Successful TRPV1 antagonist treatment for cardiac hypertrophy and heart failure in mice

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Abbreviations: TRPV1, transient receptor potential vanilloid type 1; BCTC, (4-(3-Chloro-2-pyridinyl)-N-[4-(1,1-dimethylethyl)phenyl]-1-piperazinecarboxamide); TAC, transverse aortic constriction; LVIDd, left ventricular end-diastolic; LVIDs, left ventricular end-systolic; EF, ejection fraction

Heart failure is becoming a global epidemic. It exerts a staggering toll on quality of life, and substantial medical and economic impact. In a pre-clinical model of cardiac hypertrophy and heart failure, we were able to overcome loss of heart function by administering the TRPV1 antagonist BCTC (4-(3-Chloro-2-pyridinyl)-N-[4-(1,1-dimethylethyl)phenyl]-1-piperazinecarboxamide). The results presented here identify TRPV1 antagonists as new treatment options for cardiac hypertrophy and heart failure.

Introduction

Studies on the non-selective cation channel TRPV1 (transient receptor potential vanilloid channel, subfamily V, member 1), have implicated its activation in numerous cardiovascular diseases, and our recently published study shows that functional knockout of Trpv1 protects heart function in modeled cardiac hypertrophy.1-3 TRPV1 has an ancient and extensive pharmacopoeia.4,5 Here, we used the highly selective and potent TRPV1 antagonist BCTC,6 to test whether in vivo pharmacological inhibition could elicit a similar protection in heart function to the protection we recently published study shows that functional knockout of Trpv1.3 We reasoned that this study would definitively argue for, or against, the use of TRPV1 as a therapeutic target in heart failure, since if no therapeutic protection were observed with a drug regimen then protection observed in the Trpv1 knockout could be attributed to developmental preconditioning or protection. Alternatively, if protection occurs with the drug regimen, then the extensive pharmacopoeia of TRPV1 becomes available to focus on a new therapeutic target area. This study also sought to resolve whether potential cardiovascular side effects should be factored into prescription of TRPV1 antagonists for analgesic or other use.

Results

Multiple TRPV1 antagonists are clinically effective as analgesics and have been tested in Phase I-III human trials.4 We chose the highly potent TRPV1 antagonist BCTC, with shown efficacy in neuropathic pain, and deployed it in a model system where we previously showed that genetic deficiency in Trpv1 protects from loss of heart function.6-8 Measurements of left ventricular end-diastolic (LVIDd) and end-systolic (LVIDs) internal dimensions reveal that vehicle treated mice have significantly more dilated ventricles than BCTC-treated mice from nine to 36 d after TAC treatment (LVIDd, Fig. 1A), representing a 12.8% (± 1.2) difference at 36 d. Sham mice, treated with vehicle or BCTC, showed no significant difference in their LVIDd over the experimental time period (Fig. S1A). Most notably, percentage ejection fraction is significantly (12.6% ± 0.7) better at 30 six days post TAC in BCTC than in vehicle treated mice (Fig. 1B), as is percentage fractional shortening (10% ± 0.7) (Fig. S1D) indicating significantly better heart function. BCTC TAC treated mice also show a significantly slower rate of change in ejection fraction (rate decreased by 59.9% ± 9.8), fractional shortening (rate decreased by 44.8% ± 11.7), and structural change (LVIDd, mm/day) (rate decreased by 12.8% ± 1.2) compared with rates observed in vehicle treated TAC treated mice (Fig. S1B, C and E).

Gravimetric data show that hearts from BCTC treated TAC mice weigh 13.9% (± 1.7) less than vehicle treated TAC mice, a significant difference, and BCTC and vehicle treated sham surgery mice show no significant weight difference (Fig. 1C). Histological analysis of heart sections shows that vehicle treated TAC mice have a significantly larger (49.5% ± 4.8) cardiomyocyte cross sectional area than BCTC treated TAC mice, and that BCTC and vehicle treated sham surgery mice show no significant difference in cell size (Fig. 1D and E). BCTC drug treatment...
therefore maintains heart structure and function during cardiac hypertrophy and heart failure. This protective performance of BCTC should be viewed in the context of human clinical data showing that the hazard ratio for all-cause mortality increases by 39% for every 10% reduction in ejection fraction (albeit below 45%) and that the estimated risk of a cardiac event is doubled for every 10% decrease in ejection fraction.

