Synthesis and Biological Evaluation of Novel 4-Phenylaminobenzofuro[2,3-d]pyrimidine Derivatives

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(Received on 8th April 2019, accepted in revised form 21st November 2019)

Summary: A series of novel 4-phenylaminobenzofuro[2,3-d]pyrimidine derivatives had been prepared and assessed for their *in vitro* antiproliferative activities against three lung cancer cell lines (A549, H460 and H1975). The bioassay results showed most of the designed compounds exhibited potential antiproliferation activities. Among them, compound **8f** exhibited remarkable inhibitory activity against A549 and H460 cell lines with IC_{50} value of 2.54 μM and 2.68 μM, respectively, which was comparable to that of the positive control sorafenib (IC_{50} = 2.69 μM for A549 and 3.71 μM for H460). AO/EB staining suggests that compound **8f** could induce apoptosis in A549 cells. Furthermore, cell cycle analyses show that compound **8f** increased G_0/G_1 A549 cells arrest in a concentration-dependent manner. The preliminary structure-activity relationships (SARs) studies indicated that mono-electron-withdrawing groups (mono-EWGs) on the phenyl ring are positive on the antitumor activity.

Keywords: Synthesis, 4-phenylaminobenzofuro[2,3-d]pyrimidine, Antitumor activity, Structure–activity relationship.

Introduction

Lung cancer is one of the most devastating types of malignant tumors, responsible for 28% of all cancer deaths [1-4]. Despite the efforts to discover and develop small molecule drugs in the last decade, development of more effective inhibitors with improved tumor selectivity, efficiency, drug-resistant, and safety remains desirable [5-7].

To our knowledge, compounds containing 4-anilinopyrimidine scaffold often have a wide range of biological activities, especially in antitumor drugs [8, 9]. Pazopanib [10], a well-known tyrosine kinase inhibitor, potently blocks tumour growth and inhibits angiogenesis. It was approved by FDA (19 October 2009) for the treatment of advanced/metastatic renal cell carcinoma and advanced soft tissue sarcomas. Ceritinib [11] and Brigatinib [12] have received much attention since they were launched, bringing a lot of good news to lung cancer patients. The EGFR kinase inhibitors such as gefitinib [13], afatinib [14] and rociletinib [15] whose structure contain the scaffolds of 4-anilinopyrimidine have yielded promising results, providing an ideal treatment for patients. However, acquired drug-resistant lung cancer due to EGFR mutations or ALK rearrangement was an urgent problem to be solved. Based upon the prospect of 4-anilinopyrimidine derivatives in the field of anti-tumor drugs, a novel series of 4-phenylaminobenzofuro[2,3-d]pyrimidines were designed and synthesized. Structural modification was carried out with various substituents on the terminal aromatic ring to development more effective drugs overcoming the mutations.

Experimental

Chemistry

General

All reagents used in the synthesis were obtained commercially and used without further purification unless otherwise specified. Melting points were determined on a Beijing Taike X-4 Microscopy Melting Point apparatus and the temperature was uncorrected. ^{1}H NMR spectra were performed on a Bruker Biospin 600 MHz or 400 MHz NMR spectrometer with TMS as the internal standard. The chemical shift values were expressed in ppm. IR spectra were performed on a PerkinElmer FTIR spectrometer (KBr pellets). MS were measured on an Agilent 6460 QQQ mass spectrometer. Elemental analysis was carried out on a Carlo Erba 1108 analyser and are found within the range of

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theoretical value. Compounds **6** was synthesized according to reported procedures [16].

Synthesis of methyl 4-[(4-aminophenyl)amino]benzofuro[2,3-d]pyrimidine-6-carboxylate **(7)**

To the mixture of 6 (1.0 g, 2.7 mmol), iron powder (0.2g, 4.1 mmol), hydrochloric acid was heated in ethanol (2.0 mL) with refluxing for 6 h. After completion, the resulting mixture was filter, and the filtrate was concentrated under reduced pressure until precipitate appeared. The precipitate was filtered and dried to provide the methyl 4-[(4-aminophenyl)amino]benzofuro[2,3-d] pyrimidine-6-carboxylate **(7)** (0.83 g, 90.5%). IR (KBr, cm^{-1}): 3443, 3351, 3214, 2923, 1710, 1596, 1514, 1418, 1078; MS (ESI) m/z (%): 335.1 $[M-H]^-$. Analytical Calculated for $C_{18}H_{14}N_4O_3$ (%): C, 64.67; H, 4.22; N, 16.76; found (%): C, 64.79; H, 4.31; N, 16.85.

