RESEARCH ARTICLE

A carvedilol analogue, VK-II-86, prevents hypokalaemia-induced ventricular arrhythmia through novel multi-channel effects

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Background and Purpose: QT prolongation and intracellular Ca2+ loading with diastolic Ca2+ release via ryanodine receptors (RyR2) are the predominant mechanisms underlying hypokalaemia-induced ventricular arrhythmia. We investigated the antiarrhythmic actions of two RyR2 inhibitors: dantrolene and VK-II-86, a carvedilol analogue lacking antagonist activity at β-adrenoceptors, in hypokalaemia.

Experimental Approach: Surface ECG and ventricular action potentials (APs) were recorded from whole-heart murine Langendorff preparations. Ventricular arrhythmia incidence was compared in hearts perfused with low [K+] and those pretreated with dantrolene or VK-II-86. Whole-cell patch clamping was used in murine and canine ventricular cardiomyocytes to study effects of dantrolene and VK-II-86 on AP parameters in low [K+] and effects of VK-II-86 on the inward rectifier current (IKr), late sodium current (INa_L) and the L-type Ca2+ current (ICa). Effects of VK-II-86 on IKr were investigated in transfected HEK-293 cells. A fluorogenic probe quantified the effects of VK-II-86 on oxidative stress in hypokalaemia.

Key Results: Dantrolene reduced the incidence of ventricular arrhythmias induced by low [K+] in explanted murine hearts by 94%, whereas VK-II-86 prevented all arrhythmias. VK-II-86 prevented hypokalaemia-induced AP prolongation and depolarization but did not alter AP parameters in normokalaemia. Hypokalaemia was associated with decreased IK1 and IKr, and increased INa_L and ICa. Effects of VK-II-86 on IKr were investigated in transfected HEK-293 cells. A fluorogenic probe quantified the effects of VK-II-86 on oxidative stress in hypokalaemia.

Conclusions and Implications: VK-II-86 prevents hypokalaemia-induced arrhythmogenesis by normalizing calcium homeostasis and repolarization reserve. VK-II-86 may provide an effective treatment in hypokalaemia and other arrhythmias caused by delayed repolarization or Ca2+ overload.

Abbreviations: AP, action potential; APD, action potential duration; BDVT, bidirectional ventricular tachycardia; CAMKII, Ca2+/calmodulin dependent protein kinase II; CPVT, catecholaminergic polymorphic ventricular tachycardia; DAD, delayed afterdepolarization; EAD, early afterdepolarization; ICa, L-type Ca2+ current; IK1, rapidly activating delayed rectifier K+ current; INa_L, late Na+ current; Ito, transient outward current; IH, Krebs–Henseleit solution; NSVT, non-sustained ventricular tachycardia; PMVT, polymorphic ventricular tachycardia; RMP, resting membrane potential; RV, right ventricular; SR, sarcoplasmic reticulum; TdP, torsade de pointes; VF, ventricular fibrillation; VT, ventricular tachycardia.

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1 | INTRODUCTION

Hypokalaemia is the most common electrolyte disturbance encountered clinically, with an estimated prevalence of 14% in outpatients and 20% in inpatients (Gennari, 1998; Kardalas et al., 2018). Hypokalaemia is an independent risk factor for mortality and can cause life-threatening ventricular arrhythmia. In a study of hospitalized patients with a serum potassium concentration \([K^+] < 3.0\) mEq/L, mortality was 20.4%, 10 times higher than in patients without hypokalaemia (Patel et al., 2001). A retrospective analysis of patients presenting with sustained ventricular tachycardia (VT) or ventricular fibrillation (VF) showed that hypokalaemia was present in 35.7% of these patients, with 13.6% presenting with a serum \([K^+] < 3.0\) mEq/L. Gastrointestinal illness and recent changes to diuretic therapy were significantly correlated with the risk of VT/VF, probably because these are frequent causes of hypokalaemia (Laslett et al., 2020).

Close monitoring of serum potassium can be logistically difficult and hypokalaemia is frequently mismanaged (Patel et al., 2001). Moreover, there is evidence to suggest that potassium supplements alone may be insufficient to reverse the increased mortality associated with hypokalaemia (Siscovick et al., 1994). In a retrospective observational study of heart failure patients, potassium supplements did not significantly alter mortality, but were associated with a significant increase in cardiovascular hospitalizations and heart failure progression (Ekundayo et al., 2010). There is therefore an unmet need for a safe treatment for the prevention of hypokalaemia-induced arrhythmia.

Hypokalaemia-induced arrhythmia has been attributed to reduction in key repolarizing potassium channel currents in the heart such as \(I_{Kr}\) (Fierek & Giles, 1995; Kileen et al., 2007) and \(I_{NaL}\) (Guo et al., 2009; Numaguchi et al., 2000; Sanguinetti & Jurkiewicz, 1992; Scamps & Carmellet, 1989). This results in slowing of action potential (AP) repolarization and the formation of early afterdepolarizations (EADs). These EADs are reflected on the surface ECG as closely coupled triggered beats in the context of a prolonged QTc interval.

However, the role of intracellular \(Ca^{2+}\) loading in the pathogenesis of hypokalaemia-induced arrhythmia (Tazmini et al., 2020) has been highlighted in recent studies. Hypokalaemia has been shown to inhibit Na\(^+\)/K\(^+\) ATPase (Aronsen et al., 2015). This causes the accumulation of intracellular Na\(^+\) ions, which inhibits the Na\(^+\)/Ca\(^{2+}\) exchanger (NCX) from extruding Ca\(^{2+}\) ions from cardiomyocytes. Excess intracellular Ca\(^{2+}\) ion concentration then activates Ca\(^{2+}\)/calmodulin dependent protein kinase II (CaMKII), an enzyme that facilitates Ca\(^{2+}\) loading of the sarcoplasmic reticulum (SR). SR overloading with Ca\(^{2+}\) allows diastolic release of Ca\(^{2+}\) back into the cytoplasm through ryanodine receptors (RyR2), causing EADs and delayed afterdepolarizations (DADs). Pezhouman et al demonstrated in rabbit and rat hearts, supplemented by in silico data, that the most important arrhythmogenic mechanism in hypokalaemia is activation of CaMKII, rather than reduced repolarization reserve (Pezhouman et al., 2015). CaMKII not only phosphorylates \(I_{Ca}\), phospholamban and RyR2, facilitating SR loading and diastolic Ca\(^{2+}\) release, but also increases late Na\(^+\) current \((I_{Na,L})\), thus further contributing to AP prolongation and intracellular Na\(^+\) accumulation. CaMKII thereby initiates a positive feedback loop of progressive intracellular Na\(^+\) and Ca\(^{2+}\) loading. Pezhouman et al demonstrated up-regulation of CaMKII in hypokalaemia and showed that inhibition of CaMKII, \(I_{Na,L}\), or \(I_{Ca}\) prevented triggered activity in hypokalaemic mouse and rabbit hearts, even in the presence of dofetilide, an \(I_{K}\) blocker used to reduce repolarization reserve (Pezhouman et al., 2015).
CaMKII is ubiquitously expressed in humans, making its inhibition problematic. There is no selective INa,L inhibitor approved for anti-arrhythmic use in humans and ICa blockers are often contraindicated due to negative ionotropy (Girouard et al., 2017). The aim of this study was to assess another drug target to ameliorate the Ca\(^{2+}\) handling problems contributing to arrhythmogenesis in the setting of hypokalaemia. The two candidates we have evaluated are inhibitors of RyR2, dantrolene and the carvedilol analogue VK-II-86. Dantrolene’s ability to prevent Ca\(^{2+}\)-mediated arrhythmias through RyR2 inhibition has been demonstrated in rabbits with heart failure (Maxwell et al., 2012) and in mice carrying catecholaminergic polymorphic ventricular tachycardia (CPVT) mutations (Suetomi et al., 2011; Uchinoumi et al., 2010). VK-II-86 has also been shown to be anti-arrhythmic in CPVT mice, (Zhou et al., 2011) and rats overdosed with digoxin (Gonano et al., 2018). VK-II-86 prevented Ca\(^{2+}\) waves in rats without affecting SR Ca\(^{2+}\) load or levels of phosphorylated or oxidized CaMKII, phospholamban or RyR2, suggesting direct inhibition of RyR2 (Gonano et al., 2018).

