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Novel Pyrimidines as Multi-target PTK Inhibitors for the Treatment of Idiopathic Pulmonary Fibrosis(IPF)

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Abstract: A new class of pyrimidine derivatives were identified as potent Protein tyrosine kinase (PTK) inhibitors for the treatment of Idiopathic pulmonary fibrosis (IPF). Most of these small-molecule inhibitors displayed strong enzymatic activity against BTK and JAK3 kinases at concentrations lower than 10 nM. The representative **6a** also exhibited high inhibitory potency of BTK kinase family, JAK kinase family, as well as ErbB4, at a concentration of 10 nM, achieving the inhibition rates higher than 57%. Additionally, the in vivo biological evaluation showed that **6a** could remarkably reduce the severity of the IPF disease. All these investigations suggested that the multi-PTK inhibitor **6a** may serve as a promising agent for the treatment of IPF.

Idiopathic pulmonary fibrosis (IPF), characterized by scarring of the lung tissue, could lead to serious shortness of breath, dry cough, inspiratory bibasilar crackles, and fatigue.[1,2] As a progressive, severely debilitating disease, the median survival time of this fatal lung disease is only between 3 and 5 years from diagnosis. Unfortunately, the etiology and pathology of IPF have not been elucidated to date.[3-6] Generally, IPF is thought to result from epithelial cell injury and the subsequent unconstrained fibrotic process. Pirfenidone (**1**, Esbriet®) and nintedanib (**2**, Ofev®) are the only two drugs that have been approved by the U.S. Food and Drug Administration (FDA) for the treatment of IPF. Pirfenidone works by reducing lung fibrosis through downregulation of the production of growth factors and procollagens I and II, [7,8] while nintedanib, is a well-known multi-protein tyrosine kinase (PTK) inhibitor, which can inhibit the enzymatic activity of VEGFR1, VEGFR2, VEGFR3, FGFR1, FGFR2, FGFR3, PDGFα, and PDGFβ. [9-11] GLPG1690 (**3**),[12] a recently discovered autotaxin inhibitor, showed great efficacy in a bleomycin(BLM)-induced pulmonary fibrosis model in mice and in reducing extracellular matrix deposition in the lung. GLPG1690 is now currently being evaluated in an exploratory phase Ⅲ study in idiopathic pulmonary fibrosis patients (ClinicalTrials.gov Identifier: NCT03733444) (Figure 1). Imatinib (**4**) is another novel multi-target inhibitor of *v*-Abl, *c*-Kit and PDGFR, with IC_{50} of 0.6 μM, 0.1 μM and 0.1 μM in cell-free or cell-based assays, respectively. Study also revealed that imatinib displayed potential efficacy to treat fibrotic lung disease (ClinicalTrials.gov Identifier: NCT00131274). Although these agents could reduce the progression of IPF, their therapeutic effect in IPF is only moderate and do not halt or reverse the deterioration resulting from IPF.[13] Accordingly, the identification of more effective agents or drugs with new action mechanism against IPF is still an urgent task.

Protein tyrosine kinases have been shown to participate in a variety of signaling pathways involved in cellular homeostasis processes that are critical to the pathogenesis of pulmonary fibrosis.[14] It has also been found that PTKs regulate lung myofibroblast proliferation *via* various growth factors, including TGFs, EGF, PDGFRs, FGFRs, and VEGFRs.[15,16] For example, the novel EGFR inhibitor gefitinib (**5**, Figure. 1), which has been approved by the US FDA in 2003 for the treatment of non-small cell lung cancer (NSCLC), also could produce a significant protective effect on lung fibrosis induced by BLM *via* EGF. [15]

