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Synthesis and biological evaluation of 4-oxoquinoline-3-carboxamides derivatives as potent anti-fibrosis agents

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ABSTRACT

Thirty-one 4-oxoquinoline-3-carboxamides derivatives were synthesized and evaluated for their anti-fibrotic activities by the inhibition of TGF-β1-induced total collagen accumulation and anti-inflammatory activities by the inhibition of LPS-stimulated TNF-α production. Among them, three compounds (**10a**, **10l** and **11g**) exhibited potent inhibitory effects on both TGF-β1-induced total collagen accumulation and LPS-stimulated TNF-α production. Furthermore, oral administrations of **10l** at a dose of 20 mg/kg/day for 4 weeks effectively alleviated lung inflammation and injury, and decreased lung collagen accumulation in bleomycin-induced pulmonary fibrosis model. Histopathological evaluation of lung tissue confirmed **10l** as a potential, orally active agent for the treatment of pulmonary fibrosis.

Pulmonary fibrosis (PF), characterized by the disruption of normal lung tissue architecture and loss of pulmonary function, is one of the major clinical problems of cystic fibrosis and chronic obstructive pulmonary disease.[1–4] Its incidence most recently has been estimated to be between 14 and 42.7 per 100,000 and has been increasing,[5–7] the expected survival time of patients suffered from PF is estimated to be 2.5–3.5 years.[8] However, the pathogenesis of PF is still unknown, the excessive deposition of extracellular matrix (ECM) in lung tissues caused by inflammation has been recognized as a crucial reason leading to the destruction of the lung architecture.[9,10]

A number of studies have documented that cytokines such as TGF-β1, TNF-α play key roles during the pathogenesis of the PF.[11] TGF-β1 is a fibrogenic cytokine involved in pathological fibrosis of PF by regulating extracellular matrix (ECM) deposition in the response to lung tissues injury.[12–14] TNF-α, an important pro-inflammatory cytokine released by macrophages and lymphocytes, involved in regulating the inflammation respond to lung tissue injury.[15]

Ivacaftor (Fig. 1), a 4-oxoquinoline-3-carboxamide compound, has been approved as a small-molecule drug to treat cystic fibrosis,[16] which is studied for chronic obstructive pulmonary disease in clinic.[17] Treatment with *Ivacaftor* had significant and sustained improvement in their lung function in cystic fibrosis.[18,19] In our preliminary studies, *Ivacaftor* was also observed to reveal in vitro anti-fibrotic activities by the inhibition of TGF-β1-induced total collagen accumulation in rat fibroblast cells (NRK-49F), which has been recognized an effect of good and convenient origin for anti-fibrotic agent in vitro screening model.[20] Therefore, these pharmacological properties of *Ivacaftor* provided us the impetus to develop novel and potent anti-fibrotic agents containing 4-oxoquinoline-3-carboxamide moiety by using *Ivacaftor* as a lead.

Ivacaftor derivatives (**9a**–**9f**) were synthesized according to previous reported procedures (Scheme 1).[21–23] The 2,4-di-*tert*-butylphenol **1** was treated with methyl chloroformate to give **2**. Nitration of **2** with cooled mixture of HNO_3 and H_2SO_4 (v:v 1:1) obtained **3**. Treatment of **3** with 10% Pd/C, $HCOONH_4$ yielded **4**. The pivotal 4-quinolones intermediates **5a**–**5f** were obtained by the condensation of appropriate anilines with diethyl ethoxymethylenemalonate, followed by the cyclization of the intermediate to give **6a**–**6f**. Compound **6a**–**6f** were hydrolyzed to the corresponding acid **7a**–**7f**, the intermediates **7a**–**7f** were reacted with **4** to afford **8a**–**8f**, further deprotected to give **9a**–**9f**. *Ivacaftor* analogues **10a**–**10o** and **11a**–**11j** were outlined in Scheme 2. The intermediate **7e** was treated with anilines to afford the desired products

