Anti-fibrotic effects of soluble guanylate cyclase stimulators and activators: A review of the preclinical evidence

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ABSTRACT

It is now well established that the NO-sGC-cGMP signal transduction system mediates many different physiological functions in almost every conceivable organ system; this has been best characterized in the cardiovascular system where NO-driven cGMP production exerts a plethora of cytoprotective and anti-atherogenic effects, including dilatation, inhibition of vascular smooth muscle proliferation, blockade of leukocyte recruitment, and anti-platelet activity. Accordingly, dysfunctional NO-sGC-cGMP mediated signaling is perceived as the underlying pathophysiological cause of many cardiovascular and non-cardiovascular diseases. Due to the fundamental role of sGC in the signaling pathways triggered by NO, novel sGC modulators have been identified that directly stimulate both heme-containing as well as heme-free sGC, the so-called sGC activators and sGC stimulators, respectively. The beneficial effects of this new family of sGC modulators extend beyond vasodilation, and their potential in other cardio-vascular diseases aside from pulmonary arterial hypertension is promising. In animal models of hypertension and heart failure, reno-protective effects, attenuated cardiac fibrosis, and attenuated hypertrophy independent of hemodynamic effects have been shown. During recent years it has become obvious that cGMP increase by sGC modulators exerts direct anti-fibrotic efficacy in various organs as well as the skin. This review will provide an overview of the preclinical in vitro and in vivo studies for different fibrotic disorders including chronic renal, cardiac, liver, and lung fibrosis, as well as sclerosis and wound healing. Moreover, this review provides evidence for a new mode of action of sGC modulators and its implication for clinical investigations in the treatment of fibrotic disorders such as pulmonary fibrosis and skin fibrosis.

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1. Signal transduction and pharmacotherapy

1.1. The NO-sGC-cGMP pathway

The nitric oxide (NO)-soluble guanylate cyclase (sGC)-cyclic guanosine monophosphate (cGMP) pathway, an important therapeutic target for cardiopulmonary disease [1], was first elucidated in 1980 [2]. This pathway is also an important target for cardiopulmonary disease [2]. Key to understanding this pathway was the discovery of NO as the "endothelium-derived relaxing factor" that is released from the endothelium, which increases levels of cGMP through the activation of sGC and induces vascular smooth muscle relaxation [3–6]. The implications for this pivotal research in cardiovascular signaling led to Furchgott, Ignarro, and Murad receiving the Nobel Prize for Physiology or Medicine in 1998.

Binding of NO to its receptor, sGC, stimulates the synthesis of cGMP from guanosine triphosphate, which substantially regulates vascular tone. In addition, effects on proliferation, fibrosis, and inflammation in the cardiopulmonary system and beyond are described [5,7–9]. The effects of cGMP are mediated by several downstream targets, most importantly the cGMP regulated protein kinase (PKG), which phosphorylates key components of different downstream pathways and mediates the cGMP signal [10,11]. cGMP-regulated phosphodiesterases (PDEs) and cGMP-regulated ion-channels (CNGCs) also contribute to these effects [12,13]. Thus, PKG, PDEs, and CNGCs translate the cGMP signal at the molecular level on different intracellular targets and ultimately trigger
1.2. Organic nitrates

Organic nitrates serve as NO donors to activate the NO-sGC-cGMP pathway by bioconversion. These NO donors have been used as vasodilators to treat vascular dysfunction [1]. NO is synthesized by conversion of L-arginine via the enzyme nitric oxide synthase (NOS) (Fig. 1). This process can be inhibited using asymmetric dimethyl-arginine (ADMA) and NG-methyl-L-arginine (L-NMMA) [15]. Disruption of NO synthesis, which leads to interruption of NO-sGC-cGMP signaling, has been shown to be largely responsible for vasoconstriction of coronary vessels. This results in reduced conduit vessel diameter and coronary blood flow as well as an increase in coronary vascular resistance [16]. This is clinically associated with angina pectoris and pulmonary hypertension (PH), which is characterized by endothelial dysfunction, altered smooth muscle cell growth, and impaired production of vasoactive mediators) [1,17–21]. Organic nitrates such as nitroglycerin were the earliest pharmacotherapies to target NO-sGC-cGMP signaling and have been used for over a century to treat cardiovascular disease [1,7,22,23].

1.3. PDE5 inhibitors

Phosphodiesterase-5 (PDE5) inhibitors entered into clinical practice for treatment of erectile dysfunction in 1999 and for the treatment of PAH in 2005 [2]. PDE5 inhibitors block the downstream degradation of cGMP to GMP, increasing intracellular cGMP levels. However, the efficacy of PDE5 inhibitors is dependent on the presence of sufficient amounts of endogenous NO to activate sGC and generate cGMP [2]. Thus, low endogenous NO/cGMP production significantly limits or impairs the effects of PDE5 inhibitors.