## 2 | METHODS

### 2.1 | Design of experiments

The efficacy of dantrolene and VK-II-86 in preventing ventricular tachyarrhythmia was evaluated in a murine whole-heart Langendorff model of hypokalaemia. Isolated murine and canine ventricular cardiomyocytes were then used in whole-cell patch clamp experiments to investigate the effect of the drugs on action potential (AP) morphology. As VK-II-86 proved the more effective antiarrhythmic, we evaluated the effects of VK-II-86 on ion channel currents involved in Ca\(^{2+}\) handling and repolarization, including I\(_{\text{Kg2.1}}\) (I\(_{\text{kq2.1}}\)), late sodium current (I\(_{\text{Na,L}}\)), L-type Ca\(^{2+}\) current (I\(_{\text{Ca}}\)) and transient outward current (I\(_{\text{to}}\)) at normal (4 mM) and low (3 mM) [K\(^{+}\)]. Canine and murine cardiomyocytes were studied because there is evidence to suggest that due to a short AP duration, EADs in mice may be caused by reactivation of non-equilibrium I\(_{\text{Na,L}}\) (Edwards et al., 2014) rather than Ca\(^{2+}\) overload and release through RyR2, as in larger species (Edwards et al., 2014; Tazmini et al., 2020). I\(_{\text{Ca}}\) was studied in canine myocytes only, I\(_{\text{to}}\) in murine myocytes only. The effect of VK-II-86 on I\(_{\text{Ca}}\) (K\(_{\text{v}}\) 11.1) in normal and low [K\(^{+}\)] was evaluated in HEK-293 (HEK) cells (RRID: IMSR_JAX:000664). This strain of wild-type mice was chosen due to its widespread use and availability within basic science research. Mice were housed in the institutional animal care facility, no more than 5 to a cage, with standard pellet food, water pouches, bedding, nest material and a cardboard tube for play or shelter. All mice used were assessed to be sub-threshold within the protocol severity assessment before being killed by cervical dislocation. Canine cardiomyocytes were isolated from adult mongrel dogs, of either sex, 8 to 12 months of age, 25–30 kg (Covance, Princeton, NJ, USA). Canines were housed with Covance and the hearts transported after death, in ice-cold cardioplegic solution to the study institution within 30 min.

### 2.2 | Animals

All animal care and experimental protocols conformed to the Guide for Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee. Experiments and animal care protocols. Animal studies are reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020) and with the recommendations made by the British Journal of Pharmacology (Lilley et al., 2020).

Data were obtained from murine whole-heart Langendorff preparations and single cells isolated from adult wild-type C57BL6/J mice, of either sex, 3 to 5 months of age (Jackson Laboratories, Bar Harbor, ME, USA; RRID:IMSR_JAX:000664). This strain of wild-type mice was chosen due to its widespread use and availability within basic science research. Mice were housed in the institutional animal care facility, no more than 5 to a cage, with standard pellet food, water pouches, bedding, nest material and a cardboard tube for play or shelter. All mice used were assessed to be sub-threshold within the protocol severity assessment before being killed by cervical dislocation. Canine cardiomyocytes were isolated from adult mongrel dogs, of either sex, 8 to 12 months of age, 25–30 kg (Covance, Princeton, NJ, USA). Canines were housed with Covance and the hearts transported after death, in ice-cold cardioplegic solution to the study institution within 30 min.

### 2.3 | Synthesis of VK-II-86, a carvedilol analogue

VK-II-86 was synthesized from commercially available precursors using the methods described in previous studies (Wu et al., 2009; Xu et al., 2018). The exact methodology we used is described here with reference to Scheme 1:

\[
\text{m-CPBA} \quad (5.17 \text{ g}, \quad 0.03 \text{ mol}) \quad \text{was added portion-wise to} \quad 4\text{-bromobut-1-ene} \quad (1) \quad (2 \text{ ml}, \quad 0.02 \text{ mol}) \quad \text{in} \quad \text{CH}_2\text{Cl}_2 \quad (20 \text{ ml}) \quad \text{at} \quad 0^\circ \text{C}. \quad \text{The}
\]

**SCHEME 1** Synthetic pathway of VK-II-86. (1): 4-bromobutane, (2): 2-(2-bromoethyl) oxirane, (3): 4-hydroxycarbazole, (4): 4-(2-oxiran-2-yl) ethoxy-ethoxy-carbazole, (5): 2-(2-methoxyphenoxo) ethylamine.
reaction was stirred at room temperature for 24 h. The reaction mixture was neutralized with 0.1 N sodium hydroxide, extracted with CH₂Cl₂ and dried over Na₂SO₄. The solvents were removed to give 2-(2-bromoethyl)oxirane (2) as a colourless oil in 90% yield, which was used without further purification in the next step (Wu et al., 2009).

A solution of 2-(2-bromoethyl)oxirane (2) (0.27 ml, 2.9 mmol) in aqueous NaOH (2.42 mmol, 1 m) was added to 4-hydroxy carbazole (3) (445.29 mg, 2.42 mmol) in DMSO. The reaction mixture was stirred for 24 h at 60°C, cooled to 20°C and diluted with 25 ml of water. The reaction mixture was extracted with ethyl acetate (3 x 20 ml), and the collected organic phase was washed with brine, dried (Na₂SO₄) and concentrated. Purification by silica-gel flash chromatography eluting with ethyl acetate-hexane gave 4-(2-(oxiran-2-yl)ethoxy-ethyl)oxycarbazole (4) (60%) (Zhou et al., 2011). In the last step, 4-(2-(oxiran-2-yl)ethoxy-ethyl)oxycarbazole (4) (107.15 mg, 0.42 mmol) was reacted with 2-(2-methoxynaphthoxy) ethylamine (5) (83.6 mg, 0.50 mmol) in ethanol and refluxed for 24 h (Xu et al., 2018). The reaction mixture was cooled to room temperature, filtered through Celite and concentrated prior to purification by flash chromatography on silica-gel (10% isopropyl alcohol in dichloromethane) to give VK-II-86 (132.3 mg, 75%). VK-II-86 as the HCl salt was produced by dissolving the free base VK-II-86 in acetonitrile and acidification with 1 M HCl. The aqueous phase was freeze-dried (Na₂SO₄) and concentrated. Purification by silica-gel flash chromatography eluting with ethyl acetate-hexane gave 4-(2-(oxiran-2-yl)ethoxy-ethyloxycarbazole (4) (60%) (Zhou et al., 2011).

