Anti-fibrotic mechanisms of angiotensin AT2-receptor stimulation

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ABSTRACT

The angiotensin AT\textsubscript{2}-receptor is a main receptor of the protective arm of the renin-angiotensin-system. Understanding of this unconventional G-protein coupled receptor has significantly advanced during the past decade, largely because of the availability of a selective non-peptide AT\textsubscript{2}-receptor agonist, which allowed the conduct of a multitude of studies in animal disease models.

This article reviews such preclinical studies that in their entirety provide strong evidence for an anti-fibrotic effect mediated by activation of the AT\textsubscript{2}-receptor. Prevention of the development of fibrosis by AT\textsubscript{2}-receptor stimulation has been demonstrated in lung, heart, blood vessels, kidney, pancreas and skin. In lung, AT\textsubscript{2}-receptor stimulation was even able to reverse existing fibrosis.

The article further discusses intracellular signalling mechanisms mediating the AT\textsubscript{2}-receptor coupled anti-fibrotic effect, including activation of phosphatases and subsequent interference with pro-fibrotic signalling pathways, induction of matrix-metalloproteinases, and hetero-dimerisation with the AT\textsubscript{1}-receptor, the TGF-\textbetaRII-receptor or the RXFP1-receptor for relaxin.

Knowledge of the anti-fibrotic effects of the AT\textsubscript{2}-receptor is of particular relevance, because drugs targeting this receptor have entered clinical development for indications involving fibrotic diseases.

Key Words

Angiotensin, angiotensin AT\textsubscript{2}-receptor, fibrosis, fibrotic disease
Background

The birth-hour of the renin-angiotensin-system (RAS) dates back to 1898, when Robert Tigerstedt identified renin as a substance contained in renal cortex extracts (derived from rabbits), which, when injected intravenously into another rabbit led to a sustained increase in blood pressure.¹ This association of the RAS with a cardiovascular effect set the tone for RAS research for almost 100 years to follow. During this time, identification of all components of the RAS [the active hormone angiotensin II (Ang II), the precursors angiotensinogen and angiotensin I, the enzymes renin and angiotensin converting enzyme (ACE)] together with a multitude of studies which thoroughly investigated the vascular and renal effects of Ang II and later also aldosterone, cumulated in the discovery and development of some of the most effective and most commonly used drugs in cardiovascular medicine: ACE inhibitors, angiotensin AT₁-receptor (AT₁R) blockers (ARBs) and aldosterone antagonists.²

In the 1990s, at a time, when it almost seemed as if everything essential regarding the RAS had been explored, four unexpected key findings opened up an entirely new era in RAS research. These findings were:

(1) The identification of different subtypes of angiotensin receptors, mainly the AT₁R and the angiotensin AT₂-receptor (AT₂R);³,⁴

(2) The realisation that the AT₁R and the AT₂R mediate opposing effects;⁵,⁶

(3) The discovery that Ang II through its receptor subtypes does not only act on the vasculature, kidneys and CNS for the regulation of blood pressure and fluid volume, but that it also has direct effects on inflammation, fibrosis, organ remodelling, cell proliferation, cell differentiation, and apoptosis in the above mentioned organs, and also in non-cardiovascular organs such as the eyes, skin, pancreas and liver;⁷
(4) the identification of angiotensin fragments such as angiotensin 1-7 (Ang 1-7) as being biologically active, having their own receptors (in the case of Ang 1-7, the receptor Mas) and counteracting AT\(_1\)R mediated actions of Ang II.\(^8\)

As a result of these four key findings, it is now generally accepted that apart from the classical arm of the RAS (mainly represented by Ang II acting on the AT\(_1\)R), there is also a so-called “protective arm of the RAS”. The Ang II/\(\text{AT}_2\)R and angiotensin converting enzyme-2 (ACE2)/Ang 1-7/Mas systems are the most established components of the protective RAS.\(^7\) A more recently identified component is alamandine, which acts via the Mas-related G-protein coupled receptor D (MrGD) to exert effects on the cardiovascular system, including anti-fibrotic effects in the heart.\(^9\) However, physiological actions of alamandine/MRGD are far from being established. The mediators and receptors of the protective arm of the RAS have been shown in numerous studies and multiple preclinical disease models to mediate tissue protective effects such as anti-inflammation [first described in 2001 by Wu et al.\(^{10}\)], anti-fibrosis [first described in 1998 by Ma et al. \(^{11}\)], anti-apoptosis [first described in 2002 by Grammatopoulos et al.\(^{12}\)] and neuroprotection [first described in 1998 by Lucius et al.\(^{13}\)]. Based on these data, the protective arm of the RAS can be regarded as an endogenous program for tissue protection, repair and regeneration.

When in the late 1990s the existence of a protective RAS became evident and generally accepted, the idea developed to make therapeutic use of this protective program by developing \(\text{AT}_2\)R and Mas agonists for clinical use. Consequently, several drug development projects have been initiated in the meantime, some of which have been progressed to Phase II clinical studies. Interestingly, primary indications for \(\text{AT}_2\)R- and Mas-agonists are not necessarily in cardiovascular diseases. Rather, \(\text{AT}_2\)R-agonists are being developed for Idiopathic Pulmonary Fibrosis (IPF) (www.vicorepharma.com) and
cancer (www.morphosys.com); a Mas-agonist is being developed for Duchenne Muscular Dystrophy (www.tarixpharma.com). The clinical development for IPF is based on a number of studies showing therapeutic effects of AT2R-agonists in preclinical models of fibrotic diseases – studies which also identified various anti-fibrotic mechanisms elicited by this receptor.14–16

This review will focus on the anti-fibrotic properties of the AT2R by providing general information about the receptor, by summarising findings from preclinical studies in models of fibrotic diseases and by a detailed description of the molecular mechanisms resulting in AT2R-mediated, anti-fibrotic effects.