General method for the synthesis of target compounds **8a–l**

The intermediate **7** (1.0 g, 3.0 mmol), aromatic carboxylic acid (3.9 mmol), 2-(7-Azabenzotriazol-1-yl)-N,N,N',N'-tetramethyluronium hexafluorophosphate(HATU) (1.48 g, 3.9 mmol) and triethylamine (0.6 g, 6.0 mmol) were stirred together in *N*,*N*-dimethylformamide (5 mL) at room temperature for 8 h. The mixture was poured with saturated sodium carbonate solution (30 mL) and subsequently extracted with dichloromethane. The combined organic layer was washed with saturated NaCl solution, dried over anhydrous Na_2SO_4, filtered and concentrated. The crude product was purified by flash silica chromatography and dried under vacuum to give **8a–l** as white solids**.**

Methyl 4-[(4-benzamidophenyl)amino]benzofuro[2,3-d]pyrimidine-6-carboxylate (**8a**).

Yield 73.8%; M.p.: 260-262 °C; IR (KBr, cm^{-1}): 3435, 2956, 2923, 2851, 1717, 1640, 1614, 1583, 1514, 1063; ^{1}H NMR (600 MHz, DMSO-d_6) δ 10.33 (s, 1H), 9.76 (s, 1H), 8.86 (s, 1H), 8.54 (s, 1H), 8.13 (d, *J* = 8.6 Hz, 1H), 8.00 (d, *J* = 7.3 Hz, 2H), 7.86 (t, *J* = 9.2 Hz, 3H), 7.68 – 7.51 (m, 5H), 3.91 (s, 3H); MS (ESI) m/z (%): 437.1 $[M-H]^-$. Analytical Calculated for $C_{25}H_{18}N_4O_4$ (%): C, 68.49; H, 4.14; N, 12.78; found (%): C, 68.70; H, 4.23; N, 12.91.

Methyl 4-{[4-(pyrazine-2-carboxamido)phenyl]amino}benzofuro[2,3-d]pyrimidine-6-carboxylate (**8b**).

Yield 74.1%; M.p.: 314-316 °C; IR (KBr, cm^{-1}): 3431, 2923, 2851, 1706, 1679, 1605, 1556, 1522, 1064,1017; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.80 (s, 1H), 9.76 (s, 1H), 9.31 (s, 1H), 9.00 – 8.75 (m, 3H), 8.52 (s, 1H), 8.12 (d, *J* = 8.7 Hz, 1H), 7.94 (d, *J* = 8.8 Hz, 2H), 7.85 (d, *J* = 8.4 Hz, 1H), 7.58 (d, *J* = 8.9 Hz, 2H), 3.88 (s, 3H). Analytical Calculated for $C_{23}H_{16}N_6O_4$ (%): C, 62.72; H, 3.66; N, 19.08; found (%): C, 62.79; H, 3.74; N, 19.21.

Methyl 4-{[4-(picolinamido)phenyl]amino}benzofuro[2,3-d]pyrimidine-6-carboxylate (**8c**).

Yield 76.2%; M.p.: 264-266 °C; IR (KBr, cm^{-1}): 3431, 2917, 2846, 1714, 1684, 1610, 1579, 1535, 1061; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.70 (s, 1H), 9.75 (s, 1H), 8.85 (s, 1H), 8.74 (d, *J* = 4.6 Hz, 1H), 8.51 (s, 1H), 8.21 – 8.03 (m, 3H), 7.95 (d, *J* = 8.7 Hz, 2H), 7.85 (d, *J* = 8.6 Hz, 1H), 7.73 – 7.63 (m, 1H), 7.57 (d, *J* = 8.8 Hz, 2H), 3.87 (s, 3H); MS (ESI) m/z (%): 438.0 $[M-H]^-$. Analytical Calculated for $C_{24}H_{17}N_5O_4$ (%): C, 65.60; H, 3.90; N, 15.94; found (%): C, 65.83; H, 4.01; N, 15.99.

Methyl 4-{[4-(thiophene-2-carboxamido)phenyl]amino}benzofuro[2,3-d]pyrimidine-6-carboxylate (**8d**).

Yield 78.6%; M.p.: 277-279 °C; IR (KBr, cm^{-1}): 3431, 3260, 2923, 2851, 1701, 1650, 1600, 1547, 1511, 1209, 1102; ^{1}HNMR (400MHz, DMSO-d_6) δ 10.29 (s, 1H), 9.73 (s, 1H), 8.83 (s, 1H), 8.51 (s, 1H), 8.11 (dd, *J* = 8.6, 1.6Hz, 1H), 8.03 (d, *J* = 2.9Hz, 1H), 7.90 – 7.80 (m, 2H), 7.76 (d, *J* = 8.9Hz, 2H), 7.56 (d, *J* = 8.8Hz, 2H),7.29–7.16 (m, 1H), 3.88 (s, 3H); MS (ESI) m/z (%): 443.0 $[M-H]^-$. Analytical Calculated for $C_{23}H_{16}N_4O_4S$ (%): C, 62.15; H, 3.63; N, 12.61; found (%): C, 62.21; H, 3.84; N, 12.76.

