Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.
Assessment of proarrhythmogenic risk for chloroquine and hydroxychloroquine using the CiPA concept

Urs Thomet a, Bogdan Amuzescu b,*, Thomas Knott c, Stefan A. Mann d, Kanigula Mubagwa e,f, Beatrice Mihaela Radu b

a Anaxon AG, Brünnenstrasse 90, 3018, Bern, Switzerland
b Dept. Anatomy, Animal Physiology & Biophysics, Faculty of Biology, University of Bucharest, Splaiul Independenței 91-95, 050095, Bucharest, Romania
c Cytocentrics BioScience Inc., 3463 Magic Drive, San Antonio, TX, 78229, USA
d Cytocentrics Bioscience GmbH, Nattermannallee 1, 50829, Cologne, Germany
e Dept. Cardiovascular Sciences, Faculty of Medicine, K U Leuven, B-3000, Leuven, Belgium
f Dept. Basic Sciences, Faculty of Medicine, Université Catholique de Bukavu, Bukavu, DR Congo

ARTICLE INFO

Keywords:
Chloroquine
Comprehensive in vitro proarrhythmia assay
Cytocentering automated patch-clamp
Human induced pluripotent stem cell-derived cardiomyocyte
Hydroxychloroquine
Proarrhythmogenic risk predictor

ABSTRACT

Chloroquine and hydroxychloroquine have been proposed recently as therapy for SARS-CoV-2-infected patients, but during 3 months of extensive use concerns were raised related to their clinical effectiveness and arrhythmogenic risk. Therefore, we estimated for these compounds several proarrhythmogenic risk predictors according to the Comprehensive in vitro Proarrhythmia Assay (CiPA) paradigm. Experiments were performed with either CytoPatch™2 automated or manual patch-clamp setups on HEK293T cells stably or transiently transfected with hERG1, hNav1.5, hKir2.1, hKv7.1-hMinK, and on Pluricyte® cardiomyocytes (Ncardia), using physiological solutions. Dose-response plots of hERG1 inhibition fitted with Hill functions yielded IC50 values in the low micromolar range for both compounds. We found hyperpolarizing shifts of tens of mV, larger for chloroquine, in the voltage-dependent activation but not inactivation, as well as a voltage-dependent block of hERG current, larger at positive potentials. We also found inhibitory effects on peak and late INa, and on ICaM, with IC50 of tens of μM and larger for chloroquine. The two compounds, tested on Pluricyte® cardiomyocytes using the β-escin-perforated method, inhibited fnet, ICaM, INa peaks, but had no effect on freg. In current-clamp they caused action potential prolongation. Our data and those from literature for INa were used to compute proarrhythmogenic risk predictors Bnet (Mistry HB, 2018) and Qnet (Dutta S et al., 2017), with hERG1 blocking/unblocking rates estimated from time constants of fractional block. Although the two antimalarials are successfully used in autoimmune diseases, and chloroquine may be effective in atrial fibrillation, assays place these drugs in the intermediate proarrhythmogenic risk group.

1. Introduction

Chloroquine and hydroxychloroquine are two classical quinoline antimalarials derived from quinine, extracted by indigenous inhabitants of South America from the bark of Cinchona officinalis and used for centuries as a remedy for chills and common cold. Chloroquine was synthesized in 1934 by Johann (Hans) Andersag at Bayer A.G. by replacing the acridine with a quinoline ring in quinacrine (Atabrine) and was used under the name Resochin; in 1945 it was rediscovered by E.K. Marshall and received its current name (Krafts et al., 2012), remaining for decades a first-line antimalarial therapy. The mechanisms of action of quinoline antimalarials in autoimmune diseases are complex (Chew et al., 2020; Haladjy et al., 2018; Wallace, 1989).

The antiviral activity of chloroquine and hydroxychloroquine is deemed to result from accumulation of the protonated charged form in the acidic environment of endosomes, lysosomes and Golgi vesicles, leading to increased internal endosomal pH, thus preventing their use by viral particles as entry gateways (Ducharme and Farinotti, 1996).

Chloroquine has been tested in vitro or in vivo and found effective against Zika virus (Li et al., 2017a), influenza A H5N1 (avian flu) (Yan et al., 2013), Dengue virus type 2 (Farias et al., 2015), and some coronaviruses including OC43 (Keyaerts et al., 2009) and SARS-CoV-1 (Keyaerts et al., 2020).

* Corresponding author. Dept. Biophysics & Physiology, Faculty of Biology, University of Bucharest, Splaiul Independenței 91-95, 050095, Bucharest, Romania.
E-mail address: bogdan@biologie.kappa.ro (B. Amuzescu).

https://doi.org/10.1016/j.ejphar.2021.174632
Received 2 May 2021; Received in revised form 29 October 2021; Accepted 11 November 2021
Available online 14 November 2021
0014-2999/© 2021 Elsevier B.V. All rights reserved.
Preliminary in vitro studies of effectiveness against SARS-CoV-2 found EC50 values of 1.13–5.47 μM for chloroquine (Wang et al., 2020; Yao et al., 2020) or, and 0.72–1.38 μM for hydroxychloroquine (Yao et al., 2020; Yuan et al., 2020). These values are higher than maximal free plasma therapeutic concentrations (Cmax) estimated at 0.41 μM for chloroquine (Borsini et al., 2012) and 0.495 μM (215 ng/ml) for hydroxychloroquine (Yao et al., 2020) taken in usual doses. Although initial small-scale clinical trials with chloroquine/hydroxychloroquine on coronavirus-infected patients reported positive results, subsequent randomized unbiased trials were negative (Gasmi et al., 2021; Hernandez et al., 2020; Horby et al., 2020; Khashour et al., 2021). Moreover, severe cardiac arrhythmias associated with QT prolongation were reported by many studies (Borba et al., 2020; Chorin et al., 2020; Gerard et al., 2020; Mercuro et al., 2020; Roden et al., 2020; Tleyjeh et al., 2020), including a retracted Lancet paper (Mehra et al., 2020; Piller, 2021). As a consequence, on June 15, 2020, the FDA revoked the EUA for hydroxychloroquine and chloroquine in clinical trials on coronavirus-infected patients (US Food and Drug Administration, 2020).

Given the arrhythmogenic effects of 4-aminopyridine antimalarials (Mubagwa, 2020; Vicente et al., 2019), and the fact that SARS-CoV-2-infected patients often present myocardial injury by multiple mechanisms (Topol, 2020) and are highly susceptible to arrhythmias, the aim of our study was to perform an in-depth in vitro exploration of pharmacological effects of chloroquine and hydroxychloroquine on multiple human cardiac ion channels and to compute several proarrhythmogenic risk predictors following the principles and methods emerged from the CIPA initiative, a new paradigm for cardiac safety pharmacology (Fermini et al., 2016; Sager et al., 2014).

2. Materials and methods

2.1. Electrophysiology experiments on human ion channels expressed in heterologous cell lines

We used for experiments HEK293T cell lines stably or transiently expressing human cardiac ion channel subunits genes: stably expressed hERG1, inducible hNav1.5, inducible hKv7.1−hMink, hKir2.1 included in a plasmid (pCWXPGR-pTF-Kir2.1, a gift from Patrick Salmon: Addgene plasmid #114284; http://n2t.net/addgene:114284; RRID:Addgene_114284). Transient transfection was done with LipoD293 reagent (Merck KGaA, Darmstadt, Germany) estimated at 3 μM for chloroquine (Borsini et al., 2012) and 0.495 μM for hydroxychloroquine (Yao et al., 2020) in a plasmid (pCWXPGR-pTF-Kir2.1, a gift from Patrick Salmon: Addgene plasmid #114284; http://n2t.net/addgene:114284; RRID:Addgene_114284). Transient transfection was done with LipoD293™ reagent (SigmaGen® Laboratories) following manufacturer’s instructions. Cell lines were seeded in multi-well plates and cultured in standard conditions (5% CO2, 37 °C) with either automated or manual patch-clamp experiments performed at room temperature (RT: 22–25 °C) with either automated or manual patch-clamp setups. For automated patch-clamp we used CytoPatch™ Cytocentering® (Stett et al., 2003) platforms with dual-channel microfluidic chips with quartz pipette tips (2.5 μm inner diameter), as described previously (Airini et al., 2019; Mann et al., 2019; Scheel et al., 2014). For manual patch-clamp experiments we used classical setups equipped with WPC-100 resistive feedback amplifiers (ESF electronic, Göttingen, DE) and pipettes pulled from borosilicate glass capillaries (GC150F-10; Harvard Apparatus, USA) with resistance of 2–3 MΩ in the whole-cell configuration on cells perfused with a home-made solution application system at a flow rate of 100–200 μl/min, as described previously (Airini et al., 2019; Iordache et al., 2016). For hNav1.5 and hKv7.1 experiments, the continuous perfusion flow rate was approximately 1 ml/min and series resistance compensation was 50–80%.

