Allosteric Modulation of K$_{\text{11.1}}$ (hERG) Channels Protects Against Drug-Induced Ventricular Arrhythmias

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**Background**—Ventricular arrhythmias as a result of unintentional blockade of the K$_{\text{11.1}}$ (hERG [human ether-à-go-go–related gene]) channel are a major safety concern in drug development. In past years, several highly prescribed drugs have been withdrawn for their ability to cause such proarrhythmia. Here, we investigated whether the proarrhythmic risk of existing drugs could be reduced by K$_{\text{11.1}}$ allosteric modulators.

**Methods and Results**—Using [3H]dofetilide-binding assays with membranes of human K$_{\text{11.1}}$-expressing human embryonic kidney 293 cells, 2 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$_{\text{11.1}}$ channel, with LUF7244 showing the strongest effect at 10 μmol/L. The K$_{\text{11.1}}$ affinities of typical blockers (ie, dofetilide, astemizole, sertindole, and cisapride) were significantly decreased by LUF7244. Treatment of confluent neonatal rat ventricular myocyte (NRVM) monolayers with astemizole or sertindole caused heterogeneous prolongation of action potential duration and a high incidence of early afterdepolarizations on 1-Hz electric point stimulation, occasionally leading to unstable, self-terminating 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.

**Conclusions**—Allosteric modulation of the K$_{\text{11.1}}$ channel efficiently suppresses drug-induced ventricular arrhythmias in vitro by preventing potentially arrhythmogenic changes in action potential characteristics, raising the possibility to resume the clinical use of unintended K$_{\text{11.1}}$ blockers via pharmacological combination therapy.

**Key Words:** arrhythmias, cardiac ‧ cardiotoxicity ‧ cell culture techniques ‧ myocytes, cardiac ‧ potassium voltage-gated channel, subfamily H, member 2 ‧ radioligand assay ‧ voltage-sensitive dye imaging

**Drug-induced ventricular arrhythmias (DIVAs)** are a frequently encountered clinical problem, which has resulted in restricted use or market withdrawal of existing cardiac and noncardiac drugs and still represents a major obstacle for the development of new drugs. Inhibition of the rapid component of the delayed rectifier K$^+$ current ($I_{\text{Kr}}$) has been identified as the major culprit in the development of DIVAs. The consequential slowing of cardiac repolarization, manifested in the surface ECG as a prolongation of the QT interval, increases the likelihood of early afterdepolarizations (EADs), which may give rise to ectopic beats. Drug-induced $I_{\text{Kr}}$ blockade also increases spatial dispersion of repolarization and refractoriness, thereby further increasing proarrhythmic risk. 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.
WHAT IS KNOWN

- Drug-induced long QT syndrome is characterized by prolongation of the QTc interval and associated with a distinctive form of polymorphic ventricular tachycardia known as Torsades de Pointes that may lead to palpitations, syncope, seizures, cardiac arrest and sudden cardiac death. Consequently, drug-induced long QT syndrome poses a major hurdle to the development of new drugs and has led to the restricted use or market withdrawal of a wide range of existing drugs.
- The major cause of drug-induced long QT syndrome is the slowed cardiac repolarization due to unintended binding of drugs to the central cavity of the Kv11.1 channel resulting in obstruction of outward K+ flow through the channel.

WHAT THE STUDY ADDS

- This study introduces allosteric modulation of the Kv11.1 channel as a new and effective strategy to reduce the unintended binding of drugs to the channel’s central cavity and to prevent the proarrhythmic changes associated with this binding.
- Allosteric modulators of the Kv11.1 channel may open new perspectives for the clinical use of drugs that were recalled from the market or have not been further approved because of their Kv11.1-blocking side effects via combination therapy.

The Kv11.1 protein, which is encoded by the KCNH2 gene (also known as ether-à-go-go–related gene 1 [ERG or ERG1]), represents the pore-forming α-subunit of the Ikr channel. Several structural features of Kv11.1 render the central cavity of the Ikr channel particularly susceptible to blockade by a heterogeneous collection of chemical compounds, including various noncardiac drugs (eg, astemizole, sertindole, and cisapride).

An obvious strategy to reduce the proarrhythmic risk of drugs with unintended Ikr-blocking effects is by lowering their Kv11.1 affinities via chemical modifications. Alternatively, supplementary drugs that decrease the proarrhythmic risk of inadvertent Kv11.1 blockers can be developed potentially allowing (1) reintroduction of medicines previously recalled from the market because of their Kv11.1-related cardiotoxicity and (2) admission of new drugs with fortuitous Ikr-blocking activity. Paradoxically, screening of drugs for possible Ikr-blocking side effects has resulted in the serendipitous discovery of various Kv11.1 activators. Besides their potential usefulness in treating inherited long QT syndrome, these Kv11.1 activators may also be used to counteract Kv11.1 blockade–associated DiVAs. Indeed, Kang et al showed that the action potential (AP) duration (APD)–prolonging effect of the If channel, class III antiarrhythmic drug dofetilide could be counterbalanced by the first identified synthetic Kv11.1 activator designated RPR260243. However, because of their APD-shortening effect, Kv11.1 activators may induce short QT syndrome. Recently, Potet et al described a compound designated VU0405601 that on pretreatment significantly reduced the APD-prolonging effect of dofetilide in Langendorff-perfused rabbit hearts and dose-dependently mitigated the Kv11.1-blocking effects of 7 different drugs in human embryonic kidney 293 (HEK293) cells stably overexpressing the human KCNH2 gene (HEK293Kv11.1 cells). VU0405601 exerted its effects on the Kv11.1 channel in whole-cell voltage-clamp experiments using HEK293Kv11.1 cells only when applied extracellularly. This suggests that VU0405601 binds to the extracellular domain of the Kv11.1 channel rather than to its central cavity, leading us to hypothesize that VU0405601 counteracts the APD-prolonging effect of Kv11.1 blockers by an allosteric mechanism.

Allosteric modulators bind their targets at a site topologically different from that of the endogenous ligand. From this so-called allosteric site, they generally display higher selectivity across receptor subtypes and thus provide a safer pharmacological profile than ligands binding to the orthosteric site. Notably, because the Kv11.1 channel does not have an endogenous ligand, the authors refer to the site where typical blockers (ie, dofetilide and astemizole) bind as the orthosteric site. Although allosteric modulators targeting ligand-gated ion channels and G protein–coupled receptors have been well established as research tools and therapeutic agents, little progress has been made in the discovery and clinical development of such compounds for voltage-gated ion channels.

