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Synthesis and biological evaluation of 2,4-diaminopyrimidines as selective Aurora A kinase inhibitors

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ABSTRACT

The Aurora kinases are a family of serine/threonine kinases that interact with components of the mitotic apparatus and serve as potential therapeutic targets in oncology. Here we synthesized 15 2,4-diaminopyrimidines and evaluated their biological activities, including antiproliferation, inhibition against Aurora kinases and cell cycle effects. These compounds generally exhibited more potent cytotoxicity against tumor cell lines compared with the VX-680 control, especially compound **11c**, which showed the highest cytotoxicities, with IC_{50} values of 0.5–4.0 μM. Compound **11c** had more than 35-fold more selectivity for Aurora A over Aurora B, and molecular docking analysis indicated that compound **11c** form better interaction with Aurora A both from the perspective of structure and energy. Furthermore, compound **11c** induced G2/M cell cycle arrest in HeLa cells. This series of compounds has the potential for further development as selective Aurora A inhibitors for anticancer activity.

1. Introduction

The Aurora kinase family is a subfamily of serine/threonine kinases that is essential for the regulation of centrosome maturation, mitotic spindle formation, chromosome segregation and cytokinesis during mitosis [1,2]. The family includes three kinases designated as Aurora A, B, and C, which are very closely related in the kinase domain sequence. However, these kinases have quite different and nonoverlapping functions during mitosis [3]. Aurora A regulates the cell cycle and is associated with late S phase and entry into the M phase. It associates with the spindle poles and is involved in both centrosomal and acentrosomal spindle assembly [4,5]. Aurora B localizes to the centromeres in prometaphase, and then relocates to the spindle midzone at anaphase. It has functions associated with histone phosphorylation and chromatin condensation in prophase, chromosome alignment and segregation, regulation of a mitotic checkpoint at metaphase and a role in cytokinesis [6]. Aurora C has similar functions as Aurora B [7].

The expressions of Aurora A and Aurora B are elevated in a variety of human cancers and are associated with poor prognosis [8]. The potential roles of Aurora kinases in regulating cell mitosis and tumorigenesis make them attractive targets for anticancer therapy [9]. Many Aurora kinase inhibitors have been developed and introduced into clinical trials, including VX-680/MK-0457, ZM447439, Hesperadin, MLN8054, MLN8237, and AZD1152 (Fig. 1) [10–12].

ZM447439 [13], Hesperadin [14] and VX-680/MK-0457 [15] were the first generation of Aurora kinase inhibitors. These three small molecule chemical inhibitors occupy the ATP-binding site in Aurora kinases to inhibit catalytic activity. Unlike pan-Aurora kinase inhibitors, MLN8054 and MLN8237 are ATP-competitive and reversible Aurora A selective inhibitors, and are approximately 40-fold and 200-fold more sensitive towards Aurora A, respectively, compared with Aurora B [12]. MLN8237 is more potent than MLN8054 and causes less benzodiazepine-like side effects based on structure modulation by the addition of a methoxy group to either end of the MLN8054 molecule [16,17]. AZD1152 is an Aurora B selective inhibitor that showed 1000-fold selectivity for Aurora B over Aurora A and a panel of 50 additional kinases in enzymatic assays

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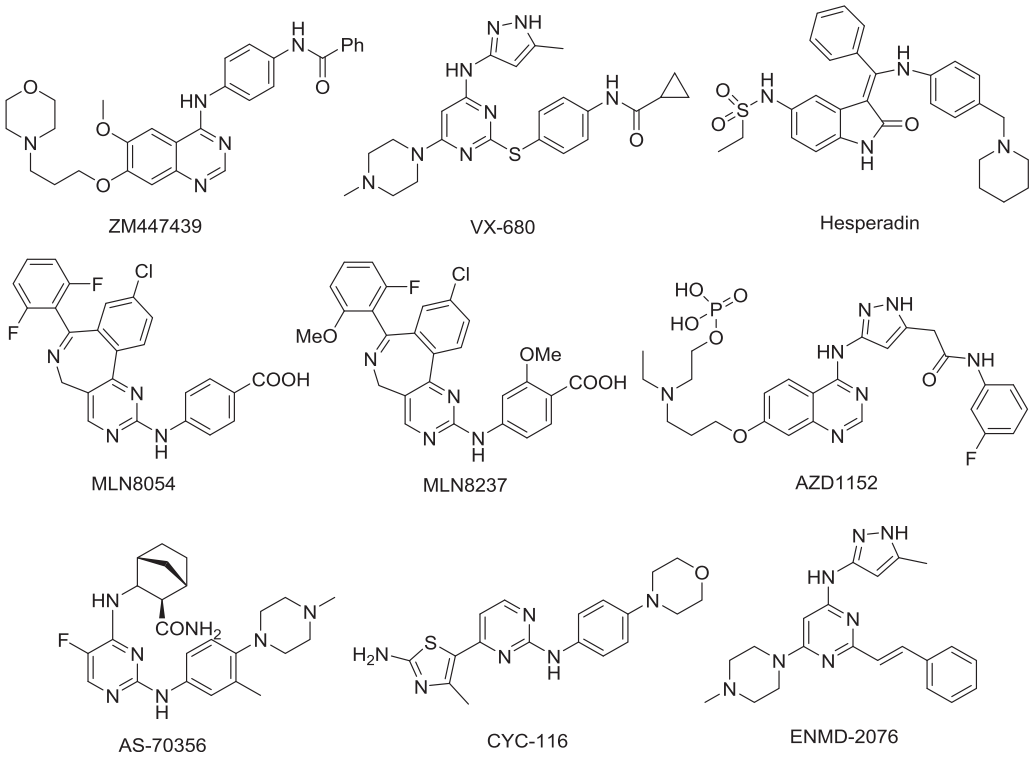


Fig. 1. Aurora kinase inhibitors.

[18–21]. It thus still remains uncertain how exactly aurora A and B pan- or monospecific inhibitors induce tumor cell death and which type of inhibitor will be preferable from a therapeutic viewpoint. Recently, the research of novel selective Aurora inhibitors has become a new trend, and a lot of new active compounds have been developed [22–24].

Pyrimidine is the important pharmacology core in many Aurora inhibitors, such as VX-680, ENMD-2076, CYC-116 and ENMD-2076 [12]. To identify additional effective Aurora inhibitors, we designed a series of 2,4-diaminopyrimidine compounds, our modeling studies suggested that the pyrimidine core as well as the secondary aromatic amine of the compounds form hydrogen bonds with the hinge region of the kinase domain and show selectively inhibition to Aurora A over Aurora B. Introduction of cyclopentyl amine on the C-4 in pyrimidine can adopt a binding mode similar to VX-680 [25]. Furthermore, the differences of F, Cl, Br and NO_2 at 5-C of pyrimidine was to investigate the effects of the electron-withdrawing on anti-proliferation and inhibition of Aurora kinase. Herein, we reported the synthesis, and evaluated their anti-proliferation activities, inhibition of Aurora kinase and effects on the cell cycle.

2. Results and discussion

2.1. Chemistry

The general synthetic routes for intermediate anilines **4a**–**b** and **7a**–**b** are illustrated in Schemes 1 and 2, respectively. Treatment of *p*-aminobenzoic acid **1a** with ditertbutyl dicarbonate ($(BOC)_2O$) afforded 4-Boc-amino-benzoic acid **2a**, and then condensation of compound **2a** with *N*-methyl-4-amino-piperidine generated **3a** under condensing agent tri(dimethylamino)benzotriazol-1-yloxyphos phonium hexafluorophosphate (BOP) in the presence of *N,N*-diisopropylethylamine (DIPEA). Finally, removal of the protecting group provided aniline **4a** in the dichloromethane solution of trifluoroacetic acid [26]. To obtain compound **4b**, 3-methoxy-4-amino-benzoic acid (**1b**), as the raw material, the reaction process was similar to preparing **4a**. Another intermediate aniline **6a**–**b** was prepared by substituted reaction of 4-chloro-1-nitrobenzene with morpholine or 4-methyl-piperazine, and the nitro was reduced by catalytic hydrogen under the catalysis of 10% Pd/C [27].

