Synthesis and biological evaluation of pyrido[2,3-d]pyrimidine-2,4-dione derivatives as eEF-2K inhibitors

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A small molecule library of pyrido[2,3-d]pyrimidine-2,4-dione derivatives 6–16 was synthesized from 6-amino-1,3-disubstituted uracils 18, characterized, and screened for inhibitory activity against eukaryotic elongation factor-2 kinase (eEF-2K). To understand the binding pocket of eEF-2K, structural modifications of the pyrido[2,3-d]pyrimidine were made at three regions (R1, R2, and R3). A homology model of eEF-2K was created, and compound 6 (A-484954, Abbott laboratories) was docked in the catalytic domain of eEF-2K. Compounds 6 (IC50 = 420 nM) and 9 (IC50 = 930 nM) are found to be better molecules in this preliminary series of pyrido[2,3-d]pyrimidine analogs. eEF-2K activity in MDA-MB-231 breast cancer cells is significantly reduced by compound 6, to a lesser extent by compound 9, and is unaffected by compound 12. Similar inhibitory results are observed when eEF-2K activity is stimulated by 2-deoxy-D-glucose (2-DOG) treatment, suggesting that compounds 6 and 9 are able to inhibit AMPK-mediated activation of eEF-2K to a notable extent. The results of this work will shed light on the further design and optimization of novel pyrido[2,3-d]pyrimidine analogs as eEF-2K inhibitors.

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1. Introduction

Eukaryotic elongation factor-2 kinase (eEF-2K, EC 2.7.11.20) is an atypical Ser/Thr-protein kinase, and is also known as a calcium/calmodulin-dependent protein kinase-III (CaM kinase-III). eEF-2K is not structurally similar to conventional protein kinases, and the structure of eEF-2K has not been reported. eEF-2K phos- phorylates eEF-2 at Thr-56 and Thr-58, and inactivates eEF-2, and thus regulates protein synthesis at the elongation step of translation. 1,2 Nutrients and growth factors inactivate eEF-2K whereas starvation activates it. 3 In 2013, Leprivier et al. 4 demonstrated a crucial role for eEF-2K in protecting normal cells from nutrient deprivation through inhibition of eEF-2, and they suggested how cancer cells could exploit this pathway by activating eEF-2K to adapt to metabolic stress. eEF-2K has been reported to be upregulated in various malignant cells such as breast cancer cells, 5 malignant glioma cells, 6 HL60 leukemia cells, 7 and pancreatic cancer (PaCa) cells, 8 and has been identified as a potential cancer-therapeutic target. 9

Several small molecule inhibitors of eEF-2K have been reported (Fig. 1), including: Rottlerin 1, which is found to be a non specific and cytotoxic inhibitor, 10 1,3-selenazine analog 2, which is suggested to block phosphorylation of eEF-2 in cells and to react with cysteine residues, 11 the imidazolium derivative (NH125) 3, 12 which does not inhibit eEF-2K in cells, 13,16 and thieno[2,3-b]pyri- dine 4, which is an ATP-competitive inhibitor that lacks potency in cells. 14 Recently, high-throughput screens for eEF-2K inhibitors by us, 15 and by Abbott laboratories, 16 revealed thiopyran-dicarbo-nitrile analog 5, and pyrido-pyrimidinedione derivative A-484954 (compound 6), respectively. A-484954, a small molecule discovered by Abbott, is an ATP-competitive and cell-permeable inhibitor of eEF-2K (IC50 = 0.28 μM)

We are interested in developing a more potent and selective eEF-2K inhibitor to probe the function of eEF-2K in the regulation of protein synthesis. In this paper, we report the synthesis of a
preliminary series of pyrido[2,3-d]pyrimidine-2,4-dione derivatives 6–16 and their inhibitory activity against eEF-2K to explore structure–activity relationships (SAR) of A-484954. Moreover, a homology model has been generated and used to dock compound 6 to provide insight into its binding in the catalytic domain of eEF-2K.

