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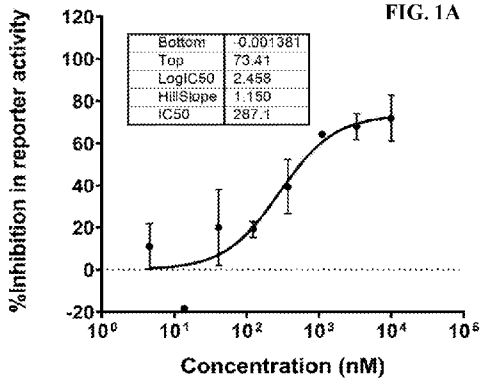
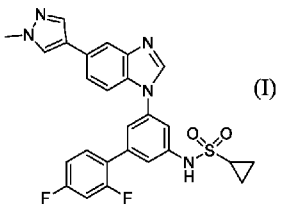
(54) **Title:** METHOD OF TREATING FIBROTIC DISEASES USING PROTEIN KINASE INHIBITOR

FIG. 1A





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(57) **Abstract:** The present disclosure relates to a method of treating or delaying the progression of fibrosis in a subject comprising administering the compound of formula (I) or a pharmaceutically acceptable salt thereof. The present disclosure also relates to a method for administering the compound of formula (I) or a pharmaceutically acceptable salt thereof to a lung.

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METHOD OF TREATING FIBROTIC DISEASES USING PROTEIN KINASE INHIBITOR

This application claims the benefit of Indian provisional application number 202341037798, filed on June 01, 2023, the specifications of which are hereby incorporated by reference in their entirety.

FIELD OF THE INVENTION

The present invention relates to a method of treating or delaying the progression of fibrosis in a subject comprising administering the compound of formula (I) or a pharmaceutically acceptable salt thereof to the subject.

BACKGROUND OF THE INVENTION

Fibrosis is a pathological feature of most chronic inflammatory diseases. Fibrosis, or scarring, is defined by the accumulation of excess extracellular matrix components. If highly progressive, the fibrotic process eventually leads to organ malfunction and death. Multiple organs can develop fibrosis, including the liver, kidney, heart, and lung. The main mediators of fibrosis are myofibroblasts, which upon activation, produce excessive amounts of collagen. Persistent abnormal activation of myofibroblasts can be activated by various mechanisms, like paracrine signals, autocrine factors, or pathogen-associated molecular patterns. Over the years, several important regulators of fibrosis have been identified, like vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), discoidin domain receptor (DDR), cytokines, chemokines, caspases, and the like and therefore can serve as targets for potential anti-fibrotic drugs.

Fibrosis is considered a double-edged sword in cancers. In many types of cancer, the tumor microenvironment (TME) undergoes significant remodelling, which can include the deposition of fibrotic tissue by cancer-associated fibroblasts (CAFs) and mesenchymal stem cells (MSCs). This tumor-associated fibrosis can promote tumor growth, invasion, and metastasis by providing structural support to the tumor, creating a barrier to immune cell infiltration, and promoting angiogenesis (Chandler *et al.*, Transl. Res. 2019). Cancer treatments, such as radiation therapy and certain chemotherapy drugs, can cause fibrosis in the tissues surrounding treated areas. Thus, fibrosis can lead to long-term complications and impair the function of affected organs (Boulter *et al.,* BJC 2021). Chronic inflammation and fibrosis

in certain organs, such as the liver (cirrhosis) or lungs (pulmonary fibrosis), can increase the risk of developing cancer. The tissue damage and repair processes associated with fibrosis can create a microenvironment that promotes the initiation and progression of cancer (Wu *et* al., Cancers 2022).

The treatment of fibrosis encompasses both anti-fibrotic and anti-inflammatory medications. Anti-inflammatory medication includes corticosteroids like prednisone and various immunosuppressants. Anti-fibrotic medication includes nintedanib and pirfenidone.

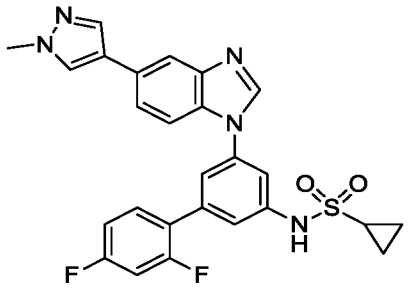
Nintedanib acts by blocking multiple signaling pathways in the body. It blocks receptor tyrosine kinases (RTKs) and non-receptor tyrosine kinases (nRTKs). The RTKs inhibited by the nintedanib include those involved in the growth and development of cells (PDGFR, FGFR, VEGFR). Non-receptor tyrosine kinases (nRTKs) such as Lck, Lyn, and Src kinases were also inhibited by nintedanib. Nintedanib attaches to specific parts of these molecules, preventing them from sending signals (USFDA label).

The mechanism of pirfenidone is not known, however, it is presumed to inhibit TGF-β, collagen synthesis, and fibroblast proliferation. Its anti-fibrotic effects are linked to the inhibition of both the production and activity of TGF-β1, a key regulator of extracellular matrix (ECM) protein deposition. Pirfenidone also exerts its activity by suppressing collagen type-1 mRNA expression. The TGF-β plays a crucial role in fibrosis, including alpha-smooth muscle actin (α-SMA), pro-collagen (Col)-I mRNA, and protein levels (Antar SA *et. al*, Investigating the possible mechanisms of pirfenidone to be targeted as a promising anti-inflammatory, anti-fibrotic, anti-oxidant, anti-apoptotic, anti-tumor, and/or anti-SARS-CoV-2. Life Sci. 2022 Nov 15;309:121048. doi: 10.1016/j.lfs.2022.121048. Epub 2022 Oct 7. PMID: 36209833).

The known anti-fibrotic medications, which are a multi-tyrosine kinase inhibitor and TGF-β inhibitor were only able to decelerate the progression of fibrosis, they are not curative and cannot completely halt the disease progression. (Man RK *et. al*, A Comparison of the Effectiveness of nintedanib and pirfenidone in treating idiopathic pulmonary fibrosis: A Systematic Review. Cureus. 2024 Feb 15;16(2):e54268. doi: 10.7759/cureus.54268. PMID: 38500898) & (Amati F *et al*, Efficacy of pirfenidone and nintedanib in interstitial lung diseases other than idiopathic pulmonary fibrosis: A Systematic Review. Int J Mol Sci. 2023 Apr 25;24(9):7849. doi: 10.3390/ijms24097849. PMID: 37175556.)

The compound N-(2',4'-difluoro-5-(5-(1-methyl-1H-pyrazol-4-yl)-1H-benzo[d]imidazol-1-yl)-[1,1'-biphenyl]-3-yl) cyclopropane sulfonamide also referred to as

"compound of formula (I)" or "Compound 1" and derivatives thereof have been disclosed in WO2013/053983.



(I)

Compound 1 is an orally bioavailable clinical stage spectrum kinase inhibitor that inhibits the activity of oncogenic pathway-related kinases.

There is a need to develop new compounds that can be used to treat or delay the progression of fibrosis.

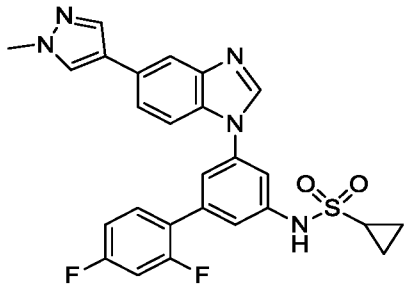
SUMMARY OF THE INVENTION

The present application is based on an unexpected technical effect that Compound 1 can be used to effectively treat or delay the progression of fibrosis, in particular, idiopathic pulmonary fibrosis (IPF) which is a rare, progressive illness of the respiratory system. As shown in Example 1, compared to known drugs pirfenidone and nintedanib, it is unexpected to find that Compound 1 exhibits dose dependent inhibition of β-catenin reporter activity. As such, compound 1 specifically modulates the Wnt/β-catenin pathway through DDR1 and SIK2.

In addition, as demonstrated in Example 2, Compound 1 exhibits superior ability to downregulate p-LRP6 (low-density lipoprotein receptor-related protein 6), p-HDAC 4/5/7 (histone deacetylase 4/5/7), and c-Myc when compared to known SIK2 and DDR1 inhibitors.

Furthermore, as described in Example 3, Compound 1 demonstrates compelling efficacy in the pulmonary fibrosis model even compared to the known drug pirfenidone, which offers a significant opportunity for this compound in fibrosis, cancer-associated fibrosis, or fibrotic cancer.

In one aspect, the present invention provides a method of treating or delaying the progression of fibrosis in a subject comprising administering to the subject a compound represented by formula (I),



(I) ;

or a pharmaceutically acceptable salt thereof.

In another aspect, the present invention provides a method comprising the step of administering to a lung of a subject suffering from fibrosis, a therapeutically effective amount of compound represented by formula (I) or a pharmaceutically acceptable salt thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1A illustrates the inhibition percentage of fibrosis marker at various concentrations of Compound 1 in reporter assay in the HCT-116 cell line.

FIG. 1B illustrates the inhibition percentage of fibrosis marker at various concentrations of pirfenidone in reporter assay in the HCT-116 cell line.

FIG. 1C illustrates the inhibition percentage of fibrosis marker at various concentrations of nintedanib in reporter assay in the HCT-116 cell line.

FIG. 1D illustrates the inhibition percentage of fibrosis marker at various concentrations of imatinib in reporter assay in the HCT-116 cell line.

FIG. 1E illustrates the inhibition percentage of fibrosis marker at various concentrations of sunitinib in reporter assay in the HCT-116 cell line.

FIG. 1F illustrates the inhibition percentage of fibrosis marker at various concentrations of erdafitinib in reporter assay in the HCT-116 cell line.

FIG. 2A illustrates the layers of the transfer cassette for Western blot.

FIG. 2B illustrates the arrangement of the transfer tank.

Fig. 2C illustrates the Western blotting of p-LRP6 and p-HDAC4/5/7 with Compound 1, ARN-3236, and DMSO.

Fig. 2D illustrates the Western blotting of c-Myc and IL-18 with Compound 1, DDR1-IN-1, and DMSO.

FIG. 3A illustrates the change in albumin levels in C57BL/6 mice that received Compound 1 (10 mg/kg/day, 20 mg/kg/day, and 40 mg/kg/day) and pirfenidone (PF) (200 mg/kg/day) at various treatment days.

FIG. 3B illustrates the change in total protein levels in C57BL/6 mice that received Compound 1 (10 mg/kg/day, 20 mg/kg/day, and 40 mg/kg/day) and pirfenidone (PF) (200 mg/kg/day) at various treatment days.

FIG. 4A illustrates the change in TGF-β levels in C57BL/6 mice that received Compound 1 (10 mg/kg/day, 20 mg/kg/day, and 40 mg/kg/day) and pirfenidone (PF) (200 mg/kg/day) at various treatment days.

FIG. 4B illustrates the change in interleukin-6 (IL-6) levels in C57BL/6 mice that received Compound 1 (10 mg/kg/day, 20 mg/kg/day, and 40 mg/kg/day) and pirfenidone (PF) (200 mg/kg/day) at various treatment days.

FIG. 4C illustrates the change in tumour necrosis factor-α (TNF-α) levels in C57BL/6 mice that received Compound 1 (10 mg/kg/day, 20 mg/kg/day, and 40 mg/kg/day) and pirfenidone (PF) (200 mg/kg/day) at various treatment days.

FIG. 5 illustrates the change in the lung histological characteristics in C57BL/6 mice that received Compound 1 (10 mg/kg/day, 20 mg/kg/day, and 40 mg/kg/day) and pirfenidone (PF) (200 mg/kg/day) at various treatment days.

FIG. 6A illustrates the change in the histological characteristics by Picrosirius Red staining of lung sections of mice that received Compound 1 (10 mg/kg/day, 20 mg/kg/day, and 40 mg/kg/day) and pirfenidone (PF) (200 mg/kg/day) at various treatment days.

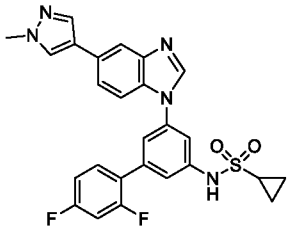
FIG. 6B illustrates the change in the percentage of fibrotic area of mice that received Compound 1 (10 mg/kg/day, 20 mg/kg/day, and 40 mg/kg/day) and pirfenidone (PF) (200 mg/kg/day) at various treatment days, where ****$p<0.0001$ vs NC, ###$p<0.001$ and ####$p<0.0001$ vs DC. NC-Normal control, DC- Disease control, PF-Pirfenidone (200 mg/kg).

FIG. 7A illustrates the change in the histological characteristics by Masson's Trichrome staining of lung sections of mice that received Compound 1 (10 mg/kg/day, 20 mg/kg/day, and 40 mg/kg/day) and pirfenidone (PF) (200 mg/kg/day) at various treatment days.

FIG. 7B illustrates the change in the percentage of fibrotic area of mice that received Compound 1 (10 mg/kg/day, 20 mg/kg/day, and 40 mg/kg/day) and pirfenidone (PF) (200 mg/kg/day) at various treatment days, where ****$p<0.0001$, #$p<0.05$, ####$p<0.0001$.

DETAILED DESCRIPTION OF THE INVENTION

In one embodiment, the present invention provides a method of treating or delaying the progression of fibrosis in a subject comprising administering to the subject a compound represented by formula (I),



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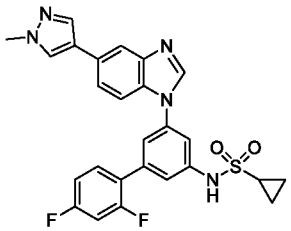
or a pharmaceutically acceptable salt thereof.

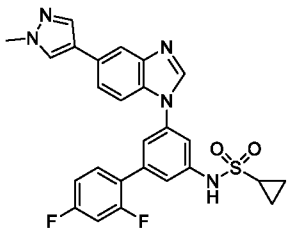
In one embodiment, the present invention provides a compound for use in treating or delaying the progression of fibrosis, wherein the compound is represented by formula (I),



(I) ;

or a pharmaceutically acceptable salt thereof.

In one embodiment, the present invention provides use of a compound represented by formula (I),



(I) ;

or a pharmaceutically acceptable salt thereof, in the manufacture of a medicament for treating or delaying the progression of fibrosis.

In one embodiment, the compound of formula (I) is an oral, selective kinase inhibitor targeting DDR1, SIK2, and related RTKs (FGFR, VEGFR, PDGFR, and RET). The selectivity profile of the compound of formula (I) makes it suitable for the treatment or delay in the progression of fibrosis and a wide variety of cancers.

