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Synthesis and Activity Evaluation of Nasopharyngeal Carcinoma Inhibitors Based on 6-(pyrimidin-4-yl)-1*H*-indazole

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Abstract: Human nasopharyngeal carcinoma is a common head and neck malignancy with high incidence in Southeast Asia and Southern China. It is necessary to develop safe, effective and inexpensive anti-cancer agents to improve the therapeutics of patients with nasopharyngeal carcinoma. A series of small molecular compounds based on 6-(pyrimidin-4-yl)-1*H*-indazole were synthesized and evaluated for antiproliferative activities against human nasopharyngeal carcinoma cell line SUNE1. Compound **6b**, **6c**, **6e** and **6l** showed potent antiproliferative activities similar to positive control drug cisplatin in vitro with lower nephrotoxicity than it. **6l** was selected for further study. It was found that **6l** induced mitochondria-mediated apoptosis and G_2/M phase arrest in SUNE1 cells. Furthermore, **6l** at 10 mg/kg can

suppress the growth of an implanted SUNE1 xenograft with a TGI% (tumor growth inhibition) value of 50% and did not cause serious side effects in BALB/c nude mice. This study suggests that 6-(pyrimidin-4-yl)-*1H*-indazole derivatives are a series of small molecule compounds with anti-nasopharyngeal carcinoma activities.

Keywords: nasopharyngeal carcinoma, indazole, antiproliferative, apoptosis, phase arrest

Introduction

Human nasopharyngeal carcinoma (*NPC*) is a common head and neck malignancy prominently in Southeast Asia and Southern China. Though trends in declining incidence and mortality of *NPC* were reported, age-standardized rates in incidence remained 20/100,000 in southern China in 2012.[1] Due to its high radio sensitivity, the standard treatment for *NPC* is radiotherapy, in fact local radiation and surgery may provide good control of *NPC*. However, the prognosis of patients with *NPC* still stays poor due to the advanced stage at the time of diagnosis, regional relapse, and distant metastasis.[2] Conventionally, chemotherapy is given concurrently with radiotherapy for treating locally advanced disease or distant metastasis. The main chemotherapeutic agents of nasopharyngeal carcinoma are cisplatin, fluorouracil, paclitaxel, docetaxel and so on. The curative effects of chemotherapy for advanced *NPC* patients remain unsatisfactory and the overall survival of recurrent or advanced *NPC* patients is usually poor, with reported median survival range from 7.2 to 22 months.[3] Moreover, adverse effects, including upper gastrointestinal impairment and bone marrow suppression, depressed the toleration and limited the clinical use of chemotherapeutic agents.[4] These urge us to explore new strategies based on molecular mechanisms and the disease characteristics to improve the therapeutics of patients with *NPC*.

Indazole is an important intermediate, derivatives of which have shown a wide spectrum of biological activities such as anti-bacterial, anti-schizophrenia, cytotoxic effect against cancer cell lines and anti-tumor.[5-9] Recently, cytotoxic effect against

cancer cell lines and anti-tumor activity of a variety of indazole derivatives were reported worldwide (Fig. 1). A indazole-based covalent inhibitor **1** which targeted drug-resistant *EGFR* exhibited potent inhibition to non-small cell lung cancers cells H1975 (the EGFR-L858R/T790M drug-resistant cell line) and HCC827 (the EGFR-delE746_A750 activating mutation cell line) with IC_{50} values of 694 nM and 459 nM, respectively.[10] Its suppression on phosphorylation of EGFR and downstream cascade was demonstrated by Western blot analysis. Fibroblast growth factor receptor inhibitor **2** based on 1*H*-indazol-3-amine showed wonderful antiproliferative activity to KG1 cell lines and SNU16 cell lines, IC_{50} of which were 25.3 ± 4.6 nM and 77.4 ± 6.2 nM respectively.[11] Multi-target RTKs inhibitor **3** bearing 1*H*-indazole-3-amine displayed antiproliferative activity to a broad spectrum of cancer cells with IC_{50} values ranging from 1.80 μM to 13.26 μM, such as HepG2, SMMC-7721 and MIAPaCa-2.[9] In addition, some skeletons based on indazole or containing indazole with anti-tumor activities have been reported.[12-16] These studies show that indazole derivatives have promising potential for applications in pharmaceutical industry.

Aminoindazole compound **4** identified by researchers at GlaxoSmithKline was reported as a potent leadlike *PDK1* inhibitor.[17] Compound **5 (GSK2334470)** with higher potency and selectivity against *PDK1* was developed after further optimization and its cytotoxic effect against cancer cell lines was demonstrated (IC_{50} against MM.1R = 4.89 μM).[18-21] Based on its antiproliferative activities and our effort to develop safe and effective nasopharyngeal carcinoma inhibitors, the antiproliferative activity to human nasopharyngeal carcinoma cell line SUNE1 of **GSK2334470** was measured. It only displayed moderate antiproliferative activities to SUNE1 cell line (IC_{50} = 24.52 ± 2.73 μM). Making it as a lead compound, we attempted to improve its potency against SUNE1 cell line. It was found that introduction of substituent on nitrogen site at position 2 of pyrimidine ring slightly improve cytotoxic effect (The IC_{50} of **6d** is 20.28 ± 1.12 μM). Therefore we introduced a variety of substituents on this position to improve potency of lead compound, and synthesized a series of derivatives **6a-m** and **7a**. It was demonstrated that indazole derivatives we

synthesized can suppress the growth of SUNE1 cell line in vitro with lower nephrotoxicity than cisplatin. Preliminary research indicates that these compounds inhibited the growth of SUNE1 cells by mitochondria-mediated apoptosis and G_2/M phase arrest. And **6l** can suppress the growth of an implanted SUNE1 xenograft without serious side effects in BALB/c nude mice. Herein, we will report the synthesis and activity evaluation of these compounds.

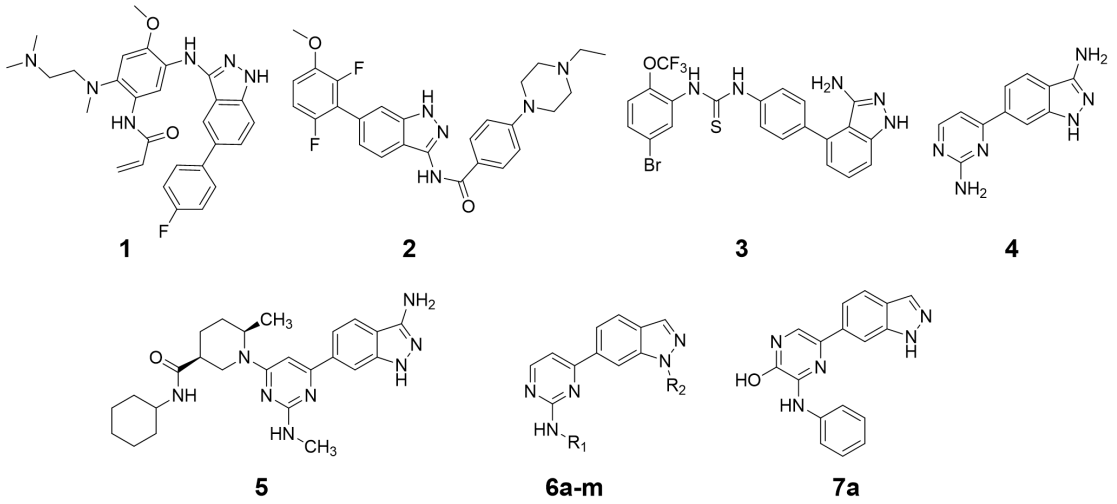


Fig. 1. Chemical structures of indazole derivatives **1**-**5** with cytotoxic effect and new compounds **6a-m** and **7a**.

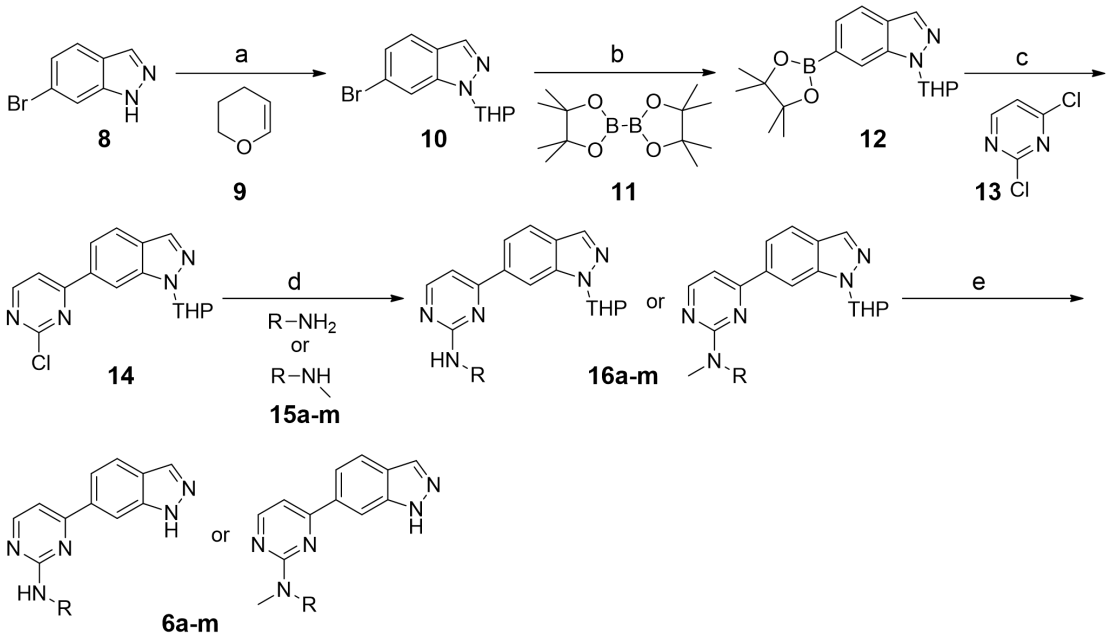
Results and discussion

Chemistry

The general synthesis for compound **6a-m** is shown in Scheme 1. For protecting the 1-amino group, 6-bromoindazole (**8**) was heated to reflux with dihydropyran (**9**), pyridinium 4-toluenesulfonateto and p-toluenesulfonic acid monohydrate in dichloromethane to obtain compound **10**.[22] Compound **12** was generated from **10** and bis(pinacolato)diboron (**11**) with potassium acetate as a base via classic palladium-catalyzed Miyaura borylation.[23,24] The Suzuki reaction was performed on compound **12** and 2,4-dichloropyrimidine (**13**) using $Pd(PPh_3)_4$ as the catalyst to

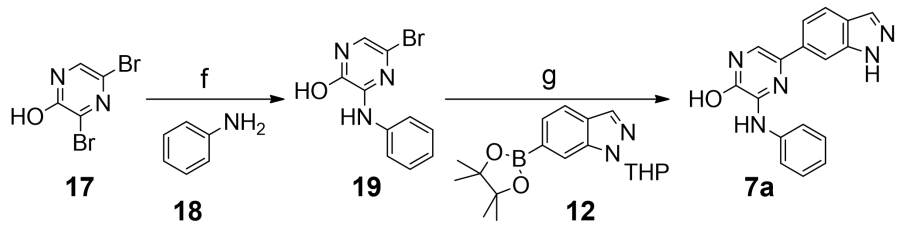
generate the intermediate **14**.[25] To produced compounds **16a-m**, coupling of the intermediate **14** with various amines (**15a-m**) was performed under one of the typical Buchwald-Hartwig amination reaction conditions with tris(dibenzylideneacetone)dipalladium(0) ($Pd_2(dba)_3$) and RuPhos as a ligand in the presence of caesium carbonate in refluxing 1,4-dioxane.[23,26] The THP group was removed from **16a-m** with HCl in ethanol to give the target compound **6a-m**.[27]

Scheme 2 shows the synthesis of compound **7a.** Nucleophilic substitution of 3,5-dibromopyrazin-2-ol (**17**) with aniline (**18**) produced compound **19**.[23,26] Finally, compound **7a** was prepared from **19** and **12** via Suzuki reaction.[25]



Reagents and conditions: (a) PPTS, p-toluenesulfonic acid monohydrate, dichloromethane, 60 ºC reflux; (b) 1,1'-Bis(diphenylphosphino)ferrocene-palladium(II)dichloride dichloromethane complex, KOAc, 1,4-dioxane, 110 ºC reflux; (c) $Pd(PPh_3)_4$, saturated sodium bicarbonate solution, 1,4-dioxane, 95 ºC; (d) $Pd_2(dba)_3$, RuPhos, Cs_2CO_3, 1,4-dioxane, 110 ºC; (e) HCl, ethanol; 70 ºC.

Scheme 1. Synthesis of 6a-m



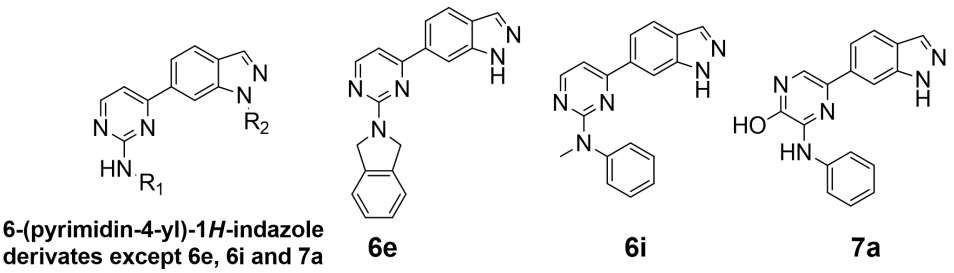
Reagents and conditions: (f) camphorsulfonic acid, isopropanol, 82 ºC; (g) i) $Pd(PPh_3)_4$, saturated sodium bicarbonate solution, 1,4-dioxane, 110 ºC reflux; ii) HCl, ethanol; 70 ºC.

Scheme 2. Synthesis of 7a

Antiproliferative activity and nephrotoxicity

The synthesized derivatives **16b-d**, **6a-m** and **7a** were evaluated for antiproliferative activities against SUNE1 cells using CCK8 assay. Cisplatin was included as the positive control drug. Cytotoxicity of these compounds against MDCK cells were also determined to evaluate nephrotoxicity. The results were listed in Table 1. The most active compounds were **6b**, **6c**, **6e** and **6l**. Similar to cisplatin, they displayed antiproliferative potency in the low micromolar range (IC_{50} = 4.34-9.27 μM). Compounds **16b**, **16c**, **16d**, **6d**, **6f**, **6i** and **6m** possessed modest activity with IC_{50} range 20.28-34.98 μM. The rest of the compounds showed no activities on SUNE1 (IC_{50} > 100 μM). CC_{50} Values in Table 1 showed that most of the compounds demonstrated lower cytotoxicity than cisplatin (CC_{50} = 8.45 ± 0.68 μM) on MDCK cell line, which indicated that they possess low nephrotoxicity.