1.4. sGC modulators

The more recently developed sGC modulators, which stimulate and activate sGC in an NO-independent manner, have been shown to have broad treatment potential. The sGC modulators as a class comprise sGC stimulators and sGC activators. The sGC stimulators bind to the heme-containing sGC and act heme-dependently, while the sGC activators preferentially bind to oxidized sGC and act heme-independently [7,24,25]. The sGC stimulators, such as BAY 41-2272, BAY 41-8543, BAY 60-4552, riociguat (BAY 63-2521), or vericiguat (BAY 102-1189) have a dual mode of action: they act in synergy with NO to restore the NO-sGC-cGMP pathway by sensitizing sGC in low-NO environments and directly stimulate sGC to synthesize cGMP independent of NO [26–29]. Both mechanisms of sGC stimulation lead to substantial elevation of cGMP in low-endogenous NO and low-cGMP environments.

Riociguat (BAY 63-2521) is the first sGC stimulator that has made a successful transition from animal experiments to controlled clinical studies in human patients with PH. In 2013, riociguat (Adempas®) was approved by the Food and Drug Administration and European Medicines Agency for the treatment of these two forms of PH: inoperable recurrent or persistent chronic thromboembolic pulmonary hypertension and pulmonary artery hypertension (PAH) [28]. Because cGMP elevation has been associated with anti-fibrotic, anti-proliferative, and anti-inflammatory effects, sGC modulators may possess treatment potential beyond vaso-relaxation in fibrotic disorders. This review will describe the anti-
fibrotic effects of cGMP elevation that have been demonstrated in both in vitro and in vivo preclinical studies; some of the known hallmarks of fibrosis investigated in these studies are listed in Table 1.

2. Molecular mechanisms of fibrosis

There are numerous etiologies of internal organ and tissue fibrosis—tobacco and pollutants may induce pulmonary fibrosis; viral hepatitis and/or ethanol abuse may precipitate liver fibrosis; metabolic syndrome, oxidative stress, pressure overload, acute myocardial or renal infarction may result in cardiac and/or renal fibrosis; and autoimmune disorders contribute to fibrotic disorders such as systemic sclerosis. However, despite differing initiating stimuli, the etiopathogenesis of organ fibrosis is quite homogenous, with common molecular pathways underlying fibrotic diseases.

Generally, fibrosis can be initiated by injury to any one of numerous and diverse forms of tissue, which universally result in inflammation characterized by infiltration and activation of pro-inflammatory mediators including macrophages, platelets, leukocytes, neutrophils and T-cells to areas of injury [30,31]. In vivo experimental data in rat pulmonary injury models from the Maurer research group in 1991 showed that exposure to dust particles results in recruitment of alveolar macrophages. These macrophages release pro-inflammatory cytokines including interleukin-1 (IL-1) and tumor necrosis factor (TNF), both important for the recruitment of other pro-inflammatory cells. The macrophages also secrete fibronectin which serves as both a chemoattractant and stimulator of fibroblast proliferation. Chronic dust exposure resulted in fibrosis, characterized by collagen deposition [32]. This phenomenon has also been confirmed using human lung epithelial cells; exposure to dust resulted in release of IL-1β, basic fibroblast growth factor (bFGF), and high mobility group box 1, as well as fibroblast proliferation [33]. IL-1 and TNF-mediated fibrosis has been reported in skin and other organs as well [34]. Administration of TNF or IL-1 in animal models results in accumulation and infiltration of neutrophils, macrophages and lymphocytes as well as pulmonary fibrosis accompanied by transforming growth factor (TGF)-β induction and myofibroblast formation characterized by α-smooth muscle actin (α-SMA) [35]. Induction of TGF-β results in the activation of the SMAD3 signaling pathway and myofibroblast formation [36]. In pulmonary fibrosis, exposure to TGF-β facilitates epithelial-mesenchymal transition thereby serving as a possible source of myofibroblasts [37]. Genetic depletion of SMAD3 inhibits the accumulation of fibroblasts, formation of myofibroblasts, deposition of collagen, and fibrosis [38]. TGF-β has been shown to directly induce transcription of α-SMA, a key component of the differentiation of fibroblasts into myofibroblasts [39]. Myofibroblasts further secrete TGF-β which creates a feed-forward loop thereby increasing inflammatory-mediated production of myofibroblasts. The proliferation of myofibroblasts and extracellular matrix (ECM) accumulation is accompanied by signaling through integrins and other cellular receptors that lead to pathological changes in tissue architecture and organ dysfunction (Fig. 2) [30,31]. The exact mechanism of TGF-β-mediated activation of fibrosis and the anti-fibrotic role of sGC-cGMP pathway in treating this disease is still not well understood. However, it was shown that the NO-sGC-cGMP pathway plays an important role in fibroblast-to-myofibroblast differentiation. The treatment of fibroblasts with TGF-β reduced NOS activity and therefore NO levels, whereas NO donors inhibited myofibroblast differentiation [40,41]. It was also recently demonstrated that myofibroblast-to-fibroblast redifferentiation is enhanced by increases in cGMP, which could also contribute to the antifibrotic effects of cGMP [42].

