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RESEARCH PAPER

Characterization of a novel multifunctional resveratrol derivative for the treatment of atrial fibrillation

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BACKGROUND AND PURPOSE

Atrial fibrillation (AF) is the most common cardiac arrhythmia and is associated with an increased risk for stroke, heart failure and cardiovascular-related mortality. Candidate targets for anti-AF drugs include a potassium channel $K_v1.5$, and the ionic currents I_{KACh} and late I_{Na}, along with increased oxidative stress and activation of NFAT-mediated gene transcription. As pharmacological management of AF is currently suboptimal, we have designed and characterized a multifunctional small molecule, compound 1 (C1), to target these ion channels and pathways.

EXPERIMENTAL APPROACH

We made whole-cell patch-clamp recordings of recombinant ion channels, human atrial I_{Kur}, rat atrial I_{KACh}, cellular recordings of contractility and calcium transient measurements in tsA201 cells, human atrial samples and rat myocytes. We also used a model of inducible AF in dogs.

KEY RESULTS

C1 inhibited human peak and late $K_v1.5$ currents, frequency-dependently, with IC_{50} of 0.36 and 0.11 $\mu mol \cdot L^{-1}$ respectively. C1 inhibited I_{KACh} (IC_{50} of 1.9 $\mu mol \cdot L^{-1}$) and the $Na_v1.5$ sodium channel current (IC_{50}s of 3 and 1 $\mu mol \cdot L^{-1}$ for peak and late components respectively). C1 (1 $\mu mol \cdot L^{-1}$) significantly delayed contractile and calcium dysfunction in rat ventricular myocytes treated with 3 $nmol \cdot L^{-1}$ sea anemone toxin (ATX-II). C1 weakly inhibited the hERG channel and maintained antioxidant and NFAT-inhibitory properties comparable to the parent molecule, resveratrol. In a model of inducible AF in conscious dogs, C1 (1 $mg \cdot kg^{-1}$) reduced the average and total AF duration.

CONCLUSION AND IMPLICATIONS

C1 behaved as a promising multifunctional small molecule targeting a number of key pathways involved in AF.

Abbreviations

AERP, atrial effective refractory period; AF, atrial fibrillation; BCL, basic cycle length; CABG, coronary artery bypass graft surgery; C1, compound 1; DPPH, diphenylpicrylhydrazyl; NRVM, neonatal rat ventricular myocytes

Introduction

Atrial fibrillation (AF) is the most common type of cardiac arrhythmia and the incidence of AF is increasing with an ageing population (Naccarelli *et al.*, 2009; McManus *et al.*, 2012). While AF does not generally induce sudden cardiac death, AF increases the risk of stroke fivefold (Wolf *et al.*, 1991; Hart and Halperin, 2001; McManus *et al.*, 2012), can cause tachycardia-induced cardiomyopathy (Nerheim *et al.*, 2004) and adverse electrical and structural remodelling of the heart (Franz *et al.*, 1997; Anter *et al.*, 2009). Furthermore, ~30% of patients undergoing coronary artery bypass graft (CABG) surgery develop transient post-operative AF (Andrews *et al.*, 1991; Mathew *et al.*, 2004). Current pharmacotherapy of AF is still suboptimal because some of the drugs used for rhythm control also display significant risk for acquired long QT syndrome and Torsades des Pointes arrhythmias (Taira *et al.*, 2010) and may increase the incidence of vascular events (Connolly *et al.*, 2011). Therefore, there is a significant need for the development of improved therapeutic agents for AF.

Over the past decade, studies on the cellular pathways in AF have revealed potential therapeutic targets to develop new anti-AF drugs. One important target is the ultra-rapid potassium channel (I_{Kur}/$K_v1.5$; channel and receptor nomenclature follows Alexander *et al.*, 2013) preferentially expressed in the atria, but not the ventricle (Wang *et al.*, 1993; Gaborit *et al.*, 2007; Tamargo *et al.*, 2009; Ravens and Wettwer, 2011). In addition to $K_v1.5$ channel, I_{KACh} (Ehrlich and Nattel, 2009), voltage-gated sodium channels (Burashnikov *et al.*, 2007; Burashnikov and Antzelevitch, 2012), oxidative stress (Carnes *et al.*, 2001; Mihm *et al.*, 2001) and activation of the transcription factor NFAT (Lin *et al.*, 2004) have all been implicated in AF development. Given the complex aetiology of AF, it has been suggested that drugs targeting multiple pathways involved in AF development may be more effective (Dobrev and Nattel, 2010; Dobrev *et al.*, 2012). To date, AF drugs have been exclusively targeted towards ion channels. However, non-ionic remodelling events also likely contribute to the initiation and maintenance of AF as well. It has been further suggested that targeting maladaptive remodelling events in AF may also be required for effective control of AF (Burashnikov and Antzelevitch, 2011). Therefore, based on these criteria, a multifunctional small-molecule drug for AF should ideally possess the following properties: (i) frequency-dependent $K_v1.5$ inhibition; (ii) I_{KACh} inhibition; (iii) late sodium current inhibition; (iv) lack of hERG channel inhibition; (v) display atrial specificity, that is, no effect on repolarization and excitation–contraction (EC) coupling in ventricular tissue; (vi) antioxidant properties; (vii) NFAT inhibition; and (viii) functional efficacy in a large-animal model of inducible AF.

Resveratrol is a bioactive polyphenol found in red grape products that has been investigated extensively for its cardioprotective and anti-ageing properties (Brisdelli *et al.*, 2009; Dolinsky and Dyck, 2011). We have previously shown that resveratrol (i) inhibits NFAT activation and hypertrophic remodelling in cardiac tissue (Chan *et al.*, 2008; Dolinsky *et al.*, 2009) and (ii) inhibits the late I_{Na} current preferentially over peak current (Wallace *et al.*, 2006). Based on these multifunctional properties, the aim of the present study was to synthesize, using resveratrol as the parent molecule, a novel small molecule called compound 1 (C1) and to characterize its antioxidant, NFAT-inhibitory effects and electrophysiological profile with respect to $K_v1.5$, I_{KACh}, late I_{Na} and hERG. We also determined its *in vivo* efficacy in a model of inducible AF in dogs.

Methods

Chemical synthesis of compounds

Details of the chemical synthesis used to generate four unique resveratrol hybrid bis-ortho-substituted biphenyl diamide derivatives are provided in Supporting Information Appendix S1.

Cell transfection

The human $Na_v1.5$ and hERG clones were generously provided by Dr Arthur Brown (Case Western Reserve University, USA) and Dr Henry Duff (University of Calgary, Canada) respectively. Expression vectors were co-transfected into the tsA201 cell line (Sigma Aldrich, St Louis, MO, USA) with a GFP expression vector using the calcium phosphate precipitation technique. TsA201 cells are a transformed HEK293 cell line expressing the temperature-sensitive antigen to increase expression levels of recombinant proteins. Transfected cells were then used within 48 h. All *in vitro* experiments were performed at room temperature (20–22°C).

$K_v1.5$ currents

Recordings were made from single tsA201 cells stably expressing the human heart *$K_v1.5$* gene. Pipettes were pulled from borosilicate glass capillary tubing (Warner Instruments, Hamden, CT, USA) and tips were fire-polished, producing resistances of 1–3 MΩ. The pipette solution contained (in mmol·L^{-1}): 140 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES and 10 EGTA. The pH was adjusted to 7.3 with KOH. A concentration of 2 mmol·L^{-1} MgATP was added immediately before use. Cells were bathed in extracellular solution contained (in mmol·L^{-1}): 135 NaCl, 5.4 KCl, 1 $CaCl_2$, 1.2 $MgCl_2$, 10 HEPES and 5 glucose (pH adjusted to 7.4 with NaOH). Solutions were applied to cells using a multi-input perfusion pipette (switch time <2 s). Once a giga-seal was formed, the patch was ruptured and the whole-cell voltage-clamp technique was used to record $K_v1.5$ currents. Currents were elicited by 120 ms duration test pulses to +40 mV from a holding potential of –100 mV with a cycle length of 0.3, 1 or 3 Hz respectively. Data were acquired using an Axopatch 200B patch-clamp amplifier and Clampex 8.2 software (Axon Instruments, Foster City, CA, USA).

$Na_v1.5$ currents

Single tsA201 cells transfected with mammalian expression vectors encoding the human heart $Na_v1.5$ channel were used for recording of whole-cell $Na_v1.5$ currents, as described previously (Wallace *et al.*, 2006). Voltage steps from a holding potential of –120–40 mV every 10 mV was used to generate the current–voltage (I –V) relationship for peak I_{Na}.

hERG currents

tsA201 cells were transfected with a mammalian expression vector encoding the hERG channel clone ($K_v11.2$) as

described for $Na_v1.5$ channels above and hERG currents were recorded as described previously (Kikuchi *et al.*, 2005).

