Novel Pirfenidone Derivatives: Synthesis, and Biological Evaluation

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Research Article

Keywords: pirfennidone, synthesis, derivatives, pulmonary fibrosis

Posted Date: April 7th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1509281/v1

Abstract

In order to discover novel anti-pulmonary fibrosis agents, a series of new pirfennidone derivatives were designed and synthesized. All compounds have been investigated for their anti-pulmonary activity and confirmed by ^{13}C and ^{1}H nuclear magnetic resonance and high-resolution mass spectrometry. Preliminary studies on its biological activity showed that all target compounds showed different degrees of inhibition on pulmonary fibrosis, and most of the derivatives were significantly better than pirfenidone.

Introduction

Pulmonary fibrosis (PF) is a chronic, progressive, fibrotic lung disease, that is a large family of interstitial lung diseases. The pathological process of PF is characterized by diffuse damage to vascular endothelial cells and alveolar epithelial cells by early pathogenic factors, causing alveolar inflammation, persistence of immune-mediated lung inflammation, and a series of immune-related factors. Various signal transduction pathways expand tissue damage, a large number of fibroblasts aggregate, drive collagen and other extracellular matrix (ECM) abnormal deposition and tissue contraction, normal alveolar tissue damage, abnormal repair leads to structural abnormalities and dysfunction[1, 2]. idiopathic pulmonary fibrosis(IPF) is the most common form of pulmonary fibrosis. IPF is characterized by progressive fibrosis, excessive matrix deposition leading to the ultimate destruction of lung structure Fatal damage to lung function. IPF clinical manifestations are heterogeneous but the median survival after diagnosis is only 2.5–3.5 years[3, 4]. In recent years, with the understanding of its pathogenesis, two PF therapeutic drugs (Pirfenidone and Nintedanib) have been approved for marketing worldwide[5].

Pirfenidone(PFD) is an oral small molecule compound that is anti-fibrotic, anti-inflammatory, and antioxidant[6], It was first synthesized by Margolin in the United States in 1974(Fig. 1), The initial study found that it has a certain anti-inflammatory effect, and later found that PFD has anti-fibrotic effect, and the adverse reactions are small. The anti-inflammatory activity of pirfenidone has been confirmed in a mouse model of septic shock[7]. PFD was approved for marketing in Japan in November 2008 for the treatment of idiopathic pulmonary fibrosis[8], whose mechanism of action is to inhibit the TGF-βpathway, TGF-β is one of the most studied profibrotic cytokines, and pirfenidone has anti-fibrotic activity by inhibiting TGF-β[9, 10].

Although pirfenidone is a potential drug for the treatment of IPF, oral pirfenidone can cause systemic side effects such as nausea, anorexia, dizziness, rash, liver dysfunction, and phototoxicity, among which phototoxic reactions are major adverse reactions. Therefore, it is necessary to structurally modify pirfenidone to obtain novel compounds with less side effects and higher biological activity. For example, Ma et al.[11] introduced alkoxy substituents that included terminal amines of different lengths at the C-4 position of the benzene ring to determine the influence of the size of the linker and different amines on the biological activity of the compound. Some derivatives were revealed to have excellent antifibrotic activities.

Studies have shown that the C_5-methyl group of PFD is much less active when it is rapidly metabolized to carboxylic acid. Thus, it is possible to prepare a compound having high anti-fibrotic activity by preventing metabolism in vivo[12]. Analyze the structural activity relationship of other drugs for the treatment of pulmonary fibrosis, some drugs act via suppressing the mechanism of the migration and proliferation of fibroblasts, such as sildenafil, zileuton, and nintedanib. Moreover, a kind of traditional Chinese medicine that contains colchicine has antifibrotic activity. It can be seen that these compounds apparently all have an amide bond structure. Therefore, based on the original lactam structure of pirfenidone, an amino group or a thioamino group having an amino bond is introduced at the 4-position of the benzene ring to enhance the biological activity of the compound. Taking bioisosterism into account, imidazole (imidazoline) and pyrazoline rings were introduced on the 4-position of the benzene ring to obtain further ideal compounds. The last, taking the pharmacophore into consideration, an activated drug molecule was combined with a receptor target to form a bioactive structure of which the geometry and energy match each other.

Based on the above analysis, hydrophobic groups and nitrogen-containing heterocycles were introduced on the 4-position of the benzene ring to give a series of new compounds with better activity than that of pirfenidone.

