Drug-induced and transgenic LQTS rabbit models with reduced repolarization reserve to study proarrhythmic drug effects

PhD Thesis

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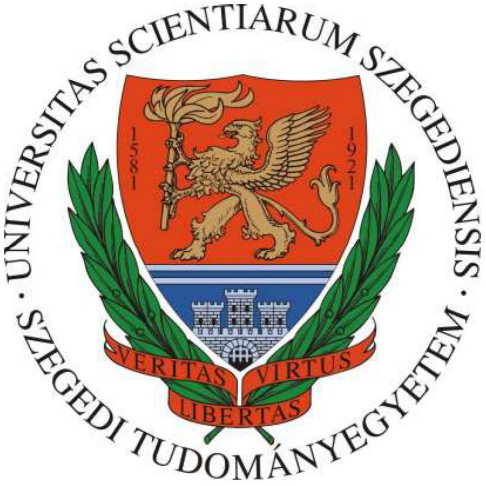

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List of Abbreviations

APD: action potential duration

AR: arrhythmia

AV: atrio-ventricular

CL: cycle length

ECG: electrocardiogram

HERG: human ether-a-go-go related gene encoded α-subunit of the I_{Kr}-conducting potassium channel

$I_{Ca,L}$: L-type calcium current

I_{Kr}: rapid delayed rectifier potassium current

I_{Ks}: slow delayed rectifier potassium current

I_{K1}: inward rectifier potassium current

K^+: potassium ion

KCNE1: β-subunit of the I_{Ks}-conducting potassium channel

KH: Krebs-Henseleit solution

LQTS: long QT syndrome

LQT2: long QT syndrome type 2

LQT5: long QT syndrome type 5

LQT2-5: combined long QT syndrome type 2 and type 5

LV: left ventricle

MAP: monophasic action potential

QT: QT interval on the ECG

RR: interval between two consecutive R-waves on the ECG

SCD: sudden cardiac death

STV_{QT}: short-term variability of QT interval

TdP: Torsades de Pointes polymorphic ventricular tachycardia

VEB: ventricular extra beat(s)

VER: ventricular escape rhythm

VT: ventricular tachycardia

VF: ventricular fibrillation

WT: wild type

Summary of the Thesis

Proarrhythmia - the triggering of arrhythmias following drug therapy - is a rare, but potentially lethal side-effect of various drugs, and therefore, a major safety concern during drug development. Most often proarrhythmia is caused by the drugs' potential to interact with various K^+-channels in the heart, leading to a prolongation of cardiac repolarization that is usually observed on the ECG as prolonged QT interval (drug-induced acquired long QT syndrome; aLQTS). Although drug-induced long-QT-related proarrhythmia is most frequently found in patients with impaired cardiac repolarization due to disease-induced structural and/or electrophysiological remodelling of the heart; most cellular, tissue and whole animal model systems used for drug safety screening are based on normal, healthy models. This approach has serious limitations; therefore, novel animal models that mimic the pathophysiological conditions under which drugs display the highest proarrhythmic risk - such as models with impaired cardiac repolarization - would be desirable for proarrhythmia safety testing.

The aims of the present study:

Drug-induced (HMR-1556 to block I_{Ks}) acquired LQTS, and various transgenic (congenital) LQTS rabbit models with impaired cardiac repolarization due to cardio-selective overexpression of loss-of-function mutations of human KCNH2 (HERG-G628S, α-subunit of I_{Kr}, loss of I_{Kr}, LQT2), KCNE1 (KCNE1-G52R, β-subunit of I_{Ks}, decreased I_{Ks}, LQT5)[1] or both KCNQ1 and KCNE1 transgenes (LQT2-5) were used to investigate:

- the proarrhythmic potential of SZV-270, a novel antiarrhythmic drug candidate with combined Class I/B and Class III effects (acquired LQTS model).
- the electrophysiological characteristics of a newly generated, double-transgenic LQT2-5 rabbit model
- the utility of transgenic LQT2, LQT5 and LQT2-5 rabbit models for more reliable prediction of drug-induced ventricular arrhythmias

Main findings:

The acquired LQTS rabbit proarrhythmia model with pharmacologically reduced repolarization reserve (by the I_{Ks} inhibitor HMR-1556) was able to predict the known torsadogenic potential of the I_{Kr} blocker dofetilide, while indicated no SZV-270-induced proarrhythmia risk. This advantageous electrophysiological effect of the SZV-270 -

prolongation of ventricular repolarization without increased arrhythmia risk - is assumed to be attributed to its combined I_{Kr} (Class III) and I_{Na} (Class I/B) blocking characteristics.

Transgenic LQTS rabbit models reflected patients with clinically ‘silent’ - normal QT interval (LQT5) - or 'manifest' - prolonged QT interval (LQT2 and LQT2-5) - impairment in cardiac repolarization reserve capacity due to different pathomechanisms. The LQTS animals were more sensitive in detecting I_{Kr} - (LQT5) or I_{K1}/I_{Ks} - (LQT2 and LQT2-5) blocking properties of drugs compared to healthy wild type (WT) animals. Impaired QT-shortening capacity at fast heart rates was observed due to disturbed I_{Ks} function in LQT5 and LQT2-5. Importantly, the transgenic LQTS models did not only show more pronounced changes in different proarrhythmia markers in response to potassium channel blockers but also exhibited higher incidence, longer duration and more malignant type of *ex vivo* arrhythmias than WT.

Conclusions:

Drug-induced and transgenic LQTS rabbit models reflect human pathophysiological settings - patients with reduced repolarization reserve - that favour drug-induced arrhythmia formation. As they demonstrate increased sensitivity to different specific ion-channel blockers (I_{Kr}-blockade in LQT5 or in HMR-1556 induced acquired LQTS model, I_{K1} - and I_{Ks} - blockade in LQT2 and LQT2-5), their combined use could provide more reliable, and more thorough prediction of (multi-channel-based) pro-arrhythmic potential of novel drug candidates especially in the setting of impaired cardiac repolarization reserve.

1. Introduction

The reliable prediction of proarrhythmic potential of novel drug candidates relies heavily on suitable preclinical testing platforms/model systems that are currently far from being ideal/sufficient.

In this thesis, an introduction into the field of proarrhythmia research will be given with special attention to the (patho)physiological aspects of drug-induced arrhythmia development that are important to better understand the limitations of the currently applied safety testing approaches. Then, the importance of a relatively recent concept in the field - the use of animal models with impaired repolarization - will be highlighted/demonstrated by presenting our findings on the assessment of the proarrhythmic potential of a novel antiarrhythmic drug candidate, SZV-270, using the drug-induced LQTS rabbit proarrhythmia model with pharmacologically (HMR-1556 to block I_{Ks}) reduced repolarization reserve. This will be followed by introducing the transgenic LQTS rabbits with genetically engineered reduction of repolarization reserve. The detailed description of the electrophysiological characteristics of these LQTS rabbit models – including a newly generated LQT2-5 line - will be presented. The pharmacological proof-of-principle studies, demonstrating the advantages of these LQTS models over the currently used healthy animals in better detection of drug-induced proarrhythmia, will be explained.

1.1 Proarrhythmia and drug development

Proarrhythmia - the development of new arrhythmias following drug treatment - was first described in patients receiving the antiarrhythmic drug, quinidine [2]. In the late 80's and early 90's, the CAST (Cardiac Arrhythmia Suppression Trial) and SWORD (Survival with Oral D-Sotalol) studies [3, 4] demonstrated that Vaughan-Williams Class IC (Na^+-current blocker) and Class III (rapid delayed-rectifier K^+-current blocker) antiarrhythmic drugs can both provoke ventricular arrhythmias that can lead to sudden cardiac death (SCD) and thereby, can increase mortality in sensitive subsets of patients.

Proarrhythmia, this rare but lethal side-effect that was initially considered as being caused by "anti"-arrhythmic cardiac drugs [2, 4, 5], however, also occurs in relation to the use of a variety of other, non-cardiac drugs [6-8]. It has been estimated that around 20-60% of novel chemical entities have the potential to modulate the function of cardiac ion

channels and therefore, to disturb normal cardiac electrical function [9]. Therefore, proarrhythmia has become a major concern for patients, physicians and the pharmaceutical industry. Although no less than 2-3% of all marketed drugs have the potential to alter cardiac electrical functions by - in most cases - prolonging cardiac repolarization [10], the (documented) incidence of potentially lethal drug-induced ventricular tachycardia (VT) (typically Torsades de Pointes VT, TdP) is very low (1:10.000 for non-cardiovascular drugs) [11], and therefore, very hard to predict reliably [12]. In recent decades, TdP-induced SCD cases were associated with a wide range of commonly used drugs (anti-psychotics, anti-depressants, antihistamines and antibiotics) [12-14] and many of them (such as cisapride, astemizole, terfenadine, grepafloxacin) have been withdrawn from the market [15-17] causing significant financial loss for the pharmaceutical industry.

This drove the scientific community to try to better understand the underlying cellular electrophysiological mechanisms of proarrhythmia formation and to develop novel, more reliable safety screening tools.

1.2 Cardiac ionic currents involved in proarrhythmia development

The electrophysiological processes in the heart are determined by ordered initiation of excitatory stimuli in specialised rhythm generator region(s) (normally in the sinoatrial (SA) node) and their fast propagation through the His-Purkinje conduction system that ultimately result in rapid depolarisation and subsequent slow repolarization of the atrial and ventricular 'working' cardiomyocytes. This regulated change of transmembrane potential in time - action potential (AP) - reflects the sequential activation and inactivation/deactivation of various transmembrane voltage- and ligand-gated ion channels and transporters. The ionic movements through the transmembrane ion channels are driven by the actual transmembrane electrochemical gradient.

The action potential of the 'working' cardiomyocyte has been divided into five phases. In phase 0, when the excitation threshold is exceeded, the voltage-sensitive sodium-channels are quickly opened and the cells are depolarised by a rapid inflow of Na^+-ions generating a large and fast transient inward Na^+-current (I_{Na}). This current is also responsible for the velocity of impulse propagation through the His-Purkinje system, working atrial and ventricular myocytes ('fast-response' tissues) and as such, contributes to the safely

synchronised activation of the entire heart. In the sinoatrial and atrioventricular (AV) nodes, the depolarisation is governed mainly by the voltage-dependent activation of the L-type Ca^{++}-channels that has, compared to the fast I_{Na}, slower activation and inactivation kinetics ('slow-response' tissue). Activation of the L-type Ca^{++}-channel - both in 'fast- and slow-response' tissues -, and the consequent Ca^{++} ion inflow ($I_{Ca,L}$) is crucial in triggering the Ca^{++}-dependent Ca^{++}-release from the intracellular Ca^{++} storages, that ultimately initiates the contraction of the cardiac cells (excitation-contraction coupling). In human cardiac cells, the fast depolarisation is followed by an initial transient repolarization (phase 1, governed by the transient outward potassium current, I_{to}) and then by a prominent plateau (phase 2) phase. During the plateau phase, the repolarising effects of the different outward potassium currents are largely counterbalanced by the depolarising inward (i) L-type Ca^{++}, (ii) late (or window) -sodium ($I_{Na,late}$) and (iii) Na^{+}/Ca^{++}-exchanger (I_{NCX}) currents. Phase 3 is the terminal part of repolarization, in which the outward potassium currents gradually overcome the decreasing inward currents enabling the cells to relatively quickly regain their initial resting membrane potential. The main outward voltage-gated potassium currents that play crucial role in the repolarization processes are the rapid and slow components of the delayed rectifier K^{+}-current (I_{Kr} and I_{Ks}, respectively) and the inward rectifier K^{+}-current (I_{K1}). Phase 4 represents the membrane potential during diastole in working cardiomyocytes. In the 'slow-response tissues' (SA and AV-nodes), phase 4 shows a characteristic spontaneous 'diastolic depolarisation' governed by the inward 'funny' (I_f) current that is activated by hyperpolarisation of the membrane potential. The density of this current determines the slope of the diastolic depolarisation and thereby, the heart rate as well.

These ion channels are composed of different transmembrane proteins: pore forming alpha (α) and channel function modulating beta (β) and/or gamma (γ) subunits. I_{Kr} or I_{Ks}, for example, are conducted by channels that are composed of the pore forming HERG or KvLQT1 α-, and the modulatory MIRP (and additionally MinK) or MinK (and additionally MIRP) β-subunits, respectively. It is well known, that significant spatial (transmural, apico-basal and inter-ventricular) differences in the expressions of these channel proteins and in the densities of ionic currents they carry exist, explaining the slightly distinct electrophysiological characteristics of different regions. It is important to highlight that this already pre-existing ionic current heterogeneity can be further aggravated by external (and internal) factors, such as by drugs, strongly contributing to their proarrhythmic potential.

Any drug-induced alteration in the function of these ion channels can impair the fine-tuned balance of depolarising and repolarising processes – leading to either shortened or prolonged AP - that consequently favour arrhythmia formation.

1.3 The concept of 'repolarization reserve' and its implications in proarrhythmia research

The safety of cardiac repolarization – as a vital biological function – is relatively well preserved, since many different potassium channels are involved in the process (in a redundant way). Therefore, the reduction or loss of one repolarizing potassium current may not lead to excessive AP lengthening, since cardiomyocytes may be able to maintain sufficient repolarization via compensatory increase of non-affected 'reserve' outward K^+-currents [18, 19]. This latter term is called cardiac 'repolarization reserve' and was first introduced by Roden [18] and was, later on, proven experimentally by Varro et al. [20, 21]. The key players of the repolarization reserve are I_{Kr}, I_{Ks} (influenced by the sympathetic activity), I_{K1} and presumably I_{to} [20-23].

It is important to note, that in regard to the robustness of cardiac repolarization reserve capacity, huge inter-individual differences exist, which represents one of the major challenges in reliable proarrhythmia screening. Individuals with subtle decrease in their repolarization reserve capacity due to slight reduction in one of their repolarizing potassium currents, for example, do not necessarily show signs of impaired repolarization – such as prolonged QT interval on the ECG –, since other K^+-currents may be able to compensate. Patients with these 'hidden' repolarization disturbances can then be especially sensitive for further - even minor - repolarization prolonging drug effects and can demonstrate very surprising and unpredictable life-threatening arrhythmia development. Gender, sex hormones, K^+ homeostasis, and - most importantly - certain diseases are the main factors that highly influence the individual proarrhythmia susceptibility by impairing cardiac repolarization reserve capacity.

Apart from a decrease in serum K^+ concentration that results in a massive prolongation of cardiac repolarization, cardiovascular and metabolic diseases such as congestive heart failure [24], cardiac hypertrophy, hypertrophic and dilated cardiomyopathy [25], myocardial ischemia [26], congenital LQTS [27], or diabetes mellitus [28, 29] play the most important

roles in decreasing repolarization reserve capacity. These conditions usually lead to downregulation of various repolarising outward - mainly I_{Kr}, I_{Ks} – or upregulation of certain depolarising inward - $I_{Na,late}$, I_{NCX} - currents that result in an increased arrhythmia susceptibility.

Sex hormones can also significantly alter the individual's repolarization reserve capacity. Women are at higher risk for drug-induced prolongation of repolarization and TdP [30-33] due to sex hormone effects on cardiac ionic currents/channels [34, 35]: Estrogen reduces repolarization reserve by decreasing I_{Ks} [36] and I_{Kr} [37, 38] and by increasing $I_{Ca,L}$ [39, 40] and NCX expression [41]; therefore, it is considered as proarrhythmic. In contrast, testosterone and progesterone both increase repolarization reserve by increasing I_{Ks} (testosterone [42] / progesterone [43]), I_{K1} and I_{Kr} (testosterone) [42], decreasing $I_{Ca,L}$ (testosterone [43] / progesterone [39, 40]), and upregulating SERCA expression (progesterone [39, 40, 44]), thereby exerting a protective, 'anti-arrhythmic' effect against drug-induced proarrhythmia [39, 40]). These observations have consequences for proarrhythmia research, as female animal models (or animal models with altered hormonal states) might be particularly sensitive in detecting potential ion channel blocking properties of drug candidates.

1.4 Mechanisms and clinical appearance of proarrhythmia

Most often, proarrhythmia is mechanistically based on prolongation of the cardiac repolarization (acquired LQTS) as a result of the drug's potential to inhibit cardiac potassium (mostly I_{Kr} / HERG) currents [14]. This drug-induced repolarization prolongation is not strictly dose-dependent and shows huge inter-individual differences [15] too, therefore, it is very hard to predict reliably. As this type of proarrhythmia (repolarization prolongation related) constitutes - by far - the highest significance for patients, physicians and for the pharmaceutical industry due to its dreadful and unpredictable nature, the present thesis work is focused on studying drug-induced LQTS-related proarrhythmia.

It is widely accepted, that both arrhythmia initiating (triggering) and maintaining mechanisms are involved in the pathogenesis of drug-induced LQTS-related arrhythmia formation. Prolongation of the cardiac repolarization lengthens the duration of phase 2 and 3 repolarization, thereby making the cells more vulnerable to re-activation of depolarising

inward currents such as I_{Na}, I_{Ca} or inward I_{NCX}. This favours the development of early after depolarizations (EADs) [45] - primarily in the Purkinje fibres [46, 47] - that is believed to serve as a typical arrhythmia initiating (triggering) mechanism [48] in drug-induced proarrhythmia. Ca^{++}-handling abnormalities [49-53] and increased sympathetic activity [54, 55] – through activation of $I_{Ca,L}$ - can also play role in EAD development. Drug-induced repolarization prolongation typically shows markedly increased spatial and temporal dispersion leading to an increased heterogeneity of the cardiac refractoriness which serves as an arrhythmia maintaining 'substrate' mechanism. The '4 dimensions' of this dispersion are: (i) transmural [56, 57], (ii) apico-basal [58, 59] and (iii) interventricular dispersion of repolarization [60-62] and (iv) temporal dispersion [63, 64].

On the ECG, drug-induced prolongation of the QT interval and EAD-s 'triggered' ventricular extrasystoles (VES) can first be observed with a typical short-long-short cycle-lengths that very often precedes the occurrence of more serious forms of proarrhythmia. Depending on the severity of the coexisting arrhythmia maintaining 'substrates', VES can initiate more complex forms of arrhythmias such as couplets, triplets, salvos or in worst case scenario, a characteristic drug-induced polymorphic ventricular tachycardia, Torsade de Pointes (TdP). TdP then may degenerate into ventricular fibrillation (VF) and can lead to sudden cardiac death (SCD) [12-14].

1.5 Current proarrhythmia safety screening approaches and their limitations

To prevent unacceptable human fatalities (SCD) related to drug therapy of non-life-threatening diseases, the International Council for Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guidelines (ICH-S7B, 2005 [65]; ICH-E14, 2005 [66]) were proposed for rigorous safety testing. In compliance with these guidelines, all new drug candidates have to undergo a sophisticated step-wise, combined pre-clinical and clinical safety testing protocol [67]. These include the assessment of the drug effects on: (i) HERG (I_{Kr}) function in heterologous expression systems such as Xenopus oocytes, HEK293 or CHO-cells stably transfected with HERG (ii) action potentials in isolated cardiomyocytes or tissue preparations derived from midmyocardium or Purkinje fibers from rabbits, guinea pigs or dogs *in vitro*, (iii) monophasic action potentials (MAP) in Langendorff-perfused wild type rabbit hearts *ex vivo*, (iv) ECG (QT) assays in anaesthetised dogs *in vivo*

and (v) computational [68-70] risk prediction platforms *in silico* (integrated risk assessment [IRA] approach), followed by clinical ECG studies. In principle, the cheaper, high throughput *in silico* and *in vitro* assays are used at the initial drug developmental phase for safety screening ('frontloading'), while the more sophisticated but lower throughput assays using more complex *in vivo* animal models are utilised at later stages for testing selected promising compounds.

1.5.1 Proarrhythmia markers

Since drug-induced proarrhythmia is a relatively rare event, the direct measurement of arrhythmia development as a 'hard end-point' in relation to drug administration is very hard and only possible by utilising sensitized model systems. Therefore, usually different proarrhythmia markers and score systems are employed to try to increase the predictive value of the overall assessment [14, 63, 71, 72] with moderate to limited success [73].

As clinical observations suggested that drug-induced proarrhythmia mostly occurred in the setting of prolonged ventricular repolarization / QT prolongation [13, 14], pre-clinical and clinical safety tests have for a long time focussed on APD and QT interval prolongation as a surrogate marker for proarrhythmia risk [66]. However, it has been known for some time that numerous drugs that block I_{Kr}/HERG and prolong APD/QT rarely cause TdP - while others causing less pronounced APD/QT prolongation carry a significant pro-arrhythmic risk [64, 74-77]. Indeed, the extent of QT prolongation did not predict serious ventricular arrhythmias and/or SCD in different rabbit and dog experimental models [64, 75, 78-80] or in patients with [81-85] or without [86-88] congenital/structural heart disease. Therefore, a number of different electrophysiological parameters and score systems have been suggested to use as prognostic markers for TdP and SCD risk evaluation both in animal experiments and in the clinical setting. These markers and scores systems do not only assess the prolongation of ventricular repolarization but also take into consideration the drug's effect on the spatial and temporal dispersion of repolarization.

