Cardiac electrophysiological studies on the antiarrhythmic and hidden cardiotoxic effects of different compounds

Ph.D. Thesis

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LIST OF ABBREVIATIONS

AERP	atrial effective refractory period
AF	atrial fibrillation
APA	action potential amplitude
APD_{10}	action potential duration at 10% of repolarization
APD_{25}	action potential duration at 25% of repolarization
APD_{50}	action potential duration at 50% of repolarization
APD_{75}	action potential duration at 75% of repolarization
APD_{90}	action potential duration at 90% of repolarization
CT	conduction time
EAD	early afterdepolarization
ERP	effective refractory period
I_{Ca}	voltage-dependent calcium current
I_{K1}	inward rectifier potassium current
I_{Kr}	rapidly activating delayed rectifier potassium current
I_{Ks}	slowly activating delayed rectifier potassium current
I/R	ischemia/reperfusion
I_{to}	transient outward potassium current
QTc	frequency corrected QT interval
RMP	resting membrane potential
sIPC	simulated ischemic preconditioning
sI/R	simulated ischemia/reperfusion
TdP	Torsades de Pointes polymorphic ventricular tachycardia
SEM	standard error of the mean
VF	ventricular fibrillation
V_{max}	maximum rate of depolarization

SUMMARY OF THE THESIS

Background and aims of the study:

Following the disappointing results of the CAST and SWORD clinical trials showing that Class I/C and pure Class III antiarrhythmic drugs increased mortality in patients with myocardial infarction and reduced left ventricular function, the strategies for development of new antiarrhythmics changed fundamentally. Instead of developing 'pure' channel blockers, novel approaches have aimed at synthesizing agents with combined Class I/B and III antiarrhythmic characteristics, as such 'multichannel-blocking' properties had been considered to possess a safer proarrhythmia profile. Therefore, the first aim of this study was to investigate the antiarrhythmic, proarrhythmic and cardiac electrophysiological effects of SZV-270, a novel agent that was synthesized by our collaborators from two well known pure Class I/B and III antiarrhythmic agents, mexiletin and D-Sotalol, respectively.

For the pharmaceutical industry, development of novel preclinical screening methods enabling more reliable prediction of the proarrhythmic potential / cardiotoxicity of novel drug candidates is also crucial. Unexpected clinical cardiotoxicity is still the leading cause of discontinuation of clinical trials and the withdrawal of drugs from the market despite great efforts to detect cardiotoxicity in the preclinical phase of drug development programs. Such cardiotoxic effects remain undetected during preclinical and early clinical safety studies and they may manifest in the presence of cardiac diseases e.g., in myocardial ischaemia/reperfusion conditions; therefore, this phenomenon was termed "hidden cardiotoxicity". Little is known about the mechanism behind of this phenomenon, moreover, hidden cardiotoxicity cannot be revealed by the routinely used preclinical studies on healthy animals or tissues. Therefore, the second main aim of this study was to characterize a preclinical model for assessing the possible hidden cardiotoxic effects of different drugs. For this aim, we used rofecoxib, a compound withdrawn from the market because of its cardiovascular side effects.

Main findings:

SZV-270 exerted Class III antiarrhythmic effects by prolonging repolarization and the effective refractory period in dog and rabbit multicellular preparations, in a concentration dependent manner. In the higher applied concentration, SZV-270 exerted Class I/B antiarrhythmic effect in both species: it significantly decreased V_{max} at cycle lengths shorter than 1000 ms. In Purkinje fibers, at 2 Hz stimulation frequency the compound significantly lenghtened repolarization, however, this prolongation was smaller after the application of the

larger concentration than that was seen following the application of the smaller concentration. The larger concentration decreased APD_{50} significantly. SZV-270 reduced V_{max} significantly in a concentration dependent manner. SZV-270 significantly lengthened atrial action potentials in a concentration dependent manner, and markedly increased atrial effective refractory period and significantly reduced the incidence of atrial fibrillation in conscious dogs. SZV-270 blocked I_{Kr} significantly in relatively low, 100 and 500 nM concentrations. This result is consistent with the APD lengthening and frequency corrected QT interval (QTc) prolonging effect of the drug. SZV-270 had no effects on the other transmembrane currents.

Rofecoxib treatment did not alter any of the observed electrophysiological parameters in normoxic conditions. After 30 min of simulated ischemia, APD_{90} was shortened significantly in all groups compared to the respective normoxic groups. Importantly, in the presence of sI/R rofecoxib increased APD_{90} in a concentration-dependent manner after reperfusion following the 30 min. simulated ischemia. In the sIPC group, these effects of rofecoxib on repolarization were not observed during reperfusion. 30 min. of simulated ischemia caused an increase of CT in all groups, while V_{max} was decreased significantly in the rofecoxib treated groups, possibly indicating reduced sodium channel function after rofecoxib administration in only ischemic conditions.

Conclusions:

SZV-270 significantly reduced the incidence of atrial fibrillation and prolonged atrial effective refractory period in a conscious dog model of atrial fibrillation. Our cellular electrophysiological investigations revealed that SZV-270 exerted its antiarrhythmic effects via combined Class I/B and Class III actions.

We demonstrated, that the hidden cardiotoxicity of rofecoxib can be revealed by preclinical cardiotoxicity testing while using experimental I/R models. Moreover, IPC might protect against the hidden cardiotoxic effects of rofecoxib in vitro. These results show that cardiac safety testing with simple preclinical models of I/R injury uncovers the hidden cardiotoxicity of rofecoxib and might reveal hidden cardiotoxicity of other drugs

1. INTRODUCTION

Cardiovascular diseases remain the leading causes of mortality in the developed world. Approximately 18 million lives are lost annually due to sudden cardiac death, most commonly caused by severe ventricular arrhythmias degenerating into ventricular fibrillation (VF) (Shomanova *et al.*, 2020). Atrial fibrillation (AF), the most prevalent sustained cardiac arrhythmia (Kannel *et al.*, 1982; Andrade *et al.*, 2014), is also associated with significant morbidity and mortality, leading to stroke (Lip *et al.*, 2011) and heart failure (Larned and Laskar, 2009).

Unfortunately, the therapy of VF and/or AF can't be considered as optimal in terms of the following two major clinical aspects: i) pharmacological therapy has limited efficacy (Andrade *et al.*, 2014) and ii) antiarrhythmic drugs can exhibit marked proarrhythmic potential due to their cardiac ventricular electrophysiological adverse effects (Fenichel et al., 2004). Therefore, there is an unmet need for novel, more effective and safer antiarrhythmic drugs. Importantly, this goal can only be achieved if novel, more reliable drug safety screening methods are developed which would allow more reliable prediction of potential cardiac side effects (cardiotoxicity) – such as proarrhythmia - of novel drug candidates. To better understand the limitations of the currently used antiarrhythmic drugs, a brief overview of the basics of cardiac cellular electrophysiology and the classification of the antiarrhythmias is provided in the following subchapters. Also, a short introduction of current limitations on cardiotoxicity assessment will be given.

1.1 The cardiac action potential

The cardiac action potential is a transmembrane potential change, with an amplitude ranging between 60 to 120 mV caused by ions flowing through transmembrane ion channels, via their dynamic and simultaneous opening and closing (Banyasz *et al.*, 2012, 2011). It starts from a negative value, i.e. the resting membrane potential (RMP) in working myocardial cells, or maximal diastolic potential in spontaneously depolarizing cells (Hoffman and Cranefield, 1960), ranging from -95 to -40 mV. Similar to other excitable cells, the RMP is primarily generated by the inwardly rectifying K^+ currents and can be predicted by the Nernst equation from the uneven distribution of mainly K^+ ions across the cell membrane. The electrogenic ATP dependent Na^+ /K^+ pump also contributes to the RMP, by exporting 3 Na^+ and importing 2K^+ (Gadsby, 1985; Gao *et al.*, 2005; Attwel *et al.*, 1981; Lee, 1996). In normal circumstances, the effective refractory period (ERP) - defined as the shortest time interval

needed before a new stimulus (or an early extrasystole) can create a new action potential - is determined by the action potential duration. The relation between APD and ERP is disrupted in pathological circumstances, for example, in hyperkalemia, leading to post-repolarization refractoriness (Shaw and Rudy, 1997). In the context of the cardiac action potential, two aspects should be highlighted. The cardiac action potential is not uniform, it has a different configuration in different regions of the heart, and therefore various action potentials should be considered and discussed separately. There are significant differences between species (Clauss *et al.*, 2019), even when action potentials are measured from similar regions of the heart.

The cardiac action potential is divided into five phases (**Figure 1**). In phase 0, cardiomyocytes are depolarized by a rapid inflow of Na^+ ions generating a significant and fast inward Na^+ current (I_{Na}). This current determines also the velocity of impulse propagation through the His-Purkinje system, working atrial and ventricular myocytes ("fast response" action potentials). In sino-atrial and atrioventricular node primarily the Ca^{2+} current (I_{Ca}) is responsible for depolarization ("slow response" action potentials). Phase 1 is the initial part of repolarization, primarily governed by the transient outward current (I_{to}). In phase 2, (plateau phase), the late component of the inward sodium current ($I_{Na,Late}$) opposes the outward repolarizing currents i.e. the slow and rapid components of the delayed rectifier K^+ current (I_{Ks} and I_{Kr}) and the inward rectifier K^+ current (I_{K1}). Phase 3 is the terminal phase of repolarization, which differs from phase 2 for its faster repolarization rate, the outward progressively overcome the inward currents, enabling fast repolarization of the action potential. The loss of one repolarizing current may not lead to excessive AP lengthening, since other unimpaired K^+ currents may provide sufficient repolarizing capacity (Roden, 1998; Varro *et al.*, 2000; Biliczki *et al.*, 2002; Jost *et al.*, 2005; Banyasz *et al.*, 2007), i.e. there is a redundancy of the repolarization ("repolarization reserve"). The key players of the reserve are I_{Kr}, I_{Ks}, I_{K1}, and possibly I_{to} (Varro *et al.*, 2000; Biliczki *et al.*, 2002; Jost *et al.*, 2005; Banyasz *et al.*, 2007). Phase 4 describes membrane potential during diastole. Time-independent (or background) currents may also contribute to the whole action potential course i.e. the Na^+/K^+ pump current (I_{NaK}), the Na^+/Ca^{2+} exchanger (I_{NCX}) and the ATP-sensitive poatssium current ($I_{K,ATP}$). In the sino-atrial node heart rate is regulated by spontaneous electrical pacemaker activity primarily controlled by the I_f current (DiFrancesco, 1995). This current determines the slope of diastolic depolarization and cardiac frequency, and its inhibition leads to the reduction of heart rate. The "funny" (I_f) current was named because its

strange characteristics, including that of being an inward current that is activated on hyperpolarization and not on depolarization like other known currents.

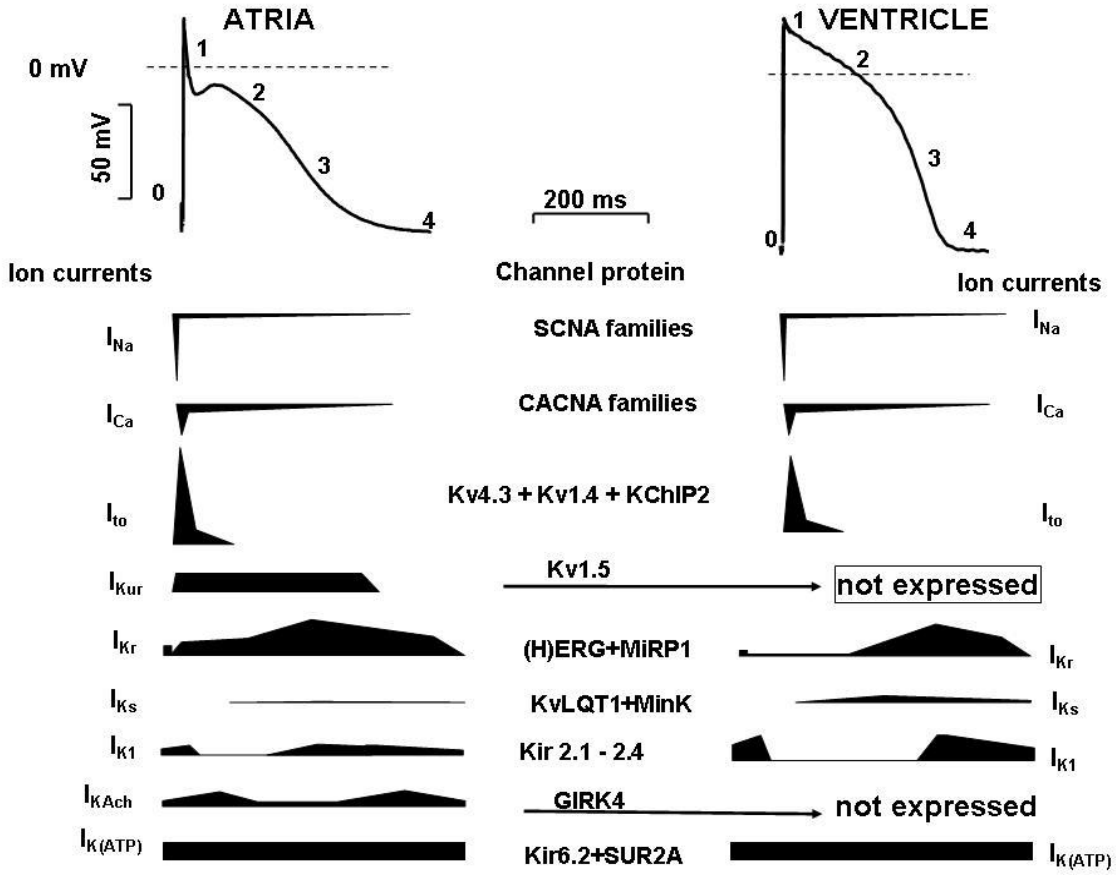

Figure 1. Phases of atrial and ventricular action potentials and underlying ionic currents. The numbers refer to the phases of the action potential. In each current profile the horizontal line represents the zero current level; inward currents are below the line and outward currents are above it (Jost, 2009).

1.2. Antiarrhythmic drugs

Antiarrhythmic agents were originally classified by the Vaughan-Williams classification (**Table 1**) based on their dominant electrophysiological effect (Singh and Williams, 1999; Singh, 1972; Nattel and Singh, 1999) **(Figure 2.)**.

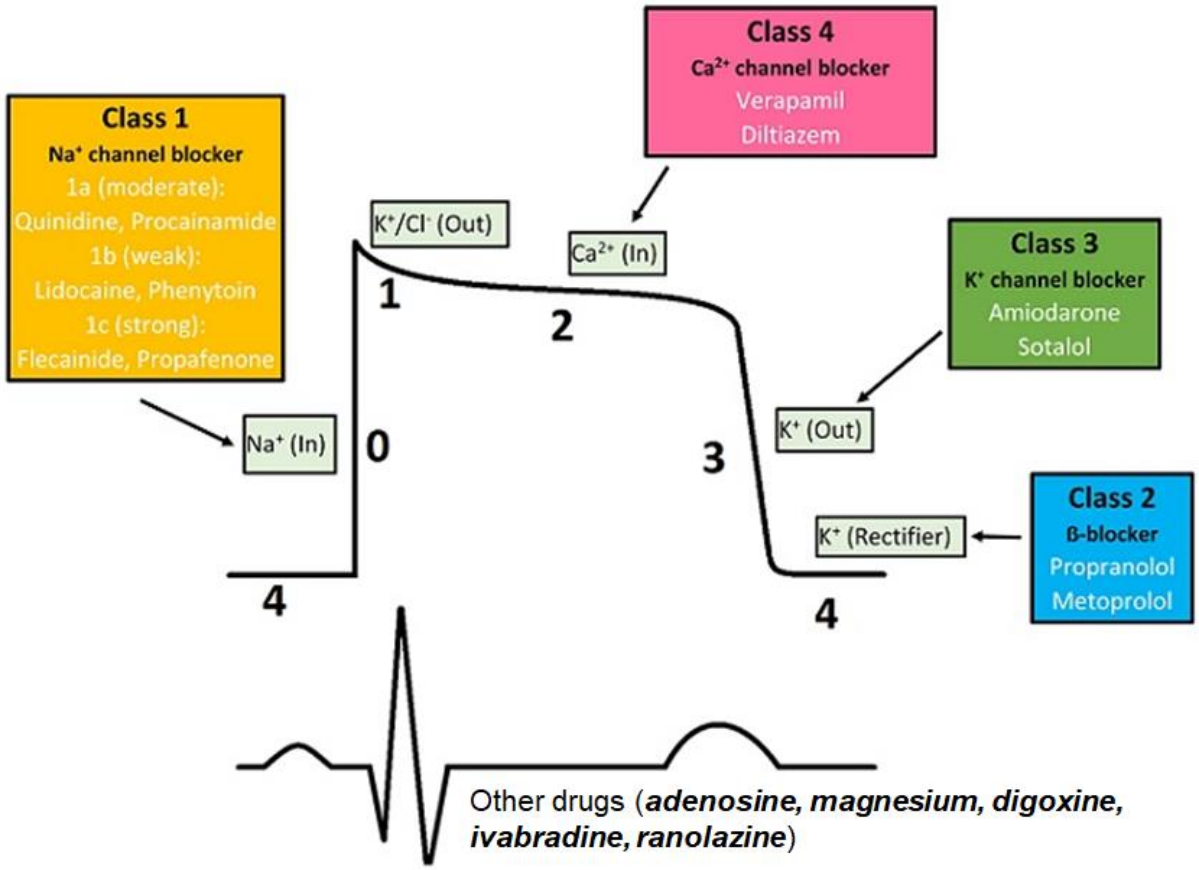

Figure 2. Schematic illustration of the main mechanisms of action of antiarrhythmic drugs according to the Vaughan-Williams classification

In time, a growing body of evidence confirmed that most antiarrhythmic drugs act on multiple ion-channels and/or receptors, therefore, it became obvious that the Vaughan-Williams classification is oversimplified and needed major revision. The revised version of this classification that was proposed on a meeting held in Sicily has become known as the 'Sicilian gambit'. This classification uses the former Vaughan-Williams classification as a frame to divide drugs into main groups based on their main electrophysiological effects, but it also provides further subclassifications of the agents based on their exact mode of action on different ion channels, transmembrane pumps and receptors which may have important clinical relevances (Table 1). A brief summary of the Vaughan-Williams Classes with extra attention given to the 'Sicilian gambit' approach is provided in the following subchapters.