Results And Discussion

Chemistry

Scheme 1 shows the general preparation procedure for these pirfenidone derivatives. Initially, a diazotization reaction between 2-aminopyridine and $NaNO_2$ in 50% H_2SO_4 afforded **2**, after which the more stable forms **3** was obtained via keto–enol tautomerism. The yield was low because of the high solubility of the product in water in the literature procedure[13]. Therefore, in order to avoid losses in the process of extraction, water was removed *in vacuo* and the residue was washed with ethyl acetate in a sand core funnel, after which the filtrate was concentrated to afford the crude product **3**. The yield was markedly increased.

4 was synthesized from **3** and 4-bromobenzaldehyde via an Ullmann coupling reaction. The improved conditions, which were based on a report by Sugahara et al., [14]comprised 0.25 equiv. CuI and a reaction time of less than 2 h. Considering the lower reactivity of 4-chlorobenzaldehyde and the higher price of 4-iodobenzaldehyde, 4-bromobenzaldehyde was chosen as the reagent.

4 reacted with different agents to generate the target derivatives **5a–5g**, respectively. Ashok et al.[15] reported the treatment of o-phenylenediamine with aldehyde using Fe/MgO as an oxidizing agent to prepare benzimidazoles. Lin et al.[16] reported a method for the preparation of a benzimidazole derivative that involved the treatment of 4-methyl-1,2-phenylenediamine with benzaldehyde in dioxane with air as an oxidizing agent. Considering the inconvenience of working with Fe/MgO and the toxicity of dioxane,

we chose air as the oxidizing agent, ethanol as the solvent, and a little glacial acetic acid as a catalyst, and the yield obtained was 84%.

5a–5c were synthesized according to the method described by Ishihara[17], but the reaction could not proceed to completion with a catalytic amount of iodine. An equivalent amount of iodine was added, improvements to the procedure made the reaction proceed to completion, and the yield exceeded 90%. The structures of all derivatives were confirmed by ^{1}H nuclear magnetic resonance (NMR), ^{13}C NMR, and high-resolution mass spectrometry (HRMS).

Biological Activity

Determination of the effect of the target compounds on 3T3L1 cells by an MTT assay

The inhibitory activities of the synthesized pirfenidone derivatives**5a–5g** against the growth of 3T3L1 cells were determined using a standard MTT assay with pirfenidoneas a positive control (the inhibition rate for blank controls was 0) at concentrations of 0.5 m mol/L, 1 m mol/L, and 2 m mol/L after 24 and 48 h, respectively. The results are listed in **Fig. 2**.

Measurement of the secretion of fibronectin by 3T3L1 cells using ELISA kits

Fibronectin is an important part of extracellular matrix (ECM) and has an extremely significant role in the proliferation of cells, the Fn of excessive accumulation can be observed in the ECM of fibrosis, so it is necessary to evaluate new compounds by Fn values.

On the basis of the results for the inhibitory activity of pirfenidone and its derivatives on the proliferation of 3T3L1 cells, certain compounds with higher activity were selected for further research. In this research, the inhibitory activity of the compounds on the secretion of fibronectin by 3T3L1 cells was tested at concentrations of 0.5 m mol/L, 1 m mol/L, and 2 m mol/L after 48 h with pirfenidone as a positive control (the fibronectin concentration for blank controls was 1389.10 ± 9.19 ng/mL). The results are listed in **Fig. 3**.

Discussion

Inhibitory activities of pirfenidone derivatives against the growth of 3T3L1 cells

The percentage inhibition rates of the growth of 3T3L1 cells for the pirfenidone derivatives after 24 h are shown in **Fig. 2**, whereas the values after 48 h are shown in **Fig. 3**.As can be seen from Fig. 2, the

inhibitory activities of pirfenidone and its derivatives against the growth of 3T3L1 cells increased with an increase in the concentration of the compounds after 24 h.

Obviously, the inhibition rates of the growth of 3T3L1 cells for pirfenidone and its derivatives increased with an increase in the concentration of the compounds. After 48 h, **5a** displayed very high inhibitory activity against 3T3L1 cells, with inhibition rates of 95.36 ± 0.09%, at concentrations of 2 m mol/L. Furthermore, the inference that the inhibitory effect on 3T3L1 cells increased over time can be drawn by combining **Fig. 2, Fig. 3**.