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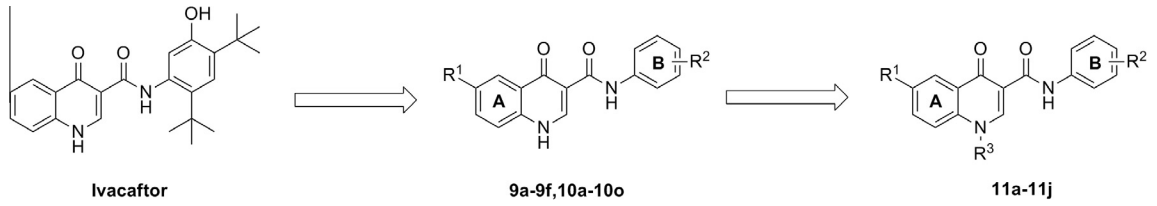
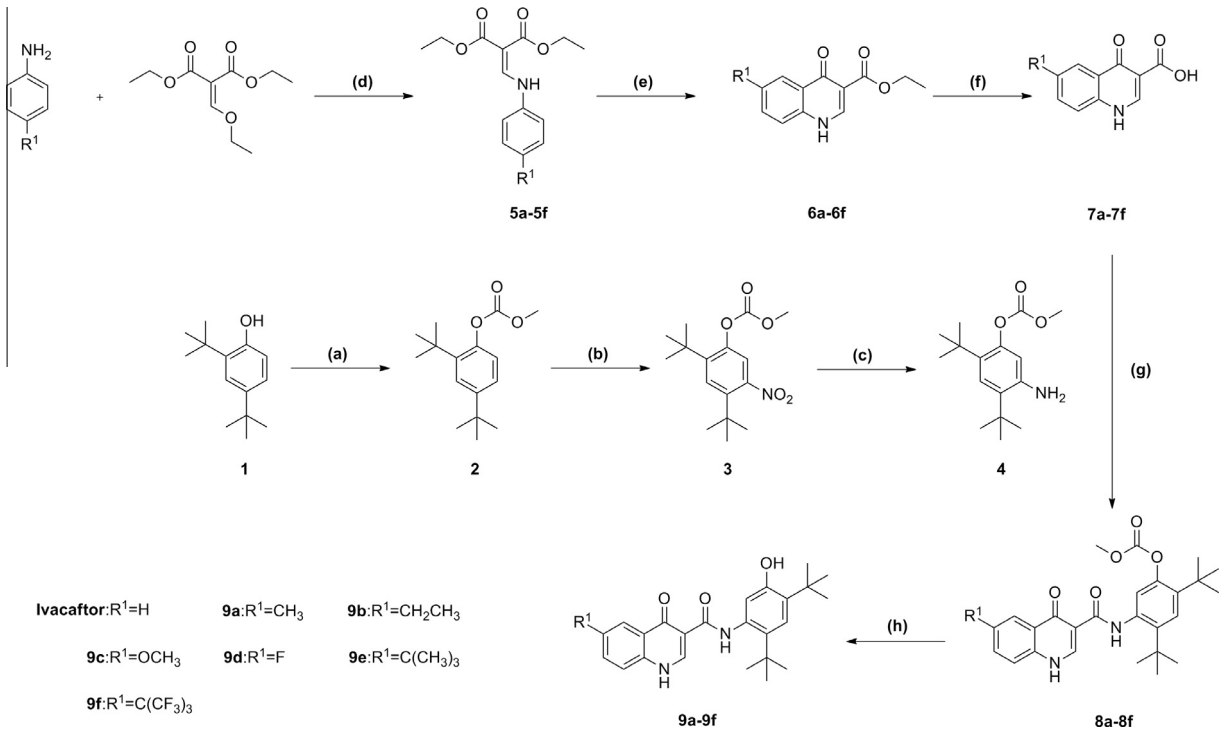
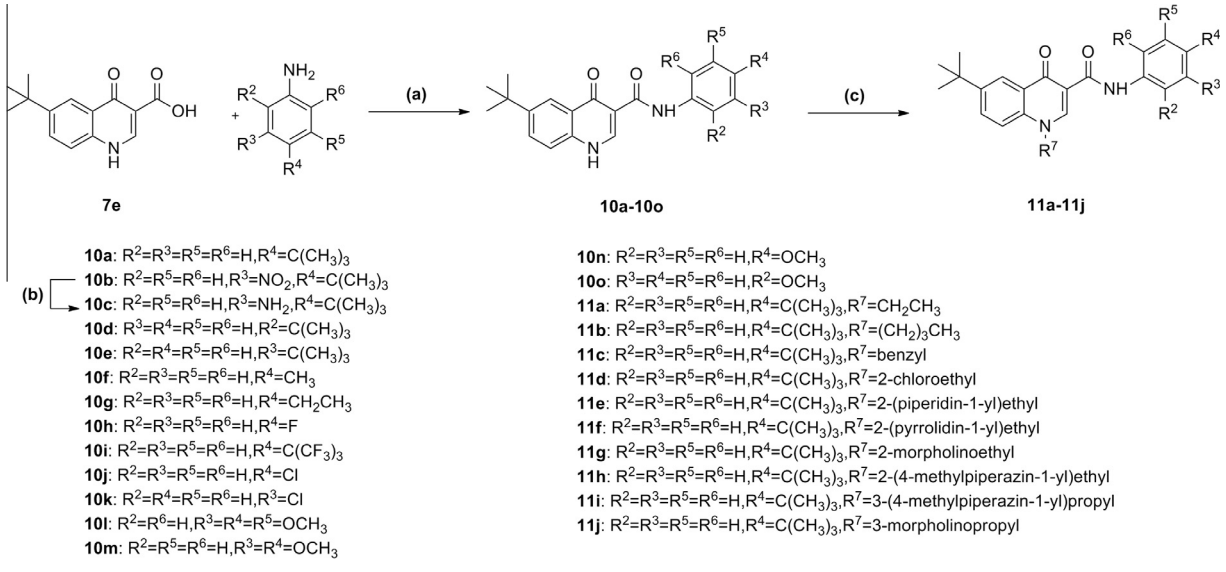


