Syntheses and Cell-Based Phenotypic Screen of Novel 7-Amino pyrido[2,3-d]pyrimidine-6-carbonitrile Derivatives as Potential Antiproliferative Agents

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Received: 12 January 2012; in revised form: 17 February 2012 / Accepted: 20 February 2012 / Published: 24 February 2012

Abstract: A series of N-3-substituted 7-aminopyrido[2,3-d]pyrimidin-6-carbonitrile derivatives was readily synthesized and their anti-proliferative activities on five types of tumor cells were evaluated through a cell-based phenotypic screening approach. Compound 3k was found to be potent on human colon cancer SW620 cells with an IC_{50} value of 12.5 μM. Structural optimization of compound 3k led to compound 4a with improved anti-proliferative potency on SW620 cells with an IC_{50} value of 6.9 μM. Further cell-cycle analyses suggested that compound 4a induced apoptosis of SW620 cells in a concentration-dependent manner.

Keywords: 7-aminopyrido[2,3-d]pyrimidin-6-carbonitrile derivatives; cell-based phenotypic screening; anti-tumor activity; structure-activity relationship (SAR); apoptosis
1. Introduction

Chemotherapy is one of the most commonly used treatment options for malignant tumors, especially for unresectable patients [1]. Improvements in treatment and prevention have led to a decrease in cancer deaths, but the number of new diagnoses continues to rise. New classes of therapies targeting specific proteins perturbed in cancers have been heralded as “smart drugs” that more effectively target the disease than current chemotherapeutic regimes such as doxorubicin, cisplatin and fluorouracil [2]. However, disappointing results in recent clinical trials indicate that a major challenge of target-based drug discovery approaches is overcoming target mechanism heterogeneity among patients and inherent or acquired drug resistance [3]. Consequently, current cancer drug discovery approaches are not appropriately tailored to complex disease mechanism(s) [4]. Phenotypic screening, widely used to find new drugs in old days, is arguably associated with the more humble recognition that you really don’t believe you understand the mechanism [5]. If you can establish a way to make a wayward cell less harmful, the mechanism may not matter all that much. Between 1999 and 2008, the contribution of phenotypic screening to the discovery of first-in-class small-molecule drugs exceeded that of target-based approaches—with 28 and 17 of these drugs coming from the two approaches, respectively [5].

Our research group focused our attention on the design, synthesis and cell-based phenotypic screening of novel tumor growth inhibitors and apoptosis inducer as potential anti-proliferative agents. In recent years nitrogen-containing heteroaromatic species of biological significance have attracted considerable research interest as these entities constitute the core structure of numerous pharmaceuticals. Pyrimidine derivatives have shown remarkable activity as PDE4 inhibitors, antileukemia, bronchodilators, vasodilators, antiallergic, antihypertensive and anticancer agents [6–12]. In the past few years, several synthetic methodologies for 7-aminopyrido[2,3-d]pyrimidine-6-carbonitrile derivatives have been developed [13]. However, the antitumor activities of these compounds have not been fully explored.

In this article we describe a novel series of N-3-substituted 7-aminopyrido[2,3-d]pyrimidine-6-carbonitrile derivatives (Figure 1) and a cell-based phenotypic evaluation of their anti-tumor activities by the MTT method. The cell-cycle analysis of the most potent compound 4a is presented. Their preliminary structure–activity relationships (SARs) are also discussed.

Figure 1. Structure of 7-amino-pyrido[2,3-d]pyrimidine-6-carbonitrile derivatives.

2. Results and Discussion

2.1. Chemistry

The general synthetic approach to the desired final compounds 3a–s is outlined in Scheme 1.
Scheme 1. Synthetic route of compounds 3a–s.

\[
\begin{align*}
\text{ArCHO} & \quad \text{(i)} \quad \text{malononitrile, 6-amino-1-methyluracil or 6-aminouracil, cat.} \\
\text{triethylbenzylammonium chloride (TEBAC), ethylene glycol for 2a–e or H}_2\text{O for 2f–i, 100 °C for} \\
\text{5–24 h. (ii) R}_2\text{-X, K}_2\text{CO}_3, \text{DMF, 60 °C for 3a–i, 3r–s; r.t. for 3j–q.}
\end{align*}
\]

The core structure of 7-aminopyrido[2,3-d]pyrimidine-6-carbonitrile derivatives, was built via a reported multi-component reaction [14] with minor revisions. Briefly, the key intermediates 2a–i were readily synthesized in parallel with an appropriate aromatic aldehyde, 6-methyl-1-amino uracil/6-aminouracil and malononitrile as starting materials in the presence of TEBAC. The final compounds 3a–s were thus obtained by N-3 alkylation of the precursors 2 with R\textsuperscript{2}-X in the presence of potassium carbonate in DMF. To note, derivatives 3a–i and 3r–s were obtained at 60 °C, whereas the reaction at room temperature was found to be optimal to afford compounds 3j–q. The structures of 3a–s were fully characterized and identified by \textsuperscript{1}H-NMR, \textsuperscript{13}C-NMR and HR-MS analyses.

As illustrated in Scheme 2, to find a more potent compound, derivatives 4a–e were prepared starting from 2g or 2j in the presence of K\textsubscript{2}CO\textsubscript{3} or triethylamine at room temperature in DMF. Compounds 4a–e were fully characterized and identified by \textsuperscript{1}H-NMR, \textsuperscript{13}C-NMR and HR-MS before biological evaluation.

Scheme 2. Synthetic route of compounds 4a–e.

\[
\begin{align*}
\text{scheme 2} & \quad \text{synthetic route of compounds 4a–e.} \\
2g: & \quad R^4 = \text{OMe} \\
2j: & \quad R^4 = \text{H}
\end{align*}
\]

Reagents and conditions: R\textsuperscript{2}-X (X=Cl, Br), K\textsubscript{2}CO\textsubscript{3}, (triethylamine for 4e), DMF, r.t.

2.2. Anti-proliferative Activities

All the final compounds underwent primary phenotypic screening for their inhibitory activity against A549, HepG2, SW620, Skov-3 and HeLa tumor cells at 40 μM using the MTT assay. For comparison, the data of cisplatin are also included. As shown in Table 1, some derivatives (compounds 3j–s) exerted potent or moderate inhibitory activities against the five assayed tumor cell lines at 40 μM (Table 1). The inhibitory activities of most compounds on SW620 cells were slightly more potent than...
those for human lung carcinoma A549 cells, human hepatocellular liver carcinoma HepG2 cells, human cervical carcinoma epithelial HeLa cells and human ovarian cancer Skov-3 cells. In our initial efforts to search for novel potent anticancer agents, a caffeine moiety, reported to suppress the proliferation of various cancer cell lines and transformed cell lines [15,16], was covalently coupled to the 7-aminopyrido[2,3-d]pyrimidine-6-carbonitrile scaffold through a two-carbon linker at the N-3 position. Unfortunately, the obtained compound 3a demonstrated low inhibitory effects on the five tested tumor cell lines. Further modification of compound 3a with various substituents at the C-5 position of the pyrido[2,3-d]pyrimidine scaffold showed no improvement in activities.

