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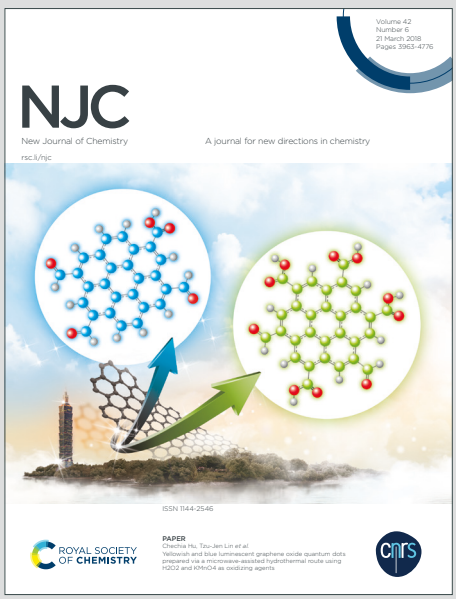

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Design, synthesis and biological evaluation of novel pteridinone derivatives as potent dual inhibitors of PLK1 and BRD4

Yinliang Qi[a], Le Xu[a], Zhiwei Li[a], Ping Gong[a], Tao Hu[b], Bixi Yin[b], Mingze Qin[a], Yajing Liu*[a], Yanfang Zhao*[a], Yunlei Hou*[a,b]

[a]*School of Pharmaceutical Engineering, Shenyang Pharmaceutical University, 103 Wenhua Road, Shenhe District, Shenyang, 110016, China.*

[b]*Yangtze River Pharmaceutical Group Co., Ltd, No.1, South Yangtze River Road, Taizhou 225321, Jiangsu Province, China*

Abstract: To develop novel simultaneous inhibition of PLK1 and BRD4 bromodomain by a single molecule, three series of novel pteridinone derivatives were designed, synthesized and evaluated for their biological activity. Most compounds exhibited moderate to excellent cytotoxic activity against A549, HCT116, PC-3 and MCF-7 cell lines. The most promising compound **III$_4$** showed high antiproliferative effects on four cell lines with IC_{50} values of 1.27 μM, 1.36 μM, 3.85 μM and 4.06 μM, respectively. The enzymatic assay identified **III$_4$** as a potent PLK1 and BRD4 dual inhibitor with % inhibition values of 96.6 and 59.1, respectively. Furthermore, to clarify the anticancer mechanism of target compounds, further explorations in the bioactivity were conducted. The results showed that compound **III$_4$** obviously inhibited proliferation of HCT-116 cell lines, induced a great decrease in mitochondrial membrane potential leading to apoptosis of cancer cells, suppressed the migration of tumor cells, and arrested S phase of HCT116 cells.

Key words: dual inhibitors; antitumor; synthesis; pteridinone derivatives

1. Introduction

Cancer remains the second leading disease after cardiovascular of our time owing to its manifestation through the aberrant regulation of multiple signaling pathways [1]. The effective strategy to inhibit tumor cell proliferation is to inhibit one or more of the proteins involved in these related signaling pathways. However, single target inhibitors may function well *in vitro*, the upregulation of compensatory signaling pathways often compromises their efficacies in cells [2]. Thus, the dual pharmacology inhibitor may increase potency for two disease relevant targets/pathways while decreasing toxicity.

Polo-like kinase 1 (PLK1), a serine–threonine kinase, plays a key role in mitosis and has been demonstrated to be required in centrosome maturation and establishment of a bipolar spindle [3]. PLK1 was overexpressed in many different tumor types including lung, colon, prostate, ovary, breast, head and neck squamous cell carcinoma, melanoma, and overexpression often correlates with poor prognosis [4]. The molecular mechanism responsible for reciprocal activation between PLK1 and MYC had been identified [5]. Because of its essential role in cell proliferation, there is a high level of interest and an

*Corresponding author. E-mail address: houyunlei901202@163.com(Y.L. Hou). yanfangzhao@126.com (Y.F. Zhao). lyjpharm@126.com (Y.J. Liu).

increasing effort to identify and develop small-molecule inhibitors of PLK1. This led to a number of different compounds to be designed (**Fig. 1**) which are currently under clinical investigation [6-7].

Bromodomain-containing protein 4 (BRD4), belonging to the bromodomain and extra-terminal domain (BET) family of proteins. Inhibition of BRD4 has recently emerged as an essential transcriptional co-regulator of MYC, and inhibition of the bromodomain has been shown to be an effective therapeutic approach to target dysregulated MYC in neuroblastoma [8-10]. Over the past decade, the number of small-molecule BET inhibitors have expanded dramatically. Some of them (**Fig. 1**) have been enrolled into different phases of human clinical trials including RVX-208, I-BET762 and (+)-JQ1. Although, several compounds have progressed to clinical trials for adult malignancies, the discovery of novel potent inhibitors still attracts many attentions due to their therapeutic potential for various human diseases [11-12]. Listed below are structure and the different functions of BRD4 protein.

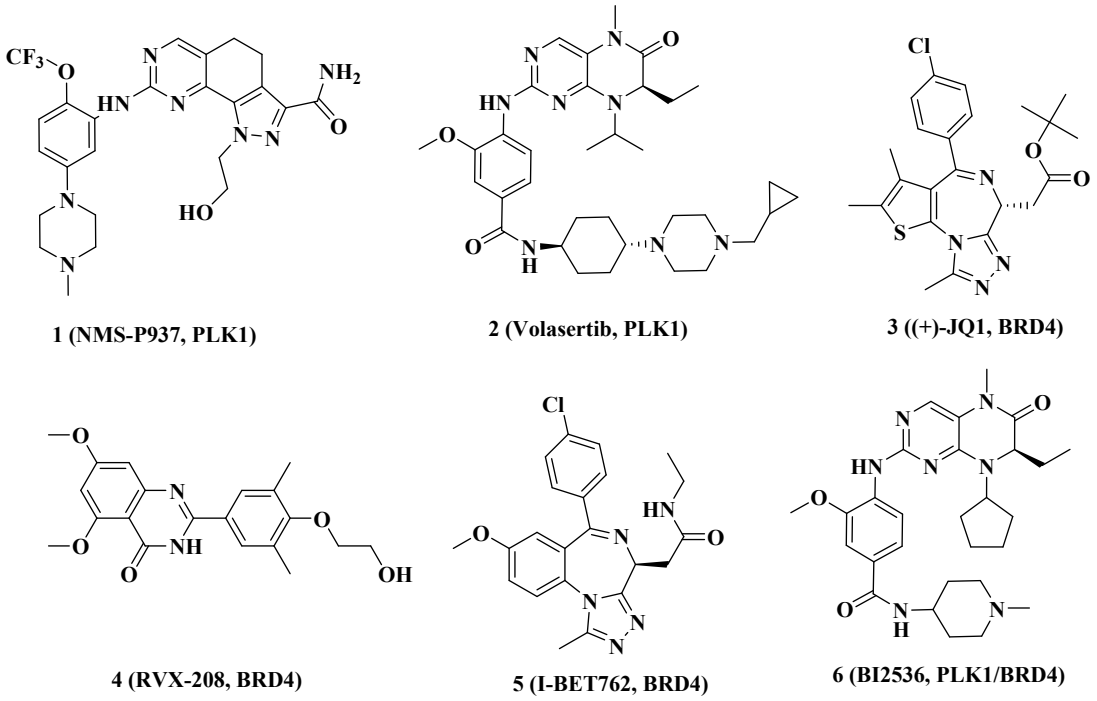

Fig. 1. Chemical structures of selected PLK1 inhibitors (structures 1-2), BRD4 inhibitors (structures 3-5) and PLK1/BRD4 inhibitor (structure 6) that are currently being evaluated in clinical trials.

Recently, the studies indicated that the mechanism of the PLK1 and BRD4 inhibitors was associated with the expression of the MYC oncogene, while markedly inhibiting cancer cell growth and metastasis in hepatocellular carcinoma and neuroblastoma *in vivo*. Tontsch-Grunt *et al* [13]. reported that the BET inhibitor BI 894999 with PLK inhibitor volasertib exerted synergistic activity of in AML *in vitro* and *in vivo*. Liu *et al* [14]. discovered that the PLK1 inhibition enhanced the efficacy of BET epigenetic reader blockade in castration resistant prostate cancer. Together, these preclinical findings provide evidence for the strong synergistic effect of PLK1 inhibitor and BRD4 inhibitor, warranting future studies in patients with cancer to investigate this paradigm. In order to achieve this synergistic effect, the single agent combinatorial inhibition of

PLK1 and BET bromodomains was an effective process to develop new antineoplastic drugs. Rationally designed dual PLK1/BRD4 inhibitor carries the promise to deliver drugs that are more efficacious in cells, as well as overcome the drawbacks associated with multidrug regimens.

Meanwhile, Knapp and Schönbrunn independently discovered that the PLK1 inhibitor BI-2536 (**6**) is also a potent inhibitor of BRD4 [15-16]. In the binding complex of BI-2536 with PLK1 and BRD4, the pteridinone moiety (A) occupied the active domain and retained to exert the key hydrogen bond interactions with Cys 133, Asp 194 and Gln 85, Asn 140 amino acid residues, respectively [17]. However, according to the cocrystal structure result the 5-methyl and 6-carbonyl groups of pteridinone moiety did not occupied the ATP cavity, completely. In our previous work, we found that the incorporation of triazolo or tetrazolo group fit into the pteridinone frameworks maintained a certain inhibition activity against PLK1 [18]. Therefore, in this work, we hope to obtain dual inhibitor of PLK1 and BRD4, which persisted the incorporation of triazolo or tetrazolo group into the pteridinone frameworks (A). On the other hand, the methoxy group on the aromatic ring moiety (B) was important to maintain kinase selectivity against PLK1/BRD4, so methoxy group was introduced. Meanwhile, the 'necessary nitrogen atom' is prone to be a versatile high-impact design element to achieve pharmacological profile. In this perspective, the 2-alkoxypyridin-3-yl motif was attached to B moiety expecting to exert extra interaction between pyridinyl motif with PLK1 or BRD4 catalytic domain. Furthermore, the hydrophilic "tail" piperidine (C) motif exposing to the solvent has been investigated extensively in recent years [19], indicating that modification on the "tail" (C) could be tolerant to maintain efficacies (**Fig. 2**).

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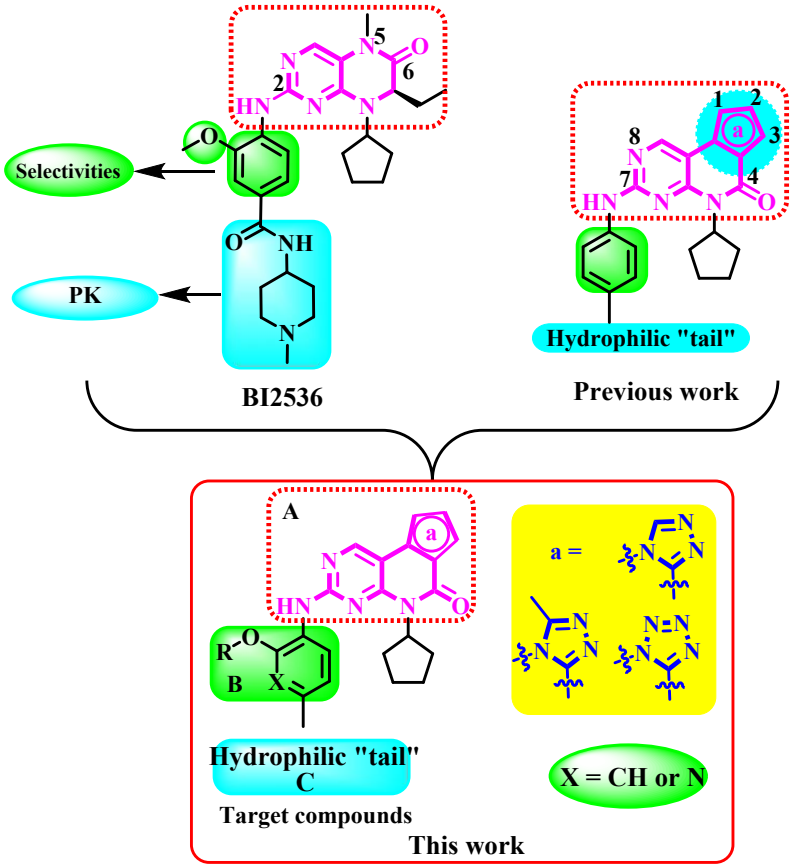


Fig. 2. Design strategy for the target compounds.

