# Investigation of the endogenous contributing factors of proarrhythmia and myocardial contractility

**PhD thesis**

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**Hungary**

**2008**

## THE THESIS IS BASED ON THE FOLLOWING PAPERS

**I. AS Farkas**, K Acsai, N Nagy, A Tóth, L Dézsi, S Orosz, T Forster, M Csanády, JG Papp, A Varró, A Farkas. Importance of extracardiac $\alpha_1$-adrenoceptor stimulation in assisting dofetilide to induce torsade de pointes in rabbit hearts. *European Journal of Pharmacology,* 537 (2006) 118-125.
Impact factor: 2.376

**II. AS Farkas**, K Acsai, N Nagy, A Tóth, F Fülöp, G Seprényi, P Birinyi, PP Nánási, T Forster, M Csanády, JG Papp, A Varró, A Farkas. $Na^+/Ca^{2+}$ exchanger inhibition exerts a positive inotropic effect in the rat heart, but fails to influence the contractility of the rabbit heart. *British Journal of Pharmacology,* 154 (2008) 93-104.
Impact factor: 3.767

**III. Farkas AS**, Balázs M, Acsai K, Tóth A, Pálinkás A, Csanády M, Forster T, Papp Gy, Varró A, Farkas A. A $Na^+/Ca^{2+}$ exchanger gátlás eltérő hatása az izolált patkány és nyúl szív kontraktilitására. *Cardiologica Hungarica,* 36 (2006) 92-96.

**IV. AS Farkas**, P Makra, N Csík, S Orosz, MJ Shattock, F Fülöp, T Forster, M Csanády, JG. Papp, A Varró, A Farkas. The role of The $Na^+/Ca^{2+}$ exchanger, $I_{Na}$ and $I_{CaL}$ in the genesis of dofetilide-induced torsades de pointes in isolated, AV-blocked rabbit hearts. *British Journal of Pharmacology,* in press.
Impact factor: 3.767

**V. AS Farkas**, L Rudas, P Makra, N Csík, I Leprán, T Forster, M Csanády, JG. Papp, A Varró, A Farkas. Endogenous factors and predictive parameters of dofetilide-induced torsades de pointes in anaesthetized, $\alpha_1$-adrenoceptor-stimulated rabbits. *Submitted for publication.*

## INTRODUCTION

The long QT syndrome (LQTS) is caused either by inherited ‘channelopathies’ or by acquired factors, *e.g.* cardiac or non-cardiac drugs that hinder the process of repolarization of the myocytes. The most dangerous consequence of the disturbed repolarization is the occurrence of a polymorphic ventricular tachycardia known as torsades de pointes (TdP), which can deteriorate into ventricular fibrillation. Thus, the pro-arrhythmic liability of any drug under development must be assessed so as to avoid the occurrence of drug-induced life-threatening arrhythmias, for example TdP, during pharmacotherapy.

A number of hypotheses have been proposed as concerns the generation of TdP, but the exact mechanism of this arrhythmia is still not clear. In the most widely accepted theory, the mechanism of TdP initiation involves the early afterdepolarization- (EAD) induced ectopic beat, while the mechanism of the arrhythmia maintenance involves the reentry.

The most commonly used animal model for the in vivo screening of drug-induced pro-arrhythmia is the $\alpha_1$-adrenoceptor-stimulated anaesthetized rabbit model of the acquired long QT syndrome. In the model, the ability of a test agent to evoke TdP is evaluated during co-administration of a ‘priming’ substance, the selective $\alpha_1$-adrenoceptor agonist methoxamine or phenylephrine. The role of the $\alpha_1$-adrenoceptor-stimulation, which makes the rabbits susceptible for the development of TdP in the presence of a repolarization prolonging drug, is not completely understood. It may be hypothesized that reflex bradycardia and/or an increased ventricular stretch, which develop as a consequence of $\alpha_1$-adrenoceptor-mediated peripheral vasoconstriction and increased resistance, are the critical sensitizing factors. However, this hypothesis has not been proven yet. Despite the frequent use, the $\alpha_1$-adrenoceptor-sensitized anaesthetized rabbit model of TdP has not been characterized sufficiently to date, and the mechanism of TdP genesis is not known. Furthermore, there is a great demand to find parameters having great predictive value for the occurrence of drug-induced arrhythmias.