In this study, in vitro radioligand-binding assays were used to investigate whether 2 previously reported compounds (ie, VU04056011 and ML-T53113) and a new compound designated LUF7244 could allosterically modulate binding of the potent Kv11.1 blocker dofetilide to the channel’s central cavity. Radioligand-binding assays were also used to study LUF7244’s influence on the interaction between (1) the Kv11.1 channel and 3 different blockbuster drugs (ie, astemizole, sertindole, and cisapride) that have been withdrawn from the market because of their Kv11.1-related cardiotoxicity and (2) astemizole and its intended target, the human histamine H1 receptor (see Data Supplement). 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 any of the 3 blockbuster drugs. Electrophysiological parameters analyzed included conduction velocity, 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 NRVMs were investigated (see Data Supplement). The results of this study indicate that negative allosteric modulation of the Kv11.1 channel may provide a safe and effective means to prevent the proarrhythmic effects of Ikr blockers that bind to the channel’s central cavity.
Materials and Methods

Radioligand-Binding Studies

Radioligand displacement assays and kinetic dissociation assays were performed on membranes of HEK293Kv11.1 cells using [3H]dofetilide as radioligand essentially as described earlier.15

Isolation and Culture of NRVMs

NRVMs were isolated from the hearts of neonatal rats and cultured as previously detailed.16 The use of these animals was 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.

Immunocytochemical Analyses

NRVMs were plated on fibronectin-coated, 15-mm diameter round glass coverslips at a density of 4x10⁴ cells. At day 9 of culture, the cells were washed with ice-cold phosphate-buffered saline (PBS), fixed in buffered 4% formaldehyde, and permeabilized with 0.1% Triton X-100 in PBS. Next, the cells were double-immunostained for Kv11.1 and α-actinin. 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) lasted for 2 hours. To visualize their nuclei, the cells were incubated with 10 μg/mL Hoechst 33342 in PBS. After each processing step, the cells were washed with PBS. Coverslips were mounted in VECTASHIELD mounting medium. Photomicrographs were obtained using a Nikon Eclipse 80i digital color camera-equipped fluorescence microscope.

Reverse Transcription–Quantitative Polymerase Chain Reaction Analyses

Total RNA extracted from NRVMs and from neonatal rat cardiac fibroblasts (maintained in NRVM medium) using the Qiagen RNeasy Mini kit was reverse transcribed with the Bio-Rad iScript cDNA synthesis kit, and the resulting cDNA was amplified by polymerase chain reaction using the Bioline SensiFAST SYBR NO-ROX kit. Polymerase chain reaction amplifications of rat KCNH2- and rat 18S rRNA (Rn18s)-specific cDNA (for primer sequences, see Data Supplement) were performed in a Bio-Rad CFX96 Touch Real-Time PCR detection system using a 2-step cycling protocol (20–40 cycles of 95°C 10 s, 60°C 30 s) after a 5-minute incubation at 95°C. Quantitative analyses were based on the 2−ΔΔCT method using Bio-Rad CFX Manager software.

Optical Mapping Experiments

Optical mapping experiments were done in confluent monolayers of NRVMs using the potentiometric dye di-4-ANEPPS [4-(2-(6-(dibutylamino)-2-naphthalenyl)ethenyl)-1-(3-sulfopropyl) pyridinium hydroxide, inner salt] as voltage indicator following previously described methods.17 To validate the experimental model, cells were incubated for 20 minutes in culture medium containing 0, 10, 30, 100, or 300 nmol/L of the human histamine H₁ receptor antagonist and unintended Kv11.1 blocker astemizole and dimethylsulfoxide at a final concentration of 0.03%. In a subsequent experiment, NRVM cultures were first exposed for 30 minutes to 10 μmol/L LUF7244 or its solvent (ie, culture medium containing 0.1% dimethylsulfoxide). Next, astemizole (final concentration of 100 nmol/L) or vehicle was added to the culture medium raising the dimethylsulfoxide concentration to 0.13%. After an incubation period of 30 minutes

Figure 1. Characterization of allosteric modulators of dofetilide binding to the Kv11.1 channel in a [3H]dofetilide-binding assay performed with membranes of HEK293Kv11.1 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 Kv11.1 channel after 6 minutes of dissociation induced by 10 μmol/L dofetilide in the absence (control) or presence of 10 μmol/L of VU0405601, ML-T531, or LUF7244. The specific binding of [3H]dofetilide in the absence of the test compounds was set as B₀ (control), whereas the specific binding in their presence was set as B. **P<0.01. D, Time-dependent dissociation of [3H]dofetilide induced by 10 μmol/L dofetilide in the absence (control) or presence of 50 μmol/L VU0405601, ML-T531, or LUF7244.
at 37°C, optical recordings were started in the continued presence of the appropriate vehicle/drug combinations.

Data Analysis
Radioligand-binding assay data were analyzed with GraphPad Prism 5.0 (GraphPad Software). Half-maximal inhibitory concentrations (ie, IC₅₀ values), apparent inhibitory binding constants (Kᵢ values), dissociation rate constants (kₐ), and half-maximal effective concentrations (ie, EC₅₀ values) were calculated as previously described. Values obtained from the radioligand-binding assays are from 3 different experiments each consisting of at least 2 independent samples. Data are expressed as mean±standard error of the mean (SEM) for the radioligand-binding assays or as mean±standard deviation (SD) for the optical voltage mapping experiments. Statistical analysis was done using nonparametric tests. The Mann–Whitney U test and the Kruskal–Wallis test were used for comparing 2 groups and >2 groups, respectively.

See the Data Supplement for more details about the materials and experimental procedures.

Results
Characterization of Allosteric Modulators of [³H] Dofetilde Binding to the Kᵥ11.1 Channel
The interaction of 2 previously reported ligands VU405601 and ML-T531 as well as the newly designed and synthesized compound LUF7244 (Figure 1A) with the human Kᵥ11.1 channel was studied in different [³H]dofetilde-binding assays. As shown in Figure 1B and Table I in the Data Supplement, all 3 compounds reduced [³H]dofetilde binding to the Kᵥ11.1 channel with relatively low affinities, that is, with IC₅₀ values of 7.8±0.4, 12±1, and 3.9±0.7 μmol/L for VU405601, ML-T531, and LUF7244, respectively. Moreover, all displacement curves demonstrated Hill coefficients different from unity (ie, −1.1±0.03 for ML-T531, −1.2±0.01 for VU405601, and −1.3±0.1 for LUF7244), implying that VU0405601, ML-T531, and LUF7244 might not competitively displace [³H]dofetilde from the Kᵥ11.1 channel but may bind elsewhere to the channel protein to allosterically modulate radioligand binding.

