Synthesis and anticancer activities of diverse furo[2,3-d]pyrimidine and benzofuro[3,2-d]pyrimidine derivatives

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ABSTRACT
A series of diverse furo[2,3-d]pyrimidines (2a–2b, 4a–4d and 8a–8c) and benzofuro[3,2-d]pyrimidines (12a–12c) were synthesized and screened for their antitumor effects against HepG2, Bel-7402 and HeLa cell lines \textit{in vitro}. Representatively, 4a, with an IC\textsubscript{50} of 0.70 \textmu M, exhibited the best antitumor activity against the tested HepG2 cell lines. Molecular docking investigation further revealed the possible binding modes of compound 4a with receptor tyrosine kinase. Preliminary results indicated that the title compounds were helpful as leading structures for preparing a new antitumor drug.

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**Introduction**

Cancer is a serious disease worldwide that threatens human health and reduces patient quality of life. Although chemotherapy has some effects for cancer treatment, many patients still suffer from drug resistance and different degrees of side effects. Therefore, the development of new anticancer drugs is very important.

Heterocycles have been considered templates for drug design and play a significant role in the discovery of anticancer drugs. To date, many furo[2,3-\(d\)]pyrimidines have been reported in the literature to be antitumor active and could be potential tyrosine kinase inhibitors (RTKs). Previously, we discovered that 2,4-diamino-furo[2,3-\(d\)]pyrimidine exhibited potent anticancer activities, suggesting that 2,4-diamino-furo[2,3-\(d\)]pyrimidine could be a highly privileged moiety for the development of novel anticancer drugs (Fig. 1). As a continuation of our studies on the field of synthesis and screening new heterocyclic compounds with antitumor activities, herein, we wish to further report an efficient synthesis and antitumor activities of a series of diverse furo[2,3-\(d\)]pyrimidine and benzofuro[3,2-\(d\)]pyrimidine derivatives.

**Results and discussion**

**Chemistry**

The key intermediate of ethyl 3,4-dihydro-6-methyl-4-oxo-2-arylamino-furo[2,3-\(d\)]pyrimidine-5-carboxylate 1 can be viewed as an initial aza-Wittig reaction starting from diethyl 2-amino-5-methylfuran-3,4-dicarboxylate. The direct reaction of 1 with halohydrocarbon provided ethyl 4-alkoxy-6-methyl-2-arylamino-furo[2,3-\(d\)]pyrimidine-5-carboxylate 2a-2b in good yields in the presence of K\(_2\)CO\(_3\). Compound 3, prepared as described previously, was reacted with amine (amino-acid ester) using tritylamine as a base to give furo[2,3-\(d\)]pyrimidine derivatives 4a-4d, and the synthetic diagram is represented in Scheme 1.

To obtain various substituted furo[2,3-\(d\)]pyrimidine derivatives, the reaction of 3a with morpholine gives compound 5, and then by hydrolyzation and acidification reactions with sodium hydroxide and hydrochloric acid, respectively, 5 was further converted to 6-methyl-4-morpholino-2-(phenylamino)-furo[2,3-\(d\)]pyrimidine-5-carboxylic acid 6. The reaction of 6 with SOCl\(_2\) generates functionalized intermediate 7. In the presence of triethylamine, 7 reacted with morpholine and amino acid ester to give 8a–8c (Scheme 2).

**Figure 1.** Structural modification of diverse furo[2,3-\(d\)]pyrimidine and benzofuro[3,2-\(d\)]pyrimidine derivatives.
According to the effect of the molecular skeleton and substituent group on the biological activity of drugs, benzofuro[3,2-d]pyrimidine derivatives were synthesized by a process similar to that described above from the starting material of the ethyl 3-amino benzofuran-2-carboxylate and aza-wittig reactions (Scheme 3).

**Biological evaluation**

**In vitro anticancer activity**

The antitumor activities of the synthesized compounds 2a–2b, 4a–4d, 8a–8c and 12a–12c were preliminarily evaluated against three human cancer cell lines, Bel-7402, HeLa and HepG-2, in vitro using a standard 2-(2-methoxyl-4-nitrophenyl)- 3-(4-nitrophenyl)-5-(2,4-disulfonyl-benzene)-2H-tetrazoliummonosodium salt (CCK8) assay after
exposure of cells to the tested compounds for 48 h with 5-FU as a positive control experiment and solvent as a negative control experiment. All experiments were performed in triplicate.

