Istaroxime metabolite PST3093 selectively stimulates SERCA2a and reverses disease-induced changes in cardiac function

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Abbreviations: AHF, acute heart failure; AP, action potential; APD, action potential duration; AUC_last, the area under the drug plasma levels curve from the start of drug administration to the time of final sampling; Ca_{rest}, resting Ca^{2+} at 20s pause end; Ca_{SR}, sarcoplasmic reticulum Ca^{2+} content; CaT, Ca^{2+} transient; C_{max}, maximum observed concentration; CO, cardiac output; CPA, cyclopiazonic acid; DT, deceleration time; EDV, end diastolic volume; ESV, end systolic volume; E_{diast}, diastolic potential; EF, ejection fraction; ER, excitation-release; F_0, reference fluorescence; FS, fractional shortening; HF, heart failure; HR, heart rate; I_{CaL}, L type Ca^{2+} current; I_{NaK}, Na^+/K^+ ATPase current; i.p., intraperitoneal administration; i.v., intravenous administration; IVSTd or
IVSTs, interventricular septum thickness in diastole or systole; K_dCa, Ca^{2+} dissociation constant; LD_{50}, in vivo dose causing 50% mortality; LV, left ventricle; LVEDD or LVESD, LV end-diastolic or systolic diameter; NCX, Na^+/Ca^{2+} exchanger; PKs, pharmacokinetics; PLN, phospholamban; PWTd or PWTs, posterior wall thickness in diastole or systole; SERCA, sarcoplasmic reticulum Ca^{2+} ATPase; SR, sarcoplasmic reticulum; STV, short term variability; STZ, streptozotocin; SV, stroke volume; SW, septal diastolic wall thickness; T_{0.5}, Ca^{2+} transient decay half-time or elimination half life time; \tau_{decay}, time-constant of Ca^{2+} decay; TDI, Tissue Doppler Imaging; T_{max}, the time of maximum observed concentration; V_{max}, maximal hydrolytic velocity.

**Section:** Cardiovascular
Abstract:

Heart failure (HF) therapeutic toolkit would strongly benefit from the availability of ino-lusitropic agents with a favorable pharmacodynamics and safety profile. Istaroxime is a promising agent, which combines Na\(^+\)/K\(^+\) pump inhibition with SERCA2a stimulation; however, it has a very short half-life and extensive metabolism to a molecule, named PST3093. The present work aims to investigate whether PST3093, still retains the pharmacodynamic and pharmacokinetic properties of its parent compound. We studied PST3093 for its effects on SERCA2a and Na\(^+\)/K\(^+\) ATPase activities, Ca\(^{2+}\) dynamics in isolated myocytes and hemodynamic effects in an in-vivo rat model of diabetic (streptozotocin (STZ)-induced) cardiomyopathy. Istaroxime infusion in HF patients led to accumulation of PST3093 in the plasma; clearance was substantially slower for PST3093 than for istaroxime. In cardiac rat preparations PST3093 did not inhibit the Na\(^+\)/K\(^+\) ATPase activity, but retained SERCA2a stimulatory activity. In in-vivo echocardiographic assessment, PST3093 improved overall cardiac performance and reversed most STZ-induced abnormalities. PST3093 i.v. toxicity was considerably lower than that of istaroxime and it failed to significantly interact with 50 off-targets. Overall, PST3093 is a “selective” SERCA2a activator, the prototype of a novel pharmacodynamic category with a potential in the ino-lusitropic approach to HF with prevailing diastolic dysfunction. Its pharmacodynamics are peculiar and its pharmacokinetics are suitable to prolong the cardiac beneficial effect of istaroxime infusion.

Significance statement

Heart failure (HF) treatment would benefit from the availability of ino-lusitropic agents with a favourable profile. PST3093 is the main metabolite of istaroxime, a promising agent combining Na\(^+\)/K\(^+\) pump inhibition and SERCA2a stimulation. PST3093 shows a longer half-life in human circulation compared to parent drug, activates SERCA2a, doesn’t inhibit Na\(^+\)/K\(^+\) pump and improves cardiac performance in a model of diabetic cardiomyopathy. Overall, PST3093 as
selective SERCA2a activator can be considered the prototype of a novel pharmacodynamic category for HF treatment.

**Introduction**

Heart failure (HF) is characterized by abnormal Ca\(^{2+}\) distribution among subcellular compartments, which contributes to impaired contractility and relaxation (Bers and Despa, 2006), facilitates arrhythmias (Zaza and Rocchetti, 2015) and, in the long run, contributes to myocardial remodeling (Nakayama et al., 2007). Evidence of a deficient SERCA2a activity in HF dates to the 70’s (Suko et al., 1970; Sulakhe and Dhalia, 1971). Since then, many studies confirmed this finding (Gwathmey et al., 1987; Arai et al., 1993; Kranias and Hajjar, 2012) showing that the impaired SERCA2a activity can often result from an over-inhibition by phospholamban (PLN) (Haghighi et al., 2001; Del Monte et al., 2002). Loss of SERCA2a function accounts for abnormal distribution of intracellular Ca\(^{2+}\) with numerous detrimental consequences. Interventions currently available to modulate myocyte Ca\(^{2+}\) handling (e.g. amines, PDE inhibitors etc.) stimulate SERCA2a, but they do so in the context of a multi-target action, thus resulting in untoward effects. Selective SERCA2a enhancement would afford inotropic and lusitropic effects without the drawbacks of the multi-target action (Zaza and Rocchetti, 2015). Accordingly, the use of SERCA2a stimulation in HF therapy is receiving considerable attention and many attempts to selectively stimulate SERCA2a activity with gene therapy or “small molecule” agents have been reported (Kho et al., 2011; Kaneko et al., 2017; Schaaf et al., 2020). Nonetheless, for reasons other than refutation of the principle, none of these attempts has been successfully translated into the clinic. The only exception is istaroxime, a small-molecule drug, identified as SERCA2a enhancer by our group (Rocchetti et al., 2005) and currently under clinical development for the treatment of acute HF (Shah et al., 2009; Carubelli et al., 2020). Istaroxime has a double mechanism of action: it inhibits the Na\(^{+}/K^{+}\) pump (Micheletti et al., 2002) and activates SERCA2a (Rocchetti et al., 2005). Thus, istaroxime increases overall cell Ca\(^{2+}\) content while promoting rapid Ca\(^{2+}\) sequestration into the sarcoplasmic reticulum (SR). Notably, at variance with Na\(^{+}/K^{+}\) pump blockade alone, this neither facilitates spontaneous Ca\(^{2+}\) release from
the SR (Alemanni et al., 2011), nor increases myocardial oxygen demand (Sabbah et al., 2007). Thus, istaroxime may improve systolic and diastolic performance (Shah et al., 2009) without promoting arrhythmia or ischemia (Gheorghiade et al., 2008; Carubelli et al., 2020). However, istaroxime has a plasma half-life of less than 1 hour, because of extensive hepatic metabolism to a molecule, named PST3093 (Gheorghiade et al., 2008; Carubelli et al., 2020); this restricts istaroxime usage to acute intravenous therapy.

The present work aims to investigate whether PST3093 may, on its own, be endowed with pharmacological activity. To this end, PST3093 has been synthesized and compared to istaroxime and digoxin (as reference compounds) in experimental setups at different levels of biological organization and in the context of disease-induced dysfunction.

The data here reported indicate that PST3093 shows a longer half-life in human circulation compared to parent drug, it stimulates SERCA2a activity but, at variance with istaroxime it does not inhibit the Na⁺/K⁺ ATPase. This pharmacodynamic profile translates to positive inotropy and lusitropy in an in-vivo disease model characterized by SERCA2a downregulation. Therefore, PST3093 qualifies as a “pure” SERCA2a activator, able to improve cardiac mechanical performance in-vivo.

**Materials and Methods**

The study aims to a comprehensive characterization of PST3093 (vs istaroxime, its parent compound) as an ino-lusitropic agent to be used chronically in the treatment of HF. The evaluations performed and their scope are summarized in Table 1. Further detail on methods is given in the Online Supplement.

The animal study protocols were approved by the Institutional Review Board of Milano Bicocca (29C09.26 and 29C09.N.YRR protocol codes approved on January 2021 and June 2018 respectively) and Chang Gung (CGU107-068 protocol code approved on June 2018) Universities in
accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health.

Disease model

Streptozotocin (STZ)-induced diabetes was selected as a pathological model because of its association with reduced SERCA2a function (Torre et al., 2021) and relevance to diastolic dysfunction (Valero-Muñoz et al., 2017), for which a lusitropic action may be more relevant. Diabetes was induced in Sprague Dawley male rats (150-175 g) by a single STZ (50 mg kg⁻¹) i.v. injection (STZ group). Control (healthy group) rats received STZ vehicle (citrate buffer). Fasting glycaemia was measured after 1 week and rats with values >290 mg dL⁻¹ were considered diabetic (Torre et al., 2021). Rats were euthanized by cervical dislocation under anaesthesia with ketamine-xylazine (130-7.5 mg kg⁻¹ i.p) 9 weeks after STZ injection.

Pharmacokinetics in humans

Pharmacokinetics (PKs) of istaroxime and its metabolite PST3093 was assessed in 30 HF patients infused for 6 hours with istaroxime at 1.0 μg/kg/min (secondary analysis of the HORIZON-HF study, NCT00616161) (Gheorghiade et al., 2008). Blood samples were taken before, during, and up to 18 hours after starting the infusion. The lowest concentration resolved by the technique was 2.6 ng mL⁻¹ for istaroxime and 2.9 ng mL⁻¹ for PST3093, lower values were considered as zero. PKs parameters were estimated using the dedicated software Kinetica (version 4.4, Thermo Electron Corp., Waltham, Massachusetts). Samples were excluded from the analysis if contamination was suspected, or ≥ 2 consecutive samples were missing. The following PKs parameters were estimated: maximum observed concentration (C_{max}), the time of maximum observed concentration (T_{max}), the elimination half life time (T_{0.5}) and the area under the concentration curve from the start of istaroxime administration to the time of final sampling (AUC_{last}).

