Identification and Characterization of a Compound That Protects Cardiac Tissue from Human Ether-à-go-go-related Gene (hERG)-related Drug-induced Arrhythmias*

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Franck Potet‡‡, Amanda N. Lorinc¶¶, Sebastien Chaigne‡, Corey R. Hopkins§§, Raghav Venkataraman¶, Svetlana Z. Stepanovic¶, L. Michelle Lewis§, Emily Days§, Veniamin Y. Sidorov¶, Darren W. Engers¶, Beiyan Zou**,**, David Afshartous‡‡, Alfred L. George, Jr.††††, Courtney M. Campbell§§§, Jeffrey R. Balser¶, Min Li**, Franz J. Baudenbacher§, Craig W. Lindsley§, C. David Weaver¶, and Sabina Kupershmidt**

From the ‡Department of Anesthesiology, the ¶¶Center for Neuroscience Drug Discovery, the §§Department of Biomedical Engineering, Institute for Chemical Biology, **Department of Biostatistics, §§Department of Pharmacology, and **‡‡Department of Medicine, Vanderbilt University, Nashville, Tennessee 37232 and the **Johns Hopkins Ion Channel Center, School of Medicine, Johns Hopkins University, Baltimore, Maryland 21205

Background: Inhibition of the cardiac hERG channel by essential pharmaceuticals is unpredictable and leads to fatal arrhythmias.

Results: Pretreatment with a newly identified compound, VU0405601, reduces sensitivity of hERG to inhibition by multiple blockers and prevents arrhythmias.

Conclusion: hERG-related arrhythmias are amenable to preventive therapy.

Significance: A novel approach at ion channel modulation that impacts drug discovery and safety concerns is outlined.

The human Ether-à-go-go-related gene (hERG)-encoded K⁺ current, Iₖ, is essential for cardiac repolarization but is also a source of cardiotoxicity because unintended hERG inhibition by diverse pharmaceuticals can cause arrhythmias and sudden cardiac death. We hypothesized that a small molecule that diminishes Iₖ, block by a known hERG antagonist would constitute a first step toward preventing hERG-related arrhythmias and facilitating drug discovery. Using a high-throughput assay, we screened a library of compounds for agents that increase the IC₅₀ of dofetilide, a well characterized hERG blocker. One compound, VU0405601, with the desired activity was further characterized. In isolated, Langendorff-perfused rabbit hearts, optical mapping revealed that dofetilide-induced arrhythmias were reduced after pretreatment with VU0405601. Patch clamp analysis in stable hERG-HEK cells showed effects on current amplitude, inactivation, and deactivation. VU0405601 increased the IC₅₀ of dofetilide from 38.7 to 76.3 nM. VU0405601 mitigates the rate of return to the steady state and reduces the sensitivity of hERG to inhibitors.

The hERG³ gene product forms the pore-forming subunit of the delayed rectifier current Iₖ, an important repolarizing current in the human heart (1, 2). Diminished repolarization either through genetic mutations in proteins that shape the action potential (AP), including ion channels or through inhibition by a pharmaceutical agent, can lead to AP prolongation and lengthening of the QT interval on the surface ECG. Mutations in the hERG gene (KCNH2) cause the relatively rare congenital long QT syndrome (LQTS), which can result in the Torsades de Pointes arrhythmia and sudden cardiac death (3, 4). Moreover, inhibition of Iₖ, either through class III antiarrhythmic drugs (5), through unintended block by agents prescribed for non-cardiac conditions (6–8), or through over-the-counter drugs (9) can lead to the acquired LQTS (10).

Consequently, arrhythmias associated with hERG inhibition have led to the withdrawal of otherwise successful drugs from the market. Indeed, the Food and Drug Administration has issued guidelines (ICH S6 and S7A) that require evaluation of novel chemical entities for their potential to induce QT prolongation early in drug development (11). It has been estimated that 50–70% of all lead compounds are eliminated at early stages due to hERG-related safety issues (12), thereby limiting the number of drugs that enter the development pipeline. On the other hand, hERG inhibition is not always predictive of proarrhythmic potential; for example, experience with clinically safe drugs like verapamil (13), which blocks hERG but does not alter APs due to simultaneous effects on calcium channels (14, 15), argues that it can be counterproductive to categorically avoid all hERG blockers. We hypothesized that a small mole-

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2 To whom correspondence should be addressed: Dept. of Anesthesiology, Research Division, Vanderbilt University School of Medicine, P-445 MRBIV Langford, 13th floor, Nashville, TN 37232. Tel.: 615-936-2586; Fax: 615-936-0456; E-mail: sabina.kupershmidt@vanderbilt.edu.

3 The abbreviations used are: hERG, human Ether-à-go-go-related gene; AP, action potential; APD, action AP duration; LQTS, long QT syndrome; PCL, pacing cycle length; HATU, 2-(1H-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate methanaminium; Iₖ, sodium current; IKs, slow component of the delayed rectifier current; IKur, ultra-rapid component of the K⁺ current.
cule that diminishes \( I_{Kr} \) block by a known hERG antagonist would constitute a first step toward preventing hERG-related arrhythmias and facilitate drug discovery. We identified one lead compound, VU0405601, via high-throughput screening, utilizing a fluorescence-based thallium (Tl\(^+\)) influx assay (16) for agents that increased the IC\(_{50}\) of dofetilide, a class III antiarrhythmic (17).