Methyl 4-{[4-(2-fluorobenzamido)phenyl]amino}benzofuro[2,3-d]pyrimidine-6-carboxylate (**8e**).

Yield 83.2%; M.p.: 249-252 °C; IR (KBr, cm^{-1}): 3436, 2917, 2851, 1716, 1650, 1614, 1584, 1543, 1209, 1097; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.47 (s, 1H), 9.76 (s, 1H), 8.82 (s, 1H), 8.51 (s, 1H), 8.11 (d, *J* = 10.3 Hz, 1H), 7.85 (d, *J* = 8.6 Hz, 1H), 7.76 (d, *J* = 8.7 Hz, 2H), 7.71 – 7.64 (m, 1H), 7.62 – 7.50 (m, 3H), 7.41 – 7.28 (m, 2H), 3.89 (s, 3H); MS (ESI) m/z (%): 455.1 $[M-H]^-$. Analytical Calculated for $C_{25}H_{17}FN_4O_4$ (%): C, 65.79; H, 3.75; N, 12.28;

found (%): C, 65.96; H, 3.84; N, 12.41.

Methyl 4-{[4-(4-fluorobenzamido)phenyl]amino}benzofuro[2,3-d]pyrimidine-6-carboxylate (**8f**).

Yield 82.4%; M.p.: 288-291 °C; IR (KBr, cm^{-1}): 3440, 2961, 2923, 1720, 1643, 1615, 1518, 1462, 1254, 1119, 1063; ^{1}H NMR (600 MHz, DMSO-d_6) δ 10.34 (s, 1H), 9.75 (s, 1H), 8.86 (s, 1H), 8.53 (s, 1H), 8.19 – 8.03 (m, 3H), 7.89 – 7.79 (m, 3H), 7.59 (d, *J* = 8.7 Hz, 2H), 7.39 (t, *J* = 8.8 Hz, 2H), 3.91 (s, 3H); MS (ESI) m/z (%): 455.1 $[M-H]^-$. Analytical Calculated for $C_{25}H_{17}FN_4O_4$ (%): C, 65.79; H, 3.75; N, 12.28; found (%): C, 65.98; H, 3.83; N, 12.43.

Methyl 4-{[4-(4-chlorobenzamido)phenyl]amino}benzofuro[2,3-d]pyrimidine-6-carboxylate (**8g**).

Yield 80.3%; M.p.: 321-323 °C; IR (KBr cm^{-1}): 3461, 3414, 2917, 2846, 1722, 1614, 1602, 1544, 1514, 1253, 1097; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.37 (s, 1H), 9.74 (s, 1H), 8.84 (s, 1H), 8.51 (s, 1H), 8.16 – 8.06 (m, 1H), 8.00 (d, *J* = 8.5 Hz, 2H), 7.92 – 7.75 (m, 3H), 7.67 – 7.50 (m, 4H), 3.88 (s, 3H); MS (ESI) m/z (%): 471.1 $[M-H]^-$. Analytical Calculated for $C_{25}H_{17}ClN_4O_4$ (%): C, 63.50; H, 3.62; N, 11.85; found (%): C, 63.67; H, 3.75; N, 11.93.

methyl 4-{[4-(4-(trifluoromethylbenzamido)phenyl]amino}benzofuro[2,3-d]pyrimidine-6-carboxylate (**8h**).

Yield 77.2%; M.p.: 287-289 °C; IR (KBr, cm^{-1}): 3417, 2923, 2846, 1722, 1615, 1639, 1516, 1403, 1209, 1064; ^{1}H NMR (400 MHz, DMSO- d_6) δ 10.53 (s, 1H), 9.75 (s, 1H), 8.85 (s, 1H), 8.52 (s, 1H), 8.20 – 8.07 (m, 3H), 7.92 (d, *J* = 8.3 Hz, 2H), 7.88 – 7.80 (m, 3H), 7.58 (d, *J* = 8.8 Hz, 2H), 3.88 (s, 3H) ; MS (ESI) m/z (%): 505.1 $[M-H]^-$. Analytical Calculated for $C_{26}H_{17}F_3N_4O_4$ (%): C, 61.66; H, 3.38; N, 11.06; found (%): C, 61.79; H, 3.50; N, 11.14.

Methyl 4-{[4-(3-fluorobenzamido)phenyl]amino}benzofuro[2,3-d]pyrimidine-6-carboxylate (**8i**).