Table 1. Antiproliferative activity and nephrotoxicity of cisplatin, **16b-d**, **6a-m** and **7a** in vitro



compounds	R_1	R_2	IC_{50} (μM)[a]	CC_{50} (μM)[b]
cisplatin			6.16 ± 0.73[c]	8.45 ± 0.68
16b	m-tolyl	Tetrahydro-2*H*-pyran-2-yl	20.91 ± 0.85	*ND*[d]

16c	1-methyl-1*H*-pyrazol-4-yl	Tetrahydro-2*H*-pyran-2-yl	24.78 ± 1.53	*ND*
16d	2-fluorophenyl	Tetrahydro-2*H*-pyran-2-yl	34.98 ± 2.76	*ND*
6a	p-tolyl	H	> 100	> 200
6b	m-tolyl	H	4.34 ± 0.26	44.63 ± 5.57
6c	pyridin-3-yl	H	8.28 ± 0.99	33.19 ± 3.37
6d	2-fluorophenyl	H	20.28 ± 1.12	> 100
6e		H	9.27 ± 0.54	43.70 ± 3.68
6f	4-methylthiazol-2-yl	H	33.72 ± 2.85	> 200
6g	3-fluorophenyl	H	> 100	> 200
6h	3-chlorophenyl	H	> 100	> 200
6i	phenyl	H	26.08 ± 0.90	58.32 ± 4.84
6j	pyrimidin-2-yl	H	> 100	> 200
6k	5-methylpyridin-2-yl	H	> 100	> 200
6l	3-aminophenyl	H	6.37 ± 0.93	19.38 ± 2.68
6m	thiazol-2-yl	H	26.23 ± 1.63	> 200
7a			24.55 ± 3.02	68.80 ± 2.05

[a]Antiproliferative activities against SUNE1 cells. [b]Cytotoxicity against MDCK cells. [c]Values shown are the *mean* ± *SD* (n =3). [d]*ND* is not determined.

The structure-activity relationship

The structure-activity relationship of these compounds can be analyzed by IC_{50} values and chemical structure (Table 1). As for R_1 substituent, phenyl (**6b**), pyridin-3-yl (**6c**), isoindolin-2-yl (**6e**) and 3-aminophenyl (**6l**) contributed most to antiproliferative activities. Group thiazol-2-yl (**6m**) contribute to activities. But pyrimidin-2-yl (**6j**) made compound lose its activities. Compounds with a substitution

at the 3-position of the benzene ring (**6b**, **6l**) were superior to those with substitutions at the 2- (**6d**) and 4-positions (**6a**, **6k**) on the phenyl group. Compounds containing electron-donating group (**6b**, **6l**) indicated more potent activity than compounds containing electron-withdrawing group (**6g**, **6h**). As for R_2 substituent, substitution of tetrahydropyran ring with hydrogen enhances the activity (**6b** > **16b**, **6c** > **16c**, **6d** > **16d**).

*Dynamic changes of cell morphology after compound **6l** administration*

6l was selected for further study. In order to pinpoint the onset time and process of compound **6l**, we observed the morphological changes of SUNE1 cells dynamically with YeeSpec Automatic microscopic imaging system. Cell death began to occur at 17 h after compound **6l** administration compared with before treatment. More than half cells died by 24 h and all cells died by 48 h. A large number of cell debris can be found in the cell culture medium (Fig. 2). These results suggested that the duration of compound **6l** induced SUNE1 cell death was 17-48 h after compound **6l** administration.

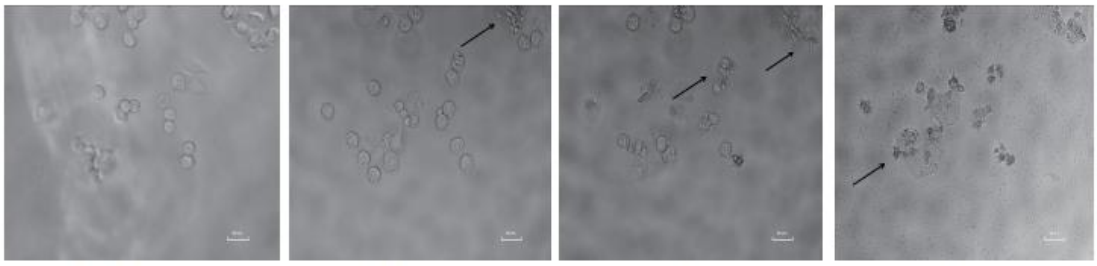


Fig. 2. Dynamic changes of cell morphology were observed by YeeSpec Automatic microscopic imaging system at the time point of 17 h, 24 h and 48 h after **6l** administration.

*Dynamic monitoring antiproliferative activity of compound **6l** via xCELLigence system*

To further characterize compound **6l**, SUNE1 cells exposed to selected compounds were dynamically monitored for over 70 h using real-time electrical impedance as a measure of viable cell number. After compound **6l** administration, the *CI* (cell index) value of SUNE1 cells slightly increase within 24 h, slowly decreased over time and dropped down to baseline by 82 h (Fig. 3). The antiproliferative activity of compound **6l** was strengthened with the increase of concentration. These data suggested that compound **6l** kill tumor cells in a time-and dose-dependent manner.

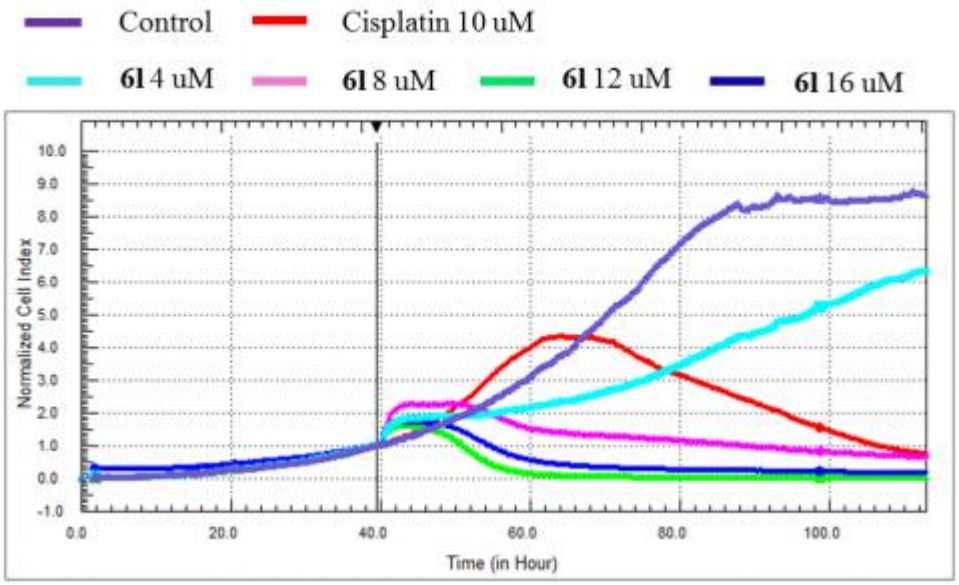


Fig. 3. SUNE1 cells exposed to compound **6l** were dynamically monitored over 70 h using real-time electrical impedance as a measure of viable cell number.

*Compound **6l** induced apoptosis of nasopharyngeal carcinoma cell*

To further evaluate the cytotoxicity of compound **6l**, we tested whether **6l** induces the cell death via apoptosis. SUNE1 cells were treated with compound **6l** at different concentrations of 2 μM, 4 μM, 6 μM, and 8 μM (Fig. 4). The apoptotic cells were measured by double staining of Annexin-V and propidium iodide (*PI*) at different time of 12 h, 24 h, 36 h, and 48 h. No obvious change of the percentage of

early apoptotic cells were observed at different time point, but the percentage of late apoptotic cells gradually increased over time, with measured values 11.8%, 16.7%, 18.5% and 23.5% at the time point of 12 h, 24 h, 36 h, and 48 h, respectively. Moreover, the percentage of apoptotic cells rose gradually with the increase of **6l** concentration. 15.6%, 14.5%, 25.4% and 43.5% early apoptotic cell were induced at the different dose of 2 μM, 4 μM, 6 μM, and 8 μM, respectively. Therefore, the antiproliferative effect of compound **6l** is caused by inducing cell apoptosis.

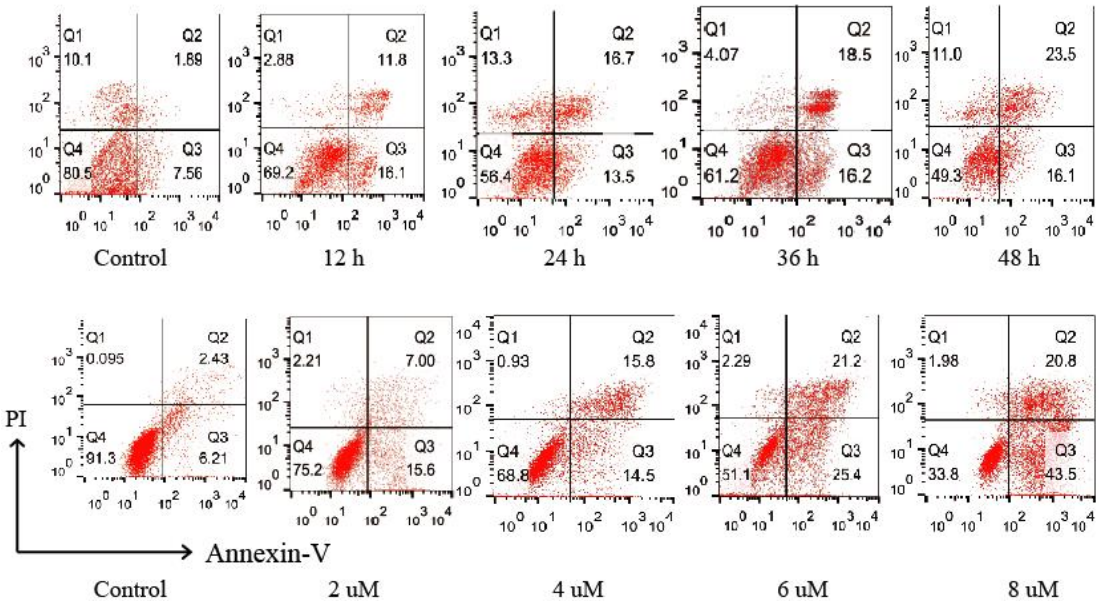


Fig. 4. SUNE1 cells were treated with compound **6l** at the different concentrations of 2 μM, 4 μM, 6 μM, and 8 μM. The apoptotic cells were measured by double staining of Annexin-V and *PI* at different time points 12 h, 24 h, 36 h, and 48 h.

*Compound **6l** induced mitochondria-mediated apoptosis in SUNE1 cells*

To determine whether **6l** treatment could induce apoptosis through the mitochondrial pathway, normally growing SUNE1 cells were treated with different concentrations of **6l**. After 48 h, the cells were stained with Rhodamine 123. The mitochondrial membrane potential decreased significantly with the increase of **6l**

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concentration compared with the untreated group (Fig. 5). These results suggested that **6l** may induce apoptosis through the mitochondrial pathway.

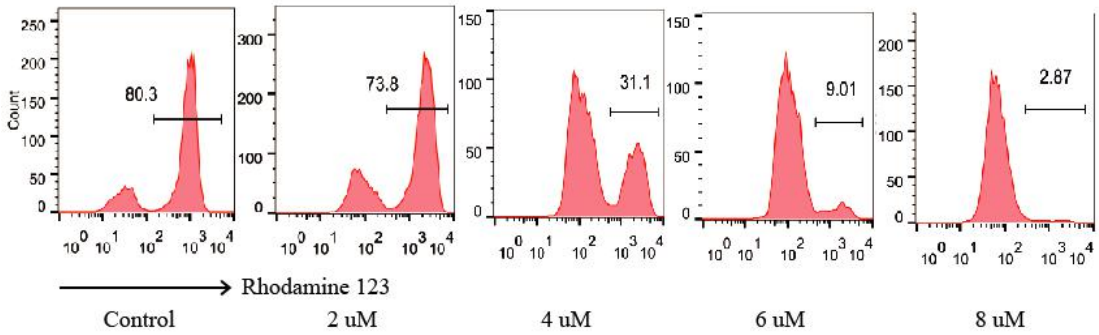

Fig. 5. The mitochondrial membrane potential (*MMP*) was analyzed using the fluorescent dye, Rhodamine 123. SUNE1 cells were seeded on glass coverslips and treated with different concentrations of **6l**. Twenty-four hours after incubation, the cells were fixed with rhodamine 123 for 30 min, the mean fluorescence intensities of the granulosa cells were determined using Flow Cytometer.

*Compound **6l** induce G_2/M phase arrest in SUNE1 cells dose-dependently*

Cell cycle arrest is an important sign for the inhibition of proliferation and the series of events take place in cells, leading to cell division and replication. Cells were washed with *PBS*, fixed with ice-cold 70% ethanol overnight, stained with *PI*, and analyzed for cell cycle distribution by flow cytometry. As shown in Fig. 6, the percent of G_2/M-phase cells rose gradually with the increase of concentration. The results indicate that compound **6l** can induce G_2/M phase arrest in SUNE1 cells dose-dependently.

