **Table 1-3** and **S1** with ribociclib as the positive control. At first, we introduced isothiocyanate to the para position of the benzene to obtain compound **34** to explore the effect of isothiocyanate group as the hydrophobic moiety on inhibition activity against CDK9. Compared with ribociclib, the activity of **34** against CDK9 was improved significantly. Meanwhile, the activity of **34** against CDK6 was decreased and CDK4 activity was still high. Preliminary design of **34** supports our hypothesis, that hydrophobic substituent in the tail might improve inhibitory activity against CDK9.

Further optimization was proceeding. When Ar is a phenyl with R^1 = H, compounds **9a**~**9e** with linkers containing *N*-alkyl-sulfonamide and **n** ranging from 1 to 5, exhibit more potent CDK9 activity compared to ribociclib (**Table 1**). Due to the decreasing activity against CDK4 and CDK6, Compound **9d** and **9e** (n = 4 or 5) were selected to test the IC_{50} value to further identify CDK9 activity (**Table 3,** IC_{50} = 9 nM), more than 20 times than ribociclib. The preliminary results indicated that extension of the linker might be helpful to promote the specificity of CDK9. When

linkers were varied to *N*-alkyl-amide, the similar results on CDK9 were shown in compound **10aa~10ae** (**Table 1**), and no obvious difference on CDK4 and CDK6 except compound **10ae** (n = 5). Then we changed back the phenyl to pyridine ring (compound **10ba~10be**) to determine the role of the pyridine ring of ribociclib for the activity against CDK9. It turned out that pyridine had decreased the efficiency against CDK9 (**Table 1**), compared to compounds **10aa~10ae**. Modification of fluorine atom to the phenyl ring resulted in analogues **10ca~10ce** (**Table 1**), which retained the potent potency against CDK9 (IC_{50}: 6 nM to 50 nM, **Table 3**), while decreased inhibitory efficiency against CDK4 and CDK6. Hydroxyl at the same position (**10da~10de**, **Table 1**) displays high selective activity on CDK9. Unfortunately, **10da~10de** lost about several times activity against CDK9 compared to the compounds with same length linker, such as **10da** (**Table 3,** IC_{50}: 68 nM, R^1 = OH) vs **10ca** (**Table 3,** IC_{50}: 10 nM, R^1 = F), and especially **10dd** and **10de** show weak activity to CDK9 as similar as ribociclib (IC_{50}: 197 nM). The substituent on the R^1 (F or OH) most likely contributes to the selectivity on CDK9, but F atom retains the high inhibition activity on CDK9 (**10ca~10ce**). Through observed, compound **9d**, **9e**, **10ae**, **10ce**, **10dd**, **10de** (Ar = phenyl, n = 4 or 5) indicate more potent efficacy selective activity on CDK9 than other same series compounds (n = 1~3). Therefore, it was noteworthy that the selectivity on CDK9 might be also correlated with the length of carbon linker and seven carbon atoms (n = 5) was optimal.

Five compounds **21a**~**21e** (**Table 1**) were thus made to further confirm the result for the selectivity on CDK9. The results also showed the compound **21e** (**Table 3,** IC_{50}: 11 nM, n = 5) with 8-isothiocyanatooctanamide linker exhibited better selectivity than compound **21a~21d** (n = 1~4) and it is a potent and selective CDK9 inhibitor. As we designed, a more flexible and bigger group might be required at the position of the linker resulting in potent selectivity against CDK9. Then the position of the linker moving from 4- to 3- or 2- on the phenyl to obtain the compounds **21f** or **21g** (**Table 2**) based on the structure of the compound **21e**. The enzymatic activity of **21f (Table 3,** IC_{50}: 15 nM,**)** was no evident change compared with **21e**, but all the three kinases activity of **21g** were lost, maybe because the bulky linker on the 2-position of the phenyl was not tolerated in the binding site. Addition of the methylene between the amide and phenyl group (**22**) decreased significantly the selectivity on CDK9 (**Table 2**). Directly adding an 8-isothiocyanatooctanamide linker to the ribociclib (**31**) maintained the similar activity in the comparison of ribociclib and lost the selectivity on CDK9. In order to obtain additional SAR information, fluorine atom or trifluoromethyl was introduced to the phenyl on the basis of compound **21e** to create the compound **32a**~**32c** (**Table 2**). Compound **32a** (**Table 3,** IC_{50}: 25 nM) exhibited a similar inhibitory potency and selectivity to that of **10ce** in the enzymatic assays due to their similarity on structure with **10ce**. While changing the position of the fluorine atom to the 3-position, **32b**, showed a decreased inhibitory efficiency and selectivity against CDK9. Further replacement of trifluoromethyl (**32c**)

compared to **32a**, however, resulted in a significant decrease in activity on the three kinases. Besides the linkers mentioned above, we made another compound that the Ar in the linker was excluded (**33**), which lost its activity in the kinase-inhibitory assays. In addition, we tried to change the core structure of pyrrole to triazole (**20**), only showed moderate potency against CDK9 (**Table 2**). So we still focus on the core structure of pyrimidine in this study. In all, we selected some compounds with high potent activity against CDK9 as well as high selectivity for CDK9 to detect the cell inhibition.

2.4. Cellular Assay.

Since our goal here is to discover CDK9 inhibitors with high potency and selectivity for NSCLC treatment, we screened compounds exhibiting better kinase inhibitory to do the preliminary cell inhibition assays. Compounds except **10ba-10be**, **10dd**, **10de**, **20**, **21g**, **31**, **32b**, **32c** and **33** were chosen to test their cellular cytotoxic activity (**Table S2 and S3**). NSCLC cell line A549 and H1299 inhibition profiling assay with fixed concentrations of 10 μM and 1 μM was first carried out. We found that compound **10ae** and **21e** had significant NSCLC cell inhibitory activity. We further detected the selectivity of the two compounds against other cancer cells, including breast cancer cell (MCF7), hepatocarcinoma cell (HepG2) and cervical cancer cell (SiHa) (**Table S4**). The selectivity for NSCLC of **21e** against other cancer cell lines is higher than that of **10ae**. Among these compounds, **21e**, bearing carbon atom at X position and hydrogen atom at R_1 position with linker as

8-isothiocyanatooctanamide (**Table 1**), showed the high potency and selectivity for NSCLC cell line against other cancer cells. Therefore, **21e** was then carried out further in in-depth *in vitro* and *in vivo* anti-NSCLC studies.

The anti-viability activities of **21e** was tested against a panel of 14 tumor cell lines from different origins including NSCLC, breast cancer, hepatocarcinoma, cervical cancer, leukemia and lymphoma using CCK8 assay. Compound ribociclib and SFN were used as positive controls. As denoted in **Table 4**, compound **21e** displayed exceptional potency against NSCLC cell lines, especial A549 and H1299 with IC_{50} values less than 0.5 μM. In the drug-resistant NSCLC cell line H1975, compound **21e** also exhibited good inhibition potency with an IC_{50} value of 0.837 μM. In HCC827 and PC-9 cell lines, compound **21e** displayed higher inhibition potency than two positive controls. In other cell lines, compound **21e** exhibited weak or no inhibition potency. These results demonstrated the good cellular selectivity of compound **21e**. Based on the high inhibitory activity, two typical NSCLC cell lines A549 and H1299 were selected for further comparative studies.

2.5. Kinase Selectivity Profile of Compound 21e.

To assess the kinase inhibitory activity and selectivity, the promising compound **21e** was investigated against a series of 381 kinases by the Eurofins kinases profiling at a single concentration of 1 μM (**Figure 2** and **Table S5**). Compound **21e** showed poor inhibition at the concentration against most of the kinases. A panel of CDKs family

kinases is listed in **Table 5**. Interestingly, our compound **21e** effectively inhibited the activities of CDK9/cyclinT with IC_{50} value of 11 nM over other CDKs (such as IC_{50} of CDK4/cyclinD at 148 nM and CDK6/cyclinD at 145 nM), indicating that compound **21e** is a potent CDK9 inhibitor. While CDK4/6 selective inhibitor ribociclib was detected with IC_{50} values of 13 nM, 71 nM and 197 nM against CDK4, CDK6, and CDK9 respectively. Based on the kinase-activity results, further IC_{50} values of selected kinases with high inhibitory rate over 95% at 1μM were examined and also shown in **Table 5**. IC_{50} values of three human protein kinases were less than 50 nM, all of which are linked with malignant tumor and autoimmune diseases.

2.6. Molecular Modeling of Compound 21e.

Predicted binding modes of compound **21e** in CDK9 (PDB code: 4BCF) and CDK6 (PDB code: 4EZ5) were carried out by Discovery Studio 3.1. Hydrogen atoms were added by Gold (version 5.0). The results of docking were created to image edited by PyMOL.

The compound **21e** is a potent and selective CDK9 inhibitor with IC_{50} value of 11 nM while ribociclib with CDK9 IC_{50} value of 197 nM. For comparison, the binding mode of **21e** was superimposed on that of ribociclib. As shown in **Figure 3A**, compound **21e** binds to the ATP-binding sites of CDK9 in similar orientation to that of ribociclib. In both two binding models, each aminopyrimidine of two compounds forms two key hydrogen bonds to the backbone CYS106 residue. Importantly, an

additional hydrogen bond exists between the isothiocyanate group of the compound **21e** and HIS108 residue. This closer binding may explain the better binding affinity of compound **21e** to CDK9, about 15-fold more than ribociclib.

Since there is no 3D structure of CDK4-ligand complex reported at present, we docked compound **21e** into the CDK6 to attempt to account for the reason of the selectivity of CDK9 among CDKs family. As shown in **Figure 3B**, **21e** adopts a similar binding mode within the CDK9 and CDK6 ATP binding sites and occupy the similar positions. In both CDK9 and CDK6, **21e** hydrogen-bonds with the kinase hinge regions. The 2-aminopyrimidine formed two hydrogen bonds with the CYS106 residue in CDK9 (VAL101 in CDK6). At the back of the ATP binding site the pyrrolo-[2,3-*d*]-pyrimidine group exploits the hydrophobic region close to the gatekeeper residue PHE103 to form a favorable π-π interaction in CDK9. At the front of the ATP binding pocket, the greater flexibility of the ATP-binding site of CDK9 enables the large flexible isothiocyanate of **21e**, to be well accommodated by CDK9. In contrast, the predicted binding mode of **21e** bound to CDK6 shows that the CDK6 binding pocket is too crowded for **21e (Figure S1)**. Therefore, CDK9 has a more flexible ATP binding pocket than CDK6. The differences in the ability of the CDKs to readily adapt inhibitors offer an explanation for the high potency and selectivity of **21e** toward CDK9.

2.7. 21e Significantly Suppressed the Colony-formation Ability of NSCLC Cells.

To complement the results from short-time treatments, long-term colony formation assay was then performed to assess the cytoreductive activity of **21e**. Colony formation assay assesses the effect of a therapy on clonogenic survival which is one of the most important parameter of therapy efficacy in oncology. As shown in **Figure 4A**, **21e** dose-dependently decreased the formation of colonies in all the two NSCLC cell lines, while ribociclib and SFN reduced slightly.

2.8. 21e Induced the Cell Cycle Progress at G2/M Phase of NSCLC Cells.

To understand how **21e** inhibits cell proliferation, cell cycle analysis was performed. The effect of **21e** on cell cycle distribution was assessed in the A549 and H1299 cell lines. The results indicated that **21e** dose-dependently blocked the cell cycle at G2/M phase compared to the vehicle-treated cells (**Figure 4B**). Of note, **21e** at 1 μM was able to increase the proportion of the cells in the G2/M phase drastically from approximate 19.0% (DMSO) to 34.4%, and decreased the corresponding fraction of the cells in the S phase from about 24.5% (DMSO) to 11.2% and basically unchanged in the G0/G1 phase in H1299 (**Figure 4B**). Although the G2/M period was only slightly increased in A549, it is consistent with the results of H1299. Therefore, our results suggest that **21e** inhibits cell proliferation by arresting cells in G2/M phase of the cell cycle.

2.9. 21e Induced Caspase-dependent Apoptotic Cell Death of NSCLC Cells.

To determine whether **21e** induces apoptosis cell death, we performed Annexin V-FITV/PI staining. As shown in **Figure 4C**, **21e** treatment for 48 h induced apoptosis of A549 cells in a concentration-dependent manner. **21e** at 0.25 to 1 μM increased the apoptotic (Q2 late apoptotic + Q3 early apoptotic) cells by approximately 1.4-1.6-fold, compared with the control. Similar results were observed in H1299, with about 2.2-3.4-fold increase.

Bcl2-family members, including anti-apoptotic and pro-apoptotic proteins, are key players in the regulation of apoptosis.[44] To determine how **21e** induces apoptosis, we next examined whether **21e** could alter the expression of anti-apoptotic (Bcl2) and pro-apoptotic (Bim) proteins in the cells. As shown in **Figure 4D**, **21e** treatment with different concentration for 24 h markedly downregulated the expression levels of Bcl2 and meanwhile upregulated the Bim level. Furthermore, we observed that **21e** increased the cleavage of caspase 3 (**Figure 4D**), indicating activation of caspase 3. Therefore, our results indicated that **21e** induced caspase-dependent apoptotic cell death by upregulating the anti-apoptotic protein Bcl2 and downregulating the pro-apoptotic protein Bim in NSCLC cells.

2.10. 21e Suppressed the CDK9 Downstream Signaling Proteins of NSCLC cells.

CDK9 and cyclin T form the P-TEFb complex that was identified as an activity controlling the early phase of transcription elongation *via* release of RNAPII from inherent promoter-proximal pause sites.[45] CDK9 also phosphorylates the

carboxyterminal heptarepeat (consensus sequence $Y_1S_2P_3T_4S_5P_6S_7$) within CTD of the largest subunit of RNAPII. RNAPII is recruited into the preinitiation complex with a hypophosphorylated CTD, and the CTD is phosphorylated on Ser5-P during initiation and then on Ser2-P during elongation.[46] The latter work is performed by CDK9.[47] The level of phosphorylation at Ser2-P increases towards the 3'-ends of genes, which correlates to binding of termination and RNA processing factors to the CTD.[48] Then Ser2-P at promoter-proximal sites further helps to release RNAPII from initiation and early elongation complexes.[49]

Thus, the effects of CDK9 inhibition by **21e** in A549 and H1299 cells were investigated. Firstly, we performed Western blot assay to evaluate the protein levels of CDK9 inhibition (**Figure 5**). The abundance of CDK9 enzymes itself was not affected by compound **21e**. **21e** slightly suppressed the expression of cyclin T1 in the two cell lines. To evaluate whether the antiproliferative activity of compound **21e** is caused by inhibition of cellular CDK9, we selected inhibition of Rb phosphorylation at the CDK-specific sites Ser 807/811 and Ser 780. The results showed that compound **21e** effectively suppressed phosphorylation of Rb Ser 807/811 and Ser 780 at 0.25 μM, which confirmed that CDK9 is a target of **21e** (**Figure 5**). Furthermore, one established cellular target of CDK9 is Ser2 within the RNA polymerase CTD which becomes phosphorylated (Ser2-P) during transcription elongation. After treated with **21e**, both Ser2-P and Ser5-P were dramatically inhibited compared with DMSO

treatment (**Figure 5**). Both analyses suggest that CDK9 is the main driver for Ser2-P and cell proliferation inhibition.

2.11. 21e Downregulated the Stem Cell Properties of NSCLC Cells.

There are mainly three methods for the identification of CSCs or CSC-like properties: (1) identifying the side population (SP) in cancer cells, which enriches CSC-like properties; (2) determining the growth properties of cells in serum-free suspension culture;[50] (3) using of CSCs surface markers, such as $CD44^{+}CD24^{-}$, CD133, $CD44^{+}/EpCAM^{+}$.[51, 52] Herein, in A549 and H1299 cells, we validated that **21e** could decrease the sphere formation and the ratios of SP in a dose-dependent manner. In sphere formation assay, **21e** decreased the number of spheres formed by A549 and H1299 cells, in comparison with the respective control, while SFN did not present inhibition at the concentration of 1μM (**Figure 6A**). Consistent with these observations, a side population that shows a higher efflux of DNA-binding dye Hoechst 33342, as determined by flow cytometry, which is enriched with hematopoietic stem cells (HSCs).[53] To some extent, the tumor cells with high side population characteristics represents cancer stem cell.[54] We found that **21e** reduced the percentage of the side population in A549 and H1299 cells, as compared to the cells treated with DMSO (**Figure 6B**). Furthermore, overexpression of Oct4 (Octamer-binding transcription factor 4), SOX2 (SRY (sex determining region Y)-box 2), Nanog, Klf4 (Kruppel-like factor 4), can induce somatic cells to acquire pluripotency.[55] These proteins also serve as the CSC markers.[56, 57] We next

investigated the effect of **21e** on these CSC markers. Western blot analysis confirmed that the expression level of stemness markers, including SOX2, Oct4, Nanog and Klf4, was decreased in A549 and H1299 cells (**Figure 6C**). These results demonstrated that **21e** suppressed the stem cell-like properties of NSCLC cells.

2.12. Pharmacokinetics of Compound 21e in Liver Microsomes of Multiple Species.

The metabolic stability study of compound **21e** was assessed using human liver microsomes (HLM), dog liver microsomes (DLM), mouse liver microsomes (MLM), rat liver microsomes (RLM, **Table 6**). The terminal half-lives ($t_{1/2}$) were moderate in these four kinds of microsomes (ranging from 23.1 min to 40.8 min). In particularly, compound **21e** showed more stable in rat liver microsomes than others (Cl_{int} = 0.057). The moderate pharmacokinetics in liver microsomes of compound **21e** were considered acceptable level for a drug development perspective.

2.13. *In Vivo* Anti-tumor Activity and Mechanism of Action of 21e.

To evaluate the anti-tumor activity of **21e** *in vivo*, H1299 xenograft model was used. In this model, H1299 cells were injected subcutaneously into abdomen of each nude mouse. The mice were randomized into five groups (5 mice per group) when the tumors grew to a size of ~ 120mm^3. Next, the animals were orally given three doses (20, 40, and 80 mg Kg^{-1}) of **21e**, one dose (80 mg Kg^{-1}) of ribociclib (positive control), or vehicle (control) every day. As shown in **Figure 7A**, the growth of xenograft

tumors was significantly inhibited by **21e** in a dose-dependent manner. Tumor growth inhibitions of 62.0%, 89.7%, 94.7% were observed in the H1299 model at doses of 20, 40, and 80 mg Kg^{-1}, respectively. In contrast, ribociclib (80 mg/kg) as the positive control was less potent (59.5%) than **21e** at the same dose. Of note, no obvious toxicity was observed in all the treated groups (**Figure 7B**). In **Figure 7C**, an obvious decrease in tumor size was also observed at the end of observation. The average tumor weight (**Figure 7D**) of the **21e**-treated group (80 mg Kg^{-1}) was 0.18 g which was less than that of ribociclib-treated group (0.49 g) and control group (2.17 g).

To elucidate the mechanism of anti-tumor action of **21e** *in vivo*, immunohistochemical analysis was conducted using H1299 tumor samples collected from mice treated with vehicle, **21e** and ribociclib, respectively. As shown in **Figure 7E**, **21e** remarkably inhibited the phosphorylation of Rb (Ser 780) and RNAPII CTD (Ser 5), and led to almost unchanged CDK9 and cyclin T1 compared with the vehicle, consistent with *in vitro* results. Moreover, **21e** reduced the expression of stemness marker Oct4. Taken together, our results indicated that **21e** suppressed H1299 xenograft growth in mice by inhibiting CDK9 signaling and tumor CSCs, leading to inhibition of cell proliferation and the stem cell properties.

3. CONCLUSION

In this study, a series of new CDK9 inhibitors bearing isothiocyanate were designed and synthesized. SAR study of these compounds was discussed based on

enzymatic and cellular activities. In cellular assays, compound **21e** exhibited considerable anti-viability potencies against human NSCLC cell lines and good selectivity against other cancer cells. It could significantly decrease the number of colonies, induced NSCLC cell apoptosis, and arrested the cell cycle in the G2 phase. Meanwhile, **21e** significantly decreased cancer stem-like cells fraction, tumor sphere formation and stemness markers expression of NSCLC cells. In H1299 xenograft mouse models, **21e** displayed potent anti-tumor activities. Immunohistochemistry of tumor tissues showed that **21e** efficiently inhibited CDK9 signaling and CSCs marker. This work offer a new potential approach based on single target CDK9 to inhibit stem cell for treatment of NSCLC.

4. EXPERIMENTAL SECTION

4.1. General Methods for Chemistry.

The commercially obtained chemicals were used directly without further purification. Solvents were purified and distilled following the standard procedures. All the reactions were monitored by thin-layer chromatography (TLC). The NMR spectra were taken on a Bruker AV-400 MHz spectrometer (400 MHz for ^{1}H and 101 MHz for ^{13}C) and chemical shifts were expressed in ppm downfield using tetramethylsilane as the internal standard. High-resolution mass spectra (HRMS) were performed on a VG ZAB-HS mass spectrometer under eluectron spray ionization (ESI). All the derivatives for testing bioactivity were purified to >95% purity which

was determined by HPLC analysis on a Shimadzu Prominence-i LC-2030C 3D system (column, InertSustain C18, 4.6 mm × 250 mm, 5 μM; mobile phase, gradient elution of methanol/H_2O (90:10); low rate, 1.0 mL/min; UV wavelength, 190−800 nm; temperapture, 40 °C; injection volume, 10 μL). Melting point were tested on the Mettler Toledo MP50 Melting Point System that can present results with a decimal precision.

4.1.1.

7-Cyclopentyl-2-((4-(N-(3-isothiocyanatopropyl)sulfamoyl)phenyl)amino)-N,N-dimeth yl-7H-pyrrolo[2,3-d]pyrimidine-6-carboxamide ***(9a).*** To a stirred solution of **7a** (382 mg, 0.65mmol) in DCM, trifluoracetic acid (3 mL) was added dropwise in ice-water bath, then stirred 1 h at RT. After removing solvent in vacuo, the residue was dissolved in n-butanol (10 mL) and washed with saturated Na_2CO_3 aquous solution (10 mL). The organic layer was concentrated under reduced pressure to use directly without purification. After dissolving the resulting light-yellow oil in THF (5 mL), carbon disulphide (800 μL, 20 equiv), DCC (148 mg, 0.72 mmol) were added in successively, stirred overnight at RT. The solvent was concentrated in vacuo and crude product was purified by silica gel column chromatography to give title compound **9a** in 57% yield as light-yellow solid; mp 176.3 °C. IR (film): 3234.3, 3151.2, 3122.5, 3032.9, 2995.9, 2950.2, 2873.1, 2194.4, 2163.2, 2067.2, 1604.7, 1588.8, 1567.7, 1546.2, 1527.4, 1483.7, 1428.6, 1401.7, 1353.8, 1328.1, 1244.5, 1176.4, 1155.8, 1138.5, 1091.3, 1027.4 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 8.68 (s, 1H, 4-H), 8.06 (s,

1H, NH), 7.89 (d, *J* = 8.8 Hz, 2H, 3', 5'-H), 7.80 (d, *J* = 8.8 Hz, 2H, 2', 6'-H), 6.47 (s, 1H, 5-H), 5.44 (t, *J* = 6.3 Hz, 1H, NH), 4.79 (m, 1H, CH), 3.62 (t, *J* = 6.3 Hz, 2H, CH_2), 3.15 (s, 6H, $2CH_3$), 3.09 (q, *J* = 6.4 Hz, 2H, CH_2), 2.61 – 2.52 (m, 2H, CH_2), 2.11 – 2.00 (m, 4H, $2CH_2$), 1.88 (p, *J* = 6.5 Hz, 2H, CH_2), 1.71 (q, *J* = 6.1 Hz, 2H, CH_2); ^{13}C NMR (101 MHz, $CDCl_3$) δ 163.9 (1C, Ar-C), 154.4 (1C, C=O), 151.7 (1C, Ar-C), 151.5 (1C, Ar-C), 144.6 (1C, Ar-C), 133.0 (1C, Ar-C), 131.9 (1C, Ar-C), 131.0 (1C, S=C=N), 128.5 (2C, Ar-C), 117.8 (2C, Ar-C), 113.3 (1C, Ar-C), 101.0 (1C, Ar-C), 58.2 (1C, CH), 42.3 (1C, CH_2), 40.1 (1C, CH_2), 39.5 (2C, $2CH_3$), 35.3 (1C, CH_2), 30.3 (2C, $2CH_2$), 24.8 (2C, $2CH_2$). HRMS (ESI, m/z) calcd for $C_{24}H_{30}N_7O_3S_2^+$ [M + H]$^+$, 528.1846, found 528.1851. HPLC purity 97% (t_R = 3.68 min).

