Probenecid improves cardiac function in subjects with a Fontan circulation and augments cardiomyocyte calcium homeostasis

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Abstract

Subjects with functionally univentricular circulation who have completed staged single ventricle palliation, with the final stage culminating in the Fontan procedure, are often living into adulthood. However, high morbidity and mortality remain prevalent in these patients, as diastolic and systolic dysfunction of the single systemic ventricle are linked to Fontan circulatory failure. We presently investigated the effects of probenecid in post-Fontan patients. Used for decades for the treatment of gout, probenecid has been shown in recent years to positively influence cardiac function via effects on the Transient Receptor Potential Vanilloid 2 (TRPV2) channel in cardiomyocytes. Indeed, we observed that probenecid improved cardiac function and exercise performance in patients with a functionally univentricular circulation. This was consistent with our findings from a retrospective cohort of patients with single ventricle physiology where TRPV2 expression was increased. Experiments in isolated cardiomyocytes associated these positive actions to augmentation of diastolic calcium homeostasis.

Introduction

Probenecid has been used in clinical practice for over fifty years in the treatment and prevention of gout as well as for increasing serum levels of antibiotics and antiviral drugs in adults and children [1, 2]. More recently, probenecid has been found to have inotropic and lusitropic properties in vivo under baseline conditions and dramatically improved contractility after ischemia [3, 4]. Subsequently, probenecid was also shown to improve both systolic and diastolic function in adult patients with heart failure with reduced ejection fraction (HFrEF) [5].

Accumulating data have linked these actions of probenecid to its potent agonism of the stretch-activated Transient Receptor Potential Vanilloid 2 (TRPV2) Ca^{2+} channel [6]. Such actions have been demonstrated both in transfected cells and in native cardiomyocytes and closely linked to effects on modulating Ca^{2+} handling [7,8,9]. Crucially, TRPV2 has been shown to be upregulated in response to stress and to play an important role in maintaining cardiac structure [10]. It has also been found to play an important role in translational models of cardiovascular disease including cardiomyopathy associated with muscular dystrophy, but has not been studied in congenital heart disease [11,12,13]. These studies prompted us to further assess the efficacy of probenecid in the treatment of patients with functionally univentricular circulations who have completed staged single ventricle palliation, with the final stage being the Fontan procedure.

Single ventricle patients with Fontan circulation have a hemodynamic arrangement that places excessive burden on the single ventricular myocardium. In the prototypical single ventricle defect, hypoplastic left heart syndrome (HLHS), the single ventricle is of right ventricular morphology and must pump blood through the arterial circulation. Pulmonary blood flow is dependent on passive filling through the systemic venous compartment, which can be impeded by diastolic dysfunction of the single ventricle. The net result is a grossly abnormal hemodynamic environment for the ventricle with high effective afterload, abnormal ventriculo-arterial coupling, markedly deranged exercise stress responses, and a high risk for the development of heart failure [14]. In addition, ventricular compliance is abnormal in up to 30% of patients and can be unmasked with relatively small fluid challenges [15]. This abnormal diastolic behavior has now been linked with poor outcomes manifesting as Fontan failure, leading to poor quality of life including end organ dysfunction and consideration of heart transplantation [15].

Systolic function can also be impaired in this condition, and cardiac magnetic resonance imaging with tissue tracking (CMR-TT) recently demonstrated prognostic value in myocardial strain. Indeed, Ishizaki and colleagues found that global longitudinal strain (GLS) using CMR-TT was an independent predictor of major adverse cardiac events (MACE) [16]. Cardiac magnetic resonance (CMR) is the reference standard to evaluate ventricular volume, mass, and function of the single ventricle [17, 18]. CMR has been shown to be a valuable tool to predict adverse outcomes in Fontan patients and offers important information on Fontan pathway anatomic details and branch pulmonary artery anatomy [19, 20].

Markers of systolic and diastolic dysfunction by CMR are associated with marked reduction in exercise capacity and progressive decline in non-cardiac organ function that worsens with increasing age and is associated with worse outcomes [21]. These hemodynamic and clinical findings have become more relevant as it is now expected that over 70% of newborns with HLHS may reach adulthood [22].

The goal of this study was to test the hypothesis that oral probenecid therapy can improve systolic and diastolic function in vivo and result in improvement in exercise capacity in post-Fontan patients > 12 years of age. Indeed, even relatively small changes in myocardial performance in these patients may lead to significant clinical effect with potential improvement in symptomatology and myocardial performance. We further hypothesized that the potential protective actions of probenecid are linked to effects on cardiomyocyte Ca^{2+} homeostasis through increased TRPV2 expression in patients with single ventricle physiology.

