DOI:
10.1111/bph.15764

Document Version
Peer reviewed version

OCT2013, AN ISCHAEMIA-ACTIVATED ANTIARRHYTHMIC PRODRUG, DEVOID OF THE SYSTEMIC SIDE EFFECTS OF LIDOCAINE

Short title:

Novel ischaemia-activated antiarrhythmic prodrug

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Word count:

7473 (excluding figure legends and references) (2891 words excluding methods, figure legends and references)

Acknowledgements:

No non-author contributions to acknowledge. Funding was supplied by The Academy of Medical Sciences and Medical Research Council.

Author contributions:

Conceptualisation: SO, LHP, MJC, MBS. Methodology: LMH, MBS, JW, FM, LP, MJC. Investigation: LMH, MBS, LMS, PT, FM, RAC, KNT, JW. Data Analysis: LMH, MBS, FM, JW, CDEW, LP, MJC. Funding Acquisition: LMH, MBS, MJC. Supervision: MJC, MBS, KTM, NSP. Writing – original draft: LMH, MBS, LHP, MJC. Writing – review and editing: LMH, MBS, LHP, MJC, RAC, KNT, JF, FM, KTM, SO, NSP

Conflict of Interest Statement:

SO, LHP, MJC and MBS are shareholders in BioTherics Limited, a private company that holds a patent on the use of OCT2013 as an antiarrhythmic. SO is a director of BioTherics Limited. All other authors have no conflict of interest to declare.

Data and materials availability:

The data that support the findings of this study are available from the corresponding author upon request.

Abstract:

Background and Purpose: Sudden cardiac death (SCD) caused by acute myocardial ischaemia and ventricular fibrillation (VF) is an unmet therapeutic need. Lidocaine suppresses ischaemia-induced VF, but utility is limited by side effects and a narrow therapeutic index. Here we characterise OCT2013, a putative ischaemia-activated prodrug of lidocaine.

Experimental Approach: The rat Langendorff-perfused isolated heart, anaesthetised rat and rat ventricular myocyte preparations were utilised in a series of blinded and randomised studies to investigate the antiarrhythmic effectiveness, adverse effects and mechanism of action of OCT2013, compared with lidocaine.

Key Results: In isolated hearts, OCT2013 and lidocaine prevented ischaemia-induced VF equi-effectively, but OCT2013 did not share lidocaine's adverse effects (PR widening, bradycardia and negative inotropy). In anesthetised rats, i.v. OCT2013 and lidocaine suppressed VF and increased survival equi-effectively; OCT2013 had no effect on cardiac output even at 64 $mg.kg^{-1}$ i.v., whereas lidocaine reduced it even at 1 $mg.kg^{-1}$. In neonatal rat ventricular myocytes, OCT2013 had no effect on Ca^{2+} handling whereas lidocaine impaired it. In paced isolated hearts, lidocaine caused rate-dependent conduction slowing and block, whereas OCT2013 was inactive. However, during regional ischaemia, OCT2013 and lidocaine equi-effectively hastened conduction block. Chromatography and mass spectrometry analysis revealed that OCT2013, detectable in normoxic OCT2013-perfused hearts, became undetectable during global ischaemia, with lidocaine becoming detectable.

Conclusions and Implications: OCT2013 is inactive but is bioreduced locally in ischaemic myocardium to lidocaine, acting as an ischaemia-activated and ischaemia-selective

antiarrhythmic prodrug with a large therapeutic index, mimicking lidocaine's benefit without adversity.

Bullet point summary:

What is already known:

- Sudden cardiac death caused by ischaemia-induced ventricular fibrillation (VF) is an unmet therapeutic need.
- Lidocaine suppresses ischaemia-induced VF, but adverse effects and a narrow therapeutic window limit its utility.

What this study adds:

- OCT2013 and lidocaine prevented ischaemia-induced VF equi-effectively, but OCT2013 had no adverse effects.
- OCT2013 was converted to lidocaine in the ischaemic myocardium leading to ischaemia-selective rate-dependent conduction block.

Clinical significance:

- OCT2013 is a prototype novel ischaemia-activated ischaemia-selective antiarrhythmic prodrug devoid of adverse effects.
- This unique profile has the potential to obtain antiarrhythmic prophylaxis against sudden cardiac death.

Keywords:

Antiarrhythmic, Cardiac, Ischaemia, Prodrug, Lidocaine, Ventricular fibrillation

Main text:

INTRODUCTION

Sudden cardiac death (SCD), due to ischaemia-induced lethal ventricular fibrillation (VF) is part of the syndrome of acute myocardial infarction (AMI) and represents a substantial clinical burden (Huikuri, Castellanos, & Myerburg, 2002; Myerburg & Junttila, 2012; Wong et al., 2019). There are no antiarrhythmic drugs currently available for out-of-hospital pre-treatment, where the greatest impact on mortality would be obtained (Priori et al., 2015). This is because, among the agents previously tested, some lacked efficacy because they increased mortality (CAST Investigators, 1989; Waldo et al., 1996), and the remainder lacked efficacy because doses were kept low to avoid adverse drug reactions (ADRs) (Køber et al., 2000; The Danish Study Group on Verapamil in Myocardial Infarction, 1990; The Multicenter Ditiazem Postinfarction Group, 1988). In a smaller cohort of AMI survivors, those at higher risk of VF and SCD, amiodarone and mexiletine are sometimes administered but their risk/benefit ratio is poor (IMPACT Research Group, 1984; Julian et al., 1997; Pandya et al., 2016; Rutledge, Harris, & Amsterdam, 1985). It has been proposed that a drug with selectivity of action for the arrhythmogenic ischaemic myocardium may address the unmet therapeutic need to suppress ischaemia-induced VF (Bain et al., 1997; Barrett, Hayes, & Walker, 1995; Barrett et al., 2000; Farkas, Qureshi, & Curtis, 1999; Walker & Guppy, 2003).

We set out to create an ischaemia-activated antiarrhythmic prodrug of lidocaine. Lidocaine is a cardiac $Na_v1.5$ channel blocker (Bean, Cohen, & Tsien, 1983). Its clinical use as an antiarrhythmic is restricted to the in-hospital setting via the i.v. route, and it does not increase survival (Aj, Anand, & Bangdiwala, 2015; Herlitz et al., 1997; Sadowski et al., 1999), in part due to dose-limiting ADRs in the central nervous system and in the heart (Aj et al., 2015; Pfeifer, Ph, Greenblatt, & Koch-weser, 1976). Nevertheless, extensive nonclinical

data show that the $Na_v1.5$ channel blocking properties of lidocaine can prevent ischaemia-induced re-entrant conduction and halt lethal arrhythmogenesis during acute myocardial ischaemia (Barrett et al., 1995; Bergey, Nocella, & McCallum, 1982; Canyon & Dobson, 2004; Farkas & Curtis, 2002; Sarraf, Barrett, & Walker, 2003).

OCT2013 is an aliphatic amine N-oxide that is converted to lidocaine via two-electron reduction (Fig. S1) mediated enzymically and non-enzymically by processes inhibited by oxygen (Patterson, 1993). Our hypothesis is that because ischaemia renders myocardial tissue hypoxic (e.g., Marshall, Parratt, & Ledingham, 1974), OCT2013 will be converted to lidocaine locally in the reducing environment of the ischaemic myocardium. This may be sufficient to obtain an ischaemia-selective conduction block during ventricular tachyarrhythmias, and suppression of VF, mimicking the beneficial effects of lidocaine but without lidocaine's myocardial and extracardiac ADRs. To test this, we used a multifaceted approach, employing *in vitro*, *ex vivo* and *in vivo* techniques. The findings identified OCT2013 as a first-in-class drug with novel effectiveness and safety.

METHODS

Experimental design

Experiments were performed in line with published guidelines, with randomization to treatment and blinded investigation and analysis of data (Curtis et al., 2018, 2015). Blinding was achieved in several different ways. To make up drug solutions, stocks of different types were prepared by the person undertaking the experimental lab work (the 'operator'). Solutions were prepared in identical storage containers, labelled, and a record of the labels written in a blinding book, by the operator. A second person then selected a code name (for example, a single letter) for each solution before they removed the original label of the storage vessel, replaced it with the code name and recorded it in the blinding book. The

relabelled stock solutions were used by the operator to prepare solutions for experimentation, guided by a randomisation table (prepared separately) (Curtis et al., 2013). A test solution or other intervention was selected by reference to the randomisation table. Blinding was maintained until after data had been collected, collated and analysed, and any excluded preparations or animals had been replaced according to predetermined exclusion criteria (Hesketh et al., 2020). Investigational endpoints were all determined prior to the start of each study. Other experimental design details varied in line with the model used and variable investigated. Group size selection was based on published principles outlined for pharmacological investigation (Curtis et al., 2018, 2015) which recommend that estimates based on power analysis when effect size is uncertain be increased by 50% to mitigate against type 2 error. Thus, for example, HPLC studies, with their high level of precision, required n=6 hearts/group whereas Langendorff-perfused isolated heart studies investigating Gaussian-distributed physiological variables (heart rate, ECG intervals) required n=9 hearts/group, and studies investigating binomially-distributed variables (ischaemia-induced VF) required a sample size of n=12 (or n=20 in order to preclude false negatives when weak effects were anticipated) (Andrag & Curtis, 2013). Details for protocol length, sample size and drug interventions are summarised in Fig. S2.

ARRIVE, ethical, legal and experimental requirements

All animal surgical procedures and perioperative management were carried out in accordance with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication, 8th Edition, 2011), under assurance number A5634-01, and the EU Directive 2010/63/EU or UK Home Office Guide on the Operation of the Animals (Scientific Procedures) Act 1986. Animal housing and husbandry were as previously described (Andrag & Curtis, 2013) in compliance with ARRIVE 2.0 (du Sert et al., 2020).