Human atrial I_{Kur} currents

For experiments on human atrial I_{Kur} ($K_v1.5$) atrial tissue was collected from 7 male patients aged 40-75 years undergoing CABG surgery at the University Hospital, University of Alberta, Canada. We obtained prior ethical approval from the University of Alberta human ethics board and informed patient consent. The atrial appendage samples were collected during surgery and enzymically dissociated into single atrial myocytes as described below. Human atrial appendage samples were cut into small pieces and washed 3 times for 10 min with 10ml of a calcium-free Tyrode's solution in a shaking water bath at 32°C. The sample was enzymically digested for 45 min in 10 ml of Tyrode's solution containing 20 mg Type II collagenase (Worthington, Lakewood NJ, USA) and 4 mg protease type XIV (Sigma Aldrich) in a shaking water bath at 32°C. After 45 min the supernatant is removed and 10 ml solution containing 20 mg Type II collagenase only was added and the sample further digested for 10 min at 32°C. Isolated cells are removed in the supernatant, centrifuged and resuspended in Tyrode's solution. I_{Kur} currents were then measured using a modification of the whole-cell recording protocol to inactivate I_{to} currents, as described previously (Cai *et al.*, 2007).

Animals

All animal care and experimental procedures involving adult and neonatal rats complied with the guidelines of the Canadian Council for Animal Care and the project was approved by the University of Alberta research ethics office. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A total of 15 adult rats, 69 neonatal rats and 5 dogs were used in the experiments described here.

Rat atrial I_{KACh} and recombinant I_{K1} currents

Rat atrial myocytes were isolated as described previously (Komukai *et al.*, 2002) from 15 adult male Sprague-Dawley rats (150-200 g) obtained from the animal facility at Biological Sciences, University of Alberta. Whole-cell recordings of carbachol-induced I_{KACh} currents were made using the same solutions used for $K_v1.5$ and a voltage step protocol from −110 to +50 mV with a holding potential of −80 mV. I_{K1} whole-cell currents were measured in tsA201 cells transiently transfected with the $K_{IR}2.1$ clone using the same solutions and voltage protocols used for I_{KACh} currents.

Cell shortening and calcium transient recordings

These were measured in rat ventricular myocytes prepared from adult Sprague-Dawley rats killed by an overdose of pentobarbital (150 mg kg^{-1}, i.p.). The hearts were removed and right ventricular myocytes were then obtained by enzymic dissociation using standard protocols, which have been described previously (Bouchard *et al.* 1993; Light *et al.* 1998). Cell shortening and calcium transients were measured using standard procedures previously published by our laboratory (Baczko *et al.*, 2005; Wallace *et al.*, 2006).

NFAT reporter assay

Neonatal rat ventricular myocytes (NRVM) were isolated from the hearts of 1-3-day-old neonatal rat pups and cultured in DMEM/F-12, containing 5% fetal bovine serum, 10% horse serum, 50 µg ml^{-1} gentamicin, and 1% penicillin-streptomycin for 16h, as described previously (Kovacic *et al.* 2003). 24h prior to the initiation of experiments, the medium was changed to serum-free DMEM/F-12 containing 50 µg ml^{-1} gentamicin supplemented with 1× insulin, transferrin, sodium selenite (ITS)+3 liquid media supplement (Chan *et al.*, 2008). All subsequent treatments of the myocytes were performed in this serum-free medium for either 1 or 24 h. NRVM were infected with Ad.GFP or Ad.NFAT-Luc-Promoter adenovirus (Seven Hills Bioreagents, Cincinnati, OH, USA) at an infection multiplicity of 10. Cells were treated 24 h post-infection with vehicle or varying concentrations of C1 or resveratrol and/or angiotensin II (1 µmol·L^{-1} for 24 h). Cells were harvested with the reporter lysis buffer supplied in the luciferase assay system kit (Promega, Madison, WI, USA) and luminescence according to the manufacturer's instructions.

Anti-oxidant assay

A diphenylpicrylhydrazyl (DPPH) assay (Pyrzynska and Pekal, 2013) was used to determine the antioxidant capacity of C1 and resveratrol. A ethanolic solution of H_2O_2 (200 nmol·L^{-1}) was mixed with DPPH alone, or DPPH with either resveratrol or C1 at various concentrations. The final concentration of ethanol in the experimental solution was 0.1%. The absorbance of these solutions was measured at 517 nm, with a lower absorbance representing an increase in antioxidant capacity.

Model of induced AF in vivo

All experiments were carried out in compliance with the Guide for the Care and Use of Laboratory Animals (USA NIH publication No 85–23, revised 1996) and approved by the Department of Animal Health and Food Control of the Ministry of Agriculture and Rural Development, Hungary (XIII/01031/000/2008). Adult male Beagle dogs (age: 24-26 months, weight: 14–16 kg; supplied by the University of Szeged Experimental Animal Facility, Hungary) were anaesthetized with ketamine (Richter Gedeon Ltd., Hungary; induction: 10 mg kg^{-1}, maintenance: 2 mg kg^{-1}, every 20 min) and xylazine (CP-Pharma Handelsges, Germany; induction: 1 mg kg^{-1}, maintenance: 0.2 mg kg^{-1}, every 20 min) and were mechanically ventilated. The implantation procedure was carried out under antibiotic coverage as follows: amoxicillin/clavulanic acid (1000 mg/200 mg i.v.; Richter Gedeon Ltd.) and gentamicin (40 mg i.v.; Sandoz GmbH, Kundl, Austria) given before the operation, amoxicillin/clavulanic acid (500 mg/125 mg orally, twice a day for 5 days; Augmentin 500mg/125mg®; GlaxoSmithKline Ltd., Hungary) given following the operation. For peri-operative analgesia metamizole sodium (1000 mg i.v., 1g/2ml; Sanofi-Aventis Hungary Ltd., Hungary) and tramadol (50 mg i.v., TEVA Ltd., Hungary, under licence from Grünenthal GmbH, Aachen, Germany) were administered.

The procedure involved the implantation of two bipolar pacemaker electrodes (Synox SX 53-JBP® and Synox SX 60/15-BP®, Biotronik Hungary Ltd., Hungary) into the right atrial appendage and apex of the right ventricle, the electrodes were connected to pacemakers (Logos DS® and Philos S®, Biotronik Hungary Ltd., Hungary) in subcutaneous pockets in the neck area, radiofrequency catheter ablation of the AV node was performed to avoid high atrial pacing rates propagating into the ventricles. The ventricular pacemaker was set between 80 to 90 beats/min, following the baseline heart rate of the dog before the operation.

Following recovery from surgery (3 days), high frequency right atrial pacing was started at 400 beats/min, maintained for 6 to 7 weeks before the experiments to allow electrical remodeling of the atria (monitored by the measurement of the right atrial effective refractory period (AERP) every second day). The AERPs were measured at basic cycle lengths (BCL) of 150 and 300 ms with a train of 10 stimuli (S1) followed by an extrastimulus (S2), with the AERP defined as the longest S1S2 interval that did not produce a response. AERP shorter than 80 ms could not be measured in conscious animals (pacemaker measurement limit). No animals were lost between pacemaker, pacemaker electrode implantation and experimental use.

On the day of the experiment atrial pacing was stopped, continuous recording of the electrocardiogram commenced using precordial leads and the AERP was measured. A control set (25 times) of 10-second long rapid atrial bursts (800 beats/min, at twice threshold) were performed in order to induce atrial fibrillation in conscious dogs preceded by a bolus infusion of vehicle (20 mL of a mixture of DMSO + β-hydroxypropyl-cyclodextrin + saline (all from Sigma, St. Louis, USA), DMSO concentration less than 0.1%, infused in 15 min). During the control 25 bursts and subsequent AF episodes, a continuous infusion of vehicle was maintained (in a volume of 1.7 mL/kg/min). Following the measurement of AERP, Compound 1 (C1) was infused in a dose of 0.3 mg/kg (in 15 min bolus + maintenance) and AF was again induced 25 times. An identical procedure was repeated in every dog with 1 mg/kg dose of C1. The incidence of AF, the total duration of AF, the average duration of AF episodes were measured along with changes in atrial refractory period and QT interval. QT intervals were measured on dogs with pacemaker implantation before the 12th burst and were not corrected for heart rate since QT measurements were made at heart rate set to 80 beats/min by the ventricular pacemaker in each animal. Experiments were done in freely moving conscious dogs so that any interference from anesthetics could be ruled out.

HPLC assay of plasma levels of C1

Blood samples were collected from the vena cephalica from the right foreleg for later determination of the plasma levels of C1, before and after administration of C1 infusion at designated time points. For each time point (5 min before the administration of 0.3 mg/kg C1, 30 min after the administration of 0.3 and 1 mg/kg C1) 4 mL of blood was taken into tubes containing lithium heparin. The tubes were immediately centrifuged at 4 °C for 15 min at 3000g and the separated plasma was stored in a freezer at –20°C.

