Anti-TGF-β effects of telmisartan occurs independent of the angiotensin II receptor

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# Table of Contents

Abstract .................................................................................................................................................. 2

Abbreviations ........................................................................................................................................ 3

Introduction ............................................................................................................................................ 4

Method .................................................................................................................................................. 10
  Mitral valve tissue .............................................................................................................................. 10
  Isolation and culture of VIC .............................................................................................................. 10
  Immunofluorescence .......................................................................................................................... 10
  Quantitative real-time polymerase chain reaction (PCR) ................................................................. 11
  Western blotting .................................................................................................................................. 11
  Antibodies ........................................................................................................................................... 11
  Chemical inhibitors and pharmaceuticals ............................................................................................ 12
  Statistical analysis .............................................................................................................................. 12

Results .................................................................................................................................................. 13
  Evaluating the properties of VIC isolates .......................................................................................... 13
  Evaluation of TGF-β response between different VIC-lines .............................................................. 14
  TGF-β activated p38 but had no effect on JNK1 and PI3K ............................................................... 16
  Telmisartan inhibited TGF-β induced COL1A1 and ELN expression ............................................. 16
  Dissecting the effects of telmisartan and AT1 blockage on signaling .............................................. 17
  Potential role of PPAR-γ ................................................................................................................... 18

Discussions .......................................................................................................................................... 19

Conclusions ......................................................................................................................................... 21

Acknowledgments .............................................................................................................................. 22

References ........................................................................................................................................... 23

Supplemental data .............................................................................................................................. 26
Abstract

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Introduction
Mitrval valve prolapse (MVP) is the most common cardiac valve abnormality in the industrialized world. Dysregulation of TGF-β signaling has been implicated in myxomatous degeneration where valvular interstitial cells (VIC) appear to play a primary role. Angiotensin receptor blockers (ARB) are primarily pharmacological target for angiotensin II receptor type 1 (AT1) but ARBs also modulate TGF-β signaling through an uncharacterized mechanism. ARB such as telmisartan can induce peroxisome proliferator-activated receptor (PPAR)-γ activity but known PPAR-γ agonists have also been shown to modulate TGF-β signaling. Our hypothesis is that telmisartan modulates TGF-β signaling through PPAR-γ independently of AT1.

Materials and methods
Operative specimens were obtained from 8 MVP patients undergoing mitral valve repair. VICs were isolated and propagated. Immunofluorescence, western blotting and quantitative real time PCR were used in the experiments to evaluate the anti-TGF-β effects of telmisartan.

Results
The VICs were positive for vimentin, SM22α and α-smooth muscle actin compared to smooth muscle cells (SMC). The same cells were negative for angiotensin II receptors type 1 and type 2 (AT1 and AT2) compared to SMCs and fibroblasts. The TGF-β activated p38 but had no effect on the activation on JNK-1 or PI3K, which were constitutively active. Telmisartan significantly decreased TGF-β induced collagen 1 (COL1A1) and elastin (ELN) expression in the VICs and the PPAR-γ agonist, PGJ2, did the same. However, the PPAR-γ antagonist GW9962 did not inhibit the anti-TGF-beta effects of telmisartan on COL1A1 and ELN expression. The PPAR-γ agonist pioglitazone did not significantly decrease TGF-β induced COL1A1 and ELN expression. TGF-β activated Smad2 independently of telmisartan and AT1. The ERK and p38 signaling pathways appeared to be activated by telmisartan but independent of AT1.

Conclusion
TGF-beta activates Smad2 and p38 in cultured VIC and induces extracellular matrix expression. VICs do not bind angiotensin II possibly due to absence of AT1 and AT2. Telmisartan effectively inhibits TGF-beta signaling independent of AT1, which appears to occur through PPAR-γ, but needs further verification.
Abbreviations

Ang II: Angiotensin II alexa fluor® 488 conjugate
ARB: Angiotensin receptor blocker
AT1: Angiotensin II receptor type 1
AT2: Angiotensin II receptor type 2
COL1A1: Collagen 1
aVIC: Activated valvular interstitial cell
ECM: Extracellular matrix
ELN: Elastin
FB: Fibroblast
MVP: Mitral valve prolapse
PCR: Polymerase chain reaction
PPAR: Peroxisome proliferator-activated receptor
qVIC: Quiescent valvular interstitial cell
SMC: Smooth muscle cell
TGF-β: Transforming growth factor – β
VIC: Valvular interstitial cell
Introduction

Mitral valve prolapse (MVP) is a pathological condition where a diseased, usually thickened, mitral valve prolapses into the left atrium during the systolic phase of the cardiac cycle. It is further defined as displacement of leaflet tissue superiorly into the left atrium past the mitral annular plane[1]. It is a primary form of myxomatous degeneration and is the most common cardiac valve abnormality in the industrialized world. Early estimates suggested that the prevalence ranged from 5-15% of the general population, but recent studies suggest this to be an overestimation and the true lifetime prevalence is estimated to be 0.6-2.4%[2].