**EXPERIMENTAL PROCEDURES**

**Thallium-based Fluorescence Assay for High-throughput Screen**—Assay development and high throughput screening were carried out according to the procedures detailed in AID 1511 (pubchem.ncbi.nlm.nih.gov) and published earlier (18).

**Thallium Flux Measurements in hERG-HEK Cells**—20,000 HEK-293 cells/well stably expressing hERG were plated in 20 \( \mu l/well \) Dulbecco’s modified Eagle’s medium containing 10% (v/v) fetal bovine serum and 2 mM Glutamax (Invitrogen) on poly-D-lysine-coated 384 well plates (BD Biosciences) and incubated overnight at 37 °C in a 5% CO\(_2\) incubator. The next day the medium was replaced with 20 \( \mu l/well \) Hanks’ balanced salt solution containing 20 mM HEPES, pH 7.3. Data were normalized by dividing each data point from the second FDSS protocol by the base-line values observed; only then did we apply dofetilide (or other hERG inhibitors) and continue the pulse protocols. The tail currents observed; only then did we apply dofetilide (or other hERG inhibitors) and continue the pulse protocols. The tail currents were normalized against peak current (fully agonist-enhanced) levels before drug application and plotted against concentration. In all experiments where we examine the test drug (VU0405601 or PD-118057) in the presence of a given hERG inhibitor, the test drug was applied to the bath first, and the concentrations listed in each figure legend were applied to the bath solution followed by repetitive depolarizing pulses to +10 mV (2 s) from a holding potential of −80 mV (6 s) and by a hyperpolarizing pulse of −50 mV (2 s). Test-pulse and tail currents amplitude were normalized to control levels before drug application and plotted against concentrations. In all experiments where we examine the test drug (VU0405601 or PD-118057) in the presence of a given hERG inhibitor, the test drug was applied to the bath first, and the pulse protocols were applied until the full (agonist) effects were observed; only then did we apply dofetilide (or other hERG inhibitors) and continue the pulse protocols. The tail currents were normalized against peak current (fully agonist-enhanced) levels rather than normalizing against agonist-naïve/untreated current levels.

**Current Biophysics**—Effects of VU0405601 on the voltage dependence of channel activation were assessed by measuring tail currents at −50 mV after pulses ranging from −80 to 60 mV. Values were normalized to the peak value of each condition. The curves were fitted with the Boltzmann function.

To measure the kinetics of inactivation, we used a triple pulse voltage protocol. To bypass the slow hERG activation process, channels were first fully activated and inactivated by a 3-s step...
to 60 mV. This was followed by a brief 10-ms step to −130 mV during which the channels recovered from inactivation without appreciable deactivation. The third depolarizing step to various potentials induces channel transition from open to inactivated states. The decay of current traces during the third pulse was fitted with a single exponential function to generate the time constant of inactivation. Because the rate of hERG activation is slower than inactivation, the time course of activation cannot be directly measured from the test pulse currents. However, the outward tail currents signify channels recovered from inactivation, and we used the growth of the peak tail current amplitude after increasing durations of depolarizing pulses to infer the time course of channel activation. To this end the membrane voltage was stepped to 10 mV for durations varying from 50 to 2000 ms in 100-ms increments (“envelope” protocol). The time course of growth of peak tail current amplitude was fit with a single exponential function to generate the activation time constant (τ).

To assess hERG recovery from inactivation, we depolarized the cells to 60 mV for 2 s and then returned to potentials from −120 to −100 mV for 2 s to generate inward tail currents. Recovery from inactivation was determined by fitting the initial rising phase of these tail currents before deactivation occurred to a single exponential function and plotted as function of voltage.

Statistics—Data were acquired using pCLAMP9 software (Axon Instruments, Foster City, CA). Both pCLAMP and Origin (Microcal, Northampton, MA) programs were used in analyzing and plotting data. Patch clamp measurements are presented as the means ± S.E. Statistical significance of the observed effects was assessed by means of t test or paired t test. A value of p < 0.05 was considered significant.

Isolated Langendorff Preparation—All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health and were approved in advance by the Vanderbilt Institutional Animal Care and Use Committee at Vanderbilt University (protocol number M/10/356).

Four-month-old New Zealand White rabbits weighing 3–4 kg were used for all experiments. Rabbits were pre-anesthetized with 50 mg/kg ketamine via intramuscular injection followed by 2–5% inhaled isoflurane and oxygen. 2000 units of heparin were injected via an ear vein to prevent coagulation. After assurance of adequate anesthesia, a midsternal incision was performed, and the heart was removed. The aorta was cannulated and connected to a constant pressure perfusion system with Tyrode’s solution (130 mM NaCl, 4 mM KCl, 23 mM NaHCO3, 1.5 mM NaH2PO4, 1 mM MgCl2, 2 mM CaCl2, and 10 mM glucose (Fisher)), warmed to 37 °C, and bubbled with 95% oxygen, 5% carbon dioxide. 3.4 μM Blebbistatin (Sigma), a myosin-ATPase inhibitor for excitation-contraction uncoupling, was added to the perfusate to avoid motion artifacts.

