Online supplement

SAR340835, a novel selective NCX inhibitor, improves cardiac function and restores sympathovagal balance in heart failure.

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Online supplement

MATERIALS AND METHODS

Ethical approvals

All the procedures described in the present study were performed in agreement with the European regulation (2010/63/UE) and under the approval and control of SANOFI's ethics committee. All procedures were performed in AAALAC-accredited facilities in full compliance with the standards for the care and use of laboratory animals and in accordance either with the French Ministry for Research or with the German animal protection law.

In vitro characterization of SAR296968, the active principle of SAR340835

Effects of SAR296968 on CHO cells expressing sodium-calcium exchanger isoforms (NCX1, NCX2 and NCX3)

In vitro potency on the NCX isoforms was assessed by a cell-based calcium mobilization assay on Chinese hamster ovary (CHO) cell lines expressing either NCX1, NCX2 or NCX3 with a fluorescent imaging plate reader (FLIPR). The recombinant CHO-K1 cell lines stably expressing, respectively, the human NCX1, NCX2 and NCX3 were delivered by Steinbeis-Transferzentrum für Angewandte Biologische Chemie, Mannheim. Five recombinant CHO-K1 cell lines stably expressing dog, guinea-pig, pig, rabbit or rat NCX1 were generated in-house. The cells were kept in continuous culture under standard conditions (37°C, air supplemented with 5% CO_2) in HAM'S-F12 medium plus glutamine supplemented with 10% fetal calf serum (FCS) and 450 µg/ml G418. Cells were passaged every 3-4 days after detachment using a Trypsin solution and reseeded at a concentration of 150.000 cells/ml.

The assay was based on the measurement of the intracellular Ca^{2+}-concentration using the Ca^{2+}-sensitive dye Fluo4-AM. The ionophore Gramicidin (G-5002, SIGMA) was added to the cells during the measurements in the FLIPR, which elevates the intracellular Na^{+}-concentration and in turn leads to an increase in NCX activity in the "reverse" transport mode (Na^{+} out, Ca^{2+} in) resulting in an intracellular Ca^{2+} accumulation and a proportional increase in Fluo4-AM fluorescence. The increase in fluorescence was measured in cells being pre-incubated or not with SAR296968.

Fluorescence kinetics reflected NCX activity. To calculate the potency of NCX inhibition, the kinetics of the Fluo4-AM fluorescence increase was measured under conditions with fully active NCX or without NCX activity. The normal assay buffer is used as "high control" (100% NCX activity) and the assay buffer with 10 µM of a potent internal NCX inhibitor A000135933 (Hug *et al.* 2009) was used as "low control" (0% NCX activity). The inhibition of NCX was calculated in reference to the controls (0% inhibition = "low control", 100% inhibition = "high control") with the following formula:

Inhibition (%) 100 x [1 - (sample – low control)/(high control – low control)]

Selectivity profile of SAR296968

An extended profiling of SAR296968 was carried out by Eurofins Cerep SA (Targets listed in supplementary Table 4) using receptor-binding, ion channel-binding and enzyme assays. The binding of SAR296968 on these targets was assessed either by enzyme immunoassays, fluorometric, photometric, HTRF or radiometric assays. Functional assays were performed for BZD (benzodiazepine), 5-HT2B (serotonin type 2b), PR (progesterone) receptors, NE (norepinephrine) transporter and DA (dopamine) transporter.

Patch-clamp studies in guinea-pig cardiomyocytes

Activity of SAR296968 on the endogenous NCX (Iti), calcium (ICa) and sodium (INa) currents were investigated in normal guinea pig cardiac myocytes.

Preparation of single cardiomyocytes.

Guinea-pigs of either sex (Dunkin-Hartley Pirbright White, Möllegaard, Denmark, weight approximately 400 g) were sacrificed by concussion and exsanguination. The heart was quickly removed and retrogradely perfused via the aorta at 37°C: first for 5 min with Tyrode solution (in mM): NaCl 143, KCl 5.4, $MgCl_2$ 0.5, NaH_2PO_4 0.25, HEPES 5 and glucose 10, pH adjusted to 7.2 with NaOH; then the perfusion was continued with the same Tyrode solution, which now contained 0.015 mM $CaCl_2$ and 0.03% collagenase (type CLS II, Biochrom KG, Berlin, Germany) until tissue softened (~5-7 min). Thereafter, the heart was washed with storage solution containing (in mM) L-glutamic acid 50, KCl 40, taurine 20, KH_2PO_4 20, $MgCl_2$ 1, glucose 10, HEPES 10, EGTA 2 (pH 7.2 with KOH). The ventricle was cut into small pieces and myocytes were dispersed by gentle shaking. Cells were then filtered through a nylon mesh. Thereafter cells were washed twice by sedimentation and kept at room temperature in the same storage solution as described above.

Whole-cell patch-clamp recordings of the NCX currents

The whole-cell patch clamp technique (Hamill OP *et al.* 1981) was employed to measure ion currents from single isolated cardiomyocytes. Whole-cell currents were recorded with an EPC-10 patch clamp amplifier (HEKA Elektronik, Lambrecht, Germany) and Pulse software (HEKA, Lambrecht, Germany). A small aliquot of cell-containing solution was placed in a perfusion chamber and after a brief period allowing cell adhesion to the bottom, the chamber was continuously perfused with bathing solution (in mM): NaCl 140, KCl 4.7, $CaCl_2$ 1.3, $MgCl_2$ 1.0, HEPES 10, Glucose 10, pH adjusted to 7.4 with NaOH. Patch pipettes were pulled from borosilicate glass capillaries (Hilgenberg, Malsfeld, Germany) using a DMZ-Universal puller (Zeitz-Instruments, Munich, Germany) and were heat polished. When filled with pipette solution, they had a resistance of 2-3 MΩ. Offset voltages generated when the pipette was inserted in NaCl solution (1-5 mV) were zeroed before formation of the seal. After formation of the whole-cell mode, the series resistance was compensated by 40-60% and the electrical capacitance caused by the cell membrane was compensated by the EPC-10 compensation network. The cell capacitance amounted to 120-180 pF and the series resistance was 5-10 MΩ. The cell potential was -70 to -80 mV. After formation of the whole-cell voltage-clamp configuration, myocytes were kept at the holding potential of -80 mV. All patch-clamp experiments were performed under continuous perfusion of the cells with solution heated to 36±1°C. The pipette solution had the following composition (in mM): CsOH 160, CsCl 20, NaOH 20, $CaCl_2$ 29, $MgCl_2$ 2, TEA-Cl 20, aspartic acid 42, EGTA 42, ATP-Mg-salt, 10, HEPES 10, pH= 7.2 with CsOH. After obtaining the whole-cell mode, the bath solution was exchanged for the NCX-solution (in mM): NaCl 140, $CaCl_2$ 2, $MgCl_2$ 2, CsCl 2, BaCl2 1, NaH_2PO_4 0.33, nisoldipine 0.002, ouabain 0.02, HEPES 10, pH = 7.4 with NaOH.

