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# Serine-Threonine Kinase inhibition as antifibrotic therapy: TGF-β and ROCK inhibitors

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**Highlights.**

- **Serine-Threonine kinases are a large group of enzymes that regulate multiple intracellular processes.**
- **TGF-β Receptors and ROCK are Serine-Threonine kinases that play crucial roles in the development and maintenance of fibrosis.**
- **Novel TGF-β and ROCK inhibitors are promising potential antifibrotic therapeutic agents.**

**Abstract**

Serine-threonine kinases mediate the phosphorylation of intracellular protein targets, transferring a phosphorus group from an ATP molecule to the specific amino acid residues within the target proteins. Serine-threonine kinases regulate multiple key cellular functions. From this large group of kinases, transforming growth factor beta (TGF-β) through the serine-threonine activity of its receptors and Rho kinase (ROCK) play an important role in the development and maintenance of fibrosis in various human diseases, including systemic sclerosis. In recent years, multiple drugs targeting and inhibiting these kinases, have been developed, opening the possibility of becoming potential antifibrotic agents of clinical value for treating fibrotic diseases. This review analyzes the contribution of TGF- β and ROCK-mediated serine-threonine kinase molecular pathways to the development and maintenance of pathological fibrosis and the potential clinical use of their inhibition.

**Keywords:**

**Serine-Threonine kinase, kinase inhibitors, fibrosis, antifibrotic treatment, TGF-β, ROCK, TGF-β inhibitors, ROCK inhibitors.**

**Word count:4099**

## Introduction.

Protein kinases are key regulators in a wide array of physiological processes ranging from cellular growth and division to apoptosis (1, 2). Their effects are mediated by phosphorylation of diverse intracellular protein targets, a process that involves the transposition of a phosphorus (as a phosphorylium ion) from an ATP molecule to the specific amino acid residues within the target protein (2-4). Target phosphorylation by protein kinases is crucial in activating intracellular pathways and is precisely regulated as it can be subsequently reversed by phosphoprotein phosphatases (5, 6). A schematic representation of the mechanism of action of serine-threonine kinases is displayed in Fig 1A. Dysregulation of the normal dynamic phosphorylation-dephosphorylation equilibrium plays a role in numerous pathological conditions including inflammatory diseases, cancer, metabolic diseases, and fibrosis.

Depending on the type of amino acid targeted by the protein kinases, they have been classified as serine-threonine, tyrosine, aspartic-glutamic acid, histidine, and mixed amino acid kinases (2, 7, 8). Despite of being numerous, only few serine-threonine kinases have been explored as potential drug targets, suggesting that drug discovery targeting these enzymes may still be in its infancy opening a vast opportunity for the development of novel therapeutic interventions.

Considering the unmet need for effective antifibrotic treatments for systemic sclerosis (SSc), morphea, and other rheumatic diseases with an associated fibrotic phenotype such as interstitial lung disease associated with rheumatoid arthritis; we focus this review on TGF-β and Rho/ROCK serine-threonine kinase-mediated pathways given their relevance in the pathogenesis of the fibrotic process. TGF-β was among the earliest and is the most potent profibrotic factor discovered (9, 10). Owing to its wide cellular distribution, TGF-β plays a crucial role on the development and maintenance of pathologic fibrosis, whereas ROCK was found to be key in the organization of the

cytoskeleton (11, 12). More recently was discovered that ROCK and TGF-β receptors following their activation exert their action through serine-threonine phosphorylation. However, only in the last decade, the recent advances in the development of specific kinase inhibitors targeting these pathways have allowed effective potential therapeutic interventions. These advances have resulted in the recent approval by regulatory agencies' of nintedanib for SSc-related interstitial lung disease (SSc-ILD) and fibrotic ILD phenotype associated with other rheumatic diseases (13-15). However, given the inherent redundancy of the fibrotic molecular pathways, we are currently still far from the goal of reversing or stopping fibrosis and the development of novel antifibrotic drugs to improve clinical outcomes is largely needed.