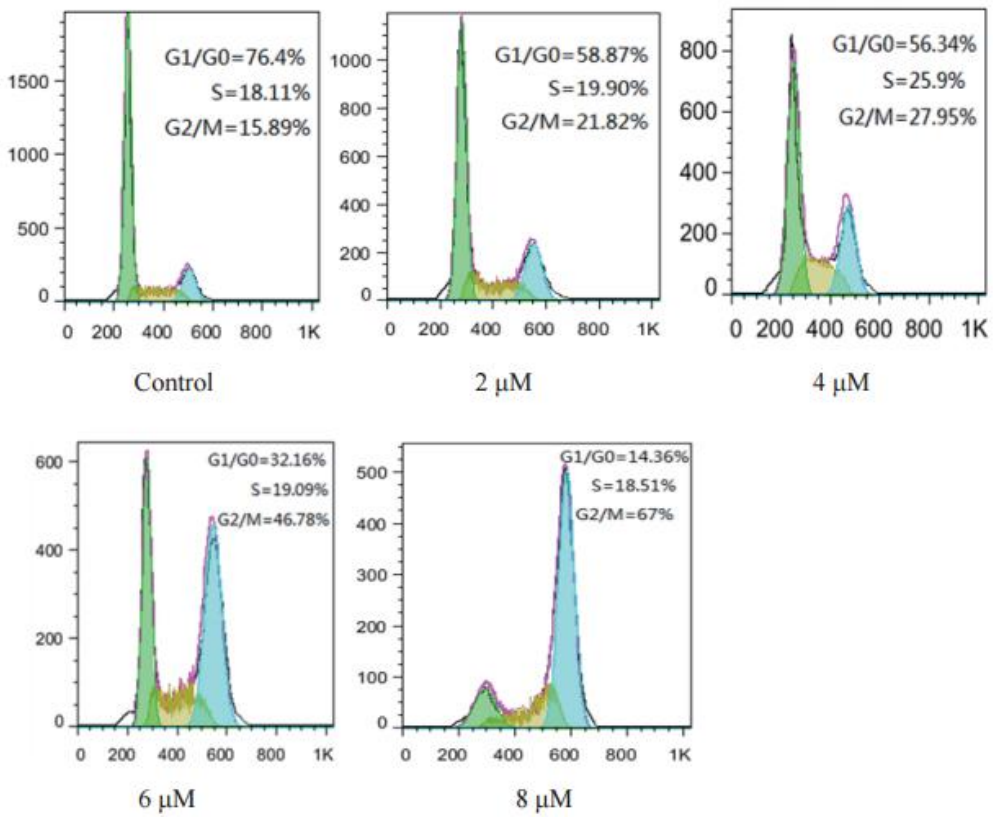

Fig. 6. SUNE1 cells treated with compound **6l** at the different concentrations of 2 μM, 4 μM, 6 μM, and 8 μM were analyzed for cell cycle distribution by flow cytometry.

*In vivo anti-tumor activity of **6l***

To evaluate the in vivo anti-tumor activity of compound **6l**, a human nasopharyngeal carcinoma xenograft model established by inoculating subcutaneously BALB/c nude mice with SUNE1 cells was used. Mice in treatment group were intraperitoneally treated with **6l** at a dose of 10 mg/kg everyday for 14 consecutive days (Fig. 7). As shown in Fig. 7 a) and b), **6l** at 10 mg/kg could significantly suppress the growth of tumor in vivo ($p = 0.003 < 0.05$), and the TGI% (tumor growth inhibition) value was 50% at day 14. In addition, weight change and other serious side effects were not observed during **6l** administration (Fig. 7 c)). Therefore, these results demonstrate the safety and efficacy of **6l** in vivo.

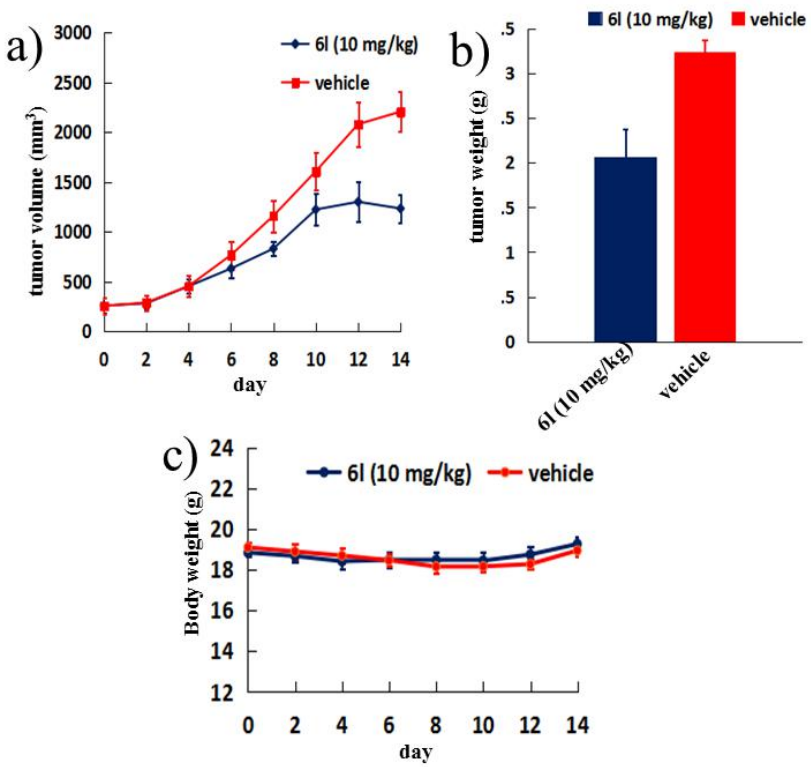

Fig. 7. In vivo anti-tumor activity of **6l**. a) The average tumor volume of treatment group and vehicle group mice during **6l** administration. b) The average tumor weight of treatment group and vehicle group mice at day 14. c) The average body weight of treatment group and vehicle group mice during **6l** administration. All the values were shown as *mean* ± *SD* (n = 7 per group), * $P < 0.05$.

Conclusion

In summary, we synthesized a series of indazole derivatives. In this research, we not only evaluated the inhibition of these compounds against nasopharyngeal carcinoma in vitro and vivo, but also preliminarily investigated the action mechanism. Some compounds (**6b**, **6c**, **6e**, **6l**) showed potent inhibition against SUNE1 cells in vitro .with lower nephrotoxicity than cisplatin. Compound **6l** was found to induce SUNE1 cell death at the time point of 17 h, 24 h and 48 h after administration and exert antiproliferative activity in a time- and dose-dependent manner. Further study demonstrated that **6l** induced mitochondria-mediated apoptosis and G_2/M phase arrest

in SUNE1 cells. To our delight, **6l** at 10 mg/kg showed moderate anti-tumor activity without serious side effects in vivo. Therefore, 6-(pyrimidin-4-yl)-1*H*-indazole derivatives are a series of small molecule compounds with potent anti-nasopharyngeal carcinoma activities.

Experimental Section

Chemical material and equipment

Reagents for synthesis were obtained commercially, unless otherwise noted. Pyran was dried by distillation from sodium. Flash chromatography was performed on silica gel 60 (230-240 mesh, 60 Å) with the indicated eluents. ^{1}H NMR was recorded at 400 or 500 MHz and ^{13}C NMR at 100 or 125 MHz on a Bruker DPX-400 or DPX-500 instrument. Mass spectra were recorded with Bruker MicroTof. The purity of all compounds was determined by a Shimadzu LC-20AT HPLC system with detection at 254 and 365 nm and C18-bonded silica column (InertSustain, 4.6 × 150 mm). The column was perfused at a flow rate of 1 mL/min with methanol, and a linear gradient from 10 to 90% of water over 120 min was adopted for elution.

Chemistry

Synthesis of ***6-bromo-1-(tetrahydro-2H-pyran-2-yl)-1H-indazole*** **(10)**: To a solution of 6-bromoindazole (10 mmol, 1970 mg), PPTS (1.5 mmol, 380 mg), and p-toluenesulfonic acid monohydrate (2.5 mmol, 430 mg) in dichloromethane (30 mL) was added dihydropyran (6 mL) and the mixture was refluxed under argon at 60 ºC overnight. After cooling, the mixture was extracted with dichloromethane, washed with saline. The organic layer was collected and the solvent was removed under

vacuum. The crude product was purified by silica gel column chromatography (hexane / ethyl acetate 5 : 1) to give yield white solid as product (1886 mg, yield 67%).

Synthesis of ***1-(tetrahydro-2H-pyran-2-yl)-6-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1H-indazole*** **(12)**: To a solution of 6-bromo-1-(tetrahydro-2*H*-pyran-2-yl)-1*H*-indazole (10.62 mmol, 2987 mg), bis(pinacolato)diboron (12.75 mmol, 3238 mg), KOAc (49.83 mmol, 4900 mg) and 1,1'-Bis(diphenylphosphino)ferrocene-palladium(II)dichloride dichloromethane complexsaline (1.00 mmol, 817 mg) in 1,4-dioxane (30 mL) was refluxed at 110 ºC for 3 h under argon atmosphere. Then, the reaction mixture was extracted with ethyl acetate, washed with saline. The organic phase was collected and the solvent was removed under vacuum. The crude product was purified by silica gel chromatography (hexane / ethyl acetate 5 : 1) to give yield white solid as product (2728 mg, yield 78.34%).

Synthesis of ***6-(2-chloropyrimidin-4-yl)-1-(tetrahydro-2H-pyran-2-yl)-1H-indazole*** **(14)**: To a solution of 1-(tetrahydro-2*H*-pyran-2-yl)-6-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1*H*-indazole (12.18 mmol, 4 g), 2,4-dichloropyrimidine (18.32 mmol, 2710 mg) and $Pd(PPh_3)_4$ (600 mg) in 1,4-dioxane (30 mL) was added saturated sodium bicarbonate solution (18 mL) and the reaction mixture protecting with argon was stirred at 95 ºC overnight. After cooling, the mixture was extracted with ethyl acetate, washed with saline. The organic phase was collected and the solvent was removed under vacuum. The crude product was purified by silica gel

chromatography (hexane / ethyl acetate 3 : 1) to give yield white solid as product (2.913 g, yield 76.17%).

General procedure for synthesis of ***16a-m***: A mixture of 6-(2-chloropyrimidin-4-yl)-1-(tetrahydro-2*H*-pyran-2-yl)-1*H*-indazole (0.15 mmol, 48 mg), corresponding compound **15a-m** (0.30 mmol), $Pd_2(dba)_3$ (0.025 mmol, 23 mg), RuPhos (0.028 mmol, 13 mg) and Cs_2CO_3 (0.5 mmol, 163 mg) in 1,4-dioxane (5 mL) protecting with argon was heated at 110 ºC overnight. After cooled to room temperature, the reaction mixture was extracted with ethyl acetate, washed with saline. The organic phase was collected and the solvent was removed under vacuum. All the crude product except **16l** was purified by silica gel chromatography (hexane / ethyl acetate 3 : 1). **16l** was purified by silica gel chromatography (hexane / ethyl acetate 1 : 1).

4-(1-(tetrahydro-2H-pyran-2-yl)-1H-indazol-6-yl)-N-p-tolylpyrimidin-2-amine ***(16a)***. 32 mg of **16a** as yellow solid, yield 55%. Purity 98.80%. Mp 218-219 ºC. ^{1}H NMR (500 MHz, DMSO) δ 9.59 (s, 1H), 8.56 (d, *J* = 5.2 Hz, 1H), 8.52 (s, 1H), 8.19 (s, 1H), 7.97 (d, *J* = 8.5 Hz, 1H), 7.91 (d, *J* = 8.5 Hz, 1H), 7.72 (d, *J* = 8.4 Hz, 2H), 7.51 (d, *J* = 5.2 Hz, 1H), 7.13 (d, *J* = 8.3 Hz, 2H), 5.97 (dd, *J* = 9.6, 2.1 Hz, 1H), 3.93 (d, *J* = 11.3 Hz, 1H), 3.78 (td, *J* = 11.4, 4.5 Hz, 1H), 2.47 – 2.41 (m, 1H), 2.27 (s, 3H), 2.10 – 1.99 (m, 2H), 1.78 (dd, *J* = 24.3, 12.3 Hz, 1H), 1.64 – 1.58 (m, 2H), ^{13}C NMR (151 MHz, DMSO) δ:164.13, 160.71, 159.56, 140.00, 138.49, 135.49, 134.03, 130.75, 129.36, 126.02, 121.77, 120.42, 119.71, 109.54, 108.65, 84.69, 67.18, 29.45, 25.28, 22.79, 20.89; MS (ESI-TOF), for $C_{23}H_{23}N_5O$ $[M+H]^+$ calculated 386.20, found

386.17.

4-(1-(tetrahydro-2H-pyran-2-yl)-1H-indazol-6-yl)-N-m-tolylpyrimidin-2-amine ***(16b)***. 40 mg of **16b** as yellow solid, yield 69%. Purity 99.13%. Mp 73-74 ºC. ^{1}H NMR (500 MHz, DMSO) δ 9.63 (s, 1H), 8.58 (d, *J* = 5.2 Hz, 1H), 8.52 (s, 1H), 8.19 (s, 1H), 7.99 (d, *J* = 8.5 Hz, 1H), 7.92 (d, *J* = 8.5 Hz, 1H), 7.69 (d, *J* = 7.2 Hz, 2H), 7.53 (d, *J* = 5.2 Hz, 1H), 7.20 (t, *J* = 8.1 Hz, 1H), 6.80 (d, *J* = 7.4 Hz, 1H), 5.98 (dd, *J* = 9.5, 2.3 Hz, 1H), 3.91 (d, *J* = 11.3 Hz, 1H), 3.83 – 3.73 (m, 1H), 2.48 – 2.41 (m, 1H), 2.32 (s, 3H), 2.10 – 1.98 (m, 2H), 1.82 – 1.72 (m, 1H), 1.64 – 1.55 (m, 2H), ^{13}C NMR (151 MHz, DMSO) δ:164.23, 160.68, 159.54, 140.99, 140.07, 138.02, 135.51, 134.08, 128.79, 126.00, 122.67, 121.79, 120.46, 120.00, 116.67, 109.47, 108.92, 84.39, 67.06, 29.43, 25.28, 22.72, 21.91; MS (ESI-TOF), for $C_{23}H_{23}N_5O$ $[M+H]^+$ calculated 386.20, found 386.15.

4-(1-(tetrahydro-2H-pyran-2-yl)-1H-indazol-6-yl)-N-(pyridin-3-yl)pyrimidin-2-amine ***(16c)***. 34 mg of **16c** as yellow solid, yield 61%. Purity 98.02%. Mp 177-178 ºC. ^{1}H NMR (500 MHz, DMSO) δ 9.92 (s, 1H), 9.00 (d, *J* = 2.4 Hz, 1H), 8.63 (d, *J* = 5.2 Hz, 1H), 8.53 (s, 1H), 8.31 (d, *J* = 8.4 Hz, 1H), 8.19 (s, 2H), 8.02 – 7.96 (m, 1H), 7.92 (d, *J* = 8.4 Hz, 1H), 7.61 (d, *J* = 5.2 Hz, 1H), 7.36 (dd, *J* = 8.3, 4.6 Hz, 1H), 5.98 (dd, *J* = 9.6, 2.2 Hz, 1H), 3.93 (d, *J* = 11.4 Hz, 1H), 3.85 – 3.75 (m, 1H), 2.48 – 2.41 (m, 1H), 2.10 – 1.98 (m, 2H), 1.85 – 1.70 (m, 1H), 1.65 – 1.56 (m, 2H), ^{13}C NMR (151 MHz, DMSO) δ:164.44, 160.49, 159.71, 142.81, 141.44, 140.01, 137.75, 135.23, 134.05, 126.09, 125.92, 123.78, 121.86, 120.42, 109.65, 84.61, 67.13, 29.44, 25.28, 22.73; MS (ESI-TOF), for $C_{21}H_{20}N_6O$ $[M+H]^+$ calculated 373.18, found 373.13.