Imperial College Ethical Review Committee or King's College London Ethical Review Committee, where appropriate, authorized the project licence.

Techniques used in the study

Rat Langendorff-perfused isolated heart technique

Male Wistar rats (290-500 g) were anaesthetized with a lethal dose of sodium pentobarbitone (170 $mg.kg^{-1}$ i.p.) and heparinized with 160 $IU.kg^{-1}$ sodium heparin to prevent blood clotting. Once a surgical level of anaesthesia was confirmed by removal of the pedal reflex, hearts were excised and arrested in ice-cold (4°C) Krebs' perfusion solution modified to contain (mM) NaCl 118.5, $NaHCO_3$ 25.0, $MgSO_4$ 1.2, NaH_2PO_4 1.2, $CaCl_2$ 1.4, KCl 3.0, and glucose 11.1 (perfusate salts purchased from VWR International, UK). Throughout, we refer to this modified Krebs' solution as *start solution*. All perfusion solutions were filtered before use (5-μm pore size) to remove particulate matter. Hearts were perfused via the ascending aorta with start solution warmed to 37°C and gassed with a combination of 95% O_2 and 5% CO_2 to achieve a pH of 7.4. The solution is nominally normoxic, meaning the pO_2 is in the region of 600 mmHg, allowing for normal heart function in the absence of haemoglobin, according to Henry's law and as previously validated (Yamada, Hearse, & Curtis, 1990). Perfusion pressure was approximately 80 mmHg, achieved by delivering solutions via a gravity-fed constant pressure system. Coronary flow ($ml.min^{-1}$) was measured by weighing coronary effluent collected over timed intervals (1 ml = 1 g) and corrected for heart weight ($ml.min^{-1}.g^{-1}$).

In all Langendorff studies the control groups were perfused with a test solution made from start solution modified to contain drug vehicle (0.1% ethanol).

To detect changes in heart rate and rhythm, a unipolar electrode was inserted into the apex of the heart and was connected to a PowerLab system (Powerlab 4/35 and Animal Bio

Amp, ADInstruments, UK; sampling rate 4 kHz) and Labchart software (v.7, ADInstruments, UK). PR interval (ms), QT_{90} interval (QT interval at 90% repolarisation) (ms) and heart rate (beats.min^{-1}) were recorded only when hearts were in sinus rhythm.

Induction of regional ischaemia by coronary artery ligation *in vitro*

In Langendorff experiments that involved regional ischaemia, a 4-0 silk suture (Ethicon) was sewn around the left anterior descending (LAD) coronary artery 1-2 mm below the left atrial appendage (accessed by lifting the left atrium), threaded through a polyethene tube, left loose and later tightened to achieve a region of ischaemia in the range of 35-60% of the total ventricular weight (TVW) in order to maximise the likelihood of VF which is dependent on ischaemic zone (IZ) size (Ridley, Yacoub, & Curtis, 1992). IZ size was quantitated at the end of the designated period of reperfusion using 1 mg.ml^{-1}, Patent blue VF sodium salt in 0.9% NaCl solution, with re-occlusion trapping the dye in the IZ, and reverting to dye-free test solution to wash out dye from the nonischaemic zone followed by dissection (Curtis & Hearse, 1989). In experiments where myocardial tissue samples were required for drug extraction and quantitation, contamination from the blue dye was avoided by quantifying IZ size from percent reduction in coronary flow at +1 min after LAD ligation versus -1 min prior, a method previously validated in this preparation (Curtis & Hearse, 1989). ECG analysis and arrhythmia definitions followed the Lambeth Conventions II guidelines (Curtis et al., 2013).

Induction of global ischaemia via cessation of flow

In Langendorff experiments that involved global myocardial ischaemia, the perfusion inflow line was clamped to cause complete cessation of coronary flow and hearts were immediately submerged in warmed start solution (37°C) to maintain a constant temperature for the duration of global ischaemia.

Use of an intraventricular balloon for assessment of contractile function

In Langendorff experiments that involved assessment of contractile function, an intraventricular balloon (IVB) made from compliant and non-elastic material was inserted into the left ventricle by way of the mitral valve, accessed by incising the left atrium (Wilder et al., 2016). The IVB was attached to a pressure transducer connected to a PowerLab system (PowerLab 4/35 and Bridge Amp, ADInstruments, UK; sampling rate 4kHz) and Labchart software (v.7, ADInstruments, UK). The pressure transducer was calibrated each day of use using a sphygmomanometer. The IVB was inflated slightly (~0.01 ml) with saline (0.9% NaCl,) until a pressure could just be detected, and the value (arbitrary) on the syringe noted as the 'zero volume', and this value was used as the reference point for later IVB inflations (Wilder et al., 2016). The IVB was then further inflated with saline until a developed pressure of >100 mmHg was achieved, while diastolic pressure remained <10 mmHg. The volume added to the 'zero volume' to achieve this was recorded and defined as the 'working volume'(Wilder et al., 2016). Diastolic pressure and developed pressure (systolic pressure - diastolic pressure) were recorded from the pressure trace on Labchart at pre-determined timepoints according to the experimental protocol (see below). Contractile function was also assessed by construction of a Starling curve, by deflating the balloon to zero volume and then increasing the volume by 0.02 ml increments up to a maximum total volume of 0.14 ml or a diastolic pressure ≤20 mmHg (whichever reached first), allowing the resultant changes in developed and diastolic pressures to plateau for at least 5 consecutive beats following cessation of any transient arrhythmia that sometimes occurs with balloon inflation (Wilder et al., 2016). The gradient between pressure (developed and diastolic) and added volume was determined in each heart, and group means calculated.

Use of epicardial pacing to measure ventricular conduction time and conduction block

In Langendorff experiments that involved measurement of ventricular conduction times and conduction block, silver bipolar pacing wires were inserted into the left ventricular apex. The wires were attached to a DS3 Isolated Constant Current Stimulator (DS3, Digitimer) and a Powerlab system (PowerLab 4/35 and Bridge Amp, ADInstruments, UK; sampling rate 4kHz) connected to Labchart software (v.7, ADInstruments, UK). The current (mA) used to stimulate a heart during an epicardial pacing protocol differed based on the investigational endpoint, but in each case was determined based on the pacing threshold of the given heart. The pacing threshold was the minimum current required to pace the heart at a frequency of 6.7 Hz (400 beats.min^{-1}) and was determined 2 min prior to commencement of an epicardial pacing protocol by incrementally increasing the current supplied to the heart, from 0 mA until the heart began to pace.

Once the pacing threshold value for an individual heart was established, this value was doubled to set the constant current used for the subsequent epicardial pacing protocols. An epicardial pacing protocol was then run to determine myocardial conduction time (ms) and the occurrence of conduction block. The pacing protocol consisted of 6 pacing rates run for sequential 15 s time periods (400, 500, 600, 700, 800 and 900 beats.min^{-1}). Langendorff-perfused isolated hearts were set up for coronary ligation, epicardial pacing wires were placed 2 mm rostral to the apex on the anterior left surface, and an ECG electrode was placed 2mm rostral to apex on the right surface. This ensured that the heart could be paced from within the IZ and that the ECG electrode was located within the uninvolved zone (UZ) when regional ischaemia was induced. Myocardial conduction time was defined as the delay between the pacing stimulus and the appearance of the subsequent paced ventricular complex (Cascio, Foster, Buchanan Jr., Johnson, & Gettes, 1987). Myocardial conduction block was defined as

an instance when the pacing stimulus was not followed by a resultant paced ventricular complex (Cascio et al., 1987).

Antiarrhythmic assessment in vivo in anaesthetised rats

When antiarrhythmic effects were evaluated *in vivo*, adult male Sprague Dawley rats, weighing 500-750 g, were anaesthetised using 5% isoflurane to allow for shaving of the chest, under-fore-limb areas, thighs and neck, and intubation via an endotracheal tube (blunted 14G intravenous cannula, Venflon). Intubated animals were attached to a Harvard Ventilator (Harvard Apparatus, Massachusetts, USA), and mechanically ventilated initially with 5% isoflurane at a rate of 90 breaths.min^{-1}, with a tidal volume of 3 ml, before the isoflurane concentration was reduced to a maintenance level of approximately 1.5% for the remainder of the experiment. The rat was repositioned and secured in the left lateral position and a 3-lead ECG was attached to the animal subcutaneously at the right thigh, right under-fore-limb and left lower abdomen. The ECG was connected to a Powerlab system (PowerLab 4/35 and Bridge Amp, ADInstruments, UK; sampling rate 4kHz) using to Labchart software v.7 (ADInstruments, UK). A 22G intravenous cannula (Venflon) was inserted into the right internal jugular vein for drug administration via a Standard Infusion Only Pump 11 Elite infusion pump (Harvard Apparatus, Massachusetts, USA). Lidocaine and OCT2013 solutions were prepared in Becton Dickinson 10 ml syringes, diluted to the desired concentration in medical grade saline (0.9% NaCl). A thoracotomy via the 4th intercostal space was carried out, ensuring an optimal view of the left atrium, left atrial appendage and left ventricular apex. Ligation of the LAD coronary artery was performed using a 6-0 prolene needled suture (Ethicon) 1-2 mm below the corner of the left atrium to achieve a large left ventricular ischaemic region, capable of inducing a high incidence of VF within 5 min of ischaemia onset (Curtis, 1998). Ligation was confirmed through observational changes in left ventricular contractility and epicardial pallor. The occurrence of VF was determined during

30 min of LAD ligation. If VF occurred, up to 3 attempts at defibrillation were tried using a small custom-built rat defibrillator (Ordodi et al., 2006). If a rhythm incompatible with life, either VF or asystole, was present 2 min after a 3rd shock the rat was deemed to have not survived. Heart rate and PR and QT_{90} intervals were recorded throughout the protocol at 5 min intervals. Animals alive at the end of the protocol were culled by cervical dislocation.