Pharmacodynamics 1: SERCA and Na⁺/K⁺ ATPase activities in cell-free preparations

Total ATPase activity was assessed by measuring the rate of ^{32}P-ATP release (μmol min⁻¹) at 37°C.
The inhibitory effect of compounds on Na⁺/K⁺ ATPase activities was tested, at multiple concentrations, on suspensions of the enzyme α1 isoform from dog kidney by measuring ³²P-ATP hydrolysis as previously described (Ferrandi et al., 1996). Na⁺/K⁺ ATPase activity was identified as the ouabain (1 mM)-sensitive component of total one; compound efficacy was expressed as the concentration exerting 50% inhibition (IC₅₀).

Measurements on SERCA2a activities were performed in whole tissue homogenates (rat) or in cardiac SR enriched microsomes (guinea-pig), including SERCA2a and PLN (Torre et al., 2021). To test for PLN involvement in the effect of compounds, SERCA1 activity was also measured in PLN-free microsomes (from guinea-pig skeletal muscle) before and after reconstitution with the PLN₁₋₃₂ inhibitory fragment at a ratio PLN:SERCA of 300:1. The SERCA component, identified as the cyclopiazonic acid (CPA, 10 µM)-sensitive one, was measured at multiple Ca²⁺ concentrations (100-2000 nM) as ³²P-ATP hydrolysis (Micheletti et al., 2007) and Ca²⁺ dose-response curves were fitted to estimate SERCA maximal hydrolytic velocity (Vₘₐₓ, μmol min⁻¹ mg⁻¹ protein) and Ca²⁺ dissociation constant (Kₐ,Ca, nM). Either an increase of Vₘₐₓ or a decrease of Kₐ,Ca (increased Ca²⁺ affinity) stand for enhancement of SERCA function.

Pharmacodynamics 2: off-target actions

To predict potential off-target actions of PST3093, its interaction with a panel of 50 ligands, potentially relevant to off-target effects, was carried out by Eurofins (Taiwan) on crude membrane preparations according to Eurofins described procedures. PST3093 was tested at the concentration of 10 µM.

Pharmacodynamics 3: effects on Iₐₕ and intracellular Ca²⁺ dynamics in healthy and diseased myocytes

To ensure stabilization of drug effect, isolated myocytes were analyzed after incubation with PST3093 or vehicle (control) for at least 30 min. Difference between means was thus tested by group comparison. The experiments were performed at 35 °C.
Na⁺/K⁺ ATPase current (I_NaK) was recorded (V-clamp) in isolated rat left ventricular (LV) myocytes as the holding current at -40 mV under conditions enhancing I_NaK and minimizing contamination by other conductances (Rocchetti et al., 2003; Torre et al., 2021). I_NaK inhibition by the compounds was expressed as percent reduction of ouabain (1 mM)-sensitive current; efficacy was expressed as the compound concentration exerting 50% inhibition (IC50) effect.

Intracellular Ca²⁺ dynamics were evaluated by Ca²⁺-dependent fluorescence (Fluo4-AM) in isolated LV myocytes and quantified by normalized units (F/F₀). Properties of voltage-induced Ca²⁺ transients (CaT, amplitude and decay kinetics) and caffeine-induced ones (estimating total SR Ca²⁺ content, Ca₅₀SR) were assessed in intact myocytes, field-stimulated at 2 Hz. A caffeine puff was applied 20 seconds (electronic timing) after a V-triggered loading sequence at 2 Hz. Resting Ca²⁺ (Ca₀rest) was measured just before caffeine application to reflect the equilibrium value of cytosolic Ca²⁺. In V-clamped myocytes, SR Ca²⁺ uptake rate was evaluated through a “SR loading” voltage protocol, specifically devised to examine the system at multiple levels of SR Ca²⁺ loading and to rule out Na⁺/Ca²⁺ exchanger (NCX) contribution. Current through L-type Ca²⁺ channel (I_CaL) was simultaneously recorded and the excitation–release (ER) “gain” was calculated as the ratio between CaT amplitude and Ca²⁺ influx through I_CaL up to CaT peak (Rocchetti et al., 2005) (protocol in Figure S1).

Pharmacodynamics 4: effect on electrical activity of normal myocytes

Action potentials (APs) were recorded by patch-clamp (I-clamp) from isolated guinea-pig LV myocytes, selected because of the AP similarity to the human one (Odening et al., 2021), under Tyrode superfusion. AP duration at 50% and 90% repolarization (APD₅₀ and APD₉₀) and diastolic potential (E_dia) were measured 1) during steady state pacing at several rates; 2) dynamically upon stepping between two rates (APD₉₀ adaptation). During steady state pacing, short-term APD₉₀ variability (STV) was calculated from 20-30 subsequent APD₉₀ values according to Eq. 1 (Altomare et al., 2015):
\[ STV = \sum (|APD(n+1) - APDn|)/[nbeats \times \sqrt{2}] \]  

(Eq. 1)

The kinetics of APD\(_{90}\) adaptation was quantified by the time constant (\(\tau\)) of exponential data fitting.

**Pharmacodynamics 5: effect on in-vivo hemodynamics of diseased hearts**

Healthy and STZ rats were studied by transthoracic echocardiographic under urethane anaesthesia (1.25 g kg\(^{-1}\) i.p.) by means of a Mindray M9 ultrasound machine (Mindray Co, Shen Zhen, China) equipped with a P10-4s phased ultrasound transducer. Left-ventricular end-diastolic (LVEDD) and end-systolic (LVESD) diameter, posterior wall (PWT) and interventricular septal (IVST) thickness were measured according to the American Society of Echocardiography guidelines (Lang et al., 2006). The Teichholz formula \((7/ (2.4+ D) \times D^3, D = \text{linear LV diameter})\) was used to calculate LV end-diastolic volume (EDV) and end-systolic volume (ESV). Stroke volume (SV) was calculated as the difference between EDV and ESV. LV ejection fraction (EF) was calculated as SV/EDV and expressed in % (Tournoux et al., 2011). Fractional shortening was calculated as \(FS = (\text{LVEDD-LVESD})\times\text{LVEDD}^{-1}\) and expressed in %. Trans-mitral flow velocity was measured (pulsed Doppler) to obtain early and late filling velocities (E, A waves) and E wave deceleration time (DT). DT was also normalized to E wave amplitude (DT/E ratio). Peak myocardial systolic (s’) and diastolic velocities (e’ and a’), were measured at the mitral annulus by Tissue Doppler Imaging (TDI). Two-dimensional LV mass and its relative index to body weight, were estimated in healthy and STZ rats.

PST3093 was i.v. infused at 0.22 mg kg\(^{-1}\) (0.16 ml min\(^{-1}\)); echocardiographic parameters were measured before and at 15 and 30 min during the infusion. Istaroxime (0.22 mg kg\(^{-1}\)) and digoxin (0.11 mg kg\(^{-1}\)), both infused for 15 min, were used as comparators. A previous study in the same setting demonstrated that neither urethane anaesthesia, nor the infusion volumes per se affected echocardiographic parameters (Torre et al., 2021).

**Pharmacodynamics 6: in-vivo acute toxicity**

Acute toxicity of PST3093 was preliminarily evaluated in male Albino Swiss CD-1 mice by identifying the dose causing 50% mortality (LD\(_{50}\), mg kg\(^{-1}\) body weight) at 24 hours after i.v.
injection. PST3093 was dissolved in DMSO and injected at 50, 100, 200, and 250 mg kg\(^{-1}\) (4 animals for each group). Higher PST3093 dose levels were not tested due to the solubility limit of the compound; control animals received the vehicle only (DMSO).

Acute toxicity of istaroxime in male CD1 mice was also evaluated for comparison, by testing i.v. dose levels of 15, 22, 27 and 33 mg kg\(^{-1}\) (5 animals for each group). Control animals received the vehicle only (saline).

**Statistical analysis**

Individual means were compared by paired or unpaired \(t\)-test; multiple means were compared by one or two-way ANOVA for repeated measurements (RM) plus post-hoc Tukey’s multiple comparisons; drug-induced changes in overall curve steepness were defined according to significance of the “factor X group” interaction. Data are reported as mean ± SEM; \(p<0.05\) defined statistical significance of differences in all comparisons. Number of animals (N) and/or cells (n) are shown in each figure legend.

**Chemicals**

Istaroxime \{PST2744: \([E,Z]-3-[\text{(2-aminoethoxy)imino}]\text{-androstane-6,17-dione hydrochloride}\)\} and its metabolite PST3093 \{\((E,Z)-[\text{(6-beta-hydroxy-17-oxoandrostan-3-ylidene)amino}]\text{oxyacetic acid}\)\} were synthesized and produced at Prassis Research Institute and Sigma-Tau Pharmaceutical Company and then by CVie Therapeutics Limited and WindTree Therapeutics. The batch of PST3093 utilized for \textit{in-vitro} and \textit{in-vivo} studies was a 1:1 mixture of oxime E:Z isomers. It was synthesized according to standard procedures, characterized by NMR spectroscopy and its purity (about 95%) was assessed by HPLC (Figure S2). Digoxin was purchased from Sigma-Aldrich. Istaroxime (PubChem CID: 9841834); digoxin (PubChem CID: 2724385).