The angiotensin AT2-receptor

The angiotensin AT2R is a seven transmembrane domain receptor, containing most of the conserved motifs of a class A G-protein coupled receptor (GPCR).17,18 Both functional and co-precipitation studies have suggested that AT2R can signal via Gi proteins.19–22 However, in contrast to many GPCR, AT2R have not been shown to signal through canonical GPCR signaling mechanisms such as modulation of cAMP or phospholipase C/Ca2+.23,24 The latter findings might be explained by a recent structural study which indicated that helix VIII of the AT2R stabilizes the conformation of the receptor in its active state, and at the same time covers binding sites for G-proteins and β-arrestin, preventing conventional GPCR signaling.25 While conventional GPCR signaling very much involves Ca2+-dependent kinase driven signaling pathways, several distinct intracellular signaling mechanisms have been described for AT2R, which often are tissue-specific.26,27 Key signaling mechanisms of the AT2R that likely play a role in the anti-fibrotic actions of this receptor involve activation of protein phosphatases such as Src homology region 2 domain-containing phosphatase-1 (SHP-1), serine-threonine
phosphatase-2A (PP2A) and Map kinase phosphatase-1 (MKP-1). AT₂R-mediated activation of the tyrosine phosphatase SHP-1 is dependent on the formation of a complex consisting of SHP-1, the kinase c-Src (which phosphorylates and thereby activates SHP-1), and the AT₂R. Gaι also seems to be involved in this process. The complex forms at the 3rd intracellular loop of the AT₂R, which has been described to be essential for AT₂R signaling. The AT₂R-induced activation of PP2A and MKP-1 has not yet been studied in such detail, but it was demonstrated that the AT₂R-mediated increase in PP2A activity in neurons involved a pertussis toxin-sensitive G-protein, potentially Gi. Activated SHP-1, PP2A and MKP-1 interfere in an inhibitory way with kinase-driven signaling cascades coupled to the AT₁R or to receptors for cytokines (including TGFβ) or growth factors that play stimulatory roles in fibrotic processes.

It is also well known that AT₂R couple to activation of endothelial nitric oxide synthase (eNOS), with subsequent generation of nitric oxide (NO) and cyclic GMP (cGMP). A recent study using endothelial cells indicates that the activation of eNOS is mediated through Akt-mediated phosphorylation plus serine/threonine and tyrosine phosphatase-mediated dephosphorylation events. This is important because eNOS activation and NO production can lead to inhibition of the synthesis of TGF-β, which constitutes an AT₂R-mediated anti-fibrotic mechanism. In addition to phosphatase/kinase and NO-mediated signaling pathways, a number of investigations have indicated that AT₂R activation can also lead to stimulation of phospholipase A2 (PLA2) and generation of arachidonic acid (AA). While there is an association of PLA2/AA signaling with certain fibrotic mechanisms, there is no evidence that links AT₂R activation of PLA2/AA pathways to anti-fibrotic effects.

These unique signaling mechanisms of the AT₂R, i.e. its inhibitory interference with potentially pathological, kinase-driven signaling cascades and also its stimulation of NO
pathways, helps to explain why selective stimulation of a single receptor (i.e. the AT$_2$R) can mediate a whole variety of protective actions such as anti-inflammation, anti-proliferation, anti-hypertrophy and anti-fibrosis.

Evidence from preclinical studies for anti-fibrotic effects of AT$_2$-receptor stimulation

Prevention or even reversal of tissue fibrosis by AT$_2$R stimulation has been demonstrated in numerous preclinical disease models involving various organs such as lung, kidney, vascular wall, heart, pancreas and skin.$^{14}$ These preclinical studies are reviewed, with respect to the affected organ, in the following paragraphs. In addition, the in vivo studies that involved the use of AT$_2$R agonists are summarized in Table 1, where it can be seen that the studies were performed exclusively using the small molecule agonist Compound 21 [C21].$^{42}$

Lung

Two preclinical studies have demonstrated that pharmacological activation of the AT$_2$R by C21 prevents and reverses pulmonary fibrosis.

In a first study, pulmonary fibrosis was induced in Sprague Dawley (SD) rats by a single subcutaneous injection of monocrotaline (50 mg/kg).$^{15}$ Treatment with either C21 (0.03 mg/kg per day) or vehicle via the intraperitoneal (i.p.) route was initiated two weeks after monocrotaline application, when pulmonary fibrosis was fully established. Daily i.p. treatment with C21 or vehicle was then continued for another two weeks (from week 2 to 4 after monocrotaline application). Treatment with C21 not only prevented, but reversed pulmonary interstitial and perivascular fibrosis, i.e. the area of fibrosis was smaller at the end of treatment than when treatment began. This effect was completely
abolished by co-administration of the AT$_2$R antagonist PD123319 (3 mg/kg/day, i.p.), demonstrating that the effect of C21 was AT$_2$R dependent. In addition, C21 treatment decreased TGF-β mRNA expression, which was strongly increased in monocrotaline/vehicle treated animals, to levels in healthy (non monocrotaline-treated) animals.$^{15}$

In a second study by the same group, pulmonary fibrosis was induced in male SD rats by a single intra-tracheal installation of bleomycin (4U/kg).$^{16}$ In a pilot study, the authors first demonstrated that as soon as 3 days after bleomycin installation, the formation of fibrotic tissue in the lungs was already clearly detectable. Subsequently, the authors tested the therapeutic effect of C21 (0.03 mg/kg/day i.p.) in both prevention- and treatment protocols. In the prevention protocol, application of C21 or vehicle was started on the day of bleomycin installation; in the treatment arm application of C21/vehicle was started 3 days after bleomycin application, when fibrosis had already developed. In both study arms, treatment was continued up to day 14 after bleomycin installation. In the prevention arm, C21 significantly attenuated the formation of pulmonary fibrosis as estimated by Ashcroft score and by quantifying the fibrotic area (based on picrosirius red staining). In the treatment arm, the progression of fibrosis was almost completely halted from the day of treatment start, i.e. day 3 after bleomycin installation. Hydroxyproline content of the lungs was reduced to levels in healthy animals by C21 treatment in both the prevention and in the treatment arm. Furthermore, mRNA levels of fibrosis markers and mediators (collagen 1 and 3, CTGF, IL-13) and of an inhibitor of ECM degradation, TIMP (tissue inhibitor of matrix-metalloproteinases), which were elevated after bleomycin treatment, were significantly reduced – partly to baseline levels – in the C21 treated rats.$^{16}$
Importantly, in both the monocrotaline and the bleomycin studies, AT$_2$R-stimulation by C21 significantly lowered right ventricular systolic pressure, thus indicating a positive, therapeutic effect on pulmonary hypertension.$^{15,16}$ This effect is remarkable inasmuch as the drugs which are currently approved for the treatment of idiopathic pulmonary fibrosis, pirfenidone and nintedanib, do not affect pulmonary blood pressure, and also because pulmonary hypertension is a major cause of death in pulmonary fibrosis.$^{43}$ The mortality associated with pulmonary hypertension is largely due to right ventricular failure. Interestingly, the studies by Bruce et al. and Rathinasabapathy et al. both also looked at right ventricular (RV) pathology resulting from pulmonary hypertension due to pulmonary fibrosis.$^{15,16}$ Both studies reported that AT$_2$R-stimulation prevented the development of RV hypertrophy and improved cardiac function. Bruce et al. additionally demonstrated a complete prevention of the formation of RV fibrosis.$^{15}$