Methods

Clinical Trial Design

This was a randomized, double-blind, crossover, placebo-controlled, single-center trial with each patient serving as their own control. The trial protocol (ClinicalTrials.gov Identifier: NCT03965351) was designed and written by the study principal investigators and was approved by the local institutional review board. Upon completion of the study, the trial data were analyzed by an independent academic statistician.

Study Population

The study goal was to recruit 17 subjects based on published data for ejection fraction (EF) change from a prior randomized crossover designed study of adult patients with HFrEF assuming a power of 80%, $\alpha = 0.05$, and a 2-sided test [5]. Despite promotion in the center and the tristate area only 8 subjects were recruited. Of these, 6 completed the full trial, 1 completed only the placebo arm, and 1 had no follow-up data and was excluded from the analysis (Fig. 1), resulting effectively in a pilot trial. Subjects were required to be 12 years of age or older, to have functionally univentricular congenital heart disease palliated with a Fontan procedure, and to exhibit impaired systolic ventricular function as assessed by preexisting echocardiographic or CMR studies. Inclusion criteria were single left ventricle EF by CMR or echocardiography assessment of $< 50\%$ or single right ventricle EF by CMR of $< 45\%$. The exclusion criteria included subjects with any documented flow obstruction, moderate or greater atrioventricular valve regurgitation, impaired renal function, and those who exhibited clinical instability. Patients with atriopulmonary Fontan, concurrent pregnancy, sulfonamide allergy, or known G6PD deficiency were also excluded.

Trial Protocol

Eligible subjects were provided written informed consent or assent for minors and then randomized using a preprinted randomization table. Patients then received either probenecid or placebo for 4 weeks of treatment according to their randomization group, after which the same testing was repeated. Following 4 weeks of washout, patients underwent the same testing procedures as during the first period of study but with the alternate treatment. All patients received a baseline assessment immediately preceding initiation of probenecid or placebo. This included symptom reporting, blood work, exercise testing (a maximal ramp-incremental cardiopulmonary exercise test to determine aerobic capacity, and a constant power endurance test at 80% of aerobic capacity to determine exercise tolerance), and MRI.

Dosing

Patients received 4 weeks of oral probenecid therapy with the following dosing regimen based on previously published data ([23]):

- For patients (adults and pediatric) ≥ 40 kg: 500 mg orally twice daily.

- For children 12–17 years old and < 40 kg: 250 mg orally twice daily.

Data Collection

At each visit, subjects who qualified for the study underwent physical examination were evaluated for New York Heart Association status, and completed a symptom-limited cardiopulmonary exercise stress test. An ECG and a non-contrast CMR were also obtained at each visit. At the end of each visit, blood was drawn for renal function and safety laboratory tests including liver function tests and N-terminal pro b-type natriuretic peptide.

Cardiac Magnetic Resonance (CMR)

Image acquisition: Cardiac magnetic resonance imaging was performed on all eligible study patients at baseline and repeated at the intervals specified above. Studies were performed on non-sedated patients while freely breathing utilizing Philips Ingenia 1.5 T research magnets. A standard protocol for image acquisition included localizer images for planning purposes followed by steady-state free precession (SSFP) cine imaging performed in the vertical long-axis as well as horizontal long-axis and short-axis stacks at a slice thickness of 6–8 mm with sufficient coverage to quantify atrial and ventricular volumes in both systole and diastole. Through-plane phase-contrast imaging was performed at the level of the AV valve leaflet tips at end-diastole as well as at the ventricular outlet for verification of volumetric analysis. Velocity encoding was optimized to avoid flow aliasing while maximizing flow velocity fidelity. Additional phase-contrast imaging through the branch pulmonary arteries and/or pulmonary veins was performed to quantify relative pulmonary and systemic blood flow. During the initial scan, a SSFP respiratory-navigated 3D whole-heart sequence was also performed during late diastole to characterize any additional extracardiac abnormalities which might have influenced image interpretation.

Image analysis: All study images were reviewed and interpreted by a single investigator with expertise in congenital CMR imaging. Institutional post-processing software (Qmass MR and Qflow, Medis) was utilized for off-line image analysis. Contouring of the single ventricle at end-systole and end-diastole was performed to quantify ventricular volumes and calculation of ejection fraction as a measure of global systolic function. Additional off-line processing of horizontal long-axis tissue strain and strain rate was performed using CVI 42 Tissue Tracking software (Circle Cardiovascular Imaging, Calgary, Canada) for further characterization of myocardial mechanics [24, 25].

