Tetrandrine reverses human cardiac myofibroblast activation and myocardial fibrosis

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Submitted 23 February 2015; accepted in final form 3 April 2015

Teng G, Svystonyuk D, Mewhort HE, Turnbull JD, Belke DD, Duff HJ, Fedak PW. Tetrandrine reverses human cardiac myofibroblast activation and myocardial fibrosis. Am J Physiol Heart Circ Physiol 308: H1564–H1574, 2015. First published April 11, 2015; doi:10.1152/ajpheart.00126.2015.—Tetrandrine (TTD) is a calcium channel blocker with documented antifibrotic actions. In this study, for the first time, we identified that TTD can directly prevent in vitro human cardiac myofibroblast activation and limit in vivo myocardial fibrosis. In vitro, cardiac myofibroblasts from human atrial biopsies (N = 10) were seeded in three-dimensional collagen matrices. Cell collagen constructs were exposed to transforming growth factor-β1 (10 ng/ml), with or without TTD (1 and 5 μM) for 48 h. Collagen gel contraction, myofibroblast activation (α-smooth muscle actin expression), expression of profibrotic mRNAs, and rate of collagen protein synthesis were compared. TTD decreased collagen gel contraction (79.7 ± 1.3 vs 60.1 ± 8.9%, P < 0.01), α-smooth muscle actin expression (flow cytometry), collagen synthesis (1H]proline incorporation), and collagen mRNA expression. Cell viability was similar between groups (annexin positive cells: 1.7 vs. 1.4%). TTD inhibited collagen gel contraction in the presence of T-type and L-type calcium channel blockers, and the intracellular calcium chelator BAPTA-AM (15 μM), suggesting that the observed effects are not mediated by calcium homeostasis. In vivo, Dahl salt-sensitive hypertensive rats were treated with variable doses of TTD (by intraperitoneal injection over 4 wk) and compared with untreated controls (N = 12). Systemic blood pressure was monitored by tail cuff. Myocardial fibrosis and left ventricular compliance were assessed by histology and passive pressure-volume analysis. Myocardial fibrosis was attenuated compared with untreated controls (%collagen area: 9.4 ± 7.3 vs 2.1 ± 1.0%, P < 0.01). Left ventricular compliance was preserved. In conclusion, TTD reverses human cardiac myofibroblast activation and myocardial fibrosis, independent of calcium channel blockade.

Tetrandrine (TTD) is an emerging therapeutic for patients at risk of organ fibrosis. TTD has documented antihypertensive, antifibrotic, and anti-inflammatory effects (13). TTD is extracted from the root of Stephania tetrandra and has been used as an herbal therapy in Chinese medicine for hundreds of years. TTD has been administered to human patients for hypertension, cancer, liver fibrosis, and other diseases (11, 13, 15). To date, there are no clinical reports of TTD use for cardiovascular diseases in North America (16). In animal studies, TTD has shown beneficial effects on limiting cardiac myocardial remodeling in response to both pressure overload and coronary ischemia (4, 24, 25, 28, 29, 36, 37). In such models of human disease, cardiac fibrosis is attenuated in response to systemic TTD therapy (25). However, the utility and mechanisms underlying the effects of TTD on cardiac fibrosis are poorly understood. TTD is known to exert hemodynamic effects by lowering blood pressure though blockade of calcium channels (10, 13). It is unclear if TTD acts directly on the cardiac myofibroblast to influence its activity.

In the present study, we examined both in vitro and in vivo effects of TTD on TGF-β1-induced cardiac myofibroblast-mediated ECM remodeling and myocardial fibrosis. Using human cardiac myofibroblasts in three-dimensional (3D) collagen matrices, we examined the direct effects of TTD on cardiac myofibroblast activity and cell-mediated ECM remodeling. Using a rodent model, we assessed the effects of systemic TTD therapy on hypertension-induced cardiac fibrosis and the relationship of hemodynamics to antifibrotic effects.