The mitral valve prevents backward flow of the blood from the left ventricle to the atrium during systole. When the pressure in the left ventricle decreases the mitral valve opens and enables the blood to fill the ventricle during diastole. About 80% of the blood flow fills the ventricle during the period of rapid filling of the ventricle before the atrium contracts. The contraction of the atrium adds the remaining 20% of the blood flow. When the myocardium in the ventricle contracts and the pressure rises in the left ventricle the mitral valve closes so that the blood will not flow back in to the atrium[3].

The complexity of the mitral valve anatomy and histology are reflected in the various structures necessary to maintain normal function of the mitral valve. The structures include the leaflets, the annulus, the chordae tendinae, the papillary muscles, the left ventricle and the left atrium. The mitral valve itself is made of two leaflets, anterior and posterior. The posterior leaflet is made of three distinct scallops, lateral, middle and medial, the middle scallop is often the largest but there can be individual variability[1]. Three well-defined tissue layers, fibrosa, spongiosa and atrialis make up each leaflet of the mitral valve. The fibrosa, the layer facing the ventricle, is mainly composed of densely packed collagen fibers that are arranged in parallel. The spongiosa, the middle layer, is composed of loosely arranged collagen and proteoglycans. The atrialis, the layer facing the atrium, is composed of elastin fibers[4].

MVP is often benign and sometimes presents with a lone click (although MVP can be without any heart murmurs) on auscultation because of a sudden tension of the diseased valves and chordae tendineae as the valves attempt to close. This should be confirmed by a two-dimensional echocardiography but the specificity of this echocardiogram is not absolute. Other symptoms (atypical chest pain, exertional
dyspnea, palpitations, syncope, and anxiety) and clinical findings (low blood pressure, leaner build, and electrocardiographic repolarization abnormalities) may also be associated with MVP and is then called MVP syndrome[5].

MVP is a leading cause of isolated mitral regurgitation but other severe complications include infective endocarditis, stroke, sudden death, chordal rupture, arrhythmias and heart failure. Some patients with MVP develop one of the severe complications, like severe mitral regurgitation, congestive heart failure or sudden death and the majority of them are 45 years or older[6]. Those with severe mitral regurgitation may need surgery (mitral valve repair or replacement), which is done with open-heart surgery or a minimally invasive approach and is a major intervention for the body. There is no medication that prevents progression of MVP and the only approved method for treating severe MVP is surgical intervention[7]. It has been demonstrated that mitral valve repair is the optimal surgical treatment for patients with severe mitral regurgitation rather then mitral valve replacement when that approach is possible. When the two methods are compared the repair shows lower perioperative mortality, improved survival, better presentation of postoperative left ventricle function and lower long-term morbidity[8].

Although interest in techniques for minimally invasive approach for mitral valve surgery is growing it has still not shown its superiority over the classic approach. The minimally invasive techniques appear to be associated with decreased bleeding, atrial fibrillation, sternal wound infection, shorter intensive care unit stay, reduced time to return to normal activity and more. However, these minimally invasive techniques may also come with increased risk of stroke, aortic dissection or aortic injury, phrenic nerve palsy, increased cross clamp and procedure time and more. These methods are currently under investigation[9].

Initial clinical assessment for patients with mitral regurgitation reviews symptoms of heart failure and physical signs of severe mitral regurgitation[7]. Although these signs can be helpful to evaluate the severity of the mitral regurgitation it is not sufficiently specific. Echocardiography is the primary method of evaluation and provides crucial information like the assessment of severity, mechanism of the regurgitation, reparability and consequences of the mitral regurgitation. Indication for surgery is for symptomatic patients with acute mitral regurgitation and for patients with symptoms from chronic mitral regurgitation and have no contraindications to surgery. For patients that have asymptomatic mitral regurgitation each case needs to be evaluated individually for suitability for surgery[8].
The pathology of MVP may be considered from the macroscopic and microscopic perspectives. Macroscopic changes of the affected leaflets are enlargement, thickening, redundancy and rubbery texture. The chordae tendineae may be elongated, thinned and sometimes ruptured[10]. Histological changes include expansion of the spongiosa with accumulation of proteoglycans, collagen fragmentation in the fibrosa and fragmentation of elastin in the atrialis. There is also evidence that these myxomatous degenerated heart valves have increased expression of proteolytic enzymes and activated myofibroblast-like cells called valvular interstitial cells (VIC). This suggests a dysregulation of matrix metabolism that modulates the abnormalities in collagen and other extracellular matrix (ECM) components[4].