Cardiopulmonary Exercise Tests (CPET)

CPET acquisition: Each participant underwent both a pre- and post-intervention cardiopulmonary exercise test at Cincinnati Children's Hospital. Participants performed a maximal ramp-incremental exercise test with a ParvoMedics Exercise cart. Baseline spirometry was performed and FEV1 was used to estimate baseline maximum voluntary ventilation (MVV) by $MVV = FEV1 \times 40$. The test consisted of a three-minute rest period followed by progressive increasing power until exercise intolerance as previously described [26]. Immediately following intolerance, pedaling resistance was reduced to zero and the participant continued for another 5 min to allow recovery variables to be collected (recovery phase). Power output, gas exchange, ventilation, EKG, BP, and pulse oximetry were monitored and recorded. Submaximal exercise parameters were derived from this collected dataset for further analysis. These include [oxygen] uptake efficiency slope, ventilatory anaerobic thresholds, and Ve/VCO_2 slope [27].

Safety

The study was conducted under the oversight of a Data and Safety Monitoring Board. Any potential serious or non-serious adverse events were reported and were promptly adjudicated. No serious adverse reactions occurred in any of the study patients related to probenecid.

Statistical Analysis for Clinical Study

Baseline values for the whole study population were evaluated prior to the first treatment per subject, with any baseline differences assessed using Fisher's Exact tests or Wilcoxon Rank-Sum tests for categorical and continuous variables, respectively. For each arm of the protocol (placebo or Probenecid), 4-week changes in outcome variables were calculated relative to the treatment-specific baseline. The mean changes of outcome variables on placebo and Probenecid were each evaluated relative to no change using unpaired t tests, as well as relative to each other using paired t tests. General linear models were employed to test for order-of-treatment effects, as well as the influence of any covariates that were not balanced at baseline. As none of these variables were significant in any model, unadjusted differences are presented throughout. p values ≤ 0.05 were considered significant.

Human Subjects for Banked Tissue Analysis

Subjects included in this portion of the study were males and females less than 18 years of age, consisting of all ethnic backgrounds who gave informed consent and donated their hearts at the

time of transplant to the Institutional Review Board-approved Investigations of Pediatric Heart Disease tissue bank at the University of Colorado Anschutz Medical Campus. Patients were excluded from the study if they were greater than 18 years of age, were not morphologic single right ventricles in the case of single ventricle (SV) subjects, or if there was inadequate RV tissue available in the Tissue Bank. Samples from biventricular non-failing control (BVNF) subjects with normal heart structure and function originated from brain dead organ donors whose hearts were not able to be transplanted for technical reasons (e.g., size or blood type mismatch). SV patient samples were considered “failing” (SVHF) and were included in this study if they had decreased ventricular function and/or ventricular dilation on echocardiogram, had signs and symptoms of heart failure, including protein losing enteropathy or plastic bronchitis, or evidence of diastolic dysfunction of the single ventricle on cardiac catheterization prior to transplant. Single ventricle patients were considered “non-failing” (SVNF) if they underwent primary transplant rather than single ventricle surgical palliation and had normal cardiac function at the time of transplant. SV patients had no clinical evidence of inherited cardiomyopathies, though genetic screening for these disorders was not always performed. At cardiac explant, heart tissue was immediately cooled in ice-cold oxygenated Tyrodes in the operating room. Ventricular tissue and septum were rapidly dissected, flash frozen, and stored at – 80 °C until further use.

Western Blots

Western blots were performed as described [28]. Protein was isolated from 10- to 25-mg frozen RV tissue in isoelectric focusing buffer at 4 °C. Blots were ponceau stained for total protein, and an anti-VRL1 (Chemicon International Inc) primary antibody was used to detect TRPV1. Blots were quantified using ImageJ (U.S. National Institutes of Health).

RNA Isolation and RT-qPCR

Human RV samples were homogenized in Qiazol, RNA was extracted using the RNeasy plus mini kit (Qiagen), and following extraction, RNA was treated with TURBO DNase (Thermo Fisher Scientific) as per the manufacturer’s protocol. cDNA was synthesized by using the Applied Biosystems High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) according to the manufacturer’s instructions. Gene expression was measured by RT-qPCR as described [29], with Power Sybr Green PCR Master Mix (Life Technologies). Expression levels of all transcripts were normalized to 18S rRNA, and are presented on a Log₂ scale.

Cardiomyocyte Isolation and Live Cell Ca^{2+} Recording

Animal experiments were approved by the Norwegian Animal Research Authority and performed according to the Norwegian Animal Welfare Act and NIH Guidelines. Left ventricular cardiomyocytes were isolated as described previously [30]. In brief, hearts were quickly harvested from deeply anesthetized adult male Wistar rats (250–350 g), cannulated through the aorta on a constant flow Langendorff setup, and perfused with isolation buffer containing (in mmol/L): 130 NaCl, 25 Hepes, 0.5 MgCl_2 , 5.4 KCl, 0.4 NaH_2PO_4 , 22 glucose, pH 7.4. After washing away the blood, hearts were digested by perfusion with 1.8 mg/mL type 2 collagenase (Worthington Biomedical Corp., Lakewood, NJ, USA) solution for 10–12 min. The left ventricle was dissected into small pieces and agitated with 0.2 mg DNase (Worthington) and 250 μL BSA (40 mg/mL). Cells were filtered and settled. The pellet was washed twice and Ca^{2+} was gradually increased to 0.2 mM.

