Synthesis and evaluation of new pirfenidone derivatives as anti-fibrosis agents†

Chenxi Gu,a Wei Li,a Qing Ju,b Han Yao,a Lisheng Yang,a Baijiao An,*b Wenhao Hu*a and Xingshu Li,c,d

Two series of new pirfenidone derivatives, in which phenyl groups or benzyl groups are attached to the nitrogen atom of the pyridin-2(1H)-one moiety were synthesized and evaluated as anti-fibrosis agents. Among them, compound 5d, with a (3)-2-(dimethylamino) propanamido group in the R2 position (series 1) exhibited 10 times the anti-fibrosis activity (IC50: 0.245 mM) of pirfenidone (IC50: 2.75 mM). Compound 9d (series 2) gave an IC50 of 0.035 mM against the human fibroblast cell line HFL1. The mechanism of the optimal compound inhibiting fibrosis was also studied.

Results and discussion

Chemistry

For example, in the presence of potassium carbonate, coupling reaction of 5-(trifluoromethyl)pyridin-2(1H)-one and 1-fluoro-4-nitrobenzene gave compound 2a, which was reduced by tin dichloride to provide compound 3a. Compound 4a was obtained by the reaction of 3a with acetic anhydride. On the other hand, compound 5a was obtained by the reaction of 3a with 2-(4-methylpiperazin-1-yl)acetic acid in the presence of a dehydrating agent HATU.
acetamido at the R₂ position, provided 34.2% inhibition rate at a concentration of 0.5 mM which is nearly the same as that of pirfenidone at the concentration of 1.0 mM. Furthermore, compounds 5a, 5c, and 5d, with 2-(dimethylamino)prop-2-yloxy or 2-(4-methylpiperazin-1-yl)acetamido at the R₂ position, show better results. Compound 5d, with (S)-2-(dimethylamino)prop-2-yloxy or 2-(4-methylpiperazin-1-yl)acetamido at the R₂ position, provides the half activity (0.490 mM) compared to its isomer. Compounds 7a, 9a–9d, 10a–10d, and 11a–11b, where the benzyl groups are attached to the nitrogen atom of the pyridin-2(1H)-one, show more potent activities with the IC₅₀ values ranging from 0.069 to 0.441 mM besides 7a (with 40.95% inhibition rate at a concentration of 1 mM). Further research on the relationship between structure and activity showed that both the R₂ group and the chirality of the R₂ group have a great influence on the activity. Compounds 9a, 9c, 11a and 11b with acetamido or N-methylacetamido at the R₂ position gave IC₅₀ values of 0.132–0.145 mM which were much better than that of 4a, 4b, with the same group at the same position in a previous compound series (compounds 1–7). On the other hand, activity changes are not as pronounced as in the previous series when R₂ groups were changed to 2-(dimethylamino)prop-2-yloxy or 2-(4-methylpiperazin-1-yl)acetamido (compounds 10a–10c, 0.273 to 0.441 mM). It is worth pointing out that the change of chiral configuration still exists for the activity; compound 10b, with a (S)-2-(dimethylamino)prop-2-yloxy or 2-(4-methylpiperazin-1-yl)acetamido group at the R₂ position, shows an IC₅₀ of 0.310 mM, which is better than its enantiomer 10c (0.441 mM). It is a delight to us that the enantiomer at the R₂ position, shows more excellent performance and was selected as the optimal compound for the further evaluation of drug availability.

**Antiproliferative activity of compounds 9b and 9d against the human fibroblast cell line**

To further study the antifibrotic activity of synthesized compounds, compounds 9b and 9d were used for the study of antiproliferative activity against the human fibroblast cell line HFL1 with CCK8 assay and the results are shown in Fig. 1A; compounds 9b and 9d displayed better antiproliferative activity with IC₅₀ values of 0.048 and 0.035 mM compared with the use of NIH3T3 cell lines, respectively. The fixed cell morphology showed the cells in the untreated group with a clear outline, while the cells began to shrink and become round after addition of the compound 9b or 9d, which shows that the proliferation of HFL1 and NIH3T3 was effectively inhibited when the cell lines were treated with 9b and 9d at a concentration of 0.050 mM for 24 h (Fig. 1B). Compound 9d showed excellent performance and was selected as the optimal compound for the further evaluation of drug availability.
Compound 9d inhibition of NHI3T3 cell migration

In pulmonary fibrosis, the key fibrogenic factor TGF-β1 induces the occurrence of epithelial–mesenchymal transition (EMT). As compound 9d exhibited the best results in inhibition of anti-proliferative activity against both the mouse fibroblast NIH3T3 cell line and human fibroblast cell line HFL1, we evaluated the effects of 9d on TGF-β1-induced EMT in NHI3T3 cells. First of all, a transwell assay was performed to evaluate the influence of 9d on the migration capacity of 3T3 cells. As shown in Fig. 2A, treatment with 9d markedly inhibited 3T3 cell migration. The data in Fig. 2C showed that TGF-β1 can effectively promote cell migration with an OD value of about 1.7 (the OD value of the control is 1.0). In contrast, compound 9d provided an OD value of about 0.35, and an OD value of about 0.8 was obtained in 9d + TGF-β1, indicating that compound 9d can inhibit the cell migration effectively. Similar results were also obtained for the cell migration inhibition rates of B and C in Fig. 2. Therefore, compound 9d could attenuate pulmonary fibrosis by inhibiting TGF-β1-induced EMT.