2. Results and discussion

2.1. Chemistry

Pyrido[2,3-d]pyrimidine-2,4-dione derivatives 6–16 were prepared according to Schemes 1–3. Direct alkylation of commercially available uracil derivatives 17 with either ethyl iodide or n-propyl iodide in the presence of 10–15% aqueous NaOH afforded the corresponding 1,3-disubstituted-6-aminouracils 18c–18e in 40–53% yield (Scheme 1). To construct the pyridine ring of pyrido[2,3-d]pyrimidine 6, uracil 18c was treated with the Vilsmeier reagent 19, which was generated in situ from phosphoryl chloride in dimethyl formamide, followed by solvent evaporation and treatment of the residue with triethylamine and cyanoacetamide in ethan-ol. However, addition of traces of water present in the reaction mixture to the hydrochloride salt 20c resulted in hydrolyzed product 21 in 66% yield, but not the required pyrido[2,3-d]pyrimidine 6 (Scheme 2).

Alternatively, when a catalytic amount of dimethyl formamide and oxalyl chloride were used to prepare the Vilsmeier reagent 22, and the resulting HCl salt 20 was treated with triethylamine and cyanoacetamide in ethanol, compound 6 was isolated in 78% yield over the two steps (Scheme 3). Other pyrido[2,3-d]pyrimidine-2,4-dione derivatives 7, 9, 12–16 were also synthesized using this procedure. Compound 8 was isolated in 47% yield by direct methylation of compound 7 in presence of KO‘Bu as a base. On treatment with sodium hydride and p-nitrobenzylbromide, compound 7 resulted in 61% of benzylated product 10 as an off-white solid, which was reduced with SnCl2 in an ethyl acetate and methanol mixture to afford amine 11 in 70% yield.

2.2. Computer homology modeling

The structure of eEF-2K was generated using homology modeling with Dictyostelium myosin heavy chain kinase A (MHCK A) as the template, and compound 6 was docked into the ATP-binding pocket of eEF-2K (Fig. 2). The inhibitor 6 is shown in cyan. The protein is shown in cartoon representation, with the hinge region in purple and the Gly-rich loop in blue. The binding of inhibitor 6 involves hydrogen bonds to residues K170, I232 and G234. The hydrophobic cyclopropyl and ethyl groups are buried deep inside the adenine-binding pocket and underneath the Gly-rich-loop, respectively. Docking was performed using Golds.15 with ChemPLP fitness function.20 Twenty independent docking attempts were performed for the ligand using generic algorithm. The results of each attempt were ranked according to the ChemPLP fitness score and were virtually inspected. The highest ranked pose of the ligand also happened to be very similar to the most of the high-ranking poses that resulted from the twenty attempts (data not shown). This suggested that the docking attempts reach an optimal state, and thus supporting the likelihood of this pose in docking. Details on generating the homology model can be found in our recent work.21

2.3. Biological activity

2.3.1. In vitro activity against recombinant eEF-2K

It has been reported that compound 6 (A-484954, Abbott labs), inhibits eEF-2K activity in vitro with an IC50 value of 0.28 µM.16 In addition to compound 6, we examined a series of pyrido[2,3-d]pyrimidine-2,4-dione derivatives (7–16) to evaluate their potential in terms of modification of the initial compound, and to probe their structure–activity relationships. A radioactive assay utilizing [γ-32P]ATP was used to assess the inhibition of recombinant human eEF-2K by the compounds, where kinase activity was determined by measuring the rate of incorporation of 32P into a peptide substrate.22,23

The inhibition found for target compounds 6–16, 18c, and 21 against recombinant eEF-2K activity in vitro are summarized in Table 1. Compound 6 has an IC50 of 0.42 µM, which differs from the reported value of 0.28 µM (Fig. 3).16 This can be accounted for by the difference in assay conditions, where our assay uses 10 µM ATP compared to 5 µM ATP by Chen et al. Because the compound is reported to be an ATP-competitive inhibitor, it is expected that an increase in ATP concentration in the assay would increase the IC50 value. When the R3 group on the scaffold is modified from hydrogen to benzyl derivatives, the potency increased from hydrogen (IC50 = 6.6 µM) to ethyl (IC50 = 0.42 µM) and was then decreased to benzyl derivatives (IC50 >25 µM). Complete loss of inhibitory activity is observed on changing the CONH2 group (IC50 = 0.42 µM) to CSNH2, CN and CO2Et (compounds 12, 13, and 14, respectively) (IC50 >25 µM). Moderate loss of activity is observed when the cyclopropyl group (IC50 = 0.42 µM) at R3 is changed to an ethyl group (IC50 = 1–2 µM). The inactivity of
Scheme 2. Hydrolysis of hydrochloride salt 20c.