1.5.1.1 Spatial dispersion of repolarization

Spatial dispersion of cardiac repolarization in transmural (e.g. between endo-, mid- and epicardium) and apico-basal as well as in interventricular (e.g. between LV and RV) directions [89-92] have been identified for a long time, and increase in these repolarization gradients

are considered to serve as an arrhythmia maintaining 'substrate' mechanism and therefore, to play a role in proarrhythmia development [59, 93, 94].

Transmural dispersion of repolarization (TDR), originally described *ex vivo* as the APD difference between endo-, mid- and epicardium, could also be measured *in vivo* as $T_{peak\text{-}end}$ on the ECG: it is the length from the peak of the T wave (T_p) to the end (T_e) in precordial leads. $T_{peak\text{-}end}$ has been suggested to be used as an indicator of arrhythmic risk [59, 93-95].

Similar to TDR, the role of increased apico-basal and interventricular dispersions in re-entry type arrhythmogenesis were also confirmed by numerous studies using animal models [59, 94, 96, 97]. Usually, the standard deviation (SD) of the QT times or alternatively, the difference between the longest and shortest QT intervals measured on conventional 12 lead surface ECG (SD of QT or $QT_{max\text{-}min}$, respectively) or their 'cellular' equivalents (SD of APD or $APD_{max\text{-}min}$) are used as markers reflecting the apico-basal and interventricular dispersions of repolarization. Increase in these parameters are considered arrhythmogenic [59, 94, 96, 97].

1.5.1.2 Temporal dispersion of repolarization

Restitution, a form of temporal repolarization dispersion [98, 99], characterizes the adaptation of APD or QT to different stimulation cycle lengths or heart rates with a steeper APD or QT restitution indicating a higher pro-arrhythmic risk [100].

Another form of the temporal dispersion – or instability – of repolarization is the temporal APD-instability [63] or the short-term variability of the QT interval (STV_{QT}) [64]. Higher arrhythmogenic potential in case of increased APD-instability was described by numerous animal experimental [63] and clinical studies [101-103]. In regards to the STV_{QT}, a growing body of evidence has repeatedly demonstrated both in animal proarrhythmia models [64, 79, 80] and clinical settings [81, 82, 104, 105] that increased STV_{QT} is superior to QT prolongation in predicting proarrhythmic risk. These data strongly support the use of APD-instability and STV_{QT} as reliable proarrhythmia markers.

1.5.1.3 Other proarrhythmia markers

APD triangulation - calculated as APD_{90}-APD_{30} - refers to the steepness of the action potential during phase 3 repolarization where I_{Kr} and I_{Ks} are the main active ion currents and describes proarrhythmic drug-induced changes in AP shape. If drugs mainly slow repolarization during phase 3 (primarily due to their I_{Kr} or I_{Ks} blocking properties), the action

potential becomes more triangular which is considered to be an important indicator of proarrhythmia [63].

Reverse use-dependence illustrates the disproportionately more pronounced drug-induced prolongation of QT (or APD) at lower heart rates compared to higher frequencies leading to steeper QR – RR (or APD – stimulatory cycle length) relationship. In case of pronounced reverse use-dependency, the heart is prone to repolarization over prolongation especially at lower heart rates with a consequently increased risk for EAD and re-entry formation.

1.5.2 Current models in proarrhythmia screening

Drug effects on various cardiac ion-currents can be studied by the patch clamp technique [106] using freshly isolated cardiomyocytes or heterologous expression cell systems, such as the human embryonic kidney cells (HEK), Chinese hamster ovary cells (CHO) and Xenopus laevis oocytes. These expression cell systems are transfected with human DNA encoding the ion channels which are to be studied [107, 108]. As drug-induced HERG (I_{Kr}) blockade is assumed to be the single most important factor responsible for most proarrhythmia, the patch clamp technique - either its manual or automated form - is generally used to identify the HERG blocking property of drugs. Nowadays, novel HERG assays have also been introduced, such as an antibody-based chemiluminescent assay called HERG-Lite [109] and the rubidium [110, 111] or thallium [112] efflux assays. The use of human induced pluripotent stem cell derived cardiomyocytes (iPSC-CM) to detect even patient/disease-specific drug effects at the ion-channels level has recently become an increasingly promising approach [113-115]. Although these techniques provide inevitably important information about the drug effects on the HERG or other ion-channels, important pro-arrhythmogenic factors such as spatial dispersion, triangulation, reverse use-dependence and instability are completely left out of the picture.

Pro-arrhythmic factors such as the change in AP shape (triangulation) and in repolarization length (APD) at different stimulatory frequencies (reverse use-dependence), on the other hand, can be studied by the intracellular AP measurement assay using Purkinje fibre and papillary muscle tissues preparations isolated from guinea pigs, rabbits or dogs [116]. Additionally, the spatial and temporal dispersion of repolarization can also be investigated by the canine [117] or rabbit [108, 118] arterially-perfused left ventricular

wedge preparation, that enables the measurement of an ECG as well as epicardial, midmyocardial and endocardial APs, giving more insight into the possible mechanisms of proarrhythmia [72]. These model systems have moderate throughput, therefore, are mainly used to clarify proarrhythmic potential of drugs with complex, multichannel-blocking properties [116].

Another very commonly used model system for proarrhythmia research is the Langendorff-perfused (rat, guinea pig or rabbit) heart or its automated version, the SCREENIT system [63] that has been used to test the arrhythmic potential of hundreds of drugs. In this method, the heart is retrogradely perfused over the aorta and epicardial and endocardial electrodes are used to derive monophasic action potentials (MAPs). This well-established system enables the direct measurement of arrhythmia formation (EADs, VES, R-on-T, TdP and VF) as well as temporal instability, TDR, reverse-use dependence and triangulation.

Animals, either anesthetised or free-moving (telemetric), can be used *in vivo* to measure the drug-induced changes in different ECG parameters (QT, QT dispersion, $T_{peak\text{-}end}$, STV_{QT}, etc.). Since proarrhythmia formation is a relatively rare event in normal, healthy subjects, these models must be sensitized - by impairing their cardiac repolarization reserve, for example - in order to be able to directly detect drug induced arrhythmia events. Animal models with cardiac diseases that indirectly reduce repolarization reserve, could, for example, represent such a sensitized model system. The volume overload chronic atrioventricular block (CAVB) dog is one of the best characterised such disease model [119, 120], in which AV-block leads to electrical (decreased I_{Ks} and partly reduced I_{Kr}) and structural remodelling of the heart [121]. This makes the CAVB model very susceptible to HERG/I_{Kr}-blocker induced TdP formation [108, 121-123]. Another well-established proarrhythmia model is the methoxamine (α1-adrenergic receptor agonist) sensitized anesthetised rabbit that is particularly prone to develop drug-induced ventricular tachycardia when exposed to HERG/I_{Kr}-blocking drugs [124, 125]. Finally, repolarization reserve could also be reduced by pharmacological inhibition of outward potassium current(s). Most commonly, E4031 [126-128] and dofetilide [129-131] or HMR-1556 [80] are used to inhibit the HERG/I_{Kr} or I_{Ks} -currents in *ex vivo* Langendorff-perfused rabbit hearts or *in vivo* in rabbits or dogs and thereby, to induce acquired LQT2 or LQT1-like conditions. All of these sensitized animal models, however, have significant limitations due to the: (i) high

cost, special expertise and substantial time that are required to generate these models (CAVB dog), (ii) possible interference of the applied anaesthetic agent with repolarising ion-channels and also potential cross-reaction between the test drug and α1-adrenergic receptors in case of the methoxamine-sensitised rabbit model, (iii) need of continuous drug administration to maintain the desired channel-blockade that makes long-term experiments very costly or impossible to carry out in models with drug-induced LQTS. Therefore, the generation of stable animal lines with impaired repolarization reserve in a species that closely reflect human cardiac electro(patho)physiological features, are highly desirable.

1.5.3 Limitations of the currently applied proarrhythmia safety screening approaches

Although no (documented) drug induced SCD cases have been reported in connection to the use of newly marketed drugs since the introduction of the ICH guidelines [65, 66], still, they are far from being either ideal or completely up-to-date approaches. In one hand, application of its rigorous regulations over the last two decades has most likely led to unnecessary termination of development of many promising drug candidates that could have - at least labelled with special restrictions - reached the market. On the other hand, these dated regulations do not fully take into account many important, newly understood pathophysiological features related to proarrhythmia formation, such as: (i) its multi-channel-based nature and (ii) the significance and need of utilizing new models with impaired repolarization reserve.

In spite of our present knowledge, that proarrhythmia is multi-channel-based, routine proarrhythmia safety tests are still largely focused on detection of HERG-blocking potential of test compounds as it is considered as the most important factor responsible for LQTS-related arrhythmogenesis ('HERG-centric' paradigm). As a result, this approach leads to elimination of potentially promising drug candidates from the developmental pipeline solely on the basis of their potential to block the HERG channel [132-134].

Based on the above, pharmaceutical industry has started the Comprehensive *in vitro* Proarrhythmia Assay (CiPA) initiative that involves - among others - the systematic measurement of the effect of drug candidates on multiple human cardiac ion currents (I_{Kr}, I_{Ks}, $I_{Na,peak}$, $I_{Na,late}$, I_{K1}, I_{to}, $I_{Ca,L}$) in heterologous expression systems [113, 135, 136]. The CiPA initiative is undoubtedly a promising approach; however, its overall implementation is not sufficient yet.

The other major area of limitation in current proarrhythmia safety screening is, that despite the fact that drug-induced TdP occurs mostly in patients with reduced cardiac repolarization reserve, current safety assessments - in principle - still rely mainly on tests performed on healthy animals with intact repolarization or on their tissues / cells [65, 66]. The lack of reliable, cheap, easy-to-use and stable animal models with impaired cardiac repolarization could – at least partly – explain why this important aspect of drug-induced arrhythmogenesis is barely reflected in currently applied screening approaches.

Based on the above, novel animal models, (i) with increased sensitivity to channel blockers other than only HERG, such as I_{Ks} or I_{K1}, and (ii) representing impairment in their cardiac repolarization reserve are needed to be employed in proarrhythmia research in order to overcome the limitations of current safety screening tests.

1.6 Rabbit as a suitable model system for proarrhythmia research

Cardiac electrical function - especially regarding cardiac repolarization processes - shows large inter-species variability. The rabbit has a prominent role in arrhythmia research, since its cardiac electrophysiological characteristics are much closer to humans than that of other frequently used small animals like mice or rats [137-140] in many aspects such as: (i) similar potassium currents (mainly I_{Kr} and I_{Ks}) convey the cardiac repolarization in rabbits and humans and therefore, (ii) the shape of the action potential [140] is similar to those in humans. Furthermore, the myocardial mechanical function [141], the relative effective heart size relating cardiac mass to the frequency of VF [142] and their responses to pharmacological interventions [143] show very close resemblance to human cardiac physiology. Additional advantages of the rabbit models are that they are relatively cheap, easy to handle and to breed and can be modified genetically. Based on above, rabbit models, especially the LQTS animals with increased sensitivity due to reduced repolarization reserve, could provide a promising safety screening platform for proarrhythmia research.

1.6.1 Drug-induced acquired LQTS rabbit proarrhythmia model with pharmacologically (HMR-1556 to block I_{Ks}) reduced repolarization reserve

As mentioned above, the chronic atrioventricular block (CAVB) dog model is one of the best characterised and most sensitive *in vivo* proarrhythmia model to detect the HERG/I_{Kr} blocking-related torsadogenic potential of drugs. In this model, downregulation of the slow

component of the delayed rectifier K^+ current (I_{Ks}) was found to be largely responsible for the increased susceptibility of the model to HERG blocking drug effects, which suggested the vital role of the I_{Ks} in the repolarization reserve. Based on these results, Lengyel et al. aimed to assess the role of I_{Kr} and I_{Ks} in the repolarization reserve capacity in the rabbit heart *in vivo*, and demonstrated that inhibition of either I_{Ks} or I_{Kr} on their own did not result in significant arrhythmia development and caused no or minimal change in QT interval or STV_{QT}; while their combined inhibition led to significant increase in these proarrhythmia markers (especially in STV_{QT}) and most importantly, resulted in unexpectedly high incidence of TdP formation. The authors suggested that pharmacological inhibition of I_{Ks} (by HMR-1556) in the rabbit leads to – as phrased in their original work - 'compromised' (impaired) repolarization reserve, and such animal model could serve as simple and sensitive tool to detect the torsadogenic potential of drugs with I_{Kr} blocking characteristics. In our present work, we used this drug-induced LQTS rabbit proarrhythmia model with pharmacologically (HMR-1556 to block I_{Ks}) reduced ('compromised') repolarization reserve to study the proarrhythmic potential of SZV-270, a novel antiarrhythmic drug candidate.

1.6.2 Novel transgenic LQTS rabbit models with genetically engineered reduction of repolarization reserve

Although the drug-induced acquired LQTS rabbit proarrhythmia model provided proof-of-concept evidence regarding the value of models with impaired repolarization reserve in proarrhythmia safety testing, the reduction of repolarising currents by pharmacological interventions has many disadvantages such as : (i) incomplete selectivity of the applied channel-blockers, (ii) not suited for long term proarrhythmia studies and (iii) they fail to mimic chronic remodelling processes that occur in patients with long term repolarization disturbances.

Therefore, to overcome the above detailed drawbacks of the acquired LQTS models and to better model pathophysiology of (human LQTS) patients with long term reduction in repolarization reserve, several transgenic LQTS rabbit models have recently been generated by over-expressing loss-of-function mutants of human *KCNH2* (*HERG-G628S*, α-subunit of I_{Kr}, loss of I_{Kr}, LQT2) [55] or *KCNE1* (*KCNE1-G52R*, β-subunit of I_{Ks}, decreased I_{Ks}, LQT5) [1] in the rabbit heart. These rabbit models have substantially helped to increase our understanding about different LQTS-related arrhythmia mechanisms.

The LQT2 model mimics human LQTS with severely prolonged QT - particularly at slower heart rates -, spontaneous TdP and in rare cases SCD, thus representing the first transgenic animal model mimicking the complete electrical phenotype of human LQT2. Studies in transgenic LQT2 rabbits highlighted the importance of an enhanced (spatial and temporal) dispersion of repolarization in LQTS-related arrhythmogenesis. The role of: (i) increased sympathetic activity [40, 54, 55], (ii) sex hormones [35, 39, 40] or (iii) anaesthetic drugs [144], for example, in repolarization-prolongation based arrhythmogenesis were also thoroughly investigated using these rabbit models.

The LQT5 model, on the other hand, reflects 'silent' LQTS, in which the slight reduction of repolarization reserve does not lead to clinically manifest QT prolongation but increases vulnerability to repolarization-prolonging I_{Kr}-blocking drugs, which promotes drug-induced TdP [1].

Although the increased sensitivity of these different LQTS rabbit models to various repolarization prolonging (drug) effects have already suggested their potential benefits/advantages in drug-induced proarrhythmia risk stratification over the healthy, wild type animals, the systematic assessment of the (proarrhythmic) effects of various specific potassium channel-blockers in these models in comparison with WT animals has never been done before. Therefore, in this study, we aimed to fill this gap.

2. Aims of the studies

2.1 Aim 1: To investigate the proarrhythmic potential of a novel antiarrhythmic drug candidate, SZV-270, with combined Class III and Class I/B effects

To investigate if the repolarization (QT) prolonging (I_{Kr} blocker Class III) effect of SZV-270 is not associated with increased proarrhythmia risk due to its I_{Na} blocker (Class I/B) characteristic, the drug-induced (HMR-1556 to block I_{Ks}) aLQTS rabbit proarrhythmia model with pharmacologically reduced repolarization reserve was used. Indirectly, this set of experiments also aimed to assess the value of *in vivo* proarrhythmia models with impaired repolarization reserve in predicting proarrhythmic side-effects of novel drug candidates.

2.2 Aim 2: Generation and electrophysiological characterization of a new, double-transgenic (LQT2-5) rabbit model

LQT2-5 rabbit models were generated to mimic diseased conditions with decreased I_{Ks} function in the setting of impaired repolarization reserve and to study the role of decreased I_{Ks} in drug-induced LQTS-related arrhythmogenesis particularly in response to increased sympathetic activity.

2.3 Aim 3: To study the value of transgenic LQT2, LQT5 and LQT2-5 rabbit models in better prediction of drug-induced ventricular arrhythmias

Various LQTS rabbit models with different mechanisms accounting for reduced repolarization reserve were utilized to study if their combined use could provide a more detailed and more reliable assessment of the (multi-channel-based) pro-arrhythmic potential of drug-candidates.

3. Materials and Methods

All animal experiments were performed in compliance with EU legislation (directive 2010/63/EU), the German (TierSchG and TierSchVersV) and Hungarian animal welfare laws, after approval by the local Institutional Animal Care and Use Committees in Germany (Regierungspraesidium Freiburg; approval number G14/111) and Hungary (Department of Animal Health and Food Control of the Csongrád County Government Office; approval number XIII/4227/2016).

3.1 Proarrhythmia studies using the drug induced acquired LQTS rabbit model

To assess the proarrhythmic potential of SZV-270, a novel antiarrhythmic drug candidate, the anaesthetised drug-induced acquired LQTS rabbit proarrhythmia model (described in detail by Lengyel et al. [80]) with pharmacologically reduced repolarization reserve was used. Briefly, the rabbits were anaesthetised by IV thiopentone (50 $mg.kg^{-1}$ BW) and ventilated artificially (Harvard rodent ventilator, model 683, Harvard Apparatus, South Natick, MA, USA) through a tracheal tube. The left carotid artery and the right jugular vein were cannulated to monitor blood pressure and administer drugs iv., respectively. After 15 minutes of stabilisation period ('Control'), the animals were randomly enrolled into 3 groups. Group 1 ('Dofetilide') received the I_{Kr} blocker dofetilide (25 $\mu g.kg^{-1}$ BW) in a volume of 2 $mL.kg^{-1}$ BW in 5 minutes. Group 2 ('HMR + Dofetilide') and group 3 ('HMR + SZV-270') were administered the I_{Ks} blocker HMR-1556 (0.1 $mg.kg^{-1}$ BW, [145]) in combination with either dofetilide (25 $\mu g.kg^{-1}$ BW) or SZV-270 (0.3 $mg.kg^{-1}$ BW) in a volume of 2 $mL.kg^{-1}$ BW. The ECG was recorded using subcutaneous needle electrodes (lead I, II, III), was digitized and stored on a computer for off-line analysis using National Instruments data acquisition hardware (National Instruments, Austin, Texas, USA) and SPEL Advanced Haemosys software (version 3.2, MDE Heidelberg GmbH, Heidelberg, Germany). The heart rate corrected QT interval (QTc) was calculated by a formula specifically worked out for anaesthetized rabbits [146], as follows: QTc = QT – (0.704 * (RR-250)). To assess the drug-induced changes in temporal (beat-to-beat) instability of repolarization, a marker for proarrhythmia, the short term variability of QT (STV_{QT}) was calculated by measuring 31 consecutive QT-s and using the following equation: $STV_{QT} = \sum |D_{n+1} - D_n| (30 \times \sqrt{2}) - 1$, where D is the duration of the QT intervals

[101]. Arrhythmias were diagnosed in accordance with the revised Lambeth conventions [147].

3.2 Proarrhythmia studies using the transgenic LQTS rabbit models

3.2.1 Generation of transgenic LQT2, LQT5 and LQT2-5 rabbit models

To better model the chronic electrophysiological changes (reduced repolarization reserve) that occur in patients most susceptible for drug-induced arrhythmias, several transgenic LQTS rabbit models were generated for the first time by Brunnel (LQT2) [55] and Major (LQT5) [1] in 2008 and 2016, respectively. These LQTS models were engineered by beta-myosin heavy chain promoter-driven cardio-selective over-expression of mutated human genes encoding for voltage-gated K^+-channels such as KCNH2/HERG, α-subunit for I_{Kr} (HERG-G628S, LQT2) or KCNE1/MinK, β-subunit for I_{Ks} (KCNE1-G52R, LQT5) [1, 55]. The co-assembly of over-expressed mutated and normal channel subunits disrupts the overall ion channel function ('dominant-negative' strategy), that leads to complete lack of I_{Kr} in LQT2 [55] and decreased function of the I_{Ks} in LQT5 [1].