**Results**
Chemical structure of PST3093

PST3093 is the final metabolite of istaroxime (Gheorghiade et al., 2008); its chemical structure is shown in Figure 1A. Compared to istaroxime, PST3093 retains the oxime moiety at position 3 with the amino-chain oxidized into a carboxylic chain, while the 6-keto group of istaroxime is stereoselectively reduced to a 6β-hydroxyl group.

Pharmacokinetics (PKs)

Figure 1B shows istaroxime and PST3093 plasma levels over a 6-hours istaroxime infusion at 1 µg kg\(^{-1}\) min\(^{-1}\) and up to 12 hours after discontinuation of infusion in HF patients (Gheorghiade et al., 2008). All patients showed measurable istaroxime levels until 10 min after stopping the infusion, drug levels decreased rapidly thereafter and just one patient had a quantifiable istaroxime level 2 hours after the end of the infusion. PST3093 plasma levels increased with a lag from the start of istaroxime infusion (as expected for a metabolite) being detectable in all patients from 1 hour after the start of infusion. Plasma PST3093 levels remained detectable long after discontinuation of the infusion, up to the last sample at 12 hours after wash out (Figure 1C). The data suggest that, if istaroxime infusion had continued beyond 6 hours, the metabolite would have accumulated further.

In quantitative terms, PST3093 had a plasma half-life (T\(_{0.5}\)) of about 9 hours, i.e. substantially longer than that of istaroxime (less than 1 hour), leading to a huge enhancement of the AUC\(_{\text{last}}\) index for PST3093; while the maximal observed concentration (C\(_{\text{max}}\)) of PST3093 was similar to istaroxime one at this infusion rate, the time of its observation (T\(_{\text{max}}\)) was longer for PST3093 in comparison to istaroxime (Figure 1C).

Effect of PST3093 on Na\(^+\)/K\(^+\) ATPase

Compounds effects on Na\(^+\)/K\(^+\) ATPase activity were tested in a range of concentrations from 10\(^{-9}\) to 10\(^{-4}\) M (Figure 2A). Na\(^+\)/K\(^+\) ATPase from dog kidney had a specific baseline activity of 14 μmol min\(^{-1}\) mg\(^{-1}\) protein. The reference compound istaroxime inhibited Na\(^+\)/K\(^+\) ATPase activity with IC\(_{50}\) of 0.14 ± 0.02 μM in dog kidney (Figure 2A), which corresponds to a higher affinity as
compared to that observed in rat renal preparations (IC$_{50}$ of 55 ± 19 μM from (Torre et al., 2021)). PST3093 did not inhibit the Na$^+$/K$^+$ ATPase activity up to 100 μM, the maximal tested concentration (Figure 2A).

PST3093 effects on Na$^+$/K$^+$ ATPase current (I$_{NaK}$) were furtherly evaluated in rat LV myocytes in comparison to istaroxime (Figure 2B). The estimated IC$_{50}$ for I$_{NaK}$ inhibition by istaroxime was 32 ± 4 μM, (from (Torre et al., 2021)); for PST3093 a barely detectable inhibition (9.2 ± 1.1%) was observed at the limit concentration for solubility (100 μM), an effect that can be considered insignificant.

Effect of PST3093 on SERCA ATPase activity

Effects on SERCA2a activity in normal and diseased myocardial preparations

In STZ rat preparations (N=30), baseline SERCA2a V$_{max}$ was lower (by -27%) than in healthy ones (N=29) (0.199±0.01 vs 0.272±0.01 μmol*min$^{-1}$*mg$^{-1}$ protein, p<0.05), with no difference in K$_d$Ca (448±35 vs 393±22 nM, NS), similarly to what reported recently in the same setting (Torre et al., 2021). As also reported previously (Ferrandi et al., 2013), the response of enzyme kinetics parameters to modulation was species-specific: whereas in rat preparations both PST3093 and istaroxime (Figure 3) increased V$_{max}$, in guinea-pig ones the compounds decreased K$_d$Ca instead (Table S1).

Over the whole range of concentration tested (0.1 - 1μM), PST3093 and istaroxime failed to affect ATPase Ca$^{2+}$-dependency in healthy rat preparations (Figure 3A,C), but similarly increased V$_{max}$ in STZ ones (e.g. +22% and +20%, respectively at 300 nM) with thresholds at 100 nM and 300 nM for PST3093 and istaroxime, respectively (Figure 3B,D); SERCA2a K$_d$Ca in rat preparations was affected by neither istaroxime nor PST3093. Thus, PST3093 and istaroxime displayed similar potency in ameliorating disease-induced depression of SERCA2a ATPase activity.

PST3093 and istaroxime effect was also detected in preparations from normal guinea-pig hearts, where they both reduced the SERCA2a K$_d$Ca value by about 20% at 100 nM (Table S1).
To summarize, PST3093 and istaroxime equally stimulated SERCA2a ATPase activity in preparations including PLN. Regardless of the kinetic parameter affected, a stimulatory effect was present in healthy guinea-pig microsomes and in rat homogenates from the STZ disease model.

Dependency of SERCA stimulation by PST3093 on PLN

In a range of concentrations from 30 to 1000 nM, PST3093 and istaroxime failed to affect SERCA1 activity in the absence of PLN in skeletal muscle preparations (Figure 4A,C). Reconstitution with the PLN1-32 fragment markedly reduced SERCA1 affinity for Ca\(^{2+}\) (K\(_{\text{dCa}}\) increased by 23-26%) (Figure 4B,D). Under this condition, both PST3093 (Figure 4B) and istaroxime (Figure 4D) dose-dependently reversed PLN-induced shift in K\(_{\text{dCa}}\) with an EC\(_{50}\) of 39 nM and 40 nM respectively.

PST3093 interaction with targets other than SERCA

The targets panel (50 items) included membrane receptors, key enzymes, ion channels and transporters, relevant to potential off-target cardiac and extra cardiac effects (list in Table S2); PST3093 was tested at the concentration of 10 μM. None among the 50 items met criteria for significance of interaction. Thus, at least for the ligands shown in Table S2, no off-target action of PST3093 is expected.

Effects of PST3093 on intracellular Ca\(^{2+}\) dynamics in cardiac myocytes

Effect on intracellular Ca\(^{2+}\) dynamics under field-stimulation

Ca\(^{2+}\) dynamics were analysed in field stimulated (2 Hz, Figure 5) rat LV myocytes isolated from healthy or STZ rats.

STZ myocytes had a lower SR Ca\(^{2+}\) content (Ca\(_{\text{SR}}\)) and a slower CaT decay than healthy ones; however, CaT amplitude remained unchanged. These changes are compatible with reduced SERCA2a function, possibly compensated by APD prolongation, known to increase cell Ca\(^{2+}\) content (Torre et al., 2021). Whereas in healthy myocytes 1 μM PST3093 failed to affect any of the Ca\(^{2+}\) dynamics parameters, in STZ-myocytes PST3093 reduced the quiescence Ca\(^{2+}\) (Ca\(_{\text{rest}}\)) prior to
caffeine application and partially restored Ca\textsubscript{SR} and CaT decay. Moreover, PST3093 restored in STZ-myocytes the distribution of Ca\textsubscript{SR} values peculiar of healthy ones. Comparable results have been obtained with istaroxime at a concentration marginally affecting Na\textsuperscript{+}/K\textsuperscript{+} ATPase (Torre \textit{et al.}, 2021). Taken together, this observation suggests that PST3093 improved Ca\textsuperscript{2+} sequestration into the SR during the post-train quiescence period.


\textit{Effect on SR Ca\textsuperscript{2+} uptake function under NCX inhibition}

STZ-induced changes in repolarization affect Ca\textsuperscript{2+} handling in a direction masking SERCA2a downregulation (Torre \textit{et al.}, 2021). Thus, the “SR loading” protocol (see methods and Figure S1) was performed under V-clamp and used to assess SR Ca\textsuperscript{2+} uptake under conditions emphasizing SERCA2a role (NCX inhibition). In STZ myocytes, as compared to healthy ones, SR reloading was significantly depressed (both in terms of CaT amplitude and ER gain) and CaT decay was slower at all time-points during reloading (Figure 6A). These changes are compatible with depressed SERCA2a function (Torre \textit{et al.}, 2021). PST3093 (1 µM) was tested in STZ myocytes (Figure 6B), where it sharply accelerated CaT decay, restoring the profile observed in healthy myocytes. Albeit less evident, drug-induced changes in CaT and ER gain pointed in the same direction. Comparable results have been obtained with istaroxime at a concentration marginally affecting Na\textsuperscript{+}/K\textsuperscript{+} ATPase (Torre \textit{et al.}, 2021).

Overall, PST3093 restored SR function in diseased myocytes, i.e. the context of the pathological cellular environment, most likely through SERCA2a enhancement.


\textit{Effects of PST3093 on cellular electrical activity}

To assess the electrophysiological safety of PST3093, its effects on AP of LV myocytes were investigated. Guinea-pig myocytes were used, instead of rat ones, because their AP is closer to the human one.

PST3093 (100 nM) marginally reduced APD\textsubscript{50} at all pacing rates, leaving the other AP parameters unchanged (Figure 7A-B). Notably, also APD rate-dependency at steady-state and the kinetics of
APD adaptation following a step change in rate, were unaffected by the agent (Figure 7C). STV of APD$_{90}$, a reporter of repolarization stability, was also unaffected by PST3093 at all pacing rates (Figure 7D-E). Except for the absence of APD$_{50}$ reduction, similar results were obtained with PST3093 at 1 µM (Figure S3).

