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Yu, Z.

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Author: Yu, Zhiyi

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Chapter 4

Allosteric modulation of K_v11.1 (hERG) channels protects against drug-induced ventricular arrhythmias

*Zhiyi Yu**

*Jia Liu**

Jacobus P. D. van Veldhoven

Adriaan P. IJzerman

Martin J. SchaliJ

Daniël A. Pijnappels

Laura H. Heitman

Antoine A. F. de Vries

**These authors contributed equally to this chapter.*

Abstract

Drug-induced ventricular arrhythmias (DiVAs) due to unintentional blockade of the $K_v11.1$ (hERG) channel are a major safety concern in drug development. In the past years, several highly prescribed drugs have been withdrawn for their ability to cause life-threatening arrhythmias. Here, we investigated whether the proarrhythmic risk of existing drugs could be reduced by $K_v11.1$ allosteric modulators. Using [3H]dofetilide binding assays with membranes of human $K_v11.1$ -expressing HEK293 cells, two existing compounds (VU0405601 and ML-T531) and a newly synthesized compound (LUF7244) were found to be negative allosteric modulators of dofetilide binding to the $K_v11.1$ channel with LUF7244 showing the strongest effect at 10 μM . The $K_v11.1$ affinity of typical blockers (i.e. dofetilide, astemizole, sertindole and cisapride) were significantly decreased by LUF7244. Treatment of neonatal rat ventricular myocyte (NRVM) monolayers with astemizole or sertindole caused heterogeneous prolongation of action potential (AP) duration and a high incidence of early afterdepolarizations upon 1-Hz electrical point stimulation, occasionally leading to unstable, self-terminating reentrant tachyarrhythmias. Pretreatment of NRVMs with LUF7244 prevented these proarrhythmic effects. NRVM monolayers treated with LUF7244 alone displayed electrophysiological properties indistinguishable from those of untreated NRVM cultures. Prolonged exposure of NRVMs to LUF7244 or LUF7244 plus astemizole did not affect their viability, excitability and contractility as assessed by molecular, immunological and electrophysiological assays. Conclusively, allosteric modulation of the $K_v11.1$ channel efficiently suppresses DiVAs *in vitro* by preventing potentially arrhythmogenic changes in AP characteristics, raising the possibility to resume the clinical use of unintended $K_v11.1$ blockers via pharmacological combination therapy.

Introduction

Drug-induced ventricular arrhythmias (DiVAs) are a frequently encountered clinical problem, which has resulted in the restricted use or market withdrawal of existing cardiac and non-cardiac drugs and still represents a major obstacle for the development of new drugs.¹ Inhibition of the rapid component of the delayed rectifier K^+ current (I_{Kr}) has been identified as the major culprit in the development of DiVAs. The consequential slowing of cardiac repolarization, manifested in the surface electrocardiogram as a prolongation of the QT interval, increases the likelihood of early afterdepolarizations (EADs), which may give rise to ectopic beats. Drug-induced I_{Kr} blockade also increases spatial dispersion of repolarization and refractoriness due to differences in relative I_{Kr} and I_{Ks} (the slow component of the delayed rectifier K^+ current) densities between cardiomyocytes in different regions of the ventricular wall.² Together these electrophysiological alterations promote the development of a special type of polymorphic ventricular arrhythmias known as “Torsades de Pointes” (TdPs), which mostly resolve spontaneously but occasionally degenerate into fatal ventricular fibrillation.³

The molecular correlate of I_{Kr} is the $K_v11.1$ protein encoded by the *KCNH2* gene (also known as ether-à-go-go-related gene 1 [ERG or ERG1]), possibly in combination with the MiRP1 or MinK protein, which are encoded by the *KCNE1* and *KCNE2* gene, respectively.⁴ $K_v11.1$ represents the pore-forming α subunit of the I_{Kr} channel, while MiRP1 and MinK are thought to be accessory β subunits involved in the modulation of its activity. Several structural features of $K_v11.1$ render the I_{Kr} channel particularly susceptible to blockade by a heterogeneous collection of chemical compounds including various non-cardiac drugs (e.g., astemizole, sertindole and cisapride).

An obvious strategy to reduce the proarrhythmic risk of drugs with unintended I_{Kr} blockade is by lowering their $K_v11.1$ affinity via chemical modifications. Alternatively, supplementary drugs that lessen the proarrhythmic risk of inadvertent $K_v11.1$ blockers can be developed potentially allowing the (i) reintroduction of medicines previously recalled from the market because of their $K_v11.1$ -related cardiotoxicity and (ii) admission of new drugs with fortuitous I_{Kr} -blocking effects. Paradoxically, screening of drugs for possible I_{Kr} -blocking side effects has resulted in the serendipitous discovery of various $K_v11.1$ activators.⁵ Besides their potential usefulness in treating inherited long QT syndrome (LQTS), these $K_v11.1$ activators may also be used to counteract $K_v11.1$ blockade-associated DiVAs. Indeed, Kang *et al.* showed that the action potential (AP) duration (APD)-prolonging effect of the I_{Kr} -blocking, class III antiarrhythmic drug dofetilide could be counterbalanced by the first identified synthetic $K_v11.1$ activator designated

RPR260243.⁶ However, due to the APD-shortening effect, $K_v11.1$ activators carry the risk of possibly inducing short QT syndrome.⁵ Recently, Potet *et al.* described a compound designated VU0405601 that upon pretreatment significantly reduced the APD-prolonging effect of dofetilide in Langendorff-perfused rabbit hearts and dose-dependently mitigated the $K_v11.1$ -blocking effects of seven different drugs in a fluorescence-based thallium flux assay performed with HEK293 cells stably overexpressing the human *KCNH2* gene (HEK293 $K_v11.1$ cells).⁷ VU0405601 exerted its effects on the $K_v11.1$ channel in whole-cell voltage clamp experiments using HEK293 $K_v11.1$ cells only when applied extracellularly. This suggests that VU0405601 binds to the extracellular domain of the $K_v11.1$ channel rather than to its central cavity leading us to hypothesize that VU0405601 counteracts the APD-prolonging effect of $K_v11.1$ blockers by an allosteric mechanism.

Allosteric modulators bind to their targets at a site topologically different from that of the endogenous ligand. Of note, since the $K_v11.1$ channel does not have an endogenous ligand, the authors refer to the site where typical blockers (i.e. dofetilide and astemizole) bind as the orthosteric site. From this so-called allosteric or regulatory site they generally display higher selectivity across receptor subtypes and thus provide a safer pharmacological profile than ligands binding to the orthosteric or active site.⁸ Whereas allosteric modulators targeting ligand-gated ion channels and G protein-coupled receptors have been well established as research tools and therapeutic agents,⁸⁻¹⁰ very little progress has been made in the discovery and clinical development of such compounds for voltage-gated ion channels, in particular the $K_v11.1$ channel.

In this study, different *in vitro* radioligand binding assays were used to investigate whether two previously reported compounds (i.e. VU0405601⁷ and ML-T531¹¹) and a new compound designated LUF7244 could allosterically modulate the binding of the potent $K_v11.1$ blocker dofetilide to the channel's central cavity.^{12,13} Radioligand binding assays were also employed to study LUF7244's influence on the interaction between (i) the $K_v11.1$ channel and three different blockbuster drugs (i.e. astemizole, sertindole and cisapride) that have been withdrawn from the market due to their $K_v11.1$ -related cardiotoxicity¹⁴ and (ii) astemizole and its intended target, the human histamine H_1 receptor (hH₁R). The radioligand binding assays were complemented with optical voltage mapping experiments in confluent monolayers of neonatal rat ventricular myocytes (NRVMs). These experiments were performed in the absence and presence of LUF7244 and/or any of the three blockbuster drugs. Electrophysiological parameters analyzed included conduction velocity (CV), APD at 40 and 90% repolarization (APD₄₀ and APD₉₀, respectively), APD dispersion and EAD incidence. In addition, the effects of LUF7244 alone and together with astemizole on the viability, excitability and

contractility of the NRVMs were investigated. The results of this study indicate that negative allosteric modulation of the $K_v11.1$ channel may provide a safe and effective means to prevent the proarrhythmic effects of I_{Kr} blockers that bind to the channel's central cavity.

Materials and methods

Materials

Dofetilide was synthesized in-house as previously described.¹⁵ Bovine serum albumin (BSA, fraction V) and the fortuitous $K_v11.1$ blockers astemizole, sertindole and cisapride were purchased from Sigma-Aldrich (St. Louis, MO). Tritium-labeled dofetilide (specific activity: 82.3 Ci·mmol⁻¹) and astemizole (specific activity: 78.4 Ci·mmol⁻¹) were obtained from PerkinElmer (Groningen, The Netherlands). The synthesis of VU0405601, ML-T531, as well as the design and synthesis of LUF7244 and its derivatives will be detailed elsewhere. G418 was purchased from Stratagene (Cedar Creek, TX). All other chemicals were of analytical grade and obtained from standard commercial sources. HEK293 $K_v11.1$ cells were kindly provided by Dr. Eckhard Ficker (Case Western Reserve University, Cleveland, OH).¹⁶ Expression plasmid pcDNA3.1-hH₁Rwt was a gift of Prof. Thue W. Schwartz (University of Copenhagen, Copenhagen, Denmark). All animal experiments were approved by the Animal Experiments Committee of Leiden University Medical Center and conformed to the Guide for the Care and Use of Laboratory Animals as stated by the US National Institutes of Health.

Radioligand binding assays

Cell culture and membrane preparations

HEK293 $K_v11.1$ cells were cultured and membranes were prepared and stored as described previously.¹⁷

HEK293 cells were cultured in a humidified atmosphere at 37 °C and 7% CO₂ in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, D6546) supplemented with 10% fetal bovine serum (Sarstedt, Nümbrecht, Germany), 50 IU·mL⁻¹ penicillin (Sarstedt) and 50 µg·mL⁻¹ streptomycin (Sarstedt). When the cells had reached 50-60% confluence, the culture medium was refreshed and 500 µL of 150 mM NaCl containing 15 µg of pcDNA3.1-hH₁Rwt DNA and 45 µg of linear 25-kDa polyethylenimine (Polysciences Europe, Eppelheim, Germany) was added per 100-mm culture dish (Sarstedt). Cells were harvested 48 h after transfection. Membranes of HEK293 cells expressing hH₁R (HEK293hH₁R cells)

were prepared and stored as described for HEK293K_v11.1 cells except that incubation buffer I (50 mM Tris-HCl [pH 7.4]) was used instead of incubation buffer II (10 mM HEPES-NaOH [pH 7.4], 130 mM NaCl, 60 mM KCl, 0.8 mM MgCl₂, 1 mM EGTA, 10 mM glucose, 0.1% BSA).

Radioligand displacement assays

[³H]Dofetilide binding assays for the K_v11.1 channel were performed in incubation buffer II as described previously.¹⁷ Briefly, membrane aliquots containing 20 µg protein were incubated with 5 nM [³H]dofetilide in a total volume of 100 µl at 25 °C for 1 hour. Radioligand displacement experiments were carried out with various concentrations of the test compounds. Total binding was determined in the presence of unsupplemented incubation buffer II, whereas non-specific binding was evaluated in incubation buffer II containing 10 µM astemizole. Displacement experiments with different concentrations of dofetilide, astemizole, sertindole and cisapride were conducted in the absence (control) or presence of 10 µM LUF7244. Incubations were terminated by dilution with ice-cold wash buffer (25 mM Tris-HCl [pH 7.4], 130 mM NaCl, 60 mM KCl, 0.8 mM MgCl₂, 0.05 mM CaCl₂, 0.05% BSA). Free radioligand was separated from bound [³H]dofetilide by rapid filtration through a UniFilter-96 GF/B microplate using a PerkinElmer MicroBeta Filtermate-96 harvester. The radioactivity was determined using a Wallac 1450 MicroBeta TriLux liquid scintillation counter (PerkinElmer) after extraction with 25 µl MicroScint 20 (PerkinElmer).