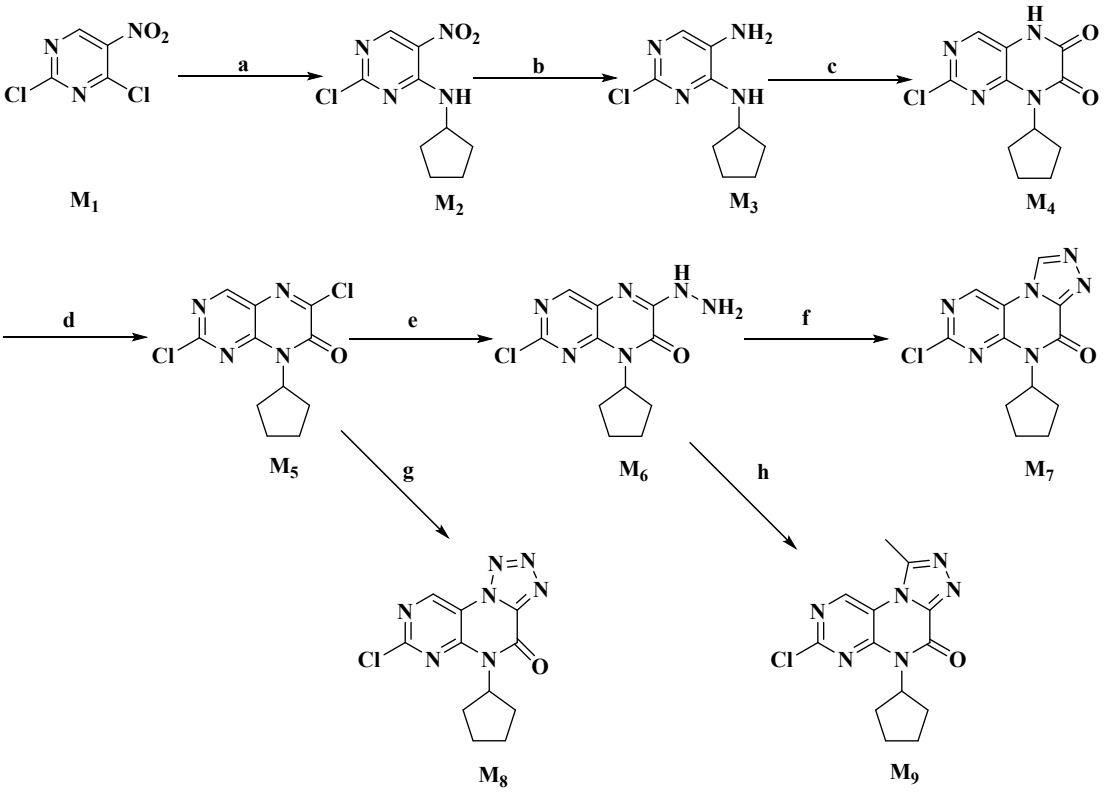
Meanwhile, compound **III$_2$** contained similar structural feature with BI2536 including hydrophilic aniline side chain and pteridinone fragments. In order to better understand the anti-tumor mechanisms, molecular docking models of **III$_2$** were performed based upon the cocrystal structure of BI2536 with PLK1 and BRD4. Compound **III$_2$** occupied the kinase domain in a similar manner to BI2536. Combining with the results of docking analysis, PLK1 and BRD4 were very likely to be the potential drug targets of these pteridinone derivatives.

In this study, we utilized the scaffold of BI-2536 to develop three series of novel pteridinone derivatives with varied inhibitory activities against PLK1 and BRD4 in order to perform structure-activity relationship (SAR) studies, as well as understand the effect of inhibiting both proteins (PLK1 and BRD4) with a single agent in cancer. All compounds were subsequently assayed for anti-proliferative activities *in vitro* against four cancer cell lines A549, PC-3, HCT116 and MCF-7. Based on the anti-proliferative results, potent compounds were selected for further *in vitro* enzymatic inhibitory studies. To further clarify the primary mechanism, **III$_4$** was taken forward and examined by *in vitro* the migration, apoptosis and cell cycle analysis of HCT116 cells.

2. Chemistry

A series of novel pteridinone derivatives linked with nitrogen heterocycle at N-5, C-6 position and different aniline at C-2 position were synthesized. The general synthetic routes of the title compounds were depicted in **Scheme 1-3**. Starting from

commercially available 2,4-dichloro-5-nitropyrimidine **M_1** reacted with cyclopentylamine to afford compound **M_2**, which was reduced by using iron powder and catalytic amounts of concentrated HCl in EtOH/H_2O to obtain amide intermediate **M_3**. The intermediate **M_4** was prepared from **M_3** by treatment with ethyl oxalyl monochloride in acetone. Then carbonyl in compound **M_4** was chlorinated by thionyl chloride to afford **M_5**. Intermediate **M_5** was converted to tetrazole product **M_8** using NaN_3 as a cyclization reagent in DMF at 0 °C. Subsequently, the intermediate M_6 was available *via* hydrazinolysis of intermediate **M_5** with 80% hydrazine monohydrate in EtOH at 40 °C. The cyclization of the triazole intermediates **M_7** and **M_9** on compounds **M_6** were performed with trimethyl orthoformate or triethyl orthoformate under 80 °C, respectively. Side chain anilines M_{12} were prepared in two steps as shown in **Scheme 2**. Aromatic nucleophilic substitution of chlorine in 4-chloro-2-methoxy-1-nitrobenzene or 6-chloro-2-methoxy-3-nitropyridine by secondary amines were readily achieved in DMF at 40 °C. The resulting nitrobenzenes were reduced to anilines using Pd/C in EtOH. Subsequently, amination of **M_7**, **M_8**, and **M_9** by corresponding aryl amines **M_{12}** with *p*-toluenesulfonic acid as catalyst furnished target compounds **I_1-I_{14}**, **II_1-II_6** and **III_1-III_{14} (Scheme 3)**.



Scheme 1. General scheme for the synthesis of key intermediates **M_7**, **M_8** and **M_9**; Reagents and conditions: (a) Cyclopentylamine, $NaHCO_3$, DCM, 25 °C, 10 h; (b) Fe powder, HCl (cat.), EtOH/H_2O, reflux, 2 h; (c) i: Ethyl oxalyl monochloride, K_2CO_3, acetone, 2 h; ii: TEA, EtOH, 100 °C, 4 h; (d) $SOCl_2$, DMF (cat.), reflux, 2 h; (e) $NH_2NH_2{\cdot}H_2O$, EtOH, 40 °C, 2 h; (f) $HC(OEt)_3$, 80 °C, 2 h; (g) NaN_3, DMF, 0 °C, 10 h; (h) $CH_3HC(OEt)_3$, 80 °C, 2 h.

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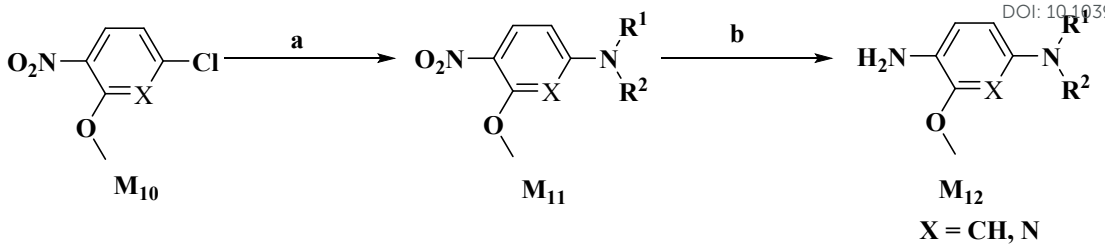
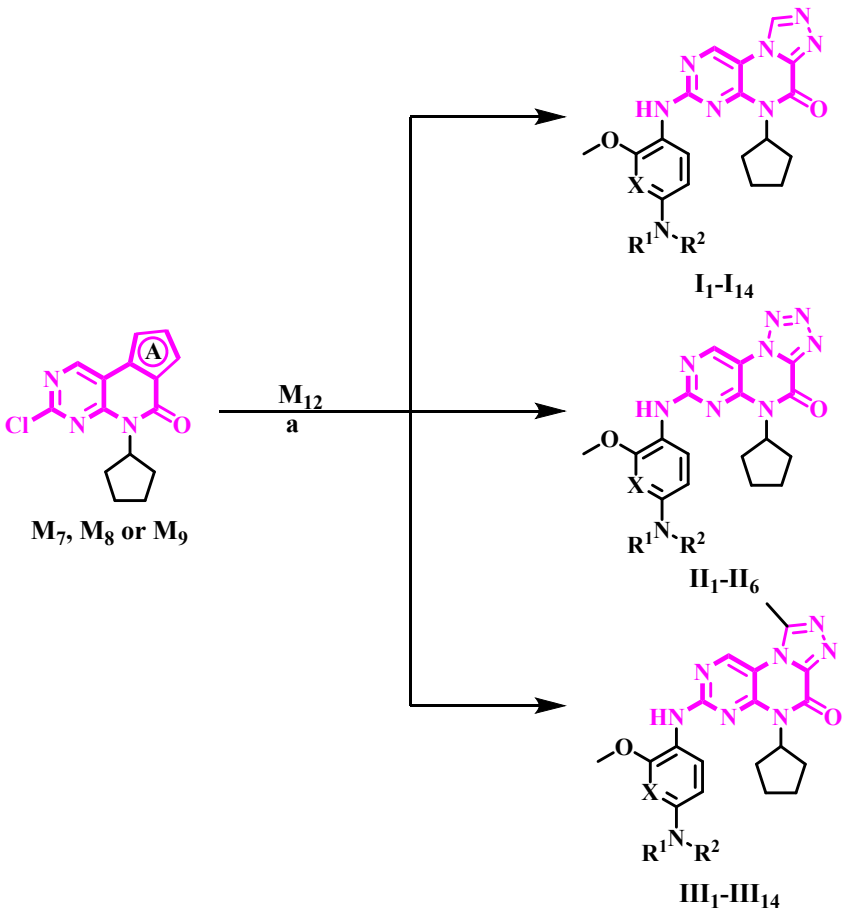


Scheme 2. Preparation of the side chain anilines; Reagents and conditions: (a) Amines R^1R^2NH, K_2CO_3, DMF, 40 °C, 6 h; (b) H_2, 10 % Pd/C, EtOH, 50 °C, 5 h.



Scheme 3. General scheme for the synthesis of target compounds; Reagents and conditions: (a) *p*-Toluenesulfonic acid, 1-butanol, 100 °C, 15 h.

3. Results and discussion

3.1. In vitro antiproliferative activity

To evaluate *in vitro* antitumor activities, all synthesized compounds (**I_1-I_{14}**, **II_1-II_6**, and **III_1-III_{14}**) were investigated against a panel of cancer cell lines, including A549 (human lung adenocarcinoma), HCT116 (human colorectal cancer), PC-3 (human prostate cancer) and MCF-7 (human breast cancer) cells by the MTT assay. Meanwhile, JQ1 and BI2536 were served as positive controls. The results were expressed as half-maximal inhibitory concentration (IC_{50}) values and summarized in **Table 1**. Most of pteridinone derivatives **I_1-I_{14}** showed moderate to significant cytotoxic activities

against the different cancer cell lines. Several of these compounds have equivalent effects to (+)-JQ1 on one or more cancer cell lines, which suggested that the combination of 7-aminoaryl-[1,2,4]triazolo[4,3-*f*]pteridinone framework and hydrophilic "tail" moiety exhibit potent synergistic antitumor effect. Preliminary SARs indicated that the introduction of different amino "tail" groups at the C-7 position of pteridinone moiety had a significant influence on activity. Compounds bearing substituted piperazine moiety (**I_1** and **I_8**) displayed excellent anti-tumor activities in the sigle-digit micromolar range against A549 and HCT116 cells. Notably, one additional nitrogen atom can be inserted into the position of 7- aminoaryl with maintaining activities (**I_8** *vs* **I_9**). However, compounds I_{14} containing an unsubstituted piperazine, compounds **I_5-I_6** containing a morpholine and **I_{12}-I_{13}** containing a hydroxyethyl piperazine respectively led to the diminished potency, which indicated that the presence of alkyl substituted piperazine was a critical factor in anti-proliferative activities. Especially, the better promising compound **I_4** displayed equivalent potency with (+)-JQ1 against A549, HCT116, PC-3 and MCF-7 cells with IC_{50} values of 4.12 μM, 6.73 μM, 4.61 μM and 4.22 μM, respectively. Overall, SARs studies identified the 7-amino-[1,2,4]triazolo[4,3-*f*]pteridinone moiety bearing piperazine "tails" moieties harboring cationic nitrogen atoms with favorable potency.

