Testing novel pharmacological strategies for the management of atrial fibrillation in a large animal experimental model

PhD Thesis

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List of Abbreviations

AP	action potential
APD	action potential duration
AERP	atrial effective refractory period
AF	atrial fibrillation
AV	atrioventricular
ATX-II	sea anemone toxin
BCL	basic cycle length
bpm	beats per minute
CaMKII	calcium/calmodulin kinase 2
CHA_2DS_2-VASc	congestive heart failure, hypertension, age≥75 (doubled), diabetes, stroke (doubled), vascular disease, age 65-74, sex category (female)
CI	contraindication
CKD	chronic kidney disease
CRT	cardiac resynchronisation therapy
DAD	delayed afterdepolarization
EAD	early afterdepolarization
EC coupling	excitation-contraction coupling
ECG	electrocardiogram
ERP	effective refractory period
ESC	European Society of Cardiology
HAS-BLED	hypertension, abnormal renal/liver function (1 point each), stroke, bleeding history or predisposition, labile INR, elderly (≥65), drugs/ alcohol concomitantly (1 point each)
hERG	human ether-a-go-go-related gene
HF	heart failure
$I_{Ca,L}$	L-type calcium current
IHD	ischemic heart disease
I_{K1}	inward-rectifier potassium current
$I_{K,ACh}$	acetylcholine-regulated inward-rectifier potassium current
I_{Kr}	rapid delayed-rectifier potassium current

I_{Ks}	slow delayed-rectifier potassium current
I_{Kur}	ultrarapid delayed-rectifier potassium current
$I_{Na,late}$	late voltage-sensitive sodium current
I_{to}	transient outward current
LA	left atrium
LQTS	long QT syndrome
NCX	sodium-calcium exchanger
NFAT	nuclear factor of activated T-cells
NOAC	non-vitamin K oral anticoagulant
PKA	protein kinase A
PM	pacemaker
PV	pulmonary vein
PVI	pulmonary vein isolation
RA	right atrium
RAA	right atrial appendage
RyR2	ryanodine receptor 2
RV	right ventricle
SERCA2a	sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase 2a
SR	sinus rhythm
TdP	torsades de pointes chaotic ventricular tachycardia

Summary of the Thesis

Atrial fibrillation (AF) is the most frequently encountered chronic arrhythmia, associated with increased morbidity and mortality due to thromboembolic complications and concomitant heart failure. Its incidence and prevalence is rapidly increasing with the aging of the population. There is a great unmet need for safer and more effective pharmacological AF therapy, since drugs currently used for rhythm control may significantly increase the risk for Torsades de Pointes (TdP) arrhythmias due to their ventricular electrophysiological effects; can promote adverse vascular events; can exhibit reduced efficacy in persistent AF; and the most effective antiarrhythmic drug, amiodarone, has serious extracardiac side effects following chronic administration. In addition, as part of the pathological electrical remodeling in AF, the expression of numerous ion channels and exchangers is altered that can modify the arrhythmia substrate and increase triggered activity resulting in AF to become self-sustaining, and remodeling also significantly alters potential drug targets.

One of the possible approaches to improve pharmacotherapy of AF is the identification of drug targets ideally expressed only in atrial tissue, since atrial selective ion channel modulation would be devoid of ventricular proarrhythmic adverse effects. In addition, another approach has also emerged in recent years: the parallel modulation of different ion channels and cellular pathways implicated in the initiation and maintenance of atrial fibrillation.

As the first step in the studies described in this PhD thesis, a large animal model of experimental AF based on chronic right atrial tachypacing was established in our laboratory. In the first set of experiments, the atrial selective $I_{K,ACh}$ blocker tertiapin-Q (TQ) significantly reduced the incidence of right atrial burst induced AF, reduced the duration of AF episodes, increased right atrial effective refractory period in conscious dogs subjected to chronic right atrial tachypacing. These effects were compared to those of two drugs used in the clinical management of AF, propafenone (Class IC) and dofetilide (Class III). In right atrial trabeculae isolated from these dogs, TQ prolonged the action potential duration (APD) at all percentages of repolarization, while dofetilide only prolonged APD_{90}. Propafenone increased conduction time but did not influence the APD. The second set of experiments evaluated the *in vitro* and *in vivo* effects of Compound 1 (C1), a novel multifunctional resveratrol derivative developed by our collaborators. C1 possessed I_{Kur}, $I_{K,ACh}$, $I_{Na,peak}$, $I_{Na,late}$ blocking effects, inhibits NFAT and had antioxidant effects similar to resveratrol. In conscious dogs C1 significantly reduced

the total and average duration of AF episodes while prolonging the atrial effective refractory period (AERP). Importantly, neither TQ nor C1 prolonged the QT interval in conscious dogs.

In summary, these results suggest that atrial selective $I_{K,ACh}$ blockers may play an important role in the future pharmacological management of AF, and C1 can be a starting point for further development of compounds modulating multiple targets for improved pharmacological treatment of AF.

1. Introduction

1.1. Atrial fibrillation – epidemiology, definition, classification, causes and consequences

Atrial fibrillation (AF) is the most common cardiac arrhythmia. AF is rarely life-threatening by itself, but it may lead to very serious complications such as cardioembolic stroke or other, "peripheral" thromboembolism (Wolf et al. 1991) and heart failure (HF) (Wang et al. 2003). It can occur at any stage of life, but its incidence and prevalence is increasing with aging and it is more common in developed countries. In the European Union, the prevalence of AF is 1.7% in women, 1.3% in men among the 55-59 years old, and 16.1% in women, 24.2% in men who are at least 85. The prevalence will likely double by the year of 2060 (Krijthe et al. 2013). Another estimate predicts 14-17 million AF patients in Europe by 2030 with a yearly incidence rate of 120 000-215 000. This increase is partly due to the better and earlier diagnosis of the so-called "silent" or "lone" AF (Zoni-Berisso et al. 2014). Thus, AF and the co-existing and common risk factor-related cardiovascular and other morbidities and their joint complications will impose a huge public health and economic burden on an already aging society.

AF is characterized by very rapid and chaotic atrial electrical activity with the accompanying unsynchronized subtle contractions of groups of myocytes rather than the coordinated systolic activity of the whole atria. Its frequency is variable but usually >300 "beats" per minute (bpm), so the cycle length <200 msec. AF is traditionally called "arrhythmia absoluta" since the absolutely irregular ventricular rhythm that follows: not all atrial stimuli are conducted through the atrioventricular (AV) node because of its longer effective refractory period (ERP), and its conduction velocity is also variable for several reasons (e.g. changes in autonomic tone). The very rapid supraventricular activation therefore does not lead to the same rapid ventricular rate (otherwise it could be quickly lethal as it would possibly occur in the case of a patient with accessory AV pathways) (Pappone et al. 2014). Nevertheless, in the majority of cases the heart rate is irregular and indeed tachycardic (>100 bpm at rest), which can cause hemodynamic-related symptoms in a short time, then tachycardia-induced cardiomyopathy can develop in the long run.

Currently, AF is classified according to its duration. AF is paroxysmal when it terminates within 7 days (either spontaneously or after medical intervention), persistent when it lasts >7 days, long-standing persistent when continues for >1 year, and AF is permanent in case the patient and/or the clinician decides not to follow rhythm control strategy, i.e. conversion of AF to normal sinus rhythm (SR) and maintenance of SR proactively (Kirchhof et al. 2016). It is important that long-standing persistent and permanent AF are not necessarily pathophysiologically distinct. Most patients start with short paroxysms (which are usually undetected) then develop more and more sustained episodes in parallel with disease progression, so the clinical classification may be used as "staging" at the same time.

The main risk factors of AF are advanced age, male gender, obesity, tall stature and lean body mass (Fenger-Grøn et al. 2017), arterial hypertension, hypercholesterolemia and atherosclerosis, diabetes mellitus, chronic kidney disease (CKD), chronic obstructive pulmonary disease, sleep apnea syndrome, vigorous training, and smoking (Andrade et al. 2014). The most common causes (and also risk factors) of AF are ischemic heart disease (IHD), coronary artery disease, valvular heart disease, cardiomyopathy of any origin, acute and chronic heart failure (Maisel and Stevenson 2003; Wang et al. 2003), congenital heart disease, genetic predisposition ("familial" AF) (Christophersen et al. 2013), hyperthyroidism, metabolic/storage diseases involving the heart, cardiac inflammation of any kind, cardiac surgery ("postoperative" AF), sustained atrial pacing (by e.g. pacemaker (PM) or cardiac resynchronization therapy (CRT) device), toxic and/or drug adverse effects (e.g. alcohol, adenozine, sympathomimetics, corticosteroids, cardiac glycosides) (Kaakeh et al. 2012).

Some of these AF promoting diseases occur at the same time. They progress and interact with each other, so their contribution to AF development can be dynamic and individually diverse. Vice versa, AF can promote/deteriorate some of these maladies. Furthermore, AF itself is also generally deemed as a promoting factor in the development of its own substrate. It is highly possible that we cannot consider AF patients to be a homologous cohort. Therefore, there is a growing expectation towards the research and cardiology communities to specify and describe particular AF phenotypes, whereby their key mechanisms responsible for the arrhythmia can be identified in the future (Heijman et al. 2016; Schotten et al. 2016).

1.2. Atrial arrhythmogenic remodeling

Due to the official restriction on the volume of the thesis, primarily well-established and consistent (e.g. animal vs. human studies) factors of AF development are discussed here. Like any other sustained tachyarrhythmia, AF requires two prerequisite phenomena, the so-called arrhythmic "substrate" and the arrhythmia "trigger". Atrial fibrillation associated remodeling of the atria leads to atrial APD shortening (Franz et al. 1997) and triangulation, with consequently shortened atrial effective refractory period ("substrate"). The trigger event is usually ectopic activity (e.g. originating from the pulmonary veins) and initiates the atrial tachycardia or AF episode and the substrate (electrical then structural remodeling as well) makes its maintenance possible. Delayed afterdepolarizations (DADs) can be caused by abnormal Ca^{2+} handling (see later) in diastole (in phase 4) and can lead to Na^{+} channel activation if reaching the threshold potential. The shortened APD/ERP and abnormal/irregular conduction (blocks; ischemia, fibrosis) are the two main reentry promoting factors. Substantially decreased ERP (as one of the results of remodeling) stabilizes multiple reentries thereby contributing to the maintenance of AF.

Changes in the density and/or function of sarcolemmal ion channels together with maladaptive alterations in Ca^{2+} homeostasis, signal transduction, and protein modulation constitute electrical remodeling. These processes play a key role in AF initiation and occur at the early stage of the disease. The first AF episodes are rather self-limiting and last seconds/minutes: AF is paroxysmal. The causes of AF further exist and the AF episodes per se contribute to the progression of the disease (Wijffels et al. 1995). As more pronounced changes, such as alterations in gene expression, accumulation of subepicardial adipose tissue and its fibrotic infiltration, and inflammation (Haemers et al. 2017) emerge (structural remodeling), the AF episodes last much longer (days-months) and rarely cease spontaneously: clinically persistent AF develops.

In the early phase of AF, paroxysmal episodes generated in the pulmonary veins (PVs)-left atrium (LA) junctions arise in most patients. These sites have some distinct properties which promote rapid ectopic impulse generation as well as re-entry. The strands of atrial cardiomyocytes stretch into the lumen of the PVs, the networks of myocytes in the PV ostia are rather disorganized with sudden changes in fibre orientation compared to nearby atrial tissue and with irregular connections between them. They possess some particular

electrophysiological properties – such as reduced I_{K1} and $I_{Ca,L}$ current, increased I_{Kr}, I_{Ks}, and I_{SK} (Ehrlich et al. 2003). These lead to shorter APD/ERP, AP triangulation, and slower conduction because of the reduced resting membrane potential (smaller I_{K1}) decreases Na^+ channel availability in the PV-LA junctions, which altogether form a basis for re-entry. The prominent role of the PVs in paroxysmal AF is underpinned by the fact that catheter ablation of these sites (PV isolation; PVI) has the greatest efficacy in lasting maintenance of SR in most patients (Haissaguerre et al. 1998). Moreover, the dominant frequencies in these patients during an AF episode are greater in the LA than in the RA revealed by an electrophysiological study also suggesting the left-sided origin of the arrhythmia (Lazar et al. 2004).

Whatever is the source of the AF paroxysms, high atrial frequencies cause rapid (at first protective) intracellular changes. High excitation rates (with the accompanying Ca^{2+} entry during systoles) would lead to increased contraction force and Ca^{2+}-overload, therefore the cells try to defend themselves by quick L-type Ca^{2+} current ($I_{Ca,L}$) reduction. Initial increase in intracellular – subsarcolemmal – Ca^{2+} concentration promptly leads to reduced $I_{Ca,L}$ (direct Ca^{2+} binding to the channel), later lasting reduction of $I_{Ca,L}$ density as well as increase in important outward currents (e.g. inward-rectifier K^+ current - I_{K1}, constitutively active ACh-regulated inward-rectifier K^+ current - $cI_{K,ACh}$) occurs through different parallel pathways involving activation of Ca^{2+}-dependent intracellular factors: Ca^{2+}/calmodulin, calcineurin/nuclear factor of activated T-cells (NFAT) (Lin et al. 2004), and calpain among others.