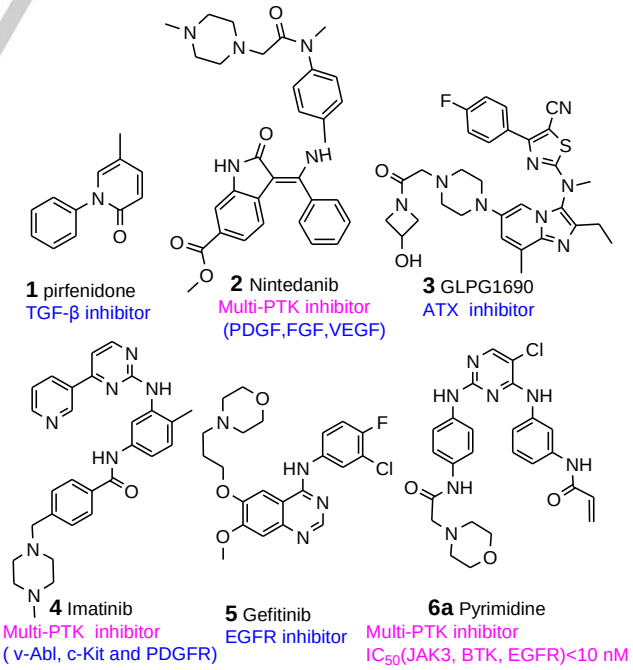
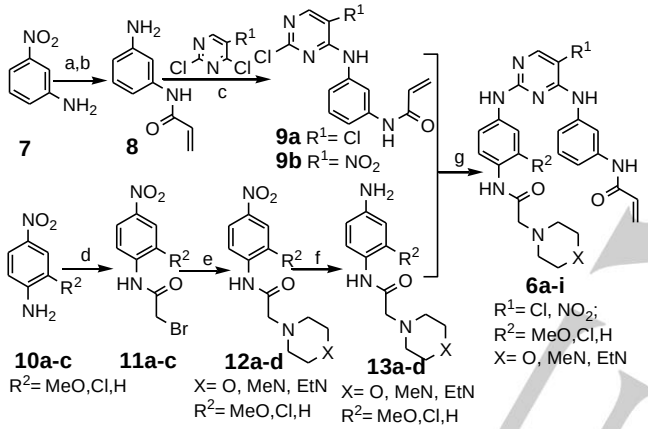


Figure 1. Chemical structures of the novel anti-IPF agents

Janus kinase 3 (JAK3), which belongs to the janus family of kinases, is involved in signal transduction by receptors that employ the common gamma chain (γc) of the type I cytokine

receptor family (e.g. IL-2R, IL-4R, IL-7R, IL-9R, IL-15R, and IL-21R). Signaling through the IL-15 receptor is known to be important in the development.[17,18] Recent studies showed that JAK-STAT signaling play an important role in the pathogenesis of pulmonary fibrosis, and thus inhibition JAK-STAT signaling in fibroblasts may be effective in the treatment of fibrosis.[19-22] Bruton's tyrosine kinase (BTK) is a type of kinase protein expressed in B lymphocytes and T cells.[23] BTK deficiency in humans is associated with reduction in TNF-α production by activated monocytes. Thus, inhibition of BTK activity can provide a useful treatment for allergic disorders, autoimmune, and/or inflammatory diseases such as, rheumatoid arthritis, idiopathic thrombocytopenic purpura, asthma, and other related disorders.[24,25] Due to our great interest in the identification of potent BTK inhibitors for the treatment of B-cell lymphoma,[26-28] we accidentally obtained a class of pyrimidine derivatives, which exhibited strong activity against BTK, JAK3, and EGFR kinases at a drug concentration of 10 nM or less. Considering the important role of these kinase in the development of IPF, we believe that these multi-PTK inhibitors are effective in treatment of the IPF disease. Therefore, herein, we describe in detail these pyrimidine derivatives, along with their synthesis, and the data of their biological activity against IPF *in vivo* and *in vitro*.



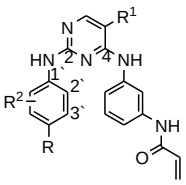
Scheme 1. Synthetic route of title compounds **6a-i**. *Reagents and conditions*: (a) acryloyl chloride, $NaHCO_3$, CH_3CN, 0 °C, 0.5 h, 95%; (b) Fe-NH_4Cl, MeOH-H_2O, 2 h, 70 °C, 81%; (c) $ArNH_2$, DIPEA, 1,4-dioxane, 60 °C, 2 h, 82-91%; (d) bromoacetyl bromide, $NaHCO_3$, CH_3CN, 0 °C, 0.5 h, 97%; (e) morpholine, K_2CO_3, KI, CH_3CN, reflux, 5 h, 81%; (f) Fe-NH_4Cl, MeOH-H_2O, 2 h, 70 °C, 82%; (g) TFA, 2-BuOH, 100°C, 12 h, 16-40%.