Although the histologic changes are well known, the pathogenesis of myxomatous degeneration is not well understood. MVP seems to be more common in individuals with certain genetic mutations or in some families. Sometimes MVP shows familial pattern and better understanding of three-dimensional mitral valve shape with 3D echocardiogram has improved diagnostic specificity. Because of this increased diagnostic specificity it was possible to do genetic studies for this disease. Both idiopathic and nonsyndromic familial myxomatous MVP has been linked to chromosome 16 (MMPVI). Autosomal dominant form of familial MVP has been linked to MMVP2 on chromosome 11p15.4 and 13q31.3-q32.1. Although MVP has been linked to several genetic mutations it is thought that the condition is rather the end result from a multiple genetic and molecular pathways than a condition that can be linked to one specific mutation. Genetic disorders such as Marfan syndrome can point out the importance of certain pathways in the pathogenesis of this condition[11].

Although MVP is not as common as aortic root dilation and ectopia lensis in Marfan syndrome, many such patients will need a mitral valve replacement or repair in their lifetime. Variability exists in families with the same mutation in the prevalence in MVP, mitral regurgitation and mitral valve thickening. Individuals with Marfan syndrome have heterozygous FBNI mutation (dominantly inherited), which leads to systemic pleiotropic manifestations in the connective tissue. This leads to upregulation of transforming growth factor (TGF)-β in the tissues that are affected. The affected protein from the mutation, fibrillin-1, is similar to latent TGF-β binding proteins, which are the proteins that bind the protein precursors for TGF-β and thus preventing too much TGF-β signaling. It is thought that fibrillin-1 binds to the precursor TGF-β proteins and stabilizes them so when this protein is defected,
because of the *FBNI* mutation, there is less regulation of TGF-β signaling[12]. Treatment of TGF-β neutralizing antibody in mice with *fbn1* mutation (which shows similar phenotype as Marfan syndrome in humans) successfully normalized the mitral valve in the mice[13]. This supports the theory that myxomatous degeneration might partly be caused by increase in TGF-β signaling[12].

Both syndromic and sporadic MVP seems to be linked to TGF-β, but it stimulates activity and matrix formation by the VICs[11]. The VICs are the main cells in the mitral valve and other heart valves and they are responsible for maintaining the structural integrity of the heart valve. These VICs can be divided into 5 phenotypes but only two of them will be mentioned here, the quiescent VICs (qVIC) and the activated VICs (aVIC). The qVICs are the cells that are thought to maintain the normal function and structure of the heart valve. They are also thought to regulate low-grade matrix synthesis and degradation, but this is yet to be confirmed accurately. Normal VICs in a human adult are thought to be quiescent but the mitotic rate is unknown. It appears that a healthy qVIC can transform into an aVIC when the valve undergoes pathological injury or abnormal hemodynamic/mechanical stress. qVIC activation can also occur through stimulation from certain growth factor stimulation[14]. aVICs have some features of myofibroblasts, like increased contraction, prominent stress fibers and other contractile proteins. The marker for aVICs is α-smooth muscle actin, which is absent, or found in a much lower quantities in qVICs (relative to aVICs)[15]. Activation of the qVICs is associated with features that are seen in the wound healing process, such as increased extracellular matrix secretion and degradation, expression of metalloproteinases and tissue inhibitors of matrix metalloproteinases[14]. In addition, aVICs increase their secretion of cytokines and the most notable of these is increased secretion of TGF-β[16]. In a normal heart valve most aVICs go through apoptosis but when an imbalance of this process occurs they continue to be active in the heart valve. Dysfunction in apoptosis and imbalance in extracellular matrix production may therefore give rise to cardiac valve disease and be an important therapeutic target for treating or preventing cardiac valve disease[14].