ANTIARRHYTHMIC DRUG ACTIONS

Vaughn-Williams Class	DRUG	ECG Changes	CHANNELS			RECEPTORS				Clinical Effects			
			Ca^{++}	Na^{+}	K^{+}	α	β	ACh	Ado	Pro-Arrhy	Extra Cardiac	LV FX	Heart Rate
A	Quinidine	A		Ⓜ	Ⓜ	Ⓛ		Ⓜ		Ⓗ	Ⓜ		
	Procainamide			Ⓜ	Ⓜ					Ⓜ	Ⓗ		
	Disopyramide (Norpace)			Ⓜ	Ⓜ			Ⓜ		Ⓛ	Ⓜ	↓↓	
B	Lidocaine (Xylocaine)	B		Ⓛ						Ⓛ	Ⓜ		
	Mexiletine (Mexitil)			Ⓛ						Ⓛ	Ⓜ		
C	Propafenone (Rythmol)	C		Ⓗ			Ⓜ			Ⓜ	Ⓛ	↓↓	↓
	Flecainide (Tambocor)			Ⓗ						Ⓗ	Ⓛ	↓↓	
II	β-Adrenergic antagonists						Ⓗ			Ⓛ	Ⓛ	↓	↓↓
III	Dronedarone (Multaq)		Ⓛ	Ⓛ	Ⓗ	Ⓜ	Ⓜ	Ⓜ		Ⓛ	Ⓗ	↓	↓
	Amiodarone (Cordarone)		Ⓛ	Ⓛ	Ⓗ	Ⓜ	Ⓜ	Ⓜ		Ⓛ	Ⓗ		↓
	Sotalol (Betapace)				Ⓗ		Ⓗ			Ⓗ	Ⓛ	↓	↓
	Ibutilide (Corvert)			△	Ⓗ					Ⓗ	Ⓛ		
	Dofetilide (Tikosyn)				Ⓗ					Ⓗ	Ⓛ		
IV	Verapamil (Calan, Isoptin)		Ⓜ							Ⓛ	Ⓛ	↓↓	↓
	Diltiazem (Cardizem)		Ⓜ							Ⓛ	Ⓛ	↓	↓
Misc	Adenosine (Adenocard)								△	Ⓛ	Ⓛ		↓

Antagonist relative potency L = Low M = Moderate H = High	△ = Agonist ● = ECG Changes related to Ca^{++} channel block ● = ECG Changes related to Na^{+} channel block ● = ECG Changes related to K^{+} channel block

Table 1. A modification of the Sicilian gambit drug classification. Abbreviations: PR interval (red), QRS interval (yellow), and QT interval (blue). The target ion channels (calcium, sodium, and potassium) and receptors (α-adrenergic (α), β-adrenergic (β), cholinergic (ACh), and adenosinergic (Ado) for antiarrhythmic drugs are listed in columns with the relative potency for each agent indicated as the letters H, M, and L for "high," "medium," and "low." Clinical effects are listed next: proarrhythmic (ProArrhy), extracardiac adverse effects (Extra Card), inotropic (left ventricular function, LV FX), and chronotropic (Heart Rate). The direction of the arrows indicates the direction of effects; the number of arrows indicates the magnitude. ECG, electrocardiogram.

1.2.1. Class I antiarrhythmic drugs

These agents inhibit the fast Na^{+} channels responsible for the upstroke and rapid conduction of the cardiac action potential. On the ECG, this effect may be reflected as widening of the P wave, widening of the QRS complex, prolongation of the PR interval, or a combination. They are able not only to slow but to block intracardiac conduction and induce arrhythmias. This proarrhythmic adverse effect has become widely known, and has led to a

reduction in the usage of these compounds, especially in patients with structural heart disease. Class I antiarrhythmic agents usually cause post-repolarization refractoriness, when the refractory period extends well beyond phase 3, even after complete repolarization. Class I drugs are subdivided based on the dissociation kinetics on the Na^+ channel, that determine the heart rates at which their electrophysiologic effects become manifest.

Class I/A drugs have intermediate dissociation kinetics, their conduction slowing effects may or may not be obvious on an ECG obtained during normal rhythm at normal rates. Class I/A drugs also block repolarizing K^+ channels, prolonging the repolarization and refractory periods (the interval from the beginning of the AP until the fiber is able to conduct another AP). On the ECG, this effect is manifested as QT-interval prolongation even at normal rates. Quinidine - extracted from the cinchona plant - is used by the 1920s, as an antiarrhythmic compound to maintain sinus rhythm after the conversion from atrial fibrillation or flatter, and to prevent the recurrence of ventricular tachycardia or fibrillation (Bozic *et al.*, 2018, Serdoz *et al.*, 2019). Due to increased risk of ventricular arrhythmia and sudden death, as well as a number of other adverse effects and drug interactions, quinidine was withdrawn from use and became unavailable in many countries (Bozic *et al.*, 2018). Procainamide is widely used alone or in combination with class I agents (eg, mexiletine) to prevent symptomatic nonsustained ventricular tachycardia or recurrent ventricular tachycardia. Procainamide is also used for short-term treatment of ventricular tachycardia and a variety of supraventricular tachycardias, primarily atrial fibrillation and flutter. A number of systemic side effects limit it's usefullness, mainly lupus-like syndrome, gastrointestinal disturbances, and autoimmune blood dyscrasias. (Ellenbogen et al, 1993). Disopyramide is an effective antiarrhythmic agent in the treatment of ventricular and supraventricular arrhythmias (Brogden and Todd, 1987), and used primarily to treat ventricular ectopic systoles (Taylor and Pappas, 1986).

Class I/B drugs exhibit fast dissociation kinetics. They express their electrophysiologic effects only at fast heart rates (use-dependent block), because Na^+ channels spend more time in activated and inactivated states, and the diastolic time for recovery from drug-induced block is shortened. Class I/B drugs have stronger effect in ischaemic, depolarized cardiac tissues (voltage-dependent block), because membrane depolarization inactivates Na^+ channels and slows recovery from block (Zipes and Jalife, 2014). Thus, an ECG recorded during normal rhythm at normal rates usually shows no signs of conduction slowing. Lidocaine is considered the drug of choice in the treatment of ventricular arrhythmias in the prehospital and early hospital phase of acute myocardial infarction (Zehender *et al.*, 1990). It has been

highly effective in terminating ventricular premature beats and ventricular tachycardia occurring during general surgery, during and after cardiac surgery, following acute myocardial infarction, and in the course of digitalis intoxication. It has also been suggested for the prevention and treatment of ventricular arrhythmias occurring during cardiac catheterization (Collinsworth *et al.*, 1974). Intramuscular lidocaine for prevention of fatal arrhythmias in the pre-hospital phase of acute myocardial infarction was tested and recommended in two clinical trials. Results of both trials showed a beneficial prophylactic antifibrillatory effect of lidocaine in the pre-hospital phase of suspected acute myocardial infarction (Valentine *et al.*; 1974; Koster *et al.*, 1985;). Lidocaine is used only by the intravenous route. Mexiletine has a similar chemical structure and antiarrhythmic activity to lidocaine, and effective in the treatment of ventricular extrasystole and tachycardia, following oral administration. Intravenous mexiletine and lidocaine can be effective in suppression and prevention of haemodynamically stable ventricular tachycardia as well as for prevention of recurrent ventricular fibrillation. In patients not responding to conventional beta-blocker therapy, mexiletine may be used as alternative treatments (Dan et *al.*, 2018). Mexiletine is effective for shortening QTc interval in LQT3 syndrome. (Prior *et al.*, 2015; Chorin *et al.*, 2017). The drug suppresses ventricular ectopy in the acute phase of myocardial infarction, and effective for some patients in whom lidocaine has failed. Mexiletine has little effect on atrial refractory period, consistent with its lack of effect in atria1 fibrillation or flutter. Adverse reactions limit the use of mexiletine in approximately 20% of patients. Gastrointestinal and central nervous system side effects are the most common. (Woosley *et al.*, 1984; Fenster and Comess, 1986). Mexiletine and disopyramide should also be avoided in post-myocardial infarction patients (Dan et *al.*,2018).

Class I/C drugs have slow dissociation kinetics, they express their electrophysiologic effects at all heart rates. Thus, an ECG recorded during normal rhythm at normal heart rates usually shows conduction slowing. Propafenone is an effective agent in the treatment of premature ventricular complexes, ventricular couplets and nonsustained ventricular tachycardia, and showed efficacy in the treatment of ventricular fibrillation and sustained ventricular tachycardia. Propafenone has marked efficacy in the treatment of Wolff-Parkinson-White syndrome, and it can convert effectively the atrial fibrillation to sinus rhythm (Bryson *et al.*, 1993; Capucci and Boriani 1995). Propafenone is also effective in suppressing ventricular premature complexes and nonsustained ventricular tachycardias. However, because of potential proarrhythmic effects, propafenone does not seems to be a first choice as a prophylactic agent for malignant ventricular arrhythmias (Capucci and Boriani

1995). Flecainide is an effective agent for the pharmacological cardioversion of atrial fibrillation, cathecolaminergic polymorphic ventricular tachycardia, supraventricular tachyarrhythmias and ventricular pre-excitation (Paolini *et al*., 2019) Potential cardiac side effects of flecainide include proarrhythmia, negative inotropic effects and conduction abnormalities. The most common non-cardiac side effect is dizziness, followed by difficulty focusing and blurred vision (Tamargo *et al*., 2012). Encainide is an antiarrhythmic agent which has been used in the treatment of life-threatening ventricular arrhythmias, symptomatic ventricular arrhythmias and supraventricular arrhythmias (Brogden and Todd, 1987). The most common adverse effect are blurred vision, nausea, heart block, and proarrhythmic effects (Tordjman and Estes, 1987). Class I/B drugs and Class I/C drugs do not block K^+-channels directly. Class I drugs can be useful in supraventricular and ventricular tachyarrhythmias also, but they are not generally recommended for patients with structural heart disease. The Cardiac Arrhythmia Suppression Trial (CAST) showed that encainide and flecainide, two Class I/C Na^+ channel blocker antiarrhythmic drugs, increased mortality rates compared to placebo due to proarrhythmic adverse effects in patients with decreased left ventricular function after myocardial infarction (The CAST Investigators, 1989). Therefore, in subsequent years following the publication of these results, the interest of drug development for treatment of ventricular tachycardia (VT) and atrial fibrillation (AF) has been shifted toward those compounds that prevent and terminate re-entry arrhythmias by prolonging the action potential duration.

1.2.2. Class II antiarrhythmic drugs

Class II drugs are β-adrenergic receptor blockers. They are used not only for the management of cardiac arrhythmias (Kühlkamp *et al.*, 2002), but also for hypertension, angina pectoris, congestive heart failure, essential tremor, glaucoma, migraine prophylaxis and myocardial infarction. As antiarrhythmic agents they suppress β-adrenergic signaling in the heart by competitively inhibiting agonist binding to β-adrenergic receptors. They differ from most other antiarrhythmic agents by not directly modifying ion channel function; rather, they prevent the arrhythmia-promoting effects of β-adrenergic stimulation. They are useful in preventing sudden death due to ventricular tachyarrhythmias associated with acute myocardial ischemia, congenital long QT syndrome, and congestive heart failure. β-blockers affect predominantly tissues with "slow response" action potentials (SA and AV nodes), where they decrease the rate of automaticity and slow conduction velocity. Thus, the heart rate is decreased and the PR interval is lengthened on the ECG. Class II drugs are used primarily to

treat supraventricular tachycardias (SVT), including sinus tachycardia, AV nodal reentry, atrial fibrillation, and atrial flutter. β-blockers are able to prevent atrial fibrillation effectively after coronary artery bypass surgery, and they can control the ventricular rate during atrial fibrillation (Kühlkamp *et al.* 2002) at rest and during excercise (Abrams *et al.*, 1985; Atwood *et al.*, 1987). These drugs are also used to treat VTs to raise the threshold for ventricular fibrillation (VF) and reduce the ventricular proarrhythmic adverse effects of β-adrenoceptor stimulation. Propranolol supresses ventricular tachycardia and ventricular ectopic depolarizations (Woosley *et al.*, 1979) and terminate supraventricular tachyarrhythmias effectively, similar to esmolol, an ultra-short-acting β-adrenergic blocking agent (elimination half-life: 9 min). Esmolol effectively and rapidly controls the heart rate in patients with supraventricular tachyarrhythmias (equal to propranolol) and in patients with acute myocardial ischemia. Furthermore, because of its short half-life, esmolol offers excellent benefits in the treatment of critically ill patients. Esmolol is well tolerated by patients for whom beta blockers in general would be unsuitable. (Abrams et al., 1985; Sung *et al.*, 1986). Metoprolol is effective and relatively safe for the prevention of postoperative atrial fibrillation in cardiac surgery patients (Norhayati *et al*., 2020).

1.2.3. Class III antiarrhythmic drugs

Class III antiarrhythmic agents prolong the cardiac action potential duration (APD) by blocking one or more potassium channels (Brendorp *et al.*, 2001). A significant number of non-cardiac agents cause lengthening of APD in both ventricular muscle cells and Purkinje fibers through similar mode of action (Pinney *et al.*, 1995; Gintant *et al.*, 2001; Antzelevitch *et al.*, 1996; Drici and Barhanin, 2000; Rampe and Murawsky, 1997). These drugs have effects also on tissues with slow and fast response action potentials. Because repolarization and refractoriness are prolonged, rate of automaticity is reduced. QT-interval prolongation is the dominant effect on the ECG. Class III drugs have been evaluated in different settings: primary and secondary prevention of ventricular arrhythmias, and in the treatment of atrial fibrillation or flutter, and they are effective in converting atrial fibrillation to sinus rhythm and for the maintenance of sinus rhythm after conversion (Brendorp *et al.*, 2002; Weirich and Antoni, 1998). Class III drugs have a risk of proarrhythmic side effects, especially Torsades de Pointes ventricular tachycardia (Waldo *et al.*, 1996; Brendorp *et al.*, 2001; Hohnloser and Woosley 1994). The reason behind this is that these compounds have a reverse use-dependent effect on the action potential duration, lenghtening the repolarization more at slower heart rates, leading to early afterdepolarization (EAD). (Nair and Grant, 1997; Vos *et al.*, 1995;

Hondeghem and Snyders, 1990). Data suggest that the combination of two factor induce Torsades de Pointes: (i) generation of EADs if repolarisation prolongs excessively, leading to EAD-induced triggered activity (Antzelevitch *et al*., 1994; Burashnikov *et al.*, 1998; Carlsson *et al.*, 1993; Viswanathan *et al.*, 1999) and (ii) existing or induced repolarization heterogeneity throughout the myocardium. (Antzelevitch *et al.*, 1996; Surawicz *et al*., 1989; Yan *et al.*, 1989). EADs have been shown in vitro to manifest mainly in the Purkinje fibers and in the subendocardial/middle cell layer of the ventricle. Bradycardia, hypokalaemia, hypomagnesemia, ventricular hypertrophy, female gender, and repolarisation prolongation increase the risk of EADs (Elming *et al.*, 2005). Repolarisation prolongation is affected variously throughout the myocardial layers. Animal (Antzelevitch *et al*, 1996; Yan *et al.*, 1998; Roden *et al.*, 1985) and human (Drouin *et al*., 1995) in vitro experiments have shown a more pronounced bradycardia-induced prolongation of APD in the Purkinje fibers and in the M-cells compared to other layers, with an even more pronounced prolongation in these cells in hypokalaemia and when exposed drugs prolonging repolarisation. Thus, the combination of EAD triggered activity in a substrate of cells in different states of repolarisation seems to increase the risk of re-entry arrhythmia (el Sherif *et al.*, 1996). It wold be favourable to prevent these proarrhythmic side effects by using combination.