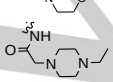
The synthetic route of the title compounds **6a-i** is depicted in Scheme 1.[29,30] Based on our previously reported method, the 2-chloropyrimidine derivatives **9a,b**, which were the key intermediates to prepare the pyrimidine template, were conveniently synthesized by acylation, reduction, and nucleophilic substitution reactions, with 70% overall yields for the three steps. Additionally, the key intermediates, *C*-2 aniline side chains **13-d**, were generally synthesized *via* acylation, substitution and reduction reactions starting from the commercial material 4-nitroanilines **10a-c**. Ultimately, by coupling anilines **13a-d** with the pyrimidines **9a,b** under trifluoroacetic acid(TFA) conditions at reflux temperature, the desired molecules **6a-i** were produced with yields in the range of 16-40%.

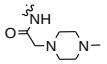
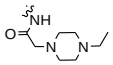
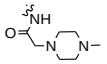
Kinase can react with ATP-binding substrate, and then activates disease-related signaling pathways. While ATP is converted to ADP. According to this mechanism, the ADP-Glo™ Kinase Assay (Promega, US), which is a luminescent ADP detection assay, provides a universal, homogeneous, high-throughput screening method to measure kinase activity by quantifying the amount of ADP produced during a kinase reaction.[31-34] All the title molecules were biologically evaluated for their activity against BTK and JAK3 enzymes using this method. The assay results are presented in Table 1. Clearly, most of these compounds exhibited strong inhibitory activity against both BTK and JAK3 enzymes at concentrations around 10 nM. In terms of their activity against BTK kinase, more than half of the title molecules (**6a**,**c**,**d**,**g**, and **i)** had IC_{50} values lower than 1 nM. The representative molecule, **6a,** displayed the strongest enzymatic activity against BTK kinase, with an IC_{50} value of 0.6 nM. As for their activity against JAK3 enzyme, compounds **6a**, **6c**, and **6d** exhibited very high inhibitory activity, with IC_{50} values of 0.59, 1.08, and 1.22 nM, respectively. Notably, compound **6a**, the strongest BTK inhibitor, also has the most potent inhibitory activity against JAK3 kinase, with an IC_{50} value of 0.59 nM. Moreover, compound **6a** has low toxicity against the normal human bronchial epithelioid (HBE) cells at concentration lower than 2.78 μM. In addition, compound **6a** displayed a moderate ClogP value (4.46), showing its good overall capability to penetrate the cell membrane.[35,36]

All these test results clearly indicated that these structural modifications, which were inspired by our previous structure and activity relationship (SAR) explorations are rational and effective. Moreover, this study further confirmed the benefit of 1-substitutedacetamide linker in the *C*-2 aniline, and revealed that the morpholine substituent (**6a,c,d,h**) in the *C*-2 aniline side chain of the pyrimidine core is more favorable than the piperazine ring (**6b,e,f,g**) at the same position. Apparently, **6c** (0.6, 1.08 nM) increased 2-12 times higher capability than **6e** (12.5, 14.0 nM) and **6f** (14.9, 1.82 nM) for inhibiting both BTK and JAK3 enzymatic activity. In terms of R2 group, the methyl(**6d**) and methoxy substituents(**6c**) exhibited equivalent anti-enzymatic activity to **6a**(H). While the nitro group (**6h**) in R1 produced strong inhibitory effect on BTK kinase(0.6 nM), but 14.5 times lost activity against JAK3(8.53 nM) compared with **6a** (0.59 nM). Although the dibutylamine substituent (**6h**) is effective for inhibiting BTK (0.6 nM) and JAK3 (4.9 nM) enzymatic activity, its ClogP value is very high (6.78), indicating its weak ability to penetrate lung tissue.