For this review the Pubmed database was searched utilizing several combinations of the keywords "serine threonine kinases", "inhibition", "fibrosis" including studies published in English language, from 01/01/2010 to date. In a second step, the review was restricted to publications containing the terms "TGF-β" and "ROCK" and inhibitors tested on phase 1 to phase 3 clinical trials.

## TRANSFORMING GROWTH FACTOR BETA, (TGF-β) AND TGF-β RECEPTOR FAMILY

TGF-β comprises a large family of pleiotropic proteins, coded by thirty-three different genes, that regulate multiple developmental and physiological processes ranging from embryogenesis and cell differentiation to inflammation and wound repair (16, 17). The interaction of TGF-β molecules with their respective transmembrane receptors triggers the phosphorylation-mediated activation of the receptor serine-threonine kinase and the initiation of multiple intracellular pathways that involve numerous molecular mediators (18).

TGF-β1 is considered the most important among the TGF-β family of proteins, owing to its participation in numerous and crucial cell functions. Following TGF-β1 synthesis and before being secreted, an important cleavage occurs between amino acids 278 and 279, resulting in the

separation of a C-terminal product, the latency-associated peptide (LAP), and an N-terminal region, the mature TGF-β (19, 20).These domains, however, continue to be maintained together by non-covalent bonds. The LAP plays a double role, facilitating TGF-β secretion but at the same time keeping it in an inactive state. Consequently, TGF-β is secreted to the extracellular compartment as an inactive dimer (21, 22) with the LAP preventing it from direct interaction with its receptors. Once secreted the TGF-β-LAP complex binds to extracellular matrix (ECM) proteins, most prominently fibrillin (23). To become activated and able to interact with its receptors, the ECM-bound TGF-β complex undergoes a series of events, including cleavage by serine proteases such as matrix-metalloproteases (MMPs) and the tolloid-like family of proteases such as the BMP-1 (18, 22, 24). An alternative activation of TGF-β is mediated by the interaction of an integrin receptor (αvβ6 in fibroblasts) (25) which can produce mechanical stress forces causing changes in the LAP quaternary structure that allow the release of TGF-β molecules (26).

Following its release from the LAP complex, TGF-β binds to the transmembrane receptor TGF-β receptor II (TβRII) that is expressed in all human cells studied. This ligand binding recruits the co-receptor TβRI and phosphorylates a serine/threonine residue of the TGF-βRI. Following phosphorylation, the TGF-βRI forms heterodimers with TβRII and becomes functionally activated. Five types of TβRII and seven TβRI receptors have been described in different human cells types. The TβRI are known as activin receptor-like kinase (ALK) 1 to 7 (27-30).

Upon its activation, TβRI phosphorylates various members of the Smad family of intracellular proteins, specifically, Smad2 and Smad3. These are collectively termed receptor-activated Smads (R-SMADs). Following phosphorylation, R-Smads bind with the co-Smad, Smad4, forming a molecular complex that is able to translocate into the nucleus and associate with transcription factors modulating the expression of target genes (31). These pathways are schematically represented in Figure 2 and are known as the TGF-β canonical pathway. In parallel to these canonical pathways, TβRI also activates non-canonical pathways that contribute to the

fibrotic phenotype, such as JAK/STAT, ERK, MAPK, and c-ABL (Figure 2). Although these pathways are involved in numerous cellular functions and molecular pathways, including fibrotic pathways they will not be discussed further because they are associated with tyrosine kinase phosphorylation (32).