N-(2-fluorophenyl)-4-(1-(tetrahydro-2H-pyran-2-yl)-1H-indazol-6-yl)pyrimidin-2-ami ne ***(16d)***. 7 mg of **16d** as yellow solid, yield 12%. Purity 96.93%. Mp 92-93 ºC. ^{1}H NMR (500 MHz, DMSO) δ 9.18 (s, 1H), 8.54 (d, *J* = 5.2 Hz, 1H), 8.47 (s, 1H), 8.17 (s, 1H), 7.93 (dd, *J* = 11.9, 4.5 Hz, 1H), 7.89 – 7.84 (m, 2H), 7.54 (d, *J* = 5.2 Hz, 1H), 7.30 – 7.24 (m, 1H), 7.23 – 7.13 (m, 2H), 5.92 (dd, *J* = 9.6, 2.0 Hz, 1H), 3.89 (t, *J* = 11.0 Hz, 1H), 3.75 (td, *J* = 11.2, 3.7 Hz, 1H), 2.47 – 2.39 (m, 1H), 2.06 – 1.96 (m, 2H), 1.81 – 1.71 (m, 1H), 1.63 – 1.55 (m, 2H), ^{13}C NMR (151 MHz, DMSO) δ:164.20, 160.91, 159.65, 156.41, 154.78, 139.98, 135.27, 134.02, 126.04, 125.64, 125.14 , 124.56, 121.72, 120.38, 116.00 , 109.51, 109.15, 84.64, 67.18, 29.43, 25.28, 22.77; MS (ESI-TOF), for $C_{22}H_{20}FN_5O$ $[M+H]^+$ calculated 390.17, found 390.00.

1-(tetrahydro-2H-pyran-2-yl)-6-(2-(isoindolin-2-yl)pyrimidin-4-yl)-1H-indazole ***(16e)***. 40 mg of **16e** as yellow solid, yield 67%. Purity 97.15%. Mp 119-121 ºC. ^{1}H NMR (500 MHz, DMSO) δ 8.68 (d, *J* = 5.2 Hz, 1H), 8.58 (s, 1H), 8.47 (d, *J* = 7.9 Hz, 1H), 8.20 (s, 1H), 8.03 (d, *J* = 8.5 Hz, 1H), 7.94 (d, *J* = 8.5 Hz, 1H), 7.59 (d, *J* = 5.2 Hz, 1H), 7.29 – 7.20 (m, 2H), 6.94 (t, *J* = 7.4 Hz, 1H), 6.04 – 5.98 (m, 1H), 4.30 (t, *J* = 8.6 Hz, 2H), 3.94 (d, *J* = 11.3 Hz, 1H), 3.85 – 3.76 (m, 1H), 3.20 (t, *J* = 8.6 Hz, 2H), 2.44 (dd, *J* = 12.8, 3.5 Hz, 1H), 2.05 (dt, *J* = 16.5, 4.1 Hz, 2H), 1.79 (dt, *J* = 16.3, 11.9 Hz, 1H), 1.60 (dd, *J* = 17.5, 4.7 Hz, 2H), ^{13}C NMR (126 MHz, DMSO) δ:164.08, 159.36, 143.79, 140.02, 135.50, 134.03, 132.90, 127.37, 126.11, 125.30, 121.88, 120.46, 115.25, 109.71, 108.50, 84.71, 67.13, 49.09, 29.43, 27.11, 25.32, 22.71; MS (ESI-TOF), for $C_{24}H_{23}N_5O$ $[M+H]^+$ calculated 398.20, found 398.16.

4-(1-(tetrahydro-2H-pyran-2-yl)-1H-indazol-6-yl)-N-(4-methylthiazol-2-yl)pyrimidin-

*2-amine **(16f)***. 44 mg of **16f** as yellow solid, yield 75%. Purity 98.94%. Mp 212-213 ºC. ^{1}H NMR (600 MHz, DMSO) δ 11.59 (s, 1H), 8.81 – 8.64 (m, 2H), 8.21 (s, 1H), 8.06 (d, *J* = 8.4 Hz, 1H), 7.95 (d, *J* = 8.4 Hz, 1H), 7.70 (d, *J* = 5.2 Hz, 1H), 7.13 (s, 1H), 6.04 – 5.93 (m, 1H), 3.92 (t, *J* = 15.5 Hz, 1H), 3.78 (dd, *J* = 14.7, 10.1 Hz, 1H), 2.35 (d, *J* = 16.2 Hz, 1H), 2.09 – 2.02 (m, 2H), 1.81 – 1.71 (m, 1H), 1.62 (d, *J* = 3.4 Hz, 2H), ^{13}C NMR (151 MHz, DMSO) δ:157.78, 140.03, 134.79, 134.10, 126.22, 121.95, 120.63, 110.15, 84.81, 67.23, 29.36, 25.29, 22.76, 11.74; MS (ESI-TOF), for $C_{20}H_{20}N_6OS$ $[M+H]^+$ calculated 393.15, found 393.09.

*N-(3-fluorophenyl)-4-(1-(tetrahydro-2H-pyran-2-yl)-1H-indazol-6-yl)pyrimidin-2-amine **(16g)***. 32 mg of **16g** as yellow solid, yield 55%. Purity 98.43%. Mp 146-147 ºC. ^{1}H NMR (500 MHz, DMSO) δ 9.97 (s, 1H), 8.63 (d, *J* = 5.2 Hz, 1H), 8.53 (s, 1H), 8.19 (s, 1H), 8.01 – 7.90 (m, 3H), 7.59 (t, *J* = 6.9 Hz, 2H), 7.33 (dd, *J* = 15.4, 8.0 Hz, 1H), 6.78 (td, *J* = 8.4, 2.4 Hz, 1H), 5.97 (dd, *J* = 9.6, 2.2 Hz, 1H), 3.92 (d, *J* = 11.3 Hz, 1H), 3.82 – 3.73 (m, 1H), 2.48 – 2.41 (m, 1H), 2.11 – 1.96 (m, 2H), 1.83 – 1.70 (m, 1H), 1.63 – 1.54 (m, 2H), ^{13}C NMR (151 MHz, DMSO) δ:164.38, 163.67, 162.08, 160.36, 159.63, 142.99, 140.03, 135.31, 134.08, 130.45, 126.07, 121.88, 120.44, 115.07, 109.59, 108.01, 105.84, 105.66, 84.53, 67.11, 29.46, 25.28, 22.76; MS (ESI-TOF), for $C_{22}H_{20}FN_5O$ $[M+H]^+$ calculated 390.17, found 390.69.

*N-(3-chlorophenyl)-4-(1-(tetrahydro-2H-pyran-2-yl)-1H-indazol-6-yl)pyrimidin-2-amine **(16h)***. 27 mg of **16h** as yellow solid, yield 44%. Purity 97.58%. Mp 147-148 ºC. ^{1}H NMR (500 MHz, DMSO) δ 9.96 (s, 1H), 8.62 (d, *J* = 5.2 Hz, 1H), 8.50 (s, 1H), 8.19 (s, 1H), 8.15 (t, *J* = 1.8 Hz, 1H), 8.00 – 7.94 (m, 1H), 7.91 (d, *J* = 8.5 Hz, 1H),

7.75 (dd, *J* = 8.2, 1.3 Hz, 1H), 7.57 (d, *J* = 5.2 Hz, 1H), 7.33 (t, *J* = 8.1 Hz, 1H), 7.01 (dd, *J* = 7.8, 1.6 Hz, 1H), 5.97 (dd, *J* = 9.5, 2.1 Hz, 1H), 3.90 (d, *J* = 11.3 Hz, 1H), 3.77 (td, *J* = 11.6, 6.9 Hz, 1H), 2.48 – 2.41 (m, 1H), 2.08 – 1.95 (m, 2H), 1.80 – 1.69 (m, 1H), 1.58 (t, *J* = 6.3 Hz, 2H), ^{13}C NMR (151 MHz, DMSO) δ:164.38, 160.32, 159.59, 142.68, 140.05, 135.30, 134.09, 133.48, 130.54, 126.06, 121.83, 121.30, 120.45, 118.53, 117.66, 109.66, 109.45, 84.34, 67.06, 29.44, 25.28, 22.72; MS (ESI-TOF), for $C_{22}H_{20}ClN_5O$ $[M+H]^+$ calculated 406.14, found 406.14.

*4-(1-(tetrahydro-2H-pyran-2-yl)-1H-indazol-6-yl)-N-methyl-N-phenylpyrimidin-2-amine **(16i)***. 21 mg of **16i** as yellow solid, yield 35%. Purity 96.67%. Mp 57-58 ºC. ^{1}H NMR (500 MHz, DMSO) δ 8.48 (d, *J* = 5.2 Hz, 1H), 8.40 (s, 1H), 8.15 (s, 1H), 7.89 (dd, *J* = 8.5, 1.0 Hz, 1H), 7.83 (d, *J* = 8.5 Hz, 1H), 7.47 – 7.41 (m, 5H), 7.24 (ddd, *J* = 10.3, 5.3, 3.3 Hz, 1H), 5.86 (dd, *J* = 9.8, 2.3 Hz, 1H), 3.89 (d, *J* = 11.4 Hz, 1H), 3.73 (td, *J* = 11.0, 3.3 Hz, 1H), 3.58 (s, 3H), 2.40 (qd, *J* = 13.1, 4.0 Hz, 1H), 2.08 – 1.93 (m, 2H), 1.81 – 1.69 (m, 1H), 1.65 – 1.50 (m, 2H), ^{13}C NMR (151 MHz, DMSO) δ:163.50, 161.86, 159.28, 145.83, 139.97, 135.43, 134.01, 129.12, 126.81, 125.99, 125.56, 121.62, 120.28, 109.39, 107.61, 84.63, 67.23, 38.52, 29.43, 25.27, 22.82; MS (ESI-TOF), for $C_{23}H_{23}N_5O$ $[M+H]^+$ calculated 386.20, found 386.04.

*4-(1-(tetrahydro-2H-pyran-2-yl)-1H-indazol-6-yl)-N-(pyrimidin-2-yl)pyrimidin-2-amine **(16j)***. 40 mg of **16j** as yellow solid, yield 71%. Purity 96.49%. Mp 121-123 ºC. ^{1}H NMR (500 MHz, DMSO) δ 10.36 (s, 1H), 8.77 – 8.55 (m, 3H), 8.19 (s, 1H), 8.04 (d, *J* = 8.3 Hz, 1H), 7.91 (d, *J* = 8.5 Hz, 1H), 7.24 (dd, *J* = 14.6, 7.3 Hz, 1H), 7.19 – 7.08 (m, 1H), 6.73 (d, *J* = 5.4 Hz, 1H), 5.96 (d, *J* = 8.5 Hz, 1H), 3.90 (t, *J* = 15.3 Hz,

1H), 3.77 (t, *J* = 12.5 Hz, 1H), 2.44 (dd, *J* = 18.0, 9.1 Hz, 1H), 2.06 – 1.96 (m, 2H), 1.83 – 1.73 (m, 1H), 1.64 – 1.56 (m, 2H), ^{13}C NMR (151 MHz, DMSO) δ:160.33 – 160.06, 159.17, 157.12, 140.04, 134.09, 129.37, 128.67, 126.14, 125.78, 121.76, 120.55, 115.73, 110.61, 109.24 – 108.53, 84.56, 67.18, 29.43, 25.28, 22.77; MS (ESI-TOF), for $C_{20}H_{19}N_7O$ $[M+H]^+$ calculated 374.17, found 374.15.

*4-(1-(tetrahydro-2H-pyran-2-yl)-1H-indazol-6-yl)-N-(5-methylpyridin-2-yl)pyrimidin-2-amine **(16k)***. 30 mg of **16k** as white solid, yield 52%. Purity 99.24%. Mp 165-166 ºC. ^{1}H NMR (500 MHz, DMSO) δ 9.78 (s, 1H), 8.64 (d, *J* = 5.2 Hz, 1H), 8.56 (s, 1H), 8.27 (d, *J* = 8.5 Hz, 1H), 8.21 – 8.15 (m, 2H), 8.00 (d, *J* = 8.5 Hz, 1H), 7.92 (d, *J* = 8.5 Hz, 1H), 7.66 – 7.60 (m, 2H), 5.98 (dd, *J* = 9.7, 2.1 Hz, 1H), 3.93 (d, *J* = 11.3 Hz, 1H), 3.84 – 3.74 (m, 1H), 2.48 – 2.41 (m, 1H), 2.26 (s, 3H), 2.09 – 1.99 (m, 2H), 1.79 (ddd, *J* = 18.2, 14.2, 6.6 Hz, 1H), 1.65 – 1.56 (m, 2H), ^{13}C NMR (151 MHz, DMSO) δ:164.23, 159.88, 159.63, 151.41, 148.12, 140.01, 138.58, 135.16, 134.06, 126.77, 126.11, 121.84, 120.43, 113.06, 109.72, 84.67, 67.18, 29.45, 25.29, 22.78, 17.72; MS (ESI-TOF), for $C_{22}H_{22}N_6O$ $[M+H]^+$ calculated 387.19, found 387.17.

*N^2-(4-(1-(tetrahydro-2H-pyran-2-yl)-1H-indazol-6-yl)pyrimidin-2-yl)pyridine-2,4-diamine **(16l)***. 40 mg of **16l** as brown liquid, yield 68%. Purity 98.96%. ^{1}H NMR (500 MHz, DMSO) δ 9.39 (s, 1H), 8.58 – 8.50 (m, 2H), 8.19 (s, 1H), 7.98 (dd, J = 8.5, 0.9 Hz,1H), 7.90 (d, J = 8.5 Hz, 1H), 7.48 (d, J = 5.2 Hz, 1H), 7.15 (d, J = 1.8 Hz, 1H), 7.00 (d, J = 8.2 Hz, 1H),6.95 (t, J = 7.9 Hz, 1H), 6.64 (t, J = 7.8 Hz, 2H), 6.23 (d, J = 7.8 Hz, 1H), 6.00 (dd, J = 9.5, 2.3 Hz, 1H),4.97 (d, J = 32.6 Hz, 2H), 3.89 (d, J = 11.5 Hz, 1H), 3.84 – 3.76 (m, 1H), 2.44 (dd, J = 12.6, 3.6 Hz, 1H),2.07 – 1.96 (m, 2H),

1.78 (td, J = 7.8, 3.1 Hz, 1H), 1.60 (dd, J = 10.0, 6.4 Hz, 2H), ^{13}C NMR (151 MHz, DMSO) δ 164.01, 160.79, 159.47, 149.35, 141.55, 140.09, 135.47, 134.05, 129.17, 125.96, 121.74, 120.49, 109.48, 108.50 (d, J = 11.8 Hz), 108.02, 105.50, 84.33, 67.03, 29.43, 25.31, 22.72; MS (ESI-TOF), for $C_{22}H_{22}N_6O$ $[M+H]^+$ calculated 387.19, found 387.19.