To generate the transgenic founder animals, the pronuclear microinjection technique was used. Superovulation was induced in wild-type (WT) rabbits using hormonal stimulation with FSH and GnRH-analogues, and inseminated oocytes were microinjected with transgenic mutant DNA-constructs and re-implanted into foster mothers [1, 55]. Mating of the resulting transgenic F0 founders with female WT rabbits resulted in vertical transmission with 50% transgenic and 50% WT offspring. To generate double-transgenic LQT2-5 rabbits, LQT2 male and LQT5 female rabbits were cross-bred [148].

3.2.2 Genotype and phenotype verification

The presence of transgene(s) in the offsprings were verified by PCR performed on genomic DNA obtained from blood drawing at the age of 40-50 days as described in detail [1, 55]. The phenotypes were verified by conventional 12-lead surface ECG in sedated rabbits at the age of 3-4 months. Sedation was performed with ketamine/xylazine (12.5/3.75 $mg.kg^{-1}$ im.). QT indexes were calculated (QTi; QTi (%) = ($QT_{observed}$ / $QT_{expected}$) * 100) as published previously [55]. Rabbits from all genotypes (WT, LQT2, LQT5, LQT2-5) with QTi of at least

95% or higher were used for further experiments (predetermined exclusion criteria; similarly to those predefined in all previous studies with these transgenic LQTS rabbit models).

3.2.3 Investigation of baseline electrophysiological characteristics of LQT2, LQT5, and LQT2-5 rabbits

To compare baseline (drug free) electrophysiological characteristics of different transgenic LQTS rabbit models to control WT animals, *in vivo* ECG - both in awake, free moving and anesthetised animals - and *ex vivo* monophasic action potential (MAP) measurements were carried out.

3.2.3.1 Telemetric ECG

For ECG monitoring of awake, unrestrained, free-moving animals, WT (n=11), LQT5 (n=11), LQT2 (n=10) and LQT2-5 (n=8) rabbits were subjected to ECG transmitter implantations (triple-lead ECG D70-EEE; Data Sciences International) [40, 55]. Subcutaneous ECG transmitter implantations were performed under general anaesthesia with ketamine and xylazine (induced with intramuscular administration of 12.5 / 3.75 $ml.kg^{-1}$ ketamine/xylazine; maintained with intravenous administration of 2.5-5 $ml.kg^{-1}.h^{-1}$ solution containing a mixture of 20 ml ketamine (25 mg/ml) and 3 ml xylazine (20 mg/ml)) as described in detail in [40, 55]. ECG recordings were started at least two weeks after device implantation to ensure adequate recovery and good healing for artefact-free ECG signals. 24 hours of continuous recording was used for measuring conventional ECG parameters such as PR, QRS, RR, QT under baseline conditions (**Figure 1A**). These parameters represent the average of 5 seconds long measurements done in every 30 minutes over the 24 hours monitoring period. To calculate the QT/RR relationship for each individual rabbit, pairs of QT and RR intervals were averaged over 5 s every 30 min during the 24-hour baseline recording period (48 pairs/animals). These QT-RR pairs were plotted and a linear regression formula (QT (y) = a * RR (x) + b) was obtained for each animal. Using this individual heart rate correction formula (QT (y) = a * RR (x) + b), individual QT expected ($QT_{expected}$ (y) = a * RR (x) + b) and QT index (QTi (%) = 100 * ($QT_{observed}$ / $QT_{expected}$)) were calculated for each animal [55]. For heart rate corrected QT intervals (QTc) calculation, a modified version (QTc = $QT_{observed}$ – (a * (RR – 250), where 'a' represents the slope of the individual QT / RR relationship) of the original Carlsson equation (QTc = $QT_{observed}$ – (0.175 * (RR – 300) for awake, free moving rabbits) [149] was used to better match the heart rate range of our

telemetrically monitored rabbits. Since no significant circadian alteration was observed in the individual's QTc and QTi values, their 24 hours averaged values were used as baseline (control) value. To obtain genotype-specific heart rate correction formulas, linear regression curves were fitted to all QT / RR pairs measured in all animals per genotype [55].

3.2.3.2 Conventional 12-lead ECG

Conventional 12-lead surface ECG was recorded to monitor conventional ECG parameters (PQ, QRS, RR, QT) at baseline in ketamine/xylazine (12.5/3.75 $ml.kg^{-1}$ im.) sedated WT (n=6), LQT5 (n=9), LQT2 (n=8) and LQT2-5 (n=8) animals. T_{peak}-T_{end} (T_{p-e}) and beat-to-beat variability of QT (short term variability of the QT interval (STV_{QT})) were calculated to assess changes in spatial and temporal heterogeneity of repolarization. T_{p-e} was measured in V3 as duration (ms) from the peak to the end of the T wave. For STV_{QT}, 31 consecutive QT were measured and STV_{QT} was calculated using the following equation: $STV_{QT} = \sum |D_{n+1} - D_n| \ (30 \times \sqrt{2}) - 1$, where D is the duration of the QT intervals [101].

3.2.3.3 Monophasic action potential (MAP) measurements ex vivo

WT (n=13), LQT5 (n=15), LQT2 (n=12) and LQT2-5 (n=11) rabbits were anesthetized with ketamine and xylazine (as described above). After additional injection of heparine (500IE iv., Braun, Germany), terminal anaesthesia was performed by intravenous administration of thiopental-sodium (40 $mg.kg^{-1}$ iv., Inresa, Germany). Immediately afterwards, beating hearts were excised and attached to a vertical Langendorff apparatus (Model IH5, Hugo Sachs Elektronik, Hugstetten, Germany).

The hearts were retrogradely perfused via the cannulated ascending aorta with warm (37°C), pre-oxygenated (95% O_2 and 5% CO_2), modified Krebs-Henseleit solution at the constant flow rate of 50ml/min [150]. The aortic pressure was kept between 80-100 mmHg. A latex balloon tipped pressure transducer was placed into the left ventricle (LV) and the end diastolic pressure (EDP) was set between 6-10 mmHg. Haemodynamic parameters (LV systolic and diastolic pressure, rate of LV pressure rise (dP/dt)) and ECG were continuously monitored during the whole experiment. Following mechanical ablation of the atrioventricular (AV) node, the heart was stimulated at basic cycle length of 500ms and 250 ms (2Hz, 120 bpm and 4Hz, 240 bpm; HSE Simulator C, type 224, Hugo Sachs Electronic, Harvard Apparatus GmbH, Hugstetten, Germany). Following the heart excision, an average of 20-30 minutes of recovery period was allowed.

Monophasic action potentials were recorded at baseline 500 and 250 ms cycle lengths of stimulation (2Hz and 4Hz, respectively) by four epicardial contact MAP electrodes positioned onto different regions of the heart: MAP1: apico-anterior, MAP2: mid-anterolateral, MAP3: base-inferolateral, and MAP4: base-inferior positions (**Figure 2A**). The duration of the monophasic action potentials at 75% of repolarization (APD_{75}) as well as the MAP triangulation (APD_{90} - APD_{30}) were measured off-line for each individual MAP electrodes (MAP1-4) and their averaged values (mean APD_{75} and mean APD_{90} - APD_{30}, respectively) were also calculated. After changing the pacing rate from 2 to 4Hz or backwards, 3 minutes of equilibration periods were allowed for the APD (stimulatory frequency) adaptation.

3.2.4 Investigation of K^+-channel blocker induced changes in pro-arrhythmia markers in WT, LQT2, LQT5 and LQT2-5 rabbits

To investigate the sensitivity of the different LQTS models to further drug-induced reduction of repolarising potassium currents, different "selective" K^+ channel blocking drugs were applied *in vivo* and *ex vivo*.

3.2.4.1 Telemetric ECG

Following 24-hour baseline recordings, dofetilide (0.02 $\mu g.kg^{-1}$ BW), barium chloride ($BaCl_2$, 0.3 $mg{\cdot}kg^{-1}$ BW), PEG-400 (0.125 $ml.kg^{-1}$ BW), HMR-1556 (0.1 $\mu g.kg^{-1}$ BW in 0.125 $ml.kg^{-1}$ BW PEG400) and combination of HMR-1556 (0.1 $\mu g.kg^{-1}$ BW) and $BaCl_2$ (0.3 $mg.kg^{-1}$ BW) were administered im. in 0.5 $ml.kg^{-1}$ BW final injection volume, one drug per subsequent day in all monitored animals (**Figure 1B**). ECGs were continuously recorded for 24-hour following each injection to monitor the changes in conventional ECG parameters.

The 24 hours averaged QTc and QTi values during baseline were used as control to assess the effect of dofetilide and barium chloride on QTc/QTi. For HMR-1556 and HMR-1556 + $BaCl_2$, the averaged QTc and QTi values measured within 5 hours after im. injection of PEG 400 vehicle administration (the time window in which the peak effect of the HMR-1556 or combined HMR-1556 + $BaCl_2$ occurred) were used as 'vehicle control' values.

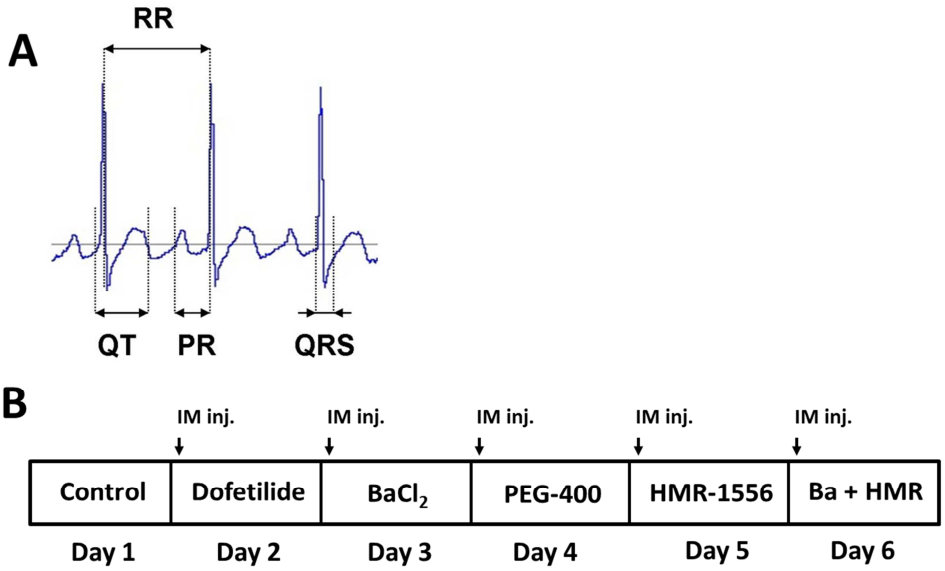

Figure 1. Schematic illustration of telemetric ECG recording with the measured parameters and illustration of the experimental protocol. (A) Telemetric ECG: RR, PR, QRS and QT intervals. (B) Experimental protocol: 24-hour ECG recordings at baseline (control) and after im. injections of dofetilide, $BaCl_2$, PEG-400, HMR-1556 and $BaCl_2$+HMR-1556.

3.2.4.2 Conventional 12-lead ECG

After (drug-free) baseline recordings, dofetilide (0.02 $\mu g.kg^{-1}$ BW), $BaCl_2$, (0.3 $mg.kg^{-1}$ BW), HMR-1556 (0.1 $\mu g.kg^{-1}$ BW), and combination of HMR-1556 and $BaCl_2$ were administered iv. as a bolus in 1 minute and (peak) changes in conventional ECG parameters (PQ, QRS, RR, QT) and proarrhythmia markers (STV_{QT}, $T_{p\text{-}e}$) were assessed after up to 20 minutes of the drugs administrations.

3.2.4.3 Monophasic action potential (MAP) measurements ex vivo

Following drug-free baseline measurements (self-control), MAPs were recorded at 500 and 250 ms cycle lengths of stimulation (2Hz and 4Hz) after a 10-minute perfusion with dofetilide (1 nM), HMR-1556 (100 nM), $BaCl_2$ (10 µM), or with combination of $BaCl_2$ (10 µM) + HMR-1556 (100 nM) (**Figure 2B**). Drug-induced changes in APD_{75} and AP-triangulation were calculated.

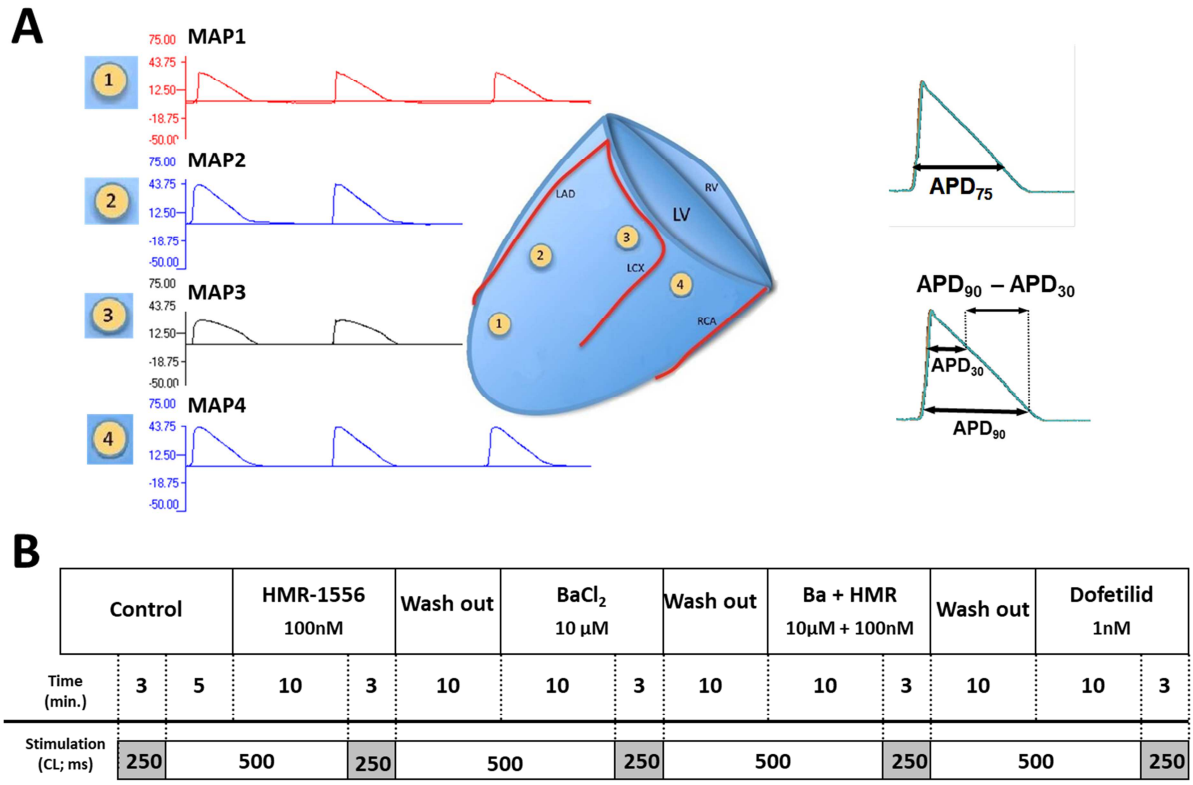

Figure 2. Schematic illustration of monophasic action potential (MAP) recordings with the measured parameters and the experimental protocol. (A) MAP recordings: Illustration of different epicardial MAP electrode positions: MAP1, apico-anterior; MAP2, mid-anterolateral; MAP3, base-inferolateral; MAP4, base-inferior. The derived MAP parameters (APD_{75}, APD_{90} and APD_{30}) are also indicated. (B) Illustration of the experimental protocol: the time course of the experiment with the applied stimulatory cycle length (CL) patterns and administered drugs are indicated.

3.2.5 Investigation of the function of I_{Ks} in vivo in WT, LQT2, LQT5 and LQT2-5 rabbits

As the sympathetic nervous system plays a major role in triggering arrhythmias in (drug-induced and acquired) LQTS – particularly in the setting of absent or impaired I_{Ks} – we aimed at investigating if similar phenomenon could be observed in the LQT2-5 and LQT5 models (with mildly reduced I_{Ks}). For these experiments, we used the sympathomimetic isoproterenol as it mimics the effect that an activation of the sympathetic nervous system will have in LQTS patients. Changes in QTi (%) resulting from iv administration of I_{Ks}-activator isoproterenol followed by I_{Ks}-blocker HMR-1556 were measured to assess I_{Ks} function in vivo.

3.2.6 Investigation of *ex vivo* arrhythmia susceptibility in isolated WT, LQT2, LQT5 and LQT2-5 rabbit hearts

Our *ex vivo* arrhythmia setting was developed based on a method described by Eckardt et al. [151], in which bradycardia, low K^+ concentration and a K^+-channel-blocker was

combined to prolong repolarization and favour arrhythmias. Arrhythmia (AR) development was provoked in Langendorff-perfused AV-ablated hearts (n=7 WT, n=8 LQT5, n=6 LQT2 and n=7 LQT2-5) – beating spontaneously in stable ventricular escape rhythm (VER) at a constant rate of 60-80 beats.min^{-1} – by perfusion with the following solutions: 5.4 mM K^+ Krebs-Henseleit (KH) (baseline I.,10 min), 2.0 mM K^+ KH (5 min), 5.4 mM K^+ KH (baseline II.,10 min), 5.4 mM K^+ KH +10 µM BaCl2 (10 min) and 2.0 mM K^+ KH +10 µM BaCl2 (5 min). ECG was continuously recorded and the duration (% of perfusion time) and incidence (as average number of AR events as well as % of total number of experiments) of arrhythmias were measured off-line. Arrhythmias were categorised by "The Lambeth Conventions (II)" [147] as ventricular extra beats (VEB; ventricular 'premature' extra beat(s) – ranging from a simple ventricular extrasystole to couplets or triplets in terms of complexity), bigeminy, ventricular tachycardia (VT) and ventricular fibrillation (VF).

3.3 Drugs

For the proarrhythmia studies using the drug-induced acquired LQTS rabbit model, dofetilide (Sigma-Aldrich, Munich, Germany) and HMR-1556 (Tocris Bioscience, Bristol, UK) were used to inhibit the rapid and slow delayed rectifier potassium current (I_{Kr} and I_{Ks}, respectively). SZV-270 was provided by Professor Péter Mátyus, Department of Organic Chemistry, Semmelweis University, Budapest, Hungary. For anaesthesia, thiopentone (Sigma Aldrich, Munich, Germany; 50 mg.kg^{-1} BW) was used.

For the *in vivo* and *ex vivo* proarrhythmia studies using the transgenic LQTS animals, the following "selective" K^+-channel-blockers were used (**Table 1**): dofetilide (Sigma-Aldrich, Munich, Germany) to inhibit I_{Kr}, HMR-1556 (Tocris Bioscience, Bristol, UK) to inhibit I_{Ks}, and barium chloride ($BaCl_2$, Sigma-Aldrich, Munich, Germany) to inhibit the inward rectifier potassium current (I_{K1}). PEG-400 (0.125 ml.kg^{-1} BW) was used as solvent to dilute HMR-1556. The *ex vivo* and *in vivo* doses of dofetilide, HMR-1556 and $BaCl_2$ were determined based on dosages described in the literature with minimal/no effect in healthy WT animals but with expected repolarization prolonging effect in the set of reduced repolarization reserve in long-QT models. Therefore, no dose-finding experiments were performed. For I_{Ks} activation, the sympathomimetic drug isoproterenol (Isuprel 0.2 mg.kg^{-1}, Hospira Inc., USA) was continuously perfused intravenously in a dose of 6-12 µg/hr (3-6ml/hr) to increase the

baseline heart rate by 20-30%. S-ketamine (Pfizer, USA) and xylazine (Bayer, Germany) were used for anaesthesia (12.5 ml.kg^{-1} / 3.5 ml.kg^{-1} i.m., followed by iv. infusion) during ECG transmitter implantation, surface ECG recording, and prior to heart extraction, since this combination does not affect cardiac repolarization [144].

Agents / Dose	Dofetilide	$BaCl_2$	HMR-1556	PEG-400
in vivo (telemetric ECG)	0.02μg*kg^{-1} BW	0.3mg*kg^{-1} BW	0.1mg*kg^{-1} BW	125μl*kg^{-1} BW
in vivo (anaesthetised)	0.02μg*kg^{-1} BW	0.1mg*kg^{-1} BW	0.03mg*kg^{-1} BW	125μl*kg^{-1} BW
ex vivo (MAP)	1nM	10μM	100nM	
Inhibited currents	I_{Kr}	I_{K1}	I_{Ks}	slight I_K

Table 1. Summary of the applied K^+-channel-blockers during proarrhythmia studies using transgenic LQTS rabbit models. Summary of K^+-blockers and their doses given to the transgenic LQTS animals and to their wild type littermate controls during *in vivo* ECG (awake, free moving telemetric and under anaesthesia) and *ex vivo* MAP measurements.