Yield 85.5%; M.p.: 292-295 °C; IR (KBr, cm^{-1}): 3434, 2917, 2851, 1720, 1640, 1614, 1585, 1401, 1122, 1061; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.37 (s, 1H), 9.75 (s, 1H), 8.86 (s, 1H), 8.52 (s, 1H), 8.12 (dd, *J* = 8.6, 1.7 Hz, 1H), 7.93 – 7.71 (m, 5H), 7.66 – 7.52 (m, 3H), 7.50 – 7.38 (m, 1H), 3.88 (s, 3H); MS (ESI) m/z (%): 455.1 $[M-H]^-$. Analytical Calculated for $C_{25}H_{17}FN_4O_4$ (%): C, 65.79; H, 3.75; N, 12.28; found (%): C, 65.86; H, 3.91; N, 12.35.

Methyl 4-{[4-(2-chlorobenzamido)phenyl]amino}benzofuro[2,3-d]pyrimidine-6-carboxylate (**8j**).

Yield 75.5%; M.p.: 228-230 °C; IR (KBr, cm^{-1}): 3414, 2923, 2846, 1709, 1616, 1596, 1517, 1425, 1207, 1109; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.56 (s, 1H), 9.75 (s, 1H), 8.85 (s, 1H), 8.50 (s, 1H), 8.11 (d, *J* = 8.5 Hz, 1H), 7.85 (d, *J* = 8.5 Hz, 1H), 7.75 (d, *J* = 8.8 Hz, 2H), 7.62 – 7.39 (m, 6H), 3.90 (s, 3H); MS (ESI) m/z (%): 471.1 $[M-H]^-$. Analytical Calculated for $C_{25}H_{17}ClN_4O_4$ (%): C, 63.50; H, 3.62; N, 11.85; found (%): C, 63.59; H, 3.81; N, 11.91.

Methyl 4-{[4-(2,4-dichlorobenzamido)phenyl]amino}benzofuro[2,3-d]pyrimidine-6-carboxylate (**8k**).

Yield 78.6%; M.p.: 296-299 °C; IR (KBr, cm^{-1}): 3441, 2931, 2826, 1729, 1680, 1598, 1501, 1449, 1210, 1069; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.59 (s, 1H), 9.75 (s, 1H), 8.86 (s, 1H), 8.50 (s, 1H), 8.11 (dd, *J* = 8.7, 1.7 Hz, 1H), 7.84 (d, *J* = 8.6 Hz, 1H), 7.79 – 7.70 (m, 3H), 7.64 (d, *J* = 8.2 Hz, 1H), 7.60 – 7.52 (m, 3H), 3.89 (s, 3H); MS (ESI) m/z (%): 505.0 $[M-H]^-$. Analytical Calculated for $C_{25}H_{16}Cl_2N_4O_4$ (%): C, 59.19; H, 3.18; N, 11.04; found (%): C, 59.30; H, 3.37; N, 11.19.

Methyl 4-{[4-(2,3,4-trimethoxybenzamido)phenyl]amino}benzofuro[2,3-d]pyrimidine-6-carboxylate(**8l**).

Yield 75.9%; M.p.: 317-320 °C; IR (KBr, cm^{-1}): 3433, 3346, 2945, 2835, 1714, 1659, 1579, 1541, 1513, 1126, 1061; ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.18 (s, 1H), 9.73 (s, 1H), 8.86 (s, 1H), 8.51 (s, 1H), 8.11 (dd, *J* = 8.7, 1.7 Hz, 1H), 7.84 (d, *J* = 8.6 Hz, 1H), 7.77 (d, *J* = 8.9 Hz, 2H), 7.58 (d, *J* = 8.9 Hz, 2H), 7.29 (s, 2H), 3.88 (br, *J* = 6.6 Hz, 9H), 3.72 (s, 3H); MS (ESI) m/z (%): 527.0 $[M-H]^-$. Analytical Calculated for $C_{28}H_{24}N_4O_7$ (%): C, 63.63; H, 4.58; N, 10.60; found (%): C, 63.72; H, 4.71; N, 10.83.

Pharmacology

MTT assay

Cells were grown in 96-well culture plates. The tested compounds of various concentrations were added into the plates at 37 ℃ with 5% CO_2. After 72 h treatment, the medium was removed. Cells were

with 20 µL fresh MTT solution for 3 - 4 h at 37 ºC. The medium was replaced by 150 µL dimethyl sulfoxide and the absorbance was measured on a microplate reader at 490 nm.