<table>
<thead>
<tr>
<th>compound</th>
<th>R¹</th>
<th>R²</th>
<th>R³</th>
</tr>
</thead>
<tbody>
<tr>
<td>18a</td>
<td>H</td>
<td></td>
<td>cyclopropyl</td>
</tr>
<tr>
<td>18c</td>
<td>Et</td>
<td></td>
<td>cyclopropyl</td>
</tr>
<tr>
<td>18d</td>
<td>n-Pr</td>
<td></td>
<td>cyclopropyl</td>
</tr>
<tr>
<td>18e</td>
<td>Et</td>
<td>Et</td>
<td></td>
</tr>
<tr>
<td>18f</td>
<td>Me</td>
<td>Me</td>
<td></td>
</tr>
</tbody>
</table>

Figure 2. Compound 6 docked into the ATP-binding pocket of eEF-2K. (A) eEF-2K is rendered using solvent accessible surface and compound 6 (sticks) is shown well fitted into the pocket. (B) Docking determined the four key residues that are shown in sticks. Hydrogen bonds are shown as blue dashed lines.
compounds 18c and 21 stresses the importance of a pyridine ring for this class of molecules. These results provide new insights, which may aid in the rational design of inhibitors for the kinase.

2.3.2. Cellular activity against eEF-2K

Chen et al. have reported that treatment of various cell lines with compound 6 inhibits eEF-2K activity in a dose-dependent manner, as indicated by reduced levels of phosphorylated eEF-2 (Thr-56). This was observed in human prostate cancer (PC3), human cervical cancer (HeLa), human non-small cell lung cancer (H460 and H1299), and rat glial (C6) cells. Their results are most prominent under conditions of nutrient deprivation (Hanks’ Balanced Salt Solution, HBSS), which is known to activate eEF-2K presumably through inhibition of the nutrient-sensitive mTOR pathway. To determine if similar inhibition was observed in breast cancer cells, compounds 6 (IC50 = 0.42 μM), 9 (IC50 = 0.93 μM), and 12 (IC50 > 25 μM) were assayed in the MDA-MB-231 cell line. The level of phosphorylated eEF-2 (Thr-56) was used to estimate the activity of eEF-2K, and as indicated in Figure 4, results of cellular inhibition are similar to the in vitro kinase assay data. In low-glucose (5.6 mM) media, eEF-2K activity is significantly reduced by the addition of compound 6, to a lesser extent by compound 9, and is unaffected by compound 12 (Fig. 4). To activate the AMPK-eEF-2K axis, 2-DOG was used. 2-DOG inhibits glycolysis and its production of ATP, thus activating AMPK, which in turn activates eEF-2K through phosphorylation at Ser-398. Similar inhibitory results are observed when eEF-2K activity is stimulated by 2-DOG treatment (Fig. 4), suggesting that compounds 6 and 9 are able to inhibit AMPK-mediated activation of eEF-2K to a notable extent.

Table 1
In vitro activity of the target compounds 6–16, 18c, and 21 against eEF-2K

<table>
<thead>
<tr>
<th>Compound</th>
<th>R1</th>
<th>R2</th>
<th>R3</th>
<th>IC50 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>H</td>
<td>CONH2</td>
<td>Cyclopropyl</td>
<td>6.6 ± 0.2</td>
</tr>
<tr>
<td>8</td>
<td>Me</td>
<td>CONH2</td>
<td>Cyclopropyl</td>
<td>6.1 ± 0.2</td>
</tr>
<tr>
<td>6</td>
<td>Et</td>
<td>CONH2</td>
<td>Cyclopropyl</td>
<td>0.42 ± 0.01</td>
</tr>
<tr>
<td>9</td>
<td>n-Pr</td>
<td>CONH2</td>
<td>Cyclopropyl</td>
<td>0.93 ± 0.03</td>
</tr>
<tr>
<td>10</td>
<td>CH2-Ph-NO2-p</td>
<td>CONH2</td>
<td>Cyclopropyl</td>
<td>&gt;25</td>
</tr>
<tr>
<td>11</td>
<td>CH2-Ph-H2-p</td>
<td>CONH2</td>
<td>Cyclopropyl</td>
<td>&gt;25</td>
</tr>
<tr>
<td>12</td>
<td>Et</td>
<td>CSNH2</td>
<td>Cyclopropyl</td>
<td>&gt;25</td>
</tr>
<tr>
<td>13</td>
<td>Et</td>
<td>CO2Et</td>
<td>Cyclopropyl</td>
<td>&gt;25</td>
</tr>
<tr>
<td>14</td>
<td>Et</td>
<td>CN</td>
<td>Cyclopropyl</td>
<td>&gt;25</td>
</tr>
<tr>
<td>15</td>
<td>Et</td>
<td>CONH2</td>
<td>Et</td>
<td>1–2</td>
</tr>
<tr>
<td>16</td>
<td>Me</td>
<td>CONH2</td>
<td>Me</td>
<td>&gt;25</td>
</tr>
<tr>
<td>18c</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>&gt;25</td>
</tr>
<tr>
<td>21</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>&gt;25</td>
</tr>
</tbody>
</table>