Some experiments on hERG1 channels were performed at physiological temperature (PT: 37 °C) using manual patch-clamp and a Peltier-controlled perfusion system similar to that described in (Reid et al., 2001), except for a new microfluidic chamber with lateral walls made of mica instead of glass and an analog instead of a computerized PID (proportional-integrative-derivative) feedback controller. The flow rate was between 100 and 200 μl/min and the temperature fluctuations of perfused solution were in the range of ±2 °C. Other manual patch-clamp experiments at PT were performed on hNav1.5 using a TC01 temperature controller and PH01 perfusion cannula (Multichannel Systems, Reutlingen, DE).

For exploring effects on hERG1 we applied multiple voltage-clamp protocols. In both automated and manual patch-clamp experiments we applied a standard 2-step voltage-clamp protocol, starting from a holding potential of –70 mV, consisting of a 2-s depolarizing step at –40 mV followed by a 2-s step at –50 mV; the peak hERG1 current during this second step was measured relative to the average current during a brief (100 ms) step at –50 mV applied prior to the first depolarizing step.

In manual patch-clamp experiments performed on hERG1 channels at RT one single concentration of drug was tested per experiment. The standard protocol was applied under control conditions in the absence of drug and when the inhibitory effect of drug reached a steady level. In between we applied a modified Milnes protocol (Milnes et al., 2010) with 10-s depolarizing pulses at 0 mV from a holding potential of –80 mV repeated at 25-s intervals. Membrane resistance was monitored with brief (100 ms) pulses to –90 mV applied before the main depolarizing pulses. After testing for the stability of current level at the end of the 10-s depolarizing step in control conditions drug application started. Current traces recorded during this step initially and at steady-state inhibition were used to compute the fractional block (Milnes et al., 2010), and plots of the inverse of time constant of fractional block vs. drug concentration were used for an onset-of-block kinetics analysis to infer the blocking and unblocking rates (Cabbalero et al., 2004; Wagner et al., 2010). In manual experiments performed at PT we applied 4 increasing concentrations of each tested drug, monitoring the peak hERG current elicited by the standard 2-step voltage-clamp protocol applied at 10-s intervals.

In automated patch-clamp experiments the standard voltage-clamp protocol was applied at 10-s intervals with monitoring and continuous plotting of peak hERG1 current, measured relative to the average current during the brief step at –50 mV preceding the main 2-s depolarizing step. After stabilization of peak current amplitude in control conditions a drug was applied consecutively at 4 progressively increasing concentrations under continuous perfusion for 4 min at each concentration to monitor inhibitory effects on peak hERG current, followed by a 5-min wash-out period to assess reversibility of drug inhibition. In a series of experiments we applied a supplementary sequence of 5 voltage-clamp protocols adapted from (Vandenberg et al., 2012) in control conditions and at each drug concentration to explore pharmacological effects on kinetics of hERG current deactivation, inactivation, recovery from inactivation, and voltage dependence of activation and inactivation. All these protocols started from a holding potential of –80 mV. For the deactivation protocol, a 500-ms depolarizing step at +40 mV was followed by 2-s steps between –50 and –160 mV in 10-mV decrements; the current traces during these steps were fitted with double exponential functions to obtain time constants of fast and slow deactivation. For the inactivation protocol, a 500-ms depolarizing step at +40 mV was followed by a brief (25 ms) step at –90 mV for fast recovery from
inactivation, and then by 200-ms depolarizing steps between −80 and +60 mV in 10-mV increments, during which the time constants of hERG current inactivation were obtained by monoexponential fits. For the recovery-from-inactivation protocol, a 500-ms depolarizing step at +40 mV was followed by 500-ms steps between +40 and −160 mV in 10-mV decrements, during which the time constants of hERG current recovery from inactivation were obtained by monoexponential fits. For the voltage dependence of activation protocol, a 500-ms depolarizing step at −30 and +140 mV in 10-mV decrements, during which channels were removed from the voltage dependence of the preceding step. For the voltage dependence of inactivation protocol, a 500-ms depolarizing step at −70 and +50 mV in 10-mV increments were followed by a 300-ms step at −50 mV during which the peak hERG current was measured relative to the average level of the first sweep (with prepulse at −70 mV), where no hERG activation occurs; the current values were normalized by dividing peak currents of all sweeps with the highest peak current and plotted against the voltages of the preceding step. For the voltage dependence of inactivation protocol, a 500-ms depolarizing step at +30 mV was followed by brief (30-ms) steps between +30 and −140 mV in 10-mV decrements, during which channels were removed from inactivation to variable degrees, and then by a second 500-ms depolarizing step at +30 mV; the peak tail currents measured at the beginning of this second depolarizing step were plotted against the voltage of the preceding step, fitted with Boltzmann charge-voltage functions, then current values were normalized relative to the upper plateau value of the fit, replotted and refitted with the same function over a range of voltages where deactivation during the 30-ms step exerted negligible effects.

For the cardiac voltage-dependent Na+ current component hNav1.5 we used a series of voltage-clamp protocols as follows. For the peak and late $I_{Na}$ measurement in the presence of ATX-II started from a holding potential of −120 mV, included a 677-ms preconditioning step at −130 mV followed by a 33-ms activation step at −20 mV, a 188-ms step at 0 mV and a 88-ms ramp to −110 mV; the repeat interval was 7400 ms. The activation protocol started from a holding potential of −110 mV, included a 677-ms preconditioning step at −130 mV followed by a 33-ms activation step between −100 mV and 0 mV in 5-mV increments (21 sweeps); the intersweep interval was 1400 ms. The inactivation protocol started from a holding potential of −110 mV, included a 677-ms preconditioning step at −140 mV up to +40 mV in 5-mV increments (21 sweeps) followed by a 33-ms activation step at −20 mV; the intersweep interval was 1400 ms.

For the slow delayed rectifier K+ current hKv7.1-hMinK we used a double-step voltage-clamp protocol starting from a holding potential of −110 mV, including a 3300-ms activation step at +20 mV followed by a 4400-ms step at −40 mV for measurement of tail current; the repeat interval was 20000 ms.

For the cardiac inward rectifier K+ current hKir2.1, transfected cells were selected based on green fluorescence via epifluorescence microscopy with blue light excitation and approached by whole-cell patch-clamp. A standard double-ramp voltage protocol, composed of a 2-s ascending ramp from −120 mV to +80 mV followed by a symmetrical descending ramp, starting from a holding potential of −70 mV, was applied repeatedly at 10-s intervals. The current level was measured at −120 mV at the beginning of the ascending ramp. After verifying current level stability in control conditions, 3 μM chloroquine or 10 μM hydroxychloroquine were applied until current inhibition reached a steady value, followed by BaCl2 1 mM. The current level in BaCl2 was subtracted from previous current measurements to assess the specific hKir2.1 current component.

2.2. Electrophysiology experiments on human induced pluripotent stem cell-derived cardiomyocytes

Ventricular-enriched human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CM Pluricyte® cardiomyocytes provided by Ncardia) were stored in liquid nitrogen, thawed, plated at high density in 24-well sterile cell culture plates precoated with fibronectin (F1141, Sigma-Aldrich) and cultured in standard conditions (5% CO2, 37°C) in humidified incubators for up to two weeks, with medium changes every second day, according to manufacturer’s instructions. After 2–3 days in culture the cardiomyocytes developed confluent spontaneously contracting monolayers. For experiments cardiomyocytes were dissociated by 7-min treatment with Accutase® at 37°C followed by 3–4 h treatment at the same temperature with 2 mg/ml Collagenase A (11 088 866 001, Roche Diagnostics) in serum-free Iscove’s Modified Dulbecco’s Medium (IMDM) mixed with standard extracellular solution 1:1. Cells were detached by gentle pipetting, briefly spun in an Eppendorf microcentrifuge, resuspended and plated on 35 mm diameter Petri dishes kept at 37°C for at least 30 min prior to experiments. Manual patch-clamp experiments were performed at room temperature as described above, with the same external and internal solutions, but in the β-escin-perfused whole-cell configuration, using 15–30 μg/ml β-escin added to the pipette solution. After perforation to an access resistance ($R_A$) of less than 15 MΩ we applied a series of 3 standard voltage-clamp protocols as described previously (Mann et al., 2019) plus the standard double-ramp protocol described above in voltage-clamp mode, and a standard current-clamp protocol with 3 brief current pulses (500 pA, up to 2.5 ms duration, at 3-s intervals). If spontaneous pacemaking was present it was suppressed by applying a faint steady hyperpolarizing current up to 30 pA (Scheel et al., 2014). This sequence of protocols was applied in control conditions, then after 3–5 min from the start of continuous perfusion with 5 μM chloroquine or 10 μM hydroxychloroquine. Voltage-clamp and current-clamp recordings (where ventricular-like action potentials (AP) were present) were analyzed to assess effects of the two compounds on different ion current components and AP parameters.