Table 1. In vitro *enzymatic activity of the title compounds* ***6a-i.*** [a]



Compd.	R	R^2	R^1	Enzymatic activity (IC_{50}, nM)[b]		Toxicity to HBE cells[c] (IC_{50}, μM)	CLogP[d]
				BTK	JAK3		
6a		H	Cl	0.60±0.08	0.59±0.09	2.78±0.87	4.46
6b		Me(3`)	Cl	1.00±0.11	6.25±1.02	0.32±0.02	3.97
6c		MeO(3`)	Cl	0.60±0.09	1.08±0.19	3.20±0.56	3.85

6d		Me(3`)	Cl	0.50±0.09	1.22±0.31	1.20±0.12	4.31
6e		MeO(3`)	Cl	12.5±1.2	14.0±2.14	1.14±0.09	2.91
6f		MeO(3`)	Cl	14.9±2.1	1.82±0.77	0.45±0.07	3.50
6g		Me(3`)	Cl	11.2±1.4	3.12±0.84	0.55±0.06	3.43
6h		H	NO_2	0.60±0.07	8.53±0.95	7.29±0.21	3.70
6i		H	NO_2	0.60±0.09	4.90±0.99	35.9±3.15	6.78
nintedanib						>10	

[a] The data were means ±SD of three separate experiments. [b] The enzymatic activity against BTK and JAK3 enzymes using the ADP-GloTM kinase assay. [c] The toxicity effects on the proliferation of normal HBE cells were determined by the CCK-8 assay. [d] CLogP means Calculated LogP which is oil-water partition coefficient.

To confirm the selectivity of these novel BTK and JAK3 dual inhibitors, the biological activity of the potent inhibitor **6a** against a panel of 411 kinase was also analyzed (SI-2) using the SelectScreen™ Biochemical Profiling Lab platform (Thermo-Fisher Scientific, Waltham, MA, USA).[37,38] The results of this analysis (Table 2) revealed that, in addition to JAK3 and BTK kinases, compound **6a** strongly binds to other kinases, including members of the BTK family (BLK, BMX and TEC) and JAK family (JAK1, JAK2), as well as ErbB4, FLT3 D835Y and TXK, at a concentration of 10 nM, reaching inhibition rates higher than 57%. Remarkably, compound **6a** also exhibited strong inhibitory potency of FLT3 (54%), FLT3 D385Y (82%), RET (59%), RET V804L (55%) and RET Y791F (50%), suggesting that this compound may be useful for the treatment of several types of cancer. From this investigation, it can be confirmed that most of these pyrimidines are pan-active kinase inhibitors, which may act with the kinase by forming the strong covalent bond between the acryloyl moiety and cysteine reside in these proteins.[39,40] While for nintedanib, its targeted kinases include all three VEGFR subtypes (IC_{50}, 13–34 nM), PDGFRa and PDGFRh (IC_{50}, 59 and 65 nM), and FGFR types 1, 2, and 3 (IC_{50}, 69, 37, and 108 nM, respectively).

Table 2. Selectivity profile of compound **6a** against a panel of 411 kinases[a]

Kinase	%[b]	Kinase	%	Kinase	%
BRAF V599E	21	AURKA (Aurora A)	52	IRAK4	15
BTK	97	AURKB (Aurora B)	50	ITK	28
RET G691S	30	AURKC (Aurora C)	33	JAK1	57
RET M918T	30	BLK	66	JAK2	57
RET V804M	51	BMX	92	JAK2	45
STK16 (PKL12)	62	EGFR	23	JAK2 V617F	33
STK33	40	EGFRL858R	17	JAK3	95
TEC	78	EGFRL861Q	30	NEK7	17
WEE1	35	EGFRT790M	47	NEK9	9
ABL1	15	EGFRT790M L858R	55	NTRK1 (TRKA)	39
ABL1 E255K	23	EGFR d746-750	89	NTRK2 (TRKB)	39
ABL1 G250E	17	ERBB4	79	NTRK3 (TRKC)	31
ABL1 T315I	22	FLT1 (VEGFR1)	2	RET	59
ABL1 Y253F	19	FLT3	54	RET V804L	55
ABL2(Arg)	11	FLT3 D835Y	82	RET Y791F	50
NUAK1 (ARK5)	50	FLT4 (VEGFR3)	38	ROS1	27
TXK	86	FLT3 ITD	58	SYK	21

[a]This test was conducted by the SelectScreen™ Biochemical Profiling Lab. Two separate experiments were performed, and the averaged data was dicipated. [b]Inhibition rate at a concentration of 10 nM, Only the inhibition rate more than 10% was showed in this table.