A very recent study by Menk et al. showed an anti-inflammatory effect of AT$_2$R-stimulation by C21 (0.03 mg/kg, i.p.) in a model of acute lung injury, indicating that anti-inflammation may contribute to the anti-fibrotic effect of AT$_2$R-agonists.$^{44}$ This is in line with an attenuation of pro-inflammatory markers (CCL2, Il-6, Tlr4, TNFα, Il-1β) in the above reviewed studies in models of pulmonary fibrosis.$^{15,16}$

**Heart**

Indirect evidence for a protective effect of the AT$_2$R in the context of cardiac remodeling and fibrosis has come from studies in genetically altered animals, in which AT$_2$R overexpression ameliorated- and AT$_2$R deletion worsened cardiac fibrosis.$^{45,46}$ However, the use of genetically altered animals has also generated conflicting results.$^{47}$ The experimental approach of direct AT$_2$R stimulation by the AT$_2$R-agonist C21 has
resulted in much more consistent findings regarding left ventricular cardiac fibrosis. These studies are reviewed in the following paragraphs.

**Post-myocardial infarction cardiac fibrosis**

In this study, Wistar rats were treated with C21 (0.03 mg/kg per day i.p.) for 6 weeks starting on the day of MI induction.\(^4^8\) C21 treatment resulted in significantly decreased collagen content in the peri-infarct cardiac tissue as estimated by picrosirius red staining and quantification of hydroxyproline content. This coincided with a significant reduction (to levels observed in healthy animals) of myocardial TGF-β1 mRNA and protein. Moreover, treatment with C21 led to the normalisation of increased serum levels of activated TGF-β1 and decreased serum levels of latent TGF-β1.\(^4^8\)

**Hypertension-induced cardiac fibrosis**

In 2012, Rehman et al. published a study, in which they investigated the effect of AT\(_2\)R stimulation in the absence or presence of an AT\(_1\)R blocker on hypertensive end organ damage in the vasculature (see more details below) and the heart.\(^4^9\) Stroke-prone spontaneously hypertensive rats (SHRsp) were treated orally with either vehicle, C21 (1 mg/kg per day mixed in chow), Losartan (10 mg/kg/day in drinking water) or the combination of C21 and Losartan for 6 weeks. This treatment with C21 significantly reduced the development of interstitial myocardial fibrosis as determined by picrosirius red staining. The effect of C21 alone was most pronounced and only significant for subendocardial, but not for myocardial and subepicardial fibrosis. The same was true for Losartan alone. However, the combination of C21 with Losartan significantly attenuated collagen formation at all three locations. It is important to note that Losartan completely prevented the naturally occurring increase in blood pressure (BP) in SHRsp, which most likely contributed to the anti-fibrotic effect, whereas C21 had no effect on BP whatsoever, but still acted in an anti-fibrotic fashion.\(^4^9\)
Kidney

As with cardiac fibrosis, initial data hinting at an attenuating effect of the AT\(_2\)R on the development of renal fibrosis came from studies in AT\(_2\)R deficient mice, which developed a more severe course of nephropathy and renal fibrosis in models of 5/6-nephrectomy and ureteral obstruction.\(^{11,50}\) Another study indicated a protective AT\(_2\)R-mediated action by demonstrating that the therapeutic effect of the AT\(_1\)R-blocker Losartan (80 mg/L, administered in drinking water) on 5/6-nephrectomy induced glomerular sclerosis was at least partially dependent on indirect AT\(_2\)R stimulation (i.e. stimulation of the unopposed AT\(_2\)R by reactively elevated Ang II levels under AT\(_1\)R blockade), since most of the beneficial effects of Losartan could be blocked by subcutaneous infusion of the AT\(_2\)R-antagonist PD123319 (15 mg/kg/day).\(^{51}\)

An anti-fibrotic effect of AT\(_2\)R stimulation was further shown in human renal proximal tubule cells \textit{in vitro}, in which treatment with the AT\(_2\)R peptide agonist CGP42112A led to inhibition of TGF\(\beta\) synthesis and acceleration of TGF\(\beta\) degradation.\(^{36}\) Furthermore, this study revealed some interesting anti-fibrotic molecular mechanisms coupled to AT\(_2\)R stimulation, which will be discussed in the section on mechanisms underlying the anti-fibrotic effect of the AT\(_2\)R.

More recently, anti-fibrotic effects of direct AT\(_2\)R stimulation by C21 have been demonstrated in various \textit{in vivo} models of diabetic nephropathy (in diabetes type 1 and 2), hypertensive nephropathy, drug-induced nephropathy and focal segmental glomerular sclerosis. These studies are reviewed in the following paragraphs.

\textbf{Diabetic nephropathy}

In Zucker diabetic fatty (ZDF) rats, a genetic model of type 2 diabetes, C21 (0.3 mg/kg per day, i.p.) and/or Losartan (10 mg/kg/day in drinking water) were administered for
15 weeks starting at 6 weeks of age. Treatment with C21 completely prevented the
development of glomerular, tubulointerstitial and perivascular fibrosis as determined
by quantification of picrosirius red staining. Remarkably, despite reducing BP to levels
10 mmHg lower than in the C21 group, Losartan only prevented glomerular and
perivascular, but not interstitial fibrosis. The anti-fibrotic effect of the combination of
C21 and Losartan tended to reduce glomerular and perivascular fibrosis in an additive
manner, but the additive effect was not statistically significant. However, by the end of
the treatment period (but not at earlier time points), only the combination of C21 with
Losartan significantly attenuated albuminuria and thus delayed the loss of therapeutic
efficacy of both drugs in monotherapy over time.