Figure 1. *Ivacaftor* and 4-oxoquinoline-3-carboxamides derivatives.



Scheme 1. General synthesis of *Ivacaftor* derivatives. Reagents and conditions: (a) methyl chloroformate, DMAP, Et_3N, DCM, rt, overnight; (b) HNO_3/H_2SO_4 (1:1), 0 °C, rt, 2 h. (c) 10% Pd/C, $HCOONH_4$, EtOH, reflux, 2 h; (d) EtOH, reflux, 4 h; (e) diphenyl ether, reflux, 1 h; (f) (i) 10% KOH, 100 °C, 3 h; (ii) 1 N HCl, pH = 4–5; (g) HATU, Et_3N, DMF, rt, overnight; (h) KOH, CH_3OH, rt, 2 h.



Scheme 2. General synthesis of *Ivacaftor* analogues. Reagents and conditions: (a) HATU, Et_3N, DMF, rt, overnight; (b) Fe powder, NH_4Cl, EtOH/H_2O (3:1), reflux, 4 h; (c) RX, Cs_2CO_3, CH_3CN, reflux, overnight.

Table 1
Inhibitory effects of compounds against TGF-β1-induced total collagen accumulation in NRK-49F cells at a concentration of 10 μM

Compds	Inhibition (%) ± SD	Compds	Inhibition (%) ± SD
Ivacaftor	64.7 ± 2.1[b]	**10j**	27.2 ± 8.3
9a	49.9 ± 8.6	**10k**	30.5 ± 5.9
9b	55.9 ± 5.5[b]	**10l**	77.1 ± 1.7[b]
9c	53.1 ± 4.7[b]	**10m**	32.6 ± 4.8[a]
9d	51.6 ± 7.3	**10n**	23.5 ± 7.0
9e	73.8 ± 3.4[a]	**10o**	47.9 ± 11.8
9f	48.7 ± 9.3	**11a**	34.3 ± 8.5
10a	81.0 ± 1.2[b]	**11b**	16.9 ± 7.7
10b	75.7 ± 1.6[b]	**11c**	9.1 ± 12.3
10c	67.9 ± 5.6[b]	**11d**	35.8 ± 6.5
10d	54.3 ± 5.5	**11e**	30.1 ± 13.5
10e	44.8 ± 4.0[a]	**11f**	44.8 ± 3.8[a]
10f	28.9 ± 17.6	**11g**	71.1 ± 6.3
10g	19.4 ± 7.2	**11h**	46.6 ± 5.9
10h	23.5 ± 5.2	**11i**	52.1 ± 5.6[a]
10i	35.3 ± 6.2	**11j**	30.2 ± 4.0

The results are the means ± SD of at least three independent experiments. [a]P <0.05, [b]P <0.01 versus the TGF-β1-treated group.

Table 2
Inhibitory effects of compounds against the production of TNF-α in LPS-induced RAW 264.7 cells at a concentration of 10 μM

Compds	TNF-α inhibition (%) ± SD	Compds	TNF-α inhibition (%) ± SD
Ivacaftor	63.2 ± 1.5[b]	**10c**	71.3 ± 7.0[a]
9e	45.7 ± 0.6[b]	**10l**	70.8 ± 3.6[b]
10a	61.6 ± 9.0	**11g**	69.5 ± 6.7[a]
10b	47.7 ± 7.7		

The results are the means ± SD of at least three independent experiments. [a]P <0.05, [b]P <0.01 versus the LPS-treated group.

Table 3
IC_{50} values against TGF-β1-induced total collagen accumulation in NRK-49F cells

Compds	IC_{50} (μM)	Compds	IC_{50} (μM)
Ivacaftor	4.0	**10l**	2.5
10a	2.0	**11g**	2.3
10c	4.1		

10a–**10o**. Treatment of **10a** with various alkyl or benzyl halides yielded the desired products **11a**–**11j**.[24]

All synthesized *Ivacaftor* derivatives were evaluated for the anti-fibrotic activities against TGF-β1-induced total collagen accumulation in NRK-49F cells. *Ivacaftor* was used as a positive control. Their initial screening data were depicted in Table 1. As for **9a**–**9f**, the replacement with different electron-donating group or electron-withdrawing group on A-ring exhibited comparable inhibitory potency to *Ivacaftor* (64.7%). Compound **9e** exhibited the most efficacious with the inhibition rate of 73.8%. The introduction of *tert*-butyl group (**10a**–**10c**) at position C-4′ on B-ring led to increased inhibitory activities. Particularly, compound **10a** exhibited the most potent inhibitory activities with the inhibition rate of 81.0%. However, the replacement of C-4′-*tert*-butyl with electron-withdrawing group (fluorine, chlorine, trifluoromethyl) or other electron-donating group (methyl, ethyl, methoxyl) significantly reduced the activity. It is surprising that the total of methoxyl group affected activities, and compound **10l** with three methoxyl groups exhibited a 3.3-fold increase in inhibitory effects (77.1%) compared to **10n** with one methoxyl group. Unfortunately, N1-substitution **10a** analogues (**11a**–**11j**) were observed much lower or moderate activities, with the exception being the N1-morpholinoethyl group (**11g**) which showed approximate inhibitory ability (71.1%) compared with *Ivacaftor*.