Expression of E-cadherin and α-SMA in NHI3T3 cells treated with 9d by immunofluorescence assay

Loss of E-cadherin expression is a hallmark of epithelial–mesenchymal transition (EMT) and is associated with an increased risk of metastases from mutant cells such as cancer cells. E-cadherin deletion not only helps tumor cells to separate

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**Table 1** Anti-proliferative activities of compounds 1–17

<table>
<thead>
<tr>
<th>Entry</th>
<th>Comp.</th>
<th>R1</th>
<th>R2</th>
<th>Inhibition% @ 1 mM</th>
<th>Inhibition% @ 0.5 mM</th>
<th>IC50 (μM)</th>
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<tbody>
<tr>
<td>1</td>
<td>BFD</td>
<td>CH3</td>
<td>H</td>
<td>35.7</td>
<td>17.9</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>2</td>
<td>4a</td>
<td>CF3</td>
<td>Acetamido</td>
<td>32.59</td>
<td>10.4</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>3</td>
<td>4b</td>
<td>CH3</td>
<td>Acetamido</td>
<td>25.7</td>
<td>1.3</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>4</td>
<td>5a</td>
<td>CF3</td>
<td>2-(4-Methylpiperazin-1-yl)acetamido</td>
<td>62.35</td>
<td>51.8</td>
<td>340.8 ± 7.3</td>
</tr>
<tr>
<td>5</td>
<td>5b</td>
<td>CF3</td>
<td>2-(Dimethylamino)acetamido</td>
<td>45.8</td>
<td>34.2</td>
<td>500</td>
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<tr>
<td>6</td>
<td>5c</td>
<td>CH3</td>
<td>(R)-(2-(Dimethylamino)propanamido)</td>
<td>52.9</td>
<td>26.8</td>
<td>490.2 ± 2.7</td>
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<tr>
<td>7</td>
<td>5d</td>
<td>CH3</td>
<td>(S)-(2-(Dimethylamino)propanamido)</td>
<td>60.9</td>
<td>58.6</td>
<td>246.7 ± 3.8</td>
</tr>
<tr>
<td>8</td>
<td>7a</td>
<td>CH3</td>
<td>H</td>
<td>40.95</td>
<td>20.58</td>
<td>&gt;500</td>
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<tr>
<td>9</td>
<td>9a</td>
<td>CF3</td>
<td>Acetamido</td>
<td>86.23</td>
<td>68.36</td>
<td>132 ± 2.3</td>
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<td>10</td>
<td>9b</td>
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<td>N-Acetylacetamido</td>
<td>90.46</td>
<td>80.57</td>
<td>87.5 ± 0.7</td>
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<tr>
<td>11</td>
<td>9c</td>
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<td>Acetamido</td>
<td>88.96</td>
<td>72.54</td>
<td>113 ± 1.9</td>
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<tr>
<td>12</td>
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<td>98.7</td>
<td>88.4</td>
<td>69.1 ± 2.7</td>
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<tr>
<td>13</td>
<td>10a</td>
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<td>2-(4-Methylpiperazin-1-yl)acetamido</td>
<td>87.69</td>
<td>66.52</td>
<td>246.3 ± 1.8</td>
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<tr>
<td>14</td>
<td>10b</td>
<td>CF3</td>
<td>(S)-(2-(Dimethylamino)propanamido)</td>
<td>64.2</td>
<td>49.4</td>
<td>310.2 ± 3.7</td>
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<tr>
<td>15</td>
<td>10c</td>
<td>CF3</td>
<td>(R)-(2-(Dimethylamino)propanamido)</td>
<td>60.23</td>
<td>37.5</td>
<td>441.6 ± 4.8</td>
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<tr>
<td>16</td>
<td>10d</td>
<td>CF3</td>
<td>2-(Dimethylamino)acetamido</td>
<td>71.3</td>
<td>52.4</td>
<td>273.1 ± 1.5</td>
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<tr>
<td>17</td>
<td>11a</td>
<td>CF3</td>
<td>N-Methylacetamido</td>
<td>78.5</td>
<td>67.9</td>
<td>145.8 ± 5.5</td>
</tr>
<tr>
<td>18</td>
<td>11b</td>
<td>CH3</td>
<td>N-Methylacetamido</td>
<td>79.4</td>
<td>66.7</td>
<td>139.6 ± 9.8</td>
</tr>
</tbody>
</table>

Values are averages (±SD) of at least three independent determinations. Values “greater than” indicate that half-maximum inhibition was not achieved at the highest concentration tested.
from each other by disrupting cell-to-cell junctions, but also induces intracellular signaling events that produce a mesenchymal state and metastatic phenotype. Mutations of gene in α-smooth muscle actin (α-SMA) cause a variety of vascular diseases, such as thoracic aortic disease, coronary artery disease, stroke, moyamoya disease, and multisystem smooth muscle dysfunction syndrome. α-SMA is currently considered a hallmark of myofibroblasts. Therefore, we chose to study the expression of E-cadherin and α-SMA in NHI3T3 cells treated with TGF-β1 and compound 9d. As shown, after 48 hours of stimulation with recombinant TGF-β1 in NHI3T3 cells treated with DMSO or 9d, immunofluorescence staining showed that the positive staining of E-cadherin decreased in the TGF-β1 group compared with the 9d-treated group and the control group, and the expression of E-cadherin was reversed after 9d treatment, higher than the control group (Fig. 3A). On the other hand, the positive staining of α-SMA increased in the TGF-β1 group compared with the control group and the 9d-treated group, and the expression of α-SMA decreased after the 9d-treatment (Fig. 3B), indicating that 9d can stifle the activation and proliferation of fibroblasts.