Inhibitor dose response assays were performed with 5 nM recombinant eEF-2K enzyme at various concentrations of the inhibitor, in the presence of 50 μM free Ca2+, 200 nM CaM and 10 μM [γ-32P]ATP, against 150 μM peptide substrate as described under the Section 4. The inhibitor was incubated with eEF-2K for 30 min at 30°C before initiating the assay with peptide and [γ-32P]ATP. Kinase activity in each case was determined by calculating the rate of phosphorylation of the peptide (μM/s), and dose-response curves for data conforming to inhibition were fit to Eq. 1.
3. Summary

In summary, a series of pyrido[2,3-d]pyrimidine-2,4-dione derivatives 6–16 was synthesized and screened against eEF-2K inhibitory activity. Ethyl group at R1, CONH2 at R2 and cyclopropyl at R3 are found to be optimal, and the pyridine ring and CONH2 group in 6 are essential for the activity of the studied compounds. Compounds 6 (IC50 = 420 nM) and 9 (IC50 = 930 nM) are found to be the best molecules in this preliminary series of pyrido[2,3-d]pyrimidine analogs. eEF-2K activity in MDA-MB-231 breast cancer cells is significantly reduced by compound 6, and to a lesser extent by compound 9. Because there is no eEF-2K crystal structure, the knowledge of the structural features from this preliminary series of analogs along with in-depth computer modeling should yield insights to develop a more potent eEF-2K inhibitor.

4. Experimental section

4.1. Chemistry

Reagents and starting materials including 17a, 17b, and 18f were purchased from various commercial sources and used without further purification unless otherwise stated. All reactions were carried out in oven- or flame-dried glassware under argon. Thin-layer chromatography (TLC) was performed using pre-coated TLC plates with silica gel 60 F254 (EMD). Melting points were recorded on a Thomas Hoover capillary melting point apparatus. NMR spectra were recorded on a Varian MR spectrometer. High-resolution mass and liquid chromatography mass spectral data were obtained at the University of Texas at Austin. Compounds were characterized by NMR and HRMS or LCMS.

4.1.1. General procedure for the synthesis of 6-amino-1-alkyl-3-alkylpyrimidine-2,4(1H,3H)-dione (Compounds 18c–18e)

A mixture of 6-amino-1-alkyl uracil (1 mmol), sodium hydroxide 10–15% (0.3 mL) and 95% EtOH (0.9 mL) was heated under reflux for 15 min and cooled to room temperature. The reaction mixture was added to a cold ethyl alcohol (2 mL) and stirred for 3–8 h. The volatiles were removed under reduced pressure and the residue was partitioned between chloroform/water (2:1 ratio v/v, 9 mL). The organic layer was washed with water, brine, dried over Na2SO4 and evaporated to dryness to give the corresponding 3-substituted 6-amino-1-alkyl-uracils 18c–18e. Unreacted starting material was recovered from the aqueous phase by evaporation to dryness. The obtained crude from organic layer was purified by flash chromatography using 0–10% methanol in dichloromethane.

4.1.1.1. 6-Amino-1-cyclopropyl-3-ethylpyrimidine-2,4(1H,3H)-dione (Compound 18c)

Prepared from 6-amino-1-cyclopropylpyrimidine-2,4(1H,3H)-dione (Compound 17a) and ethyl iodide in 40% yield as a white solid. Mp 186–187 °C. 1H NMR (400 MHz, CDCl3); δ 4.91 (s, 1H), 4.90 (br s, 2H), 3.93 (q, J = 7.0 Hz, 2H), 2.66 (m, 1H), 1.24 (m, 2H), 1.19 (t, J = 7.0 Hz, 2H), 0.99 (m, 2H). LCMS: m/z [M+H]+ 196.2.