[³H]Astemizole binding assays for the hH₁R were performed in incubation buffer I. Membrane aliquots containing 15 µg protein were incubated with 3.5 nM [³H]astemizole in a total volume of 100 µl at 25 °C for 3 h. Total binding was determined in the presence of unsupplemented incubation buffer I, whereas non-specific binding was evaluated in incubation buffer I containing 100 µM astemizole. LUF7244 displacement assays were carried out with various concentrations of this compound, while displacement assays with different concentrations of astemizole were conducted in the absence (control) or presence of 10 µM LUF7244. Incubations were terminated by dilution with ice-cold wash buffer II (50 mM Tris-HCl [pH 7.4]), and samples were obtained as mentioned above.

Kinetic dissociation assays

Kinetic dissociation assays of [³H]dofetilide were performed as described previously¹⁷ with the following modifications. Single-point dissociation experiments were conducted by addition of 10 µM dofetilide in the absence (control) or presence of 10 or 50 µM of the selected compounds after preincubating membranes

with [^3H]dofetilide at 25 °C for 2 h. After 6 min of dissociation, incubations were terminated and samples were obtained as described under “*Radioligand displacement assays*”. Traditional dissociation experiments were carried out with 10 μM dofetilide in the absence (control) or presence of 50 μM of the test compounds at 25 °C for a total period of 2 h after preincubation with the radioligand. The amounts of [^3H]dofetilide still bound to the receptor were measured at various time intervals. The concentration-dependent effect of LUF7244 was determined by addition of 10 μM dofetilide in the absence (control) or presence of different concentrations of LUF7244. After 6 min of dissociation, incubations were terminated and samples were obtained as described under “*Radioligand displacement assays*”.

Functional assays in NRVMs

Isolation and culture of NRVMs

NRVMs were isolated and cultured as described previously.¹⁸ Briefly, neonatal rats were anaesthetized with 4-5% isoflurane inhalation anesthesia. After adequate anesthesia had been confirmed by the absence of reflexes, the heart was quickly excised. Ventricles were separated from the remainder of the heart, cut into small pieces with a fine scissor and a scalpel and dissociated by collagenase type I (Worthington, Lakewood, NJ) digestion. The resulting cell suspension was applied to Primaria culture dishes (Corning Life Sciences, Amsterdam, The Netherlands) and incubated for 75 min at 37 °C in a humidified atmosphere of 5% CO_2 to allow preferential attachment of cardiac fibroblasts. The unattached cells (mainly cardiomyocytes) were collected, passed through a cell strainer (70- μm mesh pore size; BD Biosciences, Breda, The Netherlands) and applied at a density of 8×10^5 cells per well of a 24-well cell culture plate (Corning Life Sciences) to fibronectin (Sigma-Aldrich)-coated, round glass coverslips. Proliferation of residual cardiac fibroblasts (10-15%) was inhibited by incubating the cells for 2 h in culture medium containing 10 $\mu\text{g} \cdot \text{mL}^{-1}$ mitomycin C (Sigma-Aldrich) at 24 h after seeding (i.e. at day 1 of culture). Cells were subsequently maintained in a 1:1 mixture of DMEM (Life Technologies Europe, Bleiswijk, The Netherlands; catalog number: 22320) and Ham's F10 medium (Life Technologies Europe) supplemented with 5% horse serum (Life Technologies Europe), 2% BSA and sodium ascorbate to a final concentration of 0.4 mM. This so-called NRVM medium was replaced daily.

Immunocytochemical analyses

NRVMs were plated on fibronectin-coated, 15-mm diameter round glass coverslips at a density of 4×10^4 cells. At day 9 of culture, the cells were washed thrice with ice-cold phosphate-buffered saline (PBS), fixed for 30 min in buffered 4% formaldehyde (Added Pharma, Oss, The Netherlands) of 4 °C and permeabilized by a 10-minute incubation at room temperature (RT) with 0.1% Triton X-100 in PBS. Next, the cells were double-immunostained for K_v11.1 (rabbit polyclonal antibodies raised against the C terminus of human K_v11.1, Merck Millipore, Billerica, MA, catalog number: AB5930) and α -actinin (mouse monoclonal antibody, Sigma-Aldrich, clone: EA-53). Incubation with primary antibodies (diluted 1:200 in PBS-0.1% normal donkey serum) and corresponding donkey Alexa Fluor 488/568-conjugated secondary antibodies (1:400 dilution, Life Technologies Europe) lasted for 2 h. To visualize their nuclei, the cells were incubated for 10 min at RT with 10 $\mu\text{g} \cdot \text{mL}^{-1}$ Hoechst 33342 (Life Technologies Europe) in PBS. After each processing step, the cells were washed three times with PBS of RT. To minimize photobleaching, coverslips were mounted in VECTASHIELD mounting medium (Vector Laboratories, Burlingame, CA). Photomicrographs were obtained with the aid of a digital color camera-equipped fluorescence microscope (Nikon Eclipse 80i; Nikon Instruments Europe, Amstelveen, The Netherlands).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analyses

Cultures of NRVMs and of neonatal rat cardiac fibroblasts (NRCFs; maintained in NRVM medium) were washed once with ice-cold PBS after which the cells were lysed in TRIzol Reagent (Life Technologies Europe) and total RNA was isolated using the RNeasy Mini kit (QIAGEN Benelux, Venlo, The Netherlands). The RNA was reverse transcribed with the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) and the resulting cDNA was amplified by PCR using the Bioline SensiFAST SYBR No-ROX kit (GC biotech, Alphen aan den Rijn, The Netherlands). The forward and reverse primers for the amplification of rat *Kcnh2*-specific cDNA were localized in different exons and had the following sequences, respectively: 5' TAGCCTCCTCAACATCCC 3' and 5' CCATGTCTGCACTTAGCC 3'. For normalization purposes, rat 18S rRNA (*Rn18s*)-specific cDNA was amplified in parallel using the following forward and reverse primer, respectively: 5' GTAACCCGTTGAACCCATT 3' and 5' CCATCCAATCGGTAGTAGCG 3'. PCR amplifications were carried out in a CFX96 Touch Real-Time PCR detection system (Bio-Rad) using a 2-step cycling protocol (20–40 cycles of 95 °C 10 sec, 60 °C 30 sec) after a 5-minute incubation at 95 °C. Quantitative analyses were

based on the $2^{-\Delta\Delta CT}$ method using CFX Manager software (Bio-Rad). For these analyses, PCRs were performed *in triplo* on three independent samples.

Optical mapping

APs were investigated on a whole-culture scale by optical mapping using the potentiometric dye di-4-ANEPPS (Life Technologies Europe) as described previously.¹⁹ The measurements were carried out at 37 °C on 9-day-old confluent NRVM monolayer cultures (see above). Optical signals were captured using a MiCAM ULTIMA-L imaging system (SciMedia, Costa Mesa, CA). To validate the experimental model, cells were incubated for 20 min in culture medium containing 0, 10, 30, 100 or 300 nM of the hH_1R antagonist and unintended $\text{K}_v11.1$ blocker astemizole and dimethylsulfoxide (DMSO) at a final concentration of 0.03%. In a subsequent experiment, NRVM cultures were first exposed for 30 min to 10 μM LUF7244 or its solvent (i.e. culture medium containing 0.1% DMSO). Next, astemizole (final concentration of 100 nM) or vehicle was added to the culture medium raising the DMSO concentration to 0.13%. Following an incubation period of 30 min at 37 °C, optical recordings were started in the continued presence of the appropriate vehicle/drug combinations (**Figure 4A**). Optical traces were analyzed using Brain Vision Analyzer 1208 software (Brainvision, Tokyo, Japan). To minimize noise artifacts, calculations were based on the average of the signals at a selected pixel and its eight nearest neighbors. CV, APD_{40} , APD_{90} and APD_{40} dispersion were determined using NRVM cultures showing uniform AP propagation and 1:1 capture after 1-Hz local stimulation. Each of these electrophysiological parameters was the average of values obtained from 6 different positions equally distributed across the cell cultures. The effects of LUF7244 on the $\text{K}_v11.1$ -inhibiting antipsychotic drug sertindole and gastroprokinetic agent cisapride were assessed in the same way as for astemizole expect that the final concentration of sertindole and cisapride was 1 μM .

To study the effects of long-term $\text{K}_v11.1$ allosteric modulation in the absence and presence of an unintended $\text{K}_v11.1$ blocker on cardiac excitability, confluent NRVM cultures were incubated for 3 days in culture medium containing 100 nM astemizole and/or 10 μM LUF7244 prior to potentiometric dye loading, short-term drug treatment (see above) and optical mapping in the continued presence of drug(s) at culture day 9. Confluent NRVM monolayers exposed to culture medium containing vehicle (i.e. DMSO at a final concentration of 0.13%) only, served as negative controls for this experiment. Just prior to optical mapping, movies of the cells were made using a Carl Zeiss Axiovert 40C microscope equipped with a LD A-Plan 20x/0.3 Ph1 objective (Zeiss Nederland, Slidrecht, The Netherlands) to record their contractions upon electrical field stimulation at 1 Hz and 8 V using

a 15-ms rectangular pulse.

Apoptosis assay

To investigate the effects of long-term $K_v11.1$ allosteric modulation in the absence or presence of simultaneous $K_v11.1$ blockade on cell viability, externalized phosphatidylserine (as early marker of apoptosis) was detected using Alexa-568-conjugated annexin V (Life Technologies Europe). NRVM cultures in 24-well plates were treated cells for 72 h with 100 nM astemizole and/or 10 μ M LUF7244 as described under “*Optical mapping*”. Mock-treated cells and cells incubated for 24 h in culture medium containing 1 μ M doxorubicin (Sigma-Aldrich) served as negative and positive controls, respectively. Next, the cells were washed once with ice-cold PBS and incubated for 15 min at RT with annexin V conjugate diluted 40-fold in binding buffer (10 mM HEPES-NaOH [pH 7.4], 140 mM NaCl, 2.5 mM CaCl_2) of 4 °C containing 10 $\mu\text{g}\cdot\text{mL}^{-1}$ Hoechst 33342. After a single wash with binding buffer of 4 °C, photomicrographs were taken using a Leica DMI6000 B inverted microscope equipped with a Leica DFC300 FX digital color camera (both from Leica Microsystems, Rijswijk, The Netherlands).

