Electrophysiological and Pharmacological Characterization of Human Inwardly Rectifying K_{ir}2.1 Channels on an Automated Patch-Clamp Platform

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ABSTRACT

Inwardly rectifying I_{K1} potassium currents of the heart control the resting membrane potential of ventricular cardiomyocytes during diastole and contribute to their repolarization after each action potential. Mutations in the gene encoding K_{ir}2.1 channels, which primarily conduct ventricular I_{K1}, are associated with inheritable forms of arrhythmias and sudden cardiac death. Therefore, potential iatrogenic inhibition of K_{ir}2.1-mediated I_{K1} currents is a cardiosafety concern during new drug discovery and development. K_{ir}2.1 channels are part of the panel of cardiac ion channels currently considered for refined early compound risk assessment within the Comprehensive in vitro Proarrhythmia Assay initiative. In this study, we have validated a cell-based assay allowing functional quantification of K_{ir}2.1 inhibitors using whole-cell recordings of Chinese hamster ovary cells stably expressing human K_{ir}2.1 channels. We reproduced key electrophysiological and pharmacological features known for native I_{K1}, including current enhancement by external potassium and voltage- and concentration-dependent blockade by external barium. Furthermore, the K_{ir} inhibitors ML133, PA-6, and chloroquine, as well as the multichannel inhibitors chloroethylclonidine, chlorpromazine, SKF-96365, and the class III antiarrhythmic agent terikalant demonstrated slowly developing inhibitory activity in the low micromolar range. The robustness of this assay authorizes medium throughput screening for cardiosafety purposes and could help to enrich the currently limited K_{ir}2.1 pharmacology.

Keywords: safety pharmacology, CiPA initiative, sudden death, torsades de pointes, K^+ channels, ventricular arrhythmia

INTRODUCTION

Inhibition of the voltage-dependent potassium channels K_{v}11.1, also referred to as human Ether-à-go-go Related Gene (hERG), which mediates the delayed rectifier I_{Kr} potassium current in the heart, has been identified in the 1990’s as a central mechanism associated with “Torsades de Pointe” (TdP) arrhythmias and sudden cardiac death. This major cardiosafety issue caused the market withdrawal of a significant number of pharmaceuticals. Disruption of hERG function slows the repolarization phase of the cardiac action potential (AP), increasing its duration and favoring the initiation of early after depolarizations and ventricular arrhythmia, which can degenerate in TdP. In accordance with internationally harmonized guidelines, these findings prompted the systematic early cardiosafety benefit/risk assessment of new drug candidates through evaluation of their functional effects at hERG channels in vitro, along with action potential duration (APD) measurements in ex vivo ventricular conductive tissue preparations such as rabbit Purkinje fibers. These studies are combined with in vivo measurements of the time interval between Q-wave and T-wave (QT) on the electrocardiogram, typically conducted in conscious dogs, and are followed as appropriate by specific thorough QT (TQT) studies during the early clinical development phases.
Despite the fact that these guidelines resulted in no further drugs being withdrawn from the market for arrhythmia liabilities since their implementation, the consistency of the surrogate markers examined as unequivocal predictors of TdP risk has recently been challenged. In addition, their stringency when taken individually as compound prioritization criteria may have generated unnecessary attrition of otherwise efficient and innovative drugs. For example, it has been shown that among nearly one hundred preclinical compounds, hERG inhibition translates into APD prolongation in only half of the cases, with one-third revealing no activity on APD, and one-sixth actually shortening it. Moreover, it was shown that drugs either prolonging or shortening the APD could produce ventricular arrhythmia, at least in excised hearts. Similarly, a retrospective analysis of several tens of advanced candidates, which underwent TQT studies in humans, showed that the predictive value of hERG inhibition alone, while being sensitive, substantially lacks specificity.

Hence, several drugs from various pharmacological classes with diverse chemical structures do not induce proarrhythmia in clinical practice, despite being significant hERG inhibitors at therapeutically relevant concentrations. Kramer et al. suggested that, for many of these drugs, the discordance could result from compensatory inhibitory activities at depolarizing cardiac ion channels other than hERG contributing to shape the AP. Specifically, it was shown that among 55 small molecules with a clinically documented high, medium, or low torsadogenic potential, additional inhibitory activity at CaV1.2-mediated \( I_{\text{Ca,L}} \) calcium currents (and to a lesser extend at \( \text{Na}_v\text{Na}_{1.5} \)-mediated \( I_{\text{Na}} \) peak sodium currents) better describes their cardiotoxic profile compared to knowledge of hERG inhibition alone.