Numerous studies have shown that the activation of TGF-β canonical pathways promotes tissue myofibroblast accumulation through either the activation of quiescent tissue fibroblasts, or the phenotypic conversion of various cell types, including endothelial, adipose and epithelial cells, into myofibroblasts (33-36). During this trans-differentiation process, the original cell types loss their original receptors and acquire mesenchymal cell functions including the expression of smooth muscle actin (α-SMA) receptors, adhesion molecules such as cadherins, and other cytoskeletal related markers such as vimentin and paladin (37-39). Myofibroblasts have been recognized to be the crucial cellular elements involved in the development of tissue fibrosis in SSc and other fibrotic diseases. Myofibroblasts share biological features of fibroblasts, such as the ability to synthesize large amounts of ECM proteins, and display contractile cytoskeleton features of smooth muscle cells (40). These features allow the activated myofibroblasts not only to secrete excessive amounts of collagen and other ECM proteins, contributing to the fibrotic tissue stiffness, but also to respond to the surrounding tissue stiffness by biomechanical interaction. Myofibroblast contraction is transmitted by the cytoskeletal stress actin fibers trough the ECM via focal adhesions (41, 42). Conversely, ECM mechanical stress has been shown to promote latent TBG-β activation by releasing it from the LAP complex without the required proteolytic cleavage of active TGF-β from the LAP complex, also through myofibroblast response mediated by stretch-sensitive membrane channels; resulting in a subsequent increased expression of ECM proteins (43, 44). The assembly of stress actin fibers in myofibroblasts is mediated by Rho-ROCK pathways. The persistent activation of myofibroblasts causes a profound and prolonged profibrotic state in tissues, with subsequent target organ damage. Consequently,

TGF-β signaling is considered to be a crucial component in the development of various fibrotic diseases (45-50).

Given its ubiquity, it is not surprising that the TGF-β canonical pathways are tightly regulated at many levels. The regulation of the availability of TGF-β is dependent on the LAP and its release, whereas, the availability of TβRI (and its Smad substrates) is tightly controlled through ubiquitination. Furthermore, it has been postulated that endocytosis and lipid raft-caveolar internalization play a regulatory role on TβR availability (41, 51-53). Another level of regulation is exerted by phosphatases that revert the effects of the kinases. In this respect, the dephosphorylation by the protein phosphatase 1 catalytic subunit gamma (PP1c) inhibits TGF-β pathways. This phosphatase, forms a ternary complex with the subunit GADD34cx, the inhibitory Smad (I-Smad) SMAD7, and TβRI, preventing the activation of Smad2/3, thus serving as a negative feedback mechanism inhibiting TGF-β pathways. In addition, SMAD7 also recruits SMURF1, SMURF2, NEDD4–2, or WWP1, resulting in TβRI ubiquitination and degradation and a subsequent decrease in downstream signaling (18, 54).

**Inhibition of TGF-β as an antifibrotic strategy:**

Owing to the fact that TGF-β is a crucial regulator of myofibroblast differentiation and activation, and to its potent effects stimulating the expression of genes encoding fibrotic ECM molecules; it plays a crucial and most important role in the development and establishment of multiple fibrotic conditions including SSc (40, 55). Consequently, inhibition of TGF-β-initiated pathways provides a highly relevant target for the development of effective novel drugs to treat SSc and other diseases characterized by the excessive ECM deposition in the affected tissues. TGF-β signaling can be inhibited by several mechanisms including: 1) Reduction of TGF-β mRNA expression by antisense oligonucleotides (56, 57); 2) blockage of TGFβR serine-threonine Kinase activity by

small protein inhibitors; or 3) by monoclonal antibodies blocking TGF-β receptors or preventing TGF-β binding to its receptors through soluble decoy receptors (56). Most of these inhibitors have been tested in clinical trials for different types of cancer. Despite the lack of clinical trials for fibrotic diseases, it is important to review and summarize the experience available regarding the safety and tolerability of these potential antifibrotic interventions.

