Original Article

Allosteric Modulation of K_v11.1 (hERG) Channels Protects Against Drug-Induced Ventricular Arrhythmias

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Background—Ventricular arrhythmias as a result of unintentional blockade of the K_v11.1 (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 In past years, several highly prescribed drugs have been withdrawn for their ability to cause such proarrhythmia.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_v11.1 allosteric modulators.

Methods and Results—Using [^{3}H]dofetilide-binding assays with membranes of human K_v11.1-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_v11.1 channel, with LUF7244 showing the strongest effect at 10 μmol/L. The K_v11.1 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_v11.1 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_v11.1 blockers via pharmacological combination therapy.

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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.[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 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_{Kr} blockade also increases spatial dispersion of repolarization and refractoriness, thereby further increasing proarrhythmic risk.[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]

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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 K_v11.1 channel resulting in obstruction of outward K^+ flow through the channel.

WHAT THE STUDY ADDS

- This study introduces allosteric modulation of the K_v11.1 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 K_v11.1 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 K_v11.1-blocking side effects via combination therapy.

The K_v11.1 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 I_{Kr} channel.[4] Several structural features of K_v11.1 render the central cavity of the I_{Kr} 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 I_{Kr}-blocking effects is by lowering their K_v11.1 affinities via chemical modifications. Alternatively, supplementary drugs that decrease the proarrhythmic risk of inadvertent K_v11.1 blockers can be developed potentially allowing (1) reintroduction of medicines previously recalled from the market because of their K_v11.1-related cardiotoxicity and (2) admission of new drugs with fortuitous I_{Kr}-blocking activity. 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, 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, because of their APD-shortening effect, K_v11.1 activators may induce short QT syndrome.[5] 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 K_v11.1-blocking effects of 7 different drugs in human embryonic kidney 293 (HEK293) cells stably overexpressing the human *KCNH2* gene (HEK293K_v11.1 cells).[7] VU0405601 exerted its effects on the K_v11.1 channel in whole-cell voltage-clamp experiments using HEK293K_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 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.[8] Notably, because the K_v11.1 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,[8–10] 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, VU0405601[7] and ML-T531[11]) and a new compound designated LUF7244 could allosterically modulate binding of the potent K_v11.1 blocker dofetilide to the channel's central cavity.[12,13] Radioligand-binding assays were also used to study LUF7244's influence on the interaction between (1) the K_v11.1 channel and 3 different blockbuster drugs (ie, astemizole, sertindole, and cisapride) that have been withdrawn from the market because of their K_v11.1-related cardiotoxicity[14] and (2) astemizole and its intended target, the human histamine H_1 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 (APD_{40} and APD_{90}, 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 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

Radioligand-Binding Studies

Radioligand displacement assays and kinetic dissociation assays were performed on membranes of HEK293K_v11.1 cells using [^{3}H] 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 4×10^4 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 $2^{-\Delta\Delta CT}$ 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 H_1 receptor antagonist and unintended K_v11.1 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