In one embodiment, the fibrosis is mediated by Wnt/Beta-catenin signaling pathway.

In one embodiment, the fibrosis is mediated by collagen/ECM signaling pathway.

In one embodiment, the fibrosis is mediated by Discoidin domain receptor 1 (DDR1).

In one embodiment, the fibrosis is mediated by Salt-inducible kinase 2 (SIK-2).

In one embodiment, the fibrosis is mediated by both Discoidin domain receptor 1 (DDR1) and Salt-inducible kinase 2 (SIK-2).

In one embodiment, the fibrosis is one or more selected from eye fibrosis, heart fibrosis, hepatic fibrosis, lung fibrosis, pancreas fibrosis, renal fibrosis, bone marrow fibrosis, and skin fibrosis.

In one embodiment, the fibrosis is one or more selected from pulmonary fibrosis, cystic fibrosis, idiopathic pulmonary fibrosis (IPF), interstitial lung diseases (ILDs), chronic fibrosing interstitial lung diseases (ILDs) with aprogressive phenotype, systemic sclerosis-associated interstitial lung disease (SSc-ILD), radiation-induced lung injury, bridging fibrosis, liver cirrhosis, non-alcoholic hepatosteatosis (NASH), non-alcoholic fatty liver disease (NAFLD), atrial fibrosis, skeletal muscle fibrosis, myocardial fibrosis, endomyocardial fibrosis, myocardial infarction related fibrosis, glial scar, ocular fibrosis, arterial stiffness, arthrofibrosis, inflammatory bowel diseases, crohn's disease, dupuytren contracture, mediastinal fibrosis, intestinal fibrosis, myelofibrosis, Peyronie's disease, nephrogenic systemic fibrosis, progressive massive fibrosis, retroperitoneal fibrosis, scleroderma, keloids, hypertrophic scars, psoriasis, Kaposi's sarcoma, myeloid leukemia, acute myelogenous leukemia, myelodysplastic syndrome, lymphangioleiomyomatosis (LAM), chronic graft vs. host disease, polycythemia vera, essential thrombocythemia, myeloproliferative syndrome, Hansen's disease, collagenous colitis, fibrillogenesis, fibrosis of vocal cords and COVID-related pulmonary fibrosis.

In one embodiment, the fibrosis is one or more selected from pulmonary fibrosis, idiopathic pulmonary fibrosis, interstitial lung disease (ILDs), and systemic sclerosis-associated interstitial lung disease (SSc-ILD).

In one embodiment, the fibrosis is pulmonary fibrosis.

In one embodiment, the pulmonary fibrosis is idiopathic pulmonary fibrosis.

In one embodiment, the fibrosis is interstitial lung disease (ILDs).

In one embodiment, the fibrosis is systemic sclerosis-associated interstitial lung disease (SSc-ILD).

In one embodiment, the fibrosis is characterized by aberrant fibroblast proliferation and extracellular matrix deposition in the tissue of a subject.

In one embodiment, the fibrosis is further characterized by an inflammation in the tissue.

In one embodiment, the inflammation is acute or chronic inflammation.

In one embodiment, the inflammation is mediated by one or more cytokines selected from the group consisting of Tumor Necrosis Factor-alpha (TNF-a), Interleukin-6 (IL-6), and Interleukin-Iβ (IL-Iβ).

In one embodiment, pulmonary fibrosis is characterized by the presence of one or more conditions selected from an aberrant deposition of an extracellular matrix protein in a pulmonary interstitium, an aberrant promotion of fibroblast proliferation in the lung, an aberrant induction of myofibroblast differentiation in the lung, and an aberrant promotion of attachment of myofibroblasts to an extracellular matrix compared to a normal healthy control subject.

In one embodiment, the fibrosis is cancer-associated fibrosis or fibrotic cancer.

In one embodiment, the cancer-associated fibrosis or fibrotic cancer is biliary cancer, cholangiocarcinoma, bladder cancer, colorectal cancer, colon cancer, rectal cancer, colorectal adenocarcinoma, brain cancer, meningioma, glioma, astrocytoma, oligodendroglioma, medulloblastoma, breast cancer, adenocarcinoma of the breast, inflammatory carcinoma of the breast, papillary carcinoma of the breast, medullary carcinoma of the breast, kidney cancer nephroblastoma/Wilms' tumor, renal cell carcinoma, cervical cancer, squamous cell carcinoma

of the cervix, cervical adenocarcinoma, gastric cancer, esophageal cancer, gastrointestinal stromal tumor (GIST), head and neck cancer, head and neck squamous cell carcinoma, oral cancer, oral squamous cell carcinoma (OSCC), keloids, leukemia, acute lymphocytic leukemia (ALL), acute myelogenous leukemia (AML), chronic myelogenous leukemia (CML), chronic lymphocytic leukemia (CLL)), lung cancer bronchogenic carcinoma, small cell lung cancer (SCLC), non-small cell lung cancer (NSCLC), adenocarcinoma of the lung, liver cancer, hepatocellular cancer (HCC), malignant hepatoma, lymphoma, Hodgkin lymphoma (HL), non-Hodgkin lymphoma (NHL), follicular lymphoma, diffuse large B-cell lymphoma (DLBCL), mantle cell lymphoma (MCL), medulloblastoma, multiple myeloma (MM), myelodysplasia syndrome (MDS), myeloproliferative disorder (MPD), polycythemia vera (PV), essential thrombocythemia (ET), agnogenic myeloid metaplasia (AMM), primary myelofibrosis (PMF), chronic neutrophilic leukemia (CNL), hypereosinophilic syndrome (HES), neuroblastoma, neurofibroma, neurofibromatosis (NF) type 1, neurofibromatosis (NF) type 2, schwannomatosis, skin cancer, squamous cell carcinoma (SCC), keratoacanthoma (KA), melanoma, basal cell carcinoma (BCC), dermatofibroma, osteosarcoma, ovarian cancer, cystadenocarcinoma, ovarian embryonal carcinoma, ovarian adenocarcinoma, neuroendocrine cancer, gastroenteropancreatic neuroendocrine tumor (GEP-NET), carcinoid tumor, pancreatic cancer, pancreatic adenocarcinoma, intraductal papillary mucinous neoplasm (IPMN), prostate cancer, prostate adenocarcinoma, soft tissue tumors, angiolipoma, angioleiomyoma, malignant fibrous histiocytoma (MFH), liposarcoma, malignant peripheral nerve sheath tumor (MPNST), chondrosarcoma, fibrosarcoma, myosarcoma, osteosarcoma, and tumors associated with desmoplasia.

In one embodiment, the compound of formula (I) is in the form of hydrochloride (HCl) salt. In a specific embodiment, the molar ratio between the compound of formula (I) and hydrochloride is 1:1.

In one embodiment, the hydrochloride (HCl) salt of the compound of formula (I) is in crystalline form.

In one embodiment, the method of treating or delaying the progression of fibrosis further comprises administering an additional anti-fibrotic agent to the subject.

In one embodiment, the additional anti-fibrotic agent is one or more selected from an agent that blocks TGF-β signaling, an agent that inhibits activation of plasminogen activator inhibitor- 1 promoter activity, an antibody that binds to TGF-β, an antibody that binds to TGF-

β receptor I, II, or III, a kinase inhibitor, an agent that blocks connective tissue growth factor (CTGF) signaling, an agent that inhibits prolyl hydroxylase, an agent that inhibits procollagen C-proteinase, an agent that inhibits vascular endothelial growth factor VEGF, an agent that antagonizes TGF-β, an agent that antagonizes CTGF, and Corticosteroids.

In one embodiment, the additional anti-fibrotic agent is one or more selected from pirfenidone, nintedanib, silymarin, pentoxifylline, colchicine, etanercept (embrel), infliximab (remicade), tocilizumab, prednisone, mycophenolate mofetil, mycophenolic acid, and azathioprine.

In one embodiment, the compound of formula (I) or a pharmaceutically acceptable salt thereof is administered to a subject at a daily dose of 50 mg to 400 mg.

In one embodiment, the compound of formula (I) or a pharmaceutically acceptable salt thereof is administered to a subject at a daily dose of 80 mg to 350 mg.

In one embodiment, the compound of formula (I) or a pharmaceutically acceptable salt thereof is administered to a subject at a daily dose of 90 mg to 300 mg.

In one embodiment, the compound of formula (I) or a pharmaceutically acceptable salt thereof is administered to a subject at a daily dose of 50 mg.

In one embodiment, the compound of formula (I) or a pharmaceutically acceptable salt thereof is administered to a subject at a daily dose of 100 mg.

In one embodiment, the compound of formula (I) or a pharmaceutically acceptable salt thereof is administered to a subject at a daily dose of 150 mg.

In one embodiment, the compound of formula (I) or a pharmaceutically acceptable salt thereof is administered to a subject at a daily dose of 200 mg.

In one embodiment, the compound of formula (I) or a pharmaceutically acceptable salt thereof is administered to a subject at a daily dose of 250 mg.

In one embodiment, the compound of formula (I) or a pharmaceutically acceptable salt thereof is administered to a subject at a daily dose of 300 mg.

In one embodiment, the compound of formula (I) or a pharmaceutically acceptable salt thereof is administered to a subject at a daily dose of 350 mg.

In one embodiment, the compound of formula (I) or a pharmaceutically acceptable salt thereof is administered to a subject at a daily dose of 400 mg.

In one embodiment, the compound of formula (I) or a pharmaceutically acceptable salt thereof is administered to a subject daily as a continuous regimen for a 21-day cycle.

In one embodiment, the compound of formula (I) or a pharmaceutically acceptable salt thereof is administered to a subject as an interrupted regimen for a 21-day cycle.

In one embodiment, the interrupted regimen comprises two weeks of daily administration followed by one week off.

In one embodiment, the compound of formula (I) or a pharmaceutically acceptable salt thereof is administered to a subject for repeated 21-day cycles till the complete remission of the disease.

In one embodiment, the compound of formula (I) or a pharmaceutically acceptable salt thereof is administered to a subject in the range of 2 cycles to 10 cycles.

In one embodiment, the compound of formula (I) or a pharmaceutically acceptable salt thereof is administered to a subject in the range of 2 cycles to 5 cycles.

In one embodiment, the compound of formula (I) or a pharmaceutically acceptable salt thereof is administered to a subject in the range of 2 cycles to 4 cycles.

In one embodiment, the compound of formula (I) or a pharmaceutically acceptable salt thereof is administered to a subject for at least two 21-day cycles.

In one embodiment, the compound of formula (I) or a pharmaceutically acceptable salt thereof is administered to a subject for at least one 21-day cycle.

In one embodiment, wherein each cycle comprises 21 days of administration of the compound of formula (I) or a pharmaceutically acceptable salt thereof.

In one embodiment, wherein each cycle comprises 21 days of administration of the compound of formula (I) or a pharmaceutically acceptable salt thereof with interruption.

In one embodiment, the interruption is in the range of 1 days to 7 days.

In one embodiment, the subject is a mammal.

In one embodiment, the subject is human.

In one embodiment, the compound of formula (I) or a pharmaceutically acceptable salt thereof is administered to mice at a daily dose of 10 mg/kg/day to 60 mg/kg/day.

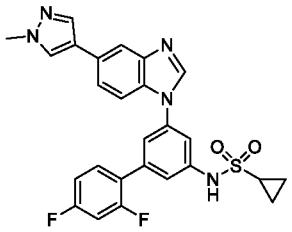
In one embodiment, the compound of formula (I) or a pharmaceutically acceptable salt thereof is administered to mice at a daily dose of 40 mg/kg/day.

In one embodiment, the compound of formula (I) or a pharmaceutically acceptable salt thereof is administered to mice at a daily dose of 20 mg/kg/day.

In one embodiment, the compound of formula (I) or a pharmaceutically acceptable salt thereof is administered to mice at a daily dose of 10 mg/kg/day.

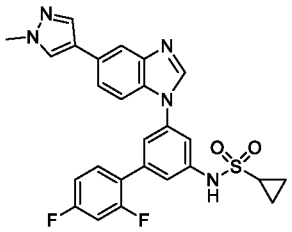
In another aspect, the present invention provides a method of administration of a compound of formula (I) or a pharmaceutically acceptable salt thereof to a lung of a subject suffering from fibrosis.

In one embodiment, the method of treating or delaying the progression of fibrosis, comprising the step of administering to a lung of a subject suffering from fibrosis a therapeutically effective amount of compound represented by formula (I) or a pharmaceutically acceptable salt thereof:



(I)

In one embodiment, the compound for use in treating or delaying the progression of fibrosis, comprising the step of administering to a lung of a subject suffering from fibrosis a therapeutically effective amount of compound represented by formula (I) or a pharmaceutically acceptable salt thereof:



(I)

In one embodiment, the compound of formula (I) is in the form of a solution.

In one embodiment, the compound of formula (I) is in the form of suspension or nanosuspension.

In one embodiment, the compound of formula (I) is in the form of a liposome formulation.

In one embodiment, the compound of formula (I) is in the form of powder.

In one embodiment, the compound of formula (I) is in the form of powder for inhalation.

In one embodiment, the compound of formula (I) is administered through intratracheal, pulmonary, or oropharyngeal route.

In one embodiment, the method further comprises an additional anti-fibrotic agent.

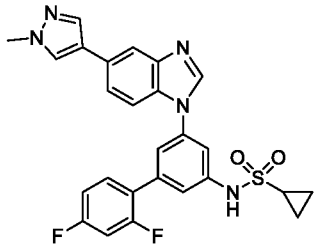
In one embodiment, the additional anti-fibrotic agent is one or more selected from an agent that blocks TGF-β signaling, inhibits activation of plasminogen activator inhibitor- 1 promoter activity, an antibody that binds to TGF-β, an antibody that binds to TGF-β receptor I, II, or III, a kinase inhibitor, an agent that blocks connective tissue growth factor (CTGF) signaling, an agent that inhibits prolyl hydroxylase, an agent that inhibits procollagen C-proteinase, an agent that inhibits vascular endothelial growth factor VEGF, an agent that antagonizes TGF-β, an agent that antagonizes CTGF, and Corticosteroids.

In one embodiment, the additional anti-fibrotic agent is one or more selected from pirfenidone, nintedanib, silymarin, pentoxifylline, colchicine, etanercept, infliximab, tocilizumab, prednisone, mycophenolate mofetil, mycophenolic acid, treprostinil, belumosudil, and azathioprine.

In one embodiment, the additional anti-fibrotic agent is administered through oral, parenteral, intravenous, intraperitoneal, oropharyngeal, and pulmonary routes.

In yet another aspect, the present invention provides a composition for pulmonary administration.