Voltage ramps were applied from -120 mV to 60 mV in 1 s with frequency of 0.1 Hz. When currents were stable in the NCX solution, $NiCl_2$ (5 µM) was added to the bathing solution, causing complete and reversible inhibition of the NCX current. After wash-out of $NiCl_2$ SAR296968 was added at 10 nM concentration and the current was recorded after 5 min. Then the SAR296968 concentration was increased stepwise and recording time at each concentration was 5 min. Finally, $NiCl_2$ was added and current was recorded after 2 min.

Whole-cell patch-clamp recordings of the calcium and sodium currents

Conditions of whole-cell patch-clamp recordings of the calcium and sodium currents were as described for recording of NCX currents with the following differences. After establishing the whole cell configuration, the series resistance was usually < 10 MΩ and was compensated for voltage error due to the series resistance of the patch pipette. Data was acquired at 6.67 kHz and filtered with 2.87 kHz. All experiments were performed at room temperature. The pipette solutions for recording of voltage dependent Ca^{2+} and Na+ currents were either (in mM): CsOH 130, NaCl 8, MgCl2x6H2O 1, EGTA 10, HEPES 10, pH = 7.3 with CsOH or methanesulfonic acid (osmolarity 285 mOsmol), or (in mM): CsOH 130, CsCl 8, MgCl2x6H2O 1, MgATP 4, EGTA 10, HEPES 10, pH = 7.3 with CsOH or methanesulfonic acid (osmolarity 285 mOsmol). The Na+ free bath solution was (in mM): NMDG 125, CsCl 5, MgCl2 x 6H2O 1, Glucose 11.5, HEPES 10, TEA 20, CaCl2 1.8, pH = 7.4 with HCl/CsOH. For recording of voltage dependent Na^+ currents the pipette solution was (in mM): CsOH 130, CsCl 8, MgCl2 x 6H2O 1, MgATP 4, EGTA 10, HEPES 10, pH = 7.3 with CsOH or methanesulfonic acid (osmolarity 285 mOsmol), and the bath solution was (in mM): NMDG 125, CsCl 5, MgCl2x6H2O 1, Glucose 11.5, HEPES 10, TEA 20, CaCl2 1.8, NaCl 20, NiCl 0.1, Nifedipine 0.01, pH = 7.4 with HCl/CsOH.

For recording of voltage dependent Ca^{2+} currents cells were held at 80 mV and currents were routinely measured every 3 s by applying 500 ms voltage pulses to +10 mV. Current amplitudes were determined as maximal inward currents at +10 mV immediately before compound application and at the end of the experiment (≥1 min in the presence of compound). For recording of voltage dependent Na^+ currents, cells were held at 80 mV and currents were routinely measured every 5 s by applying 30 ms voltage pulses to +30 mV. Current amplitudes were determined as maximal inward currents at +30 mV immediately before compound application and at the end of the experiment (≥1.5 min in the presence of compound).

The difference between the current before application of the compound (control) and the last application of $NiCl_2$ was considered to be 100 %. The percent inhibition of the current at each concentration was evaluated related to this 100% value. The arithmetical mean ± standard error of the mean (SEM) of the percent inhibition data was obtained from the different experiments at each SAR296968 concentration.

Effects of SAR296968 on atrial and ventricular arrhythmias

The anti-arrhythmic properties of the NCX inhibitor were assessed in a battery of *in vitro* and *in vivo* models, using the active principle, SAR296968.

Delayed afterdepolarization (DADs)-related arrhythmias

The efficacy against DADs-related arrhythmias was tested in both guinea pig papillary muscles and left atria under calcium overload condition. Both preparations were mounted in an organ bath heated at 37°C, with one side on a hook connected to a pressure transducer (ISOTEC, Hugo Sachs Elektronik – Havard Apparatus, March-Hugstetten, Germany), and the other side fastened

with a small metallic tube to which gently negative pressure was applied. Action potentials (APs) were recorded by means of a glass microelectrode, filled with 3 M KCl solution. The electrodes were fabricated from borosilicate glass (item number: 1B150F-4, World Precision Instruments, Sarasota, USA) by means of a microelectrode puller (DMZ-Universal Puller, Zeitz Instruments, Martinsried, Germany). The electrodes had an electrical resistance of 5 to 10 megaohms. The electrical signal was recorded with an amplifier (Model 309, Harvard Apparatus GmbH, March-Hugstetten, Germany) and stored in a computer system.
Guinea-pig papillary muscles developed spontaneous contractions (DADs) when exposed to high extracellular calcium concentration (5.5 mM $CaCl_2$) and zero extracellular potassium concentration combined to bursts of rapid electrical pacing (4 Hz). These spontaneous contractions were counted over 6 seconds after pacing cessation, repeated before and 30 minutes after applying the active principle SAR296968 (1 and 3 µM) or the vehicle (0.3% DMSO). Additionally, the protective effect of SAR296968 (3µM) against guinea pig left atria spontaneous arrhythmic contractions was investigated. The arrhythmic contractions were induced after applying SAR296968 (3µM) or the vehicle (0.3% DMSO in saline) by combining a rapid electrical pacing and treatment with isoprenaline (1µM) and were counted over 6 seconds after termination of a rapid electrical pacing. In both models, contractility (dP/dt_{max}), relaxation (dP/dt_{min}) and action potential duration (APD) were measured using the computer software (ISO-2, MFK, Niedernhausen, Germany) at baseline and under SAR296968 exposure. For measurement of contractile force, the optimal tension to the isolated muscles as preload for force development was determined for each preparation.