Amiodarone - originally developed as a vasodilator – is classified electrophysiologically as a Class III antiarrhythmic, it also has both nonspecific antisympathetic and direct, fast channel-membrane effects (Freedman and Somberg, 1991). Amiodarone markedly prolongs the action potential duration (and the QT interval on the ECG) by blockade of I_{Kr}. During chronic administration, I_{Ks} is also blocked. The action potential duration is prolonged uniformly over a wide range of heart rates; that is, the drug does not have reverse use-dependent action. In spite of its present classification as a class III agent, amiodarone also significantly blocks inactivated sodium channels. Its action potential prolonging action reinforces this effect. Amiodarone also has weak adrenergic and calcium channel-blocking actions. Consequences of these actions include slowing of the heart rate and AV node conduction (Sloskey, 1983; Zipes *et al.*, 1984). The drug suppresses recurrences of cardiac tachyarrhythmias in a high percent of patients, in the range of 80% or more for most supraventricular tachycardias and in about 66% of patients with ventricular tachyarrhythmias, sometimes requiring addition of a second antiarrhythmic agent. Amiodarone may prove beneficial in the primary prevention of atrial fibrillation, to convert fibrillation, to prevent recurrency, and finally, to reduce a fast ventricular rate that does not respond to standard therapy. Side effects, particularly when high doses are used, may limit amiodarone's usefulness and include skin, corneal, thyroid,

pulmonary, neurologic, gastrointestinal and hepatic dysfunction. Aggravation of cardiac arrhythmias occurs but serious arrhythmias are caused in less than 5% of patients (Zipes *et al.*, 1984; Gjesdal, 2008; Jukić *et al.*, 2015). The most serious side effect of amiodarone is pulmonary fibrosis. Although the occurrence of this complication has decreased with the use of lower doses, it can occur with any dose (Wolkove and Baltzan, 2009).

The SWORD (Survival With Oral D-sotalol) trial was terminated by the Steering Committee because of an excess mortality among the patients randomized to d-sotalol. A total of 3121 patients had been randomized in the SWORD trial with a mean follow-up of 146 days. The Data and Safety Monitoring Board recommended early termination of SWORD because the boundary for harm had been crossed and statistical significance had been reached (p=0.006). There was an increased mortality among the 1549 patients receiving d-sotalol (78 deaths, 5.0%) compared to the 1571 receiving placebo (48 deaths, 3.0%). This difference was present across all subgroups, even though it was markedly more present in some compared to others. The conclusion of SWORD trial is that d-sotalol causes harm in patients with a prior myocardial infarction (Schwartz, 1995).

Dofetilide, like sotalol, is a methane sulfonanilide derivative (Larsen and Lish, 1964) and it has a significant blocking effect on the rapid component of the delayed rectifier potassium current (I_{Kr}). It also increases the activity of the late sodium current at lower concentration; therefore, it prolongs action potential duration (APD) in atrial, ventricular, and Purkinje cells. It also increases the atrial effective refractory period (ERP) more than the ventricular ERP (Mounsey and DiMarco, 2000; Grant, 2009). Thus, it is more effective in atrial arrhythmias that are due to re-entry than other mechanisms (Shenasa and Shenasa, 2016). It is important to note that dofetilide did not adversely affect mortality in high-risk patient group with recent myocardial infarction, chronic heart failure, and reduced ejection fraction. Furthermore, dofetilide did not pose any adverse effects on survival in patients with supraventricular arrhythmias (Pritchett and Wilkinson, 1999). An important concern on the use of dofetilide is its torsadogenic effects (Gordon, 1999).

1.2.4. Class IV antiarrhythmic drugs

Class IV compounds block the L-type Ca^{2+} channel, and depress Ca^{2+}-dependent APs in tissues with "slow response" action potentials and thus decrease the rate of automaticity, slow conduction velocity, and prolong refractory period. Heart rate is decreased, the PR interval is lengthened, and the AV node transmits rapid atrial depolarizations at a lower frequency. These agents are used mainly to treat supraventricular tachycardias or

tachyarrhythmias. It consistently slows and regularises the ventricular response in atrial fibrillation, and usually increases the degree of AV-nodal block in atrial flutter though it occasionally induces a return to sinus rhythm. Given orally it is useful for the prophylaxis of atrioventricular reentry tachycardia, and also in modulating the atrioventricular nodal response in atrial fibrillation. Favourable response in ventricular tachycardia is exceptional and then seen in specific benign varieties. Verapamil is the agent of choice for the termination of paroxysmal supraventricular tachycardia (Krikler, 1986; Kuhn, 1981). Diltiazem has shown efficacy in the treatment of unstable angina, hypertension, and supraventricular tachyarrhythmias The drug has also been used intravenously to terminate supraventricular tachycardias and to control the ventricular response to atrial fibrillation or flutter. Atrioventricular block although rare, is the most frequent serious adverse event related to diltiazem therapy and may be exacerbated by coadministration of beta-adrenoceptor antagonists, especially in the elderly (Markham and Brogden, 1993; Chaffman and Brogden, 1985).

1.2.5. Class V antiarrhythmic drugs

Class V drugs are specific bradycardic agents with sino-atrial pacemaker current (I_f) blocking property. I_f plays an important role in spontaneous diastolic depolarization (DiFrancesco, 1995). The current is conducted by hyperpolarization-activated cyclic nucleotide-gated (HCN) channels. Inhibition of the pacemaker currrent reduces the steepness of diastolic depolarization. Thus, heart rate is reduced and the RR interval is widened on the ECG. Ivabradine is a selective inhibitor of I_f current, which has been shown to effectively suppress inappropriate sinus tachycardia, junctional tachycardia, atrial tachycardia, and ventricular ectopy in humans (Kohli *et al.*, 2020). In isolated rabbit hearts, a significant suppression of atrial fibrillation was described by ivabradine (Frommeyer *et al.*, 2017). Furthermore, a significant slowing of atrioventricular conduction in the presence of atrial fibrillation was reported for ivabradine (Verrier *et al.*, 2014; Verrier *et al.*, 2015). Long-term reduction of heart-rate with ivabradine in patients with chronic coronary syndrome improved diastolic function and reduced mean aortic flow velocity (Hohneck et al., 2021). In heart failure, reduction of heart-rate with ivabradine is important for the improvement of clinical outcomes (Swedberg *et al.*, 2010).

1.3 SZV-270: a novel compound with combined Class I/B and Class III antiarrhythmic effects

Following the significant setbacks for pharmacological prevention of ventricular arrhythmias that were provided by the Cardiac Arrhythmia Suppression Trials (The Cardiac Arrhythmia Suppression Trial Investigators, 1989; The Cardiac Arrhythmia Suppression Trial II Investigators, 1992) and the Survival with Oral D-Sotalol trial (Waldo *et al.*, 1996), where sodium channel blocker Class I/C and potassium channel blocker Class III compounds - instead of improving clinical outcome - increased mortality in post-myocardial infarction patients with reduced ejection fraction, the attention shifted towards potential new antiarrhythmic drugs with more complex ion channel and receptor modulatory effects.

One promising approach to safer and more effective pharmacological arrhythmia management is the use novel compounds that exhibit more complex actions and modulate several ionic currents. Indeed, amiodarone, a compound affecting several ionic currents, remains one of the most effective antiarrhythmic drugs both for the management of AF and severe ventricular arrhythmias (Mujovic *et al.*, 2020), however, especially during its chronic application, it exhibits severe extracardiac adverse effects (Hilleman *et al.*, 1998; Mujović, 2020). Class III antiarrhythmic drugs prolong myocardial repolarization and can effectively reduce re-entry arrhythmias (Hashimoto *et al.*, 1995; Hohnloser *et al.*, 1995; Fei and Frame, 1996), however, they can also provoke Torsades de Pointes (TdP) tachycardia (Verduyn *et al.*, 1997) and D-sotalol increased mortality in post-myocardial infarction patients (Waldo *et al.*, 1996). Despite its significant QT prolonging effect, amiodarone has a relatively low torsadogenic adverse effect (Hohnloser *et al.*, 1994; Belardinelli *et al.*, 2003; Thomsen *et al.*, 2004), possibly due to decreased transmural dispersion of repolarization and inhibition of early afterdepolarization (EAD) formation following amiodarone administration (Sicouri *et al.*, 1997), similarly to Class I/B antiarrhythmic drugs (Shimizu and Antzelevitch, 1997; Assimes and Malcolm, 1998). **Therefore, the development of novel compounds with complex actions exhibiting combined Class I/B and Class III effects and devoid of severe extracardiac adverse effects, that are effective against both supraventricular and ventricular arrhythmias, is justified.**

In line with this concept, SZV-270 (**Figure 3**), a novel agent was synthesized by our collaborators from two well-known pure Class I/B and III antiarrhythmic agents, mexiletine and D-Sotalol, respectively. The lack of beta receptor antagonist activity of the compound has already been confirmed, but no other pharmacodynamic or pharmacokinetic characteristics have been investigated so far. In the first study described in this PhD thesis, we aimed to

characterize the cardiac electrophysiological properties of SZV-270 (**Figure 3**) in rabbit and canine atrial and ventricular preparations. Also, its effects on atrial fibrillation were tested in dogs with chronic atrial tachypacing-induced atrial remodelling. In case of favourable electrophysiological effects, the authors would recommend further studies aiming to fully characterise the compound in terms of its pharmacodynamic, pharmacokinetic and toxicological properties.

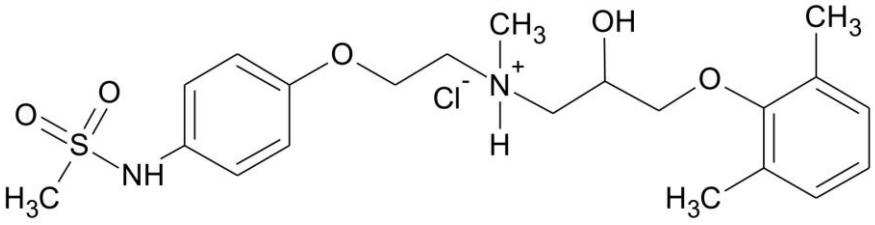

Figure 3. Chemical structure of SZV-270.

1.4 The concept of „hidden cardiotoxicity"

Unexpected clinical cardiotoxicity – often seen as proarrhythmia, a drug-induced '*de novo'* arrhythmia - is still the leading cause of discontinuation of clinical trials and the withdrawal of drugs from the market despite great efforts, such as the implementation of the ICH (International Council for Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use) guidelines (ICH-S7B and ICH-E14) that were proposed for rigorous safety testing to detect cardiovascular adverse effects in the preclinical phase of drug development programs (Madonna *et al.*, 2015).

Since cardiotoxicity often remains 'hidden' in normal/healthy individuals but manifests under cardiac diseased conditions – such as in myocardial ischaemia -, this phenomenon is termed as "hidden cardiotoxicity" (Ferdinandy *et al.*, 2019). Hidden cardiotoxicity often manifests as ischemia-related lethal myocardial injury and/or as I/R-induced arrhythmias and/or as cardiac dysfunction. Thus, drugs with hidden cardiotoxic properties may present a serious risk to patients as drugs with overt cardiotoxicity, such as certain cancer treatments (Yeh *et al.*, 2019). The mechanisms of hidden cardiotoxicity may include the activation of apoptosis, or pro-arrhythmic processes during cardiac I/R, as well as the inhibition of cardioprotective signaling pathways (e.g., ischemic conditioning-induced protection), either of which may be aggravated by the presence of cardiovascular comorbidities (Ferdinandy *et al.*, 2019) (**Figure 4**). Nearly 500 medicinal products were withdrawn from the market between 1953 and 2013 (Onakpoya *et al.*, 2016), the majority of which is related to cardiac adverse events (Madonna *et al.*, 2015). Moreover, an estimated

197,000 deaths are attributed to adverse drug reactions in the European Union each year (Pontes *et al.*, 2014). Hidden cardiotoxicity remains undetected in the preclinical and early clinical phases of drug development, since the current guidelines only require the assessment of drug safety in healthy animals (International Conference on Harmonisation. Guidance on M3(R2), 2010; Guideline on Repeated Dose Toxicity Corr, 2019). In addition, preclinical and clinical cardiac electrophysiological safety test guidelines advocate the use of healthy animals, tissues, and healthy human volunteers for the assessment of the pro-arrhythmic adverse effects of compounds in development and these tests do not represent patients with increased arrhythmia susceptibility (International Conference on Harmonisation. Guidance on E14, 2005; International Conference on Harmonisation. Guidance on S7B, 2005). However, in clinical trials, cardiotoxic adverse events occur in an unpredictable manner, often in patients with cardiac diseases and/or with cardiovascular comorbidities, e.g., hyperlipidemia, hyperglycemia, hypertension, aging, or inflammatory diseases (Page *et al.*, 2016, Chao *et al*,. 2013). Toxic effects of drugs can be 'hidden' when safety testing is only carried out in healthy heart or tissue, but may manifest in the diseased state ('hidden toxicity') (Ferdinandy *et al.*, 2019). Indeed, the guidelines for the treatment of heart failure by the American College of Cardiology Foundation/American Heart Association recommend avoiding the use of certain medications in heart failure, e.g., cyclooxygenase-2 (COX-2) inhibitors, since they may exacerbate underlying myocardial dysfunction (Yancy *et al.*, 2013).

Rofecoxib, which is a COX-2 inhibitor, was withdrawn from the market due to an increased risk of cardiovascular prothrombotic events being observed in the VIGOR and APPROVe trials (Bombardier *et al.*, 2000, Bresalier *et al.*, 2005). Later, in a meta-analysis that included 116,094 participants, it was shown that the use of rofecoxib was associated with an increased risk of arrhythmias (Zhang *et al.*, 2006). Several other mechanisms have been proposed for rofecoxib-induced cardiotoxicity, such as the inhibition of protection against I/R injury, prevention of production of epi-lipoxins, increase in blood pressure, and inhibition of vascular remodeling, however, none of those has been detected during preclinical safety assessment (Salinas *et al.*, 2007). The fact that the cardiotoxic effects of rofecoxib remained hidden in preclinical studies and was only revealed in phase 4 clinical studies and by a following metaanalysis and the enormous costs of long-term cardiovascular outcome trials required to reveal hidden cardiotoxicity suggest that more sensitive screening methods are required for toxicity studies. These models would ideally mimic diseased conditions - such as myocardial ischaemia reperfusion (I/R) - in which most of the drug-induced hidden cardiotoxicity manifests in clinical settings. The in vivo rat, guinea pig, rabbit or dog models

of myocardial I/R are typically carried out by reversibly obstructing and then restoring the blood flow in one of the main coronary arteries of the heart to induce temporal myocardial ischaemia and reperfusion (**Figure 5.**). This method is a gold standard technique to study the mechanisms of I/R-related myocardial injuries, and arrhythmias and to test drugs with potential myocardioprotective effects. The I/R model can also be used to study the mechanisms of highly effective cardioprotective mechanisms evoked by different so-called'conditioning' stimuli (**Figure 5.**). For more details about the myocardial I/R models, the mechanisms of I/R-induced myocardial injuries and arrhythmias and the possible cardioprotective effects of different conditioning methods, the author here would only like to refer to some excellent reviews in the field (e.g.: Theodore Kalogeris *et al*., 2017; Sean M. Davidson *et al*., 2019) due to the limitations of the current thesis work. Based on the above, in the second part of this thesis, we aimed to characterize a novel preclinical *in vitro* I/R model for assessing the possible hidden cardiotoxic effects of different drugs. To validate our model, we used rofecoxib, a compound withdrawn from the market because of its cardiovascular side effects (Bombardier *et al*., 2000, Bresalier *et al*., 2005).