The ongoing comprehensive in vitro proarrhythmia assay (CiPA) initiative is a public–private collaboration put in place a few years ago with the objective to propose better ways to predict the proarrhythmic potential of preclinical compounds. This endeavor proposes to address the cardiotoxic risk of a compound by combining its inhibitory profile at multiple cardiac ion channels with the predictions of an in silico model of human ventricular electrophysiology previously trained with the inhibitory profile of clinical drugs with documented high, medium, or low torsadogenic potential. The predictions would then be optionally compared with actual measurements on stem cell-derived cardiomyocytes before being assessed by electrocardiography monitoring during the early phases of clinical development. The panel of channels selected for the CiPA profiling comprise the depolarizing CaV1.2- and \( \text{Na}_v\text{Na}_{1.5} \)-mediated \( I_{\text{Ca,L}} \) and \( I_{\text{Na}} \) peak and late currents, as well as the repolarizing \( \text{K}_v\text{K}_\text{11.1}, \text{K}_v\text{K}_7.1, \text{K}_v\text{K}_4.3, \text{K}_v\text{K}_2.1 \)-mediated \( I_{\text{Kr}}, I_{\text{Ks}}, I_{\text{to}}, \text{and } I_{\text{K1}} \) currents. In this study, we have developed a medium throughput screening assay based on automated whole-cell patch-clamp recording of \( I_{\text{K1}} \)-mediated \( I_{\text{K1}} \) currents.

Inwardly rectifying \( \text{K}_v\text{K}_2.1 \) potassium channels belong to a family of transmembrane proteins sharing the property to flux \( \text{K}^+ \) ions more readily in the inward than in the outward direction. This “biological diode”-like behavior is linked to their voltage-dependent block by cytoplasmic polyamines or magnesium ions, which plug their conduction pore at depolarized voltages. K\(_v\)2.1 and other members of the \( \text{K}_v\text{K}_2.x \) subfamily display strong rectification properties and contribute thereby to the control of the membrane resting potential in excitable tissues by constantly dragging the membrane toward the \( \text{K}^+ \) equilibrium potential \( E_K \). They also support repolarization following AP firing (reviewed in refs. 18–21).

Despite the dearth of selective and potent pharmacologic agents, and the fact that several different \( \text{K}_v\text{K}_2.x \) subunits expressed in the heart can assemble into functional heterotetramers, convergent lines of evidence concur to support a primary role for \( \text{K}_v\text{K}_2.1 \) subunits to conduct \( I_{\text{K1}} \) in cardiac ventricles. First, \( I_{\text{K1}} \) currents are significantly reduced in rat ventricular cardiomyocyte exposed to antisense oligonucleotides targeting \( \text{K}_v\text{K}_2.1 \) mRNAs. Second, no \( I_{\text{K1}} \) currents can be recorded from cardiac cells in neonate \( \text{K}_v\text{K}_2.1 \) knockout mice during the few hours they survive. Third, \( I_{\text{K1}} \) can be enhanced or decreased in guinea pig hearts treated with adenovirus encoding wild-type \( \text{K}_v\text{K}_2.1 \) or a dominant-negative mutant \( \text{K}_v\text{K}_2.1 \) protein, respectively. Fourth, a clear translational path links rare human hereditary diseases involving loss- or gain-of-function mutations in the \( \text{KCNJ2} \) gene encoding \( \text{K}_v\text{K}_2.1 \) subunits with cardiac rhythm abnormalities.

One important aspect of the evaluation of drug effect at multiple cardiac ion channels pertains to adopting profiling methods reliably assessing the intrinsic activity of test articles using unbiased functional readouts. Authier et al. recently published a survey on current practices in the pharmaceutical industry, indicating that patch-clamp electrophysiology on human ion channels expressed in cell lines is the most common approach to the CiPA ion channel strategy. The development in the last decade of automated planar patch-clamp systems providing gigaseal quality recordings allows sufficient throughput to adequately expedite early cardiotoxicity support for the curtailed optimization cycles of modern medicinal chemistry. In this study, we undertook the validation of a \( \text{K}_v\text{K}_2.1 \) assay on such a platform, allowing rapid quantification of drug inhibitory activity to complement an integrated drug discovery cardiotoxicity liability assessment panel.
MATERIALS AND METHODS

**K**<sub>ir</sub>2.1 Cell Line

A Chinese hamster ovary (CHO) cell line stably expressing the human *KCNJ2* gene product (GenBank NM_000891.2) under the control of a tetracycline-inducible promoter was obtained from a commercial source and cultivated according to the vendor instructions (Charles River; Cat. No. CT6127). The cells were grown in a humidified 95% air/5% CO<sub>2</sub> atmosphere in Dulbecco's modified Eagle's medium/F-12 Nutrient Mixture (ThermoFisher Scientific) supplemented with 10% fetal bovine serum and the appropriate selection antibiotics (0.01 mg/mL blasticidin and 0.4 mg/mL zeocin). For stock cultures, cells were grown in T175 flasks and passaged every 2.5 million cells per T175 flask filled with 25–30 mL growth medium. Expression of the Kir2.1 channels was obtained by overnight induction with 1 μg/mL doxycycline added to the growth medium. On the day of recording, detached cells were spun down, washed, resuspended to 5–8 million cells/mL in a glucose-containing extracellular buffer, and placed in the cabinet of a QPatch® 48X workstation (Sophion Bioscience, Denmark). This standalone instrument comprises a robotic pipetting arm ensuring distribution of cell suspensions into disposable 48-well recording plates (QPlates®) and the sequential application of drug solutions at final test concentration, while whole-cell patch-clamp is maintained without interruption.