Data analysis

Data of the radioligand binding assays were analyzed with Prism 5.0 (Graph-Pad, San Diego, CA). Half maximal inhibitory concentrations (i.e. IC_{50} values) in displacement assays were directly obtained from non-linear regression analysis of dose-response curves. Apparent inhibitory binding constants (K_i values) were derived from the IC_{50} values according to the Cheng-Prusoff relationship²⁰: $K_i = \text{IC}_{50}/(1 + [\text{L}^*]/K_D)$, where $[\text{L}^*]$ is the concentration of radioligand and K_D is its dissociation constant determined by saturation assay²¹. Dissociation rate constants (k_{off}) were obtained by computer analysis of the exponential decay of [^3H] dofetilide bound to the $K_v11.1$ channel. Half maximal effective concentrations (i.e. EC_{50} values) from kinetic dissociation assays were calculated by non-linear regression analysis of concentration-effect curves of dissociation in the presence of different concentrations of unlabeled ligands. All values obtained from radioligand binding assays in this study are means of at least three independent experiments performed in duplicate. The number of samples per experimental group in the optical mapping experiments varied from 5 to 24 as indicated. Whenever appropriate, data are expressed as mean $\pm$ standard error of the mean (SEM) or as mean $\pm$ standard deviation (SD). Statistical analysis was performed with a two-tailed unpaired Student's t-test.

Results

Characterization of allosteric modulators of [³H]dofetilide binding to the K_v11.1 channel

The interaction of two previously reported ligands VU0405601 and ML-T531 as well as the newly designed and synthesized compound LUF7244 (**Figure 1A**) with the human K_v11.1 channel was studied in different [³H]dofetilide binding assays. As shown in **Figure 1B** and **Table 1**, all three compounds reduced [³H]dofetilide binding at the K_v11.1 channel with relatively low affinity, i.e. with IC₅₀ values of $7.8 \pm 0.4 \mu\text{M}$, $12 \pm 1 \mu\text{M}$ and $3.9 \pm 0.7 \mu\text{M}$ for VU0405601 and ML-T531 and LUF7244, respectively. Moreover, all displacement curves demonstrated Hill coefficients significantly different from unity (data not shown), implying that VU0405601, ML-T531 and LUF7244 might not competitively displace [³H]dofetilide from the K_v11.1 channel but may occupy distinct binding pockets in the channel protein to allosterically modulate binding of the radioligand.

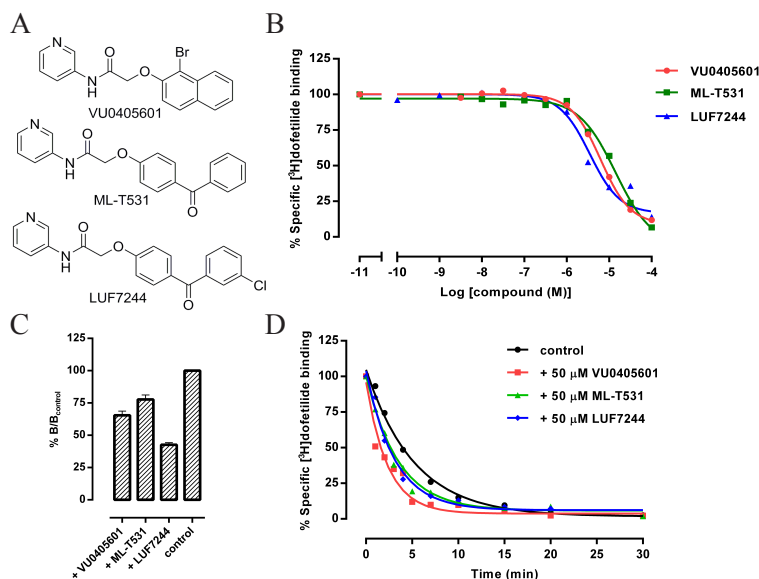


Figure 1. Characterization of allosteric modulators of dofetilide binding to the K_v11.1 channel in a [³H]dofetilide binding assay performed with membranes of HEK293K_v11.1 cells. **(A)** Chemical structures of VU0405601, ML-T531 and LUF7244. **(B)** Displacement curves of VU0405601, ML-T531 and LUF7244. **(C)** Percentage specific binding of [³H]dofetilide to the K_v11.1 channel after 6 min of dissociation induced by 10 μM dofetilide in the absence (control) or presence of 10 μM of VU0405601, ML-T531 or LUF7244. The specific binding of [³H]dofetilide in the absence of the test compounds was set as B_{control} , while the specific binding in their presence was set as B . **(D)** Time-dependent dissociation

of [^3H]dofetilide induced by 10 μM dofetilide in the absence (control) or presence of 50 μM VU0405601, ML-T531 or LUF7244. Experiments were performed at 25 $^{\circ}\text{C}$ using 20 μg of HEK293K $_{\text{v}}$ 11.1 membrane protein.

Subsequently, single-point dissociation assays were performed to screen for allosteric effects of these compounds on the binding of [^3H]dofetilide to the K $_{\text{v}}$ 11.1 channel. The results are summarized in **Figure 1C** and **Table 1**. At a concentration of 10 μM , VU0405601, ML-T531 and LUF7244 significantly increased the dissociation of [^3H]dofetilide from the K $_{\text{v}}$ 11.1 channel, indicating that these compounds are negative allosteric modulators of dofetilide binding to the channel. LUF7244 appeared to be the most potent negative allosteric modulator with $43 \pm 2\%$ dofetilide binding left compared to control conditions, while 10 μM VU0405601 and ML-T531 reduced dofetilide binding to $66 \pm 3\%$ and $78 \pm 3\%$, respectively.

The allosteric effects of VU0405601, ML-T531 and LUF7244 on the K $_{\text{v}}$ 11.1 channel were further investigated in traditional radioligand dissociation experiments to determine whether co-administration of these compounds with an excess unlabeled dofetilide would change the dissociation rate of [^3H]dofetilide from the K $_{\text{v}}$ 11.1 channel. In order to obtain larger effects, the three compounds were tested at a concentration of 50 μM instead of 10 μM as used in the single-point dissociation assay. As shown in **Figure 1D** and **Table 1**, all compounds significantly accelerated the dissociation of dofetilide, in line with the results from the single-point dissociation experiments. The off-rate of [^3H]dofetilide was allosterically increased 2.2-fold (from 0.22 ± 0.02 to $0.49 \pm 0.09 \text{ min}^{-1}$) with 50 μM VU0405601. The k_{off} value of [^3H]dofetilide rose to $0.33 \pm 0.03 \text{ min}^{-1}$ in the presence of 50 μM LUF7244, which was comparable to the effect of ML-T531 ($k_{\text{off, dofetilide}} = 0.30 \pm 0.02 \text{ min}^{-1}$).

Table 1. The half maximal inhibitory concentrations (IC_{50}), percentage specific binding of [^3H]dofetilide to the K $_{\text{v}}$ 11.1 channel after 6 minutes of dissociation in the absence or presence of 10 μM of the indicated compounds ($\%B/B_{\text{control}}$), and dissociation rates (k_{off}) of [^3H]dofetilide in the absence or presence of 50 μM of the indicated compounds.

	IC_{50} (nM)	$\%B/B_{\text{control}}$	k_{off} (min^{-1})
Dofetilide	-	100 ± 0	0.22 ± 0.02
+ VU0405601	7757 ± 350	66 ± 3 (***)	0.49 ± 0.09 (**)
+ ML-T531	12000 ± 1213	78 ± 3 (***)	0.30 ± 0.02 (**)
+ LUF7244	3855 ± 724	43 ± 2 (***)	0.33 ± 0.03 (**)

Values are means ($\pm$ SEM) of at least three independent assays performed in duplicate. (** $P < 0.01$ versus control; *** $P < 0.001$ versus control)

Effects of LUF7244 on the binding of typical K_v11.1 blockers to the channel

Since LUF7244 was the most potent amongst the three allosteric modulators at the lower test concentration of 10 μ M and may therefore have the best safety profile, its potency to increase the dissociation of [³H]dofetilide from the K_v11.1 channel was investigated. The corresponding concentration-effect curve is shown in **Figure 2A**. From this graph, the modulatory potency (i.e. the EC₅₀) of LUF7244 was calculated to be 4.6 ± 0.8 μ M. Notably, LUF7244 could not completely abrogate [³H]dofetilide binding by accelerating its dissociation from the K_v11.1 channel.

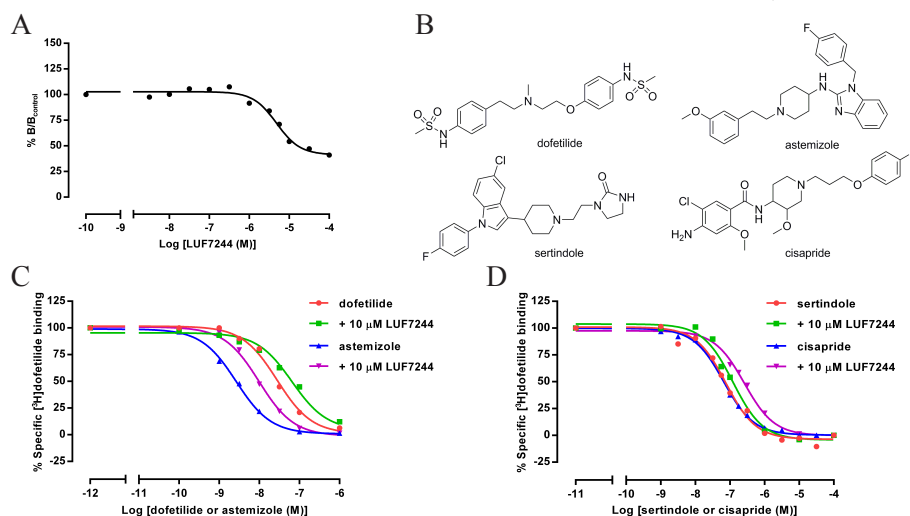


Figure 2. Assessment of the effects of LUF7244 on the binding of K_v11.1 blockers to the channel in a [³H]dofetilide binding assay performed with membranes of HEK293K_v11.1 cells. **(A)** Effect of different concentrations of LUF7244 on the dofetilide-induced dissociation of [³H]dofetilide from the K_v11.1 channel. After preincubating of HEK293K_v11.1 cell membranes with [³H]dofetilide, radioligand dissociation was induced by 10 μ M dofetilide in the absence or presence of different concentrations of LUF7244 and the incubation was terminated after 6 min. The results are expressed as the ratio of the specific binding of [³H]dofetilide in the presence of 10 μ M dofetilide plus various concentrations of negative allosteric modulators (*B*) over that in the presence of 10 μ M dofetilide alone (*B*_{control}). **(B)** Chemical structures of dofetilide, astemizole, sertindole and cisapride. **(C)** Displacement curves of dofetilide and astemizole in the absence or presence of 10 μ M LUF7244. **(D)** Displacement curves of sertindole and cisapride in the absence or presence of 10 μ M LUF7244. Experiments were performed at 25 °C using 20 μ g of HEK-293K_v11.1 membrane protein.

To investigate the effects of LUF7244 on the binding affinity of other com-

pounds besides dofetilide to the $K_v11.1$ channel, three additional $K_v11.1$ blockers (i.e. astemizole, sertindole and cisapride) from distinct therapeutic classes were selected (**Figure 2B**). As shown in **Figure 2C** and **2D**, the [3 H]dofetilide displacement curves of all four drugs were shifted rightwards in the presence of 10 μ M LUF7244, implicating that their $K_v11.1$ affinity were diminished by this negative allosteric modulator. The K_i values of dofetilide, astemizole, sertindole and cisapride in the absence or presence of 10 μ M LUF7244 are listed in **Table 2**. LUF7244 most strongly modulated cisapride binding to the $K_v11.1$ channel, increasing its K_i value by 4.0-fold from 21 ± 1 to 85 ± 7 nM. Similarly, the $K_v11.1$ affinity of astemizole, dofetilide and sertindole were reduced by 3.2-, 2.6- and 2.1-fold in the presence of LUF7244. Thus, the negative allosteric effect of LUF7244 on the $K_v11.1$ channel significantly lowered the channel's affinity for several chemically and therapeutically distinct $K_v11.1$ blockers.