Isolated cardiomyocytes were loaded with 20 μM Fluo 4 (Thermo Fisher Scientific) for 15 min and plated on polylysine-coated coverslips mounted in an open chamber. For probenecid-treated cells, 1 $\mu\text{mol/L}$ probenecid was present during Fluo-4 incubation and the following procedures. Cells were placed in a perfusion chamber and mounted on the stage of an inverted microscope (Observer D1, Zeiss, Germany) and imaged via a 63 x/1.2 W water objective (Objective C-Apochromat, Zeiss, Germany). Cells were continuously superfused with Hepes Tyrode buffer (37 °C) containing (in mmol/L): 140 NaCl, 5.4 KCl, 1.8 CaCl_2 , 0.5 MgCl_2 , 5.0 Hepes, 5.5 glucose, 0.4 NaH_2PO_4 , pH 7.40. Field stimulation was performed via platinum electrodes at accelerating frequencies between 0.5 and 5 Hz, allowing time for steady state to be reached at each frequency. In follow-up experiments, steady-state 1 Hz pacing was followed by rapid application of 10 mM caffeine to empty the sarcoplasmic reticulum (SR) of Ca^{2+} . The Fluo-4 signal > 510 nm was detected by a high-speed camera (Digital CMOS camera C11440-22CU, Hamamatsu, Japan) or by a photomultiplier (01–610, Photon Technology International, Birmingham, NJ, USA). Ca^{2+} recordings were analyzed using ImageJ (NIH) and Clampfit (Axon Instruments) software. Ca^{2+} transient magnitude was calculated by normalizing peak fluorescence to resting fluorescence (F_0). The time constant (τ) was calculated from a single exponential fit of the Ca^{2+} transient decay. The rate of SR Ca^{2+} reuptake, an estimate of SERCA activity, was calculated as the difference between the rate constant ($1/\tau$) of the steady-state Ca^{2+} transient at 1 Hz and the caffeine-induced Ca^{2+} transient [31].

Microsomal Ca²⁺ Uptake, Leak, and Release

Ca²⁺ handling was additionally examined using crude homogenates from rat left ventricle, as described in Kolstad et al. [32]. Briefly, fresh ventricular tissue was homogenized with a Polytron 1200 (Kinematica AG, Luzern, Switzerland; 25,000 rpm for 3 × 20 s) in ice-cold buffer containing (in mmol/L): 300 sucrose, 5 NaN₃, 1 EDTA, 40 L-histidine, 40 Tris HCl, and protease inhibitors at pH 7.9. Homogenates were frozen in liquid nitrogen and stored at -80 °C until use.

Ca²⁺ uptake and release were measured in 2.2 mL of assay buffer, containing (in mmol/L): 165 KCl, 22 Hepes, 7.5 oxalate, 11 NaN₃, 0.0055 TPEN, 4.5 MgCl₂, 9 Tris HCl, and 0.002 fura-2 salt (pH 7.0, 37 °C). Ca²⁺ fluxes were monitored with an LS50B luminescence spectrometer (Perkin Elmer Ltd, Beaconsfield, Buckinghamshire, United Kingdom) after addition of 100 µL of homogenate. Ca²⁺ uptake was initiated by addition of Na₄ATP (2.2 mmol/L), and then blocked by application of thapsigargin (1.5 mmol/L) to assess RyR leak. Releasable SR Ca²⁺ content was assessed by applying the RyR opener 4-chloro-m-cresol (4-CMC; 5.5 mmol/L) and measuring the amplitude of Ca²⁺ release.

Results

Baseline clinical, structural, laboratory, and functional data prior to first treatment are presented in Table 1. Briefly, the subjects were 71% male, median age was 29, and most (71%) presented with class 1 NYHA symptoms. As expected in this population, initial palliative procedures were common; half had had a prior Blalock–Taussig (BT) shunt as either the primary or secondary palliative surgery, and more than half had undergone a bidirectional Glenn procedure. All subjects were hemodynamically stable at the start of the study. For all participants combined, the median [Interquartile range, IQR] EF derived via CMR was 47% [41%, 52%], the ventricular volume was dilated with a median end diastolic volume of 157 [131, 196] mL/m², and estimated ventricular mass was 96 [80, 120] gms. Despite small sample numbers, characteristics of participants assigned first to placebo versus probenecid did not differ with the exception of weight (those starting on placebo were heavier), circumferential strain in systole (those starting on placebo were worse), and max VO₂ in absolute terms of mL/min (those starting on placebo were higher); max VO₂ adjusting for weight (mL/kg/min) did not differ by initial treatment.