MATERIALS AND METHODS

Human cardiac myofibroblast isolation and expansion. Right atrial appendages were obtained from consenting patients (N = 10) undergoing cardiac surgery at Foothills Medical Center (Calgary, AB). All experiments involving human tissue were approved by the Conjoint Health Research Ethics Board at the University of Calgary and conform to the Declaration of Helsinki. Biopsies were placed in Iscove’s modified Dulbecco’s medium (IMDM; Lonza, Walkersville, MD), washed in phosphate-buffered saline (PBS; Lonza, Walkersville, MD), minced into 1- to 3-mm fragments, and then plated as explants onto collagen-coated (Sigma-Aldrich, St. Louis, MO) 100-mm Petri dishes (Falcon, Corning, NY), with IMDM supplemented with 10% fetal bovine serum (Gibco by Life Technologies, Burlington, ON) plus 50,000 units of penicillin-streptomycin (Life Technologies, Burlington, ON). Explant tissue was cultured at 37°C in 5% CO2, with the medium replaced every 4 days. Cells were harvested by washing explants with PBS and then treating enzymatically with 3 ml trypsin for 3–5 min at 37°C.

Characterization of human cardiac myofibroblasts by immuno-histochemistry. Cells were seeded on coverslips and fixed using 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA) and blocked in blocking buffer containing 2% goat serum (Jackson ImmunoResearch Laboratories, West Grove, PA) and 1% bovine serum albumin.
albumin (Sigma-Aldrich, St. Louis, MO) and permeabilized in 0.1% Triton X-100 (EMD Millipore, Toronto, ON). The following primary antibodies were used at a dilution of 1:300: mouse anti-fibronecin (Calbiochem, EMD Biosciences, La Jolla, CA), rabbit anti-actin, rabbit anti-tropinin I, rabbit anti-desmin, or rabbit anti-von Willebrand factor (all from Santa Cruz Biotechnology, Dallas, TX). Secondary antibodies used were either Alexa Fluor 555 goat anti-mouse or Alexa Fluor 555 goat anti-rabbit (Life Technologies, Burlington, ON), at a dilution of 1:500. Coverslips were mounted onto microscope glass slides in Prolong Gold Antifade Reagent (Life Technologies, Burlington, ON) containing diamidinophenylindole for nuclear visualization. All fluorescent images were captured using confocal laser microscopy (LSM 5, Carl Zeiss Canada, Toronto, ON) and processed using Zen 2007 software.

**Collagen gel contraction assay.** Collagen gel contraction assay was performed as previously described (20). Five hundred microliters of IMDM, either alone (serum-free medium) or containing 10 ng/ml human recombinant TGF-β1 (Gibco by Life Technologies, Burlington, ON), with or without TTD (Sigma-Aldrich, St. Louis, MO) was added to each well. To determine whether TTD affected myofibroblast activity independent of Ca2+ influx or intracellular Ca2+ level, either a T-type calcium channel blocker nifedipin, L-type calcium channel blocker verapamil, or BAPTA-AM was added with TTD to the collagen matrix contraction model. Serial images of the collagen matrix dimensions were obtained from the time of release (baseline) and at 24, 48, and 72 h.

**Cell isolation from collagen matrices.** Cells were harvested from collagen matrices after 48 h culture and assessment of gel contraction. Cells were isolated by incubating the matrix in 100 μl of 500 U/ml collagenase type II (Worthington, Lakewood, NJ). Cells were then collected by centrifugation.

**Flow cytometry.** The cell pellet harvested was resuspended, and cells were fixed in ice-cold methanol followed by washing with PBS + 1% bovine serum albumin. Subsequently, cells were permeabilized by incubation with PBS + 1% Triton-X. An additional 2 ml of PBS + 1% Triton-X were added to the cell suspension, and cells were centrifuged at 2,000 rpm for 5 min. Cells were stained with anti-α-smooth muscle actin (SMA) (Abcam, Toronto, ON) conjugated with FITC (1:200 dilution; Sigma-Aldrich, St. Louis, MO). α-SMA expression was measured using a flow cytometer (LSRII, Becton Dickinson).

**Annexin V-propidium iodide binding assay.** Cells were resuspended in 500 μl of cold binding buffer. Cell suspensions were then stained with annexin V and propidium iodide (BD Pharmingen, Becton Dickinson, San Jose, CA), according to the manufacturer’s instructions. Annexin V expression and propidium iodide positive cells were quantified by flow cytometry.