**Antisense Oligonucleotides:**

Antisense Oligonucleotides (ASOs) are small-sized single-stranded nucleotides that based on their sequence homology bind to their complementary mRNA inducing translational arrest by inhibition of ribosomal activity and interference with mRNA, resulting in marked downregulation of the expression of the genes encoding the target protein (58). The therapeutic potential of ASOs, however, has been limited because unmodified ASOs are rapidly degraded by nucleases present in biological fluids and poorly internalization through the cell membrane. To address these major problems, various chemical modifications have been developed to enhance nuclease resistance, prolong tissue half-life, increase affinity and potency and reduce non-sequence-specific toxicity (59). These modified ASOs have been tested clinically in various malignancies but have not been applied to fibrotic disorders.

***Trabedersen (AP 12009):***

Trabedersen is an ASO designed to be complementary to the human TGF-β2 mRNA, causing a marked decrease in TGF-β production. Clinically, this antisense compound has been assessed in three Phase I/II-studies for the treatment of patients with recurrent or refractory malignant glioma. Although patients tolerated the administration of Trabedersen without any major adverse event, it should be noted that this compound was administered intratumorally (60). A large phase III clinical trial has been initiated (Clinical trial identifier NCT00761280) to evaluate the intratumoral

infusion, every other week, for a maximum of 21 weeks in patients with recurrent or refractory anaplastic astrocytoma or secondary glioblastoma vs standard chemotherapy.

Another antisense nucleotide compound against TGF-β1 mRNA (AP 11014) is currently in pre-clinical development for non-small cell lung cancer (NSCLC) and prostate cancer (61, 62).

**Small molecule inhibitors:**

Small molecule inhibitors are ATP-competitive specific inhibitors of the serine/threonine kinase activity. Schematic representation of the mechanism of action of serine-threonine kinase inhibitors is displayed in Fig 1B. In the case of TGF-β, they specifically inhibit the Smad pathway by occupying the ATP binding space of the TGF-β kinase domain (ALK5 or ALK1), blocking the phosphorylation of Smad2/Smad3 proteins and the TGF-β downstream canonical activation pathways (63). Given the fact that in fibroblasts the TβRI implicated in overproduction of fibrotic tissue macromolecules are ALK5, we will review exclusively the ALK-5 inhibitors in this section. ALK-5 inhibitors have been reported to exhibit antifibrotic effects in lung fibrosis models by strongly inhibiting the differentiation of fibroblasts into myofibroblasts (64-66).

***Galunisertib (LY2157299)***

Galunisertib is a small molecule inhibitor of ALK5-TβRI that strongly inhibits Smad2 phosphorylation, downregulating TGF-β-activated Smad and non-Smad pathways in pancreatic, lung, ovarian, and colorectal cancer cells (54, 67, 68). In early clinical trials, this small molecule has displayed an acceptable safety profile, with no evidence of cardiac toxicity, a primary concern with first-generation TGF-β inhibitors (69). However, cases of thrombocytopenia and pulmonary embolism have been described in a trial that tested it in patients with glioma, being the main dose-limiting toxicity effects (70). The safety of galunisertib was confirmed by multiple studies, either as a monotherapy or in combination with chemotherapy or checkpoint inhibitors for different types of solid organ malignancies (71-73). Although there are no studies of galunisertib as an antifibrotic

drug, it is a promising antifibrotic molecule owing to its potent effects inhibiting the TGF-β canonical pathways.

***Vactosertib (EW-7197)***

Vactosertib is a novel inhibitor of ALK5-TβRI. It has been recently developed as a more potent and specific inhibitor than galunisertib. Vactosertib demonstrated strong inhibition of TGF-β1-induced Smad signaling in breast tumor cells. (74-76). It is currently being tested in early-phase clinical trials for several cancer types in combination with chemotherapy or immune checkpoints inhibitors. Very interestingly, Vactosertib is being currently studied in combination with imatinib, a tyrosine kinase inhibitor for patients with advanced desmoid tumors (Clinical trial NCT03802084). This combination would be expected to also exert a robust antifibrotic effect considering that imatinib has been shown to block non-Smad fibrotic pathways and, therefore, may potentiate the antifibrotic effects of Vactosertib caused by Smad signaling inhibition.