Table 1. Inhibition of compounds 3a–s on five types of tumor cells at 40 μM.

| Compd. | R¹ | R² | Ar | SW620 (% inhibition) | A549 (% inhibition) | SKOV-3 (% inhibition) | HepG2 (% inhibition) | HeLa (% inhibition) |
|--------|----|----|----|----------------------|---------------------|----------------------|---------------------|---------------------|
| 3a     | H  | —  | Phenyl | 9 ± 2.3 | 10 ± 2.6 | NT b | NT | 37 ± 3.0 |
| 3b     | H  | —  | 4-Cl-Ph- | 8 ± 1.4 | 3 ± 1.5 | 12 ± 2.2 | NT | 18 ± 2.1 |
| 3c     | H  | —  | 3,4-di MeO-Ph- | 11 ± 0.9 | 22 ± 0.5 | NT | 29 ± 2.8 | 29 ± 2.1 |
| 3d     | H  | —  | 3-MeO-4-OH-Ph- | 13 ± 3.0 | 9 ± 2.2 | 18 ± 2.4 | 10 ± 1.8 | 20 ± 1.1 |
| 3e     | H  | —  | 4-Br-Ph- | 27 ± 1.2 | 28 ± 2.9 | NT | 21 ± 2.8 | 34 ± 1.6 |
| 3f     | Me | —  | Phenyl | 7 ± 1.7 | NT | NT | NT |  |
| 3g     | Me | —  | 3-MeO-4-OH-Ph- | 3 ± 1.3 | NT | NT | NT | 17 ± 3.1 |
| 3h     | Me | —  | 3,4-di MeO-Ph- | 10 ± 0.8 | 16 ± 1.7 | NT | 17 ± 2.2 | 23 ± 0.7 |
| 3i     | Me | —  | Thiazolyl | NT | NT | NT | NT |  |
| 3j     | Me | 2-methylbenzyl | 3,4-di MeO-Ph- | 14 ± 2.1 | NT | NT | NT | NT |
| 3k     | Me | 2-methylbenzyl | 3-MeO-4-(2-Me-BnO)-Ph | 85 ± 2.8 | 26 ± 3.6 | NT | 28 ± 3.3 | 9 ± 4.0 |
| 3l     | Me | 2-methylbenzyl | Phenyl | 36 ± 0.9 | NT | NT | 10 ± 3.8 | NT |
| 3m     | Me | 2-fluorobenzyl | Phenyl | 48 ± 4.3 | 52 ± 2.3 | 32 ± 2.1 | 20 ± 1.9 | NT |
| 3n     | Me | 3-fluorobenzyl | Phenyl | 42 ± 3.1 | 55 ± 1.1 | NT | 16 ± 0.5 | 14 ± 2.8 |
| 3o     | Me | 2-(4-fluorophenyl)-2-oxoethyl | Phenyl | 38 ± 1.7 | 22 ± 3.9 | 8 ± 2.5 | 24 ± 5.4 | 23 ± 1.9 |
| 3p     | Me | 2-(4-methoxyphenyl)-2-oxoethyl | Phenyl | 21 ± 0.9 | 38 ± 2.8 | 38 ± 1.0 | 20 ± 1.4 | NT |
| 3q     | Me | Propargyl | Phenyl | 25 ± 3.7 | 28 ± 1.6 | 7 ± 2.9 | 20 ± 3.3 | 19 ± 0.8 |
| 3r     | Me | Cyclopentyl | Phenyl | 52 ± 2.2 | NT | NT | 21 ± 1.7 | 14 ± 3.6 |
| 3s     | Me | Butyl | Phenyl | 35 ± 1.5 | 20 ± 1.8 | NT | 13 ± 4.5 |  |
| Cis c  | —  | —  | —  | 72 ± 2.1 | 68 ± 1.8 | 67 ± 1.9 | 80 ± 2.8 | 80 ± 3.5 |

a IN = inhibition, measured 48 h after treatment with compounds 3a–s (40 μM). Results are given in concentrations of 40 μM after continuous exposure of 48 h and show means ± SEM values of three-independent experiments. IR = OD(C) – OD(S) / OD(C); b NT denotes not tested; c Cis denotes cisplatin.

This finding could have been due to the excessive numbers of hydrophilic nitrogen atoms in the caffeine substituent. Hence, coupling caffeine with the pyrido[2,3-d]pyrimidine scaffold did not
work as expected. Thus, we turned to link more lipophilic groups at N-3 position to investigate whether thus obtained derivatives would inhibit tumor cell proliferation more effectively. Compounds 3j–s, with certain lipophilic alkyl or aryl groups incorporated into the N-3 position, exhibited higher inhibition values (Table 1) as proposed.

To obtain more accurate data on the anti-proliferative activities of derivatives 3j–s, we conducted an MTT assay against SW620 tumor cells to measure their IC\textsubscript{50} values. The concentrations of the assayed compound are in a range from 2.5 to 80 \( \mu \)M. As shown in Table 2, derivative 3k was found to be the most potent one against human colon cancer cells SW620 at low micromolar level, with an IC\textsubscript{50} value of 12.5 \( \mu \)M, comparable to that of cisplatin. However, compound 3k exhibited extremely poor inhibitory potency for A549, Skov-3, HepG-2 and HeLa cell lines (Table 1).

### Table 2. IC\textsubscript{50} values of compounds 3j–s on SW620 cells.

| Compd. | IC\textsubscript{50}\textsuperscript{a}(\( \mu \)M) | Compd. | IC\textsubscript{50}(\( \mu \)M) | Compd. | IC\textsubscript{50}(\( \mu \)M) | Compd. | IC\textsubscript{50}(\( \mu \)M) |
|--------|-----------------|--------|-----------------|--------|-----------------|--------|-----------------|
| 3j     | >80             | 3p     | 59.6 ± 2.1      | 3m     | 65.1 ± 1.1      | 3s     | >80             |
| 3k     | 12.5 ± 0.7      | 3q     | 71.9 ± 1.5      | 3n     | 79.6 ± 2.7      | Cis\textsuperscript{b} | 9.5 ± 0.5\textsuperscript{c} |
| 3l     | 76.1 ± 1.9      | 3r     | 29 ± 0.9        | 3o     | 71.5 ± 1.8      |

\textsuperscript{a}IC\textsubscript{50} denotes half maximal inhibitory concentration. Values are means ± SEM of three independent experiments; \textsuperscript{b}Cis denotes cisplatin; \textsuperscript{c}The IC\textsubscript{50} value is comparable to the reported in the literature [17].