**Cell proliferation assay.** Human cardiac myofibroblasts (20,000 in 80 μl of collagen matrix) were seeded into each well of a 96-well culture plate. In the presence of TGF-β1 (10 ng/ml; Gibco by Life Technologies, Burlington, ON) in serum-free medium, cells were treated with 0, 1, or 5 μM of TTD for 48 h. Cell proliferation was measured using WST-1 (Roche Life Science, Indianapolis, IN). The cell proliferation assay was performed as previously described (20). Five hundred microliters of WST-1 was added to the medium of each well, and absorbance at 450 nm was measured using a microplate reader.

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**Quantitative RT-PCR.** Messenger RNA (mRNA) was isolated using an RNeasy kit (Qiagen, Montreal, QC). mRNA was converted to cDNA using a Quantitect reverse transcription kit (Qiagen, Montreal, QC), according to the manufacturer’s protocol. Gene expression levels were obtained by real-time PCR using a Quantitect SYBR Green PCR kit (Qiagen, Montreal, QC) and the following primer pairs: ACTA2, tcaatggcaccgtag (forward) and gaaggaatgacccgcatc (reverse); COL1 A1, ccaatctgctcccaagag (forward) and tcaaaaaaagaggagattg (reverse); COL1 A2, ctgcagaaacagcatt (forward) and gggtgagctctaatg (reverse).

Gene expression levels were measured with reference to 18s ribosomal mRNA (Quantitect, Qiagen, Montreal, QC). [3H]proline incorporation. To measure collagen synthesis, 1 micro Ci/ml [3H]proline was added to the cell culture media in the presence of TGF-β1 ± TTD. After 24 h, the unincorporated extracellular proline was aspirated from the culture media. One milliliter of cold trichloroacetic acid (5% wt/vol) was added to solubilize intracellular free [3H]proline and precipitate [3H]proline incorporated into collagen. One-half milliliter of 0.4 N NaOH was added to solubilize the precipitated proteins. An aliquot of the final solution was added to scintillation vials to determine [3H]proline incorporated into protein (largely collagen). The protein content of each sample was determined using the Bradford method, and the [3H]proline signal was normalized to the total amount of protein in each sample.

**Reagents and preparations.** TTD (Sigma-Aldrich Chemical) and BAPTA-AM (Calbiochem, EMD Biosciences, La Jolla, CA) were prepared as stock solutions in DMSO. The T-type calcium channel blocker, nifedipin (Hoffmann La Roche, Basel, Switzerland), was prepared as a stock solution in deionized water, and the L-type calcium channel blocker, verapamil (Sigma-Aldrich, St. Louis, MO), was prepared as a stock solution in ethanol.

**Assessment of myofibroblast activation and ECM remodeling in individual cells.** To assess individual cells within 3D collagen matrices, we adapted a novel cell culture platform from Mohammadi and coworkers that employs thin collagen gels supported by rigid noncontractile nylon grids (19). Individual myofibroblasts were assessed for cell activation, and their local effects on adjacent ECM remodeling by image analysis. Type I bovine dermal collagen (5.9 mg/ml, Advanced Biomatrix, San Diego, CA) was diluted to a working concentration of 1.0 mg/ml and neutralized to a pH of ~7.4 using 0.1 N NaOH. Collagen-coated squares were gently detached from the culture dish using warm PBS, inverted, and floated in serum-free IMDM culture medium with 10 ng/ml human recombinant TGF-β1, with or without TTD (0, 1, and 5 μM). Cells were seeded at low density (1 × 104 cells), allowing single cells in each grid space. Treatments were maintained for 24 h.

Differences in myofibroblast morphology (cell extension length and circularity) were assessed. Myofibroblasts within each microgel were stained with Alexa Fluor 488 phalloidin (Life Technologies, Burlington, ON), and cell nuclei were stained with diamidinophenylindole. Cell extension length was measured from the center of the cell to the tip of a given cell extension using ImageJ software (version 1.48, National Institutes of Health). Measurement of cell circularity was performed using the Multi-Cell Outliner plug-in for ImageJ. By this method, values approaching 1 are representative of round cell morphology, while values approaching 0 are considered increasingly nonround (5). Collagen fiber alignment was performed to quantify local ECM remodeling. Collagen fiber images were imaged using confocal reflectance microscopy with a confocal laser microscope (LSM5, Carl Zeiss, Toronto, ON). From the resultant intensity curve, a collagen fiber alignment index is calculated as the area under the curve bound by the peak intensity value ± 10°. Larger index values indicate increased ECM remodeling.