Drug design of novel ALK5- TβRI inhibitors is evolving and new drugs such as LY3200882 are being added to the ones with more advanced clinical development (77, 78)

**Monoclonal receptor Antibodies and Fusion Constructs:**

Neutralizing antibodies to TGF-β receptors have shown to prevent organ fibrosis in different animal models (79, 80). This strategy for blocking TGF- β has been explored very early in clinical trials, including SSc patients.

***Metelimumab (CAT-192 OK JV)***

Metelimumab is an IgG4 monoclonal antibody with specific neutralizing activity against TGFβ1. The first clinical trial of TGF-β neutralizing antibodies in SSc patients compared the administration of metelimumab intravenously at baseline and at weeks 6, 12, and 18; at three different doses versus placebo in 45 patients with early (defined as having the disease for 18 months or less) and

progressive diffuse cutaneous SSc (dc-SSc). In this trial, a change in modified Rodnan Skin Score (mRSS) at 24 weeks was the primary outcome. This trial failed to show improvement in primary and secondary outcomes. Four deaths were recorded during the study, all of them were reported in the study drug arm but none was attributed to the study drug. It has been suggested that blockage of TGF-β in very early active phases of SSc, may have cause unintended effects by blocking the anti-inflammatory effect of TGF-β (55, 81)

The most common adverse event leading to drug discontinuation was disease progression and worsening dyspnea on exertion. The limited receptor specificity and the low binding affinity of this antibody, as well as the co-existence of substantial skin T cell infiltrates without additional immunosuppression, may have negatively affected the outcome (47, 81).

***Fresolimumab (GC1008)***

Fresolimumab is a fully human monoclonal antibody directed against TGF-β1, 2 and 3. Fresolimumab demonstrated acceptable safety and preliminary evidence of antitumor activity in a phase I trial on patients with previously treated malignant melanoma or renal cell carcinoma, in a phase II trial on 13 patients with malignant pleural mesothelioma, and in a phase II trial on 23 patients with metastatic breast cancer (82-84).

In an open label trial, enrolling patients with early dc-SSc, fifteen patients received Fresolimumab in two 1 mg/kg dose at weeks 0 and 4, or one 5 mg/kg initial dose. The analysis of treated patients demonstrated rapid inhibition of TGF-β-regulated gene expression, measured as the expression of the TGF-β dependent genes THBS-1 and COMP; along with parallel and statistically significant improvement in the mRSS from week 4 to 17 in comparison to the mRSS at baseline (85). A remarkable observation was that the mRSS returned to values near baseline at week 24.

***AVID200***

AVID200 is a receptor ectodomain TGF-β trap fused with an Fc antibody domain that inhibits TGF-β1 and 3 at picomolar concentrations. (86, 87). It has been evaluated in an open label, dose-escalation Phase I clinical trial (NCT03831438) to determine its safety, pharmacokinetics, pharmacodynamics, and anti-fibrotic activity in patients with diffuse SSc. At the time of the first interim report, six patients with dc-SSc, mRSS equal or greater than 15 and less than 5 years of disease duration had been recruited. All patients showed improvement of mRSS at 6 weeks and four out of six patients continued to display an improved mRSS at 12 weeks after the last administered dose compared to baseline, and in five patients there was a decrease in the expression of pre-defined biomarkers of activated TGF-β. No serious adverse events were recorded except for mild dizziness, increased serum levels of muscle enzymes and anemia (88).