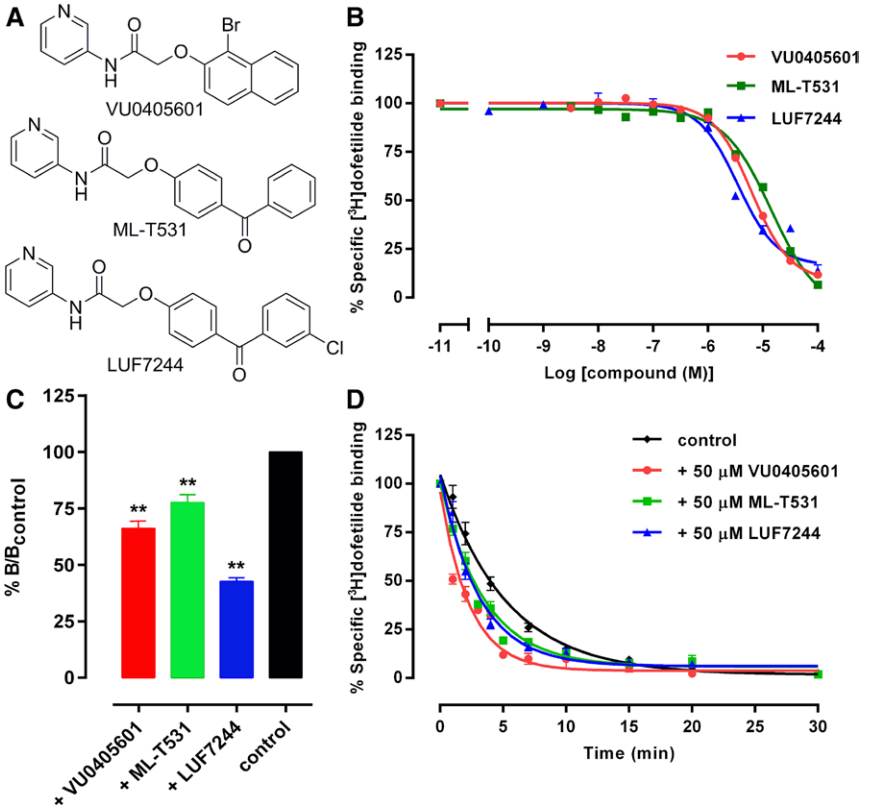

Figure 1. Characterization of allosteric modulators of dofetilide binding to the K_v11.1 channel in a [^{3}H]dofetilide-binding assay performed with membranes of HEK293K_v11.1 cells. **A**, Chemical structures of VU0405601, ML-T531, and LUF7244. **B**, Displacement curves of VU0405601, ML-T531, and LUF7244. **C**, Percentage-specific binding of [^{3}H]dofetilide to the K_v11.1 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 [^{3}H]dofetilide in the absence of the test compounds was set as $B_{control}$, whereas the specific binding in their presence was set as *B*. ***P*<0.01. **D**, Time-dependent dissociation of [^{3}H]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, IC_{50} values), apparent inhibitory binding constants (K_i values), dissociation rate constants (k_{off}), and half-maximal effective concentrations (ie, EC_{50} values) were calculated as previously described.[18] 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 [^{3}H] Dofetilide Binding to the K_v11.1 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 K_v11.1 channel was studied in different [^{3}H]dofetilide-binding assays. As shown in Figure 1B and Table I in the Data Supplement, all 3 compounds reduced [^{3}H]dofetilide binding to the K_v11.1 channel with relatively low affinities, that is, with IC_{50} values of 7.8±0.4, 12±1, and 3.9±0.7 µmol/L for VU0405601, 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 VU0405601, and −1.3±0.1 for LUF7244), implying that VU0405601, ML-T531, and LUF7244 might not competitively displace [^{3}H]dofetilide from the K_v11.1 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 [^{3}H]dofetilide to the K_v11.1 channel. At a concentration of 10 µmol/L, VU0405601, ML-T531, and LUF7244 significantly increased the dissociation of [^{3}H]dofetilide from the K_v11.1 channel, indicating that these compounds are negative allosteric modulators of dofetilide 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% dofetilide binding left compared with control conditions, whereas 10 µmol/L VU0405601 and ML-T531 reduced dofetilide binding to 63±3% and 77±3%, respectively.

The allosteric effects of VU0405601, ML-T531, and LUF7244 on the K_v11.1 channel were further investigated in traditional radioligand dissociation experiments to determine whether coadministration of these compounds with an excess unlabeled dofetilide would change the dissociation rate of [^{3}H]dofetilide from the K_v11.1 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 dofetilide, in line with the results from the single-point dissociation experiments. The off-rate of [^{3}H]dofetilide was allosterically increased 2.0-fold (from 0.21±0.02 to 0.42±0.04 min^{-1}) with 50 µmol/L VU0405601. The k_{off} value of [^{3}H]dofetilide 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 ($k_{off,dofetilide}$=0.30±0.03 min^{-1}).

Effects of LUF7244 on the Binding of Typical K_v11.1 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 [^{3}H]dofetilide from the K_v11.1 channel was investigated. From the corresponding concentration–effect curve (Figure 2A), the modulatory potency (ie, the EC_{50}) of LUF7244 was calculated to be 4.6±0.8 µmol/L. Notably, LUF7244 could not completely abrogate [^{3}H]dofetilide binding by accelerating its dissociation from the K_v11.1 channel.

To investigate the effects of LUF7244 on the binding affinities of other compounds besides dofetilide to the K_v11.1 channel, 3 additional K_v11.1 blockers (ie, 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 4 drugs were shifted rightwards in the presence of 10 µmol/L LUF7244, implicating that their K_v11.1 affinities were diminished by this negative allosteric modulator. The K_i values of dofetilide, 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 K_v11.1 channel, increasing its K_i value by 4.0-fold from 21±1 to 85±6 nmol/L. Similarly, the K_v11.1 affinities of astemizole, dofetilide, 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 K_v11.1 channel significantly lowered the channel's affinities for several chemically and therapeutically distinct K_v11.1 blockers.