In another study, type 2 diabetes was induced in Wistar rats by high fat diet (HFD)
combined with a single application of low dose (35 mg/kg i.p.) streptozotocin. After
24 days of HFD and proof of established diabetes, animals were treated for 14 days with
either vehicle, C21 (0.3 mg/kg p.o.), telmisartan (10 mg/kg p.o.) or a combination of
C21 + telmisartan. The authors tested a number of markers of fibrosis, inflammation
and apoptosis, the majority of which were significantly improved by C21 and
telmisartan alone. As in the aforementioned study, the combination of C21 with the ARB
led to an additive effect, which in this study in most cases was significantly better than
the treatment effect of the single drugs.

In a model of type 1 diabetes (diabetes induced by 5-daily injections of 55 mg/kg
streptozotocin in apolipoprotein E-deficient (ApoE−/−) mice, treatment with 1
mg/kg/day of C21 by oral gavage for 20 weeks significantly decreased mesangial area,
glomerulosclerotic injury, and albuminuria as well as markers of inflammation and
markers of fibrosis such as glomerular collagen I, collagen IV and total collagen
content. The effect of C21 on ECM was accompanied by a significant lowering of the
pro-fibrotic mediators TGF-β and CTGF. At the same time, expression and activity of the ECM-degrading enzymes MMP2 and -9 were increased in diabetic animals treated with C21. Blood pressure and metabolic markers were not altered by C21.54

**Drug-induced nephropathy**

- **Cyclosporine-induced nephropathy**

While development and approval of the immune-suppressant cyclosporine constituted a breakthrough for long-term organ survival in renal transplantation, at the same time cyclosporine is nephrotoxic and can harm the transplanted organ primarily by tubulointerstitial fibrotic remodeling.55 To test a potential protective effect of AT₂R-stimulation in cyclosporine-associated nephropathy, this condition was induced in male Sprague Dawley rats by daily injections of cyclosporine-A (15 mg/kg) for 8 or 28 days in addition to a low salt diet, which was initiated 10 days before the actual experimental period. Treatment with C21 (0.3 mg/kg i.p. per day) significantly reduced tubulointerstitial and glomerular fibrosis, renal macrophage infiltration and collagen I content after 28 days, but not after 8 days of treatment.56

- **Doxorubicin-induced nephropathy**

A common complication of chemotherapeutic therapy with doxorubicin is cardiac and renal toxicity. Hrenák et al. tested a potential protective effect of AT₂R-stimulation in Wistar rats, which received a single injection of doxorubicin (5 mg/kg i.v.) followed by a 4-week oral treatment with C21 (0.3 mg/kg/day), an ACE-inhibitor (captopril; 100 mg/kg/day) or an ARB (Olmesartan; 10 mg/kg/day) for comparison.57 After four weeks of drug administration, all treatments attenuated oxidative stress and loss of glomeruli. However, remarkably, while captopril and Olmesartan caused the expected significant reductions in blood-pressure, which most likely contributed to the therapeutic effect,
C21 was blood-pressure neutral, but still had the same protective effect on doxorubicin-induced renal damage.\textsuperscript{57}

**Focal segmental glomerulosclerosis**

Treatment with C21 was successfully tested in a model of focal segmental glomerulosclerosis (FSGS), which was induced by feeding Obese Zucker rats a high salt diet.\textsuperscript{58} In this 2-week study, rats were treated with 1 mg/kg/day C21 applied by subcutaneous infusion via osmotic minipump. All manifestations of FSGS, which were severe glomerulosclerosis, interstitial fibrosis, a decline in estimated glomerular filtration rate as well as an increase in urinary leak and tubular damage, were significantly improved by AT\textsubscript{2}R stimulation.\textsuperscript{58}

**Renal inflammation**

In addition to the above reviewed publications, which reported an anti-fibrotic effect of AT\textsubscript{2}R stimulation in various preclinical models of renal disease, several other studies have demonstrated an anti-inflammatory effect of AT\textsubscript{2}R stimulation in kidney disease. Since inflammation commonly precedes tissue fibrosis, these studies are also briefly reviewed below.

- **Hypertension-induced nephropathy**

  The effectiveness of treatment with C21 in hypertensive nephropathy was tested in SHRsp. Oral treatment with 10 mg/kg C21 in a slow-release preparation significantly delayed the development of proteinuria and reduced renal inflammatory markers.\textsuperscript{59}
- Renovascular hypertension

Matavelli et al. published two articles, which investigated the effect of AT$_2$R-stimulation on renal inflammation.$^{60,61}$ The first of these publications looked at early inflammation in the clipped kidney 4 days after interruption of blood flow in a 2-kidney, 1-clip model of renovascular hypertension in Sprague Dawley rats.$^{60}$ Increased renal expression levels of TNF-$\alpha$, IL-6 and TGF-$\beta$1 and decreased levels of NO and cGMP in the clipped kidney were significantly improved by C21 (0.3 mg/kg i.p.). This effect was at least partially blocked by PD123319 (10 mg/kg; subcutaneous infusion).

- Diabetes-induced acute renal inflammation

The second study by Matavelli et al. was performed in Sprague-Dawley rats with streptozotocin (STZ)-induced diabetes.$^{61}$ Treatment with C21 (0.3 mg/kg/day via subcutaneous osmotic minipump), which was initiated the day after STZ injection and continued for 4 weeks, resulted in a significant attenuation of elevations in the pro-inflammatory mediators TNF-$\alpha$ and IL-6 in interstitial fluid and on mRNA level. In addition, decreased levels of NO and cGMP in diabetic rats were rescued by AT$_2$R-stimulation. Functionally, treatment with C21 significantly reduced the enhanced albumin to creatinine ratio in diabetic animals.$^{61}$

At this point, it should be stressed again that in all animal models with increased BP, treatment with an AT$_2$R-agonist was BP neutral, i.e. the therapeutic effects of AT$_2$R-stimulation on renal pathology were not dependent on a reduction in BP.