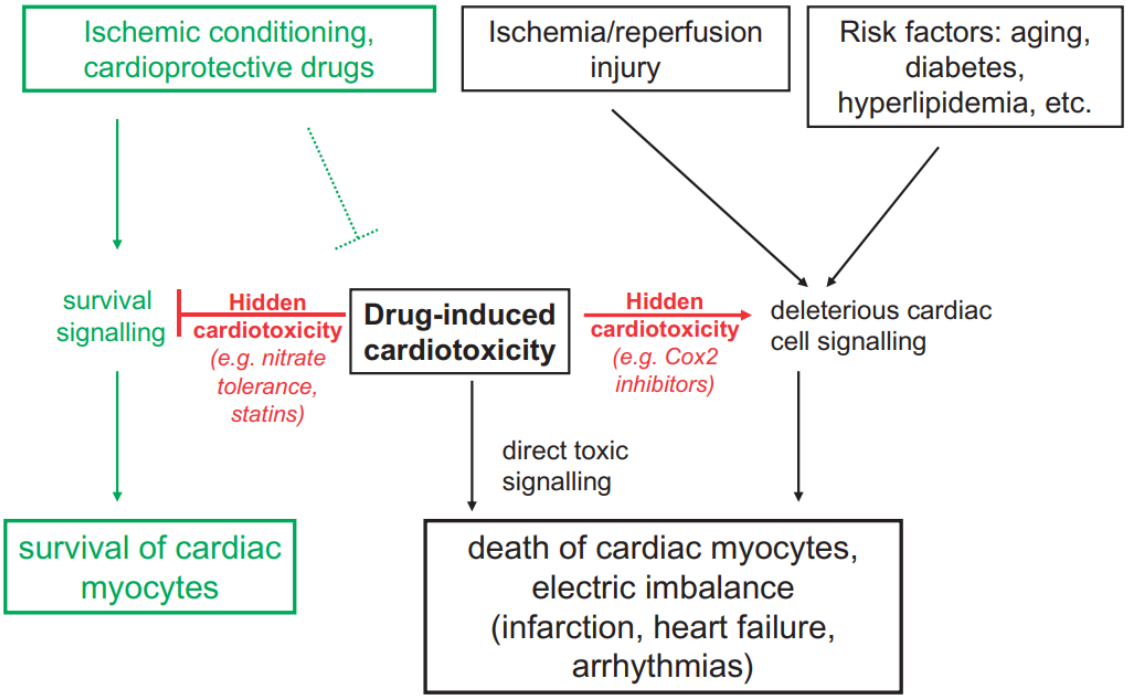

Figure 4. Schematic illustration of the influence of cardioprotective factors (ischaemic conditioning, drugs), ischemia/reperfusion injury and cardiovascular risk factors on cardiotoxic effects of drugs. (original figure: Ferdinandy *et al*., 2019).

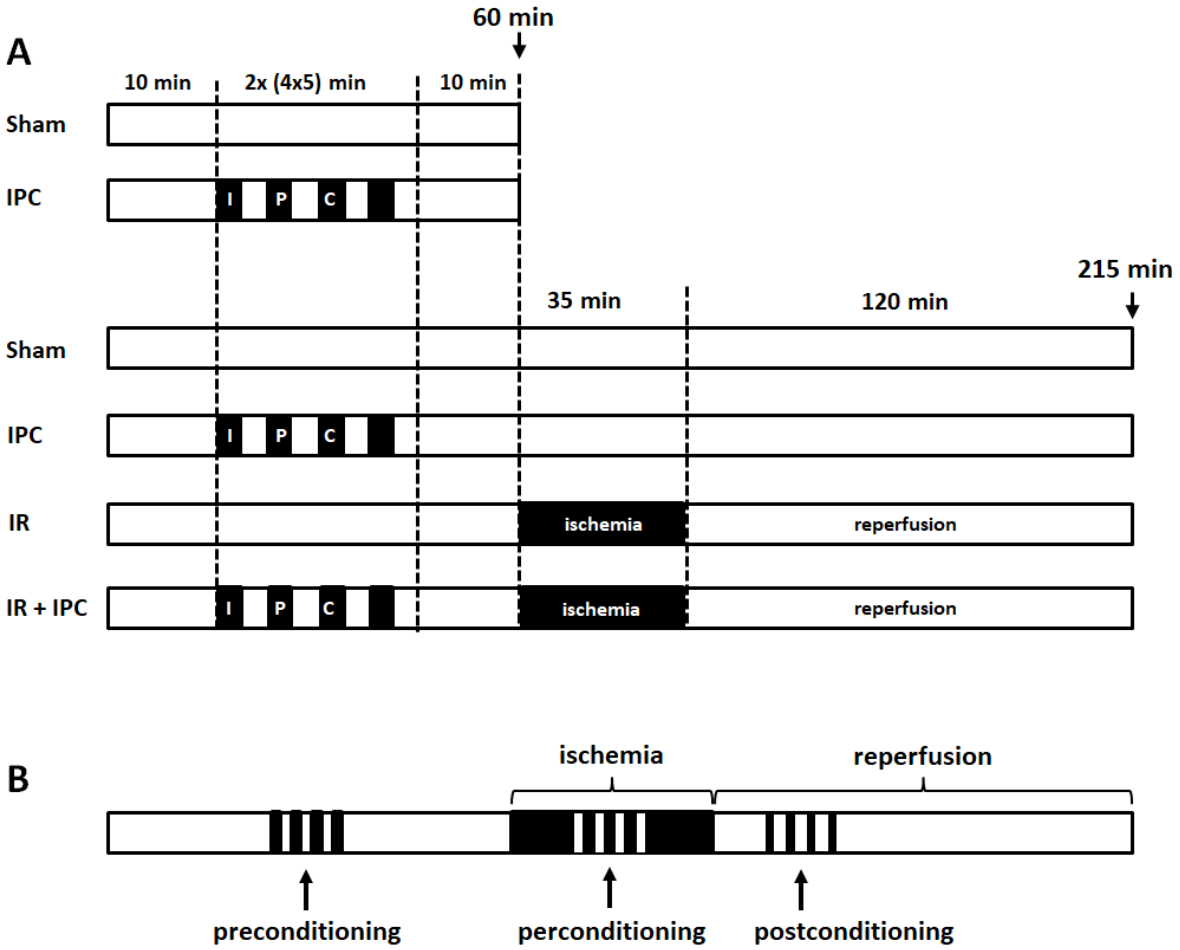

Figure 5. A. Schematic illustration of a typical experimental protocol for *in vivo* experiments studying the effects of preconditioning on cardiac ischemia/reperfusion-induced injuries and/or arrhythmias. Abbreviations: SHAM: control animals undergoing the same procedure(s) as 'non-control' groups except specific interventions that are to be studied, IPC: ischaemic preconditioning, IR: ischemia/reperfusion. **B.** Schematic diagram illustrating the key temporal aspects of pre-, per-, and post-conditioning.

2. AIMS OF THE STUDIES

2.1 Aim 1: Investigation of the cardiac electrophysiological effects of SZV-270

To investigate the cardiac electrophysiological and antiarrhythmic effects of a new compound, SZV-270 on multicellular preparations, enzymatically isolated cardiomyocytes, and in conscious dogs with electrical atrial remodeling induced by atrial tachypacing.

2.2 Aim 2: Investigation of the possible hidden cardiotoxicity of rofecoxib

To test the hidden cardiotoxic properties of rofecoxib by using an *in vitro* preclinical model of ischaemia/reperfusion injury, and establish the basis of a new method for safety pharmacology.

3. MATERIALS AND METHODS

3.1 Ethical issues

All animal care and the described experiments complied with the Guide for the Care and Use of Laboratory Animals (U.S.A. NIH publication No 85-23, revised 1996) and conformed to the the Directive 2010/63/EU of the European Parliament. The experimental protocols had been approved by the Ethical Committee for the Protection of Animals in Research of the University of Szeged, Szeged, Hungary (I-74-18-2016; I-74-15/2017; I-74-24/2017); and also by the Department of Public Health and Food Chain Safety at the Csongrád County Government Office (XIII/4227/2016; XIII/3330/2017; XIII/3331/2017).

3.2 Cardiac electrophysiological studies with SZV-270

3.2.1 Atrial fibrillation following chronic atrial tachypacing in conscious dogs

Atrial fibrillation was induced in male Beagle dogs (n=6) weighing 12-15 kg as described previously (Baczkó *et al.*, 2014). In brief, two bipolar pacemaker electrodes (Synox SX 53-JBP and Synox SX 60/15-BP, Biotronik Hungary Ltd., Hungary) were implanted into the right atrial appendage and the apex of the right ventricle were connected to pacemakers (Logos DS and Philos S, Biotronik Hungary Ltd., Hungary) placed in subcutaneous pockets in the neck area. The implantation was followed by radiofrequency catheter ablation of the AV node. Following a 5-day recovery from surgery, right atrial tachypacing was started at 400 beats/min (ICS 3000 Programmer, Biotronik Hungary Ltd., Hungary), maintained for 6 weeks before the experiments to induce atrial electrical remodeling (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 300 ms with a train of 10 stimuli (S1) followed by an extrastimulus (S2), with the AERP defined as the longest S1-S2 interval that did not produce a response.

On the day of the experiment atrial pacing was stopped, continuous recording of the electrocardiogram started using precordial leads and the AERP was measured. A control set of 10-second-long rapid atrial bursts (25 times, 800 beats/min, at twice threshold) were performed to induce atrial fibrillation in conscious dogs preceded by an infusion of vehicle in 15 min. Following the measurement of AERP, additional sets of atrial bursts were applied subsequent to SZV-270 (0.3 mg/kg), or dofetilide (Sigma-Aldrich, 25 μg/kg), i.v. administration. At least 5 days were allowed for washout between *in vivo* experiments with the two compounds. Intravenous infusions were performed using a programmable infusion

pump (Terufusion TE-3, Terumo Europe, Leuven, Belgium). The ECG was recorded using precordial leads, using SPEL Advanced Haemosys software (version 3.2, MDE Heidelberg GmbH, Heidelberg, Germany) as described above. The AERP and the incidence of AF were measured and calculated. Experiments were performed in unrestraint conscious dogs, therefore any effects of anesthetics (Freeman *et al.*, 1990; Baczkó *et al.*, 1997) on AERP and AF could be ruled out.

3.2.2 Action potential (AP) recordings with the conventional microelectrode technique

3.2.2.1. AP measurements from canine atrial trabeculae

Male Beagle dogs (weighing 12-15 kg; n=6) were sedated (xylazine, 1 mg/kg, i.v. and ketamine, 10 mg/kg, i.v.) and anesthetized (pentobarbital, Sigma-Aldrich, 30 mg/kg i.v.), their hearts were 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 the solution was set between 7.35 and 7.4 when saturated with the mixture of 95% oxygen and 5% CO_2 at 37 ºC. Isolated right atrial trabeculae were obtained, individually mounted in a tissue chamber and stimulated as decribed previously (Juhász et al., 2018) **(Figure 6.)**. The maximal rate of depolarization (V_{max}), maximum diastolic potential, action potential amplitude, and action potential duration measured at 90% of repolarization (APD_{90}) were evaluated off-line, applying stimulation with a constant basic cycle length (BCL) of 500 ms.

3.2.2.2. AP measurements from canine and rabbit right ventricular papillary muscles and in canine Purkinje fibers

Male Beagle dogs (weighing 12-15 kg; n=7) and white rabbits (weighing 2-3 kg; n=6) were used for the experiments. Right ventricular papillary muscle tips were obtained, mounted and stimulated using the conventional microelectrode technique as described previously (Jost *et al.*, 2013; Kohajda *et al.*, 2016). The preparations were stimulated (HSE stimulator type 215/II) initially at a constant cycle length of 500 ms (canine Purkinje fibers) or 1000 ms (canine and rabbit papillary muscle), with rectangular constant current pulses 2 ms in duration. The current pulses were isolated from ground and delivered through bipolar platinum electrodes. Transmembrane potentials were recorded with the use of conventional 5–20 MΩ, 3 M KCl-filled microelectrodes connected to the input of a high-impedance electrometer (Biologic Amplifier VF 102, Claix, France). The first derivative of transmembrane potential (dV/dt_{max}) was obtained electronically with a Biologic DV-140

(Claix, France) differentiator. At least 60 min. was allowed for each preparation to equilibrate during continuous superfusion with modified Locke's solution, warmed to 37˚C before the experimental measurements commenced. The following types of stimulation in the course of the experiments were applied: stimulation with a constant cycle length of 1000 or 500 ms (1 or 2 Hz); stimulation with different constant cycle lengths ranging from 300 to 5000 ms taking the measurements after the 25th beat. The preparations were then superfused with the solution containing 1 μM SZV-270 for 40–60 min. before the pacing protocol was repeated and the parameters were measured again, then superfusion continued with 5 μM SZV-270 for another 40-60 min. and measurements were repeated. Efforts were made to maintain the same impalement throughout each experiment. In case an impalement became dislodged, however, adjustment was performed and the experiment continued if AP characteristics of the re-established impalement deviated less than 5% from the previous measurement.

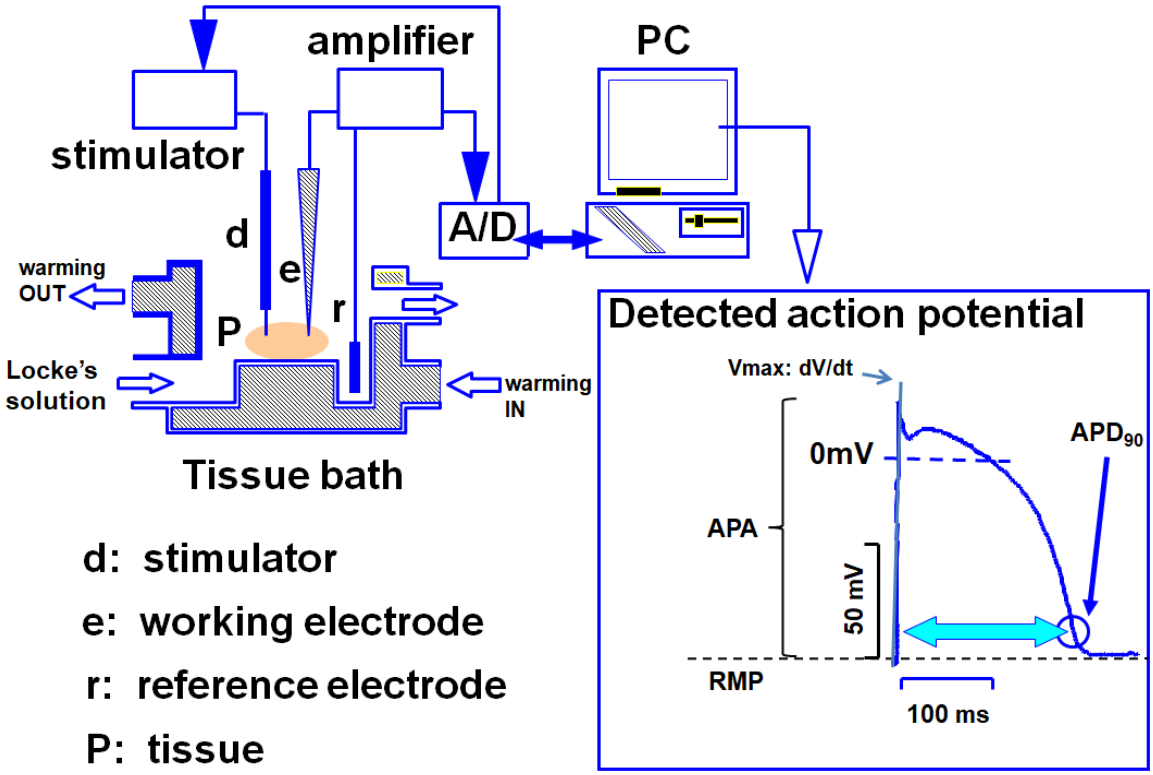

Figure 6. Schematic illustration of a typical conventional microelectrode set-up used to detect action potentials derived from cardiac tissue slices *in vitro*. Abbreviations: APA: AP amplitude (mV), RMP: resting membrane potential (mV), Vmax: maximal speed of depolarisation (V/s), APD90: AP duration at 90% of the maximal repolarisation (ms), A/D: analogue/digital converter

3.2.3 Whole cell patch-clamp studies

Isolated ventricular cardiomyocytes were obtained from male rabbits (weighing 2-3 kg) by enzymatic dissociation as described previously (Major *et al.*, 2016). A drop of cell suspension was placed into a transparent recording chamber mounted on the stage of an inverted microscope (Olympus IX51, Olympus, Tokyo, Japan), and myocytes were allowed to settle and adhere to the bottom of the chamber for at least 5 minutes before superfusion was initiated (**Figure 7**). HEPES buffered Tyrode's solution was used as the normal superfusate. This solution contained (in mM): NaCl 144, NaH_2PO_4 0.4, KCl 4.0, $CaCl_2$ 1.8, $MgSO_4$ 0.53, Glucose 5.5, and HEPES 5.0 at pH of 7.4. Patch clamp micropipettes were made from borosilicate glass capillaries using a P-97 Flaming/Brown micropipette puller (Sutter Co, Novato, CA, USA). The electrodes had 1.5-2.5 MΩ resistances when filled with pipette solution that contained (in mM): KOH 110, KCl 40, K_2ATP 5, $MgCl_2$ 5, EGTA 5, GTP 0.1 and HEPES 10, during K^+ current measurements. Aspartic acid was used to adjust the pH of the pipette solution to 7.2. The L-type calcium current ($I_{Ca,L}$) was recorded in HEPES-buffered Tyrode's solution supplemented with 3 mM 4-aminopyridine. A special pipette solution was used containing (in mM: KOH 40, KCl 110, TEACl 20, MgATP 5, EGTA 10, HEPES 10 and GTP 0.25, pH was adjusted to 7.2 by KOH.