Acridine orange/ethidium bromide (AO/EB) staining

A549 cells were seeded in 24-well plates (1x 10^6 cells /well), and then the cells were incubated for 24 h. Cells were treated with **8f** at concentrations 0 µM, 0.025 µM, 0.25 µM and 2.5 µM for 48 h, cells were collected, washed with phosphate buffer saline (PBS) that stored at 4 °C. Acridine orange/ethidium bromide (AO/EB) mixed solution 1.0 µL (100 µg/mL AO and 100 µg/mL EB) was added to each suspension, and then stained for 10 min, covered with a coverslip. The morphology of apoptotic cells was observed by fluorescent microscope(Olympus, Tokyo, Japan).

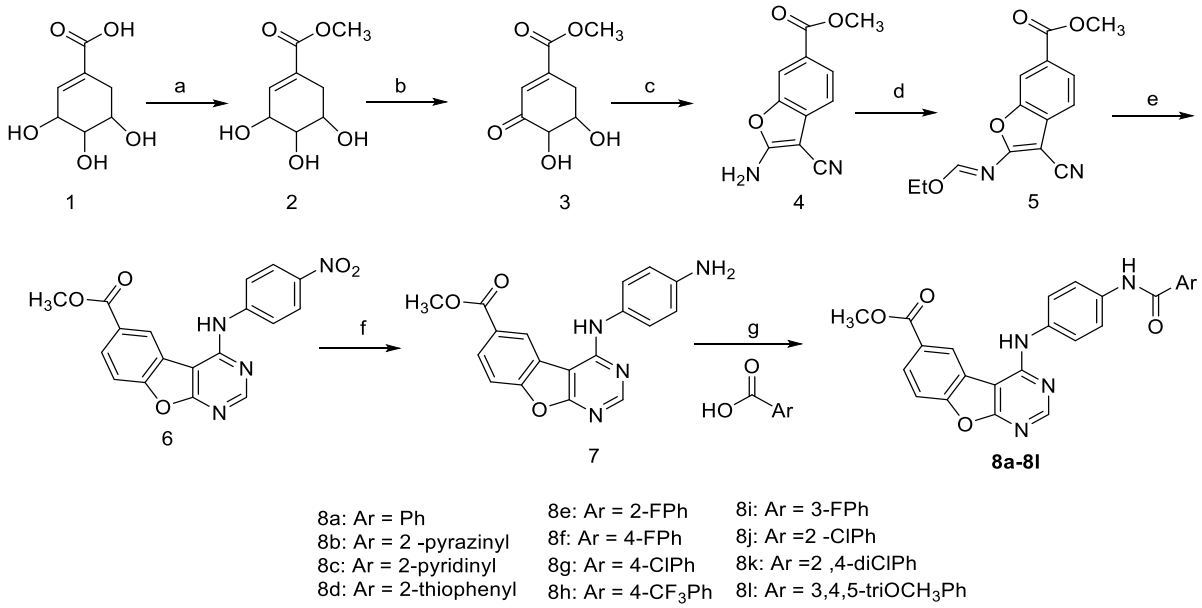
Cell cycle Progression

A549 cells were seeded in 6-well plates (1x 10^6 cells /well). After 24 h of incubation for attachment, cells were treated with **8f** at concentrations 0 µM, 0.4 µM, 2.0 µM and 10.0 µM in 2.0 mL of serum complete media. After another 24 h, media containing any floating cells were collected and combined with adherent cells that were detached by brief trypsinization (0.25% trypsin-EDTA). Cell pellets were washed with 1.0 mL of ice-cold phosphate buffer saline (PBS) and then resuspended in 1.0 mL of 70% EtOH and then kept at 4 °C overnight. Then, the cells were stained using propidium iodide (PI) for 30 min in dark. The cell cycle progression was analyzed for DNA content by a FACScan flow cytometer.

Results and Discussion

Chemistry

The synthetic methods for compounds **8a** – **8l** are outlined in Scheme-1. The key intermediates methyl 4-[(4-nitrophenyl)amino]benzofuro[2,3-d]pyrimidine -6-carboxylate (**6**) were obtained in six steps according to reported procedures[16, 17]. The intermediate **6** was reduced with iron powder in the mixture of ethanol and hydrochloric acid to obtain the key intermediate **7**, which was engaged in a condensation reaction with heterocyclic ring or benzene ring bearing a carboxylic acid to give the target compounds (**8a** – **l**). Compounds **8a** – **8l** were appropriately established by spectroscopic and analytical methods. IR, ^{1}H NMR, MS and elemental analyses of the target compounds confirmed their structural integrity.



Scheme-1: Reagents and conditions: (a) CH_3OH, H_2SO_4, 22 °C, 48 h (b) IBX, THF, 22 °C, 4 h (c) malononitrile, H_2O, 85 °C, 3 h (d) $CH(OEt)_3$, $(CH_3CO)_2O$, 120 °C, 4 h (e), 4-nitroaniline, AcOH, refluxed, 13 h (f) Fe, CH_3CH_2OH, refluxed, 6 h (g) acids, DMF, HATU, Et_3N.