In an embodiment, the composition comprises a compound represented by a compound of formula (I) or a pharmaceutically acceptable salt thereof;



(I)

In one embodiment, the composition is in the form of a solution.

In one embodiment, the composition is in the form of suspension or nanosuspension.

In one embodiment, the composition is in the form of a liposome formulation.

In one embodiment, the composition is in the form of powder.

In one embodiment, the composition is in the form of powder for inhalation.

In one embodiment, the composition is administered orally, intratracheally, parenterally, intravenously, intraperitoneally, or oropharyngeally.

In one embodiment, the composition is administered intratracheally or oropharyngeally.

In one embodiment, the composition is administered oropharyngeally.

In one embodiment, the composition is administered as a single dose.

In one embodiment, the composition is administered as a plurality of doses.

In one embodiment, the composition is administered as a plurality of doses over a period of time.

In one embodiment, the period of time is a day.

In one embodiment, the composition further comprises an additional anti-fibrotic agent.

In one embodiment, the additional anti-fibrotic agent is one or more selected from an agent that blocks TGF-β signaling, inhibits activation of plasminogen activator inhibitor- 1 promoter activity, an antibody that binds to TGF-β, an antibody that binds to TGF-β receptor I, II, or III, a kinase inhibitor, an agent that blocks connective tissue growth factor (CTGF) signaling, an agent that inhibits prolyl hydroxylase, an agent that inhibits procollagen C-

proteinase, an agent that inhibits vascular endothelial growth factor VEGF, an agent that antagonizes TGF-β, an agent that antagonizes CTGF, and Corticosteroids.

In one embodiment, the additional anti-fibrotic agent is one or more selected from pirfenidone, nintedanib, silymarin, pentoxifylline, colchicine, etanercept (embrel), infliximab (remicade), tocilizumab, prednisone, mycophenolate mofetil, mycophenolic acid, treprostinil, belumosudil and azathioprine.

In one embodiment, the present invention provides a method of preventing or slowing the progression of or treating disease, condition, or pathologic process characterized by aberrant fibroblast proliferation and extracellular matrix deposition in a tissue of a subject comprising administering to the subject in need thereof an effective amount of a compound represented by formula (I) or a pharmaceutically acceptable salt thereof.

In one embodiment, pulmonary fibrosis results from an allergic reaction, inhalation of environmental particulates, smoking, a bacterial infection, a viral infection, mechanical damage to the lung of the subject, lung transplantation rejection, an autoimmune disorder, a genetic disorder, or a combination thereof.

The efficacy of compound 1 of the present disclosure in modulating the Wnt/β-catenin signalling pathway was compared with the pirfenidone and nintedanib (FDA-approved drugs for idiopathic pulmonary fibrosis) and imatinib, sunitinib, and erdafitinib (drugs approved as multi-tyrosine kinase inhibitors).

The efficacy of compound 1 was evaluated by using *in-vitro* cellular reporter assay studies, western blot, and bleomycin-induced mouse model. The studies confirmed that Compound 1 of the present disclosure, shows enhanced efficacy when compared to the pirfenidone, nintedanib, imatinib, sunitinib, and erdafitinib. Further, the histological studies show a decrease in fibrotic area, characterized by less collagen, suggesting that Compound 1 of the present disclosure is more effective in comparison with known compounds.

As used herein the term "subject" means a mammal in need of treatment, *e.g.*, companion animals (*e.g.*, dogs, cats, and the like), farm animals (*e.g.*, cows, pigs, horses, sheep, goats, and the like) and laboratory animals (*e.g.*, rats, mice, guinea pigs and the like). Typically, the subject is a human in need of treatment.

An "effective amount" of the Compound of formula (I), or a pharmaceutically acceptable salt, is an amount sufficient to provide a therapeutic benefit in preventing or slowing

the progression of or treating disease, condition, or pathologic process characterized by aberrant fibroblast proliferation and extracellular matrix deposition. The terms "therapeutically effective amount" and "effective amount" are used interchangeably. The term "effective amount" can encompass an amount that improves overall therapy, reduces or avoids symptoms, signs, or causes of the condition. In certain embodiments, an effective amount is an amount sufficient for eliciting therapeutic effects in the treatment of a fibrotic disease cancer (including lung fibrosis as further described herein).

The precise amount of the Compound of formula (I) (or pharmaceutically acceptable salt thereof), administered to a subject will depend on various factors, such as the given drug, the pharmaceutical formulation, the route of administration, the type of disease or disorder, the identity of the subject being treated, and the like, but can nevertheless be routinely determined by one skilled in the art. For example, the determination of an effective amount will also depend on the degree, severity, and type of disease progression. The skilled artisan will be able to determine appropriate dosages depending on these and other factors.

An effective amount of the Compound of formula (I), a pharmaceutically acceptable salt thereof is generally in the range from 1 mg/kg to 100 mg/kg of body weight of the recipient (mammal) per day and particularly typically in the range from 1 to 40 mg/kg of body weight per day.

The term "treat" means decrease, suppress, attenuate, diminish, arrest, or stabilize the development or progression of a disease/disorder characterized by aberrant fibroblast proliferation and extracellular matrix deposition in a tissue or improve the symptoms associated with the disease/disorder. Treatment may also be continued after symptoms have resolved, for example, to reduce the likelihood of or delay their recurrence.

The synthetic procedures for the preparation of Compound 1, described in the present invention, were disclosed in international publication No. WO 2013/053983 dated 18th April 2013. The contents of this publication are hereby incorporated by reference in their entirety.

EXPERIMENTAL DETAILS

Example – 1: In-vitro cellular reporter assay studies

The effect of Compound 1 of the present disclosure on the Wnt/β-catenin signaling pathway was evaluated in HCT-116 (colorectal cancer cell line), which overexpresses DDR1 and has a constitutive expression of Wnt/β-catenin pathway. DDR1 is a tyrosine kinase receptor

activated by collagen and modulates the Wnt/β-catenin pathway through phosphorylation of its downstream target, BCR. The activation of this pathway leads to the production of key fibrotic marker TGF-β.

The effect of Compound 1 was compared with pirfenidone and nintedanib (FDA-approved drugs for idiopathic pulmonary fibrosis) and Imatinib, Sunitinib, and Erdafitinib (drugs approved as multi-tyrosine kinase inhibitors).

The HCT-116 cells were transfected with T-cell factor (TCF) reporter plasmid driving the expression of firefly luciferase reporter and subsequently treated with different concentrations of Compound 1, pirfenidone, nintedanib, imatinib, sunitinib, erdafitinib and % inhibition in a reporter activity was measured 24 h post-treatment.

Reagent preparation

- The kit containing 1 bottle (100 ml) of Dual-Glo® Luciferase Buffer and a pack of Dual-Glo® Luciferase Substrate (1 bottle, lyophilized powder form) were mixed to produce the Dual-Glo® Luciferase Reagent. After the substrate was thoroughly dissolved, the contents were mixed by inversion, aliquoted into 15 ml falcon tubes, and stored at -80°C.
- The Dual-Glo® Stop & Glo® Buffer was aliquoted in a 15 ml falcon tube and kept at -80°C.
- The Renilla substrate (Dual-Glo® Stop & Glo® Substrate) was stored at -20°C.
- On the day of the experiment, the buffers were thawed at room temperature (RT).
- The Renilla substrate (Dual-Glo® Stop & Glo® Substrate) (1:100) was added to the Dual-Glo® Stop & Glo® Buffer just before adding to the cells.
- 10 M lithium chloride (LiCl) solution was made in HPLC water and filter sterilized with a 0.2 µm syringe filter.

Procedure

Day 1: Seeding

HCT-116 cells were seeded at a seeding density of 12000 cells/well in a 96-well plate in 90 µl of McCoy's 5A medium containing 10% FBS (Foetal Bovine Serum) and 1% Penstrep (Penicillin-Streptomycin). The cells were incubated at 37°C, 5% CO_2 overnight.

Day 2: Transfection

Opti-MEM™ I Reduced Serum Medium and FuGENE® HD Transfection Reagent were brought to room temperature (RT) and the transfection solution was prepared as per the volume and concentrations mentioned below

Ingredient	Quantity
Opti-MEM	Upto 5 µL/well
TOPFlash plasmid	100 ng/well
pGl4.73 [hRluc/SV40] vector	10 ng/well
Fugene@HD	3µL/µg of DNA

First, the plasmids were added to Opti-MEM™ I Reduced Serum Medium and vortexed. FuGENE® HD Transfection Reagent was then vortexed briefly and added directly to Opti-MEM™ I Reduced Serum Medium containing the plasmids in the ratio of 3:1. The solution was mixed well with 1 mL pipette and incubated at RT for 15 min. 5 µL of this solution was then added per well of cells (assay plate) and mixed well by pipetting. The assay plate was then gently swirled and incubated at 37 ºC and 5% CO_2 for 24 h.

Day 3: Treatment

Medium containing the transfection solution in the assay plate was discarded and 90 µL of fresh complete medium containing 0.5 mM LiCl was added to the assay plate.

Dilution of compounds

The starting concentration for Compound 1 was 10 µM and serially diluted 3-fold dilution with an 8-point dose-response curve (DRC). The initial drug dilutions were made in 100% dimethylsulfoxide (DMSO). An intermediate dilution of 100X was made in McCoy's 5A containing 0.5mM LiCl and 10 µl of this intermediate solution was added to the cells to achieve a 10X dilution.

The starting concentration for pirfenidone was 10 mM and a 10-fold dilution of this compound was made to achieve a 5-point dose response curve (DRC). The initial drug dilutions were made in 100% DMSO. An intermediate dilution of 100X was made in culture medium and 10 µl of this intermediate solution was added to the cells to achieve a 10X dilution.

The starting concentration for nintedanib was 1 µM and a 10-fold dilution of this compound was made to achieve a 5-point dose response curve (DRC). The initial drug dilutions

were made in 100% DMSO. An intermediate dilution of 100X was made in culture medium and 10 µl of this intermediate solution was added to the cells to achieve a 10X dilution.

The starting concentration for imatinib was 10 µM and a 10-fold dilution of this compound was made to achieve a 5-point dose response curve (DRC). The initial drug dilutions were made in 100% DMSO. An intermediate dilution of 100X was made in culture medium and 10 µl of this intermediate solution was added to the cells to achieve a 10X dilution.

The starting concentration for sunitinib was 100 nM and a 10-fold dilution of this compound was made to achieve a 5-point dose response curve (DRC). The initial drug dilutions were made in 100% DMSO. An intermediate dilution of 100X was made in culture medium and 10 µl of this intermediate solution was added to the cells to achieve a 10X dilution.

The starting concentration for erdafitinib was 10 nM and a 2-fold dilution of this compound was made to achieve a 5-point dose response curve (DRC). The initial drug dilutions were made in 100% DMSO. An intermediate dilution of 100X was made in culture medium and 10 µl of this intermediate solution was added to the cells to achieve a 10X dilution.

The final DMSO concentration was maintained at 0.1%. The cells were incubated for 24 hr at 37°C, 5% CO_2.

Day 4: Termination and analysis

Dual-Glo® Luciferase Reagent and Dual-Glo® Stop & Glo® Buffer were brought to room temperature. 50 µL of Dual-Glo® Luciferase Reagent was added to each well and the assay plate was wrapped in an aluminium foil. The plate was incubated on a shaker (100 rpm) at room temperature for 20 min and reading was taken using a Clariostar^{PLUS} multimode plate reader to obtain a firefly luciferase signal. Renilla substrate (Dual-Glo® Stop & Glo® substrate) was added to Dual-Glo® Stop & Glo® Buffer in the ratio of 1:100 then the mix was vortexed briefly and added 50 µl to each well of the assay plate. The plate was wrapped in an aluminium foil and incubated again for 20 minutes on the shaker (100 rpm) at room temperature. The luminescence reading was taken using a Clariostar^{PLUS} reader to obtain renilla luciferase signal.

Analysis

The firefly luciferase signal was normalized to the renilla luciferase signal for each respective well to get the firefly signal per cell (normalization factor). The percentage (%) reporter activity was calculated for each lane with respect to DMSO using the normalization

factor obtained [normalized firefly signal/average DMSO firefly signal) *100]. The Mean and SD of % reporter activity for each condition were calculated and the data was represented taking DMSO reporter activity to be 100%.

The amount of inhibition of luciferase reporter activity with Compound 1, pirfenidone, nintedanib, imatinib, sunitinib, and erdafitinib at various concentration levels was provided below (illustrated in Fig. **1A** to **1F** respectively).

Compound 1:

Compound	Conc (µM)	Normalized firefly signal			% Average inhibition w.r.t DMSO
DMSO	0	3.12	4.60	2.15	-
Compound 1	0.004	0.823893	0.977507	0.547752	24.64612
	0.013	1.19682	1.198539	0.67598	-18.3403
	0.04	0.680167	0.939148	0.482259	29.77681
	0.12	0.775955	0.837642	0.843926	16.92378
	0.37	0.648195	0.721759	0.467718	44.86939
	1.11	0.368294	0.36415	0.355111	64.46561
	3.33	0.394989	0.301794	0.278545	71.32894
	10	0.403495	0.265663	0.184618	77.75432

Pirfenidone:

Compound	Conc (mM)	Normalized firefly signal			% Average inhibition w.r.t DMSO
DMSO	0	1.89	1.39	1.41	-
Pirfenidone	0.0001	1.108952	2.180996	1.255374	3.046536
	0.001	1.433115	1.414711	1.209982	13.44538
	0.01	1.350643	1.470026	1.351191	11.01263
	0.1	1.318151	1.496967	1.303291	12.15276
	1	2.382823	1.825099	1.687234	-25.7459

Nintedanib:

Compound	Conc (nM)	Normalized firefly signal			% Average inhibition w.r.t DMSO
DMSO	0	1.43	1.61	1.78	-
Nintedanib	0.1	1.467941	1.469468	1.515089	8.450923
	1	1.465545	1.505222	1.467863	7.775281
	10	1.541491	1.6103	1.437344	4.648156
	100	1.55046	1.505571	1.363758	8.16677
	1000	2.474955	3.864828	2.23504	-78.1655

Imatinib:

Compound	Conc (µM)	Normalized firefly signal			% Average inhibition w.r.t DMSO
DMSO	0	1.64	1.52	1.56	-
Imatinib	0.001	1.270792	1.362057	1.3749	16.21
	0.01	1.260704	1.144594	1.283111	21.75
	0.1	1.221216	1.360318	1.422043	15.06
	1	1.337012	1.417153	1.482839	10.11
	10	1.234888	2.060212	1.410282	0.17

Sunitinib:

Compound	Conc (nM)	Normalized firefly signal			% Average inhibition w.r.t DMSO
DMSO	0	1.64	1.52	1.56	-
Sunitinib	0.01	1.037259	1.716761	1.514141	22.20
	0.1	1.307683	1.691472	1.620853	13.00
	1	2.165926	1.663445	2.404304	24.07
	10	1.456631	1.685486	1.524708	7.48
	100	1.23593	1.81169	1.751255	20.14

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Erdafitinib:

Compound	Conc (nM)	Normalized firefly signal			% Average inhibition w.r.t DMSO
DMSO	0	1.56	1.60	1.68	-
Erdafitinib	0.625	1.618882	1.723597	1.687072	-4.0346
	1.25	1.700824	1.631072	1.674531	-3.55632
	2.5	1.644758	1.649913	1.622384	-1.70768
	5	1.573399	1.569785	1.649802	0.858635
	10	1.352704	1.507696	1.615762	7.412044

From this experiment, it was found that Compound 1 exhibited dose-dependent inhibition of β-catenin reporter activity in HCT-116 cells with an IC_{50} of 287.1 nM, illustrated in **Fig. 1A**.