Compounds **9e**, **10a**–**10c**, **10l** and **11g** were chosen to investigate inhibitory effects of TNF-α production in LPS-induced RAW 264.7 cells.[25,26] The results were summarized in Table 2. Four compounds **10a**, **10c**, **10l** and **11g** were chosen to further investigate the inhibitory activity against TGF-β1-induced total collagen accumulation in NRK-49F cells. As shown in Table 3, they possessed potent inhibitory activity with IC_{50} of 2.0 μM, 4.1 μM, 2.5 μM and 2.3 μM, respectively.

The visible mode of extracellular collagen deposition is established upon Picro-Sirius red (PSR) staining to text inhibitory activity of **10a**, **10c**, **10l** and **11g** against TGF-β1-induced total collagen accumulation. As depicted in Figure 2, treatment with **10a**, **10c**, **10l** and **11g** at a concentration of 3 μM, the total collagen accumulation was alleviated remarkably (Fig. 2A4–E4). It is interesting to note that the total collagen accumulation was hardly visualized in the presence of **10l** and **11g** at a concentration of 5 μM (Fig. 2D6 and E6).

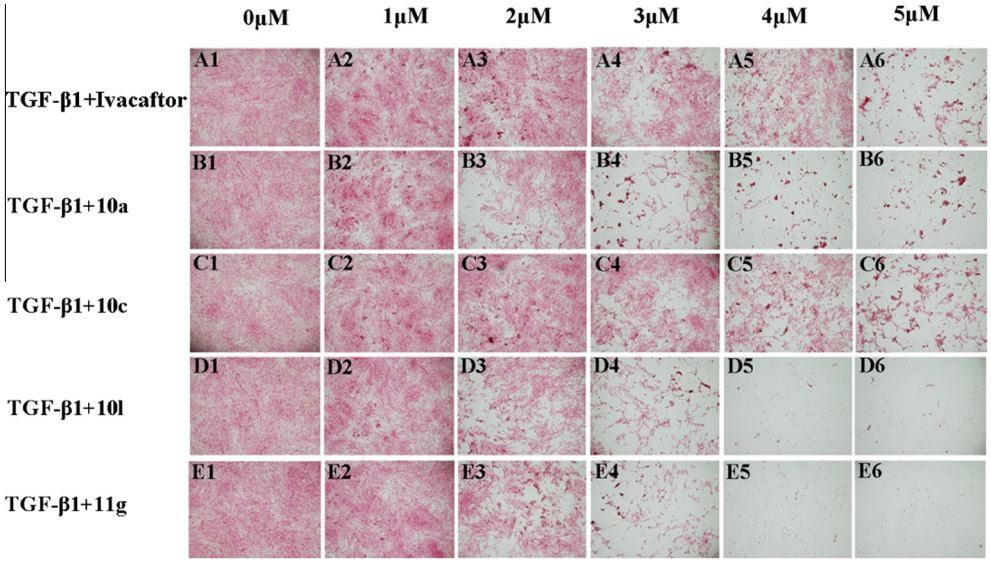


Figure 2. Picro-Sirius red (PSR) staining for the change of TGF-β1-induced total collagen accumulation in NRK-49F cells.