2.4 | Solutions

The concentrations for all solutions listed are shown in mM. Krebs–Henseleit (KH) solution: 118 NaCl, 24 NaHCO₃, 10 D-glucose, 1.2 NaH₂PO₄, 2 Na pyruvate, 2 CaCl₂, 4 KCl and 1.2 MgSO₄, pH to 7.4. The cardioplegic solution was a modified Krebs–Henseleit solution with KCl = 16 mM. The isolation solution contained 134 NaCl, 10 HEPES, 11 D-glucose, 4 KCl, 1.2 MgSO₄ and 1.2 NaH₂PO₄, pH to 7.4. The taurine buffer solution contained: 140 NaCl, 10 HEPES, 11 D-glucose, 50 taurine, 1 CaCl₂, 1.2 MgCl₂ and 4 KCl, pH to 7.4. The internal solution for single cell AP and I_K1 recordings contained: 140 KCl, 10 HEPES, 5 Na₂-ATP, 2 MgCl₂, 1 EGTA, pH to 7.2. The internal solution for I_Na_L recordings contained: 15 NaCl, 120 CsF, 4 Na₂-ATP, 10 HEPES, 10 EGTA, 1 MgCl₂, 5 KCl, pH to 7.4. Internal solution for LTCC recordings contained: 120 CsCl, 1 MgCl₂, 10 EGTA, 5 Mg-ATP, 10 HEPES, ScCaCl₂, pH to 7.2 with CsOH. Internal solution for I_K1 recordings contained: 140 NaCl, 10 HEPES, 10 D-glucose, 4 KCl, 1 MgCl₂ and 1.8 CaCl₂, pH to 7.4. External solution for I_K1 recordings contained: 140 NaCl, 10 HEPES, 10 D-glucose, 4 KCl, 1 MgCl₂, 1.8 CaCl₂ and 1 CoCl₂. External solution for I_Na_L contained: 127.8 NaCl, 5 NaOH, 10 HEPES, 0.5 CaCl₂, 1.5 MgCl₂, 10 D-glucose, 5 TEA, 2 4-AP, 0.5 BaCl₂, 1 CoCl₂, pH to 7.4. External solution for LTCC recording contained: 140 NaCl, 10 HEPES, 10 D-glucose, 4 KCl, 1 MgCl₂, 2 CaCl₂, 2 4-AP and 0.1 BaCl₂. The KC concentration [K⁺] was altered from 4 mM (normal) to 2 mM (low) in the Krebs–Henseleit (KH) solution used in the whole-heart Langendorff experiments to mimic hypokalaemia. The 2 mM [K⁺] was chosen as the concentration to replicate hypokalaemia because the normal range of [K⁺] in C57BL/6 mice is 3.5–4.4 mM (Otto et al., 2016) and 2 mM [K⁺] induces arrhythmia reliably and quickly in Langendorff-perfused mouse hearts (Killeen et al., 2007). Speed of induction is important to eliminate any confounding effects of ischaemia-induced arrhythmia.

The [K⁺] used in external patch clamp solutions was modified from 4 mM in control experiments to 3 mM to model hypokalaemia. We avoided using 2 mM [K⁺] in isolated cardiomyocytes because this level of hypokalaemia resulted in a high level of contracture and cell death.

2.5 | Murine Langendorff-perfused whole heart model of hypokalaemia

Mice were anticoagulated by i.p. injection with 300 I.U. kg⁻¹ of unfractionated heparin sodium, 1 min before killing by cervical dislocation. The heart was excised and placed in cardioplegic solution. The aorta was then cannulated with a custom-made cannula and attached to the Langendorff perfusion system. Hearts were retrogradely perfused at 2 ml min⁻¹ with oxygenated KH solution, maintained at 37°C. Perfusion pressure was maintained at 60–70 mm-Hg during experiments.

Pseudo-ECGs were recorded from the isolated mouse hearts using silver chloride (Ag/AgCl) electrodes placed 0.5 cm from the heart surface, connected to an extracellular amplifier (World Precision Instruments, Sarasota, FL, USA). Micromanipulators were used to place floating microelectrodes sub-epicardially to simultaneously record APs. Floating microelectrodes were made from glass micropipettes pulled and filled with 2.7 M KCl internal solution (resistance = 20–40 MΩ) and attached via Teflon-coated Ag/AgCl electrodes to “Ultra Quiet Intracellular Amplifiers” (World Precision
Instruments). An analogue to digital converter (CED 1401-3) was used to record the electrophysiological signals, which were analysed using Spike 2 for Windows software (Cambridge Electronic Design, Cambridge, UK).

After a 30-min equilibration period, the concentration of KCl in the KH solution was maintained at 4 mM or reduced to 2 mM to mimic hypokalaemia. After a further 15 min of perfusion, accounting for the dead space in the tubing, a 30-min period of simultaneous pseudo-ECG and AP recording was commenced. Dantrolene (1 μM-Ryanodex®) or VK-II-86 (1 μM) was added to the KH perfusate at the start of the 30-min equilibration period or omitted in control experiments. Arrhythmia burden was calculated by recording the number of episodes of non-sustained (NSVT) and sustained ventricular tachycardia (VT) within a 30-min perfusion period. NSVT was defined as a series lasting ≥3 ventricular ectopic beats, > 800 bpm, lasting for less than 30 s. A series lasting ≥30 s was considered a sustained ventricular tachycardia.

2.6 | Single cell isolation of murine and canine cardiomyocytes

Mouse hearts were attached to a Langendorff perfusion system as described above. Canines were anaesthetised with isoflurane; their hearts were excised following retrograde aortic flush of heparin (1000 U L⁻¹) and stored briefly in ice-cold cardioplegic solution. A right ventricular (RV) wedge was excised from the canine heart and an acute marginal branch of the right coronary artery cannulated. The RV wedge was then attached to the Langendorff perfusion system. The murine heart or canine ventricular wedge were perfused at 37°C with isolation solution for 5 min at 5 ml min⁻¹ for the murine heart and 15 min at 10 ml min⁻¹ for the canine wedge. Collagenase (Type 2: 0.6 mg ml⁻¹; Worthington, Lakewood, NJ, USA) and protease (0.075 mg ml⁻¹, type XIV, Sigma) were added to the perfusate and the tissue digested for 3–4 min in the murine and 25–35 min in the canine preparations, respectively. The heart was then perfused with a tauinine buffer solution for 10 min in the mouse preparation and 20 min for the canine preparation. The murine heart tissue was dismounted from the cannula, the atria discarded, and the ventricles minced and filtered through 200-μm gauze to collect intact cardiomyocytes. In the case of the RV canine wedge, thin slices of tissue were cut from the epicardial surface, minced, and filtered through 200-μm gauze. Myocytes were stored at room temperature in a modified Tyrode's solution containing 1 mM Ca²⁺.