Effect of Probenecid on Cardiac Function

Functional parameters demonstrated changes only in those derived from strain (Table 2). Specifically, both systolic and diastolic longitudinal strain demonstrated an improvement with probenecid treatment as shown in Table 2 and Fig. 2a. The longitudinal strain in systole improved by a median of -2.0% [IQR -3.0% , -2.0%] on probenecid ($p = 0.03$ compared to no change), but did not change after placebo (median 1.0% , IQR -1.0% , 2.0% , $p = 0.01$ comparing probenecid to placebo). This improvement in strain was similar regardless of baseline longitudinal strain (Fig. 2b, $p = 0.2$ for slope). Diastolic longitudinal strain also marginally improved following probenecid treatment (0.14% [0.08% , 0.19%], $p = 0.06$), compared with no change. Circumferential strain in systole was also marginally different between placebo and probenecid treatments ($p = 0.06$). Representative images are shown in Fig. 2c.

Effect of Probenecid on Exercise Capacity

With probenecid treatment, exercise parameters revealed an improvement in peak VO_2 of 40 [40 , 110] mL/min ($p = 0.06$ compared with no change) and indexed VO_2 of 1 [1 , 4] mL/kg/min ($p = 0.06$) compared with no change, although neither of these parameters was statistically different in comparison to changes observed in placebo controls (Table 2). Maximal work in watts, VE/VCO_2 slope, and O_2 uptake efficiency slope did not differ with probenecid treatment (Fig. 3a). Representative graphs are shown in Fig. 3b.

Effect of Probenecid on Laboratory Values

None of the laboratory values assessed, including NT pro-BNP, creatinine, ALT, and AST, changed significantly under either probenecid or placebo treatments, and did not differ between the treatment arms (Table 2).

TRPV2 Expression in Myocardial Samples

TRPV2 mRNA and protein expression were quantified in myocardial samples obtained from a retrospective cohort of banked samples, including samples from biventricular normal controls (BVNF) and samples with single right ventricle morphology (non-failing single ventricles, SVNf and failing single ventricles, SVHF). Patient characteristics for this cohort are presented in Table 3. There was significant upregulation of TRPV2 mRNA expression in the SV groups compared to the BV group, and an increase in TRPV2 protein in SVNf samples (Fig. 4).

Effect of Probenecid on Cardiomyocyte Calcium Homeostasis

To examine mechanisms underlying the beneficial effects of probenecid in patients, calcium-handling studies were performed in isolated cardiomyocytes loaded with fluo-4 AM.

Representative recordings (Fig. 5a) and mean data (Fig. 5b–d) showed that Ca^{2+} transients declined more rapidly following incubation with 1 $\mu\text{mol/L}$ probenecid treatment, across a range of stimulation frequencies. There was no difference in the magnitude of the Ca^{2+} transients (Fig. 5c), however, probenecid treatment inhibited buildup of diastolic Ca^{2+} levels at increasing pacing frequencies (Fig. 5d, control cells: $n = 11$, probenecid-treated cells: $n = 12$). Thus, there were clear indications that probenecid augments diastolic calcium homeostasis, consistent with the observed clinical data.

Augmented diastolic Ca^{2+} handling could result from increased SR Ca^{2+} reuptake by SERCA and/or greater Ca^{2+} extrusion of the cell by the Na^{+} - Ca^{2+} exchanger and plasmalemmal Ca^{2+} ATPase. Rapid application of 10 mM caffeine revealed similar magnitude transients following probenecid treatment, consistent with unaltered SR Ca^{2+} content (Fig. 6a). The rate of decay of the caffeine transients was also similar, indicating unaltered rates of Ca^{2+} extrusion (Fig. 6b, c, and d). This finding suggests that more rapid Ca^{2+} transient decay in probenecid-treated cells is due to increased SERCA activity, calculated by comparing decline rates for transients elicited by field stimulation or caffeine (Control cells: $n = 14$, probenecid-treated cells: $n = 13$).

Further experiments in ventricular homogenates showed that probenecid did not alter the rate of oxalate-supported Ca^{2+} uptake following addition of ATP (fura-2 fluorescence, Fig. 6e). There was also no change in Ca^{2+} leak rate, assessed by introduction of thapsigargin (TG), or in releasable vesicular Ca^{2+} content assessed by application of the RyR opener CMC ($n = 3$, 3 in control, probenecid) (Fig. 6f, g, and h). This finding suggests that the augmentation of SERCA activity observed in intact cells is not due to a direct effect on the pump but indirectly, likely through TRPV2 channel-dependent calcium flux in the microenvironment surrounding SERCA.