Analysis of K_v11.1 Protein Expression in NRVMs

Next, the electrophysiological consequences of allosteric modulation of the binding of typical K_v11.1 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.[19] 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 3C). The K_v11.1 signal had a punctate or linear appearance and was concentrated around nuclei and along the sarcolemma (Figure 3C). No significant K_v11.1 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

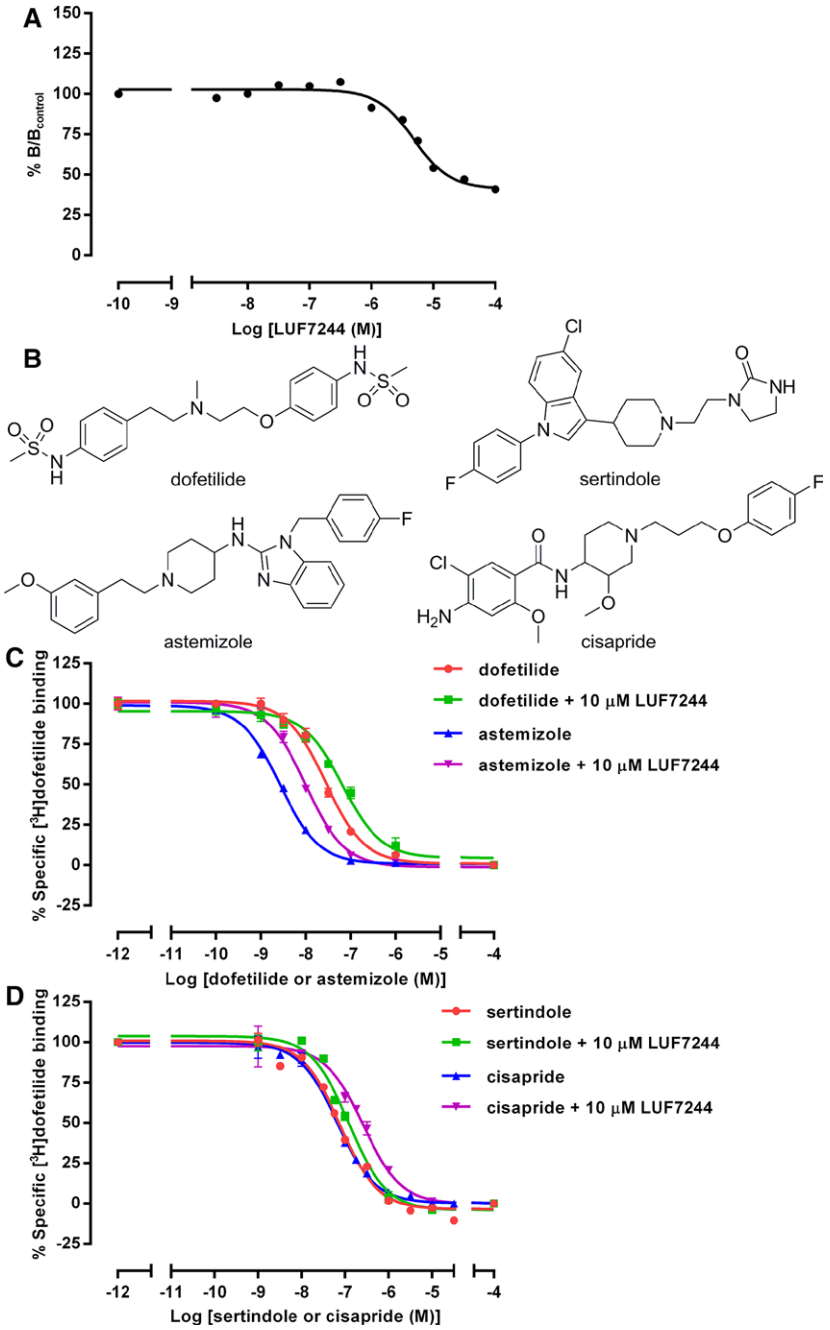

Figure 2. Assessment of the effects of LUF7244 on the binding of K_v11.1 blockers to the channel in a [^{3}H]dofetilide-binding assay performed with membranes of HEK293K_v11.1 cells. **A**, Effect of different concentrations of LUF7244 on the dofetilide-induced dissociation of [^{3}H]dofetilide from the K_v11.1 channel. After preincubating HEK293K_v11.1 cell membranes with [^{3}H]dofetilide, radioligand dissociation was induced by 10 μmol/L dofetilide in the absence or presence of different concentrations of LUF7244, and the incubation was terminated after 6 minutes. Results are expressed as the ratio of the specific binding of [^{3}H]dofetilide in the presence of 10 μmol/L dofetilide plus various concentrations of negative allosteric modulators (*B*) over that in the presence of 10 μmol/L 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 μmol/L LUF7244. **D**, Displacement curves of sertindole and cisapride in the absence or presence of 10 μmol/L LUF7244.

Kcnh2 mRNA expression in NRVMs than in neonatal rat cardiac fibroblasts (Figure 3D).

Electrophysiological Consequences of K_v11.1 Blockade by Astemizole in NRVMs

Because of its relatively high specificity for the K_v11.1 channel,[20] astemizole was selected to study the electrophysiological consequences of K_v11.1 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±5ms; 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