2.7 | HEK-293 cell transfection

HEK-293 cells (Sigma) were maintained in EMEM media (ATCC, Manassas, VA USA) with 10% fetal bovine serum and penicillin-streptomycin (Sigma) at 37°C with 5% CO₂ in air. HEK-293 cells were transfected with KCNH2 tagged with eGFP in pcDNA 3.1 using FuGENE HD (Active Motif, Carlsbad, CA, USA) and studied 48 h later.

2.8 | Whole-cell patch clamp measurements of action potentials, I_{K1}, I_{Na_L}, and I_{Ca} in dissociated canine and murine ventricular myocytes and I_{Kur} in HEK-293 cells

Cells were placed in a chamber containing the relevant external solution on the stage of an inverted microscope (Diaphot, Nikon Instruments, Melville, NY, USA) and maintained at 37°C, except for experiments measuring I_{Na_L} and I_{Kur}, which were performed at room temperature. APs were recorded in current clamp mode using an Axopatch 200B amplifier (Molecular Devices, San Jose, CA, USA) and micropipettes fabricated from borosilicate glass (World Precision Instruments). Pipette resistance was 2.0–3.0 MΩ when filled with internal solution. Cells were stimulated with a 2 ms square pulse (4–6 pA) at a frequency of 0.5 Hz. Recordings of I_{K1}, I_{Na_L}, and I_{Ca} were made using the whole-cell configuration in voltage clamp mode. Voltage clamp protocols (generated using pCLAMP v10.7 software, RRID:SCR_011323) used in individual experiments are detailed in their associated figures within the paper. Signals were acquired at 10 kHz with pCLAMP v10.7 software for offline analysis using Clampfit v10.7.

2.9 | Fluorescence microscopy of dissociated murine cardiomyocytes loaded with a fluorogenic oxidative stress reagent

Isolated murine cardiomyocytes were plated on 35-mm glass bottomed dishes (MatTek, the coat the night before with Matrigel (Corning, Corning, NY, USA). Cells were incubated for 30 min at 37°C. Plates were then washed with PBS to remove any dead cells that had not adhered to the Matrigel. 2 mL of same external solution used for AP and I_{Kur} patch-clamping experiments (4 mM [K⁺]) was added to the dishes along with 5 μM CellROX™ green (Invitrogen, Paisley, UK). Dishes were then incubated for 30 min at 37°C. Cells were then washed with PBS and the fresh external solution applied with varying concentrations of [K⁺], with or without 1 μM VK-II-86 or 50 μM H₂O₂. Dishes were therefore divided into the following groups: 4 mM [K⁺], 4 mM [K⁺] & 50 μM H₂O₂ as a positive control, 3 mM [K⁺], 3 mM [K⁺] & 1 μM VK-II-86. After 30 min of incubation at 37°C in their various conditions, dishes were imaged on a fluorescent microscope (Cairn, Faversham, UK) in brightfield mode and with a wavelength of 470 nm. Total fluorescence of viable (rod-shaped) cells was analysed using Image J (NIH, RRID:SCR_003070) and compared between dishes/groups.

2.10 | Study design and statistical analysis

Randomization by means of a random number generator producing an odd or even number was used to determine which mice were exposed to the various groups used for whole-heart Langendorff experiments.
Enzymically isolated cells were exposed to all independent variables for each experiment, therefore randomization was not necessary for single cell experiments. As the experiments were performed and analysed as part of the learning process for the first author’s PhD, the study was not blinded.

There was a relative shortage of canine hearts available for this study because cardiomyocytes were isolated from spare ventricular wedges from hearts primarily being used for another NIH funded study, and the institution was experiencing a general shortage in supply. Canine patch-clamp experiments therefore analysed data from cardiac myocytes isolated from less than five dogs, with cardiac myocytes treated as independent units (n). Although not statistically tested, these data from canine hearts are still thought to be useful due to the distribution of the data and the similarity of results to the comparative murine cardiomyocytes, which were isolated from at least five hearts. The one exception to this were the experiments measuring \( I_{\text{Na,L}} \), since these experiments are technically very demanding, requiring seal formation with virtually no leak for accurate \( I_{\text{Na,L}} \) measurement, and this was only achieved in three murine and two canine hearts, respectively. The technical difficulties of patch clamping enzymically isolated cardiomyocytes, especially those exposed to low potassium meant that numbers of cells were often unequal between comparative groups. The lack of preceding data on RyR2 inhibitors in hypokalaemia-induced arrhythmia in mice prevented prior power calculations. However, the authors aimed for a minimum \( n \) of five for each comparison group. Otherwise, the data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2018).

Statistical comparison of groups for AP duration (Figure 3c) was performed by calculating the log_{10} value of the AP duration for individual cells and performing a two-way ANOVA. Logarithms were calculated prior to performing the two-way ANOVA to satisfy the requirements of ANOVA due to the spread of the data within these groups. Statistical comparison of groups for the inward component of \( I_{\text{K1}} \) (Figure 5) was performed by calculating the gradient of the straight line between voltages –140 to –90 mV, and the groups and paired so as to test one independent variable at a time: 3 mM \([K^+]\) compared with 4 mM \([K^+]\); 3 mM \([K^+]\) compared with 3 mM \([K^+]\) & VK-II-86; 4 mM \([K^+]\) compared with 4 mM \([K^+]\) and VK-II-86. The one-way ANOVA or the Kruskal–Wallis one-way analysis of variance on ranks was applied depending on whether groups were normally distributed. For the outward component of \( I_{\text{Ks}} \), the mean voltage between –90 and –40 mV was calculated for each cell and the relevant groups similarly compared using the one-way ANOVA or the Kruskal–Wallis one-way analysis of variance on ranks. The peak density of \( I_{\text{Ks}} \) at the physiologically relevant voltage of 10 mV (Figure 8) was compared between groups using the two-way ANOVA. The level of oxidative stress was compared between groups of myocytes using the one-way ANOVA (Figure 9). The mean peak density of \( I_{\text{nK}} \) was compared between groups at the physiologically relevant voltage of 40 mV using a t-test to compare 4 mM\([K^+]\) with 3 mM \([K^+]\). Exponential curves were fitted to the \( I_{\text{nK}} \) traces from each cell and \( I_{\text{nK}} \) compared between groups using the Mann–Whitney U rank sum test (Figure S1). A \( P < 0.05 \) value was considered statistically significant. Where ANOVA tests were used, post hoc group comparisons were performed only when tests for normality and equal variance were passed. Data are expressed as mean ± SEM. Statistical analysis was performed using SigmaPlot 13.0 (Systat Software, RRID: SCR_003210).