**RAS HOMOLOG (Rho) FAMILY-ASSOCIATED PROTEIN KINASE**

The Rho family of GTP-ases comprise a group of phylogenetically well-preserved small signaling G proteins that regulate multiple aspects of intracellular actin dynamics. RhoA, a member of this family that has been extensively studied, plays a crucial role in the modeling and organization of the cytoskeleton. Consequently, it participates in cell adhesion, migration, division and transformation, and other functions such as apoptosis and metabolism (89, 90). The Rho-associated protein kinase (ROCK) is an important downstream effector of RhoA with serine/threonine kinase activity (91). The description of the crystal structure of ROCK (92, 93), ignited the discovery of numerous novel scaffold ROCK inhibitors, and improved the understanding of the mechanisms of action of previously described direct and indirect ROCK inhibitors such a fasudil and statins (94-96).

There are two isoforms of ROCK; ROCK1 and ROCK2, with high overall sequence homology (97) and up to 92% of homology of sequences in their kinase domains. (97, 98). Both isoforms are

widely distributed in different tissues (90, 97, 99). Recent studies have actually shown that ROCK1 and ROCK2 have a different intracellular compartmental distribution with a high level of functional coordination, as demonstrated when driving cell polarity and synapse formation (100).

ROCK exerts its key regulatory effect on the cytoskeleton by several mechanisms: 1) activating the LIM kinase, which inactivates actin-depolymerization activity, favoring elongation, stabilization and increase density of the actin network. The increased number of stable actin filaments reduces cell migration (101); 2) ROCK activates the motor protein myosin II by phosphorylation of the myosin light chain (MLC), leading to actin-myosin stress fiber formation and resulting in the net shortening of actin fibers; and 3) through the activation of other proteins that are also important regulators of the actin-myosin interaction such as adducing, ezrin/radixin/moesin (ERM), and forming homology domain protein 1 (FHOD1), among others.

As discussed above, the differentiation of myofibroblasts and their ECM protein production are strongly stimulated by external mechanical forces not only during the normal process of scar formation but also during pathological fibrotic disorders such as SSc. Interactions between the stress actin fibers of the myofibroblast cytoskeleton and the tissue mechanical forces are translated into biochemical signals that increase the transcription of genes involved in contraction, migration, and ECM overproduction and accumulation (102). In this regard, RhoA/ROCK signaling pathways (diagrammatically illustrated in Figure 3) play an essential role by controlling myofibroblast differentiation and transduction of mechanical forces (103). Inhibition of RhoA/ROCK has been shown to halt TGF-β induced myofibroblast differentiation (104-106) through inhibition of serum response factor (SRF), a member of the MADS box-containing transcription factor family. This effect limits the availability of its co-factor MRTF that is linked to non-polymerized actin (G-actin) when the myofibroblast is not able to maintain the actin stress fibers. Blockage of this pathway halts the myofibroblast activation and differentiation caused by TGF-β, PDGF, and lysophosphatidic acid (LPA) in myofibroblasts from healthy donors and SSc

patients (107, 108). Another important pro-fibrotic pathway that is activated by Rho/ROCK actin stress fiber formation involves the Yes-associated protein (YAP) and its transcriptional co-activator TAZ. In myofibroblasts, the stiffness of their surrounding ECM can mechano-activate YAP/TAZ, promoting the excessive ECM proteins production (109). This results in a further increase in tissue stiffness, thus establishing a feed-forward loop of fibroblast activation and tissue fibrosis. The regulation of this pathway is not yet fully elucidated. (110, 111)

ROCK also has a role in the downstream signal translation of the TGF-β canonical pathway as ROCK inhibition prevents Smad2/3 phosphorylation and inhibits collagen I production (111-113). This effect has been demonstrated to halt fibrosis in fibroblasts from keloids and from affected dermis from SSc patients, as well as in various animal models of fibrosis. Furthermore, ROCK1 can directly phosphorylate JAK2, an important non-canonical TGF-β pathway (114). Another important process of myofibroblast generation, mediated by TGF-β and ROCK is the endothelial mesenchymal transdifferentiation or EndoMT(33), a process that requires the activation of transcription factors such as Snail, Slug, and Twist through TGF-β Smad and non-Smad pathways. ROCK-dependent increased free SRF (Figure 3) induce Snail expression, contributing to the EndoMT process. (33, 115)

