Final research report

Disease-specific effects of novel, selective NCX1 inhibitors

This project was an ANN type international Hungarian-Austrian co-operational grant planned to be jointly performed with the group of Dr. Gudrun Antoons from the Department of Cardiology, University of Graz Austria. In order to conform of the rules of the National Research, Development and Innovation Office (NKFI-OTKA), the co-operational partner should also conduct a mirror project in her/his research facility funded by is/her National Grant Agency. The mirror grant in principle should have been conducted by Dr. Gudrun Antoons, however she unexpectedly had to give up her position in Graz, and returned to The Netherlands. The project initially was planned for three years (01.11.2014-31.10.2017), but in August, 2017 we needed to opt for a one year extension. This extension applied on August, 2017 was necessary due to several participants also unexpected leaving the project. One of our senior researcher, the co-leader of the project Dr. Károly Acsai, who was also responsible for the majority of cellular research work (patch-clamping and Ca^{2+}-transient measurements) left the Department of Pharmacology of the University of Szeged and returned to the industry. Several more, younger investigators also left the project (and the Department9 for various job-related purposes. The well trained professional working hands were rather hard to replace, therefore we had to apply for the extension, granted in September, 2017, by the Board for Medical and Biological Sciences Panel.

Based on the research plan the project was divided into two major subtopics (ST). During the four years of the project the following selected main results were obtained:

Subtask 1. *What is the primary effect of selective NCX1 inhibition on the intracellular Ca^{2+} homeostasis and its regulatory/adaptive mechanisms? A.) How the level of inhibition is modulated by intra/extracellular ion concentrations? B.) Is the level of inhibition depending on transport direction? C.) What is the role of subsarcolemmal Ca^{2+} and Na^{+} microdomains in development of inhibition?*

The cardiac sodium/calcium exchanger (NCX1) is considered as *the* primary transmembrane transport mechanism that controls Ca^{2+}-homeostasis in the heart. Due to lack of specific inhibitor agents its contribution to cardiac repolarization has not yet been directly studied, so that an urgent need exists for highly selective compounds. Therefore, in a study detailed electrophysiological effects of two new NCX inhibitors – GYKB-6635 and ORM-10962 – on

the NCX1, L-type Ca^{2+} and critical repolarizing K^+ currents, as well as the inhibition-induced shifts in the parameters of the action potential (AP) were investigated.

- *Investigation of the effect of ORM -10962 on mammalian hearts*

In this work the modulatory effects of ORM-10962, a novel highly selective NCX inhibitor (Orion, Finnland) were studied on the cardiac NCX current and automaticity in experimentally induced arrhythmias. The selectivity of the drug on various transmembrane ionic currents (including the L-type Ca^{2+} current, the main repolarizing K^+ currents, late sodium current, Na^+/K^+ pump and pacemaker current) has also been investigated. Ion currents and action potential were recorded by either applying the whole-cell patch-clamp technique in canine single ventricular cells (CM) or using the standard (sharp) microelectrode technique in rabbit cardiac preparations, respectively. The inhibitory effects of ORM-10962 were studied in anesthetized guinea-pigs in ouabain (10 µg/kg/min i.v.) induced arrhythmias, and ischemia-reperfusion (IR) induced arrhythmias in anesthetized rats.

We found that ORM-10962 significantly reduced both the inward and outward NCX currents with estimated EC_{50} values of 55 nM and 67 nM, respectively. The compound, even at very high concentration of 1 µM did not change significantly the amplitude of I_{CaL} in CM, neither had apparent influence on inward rectifier, transient outward, rapid and slow delayed rectifier potassium currents, as well, as on the late sodium and Na^+/K^+ pump currents. The ORM-10962 slowed the automaticity in Purkinje fibres and sinus nodes in dogs and rabbits, without significantly altering the ivabradine sensitive pacemaker current. The magnitude of the pharmacologically (digoxin) induced delayed afterdepolarizations was also significantly decreased in canine Purkinje fibres by 1 µM ORM-10962. Furthermore, in anesthetised guinea pigs 0.3 mg/kg ORM-10962 pre-treatment (i.v. 10 min before ouabain infusion started) significantly delayed (by about 50%) the development of ventricular extrasystoles and (by about 30 %) the ventricular tachycardia. On the contrary, however, in anesthetised rats ORM-10962 pre-treatment did not result in an apparent change in either development, or severity of the IR induced arrhythmias.