Further studies were performed to examine the effect of shifting the 1,2,3-triazole motif of **I_1-I_{14}** series compounds to the tetrazolo series compounds (**II_1-II_6**). The results displayed that most of the tetrazolo series compounds had lower cytotoxicity than 1,2,3-triazole series. Interestingly, **II_6** was maintained approximately cytotoxicity in A549, HCT116, PC-3, MCF-7 cells with IC_{50} values of 4.87 μM, 2.09 μM, 9.30μM, and 5.40 μM, respectively.

In our previous study, we found that the methyl group acts as an essential factor in the molecular recognition of endogenous and exogenous substrates by means of bioreceptors. This can lead to multiple biological effects, such as selectivity among bioreceptors, increased the binding affinity, and protection against enzyme metabolism. Accordingly, we next further explored the SAR resulting from a methyl group to the 1 position of 7-amino-[1,2,4]triazolo[4,3-f]pteridinone core on the cytotoxic activity. We synthesized a series of compounds (**III_1-III_{14}**) containing the methyl group. These methyl-substituted compounds show superior cytotoxic activities over earlier compounds against the different cancer cell lines, suggesting that not only the hydrocarbyl substituted piperazine "tail" but also the methyl-[1,2,4]triazolo moiety play an important role in cytotoxic activities. Moreover, the examination of SARs indicated that these analogs had similar SARs as summarized before.

In conclusion, the most potent compound **III_4** showed promising cytotoxicity against A549, HCT116, PC-3 and MCF-7 cell lines with IC_{50} values of 1.27 μM, 1.36 μM, 3.85 μM and 4.06 μM, respectively. The potency have equivalent effects to (+)-JQ1. However, the potency was slightly weaker than BI2536. These encouraging results provided a valuable lead compound **III_4** and highlighted the potential for further development of novel pteridinone derivatives as potent antitumor agents.

Table 1.

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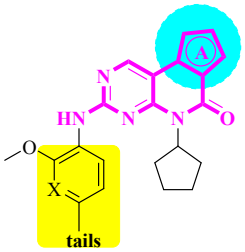
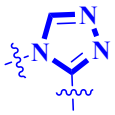
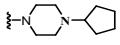
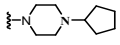
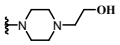
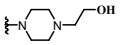
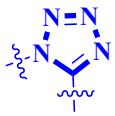
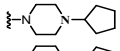
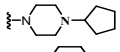
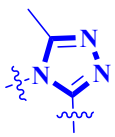
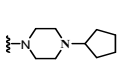
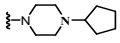
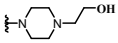
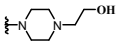
Structures and cytotoxicity of compounds (**I_1-I_{14}**, **II_1-II_6**, and **III_1-III_{14}**).



A ring	Compd.	X	"tail"	IC_{50} [a] (μM) ± SD [b]			
				A549	HCT116	PC-3	MCF-7
	I_1	CH		3.24 ± 0.02	4.57 ± 0.05	3.80 ± 0.06	6.20 ± 0.09
	I_2	N		5.7 ± 0.1	3.52 ± 0.01	14.72 ± 0.05	16.69 ± 0.03
	I_3	CH		>20	>20	>20	>20
	I_4	N		4.12 ± 0.04	6.73 ± 0.03	4.61 ± 0.08	4.22 ± 0.08
	I_5	CH		>20	>20	>20	>20
	I_6	N		>20	>20	>20	>20
	I_7	CH		15.78 ± 0.08	8.48 ± 0.05	13.07 ± 0.06	10.88 ± 0.02
	I_8	CH		6.66 ± 0.07	3.14 ± 0.08	4.32 ± 0.01	5.86 ± 0.05
	I_9	N		4.59 ± 0.01	4.07 ± 0.08	9.35 ± 0.03	11.50 ± 0.06
	I_{10}	CH		17.00 ± 0.05	9.60 ± 0.02	14.98 ± 0.08	17.85 ± 0.04
	I_{11}	N		7.51 ± 0.08	3.53 ± 0.05	10.40 ± 0.05	9.43 ± 0.07
	I_{12}	CH		>20	>20	>20	>20
	I_{13}	N		>20	>20	>20	>20
	I_{14}	CH		>20	>20	>20	>20
	II_1	CH		5.06 ± 0.09	10.41 ± 0.03	7.99 ± 0.03	9.31 ± 0.06
	II_2	CH		>20	>20	>20	>20
	II_3	N		11.43 ± 0.02	5.26 ± 0.04	16.36 ± 0.06	>20
	II_4	CH		>20	>20	>20	>20
	II_5	N		>20	>20	>20	>20
	II_6	CH		4.87 ± 0.03	2.09 ± 0.06	9.30 ± 0.05	5.40 ± 0.07
	III_1	CH		3.43 ± 0.03	4.28 ± 0.08	4.46 ± 0.02	5.43 ± 0.06
	III_2	N		3.76 ± 0.06	5.23 ± 0.08	6.32 ± 0.03	3.06 ± 0.07
	III_3	CH		15.7 ± 0.05	6.84 ± 0.05	>20	13.28 ± 0.07
	III_4	N		1.27 ± 0.09	1.36 ± 0.05	3.85 ± 0.03	4.06 ± 0.01
	III_5	CH		>20	>20	>20	>20
	III_6	N		>20	>20	>20	>20

III_7	CH		2.37 ± 0.09	3.11 ± 0.02	10.42 ± 0.01	7.17 ± 0.02
III_8	CH		>20	9.42 ± 0.09	17.48 ± 0.04	>20
III_9	N		3.65 ± 0.05	2.08 ± 0.07	6.02 ± 0.04	6.88 ± 0.05
III_{10}	CH		4.79 ± 0.04	3.71 ± 0.07	8.25 ± 0.06	12.07 ± 0.03
III_{11}	N		4.22 ± 0.01	3.30 ± 0.03	8.54 ± 0.09	13.05 ± 0.03
III_{12}	CH		>20	>20	>20	>20
III_{13}	N		>20	>20	>20	>20
III_{14}	CH		>20	>20	>20	>20
BI2536			0.05 ± 0.01	1.42 ± 0.04	0.46 ± 0.06	0.03 ± 0.09
JQ1			4.58 ± 0.04	0.79 ± 0.06	3.69 ± 0.05	6.88 ± 0.07

[a] Values are the means of at least three independent experiments. [b] SD: standard deviation.

3.2. Enzyme activity research

Based on the cellular assays, potent compounds were selected for further *in vitro* PLK1 and BRD4 % inhibition at 1 μM. The results were summarized in **Fig. 3**. Most of compounds inhibited PLK1 and BRD4 kinases with % inhibition values ranging from 44.6% to 96.6% and from 12.4% to 59.1%, respectively. In parallel with cellular results, inhibitors bearing the 1-methyl-1,2,4-triazole at **A** ring of pteridinone core were found to more potent than corresponding 1,2,4-triazole or tetrazolo analogues, which further proved 7-amino-1-methyl-triazolo[4,3-*f*]pteridin-4(5*H*)-one a favorable building block to increase the potency. Even though it showed more selectivity against PLK1, the inhibitory activity of **III_4** was still less than BRD4. These results indicated that three series of novel compounds worth further studying as new potential anticancer agent for the treatment of human cancers, and the BRD4 inhibitory activities can be increased by the structural modification.

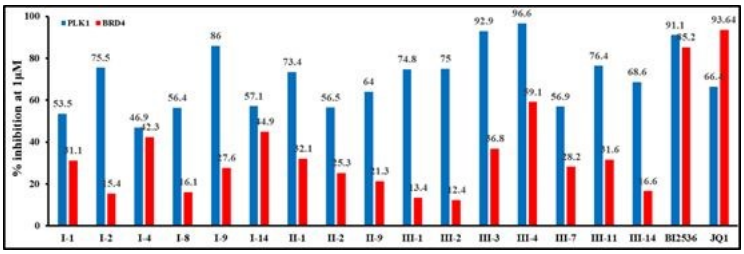


Fig. 3. Enzymatic activities of the target compounds.

3.3. Preliminary study on mechanism of action

To investigate the molecular mechanisms of action preliminarily, cell apoptosis analysis of HCT116 cells treated with the optimal compound **III_4** was performed using Annexin-V and propidium iodide (PI) double staining by flow cytometry and treating the HCT-116 cells with 1.0 μM, 3.0 μM and 9.0 μM of **III_4** for 24 h. As shown in **Fig. 4**, compound **III_4** was very effective in the induction of apoptosis in a dose-dependent manner. Compound **III_4** proved to induce apoptosis by 68.3 % as compared to 14.6% of apoptotic cells in the untreated control (**Table 2**).

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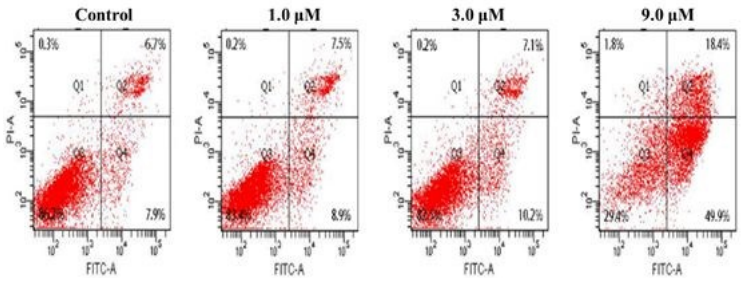

Fig. 4. Compound III_4 induced apoptosis in HCT-116 cells. Cells were treated with various concentrations of III_4 for 24 h and then analyzed the Annexin V-FITC/PI staining test by flow cytometry analysis. Values represent the mean ± S.D, n = 3. P <0.05 versus the control. The percentage of cells in each part is indicated.

Table 2.

Percent of cell death induced by compound **III$_4$** (9.0 μM and 0 μM) on HCT-116 cells.

Concentrations (**III$_4$**)	Apoptosis (%)			Necrosis (%)
	Total	Early	Late	
9.0 μM	68.3± 0.2	18.4±0.3	49.9 ± 0.1	1.8 ± 0.2
0 μM	14.6± 0.2	6.7±0.3	7.9 ± 0.1	0.3 ± 0.2

3.4 Migration experiment research

Since migration is an important characteristic for metastatic cancers, the effect of compound **III$_4$** on migration of cancer cells was investigated by the wound healing assay. As shown in **Fig. 5**, treatment of HCT116 cells with compound **III$_4$** at indicated concentrations markedly suppressed the wound healing in a concentration-dependent manner.

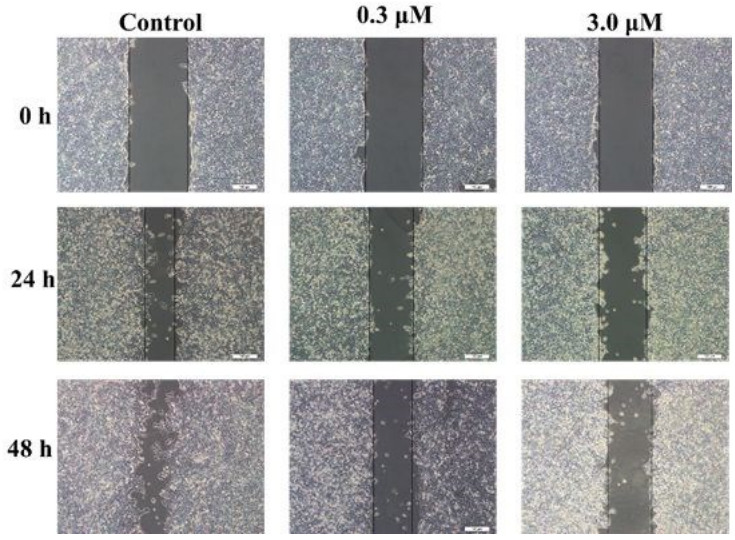

Fig. 5. *In vitro* wound healing assay on HCT116 cells. Phase contrast images were obtained by the treatment of compounds III_4 at indicated concentrations for 0, 24 and 48 h.