Automated Patch–Clamp

All recordings were performed at room temperature. Experiments aimed at characterizing the electrophysiological properties of the currents were conducted on biochips endowed with a unique pinhole orifice engineered at the bottom of each of the 48 wells of disposable measurement plates (i.e., single-hole QPlates). Currents were activated by application of a series of 500 ms-long square voltage pulses delivered every 90 s and incremented in 5 mV steps from a holding potential of −20 mV. Currents measured over 30 ms at the end of each step served to plot current-voltage (I–V) relationships. In some experiments, the external potassium [K]<sup>+</sup> concentration was changed, while maintaining internal [K]<sup>+</sup> constant to change the K<sup>+</sup> equilibrium potential. In other single-hole recordings, the I–V relationships were established in an external buffer supplemented with various concentrations of alkaline or alkaline-earth metal ions.

Experiments aimed at characterizing the pharmacology of small molecules were conducted with 48-well measurement plates, each fitted with biochips perforated with 10 holes, so that each recording site sums up the signal from multiple cells (i.e., multi-hole QPlates). The voltage protocol used was a step-ramp protocol applied every 30 s, which first stepped the membrane from a holding potential of −20 mV down to −120 mV for 500 ms, then ramped it up to 0 mV over 1 s, and eventually stepped the membrane potential back to −20 mV, where the cells were held until the next voltage cycle. This protocol allowed for long-time (30 min) recordings comprising a stable baseline and ample space for the application of multiple drug concentrations in a row. Moreover, the voltage ramp permitted to check the recording quality over time by monitoring the stability of the zero-current reversal potential value. The size of the inward current measured at the end of the initial hyperpolarizing 500 ms-step to −120 mV was used to evaluate drug effect.

For all experiments, the intracellular recording buffer contained (in mM) 5.37 CaCl<sub>2</sub>, 1.75 MgCl<sub>2</sub>, 10 ethylene glycol tetraacetic acid, 10 HEPES, 120 KCl, and 4 Na<sub>2</sub>-ATP (pH 7.2, 300 mM) and the standard extracellular recording buffer contained (in mM) 2 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 10 HEPES, 4 KCl, 145 NaCl, and 10 Glucose (pH 7.4, 310 mM).

**Drugs**

All test articles were from Sanofi’s internal libraries, except ML133 (CAS Number 1222781-70-5; Wang et al.<sup>31</sup>), which was obtained from a commercial source (Sigma-Aldrich, Saint-Quentin-Fallavier), and PA-6 (CAS Number 1199627-07-0, Takanari et al.<sup>32</sup>), a pentamidine derivative, which was synthesized following published procedures and references therein.<sup>32,33</sup> Figure 5 depicts the structural formula and IUPAC chemical names of ML133, PA-6, and the five other small-molecule inhibitors examined in this study. Compounds were dissolved as 10, 50, or 100 mM concentrated stocks in dimethyl sulfoxide (DMSO) using sonication as needed for complete dissolution, then aliquoted into capped polypropylene vials, and kept frozen at −20°C until use. On the day of study, stock solutions were diluted to the final concentrations intended for cell applications in standard 96-well microtiter plates (MTP) containing the extracellular buffer supplemented with 0.06% Pluronic F-68<sup>TM</sup> (Gibco, ThermoFisher Scientific). The latter surfactant was added to help slow unseen precipitation (if any) of poorly soluble drugs in the MTP wells upon dilution.<sup>34</sup> The final drug solutions also contained 0.3% residual DMSO from the dilution processes. The test article solutions were typically applied to the cells within 30 min of preparation. Concentration–response data were either
obtained from the cumulative applications of six increasing concentrations to the same cells within a given recording well, each concentration being exposed for 3 min, or by applying a single different concentration into given wells with an exposure duration extended to 15 min.

Data Handling
Current amplitude measurements and normalization for the fitting of concentration–response curves were done with Sophion’s dedicated analysis software (Odense 5.6.4). Time course of current amplitude was visualized offline and analyzed following optional rundown compensation as needed using built-in routines proprietary to this software (uncorrected rundown measured in \(N=5\) vehicle-treated cells did not exceed 1% per min in single application experiments). All recordings for pharmacological determinations were concluded by the application of a supramaximal concentration (3 mM) of barium as full block. Residual leak currents remaining under full block (if any) were subtracted from precedent data points. A professional graphing package (Prism 7.02; GraphPad Software) was used for drawing exported current traces, plotting I–V curves, and performing statistical calculations. Half-maximal inhibitory concentrations (IC\(_{50}\)) were obtained by fitting the average of the two or the five last current amplitude readings at the end of each drug concentration exposure period in a cumulative or noncumulative application mode, respectively. A classical four-parameter Hill equation following normalization with respect to predrug baseline readings was used for data fitting to a sigmoidal curve with minimum and maximum constrained to 0% and 100%, respectively. Estimated IC\(_{50}\) and Hill coefficient (n\(_H\)) values are reported along with their estimated 95% confidence interval (CI). Mean of individual replicates is represented with their calculated standard deviations shown as error bars on graphs where appropriate.