The cardiac $Na^+/Ca^{2+}$ exchanger (NCX) is considered to play an important role in $Ca^{2+}$ handling of cardiac myocytes. In forward mode, the NCX extrudes $Ca^{2+}$ from the cell and brings $Na^+$ into the cytosol with a ratio of 1:3, respectively; in reverse mode it operates in the opposite direction. This exchange of 3 positive charges for 2 positive charges makes the exchanger electrogenic. The NCX may be a potential contributor of EADs, delayed

afterdepolarizations (DADs) and dispersion of ventricular repolarization, thus NCX may have a role in the development of TdP. The operation of NCX may also influence the heart muscle contractility. The intracellular $Ca^{2+}$ level depends on the amount of $Ca^{2+}$ entering the myocyte and the amount of $Ca^{2+}$ removed from the myocyte. The major pathway for $Ca^{2+}$ extrusion is the forward mode of NCX. Thus, inhibition of the NCX may influence the cardiac muscle contractility.

***Aims of the study***

The main aim of our investigations was to identify the endogenous contributing factors of drug-induced TdP. The NCX, which was thought to be one of the key factors of the genesis of TdP, was examined in order to determine its contribution to i) TdP development and ii) cardiac contractility.

The aim of '*in vitro* $\alpha_1$-adrenoceptor stimulation and stretch study' was to investigate whether triggered arrhythmias induced by a constant high level of left ventricular stretch are able to initiate TdP in the presence of functional reentries caused by dofetilide, which is a blocker of the rapid component of the delayed rectifier potassium current ($I_{Kr}$), in the presence or the absence of $\alpha_1$-adrenoceptor stimulation in isolated Langendorff-perfused rabbit hearts.

The aims of the '*in vivo* dofetilide study' were to identify endogenous factors of drug-induced TdP and to examine the mechanism of TdP genesis in anaesthetized, $\alpha_1$-adrenoceptor-stimulated rabbits. Our study investigated whether the occurrence of dofetilide-induced TdP is predicted by the basic haemodynamics (*i.e.* the blood pressure and the heart rate), the blood gases, the frequency of the preceding arrhythmias, the repolarization-related parameters or the activity of the autonomic nervous system in pentobarbital anaesthetized, $\alpha_1$-adrenoceptor-stimulated rabbits.

The potential role of the NCX in the genesis of drug-induced TdP has been examined in the 'NCX arrhythmia study'. Accordingly, the effect of the inhibition of the NCX by a selective NCX inhibitor (SEA0400) was investigated on the occurrence of dofetilide-induced TdP in isolated, Langendorff-perfused, atrioventricular nodal (AV)-ablated rabbit hearts. Our results show that NCX inhibition with SEA0400 did not reduce the incidence of dofetilide-induced TdP. Since this result questions either the role of NCX in TdP or the validity of this model, we conducted a second series of experiments aimed at

validating the model with drugs known to alleviate TdP in other experimental models and in man. Thus, the anti-arrhythmic effect of the inhibition of the L-type $Ca^{2+}$ current ($I_{CaL}$) by verapamil and the inhibition of the $Na^{+}$ current ($I_{Na}$) by lidocaine was tested against dofetilide-induced TdP in isolated, Langendorff-perfused, AV-ablated rabbit hearts.

Our objective was in the 'NCX contractility study' to examine the effects of selective NCX inhibition by SEA0400 on the cardiac muscle contractility in isolated Langendorff-perfused hearts in the setting of the long action potential of the rabbit and in the short action potential of the rat. To clarify the inhibitory effect of SEA0400 on the reverse and the forward modes of the NCX in the rabbit and in the rat isolated myocardial cell, $I_{Na/Ca}$ was examined by patch clamp technique. NCX protein densities of the two species were compared to clarify whether interspecies differences in the contractile function can be explained by differences in NCX expression.

## METHODS

### *Animals*

Female New Zealand White rabbits were used for the *in vivo* TdP experiments. *In vitro* Langendorff TdP experiments were performed on hearts excised from female New Zealand White rabbits to assess the role of stretch and the inhibition of NCX on the genesis of TdP. The in vitro cardiac muscle contractility experiments were carried out on male New Zealand White rabbits and male Sprague-Dawley rats.