4.1.2.

7-Cyclopentyl-2-((4-(N-(4-isothiocyanatobutyl)sulfamoyl)phenyl)amino)-N,N-dimethyl-7H-pyrrolo[2,3-d]pyrimidine-6-carboxamide ***(9b).*** Compound **9b** was prepared using **7b** instead of **7a** in the similar manner of compound **9a** to afford the title product as light-yellow solid in 65% yield; mp 185.9 °C. IR (film): 3354.2, 3260.1, 3108.5, 2948.4, 2867.7, 2180.7, 2095.4, 2065.7, 1908.1, 1732.9, 1626.3, 1605.9, 1587.2, 1563.6, 1541.9, 1521.2, 1485.8, 1428.1, 1396.5, 1358.9, 1333.3, 1290.0, 1248.4, 1186.4, 1158.6, 1135.8, 1090.7, 1074.3, 1025.1 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.67 (s, 1H, 4-H), 8.06 (s, 1H, NH), 7.89 (d, *J* = 8.5 Hz, 2H, 3', 5'-H), 7.80 (d, *J* = 8.5 Hz, 2H, 2', 6'-H), 6.47 (s, 1H, 5-H), 5.28 (t, *J* = 6.3 Hz, 1H, NH), 4.78 (m, 1H, CH), 3.49 (t, *J* = 6.3 Hz, 2H, CH_2), 3.15 (s, 6H, $2CH_3$), 3.00 (q, *J* = 6.5 Hz, 2H),

2.57 (dd, J = 13.0, 7.4 Hz, 2H, CH_2), 2.05 (dd, J = 19.4, 8.0 Hz, 4H, $2CH_2$), 1.71 (d, J = 8.3 Hz, 4H, $2CH_2$), 1.65 – 1.57 (m, 2H, CH_2); ^{13}C NMR (101 MHz, $CDCl_3$) δ 163.9 (1C, Ar-C), 154.5 (1C, C=O), 151.7 (1C, Ar-C), 151.5 (1C, Ar-C), 144.6 (1C, Ar-C), 133.0 (1C, Ar-C), 132.1 (1C, S=C=N), 131.2 (1C, Ar-C), 128.4 (2C, Ar-C), 117.8 (2C, Ar-C), 113.3 (1C, Ar-C), 101.0 (1C, Ar-C), 58.2 (1C, CH), 44.7 (1C, CH_2), 42.4 (1C, CH_2), 39.5 (2C, $2CH_3$), 30.3 (2C, $2CH_2$), 27.1 (1C, CH_2), 26.8 (1C, CH_2), 24.8 (2C, $2CH_2$). HRMS (ESI, m/z) calcd for $C_{25}H_{32}N_7O_3S_2^+$ $[M + H]^+$, 542.2003, found 542.2007. HPLC purity 99% (t_R = 3.79 min).

4.1.3.

7-Cyclopentyl-2-((4-(N-(5-isothiocyanatopentyl)sulfamoyl)phenyl)amino)-N,N-dimethyl-7H-pyrrolo[2,3-d]pyrimidine-6-carboxamide ***(9c).*** Compound **9c** was prepared using **7c** instead of **7a** in the similar manner of compound **9a** to afford the title product as light-yellow solid in 63% yield; mp 77.7 °C. IR (film): 3270.8, 3182.4, 3109.0, 2941.6, 2866.6, 2178.3, 2099.6, 2000.1, 1624.1, 1606.4, 1587.1, 1563.3, 1519.9, 1486.8, 1427.4, 1398.2, 1355.7, 1322.5, 1248.0, 1138.6, 1092.0, 1024.2 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.67 (s, 1H, 4-H), 8.04 (s, 1H, NH), 7.89 (d, J = 8.7 Hz, 2H, 3', 5'-H), 7.81 (d, J = 8.7 Hz, 2H, 2', 6'-H), 6.47 (s, 1H, 5-H), 5.12 (t, J = 6.2 Hz, 1H, NH), 4.77 (m, 1H, CH), 3.45 (t, J = 6.5 Hz, 2H, CH_2), 3.15 (s, 6H, $2CH_3$), 2.97 (q, J = 6.7 Hz, 2H, CH_2), 2.63 – 2.52 (m, 2H, CH_2), 2.11 – 2.00 (m, 4H, $2CH_2$), 1.70 (d, J = 6.3 Hz, 2H, CH_2), 1.66 – 1.59 (m, 2H, CH_2), 1.51 (q, J = 7.2 Hz, 2H, CH_2), 1.40 (dq, J = 8.9, 3.6 Hz, 2H, CH_2); ^{13}C NMR (101 MHz, $CDCl_3$) δ 163.9 (1C, Ar-C), 154.5

(1C, C=O), 151.7 (1C, Ar-C), 151.5 (1C, Ar-C), 144.5 (1C, Ar-C), 133.0 (1C, Ar-C), 131.4 (1C, Ar-C), 130.0 (1C, S=C=N), 128.4 (2C, Ar-C), 117.7 (2C, Ar-C), 113.3 (1C, Ar-C), 101.0 (1C, Ar-C), 58.2 (1C, CH), 45.0 (1C, CH_2), 42.9 (1C, CH_2), 39.5 (2C, $2CH_3$), 30.3 (2C, $2CH_2$), 29.5 (1C, CH_2), 29.0 (1C, CH_2), 24.8 (2C, $2CH_2$), 23.7 (1C, CH_2). HRMS (ESI, m/z) calcd for $C_{26}H_{34}N_7O_3S_2^+$ $[M + H]^+$, 556.2159, found 556.2155. HPLC purity 98% (t_R = 3.93 min).

4.1.4.

7-Cyclopentyl-2-((4-(N-(6-isothiocyanatohexyl)sulfamoyl)phenyl)amino)-N,N-dimethyl-7H-pyrrolo[2,3-d]pyrimidine-6-carboxamide ***(9d).*** Compound **9d** was prepared using **7d** instead of **7a** in the similar manner of compound **9a** to afford the title product as light-yellow solid in 82% yield; mp 169.5 °C. IR (film): 3627.0, 3486.6, 3234.0, 3170.1, 3081.1, 3033.1, 2995.6, 2936.7, 2860.3, 2177.2, 2092.5, 1607.3, 1591.7, 1569.3, 1556.1, 1530.7, 1484.4, 1427.4, 1402.1, 1356.5, 1328.3, 1244.0, 1158.5, 1138.9, 1093.1, 1028.6 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 8.67 (s, 1H, 4-H), 8.00 (s, 1H, NH), 7.89 (d, *J* = 8.7 Hz, 2H, 3', 5'-H), 7.80 (d, *J* = 8.6 Hz, 2H, 2', 6'-H), 6.46 (s, 1H, 5-H), 5.02 (t, *J* = 6.3 Hz, 1H, NH), 4.79 (m, 1H, CH), 3.45 (t, *J* = 6.5 Hz, 2H, CH_2), 3.15 (s, 6H, $2CH_3$), 2.95 (q, *J* = 6.7 Hz, 2H, CH_2), 2.62 – 2.52 (m, 2H, CH_2), 2.13 – 2.01 (m, 4H, $2CH_2$), 1.71 (p, *J* = 6.6, 6.1 Hz, 2H, CH_2), 1.62 (t, *J* = 7.0 Hz, 2H, CH_2), 1.52 – 1.45 (m, 2H, CH_2), 1.32 (dt, *J* = 9.3, 5.5 Hz, 4H, $2CH_2$); ^{13}C NMR (101 MHz, $CDCl_3$) δ 164.0 (1C, Ar-C), 154.5 (1C, C=O), 151.8 (1C, Ar-C), 151.5 (1C, Ar-C), 144.4 (1C, Ar-C), 133.0 (1C, Ar-C), 131.5 (1C, Ar-C), 129.9 (1C,

S=C=N), 128.4 (2C, Ar-C), 117.7 (2C, Ar-C), 113.3 (1C, Ar-C), 101.0 (1C, Ar-C), 58.2 (1C, CH), 45.0 (1C, CH_2), 43.1 (2C, $2CH_3$), 34.0 (1C, CH_2), 30.3 (2C, $2CH_2$), 29.8 (1C, CH_2), 29.5 (1C, CH_2), 26.2 (1C, CH_2), 25.9 (1C, CH_2), 24.8 (2C, $2CH_2$). HRMS (ESI, m/z) calcd for $C_{27}H_{36}N_7O_3S_2^+$ $[M + H]^+$, 570.2316, found 570.2314. HPLC purity 99% (t_R = 4.11 min).

4.1.5.

7-Cyclopentyl-2-((4-(N-(7-isothiocyanatoheptyl)sulfamoyl)phenyl)amino)-N,N-dimethyl-7H-pyrrolo[2,3-d]pyrimidine-6-carboxamide ***(9e).*** Compound **9e** was prepared using **7e** instead of **7a** in the similar manner of compound **9a** to afford the title product as light-yellow solid in 89% yield; mp 186.1 °C. IR (film): 3378.7, 3136.3, 3085.7, 2941.2, 2908.7, 2862.0, 2176.7, 2139.2, 2114.1, 1735.7, 1625.1, 1603.6, 1583.4, 1561.5, 1546.4, 1506.9, 1477.8, 1462.6, 1420.3, 1400.5, 1344.8, 1312.1, 1271.1, 1247.3, 1181.5, 1151.0, 1138.6, 1112.0, 1091.6, 1079.2, 1059.3 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 8.68 (s, 1H, 4-H), 7.95 (s, 1H, NH), 7.89 (d, *J* = 8.6 Hz, 2H, 3', 5'-H), 7.81 (d, *J* = 8.5 Hz, 2H, 2', 6'-H), 6.46 (s, 1H, 5-H), 4.90 (t, *J* = 6.2 Hz, 1H, NH), 4.77 (m, 1H, CH), 3.44 (t, *J* = 6.6 Hz, 2H, CH_2), 3.15 (s, 6H, $2CH_3$), 2.95 (q, *J* = 6.8 Hz, 2H, CH_2), 2.58 (qd, *J* = 9.1, 6.3, 4.0 Hz, 2H, CH_2), 2.12 – 2.03 (m, 4H, $2CH_2$), 1.71 (q, *J* = 6.1 Hz, 2H, CH_2), 1.63 (d, *J* = 7.2 Hz, 2H, CH_2), 1.47 (t, *J* = 7.1 Hz, 2H, CH_2), 1.35 – 1.24 (m, 6H, $3CH_2$); ^{13}C NMR (101 MHz, $CDCl_3$) δ 163.9 (1C, Ar-C), 154.6 (1C, C=O), 151.8 (1C, Ar-C), 151.5 (1C, Ar-C), 144.4 (1C, Ar-C), 133.0 (1C, Ar-C), 131.6 (1C, Ar-C), 129.8 (1C, S=C=N), 128.5 (2C, Ar-C), 117.7 (2C,

Ar-C), 113.3 (1C, Ar-C), 101.0 (1C, Ar-C), 58.2 (1C, CH), 45.1 (1C, CH_2), 43.2 (1C, CH_2), 39.5 (2C, $2CH_3$), 30.3 (2C, $2CH_2$), 29.9 (1C, CH_2), 29.6 (1C, CH_2), 28.4 (1C, CH_2), 26.5 (1C, CH_2), 26.4 (1C, CH_2), 24.8 (2C, $2CH_2$). HRMS (ESI, m/z) calcd for $C_{28}H_{38}N_7O_3S_2^+$ $[M + H]^+$, 584.2472, found 584.2467. HPLC purity 98% (t_R = 4.41 min).

4.1.6.

7-Cyclopentyl-2-((4-((3-isothiocyanatopropyl)carbamoyl)phenyl)amino)-N,N-dimethyl-7H-pyrrolo[2,3-d]pyrimidine-6-carboxamide ***(10aa).*** Compound **10aa** was prepared using **8aa** instead of **7a** in the similar manner of compound **9a** to afford the title product as white solid in 33% yield; mp 205.6 °C. IR (film): 3355.2, 3303.7, 13180.6, 3107.8, 13068.2, 6042.1, 3015.4, 2950.4, 2922.6, 12871.7, 2177.7, 2108.5, 2082.7, 1731.0, 1623.8, 1611.5, 589.6, 1549.2, 1519.7, 1502.8, 1483.8, 1428.2, 1403.6, 1337.0, 1301.8, 1285.0, 1246.1, 1186.8, 1159.1, 1139.5, 1045.2, 1024.6 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6) δ 9.85 (s, 1H, NH), 8.79 (s, 1H, 4-H), 8.41 (t, *J* = 5.6 Hz, 1H, NH), 7.92 (d, *J* = 8.4 Hz, 2H, 3', 5'-H), 7.82 (d, *J* = 8.5 Hz, 2H, 2', 6'-H), 6.61 (s, 1H, 5-H), 4.76 (p, *J* = 8.9 Hz, 1H, CH), 3.74 (t, *J* = 6.4 Hz, 2H, CH_2), 3.35 (q, *J* = 6.3 Hz, 2H, CH_2), 3.06 (s, 6H, $2CH_3$), 2.49 – 2.43 (m, 2H, CH_2), 2.10 – 1.98 (m, 4H, $2CH_2$), 1.89 (p, *J* = 6.6 Hz, 2H, CH_2), 1.69 (q, *J* = 6.0 Hz, 2H, CH_2); ^{13}C NMR (101 MHz, DMSO-d_6) δ 166.0 (1C, C=O), 162.8 (1C, Ar-C), 155.0 (1C, C=O), 152.1 (1C, Ar-C), 151.0 (1C, Ar-C), 143.7 (1C, Ar-C), 132.1 (1C, Ar-C), 127.9 (2C, Ar-C), 127.2 (1C, S=C=N), 126.1 (1C, Ar-C), 116.8 (2C, Ar-C), 112.0 (1C, Ar-C), 100.6 (1C, Ar-C),

57.0 (1C, CH), 42.8 (1C, CH_2), 39.4 (2C, $2CH_3$), 36.3 (1C, CH_2), 34.6 (1C, CH_2), 29.6 (2C, $2CH_2$), 24.2 (2C, $2CH_2$). HRMS (ESI, m/z) calcd for $C_{25}H_{30}N_7O_2S^+$ $[M + H]^+$, 492.2176, found 492.2180. HPLC purity 99% (t_R = 6.52 min).

4.1.7.

7-Cyclopentyl-2-((4-((4-isothiocyanatobutyl)carbamoyl)phenyl)amino)-N,N-dimethyl-7H-pyrrolo[2,3-d]pyrimidine-6-carboxamide ***(10ab).*** Compound **10ab** was prepared using **8ab** instead of **7a** in the similar manner of compound **9a** to afford the title product as white solid in 80% yield; mp 182.1 °C. IR (film): 3328.6, 3279.8, 3181.1, 3106.3, 3014.0, 2943.6, 2869.3, 2187.1, 2084.8, 1623.2, 1609.6, 1588.0, 1543.3, 1517.2, 1426.3, 1403.1, 1357.6, 1301.0, 1286.5, 1251.8, 1185.0, 1125.0, 1023.1 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6) δ 9.85 (s, 1H, NH), 8.79 (s, 1H, 4-H), 8.36 (t, *J* = 5.7 Hz, 1H, NH), 7.95 – 7.88 (m, 2H, 3', 5'-H), 7.81 (d, *J* = 8.9 Hz, 2H, 2', 6'-H), 6.61 (s, 1H, 5-H), 4.76 (p, *J* = 8.9 Hz, 1H, CH), 3.71 (t, *J* = 6.3 Hz, 2H, CH_2), 3.28 (t, *J* = 6.2 Hz, 2H, CH_2), 3.05 (s, 6H, $2CH_3$), 2.47 (m, 2H, CH_2), 2.05 – 1.96 (m, 4H, $2CH_2$), 1.73 – 1.58 (m, 6H, $3CH_2$); ^{13}C NMR (101 MHz, DMSO-d_6) δ 165.8 (1C, C=O), 162.8 (1C, Ar-C), 155.0 (1C, C=O), 152.1 (1C, Ar-C), 151.0 (1C, Ar-C), 143.6 (1C, Ar-C), 132.1 (1C, Ar-C), 127.8 (2C, Ar-C), 127.1 (1C, S=C=N), 126.3 (1C, Ar-C), 116.9 (2C, Ar-C), 112.0 (1C, Ar-C), 100.6 (1C, Ar-C), 57.0 (1C, CH), 44.5 (1C, CH_2), 38.2(2C, $2CH_3$), 34.6 (1C, CH_2), 29.6 (2C, $2CH_2$), 26.9 (1C, CH_2), 26.5 (1C, CH_2), 24.2 (2C, $2CH_2$). HRMS (ESI, m/z) calcd for $C_{26}H_{32}N_7O_2S^+$ $[M + H]^+$, 506.2333, found 506.2335. HPLC purity 99% (t_R = 6.60 min).

4.1.8.

7-Cyclopentyl-2-((4-((5-isothiocyanatopentyl)carbamoyl)phenyl)amino)-N,N-dimethyl -7H-pyrrolo[2,3-d]pyrimidine-6-carboxamide ***(10ac).*** Compound **10ac** was prepared using **8ac** instead of **7a** in the similar manner of compound **9a** to afford the title product as white solid in 80% yield; mp 177.7 °C. IR (film): 3314.3, 3184.0, 3108.1, 3066.5, 3013.8, 2940.2, 2869.6, 2185.2, 2090.1, 1623.1, 1611.0, 1588.6, 1549.8, 1483.8, 1425.6, 1403.0, 1342.3, 1300.5, 1128.8, 1248.9, 1140.3, 1021.2 cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.82 (s, 1H, NH), 8.79 (s, 1H, 4-H), 8.31 (t, *J* = 5.8 Hz, 1H, NH), 7.91 (d, *J* = 8.5 Hz, 2H, 3', 5'-H), 7.81 (d, *J* = 8.5 Hz, 2H, 2', 6'-H), 6.61 (s, 1H, 5-H), 4.77 (q, *J* = 8.9 Hz, 1H, CH), 3.67 (t, *J* = 6.5 Hz, 2H, CH_2), 3.27 (t, *J* = 6.5 Hz, 2H, CH_2), 3.05 (s, 6H, $2CH_3$), 2.49 – 2.43 (m, 2H, CH_2), 2.01 (m, 4H, $2CH_2$), 1.68 (q, *J* = 7.6, 7.2 Hz, 4H, $2CH_2$), 1.58 – 1.51 (m, 2H, CH_2), 1.39 (t, *J* = 7.4 Hz, 2H, CH_2); ^{13}C NMR (101 MHz, DMSO-d_6) δ 165.8 (1C, C=O), 162.8 (1C, Ar-C), 155.0 (1C, C=O), 152.1 (1C, Ar-C), 151.0 (1C, Ar-C), 143.6 (1C, Ar-C), 132.0 (1C, Ar-C), 127.8 (2C, Ar-C), 127.2 (1C, S=C=N), 126.5 (1C, Ar-C), 116.9 (2C, Ar-C), 111.9 (1C, Ar-C), 100.6 (1C, Ar-C), 57.0 (1C, CH), 44.7 (1C, CH_2), 38.8(2C, $2CH_3$), 34.6 (1C, CH_2), 29.6 (2C, $2CH_2$), 29.0 (1C, CH_2), 28.5 (1C, CH_2), 24.2 (1C, CH_2), 23.5 (2C, $2CH_2$). HRMS (ESI, m/z) calcd for $C_{27}H_{34}N_7O_2S^+$ $[M + H]^+$, 520.2489, found 520.2492. HPLC purity 99% (t_R = 6.58 min).

4.1.9.

7-Cyclopentyl-2-((4-((6-isothiocyanatohexyl)carbamoyl)phenyl)amino)-N,N-dimethyl-

7H-pyrrolo[2,3-d]pyrimidine-6-carboxamide ***(10ad).*** Compound **10ad** was prepared using **8ad** instead of **7a** in the similar manner of compound **9a** to afford the title product as white solid in 40% yield; mp 186.7 °C. IR (film): 3322.9, 3280.0, 3188.4, 3107.6, 3013.8, 2932.1, 2857.6, 2183.2, 2109.0, 2087.5, 1625.3, 1611.6, 1588.6, 1549.4, 1520.0, 1486.8, 1426.7, 1404.0, 1364.0, 1301.4, 1288.7, 1253.8, 1186.1, 1141.9, 1088.0, 1021.1 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6) δ 9.82 (s, 1H, NH), 8.79 (s, 1H, 4-H), 8.28 (t, *J* = 5.8 Hz, 1H, NH), 7.91 (d, *J* = 8.5 Hz, 2H, 3', 5'-H), 7.80 (d, *J* = 8.5 Hz, 2H, 2', 6'-H), 6.61 (s, 1H, 5-H), 4.74 (q, *J* = 8.9 Hz, 1H, CH), 3.66 (t, *J* = 6.5 Hz, 2H, CH_2), 3.25 (q, *J* = 6.6 Hz, 2H, CH_2), 3.06 (s, 6H, $2CH_3$), 2.49 – 2.43 (m, 2H, CH_2), 2.01 (m, 4H, $2CH_2$), 1.67 (dt, *J* = 20.9, 6.9 Hz, 4H, $2CH_2$), 1.52 (q, *J* = 7.0 Hz, 2H, CH_2), 1.36 (dp, *J* = 11.4, 6.1, 5.5 Hz, 4H, $2CH_2$); ^{13}C NMR (101 MHz, DMSO-d_6) δ 165.7 (1C, C=O), 162.8 (1C, Ar-C), 155.0 (1C, C=O), 152.1 (1C, Ar-C), 151.0 (1C, Ar-C), 143.5 (1C, Ar-C), 132.0 (1C, Ar-C), 127.8 (2C, Ar-C), 127.2 (1C, S=C=N), 126.5 (1C, Ar-C), 116.9 (2C, Ar-C), 111.9 (1C, Ar-C), 100.6 (1C, Ar-C), 57.0 (1C, CH), 44.7 (1C, CH_2), 39.0(2C, $2CH_3$), 34.6 (1C, CH_2), 29.6 (2C, $2CH_2$), 29.2 (1C, CH_2), 29.1 (1C, CH_2), 25.8 (1C, CH_2), 25.7 (1C, CH_2), 24.2 (2C, $2CH_2$). HRMS (ESI, m/z) calcd for $C_{28}H_{36}N_7O_2S^+$ $[M + H]^+$, 534.2646, found 534.2651. HPLC purity 99% (t_R = 6.71 min).

4.1.10.

7-Cyclopentyl-2-((4-((7-isothiocyanatoheptyl)carbamoyl)phenyl)amino)-N,N-dimethyl -7H-pyrrolo[2,3-d]pyrimidine-6-carboxamide ***(10ae).*** Compound **10ae** was prepared

using **8ae** instead of **7a** in the similar manner of compound **9a** to afford the title product as white solid in 68% yield; mp 160.1 °C. IR (film): 3331.3, 3305.5, 3183.0, 3106.9, 3066.2, 3013.8, 2934.7, 2857.1, 2179.3, 2088.9, 1623.2, 1610.9, 1588.2, 1546.5, 1517.8, 1486.4, 1426.5, 1403.1, 1360.0, 1301.3, 1249.0, 1185.9, 1141.6, 1086.7, 1058.7, 1023.7 cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.84 (s, 1H, NH), 8.79 (s, 1H, 4-H), 8.29 (t, *J* = 5.7 Hz, 1H, NH), 7.91 (d, *J* = 8.5 Hz, 2H, 3', 5'-H), 7.81 (d, *J* = 8.5 Hz, 2H, 2', 6'-H), 6.61 (s, 1H, 5-H), 4.75 (p, *J* = 8.9 Hz, 1H, CH), 3.64 (t, *J* = 6.5 Hz, 2H, CH_2), 3.24 (q, *J* = 6.7 Hz, 2H, CH_2), 3.05 (s, 6H, $2CH_3$), 2.50 – 2.42 (m, 2H, CH_2), 2.05 – 1.96 (m, 4H, $2CH_2$), 1.66 (dt, *J* = 28.4, 7.0 Hz, 4H, $2CH_2$), 1.52 (t, *J* = 7.0 Hz, 2H, CH_2), 1.31 (t, *J* = 5.2 Hz, 6H, $3CH_2$); ^{13}C NMR (101 MHz, DMSO-d_6) δ 165.7 (1C, C=O), 162.9 (1C, Ar-C), 155.1 (1C, C=O), 152.1 (1C, Ar-C), 151.0 (1C, Ar-C), 143.6 (1C, Ar-C), 132.0 (1C, Ar-C), 127.8 (2C, Ar-C), 127.2 (1C, S=C=N), 126.5 (1C, Ar-C), 116.9 (2C, Ar-C), 111.9 (1C, Ar-C), 100.6 (1C, Ar-C), 57.0 (1C, CH), 44.7 (1C, CH_2), 39.1 (2C, $2CH_3$), 34.6 (1C, CH_2), 29.6 (2C, $2CH_2$), 29.2 (1C, CH_2), 29.2 (1C, CH_2), 28.0 (1C, CH_2), 26.4 (1C, CH_2), 26.0 (1C, CH_2), 24.2 (2C, $2CH_2$). HRMS (ESI, m/z) calcd for $C_{29}H_{38}N_7O_2S^+$ $[M + H]^+$, 548.2802, found 548.2809. HPLC purity 99% (t_R = 6.85 min).