3.5 Cell cycle analysis.

The cell cycle analysis was performed to investigate the prevention of proliferation in HCT116 cells with the most potent compound **III$_4$**. After treatment of HCT116 cells

with compound **III$_4$** for 24 h at indicated concentrations (0.3, 1.0, 9.0 μM), the cells were fixed and stained with PI, the DNA content was analyzed by flow cytometry. The obtained results were compared with non-treated HCT116 cells, as control. As shown in **Figs. 6** and **7**, treatment of HCT116 cells with **III$_4$** at 0.3, 1.0, and 9.0 μM concentrations increased the percentage of S-phase cells from 18.83% (as control group) to 19.10%, 20.02%, and 37.17%, respectively. These results confirmed that compound **III$_4$** significantly caused S-phase arrest in HCT116 cells.

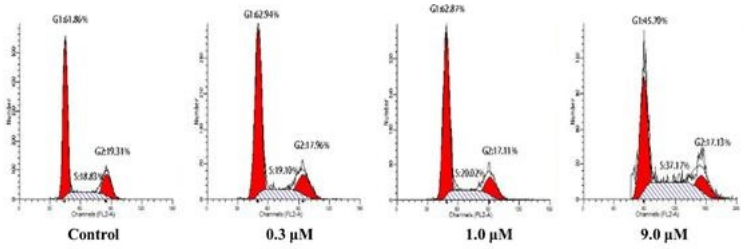

Fig. 6. Effect of compound III$_4$ on the cell cycle distribution of HCT116 cells.

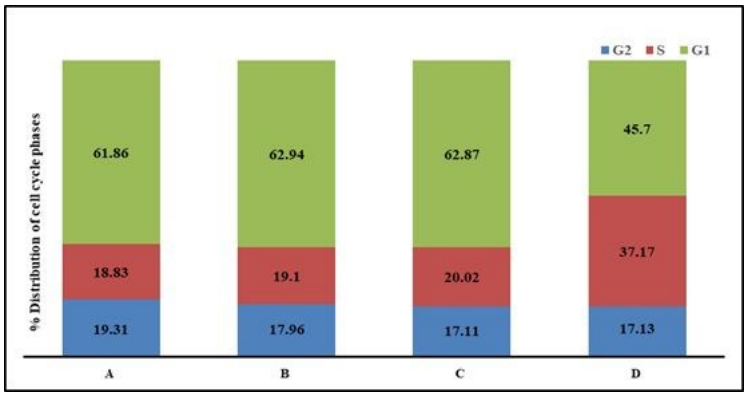

Fig. 7. Quantitative analysis of cell cycle distributions; (A) Non-treated cells as control group; (B) treated with III$_4$ at 0.3 μM; (C) treated with III$_4$ at 1.0 μM; (D) treated with III$_4$ at 9.0 μM.

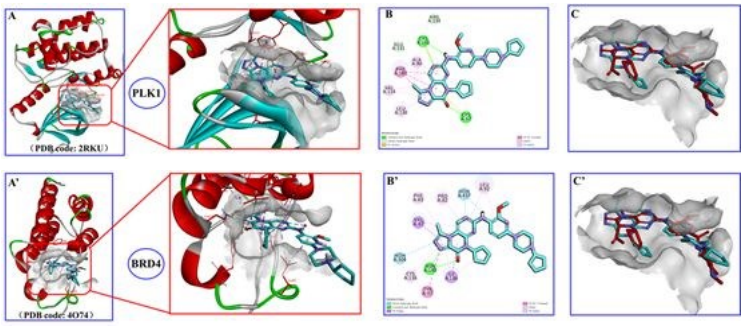

Fig. 8. The binding models of **III$_4$** with PLK1 and BRD4. (A and A') Predicted binding conformation for **III$_4$** (blue sticks) in the binding site cavity of PLK1 (PDB code: 2RKU) and BRD4 (PDB code: 4O74), (B and B') 2D diagram of the interaction between **III$_4$** and the binding site cavity of PLK1 and BRD4, (C and C') **III$_4$** overlapping with BI2536 (red sticks).

*3.6 Binding Mode of with **III$_4$** with PLK1 and BRD4*

In order to better understand the binding mode, molecular docking models of **III$_4$** was performed based upon the cocrystal structure with BI5236 of PLK1 (PDB code: 2RKU) and BRD4 (PDB code: 4O74), respectively.

As shown in **Fig. 8**, **III$_4$** occupied the kinase domain in a similar manner to BI2536 with PLK1 and BRD4, respectively. In the PLK1 kinase domain, **III$_4$** was found to form three hydrogen bonds *via* the 8-position nitrogen atom, the 7-position NH and the 4-position oxygen atom of carbonyl of the 7-amino-1-methyl-[1,2,4]triazolo[4,3-*f*]pteridinone nuclei with Cys 133 and Cys 67, respectively (**Fig. 8A**). In the BRD4 kinase domain, **III$_4$** was found to form two water-mediated hydrogen bonds with the 2-position NH, the 7-position NH and the 8-position nitrogen atom; and one hydrogen bond *via* the 3-position NH and the 4-position oxygen atom of carbonyl (**Fig. 8A'**. The methoxy group on the aromatic ring might be necessary to modulate binding affinity against the PLK1/BRD4 protein. These results could provide a molecular level foundation to illustrate that III4 could bind well at the active site of PLK1/BRD4 kinases and it is a potential inhibitor of PLK1/BRD4.

4. Conclusion

In the present study, three series of novel potent anticancer agents were designed and synthesized. The results of antiproliferative activities indicated that **III$_4$** significantly exhibits inhibitory activity against A549, HCT116, PC-3 and MCF-7 cell lines with IC_{50} values of 1.27 μM, 1.36 μM, 3.85 μM and 4.06 μM, respectively. Combined with the results of the molecular docking and enzymatic studies, the PLK1 and BRD4 were very likely to be the drug targets of compound **III$_4$**. Furthermore, to clarify the mechanism of the anticancer activity of the pteridinone molecule, the flow cytometry and wound-healing assays confirmed that **III$_4$** induced cell apoptosis. Finally, cell cycle analysis of **III$_4$** by flow cytometry showed cell cycle arrest in S phase, DNA fragmentation and alteration in mitochondrial membrane potential by compound **III$_4$** for the three concentrations tested. In summary, the SARs studies together with the pharmacological assays on novel pteridinone analogues identified **III$_4$** as a promising anti-cancer agent, which will lead to the promising development of new drugs.

5. Experimental procedures

5.1 *Chemistry*

Unless otherwise specified, all reagents and solvents were purchased from commercial sources and used as received. All melting points were obtained on a Mettler Point MP70 apparatus (Mettler, Toledo, Switzerland) and uncorrected. Mass spectra (MS) were acquired on an Agilent 1100 LC-MS (Agilent, palo Alto, CA, USA) in ESI mode. Reactions were monitored on silica plates (F-254) by thin-layer chromatography (TLC) and visualized under UV light. ^{1}H NMR (600 MHz) spectra were performed on a Bruker spectrometers (Bruker Bioscience, Billerica, Massachusetts, USA). The chemical shift (δ) of dimethyl sulfoxide-d_6 (DMSO-d_6) is based on 2.5ppm at 303 K and TMS Conducted was used as an internal standard. Column chromatography was performed using prepacked Qingdao Ocean Chemical Company (Qingdao, Shandong, China) silica gel (200-300 mesh).

*5.1.1 General procedure for preparation of compounds (**M$_2$-M$_9$**)*

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*5.1.1.1 2-chloro-N-cyclopentyl-5-nitropyrimidin-4-amine(**M_2**)*

2,4-Dichloro-5-nitropyrimidine *(**M_1**)* (30.0 g, 156 mmol) and $NaHCO_3$ (26.2g, 312 mmol) were dissolved in DCM (200 mL). Cyclopentylamine (15.9 g, 187 mmol) was dissolved in DCM (300 mL) and then added at 0 ºC. The reaction mixture was stirred for 10 h at room temperature and then organic was washed by water, and brine. The combined organic phase was dried over Na_2SO_4, filtered, and the solvent evaporated to obtain 30.95g of ***M_2*** as a yellow solid, m.p.: 125.2 – 126.6 °C. Yield: 82%; MS (ESI) m/z: 243.3 $[M+H]^+$.

*5.1.1.2 2-chloro-N4-cyclopentylpyrimidine-4,5-diamine (**M_3**)*

To a suspension of compound ***M_2*** (30.0 g, 124 mmol) in 300 mL of 95% ethanol was added HCl (1 mL), and Fe Powder (34.7 g, 620 mmol) in batches. The mixture was refluxed for 2 h. After completion of the reaction as indicated by TLC, the solution was then filtered hot through Celite. The filtrate was concentrated in a vacuum to afford pure product and then purified by silica gel column chromatography to obtain 18.42 g of ***M_3*** as a white solid, m.p.: 128.2 – 130.0 °C. Yield: 70.1%; MS (ESI) m/z: 213.2 $[M+H]^+$.

*5.1.1.3 2-chloro-8-cyclopentyl-5,8-dihydropteridine-6,7-dione (**M_4**)*

To a stirred solution of compound ***M_3*** (15.0 g, 71 mmol) in acetone (150 mL) was added K_2CO_3 (11.6 g, 85 mmol) and ethyl oxalyl monochloride (10.6g, 78 mmol). The mixture was stirred at room temperature for 2 h. The solution was filtered and the filtrate was concentrated under reduced pressure. In a pressure flask, the crude ketoester was dissolved in absolute EtOH (150 mL) and then TEA (8.6 g, 85mmol) was added. The mixture was heated for an additional 4 h at 100 °C. After indicating the completion of the reaction by TLC, the solution was filtered to obtain 16.4 g of ***M_4*** as a white solid, m.p.: 210.1 – 212.2 °C. Yield: 86.9%; MS (ESI) m/z: 264.9 $[M-H]^-$.

*4.1.1.4 2,6-dichloro-8-cyclopentylpteridin-7(8H)-one (**M_5**)*

DMF added to a solution of compound ***M_4*** (15.0 g, 56 mmol) in $SOCl_2$ (75 mL), and the resulting mixture was maintained at this temperature for 2 h. The solvent was concentrated in vacuum and the reaction mixture was poured into 200 mL ice water, the resulting precipitate was filtered and dried to obtain 14.8 g of ***M_5*** as a white solid, m.p.: 269.0 – 279.3 °C. Yield: 93.1%; MS (ESI) m/z: 285.26 $[M+H]^+$.

*5.1.1.5 2-chloro-8-cyclopentyl-6-hydrazinylpteridin-7(8H)-one (**M_6**)*

A mixture of ***M_5*** (14.0 g, 49 mmol) and 80% hydrazine monohydrate (13.7 g, 73.5 mmol) in EtOH (200 mL) was stirred at 40 °C for 2h. After indicating the completion of the reaction by TLC, a white solid appeared after most solvents were evaporated to dryness under reduced pressure. The resulting precipitate was filtered off, washed with water, and dried under vacuum to afford 12.0 g of ***M_6*** as a white solid, m.p.: 210.7 – 212.1 °C. Yield: 85.8%; MS (ESI) m/z: 279.0 $[M-H]^-$.

5.1.1.6 7-chloro-5-cyclopentyl-[1,2,4]triazolo[4,3-f]pteridin-4(5H)-one (***M_7***)

To a stirred solution of compound ***M*****$_6$** (5.0 g, 17.8 mmol) in trimethyl orthoformate (50 mL). The reaction mixture was stirred at 80 °C for 2h, cooled to room temperature. The resulting precipitate was filtered off, washed with diethyl ether, and dried to afford 4.7 g of ***M*****$_7$** as a white solid, m.p.: 251.8 – 253.1 °C. Yield: 89.9%; MS (ESI) m/z: 313.1 $[M+Na]^+$. ^{1}H NMR (DMSO-d_6, 400 MHz) δ 9.90 (s, 1H), 9.49 (s, 1H), 5.76 – 5.60 (m, 1H), 2.21-2.13 (m, 2H), 2.09 – 1.98 (m, 2H), 1.98 – 1.83 (m, 2H), 1.67-1.64 (m, 2H).