**Rodent hypertensive model of left ventricular hypertrophy and fibrosis.** The University of Calgary Health Science Animal Care Committee approved the experimental protocol. All procedures were performed in accordance with the Guide for the Care and Use of Experimental Animals from the Canadian council on Animal Care and the Guide for the Care and Use of Laboratory Animals (The National Academy Press, revised 1996). Male Dahl salt-sensitive (Dahl/SS) rats (N = 12) were ordered from Charles River Canada (Montreal, QC, Canada) at 5 wk of age. The animals were acclimated for 1 wk and then, starting at 6 wk of age, exclusively fed a high-salt diet (AIN-76A with 8% NaCl; TestDiet, Richmond, IN) for the remainder of the study. At 12 wk of age, rats were randomly divided into four treatment groups of N = 3 each: TTD 0, PBS only; TTD 7.5, 7.5 mg/kg TTD; TTD 15, 15 mg/kg TTD; and TTD 30, 30 mg/kg TTD.
Treatment for all groups was administered intraperitoneally twice a week for 4 wk. TTD was dissolved in 0.1 N HCl at a concentration of 15 mg/ml, and the resultant solution was neutralized with 0.1 N NaOH.

**Blood pressure assessments.** Blood pressure was measured by noninvasive tail cuff monitor (BP 2000 Blood Pressure Analysis System, Visitech Systems, Apex, NC). After an acclimation period, blood pressure was measured and recorded once per week, and an average of 20 consecutive measurements were taken over 30 min.

**Myocardial compliance and quantification of fibrosis area.** At 16 wk of age, animals were euthanized, and hearts arrested in diastole and explanted. Passive pressure distention measures of the left ventricle (LV) were performed as previously described (18). In brief, a latex balloon connected to a closed fluid-filled system housing a pressure catheter was inserted into the LV, and volumes at 30 mmHg were recorded. LV was isolated and short-axis sections were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5-μm thickness, and stained with picrosirius red. Samples were imaged under light and polarized light microscopes. To quantify the fibrotic area, six non-overlapping random images from each heart section were captured using a digital camera (Olympus DP73) at ×200 magnification (Olympus BX 53 Microscopy). ImageJ software was used to quantify fibrosis as a percent collagen staining per image area.

**Statistical analysis.** All group data are presented as means ± SD. Data were obtained from a representative experiment of which each was repeated in triplicate. When only two groups were compared, Student’s t-test was performed. For comparison of more than two groups, one-way ANOVA was used and followed by appropriate post hoc comparison tests. All statistical analyses were performed using GraphPad Prism 6.0, with P < 0.05 considered statistically significant.

**RESULTS**

**Characterization of human cardiac myofibroblasts.** Cell morphology was consistent with myofibroblasts. To further characterize the cells, we performed immunocytochemistry for fibroblast-specific markers (vimentin and fibronectin). Greater than 95% of the cultured cells stained positive for fibroblast markers (Fig. 1). Cells were also stained for other nonfibroblast cell types found in myocardium. Specifically, cells showed negative staining for von Willebrand factor (endothelial cells), troponin I (cardiomyocytes), and desmin (smooth muscle cells, skeletal muscle cells, cardiomyocytes) (Fig. 1).

**TTD attenuates human myofibroblast-mediated collagen gel contraction.** Measuring the extent of collagen gel contraction over time can elucidate the extent of myofibroblast activity and its influence on ECM remodeling (14). TGF-β was used to stimulate myofibroblast activity to induce collagen gel contraction (Fig. 2). TTD treatments at both 1 and 5 μM attenuated human cardiac myofibroblast-mediated collagen gel contraction compared with controls. TTD inhibited myofibroblast-mediated collagen gel contraction in a concentration-dependent manner over time (Fig. 2). We further explored if differences in gel contraction between groups were a result of altered cell...
TTD reverses human cardiac myofibroblast activation

**TTD attenuates human cardiac myofibroblast activation.** The activation of myofibroblasts is characterized by increased expression of α-SMA (9). We assessed α-SMA expression in cells within collagen gels by flow cytometry for protein levels and by PCR for gene expression (Fig. 4). The mean fluorescence intensities (MFI) of α-SMA expression were compared. At baseline (no TTD exposure), MFI showed high levels confirming a myofibroblast phenotype. With exposure to TTD, the MFI was decreased, suggesting transition to a less activated myofibroblast state (Fig. 4, A and B). This response was increased with higher doses of TTD. These data were further confirmed by analysis of α-SMA gene expression, which showed significant reductions after both TTD 1 and 5 μM exposure (Fig. 4C).