4.1.11.

7-Cyclopentyl-2-((5-((3-isothiocyanatopropyl)carbamoyl)pyridin-2-yl)amino)-N,N-di methyl-7H-pyrrolo[2,3-d]pyrimidine-6-carboxamide ***(10ba).*** Compound **10ba** was prepared using **8ba** instead of **7a** in the similar manner of compound **9a** to afford the

title product as white solid in 85% yield; mp 231.7 °C. IR (film): 3307.9, 3229.3, 3171.9, 3113.1, 3083.8, 3064.4, 3028.1, 2958.8, 2937.3, 2871.2, 2185.3, 2100.5, 1659.1, 1611.2, 1581.6, 1525.5, 1485.3, 1462.5, 1445.1, 1422.5, 1379.4, 1353.9, 1329.9, 1297.4, 1273.3, 1255.5, 1223.1, 1161.9, 1136.2, 1013.7 cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.21 (s, 1H, NH), 8.89 (s, 1H, 4-H), 8.80 (s, 1H, 6'-H), 8.58 (t, *J* = 5.6 Hz, 1H, NH), 8.42 (d, *J* = 8.8 Hz, 1H, 4'-H), 8.20 (dd, *J* = 8.8, 2.5 Hz, 1H, 3'-H), 6.66 (s, 1H, 5-H), 4.77 (p, *J* = 9.0 Hz, 1H, CH), 3.76 (t, *J* = 6.4 Hz, 2H, CH_2), 3.38 (q, *J* = 6.3 Hz, 2H, CH_2), 3.06 (s, 6H, $2CH_3$), 2.45 (tt, *J* = 10.1, 7.0, 5.5 Hz, 2H, CH_2), 2.01 (tq, *J* = 10.5, 6.1, 5.4 Hz, 4H, $2CH_2$), 1.91 (p, *J* = 6.6 Hz, 2H, CH_2), 1.68 (q, *J* = 6.6 Hz, 2H, CH_2); ^{13}C NMR (101 MHz, DMSO-d_6) δ 164.8 (1C, C=O), 162.7 (1C, Ar-C), 155.1 (1C, C=O), 153.8 (1C, Ar-C), 152.1 (1C, Ar-C), 150.8 (1C, Ar-C), 147.9 (1C, Ar-C), 136.5 (1C, Ar-C), 132.9 (1C, Ar-C), 127.2 (1C, S=C=N), 122.6 (1C, Ar-C), 112.8 (1C, Ar-C), 110.2 (1C, Ar-C), 100.4 (1C, Ar-C), 57.1 (1C, CH), 42.8 (1C, CH_2), 36.3 (2C, $2CH_3$), 34.6 (1C, CH_2), 29.8 (2C, $2CH_2$), 29.5 (1C, CH_2), 24.3 (2C, $2CH_2$). HRMS (ESI, m/z) calcd for $C_{24}H_{29}N_8O_2S^+$ $[M + H]^+$, 493.2129, found 493.2136. HPLC purity 99% (t_R = 6.70 min).

4.1.12.

7-Cyclopentyl-2-((5-((4-isothiocyanatobutyl)carbamoyl)pyridin-2-yl)amino)-N,N-dim ethyl-7H-pyrrolo[2,3-d]pyrimidine-6-carboxamide ***(10bb).*** Compound **10bb** was prepared using **8bb** instead of **7a** in the similar manner of compound **9a** to afford the title product as white solid in 34% yield; mp 184.4 °C. IR (film): 3324.7, 3231.3,

3171.0, 3027.0, 2943.2, 2871.4, 2170.4, 2073.6, 1659.4, 1611.3, 1584.0, 1563.7, 1524.8, 1487.0, 1463.1, 1423.5, 1380.5, 1349.3, 1307.3, 1274.3, 1222.7, 1162.2, 1136.7, 1059.4, 1015.6 cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.47 (s, 1H, NH), 9.01 – 8.78 (m, 2H, 4-H, 6'-H), 8.55 (t, *J* = 5.4 Hz, 1H, NH), 8.44 (t, *J* = 9.1 Hz, 1H, 4'-H), 8.21 (td, *J* = 7.7, 6.7, 2.5 Hz, 1H, 3'-H), 6.67 (s, 1H, 5-H), 4.77 (h, *J* = 7.7, 6.6 Hz, 1H, CH), 3.73 (t, *J* = 6.3 Hz, 2H, CH_2), 3.32 (dt, *J* = 6.6, 3.3 Hz, 2H, CH_2), 3.06 (d, *J* = 7.2 Hz, 6H, $2CH_3$), 2.46 (d, *J* = 10.0 Hz, 2H, CH_2), 2.02 (tt, *J* = 10.5, 6.2 Hz, 4H, $2CH_2$), 1.68 (dq, *J* = 21.6, 8.3, 7.9 Hz, 6H, $3CH_2$); ^{13}C NMR (101 MHz, DMSO-d_6) δ 164.6 (1C, C=O), 162.7 (1C, Ar-C), 155.1 (1C, C=O), 153.9 (1C, Ar-C), 152.1 (1C, Ar-C), 150.8, 147.8 (1C, Ar-C), 136.5 (1C, Ar-C), 132.9 (1C, Ar-C), 127.2 (1C, S=C=N), 122.8 (1C, Ar-C), 112.8 (1C, Ar-C), 110.2 (1C, Ar-C), 100.5 (1C, Ar-C), 57.1 (1C, CH), 44.5 (1C, CH_2), 38.2 (2C, $2CH_3$), 34.6 (1C, CH_2), 29.8 (2C, $2CH_2$), 26.9 (1C, CH_2), 26.4 (1C, CH_2), 24.3 (2C, $2CH_2$). HRMS (ESI, m/z) calcd for $C_{25}H_{31}N_8O_2S^+$ $[M + H]^+$, 507.2285, found 507.2281. HPLC purity 99% (t_R = 6.79 min).

4.1.13.

7-Cyclopentyl-2-((5-((5-isothiocyanatopentyl)carbamoyl)pyridin-2-yl)amino)-N,N-di methyl-7H-pyrrolo[2,3-d]pyrimidine-6-carboxamide ***(10bc).*** Compound **10bc** was prepared using **8bc** instead of **7a** in the similar manner of compound **9a** to afford the title product as white solid in 56% yield; mp 181.1 °C. IR (film): 3315.5, 3225.3, 3169.6, 3115.0, 3078.9, 3056.1, 3025.0, 2961.4, 2934.2, 2870.3, 2170.0, 2151.1,

2087.4, 1655.8, 1618.1, 1583.9, 1547.6, 1525.0, 1486.8, 1464.5, 1422.8, 1380.3, 1349.8, 1330.2, 1307.4, 1278.5, 1253.7, 1223.0, 1160.5, 1135.8, 1024.7 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6) δ 10.22 (s, 1H, NH),8.97 – 8.88 (s, 1H, 4-H), 8.85 – 8.76 (m, 1H, 6'-H), 8.50 (t, *J* = 5.7 Hz, 1H, NH), 8.42 (dd, *J* = 8.9, 4.2 Hz, 1H, 4'-H), 8.20 (dt, *J* = 8.9, 2.9 Hz, 1H, 3'-H), 6.66 (s, 1H, 5-H), 4.77 (p, *J* = 8.9 Hz, 1H, CH), 3.68 (t, *J* = 6.5 Hz, 2H, CH_2), 3.29 (q, *J* = 6.5 Hz, 2H, CH_2), 3.06 (s, 6H, $2CH_3$), 2.49 – 2.39 (m, 2H, CH_2), 2.02 (q, *J* = 9.9, 8.0 Hz, 4H, $2CH_2$), 1.69 (h, *J* = 6.8, 6.2 Hz, 4H, CH_2), 1.60 – 1.52 (m, 2H, CH_2), 1.41 (td, *J* = 8.3, 4.2 Hz, 2H, CH_2); ^{13}C NMR (101 MHz, DMSO-d_6) δ 164.5 (1C, C=O), 162.7 (1C, Ar-C), 155.0 (1C, C=O), 153.9 (1C, Ar-C), 152.1 (1C, Ar-C), 150.8, (1C, Ar-C) 147.8 (1C, Ar-C), 136.4 (1C, Ar-C), 132.8 (1C, Ar-C), 127.2 (1C, S=C=N), 122.9 (1C, Ar-C), 112.8 (1C, Ar-C), 110.2 (1C, Ar-C), 100.4 (1C, Ar-C), 57.1 (1C, CH), 44.7 (1C, CH_2), 38.8 (2C, $2CH_3$), 34.6 (1C, CH_2), 29.8 (2C, $2CH_2$), 29.0 (1C, CH_2), 28.4 (1C, CH_2), 24.3 (1C, CH_2), 23.5 (2C, $2CH_2$). HRMS (ESI, m/z) calcd for $C_{26}H_{33}N_8O_2S^+$ $[M + H]^+$, 521.2442, found 521.2449. HPLC purity 99% (t_R = 6.82 min).

4.1.14.

7-Cyclopentyl-2-((5-((6-isothiocyanatohexyl)carbamoyl)pyridin-2-yl)amino)-N,N-dimethyl-7H-pyrrolo[2,3-d]pyrimidine-6-carboxamide ***(10bd).*** Compound **10bd** was prepared using **8bd** instead of **7a** in the similar manner of compound **9a** to afford the title product as white solid in 38% yield; mp 153.0 °C. IR (film): 3348.2, 3224.3, 3155.3, 3079.1, 3057.4, 3023.6, 2920.4, 2872.7, 2855.4, 2196.4, 2108.9, 1658.3,

1618.5, 1585.0, 1562.5, 1524.6, 1485.6, 1463.2, 1421.8, 1399.2, 1379.9, 1348.5, 1330.4, 1302.7, 1275.8, 1253.6, 1154.7, 1134.3, 1054.2, 1017.7 cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.26 (s, 1H, NH), 8.90 (s, 1H, 4-H), 8.80 (s, 1H, 6'-H), 8.47 (t, *J* = 5.6 Hz, 1H, NH), 8.42 (d, *J* = 8.8 Hz, 1H, 4'-H), 8.20 (dd, *J* = 9.0, 2.5 Hz, 1H, 3'-H), 6.66 (s, 1H, 5-H), 4.77 (p, *J* = 8.9 Hz, 1H, CH), 3.66 (t, *J* = 6.5 Hz, 2H, CH_2), 3.27 (q, *J* = 6.7 Hz, 2H, CH_2), 3.06 (s, 6H, $2CH_3$), 2.49 – 2.40 (m, 2H, CH_2), 2.00 (td, *J* = 7.3, 3.9 Hz, 4H, $2CH_2$), 1.67 (dt, *J* = 18.6, 6.8 Hz, 4H, $2CH_2$), 1.54 (p, *J* = 7.1 Hz, 2H, CH_2), 1.37 (tt, *J* = 8.7, 4.0 Hz, 4H, $2CH_2$); ^{13}C NMR (101 MHz, DMSO-d_6) δ 164.4 (1C, C=O), 162.7 (1C, Ar-C), 155.0 (1C, C=O), 153.9 (1C, Ar-C), 152.1 (1C, Ar-C), 150.8 (1C, Ar-C), 147.8 (1C, Ar-C), 136.4 (1C, Ar-C), 132.8 (1C, Ar-C), 127.2 (1C, S=C=N), 122.9 (1C, Ar-C), 112.8 (1C, Ar-C), 110.2 (1C, Ar-C), 100.5 (1C, Ar-C), 57.1 (1C, CH), 44.7 (1C, CH_2), 39.0 (2C, $2CH_3$), 34.6 (1C, CH_2), 29.8 (2C, $2CH_2$), 29.2 (1C, CH_2), 29.1 (1C, CH_2), 25.8 (1C, CH_2), 25.7 (1C, CH_2), 24.3 (2C, $2CH_2$). HRMS (ESI, m/z) calcd for $C_{27}H_{35}N_8O_2S^+$ $[M + H]^+$, 535.2598, found 535.2599. HPLC purity 97% (t_R = 4.55 min).

4.1.15.

7-Cyclopentyl-2-((5-((7-isothiocyanatoheptyl)carbamoyl)pyridin-2-yl)amino)-N,N-di methyl-7H-pyrrolo[2,3-d]pyrimidine-6-carboxamide ***(10be).*** Compound **10be** was prepared using **8be** instead of **7a** in the similar manner of compound **9a** to afford the title product as white solid in 67% yield; mp 148.1 °C. IR (film): 3463.9, 3331.0, 3225.5, 3169.7, 3023.7, 2926.6, 2872.2, 2860.4, 2191.4, 2116.8, 1655.7, 1612.2,

1582.7, 1562.7, 1524.0, 1485.2, 1460.9, 1441.7, 1422.3, 1379.6, 1350.3, 1328.5, 1311.5, 1274.7, 1251.2, 1222.4, 1162.7, 1135.6, 1056.6, 1016.2 cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.27 (s, 1H, NH), 8.90 (s, 1H, 4-H), 8.81 (s, 1H, 6'-H), 8.48 – 8.40 (m, 2H, NH and 4'-H), 8.20 (dd, *J* = 8.9, 2.5 Hz, 1H, 3'-H), 6.66 (s, 1H, 5-H), 4.77 (p, *J* = 8.9 Hz, 1H, CH), 3.65 (t, *J* = 6.5 Hz, 2H, CH_2), 3.27 (q, *J* = 6.7 Hz, 2H, CH_2), 3.06 (s, 6H, $2CH_3$),2.45 (dp, *J* = 10.4, 7.3, 4.9 Hz, 2H, CH_2), 2.02 (qd, *J* = 9.1, 7.3, 3.6 Hz, 4H, $2CH_2$), 1.71 – 1.60 (m, 4H, $2CH_2$), 1.53 (t, *J* = 7.0 Hz, 2H, CH_2), 1.36 – 1.30 (m, 6H, $3CH_2$); ^{13}C NMR (101 MHz, DMSO-d_6) δ 164.4 (1C, C=O), 162.7 (1C, Ar-C), 155.0 (1C, C=O), 153.9 (1C, Ar-C), 152.1 (1C, Ar-C), 150.8 (1C, Ar-C), 147.8 (1C, Ar-C), 136.4 (1C, Ar-C), 132.8 (1C, Ar-C), 127.2 (1C, S=C=N), 122.9 (1C, Ar-C), 112.8 (1C, Ar-C), 110.2 (1C, Ar-C), 100.5 (1C, Ar-C), 57.1 (1C, CH), 44.7 (1C, CH_2), 39.0 (2C, $2CH_3$), 34.6 (1C, CH_2), 29.8 (2C, $2CH_2$), 29.2 (1C, CH_2), 29.1 (1C, CH_2), 28.0 (1C, CH_2), 26.4 (1C, CH_2), 26.0 (1C, CH_2), 24.3 (2C, $2CH_2$). HRMS (ESI, m/z) calcd for $C_{28}H_{37}N_8O_2S^+$ $[M + H]^+$, 549.2755, found 549.2755. HPLC purity 98% (t_R = 5.11 min).

4.1.16.

7-Cyclopentyl-2-((2-fluoro-4-((3-isothiocyanatopropyl)carbamoyl)phenyl)amino)-N,N-dimethyl-7H-pyrrolo[2,3-d]pyrimidine-6-carboxamide ***(10ca).*** Compound **10ca** was prepared using **8ca** instead of **7a** in the similar manner of compound **9a** to afford the title product as white solid in 77% yield; mp 154.5 °C. IR (film): 3439.5, 3363.2, 3307.5, 3115.7, 3012.8, 2948.9, 2922.7, 2873.2, 2178.6, 2114.7, 2088.0, 1625.7,

1593.8, 1566.3, 1539.0, 1517.8, 1493.6, 1477.2, 1432.7, 1418.4, 1355.1, 1305.2, 1272.8, 1247.5, 1192.7, 1160.9, 1138.5, 1110.4, 1066.1, 1027.1 cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.11 (s, 1H, NH), 8.79 (s, 1H, 4-H), 8.55 (t, *J* = 5.6 Hz, 1H, NH), 8.22 (t, *J* = 8.5 Hz, 1H, 3'-H), 7.77 – 7.68 (m, 2H, 5'-H, 6'-H), 6.61 (s, 1H, 5-H), 4.71 (p, *J* = 8.9 Hz, 1H, CH), 3.75 (t, *J* = 6.4 Hz, 2H, CH_2), 3.37 (t, *J* = 6.2 Hz, 2H, CH_2), 3.10 – 2.99 (m, 6H, $2CH_3$), 2.36 (tdd, *J* = 11.2, 9.8, 8.0, 5.0 Hz, 2H, CH_2), 1.99 – 1.84 (m, 6H, $3CH_2$), 1.59 (p, *J* = 5.5, 4.4 Hz, 2H, CH_2); ^{13}C NMR (101 MHz, DMSO-d_6) δ 164.8 (1C, C=O), 162.8 (1C, Ar-C), 155.0 (1C, C=O), 153.9, 152.0, 151.5, 151.1, 132.3, 131.3, 131.2, 128.4, 128.3, 127.2, 123.2, 121.2, 114.2, 114.0, 112.4, 100.4 (11C, Ar-C), 56.9 (1C, CH), 42.7 (1C, CH_2), 36.4 (2C, $2CH_3$), 34.6 (1C, CH_2), 29.9 (2C, $2CH_2$), 29.4 (1C, CH_2), 24.3 (2C, $2CH_2$). HRMS (ESI, m/z) calcd for $C_{25}H_{29}FN_7O_2S^+$ $[M + H]^+$, 510.2082, found 510.2084. HPLC purity 99% (t_R = 6.80 min).

4.1.17.

7-Cyclopentyl-2-((2-fluoro-4-((4-isothiocyanatobutyl)carbamoyl)phenyl)amino)-N,N-dimethyl-7H-pyrrolo[2,3-d]pyrimidine-6-carboxamide ***(10cb).*** Compound **10cb** was prepared using **8cb** instead of **7a** in the similar manner of compound **9a** to afford the title product as white solid in 76% yield; mp 115.6 °C. IR (film): 3322.5, 3115.9, 2944.7, 2929.0, 2869.5, 2851.7, 2185.5, 2086.6, 1736.9, 1622.0, 1589.6, 1563.6, 1542.5, 1514.2, 1479.3, 1432.1, 1412.7, 1354.0, 1307.2, 1272.2, 1233.8, 1195.5, 1136.4, 1108.8, 1088.0, 1057.1, 1022.2 cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.10 (s, 1H, NH), 8.78 (s, 1H, 4-H), 8.50 (t, *J* = 5.7 Hz, 1H, NH), 8.21 (td, *J* = 8.6, 3.8 Hz,

1H, 3'-H), 7.77 – 7.68 (m, 2H, 5'-H, 6'-H), 6.61 (s, 1H, 5-H), 4.71 (p, *J* = 9.0 Hz, 1H, CH), 3.72 (t, *J* = 6.3 Hz, 2H, CH_2), 3.33 – 3.26 (m, 2H, CH_2), 3.09 – 3.01 (m, 6H, $2CH_3$), 2.41 – 2.30 (m, 2H, CH_2), 1.98 – 1.91 (m, 2H, CH_2), 1.86 (h, *J* = 7.3, 4.8 Hz, 2H, CH_2), 1.73 – 1.67 (m, 2H, CH_2), 1.64 – 1.56 (m, 4H, $2CH_2$); ^{13}C NMR (101 MHz, DMSO-d_6) δ 164.7 (1C, C=O), 162.8 (1C, Ar-C), 155.0 (1C, C=O), 154.0, 152.0, 151.6, 151.1, 132.3, 131.2, 131.1, 128.6, 128.6, 127.2 (1C, S=C=N), 123.2, 121.3, 114.2, 114.0, 112.4, 100.4 (11C, Ar-C), 56.9 (1C, CH), 44.5 (1C, CH_2), 38.4 (2C, $2CH_3$), 33.4 (1C, CH_2), 29.9 (2C, $2CH_2$), 26.9 (1C, CH_2), 26.3 (1C, CH_2), 24.4 (2C, $2CH_2$). HRMS (ESI, m/z) calcd for $C_{26}H_{31}FN_7O_2S^+$ $[M + H]^+$, 524.2238, found 524.2244. HPLC purity 99% (t_R = 6.83 min).

4.1.18.

7-Cyclopentyl-2-((2-fluoro-4-((5-isothiocyanatopentyl)carbamoyl)phenyl)amino)-N,N-dimethyl-7H-pyrrolo[2,3-d]pyrimidine-6-carboxamide ***(10cc).*** Compound **10cc** was prepared using **8cc** instead of **7a** in the similar manner of compound **9a** to afford the title product as white solid in 64% yield; mp 115.1 °C. IR (film): 3330.3, 3114.5, 2941.4, 2869.7, 2186.2, 2091.7, 1625.7, 1590.1, 1564.8, 1510.1, 1480.7, 1431.1, 1414.6, 1355.1, 1307.0, 1274.6, 1247.3, 1234.6, 1196.2, 1141.5, 1109.7, 1087.1, 1058.6, 1022.8 cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.10 (s, 1H, NH), 8.78 (s, 1H, 4-H), 8.46 (t, *J* = 5.7 Hz, 1H, NH), 8.21 (t, *J* = 8.4 Hz, 1H, 3'-H), 7.75 – 7.69 (m, 2H, 5'-H, 6'-H), 6.61 (s, 1H, 5-H), 4.71 (p, *J* = 8.8 Hz, 1H, CH), 3.67 (t, *J* = 6.5 Hz, 2H, CH_2), 3.27 (q, *J* = 6.6 Hz, 2H, CH_2), 3.09 – 3.01 (m, 6H, $2CH_3$), 2.35 (ddd, *J* = 12.7,

9.4, 6.4 Hz, 2H, CH_2), 1.96 (dtd, *J* = 10.8, 7.6, 4.5 Hz, 2H, CH_2), 1.86 (dt, *J* = 8.2, 4.4 Hz, 2H, CH_2), 1.71 – 1.65 (m, 2H, CH_2), 1.61 – 1.52 (m, 4H, $2CH_2$), 1.40 (qd, *J* = 7.4, 6.6, 4.1 Hz, 2H, CH_2); ^{13}C NMR (101 MHz, DMSO-d_6) δ 164.6 (1C, C=O), 162.8 (1C, Ar-C), 155.0 (1C, C=O), 154.0, 152.0, 151.6, 151.1, 132.3, 131.1, 131.0, 128.7, 128.7, 127.2 (1C, S=C=N), 123.1, 121.2, 114.2, 114.0, 112.4, 100.4 (11C, Ar-C), 56.9 (1C, CH), 44.7 (1C, CH_2), 38.9 (2C, $2CH_3$), 34.6 (1C, CH_2), 29.9 (2C, $2CH_2$), 29.0 (1C, CH_2), 28.4 (1C, CH_2), 24.4 (2C, $2CH_2$), 23.5 (1C, CH_2). HRMS (ESI, m/z) calcd for $C_{27}H_{33}FN_7O_2S^+$ $[M + H]^+$, 538.2395, found 538.2402. HPLC purity 99% (t_R = 6.89 min).

4.1.19.