Discussion

Subjects with functionally univentricular physiology and a Fontan circulation now survive well into adulthood [33,34,35]. However, this comes with the cost of markedly abnormal myocardial stress responses and profound exercise intolerance [36]. Some of these adverse responses can be attributed to geometric changes in the structure of the single ventricle, coupled with intramyocardial dyssynchrony, whereas insufficient pre-load [18] may also contribute to the observed phenomena. Diastolic properties both at rest and during exercise stress have emerged as important domains in determining prognosis, and specifically the detection of late failure of the Fontan circuit [37].

The key observation of this small open-label crossover trial was that longitudinal systolic strain and to a lesser degree longitudinal diastolic strain improved significantly despite the small study population. An appreciable corresponding increment in peak oxygen consumption during exercise testing was observed in comparison to baseline (although this was not statistically significantly different from placebo). This finding is consistent with one case report and a prior clinical study in adult patients where we demonstrated improved systolic and again to a lesser degree improved diastolic function in patients with heart failure from both ischemic and non-ischemic causes [5, 12]. Furthermore, a large cohort study in adults found that probenecid therapy reduced rehospitalization rates in comparison to allopurinol, thus strongly suggesting that this therapy may be used to potentially reduce hospitalization rates in subjects with Fontan circulation and more advanced levels of heart failure [38].

Global longitudinal strain is a sensitive imaging biomarker of myocardial function and is used in multiple settings as a therapeutic target and a prognostic tool [39, 40]. In Fontan patients, global longitudinal strain as measured by CMR-TT has been shown to be an independent predictor of major adverse cardiovascular events. Importantly, we found that global longitudinal strain, a marker of systolic function, improved significantly with probenecid suggesting an improvement in intrinsic contractility following treatment [16]. In addition, we found a trend towards an improvement in diastolic strain with treatment suggesting some improvement of diastolic function consistent with augmentation of diastolic Ca^{2+} homeostasis observed in ex vivo experiments. Strain measurements have been repeatedly used as a more sensitive marker to evaluate myocardial function compared to ejection fraction in Fontan patients and other populations. The changes in strain often precede any changes in ejection fraction and thus can serve as a treatment target in heart failure [16, 18]. Longitudinal strain is used clinically in multiple settings due to reproducibility and sensitivity to many pathologies, for example, as the standard of care to evaluate ventricular function in patients after anthracycline chemotherapy [16, 41].

Our in vitro data crucially add to existing knowledge, by indicating that presently and previously reported effects of probenecid on in vivo diastolic function are linked to changes in cardiomyocyte Ca^{2+} homeostasis. We observed a faster decline of Ca^{2+} transients across a broad range of frequencies and less buildup of Ca^{2+} at higher frequencies. These effects are consistent with stimulation of SERCA activity, and an accompanying acceleration of relaxation, as our follow-up experiments ruled out an effect of probenecid on NCX [42]. Importantly, SERCA does not appear to be directly stimulated by probenecid, since SERCA-dependent Ca^{2+} uptake in ventricular homogenates was unaltered. Rather, these findings are consistent with stimulation of TRPV2 in cardiomyocytes, and a resulting Ca^{2+} influx that augments nearby SERCA activity. Indeed, Ca^{2+} is the limiting substrate for SERCA function [42].

Interestingly, we did not observe augmentation of systolic Ca^{2+} handling following probenecid treatment, despite significant improvement of systolic function in patients. It should be noted, however, that the in vivo experiments were performed in normal cardiomyocytes, and previous work has shown that mechanical stress is associated with upregulation and translocation of TRPV2 to the cell membrane [6, 13]. Indeed, we presently observed higher expression of TRPV2 in SV in comparison with BV hearts, and marginally higher expression in those with failure, that is consistent with some (but not all) prior studies that demonstrated increased TRPV2 expression in failing hearts [43, 44]. Based on these findings it is expected that probenecid likely results in more profound stimulation of TRPV2-mediated Ca^{2+} influx in diseased hearts, greater augmentation of SERCA activity, and increased Ca^{2+} release and contractility. This view is consistent with prior animal and human work demonstrating that probenecid treatment increased contractility in more diseased hearts, with parallel increases in TRPV2 expression following pressure or volume overload [43, 44].

In summary, the information provided by this study can educate future studies that may seek to personalize the dosing and timing of probenecid therapy based on degree of disease progression including severity of myocardial remodeling and/or TRPV2 channel expression and location in the cardiomyocyte. Further, these studies can also assess for potential drug–drug interactions common to this population (such as potentiation of diuretic effect) and can include a real-world population as there are very rare absolute contraindications to probenecid (i.e., children less than 2 years of age, subjects with uric acid kidney stones and those with documented hypersensitivity to the compound).

Limitations

The major limitation to this study was our inability to recruit the planned patient numbers despite repeated efforts. This small sample size therefore limited our ability to explore some of the associations of improved systolic and diastolic function and aerobic performance, and more specifically, we were not able to explore any differences between morphological RVs in comparison to LVs. However, this study serves effectively as a safety and feasibility study for larger trial designs. Future in vitro work should also be aimed at following up the in vivo work presented, with investigation of probenecid actions on diseased cardiomyocytes.