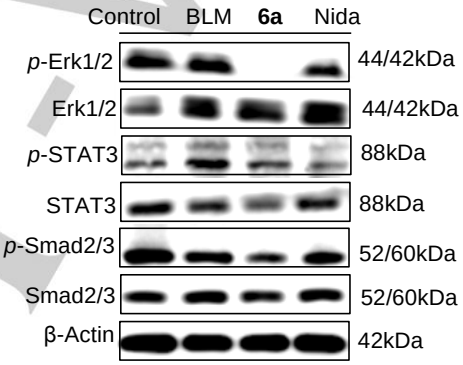


Figure 2. Compound **6a** inhibits the activation of STAT and related signaling in C57BL/6 mouse lung tissue. After administration of drug for 15 days, the lung tissue of C57BL/6 mice was grinded, homogenized and lysed to extract proteins. The protein was then performed the Western blot test according to the general test method. β-Actin is used as control, the values are relative to β-Actin. Data are presented as the mean ±SD (n = 8). $p < 0.05$.

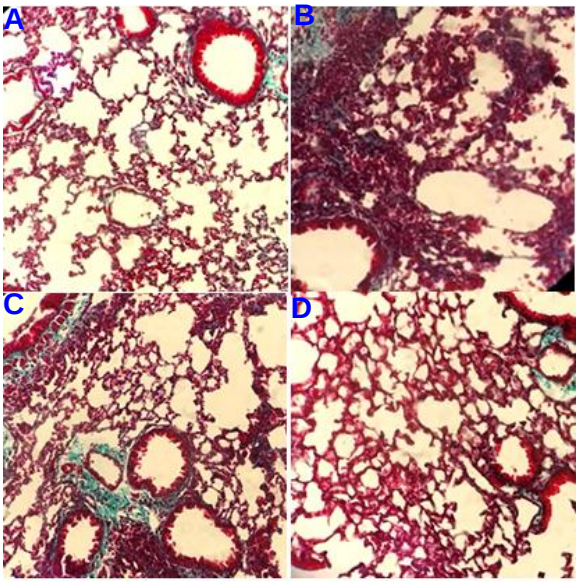


Figure 3. *In vivo* effect of 6a on BLM-induced BLM-induced pulmonary fibrosis . Mice were intratracheally instilled with BLM (1.5 mg/kg) or with vehicle only. The indicated dose (30 mg/kg) of **6a** and nintedanib were given once daily for 14 days after BLM challenge (n = 8 per group). Section of lung tissue was prepared on day 14 after BLM treatment and subjected to

Trichrome Masson staining (200×). (A: The control group, B: The BLM model group, C: The BLM model plus nintedanib group, D: The BLM model plus **6a** group).

In addition, the most active JAK3 and BTK dual inhibitor **6a** was also explored its inhibitory effects on active signaling by using western blot analysis. The novel agent nintedanib was also tested for comparsion.[41-43] As showed in Figure 2, after treating the C57BL/6 mice with both **6a** and nintedanib at a dose of 30 mg/kg daily (qd) over a 14-day period, the phosphorylation of Erk1/2, STAT3, Smad2/3 were clearly inhibited by **6a**. The particular result that the phosphorylation of Erk1/2 was almost completely repressed by compound **6a** indicates its improved inhibitory effects on Erk1/2 protein compared with that of nintedanib. However, the BTK protein was not detected in the lung tissue of this mouse model (which was not shown in this Figure 2), showing that BTK was not a key therapeutic target for IPF disease. Therefore, the multi-kinase inhibitor **6a** may play its therapeutic effects through other signaling pathway despite its strong activity against BTK kinase.