For each sample, 100 µL of plasma was combined with 100 µL of deionized water and the entire volume was loaded into individual wells of a 96-well Isolute SLE+ (supported liquid extraction) plate from Biotage (PN 820-00200-P01; Charlotte, NC, USA). The samples were eluted into a 96-well collection plate using 1 ml of *tert*-butyl methyl ether. The solvent was completely evaporated using a TurboVap 96 and samples were reconstituted in 200 µL of 0.1% formic acid in a 50/50 water/acetonitrile mixture. Analysis was performed using an Acquity UPLC-TQD (Waters) in multiple reaction monitoring mode (positive electrospray).

Data analysis

Data are expressed as mean ± SEM. In whole animal experiments, each animal served as its own control, after one-way ANOVA, the groups were compared in pairs by means of Student's *t*-test. In cell-based experiments, one-way ANOVAs or paired Student's *t*-tests were used as appropriate. A level of $P < 0.05$ was considered to be significant..

Materials

Angiotensin II, carbachol, DPPH, E-4031 and resveratrol were obtained from Sigma Aldrich. The anemone toxin ATX II, was obtained from Alomone Labs (Jerusalem, Israel).

Results

Effects of C1 on $K_v1.5$ currents

The inhibitory effects on $K_v1.5$ currents of four novel resveratrol derivatives (Figure 1A) were compared with those of the parent molecule, resveratrol. Resveratrol is a weak inhibitor of $K_v1.5$ currents (IC_{50} = 66 µmol·L^{-1}; Figure 1B). Of the four derivatives synthesized, C1 was found to be the most potent $K_v1.5$ inhibitor (IC_{50}s = 0.36 µmol·L^{-1} and 0.11 µmol·L^{-1} for peak and late current inhibition, respectively; Figure 1B–E). Compounds 2–4 displayed intermediate $K_v1.5$ peak current inhibitory potencies of 8.3, 10.9 and 11.2 µmol·L^{-1} respectively (Figure 1B). Therefore, only C1 was selected for further characterization in this study.

Frequency-dependent effects of C1 on $K_v1.5$ currents

The frequency-dependent effects of C1 were tested on whole-cell $K_v1.5$ currents in the tsA201 cell line stably expressing $K_v1.5$ channels. At 1 Hz, 0.3 µmol·L^{-1} C1 displayed a time-dependent inhibition of $K_v1.5$ currents, reaching a steady-state after 15 s ($I_{test}/I_{control}$ = 0.74 ± 0.02). In contrast, at a higher stimulation frequency of 3 Hz, the observed C1 inhibition was increased, reaching a steady-state level of 0.50 ± 0.03 when compared with control (n = 5 cells; Figure 2A,B).

Effects of C1 on human atrial I_{Kur} currents

Single atrial myocytes were isolated from human atrial appendage samples obtained during CABG surgery and whole-cell I_{Kur} currents measured. Application of C1 (0.3 µmol·L^{-1}) reversibly inhibited I_{Kur} to 31.0 ± 1.8% of

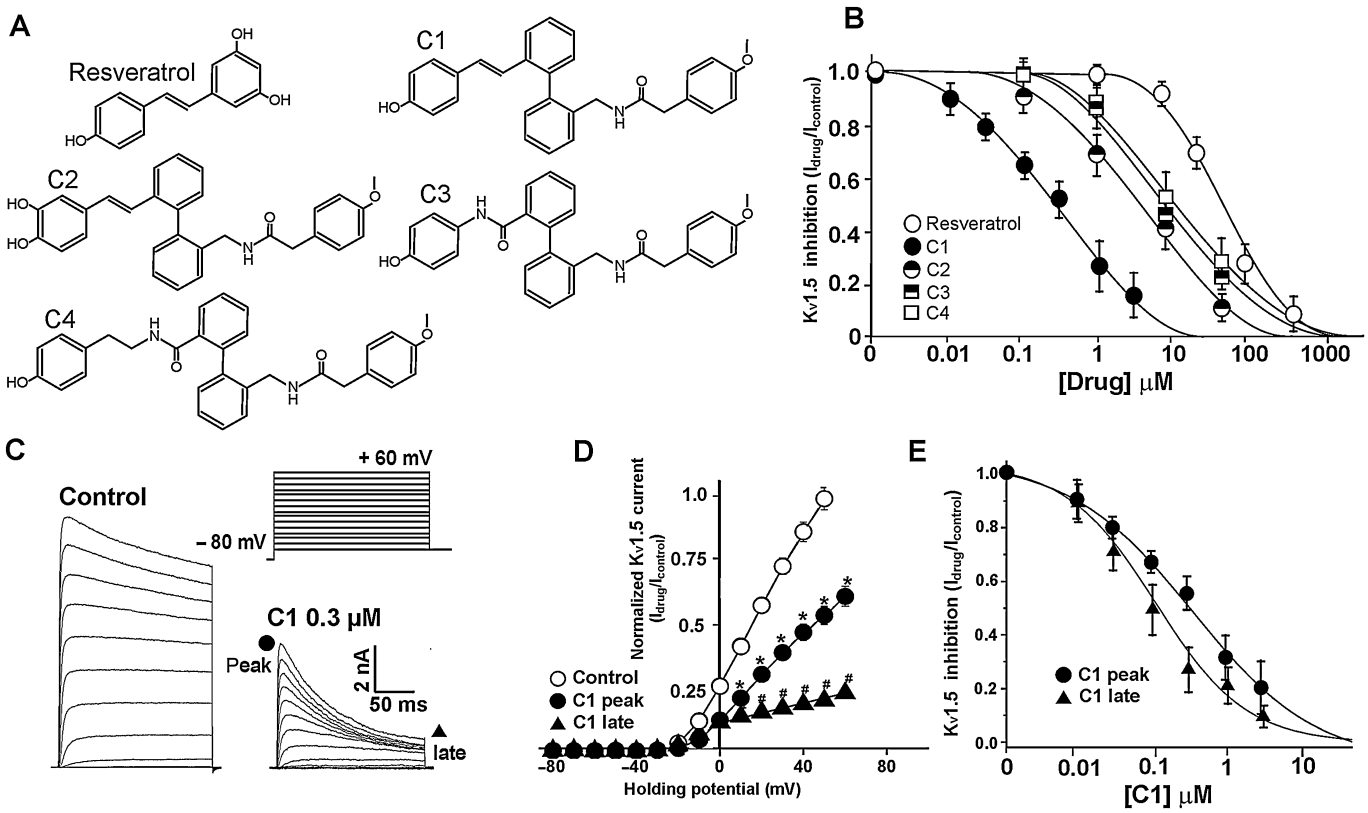

Figure 1

Effects of resveratrol and its derivatives on recombinant $K_v1.5$ current inhibition. (A) Chemical structures of resveratrol and the four resveratrol derivatives, C1–C4. (B) Concentration–inhibition curves of the effect of resveratrol and the four resveratrol derivatives (C1–C4) on $K_v1.5$ peak current amplitude (IC_{50}s = 66.0, 0.36, 8.3, 10.9 and 11.2 μmol·L^{-1}, respectively, n = 4–7 cells for each concentration). (C) Representative traces before and after application of 0.3 μmol·L^{-1} C1. (D) Current–voltage curves of $K_v1.5$ peak and late currents before and after application of 0.3 μmol·L^{-1} C1 compared with control. *P<0.05, $^{\#}$P<0.01 significantly different from control values; paired Student's t-test. (E) Concentration–inhibition curves of the effect of C1 on peak and late $K_v1.5$ current compared with control (peak current IC_{50} = 0.36 μmol·L^{-1}, late current IC_{50} = 0.11 μmol·L^{-1}, n = 4–6 cells for each concentration).

control values (n = 3 cells; Figure 2C,D), a value similar to that observed on recombinant $K_v1.5$ (Figures 1D and 2A,B).

Effects of C1 on rat atrial I_{KACh} and recombinant I_{K1} currents

Single atrial cells were isolated from rat hearts and I_{KACh} currents were elicited by application of carbachol. C1 significantly inhibited carbachol-induced rat atrial I_{KACh} currents with an IC_{50} of 1.9 μmol·L^{-1} (Figure 3). In contrast, 3 μmol·L^{-1} C1 had no significant inhibitory effect on recombinant I_{K1} (Kir2.1) whole-cell currents transiently expressed in tsA201 cells (see Supporting Information Appendix S1 for results).

Effects of C1 on human $Na_v1.5$ currents

The effects of C1 were tested on peak and late recombinant $Na_v1.5$ whole-cell currents. Application of 3 μmol·L^{-1} C1 resulted in a 50% inhibition of peak current (Figure 4A,B). To establish the concentration–response curves for peak and late $Na_v1.5$ currents, cells were treated with the sea anemone toxin (ATX-II) (3 nmol·L^{-1}) to induce the late-current component and to test the inhibitory effects of C1 at differing concentrations. C1 preferentially inhibits the late current when compared with peak current (IC_{50}s = 1.1 μmol·L^{-1} vs. 3.2 μmol·L^{-1}, respectively; Figure 4C,D).