To find a more potent compound, derivatives 4a–e were readily prepared starting from 2g or 2j in the presence of K\textsubscript{2}CO\textsubscript{3} or triethylamine at room temperature in DMF (Scheme 2). Compounds 4a–e were fully characterized and identified by \textsuperscript{1}H-NMR, \textsuperscript{13}C-NMR and HR-MS before biological evaluation. The IC\textsubscript{50} values of compounds 4a–e on SW620 tumor cells are shown in Table 3.

### Table 3. IC\textsubscript{50} values of compounds 4a–e on SW620 cells.

| Compd. | R\textsuperscript{2} | R\textsuperscript{3} | R\textsuperscript{4} | IC\textsubscript{50}\textsuperscript{a}(\( \mu \)M) |
|--------|-----------------|-----------------|-----------------|-----------------|
| 4a     |                 |                 | OMe            | 6.9 ± 0.4      |
| 4b     | "               | "               | H              | >40             |
| 4c     |                 | "               | OMe            | >40             |
| 4d     |                 | "               | H              | >40             |
| 4e     | H               |                 | OMe            | 36.8 ± 1.1     |
| Cis\textsuperscript{b} | -       | -               | -              | 9.5 ± 0.5       |

\textsuperscript{a}IC\textsubscript{50} denotes half maximal inhibitory concentration. Values are means ± SEM of three independent experiments; \textsuperscript{b}Cis denotes cisplatin.
Special emphasis was placed upon SAR studies on the 3-position of the pyrido[2,3-d]pyrimidine scaffold and 3-, 4-positions of the phenyl(Ar) at the 5-position of the scaffold (Table 3). When R⁴ was a methoxy group, R² and R³ were substituted by the benzyl or 1-phenylethanone-2-yl group, respectively, and two compounds, 4a and 4c, were obtained with notably different IC₅₀ values on SW620 cells, 6.9 μM for 4a and above 40 μM for 4c. This result suggested that the carbonyl group of the 1-phenylethanone-2-yl substituent decreased anti-proliferative activities. We next investigated if the methoxy group (R⁴) at C-5 position of the pyrido[2,3-d]pyrimidine scaffold was necessary by removing the methoxy group. Consequently, compound 4b was synthesized with a hydrogen atom for R⁴, which led to almost total loss of activity (Table 3). Thus, a methoxy group of R⁴ plays a crucial part in contributing to biological activities for pyrido[2,3-d]pyrimidine derivatives.

2.3. Cell-Cycle Analyses

Cell-cycle analyses were done by flow cytometric measurements on SW620 cells. annexin V-fluorescein isothiocyanate (FITC) was used as a marker of phosphatidylserine exposure and propidium iodide (PI) as a marker for dead cells. This combination allowed differentiation between early apoptotic cells (annexin V-positive, PI-negative), late apoptotic/necrotic cells (annexin V-positive, PI-positive), and viable cells (annexin V-negative, PI-negative).

**Figure 2.** Effects of 4a on the induction of apoptosis in SW620 cells for 48 h: a, control; b, 5 μM; c, 10 μM; d, 20 μM; e, 40 μM. Cells were stained with annexin V-FITC and PI. The total number of apoptotic cells are the sum of annexin V+/PI− (early apoptotic) and annexin V+/PI+ (late apoptotic/necrotic) cell populations.
After treatment with compound 4a at 0, 5, 10, 20 and 40 μM for 48 h, the percentage of apoptotic cells was 15.11%, 17.06%, 61.12%, 76.33% and 89.26% (Figure 2). These results indicated that the growth inhibition of SW620 cells was caused by inducing apoptosis in a concentration-dependent manner [18].

3. Experimental

3.1. General

Human cancer cell lines were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). Dulbecco’s modified Eagle’s medium (DMEM) and RPMI 1640 were purchased from Gibco (Grand Island, New York, NY, USA). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT, USA). All chemicals were commercially available and used without further purification unless otherwise stated. Column chromatography was carried out on silica gel (400 mesh, Qingdao Marine Chemical Ltd., Qingdao, China). Thin-layer chromatography (TLC) was undertaken on TLC silica gel 60 F254 plates. 1H-NMR and 13C-NMR spectra were recorded on a Bruker Avance (Varian Unity Inova) 400 MHz spectrometer using TMS as internal reference chemical shift in δ, ppm. Chemical shifts (δ) are reported in parts per million relative to tetramethylsilane (TMS) used as an internal standard, where (δ) TMS = 0.00 ppm. High-resolution mass spectrometry was carried out on a Waters Q-TOF Premier mass spectrometer.

3.2. General Procedure for the Synthesis of 2a–j

For derivatives 2a–e (Figure 3), 6-aminouracil (10 mmol) were suspended in ethylene glycol (120 mL) at 100 °C, the appropriate aldehyde (10 mmol) and propanedinitrile (10 mmol) were added in one portion.

![Figure 3. The chemical structure of compounds 2a–e.](image-url)
After the mixture turned transparent, TEBAC (150 mg) was added to the mixture as phase transfer catalyst. The resultant mixture was stirred at 100 °C for 4–7 h, cooled to room temperature, The mixture was filtered, washed with EtOH, the solids 2a–e formed were recrystallized from DMF and water. Their physical appearance, melting point and spectroscopic data were in agreement with published data.

For derivatives 2f–j (Figure 4), 6-amino-1-methyluracil (10 mmol) were suspended in water (120 mL) at 100 °C, aldehyde (10 mmol) and propanedinitrile (10 mmol) were added until the suspension almost cleared up, then the TEBAC (150 mg) was added in the mixture as phase transfer catalyst, the mixture was stirred at 100 °C for 20–24 h, cooled to room temperature, The mixture was filtered, washed with EtOH, the solids 2f–j formed were recrystallized from DMF and water. Their physical appearance, melting point and spectroscopic data were in agreement with published data.

Figure 4. The chemical structure of compounds 2f–j.

3.3. The Structure of Reagents R2-X (Figure 5) and General Procedure for the Synthesis of 1-(2-Bromoethyl)-3,7-dimethyl-1H–purine-2,6(3H,7H)-diones

Anhydrous cesium carbonate (50 mmol) and theobromine (25 mmol) were suspended in DMF (100 mL), after the mixture was stirred at room temperature for 10 min, 1,2-dibromoethane (250 mmol) was added. After completion of the reaction as monitored by TLC, the mixture was poured into water, extracted with ethyl acetate, the organic layer was washed with water twice, and dried with anhydrous sodium sulfate. The combined organic layer was evaporated to obtain a residue which was purified by column chromatography. Its physical appearance and spectroscopic data were in agreement with published data.
3.4. Syntheses of Compounds 3a–s

The corresponding compounds 2 (1 mmol) and anhydrous potassium carbonate were stirred at 60 °C (for 3f–q, room temperature) in DMF (5 mL), then R₂-X (1.1 mmol) was added. After completion of the reaction monitored by TLC, the mixture was poured into water (25 mL). It was extracted with dichloromethane (for 3f–q, ethyl acetate was used). The combined organic layer was washed with water, dried with Na₂SO₄, evaporated to obtain a residue, and purified by column chromatography.