2.11 Materials

VK-II-86 was prepared as described above. Dantrolene (Ryanodex®) was supplied by Eagle Pharmaceuticals (Woodcliff Lake, NJ, USA) and ranolazine was supplied by Sigma.

2.12 Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, and are permanently archived in the Concise Guide to PHARMACOLOGY 2021/22 (Alexander, Christopoulos, et al., 2021; Alexander, Fabbro, et al., 2021; Alexander, Kelly, et al., 2021; Alexander, Mathie, et al., 2021).

3 RESULTS

3.1 Langendorff-perfused murine hearts replicate hypokalaemia-induced arrhythmia

Under control conditions, all murine hearts perfused with 4 mM \([K^+]\) remained in sinus rhythm during the 30-min recording period (\( n = 7 \)) (Figure 1a). When perfused with 2 mM \([K^+]\), all hearts consistently exhibited early afterdepolarization (EAD)-induced triggered beats and ventricular tachyarrhythmias (\( n = 7 \)). Figure 1b shows an example of closely-coupled extrasystoles triggering an episode of polymorphic ventricular tachycardia (PMVT). Simultaneous AP and pseudo-ECG recordings confirmed that these were induced by EAD-mediated triggered activity (Figure 1b). A wide range of non-sustained and sustained ventricular tachyarrhythmias, typically found in hypokalaemic patients were produced, including polymorphic VT (PMVT), torsade de pointes (Tdp) (Figure 1c), monomorphic VT and VF. Bidirectional ventricular tachycardia (BDVT) (Figure 1d), an arrhythmia classically associated with conditions of \( Ca^{2+} \) overload: digoxin toxicity, CPVT and Andersen Tawil syndrome, also developed. The mean incidence of the different types of non-sustained and sustained ventricular tachyarrhythmias produced in the seven hearts perfused with 2 mM \([K^+]\) are shown in Figure 1e, f. PMVT was the most common sustained arrhythmia, but there was no predilection for a particular non-sustained ventricular arrhythmia.
FIGURE 1 Hypokalaemia-induced arrhythmia in a murine whole-heart Langendorff preparation. Panels (a) and (b) show simultaneously recorded subepicardial transmembrane action potentials (AP) and pseudo ECGs. Panels (c) and (d) show only the pseudo-ECG trace. (a) Sinus rhythm recorded after 30-min perfusion with Krebs Henseleit (KH) containing 4 mM $[K^+]$. (b) Recorded 30 min after perfusion with KH containing 2 mM $[K^+]$. Closely-coupled extrasystoles most likely due to early afterdepolarisation (EAD)-induced triggered activity are shown to trigger polymorphic VT (PMVT). (c) Torsade de pointes (TdP) developing in the presence of 2 mM $[K^+]$. (d) Bidirectional VT (BDVT) followed by monomorphic VT (Mono VT) developing in the presence of 2 mM $[K^+]$. Mean number of non-sustained (e) and sustained (f) arrhythmias by type recorded during 30 min of exposure to 2 mM $[K^+]$ ($n = 7$ hearts were perfused with 4 mM $[K^+]$ and $n = 7$ hearts were perfused with 2 mM $[K^+]$ KH solution). Data shown are individual values with means indicated.
3.2 | VK-II-86 is more effective than dantrolene at preventing hypokalaemia-induced ventricular arrhythmia

Dantrolene and VK-II-86 markedly reduced the number of non-sustained and sustained ventricular tachyarrhythmias in the hypokalaemic murine whole-heart model (Figure 2d,e). Ventricular tachyarrhythmias occurred in 100% of hearts perfused with 2 mM [K⁺] alone, in 71% of hearts pretreated with 1 μM dantrolene and in 0% of hearts pretreated with 1 μM VK-II-86. Figure 2a illustrates a representative example of frequent episodes of monomorphic VT, induced by EAD-mediated triggered activity in hearts perfused with 2 mM [K⁺]. By contrast, hearts perfused with 1 μM dantrolene, 30 min prior to exposure to 2 mM [K⁺], displayed EAD and delayed afterdepolarization (DAD)-induced triggered beats (Figure 2b), but the incidence of non-sustained and sustained arrhythmias was reduced by 94% (Table 1; Figure 2d,e). Pretreatment with 1 μM VK-II-86 prevented all afterdepolarizations (Figure 2c), and arrhythmia (Table 1; Figure 2d,e). VK-II-86 or dantrolene did not terminate hypokalaemia-induced arrhythmias when applied acutely to isolated hearts, post-arrhythmia development (data not shown).

3.3 | In murine and canine ventricular cardiomyocytes, VK-II-86 normalizes AP parameters and is more effective than dantrolene in preventing the development of afterdepolarizations under hypokalaemic conditions

Murine ventricular cardiomyocytes bathed in 4 mM [K⁺] external solution for 30 min prior to AP recording did not exhibit EADs or DADs (Figure 3a, left panel). However, a 30-min preincubation in external solution containing 3 mM [K⁺] resulted in marked prolongation of the AP and frequent EADs (Figure 3a, right panel). Addition of 1 μM VK-II-86 to the 3 mM [K⁺] external solution during the preincubation period prevented the development of AP prolongation and EADs/DADs (Figure 3b, left panel). However, addition of 1 μM dantrolene did not prevent the AP prolongation (Figure 3b, right panel).

Upon changing from 4 to 3 mM [K⁺], APD₉₀, APD₃₀ and APD₉₀ were significantly prolonged in murine cardiomyocytes and the resting membrane potential (RMP) also significantly depolarized (Figure 3c). Addition of 1 μM VK-II-86 to the 3 mM [K⁺] during the 30-min preincubation normalized all AP parameters and hyperpolarized the RMP (Figure 3c).

The same experiments performed in canine ventricular cardiomyocytes produced similar results: VK-II-86, but not dantrolene prevented AP prolongation and development of EADs/DADs in 3 mM [K⁺] (Figure 4a,b). As shown in Figure 4c, in 3 mM [K⁺], the APD₉₀ lengthened and the RMP depolarized. Once again, addition of 1 μM VK-II-86 to the 3 mM [K⁺] solution for 30 min normalized the APD₉₀ and RMP in canine cardiomyocytes (Figure 4c). When canine and murine ventricular cardiomyocytes were held in 4 mM [K⁺], 1 μM VK-II-86 did not alter the RMP or AP duration.