Ionic membrane currents were recorded with the Axopatch 200B patch-clamp amplifier (Molecular Devices, Sunnyvale, CA, USA) using the whole cell configuration of the patch clamp technique. Membrane currents were digitized and recorded under software control (Digidata 1440A, pClamp 10, Molecular Devices, Sunnyvale, CA, USA) after low-pass filtering at 1 kHz. The inward rectifier (I_{K1}), transient outward (I_{to}), rapid ($I_{Kr)}$ delayed rectifier potassium currents were recorded in rabbit ventricular myocytes. 1 µM nisoldipine was included in the bath solution to block $I_{Ca,L}$. When I_{Kr} was recorded, I_{Ks} was inhibited by using the selective I_{Ks} blocker HMR1556 (0.5 µM). All experiments were performed at 37 °C.

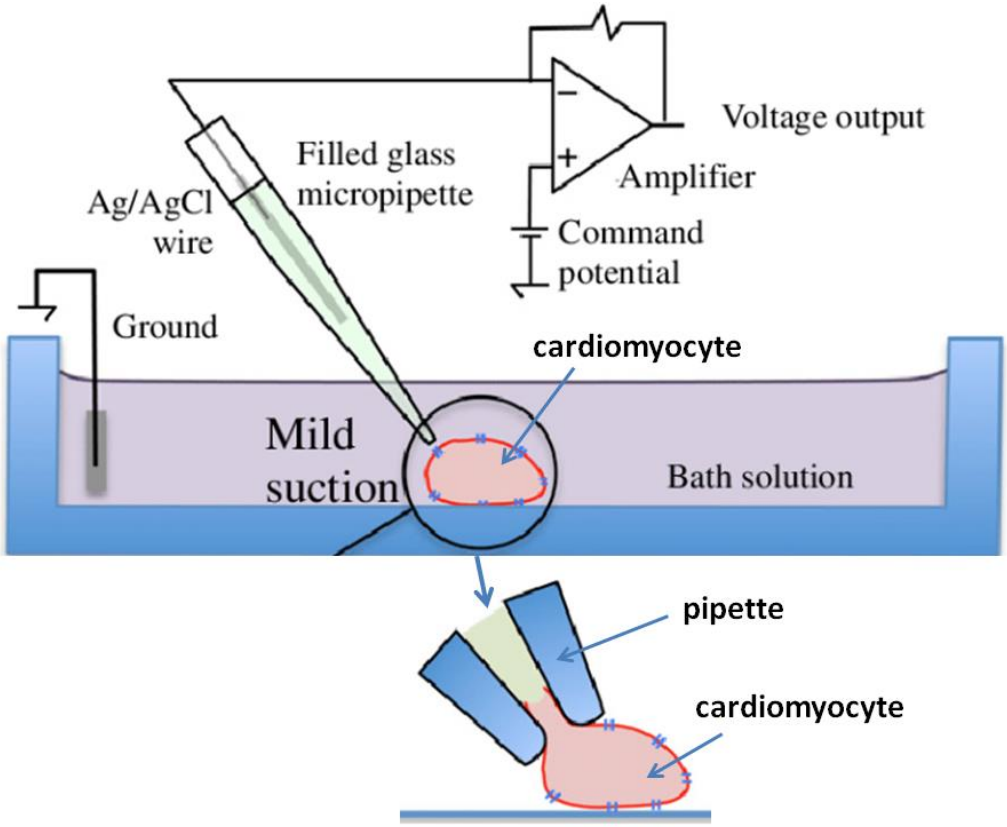

Figure 7. Schematic illustration of a patc- clamp set-up in whole cell configuration.

3.2.4 Statistical analysis for the SZV-270 study

The incidence of arrhythmias was calculated and compared by using the χ^2 method. All other data are expressed as mean ± SEM. Statistical analysis was performed using ORIGIN 8.1 (Microcal Software, Northampton, MA, USA). Differences between means were compared by ANOVA followed by Student's t-test (paired or unpaired, as appropriate). Data were considered as statistically significant when $p<0.05$.

3.3 Cardiac electrophysiological studies with rofecoxib

3.3.1 In vitro simulated ischemia/reperfusion injury study

For isolated papillary muscle experiments, male Wistar rats weighing 200–250 g were used. The rats were anesthetized with pentobarbital intraperitoneally (30 mg/kg), followed by rapid excision of the heart via thoracotomy. Left ventricular papillary muscle preparations were mounted in a tissue chamber (volume together with solution reservoir: 50 mL) and they were then continuously perfused with oxygen–saturated, HEPES-buffered Tyrode's solution (in mM: NaCl 144, NaH_2PO_4 0.4, KCl 4, $MgSO_4$ 0.53, $CaCl_2$ 1.8, glucose 5.5, HEPES 5 at pH 7.4, 37 °C). The preparations were stimulated (Hugo Sachs Electronik stimulator type 215/II, March-Hugstetten, Germany) at a cycle length of 1000 ms (frequency: 1 Hz), while

using 2 ms-long rectangular constant voltage pulses that were isolated from ground and delivered across bipolar platinum electrodes in contact with the preparation. Transmembrane potentials were recorded while using the conventional microelectrode technique. Microelectrodes that were filled with 3 M KCl and exhibiting tip resistances of 5–20 MΩ were connected to a high impedance electrometer (type 309, MDE Heidelberg GMBH, Heidelberg, Germany) coupled to a dual beam oscilloscope (Tektronix, Beaverton, OR, USA). **Figure 8** illustrates the experimental design and study protocols. Altogether, 54 animals were included in the ex vivo experiments. Papillary muscles of 6 animals/group were superfused with oxygen–saturated HEPES-buffered Tyrode's solution (normoxic solution) and were allowed to equilibrate for 60 min. before baseline measurements were taken. Throughout the experiments, measurements were taken every 2 min. Following the 60-min. equilibration period, groups of preparations were superfused with normoxic solution containing either vehicle, 1 or 10 µM rofecoxib (Normoxia groups) dissolved in DMSO for 90 min. The concentration of 1 µM was chosen for rofecoxib based on the peak plasma concentration (C_{max}) measured after a single, 5 mg kg^{-1} oral dose of rofecoxib in rats (Halpin *et al.*, 2000). The highest final concentration of DMSO following the application of 10 µM rofecoxib was 0.2% in the solution. Following the 60-min. baseline superfusion, groups of preparations were superfused with normoxic solution for 30 min. then with nitrogen-saturated and HEPES-buffered solution (ischemic solution, in mM: NaCl 144, NaH_2PO_4 0.4, KCl 4, $MgSO_4$ 0.53, $CaCl_2$ 1.8, 2-deoxy-D-glucose 5.5, HEPES 5 at pH 6.9, and 37 °C) for 30 min., and then with normoxic solution for 30 min., all containing either vehicle, 1 or 10 µM rofecoxib (sI/R groups) to induce simulated I/R (sI/R). In additional groups of preparations, sIPC (sIPC groups) was performed before 30 min. ischemia by using the following protocol: three times 5-min. simulated ischemia with intermittent 5 min. reperfusion periods. Before index ischemia, the last reperfusion lasted 15 min.

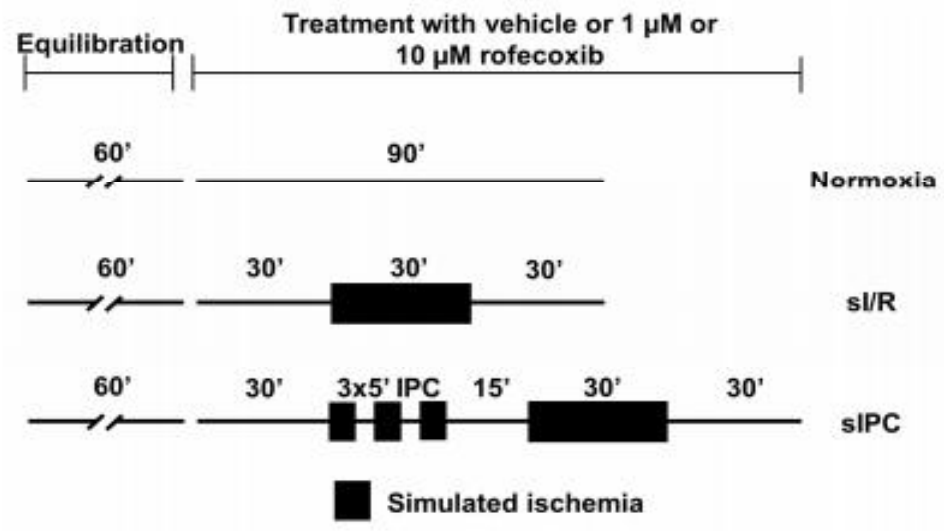

Figure 8. *Ex vivo* simulated ischemia/reperfusion (sI/R) injury study protocol: action potential parameters were measured in isolated rat left ventricular papillary muscle in normoxic, sI/R and simulated ischemic preconditioning (sIPC) conditions in the presence of vehicle or 1 or 10 μM rofecoxib, respectively.

3.3.2 Evaluation of action potential parameters

Unbiased evaluation of action potential parameters was achieved by automatic evaluation while using software that was developed in Department of Pharmacology and Pharmacotherapy, University of Szeged (Hugo Sachs Electronic-Action Potential Evaluation System): V_{max}, CT, RMP, APA, APD at 75 and 90% of repolarization (APD_{75} and APD_{90}, respectively). The maintenance of the same impalement throughout each experiment was attempted. However, in case an impalement was dislodged, electrode adjustment was performed, and the experiment was terminated and all data were excluded from analysis if the action potential characteristtics of the re-established impalement deviated by more than 5% from the previous measurement.

3.3.3 Statistical analysis for the rofecoxib study

The difference between treatment groups was evaluated while using two-way ANOVA or one-way ANOVA followed by Fisher LSD post hoc tests with multiple comparisons. We used GraphPad Prism (version 6.0, GraphPad Software, California, USA) and R (version 3.4) with the lme4 library. We claimed that the differences were statistically significant when $p < 0.05$.

4. RESULTS

4.1 Antiarrhythmic and cardiac electrophysiological effects of SZV-270

4.1.1 Effects of SZV-270 and dofetilide on burst-induced atrial fibrillation in conscious dogs

Before starting the chronic rapid atrial pacing at 400 beats/min in conscious dogs, the right atrial effective refractory period (AERP) values in these animals were 128 ± 3.2 ms (n=6, at basic cycle length of 300 ms). The AERP significantly decreased to 88 ± 2.8 ms after 6 weeks of rapid right atrial pacing, indicating marked electrical remodeling of the right atrium. In all dogs, the effects of SZV-270 on AERP and incidence of AF were compared to that of the I_{Kr} blocker dofetilide. As **Figure 9** illustrates, both SZV-270 and dofetilide markedly increased AERP and significantly reduced the incidence of AF in conscious dogs.

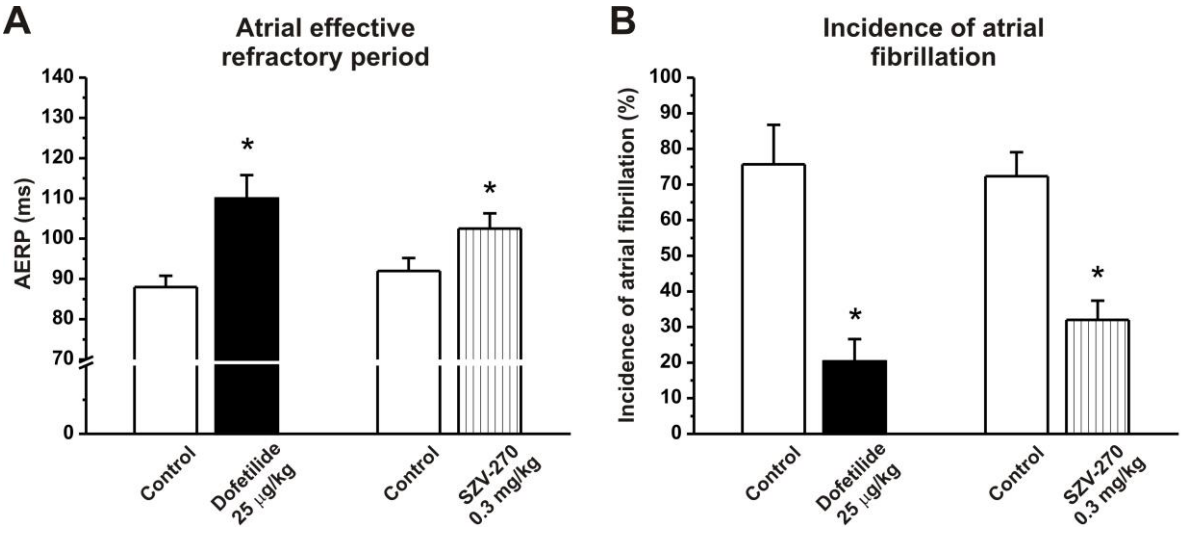

Figure 9. Effect of the selective I_{Kr} blocker dofetilide (25 μg/kg, i.v.) and SZV-270 (0.3 mg/kg, i.v.) on atrial fibrillation in conscious dogs with electrical atrial remodeling induced by atrial tachypacing. **(A)** Both dofetilide and SZV-270 increased right atrial effective refractory period (AERP) significantly. **(B)** Both dofetilide and SZV-270 reduced the incidence of atrial fibrillation (AF) significantly. AERP was measured at basic cycle length of 300 ms. Values are mean ± SEM; n=4-6 animals/group; *p<0.05 vs control values.

4.1.2 Effects of SZV-270 on action potentials in rabbit and canine right ventricular papillary muscles

In our experiments, we studied the possible mechanisms responsible for the atrial and ventricular antiarrhythmic effects of SZV-270. First, the effects of SZV-270 (1 and 5 μM) were investigated using the conventional microelectrode technique on the action potential configuration in rabbit and canine right ventricular papillary muscle preparations. As **Table 2 and 3** shows, SZV-270 did not alter resting membrane potential (RMP), action potential amplitude (APA) and the maximum rate of depolarization (V_{max}) in rabbit and dog papillary muscle at 1 Hz stimulation frequency However, SZV-270 exerted Class III antiarrhythmic effects by prolonging the repolarization at 50%, 75% and 90% (APD_{50}, APD_{75} and APD_{90}) and the effective refractory period in both species, in a concentration dependent manner (**Figure 10A**, **Figure 11A**, **Table 2 and 3**). The cycle length dependent effects of SZV-270 (1 and 5 μM) were also investigated in rabbit right ventricular papillary muscle preparations (**Figures 10B and C**). In the higher applied concentration, SZV-270 exerted Class I/B antiarrhythmic effect in both species: it significantly decreased V_{max} at cycle lengths shorter than 1000 ms (**Figure 10B** and **Figure 11B**).

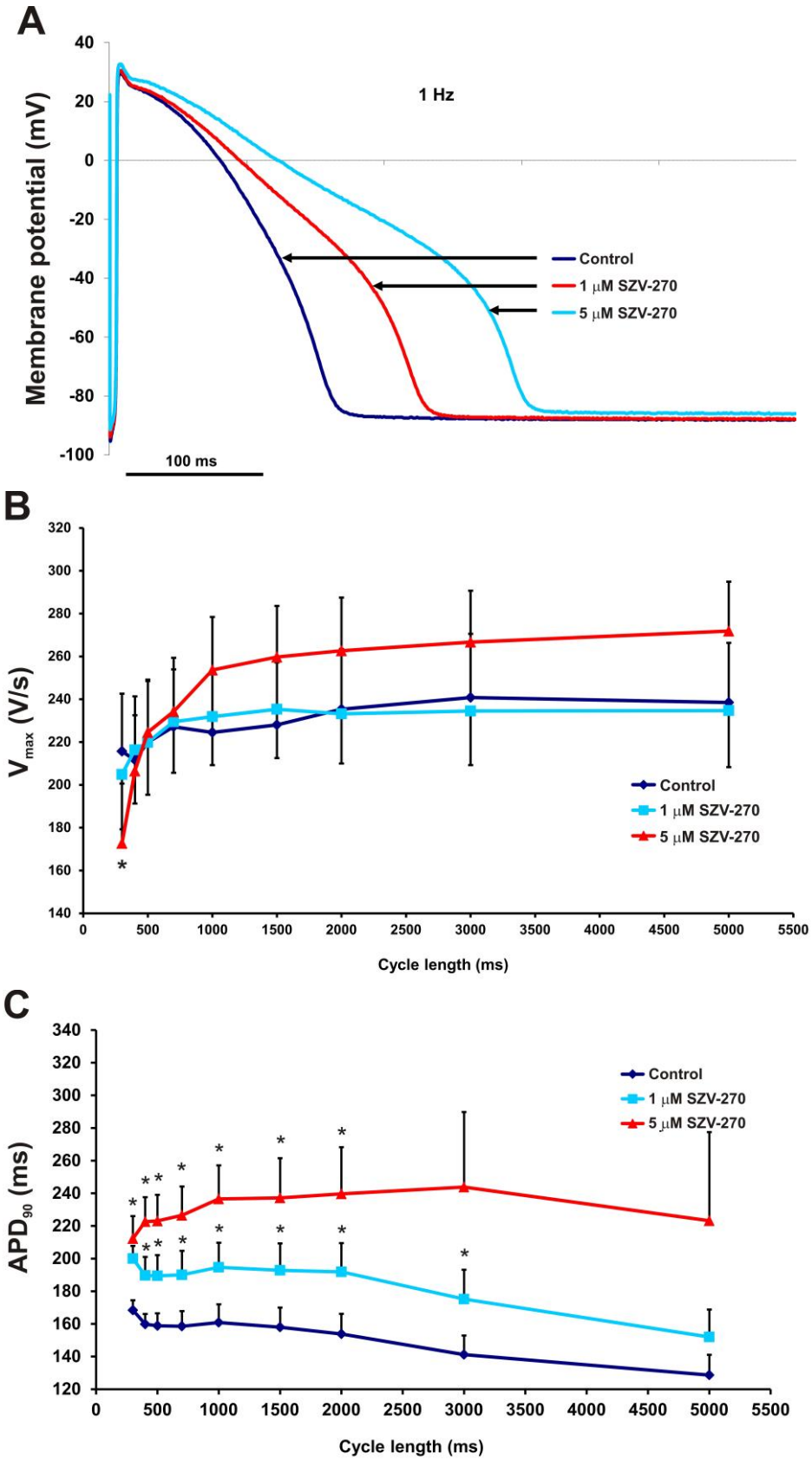

Figure 10. Effect of SZV-270 (1 and 5 μM) on the action potential parameters, on V_{max} and APD_{90} at different stimulation cycle lengths recorded from **rabbit right ventricular papillary muscle** preparations. **(A)** SZV-270 prolonged the APD_{90} in rabbit right ventricular papillary muscle. **(B)** SZV-270 (5 μM) significantly reduced V_{max} at 300 ms cycle length, **(C)** and both concentrations significantly prolonged APD_{90} at cycle lengths shorter than 3000 ms. Values are means ± SEM. n=6, *p<0.05 vs. control values.

