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

Summary of PhD Thesis

Tibor Hornyik, MD



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LIST OF FULL-LENGTH PUBLICATIONS RELATED TO THE SUBJECT OF THE THESIS

Hornyik T, Castiglione A, Franke G, Perez-Feliz S, Major P, Hiripi L, Koren G, Bősze Zs, Varró A, Zehender M, Brunner M, Bode C, Odening E. K., Baczkó I. Transgenic LQT2, LQT5, and LQT2-5 rabbit models with decreased repolarisation reserve for prediction of drug-induced ventricular arrhythmias. BRITISH JOURNAL OF PHARMACOLOGY 177 pp. 3744-3759. , 16 p. , **2020**, **IF (2019): 7.73** (Q1/D1)

Varga R, **Hornyik T**, Husti Z, Kohajda Zs, Krajsovsky G, Nagy N, Jost N, Virag L, Talosi L, Mátyus P, Varro A, Baczko I. Antiarrhythmic and cardiac electrophysiological effects of SZV-270, a novel compound with combined Class I/B and Class III effects, in rabbits and dogs, CANADIAN JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY **2021**, 99(1): 89-101, **IF (2019): 1.946** (Q2)

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OTHER FULL PUBLICATIONS

Baczko I*; **Hornyik T***, Brunner M, Koren G, Odening K. Transgenic Rabbit Models in Proarrhythmia Research. Frontiers in Pharmacology 11: Paper 853, 18 p. (**2020**), **IF: 4.225** (Q1)

*shared first authorship

Juhasz V, **Hornyik T**, Benak A, Nagy N, Husti Z, Pap R, Saghy L, Virag L, Varro A, Baczko I. Comparison of the effects of $I_{K,ACH}$, I_{Kr} and I_{Na} block in conscious dogs with atrial fibrillation and on action potentials in remodeled atrial trabeculae Canadian Journal of Physiology and Pharmacology 96(1): 18-25. (**2018**), **IF: 1.54** (Q2)

Kohajda Z, Farkas-Morvay N, Jost N, Nagy N, Geramipour A, Horvath A, Varga R, **Hornyik T**, Corici C, Acsai K, Horvath B, Prorok J, Ordog B, Déri Sz, Toth D, Levijoki J, Pollesello P, Koskelainen T, Otsomaa L, Toth A, Baczko I, Lepran I, Nanasi PP, Papp JG, Varro A, Virag L. The Effect of a Novel Highly Selective Inhibitor of the Sodium/Calcium Exchanger (NCX) on Cardiac Arrhythmias in In Vitro and In Vivo Experiments. PloS ONE 11: (11) Paper e0166041. 28 p. (2016), IF: 3.11 (Q1)

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Pueyo E, Husti Z, **Hornyik T**, Baczkó I, Laguna P, Varro A, Rodriguez B. Mechanisms of ventricular rate adaptation as a predictor of arrhythmic risk American Journal of Physiology-Heart and Circulatory Physiology: 298(5): H1577-H1587. (2010), IF: 3.881 (Q1)

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ABBREVIATIONS

APD:	action potential duration
CL:	cycle length
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
KCNE1:	β -subunit of the I_{Ks} -conducting potassium channel
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
MAP:	monophasic action potential
QT:	QT interval in surface 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

INTRODUCTION

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 mechanistically based on prolongation of the cardiac repolarization that is usually observed on the ECG as prolonged QT interval (acquired Long QT Syndrome, LQTS). This 'acquired LQTS' predisposes to Torsades-de-Pointes (TdP) ventricular tachycardia that can lead to sudden cardiac death (SCD). In recent decades, TdP-induced SCD cases were associated with a wide range of commonly used drugs (anti-psychotics, anti-depressants, antihistamines and antibiotics) and many of them have been withdrawn from the market causing significant financial loss for the pharmaceutical industry. Therefore, the need to minimize the proarrhythmic risk of novel drug candidates is overwhelming; however, it remains largely unmet.

