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A new anti-fibrotic drug attenuates cardiac remodeling and systolic dysfunction following experimental myocardial infarction

Yuan Zhang [a,1,2], Maros Elsik [b,1,2], Amanda J. Edgley [a,2], Alison J. Cox [a,2], Andrew R. Kompa [b,2], Bing Wang [b,2], Christina Yan Ru Tan [a,2], Fay L. Khong [a,2], David I. Stapleton [c,2], Steven Zammit [d,2], Spencer J. Williams [d,2], Richard E. Gilbert [e,2], Henry Krum [b,2], Darren J. Kelly [a,f,*,2]

[a] Department of Medicine, St. Vincent's Hospital, The University of Melbourne, Australia
[b] Monash Centre of Cardiovascular Research and Education in Therapeutics, Monash University, Melbourne, Australia
[c] Department of Physiology, University of Melbourne, Australia
[d] School of Chemistry and Bio 21 Institute of Molecular Science and Biotechnology, The University of Melbourne, Australia
[e] Keenan Research Centre in the Li Ka Shing Knowledge Institute, St. Michael's Hospital, Toronto, Ontario, Canada
[f] Fibrotech Therapeutics Pty. Ltd, Melbourne, Australia

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ABSTRACT

Background: Pathological deposition of extracellular matrix in the non-infarct zone (NIZ) of the ventricle post myocardial infarction (MI) is a key contributor to cardiac remodeling and heart failure. FT011, a novel antifibrotic compound, was evaluated for its efficacy in neonatal cardiac fibroblasts (NCF) and in an experimental MI model.
Methods and results: Collagen synthesis in NCF was determined by ^{3}H-proline incorporation following stimulation with TGF-β or angiotensin II (Ang II). FT011 inhibited collagen synthesis to both agents in a dose dependent manner. *In vivo*, *Sprague Dawley* rats underwent left anterior descending coronary artery ligation or sham surgery and were randomized one week later to receive either FT011 (200 mg/kg/day) or vehicle for a further 4 weeks. Echocardiography and cardiac catheterization were performed, and tissues were collected for histological analysis of collagen, myocyte hypertrophy, interstitial macrophage accumulation and Smad2 phosphorylation. mRNA expression of collagens I and III and TGF-β was measured using *in situ* hybridization and RT-PCR, respectively. FT011 treatment was associated with improved cardiac function (increased ejection fraction, fraction shortening and preload recruitable stroke work) and myocardial remodeling (reduced left ventricular diameter and volume at both end diastolic and systolic) compared with vehicle treatment. FT011 significantly reduced collagen matrix deposition, myocyte hypertrophy and interstitial macrophage infiltration, and mRNA expression of collagens I and III in NIZ compared with vehicle treatment.
Conclusion: Anti-fibrotic therapy with FT011 in MI rats attenuated fibrosis and preserved systolic function.
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1. Introduction

Chronic heart failure (CHF) following myocardial infarction (MI) represents a major cause of morbidity, hospitalization and premature death, despite current treatment strategies. MI may result in functional and structural changes in the left ventricle (LV), termed ventricular remodeling. Immediately following MI, impairment of LV function and reduced cardiac output trigger compensatory activation of various local and systemic neurohormonal systems such as the renin-angiotensin-aldosterone, and sympathetic nervous systems, as well as pro-inflammatory and pro-fibrotic cytokines in an effort to restore function. Chronic activation of these systems results in LV remodeling, characterized by necrosis and thinning of the infarct myocardium, LV chamber dilation and hypertrophy of the viable myocardium, all of which ultimately contribute to the clinical syndrome of CHF.

At the cellular level, ventricular remodeling is associated with myocyte hypertrophy, apoptosis, fibroblast proliferation and increased deposition of fibrillar collagen (fibrosis). Fibrosis is a key contributor to the cardiac remodeling that occurs post-MI. Initially, deposition of collagen plays a crucial role in maintaining the structural integrity of the LV wall in the infarct zone (preventing ventricular rupture) and this is termed replacement or reparative fibrosis. However, during recovery from MI, reactive fibrosis is also initiated, resulting in pathological collagen deposition in sites remote from and around the border of the infarct region contributing to the progression to heart failure [1].