Cell proliferative assay and structure-activity relationships

The effects of all the newly synthesized compounds (**8a~l**) on cell antiproliferation were evaluated against three human lung cancer cell lines (A549, H460 and H1975) using the standard MTT-based assay *in vitro*, with sorafenib and gefitinib used as the positive control. The IC_{50} of the compounds against these cancer cells were presented in Table 1. All the test compounds showed moderate-to-excellent antiproliferative activities against different cancer cells and some compounds showed more or similar activities in comparison with sorafenib against certain cancer lines. Among the tested compounds, compound **8f** showed potent anticancer activity with IC_{50} values of 2.54 μM, 2.68 μM and 6.19 μM against A549, H460 and H1975 cell lines, respectively, which were comparable to the positive control.

Table-1: In vitro anticancer activities (IC_{50}, μM) of all compounds against tumor cell lines.

Compounds	Structure	Cell lines (IC_{50}, μM) [a]		
	Ar	A549	H460	H1975
8a	**Ph**	**8.05**	**5.67**	**9.56**
8b	**2 -pyrazinyl**	**10.49**	**5.69**	**16.19**
8c	**2-pyridinyl**	**10.73**	**2.91**	**14.03**
8d	**2-thiophenyl**	**11.15**	**9.05**	**9.46**
8e	**2-FPh**	**4.39**	**9.49**	**13.39**
8f	**4-FPh**	**2.54**	**2.68**	**6.19**
8g	**4-ClPh**	**3.25**	**2.74**	**5.89**
8h	**4-CF_3Ph**	**35.33**	**14.51**	**22.48**
8i	**3-FPh**	**4.01**	**3.03**	**9.05**
8j	**2 -ClPh**	**4.19**	**13.46**	**9.31**
8k	**2 ,4-diClPh**	**3.42**	**12.58**	**10.24**
8l	**3,4,5-triOCH_3Ph**	**20.10**	**10.85**	**10.47**
gefitinib	-	**6.05**	**ND***	**9.86**
sorafenib	-	**2.69**	**3.71**	**ND***

[a] The values are an average of two separate determinations.
* ND = Not Detected

Initially, our effort towards exploration the SAR of the molecule was started with replacing the Ar with different aromatic rings to find a suitable rigid structure. As seen from the data on Table-1, The aromatic ring substituted with phenyl (**8a**) exerted a better activity, compared to 2-pyrazinyl (**8b**), 2-pyridinyl (**8c**) or 2-thiophenyl (**8d**). Accordingly, we next focused SAR exploration on the compounds possessing phenyl.

To further study the effect of the phenyl ring on cell antiproliferation, different substitutions at the phenyl ring were investigated. As shown in table-1, The results indicated that the equipment of mono-electron-withdrawing groups (mono-EWGs) showed a positive effect on the antiproliferative activity, such as compound **8f** (Ar = 4-FPh, IC_{50} = 2.54 μM against A549), **8g** (Ar = 4-ClPh, IC_{50} = 2.74 μM against H460), which are better than that of compound **8a** (Ar = Ph, IC_{50} = 8.05 μM against A549). However, replacement of the mono-EWGs on phenyl ring with other groups significantly decreased the activity such as strong-EWGs (**8h**, Ar = 4-CF_3Ph), double-EWGs (**8k**, Ar = 2,4-diClPh) or triple-electron-donating groups (EDGs) (**8l**, Ar = 3,4,5-triOCH_3Ph). It indicated that suitable electron density and steric hindrance were Critical for the activity. Moreover, the compounds substituted at 4-position on phenyl ring were preferred for better activity that was evidenced by **8f** (Ar = 4-FPh) and **8g** (Ar = 4-ClPh) showed much lower IC_{50} values compared to **8e** (Ar = 2-F), **8i** (Ar = 3-F) and **8j** (Ar = 2-ClPh).

AO/EB staining

Compound **8f** was further confirmed by an AO/EB staining assay [18] to assess the apoptosis-induction ability. Fig 1 showed that compound **8f (**0.025 μM, 0.25 μM and 2.5 μM**)** induced morphological changes and characteristic of apoptosis. The morphological changes such as cell volume shrinkage, membrane blebbing, chromatin condensation and apoptotic body formation (bright green nucleus with condensed chromatin and condensed orange chromatin means early apoptosis cells and last apoptosis cells, respectively.). As a comparison, the untreated control cells showed normal morphology and stained in green. These data clearly demonstrated that the ability of compound **8f** to induce apoptosis was related to the concentrations.