7-Amino-3-(2-(3,7-dimethyl-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-1-yl)ethyl)-2,4-dioxo-5-phenyl-1,2,3,4-tetrahydropyrido[2,3-d]pyrimidine-6-carbonitrile (3a) White solid; Yield: 40.1%; ¹H-NMR (DMSO-d₆) δ 11.11 (s, 1H), 8.00 (s, 1H), 7.65 (s, 2H), 7.41 (d, J = 2.4 Hz, 2H), 7.26 (t, J = 2.8 Hz, 1H), 7.15 (t, J = 3.2 Hz, 2H), 4.47 (s, 2H), 4.26 (s, 2H), 3.79 (s, 3H), 3.35 (s, 3H); ¹³C-NMR (DMSO-d₆): δ 160.78, 159.91, 158.91, 155.46, 155.00, 154.56, 150.99, 150.54, 150.19, 148.17, 142.84, 136.65, 128.23, 127.65, 127.37, 115.24, 106.37, 98.89, 98.22, 88.61, 87.80, 33.08, 29.34; HRMS: calcd. for C₂₃H₁₉N₉O₄⁺ [M+Na⁺]: 508.1458, found: 508.1486.

7-Amino-5-(4-chlorophenyl)-3-(2-(3,7-dimethyl-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-1-yl)ethyl)-2,4-dioxo-1,2,3,4-tetrahydropyrido[2,3-d]pyrimidine-6-carbonitrile (3b) White solid; Yield: 57.3%; ¹H-NMR (DMSO-d₆) δ 11.17 (s, 1H), 8.00 (s, 1H), 7.69 (s, 2H), 7.49 (d, J = 8.8 Hz, 2H), 7.19 (d, J = 8.8 Hz, 2H), 4.46 (d, J = 3.2 Hz, 2H), 4.26 (d, J = 3.2 Hz, 2H), 3.79 (s, 3H), 3.35 (s, 3H); ¹³C-NMR (DMSO-d₆): δ 159.83, 158.82, 157.56, 154.99, 154.55, 150.98, 150.49, 148.18, 142.84, 135.53, 133.06, 129.41, 127.78, 114.93, 106.37, 98.84, 87.62, 67.19, 60.70, 59.72, 33.08, 29.25, 19.93; HRMS: calcd. for C₂₃H₁₈ClN₉O₄⁺ [M+Na⁺]: 542.1068, 544.1038, found: 542.1012, 544.1064.

7-Amino-5-(3,4-dimethoxyphenyl)-3-(2-(3,7-dimethyl-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-1-yl)ethyl)-2,4-dioxo-1,2,3,4-tetrahydropyrido[2,3-d]pyrimidine-6-carbonitrile (3c) White solid; Yield: 32.2%; ¹H-NMR (DMSO-d₆) δ 11.09 (s, 1H), 8.00 (s, 1H), 7.58 (s, 2H), 6.99 (d, J = 8.4 Hz, 1H), 6.76 (d, J = 2 Hz, 1H), 6.71 (dd, J = 8 Hz, J = 2 Hz, 1H), 4.46 (m, 2H), 4.25 (s, 2H), 3.81 (s, 3H), 3.79 (s, 3H), 3.72 (s, 3H), 3.35 (s, 3H); ¹³C-NMR (DMSO-d₆): δ 159.87, 158.73, 158.00, 154.56, 151.00, 150.53, 148.93, 148.71, 148.22, 142.82, 128.73, 120.18, 115.34, 111.79, 110.88, 106.38, 99.03, 88.07, 67.19,
7-Amino-3-(2-(3,7-dimethyl-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-1-yl)ethyl)-5-(4-hydroxy-3-methoxyphenyl)-2,4-dioxo-1,2,3,4-tetrahydropyrido[2,3-d]pyrimidine-6-carbonitrile (3d) White solid; Yield: 30.5%; \(^1\)H-NMR (DMSO-d\(_6\)) \(\delta\) 11.07 (s, 1H), 9.25 (s, 1H), 7.53 (s, 2H), 6.80 (d, \(J = 8\) Hz, 1H), 6.73 (d, \(J = 1.6\) Hz, 1H), 6.58 (dd, \(J = 7.6\) Hz, \(J = 1.2\) Hz, 1H), 4.46 (d, \(J = 19.6\) Hz, 2H), 4.25 (s, 2H), 3.79 (s, 3H), 3.73 (s, 3H), 3.35 (s, 3H); \(^1\)C-NMR (DMSO-d\(_6\)): \(\delta\) 159.88, 159.05, 158.75, 155.02 (2C), 150.98, 150.54, 148.18(2C), 146.95, 146.76, 142.82, 127.23, 120.61, 114.83(2C), 112.32, 106.37, 99.03, 88.11, 55.67(2C), 33.07, 29.23, 19.92; HRMS: calcd. for C\(_{25}\)H\(_{23}\)N\(_9\)O\(_6\) \([\textrm{M+Na}^+\]): 568.1669, found: 568.1703.

7-Amino-5-(4-bromophenyl)-3-(2-(3,7-dimethyl-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-1-yl)ethyl)-2,4-dioxo-1,2,3,4-tetrahydropyrido[2,3-d]pyrimidine-6-carbonitrile (3e) White solid; Yield: 27.7%; \(^1\)H-NMR (DMSO-d\(_6\)) \(\delta\) 11.18 (s, 1H), 8.00 (s, 1H), 7.64 (s, 2H), 7.63 (d, \(J = 8.4\) Hz, 1H), 7.13 (d, \(J = 8.4\) Hz, 1H), 4.46 (s, 2H), 4.25 (s, 2H), 3.79 (s, 3H), 3.36 (s, 3H); \(^1\)C-NMR (DMSO-d\(_6\)): \(\delta\) 159.83, 158.83, 157.57, 155.00, 154.56, 150.99, 150.50, 148.19, 142.86, 135.94, 130.76, 129.57, 121.72, 114.93, 106.37, 98.79, 87.53, 59.72(2C), 33.09, 29.34, 20.73, 14.05; HRMS: calcd. for C\(_{23}\)H\(_{18}\)BrN\(_9\)O\(_4\) \([\textrm{M-H}^+\]): 562.0587, 564.0566, found: 562.0511, 564.0612.