Haemodynamic ADR assessment in vivo in anaesthetised rats

When acute adverse effects of drugs on haemodynamic function was assessed *in vivo*, adult male Sprague Dawley rats, weighing 500-750 g, were anaesthetised using 5% isoflurane. The ECG was recorded as in antiarrhythmic *in vivo* studies (above). A left parasternal incision was made, and limited thoracotomy was performed in the 5th intercostal space. A 1.9F Scisense pressure-volume (PV) catheter was then inserted into the heart at the left ventricular apex. Data were collected via the ADVantange acquisition system (Scisense Inc., Ontario, Canada) connected to Labchart software (v.7, ADInstruments, UK). Four ECG cycles were averaged, and 10 PV loop cycles were averaged and analysed using the PV loop Analysis Module on Labchart (v.7, ADInstruments, UK). A jugular i.v. line was set up for cumulative dose drug administration (above) for detection of the threshold dose evoking ADRs. Animals alive at the end of the protocol were killed by cervical dislocation.

Sarcomere length and intracellular Ca^{2+} in isolated ventricular myocytes

When adverse effects of drugs on contractile function were evaluated in isolated left ventricular myocytes, cells were isolated from male Sprague Dawley rats as previously described (Sato, O'Gara, Harding, & Fuller, 2005). Myocytes were loaded with the ratiometric Ca^{2+}-sensitive fluorescent dye Fura-2 AM (5 µM) (Invitrogen, Life Technologies Ltd) for 15 min at room temperature. Thereafter, the cell suspension was centrifuged at 400 rpm and myocytes were resuspended in a diluted Dulbecco's Modified Eagle's Medium

containing 1 mM Ca^{2+} and were further incubated at room temperature for at least 30 min to allow for de-esterification of the Ca^{2+} indicator.

Effects of drugs were examined at 37°C. Myocytes were visualised on a TE300 Nikon 40x oil-immersed objective microscope coupled to an IonOptix system (IonOptix Ltd). Cells were initially field-stimulated (20 V) at 1 Hz for a minimum of 2 min to achieve steady-state Ca^{2+} handling in Tyrode's solution containing NaCl 137 mM, KCl 5.4 mM, glucose 10 mM, N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid 10 mM, $MgCl_2$ 1 mM, $CaCl_2$ 1 mM; pH adjusted to 7.4 with 1M NaOH. Only cells with clear striations and non-spontaneous contractions were used. Sarcomere length and intracellular Ca^{2+} were measured simultaneously using the IonOptix MyoCam-S and μStep light source, respectively. A myocyte was positioned parallel within the field of view ensuring that the striation pattern was vertically aligned. The iris of the cell framing adaptor was adjusted around the perimeter of the cell to optimise the selected cell fluorescence. A region of interest was selected to measure a minimum of 10 sarcomeres, maximising measurement accuracy.

Background autofluorescence (using an average from unloaded cells) was subtracted. The F/F0 value is the fluorescence intensity of the ratio relative to the baseline (diastolic) level. The ratio was derived conventionally from the emitted fluorescence following excitation at 360 nm divided by that emitted at 380 nm. Fura2 does not have the quantal efficiency of a single wavelength dye like Fluo3 or 4. These dyes produce F/F0 values at the peak of a cardiac muscle cell Ca^{2+} transient of 4-6. We used a ratiometric Ca^{2+} indicator to reduce the impact of indicator extrusion and bleaching during experiments.

Transients were analysed using the IonWizard software (v.6.6.10.125 (x64)). For each experimental condition, the amplitude of ten transients were averaged to calculate sarcomere length shortening (%) and the F/F0. In addition, time to 90% restitution of systolic Ca^{2+} was

used as a measure of diastolic cellular Ca^{2+} uptake (Sankaranarayanan, Kistamás, Greensmith, Venetucci, & Eisner, 2017).

Conduction assessment with neonatal rat ventricular myocyte microelectrode arrays

When microelectrode arrays were used to evaluate the effects of drugs on conduction, neonatal rat ventricular myocytes (NRVMs) at age P0-P2 were isolated using a GentleMACs system (Miltenyi Biotec) using the protocol supplied with the enzyme kit. After a pre-plating step to purify the myocytes, 2 x 10^5 cells were seeded onto microelectrode arrays (MEAs) in a 30 µl drop of medium over the electrodes in the centre of the array. After allowing 30 min for the cells to attach, the medium was topped up. Recordings were taken 3-4 days after plating cells. The MEA array (MultiChannel Systems, GmbH) consisted of an 8 x 8 array of unipolar electrodes (100 µm diameter, 700 µm inter-electrode distance). Any electrode can be used to either record or stimulate. Cells were paced from the top line of electrodes at 1 Hz at 120% of threshold. A custom in-house MatLab code was used to assign local activation times, compute average inter-electrode conduction velocities, and draw activation maps (Chowdhury et al., 2018).

UHPLC-MS/MS measurement of OCT2013 and lidocaine in perfused myocardium

For detection of OCT2013 and lidocaine, the UHPLC-MS/MS used was a Waters Xevo TQS-micro triple quadrupole mass spectrometer with an electrospray ionisation source (operated in positive mode), coupled to an Acquity H Class LC system. Data were acquired using MassLynx V4.1 software. Separations were conducted on a Waters ACQUITY UHPLC™ BEH C_{18} column (2.1x50 mm, 1.7 µm), maintained at 40°C. Binary gradient profiles were developed using water (Optima™ LC-MS grade, Fisher) with 0.1% formic acid (LC-MS grade, Fisher) (A) and methanol (Optima™ LC-MS grade, Fisher) with 0.1% formic acid (B) at a flow rate of 200 µl.min^{-1}. Separations were conducted under the following chromatographic: 100% solvent A for 0.5 min, decreased to 10% in 4.5 min and subsequently

to 0% in 0.1 min, maintained for 1.9 min before being increased over 0.2 min to 100%. Column equilibration time was 3 min and the total run time of 10 min. The sample injection volume was 20 µl. Mass spectrometer parameters were as follows: capillary voltage 3.1 kV, desolvation temperature 600°C, cone gas flow 1 $l.h^{-1}$, desolvation gas flow 1000 $l.h^{-1}$ and dwell time 46 ms per analyte. Analyte quantitation was performed using the multiple reaction monitoring method shown in Table S1.

Stock solutions (1 $mg.ml^{-1}$) of lidocaine, OCT2013 and internal standard (IS) (lidocaine-d10 or (2-[bis(1,1,2,2,2-pentadeuterioethyl)amino]-*N*-(2,6-dimethylphenyl)acetamide)) used for construction of calibration curves were prepared in methanol and stored at -20°C. These stock solutions were used to generate a calibration graph for OCT2013 and lidocaine over the concentration range 0.1-500 $ng.ml^{-1}$. A standard calibration curve was constructed for both lidocaine and OCT2013 on the same day that test samples were analysed. The response generated was linear throughout the concentration range used for both analytes (0.1-500 $ng.ml^{-1}$), and the coefficient of determination (r^2) was 0.999166 for OCT2013 (Fig. S3A) and 0.999052 for lidocaine (Fig. S3B).

Following completion of each heart perfusion protocol (details below), samples of frozen myocardial ventricular tissue, weighing approximately 300 mg, were dissected and stored within CryoTube Vials (Thermo Scientific, US) in liquid nitrogen until subsequently prepared for UHPLC-MS/MS analysis. Frozen tissue samples were cut to obtain portions of 20-30 mg in weight. Tissue samples were then transferred into individual round bottom Eppendorf tubes (2 ml) containing 920 µl methanol, 70 µl H_2O_2 (30 g/100 ml, 9.8 M), 10 µl IS (100 $ng.ml^{-1}$ in methanol) and weighed. These samples were moved into cold plates and homogenised using a TissueLyser II homogeniser for 30 s at 30 Hz before being centrifuged for 5 min at 14000 rpm at 4°C. Each sample (50 µl) was then transferred into individual

UHPLC injection vials containing 250 µl of 0.2% formic acid in water, subsequently vortexed and injected into the UHPLC-MS/MS system (20 µl injection volume).

Exclusion criteria

In Langendorff experiments hearts were excluded from analysis if, during perfusion with start solution, coronary flow was less than 7 $ml.min^{-1}.g^{-1}$ or greater than 20 $ml.min^{-1}.g^{-1}$, or heart rate was less than 200 $beats.min^{-1}$, and in the coronary ligation cohort, hearts were excluded if the IZ size was outside the range of 35-60% of the TVW, all as per previous studies (Andrag & Curtis, 2013; Clements-Jewery, Kanaganayagam, Kabra, & Curtis, 2006; Wilder et al., 2016). Excluded hearts were replaced, while maintaining blinding, to maintain equal group sizes, unless their inclusion would not affect the outcome of the study (i.e. in a heart experiencing VF despite a small IZ of <35% (Curtis & Hearse, 1989)) thus mitigating against needless animal use.

Protocols for Langendorff experiments

All experimental protocols are summarised in Fig. S2.