RESULTS AND DISCUSSION
Electrophysiological Characterization
The upper part of Figure 1A illustrates the typical appearance of whole-cell currents recorded from stably transfected CHO cells induced to express the human KCNJ2 gene product. Inward or outward currents reaching steady state within a 100 ms developed when the membrane was stepped tens of mV below or above the \(E_K\) equilibrium potential (Fig. 1A). At more depolarized potentials, the current–voltage (I–V) relationship displayed a strongly inward rectifying profile. This profile fully concurs with the I–V outline of native ventricular \(I_{K1}\) currents. Specifically, the amplitudes of the inward currents below \(E_K\) grew linearly in proportion to the negativity of the membrane, reaching relatively large values at strong hyperpolarization (e.g., \(-2.3 \pm 0.4\) nA at \(-120\) mV, \(N=10\), Fig. 1B), whereas the positive currents above \(E_K\) culminated at potentials around \(-60\) to \(-50\) mV (e.g., \(0.31 \pm 0.09\) nA at \(-60\) mV, \(N=10\), Fig. 1B). Virtually no currents were detected in induced cells at potentials more positive than \(-20\) mV (Fig. 1B) nor at any potential in noninduced CHO cells (Fig. 1A bottom), even when the latter were stepped to very negative potentials (e.g., \(0.07 \pm 0.05\) nA at \(-120\) mV, \(N=10\)), indicating the absence of endogenous \(I_{K1}\)-like currents in the host CHO cell line. The success rate of exploitable recordings obtained from single-hole QPlates routinely exceeded half of the 48

![Fig. 1. Inward and outward currents in CHO cells stably expressing the human KCNJ2 gene recorded with single-hole QPlates. (A) Family of representative current traces obtained by stepping the membrane from a holding of \(-20\) mV to voltages ranging from \(-120\) to \(0\) mV in a cell exposed overnight to doxycycline (top left) or in a noninduced cell (bottom left). (B) Mean steady-state currents plotted as a function of voltage for \(N=10\) induced (closed circles) and noninduced (gray squares) cells. Error bars represent standard deviation and are smaller than symbol size in noninduced cells. (C) Descriptive statistics of steady-state current size at \(-120\) mV collected from \(N=65\) cells sampled from three single-hole QPlates. Frequency distribution was constructed at a binwidth of 0.25 nA. CHO, Chinese hamster ovary.]
cells. The inset depicts a linear regression fit of the log_{10} of [K^{+}]_{out} concentration to the membrane potential values at which no current is observed (i.e., zero-current reversal potential).

For example, statistics derived from N=3 single-hole QPlates indicated that, from 126 cells, which successfully entered whole-cell mode (i.e., 88% of the 144 theoretically possible), a total of N=65 cells formerly exhibiting seal resistances well above 200 MΩ provided complete I–V curves after 30-min recording, demonstrating that 52% of the cells recovered satisfactorily from clamp engagement and remained stable during repeated voltage stepping cycles. Figure 1C illustrates the current size frequency distribution for this sample of 65 cells. The median inward steady-state current amplitude at −120 mV was −2.4 nA, along with an interquartile range spreading from −1.7 to −2.9 nA.

A prominent property of currents flowing through Kir2.x channels is their activation by external potassium [K^{+}]_{out}. This counterintuitive behavior (given the decreased electrochemical gradient when [K^{+}]_{out} is increased) results from an increased open-channel conductance in response to elevation of external K^{+}. In the experiments illustrated in Figure 2, [K^{+}]_{out} was varied over four concentrations ranging from 2 to 20 mM, while internal potassium was kept constant at [K^{+}]_{in}=120 mM. The I–V relationships outlined by the four pairs of buffers exhibited a rightward shift proportional to [K^{+}]_{out}, along with a concomitant increase in the size of both the inward and outward currents. The latter culminated at 0.12±0.06, 0.26±0.08, 0.37±0.10, and 0.74±0.20 nA when the external buffer contained 2, 4, 10, and 20 mM [K^{+}]_{out}, respectively (N=5–10 cells per condition, Fig. 2). Beyond these maxima, the I–V curves presented regions of negative slope conductance until complete extinction of the currents. Their steepness was also proportional to [K^{+}]_{out}, and the superposition of the outward currents in these voltage regions of the I–V curves draw a series of typical “cross-over” patterns as previously described by others.

In response to the increasing [K^{+}]_{out}, the zero-current potential of each I–V relationship shifted rightward from −92±1.2 mV at 2 mM [K^{+}]_{out} to −75±2.7 mV, −56±1.5 mV, and −36±1.2 mV for 4, 10, and 20 mM [K^{+}]_{out}, respectively. Linear regression of these data as a function of log [K^{+}]_{out} yielded a highly significant (r^2=0.992) fit (Fig. 2, inset) with an estimated 54±3.4 mV shift of the zero-current potential per tenfold variation of external [K^{+}]_{out}, in good agreement with the Nernst equation prediction of 58 mV per decade.

Taken together, the electrophysiological features summarized in Figures 1 and 2 demonstrate rectification properties and activation by external potassium typical of Kir2.1-mediated I_{K1} currents. Of note, the high yield of decent currents collected simultaneously allowed to expedite the characterization of a number of electrophysiological features with minimal need of experiments.