7-Cyclopentyl-2-((2-fluoro-4-((6-isothiocyanatohexyl)carbamoyl)phenyl)amino)-N,N-dimethyl-7H-pyrrolo[2,3-d]pyrimidine-6-carboxamide ***(10cd).*** Compound **10cd** was prepared using **8cd** instead of **7a** in the similar manner of compound **9a** to afford the title product as white solid in 52% yield; mp 131.6 °C. IR (film): 3330.8, 3109.8, 3057.3, 2928.8, 2856.6, 2173.8, 2089.6, 1624.9, 1589.6, 1564.0, 1547.8, 1516.9, 1479.8, 1430.8, 1414.2, 1357.5, 1305.6, 1275.4, 1233.4, 1195.9, 1134.5, 1108.9, 1020.2 cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.10 (s, 1H, NH), 8.78 (s, 1H, 4-H), 8.43 (t, *J* = 5.6 Hz, 1H, NH), 8.19 (td, *J* = 8.5, 3.4 Hz, 1H, 3'-H), 7.76 – 7.67 (m, 2H, 5'-H, 6'-H), 6.61 (s, 1H, 5-H), 4.71 (p, *J* = 8.9 Hz, 1H, CH), 3.68 – 3.62 (m, 2H, CH_2), 3.26 (q, *J* = 6.6 Hz, 2H, CH_2), 3.08 – 3.00 (m, 6H, $2CH_3$), 2.41 – 2.30 (m, 2H, CH_2), 1.96 (ddt, *J* = 9.5, 7.0, 3.4 Hz, 2H, CH_2), 1.90 – 1.81 (m, 2H, CH_2), 1.67 – 1.51 (m,

6H, $3CH_2$), 1.36 (dq, *J* = 9.6, 5.9, 5.3 Hz, 4H, $2CH_2$); ^{13}C NMR (101 MHz, DMSO-d_6) δ 164.5 (1C, C=O), 162.8 (1C, Ar-C), 155.0 (1C, C=O), 154.1, 152.0, 151.6, 151.1, 132.3, 131.1, 131.0, 128.8, 128.7, 127.2 (1C, S=C=N), 123.1, 121.3, 114.2, 114.0, 112.4, 100.4 (11C, Ar-C), 56.9 (1C, CH), 44.7 (1C, CH_2), 39.8 (2C, $2CH_3$), 34.6 (1C, CH_2), 29.9 (2C, $2CH_2$), 29.2 (1C, CH_2), 29.0 (1C, CH_2), 25.8 (1C, CH_2), 25.7 (1C, CH_2), 24.4 (2C, $2CH_2$). HRMS (ESI, m/z) calcd for $C_{28}H_{35}FN_7O_2S^+$ $[M + H]^+$, 552.2551, found 552.2547. HPLC purity 99% (t_R = 7.06 min).

4.1.20.

7-Cyclopentyl-2-((2-fluoro-4-((7-isothiocyanatoheptyl)carbamoyl)phenyl)amino)-N,N-dimethyl-7H-pyrrolo[2,3-d]pyrimidine-6-carboxamide ***(10ce).*** Compound **10ce** was prepared using **8ce** instead of **7a** in the similar manner of compound **9a** to afford the title product as white solid in 19% yield; mp 112.2 °C. IR (film): 3321.4, 3174.1, 3115.0, 3013.0, 2932.8, 2854.5, 2168.9, 2112.0, 2080.7, 1624.6, 1601.4, 1589.5, 1565.2, 1540.2, 1509.8, 1483.8, 1430.0, 1411.1, 1331.2, 1302.6, 1272.9, 1246.6, 1229.5, 1194.8, 1143.0, 1108.7, 1056.5, 1023.5 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.77 – 8.68 (m, 2H, 4-H, NH), 7.62 – 7.52 (m, 3H, 3'-H, 5'-H, 6'-H), 6.46 (s, 1H, 5-H), 6.16 (t, *J* = 5.8 Hz, 1H, NH), 4.80 (q, *J* = 8.9 Hz, 1H, CH), 3.51 (t, *J* = 6.5 Hz, 2H, CH_2), 3.45 (q, *J* = 6.8 Hz, 2H, CH_2), 3.16 (s, 6H, $2CH_3$), 2.63 – 2.53 (m, 2H, CH_2), 2.12 – 2.02 (m, 4H, $2CH_2$), 1.75 – 1.63 (m, 6H, $3CH_2$), 1.47 – 1.37 (m, 6H, $3CH_2$); ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.3 (1C, C=O), 164.0 (1C, Ar-C), 154.5 (1C, C=O), 152.7, 151.9, 151.7, 150.3, 132.9, 132.0, 131.9, 127.4, 127.3 (1C, S=C=N), 123.0,

123.0, 118.4, 113.8, 113.6, 113.4, 101.0 (11C, Ar-C), 58.2 (1C, CH), 45.2 (1C, CH_2), 40.2 (2C, $2CH_3$), 34.1 (1C, CH_2), 30.3 (1C, CH_2), 30.0 (1C, CH_2), 29.8 (2C, $2CH_2$), 28.6 (1C, CH_2), 26.9 (1C, CH_2), 26.6 (1C, CH_2), 24.8 (2C, $2CH_2$). HRMS (ESI, m/z) calcd for $C_{29}H_{37}FN_7O_2S^+$ $[M + H]^+$, 566.2708, found 566.2706. HPLC purity 99% (t_R = 7.24 min).

4.1.21.

7-Cyclopentyl-2-((2-hydroxy-4-((3-isothiocyanatopropyl)carbamoyl)phenyl)amino)-N,N-dimethyl-7H-pyrrolo[2,3-d]pyrimidine-6-carboxamide ***(10da).*** Compound **10da** was prepared using **8da** instead of **7a** in the similar manner of compound **9a** to afford the title product as white solid in 15% yield; mp 214.1 °C. IR (film): 3398.4, 3370.6, 3246.4, 3118.3, 3014.7, 2945.5, 2916.9, 2871.9, 2185.8, 2113.9, 2089.9, 1629.0, 1595.8, 1546.8, 1505.7, 1481.0, 1418.0, 1358.2, 1310.8, 1287.1, 1241.1, 1213.3, 1197.8, 1164.7, 1134.8, 1088.1, 1022.2 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6) δ 10.38 (s, 1H, OH), 8.79 (s, 1H, 4-H), 8.46 – 8.39 (m, 2H, 2NH), 8.03 (s, 1H, 3'-H), 7.41 (m, 2H, 5'-H, 6'-H), 6.63 (s, 1H, 5-H), 4.75 (t, *J* = 8.8 Hz, 1H, CH), 3.74 (t, *J* = 6.4 Hz, 2H, CH_2), 3.33 (m, 2H, CH_2), 3.10 – 3.02 (m, 6H, $2CH_3$), 2.47 (d, *J* = 9.5 Hz, 2H, CH_2), 2.01 (ddt, *J* = 13.1, 9.4, 6.0 Hz, 4H, $2CH_2$), 1.89 (q, *J* = 6.5 Hz, 2H, CH_2), 1.69 (q, *J* = 7.0 Hz, 2H, CH_2); ^{13}C NMR (101 MHz, DMSO-d_6) δ 166.1 (1C, C=O), 162.8 (1C, Ar-C), 154.7 (1C, C=O), 152.2 (1C, Ar-C), 151.1 (1C, Ar-C), 145.1 (1C, Ar-C), 132.3 (1C, Ar-C), 131.3 (1C, Ar-C), 127.2 (1C, Ar-C), 127.1 (1C, S=C=N), 118.3 (1C, Ar-C), 116.3 (1C, Ar-C), 113.7 (1C, Ar-C), 112.1 (1C, Ar-C), 100.7 (1C, Ar-C),

57.1(1C, CH), 42.8 (1C, CH_2), 36.3 (2C, $2CH_3$), 34.6 (1C, CH_2), 29.6 (2C, $2CH_2$), 29.6 (1C, CH_2), 24.2 (2C, $2CH_2$). HRMS (ESI, m/z) calcd for $C_{25}H_{30}N_7O_3S^+$ [M + H]$^+$, 508.2125, found 508.2127. HPLC purity 99% (t_R = 4.02 min).

4.1.22.

7-Cyclopentyl-2-((2-hydroxy-4-((4-isothiocyanatobutyl)carbamoyl)phenyl)amino)-N, N-dimethyl-7H-pyrrolo[2,3-d]pyrimidine-6-carboxamide ***(10db).*** Compound **10db** was prepared using **8db** instead of **7a** in the similar manner of compound **9a** to afford the title product as white solid in 53% yield; mp 165.6 °C. IR (film): 3540.6, 3406.8, 3232.8, 3121.3, 3014.2, 2946.6, 2870.1, 2169.1, 2091.9, 1620.1, 1595.8, 1555.6, 1519.6, 1505.1, 1482.5, 1418.3, 1355.9, 1309.3, 1285.4, 1242.9, 1216.4, 1197.6, 1161.9, 1136.1, 1056.1, 1023.8 cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.36 (s, 1H, OH), 8.78 (s, 1H, 4-H), 8.43 (d, *J* = 8.3 Hz, 1H, NH), 8.35 (t, *J* = 5.8 Hz, 1H, NH), 8.02 (s, 1H, 3'-H), 7.42 – 7.37 (m, 2H, 5'-H, 6'-H), 6.63 (s, 1H, 5-H), 4.79 – 4.72 (m, 1H, CH), 3.71 (t, *J* = 6.4 Hz, 2H, CH_2), 3.27 (q, *J* = 6.5 Hz, 2H, CH_2), 3.05 (d, *J* = 9.4 Hz, 6H, $2CH_3$), 2.49 – 2.42 (m, 2H, CH_2), 2.04 – 1.97 (m, 4H, $2CH_2$), 1.72 – 1.65 (m, 4H, $2CH_2$), 1.63 – 1.56 (m, 2H, CH_2); ^{13}C NMR (101 MHz, DMSO-d_6) δ 165.9 (1C, C=O), 162.8 (1C, Ar-C), 154.7 (1C, C=O), 152.2 (1C, Ar-C), 151.1 (1C, Ar-C), 145.1 (1C, Ar-C), 132.2 (1C, Ar-C), 131.2 (1C, Ar-C), 127.3 (1C, Ar-C), 127.1 (1C, S=C=N), 118.2 (1C, Ar-C), 116.4 (1C, Ar-C), 113.7 (1C, Ar-C), 112.1 (1C, Ar-C), 100.7 (1C, Ar-C), 57.1 (1C, CH), 44.5 (1C, CH_2), 38.2 (2C, $2CH_3$), 34.6 (1C, CH_2), 29.7 (2C, $2CH_2$), 26.9 (1C, CH_2), 26.4 (1C, CH_2), 24.2 (2C, $2CH_2$). HRMS (ESI, m/z)

calcd for $C_{26}H_{32}N_7O_3S^+$ $[M + H]^+$, 522.2282, found 522.2282. HPLC purity 98% (t_R = 4.10 min).

4.1.23.

7-Cyclopentyl-2-((2-hydroxy-4-((5-isothiocyanatopentyl)carbamoyl)phenyl)amino)-N, N-dimethyl-7H-pyrrolo[2,3-d]pyrimidine-6-carboxamide ***(10dc).*** Compound **10dc** was prepared using **8dc** instead of **7a** in the similar manner of compound **9a** to afford the title product as white solid in 64% yield; mp 177.9 °C. IR (film): 3414.4, 3215.4, 3118.0, 2935.4, 2869.1, 2170.7, 2106.5, 1621.6, 1595.5, 1547.8, 1504.7, 1480.4, 1416.8, 1356.1, 1328.5, 1309.2, 1285.0, 1241.6, 1215.9, 1199.4, 1180.3, 1160.2, 1135.3, 1085.4, 1056.4, 1021.9 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6) δ 10.34 (s, 1H, OH), 8.78 (s, 1H, 4-H), 8.42 (d, *J* = 8.3 Hz, 1H, NH), 8.31 (d, *J* = 5.8 Hz, 1H, NH), 8.02 (s, 1H, 3'-H), 7.39 (d, *J* = 11.9 Hz, 2H, 5'-H, 6'-H), 6.63 (s, 1H, 5-H), 4.80 – 4.72 (m, 1H, CH), 3.67 (t, *J* = 6.6 Hz, 2H, CH_2), 3.25 (q, *J* = 6.6 Hz, 2H, CH_2), 3.05 (d, *J* = 7.6 Hz, 6H, $2CH_3$), 2.46 (m, 2H, CH_2), 2.02 (q, *J* = 7.1, 6.7 Hz, 4H, $2CH_2$), 1.68 (q, *J* = 7.3 Hz, 4H, $2CH_2$), 1.54 (t, *J* = 7.5 Hz, 2H, CH_2), 1.42 – 1.35 (m, 2H, CH_2); ^{13}C NMR (101 MHz, DMSO-d_6) δ 165.8 (1C, C=O), 162.8 (1C, Ar-C), 154.7 (1C, C=O), 152.2 (1C, Ar-C), 151.1 (1C, Ar-C), 145.0 (1C, Ar-C), 132.2 (1C, Ar-C), 131.1 (1C, Ar-C), 127.4 (1C, Ar-C), 127.2 (1C, S=C=N), 118.2 (1C, Ar-C), 116.4 (1C, Ar-C), 113.7 (1C, Ar-C), 112.0 (1C, Ar-C), 100.7 (1C, Ar-C), 57.1 (1C, CH), 44.7 (1C, CH_2), 38.8 (2C, $2CH_3$), 34.6 (1C, CH_2), 29.6 (2C, $2CH_2$), 29.0 (1C, CH_2), 28.5 (1C, CH_2), 24.2 (1C, CH_2), 23.5 (2C, $2CH_2$). HRMS (ESI, m/z) calcd for $C_{27}H_{34}N_7O_3S^+$ [M +

H]$^+$, 536.2438, found 536.2437. HPLC purity 99% (t_R = 4.22 min).

4.1.24.

7-Cyclopentyl-2-((2-hydroxy-4-((6-isothiocyanatohexyl)carbamoyl)phenyl)amino)-N, N-dimethyl-7H-pyrrolo[2,3-d]pyrimidine-6-carboxamide ***(10dd).*** Compound **10dd** was prepared using **8dd** instead of **7a** in the similar manner of compound **9a** to afford the title product as white solid in 58% yield; mp 204.7 °C. IR (film): 3411.8, 3345.5, 3208.6, 3015.7, 2967.5, 2920.1, 2871.3, 2855.0, 2188.0, 2123.0, 1633.6, 1615.2, 1597.8, 1553.2, 1507.4, 1480.9, 1419.6, 1342.6, 1330.3, 1315.2, 1286.2, 1238.3, 1219.4, 1199.5, 1155.1, 1134.8, 1054.1, 1022.3 cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.34 (s, 1H, OH), 8.78 (s, 1H, 4-H), 8.43 (d, *J* = 8.4 Hz, 1H, NH), 8.27 (t, *J* = 5.6 Hz, 1H, NH), 8.02 (s, 1H, 3'-H), 7.40 (d, *J* = 14.0 Hz, 2H, 5'-H, 6'-H), 6.62 (s, 1H, 5-H), 4.77 (q, *J* = 8.9 Hz, 1H, CH), 3.65 (t, *J* = 6.5 Hz, 2H, CH_2), 3.23 (q, *J* = 6.7 Hz, 2H, CH_2), 3.05 (s, 6H, $2CH_3$), 2.47 (m, 2H, CH_2),2.01 (p, *J* = 8.8, 7.6 Hz, 4H, $2CH_2$), 1.67 (dt, *J* = 20.9, 7.0 Hz, 4H, $2CH_2$), 1.51 (q, *J* = 7.1 Hz, 2H, CH_2), 1.39 – 1.30 (m, 4H, $2CH_2$); ^{13}C NMR (101 MHz, DMSO-d_6) δ 165.8 (1C, C=O), 162.8 (1C, Ar-C), 154.7 (1C, C=O), 152.2 (1C, Ar-C), 151.1 (1C, Ar-C), 145.1 (1C, Ar-C), 132.2 (1C, Ar-C), 131.1 (1C, Ar-C), 127.5 (1C, Ar-C), 127.2 (1C, S=C=N), 118.2 (1C, Ar-C), 116.4 (1C, Ar-C), 113.7 (1C, Ar-C), 112.1 (1C, Ar-C), 100.7 (1C, Ar-C), 57.1 (1C, CH), 44.7 (1C, CH_2), 39.0 (2C, $2CH_3$), 34.6 (1C, CH_2), 29.7 (2C, $2CH_2$), 29.2 (1C, CH_2), 29.1 (1C, CH_2), 25.8 (1C, CH_2), 25.8 (1C, CH_2), 24.3 (2C, $2CH_2$). HRMS (ESI, m/z) calcd for $C_{28}H_{36}N_7O_3S^+$ [M + H]$^+$, 550.2595, found 550.2598. HPLC purity 99%

(t_R = 4.51 min).

4.1.25.

7-Cyclopentyl-2-((2-hydroxy-4-((7-isothiocyanatoheptyl)carbamoyl)phenyl)amino)-N, N-dimethyl-7H-pyrrolo[2,3-d]pyrimidine-6-carboxamide ***(10de).*** Compound **10de** was prepared using **8de** instead of **7a** in the similar manner of compound **9a** to afford the title product as white solid in 54% yield; mp 188.3 °C. IR (film): 3420.2, 3314.1, 3204.5, 3015.2, 2929.3, 2855.6, 2170.3, 2109.6, 1623.5, 1596.6, 1547.7, 1506.0, 1483.3, 1417.8, 1358.9, 1309.3, 1285.2, 1257.2, 1239.6, 1220.6, 1198.1, 1159.2, 1136.5, 1056.7, 1023.1 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6) δ 10.28 (s, 1H, OH), 8.79 (s, 1H, 4-H), 8.41 (d, *J* = 8.8 Hz, 1H, NH), 8.30 – 8.16 (m, 1H, NH), 8.02 (d, *J* = 10.0 Hz, 1H, 3'-H), 7.40 (q, *J* = 9.3, 7.9 Hz, 2H, 5'-H, 6'-H), 6.63 (s, 1H, 5-H), 4.75 (dd, *J* = 18.0, 9.3 Hz, 1H, CH), 3.70 – 3.61 (m, 2H, CH_2), 3.25 (dd, *J* = 12.2, 5.7 Hz, 2H, CH_2), 3.07 (d, *J* = 10.7 Hz, 6H, $2CH_3$), 2.47 (m, 2H, CH_2), 2.01 (q, *J* = 10.5, 8.9 Hz, 4H, $2CH_2$), 1.75 – 1.61 (m, 4H, $2CH_2$), 1.53 (q, *J* = 7.6 Hz, 2H, CH_2), 1.35 (d, *J* = 10.0 Hz, 6H, $3CH_2$); ^{13}C NMR (101 MHz, DMSO-d_6) δ 165.8 (1C, C=O), 162.8 (1C, Ar-C), 154.7 (1C, C=O), 152.2 (1C, Ar-C), 151.1 (1C, Ar-C), 145.1 (1C, Ar-C), 132.2 (1C, Ar-C), 131.1 (1C, Ar-C), 127.5 (1C, Ar-C), 127.2 (1C, S=C=N), 118.2 (1C, Ar-C), 116.4 (1C, Ar-C), 113.7 (1C, Ar-C), 112.1 (1C, Ar-C), 100.7 (1C, Ar-C), 57.1 (1C, CH), 44.7 (1C, CH_2), 39.0 (2C, $2CH_3$), 38.8 (1C, CH_2), 34.6 (1C, CH_2), 29.6 (2C, $2CH_2$), 29.2 (1C, CH_2), 28.0 (1C, CH_2), 26.4 (1C, CH_2), 26.0 (1C, CH_2), 24.2 (2C, $2CH_2$). HRMS (ESI, m/z) calcd for $C_{29}H_{38}N_7O_3S^+$ $[M + H]^+$, 564.2751, found

564.2755. HPLC purity 98% (t_R = 4.84 min).

4.1.26.

N-(3-((3-Cyclopentyl-3H-[1,2,3]triazolo[4,5-d]pyrimidin-5-yl)amino)phenyl)-8-isothiocyanatooctanamide ***(20).*** Compound **20** was prepared using 1**7** instead of **7a** in the similar manner of compound **9a** to afford the title product as white solid in 49% yield; mp 92.8 °C. IR (film): 3268.9, 3191.5, 3154.7, 3100.5, 3051.8, 2929.9, 2853.8, 2190.4, 2110.8, 1659.2, 1604.7, 1588.0, 1548.2, 1515.1, 1499.1, 1459.8, 1420.5, 1406.9, 1363.2, 1306.1, 1217.0, 1180.4, 1112.6, 1086.8, 1044.7 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6) δ 10.16 (s, 1H, NH), 9.81 (s, 1H, NH), 9.31 (s, 1H, 7-H), 7.76 (d, *J* = 8.5 Hz, 2H, 3', 5'-H), 7.56 (d, *J* = 8.5 Hz, 2H, 2', 6'-H), 5.24 (p, *J* = 7.1 Hz, 1H, CH), 3.65 (t, *J* = 6.5 Hz, 2H, CH_2), 2.30 – 2.18 (m, 6H, $2CH_3$), 1.94 (dt, *J* = 11.2, 4.6 Hz, 2H, CH_2), 1.75 (dd, *J* = 10.3, 5.3 Hz, 2H, CH_2), 1.64 – 1.58 (m, 4H, $2CH_2$), 1.32 (m, 6H, $3CH_2$); ^{13}C NMR (101 MHz, DMSO-d_6) δ 170.8 (1C, C=O), 158.0 (1C, Ar-C), 152.9 (1C, Ar-C), 149.5 (1C, Ar-C), 134.9 (1C, Ar-C), 134.2 (1C, Ar-C), 131.4 (1C, Ar-C), 127.2 (1C, S=C=N), 119.6 (2C, Ar-C), 119.3 (2C, Ar-C), 58.2 (1C, CH), 44.7 (1C, CH_2), 36.3 (1C, CH_2), 33.3 (1C, CH_2), 31.6 (2C, $2CH_2$), 29.2 (1C, CH_2), 28.5 (1C, CH_2), 28.0 (1C, CH_2), 25.9 (1C, CH_2), 24.3 (2C, $2CH_2$).HRMS (ESI, m/z) calcd for $C_{24}H_{31}N_8OS^+$ $[M + H]^+$, 479.2336, found 479.2341. HPLC purity 99% (t_R = 5.63 min).

4.1.27.

7-Cyclopentyl-2-((4-(4-isothiocyanatobutanamido)phenyl)amino)-N,N-dimethyl-7H-p

yrrolo[2,3-d]pyrimidine-6-carboxamide ***(21a).*** Compound **21a** was prepared using **18a** instead of **7a** in the similar manner of compound **9a** to afford the title product as white solid in 20% yield; mp 173.3 °C. IR (film): 3306.1, 3293.3, 3140.1, 3080.5, 3041.3, 3013.0, 2952.6, 2923.8, 2872.1, 2185.5, 2185.5, 1736.2, 1666.6, 1650.7, 1605.1, 1568.9, 1550.5, 1511.6, 1484.9, 1427.8, 1397.4, 1353.4, 1335.6, 1297.8, 1286.9, 1242.4, 1219.7, 1172.7, 1136.3, 1084.5, 1026.1 cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.86 (s, 1H, NH), 9.42 (s, 1H, NH), 8.72 (s, 1H, 4-H), 7.74 (d, *J* = 8.5 Hz, 2H, 3', 5'-H), 7.51 (d, *J* = 8.5 Hz, 2H, 2', 6'-H), 6.56 (s, 1H, 5-H), 4.72 (p, *J* = 9.3 Hz, 1H, CH), 3.75 (t, *J* = 6.7 Hz, 2H, CH_2), 3.05 (s, 6H, $2CH_3$), 2.41 (d, *J* = 7.3 Hz, 4H, $2CH_2$), 1.97 (dt, *J* = 14.4, 7.7 Hz, 6H, $3CH_2$), 1.71 – 1.60 (m, 2H, CH_2); ^{13}C NMR (101 MHz, DMSO-d_6) δ 169.3 (1C, C=O), 162.9 (1C, Ar-C), 155.5 (1C, C=O), 152.0 (1C, Ar-C), 151.3 (1C, Ar-C), 136.4 (1C, Ar-C), 132.7 (1C, Ar-C), 131.4 (1C, Ar-C), 127.5 (1C, S=C=N), 119.5 (2C, Ar-C), 118.5 (2C, Ar-C), 111.3 (1C, Ar-C), 100.6 (1C, Ar-C), 56.9 (1C, CH), 44.4 (1C, CH_2), 32.8 (2C, $2CH_3$), 29.5 (2C, $2CH_2$), 25.2 (1C, CH_2), 24.1 (2C, $2CH_2$). HRMS (ESI, m/z) calcd for $C_{25}H_{30}N_7O_2S^+$ $[M + H]^+$, 492.2176, found 492.2183. HPLC purity 99% (t_R = 6.47 min).

4.1.28.