Nevertheless, future additional studies on the cellular and molecular level are needed to fully characterize how the anti-fibrotic effects of cGMP are mediated further downstream. However, there is robust evidence supporting an anti-fibrotic mode of action, which will be reviewed below.

2.1. Anti-fibrotic mechanisms of cGMP

There are several common mechanisms by which cGMP elevation can elicit anti-fibrotic effects (Fig. 3). These mechanisms have been investigated in various in vitro models of fibrotic diseases using either primary human fibroblasts or stable cell lines. One

Table 1

<table>
<thead>
<tr>
<th>Description</th>
<th>In vitro</th>
<th>In vivo models</th>
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<tbody>
<tr>
<td>ECM accumulation (collagen, fibronectin)</td>
<td>Changes in TGF-β expression (increase in TGF-β1; decrease in TGF-β3)</td>
<td>Liver</td>
</tr>
<tr>
<td></td>
<td>Fibroblast-to-myofibroblast differentiation markers (α-SMA, CTGF,</td>
<td>Kidney: perivascular and interstitial fibrosis, glomerulosclerosis</td>
</tr>
<tr>
<td></td>
<td>calponin-1, IGFBP3)</td>
<td>Heart: perivascular and interstitial fibrosis, cardiomyocyte hypertrophy</td>
</tr>
<tr>
<td></td>
<td>TGF, transforming growth factor; TIMP-1</td>
<td>Lung: dermal thickening</td>
</tr>
</tbody>
</table>

Abbreviations: CTGF, connective tissue growth factor; ECM, extracellular matrix; IGFBP3, insulin-like growth factor binding protein 3; PAI-1, plasminogen activator inhibitor-1; α-SMA, α-smooth muscle actin; TGF, transforming growth factor; TIMP-1, tissue inhibitor of metalloprotease-1.
common anti-fibrotic mechanism of cGMP elevation is the inhibition of the ECM production. The addition of 8-bromo-cGMP (300 μmol/L) significantly decreased both basal and TGF-β-stimulated collagen production as measured by [3H]proline incorporation in cultured human dermal fibroblasts from burn patients (either normal fibroblasts or derived from hypertrophic scar) [40].

In addition, dermal fibroblasts from healthy individuals and patients with systemic scleroderma (SSc) were used to demonstrate significant reductions in TGF-β1-induced collagen production (measured by the Sircol collagen assay) and mRNA expression for procollagens col1a1 and col1a2 with the sGC stimulator BAY 41-2272 (1 μmol/L or 10 μmol/L) [43,44]. Like BAY 41-2272, 8-bromo-cGMP also inhibited TGF-β1-induced increases in collagen mRNA and protein in human dermal fibroblasts [44].

Similar results have been observed in lung and renal fibroblasts. The sGC stimulator BAY 41-2272 caused significant, dose-dependent reductions in collagen I and fibronectin in TGF-β-stimulated human lung fibroblasts [45]. Exposure to the cGMP analog 8-bromo-cGMP (1 μmol/L) caused a significant decrease in basal collagen synthesis as measured by hydroxyproline incorporation in rat renal interstitial fibroblasts [46]. As such, the sGC modulators as a class may hold potential to inhibit fibrosis by attenuating collagen production via suppression of TGF-β stimulation.

Another mechanism by which cGMP elevation inhibits fibrosis is through the inhibition of fibroblast-to-myofibroblast differentiation. This differentiation is a key step in the fibrogenic process, leading to expression of ECM protein. In a study in human dermal fibroblasts, BAY 41-2272 was shown to both inhibit and reverse TGF-β1-induced fibroblast-to-myofibroblast differentiation. Whether it was co-incubated with TGF-β1 or added to TGF-β1-pretreated cells, BAY 41-2272 reduced the TGF-β1-induced increases in mRNA and protein for the fibroblast-to-myofibroblast differentiation markers α-SMA, calponin-1, and insulin-like growth factor binding protein 3 [44].