Early afterdepolarizations (EADs)-related arrhythmias in isolated rabbit heart
The efficacy of SAR296968 against EADs was tested in isolated rabbit heart perfused according to the Langendorff method. The sinus node was crushed allowing low pacing stimulation. The model recapitulated Long QT syndromes (LQT) induced during 2 successive runs by combining either an activator of the late Na^+ channel (veratridine 0.5 µM, LQT2 model) or a hERG blocker (sotalol 50 µM, LQT3 model) with a period of bradycardia (40 bpm) and low potassium conditions (K^+ 1.5 mmol/L instead of 4 mmol/L;). The second run was repeated under treatments with either 0.3 or 1 µM SAR296968 or the vehicle (0.25% DMSO). The number of heart preparations in which intermittent or continuous Torsades de Pointes (TdP) episodes were observed during the period of hypokalemia and bradycardia was evaluated for each tested treatment. ECG parameters, recorded from electrogram obtained in a derivation-II like electrodes positioning, were measured at 80 and 40 bpm of pacing before and after treatments; they included QRS, QT, Tp-Te, a marker of the transmural dispersion of ventricular repolarization, evaluated by the time difference (ms) between the peak (Tp) and end (Te) of T wave (Antzelevitch *et al.* 1999).

Left atria vulnerability in anesthetized pigs
The anti-arrhythmic property of SAR296968 was further investigated by measuring the left atria vulnerability (LAV) in pentobarbital-anesthetized pigs. The purpose of this investigation was to find out what the effect of NCX-inhibition is on atrial refractoriness and electrically induced atrial arrhythmias. Pigs were premedicated with 2 mL Rompun® 2% i.m. (xylazine HCl, 23.3 mg/mL) and 1mL of Zoletil100® (100 mg/mL; 50 mg/mL Tiletamine and 50 mg/mL Zolazepam) and anesthetized with an i.v.-bolus of 3 mL Narcoren® (pentobarbital, 160 mg/mL) followed by a continuous intravenous infusion of 12-17 mg/kg/h pentobarbital. Pigs were ventilated with

room air and oxygen by a respirator. Blood gas analysis (pO2; pCO2) was performed at regular time intervals to control the oxygen supply via the respirator in order to maintain pO2 >100 mm Hg and pCO2 < 35-40 mm Hg. A left thoracotomy was performed at the fifth intercostal space, the lung retracted, the pericardium incised and the heart suspended in a pericardial cradle. The atrial effective refractory period, determined by the S1-S2 method, and cardiac contractility (dP/dt_{max}) were monitored at baseline and under treatment with SAR296968 (1.5 mg/kg over 20 minutes) solved in a mixture of DMSO (1mL) and PEG400 (9 mL). LAV was determined as described previously (Wirth *et al.* 2007). Briefly the S1-S2 stimulation procedure induced short self-terminating episodes of atrial tachycardia (fibrillation or flutter). The number of atrial repetitive action potentials following the premature beat S2 had to exceed 4 for a full score (1). Three or 4 repetitive action potentials were counted as a half score (0.5). The procedure was applied while increasing the coupling S1-S2 interval by 5 ms and repeated at 3 basic cycle lengths (150, 200 and 250 bpm). A total of 45 S1-S2 stimulation procedures were repeated before and after infusion of SAR296968 in 8 pigs. A separate control group of 7 pigs was performed according to the same protocol with infusion of the vehicle.

Effect of SAR340835 on cardiac hemodynamics in normal and HF dogs

Animal and Surgical Procedure

Twelve adult mongrel dogs (body weight 27 to 31 kg) were implanted with telemetry devices (L21-F2, Datasciences International, US). Six of them were additionally equipped with a pacemaker (Adapta® model, Medtronic, MI, USA) with bipolar epicardial Pacing Lead (CapSure® Epi, Medtronic, MI, USA) for induction of heart failure by tachypacing.

Dogs were sedated with acepromazine (0.75 mg/kg, IM, Calmivet® 1%, Vetoquinol, Magny-Vernois, France), 20 minutes before the induction of anaesthesia with an intravenous bolus of thiopental (20 mg/kg, IV, Thiopental® Inresa, Bartenheim, France). Anaesthesia was maintained, after intubation, with isoflurane (Isoflo® 2%, Zoetis, Malakoff, France).

The telemetry implant was placed under aseptic conditions between 2 abdominal muscular layers on the flank. Catheters and electrocardiogram (ECG) leads were tunneled until the 6th intercostal space. After a thoracotomy (5th intercostal space), the left ventricular pressure (LVP) catheter was introduced into the left ventricle via the apex (for the measurement of left ventricular pressure) and the aortic pressure (AP) catheter into the thoracic descending aorta (for the measurement of the aortic pressure). The 2 ECG leads were sutured on the left ventricle, one on the apex and one near the left atria. A pacemaker was implanted in 6 mongrels for the induction of HF by rapid pacing. The epicardial pacing leads were sutured on the right ventricle and wires were externalized in the inter-scapular area and connected to the pacemaker placed between 2 muscular layers on the back through another surgical incision. The thoracic incision was closed in layers and pneumothorax was evacuated.

Analgesia was ensured with a combination of buprenorphine (0.01 mg/kg, IM Vétergésic®, CEVA, Libourne, France) before thoracotomy, repeated in the evening of the surgical intervention and twice a day for 3 days. Then, meloxicam was administered once a day for 3 days (meloxicam, 0.2 mg/kg, IM Metacam®, Boehringer Ingelheim, Reims, France). Prophylactic antibiotherapy was ensured for 10 days by a mixture of benzylpenicillin procaine and dihydrostreptomycin (15 mg and 30 mg, IM respectively, Intramicin®, CEVA, Libourne, France). Body temperature and weight were periodically checked. Dogs were allowed to recover after surgery for a minimum period of 12 days. For drug infusion, dogs were implanted under anesthesia with a vascular access port (VAP, with 7Fr PU Catheter 60 cm SWIRL-MID-PU-C70

(Instech Solomon, Plymouth Meeting, PA, USA, Phymep, Paris France), the catheter of which being inserted into the jugular vein. The VAP was positioned into the inter-scapulae region. Dogs received post-surgical analgesia and non-steroidal anti-inflammatory drug (meloxicam, 0.2 mg/kg, IM Metacam®, Boehringer Ingelheim, Reims, France. After each surgical procedure, animals were isolated for a recovery period of 3 to 5 days.

Rapid Right Ventricular pacing-induced HF

Heart failure was induced by chronic rapid right ventricle pacing at 240 beats/min for 4 weeks with the programmable pacemaker. Baseline echocardiogram (see below) and hemodynamic recordings were performed before and at the end of the 4-week pacing period to assess the development of heart failure.