7-Cyclopentyl-2-((4-(5-isothiocyanatopentanamido)phenyl)amino)-N,N-dimethyl-7H-pyrrolo[2,3-d]pyrimidine-6-carboxamide ***(21b).*** Compound **21b** was prepared using **18b** instead of **7a** in the similar manner of compound **9a** to afford the title product as white solid in 27% yield; mp 147.0 °C. IR (film): 3667.6, 3365.5, 3252.8, 3151.9,

3100.7, 3042.6, 3012.5, 2945.3, 2917.0, 2867.8, 2867.8, 2098.7, 1693.4, 1661.9, 1635.4, 1604.3, 1551.3, 1519.3, 1484.4, 1427.3, 1402.9, 1346.2, 1310.2, 1247.9, 1158.3, 1139.2, 1085.3, 1056.9, 1026.6 cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.81 (s, 1H, NH), 9.46 (d, *J* = 2.4 Hz, 1H, NH), 8.72 (s, 1H, 4-H), 7.74 (dd, *J* = 9.0, 2.6 Hz, 2H, 3', 5'-H), 7.51 (dd, *J* = 9.1, 2.6 Hz, 2H, 2', 6'-H), 6.57 (s, 1H, 5-H), 4.72 (p, *J* = 9.0 Hz, 1H, CH), 3.75 – 3.67 (m, 2H, CH_2), 3.05 (d, *J* = 10.6 Hz, 6H, $2CH_3$), 2.48 – 2.42 (m, 2H, CH_2), 2.33 (t, *J* = 6.1 Hz, 2H, CH_2), 2.01 – 1.92 (m, 4H, $2CH_2$), 1.72 – 1.62 (m, 6H, $3CH_2$); ^{13}C NMR (101 MHz, DMSO-d_6) δ 170.2 (1C, C=O), 162.9 (1C, Ar-C), 155.6 (1C, C=O), 152.1 (1C, Ar-C), 151.3 (1C, Ar-C), 136.4 (1C, Ar-C), 132.8 (1C, Ar-C), 131.4 (1C, Ar-C), 127.2 (1C, S=C=N), 119.4 (2C, Ar-C), 118.5 (2C, Ar-C), 111.3 (1C, Ar-C), 100.7 (1C, Ar-C), 56.9 (1C, CH), 44.6 (1C, CH_2), 35.3 (2C, $2CH_3$), 34.6 (1C, CH_2), 29.6 (2C, $2CH_2$), 28.9 (1C, CH_2), 24.2 (2C, $2CH_2$), 22.2 (1C, CH_2). HRMS (ESI, m/z) calcd for $C_{26}H_{32}N_7O_2S^+$ $[M + H]^+$, 506.2333, found 506.2335. HPLC purity 98% (t_R = 6.49 min).

4.1.29.

7-Cyclopentyl-2-((4-(6-isothiocyanatohexanamido)phenyl)amino)-N,N-dimethyl-7H-pyrrolo[2,3-d]pyrimidine-6-carboxamide ***(21c).*** Compound **21c** was prepared using **18c** instead of **7a** in the similar manner of compound **9a** to afford the title product as white solid in 68% yield; mp 183.9 °C. IR (film): 3285.8, 3200.4, 3140.3, 3082.1, 3042.3, 3017.0, 2979.8, 2946.5, 2913.0, 2864.7, 2163.3, 2085.2, 1736.1, 1655.7, 1608.3, 1568.0, 1550.5, 1513.7, 1481.1, 1427.0, 1397.7, 1353.4, 1302.4, 1242.1,

1219.6, 1172.8, 1133.0, 1083.3, 1014.3 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6) δ 9.77 (s, 1H, NH), 9.45 (s, 1H, NH), 8.72 (s, 1H, 4-H), 7.75 (d, *J* = 8.4 Hz, 2H, 3', 5'-H), 7.51 (d, *J* = 8.6 Hz, 2H, 2', 6'-H), 6.56 (s, 1H, 5-H), 4.72 (p, *J* = 8.8 Hz, 1H, CH), 3.67 (t, *J* = 6.7 Hz, 2H, CH_2), 3.05 (s, 6H, $2CH_3$), 2.30 (t, *J* = 7.5 Hz, 2H, CH_2), 1.96 (s, 4H, $2CH_2$), 1.64 (p, *J* = 8.1 Hz, 6H, $3CH_2$), 1.39 (d, *J* = 10.0 Hz, 2H, CH_2); ^{13}C NMR (101 MHz, DMSO-d_6) δ 170.5 (1C, C=O), 162.9 (1C, Ar-C), 155.6 (1C, C=O), 152.1 (1C, Ar-C), 151.3 (1C, Ar-C), 136.4 (1C, Ar-C), 132.9 (1C, Ar-C), 131.4 (1C, Ar-C), 127.2 (1C, S=C=N), 119.4 (2C, Ar-C), 118.5 (2C, Ar-C), 111.3 (1C, Ar-C), 100.7 (1C, Ar-C), 56.9 (1C, CH), 44.6 (1C, CH_2), 39.9 (2C, $2CH_3$), 36.1 (1C, CH_2), 29.6 (2C, $2CH_2$), 29.1 (1C, CH_2), 25.7 (1C, CH_2), 24.4 (1C, CH_2), 24.2 (2C, $2CH_2$). HRMS (ESI, m/z) calcd for $C_{27}H_{34}N_7O_2S^+$ $[M + H]^+$, 520.2489, found 520.2487. HPLC purity 99% (t_R = 6.55 min).

4.1.30.

7-Cyclopentyl-2-((4-(7-isothiocyanatoheptanamido)phenyl)amino)-N,N-dimethyl-7H-pyrrolo[2,3-d]pyrimidine-6-carboxamide ***(21d).*** Compound **21d** was prepared using **18d** instead of **7a** in the similar manner of compound **9a** to afford the title product as white solid in 71% yield; mp 90.1 °C. IR (film): 3271.3, 3043.0, 3014.2, 2930.9, 2859.8, 2179.6, 2100.0, 1659.3, 1601.2, 1568.6, 1538.9, 1513.4, 1486.8, 1426.9, 1397.0, 1349.6, 1305.0, 1246.0, 1220.3, 1155.4, 1137.2, 1083.3, 1023.8 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6) δ 9.75 (s, 1H, NH), 9.45 (s, 1H, NH), 8.72 (s, 1H, 4-H), 7.74 (d, *J* = 8.5 Hz, 2H, 3', 5'-H), 7.51 (d, *J* = 8.6 Hz, 2H, 2', 6'-H), 6.56 (s, 1H, 5-H),

4.72 (p, *J* = 8.9 Hz, 1H, CH), 3.65 (t, *J* = 6.5 Hz, 2H, CH_2), 3.05 (s, 6H, $2CH_3$), 2.46 (m, 2H, CH_2),2.28 (t, *J* = 7.4 Hz, 2H, CH_2), 1.96 (q, *J* = 9.0, 8.3 Hz, 4H, $2CH_2$), 1.63 (dq, *J* = 18.8, 6.9 Hz, 6H, $3CH_2$), 1.35 (h, *J* = 6.2, 5.4 Hz, 4H, $2CH_2$); ^{13}C NMR (101 MHz, DMSO-d_6) δ 170.6 (1C, C=O), 162.9 (1C, Ar-C), 155.6 (1C, C=O), 152.1 (1C, Ar-C), 151.3 (1C, Ar-C), 136.3 (1C, Ar-C), 132.9 (1C, Ar-C), 131.4 (1C, Ar-C), 127.2 (1C, S=C=N), 119.4 (2C, Ar-C), 118.5 (2C, Ar-C), 111.3 (1C, Ar-C), 100.7 (1C, Ar-C), 56.9 (1C, CH), 44.7 (1C, CH_2), 39.6 (2C, $2CH_3$), 36.2 (1C, CH_2), 29.6 (2C, $2CH_2$), 29.1 (1C, CH_2), 27.9 (1C, CH_2), 25.8 (1C, CH_2), 25.0 (1C, CH_2), 24.2 (2C, $2CH_2$). HRMS (ESI, m/z) calcd for $C_{28}H_{36}N_7O_2S^+$ $[M + H]^+$, 534.2646, found 534.2649. HPLC purity 98% (t_R = 6.67 min).

4.1.31.

7-Cyclopentyl-2-((4-(8-isothiocyanatooctanamido)phenyl)amino)-N,N-dimethyl-7H-pyrrolo[2,3-d]pyrimidine-6-carboxamide ***(21e).*** Compound **21e** was prepared using **18e** instead of **7a** in the similar manner of compound **9a** to afford the title product as white solid in 43% yield; mp 167.7 °C. IR (film): 3313.5, 3259.2, 3217.0, 3149.4, 3124.5, 3091.1, 3044.2, 3011.5, 2934.4, 2914.5, 2854.8, 2178.5, 2122.4, 2095.7, 1694.1, 1603.5, 1585.3, 1549.5, 1518.1, 1486.5, 1428.2, 1402.0, 1357.2, 1339.1, 1303.3, 1289.5, 1248.3, 1166.3, 1140.6, 1083.0, 1053.8, 1026.1 cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.75 (s, 1H, NH), 9.45 (s, 1H, NH), 8.72 (s, 1H, 4-H), 7.74 (d, *J* = 8.5 Hz, 2H, 3', 5'-H), 7.51 (d, *J* = 8.5 Hz, 2H, 2', 6'-H), 6.56 (s, 1H, 5-H), 4.72 (p, *J* = 8.9 Hz, 1H, CH), 3.65 (t, *J* = 6.5 Hz, 2H, CH_2), 3.05 (s, 6H, $2CH_3$), 2.46 (m, 2H, CH_2),

2.27 (t, J = 7.4 Hz, 2H, CH_2), 1.96 (q, J = 8.7, 7.7 Hz, 4H, $2CH_2$), 1.62 (qt, J = 14.1, 7.2 Hz, 6H, $3CH_2$), 1.32 (s, 6H, $3CH_2$); ^{13}C NMR (101 MHz, DMSO-d_6) δ 170.7 (1C, C=O), 162.9 (1C, Ar-C), 155.6 (1C, C=O), 152.1 (1C, Ar-C), 151.3 (1C, Ar-C), 136.3 (1C, Ar-C), 133.0 (1C, Ar-C), 131.4 (1C, Ar-C), 127.2 (1C, S=C=N), 119.4 (2C, Ar-C), 118.5 (2C, Ar-C), 111.3 (1C, Ar-C), 100.8 (1C, Ar-C), 56.9 (1C, CH), 44.7 (1C, CH_2), 39.6 (2C, $2CH_3$), 36.3 (1C, CH_2), 29.6 (1C, CH_2), 29.2 (2C, $2CH_2$), 28.6 (1C, CH_2), 28.0 (1C, CH_2), 25.9 (1C, CH_2), 25.1 (1C, CH_2), 24.2 (2C, $2CH_2$). HRMS (ESI, m/z) calcd for $C_{29}H_{38}N_7O_2S^+$ $[M + H]^+$, 548.2802, found 548.2805. HPLC purity 99% (t_R = 6.81 min).

4.1.32.

7-Cyclopentyl-2-((3-(8-isothiocyanatooctanamido)phenyl)amino)-N,N-dimethyl-7H-pyrrolo[2,3-d]pyrimidine-6-carboxamide ***(21f).*** Compound **21f** was prepared using **18f** instead of **7a** in the similar manner of compound **9a** to afford the title product as light-yellow solid in 52% yield; mp 197.4 °C. IR (film): 3268.4, 3217.3, 3137.4, 3107.3, 3078.3, 3023.7, 2931.5, 2868.6, 2851.4, 2189.8, 2108.7, 1683.7, 1601.1, 1577.8, 1537.8, 1485.6, 1416.6, 1358.3, 1307.4, 1283.6, 1255.3, 1241.0, 1198.6, 1163.6, 1139.7, 1084.8 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.67 (s, 1H, 4-H), 7.86 (s, 1H, 2'-H), 7.63 (s, 1H, NH), 7.56 (d, J = 8.8 Hz, 2H, NH and 4'-H), 7.27 (d, J = 7.9 Hz, 1H, 6'-H), 7.20 (d, J = 8.0 Hz, 1H, 5'-H), 6.43 (s, 1H, 5-H), 4.84 (q, J = 9.0 Hz, 1H, CH), 3.49 (t, J = 6.6 Hz, 2H, CH_2), 3.16 (s, 6H, $2CH_3$), 2.55 (s, 2H, CH_2), 2.35 (t, J = 7.4 Hz, 2H, CH_2), 2.05 (dd, J = 20.9, 10.0 Hz, 4H, $2CH_2$), 1.75 – 1.65 (m, 6H,

$3CH_2$), 1.37 (d, *J* = 11.0 Hz, 6H, $3CH_2$); ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.3 (1C, C=O), 164.2 (1C, Ar-C), 155.5 (1C, C=O), 152.2 (1C, Ar-C), 151.7 (1C, Ar-C), 140.9 (1C, Ar-C), 138.7 (1C, Ar-C), 132.0 (1C, Ar-C), 129.3 (1C, Ar-C), 127.6 (1C, S=C=N), 114.6 (1C, Ar-C), 113.4 (1C, Ar-C), 112.5 (1C, Ar-C), 110.2 (1C, Ar-C), 101.1 (1C, Ar-C), 57.8 (C, CH), 45.1 (C, CH_2), 39.5 (2C, $2CH_3$), 37.7 (C, CH_2), 30.4 (2C, $2CH_2$), 29.9 (C, CH_2), 29.1 (C, CH_2), 28.6 (C, CH_2), 26.5 (C, CH_2), 25.4 (C, CH_2), 24.8 (2C, $2CH_2$). HRMS (ESI, m/z) calcd for $C_{29}H_{38}N_7O_2S^+$ $[M + H]^+$, 548.2802, found 548.2807. HPLC purity 99% (t_R = 4.85 min).

4.1.33.

7-Cyclopentyl-2-((2-(8-isothiocyanatooctanamido)phenyl)amino)-N,N-dimethyl-7H-pyrrolo[2,3-d]pyrimidine-6-carboxamide ***(21g).*** Compound **21g** was prepared using **18g** instead of **7a** in the similar manner of compound **9a** to afford the title product as light-yellow solid in 26% yield; mp 77.3 °C. IR (film): 3414.0, 3254.9, 3118.7, 3014.9, 2929.3, 2857.1, 2177.8, 2090.5, 1594.7, 1561.3, 1513.9, 1488.3, 1422.5, 1394.9, 1347.9, 1284.8, 1250.0, 1137.0, 1083.1, 1023.7 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6) δ 9.70 (s, 1H, NH), 8.70 (s, 1H, 4-H), 8.36 (s, 1H, NH), 7.98 (d, *J* = 8.2 Hz, 1H, 3'-H), 7.39 (d, *J* = 7.9 Hz, 1H, 6'-H), 7.18 (t, *J* = 7.9 Hz, 1H, 5'-H), 7.05 (t, *J* = 7.8 Hz, 1H, 4'-H), 6.56 (s, 1H, 5-H), 4.68 (q, *J* = 8.7 Hz, 1H, CH), 3.61 (t, *J* = 6.1 Hz, 2H, CH_2), 3.04 (s, 6H, $2CH_3$), 2.33 (dt, *J* = 30.8, 9.2 Hz, 4H, $2CH_2$), 1.87 (d, *J* = 39.7 Hz, 4H, $2CH_2$), 1.57 (dq, *J* = 19.5, 6.9 Hz, 6H, $3CH_2$), 1.29 (s, 6H, $3CH_2$); ^{13}C NMR (101 MHz, DMSO-d_6) δ 172.4 (1C, C=O), 163.3 (1C, Ar-C), 156.2 (1C, C=O),

152.6 (1C, Ar-C), 151.8 (1C, Ar-C), 133.8 (1C, Ar-C), 132.1 (1C, Ar-C), 129.7 (1C, Ar-C), 129.6 (1C, Ar-C), 127.6 (1C, S=C=N), 125.4 (1C, Ar-C), 123.4 (1C, Ar-C), 123.2 (1C, Ar-C), 112.4 (1C, Ar-C), 101.0 (1C, Ar-C), 57.1 (C, CH), 45.1 (C, CH_2), 36.3 (C, CH_2), 35.0 (C, $2CH_3$), 30.5 (C, $2CH_2$), 29.6 (C, CH_2), 28.8 (C, CH_2), 28.4 (C, CH_2), 26.3 (C, CH_2), 25.7 (C, CH_2), 25.0 (2C, $2CH_2$). HRMS (ESI, m/z) calcd for $C_{29}H_{38}N_7O_2S^+$ $[M + H]^+$, 548.2802, found 548.2803. HPLC purity 99% (t_R = 4.74 min).

4.1.34.

7-Cyclopentyl-2-((4-((8-isothiocyanatooctanamido)methyl)phenyl)amino)-N,N-dimethyl-7H-pyrrolo[2,3-d]pyrimidine-6-carboxamide ***(22).*** Compound **22** was prepared using **19** instead of **7a** in the similar manner of compound **9a** to afford the title product as white solid in 54% yield; mp 76.9 °C. IR (film): 3273.5, 3191.3, 3108.4, 3078.7, 2923.7, 2854.9, 2177.5, 2091.2, 1728.2, 1616.4, 1596.0, 1561.7, 1520.4, 1487.0, 1426.7, 1351.0, 1286.2, 1246.9, 1137.1, 1082.0, 1022.1 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) δ 8.63 (s, 1H, 4-H), 7.69 – 7.64 (m, 2H, 3', 5'-H), 7.42 (s, 1H, NH), 7.26 – 7.20 (m, 2H, 2', 6'-H), 6.42 (s, 1H, 5-H), 5.77 (s, 1H, NH), 4.77 (p, *J* = 8.8 Hz, 1H, CH), 4.40 (d, *J* = 5.4 Hz, 2H, CH_2), 3.48 (td, *J* = 6.6, 2.2 Hz, 2H, CH_2), 3.14 (s, 6H, $2CH_3$), 2.58 (dt, *J* = 11.8, 5.2 Hz, 2H, CH_2), 2.20 (t, *J* = 7.6 Hz, 2H, CH_2), 2.09 – 2.00 (m, 4H, $2CH_2$), 1.68 (dt, *J* = 15.3, 6.5 Hz, 6H, $3CH_2$), 1.33 (dt, *J* = 5.7, 2.7 Hz, 6H, $3CH_2$); ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.8 (1C, C=O), 164.2 (1C, Ar-C), 155.4 (1C, C=O), 152.1 (1C, Ar-C), 151.6 (1C, Ar-C), 139.7 (1C, Ar-C), 132.1 (1C, Ar-C),

131.6 (1C, Ar-C), 129.5 (1C, S=C=N), 128.7 (2C, Ar-C), 118.8 (2C, Ar-C), 112.5 (1C, Ar-C), 101.1 (1C, Ar-C), 58.0 (C, CH), 45.1 (C, CH_2), 43.4 (C, CH_2), 36.8 (2C, $2CH_3$), 30.2 (2C, $2CH_2$), 29.9 (C, CH_2), 29.8 (C, CH_2), 29.1 (C, CH_2), 28.6 (C, CH_2), 26.5 (C, CH_2), 25.7 (C, CH_2), 24.8 (2C, $2CH_2$). HRMS (ESI, m/z) calcd for $C_{30}H_{40}N_7O_2S^+$ $[M + H]^+$, 562.2959, found 562.2961. HPLC purity 99% (t_R = 4.50 min).

4.1.35.

7-Cyclopentyl-2-((5-(4-(8-isothiocyanatooctanoyl)piperazin-1-yl)pyridin-2-yl)methyl) -N,N-dimethyl-7H-pyrrolo[2,3-d]pyrimidine-6-carboxamide ***(31).*** Compound **31** was prepared using **28** instead of **7a** in the similar manner of compound **9a** to afford the title product as white solid in 72% yield; mp 134.9 °C. IR (film): 3220.1, 3190.6, 3154.0, 3084.0, 3031.7, 2945.4, 2929.1, 2854.8, 2184.5, 2099.5, 1659.4, 1637.0, 1590.3, 1558.4, 1529.8, 1470.3, 1419.6, 1392.2, 1358.4, 1316.1, 1273.9, 1259.7, 1227.9, 1195.3, 1158.2, 1133.4, 1079.3, 1059.3, 1027.3, 1011.4 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6) δ 9.55 (s, 1H, NH), 8.80 (s, 1H, 4-H), 8.19 (d, *J* = 9.1 Hz, 1H, 4'-H), 8.06 (s, 1H, 6'-H), 7.47 (d, *J* = 9.1 Hz, 1H, 3'-H), 6.60 (s, 1H, 5-H), 4.74 (p, *J* = 9.0 Hz, 1H, CH), 3.67 – 3.57 (s, 6H, $2CH_3$), 3.09 (m, 10H, $5CH_2$), 2.43 (m, 2H, CH_2), 2.34 (s, 2H, CH_2), 1.98 (p, *J* = 8.2, 7.5 Hz, 4H, $2CH_2$), 1.69 – 1.59 (m, 4H, $2CH_2$), 1.51 (t, *J* = 7.4 Hz, 2H, CH_2), 1.32 (d, *J* = 10.8 Hz, 6H, $3CH_2$); ^{13}C NMR (101 MHz, DMSO-d_6) δ 170.6 (1C, C=O), 162.8 (1C, Ar-C), 154.6 (1C, C=O), 152.0 (1C, Ar-C), 151.2 (1C, Ar-C), 146.5 (1C, Ar-C), 142.0 (1C, Ar-C), 136.0 (1C, Ar-C), 131.9 (1C, Ar-C), 127.3 (1C, S=C=N), 126.0 (1C, Ar-C), 112.5 (1C, Ar-C), 111.8 (1C, Ar-C),

100.5 (1C, Ar-C), 56.9 (1C, CH), 49.4 (1C, CH_2), 49.0 (1C, CH_2), 44.7 (1C, CH_2), 40.7 (2C, $2CH_3$), 32.1 (1C, CH_2), 29.7 (2C, $2CH_2$), 29.2 (1C, CH_2), 28.5 (1C, CH_2), 27.9 (1C, CH_2), 25.8 (1C, CH_2), 24.6 (1C, CH_2), 24.2 (2C, $2CH_2$). HRMS (ESI, m/z) calcd for $C_{32}H_{44}N_9O_2S^+$ $[M + H]^+$, 618.3333, found 618.3340. HPLC purity 98% (t_R = 5.19 min).

4.1.36.

7-Cyclopentyl-2-((2-fluoro-4-(8-isothiocyanatooctanamido)phenyl)amino)-N,N-dimethyl-7H-pyrrolo[2,3-d]pyrimidine-6-carboxamide ***(32a).*** Compound **32a** was prepared using **29a** instead of **7a** in the similar manner of compound **9a** to afford the title product as light-yellow solid in 68% yield; mp 157.4 °C. IR (film): 3247.0, 3191.0, 3137.1, 3077.9, 2951.7, 2933.0, 2856.4, 2178.0, 2102.1, 1681.9, 1606.0, 1542.2, 1519.5, 1480.7, 1422.9, 1403.0, 1350.6, 1330.8, 1291.6, 1252.4, 1212.6, 1164.2, 1139.0, 1099.4, 1084.5, 1031.8 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6) δ 10.00 (s, 1H, NH), 8.74 (s, 1H, 4-H), 8.68 (s, 1H, NH), 7.71 – 7.65 (m, 2H, 3'-H, 6'-H), 7.27 – 7.22 (m, 1H, 5'-H), 6.54 (s, 1H, 5-H), 4.64 (t, *J* = 8.7 Hz, 1H, CH), 3.65 (t, *J* = 6.5 Hz, 2H, CH_2), 3.03 (s, 6H, $2CH_3$), 2.30 (q, *J* = 10.3, 8.9 Hz, 4H, $2CH_2$), 1.93 – 1.86 (m, 2H, CH_2), 1.75 (s, 2H, CH_2), 1.61 (dt, *J* = 14.0, 6.9 Hz, 4H, $2CH_2$), 1.50 (q, *J* = 6.2, 5.8 Hz, 2H, CH_2), 1.34 (m, 6H, $3CH_2$); ^{13}C NMR (101 MHz, DMSO-d_6) δ 171.2 (1C, C=O), 162.9 (1C, Ar-C), 156.3 (1C, C=O), 155.9, 153.5, 151.9, 151.5, 135.9, 135.8, 131.5, 127.3 (1C, S=C=N), 124.8, 122.9, 122.8, 114.1, 111.6, 106.5, 106.2, 100.4 (11C, Ar-C), 56.6 (1C, CH), 44.7 (1C, CH_2), 36.3 (2C, $2CH_3$), 34.6 (1C, CH_2), 29.9

(2C, $2CH_2$), 29.2 (1C, CH_2), 28.4 (1C, CH_2), 27.9 (1C, CH_2), 25.8 (1C, CH_2), 24.9 (1C, CH_2), 24.4 (2C, $2CH_2$). HRMS (ESI, m/z) calcd for $C_{29}H_{37}FN_7O_2S^+$ $[M + H]^+$, 566.2708, found 566.2714. HPLC purity 98% (t_R = 5.11 min).

4.1.37.