Intracellular Ca^{2+}-handling is also altered in AF (Nattel and Dobrev 2012): the sarcoplasmic reticulum stores a greater amount of Ca^{2+} and this enlarged content tends to be released abnormally. Protein kinases such as calcium/calmodulin kinase 2 (CaMKII) and protein kinase A (PKA) play key roles in those alterations. The sarcoplasmic reticulum is the main Ca^{2+} storing cellular organelle which normally releases its content during systole via ryanodine receptors (RyR2; cardiac form) and is reloaded by the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA2a; cardiac form). SERCA2a works under the control of an inhibitory molecule – phospholamban (PLN). AF causes overactivity of CaMKII and PKA leading to Ca^{2+} overload of the sarcoplasmic reticulum (phosphorylated PLN separate from SERCA2a) and RyR2 dysregulation. This may cause abnormal Ca^{2+} release events during diastole which in turn can generate transient inward current (the excess Ca^{2+} is exchanged for

extracellular Na^+ via the sodium/calcium exchanger - NCX - in a ratio of 1:3) and can lead to DAD generation. In addition, phosphorylation by CaMKII alters the function of prominent ion channels ($I_{Na,late}$, $I_{Ca,L}$, I_{to}, I_{K1}) as well.

Arrhythmogenic ion channel remodeling refers to the notion that the function of several ion channels is altered in parallel with AF development and contributes mainly to the changes in the shape and duration of atrial AP seen in AF, namely APD shortening (thereby shorter ERP) and AP triangulation that favor AF initiation and perpetuation. Reduction of $I_{Ca,L}$ leads to shortening of phase 2 ("plateau") of AP (triangulation). The late – voltage-insensitive – component of the sodium current ($I_{Na,late}$) becomes more pronounced and – together with the reduced transient outward current (I_{to}) – can cause higher amplitude at the early part of AP (triangulation), higher subsarcolemmal Na^+ concentration during the plateau phase (opposing the Ca^{2+} extrusion by NCX), and may contribute to ERP shortening. Stronger I_{K1} is one of the most consistent findings in AF studies. $I_{K,ACh}$ is another inward-rectifier current which changes in AF. Normally, the channel (Kir3.1/3.4) is activated by acetylcholine (ACh; from the vagal nerve during parasympathetic activity) via muscarinic (M_2) receptors. In remodeled atria, a constitutively active subpopulation exists of this channel whereby a constitutive current ($cI_{K,ACh}$) can occur without parasympathetic stimulation (Dobrev et al. 2005). Increased I_{K1} and $cI_{K,ACh}$ – as active outward currents in the whole cardiac cycle – shortens APD/ERP further. The role of the ultrarapid delayed-rectifier K^+ current (I_{Kur}) is more controversial as it is down-regulated but its function seems to be highly related to other ion currents and may have a greater role during high frequencies. The increased slow component of the delayed rectifier potassium current (I_{Ks}) also accumulates at higher frequencies thus may significantly contribute to APD shortening (Caballero et al. 2010).

As AF persists, several other changes with more pronounced effects emerge. Atrial hypertrophy and later dilatation occurs with relative microvascular rarefaction (ischemia) (Corradi et al. 2012), increased wall-stretch (proarrhythmic), and adverse effects on hemodynamics and hemostasis develop. Adipose tissue infiltrates the atrial wall and inflammatory cells and fibroblasts also appear (Haemers et al. 2017). Activated fibroblasts mainly contribute to extracellular remodeling and fibrosis, and may electrically interact with myocytes (Andrade et al. 2014). All these alterations constitute the massive and likely irreversible structural remodeling featuring the late stage of – incurable – AF.

1.3. Summary of the current management of atrial fibrillation

According to the current guidelines of the leading cardiological societies (Kirchhof et al. 2016, January et al. 2014), treatment of AF has several mainstays: antithrombotic treatment for prevention of ischemic stroke and other thromboembolism, management of the ventricular rate by either rhythm or rate control, alleviation of the symptoms (partly overlapping with the previous one), and management of co-morbidities and risk factors (including the so-called upstream therapies).

The research and development activities are very intensive in three areas of AF management: thromboembolic prophylaxis – e.g. novel/non-vitamin K oral anticoagulant drugs (NOACs); invasive interventions for rhythm control (catheter ablation); and pharmacological rhythm control. The first two are not in the scope of the thesis (albeit important), nevertheless key developments have emerged and entered clinical stage in those fields in recent years.

It is of utmost importance in newly diagnosed AF to consider and commence anticoagulation if needed. Risk scores have been validated like CHA_2DS_2-VASc and HAS-BLED for stroke- and bleeding risk stratification, respectively (Lip et al. 2010; Pisters et al. 2010). In recent years, NOACs (dabigatran, rivaroxaban, apixaban, edoxaban) have been approved for non-valvular AF patients. Briefly, the minority of patients should not receive antithrombotic medication – female with a CHA_2DS_2-VASc score 1, male with 0 – all the others should be anticoagulated if not contraindicated. NOACs are at least non-inferior to vitamin K antagonist drugs in terms of stroke prophylaxis and seemed to be superior regarding to bleeding events. There is no need for INR control and are much less contraindications, drug-drug-, and drug-food interactions with the NOACs. Moreover, selective antidotes are emerging, e.g. idarucizumab for dabigatran. For the elderly and more co-morbid patients who have a higher HAS-BLED score and moreover, who had a previous major bleeding event, there is a “palliative” solution for stroke prevention: left atrial appendage closure (Korsholm et al. 2017).

Pulmonary vein isolation (PVI) is a catheter-based intervention whereby under anesthesia, the 4 PVs are electrically isolated in the left atrium (LA) either with radiofrequency (RF)- or cryo-ablation technique (Kuck et al. 2016) in order to exclude the ectopic foci of these veins so converting AF to sinus rhythm (SR) and maintaining it. Several other additional ablations

in the LA (linear lines, rotor-targeting, etc.) have been proposed and examined but so far clear superiority of one technique over the others has not been established (Kirchhof and Calkins 2017). PVI may be enough for patients with paroxysmal AF, but for ones with greater AF burden, catheter ablation rather conveys symptomatic improvement than true rhythm control (Wynn et al. 2016).

Other interventional procedures are AV ablation with pacemaker implantation in case of pharmacologically unmanageable ventricular rate, or AV ablation with CRT device implantation when CRT indicated (CHF). In the end, we must not forget that invasive procedures potentially have serious adverse events, and the patients' preferences should also be considered, who might prefer taking 1-2 pills each day to undergoing an operation and having at least temporarily devices implanted in their hearts.

From a physiological point of view, the best option would be the very early detection and diagnosis of AF (in respect of "AF begets AF"; Wijffels et al. 1995), then the restoration and the maintenance of sinus rhythm (SR) when applicable (there are ongoing multicenter clinical trials addressing this issue; see e.g. Kirchhof et al. 2013).

Several antiarrhythmic drugs have been used for converting AF to SR (pharmacological cardioversion) and for SR maintenance (rhythm control), but unfortunately none of them is ideal. Effectiveness of conversion is ranging from ~40% (placebo) up to 90% (flecainide, propafenone, amiodarone, vernakalant) in paroxysmal AF, however, success rate is much worse in long-lasting persistent (in other words pathophysiologically more complex) AF. Apart from efficacy, safety – cardiac and extracardiac adverse drug reactions – issues are evident, thus antiarrhythmic drugs have several contraindications (CI):

- Class IA drugs: disopyramide, procainamide (adverse reactions: hypotension/negative inotropic, Lupus-like syndrome; CI: AV block, myasthenia gravis, CKD) can be pro-arrhythmic in patients with IHD;
- Class IC propafenone (CI: cardiogenic shock, hypotension, unstable HF, AV block, bradycardia, bronchospastic disorder, myasthenia gravis, Brugada-sy) and flecainide (CI: cardiogenic shock, recent acute myocardial infarction, 2^{nd}-3^{rd} degree AV block) can also be pro-arrhythmic in IHD;
- Drugs with class III effect prolong cardiac repolarization thereby lengthen the QT interval on surface electrocardiogram (ECG); the potentially lethal drug-induced torsades de

pointes tachycardia (TdP) may occur in susceptible people: sotalol, dofetilide, ibutilide, amiodarone, dronedarone;

- The multichannel-blocker amiodarone (adverse reactions: bradycardia, AV block, hypotension) is although one of the most effective 'anti-tachycardia' agent, has rather rare but very serious and irreversible extra-cardiac side effects (pulmonary fibrosis, hyper- or hypothyroidism, hepatotoxicity, cornea and skin coloration);
- The amiodarone-like dronedarone without the iodine-containing moiety (CI: CKD, liver disease, bradycardia, LV systolic dysfunction, symptomatic HF, permanent AF) have failed to achieve a 'breakthrough' (Kaess and Ehrlich 2016);
- The recently introduced intravenous (IV) vernakalant (CI: recent acute coronary syndrome, hypotension, bradycardia, symptomatic HF, severe aortic stenosis, sinus node dysfunction, 2^{nd}-3^{rd} degree AV block) is only indicated for acute cardioversion so far.

Therefore, an ideal AF rhythm control medication is still missing. Huge efforts have been invested to develop an agent which does not target ventricular or extra-cardiac tissue thus being much safer, that optimally has several atrial targets (because of the 'multifaceted' nature of AF pathophysiology) in order to tackle several underlying remodeling mechanisms thus achieving greater efficacy.

Unfortunately, the majority of AF patients are rather elderly who live with significant cardiovascular and other co-morbidities. The older the patient, the longer the duration of the arrhythmia before detection, and the more cardiovascular diseases the patient has, the less the likelihood of the successful and permanent rhythm control is. Independently of whether rhythm control strategy is followed (paroxysmal/persistent AF) or not (permanent AF), rate control strategy is always necessary for symptomatic improvement and protection of the ventricles. Briefly, "lenient" rate control (resting heart rate <110 bpm) is acceptable as an initial therapeutic approach (Kirchhof et al. 2016; Van Gelder et al. 2010).

1.4. Novel pharmacological strategies for the future management of atrial fibrillation – multi-target approach and upstream therapies

In order to develop a new potent and safe "anti-AF" compound, one reasonable way would be targeting ion channels or mechanisms that exclusively or predominantly exist in the atria and are known to be involved in the development and progression of AF. Such atrial-selective

targets are $K_v1.5$ (I_{Kur} current) and $K_{ir}3.1/3.4$ ($I_{K,ACh}$) channels, and "atrial-selective" inhibition of I_{Na} and the role of its late component ($I_{Na,late}$) in AF have also considered.

On the one hand, the voltage-sensitive Na^+ channel (I_{Na}) may possess partly different functional properties in atria compared to ventricles since the existing different electrophysiological features (e.g. level of resting membrane potential, AP morphology) of these sites even in healthy heart, on the other hand, the late – non-inactivated – component of the Na^+ current ($I_{Na,late}$) is implicated in AF. The very rapid excitation in the atria during AF allows a "use-dependence" approach of pharmacological intervention, indeed, some cardiovascular drugs which also have $I_{Na,peak}$ and/or $I_{Na,late}$ inhibitory effect (e.g. ranolazine) may be beneficial in the reduction of AF burden even at lower concentrations in order to mitigate ventricular adverse effects (Antzelevitch and Burashnikov 2009).

The ultrarapid delayed-rectifier K^+ current I_{Kur} is an atrial-selective member of the voltage-sensitive K^+ channel subfamily. Its pore-forming subunit ($K_v1.5$) is down-regulated in remodeled atria (however there are contradictory findings) (Yue et al. 1997; Van Wagoner et al. 1997), but I_{Kur} is proposed to retain its role in repolarization thus contributing to the observed reduction of atrial APD/ERP in AF, since its function appears to be highly dependent on pacing rate and its interaction with other ion currents is also important. I_{Kur} inhibition indeed lengthens atrial APD on RA samples from paroxysmal AF patients at high stimulation rates (Ford et al. 2016).

Increased parasympathetic autonomic tone (at rest, during sleep or postprandially) is known to be a cause of an AF subtype ("vagal" AF). Acetylcholine activates M_2-receptors which open the $K_{ir}3.1/3.4$ channels via $G_{i/o}$ proteins, and the emerging repolarizing current ($I_{K,ACh}$) contributes to APD/ERP shortening thus promoting re-entrant mechanisms. Moreover, as mentioned before, a constitutively active component of $I_{K,ACh}$ current manifests parallel with AF occurrence. Following the discovery of tertiapin (Jin and Lu 1999), the first highly selective $K_{ir}3.1/3.4$ blocker, $I_{K,ACh}$ inhibition has been suggested as a promising target for AF management (Hashimoto et al. 2006, Yamamoto et al. 2014).

More recently, other potassium channels emerged as a potential drug target and they are just briefly mentioned here. The functional activity of the small-conductance Ca^{2+} activated K^+ channels ($K_{Ca}2.1$, $K_{Ca}2.2$, $K_{Ca}2.3$.) is more prominent in the atria thus demanding further research regarding their role in AF. Some of the two-P domain K^+ channels (e.g.

K_{2P}2.1/TREK-1, K_{2P}3.1/TASK-1) were implicated in AF pathophysiology and are currently investigated as well.

Unfortunately, due to the rather complex etiology in the majority of AF patients, a new antiarrhythmic agent with one "pure" atrial-selective target will unlikely be widely effective. Therefore, an ideal medicine for AF termination and for long-lasting maintenance of SR thereafter should intervene in several parts of the pathophysiology of AF. Such properties could be: "use-dependent" inhibition of atrial-selective ion currents contributing to the arrhythmogenic electrical remodeling (e.g. I_{Kur}, $I_{K,ACh}$), interruption of the progression of structural remodeling (e.g. inhibition of calcineurin/NFAT signaling, "reverse remodeling" and "upstream therapy"), reversal of the AF substrate, and absence of any adverse ventricular/proarrhythmic side effects.

2. Aims of the Studies

1. To establish a large animal *in vivo* atrial fibrillation model in the Department of Pharmacology and Pharmacotherapy for the testing of potential novel drug candidates for AF management, and to provide remodeled atrial tissue for AF mechanism studies for *in vitro* investigations.