*5.1.1.7 7-chloro-5-cyclopentyltetrazolo[1,5-f]pteridin-4(5H)-one (****M*****$_8$)*

To a suspension of ***M*****$_5$** (5.0 g, 17.5 mmol) and DMF (20 mL) was dropped NaN_3 (1.14 g, 17.5 mmol). The mixture was stirred at 0 °C for 10 h. After indicating the completion of the reaction by TLC, the reaction mixture was cooled to room temperature. Water was then added, and the solid was filtered and dried under reduced pressure to afford 4. g of ***M*****$_8$** as a white solid, m.p.: 185.4 – 186.8 °C. Yield: 83.6%; MS (ESI) m/z: 292.3 $[M+H]^+$. ^{1}H NMR (DMSO-d_6, 400 MHz,) δ 9.71 (s, 1H), 5.78 – 5.70 (m, 1H), 2.21 – 2.13 (m, 2H), 2.08 – 2.02 (m, 2H), 2.00 – 1.88 (m, 2H), 1.73 – 1.62 (m, 2H).

*5.1.1.8 7-chloro-5-cyclopentyl-1-methyl-[1,2,4]triazolo[4,3-f]pteridin-4(5H)-one (****M*** ***9)***

Compound ***M*****$_6$** (5.0 g, 17.8 mmol) was stirred in a solution of triethyl orthoformate (50 ml). The reaction was stirred at 80 ℃ for 2 hours, then cooled to room temperature. The resulting precipitate was filtered, washed with diethyl ether, and dried to provide 4.6 g of ***M*****$_9$** as a white solid, m.p.: 255.3 – 257.1 °C. Yield: 84.1%; MS (ESI) m/z: 327.2 $[M+Na]^+$.

*5.1.2 General Procedure for Preparation of (****M*****$_{11}$)*

In the case of stirring at 40 ℃, substitute amines (1.2 equiv) were added into the DMF (7 mL/g) mixture of ***M*****$_{10}$** (1 equiv) and K_2CO_3 (2.0 equiv), and then TLC was detected. After the mixture is poured into the ice water, the product is precipitated, filtered and dried to obtain the yellow solid compound M11. After the mixture is poured into the ice water, the product is precipitated, filtered and dried to obtain the yellow solid compound ***M*****$_{11}$**.

*5.1.3 General Procedure for Preparation of (****M*****$_{12}$)*

The nitro compound ***M*****$_{11}$** (1 equiv in a mixture of EtOH-H_2O, 95:5, 20 mL) was reduced with 10% Pd-carbon (5% w/w) The reaction was hydrogenated at 50 ℃ for 5 hours. The reaction was detected by TLC. After the reaction is completed, the reactant is pumped and filtered, and the filtrate is vacuum concentrated to the product ***M*****$_{12}$**.

*5.1.4 General procedure for preparation of compounds (****I*****$_1$*****-I*****$_{14}$*****,II*****$_1$*****-II*****$_6$*** ***or III*****$_1$*****-III*****$_{14}$*****)***

Compound ***M*****$_{12}$** (1.1 equiv) and *p*-toluenesulfonic acid (1 equiv) were added to the compound to a stirred solution of compound ***M*****$_7$**, ***M*****$_8$**, or ***M*****$_9$** (1 equiv) in 1-butanol, placed in a pressure bottle, and heated to 100 ° C for 15 h. The reaction mixture was quenched with saturated Na_2CO_3 aqueous solution, then extracted with DCM, washed with water and dried on anhydrous Na_2SO_4. The organic layer was concentrated under

reduced pressure. The product was further purified by fast column chromatography and dichloromethane / methanol was used as eluent to obtain ***I_1-I_{14},II_1-II_6 or III_1-III_{14}*** as a pale yellow solid.

*5.1.4.1 5-cyclopentyl-7-((2-methoxy-4-(4-methylpiperazin-1-yl)phenyl)amino)-[1,2,4] triazolo[4,3-f]pteridin-4(5H)-one(**I_1**)*

Yield: 74.7%; m.p.:242.6-244.4 °C; MS (ESI) *m/z*:476.2[M+H]$^+$; ^{1}H NMR (DMSO-d_6, 600 MHz) δ 9.71 (s, 1H), 9.10 (s, 1H), 8.73 (s, 1H), 7.29 (s, 1H), 6.64 (s, 1H), 6.52 (s, 1H), 5.58 (s, 1H), 3.75 (s, 3H), 3.17 (s, 4H), 2.21 (d, *J* = 39.5 Hz, 7H), 1.69 (s, 5H), 1.46 (s, 5H).

*5.1.4.2 5-cyclopentyl-7-((2-methoxy-6-(4-methylpiperazin-1-yl)pyridin-3-yl)amino)-[1,2,4]triazolo[4,3-f]pteridin-4(5H)-one(**I_2**)*

Yield: 65.0%; m.p.:263.0-264.9°C; MS (ESI) *m/z*:477.2[M+H]$^+$; ^{1}H NMR (DMSO-d_6, 600 MHz) δ 9.70 (s, 1H), 9.10 (s, 1H), 8.85 (s, 1H), 7.48 (s, 1H), 6.37 (s, 1H), 5.53 (s, 1H), 3.77 (s, 3H), 3.48 (s, 4H), 2.42 (s, 4H), 2.23 (s, 3H), 2.17 (s, 2H), 1.67 (s, 3H), 1.45 (s, 3H).

*5.1.4.3 5-cyclopentyl-7-((4-(4-cyclopentylpiperazin-1-yl)-2-methoxyphenyl)amino)-[1,2 ,4]triazolo[4,3-f]pteridin-4(5H)-one(**I_3**)*

Yield:74.7 %; m.p.:296.4-298.3 °C; MS (ESI) *m/z*:530.3[M+H]$^+$; ^{1}H NMR (DMSO-d_6, 600 MHz) δ 9.70 (s, 1H), 9.10 (s, 1H), 8.72 (s, 1H), 7.27 (d, *J* = 8.3 Hz, 1H), 6.63 (d, *J* = 2.1 Hz, 1H), 6.50 (dd, *J* = 8.6, 2.2 Hz, 1H), 5.57 (s, 1H), 3.74 (s, 3H), 3.17 – 3.13 (m, 4H), 2.56 (s, 4H), 2.20 – 2.13 (m, 2H), 1.82 (d, *J* = 6.1 Hz, 3H), 1.74 – 1.59 (m, 6H), 1.52 (dd, *J* = 7.1, 5.0 Hz, 3H), 1.45 (d, *J* = 11.1 Hz, 2H), 1.37 (td, *J* = 16.3, 8.0 Hz, 3H).

*5.1.4.4 5-cyclopentyl-7-((6-(4-cyclopentylpiperazin-1-yl)-2-methoxypyridin-3-yl)amin o)-[1,2,4]triazolo[4,3-f]pteridin-4(5H)-one(**I_4**)*

Yield:55.2 %; m.p.:271.5-273.2°C; MS (ESI) *m/z*:531.3[M+H]$^+$; ^{1}H NMR (DMSO-d_6, 600 MHz) δ 9.70 (s, 1H), 9.09 (s, 1H), 8.85 (s, 1H), 7.47 (d, *J* = 5.3 Hz, 1H), 6.35 (d, *J* = 8.4 Hz, 1H), 5.52 (s, 1H), 3.77 (s, 3H), 3.46 (s, 4H), 3.17 (s, 4H), 2.52 (s, 4H), 2.19 – 2.14 (m, 2H), 1.83 – 1.79 (m, 2H), 1.66 – 1.61 (m, 3H), 1.54 – 1.49 (m, 3H), 1.41 – 1.35 (m, 3H).

*5.1.4.5 5-cyclopentyl-7-((2-methoxy-4-morpholinophenyl)amino)-[1,2,4]triazolo[4,3-f]pteridin-4(5H)-one(**I_5**)*

Yield: 65.3 %; m.p.: 272.3-274.2 °C; MS (ESI) *m/z*:463.2[M+H]$^+$; ^{1}H NMR (DMSO-d_6, 600 MHz) δ 9.71 (s, 1H), 9.11 (s, 1H), 8.73 (s, 1H), 7.33 (d, *J* = 7.3 Hz, 1H), 6.66 (s, 1H), 6.53 (d, *J* = 7.7 Hz, 1H), 5.59 (s, 1H), 3.76 (s, 7H), 3.13 (s, 4H), 2.18 (d, *J* = 4.0 Hz, 2H), 1.70 (s, 3H), 1.47 (s, 2H).

*5.1.4.6 5-cyclopentyl-7-((2-methoxy-6-morpholinopyridin-3-yl)amino)-[1,2,4]triazolo [4,3-f]pteridin-4(5H)-one(**I_6**)*

Yield: 62.5%; m.p.:276.4-278.1°C; MS (ESI) *m/z*:464.2[M+H]$^+$; ^{1}H NMR (DMSO-d_6,

600 MHz) δ 9.70 (s, 1H), 9.10 (s, 1H), 8.87 (s, 1H), 7.52 (d, *J* = 6.1 Hz, 1H), 6.37 (d, *J* = 8.3 Hz, 1H), 5.54 (s, 1H), 3.78 (s, 3H), 3.74 – 3.70 (m, 4H), 3.45 – 3.41 (m, 4H), 2.17 (s, 2H), 1.67 (s, 3H), 1.45 (s, 3H).

*5.1.4.7 5-cyclopentyl-7-((2-methoxy-4-(3-methylpiperazin-1-yl)phenyl)amino)-[1,2,4]triazolo[4,3-f]pteridin-4(5H)-one(**I**$_7$)*

Yield:69.2 %; m.p.: 251.1-253.0°C; MS (ESI) *m/z*:476.2[M+H]$^+$; ^{1}H NMR (DMSO-d_6, 600 MHz) δ 9.71 (s, 1H), 9.10 (s, 1H), 8.72 (s, 1H), 7.28 (d, *J* = 5.0 Hz, 1H), 6.62 (s, 1H), 6.50 (d, *J* = 7.7 Hz, 1H), 5.58 (s, 1H), 3.75 (s, 3H), 3.57 – 3.51 (m, 3H), 2.98 (d, *J* = 11.3 Hz, 1H), 2.82 (s, 2H), 2.59 – 2.55 (m, 1H), 2.21 (dd, *J* = 25.6, 14.6 Hz, 3H), 1.69 (s, 3H), 1.46 (s, 3H), 1.05 (d, *J* = 5.7 Hz, 3H).

*5.1.4.8 5-cyclopentyl-7-((4-(4-ethylpiperazin-1-yl)-2-methoxyphenyl)amino)-[1,2,4]triazolo[4,3-f]pteridin-4(5H)-one(**I**$_8$)*

Yield: 57.2%; m.p.: 254.2-256.3°C; MS (ESI) *m/z*:490.3[M+H]$^+$; ^{1}H NMR (DMSO-d_6, 600 MHz) δ 9.71 (s, 1H), 9.10 (s, 1H), 8.76 – 8.71 (m, 1H), 7.28 (d, *J* = 5.6 Hz, 1H), 6.64 (s, 1H), 6.51 (d, *J* = 7.4 Hz, 1H), 5.58 (s, 1H), 3.75 (s, 3H), 3.17 (s, 4H), 2.51 (s, 3H), 2.40 (d, *J* = 5.8 Hz, 2H), 2.17 (s, 2H), 1.69 (s, 3H), 1.46 (s, 3H), 1.05 (s, 3H).