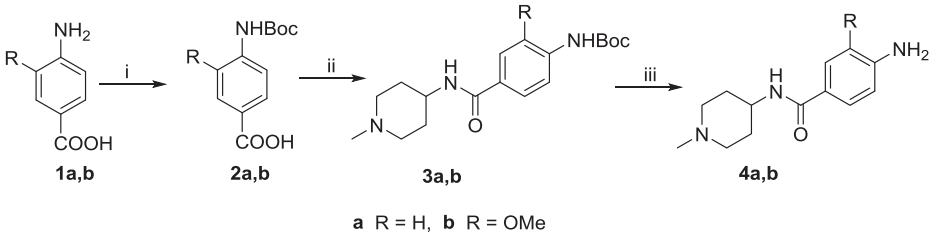
Our approach to the preparation of 2,4-diaminopyrimidines based on the double SN_2 displacement of pyrimidine is shown in Scheme 3. The displacement of the 4-chloro group of 2,4-dichloro-5-substituted pyrimidine by cyclopentyl amine, cyclopropyl amine, and n-propylamine provided **8a**–**d**, **9** and **10**, which has already been widely reported in the literature [28]. Treatment of **8a**–**d**, **9** and **10** with the different anilines **4a,b** or **7a,b** in isopropanol in the presence of hydrochloride at 80 °C gave the target compounds **11a**–**d**, **12b,c** and **13**–**19** [29]. The target compound **20** was synthesized by the nitro at C-5 of **16** and reduced with hydrogen gas under the catalysis of 10% Pd/C. The newly synthesized compounds were characterized by physicochemical and spectral means, and both analytical and spectral data of all the compounds were in full agreement with the proposed structures.

2.2. Biological activity

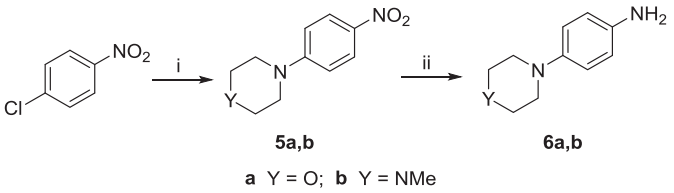
2.2.1. Cytotoxicities of compounds *11*–*20*

The *in vitro* cytotoxicities of target compounds **11**–**20** were evaluated in a panel of four human tumor cell lines (cervical carcinoma HeLa, lung carcinoma A-549, human colorectal adenocarcinoma HCT-8 and hepatic carcinoma Hep-G2 cells), with VX-680 as a reference compound. The screening procedure was based on the standard MTT method [30], and the results are summarized in Table 1.

All the target compounds showed better or equivalent



Scheme 1. Synthesis of compounds **4a**–**b**. Reagents and conditions: (i) Boc_2O, NaOH, dioxane: H_2O (2:1), 0 °C; (ii) *N*-methyl-4-amino-piperidine, BOP, DIPEA, rt; (iii) TFA: DCM = 3:7, rt.



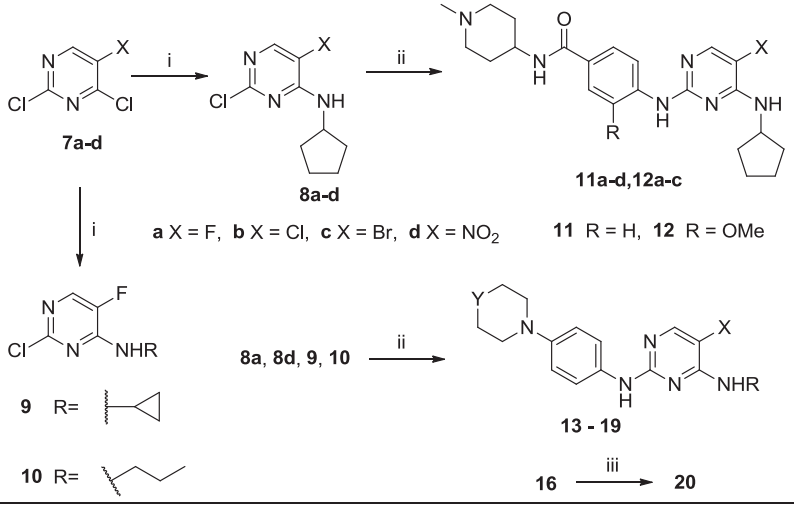
Scheme 2. Synthesis of compounds **6a**–**b**. Reagents and conditions: (i) morpholine or *N*-methylpiperazine, K_2CO_3, 80 °C, 5 h; (ii) H_2, 10% Pd/C, 1 atm, rt.

antiproliferation activity in the four human tumor cell lines compared with VX-680. Notably, compounds **11a**–**d** with different substitutions (F, Cl, Br and NO_2) at C-5 of pyrimidine showed significantly different effects in regards to cytotoxicity. The antiproliferation activity of compounds **11b** and **11c** substituted with chloride or bromide at C-5 of pyrimidine showed more potent antiproliferation effects compared with compounds **11a** and **11d**, in which C-5 in pyrimidine was substituted with fluorine and nitro, respectively. However, we did not find any obvious differences in cytotoxicities in compounds **12a**–**c**. The displacement of the anilines from **4a** to **4b** in the C-2 substitute on the pyrimidine ring led to increased or maintained cytotoxicity upon treatment of the four cell lines. However, substitution of **4a** with **7a** and **7b** in the C-2 of pyrimidine resulted in decreased antiproliferation activity of the target compounds. We next investigated the replacement of the cyclopentyl group on the C-4 in pyrimidine by different groups, such as cyclopropyl or n-propylamine. Unfortunately, all the synthesized compounds showed equivalent or lower antiproliferation activities.

From the results of the *in vitro* cytotoxic assays, we found that compound **11c** showed strong growth–inhibitory activities in the cervical carcinoma HeLa cell line. The IC_{50} value of **11c** was 0.9 μM, which was 30-fold lower than VX-680. Next we selected compounds **11c** and **12a** to explore the effects on Aurora kinases and the cell cycle in HeLa cells.

*2.2.2. Compounds **11c** and **12a** selectively inhibit Aurora A over Aurora B kinase in HeLa cells*

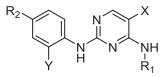
Many pyrimidine compounds, such as VX-680, ENMD-2076, CYC-116 and ENMD-2076, have entered into clinical trials as Aurora inhibitors [12]. To explore whether **11c** and **12a** had similar effects on inhibition of Aurora kinases in HeLa cells, we investigated the

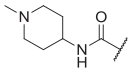
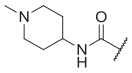
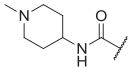
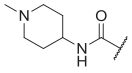
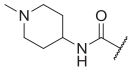
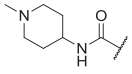
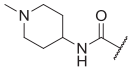


Compounds	13	14	15	16	17	18	19	20
X	F	F	NO_2	NO_2	F	F	F	NH_2
Y	O	NMe	O	NMe	O	NMe	O	NMe
R		HN			HN	HN		HN

Scheme 3. Synthetic approach of target compounds **11**–**20**. Reagents and conditions: (i) THF, 0 °C, 3 h; (ii) isopropyl alcohol, 1 drop of 37% HCl, 80 °C, 18 h; (iii) ethyl acetate, H_2, 10% Pd/C, 1 atm, 60 °C.

Table 1
The cytotoxicities of compounds **11**–**20** at drug exposure for 48 h.



Compds	X	Y	R_1	R_2	(IC_{50}, μM)[a]			
					HCT-8	A-549	HeLa	Hep-G2
11a	F	H			16.0	10.3	45.5	10.0
11b	Cl	H			4.6	1.8	1.5	2.2
11c	Br	H			3.6	0.5	0.9	2.4
11d	NO_2	H			15.6	42.3	21.7	5.8
12a	F	OMe			2.1	2.1	2.2	4.5
12b	Cl	OMe			4.4	5.3	2.7	9.3
12c	Br	OMe			12.0	2.5	3.5	4.9
13	F	H			24.0	17.3	31.4	36.5
14	F	H			29.0	37.2	20.0	25.0
15	NO_2	H			20.0	63.7	36.4	21.9
16	NO_2	H			53.7	4.6	3.8	5.2
17	F	H			40.3	8.7	6.0	33.0
18	F	H			50.0	5.1	14.2	35.0
19	F	H			73.9	8.8	7.3	20.0
20	NH_2	H			4.2	27.0	9.7	18.5
VX-680					44.6	19.4	27.3	63.4

[a] Data are the mean of three independent experiments.

effects on Aurora A and B kinases by western blot [31] and enzyme-linked immunosorbent assay (ELISA) [32].