7-Amino-3-(2-(3,7-dimethyl-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-1-yl)ethyl)-1-methyl-2,4-dioxo-5-phenyl-1,2,3,4-tetrahydropyrido[2,3-d]pyrimidine-6-carbonitrile (3f) White solid; Yield: 57.8%; \(^1\)H-NMR (DMSO-d\(_6\)) \(\delta\) 8.08 (s, 1H), 7.89 (s, 2H), 7.34 (t, \(J = 7.6\) Hz, 1H), 7.25 (t, \(J = 7.6\) Hz, 2H), 6.83 (d, \(J = 7.2\) Hz, 2H), 4.06 (m, 4H), 3.76 (s, 3H), 3.46 (s, 3H), 3.30 (s, 3H); \(^1\)C-NMR (DMSO-d\(_6\)): \(\delta\) 160.75, 159.89, 158.83, 157.57, 155.00, 154.56, 150.99, 150.50, 148.19, 142.86, 135.94, 130.76, 129.57, 121.72, 114.93, 106.37, 98.79, 87.53, 59.72(2C), 33.52, 29.96, 29.74; HRMS: calcd. for C\(_{24}\)H\(_{21}\)N\(_9\)O\(_4\) \([\textrm{M+Na}^+\]): 522.1614, found: 522.1642.

7-Amino-5-(3,4-dimethoxyphenyl)-3-(2-(3,7-dimethyl-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-1-yl)ethyl)-1-methyl-2,4-dioxo-1,2,3,4-tetrahydropyrido[2,3-d]pyrimidine-6-carbonitrile (3h) White solid; Yield: 49.5%; \(^1\)H-NMR (DMSO-d\(_6\)) \(\delta\) 8.06 (s, 1H), 7.87 (s, 2H), 7.77 (s, 1H), 6.76 (d, \(J = 8.4\) Hz, 1H), 6.62 (d, \(J = 8.4\) Hz, 1H), 6.20 (dd, \(J = 8.4\) Hz, \(J = 1.6\) Hz, 1H), 4.18 (m, 2H), 3.98 (m, 2H), 3.77 (s, 3H), 3.68 (s, 3H), 3.43 (s, 3H), 3.31 (s, 3H); \(^1\)C-NMR (DMSO-d\(_6\)): \(\delta\) 160.77, 159.84, 158.55, 154.08, 154.65, 152.05(2C), 148.79, 147.30(2C), 143.96, 127.96, 127.30, 115.68, 106.96, 98.56(2C), 88.90(2C), 33.52, 29.96, 29.74; HRMS: calcd. for C\(_{25}\)H\(_{23}\)N\(_9\)O\(_6\) \([\textrm{M+Na}^+\]): 568.1669, found: 568.1605.

7-Amino-5-(3,4-dimethoxyphenyl)-3-(2-(3,7-dimethyl-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-1-yl)ethyl)-1-methyl-2,4-dioxo-1,2,3,4-tetrahydropyrido[2,3-d]pyrimidine-6-carbonitrile (3i) White solid; Yield: 52.1%; \(^1\)H-NMR (DMSO-d\(_6\)) \(\delta\) 9.21 (s, 1H), 8.04 (s, 1H), 7.83 (s, 2H), 7.77 (s, 1H), 6.76 (d, \(J = 8.4\) Hz, 1H), 6.62 (d, \(J = 8.4\) Hz, 1H), 6.20 (dd, \(J = 8.4\) Hz, \(J = 1.6\) Hz, 1H), 4.18 (m, 2H), 3.98 (m, 2H), 3.77 (s, 3H), 3.68 (s, 3H), 3.43 (s, 3H), 3.31 (s, 3H); \(^1\)C-NMR (DMSO-d\(_6\)): \(\delta\) 160.77, 159.84, 158.55, 154.08, 154.65, 152.05(2C), 148.79, 147.30(2C), 143.96, 127.96, 127.30, 115.68, 106.96, 98.56(2C), 88.90(2C), 33.52, 29.96, 29.74; HRMS: calcd. for C\(_{25}\)H\(_{23}\)N\(_9\)O\(_6\) \([\textrm{M+Na}^+\]): 568.1669, found: 568.1605.
55.98, 55.86, 33.55, 29.96, 29.78, 21.22, 14.55; HRMS: calcd. for $C_{26}H_{25}N_9O_6^+$ $[M+Na^+]$: 582.1825, found: 582.1877.

7-Amino-3-(2-(3,7-dimethyl-2,6-dioxo-2,3,6,7-tetrahydro-1H-purin-1-yl)ethyl)-1-methyl-2,4-dioxo-5-(thiophen-2-yl)-1,2,3,4-tetrahydropyrido[2,3-d]pyrimidine-6-carbonitrile (3i) White solid; Yield: 60.3%; $^1$H-NMR (DMSO-d$_6$) $\delta$ 8.04 (s, 1H), 7.96 (s, 2H), 7.59 (d, $J$ = 4.8 Hz, 1H), 7.01 (t, $J$ = 3.2 Hz, 1H), 6.81 (d, $J$ = 2.4 Hz, 1H), 4.08 (d, $J$ = 5.2 Hz, 4H), 3.77 (s, 3H), 3.44 (s, 3H), 3.31 (s, 3H); $^{13}$C-NMR (DMSO-d$_6$): $\delta$ 161.03, 158.95, 154.12, 151.05, 149.88, 148.62, 137.76, 136.25, 130.89, 128.43, 128.02, 123.52, 116.83, 112.04, 111.80, 101.62, 90.85, 56.66, 55.89, 45.73, 30.68, 16.98; HRMS: calcd. $[M+Na^+]$: 582.1178, found: 582.1096.

7-Amino-5-(3,4-dimethoxyphenyl)-1-methyl-3-(2-methylbenzyl)-2,4-dioxo-1,2,3,4-tetrahydropyrido[2,3-d]pyrimidine-6-carbonitrile (3j) White solid; Yield: 57.8%; $^1$H-NMR (CDCl$_3$) $\delta$ 7.12 (s, 1H), 7.11 (d, $J$ = 1.2 Hz, 1H), 7.06 (m, 1H), 6.96 (d, $J$ = 8 Hz, 2H), 6.90 (dd, $J$ = 8 Hz, $J$ = 2 Hz, 1H), 6.73 (d, $J$ = 2 Hz, 1H), 5.70 (s, 2H), 5.10 (s, 2H), 3.92 (s, 3H), 3.80 (s, 3H), 3.64 (s, 3H); $^{13}$C-NMR (DMSO-d$_6$): $\delta$ 160.16(2C), 158.77, 154.22, 151.05, 149.88, 148.62, 135.76, 134.44, 130.30, 128.23, 127.02, 125.84, 125.59, 120.20, 115.53, 111.24, 110.70, 100.42, 90.35, 55.89, 55.75, 42.23, 30.27, 19.26; HRMS: calcd. for $C_{25}H_{23}N_5O_4^+$ $[M+Na^+]$: 480.1648, found: 480.1604.