Up to 60% of novel chemical entities – and no less than 2-3% of all marketed drugs - have the potential to modulate the function of cardiac ion channels and therefore, to disturb normal cardiac electrical function. The majority of these compounds inhibit the rapid delayed rectifier potassium current I_{Kr} (HERG); but interference with other ion currents such as the slow delayed rectifier potassium current I_{Ks} or the inward rectifier potassium current I_{K1} can also induce serious pro-arrhythmia ('multi-channel-based' nature). Despite this risk, current safety screening tests still mainly focus on detecting the drugs' ability to inhibit I_{Kr} while their effects on I_{Ks} or I_{K1} , for example, are not yet routinely assessed.

Furthermore, drug-induced TdP occur mostly in patients with cardiovascular and metabolic diseases that induce structural and/or electrophysiological remodelling of the heart, leading to reduction of the 'repolarisation reserve'; a term, defined as the ability of cardiomyocytes to maintain sufficient repolarisation despite repolarisation-prolonging (I_{Kr} , I_{Ks} , I_{K1} , I_{to} blocking) effects by compensation via non-affected 'reserve' outward K^+ currents. In spite of this, current safety assessments - in principle - rely still mainly on tests performed on healthy animals with intact repolarization or on their tissues / cells.

Therefore, novel animal models with remodelled myocardium and/or impaired repolarisation reserve - due to reduced I_{Kr} or I_{Ks} - that mimic the pathophysiological

conditions under which drugs usually display highest proarrhythmic risk were suggested for proarrhythmia safety testing. Among these models, the rabbit has a prominent place in arrhythmia research since its cardiac electrophysiological characteristics are much closer to humans than that of other small animals like mice or rats 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 is similar to those in humans. Furthermore, the myocardial mechanical function and their responses to pharmacological interventions show very close resemblance to human cardiac physiology.

Therefore, in this work, drug-induced (HMR-1556 to block I_{Ks}) acquired LQTS, and various transgenic (congenital) LQTS rabbit models (LQT2: HERG-G628S, loss of I_{Kr} ; LQT5: KCNE1-G52R, decreased I_{Ks} and LQT2-5, containing both mutations) with impaired cardiac repolarisation reserve were used to model different electrophysiological changes that occur in patients most susceptible for drug-induced arrhythmias. The sensitivities of these models - changes in proarrhythmia markers and *ex vivo* arrhythmia susceptibility - to various repolarisation prolonging agents ('selective' K^+ channel blockers and a novel drug candidate, SZV-270) were compared to WT animals to estimate their potential use in drug-induced proarrhythmia risk prediction/assessment.

AIMS OF THE STUDIES

1. To investigate the proarrhythmic potential of a novel antiarrhythmic drug candidate, SZV-270, with combined Class III and Class I/B effects by using the drug-induced (HMR-1556 to block I_{Ks}) acquired LQTS rabbit proarrhythmia model with pharmacologically reduced repolarisation reserve.
2. To generate and characterize a new, double-transgenic (LQT2-5) rabbit model in order 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.
3. To study the utility of various transgenic LQTS rabbit models (LQT2, LQT5 and LQT2-5) - with different mechanisms accounting for their reduced repolarisation reserve - in better prediction of drug-induced ventricular arrhythmias.

MATERIALS AND METHODS

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

Proarrhythmia studies using the drug-induced acquired LQTS rabbit model

To assess the proarrhythmic potential of SZV-270, a novel antiarrhythmic drug candidate, the drug-induced acquired LQTS rabbit proarrhythmia model was used. The rabbits were anaesthetised by iv. thiopentone (50 mg/kg) and ventilated artificially 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'), group 1 ('Dofetilide') received the I_{Kr} blocker dofetilide (25 μ g/kg) in 5 minutes while group 2 ('HMR + Dofetilide') and group 3 ('HMR + SZV-270') were administered the I_{Ks} blocker HMR-1556 (0.1 mg/kg) in combination with either dofetilide (25 μ g/kg) or SZV-270 (0.3 mg/kg). ECG was recorded using subcutaneous needle electrodes (lead I, II, III). The heart rate corrected QT interval (QTc) was calculated as follows: $QTc = QT - (0.704 * (RR-250))$. To assess the drug-induced changes in temporal (beat-to-beat) instability of repolarisation, 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. Arrhythmias were diagnosed in accordance with the revised Lambeth conventions.