Pharmacological Characterization

Cations such as Cs^{+} and Ba^{2+} are blockers of native I_{K1} currents in sheep Purkinje fibers, starfish eggs, or frog leg muscles, abolishing the influence of [K^{+}]_{out} on the I–V profile and blocking I_{K1} in a concentration- and voltage-dependent manner. These cations are fast-acting open-channel blockers, reaching steady-state inhibition of the current flow within a few seconds after external exposure. This property was used to document Kir2.1 channels when they were first isolated by expression cloning in Xenopus oocytes. Figure 3 illustrates the effects of micromolar Ba^{2+} concentrations on Kir2.1-mediated I_{K1} currents activated by a potassium.
step-ramp voltage protocol in KCNJ2 expressing CHO cells. As previously described by Kubo et al.\textsuperscript{42} external Ba\textsuperscript{2+} blocked the steady-state current more strongly toward the end of the hyperpolarizing pulses than the initial instantaneous current (Fig. 3A). Moreover, this time-dependent inhibition was also voltage dependent, the channel block increasing when the membrane was stepped to more negative potentials (Fig. 3B). Group data obtained from $N=3$ single-hole recordings indicated that the inhibition of the inward current produced by 3 $\mu$M Ba\textsuperscript{2+} reached 17\% at $-120$ mV, increasing to 48\% at $-150$ mV. Similarly, the 58\% inhibition of the current afforded by 10 $\mu$M Ba\textsuperscript{2+}at $-120$ mV rose to 77\% at $-150$ mV. These properties result in characteristic changes in the I–V relationship profile in the presence of Ba\textsuperscript{2+} (Fig. 3B) and Cs\textsuperscript{+} ions (data not shown).

Figure 3C depicts the time course of the effect of cumulative applications of increasing concentrations of Ba\textsuperscript{2+} into a multi-hole recording well. The Kir2.1-mediated $I_{K1}$ currents stabilized within several minutes after whole-cell access. As expected from a fast-acting pore blocker, the inhibitory effect of each cumulatively applied concentration of Ba\textsuperscript{2+} fully developed within the first couple of voltage-protocol cycles immediately at the beginning of each 3-min application period. The IC\textsubscript{50} value (calculated from the average normalized inward current amplitudes collected at the two last voltage steps down to $-120$ mV of each incubation period) was estimated at 8.2 $\mu$M (95\% CI: 7.6–8.8 $\mu$M, $N=6$, Fig. 4). The Hill coefficient ($n_H$) associated to the sigmoidal fit of the normalized data was close to unity ($n_H=1.03$; 95\% CI: 0.96–1.1), consistent with a mechanism involving a single ion blocking each channel pore according to first-order kinetics. Notwithstanding the voltage dependency of the blocking effect of Ba\textsuperscript{2+}, this value is in fairly good agreement with published values on Kir2.1 currents recorded in HEK cells (IC\textsubscript{50} = 7.9 $\mu$M at $-90$ mV, Shen et al.\textsuperscript{43}) or Xenopus oocytes (IC\textsubscript{50} = 16 $\mu$M at $-120$ mV, Schram et al.\textsuperscript{23}). The time course and the inhibitory effects of Cs\textsuperscript{+} and Sr\textsuperscript{2+} ions using the same cumulative concentration–response application protocol were very similarly to Ba\textsuperscript{2+},
exhibiting full inhibitory activity immediately at the beginning of each application period. These blockers were less potent than Ba$^{2+}$, yielding IC$_{50}$ values of 833 μM (95% CI: 789–880 μM, N = 12) with nH = 1.07 (95% CI: 1.0–1.1), and 4,585 μM (95% CI: 4,308–4,881 μM, N = 11) with nH = 0.92 (95% CI: 0.87–0.98), respectively (Fig. 4). The potency obtained for Cs$^+$ on human K$_{ir}2.1$ channels was one order of magnitude weaker than published data for mouse Kir2.1 overexpressed in a murine-erythroleukemia cell line (IC$_{50}$ = 91 μM at −100 mV, Abrams et al. 44), raising the possibility that K$_{ir}2.1$ expression in a conspecific rather than ectopic cell environment affects voltage dependency of Cs$^+$ blockade. We did not find quantitative pharmacological data with which to compare our potency determinations for Sr$^{2+}$ ions.

There are currently no selective small-molecule K$_{ir}2.1$ inhibitors with nanomolar potency described in the literature.22 Even in the realm of toxins isolated from the venom of poisonous species, which are a rich source of high affinity ion channel modulators, no K$_{ir}$2.1 selective peptide inhibitors have been described so far.45 We therefore went on to characterize the pharmacology of the K$_{ir}2.1$-mediated I$_{K1}$ currents expressed in CHO cells using seven small-molecule inhibitors displaying micromolar potencies in manual patch-clamp experiments (Fig. 5). We started with the aromatic diamidine derivative PA-6, 32 the methoxybenzyl-methanamide ML133, 31 and the antimalaria drug chloroquine 46 using the six-point cumulative application paradigm adopted with Ba$^{2+}$ and the other ionic pore blockers. However, the active concentrations of these weak amines did not reach full inhibition within the 3-min exposure periods. Previous studies comparing the inhibitory activity of these compounds in the inside-out and whole-cell patch-clamp configurations evidenced an accelerated inhibition rate and increased potency against K$_{ir}2.1$ currents when excised patches were assayed. These data pointed to cytoplasmic sites of actions for these three compounds rather than ectopic cell environment affects voltage dependency of Cs$^+$ blockade. We did not find quantitative pharmacological data with which to compare our potency determinations for Sr$^{2+}$ ions.