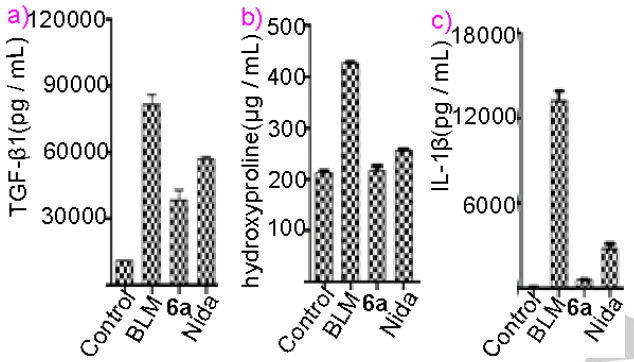


Figure 4. Compound **6a** treatment reduced BLM-induced lung inflammati去啊 Ytfgand fibrosis *in vivo* evaluation, the mediator concentrations in whole-lung homogenates were determined by ELISA: a)TGF-β1, b)hydroxyproline, and c)IL-1β in lungs homogenate were determined as described in the methods. Data are presented as the mean ±SD (n = 3).

The effects of compound **6a** on Leukotriene B4(LTB4) production and neutrophil infiltration *in vivo* were further examined using a murine experimental model of BLM-induced lung neutrophilic inflammation.[41] Nintedanib was used as a positive control. The deposition of collagen fibers was assessed by Masson's trichrome staining. The results showed in Figure 3 clearly indicated that compound **6a** remarkably reduced the severity of the inflammation compared with the control group. In addition, as showed in Figure 4, three typical biomarkers of the degree of pulmonary fibrosis (TGF-β1, IL-1β, and hydroxyproline) were also detected in the lung tissues of the experimental mice. Evidently, all the three biomarkers in the **6a**-treated groups were apparently reduced compared with those in the nintedanib-treated groups, showing its superior biological activity against IPF.

To investigate the interaction mechanism of the title molecules with BTK and JAK3 enzymes, the representative compound **6a** was docked into the ATP binding pocket of each, the BTK (PDB ID: 3GEN) [44] and JAK3 (PDB ID: 4Z16) [45] proteins. The AutoDock 4.2 software and its default parameters were used to conduct the structural analysis. [46,47] The results shown in Figure 5 indicated that the structures displayed a very special and strong covalent- and hydrogen-bond network in the ATP binding pocket. Importantly, the essential acrylamide functional group of compound **6a** forms a strong covalent bond with the amino acid Cys481 in the BTK enzyme, and the amino acid Cys909 in the JAK3 protein. In addition, the hydrogen-bond interactions formed by the *N*-2 atom of the pyrimidine core with the amino acid Leu488 in BTK, and with the amino acid Leu905 in JAK3 were also present. For compound **6a**, the carbonyl group in the *C*-2 aniline side chain also forms additional hydrogen bonds with the amino acids Leu528 in BTK and Gly908 in JAK3. Additionally, the morpholine substituent reaches into the hydrophilic section surrounded by the amino acids Val537 and Sep538 in BTK, and Val912 and Val838 in JAK3. The chlorine atom at the *C*-5 position of the pyrimidine core serves as a base to interact with the amino acid side chain of Lys430 in BTK and Leu956 in JAK3. All these important interaction forces produced the strong anti-BTK and -JAK enzymatic activity.