2. To test the effects of an atrial selective ion channel blocker in the established model on AF incidence, on the duration of AF episodes, on the right atrial effective refractory period (AERP) in conscious dogs with AF and investigation of the mechanisms of action on atrial trabeculae isolated form these animals.

3. To investigate experimental compounds with multiple mechanisms of action with parallel targeting of ion channels and cellular pathways implicated in AF in the chronic right atrial tachypacing induced conscious dog atrial fibrillation model.

3. Materials and Methods

3.1 *In vivo* model of induced AF

3.1.1. Ethical issues

The experiments complied with the Guide for the Care and Use of Laboratory Animals (U.S.A. NIH publication No 85-23, revised 1996). The protocols had been approved by the Ethical Committee for the Protection of Animals in Research of the University of Szeged, Szeged, Hungary (I-74-5-2012); and by the Department of Animal Health and Food Control of the Ministry of Agriculture (XIII/1211/2012).

3.1.2. Chronic atrial tachypacing induced AF in conscious dogs

Adult male Beagle dogs (n=5; Compound 1) (n=6; dofetilide, propafenone, tertiapin-Q) 24-26 months old, weighing 12-16 kg were used for the experiments. The dogs were accommodated to experimental personnel and equipment, every day for a week before the start of the studies. The pacemaker and pacemaker electrode implantation procedures were performed under ketamine (Richter Gedeon Ltd., Hungary; induction: 10 mg/kg i.v., maintenance: 2 mg/kg, every 20 min) + xylazine (CP-Pharma Handelsges, Germany; induction: 1 mg/kg i.v., maintenance: 0.2 mg/kg, every 20 min) general anesthesia and

mechanical ventilation was employed. For antibiotic coverage, amoxicillin/clavulanic acid (1000 mg/200 mg i.v.; Richter Gedeon Ltd.) and gentamicin (40 mg i.v.; Sandoz GmbH, Kundl, Austria) were given before the operation, amoxicillin/clavulanic acid (500 mg/125 mg orally, twice a day for 5 days; Augmentin 500mg/125mg®; GlaxoSmithKline Ltd., Hungary) was 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, respectively, 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 bpm, following the baseline heart rate of the dog before the operation. The pacemakers were programmed by the ICS 3000 Programmer (Biotronik Hungary Ltd., Hungary).

Following recovery from surgery (3-5 days), high frequency right atrial pacing was started at 400 bpm, 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 extra stimulus (S2), with the AERP defined as the longest S1-S2 interval that did not produce a response. AERP shorter than 80 ms could not be measured in conscious animals (pacemaker measurement limit).

3.1.3. In vivo experiments – Compound 1

Effects of Compound 1 (C1) on *in vivo* AF incidence and duration of AF as well as on AERP and QT interval were tested on five animals. On the day of the experiment atrial pacing was stopped, continuous recording of the ECG commenced using precordial leads and the AERP was measured. A control set (25 times) of 10-second-long rapid atrial bursts (800/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). After every bolus infusion, the AERP was again measured. 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 12^{th} burst and were not corrected for heart rate since QT measurements were made at heart rate set to 80 bpm (during each test) by the ventricular pacemaker in each animal. All intravenous infusions were performed using a programmable infusion pump (Terufusion TE-3, Terumo Europe, Leuven, Belgium). The ECG was recorded using precordial leads, was digitized and stored on a computer for off-line analysis using National Instruments data acquisition hardware (National Instruments, Austin, Texas, USA) and SPEL Advanced Haemosys software (version 3.2, MDE Heidelberg GmbH, Heidelberg, Germany). Experiments were performed in freely moving conscious dogs so that any effects of anesthetics on AERP and AF could be ruled out.

3.1.4. In vivo experiments – tertapin-Q, dofetilide, and propafenone

In another set of experiments (on other animal group; n=6), similarly performed tests were done in order to measure the *in vivo* effects of tertiapin-Q, dofetilide, and propafenone on right atrial tachypaced dogs. The differences are discussed briefly: a bolus infusion of vehicle in 15 min precedes the control set of 25-time burst pacing. Additional sets of atrial bursts were applied subsequent to either tertiapin-Q (Tocris Bioscience, Bristol, UK; 18 μg/kg then 56 μg/kg), or dofetilide (Sigma-Aldrich, 25 μg/kg), or propafenone (RYTMONORM, Mylan EPD Ltd., Hungary; 0.3 mg/kg then 1 mg/kg) i.v. administration. Again, for assessing dynamic changes in AERP, repeated measurements were taken before and 5 min after the administration of each bolus of vehicle/any tested agent, and at the end of each set (after the 25^{th} burst or at the end of the subsequent AF episode if induced). At least 4 days were allowed for washout between in vivo experiments with different compounds. Likewise, the incidence of AF, the total duration of AF, the average duration of AF episodes were measured and calculated along with changes in AERP and QT interval.

3.2. *In vitro* measurements

3.2.1. Action potential recordings from canine right atrial trabeculae with the conventional microelectrode technique

The dogs from the second group (n=6) of *in vivo* AF studies were used 4 days following their completion to allow washout of the last applied compound. Following sedation (xylazine, 1 mg/kg, i.v. and ketamine, 10 mg/kg, i.v.) and anesthesia (pentobarbital, Sigma-Aldrich, 30 mg/kg i.v.), the heart was rapidly removed through right lateral thoracotomy. The hearts were immediately rinsed in oxygenated modified Locke's solution containing (in mM): NaCl 128.3, KCl 4, $CaCl_2$ 1.8, $MgCl_2$ 0.42, $NaHCO_3$ 21.4, and glucose 10. The pH of this solution was set between 7.35 and 7.4 when saturated with the mixture of 95% O_2 and 5% CO_2 at 37^{o}C. Isolated right atrial trabeculae were obtained and individually mounted in a tissue chamber with a volume of 50 ml. The preparations were stimulated through a pair of platinum electrodes in contact with the preparation using rectangular current pulses of 2 ms duration. The stimuli were delivered at a constant BCL of 500 ms for at least 60 min allowing the preparation to equilibrate before the measurements were initiated. Transmembrane potentials were recorded using conventional glass microelectrodes, filled with 3M KCl and having tip resistances of 5-20 MΩ, connected to the input of a high impedance electrometer (type 309, MDE Heidelberg GmbH, Heidelberg, Germany) which was coupled to a dual beam oscilloscope. The conduction time, maximum diastolic potential, action potential amplitude, and action potential duration measured at 25%, 50% and 90% of repolarization (APD_{25}, APD_{50}, and APD_{90}, respectively) were evaluated off-line using a custom made software running on an IBM compatible computer equipped with an ADA 3300 analogue-to-digital data acquisition board (Real Time Devices Inc., State Collage, PA, USA) having a maximum sampling frequency of 40 kHz. Stimulation with a constant BCL of 500 ms was applied during the course of the experiments. We aimed at maintaining the same impalement throughout each experiment, however, in case the impalement became dislodged, adjustment was performed and the experiment continued if AP characteristics of the re-established impalement deviated less than 5% from the previous measurement. Due to the contractility of these preparations some of the impalements had to be repeated, therefore the maximum upstroke velocity (V_{max}) of the action potentials was not evaluated, and the conduction time was used to assess class I antiarrhythmic activity.

3.2.2. Cell shortening and calcium transient recordings

These were measured in rat ventricular myocytes prepared from adult Sprague-Dawley rats euthanized by an overdose of pentobarbital (150 mg/kg, i.p.). The hearts were removed and right ventricular myocytes were then obtained by enzymatic dissociation using standard protocols, which have been described previously (Bouchard et al. 1993; Light et al. 1998). Cell shortening and calcium transients with the calcium-sensitive fluorescent probe Calcium Green-1AM were measured using standard procedures as published previously (Baczkó et al. 2005; Wallace et al. 2006).

3.3. Compounds

Tertiapin-Q (Tocris Bioscience, Bristol, UK) was dissolved in distilled water for conventional microelectrode experiments (stock solution: 30 μM), and in saline for *in vivo* experiments. Dofetilide (Sigma-Aldrich, St. Louis, USA), was dissolved in DMSO to obtain a stock solution of 1 mM for microelectrode experiments, and the stock solution was diluted in saline for *in vivo* experiments. For microelectrode experiments, propafenone (Sigma-Aldrich) was dissolved in DMSO (stock solution: 10 mM), and in *in vivo* experiments, propafenone was applied using the commercially available 3.5 mg/ml ampule (RYTMONORM, Mylan EPD Ltd., Hungary). Each stock solution was diluted prior to the actual experiment.

C1 was dissolved in 20 mL of a mixture of DMSO + β-hydroxypropyl-cyclodextrin + saline (all from Sigma-Aldrich) before administration. The concentration of DMSO was less than 0.1%.

3.4. Statistical analysis

All data are expressed as mean ± SEM. Statistical analysis was carried out using ORIGIN 8.1 (Microcal Software, Northampton, MA, USA). In whole animal studies, differences between means were compared by one-way ANOVA followed by Student's t-test. In cell-based experiments, one-way ANOVAs or paired Student's t-tests were used as appropriate. Data were considered statistically significant when $p<0.05$.

4. Results

4.1. 1st Aim: Establishment of the large animal model of chronic atrial tachypacing induced AF in conscious dogs in the laboratory

In cooperation with our colleagues (Dr. László Sághy, Dr. Róbert Pap) from the 2nd Department of Internal Medicine and Cardiology Centre, University of Szeged, the large animal model of chronic atrial tachypacing induced atrial fibrillation was established in the *In Vivo* Electrophysiology Laboratory at the Department of Pharmacology and Pharmacotherapy, University of Szeged.

The author of this thesis played an essential role in the manual establishment of the large animal model of chronic atrial tachypacing induced AF in conscious dogs in the laboratory of his supervisor, participating in setting up the model as well as taking part in almost every pacemaker and pacemaker electrode implantation surgery for the studies performed in the laboratory of his supervisor.

Figure 1 shows representative ECG recordings from conscious dogs using precordial leads at different stages of the experiments. On **panel A**, sinus rhythm is shown, while **panel B** represents an ECG recorded in a dog following pacemaker (a ventricular and an atrial) implantation and radiofrequency catheter ablation of the atrioventricular node. At this stage, the ventricular pacemakers were set to between 80 to 90/min, depending on the resting heart rate of dogs before the implantation procedures. The illustration shows ventricular rate set to 50/min, which was used during measurements of AERP for better visibility and easier identification of atrial capture. After complete recovery from surgery, the atrial pacemaker was programmed to elicit rapid right atrial pacing at 400/min for 6 to 7 weeks to allow the development of atrial remodeling monitored by frequent AERP measurements, and this atrial tachypacing is illustrated on **Figure 1**, **panel C**. Once AERP values were around 80-90 ms, atrial fibrillation was induced by atrial burst pacing for 10 seconds at the rate of 800/min in conscious dogs, as shown on **Figure 1, panel D**.

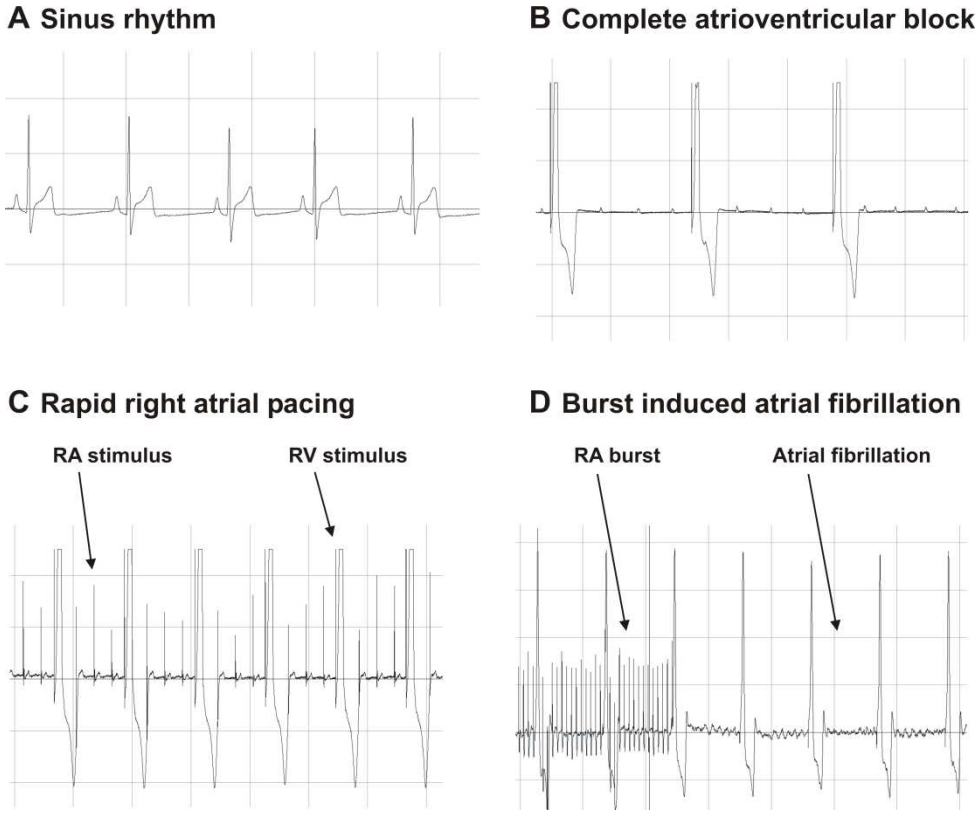

Figure 1. Representative ECG recordings from a conscious dog (control) showing **(A)** sinus rhythm (heart rate = 75/min), **(B)** complete atrioventricular block following radiofrequency catheter ablation (ventricular rate set to 50/min by ventricular pacemaker), **(C)** right atrial (RA) tachypacing at 400/min and **(D)** right atrial burst pacing (800/min) induced AF. RV: right ventricular.