Protocol for antiarrhythmic assessment of drugs administered from before the start of ischaemia

Hearts were initially perfused with start solution for 10 min to record baseline values of measured variables and apply exclusion criteria. Variables were then recorded at 5 min intervals for the remainder of the protocol. Test solution, either 15 µM lidocaine, 15 µM OCT2013 or control solution (n=12/group), was introduced from 10 min prior to the onset of 30 min of regional ischaemia, with IZ size later quantified by the dye method. The occurrence of ventricular arrhythmias was recorded throughout the experiment (Curtis et al., 2013).

Protocol for antiarrhythmic assessment of drugs administered after the start of ischaemia

Hearts were initially perfused with start solution for 10 min to record baseline values. Regional ischaemia was induced and 10 min later the perfusate was switched to one of 5 test solutions, 15 μM lidocaine, 15 μM OCT2013, 60 μM lidocaine, 60 μM OCT2013 or control solution (n=20/group) for a further 20 min before IZ size was quantified by the flow reduction method.

Protocol for contractile function assessment (in vitro)

With the IVB inflated to working volume, hearts were perfused with start solution for 10 min to establish baseline values for diastolic and developed pressures. A Starling curve was constructed. Perfusate was then switched to 15 μM lidocaine, 15 μM OCT2013 or vehicle (n=12/group) and at 10 min a second Starling curve was constructed. Regional ischaemia was then induced for 30 min followed by reperfusion for 60 min whence a third and final Starling curve was constructed. The IZ size was then determined by the dye method.

Protocol for conduction time and conduction block assessment during regional ischaemia

Values of heart rate and coronary flow were recorded 5 min before the start of left ventricular epicardial pacing, and any hearts meeting exclusion criteria were replaced. Conduction time and conduction block were recorded before and after switch to perfusion with test solution (15 μM lidocaine, 15 μM OCT2013 or vehicle, n=9/group), and at 5, 10, 20, 30 and 40 min into myocardial ischaemia. Following cessation of the protocol IZ size was quantified by the blue dye method.

Protocols for *in vivo* experiments in anaesthetised rats

All experimental protocols are summarised in Fig. S2.

Protocol for assessment of antiarrhythmic effectiveness in vivo

When ischaemia-induced arrhythmias were examined in rats *in vivo*, a bolus of 2 $mg.kg^{-1}$ lidocaine, 2 $mg.kg^{-1}$ OCT2013 or the equivalent volume of saline (n=6/group) was administered to the animal, immediately followed by the commencement of a 0.5 $mg.kg^{-1}.min^{-1}$ continuous infusion, which remained constant for the remainder of the experiment. Coronary ligation was undertaken 5 min later for a period of 30 min.

Protocol for assessment of haemodynamic ADRs in vivo

When acute haemodynamic ADRs were examined *in vivo*, rats were administered sequential i.v. boluses of lidocaine or OCT2013 (n=5/group), 1 $mg.kg^{-1}$, then a further 1, 2, 4, 8 16 and 32 $mg.kg^{-1}$, giving total drug doses of 1, 2, 4, 8, 16, 32 and 64 $mg.kg^{-1}$ respectively. The interval between boluses was 2 min. PR, QT_{90} interval and heart rate were recorded just prior to each bolus (i.e. at the end of each 2 min interval).

Protocol for isolated myocyte intracellular Ca^{2+} and sarcomere length measurement

The experimental protocol is summarised in Fig. S2. Following continuous superfusion with start solution, Tyrode's buffer (2 min at 1 Hz), ten sarcomere length and fura-2 ratio transients were recorded. Thereafter, remaining on the same myocyte, start solution was switched to test solution, Tyrode's containing either 15 µM lidocaine (n=35), 15 µM OCT2013 (n=20) or 60 µM OCT2013 (n=20). The test solution was continuously superfused for 2 min while stimulating the cell at 1 Hz. Following the incubation period, another ten sarcomere lengths and fura-2 ratio transients were acquired. The experimental solutions were sequentially reversed, thus, on the next selected cell the experiment started in the solution containing the drug in order to avoid bias relating to possible cell deterioration over time (Sikkel et al., 2013).

Protocol for assessment of conduction velocity using microelectrode arrays

The experimental protocol is summarised in Fig. S2. Recordings (10 s) were taken in Hanks Buffered Salt Solution during perfusion with start solution, and after administration of 1, 5, 10, 75 and 100 µM lidocaine or OCT2013 (n=10/group).

Protocol for measurement of ischaemia-activated conversion of OCT2013 to lidocaine

The experimental protocol is summarised in Fig. S2. Male Wistar rat (315-394 g) hearts were Langendorff perfused with start solution (as described above) for 10 min, during which baseline values of coronary flow were recorded. Perfusate was switched to 15 µM OCT2013 (n=12) for 10 min prior to either 30 min of global ischaemia (n=6), or 30 min time-matched normoxia (normal perfusion) (n=6). Hearts were submerged in warmed start solution for the duration of global ischaemia or time-matched normoxia (37°C). At the end of the protocol, the external surface of the hearts was rapidly washed with warmed start solution and whole hearts were snap frozen with cryogenic tongs previously cooled in liquid nitrogen (approximately -195°C).

Data and statistical analysis

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2018, 2015). Gaussian distributed variables were subjected to t-tests (two group comparisons), 1 way ANOVA (three or more groups) or 2 way ANOVA (changes to variables over time, across 3 or more groups) followed by either Dunnett's (comparison of groups to one control group) or Sidak's (comparison of two groups with multiple points) post hoc tests (if F was significant and data Gaussian). Binomially distributed variables (e.g. arrhythmia incidence) were compared using Fisher's exact test. Survival or 'trend over time' data (e.g. survival or time to VF in anaesthetised rats) were

analysed using the log-rank (Mantel-Cox) test. Line gradients (e.g. Starling curve analysis) were compared between groups using linear regression analysis. Statistical significance was set at $p<0.05$ for all analysis. All statistical analyses were performed on Graphpad Prism software (v.8).

Materials

OCT2013 [2-(2,6-dimethylanilino)-N,N-diethyl-2-oxoethanamine oxide hydrochloride] was synthesised by BioTherics Limited from commercially available lidocaine according to a method generally used to prepare aliphatic tertiary amine N-oxides (Craig & Purushothaman, 1970), and shown to be authentic and pure by NMR and mass spectroscopy.

Perfusion solutions containing lidocaine hydrochloride (Sigma Aldrich, UK) or OCT2013 were made by diluting concentrated stock solutions comprised of drug in a vehicle of 1:4 ethanol (VWR International, UK) and water (PURELAB ELGA Process Water, UK). Patent Blue VF Sodium Salt and Lidocaine-(diethyl-d_{10}) were purchased from Sigma-Aldrich, UK. Optima LC/MS Grade Formic Acid, Optima LC/MS Grade Water and Optima LC/MS Grade Methanol were obtained from Fisher Scientific, UK. $CaCl_2 2H_2O$, Glucose D(+), Methanol, NaCl, $NaHCO_3$, $MgSO_4 7H_2 0$, NaH_2PO_4 and KCl were purchased from VWR International, UK.

RESULTS

OCT2013 prevents ischaemia-induced VF without ADRs

Pre-treatment and continuous perfusion with OCT2013 (15 µM) or lidocaine (15 µM) reduced the incidence of VF (Fig. 1A) during 30 min of regional ischaemia in the rat Langendorff preparation. A higher concentration of OCT2013 (60 µM), and a range of concentrations of lidocaine (15 µM and 60 µM) had similar effects when administered ‘post-ligation’, from the 10^{th} min after the onset of ischaemia only (Fig. 1E). OCT2013 15 µM and

60 µM caused no ADRs and was devoid of any pharmacological activity in non-ischaemic tissue (Fig. 1B-D+F-H). In contrast, lidocaine evoked concentration-dependent ADRs including bradycardia (Fig. 1B+F), PR interval prolongation (Fig. 1C+G) and QT_{90} interval prolongation (Fig. 1D). Likewise, in separate studies in the rat Langendorff preparation, 15 µM OCT2013 was devoid of adverse actions on ventricular contractile function, whereas lidocaine had negative inotropic activity (Fig. 2B). Lidocaine and OCT2013 provided modest protection against diastolic dysfunction during reperfusion (Fig. 2F).

Studies *in vivo* in anaesthetised rats recapitulated the key findings. OCT2013 and lidocaine significantly and similarly reduced the incidence of VF (Fig. 3A+C), and increased animal survival (Fig. 3B) during 30 min regional ischaemia.

OCT2013 has no adversity in cumulative dose acute toxicity assessment

In anaesthetised rats, cumulative doses of lidocaine (2-32 $mg.kg^{-1}$) increased PR interval (Fig. 4A), caused bradycardia (Fig. 4B), and reduced cardiac output (Fig. 4D) and stroke volume (Fig. 4E). At 64 $mg.kg^{-1}$, lidocaine caused death in all animals from AV block and cardiovascular collapse. In contrast, OCT2013 caused no such ADRs up to 32 $mg.kg^{-1}$ (Fig. 4A-E). At 64 $mg.kg^{-1}$ (>30 fold its effective antiarrhythmic activity in anaesthetised rats) OCT2013 caused a modest PR widening (Fig. 4A) without any other adversity (Fig. 4B-E).

Mechanism of action is ischaemia-selective ventricular conduction block

It was hypothesized that ischaemia would transform OCT2013 from an inert substance to a mimic of lidocaine, with effects typical of those of ischaemia-selective $Na_v1.5$ channel block, and this hypothesis was tested in multiple different mechanistic studies.