*5.1.4.9 5-cyclopentyl-7-((6-(4-ethylpiperazin-1-yl)-2-methoxypyridin-3-yl)amino)-[1,2,4]triazolo[4,3-f]pteridin-4(5H)-one(**I**$_9$)*

Yield: 64.2%; m.p.:214.0-216.2 °C; MS (ESI) *m/z*:491.26[M+H]$^+$; ^{1}H NMR (DMSO-d_6, 600 MHz) δ 9.71 (s, 1H), 9.10 (s, 1H), 8.86 (s, 1H), 7.48 (s, 1H), 6.37 (s, 1H), 5.53 (s, 1H), 3.78 (s, 3H), 3.50 (d, *J* = 14.1 Hz, 4H), 2.51 (s, 4H), 2.39 (s, 2H), 2.17 (s, 2H), 1.66 (s, 3H), 1.45 (s, 3H), 1.05 (s, 3H).

*5.1.4.10 5-cyclopentyl-7-((4-(3,5-dimethylpiperazin-1-yl)-2-methoxyphenyl)amino)-[1,2,4]triazolo[4,3-f]pteridin-4(5H)-one(**I**$_{10}$)*

Yield:68.3 %; m.p.:203.2-206.7 °C; MS (ESI) *m/z*:490.3.26[M+H]$^+$; ^{1}H NMR (DMSO-d_6, 600 MHz) δ 9.71 (s, 1H), 9.11 (s, 1H), 8.73 (s, 1H), 7.28 (d, *J* = 8.1 Hz, 1H), 6.64 (d, *J* = 2.1 Hz, 1H), 6.52 (dd, *J* = 8.6, 2.3 Hz, 1H), 5.58 (s, 1H), 4.66 (s, 3H), 3.76 (s, 3H), 3.61 (d, *J* = 11.3 Hz, 3H), 2.97 (s, 3H), 2.20 – 2.14 (m, 3H), 1.69 (s, 3H), 1.46 (s, 3H), 1.09 (d, *J* = 5.8 Hz, 3H).

*5.1.4.11 5-cyclopentyl-7-((6-(3,5-dimethylpiperazin-1-yl)-2-methoxypyridin-3-yl)amino)-[1,2,4]triazolo[4,3-f]pteridin-4(5H)-one(**I**$_{11}$)*

Yield: 58.4%; m.p.:205.6-208.3 °C; MS (ESI) *m/z*:491.3.26[M+H]$^+$; ^{1}H NMR (DMSO-d_6, 600 MHz) δ 9.70 (s, 1H), 9.10 (s, 1H), 8.85 (s, 1H), 7.47 (d, *J* = 5.1 Hz, 1H), 6.38 (d, *J* = 8.4 Hz, 1H), 5.54 (s, 1H), 4.16 (d, *J* = 11.8 Hz, 3H), 3.77 (s, 3H), 2.89 (s, 3H), 2.38 (d, *J* = 12.5 Hz, 3H), 2.20 – 2.13 (m, 3H), 1.66 (s, 3H), 1.43 (s, 3H), 1.09 (d, *J* = 5.7 Hz, 3H).

*5.1.4.12 5-cyclopentyl-7-((4-(4-(2-hydroxyethyl)piperazin-1-yl)-2-methoxyphenyl)amino)-[1,2,4]triazolo[4,3-f]pteridin-4(5H)-one(**I**$_{12}$)*

Yield:55.2 %; m.p.: 273.1-275.6°C; MS (ESI) *m/z*:506.2[M+H]$^+$; ^{1}H NMR (DMSO-d_6, 600 MHz) δ 9.71 (s, 1H), 9.10 (s, 1H), 8.73 (s, 1H), 7.28 (d, *J* = 6.2 Hz, 1H), 6.64 (s, 1H), 6.51 (d, *J* = 7.8 Hz, 1H), 5.58 (s, 1H), 4.46 (s, 1H), 3.75 (s, 3H), 3.55 (d, *J* = 4.9 Hz, 2H), 3.16 (s, 4H), 2.58 (s, 4H), 2.46 (d, *J* = 5.4 Hz, 2H), 2.17 (s, 2H), 1.69 (s, 3H), 1.46 (d, *J* = 0.9 Hz, 3H).

*5.1.4.13 5-cyclopentyl-7-((6-(4-(2-hydroxyethyl)piperazin-1-yl)-2-methoxypyridin-3-yl)amino)-[1,2,4]triazolo[4,3-f]pteridin-4(5H)-one(**I$_{13}$**)*

Yield: 51.0%; m.p.:226.7-229.2°C; MS (ESI) *m/z*:507.2[M+H]$^+$; ^{1}H NMR (DMSO-d_6, 600 MHz) δ 9.71 (s, 1H), 9.10 (s, 1H), 8.86 (s, 1H), 7.47 (d, *J* = 4.9 Hz, 1H), 6.36 (d, *J* = 8.4 Hz, 1H), 5.53 (s, 1H), 4.46 (t, *J* = 5.1 Hz, 1H), 3.77 (s, 3H), 3.55 (dd, *J* = 11.3, 5.8 Hz, 2H), 3.47 (s, 4H), 2.52 (s, 4H), 2.44 (t, *J* = 6.2 Hz, 2H), 2.16 (s, 2H), 1.66 (s, 3H), 1.45 (s, 4H).

*5.1.4.14 5-cyclopentyl-7-((2-methoxy-4-(piperazin-1-yl)phenyl)amino)-[1,2,4]triazolo[4,3-f]pteridin-4(5H)-one(**I$_{14}$**)*

Yield: 52.9%; m.p.: 264.3-266.8°C; MS (ESI) *m/z*:462.2[M+H]$^+$; ^{1}H NMR (DMSO-d_6, 600 MHz) δ 9.71 (s, 1H), 9.11 (s, 1H), 8.72 (s, 1H), 7.29 (s, 1H), 6.63 (s, 1H), 6.50 (d, *J* = 6.8 Hz, 1H), 5.58 (s, 1H), 3.75 (s, 3H), 3.07 (s, 4H), 2.86 (s, 4H), 2.18 (s, 3H), 1.70 (s, 3H), 1.47 (s, 3H).

*5.1.4.15 5-cyclopentyl-7-((2-methoxy-4-(4-methylpiperazin-1-yl)phenyl)amino)tetrazolo[1,5-f]pteridin-4(5H)-one(**II$_1$**)*

Yield: 55.1%; m.p.: 221.2-222.9°C; MS (ESI) *m/z*:477.2[M+H]$^+$; ^{1}H NMR (DMSO-d_6, 600 MHz) δ 9.20 (s, 1H), 9.13 (s, 1H), 7.22 (d, *J* = 3.9 Hz, 1H), 6.65 (s, 1H), 6.53 (d, *J* = 6.8 Hz, 1H), 5.54 (s, 1H), 3.75 (s, 3H), 3.18 (s, 4H), 2.51 (s, 4H), 2.22 (d, *J* = 40.4 Hz, 5H), 1.70 (s, 3H), 1.46 (s, 3H).

*5.1.4.16 5-cyclopentyl-7-((4-(4-cyclopentylpiperazin-1-yl)-2-methoxyphenyl)amino)tetrazolo[1,5-f]pteridin-4(5H)-one(**II$_2$**)*

Yield: 57.9%; m.p.:248.2-250.1 °C; MS (ESI) *m/z*:531.3[M+H]$^+$; ^{1}H NMR (DMSO-d_6, 600 MHz) δ 9.19 (s, 1H), 9.11 (s, 1H), 7.22 (s, 1H), 6.64 (s, 1H), 6.51 (d, *J* = 8.2 Hz, 1H), 5.52 (s, 1H), 3.74 (s, 3H), 3.16 (s, 4H), 2.57 (s, 4H), 2.17 (d, *J* = 13.4 Hz, 2H), 1.82 (d, *J* = 4.5 Hz, 3H), 1.66 (dd, *J* = 21.0, 15.5 Hz, 4H), 1.56 – 1.50 (m, 3H), 1.47 – 1.32 (m, 5H).

*5.1.4.17 5-cyclopentyl-7-((6-(4-cyclopentylpiperazin-1-yl)-2-methoxypyridin-3-yl)amino)tetrazolo[1,5-f]pteridin-4(5H)-one(**II$_3$**)*

Yield: 66.2%; m.p.:219.0-220.8 °C; MS (ESI) *m/z*:532.3[M+H]$^+$; ^{1}H NMR (DMSO-d_6, 600 MHz) δ 9.19 (s, 1H), 9.11 (s, 1H), 7.22 (s, 1H), 6.64 (s, 1H), 6.51 (d, *J* = 8.2 Hz, 1H), 5.52 (s, 1H), 3.74 (s, 3H), 3.16 (s, 4H), 2.57 (s, 4H), 2.17 (d, *J* = 13.4 Hz, 2H), 1.82 (d, *J* = 4.5 Hz, 3H), 1.66 (dd, *J* = 21.0, 15.5 Hz, 4H), 1.56 – 1.50 (m, 3H), 1.47 – 1.32 (m, 5H).

*5.1.4.18 5-cyclopentyl-7-((2-methoxy-4-morpholinophenyl)amino)tetrazolo[1,5-f]pteridin-4(5H)-one(**II$_4$**)*

Yield: 64.9%; m.p.:240.4-242.2 °C; MS (ESI) *m/z*:464.2[M+H]$^+$; ^{1}H NMR (DMSO-d_6, 600 MHz) δ 9.20 (s, 1H), 9.14 (s, 1H), 7.25 (s, 1H), 6.67 (d, *J* = 2.2 Hz, 1H), 6.54 (dd, *J* = 8.6, 2.2 Hz, 1H), 5.57 (s, 1H), 3.78 – 3.76 (m, 7H), 3.15 – 3.13 (m, 4H), 2.18 (d, *J* = 6.8 Hz, 2H), 1.71 (s,3H), 1.46 (s, 3H).

*5.1.4.19 5-cyclopentyl-7-((2-methoxy-6-morpholinopyridin-3-yl)amino)tetrazolo[1,5-f]pteridin-4(5H)-one(**II$_5$**)*

Yield:45.9 %; m.p.:244.2-246.2 °C; MS (ESI) *m/z*:465.2[M+H]$^+$; ^{1}H NMR (DMSO-d_6, 600 MHz) δ 9.26 (s, 1H), 9.20 (s, 1H), 7.50 (d, *J* = 3.2 Hz, 1H), 6.39 (d, *J* = 8.4 Hz, 1H), 5.43 (s, 1H), 3.78 (s, 3H), 3.74 – 3.70 (m, 4H), 3.47 – 3.42 (m, 4H), 2.16 (s, 2H), 1.64 (s, 3H), 1.41 (s, 3H).

*5.1.4.20 5-cyclopentyl-7-((2-methoxy-4-(3-methylpiperazin-1-yl)phenyl)amino)tetrazolo[1,5-f]pteridin-4(5H)-one(**II$_6$**)*

Yield: 53.0%; m.p.:209.2-211.1 °C; MS (ESI) *m/z*:477.2[M+H]$^+$; ^{1}H NMR (DMSO-d_6, 600 MHz) δ 9.19 (s, 1H), 9.13 (s, 1H), 7.23 (s, 1H), 6.66 (d, *J* = 1.9 Hz, 1H), 6.55 – 6.52 (m, 1H), 5.53 (s, 1H), 3.75 (s, 3H), 3.62 (dd, *J* = 16.9, 12.8 Hz, 3H), 3.09 (d, *J* = 11.9 Hz, 1H), 2.97 (s, 1H), 2.90 (dd, *J* = 11.7, 9.4 Hz, 1H), 2.68 (t, *J* = 10.9 Hz, 1H), 2.38 – 2.34 (m, 1H), 2.18 (s, 2H), 1.69 (s, 3H), 1.45 (s, 3H), 1.12 (d, *J* = 6.3 Hz, 3H).

*5.1.4.21 5-cyclopentyl-7-((2-methoxy-4-(4-methylpiperazin-1-yl)phenyl)amino)-1-methyl-[1,2,4]triazolo[4,3-f]pteridin-4(5H)-one(**III$_1$**)*

Yield:57.2 %; m.p.:261.2-263.1 °C; MS (ESI) *m/z*:490.2[M+H]$^+$; ^{1}H NMR (DMSO-d_6, 600 MHz) δ 8.91 (s, 1H), 8.69 (s, 1H), 7.30 (s, 1H), 6.63 (s, 1H), 6.50 (d, *J* = 6.5 Hz, 1H), 5.59 (s, 1H), 3.75 (s, 3H), 3.15 (s, 4H), 2.90 (s, 3H), 2.48 (s, 4H), 2.24 (s, 3H), 2.17 (s, 2H), 1.69 (s, 3H), 1.47 (s, 3H).