Figure 2 illustrates the positioning of the radiofrequency ablation catheter by monitoring the intracardiac His-electrogram in an anesthetized dog during the surgery.

Figure 2. Positioning the catheter for AV node ablation: representative recording showing an intracardiac His-electrogram using the ablation catheter.

4.2. 2nd Aim: Effects of atrial selective $I_{K,ACh}$ inhibition on AF in conscious dogs

A promising and safer approach to more effective pharmacological AF management is the application of atrial selective compounds that are devoid of ventricular electrophysiological proarrhythmic adverse effects. The inhibition of the acetylcholine-regulated potassium current ($I_{K,ACh}$) represents such an atrial target. In the following set of experiments, I set out to investigate the effects of the $I_{K,ACh}$ inhibitor tertiapin-Q (TQ), a stable derivative of the honey bee venom toxin tertiapin, on experimental atrial fibrillation in conscious dogs. Importantly, these effects were compared in the same model to those elicited by the class IC antiarrhythmic drug, propafenone, and the class III compound, dofetilide, both used for rhythm control in the clinical management of AF. In order to gain insight into the mechanism of action of these compounds in our model, the electrophysiological effects of TQ, propafenone and dofetilide were also compared in right atrial trabeculae isolated from these dogs with AF.

4.2.1. Effects of tertiapin-Q, dofetilide and propafenone on burst-induced AF in conscious dogs

Rapid right atrial bursts at 800/min did not induce any AF in any of the animals before the commencement of chronic right atrial tachypacing. Infusion of the $I_{K,ACh}$ blocker tertiapin-Q dose dependently and robustly reduced the incidence of right atrial burst induced AF (**Fig. 3A**), the total duration of AF (**Fig. 3C**) and the average duration of AF episodes (**Fig. 3D**) in conscious dogs. The antiarrhythmic effect of tertiapin-Q was then compared to the Class III compound dofetilide and Class IC drug propafenone, both used in clinical settings for rhythm control in AF management. Both dofetilide (**Fig. 4A**) and propafenone (**Fig. 5A**) reduced AF incidence, the total duration of AF (**Figs. 4C** and **5C**) and the mean duration of AF episodes (**Figs. 4D** and **5D**). These results clearly show that tertiapin-Q exhibits marked antiarrhythmic effect against AF in conscious dogs, and this effect seemed to be stronger than those of dofetilide and propafenone.

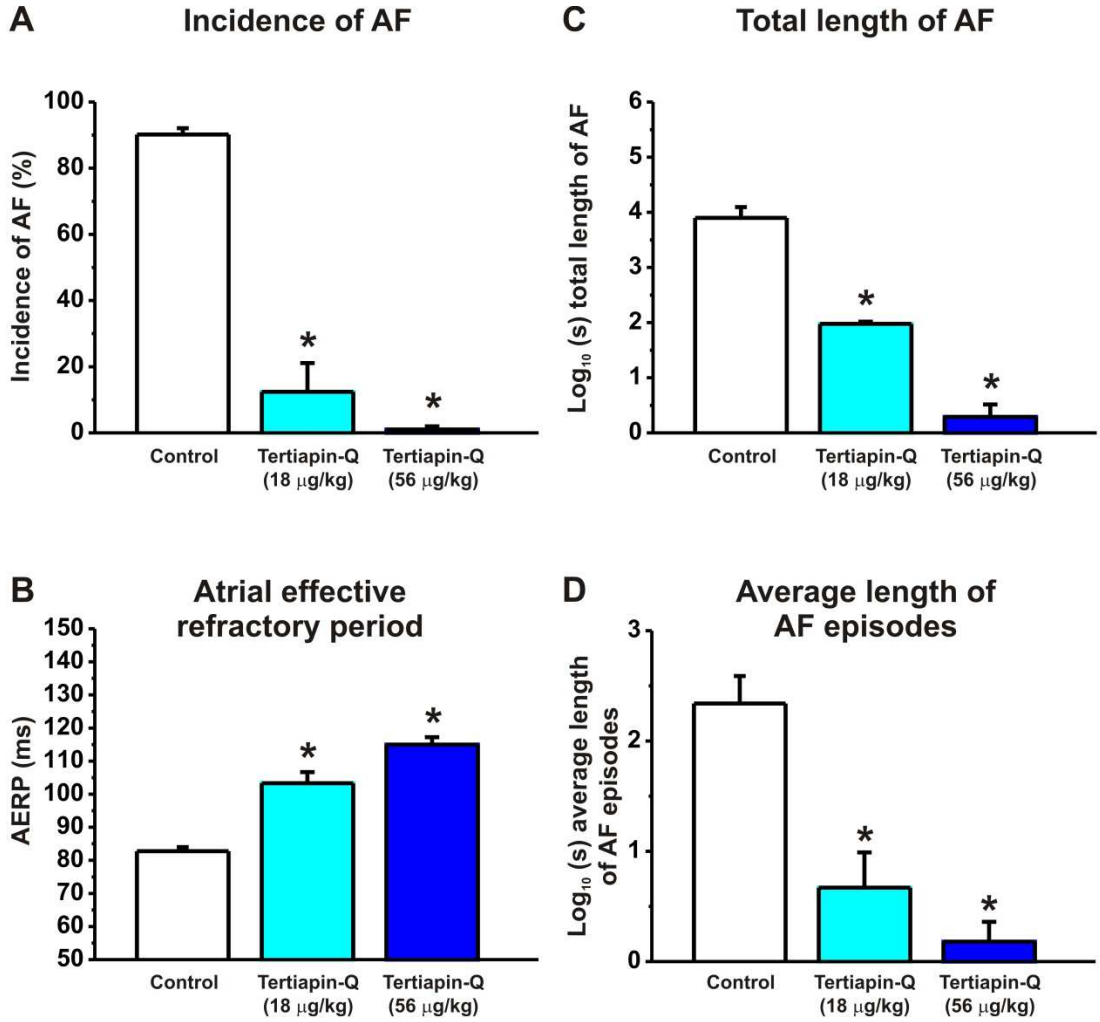

Figure 3. Effect of tertiapin-Q (TQ; 18 and 56 μg/kg, i.v.) administration on atrial tachypacing-induced experimental atrial fibrillation (AF) in conscious dogs. The data show that administration of both 18 and 56 μg/kg TQ significantly **(A)** reduced the incidence of AF, **(B)** increased the atrial effective refractory period (AERP) and decreased **(C)** the total duration of AF and the **(D)** average duration of AF episodes in conscious dogs. *$p<0.05$; n = 6. AERP shown on the figure was measured at the basic cycle length of 300 ms.

4.2.2. Effects of the $I_{K,ACh}$ blocker tertiapin-Q, the I_{Kr} blocker dofetilide and the I_{Na} blocker propafenone on right atrial effective refractory period (AERP) in conscious dogs

Before the commencement of right atrial tachypacing at 400 beats/min, right AERP was 117 ± 5.8 and 127 ± 6.4 ms in conscious dogs (n = 6; at basic cycle lengths of 150 and 300 ms respectively). Rapid right atrial pacing for 6–7 weeks markedly shortened right AERP, as shown on panel B of **Figures 3, 4,** and **5** (measured at the basic cycle length of 300 ms). AERP was significantly and dose dependently prolonged by tertiapin-Q at both cycle lengths

of 300 ms (**Fig. 3B**), and of 150 ms: 82.3 ± 1.48 ms in control vs. 93.3 ± 3.33 ms (n=6, p<0.05) following 18 μg/kg and 106.7 ± 2.11 ms (n=6, p<0.05) following 56 μg/kg, respectively). The AERP was also significantly prolonged by dofetilide (**Fig. 4B**; at 150 ms BCL: 81.0 ± 1.81 ms in control vs. 98.3 ± 3.07 ms (n=6, p<0.05) following 25 μg/kg). Only the larger dose of propafenone increased AERP at the BCL of 150 ms: 80.2 ± 0.98 ms in control vs. 85.0 ± 2.89 ms (n=6, p>0.05) following 0.3 mg/kg and 96.7 ± 3.33 ms (n=6, p<0.05) following 1 mg/kg, respectively), while the AERP was significantly increased by both propafenone doses at the cycle length of 300 ms (**Fig. 5B**).

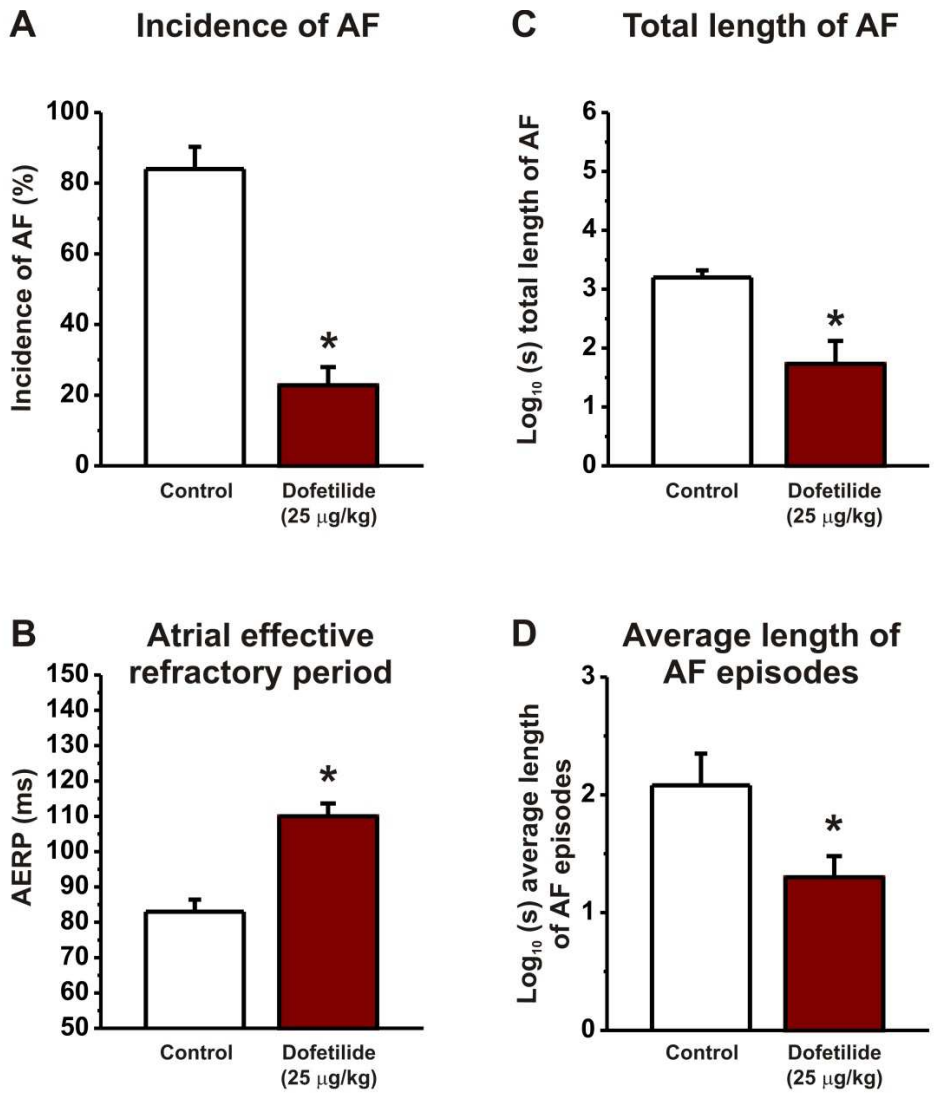

Figure 4. Effect of the administration of the class III antiarrhythmic dofetilide (25 μg/kg, i.v.) on atrial tachypacing-induced experimental atrial fibrillation (AF) in conscious dogs. The data show that dofetilide significantly **(A)** reduced the incidence of AF, **(B)** prolonged the atrial effective refractory

period (AERP), **(C)** decreased the total duration of AF and the **(D)** average duration of AF episodes in conscious dogs. *p<0.05; n = 6. AERP shown on the figure was measured at the basic cycle length of 300 ms.