### *Langendorff perfusion of the isolated hearts*

The hearts were retrogradely perfused at a constant temperature of 37°C with the modified Krebs-Henseleit buffer solution containing (in mM) NaCl 118.5, $CaCl_2$ 2.0, glucose 11.1, $MgSO_4$ 0.5, $NaH_2PO_4$ 1.2, $NaHCO_3$ 25 and KCl 3. In the NCX contractility study, Krebs-Henseleit buffer solution contained the same salts but the concentrations of $CaCl_2$, $MgSO_4$, and KCl were 2.0 mM, 1.0 mM and 4 mM, respectively.

### *Assessment of the role of $\alpha_1$-adrenoceptor stimulation and the constant left ventricular stretch in the development of torsades de pointes in isolated rabbit hearts*

In the first set of experiments, to find the appropriate dofetilide concentration in terms of the proarrhythmic potential, three groups of hearts [dofetilide 50 nM (n=8),

dofetilide 100 nM (n=9), and control (solvent of dofetilide, n=10)] were compared. TdP occurred only in the 100 nM dofetilide group and it did so with an incidence which offered scope for the examination of additional effects that can further increase the incidence of this arrhythmia. This concentration was therefore chosen and used in the remainder of the study.

In the second set of experiments, three groups of hearts (n=8 hearts in each group) were perfused with 100 nM dofetilide for 40 min after 15 min of initial perfusion with modified Krebs-Henseleit solution. In two of the three dofetilide-perfused groups of hearts, methoxamine at a concentration of 100 nM was added to the dofetilide-containing perfusion solution. In each heart, a non-elastic balloon was inserted into the left ventricle via the mitral valve and was connected to a pressure transducer. The intraventricular balloon was filled with water at a constant volume of 1.4 ml throughout the whole experiment in the group of hearts perfused only with dofetilide and the vehicle of methoxamine ('dofetilide+stretch' group) and in one of the groups of hearts perfused with dofetilide and methoxamine ('dofetilide+methoxamine+stretch' group).

Individual measurements of coronary flow, left ventricle pressure and ECG variables were made every 5 min and 1 min before and 1 min after the introduction of drug perfusion. At the end of each experiment, the atria were removed from the heart and the ventricles were weighed.

***The assessment of predictive parameters of dofetilide-induced torsades de pointes in $\alpha_1$-adrenoceptor stimulated pentobarbital anaesthetized rabbits***

30 rabbits were used for the experiments. The animals were anaesthetized intravenously with pentobarbital via the marginal vein of the left ear. The rabbits were retrospectively divided into 2 groups according to the presence or the absence of TdP, i.e. the animals that experienced TdP formed the 'TdP+' group, and the animals that did not experience this arrhythmia formed the 'TdP-' group.

After a 10-min baseline period, $\alpha_1$-adrenoceptor agonist phenylephrine infusion was started at increasing rates (*i.e.* 3, 6, 9, 12, and 15 μg/kg/min for 3, 3, 3, 3, and 5 min, respectively). From the $27^{th}$ min, dofetilide (0.02 mg/kg/min iv., for 30 min) was administered simultaneously with the background phenylephrine infusion (at a rate of 15 μg/kg/min) until the end of the experiments.

All the values for the QT interval were corrected for the heart rate, $T_{peak}$-$T_{end}$ interval and T-wave lability index were measured and determined.

The beat-to-beat variability of the QT intervals was determined from the manual measurement data on 40 consecutive QT intervals and the corresponding RR intervals in sinus rhythm ('sinus variability') and irrespectively from the presence or absence of sinus rhythm at the time point of the measurement ('real beat-to-beat variability'). Baroreflex and spectral analysis were performed with the arterial blood pressure and the ECG signal of the animals in predetermined time points.

***Examination of the role of the $Na^+/Ca^{2+}$ exchanger, $I_{Na}$ and $I_{CaL}$ in the genesis of dofetilide-induced torsades de pointes in isolated, AV-blocked rabbit hearts***

The AV node was ablated using forceps two minutes after mounting the hearts. After AV ablation the hearts were allowed to beat in their own spontaneous rhythm.