**Inhibiting ROCK as an antifibrotic strategy:**

Following the detailed description of ROCK structure and the progressive understanding of the role of ROCK in multiple pathophysiological processes including fibrosis, numerous ROCK inhibitors have been developed. Almost all ROCK inhibitors have been designed as a mirror of their earlier counterpart, tyrosine kinase inhibitors, as competitive inhibitors of their ATP binding pocket. Initially, classic ROCK inhibitors were isoquinoline-based and derivatives, but newer compounds have been recently discovered. The differences in pharmacodynamics and pharmacokinetics of these inhibitors have been comprehensively reviewed recently (94).

A recent step in improving the therapeutic window for the ROCK inhibitors was the development of ROCK2-specific inhibitors that display improved tolerance to systemic administration and cause less hypotension. Two of these compounds were studied: SAR407899, for their use in erectile dysfunction and pulmonary hypertension (NCT00914277), but their clinical development was discontinued, and the more specific ROCK2 inhibitor, SLX-2119 (Belumosudil), described in detail below was further pursued for its antifibrotic and anti-inflammatory properties (116, 117).

***Belumosudil.***

SLX-2119 (more recently labeled as KD025 or Belumosudil) was developed as an Indazole derivative ROCK inhibitor. Belumosudil is a very specific inhibitor of ROCK2 serine/threonine kinase activity with IC50 values of 60–110 nM for ROCK2 and greater than 10 μM for ROCK1 (118).

Initially, this drug was explored for the treatment of idiopathic pulmonary fibrosis (IPF), chronic graft versus host disease (cGVHD), and moderate to severe psoriasis because it inhibits ROCK2-mediated signaling pathways, which play significant roles in pro-and anti-inflammatory pathways and in immune cell responses (119-121). A genomic study in human cells demonstrated that the drug also affects oxidative phosphorylation, WNT signaling, angiogenesis, and KRAS signaling (122).

Belumosudil arrested the progression and reverted the changes of cGVHD in multiple animal models (123). In peripheral blood mononuclear cells (PBMC) isolated from patients with active cGVHD, Belumosudil showed inhibition of the secretion of interferon gamma, IL-21, IL-17, decreased phosphorylated STAT3, and reduced the protein expression of interferon regulatory factor 4 (IRF4) and B-cell lymphoma 6 (BCL6) (123).

A long-term follow-up of a phase 2 study that enrolled three sequential cohorts of patients with active cGVHD despite prior therapy totaling 54 patients, receiving 200 mg QD, 200 mg BID, and

400 mg QD of Belomosudil were followed for 33-39 weeks. The primary endpoint was the overall response rate (ORR). A remarkable 65% (51%, 77%) reduction of ORR of was achieved across the cohorts and improvement was observed in multiple organs. Remarkably, the responses were rapid and were achieved within the first 8 weeks in the majority of patients. The most common adverse events were upper respiratory infections, diarrhea, nausea, fatigue, dyspnea, and peripheral edema (120).

Belumosudil is also being tested in an ongoing trial in IPF patients (NCT02688647), expected to include about 36 IPF patients previously treated with nintedanib or pirfenidone that will be randomized to receive 400 mg QD or standard-of-care treatment. Interim data collected after 24 weeks, included 20 patients on Belumosudil and 9 patients receiving standard of care, showed that the Belumosudil arm prevented the median decline of total forced vital capacity (FVC) by 73%, and the median decline in FVC predicted compared to standard of care by 50%. A current phase II trial on SSc patients taking either belumosudil or placebo for 24 weeks followed by 24 weeks of open-label for both arms is currently underway.