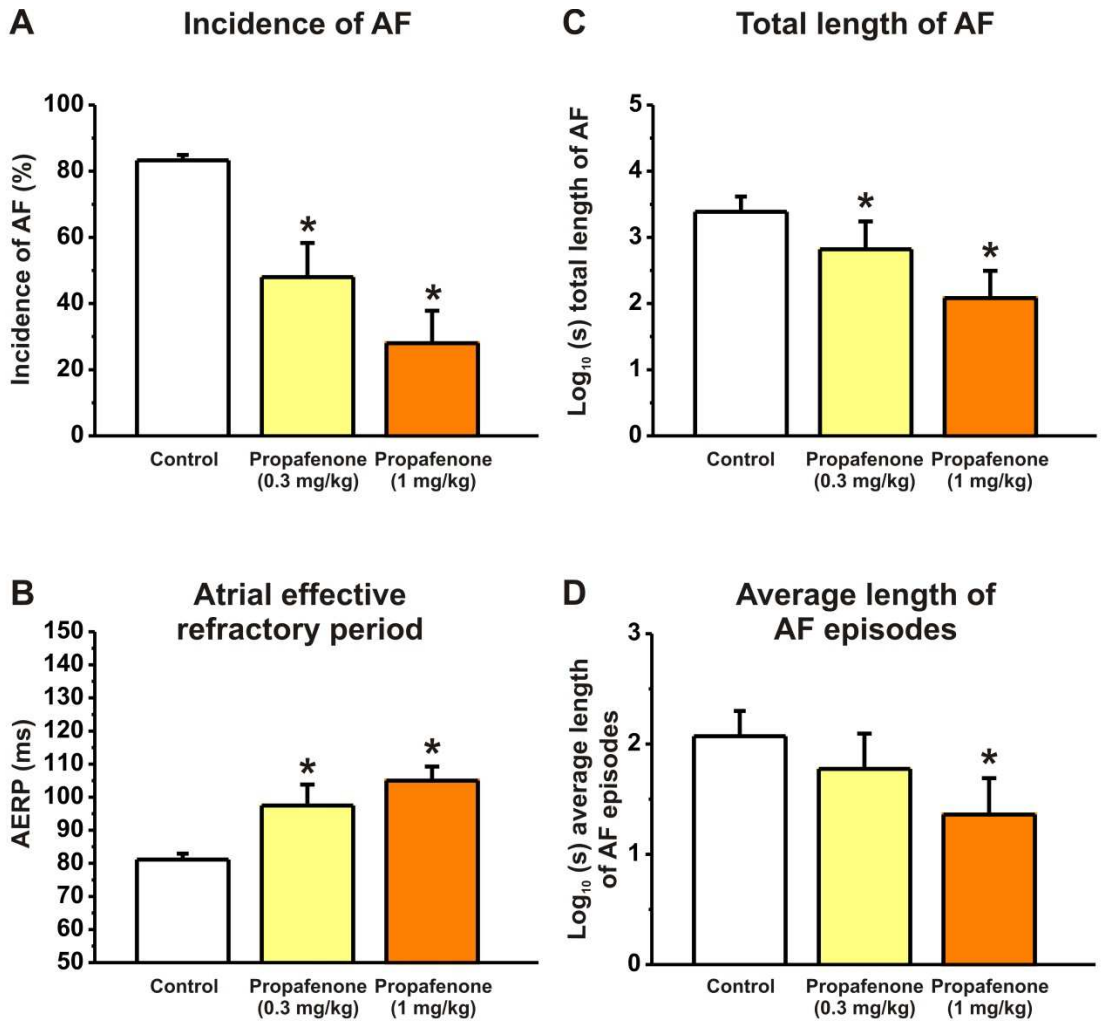

Figure 5. Effect of propafenone (0.3 and 1 mg/kg, i.v.) administration on right atrial tachypacing-induced experimental atrial fibrillation (AF) in conscious dogs. The data show that propafenone administration significantly **(A)** reduced the incidence of AF, **(B)** increased the atrial effective refractory period (AERP), **(C)** decreased the total duration of AF and the **(D)** average duration of AF episodes (only the larger dose) in conscious dogs. *p<0.05; n = 6. AERP shown on the figure was measured at the basic cycle length of 300 ms.

4.2.3. Effect of tertiapin-Q, dofetilide and propafenone on the QT interval in conscious dogs

Importantly, none of the investigated doses of tertiapin-Q prolonged the QT interval in conscious dogs, yielding 283.0 ± 10.36 ms in control vs. 281.8 ± 13.29 ms (n=6, p>0.05) following 18 μg/kg and 268.2 ± 17.75 ms (n=6, p>0.05) following 56 μg/kg, respectively.

Dofetilide (25 μg/kg) significantly prolonged the QT interval in conscious dogs, from 265.8 ± 8.68 ms in control to 302.8 ± 10.53 ms (n=6, $p<0.05$). Propafenone did not influence the duration of the QT interval: 268.0 ± 8.41 ms in control vs. 265.4 ± 9.34 ms (n=6, $p>0.05$) following 0.3 mg/kg and 272.2 ± 7.70 ms (n=6, $p>0.05$) following 1 mg/kg, respectively.

4.2.4. Effects of tertiapin-Q, dofetilide and propafenone on action potentials in atrial trabeculae isolated from dogs with AF

In order to evaluate changes in action potential parameters following chronic atrial tachypacing, in preliminary experiments from non-instrumented dogs in sinus rhythm, the following action potential parameters were measured from right atrial trabeculae: conduction time, 4.4 ± 0.3 ms; action potential amplitude, 107.1 ± 1.6 mV; diastolic potential, -87.9 ± 1.2 mV; APD_{25}, 28.7 ± 2.0 ms; APD_{50}, 64.5 ± 3.8 ms; APD_{90}, 133.5 ± 5.4 ms (n=12). These action potential duration values in uninstrumented dogs were significantly longer at all investigated percentages of repolarization than those following chronic right atrial tachypacing (see APD values following chronic atrial tachypacing as control on **Figure 6**).

Right atrial trabeculae were isolated from the dogs used for the in vivo AF studies, allowing wash-out of the last compound tested. The effects of tertiapin-Q (30 nM), dofetilide (100 nM) and propafenone (1 μM) on the action potential configuration and action potential parameters are illustrated on **Figure 6**. All measurements were performed at the cycle length of 500 ms. Tertiapin-Q significantly prolonged the action potential at all percentage of repolarization (APD_{25}, APD_{50} and APD_{90}) in right atrial trabeculae from dogs with AF (Fig. 5 bottom panel). Tertiapin-Q did not influence conduction time (4.6 ± 0.5 ms in control vs. 4.6 ± 0.3 ms following TQ, n=7, $p>0.05$), action potential amplitude (107.0 ± 1.4 mV in control vs. 107.0 ± 3.4 mV following TQ, n=7, $p>0.05$), diastolic potential (-82.9 ± 1.1 mV in control vs. -81.7 ± 1.2 mV following TQ, n=7, $p>0.05$). Dofetilide significantly prolonged the action potential duration only at 90% of repolarization (Fig. 5 bottom panel). Dofetilide did not alter conduction time (3.7 ± 1.0 ms in control vs. 3.6 ± 0.9 ms following dofetilide, n=4, $p>0.05$), action potential amplitude (109.8 ± 1.4 mV in control vs. 111.3 ± 0.8 mV following dofetilide, n=4, $p>0.05$), diastolic potential (-87.5 ± 3.4 mV in control vs. -88.3 ± 2.8 mV following dofetilide, n=4, $p>0.05$). Propafenone did not prolong the atrial action potential (**Fig. 6** bottom panel), did not influence the action potential amplitude (101.4 ± 2.7 mV in

control vs. 101.8 ± 2.2 mV following propafenone, n=5, p>0.05) or diastolic potential (-83.1 ± 0.5 mV in control vs. -84.0 ± 1.5 mV following propafenone, n=5, p>0.05), but significantly increased conduction time (3.1 ± 0.3 ms in control vs. 3.9 ± 0.3 ms following propafenone, n=5, p<0.05).

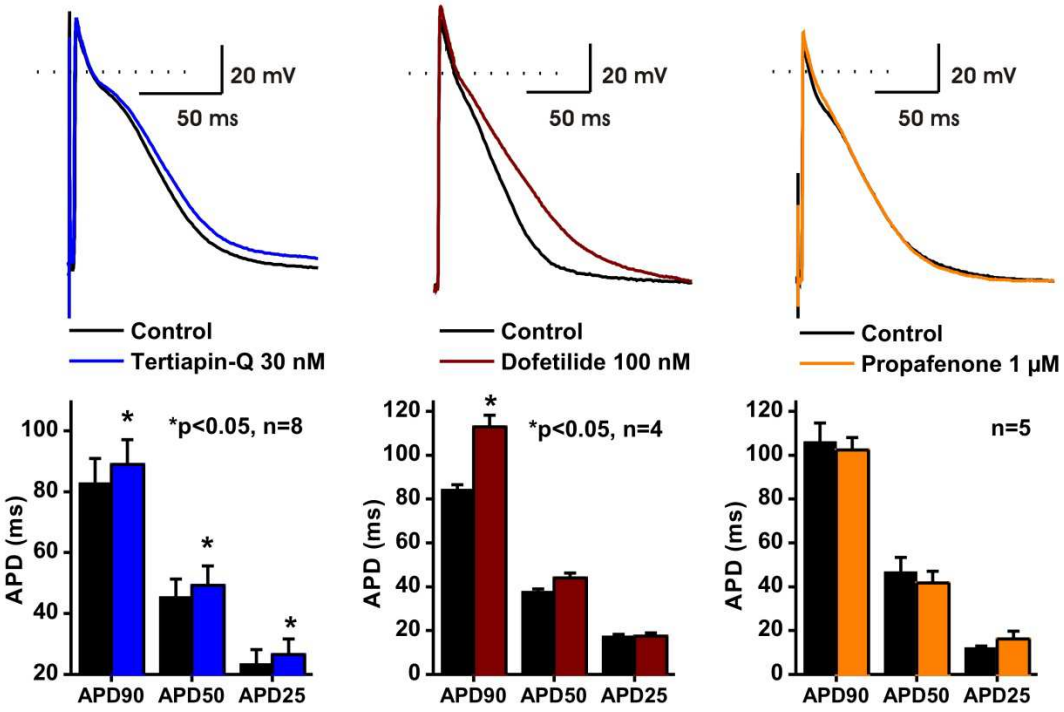

Figure 6. Effects of tertiapin-Q (TQ; 30 nM), dofetilide (100 nM) and propafenone (1 µM) on action potential durations (APD) at 90%, 50% and 25% of repolarization, respectively, measured in right atrial trabeculae isolated from dogs with AF. Top panel shows representative AP recordings and bottom panel summarizes grouped data (n=4 to 8/group). TQ prolonged APD measured at all % of repolarization. As expected, dofetilide only prolonged APD_{90} and propafenone did not influence APD. The dotted line on the top panel represents 0 mV.

4.3. 3rd Aim: Effects of a multifunctional resveratrol derivative compound on AF in conscious dogs

The complexity and large individual differences in the aetiology of AF in patients led to the suggestion that drugs targeting multiple pathways involved in AF development may be more effective. In addition to ion channels expressed only in the atria, voltage-gated sodium channels, two-pore potassium channels, oxidative stress and activation of the transcription factor NFAT have all been implicated in AF development. To date, AF drugs have been

exclusively targeted towards ion channels. However, non-ionic remodeling events also contribute to the initiation and maintenance of AF. It has been further suggested that targeting maladaptive remodeling events („upstream therapy") in AF may also be required for effective control of AF.

Therefore, in the next set of experiments I investigated the effects of a resveratrol derivative small molecule, C1 (**Fig. 7**), developed by our collaborators at the University of Alberta, Edmonton, and University of Manitoba, Winnipeg, Canada, in some *in vitro* experiments and *in vivo* on AF in conscious dogs.

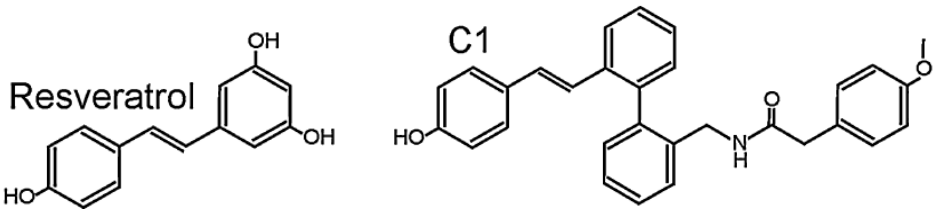

Figure 7. Chemical structures of resveratrol and the multifunctional resveratrol derivative, Compound 1 (C1).

4.3.1. Effects of C1 and the parent molecule, resveratrol on different ionic currents implicated in atrial fibrillation

The detailed characterization of the effects of C1 and resveratrol on ionic currents was carried out by our colleagues in Edmonton, Canada, however, these results must be briefly summarized here to fully appreciate the truly multifunctional nature of C1, that serves as the basis for its *in vivo* efficacy against AF. First, the inhibitory effects on $K_v1.5$ currents of four novel resveratrol derivatives were compared to resveratrol by measuring whole-cell $K_v1.5$ currents in the tsA201 cell line stably expressing $K_v1.5$ channels. Resveratrol proved to be a weak inhibitor of $K_v1.5$ currents (IC_{50} = 66 μmol/L). Four resveratrol derivatives were synthesized, and C1 was the most potent blocker of $K_v1.5$ (IC_{50}s = 0.36 μmol/L and 0.11 μmol/L for peak and late current inhibition, respectively). As **Figure 8** shows, the other related compounds (C 2–4) displayed intermediate $K_v1.5$ peak current inhibition (8.3, 10.9 and 11.2 μmol/L, respectively). Therefore, only C1 was selected for further studies.

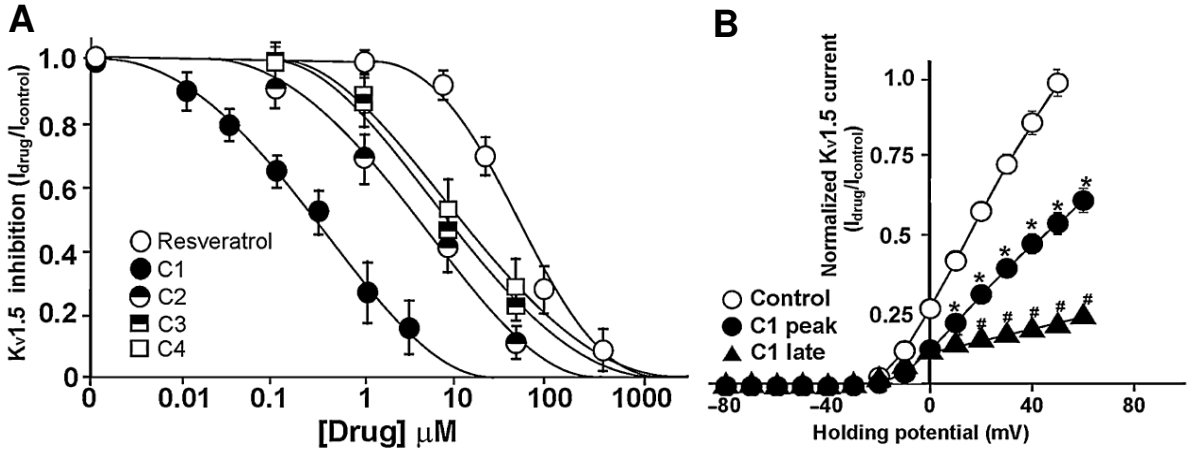

Figure 8. **(A)** Concentration-inhibition curves of the effect of resveratrol and the four resveratrol derivatives (C1–C4) on $K_v1.5$ peak current amplitude (IC_{50}s = 66.0, 0.36, 8.3, 10.9 and 11.2 μmol/L, respectively, n = 4–7 cells for each concentration). **(B)** Current-voltage curves of $K_v1.5$ peak and late currents before and after application of 0.3 μmol/L C1 compared with control. *p<0.05, #p<0.01.