Study Design in normal and pacing-induced HF dogs

The same study design was applied to normal and HF dogs. All the experiments were performed in conscious animals 4 weeks after the induction of HF by rapid pacing. Dogs were daily trained to remain quiet during the hemodynamic and echocardiography procedures before and after surgery. Before each echocardiography and telemetry monitoring session, pacing was turned off and maintained off during the whole recording period.

Each animal was subjected to 4 treatment sessions over the two following weeks with vehicle or SAR340835 infused at 250, 750 or 1500 µg/kg/h. Pacing-induced heart failure dogs received dobutamine infused at 10 µg/kg/min during an additional session for comparison purpose. SAR340835 was intravenously administered with a loading dose over 2 min (0.29 mg/kg, 0.86 mg/kg or 1.73 mg/kg for 250, 750 and 1500 µg/kg/h, respectively) followed by a IV infusion maintained for 3 hours in HF dogs and 6 hours in normal dogs (250, 750 or 1500 µg/kg/h, respectively). For simplification, doses are designated by the maintenance infusion rate in the Tables and Figures. A minimum washout period of 2 days in accordance with the short half-life of compounds was allowed between two sessions.

During each session, after a 15-minute stabilization period, telemetry signals were continuously recorded throughout the treatment infusion. Echocardiography was performed before starting the treatment infusion and over the last minutes of the 3- or 6-hour treatment infusion.

Echocardiography Measurements

Cardiac function was assessed by echocardiography using a Philips CX 50 (Philips, Amsterdam, Netherlands) with a 5-MHz phased-array transducer. Right parasternal short axis view was performed to acquire M-mode on which end-diastolic and end-systolic diameter were measured. Right parasternal long axis view was performed to record a 4-chamber view on which left ventricular end-diastolic and end-systolic contouring was performed. The two latter were used to calculate end-diastolic and end-systolic volumes with the Simpson method of disks. Fractional shortening (FS) was calculated as $100 \times (LVEDD - LVESD)/LVEDD$ and ejection fraction was calculated as $100\times (LVEDV-LVESV)/LVEDV$). Additional methodological details are provided online in Supplemental methods.

Telemetry recordings and analysis

Telemetry signals (LVP, AP, ECG) were continuously recorded throughout the experiment starting 15 minutes before and until the end of vehicle or treatment infusion at a sampling rate of 500 Hz. Measurements were averaged over at least 20 seconds-periods using HEM software

(Notocord System, Croissy, France). Several derived parameters were calculated: diastolic (DBP) and systolic (SBP) aortic blood pressure, left ventricular end-diastolic pressure (LVEDP), dP/dt_{max} and dP/dt_{min}.

Oxygen consumption (MVO2) was calculated based on echocardiographic and telemetry parameters according to the formula (*Rooke and Feigl 1982*) :

$$MV02 = 0.000408*(SBP \times HR) +0.000325*[(0.8\ SBP + 0.2\ DBP) \times HR \times SV]/BW+1.43$$

Evaluation of autonomic tone and baroreflex

The effects of SAR340835 on the autonomic nervous system (ANS) were explored. Spectral analysis of heart rate variability (HRV) was performed for the evaluation of autonomic tone in all telemetered dogs before and at the end of the 3 hours of dosing. This spectral analysis using a fast Fourier transform algorithm on sequences of 512 points (5 minutes) was performed with the HEM CsA10 software (Notocord Systems, Croissy, France).

Specific frequency bands of HRV permitted the simultaneous assessment of sympathetic (Low Frequency (LF)) and parasympathetic (High Frequency (HF)) modulation with LF/HF ratio illustrating the sympathovagal balance. Spectral powers were determined as the area under the curve calculated for the Very Low Frequency (VLF: 0.04 to 0.05 Hz), Low Frequency (LF: 0.05 to 0.15 Hz), and High Frequency (HF: 0.15 to 0.5 Hz) bands. The results are expressed in normalized units (nu) for spectral indices calculated as follows:

LF nu (%) = (LF / (LF + HF)) * 100

HF nu (%) = (HF / (LF + HF)) * 100.

To investigate the ability of heart rate changes to counteract arterial blood pressure variations, spontaneous baroreflex efficiency was evaluated using the sequence method (Gronda *et al.* 2014, Verwaerde *et al.* 1999). Briefly, sequences of at least 5 beats in which the systolic blood pressure and the RR interval changed in the same direction were identified as baroreflex sequences. A linear regression (r^2 =0,85) was applied to each sequence to calculate the value of the slope. For each evaluation time, the value of spontaneous baroreflex efficiency is the mean slope of all baroreflex sequences obtained on 512 consecutive values of systolic blood pressure and heart rate.

ECG analysis

The ECG signals of all animals were examined for any test article-related abnormality in waveform morphology. The PR interval was evaluated on each dosing day, at least at each selected time-point, over a 60 seconds-period. Examination of 2nd degree AVB was performed on the totality of the 24-hour recording of ECG.

Dog cardiomyocytes studies

Cell preparation

Four Pacing-induced Heart Failure Mongrels dogs (Marshall BioResourcesNorth Rose, New York, United States, 14-35 kg) or five Normal Beagles (14-16 kg) were used to isolate cardiomyocytes. The anesthesia was induced with nesdonal (Thiopental® Inresa, Bartenheim, France) and maintained after intubation with isoflurane (Isoflo® 2%, Zoetis, Malakoff, France). Before left lateral thoracotomy heparin injection (Choay, France: 300 UI/kg iv) was performed and the heart was perfused with ice-cold cardioplegic solution *(*Custodiol®). The inferior and superior vena cava, the pulmonary artery and the aorta were clamped, and a cannula was inserted