Currently approved pharmacological treatments for MI-associated CHF utilize drugs such as angiotensin-converting-enzyme (ACE)

* Corresponding author at: Department of Medicine St. Vincent's Hospital, Fitzroy, Victoria 3065, Australia. Tel.: +61 3 9288 2580; fax: +61 3 9288 2581.
E-mail address: dkelly@medstv.unimelb.edu.au (D.J. Kelly).
[1] Co-first authors.
[2] These authors take responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation.

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inhibitors, angiotensin II receptor antagonists, aldosterone receptor antagonists and β-blockers, all of which improve clinical outcomes, and are directly linked to improvements in ventricular function [2–5]. In addition, the beneficial effects of these agents on ventricular remodeling appear to be at least partly due to their ability to inhibit extracellular matrix deposition and reduce myocardial fibrosis [5]. However, these therapeutic strategies do not halt the disease progression.

More recently, therapeutic strategies have focused on directly inhibiting or reducing myocardial fibrosis to provide potential additional and alternate approaches to improve ventricular function post-MI. A number of direct anti-fibrotic agents, such as pirfenidone [6] and Tranilast [7] have been shown to have potential therapeutic benefits in heart disease. Previously, our group and others have explored the beneficial effects of Tranilast in kidney and heart disease in several animal studies [7–11]. We have shown that, in the diabetic heart, attenuation of extracellular matrix deposition by Tranilast is associated with inhibition of the actions of TGF-β, in particular inhibition of phosphorylated Smad2, a downstream signaling pathway of TGF-β [7–10]. However the widespread clinical use of this compound has been limited due to undesirable side effects including liver toxicity [12].

On this basis, we have previously developed a series of novel anti-fibrotic agents based on the core structure of Tranilast, with the purpose of reducing adverse side effects and maintaining or enhancing the anti-fibrotic effects based on structure–activity relationships. FT011 (Fibrotech Therapeutics, Melbourne, Australia) is our lead compound, displaying improved bioactivity compared with Tranilast [13]. FT011 has recently been approved for pre-clinical development for diabetic nephropathy, and has been investigated as a novel anti-fibrotic therapy for the treatment of experimental diabetic cardiomyopathy [13,14].

The aim of the present study was to first evaluate the potential anti-fibrotic properties of FT011 in cultured neonatal cardiac fibroblasts (NCF), and secondly, test the efficacy of FT011 in its ability to prevent myocardial fibrosis and myocyte hypertrophy, and improve function in an experimental rat model of MI-induced LV heart failure.

2. Methods

2.1. In vitro studies

2.1.1. Measurement of collagen synthesis in rat NCF

NCF were isolated from one to two day old pups with enzymatic digestion as described previously, and used at passage two [15,16].

NCF collagen synthesis assays were performed as described previously [17]. Briefly, NCF were pre-incubated for 2 h in the presence of FT011 (10–200 μM) or 0.1% DMSO (control group) in fresh DMEM/F12 before stimulation with 5 ng/ml of TGF-β or 100 nM of AngII in the presence of 1 μCi of ^{3}H-proline/well and incubated for further 48 h before harvest. ^{3}H-proline level was counted with 3 ml scintillation fluid on a β-counter to determine the level of ^{3}H-proline incorporation. Experiments were performed in triplicate.

2.2. In vivo studies

2.2.1. Animals

Animal studies were conducted with the approval of the Animal Ethics Committee of St Vincent's Hospital in accord with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Rats received normal rat chow (Certified Rodent Diet #5002, LabDiet, USA) and drinking water *ad libitum*. All animals were housed in a stable environment maintained at 22 ± 1 °C with a 12-h light/dark cycle commencing at 6 am.

2.2.2. Myocardial infarction and study design

Seventy male Sprague Dawley (SD) rats in total weighing 200–250 g were randomized to undergo left anterior descending coronary artery (LAD) ligation or sham surgery as described previously [18]. Briefly, animals were anesthetized with isoflurane, intubated and the thoracic cavity opened. The pericardial sac was torn open and a 6-0 prolene suture was used to ligate the LAD. Visible blanching and hypokinesis of the anterior wall of the left ventricle and swelling of the left atrium are indicative of successful ligation. Sham operations consisted of the same procedure except that the suture was passed through the myocardium beneath the LAD without ligation.

Echocardiography was performed on all animal groups 2 days post-MI surgery (base-line). On day 7 after surgery, sham and MI groups were randomized to receive either treatment with FT011 (100 mg/kg b.i.d. gavage) or vehicle (0.1% carboxy-methyl cellulose) for 4 weeks. We have previously examined the safety profile of FT011 within the dose range used in the present study [13,14]. Cardiac function was assessed by echocardiography and cardiac catheterization prior to sacrificing at day 35 after surgery.