The paucity of PST3093 effects on the AP is consistent with the absence of hits in the analysis of PST3093 interaction (up to 10 $\mu$M) with molecular targets other than SERCA2a, among them ion channels and transporters (Table S2).

In vivo acute toxicity in mice

*In vivo* toxicity after i.v. injection was investigated in mice for PST3093 and istaroxime. PST3093 was well tolerated and did not cause death up to 250 mg/kg. The LD$_{50}$ was not calculated since no deaths occurred at the maximal usable dose (limited by solubility). The LD$_{50}$ for istaroxime was 23.06 mg/kg. The main signs of toxicity were prostration, gasping and convulsions. In most of the animals, death occurred within 5 min after istaroxime administration. Post-mortem examination revealed pulmonary oedema and/or haemorrhages and generalized organ congestion. No remarkable alterations were found in the surviving animals.

Therefore, when i.v. administered, PST3093 was far less toxic than istaroxime, a result ascribable to its lack of effects on the Na$^+$/K$^+$ ATPase.

Modulation of cardiac function in vivo in rats with diabetic cardiomyopathy

In-vivo cardiac function was evaluated by echocardiography. In light of the uncertainty inherent to the mechanistic interpretation of drug effect on individual echo indexes, this set of experiments was designed to test whether PST3093 was able to reverse the derangements peculiar of the STZ disease model. To this end, the STZ model had to be first characterized.

Features of the disease model
Fasting hyperglycaemia, polydipsia, polyuria and polyphagia ensued 1 week after STZ injection; none of these symptoms was observed in healthy rats. Eight weeks after STZ, total body weight (BW) was substantially lower in STZ rats; LV mass (by echo) was reduced in absolute value but, when normalized to BW, was not significantly different in STZ rats (Table S3). The STZ model was largely characterized in a previous work of ours (Torre et al., 2021).

In this study a comprehensive echocardiographic analysis of STZ rats in comparison to healthy ones was performed. Figure 8 compares some echo indexes in STZ vs healthy rats; Table S4 lists all the measured echo parameters in the two groups. Heart rate (HR) was lower in STZ rats (-20%; p<0.05) and stroke volume (SV) unchanged; nonetheless, differences in cardiac output (CO) between the two groups did not achieve significance. **Systolic indexes:** in STZ rats LV end-systolic diameter (LVESD) was larger and the ejection fraction (EF) reduced; fractional shortening (FS) and systolic tissue velocity (s’) were depressed. **Diastolic indexes:** in STZ rats LV end-diastolic diameter (LVEDD) tended to be larger; peak E wave velocity (E) was slightly smaller and A wave velocity (A) was unchanged (E/A unchanged); E wave deceleration time (DT) was unchanged in absolute, but the DT/E ratio increased. Changes in early (e’) and late (a’) diastolic tissue velocities paralleled those in E and A waves; therefore, the e’/a’ and E/e’ ratios did not differ between STZ and healthy rats.

To summarize, in STZ rat echocardiographic abnormalities were rather subtle; nonetheless, 11 out of 21 indexes were significantly affected indicating derangements in both systolic and diastolic function.

**Drug effects in the disease model**

The *in vivo* acute effect of PST3093 (0.22 mg·kg⁻¹·min⁻¹) on echo indexes of STZ rats was investigated at 15 and 30 minutes of infusion (Figure 9). Data at 15 min were also obtained with istaroxime (0.22 mg·kg⁻¹·min⁻¹) and digoxin (0.11 mg·kg⁻¹·min⁻¹) (Table 2). Overall, PST3093 increased SV volume and, albeit unchanged HR, it increased CO (Figure 9). **Systolic indexes:**
PST3093 tended to decrease LVESD and increased FS, EF and s’. Diastolic indexes: PST3093 increased LVEDD, E, A and e’ (E/A and E/e’ unchanged, Table 2); DT and DT/E were reduced. PST3093 effect was almost complete at 15 min of infusion, only minor increments were observed at 30 min (Figure 9). Collectively, PST3093 improved overall cardiac function, both systolic and diastolic, beyond simple recovery of STZ-induced derangements. As shown in Figure 9, PST3093 “reversed” STZ-induced changes in 7 out of 11 indexes; moreover, five additional indexes, unaffected by STZ, were changed by PST3093 in a direction compatible with positive inotropy/lusitropy (Table 2).

PST3093 effects were only partially shared by istaroxime at the same infusion rate (Table 2). Istaroxime failed to increase SV and systolic indexes (SV, FS, EF, s’); it increased CO but, at variance with PST3093, this was because HR increased. Similar to PST3093, istaroxime shortened DT, DT/E and increased e’, a’ and A; however, it did not change E, thus reducing E/e’. Digoxin (Table 2), as expected from its inotropic effect, increased systolic indexes (FS, EF and s’); however, at variance with PST3093, it did not affect SV or CO. Notably, also digoxin improved diastolic function to some extent: E/A, DT and DT/E were reduced and e’ was increased.
Discussion

In the present study, we have investigated effects of the istaroxime metabolite PST3093 at molecular, cellular and \textit{in-vivo} levels. The interest in this molecule is motivated by 1) the possibility that it may actually contribute to (i.e. be endowed with) the unique mechanism of action and interesting therapeutic profile of istaroxime (inotropy and lusitropy at low proarrhythmic risk, confirmed in phase 2 clinical trials) (Sabbah \textit{et al.}, 2007; Gheorghiade \textit{et al.}, 2008; Shah \textit{et al.}, 2009; Carubelli \textit{et al.}, 2020) and 2) the possibility it would afford to test the clinical benefit associated specifically with the rescue of SERCA2a depression, which is widely recognized as the basis for many among HF abnormalities.

PST3093 effect has been tested in three experimental settings with incremental level of biological organization, including \textit{in-vivo} measurements from diseased hearts. The consistency of effects across these three sets of experiments confers robustness to the findings.

The results of molecular studies indicate that PST3093 differs from istaroxime because it is devoid of any inhibitory activity on the Na$^+$/K$^+$ ATPase, while retaining SERCA2a stimulatory action. This identifies PST3093 as a “selective” SERCA2a activator. The results also indicate that, similar to istaroxime (Ferrandi \textit{et al.}, 2013), PST3093 may act by weakening SERCA-PLN interaction. In rat preparations, PST3093 (and istaroxime) increased SERCA2a $V_{\text{max}}$. Albeit this apparently conflicts with the notion that interference with PLN should decrease the $K_d$Ca instead (Brittsan \textit{et al.}, 2003), the same pattern coexisted with evidence, by several independent approaches, of istaroxime antagonism of SERCA2a-PLN interaction (Ferrandi \textit{et al.}, 2013). The reason for this apparent discrepancy is unclear; the observation that PST3093 effect on SR Ca$^{2+}$ uptake was present in rat cardiac myocytes (i.e. at physiological Ca$^{2+}$ concentrations) suggests that it may reside in specificities of the microsomal preparation.

Investigations in intact ventricular myocytes confirm a negligible effect of PST3093 on Na$^+$/K$^+$ pump function. Furthermore, PST3093 abolished STZ-induced intracellular Ca$^{2+}$ abnormalities.
likely dependent on SERCA2a downregulation. While PST3093 clearly affected several Ca\textsuperscript{2+} cycling parameters under V-clamp conditions (Figure 6), its effects in field stimulated cells were apparently small (Figure 5). As we have previously shown in this experimental model (Torre et al., 2021), evaluation of Ca\textsuperscript{2+} handling without controlling membrane potential (field stimulation) and without disabling competing mechanisms (NCX) may be unsuitable to detect SERCA2a activation. This is why we also performed experiments under V-clamp and with disabled NCX function, shown in Figure 6. Field-stimulation experiments are nonetheless informative because they better represent drug effects at the cell level under “physiological” conditions. In particular, the reduction in Ca\textsubscript{rest} indicates that PST3093 allows the other Ca\textsuperscript{2+} cycling parameters to be preserved (or even showing a trend to improve) at a lower cytosolic Ca\textsuperscript{2+} level. Considering that all Ca\textsuperscript{2+} homeostatic mechanisms are in place in this setting, this is precisely what should be expected from pure SERCA2a activation (Alemanni et al., 2011; Zaza and Rocchetti, 2015), i.e. improved subcellular Ca\textsuperscript{2+} compartmentalization. That this apparently small change in myocyte physiology has an impact on \textit{in-vivo} cardiac performance is shown by the \textit{in-vivo} echo measurements (Figure 9).

The present \textit{in-vivo} studies were conducted by echocardiography in a disease model characterized by impairment of SERCA2a function (Choi et al., 2002; Torre et al., 2021). In this model, PST3093 infusion improved overall cardiac performance (SV and CO); both systolic and diastolic indexes were positively affected by the agent. Mechanistic interpretation of echocardiographic indexes is often ambiguous. For instance, both DT prolongation and shortening have been associated with deterioration of diastolic function (Eren et al., 2004; Sabbah et al., 2007). These puzzling observations can be interpreted by considering the contribution to DT of opposing factors, each prevailing in a specific condition (Mossahebi et al., 2015). At any rate, whenever HF was associated with DT shortening, istaroxime (having PST3093 as a metabolite) prolonged it (Sabbah et al., 2007; Shah et al., 2009). A further difficulty may arise from the expectation that ino-lusitropy may increase atrial contraction (A amplitude) and ventricular relaxation (E amplitude) at the same time, thus conceivably making their ratio (E/A) unable to detect drug effects on diastolic function.
A similar consideration applies to the E/e’ index. An approach to the interpretation of drug effects, independent of mechanistic models, is to check whether the drug counters disease-induced abnormalities. In the case of PST3093, this was true for the majority of indexes (7 out of 11), the most notable exception being a small further increase in LVEDD. While increments in LVEDD are usually associated with deterioration of systolic function, PST3093 tended to decrease LVESD instead. The LVEDD increment was indeed associated with increased SV, and EF to which it likely contributed.