3.4 Statistical analysis

Data are expressed either as mean ± SEM or median with the lower (25th percentile) and upper (75th percentile) quartiles and the minimum and maximum values. Statistical analyses were performed by Prism 8.0 (Graphpad, San Diego, USA). Graphs were created by Prism 8.0. Normal distribution of all data was checked prior to statistical analysis. To analyse normally distributed data, the following parametric tests were used: paired *t*-test for before vs. after treatment comparisons, one-way ANOVA for genotype-specific comparisons and repeated-measure ANOVA for intra-group comparisons (f. ex. for regional comparisons between MAP1-4 parameters). Post-hoc Tukey tests were conducted only if F was significant and there was no variance inhomogeneity. For not normally distributed data, non-parametric tests were used: Wilcoxon Rank - Sum test for before vs. after treatment comparisons or Kruskal-Wallis test for genotype-specific comparisons. Chi-square test was used to compare arrhythmia incidence between different groups or genotypes. Levels of significance were abbreviated as * for $p < 0.05$ for inter-genotype comparison and as # for $p < 0.05$ for before vs. after treatment.

4. Results

4.1 1st Aim: Assessment of the proarrhythmic potential of a novel antiarrhythmic drug candidate, SZV-270

Antiarrhythmic drugs with Class III (I_{Kr} blocker) electrophysiological characteristics could cause proarrhythmia, unless this effect is counterbalanced by the drugs' effect on other cardiac ion currents. Therefore, to test whether SZV-270 with combined Class III (I_{Kr} blocker) and Class I/B (I_{Na} blocker) characteristics had proarrhythmic effect in susceptible individuals, its torsadogenic potential was assessed using the anaesthetised drug-induced acquired LQTS rabbit model with impaired repolarization reserve. First, the repolarization reserve in the model was reduced by the I_{Ks} blocker HMR-1556. Then the effect of SZV-270 on proarrhythmia markers (QTc and STV_{QT}) and TdP development was evaluated and compared to that of dofetilide, a known torsadogenic drug with pure I_{Kr} blocker (Class III) characteristics. The effect of dofetilide on its own (without HMR-1556) was also assessed.

PQ intervals were not changed by dofetilide (65.7 ± 1.68 ms at baseline *vs* 66.3 ± 1.38 ms following dofetilide administration, $p>0.05$), HMR-1556, or combined HMR-1556 and dofetilide treatment (63.3 ± 2.56 ms following HMR-1556 and 63.5 ± 2.95 ms following HMR-1556+dofetilide combination *vs* 62.7 ± 2.62 ms at baseline, respectively, all $p>0.05$), or in the SZV-270 administered group (62.1 ± 1.38 ms following HMR-1556 and 62.3 ± 1.62 ms following HMR-1556+SZV270 combination *vs* 63.3 ± 1.35 ms at baseline, respectively, all $p>0.05$). The RR interval was not affected by dofetilide (206 ± 10.2 ms at baseline *vs* 214 ± 5.8 ms following dofetilide, $p>0.05$) or HMR-1556 alone, however, it was increased by the combination of HMR-1556 and dofetilide (210 ± 6.1 ms at baseline *vs* 212 ± 6.1 ms following HMR-1556, $p>0.0.5$, and 227 ± 5.1 ms following HMR-1556+dofetilide, $p<0.05$) or the combination of HMR-1556 and SZV-270 (213 ± 8.3 ms at baseline *vs* 218 ± 4.7 ms following HMR-1556, $p>0.0.5$, and 261 ± 8.1 ms following HMR-1556+SZV270, $p<0.05$).

After HMR-1556 administration, SZV-270 significantly widened the QRS interval, while no other drug had any effect on this parameter (**Figure 3A**). The heart rate corrected QT interval (QTc) was not changed by the I_{Ks}-blocker HMR-1556 but was prolonged significantly - in a similar extent - when dofetilide or combination of HMR-1556 and dofetilide or SZV-270 was administered (**Figure 3B**). STV_{QT}, a novel proarrhythmia marker that has recently been

suggested as being superior over QTc in predicting TdP [82, 104], was increased only by dofetilide or (even more pronouncedly) combined HMR-1556 + dofetilide but remained unchanged when SZV-270 was administered in combination with HMR-1556 (**Figure 3C**). These changes in STV_{QT} were in good correlation with the incidence of TdP formation in these groups: dofetilide alone or especially when given in combination with HMR-1556 markedly increased TdP incidence while SZV-270 administration (after HMR-1556) did not evoke any TdP (**Figure 3D**). Therefore, it is important to highlight, that after reduction of the cardiac repolarization reserve by HMR-1556, both SZV-270 and dofetilide did prolonge the QTc in a similar extent, however – as opposed to dofetilide - this repolarization prolongation in case of SZV-270 did not increase the beat-to-beat variability of QT (STV_{QT}) and most importantly, did not lead to any TdP formation. These results suggest no proarrhythmic potential of SZV-270.

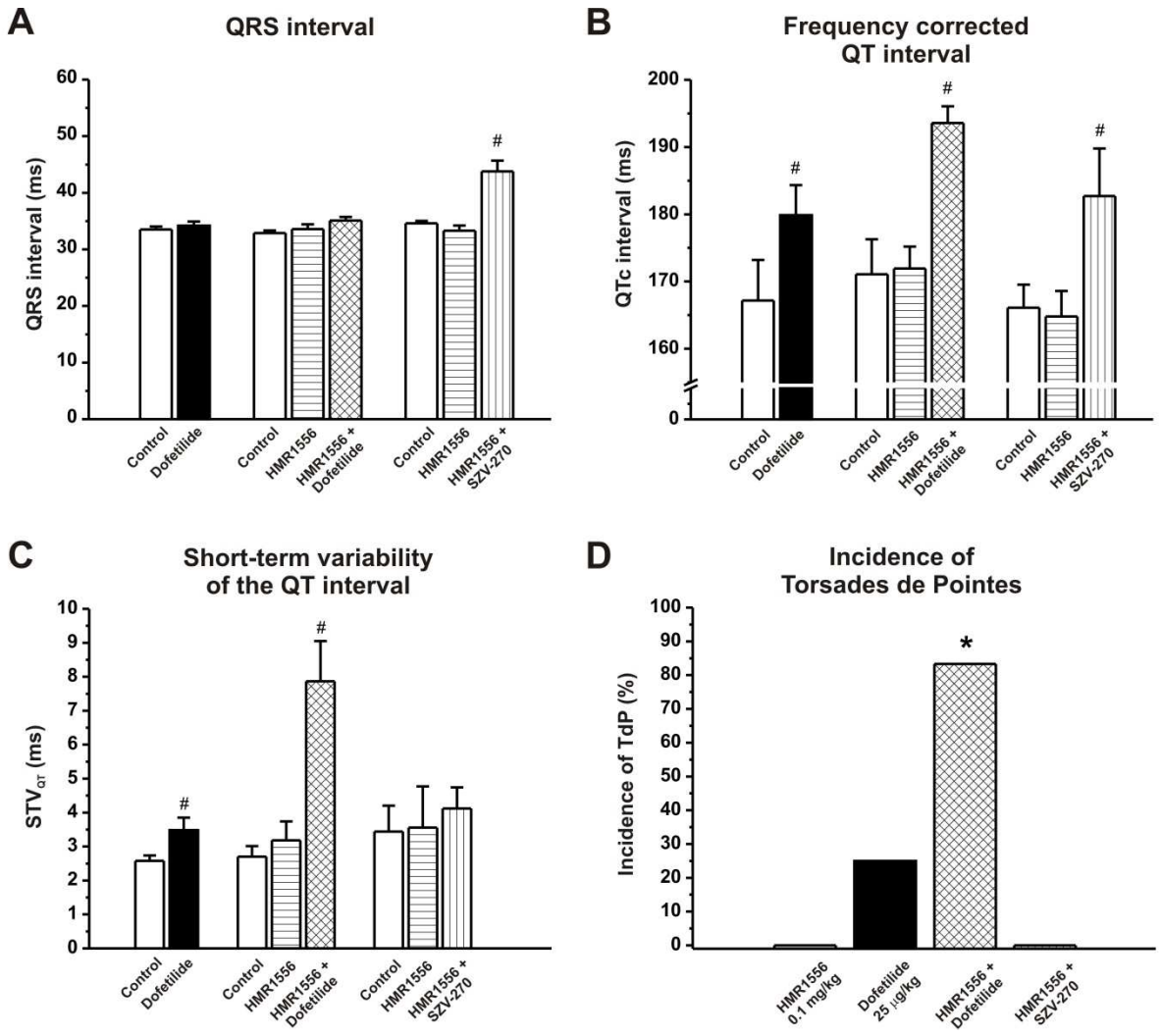

Figure 3. The effects of the I_{Ks} blocker HMR-1556, the I_{Kr} blocker dofetilide and SZV-270 on different ECG parameters and the incidence of Torsades de Pointes (TdP) arrhythmia in an anesthetized rabbit proarrhythmia model. (A) Only SZV-270 widened the QRS interval, while (B) the frequency corrected QT interval (QTc) was prolonged in a similar extent by dofetilide, the combination of HMR-1556+dofetilide and HMR-1556+SZV270. (C) Despite prolonging QTc, the combination of HMR-1556+SZV270 did not increase the short-term variability of the QT interval (STV_{QT}). (D) In parallel with a markedly and significantly increased STV_{QT}, only the combination of HMR-1556+dofetilide led to a high incidence of TdP. SZV-270 did not show any proarrhythmic activity in this model with impaired repolarization reserve. Values are mean ± SEM. #p<0.05 vs. baseline values within the same group; *p<0.05 vs. dofetilide group; n=8-11 in each group.

4.2 2nd Aim: Baseline characteristics of WT, LQT5, LQT2 and the new double-transgenic LQT2-5 rabbit models

4.2.1 ECG characteristics in awake, free moving and anaesthetised rabbits (in vivo)

To compare the electrophysiological features of wild type (WT) and different transgenic LQTS (LQT5, LQT2, LQT2-5) rabbits, 24-hours ECGs were recorded telemetrically in awake, free-moving animals under baseline (drug-free) condition. RR, PR and QRS were similar in all genotypes, however, pronounced genotype-specific differences were detected in repolarization: LQT2 and LQT2-5 models shown pronounced QT interval prolongation compared to WT or LQT5 (**Figure 4A**) - despite similar heart rates (**Figure 4B, Table 2A**). Similarly, heart-rate corrected QT interval (QTc) was prolonged in LQT2 and LQT2-5 rabbit models ($p < 0.05$ vs. WT or LQT5) - but did not differ between LQT5 and WT (**Figure 4B, Table 2A**).

Importantly, an increased QT/RR ratio steepness was observed in LQT2 and LQT2-5 compared to WT or LQT5 (**Figure 4C, Table 2A**), indicating a particularly pronounced QT prolongation at lower heart rates. Of note, in LQT2-5 rabbits, flatter QT-RR regression curve with slightly higher QT values at high heart rates could be observed compared to LQT2, which may be the consequence of the impaired I_{Ks} function in LQT2-5 compared to LQT2.

Similarly to free-moving animals, no genotype-differences were seen in RR, PQ or QRS in anaesthetised rabbits. Heart rate corrected QTc and proarrhythmia markers STV_{QT} and T_{peak}-T_{end} (Tp-e), however, were significantly prolonged in LQT2 and LQT2-5 as compared to WT and LQT5 (**Table 2B**).

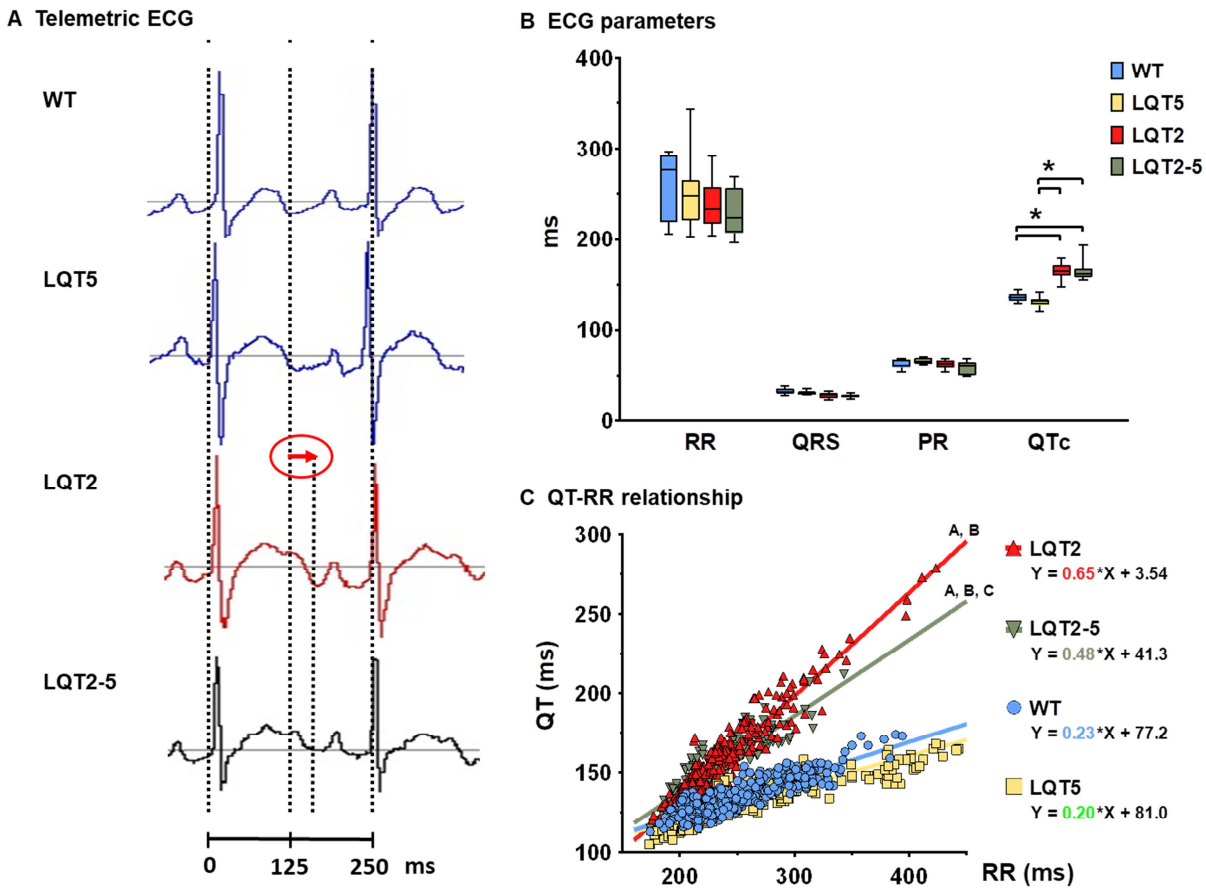

Figure 4. Baseline ECG characteristics in transgenic LQTS models *in vivo*. (A) Telemetric ECG: Representative telemetric ECG recordings (lead II) in WT, LQT5, LQT2 and LQT2-5 rabbits at comparable RR intervals. (B) ECG parameters: RR, QRS, PR, QT, and QTc intervals of awake, free-moving rabbits during 24-hours baseline (drug-free) telemetric ECG recordings in WT (n=11), LQT5 (n=11), LQT2 (n=10) and LQT2-5 (n=8) animals. * $p < 0.05$ inter-genotype comparison. Data are shown as median, the lower (25^{th} percentile) and upper (75^{th} percentile) quartiles, the minimum and maximum values. (C) QT/RR relationship: QT/RR relationships of WT (n=11), LQT5 (n=11), LQT2 (n=10) and LQT2-5 (n=8) rabbits. Colour coded lines indicate linear regression curves best-fit to all data points per genotype. Linear regression formulas with QT/RR ratio steepness (as colour coded numbers) are also shown. A $p < 0.05$ vs. WT; B $p < 0.05$ vs. LQT5; C $p < 0.05$ vs. WT.

A Mean RR, QRS, PR, QT, QTc intervals and QT/RR ratio of awake, free-moving rabbits during 24-hours baseline telemetric ECG recordings

Genotype (No. of animals)	RR	QRS	PR	QT	QTc	QT/RR steepness
WT (11)	265.5 ±10.9	33.2 ±1.2	64.4 ±1.9	136.5 ±2.9	136.8 ±1.6	0.23 ±0.007
LQT5 (11)	248.9 ±11.9	31.6 ±0.9	66.1 ±1.3	129.1 ±2.9	131.6 ±1.7	0.20 ±0.005
LQT2 (10)	239.4 ±9.3	29.0 ±1.1	62.6 ±1.7	153.9 [A,B] ±6.1	165.4 [A,B] ±2.9	0.65 [A,B] ±0.013
LQT2-5 (8)	230.2 ±9.3	28.0 ±0.9	59.0 ±3.1	151.7 [A,B] ±4.7	165.7 [A,B] ±4.2	0.48 [A,B] ±0.017

B Mean RR, QT, QTc, T_{peak}-T_{end} and STV_{QT} of anesthetized rabbits at baseline ECG recordings

Genotype (No.;animals)	RR	QT	QTc	STV_{QT}	Tp-e
WT 18 (6)	347.2 ±10.4	159.3 ±3.5	137.0 ±2.4	1.9 ±0.1	29.8 ±0.8
LQT5 27 (9)	359.9 ±6.0	167.7 ±1.6	145.7 ±1.2	1.9 ±0.1	30.6 ±0.6
LQT2 20 (8)	357.8 ±7.7	233.1 [A,B] ±8.2	163.0 [A,B] ±5.2	2.8 [A,B] ±0.1	40.9 [A,B] ±1.4
LQT2-5 20 (8)	354.0 ±8.0	218.1 [A,B] ±5.6	168.2 [A,B] ±3.2	2.5 [A,B] ±0.1	39.2 [A,B] ±1.2

Table 2. ECG parameters at baseline. (A) Mean RR, QRS, PR, QT, QTc intervals and QT/RR steepness of awake, free-moving rabbits during 24-hours baseline (drug-free) telemetric ECG recordings. (B) Mean RR, QT, QTc intervals, STV_{QT} and T_{peak}-T_{end} of anesthetized rabbits at baseline ECG recordings. [A] $p < 0.05$ vs. WT; [B] $p < 0.05$ vs. LQT5. Numbers of animals are indicated in the Table. Data are shown as mean ± SEM.

4.2.2 Global and regional monophasic action potential (MAP) characteristics (ex vivo)

Similarly to QTc, monophasic action potential durations (APD_{75}) recorded *ex vivo* in Langendorff-perfused hearts were also significantly longer in LQT2 and LQT2-5 rabbits than in WT or LQT5 (**Figure 5A and 5C**). This genotype-difference in APD_{75} was particularly

pronounced at longer stimulation cycle length (CL), which resulted in steeper APD / CL ratio in LQT2 and LQT2-5 rabbits as in WT or LQT5 (**Figure 5B, Table 3**).

Triangulation of the action potential (APD_{90-30}), an important marker of pro-arrhythmia [63, 71] that reflects the duration of the phase 3 repolarization, was also more prominent in LQT2 and LQT2-5 than in WT or LQT5 (**Figure 5D, Table 3)**.

In addition to genotype-differences in overall repolarization characteristics (mean APD, mean APD_{90-30}), genotype-differences in regional heterogeneities of APD and AP-triangulation were also observed: significant apico-basal APD heterogeneity (e.g. shorter apical (MAP1) than basal (MAP3/4) APD_{75}) was observed in all transgenic rabbits but not in WT animals (**Figure 6A**). Furthermore, AP triangulation (APD_{90-30}) was more pronounced in LV apex than in base in LQT5 and LQT2-5 rabbits (**Figure 6B**).

In summary, baseline overall AP parameters (APD_{75}, APD_{90-30} and APD_{75}/CL ratio) were not significantly different in LQT5 models compared to WT but were similarly prolonged in LQT2 and LQT2-5 models. Assessment of regional AP parameters, however, revealed further genotype-specific repolarization disturbances: (i) in LQT5, LQT2, and LQT2-5 models an apico-basal heterogeneity of repolarization (APD) was detected, (ii) regional heterogeneity in APD / CL ratio was measured in LQT2 but not in LQT2-5 and (iii) regional differences in AP triangulation was detected in LQT2-5 but was absent in LQT2.