In isolated single rat atrial cells, $I_{K,ACh}$ currents were induced by carbachol (**Fig. 9A**). C1 significantly inhibited carbachol-induced rat atrial $I_{K,ACh}$ currents with an IC_{50} of 1.9 μmol/L (**Fig. 9B**).

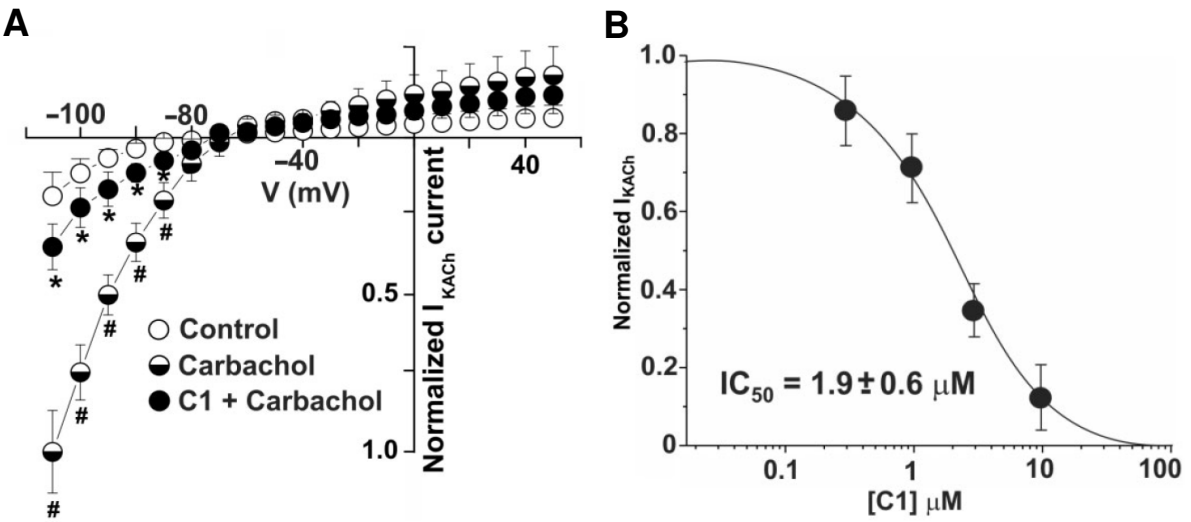

Figure 9. Effects of C1 on rat atrial $I_{K,ACh}$ currents. **(A)** Current –voltage curves of control, carbachol and carbachol + 3 μmol/L C1 (n = 5 cells, currents normalized to control values). *p<0.05, #p<0.01 compared to control values. **(B)** Concentration-inhibition curve of the effect of C1 on $I_{K,ACh}$ current (n = 4-6 cells for each concentration).

To test the effects of C1 on the peak and late recombinant sodium current, $Na_v1.5$ whole-cell currents were measured. 3 μmol/L C1 resulted in a 50% inhibition of peak current (**Fig. 10A**). To obtain concentration–response curves for peak and late $Na_v1.5$ currents, cells were treated with the sea anemone toxin (ATX-II; 3 nmol/L) to induce the late-current component and to test the inhibitory effects of C1 at different concentrations (**Fig. 10B**). C1 preferentially inhibited the late current when compared with peak current (**Fig. 10C**; IC_{50}s = 1.1 μmol/L vs. 3.2 μmol/L, respectively).

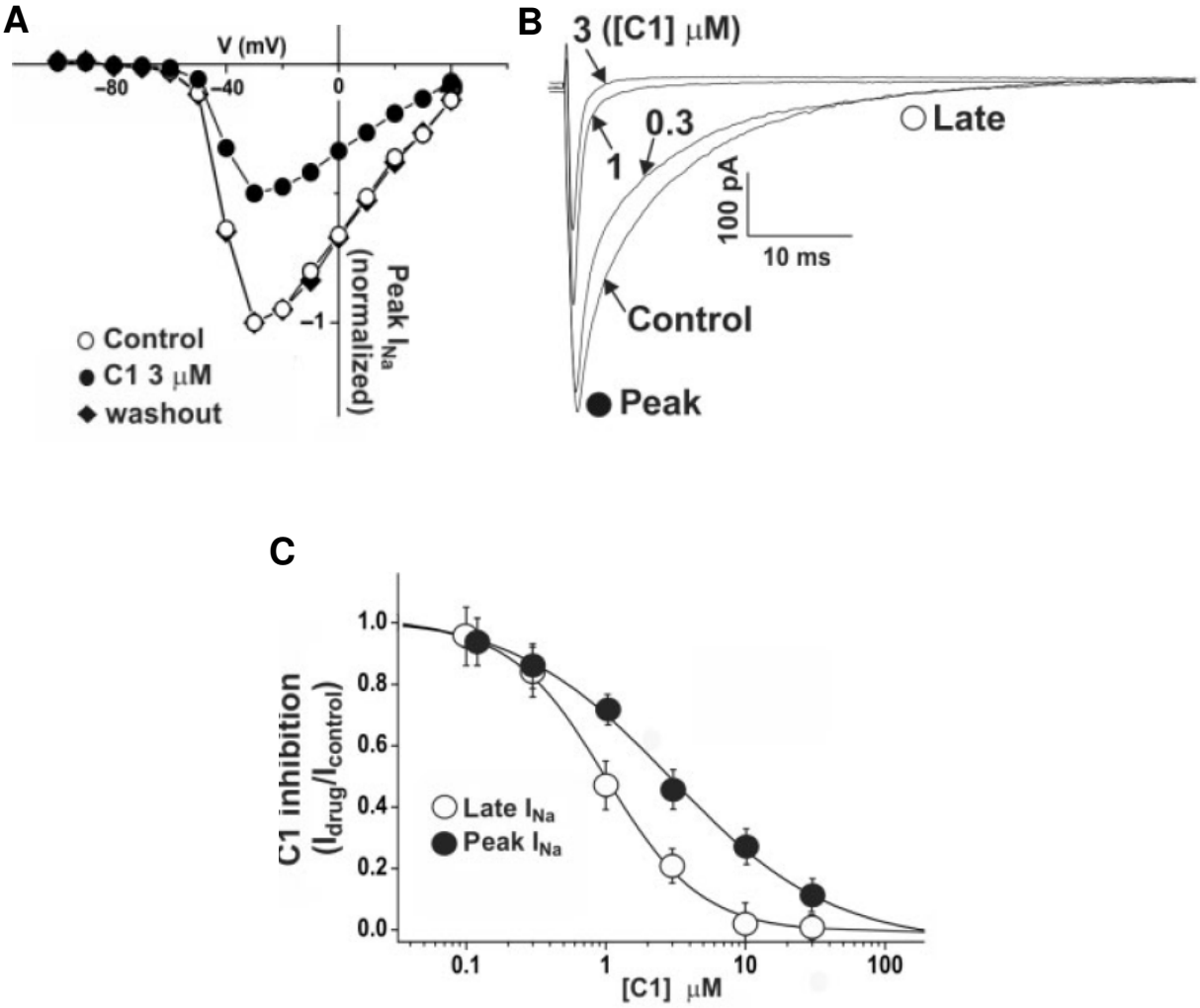

Figure 10. Effect of C1 on $Na_v1.5$ currents. 3 nmol/L ATX-II was added to induce late sodium current where late current recordings are indicated. **(A)** Current-voltage curves before and upon application of 3 μmol/L C1 and after washout, normalized to control values. **(B)** Representative traces showing control and the effect of 0.3, 1 and 3 μmol/L C1. **(C)** Concentration-inhibition curve of the effect of C1 on peak and late $Na_v1.5$ current (late current IC_{50} = 1.1 μmol/L, peak current IC_{50} = 3.2 μmol/L; n = 4-7 cells for each concentration).

Inhibition of the hERG (I_{Kr}, $K_v11.1$) may result in arrhythmogenic QT prolongation, therefore, the effects of C1 on recombinant whole-cell hERG channel currents were tested. Construction of concentration-inhibition curves revealed that C1 was a weak inhibitor of peak and tail hERG currents with IC_{50}s of 30 and 25μmol/L, respectively, and these values were 100-fold higher than those inhibiting peak and late $K_v1.5$ currents.

4.3.2. The antioxidant and NFAT inhibitory effects of C1

Antioxidant therapy has been shown to reduce the incidence of post-operative AF in patients, and reactive oxygen species (ROS) also directly activate $K_v1.5$ current. As resveratrol is a known antioxidant, we compared the antioxidant properties of C1 with resveratrol. At 10 μmol/L, resveratrol and C1 displayed significant antioxidant effects (0.59 ± 0.04 vs. 0.77 ± 0.02 of maximal DPPH 517 nm absorbance signal). At 100 μmol/L, these values were 0.09 ± 0.02 and 0.19 ± 0.02 for resveratrol and C1, respectively. It has been shown that resveratrol inhibits NFAT activation induced by phenylephrine contributing to a reduction in maladaptive hypertrophy in neonatal rat ventricular myocytes. Accordingly, the effects of C1 on NFAT activation in these cells were tested. Treatment with 0.01-25 μmol/L of C1 resulted in a significant reduction in NFAT activity when compared to no treatment (**Fig. 11A**). These results revealed that C1 significantly reduced NFAT activity at concentrations of 0.1 μmol/L and higher, with 1 μmol/L of C1 exhibiting half-maximal inhibition. C1 also significantly reduced Angiotensin-II-induced activation of NFAT (**Fig. 11B**).

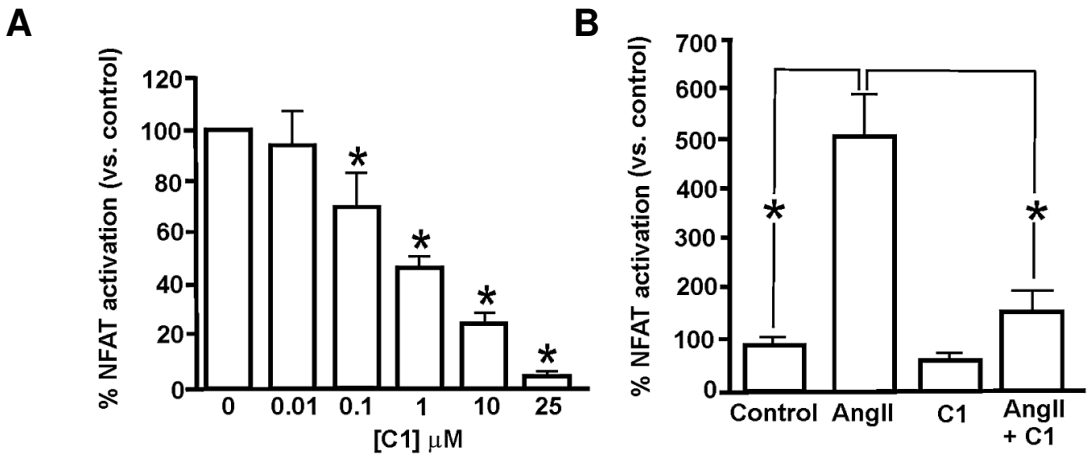

Figure 11. **(A)** Grouped concentration NFAT-inhibition data for C1. **(B)** C1 significantly inhibits Angiotensin-II-mediated NFAT activation in neonatal rat ventricular myocytes. *p<0.05; n = 6.

4.3.3. Effects of C1 on calcium transients and cell shortening in isolated rat ventricular myocytes

A desirable property of potential compounds for AF treatment is a minimal effect on EC coupling in ventricular myocytes. Single rat ventricular myocytes were field-stimulated at 1 Hz. Cell contractility and calcium transients were then measured by edge detection and calcium imaging. Application of 3 μmol/L C1 had no significant effect on diastolic resting cell length, calcium levels or peak systolic contractility or Ca^{2+} transient amplitude (**Fig. 12**).

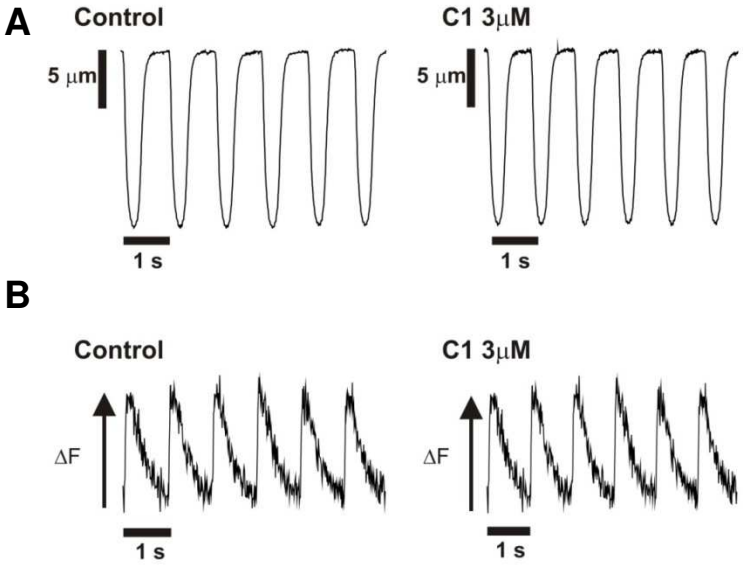

Figure 12. **(A)** C1 did not influence diastolic resting cell length and peak systolic contractility. **(B)** Representative calcium transient recordings show the lack of effect of 3 μmol/L C1 on Ca^{2+} transient.