It was observed that Compound 1 inhibited the luciferase reporter activity in a dose-dependent manner whereas pirfenidone, nintedanib, imatinib, sunitinib, and erdafitinib did not show any effect on the reporter activity, illustrated in **Fig. 1B – 1F**, suggesting Compound 1 can effectively modulate Wnt/ β-catenin pathway through DDR1 and SIK2. Thus Compound 1 is effective in fibrosis treatment.

It is evident from the above β-catenin reporter assay that Compound 1 of the present disclosure inhibits DDR1 thus blocking the Wnt/β-catenin pathway, which results in the downregulation of fibrotic markers.

Example – 2: Western Blot

The effect of Compound 1 of the present disclosure was evaluated for its activity on the downstream targets of DDR1-SIK2-Wnt/β-catenin pathway i.e. p-LRP6, p-HDAC 4/5/7 in a SIK2-overexpressing triple-negative breast cancer cell line, BT549, and c-Myc and IL-18 in a DDR1-overexpressing colorectal cancer cell line, HCT-116. The activity of the compound of formula I was compared with the known DDR1 and SIK2 inhibitors i.e. ARN-3236 (SIK2 inhibitor) and DDR1-IN-1 (DDR1 inhibitor).

The effect of Compound 1 of the present disclosure was evaluated for its activity on the DDR1-SIK2-Wnt/β-catenin pathway. The activity was evaluated by detection/expression of biomarkers such as p-LRP6, p-HDAC 4/5/7 for SIK2; c-Myc for IL-18 for DDR1.

Preparation of Reagents and Solution

- Concentrated sodium hydroxide solution (NaOH)
- Concentrated hydrochloride solution (HCl)
- **1.5M Tris pH 8.8 Buffer (150 mL)**: 35.46 gm of Trizma hydrochloride was dissolved in 70 mL of Milli-Q water by placing it in a magnetic stirrer for approximately 1 hour. The pH was adjusted to 8.8 using concentrated NaOH and concentrated HCl solutions. The buffer was mixed well after each pH adjustment. The volume was made up to 150 mL with Milli-Q water. The pH was checked again and adjusted to 8.8 with the addition of NaOH and HCl. It was mixed well and stored at RT.
- **1M Tris pH 6.8 Buffer (25 mL)**: 3.94 gm of Trizma hydrochloride was dissolved in 15 mL Milli-Q water by placing it in a magnetic stirrer for approximately 1 hour. The pH was adjusted to 6.8 using concentrated NaOH and concentrated HCl solutions. The buffer was mixed well after each pH adjustment. The volume was made up to 150 mL with Milli-Q water. The pH was checked again and adjusted to 6.8 with the addition of NaOH and HCl. It was mixed well and stored at RT.
- **10X Wash Buffer (1000 mL)**: 24.23 gm of Trizma hydrochloride and 88.06 gm of NaCl were added in 800 mL Milli-Q water. The solution was mixed well by shaking until the chemicals were completely dissolved. The pH was adjusted to 7.6 using concentrated NaOH and concentrated HCl solutions. The buffer was mixed well after each pH adjustment. The volume was made up to 1000 mL using Milli-Q water. The pH was checked again and adjusted to 7.6 with the addition of NaOH and HCl. It was mixed well and stored at RT.
- **10% (w/v) ammonium persulfate (APS) solution (1 mL aliquots)**: 1 gm of APS crystals were dissolved in 5 mL of Milli-Q water. Once dissolved, the volume was made up to 10 mL with Milli-Q water and mixed well. 1 mL of the prepared solution was aliquoted in 1.5 mL microcentrifuge tubes, covered in aluminium foil, and stored at -20°C freezer. The tube was thawed at RT before use.
- **10% (w/v) sodium dodecyl sulfate (SDS) solution (200 mL)**: 20 gm of SDS crystals were dissolved in 125 mL of Milli-Q water and mixed well by shaking for 10 – 15 minutes. The solution was stored at RT overnight for the complete dissolution of the SDS crystals. The next day, the volume was made to 200 mL using Milli-Q water. The prepared solution was stored at RT.
- **70% (v/v) IPA solution**: 15 mL of Milli-Q water was added to 35 mL of IPA solution

and mixed well. The prepared solution was stored at RT.

- **Ponceau stain**: 0.1 gm of Ponceau powder was dissolved in 2.5 mL of glacial acetic acid. The stain was completely dissolved by adding 10 – 15 mL of Milli-Q water. Once completely dissolved, the volume was made to 50 mL with Milli-Q water. The prepared solution was stored at RT.
- **PBS**: To prepare 1000 mL of PBS, one sachet of PBS was emptied into a glass bottle and dissolved in 800 mL of HPLC grade water. The volume was made up to 1000 mL with water. The same was autoclaved and stored at RT inside the laminar hood.
- **Lysis buffer solution**: To RIPA lysis buffer, 1X protease inhibitor cocktail, 1X phosphatase inhibitor cocktail, and 1X PMSF were added before use.
- **Running buffer (per tank, per 4 gels) (1300 mL)**: 130 mL of 10X Tris-Glycine-SDS buffer was diluted in 1170 mL of Milli-Q water and mixed well. The solution was stored at RT.
- **Transfer buffer (per tank with the use of ice pack) (per 2 gels) (1500 mL)**: 150 mL of 10X Tris-Glycine buffer was diluted in 1050 mL of Milli-Q water. 350 mL of HPLC-grade methanol was added to it. The solution was stored in a cold room or -20°C freezer until use (must be used within 3 hours before crystallization of the solution).
- **1X TBST (1000 mL)**: 100 mL of 10X TBST solution was diluted in 900 mL of Milli-Q water and mixed well. 1000 µL of Tween-20 was added to it and mixed well until Tween-20 completely dissolved. The solution was stored at RT. The solution must be used within a day.
- **Primary antibody dilutions**: 10 µL of Tween-20 was added to 10 mL of Intercept TBS Blocking buffer. It was mixed by vortexing. 10 µL each of p-LRP6, p-HDAC 4/5/7, and IL-18 were added to 3 separate 10 mL blocking buffer containing Tween-20. 5 µL of c-Myc was added to another 10 mL blocking buffer containing Tween-20.
- **Secondary antibody dilutions (1:10000)**: 10 µL of Tween-20 was added to 10 mL of Intercept TBS Blocking buffer. 1 µL each of IRDye 800CW Goat anti-Rabbit IgG Secondary antibody and IRDye 680RD Goat anti-Mouse IgG Secondary Antibody was added to 2 separate tubes of blocking buffer containing Tween-20.
- **McCoy's 5A complete medium**: To 445 mL of McCoy's 5A medium, 50 mL FBS and 5 mL of Penstrep were added. The prepared medium was stored at 2-8 °C.
- **RPMI-1640 complete medium**: To 445 mL of RPMI-1640, 50 mL FBS and 5 mL of Penstrep were added. The prepared medium was stored at 2-8 °C. Human insulin

(1:1000) was freshly added when the medium was used to culture cells.

Procedure

Experimental Design

Culture of HCT-116 and BT-549 cells

HCT-116 cells (ATCC) and BT549 cells (triple-negative breast cancer cell line) running culture was maintained at 37°C and 5% CO_2 in McCoy's 5A complete medium and RPMI-1640 complete medium respectively in T-75 flasks. For subculturing the cells, the media was aspirated, and the cells were washed with PBS followed by their trypsinization with 3 mL of 1X Trypsin EDTA solution at 37 °C and 5% CO_2 for 5 min. The residual trypsin activity was neutralized with 10 mL of fresh medium. The cell suspension was centrifuged at 1200 rpm for 5 min and the supernatant was discarded to obtain the cell pellet. The cell pellet was resuspended in 1 mL of complete medium and cell density was determined by counting the number of cells.

Cell Seeding (Day 1)

0.2 million cells of HCT-116 and 0.4 million cells of BT-549 cells were seeded onto 6-well plates and the volume of each well was adjusted to 2 mL with fresh medium. The plates were incubated at 37 °C and 5% CO_2 for 24 h.

Treatment of compounds (Day 2)

After incubating the plates for 24 h, the cells were treated with either 3 µM, 1 µM, 0.33 µM of Compound 1, and 4 µM of ARN-3236 (CAS No. 1613710-01-2) for BT549 and the same concentrations of Compound 1 and 1 µM of DDR1-IN-1 (CAS No. 1449685-96-4) for HCT-116. The final DMSO concentration was 0.1%. The plates were then gently swirled and incubated at 37 °C and 5% CO_2 for 24 h.

Cell lysis and protein extraction (Day 3)

After 24 h, the media from the cells were aspirated out and the cells were washed with PBS. To these cells, 60 µL of lysis buffer was added and cells were incubated on ice for 5 minutes. Following that, the cells were scrapped with a scraper on ice until all the cells were collected for lysis. The cell lysates were transferred to 1.5 mL microcentrifuge tubes and incubated on ice for 20 min with occasional vortexing every 5 min. They were then sonicated for 30 sec, and then incubated on ice for 30 sec. This step was repeated twice. Following

sonication, lysates were centrifuged at 15000 rpm at 4°C for 15 minutes. The supernatant containing protein was collected for further evaluation.

In a 96-well plate, 25 µL of bovine serum albumin (BSA) standards were added. 5 µL of protein samples were added to 20 µL of HPLC grade water in separate wells. For blanking BSA standards, 25 µL of HPLC grade water and for blanking protein samples, 20 µL of HPLC grade water along with 5 µL lysis buffer were added to separate wells. From the BCA kit, BCA solution was prepared by addition of solution A to solution B in the ratio of 1:100. 200 µL of this solution was then added to the samples, standards, and blanks, and incubated at room temperature (RT) for 20 minutes in the dark.

The absorbance of the samples was measured at 562 nm using a multi-plate reader, and the concentrations of the protein samples were measured using the slope obtained from the BSA standard curve. The proteins were stored at -80°C until further evaluation.

Preparation of gels

Resolving solution (7.5%, for p-LRP6, p-HDAC 4/5/7): The following reagents were added in the following order. APS and TEMED were added only before adding the resolving gel solution into the gel casting compartment. They are all mixed well by gently inverting the centrifuge tube. The formation of air bubbles was avoided.

SL. No.	Reagent	Volume (mL)
1	HPLC grade water	19.6
2	30% Acrylamide solution	10
3	1.5M Tris pH 8.8 buffer	10
4	10% (w/v) SDS solution	0.4
5	10% (w/v) APS solution	0.4
6	TEMED solution	0.04

Resolving solution (12%, for IL-18, c-Myc): The following reagents were added in the following order. APS and tetramethylethylenediamine (TEMED) were added only before adding the resolving gel solution into the gel casting compartment. They are all mixed well by gently inverting the centrifuge tube. The formation of air bubbles was avoided.

Sl. No.	Reagent	Volume (mL)
1	HPLC grade water	3.3
2	30% Acrylamide solution	4
3	1.5M Tris pH 8.8 buffer	2.5
4	10% (w/v) SDS solution	0.1
5	10% (w/v) APS solution	0.1
6	TEMED solution	0.01

Once prepared, the resolving gel was immediately added to the gel casting compartment.

Stacking gel solution (5%): The following reagents were added in the following order. APS and TEMED were added only before adding the resolving gel solution into the gel casting compartment. They are all mixed well by gently inverting the centrifuge tube. The formation of air bubbles was avoided.

Sl. No.	Reagent	Volume (mL)
1	HPLC grade water	11.2
2	30% Acrylamide solution	2.64
3	1.5M Tris pH 8.8 buffer	2
4	10% (w/v) SDS solution	0.16
5	10% (w/v) APS solution	0.16
6	TEMED solution	0.016

Once prepared, the stacking gel was immediately added to the gel casting compartment.

Gel Electrophoresis

4 clean and dry-casting glass plate pairs were placed on 4 dry casting frames. It was ensured that the bottom surface of the glass plate pairs was equal and even. Each casting plate pairs were locked onto casting frames. They were placed on casting stands and their positions were locked. HPLC grade water was added to each of the gel-casting compartments to check for any leakage of water. If there was no leakage, the compartment was dried, and the corners were dabbed with tissue paper to remove any residual water.

7.5% or 12% resolving gel (depending on the proteins to be probed) was then added to the gel casting compartment until 3/4th the volume of the casting compartment. 1 mL of 70%

IPA solution was added to the resolving gel using a 1 mL pipette to ensure the removal of any bubbles. The gel casting setup was rested for 30 minutes until the gel solidified. The IPA solution was then discarded by inverting the gel casting setup. The corners were dabbed with tissue papers to remove any residual IPA. 1 mL of HPLC grade water was layered on top of the resolving gel using a 1 mL pipette to ensure the removal of excess IPA. The water was discarded by inverting the casting set and the corners were dabbed with tissue paper to remove any residual water.

5% stacking gel was then added to the resolving gel until it filled the casting compartment of each gel casting setup. 10 well combs were placed immediately and gently onto the stacking gel and the casting setup was rested for approximately 30 minutes until the stacking gel solidified. Once solidified, each casting plate pairs containing resolving and stacking gels were washed to remove any leaked gels.

The gels were gently placed in the running modules, followed by placing the nodules in the running tank aligning the anode and cathode electrodes. The running tanks were filled with running buffers up to the 4-gel mark. The inserted combs were then gently removed from the casted gel. It was ensured that the tanks were filled with running buffers.

The proteins were taken out from -80°C and thawed in an ice bath for approximately 5 minutes. To 50 µg of protein, 1X Pierce Lane Marker Reducing Sample Buffer was added and the volume was made up to 25 mL by RIPA lysis buffer. The proteins were then boiled at 95°C for 10 minutes. The samples were then vortexed and given a short spin.