2.3. Cardiac function

2.3.1. Echocardiography

Transthoracic echocardiography was performed under light anesthesia (xylazine 0.5 mg/100 g, ketamine 3.75 mg/100 g) using a Vivid 7 Dimension (GE Vingmed, Horten, Norway) echocardiography machine with a 10 MHz phased array probe as described previously [19]. Electrocardiographic data were acquired simultaneously. Parasternal short-axis and long axis views were obtained. M-mode echocardiography was performed by using a parasternal short-axis view at the level of the papillary muscles. LV internal diameters at end diastole (LVIDd) and end systole (LVIDs) were obtained. Fractional shortening (FS%) was calculated according to the formula: FS% = (LVEDD − LVESD)/LVEDD × 100, where LVEDD and LVESD are LV end-diastolic and end-systolic diameters, respectively, as previously described. Left ventricular end-diastolic (LVEDV) and end-systolic volumes (LVESV) obtained from the parasternal long axis view were calculated according to a single plane area-length method [20].

All parameters were assessed using an average of three beats, and calculations were made in accordance with the American Society of Echocardiography guidelines [21]. Data were acquired and analyzed by two blinded observers (inter-observer) using EchoPAC (GE Vingmed) offline processing. Inter-observer differences were calculated as the difference between two observations divided by the mean of the observations and were expressed as percentages [22,23].

2.3.2. Cardiac catheterization

Post echocardiography, left ventricular catheterization was performed as described previously [21]. Briefly, animals were intubated and ventilated. A 2F miniaturized combined conductance catheter-micromanometer (Model SPR-838 Millar instruments, Houston, TX) was inserted into the right carotid artery to obtain aortic blood pressure, and then advanced into the left ventricle until stable pressure–volume (PV) loops were obtained [24]. Elastic bands were placed around the inferior vena cava and portal vein to reduce cardiac preload. All PV loops were obtained with the ventilator turned off and the animal apnoeic. (Millar analysis software (PVAN 3.5) was used to calculate central aortic pressure (CAP), the slope of the end diastolic pressure volume relationship (EDPVR) and the slope of the preload recruitable stroke work relationship (PRSW).

2.4. Tissue preparation

Post-cardiac catheterization, the heart and lungs were excised, dissected and weighed. The mid-section of the heart was preserved initially in 10% formaldehyde and then embedded in paraffin for histology and immunohistochemical stain.

2.5. Histopathology

Histopathological changes in the heart were assessed in a masked protocol. Sections were stained with haematoxylin & eosin (H & E) to determine the size of cardiomyocytes, and picrosirius red to demonstrate collagenous matrix.

2.5.1. Infarct size

Four micron short axis cross section at papillary muscle level of the LV was stained with Masson's trichrome for analysis of infarct size. This was determined morphologically and calculated as the ratio of scar average circumferences of the endocardium and the epicardium to LV average circumferences of the endocardium and the epicardium, as previously described [25].

2.5.2. Hypertrophy score

Myocyte hypertrophy was determined from the methods described by Fiordaliso et al. [26]. In brief, digitized images from H & E stain were acquired at a magnification of 400× using a Carl Zeiss microscope attached to AxioCamMRc5 digital camera (Carl Zeiss, North Ryde, NSW, Australia). Cardiac myocytes positioned perpendicular to the plane of the section with a visible round nucleus and cell membrane clearly outlined were selected, and the area was determined by manually tracing the cell contour. The average of the area of at least 50 myocytes was then calculated in each group.

2.6. Immunohistochemistry

Collagen types I and III, macrophage interstitial infiltration and phosphorylated Smad2 were evaluated by immunohistochemical staining as described previously [27]. In brief, 4 μm sections were incubated with specific primary antibodies: goat anti-collagen I (1:200 Southern Biotechnology Associates, Inc. Birmingham, AL 35226, USA) and mouse anti-collagen III (1:10 Biogenex, San Ramon Cal, 94583 USA), anti-ED-1 (1:300 Serotec, Raleigh NC, USA) and anti-phosphorylated Smad2 (1:300 Cell Signaling, Massachusetts, USA). Appropriate secondary antibodies were applied prior to using diaminobenzidine tetrahydrochloride for the localization of the brown peroxidase conjugates.

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