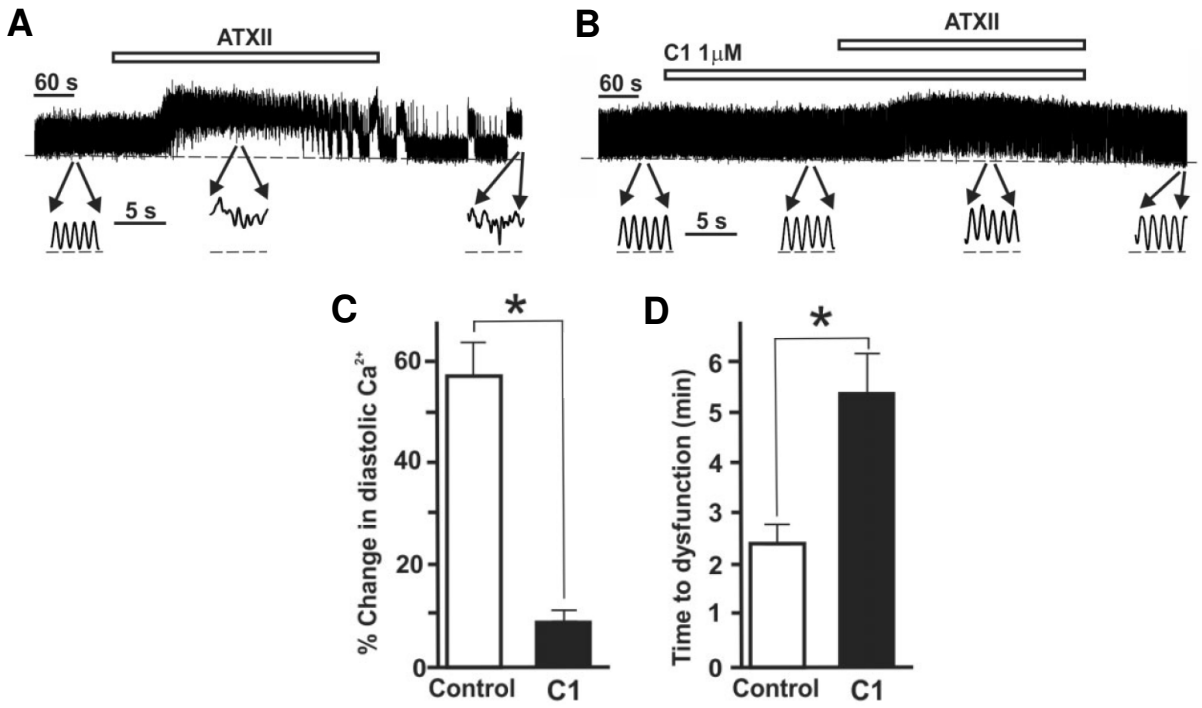

Figure 13. **(A)** Effect of 3 nmol/L ATX-II on diastolic calcium transient amplitude. **(B)** Effect of 1 μmol/L C1 pretreatment on ATX-II-induced diastolic maximal calcium transient amplitude. **(C)** Column-graph representation of the effect of 1 μmol/L C1 pretreatment on ATX-II-induced diastolic maximal calcium transient amplitude; *p<0.05. **(D)** Effect of 1 μmol/L C1 on onset of ATX-II-induced calcium transient dysfunction; *p<0.05; n = 10 cells.

The results presented in **Figure 10** 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 ATX-II. ATX-II pretreatment results in markedly elevated diastolic calcium levels and the loss of uniform calcium transients (**Fig. 13**). In contrast, application of C1 (1 μmol/L) 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 (**Fig. 13**; C1 = 5.3 ± 0.7 min vs 2.4 ± 0.4 min for control, p<0.01, n = 10 cells).

4.3.4. Effects of C1 on right atrial effective refractory period (AERP) and atrial fibrillation in conscious dogs

Right atrial effective refractory period measurements before the start of rapid atrial pacing at 400 beats/min yielded values of 118 ± 3.7 and 130 ± 3.2 ms in 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 C1; and 90.0 ± 3.16 ms at 150 ms BCL, 100 ± 3.16 ms at 300 ms BCL, respectively, following 1 mg/kg C1 (n = 5; with the exception of AERP measurement at 150 ms BCL following 0.3 mg/kg 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 C1, respectively, both $p>0.05$). However, the total duration of AF was significantly reduced by 1 mg/kg C1 administration (**Figs. 14** and **15**) and the average duration of AF episodes was also significantly decreased by 1 mg/kg C1 (**Fig. 16**). 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 concentration ranges of 0.32-0.79 and 0.7-3.0 μmol/L for the 0.3 and 1 mg/kg doses were obtained, 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 C1 and 257.3 ± 9.05 ms ($p = 0.55$) following 1 mg/kg C1.

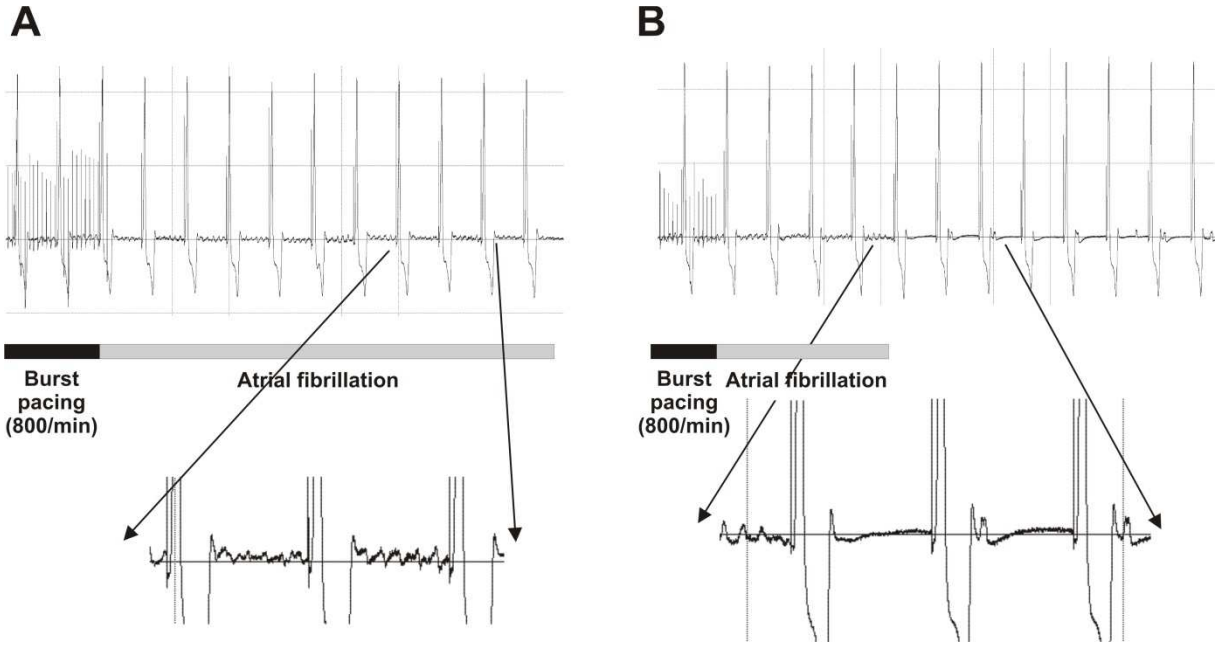

Figure 14. **(A)** Representative ECG recording from a conscious dog exhibiting AF following right atrial 800/min burst pacing (control). **(B)** Representative ECG recording from a conscious dog with a very short duration of AF in response to right atrial burst pacing following 1 mg/kg, i.v., C1 administration. Note P wave reoccurrence after cessation of AF on the magnified inset.

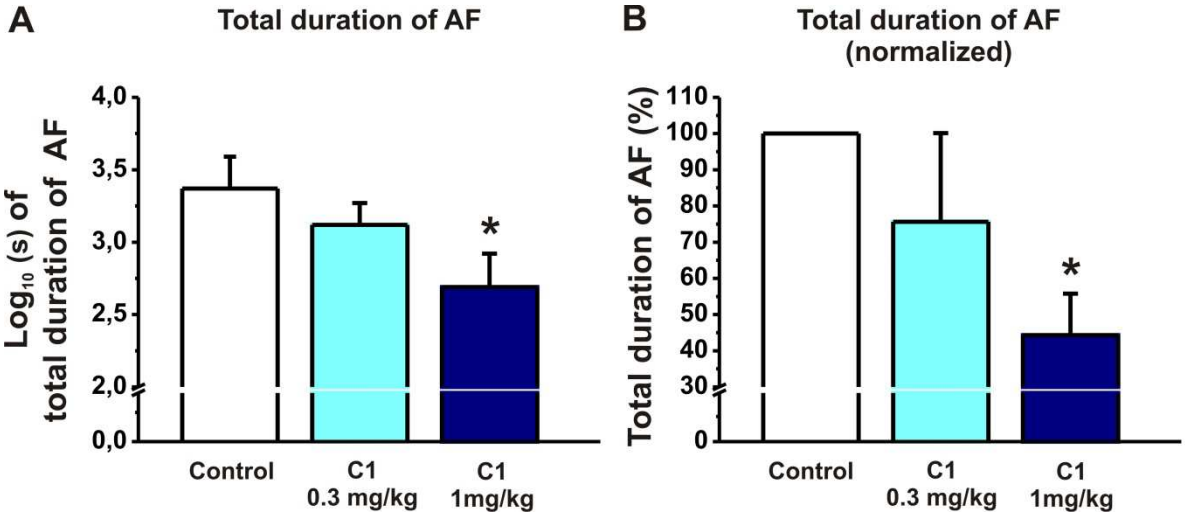

Figure 15. **(A)** C1 administration significantly reduced the total duration of atrial fibrillation only in the higher (1 mg/kg) dose in conscious dogs. **(B)** Normalized data shows that the total AF episode duration was less than 50% of that in control. *$p<0.05$; n = 5 animals.

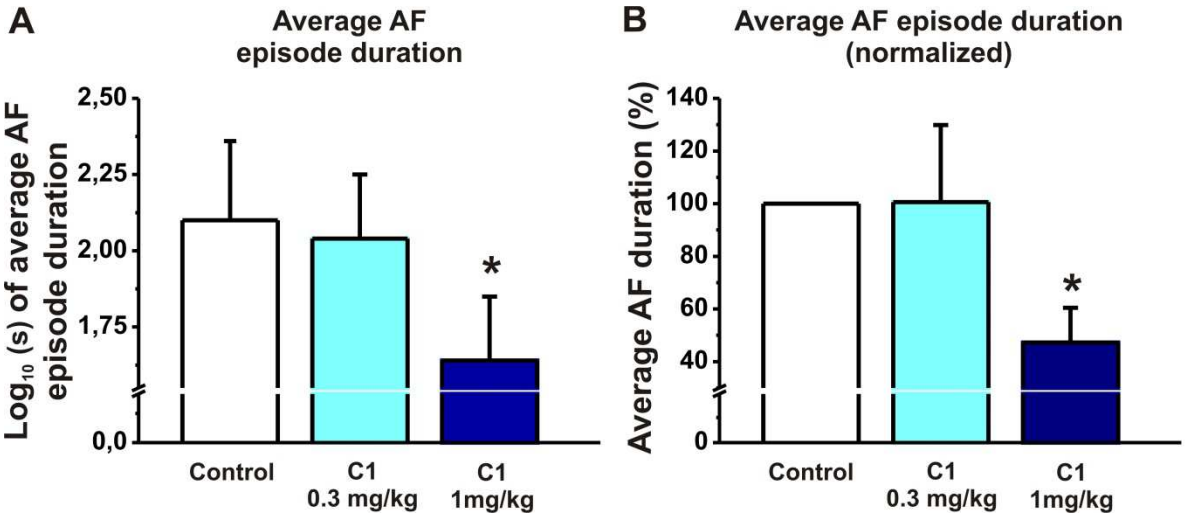

Figure 16. **(A)** C1 administration significantly reduced the average duration of atrial fibrillation only in the higher (1 mg/kg) dose in conscious dogs. **(B)** Normalized data shows that the average AF episode duration was less than 50% of that in control. *$p<0.05$; n = 5 animals.

5. Discussion

There is an unmet need for the safer and more efficacious pharmacological management of AF with compounds that lack ventricular cardiac electrophysiological (proarrhythmic) adverse effects. In the majority of *in vivo* studies characterizing drug candidates against AF, animals anesthetized with volatile and/or intravenous anesthetics were used. These anesthetic agents have their own relatively well identified effects on cardiac ion channels (Carnes et al. 1997; Heath and Terrar 1996; Morey et al. 1997; Pancrazio et al. 1993; Sakai et al. 1996) that can significantly influence the results of these antiarrhythmic studies (Freeman et al. 1990; Napolitano et al. 1996). Therefore, in this work the effects of different compounds on experimental AF were investigated in freely moving conscious dogs to avoid possible confounding effects of anesthetics. One of the approaches to more efficacious and safer pharmacological management of AF is the application of compounds acting on targets ideally expressed only in atrial tissue, since atrial selective ion channel modulation would lack ventricular proarrhythmic adverse effects. In the first set of experiments, the effects of atrial selective $I_{K,ACh}$ inhibition on AF were investigated *in vivo* and *in vitro*.