5 µL of Puregene 3 colour broad range pre-stained protein ladder was added on lane 1 of each prepared gel. 20 µL of the prepared samples containing 1X Pierce Lane Marker Reducing Sample Buffer were added to the subsequent lanes. The loading pattern of the samples was noted down. The gels were run at 70 V until the samples crossed the stacking gel. The voltage was then increased to 90 V and the proteins were allowed to run until the end of the gel (approximately 0.3 cm above the bottom portion of the gel). The run was then stopped.

WESTERN BLOT

The PVDF membrane was submerged in methanol for approximately 1 minute. It was then placed in a prepared ice-cold transfer buffer. The transfer tank was filled with this ice-cold transfer buffer until the 4-gel mark and the ice packs were placed onto it. The components in the Western Blot cassette were arranged in a specific order, illustrated in FIG. 2A. The

formation of air bubbles in between layers was avoided. The transfer cassette was placed in the trans-blot module.

The trans-blot was placed in the transfer tankaligning cathode and anode electrodes, illustrated in FIG. 2B. The transfer tank was covered with the lid in proper alignment with the anode and cathode electrodes. The electrodes were connected to the power pack. The voltage was set to 70 V and it was run for 120 minutes. The entire process was carried out in the cold room. The run button was pressed to start the transfer process. Tiny bubbles at the wire electrodes of the modules indicated the ongoing transfer process, it automatically stopped after 120 minutes.

Blocking and primary antibody dilution

The PVDF membrane was gently removed from the transfer cassette. The membrane was submerged in methanol for approximately 1 minute. It was washed thrice with Milli-Q water, with 1 minute/wash. Ponceau stain was then added onto the membrane and it was stained for approximately 2 minutes until the protein bands were visible. The membrane was again washed with Milli-Q water to remove excess Ponceau and the membrane was checked for proper transfer of the proteins. It was then washed with Milli-Q water for approximately 1 minute per wash, followed by washing with previously used transfer buffer for approximately 5 minutes to completely de-stain the membrane from Ponceau.

10 mL of Intercept TBS blocking buffer was added to each of the staining boxes and the membranes were placed in these boxes such that the side of the membrane that was in contact with the gel faced upwards. The boxes were placed in a platform rocker and incubated for 1 hour at RT.

After 1 hour, the blocking buffer was collected and immediately 10 mL of required primary antibodies were added to each of the membranes. It was ensured that at any point the membrane wasn't left dry. The staining boxes were incubated on a platform rocker overnight in the cold room.

Secondary antibody dilution and imaging of blots (Day 4)

The primary antibody solutions were collected and stored in a freezer at -20°C. The membranes were washed with 10 mL 1X TBST buffer (Tris-buffered saline with 0.1% Tween® 20 Detergent) 5 times, with each wash being 25 minutes. After each wash, the TBST solution was discarded, and a fresh solution was added. 10 mL of secondary antibody was then added

to the membranes, and they were incubated on the platform rocker for 2 hours at RT. The secondary antibodies were then discarded, and the membranes were again washed with 10 mL 1X TBST buffer 5 times, with 25 min/wash.

After the fifth wash, 10 mL of 1X TBST buffer was added to the staining boxes. Then the membranes were dried off with tissue paper by sandwiching them in between tissue papers and gently rolling the tissue papers. The dried membranes were used for image development of obtained infrared bands using an Odessey infrared scanner (with 800 nm for p-LRP6, p-HDAC 4/5/7, IL-18, c-Myc, and 700 nm for β-actin), illustrated in FIG. 2C and 2D.

It is evident from the above results that Compound 1 downregulates p-LRP6, p-HDAC 4/5/7, and c-Myc more significantly when compared with the known SIK2 inhibitor and DDR1 inhibitor. Additionally, IL-18 shows a significant upregulation in HCT-116 cells upon Compound 1 treatment, but the effect is not seen with the known DDR1 inhibitor.

Example – 3: Anti-fibrotic effects in Mouse Bleomycin (BLM) Induced Pulmonary Fibrosis model

Six to eight-week-old male C57BL/6 N mice (specific-pathogen-free (SPF) grade, 20 g to 25 g) were acclimatized to experimental conditions and randomly distributed into 6 groups of 10 animals.

G1: Normal control (NC)

G2: BLM disease control group (DC)

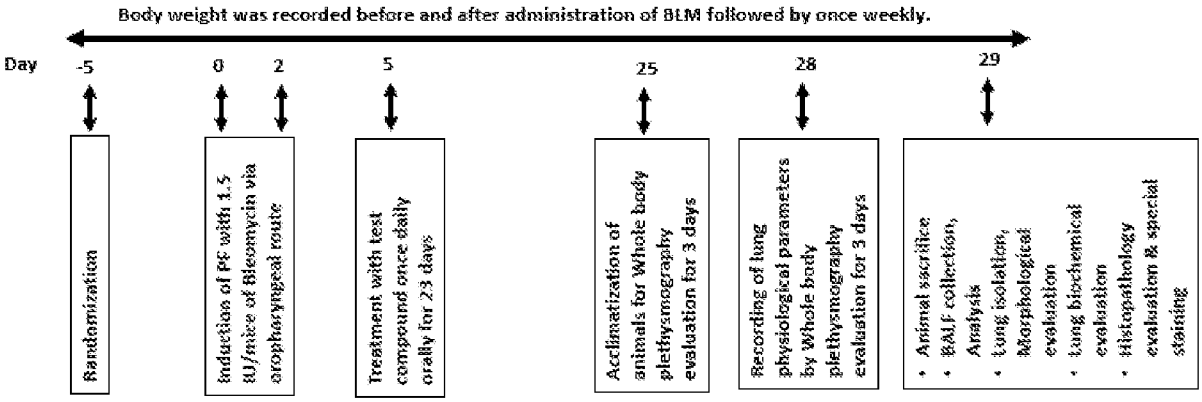
G3: BLM + Compound 1, 10 mg/kg/day

G4: BLM + Compound 1, 20 mg/kg/day

G5: BLM + Compound 1, 40 mg/kg/day

G6: BLM + pirfenidone (PF), 200 mg/kg/day (Standard drug)

Study Design



The Bleomycin (BLM) injection was used for induction of Pulmonary Fibrosis. Mice were oropharyngeally administered two doses of Bleomycin at 1.5 IU/mice (Days 0 and 2). All experiment animals were induced with BLM except the normal control group which were oropharyngeally administered with normal saline. The decrease in body weight after BLM lung installation is indicative of initiation of lung injury, and pro-inflammatory cytokines elevation due to severe inflammatory condition.

The animals were dosed with the indicated dose from Day 5 i.e. after 5 days of administration of bleomycin daily for 23 days. At the end of the study i.e. Day 28, lung physiological parameters were evaluated by whole-body plethysmography. After completion of the study, on Day 29, the animals were sacrificed and evaluated for lung morphological evaluation, lung biochemical evaluation, and histopathological evaluation.

All treatments were well tolerated without any treatment related clinical signs and/or body weight loss. Treatment with pirfenidone (PF) and Compound 1 resulted in dose dependent effect on lung function parameters, lung morphology, lung weight index**,** BALF counts (total cell, lymphocytes, neutrophils population, albumin, and total protein levels (FIG. 3A & 3B)).

The detailed observations for each of the parameters are provided below.

Effect of Compound 1 and pirfenidone on lung function parameters in bleomycin-induced fibrotic mice

a) Breath frequency

S. No.	Groups	Breath frequency (f) (bpm)
1	NC	437.96 ± 22.96
2	DC	430.47 ± 11.16

3	Compound 1 at 10 mg/kg	472.95 ± 9.61
4	Compound 1 at 20 mg/kg	465.31 ± 11.76
5	Compound 1 at 40 mg/kg	451.99 ± 9.81
6	Pirfenidone (PF) - 200 mg/kg/day	469.68 ± 13.35

b) Minute Volume

S. No.	Groups	Minute volume (ml)
1	NC	82.07 ± 6.82
2	DC	65.51 ± 7.53
3	Compound 1 at 10 mg/kg	75.92 ± 4.18
4	Compound 1 at 20 mg/kg	89.39 ±4.92
5	Compound 1 at 40 mg/kg	78.35 ± 7.25
6	Pirfenidone (PF) - 200 mg/kg/day	89.88 ± 16.18

Effect of Compound 1 and Pirfenidone on Lung Weight Index in bleomycin induced fibrotic mice

S. No.	Groups	Lung weight index
1	NC	0.66 ± 0.04
2	DC	1.41 ± 0.20
3	Compound 1 at 10 mg/kg	1.38 ± 0.44
4	Compound 1 at 20 mg/kg	0.95 ± 0.14
5	Compound 1 at 40 mg/kg	1.01 ± 0.24
6	Pirfenidone (PF) - 200 mg/kg/day	0.87 ± 0.05

Effect of Compound 1 and Pirfenidone on BALF cell count in bleomycin-induced lung fibrotic mice

a) Total cell count

S. No.	Groups	No. of cells (1 x 10^5)/ml
1	NC	0.63 ± 0.12
2	DC	3.86 ± 1.26
3	Compound 1 at 10 mg/kg	1.50 ± 0.38
4	Compound 1 at 20 mg/kg	2.24 ± 0.80
5	Compound 1 at 40 mg/kg	2.04 ± 0.63
6	PF	2.81 ± 0.61

b) Lymphocytes

S. No.	Groups	%Lymphocytes
1	NC	32.00 ± 4.16
2	DC	91.25 ± 4.09 ****
3	Compound 1 at 10 mg/kg	88.33 ± 4.41
4	Compound 1 at 20 mg/kg	82.00 ± 3.06
5	Compound 1 at 40 mg/kg	60.33 ± 3.18 ###
6	PF	60.33 ± 2.91 ###

Effect of Compound 1 and Pirfenidone on Albumin and total protein levels in bleomycin induced lung fibrotic mice

a) Albumin

S. No.	Groups	Albumin (gm%)
1	NC	0.18 ± 0.02
2	DC	0.89 ± 0.15 ****
3	Compound 1 at 10 mg/kg	0.81 ± 0.05
4	Compound 1 at 20 mg/kg	0.52 ± 0.05 #
5	Compound 1 at 40 mg/kg	0.28 ± 0.03 ###
6	PF	0.39 ± 0.06 ##

a) Protein

S. No.	Groups	Total protein (mg/ml)
1	NC	2.61 ± 0.18
2	DC	5.83 ± 0.51 ****
3	Compound 1 at 10 mg/kg	5.47 ± 0.51
4	Compound 1 at 20 mg/kg	4.66 ± 0.08
5	Compound 1 at 40 mg/kg	3.23 ± 0.16 ###
6	PF	3.94 ± 0.29 ##

Compound 1 resulted in dose-dependent effect on albumin and total protein content in mice, illustrated in **FIG. 3A** and **3B**.

Effect of Compound 1 and Pirfenidone on IL-6, TNF-α, and TGF-β in bleomycin induced lung fibrotic mice

a) TGF-β levels

S. No.	Groups	TGF-β levels (pg/mg of protein)
1	NC	100.22 ± 24.39
2	DC	256.79 ± 71.90
3	Compound 1 at 10 mg/kg	105.61 ± 42.47
4	Compound 1 at 20 mg/kg	91.29 ± 23.64#
5	Compound 1 at 40 mg/kg	147.93 ± 13.25
6	PF	55.39± 3.36#

b) IL-6 levels

S. No.	Groups	IL-6 levels (pg/mg of protein)
1	NC	214.86 ± 38.80
2	DC	394.26 ± 88.12
3	Compound 1 at 10 mg/kg	135.90 ± 42.55 ##
4	Compound 1 at 20 mg/kg	174.16 ± 24.23 #
5	Compound 1 at 40 mg/kg	148.74 ± 48.02 ##
6	PF	134.42 ± 26.36 ##

c) TNF-α levels

S. No.	Groups	TNF-α levels (pg/mg of protein)
1	NC	47.22 ± 7.53
2	DC	89.63 ± 7.96
3	Compound 1 at 10 mg/kg	39.53 ± 18.32 ##
4	Compound 1 at 20 mg/kg	31.03 ± 3.74 ###
5	Compound 1 at 40 mg/kg	33.12 ± 9.45 ##
6	PF	29.24 ± 6.74 ###

The lung tissue pro-inflammatory cytokines mainly TGF-β, IL-6, and TNF-α levels indicate antifibrotic effects, illustrated in FIG. 4A, 4B, and 4C respectively.

The H&E staining of Compound 1 treated groups and pirfenidone (standard drug) clearly demonstrated dose dependent reduction in lung histological abnormalities in BLM-induced fibrotic lung sections. There was a significant increase in the extracellular matrix deposition and decreased alveolar spaces in fibrotic lung tissues. The characteristic fibrotic features were found to be gradually reduced in a dose-dependently manner with Compound 1 treated groups and PF treated group with the maximum reduction in histological features observed with Compound 1 at 40 mg/kg/day dose group. The Ashcroft scoring of lung fibrosis also indicated a similar kind of damage in the BLM group and protective effects in Compound 1 and PF treated animals, illustrated in FIG. 5.

Histology studies

The effect of Compound 1 and pirfenidone on extracellular matrix deposition of collagen in lung tissue was evaluated by collagen specific stains (*Picrosirius red staining and Masson's Trichrome staining).*

Picrosirius red staining

The lung sections obtained from the mice after the completion of the study were stained with Picrosirius red staining (https://ihcworld.com/2024/01/26/sirius-red-staining-protocol-for-collagen/, last accessed on May 30, 2024) and the stained tissues were observed for the presence of collagen under a bright field and polarized microscope at 20X magnification, illustrated in FIG. 6A and 6B.

It is evident from Figures 6A and 6B that the Picrosirius red staining of lung sections, their colorimetric images, and corresponding Polarized microscopy images demonstrated a significant accumulation of collagen in the lung tissue of BLM-induced fibrotic mice. The mice treated with the Compound of formula (I) at various dosages of the present disclosure indicate dose-dependent decreased collagen content in the lungs and pirfenidone (PF) treated animals produced marked amelioration of collagen deposition in lung sections. Moreover, the Compound of formula (I) when administered at 40 mg/kg shows a reduced amount of fibrotic area when compared with pirfenidone (PF) treated animals. The image-based quantification of Picrosirius red stained images indicates the protective effect of the Compound of formula (I).