HeLa cells were treated with various concentrations of compound **11c** (10 nM, 25 nM, and 50 nM) for 12 h. The expression levels of Aurora A and Aurora B were decreased in HeLa cells upon exposure to **11c** in a dose-dependent manner. We also observed that the effect of **11c** on Aurora A was more potent than on Aurora B, indicating that compound **11c** showed selectivity of inhibition of Aurora A over Aurora B (Fig. 2A and C). Compound **12a** showed the same effects of **11c**, but its potency was weaker than **11c** (Fig. 2B and D).

The selective effects of compounds **11c**, **12a** and VX-680 on Aurora A kinase inhibition were also confirmed by ELISA, as shown in Fig. 3. When HeLa cells were treated with less than 5 nM of compound **11c** for 12 h, the expression levels of Aurora A and B showed no significant changes. When the concentration of **11c** was increased to more than 10 nM, the expression levels of Aurora A and B protein rapidly decreased in HeLa cells, and Aurora A protein was reduced more than that of Aurora B. From Fig. 3A, we easily obtained the IC_{50} values of **11c** for Aurora A and B as 0.012 μM and 0.430 μM, respectively. These results indicate that compound **11c** was more than 35-fold more selective for Aurora A compared with Aurora B in HeLa cells. We also obtained IC_{50} values of **12a** for Aurora A and B of 0.043 μM and 0.395 μM, respectively, and the selectivity of **12a** for Aurora A was only 9-fold over Aurora B. However, the IC_{50} values of VX-680 for Aurora A and B was 0.261 μM and 0.453 μM, respectively, and no obvious selectivity of VX-680 for Aurora A over Aurora B. Obviously, the strong growth–inhibitory activities of compound **11c** and **12a** than VX-680 were in accordance with their better selectivity of Aurora A

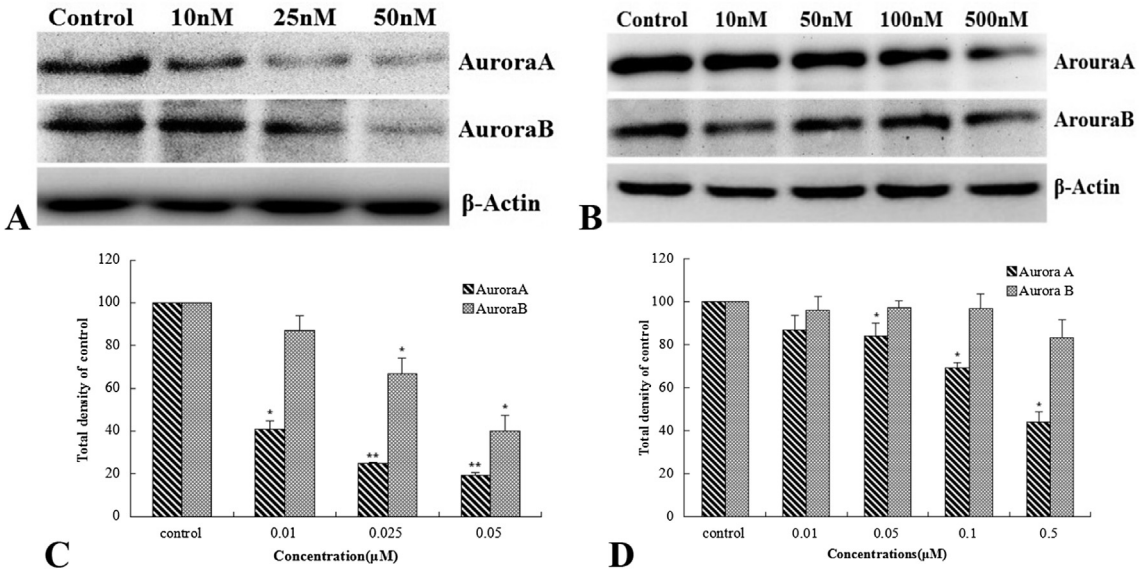
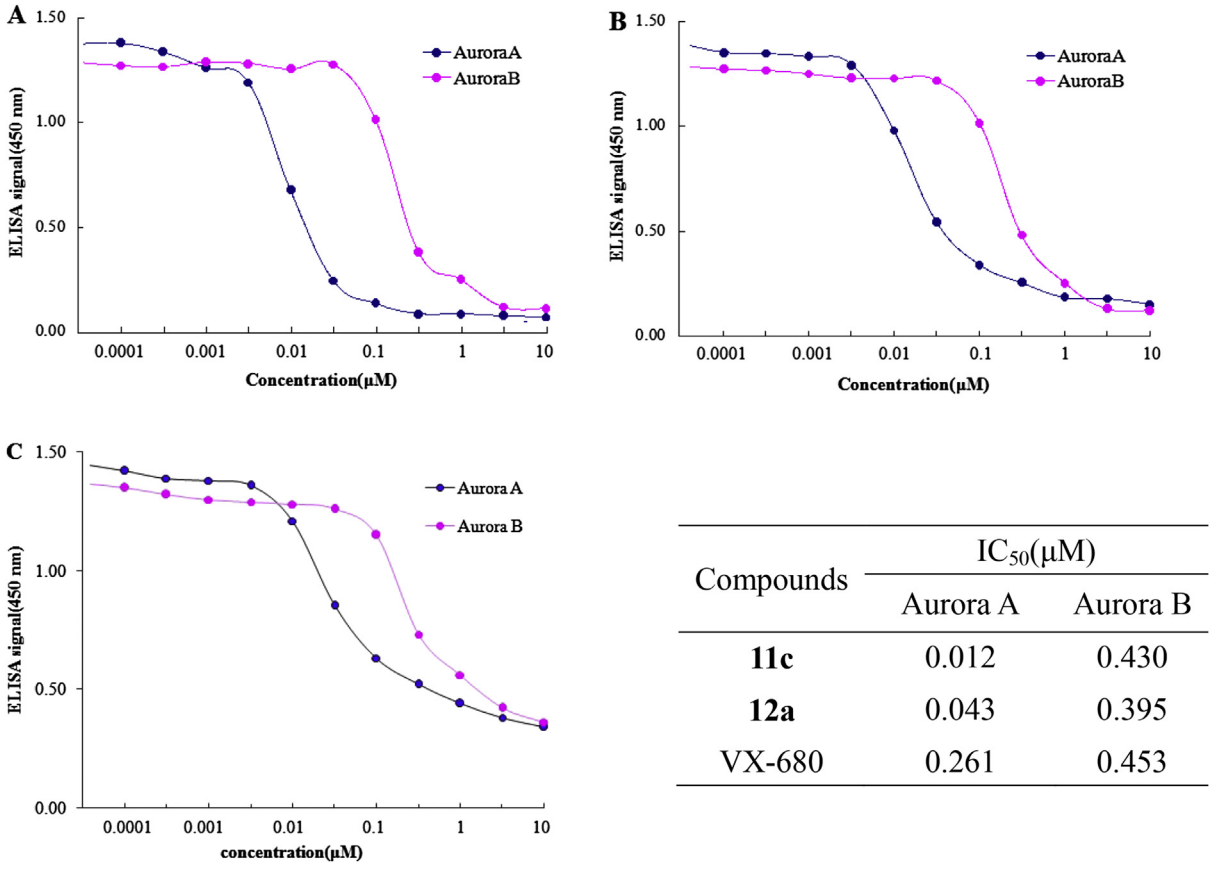


Fig. 2. Western blot for selectivity of inhibition of Aurora A and Aurora B with compound **11c** (A, C) and **12a** (B, D) in HeLa cells.



Compounds	IC_{50}(μM)	
	Aurora A	Aurora B
11c	0.012	0.430
12a	0.043	0.395
VX-680	0.261	0.453

Fig. 3. Compound **11c** (A), **12a** (B) and VX-680 (C) selectively inhibits Aurora A over Aurora B in HeLa cells.

over Aurora B.