3.4 | In both murine and canine ventricular cardiomyocytes, 1 μM VK-II-86 prevents the low-[K⁺]-induced changes in both the inward and outward components of Iₖ₁ (Iₖir₂.1)

Canine and murine cardiomyocytes pre-incubated in 3 mM [K⁺] demonstrated a marked reduction in Iₖ₁ (Figure 5a), which was prevented by the addition of 1 μM VK-II-86 to the external solution (Figure 5b). The I-V curves (Figure 5c) recorded in murine cardiomyocytes showed that both the inward and outward components of Iₖ₁, responsible for maintaining the RMP and the APD₉₀ respectively, were significantly reduced in 3 mM [K⁺]. VK-II-86 (1 μM) prevented the hypokalaemia-induced decrease of Iₖ₁ density (Figure 5c). The inset in Figure 5c shows a magnification of the portion of the I-V curves for Iₖ₁ positive to the equilibrium potential (Eₖ), that is, the outward component of Iₖ₁ during rectification. The mean outward current between ~80 and ~40 mV was markedly reduced in 3 mM [K⁺], and was increased by the addition of VK-II-86 to the solution. Neither the inward nor outward components of Iₖ₁ were similarly altered by the addition of VK-II-86 to cells exposed to 4 mM [K⁺] (Figure 5c). In canine cardiomyocytes, the inward and outward components of Iₖ₁ were similarly reduced in 3 mM [K⁺] and restored by the addition of VK-II-86 (Figure 5d). Once again, the addition of VK-II-86 to 4 mM [K⁺] external solution did not alter Iₖ₁ density in canine cardiomyocytes. (Figure 5d)

3.5 | In both murine and canine ventricular cardiomyocytes, hypokalaemic conditions increase the late sodium current (Iₙₐ,L) and VK-II-86 prevents this increase

The upper panel of Figure 6 shows superimposed whole-cell voltage clamp traces of the late sodium current (Iₙₐ,L) recorded from murine cells pre-incubated for 30 min in either 4 mM [K⁺], 3 mM [K⁺] or 3 mM [K⁺] plus 1 μM VK-II-86. The Iₙₐ,L was larger in the 3 mM [K⁺] solution, and this increase was prevented by the addition of VK-II-86. Similar changes occurred in canine cardiomyocytes. Figure 6 (bottom panel) shows composite data from both murine and canine cells. In murine cardiomyocytes (Figure 6, bottom left), the mean Iₙₐ,L density increased more than four-fold on changing from 4 mM [K⁺] to 3 mM [K⁺]. This increase was prevented by the addition of 1 μM VK-II-86, yielding a mean Iₙₐ,L density similar to the Iₙₐ,L density measured following addition of 10 μM ranolazine to the 3 mM [K⁺] solution. In canine cells, (Figure 6, bottom right) Iₙₐ,L increased by five-fold 500% on changing from 4 mM [K⁺] to 3 mM [K⁺]. Addition of 1 μM VK-II-86 to the 3 mM [K⁺] solution reversed these increases in mean Iₙₐ,L density. There was no difference in Iₙₐ,L density between cells pre-incubated in 4 mM [K⁺] compared with that in cells pre-incubated in 4 mM [K⁺] plus VK-II-86 in either murine or canine cells (Figure 6, bottom panels).
FIGURE 2 VK-II-86 is more effective than dantrolene at preventing hypokalaemia-induced ventricular arrhythmia. Each panel shows a subepicardial transmembrane action potential (AP) and a pseudo-ECG simultaneously recorded after 30 min of perfusion with Krebs–Henseleit (KH) solution containing 2 mM [K$^+$] in the absence and presence of dantrolene (DAN) or VK-II-86. (a) Extrasystolic activity likely arising from EAD-induced triggered activity and runs of VT in 2 mM [K$^+$] ($n=7$). (b) Recorded 30 min after perfusion with KH containing 2 mM [K$^+$] + 1 μM dantrolene ($n=6$). Extrasystolic activity probably arising from EAD and delayed afterdepolarization (DAD)-triggered activity persist, but no runs of VT were observed; (c) recorded 30 min after perfusion with KH containing 2 mM[K$^+$] + 1 μM VK-II-86 ($n=7$). All arrhythmic activity was suppressed. (d, e) Number of episodes of non-sustained and sustained arrhythmia recorded during 30 min of exposure to 2 mM [K$^+$] in the absence and presence of 1-μM dantrolene or 1 μM VK-II-86. Data shown are individual values with group medians indicated.
3.6 In canine ventricular cardiomyocytes, hypokalaemic conditions increased L-type Ca\(^{2+}\) (IC\(_{Ca}\)) current density and accelerated the channel's recovery from inactivation.

Canine ventricular cardiomyocytes pre-incubated for 30 min in 3 mM [K\(^{+}\)] demonstrated a 77% larger IC\(_{Ca}\) peak density than those bathed in 4 mM [K\(^{+}\)] external solution (Figure 7a,b). The addition of 1 μM VK-II-86 to the 3 mM [K\(^{+}\)] solution prevented this increase (Figure 7b, left panel) The recovery from inactivation also increased in 3 mM [K\(^{+}\)] and this effect of hypokalaemia was also prevented by the addition of VK-II-86 (Figure 7b, right panel). There was no significant difference in IC\(_{Ca}\) peak density or its recovery from inactivation between cells pre-incubated in 4 mM [K\(^{+}\)] compared with cells pre-incubated in 4 mM [K\(^{+}\)] plus VK-II-86.

### TABLE 1

|                      | 2 mM [K\(^{+}\)] | 2 mM [K\(^{+}\)] and 1 μM dantrolene | 2 mM [K\(^{+}\)] and 1 μM VK-II-86 |
|----------------------|-------------------|--------------------------------------|-----------------------------------|
| Non-sustained ventricular arrhythmia | 36 (22–66)       | 1 (0–8)                              | 0 (0–0)                           |
| Sustained ventricular arrhythmia     | 2 (1–4)           | 0 (0–1)                              | 0 (0–0)                           |

**FIGURE 3** In murine ventricular cardiomyocytes, VK-II-86 normalizes action potential parameters and is more effective than dantrolene in preventing afterdepolarisations in hypokalaemic conditions.

(a) Representative action potential traces recorded from isolated mouse ventricular myocytes incubated for 30 min in an external solution containing normal (4 mM, \(n = 10\)) or low (3 mM, \(n = 10\)) [K\(^{+}\)]. In 3 mM [K\(^{+}\)], action potential prolongation and EADs were observed. (b) Representative action potential traces recorded after 30 min of incubation in external solution containing 3 mM [K\(^{+}\)] with 1μM VK-II-86 (\(n = 6\)) or 1 μM dantrolene (\(n = 9\)). VK-II-86 normalized the action potential duration and suppressed all arrhythmic activity. In the presence of 1 μM dantrolene, EADs and DAD activity persisted. (C) Composite data showing that in murine cardiomyocytes, 1 μM VK-II-86 reverses the effect of low [K\(^{+}\)] to prolong APD\(_{90}\), APD\(_{50}\), APD\(_{90}\) and resting membrane potential (RMP), but does not alter action potential parameters when added to 4 mM [K\(^{+}\)] extracellular solution (cells isolated from 5 mice). Data shown are individual values with group means indicated. *P<0.05, significantly different as indicated
In HEK-293 cells transiently transfected with KCNH2, IKr is dramatically reduced under hypokalaemic conditions, whereas IKr is significantly increased in the presence of 1 μM VK-II-86, in both low and normal [K+] solutions. Figure 8 presents data obtained from HEK cells expressing IKr. In 3 mM [K+], the IKr tail current density at 10 mV was 60% smaller than that in 4 mM [K+]. In 3 mM [K+], action potential prolongation and EADs were observed. (B) Representative action potential traces recorded after 30 min of incubation in external solution containing 3 mM [K+] with 1 μM VK-II-86 (n = 13) or 1 μM dantrolene (n = 7). VK-II-86 normalized the action potential duration and suppressed all arrhythmic activity. In the presence of 1 μM dantrolene, EADs and DAD activity persisted. (C) Composite data showing that in canine cardiomyocytes, 1 μM VK-II-86 reverses the effect of low [K+] to prolong APD90 and depolarise the resting membrane potential (RMP), but does not alter action potential parameters when added to 4 mM [K+] extracellular solution. (cells isolated from four dogs). Data shown are individual values with group means indicated.