First it was established that the molecular properties typical of $Na_v1.5$ channel block exhibited by lidocaine were not shared by OCT2013 during normoxia. In neonatal rat cultured ventricular myocyte monolayers, lidocaine reduced conduction velocity at 50 µM

whereas OCT2013 had no effect even at 100 µM (Fig. 5A+B). Likewise, lidocaine 15 µM caused a marked reduction in myocyte contractility and systolic calcium release and diastolic calcium uptake, in neonatal rat ventricular myocytes, whereas OCT2013 at 15 µM or 60 µM did not affect these variables (Fig. 6A-I).

Next we tested the hypothesis that in whole hearts (Langendorff perfusion) OCT2013 would mimic the typical rate-dependent effects of lidocaine on conduction (Davis, Matsubara, Scheinman, Katzung, & Hondeghem, 1986; Matsubara, Clarkson, & Hondeghem, 1987) but, unlike lidocaine, only during regional ischaemia. During normoxia 15 µM OCT2013 had no effect on conduction time, whereas 15 µM lidocaine caused a conduction slowing at pacing rates of 600-900 beats.min^{-1} (Fig. 5C). Conduction time was progressively slowed 5 to 10 min after the onset of regional ischaemia in controls and this effect was enhanced by increasing pacing rates (400-900 beats.min^{-1}) (Fig. 5C). OCT2013 and lidocaine exacerbated this slowing at every pacing rate, and to a similar degree (Fig. 5C). Ischaemia altered the rate dependence such that maximum conduction slowing was obtained by increasing rate from 400 to only 500 beats.min^{-1} (Fig. 5C). In the absence of drug or ischaemia there was no conduction block in any heart at pacing rates up to 900 beats.min^{-1} (Fig. 5D). In the absence of ischaemia, there was only one episode of conduction block in one heart perfused with OCT2013, and only when paced at 900 beats.min^{-1}, whereas lidocaine readily caused rate-dependent conduction block, with almost half the group of hearts experiencing some block when paced at 900 beats.min^{-1} (Fig. 5D). During ischaemia, rate-dependent conduction block occurred in control hearts. This block was markedly exacerbated by lidocaine within 5 min of ischaemia onset, an effect mimicked precisely by OCT2013 (Fig. 5D). By 10 min of ischaemia, when arrhythmia severity peaks in this preparation, lidocaine and OCT2013 caused conduction block in most hearts even at low pacing rates (400-600 beats.min^{-1}), whereas block was less frequent in controls (Fig. 5D). However, by 20

min after ischaemia onset, when arrhythmia severity begins to wane, rate-dependent conduction block in controls had become so marked that that all three groups resembled one another (Fig. 5D).

OCT2013 is an ischaemia-activated prodrug of lidocaine

Since we had shown that ischaemia converted OCT2013 into *a mimic* of lidocaine, we next sought whether ischaemia converted OCT2013 to lidocaine, itself, in Langendorff-perfused rat hearts, using UHPLC-MS/MS analysis. Hearts subjected to global ischaemia showed almost complete loss of OCT2013 trapped in the coronary vasculature during cessation of perfusion compared with the amount of OCT2013 found in the normoxic perfused hearts, and the loss of OCT2013 was accompanied by the appearance of lidocaine (Fig. 7) albeit the apparent stoichiometry of the conversion was not 1:1. In contrast, minimal formation of lidocaine (less than 6% of that in ischaemic hearts) was present in hearts snap frozen during normoxic perfusion with OCT2013, indicating the intrinsic resistance of OCT2013 to bioreduction under normoxic conditions (Fig. 7).

DISCUSSION AND CONCLUSIONS

Sudden cardiac death, an unmet therapeutic need

Despite clear therapeutic need (Huikuri et al., 2002; Myerburg & Junttila, 2012; Wong et al., 2019), there have been no novel pharmacological interventions introduced for the treatment of lethal ventricular arrhythmias associated with AMI in decades. Current guidelines support the use of β-blockers to reduce the risk of SCD in patients diagnosed with ischaemia (Priori et al., 2015) but their impact on SCD has been limited (Kezerashvili, Marzo, & De Leon, 2012). Amiodarone (class 3) is the most widely used second line pharmacological therapy for ventricular arrhythmias but evidence for mortality reduction is weak (Priori et al., 2015). In addition, amiodarone and mexiletine (class 1b) have narrow, if barely discernible, therapeutic

windows owing to serious ADRs (IMPACT Research Group, 1984; Julian et al., 1997; Pandya et al., 2016; Rutledge et al., 1985). Cardiological intervention in AMI is currently focused on reperfusion and electrical defibrillation (Ibanez et al., 2018; Priori et al., 2015), i.e., in-hospital events. A very safe antiarrhythmic drug would be required to reach other lower-risk groups (out-of-hospital) that represent a larger numerical cohort of SCD victims (Huikuri et al., 2002). Although prevention of VF in this cohort represents a major unmet therapeutic need, the multiple failures of prior clinical trials (CAST Investigators, 1989; Køber et al., 2000; Waldo et al., 1996) appears to have resulted in the current perception that no antiarrhythmic drug has (or will have) any value for SCD prevention (Al-Gobari, Al-Aqeel, Gueyffier, & Burnand, 2018).

The concept of an ischaemia-activated antiarrhythmic prodrug of lidocaine

We sought to address the problem by creating an ischaemia-activated prodrug of an antiarrhythmic. We selected lidocaine for two reasons. Firstly, as a tertiary amine, lidocaine has scope for chemical modification to the corresponding N-oxide, to provide an inert drug that may be converted to active drug in ischaemic tissue (Patterson, 1993), something not possible for some other antiarrhythmics, including mexiletine. Second, although lidocaine itself has a range of ADRs, it is a highly effective inhibitor of ischaemia-induced VF according to numerous animal model studies (Bergey et al., 1982; Canyon & Dobson, 2004; Farkas & Curtis, 2002; Sarraf et al., 2003), owing to its high affinity for the $Na_v1.5$ Na^+ channel in its activated/inactivated state (Matsubara et al., 1987) and consequent inherent tachycardia- and ischaemia-selectivity (Davis et al., 1986; Hondeghem, 1987; Hondeghem & Katzung, 1984; Matsubara et al., 1987), which converts ischaemia-induced conduction slowing into antiarrhythmic conduction block (Hondeghem, 1987; Hondeghem & Cotner, 1978). Thus, an ischaemia-activated lidocaine prodrug was conceived as a plausible candidate.

Proof of concept

OCT2013 was shown to mimic the ability of lidocaine to suppress ischaemia-induced VF, *in vivo,* in the anesthetised rat, and *in vitro,* but without the archetypal ADRs found to be caused by lidocaine (Wilson, Soei, Bezstarosti, Lamers, & Verdouw, 1993) that include bradycardia, PR prolongation, negative inotropy and Ca^{2+} mishandling, effects mediated outside the ischaemic region (Bergey et al., 1982; Canyon & Dobson, 2004; Farkas & Curtis, 2002). During reperfusion OCT2013 and lidocaine offered some protection against diastolic dysfunction. Chemical analysis of hearts showed that ischaemia resulted in the loss of OCT2013 and the concomitant appearance of lidocaine (hitherto absent during normoxic perfusion). Together these data confirm that OCT2013 is an ischaemia-activated antiarrhythmic mimic of lidocaine and an ischaemia-activated antiarrhythmic prodrug of lidocaine.

Mechanism of antiarrhythmic activity of OCT2013

Further studies identified a plausible mechanism for OCT2013's suppression of ischaemia-induced VF, namely the ischaemia-selective mimicry of lidocaine's hastening of rate-dependent conduction bock during ischemia. Evidence for this was (i) lack of effect of OCT2013 on conduction velocity during normoxic perfusion compared with rate-dependent conduction slowing by lidocaine and (ii) marked exacerbation of ischaemia-induced rate-dependent conduction slowing versus controls that became rate-dependent conduction block as ischaemia progressed, with OCT2013 and lidocaine possessing almost identical activity. Rate-dependent conduction block progressed more slowly during ischaemia in controls with values matching OCT2013 and lidocaine values 20 min after ischemia onset, a time when the severity of ischaemia-induced arrhythmias diminishes in this model, (Curtis, 1998). Thus, OCT2013 mimicked the actions of lidocaine's that are attributable Na^+ channel block, and which account for its antiarrhythmic effects (Kléber, Janse, Wilmsschopmann, Wilde, &

Coronel, 1986), hastening conduction block in the ischaemic region to narrow the temporal window of vulnerability to re-entry, the well-established basis for the antiarrhythmic action of lidocaine during ischaemia (Hondeghem, 1987; Hondeghem & Cotner, 1978), while lacking any measurable pharmacological activity during normoxia.

Effectiveness and safety of OCT2013 *in vivo*

Key findings were confirmed in the anesthetised rat, with OCT2013 devoid of effect on important haemodynamic variables including cardiac output, stroke volume, heart rate and PR interval, at doses up to 16 times that which suppressed ischaemia-induced VF (2 $mg.kg^{-1}$). In contrast, lidocaine produced adverse effects at 1 mg/kg. The fact that all rats treated with 64 $mg.kg^{-1}$ of lidocaine died (of conduction block with slow atrial activity continuing but ventricular standstill) whilst all rats treated with OCT2013 at the same dose survived highlights the substantial differences in the translational therapeutic window of these drugs.