Vasculature

In 2012, two studies were published back-to-back in Hypertension, which both investigated the effect of AT$_2$R-stimulation on fibrotic remodeling of the aortic wall in response to hypertension.$^{49,62}$ One of these studies was performed in stroke-prone
spontaneously hypertensive rats (SHRsp), the other one in Wistar rats, which developed hypertension due to chronic treatment with the nitric oxide synthase inhibitor L-NAME. SHRsp were treated for 6 weeks with 1 mg/kg/day C21 mixed in chow, whereas L-NAME hypertensive Wistar rats were treated orally by having them lick a 0.3 mg/kg solution of C21 in aqua ad injectabilia from a pipette. In both models, AT2R-stimulation significantly reduced aortic collagen content and vascular stiffness compared to control animals. In the L-NAME study, this morphological finding correlated with an improvement of pulse wave velocity in the C21 treated rats. Both studies also included a group treated with an ARB (Losartan or Olmesartan, respectively; both dosed at 10 mg/kg p.o.) and a group treated with a combination of ARB and C21. In the vast majority of cases, treatment with an ARB or with C21 yielded the same effect on markers of vascular remodeling; the only exceptions were media/lumen ratio and isobaric media stress in mesenteric arteries and media collagen fraction in coronary arteries in the SHRsp study, as well as elastic modulus in the L-NAME study, which were significantly improved by the ARB alone. The otherwise same therapeutic effect of ARB or AT2R-agonist was still remarkable, since the ARBs – as expected – had a significant anti-hypertensive effect in these hypertension animal models, while treatment with C21 did not alter BP at all, i.e. the beneficial effect of ARBs was supported by a lowering of BP whereas the effect of AT2R-stimulation was BP independent. Another interesting observation of these studies was the lack of an additive effect in the ARB/C21 combination groups. The only exceptions to this rule were media collagen fraction in aortas of SHRsp and aortic hydroxyproline content in the L-NAME model – remarkably both markers of collagen accumulation.

A further attenuating effect of AT2R-agonists on vascular fibrotic processes was observed in atherosclerotic aortas from diabetic (STZ-induced) ApoE−/− mice, in which

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C21 (1 mg/kg/day, oral gavage) reduced the increases in TGF-β gene expression back to levels found in non-diabetic animals. The combination of AT₂R-agonist and an ARB (Candesartan, 4 mg/kg/day, oral gavage) had no additive effect in this study. Overexpression of the AT₂R by injection of an adeno-associated virus (AAV) type 2 vector produced a similar reduction in collagen content in atherosclerotic aortas of low-density lipoprotein receptor knockout mice.

Pancreas

A major reason for loss of pancreatic organ function in chronic pancreatitis is pancreatic fibrosis. Ulmasov et al. published a study in 2008, in which they induced pancreatic inflammation and fibrosis by repetitive i.p. injections of cerulein every 2nd day over one week in C57BL/6 wild-type (WT) or male and female AT₂R-deficient mice. Tissue was analyzed 10 days after start of first cerulein injection. The authors found that AT₂R deficiency resulted in more severe fibrosis (i.e. significantly increased collagen I and hydroxyproline content), enhanced activation of pancreatic stellate cells and stronger expression of the main pro-fibrotic cytokine, TGFβ, on mRNA and protein level. The authors concluded that in pancreatic fibrosis, the AT₂R exerts protective, anti-fibrogenic effects. These results still await confirmation in a study involving direct AT₂R stimulation.

Dupuytren disease

Dupuytren disease is a chronic affliction of the subcutaneous tissue of the palms that can lead to a significant impairment of motility of the 3rd to 5th fingers. AT₂R expression has been reported to be strongly increased in tissue samples taken at fasciectomy in patients with Dupuytren disease thus suggesting that targeting the AT₂R for the
treatment of this disease may be particularly promising. This hypothesis has been tested in a xenograft model, in which cord specimens derived from patients undergoing open partial fasciectomy were implanted under the dorsal skin of nude mice. From day 5 to 15 after implantation, mice were treated with C21 (0.01 mg/kg/day, i.p.), which resulted in an inhibition of fibroblast to myofibroblast transition (indicated by a significantly reduced expression of $\alpha$-smooth muscle actin) and proliferation (measured by Ki67 staining).

**In vitro experiments in human primary cells**

In addition to evidence for an anti-fibrotic effect of AT$_2$R stimulation derived from animal studies, experiments in human primary cells in vitro suggest that this effect can potentially be translated into the human situation. In the above cited study, treatment of human primary dermal fibroblasts with C21 inhibited the expression of the fibrosis-relevant mediators CTGF, fibroblast specific protein-1, TGF-β1, SMAD3, and SMAD4 at the mRNA level.

**Preclinical studies for anti-fibrotic effects of AT$_2$-receptor: Knowledge gaps and future directions**

Based on the above paragraphs, and on the evidence displayed in Table 1, there are now a myriad of preclinical studies which indicate potential clinical indications for AT$_2$R agonists in fibrotic diseases (see Figure 1). Despite the preclinical studies that have already been performed, it is clear that a number of questions remain about the effects of AT$_2$R agonists in fibrotic diseases, and whether these agents might ultimately become effective therapeutics. One primary issue is that most of the studies already performed have been prevention studies, i.e. the AT$_2$R agonist application begins before or at the
same time as the induction of the disease state (Table 1). While some approaches have involved treatment protocols that have been effective in alleviating disease (Table 1), the treatment approach should be used in many more cases, and studies should also include more than one dose of the agonist to help determine effectiveness against a particular disease state. A further point is that many of the studies require long-term treatment with the agonist to produce a beneficial effect. This is probably not surprising, as fibrotic diseases are slowly developing processes and the protective anti-fibrotic/anti-inflammatory mechanisms that are induced by AT2R agonists require long-term changes in gene/protein expression (see next section).