Optical Imaging—The hearts were loaded with 200 μM of the transmembrane potential-sensitive fluorophore Di-4-ANEPPS (Biotium) to allow for optical imaging. Fluorescence was excited by a 432-nm argon laser (Coherent Inc.) focused onto the heart by 4 liquid light guides and recorded on a 1000 frame/s 128 × 128 pixel camera through a long-pass red optical filter (Tiffen). ECGs and APs were continuously recorded and monitored.

Pacing Protocol—Hearts were electrically paced using a unipolar platinum electrode positioned at the center of the left ventricle. Hearts were paced as follows at increasing rates; using an S1 stimulus and starting at a pacing cycle length (PCL) of 300 ms, hearts were paced for 1 min each at PCLs of 250, 200, 170, 150, 130, 120, and 110-ms duration or until premature ventricular contractions lasting ≥4 beats or loss of capture occurred. At each PCL and for each heart, we recorded optical images for 3 s to establish base line conditions before drug application (Con, Fig. 1). After control conditions were established, we added either 100 nM dofetilide or 5 μM VU0405601 before repeating the same pacing protocol. In a third condition (VU0405601>Dof, Fig. 1, B and C), the VU0405601-treated hearts was perfused with 100 nM dofetilide before repeating the pacing protocol. After each drug addition, hearts were perfused for 10 min before resumption of the pacing protocol. The pacing protocol was applied 5 times per heart and per condition. 10 or 11 individual rabbit hearts were used for the VU0405601-pretreated and the dofetilide-only-treated groups, respectively. Between pacing protocols, sinus rhythm was reestablished either spontaneously or after cardioversion with a defibrillator (150–300 V).

Filtering—Data were analyzed offline in MATLAB (Mathworks) using custom algorithms. Recordings were initially processed with a temporal 3-frame moving average filter and a spatial rotationally symmetric Gaussian low-pass filter with an S.D. of 1 applied to a 3 × 3 matrix.

AP Duration (APD) Calculations (PCL 300 and PCL 150)—A point was selected at the center of the left ventricle. A linear trace of fluorescence was generated by spatially averaging a 5 × 5 window about the selected point for each frame. The algorithm then scanned through 6 successive beats to determine APD 50, 70, and 90. This analysis was done at different PCLs for every heart.

Statistical Considerations—Data on arrhythmia occurrence were analyzed at both the rabbit heart and trial level. At the rabbit heart level, the binary end point equals one if at least one arrhythmia occurred over five trials, and zero otherwise. Group differences were assessed via Fisher’s Exact Test. At the trial level the binomial end point represents the number of arrhythmias over five trials, and group differences were assessed via a binomial model with logit link function. Both approaches yielded similar results. Group differences in APD were assessed via a mixed-effects model with random effects for rabbit heart to account for repeated APD measurements.

Preparation of Rabbit Myocytes—Left ventricular myocytes were isolated from hearts of male New Zealand White rabbits (6–7 lbs from Charles River Canada) using the method of Basani et al. (20) with slight modifications. Briefly, rabbits were intravenously anesthetized with sodium pentobarbital. Hearts were excised quickly and arrested on ice for 5 min in nominally calcium free minimum essential medium solution (Joklik modification; Sigma). Hearts were Langendorff-perfused at 37 °C for 10–15 min in the same minimum essential medium solution gassed with 95% O2, 5% CO2, and then perfusion was switched to minimum essential medium solution containing...
0.05 mg/ml Liberase TH (Roche Applied Science). When sampling of the ventricle yielded a single cell suspension of rod-shaped myocytes (12–17 min), the free wall of the left ventricle was removed and placed in minimum essential medium solution containing 1% bovine serum albumin. The tissue was minced, gently triturated, and filtered. Single cells were stored at 37 °C and used within 4 h. The experimental procedure for isolation of myocytes was approved by Vanderbilt University Institutional Animal Care and Use Committee (protocol number M/08/507).

**Action Potential Recording**—Myocytes were perfused with a 37 °C solution containing 137 mM NaCl, 5.4 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 2 mM HEPES, and 10 mM glucose adjusted to pH 7.4 with NaOH. APs were recorded using a standard glass microelectrode filled with 110 mM potassium glutamate, 1 mM MgATP adjusted to pH 7.2 with KOH. Myocytes were allowed to equilibrate at a stimulation rate of 1 Hz for 5 min before recordings. APs were generated with the same amplifier, and acquisition instrumentation were employed for hERG voltage-clamp experiments.

**Chemical Synthesis**—All NMR spectra were recorded on a 400-MHz AMX Bruker NMR spectrometer. ¹H Chemical shifts are reported in δ values in ppm downfield with the deuterated solvent as the internal standard. Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, br = broad, m = multiplet), integration, coupling constant (Hz). Low resolution mass spectra were obtained on an Agilent 1200 series with UV detection at 215 and 254 nm, high resolution mass spectra were recorded on a Waters Q-TOF API-US plus Acquity system with electrospray ionization. Analytical HPLC was performed on an Agilent 1200 series with UV detection at 215 and 254 nm, LCMS: RT 0.709 min, >98% at 215 and 254 nm, m/z = 357.0 [M + H]⁺. The residue was purified by reverse phase liquid column chromatography (30–75% acetonitrile:water with 0.1% trifluoroacetic acid) to afford the desired product (91 mg, 71%). LCMS: Rₜ = 0.765 min, >98% at 215 and 254 nm, m/z = 357.0 [M + H]⁺.