into the aorta to retro-infuse the ice-cold-cardioplegic solution (Custodiol®) to induce rapidly cardiac arrest. After excision, the heart was stored in Custodiol®.
Normal and HF dogs' cardiomyocytes were isolated by the Langendorff technique as previously published (Volders *et al.* 1999, Molina *et al.* 2014). Left anterior coronary artery was cannulated and the left ventricle was washed for 20 minutes with an isolation solution (35 mM NaCl, 4.75 mM KCl, 1.2 mM KH2PO4, 10 mM Dextrose (D-Glucose), 134 mM Saccharose, 16 mM Na2HPO4, 25 mM NaHCO3, 10 mM HEPES). The flow was set to 60 mL/min and left ventricle digested for 10 to 20 min with isolation solution complemented with 0.5% BSA (#0881066, MP Biomedicals) and 1mg/ml of collagenase A (#11088793001, Roche). The epicardial layer was removed and finely chopped in Wash Solution (130 mM NaCl, 4.8 mM KCl, 1.2 mM KH2PO4, 5 mM Dextrose (D-Glucose), 25 mM HEPES, 1% BSA (A7906, Sigma-Aldrich)). The supernatant was removed, and cells resuspended three more times with Wash solution: Calcium Medium mix at increasing [Ca2+] from 0.3 to 1.2 mM. Freshly isolated cells in Calcium Medium (MEM: M 4780; Sigma, St Louis, MO USA) containing 1.2 mM [Ca2+] supplemented with 2.5% foetal bovine serum (N4762; Sigma), 1% penicillin–streptomycin (15140-122, Gibco), 20 mM HEPES (pH 7.6), were plated on 35 mm, laminin-coated culture dishes (10 mg/mL) at a density of 20103 cells per dish. After 1 h the medium was replaced by 1 mL of FBS-free medium.

Sarcomere shortening in dog cardiomyocytes
Sarcomere dynamics were recorded from cardiomyocytes using video-based cell geometry (IonOptix systems, Dublin, Ireland). Laminin-coated coverslips containing adherent cardiomyocytes were washed with Tyrode Solution containing 121 mM NaCl, 5.4 mM KCL, 4.0 mM NaHCO3, 0.8 mM Na2HPO4, 1.8 mM MgCl2, 5 mM glucose, 10 mM HEPES, 5 mM Na pyruvate and 1.8 mM CaCl2 (pH 7.4). Each coverslip was placed in a perfusion chamber (FHD chamber, IonOptix) mounted on the stage of an inverted microscope (Motic AE30/31).
Each coverslip was exposed to only one concentration and only one cardiomyocyte was recorded per coverslip. Signals were continuously recorded for the duration of the experiment. The myocytes were field stimulated at 0.5 Hz with suprathreshold voltage with a bipolar pulse of 3 ms duration (Myopacer stimulator, Ionoptix) using a pair of platinum wires placed on opposite sides of the chamber. All measurements were performed at room temperature. After a 100 stabilization period in Tyrode Solution (vehicle), perfusion was switched to SAR296968, dobutamine or Vehicle for 6 min. Myocytes were continuously perfused with Tyrode solution containing either SAR296968 (0.3, 1, 3 or 10 µM), dobutamine (1µM) or vehicle (0.3% BSA and 0.1% DMSO in Tyrode solution) depending on the group.
Parameters of interest were captured at the end of stabilization period (baseline) and at the end of SAR296968, dobutamine or vehicle perfusion period. Ten peaks were averaged at each time-points and analyzed for sarcomere length with IonWizard 6.3 software (IonOptix). Parameters quantifying sarcomere dynamics were deduced from this analysis. Contraction velocity (µm/s) was calculated as the maximum rate of change in sarcomere length during the contraction phase. The peak height (µm), which represents the amplitude of the sarcomere shortening, was calculated by subtracting sarcomere length at minimum value (contracted state) to sarcomere length at maximum value (relaxed state). The peak height measured under exposure to SAR296968 was normalized to the measurement performed before SAR296968 infusion in the same cell as followed: $Ratio\ Peak\ Height = Peak\ Height_{SAR} / Peak\ Height_{baseline}$. Parameters of diastolic function were Relaxation Velocity (µm/s) which was calculated as the maximum rate of change in sarcomere length during the relaxation phase and the Time to 50% relaxation.

Effects of SAR296968 on dog cardiomyocytes

Averaged results (Table S4) obtained from 3-4 hearts from pacing-induced HF dogs indicate that the amplitudes and velocities of sarcomere shortening were significantly increased by SAR296968. The inotropic effect is further illustrated in a representative superimposed trace (Figure S1).

Sarcomere shortening ratio values showed a significant increase in the SAR296968-treated groups reaching 1.69 and 2.12-fold at 3 and 10 µM, respectively vs. the vehicle group. The significant positive inotropic effect observed with dobutamine, tested as a reference positive control, was in the same range (about 2-fold of vehicle effect). Concomitantly the contraction velocity was amplified but did not reach significance. SAR296968 induces a significant increase in relaxation velocity at 10µM like dobutamine but none of the drugs showed any effect on the Time to 50% relaxation (Table S4).

The positive inotropic effects obtained with SAR296968 in HF dog cardiomyocytes were not observed in cardiomyocytes isolated from normal dog (Table S5). In the latter study, sarcomere shortening was unchanged by SAR296968 but increased by dobutamine (about 3-fold of vehicle effect).

Figure S1. Evaluation of positive inotropic effect of SAR296968 on isolated canine ventricular cardiomyocytes under HF conditions. (A) Representative traces of Sarcomere Length (SL) shortening under basal conditions (red) and after exposure to 3 µM SAR296968 (blue). The cells were paced using field stimulation at 0.5 Hz. (B) Pooled data from 3-4 canine hearts showing contractile parameters following treatment with SAR296968, Dobutamine or Vehicle. For each graph, individual values, median and interquartile range are represented. p-value significant at 5% level (comparison versus Vehicle; *: Dunnett's test for SAR296968 / #: Student test for Dobutamine). Further statistical details are available in Table 4.