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Acknowledgements

We would like to acknowledge the Heart Transplant Team at Children's Hospital Colorado, especially Drs. David Campbell, Max Mitchell, and James Jagers for their assistance with obtaining explanted heart tissue used for the retrospective analysis. We would also like to acknowledge the Pediatric Cardiovascular Research Laboratory team at the University of Colorado Anschutz Medical Campus for the rapid dissection and transportation of fresh heart tissue for protein and mRNA studies.

Funding

This study was funded by AHA Award #17GRNT33400014 (Co-funded by The Children's Heart Foundation, J.R, J.G.W, and G.V). Additional funding was provided by the European Union's Horizon 2020 research and innovation program (Consolidator grant, W.E.L. and M.L.) under grant agreement No. 647714, The Norwegian Institute for Public Health (J.L.), and Oslo University Hospital Ullevål (P.K.L.). A.M.G is supported by the NIH/NCATS Colorado CTSI KL2, KL2-TR002534. S.D.M is supported by NIH/NHLBI R01s HL126928 and HL107715, and the Jack Cooper Millisor Chair in Pediatric Heart Disease.

Tables

Table 1: Baseline characteristics of study population, prior to first treatment

	Overall	First treatment: Placebo	First treatment: Probenecid	p-value
N	7	4	3	
Sex (% M)	5 (71%)	3 (75%)	2 (67%)	1.00
Age (years)	28.8 [12.4, 36.4]	27.4 [14,8, 36.4]	28.8 [12.4, 31.9]	0.48
Underlying Diagnosis				
DILV	3 (43%)	1 (25%)	2 (67%)	0.49
Tricuspid Atresia	2 (29%)	1 (25%)	1 (33%)	1.00
Pulmonary Atresia	1 (14%)	1 (25%)	0 (0%)	1.00
AVSD	1 (14%)	1 (25%)	0 (0%)	1.00
First palliative surgery type:				
Blalock-Taussig (BT) Shunt	3 (43%)	1 (25%)	2 (67%)	0.49
Bidirectional Glenn	2 (29%)	2 (50%)	0 (0%)	0.43
Norwood	1 (14%)	0 (0%)	1 (33%)	0.43
Pulmonary artery banding	2 (29%)	2 (50%)	0 (0%)	0.43
Second palliative surgery type:				
Blalock-Taussig (BT) Shunt	1 (14%)	1 (25%)	0 (0%)	1.00
Bidirectional Glenn	2 (29%)	1 (25%)	1 (33%)	1.00
Third palliative surgery type:				

Atrio-ventricular valve surgery	1 (14%)	0 (0%)	1 (33%)	0.43
Arrhythmia:				
Atrial tachycardia before the study	1 (14%)	1 (25%)	0 (0%)	1.00
NYHA Class				
1	5 (71%)	2 (50%)	3 (100%)	0.43
2	2 (29%)	2 (50%)	0 (0%)	
Vital status				
Height (cm)	165 [153, 178]	168.5 [165, 173]	162 [153, 178]	0.48
Weight (kg)	64.5 [48, 132]	89.2 [64.5, 132.3]	60.3 [48, 64]	0.03
BMI (kg/m ²)	24.4 [19.0, 44.2]	32.7 [21.8, 44.2]	20.5 [19.0, 24.4]	0.08
Systolic BP (mmHg)	110 [81, 128]	111 [108, 128]	96 [81, 117]	0.29
Diastolic BP (mmHg)	63 [50, 76]	64 [50, 76]	61 [56, 64]	0.48
Heart rate (bpm)	81 [68, 108]	85 [74, 98]	74 [68, 108]	0.59
O ₂ saturation (%)	91 [80, 99]	93 [80, 94]	89 [89, 99]	1.00

*n (%) or median (interquartile range) presented. P-value from Fisher exact test or Wilcoxon rank-sum test, respectively.

Table 2. Changes in key outcomes by group

Measure	Placebo (n=7)		Probenecid (n=6)		Probenecid vs. Placebo
	Difference from baseline ^a	p-value vs. no change ^b	Difference from baseline ^a	p-value vs. no change ^b	p-value ^c
Systolic/Structural					
EDV (left), ml	6.36 ± 3.07	0.08	3.11 ± 4.83	0.55	0.90
ESV (left), ml	5.0 ± 2.3	0.08	2.16 ± 2.90	0.5	0.36