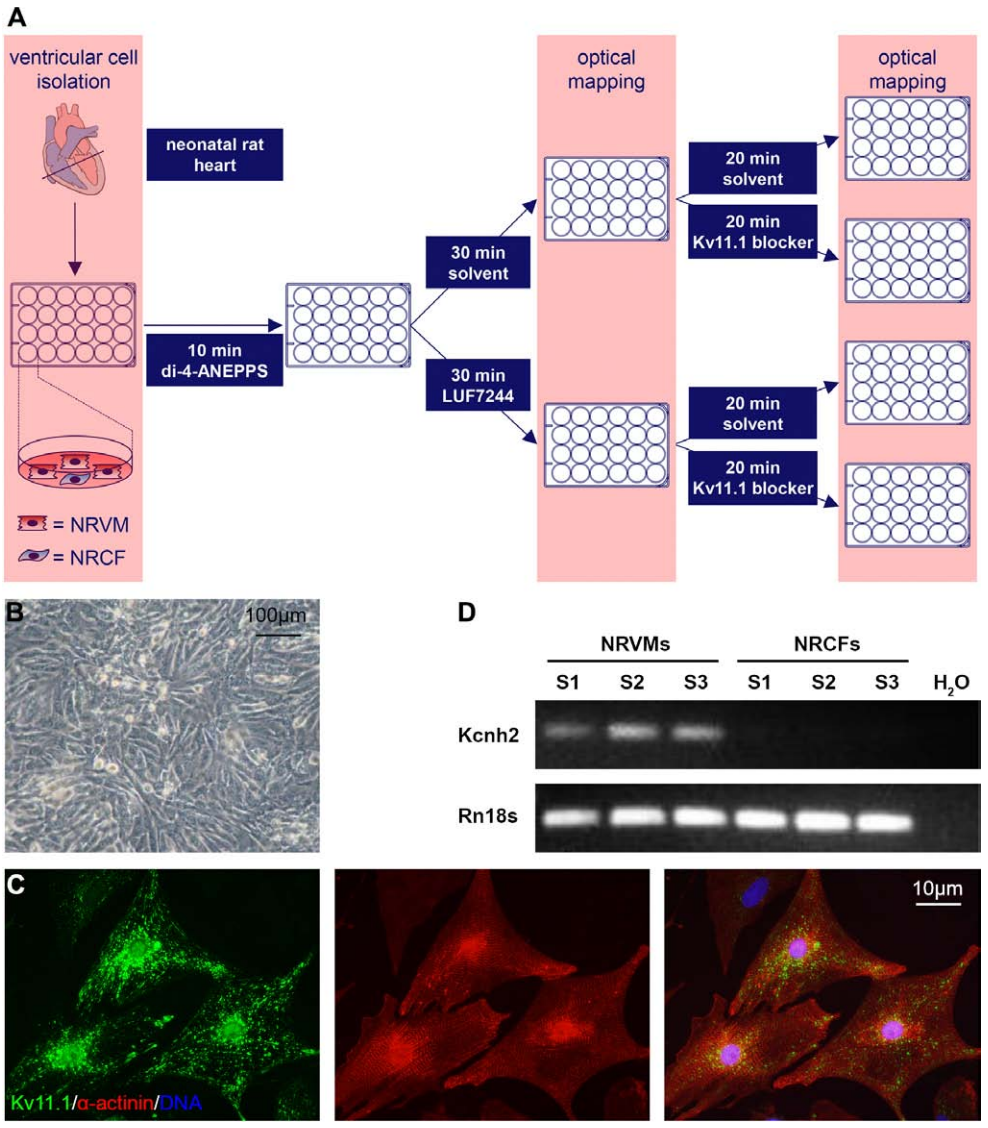

Figure 3. Biochemical characterization of the neonatal rat ventricular myocyte (NRVM) model. **A**, Basic setup of the optical voltage mapping experiments. **B**, Phase-contrast image of a typical confluent NRVM monolayer used for optical voltage mapping. **C**, Immunocytological analysis of K_v11.1 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 noncardiomyocytes. **D**, Analysis of *Kcnh2* mRNA levels in NRVMs and neonatal rat cardiac fibroblasts (NRCFs) by reverse transcription–polymerase chain reaction.

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 proarrhythmic effects of K_v11.1 blockers like astemizole.

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 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 K_v11.1 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