**REACTION 1**

2-((1-Bromonaphthalen-2-yl)oxy)-N-(pyridin-2-yl)acetamide (VU0453659, 3)—Compound was prepared in a similar manner to compound 1. LCMS: Rₜ = 0.931 min, >98% at 215 and 254 nm, m/z = 358.0 [M + H]⁺.

2-((1-Bromonaphthalen-2-yl)oxy)-N-(pyrimidin-5-yl)acetamide (VU0453657, 4)—Compound was prepared in a similar manner to compound 1. LCMS: Rₜ = 0.777 min, >98% at 215 and 254 nm, m/z = 357.0 [M + H]⁺.

2-((1-Bromonaphthalen-2-yl)oxy)-N-(pyridin-3-yl)acetamide (VU0453656, 5)—Compound was prepared in a similar manner to compound 1. LCMS: Rₜ = 0.634 min, >98% at 215 and 254 nm, m/z = 357.0 [M + H]⁺.

2-(Bromophenoxy)-N-(pyridin-3-yl)acetamide (VU0453659, 6)—Compound was prepared in a similar manner to compound 1. LCMS: Rₜ = 0.511 min, >98% at 215 and 254 nm, m/z = 307.1 [M + H]⁺.

The reaction was cooled in an ice bath, and 1 N HCl was added until a precipitate was evident. After filtration and washing with H₂O (10 ml), the product was obtained (2.7 g, 96% yield over two steps). LCMS: Rₜ = 0.709 min, >98% at 215 and 254 nm, m/z = 281.0 [M + H]⁺. See Reaction 2; DMF is N,N-dimethylformamide.

To a solution of compound 1-methyl (0.36 mmol, 1.0 eq) and Et₃N (0.54 mmol, 1.5 eq) in N,N-dimethylformamide (3 ml) was added 3-amipopyridine (0.39 mmol, 1.1 eq) and HATU (0.39 mmol, 1.1 eq) sequentially. After 16 h at room temperature, the reaction was added to EtOAc:H₂O (1:1, 50 ml). The organic layer was separated and washed with water (2 × 15 ml), brine (10 ml), dried (MgSO₄), filtered, and concentrated under reduced pressure. The residue was purified by reverse phase liquid column chromatography (30–75% acetonitrile:water with 0.1% trifluoroacetic acid) to afford the desired product (91 mg, 71%). LCMS: Rₜ = 0.765 min, >98% at 215 and 254 nm, m/z = 357.0 [M + H]⁺. See Reaction 2; DMF is N,N-dimethylformamide.
RESULTS

We used a high-throughput fluorescence-based Tl\(^+\) influx assay (16, 18), to test our hypothesis that a small chemical agent could modulate the sensitivity of the hERG channel to a known inhibitor. To this end we screened a library of compounds for agents that increased the IC\(_{70}\) of dofetilide, a well characterized hERG inhibitor (21, 22) in heterologous cells that stably expressed hERG currents. Details of the screen have been described (PubChem AID1511). We isolated one compound with the desired properties, VU0405601, and further characterized it as follows.

**VU0405601 Protects Cardiac Tissue from Dofetilide-induced Ventricular Tachycardia**—Determining the relative clinical risk of hERG-related arrhythmias requires the use of a complex cardiac model. In rabbits, the cardiac AP and ECG are similar to the human heart, and the hERG current, I\(_{Kr}\), is prominent (23). We thus tested the hypothesis that VU0405601 can protect cardiac tissue from the proarrhythmic effects of a known hERG inhibitor, dofetilide, using isolated Langendorff-perfused rabbit hearts (24–26). Cardiac pacing using an S1 stimulus train was initiated at a PCL of 300 ms, and APs were recorded in hearts loaded with Di-4-ANEPPS using epifluorescence. 5 \(\mu\)M VU0405601 alone did not change APD compared with untreated hearts (Fig. 1A). As predicted, dofetilide lengthened APD, but pretreatment with VU0405601 compensated for this effect (Fig. 1, A and B). PCL was reduced in progressively shorter decrements to 130 ms until predetermined endpoints (arrhythmias), defined as loss of capture or premature ventricular contractions were achieved. Fig. 1C shows the frequency of premature ventricular contractions induced at a PCL of \(\geq\)130 ms without drug (Con) after a 30-min perfusion of 100 \(\mu\)M dofetilide or 5 \(\mu\)M VU0405601 alone as well as when VU0405601 was administered 30 min before dofetilide (VU0405601>Dof). With dofetilide alone, 10 of 11 hearts experienced arrhythmias compared with 2 of 10 when VU0405601 was preadministered (0.019). This difference was even more striking when individual attempts at arrhythmia induc-
Novel Approaches to Antiarrhythmic Therapy

Fig. 2D shows the effect of dofetilide on normalized peak tail currents recorded at −50 mV with and without VU0405601. After 15 min of pulsing, only 11% of the current remained when dofetilide alone was applied, whereas with 50 μM (or 5 μM) VU0405601, 49% (or 36%) remained. Application of VU0405601 increased the IC50 of dofetilide from 38.7 to 76.3 nM.