3.7 In HEK-293 cells transiently transfected with KCNH2, IKr is dramatically reduced under hypokalaemic conditions, whereas IKr is significantly increased in the presence of 1 μM VK-II-86, in both low and normal [K+] solutions.

3.8 In murine ventricular cardiomyocytes, VK-II-86 did not significantly alter Ito during hypokalaemic conditions.

The canine AP data in Figure 4 showing that APD90 is not significantly altered in normal or low [K+] with or without VK-II-86 and the retained spike and dome AP morphology demonstrates that Ito is not likely to be significantly altered. Unfortunately, the study’s supply of canine hearts ended before this could be formally measured using patch clamp techniques. However, whole-cell voltage-clamp experiments in isolated murine ventricular cardiomyocytes, in which Ito is the major repolarising current demonstrated that...
although peak $I_{\text{to}}$ density at 40 mV was significantly decreased in hypokalaemic conditions, VK-II-86 did not significantly alter $I_{\text{to}}$ density in either low or normal $[K^+]_e$ (Figure S1). VK-II-86 did significantly accelerate the rate of inactivation of $I_{\text{to}}$ in both normal and low $[K^+]_e$ (Figure S1), reducing total $I_{\text{to}}$ charge and a faster recovery from activation, but the translation of these results to human heart rates is tenuous.

3.9 | In murine cardiomyocytes, VK-II-86 prevents the increase in oxidative stress that occurs during exposure to low $[K^+]_e$

Data in Figure 9 show pooled mean fluorescent densities of murine ventricular cardiomyocytes isolated from 5 mouse hearts. Cells from each heart were loaded with a fluorogenic oxidative stress
reagent prior to 30 min of incubation in either normal [K+]±H2O2 as a positive control or low [K+]±VK-II-86. Oxidative stress significantly increased for cells incubated in low [K+] (P<0.05), but coincubation with VK-II-86 prevented the rise in oxidative stress (Figure 9).

4 | DISCUSSION

Our results demonstrate for the first time that RyR2 inhibition is an effective method of reducing the frequency and severity of ventricular arrhythmias in the context of hypokalaemia. This could have significant benefits to morbidity and mortality for patients susceptible to hypokalaemia, particularly those with heart failure. However, a multichannel approach is more effective than selective RyR2 inhibition. Pretreatment with dantrolene and VK-II-86, both previously shown to inhibit RyR2 (Gonano et al., 2018; Maxwell et al., 2012; Suetomi et al., 2011; Uchinoumi et al., 2010; Zhou et al., 2011), significantly reduced the incidence of ventricular arrhythmias in our whole-heart murine Langendorff model of hypokalaemia. VK-II-86 was the more effective antiarrhythmic compound, as it was able to prevent all triggered activity, sustained and non-sustained ventricular arrhythmias. Further investigation into the effects of VK-II-86 on whole-cell ion channel currents revealed beneficial multichannel effects on key currents involved in hypokalaemia-induced arrhythmia. VK-II-86 prevented the detrimental effects of hypokalaemia on IK1, INa,L, ICa, and IK, thus preventing the deleterious effects of hypokalaemia on the ventricular AP.
In canine ventricular cardiomyocytes, which have an AP morphology very similar to that of humans (de Boer & Stengl, 2018), the AP shape and intervals were not significantly altered by VK-II-86, when bathed in external solution containing normal $[\text{K}^+]$. The same was true for murine cardiomyocytes, despite the fact that the main repolarizing current in mice is the transient outward current ($I_{\text{to}}$) instead of $I_{\text{Kr}}$ (Nerbonne, 2004). The lack of an effect of VK-II-86 on normal AP morphology at $4 \text{ mM} [\text{K}^+]$ in two contrasting species suggests that VK-II-86 is less likely to have pro-arrhythmic side effects, than other drugs that alter AP morphology at baseline. VK-II-86 caused a slight reduction in peak density of $I_{\text{Ca}}$ in $4 \text{ mM} [\text{K}^+]$, so an investigation of ionotropic effects would be prudent. A previous study of VK-II-86 in rat cardiomyocytes demonstrated no effect of VK-II-86 on cell ionotropy, in the presence of ouabain (Gonano et al., 2018).

The ability of VK-II-86 to prevent the hypokalaemia-induced reduction of $I_{\text{K1}}$ is a functionally important feature of the drug. The inward component of $I_{\text{K1}}$ stabilizes the RMP and its outward component contributes to terminal repolarization of the action potential.

**FIGURE 7** In canine ventricular cardiomyocytes, hypokalaemic conditions increase L-type $\text{Ca}^{2+}$ current (LTCC) density and accelerate channel recovery from inactivation. (a) Representative whole-cell L-type $\text{Ca}^{2+}$ channel current traces recorded from canine ventricular myocytes by applying the voltage-clamp protocol shown. Cells were pre-incubated for 30 min in external solution containing either 3 or 4 mM $[\text{K}^+]$ in the absence or presence of 1 µM VK-II-86. (b) Mean peak I-V relationship for LTCC in canine ventricular myocytes normalized to cell capacitance (left) and mean recovery from inactivation of the LTCC under the various conditions shown (right). The voltage-clamp protocol used to measure recovery from inactivation is shown above the right hand graph. Cells isolated from three dogs: 4 mM $[\text{K}^+] n = 6$; 4 mM $[\text{K}^+]$ and VK-II-86 $n = 6$; 3 mM $[\text{K}^+] n = 7$; 3 mM $[\text{K}^+]$ and VK-II-86 $n = 9$. Data shown are means ± SEM.
Loss-of-function mutations in KCNJ2, the gene encoding the channel carrying a substantial proportion of ventricular $I_{K1}$, are associated with congenital Andersen Tawil syndrome, a rare condition with a pro-arrhythmic phenotype. A reduction of $I_{K1}$ has also been found in pro-arrhythmic acquired disease such as advanced heart failure (Beuckelmann et al., 1993). An in silico model of a guinea pig ventricular myocyte demonstrated that reduced $I_{K1}$ results in a depolarized RMP, favouring $Ca^{2+}$ leak into the cell. The electrogentic sodium-calcium exchanger (NCX), in its efforts to export the excess $Ca^{2+}$ could depolarize the cell, contributing to the development of EADs and triggered arrhythmia (Silva & Rudy, 2003). In our study, canine ventricular myocytes in low $[K^+]_o$ showed a substantial lengthening of the APD$_{90}$ and depolarization of the RMP, indicating a probable key role of $I_{K1}$ in the pathogenesis of EAD formation in