Subsequently, single-point dissociation assays were performed to screen for allosteric effects of these compounds on the binding of [³H]dofetilde to the Kᵥ11.1 channel. At a concentration of 10 μmol/L, VU405601, ML-T531, and LUF7244 significantly increased the dissociation of [³H]dofetilde from the Kᵥ11.1 channel, indicating that these compounds are negative allosteric modulators of dofetilde binding to the channel (Figure 1C; Table I in the Data Supplement). LUF7244 appeared to be the most potent negative allosteric modulator with 44±2% dofetilde binding left compared with control conditions, whereas 10 μmol/L VU405601 and ML-T531 reduced dofetilde binding to 63±3% and 77±3%, respectively.

The allosteric effects of VU405601, ML-T531, and LUF7244 on the Kᵥ11.1 channel were further investigated in traditional radioligand dissociation experiments to determine whether coadministration of these compounds with an excess unlabeled dofetilde would change the dissociation rate of [³H]dofetilde from the Kᵥ11.1 channel. To obtain larger effects, the 3 compounds were tested at a concentration of 50 μmol/L, instead of 10 μmol/L, as used in the single-point dissociation assays. As shown in Figure 1D and Table I in the Data Supplement, all compounds significantly accelerated the dissociation of dofetilde, in line with the results from the single-point dissociation experiments. The off-rate of [³H]dofetilde was allosterically increased 2.0-fold (from 0.21±0.02 to 0.42±0.04 min⁻¹) with 50 μmol/L VU405601. The kₐff value of [³H]dofetilde rose to 0.33±0.02 min⁻¹ in the presence of 50 μmol/L LUF7244, which was comparable to the effect of ML-T531 (kₐff,dofetilde=0.30±0.03 min⁻¹).

Effects of LUF7244 on the Binding of Typical Kᵥ11.1 Blockers to the Channel
Because LUF7244 was the most potent among the 3 allosteric modulators at the lower test concentration of 10 μmol/L and may therefore have the best safety profile, its potency to increase the dissociation of [³H]dofetilde from the Kᵥ11.1 channel was investigated. From the corresponding concentration–effect curve (Figure 2A), the modulatory potency (ie, the EC₅₀ of LUF7244 was calculated to be 4.6±0.8 μmol/L. Notably, LUF7244 could not completely abrogate [³H]dofetilde binding by accelerating its dissociation from the Kᵥ11.1 channel.

To investigate the effects of LUF7244 on the binding affinities of other compounds besides dofetilde to the Kᵥ11.1 channel, 3 additional Kᵥ11.1 blockers (ie, astemizole, sertindole, and cisapride) from distinct therapeutic classes were selected (Figure 2B). As shown in Figure 2C and 2D, the [³H]dofetilde displacement curves of all 4 drugs were shifted rightwards in the presence of 10 μmol/L LUF7244, implicating that their Kᵥ11.1 affinities were diminished by this negative allosteric modulator. The Kᵢ values of dofetilde, astemizole, sertindole, and cisapride in the absence or presence of 10 μmol/L LUF7244 are listed in Table II of the Data Supplement. LUF7244 most strongly modulated cisapride binding to the Kᵥ11.1 channel, increasing its Kᵢ value by 4.0-fold from 21±1 to 85±6 nmol/L. Similarly, the Kᵥ11.1 affinities of astemizole, dofetilde, and sertindole were reduced by 3.8-, 3.2-, and 2.2-fold in the presence of LUF7244. Thus, the negative allosteric effect of LUF7244 on the Kᵥ11.1 channel significantly lowered the channel’s affinities for several chemically and therapeutically distinct Kᵥ11.1 blockers.

Analysis of Kᵥ11.1 Protein Expression in NRVMs
Next, the electrophysiological consequences of allosteric modulation of the binding of typical Kᵥ11.1 blockers to the channel by LUF7244 (see Figure 3A for experimental setup) were examined in confluent monolayers of NRVMs (Figure 3B) as relevant in vitro model for studying cardiac arrhythmias. Double immunostaining for Kᵥ11.1 and sarcomeric α-actinin showed that all cardiomyocytes in the NRVM cultures expressed the Kᵥ11.1 protein (Figure 3C). The Kᵥ11.1 signal had a punctate or linear appearance and was concentrated around nuclei and along the sarcolemma (Figure 3C). No significant Kᵥ11.1 protein expression was observed in the low percentage of α-actinin⁺ cells (mainly neonatal rat cardiac fibroblasts) present in the NRVM cultures. Consistently, comparison of Kcnh2 transcript levels between NRVMs and neonatal rat cardiac fibroblasts by reverse transcription–polymerase chain reaction analysis demonstrated ±40-fold higher
Kcnh2 mRNA expression in NRVMs than in neonatal rat cardiac fibroblasts (Figure 3D).

Electrophysiological Consequences of Kv,11.1 Blockade by Astemizole in NRVMs

Because of its relatively high specificity for the Kv,11.1 channel, astemizole was selected to study the electrophysiological consequences of Kv,11.1 blockade by optical voltage mapping (Figure 4). NRVMs treated with astemizole displayed a concentration-dependent increase in APD and EAD incidence (Figure I in the Data Supplement). As displayed in Figure 4A and 4B, 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 nmol/L astemizole (n=24, P<0.0001 and P<0.001, respectively). Furthermore, exposure to 100 nmol/L astemizole resulted in the occurrence of EADs in 25% of the NRVM cultures, whereas no EADs were observed under control conditions (Figure 4C and 4D). As shown in Figure 4E and 4F, the APD_{40} dispersion between NRVMs in the presence of 100 nmol/L astemizole was significantly higher than that in its absence (39±11 versus 16±5 ms; P<0.0001), indicative of aggravated repolarization heterogeneity because of inhibition of I_{Kr} by astemizole. Occasionally, the astemizole-induced APD prolongation resulted in short-lasting, irregular tachyarrhythmias (Figure II in the Data Supplement) reminiscent of spontaneously terminating TdP episodes. Importantly, as indicated by the activation maps (Figure 4G) and corresponding quantitative analysis (Figure 4H), the conduction velocity in NRVM cultures was not
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significantly influenced by 100 nmol/L astemizole (20±4 cm/s versus 21±3 cm/s in control cultures; \( P = 0.1254 \)). Collectively, these data validate the utility of NRVM cultures as an in vitro model for investigating the AP-prolonging and associated pro-arrhythmic effects of Kv11.1 blockers like astemizole.