As indicated in Table 1, all the compounds showed potential anticancer activity against the three cell lines. The most promising compound, 4a, exhibited the best anti-tumor activity against the HepG2 cell line with an IC$_{50}$ of 0.70 μM, and 2b, 4d, 8c, and 12c had a broad spectrum of cytotoxicity against the HepG2, HeLa and Bel-7402 cell lines with an IC$_{50}$ range of 4.5 – 38.5 μM. Meanwhile, the results also revealed that 2a, 8b, 12b, and 4b presented a certain protective effect on HepG2 and HeLa cell lines.

**Molecular dynamics simulation**

To obtain the stable binding mode of 4a with Abl, molecular dynamics (MD) simulation was performed based on the 4a-EGFR complex structure obtained from the docking results. We used the antechamber module of the Amber16 program$^{[14]}$ to assign bcc charges for the atoms of 4a. The topology and coordinate files of the 4a-EGFR complex were built with the Leap module in the Amber16 program. The AMBER ff14SB force field was used for amino acid residues, and the AMBER gaff force field was used for 4a$^{[15]}$. Cl$^-$ or Na$^+$ was added to neutralize the system. All the molecules were solvated in a rectangular box of TIP3P waters extended at least 10 Å in each direction from the solute.$^{[16]}$ Energy minimization was performed on the system before the MD simulation. The MD simulation was carried out by employing the periodic boundary condition with the NPT ensemble to avoid edge effects. First, 10 picoseconds (ps) simulation was performed on water and ions to obtain an equilibrated solvent environment. Second, the temperature of the system was gradually heated from 10 to 298 K for 50 ps. Finally, to obtain a stable MD trajectory, the systems were run for 5.5 nanoseconds (ns) at 298 K and constant pressure. During the MD simulation, we used the SHAKE algorithm to constrain all covalent bonds involving hydrogen atoms.$^{[16]}$ The particle mesh Ewald (PME) algorithm was used to handle van der Waals (vdW) energy terms as well as the long-range electrostatic interactions with a cutoff distance of 10 Å. The 2.0 femtoseconds
(fs) were used as the time step during the MD simulations, and the coordinates were collected every 1 ps.

**Free energy calculation**

The MM-PBSA method was used to calculate the binding free energy ($\Delta G_{\text{bind}}$) between the receptor and ligand.[17] This value was obtained by calculating the differences in free energies between the ligand–receptor complex ($G_{\text{cpx}}$) and the unbound receptor ($G_{\text{rec}}$) and ligand ($G_{\text{lig}}$) as follows:

$$\Delta G_{\text{bind}} = G_{\text{cpx}} - (G_{\text{rec}} + G_{\text{lig}})$$ (1)

$\Delta G_{\text{bind}}$ consists of the molecular mechanical (MM) gas-phase binding energy ($\Delta E_{\text{MM}}$), solvation free energy ($\Delta G_{\text{sol}}$) and entropic contribution ($-T\Delta S$):

$$\Delta G_{\text{bind}} = \Delta E_{\text{MM}} + \Delta G_{\text{sol}} - T\Delta S$$ (2)

The $\Delta E_{\text{MM}}$ includes two parts, the electrostatic energies ($\Delta E_{\text{ele}}$) and van der Waals interactions ($\Delta E_{\text{vdW}}$):

$$\Delta E_{\text{MM}} = \Delta E_{\text{ele}} + \Delta E_{\text{vdW}}$$ (3)

The $\Delta G_{\text{sol}}$ is made up of electrostatic contribution ($\Delta G_{\text{PB}}$) and nonelectrostatic contribution ($\Delta G_{\text{np}}$) to the solvation free energy. $\Delta G_{\text{PB}}$ is calculated by the Poisson–Boltzman (PB) method using the MM_PBSA module in the amber16 program. $\Delta G_{\text{np}}$ is determined by the solvent accessible surface area.[18]

$$\Delta G_{\text{sol}} = \Delta G_{\text{PB}} + \Delta G_{\text{np}}$$ (4)

For the entropic contribution, an empirical method[19] was used, which consists of two subitems, the solvation entropy change ($\Delta S_{\text{sol}}$) and conformational entropy change ($\Delta S_{\text{conf}}$):

$$\Delta S = \Delta S_{\text{sol}} + \Delta S_{\text{conf}}$$ (5)