				4 9	
Stroke volume (left), ml	1.36 ± 3.04	0.6 7	0.96 ± 3.59	0 . 8 0	0.66
EF% (left)	- 1.44 ± 1.31	0.3 1	- 0.60 ± 1.37	0 . 6 8	0.53
LV Mass (g)	- 2.18 ± 3.00	0.4 9	- 1.62 ± 3.34	0 . 6 5	0.50
Cardiac output	0.11 ± 0.30	0.7 3	0.18 ± 0.39	0 . 6 7	0.50
Longitudinal strain %, systole	0.57 ± 0.65	0.4 1	- 2.50 ± 0.56	0 . 0 0 7	0.00 9
Circumferential strain %, systole	- 0.83 ± 0.31	0.0 4	1.33 ± 0.95	0 . 2 2	0.06
Radial strain %, systole	-1.0 ± 3.6	0.7 9	4.83 ± 3.53	0 . 2 3	0.61

Longitudinal strain rate%, diastole	0.19 ± 0.27	0.5 1	0.15 ± 0.06	0 . 0 7	0.23
Circumferential strain rate%, diastole	0.16 ± 0.28	0.6 0	0.13 ± 0.11	0 . 2 9	0.43
Radial strain rate %, diastole	0.37 ± 0.82	0.6 7	0.33 ± 0.96	0 . 7 4	0.64
Clinical/Laboratory					
Max VO ₂ , abs, ml/min	32.0 ± 68	0.6 6	112 ± 44	0 . 0 6	0.35
Max VO ₂ , index, ml/kg/min	1.0 ± 0.95	0.3 5	2.2 ± 0.73	0 . 0 4	0.37
Max work, watts	-8.6 ± 9.8	0.4 3	0.40 ± 1.9	0 . 8 5	0.47
Max VE/VCO ₂ slope	3.0 ± 2.32	0.2 7	- 0.20 ± 0.97	0 . 8 5	0.10
Max O ₂ uptake efficiency slope	- 79.2	0.5 8	1.4 ± 33	0 .	0.60

	± 133			9 7	
NYHA	0.14 ± 0.14	0.3 6	0.17 ± 0.31	0 . 6 1	1.00
NT pro-BNP, pg/ml	43.1 ± 51.8	0.4 4	- 14.5 ± 21.9	0 . 5 4	0.82
Creatinine, mg/dl	0.02 ± 0.02	0.3 8	0.11 ± 0.06	0 . 1 2	0.20
ALT	-3.0 ± 2.4	0.2 7	-2.3 ± 4.9	0 . 6 5	0.92
AST	11.9 ± 16.2	0.4 9	-8.3 ± 7.1	0 . 2 9	0.37

^a Difference of 4-week measurement from treatment-specific baseline (mean ± SE) presented.

^b Test for difference from baseline within group by unpaired t-test

^c Test for difference between Probenecid and placebo groups by paired t-test adjusting for crossover design (n=6 pairs)

Fig. 1 CONSORT diagram showing enrollment, recruitment, and subjects that completed the study

Fig. 2. Effect of probenecid on systolic function. **a** Change in global longitudinal strain (%) in systole and diastole. **b** Relationship between change in global longitudinal strain and baseline global longitudinal strain after probenecid and placebo. **c** Representative figures. (a compared with no change; b compared with placebo)

Fig. 3. Effect of probenecid on exercise parameters. **a** Change in Max VO_2 (maximum rate of oxygen consumption); Max work (maximum work); Max VE/VCO_2 slope (slope of the linear relationship between ventilation and carbon dioxide output). **b** Representative graphs, with red square marking Max VO_2 . (a compared with no change; b compared with placebo)

Fig. 4. Change in TRPV2 expression in samples from biventricular non-failing controls (BVNF), single ventricle non-failing samples (SVNF), and single ventricle failing samples (SVHF). **a** relative mRNA expression in all three groups. **b** relative protein expression in all three groups and **c** representative western blots.

Fig. 5. Effects of probenecid on cardiomyocyte Ca^{2+} homeostasis. **a** Representative recordings of fluo-4 Ca^{2+} transients across a range of stimulation frequencies. **b** and **c** Mean measurements of Ca^{2+} transient magnitude and decay kinetics. **d** Change in resting Ca^{2+} levels, presented as diastolic fluorescence normalized to 0.5 Hz

Fig. 6. Probenecid augments SERCA function in cardiomyocytes. **a** Representative recordings for caffeine-elicited Ca^{2+} transients following 1 Hz pacing. **b** and **c** Mean measurements of caffeine decay kinetics and release magnitude. **d** SERCA activity was estimated by comparing the decay of caffeine and 1 Hz transients. **e** Oxalate-supported monitoring of Ca^{2+} uptake into SR homogenates was assessed upon addition of ATP, Ca^{2+} leak was measured by application of thapsigargin (TG), and releasable SR content was assessed by application of CMC. **f**, **g**, and **h** Mean measurements showed no effect of probenecid, supporting that SERCA stimulation in intact cardiomyocytes is indirect