Sildenafil was also shown to elevate cGMP levels and have similar anti-fibrotic effects in a study in dermal fibroblasts from SSc patients [47]. TGF-β1 also induced an increase in the protein expression of the fibroblast-to-myofibroblast differentiation markers α-SMA and connective tissue growth factor in normal human lung fibroblasts and human lung fibroblast cell lines [48]. While treatment with the PDE5 inhibitor sildenafil did not block either basal or TGF-β1-induced α-SMA protein expression in human lung fibroblast cell lines or normal human fibroblasts, the combination of sildenafil plus the sGC stimulator BAY 41-2272 prevented the TGF-β1-induced increase in α-SMA protein expression [48]. Similar results were seen with sildenafil combined with the sGC activator cinciguat plus 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one, which potentiates the effects of cinciguat [46]. In summary, these data suggest that elevation of cGMP by sGC modulators or PDE5 inhibitors could block TGF-β-induced fibroblast-to-myofibroblast differentiation in dermal and lung fibroblasts. This inhibition may further the anti-fibrotic effects of cGMP elevation, as myofibroblast-driven ECM production would subsequently be limited.

More recent findings showed significant, dose-dependent reductions in α-SMA in TGF-β-stimulated human lung fibroblasts when treated with BAY 41-2272. In addition, BAY 41-2272 abolished the TGF-β1-induced increase in proliferation [45]. In neonatal rat cardiac fibroblasts, 8-bromo-cGMP was shown to significantly reduce Ang II-stimulated protein synthesis, indicating inhibition of cell growth [49]. The sGC activator cinciguat (BAY 58-2667) and the sGC stimulator BAY 41-2272 had anti-hypertrophic effects in endothelin-1-stimulated neonatal rat cardiomyocytes, with significant reductions in cell size and protein synthesis at doses of 0.01–0.3 μmol/L [50]. Both compounds also inhibited neonatal rat cardiac fibroblast proliferation, but only at higher concentrations (10 μmol/L) [50].

Finally, exposure to the cGMP analog 8-bromo-cGMP (1 mmol/L) caused significant decreases in serum-stimulated mitogenesis and basal cell growth in rat renal interstitial fibroblasts [46]. In addition to blocking TGF-β-driven collagen production and fibroblast-to-myofibroblast differentiation, sGC modulators may also induce anti-fibrotic effects by inhibiting cellular proliferation.

TGF-β has been shown to inhibit induction of iNOS via the SMAD signaling pathway and regulate NO production in vascular and pulmonary smooth muscle cells [51,52]. Vyas-Read et al. have explored the interplay between the anti-fibrotic effects of NO and the pro-fibrotic effects of TGF-β. Inhibition of endogenous NOS resulted in spontaneous epithelial-mesenchymal transition, α-SMA expression, and fibroblast-like morphology. Exogenous NO attenuated TGF-β-mediated epithelial-mesenchymal transition of pulmonary fibrosis [53]. The NO and the NO/cGMP pathway inhibit TGF-β/SMAD signaling by promoting proteasome degradation of SMAD2 [54].

3. sGC modulators in organ fibrosis

3.1. Renal and cardiac fibrosis

Two of the characteristic fibrotic changes associated with glomerulonephritis are mesangial cell proliferation and ECM accumulation [55]. In a rat model of anti-Thy1-induced glomerulonephritis, treatment with the sGC stimulator BAY 41-2272 caused a 4-fold increase in glomerular cGMP levels on day 6 and significantly reduced total glomerular proliferation, proliferation in the mesangial cell component of the glomerulus, and glomerular ECM accumulation as measured by immunostaining for collagen IV and for fibronectin compared with placebo [55]. Proteinuria, a marker for renal disease, was also significantly lower with BAY 41-2272 compared with placebo (on day 2 and day 6) [55]. Additionally, aortic remodeling was ameliorated in a rat model of mild uremia when treated with BA 41-8543; similar results were not seen with hydralazine [56]. These results demonstrate the anti-fibrotic effects
of cGMP elevation in an experimental model of inflammation-driven, acute renal fibrosis and result in a functional renal improvement.

BAY 41-2272 was also evaluated in a rat model of anti-Thy1-induced chronic progressive glomerulosclerosis, which develops several months after anti-Thy1 injection [57]. In this model, BAY 41-2272 treatment significantly reduced anti-Thy1-induced ECM accumulation; mRNA and protein expression of TGF-β1, fibronectin, and tissue inhibitor of metalloproteinase-1 (TIMP-1) in tubulointerstitial tissue; and protein expression of fibronectin in glomerular tissue [57]. BAY 41-2272 also significantly reduced macrophage infiltration, tubulointerstitial and glomerular cell proliferation, and cortical P-selectin expression [57]. Consistent with the effects on the ECM, renal function in the disease model, as assessed by plasma creatinine, plasma urea, creatinine clearance, and blood hematocrit, was preserved with BAY 41-2272 [57]. Besides the sGC stimulator BAY 41-2272, the sGC activator cinaciguat also had anti-fibrotic effects in a rat model of chronic renal failure, 5/6 nephrectomy (5/6 NX) [58]. Cinaciguat decreased glomerulosclerosis and renal perivascular and interstitial fibrosis; it also decreased the cardiac media-lumen ratio and mean cardiomyocyte diameter, but had no significant effect on cardiac perivascular or interstitial fibrosis [58].