2.3. Solutions and chemicals

The standard external solution contained (in mM): NaCl 140, KCl 2.5, MgCl2 2, CaCl2 2, HEPES 10, D-glucose 10, sucrose 20, pH 7.40 at 25°C titrated with NaOH. CaCl2, MgCl2, glucose and sucrose were added on the day of the experiment from 1 M aqueous stock solutions. The internal (pipette) solution had the following composition (in mM): KCl 140, NaCl 5, CaCl2 1, MgCl2 2, EGTA 11, HEPES 10, pH 7.20 at 25°C titrated with KOH. Both solutions were sterile filtered after preparation with 0.22 μm polyvinyl filters (Merck Millipore). For hNav1.5 recordings the external solution contained (in mM): NaCl 140, KCl 4, CaCl2 5, MgCl2 1, HEPES 10, pH 7.4 with NaOH, and the internal solution contained (in mM): CsF 60, CsCl 50, NaCl 10, HEPES 10, EGTA 20, pH 7.20 with KOH. For hKv7.1 recordings we used the same external solution as for hNav1.5 and an internal solution containing (in mM): CsF 60, CsCl 50, NaCl 10, HEPES 10, EGTA 20, pH 7.20 with KOH. For automated patch-clamp experiments the osmolality of external solution was adjusted to 325 mosm/kg H2O and that of internal solution to 290 mosm/kg H2O. External solution and deionized water for the automated patch-clamp equipment were degassed overnight by magnetic stirring into bottles connected to a low-grade vacuum pump or by brief boiling in a microwave oven followed by vigorous mechanical shaking to remove air bubbles. For perforated patch-clamp experiments on Pluricyte® hiPSC-CM, β-escin (Sigma E1378) was added freshly to the internal solution on the day of experiment from a 3-mM stock solution in water. Chloroquine sulfate (C1650000) and hydroxychloroquine sulfate (Y0001839) were purchased as European Pharmacopoeia certified reference standard compounds from European Directorate for the Quality of Medicines & HealthCare. Aqueous stock solutions (10 mM) were prepared and stored at −22°C for several weeks. ATX-II (Alomone labs STA-700) was dissolved in external solution at a final concentration of 20 nM. All other chemical reagents were from Sigma-Aldrich or Merck Millipore unless otherwise specified.

2.4. Data analysis

Throughout the paper data are reported as mean ± SD or mean ± SEM, as appropriate, with n indicating the number of cells recorded in
each experimental condition. Statistical significance was tested via Student’s t tests or one-way ANOVA, their non-parametric variants if normality condition was not met, or regression analysis, using a critical level $\alpha = 0.05$. For experiments on cell lines expressing human cardiac ion channels the selection criteria were a membrane resistance $> 0.2 \, \Omega$, an access resistance $< 20 \, \Omega$, and a holding current $< 100 \, \mu A$ ($< 300 \, \mu A$ for hK1.21) at $-70 \, mV$. For hPSC-CM experiments the selection criteria were an access resistance $< 20 \, \Omega$ (in most experiments it decreased $< 10 \, \mu A$) and an initial holding current $< 200 \, \mu A$ (although for most cardiomycocytes it was $< 100 \, \mu A$) at $-60 \, mV$. Automated analysis of voltage-clamp and current-clamp recordings in hPSC-CM was performed as described previously (Mann et al., 2019). Half-inhibitory concentrations (IC50) and Hill coefficients $n_H$ for chloroquine and hydroxychloroquine effects on different cardiac ion channels obtained in experiments on cell lines and hPSC-CM, together with blocking/unblocking rates and activation voltage shifts for hERG1 channels were used to compute proarrhythogenic risk predictors $b_{het}$ (Mistry, 2018, 2019) and $Q_{net}$ (Dutta et al., 2017). For $Q_{net}$ we used a modified C++ script of the O’Hara-Rudy 2011 model (O’Hara et al., 2011) including the Markov hERG gating model proposed by others (Li et al., 2017b) but with open bound and inactivated open bound states removed, using instead direct experimental estimates of blocking and unblocking rates.

3. Results

3.1. Blocking effects of chloroquine and hydroxychloroquine on hERG1 channels

Both chloroquine and hydroxychloroquine exerted strong inhibitory effects on peak hERG current amplitude measured with the standard manual two-step hERG voltage-clamp protocol (Fig. 1 a,b). During the first 2-s depolarizing step at $+40 \, mV$ hERG channels open and then inactivate, while during the second 2-s step at $-50 \, mV$ the channels exit the inactivated state very quickly (in a few milliseconds), giving rise to a specific tail peak hERG current. Measurements of hERG peak current amplitude were done relative to the current level during the 100-ms step at $-50 \, mV$ preceding the main depolarizing step. Similar results were obtained using the same voltage protocol in automated patch-clamp experiments (CytoPatchTM) in the whole-cell configuration on HEK293T cells stably expressing hERG1. Fig. S1 of Supplemental files illustrates the time lines of corrected peak hERG current measured in automated patch-clamp experiments at 10-s intervals with application of four consecutively increasing concentrations of chloroquine or hydroxychloroquine, as well as representative current traces recorded in these experiments initially and at each concentration when steady-state inhibition was obtained. Tables S1–S4 of Supplemental files show relative hERG current inhibition by each drug concentration tested in individual automated and manual patch-clamp experiments, as well as average values $\pm SD$. These average values were plotted in dose-response graphs shown in Fig. 1 c,d and were fitted with Hill functions, yielding similar half-inhibitory concentrations (IC50) for each drug in automated vs. manual experiments at RT: for chloroquine IC50 $= 1.82 \, \mu M$ ($n_H = 1.36$) for automated patch-clamp vs. IC50 $= 1.85 \, \mu M$ ($n_H = 0.90$) for manual patch-clamp, and for hydroxychloroquine IC50 $= 3.42 \, \mu M$ ($n_H = 1.02$) for automated patch-clamp vs. IC50 $= 4.79 \, \mu M$ ($n_H = 0.85$) for manual patch-clamp. For chloroquine at PT in manual patch-clamp experiments we obtained IC50 $= 1.29 \, \mu M$ ($n_H = 1.25$) (Fig. 1c and Table S2 of Supplemental files).

In manual patch-clamp experiments only (at RT) we also applied a modified Milnes protocol (Milnes et al., 2010) consisting of 10-s depolarizing steps at $0 \, mV$ from a holding potential of $-80 \, mV$, repeated at 25-s intervals, to study the onset-of-block kinetics. In Fig. 2 a,b the first traces (marked in red) were recorded initially with this protocol in control conditions, and the subsequent ones in the presence of the blocker, until current inhibition reached a steady-state. By plotting the difference between the initial current and the current in the presence of blocker at steady state (marked in magenta) divided by the former against time we obtained the kinetics of fractional block. The inverse of the monoexponential time constant of fractional block represents the sum of the microscopic blocking and unblocking rates. Therefore by plotting this value at different drug concentrations and applying linear fits we could directly estimate the blocking rates $k_B$ as slopes of these linear fits and the unblocking rates $k_U$ as Y axis intercepts, as shown in Fig. 2 c,d. This method represents the onset-of-block kinetics analysis (Caballero et al., 2004; Wagner et al., 2010). By applying it we obtained for chloroquine $k_B = 0.366 \, \mu M^{-1}\cdot s^{-1}$, $k_U = 0.108 \, s^{-1}$, and for hydroxychloroquine $k_B = 0.213 \, \mu M^{-1}\cdot s^{-1}$, $k_U = 0.566 \, s^{-1}$.