In the first set of experiments, four groups (n=8 hearts in each group) [dofetilide 100 nM (DOF1), SEA0400 1.0 μM + dofetilide 100 nM (SEA+DOF), and control groups: distilled water ($H_2O$) and dimethyl sulfoxide, solvent of dofetilide and SEA0400, (DMSO)] were compared. $H_2O$ control group was involved as DMSO may affect the repolarization.

Since SEA0400 administration did not decrease the incidence of dofetilide-induced TdP, a second set of experiments was designed to examine whether it is possible to reduce dofetilide-induced TdP with other drugs in the applied model. Lidocaine and verapamil were chosen as test drugs as they could successfully reduce the incidence of drug-induced TdP in other experimental models. The second set of experiments comprised three groups of hearts: i) 100 nM dofetilide (DOF2), ii) 30 μM lidocaine+100 nM dofetilide (LID+DOF), iii) 750 nM verapamil+100 nM dofetilide (VER+DOF).

The beat-to-beat variability of the QT and RR intervals were measured as described above.

***Analysis of the effect of $Na^+/Ca^{2+}$ exchanger inhibition on the cardiac muscle contractility in isolated rat and rabbit hearts***

The contractile function of the left ventricle in isolated rabbit and rat hearts was measured by using a non-elastic balloon filled with water, connected to a pressure transducer. The balloon was inserted into the left ventricle via an incision in the left atrium and the mitral valve. The ventricular systolic, end-diastolic pressures and the developed pressure (i.e, the systolic pressure minus the diastolic pressure) were recorded.

A control group (which received the solvent of SEA0400) and the three treated groups (which received the different concentrations of SEA0400: 0.1, 0.3, and 1.0 μM) were compared as concerns both the rabbit heart and the rat heart. Each of the groups in the rabbit study contained 10 hearts, and each of the four groups in the rat study contained 12 hearts.
Each heart was set up under Krebs perfusion and the balloon was inserted into the left ventricle. Then, 0.1-ml increments in the rabbit heart and 0.02-ml increments in the rat heart were added to the balloon volume in every minute to reach the maximum developed pressure (a Starling curve was constructed), the final diastolic pressure not being allowed to exceed 10 mm Hg. Then whole procedure was repeated in the presence of the test drug.

To examine the effects of SEA0400 on the NCX current, the whole-cell configuration of the patch clamp technique was applied. $I_{Na/Ca}$ was recorded by using ramp pulses.

Confocal laser scanning microscopy was applied for the quantification of the NCX on the sarcolemmal surface of cardiac myocyte. The fluorescent images of 25 randomly selected rat and 25 rabbit cardiac myocytes, in each case derived from 4 animals, were subjected to immunofluorescent profile analysis.

## RESULTS AND DISCUSSION

### *In vitro $\alpha_1$-adrenoceptor stimulation and stretch study*

In the first set of experiments, spontaneous TdP occurred only in the 100 nM dofetilide group and it did so with a relatively low incidence, which offered scope for the examination of additional effects that can further increase the incidence of this arrhythmia.

In the second set of experiments, neither the sustained load-induced left ventricular stretch nor methoxamine nor the in combination increased the incidence of dofetilide-provoked TdP.

Our '*in vitro* $\alpha_1$-adrenoceptor stimulation and stretch study' indicated that neither a sustained load-induced constant high level of left ventricular stretch nor $\alpha_1$-adrenoceptor stimulation nor their co-application, promote the generation of TdP in the setting of prolonged repolarization in isolated, Langendorff-perfused rabbit hearts. The roles of intracardiac $\alpha_1$-adrenoceptor stimulation and a sustained load-induced left ventricular stretch in the provocation of TdP in the rabbit are therefore questionable. In contrast, our results

suggest the importance of extracardiac $\alpha_1$-adrenoceptor stimulation in TdP development in the *in vivo* rabbit heart.

***Predictive parameters in the 'in vivo dofetilide study'***

TdP and ventricular fibrillation did not occur before the dofetilide infusion. The incidence of all kind of arrhythmias (ventricular premature beat, bigeminy, salvo, ventricular tachycardia) was higher, but not significantly, in the 'TdP+' group before dofetilide administration. There was no statistical difference in the onset times of these arrhythmias.