Analysis of classical hypertrophic, fibrotic, inflammatory, and tissue remodeling markers indicates significantly less atrial natriuretic peptide (ANP, Nppa), brain natriuretic peptide (BNP, Nppb), Collagen (Col3a1), metallopeptidase 9 (Mmp9), and interleukin 6 (IL-6) transcript expression in heart tissues derived from BCTC treated TAC mice than vehicle treated TAC control mice, while BCTC and vehicle treated sham surgery mice show no significant differences (Fig. 2A–E). BCTC treatment therefore appears to protect the heart from inducing the hypertrophic transcriptional programs associated with loss of function. Markers of fibrosis, inflammation and tissue remodeling associated with stiffening of the heart are reduced, correlating with the above structure and functional data, as well as with reduced caspase 3 cleavage, indicating 61.2% (± 10.6) less apoptosis in BCTC treated TAC mice than vehicle treated TAC control mice (Fig. 2F).

**Discussion**

Our focus on TRPV1 as a target in heart failure has been predicted on the fact that this fascinating ion channel represents a
unique nexus, integrating multiple signals and inputs that are associated with cardio-hypertrophic conditions (including pH, \(^{11}\) anandamide concentration, \(^{12}\) activation by PKC phosphorylation, \(^{13,14}\) mechanosensitivity \(^{15}\) and baroreflex \(^{16}\)). Moreover, it is expressed in multiple target tissues and cell types in the heart, \(^{3}\) There are multiple scenarios within the literature in which TRPV1 regulation could impact cardio-pathologies. Our initial hypothesis was an immunological regulation, as TRPV1 knockout exacerbates modeled infarction, impairing post ischemic recovery, \(^{17}\) as well as enhancing local inflammation and accelerating the onset of systemic inflammatory response syndrome. \(^{18}\) This parallels mast cell induction of cardiac hypertrophy and cardiac fibrosis, \(^{19}\) with mast cell knockout impacting repair responses in infarcted hearts. \(^{20}\) From a neuronal and excitable tissue perspective the literature identifies TRPV1 as a molecular sensor to detect tissue ischemia, \(^{21}\) as responsible for excitation of cardiac nociceptors by bradykinin, \(^{22}\) coupling myocardial blood flow to cardiac metabolism, \(^{23}\) and regulating vascular tension. \(^{24}\) TRPV1 is therefore putatively involved in both immunological and neuronal, and excitable tissue, cardiac responses, and depending on the cardiac pathology could present differing pathological outcomes. What is clear is that TRPV1 responds to multiple pathological inputs.

While this broad implication in heart function gives us an exploitable therapeutic advantage for TRPV1 antagonism, it is associated with a significant challenge in trying to establish mechanism. The efficacy of TRPV1 antagonism that we show here may represent the integration of inhibition of multiple contributing pathways, or the dominating pathophysiological role of one TRPV1 input pathway, remaining to be elucidated. Thus the efficacy of the BCTC antagonist against multiple TRPV1 modalities\(^ {4}\) may be an advantage for this study, where we seek therapeutic advantage, but single pathway antagonists and tissue/cell specific knockouts may be required to establish a definitive mechanism of action.

A billion dollar pharmacopeia of TRPV1 antagonists is awaiting deployment in the clinic. \(^{18}\) BCTC establishes that a subset of these may be appropriate for treatment of cardiac hypertrophy and progression to heart failure. Rapid translation to the

**Figure 2.** Real-time PCR transcriptional and western blot analysis of markers of hypertrophy, fibrosis, inflammation, tissue remodeling and apoptosis. Real-time PCR analysis of RNA transcripts from isolated heart tissues from vehicle control and BCTC treated, sham and TAC treated mice. (A) Atrial natriuretic peptide (ANP, Nppa), (*) \(p = 0.0414, n = 4, 4, 2, 5\). (B) Brain natriuretic peptide (BNP, Nppb) (**\(p = 0.022, *p = 0.0425, n = 4, 4, 2, 6\)). (C) Collagen (Col3a1) (**\(p = 0.0013, *p = 0.0054, n = 4, 4, 2, 6\)). (D) Matrix metallopeptidase 9 (Mmp9) (**\(p = 0.0018, n = 4, 4, 2, 6\)). (E) Interleukin 6 (Il-6) (**\(p = 0.0172, n = 4, 4, 2, 6\)). (F) Densitometry analysis of western blot signals corresponding to cleaved caspase 3, relative to loading control GAPDH, displayed as a ratio. Error bars are s.e.m. (**\(p = 0.0135, n = 1, 2, 2, 4\)).
Clinic may arise from the fact that so many TRPV1 antagonists have already navigated toxicology assessment and multi-phase clinical trials. Certain TRPV1 antagonists show a hyperthermic response, a side effect that has raised concerns and could potentially negate their use. However, recent studies show that repeated administration of TRPV1 antagonists attenuates this hyperthermic reaction in our mouse studies, and other work suggests that depending on the modality of the TRPV1 antagonist involved, this side effect should be eliminated. Conversely, our study raises the consideration that cardiovascular effects of TRPV1 antagonists could represent a potential side effect as these classes of compound are further developed for analgesia, itch, colitis, rhinitis and incontinence.