The anti-fibrotic effects of BAY 41-2272 in the heart were evaluated using a rat model of hypertensive cardiac disease induced by suprarenal aortic constriction, which causes pressure overload [59]. Aortic constriction resulted in fibrotic changes in the left ventricle, including an increased number of myofibroblasts, collagen accumulation, and upregulation of TGF-β1 and type 1 collagen mRNA [59]. BAY 41-2272 inhibited these effects by causing a significant decrease in the number of myofibroblasts and perivascular and interstitial collagen accumulation for TGF-β1 mRNA and type 1 collagen in the left ventricle compared with no treatment [59].

Similarly, the effect of BAY 41-2272 in pulmonary vascular remodeling was investigated in cultured human pulmonary arterial smooth muscle cells (PASMCs). Overexpression of plasminogen activator inhibitor type II (PAI-2) was shown to inhibit both proliferation and migration of PASMCs and increase apoptosis, whereas PAI-2 knockout increased proliferation and migration. BAY 41-2272 up regulated endogenous PAI-2 in PASMCs, and may therefore have potential to alleviate or reverse vascular remodeling through PAI-2 up-regulation [60].

In a comparison of sGC stimulators and activators in cardiovascular disease, the stimulator BAY 60-4552 and activator GSK2181236A were both shown to provide some benefit against hypertension-induced organ damage. In hypertensive stroke-prone rats on a high salt and fat diet, low dose BAY 60-4552 decreased urine output and improved overall survival, whereas GSK2181236A attenuated development of hypertrophy. High doses of each drug attenuated hypertrophy and improved survival, with BAY 60-4552 reducing urine output and microalbuminuria, and reducing the increase in mean arterial pressure [61].

Another in vivo study reported on the renal and cardiac anti-fibrotic effects of riociguat in the 5/6 NX model and also in a high-renin model using renin-transgenic rats given N-nitro-L-arginine methyl ester (l-NAME), an NOS inhibitor (Table 2) [62]. In the 5/6 NX model, there were significant increases in renal interstitial fibrosis, glomerulosclerosis, and cardiomyocyte diameter compared with sham controls [62]. Riociguat (15 mg/kg/day) significantly reduced renal interstitial fibrosis and cardiomyocyte diameter but had no significant effect on glomerulosclerosis [62]. In the high-renin model, riociguat (3 mg/kg/day and 10 mg/kg/day) significantly reduced renal interstitial fibrosis, glomerulosclerosis, and cardiac interstitial fibrosis [62].

The effect of riociguat on renal and cardiac fibrosis was investigated in several different in vivo models (Table 2). In a study in Dahl salt-sensitive (SS) rats maintained on a high-salt diet, which leads to chronic pressure and volume overload, Dahl SS rats treated with vehicle alone had significantly increased mRNA expression for the pro-fibrotic mediators PAI-1 and TIMP-1 in the renal cortex; these increases were abrogated by riociguat [80,63]. A similar pattern was observed in another pro-fibrotic mediator, osteopontin mRNA in the kidney and left ventricle, and for PAI-1 and TIMP-1 mRNA in the left ventricle, but the differences between the groups was not significant [63]. The salt-induced increases in plasma and urine osteopontin, PAI-1, and TIMP-1 were also decreased by riociguat [63]. Histopathologic analyses of the kidney and heart showed significant reductions in glomerulosclerosis, renal interstitial fibrosis, renal perivascular fibrosis, cardiac interstitial fibrosis, and cardiomyocyte diameter with riociguat compared with vehicle, but riociguat had no significant effect on cardiac perivascular fibrosis [63].

In summary, the in vivo data discussed here suggest that cardiac and renal fibrosis can be attenuated by increasing available cGMP via sGC modulators. The effects of these compounds are maintained throughout a variety of different etiologies. These and further investigations may warrant investigation of sGC modulators as a viable treatment for renal and cardiac fibrosis.

