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Proarrhythmic risk assessment using conventional and new *in vitro* assays

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ABSTRACT

Drug-induced QT prolongation is a major safety issue in the drug discovery process. This study was conducted to assess the electrophysiological responses of four substances using established preclinical assays usually used in regulatory studies (hERG channel or Purkinje fiber action potential) and a new assay (human-induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs)-field potential).

After acute exposure, moxifloxacin and dofetilide concentration-dependently decreased I_{Kr} amplitude (IC_{50} values: 102 μM and 40 nM, respectively) and lengthened action potential (100 μM moxifloxacin: +23% and 10 nM dofetilide: +18%) and field potential (300 μM moxifloxacin: +76% and 10 nM dofetilide: +38%) durations. Dofetilide starting from 30 nM induced arrhythmia in hiPSC-CMs. Overnight application of pentamidine (10 and 100 μM) and arsenic (1 and 10 μM) decreased I_{Kr}, whereas they were devoid of effects after acute application. Long-term pentamidine incubation showed a time- and concentration-dependent effect on field potential duration.

In conclusion, our data suggest that hiPSC-CMs represent a fully functional cellular electrophysiology model which may significantly improve the predictive validity of *in vitro* safety studies. Thereafter, lead candidates may be further investigated in patch-clamp assays for mechanistic studies on individual ionic channels or in a multicellular Purkinje fiber preparation for confirmatory studies on cardiac conduction.

1. Introduction

Non-clinical and clinical cardiovascular toxicities remain the main cause for drug discontinuation during drug development and for post-marketing drug withdrawal (Valentin, 2010). The current International Conference of Harmonization (ICH) S7B guideline (2005) recommends *in vitro* electrophysiology studies employing either single cell (e.g. heterologous expression systems) or multicellular preparations (e.g. Purkinje fiber, papillary muscle) for the evaluation of proarrhythmic liability. Purkinje fiber assay can provide valuable information on the integrated activity of multiple ion channels in the cardiac tissue (Picard et al., 2011). Nevertheless, the manual patch-clamp hERG channel assay, considered as the gold standard in patch-clamp electrophysiology for cardiac liability in the evaluation of new drugs, is nowadays preferred as *in vitro* model by pharmaceutical industry for regulatory submission. However, it is widely recognized that the extensive focus on hERG has resulted in an overly high attrition rate (false positives) in drug development (Colatsky et al., 2016).

Consequently, in order to address the limitations of the guidelines, the FDA in collaboration with pharmaceutical companies, contract research organizations or academia, initiated the Comprehensive *in vitro* Proarrhythmia Assay (CiPA) (Gintant et al., 2016) which is a nonclinical Safety Pharmacology paradigm for discovering electrophysiological mechanisms that are likely to confer proarrhythmic liability to drug candidates intended for human use (Cavero et al., 2016). The CiPA includes 3 preclinical approaches to evaluate the cardiovascular electrophysiological risk of new chemical entities: (1) an array of key cardiac ion channels, (2) the use of *in silico* models, and (3) electrophysiological activity of human-induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) (Colatsky et al., 2016; Kitaguchi et al., 2016).

The aim of the present work was to assess the electrophysiological responses of reference substances known to prolong the QT interval of the electrocardiogram using existing preclinical assays usually used in regulatory studies (hERG channel or Purkinje fiber action potential) and new assays with hiPSC-CMs

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and multielectrode arrays (MEA) technology. The acute effects of moxifloxacin and dofetilide (hERG channel blockers) were assessed on the hERG channel, Purkinje fiber action potential and hiPSC-CMs field potential and the effects of long-term incubation of pentamidine and arsenic (hERG trafficking inhibitors) were investigated on the hERG channel and hiPSC-CMs field potential.

2. Materials and methods

2.1. Test systems

2.1.1. Cells

Human embryonic kidney (HEK) 293 cells were stably transfected by the hERG clone or hNav1.5 alpha-subunit (Creacell, La Tronche, France). Human induced pluripotent stem cell derived cardiomyocytes (hiPSC-CMs, Cor 4U®) were provided by Axiogenesis (Germany). Cells were maintained at 37 °C in a 5% CO_2/95% air incubator.

The experiments were performed in accordance with French legislation concerning the importation and housing of genetically modified cells belonging to Class 1, Group I, Confinement L2 and in accordance with a currently valid license, issued by the French Ministry for Jeunesse, Education Nationale et Recherche (License N° 5286 valid up to 22 August 2019).

2.1.2. Animals

Male Beagle dogs (CEDS, France) were housed in groups in a kennel, under natural lighting. They have restricted access to food (approximately 300 g/animal/day) (Code 125 - SAFE, 89290 Augy, France) and unlimited access to water. The animal rooms were maintained in a controlled ambient temperature of 18 ± 3 °C.

The experiments were performed in accordance with French legislation concerning the protection of laboratory animals and in accordance with a currently valid license for experiments on vertebrate animals, issued by the French Ministry for Agriculture and Fisheries.