Proarrhythmia studies using the transgenic LQTS rabbit models

To better model the chronic electrophysiological changes (reduced repolarization reserve) that occur in patients most susceptible for drug-induced arrhythmias, transgenic LQTS rabbit models were generated by cardio-selective overexpression of loss-of-function mutations of human KCNH2 (HERG-G628S, loss of I_{Kr} , LQT2) or KCNE1 (KCNE1-G52R, decreased I_{Ks} , LQT5). To generate double-transgenic LQT2-5 rabbits, LQT2 male and LQT5 female rabbits were cross-bred. The presence of transgene(s) in the animals were verified by

PCR performed on genomic DNA obtained from blood drawing at the age of 40-50 days. The phenotypes were verified by conventional 12-lead surface ECG in sedated rabbits at the age of 3-4 months by calculating QT indexes (QT_i; QT_i (%) = (QT_{observed} / QT_{expected}) * 100). Rabbits from all genotypes (WT, LQT2, LQT5, LQT2-5) with QT_i of 100 ± 5% were used to study their baseline electrophysiological characteristics and I_{Ks} function as well as their sensitivity to various K⁺-channel blockers and *ex vivo* arrhythmias.

Investigation of baseline electrophysiological characteristics of transgenic LQTS rabbits

Telemetric ECG: For ECG monitoring of awake, free-moving animals, WT (n=11), LQT5 (n=11), LQT2 (n=10) and LQT2-5 (n=8) rabbits were subjected to subcutaneous ECG transmitter implantations (triple-lead ECG D70-EEE; Data Sciences International) under general anaesthesia with ketamine and xylazine (im. 12.5 / 3.75 mg/kg ketamine/xylazine; maintained with iv. administration of 2.5-5 ml/kg/hour solution containing a mixture of 20 ml ketamine and 3 ml xylazine; ketamine: 25 mg/ml; xylazine: 20 mg/ml). After two weeks of recovery period, 24 hours of continuous recording was performed for measuring conventional ECG parameters such as PQ, QRS, RR, QT at baseline. 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 (48 pairs/animals) 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 (QT_i (%) = 100 * (QT_{observed} / QT_{expected})) were calculated for each animal. The heart rate corrected QT intervals (QT_c) were calculated as QT_c = QT_{observed} - (a * (RR - 250)), where 'a' represents the slope of the individual QT / RR relationship. 24 hours averaged QT_c and QT_i 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.

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 mg/kg 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 STV_{QT} were calculated to assess changes in spatial and temporal heterogeneity of

repolarisation. T_{p-e} was measured in V3 as duration (ms) from the peak to the end of the T wave. STV_{QT} was calculated as described before.

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/xylazine (as described above) and heparinised (500IE iv., Braun, Germany). After terminal anaesthesia was performed by thiopental-sodium (40 mg/kg iv., Inresa, Germany), beating hearts were excised and attached to a vertical Langendorff apparatus (Model IH5, Hugo Sachs Elektronik, Hugstetten, Germany). The hearts were retrogradely perfused with warm (37°C), pre-oxygenated (95% O₂ and 5% CO₂) modified Krebs-Henseleit solution at the constant flow rate of 50ml/min. 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 was set between 6-10 mmHg. Following mechanical ablation of the atrioventricular (AV) node, the heart was stimulated at basic cycle length of 500ms and 250 ms. ECG was continuously monitored during the whole experiment. 20-30 minutes after the hearts excision (recovery period), monophasic action potentials (MAP) 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. The duration of the MAPs at 75% of repolarisation (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.

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

Telemetric ECG: Following 24-hour baseline recordings, dofetilide (0.02 $\mu\text{g}\cdot\text{kg}^{-1}$ BW), barium chloride (BaCl₂, 0.3 mg/kg), PEG-400 (0.125 ml/kg), HMR-1556 (0.1 $\mu\text{g}/\text{kg}$ in 0.125 ml/kg PEG400) and combination of HMR-1556 and BaCl₂ were administered im., one drug per subsequent day in all monitored animals. ECGs were continuously recorded for 24-hour following each injection to monitor the changes in conventional ECG parameters. The 24 hours averaged QTc and QT_i values during baseline were used as control to assess the effect of dofetilide and BaCl₂ on QTc/QT_i. For HMR-1556 and HMR-1556 + BaCl₂, the averaged QTc

and QT_i values measured within 5 hours after im. injection of PEG 400 vehicle administration were used as 'vehicle control' values.