Importantly, we found that the previously described hERG agonist, PD-118057, at a concentration of 10 μM (28) enhances tail currents 1.8-fold, whereas 5 μM VU0405601 enhanced tail currents only 1.5-fold (Fig. 3C). Thus, although PD-118057 was a more effective hERG potentiator at the concentrations used in Fig. 2D, it did not relieve dofetilide inhibition of hERG. The fully activated I-V relationship for hERG was determined using the protocol shown in Fig. 3D. We measured the peak amplitude of tail current over a wide range of membrane potentials after a 2-s pulse to +60 mV. The figure shows that the reversal potential was not changed after application of VU0405601, indicating no change in selectivity.

The voltage dependence of activation was assessed by studying the half-maximal activation voltage (V1/2). We determined the V1/2 by fitting the magnitude of the tail currents measured at −50 mV in response to various prepulse potentials to a Boltzmann function (Fig. 4A). VU0405601 modifies the hERG voltage dependence of activation by shifting the V1/2 from −9.8 ±

*Fig. 2. hERG currents recorded in hERG-HEK cells. A, shown is the time course of hERG current before (control) and after 50 μM VU0405601. Whole-cell currents were generated from a holding voltage of −80 mV and stepped to 10 mV for 2 s (A) followed by a tail pulse at −50 mV for 2 s (C). Dofetilide was added as indicated. Representative current traces are shown in the inset. B, shown is dose response of VU0405601 measured at 10 mV (test pulse) and the ensuing peak tail current currents (−50 mV). Current amplitudes were normalized to untreated control conditions and plotted against VU0405601 concentrations. A vertical dashed line is drawn at 5 μM. C, pulse protocol was used to assess time course of hERG inhibition. Whole-cell currents were generated from a holding voltage of −80 mV and stepped to +10 mV for 2 s followed by a tail pulse at −50 mV for 2 s. Continued pulsing with dofetilide (Dof) results in a gradual onset of hERG inhibition due to open-state block. D, hERG-HEK cells were stimulated until steady-state effects of VU0405601 or PD-119057 was achieved and inhibitor was applied subsequently. Tail currents were normalized for maximally stimulated current (pre-inhibitor) and measured at multiple time points with 100 nM dofetilide alone (open circles, n = 23) with 10 μM PD-118057 followed by 100 nM dofetilide (green triangles, n = 6), with 5 and 50 μM VU0405601 (red circles and triangles, n = 6 and 12, respectively) followed by 100 nM dofetilide, and with intracellular application of 50 μM VU0405601 followed by 100 nM dofetilide (black triangles, n = 5).
3.4 mV in controls to \(-23.6 \pm 2.5\) mV with 50 \(\mu M\) VU0405601 \((p < 0.005)\). For hERG, the growth of the peak tail current after increasing durations of depolarizing pulses is used to infer the time course of activation. VU0405601 accelerated the activation kinetics by decreasing the time constant from 899.7 to 294.2 ms (Fig. 4C).

Effects of VU0405601 on the voltage dependence of inactivation was determined using the protocol in the inset of Fig. 4B. hERG current was first fully activated and inactivated by a 3-s step to +60 mV; this was followed by stepping the membrane to a range of voltages between -130 and +60 mV for 10 ms to allow recovery from inactivation. Tail currents were recorded at 30 mV. Peak tail currents were normalized to the maximal peak currents and plotted against voltage. VU0405601 shifted the \(V_{1/2}\) of inactivation from -48.8 \(\pm 1.7\) to -64.6 \(\pm 3.7\) mV. The decay of current traces during the third pulse was fitted with a single exponential function to generate the time constant of inactivation. VU0405601 slowed the kinetics of inactivation, as reflected by the increasing time constants at more positive potentials (Fig. 4D). Recovery from inactivation was determined by fitting the initial rising phase of the tail currents before deactivation occurred to a single exponential function and plotted as function of voltage. The data showed that VU0405601 hastened the kinetics of recovery from inactivation (Fig. 4E).

Deactivation time constants were derived by fitting the decay phase of tail currents with a double-exponential function. VU0405601 selectively increased the fast component (Fig. 4F) of the time constant with increasing effects at more depolarized membrane potentials, suggesting that delayed closing of the activation gate contributes to enhanced hERG currents. We observed no modification of the slow component.

Inactivation Is a Key Requirement for VU0405601 Activity—To assess the extent to which inactivation contributes to VU0405601 enhancement of currents, we made use of a hERG channel with impaired inactivation, S631A. This variant retains K\(^+\) selectivity and exhibits a voltage dependence of activation identical to that of WT, but inactivation in S631A is shifted toward depolarized potentials by 100 mV (29). Fig. 5A shows that although S631A tail currents showed similar sensitivity to 50 \(\mu M\) VU0405601 as WT, the test pulse currents, which are a composite of the open and inactivated current component, show decreased sensitivity. These results argue that the effects of VU0405601 on the test-pulse current are in large part due to effects on the inactivated state.

VU0405601 Does Not Bind to the Intracellular Pore—Class III antiarrhythmics including dofetilide and other small molecule hERG inhibitors have been demonstrated to interact with binding sites accessible from the intracellular aspect of the channel (22, 30, 31). In contrast, peptide toxin inhibitors, including the scorpion toxin BeKm-1 interact with the S5-P loop of the extracellular vestibule (32, 33).