Acute toxicity experiment and histopathologic examination

In clinical practice, the biosafety of drugs is the primary concern, so a toxicology assessment was conducted for the preliminary evaluation of the safety of compound 9d. The KM mice, originally from Switzerland, introduced into Kunming Central Epidemic Prevention Office of China from Haffkine Institute of India in 1946, and widely used in the evaluation of drug safety and effectiveness at present, were used for the evaluation.24–26 The mice (3 male and 5 female in each group) were randomly divided into experimental group and control group, and were caudal vein injected with 9d at a dose of 100/150/300 mg kg⁻¹, and the control group was caudal vein injected with physiological saline. The results showed no death and obvious toxicological reactions in mice within 24 hours after administration. Subsequently, the mice were euthanized and histologically analyzed to assess damage of the liver, kidney, heart, and spleen. As shown in Fig. 4, no pathological abnormalities such as inflammation, injury and necrosis were observed in the treatment group.

Molecular docking

The pathogenesis of IPF is complex and the etiology is not fully clarified. It mainly includes immune inflammatory injury,
abnormal repair and fibrosis, oxidative stress, angiogenesis and remodeling. Studies\(^\text{23}\) showed that inhibiting the expression of VEGF/VEGFR-2 can reduce microvessel density, regulate protein kinase ERK1/2 by inhibiting the extracellular signal, down-regulate the levels of TNF-\(\alpha\) and TNF-\(\beta\), and inhibit the apoptosis of type II alveolar epithelial cells, so as to play an inhibitory role in the formation of pulmonary fibrosis. In order to study the possible interaction of compound 9d with VEGFR, we docked compound 9d at the active site of kinase using Schrodinger Suites 2018 software (PDB Code: 3c7q). As shown in Fig. 5, compound 9d is in the hydrophobic active cavity composed of hydrophobic amino acids that is similar to nintedanib. The pyridinone moiety of 9d forms a hydrogen bond with lys868, and the diacetylamine moiety forms a hydrogen bond with asn923, which has high affinity with the kinase. Therefore, the docking results showed that the two ends of 9d formed hydrogen bonds with kinase, which was conducive to the matching with VEGFR-2 active sites.

Conclusions

Pirfenidone is one of the two drugs approved by the FDA for the treatment of IPF at present. As pirfenidone has a poor therapeutic effect on patients with severe pulmonary fibrosis and owing to its side effects on patients, it is necessary to develop new anti-fibrosis drugs. Herein, sixteen new pirfenidone derivatives were synthesized and evaluated against the mouse fibroblast cell line (NIH3T3 cells) and human fibroblast cell line HFL1. By the screening at the cell level, two optimal compounds that can be further studied were obtained. Compound 5d, with the same ring skeleton as pirfenidone, but a (S)-2-(dimethylamino)propanamido group in the R\(_2\) position, exhibited 10 times anti-fibrosis activity of pirfenidone. Compound 9d, with benzyl groups attached to the nitrogen atom of the pyridin-2(1H)-one moiety and a N-acetylacetamido group at the R\(_2\) position, gave more than 40 times anti-fibrosis activity of pirfenidone. In addition, the synthetic route to the optimal compound in this manuscript is simple and the target compound product is easy to purify. Considering the safety limits of impurities, relevant analytical methods, including appropriate limit of quantitation (LOQ) and limit of detection (LOD), will be developed. The anti-fibrosis assay \textit{in vivo} of two optimal compounds is in progress.

Experimental

Chemistry

All reagents were analytically pure without further purification, and all chemistry solvents were of reagent-grade and were dried and freshly distilled before the necessary step. \(^1\)H NMR and \(^{13}\)C NMR spectra were recorded on a Bruker Avance III spectrometer, and chemical shifts are reported as parts per million (ppm) with tetramethylsilane as the internal standard. High-resolution mass spectra (HR-MS) were recorded using an Agilent LC-MS 6120 instrument. High-performance liquid chromatography (HPLC) was run on a TC-C18 column (4.6 \(\times\) 250 mm, 5 \(\mu\)m) with two different solvent gradients (methanol/water = 85 : 15), and a flow rate of 0.50 mL min\(^{-1}\). All synthesized compounds were determined to have at least 95% of purity by HPLC.