Conclusions

A series of new pirfenidone derivatives were synthesized and their anti-fibrotic activity was evaluated. The structure was confirmed by nuclear magnetic resonance spectroscopy, nuclear magnetic resonance carbon spectroscopy and HRMS spectroscopy. Each compound has different degrees of inhibition on pulmonary fibrosis, and most compounds have better inhibition than pirfenidone. The results of this study have reference value for the synthesis and further study of pirfenidone derivatives.

Experimental Materials And Methods

Chemistry

All reagents and solvents were commercially available and were used without further treatment unless otherwise noted. ^{1}H NMR and ^{13}C NMR spectra were recorded at 400 and 100 MHz, respectively, using a Varian Unity Inova 400 MHz instrument. Chemical shifts are stated in ppm (δ) with reference to tetramethylsilane as an internal standard. HRMS spectra were recorded with a Bruker Daltonics ESI-BioTOF Q spectrometer. Column chromatography was performed on silica gel (200–300 mesh). Thin-layer chromatography on glass slides precoated with GF-254 silica gel was used to monitor the progress of reactions.

The following equipment and reagents were used for the biological activity tests: biochemical incubators made by Changzhou Wanhe instrument manufacturing company: 150A and 250B; microplate reader: SpectraMax M5, Molecular Devices; 3T3L1 mouse embryonic fibroblasts: Cyagen Biosciences Inc.

*pyridin-2(1H)-one (*3 *)*.To a 25 mL flask were added 3.40 mL 50% sulfuric acid (v/v) and 1.00 g (10 mmol) 2-aminopyridine (**1**). The mixture turned milky white after being stirred for a few minutes below 10°C on an ice-salt bath. Then, 1.72 g (25 mmol) $NaNO_2$ in 3 mL H_2O was added dropwise, which produced an irritant gas. The pH of the resulting pale yellow solution was adjusted to 7–8 using 10% dilute sulfuric acid and the mixture was refluxed with stirring for 20 min. Water was mostly removed *in vacuo*, and the residue was mixed with a moderate amount of silica gel, concentrated to dryness, and then washed with ethyl acetate in a sand core funnel. The filtrate was concentrated to afford the crude product **3**, which was used in the following reaction without further purification.

4-(2-oxopyridin-1(2H)-yl)benzaldehyde (4 *)*.In a 25 mL round-bottom flask, a mixture of 0.10 g (1 mmol, 1 equiv.) 3, 0.17 g (1 mmol, 1 equiv.) 4-bromobenzaldehyde, 1.4 g (10 mmol, 10 equiv.) K_2CO_3, and 0.05 g (0.26 mmol, 0.25 equiv.) CuI in dimethylformamide (5 mL) was stirred under reflux for 1 h. After cooling, 30 mL water was added, and the mixture was extracted with ethyl acetate (3 × 20 mL). The organic phase was dried over anhydrous Na2SO4 and concentrated to dryness. The residue was purified by flash column chromatography (petroleum ether: ethyl acetate = 1:3 (v/v)) to give 4 as a pale yellow solid.

1-(4-(3a,4,5,6,7,7a-Hexahydro-1H-benzo[d]imidazol-2-yl)phenyl)pyridin-2(1H)-one (5a *)*.A mixture of 10 mL tert-butanol, 0.22 g (1 mmol, 1 equiv.) **4**, 0.086 g (1.2 mmol, 1.2 equiv.) 1,2-diaminopropane, 0.32 g (2.5 mmol, 2.5 equiv.) iodine, and 0.43 g (3 mmol, 3.0 equiv.) K_2CO_3 in a 25 mL round-bottom flask was stirred for 3 h at 70°C. The pH of the mixture was adjusted to 7–8, and the mixture was then concentrated *in vacuo* to give a residue, which was purified by column chromatography (petroleum ether: ethyl acetate = 1:2 (v/v) → ethanol (3–5 drops triethylamine)) to give **5b** as a yellow solid. **5b** and **5c** were obtained using procedures similar to that used for **5a**.

1-(4-(Hydrazonomethyl)phenyl)pyridin-2(1H)-one(5d *)*.In a 25 mL flask, to a solution of 0.21 g (1 mmol, 1 equiv.) **4** in 15 mL absolute ethanol 0.13 g (1.2 mmol, 1.2 equiv.) 30% hydrazine hydrate was added dropwise with 3 drops glacial acetic acid as a catalyst. The mixture was stirred for 3 h at room temperature. Removal of the solvent and recrystallization from ethanol provided **5d** as a white solid. **5e** and **5h** were obtained using procedures similar to that used for **5d**.