7-Amino-5-(3-methoxy-4-(2-methylbenzyloxy)phenyl)-1-methyl-3-(2-methylbenzyl)-2,4-dioxo-1,2,3,4-tetrahydropyrido[2,3-d]pyrimidine-6-carbonitrile (3k) White solid; Yield: 28.8%; $^1$H-NMR (CDCl$_3$) $\delta$ 7.46 (d, $J$ = 7.2 Hz, 1H), 7.26–7.21 (m, 3H), 7.13 (t, $J$ = 2.8 Hz, 2H), 7.10–7.07 (m, 1H), 7.02 (d, $J$ = 8.8 Hz, 1H), 6.97 (d, $J$ = 7.6 Hz, 1H), 6.88 (dd, $J$ = 8 Hz, $J$ = 2Hz, 1H), 6.78 (d, $J$ = 1.6 Hz, 1H), 5.71 (s, 2H), 5.15(s, 2H), 5.13 (s, 2H), 3.80 (s, 3H), 3.66 (s, 3H), 2.41 (s, 3H), 2.39 (s, 3H); $^{13}$C-NMR (DMSO-d$_6$): $\delta$ 160.88, 159.84, 158.73, 154.00, 152.80(2C), 148.71, 148.59, 137.28, 135.40, 135.29, 135.26, 130.56, 130.26, 129.97, 129.33, 128.63, 126.91, 126.26, 125.04, 120.46, 116.11, 112.76, 112.47, 99.29, 89.36, 68.96, 55.98, 42.23, 30.24, 19.13, 18.94; HRMS: calcd. for $C_{32}H_{29}N_5O_4^+$ $[M-H^+]$: 546.2141, found: 546.2157.

7-Amino-1-methyl-3-(2-methylbenzyl)-2,4-dioxo-5-phenyl-1,2,3,4-tetrahydropyrido[2,3-d]pyrimidine-6-carbonitrile (3l) White solid; Yield: 65.8%; $^1$H-NMR (CDCl$_3$) $\delta$ 7.48 (t, $J$ = 3.2 Hz, 3H), 7.28 (d, $J$ = 3.6 Hz, 2H), 7.11 (d, $J$ = 3.6 Hz, 2H), 7.07 (m, 1H), 6.90 (d, $J$ = 7.6 Hz, 1H), 5.71 (s, 2H), 5.08 (s, 2H), 3.65 (s, 3H), 2.35 (s, 3H), 13C-NMR (DMSO-d$_6$): $\delta$ 160.49, 159.92, 158.82, 154.24, 151.32, 150.06, 148.77, 136.18, 135.63, 134.28, 130.26, 129.19, 128.32, 127.07, 126.99, 125.91, 125.35, 115.19, 100.56, 90.60, 42.20, 30.26, 19.21; HRMS: calcd. for $C_{23}H_{19}N_5O_2^+$ $[M+H^+]$: 398.1617, found: 398.1685.

7-Amino-3-(2-fluorobenzyl)-1-methyl-2,4-dioxo-5-phenyl-1,2,3,4-tetrahydropyrido[2,3-d]pyrimidine-6-carbonitrile (3m) White solid; Yield: 60.2%; $^1$H-NMR (CDCl$_3$) $\delta$ 7.51 (t, $J$ = 2.8 Hz, 3H), 7.38 (dd, $J$ = 8.4 Hz, $J$ = 5.2 Hz, 2H), 7.26 (s, 2H), 6.93 (t, $J$ = 8.8 Hz, 2H), 5.68 (s, 2H), 5.03 (s, 2H), 3.62 (s, 3H), 2.18 (s, 3H); $^{13}$C-NMR (DMSO-d$_6$): $\delta$ 160.49, 159.94, 159.87, 158.70, 154.12, 150.80, 136.14, 131.03, 130.98, 129.25, 128.95, 128.92, 128.35, 127.10, 123.98(2C), 115.42, 115.14, 100.37, 90.26, 43.84, 38.67, 30.21; HRMS: calcd. for $C_{22}H_{16}FN_5O_2^+$ $[M-H^+]$: 400.1210, found: 400.1248.
7-Amino-3-(3-fluorobenzyl)-1-methyl-2,4-dioxo-5-phenyl-1,2,3,4-tetrahydropyrido[2,3-d]pyrimidine-6-carbonitrile (3n) White solid; Yield: 66.7%; $^1$H-NMR (CDCl$_3$) δ 7.51 (t, $J = 3.2$ Hz, 3H), 7.28–7.19 (m, 3H), 7.14 (d, $J = 7.6$ Hz, 1H), 7.05 (d, $J = 10$ Hz, 1H), 6.94–6.89 (m, 1H), 5.70 (s, 2H), 5.05 (s, 2H), 3.63 (s, 3H); $^{13}$C-NMR (DMSO-d$_6$): δ 163.94, 161.49, 160.47, 159.95, 158.68, 154.07, 150.98, 139.06, 136.21, 129.82, 129.24, 128.35, 127.11, 124.43, 115.62, 115.13, 115.38, 114.41, 100.39, 90.31, 44.09, 30.22; HRMS: calcd. for C$_{22}$H$_{16}$FN$_5$O$_2$ [M-H$^+$]: 400.1210, found: 400.1296.

7-Amino-3-(2-(4-fluorophenyl)-2-oxoethyl)-1-methyl-2,4-dioxo-5-phenyl-1,2,3,4-tetrahydropyrido[2,3-d]pyrimidine-6-carbonitrile (3o) Yellow solid; Yield: 48.0%; $^1$H-NMR (CDCl$_3$) δ 7.95 (dd, $J = 8.8$, $J = 5.2$ Hz, 2H), 7.46 (t, $J = 3.6$Hz, 3H), 7.26 (d, $J = 9.2$Hz, 2H), 7.12 (t, $J = 8.6$ Hz, 2H), 5.74 (s, 2H), 5.32 (s, 2H), 3.66 (s, 3H); $^{13}$C-NMR (DMSO-d$_6$): δ 191.68, 166.53, 160.90, 160.12, 158.37, 154.23, 151.00, 137.43, 131.60, 131.53, 128.64, 128.23(2C), 127.68(3C), 116.55, 116.41, 115.65, 98.69, 89.53, 47.82, 30.20; HRMS: calcd. for C$_{23}$H$_{16}$FN$_5$O$_3$ [M-H$^+$]: 428.1159, found: 428.1105.