Genotype (no. of animals)	Cycle lengths of stimulation: 250ms APD_{75} (ms)	250ms APD_{90-30} (ms)	500ms APD_{75} (ms)	500ms APD_{90-30} (ms)	APD_{75}/CL ratio
WT (15)	86.4 ±3.0	62.8 ±1.9	121.2 ±3.7	78.4 ±2.7	0.14 ±0.018
LQT5 (17)	79.5 ±2.7	58.5 ±1.0	112.5 ±3.7	73.0 ±2.0	0.13 ±0.017
LQT2 (14)	102.3^{A,B} ±4.6	70.4^{B} ±3.5	152.6^{A,B} ±6.3	94.9^{A,B} ±4.1	0.20^{A,B} ±0.029
LQT2-5 (11)	98.6^{A,B} ±5.2	66.0^{A,B} ±1.4	152.5^{A,B} ±5.2	82.1 ±2.7	0.22^{A,B} ±0.029

Table 3. APD_{75}, APD_{90-30} and APD_{75} / CL ratio values at baseline. A $p < 0.05$ vs. WT; B $p < 0.05$ vs. LQT5. Numbers of animals are indicated in the Table. Data are shown as mean ± SEM.

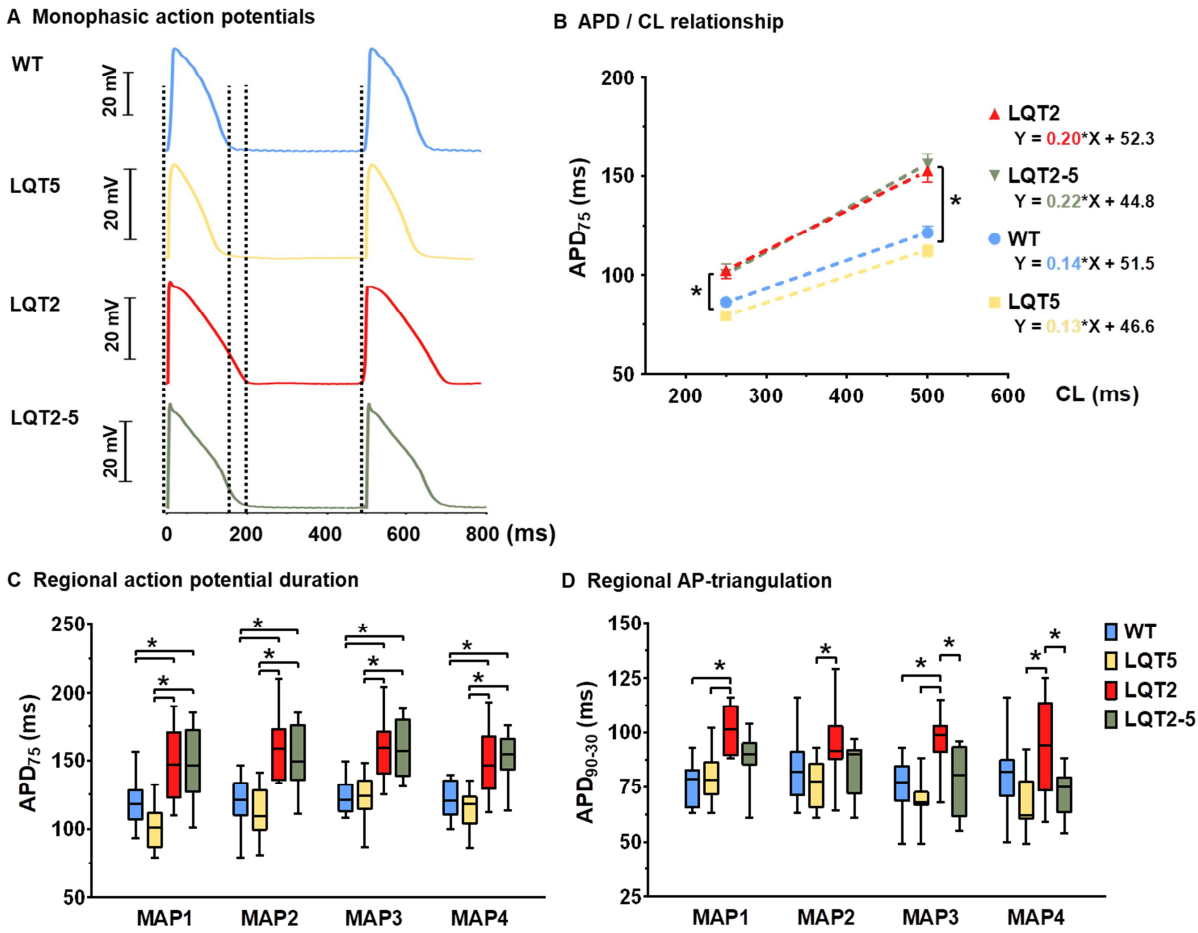

Figure 5. Baseline action potential parameters *ex vivo*. (A) Monophasic action potentials: representative MAP recordings in WT, LQT5, LQT2 and LQT2-5 rabbits' hearts recorded at 500 ms cycle length of stimulation. (B) APD/CL relationship: cycle length (CL) dependence of averaged APD_{75} (representing the averaged APD75 values measured simultaneously by four epicardial electrodes of n=15 WT, n=17 LQT5, n=14 LQT2, n=11 LQT2-5). Data are shown as mean ± SEM. (C) Regional action potential duration: action potential durations were defined as APD_{75}. WT, n=15; LQT5, n=17; LQT2, n=13 and LQT2-5, n=11. (D) Regional AP-triangulation: AP-triangulation was defined as APD_{90}-APD_{30}. WT, n=12; LQT5, n=15; LQT2, n=13 and LQT2-5, n=9. * $p < 0.05$ inter-genotype comparison. Data are shown as median, the lower (25th percentile) and upper (75th percentile) quartiles, the minimum and maximum values. Abbreviations: MAP1: apico-anterior, MAP2: mid-anterolateral, MAP3: base-inferolateral and MAP4: base-inferior positions.

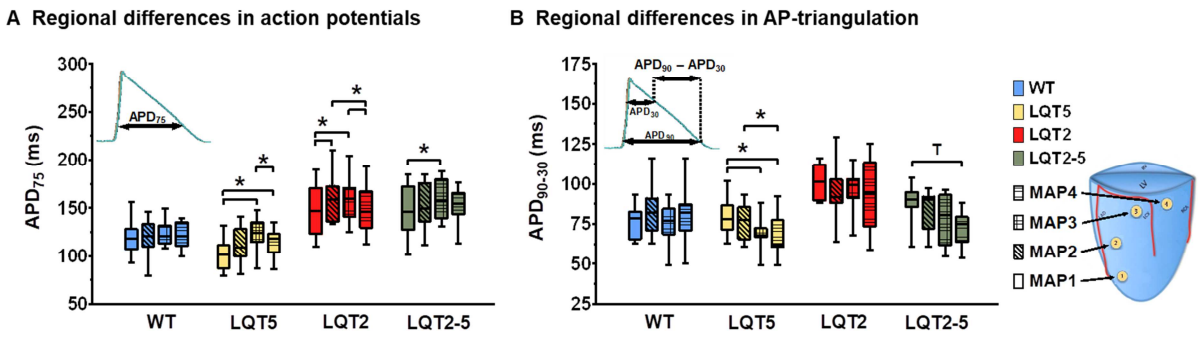

Figure 6. Regional heterogeneities of baseline action potential parameters *ex vivo*. Regional differences in (A) action potentials (APD_{75}) and (B) AP triangulation. WT, n=13-15; LQT5, n=15-17; LQT2, n=13-14 and LQT2-5, n=9-12. * $p < 0.05$ inter-regional comparison. Data are shown as median, the lower (25^{th} percentile) and upper (75^{th} percentile) quartiles, the minimum and maximum values. Abbreviations: MAP1: apico-anterior, MAP2: mid-anterolateral, MAP3: base-inferolateral and MAP4: base-inferior positions.

4.3 3rd Aim: Genotype differences in K^+-channel blocker induced changes in proarrhythmia markers and *ex vivo* arrhythmia formation

To investigate the sensitivity of the different models with impaired repolarization reserve to further drug-induced reduction of repolarising potassium currents, different "selective" K^+-channel blocking drugs were applied *in vivo* and *ex vivo* as well.

4.3.1 K^+-channel blocker effects on in vivo proarrhythmia markers

In awake, free moving animals, i.m. administration of low-dose dofetilide (slight I_{Kr}-blockade) prolonged QTc only in LQT5 but not in healthy WT, nor in LQT2 and LQT2-5 rabbits that both lack I_{Kr} (**Figure 7A**). I_{K1}-blocker $BaCl_2$ prolonged QTc in all groups (**Figure 7A**); this effect, however, was particularly pronounced in LQT2 rabbits ($p < 0.05$ vs. WT, LQT5 and LQT2-5). I_{Ks}-blockade alone (HMR-1556) did not have any significant effect on QTc in any genotype. Combined blockade of I_{K1} ($BaCl_2$) and I_{Ks} (HMR-1556), in contrast, prolonged QTc in all groups (**Figure 7A**).

In anaesthetised animals, similar changes in QTc were observed (**Figure 7B**). I_{K1}-blocker $BaCl_2$ prolonged QTc significantly in all genotypes, but this effect was more prominent in LQT2 and LQT2-5 as in WT or LQT5. HMR-1556 and HMR-1556 + $BaCl_2$ effects were similar as in free moving animals. Dofetilide prolonged QTc in LQT5; surprisingly, however, it also prolonged QTc in LQT2-5. Pro-arrhythmia markers STV_{QT} and T_{peak}-T_{end} were more pronouncedly affected by K^+-channel-blockers in LQTS rabbits with impaired repolarization

reserve: Dofetilide and HMR-1556 increased STV_{QT} and prolonged T_{peak}-T_{end} only in LQT5 and LQT2-5 rabbits (**Figure 7C and 7D**). $BaCl_2$- and combined HMR-1556 and $BaCl_2$-induced increases in STV_{QT} and T_{peak}-T_{end} were more pronounced in all LQTS animals than in WT (**Figure 7C-D**).

Important to note, series of VEBs and non-sustained VTs were observed in one LQT2 and one LQT2-5 rabbit – which both had exceptionally severe phenotype (QTi > 110%) even at baseline – during $BaCl_2$ exposure, demonstrating increased *in vivo* arrhythmia susceptibility to K^+-channel-blockers (**Figure 7E**).

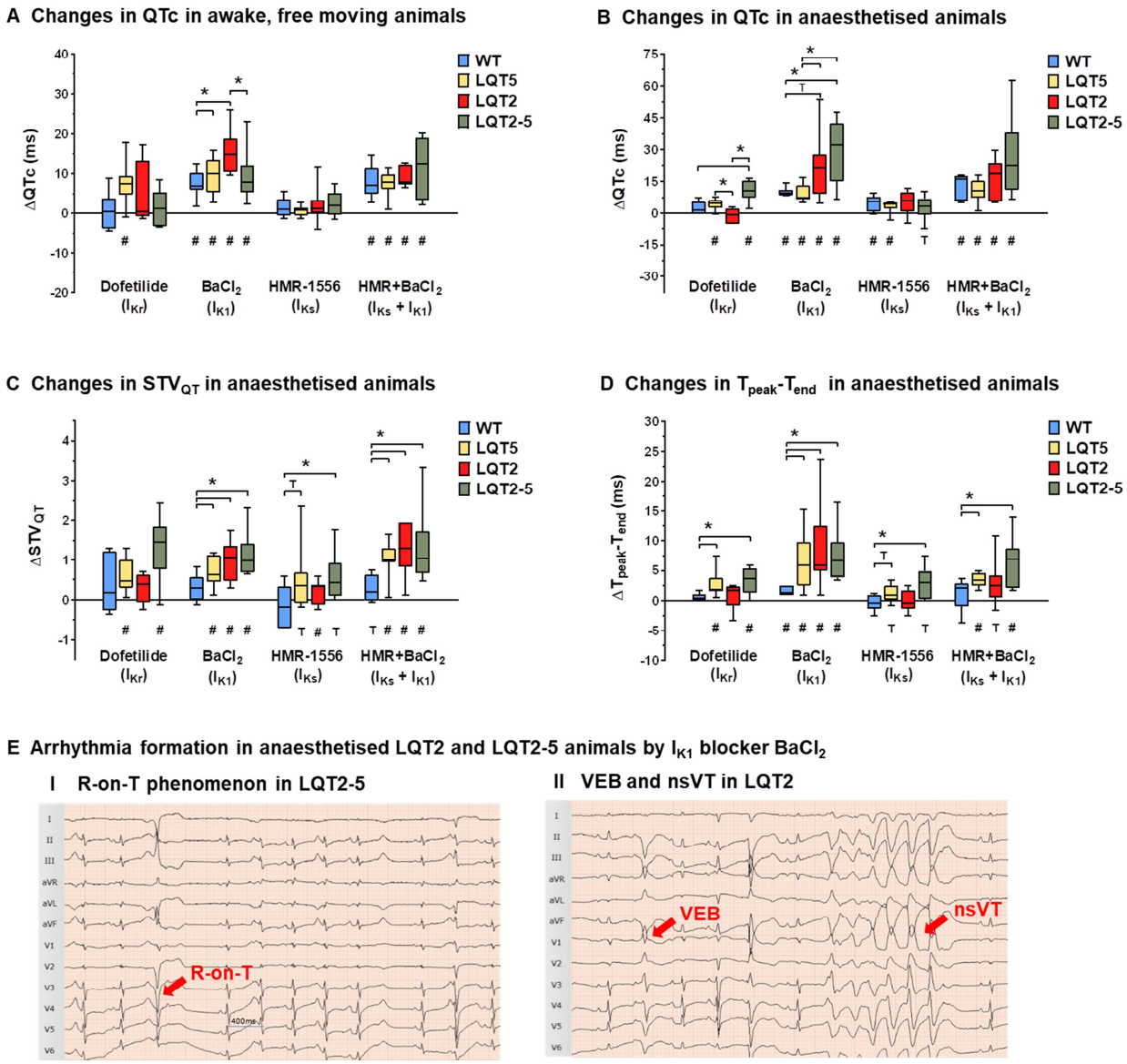

Figure 7. I_K-blocker-induced changes in ECG parameters and arrhythmia formation *in vivo.* (A) Changes in QTc in awake, free moving animals. n=6-8 in each genotype. Changes in QTc (B), STV_{QT} (C) and T_{peak}-T_{end} (D) in anaesthetized animals. Box and whisker graphs show maximal changes in the indicated parameters after i.m. injection of I_{Kr}, I_{K1}, I_{Ks} and I_{Ks} + I_{K1}-

blockers dofetilide, $BaCl_2$, HMR-1556, and HMR+$BaCl_2$, respectively in WT, LQT2, LQT5, and LQT2-5 rabbits. * $p < 0.05$ inter-genotype comparison, # $p < 0.05$ vs. baseline, n=6-9 in each genotype. Data are shown as median, the lower (25^{th} percentile) and upper (75^{th} percentile) quartiles, the minimum and maximum values. (E) Exemplary ECG recordings demonstrate I_{K1}-blocker $BaCl_2$ induced ventricular extra beat (VEB) with R-on-T phenomenon in LQT2-5 (I) and VEB and non-sustained ventricular tachycardia (nsVT) in LQT2 (II) rabbits *in vivo*.

4.3.2 I_{Ks} function in the different LQTS models in vivo

The sympathomimetic isoproterenol was administered to activate I_{Ks} and to investigate differences in cardiac repolarization, which may occur upon sympathetic activation in the different LQTS rabbit models. Normally, QT-shortening is observed as a consequence of physiological QT adaptation, a process in which the interplay between simultaneously activated repolarising I_{Ks} (QT-shortening) and depolarizing $I_{Ca,L}$ (QT-prolongation) plays a major role [54]. Due to the presence of the mutant *KCNE1* (*KCNE1-G52R*) encoding an abnormal beta-subunit of the I_{Ks}-conducting channel complex in LQT5 and LQT2-5, the malfunctioning I_{Ks} could not counterbalance the QT-prolonging effect of activated $I_{Ca,L}$, thus resulting in significantly more pronounced QT-prolongation in LQT5 and LQT2-5 than in WT and LQT2 with normally functioning I_{Ks} (**Figure 8A**). On the other hand, I_{Ks}-blocker HMR-1556-induced QT-prolongation was much more prominent in WT or LQT2, in which the normally functioning I_{Ks} was properly 'pre'-activated by isoproterenol, as in LQT5 or LQT2-5, in which I_{Ks} could not be 'pre'-activated (**Figure 8B**).

These results suggest impaired I_{Ks} function in LQT5 and LQT2-5; a fact that should be taken into consideration when testing pro-arrhythmic potential of drugs at different sympathetic activity levels of the animal models.

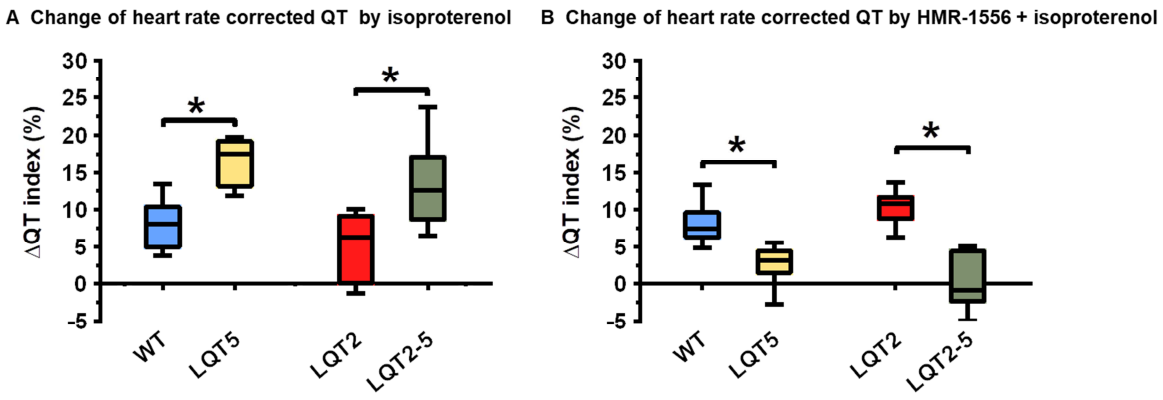

Figure 8. I_{Ks} function *in vivo.* Change of heart rate corrected QT (QTindex (QTi) = $100*(QT_{observed}/QT_{expected})$) after i.v. administration of isoproterenol (A) and HMR-1556 +

isoproterenol (B) in WT, LQT5, LQT2 and LQT2-5 animals. * $p < 0.05$ inter-genotype comparison, n=6-9 in each group. Data are shown as median, the lower (25^{th} percentile) and upper (75^{th} percentile) quartiles, the minimum and maximum values.

4.3.3 K^+-channel blockers effects on monophasic action potential (MAP) characteristics ex vivo

To investigate global and regional sensitivity of the different hearts with impaired repolarization reserve to further drug-induced reduction of K^+-currents, hearts were Langendorff-perfused with different "selective" potassium channel-blockers.

Following perfusion with a very low concentration of the I_{Kr}-blocker dofetilide (1nM), a slight prolongation of mean APD_{75} were observed in all groups (**Figure 9A**). However, since this prolongation was very subtle, below a "threshold" of 10 ms, it is likely due to the experimental setup and not of clinical relevance. I_{Ks}-blocker HMR-1556 (100 nM) induced a more pronounced APD_{75} prolongation in LQT2 and LQT2-5 than in WT or LQT5 hearts (**Figure 9A**). Similarly, I_{K1}-blocker $BaCl_2$ (10 µM) or combined I_{K1}/I_{Ks}-blockade by $BaCl_2$ (10 µM) + HMR-1556 (100 nM) prolonged APD_{75} significantly more in LQT2 and LQT2-5 than in WT or in LQT5 (**Figure 9A**). This prolongation of APD was particularly pronounced at slower rates, leading to an increased APD/CL ratio steepness upon I_{K1}-, I_{Ks}- or combined I_{K1}/I_{Ks}-blockade in LQT2 and LQT2-5 (**Figure 9B**).

Importantly, mean AP triangulation (APD_{90-30}) was more pronounced following I_{K1}- or combined I_{K1}/I_{Ks}-blockade in LQT2 and LQT2-5 than in WT (**Figure 9C**).