Parameter	Control	SZV-270 1 µM	SZV-270 5 µM
RMP (mV)	-86.3 ± 1.8	-84.2 ± 1.2	-85.2 ± 1.4
APA (mV)	115.7 ± 1.8	112.8 ± 3.3	113.5 ± 2.5
APD_{10} (ms)	54.8 ± 7.2	49.6 ± 7.4	50.8 ± 9.1
APD_{25} (ms)	105.2 ± 11.5	108.2 ± 14.6	114.5 ±16.2
APD_{50} (ms)	152.3 ± 15.4	178.7 ± 18.9*	221.2 ± 23.9*
APD_{75} (ms)	174.3 ± 15.6	209.0 ± 18.6*	263.7 ± 23.9*
APD_{90} (ms)	183.3 ±15.4	219.3 ± 18.5*	274.5 ± 23.7*
V_{max} (V/s)	229.8 ± 24.3	231.2 ± 23.2	241.0 ± 22.1
ERP (ms)	174.7 ± 14.7	223.8 ± 20.4*	277.5 ± 27.5*

Table 2. Effect of SZV-270 (1 and 5 µM) on the action potential parameters **in rabbit right ventricular papillary muscle** preparations (n=6). Stimulation frequency = 1 Hz; RMP = resting membrane potential; APA = action potential amplitude; $APD_{10–25–50–75–90}$ = action potential duration at 10, 25, 50, 75 and 90% of repolarisation; V_{max} = maximal rate of depolarization; ERP = effective refractory period. Results are expressed as means ± SEM; *$p<0.05$.

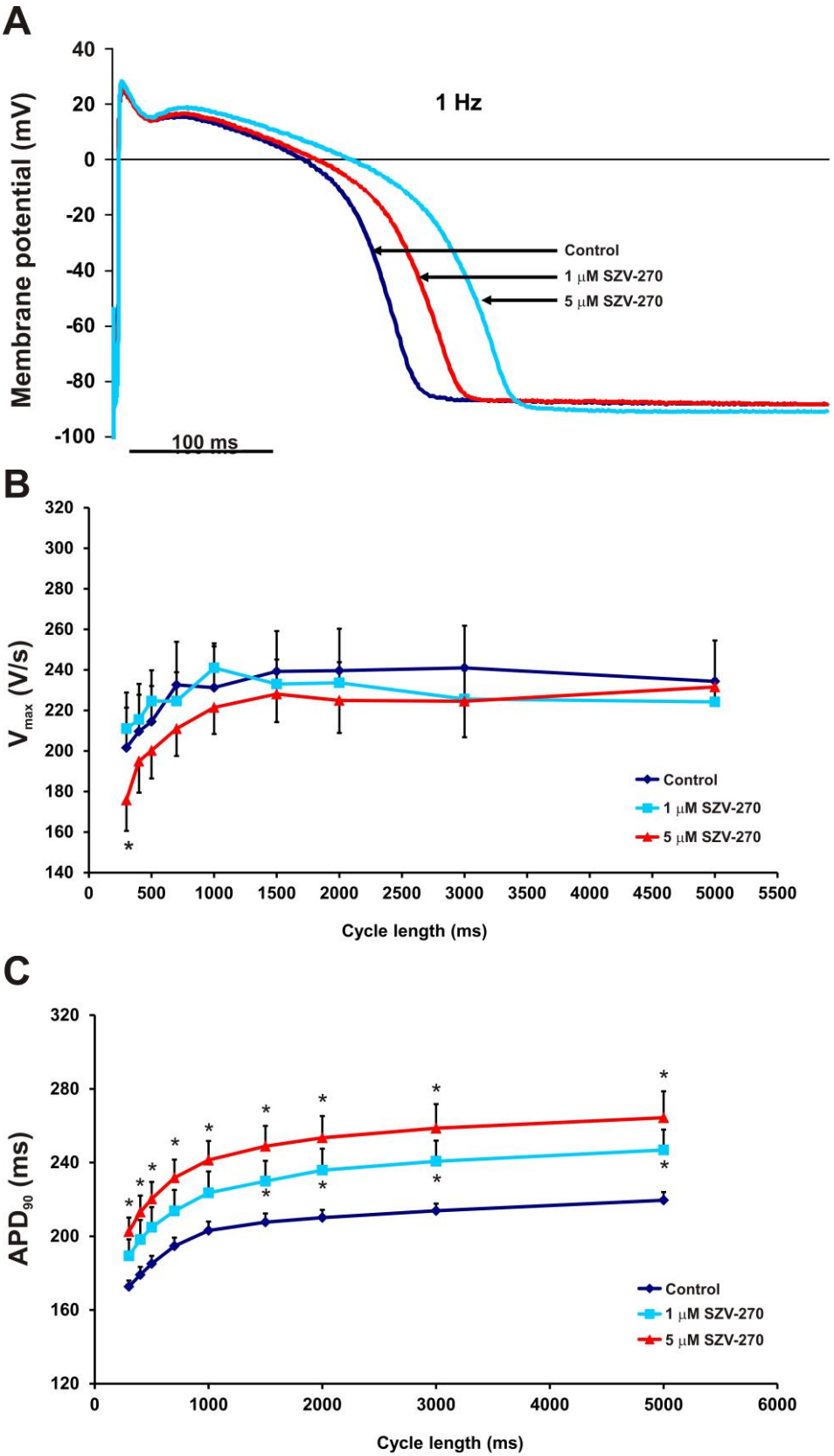

Figure 11. Effect of SZV-270 (1 and 5 μM) on the action potential parameters, on V_{max} and APD_{90} at different stimulation cycle lengths recorded from **dog right ventricular papillary muscle** preparations. (**A**) SZV-270 prolonged the action potential in canine right ventricular papillary muscle. (**B**) SZV-270 (5 μM) significantly reduced Vmax at 300 ms cycle length, (**C**) and both concentrations significantly prolonged APD_{90} in these preparations. Values are means ± SEM. n=6, *p<0.05 vs. control values.

Parameter	Control	SZV-270 1 µM	SZV-270 5 µM
RMP (mV)	-84.9 ± 1.0	-85.4 ± 1.0	-83.9 ± 1.1
APA (mV)	105.4 ± 1.3	107.3 ± 1.3*	106.0 ± 2.4
APD_{10} (ms)	60.9 ± 13.3	66.3 ± 14.2	58.9 ±16.7
APD_{25} (ms)	133.0 ± 6.9	143.9 ± 8.0*	149.4 ± 7.7
APD_{50} (ms)	180.0 ± 6.8	198.3 ± 9.9*	208.0 ±11.4
APD_{75} (ms)	201.6 ± 7.5	222.6 ± 11.0*	240.7 ± 10.4*
APD_{90} (ms)	211.4 ± 7.9	233.6 ± 11.2*	251.7 ± 10.6*
V_{max} (V/s)	208.0 ± 8.9	220.6 ± 13.0	212.0 ± 12.7
ERP (ms)	223.4 ± 8.3	250.0 ± 14.1*	263.4 ± 13.1*

Table 3. Effect of SZV-270 (1 and 5 µM) on the action potential parameters in **dog right ventricular papillary muscle** preparations (n=7). Stimulation frequency = 1 Hz; RMP = resting membrane potential; APA = action potential amplitude; $APD_{10–25–50–75–90}$ = action potential duration at 10, 25, 50, 75 and 90% of repolarisation; V_{max} = maximal rate of depolarization; ERP = effective refractory period. Results are expressed as means ± SEM; *$p<0.05$.

4.1.3 Effects of SZV-270 on action potentials in canine Purkinje fibers

In dog Purkinje fibers SZV-270 did not influence the RMP or the APA (**Table 4**). SZV-270 showed more complex effects on the repolarization in Purkinje fibers compared to papillary muscle preparations. As shown in **Table 4**, at 2 Hz stimulation frequency the compound significantly lenghtened the APD_{75} and APD_{90}, however, the APD prolongation was smaller after the application of the larger concentration than that was seen following the application of the smaller concentration. The larger concentration decreased APD_{50} significantly. SZV-270 reduced V_{max} significantly in a concentration dependent manner (**Table 4**).

Parameter	**Control**	**SZV-270 1 µM**	**SZV-270 5 µM**
RMP (mV)	-89.3 ± 0.8	-90.2 ± 0.6	-89.2 ± 0.6
APA (mV)	125.0 ± 1.9	127.2 ±1.5	123.7 ± 1.5
APD_{10} (ms)	1.8 ± 0.15	1.8 ± 0.17	1.7 ± 0.21
APD_{25} (ms)	32.6 ± 9.6	30.5 ± 10.0	24.6 ± 7.2
APD_{50} (ms)	174.7 ±11.1	186.8 ± 13.9	144.2 ± 12.1*
APD_{75} (ms)	229.3 ± 6.3	271.5 ± 9.6*	245.5 ± 7.1*
APD_{90} (ms)	250.0 ± 6.3	301.0 ± 10.2*	285.0 ± 9.1*
V_{max} (V/s)	730.8 ± 67.6	704.7 ± 64.3*	684.8 ± 43.4*

Table 4. Effect of SZV-270 (1 and 5 µM) on the action potential parameters in **canine Purkinje fibers** (n=6). Stimulation frequency = 2 Hz; RMP = resting membrane potential; APA = action potential amplitude; $APD_{10–25–50–75–90}$ = action potential duration at 10, 25, 50, 75 and 90% of repolarisation; V_{max} = maximal rate of depolarization; *$p<0.05$

4.1.4 Effects of SZV-270 on action potentials in canine right atrial trabeculae

The effects of SZV-270 (1 and 5 µM) on atrial action potentials were studied on dog atrial trabeculae. SZV-270 did not change the RMP, APA and V_{max} at 2 Hz stimulation frequency in canine atrial preparations (**Table 5**). Importantly, SZV-270 significantly lengthened atrial action potentials (**Figure 12A**), APD_{50}, APD_{75} and APD_{90} in a concentration dependent manner (**Table 5**). These effects can, at least in part, the reason for the observed atrial antiarrhythmic effects of SZV-270 in our canine AF model.

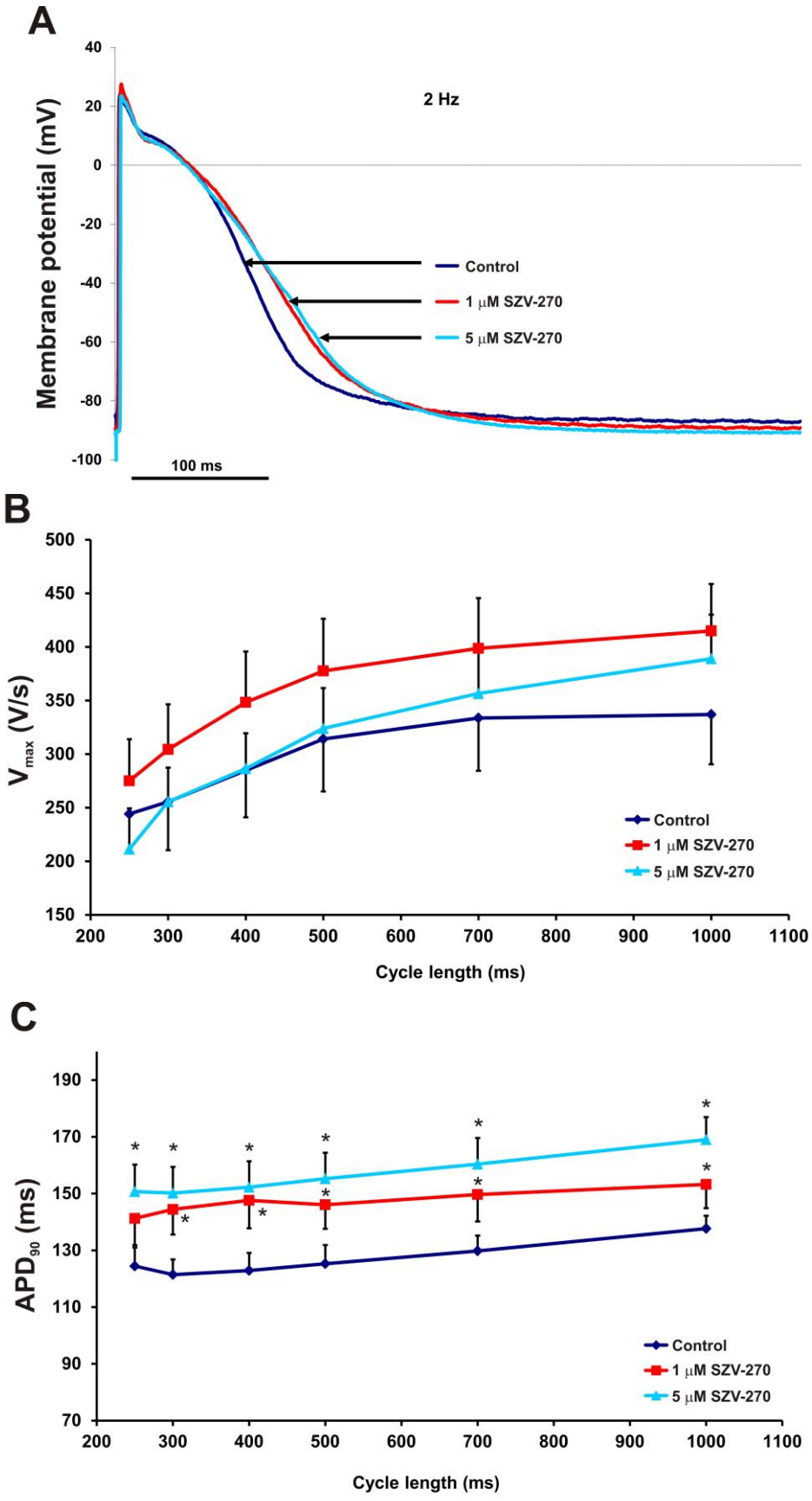

Figure 12. The effects of SZV-270 (1 and 5 μM) on the action potential parameters, on V_{max} and APD_{90} at different stimulation cycle lengths recorded from isolated **canine right atrial trabeculae**. **(A)** SZV-270 prolonged the action potential in dog atrial trabeculae. **(B)** SZV-270 did not significantly alter V_{max}, however, **(C)** prolonged APD_{90} significantly in these preparations. Values are means ± SEM. n=6, *p<0.05 vs. control values.