The study provided evidence for a strong and highly selective NCX-inhibitory activity of ORM-10962. In addition it is suggested that specific inhibition of the NCX current may also influence normal pacemaker function (Ca^{2+}-clock hypothesis) and may substantially contribute to prevention of DAD induced arrhythmias, *in vivo*. Nonetheless, the nature of its effect on ischemia-reperfusion arrhythmias is still uncertain.

The results of the study were published in *PLoS One*: Kohajda *et al*, PLoS One, 11(11): e0166041. doi: 10.1371/journal.pone.0166041, 2016).

- *Investigation of the effect of GYKB-6635, a newly synthesised NCX blocker mammalian hearts*

In the next study, the electrophysiological effects of GYKB-6635, a novel NCX inhibitor, on the NCX, L-type calcium and main repolarizing potassium currents as well as action potential (AP) parameters were investigated. Ion currents and AP recordings were investigated in canine heart at 37°C by applying the whole-cell patch clamp and standard microelectrode techniques, respectively.

The effects of GYKB-6635 were also studied in ouabain induced arrhythmias in isolated guinea-pig hearts. 1 μM GYKB significantly reduced both the inward and outward NCX currents (57% and 58%, respectively). Even at a high concentration (10 μM), GYKB-6635 did not change the I_{CaL}, the maximum rate of depolarization (dV/dt_{max}), the main repolarizing K^+ currents and the main AP parameters. GYKB-6635 pre-treatment significantly delayed the time to development of ventricular fibrillations (by about 18 %).

It was concluded that GYKB-6635 is a potent and highly selective inhibitor of the cardiac NCX and in addition it may also contribute to prevention of DAD based arrhythmias.

The major results of this study were published in *Canadian J Physiol and Pharmacol* (Geramipour *et al*, Can J Physiol Pharmacol, 94: 1090-1101, 2016;

- *Inotropic effect of NCX inhibition depends on the relative activity of the reverse NCX assessed by a novel inhibitor ORM-10962 on canine ventricular myocytes.*

Na^+/Ca^{2+} exchanger (NCX1) is the main Ca^{2+} transporter in cardiomyocytes. Therefore, its inhibition could be expected to exert a significant positive inotropic action via accumulation of the cytosolic Ca^{2+} (i.e. increasing $[Ca^{2+}]_i$). However, upon selective inhibition of NCX we only could observe a marginal positive inotropic effect, which effect was enhanced following facilitation of the forward activity. In this study we attempted to clarify the major underlying mechanism of the limited inotropic action of selective NCX1 inhibition by a novel inhibitor ORM-10962 on canine ventricular myocytes.

1μM ORM-10962 reduced the Ca^{2+} content of sarcoplasmic reticulum (SR) when the reverse NCX was favoured, while SR Ca^{2+} content was increased by ORM-10962 under conditions favouring the forward activity (elevation of $[Ca^{2+}]_i$). In the absence of Ca^{2+} release

from the SR L-type Ca^{2+} current (I_{CaL}) was not affected by 1μM ORM-10962, while during normal Ca^{2+} cycling I_{CaL} was suppressed by ORM-10962. The apparent degree of forward NCX1 inhibition was strongly dependent on the elevation of $[Ca^{2+}]_i$, suggesting that an increased driving force for forward NCX may also limit the accumulation of $Ca^{2+}{}_i$. We concluded that in healthy myocardium the possible positive inotropic potential of NCX1 inhibition can be considerably weaker than expected earlier based on theoretical assumptions. The underlying mechanism may involve the autoregulation of Ca^{2+} handling and/or preserved inducibility of forward I_{NCX} by high $[Ca^{2+}]_i$. The limited efficacy of selective NCX1 inhibition found in undiseased myocardium requires further studies conducted in the failing heart, allowing a correct evaluation of the potential therapeutic value of selective NCX inhibitors in the treatment of heart failure.

The results of this study were published in *Eur J Pharmacol* (Oravecz *et al*, European Journal of Pharmacology, 818:278-286, 2018).