**FIGURE 8**  In HEK cells transiently transfected with KCNH2, $I_{K1}$ ($K_r$, 11.1) is reduced under hypokalaemic conditions, whereas $I_{K1}$ is significantly increased in the presence of 1-$\mu$M VK-II-86 in external solution containing either normal (4 mM) or low (3 mM) $[K^+]_o$. (a) Representative whole cell traces of $I_{K1}$ recorded in external solution containing either 4 mM or 3 mM $[K^+]_o$ in the absence and presence of 1-$\mu$M VK-II-86. (b) Composite data of $I_{K1}$ tail currents (normalized to cell capacitance) for HEK cells pre-incubated for 30 min in the various external solutions shown. Cells from 2 transfections: 4 mM $[K^+]_o$ $n=6$; 4 mM $[K^+]_o$ and VK-II-86 $n=5$; 3 mM $[K^+]_o$ $n=7$; 3 mM $[K^+]_o$ and VK-II-86 $n=5$. Peak current is statistically compared at 10 mV. Data shown are means ± SEM. *$P<0.05$, significantly different as indicated.
larger mammals during hypokalaemia. Whole-cell voltage clamp studies demonstrated a significant reduction of both the inward and outward components of $I_{K1}$ in low $[K^+]$, which were prevented in the presence of VK-II-86.

We found a significant depolarization of the RMP in the ventricular AP for both murine and canine ventricular tissues and cells. This contrasts with previous studies reporting that hypokalaemia results in hyperpolarization of RMP (Aronsen et al., 2015; Bouchard et al., 2004; Tazmini et al., 2020). These studies all investigated the effects of acute hypokalaemia on RMP, but there are no published studies of the effects of chronic hypokalaemia on RMP. In our study, the whole-heart and isolated ventricular myocytes were exposed to low $[K^+]$ for a period of 30-min, which may be enough to reflect an effect of altered trafficking of $I_{K1}$. Hypokalaemia also alters the trafficking of other potassium channels, such as $K_r$, carrying $I_{Ks}$ (Guo et al., 2009), but to date, no studies have addressed the effects of hypokalaemia on the trafficking of Kir 2.1 channels. We are embarking on further study to address this question. The depolarized RMP after 30-min of low $[K^+]$ exposure may also be due to a combination of reduced conductance of the $I_{K1}$ channel, as well as a CaMKII-mediated increase of inward currents, such as $I_{Ca}$ and $I_{Na,L}$, which could shift the membrane to more depolarized potentials. In the present study, 1 $\mu$M VK-II-86 in both murine and canine models slightly hyperpolarized the resting membrane potential in low $[K^+]$. This could also be beneficial in reducing intracellular $Ca^{2+}$ loading, because a hyperpolarized RMP favours the forward mode of NCX (Tazmini et al., 2020), and would therefore enable more $Ca^{2+}$ extrusion from the cell.

Up-regulated $I_{K1}$ is pro-arrhythmic and has been implicated in both congenital and acquired arrhythmia. Gain-of-function mutations

**Figure 9** In murine ventricular cardiomyocytes, hypokalaemic conditions increase oxidative stress, whereas co-incubation with VK-II-86 prevents this. (a): Typical murine ventricular cardiomyocytes pictured in both bright field mode (left-hand pictures) and $\lambda = 470$ nm (right-hand pictures) after loading with the fluorogenic oxidative stress reagent CellRox™ green and incubated in the conditions shown. (b) Collated data from $n = 5$ murine hearts. Data points are pooled averages of the mean fluorescence density, adjusted for cell surface area of cardiomyocytes, incubated with a fluorogenic oxidative stress reagent. Each data point represents the mouse from which pooled averages were calculated. Data shown are individual values with means ± SD. *$P<0.05$, significantly different as indicated.
in KCNJ2 are associated with congenital AF (Xia et al., 2005) and short QT syndrome type 3 (Priori et al., 2005). Up-regulated IKc has been found in acquired AF (Dobrev et al., 2002) and has been shown to stabilize rotors in both AF and ventricular fibrillation (Dhamoon & Jalife, 2005; Samie et al., 2001). Therefore, it is advantageous that, in the present study, VK-II-86 did not significantly up-regulate the inward or outward components of IKc, in normal [K+].

Canine and murine cardiomyocytes both showed an increase in INa-L when incubated in low [K+]. This finding is in agreement with those of Pezhouman et al. (2015), who demonstrated that low [K+], through inhibition of Na+/K+ ATPase and subsequent intracellular Ca2+ loading, induced CaMKII activation and led to a positive feedback loop by up-regulating INa-L and ICa. The finding of increased INa-L in hypokalaemia also has implications as to why hypokalaemia is proarrhythmic in patients with the Long QT syndrome. Pezhouman et al. found that pretreatment with the INa-L inhibitor GS-967 and the ICa blocker nifedipine prevented the development of EADs and ventricular arrhythmia. It is therefore encouraging that VK-II-86 prevented this increase. As IK1, INa-L and ICa channels are all altered by oxidative stress (Bérubé et al., 2001; Hamilton et al., 2020; Wagner et al., 2011; Xie et al., 2009), it is plausible that the ability of VK-II-86 to prevent oxidative stress during hypokalaemia. VK-II-86 targets key ion channels involved in intracellular Ca2+ loading and repolarization including those carrying IKc, IKr, ICa, INa-L and RyR2. VK-II-86 affected these channels only under conditions of hypokalaemia, preventing their dysfunction when exposed to low [K+]. VK-II-86 may provide an effective therapeutic alternative for other conditions complicated by arrhythmia secondary to Ca2+ overload, such as heart failure, CPVT and Andersen Tawil Syndrome.

In conclusion, the results presented here provide proof of concept for a novel anti-arrhythmic drug, VK-II-86, capable of preventing hypokalaemia-induced arrhythmogenesis because of its multi-ion channel effects, and its ability to prevent oxidative stress during hypokalaemia. VK-II-86 targets key ion channels involved in intracellular Ca2+ loading and repolarization including those carrying IKc, IKr, ICa, INa-L and RyR2. VK-II-86 affected these channels only under conditions of hypokalaemia, preventing their dysfunction when exposed to low [K+]. VK-II-86 may provide an effective therapeutic alternative for other conditions complicated by arrhythmia secondary to Ca2+ overload, such as heart failure, CPVT and Andersen Tawil Syndrome.

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AUTHOR CONTRIBUTIONS

V.M.R. contributed to experimental design, carried out the experiments, data analysis, and drafted the paper. I.A. synthesized the VK-II-86 under the supervision of S.F. C.A. supervised the study. H.B.M. contributed to experimental design, provided experimental and analysis training for V.M.R and supervised the project. L.V. supervised the project and obtained study funding.
CONFLICT OF INTEREST
Dr Antzelevitch is a consultant to and has received research funds from Novartis Institutes for BioMedical Research, Inc, Trevena Pharmaceuticals Inc and Praxis Pharmaceuticals. The other authors report no conflicts of interest.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR
Declaration of transparency and scientific rigour This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJPs guidelines for Design and Analysis, and Animal Experimentation, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

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