\(1-(4\text{-Nitrophenyl})-5\text{-}(trifluoromethyl)pyridin-2(1H)-one\) (2a)

To a solution of 5-(trifluoromethyl)pyridin-2(1H)-one 1a (1.63 g, 10 mmol) in DMF (20 mL), K\(_2\)CO\(_3\) (2.07 g, 15 mmol) and 1-
N-(4-(2-oxo-5-(trifluoromethyl)pyridin-1(2H)-yl)phenyl)acetamide (4a)

Under nitrogen, compound 3a (0.51 g, 2 mmol) was added in a solution of acetic anhydride (3 mL). The mixture was kept at 125 °C for 2 h and the excess acetic anhydride was removed under reduced pressure. The residue was purified by column chromatography on silica gel to give 4a (0.48 g, 81% yield). ¹H NMR (500 MHz, DMSO-d₆): δ 10.15 (s, 1H), 8.21 (s, 1H), 7.74 (d, J = 9.6 Hz, 1H), 7.69 (d, J = 8.5 Hz, 2H), 7.38 (d, J = 8.6 Hz, 2H), 6.63 (d, J = 9.6 Hz, 1H), 2.08 (s, 3H). ¹³C NMR (125 MHz, DMSO-d₆): δ 168.6, 160.9, 139.5 (q, J = 5.0 Hz), 135.6, 134.5, 127.2, 124.8, 122.6, 121.2, 119.1, 24.0. HRMS (ESI) m/z: calculated for C₁₄H₁₁F₃N₂O₂ [M + Na⁺] 319.0665; found 319.0666.

5-Methyl-1-(4-nitrophenyl) pyridin-2(1H)-one (2b)

To a solution of 5-methylpyridin-2(1H)-one 1b (1.09 g, 10 mmol) in MeCN (20 mL), K₂CO₃ (2.07 g, 15 mmol) and 1-fluoro-4-nitrobenzene (1.48 g, 10.5 mmol) were added. The reaction mixture was refluxed at 85 °C for 5 h under nitrogen. After cooling to room temperature, the reaction mixture was concentrated with a rotary evaporator. Then the residue was diluted with water and extracted with ethyl acetate. The combined organic layers were washed with brine and then dried over Na₂SO₄. The solvent was removed under reduced pressure and the residue was purified by flash column chromatography on silica gel to give 2b as a brown solid (1.61 g, 70% yield). ¹H NMR (400 MHz, CDCl₃): δ 7.87–7.91 (m, 2H), 7.31 (d, J = 8.8 Hz, 2H), 7.48 (d, J = 9.6 Hz, 2H), 7.39 (d, J = 8.6 Hz, 2H), 6.63 (d, J = 9.6 Hz, 1H), 2.08 (s, 3H). ¹³C NMR (125 MHz, CDCl₃): δ 16.0 (d, J = 2.1 Hz), 127.8, 125.1, 123.0.

1-(4-Aminophenyl)-5-methylpyridin-2(1H)-one (3a)

Concentrated hydrochloric acid (4.5 mL) was added to a solution of 1-(4-nitrophenyl)-5-methylpyridin-2(1H)-one 2a (0.57 g, 2 mmol) in MeOH (15 mL), and then SnCl₂ · 2H₂O was added at 0 °C. The reaction mixture was stirred for 1 h at 40 °C. After cooling to room temperature, a saturated sodium carbonate solution was added. The mixture was filtrated and the filtrate was extracted with ethyl acetate (15 mL x 3). The combined organic layers were washed with brine and then dried over Na₂SO₄. After filtration, the solvent was concentrated under reduced pressure and the residue was purified by flash column chromatography on silica gel to give 3a (0.4 g, 79% yield). ¹H NMR (500 MHz, CDCl₃): δ 7.73 (d, J = 0.75 Hz, 1H), 7.49 (dd, J = 9.6, 2.7 Hz, 1H), 7.13–7.10 (m, 2H), 6.73–6.68 (m, 3H). ¹³C NMR (125 MHz, CDCl₃): δ 162.1, 147.4, 138.3 (q, J = 5.3 Hz), 135.2 (d, J = 2.2 Hz), 130.50, 127.2, 122.1, 115.2.

1-(4-Aminophenyl)-5-methylpyridin-2(1H)-one (3b)

Compound 3b was obtained by the same method as 3a (0.36 g, 90% yield). ¹H NMR (500 MHz, CDCl₃): δ 7.23 (dd, J = 9.3, 2.6 Hz, 1H), 7.12–7.09 (m, 3H), 6.71–6.68 (m, 2H), 6.58 (d, J = 9.3 Hz, 1H), 2.08 (d, J = 0.8 Hz, 3H). ¹³C NMR (125 MHz, CDCl₃): δ 162.2, 146.7, 142.4, 136.1, 131.9, 127.3, 121.2, 115.2, 114.6, 17.0.

N-(4-(2-oxo-5-(trifluoromethyl)pyridin-1(2H)-yl)phenyl)acetamide (4b)

Compound 4b was obtained by the same method as 4a (0.39 g, 80% yield). ¹H NMR (500 MHz, DMSO-d₆): δ 10.11 (s, 1H), 7.67 (d, J = 8.5 Hz, 2H), 7.40 (s, 1H), 7.37 (d, J = 9.3 Hz, 1H), 7.30 (d, J = 8.5 Hz, 2H), 6.41 (d, J = 9.3 Hz, 1H), 2.07 (s, 3H), 2.04 (s, 3H). ¹³C NMR (125 MHz, DMSO-d₆): δ 168.5, 160.5, 142.9, 138.9, 136.2, 135.7, 127.0, 120.1, 119.1, 113.9, 24.0, 16.3. HRMS (ESI) m/z: calculated for C₁₄H₁₄F₃N₂O₂ [M + H⁺] 243.1128; found 243.1140.