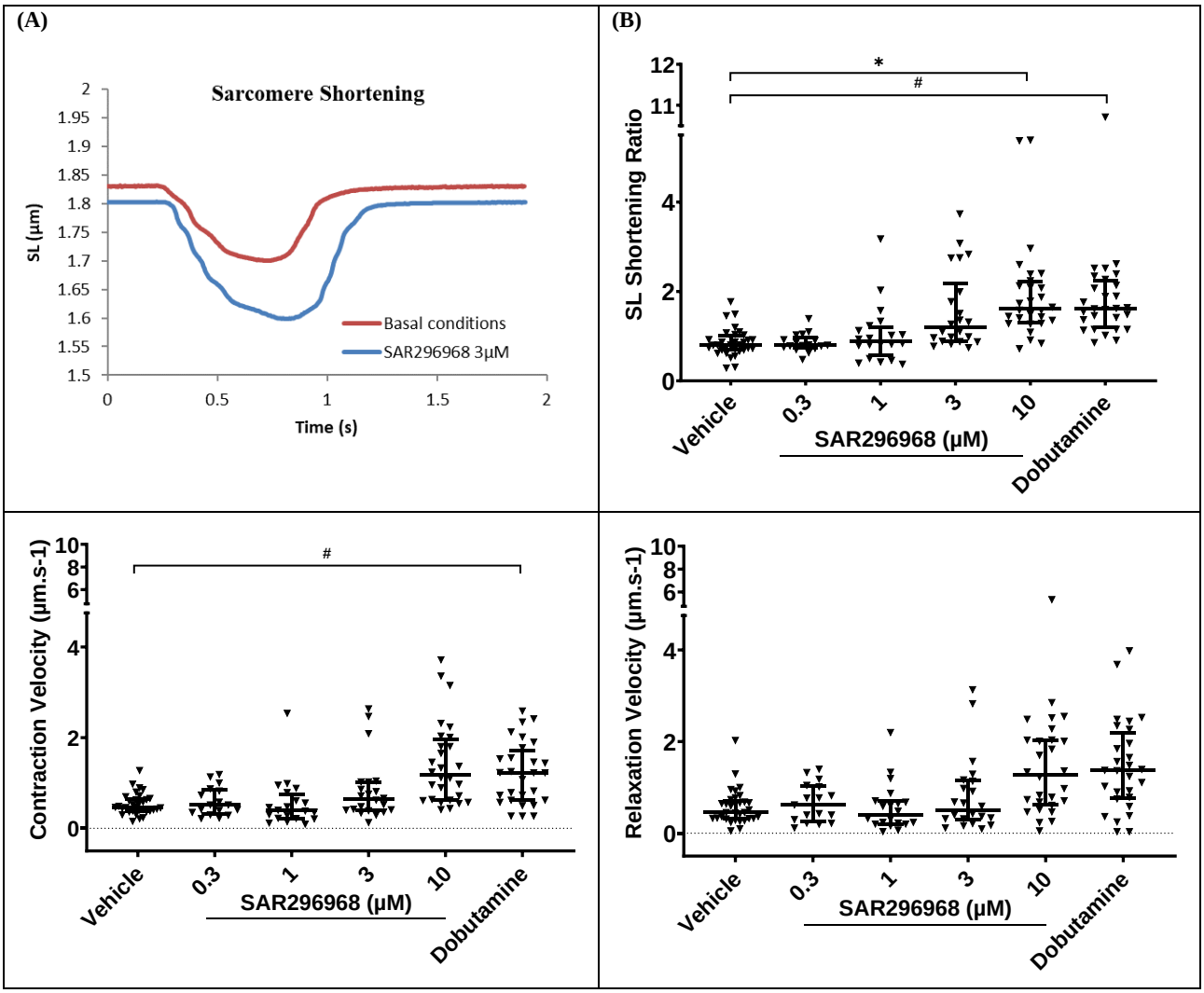

Table S1. Echocardiographic parameters were determined as described below:

Right parasternal short axis view was performed to acquire M-mode on which end-diastolic, end-systolic, fractional shortening and diameter aortic surface were measured			
Right parasternal short axis	Left Ventricular End Diastolic Diameter	LV EDD	mm
	Left Ventricular End Systolic Diameter	LV ESD	mm
	Left Ventricular Fractional Shortening[a]	LV FS	%
Right parasternal short axis	Aortic surface	Ao surf	cm^2
Right parasternal long axis view where end diastolic, end systolic volume were measured and Left ventricle ejection fraction was measured			
Right parasternal long axis view	Left Ventricular End Diastolic Volume	LV EDV	mL
	Left Ventricular End Systolic Volume	LV ESV	mL
	Left Ventricular Ejection Fraction[b]	LV EF	%
Left parasternal long axis view where Pulse wave Doppler was performed to measure Aortic and Mitral flow			
Pulse wave Doppler – aortic flow			
Left parasternal long axis view	Velocity time integral	VTI	cm
	Aortic Ejection Time	Ao ET	ms
Pulse wave Doppler – mitral flow			
Left parasternal long axis view	Peak velocity of early diastolic transmitral flow	E peak	cm/s
	Peak velocity of late transmitral flow	A peak	cm/s
	Deceleration time	DT	ms
	Duration of the A Wave	MV A duration	ms
	Ratio E/A	E/A ratio	-
Parameters calculated			
-	Stroke volume [c]	SV	mL
-	Cardiac output [d]	CO	L/min

a LV FS = [(EDD-ESD)/EDD]

b LV EF = [(EDV-ESV)/EDV]

c SV = [VTI * Ao surf]

d CO = [SV * HR] where HR is measured during the VTI measure.

Table S2. Effects of SAR296968 (1 and 3 µM) on contractility parameters and action potential duration in guinea pig papillary muscles (median +/- median absolute deviation)

	Guinea-pig papillary muscles		
	Vehicle	**SAR296968 1 µM**	**SAR296968 3 µM**
	mean ± sem	**mean ± sem**	**mean ± sem**
Maximal force of contraction (µN)	2443 ± 444	3779 ± 526	4419 ± 1029
dP/dt max (µN/ms)	38 ± 6	49 ± 6	56 ± 12
dP/dt min (µN/ms)	-32 ± 5	-43 ± 6	-46 ± 10
APD90 (ms)	185 ± 6	157 ± 15	163 ± 10

Table S3a. Effect of SAR296968 on the left ventricular repolarization and the QRS interval in isolated rabbit heart pretreated with sotalol or veratridine.