Table 2. The $K_v11.1$ affinity (K_i) of dofetilide, astemizole, sertindole and cisapride in the absence (control) or presence of 10 μ M LUF7244.

	K_i (nM)	$K_{i, + 10 \mu\text{M LUF7244}}$ (nM)	Fold Change
Dofetilide	6.0 ± 0.5	16 ± 4 (**)	2.6
Astemizole	1.2 ± 0.2	3.7 ± 0.1 (***)	3.2
Sertindole	20 ± 2	40 ± 4 (**)	2.1
Cisapride	21 ± 1	85 ± 7 (***)	4.0

Values are means ($\pm$ SEM) of at least three independent assays performed in duplicate. (** $P < 0.01$ versus control; *** $P < 0.001$ versus control)

Effects of LUF7244 on the binding of [3 H]astemizole to the hH_1R

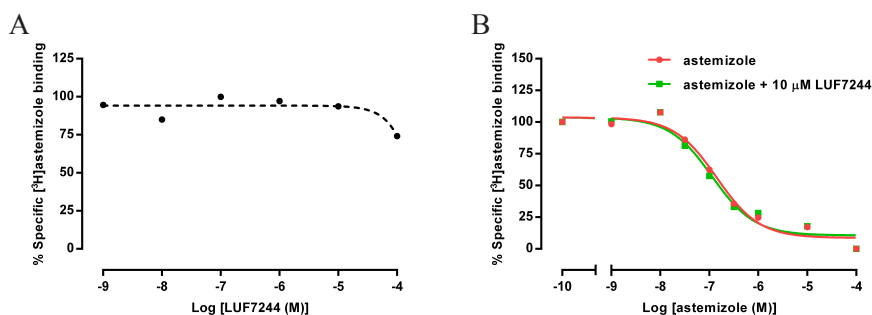


Figure 3. Assessment of the effects of LUF7244 on the binding of astemizole to the hH_1R in a [3 H]astemizole binding assay performed with membranes of HEK293 hH_1R cells. **(A)** Displacement curve of LUF7244. **(B)** Displacement curves of astemizole in the absence or presence of 10 μ M LUF7244. Experiments were performed at 25 $^{\circ}$ C using 15 μ g of HEK293 hH_1R membrane protein.

To be of practical use in preventing DiVAs, LUF7244 should not interfere with the desired activities of unintended $K_v11.1$ blockers. We therefore tested whether LUF7244 affected binding of the antihistamine drug astemizole to the hH_1R . As displayed in **Figure 3A**, LUF7244 did not decrease the binding of [3H]astemizole to the hH_1R . Furthermore, the displacement curve of astemizole binding to the hH_1R was not significantly shifted rightwards by LUF7244 (**Figure 3B**). Thus, LUF7244 reduces the affinity of the $K_v11.1$ channel for astemizole without affecting the binding of astemizole to its intended target receptor (i.e. the hH_1R).

Analysis of $K_v11.1$ protein expression in NRVMs

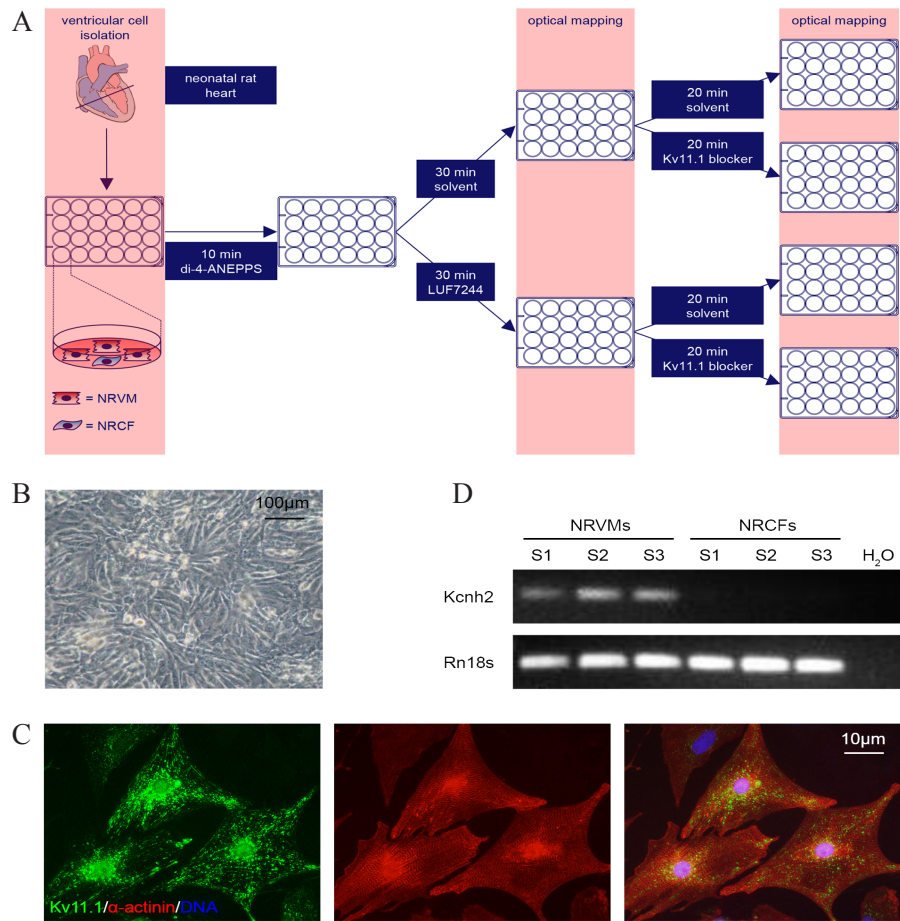


Figure 4. Biochemical characterization of the NRVM model. **(A)** Basic experimental set-up of the optical voltage mapping experiments. **(B)** Phase-contrast image of a typical confluent NRVM monolayer used for optical voltage mapping. **(C)** Immunocytochemical analysis of $K_v11.1$ protein expression in NRVM cultures. The $K_v11.1$ protein (green) is mainly located around the nucleus (blue) and at the sarcolemma of the α -actinin (red)-positive

NRVMs and hardly detectable in the α -actinin-negative non-cardiomyocytes. **(D)** Analysis of *Kcnh2* mRNA levels in NRVMs and NRCFs by RT-PCR.

Next, the electrophysiological consequences of allosteric modulation of the binding of typical $K_v11.1$ blockers at the channel by LUF7244 (see **Figure 4A**) was examined in confluent monolayer cultures of NRVMs (**Figure 4B**) as relevant *in vitro* model for studying cardiac arrhythmias.²² Double immunostaining for $K_v11.1$ and sarcomeric α -actinin showed that all cardiomyocytes in the NRVM cultures expressed the $K_v11.1$ protein (**Figure 4C**). The $K_v11.1$ signal had a punctate or linear appearance and was concentrated around nuclei and along the sarcolemma (**Figure 4C**). No significant $K_v11.1$ protein expression was observed in the low percentage of α -actinin⁺ cells (mainly NRCFs) present in the NRVM cultures. Consistently, comparison of *Kcnh2* transcript levels between NRVMs and NRCFs by RT-qPCR demonstrated 39-fold higher ($n=3$, $P < 0.01$) *Kcnh2* mRNA expression in NRVMs than in NRCFs (**Figure 4D**).

Electrophysiological consequences of $K_v11.1$ blockade by astemizole in NRVMs

Due to its relatively high specificity for the $K_v11.1$ channel,²³ astemizole was selected to study the electrophysiological consequences of $K_v11.1$ blockade by optical voltage mapping. NRVMs treated with astemizole displayed a concentration-dependent lengthening of APD and EAD incidence (**Figure 5** and **Figure 6**). As displayed in **Figure 5A** and **5B**, the APD_{40} and APD_{90} values of NRVMs were increased from 112 ± 16 and 295 ± 62 ms in vehicle-treated cultures ($n = 24$) to 156 ± 39 and 355 ± 66 ms in cultures containing 100 nM astemizole ($n = 24$, $P < 0.001$ and $P < 0.01$), respectively. Furthermore, exposure to 100 nM astemizole resulted in the occurrence of EADs in 25% of the NRVM cultures, whereas no EADs were observed under control conditions (**Figure 5C** and **5D**). Intriguingly, results presented in **Figure 5E** and **5F** demonstrated that the APD_{40} dispersion between NRVMs in the presence of 100 nM astemizole was significantly higher than that in its absence (39 ± 11 versus 16 ± 5 ms; $n = 24$, $P < 0.001$), indicative of aggravated repolarization heterogeneity due to the inhibition of I_{Kr} by astemizole. Occasionally, the astemizole-induced EADs resulted in short episodes of reentrant excitation (**Figure 7**) reminiscent of spontaneously terminating TdP episodes. Nonetheless, as indicated by the activation maps shown in **Figure 5G** and corresponding quantitative analysis depicted in **Figure 5H**, the CV in NRVM cultures was not significantly influenced by 100 nM astemizole (20 ± 4 cm·s⁻¹ vs 21 ± 3 cm·s⁻¹ in control cultures; $n = 10$, $P = 0.48$). Overall, these data validate the utility of NRVM cultures as an *in vitro* model for investigating the AP-prolonging

and associated proarrhythmic effects of $K_v11.1$ blockers like astemizole.