2.2.3. *Aurora kinase binding model of compound **11c***

To gain insight into the interaction of compound **11c** with Aurora A and Aurora B, docking simulation was performed using the Autodock 4.2 [33] with Lamarckian Genetic Algorithm [34]. All the figures displaying the docking results were obtained using the scientific software Pymol [35]. AutoGrid was used to produce grids based on the position of the ligand in the proteins (PDB code 3D14 for Aurora A and 4C2V for Aurora B). In the docking process, the protein was considered to be rigid, while the ligand was considered flexible. The ligand **11c** was docked into the appropriate binding

pocket of Aurora A and Aurora B using the autodock module and the calculated binding energy was −10.02 kcal/mol for Aurora A and −9.05 kcal/mol for Aurora B, respectively. The resulting docking poses are shown in Fig. 4. According to the binding energy, **11c** is more sensitive to Aurora A than Aurora B.

Overall, the binding pockets of Aurora A and Aurora B are highly hydrophobic. Thus, hydrophobic interaction is the main driving force for the binding of **11c** to Aurora A and Aurora B. In addition to the hydrophobic interactions, **11c** can also form several important H-bonds. For example, in Aurora A, **11c** can form two H-bonds with Glu62 and Asp155. In Aurora B, the oxygen atom in the amide group and the nitrogen atom in the piperidine ring of **11c** can form two H-bonds with Lys122. From the distance of the two atoms that form hydrogen bonds, Aurora A can form stronger hydrogen-bonding interactions compared with Aurora B. Additionally, from the shape of the binding pocket, **11c** can fit much better with the binding pocket of Aurora A. These differences may explain why compound **11c** binds better with Aurora A compared with Aurora B.

2.2.4. Compound *11c* induces cell cycle arrest in G2/M phase

The Aurora A inhibitor induces common phenotypic effects such as G2/M accumulation, spindle defects and chromosome misalignment, and has enabled the identification of previously unknown Aurora A-regulated cellular functions [36]. Many studies have shown that VX-680 induces cell cycle arrest in the G2/M phase. To determine whether compound **11c** has similar effects on tumor cells, we investigated its effects on cell cycle progression using fluorescence-activated cell sorting analysis of HeLa cells stained with propidium iodide [37] (Fig. 5).

Treatment of HeLa cells with **11c** resulted in a dose-dependent accumulation of cells in the G2/M phase with a concomitant decrease in the population of G1 phase cells. After 12 h of treatment with 2 μM or 5 μM of **11c**, the percentages of cells in G2/M phase arrest were 33.2% and 43.5%, respectively, compared with 10.2% in untreated cultures. These results demonstrate that **11c** interfered with cell proliferation by arresting the cell cycle in G2/M.

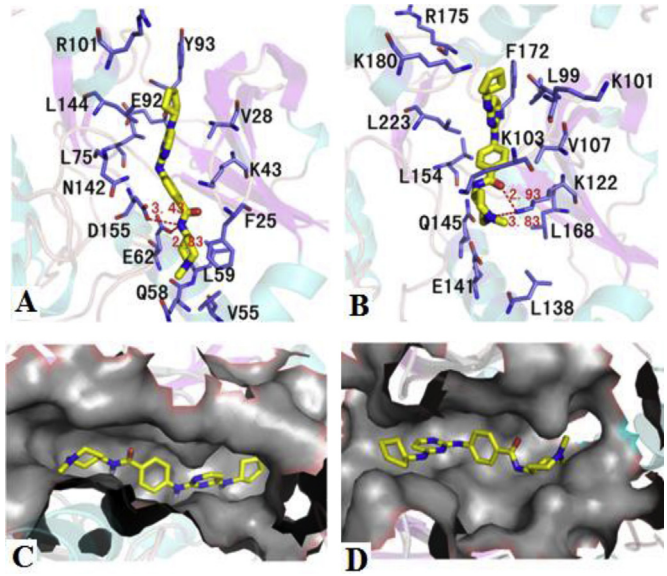


Fig. 4. The binding mode of **11c** with Aurora A and Aurora B. A) The interaction between **11c** and Aurora A. B) The interaction between **11c** and Aurora B. C) The binding pocket of Aurora A was shown in surface. D) The binding pocket of Aurora B was shown in surface (To show the binding pocket clearly, K103 and F104 weren't shown). The surface is colored by gray.

2.2.5. Compound *11c* activates cyclin B1

Cyclin B is a member of the cyclin family and regulates the progression of cells into and out of M phase [38]. Thus, we examined the effect of **11c** on the expression of cyclin B in HeLa cells [39]. As shown in Fig. 6, treatment of cells with **11c** resulted in an apparent increase of cyclin B, demonstrating that compound **11c** can interfere with cell cycle progression. This conclusion is consistent with the cell cycle analysis results.

3. Conclusions

Aurora kinases have been of interest as potential therapeutic targets in oncology. Here we describe a series of 2,4-diaminopyrimidine small molecule inhibitors that exert their cytotoxic activities in human tumor cell lines through inhibition of Aurora kinases. We specifically demonstrate that compound **11c** was selective for inhibition of Aurora A over Aurora B in HeLa cells, and the molecular docking analysis revealed that **11c** form better interaction with Aurora A than that with Aurora B. Treatment of HeLa cells with compound **11c** also results in G2/M accumulation. These results suggest that these compounds have potential for further development *in vivo* as anticancer agents.

4. Experiment

4.1. Chemistry

All starting materials and regents were purchased commercially and used without further purified, unless otherwise stated. All reactions were monitored by thin layer chromatograph (TLC) on silica gel GF254 (0.25 mm thick). Column chromatography (CC) was performed on Silica Gel 60 (230–400 mesh, Qingdao Ocean Chemical Ltd., China). Melting points were determined in Kofler apparatus and were uncorrected. IR spectra were measured on a NicoLET iS5 spectrometer on neat samples placed between KBr plates. ^{1}H NMR and ^{13}C NMR spectra were recorded with a Varian Mecury-400BB or Mecury-600BB spectrometer with TMS as an internal standard, all chemical shift values are reported as ppm. Mass spectra were recorded on a Bruker Dalton APEXII49e and Esquire6000 (ESI-ION TRAP) spectrometer with ESI source as ionization, respectively.

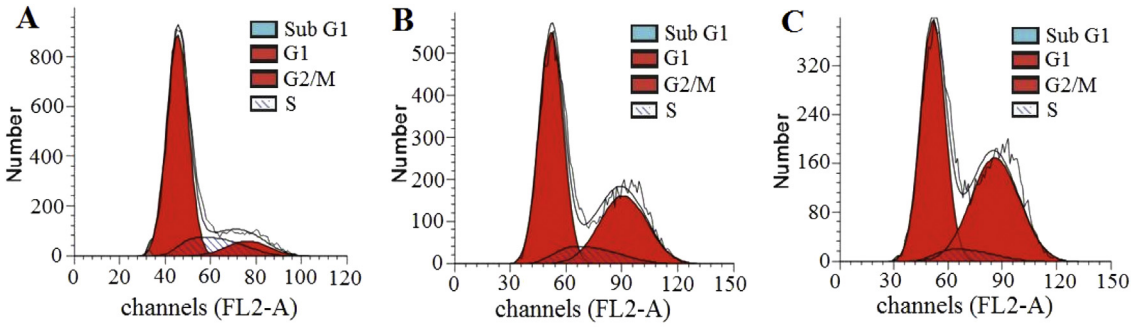
4.2. Synthesis of anilines *4a,b*

4.2.1. 4-[(tert-Butoxycarbonyl)amino]benzoic acid (*2a*)

To a mixture of 4-aminobenzoic acid **1a** (5.00 g, 36.5 mmol) in dioxane (70 mL) and water (35 mL) were added NaOH (1.46 g, 36.5 mmol) followed by di-tert-butyl dicarbonate (11.9 g, 54.8 mmol). The reaction mixture was stirred at room temperature for 24 h. Solvent was removed by rotary evaporation, and 3 N aqueous hydrochloric acid was added dropwise to the residue to adjust pH 3. A precipitate was obtained, collected, washed with water, and dried to provide **2a** (8.28 g, 96%) as a solid.