*5.1.4.22 5-cyclopentyl-7-((2-methoxy-6-(4-methylpiperazin-1-yl)pyridin-3-yl)amino)-1-methyl-[1,2,4]triazolo[4,3-f]pteridin-4(5H)-one(**III$_2$**)*

Yield: 78.1%; m.p.:257.2-258.9 °C; MS (ESI) *m/z*:491.2[M+H]$^+$; ^{1}H NMR (DMSO-d_6, 600 MHz) δ 8.91 (s, 1H), 8.85 (s, 1H), 7.49 (d, *J* = 1.9 Hz, 1H), 6.37 (d, *J* = 8.3 Hz, 1H), 5.55 (s, 1H), 3.78 (s, 3H), 3.48 (s, 4H), 2.90 (s, 3H), 2.43 (s, 4H), 2.24 (s, 3H), 2.17 (d, *J* = 2.0 Hz, 2H), 1.67 (s, 3H), 1.46 (s, 3H).

*5.1.4.23 5-cyclopentyl-7-((4-(4-cyclopentylpiperazin-1-yl)-2-methoxyphenyl)amino)-1-methyl-[1,2,4]triazolo[4,3-f]pteridin-4(5H)-one(**III$_3$**)*

Yield: 55.8%; m.p.:283.8-285.7 °C; MS (ESI) *m/z*:544.3[M+H]$^+$; ^{1}H NMR (DMSO-d_6, 600 MHz) δ 8.91 (s, 1H), 8.69 (s, 1H), 7.28 (d, *J* = 6.0 Hz, 1H), 6.62 (s, 1H), 6.49 (dd, *J* = 8.4, 1.3 Hz, 1H), 5.59 (s, 1H), 3.75 (s, 3H), 3.14 (s, 4H), 2.89 (s, 3H), 2.57 (s, 4H), 2.17 (s, 2H), 1.82 (d, *J* = 5.6 Hz, 2H), 1.74 – 1.58 (m, 6H), 1.57 – 1.41 (m, 5H), 1.40 – 1.34 (m, 2H).

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*5.1.4.24 5-cyclopentyl-7-((6-(4-cyclopentylpiperazin-1-yl)-2-methoxypyridin-3-yl)amino)-1-methyl-[1,2,4]triazolo[4,3-f]pteridin-4(5H)-one(**III$_4$**)*

Yield:54.3 %; m.p.: 246.8-248.7°C; MS (ESI) *m/z*:545.3[M+H]$^+$; ^{1}H NMR (DMSO-d_6, 600 MHz) δ 8.91 (s, 1H), 8.83 (s, 1H), 7.48 (d, *J* = 3.8 Hz, 1H), 6.34 (d, *J* = 8.4 Hz, 1H), 5.54 (s, 1H), 3.77 (s, 3H), 3.46 (s, 4H), 2.89 (s, 3H), 2.51 (d, *J* = 3.0 Hz, 4H), 2.16 (d, *J* = 0.7 Hz, 2H), 1.82 (d, *J* = 4.8 Hz, 2H), 1.70 – 1.58 (m, 5H), 1.56 – 1.47 (m, 3H), 1.45 (d, *J* = 5.3 Hz, 1H), 1.39 (dd, *J* = 12.0, 3.9 Hz, 3H).

*5.1.4.25 5-cyclopentyl-7-((2-methoxy-4-morpholinophenyl)amino)-1-methyl-[1,2,4]triazolo[4,3-f]pteridin-4(5H)-one(**III$_5$**)*

Yield: 55.7%; m.p.:284.6-286.3 °C; MS (ESI) *m/z*:477.2[M+H]$^+$; ^{1}H NMR (DMSO-d_6, 600 MHz) δ 8.92 (s, 1H), 8.72 (s, 1H), 7.32 (d, *J* = 7.9 Hz, 1H), 6.66 (d, *J* = 2.3 Hz, 1H), 6.52 (dd, *J* = 8.7, 2.3 Hz, 1H), 5.60 (s, 1H), 3.76 (t, *J* = 4.6 Hz, 7H), 3.13 – 3.11 (m, 4H), 2.90 (s, 3H), 2.17 (dd, *J* = 15.5, 8.0, 3.3 Hz, 2H), 1.70 (s, 4H), 1.47 (s, 2H).

*5.1.4.26 5-cyclopentyl-7-((2-methoxy-6-morpholinopyridin-3-yl)amino)-1-methyl-[1,2,4]triazolo[4,3-f]pteridin-4(5H)-one(**III$_6$**)*

Yield: 64.9%; m.p.: 246.6-248.5°C; MS (ESI) *m/z*:478.2[M+H]$^+$; ^{1}H NMR (DMSO-d_6, 600 MHz) δ 8.92 (s, 1H), 8.86 (s, 1H), 7.53 (d, *J* = 7.6 Hz, 1H), 6.37 (d, *J* = 8.4 Hz, 1H), 5.56 (s, 1H), 3.78 (s, 3H), 3.75 – 3.69 (m, 4H), 3.46 – 3.40 (m, 4H), 2.89 (s, 3H), 2.17 (dd, *J* = 15.2, 8.2 Hz, 3H), 1.67 (s, 3H), 1.46 (s, 3H).

*5.1.4.27 5-cyclopentyl-7-((2-methoxy-4-(3-methylpiperazin-1-yl)phenyl)amino)-1-methyl-[1,2,4]triazolo[4,3-f]pteridin-4(5H)-one(**III$_7$**)*

Yield: 63.6%; m.p.:253.5-255.4°C; MS (ESI) *m/z*:490.2[M+H]$^+$; ^{1}H NMR (DMSO-d_6, 600 MHz) δ 8.91 (s, 1H), 8.71 (s, 1H), 7.30 (d, 1H), 6.63 (s, 1H), 6.51 (s, 1H), 5.60 (s, 1H), 3.76 (s, 3H), 3.55 (d, *J* = 13.4 Hz, 3H), 3.02 (d, *J* = 9.4 Hz, 1H), 2.92 – 2.89 (m, 3H), 2.87 (s, 2H),2.63 – 2.57 (m, 1H), 2.25 (d, *J* = 9.6 Hz, 1H), 2.18 (s, 1H), 1.70 (s, 3H), 1.46 (s, 3H), 1.10 – 1.04 (m,3H).

*5.1.4.28 5-cyclopentyl-7-((4-(4-ethylpiperazin-1-yl)-2-methoxyphenyl)amino)-1-methyl-[1,2,4]triazolo[4,3-f]pteridin-4(5H)-one(**III$_8$**)*

Yield: 69.1%; m.p.:264.3-266.8 °C; MS (ESI) *m/z*:504.3[M+H]$^+$; ^{1}H NMR (DMSO-d_6, 600 MHz) δ 8.92 (s, 1H), 8.72 (s, 1H), 7.29 (d, *J* = 4.9 Hz, 1H), 6.64 (d, *J* = 2.1 Hz, 1H), 6.51 (dd, *J* = 8.6, 2.2 Hz, 1H), 5.59 (s, 1H), 3.51 (s, 3H), 3.16 (s, 4H), 2.90 (s, 3H), 2.53 (s, 4H), 2.40 (dd, *J* = 13.9, 7.0 Hz, 2H), 2.18 (dd, *J* = 14.1, 6.9 Hz, 2H), 1.69 (s, 3H), 1.47 (s, 3H), 1.05 (t, *J* = 7.1 Hz, 3H).

*5.1.4.29 5-cyclopentyl-7-((4-(4-ethylpiperazin-1-yl)-2-methoxyphenyl)amino)-[1,2,4]triazolo[4,3-f]pteridin-4(5H)-one(**III$_9$**)*

Yield: 51.9%; m.p.:227.7-231.4 °C; MS (ESI) *m/z*:505.3[M+H]$^+$; ^{1}H NMR (DMSO-d_6, 600 MHz) δ 8.92 (s, 1H), 8.85 (s, 1H), 7.48 (s, 1H), 6.37 (s, 1H), 5.54 (s, 1H), 3.78 (s, 3H), 3.48 (s, 4H), 2.90 (s, 3H), 2.44 (d, *J* = 68.0 Hz, 6H), 2.17 (s, 2H), 1.66 (s, 3H), 1.45 (s, 3H), 1.05 (s, 3H).

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*5.1.4.30 5-cyclopentyl-7-((4-(3,5-dimethylpiperazin-1-yl)-2-methoxyphenyl)amino)-1-methyl-[1,2,4]triazolo[4,3-f]pteridin-4(5H)-one(**III$_{10}$**)*

Yield:66.9 %; m.p.:259.3-261.8 °C; MS (ESI) *m/z*:504.3[M+H]$^+$; ^{1}H NMR (DMSO-d_6, 600 MHz) δ 8.91 (s, 1H), 8.70 (s, 1H), 7.28 (d, *J* = 9.1 Hz, 1H), 6.63 (d, *J* = 2.2 Hz, 1H), 6.50 (dd, *J* = 8.7, 2.3 Hz, 1H), 5.59 (s, 1H), 3.75 (s, 3H), 3.59 (d, J = 9.6 Hz, 3H), 2.99 – 2.91 (m, 3H), 2.90 (s, 3H), 2.32 – 2.21 (m, 3H), 2.18 (dd, *J* = 8.7, 5.8 Hz, 3H), 1.68 (s, 3H), 1.46 (s, 3H), 1.07 (d, *J* = 5.7 Hz, 3H).

*5.1.4.31 5-cyclopentyl-7-((6-(3,5-dimethylpiperazin-1-yl)-2-methoxypyridin-3-yl)amino)-1-methyl-[1,2,4]triazolo[4,3-f]pteridin-4(5H)-one(**III$_{11}$**)*

Yield: 53.5%; m.p.:259.3-261.8 °C; MS (ESI) *m/z*:504.3[M+H]$^+$; ^{1}H NMR (DMSO-d_6, 600 MHz) δ 8.91 (s, 2H), 8.84 (s, 2H), 7.47 (s, 2H), 6.37 (d, *J* = 8.4 Hz, 1H), 5.55 (s, 1H), 4.14 (d, *J* = 11.3 Hz, 3H), 3.78 (s, 3H), 2.90 (s, 3H), 2.84 (s, 3H), 2.33 (s, 3H), 2.18 (dd, *J* = 16.5, 9.0 Hz, 3H), 1.66 (s, 3H), 1.46 (s, 3H), 1.07 (d, *J* = 5.5 Hz, 3H).

*5.1.4.32 5-cyclopentyl-7-((4-(4-(2-hydroxyethyl)piperazin-1-yl)-2-methoxyphenyl)amino)-1-methyl-[1,2,4]triazolo[4,3-f]pteridin-4(5H)-one(**III$_{12}$**)*

Yield:56.9 %; m.p.: 273.1-275.6°C; MS (ESI) *m/z*:520.3[M+H]$^+$; ^{1}H NMR (DMSO-d_6, 600 MHz) δ 8.91 (s, 1H), 8.72 (s, 1H), 7.28 (s, 1H), 6.63 (d, *J* = 2.5 Hz, 1H), 6.50 (dd, *J* = 8.7, 2.5 Hz, 1H), 5.32 (s, 1H), 4.46 (s, 1H), 3.75 (s, 3H), 3.55 (d, *J* = 5.5 Hz, 2H), 3.15 (s, 4H), 2.90 (s, 3H), 2.59 (s, 4H), 2.46 (s, 2H), 2.17 (dd, *J* = 14.6, 7.2 Hz, 2H), 1.68 (s, 3H), 1.46 (d, *J* = 6.9 Hz, 3H).