Fig 1. AO/EB stained apoptosis of A549 cell lines. A549 cells were treated with compound **8f** at 0.025 μM, 0.25 μM and 2.5 μM for 48 h. The white arrow indicates normal cells, the blue arrow indicates early apoptotic cells and the red arrow later apoptotic cells.

Cell cycle assays

Generally，anticancer drugs could interact with cells resulted cell growth arrest or cell death. We next tested how the compound **8f** affect on cell cycles in the A549 lung cancer cells. Human lung cancer cells A549 were treated with different concentrations of compound **8f** at 0 μM, 0.4 μM, 2.0 μM and 10.0 μM for 24 h. The results were presented in Fig. 2. The results from the Fig 2 showed that thepercentage of A549 cells in G_0/G_1 phase from 63.34% in control accumulated to 76.57 % at 0.4 μM, 82.06 % at 2.0 μM and 85.67 % at 10.0 μM, respectively. These data indicated that compound **8f** induce cell cycle arrest in G_0/G_1 phase in A549 cells in a dose dependent manner.

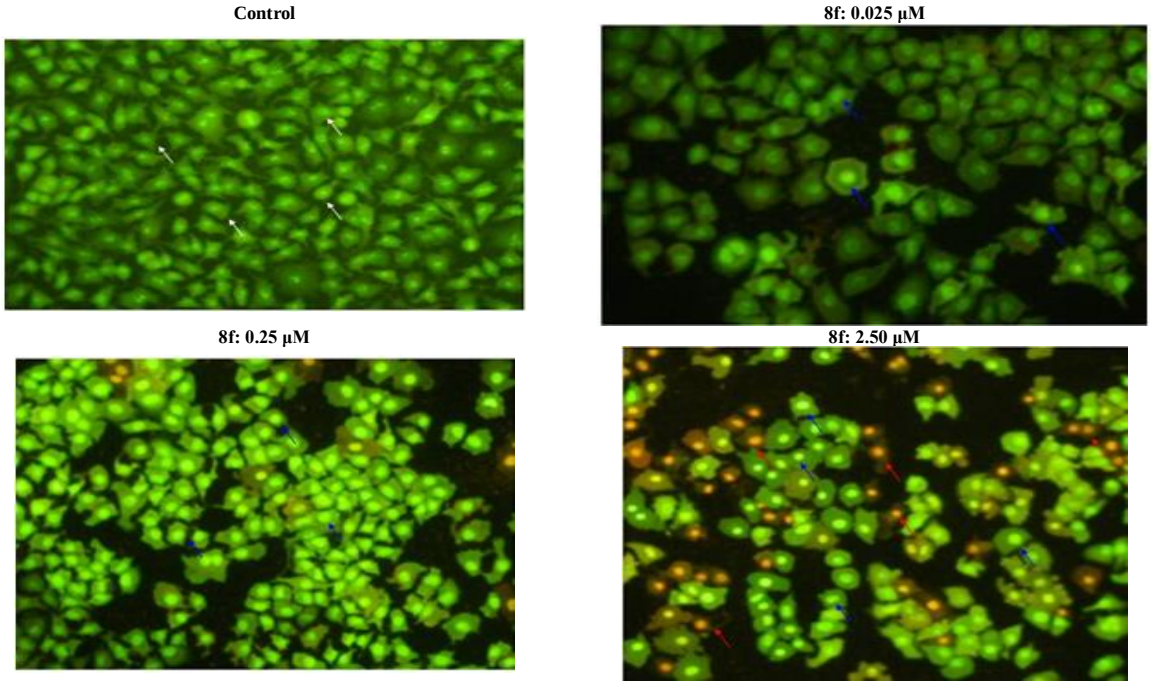
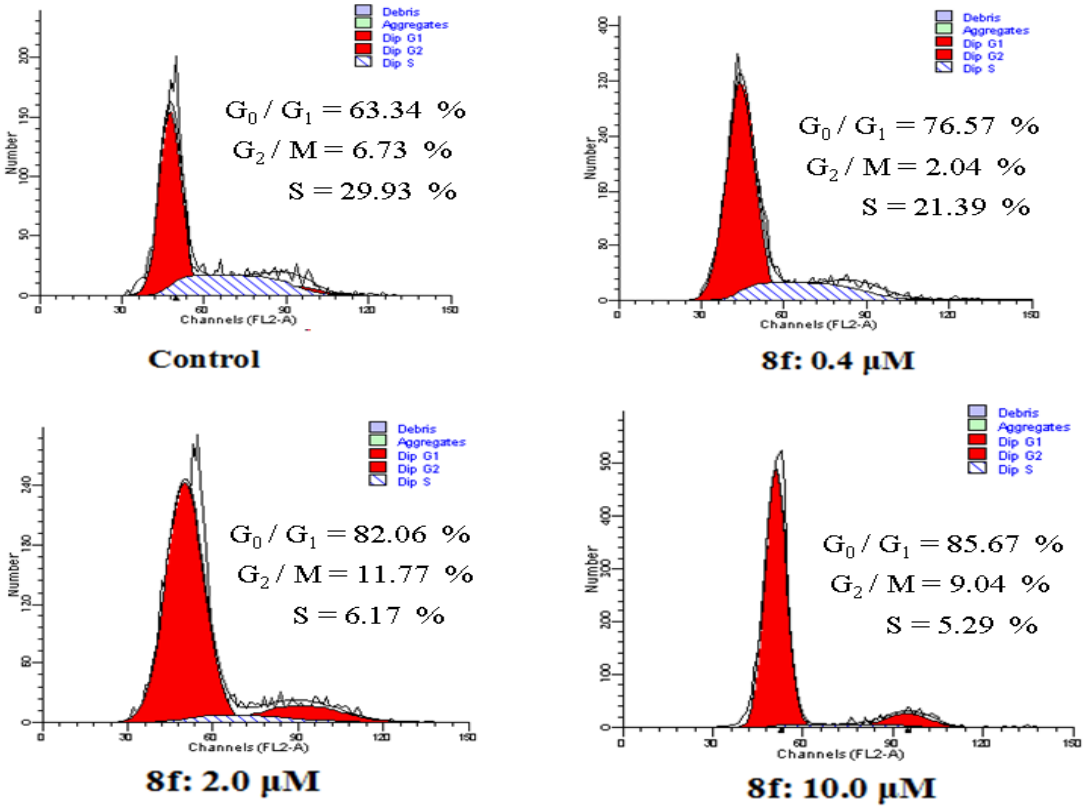