TGF-β is a superfamily of proteins that initiates their responses through binding to and activating a cell surface receptor, which has intrinsic serine/threonine kinase receptors. Each member of the TGF-β superfamily binds both to type 1 and 2 receptors in a specific combination. When the ligand binds to the receptors type 1 and 2, the receptors assemble in complexes in which the type 2 receptor phosphorylates the type 1 receptor. This is essential for TGF-β signaling because now the type 1
receptor is activated. The activated type 1 receptor kinase subsequently transduces its signals through the phosphorylation of receptor-regulated Smads[17]. In cultured VICs TGF-β seems to regulate extracellular matrix production that is similar to wound healing and proliferation through Smad2 and Smad3[14] but also through p38 signaling but seems to be independent of ERK signaling[18]. TGF-β has a significant effect on the extracellular matrix: it increases cell differentiation without apoptosis, increases the expression on α-smooth muscle actin and promotes stress fiber formation[19]. This is consistent with a myxomatous degeneration so this might suggest that TGF-β is connected to the pathogenesis of myxomatous degeneration and thus to MVP[4].

Telmisartan and losartan are angiotensin II receptor blockers (ARB), which specifically blocks angiotensin II receptor type 1 (AT1)[20]. Reduction of vascular resistance is desirable for patients with mitral regurgitation and losartan has been implicated with decrease in mitral regurgitation among patients with diagnosed mitral regurgitation[21]. Losartan has been shown to block TGF-β signaling and it has proved to be beneficial for treating aortic aneurysms in patients with Marfan syndrome. ARBs do not stop aortic growth but they significantly reduce the pathologic rate of increase in the diameter of the aorta[22]. The cause for these effects is believed to be the result of the ability of ARBs to modulate TGF-β signaling[23]-[24].

Clinical trials demonstrate that losartan lowers the risk for type II diabetes compared with other antihypertensive treatments[25]. There is also evidence that ARBs increase insulin sensitivity in rats[26], which suggests that ARBs somehow influence the regulation of insulin sensitivity. Studies show that ARBs can induce the activity of peroxisome proliferator-activated receptor (PPAR)-γ independent from the effect of the angiotensin II receptor blockage[27]-[28]. The PPAR-γ regulates adipocyte differentiation, fatty acid storage and glucose metabolism. PPARs are heterodimers, which in association with co-activator complex, binds to peroxisome proliferator responsive elements in the promoter region of a target gene. Activation of PPAR (that is, when the ligand binds to the PPAR) leads to transactivation and transexpression of selected genes. PPAR-γ is expressed in many organs in the body including the heart, muscle, kidneys and more. PPAR-γ agonists improve insulin resistance and because of these effects PPAR-γ is a target for antidiabetic drugs. Thiazolidinedione, which is a PPAR-γ agonist, was the first drug of it’s kind but later rosiglitazone and pioglitazone were also available[29].
In recent studies PPAR-γ agonists have been shown to inhibit TGF-β induced expression of chemokines in renal tubular epithelial cells[30]. Furthermore PPAR-γ agonists have also been implicated with decrease in TGF-β stimulated myofibroblast development and collagen production in pulmonary fibrosis[31]. This suggests that PPAR-γ activation might be beneficial for inhibiting TGF-β induced ECM components expression, like collagen and elastin, in VICs. Modulation of TGF-β signaling with ARB has shown to attenuate TGF-β induced basal ECM production in cultured mitral valve tissue[18]. However the mechanism of that modulation of TGF-β signaling is not quite clear.

In summary MVP is a disorder involving the mitral valves with a lifetime prevalence of 2% in western culture. MVP is a leading cause of isolated mitral regurgitation and is associated with multiple cardiac complications. MVP is a primary form of myxomatous degeneration, which involves expansion of tissue layers, fragmentation of collagen and elastin, increased expression of proteolytic enzymes and activation of VICs. The etiology of MVP is unknown but there are indications that dysregulation of TGF-β signaling is an important part of the pathogenesis. Previous studies have confirmed that TGF-β regulates ECM synthesis in VICs and that TGF-β induced effects on ECM components can be inhibited with ARB treatment. There is evidence that ARBs can induce the activity of PPAR-γ independent of AT1 but PPAR-γ agonists can modulate TGF-β signaling. We hypothesized that telmisartan mediated modulation of TGF-β signaling occurs through PPAR-γ independent of AT1.
Method

Mitral valve tissue

Human tissues were obtained using research protocols approved by the institutional review boards of Yale University, Veterans Affairs Connecticut Healthcare System and/or the New England Organ Bank. Myxomatous mitral valve tissue was obtained from patients undergoing repair for severe mitral regurgitation with resection of the central scallop of the posterior leaflet. A total of 8 diseased tissue samples were collected. Experienced cardiac surgeons (Arnar Geirsson, Sabet Hashim and George Tellides) determined the diagnosis of myxomatous mitral valve disease by gross inspection and visually excluded other mitral valve pathology including stenosis, calcific degeneration and endocarditis as well as any uncertain diagnosis of myxomatous degeneration. A portion of tissue was submitted to surgical pathology confirming myxomatous degeneration changes in all samples used. Tissues were placed immediately in cold saline on ice and processed.