To investigate whether VU0405601 acts within the inner pore region, we applied VU0405601 through the intracellular pipette solution but observed no enhancement of hERG current or attenuation of dofetilide block (Figs. 5A and 2D). Thus,
VU0405601 does not bind from the intracellular side. To investigate possible interaction with extracellular channel residues, we made use of the peptide toxin BeKm-1. When we pretreated hERG-HEK with either BeKm-1 or dofetilide at levels (50 nM) that incompletely inhibit currents over the time period measured (5–7 min), we found that only BeKm-1 application prevents VU0405601 agonist effects. Moreover, mutating the F656 residue, which is crucial for hERG inhibitor binding (30) at the inner cavity, did not diminish the VU0405601 effect (Fig. 5A). In combination, these data indicate that VU0405601 binds extracellularly and that alleviation of hERG inhibition is allosteric.

Specificity—As an initial step to address compound specificity, we determined the effects of VU0405601 on APs in freshly isolated rabbit ventricular cardiomyocytes using the whole-cell patch clamp technique. APs were evoked using whole-cell current clamp by repeated injection of brief depolarizing current pulses at a frequency of 1 Hz. The effect of VU0405601 on resting membrane potential, AP amplitude, AP duration at 50% (APD_{50}), and 90% (APD_{90}) repolarization were determined.
Typical AP recordings in the absence or presence of VU0405601 (5 and 50 μM) are shown in Fig. 6, A and B. VU0405601 had no effect on resting membrane potential or AP amplitude. At 5 μM, VU0405601 had minor effects on APD. This agrees with our findings with APD measured in the whole heart (Fig. 1, A and B). However, as would be expected from the EC50 relationship (Fig. 2B), which showed robust IKr potentiation at that concentration, 50 μM VU0405601 shortened APDs in isolated cardiomyocytes (Fig. 6A). At this concentration, 50 μM VU0405601 was co-applied with 1 μM dofetilide to inhibit IKr, we noted a much smaller shortening in APD (12 ± 4% and 10 ± 2%; n = 3 for APD50 and APD90, respectively; Fig. 6, A and B, p < 0.05, t test). This suggests that VU0405601 has small effects on the AP when IKr is blocked and thus primarily impacts IKr.

We next tested the effects of 50 μM VU0405601 on individual voltage-gated currents that play a role in shaping the cardiac AP. cDNAs coding for Kv1.5, Nav1.5, and IKs ( KCNQ1 plus KCNE1 ) currents were transiently expressed in heterologous cells, and the effects of 50 μM VU0405601 on current amplitudes were tested. We observed small to no effects on these currents, indicating that the compound mainly impacts hERG (Fig. 6D).

In addition, VU0405601 was tested at a concentration of 10 μM in a commercially available “lead profiling screen” (Ricerca Biosciences). 68 different targets (G-protein coupled receptors, ion channels, and transporters) were screened for reactivity with VU0405601 in biochemical radioligand binding assays. Ion channel targets included the L- and N-type Ca2+ channels, IKATP, and hERG. Significant responses, defined as inhibition of radio ligand binding ≥50%, were not noted for any of the targets other than the adenosine A2A receptor (64% inhibition) or the 5-HT3 receptor (92% inhibition).

Structure-Activity Relationships—To assign structure-activity relationships, we designed chemical modifications (Fig. 7A) that we then tested in in vitro cell-based electrophysiology assays. We characterized derivative chemical compounds with respect to effects on hERG-activating and tail current amplitudes (Fig. 7B) as well as dofetilide sensitivity (Fig. 7C). We identified the 3-pyridyl and naphthyridine ring systems as critical for protection from dofetilide inhibition. Replacing the 3-pyridyl with phenyl substituent led to a loss of activity (VU0405601, 1 versus VU0418705, 2). In addition, pyridyl regioisomers (2-pyridyl, VU0453657, 4; 4-pyridyl, VU0453658, 5) were inactive as well as 4-pyrimidyl (VU0453656, 3) and 5-thiazole (VU0453660, 7) replacement groups. Changes to the right-side of the molecule also showed limited functional group tolerance. Deletion of the naphthyl moiety (VU0453659, 6) led to an inactive compound. Removal of the 1-bromo group (VU0453661, 8) was inactive as well as moving the bromo to the 3-position (VU0455655, 10). However, the 1-bromo substituent could be replaced with either a methyl (VU0453653, 9) or chloro (VU0456912, 11) and maintain activity similar to VU0405601, 1.

Because most clinically relevant hERG inhibitors act at or close to a shared intracellular site (34–39), accessing the inner pore from the cytoplasmic aspect (21), we hypothesized that VU0405601 would exhibit the same effect against other antagonists and tested a second hERG inhibitor, the anti-emetic droperidol (6). Fig. 3B shows that VU0405601 application increases the droperidol IC50 from 0.35 to 40 μM. We subsequently utilized the Tl+ flux assay to investigate whether VU0405601 is effective against other inhibitors that bind the inner pore site. We tested VU0405601 and representative active (VU0455653, VU0456912) and inactive (VU0453659) congeners in the Tl+ flux assay (40) against a panel of six additional therapeutic agents that inhibit hERG as a side effect (Table 1). The table lists the concentrations of hERG potentiators required to counteract the blockade of an IC50 concentration of each of the hERG inhibitors and return the data traces to a form indistinguishable from vehicle control traces. The active compounds from the VU series were substantially more effective than the inactive analog, VU0453659, and the reference hERG potentiator, PD118057. In summary, these results indicate that VU0405601 effects are generalizable to inhibitors that bind the hERG inner pore region.