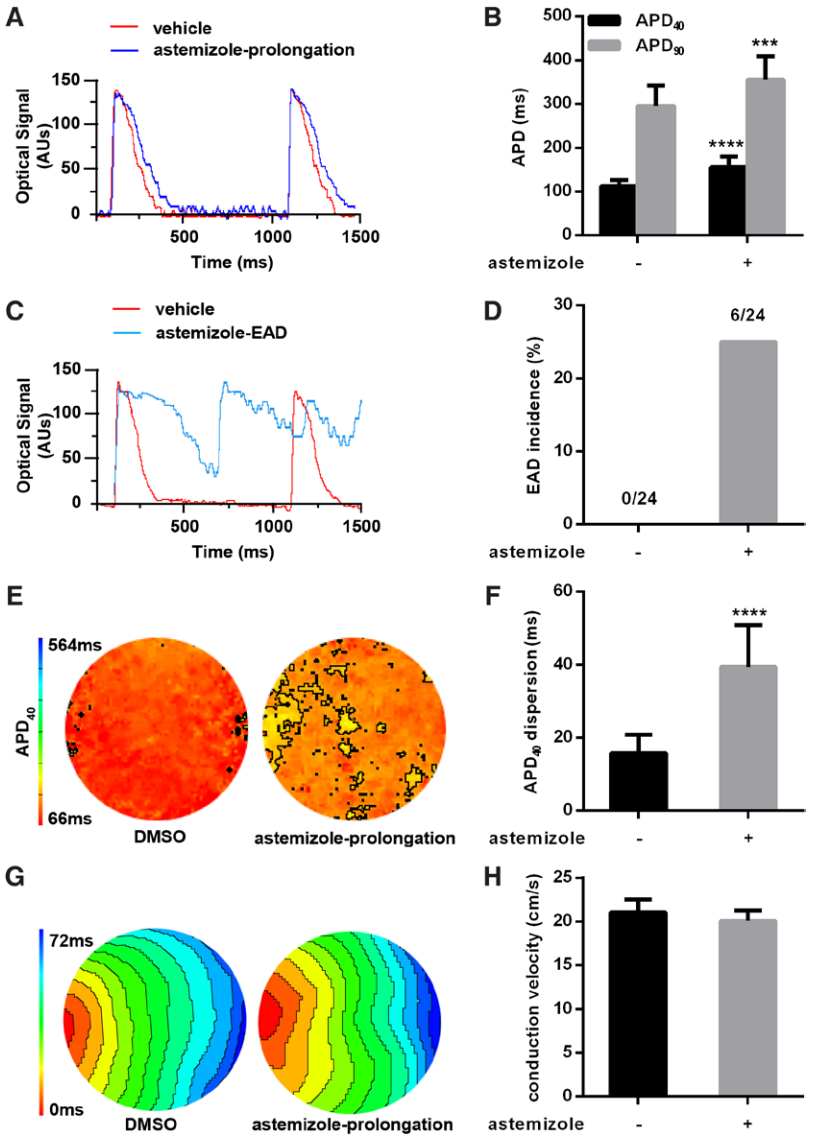

Figure 4. Electrophysiological characterization of the neonatal rat ventricular myocyte (NRVM) model by optical voltage mapping after 1 Hz electric stimulation. Representative filtered optical signal traces (**A** and **C**), corresponding action potential duration (APD) dispersion and activation maps (**E** and **G**, respectively), and quantitative analyses (bar graphs in **B**, **D**, **F,** and **H**) of NRVM cultures exposed for 30 minutes to 100 nmol/L astemizole or vehicle (control) and subjected to optical voltage mapping immediately afterward. Astemizole treatment resulted in APD prolongation (APD_{40} and APD_{90}, **A** and **B**), the occurrence of early afterdepolarizations (EADs; **C** and **D**), and an increase in APD_{40} dispersion (**E** and **F**) but did not significantly alter conduction velocity (CV; **G** and **H**). ***P<0.001 and ****P<0.0001. DMSO indicates dimethylsulfoxide.