With the exception of guinea-pig, PST3093 efficacy on SERCA2a function in the diseased condition consistently contrasted with the lack of effect in healthy preparations (Figures 3 and 5). This suggests that SERCA2a function, while not strictly limiting in health, may become so whenever its “reserve” is diminished. This view may not clash with the clear-cut effect of PLN knock-out in healthy murine myocytes; indeed, SERCA2a modulation by PST3093 may be, albeit functionally significant, subtler than complete PLN ablation.

While failing to increase the amplitude of CaT, PST3093 improved echo indexes of systolic function. Mechanisms at two levels may account for this observation: at the intracellular level, increased compartmentation of Ca^{2+} within the SR may improve the energetic efficiency of Ca^{2+} cycling (Shannon et al., 2001); at the organ level, improved relaxation may increase preload, with its well-known impact on systolic force (Shiels and White, 2008). Indeed, normalization of diastolic function in HF patients with preserved ejection fraction, may restore cardiac output irrespective of changes in the latter (Tobushi et al., 2017). On the other hand, digoxin, whose mechanism of action is purely inotropic, accelerated early relaxation (DT shortening and e’ increase). This is consistent with systo-diastolic coupling, i.e. the contribution to early relaxation of elastic restitution (recoil) of systolic force (Burns et al., 2009).
Beside affording inotropy and lusitropy, SERCA2a stimulation may improve intracellular Ca\(^{2+}\) compartmentalization, with potential long-term effects on energetic efficiency and biology of cardiac myocytes (Zaza and Rocchetti, 2015).

PST3093 is remarkably less toxic than istaroxime, which, in turn, has a lower proarrhythmic risk as compared to digoxin (Micheletti et al., 2002). We speculate that the low PST3093 toxicity, relative to istaroxime, may be due to its failure to inhibit the Na\(^{+}/K^{+}\) pump. Absence of interaction with 50 cardiac and non-cardiac targets commonly involved in drug toxicity provides at least a first level evidence of PST3093 suitability as a therapeutic agent.

**Limitations**

Whereas, in the *in-vivo* experiments PST3093 effects generally achieved a maximum at 30 min of infusion, istaroxime infusion period was limited to 15 min. Our previous study (Torre et al., 2021) indicates that a 15 min infusion is sufficient for modulation of diastolic parameters by istaroxime. This time-point was selected in the present study to minimize metabolism to PST3093, thus allowing it to differentiate istaroxime’s own effect from that of its metabolite. Nonetheless, istaroxime effects reported here might differ from the steady-state ones, described in previous studies (Sabbah et al., 2007; Shah et al., 2009; Carubelli et al., 2020), to which PST3093 (the metabolite) might actually contribute.

Echo parameters are sensitive to changes in blood pressure, which was not directly measured. Nonetheless, previous in-vivo studies ruled out the effect of infused istaroxime and, implicitly, of PST3093, on pulmonary and peripheral resistances (Gheorghiade et al., 2008).

Translation of the present *in-vivo* results to human therapy has to consider differences between clinical HF and the STZ rat model, which has specific hemodynamic features (Mihm et al., 2001). However, consistency of the istaroxime effect reported here with that described in HF patients (Carubelli et al., 2020) supports this translation.

**Therapeutic relevance and perspective**
The results of this study identify PST3093 as a prototype “selective” (i.e. devoid of Na⁺/K⁺ pump inhibition) SERCA2a activator. This may entail significant differences from the already characterized pharmacodynamic profile of istaroxime.

In the case of istaroxime, lack of the proarrhythmic effect (expected from Na⁺/K⁺ ATPase inhibition (Rocchetti et al., 2005)) is likely due to SERCA2a stimulation. Indeed, the latter may reduce the occurrence of “Ca²⁺ waves” and the resulting “triggered activity” (Bai et al., 2013; Zaza and Rocchetti, 2015; Fernandez-Tenorio and Niggli, 2018). It is logical to predict that a pure SERCA2a activator may exert substantial antiarrhythmic effects, at least under the common conditions characterized by SR instability (e.g. HF). On the other hand, Na⁺/K⁺ pump inhibition may contribute to inotropy; thus, at least theoretically, PST3093 should increase systolic force less than istaroxime. The present results argue for a PST3093 effect on global cardiac function, including positive inotropy. Moreover, compared to istaroxime, PST3093 has a much longer half-life that, per se, may also prolong the beneficial hemodynamic effect of istaroxime infusion.

Overall, PST3093 acting as “selective” SERCA2a activator can be considered the prototype of a novel pharmacodynamic class for the ino-lusitropic approach of HF. After more than 50 years from the suggestion of the involvement of a reduced SERCA2a function as a cause of the depressed cardiac function and the increased arrhythmias in HF, we may have the possibility to prove this hypothesis and provide a “causal” and selective therapy for HF patients.
Author Contributions

Participated in research design: Rocchetti, Zaza, Peri, Ferrari and Bianchi. Conducted experiments: Arici, Ferrandi, Barassi, Hsu and Chang. Performed data analysis: Arici, Ferrandi, Torre, Luraghi and Ronchi. Wrote or contributed to the writing of the manuscript: Rocchetti and Zaza.

References


American Heart Association; European Association of Echocardiography; European So. Eur J Echocardiogr 7:79–108.


Sulakhe P V., and Dhalia NS (1971) Excitation-contraction coupling in heart. VII. Calcium


Footnotes

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Manuscript is posted on BioRxiv (https://www.biorxiv.org/content/10.1101/2021.08.17.455204v1).

Conflicts of Interest

MF and PB are Windtree employees, PF and GB are Windtree consultants, S-CH is an employee of CVie Therapeutics Limited. All the other Authors declare no conflict of interest.
Figure legends

Figure 1. Chemical structure of istaroxime and its metabolite PST3093 (A) and PKs in humans (B-C). A) The main metabolic pathways of istaroxime are reduction of the carbonyl in position 6 catalyzed by carbonyl reductases and oxidative deamination of the primary amino group catalyzed by MAOs or tissue-bound semicarbazide-sensitive amine oxidase. B) Istaroxime (N=22) and PST3093 (N=29) plasma levels evaluated over 6 hours istaroxime infusion in HF patients at 1 μg/kg/min and up to 12 hours after wash-out (from HORIZON-HF study, NCT00616161). C) Statistics of the maximal observed concentration (C\text{max}), the time of C\text{max} (T\text{max}), the area under the curve from the start of drug administration to the time of final sampling (AUC\text{last}) and the plasma half-life (T\text{0.5}); *p<0.05 vs istaroxime (unpaired t-test). Data are the mean ± SEM.

Figure 2. Modulation of Na\textsuperscript{+}/K\textsuperscript{+} ATPase activity. A) inhibition of Na\textsuperscript{+}/K\textsuperscript{+} ATPase activity by istaroxime and PST3093 in dog renal preparations (N=3); B) concentration-response curves for I\textsubscript{NaK} inhibition by PST3093 (n=15) and istaroxime (modified from (Torre et al., 2021), this work is available under a CC-BY-NC license) in rat LV myocytes; I\textsubscript{NaK} recording under increasing concentrations of PST3093 and finally to ouabain (OUA as reference) is shown on the right. Data are the mean ± SEM.

Figure 3. Modulation of SERCA2a ATPase activity in healthy and diseased (STZ) preparations. Effect of PST3093 (N=5-11) (A-B) and istaroxime (N=5-16) (C-D) on SERCA2a V\text{max} estimated from Ca\textsuperscript{2+}-dose response curves in cardiac homogenates from healthy and diabetic (STZ) rats. Internal controls (ctr) are provided. Data are the mean ± SEM. * p<0.05 vs ctr (RM one-way ANOVA plus post-hoc Tukey’s multiple comparisons test or paired t-test).

Figure 4. Modulation of SERCA1 ATPase activity. Concentration-dependency of PST3093 and istaroxime modulation of SERCA1 K\text{d}Ca in guinea-pig skeletal muscle microsomes containing SERCA1 alone (A, C) (N=5,6) and after reconstitution with the PLN\textsubscript{1-32} fragment (B, D) (N=5,6).
Data are the mean ± SEM. *p<0.05 (RM or mixed model one-way ANOVA plus post-hoc Tukey’s multiple comparisons).

**Figure 5. Modulation of intracellular Ca\(^{2+}\) handling in field stimulated myocytes from healthy and diseased (STZ) hearts.** A) Representative recordings of Ca\(^{2+}\) transients (CaT) triggered by steady-state electrical stimulation at 2Hz in intact cells, followed after 20 secs by caffeine-induced Ca\(^{2+}\) release measuring SR Ca\(^{2+}\) content (Ca\(_{SR}\)). PST3093 (1 µM) was tested in normal (healthy) and diseased (STZ) myocytes. B) Statistics for CaT amplitude, Ca\(_{SR}\), Ca\(^{2+}\) at pause end (Ca\(_{rest}\)), time for 50% CaT decay (T\(_{0.5}\)). CTR N=3 (n=28 w/o PST3093, n=31 with PST3093), STZ N=4 (n=28 w/o PST3093, n=30 with PST3093). *p<0.05 (one-way ANOVA plus post-hoc Tukey’s multiple comparisons).