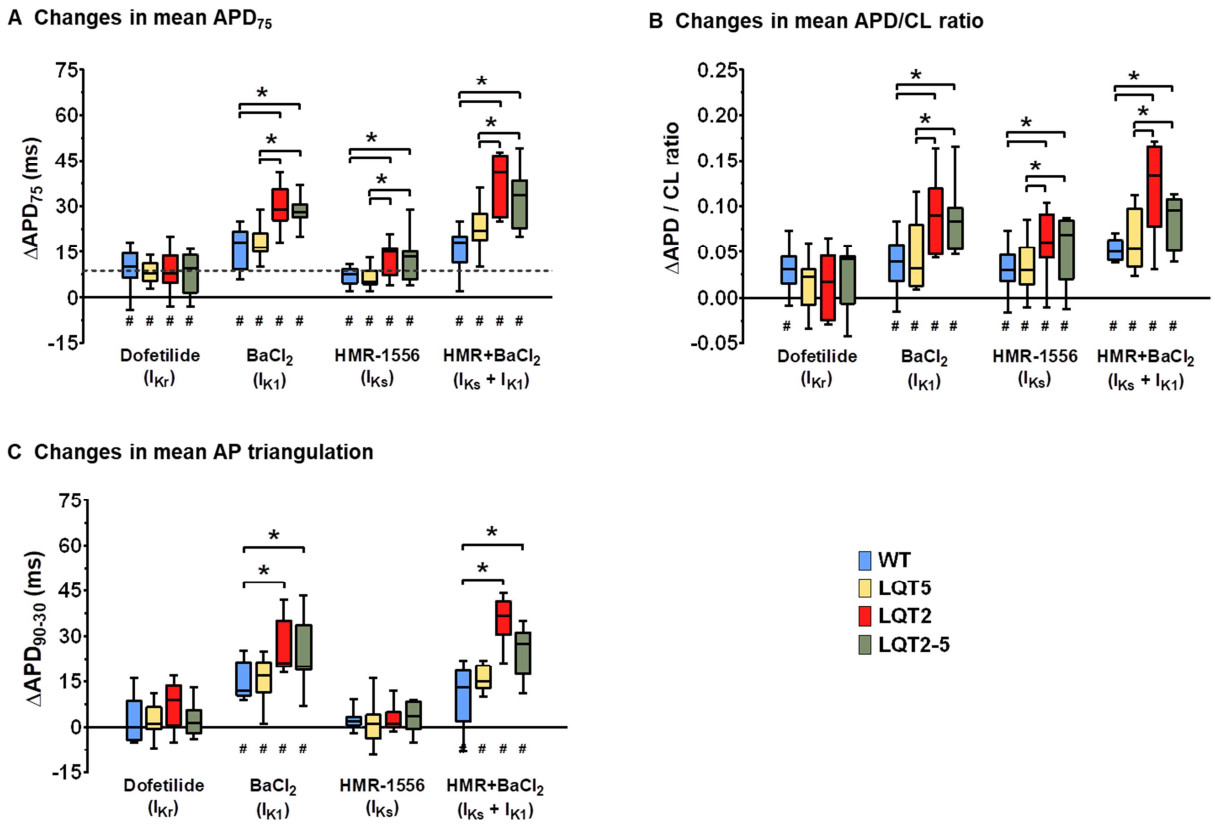

Figure 9. I_K-blocker-induced changes in action potential parameters *ex vivo.* Bar graphs of mean (average MAP1-4) changes in (A) action potential duration (ΔAPD_{75}), (B) APD/CL ratio (ΔAPD/CL ratio), and (C) triangulation of action potential ($\Delta APD_{90\text{-}30}$). * $p < 0.05$ inter-genotype comparison, # $p < 0.05$ vs. baseline, n=6-7 in each group. Data are shown as median, the lower (25th percentile) and upper (75th percentile) quartiles, the minimum and maximum values.

4.3.4 Genotype differences in low potassium and K$^+$-channel blocker induced arrhythmia development ex vivo

To investigate if LQTS models could be better used for detection of drug-induced pro-arrhythmias than WT animals, arrhythmias were provoked in AV-ablated hearts by perfusion with low $[K^+]_o$ KH solution and/or I_{K1}–blocker $BaCl_2$. At baseline (5.4 mM $[K^+]_o$ KH I.), the AV-ablated hearts were beating on their own stable ventricular escape rhythm (VER, heart rates in average 69.1±3.5 in all groups). No major arrhythmia events were observed.

5 min perfusion with 2.0 mM $[K^+]_o$ KH resulted in longer duration of Bigeminy and VTs in transgenic animals with reduced repolarization reserve than in healthy WT (% of perfusion time; Bigeminy: LQT2 38.8±11.7, LQT2-5 37.9±7.0 vs. WT 11.1±6.8; VT: LQT2 25.0±11.1, LQT2-5 30.2±10.5 vs. WT 1.7±1.1; all $p<0.05$, **Figure 10A**). The effects were reversible, and

the original VER was regained in all group after 10 min perfusion with normal KH solution (5.4 mM $[K^+]_o$ KH II. as 2nd baseline).

I_{K1}–blocker $BaCl_2$ induced longer duration and higher incidence of arrhythmias in LQT2 and LQT2-5 rabbits than in WT (**Figure 10A-B**). Combined $BaCl_2$ and 2mM $[K^+]_o$ perfusion resulted in even more pronounced (longer duration and higher incidence of) arrhythmia formation in transgenic animals than in WT (total duration of all AR events as % of perfusion time: LQT5 53.7±11.3, LQT2 86.3±5.3, LQT2-5 83.0±5.1 vs. WT 16.2±5.9; average incidence [No.] of all AR events; LQT2 52.0±16.1, LQT2-5 46.9±13.2 vs. WT 3.6±1.6; all $p<0.05$; **Figure 10A-B**).

Overall, more malignant type of arrhythmia development (VT and VF) was seen in LQT2 and LQT2-5 than in LQT5 or WT (occurrence of AR: $BaCl_2$: LQT2 (VT: 100%) and LQT2-5 (VT: 86%, VF: 57%) vs. WT (VT: 0%, VF: 0%); $BaCl_2$ + 2mM $[K^+]_o$: LQT2 (VT: 100%, VF: 60%) and LQT2-5 (VT: 83%, VF: 57%)) vs. WT (VT: 0%, VF: 0%); all $p<0.05$, **Figure 11**).

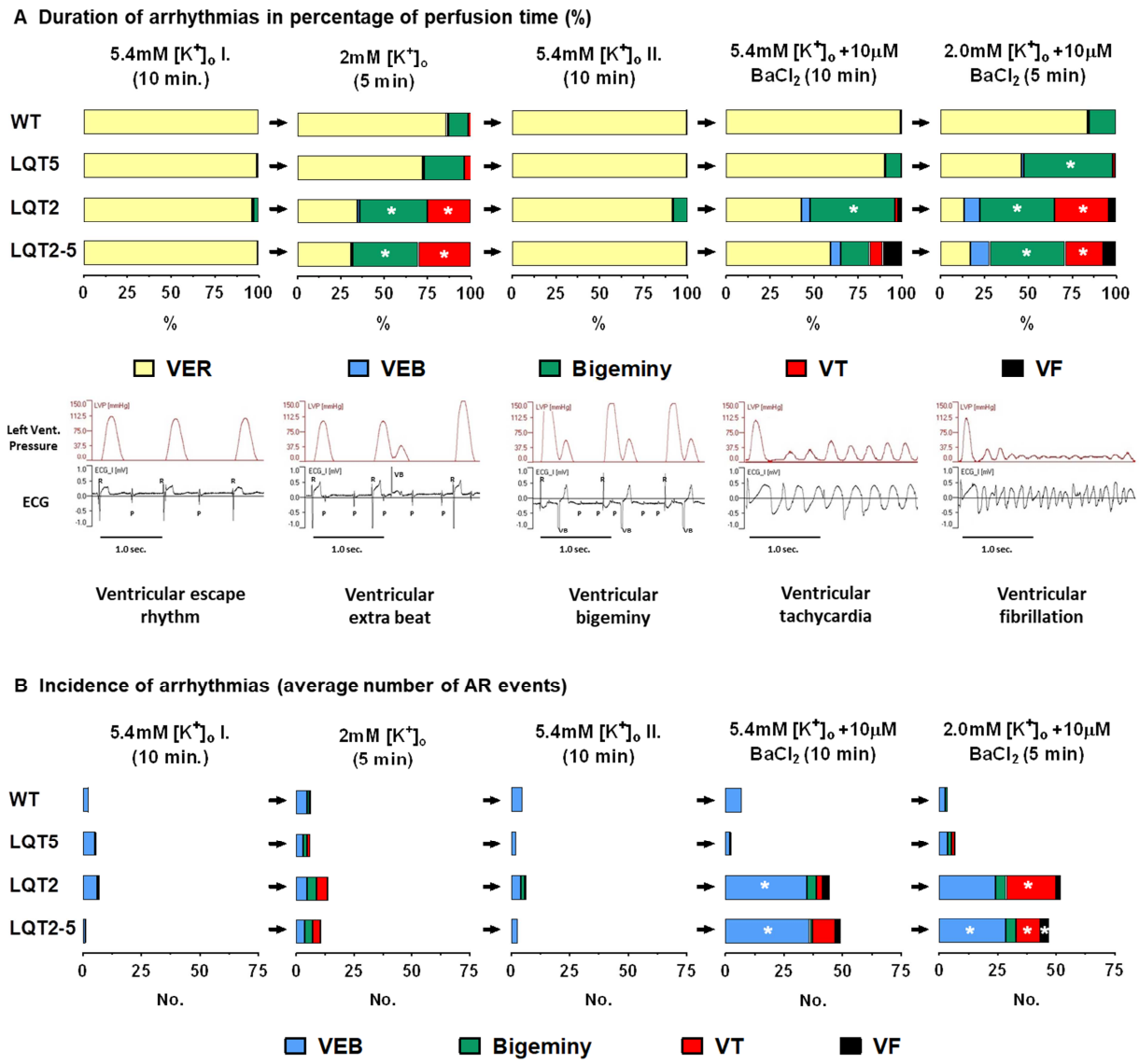

Figure 10. Low potassium- and drug-induced *ex vivo* arrhythmia development. Graphs indicating the duration (% of perfusion time) (A) and incidence (average number of events) (B) of arrhythmias provoked by perfusion of the AV-ablated non-stimulated hearts with low $[K^+]_o$ (2 mM) KH, I_{K1}-blocker $BaCl_2$ (10 μM) or with their combination in WT (n=7), LQT5 (n=8), LQT2 (n=6) and LQT2-5 (n=7) animals. Inlets show representative left ventricular pressure curves and ECG recordings of ventricular escape rhythm (VER), ventricular extra beats (VEB), bigeminy, ventricular tachycardia (VT) and ventricular fibrillation (VF). * $p < 0.05$ inter-genotype comparison.

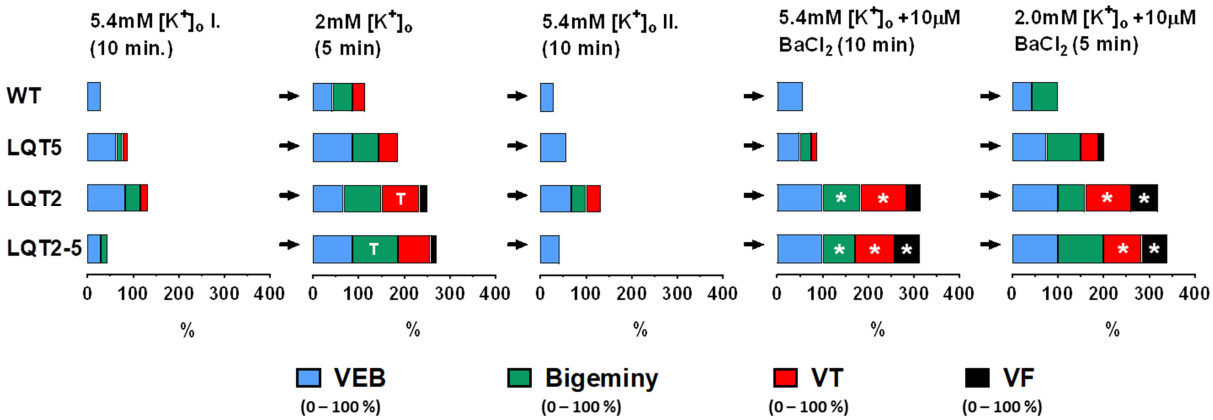

Figure 11. Low potassium- and drug-induced *ex vivo* arrhythmia incidence. Graphs indicating the incidence (% of the total number of experiments) of arrhythmias provoked by perfusion of the AV-ablated non-stimulated hearts with low $[K^+]_o$ (2 mM) KH, I_{K1}-blocker $BaCl_2$ (10 µM) or with their combination in WT (n=7), LQT5 (n=8), LQT2 (n=6) and LQT2-5 (n=7) animals. * $p < 0.05$ inter-genotype comparison. Data are shown as mean.

5. Discussion

5.1 Animal models with reduced repolarization reserve in proarrhythmia research

Drug-induced proarrhythmia mostly occurs in patients with cardiovascular and metabolic diseases that induce structural and/or electrophysiological remodelling of the heart leading to impaired repolarization reserve capacity [18, 19]. Therefore, animal models with reduced repolarization reserve are expected to be more susceptible in detecting proarrhythmic potential of drugs than models with intact repolarization.

The volume overload chronic atrioventricular block (CAVB) dog and the methoxamine (α1-adrenergic receptor agonist) sensitised anesthetised rabbit models provided proof-of-principle evidence that animal models with disease related secondary reduction of cardiac repolarization reserve are particularly susceptible to HERG/I_{Kr}-blocker induced TdP formation and hence, could be used for proarrhythmia safety testing. None of these models, however, are routinely used for predicting drug-related arrhythmogenesis.

Another way to reduce repolarization reserve is the pharmacological inhibition of outward potassium current(s) such as the I_{Kr} or I_{Ks} by E4031 [126-128] and dofetilide [129-131] or HMR-1556 [80], respectively, in *ex vivo* Langendorff-perfused rabbit hearts or *in vivo* in rabbits and dogs. Since HERG/I_{Kr} blockade plays an inevitably important role in most drug-induced proarrhythmia formation and animal models with decreased I_{Ks} function (such as the CAVB dog) are especially sensitive in detecting torsadogenic potential of drugs with HERG/I_{Kr} blocking characteristics, in this work we choose the anaesthetised, acquired LQTS rabbit model with pharmacologically (HMR-1556) decreased I_{Ks} function (reduced repolarization reserve) to study the proarrhythmic potential of SZV-270.

Lastly, reduction of repolarization reserve can also be achieved by the generation of transgenic LQTS animals with genetically altered cardiac ion channels. The first LQTS rabbit models were generated by over-expression of the human mutant *KCNQ1*/KvLQT1 (*KvLQT1-Y315S*, loss of I_{Ks}, LQT1) or *KCNH2*/HERG (*HERG-G628S*, loss of I_{Kr}, LQT2) in the heart [55]. These LQT1 and LQT2 models mimic human LQTS with severely prolonged QT, spontaneous TdP and in rare cases SCD (in LQT2).

Later, the LQT5 transgenic rabbit model was generated by cardio-selective over-expression of human mutant *KCNE1* (*KCNE1*-G52R, impaired I_{Ks}) [1]. This model reflects 'silent' LQTS, in which the slight reduction of repolarization reserve does not lead to clinically manifest QT prolongation but increases vulnerability to repolarization-prolonging drugs [1].

In this work, we investigated the potential benefits of using drug-induced acquired or different transgenic LQTS rabbit models with reduced repolarization reserve in pro-arrhythmia research. We provided evidence that drug-induced and transgenic LQTS rabbit models were not only able to more reliably detect I_K-blocker induced changes in different proarrhythmia markers but also demonstrated increased drug-induced arrhythmia susceptibility compared to healthy animals. Therefore, animals with pharmacologically or genetically reduced repolarization reserve due to different mechanisms could be used to assess the pro-arrhythmic potential of drug candidates in a more complex and more reliable manner.

First, in line with earlier findings [80], we confirmed that the anaesthetised acquired LQTS rabbit model with pharmacologically decreased I_{Ks} could be used to reliably detect the torsadogenic potential of drugs with pure I_{Kr} blocking characteristics. Second, we provided evidence that similar to the pharmacologically sensitized acquired LQTS model, transgenic LQT5, LQT2 and LQT2-5 models with genetically engineered reduction in I_{Ks} and/or I_{Kr}, were also able to more reliably detect I_K-blocking properties of drugs, e.g., blockade of I_{Kr} ,I_{K1} and I_{Ks} than healthy animals.

5.2 Drug-induced acquired LQTS rabbit model with pharmacologically reduced repolarization reserve

It has been known for a long time that I_{Ks} plays a vital role in repolarization reserve [19] and its dysfunction is associated with increased sensitivity to drug-induced proarrhythmias, especially in the setting of elevated sympathetic tone [54, 55]. Most obviously, reduced I_{Ks} function was found to be a causal link to the increased arrhythmogenicity in LQT1. Downregulation of the I_{Ks} was found in other human diseased conditions as well (such as in chronic heart failure [24] or diabetes mellitus [28, 29]) that were also associated with high proarrhythmia risk. Based on these (patho)physiological findings, different animal models with decreased I_{Ks} function were generated for proarrhythmia research. The CAVB dog, or

the I_{Ks} -blocker HMR-1556-induced anaesthetised (acquired LQTS) rabbit models with impaired I_{Ks} function, for example, demonstrated markedly increased sensitivity to drugs with HERG/I_{Kr} blocking characteristics and therefore were suggested to use for detecting torsadogenic side-effects of drug candidates. Our results on studying the proarrhythmic potential of SZV-270, a novel antiarrhythmic drug candidate, using the HMR-1556-induced impaired repolarization reserve (acquired LQTS) rabbit model are in line with these earlier findings: I_{Kr} blocker dofetilide administration to healthy (control) animals led to relatively low TdP incidence while applying the dofetilide to animals in which I_{Ks} had been reduced by HMR-1156 resulted in almost 90% of TdP formation. SZV-270 with mixed I_{Kr} (Class III) and I_{Na} (Class I/B) blocking characteristics on the other hand, did not provoke any TdP in HMR-1556 sensitized rabbits. This beneficiary electrophysiological effect of SZV-270 – repolarization prolongation without the increase in proarrhythmia risk - is most likely attributed to its Class I/B characteristic, that – by inhibiting the $I_{Na,late}$ as well - could oppose the I_{Kr} inhibition related - proarrhythmic - repolarization over prolongation at low heart rates [152]. Based on our results, combined Class III and Class I/B antiarrhythmic drugs such as the SZV-270, - as proposed by earlier hypotheses as well [152] – with no/minimal proarrhythmic potential could represent a safe, novel therapeutic strategy in treating re-entry type ventricular arrhythmias.

5.3 Transgenic LQTS rabbit models with genetically reduced repolarization reserve

5.3.1 Baseline characteristics of transgenic LQTS rabbit models

Both arrhythmia initiating (triggering) and maintaining (substrate) mechanisms have been showed to play an important role in the arrhythmogenesis in (acquired and genetic) LQTS patients. Increased temporal instability and regional heterogeneity of repolarization are considered to form the electrical "substrate" that facilitates re-entry formation, while increased sympathetic nervous system activity serves as the "trigger" for early afterdepolarizations and mediates additional acute effects on I_{Ks}- and $I_{Ca,L}$-currents [55, 96, 153].

LQT5 rabbits showed no overall repolarization prolongation (no increase in QT interval), however, they demonstrated increased apico-basal heterogeneity of APD and AP-

triangulation compared to WT indicating slight regional repolarization disturbances at baseline. This is in good agreement with the first characterisation of the model by Major et al. [1], in which accelerated I_{Ks} and I_{Kr} deactivation kinetics and only slightly increased baseline STV_{QT} were described. Therefore, LQT5 rabbits could be a model to mimic 'silent' LQTS condition with nearly normal baseline phenotype.

In contrast, LQT2 and LQT2-5 rabbits demonstrated a pronounced, temporally and spatially (regionally) heterogeneous repolarization prolongation already at baseline: prolonged QTc/APD, steeper QT/RR slope, increased temporal instability (STV_{QT}), transmural ($T_{peak\text{-}end}$) and apico-basal heterogeneity of repolarization and more AP-triangulation – similarly to human LQTS patients – were found. These characteristics form an 'arrhythmia substrate' in these models that favour re-entry-based drug-induced proarrhythmia formation especially during bradycardia. Interestingly, the overall severity of the phenotype was similar in LQT2-5 and LQT2 at baseline. The I_{Ks} function, however, was more impaired in LQT2-5 rabbits with decreased QT-shortening capacity at higher heart rates.

5.3.2 Utility of transgenic LQTS rabbit models to detect K^+-channel blocking effects on pro-arrhythmia markers

Drug-induced proarrhythmia occurs most frequently in patients harbouring repolarization disturbances (such as in LQTS, chronic heart failure or diabetes mellitus) as these (diseased) individuals are particularly sensitive to drugs that (further) disturb cardiac repolarization [154]. Similar to LQTS patients, different LQTS rabbits mimicking human LQTS characteristics demonstrated increased sensitivity to repolarization-prolonging side-effects of known K^+-channel-blockers.

LQT1 rabbits – with complete lack of I_{Ks} – showed more pronounced sensitivity to I_{Kr} blocking drug effects than healthy, WT animals with intact repolarization and therefore, were proposed as useful tools for reliable prediction of (I_{Kr}-blocker) drug-induced arrhythmias [155]. Similarly, in line with earlier findings [1], LQT5 animals with mild impairment in I_{Ks} were also more sensitive to I_{Kr}-blocker dofetilide than healthy, WT rabbits demonstrating more pronounced increase in QTc, $T_{peak\text{-}end}$ and STV_{QT}.