For a potential future clinical use of AT2R agonists in fibrotic diseases, an important consideration will be, whether a standard treatment for the respective disease already exists with the consequence that AT2R agonists would have to be applied “on top”, or whether AT2R agonists can potentially be given as mono-therapy. This is of importance since for several of the above-mentioned diseases – in particular fibrotic kidney diseases 68 – ARBs are standard of care, whereas an additive, beneficial effect of AT2R agonists and ARBs could not consistently be shown in preclinical disease models. Therefore, more studies testing a potential additive effect of AT2R agonists in combination with standard treatment are warranted.

**Mechanisms underlying the anti-fibrotic effect of AT2-receptor stimulation**

The intracellular signaling mechanisms underlying the anti-fibrotic action of the AT2R are multiple (Figure 2).

A key to the anti-fibrotic effect is the inhibition of expression and signaling of the profibrotic cytokine TGF-β, resulting in an attenuation of extracellular matrix (ECM; e.g. collagen) synthesis.69 This mechanism of action of AT2R-stimulation is similar to the
mechanism of action of the approved anti-fibrotic drug pirfenidone (Figure 2).\textsuperscript{70} AT\textsubscript{2}R stimulation has been shown in various animal models (i.e. pulmonary fibrosis, cardiac scar formation after MI, diabetic nephropathy, Dupuytren disease) to have a strong, inhibitory effect on pathologically induced increases in TGF-β expression at both the mRNA and protein levels, often reducing TGF-β expression back to levels seen in healthy tissue.\textsuperscript{15,48,54,67} Strong AT\textsubscript{2}R-mediated inhibition of pro-fibrotic TGF-β signaling is further indicated by the reversal of increased expression of CTGF, which is downstream of TGF-β receptor stimulation, e.g. in pulmonary fibrosis, diabetic nephropathy or Dupuytren disease.\textsuperscript{16,54,67,71} This effect seems to involve an accelerated degradation of TGF-β as well as an inhibitory effect on TGF-β synthesis by decreasing activity of the transcription factor early growth response gene-1 (EGR-1), which controls transcription of TGF-β.\textsuperscript{36,72} Since EGR-1 is also in control of synthesis of platelet derived growth factor-BB (PDGF-BB), the AT\textsubscript{2}R-mediated inhibition of EGR-1 results in an additional anti-fibrotic mechanism by attenuating PDGF-BB-induced proliferation of fibroblasts and hepatic stellate cells as well as ECM synthesis by these cells.\textsuperscript{73}

Accelerated degradation of TGF-β by AT\textsubscript{2}R-stimulation seems to involve dimerisation of the AT\textsubscript{2}R with the receptor for TGF-β, TGF-βRII.\textsuperscript{36} Dimerisation with the AT\textsubscript{1}R is also a way by which the AT\textsubscript{2}R inhibits AT\textsubscript{1}R signaling, which is pro-fibrotic.\textsuperscript{74,75} Furthermore, the AT\textsubscript{2}R forms hetero-dimers with the receptor for relaxin, RXFP1.\textsuperscript{76} RXFP1 is a drug target of current interest, exerting when stimulated anti-fibrotic effects by prevention of phosphorylation of SMAD2, resulting in inactivation and inhibition of complex formation of SMAD2 with SMAD3.\textsuperscript{77} Interestingly, signaling of RXFP1 seems to be entirely dependent on RXFP1/AT\textsubscript{2}R dimerisation, since in AT\textsubscript{2}R-deficient mice or during antagonism of the AT\textsubscript{2}R, relaxin is non-functional as an anti-fibrotic.\textsuperscript{76}
In addition to this indirect interference of the AT$_2$R with TGF-β signaling through relaxin, there are also direct mechanisms, by which AT$_2$R-stimulation inhibits effects elicited by TGF-β. Some of these mechanisms have their origin in the AT$_2$R-mediated activation of protein phosphatases, which in turn dephosphorylate various targets within pro-fibrotic signaling cascades. For example, AT$_2$R-mediated dephosphorylation inhibits ERK1/2, which is of relevance for the AT$_2$R-mediated inhibition of non-canonical, ERK1/2 dependent TGF-β signaling as shown in a model of Marfan syndrome, in which TGF-β signaling is enhanced and is part of the pathomechanism. Another example of the importance of AT$_2$R-mediated activation of phosphatases is the inhibition of receptor tyrosine kinase activity by dephosphorylation. Tyrosine kinase receptors are the prevailing type of receptor for growth factors such as PDGF-BB, fibroblast growth factor (FGF) or vascular endothelial growth factor (VEGF), which play a key role in the pathogenesis of fibrotic diseases. The therapeutic relevance of targeting tyrosine kinase receptors has become apparent in recent years due to the approval of nintedanib, a triple receptor tyrosine kinase inhibitor (inhibiting receptors for PDGF, FGF and VEGF), for the treatment of IPF (Figure 2). AT$_2$R-induced dephosphorylation and subsequent inactivation has been shown experimentally for the tyrosine kinase receptor for insulin. Experimental proof of dephosphorylation of other tyrosine kinase receptors or of the serine/threonine kinase receptors for TGF-β is still lacking, but trans-inactivation of kinase receptor coupled signaling by the AT$_2$R or its binding protein ATIP has been shown for the receptors for insulin, EGF and bFGF. Moreover, the inhibitory effect of the AT$_2$R on collagen synthesis was reported to be dependent on phosphatase activation.
In addition to the inhibitory effect on growth factor signaling, AT$_2$R-mediated activation of phosphatases and subsequent dephosphorylation has also been identified as a mechanism for inhibition of NF-κB, the key transcription factor for pro-inflammatory mediators (for more details see next paragraph).$^{31}$ and as a mechanism of eNOS activation.$^{35}$ The latter is of relevance, since eNOS activation and NO production seem essential for some AT$_2$R-related anti-fibrotic effects such as inhibition of TGF-β synthesis,$^{36}$ increase in matrix-metalloproteinase expression$^{90,91}$ or relaxin signaling.$^{76}$ Moreover, NO itself acts anti-fibrotic via generation of cGMP.$^{92}$