4.2.2. 4-[N-(tert-Butoxycarbonyl)amino]-N-(4-N-methyl-piperazinyl)benzamide (*3a*)

To a solution of **2a** (3.6 g, 15 mmol) in dry DMF (50 mL) were added BOP (8.0 g, 18 mmol) and DIPEA (9.5 mL, 18 mmol). After stirring at room temperature for 1 h, 4-*N*-methyl-piperazine (2.5 mL, 20 mmol) was added dropwise and the reaction continued for 24 h at room temperature under argon. The product was then extracted with ethyl acetate (5 × 50 mL). The combined organic extracts were washed with brine (2 × 40 mL), dried over sodium sulfate, filtered, and the solvent removed. Purification was achieved by flash chromatography (dichloromethane/methanol 20:1 by



	control (A)	2 μM (B)	5 μM (C)
G1 (M1)	73.0	56.2	50.5
S (M2)	16.6	10.6	6.0
G2/M (M3)	10.2	33.2	43.5

Fig. 5. Effect of **11c** on cell cycle progression. (A) Control HeLa cells, (B) HeLa cells treated with 2 μM **11c** for 12 h, (C) HeLa cells treated with 5 μM **11c** for 12 h.

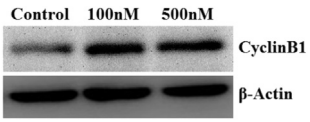


Fig. 6. Western blotting analysis of expression of cyclin B1 in HeLa cells treated with **11c**.

volume) to yield pure **3a** (4.0 g, 80%).

*4.2.3. 4-Amino-N-(4-N-methyl-piperazinyl)benzamide (**4a**)*

A solution of **3a** (2.88 g, 8.6 mmol) in 7:3 dichloromethane:trifluoroacetic acid (40 mL) was stirred at room temperature for 1 h. The solvent was evaporated in vacuo, and diethyl ether (30 mL) was added. The precipitate was collected, washed with ether and dried to provide white solid **4a** (1.86 g, 93%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.74 (d, J = 7.6 Hz, 1H), 7.57 (d, J = 8.8 Hz, 2H), 6.52 (d, J = 8.4 Hz, 2H), 5.56 (s, 2H), 3.73–3.67 (m, 1H), 2.83 (d, J = 11.6 Hz, 2H), 2.22 (s, 3H), 2.16–2.02 (m, 2H), 1.73 (d, J = 10.0 Hz, 2H), 1.53–1.04 (m, 2H).

*4.2.4. 4-Amino-3-methoxyl-N-(4-N-methyl-piperazinyl)benzamide (**4b**)*

From compound **1b** (1.67 g, 10 mmol), a similar procedure as that described for **4a** gave pure **4b** (2.02 g, 77% for 3 steps) as a white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.79 (d, J = 8.0 Hz, 1H), 7.29 (dd, J = 5.6 Hz, 2H), 6.59 (dd, J = 5.6 Hz, 1H), 5.20 (s, 2H), 3.80 (s, 3H), 3.73–3.65 (m, 1H), 3.75 (d, J = 11.6 Hz, 2H), 2.15 (s, 1H), 1.91–1.88 (m, 2H), 1.72 (d, J = 10.2 Hz, 2H), 1.57–1.51 (m, 2H).

*4.3. Synthesis of anilines **6a,b***

*4.3.1. 4-morpholino nitrobenzene (**5a**)*

To a mixture of nitro chlorobenzene (4.7 g, 30 mmol), morpholine (4.25 mL, 48 mmol) in dioxane (50 mL) and water (25 mL) were added potassium carbonate (3.6 g, 35.9 mmol). The reaction mixture was stirred at 80 °C for 5 h. White solid was removed by filtration, the solvent was removed in vacuo to provide yellow solid. Solid was purified by column chromatography (pure CH_2Cl_2) to provide 4-morpholino nitrobenzene **5a** (2.3 g, 40%).^{1}H NMR (400 MHz, $CDCl_3$) δ 8.14 (dd, J = 7.2, 2.0 Hz, 2 H), 6.84 (dd, J = 7.6, 2.0 Hz, 2 H), 3.87 (t, J = 4.8 Hz, 4 H), 3.41 (t, J = 5.2 Hz, 4 H).

*4.3.2. 4-morpholinyl aniline (**6a**)*

To a mixture of **5a** (2.13 g, 10 mmol) in dry ethyl acetate was added 10% Pd/C (0.5 g), following the reaction mixture was stirred under hydrogen gas at 80 °C for 12 h. The reaction mixture was concentrated in vacuo and the crude product was purified by column chromatography (dichloromethane/methanol 15:1 by volume) to give 4-morpholinyl aniline **6a** (1.5 g, yield 85%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.81–6.77 (m, 2H), 6.68–6.64 (m, 2H), 3.85–3.83 (m, 4H), 3.03–3.01 (m, 4H).

*4.3.3. 4-(4-methyl-piperazin-1-yl) aniline (**6b**)*

From nitro chlorobenzene (4.7 g, 30 mmol), a similar procedure as that described for **6a** gave pure **6b** (2.40 g, 42% for 2 steps) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.81 (d, J = 8.8 Hz, 2H), 6.80 (d, J = 8.2 Hz, 2H), 3.06 (t, J = 4.8 Hz, 4H), 2.57 (t, J = 4.8 Hz, 4H), 2.34 (s, 3H).

*4.4. General synthetic procedure of compounds **8–10***

To a solution of 2,4-dichloro-5-fluoropyrimidine **7a** (0.83 g, 5 mmol) in THF (10 mL) at 0 °C was slowly added THF solution of cyclopentylamine (0.74 mL, 7.5 mmol). The resulting mixture was stirred at 0 °C for 3 h (monitored by TLC). The solvent was removed in vacuo and water (20 mL) was added and extracted with CH_2Cl_2 (3 × 10 mL). Combined organic phase was washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated. The crude product was purified by column chromatography on silica gel (dichloromethane/methanol 50:1) to give **8a** (0.83 g, 77%) as a solid.

Similar procedure as that described for **8a** gave pure **8b–d**, **9** and **10** as a solid.

*4.4.1. General synthetic procedure of compounds **11–19***

*4.4.1.1. 4-((4-(cyclopentylamino)-5-fluoro-2-yl)amino)-N-(1-methylpiperidin-4-yl) benzamidepyrimidine (**11a**).* A mixture of **8a** (130 mg, 0.6 mmol), **4a** (115 mg, 0.5 mmol), 5 mL isopropyl alcohol, and 1 drop of 37% HCl was refluxed at 80 °C for 18 h. Solvents were

evaporated in vacuo and the residue purified by column chromatography on silica gel (dichloromethane/methanol 20:1) gave desired product **11a** 172 mg. Yield: 83%; White solid; m.p.: 190–192 °C; IR (KBr, cm^{-1}) 3456, 3287, 2945, 1635, 1611, 1543, 1489, 1377, 1347, 852, 770; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.41 (s, 1H), 7.97 (d, J = 7.6 Hz, 1H), 7.87 (d, J = 4.0 Hz, 1H), 7.80 (d, J = 8.8 Hz, 2H), 7.73 (d, J = 9.2 Hz, 2H), 7.40 (d, J = 6.8 Hz, 1H), 4.37–4.30 (m, 1H), 3.74–3.66 (m, 1H), 2.75 (d, J = 11.6 Hz, 2H), 2.14 (s, 3H), 2.01–1.88 (m, 4H), 1.74–1.71 (m, 4H), 1.63–1.51 (m, 6H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 165.8, 155.9, 152.2 (d, J = 12.0 Hz, 1C), 144.4, 141.5 (d, J = 244.0 Hz, 1C), 138.9 (d, J = 19.0 Hz, 1C), 128.3 (2C), 126.6, 117.0 (2C), 55.1 (2C), 52.3, 46.8, 46.4, 32.5, 32.1, 24.1; HRMS (ESI) 413.2472 for $[M+H]^+$ (calcd 413.2460 for $C_{22}H_{30}FN_6O$).