Parameter	Control	SZV-270 1 µM	SZV-270 5 µM
RMP (mV)	-85.7 ± 1.2	-85.2 ± 1.6	-85.5 ± 1.1
APA (mV)	109.0 ± 1.0	109.3 ± 1.6	111.5 ± 2.5
APD_{10} (ms)	9.0 ± 0.7	9.2 ± 0.5	9.5 ± 0.8
APD_{25} (ms)	43.8 ± 5.1	46.6 ± 4.6	47.4 ± 4.2
APD_{50} (ms)	74.0 ± 5.8	81.8 ± 8.0*	83.8 ± 6.6*
APD_{75} (ms)	100.2 ± 4.8	115.0 ± 8.8*	120.5 ± 8.0*
APD_{90} (ms)	130.8 ± 4.1	156.0 ± 9.6*	165.0 ± 9.4*
V_{max} (V/s)	299.8 ± 38.8	343.0 ± 37.8	347.2 ± 45.8

Table 5. Effect of SZV-270 (1 and 5 µM) on the action potential parameters **in canine right atrial trabecular preparations** (n=6). Stimulation frequency = 2 Hz; RMP = resting membrane potential; APA = action potential amplitude; $APD_{10–25–50–75–90}$ = action potential duration at 10, 25, 50, 75 and 90% of repolarisation; V_{max} = maximal rate of depolarization. Results are expressed as means ± SEM; *$p<0.05$.

4.1.5 Effects of SZV-270 on various transmembrane ionic currents in isolated rabbit ventricular myocytes

To investigate the cellular mechanisms underlying the observed in vivo and in vitro effects of SZV-270, rabbit right ventricular myocytes were isolated and the effects of the drug were observed on I_{Kr}, I_{K1}, I_{to} and $I_{Ca,L}$ using the the whole cell configuration of the patch-clamp technique. As **Figure 13** shows, SZV-270 blocked I_{Kr} significantly in relatively low, 100 and 500 nM concentrations. This result is consistent with the APD lengthening and QTc prolonging effect of the drug. SZV-270 had no effects on the other transmembrane currents, I_{K1} (**Figure 14A**), I_{to} (**Figure 14B**) and $I_{Ca,L}$ (**Figure 15**), even at the high (10 µM) concentration.

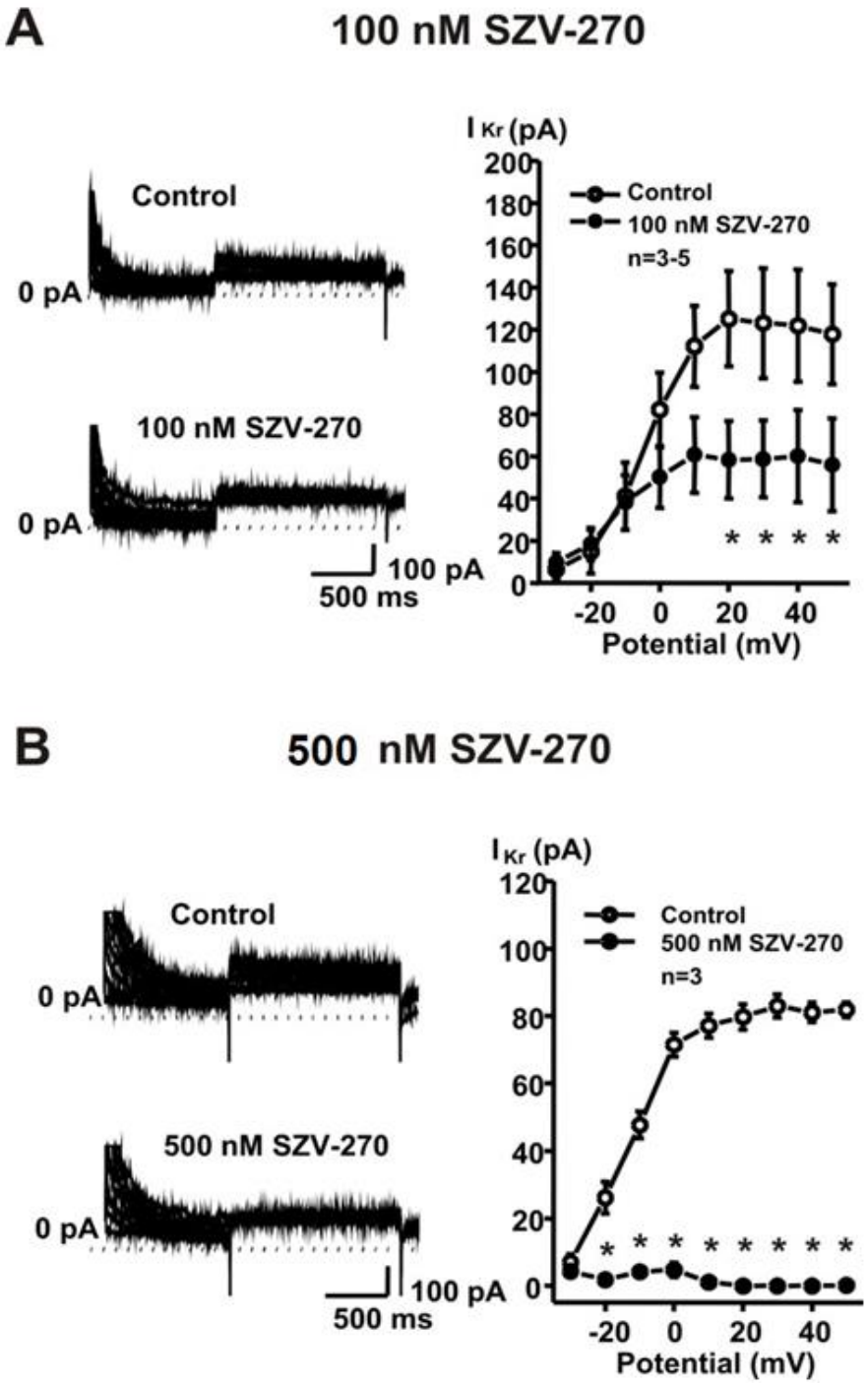

Figure 13. The effect of SZV-270 on the rapid component of the delayed rectifier potassium current (I_{Kr}). SZV-270 inhibited the I_{Kr} tail current in a concentration dependent manner (**panel A**: effects of 100 nM, **panel B**: effects of 500 nM SZV-270). Left subpanels show original current traces in control conditions and following application of 100 and 500 nM SZV-270. Graphs on the right show the respective current-voltage relationships. Values are means ± SEM. n=3-5, *p<0.05 vs corresponding data point in control conditions.

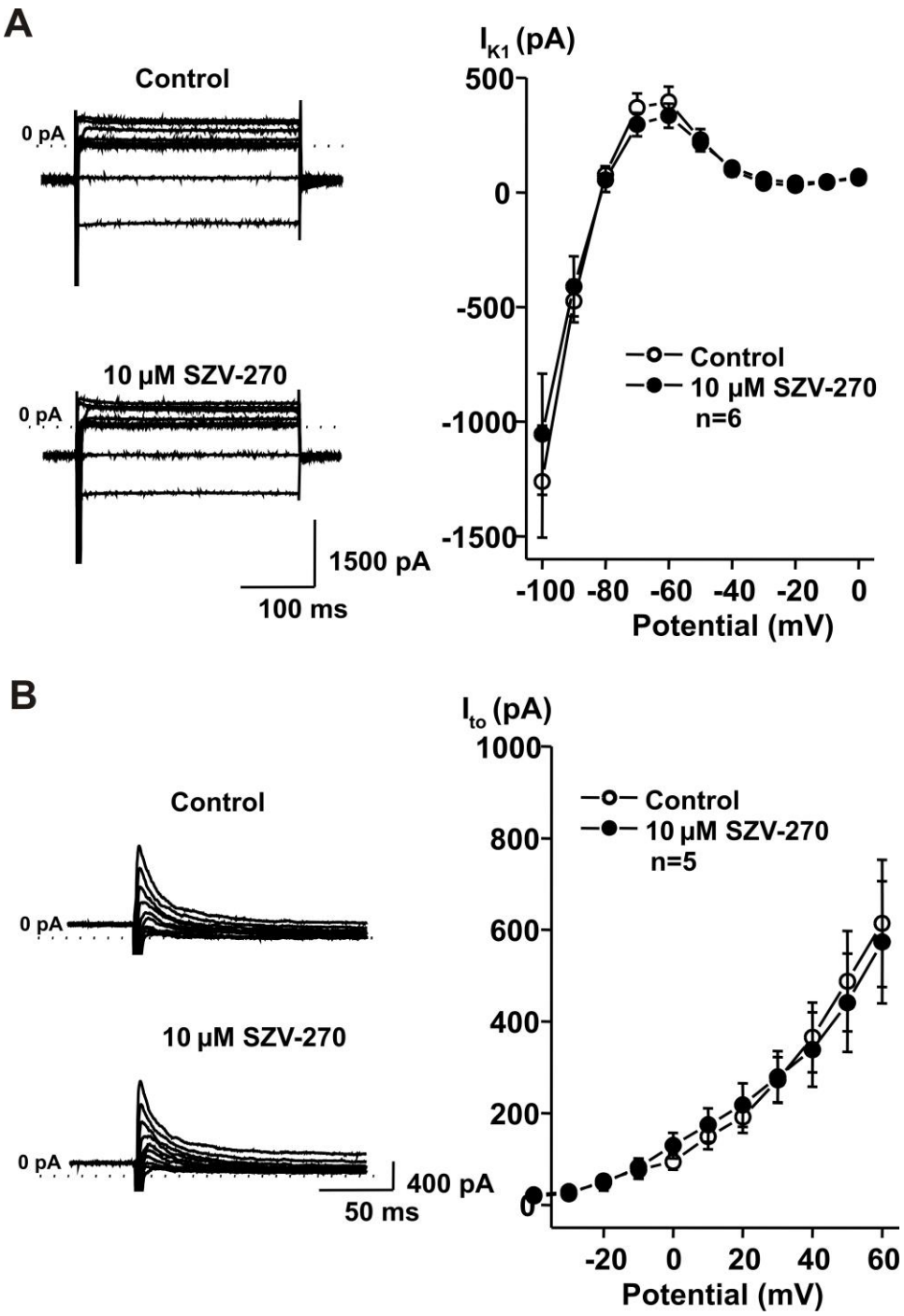

Figure 14. SZV-270 did not influence **(A)** I_{K1} or **(B)** I_{to} even at the high concentration of 10 µM in isolated rabbit right ventricular cardiomyocytes. Left panels depict original current traces recorded in control conditions and in the presence of 10 µM SZV-270. Right panels show the current-voltage relationships. Values are means ± SEM. n=5-6, all $p>0.05$.

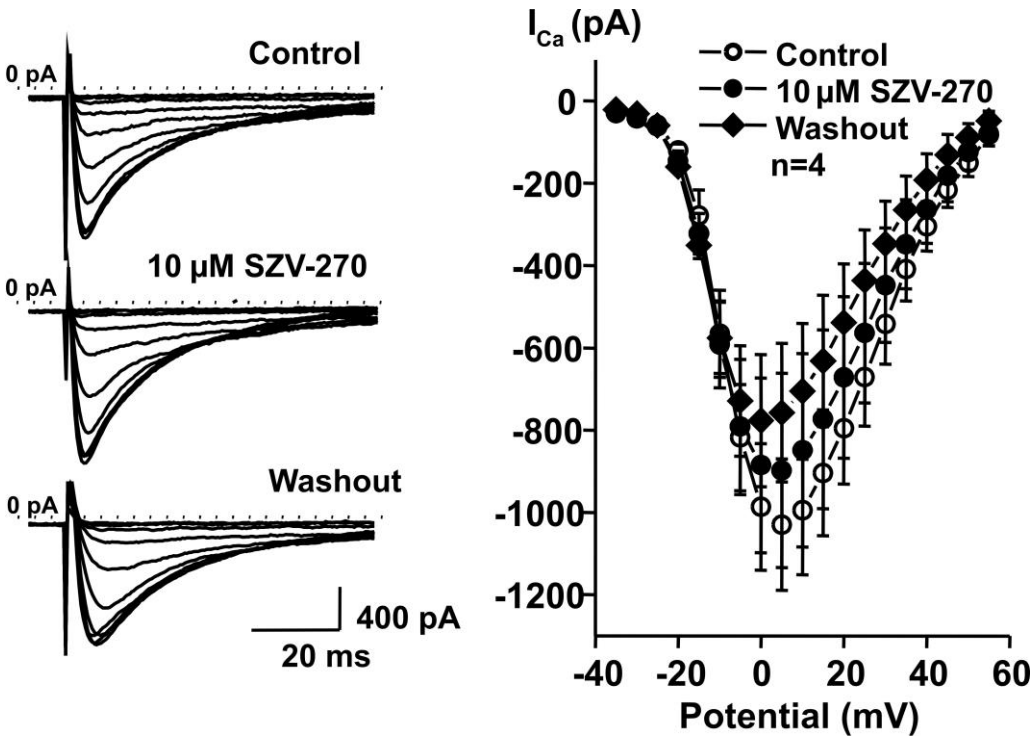

Figure 15. SZV-270 did not influence $I_{Ca,L}$ even at the high concentration of 10 µM in isolated rabbit right ventricular cardiomyocytes. Left panels depict original current traces recorded in control conditions, in the presence of 10 µM SZV-270 and following washout. Right panel shows the current-voltage relationship. Values are means ± SEM. n=4, all $p>0.05$.

4.2 Effects of rofecoxib on action potential in rat isolated papillary muscles

In order to investigate the effects of rofecoxib on cardiac action potential parameters, we performed in vitro simulated ischemia/reperfusion (sI/R) and sIPC experiments on isolated rat left ventricular papillary muscles. Rofecoxib treatment did not alter any of the observed electrophysiological parameters, including APD_{90} (**Figure 16 A, B**) in normoxic conditions. After 30 min. of simulated ischemia, APD_{90} was shortened significantly (**Figure 16**) in all groups compared to the respective normoxic groups. Importantly, in the presence of sI/R rofecoxib increased APD_{90} in a concentration-dependent manner (**Figure 16**) after reperfusion following the 30 min. simulated ischemia. In the sIPC group, these effects of rofecoxib on repolarization were not observed during reperfusion (**Figure 16**). The effects of rofecoxib on the resting membrane potential (RMP), conduction time (CT), action potential amplitude (APA), and maximum rate of depolarization (V_{max}) in sI/R are shown in **Table 6**. 30 min. of simulated ischemia caused an increase of CT in all groups, while V_{max} was decreased significantly in the rofecoxib treated groups (**Table 6**), possibly indicating reduced sodium channel function after rofecoxib administration in only ischemic conditions. The fact that rofecoxib did not influence repolarisation duration on its own in the control group (without

ischaemia/reperfusion), but its repolarisation prolonging effects was definitely manifested at the end of the reperfusion period indicate that rofecoxib could indeed alter repolarisation processes following an ischaemic event. Importantly, we also found that these rofecoxib-induced potentially harmful post-ischaemic repolarisation disturbances could be prevented by ischaemic preconditioning.

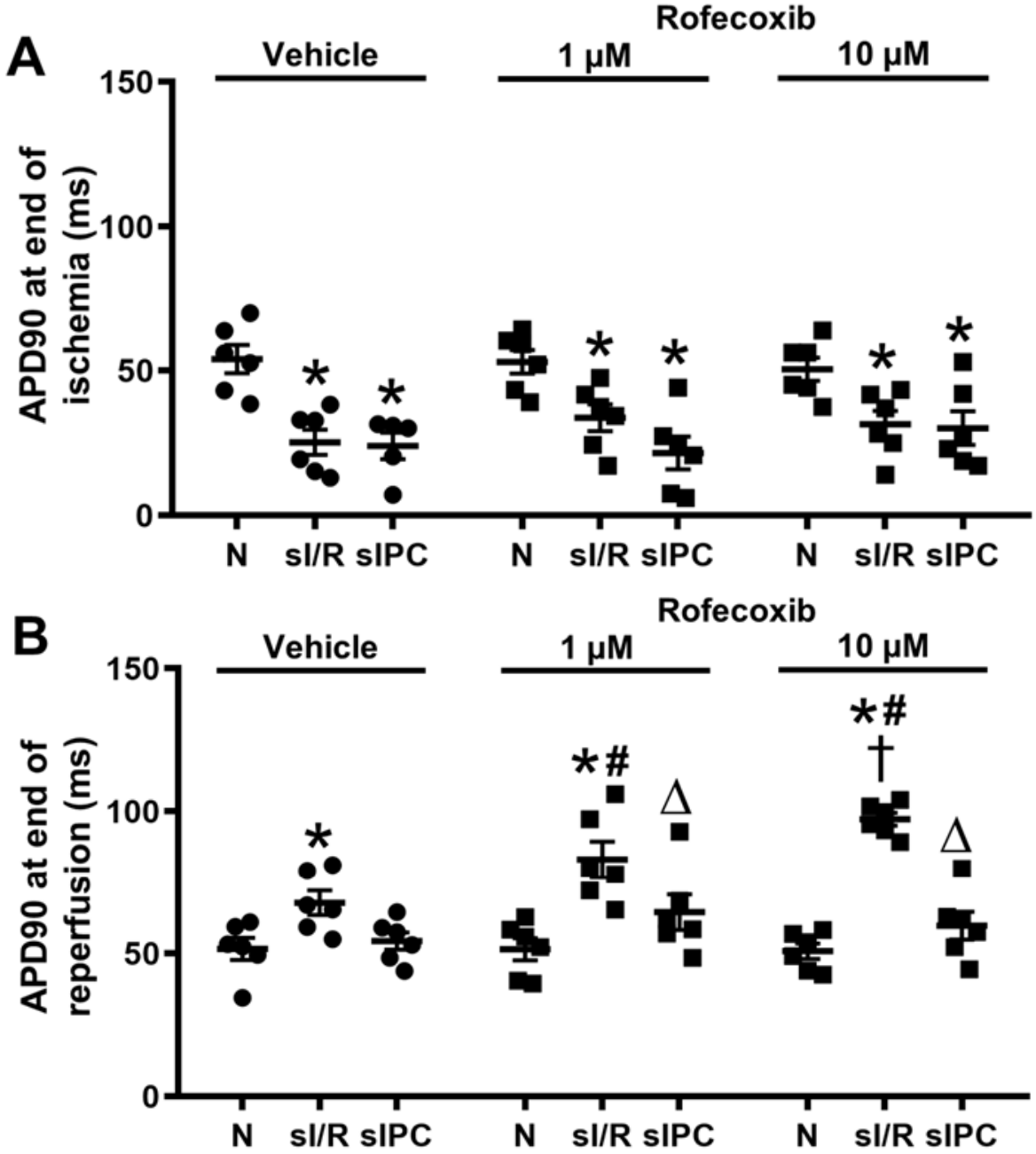

Figure 16. (A) Action potential duration at 90% repolarization (APD_{90}) decreased by the end of 30 min. simulated ischemia in the simulated ischemia/reperfusion groups (sI/R) and simulated ischemic preconditioning groups (sIPC) as compared to the normoxia (N) group. **(B)** Rofecoxib increased the APD_{90} in adult rat isolated papillary muscles at the end of reperfusion and this effect was reversed by sIPC (*p<0.05 vs. corresponding normoxia group, #p < 0.05 vs. sI/R + vehicle,†p < 0.05 vs. sI/R + 1 μM rofecoxib, Δp < 0.05 vs. corresponding sI/R group, n = 5–6).