Effects of C1 on hERG currents

Inhibition of the hERG (I_{Kr}, $K_v11.2$) is highly undesirable and may result in arrhythmogenic QT prolongation (Sanguinetti *et al.*, 1996; Sanguinetti and Tristani-Firouzi, 2006; Taira *et al.*, 2010). Therefore, we tested the effects of C1 on recombinant whole-cell hERG channel currents. hERG currents were positively identified by their characteristic inactivation at positive holding potentials, large tail currents and inhibition by 1 μmol·L^{-1} E-4031 (Figure 5A). Construction of concentration–inhibition curves revealed that C1 is a weak inhibitor of peak and tail hERG currents (Figure 5B,C), with IC_{50}s of 30 and 25 μmol·L^{-1}, respectively, values that are 100-fold higher than those inhibiting peak and late $K_v1.5$ currents.

C1 antioxidant capacity and NFAT activation

Antioxidant therapy has been shown to reduce the incidence of post-operative AF in humans (Carnes *et al.*, 2001), and reactive oxygen species (ROS) also directly activate $K_v1.5$

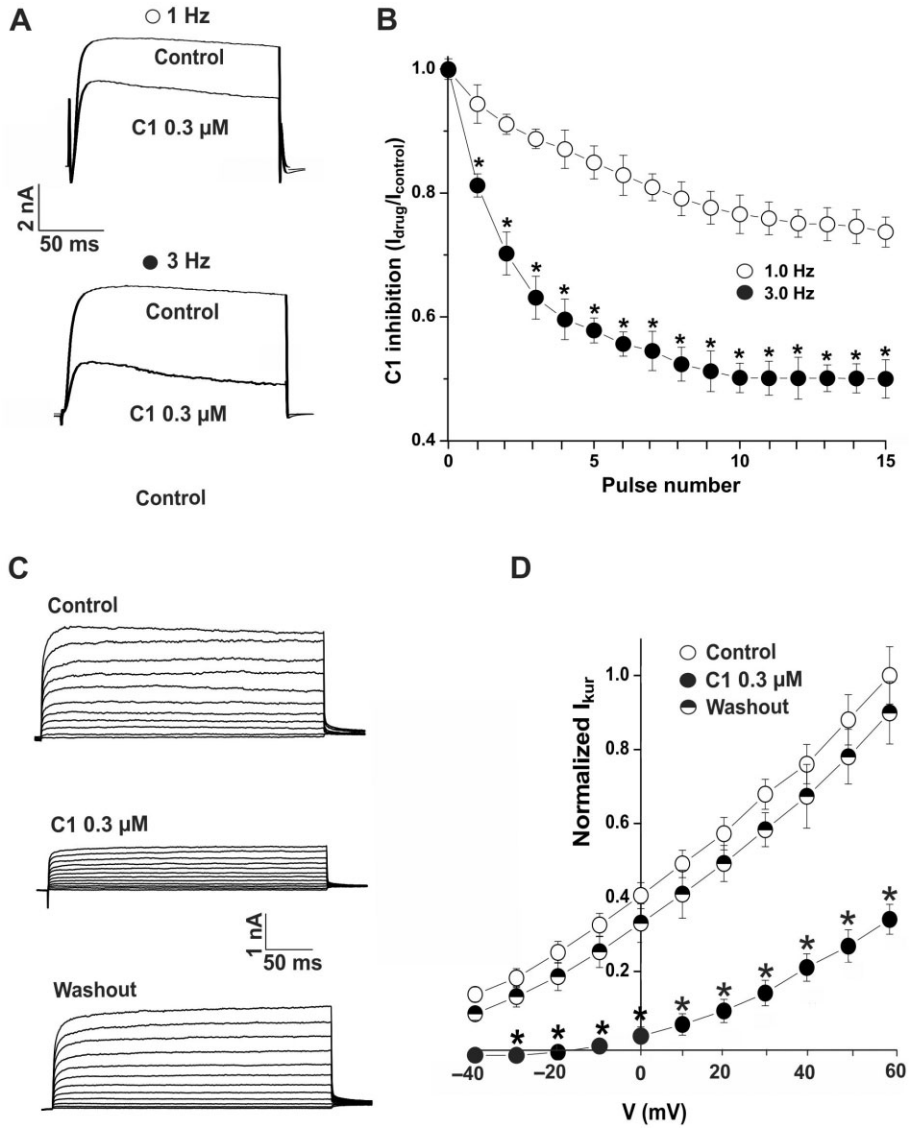

Figure 2

Effects of C1 on human atrial I_{Kur}. (A) Representative traces of frequency-dependent effect on steady-state current by application of 0.3 μmol·L^{-1} C1 compared with controls (n = 5). Top: Effect on steady-state current at 1 Hz stimulation ($I_{test}/I_{control}$ = 0.74 ± 0.02). Bottom: Effect at 3 Hz stimulation ($I_{test}/I_{control}$ = 0.50 ± 0.03). (B) Use-dependent effects of 0.3 μmol·L^{-1} C1 on I_{Kur} at 1 and 3 Hz stimulation. *P < 0.05, significantly different from control values; paired Student's t-test. (C) Representative traces of effects of 0.3 μmol·L^{-1} C1 on I_{Kur} before and after application, and recovery after washout. (D) Current–voltage curve of the effect of the application of 0.3 μmol·L^{-1} C1 on I_{Kur} current, and after washout, compared with control, n = 5 cells). *P < 0.05, significantly different from control values; paired Student's t-test.

channels (Caouette *et al.*, 2003). As resveratrol is a known antioxidant, we compared the antioxidant properties of C1 with resveratrol. At 10 μmol·L^{-1}, resveratrol and C1 displayed significant antioxidant capacities (0.59 ± 0.04 vs. 0.77 ± 0.02 of maximal DPPH 517 nm absorbance signal; Figure 6A). At 100 μmol·L^{-1}, these values were 0.09 ± 0.02 and 0.19 ± 0.02 for resveratrol and C1 respectively.

We have previously shown that resveratrol inhibits NFAT activation induced by phenylephrine contributing to a reduction in maladaptive hypertrophy in NRVMs (Chan *et al.*, 2008). Accordingly, we tested the effects of C1 on NFAT activation in NRVMs. Treatment of NRVMs with 0.01–25 μmol·L^{-1} of C1 resulted in a significant reduction in NFAT activity when compared to no treatment (Figure 6B). These experiments revealed that C1 significantly reduced NFAT activity at concentrations of 0.1 μmol·L^{-1} and higher, with 1 μmol·L^{-1} of C1 exhibiting half-maximal inhibition (Figure 6B). As 1 μmol·L^{-1} of C1 inhibited NFAT activation by 50%, we used this concentration to determine whether C1 was able to inhibit NFAT activation in response to a pro-hypertrophic stimuli. NRVMs were infected as above and treated with vehicle, AngII (1 μmol·L^{-1}), C1 (1 μmol·L^{-1}) or AngII + C1 (1 μmol·L^{-1}) for 24 h. As expected, treatment of NRVM with 1 μmol·L^{-1} of AngII significantly increased NFAT