	Rate (bpm)	Vehicle (n=6)		SAR296968 0.3 µM (n=6)		SAR296968 1 µM (n=6)	
QRS (ms)							
Baseline	80	35 ± 2		37 ± 4		37 ± 1	
First run (Sotalol)	80	37 ± 2		36 ± 3		38 ± 1	
Second run (Sotalol + treatment)	80	38 ± 1		36 ± 2	*ns*	39 ± 2	*ns*
	40	37 ± 1		38 ± 3	*ns*	39 ± 1	*ns*
QT (ms)							
Baseline	80	201 ± 10		195 ± 8		191 ± 7	
First run (Sotalol)	80	258 ± 17		257 ± 25		238 ± 29	
Second run (Sotalol + treatment)	80	299 ± 30		253 ± 13	*	209 ± 7	*
	40	330 ± 30		214 ± 17	*	170 ± 6	*
Tp-Te (ms)							
Baseline	80	20 ± 2		20 ± 2		23 ± 2	
First run (Sotalol)	80	31 ± 3		29 ± 5		28 ± 4	
Second run (Sotalol + treatment)	80	38 ± 9		28 ± 4	*ns*	34 ± 4	*ns*
	40	58 ± 27		31 ± 6	*	38 ± 6	*ns*
QRS (ms)							
Baseline	80	39 ± 2		36 ± 1		39 ± 1	
First run (veratridine)	80	41 ± 1		36 ± 2		39 ± 1	
Second run (veratridine + treatment)	80	41 ± 3		36 ± 2	*ns*	40 ± 2	*ns*
	40	40 ± 3		37 ± 1	*ns*	40 ± 2	*ns*
QT (ms)							
Baseline	80	205 ± 3		194 ± 11		196 ± 8	
First run (veratridine)	80	395 ± 9		357 ± 21		390 ± 30	
Second run (veratridine + treatment)	80	397 ± 6		344 ± 15	*	356 ± 19	*
	40	486 ± 15		409 ± 12	*	418 ± 24	*ns*
Tp-Te (ms)							
Baseline	80	20 ± 1		20 ± 2		24 ± 4	
First run (veratridine)	80	56 ± 3		50 ± 10		45 ± 9	
Second run (veratridine + treatment)	80	53 ± 5		51 ± 6	*ns*	43 ± 9	*ns*
	40	51 ± 5		43 ± 2	*ns*	69 ± 8	*ns*

Table S3b. Effect of SAR296968 on left and right atria refractory periods in anesthetized pigs at pacing rate of either 150, 200 or 250 bpm.

	Left atrium AERP			Right atrium AERP		
BCL (bpm)	**150**	**200**	**250**	**150**	**200**	**250**
Baseline	113 ± 9	106 ± 8	100 ± 7	181 ± 9	162 ± 8	148 ± 9
Vehicle	110 ± 9	105 ± 7	101 ± 6	186 ± 12	170 ± 12	150 ± 10
Baseline	137 ± 4	128 ± 4	118 ± 3	190 ± 6	173 ± 8	161 ± 8
SAR296968	140 ± 7	125 ± 7	117 ± 6	188 ± 8	176 ± 8	160 ± 9

Table S4. Effect of SAR296968 on contraction and relaxation of failing cardiomyocytes isolated from pacing-induced HF dogs (median +/- median absolute deviation); dobutamine was tested as a positive control; p-value significant at 5% level (comparison versus Vehicle; *: Dunnett's test for SAR296968 / #: Student test for dobutamine).

Treatment	Vehicle	SAR296968 (µM)				dobutamine 1µM
		0.3	1	3	10	
N (Dog Heart)	4	3	4	4	4	4
n (cells)	33	17	20	22	28	28
sarcomere shortening (µm) before treatment	0.127 +/- 0.029	0.129 +/- 0.050	0.105 +/- 0.042	0.07 +/- 0.026	0.118 +/- 0.058	0.091 +/- 0.026
sarcomere shortening (µm) after 6 minutes of treatment's perfusion	0.102 +/- 0.027	0.103 +/- 0.053	0.093 +/- 0.038	0.095 +/- 0.030	0.188 +/- 0.066	0.169# +/- 0.067
sarcomere shortening ratio	0.803 +/- 0.122	0.798 +/- 0.098	0.888 +/- 0.294	1.190 +/- 0.316	1.617* +/- 0.404	1.618# +/- 0.464
contraction velocity (µm/s)	0.466 +/- 0.113	0.516 +/- 0.220	0.392 +/- 0.178	0.642 +/- 0.246	1.18 +/- 0.586	1.225# +/- 0.578
relaxation velocity (µm/s)	0.454 +/- 0.185	0.617 +/- 0.397	0.401 +/- 0.223	0.51 +/- 0.340	1.283 +/- 0.739	1.371 +/- 0.607
time to relax 50% (s)	0.305 (n=31) +/- 0.182	0.191 +/- 0.079	0.277 (n=18) +/- 0.102	0.235 (n=20) +/- 0.146	0.247 (n=24) +/- 0.139	0.222 +/- 0.118

Median +/- Median Absolute Deviation

Table S5. Effect of SAR296968 on contraction and relaxation of Normal cardiomyocytes from Beagles (median +/- median absolute deviation); dobutamine as positive control; p-value significant at 5% level (comparison versus Vehicle; *: Dunnett's test for SAR296968 / #: Student test for dobutamine). The protocol is the same as used for HF cardiomyocytes and it is described in Material and Methods.

Treatment	Vehicle	SAR296968 (μM)			dobutamine 1μM
		1	3	10	
N (Dog Heart)	5	4	5	5	5
n (cells)	16	12	16	18	12
sarcomere shortening (μm) before treatment	0.128 +/- 0.052	0.151 +/- 0.053	0.129 +/- 0.027	0.143 +/- 0.041	0.092 +/- 0.041
sarcomere shortening (μm) after 6 minutes of treatment's perfusion	0.142 +/- 0.052	0.132 +/- 0.060	0.143 +/- 0.028	0.141 +/- 0.040	0.250# +/- 0.039
sarcomere shortening ratio	0.843 +/- 0.258	0.917 +/- 0.259	0.894 +/- 0.373	1.075 +/- 0.265	2.249# +/- 0.674
contraction velocity (μm/s)	-0.699 +/- 0.414	-0.953 +/- 0.567	-1.032 +/- 0.476	-1.413 +/- 0.672	-3.004 +/- 1.164
relaxation velocity (μm/s)	0.782 +/- 0.544	1.069 +/- 0.730	0.896 +/- 0.646	1.260 +/- 0.736	2.995 +/- 0.560
time to relax 50% (s)	0.381 +/- 0.123	0.380 +/- 0.076	0.349 +/- 0.077	0.310 +/- 0.048	0.310 +/- 0.059

Median +/- Median Absolute Deviation

Table S6. CEREP selectivity profile (78 targets)