**Effects of LUF7244 on Kv11.1 Blockade–Associated Proarrhythmic Changes in NRVM Cultures**

To investigate the functional consequences of negative allosteric modulation of Kv11.1’s interaction with typical Kv11.1 blockers, di-4-ANNEPS-loaded NRVM cultures were incubated for 20 minutes in culture medium containing 10 μmol/L LUF7244 before addition of astemizole, sertindole, or cisapride. Because the \( K_i \) values of sertindole and cisapride for Kv11.1 are >10× higher than that of astemizole (Table II in the Data Supplement), these drugs were tested at a final concentration of 1 μmol/L instead of 100 nmol/L as was used for astemizole. After incubation for 30 minutes, the NRVMs were optically mapped. As shown in Figure 5A, the APD-prolonging and EAD-promoting effects of astemizole were effectively suppressed by LUF7244. APD\(_{40}\) and APD\(_{90}\) were significantly shortened from 156±39 to 118±18 ms (n=24; \( P < 0.0001 \)) and from 355±66 to 282±63 ms (n=24; \( P < 0.001 \)), respectively, that is, LUF7244 was able to reduce APD\(_{40}\) and APD\(_{90}\) to control values (Figure 5B). Moreover, in the presence of 10 μmol/L LUF7244, EADs were no longer observed in NRVM cultures exposed to 100 nmol/L astemizole (Figure 5C). LUF7244 also prevented the increase in APD\(_{40}\) dispersion caused by astemizole (Figure 5D and 5E). Importantly, the conduction velocities in 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 μmol/L, LUF7244 per se did not

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Figure 3. Biochemical characterization of the neonatal rat ventricular myocyte (NRVM) model. A, Basic setup 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 Kv11.1 protein expression in NRVM cultures. The Kv11.1 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 noncardiomyocytes. D, Analysis of Kcnh2 mRNA levels in NRVMs and neonatal rat cardiac fibroblasts (NRCFs) by reverse transcription–polymerase chain reaction.

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reduce APD or significantly affect APD_40 dispersion (Figure 5F–5H; Figure III in the Data Supplement), suggesting that this negative allosteric modulator poses little, if any, risk for the development of arrhythmias associated with abnormal APD shortening. Similar results of LUF7244 were obtained in NRVM cultures exposed to sertindole or cisapride (Figure IV in the Data Supplement). LUF7244 can thus suppress the proarrhythmic side effects of drugs from different therapeutic classes by allosteric modulation of the Kv11.1 channel without exerting, by itself, any obvious adverse electrophysiological effects on cardiomyocytes.

**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 Kv11.1 channel and the class III antiarrhythmic agent dofetilide, as well as the unintended Kv11.1 blockers astemizole, sertindole, and cisapride. VU405601, ML-T531, and LUF7244 exerted their negative effects on the binding of typical Kv11.1 blockers to the channel’s central cavity by an allosteric mechanism. Importantly, LUF7244 decreased the Kv11.1 affinity of astemizole without influencing its affinity at the human histamine H1 receptor, being astemizole’s intentional target (Figure V in the Data Supplement). 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 Kv11.1 blockade-related proarrhythmic risk. Pretreatment of NRVMs with 10 μmol/L 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 (Figure VI in the Data Supplement). These findings provide a rationale for further exploring allosteric modulation as a strategy to prevent DiVAs.
Many different methods have been exploited to assess drugs for their Kv11.1 blockade-associated arrhythmogenicity. The interaction between a drug and the Kv11.1 channel is usually first investigated by computational and biochemical assays. Next, the Kv11.1 liabilities of suspicious drugs are typically evaluated by electrophysiological measurements on Chinese hamster ovary or HEK293 cells expressing the human Kv11.1 channel. Despite their practical advantages, these nonexcitable cellular models do not recapitulate the complex regulatory circuits governing Kv11.1 channel activity in cardiomyocytes and are unsuitable for studying AP generation and propagation. To overcome these shortcomings, human-induced pluripotent stem cell–derived cardiomyocytes are now often used for cardiotoxicological screenings. However, this is the first study using NRVMs to test compounds for their ability to prevent DiVAs resulting from unintended Kv11.1 blockade.

Although mechanistic insight into DiVAs is limited, there is broad consensus about spatial dispersion of repolarization and EAD-induced triggered activity providing the substrate and trigger for the genesis of drug-induced TdP, respectively. The EADs typically arise during phase 2 of the cardiac AP because of drug-dependent decreases in $I_{Kr}$, causing increases of APD and QT interval. In support of the validity

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\text{NRVM Monolayers as Model for Studying Drug-Induced Long QT Syndrome}
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![Figure 5. Assessment by optical voltage mapping of the ability of LUF244 to counteract the proarrhythmic effects of astemizole on neonatal rat ventricular myocyte (NRVMs). Representative filtered optical signal traces (A and F), action potential (AP) duration (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 nmol/L astemizole or with 10 μmol/L LUF244 followed by 100 nmol/L astemizole immediately before optical voltage mapping. Pretreatment of NRVM cultures with LUF244 completely prevented the astemizole-induced APD prolongation (APD$_{40}$ and APD$_{90}$; B), occurrence of early afterdepolarizations (EADs; C), and increase in APD$_{40}$ dispersion (D and E). Treatment of NRVM cultures with 10 μmol/L LUF244 only did not change AP morphology (F), APD (G), or APD$_{40}$ dispersion (H). ***P<0.001 and ****P<0.0001. DMSO indicates dimethylsulfoxide.](http://circep.ahajournals.org/).
of our model, astemizole dose-dependently increased APD and EAD incidence in monolayers of NRVMs (Figure I in the Data Supplement). Also, sertindole and cisapride had APD-prolonging effects (Figure IVA and IVD in the Data Supplement), and each of the 3 fortuitous K_{1.1} blockers increased APD dispersion (Figure 4F; Figure IVC and IVF in the Data Supplement). Although 100 nmol/L astemizole, 1 μmol/L sertindole, and 1 μmol/L cisapride increased APD and APD dispersion to a similar extent, cisapride did not significantly increase EAD incidence in contrast to the other 2 drugs (Figure 4D; Figure IVB and IVE in the Data Supplement). This finding may be explained by cisapride’s inhibitory effect on the I_{Ca,L} in NRVMs.25

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.26,27 Although we did observe several instances of short-lasting, irregular tachyarrhythmias exclusively in NRVM cultures exposed to K_{1.1} blockers, they did not allow us to make specific claims about the underlying electrophysiological mechanisms. Pretreatment with LUF7244 rendered NRVM monolayers unsusceptible to the K_{1.1}-blocking effects of astemizole, sertindole, and cisapride. In the presence of LUF7244, these K_{1.1} blockers no longer caused heterogeneous APD prolongation and, because of 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_{Ks} 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_{1.1}-related cardiotoxicity.