5-methyl-1-phenyl-2(1H)-pyridone (pirfenidone).Yellow solid, yield: 76%, m.p. 121–123°C. ^{1}H NMR (400 MHz, DMSO) δ 7.49 (t, *J* = 7.4 Hz, 2H), 7.44–7.32 (m, 5H), 6.43 (d, *J* = 9.3 Hz, 1H), 2.03 (s, 3H). ^{13}C NMR (101 MHz, DMSO) δ 160.41, 142.98, 141.01, 136.04, 128.96, 127.92, 126.71, 120.21, 114.01, 16.30.

1-(4-(3a,4,5,6,7,7a-Hexahydro-1H-benzo[d]imidazol-2-yl)phenyl)pyridin-2(1H)-one (5a *)*.Yellow solid, yield: 64%, m.p. 254–256°C. ^{1}H NMR (400 MHz, DMSO) δ 8.07 (d, *J* = 8.6 Hz, 2H), 7.83–7.69 (m, 3H), 7.59–7.52 (m, 1H), 6.51 (d, *J* = 9.3 Hz, 1H), 6.38 (td, *J* = 6.7, 1.1 Hz, 1H), 3.50–3.37 (m, 1H), 3.08 (dd, *J* = 14.5, 7.2 Hz, 1H), 2.57 (dd, *J* = 11.1, 6.1 Hz, 4H), 1.75 (s, 1H), 1.44 (dd, *J* = 16.8, 11.7 Hz, 4H). ^{13}C NMR (101 MHz, DMSO) δ 163.61, 160.91, 145.35, 141.14, 138.39, 129.26, 127.76, 122.32, 120.64, 106.21, 56.01, 53.84, 31.94, 25.33, 23.97, 18.45. HRMS (ESI) calcd for $C_{18}H_{19}N_3O$ $[M + H]^+$ 294.1607, found 294.1604.

1-(4-(4,5-Dihydro-1H-imidazol-2-yl)phenyl)pyridin-2(1H)-one (5b *)*.Yellow solid, yield: 56%, m.p. 237–239°C. ^{1}H NMR (400 MHz, DMSO) δ 8.00 (d, *J* = 8.6 Hz, 2H), 7.69 (dd, *J* = 12.3, 5.2 Hz, 3H), 7.57–7.51 (m, 1H), 6.50 (d, *J* = 9.2 Hz, 1H), 6.37 (td, *J* = 6.7, 1.1 Hz, 1H), 3.56–3.10 (m, 4H), 1.87 (s, 1H). ^{13}C NMR (101 MHz, DMSO) δ 170.27, 163.97, 160.98, 144.55, 141.07, 138.51, 128.93, 127.49, 120.63, 106.14, 45.90, 45.74. HRMS (ESI) calcd for $C_{14}H_{13}N_3O$ $[M + H]^+$ 240.1138, found 240.1138.

1-(4-(1,4,5,6-Tetrahydropyrimidin-2-yl)phenyl)pyridin-2(1H)-one (5c *)*.Yellow solid, yield: 56%, m.p. 146–148°C. ^{1}H NMR (400 MHz, DMSO) δ 8.10 (d, *J* = 8.6 Hz, 2H), 7.83 (d, *J* = 8.4Hz, 2H), 7.65 (d, *J* = 8.4 Hz, 1H), 7.57–7.51 (m, 1H), 6.48 (d, *J* = 9.3 Hz, 1H), 6.37 (td, *J* = 6.7, 1.1 Hz, 1H), 3.53–3.41 (m, 6H), 2.94 (dd,

J = 14.7, 7.4 Hz, 1H). ^{13}C NMR (101 MHz, DMSO) δ 170.34, 165.08, 162.01, 161.64, 159.06, 141.64, 138.91, 129.13, 128.63, 127.82, 120.99, 106.76, 46.31, 37.27, 35.33. HRMS (ESI) calcd for $C_{15}H_{15}N_3O$ $[M + H]^+$ 254.1294, found 254.1292.