4-(1-(tetrahydro-2H-pyran-2-yl)-1H-indazol-6-yl)-N-(thiazol-2-yl)pyrimidin-2-amine ***(16m)***. 37 mg of **16m** as yellow solid, yield 65%. Purity 96.74%. Mp 206-208 ºC. ^{1}H NMR (600 MHz, DMSO) δ 11.83 (s, 1H), 8.76 (d, *J* = 16.8 Hz, 1H), 8.22 (s, 1H), 8.11 (d, *J* = 7.0 Hz, 1H), 7.97 (d, *J* = 7.4 Hz, 1H), 7.75 (d, *J* = 17.5 Hz, 1H), 7.48 (d, *J* = 18.5 Hz, 1H), 7.21 (s, 1H), 6.65 (d, *J* = 7.1 Hz, 1H), 5.99 (d, *J* = 8.5 Hz, 1H), 3.99 (s, 1H), 3.82 (s, 1H), 2.12 (t, *J* = 33.2 Hz, 3H), 1.78 (d, *J* = 33.5 Hz, 1H), 1.54 (d, *J* = 53.1 Hz, 2H), ^{13}C NMR (151 MHz, DMSO) δ:160.04, 157.79, 156.62, 139.92, 138.60, 134.68, 133.99, 126.31, 121.94, 120.62, 112.61, 110.35, 107.59, 85.19, 67.39, 29.47, 25.33, 22.21; MS (ESI-TOF), for $C_{19}H_{18}N_6OS$ $[M+H]^+$ calculated 379.13, found 379.07.

General procedure for synthesis of ***6a-m***: **16a-m** (0.45 mmol) was dissolved in ethanol (5 mL) in a 25 mL round-bottom flask. Then, 4 mol/L HCl in 1,4-dioxane (0.3 mL) was added to the mixture and the mixture was heated at 70 ºC for 2 h. After cooled to room temperature, $NaHCO_3$ solution was added to the reaction mixture until pH = 8. The mixture was extracted with ethyl acetate, washed with saline. The organic phase was collected and the solvent was removed under vacuum. The crude product was purified by silica gel chromatography.

4-(1H-indazol-6-yl)-N-p-tolylpyrimidin-2-amine ***(6a)***.

The crude product was purified by silica gel chromatography (cyclohexane / ethyl acetate 2 : 1).

88 mg of **6a** as yellow solid, yield 65%. Purity 96.63%. Mp 222-224 ºC. ^{1}H NMR (400 MHz, DMSO) δ:13.33 (s, 1H), 8.53 (d, *J* = 5.2 Hz, 1H), 8.35 (s, 1H), 8.14 (s, 1H), 7.89 (s, 2H), 7.72 (d, *J* = 8.4 Hz, 2H), 7.65-7.58 (m, 2H), 7.57-7.51 (m, 1H), 7.74 (d, *J* = 5.2 Hz,1H), 7.13 (d, *J* = 8.3 Hz, 2H), 2.27 (s,3H), ^{13}C-NMR (101 MHz, DMSO) δ 109.89, 110.66, 120.94, 122.72, 126.10, 130.52, 130.64, 130.75, 132.01, 133.27, 133.37, 133.84, 135.43, 136.46, 139.92, 141.98, 160.81, 162.09, 165.77; MS (ESI-TOF), for $C_{18}H_{15}N_5$ [M] calculated 301.13, found 301.25.

4-(1H-indazol-6-yl)-N-m-tolylpyrimidin-2-amine ***(6b)***.

The crude product was purified by silica gel chromatography (cyclohexane / ethyl acetate 2 : 1).

102 mg of **6b** as yellow solid, yield 75%. Purity 98.64%. Mp 191-192 ºC. ^{1}H NMR (500 MHz, DMSO) δ:13.38 (s, 1H), 9.61 (s, 1H), 8.55 (d, *J* = 5.2 Hz, 1H), 8.36 (s, 1H), 8.15 (s, 1H), 7.90 (s, 2H), 7.70 (d, *J* = 8.1 Hz, 1H), 7.47 (d, *J* = 5.2 Hz, 1H), 7.19 (t, *J* = 7.8 Hz, 1H), 6.79 (d, *J* = 7.4 Hz, 1H), 2.32 (s, 3H), 1.38 (s, 2H); ^{13}C NMR (126 MHz, DMSO) δ 164.38, 160.68, 159.49, 141.04, 140.59, 138.05, 135.06, 134.09, 128.82, 124.77, 122.61, 121.39, 119.95, 119.60, 116.63, 109.29, 108.74, 21.92; MS (ESI-TOF), for $C_{18}H_{15}N_5$ [M] calculated 301.13, found 301.74.

4-(1H-indazol-6-yl)-N-(pyridin-3-yl)pyrimidin-2-amine ***(6c)***.

The crude product was purified by silica gel chromatography (hexane / ethyl acetate

1 : 1).

109 mg of **6c** as white solid, yield 52.43%. Purity 97.38%. Mp 239-240 ºC. ^{1}H NMR (500 MHz, DMSO) δ:13.39 (s, 1H), 9.90 (s, 1H), 9.02 (d, *J* = 2.5 Hz, 1H), 8.59 (d, *J* = 5.2 Hz, 1H), 8.36 (s, 1H), 8.27 (ddd, *J* = 8.4, 2.5, 1.5 Hz, 1H), 8.19 (dd, *J* = 4.6, 1.3 Hz, 1H), 8.16 (s, 1H), 7.92–7.87 (m, 2H), 7.55 (d,J = 5.2 Hz, 1H), 7.35 (dd, *J* = 8.3, 4.6 Hz, 1H), ^{13}C-NMR (DMSO, 125.67 MHz): 109.40, 109.53, 119.61, 121.48, 123.83, 124.82, 125.86, 134.10, 134.32, 137.30, 140.57, 141.29, 142.73, 159.64, 160.49, 164.67; MS (ESI-TOF), for $C_{16}H_{12}N_6$ $[M+H]^+$ calculated 289.12, found 289.12.

N-(2-fluorophenyl)-4-(1H-indazol-6-yl)pyrimidin-2-amine ***(6d)***.

The crude product was purified by silica gel chromatography (cyclohexane / ethyl acetate 3 : 1).

96 mg of **6d** as yellow solid, yield 70%. Purity 96.28%. Mp 154-155 ºC. ^{1}H NMR (500 MHz, DMSO) δ:13.34 (s, 1H), 9.16 (s, 1H), 8.50 (d, *J* = 5.2 Hz, 1H), 8.30 (s, 1H), 8.13 (s, 1H), 7.89-7.80 (m, 3H), 7.48 (d, *J* = 5.2 Hz, 1H), 7.26 (ddd, *J* = 11.0, 8.0, 1.5 Hz, 1H), 7.20 (td, *J* = 7.6, 1.5 Hz, 1H), 7.18-7.13 (m, 1H), ^{13}C-NMR (DMSO, 125.67 MHz): 109.05, 109.31, 115.95, 116.11, 121.33, 124.60, 124.62, 124.75, 125.17, 125.66, 134.06, 134.88, 140.56, 154.63, 156.58, 159.57, 160.95, 164.47; MS (ESI-TOF), for $C_{17}H_{12}FN_5$ [M] calculated 305.11, found 305.87.

6-(2-(isoindolin-2-yl)pyrimidin-4-yl)-1H-indazole ***(6e)***.

The crude product was purified by silica gel chromatography (hexane / ethyl acetate 3 : 1).

92 mg of **6e** as yellow solid, yield 65%. Purity 95.72%. Mp 96-98 ºC. ^{1}H NMR (500 MHz, DMSO) δ:13.34 (s, 1H), 8.65 (d, *J* = 5.2 Hz, 1H), 8.44 (d, *J* = 8.0 Hz, 1H), 8.39(s, 1H), 8.16(s, 1H), 7.93(p, J=8.5Hz, 2H), 7.53(d, J=5.2Hz, 1H), 7.27-7.20(m, 2H), 6.93(t, *J* = 7.3 Hz, 1H), 4.30 (t, *J* = 8.7 Hz, 2H), 3.20 (t, *J* = 8.6 Hz, 2H), 1.38 (s, 1H), ^{13}C-NMR (DMSO, 125.67 MHz): 27.09, 49.12, 108.43, 109.43, 115.23, 119.67, 121.50, 121.82, 124.81, 125.30, 127.46, 132.85, 134.10, 135.13, 140.64, 143.82, 159.31, 159.38, 164.32; MS (ESI-TOF), for $C_{19}H_{15}N_5$ $[M+H]^+$ calculated 314.14, found 314.83.

4-(1H-indazol-6-yl)-N-(4-methylthiazol-2-yl)pyrimidin-2-amine ***(6f)***.

The crude product was purified by silica gel chromatography (dichloromethane / methanol 10 : 1).

128 mg of **6f** as yellow solid, yield 92%. Purity 97.68%. Mp > 300 ºC. ^{1}H NMR (500 MHz, DMSO) δ:13.52 (s, 1H), 11.54 (s, 1H), 10.34 (s, 2H), 8.66 (d, *J* = 5.2 Hz, 1H), 8.53 (s, 1H), 8.16 (s, 1H), 7.98 (dd, *J* = 8.5, 1.2 Hz, 1H), 7.93 (d, *J* = 8.5 Hz, 1H), 7.64 (d, *J* = 5.2 Hz, 1H), 7.12(d, *J* = 1.2 Hz, 1H), 2.40 (d, *J* = 0.9 Hz, 3H), ^{13}C-NMR (DMSO, 125.67 MHz): 11.69, 45.73, 110.07, 119.76, 121.49, 124.86, 125.40, 134.00, 134.34, 135.45, 157.76, 158.29, 159.71, 164.66; MS (ESI-TOF), for $C_{15}H_{12}N_6S$ $[M+H]^+$ calculated 309.09, found 308.93.

N-(3-fluorophenyl)-4-(1H-indazol-6-yl)pyrimidin-2-amine ***(6g)***.

The crude product was purified by silica gel chromatography (cyclohexane / ethyl acetate 2 : 1).

99 mg of **6g** as white solid, yield 72%. Purity 98.41%. Mp 244-245 ºC. ^{1}H NMR (400

MHz, DMSO) δ:13.38 (s, 1H), 9.93 (s, 1H), 8.60 (d, *J* = 5.2 Hz, 1H), 8.35 (s, 1H), 8.16 (s, 1H), 7.95–7.85 (m, 3H), 7.60 (d, *J* = 8.2 Hz, 1H), 7.53 (d, *J* = 5.2 Hz, 1H), 7.33 (dd, *J* = 15.4, 8.0 Hz, 1H), 6.77 (td, *J* = 8.4, 2.4 Hz, 1H), ^{13}C-NMR (DMSO, 100.61 MHz): 106.94, 107.20, 119.21, 110.78, 110.89, 116.38, 120.98, 122.82, 126.18, 131.87, 135.46, 136.27, 141.95, 144.35, 144.47, 160.84, 161.75, 163.03, 165.42, 166.07; MS (ESI-TOF), for $C_{17}H_{12}FN_5$ $[M+H]^+$ calculated 306.12, found 306.83.

N-(3-chlorophenyl)-4-(1H-indazol-6-yl)pyrimidin-2-amine ***(6h)***.

The crude product was purified by silica gel chromatography (cyclohexane / ethyl acetate 3 : 1).

110 mg of **6h** as white solid, yield 76%. Purity 97.34%. Mp 240-241 ºC. ^{1}H NMR (400 MHz, DMSO) δ:13.38 (s, 1H), 9.92 (s, 1H), 8.60 (d, *J* = 5.2 Hz, 1H), 8.35 (s, 1H), 8.13 (d, *J* = 25.2 Hz, 2H), 7.90 (s, 2H), 7.77 (d, *J* = 8.1 Hz, 1H), 7.54 (d, *J* = 5.2 Hz, 1H), 7.33 (t, *J* = 8.1 Hz, 1H), 7.00 (d, *J* = 7.9 Hz, 1H), ^{13}C-NMR (DMSO, 100.61 MHz): 110.81, 110.92, 118.95, 119.86, 120.98, 122.62, 122.81, 126.19, 131.94, 134.86, 135.46, 136.23, 141.95, 144.09, 160.85, 161.71, 166.06; MS (ESI-TOF), for $C_{17}H_{12}ClN_5$ $[M+H]^+$ calculated 322.79, found 322.05.

4-(1H-indazol-6-yl)-N-methyl-N-phenylpyrimidin-2-amine ***(6i)***.

The crude product was purified by silica gel chromatography (hexane / ethyl acetate 3 : 1).

84 mg of **6i** as white solid, yield 62%. Purity 96.73%. Mp 168−169 ºC. ^{1}H NMR (500 MHz, DMSO) δ:13.29 (s, 1H), 8.44 (d, *J* = 5.2 Hz, 1H), 8.25 (s, 1H), 8.12 (s, 1H), 7.86–7.79 (m, 2H), 7.44–7.39 (m, 5H), 7.25–7.20 (m, 1H), 3.58 (s, 3H), 1.38 (s, 1H),

^{13}C-NMR (DMSO, 125.67 MHz): 26.81, 38.65, 107.60, 109.17, 119.53, 121.26, 124.71, 125.57, 126.77, 129.16, 134.03, 135.11, 140.56, 145.87, 159.20, 161.88, 163.88; MS (ESI-TOF), for $C_{18}H_{15}N_5$ $[M+H]^+$ calculated 302.14, found 302.13.

4-(1H-indazol-6-yl)-N-(pyrimidin-2-yl)pyrimidin-2-amine ***(6j)***.

The crude product was purified by silica gel chromatography (dichloromethane / methanol 10 : 1).

83 mg of **6j** as white solid, yield 64%. Purity 97.13%. Mp 273-274 ºC. ^{1}H NMR (400 MHz, DMSO) δ:13.34 (s, 1H), 10.29 (s, 1H), 8.65 (d, *J* = 5.2 Hz, 1H), 8.62 (d, *J* = 4.8 Hz, 2H), 8.42 (s, 1H), 8.14 (s, 1H), 7.91 (dt, *J* = 20.9, 4.7 Hz, 2H), 7.68 (d, *J* = 5.3 Hz, 1H), 7.07 (t, *J* = 4.8 Hz, 1H), ^{13}C-NMR (DMSO, 100.61 MHz): 111.01, 112.62, 116.94, 121.05, 122.69, 126.18, 135.44, 136.04, 141.99, 142.20, 142.49, 159.93, 160.88, 160.95, 161.01, 165.82; MS (ESI-TOF), for $C_{15}H_{11}N_7$ $[M+H]^+$ calculated 290.12, found 290.11.