### 3.2. Liver fibrosis

The sGC activator BAY 60-2770, a close chemical analog of cinaciguat, was evaluated in two rat models of liver fibrosis. In both the pig serum model and the carbon tetrachloride model, there

<table>
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<tr>
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<th>Anti-fibrotic effects</th>
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<tr>
<td>Renal and cardiac fibrosis</td>
<td>Reductions in osteopontin, PAI-1, TIMP-1 expression in renal cortex, left ventricle, plasma, and urine.</td>
<td>[63]</td>
</tr>
<tr>
<td>Dahl SS rat model</td>
<td>Reductions in glomerulosclerosis, renal interstitial fibrosis, renal perivascular fibrosis, cardiac interstitial fibrosis, and cardiomyocyte diameter</td>
<td>[62]</td>
</tr>
<tr>
<td>5/6 NX rat model</td>
<td>Reduction in renal interstitial fibrosis</td>
<td>[62]</td>
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<tr>
<td>High renin rat model</td>
<td>Reductions in renal interstitial fibrosis, glomerulosclerosis, and cardiac interstitial fibrosis</td>
<td>[62]</td>
</tr>
<tr>
<td>Dermal fibrosis</td>
<td>Reductions in dermal thickness, collagen, and α-SMA-positive myofibroblasts</td>
<td>[84]</td>
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<tr>
<td>Bleomycin-induced mouse model</td>
<td>Reductions in dermal thickness, collagen, and α-SMA-positive myofibroblasts</td>
<td>[84]</td>
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<tr>
<td>Pulmonary fibrosis</td>
<td>Reductions in dermal thickness, collagen, and α-SMA-positive myofibroblasts</td>
<td>[84]</td>
</tr>
<tr>
<td>5/6 NX rat model</td>
<td>Reductions in PH, right ventricle hypertrophy, pulmonary fibrosis, and pulmonary inflammation</td>
<td>[77]</td>
</tr>
</tbody>
</table>

Abbreviations: NX, nephrectomy; PAI-1, plasminogen activator inhibitor-1; PH, pulmonary hypertension; α-SMA, α-smooth muscle actin; SS, salt-sensitive; TGF, transforming growth factor; TIMP-1, tissue inhibitor of metalloproteinase-1; TSK-1, tight skin 1.
were increases in fibrous collagen as measured by Sirius-Red staining (5.5-fold and 5-fold, respectively), and total collagen as measured by hydroxyproline content (2-fold and 3-fold, respectively) [64]. In the pig serum model, BAY 60-2270 at either 0.1 mg/kg or 0.3 mg/kg had an anti-fibrotic effect, significantly reducing the levels of both fibrous and total collagen compared with untreated controls [64]. In the carbon tetrachloride model, only BAY 60-2270 at 0.3 mg/kg caused a significant reduction in fibrous and total collagen [64].

In the bile duct ligation rat model of liver fibrosis, treatment with the sGC stimulator BAY 41-2272 caused a significant decrease in collagen as measured by hydroxyproline content and Sirius-Red staining [65]. This was accompanied by corresponding reductions in the mRNA expression of fibrotic markers including fibrillar collagen type 1α and type 3α, latent binding protein 2, and α-SMA [65]. This suggests that sGC modulators may be clinically viable therapy for liver fibrosis. The anti-fibrotic effects of these drugs are still in the early phase of preclinical investigation in liver fibrosis models.

3.3. Pulmonary fibrosis

Studies using mouse models have shown that injury to alveolar epithelial cells induces the development of fibrosis and myofibroblast differentiation and apoptosis in a bleomycin mouse model [66–68]. Moreover, apoptosis was shown to occur in alveolar epithelial cells adjacent to myofibroblasts in tissue sections of fibrotic human lung, suggesting a feedback loop by which injury to alveolar epithelial cells caused by myofibroblasts can induce additional myofibroblast differentiation [30,69]. Although the myofibroblasts present in pulmonary fibrosis may putatively arise from a number of different cell lineages, a recent study using the bleomycin-induced mouse model supports a role for lung pericytes and resident fibroblasts as the lung myofibroblast progenitor cells [70]. The fibrotic changes that occur in the lung parenchyma are responsible for the characteristic histopathology associated with idiopathic pulmonary fibrosis (IPF), namely honeycombing, dense collagen deposition, and foci created by the proliferation of fibroblasts or myofibroblasts [71]. These histopathologic changes are accompanied by clinical symptoms such as dyspnea and reduced lung function that worsen either progressively or acutely and ultimately lead to death [71].

In pulmonary fibrosis, TGF-β1 appears to play a role in mediating the epithelial-to-mesenchymal transition of alveolar epithelial cells to myofibroblasts [72]. Latent TGF-β1 was shown to be activated by the binding of fibronectin to integrin αvβ6 in alveolar epithelial cells from transgenic mice [72]. There is also evidence that reactive oxygen species (ROS) generated by NADPH oxidase signaling play a role in pulmonary fibrosis. In primary human lung fibroblasts from patients with IPF, peroxide treatment induced the expression of the fibroblast-to-myofibroblast differentiation marker α-SMA and increased the expression of type I collagen; high ROS levels were present in IPF fibroblasts [73]. Endothelin-1 also appears to be involved in mediating IPF based on studies showing the upregulation of endothelin-1 in patients with IPF and in a bleomycin-induced model of pulmonary fibrosis [74,75]. IPF, unlike other fibrotic disorders, does not appear to have an inflammatory component [76].