Isolation and culture of VIC

VIC were isolated by digesting mitral valve tissue in 125 U/mL collagenase XI, 60 U/mL hyaluronidase I, 60 U/mL DNase 1, and 450 U/mL collagenase I (Sigma-Aldrich) in PBS at 37 °C for 3 hours. They were passed through a 70-μm cell strainer and then suspended and cultured in M199 medium supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen).

The VICs were treated with TGF-β (1 ng/ml) for 30 or 60 minutes for signaling studies and 18 hours for expression analysis. The VICs were treated with PPAR-γ agonists or antagonist for 24 hours for expression analysis but with different concentrations: pioglitazone (10 μM), PGJ2 (10 μM) and GW9962 (2 μM). Telmisartan treatment lasted 1 hour both for expression analysis and signaling studies.

Immunofluorescense

The cells were cultured on a cover glass slides and were fixed in 2 ml of PFA (pH 7.0 NaOH in PBS) for 15 min and then washed 1x with PBS. The cells were permeabilized by incubating the cells for 15 min on ice with 2 mL of 0.1% Triton X-100 in PBS and then washed 3x in PBS. The cells were incubated for 1 hour in blocking buffer (2% BSA in PBS) and then incubated in primary antibody in blocking
buffer (in dilution) at 4°C overnight. The next day the cells were washed for 5 minutes x 5 and then incubated with fluorescence-conjugated secondary antibody in blocking buffer in a dark humidity chamber at 4°C for 1 hour. The cells were washed for 5 minutes x 6 in PBS and the samples were mounted by inverting the cover slides with the cultured cells onto mounting medium on glass slides. The samples were then analyzed using a microscope with Axiovert 200M microscopy system with AxioVision 4.6 software (Carl Zeiss MicroImaging). The pictures were further analyzed by using the software Volocity.

Antibodies that were used for IF were as follows: α-smooth muscle actin, vimentin, SM22α and Angiotensin II alexa fluor® 488 conjugate.

**Quantitative real-time polymerase chain reaction (PCR)**

RNA was extracted from cultured VICs by using RNeasy mini kits (Qiagen) with on-column DNA digestion according to the manufacturer’s protocol. Reverse transcription was performed using Multiscribe RT reagents (Applied Biosystems). Quantitative real-time PCR was performed using commercially available Applied Biosystem probes (COL1A1, ELN, GAPDH), Taqman Mastermix, and a thermal cycler (iQ5; Bio-Rad Laboratories). Samples were analyzed in duplicate and target gene expression was normalized to GAPDH.

**Western blotting**

Proteins were extracted from cultured VICs and separated by 5% and 10% SDS-PAGE. Immunoblotting was performed in standard fashion using monoclonal antibodies to SM22α, p38, P-p38, PI3K, P-PI3K, JNK1, P-JNK1, ERK, P-ERK, Smad2 and p-Smad2. β-actin was used as a loading control.

**Antibodies**

The following antibodies were used: α-smooth muscle actin (Sigma), vimentin (Santa Cruz), SM22α (Santa Cruz), Smad2 (Cell Signaling Technology), p-Smad2 (Cell Signaling Technology), PI3K (Santa Cruz), p-PI3K (Santa Cruz), p38 (abcam), p-p38 (Santa Cruz), ERK (Cell Signaling Technology), p-ERK (Cell Signaling Technology), JNK1 (Cell Signaling Technology), p-JNK1 (Santa Cruz), Anti-Angiotensin II Type 1 Receptor antibody (abcam) and Angiotensin II alexa fluor® 488 conjugate (Invitrogen).
Chemical inhibitors and pharmaceuticals

The following pharmaceuticals were used: TGF-β (R&D system), telmisartan (Sigma), PGJ2 (Cayman chemicals), pioglitazone (Cayman chemicals) and GW9962 (Cayman chemicals).

Statistical analysis

Unpaired t-test was used to compare means of two experimental groups; the p values were two-tailed and p values <0.05 were considered to indicate statistical significance. Data presented are mean ± S.E.M. Statistical analyses were performed using Prism 5 (GraphPad Software).
Results

Evaluating the properties of VIC isolates

Immunofluorescence was performed to determine the properties of the VICs. The cells were positive for vimentin, α-smooth muscle actin and SM22α compared to smooth muscle cells (SMC; Figure 1A-C). There was no binding of the Angiotensin II alexa fluor® 488 conjugate (Ang II) in the VICs compared to SMCs and fibroblasts (FB) and suggesting that the cells are negative for AT1 and angiotensin II receptor type 2 (AT2) (Figure 1D).