Fig. 1: AO/EB stained apoptosis of A549 cell lines. A549 cells were treated with different concentrations of compound **8f** for 24 h. The white arrow indicates normal cells, the blue arrow indicates early apoptotic cells and the red arrow later apoptotic. Cells.

A



B

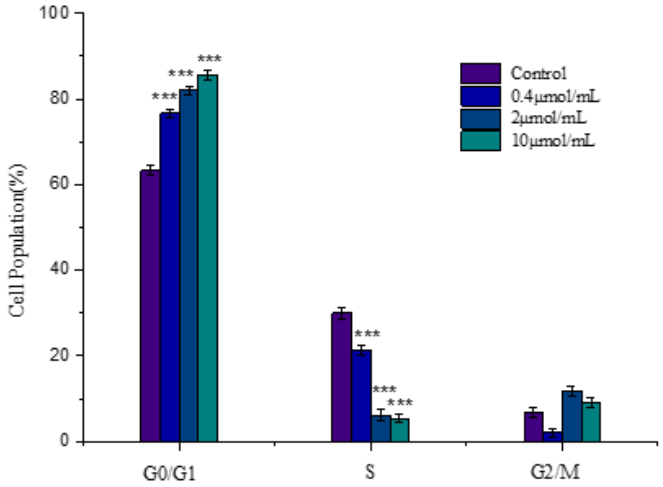


Fig. 2: Effects of compound **8f** on the A549 cells cycle. Cells were treated with compound 8c (0.4, 2, and 10 μmolm/L) for 24 h. (A) Effects of compound **8f** on the cell-cycle distribution of A549 cells. (B) Quantitative analysis of cell-cycle phase. (***) indicate significant difference ($p < 0.001$); analysis of variance [ANOVA] followed by Dunnett's test compared with control group.

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Conclusion

In summary, twelve novel 4-anilinobenzofuro[2,3-d]pyrimidine derivatives were designed, synthesized and evaluated for their biological activitives. The screening of cytotoxicity led to the identification of a most promising compound **8f** with IC_{50} values of 2.54 μM, 2.68 μM and 6.19 μM against A549, H460 and H1975 cell lines, respectively, representing a promising lead for further optimization. The initial SARs analysis disclosed that mono-electron-withdrawing groups (mono-EWGs) on the phenyl ring were more preferred. Meanwhile, AO/EB assays and cell cycle assays on A549 cells results indicated that compound **8f** could induce cells apoptosis and arrest in G_0/G_1 phase in a dose dependent manner. Further studies on structural optimization (especially methyl ester on benzofuro[2,3-d]pyrimidine) and biological activities about these derivatives are still underway in our laboratory and will be reported in the future.

Acknowledgements

The authors thank the financial support of Shenyang Science & Technology project (project no. 18-013-0-03) and Natural Science Foundation of Liaoning Province (project no. 2019-ZD-0191).

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