7-Cyclopentyl-2-((3-fluoro-4-(8-isothiocyanatooctanamido)phenyl)amino)-N,N-dimethyl-7H-pyrrolo[2,3-d]pyrimidine-6-carboxamide ***(32b).*** Compound **32b** was prepared using **29b** instead of **7a** in the similar manner of compound **9a** to afford the title product as light-yellow solid in 59% yield; mp 134.3 °C. IR (film): 3258.0, 3148.2, 3080.7, 3056.7, 3012.1, 2941.8, 2916.9, 2849.3, 2182.0, 2162.3, 2085.4, 1696.1, 1614.0, 1598.6, 1548.8, 1518.9, 1487.6, 1427.6, 1410.8, 1396.9, 1357.3, 1336.1, 1296.0, 1271.9, 1250.2, 1211.2, 1161.6, 1140.6, 1106.8, 1081.8, 1053.9, 1028.6 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6) δ 9.73 (s, 1H, NH), 9.47 (s, 1H, NH), 8.77 (s, 1H, 4-H), 8.01 (d, *J* = 14.3 Hz, 1H, 5'-H), 7.59 (t, *J* = 8.9 Hz, 1H, 2'-H), 7.38 (d, *J* = 8.8 Hz, 1H, 6'-H), 6.60 (s, 1H, 5-H), 4.71 (q, *J* = 8.9 Hz, 1H, CH), 3.65 (t, *J* = 6.6 Hz, 2H, CH_2), 3.06 (s, 6H, $2CH_3$), 2.46 (m, 2H, CH_2),2.33 (t, *J* = 7.4 Hz, 2H, CH_2), 1.99 (dq, *J* = 12.9, 7.2 Hz, 4H, $2CH_2$), 1.63 (dp, *J* = 20.9, 7.3, 6.7 Hz, 6H, $3CH_2$), 1.32 (m, 6H, $3CH_2$); ^{13}C NMR (101 MHz, DMSO-d_6) δ 171.3 (1C, C=O), 162.8 (1C, C=O), 155.5, 155.1, 153.1, 152.1, 151.0, 138.8, 138.7, 131.9, 127.3, 125.2 (1C, S=C=N), 118.7, 118.5, 113.5, 111.8, 105.0, 104.7, 100.6 (11C, Ar-C), 57.1 (1C, CH), 44.7 (1C, CH_2), 35.5 (2C,$2CH_3$), 34.6 (1C, CH_2), 29.5 (2C, $2CH_2$), 29.2 (1C, CH_2), 28.4 (1C, CH_2), 27.9 (1C, CH_2), 25.8 (1C, CH_2), 25.0 (1C, CH_2), 23.9 (2C, $2CH_2$). HRMS (ESI, m/z)

calcd for $C_{29}H_{37}FN_7O_2S^+$ $[M + H]^+$, 566.2708, found 566.2705. HPLC purity 98% (t_R = 4.77 min).

4.1.38.

7-Cyclopentyl-2-((4-(8-isothiocyanatooctanamido)-2-(trifluoromethyl)phenyl)amino)-N,N-dimethyl-7H-pyrrolo[2,3-d]pyrimidine-6-carboxamide ***(32c).*** Compound **32c** was prepared using **29c** instead of **7a** in the similar manner of compound **9a** to afford the title product as light-yellow solid in 55% yield; mp 155.6 °C. IR (film): 3298.0, 3259.5, 3185.7, 3132.1, 3078.8, 3013.3, 2944.2, 2926.3, 2858.2, 2185.4, 2103.0, 1690.6, 1606.5, 1579.8, 1547.6, 1508.3, 1477.5, 1445.7, 1421.5, 1348.2, 1324.9, 1294.1, 1254.4, 1233.1, 1152.2, 1128.9, 1052.3 cm^{-1}. ^1H NMR (400 MHz, DMSO-d_6) δ 10.16 (s, 1H, NH), 8.65 (s, 1H, 4-H), 8.41 (s, 1H, NH), 8.10 (s, 1H, 3'-H), 7.80 (dd, *J* = 8.7, 2.5 Hz, 1H, 5'-H), 7.66 (t, *J* = 7.9 Hz, 1H, 6'-H), 6.53 (s, 1H, 5-H), 4.61 (p, *J* = 8.4 Hz, 1H, CH), 3.66 (t, *J* = 6.4 Hz, 2H, CH_2), 3.02 (s, 6H, $2CH_3$), 2.34 (t, *J* = 7.3 Hz, 2H, CH_2), 2.14 (dt, *J*= 12.6, 6.1 Hz, 2H, CH_2), 1.84 (ddd, *J* = 15.8, 7.9, 4.4 Hz, 2H, CH_2), 1.60 (dt, *J* = 25.7, 8.4 Hz, 6H, $3CH_2$), 1.44 – 1.29 (m, 8H, $4CH_2$); ^{13}C NMR (101 MHz, DMSO-d_6) δ 171.5 (1C, C=O), 162.9 (1C, Ar-C), 157.6 (1C, C=O), 152.0, 151.5, 136.5, 132.8, 131.4, 129.9, 127.2 (1C, S=C=N), 125.2, 122.8, 122.5, 116.2, 111.5, 100.4 (12C, Ar-C and CF_3), 56.4 (1C, CH), 44.7 (1C, CH_2), 36.3 (2C, $2CH_3$), 34.6 (1C, CH_2), 30.0 (1C, CH_2), 29.2 (2C, $2CH_2$), 28.4 (1C, CH_2), 28.0 (1C, CH_2), 25.9 (1C, CH_2), 24.9 (1C, CH_2), 24.5 (2C, $2CH_2$). HRMS (ESI, m/z) calcd for $C_{30}H_{37}F_3N_7O_2S^+$ $[M + H]^+$, 616.2676, found 616.2672. HPLC purity 98% (t_R = 5.32

min).

4.1.39.

7-Cyclopentyl-2-(2-(8-isothiocyanatooctanoyl)hydrazinyl)-N,N-dimethyl-7H-pyrrolo[2,3-d]pyrimidine-6-carboxamide ***(33).*** Compound **33** was prepared using **30** instead of **7a** in the similar manner of compound **9a** to afford the title product as light-yellow solid in 36% yield; mp 116.5 °C. IR (film): 3232.3, 3117.2, 2932.3, 2855.9, 2179.3, 2088.4, 1686.1, 1601.9, 1536.3, 1479.4, 1428.3, 1397.0, 1356.9, 1251.7, 1150.2, 1135.8, 1085.1, 1026.9 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.62 (s, 1H, 4-H), 7.87 (s, 1H, NH), 7.17 (s, 1H, NH), 6.41 (s, 1H, 5-H), 4.75 (p, *J* = 8.7 Hz, 1H, CH), 3.54 – 3.43 (m, 2H, CH_2), 3.08 (s, 6H, $2CH_3$), 2.43 – 2.28 (m, 4H, $2CH_2$), 2.06 – 1.94 (m, 4H, $2CH_2$), 1.70 (dd, *J* = 10.8, 6.6 Hz, 6H, $3CH_2$), 1.44 – 1.28 (m, 6H, $3CH_2$); ^{13}C NMR (101 MHz, $CDCl_3$) δ 179.6 (1C, C=O), 172.3 (1C, C=O), 164.1 (1C, Ar-C), 158.6 (1C, Ar-C), 152.5 (1C, Ar-C), 151.6 (1C, Ar-C), 132.5 (1C, S=C=N), 113.7 (1C, Ar-C), 100.8 (1C, Ar-C), 57.6 (1C, CH), 45.1 (1C, CH_2), 39.5 (2C, $2CH_3$), 34.3 (1C, CH_2), 30.9 (2C, $2CH_2$), 30.8 (1C, CH_2), 30.0 (1C, CH_2), 29.2 (1C, CH_2), 28.7 (1C, CH_2), 26.5 (1C, CH_2), 25.3 (2C, $2CH_2$). HRMS (ESI, m/z) calcd for $C_{23}H_{34}N_7O_2S^+$ $[M + H]^+$, 472.2489, found 472.2493. HPLC purity 98% (t_R = 3.76 min).

4.1.40.

7-Cyclopentyl-2-((4-isothiocyanatophenyl)amino)-N,N-dimethyl-7H-pyrrolo[2,3-d]pyrimidine-6-carboxamide ***(34).*** Compound **34** was prepared using **26d** instead of **7a** in the similar manner of compound **9a** to afford the title product as light-yellow solid in

63% yield; mp 210.5 °C. IR (film): 3322.9, 3252.7, 3177.7, 3109.6, 3043.7, 3011.8, 2954.1, 2928.0, 2849.6, 2171.3, 2088.8, 1624.4, 1608.3, 1595.0, 1566.3, 1534.9, 1485.3, 1432.9, 1402.1, 1352.8, 1324.3, 1310.8, 1271.6, 1243.1, 1168.0, 1141.0, 1110.1, 1087.2, 1067.6, 1025.4 cm^{-1}. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.86 (d, *J* = 2.2 Hz, 1H, NH), 8.79 (d, *J* = 2.2 Hz, 1H, 4-H), 7.93 – 7.88 (m, 2H, 3', 5'-H), 7.39 (dd, *J* = 8.9, 2.3 Hz, 2H, 2', 6'-H), 6.61 (d, *J* = 2.2 Hz, 1H, 5-H), 4.74 (p, *J* = 8.9 Hz, 1H, CH), 3.10 – 3.02 (m, 6H, $2CH_3$), 2.42 (m, 2H, CH_2), 2.02 – 1.92 (m, 4H, $2CH_2$), 1.66 (m, 2H, CH_2); ^{13}C NMR (101 MHz, DMSO-d_6) δ 162.8 (1C, Ar-C), 154.9 (1C, C=O), 152.1 (1C, Ar-C), 151.0 (1C, Ar-C), 140.8 (1C, Ar-C), 132.1 (1C, Ar-C), 131.4 (1C, S=C=N), 126.4 (2C, Ar-C), 121.5 (1C, Ar-C), 118.6 (2C, Ar-C), 112.0 (1C, Ar-C), 100.6 (1C, Ar-C), 57.0 (1C, CH), 33.4 (2C, $2CH_3$), 29.7 (2C, $2CH_2$), 24.1 (2C, $2CH_2$). HRMS (ESI, m/z) calcd for $C_{21}H_{23}N_6OS^+$ $[M + H]^+$, 407.1649, found 407.1656. HPLC purity 98% (t_R = 7.57 min).

4.2. Biology Methods.

4.2.1. Cell lines and Cell Culture.

All the human cancer cell lines were obtained from ATCC. Cells were cultured in DMEM (BioInd) or RPMI-1640 (BioInd) medium according to the instructions from ATCC, with the medium containing 10% FBS (BioInd), 1% antibiotics (penicillin and streptomycin) at 37 °C in an atmosphere of 5% CO_2.

4.2.2. Cytotoxicity Assay.

A Cell Counting Kit-8 assay (Promega, WI) was preformed to the drug cytotoxicity against the human cell lines. Cells were treated with various concentrations of **21e**, ribociclib or SFN in 96-well culture plates for 72 h in final volumes of 200 μL (3000 cells per well for adhere cells and 10,000 cells per well for suspension cells). 20 μL of CCK-8 was added to each well and incubated for 1-4 hours. Finally, absorbance values of test wells (A_S), control wells (A_C), and blank wells (A_b) were readings at a wavelength of 450 nm taken on a spectrophotometer (Promega, WI). Inhibition ratio was calculated as follows: $[(A_C - A_S)/ (A_C - A_b)] \times 100\%$. IC_{50} values were calculated using GraphPad 6.0 software.

4.2.3. Cell Cycle Assay.

A549 and H1299 were plated on 6-well culture plates at a density of 5×10^5 cells/mL. The cells were treated with the indicated concentrations of **21e**, ribociclib or SFN for 24 h after they adherence. Cells were washed with PBS for three times and then fixed with ice cold 75% ethanol overnight. The fixed cells were then washed with PBS and stained with propidium iodide (50 mg/mL) in the presence of RNase A (0.5 mg) for 30 min at 37 °C. The stained cells were then subjected to flow cytometry (Modfit, BD) for cell cycle analysis.

4.2.4. Annexin V-FITC/ PI Apoptosis Assay.

Cells at a density of 3×10^5 cells/mL were seeded in 6-well plates and treated with compounds at different concentrations for 48 h. The cells were then harvested and

washed twice with cold PBS. Then the cells were subjected to an Annexin V/PI Apoptosis Detection kit (BD Biosciences) for staining according to manufacture's instructions, and finally analyzed by flow cytometry (Modfit, BD).

4.2.5. Western Blotting.

Protein extraction and western blotting methods were performed as described previously.[58] The antibodies used in this study including anti-β-actin (Santa Cruz; sc-47778), anti-Bcl-2 (BD; #51-6511GR), anti-Bim (BD; #559685), anti-Caspase-3 (Santa Cruz; sc-7148), anti-CDK9 (CST; #2316), anti-Cyclin T1(CST; #81464), anti-Cyclin D1 (CST; #2978), anti-Rb (CST; #9309), anti-phospho-Rb $^{Ser807/811}$ (CST; #8516), anti- phospho-Rb ^{Ser780} (CST; #9307), anti-RNA polymerase II CTD (Abcam; ab817), anti-RNA polymerase II CTD $^{phospho\ S5}$ (Abcam; ab5131), anti-RNA polymerase II CTD $^{phospho\ S2}$ (Abcam; ab5095), anti-Klf4 (CST; #4038), anti-SOX2 (Santa cruz; sc-20088), anti-Oct4 (Abcam;ab19857), anti-Nanog (Abcam; ab80892).

4.2.6. Colony Formation Assay.

A549 and H1299 cells treated with indicated compounds of **21e** were washed with PBS, trypsinized, and reseeded into 6-well plates at 300 cells per well. The colonies were allowed to form for 10-14 days. At the end of the culture, cells were washed with PBS, fixed with methanol for 30 min, then stained with 0.5% crystal violet overnight. After careful washing, the images were taken.

4.2.7. Sphere Formation Assay.

A549 and H1299 cells with different concentration of compounds were seeded in low-adherent 48-well culture plates (Corning, NY, USA) at 1 $\times 10^3$ cells per well, and incubated under serum-free condition in RPMI 1640 (BioInd) containing 20 ng/mL of B27 (Invitrogen, CA, USA), 20 ng/mL of epidermal growth factor (EGF) (Invitrogen, Carlsbad, CA, USA), 20 ng/mL of basic fibroblast growth factor (bFGF) (Invitrogen, Carlsbad, CA, USA) and 1% of penicillin-streptomycin (HyClone, Logan City, Utah, USA). After incubation at 37 °C in a 5% CO_2 incubator for 10-14 days, pictures were taken under a microscope and the number of spheres was counted in three separate fields.

4.2.8. Side Population Assay.

Lung cancer cell lines cultured in 6-well plates were live stained with 5 μg/mL Hoechst 33342 in 1 mL of buffer (2 % FBS in PBS) at 37 °C with 5% CO_2 for 1 h. The ABCG2 blocker FTC (for A549, 10 mM, Sigma F9054) or resperin (for H1299, 5 mM, Sigma R0875) was added to the blocker control well for each condition 30 min before addition of Hoechst33342. Subsequently, the supernatant was removed, and the cells were scraped in RPMI-1640 and transferred to tubes. Cells were collected by centrifugation at 1,000 rpm, 4°C for 5 minutes, and resuspended in 1 mL of cold buffer (2 % FBS in PBS). Propidium iodide (PI) was added to the buffer at the 1 μg/mL final concentration. Flow cytometric analysis was performed on BD FACS (BD Biosciences). The Hoechst 33342 dye was excited at 355 nm, and fluorescence was measured with both a 670/50 filter (Hoechst Red) and a 450/50 filter (Hoechst

Blue). The side population was gated for each condition based on the population of cells that disappeared in the blocker control for this condition.

4.2.9. Antitumor Activity *in Vivo*.

All animal studies were conducted under the approval of the Experimental Animal Management Committee of Nankai University. 6- to 8-week-old female BALB/c nude mice were purchased from Beijing HFK Bioscience Company. Human Lung cancer cells H1299 were harvested during the exponential-growth phase, washed 3 times with serum-free medium, followed by resuspension at a concentration of 1×10^7 per mL. A total of 100 μL of cell suspension was injected into SCID mice subcutaneously. After the tumors had grown to 100−120 mm^3, all the mice were randomized into 5 groups (5 mice for each group) and dosed with **21e** (20, 40, or 80 mg kg^{-1} d^{-1}), ribociclib (80 mg kg^{-1} d^{-1}), or vehicle. The compounds were dissolved in DMSO and administered orally. Mice were monitored for side effects every day. Body weights and tumor size were determined every other day. Tumor measurements were used using a digital vernier caliper, and the volumes were determined using the following calculation: ($short^2$) × long × 0.5. Inhibition rate of tumor growth was calculated using the following formula: 100 × {1 - [(tumor volume $_{final}$-tumor volume $_{initial}$) for **21e**-treated group]/ [(tumor volume $_{final}$-tumor volume $_{initial}$) for the vehicle-treated group]}.

4.2.10. Hematoxylin and Eosin (H&E) and Immunohistochemistry Staining.

H&E staining was performed on the formalin-fixed, paraffin-embedded orthotopic mice tumor tissues. Tumor tissue sections were deparaffinized, counterstained with hematoxylin and eosin, then observed under a light microscopy (Olympus). For immunohistochemistry assay, ormalin-fixed, paraffin-embedded 5-μm tumor tissue sections were de-paraffinized and rehydrated through a graded series of ethanol and incubated with primary antibody of CDK9, cyclinT1, P-Rb (Ser780), RNA polymerase II CTD, RNA polymerase II CTD (Ser5), Oct4 diluted 1:100 at 4 °C overnight, then tissues were incubated with biotin-labeled secondary antibody (Vector Laboratories. Inc. Burlingame. CA) at room temperature for 1 hour. Sections were incubated with ABC-peroxidase and diaminobenzidine (DAB), counterstaining with hematoxylin and observed under a light microscopy (Olympus).

4.2.11. Statistical Analysis.

Statistical analysis results were analyzed values by GraphPad Prism version 6.0 software. For Student *t* test and ANOVA $P< 0.05$ was considered statistically significant. Values were expressed as means ± SEM. Significance was determined by χ^2 test, others were determined by Student's t-test. A value of $P < 0.05$ was used as the criterion for statistical significance. ***indicates significant difference with $P < 0.001$, ** indicates $P < 0.01$, * indicates $P< 0.05$.

4.2.12. Microsomal Stability Studies.

The metabolic stability was assessed using human liver microsomes (HLM, Gentest, 452161, Mixed Gender), dog liver microsomes (DLM, Gentest, 452601, male), mouse liver microsomes (MLM, Gentest, 452501, male), rat liver microsomes (RLM, Gentest, 452701, male). The tested compounds (with concentration of 2 μM) was incubated with 150 μL of microsomes (1 mg/mL), 75 μL of 3 mM $MgCl_2$ and 200 μL of 0.1M phosphate buffer (pH = 7.4) at 37 °C. After 5 min of preincubation, reactions were initiated by the addition of 75 μL NADPH (3 mM). Aliquots (n = 3) were taken at 0, 10, 20, 30, 45 and 60 min, and part of the reaction solution (100 μL) immediately terminated by adding 200 μL ice acetonitrile. Samples were centrifuged and the supernatant fractions analyzed by HPLC.

ASSOCIATED CONTENT

Supporting Information

Enzymatic Inhibition (%) at 500 nM of Selected Compounds; *In vitro* cell growth inhibition (%) of selected compounds against NSCLC cell line A549; *in vitro* cell growth inhibition (%) of selected compounds against NSCLC cell line H1299; *in vitro* cell growth inhibition (%) of selected compounds against NSCLC cell line A549; *in vitro* cell growth inhibition (%) of compound **10ae** and **21e** against multiple cancer cell lines; kinase profiling results of compound **21e**; comparison of the predicted binding modes of compound **21e** with CDK9 and CDK6; general methods for chemistry; copy of 1H- and 13C-NMR spectra for each compound; copy of HRMS Spectra for compound **21e**; HPLC purity analysis for compound **21e** (**PDF**)

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ABBREVIATIONS USED

CDK9, cyclin-dependent kinase 9; NSCLC, non-small-cell lung cancer; P-TEFb, positive transcription elongation factor b; CTD, C-terminal domain; SFN, sulforaphane; SAR, structure-activity relationship; ATP, adenosine triphosphate; PDB, Protein Data Bank; SP, side population; HSCs, hematopoietic stem cells; Oct4, Octamer-binding transcription factor 4; SOX2, SRY (sex determining region Y)-box 2; Klf4, Kruppel-like factor 4; H&E, Hematoxylin and Eosin; HLM, human liver microsomes; DLM, dog liver microsomes; MLM, mouse liver microsomes; RLM, rat liver microsomes; $t_{1/2}$, terminal half-lives; CL, clearance; TLC, thin-layer chromatography; HRMS, High-resolution mass spectra; ESI, eluectron spray

ionization; DMF, *N*,*N*-dimethylformamide; DIPEA, *N*,*N*-diisopropylethylamine; DMSO, dimethyl sulfoxide; EDCI, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; DMAP, 4-(dimethylamino)pyridine; DIEA, diisopropylethylamine; BINAP, 2,2'-bis (diphenylphosphino)-1,1'-binaphthyl; THF, tetrahydrofuran; HOBt, 1-Hydroxybenzotriazole; DCC, Dicyclohexylcarbodiimide; DCM, dichloromethane.

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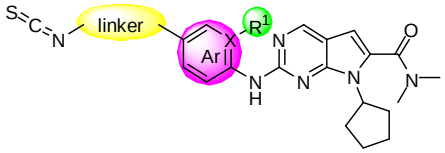
Table 1. Structure and Enzymatic Inhibition Activity Evaluation of Compounds **34**, **9a~9e**, **10aa~10de, 21a~21e**[a]



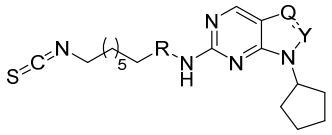
Formula I

Compds.	linker	X	R^1	Inhibition (%) at 1 μM		
				CDK4	CDK6	CDK9
Ribociclib				101 (IC_{50} = 13 nM)	86 (IC_{50} = 71 nM)	84 (IC_{50} = 197 nM)
34	-	C	H	94	72	96
9a	n = 1	C	H	90	89	97
9b	n = 2	C	H	90	92	98
9c	n = 3	C	H	91	89	98
9d	n = 4	C	H	89	81	96
9e	n = 5	C	H	86	77	97
10aa	n = 1	C	H	99	94	98
10ab	n = 2	C	H	98	93	99
10ac	n = 3	C	H	97	94	97
10ad	n = 4	C	H	98	92	96
10ae	n = 5	C	H	83	85	97
10ba	n = 1	N	-	95	89	84
10bb	n = 2	N	-	95	88	85

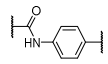
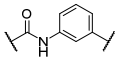
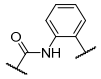
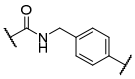
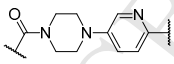
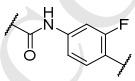
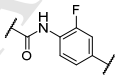
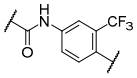
10bc	n = 3	N	-	94	87	87
10bd	n = 4	N	-	95	82	73
10be	n = 5	N	-	84	64	73
10ca	n = 1	C	F	86	82	97
10cb	n = 2	C	F	89	86	96
10cc	n = 3	C	F	86	81	96
10cd	n = 4	C	F	89	78	94
10ce	n = 5	C	F	67	52	92
10da	n = 1	C	OH	46	68	94
10db	n = 2	C	OH	55	61	91
10dc	n = 3	C	OH	59	60	92
10dd	n = 4	C	OH	45	47	81
10de	n = 5	C	OH	37	42	84
21a	n = 1	C	H	98	93	98
21b	n = 2	C	H	97	93	97
21c	n = 3	C	H	96	90	97
21d	n = 4	C	H	97	86	99
21e	n = 5	C	H	87	79	98

[a]Inhibition activities were determined using the KinaseProfiler of Eurofins. The data represent the mean values of two independent experiments.

Table 2. Structure and Enzymatic Inhibition Activity Evaluation of Compounds **20, 21f~21g, 22**, **31, 32a~32c, 33**[a]



Formula II

Compds.	R	Y	Q	Inhibition (%) at 1 μM		
				CDK4	CDK6	CDK9
Ribociclib				101 (IC_{50} = 13 nM)	86 (IC_{50} = 71 nM)	84 (IC_{50} = 197 nM)
20		N	N	19	42	78
21f			C	80	74	96
21g			C	14	1	11
22			C	93	81	95
31			C	98	93	58
32a			C	72	49	95
32b			C	86	69	87
32c			C	63	1	72
33			C	14	NA	17

[a]Inhibition activities were determined using the KinaseProfiler of Eurofins. The data represent the mean values of two independent experiments

Table 3. Further Enzymatic Inhibition Activity Evaluation (IC_{50} value) of Selected Compounds [a]

Compds.	**IC_{50} value (nM)**	**Compds.**	**IC_{50} value (nM)**
Ribociclib	197	**10ce**	50
9d	9	**10da**	68
9e	9	**10db**	42
10ae	12	**10dc**	33
10ca	10	**21e**	11
10cb	6	**21f**	15
10cc	11	**32a**	25
10cd	34		

[a]IC_{50} value were determined using the KinaseProfiler of Eurofins. The data represent the mean values of two independent experiments.