**TTD suppresses collagen synthesis and gene expression.** To further define the effects of TTD on the fibrosis pathway, we next examined collagen synthesis and gene expression levels in isolated human cardiac myofibroblasts exposed to increasing levels of TTD. TTD suppressed collagen synthesis and gene expression in a dose-dependent manner (Fig. 5).

**TTD inhibits human cardiac myofibroblast-mediated ECM remodeling.** Using a novel floating nylon-grid supported thin collagen gel culture platform system, myofibroblast activation and local ECM remodeling around isolated single cells was imaged using confocal microscopy and quantified by image analysis (Fig. 6). In the absence of TTD, human cardiac myofibroblasts stimulated by TGF-β1 were observed to have a stellate morphology with numerous cell extensions and local ECM remodeling around each extension (Fig. 6, A, B, and D). TTD altered the morphology of the cell toward a spherical shape in a dose-dependent relationship, and ECM remodeling around each cell extension was significantly attenuated (Fig. 6, A, C, and D), suggesting that TTD directly suppresses myofibroblast activation and myofibroblast-mediated ECM remodeling.

**Calcium channel blockade does not mediate TTD effects on myofibroblast activity.** Voltage-gated Ca2+ channels, known to exist in excitable cells, such as neurons and muscle cells, are also present in fibroblasts and may contribute to their activation (26, 30). TTD is a unique calcium channel blocker with specific inhibitory effects on L-type and T-type Ca2+ channels (13, 32). We explored if the effects of TTD on human cardiac myofibroblast-mediated collagen gel contraction was a consequence of calcium channel blockade. We assessed the effects of TTD in the presence and absence of a T-type calcium channel blocker, mibefradil (Fig. 7A) and an L-type calcium channel blocker, verapamil (Fig. 7B). Selective calcium channel blockade influenced but did not completely eliminate the effects of TTD on human cardiac myofibroblast-mediated collagen gel contraction. These data suggest that TTD effects in myofibroblast activation are not completely dependent on its calcium channel blockade activity. Given that other calcium channels are known to mediate myofibroblast activity, such as transient receptor potential channels (40), we further explored calcium channel signaling by using BAPTA-AM, a cell-permeant chelator, to control intracellular Ca2+. In the presence of BAPTA-AM, we continued to observe a suppressive effect of TTD on human cardiac myofibroblast-mediated collagen gel contraction (Fig. 7C). These data indicate that TTD influences human cardiac myofibroblast activity through mechanisms that are not dependent on calcium channel blockade or intracellular calcium.