In 2011, a study was designed to explore the treatment of pulmonary fibrosis (PF) using pharmacologic manipulation of the sGC pathway. PF was induced using bleomycin. After recovery from induction, mice were treated for 2 weeks with the sGC stimulator riociguat, the PDE5 inhibitor sildenafil, or a combination; at which point pulmonary hemodynamics were evaluated. Riociguat was shown to attenuate PF, PH, and right heart hypertrophy. This effect was superior compared with mice treated with sildenafil [77]. In another study, riociguat (1 mg/kg/day) significantly reduced PH, right ventricle hypertrophy, pulmonary fibrosis, and pulmonary inflammation (Table 2) [77]. While treatment with sildenafil also reduced PH and right ventricle hypertrophy, there was no significant effect on pulmonary fibrosis or inflammation [77].

3.4. Dermal fibrosis

The antifibrotic mechanisms of sGC modulators were noted in internal organs such as the kidney, lung, and liver based on the well-characterized effects of cGMP elevation in cardiovascular, cardiopulmonary, and cardioenal studies. Studies examining the role of cGMP elevation in dermal fibrosis are much more recent, and have yielded promising data.

TGF-β is considered the primary driver of fibrosis in SSC [78]. Additional key players in the fibrotic mechanisms of SSC include the TGF-β, TGF-β receptor 1 (TGF-βR1) and TGF-β receptor II (TGF-βRII) signaling pathway. Interestingly, the inhibitory effects of the sGC stimulators were dependent on the sGC/cGMP independent of canonical TGF-β effects of sGC modulators.

Beyer et al. explored the link between TGF-β–mediated dermal fibrosis and the anti-fibrotic effects of sGC modulators. BAY 41-2272 reduced TGF-β–mediated activation of dermal fibroblasts from both healthy and SSC individuals. sGC stimulation inhibited TGF-β–mediated fibroblast-to-myofibroblast differentiation. Genetic knockout of sGC in fibroblasts attenuated the anti-fibrotic effects of BAY 41-2272 on TGF-β–mediated collagen release in dermal fibroblasts, suggesting specificity of sGC. Similar results were observed using 8-Bromo–cGMP suggesting that the effects of sGC stimulators were dependent on the sGC/cGMP signaling pathway. Interestingly, the inhibitory effects of the sGC stimulator BAY 41-2272 on TGF-β–mediated dermal fibrosis was independent of canonical TGF-β–cGMP signaling. Rather, sGC stimulation by BAY 41-2272 inhibited TGF-β–mediated activation of ERK [44].

In the bleomycin-induced mouse model of dermal fibrosis, intradermal injection of bleomycin caused a significant increase in skin fibrosis, characterized by dermal thickening, increase of dermal hydroxyproline content and increase of the number of dermal fibroblasts. The sGC stimulator BAY 41-2272 (1 mg/kg BID or 3 mg/kg BID) caused dose-dependent decreases in dermal thickening, dermal hydroxyproline content, and α-SMA-positive myofibroblasts compared with sham-treated bleomycin-induced mice [43]. The sGC stimulator riociguat, like BAY 41-2272, caused dose-dependent anti-fibrotic effects in the bleomycin-induced mouse model, with decreases in dermal thickness, dermal hydroxyproline, and dermal α-SMA-positive myofibroblasts (Table 2) [84]. Consistent with findings from the sGC stimulators BAY 41 and riociguat, there was also a moderate effect with the PDE5 inhibitor sildenafil in this animal model. However, the efficacy of sildenafil appears to be lower compared with sGC stimulators. These results demonstrate that the decrease of skin fibrosis in the bleomycin model is mediated by cGMP and that low NO/cGMP production in this model may limit the efficacy of PDE5 inhibitors.

While the bleomycin-induced model represents an inflammation-driven dermal fibrosis, the tight skin 1 (TSK-1) mouse model represents inflammation-independent, established dermal fibrosis [43]. In the TSK-1 model, BAY 41-2272 caused
dose-dependent decreases in dermal thickening, hydroxyproline content, and α-SMA-positive myofibroblasts [43]. BAY 41-2272 showed similar anti-fibrotic effects in a mechanistic model of dermal fibrosis induced by TGF-β receptor I overexpression in mice [44]. Similar results were seen again with riociguat in the TSK-1 model (Table 2): there were significant, dose-dependent reductions in dermal thickening and α-SMA-positive myofibroblasts with riociguat 0.3, 1, and 3 mg/kg BID and in dermal hydroxyproline content with riociguat 1 or 3 mg/kg BID [84]. The effects of sildenafil were lower compared with the sGC stimulator riociguat in the TSK-1 mouse model as well.