**DISCUSSION**

Although the hERG potassium channel was initially characterized as the cause for the chromosome 7-linked congenital
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FIGURE 6. Effects of VU0405601 on AP measured in isolated rabbit myocytes when I_{Kr} is blocked. Evoked APs were measured from freshly isolated rabbit ventricular cardio-myaocytes (A) in the absence or presence of VU0405601 (5 and 50 μM). The percentage reductions in APD_{50} and APD_{90} with 5 μM VU0405601 were 4 ± 3 and 5 ± 2%, respectively (p = not significant, paired t test; n = 7). With 50 μM VU0405601 APD_{50} and APD_{90} were reduced by 35 ± 6 and 32 ± 4%, respectively (p < 0.05, paired t test; n = 5). B, dofetilide (Dof; 1 μM) attenuates the AP-shortening effects of 50 μM VU0405601. In the absence of dofetilide alone, APD_{50} and APD_{90} were prolonged by 22 ± 6 and 18 ± 7%, respectively (p < 0.05, paired t test; n = 3). C, with 5 μM VU0405601 with dofetilde resulted in a modest effect on APD compared with VU0405601 alone: 12 ± 4 and 10 ± 2% for APD_{50} and APD_{90}, respectively (p < 0.05, t test; n = 3). D, VU0405601 effects on individual cardiac ion channels were recorded in transfected CHO cells. C, raw current traces are shown. Voltage protocols as indicated. Current was measured at the arrow. Gray lines are with VU0405601. D, effects of 50 μM VU0405601 on current amplitudes are shown.

LQTS2, the acquired LQTS represents a far more significant clinical problem. The overwhelming majority of acquired LQTS is due to selective inhibition of hERG and aggravated by co-morbidities (41). One poignant example is the non-sedating anti-histamine blockbuster drug, Seldane (terfenadine) that was withdrawn from the market in 1998 after it had been prescribed to millions of patients, causing dozens of cardiac events (42). This event rate is undetectable even in large scale clinical trials. In response, the Food and Drug Administration issued guidelines (for review, see Ref. 43) that requires evaluation of drugs for their potential to induce QT prolongation early in development. Nonetheless, these guidelines remain somewhat unsatisfying because QT prolongation is not always a reliable indicator of proarhythmic potential (13). For example, useful compounds proven to be safe in the clinic (verapamil) would have been screened out under these guidelines. We hypothesized that a means to improve drug safety would be to identify a method that would prevent block of the hERG-related current, I_{Kr}. We used a high throughput Ti^{+} flux assay to identify a candidate compound, VU0405601, with the desired activity. VU0405601 caused increased fluorescence in the initial Ti^{+} flux assay, indicating relief of hERG inhibition, and in subsequent, cell-based electrophysiology assays, we determined that VU0405601 increased the IC_{50} of the hERG inhibitor, dofetilide (24–26).

To test whether VU0405601 protects cardiac tissue from proarhythmic effects induced by hERG inhibition, we recorded ECGs and APs through epifluorescent imaging of isolated, Langendorff-perfused rabbit hearts. Initially, we identified a dose of VU0405601 that by itself did not lead to changes in APD or incidents of arrhythmias (Fig. 2). Significantly, treatment of isolated hearts with VU0405601 before application of the proarhythmic hERG inhibitor dofetilide led to a decrease in pacing-induced arrhythmias compared with hearts treated with dofetilide alone. Our data indicate that VU0405601 has the characteristics of a compound that protects against arrhythmias induced by hERG inhibition.

On its own, VU0405601 enhances hERG test-pulse and tail current with an EC_{50} around 11.4 μM. Multiple biophysical properties of the hERG current are affected by treatment with 50 μM VU0405601; the V_{1/2} of activation is shifted toward more negative potentials, whereas the V_{1/2} of inactivation is shifted toward more depolarized potentials, thus increasing availability of the channel. The kinetics of activation are accelerated, inactivation is slowed, deactivation is delayed, and recovery from inactivation is hastened. These changes would be expected to increase current amplitude, and we did indeed observe enhanced activating as well as tail currents.

A potential drawback for development of a compound that exhibits hERG agonist effects, in addition to protecting from hERG inhibition, is that current increases may also be proarhythmic, leading to the short QT syndrome (44–46). However, we identified a concentration of VU0405601 (5 μM) that protects from dofetilide-induced arrhythmias (Fig. 1C) with only small changes in current amplitude (Fig. 2B). Hence, our approach did not cause the APD shortening that would be the expected consequence of hERG potentiation after VU0405601 application in isolated rabbit hearts (Fig. 1, A and B) or in cardiomyocytes (Fig. 6A). Nevertheless, we have taken initial steps to refine the activity of the compound through medicinal chemistry approaches, with the goal to minimize agonist effects while at the same time optimizing protection from hERG inhibition (Fig. 7).