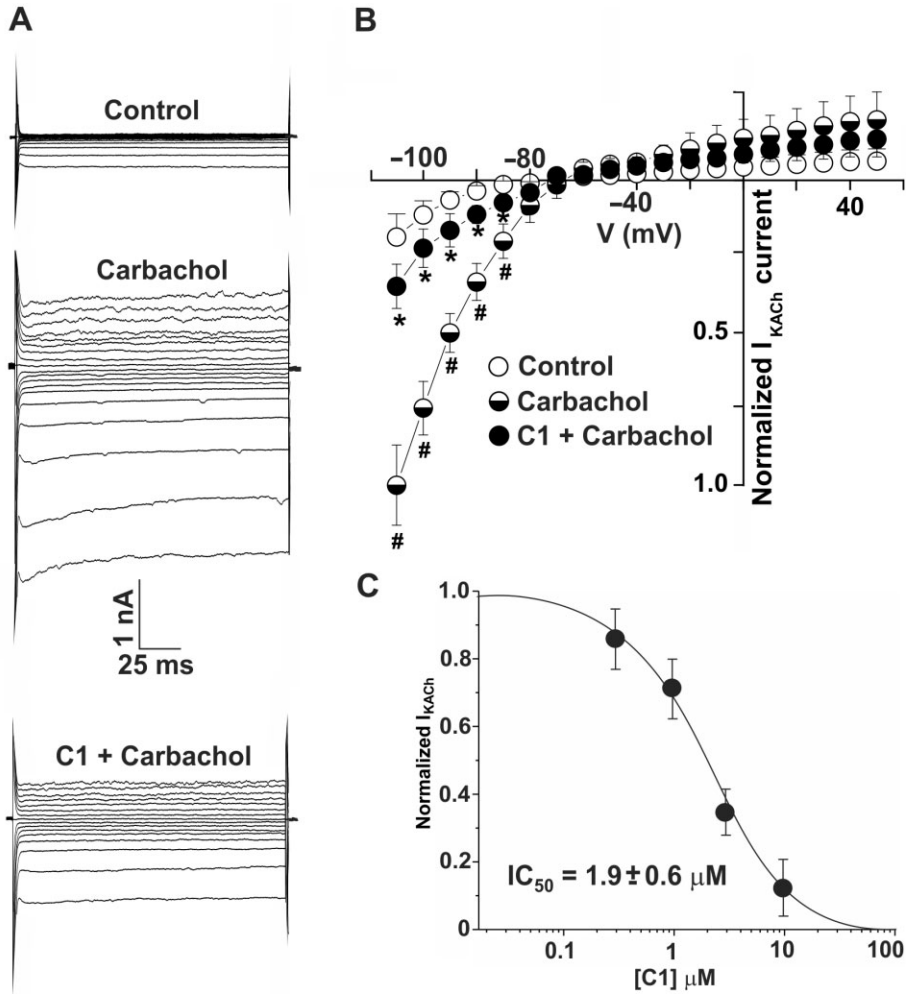

Figure 3

Effect of C1 on rat atrial I_{KACh} currents. (A) Representative traces of the effects of 3 μmol·L^{-1} C1 on I_{KACh} whole-cell currents induced by 10 μmol·L^{-1} carbachol. (B) Grouped data current–voltage curves of control, carbachol and carbachol + 3 μmol·L^{-1} C1 (n = 5 cells, currents normalized to control values). *P < 0.05, $^{\#}P$ < 0.01 significantly different from control values; paired Student's t-test. (C) Concentration–inhibition curve of the effect of C1 on I_{KACh} current (n = 4–6 cells for each concentration).

activation by 504 ± 73%, whereas 1 μmol·L^{-1} of C1 inhibited NFAT activation by approximately 50%. C1 also significantly reduced AngII-induced activation of NFAT to 160 ± 23% of control values (Figure 6C.).

Effects of C1 on ventricular myocyte excitation–contraction coupling

A desirable property of potential compounds for AF is a minimal effect on EC coupling in ventricular myocytes. Single rat ventricular myocytes were field-stimulated at 1 Hz; contractility and calcium transients were then measured by edge detection and calcium imaging. Application of 3 μmol·L^{-1} C1 had no significant effect on (i) diastolic resting cell length or calcium levels or (ii) peak systolic contractility or calcium transient amplitude (Figure 7A,B).

The results presented in Figure 3 indicate that C1 inhibits the late component of the cardiac voltage-gated sodium channel current compared with peak current. Therefore, the effects of C1 were tested in a single-ventricular myocyte model of late sodium current-induced disturbances in calcium homeostasis by pretreatment of myocytes with 3 nmol·L^{-1} ATX-II. ATX-II pretreatment results in markedly elevated diastolic calcium levels and the loss of uniform calcium transients (Figure 7C,E,F). In contrast, application of C1 (1 μmol·L^{-1}) significantly reduced the magnitude of ATX-II-induced diastolic calcium elevations (C1 = 5.9 ± 2.9% vs. control = 57.8 ± 5.9% of maximal transient amplitude, P < 0.01, n = 5 cells) and significantly delayed the onset of calcium transient dysfunction (C1 = 5.3 ± 0.7 min vs 2.4 ± 0.4 min for control, P < 0.01, n = 10 cells, Figure 7D, E, F).

Effects of C1 in a model of inducible AF in dogs

Right atrial effective refractory period (AERP) measurements before the commencement of rapid atrial pacing at 400 beats·min^{-1} yielded values of 118 ± 3.7 and 130 ± 3.2 ms in

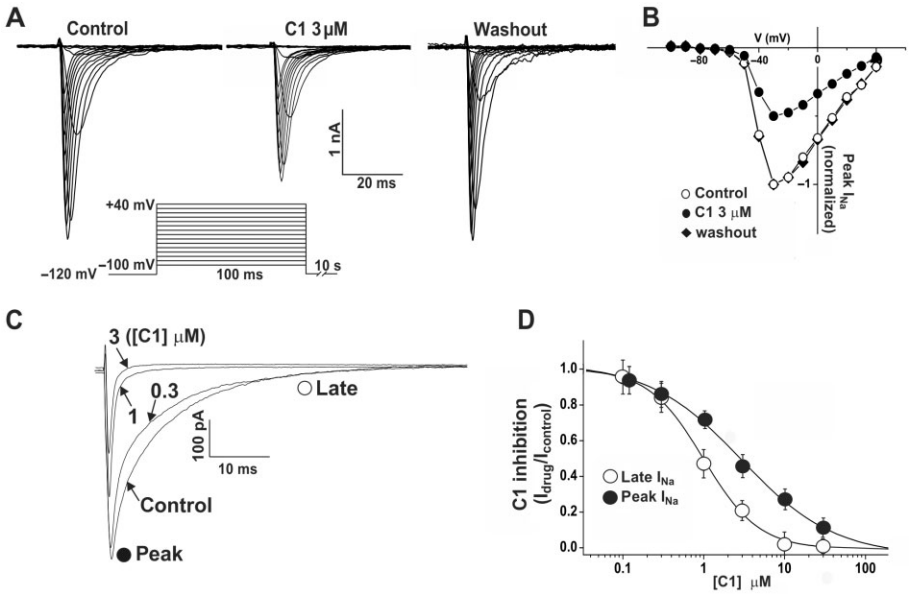

Figure 4

Effect of C1 on $Na_v1.5$ currents. 3 nmol·L^{-1} ATX-II was added to induce the late sodium current component where late current recordings are indicated. (A) Representative traces of effects on $Na_v1.5$ current with control, on application of 3 μmol·L^{-1} C1, and after washout. (B) Current–voltage curves on application of 3 μmol·L^{-1} C1 and after washout, normalized to control values. (C) Representative traces of control and the effect of application of 0.3, 1 and 3 μmol·L^{-1} C1. (D) Concentration–inhibition curve of the effect of C1 on peak and late $Na_v1.5$ current (late current IC_{50} = 1.1 μmol·L^{-1}, peak current IC_{50} = 3.2 μmol·L^{-1}, n = 4–7 cells for each concentration).

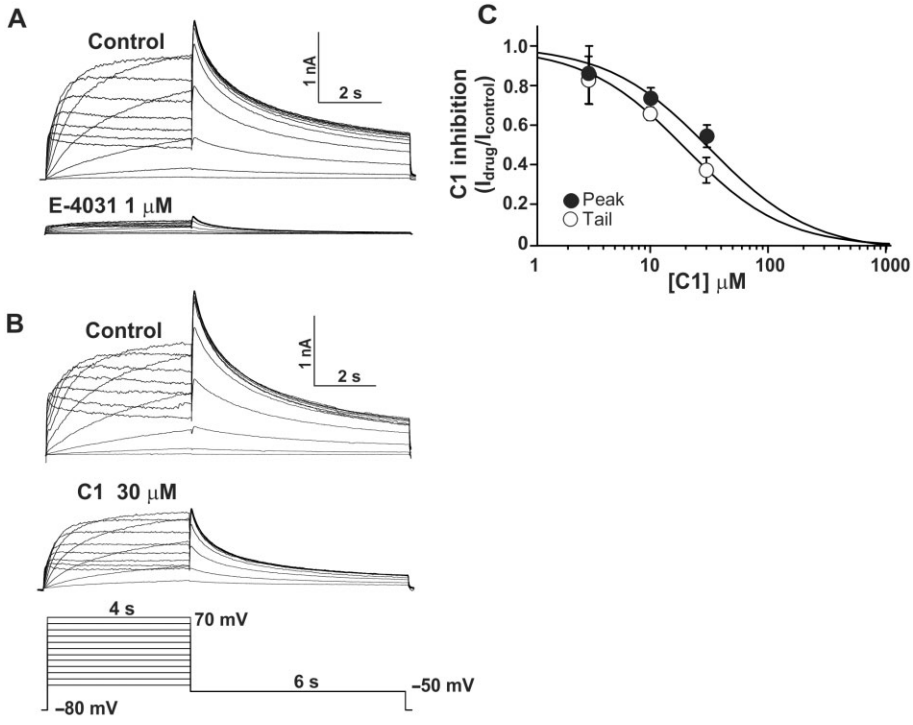

Figure 5

Effects of C1 on hERG currents. (A) Identification of hERG currents by inactivation at positive holding potential, tail currents and inhibition by 1 μmol·L^{-1} E-4031. (B) Representative traces before and after application of 30 μmol·L^{-1} C1. (C) Concentration–inhibition curve of the effect of C1 on hERG peak and tail currents (peak current IC_{50} = 30 μmol·L^{-1}, tail current IC_{50} = 25 μmol·L^{-1}, n = 4–6 cells for each concentration).