*4.4.1.2. 4-((5-chloro-4-(cyclopentylamino)-2-yl)amino)-N-(1-methylpiperidin-4-yl) benzamidepyrimidine (**11b**).* From compound **8b** (105 mg, 0.5 mmol), **4a** (105 mg, 0.5 mmol), a similar procedure as that described for **11a** gave pure **11b** as a white solid 156 mg. Yield: 72%; White solid; m.p.: 184–186 °C; IR (KBr, cm^{-1}) 3409, 3287, 2935, 1628, 1576, 1526, 1508, 1380, 848, 771; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.47 (s, 1H), 8.15 (d, J = 8.0 Hz, 1H), 7.98 (s, 1H), 7.80 (d, J = 8.8 Hz, 2H), 7.74 (d, J = 8.8 Hz, 2H), 6.88 (d, J = 7.2 Hz, 1H), 4.42–4.35 (m, 1H), 3.75–3.66 (m, 1H), 2.75 (d, J = 12.0 Hz, 2H), 2.14 (s, 3H), 2.02–1.88 (m, 4H), 1.74–1.71 (m, 4H), 1.66–1.59 (m, 6H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 165.7, 158.1, 157.6, 153.4, 144.0, 128.3 (2C), 127.0, 117.6 (2C), 104.6, 55.1 (2C), 52.8, 46.8, 46.4, 32.4, 32.0, 24.1; HRMS (ESI) 429.2174 for $[M+H]^+$ (calcd 429.2164 for $C_{22}H_{30}ClN_6O$).

*4.4.1.3. 4-((5-bromo-4-(cyclopentylamino)-2-yl)amino)-N-(1-methylpiperidin-4-yl) benzamidepyrimidine (**11c**).* From compound **8c** (154 mg, 0.56 mmol), **4a** (130 mg, 0.56 mmol), a similar procedure as that described for **11a** gave pure **11c** 166 mg. Yield: 63%; White solid; m.p.: 194–196 °C; IR (KBr, cm^{-1}) 3407, 3218, 2950, 1630, 1604, 1554, 1510, 1494, 1336, 853, 762; ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.46 (s, 1H), 8.05 (s, 1H), 8.00 (d, J = 7.6 Hz, 1H), 7.80 (d, J = 8.8 Hz, 2H), 7.75 (d, J = 8.8 Hz, 2H), 6.60 (d, J = 3.2 Hz, 1H), 4.42–4.35 (m, 1H), 3.75–3.66 (m, 1H), 2.75 (d, J = 11.6 Hz, 2H), 2.14 (s, 3H), 2.02–1.88 (m, 4H), 1.74–1.71 (m, 4H), 1.66–1.59 (m, 6H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 165.9, 158.4, 157.6, 153.4, 144.0, 128.2 (2C), 127.0, 117.6 (2C), 104.6, 55.2, 52.9, 46.8, 46.4, 32.4, 32.0, 24.1; HRMS (ESI) 473.1673 for $[M+H]^+$ (calcd 473.1659 for $C_{22}H_{30}BrN_6O$).

*4.4.1.4. 4-((4-(cyclopentylamino)-5-nitro-2-yl)amino)-N-(1-methylpiperidin-4-yl) benzamidepyrimidine (**11d**).* From compound **8d** (120 mg, 0.5 mmol), **4a** (95 mg, 0.5 mmol), a similar procedure as that described for **11a** gave pure **11d** 90 mg. Yield: 60%; yellow solid; m.p.: 198–200 °C; IR (KBr, cm^{-1}) 3423, 2968, 1616, 1585, 1543, 1503, 1418, 1386, 1227, 787, 768; ^{1}H NMR (600 MHz, DMSO-d_6) δ 10.39 (s, 1H), 9.05 (s, 1H), 8.31 (d, J = 6.6 Hz, 1H), 7.92–7.80 (m, 2H), 7.76 (s, 1H), 7.73 (d, J = 8.4 Hz, 1H), 3.71–3.67 (m, 1H), 3.60–3.56 (m, 1H), 2.98 (d, J = 6.6 Hz, 4H), 2.37 (s, 3H), 1.90–1.82 (m, 2H), 1.76–1.68 (m, 2H), 1.30–1.28 (m, 2H), 1.18–1.13 (m, 6H); ^{13}C NMR (150 MHz, DMSO-d_6) δ 165.0, 159.5, 158.0, 153.1, 139.9, 127.9, 127.8, 123.1, 121.6 (2C), 119.8, 53.5 (2C), 45.5, 42.7, 42.3, 30.3, 30.2, 21.5; HRMS (ESI) 440.2417 for $[M+H]^+$ (calcd 440.2405 for $C_{22}H_{30}N_7O_3$).

*4.4.1.5. 4-((4-(cyclopentylamino)-5-fluoro-2-yl)amino)-3-methoxy-N-(1-methylpiperidin- 4-yl)benzamidepyrimidine (**12a**).* From compound **8a** (85 mg, 0.4 mmol), **4b** (86 mg, 0.33 mmol), a similar procedure as that described for **11a** gave pure **12a** 61 mg. Yield: 42%; White solid; m.p.: 194–196 °C; IR (KBr, cm^{-1}) 3391, 2947, 1632, 1611, 1596, 1566, 1499, 1477, 776, 661; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.46 (d, J = 8.8 Hz, 1H), 8.10 (d, J = 7.6 Hz, 1H), 7.89 (d, J = 3.6 Hz, 1H), 7.57 (s, 1H), 7.52–7.47 (m, 3H), 4.35–4.29 (m, 1H), 3.92 (s, 3H), 3.76–3.68 (m, 1H), 2.76 (d, J = 11.2 Hz, 2H), 2.15 (s, 3H), 1.99–1.88 (m, 4H), 1.75–1.72 (m, 4H), 1.63–1.55 (m, 6H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 165.7, 155.4, 152.4 (d, J = 12.0 Hz, 1C), 146.9, 141.8 (d, J = 245.0 Hz, 1C), 138.9 (d, J = 19.0 Hz, 1C), 132.7, 127.1, 120.7, 116.1, 109.7, 56.5, 54.9, 52.3, 46.8, 46.1, 41.8, 32.5, 31.8, 24.1, 19.0, 11.6; HRMS (ESI) 443.2571 for $[M+H]^+$ (calcd 443.2565 for $C_{23}H_{32}FN_6O_2$).

*4.4.1.6. 4-((5-chloro-4-(cyclopentylamino)-2-yl)amino)-3-methoxy-N-(1-methylpiperidin-4-yl)benzamidepyrimidine (**12b**).* From compound **8b** (138 mg, 0.6 mmol), **4b** (130 mg, 0.49 mmol), a similar procedure as that described for **11a** gave pure **12a** 70 mg. Yield: 31%; White solid; m.p.: 197–199 °C; IR (KBr, cm^{-1}) 3409, 2947, 1629, 1511, 1477, 1378, 1027, 767, 683, 638; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.41 (d, J = 9.2 Hz, 1H), 8.08 (d, J = 7.6 Hz, 1H), 7.97 (s, 1H), 7.70 (s, 1H), 7.49 (d, J = 1.6 Hz, 1H), 7.47 (s, 1H), 6.97 (d, J = 7.2 Hz, 1H), 4.40–4.33 (m, 1H), 3.92 (s, 3H), 3.77–3.67 (m, 1H), 2.76 (d, J = 11.6 Hz, 2H), 2.15 (s, 3H), 2.01–1.89 (m, 4H), 1.76–1.66 (m, 4H), 1.64–1.54 (m, 6H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 165.5, 157.7, 157.6, 153.4, 147.3, 132.2, 127.7, 120.4, 117.0, 109.8, 105.1, 56.5, 55.1, 52.8, 47.0, 46.4, 32.3, 32.0, 24.1; HRMS (ESI) 459.2281 for $[M+H]^+$ (calcd 459.2270 for $C_{23}H_{32}ClN_6O_2$).

*4.4.1.7. 4-((5-bromo-4-(cyclopentylamino)-2-yl)amino)-3-methoxy-N-(1-methylpiperidin-4-yl)benzamidepyrimidine (**12c**).* From compound **8c** (145 mg, 0.53 mmol), **4b** (130 mg, 0.49 mmol), a similar procedure as that described for **11a** gave pure **12c** 75 mg. Yield: 31%; White solid; m.p.: 204–206 °C; IR (KBr, cm^{-1}) 3395, 2947, 1632, 1612, 1597, 1572, 1505, 1378, 777, 767, 671; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.39 (d, J = 8.8 Hz, 1H), 8.09 (d, J = 7.6 Hz, 1H), 8.05 (s, 1H), 7.72 (s, 1H), 7.49 (d, J = 1.6 Hz, 1H), 7.47 (s, 1H), 6.66 (d, J = 7.2 Hz, 1H), 4.39–4.32 (m, 1H), 3.92 (s, 3H), 3.77–3.68 (m, 1H), 2.76 (d, J = 11.6 Hz, 2H), 2.15 (s, 3H), 2.01–1.89 (m, 4H), 1.76–1.65 (m, 4H), 1.63–1.53 (m, 6H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 165.6, 158.4, 158.2, 156.3, 147.5, 132.2, 127.8, 120.5, 117.2, 109.8, 94.3, 56.5, 55.1, 53.1, 47.1, 46.5, 32.4, 32.1, 24.1, 19.0; HRMS (ESI) 503.1776 for $[M+H]^+$ (calcd 503.1765 for $C_{23}H_{32}BrN_6O_2$).