4.1.1.2. 6-Amino-1-cyclopropyl-3-propylpyrimidine-2,4(1H,3H)-dione (Compound 18d)

Prepared from 6-amino-1-cyclopropylpyrimidine-2,4(1H,3H)-dione (Compound 17a) and propyl iodide in 53% yield as a white solid. Mp 154–155 °C. 1H NMR (400 MHz, CDCl3); δ 4.90 (s, 1H), 4.87 (br s, 2H), 3.88–3.70 (m, 2H), 2.72–2.60 (m, 1H), 1.61 (m, 2H), 1.24 (m, 2H), 1.01–0.95 (m, 2H), 0.92 (t, J = 7.5 Hz, 3H). LCMS: m/z [M+H]+ 210.2.

4.1.1.3. 6-Amino-1,3-diethylpyrimidine-2,4(1H,3H)-dione (Compound 18e)

Prepared from 6-amino-1-ethylpyrimidine-2,4(1H,3H)-dione (Compound 17b) and ethyl iodide in 42% yield as a white solid. Mp 192–193 °C. 1H NMR (400 MHz, DMSO-d6); δ 6.79 (br s, 2H), 4.64 (s, 1H), 3.82 (q, J = 7.0 Hz, 2H), 3.73 (q, J = 7.0 Hz, 2H), 1.09 (t, J = 7.0 Hz, 3H), 1.02 (t, J = 7.0 Hz, 3H). LCMS: m/z [M+H]+ 184.4.

4.1.2. Synthesis of 6-amino-1-cyclopropyl-3-ethyl-2,4-dioxo-1,2,3,4-tetrahydropyrimidine-5-carbaldehyde (Compound 21)

To a suspension of 18c (20 mg, 0.102 mmol) in DMF (0.6 mL) was added phosphoryl chloride (10 µL, 0.113 mmol) dropwise at below 20 °C. The reaction mixture was stirred for 30 min at room temperature. After evaporation of volatiles, the crude was taken to the next step. To a mixture of the crude in ethanol (1 mL) was added cyanoacetamide (9 mg, 0.102 mmol) and then triethylamine (17 µL, 0.123 mmol) dropwise at 0 °C. The suspension was stirred for 2 h at room temperature. Solvent was removed and the obtained crude was purified by flash chromatography using 0–5% methanol in dichloromethane to afford 21 as a white solid in 66% yield. Mp 149–150 °C. 1H NMR (400 MHz, CDCl3); δ 10.63 (br s, 1H), 9.93 (s, 1H), 6.19 (br s, 1H), 3.97 (q, J = 7.2 Hz, 2H), 2.67 (m, 1H), 1.33 (m, 2H), 1.21 (t, J = 7.2 Hz, 3H), 1.00 (m, 2H). 13C NMR (100 MHz, CDCl3) δ 188.8, 162.1, 157.9, 150.2, 91.3, 36.2, 23.5, 13.1, 9.1. LCMS: m/z [M+H]+ 224.2.

4.1.3. General procedure for the synthesis and characterization of pyrido[2,3-d]pyrimidine-2,4-dione derivatives (Compounds 6, 7, 9, 12–16)

To a solution of dimethylformamide (0.11 mmol) in dichloromethane (1.5 mL) was added oxaly chloride (0.11 mmol) dropwise at ca. 10 °C. The reaction mixture was stirred for 30 min at room temperature. Compound 18 (0.10 mmol) was added to the resulting white suspension and stirred for another 30 min. Volatiles were removed and the crude was taken to the next step without further purification. To the mixture of the crude in dry ethanol (1 mL) was added an active methylene compound such as cyanoacetamide, cyanothioacetamide, ethyl cyanoacetate, or malononitrile (0.10 mmol) and then triethylamine (0.12 mmol) dropwise at 0 °C. The suspension was allowed to room temperature and stirred for 2 h. The resulting precipitate was washed with ethanol. In the case of no precipitation, solvent was removed and the obtained crude was purified by flash chromatography (either neutral Al2O3 or silica gel) using 0–10% methanol in dichloromethane.