Our data suggest that initiating a TRPV1 antagonist regime in patients suffering from symptoms of cardiac hypertrophy, or heart failure, may protect the heart from further hypertrophy, fibrosis, apoptosis, tissue remodeling, dilation and the eventual loss of function leading to subsequent heart failure and death.

**Methods**

**Animals.** All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Hawaii. C57BL/6J (Jackson Labs) mice were housed under a 12-h light/dark cycle and fed with a standard diet and water ad libitum. Ten week-old male mice were used for all experiments. 36 d after TAC, mice were euthanized by CO2 asphyxiation for histological and molecular analysis.

**Drug administration.** The TRPV1 antagonist BCTC (4-(3-Chloro-2-pyridinyl)-N-[4-(1,1-dimethylethyl)phenyl]-1-piperazinecarboxamide) was obtained from BioTrend. Subcutaneously implanted osmotic pumps (Alzet, Model 2006) were used to systemically administer 4 mg/kg of BCTC in 21% hydroxypropyl-β-cyclodextrin / PBS or vehicle (BCTC in 21% hydroxypropyl-β-cyclodextrin / PBS) at a continuous rate of 0.15 μl/hr, equivalent to 190 μg/day for 42 d max.

**Osmotic pump installation.** Drugs can be administered through use of an osmotic pump alleviating the need for a daily injection. Briefly, long-term (up to 42 d) infusion of drugs can be accomplished by insertion of osmotic pumps without the need for repeated injection. Mice are placed under a low plane of anesthesia with an injection of Ketamine/Xylazine anesthetic (50 mg/10 mg.Kg) intraperitoneally (IP) 10 min prior to surgery. A small area between shoulder blades is shaved and sterilized with Povidine swab. A small incision is made in this area and blunt dissected below skin to allow placement of an Alzet osmotic pump (model 2006), previously loaded with the drug of choice under the skin between the shoulder blades where it is inaccessible to the mouse. Several stitches are applied to close the incision. The mouse is placed in regular housing on a warming mat until completely conscious, after which mice are then returned to regular housing room.

**Transverse aortic constriction (TAC).** Six days following osmotic pump implantation, transverse aortic constriction or sham surgery was performed as described by Rockman et al., producing left ventricular hypertrophy by constriction of the aorta. Briefly, the left side of the chest was depilated and a baseline 2-D echocardiogram was obtained. Mice were deeply anesthetized with a mixture of ketamine and xylazine. The transverse aorta between the brachiocephalic and left carotid artery was banded using 6–0 silk ligature around the vessel and a 26-gauge needle, after which the needle was withdrawn. Sham surgeries were identical apart from the constriction of the aorta.

**Doppler echocardiography.** Doppler echocardiography was performed one week post-TAC to confirm aortic constriction. Mice were anesthetized lightly with isoflurane gas and shaved. Doppler was performed using the Visualsonics Vevo 770 system. In the parasternal short-axis view, the pulsed wave Doppler sample volume was placed in the transverse aorta just proximal and distal to the site of banding. Peak velocity was traced using Vevo 770 software and the pressure gradient calculated using the simplified Bernoulli equation.

**Transthoracic echocardiography.** Transthoracic echocardiography was performed on depeilized mice using a 30 MHz transducer (Vevo 770, VisualSonics). Measurements of left ventricular end-diastolic (LVID,d) and end-systolic (LVID,s) internal dimensions were performed by the leading edge-to-leading edge convention adopted by the American Society of Echocardiography. Left ventricular ejection fraction (%EF) was calculated as [(LV Vol,d – LV Vol,s) / LV Vol,d ]x100%, and left ventricular fractional shortening (%FS) was calculated as [(LVID,d – LVID,s) / LVID,d ]x100%.

**Immunohistochemistry.** Hearts were perfused with phosphate-buffered saline (PBS) and 10% formalin in situ, collected immediately and fixed overnight in 10% formalin at 4°C. Tissues were paraffin-embedded, cut into 5 μm sections and mounted onto glass slides. For measurement of cardiomyocyte cross-sectional areas, sections were permeabilized with 0.1% Triton X-100, blocked with 1% BSA and stained with 50 μg/ml Alexa Fluor488-conjugated wheat germ agglutinin (Life Technologies) to identify sarcolemmal membranes. Nuclei were visualized with Hoechst 3649. Bright field and fluorescent images were acquired on a Zeiss Axioscope, and image analysis performed using ImageJ (NIH). Quantification was performed on ten images from two sections per heart and averaged. Cardiomyocytes from WGA-stained sections were randomly selected and traced to calculate the cross-sectional area of individual myocytes. Ten traces were performed on each of ten images from two sections per heart and averaged.