Effectiveness of post-ligation administration

Previous studies have shown that i.v. verapamil given post-ligation can access the ischaemic region of the rat heart via collateral vessels and accumulate there, with this accounting for its post-ligation antiarrhythmic activity (Curtis, MacLeod, & Walker, 1984). This is despite residual collateral flow in rat hearts being the *lowest* among animal species, amounting to a maximum of only ≈6% of the nonischaemic flow (Maxwell, Hearse, & Yellon, 1987). In the present study, OCT2013 was likewise effective when administered post-ligation, albeit less potently than by pre-treatment (as was the case with verapamil *in vivo* (Curtis et al., 1984)), consistent with a requirement for time to access and accumulate in the ischaemic region via collaterals, and subsequently be converted to lidocaine to exert its effect (Curtis, 1998). Benefit was, nevertheless, obtainable. This is encouraging since thrombolysis candidates with AMI are an obvious cohort for initial human efficacy testing, meaning that OCT2013 would

need to be able to obtain benefit when administration is begun after the onset of regional ischaemia.

Study limitations

The rat Langendorff preparation has several limitations. These include the small heart size (1 g), fast sinus rate (>300 beats.min^{-1}), and absence of blood and functional innervation. These limitations, together with the model's advantages, are well-documented (Curtis, 1998). However, the conclusions of the present study do not rely on Langendorff data and key findings were recapitulated in a whole animal model. Future studies will seek to confirm key findings in a second species with different translational relevance, and undertake chronic toxicity assessment and pharmacokinetic characterization after single and repeat dosage *in vivo*.

Conclusions

OCT2013 is a first-in-class antiarrhythmic prodrug, with ischaemia-selective actions that mimic those of lidocaine, resulting in VF prevention, but without adversity at ≥16 times the effective dose *in vivo* and ≥7 the effective concentration *in vitro* (these numbers representing the translational therapeutic index in each setting). This is far better than the profile of lidocaine, whose equivalent value was <1 according to the data in the present study, and another clinically used class 1b drug, mexiletine (value <2 according to recent data derived from the *in vitro* rat heart model used in the present study) (Hesketh et al., 2020). There are no drugs tested in animal models of SCD that have shown a profile as promising as that of OCT2013 since assessment began in the 1960s, to our knowledge.

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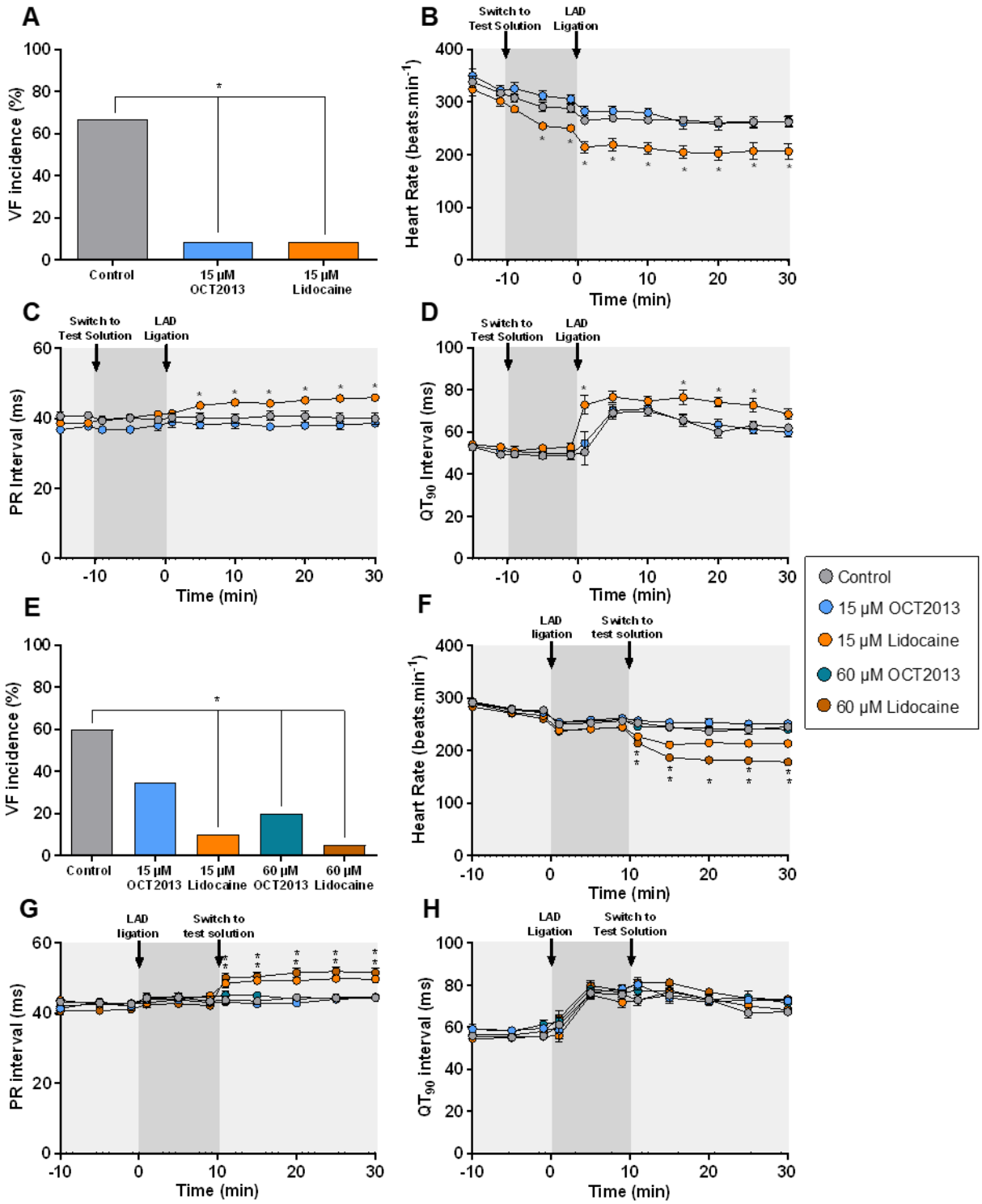

Fig. 1- Antiarrhythmic and ECG effects of OCT2013 vs lidocaine in rat Langendorff hearts.

(A,E) Incidence of VF, **(B,F)** heart rate, **(C,G)** PR and **(D,H)** QT_{90} intervals during 30 min of regional ischaemia in hearts perfused with Krebs, OCT2013 or lidocaine. **(A-D)** Perfusion with test solution started 10 min before coronary ligation (onset of ischaemia), n=12 hearts per group were required to compare drugs (15 µM each) versus control group. **(E-H)** Test solution perfusion commenced at 10 min after ligation. A higher concentration of each drug (60 µM) and increased group sizes to n=20 were implemented in the expectation of weaker drug effects with post-ligation administration. Binomially distributed variables (arrhythmia incidence) were compared using Fisher's exact test. Gaussian distributed variables (mean ± SEM) were subjected to 2 way ANOVA followed by Dunnett's post hoc tests (following demonstration that F was significant and data Gaussian). *$p<0.05$ versus Krebs' control.

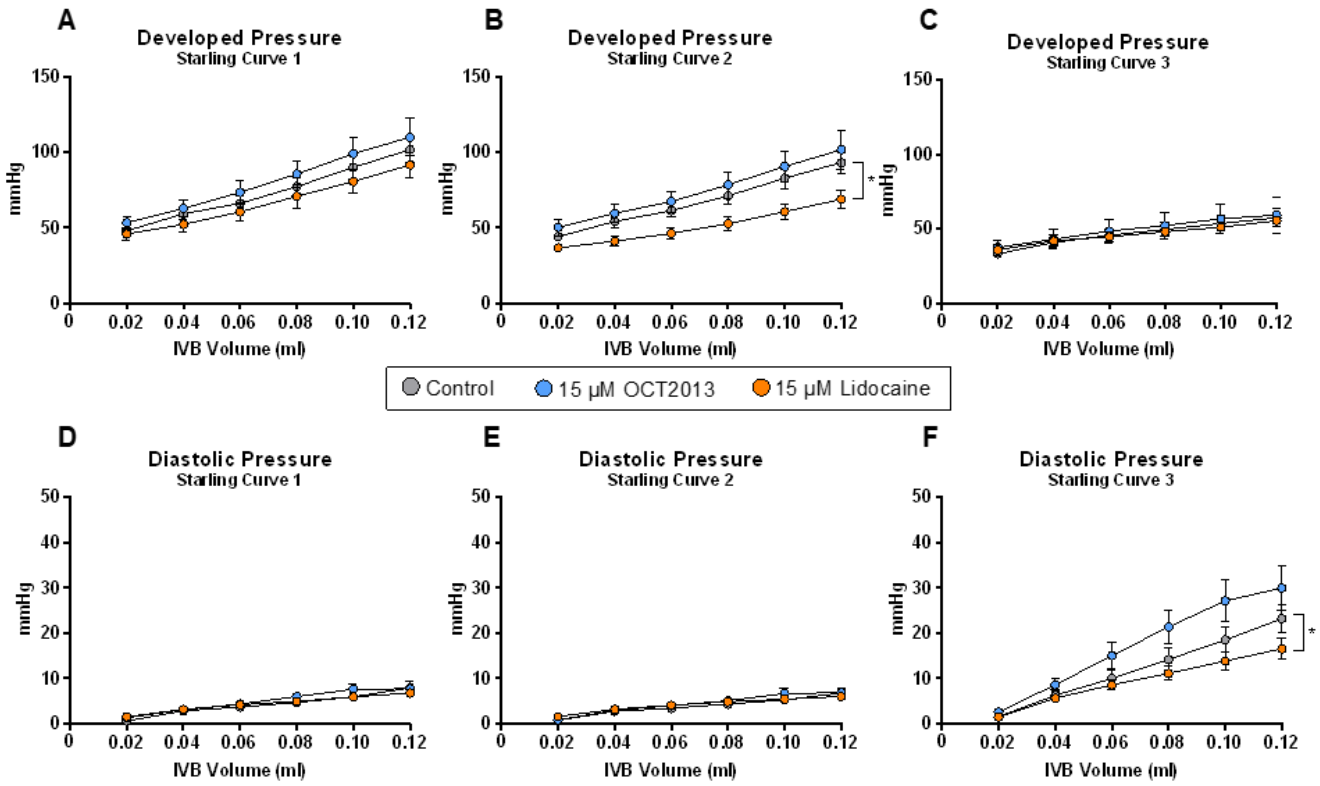

Fig. 2– Inotropic and lusitropic effects of OCT2013 in the rat Langendorff preparation assessed by IVB Starling curve analysis.