Although it has been known for a long time that inhibition of I_{K1} can lead to prolonged APD, increased resting membrane potential and early and delayed afterdepolarisations [19, 126, 156], current proarrhythmia safety tests do not focus on detecting I_{K1}-blocking

properties of novel drug candidates. Based on our results on I_{K1}-blocker $BaCl_2$, LQT2 and LQT2-5 rabbits could represent ideal models to detect such I_{K1}-blocking side-effects of drugs. This is in good agreement with our previous findings: midazolam, a well-known sedato-anxiolytic drug with I_{K1}-blocking properties, increased QT-prolongation and provoked ventricular extra beats only in the more sensitive LQT2 rabbits but not in the WT animals [144].

In our *in vivo* experiments, application of the sympathomimetic (I_{Ks} and $I_{Ca,L}$ activating) isoproterenol resulted in less I_{Ks} activation, and therefore, in more pronounced QTi-prolongation in LQT5 and LQT2-5 than in WT or LQT2, suggesting that LQT5 and LQT2-5 animals could be especially sensitive to adrenergic trigger induced repolarization over prolongation and arrhythmias. The phenotype of the LQT2-5 closely resembles LQT2, however, beside their similarity (lack of I_{Kr} in both models), the slight decrease in I_{Ks} in the LQT2-5 may provide additional insights into arrhythmias caused by sympathetic stimulation in the setting of impaired repolarization reserve. In this regard, LQT2-5 may serve as an important model for diseases with high arrhythmia risk due to an impaired I_{Ks} function – such as chronic heart failure and diabetes mellitus – in which chronic remodelling reduces not only I_{Kr} but also I_{Ks}. In contrast to LQT1, in which I_{Ks} is completely missing, in LQT5 and LQT2-5 I_{Ks} is only slightly reduced; therefore, the effect of its change – either reduction or increase – on a clinically manifest (LQT2-5) or hidden (LQT5) long QT syndrome may be studied.

5.3.3 Utility of LQTS rabbit models to detect drug-induced arrhythmias

So far, LQTS rabbits have substantially helped to increase our understanding about the role of various extrinsic (anaesthetics) [144] and intrinsic (sympathetic activity [40, 54, 55], sex hormones [35, 39, 40]) factors in repolarization-prolongation based arrhythmogenesis.

Increased sensitivity of different LQTS models to various provocation factors – such as bradycardia, hypokalaemia or K^+-channel-blockers that are crucial for drug-induced pro-arrhythmia in the clinical setting – have already suggested their potential benefits in drug-induced proarrhythmia risk stratification over the healthy, wild type animals. The systematic assessment of the proarrhythmic effects of various specific potassium channel-blockers in these models in comparison with WT animals, however, has not been performed to date. Therefore, in this study, we closed this gap.

To assess the utility of these LQTS models in detecting drug-induced arrhythmias directly (instead of only measuring changes in proarrhythmia markers), we chose the I_{K1}-blocker $BaCl_2$ in our *ex vivo* experimental setting, i) since I_{K1} plays an important role in repolarization reserve [19] and ii) since only this drug caused prolongation of repolarization in all genotypes.

Application of $BaCl_2$ increased the incidence and duration of complex ventricular extra beats and more malignant arrhythmias such as VT and VF in LQT2 and LQT2-5; while in LQT5 only bigeminy occurred; and no serious arrhythmias were observed in WT. In LQTS hearts, the pre-existing temporal and regional heterogeneity in repolarization-prolongation, which was even further aggravated by the provocation factors, increased the sensitivity for re-entry formation.

These observations are in good agreement with earlier findings [157, 158], demonstrating no arrhythmias in AV-ablated Langendorff-perfused WT hearts either at baseline or during hypokalaemia, and serious VT only when repolarization-prolonging drugs were added. Similarly, increased arrhythmia susceptibility was demonstrated in various LQTS models *in vivo*: spontaneous VT and SCD in LQT2 rabbits [55], increased TdP development in drug-induced (dofetilide+HMR-1556) acquired LQTS rabbit and dog models [80] and increased dofetilide-induced TdP in LQT5 rabbits [1].

It is important to note, the incidence of arrhythmia development in response to repolarization prolonging drugs was very low in LQTS animals *in vivo*, while during *ex vivo* experiments, LQTS hearts were much more prone to develop drug-induced arrhythmias since pro-arrhythmic factors were present: 1) the hearts were beating with a stable – very slow – ventricular escape rhythm after AV ablation, and 2) low potassium concentrations were used for the perfusion.

6. Conclusions, New Results and their Potential Significance

The acquired LQTS rabbit proarrhythmia model with pharmacologically (HMR-1556 to block I_{Ks}) reduced repolarization reserve is a suitable tool to predict the torsadogenic potential of drug candidates with I_{Kr} blocking characteristics. In our present work, this model indicated no proarrhythmic potential of the tested novel antiarrhythmic drug candidate, SZV-270.

Transgenic LQTS rabbit models reflected patients with clinically 'silent' - normal QT interval (LQT5) - or 'manifest' - prolonged QT interval (LQT2 and LQT2-5) - impairment in cardiac repolarization reserve capacity due to different pathomechanisms (decreased I_{Ks} in LQT5 and LQT2-5 and lack of I_{Kr} in LQT2 and LQT2-5).

Phenotypically, the new LQT2-5 model closely resembled LQT2; however, it also showed characteristic differences due to its decreased I_{Ks} function (impaired QT-shortening capacity at fast heart rates). Having both, decreased I_{Ks} and lack of I_{Kr}, the new LQT2-5 model may provide additional insights into arrhythmias caused by sympathetic stimulation in the setting of impaired repolarization reserve. In this regard, LQT2-5 may serve as an important model i) for diseases with high arrhythmic risk due to (remodeling-based) impaired I_{Ks} function – such as heart failure and diabetes – and ii) to investigate the effect of pharmacological reduction or increase of I_{Ks} in clinically manifest LQTS.

As LQTS animals were more sensitive in detecting I_{Kr} - (LQT5) or I_{K1}/I_{Ks} - (LQT2 and LQT2-5) blocking properties of drugs compared to healthy WT animals - demonstrating more pronounced changes in different proarrhythmia markers as well as exhibiting higher incidence, longer duration and more malignant type of *ex vivo* arrhythmias -, therefore, they are suggested to be utilized for more reliable prediction of the (multi-channel-based) pro-arrhythmic potential of novel drug candidates.

Based on our findings, the ideal proarrhythmia safety testing strategy would involve the assessment of 1) (multi-) ion channel blocking properties of new drugs (not only I_{Kr}, but also I_{K1}, I_{Ks}, potentially I_{to}) in cardiomyocytes or heterologous expression systems (CiPA initiative) in combination with 2) *in vivo* and *ex vivo* whole heart investigations using drug-induced or different transgenic LQTS and WT rabbit models. The selection of LQTS models should be based on the determined cellular ion channel effects: for I_{Kr} blocking agents, LQT5 or I_{Ks} blocked acquired LQTS models; for I_{K1} blocking drugs, LQT2; and for drugs that affect I_{Ks}

current, LQT2-5 models could be chosen as an ideal pro-arrhythmia detection platform in addition to WT animals. For *in vivo* assessment, 12-lead ECG might be sufficient to determine drug effects on various proarrhythmia markers (QTc, QT dispersion, STV_{QT}, $T_{peak-end}$, etc.); as the rate of (expected) arrhythmia would be fairly small, and attempts to directly investigate drug-induced arrhythmia incidences using ECG monitoring would be too time-consuming and too expensive. For *ex vivo* assessment, MAP measurement could provide information on pro-arrhythmia markers, and – even more importantly – whole heart arrhythmia experiments could provide direct information about the pro-arrhythmic potential of the test drug.

In these proof-of-principle experiments, LQTS rabbit models detected proarrhythmia with increased sensitivity compared to WT animals with normal repolarization, which - from a clinical point of view - is particularly important since current models with intact repolarization are clearly not sensitive enough, as the patients with reduced repolarization reserve that are at highest risk for drug-induced pro-arrhythmia are not considered and represented by relevant animal models. The author is aware, however, that further detailed assessment of the sensitivity and specificity of these LQTS models would be mandatory prior to their routine use for pro-arrhythmia screening.

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8. References

1. Major, P., et al., *A novel transgenic rabbit model with reduced repolarization reserve: long QT syndrome caused by a dominant-negative mutation of the KCNE1 gene.* Br J Pharmacol, 2016. **173**(12): p. 2046-61.
2. Selzer, A. and H.W. Wray, *Quinidine Syncope. Paroxysmal Ventricular Fibrillation Occurring during Treatment of Chronic Atrial Arrhythmias.* Circulation, 1964. **30**: p. 17-26.
3. *The cardiac arrhythmia suppression trial.* N Engl J Med, 1989. **321**(25): p. 1754-6.
4. Waldo, A.L., et al., *Effect of d-sotalol on mortality in patients with left ventricular dysfunction after recent and remote myocardial infarction. The SWORD Investigators. Survival With Oral d-Sotalol.* Lancet, 1996. **348**(9019): p. 7-12.
5. Echt, D.S., et al., *Mortality and morbidity in patients receiving encainide, flecainide, or placebo. The Cardiac Arrhythmia Suppression Trial.* N Engl J Med, 1991. **324**(12): p. 781-8.
6. Woosley, R.L., et al., *Mechanism of the cardiotoxic actions of terfenadine.* JAMA, 1993. **269**(12): p. 1532-6.
7. Wysowski, D.K. and J. Bacsanyi, *Cisapride and fatal arrhythmia.* N Engl J Med, 1996. **335**(4): p. 290-1.
8. Darpo, B., *Spectrum of drugs prolonging QT interval and the incidence of torsades de pointes.* European Heart Journal Supplements, 2001. **3**(K): p. K70-K80.
9. Danker, T. and C. Moller, *Early identification of hERG liability in drug discovery programs by automated patch clamp.* Front Pharmacol, 2014. **5**: p. 203.
10. Stansfeld, P.J., M.J. Sutcliffe, and J.S. Mitcheson, *Molecular mechanisms for drug interactions with hERG that cause long QT syndrome.* Expert Opin Drug Metab Toxicol, 2006. **2**(1): p. 81-94.
11. Yap, Y.G. and A.J. Camm, *Drug induced QT prolongation and torsades de pointes.* Heart, 2003. **89**(11): p. 1363-72.
12. Fenichel, R.R., et al., *Drug-induced torsades de pointes and implications for drug development.* J Cardiovasc Electrophysiol, 2004. **15**(4): p. 475-95.
13. Haverkamp, W., et al., *The potential for QT prolongation and pro-arrhythmia by non-anti-arrhythmic drugs: clinical and regulatory implications. Report on a Policy Conference of the European Society of Cardiology.* Cardiovasc Res, 2000. **47**(2): p. 219-33.
14. Redfern, W.S., et al., *Relationships between preclinical cardiac electrophysiology, clinical QT interval prolongation and torsade de pointes for a broad range of drugs: evidence for a provisional safety margin in drug development.* Cardiovasc Res, 2003. **58**(1): p. 32-45.
15. Farkas, A.S. and S. Nattel, *Minimizing repolarization-related proarrhythmic risk in drug development and clinical practice.* Drugs, 2010. **70**(5): p. 573-603.
16. Ferdinandy, P., et al., *Definition of hidden drug cardiotoxicity: paradigm change in cardiac safety testing and its clinical implications.* Eur Heart J, 2019. **40**(22): p. 1771-1777.
17. Stockbridge, N., et al., *Dealing with global safety issues : was the response to QT-liability of non-cardiac drugs well coordinated?* Drug Saf, 2013. **36**(3): p. 167-82.
18. Roden, D.M., *Taking the "idio" out of "idiosyncratic": predicting torsades de pointes.* Pacing Clin Electrophysiol, 1998. **21**(5): p. 1029-34.
19. Varro, A. and I. Baczko, *Cardiac ventricular repolarization reserve: a principle for understanding drug-related proarrhythmic risk.* Br J Pharmacol, 2011. **164**(1): p. 14-36.
20. Varro, A., et al., *The role of the delayed rectifier component IKs in dog ventricular muscle and Purkinje fibre repolarization.* J Physiol, 2000. **523 Pt 1**: p. 67-81.
21. Jost, N., et al., *Restricting excessive cardiac action potential and QT prolongation: a vital role for IKs in human ventricular muscle.* Circulation, 2005. **112**(10): p. 1392-9.
22. Biliczki, P., et al., *Interaction of different potassium channels in cardiac repolarization in dog ventricular preparations: role of repolarization reserve.* Br J Pharmacol, 2002. **137**(3): p. 361-8.

23. Banyasz, T., et al., *Action potential clamp fingerprints of K+ currents in canine cardiomyocytes: their role in ventricular repolarization.* Acta Physiol (Oxf), 2007. **190**(3): p. 189-98.
24. Kjekshus, J., *Arrhythmias and mortality in congestive heart failure.* Am J Cardiol, 1990. **65**(19): p. 42I-48I.
25. Decker, J.A., et al., *Risk factors and mode of death in isolated hypertrophic cardiomyopathy in children.* J Am Coll Cardiol, 2009. **54**(3): p. 250-4.
26. Dutta, S., et al., *Early afterdepolarizations promote transmural reentry in ischemic human ventricles with reduced repolarization reserve.* Prog Biophys Mol Biol, 2016. **120**(1-3): p. 236-48.
27. el-Sherif, N. and G. Turitto, *The long QT syndrome and torsade de pointes.* Pacing Clin Electrophysiol, 1999. **22**(1 Pt 1): p. 91-110.
28. McNally, P.G., et al., *Sudden death in type 1 diabetes.* Diabetes Obes Metab, 1999. **1**(3): p. 151-8.
29. Whitsel, E.A., et al., *Electrocardiographic QT interval prolongation and risk of primary cardiac arrest in diabetic patients.* Diabetes Care, 2005. **28**(8): p. 2045-7.
30. Lehmann, M.H., et al., *Sex difference in risk of torsade de pointes with d,l-sotalol.* Circulation, 1996. **94**(10): p. 2535-41.
31. Benton, R.E., et al., *Greater quinidine-induced QTc interval prolongation in women.* Clin Pharmacol Ther, 2000. **67**(4): p. 413-8.
32. Wolbrette, D.L., *Risk of proarrhythmia with class III antiarrhythmic agents: sex-based differences and other issues.* Am J Cardiol, 2003. **91**(6A): p. 39D-44D.
33. Gowda, R.M., et al., *Female preponderance in ibutilide-induced torsade de pointes.* Int J Cardiol, 2004. **95**(2-3): p. 219-22.
34. Yang, P.C. and C.E. Clancy, *Effects of sex hormones on cardiac repolarization.* J Cardiovasc Pharmacol, 2010. **56**(2): p. 123-9.
35. Odening, K.E. and G. Koren, *How do sex hormones modify arrhythmogenesis in long QT syndrome? Sex hormone effects on arrhythmogenic substrate and triggered activity.* Heart Rhythm, 2014. **11**(11): p. 2107-15.
36. Drici, M.D., et al., *Sex hormones prolong the QT interval and downregulate potassium channel expression in the rabbit heart.* Circulation, 1996. **94**(6): p. 1471-4.
37. Ando, F., A. Kuruma, and S. Kawano, *Synergic effects of beta-estradiol and erythromycin on hERG currents.* J Membr Biol, 2011. **241**(1): p. 31-8.
38. Kurokawa, J., et al., *Acute effects of oestrogen on the guinea pig and human IKr channels and drug-induced prolongation of cardiac repolarization.* J Physiol, 2008. **586**(12): p. 2961-73.
39. Odening, K.E., B.R. Choi, and G. Koren, *Sex hormones and cardiac arrest in long QT syndrome: does progesterone represent a potential new antiarrhythmic therapy?* Heart Rhythm, 2012. **9**(7): p. 1150-2.
40. Odening, K.E., et al., *Estradiol promotes sudden cardiac death in transgenic long QT type 2 rabbits while progesterone is protective.* Heart Rhythm, 2012. **9**(5): p. 823-32.
41. Chen, G., et al., *Regional genomic regulation of cardiac sodium-calcium exchanger by oestrogen.* J Physiol, 2011. **589**(Pt 5): p. 1061-80.
42. Liu, X.K., et al., *In vivo androgen treatment shortens the QT interval and increases the densities of inward and delayed rectifier potassium currents in orchiectomized male rabbits.* Cardiovasc Res, 2003. **57**(1): p. 28-36.
43. Furukawa, T. and J. Kurokawa, *Non-genomic regulation of cardiac ion channels by sex hormones.* Cardiovasc Hematol Disord Drug Targets, 2008. **8**(4): p. 245-51.
44. Moshal, K.S., et al., *Progesterone modulates SERCA2a expression and function in rabbit cardiomyocytes.* Am J Physiol Cell Physiol, 2014. **307**(11): p. C1050-7.
45. Jackman, W.M., et al., *The long QT syndromes: a critical review, new clinical observations and a unifying hypothesis.* Prog Cardiovasc Dis, 1988. **31**(2): p. 115-72.

46. Nattel, S. and M.A. Quantz, *Pharmacological response of quinidine induced early afterdepolarisations in canine cardiac Purkinje fibres: insights into underlying ionic mechanisms.* Cardiovasc Res, 1988. **22**(11): p. 808-17.
47. Ben Caref, E., et al., *Role of subendocardial Purkinje network in triggering torsade de pointes arrhythmia in experimental long QT syndrome.* Europace, 2008. **10**(10): p. 1218-23.
48. Belardinelli, L., C. Antzelevitch, and M.A. Vos, *Assessing predictors of drug-induced torsade de pointes.* Trends Pharmacol Sci, 2003. **24**(12): p. 619-25.
49. Oros, A., J.D. Beekman, and M.A. Vos, *The canine model with chronic, complete atrio-ventricular block.* Pharmacol Ther, 2008. **119**(2): p. 168-78.
50. Sipido, K.R., A. Varro, and D. Eisner, *Sodium calcium exchange as a target for antiarrhythmic therapy.* Handb Exp Pharmacol, 2006(171): p. 159-99.
51. Tweedie, D., et al., *The effect of alterations to action potential duration on beta-adrenoceptor-mediated aftercontractions in human and guinea-pig ventricular myocytes.* J Mol Cell Cardiol, 1997. **29**(5): p. 1457-67.
52. Volders, P.G., et al., *Similarities between early and delayed afterdepolarizations induced by isoproterenol in canine ventricular myocytes.* Cardiovasc Res, 1997. **34**(2): p. 348-59.
53. Farkas, A.S., et al., *The role of the Na+/Ca2+ exchanger, I(Na) and I(CaL) in the genesis of dofetilide-induced torsades de pointes in isolated, AV-blocked rabbit hearts.* Br J Pharmacol, 2009. **156**(6): p. 920-32.
54. Liu, G.X., et al., *Differential conditions for early after-depolarizations and triggered activity in cardiomyocytes derived from transgenic LQT1 and LQT2 rabbits.* J Physiol, 2012. **590**(5): p. 1171-80.
55. Brunner, M., et al., *Mechanisms of cardiac arrhythmias and sudden death in transgenic rabbits with long QT syndrome.* J Clin Invest, 2008. **118**(6): p. 2246-59.
56. Milberg, P., et al., *Transmural dispersion of repolarization as a key factor of arrhythmogenicity in a novel intact heart model of LQT3.* Cardiovasc Res, 2005. **65**(2): p. 397-404.
57. Antzelevitch, C., *Ionic, molecular, and cellular bases of QT-interval prolongation and torsade de pointes.* Europace, 2007. **9 Suppl 4**: p. iv4-15.
58. Hamlin, R.L. and A. Kijtawornrat, *Use of the rabbit with a failing heart to test for torsadogenicity.* Pharmacol Ther, 2008. **119**(2): p. 179-85.
59. Antzelevitch, C., *Drug-induced spatial dispersion of repolarization.* Cardiol J, 2008. **15**(2): p. 100-21.
60. Gallacher, D.J., et al., *In vivo mechanisms precipitating torsades de pointes in a canine model of drug-induced long-QT1 syndrome.* Cardiovasc Res, 2007. **76**(2): p. 247-56.
61. Chen, Y.J., et al., *Electropharmacologic characteristics of ventricular proarrhythmia induced by ibutilide.* J Cardiovasc Pharmacol, 1999. **34**(2): p. 237-47.
62. Haapalahti, P., et al., *Electrocardiographic interventricular dispersion of repolarization during autonomic adaptation in LQT1 subtype of long QT syndrome.* Scand Cardiovasc J, 2008. **42**(2): p. 130-6.
63. Hondeghem, L.M., L. Carlsson, and G. Duker, *Instability and triangulation of the action potential predict serious proarrhythmia, but action potential duration prolongation is antiarrhythmic.* Circulation, 2001. **103**(15): p. 2004-13.
64. Thomsen, M.B., et al., *Increased short-term variability of repolarization predicts d-sotalol-induced torsades de pointes in dogs.* Circulation, 2004. **110**(16): p. 2453-9.
65. Food and H.H.S. Drug Administration, *International Conference on Harmonisation; guidance on S7B Nonclinical Evaluation of the Potential for Delayed Ventricular Repolarization (QT Interval Prolongation) by Human Pharmaceuticals; availability. Notice.* Fed Regist, 2005. **70**(202): p. 61133-4.
66. Food and H.H.S. Drug Administration, *International Conference on Harmonisation; guidance on E14 Clinical Evaluation of QT/QTc Interval Prolongation and Proarrhythmic Potential for Non-Antiarrhythmic Drugs; availability. Notice.* Fed Regist, 2005. **70**(202): p. 61134-5.