Fig. 4. Inhibition of K$_{ir}2.1$-mediated I$_{K1}$ currents by alkali or alkaline-earth channel blockers. Ba$^{2+}$ (circles), Cs$^+$ (squares), and Sr$^{2+}$ (triangles) ions were tested as chloride salts in multi-hole QPlates. Group estimates were derived from at least N = 3 wells from overlapping concentration ranges applied cumulatively to the same cells (see legend to Fig. 3C and text for details and estimated IC$_{50}$ values with 95% confidence intervals). IC$_{50}$, half-maximal inhibitory concentrations.

Fig. 5. Chemical structure of disclosed compounds with K$_{ir}2.1$ inhibitory properties evaluated in this study: PA-6, N,N’-((pentane-1,5-diylbis(oxo))bis(4,1-phenylene)) dibenzimidamide 32; ML133, N-(4-methoxybenzyl)-1-(naphthalene-1-yl) methanamine 31; Chloroquine, 4-N-(7-chlorquinolin-4-yl)-1-N,1-diethylpentane-1,4-diamine 46; Chloroethylclonidine, N[2,6-dichloro-4-[[2-chloroethyl(methyl)amino]methyl]phenyl]-4,5-dihydro-1H-imidazol-2-amine 46; Chlorpromazine, 3-(2-chlorophenothiazin-10-yl)-N,N-dimethylpropan-1-amine 51; SKF-96365, 1-[2-(4-methoxyphenyl)-2-[3-(4-methoxyphenyl)propoxy]ethyl]imidazole 53; Terikalant, 1-[2-[4S]-3,4-dihydro-2H-chromen-4-yl]ethyl]-4-(3,4-dimethoxyphenyl)piperidine. 53
membrane permeation of their uncharged molar fraction at neutral pH. Entry by organic cation transporters has been shown for pentamidine, but it remains to be established whether such carriers are significantly expressed in CHO cells. We therefore adopted a noncumulative cell treatment protocol for the evaluation of PA-6, ML133, and chloroquine inhibitory potency. Cells were exposed to single concentrations of each compound in individual wells recorded in parallel. Figure 6A illustrates the time course of the effects of PA-6 when applied at concentrations ranging from 1.25 to 10 μM. Extension of the exposure duration to 15 min proved sufficient to reach stable inhibition for PA-6, ML133, and chloroquine. In contrast to Ba²⁺, the inhibitory action of PA-6 on the Kir2.1 current traces indicated no time dependency during the hyperpolarizing pulse, displaying identical inhibition on its instantaneous and steady-state components (Fig. 6B). The inhibitory potency of PA-6 calculated on the average current amplitudes collected over the five last voltage steps to -120 mV under drug exposure was IC₅₀ = 1.3 μM (95% CI: 1.2–1.4 μM, N=3–5, Fig. 6B) along with a Hill slope value near unity (n₅₀ = 1.2, 95% CI: 1.2–1.4, Table 1). When tested with the same prolonged 15-min exposure protocol, ML133 and chloroquine yielded micromolar potencies (Table 1). Hill coefficients were close to 1, except for ML133, suggesting a cooperative mechanism of action for this compound independent of its slow kinetics of action as proposed by others. These inhibitory activities are consistent with published data obtained using traditional manual patch-clamp. Thus, whole-cell Kir2.1 currents elicited at -110 mV in KCNJ2-transfected HEK cells were inhibited by 68% in the presence of 3 μM external PA-6, whereas ML133 showed an IC₅₀ value of 1.8 μM at -100 mV. Similarly, in feline ventricular myocytes, 3 μM chloroquine decreased Iₖ₁ currents at -100 mV by 67%.

Only a few small-molecule Kir2.1 inhibitors have their molecular mechanism of action deciphered, and this was not an objective of this study. We rather extended our efforts to assess structurally diverse Kir2.1-preferring or

**Fig. 6.** Slow inhibition of Kir2.1-mediated Iₖ₁ currents by the pentamidine derivative PA-6. (A) Four representative plots of steady-state current amplitude normalized with respect to baseline from multi-hole QPlates recordings are displayed. Induced CHO cells were first exposed to vehicle alone for the time period shown before the vertical dashed line. Then, in control experiments (open circles), the cells were exposed to vehicle alone, while in drug treatment recordings, the cells were exposed to vehicle containing 1.25 μM (closed squares), 5 μM (closed triangles), or 10 μM (closed diamonds) PA-6 (twofold dilution series). The inhibitory activity was allowed to develop over 15 min. The average two measurements at the end of the last vehicle period and the average five measurements at the end of a drug treatment period served for current amplitude normalization. A final episode in each experiment consisted in the application of a saturating concentration of Ba²⁺ as full block. (B) Representative multi-hole current traces in response to the voltage protocol shown above the traces. (C) Concentration–response curve for PA-6 inhibition using a threefold dilution series; IC₅₀ was estimated from at least N=3 wells per concentration applied individually. See Table 1 for estimated value and 95% confidence interval.
multichannel inhibitors to generate a high-quality patch-clamp data set for further pharmacological assay or protocol comparisons. Chloroethylclonidine, chlorpromazine, SKF-96365, and the class III antiarrhythmic agent terikalant are known to affect Kᵢr2.1-mediated IᵦK₁ currents in recombinant or native cell systems at micromolar concentrations. We therefore screened them with the cumulative concentration application mode and observed slow inhibition kinetics for all of them, suggesting common actions at the cytoplasmic side of the Kᵢr2.1 channels. We therefore used the single-concentration noncumulative parallel application mode also for these four compounds; results of these quantitative evaluations are reported in Table 1. Only chloroethylclonidine appeared to reach fast-enough steady-state inhibition fitting a cumulative mode evaluation, which doubled its apparent potency with an IC₅₀ value of 25 μM (95% CI: 23–28 μM, N = 3) compared to the noncumulative application mode (data not shown).