(A-C) Left ventricular developed pressure and **(D-F)** diastolic pressure at increasing IVB volumes in hearts perfused with Krebs prior to switch to test solution (Starling Curve 1), 10 min after switch to test solution comprised of Krebs, 15 µM OCT2013 or 15 µM lidocaine (Starling curve 2), and at the 60th min of reperfusion with the same test solution after 30 min regional ischaemia (Starling Curve 3). n=12 per group; mean ± SEM; *p<0.05 versus control (linear regression analysis).

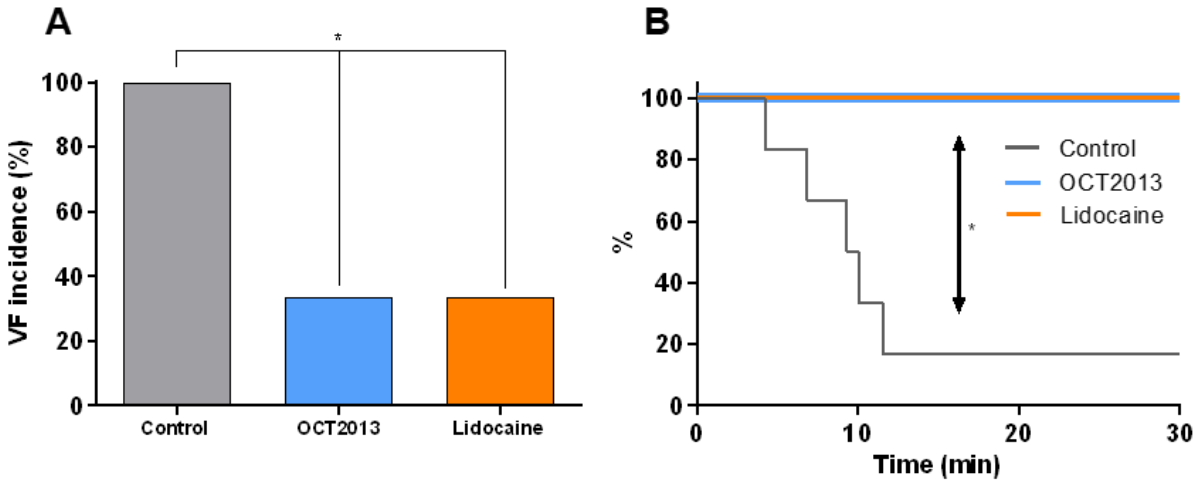

Fig. 3–Antiarrhythmic effectiveness and survival benefit of OCT2013 *in vivo*.

(A) Incidence of VF and **(B)** overall survival in anaesthetised rats administered 2 mg.kg^{-1} (+0.5 mg.kg^{-1}.min^{-1}) OCT2013, lidocaine, or saline (control). n=6 per group; Binomially distributed variables (arrhythmia incidence) were compared using Fisher's exact test. Survival was analysed using the log-rank (Mantel-Cox) test. *p<0.05 versus control.

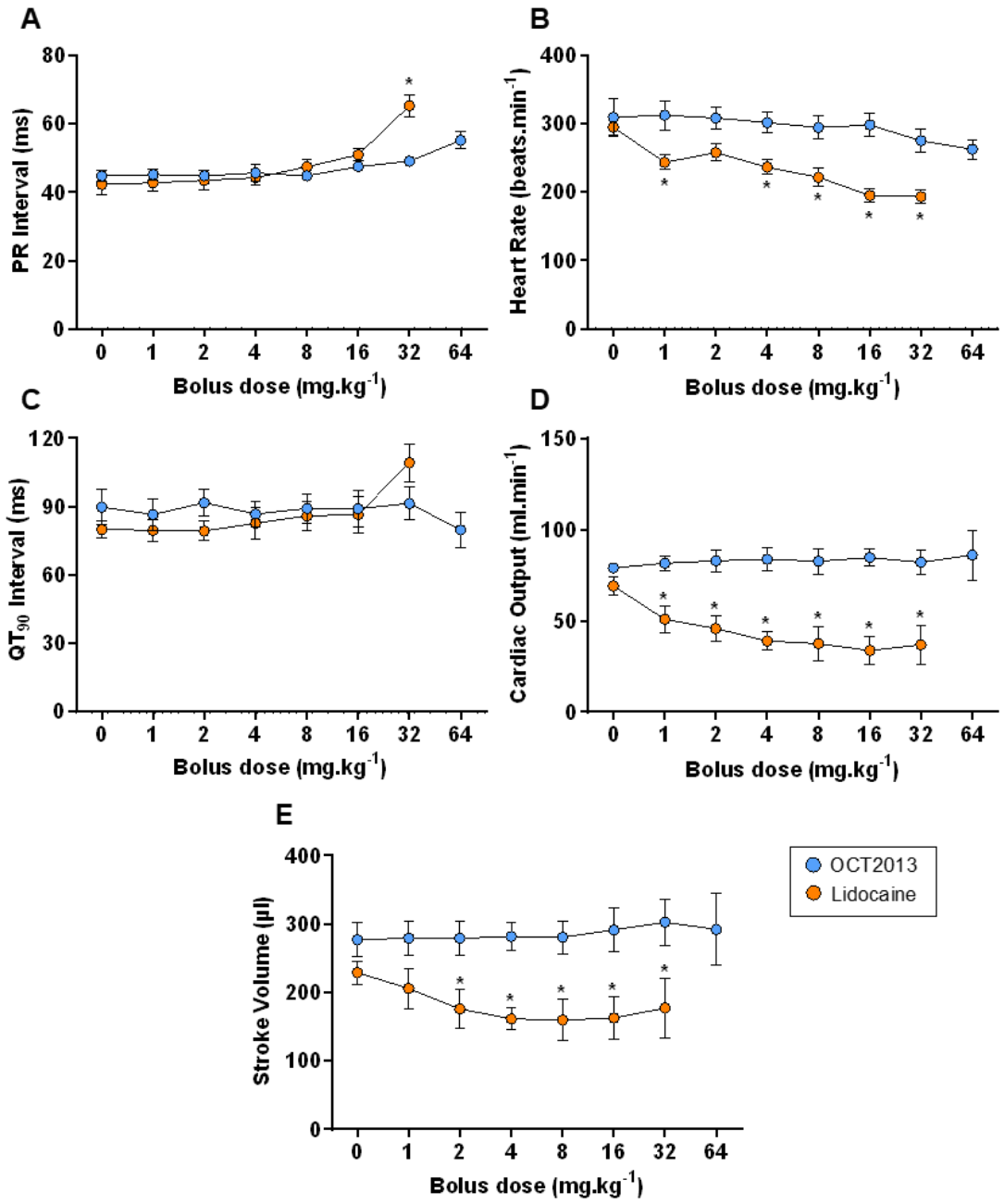

Fig. 4– Cumulative-dose toxicity of OCT2013 and lidocaine in anaesthetized rats.

(A) PR intervals, **(B)** heart rate, **(C)** QT_{90} intervals, **(D)** cardiac output and **(E)** stroke volume measured approximately 1 min 55 sec after sequential escalating drug boluses (1-64 $mg.kg^{-1}$) given at 2 min intervals. No animal survived the 64 $mg.kg^{-1}$ lidocaine dose long enough to permit data recording. n=5 per group; mean ± SEM; Variables were subjected to 2 way ANOVA followed by Sidak's post hoc tests (if F values were significant and data Gaussian). *$p<0.05$.

