SAR340835, a Novel Selective Na\(^+\)/Ca\(^{2+}\) Exchanger Inhibitor, Improves Cardiac Function and Restores Sympathovagal Balance in Heart Failure\(^5\)

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ABSTRACT

In failing hearts, Na\(^+\)/Ca\(^{2+}\) exchanger (NCX) overactivity contributes to Ca\(^{2+}\) depletion, leading to contractile dysfunction. Inhibition of NCX is expected to normalize Ca\(^{2+}\) mishandling, to limit afterdepolarization-related arrhythmias, and to improve cardiac function in heart failure (HF). SAR340835/SAR296868 is a selective NCX inhibitor for all NCX isoforms across species, including human, with no effect on the native voltage-dependent calcium and sodium currents in vitro. Additionally, it showed in vitro and in vivo antiarrhythmic properties in several models of early and delayed afterdepolarization-related arrhythmias. Its effect on cardiac function was studied under intravenous infusion at 250, 750, or 1500 \(\mu\)g/kg per hour in dogs, which were either normal or submitted to chronic ventricular pacing at 240 bpm (HF dogs). HF dogs were infused with the reference inotrope dobutamine (10 \(\mu\)g/kg per minute, i.v.). In normal dogs, NCX inhibitor increased cardiac contractility (dP/dt\(_{\text{max}}\)) and stroke volume (SV) and tended to reduce heart rate (HR). In HF dogs, NCX inhibitor significantly and dose-dependently increased SV from the first dose (+28.5%, +48.8%, and +62% at 250, 750, and 1500 \(\mu\)g/kg per hour, respectively) while significantly increasing dP/dt\(_{\text{max}}\) only at 1500 (+33%). Furthermore, NCX inhibitor significantly restored sympathovagal balance and spontaneous baroreflex sensitivity (BRS) from the first dose and reduced HR at the highest dose. In HF dogs, dobutamine significantly increased dP/dt\(_{\text{max}}\) and SV (+68.8%) but did not change HR, sympathovagal balance, or BRS. Overall, SAR340835, a selective potent NCX inhibitor, displayed a unique therapeutic profile, combining antiarrhythmic properties, capacity to restore systolic function, sympathovagal balance, and BRS in HF dogs. NCX inhibitors may offer new therapeutic options for acute HF treatment.

SIGNIFICANCE STATEMENT

HF is facing growing health and economic burden. Moreover, patients hospitalized for acute heart failure are at high risk of decompensation recurrence, and no current acute decompensated HF therapy definitively improved outcomes. A new potent, Na\(^+\)/Ca\(^{2+}\) exchanger inhibitor SAR340835 with antiarrhythmic properties improved systolic function of failing hearts without creating hypotension, while reducing heart rate and restoring sympathovagal balance. SAR340835 may offer a unique and attractive pharmacological profile for patients with acute heart failure as compared with current inotrope, such as dobutamine.

Introduction

A variety of treatments are used to improve depressed left ventricular function in heart failure (HF) during periods of acute decompensation. \(\beta\)-Adrenergic agonists, like dobutamine, are currently the gold standard for inotropic agents in acute decompensated HF (AHF); however, their clinical use...
is limited by major drawbacks. Firstly, β-adrenergic receptor desensitization requires continuous augmentation of the dose to maintain inotropic efficacy. Secondly, stimulation of adrenergic receptors induces tachycardia and increases myocardial oxygen consumption out of proportion to their positive inotropic action, potentially reducing cardiac efficiency at midterm. Finally, they exert deleterious effects on membrane electrical stability, favoring the occurrence of arrhythmias and cardiovascular death (Van Bilsen et al., 2017).

It is well known that hemodynamic alterations accompanying HF are associated with abnormal regulation of intracellular Ca\(^{2+}\), leading to electrophysiological and excitation-contraction (E-C) alterations at the cellular level. Reduction of the amplitude of intracellular Ca\(^{2+}\) transient and of its rate of decay have been reported in isolated myocytes from failing human and dog hearts (O’Rourke et al., 1999; Menick et al., 2007; Bögeholz et al., 2017). The cardiac plasma membrane Na\(^+/\)Ca\(^{2+}\) exchanger (NCX) is the main Ca\(^{2+}\) extrusion mechanism of the cardiac myocyte and is crucial for maintaining Ca\(^{2+}\) homeostasis. NCX is a key player of cardiac E-C coupling that regulates cytosolic Ca\(^{2+}\) and Na\(^+\) concentration, repolarization process, and contractility (Wei et al., 2007; Ottolia et al., 2013). Moreover, NCX expression and activity are consistently upregulated in both patients with HF (Sipido et al., 2002; Pott et al., 2011) and animal models of HF (O’Rourke et al., 1999; Goldhaber and Philipson, 2013; Bögeholz et al., 2017). This overactivity, which is associated with a reduced efficacy of sarcoplasmic reticulum Ca\(^{2+}\)-ATPase to pump cytosolic Ca\(^{2+}\) back into the sarcoplasmic reticulum during diastole, favors excessive Ca\(^{2+}\) extrusion from the cytosol, which leads to a reduction in sarcoplasmic reticulum Ca\(^{2+}\) content and thereby contributes to cardiac contractility impairment.

In addition, increased NCX current favors generation of early afterdepolarization (EAD)-related arrhythmias and delayed afterdepolarization (DAD)-related arrhythmias. Enhanced expression of NCX is recognized as one of the molecular mechanisms that increases the risk of arrhythmias during the development of HF with reduced ejection fraction (Hobai et al., 2004; Peana and Domeier, 2017). Therefore, NCX blockers have been proposed as positive inotropes and antiarrhythmic agents in the treatment of HF. The therapeutic objective in patients with HF is therefore to limit NCX overactivity to maintain adequate sarcoplasmic reticulum Ca\(^{2+}\) refilling, to improve cardiac dysfunction, and additionally to reduce the risk of arrhythmias through restoration of proper calcium-induced calcium release. Indeed, partial inhibition of NCX increases intracellular Ca\(^{2+}\) available for sarcoplasmic reticulum Ca\(^{2+}\)-ATPase and improves systolic and diastolic function in both normal and failing canine cardiomyocytes (O’Rourke et al., 1999; Hobai et al., 2004) and prevents the occurrence of EAD- and DAD-related arrhythmias, which are commonly observed in patients with HF (Kohajda et al., 2016). Only one NCX inhibitor, caldaret, has been clinically developed that aims at normalizing disturbed calcium handling in patients with either myocardial infarction or HF. Although caldaret was shown to be safe in patients, the compound was stopped due to its limited efficacy on myocardial infarction size or LV dysfunction (Bår et al., 2006). However, the limited literature about caldaret did not allow a conclusion as to whether its potency and specificity to inhibit NCX were appropriate to increase cardiac function in these patients. Recently, highly specific and potent NCX inhibitors were reported (Primessnig et al., 2019; Otsomaa et al., 2020). They clearly improved cardiac function in both the normal and HF condition in rat and rabbit and showed antiarrhythmic properties (Kohajda et al., 2016).