Figure 1

Figure 1. Immunofluorescence photos to evaluate the properties of cultured valvular interstitial cells (VIC). Scale bar is 62 µm. Negative control is in the lower right corner. (A) Immunofluorescence of VIC for vimentin with smooth muscle cells (SMC) serving as a positive control. (B) Immunofluorescence of VICs for α-smooth muscle actin with SMCs serving as a positive control. (C) Immunofluorescence of VICs for SM22α with SMCs serving as a positive control. (D)
Immunofluorescence of VICs for Angiotensin II alexa fluor® 488 conjugate (Ang II) with SMCs and fibroblasts (FB) serving as positive controls.

**Evaluation of TGF-β response between different VIC-lines**

In total, 4 cell lines were used in the experiments and all were treated for 18 hours with 1 ng/ml TGF-β. Then the cells were harvested and quantitative real-time PCR was performed to evaluate and compare their response. In 3 cell lines (VIC 12/16/11, VIC 12/22/11 and VIC 12/24/11) significant increase (p<0.05) in both in expression of collagen 1 (COL1A1) and elastin (ELN) was observed (Figure 2A-C). In one cell line (VIC 1/17/12) significant increase (p<0.05) in COL1A1 expression was observed but not in ELN expression (Figure 2D).
Figure 2. TGF-β induced expression of collagen 1 (COL1A1) and elastin (ELN) in 4 separate cultured valvular interstitial cell (VIC) lines. Relative expression was determined by quantitative real-time PCR and target gene expression was normalized to GAPDH. (A) The cell line VIC 12/16/11 showed significant increase (p<0.05) in TGF-β induced COL1A1 and ELN expression. (B) The cell line VIC 12/22/11 showed significant increase (p<0.05) in TGF-β induced COL1A1 and ELN expression. (C) The cell line VIC 12/24/11 showed significant increase (p<0.05) in TGF-β induced COL1A1 and ELN expression. (D) The cell line VIC 1/17/12 showed significant increase (p<0.05) in TGF-β induced COL1A1 and ELN expression.
expression. (D) The cell line VIC 1/17/12 showed significant increase (p<0.05) in TGF-β induced COL1A1 expression but not in ELN expression. *p<0.05, **p<0.01, ***p<0.001.

**TGF-β activated p38 but had no effect on JNK1 and PI3K**

To evaluate the effects on p38, JNK1 and PI3K the VICs were treated with TGF-β for 180, 60, 30 and 15 minutes in the concentration of 1 ng/ml. The cells were also treated with concentrations 0.1 ng/ml, 0.3 ng/ml, 1 ng/ml and 3 ng/ml for 60 minutes. The cells were harvested and western blot was performed to assess the effects of the TGF-β treatment.

TGF-β treatment had no effect on the activation of JNK1 and PI3K because these kinases were constitutively active (Figure 3). TGF-β activated the p38 kinase and showed the optimal time for treatment to be 60 minutes, but there was no apparent difference in the activation between the concentrations (Figure 3).

**Figure 3**

![Figure 3](image-url)

**Figure 3.** Dose- and time response to TGF-β, demonstrated by western blots for phosphorylated JNK1, p38 and PI3K in cultured valvular interstitial cells (VIC). Dose response was performed at 1 hour; time response at 1 ng/ml. Beta-actin served as loading control.

**Telmisartan inhibited TGF-β induced COL1A1 and ELN expression**

To evaluate the inhibition of telmisartan on TGF-β induced expression on COL1A1 and ELN, quantitative real-time PCR was performed. After 1 hour treatment with telmisartan with different concentrations (10, 20, 50 and 100 µM) followed by 18 hour treatment of TGF-β, the cells were harvested. Significant inhibition (p<0.05) was observed for both COL1A1 and ELN expression when the telmisartan concentration reached 50 and 100 µM respectively.
Figure 4. Demonstration of the anti-TGF-β effects of telmisartan on TGF-β induced expression of collagen 1 (COL1A1) and elastin (ELN) in valvular interstitial cells (VIC). Relative expression was determined by quantitative real-time PCR and target gene expression was normalized to GAPDH. *p<0.05, ***p<0.001.