**Real-time PCR.** Total RNA was isolated from homogenized hearts with TRIzol (Life Technologies) and purified with the UltraClean Tissue and Cells RNA Isolation Kit (Mo Bio Laboratories). Single-stranded cDNA was synthesized from 300ng of RNA using qScript cDNA Super Mix (Quanta Biosciences) according to the manufacturer’s instructions. mRNA levels were measured by real-time PCR with Power SYBR Green PCR Master Mix (Life Technologies) on an ABI 7900HT Fast Real-Time PCR System (Life Technologies) using the following cycling conditions: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C, 1 min at 60°C. Primer sequences are listed below. Samples were run in triplicate and
normalized to the housekeeping gene cyclophilin. Relative quantitation was performed using the comparative (ΔΔCt) method (ABI User Bulletin #2).

Primers: Nppa (ANP) AGA AAC CAG AGA GTG GCC AGA G, CAA GAC GAG GAA GAC GCC CAG; Nppb (BNP) GCG GCA TGG ATC TCC TGA AGG, CCC AGG CAG AGT CAG AAA CTG; Col3a1 (Collagen 3) A GA TGT CCT TGA TGT GCA GC, CCA CCA ATG TCA GGT GC; Mmp9 CGG CAC GCC TTT GTG TAG CA, TCG CTT GCA CTC GGG TAG GG; Il6 AAG AGG AGT GGC TAA GCA CCA A, GCA TAA CAG ACT AGG TTT GCC; Tgfbl TGG AGC AAC ATG TGG AAC TC, CAG CAG CGG GTT ACC AAG; Ppia (Cyclophilin) CAA AGT TCC AAA GAC AGC AGA AAA C, GCC ACA TGA ATC TGT GAA TAA TTC.

Western blot analysis. Protein extracts were prepared with lysis buffer (75 mM NaCl, 40 mM mM NaF, 10 mM Iodoacetamide, 50 mM HEPES, 10% IGEPAL, 0.5 mM PMSF) containing protease inhibitors (Complete Protease Inhibitor Cocktail, Roche). Total protein concentrations were determined by bicinchoninic acid (BCA) assay (Sigma-Aldrich). Thirty micrograms of protein per lane was electrophoretically separated on a pre-cast 4–12% bis-Tris gel (Life Technologies) and transferred to PVDF membrane (EMD Millipore). Immunoblots were blocked with Odyssey Blocking Buffer (LI-COR) and probed with antibodies to cleaved caspase 3 (Cell Signaling Technology, 1:1000) and GAPDH (Calbiochem, 1:10,000), and incubated with species-appropriate IRDye680 or IRDye800-conjugated secondary antibodies (LI-COR). Washes with TBS-Tween were done between incubations. Signal intensities were analyzed with the Odyssey Infrared Imaging System and quantitated using LI-COR imaging software.

Statistics. Statistical significance was evaluated by unpaired two-tailed Student’s t-test (GraphPad Prism), with p < 0.05 regarded as significant. All data are shown as mean ± SEM.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Supplemental Materials
Supplemental materials may be found here: www.landesbioscience.com/journals/article/23006
25. Gavva NR, Bannon AW, Hovland DN Jr, Lehto SG, Klionsky I, Surapaneni S, et al. Repeated administration of vanilloid receptor TRPV1 antagonists attenuates hyperthermia elicited by TRPV1 blockade. J Pharmacol Exp Ther 2007; 323:128-37; PMID:17652633; http://dx.doi.org/10.1124/jpet.107.125674

26. Reilly R, McDonald H, Purtzreren P, Joshi S, Lewis L, Pai M, et al. Pharmacology of modality-specific transient receptor potential vanilloid-1 antagonists that do not alter body temperature. J Pharmacol Exp Ther 2012; 342:416-28; PMID:22570364; http://dx.doi.org/10.1124/jpet.111.190314

27. Rockman HA, Ross RS, Harris AN, Knowlton KU, Steinhelper ME, Field LJ, et al. Segregation of atrial-specific and inducible expression of an atrial natriuretic factor transgene in an in vivo murine model of cardiac hypertrophy. Proc Natl Acad Sci U S A 1991; 88:8277-81; PMID:1832775; http://dx.doi.org/10.1073/pnas.88.18.8277