SAR340835 is a water-soluble prodrug of SAR296968, a potent, selective, short-acting NCX inhibitor (Czechtizky et al., 2013). At 30 minutes after intravenous injection, SAR340835 is totally transformed into SAR296968, its active moiety, which is responsible for its NCX inhibitory activity.

The purpose of this study was to evaluate the antiarrhythmic, hemodynamic, and cardiac effects of SAR340835 in conscious normal dogs and in dogs with rapid right ventricular pacing–induced heart failure. The antiarrhythmic properties were investigated in experimental models using mammalian species with electrophysiological properties close to human (guinea pig, rabbit, pig). To examine E-C coupling in more detail, ex vivo contractility studies in response to SAR296968 were also performed on normal and failing canine cardiomyocytes. SAR340835 profiling was performed in comparison with dobutamine, which was used as a calibrator in the rapid right ventricular pacing–induced HF dogs.

### Materials and Methods

The purpose of this study was to evaluate the therapeutic potential for AHF of SAR340835, a new potent NCX inhibitor, by successively profiling its in vitro potency and specificity, evaluating its antiarrhythmic properties, and determining the cardiodynamic profile in normal and HF dogs. As we were hypothesizing that NCX inhibition could be beneficial in patients with acute heart failure, the canine heart failure studies were performed versus dobutamine, a well known therapeutic reference for AHF. Electrophysiological investigations were performed in mammalian species with electrophysiological features close to humans, i.e., showing a marked plateau of action potential (guinea pig, rabbit, dog, pig) and in which NCX is known to behave as in human heart (Bere et al., 2006).

The successive steps of our methodological approach to fully characterize SAR340835, the prodrug of SAR296968, are illustrated by Fig. 1. A brief summary of methods is provided here. The full experimental conditions of those experiments are detailed in Supplemental Material.

### 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 Association for Assessment and Accreditation of Laboratory Animal Care International-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

An extended method for these in vitro studies is available in Supplemental Material. Briefly, in vitro potency on the NCX isoforms was assessed by a cell-based calcium mobilization assay on CHO cell lines expressing either NCX1, NCX2, or NCX3 with a fluorescent potential (guinea pig, rabbit, dog, pig) and in which NCX is known to behave as in human heart (Bere et al., 2006). They clearly improved cardiac function in both normal and HF condition in rat and rabbit and showed antiarrhythmic properties (Kohajda et al., 2016).

The successive steps of our methodological approach to fully characterize SAR340835, the prodrug of SAR296968, are illustrated by Fig. 1. A brief summary of methods is provided here. The full experimental conditions of those experiments are detailed in Supplemental Material.
Moreover, an extended profiling of SAR296968 was carried out using receptor binding, ion channel binding, and enzyme assays (Supplemental Table 6).

**Effects of SAR296968 on Atrial and Ventricular Arrhythmias**

**Confirmatory Studies.** The antiarrhythmic properties of the NCX inhibitor were assessed in a battery of in vitro and in vivo models using the active principle SAR296968. NCX inhibition was extensively described in literature to reduce the occurrence of early and delayed afterdepolarization. We performed confirmatory studies with our compound in guinea pig papillary muscles and left atria under calcium overload condition to determine its ability to reduce DAD-related arrhythmias. Furthermore, the efficacy of SAR296968 against EADs was tested in isolated rabbit heart perfused according to the Langendorff method. The extended methods for those experiments are described in Supplemental Material.

**Left Atria Vulnerability in Anesthetized Pigs.** The antiarhythmic property of SAR296968 was further investigated by measuring the left atria vulnerability (LAV) in pentobarbital-anesthetized pigs. The purpose of this investigation was to determine the effect of NCX inhibition 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 1 ml of Zoletil 100 (100 mg/ml; 50 mg/ml tiletamine and 50 mg/ml zolazepam) and anesthetized with an intravenous bolus of 3 ml Narcoren (pentobarbital, 160 mg/ml), followed by a continuous intravenous infusion of 12–17 mg/kg per hour pentobarbital. Pigs were ventilated with room air and oxygen by a respirator. Blood gas analysis (partial pressure of oxygen or pO2, partial pressure of carbon dioxide or pCO2) was performed at regular time intervals to control the oxygen supply via the respirator to maintain pO2 > 100 mm Hg and pCO2 < 35–40 mm Hg. A left thoracotomy was performed at the fifth intercostal space, the lung was retracted, the pericardium was incised, and the heart was suspended in a pericardial cradle. The atrial effective refractory period, determined by the S1-S2 method, and cardiac contractility (dP/dtmax) were monitored at baseline and under treatment with SAR296968 (1.5 mg/kg over 20 minutes) dissolved in a mixture of DMSO (1 ml) 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 after the premature beat S2 had to exceed 4 for a full score (1). Three or four repetitive action potentials were counted as a half score (0.5). The procedure was applied while increasing the coupling S1-S2 interval by 5 milliseconds and was repeated at three 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 eight pigs. The same procedure was performed on a separate control group of seven pigs 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.** In total, 12 adult mongrel dogs (body weight 27–31 kg) were implanted with telemetry devices (L21-F2; Datasciences International). Six of them were additionally equipped with a pacemaker (Adapta model; Medtronic, MI) with bipolar epicardial Pacing Lead (CapSure Epi; Medtronic) for induction of heart failure by tachypacing: 240 beats per minute for 4 weeks. For drug infusion, dogs were implanted under anesthesia with a vascular access port.

**Rapid Right Ventricular Pacing-Induced HF.** Heart failure was induced by chronic rapid right ventricle pacing at 240 beats per minute for 4 weeks with the programmable pacemaker. Baseline echocardiogram 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 trained daily 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 four treatment sessions over the following 2 weeks with vehicle or SAR340835 infused at 250, 750, or 1500 µg/kg per hour. Pacing-induced heart failure dogs received dobutamine infused at 10 µg/kg per minute during an additional session for comparison purposes. SAR340835 was intravenously administered with a loading dose over 2 minutes (0.28, 0.86, or 1.73 mg/kg for 250, 750, and 1500 µg/kg per hour, respectively), followed by an intravenous infusion maintained for 3 hours in HF dogs and 6 hours in normal dogs (250, 750, or 1500 µg/kg per hour, 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, The Netherlands) with a 5-MHz phased-array transducer. Additional methodological details are provided online in Supplemental Methods.

**Telemetry Recordings and Analysis.** Telemetry signals (LVP, ECG, aortic blood pressure) 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-second 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\text{max}, and dP/dt\text{min}.