There was no significant difference in the blood pressure values between the two groups at any time point of the measurement.

Phenylephrine reduced the heart rates and prolonged the QT intervals in both groups before the dofetilide infusion, but there were no differences in the heart rates and the QT intervals between the groups immediately before the start of the dofetilide infusions. Dofetilide infusion prolonged the QT and the rate-corrected QT intervals (QTc) in both groups, but there was no qualitative difference in the effects of the drugs on the QT and the QTc intervals at any time point in either group of animals.

There was no significant difference in the $T_{peak}$-$T_{end}$ intervals between the two groups at any of the three time points of the measurement. Phenylephrine slightly increased the TWLI in the 'TdP+' group, but the change remained insignificant.

Phenylephrine infusion increased strikingly all 'real' beat-to-beat RR and QT variability parameters as compared with baseline, which indicates that the drug induced large number of arrhythmias and electrical instability in the heart. Dofetilide administration quickly exaggerated the phenylephrine-induced increase in the 'real' beat-to-beat variability of the RR and QT intervals in both the 'TdP+' and 'TdP-' groups, but the dofetilide-induced increase in these parameters reached statistical significance only in the 'TdP+' group. All kinds of 'real' RR and QT variability parameters increased even further immediately before the occurrence of TdP in the 'TdP+' group.

Significantly higher up-BRS was found in the 'TdP+' group than in the 'TdP-' group when only a single between-group analysis was performed using the pooled baroreflex sensitivity data during the phenylephrine infusion. The mid-frequency spectral power of the systolic arterial pressure, which is a marker of the sympathetic activity, did not differ between the 'TdP+' and 'TdP-' groups at baseline and during phenylephrine infusion.

There were no statistical differences in the serum levels of $K^+$, $Na^+$ or $Ca^{2+}$, or the serum $pH^+$ or blood gas values between the two groups at baseline, during the phenylephrine infusion and at the beginning of the dofetilide infusion.

This 'in vivo dofetilide study' reported that dofetilide-induced TdP was not determined by the baseline haemodynamics, repolarization properties, autonomic status, blood gases or serum ion concentrations. However, those animals that subsequently experienced TdP had more responsive baroreflex during phenylephrine infusion before dofetilide administration, which implies that vagal nerve activity might contribute to TdP genesis. When measured in sinus rhythm, none of the repolarization-related parameters forecasted dofetilide-induced TdP at any stage of the experiment. However, when arrhythmic activity was involved in the measurement of the beat-to-beat variability of the RR and QT interval, the 'real' variability of the RR and QT intervals predicted subsequent TdP occurrence. The progressive increase in the 'real' variability of the RR and QT interval implies that irregularity of the 'preceding' non-complex ventricular arrhythmias and the electrical instability rose steeply during dofetilide administration, which may be a prerequisite for the development of complex, re-entrant arrhythmias e.g. TdP in the model.

***Examination of the role of the $Na^+/Ca^{2+}$ exchanger, $I_{Na}$ and $I_{CaL}$ in the genesis of dofetilide-induced torsades de pointes in isolated, AV-blocked rabbit hearts***

The applied mechanical AV block decreased the average ventricular heart rate. The AV ablation also led to a chaotic and irregular spontaneous ventricular rhythm, and it strikingly increased all parameters of the beat-to-beat variability of the RR intervals. In addition, the AV ablation increased all QT variability parameters, which reflected a tentative beat-to-beat repolarization.

In the first set of experiments, TdP occurred in the majority of the dofetilide-perfused hearts. NCX inhibition with SEA0400 did not reduce the incidence of dofetilide-induced TdP and did not affect the onset time of this arrhythmia. In the second set of experiments, dofetilide provoked TdP in the majority of the hearts as seen in the first set of experiments. Lidocaine significantly decreased the incidence of dofetilide-induced TdP, while verapamil completely prevented the development of this arrhythmia.