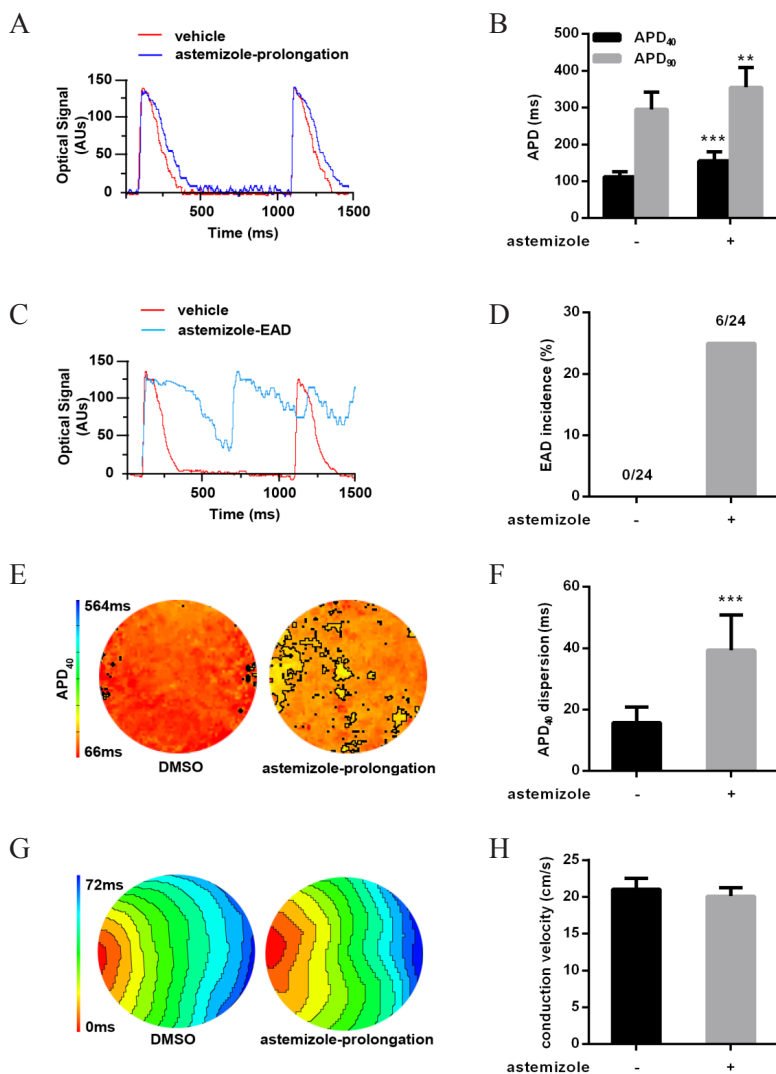


Figure 5. Electrophysiological characterization of the NRVM model by optical voltage mapping following 1-Hz electrical stimulation. Representative filtered optical signal traces (**A** and **C**), corresponding APD dispersion and activation maps (**E** and **G**, respectively) and quantitative analyses (bar graphs in **B**, **D**, **F** and **H**) of NRVM cultures exposed for 30 min to 100 nM astemizole or vehicle (control) and subjected to optical voltage mapping immediately afterwards. Astemizole treatment resulted in APD prolongation (APD₄₀ and APD₉₀, **A** and **B**), the occurrence of EADs (**C** and **D**) and an increase in APD₄₀ dispersion (**E** and **F**) but did not significantly alter CV (**G** and **H**). ** $P < 0.01$ and *** $P < 0.001$.

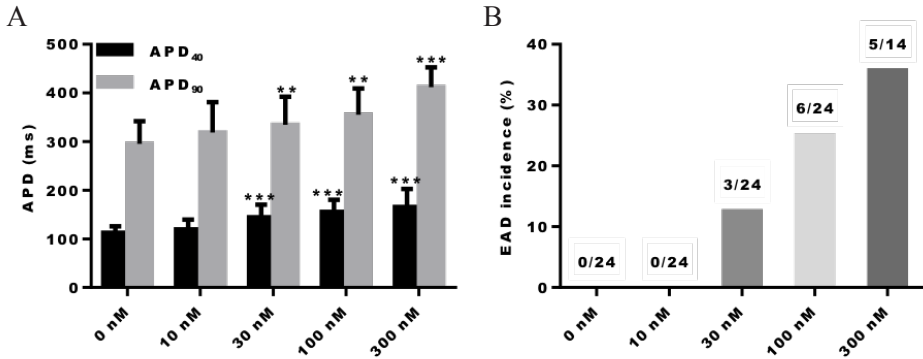


Figure 6. Assessment by optical voltage mapping of the effect of different concentrations of astemizole on the APD (APD₄₀ and APD₉₀, **A**) and EAD incidence (**B**) in NRVM monolayers. ** $P < 0.01$ and *** $P < 0.001$.

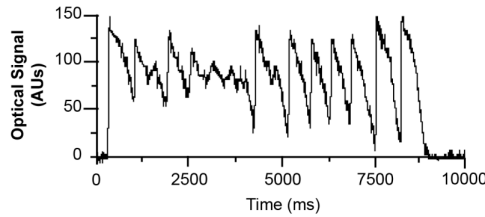


Figure 7. Optical voltage trace showing an unstable, self-terminating tachyarrhythmia in an astemizole-treated NRVM culture following 1-Hz electrical point stimulation.

Effects of LUF7244 on K_v11.1 blockade-associated proarrhythmic changes in NRVM cultures

To investigate the functional consequences of negative allosteric modulation of K_v11.1's interaction with typical K_v11.1 blockers, di-4-ANNEPS-loaded NRVM cultures were incubated for 20 min in culture medium containing 10 μ M LUF7244 before addition of astemizole, sertindole or cisapride. Since the K_i values of sertindole and cisapride for K_v11.1 are more than 10 times higher than that of astemizole (**Table 2**), these drugs were tested at a final concentration of 1 μ M instead of 100 nM as was used for astemizole. After incubation for 30 min, the NRVMs were optically mapped. As shown in **Figure 8A**, the APD-prolonging and EAD-promoting effects of astemizole were effectively suppressed by LUF7244. The APD₄₀ and APD₉₀ were significantly shortened from 156 ± 39 ms to 118 ± 18 ms ($n = 24$, $P < 0.001$) and from 355 ± 66 ms to 282 ± 63 ms ($n = 24$, $P < 0.01$), respectively, i.e. LUF7244 was able to reduce APD₄₀ and APD₉₀ to control values (**Figure 8B**). Moreover, in the presence of 10 μ M LUF7244 EADs were no longer observed in NRVM cultures exposed to 100 nM astemizole (**Figure 8C**).

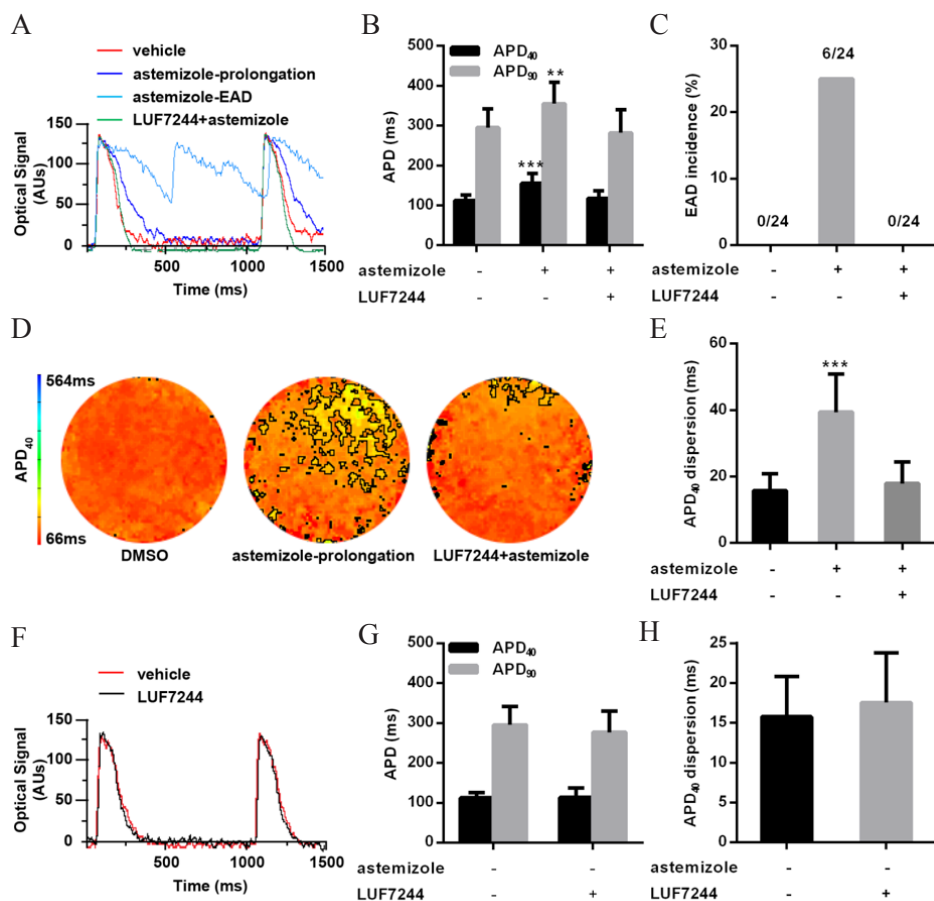


Figure 8. Assessment by optical voltage mapping of the ability of LUF7244 to counteract the proarrhythmic effects of astemizole on NRVMs. Representative filtered optical signal traces (A and F), APD dispersion maps (D) and quantitative analysis (bar graphs in B, C, E, G and H) of control NRVM cultures and of NRVM cultures that had been treated with 100 nM astemizole or with 10 μ M LUF7244 followed by 100 nM astemizole immediately before optical mapping. Pretreatment of NRVM cultures with LUF7244 completely prevented the astemizole-induced APD prolongation (APD₄₀ and APD₉₀, B), occurrence of EADs (C) and increase in APD₄₀ dispersion (D and E). Treatment of NRVM cultures with 10 μ M LUF7244 only did not change AP morphology (F), APD (G) or APD₄₀ dispersion (H). ** $P < 0.01$ and *** $P < 0.001$.

LUF7244 also prevented the increase in APD₄₀ dispersion caused by astemizole (Figure 8D and 8E). Reassuringly, the CVs in the NRVM cultures treated with vehicle, LUF7244, astemizole or LUF7244 plus astemizole did not significantly differ (data not shown). Also, at a final concentration of 10 μ M LUF7244 per se

did not reduce APD or significantly affect APD₄₀ dispersion (**Figure 8F-8H**), suggesting that this negative allosteric modulator poses very little, if any, risk for the development of arrhythmias associated with abnormal APD shortening. Very similar results of LUF7244 were obtained in NRVM cultures exposed to sertindole or cisapride (**Figure 9**). LUF7244 can thus suppress the proarrhythmic side effects of drugs from different therapeutic classes by allosteric modulation of the K_v11.1 channel without exerting, by itself, any obvious adverse electrophysiological effects on cardiomyocytes.

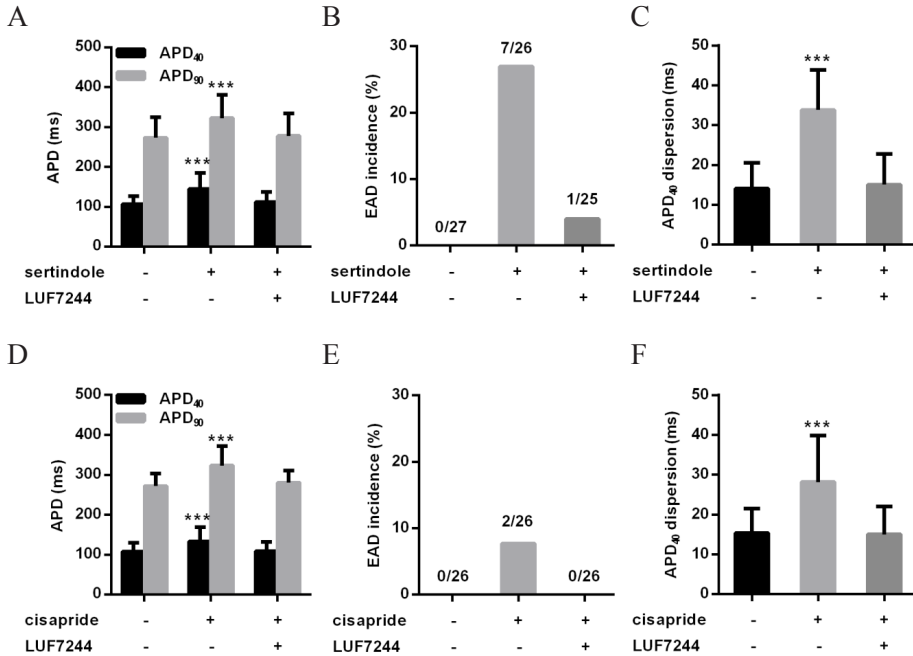


Figure 9. Assessment by optical voltage mapping of the ability of LUF7244 to counteract the proarrhythmic effects of sertindole and cisapride on NRVMs. Pretreatment of NRVM cultures with LUF7244 completely prevented the increase in APD (APD₄₀ and APD₉₀, **A** and **D**), EAD incidence (**B** and **E**) and APD₄₀ dispersion (**C** and **F**) induced by exposure of the cells to 1 μ M sertindole or cisapride. ** $P < 0.01$ and *** $P < 0.001$.