The $\Delta S_{\text{sol}}$ is obtained by the tendency of water molecules to minimize their contacts with hydrophobic groups in the protein, and $\Delta S_{\text{conf}}$ is related to the change in the number of rotatable bonds during the binding process. The entropic contribution is evaluated, and the conformational entropy change is proportional to the number ($\Delta N_{\text{rot}}$) of the lost rotatable bonds during binding:

| Table 1. Antitumor activity of compounds 2, 4, 8 and 12 (IC$_{50}$, µM)$^a$. |
|-----------------|----------|----------|----------|----------|----------|----------|
|                 | HeLa     | Bel-7402 | HepG2    | HeLa     | Bel-7402 | HepG2    |
| 2a               | 39.1     | 35.3     | <0       | 8a       | 32.5     | 40.0     | 1.5      |
| 2b               | 8.1      | 38.5     | 4.5      | 8b       | 35.2     | 136.1    | <0       |
| 4a               | 31.9     | 36.8     | 0.7      | 8c       | 24.9     | 25.7     | 24.4     |
| 4b               | <0       | 77.9     | 31.9     | 12a      | 54.0     | 54.7     | 65.3     |
| 4c               | 54.7     | 31.4     | 37.6     | 12b      | 3.3      | 153.0    | <0       |
| 4d               | 22.5     | 28.1     | 34.4     | 12c      | 14.0     | 31.3     | 31.0     |
| 5-FU             | 23.45    | –        | 34.61    |          |          |          |          |

$^a$IC$_{50}$ is the concentration of compound required to inhibit cell growth by 50% compared to an untreated control.
\[ -T\Delta S_{\text{conf}} = w(\Delta N_{\text{rot}}) \]  \hspace{1cm} (6)

in which \( w \) is a scaling factor that was set to be 1 Kcal/mol for the binding energy calculation. Thus, Eq. (1) can be written as:

\[ \Delta G_{\text{bind}} = \Delta E_{\text{MM}} + \Delta G_{\text{PB}} + \Delta G_{\text{np}} - T\Delta S_{\text{sol}} + w(\Delta N_{\text{rot}}) \]  \hspace{1cm} (7)

All other parameters in the energy calculation are the standard parameters or the default values of the Amber16 program.

**Molecular simulation result**

Approximately 5.5 ns of MD simulation was performed on the 4a-Abl complex. The Cpptraj tool in Amber16 was used to plot the root-mean-square displacement (rmsd) values (shown in Fig. 2A). The rmsd plots indicated that the systems of 4a-EGFR achieved equilibrium very quickly. The final conformations of the MD result were used to analyze the binding mode (shown in Fig. 2B). Compound 4a formed two H-bonds with residue D381, which belongs to the DFG motif of Abl. The phenyl ring at the end formed hydrophobic interactions with side chains of residues, including L370, T315 and M290, and at the same time formed \( \pi-\pi \) interactions with DFG-motif residue F382. The binding free energies were calculated by using the molecular mechanics-Poisson-

![Figure 2](image-url)

**Figure 2.** Simulated binding mode of 4a with Abl (PDB ID: 3CS9). (A) Plots of the rmsd of 4a-EGFR vs the simulation time. The black and red lines represent the rmsd of the backbone and ligand atoms, respectively. (B) The binding mode of 4a with Abl from the MD results. The ligand is represented by yellow sticks and balls, and Abl is represented by sky blue sticks and white cartoons. 4a formed two H-bonds with residue D381 (dashed red lines) and hydrophobic interactions with residues L370, T315, and M290. The phenyl ring also formed \( \pi-\pi \) interactions with DFG motif residue F382.
Boltzmann surface area (MM/PBSA)\textsuperscript{[20–22]} binding free energy calculation method (shown in Table 2).

**Conclusion**

In conclusion, we have developed an efficient synthesis for diverse furo[2,3-d]pyrimidine and benzofuro[3,2-d]pyrimidine derivatives. Bioassays indicated that these compounds possess potential antitumor activities, and promising compound 4a stood out as the most potent on HepG2 cell lines with an IC\textsubscript{50} value of 0.70 μM. A molecular simulation study predicted the possible binding mode of compound 4a with its potential target tyrosine kinase, which can help us gain a deeper understanding of the mechanism of action of our compounds to have drug effects. We hope our efforts will be helpful for further anticancer research and development.