Together with IᵦK₁ and IᵦKₛ currents flowing through the voltage-gated Kᵥ11.1 and Kᵥ7.1 channels, respectively, IᵦKᵢ currents contribute to the “repolarization reserve” of the cardiac AP, suggesting Kᵢr2.1 as a possible off-target for drug-induced cardiac side effects. Several screening assays for Kᵢr channels have previously been reported based on other readouts and formats. For example, a ratiometric fluorescence resonance energy transfer assay using cells in which Kᵢr to prevent them from spontaneously depolarizing and automatically beating. Thus, the assay we describe in this study provides a useful tool for cardiovascular safety screening. Furthermore, it provides a platform for the discovery of novel Kᵢr2.1 channel ligands as drug candidates.

**CONCLUSION**

We have validated an automated patch-clamp assay for Kᵢr2.1 channels that reproduces cardinal features known for native IᵦK₁ currents and that generates concentration–response data for inhibitor tool compounds that agree with manual electrophysiology data. This assay is ready for cardiotoxicity screening of somewhat larger compound sets advancing toward preclinical development.

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**DISCLOSURE STATEMENT**

The authors are current or former employees of Sanofi and declare no conflict of interests.

### Table 1. Potencies of Kᵢr2.1 Inhibitors

| Test article | IC₅₀ (95% CI) (μM) | Hill slope nᵦI (95% CI) |
|--------------|-------------------|------------------------|
| PA-6         | 1.3 (1.2–1.4)     | 1.2 (1.1–1.4)          |
| ML133        | 1.2 (1.1–1.3)     | 2.6 (2.2–3.1)          |
| Chloroquine  | 1.9 (1.8–2.1)     | 0.80 (0.76–0.83)       |
| Chloroethylclonidine | 46 (42–50) | 0.81 (0.75–0.87) |
| Chlorpromazine | 3.8 (3.4–4.2)    | 1.7 (1.4–2.1)          |
| SKF-96365    | 4.7 (4.1–5.4)     | 1.3 (1.1–1.5)          |
| Terikalant   | 8.4 (7.4–9.4)     | 0.98 (0.88–1.1)        |

CI, confidence interval; IC₅₀, half-maximal inhibitory concentrations.
pointes for a broad range of drugs: evidence for a provisional safety margin in drug development. Cardiovasc Res 2003;58:32–45.

7. Hammond TG, Carlson L, Davis AS, et al.: Methods of collecting and evaluating non-clinical cardiac electrophysiology data in the pharmaceutical industry: results of an international survey. Cardiovasc Res 2001;49:741–750.

8. Lu HR, Vlaminckx E, Hermans AN, et al.: Predicting drug-induced changes in QT interval and arrhythmias: QT-shortening drugs point to gaps in the ICHS7B Guidelines. Br J Pharmacol 2008;154:1427–1438.

9. Gintant G: An evaluation of hERG current assay performance: translating preclinical studies to clinical QT prolongation. Pharmacol Ther 2011;129:109–119.

10. Kramer J, Obiiero-Paz CA, Myatt G, et al.: MICE models: superior to the HERS model in predicting torsade de pointes. Sci Rep 2013;3:2100.

11. Fermini B, Hancox JC, Abi-Gerges N, et al.: A new perspective in the field of cardiac safety testing through the comprehensive in vitro arrhythmia assay paradigm. J Biomol Screen 2016;21:1–11.

12. Gintant G, Sager PT, Stockbridge N: Evolution of strategies to improve preclinical cardiac safety testing. Nat Rev Drug Discov 2016;15:457–471.

13. Sager PT, Gintant G, Turner JR, Pettit S, Stockbridge N: Rechanneling the cardiac proarrhythmia safety paradigm: a meeting report from the Cardiac Safety Research Consortium. Am Heart J 2014;167:292–300.

14. Huang H, Pugsley MK, Fermini B, et al.: Cardiac voltage-gated ion channels in safety pharmacology: review of the landscape leading to the CIPA initiative. J Pharmacol Toxicol Methods 2017;87:11–23.

15. Millard D, Dang Q, Shi H, et al.: Cross-Site reliability of human induced pluripotent stem-cell derived cardiomyocyte based safety assays using microelectrode arrays: results from a blinded CIPA pilot study. Toxicol Sci 2018;164:550–562.