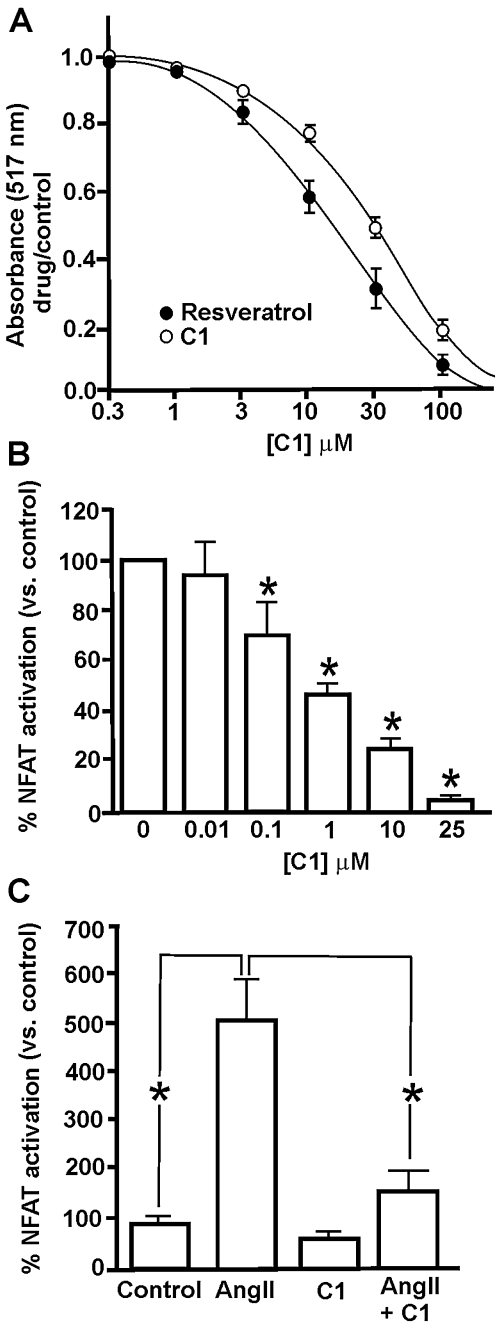

Figure 6

The antioxidant and NFAT-inhibitory effects of C1. (A) Antioxidant capacity of C1 and resveratrol expressed as a fraction of control H_2O_2 fluorescence. (B) Grouped concentration NFAT-inhibition data for C1. *$P < 0.05$, significantly different from control values; ANOVA. (C) C1 significantly inhibits AngII-mediated NFAT activation in neonatal rat ventricular myocytes. *$P < 0.05$, significantly different from control values; ANOVA.

conscious dogs (n = 5; at basic cycle lengths of 150 and 300 ms respectively). Rapid right atrial pacing for 6–7 weeks resulted in a significant decrease of right AERP, as AERP decreased below 80 ms in all five animals, and were defined as 79 ms for the following reason: the lower adjustable limit for S1–S2 intervals in the pacemakers available for this study was 80 ms, therefore the exact AERP after 7 weeks of rapid atrial pacing and immediately before C1 administration could not be determined (measured AERP values were less than 80 ms in all dogs at both basic cycle lengths (BCLs) as an S1–S2 interval of 80 ms still evoked a P wave), although statistical comparison of AERP values was not possible. AERP measurements following C1 administration yielded 87.5 ± 2.50 ms at 150 ms BCL in four animals (in one animal AERP was less than 80 ms) and 90 ± 3.16 ms at 300 ms BCL, respectively, following 0.3 mg·kg^{-1} C1; and 90.0 ± 3.16 ms at 150 ms BCL, 100 ± 3.16 ms at 300 ms BCL, respectively, following 1 mg·kg^{-1} C1 (n = 5; with the exception of AERP measurement at 150 ms BCL following 0.3 mg·kg^{-1} C1).

The incidence of AF was not influenced by administration of C1 (86.4 ± 6.4% in control vs. 71.2 ± 15.7% and 66.4 ± 15.7% following 0.3 and 1 mg·kg^{-1} C1, respectively, both $P >$ 0.05). However, as typical ECG recordings and grouped data show, the total duration of AF was significantly reduced by 1 mg·kg^{-1} C1 administration (Figure 8B–D) and the average duration of AF episodes was also significantly decreased by 1 mg·kg^{-1} C1 (Figure 8A–D). These results clearly demonstrate *in vivo* efficacy of C1 against AF in conscious dogs. HPLC analysis of blood plasma collected within 5 min of i.v. bolus injection showed that we obtained concentration ranges of 0.32–0.79 and 0.7–3.0 μmol·L^{-1} for the 0.3 and 1 mg·kg^{-1} doses respectively.

None of the investigated doses exhibited any QT interval prolonging effect in five conscious dogs, yielding 261.7 ± 9.37 ms in control vs. 260.9 ± 8.77 ms (P = 0.15) following 0.3 mg·kg^{-1} C1 and 257.3 ± 9.05 ms (P = 0.55) following 1 mg·kg^{-1} C1.

Discussion

Ionic effects of C1

As decreased atrial action potential refractory period contributes to the development of AF, the design of atrial-specific repolarization-delaying drugs has received much recent attention (Tamargo *et al.*, 2009; Dobrev and Nattel, 2010). Specifically, inhibition of the I_{Kur}/K_v1.5 repolarizing potassium channel that is predominantly expressed in atria rather than ventricle (Wang *et al.*, 1993; Gaborit *et al.*, 2007; Tamargo *et al.*, 2009) has emerged as an attractive therapeutic target (Tamargo *et al.*, 2009). Results generated in this study on recombinant K_v1.5 and human atrial myocyte I_{Kur} currents indicate that C1 is an effective inhibitor of this atrial-specific ion channel. The calculated IC_{50} values for K_v1.5 channels are in the 0.11–0.36 μmol·L^{-1} range for late and peak K_v1.5 current inhibition, which are 180- to 600-fold lower than observed for the parent molecule, resveratrol (66 μmol·L^{-1}). Furthermore, these IC_{50} values are 10- to 30-fold lower than those reported for the recently developed AF drug vernakalant that is in clinical use in Europe (Camm *et al.*, 2012). Further analysis revealed that C1 is a more effective K_v1.5 channel inhibitor at a higher stimulatory frequency of 3 Hz compared with 1 Hz, indicating that C1 may display increased inhibitory efficacy in fibrillatory tissue. Therefore, our results indicate that C1 has a favourable pharmacological