**Experimental section**

**General**

Melting points were recorded using an uncorrected X-4 digital melting point apparatus. All chemicals were commercially available, analytically pure and used without further purification. MS was measured by a high-performance liquid chromatography-triple quadrupole tandem mass spectrometer. NMR spectra were recorded in CDCl\textsubscript{3} (or DMSO-d6) using a Varian Mercury 400 spectrometer with resonances relative to tetramethylsilane (TMS) as an internal standard. Elemental analyses were recorded on a PerkinElmer CHN 2400 instrument. TLC analysis was carried out on silica gel plates GF254 (Wuhan, Geao Co.).

**Representative procedure for the synthesis of 8a–8c**

The synthesis of 2-arylamino-6-methyl-4-morpholino-furo[2,3-d]pyrimidine-5-formamide 8a–8c.

To a mixture of 6a (5 mmol), prepared as described in detail previously, in aqueous ethanol solution 50 mL, solid KOH (10 mmol) was added. The reaction mixture was stirred for 8 h at 50°C and then for an additional 1 h at room temperature after completion of the reaction, as indicated by TLC. The solution was concentrated and acidified to pH = 1 with dilute hydrochloric acid in an ice bath. The solid obtained was filtered, washed with water and dried to provide 6a.

A mixture of 6a (3 mmol) and SOCl\textsubscript{2} (1 mL) was stirred at room temperature for 12 h and then concentrated under reduced pressure to remove excessive SOCl\textsubscript{2} to give

**Table 2. Binding free energy calculation of 4a with Abl.**

| Cpd. | ΔE\textsubscript{ele} | ΔE\textsubscript{vdW} | ΔG\textsubscript{np} | ΔH | -TΔS | ΔG\textsubscript{bind} |
|------|----------------------|---------------------|------------------|-----|-------|---------------------|
| 4a   | -1.65                | -64.66              | 30.02            | -41.98 | 22.58 | -19.40              |

ΔE\textsubscript{ele}: electrostatic energies; ΔE\textsubscript{vdW}: van der Waals interaction; ΔG\textsubscript{np}: nonelectrostatic contribution; ΔG\textsubscript{PB}: electrostatic contribution; ΔH: enthalpy change, which is the sum of ΔE\textsubscript{ele}, ΔE\textsubscript{vdW}, ΔG\textsubscript{np}, and ΔG\textsubscript{PB}; -TΔS: entropic contribution; ΔG\textsubscript{bind}: binding free energy.
7a, which was used directly with further purification. Triethylamine (6 mmol) was slowly added to a stirred solution of 7a and amino acid ester (3 mmol) in 15 mL of dry CH₂Cl₂ in an ice bath. The mixture was stirred at room temperature for 20 h, concentrated, and then poured into ice water. The solid obtained was filtered and recrystallized from DMF/CH₂Cl₂ (v:v = 1:4) to give 8a–8c.

(6-Methyl-4-morpholino-2-(phenylamino)furo[2,3-d]pyrimidine-5-yl)-N-(2-ethoxycarbonylmethyl)formamide (8a). White crystals, yield: 0.94 g, 72%, m.p.: 218–220°C. ¹H NMR (400 MHz, CDCl₃): δ (ppm): 7.68 (d, J = 8.0 Hz, 2H, ArH), 7.61–7.65 (m, 1H, NH), 7.35–7.39 (m, 3H, ArH), 7.06 (t, J = 4.0 Hz, 1H, NH), 4.33 (q, J = 8.0 Hz, 2H, CH₂), 4.26 (d, J = 8.0 Hz, 2H, CH₂), 3.76 (t, J = 4.0 Hz, 4H, 2/CH₂), 3.45 (t, J = 4.0 Hz, 4H, 2/CH₂), 2.66 (s, 3H, CH₃), 1.38 (t, J = 8.0 Hz, 3H, CH₃). ¹³C NMR (100 MHz, CDCl₃): δ (ppm): 13.53, 14.22, 41.92, 49.76, 61.86, 66.26, 95.69, 110.53, 118.95, 122.30, 128.90, 139.70, 154.92, 156.09, 160.85, 163.59, 168.03, 169.90. MS (m/z, %) Anal. Calcd for C₂₂H₂₅N₅O₅ (439.18), found: 439.19 (M).

Full experimental details, ¹H and ¹³C NMR, and mass spectra data are provided in the supporting information. This material can be found via the “Supplementary Content” section of this article’s webpage.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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