16. Lopatin AN, Makhina EN, Nichols CG: Potassium channel block by cytoplasmic polyamines as the mechanism of intrinsic rectification. Nature 1994;372:366–369.

17. Matsuda H, Saigusa A, Irisawa H: Ohmic conductance through the inward rectifying K channel and blocking by internal Mg2+. Nature 1987;325:156–159.

18. Starfield PR, Nakajima S, Nakajima Y: Constitutively active and G-protein coupled inward rectifier K+ channels: Kir2.0 and Kir3.0. Rev Physiol Biochem Pharmacol 2002;145:47–179.

19. Hlibo H, Inanobe A, Furutani K, Murakami S, Findlay I, Kurachi Y: Inwardly rectifying potassium channels: their structure, function, and physiological roles. Physiol Rev 2010;90:291–366.

20. de Boer TP, Houtman MJ, Compier M, van der Heyden MA: The mammalian K(JR)2.x inward rectifier ion channel family: expression pattern and pathophysiology. Acta Physiol (Oxf) 2010;199:243–256.

21. Hille B: Ionic Channels of Excitable Membranes. Sinauer Associates, Sunderland, MA, 1992.

22. Swale DR, Kharade SV, Denton JS: Cardiac and renal inward rectifier potassium channel pharmacology: emerging tools for integrative physiology and therapeutics. Curr Opin Pharmacol 2014;15:7–15.

23. Schram G, Pourrier M, Wang Z, White M, Nattel S: Barium block of Kir2 and Kir3 potassium channels: their structure, function, and physiological roles. Physiol Rev 2010;90:291–366.

24. Nakamura TY, Artman M, Rudy B, Coetzee WA: Inhibition of rat ventricular IK1 current by barium chloride impaired Kir2.1 inward rectifier channel. J Physiol 2006;574:235–245.

25. Miale J, Marban E, Nuss HB: Functional role of inward rectifier current in heart probe by Kir2.1 overexpression and dominant-negative suppression. J Clin Invest 2003;111:1529–1536.
48. Ji Y, Veldhuis MG, Zandvoort J, et al.: PA-6 inhibits inward rectifier currents carried by V93I and D172N gain-of-function Kir2.1 channels, but increases channel protein expression. J Biomed Sci 2017;24:44.

49. Sanchez-Chapula JA, Salinas-Stefanon E, Torres-Jacome J, Benavides-Haro DE, Navarro-Polanco RA: Blockade of currents by the antimalarial drug chloroquine in feline ventricular myocytes. J Pharmacol Exp Ther 2001;297:437–445.

50. Barrett-Jolley R, Dart C, Standen NB: Direct block of native and cloned (Kir2.1) inward rectifier K+ channels by chloroethylclonidine. Br J Pharmacol 1999;128:760–766.

51. Crumb WJ, Jr., Vicente J, Johannesen L, Strauss DG: An evaluation of 30 clinical drugs against the comprehensive in vitro proarrhythmia assay (CiPA) proposed ion channel panel. J Pharmacol Toxicol Methods 2016;81:251–262.

52. Liu H, Yang L, Chen KH, et al.: SKF-96365 blocks human ether-a-go-go-related gene potassium channels stably expressed in HEK 293 cells. Pharmacol Res 2016;104:61–69.

53. Escande D, Mestre M, Cavero I, Brugada J, Kirchhof C: RP 58866 and its active enantiomer RP 62719 (terikalant): blockers of the inward rectifier K+ current acting as pure class III antiarrhythmic agents. J Cardiovasc Pharmacol 1992;20 Suppl 2:S106–S113.

54. Roden DM: Taking the "idio" out of "idiosyncratic": predicting torsades de pointes. Pacing Clin Electrophysiol 1998;21:1029–1034.

55. Solly K, Cassaday J, Felix JP, et al.: Miniaturization and HTS of a FRET-based membrane potential assay for Kir channel inhibitors. Assay Drug Dev Technol 2008;6:225–234.

56. Zaks-Makhina E, Kim Y, Aizenman E, Levitan ES: Novel neuroprotective K+ channel inhibitor identified by high-throughput screening in yeast. Mol Pharmacol 2004;65:214–219.

57. Goverse B, van der Heyden MAG, van Veen TAB, de Boer TP: The immature electrophysiological phenotype of iPSC-CMs still hampers in vitro drug screening: Special focus on IK1. Pharmacol Ther 2018;183:127–136.

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Abbreviations Used

| Abbreviation | Definition |
|--------------|------------|
| AP           | action potential |
| APD          | action potential duration |
| CHO          | Chinese hamster ovary |
| CI           | confidence interval |
| DMSO         | dimethyl sulfoxide |
| $E_K$        | potassium equilibrium potential |
| $IC_{50}$    | half-maximal inhibitory concentration |
| $I_{K_1}$    | native inwardly rectifying current |
| $K_{r2.x}$   | inward rectifier channel subunits—subfamily 2 |
| LQT-7        | long QT syndrome—subtype 7 |
| MTP          | microtiter plates |
| QT           | time interval between Q-wave and T-wave on the electrocardiogram |
| SQT-3        | Short QT syndrome—subtype 3 |
| TdP          | torsades de pointes |
| TQT          | thorough QT |