4-(1H-indazol-6-yl)-N-(5-methylpyridin-2-yl)pyrimidin-2-amine ***(6k)***.

The crude product was purified by silica gel chromatography (cyclohexane / ethyl acetate 3 : 1).

88 mg of **6k** as white solid, yield 65%. Purity 96.48%. Mp > 300 ºC. ^{1}H NMR (500 MHz, DMSO) δ:13.52 (s, 1H), 9.85 (s, 1H), 8.61 (d, *J* = 5.2 Hz, 1H), 8.39 (s, 1H), 8.25 (d, *J* = 8.5 Hz, 1H), 8.17–8.09 (m, 2H), 7.90 (d, *J* = 0.8 Hz, 2H), 7.66 (dd, *J* = 8.5, 2.1 Hz, 1H), 7.57 (d, *J* = 5.2 Hz, 1H), 3.15 (s, 2H), 2.25 (s, 3H), 1.20 (s, 1H), ^{13}C-NMR (DMSO, 125.67 MHz): 17.71, 109.59, 109.73, 113.07, 119.59, 121.43, 124.80, 126.71, 134.00, 134.67, 138.94, 140.60, 147.74, 151.33, 159.50, 159.82,

164.59; MS (ESI-TOF), for $C_{17}H_{14}N_6$ $[M+H]^+$ calculated 303.14, found 303.10.

N^2-(4-(1H-indazol-6-yl)pyrimidin-2-yl)pyridine-2,4-diamine ***(6l)***.

The crude product was purified by silica gel chromatography (hexane / ethyl acetate 1 : 1).

85 mg of **6l** as white solid, yield 62%. Purity 98.15%. Mp > 300 ºC. ^{1}H NMR (500 MHz, DMSO) δ:13.35 (d, *J* = 10.3 Hz, 1H), 9.38 (s, 1H), 8.51 (d, *J* = 5.2 Hz, 1H), 8.36 (s, 1H), 8.15 (s, 1H), 7.89 (s, 2H), 7.42 (d, *J* = 5.2Hz, 1H), 7.12 (d,J = 1.7 Hz, 1H), 7.00 (d,J = 8.2 Hz, 1H), 6.94 (t, *J* = 7.9 Hz, 1H), 6.24–6.20 (m, 1H), 4.97 (s, 2H), 2.09 (d, *J* = 16.3 Hz, 4H), 1.79 (s, 1H), 1.38 (s, 3H), ^{13}C-NMR (DMSO, 100.61 MHz): 28.18, 32.51, 106.68, 109.36, 109.74, 109.84, 110.68, 121.03, 122.70, 126.09, 126.44, 130.57, 135.42, 136.49, 141.99, 143.01, 150.64, 160.69, 162.19, 165.72, 165.91; MS (ESI-TOF), for $C_{17}H_{14}N_6$ [M] calculated 302.12, found 302.97.

4-(1H-indazol-6-yl)-N-(thiazol-2-yl)pyrimidin-2-amine ***(6m)***.

The crude product was purified by silica gel chromatography (dichloromethane / methanol 10 : 1).

112 mg of **6m** as white solid, yield 85%. Purity 95.42%. Mp > 300 ºC. ^{1}H NMR (500 MHz, DMSO) δ:8.71 (d, *J* = 5.4 Hz, 1H), 8.54 (s, 1H), 8.18 (s, 1H), 8.03 (d, *J* = 8.5 Hz, 1H), 7.95 (d, *J* = 8.5 Hz, 1H), 7.73 (d, *J* = 5.4 Hz, 1H), 7.51 (d, *J* = 3.7 Hz, 1H), 7.22 (d, *J* = 3.7 Hz, 1H), ^{13}C-NMR (DMSO, 125.67 MHz): 110.22, 110.42, 112.83, 119.950, 121.62, 125.04, 134.04, 134.09, 140.65, 157.33; MS (ESI-TOF), for $C_{14}H_{10}N_6S$ $[M+H]^+$ calculated 295.08, found 295.03.

Synthesis of ***5-bromo-3-(phenylamino)pyrazin-2-ol*** **(19)**: A mixture of

3,5-dibromo-2-hydroxypyrazine (0.2 mmol, 50 mg), aniline (0.2 mmol, 20 mL), and camphorsulfonic acid (25 mg) in isopropanol (50 mL) was heated at 82 ºC for 48 h. After cooled to room temperature, the solvent was removed under vacuum. The crude product was purified by silica gel chromatography (hexane / ethyl acetate 1 : 1) to give 5-bromo-3-(phenylamino)pyrazin-2-ol (**19**). 34 mg of **19** as yellow solid, yield 63%. Purity 96.25%. Mp > 300 ºC. ^{1}H NMR (500 MHz, DMSO) δ 12.15 (s, 1H), 9.34 (s, 1H), 7.89 (d, J = 7.9 Hz, 2H), 7.31 (t, J = 7.9 Hz, 2H), 7.03 (t, J = 7.4 Hz, 1H), 6.98 (s, 1H). ^{13}C NMR (126 MHz, DMSO) δ 151.05, 148.19, 139.45, 129.04, 123.42, 120.03, 115.59, 112.15.

Synthesis of **7a**: To a solution of 1-(tetrahydro-2*H*-pyran-2-yl)-6-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)-1*H*-indazole (0.3 mmol, 98 mg), 5-bromo-3-(phenylamino)pyrazin-2-ol (0.3 mmol, 83 mg) and $Pd(PPh_3)_4$ (0.01 mmol, 12 mg) in 1,4-dioxane (30 mL) was added saturated sodium bicarbonate solution (18 mL) and the reaction mixture was refluxed at 110 ºC for 16 h under argon atmosphere. After cooling, the mixture was extracted with ethyl acetate,washed with saline. The organic phase was collected and the solvent was removed under vacuum. Ethanol (5 mL) and 4 mol/L HCl in 1,4-dioxane (0.3 mL) were added to the mixture and the mixture was heated at 70 ºC for 2 h. After cooled to room temperature, $NaHCO_3$ solution was added to the reation mixture until pH = 8. The mixture was extracted with ethyl acetate, washed with saline. The organic phase was collected and the solvent was removed under vacuum. The crude product was purified by silica gel chromatography (hexane / ethyl acetate 1 : 1) to afford

5-(1*H*-indazol-6-yl)-3-(phenylamino)pyrazin-2-ol. *5-(1H-indazol-6-yl)-3-(phenyl amino)pyrazin-2(1H)-one* ***(7a)***. 44 mg of **7a** as yellow solid, yield 44%. Purity 94.21%. Mp > 300 ºC. ^{1}H NMR (500 MHz, DMSO) δ 13.07 (s, 1H), 12.21 (s, 1H), 9.22 (s, 1H), 8.13 (d, *J* = 7.9 Hz, 2H), 8.02 (d, *J* = 4.2 Hz, 2H), 7.75 (d, *J* = 8.5 Hz, 1H), 7.61 (dd, *J* = 8.6, 0.9 Hz, 1H), 7.49 (s, 1H), 7.37 (t, *J* = 7.9 Hz, 2H), 7.05 (t, *J* = 7.3 Hz, 1H), ^{13}C NMR (126 MHz, DMSO) δ 151.38, 148.07, 141.07, 140.35, 135.37, 133.85, 130.06, 129.04, 122.66, 122.44, 120.99, 119.77, 118.19, 113.01, 106.09; MS (ESI-TOF), for $C_{17}H_{13}N_5O$ $[M+H]^+$ calculated 304.12, found 304.08.

Cell lines and reagents

The human nasopharyngeal carcinoma cell line SUNE1 and canine kidney cell line MDCK were purchased from China Center for Type Culture Collection. SUNE1 cells were cultured in RPMI-1640 medium (Thermo Fisher Scientific, Waltham, USA) supplemented with 10% fetal bovine serum (PAN-Biotech GmbH, Germany). MDCK cells were cultured in DMEM-high glucose (Thermo Fisher Scientific, Waltham, USA) supplemented with 10% fetal bovine serum (PAN-Biotech GmbH, Germany). The cells were maintained in a humidified atmosphere with 5% CO_2 at 37 °C. The Cell Counting Kit 8 was purchased from Dojindo Laboratories. Annexin-V and PI staining for apoptosis analysis were purchased from Keygen Company. The RNase and PI staining for analysis of cell cycle distribution were purchased from Tiangen Company and MP Biomedicals, respectively.

Antiproliferative activity

The cytotoxicity of the compounds were tested against the human nasopharyngeal carcinoma cell line SUNE1. For the cytotoxic assay, cells were plated in 96-well plates (Corning， USA) at the concentration of 3×10^4 cells/well and after 24 h the composts were analyzed in six different concentrations diluted in RPMI-1640 medium: 100 μM, 50 μM, 25 μM, 12.5 μM, 6.25 μM, 3.125 μM. Cisplatin used as a positive control. Control group was treated under the same conditions of the experimental groups.

The growth of tumor cells was quantified by Cell Counting Kit 8 (Dojindo Laboratories, Japan). At the end of 48 h of incubation with the compounds, 10 μL CCK8 was added to each well. The cell survival was identified by quantifying the color intensity by absorbance measurement at the wavelength of 450 nm in a microplate spectrophotometer (EL808, Bio-Tek, USA). All experiments were performed at least three times. The IC_{50} was calculated by CalcuSyn2.0 software.

Cytotoxicity assay

The cytotoxicity of compounds for MDCK cells was measured using the Cell Counting Kit 8. Briefly, 100 μL of MDCK cells (3×10^3 cells/well) was seeded in a 96-well cell culture plate. The composts were analyzed in six different concentrations diluted in DMEM medium: 200 μM, 100 μM, 50 μM, 25 μM, 12.5 μM, 6.25 μM. At the end of 48 h of incubation with the compounds, 10 μL CCK8 was added to each well. After incubation at 37 °C for 4 h，the absorbance at 450 nm was measured with a microplate spectrophotometer (EL808, Bio-Tek, USA). The CC_{50} (the concentration of an agent causing 50% cytotoxicity) values were calculated using the Calcusyn2.0

software.

Dynamic observation of cell morphology

Dynamic changes of cell morphology after compound **6l** administration were observed by YeeSpec Automatic microscopic imaging system (YeeSpec Photoelectric Technology, Guangdong, China). The SUNE1 cells were placed under the microscope when **6l** compound was added at the concentration of 25 μM. Photographs of cell morphology were taken every 30 minutes for 72 h.

Measurements via xCELLigence system

The xCelligence system, co-developed by Roche Diagnostics and ACEA Biosciences, was widespread use in cytotoxicity assessment or pharmaceutical screening. The impedance measurement is recorded in real time and expressed as cell index (*CI*), a dimensional parameter that is indicative of cellular adherence, cell number, and morphology. Thus, kinetic profiles generated by the xCELLigence system can be used to monitor proliferation, adherence, viability, spreading, function of cell surface receptors. The xCELLigence system was operated according to the instructions in the user's manual. Briefly, SUNE1 cells (5×10^3 cells/well) were seeded into 96-well E-plates. Approximately 24 h later, when the cells were in the log growth phase, different concentrations of **6l** were added to the culture medium and CI was continuously recorded.

Flow-cytometry analysis of apoptotic cells

The apoptotic cells induced by compound **6l** were determined by FACSCalibur Flow Cytometer (BD Biosciences, USA). SUNE1 and MDCK cells were treated with

compound **6l** and collected after trypsinizing. The cells at 1 × 10^6 cells/mL were incubated with Annexin-V and *PI* staining (Keygen, Nanjing, China) for 10 min in the dark at 37 °C. The cells in binding buffer were regulated to 500 μL and calculated using a FACSCalibur Flow Cytometer (BD Biosciences, USA). Then the data were analyzed by using FlowJo 10.0 software.

Estimation of Mitochondrial membrane potential (MMP)

The *MMP* was analyzed using the fluorescent dye, Rhodamine123. A decrease in the fluorescence of Rhodamine123 indicates a loss of *MMP*. SUNE1 cells were seeded on glass coverslips and treated with different concentrations of **6l**. Twenty-four hours after incubation, the cells were fixed with rhodamine 123 for 30 min, the mean fluorescence intensities of the granulosa cells were determined using FACSCalibur Flow Cytometer (BD Biosciences, USA).

Flow cytometric analysis of cell cycle distribution

After incubation with compound **6l** for 48 h, cells were harvested and washed with cold PBS for two times. Then, the cells were fixed with 80% ice-cold ethanol at 4 °C overnight. The fixed cells were washed twice with cold *PBS* and incubated RNase (0.5 μg/uL, Tiangen, Beijing, China) for 20 min. Then the cells were stained with *PI* staining (MP Biomedicals, USA) for 30 min in the dark at 37 °C. Ten thousand events were collected for each sample and analyzed by FACSCalibur Flow Cytometer (BD Biosciences, USA).

Anti-tumor study in vivo

All animal handling and procedures complied with the ARRIVE guidelines and

were carried out in accordance with the U.K. Animals (Scientific Procedures) Act 1986 and associated guidelines. The anti-tumor study in vivo was approved by the Animal Care and Use Committee of Southern Medical University. Female BALB/c nude mice at the age of 6-week-old (Sebiona Biological Technology Co., Ltd, Guangzhou, China) were inoculated subcutaneously with 5×10^6 SUNE1 cells. When an average tumor volume reached 100-300 mm^3, the mice were randomly divided into two groups (n = 7 per group), treatment group and vehicle control group. Mice in treatment group and vehicle group were injected intraperitoneally with **6l** (dose: 10 mg/kg/day, dissolved in normal saline) and normal saline of the same quantity (approximately 0.2 mL) for 14 days respectively. Tumor diameter and body weight were measured every 2 days. At the end of the experiment, the mice were sacrificed and their tumors were harvested for recording weight.[28-33] Tumor volume was calculated by the formula: tumor volume = $0.52 \times \text{length} \times \text{width}^2$ (Length and width are the largest diameter and the smallest diameter of tumor respectively).[34] Tumor growth inhibition was calculated by the formula: tumor growth inhibition (TGI%) = $[1- (T_t - T_0)/(C_t - C_0) \times 100$, T_0 and T_t are the average tumor volumes for treatment group on day 0 and day t, respectively; C_0 and C_t are the average tumor volumes for vehicle group on day 0 and day t, respectively.[35] The data were processed and analyzed by IBM SPSS Statistics Version 20.

Declarations of interest: none.