**TTD attenuates myocardial fibrosis and preserves LV compliance in hypertensive rats independent of hemodynamic load.** We next evaluated the effects of systemic TTD administration in a clinically relevant rat model of hypertension-induced myocardial hypertrophy, fibrosis, and failure (Fig. 8). After induction of the model by high-salt diet, Dahl/SS rats were treated with escalating doses of TTD (7.5, 15, and 30 mg/kg by intraperitoneal injection over 4 wk) and compared with untreated controls. Myocardial interstitial fibrosis was significantly attenuated after 4 wk of TTD treatment compared with untreated controls. Myocardial hypertrophy, fibrosis, and failure (Fig. 8). After induction of the model by high-salt diet, Dahl/SS rats were treated with escalating doses of TTD (7.5, 15, and 30 mg/kg by intraperitoneal injection over 4 wk) and compared with untreated controls. Myocardial interstitial fibrosis was significantly attenuated after 4 wk of TTD treatment compared with untreated controls. Myocardial hypertrophy, fibrosis, and failure (Fig. 8). After induction of the model by high-salt diet, Dahl/SS rats were treated with escalating doses of TTD (7.5, 15, and 30 mg/kg by intraperitoneal injection over 4 wk) and compared with untreated controls. Myocardial interstitial fibrosis was significantly attenuated after 4 wk of TTD treatment compared with untreated controls. Myocardial hypertrophy, fibrosis, and failure (Fig. 8). After induction of the model by high-salt diet, Dahl/SS rats were treated with escalating doses of TTD (7.5, 15, and 30 mg/kg by intraperitoneal injection over 4 wk) and compared with untreated controls. Myocardial interstitial fibrosis was significantly attenuated after 4 wk of TTD treatment compared with untreated controls. Myocardial hypertrophy, fibrosis, and failure (Fig. 8). After induction of the model by high-salt diet, Dahl/SS rats were treated with escalating doses of TTD (7.5, 15, and 30 mg/kg by intraperitoneal injection over 4 wk) and compared with untreated controls. Myocardial interstitial fibrosis was significantly attenuated after 4 wk of TTD treatment compared with untreated controls. Myocardial hypertrophy, fibrosis, and failure (Fig. 8). After induction of the model by high-salt diet, Dahl/SS rats were treated with escalating doses of TTD (7.5, 15, and 30 mg/kg by intraperitoneal injection over 4 wk) and compared with untreated controls. Myocardial interstitial fibrosis was significantly attenuated after 4 wk of TTD treatment compared with untreated controls. Myocardial hypertrophy, fibrosis, and failure (Fig. 8). After induction of the model by high-salt diet, Dahl/SS rats were treated with escalating doses of TTD (7.5, 15, and 30 mg/kg by intraperitoneal injection over 4 wk) and compared with untreated controls. Myocardial interstitial fibrosis was significantly attenuated after 4 wk of TTD treatment compared with untreated controls. Myocardial hypertrophy, fibrosis, and failure (Fig. 8). After induction of the model by high-salt diet, Dahl/SS rats were treated with escalating doses of TTD (7.5, 15, and 30 mg/kg by intraperitoneal injection over 4 wk) and compared with untreated controls. Myocardial interstitial fibrosis was significantly attenuated after 4 wk of TTD treatment compared with untreated controls.

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**Fig. 2.** A: human cardiac myofibroblast-mediated three-dimensional (3D) collagen gel contraction is represented as a percent reduction in collagen matrix surface area over 72 h. Values are means ± SD; N = 4 per group. *P < 0.05. B: representative images of 3D collagen gels showing the differential contraction after 72-h of treatment. TTD, tetrandrine. Survival or proliferation from TTD exposure by measuring annexin V, propidium iodide, and WST-1 expression (Fig. 3). There was no significance difference between groups for indexes of cell death (Fig. 3, A and B), apoptosis (Fig. 3, A–C), or cell proliferation (Fig. 3D).
despite escalating doses (Fig. 8C). LV compliance was assessed by ex vivo passive pressure distention analysis and revealed a significant attenuation of myocardial stiffness with higher doses of TTD in the setting of equivalent hemodynamic load (Fig. 8C, TTD 7.5 mg/kg vs. TTD ≥ 15 mg/kg). These data suggest that, at higher doses, TTD may act on myocardial ECM and tissue remodeling, independent of its influence on hemodynamic load.

**DISCUSSION**

Approximately one-half of all patients with heart failure have preserved systolic function and suffer primarily from diastolic dysfunction characterized by progressive myocardial fibrosis and chamber stiffness. Despite the prevalence of this condition, there is a fundamental lack of effective medical therapies or specific guidelines for effective treatments to
reduce the morbidity or mortality of heart failure with preserved ejection fraction (“diastolic heart failure”) (23). Hypertension is a common cause of progressive myocardial fibrosis with diastolic dysfunction, and the prognosis is similar to that of patients with systolic dysfunction (22). The discovery and validation of therapeutic targets that directly influence myofibroblast-mediated ECM remodeling are necessary to treat this common, yet poorly understood, cardiovascular problem.

For the first time, we document a direct influence of TTD on limiting human cardiac myofibroblast activation, ECM regulation, and ECM remodeling. We provide novel mechanistic data that these direct effects are not mediated through calcium channel blockade. This observation prompted us to explore the effects of TTD in vivo, as the known antihypertensive effects may not be primarily responsible for the antifibrotic effects of TTD on the heart. Using a clinically relevant hypertensive rat model of LV hypertrophy, we showed that TTD limits myocardial fibrosis and also prevents myocardial stiffening at higher doses, even between groups with similar hemodynamic loads. Collectively, these novel observations support a direct effect of TTD on cardiac myofibroblasts that significantly influences myocardial ECM and tissue remodeling.