We also analyzed the voltage dependence of block induced by the two antimalarial drugs, by measuring the unblocked current following long (5 s) depolarizing steps at different potentials (as shown in Fig. S2 of Supplemental files), considering only those potentials where hERG channel activation is quasistatic. By fitting these relative peak hERG current vs. voltage plots (illustrated in Fig. 3) with a special Boltzmann charge-voltage function (Amuzescu et al., 2003):

$$I_B / I = 1 / (1 + \exp((-\Delta G_{\text{bind}} + zF \psi_0) / RT))$$

Fig. 1. Inhibitory effects of chloroquine and hydroxychloroquine on hERG1 channels. Standard hERG protocol applied to HEK293T cells stably expressing hERG1 approached by manual whole-cell patch-clamp initially and in the presence of chloroquine 3 $\mu M$ (a) or hydroxychloroquine 10 $\mu M$ (b). Dose-response curves for inhibition of peak hERG current elicited with the standard hERG protocol fitted with Hill functions in manual vs. automated patch-clamp experiments for chloroquine (at room temperature-RT, and physiological temperature-PT) (c) and hydroxychloroquine (d). Data points represent average values and error bars SD of multiple experiments ($n = 3$–7); individual values are listed in Tables S1–S4 of Supplemental files.
3.2. Effects of chloroquine and hydroxychloroquine on hERG1 kinetics and voltage dependence of activation and inactivation

Beyond the main blocking effects, we also explored the influence of the two antimalarials on kinetics and voltage dependence of activation and inactivation of hERG1 channels in automated patch-clamp experiments performed on HEK293T cells with stable hERG1 expression, initially in control conditions and when drug inhibition reached steady-state at each of the four concentrations tested. The protocol for voltage dependence of activation (Fig. S2 of Supplemental files) consisted in 5-s depolarizing pulses at various potentials, during which channels opened and subsequently inactivated, followed by a brief pulse at −50 mV that drove the channels rapidly out of inactivation and back in the open state. Thus the peak current amplitude during this step reflects the level of activation at the voltage of the previous step, and by plotting normalized peak current levels vs. voltage of preceding depolarizing step we could obtain the hERG current voltage-dependent activation in control conditions and at different drug concentrations (Fig. 4 a,b).

For studying the voltage dependence of inactivation of hERG1 channels (Fig. S3 of Supplemental files) we applied a 500-ms depolarizing step at +30 mV, followed by 35-ms steps at different hyperpolarized potentials, where hERG channels inactivated during the previous step recovered from inactivation in a voltage-dependent manner; the level of recovery from inactivation was assessed by measuring the peak tail current during the third voltage step at −30 mV. These peak tail current levels are easier to interpret than the peak currents during the preceding hyperpolarizing steps because they are elicited at the same voltage and thus directly reflect levels of hERG conductance activated during the previous step at different voltages. To obtain the voltage-dependent inactivation the peak tail currents were
plotted against voltage of preceding step. The plot was fitted with a Boltzmann function, then current values were normalized relative to the plateau current value of this Boltzmann function (Fig. 4 c,d). The data points in Fig. 4 are average values ± SEM of data obtained in multiple experiments in each condition; the individual data are listed in Tables S5–S8 of Supplemental files. Fig. 4 shows that both chloroquine and hydroxychloroquine (although to a lesser extent) shift the sigmoid hERG voltage-dependent activation curves in the hyperpolarizing direction. The voltage-dependent inactivation was not shifted significantly by either compound. In Fig. 5 a,b the average (±SEM) V\textsubscript{1/2} for activation and inactivation are plotted against concentration of chloroquine and hydroxychloroquine and fitted by linear regression. The slopes of the regression lines were significantly (p < 0.05) different from 0 only for activation.

We also examined the effects of the two antimalarials on hERG channels gating kinetics. The hERG deactivation protocol (Fig. S4 of Supplemental files) consisted in a 500-ms depolarizing step at +40 mV followed by long (2-s) steps at different potentials, during which channels were rapidly removed from inactivation and then deactivated. Double-exponential fits of currents recorded during these steps provided fast and slow rates of deactivation, illustrated in Fig. 6 a,b (individual values provided in Tables S9–S10 of Supplemental files). The fast deactivation rate varies quasiexponentially over the entire range of tested voltages (−50 to −160 mV), while the slow deactivation rate reaches a saturating value of 2−3 s\(^{-1}\) at approximately −90 mV.

The voltage protocol for hERG inactivation kinetics (Fig. S5 of Supplemental files) activated the channels with a 500-ms depolarizing step at +40 mV. The subsequent brief step at −90 mV quickly removed channels from inactivation, while the subsequent 200-ms depolarizing steps inactivated them again; inactivation time constants and rates were obtained by monoeponential fits of currents recorded during these steps. The voltage protocol for hERG recovery from inactivation kinetics (Fig. S6 of Supplemental files) is only slightly different from the previous one, lacking the brief intermediate step at −90 mV; hERG channels opened and inactivated during the 500-ms depolarizing step at +40 mV are removed from inactivation during subsequent hyperpolarizing steps, and the rates of recovery from inactivation are obtained from monoeponential fits of currents during this brief recovery stage. Inactivation/recovery form inactivation rates obtained in individual experiments are listed in Tables S11–S12 of Supplemental data, and average (±SEM) values of rates are plotted in Fig. 6 c,d. Theoretically there should be a continuity between the inactivation rates and the statistical significance of correlation coefficients.
recovery-from-inactivation rates, reflecting the fact that macroscopic time constants measured in these experiments are the inverse of the sum of inactivation and recovery-from-inactivation rates, as described (Vandenberg et al., 2012). Despite the lack of experimental data points at an intermediate potential range (between -50 and -90 mV), our data are compatible with a continuity of plots between the range of inactivation rates and that of recovery-from-inactivation rates. The two intersecting straight lines shown in Fig. 6 c,d represent estimates of voltage-dependent hERG inactivation and recovery-from-inactivation rates that approach asymptotically the experimental rate plots at extremities. Their point of intersection represents the half-inactivation potential ($V_{1/2}$), with similar values to those found by Boltzmann charge-voltage fits in Fig. 4 c,d.

3.3. Effects of chloroquine and hydroxychloroquine on hNav1.5 peak and late current

Both chloroquine and hydroxychloroquine exerted inhibitory effects
on peak and late \( I_{\text{Na}} \) measured in the presence of ATX-II 20 nM in HEK293 cells stably expressing hNav1.5 channels, as shown in Fig. 7 a,b and Tables S13–S14 of Supplemental files. Two measurement points were used to assess inhibitory effects on late Na\(^+\) current: immediately before jump from –20 mV to 0 mV (end of step current at –20 mV), and at the end of the voltage step at 0 mV, before the descending voltage ramp (end of tail current at 0 mV). The latter measurement point was preferred and was used further for computation of IC50 values, as shown in Fig. 7 c,d and Tables 1 and 3, yielding the following values:

- \( I_{\text{Na}} \) peak: IC50 = 159.15 \( \mu \)M (\( n_1 = 0.98 \)) at RT and IC50 = 39.32 \( \mu \)M (\( n_1 = 0.97 \)) at PT for chloroquine, IC50 = 96.23 \( \mu \)M (\( n_1 = 0.81 \)) at RT and IC50 = 42.44 \( \mu \)M (\( n_1 = 1.02 \)) at PT for hydroxychloroquine;
- \( I_{\text{Na}} \) late: IC50 = 30.37 \( \mu \)M (\( n_1 = 0.85 \)) for chloroquine, IC50 = 64.94 \( \mu \)M (\( n_1 = 0.89 \)) for hydroxychloroquine.

We also explored in separate experiments without ATX-II the effects of the two compounds on voltage dependence of activation and inactivation, and found slight but statistically significant hyperpolarizing shifts (less than 10 mV at highest concentration of drug) of half-inactivating potential \( V_{1/2} \), as shown in Figs. 8 a,b and Tables S15–S16 of Supplemental files.

### 3.4. Effects of chloroquine and hydroxychloroquine on hKv7.1•hMinK current

We explored inhibitory effects of chloroquine and hydroxychloroquine on \( I_{\text{K\(\beta\)}} \) via whole-cell experiments on HEK293 cells with inducible expression of hKv7.1•hMinK, using a standard two-step voltage protocol illustrated in Fig. 8 a,b. The plateau \( I_{\text{K\(\beta\)}} \) level measured at the end of the first depolarizing step at +20 mV was only slightly inhibited by the highest concentrations of drug applied (100 \( \mu \)M), as shown in Fig. 8 c,d and Tables S17–S18 of Supplemental files.