Masson's Trichrome staining

The lung sections obtained from the mice after the completion of the study were evaluated under Masson's Trichrome staining (https://ihcworld.com/2024/01/26/massons-

trichrome-staining-protocol-for-collagen-fibers/, last accessed on May 30, 2024) and the stained tissues were observed for the presence of collagen under a bright field and polarized microscope, illustrated in FIG. 7A and 7B.

It is evident from Figures 7A and 7B that the Masson's Trichrome staining of lung sections shows a significantly marked deposition of accumulation of collagen (blue colour) in the lung tissue of BLM-induced fibrotic mice. The mice treated with the Compound of formula (I) at various dosages of the present disclosure and pirfenidone show decreased collagen content as evident by the blue colour intensity and reduced fibrotic area. Moreover, the Compound of formula (I) when administered at 40 mg/kg shows a reduced amount of fibrotic area when compared with pirfenidone (PF) treated animals. The image-based quantification of Picrosirius red stained images indicates the protective effect of the Compound of formula (I).

Results are expressed as mean ± SEM. ****$p<0.0001$ vs NC, ###$p<0.001$ and ####$p<0.0001$ vs DC. NC-Normal control, DC- Bleomycin control, PF-Pirfenidone (200 mg/kg/day).

The analysis of various parameters across all the experimental groups as described above showed that Compound 1 at 20 mg/kg/day and 40 mg/kg/day possesses anti-fibrotic effects.

Incorporation by Reference

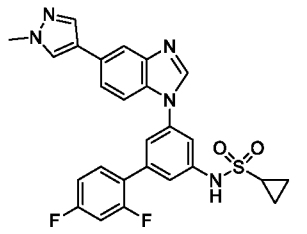
All publications and patents mentioned herein are hereby incorporated by reference in their entirety as if each individual publication or patent were specifically and individually indicated to be incorporated by reference. In case of conflict, the present application, including any definitions herein, will control.

Equivalents

While specific embodiments of the subject invention have been discussed, the above specification is illustrative and not restrictive. Many variations of the invention will become apparent to those skilled in the art upon review of this specification and the claims below. The full scope of the invention should be determined by reference to the claims, along with their full scope of equivalents and the specification, along with such variations.

CLAIMS

1. A method of treating or delaying the progression of fibrosis, in a subject comprising administering to the subject a compound represented by formula (I),



(I) ;

or a pharmaceutically acceptable salt thereof.

2. The method of claim 1, wherein the fibrosis is mediated by Wnt/Beta-catenin signaling pathway.

3. The method of claim 1, wherein the fibrosis is mediated by collagen/ECM signaling pathway.

4. The method of claim 1, wherein the fibrosis is mediated by Discoidin domain receptor 1 (DDR1).

5. The method of claim 1, wherein the fibrosis is mediated by Salt-inducible kinase 2 (SIK-2).

6. The method of claim 1, wherein the fibrosis is mediated by Discoidin domain receptor 1 (DDR1) and Salt-inducible kinase 2 (SIK-2).

7. The method of any one of claims 1-6, wherein the fibrosis is one or more selected from eye fibrosis, heart fibrosis, hepatic fibrosis, lung fibrosis, pancreas fibrosis, renal fibrosis, bone marrow fibrosis, and skin fibrosis.

8. The method of any one of claims 1-6, wherein the fibrosis is one or more selected from pulmonary fibrosis, cystic fibrosis, idiopathic pulmonary fibrosis (IPF), interstitial lung diseases (ILDs), chronic fibrosing interstitial lung diseases (ILDs) with a progressive phenotype, systemic sclerosis-associated interstitial lung disease (SSc-ILD), radiation-induced lung injury, bridging fibrosis, liver cirrhosis, non-alcoholic hepatosteatosis (NASH), non-alcoholic fatty liver disease (NAFLD), atrial fibrosis, skeletal muscle fibrosis, myocardial fibrosis, endomyocardial fibrosis, myocardial infarction related fibrosis, glial scar, ocular fibrosis, arterial stiffness, arthrofibrosis, inflammatory bowel

diseases, crohn's disease, dupuytren contracture, mediastinal fibrosis, intestinal fibrosis, myelofibrosis, Peyronie's disease, nephrogenic systemic fibrosis, progressive massive fibrosis, retroperitoneal fibrosis, scleroderma, keloids, hypertrophic scars, psoriasis, Kaposi's sarcoma, myeloid leukemia, acute myelogenous leukemia, myelodysplastic syndrome, lymphangioleiomyomatosis (LAM), chronic graft vs. host disease, polycythemia vera, essential thrombocythemia, myeloproliferative syndrome, Hansen's disease, collagenous colitis, fibrillogenesis, fibrosis of vocal cords and COVID-related pulmonary fibrosis.

9. The method of any one of claims 1-8, wherein the fibrosis is further characterized by an inflammation in the tissue.

10. The method of claim 8, wherein the pulmonary fibrosis is characterized by presence of one or more condition selected from an aberrant deposition of an extracellular matrix protein in a pulmonary interstitium, an aberrant promotion of fibroblast proliferation in the lung, an aberrant induction of myofibroblast differentiation in the lung, and an aberrant promotion of attachment of myofibroblasts to an extracellular matrix compared to a normal healthy control subject.

11. The method of claim 9, wherein the inflammation is an acute or a chronic inflammation.

12. The method of claim 9, wherein the inflammation is mediated by one or more cytokines selected from the group consisting of Tumor Necrosis Factor-alpha (TNF-a), Interleukin-6 (IL-6), and Interleukin-Iβ (IL-Iβ).

13. The method of any one of claims 1-6, wherein the fibrosis is cancer-associated fibrosis or fibrotic cancer.

14. The method of claim 13, wherein the cancer-associated fibrosis or fibrotic cancer is biliary cancer, cholangiocarcinoma, bladder cancer, colorectal cancer, colon cancer, rectal cancer, colorectal adenocarcinoma, brain cancer, meningioma, glioma, astrocytoma, oligodendroglioma, medulloblastoma, breast cancer, adenocarcinoma of the breast, inflammatory carcinoma of the breast, papillary carcinoma of the breast, medullary carcinoma of the breast, kidney cancer nephroblastoma/Wilms' tumor, renal cell carcinoma, cervical cancer, squamous cell carcinoma of the cervix, cervical adenocarcinoma, gastric cancer, esophageal cancer, gastrointestinal stromal tumor (GIST), head and neck cancer, head and neck squamous cell carcinoma, oral cancer, oral squamous cell carcinoma (OSCC), keloids, leukemia, acute lymphocytic leukemia

(ALL), acute myelogenous leukemia (AML), chronic myelogenous leukemia (CML), chronic lymphocytic leukemia (CLL), lung cancer bronchogenic carcinoma, small cell lung cancer (SCLC), non-small cell lung cancer (NSCLC), adenocarcinoma of the lung, liver cancer, hepatocellular cancer (HCC), malignant hepatoma, lymphoma, Hodgkin lymphoma (HL), non-Hodgkin lymphoma (NHL), follicular lymphoma, diffuse large B-cell lymphoma (DLBCL), mantle cell lymphoma (MCL), medulloblastoma, multiple myeloma (MM), myelodysplasia syndrome (MDS), myeloproliferative disorder (MPD), polycythemia vera (PV), essential thrombocythemia (ET), agnogenic myeloid metaplasia (AMM), primary myelofibrosis (PMF), chronic neutrophilic leukemia (CNL), hypereosinophilic syndrome (HES), neuroblastoma, neurofibroma, neurofibromatosis (NF) type 1, neurofibromatosis (NF) type 2, schwannomatosis, skin cancer, squamous cell carcinoma (SCC), keratoacanthoma (KA), melanoma, basal cell carcinoma (BCC), dermatofibroma, osteosarcoma, ovarian cancer, cystadenocarcinoma, ovarian embryonal carcinoma, ovarian adenocarcinoma, neuroendocrine cancer, gastroenteropancreatic neuroendocrine tumor (GEP-NET), carcinoid tumor, pancreatic cancer, pancreatic adenocarcinoma, intraductal papillary mucinous neoplasm (IPMN), prostate cancer, prostate adenocarcinoma, soft tissue tumors, angiolipoma, angioleiomyoma, malignant fibrous histiocytoma (MFH), liposarcoma, malignant peripheral nerve sheath tumor (MPNST), chondrosarcoma, fibrosarcoma, myosarcoma, osteosarcoma, and tumors associated with desmoplasia.

15. The method of any one of claims 1-14, wherein the compound of formula (I) is in the form of hydrochloride (HCl) salt.

16. The method of claim 15, wherein the hydrochloride (HCl) salt of the compound of formula (I) is in crystalline form.

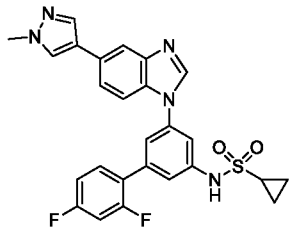
17. The method of any one of claims 1-16, wherein the method of treating or delaying the progression of fibrosis, further comprises administering an additional anti-fibrotic agent to the subject.

18. The method of claim 17, wherein the additional anti-fibrotic agent is one or more selected from an agent that blocks TGF-β signalling, an agent that inhibits activation of plasminogen activator inhibitor- 1 promoter activity, an antibody that binds to TGF-β, an antibody that binds to TGF-β receptor I, II, or III, a kinase inhibitor, an agent that blocks connective tissue growth factor (CTGF) signalling, an agent that inhibits prolyl

hydroxylase, an agent that inhibits procollagen C-proteinase, an agent that inhibits vascular endothelial growth factor (VEGF), an agent that antagonizes TGF-β, an agent that antagonizes CTGF, and corticosteroids.

19. The method of claim 17, wherein the additional anti-fibrotic agent is one or more selected from pirfenidone, nintedanib, silymarin, pentoxifylline, colchicine, etanercept, infliximab, tocilizumab, prednisone, mycophenolate mofetil, mycophenolic acid, treprostinil, belumosudil, and azathioprine.

20. A method of treating or delaying the progression of fibrosis, comprising the step of administering to a lung of a subject suffering from fibrosis a therapeutically effective amount of compound represented by formula (I) or a pharmaceutically acceptable salt thereof:



(I)

21. The method of claim 20, wherein the compound of formula (I) is in the form of a solution.

22. The method of claim 20, wherein the compound of formula (I) is in the form of suspension or nanosuspension.

23. The method of claim 20, wherein the compound of formula (I) is in the form of a liposome formulation.

24. The method of claim 20, wherein the compound of formula (I) is in the form of powder.

25. The method of claim 20, wherein the compound of formula (I) is administered through intratracheal, pulmonary, or oropharyngeal route.

26. The method of any one of claims 20-25, wherein the method further comprises an additional anti-fibrotic agent.

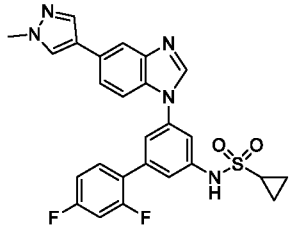
27. The method of claim 26, wherein the additional anti-fibrotic agent is one or more selected from an agent that blocks TGF-β signalling, inhibits activation of plasminogen activator inhibitor- 1 promoter activity, an antibody that binds to TGF-β, an antibody

that binds to TGF-β receptor I, II, or III, a kinase inhibitor, an agent that blocks connective tissue growth factor (CTGF) signalling, an agent that inhibits prolyl hydroxylase, an agent that inhibits procollagen C-proteinase, an agent that inhibits vascular endothelial growth factor VEGF, an agent that antagonizes TGF-β, an agent that antagonizes CTGF, and Corticosteroids.

28. The method of claim 26, wherein the additional anti-fibrotic agent is one or more selected from pirfenidone, nintedanib, silymarin, pentoxifylline, colchicine, Etanercept, infliximab, tocilizumab, prednisone, mycophenolate mofetil, mycophenolic acid, treprostinil, belumosudil and azathioprine.

29. The method of claim 26, wherein the additional anti-fibrotic agent is administered through oral, parenteral, intravenous, intraperitoneal, oropharyngeal, and pulmonary routes.

30. A compound for use in treating or delaying the progression of fibrosis, wherein the compound is represented by formula (I),



(I) ;

or a pharmaceutically acceptable salt thereof.

31. The compound, or a pharmaceutically acceptable salt thereof, for use according to claim 30, wherein the fibrosis is mediated by Wnt/Beta-catenin signaling pathway.

32. The compound, or a pharmaceutically acceptable salt thereof, for use according to claim 30, wherein the fibrosis is mediated by collagen/ECM signaling pathway.

33. The compound, or a pharmaceutically acceptable salt thereof, for use according to claim 30, wherein the fibrosis is mediated by Discoidin domain receptor 1 (DDR1).

34. The compound, or a pharmaceutically acceptable salt thereof, for use according to claim 30, wherein the fibrosis is mediated by Salt-inducible kinase 2 (SIK-2).

35. The compound, or a pharmaceutically acceptable salt thereof, for use according to claim 30, wherein the fibrosis is mediated by Discoidin domain receptor 1 (DDR1) and Salt-inducible kinase 2 (SIK-2).

36. The compound, or a pharmaceutically acceptable salt thereof, for use according to any one of claims 30-35, wherein the fibrosis is one or more selected from eye fibrosis, heart fibrosis, hepatic fibrosis, lung fibrosis, pancreas fibrosis, renal fibrosis, bone marrow fibrosis, and skin fibrosis.

37. The compound, or a pharmaceutically acceptable salt thereof, for use according to claim 30-35, wherein the fibrosis is one or more selected from pulmonary fibrosis, cystic fibrosis, idiopathic pulmonary fibrosis (IPF), interstitial lung diseases (ILDs), chronic fibrosing interstitial lung diseases (ILDs) with a progressive phenotype, systemic sclerosis-associated interstitial lung disease (SSc-ILD), radiation-induced lung injury, bridging fibrosis, liver cirrhosis, non-alcoholic hepatosteatosis (NASH), non-alcoholic fatty liver disease (NAFLD), atrial fibrosis, skeletal muscle fibrosis, myocardial fibrosis, endomyocardial fibrosis, myocardial infarction related fibrosis, glial scar, ocular fibrosis, arterial stiffness, arthrofibrosis, inflammatory bowel diseases, crohn's disease, dupuytren contracture, mediastinal fibrosis, intestinal fibrosis, myelofibrosis, Peyronie's disease, nephrogenic systemic fibrosis, progressive massive fibrosis, retroperitoneal fibrosis, scleroderma, keloids, hypertrophic scars, psoriasis, Kaposi's sarcoma, myeloid leukemia, acute myelogenous leukemia, myelodysplastic syndrome, lymphangioleiomyomatosis (LAM), chronic graft vs. host disease, polycythemia vera, essential thrombocythemia, myeloproliferative syndrome, Hansen's disease, collagenous colitis, fibrillogenesis, fibrosis of vocal cords and COVID-related pulmonary fibrosis.