Apart from the above-discussed anti-fibrotic effect, i.e. inhibition of ECM formation, AT$_2$R-stimulation also alters the expression and activation status of matrix-metalloproteinases (MMPs), which are the ECM degrading enzymes, and of tissue inhibitor of matrix-metalloproteinases (TIMP). At present, the data on MMP2/9 and TIMP regulation by AT$_2$R-stimulation are controversial. While in the context of diabetic nephropathy, an increase in MMP2/9 expression was reported,$^{54}$ in hypertension-induced cardiac fibrosis in SHRsp, expression was unchanged,$^{49}$ and in myocardial peri-infarct tissue$^{48}$ and atherosclerotic aorta,$^{64}$ pathologically increased expression and activity of MMP2/9 were reduced to baseline levels by AT$_2$R-stimulation or –overexpression. In the post-MI model, TIMP expression was enhanced.$^{48}$ These discrepancies may be partly due to a different pathological context of the fibrotic process, which can be either persistent reactive (in diabetic nephropathy, atherosclerosis, hypertensive cardiac fibrosis) or reparative (in cardiac peri-infarct tissue).$^{14}$ However, the decrease in MMP2/9 expression and activity in the atherosclerotic vessel does not adhere to this concept. Another explanation may be that in general, MMPs seem to have effects distinct from ECM degradation in the fibrotic process, such as a direct impact on synthesis of collagen or TGF-β.$^{93,94}$ Since
identification and understanding of these additional features are only at a very early stage, conclusions on functional consequences of changes in expression or activity of MMPs are generally difficult.

**Mechanisms underlying the anti-inflammatory effect of AT₂-receptor stimulation**

Similar to the anti-fibrotic effect of AT₂R-stimulation, the anti-inflammatory effect of the AT₂R, which may contribute to the prevention of subsequent development of fibrosis, seems very much dependent on activation of phosphatases and thereby crosstalk with pro-inflammatory signaling pathways (Figure 3). For example, as shown by others and us, activation of tyrosine and serine/threonine phosphatases is mandatory for the AT₂R-mediated inhibition of activity of the transcription factor for pro-inflammatory cytokines and for cyclooxygenase 2 (COX-2, the enzyme responsible for synthesis of pro-inflammatory prostaglandins), NF-κB. Wu et al described that AT₂R-stimulation induced dephosphorylation of inhibitory κB (I-κB) thus stabilising I-κB and reducing the levels of NF-κB unbound to I-κB, which is the fraction of NF-κB that can translocate to the nucleus and initiate transcription. Moreover, our own group demonstrated that the AT₂R-mediated reduction of NF-κB translocation to the nucleus is significantly attenuated in the presence of tyrosine- or serine/threonine-phosphatase inhibitors. Another pro-inflammatory signaling pathway, which is initiated by cytokines and leads to the synthesis of more cytokines, is the Janus kinases (JAK)/ Signal Transducer and Activator of Transcription proteins (STAT) pathway. A key step in this pathway is phosphorylation of STAT at tyrosine residues, which eventually enables STAT to translocate to the nucleus and act as transcription factor. As shown by Horiuchi et al, AT₂R-stimulation prevents phosphorylation of STAT1α/β, STAT 2 and STAT3 by activation of tyrosine phosphatases resulting in reduced transcriptional activity of
In addition, STAT phosphorylation at serine/threonine residues is also inhibited as a result of ERK1/2 inhibition through AT2R-stimulation. Finally, AT2R-mediated inhibition of ERK1/2 has been shown by various groups. Generally, ERK1/2 inhibition results in less activity of the transcription factor AP-1, which again leads to an attenuation of cytokine synthesis. This cascade of events has also been demonstrated to occur in response to AT2R-stimulation; specifically in tubular epithelial cells in vitro and in vivo, indirect AT2R-stimulation under AT1R-blockade led to dephosphorylation of ERK, which resulted in reduced mRNA levels of monocyte chemoattractant protein-1 (MCP-1). In vivo, the reduced levels of MCP-1 translated into attenuated interstitial inflammation and fibrogenesis. The significant role of indirect AT2R-stimulation and subsequent p-ERK dephosphorylation for the anti-inflammatory effect was proven in this study by the absence of ERK dephosphorylation / MCP-1 reduction / anti-inflammation in AT2R-deficient mice treated with an ARB. Moreover, specific knockdown of ERK in tubular epithelium by anti-sense oligodeoxynucleotides led to a similar effect as indirect AT2R-stimulation.

Clinical perspectives

The treatment of fibrotic diseases is still one of the largest unmet medical needs. There are only two drugs, nintedanib and pirfenidone, which have been recently approved for this group of diseases (specifically for IPF). However, both of these drugs have limited efficacy (no proven life prolongation) and at the same time quite serious side effects. Therefore, it is important to identify new potential drug targets with anti-fibrotic potential. The AT2R may be such a target, because – as reviewed above – it exerts anti-fibrotic effects in a multimodal way, i.e. by stimulation of the AT2R, various anti-fibrotic mechanisms are activated which in an orchestrated way prevent the...
formation of or reduce prevalent fibrosis. Several of these molecular mechanisms have been identified experimentally, and in multiple preclinical studies in various disease models and organs, it has been proven that the mechanisms translate into the \textit{in vivo} situation. However, clinical development of AT$_2$R-agonists is still at early stages [successfully finalised Phase I clinical studies for C21 (www.vicorepharma.com) and MOR107 (www.morphosys.com)], and clinical data on the translatability of the promising preclinical anti-fibrotic effects into the human situation won’t be available before 2020 at the earliest.

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**CONFLICT OF INTEREST**

UMS was part-time employed by Vicore Pharma, the company that owns C21, from September 2017 until August 2018.

The other authors have no conflicts to declare.
REFERENCES


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FIGURE LEGENDS:

**Figure 1:** Fibrotic diseases in which AT2-receptor stimulation exerts beneficial actions. This scheme illustrates the fibrotic diseases, on an organ-by-organ basis, where AT2R activation by selective AT2R agonists produces beneficial anti-fibrotic /anti-inflammatory actions. The width of the arrows to the organs indicates where there is more evidence (kidney) versus less evidence (pancreas; skin) for beneficial effects. AT2R = Angiotensin AT2 receptor

**Figure 2:** AT2-receptor coupled signalling pathways resulting in an anti-fibrotic effect: This figure illustrates the multi-modal way by which AT2R-coupled signalling mediates anti-fibrotic effects.