### 3.5. Effects of chloroquine and hydroxychloroquine on hKir2.1 current

HEK293T cells expressing hKir2.1 channels were selected based on eGFP fluorescence (the eGFP gene was present together with the hKir2.1 gene KCNJ2 on the same plasmid) and approached by whole-cell manual patch-clamp. The standard double-ramp voltage-clamp protocol (ascending ramp –120 mV to +80 mV in 2 s with symmetrical descending ramp) was applied repeatedly at 10-s intervals and the current level at –120 mV at the beginning of the ascending ramp was monitored. After verifying current stability in control conditions, chloroquine 3 \( \mu \)M or hydroxychloroquine 10 \( \mu \)M were applied until current inhibition reached steady values, followed by BaCl\(_2\) 1 mM, a strong blocker of Kir2.1 channels. Fig. 9 a,b illustrates I–V plots of current traces recorded in typical experiments initially, under drug application at steady inhibition, and during final application of BaCl\(_2\) (only the ascending voltage ramp is shown), while Fig. 9 c,d illustrates the time course of current inhibition. Current levels recorded in individual experiments are listed in Tables S19–S20 of Supplemental files. Average (±SD) steady inhibition of Kir2.1 current amounted to 33.85 ± 4.04% for 3 \( \mu \)M chloroquine and 25.46 ± 8.88% for 10 \( \mu \)M hydroxychloroquine, resulting in estimated IC50 for hKir2.1 channels of 5.86 \( \mu \)M for chloroquine and 29.28 \( \mu \)M for hydroxychloroquine, respectively (Table 1).

#### 3.6. Effects of chloroquine and hydroxychloroquine on multiple ion currents and APs recorded in Pluricyte® ventricular hiPSC-CM

Experiments on ventricular hiPSC-CM commercial preparations (Pluricyte® cardiomyocytes from NCdria) were performed by manual patch-clamp in the β-escin-perforated whole-cell patch-clamp configuration (Mann et al., 2019), as described in the Methods section. By applying the sequence of 3 standard voltage-clamp protocols (Mann et al., 2019) in control conditions initially and at 3–5 min since start of drug application we could assess specific inhibitory effects of the two compounds on peak voltage-dependent Na\(^+\) current (\( I_{\text{Na}} \)), peak L-type Ca\(^{2+}\) current (\( I_{\text{CaL}} \)), peak hERG current (\( I_{\text{Ks}} \)), and steady funny current (\( I_{\text{Kr}} \)). Furthermore, applying a standard current-clamp protocol we could estimate via automated analysis changes in parameters of externally paced APs induced by the two compounds. Tables S21–S30 of Supplemental files list drug-induced changes in ion currents and AP parameters obtained in individual experiments, and Fig. 10 illustrates changes in peak \( I_{\text{Na}} \), peak \( I_{\text{CaL}} \), and peak \( I_{\text{K}} \), as well as changes in AP shape induced by chloroquine or hydroxychloroquine in typical experiments. Chloroquine (5 \( \mu \)M) produced an average (±SD) peak \( I_{\text{Na}} \) inhibition to 90 ± 12% of control values (i.e. a 10% average inhibition), corresponding to an IC50 of 45 \( \mu \)M, average peak \( I_{\text{CaL}} \) inhibition to 86 ± 9% of control values, corresponding to an IC50 of 30.7 \( \mu \)M, and an average peak \( I_{\text{K}} \) inhibition by 62.78 ± 11.12% (to 37.22% of control values). Hydroxychloroquine (10 \( \mu \)M) produced an average (±SD) peak \( I_{\text{Na}} \) inhibition to 84 ± 16% of control values, corresponding to an IC50 of 52.5 \( \mu \)M, a peak \( I_{\text{CaL}} \) inhibition to 90 ± 12% of control values, corresponding to an IC50 of 90 \( \mu \)M, and a peak \( I_{\text{K}} \) inhibition by 70.65 ± 18.14% (to 29.35% of control values). Both compounds failed to elicit any significant effect on steady \( I_{\text{K}} \) amplitudes at the concentrations tested. In current-clamp recordings, 5 \( \mu \)M chloroquine produced on average only a mild prolongation of APD90 of externally paced APs (average APD90 104.1 ± 32.7% with chloroquine relative to control). Hydroxychloroquine 10 \( \mu \)M produced an average APD90 prolongation to 119.2 ± 82.6% of control values.

### 3.7. Estimation of proarrhythmic risk predictors \( B_{\text{net}} \) and \( Q_{\text{net}} \) for chloroquine and hydroxychloroquine

The IC50 values for chloroquine and hydroxychloroquine inhibition of several human cardiac ion channels obtained in this study (plus a few data from literature), as well as maximal effective free therapeutic plasma concentrations (EFTPC or \( C_{\text{max}} \) for the two compounds extracted also from literature are summarized in Table 1. With these pharmacological inhibition data and \( C_{\text{max}} \) values we computed the proarrhythmic risk predictor \( B_{\text{net}} \) (Mistry, 2018, 2019) using the formula:

\[
B_{\text{net}} = \sum_{i=1}^{n} R_i + \sum_{j=1}^{m} D_j
\]

where \( \Sigma R_i \) and \( \Sigma D_j \) represent the sums of relative inhibitions of repolarizing and depolarizing cardiac ion currents, defined using \( C_{\text{max}} \) (EFTPC) values:

- Relative inhibition \((R_i \text{ or } D_j) = 1 / (1 + IC50 / C_{\text{max}})\)

The \( B_{\text{net}} \) values obtained via this method (\( B_{\text{net}} = 0.09116 \) for chloroquine, \( B_{\text{net}} = 0.09176 \) for hydroxychloroquine) were compared with

---

**Table 1**

| Chloroquine | Hydroxychloroquine |
|-------------|---------------------|
| Value (\( \mu \)M) | Source | Value (\( \mu \)M) | Source |
| C\(_{\text{max}}\) 0.41 | Borsini et al. (2012) | 0.495 (215 ng/ml) | Yao et al. (2020) |
| \( I_{\text{Na}} \) peak I\(_{\text{CaL}}\) 159 | own data | 96.2 | own data |
| I\(_{\text{Na}}\) late I\(_{\text{CaL}}\) 30.4 | own data | 64.9 | own data |
| I\(_{\text{Na}}\) IC50 30.7 | own data | 90 | own data |
| I\(_{\text{CaL}}\) IC50 4600 | Wagner et al. (2019) | 4600 | analogy with chloroquine |
| I\(_{\text{Na}}\) IC50 1.82 | own data | 3.42 | own data |
| I\(_{\text{Na}}\) IC50 >100 | own data | >100 | own data |
| I\(_{\text{Na}}\) IC50 5.86 | own data | 29.28 | own data |
$B_{\text{net}}$ values of 12 compounds belonging to different proarrhythmogenic risk groups (Dutta et al., 2017) (Table 2) and were placed in the intermediate risk group.