Subtask 2. *Is there any and if exists what is the efficacy of cardioprotection of selective NCX1 inhibition in acute or chronic heart disease models in which pathological NCX1 activity is playing a major role in either the development of cardiac arrhythmias or in critical elevation (i.e. Ca^{2+} overload – acute ischemia models), or decrease (several heart failure phenotypes) of intracellular Ca^{2+} levels..*

- *Effect of the intracellular calcium concentration chelator BAPTA acetoxy-methylester on action potential duration in canine ventricular myocytes.*

Intracellular calcium concentration ($[Ca^{2+}]_i$) is often buffered under experimental conditions when the cell-permeant acetoxy-methylester form of the Ca^{2+} chelator BAPTA (BAPTA-AM) is used. This study was designed to investigate the time-dependent actions of extracellularly applied BAPTA-AM on action potential duration (APD) in cardiac cells. APs were recorded from enzymatically isolated canine ventricular myocytes with conventional microelectrodes. The effect of BAPTA-AM on the rapid delayed rectifier K^+ current (I_{Kr}) was studied using conventional voltage clamp, as well, as action potential voltage clamp techniques.

We found that APD was lengthened by 5 μM BAPTA-AM - but not by BAPTA - and shortened by the Ca^{2+} ionophore A23187 in a time-dependent manner. The APD-lengthening effect of BAPTA-AM was strongly suppressed in the presence of nisoldipine, and enhanced in the presence of BAY K8644, suggesting that a shift in the $[Ca^{2+}]_i$-dependent inactivation of

L-type Ca^{2+} current may be an important underlying mechanism. However, in the presence of the I_{Kr}-blocker dofetilide or E-4031 APD was shortened rather than lengthened by BAPTA-AM. Similarly, the APD-lengthening effect of 100 nM dofetilide was halved by pretreatment with BAPTA-AM. In line with these results, I_{Kr} was significantly reduced by extracellularly applied BAPTA-AM under both conventional voltage clamp and action potential voltage clamp conditions. This inhibition of I_{Kr} was partially reversible and was not related to the Ca^{2+} chelator effect BAPTA-AM.

The possible mechanisms involved in the APD-modifying effects of BAPTA-AM are discussed. It is concluded that BAPTA-AM has to be applied carefully to control $[Ca^{2+}]_i$ in whole cell systems because of its direct inhibitory action on I_{Kr}.

The results of this study were published in *Journal of Physiology and Pharmacology* (Horváth *et al*, J Physiol Pharmacol. 2018 Feb;69(1):99-107, 2018).

- *The investigation and characterisation of the Purkinje fibre action potential from the undiseased human heart.*

Data obtained in canine cardiac electrophysiology studies are often extrapolated to the human heart. However, as we have demonstrated in several previous studies, human ventricular action potential, due to the lower density of its K^+ currents, has less powerful repolarization reserve. Since the relevance of canine data to the human heart has not yet been fully clarified, the aim of the present study was to determine for the first time the parameter of the action potentials of undiseased human Purkinje fibres (PFs) and to compare them directly with those of dog PFs. Current, action potential and simulation data all suggest that largely differing protein expression profiles of the two species may mostly underlie these important disparities. Therefore, caution is advised when extrapolating canine PF data to human, and further estudies are required to investigate the characteristics of human PF repolarization and its possible role in ventricular arrhythmogenesis.

The results of this investigation were published in *Canadian Journal of Physiology and Pharmacology* (Nagy *et al*, Can J Physiol Pharmacol, 93, 803-810 2015).

<u>Other important data achieved:</u>

- *Pharmacological inhibition of the sodium/calcium-exchanger attenuates the hypokalemia-induced elevated cellular Calcium load and decreases the risk of arrhythmias*

Hypokalaemia (HK) markedly increases the risk of development of cardiac arrhythmias. HK decreases the activity of the Na-K-ATP-ase, leading to elevated intracellular $[Na^+]$. This increase may lead to