2.2. Patch-clamp recording

2.2.1. hERG channel test

2.2.1.1. Acute effects of proarrhythmic substances. The HEK293 cells used for the study were transferred to an experimental chamber maintained at a temperature of 35 ± 0.5 °C, as previously described (Goineau et al., 2012a). Cells were continuously superfused with Tyrode's solution (in mM: NaCl: 145/KCl: 4/HEPES: 5/glucose: 5/$CaCl_2$: 1/$MgCl_2$: 1, pH = 7.45 ± 0.05).

Ionic currents from hERG-transfected cells were measured using the whole-cell configuration of the patch-clamp technique. Glass pipettes (resistance from approximately 1.5 to 3.5 MΩ) filled with internal solution (in mM: K-gluconate: 145/Mg-gluconate: 1/EGTA: 2/HEPES: 5/K_2ATP: 2, pH = 7.20 ± 0.05) were connected to the input stage of a patch-clamp amplifier (Axon Instruments: Multiclamp 700B). Stimulation, data recording and analysis were performed using specialized Axon Instruments software (pClamp 10.5.2.6).

After rupture of the cell membrane (entering whole-cell mode), cells were stimulated every 10 s (0.1 Hz) using the following protocol: 500 ms pulse to +10 mV from a holding potential of −80 mV followed by a 500 ms pulse to −40 mV during which tail current was measured.

Once the current under control conditions was stable, recordings were taken before (control) and after addition of each test substance concentration (moxifloxacin: 10, 30, 100 and 300 μM; dofetilide: 3, 10, 30 and 100 nM, pentamidine: 30 μM or arsenic: 10 μM). The effect of each substance on tail current was monitored continuously until steady-state was reached. The peak tail current amplitude was averaged for 3 stimuli. Cell capacitance (pF) and peak tail current amplitude (pA) were measured. Peak tail current measurements were normalized using the cell capacitance as an index of cell surface. Results were calculated as percentage change from control (percentage of tail current inhibition). Six cells were tested per substance.

2.2.1.2. Long-term incubation with pentamidine or arsenic. The HEK293 cells were overnight incubated at 37 °C with a range of concentrations of pentamidine (1, 10 and 100 μM), arsenic (0.1, 1 and 10 μM) or control substance. The day after prolonged exposure (18–24 h), the whole-cell configuration was established, as described above. Once a steady-state was reached, the amplitude value was recorded for each cell. The percentage of tail current inhibition was evaluated for each test substance concentration (comparison to the mean value recorded in the vehicle control group). Twelve cells were tested per concentration.

2.2.2. hNav1.5 sodium channel test

Cells were transferred to an experimental chamber which was maintained at room temperature, as previously described (Goineau et al., 2012b). Cells were continuously superfused with an extracellular solution (in mM: NaCl 145, KCl 4.5, HEPES 10, glucose 5, $CaCl_2$ 1.5, $MgCl_2$ 1, pH of 7.35 ± 0.05). The internal solution was constituted as follows (mM): NaF 10, CsF 110, CsCl 20, EGTA 10, HEPES 10 (pH = 7.35 ± 0.05).

After rupture of the cell membrane (entering whole-cell mode), cells were stimulated every second (1 Hz) using 40 ms pulse to −30 mV from a holding potential of −100 mV during which peak current was measured. Whole-cell capacitance and series resistance were compensated.

Once the current under control conditions was stable (recording of baseline values), the test substance was added in the bath (moxifloxacin at 300 μM or dofetilide at 10 nM) and the effect was monitored continuously until steady-state was reached. The peak current amplitude was averaged for 3 consecutive stimuli. Cell capacitance (pF) and peak current amplitude (pA) were measured. Peak current measurements were normalized using the cell capacitance. Results were calculated as percentage change from control (percentage of current inhibition). Four cells were tested per substance.

2.3. Action potentials: dog Purkinje fibers

Dogs were anesthetized using sodium pentobarbital (approximately 35 mg/kg i.v.). Following thoracotomy, the heart was exposed, then quickly removed and placed in a cardioplegic solution (in mM: NaCl 118.2, KCl 30.0, $CaCl_2$ 1.8, $MgCl_2$ 1.0, $NaHCO_3$ 25.0, NaH_2PO_4 1.8, glucose 55.0, pH = 7.35 ± 0.05).

Purkinje fibers were carefully dissected from the heart, as previously described (Picard et al., 2006) and pinned on the rubber silicon base of a Plexiglas chamber bath. The fibers were then superfused at 3 ml/min with first cardioplegic solution (stimulation frequency: 120 pulses/min i.e. 2 Hz) and then with Tyrode's solution which differed from the cardioplegic solution by lower concentrations of KCl (4 instead of 30 mM) and glucose (11 instead of 55 mM). The superfused solutions were maintained at 36.5 ± 0.5 °C and continuously gassed with 95% O_2/5% CO_2. The stimulation frequency was progressively reduced to 60 pulses/min (1 Hz).

Transmembrane action potentials were recorded using an intracellular glass microelectrode, filled with 3 M KCl. The reference was an Ag/AgCl electrode plunged into the experimental bath. The microelectrode was connected to the input stage of a high impedance negative capacity neutralizing