*1-(4-(Hydrazonomethyl)phenyl)pyridin-2(1H)-one (*5d*)*.Yellow solid, yield: 69%, m.p. 164–166°C. ^{1}H NMR (400 MHz, DMSO) δ 7.75 (s, 1H), 7.63 (ddd, *J* = 6.8, 2.0, 0.5 Hz, 1H), 7.61–7.55 (m, 2H), 7.53–7.46 (m, 1H), 7.39–7.30 (m, 2H), 6.95 (s, 2H), 6.48 (dd, *J* = 9.2, 0.5 Hz, 1H), 6.30 (td, *J* = 6.7, 1.3 Hz, 1H). ^{13}C NMR (101 MHz, DMSO) δ 161.21, 140.54, 139.59, 138.98, 136.86, 136.33, 126.84, 125.41, 120.51, 105.61. HRMS (ESI) calcd for $C_{12}H_{11}N_3O$ $[M + H]^+$ 214.0981, found 214.0981; $[M + Na]^+$ 236.0800, found 236.0809.

*1-(4-((2-Phenylhydrazono)methyl)phenyl)pyridin-2(1H)-one (*5e*)*.Yellow solid, yield: 62%, m.p. 208–210°C. ^{1}H NMR (400 MHz, DMSO) δ 7.93 (s, 1H), 7.75 (d, *J* = 8.5 Hz, 2H), 7.65 (dd, *J* = 6.9, 1.7 Hz, 1H), 7.50 (ddd, *J* = 9.0, 6.6, 2.1 Hz, 1H), 7.40 (d, *J* = 8.5 Hz, 2H), 7.29–7.20 (m, 2H), 7.11 (d, *J* = 7.6 Hz, 2H), 7.02–6.88 (m, 1H), 6.76 (t, *J* = 7.2 Hz, 1H), 6.49 (d, *J* = 9.0 Hz, 1H), 6.32 (td, *J* = 6.7, 1.2 Hz, 1H). ^{13}C NMR (101 MHz, DMSO) δ 161.21, 145.60, 145.15, 140.60, 140.01, 138.95, 135.73, 135.20, 129.06, 126.99, 125.93, 121.44, 120.53, 118.97, 114.45, 112.12, 105.69. HRMS (ESI) calcd for $C_{18}H_{15}N_3O$ $[M + H]^+$ 290.1294, found 290.1292; $[M + Na]^+$ 312.1111, found 312.1104.

*2-(4-(2-Oxopyridin-1(2H)-yl)benzylidene)hydrazinecarboxamide (*5f*)*.Pale-yellow solid, yield: 66%, m.p. 230–232°C. ^{1}H NMR (400 MHz, DMSO) δ 10.38 (s, 1H), 7.89 (s, 1H), 7.85 (d, *J* = 8.5 Hz, 2H), 7.66 (dd, *J* = 6.9, 1.6 Hz, 1H), 7.51 (ddd, *J* = 9.0, 6.6, 2.0 Hz, 1H), 7.41 (d, *J* = 8.5 Hz, 2H), 6.58 (s, 2H), 6.48 (d, *J* = 9.1 Hz, 1H), 6.32 (td, *J* = 6.7, 1.2 Hz, 1H). ^{13}C NMR (101 MHz, DMSO) δ 161.16, 157.83, 156.74, 141.00, 140.66, 138.92, 138.11, 134.66, 126.97, 120.55, 105.73. HRMS (ESI) calcd for $C_{13}H_{12}N_4O_2$ $[M + H]^+$ 257.1039, found 257.1035.

*2-(4-(2-Oxopyridin-1(2H)-yl)benzylidene)hydrazinecarbothioamide (*5g*)*.Gray solid, yield: 68%, m.p. 222–224°C. ^{1}H NMR (400 MHz, DMSO) δ 11.53 (s, 1H), 8.28 (s, 1H), 8.10 (d, *J* = 8.6 Hz, 2H), 7.93 (d, *J* = 8.5 Hz, 2H), 7.66 (dd, *J* = 6.9, 1.6 Hz, 1H), 7.54–7.47 (m, 1H), 7.43 (d, *J* = 8.5 Hz, 2H), 6.49 (d, *J* = 8.9 Hz, 1H), 6.32 (td, *J* = 6.7, 1.2 Hz, 1H). ^{13}C NMR (101 MHz, DMSO) δ 178.11, 161.13, 141.65, 141.07, 140.69, 138.82, 134.02, 127.80, 127.00, 120.58, 105.78. HRMS (ESI) calcd for $C_{13}H_{12}N_4OS$ $[M + H]^+$ 273.0811, found 273.0815.