**Figure 6. Modulation of SR Ca\(^{2+}\) uptake under NCX inhibition in V-clamped myocytes from STZ hearts.** A) Disease (STZ) effect on SR Ca\(^{2+}\) loading in patch-clamped myocytes. SR Ca\(^{2+}\) loading by a train of V-clamp pulses was initiated after caffeine-induced SR depletion; NCX was blocked by Na\(^{+}\) substitution to identify SERCA2a-specific effects (see Methods and Figure S1); myocytes from healthy (N=9, n=32) and diseased hearts (STZ, N=6, n=31) are compared. B) PST3093 effect in STZ myocytes (N=4, w/o PST3093 n=18, with PST3093, n=19). Panels from left to right: CaT amplitude, Excitation-Release (ER) gain (the ratio between CaT amplitude and Ca\(^{2+}\) influx through I\(_{Ca-L}\)), time constant (τ) of CaT decay. *p<0.05 for the “interaction factor” in RM two-way ANOVA, indicating a different steepness of curves.

**Figure 7. Modulation of electrical activity in guinea pig myocytes.** The effect of 100 nM PST3093 was tested on action potential (AP) parameters and their steady-state rate-dependency in guinea pig myocytes (N=5). A) representative APs recorded at 0.5, 1, 2, 4 Hz in control (left) and with 100 nM PST3093 (right). B) Effect on the rate dependency of diastolic potential (E\(_{diast}\)) and AP duration (APD\(_{50}\), APD\(_{90}\)) (n≥24 w/o PST3093, n≥22 with PST3093). C) Effect on the time constant (τ) of APD\(_{90}\) adaptation following a step change in rate (n≥22 w/o PST3093, n≥16 with PST3093).
D) Effect on the rate-dependency of APD$_{90}$ short term variability (STV) (n≥24 w/o PST3093, n≥20 with PST3093). E) Effect on the correlation between STV of APD$_{90}$ and APD$_{90}$ values; data from 1, 2, 4 Hz were pooled. *p<0.05 for the “interaction factor” of RM two-way ANOVA. The effect of PST3093 at a higher concentration (1 µM) is reported in Figure S3.

**Figure 8. Disease (STZ) effects on in-vivo echocardiographic parameters.** Echocardiographic parameters are compared between healthy rats (N=18) and 8 weeks after STZ treatment (N=20). Top row, global function parameters: HR: heart rate; SV: stroke volume; CO: cardiac output. Mid row, systolic function parameters: LVESD: left ventricular end-systolic diameter; FS: fractional shortening; EF: ejection fraction; s’: peak systolic tissue velocity. Bottom row, diastolic function parameters: LVEDD: left ventricular end-diastolic diameter; E: E wave amplitude; DT: E wave deceleration time; DT/E: normalized DT; e’: peak early diastolic tissue velocity. *p<0.05 (unpaired t-test).

**Figure 9. PST3093 effects on in-vivo echocardiographic parameters in diseased (STZ) rats.** PST3093 was intravenously infused (0.22 mg.kg$^{-1}$.min$^{-1}$) in rats 8 weeks after STZ treatment. Echocardiographic parameters were measured before and at 15 and 30 min during drug infusion. Rows and symbols as in Figure 8; N=10 rats. Data are the mean ± SEM. *p<0.05 (RM one-way ANOVA plus post-hoc Tukey’s multiple comparisons). Effects of PST3093 on all echocardiographic parameters at 15 minutes of infusion in STZ rats in comparison to istaroxime and digoxin are reported in Table 2.
Table 1: synopsis of the studies

<table>
<thead>
<tr>
<th>Evaluation</th>
<th>Experimental model</th>
<th>Scope</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pharmacokinetics</td>
<td>Human blood samples from the clinical HORIZON-HF study on istoroxime (Gheorghiade et al., 2008).</td>
<td>To assess compatibility of pharmacokinetics with chronic usage.</td>
</tr>
<tr>
<td>Pharmacodynamics 1: effect on SERCA2a and Na⁺/K⁺ ATPase activities in cell-free preparations (enzymatic assays)</td>
<td>Tissue homogenates/microsomes from: dog kidney (Na⁺/K⁺ ATPase); rat and guinea-pig ventricle (SERCA2a); guinea pig skeletal muscle (SERCA1).</td>
<td>To assess efficacy and selectivity in modulating the ATPase proteins relevant to the ino-lusitropic effect. Demonstration of PLN-SERCA2a interaction as the molecular target.</td>
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<td>Pharmacodynamics 2: in-vitro effects for ligands potentially accounting for off-target actions</td>
<td>High throughput in vitro ligation to a panel of 50 molecular ligands.</td>
<td>To screen for a wide range of interactors potentially involved in off-target effects.</td>
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<tr>
<td>Pharmacodynamics 3: effect on Na⁺/K⁺ ATPase current and intracellular Ca²⁺ dynamics</td>
<td>Ventricular myocytes isolated from healthy and diseased rats with known SERCA2a dysfunction (STZ-diabetic rats).</td>
<td>To assess in isolated cells efficacy in restoring the SR function in diseased cardiac myocytes</td>
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<td>Pharmacodynamics 4: effect on electrical activity of normal myocytes</td>
<td>Ventricular myocytes isolated from guinea-pigs, a species with human-like repolarization.</td>
<td>To test for potential off-target effects of proarrhythmic relevance</td>
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<td>Pharmacodynamics 5: effect on in-vivo hemodynamics of diseased hearts</td>
<td>Healthy and STZ-diabetic rats; echocardiographic evaluation before and during drug infusion</td>
<td>To assess efficacy in reversing disease-induced hemodynamic abnormalities in vivo</td>
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<td>Pharmacodynamics 6: in-vivo acute toxicity</td>
<td>Healthy mice, evaluation of LD₅₀</td>
<td>To assess drug toxicity in-vivo</td>
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Table 2. Effects of PST3093 (0.22 mg.kg⁻¹.min⁻¹), istaroxime (0.22 mg.kg⁻¹.min⁻¹) and digoxin (0.11 mg.kg⁻¹.min⁻¹) on echo indexes of STZ rats at 15 minutes of infusion. Data are the mean ± SEM. *p<0.05 vs before drug infusion (paired t-test).
<table>
<thead>
<tr>
<th>Echo parameters</th>
<th>Istaroxime before</th>
<th>PST3093 before</th>
<th>Digoxin before</th>
<th>Istaroxime @ 15 min</th>
<th>PST3093 @ 15 min</th>
<th>Digoxin @ 15 min</th>
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<tr>
<td>IVSTd (mm)</td>
<td>1.91±0.09</td>
<td>1.89±0.09</td>
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<td>88±1.3 #</td>
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This article has not been copyedited and formatted. The final version may differ from this version.
Figure 1

A  istaroxime

B  ista

C  C_{max}  T_{max}  AUC_{last}  T_{0.5}
Figure 2

A  Na⁺/K⁺ ATPase activity  

B  Na⁺/K⁺ ATPase current (I_{NaK})  

Drug concentration (µM)  

Drug concentration (µM)
Figure 3

SERCA2a ATPase activity

Healthy

A

STZ

B

V_{max} (μmol·min^{-1}·mg^{-1} prot)

100 nM PST
300 nM PST
500 nM PST
1000 nM PST

ctr

V_{max} (μmol·min^{-1}·mg^{-1} prot)

100 nM PST
300 nM PST
500 nM PST
1000 nM PST

ctr


C

STZ

D

V_{max} (μmol·min^{-1}·mg^{-1} prot)

100 nM ista
300 nM ista
500 nM ista
1000 nM ista

ctr

V_{max} (μmol·min^{-1}·mg^{-1} prot)

100 nM ista
300 nM ista
500 nM ista
1000 nM ista

ctr

* * *

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* * *
Figure 4

SERCA1 ATPase activity

A

PLN-free

B

+PLN_{1-32}

C

D

K_d Ca (nM)

K_d Ca (nM)

K_d Ca (nM)

K_d Ca (nM)

ctl

100 nM PST

300 nM PST

1000 nM PST

ctl

100 nM ista

300 nM ista

1000 nM ista

PLN_{1-32}

PST (nM)

- + + + +

- + + + +

- + + + +

- + + + +

30 100 300 1000

30 100 300 1000

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Figure 5
Figure 6

A

CaT ampl

3x10^10

2x10^10

1x10^10

pulse #

0 2 4 6 8 10

B

CaT decay

Healthy

STZ

800

700

600

500

400

pulse #

0 2 4 6 8 10

STZ

STZ + PST

800

700

600

500

400

pulse #

0 2 4 6 8 10

3x10^10

2x10^10

1x10^10

pulse #

0 2 4 6 8 10

3

2

1

pulse #

0 2 4 6 8 10

3

2

1

pulse #

0 2 4 6 8 10

3

2

1

pulse #

0 2 4 6 8 10

3

2

1
Figure 7

A

B

C

D

E
“Istaroxime metabolite PST3093 selectively stimulates SERCA2a and reverses disease-induced changes in cardiac function” by Arici M & Ferrandi M et al.

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Supplementary Methods

Animal models

Male Sprague Dawley (SD) rats (150-175 gr) were used to generate STZ-induced diabetic cardiomyopathy model to test compounds in vivo and in vitro; female Dunkin-Hartley guinea pigs (175-200 g) were used for cardiac and skeletal muscle preparations (male 450-500 g) and myocytes isolation and finally, male Albino Swiss CD1 mice (30 g) were used for acute in vivo toxicity.