Effects of prolonged exposure of NRVMs to LUF7244 and/or astemizole on cell viability, excitability and contractility

To study the effects of prolonged allosteric modulation and/or blockade of the K_v11.1 channel on the viability of cardiomyocytes, 6-day-old NRVM cultures were incubated for 72 h in culture medium containing 10 μ M LUF7244 and/or 100 nM astemizole. Subsequent staining with fluorescently labeled annexin V to

detect cell surface-exposed phosphatidylserine yielded only very weak signals for cells exposed to vehicle, LUF7244, astemizole or LUF7244 plus astemizole, while NRVMs incubated for 24 h with the proapoptotic drug doxorubicin displayed strong annexin V fluorescence (**Figure 10A**). These results indicate that prolonged exposure to 10 μ M LUF7244 alone or in combination with 100 nM astemizole does not trigger programmed cell death.

Optical voltage mapping of 9-day-old NRVM cultures that had been mock-treated or treated for 72 h with 10 μ M LUF7244 and/or 100 nM astemizole produced very similar results as were obtained for cells that had only briefly been exposed to these compounds (compare **Figure 5** and **8** with **Figure 10B** and **10C**) and provided no indications for a drug-related reduction in excitability. Moreover, after electrical field stimulation cells in all four treatment groups displayed rhythmic contractions following the pacing frequency of 1 Hz (data not shown).

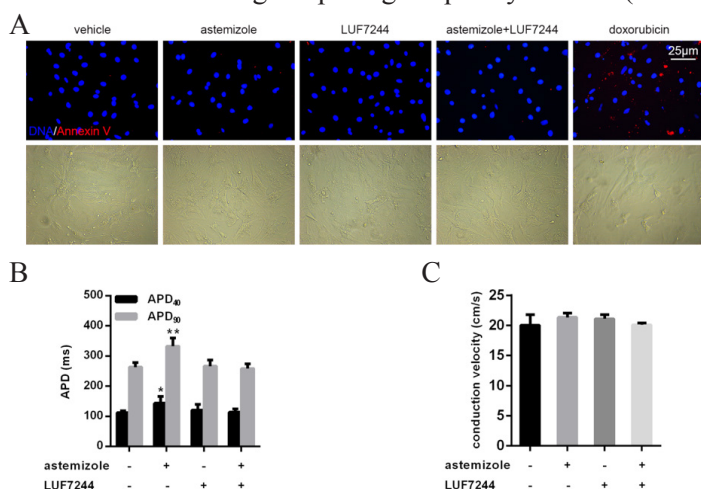


Figure 10. Effects of prolonged exposure of NRVMs to LUF7244 and/or astemizole on cell viability and excitability. **A:** Analysis by Alexa-568-conjugated annexin V staining of cell surface expression of phosphatidylserine as early marker of apoptosis. NRVM cultures exposed for 72 hours to vehicle, 10 μ M LUF7244, 100 nM astemizole or 10 μ M LUF7244 plus 100 nM astemizole showed very weak signals, while NRVMs incubated for 24 hours with 1 μ M of the proapoptotic drug doxorubicin displayed strong annexin V fluorescence. **B:** Quantitative comparison of APDs (APD₄₀ and APD₉₀) in NRVM cultures exposed for 72 hours to vehicle, 10 μ M LUF7244, 100 nM astemizole or 10 μ M LUF7244 plus 100 nM astemizole. **C:** Quantitative comparison of CV in NRVM cultures exposed for 72 hours to vehicle, 10 μ M LUF7244, 100 nM astemizole or 10 μ M LUF7244 plus 100 nM astemizole.

Discussion

Major findings

In radioligand binding assays the structurally related compounds VU0405601, ML-T531 and LUF7244 were found to weaken the interaction between the human $K_v11.1$ channel and the class III antiarrhythmic agent dofetilide as well as the unintended $K_v11.1$ blockers astemizole, sertindole and cisapride. VU0405601, ML-T531 and LUF7244 exerted their negative effects on the binding of typical $K_v11.1$ blockers to the channel's central cavity by an allosteric mechanism. Importantly, LUF7244 decreased the $K_v11.1$ affinity of astemizole without influencing its affinity at the hH_1R , being astemizole's intended target. Optical voltage mapping showed that incubation of NRVM monolayers with astemizole, sertindole or cisapride led to a significant increase in APD, APD dispersion and, except for cisapride, EAD incidence demonstrating the usefulness of this cellular model system for studying $K_v11.1$ blockade-related proarrhythmic risk. Pretreatment of the NRVMs with LUF7244 effectively prevented the proarrhythmic changes induced by astemizole, sertindole and cisapride without significantly shortening APD by itself and without adversely affecting NRVM viability, excitability and contractility. These findings provide a rationale for further exploring allosteric modulation as a strategy to prevent DiVAs.

NRVM monolayers as model for studying drug-induced LQTS

Many different methods have been exploited to assess drugs for their $K_v11.1$ blockade-associated arrhythmogenicity.^{3, 24} The interaction between a drug and the $K_v11.1$ channel is usually first investigated by computational and biochemical assays. Next, the $K_v11.1$ liability of suspicious drugs are typically evaluated by electrophysiological measurements on CHO or HEK293 cells expressing the human $K_v11.1$ channel. Despite their practical advantages, these non-excitable cellular models do not recapitulate the complex regulatory circuits governing $K_v11.1$ channel activity in cardiomyocytes and are unsuitable for studying AP generation and propagation. We hence employed NRVM monolayers to study the effects of LUF7244 on the proarrhythmic potential of three inadvertent $K_v11.1$ blockers. The reasons to specifically use NRVMs for this purpose are that these cells (i) can be relatively easily isolated and cultured, (ii) are well-characterized and (iii) do express functional $K_v11.1$ channels.²⁵ However, to the best of our knowledge, it is the first time that this model is being used to test compounds for their ability to prevent DiVAs resulting from unintended $K_v11.1$ blockade.

Although mechanistic insight into DiVAs is limited, there is broad consen-

sus about spatial dispersion of repolarization and EAD-induced triggered activity providing the substrate and trigger for the genesis of drug-induced TdP, respectively.² The EADs typically arise during phase 2 of the cardiac AP due to drug-dependent decreases in I_{Kr} causing increases of APD and QT interval. In support of the validity of our model, astemizole dose-dependently increased the APD and EAD incidence in monolayers of NRVMs (**Figure 6**). Also sertindole and cisapride had APD-prolonging effects (**Figure 9A** and **9D**) and each of the three fortuitous $K_v11.1$ blockers increased APD dispersion (**Figure 5F**, **Figure 9C** and **9F**). While 100 nM astemizole, 1 μ M sertindole and 1 μ M cisapride increased APD and APD dispersion to a similar extent, cisapride did not significantly increase EAD incidence in contrast to the other two drugs. This finding is in line with a previous study²⁶ and may be explained by cisapride's inhibitory effect on the $I_{Ca,L}$ in NRVMs.

Initially, EAD-dependent ectopic activity at multiple competing foci was thought to generate the undulating electrocardiographic patterns of TdP. Recently, meandering $I_{Ca,L}$ -mediated reentrant circuits initiated by EADs at single foci have been proposed as an alternative explanation for the highly characteristic electrocardiographic signature of TdP.^{27, 28} In support of the latter idea, exposure of NRVMs to $K_v11.1$ blockers occasionally gave rise to unstable, self-terminating reentrant tachyarrhythmias reminiscent of spontaneously terminating TdP episodes. Pretreatment with LUF7244 rendered NRVM monolayers unsusceptible to the $K_v11.1$ -blocking effects of astemizole, sertindole and cisapride. In the presence of LUF7244, these $K_v11.1$ blockers no longer caused heterogeneous APD prolongation and, due to the reduced opportunity for L-type Ca^{2+} channel reactivation, no longer gave rise to EADs. This raises the perspective to use LUF7244 as an antiarrhythmic additive to drugs with unintended I_{Kr} -suppressing effects. Thus, radioligand binding assays in combination with optical voltage mapping experiments of NRVM cultures offer convenient preclinical test systems for evaluating chemical entities that can potentially reduce $K_v11.1$ -related cardiotoxicity.

LUF7244's mode of action

The chemical structure of LUF7244 resembles those of ML-T531 and VU0405601. In a recent study, 10 μ M of ML-T531 was shown to reduce the APD of human induced pluripotent stem cells (hiPSC)-derived cardiomyocytes from an LQT1 patient to that of control cells by augmenting I_{Kr} .¹¹ However, the effects of ML-T531 on the APD of hiPSC-derived cardiomyocytes from a healthy individual and the ability of ML-T531 to inhibit the APD-prolonging effects of unintended $K_v11.1$ blockers were not investigated. Voltage clamp recordings of $K_v11.1$ -ex-

pressing CHO cells showed that ML-T531 reduces the deactivation rate of the $K_v11.1$ channel and causes a shift of its inactivation curve towards more positive voltages. Shortly after the discovery of ML-T531, VU0405601 was identified as a compound that, at a final concentration of 5 μM , protected Langendorff-perfused rabbit hearts from the proarrhythmic effects of exposure to 100 nM dofetilide.⁷ Although VU0405601 only partially reversed the dofetilide-dependent increase in APD, its administration prior to dofetilide strongly reduced the pacing-induced arrhythmia incidence from 42% to 4%, which was very close to the 2% of pacing-induced premature ventricular contractions observed in untreated hearts.⁷ Exposure of isolated rabbit ventricular myocytes to 5 μM of VU0405601 only marginally reduced APD, which is consonant with our finding that 10 μM LUF7244 did not noticeably affect the APD of NRVMs. However, at a final concentration of 50 μM , VU0405601 decreased the APD_{50} and APD_{90} of rabbit ventricular myocytes by $35 \pm 6\%$ and $32 \pm 4\%$, respectively. Patch clamp analysis of HEK293 $K_v11.1$ cells linked the APD-shortening effect of 50 μM VU0405601 to shifts in the $V_{1/2}$ of activation and inactivation and to changes in the kinetics of (de)activation and (de)inactivation causing an increase in I_{Kr} .