Table 4. Antiviability Activities of **21e** against Various Cancer Cell Lines [a]

cell line	cell type	IC_{50} (μM)		
		21e	Ribociclib	SFN
A549	NSCLC	0.450	7.455	>10
H1299	NSCLC	0.203	2.349	7.648
H1975	NSCLC	0.837	>10	>10
HCC827	NSCLC	1.071	>10	>10
PC-9	NSCLC	0.947	5.342	6.308
MCF7	breast cancer	1.739	>10	>10
MDA-MB-231	breast cancer	3.045	>10	>10
HepG2	hepatocarcinoma	3.861	>10	8.357
Hep3B	hepatocarcinoma	5.610	9.264	>10
Hela	cervical cancer	1.850	>10	>10
SiHa	cervical cancer	1.809	>10	9.953
Jurkat	leukemia	3.427	>10	>10
CCRF-CEM	leukemia	>10	>10	>10
U-937	lymphoma	1.988	6.517	>10

[a] The IC_{50} values are shown in the forms. The cytotoxic effect of compounds was assayed using CCK-8 assay with 72 h incubation. Data are from three independent experiments.

Table 5. Kinase Inhibition Profile of **21e** against Selected Protein Kinases[a]

Kinases	Inhibition (%) at 1 μM
CDK1/cyclinB(h)	58
CDK2/cyclinA(h)	58
CDK2/cyclinE(h)	75
CDK3/cyclinE(h)	82
CDK4/cyclinD3(h)	87 (IC_{50} = 148 nM)
CDK5/p25(h)	63
CDK5/p35(h)	59
CDK6/cyclinD3(h)	79 (IC_{50} = 145 nM)
CDK7/cyclinH/MAT1(h)	55
CDK9/cyclin T1(h)	98 (IC_{50} = 11 nM)
CDK12/cyclinK(h)	45
CDK13/cyclinK(h)	26
CDK14/cyclinY(h)	56
CDK18/cyclinY(h)	11
Kinases	**IC_{50} values (nM)**
Aurora-A(h)	16
Aurora-B(h)	18
BTK(h)	52
IRAK1(h)	26
MAP4K3(h)	113

[a]IC_{50} values were determined using KinaseProfiler by Eurofins. The data represent the mean

values of two independent experiments.

Table 6. Pharmacokinetics of **21e** in Liver Microsomes of Multiple Species.

	k [a]	$t_{1/2}$ (min) [b]	Cl_{int} (mL/min/mg) [c]
HLM	0.021	33.0	0.070
DLM	0.026	26.7	0.087
MLM	0.030	23.1	0.100
RLM	0.017	40.8	0.057

[a]k is the correlation of the linear regression for the determination of the kinetic constant. [b]$t_{1/2}$ is the half-life. [c]CL_{int} is the intrinsic clearance. HLM (human liver microsomes), DLM (dog liver microsomes), MLM (mouse liver microsomes), RLM (rat liver microsomes)

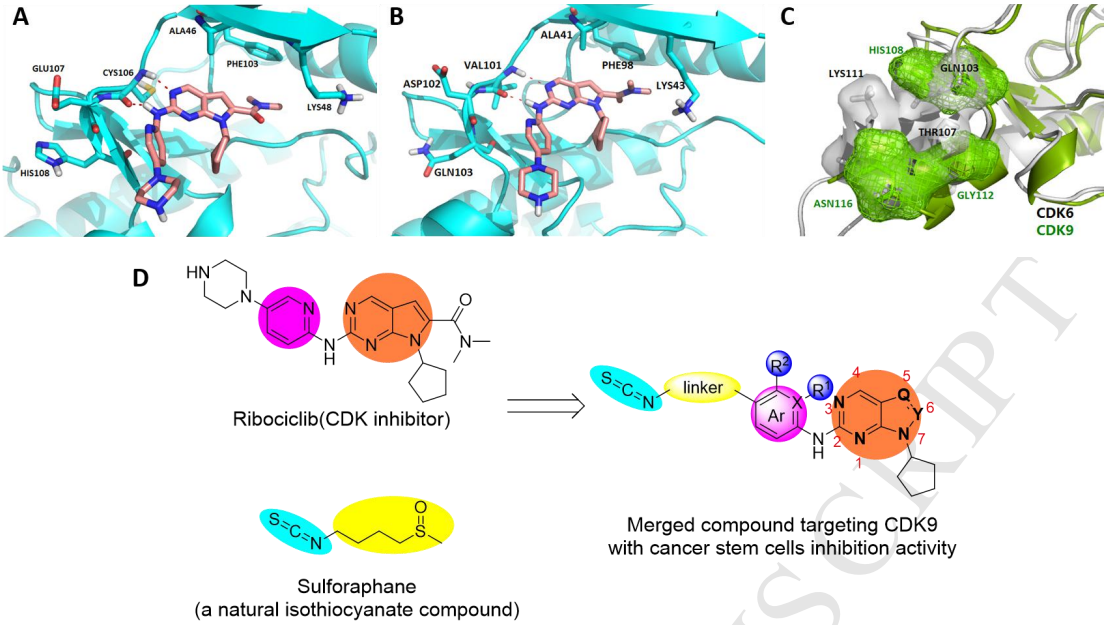
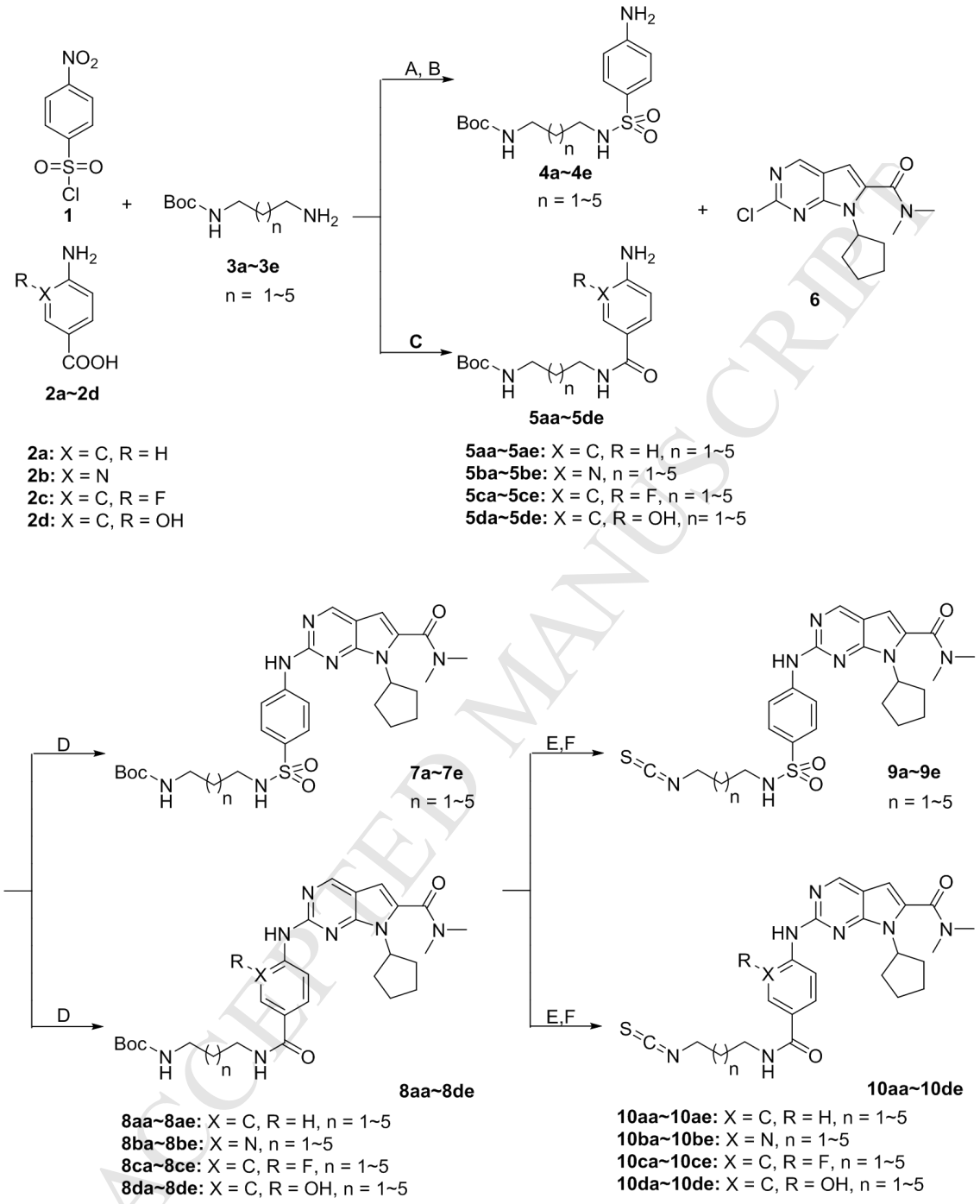


Figure 1. (**A**) Proposed binding modes of ribociclib with CDK9 (PDB code: 4BCF). (**B**) The binding modes of ribociclib with CDK6 (PDB code: 4EZ5). (**C**) Illustration of the hydrophobic pocket difference by overlaying CDK6 and CDK9. (**D**) The designed strategy of CDK9 inhibitor with cancer stem cells inhibition activity.

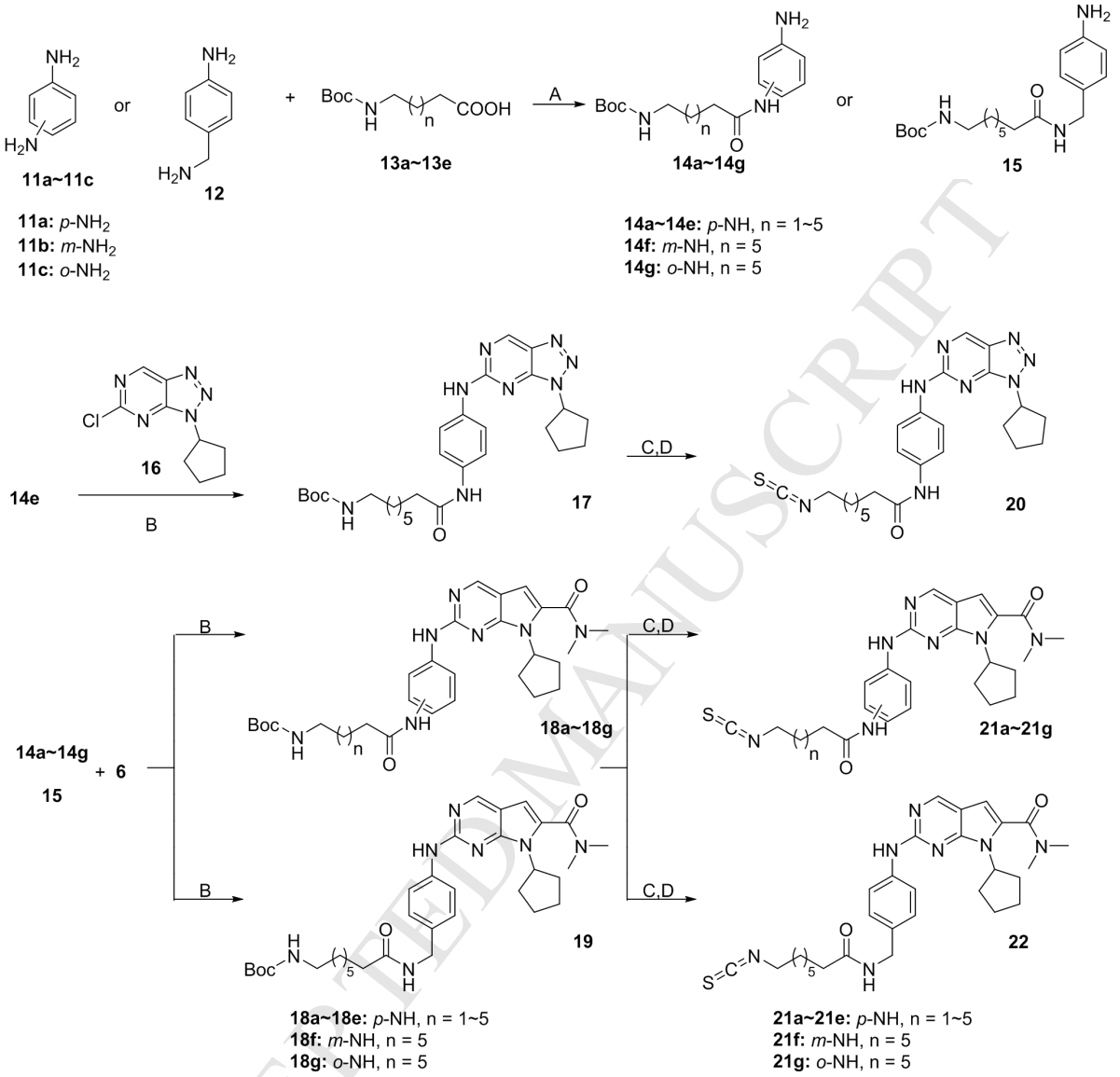
Scheme 1. Synthesis of target compounds of 9a~9e, 10aa~10de[a]



[a]Reagents and conditions: (A) DIEA (2.0 eq), DCM, rt, 3h; (B) Fe (11.0 eq), AcOH, 65 °C, 4h, yield 88% over two steps; (C) EDCI (1.5 eq), HOBt (1.2 eq), DIEA (2.0 eq), DMF, rt, overnight, yield 97%; (D) $Pd(OAc)_2$ (0.10 eq), BINAP (0.06 eq),

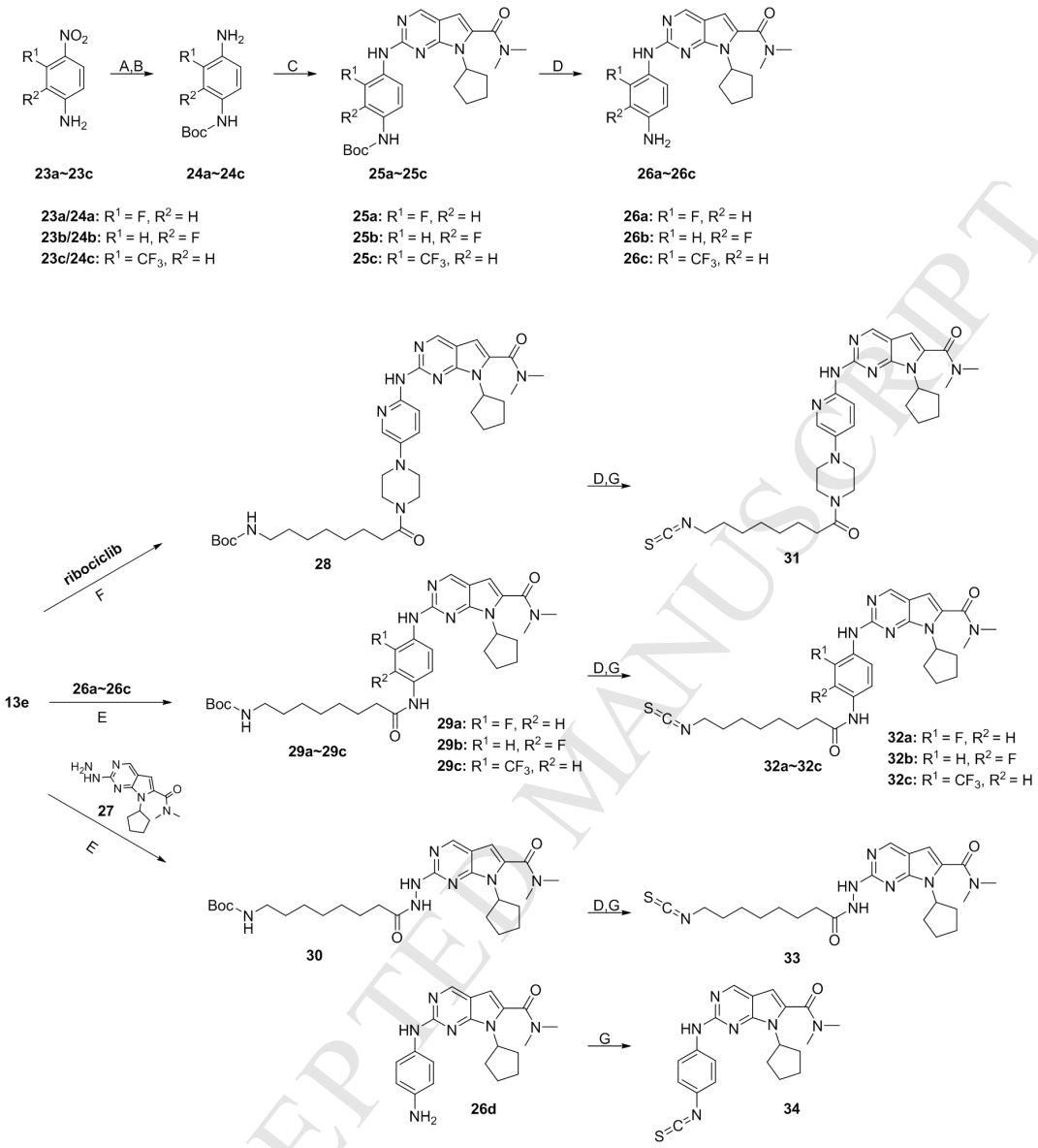
Cs_2CO_3 (2.0 eq), 1,4-dioxane, 105 °C, 7h, yield 91%; (E) CF_3COOH, DCM, 0 °C to rt, 1h; (F) CS_2 (20 eq), dicyclohexylcarbodiimide (DCC, 1.1 eq), THF, rt, overnight, yield 89%.

Scheme 2. Synthesis of target compounds of 20, 21a~21g, 22[a]



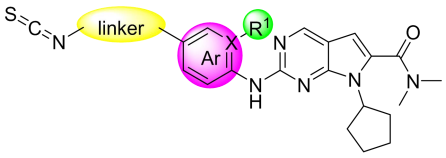
[a]Reagents and conditions: (A) EDCI (1.5 eq), HOBt (1.2 eq), DIEA (2.0 eq), DMF, rt, overnight, yield 97%; (B) $Pd(OAc)_2$ (0.10 eq), BINAP (0.06 eq), Cs_2CO_3 (2.0 eq), 1,4-dioxane, 105 °C, 7h, yield 91%; (C) CF_3COOH, DCM, 0 °C to rt, 1h; (D) CS_2 (20 eq), dicyclohexylcarbodiimide (DCC, 1.1 eq), THF, rt, overnight, yield 89%.

Scheme 3. Synthesis of target compounds of 31, 32a~32c, 33, 34[a]



[a]Reagents and conditions: (A) $(Boc)_2O$ (1.0 eq), DIEA (1.0 eq), DMAP (1.0 eq), DCM, rt, overnight; (B) Fe (11.0 eq), AcOH, 65 °C, 4h, yield 78% over two steps; (C) $Pd(OAc)_2$ (0.10 eq), BINAP (0.06 eq), Cs_2CO_3 (2.0 eq), 1,4-dioxane, 105 °C, 7h, yield 91%; (D) CF_3COOH, DCM, 0 °C to rt, 1h; (E) EDCI (1.5 eq), HOBt (1.2 eq), DIEA (2.0 eq), DMF, rt, overnight, yield 97%; (F) ribociclib (1.0 eq), EDCI (1.5 eq), HOBt (1.2 eq), DIEA (2.0 eq), DMF, rt, overnight, yield 93%; (G) CS_2 (20 eq), dicyclohexylcarbodiimide (DCC, 1.1 eq), THF, rt, overnight, yield 89%.

Table 1. Structure and Enzymatic Inhibition Activity Evaluation of Compounds **34**, **9a~9e**, **10aa~10de, 21a~21e**[a]



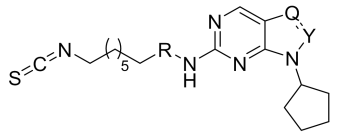
Formula I

Compds.	linker	X	R[1]	Inhibition (%) at 1 μM		
				CDK4	CDK6	CDK9
Ribociclib				101 (IC_{50} = 13 nM)	86 (IC_{50} = 71 nM)	84 (IC_{50} = 197 nM)
34	-	C	H	94	72	96
9a	n = 1	C	H	90	89	97
9b	n = 2	C	H	90	92	98
9c	n = 3	C	H	91	89	98
9d	n = 4	C	H	89	81	96
9e	n = 5	C	H	86	77	97
10aa	n = 1	C	H	99	94	98
10ab	n = 2	C	H	98	93	99
10ac	n = 3	C	H	97	94	97
10ad	n = 4	C	H	98	92	96
10ae	n = 5	C	H	83	85	97
10ba	n = 1	N	-	95	89	84
10bb	n = 2	N	-	95	88	85
10bc	n = 3	N	-	94	87	87
10bd	n = 4	N	-	95	82	73
10be	n = 5	N	-	84	64	73
10ca	n = 1	C	F	86	82	97
10cb	n = 2	C	F	89	86	96
10cc	n = 3	C	F	86	81	96
10cd	n = 4	C	F	89	78	94
10ce	n = 5	C	F	67	52	92
10da	n = 1	C	OH	46	68	94
10db	n = 2	C	OH	55	61	91
10dc	n = 3	C	OH	59	60	92
10dd	n = 4	C	OH	45	47	81
10de	n = 5	C	OH	37	42	84
21a	n = 1	C	H	98	93	98
21b	n = 2	C	H	97	93	97
21c	n = 3	C	H	96	90	97
21d	n = 4	C	H	97	86	99

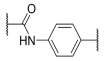
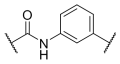
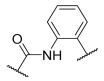
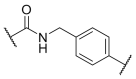
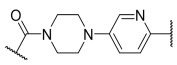
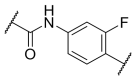
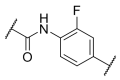
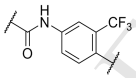
21e	n = 5	C	H	87	79	98

[a]Inhibition activities were determined using the KinaseProfiler of Eurofins. The data represent the mean values of two independent experiments.

Table 2. Structure and Enzymatic Inhibition Activity Evaluation of Compounds **20, 21f~21g, 22**, **31, 32a~32c, 33**[a]



Formula II

Compds.	R	Y	Q	Inhibition (%) at 1 μM		
				CDK4	CDK6	CDK9
Ribociclib				101 (IC_{50} = 13 nM)	86 (IC_{50} = 71 nM)	84 (IC_{50} = 197 nM)
20		N	N	19	42	78
21f			C	80	74	96
21g			C	14	1	11
22			C	93	81	95
31			C	98	93	58
32a			C	72	49	95
32b			C	86	69	87
32c			C	63	1	72
33			C	14	NA	17

[a]Inhibition activities were determined using the KinaseProfiler of Eurofins. The data represent the mean values of two independent experiments

Table 3. Further Enzymatic Inhibition Activity Evaluation (IC_{50} value) of Selected Compounds [a]

Compds.	IC_{50} value (nM)	Compds.	IC_{50} value (nM)
Ribociclib	197	**10ce**	50
9d	9	**10da**	68
9e	9	**10db**	42
10ae	12	**10dc**	33
10ca	10	**21e**	11
10cb	6	**21f**	15
10cc	11	**32a**	25
10cd	34		

[a]IC_{50} value were determined using the KinaseProfiler of Eurofins. The data represent the mean values of two independent experiments.

Table 4. Antiviability Activities of **21e** against Various Cancer Cell Lines [a]

		IC_{50} (μM)		
cell line	cell type	**21e**	Ribociclib	SFN
A549	NSCLC	0.450	7.455	>10
H1299	NSCLC	0.203	2.349	7.648
H1975	NSCLC	0.837	>10	>10
HCC827	NSCLC	1.071	>10	>10
PC-9	NSCLC	0.947	5.342	6.308
MCF7	breast cancer	1.739	>10	>10
MDA-MB-231	breast cancer	3.045	>10	>10
HepG2	hepatocarcinoma	3.861	>10	8.357
Hep3B	hepatocarcinoma	5.610	9.264	>10
Hela	cervical cancer	1.850	>10	>10
SiHa	cervical cancer	1.809	>10	9.953
Jurkat	leukemia	3.427	>10	>10
CCRF-CEM	leukemia	>10	>10	>10
U-937	lymphoma	1.988	6.517	>10

[a] The IC_{50} values are shown in the forms. The cytotoxic effect of compounds was assayed using CCK-8 assay with 72 h incubation. Data are from three independent experiments.