Biological activity

3T3L1 cell proliferation rate/inhibition rate

Cell culture. 3T3L1 cells were inoculated in cell culture medium with 10% fetal bovine serum (FBS) supplemented with 100 IU/mL penicillin and streptomycin, incubated in 5% CO_2 at 37°C, and passaged by digestion with 0.25% trypsin. Experiments were performed on passages 3–10. Pirfenidone and its derivatives were dissolved in DMSO and then filtered through a 0.22 μm membrane to remove bacteria and stored at − 20°C.

Determination of inhibition rate. 3T3L1 cells were seeded into 96-well plates in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS at a concentration of 8×10^4 per well and incubated in a 5% CO_2 atmosphere at 37°C for 24 h. Compounds were added at various concentrations (0.5, 1, and 2 m mol/L) with pirfenidone as a positive control and an equal amount of DMEM as a blank control (5 parallel wells for each group). Incubation was continued at 37°C in a 5%CO_2 incubator for 24 and 48 h, and then 20 μL MTT (5 mg/mL) was added for a further 4 h. Then, the supernatant was discarded, 150 μL DMSO was added to every well, and the culture was mixed for 10 min. Optical density (OD) values were determined at 570 nm using an enzyme-linked immunosorbent assay reader. The cell inhibition rates were calculated from the OD values as follows: percentage growth inhibition = $\{([OD]_{test} - [OD]_{control})/[OD]_{control}\} \times 100$.

Determination of fibronectin concentrations

Cell culture. 3T3L1 cells were inoculated in cell culture medium with 10% FBS supplemented with 100 IU/mL penicillin and streptomycin, incubated in 5%CO_2 at 37°C, and passaged by digestion with 0.25% trypsin. Experiments were performed on passages 3–10. Pirfenidone and its derivatives were dissolved in DMSO and then filtered through a 0.22 μm membrane to remove bacteria and stored at − 20°C.

Determination of expression of fibronectin by ELISA kits. 3T3L1 cells were seeded into 96-well plates in DMEM supplemented with 10% FBS at a concentration of 8×10^4 per well and incubated in a 5%CO_2 atmosphere at 37°C for 24 h. Compounds were added at various concentrations (100, 200, and 400 μg/mL) with pirfenidone as a positive control and an equal amount of DMEM as a blank control. Incubation was continued at 37°C in a 5%CO_2 incubator. The cell supernatant was added to the culture plate after 48 h. OD values, which were determined at 450 nm using an enzyme-linked immunosorbent assay reader, were compared with a standard curve to determine fibronectin concentrations.

Declarations

Acknowledgements The study was supported by the Open Fund of Key Laboratory of Biotechnology and Bioresources Utilization (Dalian Minzu University), Ministry of Education NO. KF2021005, China.

Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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Scheme 1

Scheme 1 is available in the Supplemental Files section.

Figures

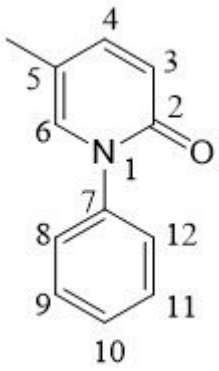

Figure 1

Structure of pirfenidone.