2-(4-Methylpiperazin-1-yl)-N-(4-(2-oxo-5-(trifluoromethyl)pyridin-1(2H)-yl)phenyl)acetamide (5a)

To a solution of 2-(4-methylpiperazin-1-yl)acetic acid in DMSO (3 mL), N-ethyl-N-isopropylpropan-2-amine (DIEPA, 0.65 g, 5 mmol) was added at 0 °C. After the mixture was stirred for ten minutes, HATU (0.57 g, 1.5 mmol) was added, and then 3a (0.25 g, 1 mmol) was added after another ten minutes. After the reaction rises to room temperature, DMF was removed under reduced pressure. The residue was purified by column chromatography on silica gel to give 5a (0.32 g, 81% yield). ¹H NMR (500 MHz, CD₂OD): δ 8.10 (s, 1H), 7.81 (d, J = 8.8 Hz, 2H), 7.75 (dd, J = 9.6, 2.6 Hz, 1H), 7.38 (d, J = 8.8 Hz, 2H), 6.72 (d, J = 9.6 Hz, 1H), 3.31 (s, 2H), 3.21 (s, 4H), 2.85 (s, 4H), 2.78 (s, 3H). ¹³C NMR (125 MHz, CD₂OD): δ 170.6, 164.0, 140.2–140.1 (m), 137.7, 137.0, 128.3, 122.5, 122.0, 61.8, 54.9, 51.7, 44.1. HRMS (ESI) m/z: calculated for C₁₉H₂₁F₃N₄O₂ [M + H⁺] 395.1689; found 395.1684.

2-(Dimethylamino)-N-(4-(2-oxo-5-(trifluoromethyl)pyridin-1(2H)-yl)phenyl)acetamide (5b)

In addition to replacing 2-(4-methylpiperazin-1-yl)acetic acid with dimethylglycine, compound 5b was obtained by the same method as 5a (0.25 g, 75% yield). ¹H NMR (500 MHz, CD₂OD): δ 8.10 (s, 1H), 7.78 (d, J = 8.8 Hz, 2H), 7.77–7.75 (m, 1H), 7.40 (d, J = 8.8 Hz, 2H), 6.73 (d, J = 9.6 Hz, 1H), 3.74 (s, 2H), 2.75 (s, 6H). ¹³C NMR (125 MHz, CD₂OD): δ 167.2, 164.0, 140.0 (q, J = 6.3 Hz), 137.7, 137.2, 128.4, 122.5, 121.7, 61.6, 45.1. HRMS (ESI) m/z: calculated for C₁₉H₂₃F₃N₃O₂ [M + H⁺] 340.1267; found 340.1276.

(R)-2-(Dimethylamino)-N-(4-(5-methyl-2-oxopyridin-1(2H)-yl)phenyl)propanamide (5c)

In addition to replacing 2-(4-methylpiperazin-1-yl)acetic acid with (R)-dimethylalanine, compound 5c was obtained by the...
same method as 5a (0.21 g, 70% yield). $^1$H NMR (500 MHz, CD$_2$OD): $\delta$: 7.77 (d, $J = 8.8$ Hz, 2H), 7.51 (dd, $J = 9.3$, 2.5 Hz, 1H), 7.40 (s, 1H), 7.36–7.33 (m, 2H), 6.58 (d, $J = 9.3$ Hz, 1H), 2.45 (s, 6H), 2.15 (s, 3H), 1.37 (d, $J = 6.9$ Hz, 3H). $^{13}$C NMR (125 MHz, CD$_2$OD): $\delta$: 173.5, 164.0, 145.4, 139.8 (q, $J = 6.3$ Hz), 138.0, 137.6, 128.2, 121.7, 121.0, 118.4, 66.3, 42.4, 16.8, 13.9. HRMS (ESI) m/z: calculated for C$_7$H$_{12}$N$_2$O$_2$ [M + H]$^+$ 300.1707; found 300.1708.

(S)-2-(Dimethylamino)-N-(4-(5-methyl-2-oxopyridin-1(2H)-yl)phenyl)propanamide (5d)

To a solution of (S)-dimethylalanine (0.26 g, 2.2 mmol) in DCM (10 mL), N-ethyl-N-isopropylpropan-2-amine (DIPEA, 1.03 g, 8.0 mmol) was added at 0 ºC. After the mixture was stirred for ten minutes, HATU (0.91 g, 2.38 mmol) was added, and then 3b (0.32 g, 1.6 mmol) was added after another ten minutes. After the reaction rises to room temperature, DCM was removed under reduced pressure. The residue was purified by column chromatography on silica gel to give 5a (0.33 g, 70% yield). $^1$H NMR (500 MHz, CD$_2$OD): $\delta$: 7.77 (d, $J = 8.8$ Hz, 2H), 7.51 (dd, $J = 9.3$, 2.4 Hz, 1H), 7.40 (s, 1H), 7.34 (d, $J = 8.8$ Hz, 2H), 6.57 (d, $J = 9.3$ Hz, 1H), 3.67 (q, $J = 6.9$ Hz, 1H), 2.48 (s, 6H), 2.14 (s, 3H), 1.38 (d, $J = 6.9$ Hz, 3H). $^{13}$C NMR (125 MHz, CD$_2$OD): $\delta$: 173.2, 163.9, 145.4, 139.8, 138.0, 137.5, 128.2, 121.7, 121.0, 118.3, 66.2, 42.4, 16.8, 13.9. HRMS (ESI) m/z: calculated for C$_{17}$H$_{21}$N$_3$O$_2$ [M + H]$^+$ 300.1707; found 300.1708.