Red lines depict pro-fibrotic pathways; green lines depict anti-fibrotic (and AT2R-induced) signalling pathways. Vertical green arrows in boxes indicate the effect of AT2R-stimulation on the respective, individual molecule. Note the central role of protein phosphatase activation (dark blue boxes) and subsequent dephosphorylation of various target proteins.

For a detailed explanation, please refer to the respective paragraph of the article.

Abbreviations: Akt = Rac-alpha serine/threonine protein kinase; AT2R = Angiotensin AT2 receptor; ATIP = Angiotensin AT2 receptor Interacting Protein; CTGF = Connective Tissue Growth Factor; cGMP = cyclic guanosine monophosphate; ECM = extracellular matrix; EGR-1 = Early Growth Response Gene 1; eNOS = endothelial nitric oxide synthase; ERK1/2 = extracellular signal-regulated kinase 1/2; FGF = Fibroblast Growth Factor; MKP-1 = mitogen-activated protein kinase phosphatase 1; MMP9 = Matrix Metalloproteinase 9; NO = Nitric Oxide; PDGF-BB = Platelet Derived Growth Factor BB; PP2A = Protein Phosphatase 2A; SHP-1 = Src homology region 2 domain-containing
phosphatase-1; SMAD 3/4 = Mothers against decapentaplegic homolog 3/4; TGFβ = Transforming Growth Factor beta; TGFβRII = Transforming Growth Factor beta receptor II; RXFP-1 = Relaxin Family Peptide Receptor 1; VEGF = Vascular Endothelial Growth Factor;

Figure 3: AT2-receptor coupled signalling pathways resulting in an anti-inflammatory effect: This figure illustrates, how AT2R-coupled signalling mediates anti-inflammatory effects. Red lines depict pro-inflammatory pathways; green lines depict anti-inflammatory (and AT2R-induced) signalling pathways. Vertical green arrows in boxes indicate the effect of AT2R-stimulation on the respective, individual molecule. As in anti-fibrotic signalling, protein phosphatase activation (dark blue boxes) and subsequent dephosphorylation of various target proteins plays a pivotal role for the AT2R-mediated effect.

For a detailed explanation, please refer to the respective paragraph of the article.

Abbreviations: Akt = Rac-alpha serine/threonine protein kinase; AT1R = Angiotensin AT1 receptor; AT2R = Angiotensin AT2 receptor; ATIP = Angiotensin AT2 receptor Interacting Protein; COX-2 = Cyclooxygenase 2; cGMP = cyclic guanosine monophosphate; eNOS = endothelial nitric oxide synthase; ERK1/2 = extracellular signal-regulated kinase 1/2; I-κB = NF-kappa-B inhibitor; JAK = Janus kinase; MKP-1 = mitogen-activated protein kinase phosphatase 1; NF-κB = Nuclear factor κB; NO = Nitric Oxide; PP2A = Protein Phosphatase 2A; SHP-1 = Src homology region 2 domain-containing phosphatase-1; STAT = signal transducer and activator of transcription protein

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<th>Organ &amp; Disease</th>
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<td>Lung – Pulmonary Fibrosis (PF)</td>
<td>SD Rat 6-weeks old, MCT-treated</td>
<td>C21 0.03 mg/kg/day, i.p., for 2 weeks: began 2 weeks after MCT-induced PF. (T)</td>
<td>Prevented and reversed pulmonary interstitial and peri-vascular fibrosis in a monocrotaline model.</td>
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<td>SD Rat 6-weeks old, BLEO-treated</td>
<td>C21 0.03 mg/kg/day, i.p., began on the same day (P) or 3 d after (T) BLEO-induced PF, for 14 d.</td>
<td>Attenuated the formation and abolished the progression of pulmonary fibrosis in a BLEO model.</td>
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<td>Acute Lung Injury</td>
<td>SD Rat 300-350 g Pulmonary lavage</td>
<td>C21 0.03 mg/kg/day, i.p., one hour before induction of lung failure. (P)</td>
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<td>Heart – Cardiac Fibrosis</td>
<td>Wistar Rat 200-220 g MI induced via LCA ligation</td>
<td>C21 1.0 mg/kg/day, i.p., began at MI induction, for 6 weeks. (P)</td>
<td>Decreased collagen content in peri-infarct cardiac fibrotic tissue post-MI. Decrease in myocardial TGF-β1 mRNA and protein.</td>
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<td>SHR-Rap Rat 6-weeks old</td>
<td>C21 1.0 mg/kg/day, in chow, and/or Losartan 10 mg/kg/day in drinking water, or a combination of the two, for 6 weeks. (P)</td>
<td>C21- or Losartan alone reduced hypertension-induced subendocardial fibrosis. C21 + Los combo also reduced myocardial and subepicardial fibrosis. C21 did not alter blood pressure.</td>
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<td>Kidney – Diabetic Nephropathy</td>
<td>Zucker Diabetic Fatty Rat 6-weeks old (Genetic model of T2D)</td>
<td>C21 0.3 mg/kg/day, s.c. and/or Losartan 10 mg/kg/day in drinking water, for 15 weeks. (P)</td>
<td>C21 completely prevented the development of glomerular, tubulointerstitial and perivascular fibrosis associated with T2DN. C21 + Los combo tended to reduce glomerular and perivascular fibrosis in an additive manner.</td>
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<td>Wistar Rat 180-180 g (T2DN induced by HFD/low dose STZ for 24 d)</td>
<td>C21 0.3 mg/kg/day, p.o. and/or Telmisartan 10 mg/kg/day, p.o., for 2 weeks. (T)</td>
<td>C21 or Telmisartan alone attenuates renal dysfunction, morphological changes, fibrosis and inflammation associated with T2DN. C21 + Telmisartan combo produced additive effects.</td>
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<td>ApoE-/- mice 6-weeks old (T1D induced by daily STZ for 5 d)</td>
<td>C21 1.0 mg/kg/day, p.o., for 20 weeks. (P)</td>
<td>C21 decreased mesangial area, albuminuria &amp; glomerulosclerosis in T2DN; also lowered markers of inflammation and pro-fibrotic mediators.</td>
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