In this study, we found that VU0405601, ML-T531 and LUF7244 displayed comparatively low $K_v11.1$ affinity with Hill coefficients significantly different from unity for their [^3H]dofetilide displacement curves. This suggests that these ligands bind to the $K_v11.1$ channel at sites distinct from that of dofetilide, indicative of an allosteric mode of action.^{10, 29, 30} The binding of a drug to a receptor at an allosteric site (i.e. a site topologically distinct from that of the test ligand) triggers a conformational change within the receptor ultimately causing an alteration of the ligand's dissociation rate from its cognate (i.e. orthosteric) binding site.^{10, 31} Altered ligand dissociation rates have been found representative of allosteric interactions in various drug targets such as muscarinic and adenosine receptors.^{10, 32-34} Consistently, VU0405601, ML-T531 and LUF7244 significantly accelerated the dissociation of dofetilide from the $K_v11.1$ channel, strengthening the conclusion that they are negative allosteric modulators of dofetilide binding to the $K_v11.1$ channel. Our finding for VU0405601 is in agreement with the results of Potet *et al.*, who presented indirect evidence that VU0405601 binds from outside to the ectodomain of the $K_v11.1$ channel,⁷ while astemizole, cisapride, dofetilide and sertindole all bind to the channel's central cavity from inside³⁵⁻³⁷. In addition, the [^3H]dofetilide displacement curves of dofetilide, astemizole, sertindole and cisapride were shifted rightwards by LUF7244 (**Figure 2C and 2D**) providing further proof for its negative allosteric effect.^{10, 34} Collectively, the results of the different radioligand binding assays provide strong evidence that VU0405601, ML-T531 and LUF7244 are negative allosteric modulators of dofetilide binding

to the $K_v11.1$ channel. Binding of VU0405601, ML-T531 and LUF7244 likely alters the 3D structure of the $K_v11.1$ channel, which decreases its affinity for typical $K_v11.1$ blockers by increasing the dissociation rates of these blockers from the channel. Of note, the vast majority of fortuitous $K_v11.1$ blockers exert their effects by occupying the central cavity of the $K_v11.1$ channel and thereby obstructing the transport of K^+ ions through the channel's pore.³⁸ There are, however, also examples of drugs that reduce I_{Kr} by inhibiting, directly or indirectly, the trafficking of $K_v11.1$ to the plasma membrane.³⁹ Given their specific mode of action, it is unlikely that the I_{Kr} -inhibiting effects of these drugs can be abolished by LUF7244 or a related compound.

Since both VU0405601 and ML-T531 dose-dependently increased the $K_v11.1$ current density and given the structural similarity of these compounds with LUF7244, it is very likely that also LUF7244 can directly act as a (allosteric) $K_v11.1$ activator. Accordingly, the ability of LUF7244 to counteract astemizole-, sertindole- and cisapride-related arrhythmogenesis may be the combined result of its inhibitory effect on the binding of these unintended $K_v11.1$ blockers to the channel and of its direct enhancing effect on the $K_v11.1$ channel's activity. However, the fact that exposure of NRVMs to 10 μ M LUF7244 alone did not significantly change AP characteristics suggests that LUF7244's $K_v11.1$ -activating activity is not of critical importance for its ability to suppress the proarrhythmic effects of inadvertent $K_v11.1$ blockers. Moreover, possible effects of LUF7244 on other cardiac ion channels besides the $K_v11.1$ channel must be considered. The absence of a noticeable change in AP shape and duration following exposure of NRVMs to LUF7244 also argues against a possible effect of this allosteric modulator on cardiac ion channels different from the $K_v11.1$ channel. In keeping with this notion, $Na_v1.5$, I_{Ks} and $K_v1.5$ activities were not affected or only slightly reduced by 50 μ M VU0405601.⁷ Likewise, ML-T531 at a final concentration of 10 μ M had a minor suppressive effect on I_{Ks} and did not influence $Na_v1.5$, $Ca_v1.2$, $K_v4.3$ or $Kir2.1$ activities.¹¹ Thus, the antiarrhythmic propensity of LUF7244 is dominated by its negative allosteric impact on the binding of typical $K_v11.1$ blockers to the channel.

The fact that LUF7244 significantly decreased the $K_v11.1$ affinity of drugs with very different chemical structures (**Figure 2B**) in membrane radioligand binding assays and prevented these drugs from causing APD prolongation in NRVMs makes it likely that LUF7244 will be effective in reducing the cardiotoxicity of a broad range of $K_v11.1$ blockers. Further support for this notion comes from the fact that VU0405601, which was here shown to inhibit dofetilide interaction with the $K_v11.1$ channel by a similar mechanism to LUF7244, could abolish the blockade of $K_v11.1$ by seven different drugs.⁷ The different degree

to which LUF7244 increased the K_i values of dofetilide, astemizole, sertindole and cisapride suggests that the sensitivity of different $K_v11.1$ blockers to conformational changes in the $K_v11.1$ proteins differs. Accordingly, different LUF7244 concentrations may be required to abrogate the proarrhythmic effects of distinct $K_v11.1$ blockers.

Although at a final concentration of 50 μM LUF7244's ability to weaken the interaction between [^3H]dofetilide and the $K_v11.1$ channel was similar to those of VU0405601 and ML-T531 (**Figure 1D**), the new modulator was much more effective than the other two compounds at a final concentration of 10 μM (**Figure 1C**). Moreover, even 50 μM VU0405601 only modestly inhibited the APD-prolonging effect of 1 μM dofetilide (K_i for human $K_v11.1$: 6.0 nM) on rabbit ventricular myocytes,⁷ while 10 μM LUF7244 totally blocked the APD prolongation caused by exposure of NRVMs to 100 nM astemizole (K_i for human $K_v11.1$: 1.2 nM). These findings together with the substantial decrease in the APD of normal rabbit ventricular myocytes caused by 50 μM VU0405601 (see above), suggests that LUF7244 may possess a more favorable safety profile than VU0405601 or ML-T531.

Clinical applicability of allosteric $K_v11.1$ modulators

A major concern and obstacle for medicinal chemists involved in the development of drugs to remedy congenital and acquired LQTS is excessive APD shortening resulting in the short QT syndrome (SQTS).^{5,40} For instance, at a concentration of 10 μM , the potent $K_v11.1$ activator ICA-105574 shortened the APD in Langendorff-perfused guinea pig hearts by 34% and induced non-TdP like ventricular arrhythmias in 2 out of 8 animals.⁴¹ Likewise, in a transgenic rabbit model of LQT1, shortening of the QTc interval by another $K_v11.1$ activator designated NS1643 was accompanied by an increased incidence of pacing-induced ventricular fibrillation.⁴² However, the fact that LUF7244 at a concentration that completely abolished the proarrhythmic effects of astemizole, sertindole and cisapride does not shorten the APD of NRVMs and therefore may not give rise to drug-induced short QT syndrome (SQTS) raises hope for its clinical applicability.

For the experiments in NRVM monolayers, the final astemizole concentration was 100 nM in most cases, while sertindole and cisapride were used at a final concentration of 1 μM . These concentrations are much higher than the therapeutic blood concentrations of these fortuitous $K_v11.1$ blockers, which are 2-50 $\mu\text{g}\cdot\text{L}^{-1}$ (i.e. 4.4-109 nM) for astemizole, 40-80 $\mu\text{g}\cdot\text{L}^{-1}$ (i.e. 86-172 nM) for cisapride and 50-100 $\mu\text{g}\cdot\text{L}^{-1}$ (i.e. 113-227 nM) for sertindole.⁴³ The proarrhythmic effects of these drugs on NRVMs were completely prevented by 10 μM (i.e. 3.7

mg·L⁻¹) LUF7244, a concentration that did not negatively influence the binding of astemizole to the hH₁R and did not noticeably affect the viability, contractility and excitability of the cardiomyocytes. Accordingly, blood LUF7244 concentrations well below 10 μM may suffice to counteract the proarrhythmic effects of unintended K_v11.1 blockers in humans. However, before LUF7244 or other allosteric modulators can be clinically applied as “co-drugs” or otherwise, they should be subjected to rigorous preclinical and clinical testing with appropriate pharmacokinetic and pharmacodynamics assessments to determine their benefit-risk profiles.

Limitations

Due to the difficulty to obtain and culture adult human ventricular myocytes, in this study NRVMs were used as 2D model system to investigate the effects of LUF7244 on K_v11.1 blockade-associated proarrhythmic changes in cardiac electrophysiology. However, adult human and neonatal rat ventricular myocytes have different AP morphologies because of qualitative and quantitative differences in the molecular components shaping the APs. Also, changes in cardiomyocyte electrophysiological properties may work out differently in a 2D cell layer than in the 3D heart. Nonetheless, studies on NRVM monolayers have greatly contributed to our current understanding of cardiac electrophysiology. Moreover, in spite of the differences in ventricular ion channel composition between humans and rats, their K_v11.1 proteins are very similar showing 95% amino acid identity for the largest isoforms. Consistently, the results of the radioligand binding assays, which were carried out with the human K_v11.1 protein, correlated very well with those of the optical mapping studies using NRVMs. Yet, ultimately, the ability of LUF7244 to counteract the proarrhythmic effects of unintended K_v11.1 blockers should be investigated in human subjects.

As mentioned above, LUF7244 did not inhibit the binding of astemizole to its intended target in radioligand binding assays and did not compromise the viability, excitability or contractility of NRVMs at a concentration sufficient to fully abrogate the proarrhythmic consequences of drug-induced K_v11.1 blockade in these cells. Despite these encouraging results, certainty about the lack of specific adverse/interfering effects of this negative allosteric modulator of K_v11.1 channel in humans can only be obtained through clinical studies. Moreover, now that the ability of LUF7244 to reduce the channel binding affinity of K_v11.1 blockers has been established, allosteric modulators with higher safety and/or efficacy than LUF7244 are likely to arise in the near future. The design of such compounds may benefit from the identification of the precise binding site of LUF7244 at the

K_v11.1 channel.

Conclusions

Allosteric modulators at the K_v11.1 channel could provide a new pharmacological treatment for drug-induced LQTS by preventing the potentially arrhythmogenic changes in AP characteristics caused by unintended K_v11.1 blockers. Through combined administration with a negative allosteric modulator, use of “old” drugs that have been banned because of their “K_v11.1 liability” may be resumed and new drugs with K_v11.1-blocking effects may not have to be excluded from clinical application.

References

1. Stockbridge, N.; Morganroth, J.; Shah, R. R.; Garnett, C. Dealing with global safety Issues. *Drug Saf.* **2013**, *36*, 167-182.
2. Antzelevitch, C. Ionic, molecular, and cellular bases of QT-interval prolongation and torsade de pointes. *Europace* **2007**, *9*, iv4-iv15.
3. Kannankeril, P.; Roden, D. M.; Darbar, D. Drug-induced long QT syndrome. *Pharmacol. Rev.* **2010**, *62*, 760-781.
4. Vandenberg, J. I.; Perry, M. D.; Perrin, M. J.; Mann, S. A.; Ke, Y.; Hill, A. P. hERG K⁺ channels: Structure, function, and clinical significance. *Physiol. Rev.* **2012**, *92*, 1393-1478.
5. Sanguinetti, M. C. hERG1 channel agonists and cardiac arrhythmia. *Curr. Opin. Pharmacol.* **2014**, *15*, 22-27.
6. Kang, J.; Chen, X.; Wang, H.; Ji, J.; Cheng, H.; Incardona, J.; Reynolds, W.; Viviani, F.; Tabart, M.; Rampe, D. Discovery of a small molecule activator of the human ether-à-go-go-related gene (hERG) cardiac K⁺ channel. *Mol. Pharmacol.* **2005**, *67*, 827-836.
7. Potet, F.; Lorinc, A. N.; Chaigne, S.; Hopkins, C. R.; Venkataraman, R.; Stepanovic, S. Z.; Lewis, L. M.; Days, E.; Sidorov, V. Y.; Engers, D. W.; Zou, B.; Afshartous, D.; George, A. L.; Campbell, C. M.; Balser, J. R.; Li, M.; Baudenbacher, F. J.; Lindsley, C. W.; Weaver, C. D.; Kupersmidt, S. Identification and characterization of a compound that protects cardiac tissue from human ether-à-go-go-related gene (hERG)-related drug-induced arrhythmias. *J. Biol. Chem.* **2012**, *287*, 39613-39625.
8. May, L. T.; Leach, K.; Sexton, P. M.; Christopoulos, A. Allosteric modulation of G protein-coupled receptors. *Annu. Rev. Pharmacol. Toxicol.* **2007**, *47*, 1-51.
9. Conn, P. J.; Christopoulos, A.; Lindsley, C. W. Allosteric modulators of GPCRs: A novel approach for the treatment of CNS disorders. *Nat. Rev. Drug Discov.* **2009**, *8*, 41-54.