1-Benzyl-5-(trifluoromethyl)pyridin-2(1H)-one (7a)

To a solution of 5-methylpyridin-2(1H)-one in DMF (6 mL), K$_2$CO$_3$ (0.41 g, 3 mmol) and benzyl bromide (0.34 g, 2 mmol) were added. The reaction was carried out at 105 ºC for 2 h and then the DMF was removed under reduced pressure. Water (10 mL) was added to the residue and the mixture was extracted with ethyl acetate (20 mL). Neutral the mixture was extracted with ethyl acetate (20 mL), concentrated hydrochloric acid (8 mL) and SnCl$_2$ 2H$_2$O (5.3 g, 23.5 mmol) were added at 0 ºC. Then the DMF was removed under reduced pressure, the residue was purified by column chromatography on silica gel to give 7a (0.34 g, 85% yield). $^1$H NMR (400 MHz, CDCl$_3$): $\delta$: 7.62 (s, 1H), 7.40 (d, $J = 9.6$, 2.6 Hz, 1H), 7.12 (d, $J = 8.3$ Hz, 2H), 6.67–6.63 (m, 3H), 5.01 (s, 2H). $^{13}$C NMR (125 MHz, CDCl$_3$): $\delta$: 162.2, 147.0, 136.6 (q, $J = 5.0$ Hz), 134.9 (d, $J = 2.5$ Hz), 130.2, 124.6, 121.5, 115.5, 52.3.

1-(Aminobenzyl)-3-(trifluoromethyl)pyridin-2(1H)-one (8a)

To a solution of 7b (1.41 g, 4.7 mmol) in 25 mL of methanol, concentrated hydrochloric acid (8 mL) and SnCl$_2$ 2H$_2$O (5.3 g, 23.5 mmol) were added at 0 ºC, and then at 40 ºC for 1 h. After the reaction temperature dropped to room temperature, saturated sodium carbonate was added until the solution was neutral. The mixture was extracted with ethyl acetate (20 mL × 3), and the combined organic phase was washed with brine, and dried over sodium sulfate. The solvents were removed under reduced pressure and the residue was purified by column chromatography on silica gel to give 8a (1.00 g, 79% yield). $^1$H NMR (500 MHz, CDCl$_3$): $\delta$: 7.62 (s, 1H), 7.40 (dd, $J = 9.6$, 2.6 Hz, 1H), 7.12 (d, $J = 8.3$ Hz, 2H), 6.67–6.63 (m, 3H), 5.01 (s, 2H). $^{13}$C NMR (125 MHz, CDCl$_3$): $\delta$: 162.2, 147.0, 136.6 (q, $J = 5.0$ Hz), 134.9 (d, $J = 2.5$ Hz), 130.2, 124.6, 121.5, 115.5, 52.3.

5-Methyl-1-(4-nitrobenzyl)pyridin-2(1H)-one (7c)

To a solution of 5-methylpyridin-2(1H)-one 1b (1.09 g, 10 mmol) in MeCN (6 mL), K$_2$CO$_3$ (2.07 g, 15 mmol) and 1-(bromomethyl)-4-nitrobenzene (2.27 g, 10.5 mmol) were added. The reaction was carried out at 85 ºC for 5 h and then the MeCN was removed under reduced pressure. Water (10 mL) was added to the residue and the mixture was extracted with ethyl acetate (2 × 20 mL). The combined organic phase was washed with brine, and dried over sodium sulfate, and then the solvents were removed under reduced pressure. The residue was purified by column chromatography on silica gel to give 7c (1.6 g, 65% yield). $^1$H NMR (400 MHz, CDCl$_3$): $\delta$: 8.19–8.17 (m, 2H), 7.45 (dd, $J = 8.8$, 2Hz), 7.24 (dd, $J = 9.3$, 2.5 Hz, 1H), 7.07 (s, 1H), 6.58 (d, $J = 9.3$ Hz, 1H), 5.19 (s, 2H), 2.07 (d, $J = 0.7$ Hz, 3H). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$: 161.8, 147.5, 144.0, 142.7, 134.5, 128.5, 124.0, 121.1, 115.9, 51.6, 17.1.

N-(4-(2-oxo-5-(Trifluoromethyl)pyridin-1(2H)-yl)methyl)phenylacetamide (9a)

Under nitrogen, acetic anhydride (3 mL) was added to the reaction bottle containing 8a (0.64 g, 75% yield). $^1$H NMR (400 MHz, CDCl$_3$): $\delta$: 7.14–7.09 (m, 3H), 7.00 (d, $J = 0.5$ Hz, 1H), 6.63–6.61 (m, 2H), 6.52 (d, $J = 9.2$ Hz, 1H), 4.97 (s, 2H), 1.99 (s, 3H). $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$: 162.0, 146.4, 141.9, 134.5, 129.7, 126.3, 120.6, 115.2, 115.0, 51.3, 17.1.
N-Acetyl-N-[(2-oxo-5-(trifluoromethyl)pyridin-1(2H)-yl) methyl]phenyl]acetamide (9b)