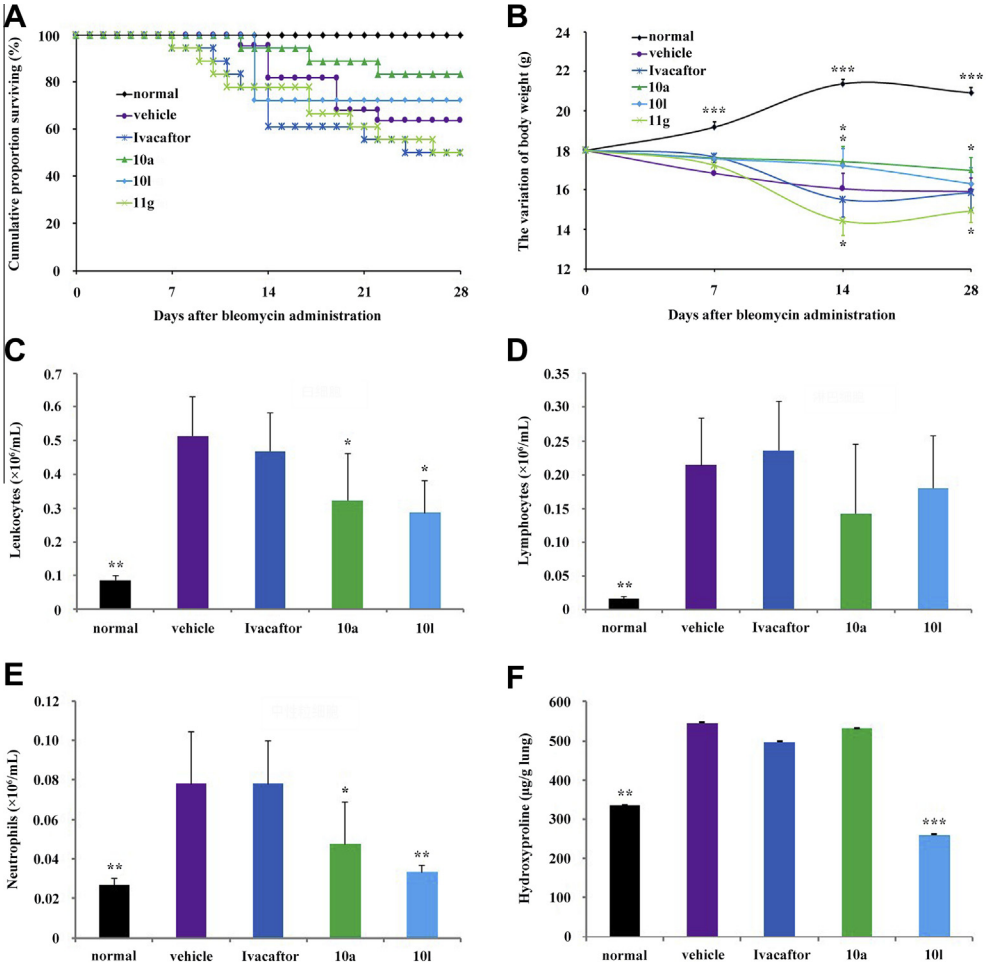


Figure 3. Treatment of bleomycin-induced lung fibrosis, effects on accumulative survival rate (A) and variation of body weight (B), effects on differential cells count in BALF (C–E) and hydroxyproline analysis in right lung tissue (F). Mice were treated with *Ivacaftor*, **10a**, **10l**, **11g** at a dose of 20 mg/kg/day. The results were expressed as the means ± SEM, $^{*}P$ <0.05, $^{**}P$ <0.01, $^{***}P$ <0.001 versus vehicle.

Pulmonary fibrosis is a chronic, progressive, and irreversible lung disease.[27,28] The severity of pulmonary fibrosis was affected by individual differences and the fibrotic progression. To evaluate the in vivo anti-fibrotic potency of **10a**, **10l** and **11g**, bleomycin-induced lung fibrosis model was employed.[29] Compounds **10a**, **10l**, **11g** and *Ivacaftor* were administered (ip) at a dose of 20 mg/kg/day prior to the bleomycin injection. Results exhibited that the cumulative proportion surviving of **10a** (83.3%), **10l** (72.2%) were higher than others on day 28 (Fig. 3A). The body weight loss was reduced more slowly during the treatment with **10a** and **10l** than others (Fig. 3B). Inflammatory cells such as leukocytes, lymphocytes and neutrophils were documented to play a key role during lung fibrotic reactions.[30] The amount of inflammatory cells in bronchoalveolar lavage fluid (BALF) are an important parameter of acute inflammatory caused by bleomycin for evaluating compounds with potential anti-fibrotic activities. As depicted in Figure 3C–E, Compound **10l** significantly reduced the inflammatory cells (54.8% for leukocytes, 15.8% for lymphocytes, 57.7% for neutrophils). Compound **10a** exhibited less effect (40.3% for leukocytes, 33.6% for lymphocytes, 39.7% for neutrophils).