7-Amino-3-(2-(4-methoxyphenyl)-2-oxoethyl)-1-methyl-2,4-dioxo-5-phenyl-1,2,3,4-tetrahydropyrido[2,3-d]pyrimidine-6-carbonitrile (3p) Yellow solid; Yield: 45.2%; $^1$H-NMR (CDCl$_3$) δ 7.90 (d, $J = 8.8$ Hz, 2H), 7.46 (t, $J = 4$ Hz, 3H), 7.27 (d, $J = 9.2$ Hz, 2H), 6.91 (d, $J = 8.8$ Hz, 2H), 5.73 (s, 2H), 5.32 (s, 2H), 3.86 (s, 3H), 3.67 (s, 3H); $^{13}$C-NMR (DMSO-d$_6$): δ 191.11, 164.13, 160.88, 160.11, 158.40, 154.22, 151.03, 137.46, 130.77(2C), 128.63, 128.23(2C), 127.69(2C), 115.68, 114.57(2C), 98.72, 89.50, 56.07(2C), 47.56, 30.18; HRMS: calcd. for C$_{24}$H$_{19}$N$_5$O$_4$ [M-H$^+$]: 440.1359, found: 440.1375.

7-Amino-1-methyl-2,4-dioxo-5-phenyl-3-(prop-2-ynyl)-1,2,3,4-tetrahydropyrido[2,3-d]pyrimidine-6-carbonitrile (3q) White solid; Yield: 65.7%; $^1$H-NMR (DMSO-d$_6$) δ 7.95 (s, 2H), 7.44 (t, $J = 3$ Hz, 3H), 7.25–7.23 (m, 2H), 7.44 (d, $J = 1.6$ Hz, 2H), 5.66 (s, 2H), 5.26–5.21 (m, 2H), 3.52 (s, 3H), 3.07 (s, 1H); $^{13}$C-NMR (DMSO-d$_6$): δ 160.82, 160.13, 157.85, 154.17, 150.50, 137.52, 128.63, 128.23(2C), 127.69(2C), 115.68, 114.57(2C), 98.72, 89.50, 56.07(2C), 47.56, 30.18; HRMS: calcd. for C$_{18}$H$_{13}$N$_5$O$_2$ [M-H$^+$]: 330.0991, found: 330.0916.

7-Amino-3-cyclopentyl-1-methyl-2,4-dioxo-5-phenyl-1,2,3,4-tetrahydropyrido[2,3-d]pyrimidine-6-carbonitrile (3r) White solid; Yield: 48.3%; $^1$H-NMR (CDCl$_3$) δ 7.51–7.49 (m, 3H), 7.25 (t, $J = 4$ Hz, 2H), 5.66 (s, 2H), 5.26–5.21 (m, 1H), 3.61 (s, 3H), 2.05–1.99 (m, 2H), 1.79–1.71 (m, 2H), 1.52–1.48 (m, 2H); $^{13}$C-NMR (DMSO-d$_6$): δ 160.73, 160.02, 159.12, 154.08, 150.70, 137.96, 128.48, 128.22(2C), 127.64(2C), 115.80, 99.37, 89.10, 52.83, 29.88, 28.37(2C), 25.66(2C); HRMS: calcd. for C$_{20}$H$_{19}$N$_5$O$_2$ [M-H$^+$]: 360.1460, found: 360.1436.

7-Amino-3-butyl-1-methyl-2,4-dioxo-5-phenyl-1,2,3,4-tetrahydropyrido[2,3-d]pyrimidine-6-carbonitrile (3s) White solid; Yield: 51.9%; $^1$H-NMR (CDCl$_3$) δ 7.50 (t, $J = 3.6$ Hz, 3H), 7.25 (t, $J = 4$ Hz, 2H), 5.67 (s, 2H), 5.26–5.21 (m, 1H), 3.61 (s, 3H), 2.05–1.99 (m, 2H), 1.90–1.85 (m, 2H), 1.79–1.71 (m, 2H), 1.52–1.48 (m, 2H); $^{13}$C-NMR (DMSO-d$_6$): δ 160.23, 159.80, 158.64, 154.01, 151.00, 136.37, 129.14, 128.31(2C), 127.00(2C), 115.26, 100.59, 90.06, 41.69, 30.10, 29.74, 20.15, 13.75; HRMS: calcd. for C$_{19}$H$_{19}$N$_5$O$_2$ [M-H$^+$] m/z 348.1460, found: 348.1488.
3.5. Synthesis of Compounds 4a–d

Compounds 2g or 2j (1 mmol) and anhydrous potassium carbonate were stirred at room temperature in DMF (5 mL). Reagents 1-(bromomethyl)-4-fluorobenzene or 2-bromo-1-(4-methoxyphenyl) ethanone (2.1 mmol) were added. After monitoring the completion of the reaction by TLC, the mixture was poured into water (25 mL). It was extracted with EA. The combined organic layer was washed with water, dried with Na₂SO₄, evaporated to obtain a residue, that was purified by column chromatography.

7-Amino-3-(4-fluorobenzyl)-5-(4-(4-fluorobenzyloxy)-3-methoxyphenyl)-1-methyl-2,4-dioxo-1,2,3,4-tetrahydropyrido[2,3-d]pyrimidine-6-carbonitrile (4a) White solid; Yield: 27.2%; ³¹H-NMR (CDCl₃) δ 7.47–7.40 (m, 4H), 7.08 (t, J = 8.6 Hz, 2H), 7.00 (d, J = 8.4 Hz, 1H), 6.94 (t, J = 8.8 Hz, 2H), 6.84 (dd, J = 8.4 Hz, J = 2 Hz, 1H), 5.67 (s, 2H), 5.15 (s, 2H), 5.05 (s, 2H), 3.83 (s, 3H), 3.62 (s, 3H); ¹³C-NMR (DMSO-d₆): δ 163.04, 162.44, 160.62, 160.33, 160.03, 159.29, 158.17, 153.77, 150.78, 148.18, 147.93, 133.88, 133.24, 133.21, 130.25, 130.17, 129.64, 119.97, 115.56, 115.33, 115.12, 114.99, 114.78, 112.36, 112.06, 98.74, 88.91, 69.13, 55.51, 29.69; HRMS: calcd. for C₃₀H₂₃F₂N₅O₄+ [M+H⁺]: 556.1796, found: 556.1856.

7-Amino-3-(4-fluorobenzyl)-5-(4-(4-fluorobenzyloxy)phenyl)-1-methyl-2,4-dioxo-1,2,3,4-tetrahydropyrido[2,3-d]pyrimidine-6-carbonitrile (4b) White solid; Yield: 24.6%; ¹³H-NMR (CDCl₃) δ 7.55 (t, J = 7.6 Hz, 1H), 7.36–7.00 (m, 11H), 5.72 (s, 2H), 5.20 (s, 2H), 5.19 (s, 2H), 3.64 (s, 3H); ¹³C-NMR (DMSO-d₆): δ 161.47, 160.30, 159.71, 158.82, 154.20, 150.79, 129.95, 129.88, 129.82, 129.04, 128.95, 128.43, 123.34, 123.98, 123.96, 123.88, 123.78, 123.64, 123.54, 115.48, 115.34, 115.32, 114.44, 100.40, 90.39, 63.64, 63.61, 38.71, 30.24; HRMS: calcd. for C₂₉H₂₁F₂N₅O₃+ [M+H⁺]: 526.1691, found: 526.1675.