Under nitrogen, acetic anhydride (5 mL) was added to the reaction bottle containing 8a (0.54 g, 2 mmol) and the reaction was kept for 4 h at 135 °C. After the excess acetic anhydride was removed under reduced pressure, the residue was purified by column chromatography on silica gel to give 9b (0.54 g, 76% yield). 1H NMR (400 MHz, CDCl3) δ: 7.73 (s, 1H), 7.48 (dd, J = 9.6, 2.6 Hz, 1H), 7.41 (d, J = 8.4 Hz, 2H), 7.17–7.14 (m, 2H), 6.69 (d, J = 9.6 Hz, 1H), 5.18 (s, 2H), 2.28 (s, 6H). 13C NMR (100 MHz, CDCl3) δ: 173.0, 161.9, 139.6, 137.1 (q, J = 5.0 Hz), 136.2, 135.5 (d, J = 2.0 Hz), 129.5, 122.0, 52.5, 27.1. HRMS (ESI) m/z: calculated for C17H15F3N3O2 [M + Na]+ 375.0927; found 375.0929.

N-(4-[(5-Methyl-2-oxopyridin-1(2H)-yl)methyl]phenyl) acetamide (9c)

Replacing 8a with compound 8b, compound 9c was obtained by the same method as 9a (0.37 g, 73% yield). 1H NMR (400 MHz, DMSO-d6) δ: 9.99 (s, 1H), 7.53–7.51 (m, 3H), 7.29–7.22 (m, 3H), 6.36 (d, J = 9.2 Hz, 1H), 4.98 (s, 2H), 2.02 (s, 3H), 2.00 (s, 3H). 13C NMR (100 MHz, DMSO-d6) δ: 168.4, 160.7, 142.4, 138.7, 136.1, 132.1, 128.3, 119.6, 119.1, 114.1, 50.5, 24.0, 16.5.

N-Acetyl-N-[(2-methyl-5-oxo-2-oxopyridin-1(2H)-yl) methyl]phenyl acetamide (9d)

Under nitrogen, acetic anhydride (5 mL) was added to the reaction bottle containing 8b (0.43 g, 2 mmol) and the reaction was kept for 4 h at 135 °C. After the excess acetic anhydride was removed under reduced pressure, the residue was purified by column chromatography on silica gel to give 9d (0.42 g, 70% yield). 1H NMR (500 MHz, CDCl3) δ: 7.38 (d, J = 8.0 Hz, 2H), 7.22 (d, J = 9.2 Hz, 1H), 7.11 (t, J = 8.0 Hz, 3H), 6.59 (d, J = 9.3 Hz, 1H), 5.15 (s, 2H), 2.27 (s, 6H), 2.06 (s, 3H). 13C NMR (125 MHz, CDCl3) δ: 172.9, 162.0, 142.5, 138.9, 133.7, 134.8, 129.1, 120.7, 115.7, 51.4, 27.0, 17.1. HRMS (ESI) m/z: calculated for C17H15F3N3O2 [M + Na]+ 321.1210; found 321.1212.

2-(2-Methylpiperazin-1-yl)-N-[(2-oxo-5-(trifluoromethyl) pyridin-1(2H)-yl)methyl]phenyl]acetamide (10a)

To a solution of 2-(2-methylpiperazin-1-yl)acetic acid in DMF (3 mL), N-ethyl-N-isopropylpropan-2-amine (DIPEA, 0.65 g, 5 mmol) was added at 0 °C. After the mixture was stirred for 10 minutes, HATU (0.57 g, 1.5 mmol) was added. After stirring for 10 minutes, 8a (0.27 g, 1 mmol) was added and the reaction was raised to room temperature. The solvent was removed under reduced pressure and the residue was purified by column chromatography on silica gel to give 10a (0.29 g, 70% yield). 1H NMR (500 MHz, CD2OD) δ: 8.24 (s, 1H), 7.65 (dd, J = 9.5, 2.6 Hz, 1H), 7.60 (d, J = 8.5 Hz, 2H), 7.32 (d, J = 8.6 Hz, 2H), 6.65 (d, J = 9.5 Hz, 1H), 5.18 (s, 2H), 3.22 (s, 2H), 2.91 (s, 4H), 2.74 (s, 4H), 2.56 (s, 3H). 13C NMR (125 MHz, CD2OD) δ: 169.2, 162.7, 138.3 (q, J = 8.5 Hz), 137.7, 135.8 (d, J = 1.6 Hz), 131.9, 128.4, 127.1, 124.5, 122.4, 120.4, 60.8, 53.8, 52.1, 51.3, 43.5. HRMS (ESI) m/z: calculated for C28H22F2N4O2 [M + H]+ 409.1846; found 409.1833.