Myocardial oxygen consumption (MVO2 in milliliters of O2/min per 100 g) was calculated using the following equation developed by Rooke and Feigl (1982):

\[
\text{MVO2} = 0.000408\times\text{SBP}\times\text{HR} + 0.000325\times\left[\left(0.8\times\text{SBP} + 0.2\times\text{DBP}\right)\times\text{HR}\times\text{SV}/\text{BW}\right] + 1.43.
\]

**Evaluation of Autonomic Tone and Baroreflex.** The effects of SAR340835 on the autonomic nervous system (ANS) were explored. Spectral analysis of heart rate variability was performed for the very low frequency (0.04–0.05 Hz), low frequency (0.05–0.15 Hz), and high frequency (0.15–0.5 Hz) bands. The results are expressed in normalized units (nu) for spectral indices calculated as follows:

- **Low Frequency (nu, %)**
  \[
  = \left(\frac{\text{Low Frequency}}{\text{Low Frequency} + \text{High Frequency}}\right)\times100;
  \]
- **High Frequency (nu, %)**
  \[
  = \left(\frac{\text{High Frequency}}{\text{Low Frequency} + \text{High Frequency}}\right)\times100.
  \]

To investigate the ability of heart rate changes to counteract arterial blood pressure variations, spontaneous baroreflex efficiency was evaluated using the sequence method (Verwaerde et al., 1999; Gronda et al., 2014). Additional methodological details are provided online in Supplemental Methods.

**ECG Analysis.** The ECG signals of all animals were examined for any test article–related abnormality in wave form morphology. The progesterone (PR) interval was evaluated on each dosing day, at least at each selected time point, over a 60-second period. Examination of second-degree AVB was performed on the totality of the 24-hour recording of ECG.

**Dog Cardiomyocyte Studies**
Detailed methods of dog cardiomyocyte studies are provided in the Supplemental Material.

**Statistical Analysis**
Detailed statistical analysis is described in Supplemental Methods.

**Results**

**In Vitro Profile of SAR296968**

**NCX Inhibition Potency of SAR296968.** In CHO cell lines expressing sodium–calcium exchanger isoforms, SAR296968 potently inhibited the human NCX1, with an IC50 of 74 nM (Fig. 2). Inhibition of human NCX2 and NCX3 occurred within similar ranges. Testing of SAR296968 on NCX1 orthologs from dog, guinea pig, pig, rabbit, and rat also showed the same range of inhibitory potency (Fig. 2).

In voltage-clamp studies in guinea pig cardiomyocytes, SAR296968 inhibited both the forward and reverse mode of the NCX current in a concentration-dependent manner with similarly high potency. For the forward mode (recorded at the potential of −90 mV), an IC50 value with 95% confidence interval of 34.9 nM (29.0; 42.0) was yielded. For the reverse mode (recorded at the potential of +45 mV), the curve fit yielded an IC50 value with 95% confidence interval of 38.9 nM (30.6; 49.5) (Fig. 2).

**Selectivity of SAR296968.** At a concentration more than 100-fold higher than the IC50 on NCX, SAR296968 had no or only a minimal effect on the native voltage-dependent calcium and sodium currents in guinea pig cardiomyocytes (Fig. 2).

The target profiling showed that SAR296968 (10 µM) had only a weak antagonist effect on 5-Hydroxytryptamine receptor 2B (IC50 = 4 µM) and benzodiazepine peripheral receptor (IC50 = 6 µM) and weak inhibition of norepinephrine uptake (IC50 > 30 µM) and dopamine uptake (IC50 = 17 µM).

No functional effects on PR (IC50 of 1.4 µM in binding assay) were reported. There are no functional assays available for androgen receptor (IC50 of 0.18 µM in binding assay), but potential effects should be limited because of the short duration of the anticipated treatment in patients (48 hours). The binding profile of SAR340835 is comparable to the one of SAR296968.

**Antiarrhythmic Properties of SAR296968**

SAR296968 was effective to reduce DAD-related arrhythmias in both models at doses that increased cardiac contractility. Thus, in guinea pig papillary muscles, a positive inotropic effect was observed at all tested SAR296968 concentrations (dP/dt\text{max} changes versus vehicle group: +29% and +47% at 1 and 3 µM SAR296968, respectively; Supplemental Table 2). In parallel, the number of arrhythmic contractions triggered by high calcium/low potassium concentrations was strongly reduced from 20.5 ± 0.0 (median ± MAD, n = 12) in the vehicle control group to 0.0 ± 0.0 at 1 µM (median ± MAD,
$n = 8, P = 0.0054$ vs. vehicle) and to $0.0 \pm 0.0$ at $3 \mu M$ (median $\pm$ MAD, $n = 8, P = 0.0054$ vs. vehicle; Table 1). Similarly, guinea pig left atria treated with $3 \mu M$ SAR296968 responded with a 1.28-fold increase in $dP/dt_{max}$ from baseline versus a 0.84-fold change recorded under vehicle treatment, and a 90% reduction of the spontaneous arrhythmic contractions induced by isoprenaline (1.5 spontaneous arrhythmic contractions after $3 \mu M$ SAR296968 (median $\pm$ MAD, $n = 10$) vs. 15.5 $\pm$ 4.5 in the separate vehicle control group (median $\pm$ MAD, $n = 10$) (Table 1)).

In isolated rabbit hearts, sotalol-induced TdP occurred in the six hearts tested when the pacing rate and the K$^+$ concentration were decreased. Their occurrence was limited by SAR296968 pretreatment at $0.3 \mu M$ (TdP in two of six hearts) or $1 \mu M$ (TdP in one of six hearts) (Fig. 3). In parallel, SAR296968 reduced in a concentration-dependent manner the sotalol prolonging effect on QT interval duration by $-46$ and $-95$ milliseconds for 80 bpm at 0.3 and 1 $\mu M$, respectively. Moreover, SAR296968 blunted the sotalol-induced increase in dispersion of ventricular repolarization, although not significantly, except for the 0.3 $\mu M$ dose at 40 bpm ($T$ wave peak to $T$ wave end) interval duration ($T_p-T_e$) reduced by $-27$ milliseconds at $0.3 \mu M$ and $-20$ milliseconds at $1 \mu M$ SAR296968). The QRS interval duration was not affected by SAR296968 administration (Supplemental Table 3). The same level of antiarrhythmic efficacy was shown when TdP were induced by veratridine application (Fig. 3), which was associated with reduction of the veratridine-induced prolongation of the QT interval duration, without affecting the dispersion of ventricular repolarization or the QRS interval (Supplemental Table 3a).

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**A**

**B**

| Species NCX | IC50 [nM] | Confidence Intervals [nM] | Number of experiments |
|-------------|-----------|---------------------------|-----------------------|
| Human NCX1  | 74        | [54; 102]                 | n=11                  |
| Human NCX2  | 23        | [17; 31]                  | n=4                   |
| Human NCX3  | 129       | [84; 199]                 | n=5                   |
| Dog NCX1    | 21        | [16; 29]                  | n=4                   |
| Guinea-pig NCX1 | 156   | [129; 188]              | n=7                   |
| Pig NCX1    | 68        | [42; 110]                 | n=3                   |
| Rabbit NCX1 | 142       | [120; 166]                | n=7                   |
| Rat NCX1    | 211       | [183; 242]                | n=7                   |

**C**

Dose/Response relationship for SAR296968 on NCX1 current

| IC50 [nM] | Confidence Intervals [nM] |
|-----------|---------------------------|
| NCX1 Forward (-90 mV) | 34.9 [29.0; 42.0] |
| NCX1 Reverse (+45 mV)  | 38.9 [30.6; 49.5] |

**D**

Effect of SAR296968 at 10 $\mu M$ on ICa and INa currents (Current change values, Mean %)

| Current change [%] | SAR296968 10 $\mu$M | Nifedipine 10 $\mu$M | Tetrodotoxin 10 $\mu$M | N |
|-------------------|----------------------|----------------------|------------------------|---|
| ICa               | -1.75 $\pm$ 5.21     | -78.70 $\pm$ 3.824   | -86.94 $\pm$ 1.70      | 7 |
| INa               | 6.76 $\pm$ 6.00      |                      |                        | 4 |

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**Fig. 2.** SAR296968 in vitro characterization. (A) Chemical structure of SAR296968 (active principle, left) and SAR340835 (prodrug, right). (B) Inhibition potency of SAR296968 on NCX isoforms in CHO cells. Effect of SAR296968 on the native NCX1 current (C) and on calcium current (ICa) and sodium current (INa) (D).
In anesthetized pigs, SAR296968 1.5 mg/kg significantly increased left ventricular contractility by 49%, raising the dP/dt max from 1423 ± 144 mm Hg/s at baseline to 2115 ± 189 mm Hg/s after infusion completion. No changes in the left or right atrial effective refractory period were recorded after SAR296968 treatment regardless of the basic cycle length (Supplemental Table 3b). However, the incidence of atrial arrhythmias (LAV) was significantly reduced after SAR296968 administration vs. 27.0 ± 6% with vehicle administration by 74% (6.56 ± 1.75 events after vehicle administration vs. 2.55 ± 1.75 events after SAR296968 administration) (Table 2).

**Fig. 3.** Effect of SAR296968 on EAD-related arrhythmias in the isolated rabbit heart model SAR296968 in isolated rabbit heart. SAR296968 treatment at 0.3 or 1 μM decreased the sotalol-induced Tdp without any proarrhythmic activity by suppression of EAD induced by veratridine.

**Effects of SAR340835 in Normal Conscious Dogs**

Infusion of SAR340835 had no effect on arterial pressure but dose-dependently decreased heart rate with a significant effect only at the highest dose (−23.4%, P = 0.0164) (Table 2). Compared with vehicle, SAR340835 significantly increased dP/dt max at 250, 750, and 1500 by +24.4% (P = 0.0479), +38.7% (P = 0.0054), and +65.6% (P = 0.0003), respectively (Fig. 4; Table 2). In parallel, stroke volume (SV) was significantly increased to an almost similar magnitude regardless of the dose (+16.5% (P = 0.0144), +21.7% (P = 0.0026), and +14.1% (P = 0.0327) at 250, 750, and 1500 μg/kg per hour, respectively (Table 2). SAR340835 increased dP/dt max and left ventricular end-diastolic volume (LVEDV) in a dose-dependent manner, only reaching statistical significance at 750 and 1500 μg/kg per hour [+27% and +36.3% increase compared with vehicle, respectively (Table 2)]. SAR340835 had no effect on calculated oxygen consumption (MVO2) (Table 2).

SAR340835 significantly increased dP/dt min at 750 and 1500 μg/kg per hour by 7.2% (P = 0.0238) and 9.6% (P = 0.0066), respectively (Table 2). However, SAR340835 did not induce any changes in the other echocardiographic parameters of diastolic function (Table 2).

**Fig. 4.** Echocardiographic changes in normal conscious dogs (n = 8). AVB, atrioventricular block; AVF, atrial fibrillation; E/A, early to late diastolic ratio; dP/dt max, maximum rate of pressure rise; EDV, end-diastolic volume; FA, foreshock activity; FV, foreshock volume; IVA, isolated ventricular arrhythmias; LAV, left atrial flutter; LVEDP, left ventricular end-diastolic pressure; LVEDV, left ventricular end-diastolic volume; MVO2, maximum oxygen consumption; PVo2, partial pressure of oxygen. "NS" signifies no statistical significance. *P < 0.05 vs. baseline.

**Effects of SAR340835 in Pacing-Induced HF Dogs**

HF Dog Model Characteristics. After 4 weeks of rapid right ventricular pacing at 240 bpm, HF dogs’ phenotype recapitulated the clinical signs of dilated cardiomyopathy and heart failure in humans (Table 3). The dogs exhibited a major increase in LVEDV from 75 ± 6 ml at baseline (i.e., before starting the pacing) to 119 ± 6 ml (P = 0.0010), a depressed contractility illustrated by a marked decrease in dP/dt max and dp/dt max/LVEDV (Table 3), and a significant and profound decrease in LVEF from 55% ± 3% to 28% ± 1% (P = 0.0001). This decrease in cardiac contractility is also reflected by a significantly reduced SV from 64 ± 2 ml to 31 ± 3 ml (P = 0.0012). HF dogs also displayed a severe diastolic dysfunction illustrated by a restrictive pattern (E/A > 1) and a reduced deceleration time (DT) from 83.6 ± 7.1 to 54.3 ± 4.4 milliseconds (P = 0.0076). In addition, ANS modifications typical of human HF were also observed.

**Table 1.** Antiarrhythmic effects of SAR296968 in animal models of arrhythmias related to early or late afterdepolarizations

| Model | Number of arrhythmic contractions in guinea pig papillary muscle | Number of arrhythmic contractions in guinea pig left atria | Number of inducible atrial fibrillation episodes in anesthetized pig |
|-------|----------------------------------------------------------------|----------------------------------------------------------|------------------------------------------------------------------|
| Vehicle | 12 | 20.5 | ± | MAD |
| SAR296968 1 μM | 8 | No arrhythmic contraction | | |
| SAR296968 3 μM | 8 | No arrhythmic contraction | | |
| Vehicle | 10 | 15.5 | ± | MAD |
| SAR296968 1 μM | 8 | 1.5 | ± | MAD |
| SAR296968 3 μM | 10 | 1.4 | ± | MAD |
| n | Mean | ± | SEM |

1. *P < 0.05 vs. baseline.
Additional pathologic features of dogs included tachycardia, loss of the normally observed respiratory sinus arrhythmia, ANS imbalance as shown by an Low Frequency/High Frequency ratio that was increased from 0.22 ± 0.12 in normal dogs to 2.29 ± 0.45 in HF dogs (P < 0.0001) and impaired baroreflex sensitivity (49 ± 3 in normal dogs vs. 24 ± 3 in HF dogs, P < 0.0001).

Effects of SAR340835 and Dobutamine on Hemodynamics of Conscious HF Dogs. The effects of SAR340835 and dobutamine on hemodynamic parameters in dogs with HF are shown in Table 4. Neither SAR340835 nor dobutamine altered systolic or diastolic arterial pressure compared with vehicle regardless of the dose tested (Table 4). SAR340835 infusion over 3 hours tended to decrease heart rate (HR) from 250 ± 150 mm Hg/s at the completion of vehicle infusion (P = 0.0125) (Fig. 5; Table 4). dP/dtmax increased in a dose-dependent manner, but it reached statistical significance only at the highest dose tested (P = 0.0125) (Fig. 5; Table 4). Infusion of dobutamine significantly increased LV dP/dtmax at 10 µg/kg per minute (59.6% vs. vehicle, P = 0.0177) (Table 4).

Table 4. As heart rate decreased with SAR340835 infusion, the increase in stroke volume translated into a moderate increase in dP/dtmax/LVEDV and LVEF without reaching significance (P = 0.0001 and 0.0031, respectively) that did not reach statistical significance (Fig. 5; Table 4). SAR340835 dose-dependently increased LVEF (+33.9%, P = 0.0182, and +41%, P = 0.0034) at 750 and 1500 µg/kg per hour, respectively. SAR340835 did not change calculated oxygen consumption (MVO2) (Table 4).

After 3 hours of infusion, dobutamine at 10 µg/kg per minute significantly increased SV by 68.8% (P = 0.0030) and CO by 75.1% (P = 0.0031) and increased LVEF by 47.3% without reaching significance (P = 0.0679). Dobutamine significantly increased calculated MVO2 by 28.14% (P = 0.0013) (Table 4).
SAR340835 significantly improved baroreflex sensitivity (BRS) in a dose-dependent manner (+55.6%, \( P = 0.0304 \); +74.9%, \( P = 0.0048 \); and +119.6%, \( P = 0.0003 \), compared with vehicle, respectively) (Fig. 5; Table 4). Dobutamine infusion did not change sympathovagal imbalance or baroreflex sensitivity as compared with vehicle (Table 4).

**Effects of SAR340835 and Dobutamine on ECG Parameters of HF Dogs.** SAR340835 infusion significantly increased PR interval duration after dose 3 in HF dogs (Table 2). Dobutamine infusion shortened PR interval significantly in HF dogs (Table 4). PR interval was 90 ± 2 milliseconds versus 101 ± 5 milliseconds in the dobutamine versus vehicle group, respectively. No AVBs were observed under SAR340835 infusion in HF dogs.

**Effects of SAR296968 on HF Dog Cardiomyocytes**

Detailed results of dog cardiomyocyte studies are provided in the Supplemental Material.

**Discussion**

The current work aimed at establishing the potential therapeutic value of SAR340835, a novel NCX inhibitor, for the treatment of AHF. We first demonstrated that SAR340835 is a potent inhibitor of NCX across the different isoforms with no effects on \( \text{Ca}^{2+} \) or \( \text{Na}^{+} \) channels and with a similar potency in reverse and forward mode. In vivo and in vitro studies showed that SAR340835 displayed antiarrhythmic effects, improved systolic function, reduced HR, and restored both sympathovagal balance and BRS in HF dogs, exerting a more pronounced beneficial cardiac effect in HF than in normal conditions without changing the calculated MVO2. Interestingly, SAR340835 pharmacodynamic profile differed notably from dobutamine, the inotrope of reference for AHF.

NCX expression and activity are upregulated in human and animal failing heart (Hasenfuss et al., 1999; Sipido et al., 2002), thereby contributing to cardiac dysfunction in HF (Hobai et al., 2004) and EAD- and DAD-related arrhythmias typically occurring in patients with HF (Sipido et al., 2002). Consistently, NCX inhibition displayed antiarrhythmic properties (Tanaka et al., 2007; Milberg et al., 2008). This was confirmed with SAR340835, which strongly inhibits the occurrence of ventricular and atrial arrhythmias in various experimental conditions, stimulating NCX inward current in guinea pig, rabbit, or pig. Thus, SAR340835 suppressed DAD-related arrhythmias at concentrations that increased cardiac contractility in guinea pig isolated papillary muscles and atria. SAR296968 efficiently reduced long QT–related arrhythmias at concentrations matching those that increased SV in HF dog. Overall, these results suggested a marked antiarrhythmic profile that is probably devoid of proarrhythmic property since no direct activity was detected on \( K^+ \), \( \text{Na}^+ \), or \( \text{Ca}^{2+} \) channels. Indeed, patch-clamp studies supported the
high selectivity of SAR296968 for NCX versus these ion channels. Beyond its antiarrhythmic effects, prolonged infusion of SAR340835 markedly improved the systolic cardiac function. Such an effect was expected with NCX inhibition (O’Rourke et al., 1999; Otsomaa et al., 2020) but was not yet demonstrated in vivo in conscious large-species models of HF. SAR340835 increased systolic function at similar exposure in normal and HF dogs but with different associated cardiovascular changes. In normal dogs, SAR340835 significantly and dose-dependently increased cardiac contractility as evaluated by dP/dt_max, whereas the increase in SV plateaued from the first dose, probably because of the naturally high cardiac performance at baseline in normal conditions. Conversely, a marked and dose-dependent increase in SV revealed the improvement of systolic function in HF dogs more clearly than the increase in dP/dt_max. The dP/dt_max estimation of cardiac contractility depends on preload and afterload, and the large difference in cardiac load conditions at baseline between normal and HF dogs might have influenced the response on dP/dt_max. Overall, SAR340835 was more efficient in improving cardiac pump function in HF than in normal condition.

In isolated HF dog cardiomyocytes, SAR296968 at the highest concentration tested significantly increased sarcoplasmic reticulum Ca^2+ content, and impaired cell contractility. Then, partial inhibition of NCX could be effective in normal canine cardiomyocytes (Hobai et al., 2008; Oravecz et al., 2018). Interestingly, partial inhibition of NCX with the exchanger inhibitory peptide XIP improved the Ca^{2+} transient amplitude more in failing than in normal canine cardiomyocytes (Hobai et al., 2004). This was not unexpected considering the reported overexpression of NCX in human (Goldhaber and Philipson, 2013) and canine failing hearts (Winslow et al., 1999; O’Rourke et al., 1999), leading to premature Ca^{2+} efflux, reduced sarcoplasmic reticulum Ca^{2+} content, and impaired cell contractility. Then, partial inhibition of NCX could be enough to regulate disturbed calcium handling due to NCX overactivity and thus better improve the cardiac contractility than in normal condition.

On the other hand, other factors besides the augmented cardiac contractility could promote an increase in SV. In HF dogs, we observed that SAR340835 corrected the autonomic tone and BRS deficiency from the first dose tested while reducing the HR, in contrast with dobutamine. These beneficial effects of SAR340835 were comparable to those reported after chronic electrical stimulation of the carotid sinus baroreflex in HF dogs (Zhang et al., 2009; Sabbah et al., 2011). Indeed, ANS disturbances have been studied extensively in the HF condition (Shen et al., 2002; Zucker et al., 2007; Zhang et al., 2009). Overall, the autonomic balance shifts from primarily dominant vagal tone in the normal condition to sympathetic predominance in HF. The pathophysiological role of sympathetic activation in HF is highlighted by the beneficial effects of β-blocker therapy. The functional weakness of parasympathetic activity was observed in clinical and experimental HF (Floras and Ponikowski, 2015), from the early stages of HF (Ishise et al., 1998), and rising with disease progression (van Bilsen et al., 2017). Furthermore, the diminished cardiac vagal activity, increased HR, and decreased BRS are predictors of high mortality rate in patients with myocardial infarction or HF (La Rovere et al.,

### TABLE 3

Hemodynamics and LV function in conscious dogs before and after development of pacing-induced heart failure (n = 5 to 6)

Autonomic nervous system was evaluated by heart rate variability analysis. All data are expressed as means ± SEM and median ± MAD for Low Frequency/High Frequency ratio.

|                         | Before Pacing Normal Dogs (n = 5 to 6) | After Pacing HF Dogs (n = 5 to 6) |
|-------------------------|---------------------------------------|----------------------------------|
| **Hemodynamics**        |                                       |                                  |
| dP/dt_{max} (mm Hg/s)   | 3082 ± 300                            | 1249 ± 55**                      |
| dP/dt_{min} (mm Hg/s)   | −3104 ± 152                           | −1487 ± 112***                   |
| LVEDP (mm Hg)           | 11.0 ± 1.6                            | 33.2 ± 3.7**                     |
| SBP (mm Hg)             | 122.3 ± 5.6                           | 104.3 ± 4.7**                    |
| DBP (mm Hg)             | 92.0 ± 6.0                            | 79.3 ± 5.5**                     |
| dP/dt_{max}/LVEDV (mm Hg per milliliter) | 43.5 ± 5.6 | 10.8 ± 1.1** |
| MVO2 (ml O2/min per 100 gram) | 13.0 ± 1.27 | 13.3 ± 1.02 |
| **Echocardiography**    |                                       |                                  |
| SV (ml)                 | 64 ± 2                                | 31 ± 3**                         |
| HR (bpm)                | 87 ± 6                                | 149 ± 9**                        |
| CO (l/min)              | 5.2 ± 0.5                             | 4.3 ± 0.4                        |
| LVEF (%)                | 55 ± 3                                | 28 ± 1***                        |
| LVEDV (ml)              | 75 ± 6                                | 119 ± 6**                        |
| E (cm/s)                | 0.85 ± 0.051                          | 0.872 ± 0.029                    |
| DT (ms)                 | 83.6 ± 7.1                            | 54.3 ± 4.4**                     |
| A (cm/s)                | 0.608 ± 0.042                         | 0.423 ± 0.027**                  |
| E/A                     | 1.41 ± 0.07                           | 2.08 ± 0.07***                   |
| **Heart rate variability** |                                       |                                  |
| Low Frequency (nu)      | 19 ± 4                                | 61 ± 3***                        |
| High Frequency (nu)     | 78 ± 5                                | 27 ± 3***                        |
| Low Frequency/High Frequency | 0.22 ± 0.12  | 2.29 ± 0.45**                   |
| BRS (mm Hg/s)           | 49 ± 3                                | 24 ± 3**                         |

*P < 0.05, **P < 0.01, and ***P < 0.001 HF vs. Normal condition.
TABLE 4
Cardiac and hemodynamic effects of SAR340835 and dobutamine in HF dogs

Autonomic nervous system was evaluated by heart rate variability analysis. All data are expressed as means ± SEM and median ± MAD for Low Frequency/High Frequency ratio (P < 0.05 vs. vehicle group; n = 3–5 dogs according to the group or parameter considered).

|                      | Vehicle Infusion | SAR340835 Infusion | Dobutamine |
|----------------------|------------------|--------------------|------------|
|                      | 250µg/kg per hour| 750µg/kg per hour  | 1500 µg/kg per hour | 10 µg/kg per minute |
| **HF Dogs**          |                  |                    |             |                     |
| **Hemodynamics**     |                  |                    |             |                     |
| dP/dt max (mm Hg/s)  | 3 to 4 1276 ± 79  | 1382 ± 225         | 1554 ± 195  | 1699 ± 208*         | 2037 ± 9*          |
| dP/dt max (mm Hg/s)  | 3 to 4 1550 ± 42  | -1643 ± 128        | -1720 ± 117 | -1726 ± 65          | -2452 ± 194*       |
| LVEDP (mm Hg)        | 3 to 4 36.6 ± 2.8 | 34.0 ± 4.4         | 30.0 ± 3.5  | 30.8 ± 3.8          | 30.3 ± 2.3*        |
| SBP (mm Hg)          | 5     109.0 ± 4.4  | 105.5 ± 5.9        | 102.4 ± 5.2 | 111.0 ± 5.3         | 106.0 ± 2.2        |
| DBP (mm Hg)          | 4 to 5 84.6 ± 4.2  | 80.0 ± 4.8         | 77.5 ± 4.7  | 83.4 ± 4.6          | 73.6 ± 3.5         |
| dP/dt max/LVEDP (mm Hg/mm per milliliter) | 3 to 4 11.4 ± 0.7 | 12.2 ± 1.5         | 13.1 ± 1.2  | 14.4 ± 1.5**        | 18.8 ± 1*          |
| MVO2 (mLO2/min per 100 gram) | 4 to 5 13.2 ± 1.06 | 13.51 ± 0.52      | 12.51 ± 0.99 | 14.01 ± 1.03       | 16.93 ± 1.19**     |
| PR interval (ms)     | 4 to 5 101 ± 5    | 103 ± 5            | 106 ± 6     | 114 ± 2*            | 90 ± 2*            |
| **Echocardiography** |                  |                    |             |                     |                     |
| SV (ml)              | 4 to 5 34 ± 3     | 44 ± 3*            | 52 ± 7**    | 56 ± 6***           | 60 ± 5*            |
| HR (bpm)             | 4 to 5 136 ± 17   | 125 ± 7            | 109 ± 20    | 110 ± 11            | 133 ± 5            |
| CO (l/min)           | 4 to 5 4.6 ± 0.2  | 5.6 ± 0.2          | 5.3 ± 0.5   | 5.8 ± 0.6           | 8.0 ± 0.7**        |
| LVEF (%)             | 4 to 5 29 ± 3     | 34 ± 3             | 40 ± 3*     | 41 ± 2**            | 43 ± 3             |
| LVEDV (ml)           | 4 to 5 111 ± 2    | 110 ± 4            | 116 ± 4     | 118 ± 2             | 109 ± 2            |
| E (cm/s)             | 4 to 5 0.912 ± 0.027 | 0.958 ± 0.071   | 0.965 ± 0.108 | 1.012 ± 0.078    | 1.088 ± 0.083      |
| DT (ms)              | 4 to 5 56.2 ± 7.1 | 56.2 ± 3.0         | 61.8 ± 5.1  | 65.6 ± 3.0          | 57.3 ± 6.6         |
| A (cm/s)             | 4 to 5 0.446 ± 0.027 | 0.472 ± 0.035   | 0.465 ± 0.044 | 0.472 ± 0.061    | 0.485 ± 0.031      |
| E/A                  | 4 to 5 2.07 ± 0.11 | 2.05 ± 0.13       | 2.16 ± 0.38 | 2.22 ± 0.22        | 2.25 ± 0.15        |
| **Heart rate variability** |                  |                    |             |                     |                     |
| Low Frequency (nu)   | 4 to 5 55 ± 4     | 31 ± 4**           | 21 ± 2 ***  | 24 ± 5***           | 48 ± 3             |
| High Frequency (nu)  | 4 to 5 35 ± 4     | 66 ± 4**           | 74 ± 4 ***  | 73 ± 5***           | 43 ± 6             |
| Low Frequency/High Frequency | 4 to 5 1.51 ± 0.40 | 0.44 ± 0.06*      | 0.28 ± 0.06 *** | 0.31 ± 0.09***  | 0.95 ± 0.14        |
| BRS (mm Hg/s)        | 4 to 5 32 ± 3     | 50 ± 4*            | 55 ± 4*     | 70 ± 4***           | 29 ± 2             |

High Frequency, parasympathetic modulation; Low Frequency, sympathetic modulation; Low Frequency/High Frequency, sympathovagal balance; nu, normalized unit.

*P < 0.05, **P < 0.01, and ***P < 0.001 SAR340835 vs. vehicle.
P < 0.05, #P < 0.01, and ##P < 0.001 dobutamine vs. vehicle.

1998; Lechat et al., 2001). Accordingly, several clinical studies have used stimulation devices, like vagal nerve stimulation or baroreceptor activation therapy, to reduce sympathetic nervous system reflex activity or promote vagal activity to restore a proper ANS balance (Singh et al., 2014). However, while the vagal nerve stimulation or baroreceptor activation therapy worked in animal models, clinical studies have been disappointing (van Bilsen et al., 2017).

The mechanisms provided by SAR340835 that lead to ANS balance restoration and the slight but consistent negative chronotropic effect are probably the end results of complex changes into electromechanical machinery underlying cardiac activity. Since HR was unaltered in NCX Knock Out mice or in mice overexpressing NCX, a direct role of NCX on pacemaker cells is unlikely (Gao et al., 2013; Kaese et al., 2017). However, NCX is an essential effector in β-adrenergic–mediated chronotropy (Gao et al., 2013; Kaese et al., 2017). Therefore, in HF conditions combining NCX and sympathetic overactivity, one might expect that NCX inhibition is likely to reduce HR, as observed in our HF dogs. However, we could not exclude that
SAR340835-mediated bradycardia could be the consequence of restored sympathovagal balance and BRS, as previously reported for other positive inotropes that increased intracellular Ca\(^{2+}\) concentrations. Na\(^+\) channel enhancer (Shen et al., 2002) and Ca\(^{2+}\) channel activator (Uechi et al., 1998) both reduced HR and enhanced BRS in pacing-induced HF dogs but not in normal dogs. Using ganglionic or \(\beta\)-adrenergic blockade, these authors demonstrated that the negative chronotropic effect was mainly due to an increased parasympathetic tone. The Ca\(^{2+}\) channel activator was hypothesized either to enhance BRS due to local alterations in the Na\(^+\) content of the vessel wall (Kunze and Brown, 1978) or to exert a central neuronal mechanism increasing the vagal tone through intracellular Ca\(^{2+}\) elevation (Uechi et al., 1998). A close mode of action can reasonably be hypothesized for SAR340835, which similarly increased intracellular Ca\(^{2+}\) and showed the same pharmacological in vivo profile, but further studies are warranted to confirm this hypothesis. Additional beneficial effects of NCX inhibitors would be related to their antiarrhythmic properties, as they did not affect the refractory periods in ECG time intervals but slightly accelerated repolarization. This contrasts with Na\(^+\) channel enhancers, which prolong action potential duration and can promote EADs and torsade de pointes, and with Ca\(^{2+}\) channel activators also known to induce EADs and DADs (Chang et al., 2012; Shroyer et al., 2013).

The attractiveness of NCX inhibition for the treatment of AHF could be compromised by the occurrence of second-degree AVB reported in normal dogs. However, SAR340835 at the highest dose tested showed only a limited prolongation effect on the PR interval in HF dogs and did not induce second-degree AVB. The high vagal tone characterizing normal dogs and the profound electrophysiological remodeling of failing hearts (including NCX overactivity) could partly explain these contrasting results between HF and normal conditions.

Overall, SAR340835 displays an attractive therapeutic profile for patients with HF, contrasting with current inotropes, reducing afterdepolarization-related arrhythmias, and improving systolic cardiac function without changing blood pressure or impairing the diastolic function. SAR340835 restored the impaired baroreflex sensitivity in HF dogs, thereby improving the balance between sympathetic and parasympathetic tone while maintaining potent \(\beta\)-adrenergic–independent inotropic effects. In addition, the slight bradycardia reported with SAR340835 could contribute to a reduction in myocardial oxygen consumption and improve cardiac efficiency, a valuable property in the setting of AHF. Accordingly, in HF dogs, the calculated MVO\(_2\) remained unchanged regardless of the dose of SAR340835, whereas it was raised with dobutamine infused in a therapeutic dose range. These promising results warrant further studies comparing SAR340835 and dobutamine at doses inducing a similar increase in cardiac contractility. Altogether, these results show that potent selective NCX inhibitors offer new therapeutic opportunities for the treatment of patients with AHF. Whether this efficacy would be observed in HF with preserved ejection fraction (HFpEF) as well as in HF with reduced ejection fraction conditions remains to be determined. Experimental studies (Kamimura et al., 2012; Primessng et al., 2019) suggested that chronic partial NCX inhibition could be beneficial in HFpEF through normalization of overactivity of NCX. Moreover, several publications support the rationale for NCX overactivity in patients with HFrEF (Pieske et al., 2002; Ashrafi et al., 2017). Next steps with SAR340835 would be to explore the potential benefit against diastolic dysfunction in HFpEF models and to characterize the safety risk and determine the safety margin before considering any clinical development in AHF.

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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% CO₂) 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 Ca²⁺-concentration using the Ca²⁺-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, Ca²⁺ in) resulting in an intracellular Ca²⁺ 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 A00013593 (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:

\[
\text{Inhibition (\%)} = 100 \times \frac{1 - (\text{sample} - \text{low control})}{(\text{high control} - \text{low control})}
\]

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 (Iit), 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, MgCl₂ 0.5, Na₂HPO₄ 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 CaCl₂ 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, KH₂PO₄ 20, MgCl₂ 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, CaCl₂ 1.3, MgCl₂ 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, CaCl₂ 29, MgCl₂ 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, CaCl₂ 2, MgCl₂ 2, CsCl 2, BaCl₂ 1, Na₂HPO₄ 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, NiCl₂ (5 µM) was added to the bathing solution, causing complete and reversible inhibition of the NCX current. After wash-out of NiCl₂ 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, NiCl₂ 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 Ca²⁺ 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, NiCl₂ 0.1, Nifedipine 0.01, pH = 7.4 with HCl/CsOH.

For recording of voltage dependent Ca²⁺ 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 NiCl₂ 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 CaCl₂) 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
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 × (LVEDD − LVESD)/LVEDD and ejection fraction was calculated as 100× (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.
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):

\[ MVO2 = 0.000408 \times (SBP \times HR) + 0.000325 \times ((0.8 \times SBP + 0.2 \times DBP) \times HR \times SV) / BW + 1.43 \]

**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 sympatovagal 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:

- \( LF\ nu\ (%) = (LF / (LF + HF)) \times 100 \)
- \( HF\ nu\ (%) = (HF / (LF + HF)) \times 100 \)

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: M4780; 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**<sub>SAR</sub> / **Peak Height**<sub>baseline</sub>. 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 | Parameter                                | Unit      |
|-----------------------------|------------------------------------------|-----------|
|                            | Left Ventricular End Diastolic Diameter  | mm        |
|                            | Left Ventricular End Systolic Diameter   | mm        |
|                            | Left Ventricular Fractional Shortening a | %         |
| Aortic surface             | Aortic surface                           | cm²       |

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 | Parameter                                | Unit     |
|---------------------------------|------------------------------------------|----------|
|                                | Left Ventricular End Diastolic Volume    | mL       |
|                                | Left Ventricular End Systolic Volume     | mL       |
|                                | Left Ventricular Ejection Fraction b      | %        |

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 | Parameter                        | Unit |
|---------------------------------|----------------------------------|------|
|                                 | Velocity time integral           | cm   |
|                                 | Aortic Ejection Time             | ms   |

**Pulse wave Doppler – mitral flow**

| Left parasternal long axis view | Parameter                                | Unit     |
|---------------------------------|------------------------------------------|----------|
|                                 | Peak velocity of early diastolic transmitral flow | cm/s     |
|                                 | Peak velocity of late transmitral flow    | cm/s     |
|                                 | Deceleration time                      | ms       |
|                                 | Duration of the A Wave                 | ms       |
|                                 | Ratio E/A                               | -        |

**Parameters calculated**

\[ a \quad \text{LV FS} = \frac{[(\text{EDD} - \text{ESD})/\text{EDD}]}{\text{EDD}} \]

\[ b \quad \text{LV EF} = \frac{[(\text{EDV} - \text{ESV})/\text{EDV}]}{\text{EDV}} \]

\[ c \quad \text{SV} = \text{VTI} \times \text{Ao surf} \]

\[ d \quad \text{CO} = \text{SV} \times \text{HR} \] 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)

|                              | Vehicle mean ± sem | SAR296968 1 µM mean ± sem | SAR296968 3 µM 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.

| BCL (bpm) | Left atrium AERP |  | Right atrium AERP |  |
|-----------|-------------------|---|-------------------|---|
|           | 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  |                      |
| n (cells)                      | 33      | 17  | 20| 22 | 28 | 28                    |
| sarcomere shortening (µm)      | 0.127 +/- 0.029 | 0.129 +/- 0.050 | 0.105 +/- 0.042 | 0.07 +/- 0.026 | 0.118 +/- 0.058 | 0.091 +/- 0.026 |
| before treatment               |         |     |   |    |    |                      |
| sarcomere shortening (µm)      | 0.102 +/- 0.027 | 0.103 +/- 0.053 | 0.093 +/- 0.038 | 0.095 +/- 0.030 | 0.188 +/- 0.066 | 0.169# +/- 0.067 |
| after 6 minutes of treatment's |         |     |   |    |    |                      |
| perfusion                      |         |     |   |    |    |                      |
| 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 +/- 0.182 | 0.191 +/- 0.079 | 0.277 +/- 0.102 | 0.235 +/- 0.146 | 0.247 +/- 0.139 | 0.222 +/- 0.118 |
| (n=31)                         |         |     |   |    |    |                      |
| (n=18)                         |         |     |   |    |    |                      |
| (n=20)                         |         |     |   |    |    |                      |
| (n=24)                         |         |     |   |    |    |                      |

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              |
| n (cells)          | 16      | 12             | 16             |
| sarcomere shortening (µm) before treatment | 0.128 +/- 0.052 | 0.151 +/- 0.053 | 0.129 +/- 0.027 | 0.143 +/- 0.041 |
|                     | 0.142 +/- 0.052 | 0.132 +/- 0.060 | 0.143 +/- 0.028 | 0.141 +/- 0.040 |
| sarcomere shortening ratio | 0.843 +/- 0.258 | 0.917 +/- 0.259 | 0.894 +/- 0.373 | 1.075 +/- 0.265 |
|                     | 0.249 +/- 0.039 | 0.250 +/- 0.039 | 0.250 +/- 0.039 | 0.250 +/- 0.039 |
| contraction velocity (µm/s) | -0.699 +/- 0.414 | -0.953 +/- 0.567 | -1.032 +/- 0.476 | -1.413 +/- 0.672 |
|                     | -3.004 +/- 1.164 | -3.004 +/- 1.164 | -3.004 +/- 1.164 | -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 | 2.995 +/- 0.560 | 2.995 +/- 0.560 | 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 | 0.310 +/- 0.059 | 0.310 +/- 0.059 | 0.310 +/- 0.059 |

Median +/- Median Absolute Deviation
### Table S6. CEREP selectivity profile (78 targets)

| Non-peptide receptors |  
|-----------------------|
| A3 (h)                | 5-HT_{1D} |
| BZD (central)         | 5-HT_{3B} (h) |
| BZD (peripheral)      | 5-HT_{3C} (h) |
| GABA_A              | 5-HT_{5} (h) |
| GABA_A_{(h)0}        | 5-HT_{4} (h) |
| kainate              | 5-HT_{4} (h) |
| H_{3} (h)            | 5-HT_{3} (h) |
| H_{4} (h)            | α (non-selective) |
| P2X                  | σ1A |
| P2Y                  |  

| Peptide receptors |  
|------------------|
| AT_{1} (h)       | MC_{4} (h) |
| AT_{2} (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)       | NMU_{2} (h) |
| GAL_{1} (h)      | β_{2} (DOP) (h) |
| GAL_{2} (h)      | δ (KOP) |
| CXCR_{2} (IL-8B) (h) | NOP (ORL1) (h) |
| CCR_{1} (h)      | sst (non-selective) |
| TNF-α (h)        | VPAC_{1} (VIP) (h) |
| CCR_{2} (h)      | V_{1a} (h) |
| MCH_{1} (h)      | V_{1b} (h) |
| MCH_{2} (h)      | V_{2b} (h) |
| MC_{3} (h)       | V_{2a} (h) |

| Nuclear receptors |  
|------------------|
| GR (h)           | AR (h) |
| ER_{α} (h)       | TR (TH) |
| PR (h)           |  

| Ion channels |  
|--------------|
| Ca_{2+} channel (L, verapamil site) | K_{ATP} channel |
| Ca_{2+} channel (N) |  

| Amine transporters |  
|--------------------|
| GABA transporter   | norepinephrine transporter (h) |
| 5-HT transporter (h) | dopamine transporter (h) |

| Kinases |  
|---------|
| CaMK_{2α} (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) |
| PDE_{4D} (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@ 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 Ratko

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 other parameters, maximal force of contraction, contractility, relaxation and action potential duration only descriptive statistics was provided. For the parameter contractility separate paired t-tests were applied on log-transformed dP/dtmax values between baseline and subsequent 3 µM SAR296968 or vehicle administration. The paired differences in means of the log-transformed dP/dtmax 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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