Conventional 12-lead ECG: After drug-free baseline recordings ('control'), dofetilide (0.02 µg/kg), BaCl₂ (0.3 mg/kg), HMR-1556 (0.1 µg/kg), and combination of HMR-1556 and BaCl₂ were administered iv. as a bolus and (peak) changes in conventional ECG parameters (PQ, QRS, RR, QT) and proarrhythmia markers (STV_{QT}, T_{p-e}) were assessed after up to 20 minutes of the drugs administrations.

Monophasic action potential (MAP) measurements ex vivo: Following drug-free baseline measurements ('control'), MAPs were recorded at 2Hz after 10 minutes perfusion with dofetilide (1 nM), HMR-1556 (100 nM), BaCl₂ (10 µM), or with combination of BaCl₂ (10 µM) + HMR-1556 (100 nM). Drug-induced changes in APD₇₅ and AP-triangulation were calculated.

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

Conventional 12-lead ECG was recorded in ketamine/xylazine sedated (as described above) WT (n=6), LQT5 (n=9), LQT2 (n=8) and LQT2-5 (n=8) animals. Isoproterenol (Isuprel 0.2 mg/kg, Hospira Inc., USA) was continuously perfused intravenously in a dose of 6-12 µg/hr to increase the baseline heart rate by 20-30%. Changes in QT_i (%) 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*.

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., in which bradycardia, low K⁺ concentration and a K⁺-channel-blocker was combined to prolong repolarisation 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 – 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 in accordance with the revised Lambeth conventions.

RESULTS

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

To test the proarrhythmic potential of SZV-270, a novel antiarrhythmic drug candidate with mixed Class III (I_{Kr} blocker) and Class I/B (I_{Na} blocker) characteristics, the repolarisation reserve in WT rabbit was first 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) characteristic. The effect of dofetilide on its own (without HMR-1556) was also assessed. Blood pressures and PQ intervals were not changed by HMR-1556, dofetilide, or combined HMR-1556 and dofetilide or SZV-270 administration. Heart rate was not affected by HMR-1556 but was slightly decreased when dofetilide or combination of HMR-1556 and dofetilide or SZV-270 were given.

After the HMR-1556 administration, SZV-270 significantly widened the QRS interval, while no other drug had any effect on this parameter. 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 (change in QTc, ms \pm SEM; dofetilide 12.4 \pm 4.2, HMR+dofetilide 21.3 \pm 2.6, HMR+SZV-270 18.1 \pm 4.7, all $p < 0.05$). STV_{QT} , a novel proarrhythmia marker that has recently been suggested as being superior over QTc in predicting TdP, was increased only by dofetilide or (even more pronouncedly) combined HMR-1556 + dofetilide (change in STV_{QT} , \pm SEM; dofetilide 1.1 \pm 0.2, HMR+dofetilide 5.1 \pm 1.9, all $p < 0.05$) but remained unchanged when SZV-270 was administered in combination with HMR-1556. 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 (TdP incidence, %; Dofetilide 25.0, HMR+dofetilide 83.3 $p < 0.05$ vs. 'control' (HMR alone)) while SZV-270 administration (after HMR-1556) did not evoke any TdP. Therefore, it is important to highlight, that after reduction of the cardiac repolarisation reserve by HMR-

1556, both SZV-270 and dofetilide did prolong the QTc to a similar extent, however – as opposed to dofetilide - this repolarisation 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 for SZV-270.

2nd Aim: Study the baseline electrophysiological characteristics of the new double-transgenic LQT2-5 as well as WT, LQT2 and LQT5 rabbit models

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

To compare the electrophysiological features of wild type (WT) and different transgenic LQTS 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 repolarisation: LQT2 and LQT2-5 models shown pronounced heart-rate corrected QT interval (QTc) prolongation compared to WT or LQT5 (QTc, ms \pm SEM; LQT2 165.4 \pm 2.9 and LQT2-5 165.7 \pm 4.2 vs. WT 136.8 \pm 1.6 or LQT5 131.6 \pm 1.7, all $p < 0.05$). Importantly, an increased QT/RR ratio steepness was observed in LQT2 and LQT2-5 compared to WT or LQT5 (QT/RR ratio, \pm SEM; LQT2 0.65 \pm 0.01 and LQT2-5 0.48 \pm 0.02 vs. WT 0.23 \pm 0.01 or LQT5 0.2 \pm 0.01, all $p < 0.05$), indicating a particularly pronounced QT prolongation at lower heart rates.