**TTD attenuates human cardiac myofibroblast activation and ECM remodeling.** When plated on plastic under two-dimensional culture conditions, cardiac fibroblasts in vitro (within 48 h) undergo a phenotypic shift to myofibroblasts (5). Accordingly, our culture conditions result in a mixed population of cells that became >90% myofibroblasts with time, consistent with the observations of others (5, 17, 27, 33). Stimulated with exogenous TGF-β1, we assessed the differential effects of TTD

![Flow cytometry analysis for α-smooth muscle actin (SMA) expression in human cardiac myofibroblasts treated with TTD (N = 3). B: treatment with TTD significantly decreased the mean fluorescence intensity, **P < 0.001 by one-way ANOVA. C: messenger RNA levels were determined by quantitative RT-PCR and showed significant reductions after 1 and 5 μM TTD treatments (N = 3).](attachment:figure4.png)
on activation of cultured human myofibroblasts within two different 3D collagen matrix platforms. TTD reversed the activation of human cardiac myofibroblasts in both of these models, and changes in ECM expression were observed to be a consequence of changes in myofibroblast activity toward a less active baseline state. Importantly, the observed decrease in myofibroblast activation occurred in the absence of exaggerated apoptosis or altered cell proliferation. We further explored the effects of TTD on human cardiac myofibroblasts using a floating (noncontractile) nylon-grid supported thin collagen gel culture platform system (19). This unique model allows myofibroblast activation and local ECM remodeling around isolated single cells to be assessed and compared between groups. We showed that increasing doses of TTD attenuated human cardiac myofibroblast activation, and, in so doing, preserved local ECM architecture and maintained ECM homeostasis.

**TTD mechanisms of action.** While TTD is a known calcium channel antagonist, the effects of TTD on myofibroblasts and ECM remodeling do not appear to be related to a hemodynamic effect alone. The evidence for this is TTD, when applied in vitro, suppresses myofibroblast activation and ECM remodeling. TTD interrupts the ability of TGF-β to induce collagen contraction when applied in vitro. TTD suppresses collagen synthesis and gene expression in vitro, and this is paralleled by less fibrosis in vivo. While TTD is a calcium channel blocker, we provide evidence that TTD suppresses myofibroblast activation in the presence of L-type and T-type calcium channel blockers. Calcium channel blockade with verapamil alone had a significant effect on myofibroblast activation, but this effect was increased in the presence of TTD. In addition, the use of the cytosolic calcium chelator BAPTA-AM suggests that a change in intracellular calcium does not completely explain TTD’s antifibrotic activity.

TTD is a unique calcium channel blocker with well-documented effects on limiting hypertension and fibrosis in animal models (4, 24, 25, 28, 29, 35–37, 39). These studies have not defined an antifibrotic mechanism for the observed effects on the heart, and the role of hemodynamic load as a mediator of the antifibrotic effects has not been compared or addressed. A direct mechanistic effect of TTD in fibroblasts is supported by studies that have examined the in vitro influence of TTD on human skin and subconjunctival fibroblasts, where suppressed SMAD signaling was observed (12, 41). TGF-β activates the SMAD-2,3 signal transduction pathway, and TTD may act to interrupt this signal transduction pathway.

**TTD prevents interstitial fibrosis and chamber stiffness in hypertensive rat model.** Likely as a consequence of reverting activated myofibroblasts toward an inactive baseline state, TTD has marked effects on regulating myocardial ECM remodeling. The observed in vitro effects of TTD on suppressing collagen expression and preserving ECM homeostasis were paralleled in vivo by decreased interstitial collagen area and reduced myocardial stiffness. We observed these effects in atrial-derived cells, and differences between atrial and ventricular-derived myofibroblasts have been documented. Atrial fibroblasts show a greater response to TGF-β in vitro and a more robust fibrotic response in vivo (3, 38). Further work should be performed to confirm our observations using human ventricular-derived cells. Our preliminary results suggest a similar response to TTD, as observed in atrial-derived cells (data not shown). Remodeling of myocardial ECM is dynamic.