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Author Contribution Statement

Jin Zhou, Peiquan Zhang and Xin Zhang performed chemical experiments, Bohong Liao, Zike Yang, Xiaowen Guo, Lingrong Peng, Huiting Mo and Jialan Zhao performed biological experiments, Zhibo Zhu concived and designed the experiments, Bohong Liao wrote the article.

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10.1002/cbdv.201800598

Graphical Abstract

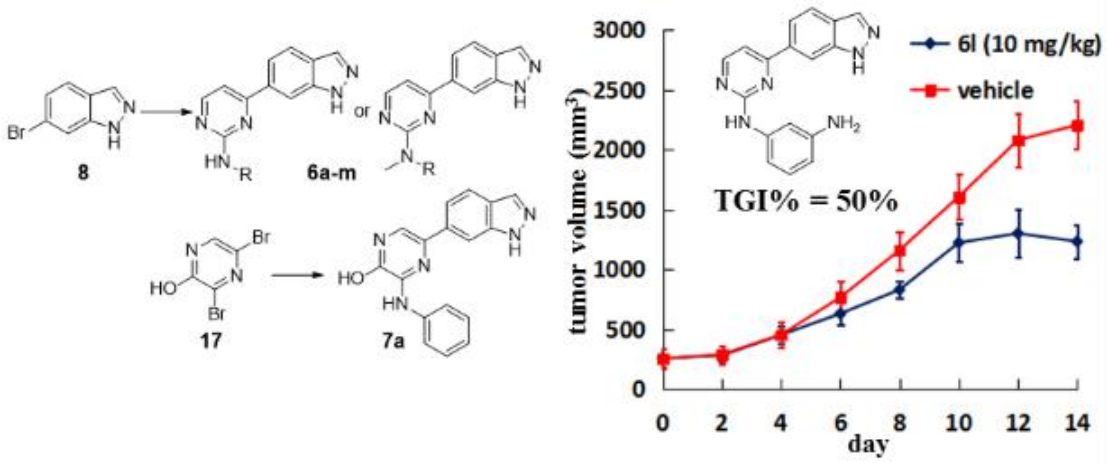

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Figure 1

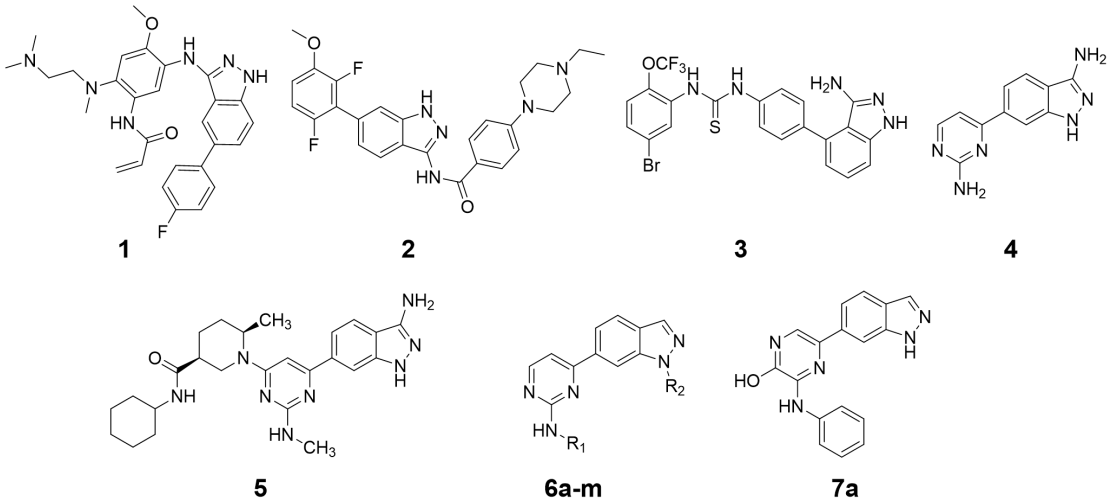

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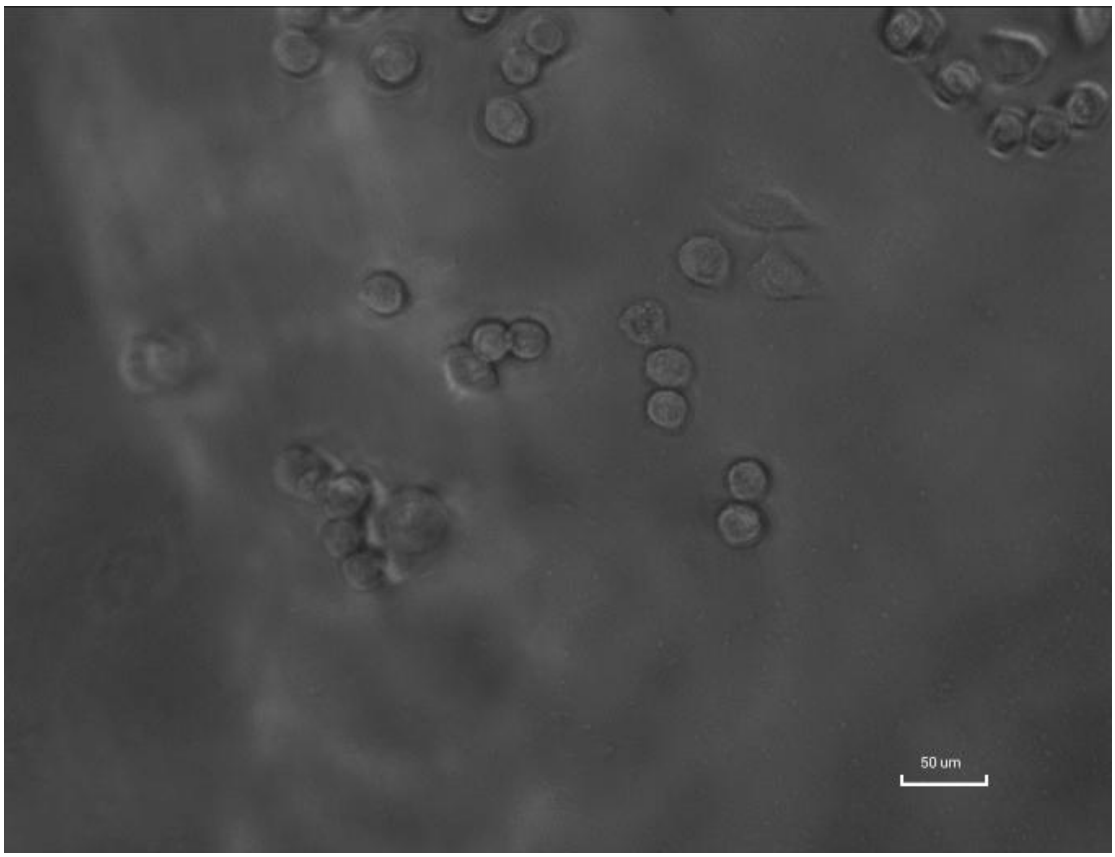
Figure 2 control



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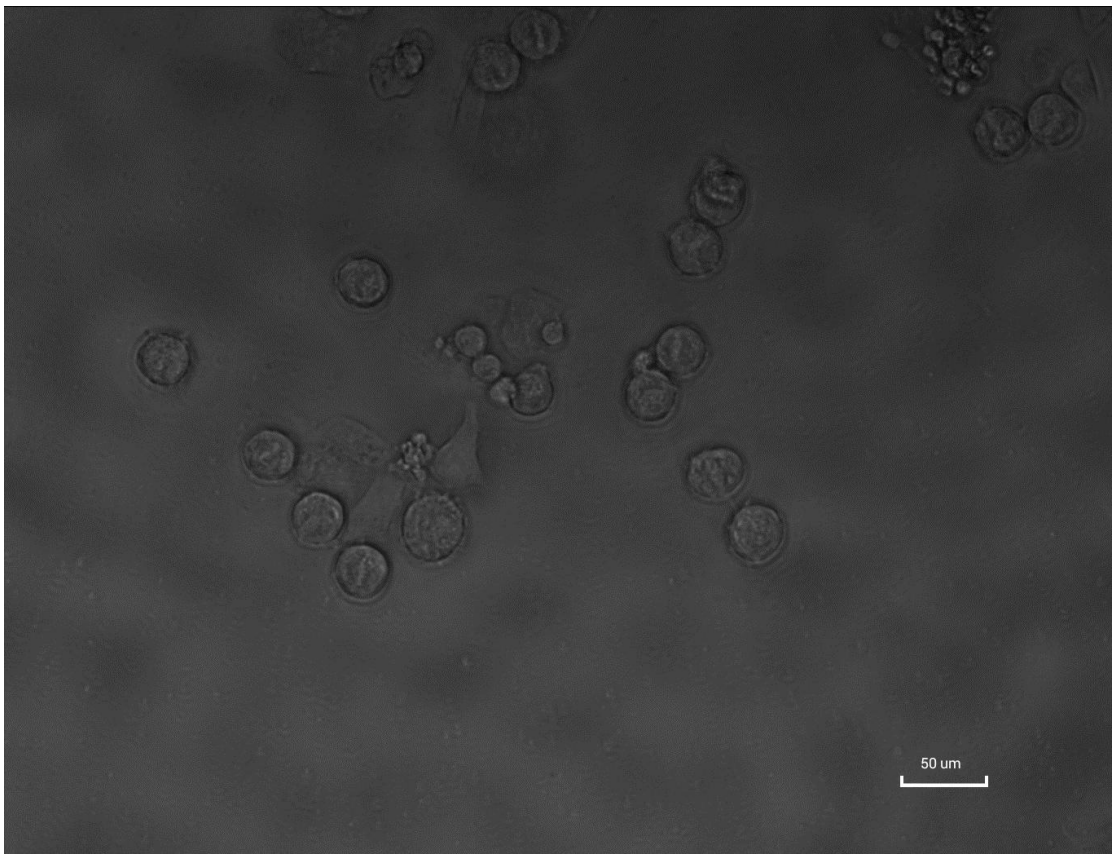

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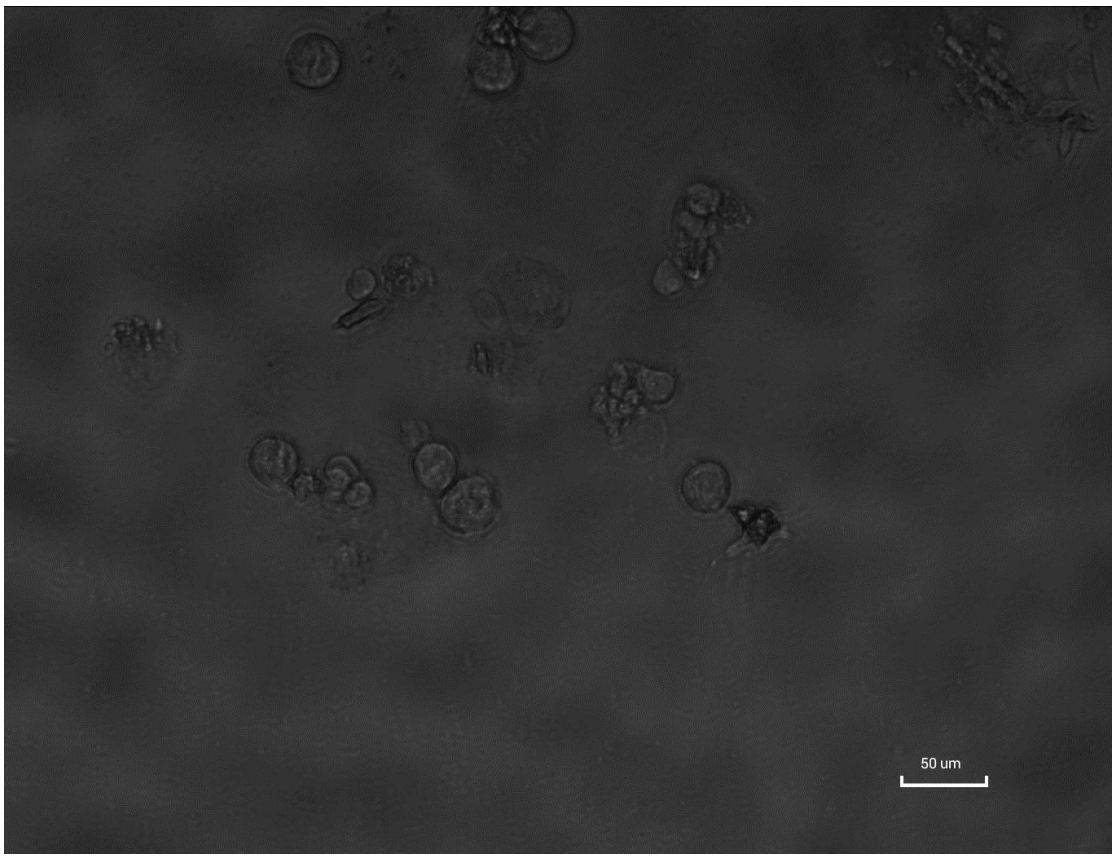

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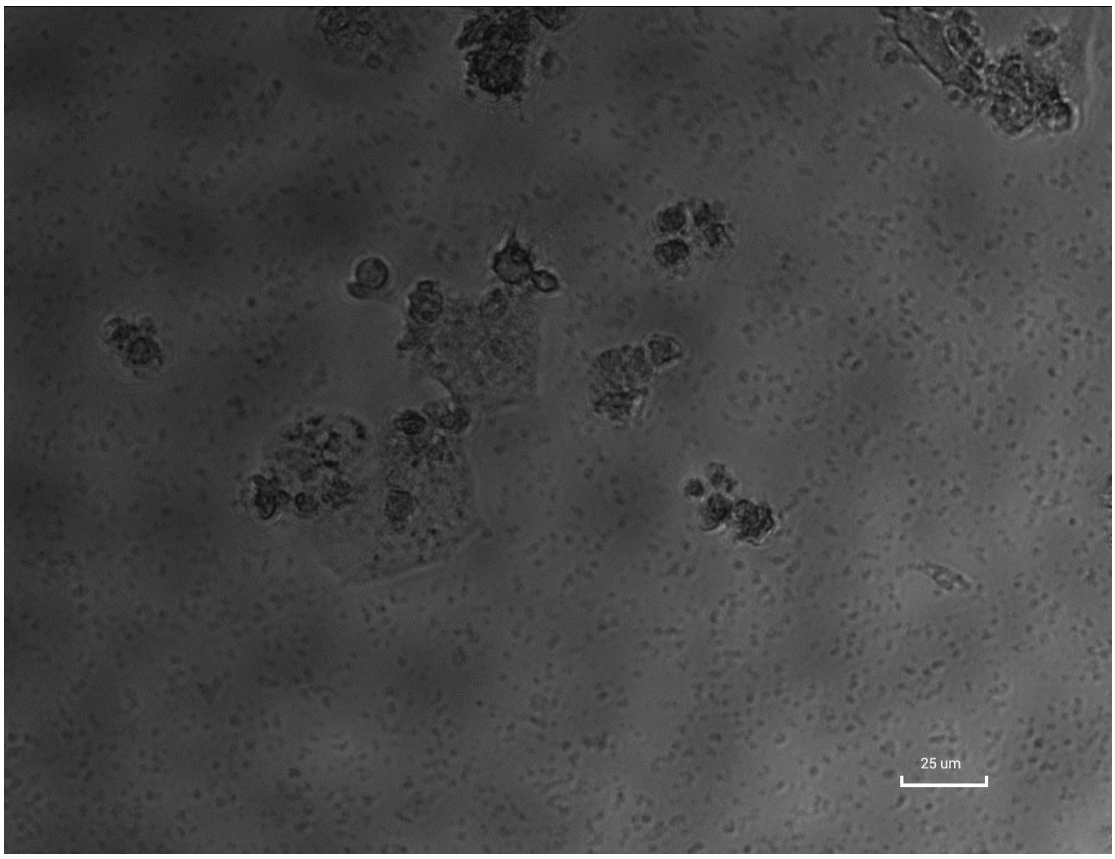