Similarly, to the 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 increased in LQT2 and LQT2-5 as compared to WT and LQT5 (STV_{QT} and T_{p-e} (ms) \pm SEM; LQT2 2.8 \pm 0.1 and 40.9 \pm 1.4, LQT2-5 2.5 \pm 0.1 and 39.2 \pm 1.2 vs. WT 1.9 \pm 0.1 and 29.8 \pm 0.8 or LQT5 1.9 \pm 0.1 and 30.6 \pm 0.6, all $p < 0.05$).

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

Similarly, to QTc, monophasic action potential durations (APD₇₅) recorded *ex vivo* in Langendorff-perfused hearts were also significantly longer in LQT2 and LQT2-5 rabbits than in WT or LQT5 (APD₇₅ at 500ms stimulatory cycle length, ms \pm SEM; LQT2 152.6 \pm 6.3 and LQT2-5 152.5 \pm 5.2 vs. WT 121.2 \pm 3.7 or LQT5 112.5 \pm 3.7, all $p < 0.05$). This genotype-difference in APD₇₅ 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 (APD/CL ratio, \pm SEM;

LQT2 0.2 ± 0.03 and LQT2-5 0.22 ± 0.03 vs. WT 0.14 ± 0.02 or LQT5 0.13 ± 0.02 , all $p < 0.05$). Triangulation of the action potential (APD_{90-30}), an important marker of pro-arrhythmia that reflects the duration of the phase 3 repolarisation, was also more prominent in LQT2 than in WT or LQT5 (APD_{90-30} at 500ms CL, $ms \pm SEM$; LQT2 94.9 ± 4.1 vs. WT 78.4 ± 2.7 or LQT5 73.0 ± 2.0 , all $p < 0.05$). In addition to genotype-differences in overall repolarisation 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. Furthermore, AP triangulation (APD_{90-30}) was more pronounced in LV apex than in base in LQT5 and LQT2-5 rabbits.

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 LQTS models to drug effects that further lengthen their cardiac repolarisation, different “selective” K⁺-channel blocking drugs were applied *in vivo* and *ex vivo* as well.

K⁺-channel blocker effects on *in vivo* proarrhythmia markers

In awake, free moving animals, im. administration of low-dose dofetilide (slight I_{Kr} -blockade) prolonged QTc only in LQT5 (change in QTc, $ms \pm SEM$; $+7.7 \pm 1.9$, $p < 0.05$) but not in healthy WT, nor in LQT2 and LQT2-5 rabbits that both lack I_{Kr} . I_{K1} -blocker BaCl₂ prolonged QTc in all groups; this effect, however, was particularly pronounced in LQT2 rabbits (change in QTc, $ms \pm SEM$; LQT2 $+15.4 \pm 2.0$ vs. WT $+7.5 \pm 1.2$, LQT5 $+9.6 \pm 1.7$ and LQT2-5 $+9.6 \pm 2.2$, all $p < 0.05$). I_{Ks} -blockade alone (HMR-1556) did not have any significant effect on QTc in any genotype. Combined blockade of I_{K1} (BaCl₂) and I_{Ks} (HMR-1556) prolonged QTc in all groups.