We proceeded further to compute another relevant proarrhythmogenic risk predictor, $Q_{\text{net}}$ (Dutta et al., 2017). For this purpose we used for numeric integration simulations an $O'Hara-Rudy$ human ventricular cardiomyocyte electrophysiology and Ca$^{2+}$ cycling model ($O'Hara$ et al., 2011) with subendocardial cardiomyocyte parameter set (cell type = 0), including a Markov gating model for hERG as defined by (Li et al., 2017b), but we simplified the pharmacodynamic component of this Markov model by removing the open bound (O*) and inactivated open bound (IO*) states and connecting directly the blocked state (C*) to the open (O) and inactivated (I) state by experimentally-derived blocking and unblocking rates (Fig. S9 of Supplemental files). The reasons for this simplifying change are multiple. First, we wanted to make use of the hERG blocking and unblocking rates for chloroquine and hydroxychloroquine estimated directly from experiments by onset-of-block kinetics analysis. Second, although the open bound and inactivated open bound states exist in reality beyond any doubt, corresponding to hERG channel conformations where the blocker molecule entered the inner vestibule without occupying the final blocking position and occluding the ion permeation pathway, in practice they are extremely difficult to distinguish from the corresponding non-bound states, featuring the same single-channel levels of conductance. The distinction based on drug trapping effects and changes in relative fractional block at consecutive applications of the Milnes protocol is also tricky because the pharmacokinetics of drug penetration into the cell is variable from one cell to another and rather unpredictable, as shown by our experiments (Fig. 2a,b). In order to use this simplified Markov model for the standard CiPA drugs, we introduced for them compound blocking rates $E_{\text{max}}(D)$, assuming that only the fraction $E_{\text{max}}(D)$ of channels in the open or inactivated state is available for drug block. Simulations with this modified cardiomyocyte electrophysiology and pharmacology model (C++ script available at https://github.com/bamuzesc/Qnet-for-CiPA.git, https://zenodo.org/record/5615548#.YXvbQ7hyGSp) were run for chloroquine, hydroxychloroquine and the standard CiPA compounds at concentrations from 1x $C_{\text{max}}$ to 25x $C_{\text{max}}$. At each concentration we ran 1000 2-s pacing cycles and collected $Q_{\text{net}}$ values for the last 10 cycles. For chloroquine and hydroxychloroquine we ran the simulations both with and without including the drug-induced voltage shifts in half-activation potential (approximated by the linear fits shown in Fig. 5).
a, b) applied to the voltage dependency of all opening and closing rates included in the hERG Markov model, but including half-activation potential shifts produced only small effects on simulated APs and $Q_{net}$ values (Tables S31–S32 of Supplemental files). We also performed simulations with the modified O’Hara-Rudy model (Li et al., 2017b) incorporated in the Nottingham AP portal (https://cardiac.nottingham.ac.uk) (Williams and Mirams, 2015), which uses an IC50-based estimation of hERG inhibition by drugs, and independently with our own script of the modified O’Hara-Rudy model (Li et al., 2017b) without pharmacodynamic component and using a hERG open-state probability weighted by hERG IC50 values for $I_{Kr}$ computation $\left[I_{Kr} = G_{Kr} \cdot \sqrt{K^-/\left[\frac{4.4 I_{KrO}}{V - E_K} \right]} \right]$. In both series of simulations we used the experimental pharmacological inhibition data for chloroquine and hydroxychloroquine exposed in Table 1, performing 1000 2-s simulation cycles for drug concentrations in the range of 0–25x $C_{max}$. The results of these IC50-based hERG inhibition simulations were very similar, as shown in Tables S31–S32 and Figs. S10–S11. However, using the experimentally measured hERG blocking/unblocking rate modeling approach resulted in higher $Q_{net}$ values, which became similar to the hERG IC50-based predicted $Q_{net}$ values for chloroquine only at high concentrations (＞20x $C_{max}$, Fig. S10). Increasing the hERG blocking rate ($k_b$) by 3x for chloroquine resulted in $Q_{net}$ values similar to those of hERG IC50-based simulations at low concentrations (1–4 x $C_{max}$, Fig. S10), while increasing hERG $k_b$ by 4.8x for hydroxychloroquine.
reached IC50-based \( Q_{\text{det}} \) predictions at 20–25 x \( C_{\text{max}} \) (Fig. S11). Complex pharmacological effects of the two compounds on AP shape obtained in different types of simulations are illustrated in Figs. S12–S17.

\( Q_{\text{det}} \) values for the two antimalarial compounds (using experimental hERG blocking/unblocking rate estimates, including drug-induced activation voltage shifts), together with values for the standard 12-compounds panel (CIPA training set) used by (Li et al., 2017) and (Dutta et al., 2017), as well as for other 16 compounds included in a CIPA validation set (Han et al., 2020; Li et al., 2020) (with inhibition data retrieved from https://github.com/FDA/CiPA/tree/Lab_Specific_Vali- dation2020), for concentrations ranging from 1 x \( C_{\text{max}} \) to 25 x \( C_{\text{max}} \), are illustrated in Fig. 11a and b. These simulations yielded the following values:

- chloroquine: \( Q_{\text{det}} \) at 4 x \( C_{\text{max}} \): 62.81 nC/μF
- hydroxychloroquine: \( Q_{\text{det}} \) at 4 x \( C_{\text{max}} \): 65.84 nC/μF

placing both compounds in the intermediate proarrhythmogenic risk group. Fig. 12 shows correlation between \( B_{\text{det}} \) and \( Q_{\text{det}} \) at 4 x \( C_{\text{max}} \) for chloroquine, hydroxychloroquine and the standard 12-compound panel used previously (Dutta et al., 2017; Li et al., 2017).

4. Discussion

In the present study we characterized inhibitory effects of chloroquine and hydroxychloroquine on human cardiac ion channels in heterologous expression systems and hiPSC-CM preparations. Assessment of inhibitory potency of these two compounds yielded IC50 values largely similar with previous reports (Table 3). Disparity with literature may be due to influence on IC50 of experimental protocol and measurement method (Gomis-Tena et al., 2020), or variable enantiomer composition (Ducharme and Farinotti, 1996; Lentini et al., 2020). IC50 values for multiple cardiac ion currents decrease with temperature increase to 37°C (Delaunois et al., 2021), although further rise to 42°C reduces hERG inhibition (TeBay et al., 2021).

hERG inhibitory effects of the two 4-aminoquinoline drugs play significant roles for their proarrhythogenic risk. Using a modified Milnes protocol (Milnes et al., 2010) to assess fractional block and the onset-of-block kinetics analysis (Caballero et al., 2004; Wagner et al., 2010) we obtained direct estimates of hERG channels blocking and unblocking rates. The relative electrical distances \( \theta \) of blocking site from interior found by us are smaller than the value of 0.57 that can be

---

### Table 3

Estimated half-inhibitory concentrations (IC50) of chloroquine and hydroxychloroquine for different cardiac ion currents from own experiments compared to data retrieved from the literature (RT - room temperature, PT - physiological temperature, SAN - sino-atrial node).

| Cardiac ion current | IC50 (μM) | Preparation | Ref. |
|--------------------|-----------|-------------|------|
| Chloroquine | | | |
| \( f_{\text{f}} \) | 1.82 at RT | 0.96 at RT | HEK293 |
| \( f_{\text{f}} \) | 1.29 at PT | 1.03 at PT | CHO |
| \( f_{\text{f}} \) | 1.47 at PT | 1.6 at PT (65% block at 3 μM) | CHO |
| \( f_{\text{f}} \) | 2.5 at RT | 7.77 | cat Purkinje fibers |
| \( f_{\text{f}} \) | >100 | >80.9 (11% block at 10 μM) | cat Purkinje fibers |
| \( f_{\text{f}} \) | >100 | >115 (8% block at 10 μM) | cat Purkinje fibers |
| \( f_{\text{f}} \) | 5.86 | 0.35 (74% block at 1 μM) | HEK293 |
| \( f_{\text{f}} \) | | | CHO |
| \( f_{\text{f}} \) | 5.86 | 1.2 | CHO |
| \( f_{\text{f}} \) | | 4.98 | CHO |
| \( f_{\text{f}} \) | 159.15 at RT | 8.48 | HEK293 |
| \( f_{\text{f}} \) | 39.32 at PT | 12.2 (45% block at 10 μM) | cat Purkinje fibers |
| \( f_{\text{f}} \) | 40.4 (42.6% block at 30 μM) | | human atrial myocytes |
| \( f_{\text{f}} \) | 64.2 at RT, 13.95 at PT | 3.05 | CHO |
| \( f_{\text{f}} \) | 109.2 at RT, 11.6 at PT | 19.75 at RT | CHO |
| \( f_{\text{f}} \) | 30.7 | 3.05 | HEK293 |
| \( f_{\text{f}} \) | 80.9 (22% block at 300 μM) | 21.25 (32% block at 10 μM) | cat Purkinje fibers |
| \( f_{\text{f}} \) | >240 (4% block at 10 μM) | | CHO |
| \( f_{\text{f}} \) | >300 | | CHO |
| \( f_{\text{f}} \) | 4600 | | CHO |
| \( f_{\text{f}} \) | >161.7 | 227.95 at RT | HEK293 |