7-Amino-5-(3-methoxy-4-(2-(4-methoxyphenyl)-2-oxoethoxy)phenyl)-3-(2-(4-methoxyphenyl)-2-oxoethyl)-1-methyl-2,4-dioxo-1,2,3,4-tetrahydropyrido[2,3-d]pyrimidine-6-carbonitrile (4c) White solid; Yield: 28.1%; ¹³H-NMR (DMSO-d₆) δ 8.01 (dd, J = 8.8 Hz, J = 6.8 Hz, 4H), 7.07 (dd, J = 8.4 Hz, J = 5.2 Hz, 4H), 6.90–6.85 (m, 2H), 6.73 (dd, J = 8.8 Hz, J = 2 Hz, 1H), 5.50 (s, 2H), 5.23 (s, 2H), 3.85 (s, 6H), 3.74 (s, 3H), 3.53 (s, 3H); ¹³C-NMR (DMSO-d₆): δ 193.22, 191.18, 164.14, 164.02, 160.90, 159.84, 158.36, 154.22, 151.04, 148.50, 148.10, 137.07(4C), 130.01, 127.77, 127.71, 120.37, 115.97, 114.59(2C), 114.50(2C), 112.97, 112.64, 98.88, 89.67, 70.87, 56.13, 56.07, 47.61, 30.18; HRMS: calcd. for C₃₄H₂₉N₅O₈+ [M+H⁺]: 658.1914, found: 658.1896.

7-Amino-5-(3-methoxy-4-(2-(4-methoxyphenyl)-2-oxoethoxy)phenyl)-1-methyl-2,4-dioxo-1,2,3,4-tetrahydropyrido[2,3-d]pyrimidine-6-carbonitrile (4d) White solid; Yield: 21.7%; ¹³H-NMR (DMSO-d₆) δ 8.01 (t, J = 9.2 Hz, 4H), 7.17 (d, J = 8.8 Hz, 2H), 7.10–7.03 (m, 4H), 6.96 (d, J = 8.8 Hz, 2H), 5.53 (s, 2H), 5.22 (s, 2H), 3.86 (s, 6H), 3.53 (s, 3H); ¹³C-NMR (DMSO-d₆): δ 193.14, 191.15, 164.13, 164.03, 160.90, 160.50, 159.94, 158.58, 158.45, 158.14, 154.26, 151.02, 130.79(2C), 130.73(2C), 129.63, 129.46, 129.39, 127.72, 127.68, 114.98, 114.54(2C), 114.30(2C), 98.82, 89.66, 70.33, 56.07(2C), 47.58, 30.19; HRMS: calcd. for C₃₃H₂₇N₅O₇+ [M+H⁺]: 606.1989, found: 606.1971.
3.6. Synthesis of Compound 4e

Compound 2g (1 mmol) and TsCl (2.1 mmol) were dissolved in DMF (5 mL) followed by the addition of triethylamine (3 mmol). The mixture was stirred at room temperature for 24 h. The reaction mixture was poured into water and extracted with DCM. The combined organic layer was washed with water and evaporated to half of its volume. The organic layer was cooled to room temperature, and the solid filtered and washed with EtOH to obtain 4-(7-amino-6-cyano-1-methyl-2,4-dioxo-1,2,3,4-tetrahydropyrido[2,3-d]pyrimidin-5-yl)-2-methoxy phenyl-4-methylbenzenesulfonate (4e) White solid; Yield: 57.0%; 1H-NMR (DMSO-d6) δ 11.24 (s, 1H), 7.89 (s, 2H), 7.62 (d, J = 8.4 Hz, 2H), 7.42 (d, J = 8 Hz, 2H), 7.21 (d, J = 8 Hz, 1H), 6.92 (d, J = 1.6 Hz, 1H), 6.84 (dd, J = 8.4 Hz, J = 1.6 Hz, 1H), 3.42 (s, 3H), 3.34 (s, 3H), 2.41 (s, 3H); 13C-NMR (DMSO-d6): δ 162.77, 160.74, 159.24, 158.29, 155.40, 151.15, 150.96, 145.90, 137.86, 137.74, 132.21, 130.15, 128.74, 123.57, 120.31, 115.66, 113.28, 99.70, 88.48, 56.07, 36.24, 29.12, 21.62; HRMS: calcd. for C23H19N5O6S+ [M+H+] : 494.1134, found: 494.1108.

3.7. Cell Proliferation Assay (MTT Assay)

Briefly, cells (2,500/well) were seeded in 96-well plates and cultured for 24 h, followed by treatment with target compounds for a further 48 h. Twenty microlitres of 5 mg/mL MTT was added per well and incubated for a further 2.5 h at 37 °C. Then the supernatant was removed, and 150 µL/well DMSO added for 15–20 min. The optical density of each well was measured at 570 nm using a SpectraMAX M5 microplate spectrophotometer (Molecular Devices, Silicon Valley, CA, USA).

3.8. Apoptosis Analyses

Briefly, 1.5 × 10^5 cells were seeded per well in a six-well plate. Twenty-four hours later, cells were treated with 4a for a further 48 h. All cells were collected, washed twice with phosphate-buffered saline (PBS) and resuspended in 100 µL binding buffer. Then cell suspensions were mixed with 5 µL annexin V-FITC and 10 µL PI, and incubated for 15 min in the dark at room temperature. After staining, 400 µL of binding buffer was added and stained cells analyzed using a flow cytometer.

4. Conclusions

Twenty-four novel N-3 substituted 7-aminopyrido[2,3-d]pyrimidin-6-carbonitrile derivatives were synthesized and evaluated as potential anticancer agents through a cell-based phenotypic screening approach. Compound 3k was found to exhibit significant potency against human colon cancer cells SW620 with an IC_{50} value of 12.5 µM. Further structural modification led to a more potent compound 4a with an IC_{50} value of 6.9 µM, which is slightly more potent than cisplatin against SW620 tumor cells. Preliminary SARs information suggested that lipophilic groups at N-3 position are preferred and a methoxy group at the R^4-position plays a crucial part. Flow cytometric analyses indicated that compound 4a acts through induction of apoptosis in human colon cancer SW620 cells. The underlying mechanism of action of compound 3k and 4a will further examined in our lab.
Acknowledgments

This work was supported by the National Major Program of China during the 12th Five-Year Plan Period (2012ZX09103-101-036). The authors gratefully thank Ms. Yong-Qiu Mao for flow cytometric measurements and fruitful discussions.

Conflict of Interest

The authors declare no conflict of interest.

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*Sample Availability:* Samples of the compounds are available from the authors.

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