In anaesthetised animals, similar changes in QTc were observed. I_{K1} -blocker BaCl₂ 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 + BaCl₂ effects were similar as in free moving animals. Dofetilide prolonged QTc in LQT5; surprisingly, however, it also prolonged QTc in LQT2-5 (change in QTc, $ms \pm SEM$; LQT5 $+4.3 \pm 0.8$, LQT2-5 $+10.9 \pm 2.0$, all $p < 0.05$). Pro-arrhythmia markers STV_{QT} and $T_{peak-T_{end}}$ were more pronouncedly affected by K⁺-channel-

blockers in LQTS rabbits with impaired repolarisation reserve: Dofetilide and HMR-1556 increased STV_{QT} and prolonged $T_{peak-T_{end}}$ only in LQT5 and LQT2-5 rabbits (dofetilide induced change in STV_{QT} , $\pm SEM$; LQT5 $+0.6 \pm 0.1$, LQT2-5 $+1.3 \pm 0.3$, all $p < 0.05$). $BaCl_2$ - and combined HMR+ $BaCl_2$ -induced increases in STV_{QT} and $T_{peak-T_{end}}$ were more pronounced in all LQTS animals than in WT ($BaCl_2$ induced change in STV_{QT} , $ms \pm SEM$; LQT5 $+0.7 \pm 0.1$, LQT2 $+1.0 \pm 0.2$, LQT2-5 $+1.1 \pm 0.2$ vs. WT $+0.3 \pm 0.1$, all $p < 0.05$). Important to note, series of VEBs and non-sustained VTs were observed in one LQT2 and one LQT2-5 rabbit during $BaCl_2$ exposure, demonstrating increased *in vivo* arrhythmia susceptibility to K^+ -channel-blockers.

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 repolarisation, 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. 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} (change in QT, $\% \pm SEM$; LQT5 $+16.5 \pm 1.2$, LQT2-5 $+13.4 \pm 2.4$ vs. WT $+8.0 \pm 1.4$, LQT2 $+5.1 \pm 2.0$, all $p < 0.05$). On the other hand, I_{Ks} -blocker HMR-1556-induced QT-prolongation was 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. These results suggest impaired I_{Ks} function in LQT5 and LQT2-5.

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

Following perfusion with very low concentration I_{Kr} -blocker dofetilide (1nM), a slight (well below the clinically relevant 10ms 'threshold') prolongation of mean APD_{75} were observed in all groups. 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 (change in mean APD_{75} , $ms \pm SEM$; LQT2 $+13.2 \pm 2.2$, LQT2-5 $+13.0 \pm 2.8$ vs. WT $+7.0 \pm 1.1$, LQT5 $+6.2 \pm 1.2$, all $p < 0.05$). 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 APD₇₅ significantly more in LQT2 and LQT2-5 than in WT or in LQT5 (BaCl₂ induced change in mean APD₇₅, ms±SEM; LQT2 +29.4±2.9, LQT2-5 +32.3±4.2 vs. WT +16.1±2.3, LQT5 +18.1±1.8, all p<0.05). 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. Importantly, mean AP triangulation (APD₉₀₋₃₀) was more pronounced following I_{K1}⁻ or combined I_{K1}/I_{Ks}-blockade in LQT2 and LQT2-5 than in WT (change in mean APD₇₅ by I_{K1}-blockade, ms±SEM; LQT2 +25.8±3.9, LQT2-5 +23.8±3.7 vs. WT +14.6±2.3, all p<0.05).

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 BaCl₂. At baseline (5.4 mM [K⁺]_o), 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 repolarisation 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). The effects were reversible, and the original VER was regained in all group after 10 min perfusion with normal (5.4 mM [K⁺]_o) KH solution (2nd baseline). I_{K1}-blocker BaCl₂ induced longer duration and higher incidence of arrhythmias in LQT2 and LQT2-5 rabbits than in WT. Combined BaCl₂ 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). 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: BaCl₂: LQT2 (VT: 100%) and LQT2-5 (VT: 86%, VF: 57%) vs. WT (VT: 0%, VF: 0%); BaCl₂ + 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).

DISCUSSION

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 repolarisation reserve capacity. Animal models such as the volume overload chronic atrioventricular block (CAVB) dog and the methoxamine (α 1-adrenergic receptor agonist) sensitised anaesthetised rabbit models provided proof-of-principle evidence that animals with disease related secondary reduction of cardiac repolarisation 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. In this work, we investigated the potential benefits of using animal models with pharmacologically (drug-induced LQTS rabbit) or genetically (transgenic LQTS rabbits) reduced repolarisation reserve in better prediction of drug-induced ventricular arrhythmias.