38. The compound, or a pharmaceutically acceptable salt thereof, for use according to any of claims 30-37, wherein the fibrosis is further characterized by an inflammation in the tissue.

39. The compound, or a pharmaceutically acceptable salt thereof, for use according to claim 37, wherein the pulmonary fibrosis is characterized by the presence of one or more conditions selected from an aberrant deposition of an extracellular matrix protein in a pulmonary interstitium, an aberrant promotion of fibroblast proliferation in the lung, an aberrant induction of myofibroblast differentiation in the lung, and an aberrant

promotion of attachment of myofibroblasts to an extracellular matrix compared to a normal healthy control subject.

40. The compound, or a pharmaceutically acceptable salt thereof, for use according to claim 38, wherein the inflammation is an acute or a chronic inflammation.

41. The compound, or a pharmaceutically acceptable salt thereof, for use according to claim 38, wherein the inflammation is mediated by one or more cytokines selected from the group consisting of Tumor Necrosis Factor-alpha (TNF-a), Interleukin-6 (IL-6), and Interleukin-Iβ (IL-Iβ).

42. The compound, or a pharmaceutically acceptable salt thereof, for use according to any one of claims 30-35, wherein the fibrosis is cancer-associated fibrosis or fibrotic cancer.

43. The compound, or a pharmaceutically acceptable salt thereof, for use according to claim 42, wherein the cancer-associated fibrosis or fibrotic cancer is biliary cancer, cholangiocarcinoma, bladder cancer, colorectal cancer, colon cancer, rectal cancer, colorectal adenocarcinoma, brain cancer, meningioma, glioma, astrocytoma, oligodendroglioma, medulloblastoma, breast cancer, adenocarcinoma of the breast, inflammatory carcinoma of the breast, papillary carcinoma of the breast, medullary carcinoma of the breast, kidney cancer nephroblastoma/Wilms' tumor, renal cell carcinoma, cervical cancer, squamous cell carcinoma of the cervix, cervical adenocarcinoma, gastric cancer, esophageal cancer, gastrointestinal stromal tumor (GIST), head and neck cancer, head and neck squamous cell carcinoma, oral cancer, oral squamous cell carcinoma (OSCC), keloids, leukemia, acute lymphocytic leukemia (ALL), acute myelogenous leukemia (AML), chronic myelogenous leukemia (CML), chronic lymphocytic leukemia (CLL), lung cancer bronchogenic carcinoma, small cell lung cancer (SCLC), non-small cell lung cancer (NSCLC), adenocarcinoma of the lung, liver cancer, hepatocellular cancer (HCC), malignant hepatoma, lymphoma, Hodgkin lymphoma (HL), non-Hodgkin lymphoma (NHL), follicular lymphoma, diffuse large B-cell lymphoma (DLBCL), mantle cell lymphoma (MCL), medulloblastoma, multiple myeloma (MM), myelodysplasia syndrome (MDS), myeloproliferative disorder (MPD), polycythemia vera (PV), essential thrombocythemia (ET), agnogenic myeloid metaplasia (AMM), primary myelofibrosis (PMF), chronic neutrophilic leukemia (CNL), hypereosinophilic syndrome (HES), neuroblastoma, neurofibroma, neurofibromatosis (NF) type 1, neurofibromatosis (NF) type 2, schwannomatosis, skin

cancer, squamous cell carcinoma (SCC), keratoacanthoma (KA), melanoma, basal cell carcinoma (BCC), dermatofibroma, osteosarcoma, ovarian cancer, cystadenocarcinoma, ovarian embryonal carcinoma, ovarian adenocarcinoma, neuroendocrine cancer, gastroenteropancreatic neuroendocrine tumor (GEP-NET), carcinoid tumor, pancreatic cancer, pancreatic adenocarcinoma, intraductal papillary mucinous neoplasm (IPMN), prostate cancer, prostate adenocarcinoma, soft tissue tumors, angiolipoma, angioleiomyoma, malignant fibrous histiocytoma (MFH), liposarcoma, malignant peripheral nerve sheath tumor (MPNST), chondrosarcoma, fibrosarcoma, myosarcoma, osteosarcoma, and tumors associated with desmoplasia.

44. The compound, or a pharmaceutically acceptable salt thereof, for use according to any one of claims 30-43, wherein the compound of formula (I) is in the form of hydrochloride (HCl) salt.

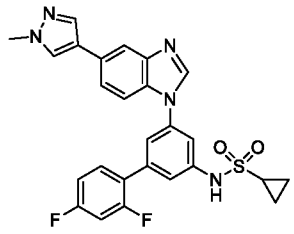
45. The compound, or a pharmaceutically acceptable salt thereof, for use according to claim 44, wherein the hydrochloride (HCl) salt of the compound of formula (I) is in crystalline form.

46. The compound, or a pharmaceutically acceptable salt thereof, for use according to any one of claims 30-45, wherein the method of treating or delaying the progression of fibrosis, further comprises administering an additional anti-fibrotic agent.

47. The compound, or a pharmaceutically acceptable salt thereof, for use according to claim 46, wherein the additional anti-fibrotic agent is one or more selected from an agent that blocks TGF-β signalling, an agent that inhibits activation of plasminogen activator inhibitor- 1 promoter activity, an antibody that binds to TGF-β, an antibody that binds to TGF-β receptor I, II, or III, a kinase inhibitor, an agent that blocks connective tissue growth factor (CTGF) signalling, an agent that inhibits prolyl hydroxylase, an agent that inhibits procollagen C-proteinase, an agent that inhibits vascular endothelial growth factor (VEGF), an agent that antagonizes TGF-β, an agent that antagonizes CTGF, and corticosteroids.

48. The compound, or a pharmaceutically acceptable salt thereof, for use according to claim 46, wherein the additional anti-fibrotic agent is one or more selected from pirfenidone, nintedanib, silymarin, pentoxifylline, colchicine, etanercept, infliximab, tocilizumab, prednisone, mycophenolate mofetil, mycophenolic acid, treprostinil, belumosudil, and azathioprine.

49. A compound for use in treating or delaying the progression of fibrosis, comprising the step of administering to a lung ~~of a subject suffering from fibrosis~~ a therapeutically effective amount of compound represented by formula (I) or a pharmaceutically acceptable salt thereof:



(I)

50. The compound, or a pharmaceutically acceptable salt thereof, for use according to claim 49, wherein the compound of formula (I) is in the form of a solution.
51. The compound, or a pharmaceutically acceptable salt thereof, for use according to claim 49, wherein the compound of formula (I) is in the form of suspension or nanosuspension.
52. The compound, or a pharmaceutically acceptable salt thereof, for use according to claim 49, wherein the compound of formula (I) is in the form of a liposome formulation.
53. The compound, or a pharmaceutically acceptable salt thereof, for use according to claim 49, wherein the compound of formula (I) is in the form of powder.
54. The compound, or a pharmaceutically acceptable salt thereof, for use according to claim 49, wherein the compound of formula (I) is administered through intratracheal, pulmonary, or oropharyngeal route.
55. The compound, or a pharmaceutically acceptable salt thereof, for use according to any one of claims 49-54, wherein the method further comprises an additional anti-fibrotic agent.
56. The compound, or a pharmaceutically acceptable salt thereof, for use according to claim 55, wherein the additional anti-fibrotic agent is one or more selected from an agent that blocks TGF-β signalling, inhibits activation of plasminogen activator inhibitor- 1 promoter activity, an antibody that binds to TGF-β, an antibody that binds to TGF-β receptor I, II, or III, a kinase inhibitor, an agent that blocks connective tissue growth factor (CTGF) signalling, an agent that inhibits prolyl hydroxylase, an agent that inhibits procollagen C-proteinase, an agent that inhibits vascular endothelial growth

factor VEGF, an agent that antagonizes TGF-β, an agent that antagonizes CTGF, and Corticosteroids.

57. The compound, or a pharmaceutically acceptable salt thereof, for use according to claim 55, wherein the additional anti-fibrotic agent is one or more selected from pirfenidone, nintedanib, silymarin, pentoxifylline, colchicine, Etanercept, infliximab, tocilizumab, prednisone, mycophenolate mofetil, mycophenolic acid, treprostinil, belumosudil and azathioprine.

58. The compound, or a pharmaceutically acceptable salt thereof, for use according to claim 55, wherein the additional anti-fibrotic agent is administered through oral, parenteral, intravenous, intraperitoneal, oropharyngeal, and pulmonary routes.

FIG. 1A

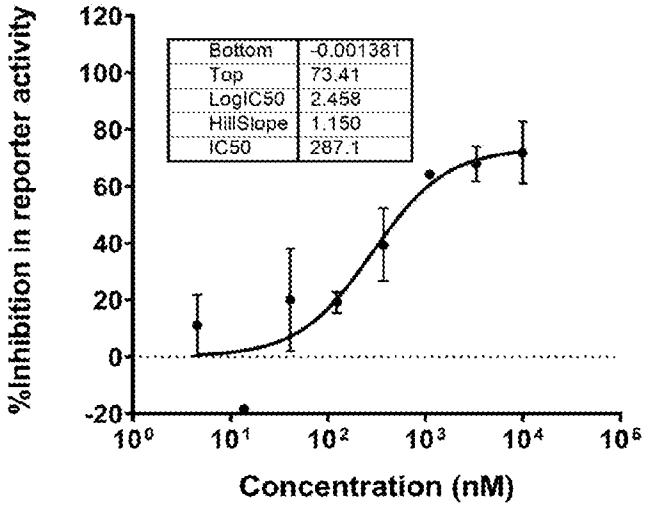

Bottom -0.001381
Top 73.41
LogIC50 2.458
HillSlope 1.150
IC50 287.1
%Inhibition in reporter activity
120
100
80
60
40
20
0
-20
10^0 10^1 10^2 10^3 10^4 10^5
Concentration (nM)

FIG. 1B

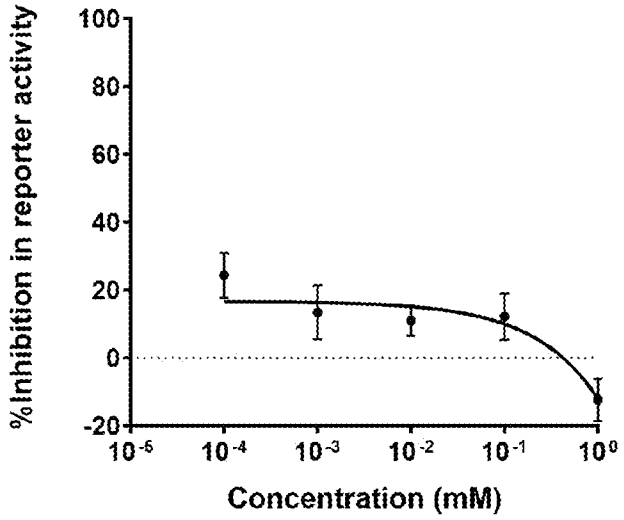

%Inhibition in reporter activity
100
80
60
40
20
0
-20
10^{-5} 10^{-4} 10^{-3} 10^{-2} 10^{-1} 10^0
Concentration (mM)

FIG. 1C

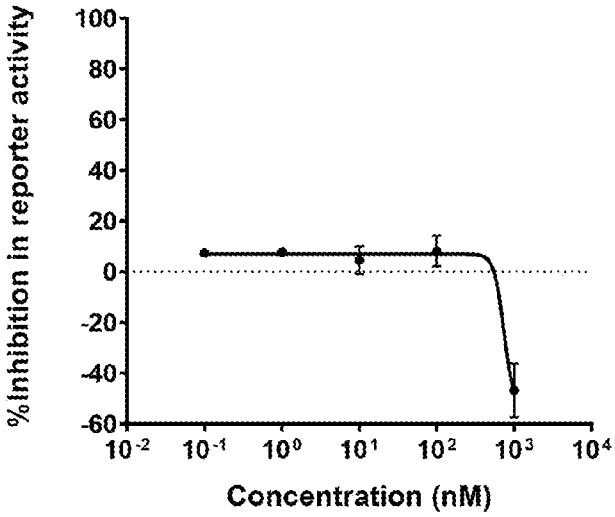

%Inhibition in reporter activity
100
80
60
40
20
0
-20
-40
-60
10-2
10-1
100
101
102
103
104
Concentration (nM)

FIG. 1D

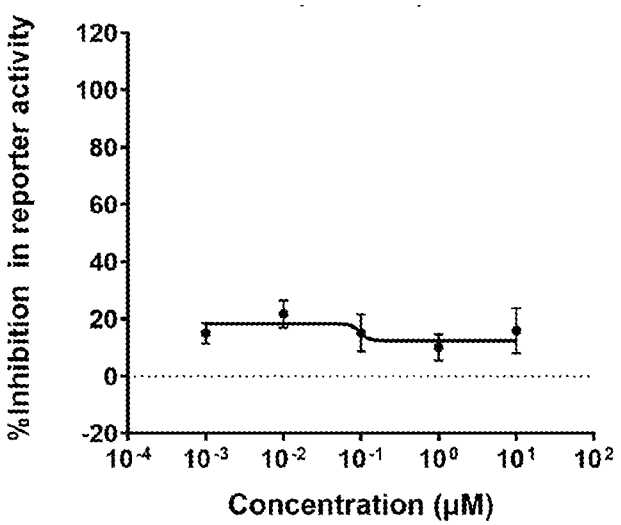

%Inhibition in reporter activity
120
100
80
60
40
20
0
-20
10-4
10-3
10-2
10-1
100
101
102
Concentration (µM)

FIG. 1E

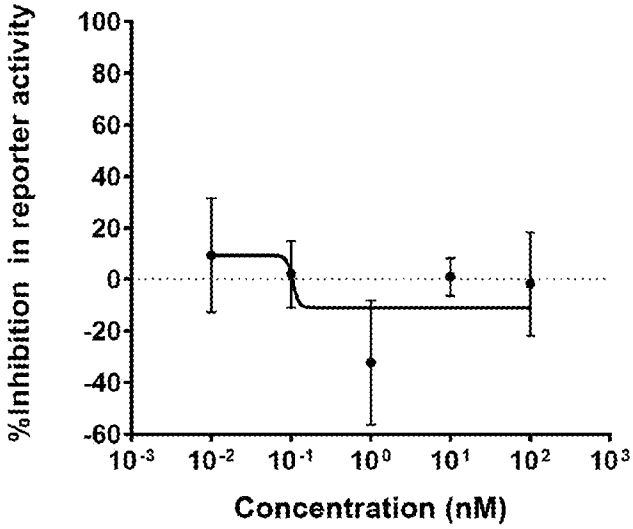

%Inhibition in reporter activity
100
80
60
40
20
0
-20
-40
-60
10-3
10-2
10-1
100
101
102
103
Concentration (nM)