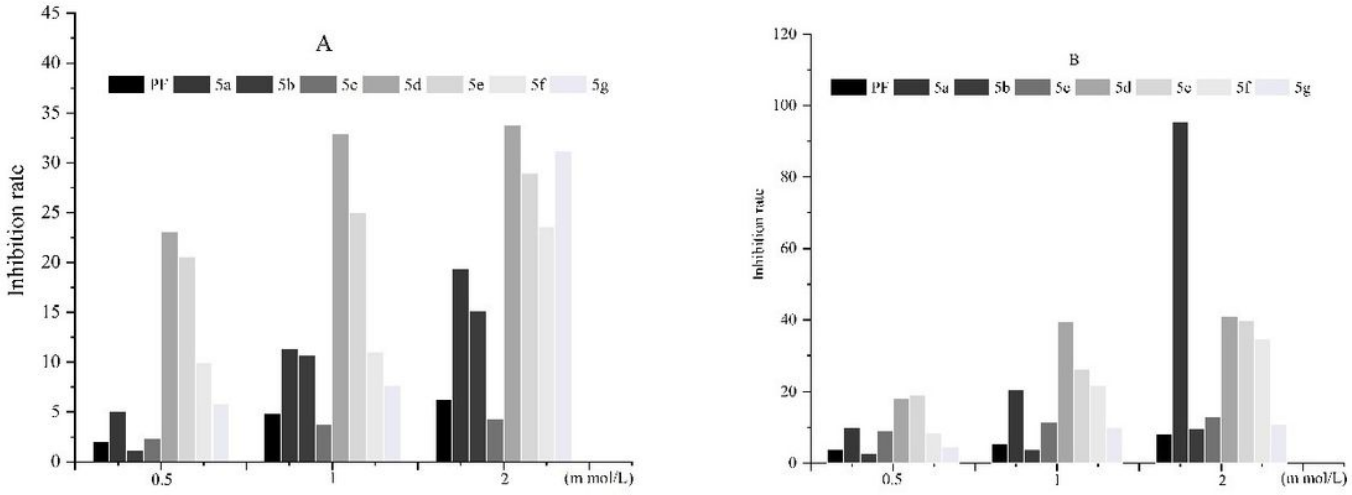

Figure 2

Different concentrations of pirfenidone and its derivatives inhibit the proliferation of 3T3L1 cells

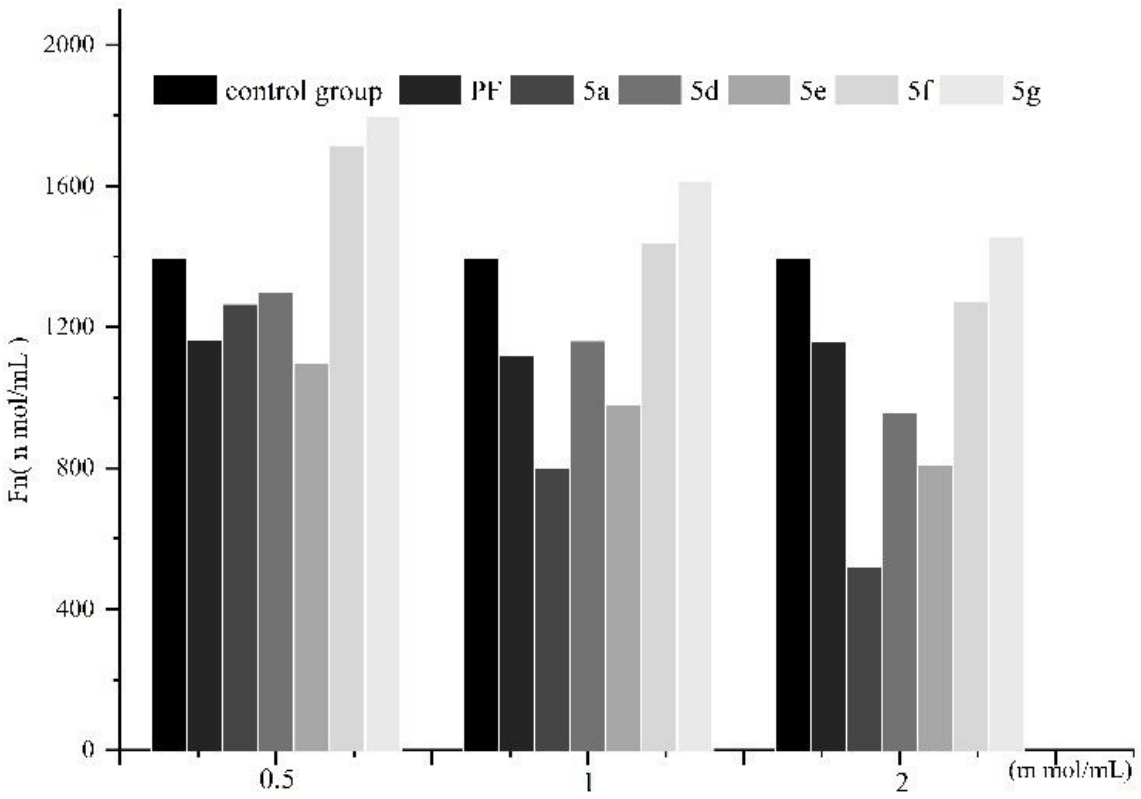

Figure 3

Inhibitory activity of pirfenidone and its derivatives at different concentrations on secretion of Fn in 3T3L1 cells (48 h later)

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supportinginformation.docx
- GraphicalAbstract.jpg
- Scheme1.jpg