Drug-induced acquired LQTS rabbit model with pharmacologically reduced repolarisation reserve

It has been known for a long time that I_{Ks} plays a vital role in the repolarisation reserve and its dysfunction is associated with increased sensitivity to drug-induced proarrhythmia, especially in the setting of elevated sympathetic tone. Downregulation (or lack) of the I_{Ks} was found in human diseased conditions - such as in chronic heart failure and diabetes mellitus (or in LQT1) - that were 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 (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 – repolarisation 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 - repolarisation over prolongation at low heart rates.

Transgenic LQTS rabbit models with genetically reduced repolarisation reserve

Baseline characteristics of transgenic LQTS rabbit models

LQT5 rabbits showed no overall repolarisation prolongation (no increase in QT interval), however, demonstrated increased apico-basal heterogeneity of APD and AP-triangulation compared to WT indicating slight regional repolarisation disturbances at baseline. This is in good agreement with the first characterisation of the model by Major et al. 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 repolarisation prolongation already at baseline: prolonged QTc/APD, steeper QT/RR slope, increased temporal instability (STV_{QT}), transmural ($T_{peak-end}$) and apico-basal heterogeneity of repolarisation and more AP-triangulation – similarly as in 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.

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

Similar to LQTS patients, different LQTS rabbits mimicking human LQTS characteristics demonstrated increased sensitivity to (K^+ -channel-blocker) drugs with repolarisation-prolonging side-effects. In our present study, - in line with earlier findings -, LQT5 animals with mild impairment in I_{Ks} were more sensitive to I_{Kr} -blocker dofetilide than healthy, WT rabbits demonstrating more pronounced increase in QTc, $T_{peak-end}$ and STV_{QT} . LQT2 and LQT2-5 rabbits with lack of I_{Kr} and impaired I_{Ks} on the other hand, exhibited particularly

pronounced sensitivity to I_{K1} blocker $BaCl_2$ compared to WT, which is in good agreement with 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. Therefore, LQT2 and LQT2-5 rabbits could represent novel models to detect such (I_{K1} -blocking) side-effects of drugs, particularly as I_{K1} -blocking properties of drugs are not assessed despite their known proarrhythmic potential.

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 QT-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 repolarisation over prolongation and arrhythmias. Furthermore, LQT2-5 could mimic diseases – such as heart failure and diabetes - with high arrhythmogenic risk due to impaired I_{Ks} in the context of clinically manifest LQTS, and - in contrast to LQT1, in which I_{Ks} is completely missing - the pro/antiarrhythmic effects of pharmacological reduction or increase of I_{Ks} could be investigated.

Utility of LQTS rabbit models to detect drug-induced arrhythmias

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 repolarisation reserve and ii) since only this drug caused prolongation of repolarisation 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 repolarisation-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, demonstrating no arrhythmias in AV-ablated Langendorff-perfused WT hearts either at baseline or during hypokalaemia, and serious VT only when repolarisation-prolonging drugs were added. Similarly, increased arrhythmia susceptibility was demonstrated in various LQTS models *in vivo*: spontaneous VT and SCD in LQT2 rabbits, increased TdP development in drug-induced

(dofetilide+HMR-1556) acquired LQTS rabbit and dog models and increased dofetilide-induced TdP in LQT5 rabbits.

CONCLUSIONS AND NEW RESULTS

1. The acquired LQTS rabbit proarrhythmia model with pharmacologically (HMR-1556 to block I_{Ks}) reduced repolarisation 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.
2. Transgenic LQTS rabbit models reflect patients with clinically 'silent' - normal QT interval (LQT5) - or 'manifest' - prolonged QT interval (LQT2 and LQT2-5) - impairment in cardiac repolarisation reserve capacity due to different pathomechanisms (decreased I_{Ks} in LQT5 and LQT2-5 and lack of I_{Kr} in LQT2 and LQT2-5).
3. Phenotypically, the new LQT2-5 model closely resembles LQT2; however, it also shows 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 repolarisation reserve. In this regard, LQT2-5 may serve as an important model i) for diseases with high arrhythmic risk due to (remodelling-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.
4. LQTS animals are 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 could be utilized for more reliable prediction of the (multi-channel-based) pro-arrhythmic potential of novel drug candidates.
5. The author is aware, 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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