elevated intracellular [Ca^{2+}] via activation of the reverse mode Na/Ca-exchanger (NCX1), which may contribute to an increased risk of cardiac arrhythmias. In this study we investigated whether the selective NCX blocker ORM-10962 could prevent the HK-induced electrophysiological changes and the associated increase of arrhythmia risk. Left ventricular pressure (LVP) and ECG were recorded in isolated guinea pig and rat hearts; action potential duration (APD) and cell shortening were determined in isolated rat papillary muscles and ventricular cardiomyocytes, respectively. HK was induced by using solutions with low [K^+] (2 mM). Since in this model HK alone was not able to induce cardiac arrhythmias, in the arrhythmia and APD experiments the Ca^{2+} level was also increased to 3 mM. NCX inhibition was achieved by 1µM ORM-10962. In isolated guinea pig hearts HK solution markedly increased LVP, indicating net cellular Ca^{2+} gain. In a separate group administration of ORM-10962 completely prevented the HK-induced LVP increase. In line with this observation ORM-10962 effectively reduced the HK-induced increase of cell shortening in isolated rat ventricular myocytes. Perfusion of isolated rat hearts with HK solution markedly elevated the total number of arrhythmias compared to the normal solution. The presence of ORM-10962 in the HK solution significantly decreased the incidence of ventricular arrhythmias. In rat papillary muscles HK solution lengthened the APD. This lengthening was significantly reduced by application of ORM-10962. We concluded that increased arrhythmia propensity induced by HK may be attenuated following NCX inhibition. Since both increased Ca^{2+} load and altered AP morphology may contribute to arrhythmia generation, further studies are needed to clarify the detailed mechanism of antiarrhythmic protection provided by NCX inhibition in HK.

This data were presented at the *Annual Congresses of Hungarian Society of Cardiology*, Balatonfüred in 2017 and 2018, and was published as meeting abstracts. (Prorok et al, *Cardiol Hung*, 47 : Suppl.C pp. C42-C43, 2017; Gazdag et al, Hungarica A 48 : Suppl.C p. C41, 2018).

- *Low resting membrane potential and low inward rectifier potassium currents are not inherent features of hiPSC-derived cardiomyocytes*

Human induced pluripotent stem cell (hiPSC) cardiomyocytes (CMs) show less negative resting membrane potential (RMP), which is attributed to small inward rectifier currents (I_{K1}). Here, I_{K1} was measured in hiPSC-CMs (proprietary and commercial cell line) cultured as monolayer (ML) or 3D engineered heart tissue (EHT) and, for direct comparison, in CMs from human right atrial (RA) and left ventricular (LV) tissue. RMP was measured in isolated cells and intact tissues. I_{K1} density in ML- and EHT-CMs from the proprietary line was similar to LV and RA, respectively. I_{K1} density in EHT-CMs from the commercial line was 2-fold smaller than in the proprietary line. RMP in EHT of both lines was similar to RA and LV. Repolarization fraction and $I_{K,ACh}$ response discriminated best between RA and LV and

indicated predominantly ventricular phenotype in hiPSC-CMs/EHT. The data indicate that I_{K1} is not necessarily low in hiPSC-CMs; technical issues may underlie low RMP in hiPSC-CMs.

This investigation was published in Stem Cell Reports (Horváth *et al, Stem Cell Reports*. 2018 Mar 13;10(3):822-833.).

Science educating, management and publishing activities

The results obtained by implementing the present project have been published in 6 full lengths research papers having the cumulative impact factor of 18.83. In addition we have published two abstracts, 3 other conference related papers and one book chapters.

A senior researcher of the project (Dr. N Jost) was invited by Springer Publishing to contribute with a chapter for a Cardiac Electrophysiology book entitled: Pathophysiology and Pharmacotherapy of Cardiovascular Disease. Eds. Jagadeesh G, Balakumar P, Maungh-U K, 2015, ISBN: 978-3-319-15960-7.

Some of the researchers involved in the project (János Prorok, Norbert Nagy) regularly gave presentations about their current work in the Institute Journal Club.

- The investigations presented in this report served as a basis for the following MSc, PhD and DSc theses:

Péter Gazdag. The selective NCX inhibition decreased the hypokalaemia induced cardiac arrhythmias in isolated hearts. Supervisor: Dr. János Prorok. *MSc thesis* defended in June, 2017.

Kinga Oravecz: The inotropic consequences of selective Na^+/Ca^{2+} exchanger inhibition is controlled by the actual transport balance. Supervisor: Dr. Norbert Nagy; *PhD thesis* defended at 17.05.2018.

András Tóth. Intracelluláris Ca^{2+}-homeosztázis változások analízise izolált szívpreparátumokon. *DSc thesis*, defended at December, 2016.

Szeged, 14 December, 2018.

András Tóth, *PhD, DSc*
principal investigator