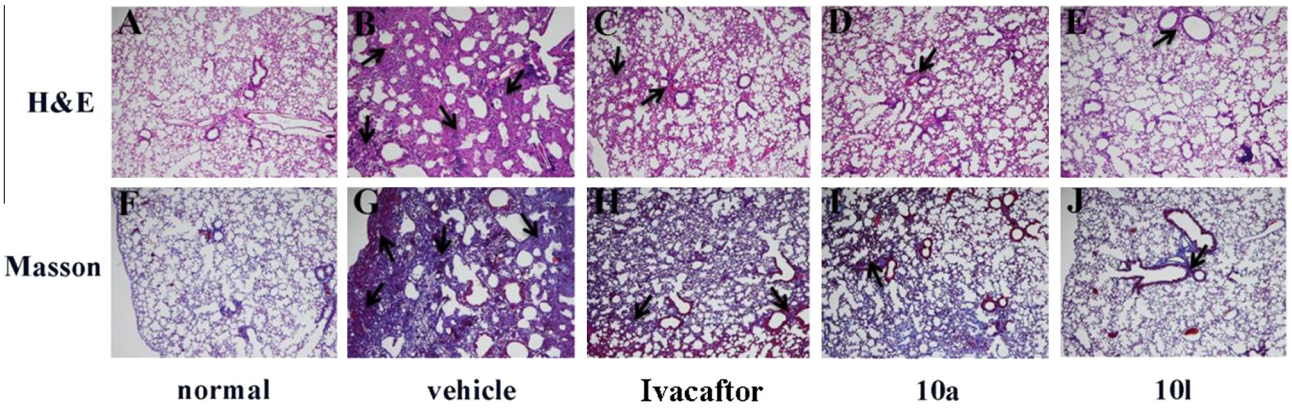


Figure 4. H&E staining and Masson staining for the histological changes of left lung tissues.

Anti-fibrotic effects of compounds can also be evaluated by measuring the content of hydroxyproline, an amino acid that is specifically found in collagen.[31] As displayed in Figure 3F, the content of hydroxyproline was significantly reduced by the value of 52.4% in the presence of **10l** at a dose of 20 mg/kg/day. However, **10a** showed slight effect.

From the results of H&E staining, massive inflammatory cells infiltrated into the alveoli and interstitium in bleomycin-induced group, the walls of the air sacs and the small blood vessels were destroyed because of inflammation (Fig. 4B) while administrations of **10l** at a dose of 20 mg/kg/day was observed with few signs of inflammatory infiltrate and a strong amelioration of bleomycin-induced injury (Fig. 4E). The effects of *Ivacaftor* and **10a** were not obvious at the same doses (Fig. 4C and D). As shown by Masson staining, excessive collagen was visualized to accumulate in lung tissue and the normal air sacs architecture was destroyed in bleomycin-induced group, treatment with **10l** resulted in significant suppression of bleomycin-induced pulmonary fibrosis, characterized by a reduction of lung collagen deposition (Fig. 4J). *Ivacaftor* and **10a** had slight effects (Fig. 4H and I).

In conclusion, thirty-one 4-oxoquinoline-3-carboxamides derivatives were synthesized to evaluate for their anti-fibrotic activities. Three derivatives (**10a**, **10l**, **11g**) exhibited potent inhibitory activity on both TGF-β1-induced total collagen accumulation and TNF-α production. Treatment with **10l** at a dose of 20 mg/kg/day clearly reduced lung inflammation and fibrosis. These observations suggest that **10l** might be an effective agent for the treatment of pulmonary fibrosis.

Acknowledgments

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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.bmcl.2014.10.071.

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