LUF7244's Mode of Action
The chemical structure of LUF7244 resembles those of ML-T531 and VU0405601. In a recent study, 10 μmol/L of ML-T531 was shown to reduce the APD of human-induced pluripotent stem cell–derived cardiomyocytes from an LQT1 patient to that of control cells by augmenting I_{Ks}.11 However, the effects of ML-T531 on the APD of human-induced pluripotent stem cell–derived cardiomyocytes from a healthy individual and ML-T531’s ability to inhibit the APD-prolonging effects of unintended K_{1.1} blockers were not investigated. Voltage clamp recordings of K_{1.1}-expressing Chinese hamster ovary cells showed that ML-T531 reduces the deactivation rate of the K_{1.1} channel and causes a shift of its inactivation curve toward more positive voltages. Shortly after the discovery of ML-T531, VU0405601 was identified as a compound that, at a final concentration of 5 μmol/L, protected Langendorff-perfused rabbit hearts from the proarrhythmic effects of exposure to 100 nmol/L dofetilide.7 Although VU0405601 only partially reversed the dofetilide-dependent increase in APD, its administration before dofetilide strongly reduced the pacing-induced arrhythmia incidence from 42% to 4%, which was close to the 2% of pacing-induced premature ventricular contractions observed in untreated hearts.7 Exposure of isolated rabbit ventricular myocytes to 5 μmol/L of VU0405601 only marginally reduced APD, which is consonant with our finding that 10 μmol/L LUF7244 did not noticeably affect the APD of NRVMs. However, at a final concentration of 50 μmol/L, VU0405601 decreased the APD_{50} and APD_{90} of rabbit ventricular myocytes by 35±6% and 32±4%, respectively. Patch-clamp analysis of HEK293K_{1.1} cells linked the APD-shortening effect of 50 μmol/L 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_{Ks}.

In this study, we found that VU0405601, ML-T531, and LUF7244 displayed comparatively low K_{1.1} affinities, with Hill coefficients significantly different from unity for their [1H]dofetilide displacement curves. This suggests that these ligands bind to the K_{1.1} channel at sites distinct from that of dofetilide, indicative of an allosteric mode of action.10,28,29 The binding of a drug to a receptor at an allosteric site (ie, 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 (ie, orthosteric) binding site.10,30 Altered ligand dissociation rates have been found representative of allosteric interactions in various drug targets, such as muscarinic and adenosine receptors.10,31–33 Consistently, VU0405601, ML-T531, and LUF7244 significantly accelerated the dissociation of dofetilide from the K_{1.1} channel, strengthening the conclusion that they are negative allosteric modulators of dofetilide binding to the K_{1.1} 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_{1.1} channel,34 whereas astemizole, cisapride, dofetilide, and sertindole all bind to the channel’s central cavity from inside.34–36 In addition, the [1H]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.10,33 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_{1.1} channel. Binding of VU0405601, ML-T531, and LUF7244 likely alters the 3D structure of the K_{1.1} channel, which decreases its affinity for typical K_{1.1} blockers by increasing the dissociation rates of these blockers from the channel. Notably, the vast majority of fortuitous K_{1.1} blockers exert their effects by occupying the central cavity of the K_{1.1} channel and thereby obstructing the transport of K⁺ ions through the channel’s pore.37 There are, however, also examples of drugs that reduce I_{Ks} by inhibiting, directly or indirectly, the trafficking of K_{1.1} to the plasma membrane.38 Given their specific mode of action, it is unlikely that the I_{Ks}-inhibiting effects of these drugs can be abolished by LUF7244 or a related compound.

The ability of LUF7244 to counteract astemizole-, sertindole-, and cisapride-related arrhythmogenesis may be the combined result of its inhibitory allosteric effect on the binding of these unintended K_{1.1} blockers to the channel and of its direct enhancing effect on the K_{1.1} channel’s activity. Indeed, at concentrations ≥25 μmol/L, LUF7244 caused APD
shortening (Figure III in the Data Supplement), suggesting that LUF7244 can directly act as (allosteric) K\(_{11.1}\) activator like VU0405601 and ML-T531.\(^7\)\(^1\) However, the fact that exposure of NRVMs to 10 \(\mu\)mol/L LUF7244 alone did not significantly change AP characteristics suggests that LUF7244’s K\(_{11.1}\)-activating activity is not of critical importance for its ability to suppress the proarrhythmic effects of inadvertent K\(_{11.1}\) blockers. The absence of a noticeable change in AP shape and duration after exposure of NRVMs to 10 \(\mu\)mol/L LUF7244 also argues against a possible effect of this allosteric modulator on cardiac ion channels different from the K\(_{11.1}\) channel. In keeping with this notion, Na\(_{1.5}\) and K\(_{1.5}\) currents and \(I_\text{Kr}\) were not affected or only slightly reduced by 50 \(\mu\)mol/L VU0405601.\(^7\) Likewise, ML-T531 at a final concentration of 10 \(\mu\)mol/L had a minor suppressive effect on \(I_\text{Kr}\), and did not influence Na\(_{1.5}\), Ca\(_{1.2}\), K\(_{4.3}\), or Kir2.1 activities.\(^1\) Thus, the antiarrhythmic propensity of LUF7244 is dominated by its negative allosteric impact on the binding of typical K\(_{11.1}\) blockers to the channel.

Because LUF7244 significantly decreased the K\(_{11.1}\) affinities of drugs with different chemical structures (Figure 2B) in radioligand-binding assays and prevented these drugs from causing APD prolongation in NRVMs, LUF7244 may be effective in reducing the cardio toxicity of a broad range of K\(_{11.1}\) blockers. Further support for this notion comes from the fact that VU0405601, which was here shown to inhibit dofetilide’s interaction with the K\(_{11.1}\) channel by a similar mechanism to LUF7244, could abolish the blockade of K\(_{11.1}\) by 7 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\(_{11.1}\) blockers to conformational changes in the K\(_{11.1}\) proteins differs. Accordingly, different LUF7244 concentrations may be required to abrogate the proarrhythmic effects of distinct K\(_{11.1}\) blockers.

Although at a final concentration of 50 \(\mu\)mol/L, LUF7244’s ability to weaken the interaction between [\(^3\)H]dofetilide and the K\(_{11.1}\) channel was similar to those of VU0405601 and ML-T531 (Figure 1D), the new modulator was much more effective than the other 2 compounds at a final concentration of 10 \(\mu\)mol/L (Figure 1C). Moreover, even 50 \(\mu\)mol/L VU0405601 only modestly inhibited the APD-prolonging effect of 1 \(\mu\)mol/L dofetilide (\(K_I\) for human K\(_{11.1}\): 6.0 \(\mu\)mol/L) on rabbit ventricular myocytes,\(^7\) whereas 10 \(\mu\)mol/L LUF7244 totally blocked the APD prolongation caused by exposure of NRVMs to 100 nmol/L astemizole (\(K_I\) for human K\(_{11.1}\): 1.2 nmol/L). These findings together with the substantial decrease in the APD of normal rabbit ventricular myocytes caused by 50 \(\mu\)mol/L VU0405601 (see above) suggest that LUF7244 may possess a more favorable safety profile than VU0405601 or ML-T531.