Non-peptide receptors	
A_3 *(h)*	5-HT_{1D}
BZD (central)	5-HT_{2B}*(h)*
BZD (peripheral)	5-HT_{2C} *(h)*
$GABA_A$	5-HT_3 *(h)*
$GABA_{B(1b)}$ *(h)*	5-HT_4e *(h)*
kainate	5-HT_6 *(h)*
H_3 *(h)*	5-HT_7 *(h)*
H_4 *(h)*	σ (non-selective)
P2X	σ_{1A}
P2Y	
Peptide receptors	
AT_1 *(h)*	MC_4 *(h)*
AT2 *(h)*	NK_1 *(h)*
BB (non-selective)	NK_2 *(h)*
B_1 *(h)*	NK_3 *(h)*
B_2 *(h)*	Y_1 *(h)*
CCK_1 (CCK_A) *(h)*	Y_2 *(h)*
CCK_2 (CCK_B) *(h)*	NTS_1 (NT_1) *(h)*
ET_A *(h)*	NMU2 *(h)*
GAL_1 *(h)*	δ_2 (DOP) *(h)*
GAL_2 *(h)*	κ (KOP)
CXCR2 (IL-8B) *(h)*	NOP (ORL1) *(h)*
CCR1 *(h)*	sst (non-selective)
TNF-α *(h)*	$VPAC_1$ (VIP_1) *(h)*
CCR2 *(h)*	V_1a *(h)*
MCH_1 *(h)*	V_{1b} *(h)*
MC_3 *(h)*	V_2 *(h)*
Nuclear receptors	
GR *(h)*	AR *(h)*
ERα *(h)*	TR (TH)
PR *(h)*	
Ion channels	
Ca_{2+} channel (L, verapamil site) (phenylalkylamine)	K_{ATP} channel
Ca_{2+} channel (N)	
Amine transporters	
GABA transporter	norepinephrine transporter (h)
5-HT transporter *(h)*	dopamine transporter (h)
Kinases	
CaMK2α *(h)*	IRK *(h)* (InsR)
Non-kinase enzymes	
COX_1 *(h)*	cathepsin L *(h)*
COX_2 *(h)*	MMP-1 *(h)*
12-lipoxygenase *(h)*	tryptase *(h)*
constitutive NOS *(h)* (endothelial)	phosphatase 1B *(h)* (PTP1B)
$PDE4D_2$ *(h)*	PLC
ACE *(h)*	MAO-B *(h)*
cathepsin D *(h)*	

For further details on assays see online information at www.eurofinsdiscoveryservices.com.

Specific Statistical Analysis

For Inhibition of NCX transport activity on CHO Cell-lines.

Single experiments for inhibition of NCX transport activity were carried out using 10 concentrations of the test compound in double determination. Half-maximal inhibitory concentrations (IC50) of the compounds for transport inhibition were calculated with internal software Biost@t SPEED V2.0 LTS using the 4-parameter logistic model according to Ratkowsky and Reedy (1986).

The adjustment was obtained by non-linear regression using the Levenberg-Marquardt algorithm in SAS v9.1.3.

For inhibition of NCX, ICa and INa currents.

The values for half-maximal inhibition (IC50) and the Hill coefficient were calculated by fitting the data points of the concentration/response curves to the logistic function. Results were obtained with internal software Biost@t-SPEED v1.3 using the 4-parameter logistic model according to Ratkowsky and Reedy (1986) with lower asymptote constrained at 0 and upper asymptote constrained at 100 (if not otherwise stated). The adjustment was obtained by non-linear regression using the Marquardt algorithm in SAS v8.2 software under UNIX. IC50 are given with their confidence interval.

For DADs-related arrhythmias models

All data are expressed as mean ± SEM or as median ± MAD. The Levene test was used to check heterogeneity of variances for both the guinea-pig papillary muscles and left atria studies. Due to heterogeneous variances in the guinea-pig papillary muscles study the Kruskal-Wallis test was applied on the parameter 'number of arrhythmic contractions' followed by Wilcoxon multiple comparisons test with Bonferroni Holm correction versus the vehicle control group. For the other parameters, maximal force of contraction, contractility, relaxation and action potential duration only descriptive statistics was provided. For the guinea-pig left atria study, a Wilcoxon test was applied on the parameter 'number of arrhythmic contractions' between SAR296968 3 µM and the vehicle control group. For the parameter contractility separate paired t-tests were applied on log-transformed dP/dt_{max} values between baseline and subsequent 3 µM SAR296968 or vehicle administration. The paired differences in means of the log-transformed dP/dt_{max} values were converted to mean ratios via the anti-log transformation. P-values < 0.05 were regarded as statistically significant.

For study on Left atrial vulnerability (LAV)

The one or two factor Levene test was used to check heterogeneity of variances. The paired t-test for SAR296968 treated animals versus baseline was used for the parameter left ventricular contractility. A two-way ANOVA with factor treatment and repeated measures for factor time (baseline and treated level) was applied on parameter LAV using heterogeneous variances for factor treatment followed by a Winer analysis for effect of factor time for each level of factor treatment. For parameter AERP a two-way ANOVA with repeated measures on factor time (baseline and treated level) and on factor BCL (basic cycle length) was applied separately for left and right atrium of the SAR296968 treated group and of the vehicle treated group correspondingly. P-values < 0.05 were regarded as statistically significant.

For in vivo experiments in normal and HF-dogs:

First, to analyze the induction of pathology, for each variable, a paired Student t-test was performed to compare parameters before and after induction of heart failure by 4 weeks of pacing.

To analyze the SAR340835 effect versus vehicle and the Dobutamine effect versus vehicle, for each variable and each objective, a one-way analysis of variance was performed on the raw data with group as fixed factor and animal as random factor.

In case of significance of the group factor, for the first objective a Dunnett's test was performed to compare each treated group to the vehicle group. For both objectives, the differences between the groups were estimated as well as their 95% CI (with Dunnett's adjustment for multiplicity for the first objective), they were expressed as a percentage of the corresponding vehicle mean for an easier interpretation, except for the LF/HF ratio parameter which was expressed as a ratio versus vehicle. The significance level was taken to 0.05. For the LF/HF ratio, the analyses were performed on log-transformed data due to heterogeneous variances.

The statistical analyses were performed using SAS® version 9.4 for Windows 7.

For Contractility evaluation on Dogs Cardiomyocytes

The statistical analyses for the in vitro part were performed to evaluate the SAR296968 and then the dobutamine (positive reference) treatment effect.

For each objective, for normal Beagles dogs a mixed model with fixed factor group and random factors animal and animal*group was performed. For HF Mongrel dogs, a mixed model with fixed factors group, system and their interaction and random factors animal and animal*group was performed was performed; then a backward elimination was applied to simplify the model. The variances heterogeneity on group factor was considered depending on the parameter. Appropriate post-hoc analysis was performed. A rank or log-transformation was applied when appropriate.

Descriptive statistics were calculated on cells. The significance level is taken to 5%, except for the interaction test for which the significance level is taken to 10%.

The statistical analyses were performed using SAS® version 9.4 for Windows 7.

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