hydroxychloroquine

| Cardiac ion current | IC50 (μM) | Preparation | Ref. |
|--------------------|-----------|-------------|------|
| \( f_{\text{f}} \) | 3.42 at RT | 3.78 at PT | CHO |
| \( f_{\text{f}} \) | 5.6 at RT (35% block at 3 μM) | 6.97 at RT | gilena pig SAN cells |
| \( f_{\text{f}} \) | 2.66 at PT | 9.7 | CHO |
| \( f_{\text{f}} \) | >100 | >300 at RT | CHO |
| \( f_{\text{f}} \) | 29.28 | 44.15 at RT | CHO |
| \( f_{\text{f}} \) | 44.15 at RT, 16.99 at PT | 21.25 (32% block at 10 μM) | cat Purkinje fibers |
| \( f_{\text{f}} \) | 62.4 at PT | >300 | CHO |
| \( f_{\text{f}} \) | 64.94 | 92.87 at RT 21.43 at PT | CHO |
| \( f_{\text{f}} \) | 90 | 7.64 | HEK293 |
| \( f_{\text{f}} \) | 22 at PT (12% block at 3 μM) | 22 at PT (12% block at 3 μM) | gilena pig SAN cells |
| \( f_{\text{f}} \) | 66.29 at RT | 372.3 at RT, >300 at PT | gilena pig SAN cells |
| \( f_{\text{f}} \) | >990 | 22 at PT (12% block at 3 μM) | CHO |
| \( f_{\text{f}} \) | >990 | 22 at PT (12% block at 3 μM) | CHO |
| \( f_{\text{f}} \) | >990 | 372.3 at RT, >300 at PT | CHO |

---
computed for chloroquine from data reported by others (Sánchez-Chapula et al., 2002).

Another result of our study is the hyperpolarizing shift in voltage dependence of hERG activation induced by chloroquine/hydroxychloroquine, while the voltage dependence of inactivation is not changed (Figs. 3 and 4 and Tables S5–S8 of Supplemental files). Similar hyperpolarizing shifts of hERG channels half-activation potential were reported for almokalant and dofetilide in studies on dissociated rabbit or guinea pig ventricular myocytes performed by Prof. Edward Carmeliet in the early 1990s (Carmeliet, 1992, 1993). Since then it was clear that the shift is genuine, not a result of reversible state-dependent block, that it can counteract blocking effects by increasing the fraction of open channels, and that it may result from drug binding to a secondary site distinct from the blocking site, with faster accessibility and different affinity (Carmeliet, 1993). This general property of drugs to exert hyperpolarizing shifts in voltage-dependent hERG activation in addition to block was named facilitation by Perry, Sanguinetti and Mitcheson (Perry et al., 2010). A helical wheel analysis of differential effects on hERG block and facilitation of S6 point mutants (Hosaka et al., 2007), together with the assumption that a hyperpolarizing shift in voltage dependence of hERG activation requires actions exerted before channel opening, leads to the conclusion that facilitation involves drug binding to secondary sites on hERG channels, distinct from the blocking site, involving S6 residues not facing the inner cavity (Gessner et al., 2010).

Drug trapping in the inner cavity by hERG channel closure has been also described (Carmeliet, 1993). Mitcheson et al. demonstrated the trapping mechanism using a hyperpolarization-activated hERG point-mutant (D540K) where MK-499 block was removed at $-160 \text{ mV}$ (Mitcheson et al., 2000). Using the Milnes protocol (Milnes et al., 2010), Windley et al. assessed the drug trapping mechanism for several hERG blockers (Windley et al., 2017). However, with a unique long-depolarization step protocol it is virtually impossible to distinguish real drug trapping (by channel closure) from apparent drug trapping by slow dissociation from a high-affinity open/inactivated blocked state.

Fig. 11. $Q_{\text{net}}$ values at concentrations between 1x and 25x $C_{\text{max}}$ computed with a modified O’Hara et al., 2011 model with the simplified hERG gating Markov model proposed by us for chloroquine, hydroxychloroquine and: (a) the standard 12-compounds set used by Li et al. 2017 and Dutta et al., (2017) (the CiPA training set); (b) a supplementary 16-compounds set used by Han et al., (2020) and Li et al., (2020) (the CiPA validation set).
conditions, without adding high-potency specific blockers for pharmacological isolation of these ion current components. For \( I_{Kr} \) the estimated IC50 values (2.96 \( \mu \)M for chloroquine and 4.15 \( \mu \)M for hydroxychloroquine) were largely similar to those yielded by both automated and manual patch-clamp experiments on hERG1-expressing HEK293 cells. Although with the currently applied protocol we could measure only the total delayed rectifier \( K^- \) current, representing a mixture of \( I_{Kr} \) and \( I_{Ks} \), usually \( I_{Kr} \) is the main component, while \( I_{Ks} \) activation requires \( \beta \)-adrenergic stimulation. In our experimental conditions we could elicit externally-triggered APs in over 40% of the hiPSC-CM (Amuzescu et al., 2021), and most of them were ventricular-like (Tables S29–S30 of Supplemental files), but in some cases we had to apply steady external hyperpolarizing currents up to 30 pA to suppress spontaneous pacemaking (Scheid et al., 2014). APD prolongation by chloroquine/hydroxychloroquine in individual experiments is consistent with that reported by others (Borsini et al., 2012; Capel et al., 2015; Filgueiras-Rama et al., 2012; Sánchez-Chapula et al., 2001) and with QT prolongation induced by chloroquine in clinical trials (Becker et al., 2021; Cardiac Arrhythmia Suppression Trial (CAST) Investigators, 1989; Cook et al., 2006; Vicente et al., 2019).

Both \( B_{net} \) (Mistry, 2018, 2019) and \( Q_{net} \) (Dutta et al., 2017; Li et al., 2017b) are reliable proarrhythmogenic risk predictors. Our study proved statistically significant correlation for chloroquine/hydroxychloroquine and the 12-compound panel used by (Li et al., 2017b).

\( Q_{net} \) values found by us for the two antimalarials (Table S31 of Supplemental files) are similar to those reported by (Delaunois et al., 2021; Whittaker et al., 2021), along with APD-prolongation and EAD-induction effects found in other simulation studies (Montnach et al., 2021; Varshneya et al., 2021). Although \( Q_{net} \) provides an in-depth mechanistic interpretation of proarrhythmogenic effects (Parikh et al., 2019), we found that the method requires supplementary precautions and avoiding simulated APs that actually feature EADs.

Clinical studies evidenced significant QT prolongation by chloroquine and hydroxychloroquine (Borba et al., 2020; Chorin et al., 2020; Roden et al., 2020; Vicente et al., 2019) and increased risk of arrhythmias (ventricular tachycardia/fibrillation, torsades-de-pointes, cardiac arrests) (Gérard et al., 2020; Grandvuillemin et al., 2021; Roden et al., 2020; Teyjeh et al., 2020), particularly at high doses and during hypokalemia (TeBay et al., 2021). Although initial in vitro efficiency results (Wang et al., 2020) and non-randomized clinical trials suggested effectiveness against coronavirus-induced cytokine storm (Gao et al., 2020; Guo et al., 2020; Jean et al., 2020; Mehta et al., 2020; Yao et al., 2020), subsequent large-scale trials failed to demonstrate any benefit of the two antimalarials for SARS-CoV-2-infected patients (Borba et al., 2020; Hernandez et al., 2020; Horby et al., 2020; Pan et al., 2021). Similar disappointing results were obtained with hydroxychloroquine administered to macaque monkeys artificially infected with SARS-CoV-2 (Maisonnasse et al., 2020). SARS-CoV-2 infection association with endothelium damage (Wadman et al., 2020) and viral myocarditis (Chang et al., 2021; Topol, 2020), in combination with possible cardiomyopathy induced by chloroquine per se (Tomesmann et al., 2013), represent further arguments against use of the two antimalarials in coronavirus-infected patients. The immunomodulatory effect of chloroquine via TLR7/TLR9 inhibition by impaired lysosomal acidification (In’t Veld et al., 2021; Kuznik et al., 2011; Tohmé and Manoury, 2014) may not be beneficial during coronavirus infection, since it was postulated that higher TLR7 expression in women vs. men via incomplete X chromosome inactivation may protect against viral infections including SARS-CoV-2 (Takahashi and Iwasaki, 2021).