Limitations
Because of the difficulty to obtain and culture adult human ventricular myocytes, NRVMs were used as 2D model system to investigate the effects of LUF7244 on K\(_{11.1}\) blockade–associated proarrhythmic changes in cardiac electrophysiology. However, ventricular adult human and neonatal rat cardiomyocytes 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\(_{11.1}\) proteins are similar, showing 95% amino acid identity for the largest isoforms. Consistently, the results of the radioligand-binding assays, which were performed with the human K\(_{11.1}\) protein, correlated well with those of the optical mapping studies using NRVMs. Yet, ultimately, the ability of LUF7244 to counteract the proarrhythmic effects of unintended K\(_{11.1}\) blockers should be investigated in human subjects.

As mentioned earlier, LUF7244 did not inhibit the binding of astemizole to its intended target in radioligand-binding assays (Figure V in the Data Supplement) and did not compromise the viability, excitability, or contractility of NRVMs (Figure VI in the Data Supplement) at a concentration sufficient to fully abrogate the proarrhythmic consequences of drug-induced K\(_{11.1}\) blockade in these cells. Despite these encouraging results, certainty about the lack of specific adverse/interfering effects in humans of this negative allosteric modulator of the K\(_{11.1}\) channel can only be obtained through clinical studies. Moreover, now that the ability of LUF7244 to reduce the channel-binding affinities of K\(_{11.1}\) blockers has been established, allosteric modulators with higher safety and efficacy than LUF7244 are likely to arise in the near future. The design of such compounds may benefit from identification of the precise binding site of LUF7244 at the K\(_{11.1}\) channel.

Conclusions
Allosteric modulators of the K\(_{11.1}\) channel could provide a new pharmacological treatment for drug-induced long QT syndrome by preventing the potentially arrhythmogenic changes in AP characteristics caused by unintended K\(_{11.1}\) blockers. Through combined administration with a negative allosteric modulator, use of old drugs that have been banned because of their K\(_{11.1}\) liabilities may be resumed, and new drugs with K\(_{11.1}\)-blocking effects may not have to be excluded from clinical application.

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Disclosures

None.

References

Allosteric Modulation of K\(_{\text{r}11.1}\) (hERG) Channels Protects Against Drug-Induced Ventricular Arrhythmias

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SUPPLEMENTAL MATERIAL

Materials and Detailed Methods

Materials
Dofetilide was synthesized in-house as previously described. 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) and astemizole (specific activity: 78.4 Ci/mmol) were obtained from PerkinElmer (Groningen, the Netherlands). The synthesis and chemical analysis of VU0405601, ML-T531, as well as the design, synthesis and chemical analysis of LUF7244 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. HEK293K_v11.1 cells, i.e., HEK293 cells stably overexpressing the human KCNH2 gene, were kindly provided by Dr. Eckhard Ficker (Case Western Reserve University, Cleveland, OH). Plasmid pcDNA3.1-hH1Rwt encoding the human histamine H_1 receptor (hH_1R) was a gift of Prof. Thue W. Schwartz (University of Copenhagen, Copenhagen, Denmark). This plasmid was used to generate HEK293hH_1R cells, i.e., cells transiently overexpressing the human HRH_1 gene (see “HEK293 Cell Culture and Transfection”).

Methods

HEK293 Cell Culture and Transfection
HEK293 cells were cultured in a humidified atmosphere at 37°C and 7% CO_2 in Dulbecco’s modified Eagle’s medium (DMEM; Sigma-Aldrich, catalog number: D6546) supplemented with 10% fetal bovine serum (Sarstedt, Nümbrecht, Germany), 50 IU/mL penicillin (Sarstedt) and 50
μg/mL 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). The resulting HEK293hH1R cells were harvested 48 hours after transfection.

**Preparation of Membrane Samples**

HEK293Kv11.1 cells were cultured and membranes were prepared and stored as detailed previously. Membranes of HEK293hH1R cells were prepared and stored as described for HEK293Kv11.1 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 with [3H]dofetilide**

[3H]Dofetilide binding assays for the Kv11.1 channel were performed in incubation buffer II as described previously. 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 filter-bound radioactivity was determined using a Wallac 1450 MicroBeta TriLux liquid scintillation counter (PerkinElmer) after extraction with 25 µL MicroScint 20 (PerkinElmer).

**Radioligand Displacement Assays with [³H]astemizole**

[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 hours. 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 (50 mM Tris-HCl [pH 7.4]). Samples were processed further as described under “Radioligand Displacement Assays with [³H]dofetilide”.

**Kinetic Dissociation Assays with [³H]dofetilide**

Kinetic dissociation assays of [³H]dofetilide were performed as described previously 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 hours. After 6 minutes of dissociation, incubations were terminated and samples were obtained as described under “Radioligand Displacement Assays with [³H]dofetilide”. 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 hours 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 minutes of dissociation, incubations were terminated and samples were obtained as described under “Radioligand Displacement Assays with [3H]dofetilide”. The non-radioabeled dofetilide was applied at a concentration 1667-fold higher than the Kᵢ for the interaction of dofetilide with Kv11.1 (Table II), to avoid a significant contribution of reassociation of the radioligand with the channel to the results of the kinetic dissociation assays.

**Isolation and Culture of Neonatal Rat Ventricular Myocytes (NRVMs)**

NRVMs were isolated and cultured as described previously. 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 minutes at 37°C in a humidified atmosphere of 5% CO₂ 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 8×10⁵ cells/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 hours in culture medium containing 10 µg/mL mitomycin C (Sigma-Aldrich) at 24 hours 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:
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 $4 \times 10^4$ cells. At day 9 of culture, the cells were washed thrice with ice-cold phosphate-buffered saline (PBS), fixed for 30 minutes 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 Kv11.1 (rabbit polyclonal antibodies raised against the C terminus of human Kv11.1, 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 hours. To visualize their nuclei, the cells were incubated for 10 minutes at RT with 10 µg/mL 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 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′ GTAACCCGTTGAACCCCATT 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 Voltage Mapping