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 K_v11.1 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 K_v11.1 channel and the class III antiarrhythmic agent dofetilide, as well as the unintended K_v11.1 blockers astemizole, sertindole, and cisapride. VU405601, 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 human histamine H_1 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 K_v11.1 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.

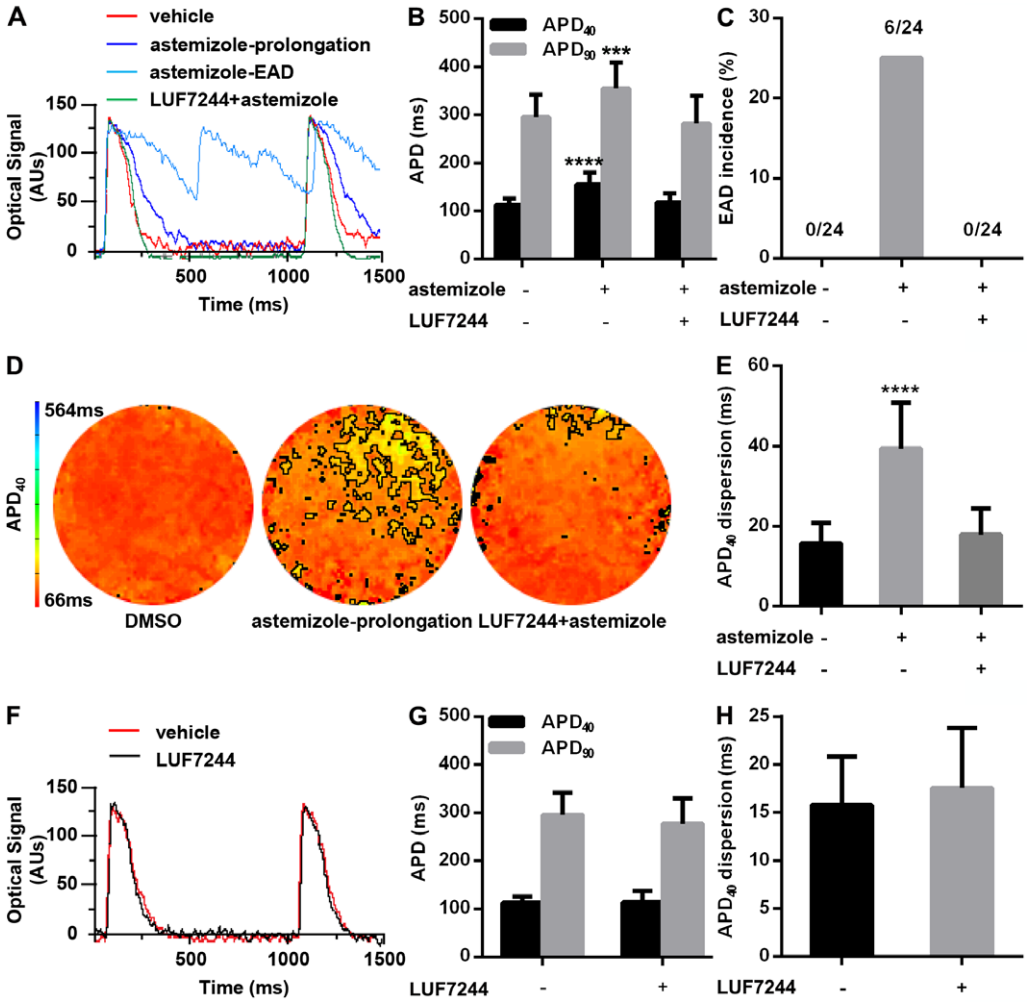

Figure 5. Assessment by optical voltage mapping of the ability of LUF7244 to counteract the proarrhythmic effects of astemizole on neonatal rat ventricular myocyte (NRVMs). Representative filtered optical signal traces (**A** and **F**), action potential (AP) duration (APD) dispersion maps (**D**), and 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 LUF7244 followed by 100 nmol/L astemizole immediately before optical voltage mapping. Pretreatment of NRVM cultures with LUF7244 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 LUF7244 only did not change AP morphology (**F**), APD (**G**), or APD_{40} dispersion (**H**). ***P<0.001 and ****P<0.0001. DMSO indicates dimethylsulfoxide.