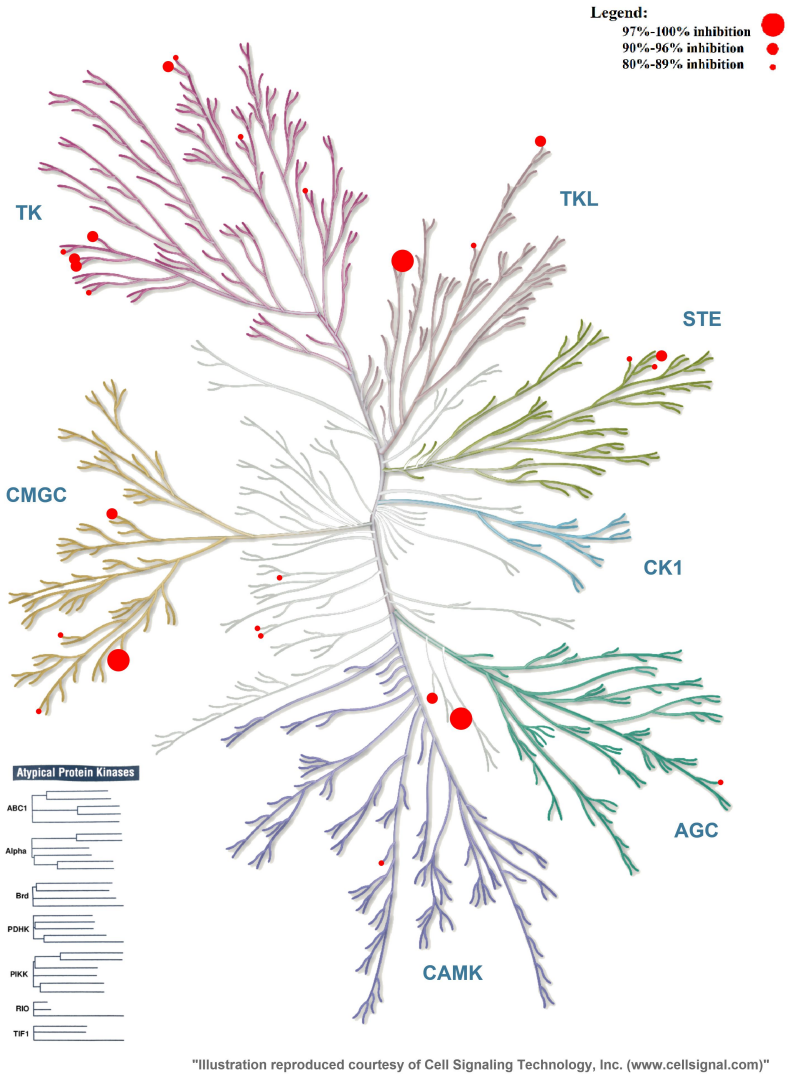


Figure 2. Kinase binding selectivity for compound **21e** shown on the human kinome dendrogram. The inhibition rates were determined using the KinaseProfiler of Eurofins. The figure was generated by using an online KinMap program (http://kinhub.org/kinmap/).

Table 5. Kinase Inhibition Profile of **21e** against Selected Protein Kinases[a]

Kinases	Inhibition (%) at 1 μM
CDK1/cyclinB(h)	58
CDK2/cyclinA(h)	58
CDK2/cyclinE(h)	75
CDK3/cyclinE(h)	82
CDK4/cyclinD3(h)	87 (IC_{50} = 148 nM)
CDK5/p25(h)	63
CDK5/p35(h)	59
CDK6/cyclinD3(h)	79 (IC_{50} = 145 nM)
CDK7/cyclinH/MAT1(h)	55
CDK9/cyclin T1(h)	98 (IC_{50} = 11 nM)
CDK12/cyclinK(h)	45
CDK13/cyclinK(h)	26
CDK14/cyclinY(h)	56
CDK18/cyclinY(h)	11
Kinases	**IC_{50} values (nM)**
Aurora-A(h)	16
Aurora-B(h)	18
BTK(h)	52
IRAK1(h)	26
MAP4K3(h)	113

[a]IC_{50} values were determined using KinaseProfiler by Eurofins. The data represent the mean values of two independent experiments.

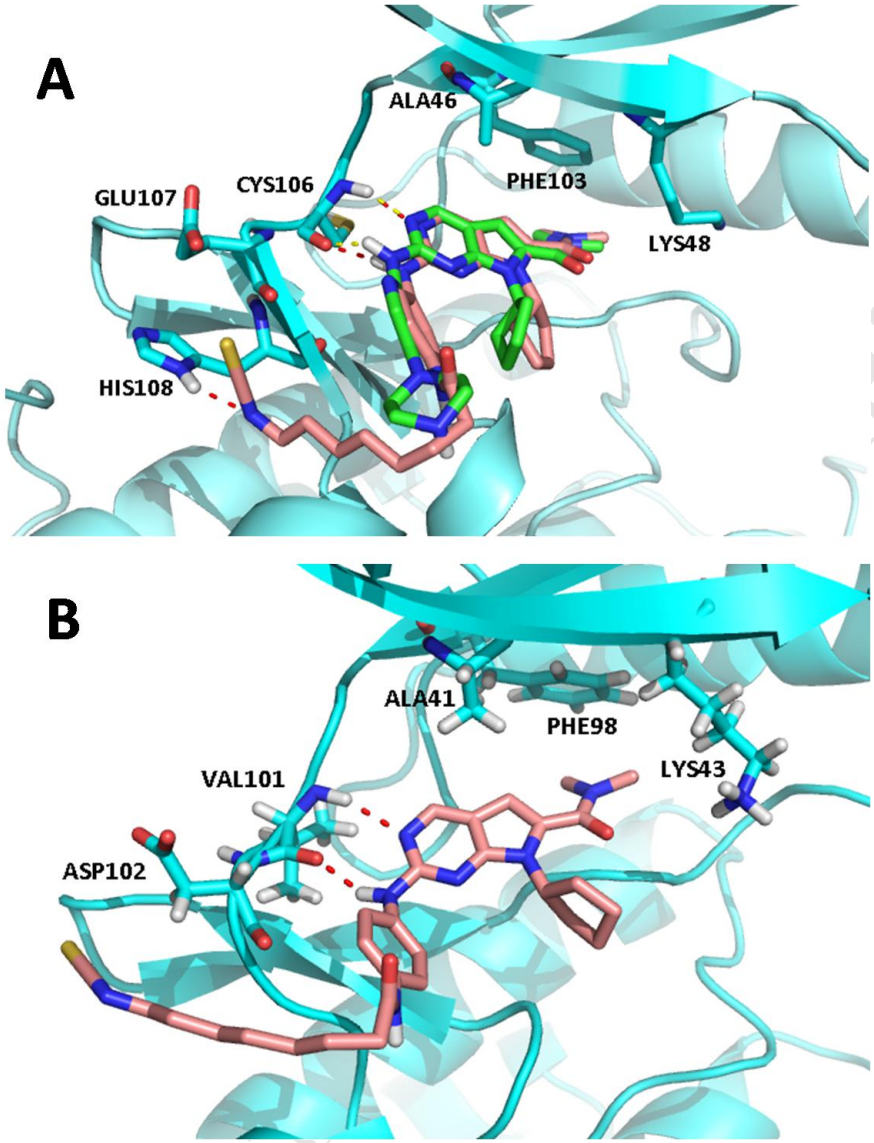


Figure 3. Representation of the predicted binding modes of compound **21e** with CDKs kinase domain. CDKs backbone is shown in cyan. Hydrogen bond is shown in red. (**A**) Proposed binding modes of compound **21e** and ribociclib with CDK9 (PDB code: 4BCF). **21e** is shown in pink, ribociclib is shown in green. (**B**) Proposed binding modes of compound **21e** with CDK6 (PDB code: 4EZ5).

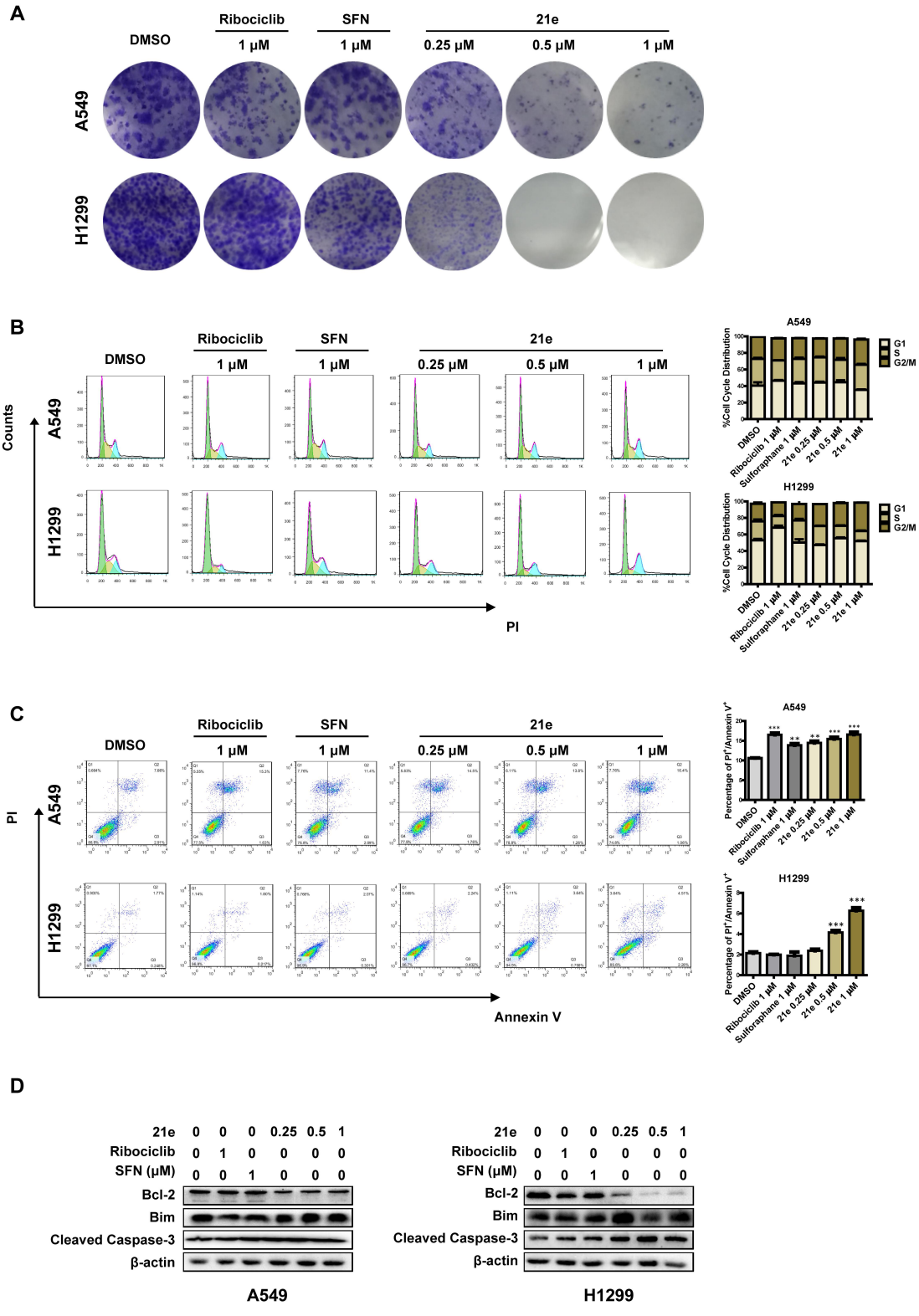


Figure 4. 21e induces G2/M phase arrest and apoptosis in NSCLC cells. (A) NSCLC cells were seeded in 6-well plates and treated with **21e**, ribociclib or SFN for 12 days and colonies were stained with crystal violet. **(B)** Influence of **21e**, ribociclib

and SFN on cell cycle progression in NSCLC cells. Representative images for A549 and H1299 treated with indicated concentrations of **21e**, ribociclib or SFN for 24 h are showed on the left, and the percentage of cell cycle distribution are presented on the right. Data shown in the histogram are means ± SD from three independent experiments. **(C) 21e** induced apoptosis in A549 and H1299 cells were harvested after treatment with different concentrations of **21e**, ribociclib and SFN for 48 h. Cells were stained using an AnnexinV-FITC Apoptosis Detection Kit. The assays were performed in triplicate. The percentage of AnnexinV-positive cells is represented for apoptosis rate. ** $P<0.01$ *vs* DMSO; *** $P<0.001$ *vs* DMSO. **(D)** A549 and H1299 cells were exposed to indicated concentrations of **21e**, ribociclib or SFN for 24 h, following by western blotting with indicated antibodies associated with apoptosis pathways.

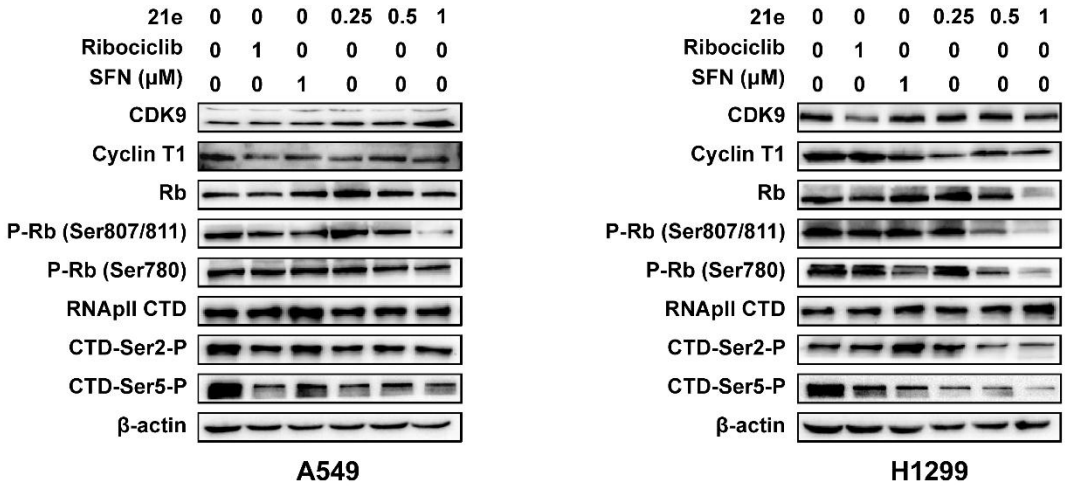


Figure 5. 21e suppressed the CDK9 and SFN downstream signaling proteins of NSCLC cells. A549 and H1299 cells treated with DMSO, 1μM ribociclib, 1μM SFN, or serial dilutions of **21e** for 24 h. Proteins extracts were separated for western blot analysis.

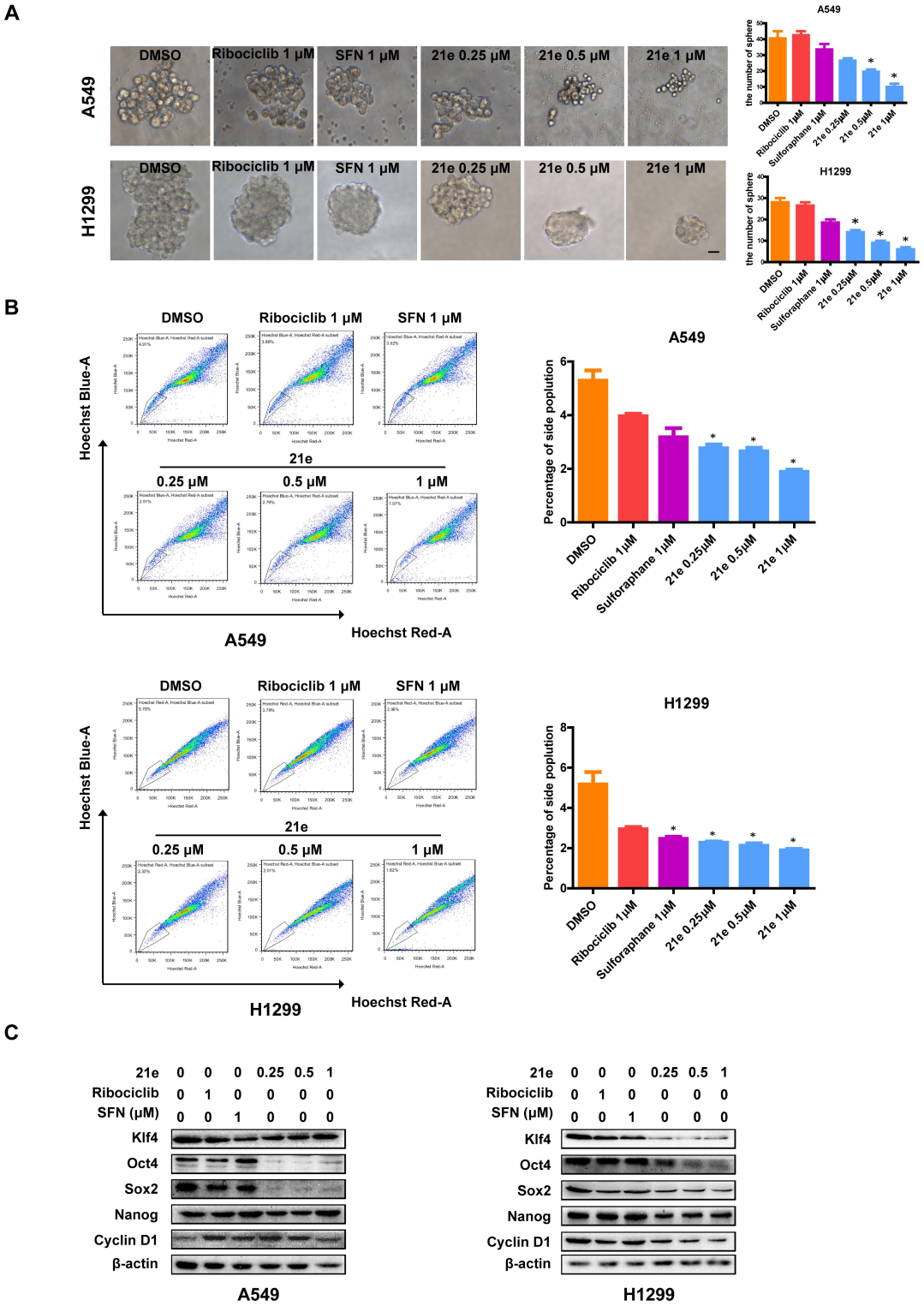


Figure 6. 21e downregulated the stem cell properties of NSCLC cells. (A) Sphere formation assay of A549 and H1299 cells were treated with indicated agents for 2 weeks. Scar bar, 100 μm. Bar graphs show quantifications of the number of the spheres, respectively. **(B)** Flow cytometry showing the percentage of the side

population detected by Hoechst 33342 staining in A549 and H1299 cells cultured in the presence of different agents. Bar graphs show quantifications of the percentage of side population on the right. * indicates $P<0.05$ in Student's *t*-test. **(C)** Western blot analysis of the stemness markers in A549 and H1299 cells using indicated agents.

Table 6. Pharmacokinetics of **21e** in Liver Microsomes of Multiple Species.

	k [a]	$t_{1/2}$ (min) [b]	Cl_{int} (mL/min/mg) [c]
HLM	0.021	33.0	0.070
DLM	0.026	26.7	0.087
MLM	0.030	23.1	0.100
RLM	0.017	40.8	0.057

[a]k is the correlation of the linear regression for the determination of the kinetic constant. [b]$t_{1/2}$ is the half-life. [c]CL_{int} is the intrinsic clearance. HLM (human liver microsomes), DLM (dog liver microsomes), MLM (mouse liver microsomes), RLM (rat liver microsomes)

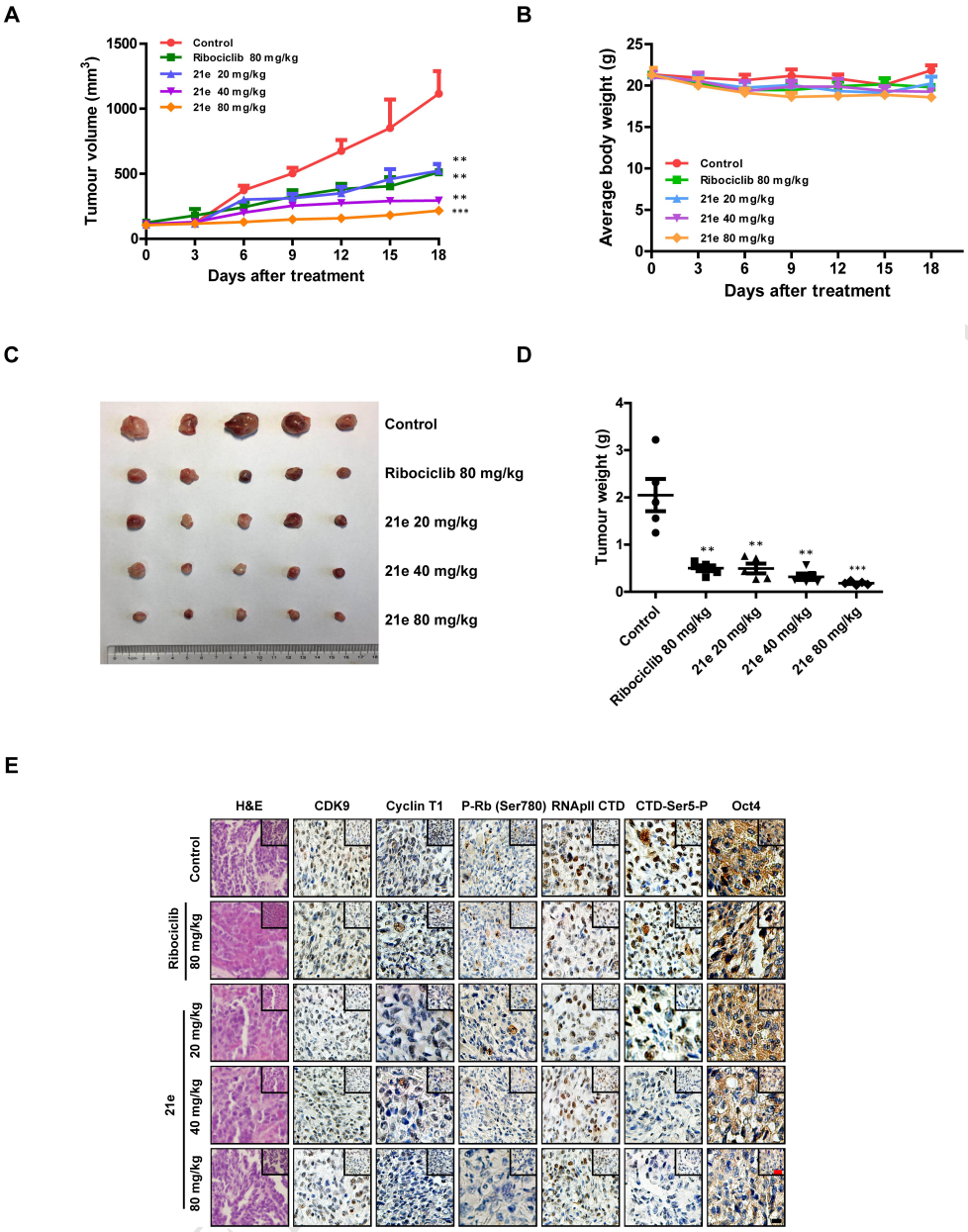


Figure 7. ***In vivo*** **anti-tumor activity and mechanism of action of 21e. (A)** Nude mice bearing H1299 tumor cells were treated with **21e**, ribociclib at the indicated doses or vehicle control alone for 21 days (N = 5 per group, mean±SD). The *P*-values were determined using Student's *t*-test. ** P <0.01, ***P<0.001. Points indicate mean tumor volumes (mm^3). Bars indicate SD. **(B)** Average body weight of xenograft tumor mice after treatment with different concentrations of **21e**, ribociclib or control. **(C, D)** At the end of the experiments, the mice were killed and the tumor were dissected and weighed. The data are expressed as the mean ± SD of groups. The representative images of isolated tumors are also shown. **(E)** Tumor tissues from mice treated with

the indicated compounds for 3 weeks, analyzed by immunohistochemistry for the indicated proteins. Red bars represent 20 μM and black bars represent 50 μM.

- A series of novel, highly potent, selective CDK9 inhibitors with cancer stem cells (CSCs) inhibition activity for non-small-cell lung cancer (NSCLC) therapy were designed and synthesized.
- **21e** potently inhibited CDK9 with IC_{50} value of 11 nM and suppressed the stemness properties of NSCLC.
- In H1299 xenograft mouse model, a once-daily dose of compound **21e** at 20 mg/kg led to significant tumor regression without obvious toxicity.