During 'treatment period', in the 1$^{st}$ set of experiments, dofetilide perfusion significantly increased the mean QTc intervals and the beat-to-beat variability of the QT interval without affecting the mean RR interval and the beat-to-beat variability of the RR

interval. NCX inhibition with SEA0400 exaggerated the dofetilide-induced increase in the mean QTc interval and further increased some of the QT variability parameters. Further, SEA0400 upon dofetilide perfusion increased the beat-to beat variability of the RR interval similarly to QT variability parameters without affecting the mean RR interval.

Verapamil on top of dofetilide perfusion significantly increased the mean RR interval i.e. decreased the ventricular heart rate as compared with that of the DOF2 group.

The 'NCX arrhythmia study' revealed that NCX neither contributed to the initiation nor assisted the maintenance of dofetilide-induced TdP in the applied in vitro experimental model, in which inhibition of the $I_{Na}$ and $I_{CaL}$ currents successfully antagonized the genesis of this arrhythmia. Neither the prolongation of the QTc interval nor the elevation of the beat-to-beat variability of the QT interval correlated to the occurrence of dofetilide-induced TdP in this model. AV ablation resulted in a chaotic idioventricular rhythm, which might make the hearts more sensitive to the proarrhythmic activity of dofetilide in the applied isolated rabbit heart model.

***Analysis of the effect of $Na^+/Ca^{2+}$ exchanger inhibition on the cardiac muscle contractility in isolated rat and rabbit hearts***

SEA0400 suppressed both the inward and the outward $I_{Na/Ca}$. $IC_{50}$ values of the inward (forward) and the outward (reverse) currents were 243 nM and 309 nM in rabbit, and 120 nM and 61 nM in rat myocytes, respectively. No significant difference was seen in the SEA0400-induced suppression of the inward and the outward $I_{Na/Ca}$.

In rabbit hearts, the applied SEA0400 concentrations (0.1, 0.3 and 1.0 μM) failed to alter the systolic, diastolic and developed pressures as compared with the control group.

In rat hearts, the applied SEA0400 concentrations increased the systolic pressure in a concentration-dependent manner both at a constant balloon volume and during the second Starling curve. The developed pressure also increased in a concentration-dependent manner during perfusion with SEA0400 since the systolic pressure increased whereas the diastolic pressure remained unchanged.

Immunohistochemistry with confocal microscopy imaging revealed similar scattered distributions of the NCX protein on the surface of the rabbit and the rat myocytes. There was no difference in the NCX protein density between the rabbit and the rat.

This 'NCX contractility study' has provided evidence that the selective inhibition of the NCX with SEA0400 increases the contractility in a concentration-dependent manner in

the isolated rat heart with a short action potential duration and a higher intracellular $Na^+$ concentration, but it does not exert an appreciable influence on the contractile force in the isolated rabbit heart with a longer action potential duration and a lower intracellular $Na^+$ concentration. These data reveal important functional interspecies differences in contractility as a result of NCX inhibition. Since rabbit action potential resembles more the human action potential than that of rat, the NCX inhibition probably does not influence the contractile force of human heart under physiological circumstances. However, the effect of NCX inhibition on cardiac contractility under pathological conditions, e.g. heart failure, needs further investigations.

## ACKNOWLEDGEMENTS

I would like to thank Professor András Varró, Head of the Department of Pharmacology and Pharmacotherapy, Faculty of Medicine, University of Szeged, for providing me with all the facilities needed to carry out my experiments, and especially for his invaluable support I have enjoyed during the last few years.

I would like to acknowledge professor Julius Gy. Papp (Division of Cardiovascular Pharmacology, Hungarian Academy of Sciences and University of Szeged) for his support and help during my PhD studies.

I would also like to thank Dr. András Farkas, my tutor and supervisor, who directed my research work for his invaluable help during my PhD studies. His teaching and encouragement were also essential to me through the first steps of my scientific career.

I wish to acknowledge professors Miklós Csanády and Tamás Forster ($2^{nd}$ Dept. of Internal Medicine and Cardiology Centre, Faculty of Medicine, University of Szeged) for their support and useful comments on my research work.

I am also much grateful to the whole Staff of the Department of Pharmacology and Pharmacotherapy in Szeged for their continuous help.

I would also like to express my gratitude to my parents for all their support and care.