Class III antiarrhythmics, including dofetilide and other small molecule hERG inhibitors that can traverse the plasma membrane have been demonstrated to interact with binding sites accessible from the intracellular aspect of the channel (22, 27, 30, 39). Drug interaction involves residues located in the S6 transmembrane domain at the base of the pore helix, including the key residues Tyr-652 and Phe-656 that are unique to hERG
VU0405601 doubles the IC$_{50}$ of dofetilide, indicating that VU0405601 changes the affinity of the dofetilide binding site. Two sets of data argue against a model where the agonist activity of VU0405601 simply compensates for the effects of the inhibitor. 1) We utilized an experimental set-up that allowed us to assess the effects of dofetilide (or other inhibitors) while accounting and correcting for the agonist effects of any test drug. To achieve this, we added the test drug (VU0405601 or PD-118057) to the bath and applied the described pulse protocols until the full (agonist) effect was observed; only then did we apply dofetilide (or other hERG inhibitors) and continue the pulse protocols. Finally, the tail currents were normalized against peak current (fully agonist-enhanced) levels rather than normalizing against agonist-naïve/untreated current levels. Under these conditions, the effects of VU0405601 are quite distinct from those of PD-118057 (Fig. 2D). 2) Despite the fact that PD-118057 shows more pronounced potentiation than VU0405601 (Fig. 3C), VU0405601 provides relatively better protection from dofetilide inhibition (Fig. 2D). If enhancement of hERG currents were to simply compensate for decreased current levels (“functional antagonism”) after inhibition by dofetilide, it follows that PD-118057 should be a “better” functional antagonist than VU0405601. However, our data support the opposite conclusion, and therefore, “functional antagonism” cannot fully explain the relief of dofetilide inhibition after VU0405601 application.

Our observation that VU0405601 reduces channel inactivation (Fig. 4B) may explain reduced dofetilide affinity for the inner cavity binding site. Thus, previous data show that for most hERG inhibitors, including dofetilide, drug binding is dependent on channel opening and develops gradually. Inhibitors have increased affinity for the open or inactivated state, and mutated channels with reduced inactivation also show decreased inhibition, although some variability has been noted (22, 27, 49–53). Additional previous studies point out the importance of residues contained in the extracellular S5-P loop on sensitivity to inhibitors (49), and a hERG variant associated with the short QT syndrome (N588K) contains a mutation in

**TABLE 1**

**Effects of hERG potentiators on hERG blockade by known hERG inhibitors**

Concentration-response relationships were obtained using the Tl$^{+}$ flux assay by testing 9 concentrations of hERG inhibitors against 10 concentrations of hERG potentiators. Shown are the concentrations of hERG potentiators required to fully counteract the blockade of an IC$_{70}$ concentration of each of the hERG inhibitors and return the data traces to a level indistinguishable from vehicle control traces.

| Activator   | Dofetilide | Terfenadine | Amiodarone | Thioridazine | Haloperidol | Droperidol | Pimozide |
|-------------|------------|-------------|------------|--------------|-------------|------------|----------|
| VU0405601   | 3.3E-06    | 3.3E-06     | 3.3E-06    | 3.3E-06      | 1E-05       | 1E-05      | 3.3E-06  |
| VU0455653   | 1E-05      | 1E-05       | 1E-05      | 1E-05        | 1E-05       | 1E-05      | 1E-05    |
| VU0453659   | Inactive   | Inactive    | Inactive   | Inactive     | Inactive    | Inactive   | Inactive |
| VU0456912   | 3.3E-06    | 3.3E-06     | 3.3E-06    | 3.3E-06      | 6E-06       | 3.3E-06    | 6E-06    |
| PD118057    | 9E-05      | 9E-05       | 3E-05      | >1E-05       | >3E-05      | >3E-05     | >3E-05   |

FIGURE 7. Structure-activity relationships. A, chemical structures of VU0405601 and its derivatives are shown. The 3-pyridyl group (red) and the halogenated naphthyridine ring (blue) are essential for activity. Effects of VU0405601 and its derivatives on hERG current amplitudes (B) and dofetilide sensitivity (C) are shown. con, control. Measurements in panel B were performed as in Fig. 2B. Measurements in panel C were performed as described in Fig. 2D.
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the S5-P loop that exhibits reduced affinity for \( I_{Ks} \) blockers (44). Our own experiments described in Fig. 5B with the outer-pore blocker BeKm1 provide evidence that the binding site for VU0405601 is located extracellularly and, therefore, different from the inner cavity site described for drugs that inhibit hERG (22, 27, 30, 39).

Because of its unique structural features, hERG is particularly sensitive to inhibition by a wide range of diverse compounds including dofetilide. However, most clinically relevant hERG inhibitors act at, or close to, a shared site (34–39), and it is likely that any findings made with dofetilide will also apply to other blockers. Our data with the anti-ectopic droperidol, which also shows reduced affinity for hERG in the presence of VU0405601, and a panel of additional therapeutic agents that block hERG (Table 1) but that show reduced efficacy in the presence of VU0405601 in the \( \text{TI}^{\text{+}} \) flux assays support this conclusion. We noted that VU0405601 only modestly increases the IC50 for hERG block compared with much larger increases for droperidol (Fig. 3). We speculate that this may be related to the fact that different blockers show different relative affinities for the open versus the inactivated states (51).

In preliminary assays we detected small or no effects on the cardiac currents \( I_{Na} \), \( I_{Ks} \), or \( I_{Kur} \). Although further investigation is required to assess the therapeutic value and safety profile of this compound, our data provide proof-of-concept that protection of hERG from inhibition by clinically useful therapeutics can be accomplished through application of a small chemical compound.

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