*4.4.1.8. N4-cyclopentyl-5-fluoro-N2-(4-morpholinophenyl)-2,4-diaminepyrimidine (**13**).* From compound **8a** (89 mg, 0.5 mmol), **6a** (143 mg, 0.75 mmol), a similar procedure as that described for **11a** gave pure **13** 120 mg. Yield: 75%; White solid; m.p.: 164–166 °C; IR (KBr, cm^{-1}), 3316, 2968, 1627, 1586, 1542, 1542, 1516, 1375, 1226, 774, 740, 655; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.71 (s, 1H), 7.76 (d, J = 3.6 Hz, 1H), 7.56 (d, J = 9.2 Hz, 2H), 7.21 (d, J = 7.2 Hz, 1H), 6.82 (d, J = 9.2 Hz, 2H), 4.32–4.29 (m, 1H), 3.71 (t, J = 4.8 Hz, 4H), 2.98 (t, J = 4.8 Hz, 4H), 1.98–1.91 (m, 2H), 1.70–1.67 (m, 2H), 1.57–1.53 (m, 4H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 156.2, 151.9 (d, J = 12.0 Hz, 1C), 145.6, 140.8 (d, J = 242.0 Hz, 1C), 138.5 (d, J = 18.0 Hz, 1C), 119.5 (2C), 115.8 (2C), 66.3, 56.2, 55.0, 51.8, 49.7, 49.2, 32.2, 23.7, 18.6; HRMS (ESI) 358.2047 for $[M+H]^+$ (calcd 358.2038 for $C_{19}H_{25}FN_5O$).

*4.4.1.9. N4-cyclopentyl-5-fluoro-N2-(4-(4-methylpiperazin-1-yl) phenyl)- 2,4-diaminepyrimidine (**14**).* From compound **8a** (150 mg, 0.7 mmol), **6b** (100 mg, 0.55 mmol), a similar procedure as that described for **11a** gave pure **14** 62 mg. Yield: 31%; White solid; m.p.: 168–170 °C; IR (KBr, cm^{-1}) 3436, 3265, 2925, 1675, 1616, 1517, 1475, 1442, 808, 566; ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.69 (s, 1H), 7.75 (d, J = 3.6 Hz, 1H), 7.56 (d, J = 8.8 Hz, 2H), 7.20 (d, J = 7.2 Hz, 1H), 6.81 (d, J = 8.8 Hz, 2H), 4.32–4.25 (m, 1H), 3.06 (s, 4H), 2.63 (s, 4H), 2.33 (s, 3H), 1.97–1.91 (m, 2H), 1.69–1.65 (m, 2H), 1.58–1.50 (m, 4H); ^{13}C NMR (150 MHz, DMSO-d_6) δ 158.7, 156.3, 152.1 (d, J = 12.0 Hz, 1C), 145.3, 140.9 (d, J = 242.6 Hz, 1C), 138.5, 134.4, 119.7, 116.4 (2C), 56.4, 54.4, 52.0, 48.9, 48.7, 45.0, 32.3, 23.8, 18.7; HRMS (ESI) 371.2360 for

$[M+H]^+$ (calcd 371.2354 for $C_{20}H_{28}FN_6$).

*4.4.1.10. N4-cyclopentyl-N2-(4-morpholinophenyl)-5-nitro-2,4-diaminepyrimidine **(15)**.* From compound **8d** (89 mg, 0.5 mmol), **6a** (161 mg, 0.75 mmol), a similar procedure as that described for **11a** gave pure **15** 120 mg. Yield: 62%; White solid; m.p.: 169–171 °C; IR (KBr, cm^{-1}) 3438, 3370, 2958, 1730, 1628, 1587, 1548, 1513, 1270, 786, 642; ^1H NMR (600 MHz, DMSO-d_6) δ 10.28 (s, 1H), 8.92 (s, 1H), 8.50 (d, J = 6.6 Hz, 1H), 7.66 (d, J = 9.6 Hz, 2H), 6.91 (d, J = 9.6 Hz, 2H), 4.44–4.42 (m, 1H), 3.72 (t, J = 4.8 Hz, 4H), 3.05 (t, J = 4.2 Hz, 4H), 2.04–2.01 (m, 2H), 1.71–1.61 (m, 2H), 1.35–1.21 (m, 4H); ^{13}C NMR (150 MHz, DMSO-d_6) δ 166.9, 158.9, 157.2, 155.1, 147.4, 131.6, 121.6, 115.1, 114.7, 66.8 (2C), 52.5, 48.7 (2C), 32.3 (2C), 23.2 (2C); HRMS (ESI) 385.1993 for $[M+H]^+$ (calcd 385.1983 for $C_{19}H_{25}N_6O_3$).

*4.4.1.11. N4-cyclopentyl-N2-(4-(4-methylpiperazin-1-yl)phenyl)-5-nitro- 2,4-diaminepyrimidine **(16)**.* From compound **8d** (145 mg, 0.6 mmol), **6b** (92 mg, 0.47 mmol), a similar procedure as that described for **11a** gave pure **16** 166 mg. Yield: 89%; White solid; m.p.: 173–175 °C; IR (KBr, cm^{-1}) 3469, 3346, 2954, 1615, 1589, 1536, 1512, 1498, 1361, 826, 783; ^1H NMR (600 MHz, DMSO-d_6) δ 10.27 (s, 1H), 8.93 (s, 1H), 8.49 (d, J = 6.6 Hz, 1H), 7.66 (d, J = 9.0 Hz, 2H), 6.91 (d, J = 9.0 Hz, 2H), 4.44–4.42 (m, 1H), 3.09 (s, 4H), 2.47 (s, 4H), 2.23 (s, 3H), 2.10–2.02 (m, 2H), 1.75–1.69 (m, 2H), 1.65–1.54 (m, 4H); ^{13}C NMR (150 MHz, DMSO-d_6) δ 158.9, 157.1, 155.1, 147.2, 130.6, 121.1 (2C), 119.8, 115.3 (2C), 54.5 (2C), 52.5, 48.2, 45.6, 32.3 (2C), 23.5; HRMS (ESI) 398.2308 for $[M+H]^+$ (calcd 398.2299 for $C_{20}H_{28}N_7O_2$).

*4.4.1.12. N4-cyclopropyl-5-fluoro-N2-(4-morpholinophenyl)-2,4-diaminepyrimidine **(17)**.* From compound **9** (140 mg, 0.75 mmol), **6a** (89 mg, 0.5 mmol), a similar procedure as that described for **11a** gave pure **17** 95 mg. Yield: 58%; White solid; m.p.: 163–165 °C; IR (KBr, cm^{-1}) 3274, 2953, 1627, 1588, 1541, 1516, 1377, 1229, 773, 636; ^1H NMR (400 MHz, DMSO-d_6) δ 7.77 (d, J = 3.6 Hz, 1H), 7.57 (d, J = 8.8 Hz, 2H), 6.92 (s, 1H), 6.90 (d, J = 9.2 Hz, 2H), 5.16 (brs, 1H), 3.87 (t, J = 4.8 Hz, 4H), 3.11 (t, J = 4.8 Hz, 4H), 2.86–2.80 (m, 1H), 0.91–0.86 (m, 2H), 0.66–0.63 (m, 2H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 156.1, 153.6 (d, J = 10.9 Hz, 1C), 146.5, 141.9 (d, J = 242.5 Hz, 1C), 138.8 (d, J = 18.9 Hz, 1C), 133.5, 120.2 (2C), 116.6 (2C), 67.0, 66.9, 51.1, 50.3 (2C), 23.4, 7.2; HRMS (ESI) 330.1729 for $[M+H]^+$ (calcd 330.1725 for $C_{17}H_{21}FN_5O$).