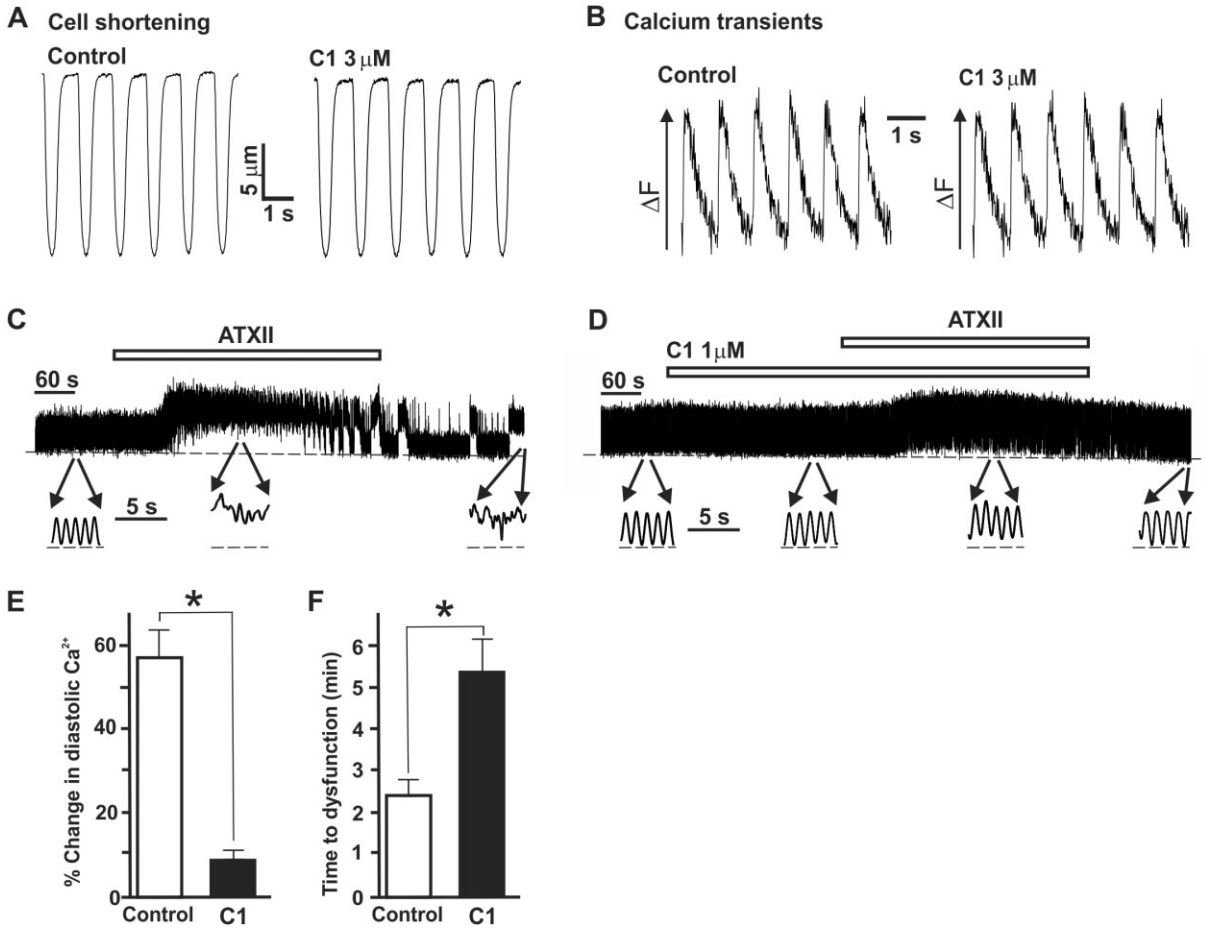

Figure 7

Effect of C1 on excitation–contraction coupling in rat ventricular myocytes. (A) Effect of 3 μmol·L^{-1} C1 on diastolic resting cell length and peak systolic contractility. (B) Representative calcium transient recordings of the effect of 3 μmol·L^{-1} C1 on transient amplitude. (C) Effect of 3 nmol·L^{-1} ATX-II on diastolic calcium transient amplitude. (D) Effect of 1 μmol·L^{-1} C1 pretreatment on ATX-II-induced diastolic maximal calcium transient amplitude. * $P < 0.01$, significantly different from control, $n = 5$ cells). (E) Column–graph representation of the effect of 1 μmol·L^{-1} C1 pretreatment on ATX-II-induced diastolic maximal calcium transient amplitude. *$P < 0.05$, significantly different from control values; paired Student's t-test (F) Effect of 1 μmol·L^{-1} C1 on onset of ATX-II-induced calcium transient dysfunction (C1 = 5.5 ± 0.8 min, control 3.1 ± 0.4), *$P < 0.05$, significantly different from control values; paired Student's t-test $n = 10$ cells).

and frequency-dependent inhibitory profile for $K_v1.5$ currents that are both desirable properties for novel AF therapeutic agents.

Recent research has suggested that inhibition of peak and late atrial sodium currents may also be an attractive strategy to suppress AF (Burashnikov *et al.*, 2007; Burashnikov and Antzelevitch, 2012). Inhibition of peak sodium current in a frequency-dependent manner may reduce the occurrence of premature action potentials and reduce the incidence of AF. Whereas the induction of the $Na_v1.5$ late current may not only increase the risk of early after depolarization (EAD)-induced arrhythmias but also lead to excessive sodium loading within cells that may contribute to chronic calcium loading that is considered to be a primary trigger for EADs and calcineurin-mediated activation of the NFAT gene transcriptional pathway that contributes to maladaptive hypertrophic remodelling observed in chronic AF (Lin *et al.*, 2004). Previous research from our group reported that the C1 parent molecule resveratrol preferentially inhibited late over peak $Na_v1.5$ currents (Wallace *et al.*, 2006; 26 μmol·L^{-1} vs. 77 μmol·L^{-1} respectively). Our results indicate that C1 inhibits the peak and late components of recombinant human heart $Na_v1.5$ currents more potently than resveratrol with IC_{50}s of 3.2 and 1.1 μmol·L^{-1} respectively. While we did not directly compare atrial and ventricular sodium currents, these findings warrant further investigation with respect to atrial-specific sodium channel inhibition by C1. However, results from ventricular myocytes indicate that C1 does not affect EC coupling at concentrations in the low micromolar range. In contrast, C1 was effective at reducing calcium-transient abnormalities caused by late sodium current induction with ATX-II. These findings suggest C1 will not interfere with ventricular EC coupling at concentrations effective at inhibiting atrial $K_v1.5$ and peak/late sodium channel currents.

Inhibition of the hERG ($K_v11.2$) potassium channel is a highly undesirable property of any new drug as significant

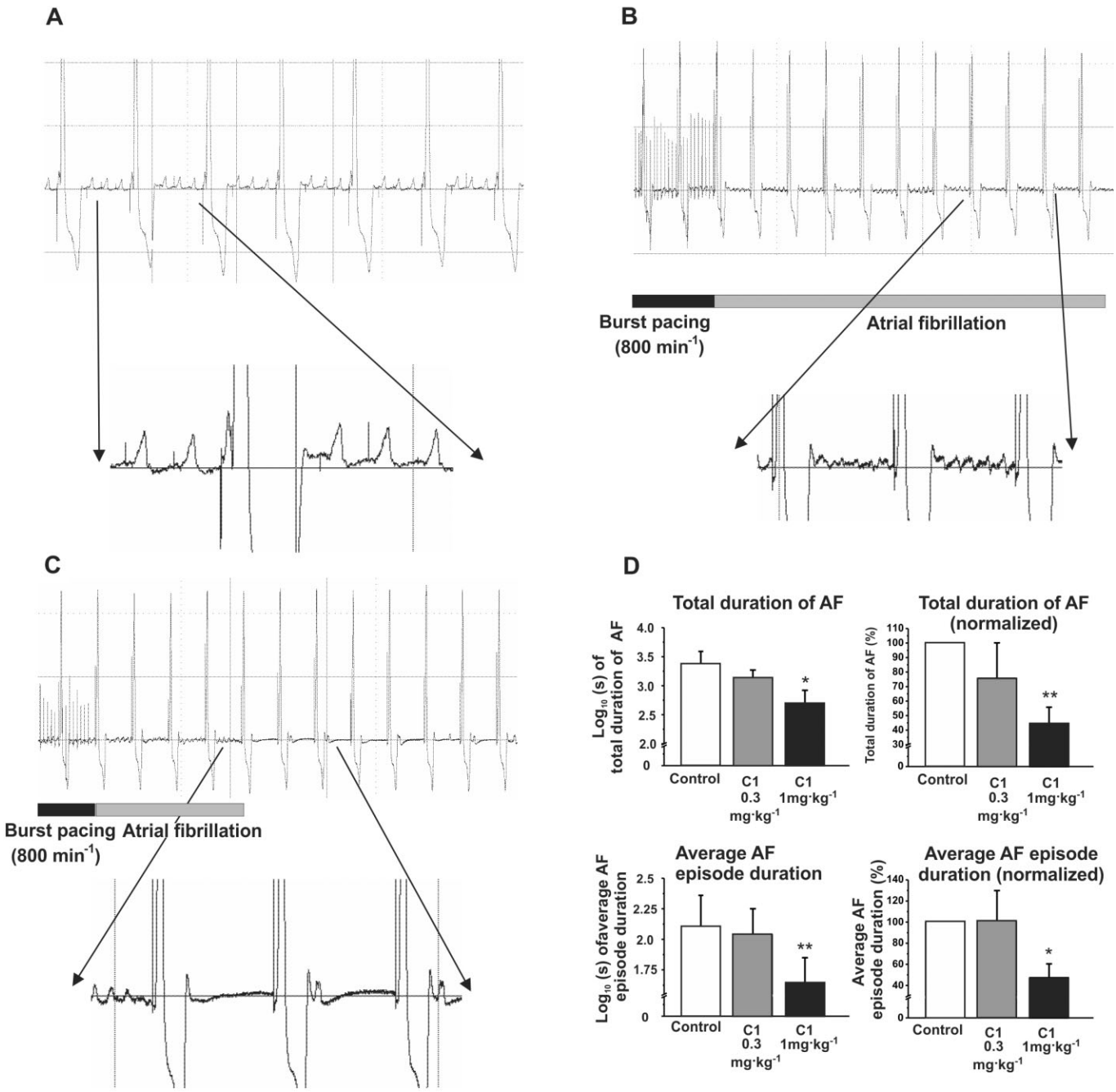

Figure 8

Effect of C1 administration on experimental atrial fibrillation (AF) in conscious dogs. (A) Representative ECG recording from a conscious dog showing right atrial tachypacing at 400 min^{-1}. (B) Representative ECG recording from a conscious dog exhibiting AF following right atrial 800 min^{-1} burst pacing (control). (C) Representative ECG recording from a conscious dog with a short duration of AF in response to right atrial burst pacing following 1 $mg{\cdot}kg^{-1}$, i.v., C1 administration. Note P wave reoccurrence after cessation of AF on the magnified inset. (D) Grouped data show that 1 $mg{\cdot}kg^{-1}$ C1 administration significantly reduced the total duration of atrial fibrillation and the average duration of AF episodes in conscious dogs. *$p < 0.05$, **$p < 0.01$; significantly different from control values, one way ANOVA, n = 5 animals

hERG inhibition can lead to excessive action potential prolongation and life-threatening Torsades des Pointes arrhythmias (Sanguinetti and Tristani-Firouzi, 2006). Accordingly, we tested the effects of C1 on recombinant hERG channel whole-cell currents and observed that C1 is a weak inhibitor of peak and tail hERG currents with IC_{50} values of 30 and 25 $\mu mol{\cdot}L^{-1}$ respectively. These results indicate that C1 is unlikely to exhibit any QT prolongation via hERG channel inhibition at concentrations shown to be effective at inhibiting $K_v1.5$, $Na_v1.5$ and I_{KACh} channels.