4.1.3.1. Synthesis of 7-amino-1-cyclopropyl-3-ethyl-2,4-dioxo-1,2,3,4-tetrahydropyrido[2,3-d]pyrimidine-6-carboxamide (Compound 6)

Isolated in 78% yield as a white solid. Mp >250 °C. 1H NMR (400 MHz, DMSO-d6); δ 8.55 (s, 1H), 8.22 (s, 2H), 7.36 (s, 2H), 3.89 (q, J = 8.0 Hz, 2H), 2.76 (m, 1H), 1.11 (t, J = 8.0 Hz, 3H), 1.08 (m, 2H), 0.86–0.71 (m, 2H). 13C NMR (100 MHz, DMSO-d6); δ 168.8, 161.1, 160.0, 153.6, 151.1, 138.3, 105.4, 99.5, 35.7, 25.8, 13.1, 9.7. HRMS: m/z calcd for C13H15O3N2 [M]+ 289.1175, found 289.1182.

4.1.3.2. Synthesis of 7-amino-1-cyclopropyl-3-ethyl-2,4-dioxo-1,2,3,4-tetrahydropyrido[2,3-d]pyrimidine-6-carboxamide (Compound 7)

Isolated in 81% yield as a yellow solid. Mp >250 °C. 1H NMR (400 MHz, DMSO-d6); δ 11.17 (s, 1H), 8.51 (s, 1H), 8.20 (s, 1H), 7.33 (s, 1H), 2.70 (m, 1H), 1.14–0.98 (m, 2H), 0.85–0.73 (m, 2H). 13C NMR (150 MHz, DMSO-d6); δ 168.8, 161.2, 160.9, 154.9, 151.1, 137.9, 105.0, 100.0, 25.0, 9.4. LCMS: m/z [M+H]+ 262.1.
4.1.3.3. Synthesis of 7-amino-1-cyclopentyl-2,4-dioxo-3-propyl-1,2,3,4-tetrahydropyrido[2,3-d]pyrimidine-6-carboxamide (Compound 9). Isolated in 66% yield as a white solid. Mp 234–235 °C. 1H NMR (400 MHz, DMSO-d6): δ 8.54 (s, 1H), 8.23 (s, 1H), 7.36 (s, 1H), 3.91–3.72 (m, 2H), 2.77 (m, 1H), 1.64–1.46 (m, 2H), 1.17–1.02 (m, 2H), 0.86 (t, J = 7.4 Hz, 3H), 0.81–0.71 (m, 2H). 13C NMR (100 MHz, DMSO-d6): δ 168.9, 161.1, 160.2, 153.6, 151.3, 138.4, 105.4, 99.4, 42.1, 25.8, 20.8, 11.3, 9.9. HRMS: m/z calcd for C13H17N3O3 [M]+ 303.1331, found 303.1331.

4.1.3.4. Synthesis of 7-amino-1-cyclopentyl-3-ethyl-2,4-dioxo-1,2,3,4-tetrahydropyrido[2,3-d]pyrimidine-6-carboxamide (Compound 10). Isolated in 72% yield as a light yellow solid. Mp >250 °C. 1H NMR (400 MHz, DMSO-d6): δ 8.57 (s, 1H), 8.23 (br s, 2H), 8.17 (d, J = 8.7 Hz, 2H), 7.56 (d, J = 8.8 Hz, 2H), 7.38 (br s, 2H), 7.16 (s, 2H), 2.85–2.71 (m, 1H), 1.12–1.06 (m, 2H), 0.90–0.73 (m, 2H), LCMS: m/z [M+H]+ 397.2.

4.1.3.11. Synthesis of 7-amino-3-(4-aminobenzyl)-1-cyclopentyl-2,4-dioxo-1,2,3,4-tetrahydropyrido[2,3-d]pyrimidine-6-carboxamide (Compound 11). A mixture of 10 (27 mg, 0.068 mmol), SnCl2 (52 mg, 0.273 mmol) in ethyl acetate/methanol (6 mL; 5:6 ratio v/v) was heated at reflux for 12 h. After cooling to room temperature, the solvent was evaporated and the residue was purified by flash chromatography (neutral Al2O3 using 0–3% methanol in dichloromethane to yield 61% of 10 as an off-white solid. Mp >250 °C. 1H NMR (400 MHz, DMSO-d6): δ 8.59 (s, 1H), 8.23 (br s, 2H), 8.21 (br s, 2H), 7.36 (br s, 2H), 7.02 (d, J = 8.3 Hz, 2H), 6.45 (d, J = 8.4 Hz, 2H), 4.98 (s, 2H), 4.86 (s, 2H), 2.77 (m, 1H), 1.09 (m, 2H), 0.77 (m, 2H), LCMS: m/z [M+H]+* 367.1.