*5.1.4.33 5-cyclopentyl-7-((6-(4-(2-hydroxyethyl)piperazin-1-yl)-2-methoxypyridin-3-yl)amino)-1-methyl-[1,2,4]triazolo[4,3-f]pteridin-4(5H)-one(**III$_{13}$**)*

Yield:67.3 %; m.p.: 231.7-234.2°C; MS (ESI) *m/z*:521.3[M+H]$^+$; ^{1}H NMR (DMSO-d_6, 600 MHz) δ 8.91 (s, 1H), 8.84 (s, 1H), 7.47 (d, *J* = 5.5 Hz, 1H), 6.35 (d, *J* = 8.4 Hz, 1H), 5.54 (s, 1H), 4.44 (t, *J* = 5.2 Hz, 1H), 3.77 (s, 3H), 3.54 (dd, *J* = 11.3, 6.0 Hz, 2H), 3.49 – 3.44 (m, 4H), 2.89 (s, 3H), 2.52 (d, *J* = 4.5 Hz, 4H), 2.43 (t, *J* = 6.3 Hz, 2H), 2.17 (dd, *J* = 16.3, 8.9 Hz, 2H), 1.66 (s, 3H), 1.45 (s, 3H).

*5.1.4.34 5-cyclopentyl-7-((2-methoxy-4-(piperazin-1-yl)phenyl)amino)-1-methyl-[1,2,4]triazolo[4,3-f]pteridin-4(5H)-one(**III$_{14}$**)*

Yield: 54.7%; m.p.: 269.3-271.8°C; MS (ESI) *m/z*:476.2[M+H]$^+$; ^{1}H NMR (DMSO-d_6, 600 MHz) δ 8.92 (s, 1H), 8.71 (s, 1H), 7.29 (d, *J* = 6.0 Hz, 1H), 6.62 (d, *J* = 2.4 Hz, 1H), 6.50 (dd, *J* = 8.6, 2.4 Hz, 1H), 5.60 (s, 1H), 3.76 (s, 3H), 3.07 (d, *J* = 4.6 Hz, 4H), 2.90 (s, 4H), 2.86 – 2.84 (m, 3H), 2.18 (s, 3H), 1.70 (s, 2H), 1.47 (s, 3H).

5.2 *Pharmacology*

5.2.1 *In vitro antiproliferative assays*

MTT assay was performed on A549, HCT116, PC-3 and MCF-7 cells to evaluate anti-proliferative activity of the target compounds, with BI-2536 as the positive controls. Cancer cell lines were cultured in minimum essential medium (MEM) supplement with

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10% foetal bovine serum (FBS).
Approximate 5×10^3 cells, suspended in MEM medium, were plated in a 96-well plate and incubated in 5% CO_2 at 37 °C for 24 h. The compounds at the specified concentration were added to the culture medium and the cell cultures were incubated for another 72 h. 10 μL of 5 mg/mL MTT solution were added to each well and incubated with cells at 37 °C for 4 h. Dissolved the formazan crystals in each well with 100 μL dimethyl sulfoxide, and measured the absorbance at 492 nm (absorbance of MTT formazan) and 630 nm (reference wave length) with an ELISA reader. The results were expressed as IC_{50}, which is the average of at least three measured values, and wrer calculated using Bacus Laboratories Incorporated Slide Scanner (Bliss) software.

5.2.2 *In vitro PLK1and BRD4 enzymatic assays*

The in vitro enzymes of the selected compounds were evaluated by the homogeneous time-resolved fluorescence (HTRF) assay.

Materials and reagents：Enzyme-labeled instrument,384 Plates, PLK1, ATP 10 mM, DTT 1 M, $MgCl_2$ 1 M, HTRF KinEASE -STK S1 kit contains STK substrate-1-biotin, Streptavidin-XL665, STK Antibody-Cryptate, 5x Enzymatic buffer, SEB, HTRF Detection buffer, Tb-labeled donor, Dye-labeld acceptor, 1xBRD TR-FRET Assay Buffer, BET Ligand, BRD4(BD1) 3ng/μL.

According to the manufacturer's instructions (http://www.cisbio.com/kinease), prepare working solutions (Compounds, TK Substrate-biotin, PLK1, ATP, Sa-XL665 and TK-Antibody-Cryptate). Adding 4 μL of the diluted compounds working solution to the reaction wells except the control well to which 4 μL of 2.5% DMSO solution was added. Then add 4 μL of Substrate-STK and ATP working solution to all wells. Adding 2 μL of PLK1enzyme working solution to all reaction wells except negative wells, and make up the volume with 2 μL of enzyme corresponding to 1 × KB in negative wells. Seal the plate with a sealing film and mix for 60 minutes at room temperature. Prepare the test solution 5 minutes before the end of the kinase reaction. After the reaction, add 10 μL of the prepared working solution of Streptavidin-XL665 and TK antibody europium cryptate to all wells. Seal the plate and mix well. After reaction at room temperature for 1h, using ENVISION instrument to detect the fluorescence. Add diluted Tb-labeled donor and dye-labeled acceptor to all wells at 5 μl per well. Add 2 μl of compound working solution to each well. Add 2 μl 1x BRD TR-FRET Assay Buffer to each of the negative and positive control wells. Add 5 μL per well of diluted BET Ligand to the positive control wells and compound wells. Add 5 μl 1x BRD TR-FRET Assay Buffer to each of the negative control wells. Add diluted BRD4 enzyme to all wells at 3 μl per well. Reaction at room temperature for 2 hours. Read fluorescence value at Ex320, Em665/615 nm wavelength.
(Ex320, Em665/615 nm). Emission Ratio(ER)= 665 nm Emission signal/ 615 nm Emission signal. Percent inhibition = (ERpositive—ER sample)/(ERpositive—ERnegative)*100%

5.2.3 *Morphology assays of apoptotic cells*

HCT116 cells were seeded in 6-well plates at a final concentration of 50 000/well and incubated for 24 hours until the cells adhered. The cells were treated with various concentrations of compound **III$_4$** and cultured for another 48 h, Control cells and treated cells were collected and washed with PBS, and then resuspended in 100μL 1*binding buffer incubated in a mixture of 5 μL Annexin V-FTIC and 5μL PI for 10 min at room temperature in a dark place. Prior to flow cytometry analysis, cells were resuspended in 400 μL 1 * binding buffer.

5.2.4 *Cell migration assay*

Cell migration assay were performed using wound-healing assay. HCT-116 cells were seeded in six-well plates at a final concentration of 1×10^6/well and grown to 100% confluence. Linear wound across the confluent cell layer by a pipette tip. HCT-116 cells were washed twice with PBS, and then incubated with culture medium containing DMSO or various concentrations of compound **III$_4$**. After 0, 24, and 48 hours of cultivating, the images were taken through a fluorescence microscope (Olympus, Tokyo, Japan).

5.2.5 *Cell cycle assays*

The effects of compound **III$_4$** on cell cycle progression were verified by using a standard propidium iodide (PI) staining procedure followed by flow cytometry analysis. HCT-116 cells were seeded in six-well plates at a final concentration of 25 000/well and incubated for 24 h. Cells treated with various concentrations of **III$_4$** and then incubated for another 24 h. The cells were harvested and washed twice with ice cold PBS, then fixed in ice-cold 70% (v/v) ethanol overnight at 4 ℃. The cells were washed again by PBS, and then the cell DNA was stained with 300 μL PI (Beyotime) for 10 min. Data collection and analysis were carried out by using a flow cytometer.

5.2.6 *Molecular docking*

Structural alignment was accomplished through the Cresset Forge v10.4.1. The PLK1 model was built using PDB code: 2RKU as template. The protein coordinate was downloaded from the Protein Data Bank (http:// www.rcsb.org/pdb/). BI2536 (PDB code: 2RKU) and BRD4 (PDB code: 4O74) were regarded as reference molecule for the computation of field points and alignment. The MCS conformation of the molecule in training set was generated by being aligned on the reference molecule. After the conformation was obtained, the highest structural arrangement score was recommended as the best alignment. Molecular field points were computed for the test set of molecule, together with the molecular field points of the training set structure were revealed to be the best alignment with the template field points. The analysis of results implemented on discovery studio visualizer software.

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References

1 R.L. Siegel, KD Miller, CA: a cancer journal for Clinicians 2016. 2016, 66 (1)，7-30.

2 L. Chen, J.L. Yap, M. Yoshioka, *ACS Med. Chem. Lett.*, 2015, 6, 764-769.

3 G Combes, I Alharbi, L G Braga, et al., Playing polo during mitosis: PLK1 takes the lead, *Oncogene*., 2017, 36 (34), 4819-4827.

4 Z. Zhang, X. Hou, C. Shao, J. Li, J.X. Cheng, S. Kuang, N. Ahmad, T. Ratliff, X. Liu, *Cancer Res*, 2014,74 (22),6635-47.

5 D. Xiao, M. Yue, H. Su, P. Ren, J. Jiang, F. Li, Y. Hu, H. Du, H. Liu, G. Qing, *Molecular Cell.*, 2016, 64 (3), 93–506.

6 T.P. Heffron, *J. Med. Chem*., 2016,59 (22), 10030-10066.

7 Z. Liu, P. Wang, H. Chen, E.A. Wold, B. Tian, A.R. Brasier, J. Zhou, *J. Med. Chem*., 2017,60 (11), 4533-4558.

8 A. Puissant, S.M. Frumm, G. Alexe, C.F. Bassil, J. Qi, Y. Chanthery, E.A. Nekritz, R. Zeid, W.C. Gustafson, P.Greninger, M.J. Garnett, U. Mcdermott, C.H. Benes, A.L. Kung, W.A. Weiss, J.E. Bradner, K. Stegmaier, *Cancer Discov.*, 2013,3 (3), 308-323.

9 J. Delmore, G.C. Issa, M.E. Lemieux, P.B. Rahl, J. Shi, H.M. Jacobs, E. Kastritis, T. Gilpatrick, R.M. Paranal, J. Qi, M. Chesi, A.C. Schinzel, M.R. Mckeown, T.P. Heffernan, C.R. Vakoc, P.L. Bergsagel, I.M. Ghobrial, P.G. Richardson, R.A. Young, W.C. Hahn, K.C. Anderson, A.L. Kung, J.E. Bradner, C.S. Mitsiades, *Cell*., 2011,146 (6), 904-917.

10 J.A. Mertz, A.R. Conery, B.M. Bryant, P. Sandy, S. Balasubramanian, D.A. Mele, L. Bergeron, R.J. Sims, *Proc. Natl. Acad. Sci.*, 2011,108 (40), 16669-16674.

11 L. Ouyang, L. Zhang, J. Liu, L. Fu, D. Yao, Y. Zhao, S. Zhang, G. Wang, G. He, B. Liu, *J. Med. Chem*., 2017, 60 (24), 9990-10012.

12 M. Zengerle, K. Chan, A. Ciulli, *ACS Chem. Biol*., 2015,10 (8), 1770-1777.

13 U. Tontschgrunt, D. Rudolph, I. Waizenegger, A. Baum, D. Gerlach, H. Engelhardt, M. Wurm, F. Savarese, N. Schweifer, N. Kraut, *Cancer Lett*., 2018, 421, 112-120.

14 F. Mao, J. Li, Q. Luo, R. Wang, Y. Kong, C. Carlock, Z. Liu, B.D. Elzey, X. Liu, *Mol Cancer Ther*., 2018, 17 (7), 1554-1565.

15 S.W. Ember, J.Y. Zhu, S.H. Olesen, M.P. Martin, A. Becker, N. Berndt, G.I. Georg, *ACS Chem. Biol*., 2014,9 (5), 1160-1171.

16 P. Ciceri, S. Muller, A. Omahony, O. Fedorov, P. Filippakopoulos, J.P. Hunt, E.A. Lasater, G. Pallares, S. Picaud, C. Wells, S. Martin, L. Wodicka, N.P. Shah, D.K. Treiber, S. Knapp, *Nat. Chem. Biol*., 2014,10 (4) , 305-312.

17 S. Liu, H. Yosief, L. Dai, H. Huang, G. Dhawan, X. Zhang, A. Muthengi, J.M. Roberts, D.L. Buckley, J.A. Perry, L. Wu, J.E. Bradner, J. Qi, W. Zhang, *J. Med. Chem.*, 2018, 61 (17), 7785-7795.

18 Y. Hou, L. Zhu, Z. Li, Q. Shen, Q. Xu, W. Li, Y. Liu, P. Gong, *Eur. J. Med. Chem.*, 2019,163, 1690-709.

19 L. Xiao, X. Yang, M. Zhan, P. Cao, S. Zheng, R. Peng, J. Han, Z. Xie, Z. Tu, C. Liao, *Eur. J. Med. Chem*., 2019, 184, 111769.