Interestingly, BAY 41-2272 was also investigated in both mouse models of established dermal fibrosis (bleomycin and TSK-1) [43]. BAY 41-2272 (3 mg/kg BID) administered during the last 3 weeks of a 6-week bleomycin induction resulted in decreases in dermal thickness compared with 6 weeks of bleomycin alone. BAY 41-2272 also reduced hydroxyproline content and α-SMA-positive myofibroblasts to levels below that of 3 weeks of bleomycin followed by 3 weeks of NaCl [43]. A regression in induced fibrosis was seen in TSK-1 mice given BAY 41-2272 (3 mg/kg BID) during weeks 5–10. Dermal thickness was reduced to below that of 5-week-old TSK-1 mice. BAY 41-2272 also reduced hydroxyproline content and α-SMA-positive myofibroblasts to levels below those of un.injected TSK-1 mice at 5 weeks [43]. Together these results suggest that sGC stimulation not only prevents the progression of fibrosis but also induces regression of already-established inflammation-dependent and inflammation-independent fibrosis since this effect was found in both pharmacologically-induced (bleomycin) and genetic (TSK-1) mouse models of dermal fibrosis.

SSc is characterized not only by excessive skin fibrosis but also vasculopathies which include Raynaud’s phenomenon and digital ulcers. The interplay between fibrosis and vasculopathy are not fully understood but the frequently concomitant symptoms exacerbate patient discomfort. As sGC modulators both increase peripheral blood flow and exert antifibrotic effects, they may be beneficial in digital ulcer healing. In an in vivo investigation of the effect of sGC stimulators on wound healing in the TSK-1 mouse model, there was a dose-dependent reduction in wound size with either BAY 41-2272 or riociguat administered 3 days prior to and following punching (Table 2) [85]. The reduction was significant for BAY 41-2272 at a dose of 3 mg/kg BID and riociguat at 1 and 3 mg/kg BID [85]. These data imply that, in addition to antifibrotic activity there is an effect on peripheral blood flow which may be of benefit in the treatment of vasculopathy in SSc, especially for the treatment of digital ulcers.

The efficacy of sGC stimulators has been explored in a mouse model of sclerodermatous chronic graft-versus-host-disease (cGVHD), accompanied by fibrosis in multiple organs. Riociguat reduced cGVHD-induced dermal fibrosis as well as fibrosis in the gastrointestinal tract. The PDE5 inhibitor sildenafil was less effective compared to riociguat. The anti-fibrotic effects of riociguat were dependent on non-canonical TGF-β/SMAD activation of ERK signaling [84].

TGF-β has been shown to be a major driver of dermal fibrosis. This has been explored in healthy and SSc dermal fibroblasts as well as multiple animal models. Using both pharmacologic and genetic methods, sGC stimulators have been shown to inhibit TGF-β-mediated dermal fibrosis in a SMAD-independent TGF-β—signaling manner. The higher efficacy of sGC stimulators, compared with PDE5 inhibitors suggests that targeting sGC directly may be of more benefit than targeting downstream components of cGMP production, potentially due to low endogenous cGMP production in fibrotic diseases. However, further preclinical and clinical studies are needed to understand in more detail these differences in efficacy.

4. Conclusion

The results of these in vitro and in vivo preclinical experiments support the anti-fibrotic efficacy of cGMP elevation in a range of fibrotic disorders. Incubation of fibroblasts from healthy humans and patients with SSc with TGF-β resulted in fibroblast activation, accompanied by production of ECM components. This observation has been replicated in vivo using pharmacologic and genetic induction of fibrosis in mouse models. PDE5 inhibitors exhibit less efficacy compared with sGC stimulators suggesting that there may be a higher clinical benefit to targeting the upstream components of the sGC–cGMP pathway. There is growing evidence that TGF-β-mediated induction of fibrosis may depend mostly on a non-canonical TGF-β2 activation of ERK signaling pathway. The majority of these studies indicated that sGC stimulators have the potential to be preventive, not curative. One possible exception is skin fibrosis, where sGC stimulators showed the potential to reverse established fibrosis. In sum, the preclinical data presented here provide the basis for an anti-fibrotic action of sGC ‘modulators’ and the rationale for clinical investigations in the treatment of fibrotic disorders such as pulmonary fibrosis and skin fibrosis; efficacy is being investigated in ongoing studies.

Conflict of interest

Peter Sandner, PhD and Johannes Peter Stasch, PhD are employees of Bayer Healthcare AG.

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