67. Haverkamp, W., et al., *The potential for QT prolongation and proarrhythmia by non-antiarrhythmic drugs: clinical and regulatory implications. Report on a policy conference of the European Society of Cardiology.* Eur Heart J, 2000. **21**(15): p. 1216-31.
68. Yang, P.C., et al., *A computational model predicts adjunctive pharmacotherapy for cardiac safety via selective inhibition of the late cardiac Na current.* J Mol Cell Cardiol, 2016. **99**: p. 151-161.
69. Ortega, F.A., et al., *Applications of Dynamic Clamp to Cardiac Arrhythmia Research: Role in Drug Target Discovery and Safety Pharmacology Testing.* Front Physiol, 2017. **8**: p. 1099.
70. Li, Z., et al., *General Principles for the Validation of Proarrhythmia Risk Prediction Models: An Extension of the CiPA In Silico Strategy.* Clin Pharmacol Ther, 2020. **107**(1): p. 102-111.
71. Hondeghem, L.M. and P. Hoffmann, *Blinded test in isolated female rabbit heart reliably identifies action potential duration prolongation and proarrhythmic drugs: importance of triangulation, reverse use dependence, and instability.* J Cardiovasc Pharmacol, 2003. **41**(1): p. 14-24.
72. Lawrence, C.L., et al., *Nonclinical proarrhythmia models: predicting Torsades de Pointes.* J Pharmacol Toxicol Methods, 2005. **52**(1): p. 46-59.
73. Park, E., et al., *Can non-clinical repolarization assays predict the results of clinical thorough QT studies? Results from a research consortium.* Br J Pharmacol, 2018. **175**(4): p. 606-617.
74. Roden, D.M., *Drug-induced prolongation of the QT interval.* N Engl J Med, 2004. **350**(10): p. 1013-22.
75. Shah, R.R. and L.M. Hondeghem, *Refining detection of drug-induced proarrhythmia: QT interval and TRIaD.* Heart Rhythm, 2005. **2**(7): p. 758-72.
76. Yang, T., D. Snyders, and D.M. Roden, *Drug block of I(kr): model systems and relevance to human arrhythmias.* J Cardiovasc Pharmacol, 2001. **38**(5): p. 737-44.
77. Zhang, S., et al., *Mechanism of block and identification of the verapamil binding domain to HERG potassium channels.* Circ Res, 1999. **84**(9): p. 989-98.
78. Amos, G.J., et al., *Potassium and calcium current blocking properties of the novel antiarrhythmic agent H 345/52: implications for proarrhythmic potential.* Cardiovasc Res, 2001. **49**(2): p. 351-60.
79. Jacobson, I., L. Carlsson, and G. Duker, *Beat-by-beat QT interval variability, but not QT prolongation per se, predicts drug-induced torsades de pointes in the anaesthetised methoxamine-sensitized rabbit.* J Pharmacol Toxicol Methods, 2011. **63**(1): p. 40-6.
80. Lengyel, C., et al., *Combined pharmacological block of I(Kr) and I(Ks) increases short-term QT interval variability and provokes torsades de pointes.* Br J Pharmacol, 2007. **151**(7): p. 941-51.
81. Hinterseer, M., et al., *Relation of increased short-term variability of QT interval to congenital long-QT syndrome.* Am J Cardiol, 2009. **103**(9): p. 1244-8.
82. Hinterseer, M., et al., *Usefulness of short-term variability of QT intervals as a predictor for electrical remodeling and proarrhythmia in patients with nonischemic heart failure.* Am J Cardiol, 2010. **106**(2): p. 216-20.
83. Maron, B.J., et al., *Assessment of QT dispersion as a prognostic marker for sudden death in a regional nonreferred hypertrophic cardiomyopathy cohort.* Am J Cardiol, 2001. **87**(1): p. 114-5, A9.
84. Strasberg, B., et al., *Familial inducible torsade de pointes with normal QT interval.* Eur Heart J, 1983. **4**(6): p. 383-90.
85. Yi, G., et al., *QT dispersion and risk factors for sudden cardiac death in patients with hypertrophic cardiomyopathy.* Am J Cardiol, 1998. **82**(12): p. 1514-9.
86. Eisenberg, S.J., et al., *Sudden cardiac death and polymorphous ventricular tachycardia in patients with normal QT intervals and normal systolic cardiac function.* Am J Cardiol, 1995. **75**(10): p. 687-92.
87. Kusano, K.F., et al., *Torsade de pointes with a normal QT interval associated with hypokalemia: a case report.* Jpn Circ J, 2001. **65**(8): p. 757-60.

88. Paltoo, B., S. O'Donoghue, and M.S. Mousavi, *Levofloxacin induced polymorphic ventricular tachycardia with normal QT interval.* Pacing Clin Electrophysiol, 2001. **24**(5): p. 895-7.
89. Abriel, H. and E.V. Zaklyazminskaya, *Cardiac channelopathies: genetic and molecular mechanisms.* Gene, 2013. **517**(1): p. 1-11.
90. Abbott, G.W., et al., *MiRP1 forms IKr potassium channels with HERG and is associated with cardiac arrhythmia.* Cell, 1999. **97**(2): p. 175-87.
91. Cordeiro, J.M., et al., *Cellular and subcellular alternans in the canine left ventricle.* Am J Physiol Heart Circ Physiol, 2007. **293**(6): p. H3506-16.
92. Szentadrassy, N., et al., *Apico-basal inhomogeneity in distribution of ion channels in canine and human ventricular myocardium.* Cardiovasc Res, 2005. **65**(4): p. 851-60.
93. Barbhaiya, C., et al., *Tpeak - Tend and Tpeak - Tend /QT ratio as markers of ventricular arrhythmia risk in cardiac resynchronization therapy patients.* Pacing Clin Electrophysiol, 2013. **36**(1): p. 103-8.
94. Guerard, N., P. Jordaan, and B. Dumotier, *Analysis of unipolar electrograms in rabbit heart demonstrated the key role of ventricular apicobasal dispersion in arrhythmogenicity.* Cardiovasc Toxicol, 2014. **14**(4): p. 316-28.
95. Meijborg, V.M., et al., *Electrocardiographic T wave and its relation with ventricular repolarization along major anatomical axes.* Circ Arrhythm Electrophysiol, 2014. **7**(3): p. 524-31.
96. Antzelevitch, C., *Role of spatial dispersion of repolarization in inherited and acquired sudden cardiac death syndromes.* Am J Physiol Heart Circ Physiol, 2007. **293**(4): p. H2024-38.
97. Cheng, J., et al., *Heterogeneous distribution of the two components of delayed rectifier K+ current: a potential mechanism of the proarrhythmic effects of methanesulfonanilideclass III agents.* Cardiovasc Res, 1999. **43**(1): p. 135-47.
98. Edwards, J.N. and L.A. Blatter, *Cardiac alternans and intracellular calcium cycling.* Clin Exp Pharmacol Physiol, 2014. **41**(7): p. 524-32.
99. Shimizu, S., et al., *Temporal and spatial dispersion of repolarization during premature impulse propagation in human intact ventricular muscle: comparison between single vs double premature stimulation.* Europace, 2000. **2**(3): p. 201-6.
100. Taggart, P. and M. Lab, *Cardiac mechano-electric feedback and electrical restitution in humans.* Prog Biophys Mol Biol, 2008. **97**(2-3): p. 452-60.
101. Berger, R.D., et al., *Beat-to-beat QT interval variability: novel evidence for repolarization lability in ischemic and nonischemic dilated cardiomyopathy.* Circulation, 1997. **96**(5): p. 1557-65.
102. Momiyama, Y., et al., *Exercise-induced T-wave alternans as a marker of high risk in patients with hypertrophic cardiomyopathy.* Jpn Circ J, 1997. **61**(8): p. 650-6.
103. Thomsen, M.B., et al., *Proarrhythmic electrical remodelling is associated with increased beat-to-beat variability of repolarisation.* Cardiovasc Res, 2007. **73**(3): p. 521-30.
104. Hinterseer, M., et al., *Beat-to-beat variability of QT intervals is increased in patients with drug-induced long-QT syndrome: a case control pilot study.* Eur Heart J, 2008. **29**(2): p. 185-90.
105. Oosterhoff, P., et al., *High-rate pacing reduces variability of repolarization and prevents repolarization-dependent arrhythmias in dogs with chronic AV block.* J Cardiovasc Electrophysiol, 2010. **21**(12): p. 1384-91.
106. Neher, E. and B. Sakmann, *Single-channel currents recorded from membrane of denervated frog muscle fibres.* Nature, 1976. **260**(5554): p. 799-802.
107. Kannankeril, P., D.M. Roden, and D. Darbar, *Drug-induced long QT syndrome.* Pharmacol Rev, 2010. **62**(4): p. 760-81.
108. Nattel, S., G. Duker, and L. Carlsson, *Model systems for the discovery and development of antiarrhythmic drugs.* Prog Biophys Mol Biol, 2008. **98**(2-3): p. 328-39.
109. Wible, B.A., et al., *HERG-Lite: a novel comprehensive high-throughput screen for drug-induced hERG risk.* J Pharmacol Toxicol Methods, 2005. **52**(1): p. 136-45.

110. Chaudhary, K.W., et al., *Evaluation of the rubidium efflux assay for preclinical identification of HERG blockade.* Assay Drug Dev Technol, 2006. **4**(1): p. 73-82.
111. Rezazadeh, S., J.C. Hesketh, and D. Fedida, *Rb+ flux through hERG channels affects the potency of channel blocking drugs: correlation with data obtained using a high-throughput Rb+ efflux assay.* J Biomol Screen, 2004. **9**(7): p. 588-97.
112. Titus, S.A., et al., *A new homogeneous high-throughput screening assay for profiling compound activity on the human ether-a-go-go-related gene channel.* Anal Biochem, 2009. **394**(1): p. 30-8.
113. Colatsky, T., et al., *The Comprehensive in Vitro Proarrhythmia Assay (CiPA) initiative - Update on progress.* J Pharmacol Toxicol Methods, 2016. **81**: p. 15-20.
114. Mehta, A., et al., *Pharmacoelectrophysiology of viral-free induced pluripotent stem cell-derived human cardiomyocytes.* Toxicol Sci, 2013. **131**(2): p. 458-69.
115. Sager, P.T., et al., *Rechanneling the cardiac proarrhythmia safety paradigm: a meeting report from the Cardiac Safety Research Consortium.* Am Heart J, 2014. **167**(3): p. 292-300.
116. Lindgren, S., et al., *Benchmarking safety pharmacology regulatory packages and best practice.* J Pharmacol Toxicol Methods, 2008. **58**(2): p. 99-109.
117. Yan, G.X. and C. Antzelevitch, *Cellular basis for the electrocardiographic J wave.* Circulation, 1996. **93**(2): p. 372-9.
118. Yan, G.X., et al., *Ventricular hypertrophy amplifies transmural repolarization dispersion and induces early afterdepolarization.* Am J Physiol Heart Circ Physiol, 2001. **281**(5): p. H1968-75.
119. Vos, M.A., et al., *Enhanced susceptibility for acquired torsade de pointes arrhythmias in the dog with chronic, complete AV block is related to cardiac hypertrophy and electrical remodeling.* Circulation, 1998. **98**(11): p. 1125-35.
120. Vos, M.A., et al., *Reproducible induction of early afterdepolarizations and torsade de pointes arrhythmias by d-sotalol and pacing in dogs with chronic atrioventricular block.* Circulation, 1995. **91**(3): p. 864-72.
121. Volders, P.G., et al., *Downregulation of delayed rectifier K(+) currents in dogs with chronic complete atrioventricular block and acquired torsades de pointes.* Circulation, 1999. **100**(24): p. 2455-61.
122. Thomsen, M.B., et al., *Assessing the proarrhythmic potential of drugs: current status of models and surrogate parameters of torsades de pointes arrhythmias.* Pharmacol Ther, 2006. **112**(1): p. 150-70.
123. Volders, P.G., et al., *Cellular basis of biventricular hypertrophy and arrhythmogenesis in dogs with chronic complete atrioventricular block and acquired torsade de pointes.* Circulation, 1998. **98**(11): p. 1136-47.
124. Carlsson, L., *The anaesthetised methoxamine-sensitised rabbit model of torsades de pointes.* Pharmacol Ther, 2008. **119**(2): p. 160-7.
125. Carlsson, L., et al., *Assessment of the ion channel-blocking profile of the novel combined ion channel blocker AZD1305 and its proarrhythmic potential versus dofetilide in the methoxamine-sensitized rabbit in vivo.* J Cardiovasc Pharmacol, 2009. **54**(1): p. 82-9.
126. Maruyama, M., et al., *Genesis of phase 3 early afterdepolarizations and triggered activity in acquired long-QT syndrome.* Circ Arrhythm Electrophysiol, 2011. **4**(1): p. 103-11.
127. Parikh, A., et al., *Ranolazine stabilizes cardiac ryanodine receptors: a novel mechanism for the suppression of early afterdepolarization and torsades de pointes in long QT type 2.* Heart Rhythm, 2012. **9**(6): p. 953-60.
128. Choi, B.R., F. Burton, and G. Salama, *Cytosolic Ca2+ triggers early afterdepolarizations and Torsade de Pointes in rabbit hearts with type 2 long QT syndrome.* J Physiol, 2002. **543**(Pt 2): p. 615-31.
129. Farkas, A.S., et al., *Importance of extracardiac alpha1-adrenoceptor stimulation in assisting dofetilide to induce torsade de pointes in rabbit hearts.* Eur J Pharmacol, 2006. **537**(1-3): p. 118-25.

130. Dhein, S., F. Perlitz, and F.W. Mohr, *An in vitro model for assessment of drug-induced torsade de pointes arrhythmia : effects of haloperidol and dofetilide on potential duration, repolarization inhomogeneities, and torsade de pointes arrhythmia.* Naunyn Schmiedebergs Arch Pharmacol, 2008. **378**(6): p. 631-44.
131. Orosz, S., et al., *Assessment of efficacy of proarrhythmia biomarkers in isolated rabbit hearts with attenuated repolarization reserve.* J Cardiovasc Pharmacol, 2014. **64**(3): p. 266-76.
132. Bouder, F., *A case study of long QT regulation: A regulatory tennis game across the Atlantic.* Journal of Risk Research, 2007. **10**(3): p. 385-412.
133. Carlsson, L., *Drug-induced torsade de pointes: the perspectives of industry.* European Heart Journal Supplements, 2001. **3**(K): p. K114-K120.
134. Pugsley, M.K., S. Authier, and M.J. Curtis, *Principles of safety pharmacology.* Br J Pharmacol, 2008. **154**(7): p. 1382-99.
135. Cavero, I. and H. Holzgrefe, *Comprehensive in vitro Proarrhythmia Assay, a novel in vitro/in silico paradigm to detect ventricular proarrhythmic liability: a visionary 21st century initiative.* Expert Opin Drug Saf, 2014. **13**(6): p. 745-58.
136. Kramer, J., et al., *MICE models: superior to the HERG model in predicting Torsade de Pointes.* Sci Rep, 2013. **3**: p. 2100.
137. Jost, N., et al., *Ionic mechanisms limiting cardiac repolarization reserve in humans compared to dogs.* J Physiol, 2013. **591**(17): p. 4189-206.
138. Nerbonne, J.M., *Molecular basis of functional voltage-gated K+ channel diversity in the mammalian myocardium.* J Physiol, 2000. **525 Pt 2**: p. 285-98.
139. Nerbonne, J.M. and R.S. Kass, *Molecular physiology of cardiac repolarization.* Physiol Rev, 2005. **85**(4): p. 1205-53.
140. Varro, A., et al., *Ionic currents and action potentials in rabbit, rat, and guinea pig ventricular myocytes.* Basic Res Cardiol, 1993. **88**(2): p. 93-102.
141. Jung, B., et al., *A quantitative comparison of regional myocardial motion in mice, rabbits and humans using in-vivo phase contrast CMR.* J Cardiovasc Magn Reson, 2012. **14**: p. 87.
142. Panfilov, A.V., *Is heart size a factor in ventricular fibrillation? Or how close are rabbit and human hearts?* Heart Rhythm, 2006. **3**(7): p. 862-4.
143. Harken, A.H., et al., *Early ischemia after complete coronary ligation in the rabbit, dog, pig, and monkey.* Am J Physiol, 1981. **241**(2): p. H202-10.
144. Odening, K.E., et al., *Pharmacogenomics of anesthetic drugs in transgenic LQT1 and LQT2 rabbits reveal genotype-specific differential effects on cardiac repolarization.* Am J Physiol Heart Circ Physiol, 2008. **295**(6): p. H2264-72.
145. Gogelein, H., et al., *Inhibition of IKs channels by HMR 1556.* Naunyn Schmiedebergs Arch Pharmacol, 2000. **362**(6): p. 480-8.
146. Batey, A.J. and S.J. Coker, *Proarrhythmic potential of halofantrine, terfenadine and clofilium in a modified in vivo model of torsade de pointes.* Br J Pharmacol, 2002. **135**(4): p. 1003-12.
147. Curtis, M.J., et al., *The Lambeth Conventions (II): guidelines for the study of animal and human ventricular and supraventricular arrhythmias.* Pharmacol Ther, 2013. **139**(2): p. 213-48.
148. Hornyik, T., et al., *Transgenic LQT2, LQT5 and LQT2-5 rabbit models with decreased repolarisation reserve for prediction of drug-induced ventricular arrhythmias.* Br J Pharmacol, 2020.
149. Carlsson, L., et al., *Proarrhythmic effects of the class III agent almokalant: importance of infusion rate, QT dispersion, and early afterdepolarisations.* Cardiovasc Res, 1993. **27**(12): p. 2186-93.
150. Ziupa, D., et al., *Pronounced effects of HERG-blockers E-4031 and erythromycin on APD, spatial APD dispersion and triangulation in transgenic long-QT type 1 rabbits.* PLoS One, 2014. **9**(9): p. e107210.
151. Eckardt, L., et al., *Drug-related torsades de pointes in the isolated rabbit heart: comparison of clofilium, d,l-sotalol, and erythromycin.* J Cardiovasc Pharmacol, 1998. **32**(3): p. 425-34.

152. Matyus, P., et al., *Novel antiarrhythmic compounds with combined class IB and class III mode of action.* Curr Med Chem, 2004. **11**(1): p. 61-9.
153. Ziv, O., et al., *Origin of complex behaviour of spatially discordant alternans in a transgenic rabbit model of type 2 long QT syndrome.* J Physiol, 2009. **587**(Pt 19): p. 4661-80.
154. Schwartz, P.J. and R.L. Woosley, *Predicting the Unpredictable: Drug-Induced QT Prolongation and Torsades de Pointes.* J Am Coll Cardiol, 2016. **67**(13): p. 1639-1650.
155. Odening, K.E., et al., *Electrophysiological studies of transgenic long QT type 1 and type 2 rabbits reveal genotype-specific differences in ventricular refractoriness and His conduction.* Am J Physiol Heart Circ Physiol, 2010. **299**(3): p. H643-55.
156. Zhang, L., et al., *Electrocardiographic features in Andersen-Tawil syndrome patients with KCNJ2 mutations: characteristic T-U-wave patterns predict the KCNJ2 genotype.* Circulation, 2005. **111**(21): p. 2720-6.
157. Frommeyer, G., et al., *Low proarrhythmic potential of citalopram and escitalopram in contrast to haloperidol in an experimental whole-heart model.* Eur J Pharmacol, 2016. **788**: p. 192-199.
158. Frommeyer, G., et al., *Severe proarrhythmic potential of risperidone compared to quetiapine in an experimental whole-heart model of proarrhythmia.* Naunyn Schmiedebergs Arch Pharmacol, 2016. **389**(10): p. 1073-80.