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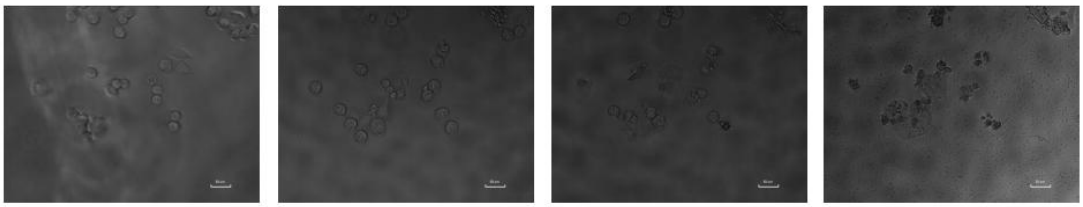

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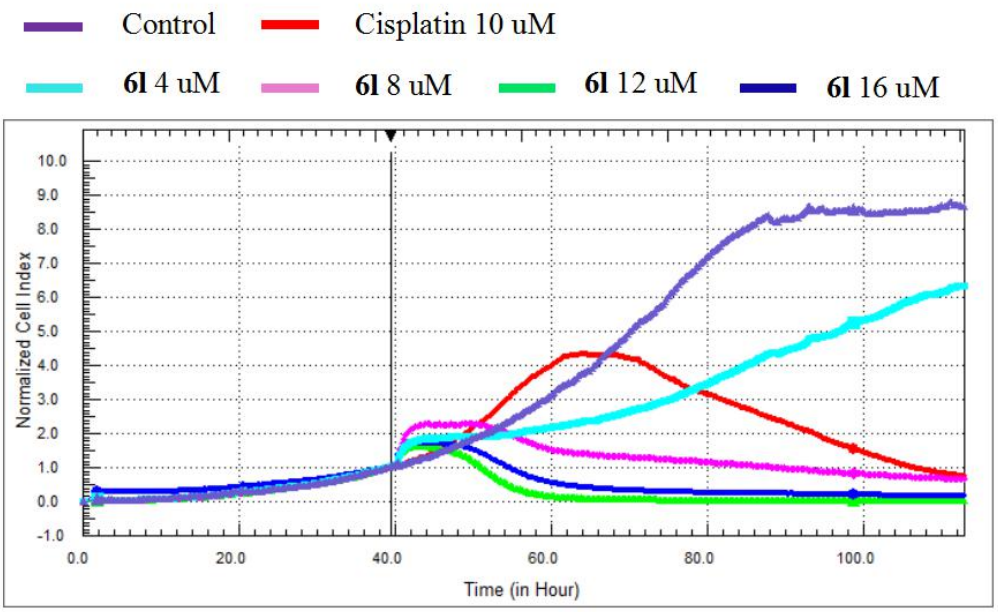

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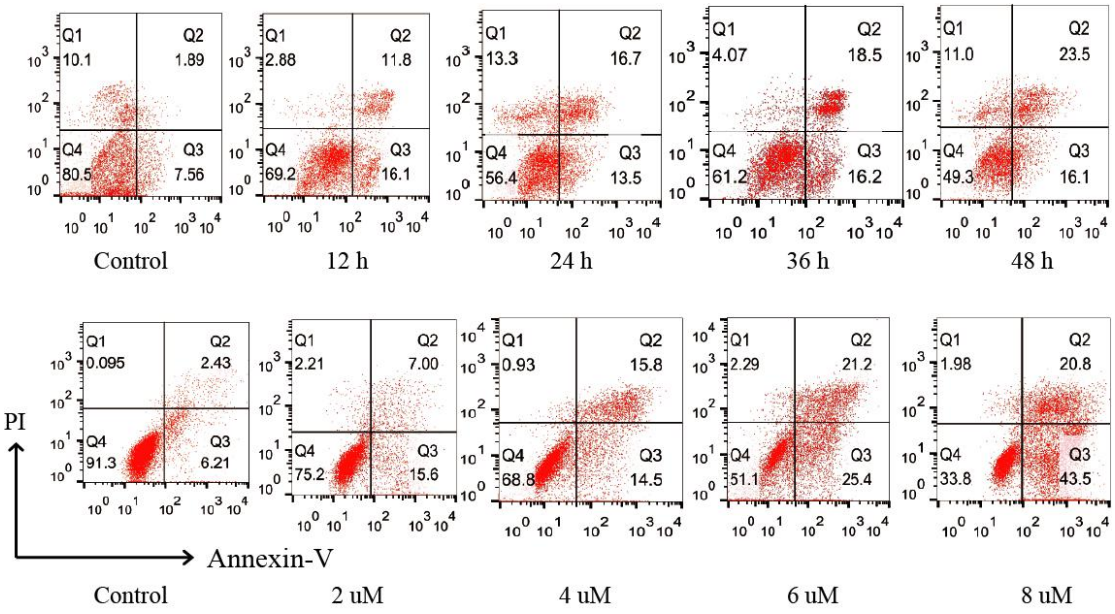

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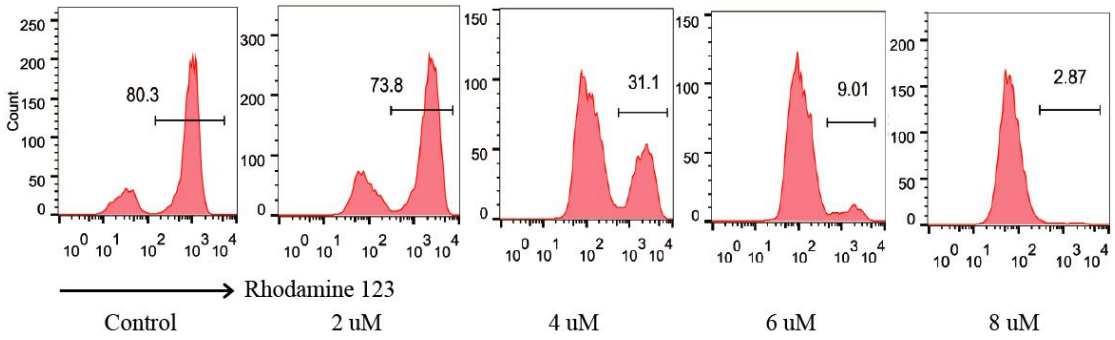

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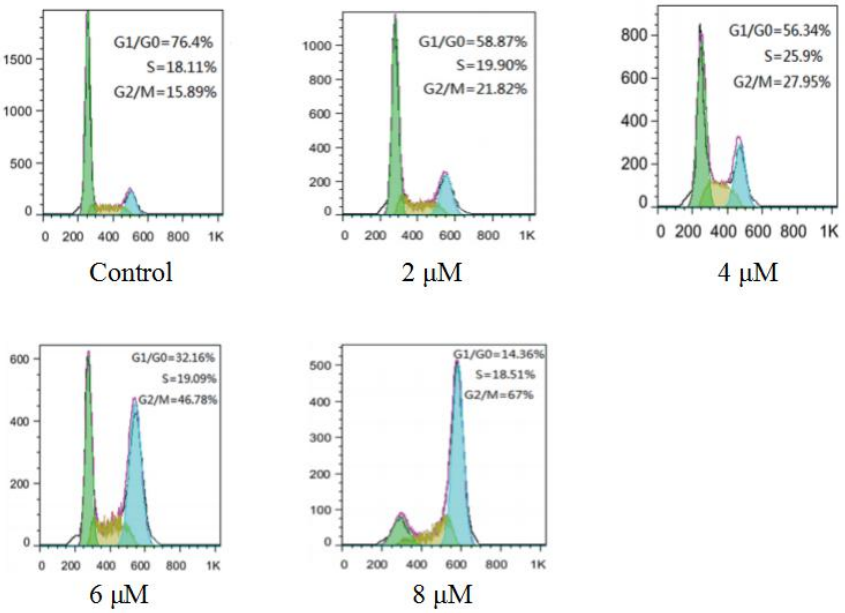

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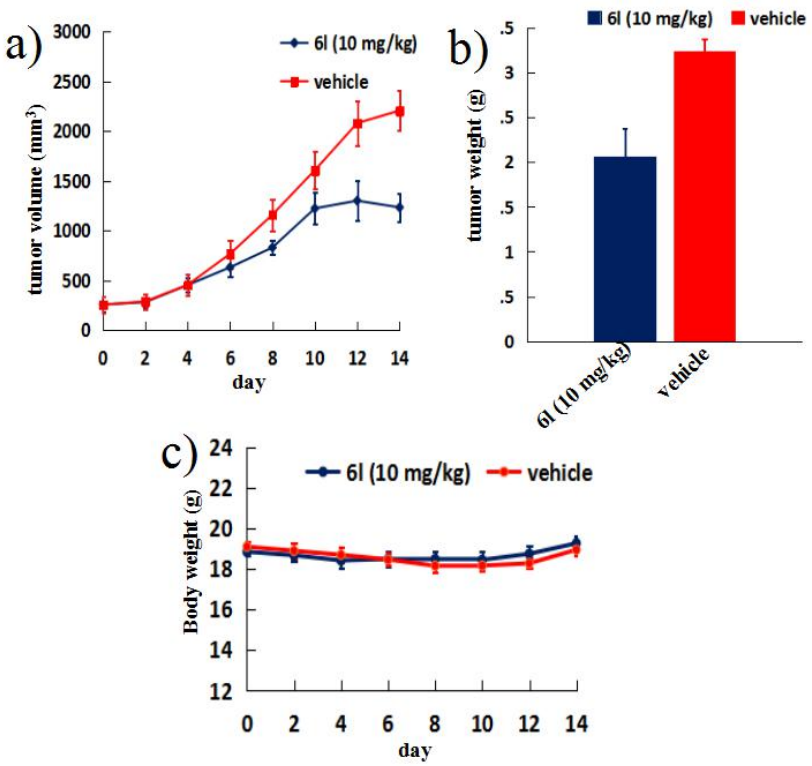

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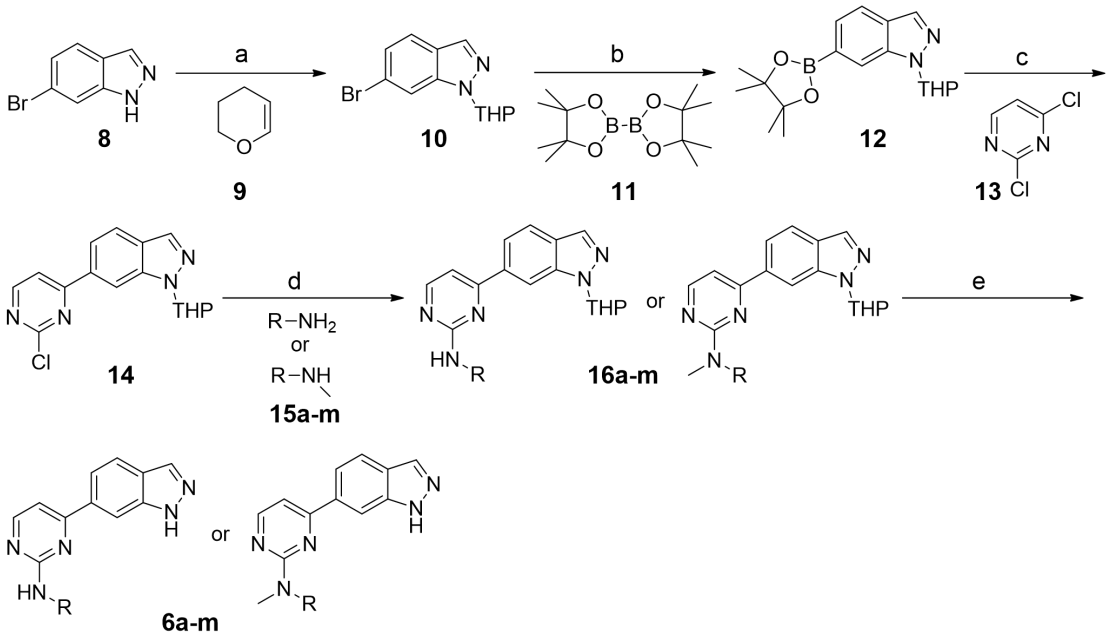
Scheme 1



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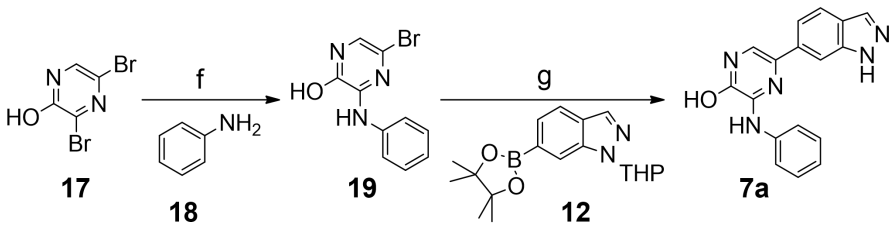

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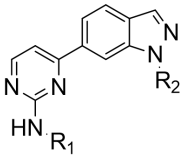

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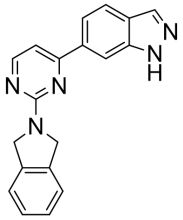
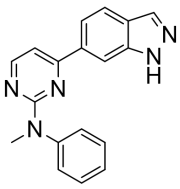
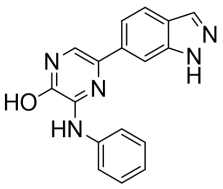


6e



6i



7a