The rapid and progressive discovery of novel serine-threonine inhibitors of crucial pro-fibrotic kinases, is opening a new potential therapeutic approach for rheumatic diseases with predominant fibrotic component such as SSc, morphea, eosinophilic fasciitis, or other diseases with associated tissue fibrosis such as interstitial lung disease associated to rheumatoid arthritis, antisynthesase syndrome, among others. It should be emphasized, however that Phase II and III trials will be needed to corroborate the utility and safety of this approach, but the recent approval by regulatory agencies of tyrosine kinase inhibitors such as nintedanib demonstrate that specific kinase inhibitors can have a therapeutic role in the treatment of fibrotic diseases with a reasonable safety profile. Inhibiting serine-threonine specific pathways allows a more specific inhibition of the fibrotic process than inhibiting upstream cytokines without the associated immune suppression,

thus rendering them potentially suitable for combination therapy with antimetabolites or cytokine inhibitors.

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**Figure 1:**
**Serine-Threonine kinases mechanism of action**
Serine-threonine Kinases (STK) transfer a phosphate group from an ATP molecule to a serine or threonine residue of their specific substrates. Serine-threonine inhibitors block the ATP cleft, abrogating the phosphorylation process.

**Figure 2:**
**TGF-β Fibrosis Pathways**
After its synthesis, TGF-β is released to the extracellular space in an inactive state linked to the LAP protein. Through interactions with fibrillin or integrin receptors, TGF-β is released from LAP and binds to its cell membrane receptor. This binding causes activation of Canonical (SMAD) and non-canonical (non-SMAD) pathways. **Canonical pathway**: the binding of TGF-β the formation of TGFβR heterodimers that phosphorylate a serine-threonine residue in SMAD2 and SMAD3 (r-SMADs). This reaction is inhibited by SMAD7. r-SMADs require to interact with SMAD4 to be translocated inside the nucleus. **Non-canonical pathway:** On the other hand, TGFβR also phosphorylate tyrosine residues activating ERK/MEK, MKK/JNK/P38, JAK/STAT and Src/c-Abl ( non-SMAD pathways).The overall effect of these intracellular pathways cause activation of myofibroblasts and increased production of extracellular matrix proteins

**Abbreviations:** TGF-β: Transforming growth Factor beta, LAP: Latency associated peptide, ERK: Extracellular regulated kinase, MEK: Mitogen-activated protein kinase, MKK: Mitogen-activated protein kinase, JNK: c-Jun N-terminal kinase, JAK: Janus kinase, c-abl: c-Abelson tyrosine kinase.

**Figure 3:**

**Rho-ROCK kinase intracellular fibrotic pathways:** G protein-Coupled Receptors (Ang II, ET-1, PDGF-BB, among others) activate ROCK through Rho activation. This pathway is inhibited by Rac. ROCK exerts its pro-fibrotic activity through multiple mediators: Activation of Adducin and ERM favors the elongation and polymerization of actin (F-Actin). This process increases the availability of free MRTF that is coupled with the non-polymeric actin (G-Actin). MRTF interacts with its co-factor, Serum Response Factor (SRF) to activate genes decreasing myofibroblast apoptosis and promoting myofibroblast differentiation and proliferation. ROCK also activates LIM Kinases, promoting stabilization of F-Actin by inhibition of its de-polymerization. Furthermore, ROCK stimulates the Myosin Light Chain kinase promoting Actin-Myosin mediated cell contraction. This contraction generates mechanical interaction between cell surface integrins and the extracellular matrix, causing release of tissue TGF-β from the LAP. In addition, ROCK also have a direct role on the activation of SMAD and JAK 2.

**Abbreviations:** Ang II: Angiotensin II, PDGF: Platelet Derived Growth Factor, ET-1:Endothelin 1, ROCK : Rho Kinase, ERM: Ezrin/radixin/moesin, MRTF: Myocardin Related Transcription Factor, SRF: Serum Response Factor TGF-β:Transforming growth Factor beta, LAP: Latency associated peptide, JAK: Janus Kinase.