![Fig. 5.](A: collagen synthesis was determined by [3H]proline incorporation. Treatment with TTD resulted in significantly decreased collagen synthesis (A) and messenger RNA collagen 1A1 (B) and 1A2 (C). DPM, disintegrations per minute. Values are means ± SD; N = 3. *P < 0.05; **P < 0.01. ***P < 0.001.)
and complex, involving changes in the amount of collagen, types of collagens, degree of collagen cross-linking, and numerous additional emerging pathways (6, 7). Importantly, changes in chamber stiffness can be influenced by both the quantity and quality of myocardial collagens (1). We observed a reduction in myocardial stiffness with higher doses of TTD, despite similar levels of collagen quantity and hemodynamic pressure loads between groups. These data may reflect preservation of ECM quality in addition to ECM quantity, resulting from a direct influence of TTD on reserving cardiac myofibroblast activity and, in so doing, preserving ECM homeostasis. These data support that TTD can influence myocardial fibrosis.

**Fig. 6.** A: confocal immunocytochemistry of human cardiac myofibroblasts (phalloidin stained; green) embedded in the 3D collagen matrix (autoreflectance; red) within a microgrid treated with varying concentrations of TTD. Morphological markers of the extent of myofibroblast activation were assessed as cell extension length (B) and cell circularity index (C) and showed attenuation of myofibroblast activation in response to increasing concentrations of TTD. D: collagen fiber alignment index demonstrates attenuation of ECM remodeling in response to increasing concentrations of TTD. Values are means ± SD. *P < 0.05.
and myocardial stiffness, independent of its effects on hemodynamic load or calcium homeostasis, likely as a consequence of direct effects on the myofibroblast. Progressive fibrosis and chamber stiffening are the hallmarks of progression to heart failure, and the effect of TTD documented in our study may have clinical relevance.

Clinical implications. We believe that data from our in vitro model has clinical relevance. Human 3D cell culture models allow for improved translation to the human condition compared with two-dimensional culture experiments using animal cells. 3D matrix constructs best reflect in vivo tissue microenvironments and permit normal physiological cell-ECM interactions (8). Our unique floating gel system with rigid lateral boundaries may best reflect in vivo physiological conditions as it mimics the influence of basement membranes (19). In this study, we used freshly isolated cardiac myofibroblasts from

Fig. 7. Human cardiac myofibroblast-mediated 3D collagen gel contraction in response to TTD in the presence of T-type Ca\(^{2+}\) channel blocker mibefradil (A), L-type Ca\(^{2+}\) channel blocker verapamil (B), and a cell-permeant chelator BAPTA-AM (C). After blocking T- or L-type Ca\(^{2+}\) channels and binding intracellular Ca\(^{2+}\), 3D collagen matrix contraction was further decreased. Values are means ± SD; N = 3. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Fig. 8. A: histologic specimens of left ventricular (LV) myocardium from untreated and TTD-treated hypertensive rats stained with picrosirius red imaged using polarized light (left) and light (right) microscopy, demonstrating decreased fibrosis in response to TTD treatment. B: interstitial collagen abundance within the LV myocardium of hypertensive rats is expressed as average %collagen per high-power field treated and was significantly reduced with TTD treatment at variable doses. *P < 0.05. C: LV volumes as measured by passive pressure distension were matched between hearts at 30 mmHg and indexed to body weight. Systolic blood pressure area under the curve (SBP-AUC) measures are also shown (inset) as an index of hemodynamic load (TTD 0, N = 3; TTD 7.5 mg/kg, N = 3; TTD ≥15 mg/kg, N = 5). As assessed by SBP-AUC, hemodynamic load was decreased by TTD treatment but was not different between 7.5 and ≥15 mg/kg groups. However, passive LV volume was significantly different between the 7.5 and ≥15 mg/kg groups, despite similar hemodynamic load, indicating altered compliance with higher doses of TTD. Values are means ± SD.
human hearts undergoing heart surgery. Santiago and coworkers (27) observed that cultured adult cardiac fibroblasts that differentiate to myofibroblasts in vitro share similar expression profiles to myofibroblasts found in vivo. We utilized an ECM composed of type I collagen, which is the primary isofrom in the human heart (34). In addition, our in vivo rat model of human disease is clinically relevant, as myocardial ECM remodeling and fibrosis is induced by hypertension, a primary cause of human diastolic heart failure with progressive myocardial fibrosis and altered chamber compliance. TTD should be explored as a potential therapy for patients with diastolic dysfunction secondary to pressure overload.

GRANTS
This work was supported by the Heart and Stroke Foundation of Alberta, NWT, and Nunavut, P. W. M. Fedak is a Clinical Investigator for Alberta Innovates-Health Solutions (AIHS). H. E. M. Mewhort receives salary support from the AIHS.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES