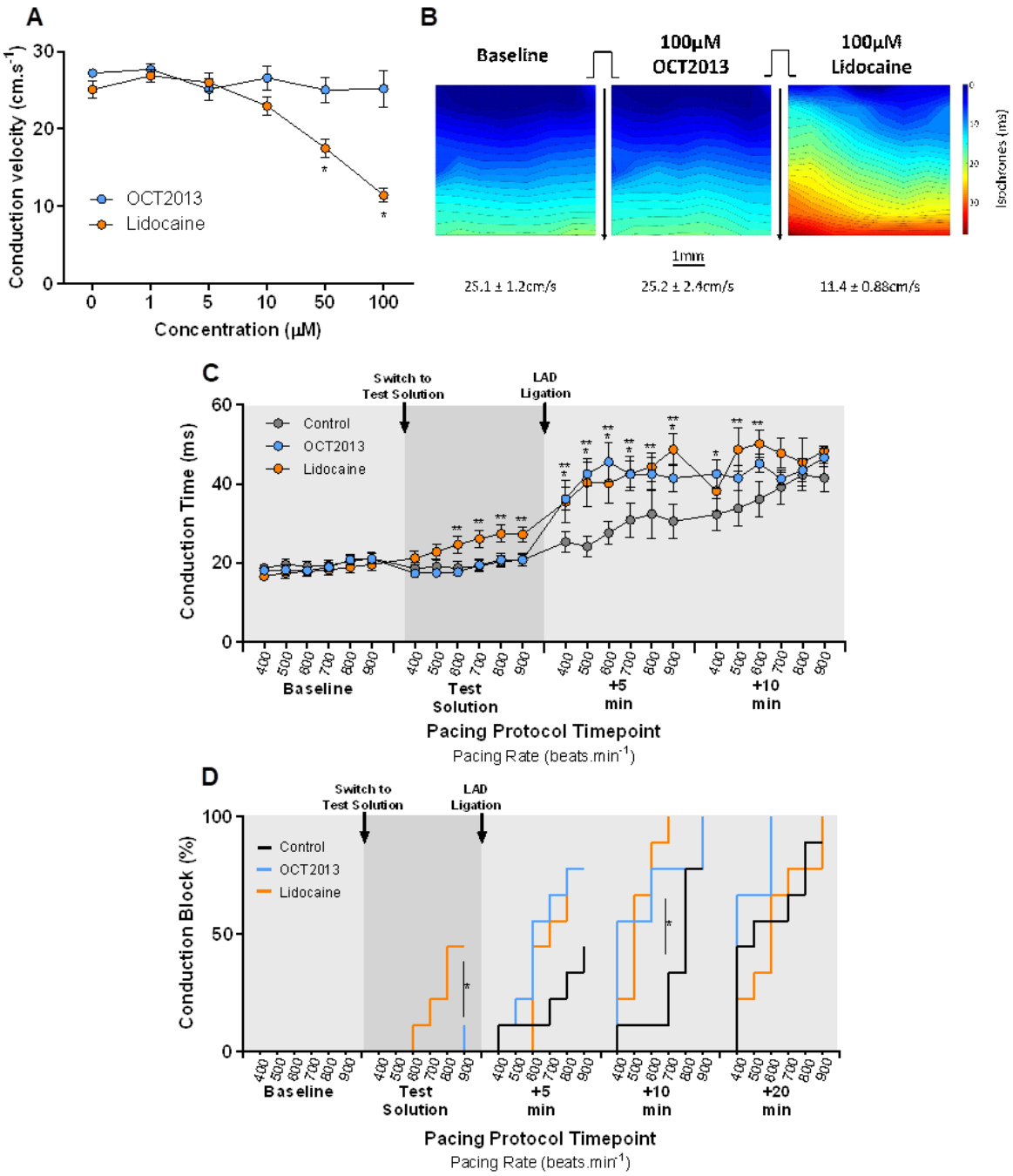

Fig. 5– Effects of OCT2013 and lidocaine on myocardial conduction in neonatal rat ventricular myocytes seeded onto MEAs and in whole Langendorff perfused rat hearts.

In MEAs, **(A)** effects of up to 100 μM OCT2013 or lidocaine (n=10/group) compared with baseline on conduction velocity ($cm.s^{-1}$) are shown together with **(B)** an example of the effect of 100 μM OCT2013 and 100 μM lidocaine. In paced (400-900 $beats.min^{-1}$) hearts the effects

15 µM OCT2013 or 15 µM lidocaine compared with Krebs' are shown on **(C)** conduction time and **(D)** occurrence of conduction block during different stages of regional ischaemia, n=9 per group. Data are **(A-C)** mean ± SEM or **(D)** survival percentage; Conduction velocity values were compared by 2 way ANOVA followed by Sidak's post hoc test (as F was significant and data Gaussian). Conduction time was evaluated by 2 way ANOVA followed by Dunnett's post hoc tests (if F was significant and data Gaussian). Incidence of conduction block was subjected to Kaplan-Meier survival analysis. *$p<0.05$.

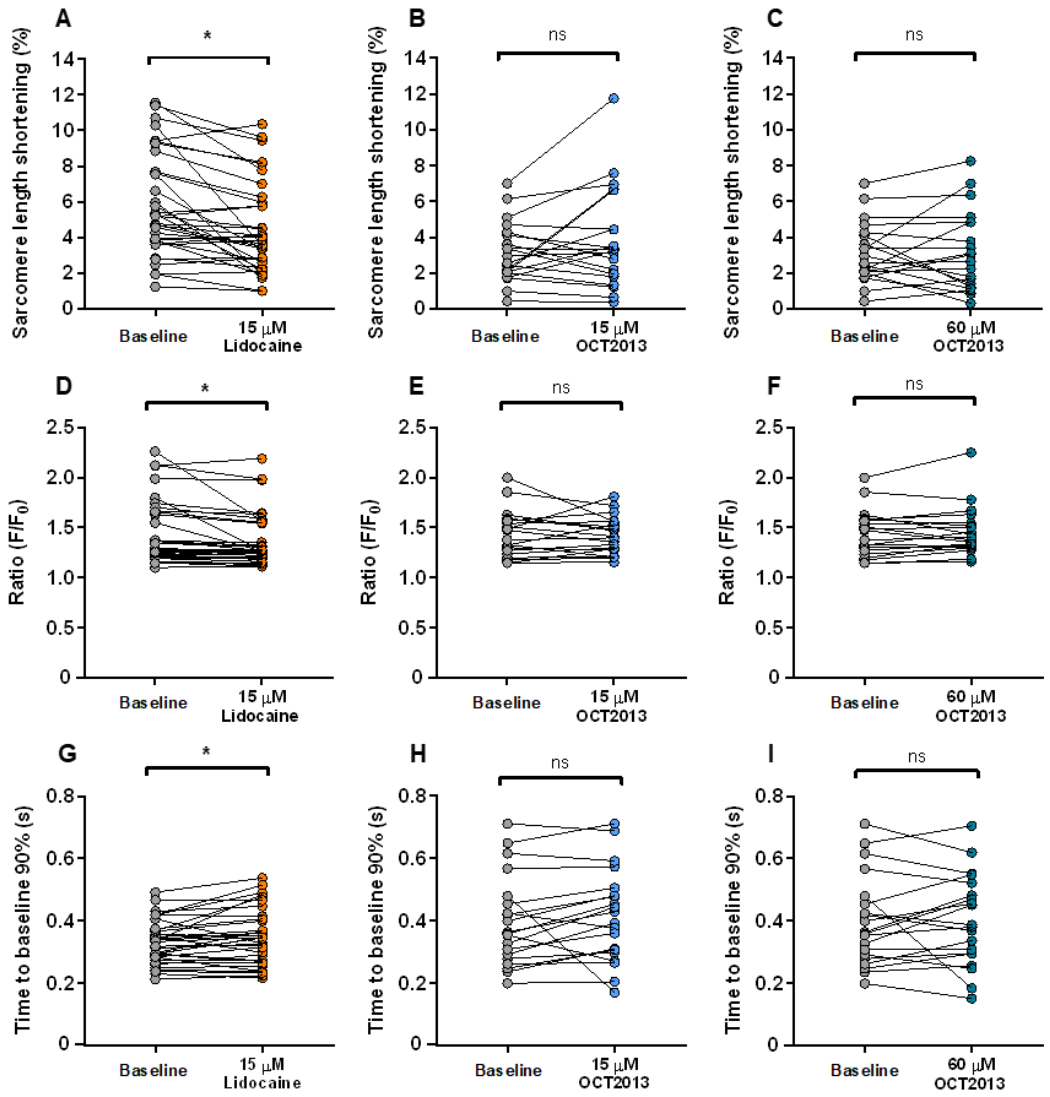

Fig. 6- Sarcomere shortening and intracellular Ca^{2+} dynamics in neonatal rat ventricular myocytes. Myocytes were loaded with the ratiometric Ca^{2+}-sensitive fluorescent dye Fura-2 AM (5 µM). Data are **(A-C)** sarcomere shortening, **(D-F)** Fura-2 AM fluorescence ratio (F/F0) and **(G-I)** time of fluorescence recovery to 90% of baseline. Measurements were taken 2 min after normal superfusion (baseline) and 2 min after the start of superfusion with test solution (15 µM lidocaine, 15 µM OCT2013 or 60 µM OCT2013). 20 single cell (15 µM OCT2013, or 60 µM OCT2013) or 35 single cell (15 µM lidocaine) recordings were made, with values averaged from 10 Ca^{2+} transients or 10 sarcomere shortenings; comparison with baseline (no treatment) was undertaken using paired t-tests. *$p<0.05$ versus baseline.

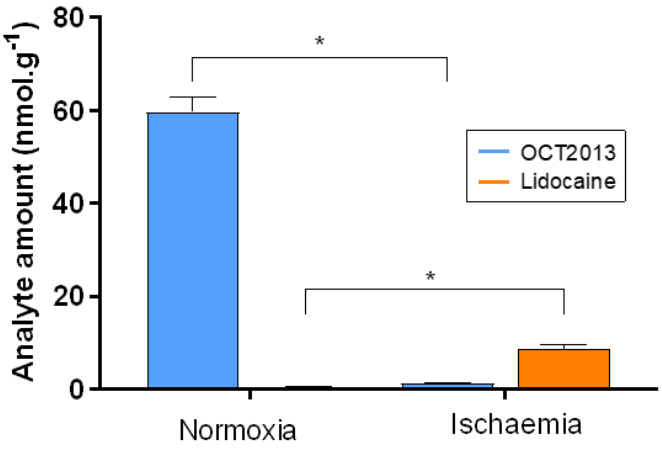

Fig. 7- The presence of OCT2013 and lidocaine in myocardial tissue.

OCT2013 and lidocaine were detected using UHPLC-MS/MS methodology in myocardial tissue samples collected from Langendorff-perfused isolated hearts, perfused with 15 µM OCT2013, after 30 min global ischaemia or time-matched normoxia. Variables (n=5 per group; mean ± SEM) were subjected to 1-way ANOVA followed by Dunnett's post hoc tests (as F was significant and data Gaussian). *$p<0.05$ versus normoxic control.