Pharmacodynamics 1: effect on SERCA2a and Na+/K+ ATPase activities in cell-free preparations (enzymatic assays)

Renal Na+/K+ ATPase purification and activity. Purification of renal Na+/K+ ATPase was performed according to the method of Jørgensen (Jørgensen, 1988). Frozen kidneys from 1-3 years-old male beagle dogs were obtained from the General Pharmacology Department of Sigma-tau, Pomezia, Italy. Kidneys were sliced and the outer medulla was dissected, pooled and suspended in a sucrose-histidine solution, containing 250 mM sucrose, 30 mM histidine and 5 mM EDTA, pH 7.2 and homogenized. The homogenate was centrifuged at 6,000 g for 15 min, the supernatant was decanted and centrifuged at 48,000 g for 30 min. The pellet was suspended in the sucrose-histidine buffer and incubated for 20 min with a sodium-dodecyl-sulphate (SDS) solution, dissolved in a gradient buffer, containing 25 mM imidazole and 1 mM EDTA, pH 7.5. The sample was layered on the top of a sucrose discontinuous gradient (10, 15 and 29.4%) and centrifuged at 60,000 g for 115 min. The final pellet was suspended in the gradient buffer. Na+/K+ ATPase activity was assayed by measuring 32P-ATP hydrolysis, as previously described (Ferrandi et al., 1996). Increasing concentrations of the standard ouabain, or tested compound, were incubated with 0.3 μg of purified dog kidney enzyme for 10 min at 37°C in 120 μl final volume of a medium, containing 140 mM NaCl, 3 mM MgCl2, 50 mM Hepes-Tris, 3 mM ATP, pH 7.5. Then, 10 μl of incubation solution containing 10 mM KCl and 20 nCi of 32P-ATP (3-10 Ci/mmol, Perkin Elmer) were added, the reaction continued for 15 min at 37°C and was stopped by acidification with 20% ice-cold perchloric acid. 32P was separated by centrifugation with activated Charcoal (Norit A, Serva) and the radioactivity was measured. Effects of increasing concentrations of the test compound were compared to ouabain, as positive standard, and to vehicle (control) at 37°C. The inhibitory activity was expressed as percent of activity in control.

SERCA ATPase activity assay. LV were dissected from rat and guinea pig of healthy and failing preparations and frozen until use. Tissues were homogenized, subjected to centrifugation to obtain SR-enriched microsomes and sarcemeric proteins were extracted, as previously described (Micheletti et al., 2007). In the case of rat hearts, cardiac homogenates were used in order to have sufficient material to replicate the experiments within a single animal. LV tissues from healthy and STZ rats were homogenized in a medium containing 300 mM sucrose, 50 mM K-phosphate, 10 mM NaF, 0.3 mM PMSF, 0.5 mM DTT (pH 7) and centrifuged at 35,000 g for 30 min. The final pellet was resuspended in the same buffer. In the case of guinea-pig hearts, LV tissues were homogenized in 4 volumes of 10 mM NaHCO3, 1 mM PMSF, 10 μg/ml aprotinin and leupeptin (pH 7) and centrifuged at 12,000 g for 15 minutes. Supernatants were filtered and centrifuged at 100,000 g for 30 min. Contractile proteins were extracted by suspending the pellets with 0.6 M KCl, 30 mM histidine, pH 7 and further centrifugation at 100,000 g for 30 min. Final pellets were reconstituted with 0.3 M sucrose, 30 mM histidine, pH 7, to obtain SR-enriched microsomes. SERCA2a ATPase activity was measured as the rate of 32P-ATP release at multiple Ca2+ concentrations in the absence and presence of test compounds, as previously described (Micheletti et al., 2007). Increasing concentrations of each compound were pre-incubated with 2 μg of cardiac preparations for 5 min at 4°C in 80 μl of a solution containing 100
mM KCl, 5 mM MgCl₂, 1 μM A23187, 20 mM Tris, pH 7.5. Then, 20 μl of 5 mM Tris-ATP containing 50 nCi of ³²P-ATP (3-10 Ci/mmol, Perkin Elmer) were added. The ATP hydrolysis was continued for 15 min at 37°C and the reaction was stopped by acidification with 100 μl of 20% ice-cold perchloric acid. ³²P was separated by centrifugation with activated charcoal (Norit A, Serva) and the radioactivity was measured.

Reconstitution of SERCA1 with PLN1-32 synthetic fragment. Adult healthy guinea-pigs were used to prepare SERCA1-enriched SR microsomes from fast-twitch hind leg muscles. Microsomes were prepared as described for SERCA2a preparations. For reconstitution experiments, SERCA1 (PLN-free) was pre-incubated with synthetic PLN1-32 fragment (canine sequence, Biomatik Corporation, Canada) in 20 mM imidazole, pH 7, at 1:300 SERCA1:PLN ratio for 30 min at room temperature. After pre-incubation, SERCA1 (PLN-free) alone, or reconstituted with PLN1-32 fragment, was utilized for SERCA activity measurement by using ³²P-ATP hydrolysis method at different Ca²⁺ concentrations in the absence and presence of increasing concentrations of tested compounds, as described for SERCA2a ATPase activity.

Pharmacodynamics 2: in-vitro effects for ligands potentially accounting for off-target actions

Analysis of PST3093 interaction with a panel of 50 ligands was carried out by Eurofins (Taiwan) on crude membrane preparations according to Eurofins described procedures. The assay is partly based on radioligand displacement (e.g. for receptors) and partly on spectrophotometric detection of change in function (e.g. for enzymes). Results were compared to appropriate reference standards; a >50% change in affinity or activity was considered as a positive hit (interaction present).

Pharmacodynamics 3: effect on Na⁺/K⁺ ATPase current and intracellular Ca²⁺ dynamics

Rat and guinea-pig LV ventricular myocytes were isolated by using a retrograde coronary perfusion method previously published (Rocchetti et al., 2003) with minor modifications. Rod-shaped, Ca²⁺-tolerant myocytes were used within 12 h from dissociation. LV myocytes were clamped in the whole-cell configuration (Axopatch 200A, Axon Instruments Inc., Union City, CA). During measurements, myocytes were superfused at 2 ml/min with Tyrode's solution containing 154 mM NaCl, 4 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES/NaOH, and 5.5 mM D-glucose, adjusted to pH 7.35. A thermostated manifold, allowing for fast (electronically timed) solution switch, was used for cell superfusion. All measurements were performed at 35 °C. The pipette solution contained 110 mM K⁺-aspartate, 23 mM KCl, 0.2 mM CaCl₂ (10⁻⁷ M calculated free-Ca²⁺ concentration), 3 mM MgCl₂, 5 mM HEPES-KOH, 0.5 mM EGTA-KOH, 0.4 mM GTP-Na⁺ salt, 5 mM ATP-Na⁺ salt, and 5 mM creatine phosphate Na⁺ salt, pH 7.3. Membrane capacitance and series resistance were measured in every cell but left uncompensated. Current signals were filtered at 2 KHz and digitized at 5 KHz (Axon Digidata 1200). Trace acquisition and analysis was controlled by dedicated software (Axon pClamp 8.0).

Na⁺/K⁺ ATPase current (I₅NaK) measurements. I₅NaK was recorded in isolated rat LV myocytes (Rocchetti et al., 2003; Alemanni et al., 2011) as the holding current recorded at -40 mV in the presence of Ni²⁺ (5 mM), nifedipine (5 μM), Ba²⁺ (1 mM) and 4-aminopyridine (2 mM) to minimize contamination by changes in Na⁺/Ca²⁺ exchanger (NCX), Ca²⁺ and K⁺ currents, respectively. Tetraethylammonium-Cl (20 mM) and EGTA (5 mM) were added to the pipette solution and intracellular K⁺ was replaced by Cs⁺. To optimize the recording conditions, I₅NaK was enhanced by increasing intracellular Na⁺ (10 mM) and extracellular K⁺ (5.4 mM). All drugs were dissolved in dimethyl sulfoxide (DMSO). Control and test solutions contained maximum 1:100 DMSO.
Intracellular Ca\textsuperscript{2+} dynamics. LV myocytes were incubated in Tyrode’s solution for 30 min with the membrane-permeant form of the dye, Fluo4-AM (10 μM), and then washed for 15 min to allow dye de-esterification. Fluo4 emission was collected through a 535 nm band pass filter, converted to voltage, low-pass filtered (100 Hz) and digitized at 2 kHz after further low-pass digital filtering (FFT, 50 Hz). After subtraction of background luminescence, a reference fluorescence (F\textsubscript{0}) value was used for signal normalization (F/F\textsubscript{0}). Cytosolic Ca\textsuperscript{2+} activity was dynamically measured in field stimulated (2 Hz) and patch-clamped rat LV myocytes. In the first case, fluorescence in diastole was used as F\textsubscript{0} for signal normalization (F/F\textsubscript{0}).