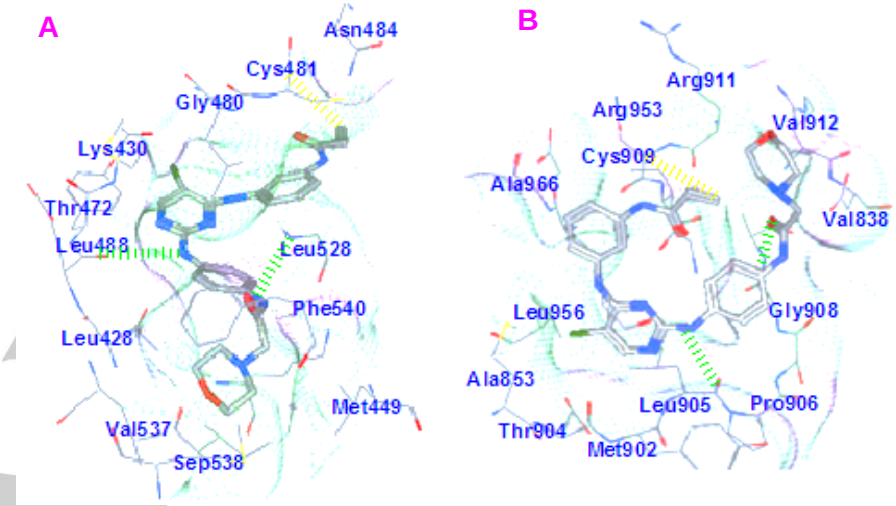


Figure 5. Putative co-structure of compound **6a** bound to the ATP binding site of BTK (PDB ID: 3GEN) [44] and JAK3 (PDB ID: 4Z16) [45].

To effectively treat the most lethal diffuse fibrosing lung disease IPF, a new series of pyrimidine derivatives were synthesized and biologically evaluated *in vivo* and *in vitro* activity in this study. Most of these small molecules displayed strong dual inhibitory effect on the BTK and JAK3 enzymatic activities at a concentration of 10 nM or less. Additionally, the most active inhibitor **6a** also exhibited strong inhibitory activity against other kinases including members of the BTK family (BLK, BMX and TEC), JAK family (JAK1, JAK2), as well as ErbB4, FLT3 D835Y and TXK at a concentration of 10 nM, with inhibition rates higher than 57%. Furthermore, compound **6a** remarkably reduced the three typical biomarkers of the degree of pulmonary fibrosis (TGF-β1, IL-1β, and hydroxyproline) in the lung tissues of the experimental mice, showing its slightly higher efficacy than that

of nintedanib in the *in vivo* biological evaluation. Overall, this contribution provides a novel multi-PTK inhibitor, **6a,** as a promising agent for the treatment of the IPF disease.

Experimental Section

Experimental details and data are included in the Supporting Information.

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Keywords: IPF • PTK • multi-target • inhibitors • pyrimidine

ABBREVIATIONS

IPF, Idiopathic pulmonary fibrosis; NSCLC, Non-small cell lung cancer; PTK, Protein tyrosine kinase, BLM, bleomycin; JAK3, Janus kinase 3; BTK, Bruton's tyrosine kinase; TFA , trifluoroacetic acid; DAPIA, *N,N*-Diisopropylethylamine; BLK, B-Lymphoid tyrosine kinase; BMX, Bone marrow tyrosine kinase on chromosome X; TEC, tyrosine kinase expressed in hepatocellular carcinoma; FLT3,Fms-like tyrosine kinase 3; RET, rearranged during transfection; CLogP, Calculated LogP(oil-water partition coefficient); Erk1/2, extracellular regulated kinases 1/2; STAT3, Signal transducers and activators of transcription 3; Smad2/3, drosophila mothers against decapentaplegic 2/3; LTB4,Leukotriene B4; VEGFR, Vascular Endothelial Growth Factor Receptor; FGFR, fibroblast growth factor receptor; PDGF, platelet derived growth factor; EGFR, epidermal growth factor receptor; ErbB4, erb-b2 receptor tyrosine kinase 4; TGFs, Transforming growth factor; EGF, Epidermal Growth factor; γc, gamma chain; IL-2R, Interleukin-2; HBE, human bronchial epithelioid.

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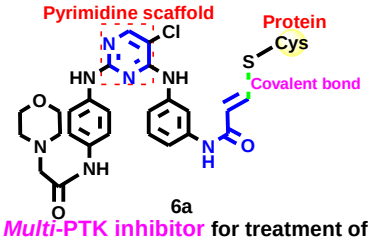

Multi-PTK inhibitor for treatment of IPF

$IC_{50(JAK3)}$=0.59 nM, $IC_{50(BTK)}$=0.6 nM

- Novel pyrimidines as potent PTK inhibitors for treatment of IPF.
- They displayed strong enzymatic activity against BTK and JAK3 kinases(IC_{50}<10 nM).
- **6a** remarkably reduced the severity of the IPF disease *in vivo* biological evaluation.