N-Methyl-N-[[(2-oxo-5-(trifluoromethyl)pyridin-1(2H)-yl) methyl]phenyl]acetamide (11a)

To a solution of compound 9a in DMF (10 mL), NaH (50 mg, 1.2 mmol) was added at 0 °C. After the mixture was stirred for 15 minutes, methyl iodide (170 mg, 1.2 mmol) was added. After the reaction rose naturally to room temperature, water (1 mL) was added. DMF was removed under reduced pressure and water was added to the residue. The mixture was extracted with ethyl acetate (10 mL × 2) and the combined organic phase was washed with brine, and dried over sodium sulfate. The solvents were removed under reduced pressure and the residue was purified by column chromatography on silica gel to give 11a (0.24 g, 75% yield). 1H NMR (500 MHz, CDCl3) δ: 7.77 (s, 1H), 7.48 (d, J = 9.5 Hz, 1H), 7.39 (d, J = 8.0 Hz, 2H), 7.21 (d, J = 7.8 Hz, 2H), 6.69 (d, J = 9.5 Hz, 1H), 5.18 (s, 2H), 3.25 (s, 3H), 1.87 (s, 3H). 13C NMR (125 MHz, CDCl3) δ: 170.4, 161.8, 144.6, 136.9 (q, J = 5.0 Hz), 135.2 (d, J = 2.5 Hz), 134.9, 129.4, 127.7,
124.2, 122.1, 121.8, 52.3, 37.1, 22.4. HRMS (ESI) m/z: calculated for C_{16}H_{18}N_{2}O_{2} [M + Na]^+ 347.0978; found 347.0977.

**N-Methyl-N-(4-(5-methyl-2-oxopyridin-1(2H)-yl)(methyl)phenyl)acetamide (11b)**

Replacing 9a with 9c, compound 11b was obtained by the same method as 11a (0.21 g, 77% yield). 1H NMR (500 MHz, CDCl₃): δ: 7.35 (d, J = 7.9 Hz, 2H), 7.23 (d, J = 9.2 Hz, 1H), 7.16 (d, J = 7.8 Hz, 2H), 7.11 (s, 1H), 6.58 (d, J = 9.2 Hz, 1H), 5.13 (s, 2H), 3.23 (s, 3H), 2.08 (s, 3H), 1.86 (s, 3H). 13C NMR (125 MHz, CDCl₃): δ: 170.4, 161.9, 144.1, 142.4, 136.3, 129.1, 127.4, 120.9, 115.6, 51.6, 37.1, 22.4, 17.1. HRMS (ESI) m/z: C_{16}H_{18}N_{2}O_{2} [M + Na]^+ 293.1260; found 293.1262.

**Biological assay**

**Cell lines and culture.** The human embryonic lung fibroblast cell line (HFL1) and the mouse embryonic lung fibroblast cell line (NH3T3) used in this study were purchased from Bokang Biotechnology Co. LTD (Qingdao, China). Cell lines were cultivated in F12K/DMEM containing 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 units per mL penicillin, and 100 μg mL⁻¹ streptomycin.

**CCK8 assay**

For cytotoxicity assay, the cells grown in the logarithmic phase were seeded into 96-well plates (5 × 10³ cells per well) for 24 h, and then exposed to different concentrations of the test compounds for 72 h. The attached cells were incubated with CCK8 (Sigma, USA) for another 4 h. Then, the absorbance at 450 nm was measured using a multifunction microplate reader (Molecular Devices, Flex Station 3), and each experiment was performed at least in triplicate. The cytotoxic effects of each compound were expressed as IC₅₀ values, which represent the drug concentrations required to cause 50% tumour cell growth inhibition, and were calculated with GraphPad Prism Software version 5.02 (GraphPad Inc., La Jolla, CA, USA).

**Anti-cell-migration study**

NH3T3 cells were plated in a 6-well culture dish at 5 × 10³ cells per dish and grown for 24 h, and the non-migrated cells were scraped off the upper surface of the membrane with a 10 μL pipette. The medium was then replaced by 10% serum 1640 medium and treated with compound 9d at the indicated concentrations for another 24 h. After washing with phosphate buffer solution (PBS), the cell images were immediately detected using a Zeiss LSM 570 laser scanning confocal microscope (Carl Zeiss, Germany). The cell migration assay was performed using Transwell inserts with an 8.0 μM pore size. The cells (5 × 10³ cells in 0.2 mL of serum-free medium) were added to the upper chamber. Various concentrations of chemicals in 0.5 L of medium with 10% FBS were applied to the lower chamber. After 9d 48 h, the migrated cells attached to the lower surface were stained with crystal violet (500 μL of 5% methanol) (Sigma-Aldrich) and allowed to incubate for 10 min. The membrane was then washed several times with PBS, and the cells that penetrated the filter were counted under a microscope.

**Immunofluorescence microscopy**

The NH3T3 cells were fixed in 4% paraformaldehyde, stained with specific primary antibodies, including those against E-cadherin and z-SMA, at 4 °C overnight and then incubated with FITC-conjugated anti-rabbit IgG (Solarbio). The nuclei were stained with DAPI. Representative micrographs were observed using a confocal laser scanning microscope (Leica, Wetzlar, Germany).

**In vivo biosafety evaluation**

Kunming mice (5–6 weeks old, 18–24 g) in pathogen-free condition, obtained from the Vital River of Beijing University, were maintained at constant room temperature and fed a standard rodent chow and water. The mice were randomly divided into experimental group and control group (n = 10, five male and five female). The mice were caudal vein injected with 9d at a dose of 100/150/300 mg kg⁻¹, and the control group was caudal vein injected with NaCl solution. The mice died after neck dissection, and the tissues of the liver, spleen and kidney were taken for histopathological examination. The major organs (liver, kidney, heart, and spleen) were excised, fixed in 4% paraformaldehyde, embedded in paraffin, and cut into 6 μm sections for hematoxylin and eosin (H&E) staining by standard procedures. Images were captured using a microscope.

**Conflicts of interest**

There are no conflicts to declare.

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**Notes and references**