5.1. Atrial selective ion channel modulation and AF: effects of the $I_{K,ACh}$ blocker tertiapin-Q on AF and action potential configuration in remodeled atrial trabeculae

The effects of the atrial selective $I_{K,ACh}$ inhibitor tertiapin-Q on AF were investigated and compared to those with dofetilide and propafenone, drugs used in the clinical setting for rhythm control in patients with AF. Also, for the first time, the effects of these compounds on atrial action potential configuration and parameters were compared in right atrial trabeculae isolated from dogs with chronic right atrial tachypacing induced AF.

Rapid atrial pacing in dogs is an established large animal AF model where tachypacing leads to electrical and structural remodeling in the atria (Morillo et al. 1995; Gaspo et al. 1997). In the present study, the electrical remodeling was monitored as the gradual decrease in AERP over the course of chronic tachypacing in our animals. In our conscious *in vivo* canine AF model, tertiapin-Q markedly and dose dependently reduced the incidence of AF, the total and average duration of AF episodes, and this effect was paralleled by a significantly increased right AERP following acute intravenous tertiapin-Q administration (**Fig. 3**). The significant prolongation by tertiapin-Q of the action potential duration at all percentages of repolarization was most likely responsible for the increased AERP in right atrial trabeculae isolated from these animals (**Fig. 6**). Tertiapin-Q is a honey bee venom toxin peptide derivative (Jin and Lu 1999) that is a highly selective inhibitor of GIRK (Kir3) channels carrying the acetylcholine-regulated potassium current, $I_{K,ACh}$ (Dascal et al. 1993; Ehrlich et al. 2004). This channel is activated via muscarinic receptors following vagal stimulation (Yamada et al. 1998) leading to atrial action potential shortening and increased atrial dispersion of repolarization (Liu and Nattel 1997), suggesting an important role for this channel in creating an arrhythmia substrate for AF (Kovoor et al. 2001; Nattel 2002). Although $I_{K,ACh}$ downregulation was found in AF patients (Brundel et al. 2001; Dobrev et al. 2001), a constitutively active component independent of muscarinic receptor activation was later identified in patients with chronic AF (Dobrev et al. 2005). In a dog model of atrial tachypacing induced AF, constitutive $I_{K,ACh}$ was also observed (Ehrlich et al. 2004). Inhibition of $I_{K,ACh}$ by tertiapin-Q increased atrial action potential duration in atrial tachycardia-remodeled canine coronary-perfused left atrial preparations and decreased atrial tachycardia inducibility (Cha et al. 2006), similarly to the APD prolongation observed in right atrial trabeculae and the *in vivo* antiarrhythmic activity following tertiapin-Q application in our

study. $I_{K,ACh}$ inhibition proved to be beneficial in previous, other canine models of AF – like aconitine and vagal nerve stimulation induced AF (Hashimoto et al. 2006), however, in these studies the effects of $I_{K,ACh}$ inhibition were tested during isoflurane and/or combined isoflurane+thiopental anesthesia (Yamamoto et al. 2014). Thiopental significantly prolonged AERP in a concentration dependent manner and caused an increase in atrial wavelength in guinea pig hearts (Napolitano et al. 1996), and isoflurane was found to have anti-fibrillatory effects in canine atria (Freeman et al. 1990). Although $I_{K,ACh}$ is also present in the ventricles (Krapivinsky et al. 1995), it is important to note that in conscious dogs tertiapin-Q did not prolong the QT interval in this study, suggesting that selective $I_{K,ACh}$ block is unlikely to provoke ventricular arrhythmias based on repolarization prolongation. The lack of QT prolongation by tertiapin-Q in this study is in agreement with previous studies showing no significant ventricular effects following $I_{K,ACh}$ block (Machida et al. 2011).

The class IC antiarrhythmic propafenone and class III antiarrhythmic dofetilide were chosen as reference molecules in this study, both compounds are used in the clinical management of AF for rhythm control (Kirchhof et al. 2016; Piccini and Fauchier 2016). Both propafenone and dofetilide reduced AF incidence, the duration of AF episodes and increased right atrial ERP in conscious dogs with right atrial tachypacing induced remodeling (**Figs. 4** and **5**). Dofetilide prolonged the atrial APD while propafenone increased atrial conduction time in right atrial trabeculae isolated from dogs with AF (**Fig. 6**). Dofetilide selectively blocks I_{Kr} in the concentration used in this study (Jurkiewicz and Sanguinetti 1993), and its beneficial effects in atrial fibrillation are based on prolongation of atrial repolarization and AERP (Allessie et al. 2001; Pedersen et al. 2001; Singh et al. 2000). However, dofetilide significantly prolongs ventricular APD as well that manifests as marked QT prolongation on the ECG, and can provoke serious ventricular arrhythmias (Wolbrette 2003; Lengyel et al. 2007). In the present study, dofetilide significantly prolonged the QT interval in conscious animals. Propafenone is a class IC antiarrhythmic drug, exhibiting I_{Na}, beta-adrenergic receptor and also hERG blocking properties (Kohlhardt and Seifert 1980; Stoschitzky et al. 2016; Mergenthaler et al. 2001), and the drug is successfully applied for rhythm control in AF management due to its conduction slowing effects (Allessie et al. 2001; Kirchhof et al. 2016). Propafenone is not recommended in patients with structural heart disease due to ventricular proarrhythmia and increased mortality (CAST Investigators 1989). Interestingly, the applied

dose and concentration of propafenone did not prolong the QT interval in conscious dogs and did not prolong APD in isolated right atrial trabeculae (Fig. 5). Therefore, propafenone most likely exerted its beneficial effects against AF via mechanisms other than hERG block in this study. The prolongation of repolarization and slowing of conduction would prevent or decrease atrial reentry following the administration of dofetilide and propafenone, respectively. Interestingly, both propafenone and dofetilide were suggested to exert $I_{K,ACh}$ blocking effects (Mori et al. 1995; Voigt et al. 2010), however, it is not clear yet to what degree these effects contribute to their beneficial effects in patients with AF. Of note, neither dofetilide nor the class IC antiarrhythmic drug flecainide had significant effects against AF in dogs anesthetized with the combination of thiopental and isoflurane (Yamamoto et al. 2014), emphasizing again the need for experiments in conscious animals.

In conclusion, the selective $I_{K,ACh}$ inhibitor tertiapin-Q significantly decreased the incidence of AF, reduced the duration of AF episodes and prolonged atrial effective refractory period in conscious dogs with chronic right atrial tachypacing induced atrial remodeling. In this model, similar effects on AF and AERP were observed following the administration of the class IC antiarrhythmic drug propafenone, and the class III compound dofetilide, both used in the clinical management of AF. In right atrial trabeculae isolated from these dogs with AF, atrial action potential durations were prolonged by tertiapin-Q and dofetilide, but not by propafenone, which increased atrial conduction time. Importantly, tertiapin-Q did not affect the QT interval, suggesting that the beneficial effects against AF are not accompanied by adverse effects on ventricular repolarization, therefore, selective $I_{K,ACh}$ inhibitors may be promising atrial selective compounds in the future management of AF.

5.2. Parallel modulation of multiple ion channels and cellular pathways implicated in AF - effects of C1, a novel compound related to resveratrol

Given the complex aetiology of AF, it has been suggested that drugs targeting multiple pathways involved in AF development may be more effective. Over the past fifteen years, studies on the cellular pathways in AF have revealed potential therapeutic targets to develop new antiarrhythmic drugs for AF management. Based on the available data in the literature, an ideal multifunctional anti-AF compound should exhibit the following effects: (i) $K_v1.5$ (I_{Kur}) inhibition in a frequency dependent manner; (ii) $I_{K,ACh}$ block; (iii) $I_{Na,late}$ inhibition; (iv) lack of I_{Kr} inhibition; (v) display atrial specificity: no effect on ventricular repolarization and

on excitation-contraction (EC) coupling in ventricular tissue; (vi) antioxidant properties; (vii) NFAT inhibition.

Inhibition of the $I_{Kur}/K_v1.5$ repolarizing K^+ channel that is predominantly expressed in atria rather than the ventricles has been identified as an attractive therapeutic target in recent years. We found that C1 was an effective inhibitor of this ion channel. The calculated IC_{50} values for $K_v1.5$ channels were in the 0.11–0.36 μmol/L range for late and peak $K_v1.5$ current inhibition, 180- to 600-fold lower than observed for the parent molecule, resveratrol (66 μmol/L). 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 2013). Inhibition of peak sodium current in a frequency-dependent manner may also be a useful strategy to manage AF by reducing the occurrence of premature action potentials and reduce the incidence of AF. Induction of the $Na_v1.5$ late current may not only increase the risk of early after depolarization (EAD)-induced arrhythmias but also might lead to excessive sodium loading within cells that can promote chronic calcium loading, a primary trigger for EADs and calcineurin-mediated activation of the NFAT gene transcriptional pathway leading to pathological hypertrophic remodeling observed in chronic AF. Our results indicate that C1 inhibited peak and late recombinant human heart $Na_v1.5$ currents more potently than resveratrol. Inhibition of the hERG ($K_v11.1$) potassium channel is a highly undesirable property of any new drug as significant I_{Kr} (hERG) inhibition can lead to excessive action potential prolongation and serious TdP arrhythmias (Sanguinetti and Tristani-Firouzi, 2006). Importantly, we observed that C1 was a weak inhibitor of hERG (peak and tail hERG currents with IC_{50} values of 30 and 25 μmol/L) 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 $I_{K,ACh}$ channels. In our experiments in conscious dogs, C1 did not prolong the QT interval in the investigated doses.

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). The results showed that C1 possessed an antioxidant capacity

similar to that of resveratrol. While the concentrations of C1 required to significantly quench H_2O_2 were 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 signaling 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). Previous research indicated that resveratrol inhibited NFAT nuclear translocation and reduced phenylephrine-induced hypertrophy in cardiac myocytes (Chan et al. 2008). Interestingly, the present results also indicated that C1 inhibited basal and angiotensin-II-induced NFAT activation at similar concentrations ($IC_{50} \sim 1$ μmol/L) 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 become remodeled.

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 (**Figs. 1** and **14**). Atrial tachycardia and flutter have also been shown to lead to similar atrial remodeling in humans (Franz et al. 1997). Therefore, the significantly decreased duration of AF (**Figs. 15** and **16**) and the increased AERP following C1 administration provides *in vivo* evidence that C1 may be beneficial in the management of certain forms of AF. Interestingly, treatment with C1 (0.3 and 1 mg/kg, 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 remodeling of the atria following chronic AF in patients and that following chronic rapid atrial pacing in animal models (Nattel et al. 2007). Remodeling 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. 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 remodeled 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 remodeling remains to be determined by additional long-term studies.

In summary, the aetiology of AF is complex, and numerous 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). These results indicate that the resveratrol derivative C1 is an effective inhibitor of several potential targets involved in AF development and confirmed that C1 was also effective at reducing the duration of AF episodes in a large animal model of inducible AF.

5.3. Limitations

The species differences regarding the relative roles of different atrial ionic currents, including $I_{K,ACh}$, in dogs and humans are not yet fully explored. In dogs subjected to chronic atrial tachypacing a constitutive $I_{K,ACh}$ has been observed (Ehrlich et al. 2004) and a constitutively active $I_{K,ACh}$ has also been identified in patients with chronic AF (Dobrev et al. 2005), suggesting a potentially important role of $I_{K,ACh}$ in AF. However, the complex etiology, and the heterogeneous mechanisms responsible for the initiation and maintenance of AF in clinical settings as opposed to chronic atrial tachypacing in dogs should be considered. Based on the above, the results obtained in the chronic atrial tachypacing induced canine experimental AF model should be extrapolated to human clinical settings with caution and further clinical studies are needed to evaluate the role of $I_{K,ACh}$ block in patients with AF.

6. Conclusions, New Results and Potential Significance

1. In cooperation with our clinician colleagues, the author of this thesis has played a key role in establishing a large animal model of chronic right atrial tachypacing induced atrial fibrillation. This model is suitable for the *in vivo* testing of novel drug candidates for the management of atrial fibrillation and also provides essential cardiac tissue for in vitro measurements aiming at the investigation of mechanisms responsible for AF initiation and maintenance.

2. It was found that the selective $I_{K,ACh}$ inhibitor tertiapin-Q significantly decreased the incidence of AF, reduced the duration of AF episodes and prolonged atrial effective refractory period in conscious dogs with chronic right atrial tachypacing induced atrial remodeling. In this model, similar but somewhat less pronounced effects on AF and AERP were observed following the administration of the class IC antiarrhythmic drug propafenone, and the class III compound dofetilide, both used in the clinical management of AF. Importantly, tertiapin-Q did not affect the QT interval, suggesting that the beneficial effects against AF were not accompanied by adverse effects on ventricular repolarization, therefore, selective $I_{K,ACh}$ inhibitors may be promising atrial selective compounds in the future management of AF.

3. In right atrial trabeculae isolated from dogs with AF, atrial action potential durations were prolonged by tertiapin-Q and dofetilide, but not by propafenone, which increased atrial conduction time. The prolongation of repolarization and slowing of conduction would prevent or decrease atrial reentry following the administration of the investigated compounds.

4. A novel resveratrol derivative small molecule (C1) developed by our colleagues at the University of Alberta, Edmonton, Canada, exhibited *in vivo* efficacy in our conscious dog AF model and its effects were based on an advantageous combination of effects on multiple ion channels and cellular pathways implicated in AF generation and maintenance. Therefore, C1 can be a starting point for further development of compounds modulating multiple targets for improved pharmacological treatment of AF.

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