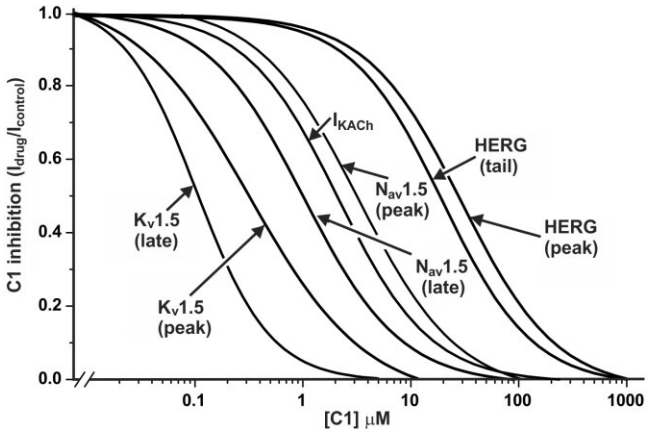

Figure 9

Concentration–inhibition curves of the effects of C1 on $K_v1.5$, I_{KACh}, $Na_v1.5$, and hERG currents. $K_{v1}.5$: Late current IC_{50} = 0.11 μmol·L^{-1}, peak current IC_{50} = 0.36 μmol·L^{-1}. I_{KACh}: IC_{50} = 1.9 μmol·L^{-1}. $Na_v1.5$: Late current IC_{50} = 1.1 μmol·L^{-1}, peak current IC_{50} = 3.2 μmol·L^{-1}. hERG: Tail current IC_{50} = 25 μmol·L^{-1}, peak current IC_{50} = 30 μmol·L^{-1}. Data replotted from Figures 1–5.

With respect to the overall inhibitory profile on ion channels tested in this study, C1 displays marked selectivity in the following order of IC_{50} values $K_v1.5$ (late) < $K_v1.5$ (peak) < $Na_v1.5$ (late) < I_{KACh} < $Na_v1.5$ (peak) << hERG (tail) < hERG (peak; Figure 9). These results are summarized in Figure 9 and indicate that C1 displays a favourable ion channel inhibitory profile that merits further characterization as a potential treatment for AF.

Non-ionic effects of C1

The generation of ROS in the fibrillating atria is likely to contribute to the disturbances in the ionic and non-ionic milieu observed in AF (Carnes *et al.*, 2001; Mihm *et al.*, 2001). Furthermore, $K_v1.5$ activity is increased in the presence of the ROS H_2O_2 (Caouette *et al.*, 2003). Resveratrol exhibits a widely described antioxidant capacity that contributes to its well-documented biological effects (Brisdelli *et al.*, 2009; Dolinsky and Dyck, 2011; Wu and Hsieh, 2011; Chung *et al.*, 2012). Our results show that C1 possesses an antioxidant capacity similar to that of resveratrol. While the concentrations of C1 required to yield significant quenching of H_2O_2 are higher than those exerting its $K_v1.5$ and $Na_v1.5$ inhibitory effects, C1 levels may accumulate in the plasma-membrane as resveratrol is known to partition into lipid membranes leading to significant tissue accumulation (Sale *et al.*, 2004). Furthermore, binding of C1 to $K_v1.5$ channels may additionally contribute to inhibition of $K_v1.5$ currents in the presence of ROS by decreasing the ROS-induced activation (Caouette *et al.*, 2003).

Activation of the NFAT transcription pathway is an important signalling pathway that contributes to the initiation of maladaptive hypertrophy and chronic AF (Cha *et al.*, 2004; Wilkins *et al.*, 2004) and activation and nuclear translocation of NFAT is increased in AF (Lin *et al.*, 2004). Our previous research indicated that resveratrol inhibited NFAT nuclear translocation and reduced phenylephrine-induced hypertrophy in cardiac myocytes (Chan *et al.*, 2008). Interestingly, our present results also indicated that C1 inhibited basal and angiotensin-II-induced NFAT activation at similar concentrations (IC_{50} ~ 1 μmol·L^{-1}) to resveratrol. While this effect may not play a major role in the reduction of AF in the acute setting, it may become more important in the prevention of transient AF conversion into chronic AF as the atria becomes remodelled.

In vivo efficacy

To test the *in vivo* efficacy of C1, we used a model of AF in chronically instrumented, conscious dogs, where electrophysiological changes favouring the development of AF were elicited by chronic rapid right atrial pacing (Figure 8A). Atrial tachypacing in dogs is an established AF model leading to electrical and structural remodelling (Morillo *et al.*, 1995; Gaspo *et al.*, 1997). Atrial tachycardia and flutter have also been shown to lead to similar atrial remodelling in humans (Franz *et al.*, 1997). Therefore, the significantly decreased duration of AF (Figure 8B–D) and possibly the increased AERP following C1 administration provides *in vivo* evidence that C1 may be beneficial in the treatment of certain forms of AF in patients.

Interestingly, treatment with C1 (0.3 and 1 mg·kg^{-1}, i.v.) did not reduce the incidence of burst-induced AF in this study; however, it significantly reduced the duration of AF episodes. Selective and non-selective I_{Kur} blocking compounds have been shown to reduce both the incidence and the duration of AF (Ford and Milnes, 2008). However, one must consider the electrical and structural remodelling of the atria following chronic AF in patients and thtat following chronic rapid atrial pacing in animal models (Nattel *et al.*, 2007). Remodelling can alter the efficacy and effective doses of compounds developed for the treatment of AF. Although no change in I_{Kur} density was previously observed in a tachypaced dog AF model (Yue *et al.*, 1997), the down-regulation of I_{Kur} has been demonstrated in humans with chronic AF (Van Wagoner *et al.*, 1997; Bosch *et al.*, 1999; Caballero *et al.*, 2010). Such down-regulation of $K_v1.5$ currents in this model, if present, might have influenced the *in vivo* effect of C1, making the I_{Kur} current-blocking effect less important in this particular model. Other compounds blocking I_{Kur} that we recently investigated in our laboratory (unpublished data) in the same model (with similar IC_{50} values for I_{Kur}) significantly decreased the incidence of burst-induced AF in i.v. doses of 3 mg·kg^{-1} or higher. A recent review summarizing data on the cardiac electrophysiological effects of novel I_{Kur} blockers also found that they were effective against AF induction in i.v. doses of 1–3 mg·kg^{-1} and higher (Ford and Milnes, 2008). In the present study, due to limits in the synthesized amount of C1 (required for several parallel *in vitro* studies), it was not feasible to investigate C1 *in vivo* at a dose of 3 mg·kg^{-1}.

Importantly, C1 did not prolong the QT interval in conscious dogs in the present study, suggesting that C1 is unlikely to provoke ventricular arrhythmias based on repolarization disturbances. These latter findings support our results on weak C1 hERG channel inhibition and a lack of effect of C1 on EC coupling in single ventricular myocytes. As this animal model represents AF in the setting of previously remodelled atria leading to an increased induction of AF, the pronounced acute effects observed with C1 are likely to be

mediated by its ion channel inhibitory and antioxidant properties rather than inhibition of NFAT activation. Whether chronic treatment with C1 reduces maladaptive remodelling remains to be determined by long-term studies.

Limitations of the study

While the effects of C1 on human atrial cell I_{Kur} were measured, the effects of C1 on human atrial cell action potentials were not studied due to the limited supply of atrial appendage tissue and the limited number of viable cells following enzymic digestion. For practical reasons, all ionic currents were measured at room temperature (20–22°C) rather than at 37°C. Moreover, we acknowledge that the cardiac rhythm status of the donor could influence the electrophysiological response to C1 in human atrial cells; however, no information was available on the cardiac rhythm status of the patients whose atrial tissue samples were used in this study. In the conscious dog model of AF, the AERP could not be measured below 80 ms as this is the shortest S1–S2 duration that can be set in the human pacemakers used in this study. The conscious animal experimental set-up did not allow us to directly measure AERP as that would have required anaesthesia and thoracotomy. Therefore, the exact statistical evaluation of the possible AERP-prolonging effect of C1 was not performed in this study.

In conclusion, the aetiology of AF is complex, and a number of ionic and non-ionic pathways contribute to the initiation and maintenance of AF (Franz *et al*., 1997; Lin *et al*., 2004). Therefore, inhibition of more than one of these pathways is likely to provide greater anti-fibrillatory efficacy than one pathway alone (Ehrlich and Nattel, 2009; Dobrev and Nattel, 2010; Dobrev *et al*., 2012). Our results indicate that the resveratrol derivative C1 is an effective inhibitor of several potential targets involved in AF development and confirmed that C1 is also effective at reducing the duration of AF episodes in a model of inducible AF in dogs.

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Conflict of interest

The authors declare no conflicts of interest. It should be noted that C1 and derivatives thereof are the subject of a US Provisional Patent application filed in May 2013.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

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Appendix S1 Chemical synthesis of compounds C1–4 and effects of C1 on recombinant IK_1 currents.