NRVM Monolayers as Model for Studying Drug-Induced Long QT Syndrome

Many different methods have been exploited to assess drugs for their K_v11.1 blockade-associated arrhythmogenicity.[3,21] 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 liabilities of suspicious drugs are typically evaluated by electrophysiological measurements on Chinese hamster ovary or HEK293 cells expressing the human K_v11.1 channel. Despite their practical advantages, these nonexcitable 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. To overcome these shortcomings, human-induced pluripotent stem cell–derived cardiomyocytes are now often used for cardiotoxicological screenings.[22] However, thus far, human-induced pluripotent stem cell–derived cardiomyocytes cannot produce dense monolayers of phenotypically homogeneous ventricular cardiomyocytes for high-resolution optical mapping.[23] We hence used NRVM monolayers to study the effects of LUF7244 on the proarrhythmic potential of 3 inadvertent K_v11.1 blockers by optical voltage mapping. The reasons to specifically use NRVMs for this purpose are that these cells (1) can be relatively easily isolated and cultured, (2) are well-characterized, and (3) express functional K_v11.1 channels.[24] However, this is the first study using NRVMs 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 consensus 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 because of 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 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_v11.1 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_v11.1 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_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, 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_{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 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_{Kr}.[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_v11.1 blockers were not investigated. Voltage clamp recordings of K_v11.1-expressing Chinese hamster ovary cells showed that ML-T531 reduces the deactivation rate of the K_v11.1 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_v11.1 cells linked the APD-shortening effect of 50 μmol/L VU0405601 to shifts in the $V_{½}$ 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 affinities, with Hill coefficients significantly different from unity for their [^{3}H]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.[10,28,29] 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_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] whereas astemizole, cisapride, dofetilide, and sertindole all bind to the channel's central cavity from inside.[34–36] In addition, the [^{3}H]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_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. Notably, 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.[37] 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.[38] 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.

The ability of LUF7244 to counteract astemizole-, sertindole-, and cisapride-related arrythmogenesis may be the combined result of its inhibitory allosteric 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. 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_v11.1 activator like VU0405601 and ML-T531.[7,11] However, the fact that exposure of NRVMs to 10 μmol/L 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. The absence of a noticeable change in AP shape and duration after exposure of NRVMs to 10 μmol/L 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 and K_v1.5 currents and I_{Ks} were not affected or only slightly reduced by 50 μmol/L VU0405601.[7] Likewise, ML-T531 at a final concentration of 10 μmol/L 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.

Because LUF7244 significantly decreased the K_v11.1 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 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's interaction with the K_v11.1 channel by a similar mechanism to LUF7244, could abolish the blockade of K_v11.1 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_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 μmol/L, LUF7244's ability to weaken the interaction between [^{3}H]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 2 compounds at a final concentration of 10 μmol/L (Figure 1C). Moreover, even 50 μmol/L VU0405601 only modestly inhibited the APD-prolonging effect of 1 μmol/L dofetilide (K_i for human K_v11.1: 6.0 nmol/L) on rabbit ventricular myocytes,[7] whereas 10 μmol/L LUF7244 totally blocked the APD prolongation caused by exposure of NRVMs to 100 nmol/L astemizole (K_i for human K_v11.1: 1.2 nmol/L). These findings together with the substantial decrease in the APD of normal rabbit ventricular myocytes caused by 50 μmol/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_v11.1 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_v11.1 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_v11.1 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_v11.1 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_v11.1 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_v11.1 channel can only be obtained through clinical studies. Moreover, now that the ability of LUF7244 to reduce the channel-binding affinities of K_v11.1 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_v11.1 channel.

Conclusions

Allosteric modulators of the K_v11.1 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_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 liabilities may be resumed, and new drugs with K_v11.1-blocking effects may not have to be excluded from clinical application.

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Disclosures

None.

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