*4.4.1.13. 5-fluoro-N2-(4-(4-methylpiperazin-1-yl)phenyl)-N4-propyl- 2,4-diaminepyrimidine **(18)**.* From compound **10** (113 mg, 0.6 mmol), **6b** (95 mg, 0.5 mmol), a similar procedure as that described for **11a** gave pure **18** 92 mg. Yield: 70%; White solid; m.p.: 166–168 °C; IR (KBr, cm^{-1}) 3385, 2965, 1683, 1618, 1519, 1440, 1379, 800, 722; ^1H NMR (600 MHz, DMSO-d_6) δ 8.73 (s, 1H), 7.77 (d, J = 4.2 Hz, 1H), 7.55 (d, J = 9.0 Hz, 2H), 7.36 (s, J = 4.8 Hz, 1H), 6.83 (d, J = 9.6 Hz, 2H), 3.330 (dd, J = 13.8, 6.0 Hz, 2H), 3.15–3.10 (m, 4H), 2.78–2.72 (m, 4H), 2.42 (s, 3H), 1.60–1.55 (m, 2H), 0.88 (t, J = 7.2 Hz, 3H); ^{13}C NMR (150 MHz, DMSO-d_6) δ156.1, 152.1 (d, J = 11.4 Hz, 1C), 144.8, 140.6 (d, J = 242.5 Hz, 1C), 138.3 (d, J = 17.3 Hz, 1C), 134.2, 119.3 (2C), 116.1 (2C), 53.8 (2C), 48.1 (2C), 44.4, 41.7, 22.1, 11.4; HRMS (ESI) 345.2206 for $[M+H]^+$ (calcd 345.2197 for $C_{18}H_{26}FN_6$).

*4.4.1.14. 5-fluoro-N2-(4-morpholinophenyl)-N4-propyl-2,4-diaminepyrimidine **(19)**.* From compound **10** (113 mg, 0.6 mmol), **6a** (89 mg, 0.5 mmol), a similar procedure as that described for **11a** gave pure **19** 166 mg. Yield: 96%; White solid; m.p.: 162–163 °C; IR (KBr, cm^{-1}) 3341, 2960, 1630, 1589, 1544, 1517, 1372, 1226, 776, 659; ^1H NMR (400 MHz, DMSO-d_6) δ 8.73 (s, 1H), 7.77 (d, J = 3.6 Hz, 1H), 7.56 (d, J = 9.2 Hz, 2H), 7.37 (t, J = 5.2 Hz, 1H), 6.82 (d, J = 8.8 Hz, 2H), 3.71 (t, J = 4.8 Hz, 4H), 3.33–3.27 (m, 2H), 2.98 (t, J = 4.8 Hz, 4H), 1.62–1.53 (m, 2H), 0.89 (t, J = 7.2 Hz, 3H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 156.1, 152.2 (d, J = 12.0 Hz, 1C), 145.5, 140.7 (d, J = 241.0 Hz, 1C), 138.4 (d, J = 17.0 Hz, 1C), 134.1, 119.4 (2C), 115.7 (2C), 66.3 (2C), 49.6 (2C), 41.7, 22.2, 11.5; HRMS (ESI) 332.1889 for $[M+H]^+$ (calcd 332.1881 for $C_{17}H_{23}FN_5O$).

*4.4.1.15. N4-cyclopentyl-N2-(4-(4-methylpiperazin-1-yl)phenyl)-2,4,5-triaminepyrimidine **(20)**.* Compound **16** (225 mg, 0.56 mmol) was dissolved in dry ethyl acetate (10 mL), added 0.1 g 10% of Pd/C. The reaction was stirred for 14 h under hydrogen gas at 60 °C, then Pd/C was filtered, the filtrate was evaporated and the solid was purified by column chromatography (dichloromethane: methanol = 15:1) to give **20** 160 mg. Yield: 80%; pink solid; m.p.: 159–161 °C; IR (KBr, cm^{-1}) 3354, 3198, 2965, 1731, 1659, 1582, 1559, 1513, 1470, 1357, 826, 764, 652; ^1H NMR (600 MHz, DMSO-d_6) δ 8.15 (s, 1H), 7.56 (d, J = 9.0 Hz, 2H), 7.31 (s, 1H), 6.77 (d, J = 9.0 Hz, 2H), 6.08 (d, J = 6.6 Hz, 1H), 2.98 (t, J = 4.8 Hz, 4H), 2.43 (t, J = 4.2 Hz, 6H), 2.19 (s, 3H), 2.00–1.97 (m, 1H), 1.88 (brs, 2H), 1.72–1.70 (m, 2H), 1.58–1.55 (m, 2H), 1.51–1.46 (m, 2H); ^{13}C NMR (150 MHz, DMSO-d_6) δ 153.8, 144.9, 138.3, 135.6, 129.1, 120.1, 118.6 (2C), 116.6 (2C), 55.3 (2C), 52.3, 49.8 (2C), 46.2, 32.9 (2C), 22.8 (2C); HRMS (ESI) 368.2570 for $[M+H]^+$ (calcd 368.2557 for $C_{20}H_{30}N_7$).

4.5. Biology

4.5.1. Cytotoxicity assays

Cells were incubated at 37 °C in a 5% CO_2 atmosphere. The MTT assay were used to determined growth inhibition. The synthetic compounds and reference compound VX-680 were dissolved in saline for five concentrations (0.001–100 mM). The A-549, HepG2, HCT-8 and HeLa cells were plated in 96-well plates and allowed to attach for 4–6 h, then exposed in quadplex well for 48 h. The media was aspirated, and 10 μL of 5 mg/mL MTT solution (dilute in sterile PBS) diluted in serum-free media was added to each well. After 4 h of incubation, the solution was centrifuged for 10 min under 2000 rpm. The supernatant was mixed with 150 μL DMSO, and shaken on an oscillator. The absorbance at λ_{490} was determined on a plate reader. IC_{50} values were determined from a log plot of percent of control versus concentration [36].

4.5.2. Western blot analysis

HeLa cells (1×10^6 cells) exposed to compound **11c** were collected into tubes and then washed with PBS. Cell pellets were lysed with lyses buffer (50 mM TriseHCl, pH 7.5, 10 mM EDTA, 0.2 M NaCl, 1.5 mM PMSF and 1% SDS). Cell lysates were boiled for 10 min, centrifuged and stored at −20 °C. Cell lysates containing 10–20 μm g protein were separated and transferred to nitro-cellulosefilters. The blots were incubated with the corresponding antibodies and developed [36].

4.5.3. Analysis of cell cycle by flow cytometry

For cell cycle analysis, we used the cervical carcinoma HeLa cell line grown in RPMI-1640 supplemented with 10% (v/v) heati-nactivated fetal calf serum, 2 mM L-glutamine, 100 units/mL penicillin, and 24 μg/mL gentamicin and incubated at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Untreated and drugtreated cells ($(3–5) \times 10^5$) were harvested and fixed overnight in 70% ethanol at 4 °C. Cells were then washed three times with PBS, incubated for 1 h with 1 mg/mL RNase A and 20 μg/mL pro-pidium iodide at room temperature, and analyzed with a flow cy-tometer (COULTER EPICS XL, USA) as described previously [35].

4.5.4. ELISA experiment

Use Purified Human Aurora A or B antibody to coat microtiter plate wells, make solid-phase antibody, then add Aurora A or B to wells, Combined Aurora A which With HRP labeled, become

antibody-antigen-enzyme-antibody complex, after washing Completely, Add TMB substrate solution, TMB substrate becomes blue color At HRP enzyme-catalyzed, reaction is terminated by the addition of a sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm [32].

4.5.5. Molecular docking study

The docking simulation was performed using the Aurodock 4.2 software. Before docking, the protein structure was minimized firstly. The crystal waters were removed and the Kollman united atom charges and polar hydrogen was added to the two proteins. The ligand in the crystal structure was used to determine the location of a docking grid box and was then removed prior to grid generation in next step. Gasteiger charges were assigned to the new constructed structures in Autodock. At the same time, the Non-polar hydrogen atoms were merged and the rotatable bonds were defined. Based on the ligand in the crystal structure, the grid maps of the protein were produced using AutoGrid module embedded in Autodock software. As result, a grid size of 60 × 60 × 66 Å points and 0.375 Å spacing were generated. Each docking process was performed in 250,000 energy evaluation with 10 conformations kept and the most favorable pose of each compound was displayed.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2015.03.044.

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