4.2. In vitro recombinant kinase inhibition assays

Recombinant human eEF-2K (expressed in bacteria in the absence of λ-phosphatase) was purified as described earlier.22,23 Inhibitor dose response assays were performed in Buffer A (25 mM HEPES (pH 7.5), 2 mM DTT, 0.15 mM bSA, 50 mM KAcO, 100 mM EGTA, 150 mM CaCl2, 200 mM CaM and 10 mM MgCl2), against 30 µM peptide substrate using 5 nM eEF-2K enzyme, 10 µM [γ-32P]ATP (100–1000 cpm/pmol) and various concentrations of the inhibitor, in a final reaction volume of 100 µL. The reaction mixture containing the inhibitor and eEF-2K was incubated at 30 °C for 30 min before initiating the assay with peptide and [γ-32P]ATP. At set time points, 10 µL aliquots were taken and spotted onto P81 cellulose filters (Whatman, 2 × 2 cm). The filter papers were then washed thrice with 50 mM phosphoric acid (15 min each wash), once with acetone (15 min), and dried. The amount of labeled peptide associated with each paper was determined by measuring the cpm on a Packard 1500 scintillation counter. Kinase activity in each case was determined by calculating the rate of phosphorylation of the peptide (μM/s), and dose-response curves for data conforming to inhibition were fit to Eq. 1.

\[ V_{\text{app, obs}} = V_{\text{app, max}} - \left( V_{\text{app, max}} \times \frac{[I]}{[I] + K_{50}} \right) + V_{\text{app, inf}} \]

(1)

The parameters are defined as follows: \( V_{\text{app, obs}} \), apparent rate; \( V_{\text{app, max}} \), apparent rate in the absence of the inhibitor (maximum rate); \( V_{\text{app, inf}} \), apparent rate at saturating concentration of the inhibitor; [I], concentration of the inhibitor; \( K_{50} \), inhibitor concentration required to achieve half maximal apparent rate. When \( V_{\text{app, obs}} \) is converted to percentage maximal activity, \( V_{\text{app, max}} \) is taken as 100.

4.3. Cellular kinase inhibition assays

4.3.1. Cell lines and culture conditions

The human breast cancer cell line MDA-MB-231 was obtained from American Type Culture Collection (Manassas, VA). Cells were...
cultured in DMEM/F12 supplemented with 10% FBS, 50 units/ml penicillin and 50 µg/ml streptomycin. Cell cultures were maintained at 37°C in a humidified incubator containing 5% CO₂. All cell culture reagents were from Invitrogen or Sigma–Aldrich.

4.3.2. Treatment of cells with stimuli and inhibitors

MDA-MB-231 breast cancer cells were seeded in 6-well plates, and treated when ~80% confluent. To activate the AMPK-eEF-2K axis, cells were treated with 2-DG as follows. Cells were washed with phosphate buffered saline (PBS, pH 7.4), and then pre-incubated in low-glucose (5.6 mM) DMEM supplemented with 10% FBS for 2 h. Then in the same low-glucose media, cells were initially treated with 75 µM compound 6, 9, or 12 for 30 min, followed by incubation ±25 mM 2-DG in the presence of the compounds for an additional 30 min. Treatments with the appropriate DMSO controls were also performed.

4.3.3. Cell lysis and western blot analysis

Following treatments, cells were washed twice in ice-cold PBS (pH 7.4), and lysed in ice-cold Buffer B [50 mM HEDES (pH 7.4), 150 mM NaCl, 1.5 mM MgCl₂, 1% Triton X-100, 1 mM EGTA, 100 mM NaF, 10 mM Na pyrophosphate, 1 mM Na₃VO₄ and 10% glycerol], supplemented with PhosSTOP Phosphatase Inhibitor Cocktail (Roche Diagnostics, Indianapolis, IN), complete EDTA-free Protease Inhibitor Cocktail (Roche Diagnostics) and Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific). Lysates were subjected to one freeze-thaw cycle, and then clarified by centrifugation at 15,000 rpm for 15 min. Total protein concentration for each sample was determined by Bradford assay (Bio-Rad Laboratories, Hercules, CA). Western blotting was carried out with appropriate HRP-conjugated secondary antibodies.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2014.06.050.

References and notes