Action potentials (APs) were investigated on a whole-culture scale by optical voltage mapping using the potentiometric dye di-4-ANEPPS (Life Technologies Europe) as described previously. The measurements were carried out at 37°C on 9-day-old confluent NRVM monolayer cultures (Figure 3B in the Main Manuscript). 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 minutes in culture medium containing 0, 10, 30, 100 or 300 nM of the hH1R antagonist and unintended Kv11.1 blocker astemizole and dimethylsulfoxide (DMSO) at a final concentration of 0.03%. In a subsequent experiment, NRVM cultures were first exposed for 30
minutes 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 minutes at 37°C, optical recordings were started in the continued presence of the appropriate vehicle/drug combinations (see Figure 3A in the Main Manuscript for a scheme of the basic experimental setup). 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. Conduction velocity (CV), AP duration (APD) at 40 and 90% repolarization (APD$_{40}$ and APD$_{90}$, respectively), APD dispersion and early afterdepolarizations (EADs) incidence 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 Kv11.1-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 Kv11.1 allosteric modulation in the absence and presence of an unintended Kv11.1 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
20×/0.3 Ph1 objective (Zeiss Nederland, Sliedrecht, the Netherlands) to record their contractions
upon electrical field stimulation using two epoxy-coated platinum electrodes with an
interelectrode spacing of 13 mm. The electrical stimulus consisted of a 1.5-ms rectangular pulse
of 12V produced at a frequency of 1 Hz by a model EV4543 miniature temporary cardiac
pacemaker (PACE Medical, Waltham, MA).

**Apoptosis Assay**

To investigate the effects of long-term Kv11.1 allosteric modulation in the absence or presence of
simultaneous Kv11.1 blockade on cell viability, externalized phosphatidylserine (as early marker
of apoptosis) was detected using Alexa Fluor-568-conjugated annexin V (Life Technologies
Europe). NRVM cultures in 24-well plates were treated cells for 72 hours with 100 nM
astemizole and/or 10 µM LUF7244 as described under “Optical Voltage Mapping”. Mock-
treated cells and cells incubated for 24 hours in culture medium containing 1 µM doxorubicin
(Sigma-Aldrich) served as negative and positive controls, respectively. Next, the cells were
washed once with ice-cold PBS and incubated for 15 minutes 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 CaCl₂)
of 4°C containing 10 µg/mL 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 GraphPad Prism 5.0 (GraphPad
Software, San Diego, CA). Half maximal inhibitory concentrations (i.e., IC₅₀ values) in
displacement assays were directly obtained from non-linear regression analysis of dose-response curves. Half maximal inhibitory concentrations (i.e., IC50 values) in displacement assays were directly obtained from non-linear regression analysis of dose-response relationships by four-parameter logistic curve fitting. Apparent inhibitory binding constants (Ki values) were derived from the IC50 values according to the Cheng-Prusoff relationship6: Ki=IC50/(1+[L*]/KD), where [L*] is the concentration of radioligand and KD is its dissociation constant determined by saturation assay7. Dissociation rate constants (koff) were obtained by computer analysis of the exponential decay of [3H]dofetilide bound to the Kv11.1 channel. Half maximal effective concentrations (i.e., EC50 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. Values obtained from the radioligand binding assays are means of three independent experiments performed in duplicate. The number of samples per experimental group in the optical voltage mapping experiments varied from 6 to 24 as indicated. Data are expressed as mean±standard error of the mean (SEM) for the radioligand binding assays or as mean±standard deviation (SD) for the optical voltage mapping experiments. Statistical analysis was done using non-parametric tests. The Mann-Whitney U test and the Kruskal-Wallis test were used for comparing two groups and more than two groups, respectively.

Results

Comparison of the Concentration-dependent Effects of LUF7244 and VU0405601 on APD in NRVMs

Given the known APD-shortening effect of VU04056018 in rabbit ventricular cardiomyocytes and the similarities in chemical structure between LUF7244 and VU0405601, a side-by-side comparison of the concentration-dependent effects of these 2 compounds on the APD of NRVMs
was performed. Consistent with the results shown in Figure 5F and 5G of the Main Manuscript, at a final concentration of 10 µM, LUF7244 did not significantly affect the APD in NRVMs as assessed by optical voltage mapping (Figure IIIA). In contrast, exposure of NRVM monolayers to 25 or 62.5 µM of LUF7244 decreased APD$_{40}$ and significantly decreased APD$_{90}$ to a similar extent. While the APD-shortening effect of LUF7244 was first observed at a final concentration of 25 µM, VU0405601 already caused a decrease of APD$_{40}$ and a significant decrease of APD$_{90}$ at a final concentration of 10 µM (Figure IIIB). Raising the VU0405601 concentration to 25 or 62.5 µM did not lead to further APD shortening. Moreover, LUF7244 and VU0405601 had very similar maximum effects on the APD. Importantly, neither LUF7244 nor VU0405601 significantly changed the conduction velocity in the NRVM cultures at the 3 concentrations tested. These findings suggest that both LUF7244 and VU0405601 can increase $I_{Kr}$ density but that VU0405601 is a more potent K$_v$11.1 activator than LUF7244.

**Effects of LUF7244 on the Binding of $[^3]$HAstemizole to the hH$_1$R**

To be of practical use in preventing drug-induced ventricular arrhythmias, LUF7244 should not interfere with the desired activities of unintended K$_v$11.1 blockers. We therefore tested whether LUF7244 affected binding of the antihistamine drug astemizole to the hH$_1$R. As displayed in Figure VA, LUF7244 did not decrease the binding of $[^3]$Hastemizole to the hH$_1$R. Furthermore, the displacement curve of astemizole binding to the hH$_1$R was not significantly shifted rightwards by LUF7244 (Figure VB). Thus, LUF7244 reduces the affinity of the K$_v$11.1 channel for astemizole without affecting the binding of astemizole to its intended target receptor (i.e., the hH$_1$R).
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 Kv11.1 channel on the viability of cardiomyocytes, 6-day-old NRVM cultures were incubated for 72 hours in culture medium containing 10 µM 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 hours with the proapoptotic drug doxorubicin displayed strong annexin V fluorescence (Figure VIA). These results indicate that prolonged exposure to 10 µM 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 hours with 10 µM 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 4 and 5 of the Main Manuscript with Figure VIB and VIC) 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 (see Supplemental Movies 1 through 4).