Dissecting the effects of telmisartan and AT1 blockage on signaling

To evaluate if the effects of telmisartan on the TGF-β pathways were altered by AT1 blockage, western blot was performed (Figure 5A-B). The VICs were treated first with AT1 antibody for 30 minutes, then with telmisartan for 60 minutes and finally with TGF-β for 30 or 60 minutes. Telmisartan decreased slightly the TGF-β induced activation of Smad2 but independent from AT1 blockage (Figure 5A). Telmisartan appeared to increase p38 activation (Figure 5B) but this activation decreased with AT1 blockage. ERK activation was observed in samples treated with telmisartan (Figure 5B).

Figure 5.

A

| TGF-β (30 min) | + | + | + | + | - |
| Telmisartan | - | - | - | - | - |
| AT1 block | - | - | - | - | - |
| Smad2 | - | - | - | - | - |
| P-Smad2 | - | - | - | - | - |
| Beta-actin | - | - | - | - | - |

B

| TGF-β (60 min) | + | + | + | + | - |
| Telmisartan | - | - | - | - | - |
| AT1 block | - | - | - | - | - |
| P38 | - | - | - | - | - |
| P-P38 | - | - | - | - | - |
| ERK | - | - | - | - | - |
| P-ERK | - | - | - | - | - |
| Beta-actin | - | - | - | - | - |

Figure 5. Western blot demonstrated the effects of telmisartan on the TGF-β pathway with angiotensin II receptor type 1 (AT1) blockage. Beta-actin served as loading control. (A) Effective activation of Smad2 after TGF-β treatment for 30 minutes. (B) Effective activation of ERK and increased activation of p38 with 60 minutes treatment of TGF-β.
Potential role of PPAR-γ

Potential role of PPAR-γ was evaluated with the inhibition of the TGF-β induced expression of COL1A1 and ELN. Known PPAR-γ agonists and antagonists were compared to the anti-TGF-β effects of telmisartan (Figure 6). The VICs were treated with PPAR-γ agonists or antagonists for 24 hours prior to 1 hour treatment with telmisartan and finally 18 hour treatment of TGF-β. Both telmisartan and PGJ2, a known PPAR-γ agonist, demonstrated anti-TGF-β effects revealed by the significant decrease in TGF-β induced expression of COL1A1 and ELN. Pioglitazone, also a known PPAR-γ agonist, did not inhibit the TGF-β induced expression of COL1A1 and ELN. The PPAR-γ antagonist, GW9962, did not inhibit the TGF-β induced COL1A1 and ELN expression. However, GW9962 did not inhibit the anti-TGF-β effects of telmisartan.

Figure 6

![Graph showing the comparison of anti-TGF-β effects of telmisartan and PPAR-γ agonists (PGJ2 and pioglitazone) or antagonists (GW99629) on TGF-β induced collagen 1 (COL1A1) and elastin (ELN) expression.](image)

*Figure 6.* Comparison of anti-TGF-β effects of telmisartan and PPAR-γ agonists (PGJ2 and pioglitazone) or antagonists (GW99629) on TGF-β induced collagen 1 (COL1A1) and elastin (ELN) expression. Relative expression was determined by quantitative real-time PCR and target gene expression was normalized to GAPDH. *p<0.05, **p<0.01.
Discussions

Despite being a relatively common disorder, the knowledge about the pathogenesis of MVP is limited. Dysregulation of TGF-β signaling has been implicated with myxomatous degeneration, which is always present in MVP. ARBs have been shown to modulate TGF-β signaling with unknown mechanism. The ARB, telmisartan, has been proven to be partial PPAR-γ agonist and thus the hypothesis was that telmisartan modulates TGF-β signaling through activation of PPAR-γ independent from AT1. This study demonstrated that telmisartan, inhibited TGF-β induced expression of COL1A1 and ELN in cultured VICs and these effects are probably independent of AT1. In addition, there were indications that telmisartan activated the nuclear receptor, PPAR-γ and modulates TGF-β signaling through it.

The VIC properties were evaluated using immunofluorescence technique. It revealed that the cells were positive for vimentin and α-smooth muscle actin compared to SMCs. This was consistent with the literature[14] and earlier research[18]. However, the VICs were also positive for SM22α which is usually found in adult SMCs and FBs[32], and was not consistent with earlier research[18]. This was confirmed with western blot, which showed that both FBs and VICs were positive for SM22α (Supplementary figure 1). There is evidence that SM22α can be found in bovine aortic VICs so this is not an isolated example[33]. There was no binding of Angiotensin II alexa fluor® 488 conjugate in the VICs compared to SMCs and FBs. This suggested the absence of AT1 and AT2 in the VICs but this would need further verification with more than one technique.