10. Christopoulos, A.; Changeux, J.; Catterall, W. A.; Fabbro, D.; Burris, T. P.; Cidlowski, J. A.; Olsen, R. W.; Peters, J. A.; Neubig, R. R.; Pin, J.-P.; Sexton, P. M.; Kenakin, T. P.; Ehlert, F. J.; Spedding, M.; Langmead, C. J. International union of basic and clinical pharmacology. XC. multisite pharmacology: Recommendations for the nomenclature of receptor allosterism and allosteric ligands. *Pharmacol. Rev.* **2014**, *66*, 918-947.
11. Zhang, H.; Zou, B.; Yu, H.; Moretti, A.; Wang, X.; Yan, W.; Babcock, J. J.; Bellin, M.; McManus, O. B.; Tomaselli, G.; Nan, F.; Laugwitz, K. L.; Li, M. Modulation of hERG potassium channel gating normalizes action potential duration prolonged by dysfunctional KCNQ1 potassium channel. *Proc. Natl. Acad. Sci. U.S.A.* **2012**, *109*, 11866-11871.
12. Diaz, G. J.; Daniell, K.; Leitza, S. T.; Martin, R. L.; Su, Z.; McDermott, J. S.; Cox, B. F.; Gintant, G. A. The [³H]dofetilide binding assay is a predictive screening tool for hERG blockade and proarrhythmia: comparison of intact cell and membrane preparations and effects of altering [K⁺]_o. *J Pharmacol. Toxicol. Methods* **2004**, *50*, 187-199.
13. Ficker, E.; Jarolimek, W.; Kiehn, J.; Baumann, A.; Brown, A. M. Molecular determinants of dofetilide block of hERG K⁺ channels. *Circ. Res.* **1998**, *82*, 386-395.
14. Aronov, A. M. Predictive in silico modeling for hERG channel blockers. *Drug Discov. Today* **2005**, *10*, 149-155.
15. Shagufita; Guo, D.; Klaasse, E.; de Vries, H.; Brussee, J.; Nalos, L.; Rook, M. B.; Vos, M. A.; van der Heyden, M. A.; IJzerman, A. P. Exploring chemical substructures essential for hERG K⁺ channel blockade by synthesis and biological evaluation of dofetilide analogues. *ChemMedChem* **2009**, *4*, 1722-1732.
16. Ficker, E.; Dennis, A. T.; Wang, L.; Brown, A. M. Role of the cytosolic chaperones Hsp70 and Hsp90 in maturation of the cardiac potassium channel hERG. *Circ. Res.* **2003**, *92*, e87-e100.
17. Yu, Z.; Klaasse, E.; Heitman, L. H.; IJzerman, A. P. Allosteric modulators of the hERG K⁺ channel: Radioligand binding assays reveal allosteric characteristics of dofetilide analogs. *Toxicol. Appl. Pharmacol.* **2014**, *274*, 78-86.
18. Pijnappels, D. A.; Schalij, M. J.; Ramkisoensing, A. A.; van Tuyn, J.; de Vries, A. A.; van der Laarse, A.; Ypey, D. L.; Atsma, D. E. Forced alignment of mesenchymal stem cells undergoing cardiomyogenic differentiation affects functional integration with cardiomyocyte cultures. *Circ. Res.* **2008**, *103*, 167-176.
19. Askar, S. F.; Ramkisoensing, A. A.; Schalij, M. J.; Bingen, B. O.; Swildens, J.; van der Laarse, A.; Atsma, D. E.; de Vries, A. A.; Ypey, D. L.; Pijnappels, D. A. Antiproliferative treatment of myofibroblasts prevents arrhythmias *in vitro* by limiting myofibroblast-induced depolarization. *Cardiovasc. Res.* **2011**, *90*, 295-304.
20. Cheng, Y.; Prusoff, W. H. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 percent inhibition (I_{50}) of an enzymatic reaction. *Biochem. Pharmacol.* **1973**, *22*, 3099-3108.

21. Yu, Z.; IJzerman, A.; Heitman, L. K_v11.1 (hERG)-induced cardiotoxicity: A molecular insight from a binding kinetics study of prototypic K_v11.1 (hERG) inhibitors. *Br. J. Pharmacol.* **2015**, *172*, 940-945.
22. Himel, H. D.; Bub, G.; Lakireddy, P.; El-Sherif, N. Optical imaging of arrhythmias in the cardiomyocyte monolayer. *Heart Rhythm* **2012**, *9*, 2077-2082.
23. Redfern, W. S.; Carlsson, L.; Davis, A. S.; Lynch, W. G.; MacKenzie, I.; Palethorpe, S.; Siegl, P. K. S.; Strang, I.; Sullivan, A. T.; Wallis, R.; Camm, A. J.; Hammond, T. G. 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*, 32-45.
24. Heijman, J.; Voigt, N.; Carlsson, L. G.; Dobrev, D. Cardiac safety assays. *Curr. Opin. Pharmacol.* **2014**, *15*, 16-21.
25. Korhonen, T.; Hänninen, S. L.; Tavi, P. Model of excitation-contraction coupling of rat neonatal ventricular myocytes. *Biophys. J.* **2009**, *96*, 1189-1209.
26. Davie, C.; Pierre-Valentin, J.; Pollard, C.; Standen, N.; Mitcheson, J.; Alexander, P.; Thong, B. Comparative pharmacology of guinea pig cardiac myocyte and cloned hERG (I_{Kr}) channel. *J. Cardiovasc. Electrophysiol.* **2004**, *15*, 1302-1309.
27. Murakawa, Y. Focal and reentrant mechanisms of torsades de pointes: EAD, reentry, or chimera? *J. Arrhythm.* **2011**, *27*, 28-37.
28. Chang, M. G.; Sato, D.; de Lange, E.; Lee, J.-H.; Karagueuzian, H. S.; Garfinkel, A.; Weiss, J. N.; Qu, Z. Bi-stable wave propagation and early afterdepolarization-mediated cardiac arrhythmias. *Heart Rhythm* **2012**, *9*, 115-122.
29. Pedigo, N.; Yamamura, H.; Nelson, D. L. Discrimination of multiple [³H]5-hydroxytryptamine binding sites by the neuroleptic spiperone in rat brain. *J. Neurochem.* **1981**, *36*, 220-226.
30. van den Nieuwendijk, A. M.; Pietra, D.; Heitman, L.; Göblyös, A.; IJzerman, A. P. Synthesis and biological evaluation of 2, 3, 5-substituted [1, 2, 4] thiadiazoles as allosteric modulators of adenosine receptors. *J. Med. Chem.* **2004**, *47*, 663-672.
31. Vauquelin, G.; Van Liefde, I. Radioligand dissociation measurements: Potential interference of rebinding and allosteric mechanisms and physiological relevance of the biological model systems. *Expert Opin. Drug Discov.* **2012**, *7*, 583-595.
32. Schober, D. A.; Croy, C. H.; Xiao, H.; Christopoulos, A.; Felder, C. C. Development of a radioligand, [³H]LY2119620, to probe the human M₂ and M₄ muscarinic receptor allosteric binding sites. *Mol. Pharmacol.* **2014**, *86*, 116-123.
33. Gao, Z.; Van Muijlwijk-Koezen, J. E.; Chen, A.; Müller, C. E.; IJzerman, A. P.; Jacobson, K. A. Allosteric modulation of A₃ adenosine receptors by a series of 3-(2-pyridinyl) isoquinoline derivatives. *Mol. Pharmacol.* **2001**, *60*, 1057-1063.
34. Dror, R. O.; Green, H. F.; Valant, C.; Borhani, D. W.; Valcourt, J. R.; Pan, A. C.; Arlow, D. H.; Canals, M.; Lane, J. R.; Rahmani, R.; Baell, J. B.; Sexton, P. M.; Chris-

- topoulos, A.; Shaw, D. E. Structural basis for modulation of a G protein-coupled receptor by allosteric drugs. *Nature* **2013**, *503*, 295-299.
35. Kamiya, K.; Niwa, R.; Mitcheson, J. S.; Sanguinetti, M. C. Molecular determinants of hERG channel block. *Mol. Pharmacol.* **2006**, *69*, 1709-1716.
36. Pearlstein, R. A.; Vaz, R. J.; Kang, J.; Chen, X.; Preobrazhenskaya, M.; Shchekotikhin, A. E.; Korolev, A. M.; Lysenkova, L. N.; Miroshnikova, O. V.; Hendrix, J.; Rampe, D. Characterization of hERG potassium channel inhibition using CoMSiA 3D QSAR and homology modeling approaches. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 1829-1835.
37. García-Ferreiro, R. E.; Kerschensteiner, D.; Major, F.; Monje, F.; Stühmer, W.; Pardo, L. A. Mechanism of block of hEAG1 K⁺ channels by imipramine and astemizole. *J. Gen. Physiol.* **2004**, *124*, 301-317.
38. Perry, M.; Stansfeld, P. J.; Leaney, J.; Wood, C.; de Groot, M. J.; Leishman, D.; Sutcliffe, M. J.; Mitcheson, J. S. Drug binding interactions in the inner cavity of hERG channels: Molecular insights from structure-activity relationships of clofilium and ibutilide analogs. *Mol. Pharmacol.* **2006**, *69*, 509-519.
39. Nogawa, H.; Kawai, T. hERG trafficking inhibition in drug-induced lethal cardiac arrhythmia. *Eur. J. Pharmacol.* **2014**, *741*, 336-339.
40. Grunnet, M.; Hansen, R. S.; Olesen, S.-P. hERG1 channel activators: A new anti-arrhythmic principle. *Prog. Biophys. Mol. Biol.* **2008**, *98*, 347-362.
41. Meng, J.; Shi, C.; Li, L.; Du, Y.; Xu, Y. Compound ICA-105574 prevents arrhythmias induced by cardiac delayed repolarization. *Eur. J. Pharmacol.* **2013**, *718*, 87-97.
42. Bentzen, B. H.; Bahrke, S.; Wu, K.; Larsen, A. P.; Odening, K. E.; Franke, G.; Biermann, J.; Peng, X.; Koren, G.; Zehender, M.; Bode, C.; Grunnet, M.; Brunner, M. Pharmacological activation of K_v11.1 in transgenic long QT-1 rabbits. *J. Cardiovasc. Pharmacol.* **2011**, *57*, 223-230.
43. Schulz, M.; Iwersen-Bergmann, S.; Andresen, H.; Schmoldt, A. Therapeutic and toxic blood concentrations of nearly 1,000 drugs and other xenobiotics. *Critical Care* **2012**, *16*, R136.