Discussion

Clinical Applicability of Allosteric Kv11.1 Modulators

A major concern and obstacle for medicinal chemists involved in the development of drugs to remedy congenital and acquired long QT syndrome is excessive APD shortening resulting in short QT syndrome (SQTS). For instance, at a concentration of 10 µM, the potent Kv11.1
activator ICA-105574 shortened the APD in Langendorff-perfused guinea pig hearts by 34% and induced non-"Torsades de Pointes"-like ventricular arrhythmias in 2 out of 8 animals.\textsuperscript{11}

Likewise, in a transgenic rabbit model of LQT1, shortening of the QTc interval by another Kv\textsubscript{11.1} activator designated NS1643 was accompanied by an increased incidence of pacing-induced ventricular fibrillation.\textsuperscript{12} 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 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 Kv\textsubscript{11.1} blockers, which are 2-50 µg/L (i.e., 4.4-109 nM) for astemizole, 40-80 µg/L (i.e., 86-172 nM) for cisapride and 50-100 µg/L (i.e., 113-227 nM) for sertindole.\textsuperscript{13} The proarrhythmic effects of these drugs on NRVMs were completely prevented by 10 µM (i.e., 3.7 mg/L) LUF7244, a concentration that did not negatively influence the binding of astemizole to the hH\textsubscript{1}R and did not noticeably affect the viability, contractility and excitability of the cardiomyocytes (see “Effects of Prolonged Exposure of NRVMs to LUF7244 and/or Astemizole on Cell Viability, Excitability and Contractility”). Accordingly, blood LUF7244 concentrations well below 10 µM may suffice to counteract the proarrhythmic effects of unintended Kv\textsubscript{11.1} blockers in humans. However, before LUF7244 or other Kv\textsubscript{11.1} 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.
References:


**Supplemental Table I:** The half maximal inhibitory concentrations (IC$_{50}$), percentage specific binding of [$^3$H]dofetilide to the Kv11.1 channel after 6 minutes of dissociation in the absence (control) or presence of 10 µM indicated compounds (%B/B$_{control}$), and dissociation rate (k$_{off}$) of [$^3$H]dofetilide in the absence (control) or presence of 50 µM indicated compounds.

<table>
<thead>
<tr>
<th></th>
<th>IC$_{50}$ (nM)</th>
<th>%B/B$_{control}$</th>
<th>k$_{off}$ (min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dofetilide</td>
<td>-</td>
<td>100±0.01</td>
<td>0.21±0.02</td>
</tr>
<tr>
<td>+VU0405601</td>
<td>7,757±350</td>
<td>63±3**</td>
<td>0.42±0.04**</td>
</tr>
<tr>
<td>+ML-T531</td>
<td>12,000±1,213</td>
<td>77±3**</td>
<td>0.30±0.03*</td>
</tr>
<tr>
<td>+LUF7244</td>
<td>3,855±724</td>
<td>44±2**</td>
<td>0.33±0.02**</td>
</tr>
</tbody>
</table>

Values for comparison are means (±SEM) of six samples except that the k$_{off}$ values of dofetilide in the absence of the indicated compounds or in the presence of 50 µM ML-T531 are derived from seven samples. *P<0.05 vs control and **P<0.01 vs control.
**Supplemental Table II**: The $K_v$11.1 affinities ($K_i$) of dofetilide, astemizole, sertindole and cisapride in the absence (control) or presence of 10 µM LUF7244.

<table>
<thead>
<tr>
<th></th>
<th>$K_i$ (nM)</th>
<th>$K_i + 10$ µM LUF7244 (nM)</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>dofetilide</td>
<td>6.2±0.3</td>
<td>20±5*</td>
<td>3.2</td>
</tr>
<tr>
<td>astemizole</td>
<td>0.97±0.04</td>
<td>3.7±0.2**</td>
<td>3.8</td>
</tr>
<tr>
<td>sertindole</td>
<td>21±2</td>
<td>47±7**</td>
<td>2.2</td>
</tr>
<tr>
<td>cisapride</td>
<td>21±1</td>
<td>85±6**</td>
<td>4.0</td>
</tr>
</tbody>
</table>

Values for comparison are means (±SEM) of six samples. *P<0.05 vs control and **P<0.01 vs control.
**Supplemental Figure I:** Assessment, by optical voltage mapping, of the effect of different concentrations of astemizole on APD40 and APD90 (A) and EAD incidence (B) in NRVM monolayers. *P<0.05, ***P<0.001 and ****P<0.0001.
**Supplemental Figure II:** Optical voltage trace showing an unstable, self-terminating tachyarrhythmia in an astemizole-treated NRVM culture following 1-Hz electrical point stimulation.
Supplemental Figure III: Assessment, by optical voltage mapping, of the effect of different concentrations of LUF7244 (A) or VU0405601 (B) on APD40 and APD90. Depicted are the pairwise average changes in APD (ΔAPD) caused by the treatment of NRVM cultures with vehicle or the indicated concentrations of LUF7244 or VU0405601 for 30 min. *P<0.05 and **P<0.01 vs vehicle-treated NRVM cultures. No significant differences in APD40 and APD90 were observed between the cell cultures exposed to 25 and 62.5 µM LUF7244 and between the NRVM monolayers treated with 10, 25 or 62.5 µM VU0405601 (n=6 per group).
**Supplemental Figure IV:** 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 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 µM sertindole (A, B and C) or 1 µM cisapride (D, E and F). *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001.
Supplemental Figure V: Assessment of the effects of LUF7244 on the binding of astemizole to the hH1R in a [3H]astemizole binding assay performed with membranes of HEK293hH1R cells. A: Displacement curve of LUF7244. B: Displacement curve of astemizole in the absence or presence of 10 µM LUF7244.
**Figure VI:** Effects of prolonged exposure of NRVMs to LUF7244 and/or astemizole on cell viability and excitability. A: Analysis by Alexa Fluor-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 µM LUF7244, 100 nM astemizole or 10 µM LUF7244 plus 100 nM astemizole showed very weak signals, while NRVMs incubated for 24 hours with 1 µM of the proapoptotic drug doxorubicin displayed strong annexin V-associated red fluorescence. The blue fluorescence corresponds to cell nuclei stained with the DNA-binding dye Hoechst 33342. B: Quantitative comparison of APD40 and APD90 in NRVM cultures exposed for 72 hours to vehicle, 10 µM LUF7244, 100 nM astemizole or 10 µM LUF7244 plus 100 nM astemizole. **P<0.01. C: Quantitative comparison of CV in NRVM cultures exposed for 72 hours to vehicle, 10 µM LUF7244, 100 nM astemizole or 10 µM LUF7244 plus 100 nM astemizole (n=6 per group).
Movie Legends:

**Movie 1:** Contractile behavior, following 1-Hz electrical stimulation, of NRVMs exposed for 72 hours to vehicle (i.e., DMSO).

**Movie 2:** Contractile behavior, following 1-Hz electrical stimulation, of NRVMs exposed for 72 hours to 10 µM LUF7244.

**Movie 3:** Contractile behavior, following 1-Hz electrical stimulation, of NRVMs exposed for 72 hours to 100 nM astemizole.

**Movie 4:** Contractile behavior, following 1-Hz electrical stimulation, of NRVMs exposed for 72 hours to 10 µM LUF7244 plus 100 nM astemizole.