The 4 VIC-lines that were used were treated with TGF-β and their TGF-β induced expression on COL1A1 and ELN compared. All 4 cell-lines showed significant increase in COL1A1 expression but 3 cell-lines showed significant increase in ELN expression. This showed that COL1A1 and ELN are TGF-β responsive genes, which has been well established in the past[34]. The cell-line that did not show significant increase in both COL1A1 and ELN (VIC 1/17/12) was excluded from the research and not used in this study.

In previous studies it has been proven that inhibition of p38 effectively abrogated TGF-β induced ECM expression while inhibition of JNK1 seemed not to have any effect[18]. This study demonstrated with western blot that the TGF-β treatment appeared to activate p38 but both JNK1 and PI3K seemed to be constitutively active. This suggests that there was something else, other than TGF-β,
that activated JNK1 and PI3K in the VICs and thus the TGF-β induced ECM expression appears not to be dependent on those pathways. However, TGF-β appeared to be necessary to activate p38 in the VICs so this might suggest that the TGF-β induced ECM expression is dependent on the p38 pathway.

Effective inhibition of TGF-β induced COL1A1 and ELN expression with telmisartan treatment was significant when the concentration of telmisartan reached 50 and 100 µM. However, there was no significant difference in the inhibition between 50 and 100 µM so it was decided to use 50 µM concentration in further experiments. When the anti-TGF-β effects of telmisartan was compared to PPAR-γ agonists only PGJ2, a known PPAR-γ agonist[35], showed significant inhibition on TGF-β induced COL1A1 and ELN expression. Pioglitazone, also a known PPAR-γ agonist[36], did not significantly inhibit the TGF-β induced expression of COL1A1 and ELN. The PPAR-γ antagonist, GW9962[37], did not inhibit the TGF-β induced COL1A1 and ELN expression as was anticipated but did not inhibit the anti-TGF-β effects of telmisartan. This gives an indication that the anti-TGF-β effects of telmisartan might be because of increased PPAR-γ activity but this needs further verification. Repetition and modulation of these experiments would be necessary to verify these findings. Pioglitazone is very unstable in its soluble form and is light sensitive; those are variables that need some consideration for future experiments. The concentration of GW9962 was 2 µM, which was considerably lower than the concentrations used for the PPAR-γ agonists so for future experiments it might be beneficial to reconsider the concentration of GW9962. There is also a possibility that PGJ2 and telmisartan modulate their anti-TGF-β effects through another unknown mechanism.

In previous studies the ARB, losartan, showed inhibition on TGF-β induced activation on Smad2 and p38 while ERK activation was not affected[18]. This study did not confirm these results entirely; instead of completely inhibiting the activation of Smad2 it only reduced the activation slightly, independently of AT1 blocking. Instead of blocking p38 activation, telmisartan appeared to increase it and this increased activation seemed to decrease with AT1 block. However, telmisartan, and the AT1 blocking effect, do not affect the TGF-β activation. Hence the anti-TGF-β effect of telmisartan might not be dependent on the p38 inhibition. The effects on ERK activation was not related to the TGF-β treatment and thus confirming the previous results that the ERK pathway does not seem to be involved in the TGF-β induced ECM expression[18].
Conclusions

These results demonstrate that telmisartan shows in-vitro anti-TGF-β effects on the ECM components, COL1A1 and ELN, in mitral VICs. These effects are maybe independent from AT1 due to the absence of AT1 in the VICs. The anti-TGF-β effects of telmisartan maybe due to its ability to activate PPAR-γ but this needs further verification.

The next set of experiments would involve estimation of dose response with the PPAR-γ antagonist, GW9962, to evaluate if GW9962 inhibits the anti-TGF-β effects of telmisartan. It would also be important to repeat the experiments that showed the anti-TGF-β effects of pioglitazone and PGJ2 due to technical difficulties in experimentations secondary to the short period of time in the lab. Verification of the absence of AT1 and AT2 in the VICs is necessary. It would be possible to do quantitative real-time PCR, western blot and fluorescence-activated cell sorting with positive control for AT1 and AT2.

Deeper understanding of the mechanisms behind the anti-TGF-β effect of telmisartan is needed to confirm the hypothesis that one of the therapeutic effects of telmisartan is to modulate TGF-β signaling through the PPAR-γ independent of AT1. If this is ultimately confirmed, medications like telmisartan or pioglitazone may be effective in slowing progression of myxomatous degeneration in patients with diagnosed MVP, thereby significantly altering the course of medical symptoms associated with MVP.
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References


Supplemental data

**Supplementary Figure 1**

**Supplementary figure 1.** Demonstrating the presence of SM22α in both valvular interstitial cells (VIC) and fibroblasts (FB) with western blot. Beta-actin is used as loading control.