In patch-clamped myocytes membrane current, whose time-dependent component mainly reflected the sarcolemmal Ca\textsuperscript{2+} current (I\textsubscript{CaL}), was simultaneously recorded. Drug effects on SR Ca\textsuperscript{2+} uptake rate were evaluated with a “SR loading protocol” specifically devised to rule out the contribution of NCX and to assess the SR Ca\textsuperscript{2+} uptake rate at multiple levels of SR Ca\textsuperscript{2+} loading (Rocchetti et al., 2005) (protocol in Figure S1). The protocol consisted in emptying the SR by a brief caffeine (10 mM) pulse and then progressively refilling it by 7-10 voltage steps (-35 to 0 mV) activating Ca\textsuperscript{2+} influx through I\textsubscript{CaL}. NCX was blocked by omission of Na\textsuperscript{+} from intracellular and extracellular (replaced by equimolar Li\textsuperscript{+} and 1 mM EGTA) solutions. The procedure is in agreement with published methods, with minor modifications (Rocchetti et al., 2005; Alemanni et al., 2011; Torre et al., 2021). Multiple parameters, suitable to quantify SR Ca\textsuperscript{2+} uptake, can be extracted from Ca\textsuperscript{2+} and I\textsubscript{CaL} response to the protocol: the time constant (τ) of cytosolic Ca\textsuperscript{2+} decay within each V-step largely reflects net Ca\textsuperscript{2+} flux across the SR membrane (the faster SR Ca\textsuperscript{2+} uptake, the smaller τ decay). Because of the steep dependency of CaT amplitude on SR Ca\textsuperscript{2+} content, the rate at which CaT amplitude increases across the subsequent pulses of the protocol reflects the rate at which the SR refills. To rule out the potential contribution of changes in I\textsubscript{CaL}, in each loading step, CaT amplitude was normalized to Ca\textsuperscript{2+} influx (estimated from I\textsubscript{CaL} integral up to CaT peak) to obtain excitation-release (ER) gain. As expected from its strong dependency on SR Ca\textsuperscript{2+} content, this parameter progressively increases during the loading protocol. Diastolic Ca\textsuperscript{2+} of the first step was used as F\textsubscript{0} for signal normalization (F/F\textsubscript{0}). Specificity of the “loading protocol” parameters in detecting SERCA2a activation is supported by the observation that they did not detect any effect of digoxin, an inotropic agent blocking the Na\textsuperscript{+}/K\textsuperscript{+} pump and devoid of SERCA2a stimulating effect (Rocchetti et al., 2005; Alemanni et al., 2011).
Figure S1. Protocol to evaluate intracellular Ca^{2+} dynamics in patch-clamped cells under Na^{+} free condition. A) Protocol outline. B) Transmembrane current (left) and Ca^{2+} transients (right) recordings during SR reloading after caffeine-induced SR depletion in patch-clamped cells. See Methods for details.
Figure S2. Analytical characterization of PST3093. A) $^1$H-NMR in DMSO (400 MHz, Bruker), B) $^{13}$C-NMR in DMSO (100 MHz, Bruker), C) HPLC profile (about 95% purity).
Figure S3. Effects of 1 μM PST3093 on electrical activity in guinea-pig myocytes. Rate dependency of AP parameters (E<sub>diast</sub>, AP<sub>D50</sub>, AP<sub>D90</sub>), AP<sub>D90</sub> adaptation kinetics and AP<sub>D90</sub> short term variability (STV) with or w/o 1 μM PST3093; N=3 (n=16 w/o PST3093, n=18 with PST3093). Bottom right: linear correlation between STV of AP<sub>D90</sub> and AP<sub>D90</sub> values with or w/o PST3093; data from 1, 2, 4 Hz were pooled (w/o PST3093 slope = 0.012, with PST3093 slope = 0.014, NS).
Table S1. Effect of PST3093 and istaroxime on SERCA2a kinetic parameters in cardiac preparations from healthy guinea-pigs. Data are mean ± SEM; N indicates the number of experiments. *p<0.05 vs control (RM one-way ANOVA with post-hoc Tukey’s multiple comparisons test or paired t-test).

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Table S2. Effect of PST3093 (10 μM) on the panel of molecular targets (Eurofins, Taiwan).

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<td>226600</td>
<td>GABA, Flunitrazepam, Central</td>
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<td>31</td>
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<td>Glucocorticoid</td>
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<td>32</td>
<td>233000</td>
<td>Glutamate, NMDA, Phencyclidine</td>
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<td>Histamine H1</td>
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<td>241000</td>
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<td>35</td>
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<td>Insulin</td>
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<td>Muscarinic M3</td>
<td>438661</td>
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<td>Muscarinic M5</td>
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<td>258730</td>
<td>Nicotinic Acetylcholine α3β4</td>
<td>438656</td>
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<td>40</td>
<td>260410</td>
<td>Opiate μ (OP3, MOP)</td>
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<td>41</td>
<td>264500</td>
<td>Phorbol Ester</td>
<td>438624</td>
<td>mouse</td>
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<td>42</td>
<td>265600</td>
<td>Potassium Channel (K_{ATP})</td>
<td>438632</td>
<td>hamster</td>
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<td>265900</td>
<td>Potassium Channel hERG</td>
<td>438633</td>
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<td>44</td>
<td>299005</td>
<td>Progesterone PR-B</td>
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<td>45</td>
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<td>Ryanodine RyR3</td>
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<td>46</td>
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<td>Serotonin (5-Hydroxytryptamine) 5-HT1, non-selective</td>
<td>438668</td>
<td>rat</td>
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<td>47</td>
<td>299007</td>
<td>Sigma σ2</td>
<td>438662</td>
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<td>Sigma σ1</td>
<td>438636</td>
<td>human</td>
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<td>49</td>
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<td>Sodium Channel, Site 2</td>
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<tr>
<td>50</td>
<td>204410</td>
<td>Transporter, Norepinephrine (NET)</td>
<td>438597</td>
<td>human</td>
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</tbody>
</table>
Table S3. Characterization of the STZ diabetic rat model. Parameters were measured after 1 or 8 weeks (wk) from STZ injection. Data are mean ± SEM. N represents the number of rats for each group. *p<0.05 vs healthy rats (unpaired t-test).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Healthy</th>
<th>STZ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 wk after vehicle</td>
<td>8 wk after vehicle</td>
</tr>
<tr>
<td>BW (g)</td>
<td>201±4.7</td>
<td>449±11.8</td>
</tr>
<tr>
<td>Glycaemia (mg/dL)</td>
<td>130±6.6</td>
<td>nd</td>
</tr>
<tr>
<td>LV mass (mm³)</td>
<td>nd</td>
<td>985±34</td>
</tr>
<tr>
<td>LV mass index (mm³/g)</td>
<td>nd</td>
<td>2,23±0,1</td>
</tr>
<tr>
<td>N</td>
<td>18</td>
<td>18</td>
</tr>
</tbody>
</table>
Table S4. Echocardiographic parameters in healthy and STZ diabetic rats. Data are mean ± SEM. N represents the number of rats for each group. *p<0.05 vs healthy rats (unpaired t-test).

<table>
<thead>
<tr>
<th>Echo parameters</th>
<th>Healthy</th>
<th>STZ</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Morphometric parameters</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IVSTd (mm)</td>
<td>2,22±0,07</td>
<td>1,73±0,1 *</td>
</tr>
<tr>
<td>PWTd (mm)</td>
<td>1,87±0,09</td>
<td>1,54±0,05 *</td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>6,6±0,14</td>
<td>6,95±0,13</td>
</tr>
<tr>
<td>IVSTS (mm)</td>
<td>2,46±0,07</td>
<td>2,26±0,1</td>
</tr>
<tr>
<td>PWTs (mm)</td>
<td>2,96±0,09</td>
<td>2,47±0,11 *</td>
</tr>
<tr>
<td>LVESD (mm)</td>
<td>3,09±0,09</td>
<td>3,54±0,1 *</td>
</tr>
<tr>
<td><strong>Systolic parameters</strong></td>
<td></td>
<td></td>
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<tr>
<td>FS (%)</td>
<td>53,08±1,17</td>
<td>48,73±1,37 *</td>
</tr>
<tr>
<td>s' (mm/s)</td>
<td>29,59±1,15</td>
<td>24,8±0,75 *</td>
</tr>
<tr>
<td>EF (%)</td>
<td>88 ± 0,8</td>
<td>85 ±1,08 *</td>
</tr>
<tr>
<td><strong>Diastolic parameters</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E (mm/s)</td>
<td>0,93±0,01</td>
<td>0,84±0,02 *</td>
</tr>
<tr>
<td>A (mm/s)</td>
<td>0,65±0,04</td>
<td>0,63±0,03</td>
</tr>
<tr>
<td>E/A</td>
<td>1,50±0,08</td>
<td>1,38±0,07</td>
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<tr>
<td>DT (ms)</td>
<td>54,61±2,1</td>
<td>56,8±2,16</td>
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<tr>
<td>DT/E</td>
<td>59,23±2,4</td>
<td>68,37±3,0 *</td>
</tr>
<tr>
<td>E/DT</td>
<td>17,41±0,77</td>
<td>15,18±0,67 *</td>
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<tr>
<td>e' (mm/s)</td>
<td>23,46±0,77</td>
<td>21,34±0,58 *</td>
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<tr>
<td>a' (mm/s)</td>
<td>24,54±1,38</td>
<td>24,3±1,13</td>
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<tr>
<td>e'/a'</td>
<td>1,01±0,06</td>
<td>0,91±0,04</td>
</tr>
<tr>
<td>E/e'</td>
<td>40,1±1,33</td>
<td>39,64±1,0</td>
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<tr>
<td><strong>Overall cardiac function</strong></td>
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<tr>
<td>HR (bpm)</td>
<td>306±10</td>
<td>248±7,4 *</td>
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<tr>
<td>LV EDV (mL)</td>
<td>0.67±0.04</td>
<td>0.77±0.04</td>
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<tr>
<td>LV ESV (mL)</td>
<td>0.08±0.01</td>
<td>0.12±0.01 *</td>
</tr>
<tr>
<td>SV (ml)</td>
<td>0.59±0.03</td>
<td>0.65±0.04</td>
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<tr>
<td>CO (ml/min)</td>
<td>178,9±10,2</td>
<td>161,1±10,9</td>
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<td>N</td>
<td>18</td>
<td>20</td>
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</tbody>
</table>
References