FIG. 1F

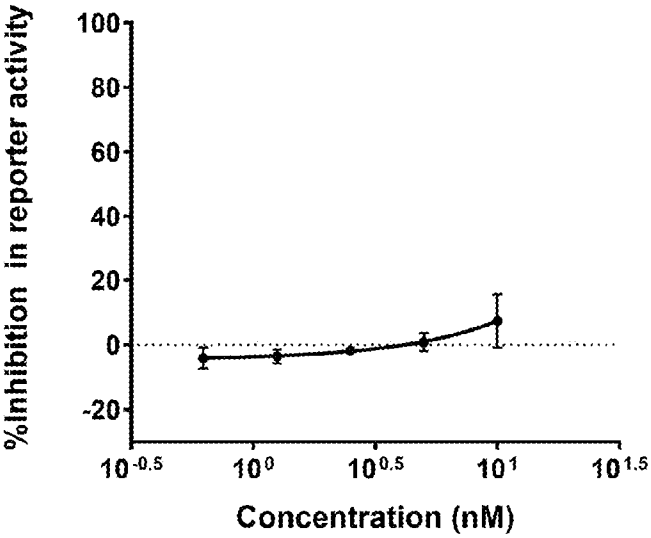

%Inhibition in reporter activity
100
80
60
40
20
0
-20
10-0.5
100
100.5
101
101.5
Concentration (nM)

FIG. 2A



FIG. 2B



FIG. 2C

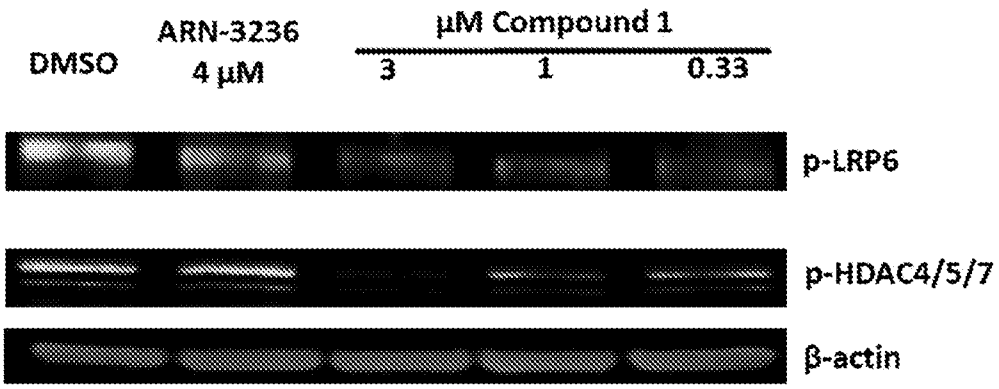


FIG. 2D

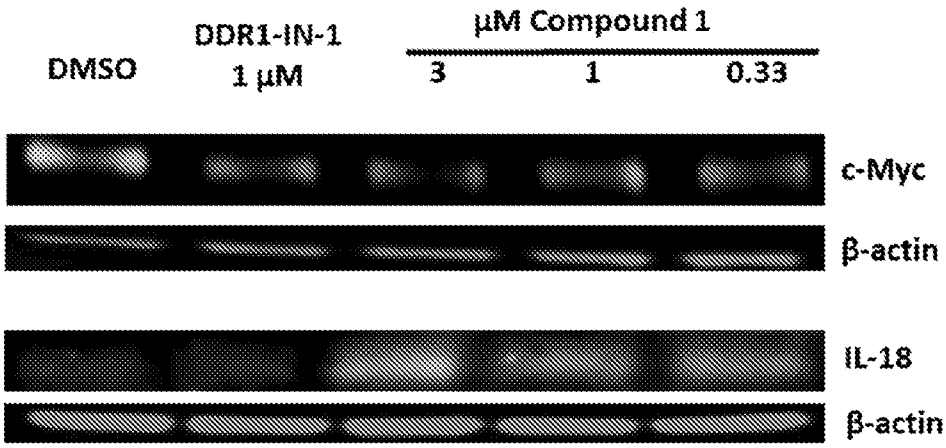

DMSO
DDR1-IN-1
1 μM
μM Compound 1
3
1
0.33
c-Myc
β-actin
IL-18
β-actin

FIG. 3A

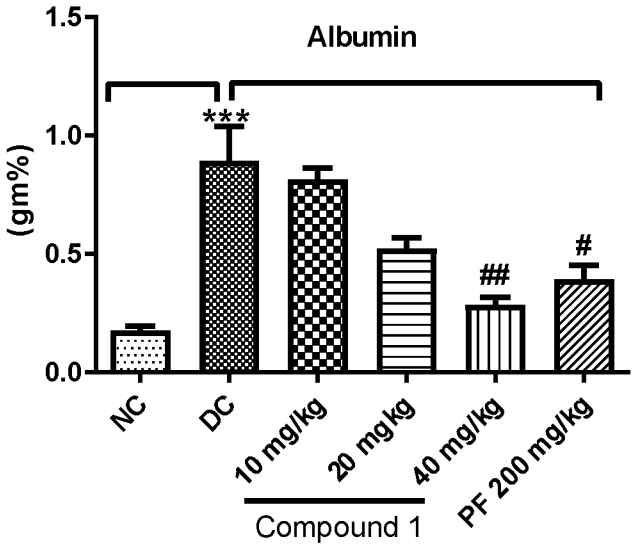

Albumin
(gm%)
1.5
1.0
0.5
0.0
***
##
#
NC
DC
10 mg/kg
20 mg/kg
40 mg/kg
PF 200 mg/kg
Compound 1

FIG. 3B

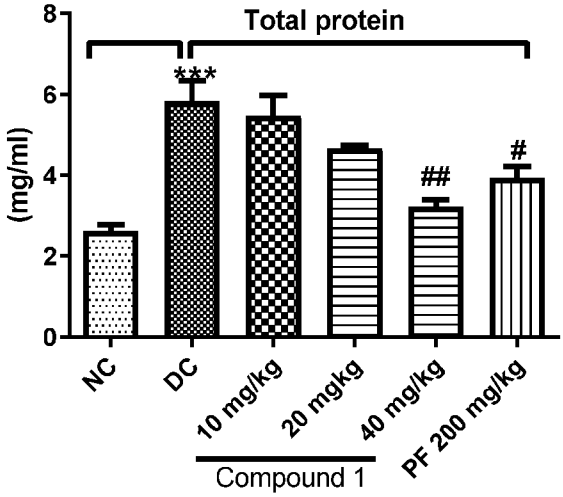


FIG 4A

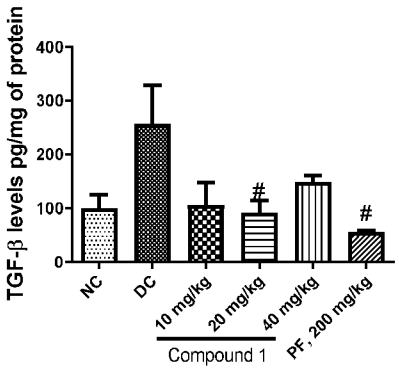


FIG 4B

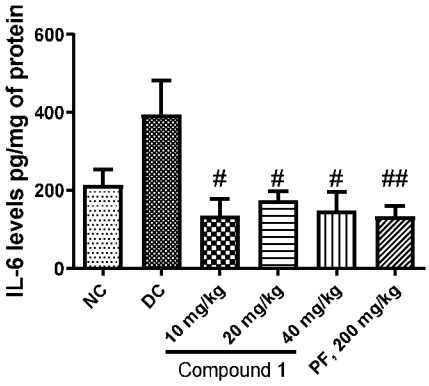


FIG 4C

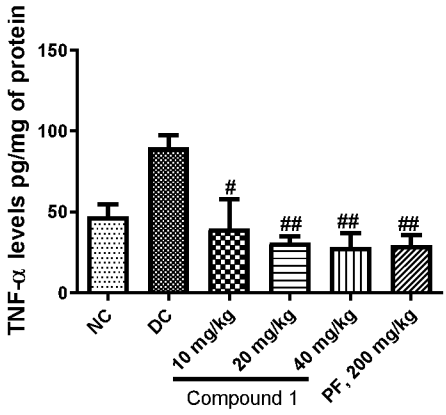


FIG. 5

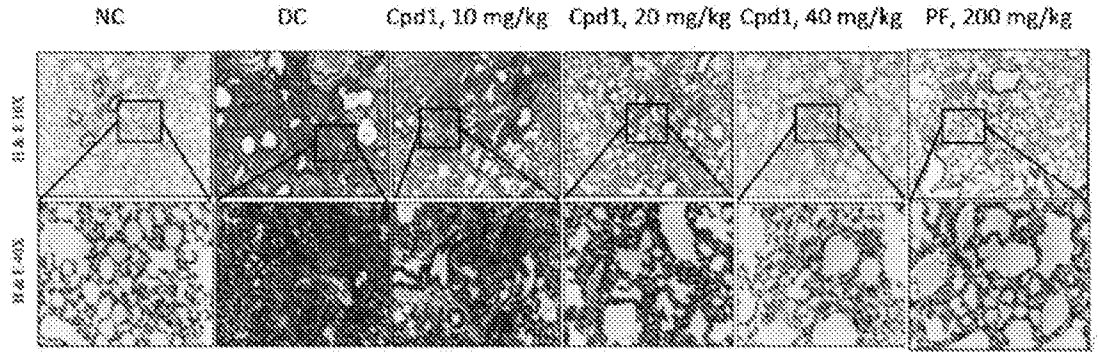


FIG. 6A

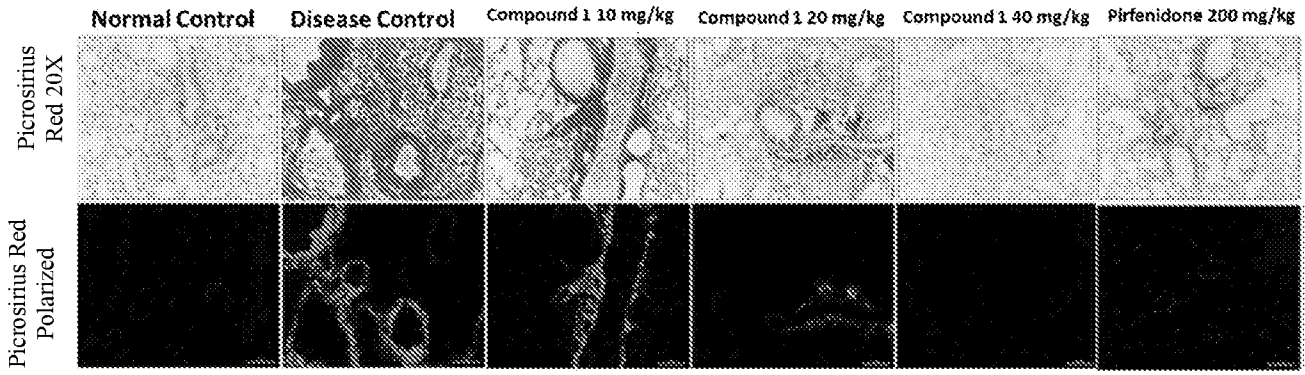


FIG. 6B

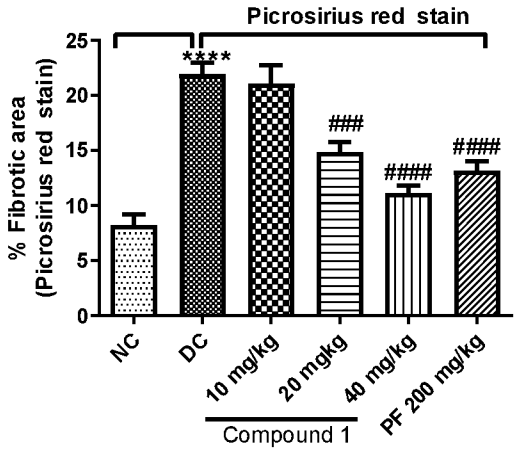


FIG. 7A

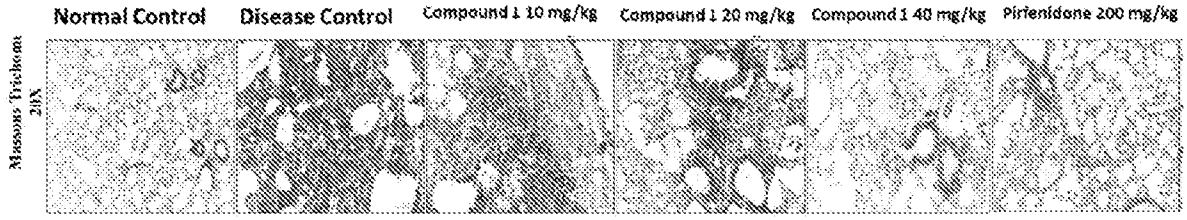
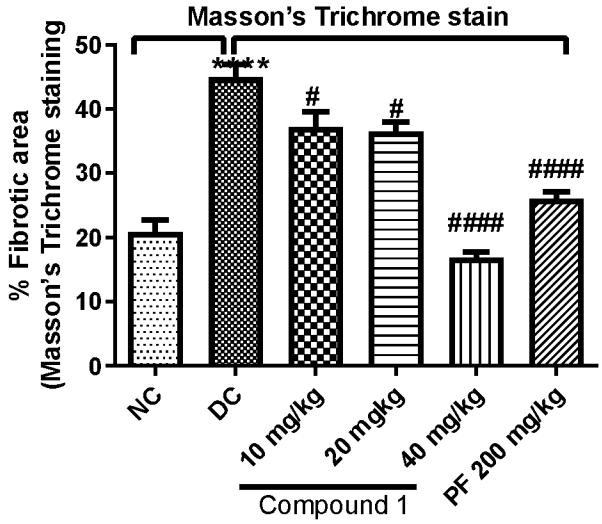

Normal Control
Disease Control
Compound 1 10 mg/kg
Compound 1 20 mg/kg
Compound 1 40 mg/kg
Pirfenidone 200 mg/kg
Masson's Trichrom 20X

FIG. 7B


Masson's Trichrome stain
% Fibrotic area (Masson's Trichrome staining
50
40
30
20
10
0
****
#
#
####
####
NC
DC
10 mg/kg
20 mgkg
40 mg/kg
PF 200 mg/kg
Compound 1