Action potential parameters	Group	Vehicle	1 µM rofecoxib	10 µM rofecoxib
APA at end of ischaemia (mV)	Normoxia	106.7 ± 2.55	110.3 ± 0.79	112.7 ± 0.98
	sI/R	97.0 ± 2.56	98.5 ± 2.01*	89.6 ± 2.47*
	sIPC	89.4 ± 5.87*	90.6 ± 5.59*	91.7 ± 7.03*
APA at end of reperfusion (mV)	Normoxia	108.6 ± 2.35	110.4 ± 0.96	112.4 ± 1.10
	sI/R	110.1 ± 1.89	109.9 ± 0.19	110.8 ± 2.23
	sIPC	106.2 ± 4.93	113.2 ± 2.98	105.7 ± 4.02
CT at end of ischaemia (ms)	Normoxia	4.8 ± 0.28	4.8 ± 0.33	4.5 ± 0.21
	sI/R	7.3 ± 0.58*	7.6 ± 0.45*	7.3 ± 0.37*
	sIPC	6.7 ± 0.67*	5.8 ± 5.58*$^{\Delta}$	6.2 ± 0.83*
CT at end of reperfusion (ms)	Normoxia	5.2 ± 0.20	4.8 ± 0.32	4.6 ± 0.22
	sI/R	4.9 ± 0.24	4.4 ± 0.31	4.5 ± 0.36
	sIPC	5.4 ± 0.69	4.9 ± 0.31	4.5 ± 0.44
RMP at end of ischaemia (mV)	Normoxia	-87.8 ± 2.96	-90.6 ± 2.03	-86.3 ± 3.00
	sI/R	-82.1 ± 1.78	-78.3 ± 3.45*	-77.0 ± 1.59
	sIPC	-80.2 ± 4.20	-78.5 ± 2.78*	-77.9 ± 7.41
RMP at end of reperfusion (mV)	Normoxia	-85.4 ± 3.00	-89.3 ± 1.51	-88.6 ± 1.74
	sI/R	-89.5 ± 1.79	-91.4 ± 2.34	-92.8 ± 1.98
	sIPC	-89.7 ± 3.35	-86.0 ± 2.08	-88.5 ± 3.79
V_{max} at end of ischaemia (V/s)	Normoxia	170.1 ± 31.76	211.4 ± 19.14	198.1 ± 17.52
	sI/R	142.5 ± 21.43	112.4 ± 10.5*	109.3 ± 12.53*
	sIPC	121.1 ± 17.75	135.4 ± 12.35*	139.9 ± 11.42*
V_{max} at end of reperfusion (V/s)	Normoxia	158.7 ± 26.09	208.2 ± 21.49	207.1 ± 16.01
	sI/R	205.9 ± 20.44	189.2 ± 7.20	174.2 ± 6.69
	sIPC	205.9 ± 20.8	172.6 ± 23.57	195.3 ± 24.28

Table 6. Action potential parameters measured in **rat papillary muscles** in normoxic, simulated ischemia/reperfusion (sI/R) and simulated ischemic preconditioning (sIPC) conditions. APA: action potential amplitude, CT: conduction time, RMP: resting membrane potential, V_{max}: maximum rate of depolarization, *$p<0.05$ vs. corresponding normoxia group, $^{\Delta}p<0.05$ vs. corresponding sI/R group, n = 5–6).

5. DISCUSSION

5.1 SZV-270 has combined Class I/B and Class III effects

A promising approach to safer and more effective pharmacological treatment of arrhythmia is useing novel compounds that exhibit more complex actions and modulate several ionic currents.

In this study, the cardiac cellular electrophysiological and in vivo antiarrhythmic effects of SZV-270, a novel compound with a structure that features Class I/B and Class III structural elements (of D-sotalol and mexiletine, **Figure 3**), were investigated in dogs and rabbits, two species used frequently in arrhythmia research.

To elucidate the mechanisms underlying the ventricular antiarrhythmic effects of SZV-270, action potential measurements were performed in rabbit and dog right ventricular papillary muscle preparations, and several ionic currents were also measured in isolated rabbit right ventricular cardiomyocytes. The compound lengthened the effective refractory period, APD_{50}, APD_{75} and APD_{90} in a concentration dependent manner in ventricular preparations in both species (**Tables 2 and 3**). Similar effects were observed with sotalol (Stroobandt *et al*., 1986; Taggart *et al.*, 1985). Furthermore, SZV-270 significantly inhibited the I_{Kr} tail current at relatively low concentrations of 100 and 500 nM (**Figure 13**). These Class III antiarrhythmic effects were supplemented by Class I/B effects of SZV-270 in the present study. In right ventricular preparations isolated from dogs and rabbits, the larger investigated concentration of SZV-270 significantly reduced V_{max} at stimulation cycle lengths shorter than 1000 ms (**Figures 10B and 11B**). In other studies, lidocaine (Rosen *et al.*, 1975) and mexiletene (Hézső *et al*, 2021; Manolis *et al*, 1990) showed similar electrophysiological effects on dog and rabbit preparations. In addition, the larger concentration of SZV-270 prolonged APD_{90} in a lesser degree and significantly shortened APD_{50} (depressed the plateau phase) in dog Purkinje fibers (**Table 4**). These effects can decrease repolarization heterogeneity in the ventricle, resembling a similar effect of amiodarone (Papp et al., 1996). Even high concentrations of SZV-270 did not affect I_{K1}, I_{to} and $I_{Ca,L}$ in rabbit right ventricular cardiomyocytes (**Figures 14 and 15**).

Based on the results of this study, SZV-270 exhibits combined Class I/B and Class III antiarrhythmic actions. The following considerations suggest that this combination is beneficial. Class III drugs prolong repolarization and the effective refractory period and are especially effective against re-entry arrhythmias (Lynch *et al.*, 1985; Hohnloser *et al.*, 1995; Fei and Frame, 1996). However, Class III compounds possess marked proarrhythmic activity: they promote EAD formation and subsequent development of TdP polymorphic ventricular

tachycardia (Buchanan *et al.*, 1993; Vos *et al.*, 1995; Gottlieb *et al.*, 1997). Drugs with ClassI/B actions, however, can reduce EAD formation (Papp *et al.*, 1996; Sicouri *et al.*, 1997) and have been shown to suppress TdP induced by pure Class III agents (Shimizu and Antzelevitch, 1997; Assimes and Malcolm, 1998). Also, the combination of the Class I/B drug mexiletine and the Class III compound sotalol prevented ventricular tachyarrhythmias in dogs with myocardial infarction (Chezalviel *et al.*, 1993). Luderitz et al. also suggested that the combination of mexiletine and sotalol was able to suppress ventricular arrhythmias more effectively than either compound alone (Luderitz *et al.*, 1991). These results strongly suggest that a compound with combined Class I/B and III effects can prevent re-entry arrhythmias with reduced risk of provoking TdP arrhythmia.

The antiarrhythmic effects of SZV-270 were tested in a conscious dog model of atrial fibrillation that is based on chronic right atrial tachypacing-induced atrial electrical remodeling (Morillo *et al.*, 1995). SZV-270 significantly reduced the incidence of burst-induced AF and prolonged the AERP (**Figure 9**). In canine right atrial trabeculae, SZV-270 prolonged the APD_{50}, APD_{75} and APD_{90} in a concentration dependent manner (**Table 5**). The effects of SZV-270 on AF in this model were comparable to those of the selective I_{Kr} blocker dofetilide, which is known as an effective compound for rhythm control in AF (Kirchhof 2016; Piccini and Fauchier 2016). Dofetilide also reduced AF incidence and increased AERP in the present study, and was shown to prolong atrial APD in atrial trabeculae isolated from dogs with chronic tachypacing induced atrial remodeling (Juhász *et al.*, 2018). The beneficial effects of dofetilide in AF were attributed to its atrial repolarization and AERP prolonging effects (Allessie *et al.*, 2001; Pedersen *et al.*, 2001). The AF incidence reducing effects of SZV-270 is also probably due to its atrial APD prolonging effects in this study.

In order to test the hypothesis, that because of the combined class I/B and Class III properties, SZV-270 has no proarrhythmic effects, we used an anesthetized rabbit proarrhythmia model. On the ECG recordings, SZV-270 widened the QRS interval, and despite QTc prolongation, the combination of HMR 1556 and SZV-270 did not increase the short-term variability of the QT interval (STV_{QT}) and did not induce TdP (Varga *et al.*, 2021). STV_{QT} is a new ECG biomarker, which shows a better correlation with the occurence of ventricular arrhythmias, than the QT prolongation, in animal experiments (Thomsen *et al.*, 2004, 2006; Lengyel *et al.*, 2007) and in clinical studies (Hinterseer *et al.*, 2008, 2009, 2010). These results of the rabbit proarrhythmia experiments are not detailed in this dissertation, because they are the part of an other thesis.

5.2 Hidden electrophysiological cardiotoxic effects of rofecoxib can be revealed in experimental models of Ischemia/Reperfusion

Detection of cardiotoxicity of drugs in early preclinical phase have great importance to increase success rate of drug development and patient safety, therefore novel safety testing methods - involving experimental models of various cardiac diseases, especially myocardial ischaemia/reperfusion - would have great benefits.

Although these additional tests will certainly increase the cost and time of preclinical safety tests, by the early detection of hidden cardiotoxicity of drugs, it will finally lead to:

- overall saving of time and cost of drug development for the pharmaceutical industry by terminating early the development of drugs with potentially cardiotoxic effects;
- increasing success rate of clinical drug development by more rational design of clinical trials to enroll patients that are not prone to manifest certain cardiotoxic side effects of a drug with potential hidden cardiotoxity in a disease condition;
- increased patient safety by preventing the clinical testing and clinical use of potentially cardiotoxic drugs in patient populations that are prone to manifest hidden cardiotoxicity. As an example, in case the potential cardiotoxic effect of rofecoxib (Vioxx) were detected by assays for hidden cardiotoxicity in the early pre-clinical phase of its development, the manufacturer company could have saved significant amount of resources spent for the development of rofecoxib and for the still ongoing legal issues related to its withdrawal from the market in 2004 (Faunce *et al.*, 2010; Mayer, 2008). In a retrospective cohort study of individuals on the expanded Tennessee Medicaid programme, users of high-dose rofecoxib were 1.70 times more likely than non-users to have coronary heart disease, among new users this rate increased to 1.93 (Ray et *al.*, 2002). Early detection of hidden cardiotoxicity of rofecoxib could have prevented the unexpected incidence of myocardial infarction of some patients taking Vioxx. However, more than a decade after its withdrawal, the mechanism of hidden cardiotoxicity of rofecoxib is still a question of debate. In a comprehensive analysis of 114 randomized trials with 116 094 participants, rofecoxib increased risks of renal events (peripheral edema, renal dysfunction, hypertension) and arrhythmia events (the vast majority were ventricular fibrillation, cardiac arrest, and sudden cardiac death) (Zhang *et al.*, 2006). However, to increase the productivity of drug development, we definitely need to increase knowledge on mechanisms and early detection of drug toxicity.

In a recent study (Brenner *et al.*, 2020), rats were treated with rofecoxib for four weeks and then subjected to 30 min. ischemia and 120 min. reperfusion to investigate hidden cardiotoxicity of rofecoxib. Rofecoxib treatment increased the mortality rate as compared to

the pooled data of other groups. In the I/R + rofecoxib group, seven animals died due to irreversible ventricular fibrillation during the ischemic period and one animal died due to a sudden drop in blood pressure during reperfusion. In the I/R + vehicle group, only one animal died due to irreversible ventricular fibrillation during the ischemic period. In the IPC + rofecoxib group, one animal died due sudden drop in blood pressure in the reperfusion period. These data imply that the increased mortality due to rofecoxib treatment can be attributed to its proarrhythmic property that only manifests following I/R (Brenner *et al.*, 2020). Myocardial tissue is more susceptible to ventricular arrhythmias during ischemia, and I/R injury may exacerbate proarrhythmic effects of drugs (Ferdinandy *et al.*, 2019).

To further analyze the hidden cardiotoxic effects of rofecoxib, we subjected left ventricular papillary muscles to sI/R. Rofecoxib did not change the action potential parameters in normoxic conditions; however, following sI/R, several, potentially proarrhythmic effects appeared. First, the APD was only significantly prolonged by rofecoxib in cardiac tissue that were subjected to sI/R. Increased spatial dispersion of repolarization between normoxic and ischemic myocardium is a critically important factor that promotes the development of ischemia-induced arrhythmias (Antzelevitch and Fish, 2001; Janse and Wit, 1989). Based on the present results, rofecoxib might further exacerbate the differences in APD between normoxic and ischemic myocardium, further increasing the arrhythmia substrate in I/R. Secondly, simulated ischemia in the presence of rofecoxib more markedly reduced action potential upstroke (characterized by decreased V_{max}) by the end of test ischemia, which only suggested an additional reduction of sodium channel function by rofecoxib in sI/R conditions, further decreasing the already slowed impulse conduction in depolarized ischemic myocardial tissue (Kleber *et al.*, 1986). These data suggest that adverse effects of COX-2 inhibitors may occur only in the presence of cardiac I/R.

Here, we demonstrated, for the first time, that rofecoxib increased acute mortality due to its proarrhythmic effect via increased APD during I/R. We also showed that rofecoxib did not interfere with the cardiprotective effect of IPC and that IPC was able to protect against rofecoxib-induced hidden cardiotoxicity.

6. CONCLUSIONS AND NOVEL FINDINGS

1. SZV-270 blocked I_{Kr} significantly in relatively low, 100 and 500 nM concentrations. This result is consistent with the APD lengthening and QTc prolonging effect of the drug. SZV-270 had no effects on the other transmembrane currents, I_{K1}, I_{to} and $I_{Ca,L}$, even at the high (10 μM) concentration.

   SZV-270 lengthened the effective refractory period, APD_{50}, APD_{75} and APD_{90} in a concentration dependent manner in dog and rabbit ventricular and atrial preparations, and Purkinje fibers. The larger investigated concentration of SZV-270 significantly reduced V_{max} at stimulation cycle lengths shorter than 1000 ms. In addition, the larger concentration of SZV-270 prolonged APD_{90} in a lesser degree and significantly shortened APD_{50} (depressed the plateau phase) in dog Purkinje fibers.

   SZV-270 significantly reduced the incidence of atrial fibrillation and prolonged atrial effective refractory period in a conscious dog model of atrial fibrillation.

2. Rofecoxib did not change the action potential parameters in normoxic conditions; however, following sI/R, several, potentially proarrhythmic effects appeared. First, the APD was only significantly prolonged by rofecoxib in cardiac tissue that were subjected to sI/R. Secondly, simulated ischemia in the presence of rofecoxib more markedly reduced action potential upstroke (characterized by decreased V_{max}) by the end of test ischemia. These results show that cardiac safety testing with simple preclinical models of I/R injury uncovers the hidden cardiotoxicity of rofecoxib and might reveal hidden cardiotoxicity of other drugs.

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