5. Conclusions

In conclusion, we have shown via automated and manual patch-clamp experiments on cell lines expressing human cardiac ion channels and hiPSC-CM multiple inhibitory effects of chloroquine and hydroxychloroquine. We also produced preliminary estimates of hERG...
blocking and unblocking rates via onset-of-block kinetics analysis. Proarrhythmogenic risk predictors $B_{0\text{eq}}$ and $Q_{\text{net}}$ computed based on these data provided similar results, placing the two compounds in the intermediate proarrhythmogenic risk group. In addition, we studied the voltage dependence of HERG block and also provided evidence for drug-induced hyperpolarizing shifts in voltage-dependent activation of HERG channels, as well as smaller hyperpolarizing shifts of voltage-dependent inactivation of hNav1.5 channels. However, these shifts applied to in silico simulations produced only small changes in $Q_{\text{net}}$ values. Overall, we proved that CPA-based methods can be combined and effectively used for proarrhythmogenic risk prediction in a real-life example.

CRediT authorship contribution statement

Urs Thomet: Conceptualization, Methodology, Investigation, Validation, Formal analysis, Data curation, Resources. Bogdan Amuzescu: Conceptualization, Methodology, Investigation, Validation, Formal analysis, Data curation, Software, Writing – original draft, Supervision. Thomas Knot: Conceptualization, Methodology, Validation, Formal analysis, Data curation, Resources, Writing – review & editing. Stefan A. Mann: Conceptualization, Methodology, Investigation, Formal analysis, Writing – review & editing. Beatrice Mihaela Radu: Conceptualization, Methodology, Validation, Formal analysis, Writing – review & editing, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. TK is CSO of CytoBioScience Inc. Part of the methods used within this study have been included in EPO patent application EP18465611.

Acknowledgements

This study was funded from Competitiveness Operational Programme 2014–2020 project P.37.675 (contract no. 146/2016). Priority Axis I, Action 1.1.4, co-financed by the European Funds for Regional Development and Romanian Government funds. The content of this publication does not necessarily reflect the official position of the European Union or Romanian Government.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejphar.2021.174632.

References

Airini, R., Iordache, F., Alexandru, D., Savu, L., Epureanu, F.B., Mihailescu, D., Amuzescu, B., Maniu, H., 2019. Senescence-induced immunophenotype, gene expression and electrophysiological changes in human amnioncytes. J. Cell Mol. Med. 23, 7233-7245. https://doi.org/10.1007/s10865-019-00465-6.
Amuzescu, B., Segal, A., Flonta, M.L., Simaels, J., Van Driessche, W., 2003. Zinc is a Proarrhythmogenic risk predictors. Cardiovasc. Toxicol. 2, 1-8. https://doi.org/10.1080/1470582031000160125.
Borba, M.G.S., Val, F.F.A., Sampaio, V.S., Alexandre, A.M.A.A., Melo, G.C., Brito, M., Mourão, M.P.G., Brito-Sousa, J.D., Baía-da-Silva, D., Guerra, M.V.F., Hajjar, L.A., Fares, K.J., Machado, P.R., Muniz, J.A., Imbeloni, A.A., da Fonseca, B.A., 2015. Antiviral drug? J. Chem. Inf. Model. 60, 1779-1815. https://doi.org/10.1021/acs.jcim.9b00354.
Bonassar, G., Colatsky, T., Li, Z., 2017. Optimization of an in silico cardiac cell model for electrophysiological insights and therapeutic potential. Heart Rhythm 12, 1816-2194. https://doi.org/10.1016/j.hrthm.2017.08.027.
Carmeliet, E., 1992. Voltage- and time-dependent block of the delayed rectifier K+ current by almokalant in rabbit ventricular myocytes. Circ. Res. 73, 857-868. https://doi.org/10.1161/01.RES.73.7.857.
Chang, W.T., Toh, H.S., Liao, C.T., Yu, W.L., 2021. Cardiac involvement of COVID-19: a comprehensive review. Am. J. Med. Sci. 361, 14-22. https://doi.org/10.1016/j.amjms.2020.10.002.
Chew, C.Y., Mar, A., Nikpour, M., Saracino, A.M., 2020. Hydroxychloroquine in dermatology: new perspectives on an old drug. Australas. J. Dermatol. 61, 1-8. https://doi.org/10.1111/ajd.13169.
Chorin, E., Dai, M., Shulman, E., Wadhwani, L., Bar-Cohen, R., Barbhaya, C., Aizer, A., Holmes, D., Bernstein, S., Spinelli, M., Park, D.S., Chiniz, L.A., Jankelson, L., 2020. The QT interval in patients with COVID-19 treated with hydroxychloroquine and azithromycin. Nat. Med. 26, 808-809. https://doi.org/10.1038/s41591-020-0889-3.
Cook, J.A., Randinitti, E.J., Bramson, C.R., Wenche, D.L., 2006. Lack of a pharmacokinetic interaction between azithromycin and chloroquine. Am. J. Trop. Med. Hyg. 74, 407-412.
Delenauer, A., Abernathy, M., Anderson, W.D., Beatte, K.A., Chaudhary, K.W., Gourley, S., 2015. Chloroquine phosphate has shown apparent efficacy in treatment of COVID-19 associated pneumonia in clinical studies. Appl. Microbiol. Biotechnol. 30, 1-8. https://doi.org/10.1007/s00253-021-11094-4.
Ducharme, J., Farinotti, R., 1996. Clinical pharmacokinetics and metabolism of chloroquine. Focus on recent advancements. Clin. Pharmacokinet. 31, 257-274. https://doi.org/10.2165/00003088-199631040-00003.
Duatu, S., Chang, K.C., Beatte, K.A., Chessa, S., Tran, P.N., Wu, W.W., Wu, M., Strauss, D.G., Colatsky, T., Li, Z., 2017. Optimization of an in silico cardiac cell model for proarrhythmia risk assessment. Front. Physiol. 8, 616. https://doi.org/10.3389/fphys.2017.00616.
Farias, K.J., Machado, P.R., Muz, J.A., Imbeloni, A.A., da Fonseca, B.A., 2015. Antiviral activity of chloroquine against dengue virus type 2 replication in Aotus monkeys. Virus Immunol. 28, 161-167. https://doi.org/10.1007/s11236-015-0681-1.
Fermini, B., Hancox, J.K., Abg-Ergestes, N., Bridl-Develay, Chaudhary, K.W., Colatsky, T., Correll, K., Chw, D., Damiano, B., Erdemli, G., Gintant, G., Imredey, J., Krömer, J., Kramer, J., Levesque, P., Li, Z., Lindquist, Å., Obejo-Paz, A., de Kamps, D., Sandra, D., Sandw, K., Stra, D., 2021. A new perspective in the field of cardiac safety testing through the comprehensive in vitro proarrhythmia assay paradigm. J. Biomol. Screen 21, 1-11. https://doi.org/10.1177/1087057120945949.
Filgueiras-Rama, D., Martins, R.P., Mironov, S., Yamazaki, M., Calvo, C.I., Ennis, S.R., Bandara, K., Nouisam, S.F., Calvo, J., Berenfeld, O., Jalife, J., 2012. Chloroquine terminates stress-induced atrial fibrillation more effectively than flecainide in the sheep heart. Circ. Arrhythm. Electrophysiol. 5, 561-570. https://doi.org/10.1161/CIRCEP.111.96620.
Gao, J., Tian, Z., Yang, X., 2020. Breakthrough: chloroquine phosphate has shown apparent efficacy in treatment of COVID-19 associated pneumonia in clinical studies. Biosk. Trends. 14, 72-73. https://doi.org/10.5587/bst.2020.01047.
Garni, A., Peana, M., Nour, A., Tyas, A., Garni, A., Garni, A., 2021. Chloroquine and hydroxychloroquine in the treatment of COVID-19: the never-ending story. Appl. Microbiol. Biotechnol. 632, 52-570. https://doi.org/10.1007/s00253-021-11094-4.
Gerd, A., Roman, S., Fresse, A., Viard, D., Parassol, N., Roman, A., Baia-da-Silva, D., Guerra, M.V.F., Hajjar, L.A., Fares, K.J., Machado, P.R., Muniz, J.A., Imbeloni, A.A., da Fonseca, B.A., 2015. Chloroquine phosphate has shown apparent efficacy in treatment of COVID-19 associated pneumonia in clinical studies. Eur. J. Pharmacol. 735, 252-59. https://doi.org/10.1016/j.ejphar.2021.01.010.
Gomi-Tena, J., Brown, B.M., Cano, J., Trenor, B., Yang, P.C., Saiz, J., Clancy, C.E., Romero, L., 2020. When does the ICSO accurately assess the blocking potency of a drug? J. Chem. Inf. Model. 60, 1779-1796. https://doi.org/10.1021/acs.jcim.0c00185.
