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In vitro ion channel profile and ex vivo cardiac electrophysiology properties of the R(-) and S(+) enantiomers of hydroxychloroquine

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ABSTRACT

Hydroxychloroquine (HCQ) is a derivative of the antimalaria drug chloroquine primarily prescribed for autoimmune diseases. Recent attempts to repurpose HCQ in the treatment of corona virus disease 2019 has raised concerns because of its propensity to prolong the QT-segment on the electrocardiogram, an effect associated with increased pro-arrhythmic risk. Since chirality can affect drug pharmacological properties, we have evaluated the functional effects of the R(-) and S(+) enantiomers of HCQ on six ion channels contributing to the cardiac action potential and on electrophysiological parameters of isolated Purkinje fibers. We found that R(-)HCQ and S(+) HCQ block human K\textsubscript{ir} and hERG potassium channels in the 1 \mu M-100 \mu M range with a 2-4 fold enantiomeric separation. Na\textsubscript{a}1.5 sodium currents and Ca\textsubscript{a}1.2 calcium currents, as well as K\textsubscript{ir}4.3 and K\textsubscript{ir}7.1 potassium currents remained unaffected at up to 90 \mu M. In rabbit Purkinje fibers, R(-)HCQ prominently depolarized the membrane resting potential, inducing autogenic activity at 10 \mu M and 30 \mu M, while S(+)HCQ primarily increased the action potential duration, inducing occasional early afterdepolarization at these concentrations. These data suggest that both enantiomers of HCQ can alter cardiac tissue electrophysiology at concentrations above their plasmatic potential in vitro.

1. Introduction

Hydroxychloroquine (HCQ, Plaquenil®) is a well-known antimalarial drug currently used in immuno-inflammatory conditions such as cutaneous and systemic lupus erythematosus or rheumatoid arthritis (Schrezenmeier and Dorner, 2020). Recent attempts to repurpose HCQ for the treatment of corona virus disease 2019 (Covid-19) have raised concerns because this drug can prolong the QT-segment on the electrocardiogram (Mazzanti et al., 2020; Oscanoa et al., 2020). Drug-induced QT interval prolongation is associated with an increased risk of a distinctive type of ventricular arrhythmias known as Torsade de Pointes (TdP) which can lead to cardiac arrest (Rodén, 2004). The mechanism underlying TdP results from a delay of the cardiac action potential repolarization towards the end of its plateau phase, which prematurely reactivates the slowly inactivating L-type Ca\textsubscript{a}1.2 calcium channels. Action potential repolarization is driven by outward potassium currents flowing through channels encoded by the human ether-a-go-go related gene (i.e. hERG channels). Inhibition of hERG function was the first molecular mechanism associated with TdP Rampe and Brown (2013); Sanguinetti et al., 1995). However, drug effects at the voltage gated Kv7.1 or the inwardly rectifying K\textsubscript{ir}2.1 channels can also influence repolarization and induce arrhythmic events. Moreover, blockade of inward Na\textsuperscript{+} and Ca\textsuperscript{2+} currents could in principle counter-balance the QT elongating effects of outward K\textsuperscript{+} currents inhibition, a hypothesis that underlies the Comprehensive in vitro Pro-Arrhythmia (CiPA) initiative aimed at refining the electrocardiographic risk...
assessment of new drugs (Gintant et al., 2016).

HCQ is a chiral 4-aminoquinoline that has been developed and marketed as a racemate. Yet, it has been documented for several racemic drugs that the pharmacology of each enantiomer can give rise to a eutomer and a distomer where the desired properties can be ascribed primarily to the Rectus- or the Sinister-enantiomer (Tucker, 2000). Chirality can also influence pharmacodynamic behavior, a feature that has been demonstrated for R(-)-HCQ and S(+)-HCQ in the mid-90's (Ducharme et al., 1994; Tett et al., 1994). Several methods have been described to obtain the enantiomers of HCQ separately, including direct synthesis using optically resolved reactants (Blaney et al., 1994) and chromatographic separation on a preparative scale (Wilson et al., 2020).

Here we have evaluated the effects of R(-)-HCQ and S(+)-HCQ in vitro electrophysiological models pertaining to cardiovascular safety pharmacology. First, we evaluated the effects of each enantiomer on six cardiac ion channels using automated patch-clamp and cloned human channels expressed in mammalian cells. Secondly, we tested the effects of R(-)-HCQ and S(+)HCQ on transmembrane currents recorded from ex vivo isolated rabbit Purkinje fibers, a native tissue that recapitulates the essential features of human ventricular conductive tissue (Baczko et al., 2016). We found that the enantiomers of HCQ selectively inhibit K$_{v}$2.1 channels and, less potently, hERG channels, and significantly alter several cardinal AP metrics at concentrations about one order of magnitude above the free plasma concentrations circulating at therapeutically effective doses.

2. Material and methods

2.1. Chiral preparative HPLC

Separation of the enantiomers of HCQ was performed using a carbamate-derivatized amylose chiral stationary phase (Chiralpak® AD, Daicel chemical industries). About 1 g hydroxychloroquine sulfate was dissolved in 200 mL of 90:10 acetonitrile/methanol containing 5% triethylamine and loaded in two halves on a 76.4 μm particle size. Elution was performed with the same solvent mix containing 0.1% triethylamine at a flow rate of 400 mL/min. Efficiency was improved by recycling the feed after the desorption of the first enantiomer. This second passage on the same column allowed complete resolution of both enantiomers. The fractions monitored at 265 nm UV were collected and evaporated to obtain free-base oils. Absolute configurations were assigned as S(+) HCQ (optical rotation OR = +76.4 [c] = 320 g/mL in DMSO at 589 nm; enantiomeric excess ee = 98.5%) and R(-)HCQ (OR = -68.1 same conditions; ee = 99%) based on published data (Ibrahim and Fell, 1990).

2.2. Patch-clamp on human cardiac ion channels

Studies were performed as described previously (Le Marois et al., 2020). Briefly, CHO cells expressing hERG or K$_{v}$2.1 minK were obtained from B Sys (Switzerland), and Cav1.2/h2/a2b1, K$_{v}$2.1 or K$_{v}$/4.3/4KbP2.2 from Charles River (USA), respectively. HEK cells expressing Nav1.5 were constructed in house. All expressions were constitutive except K$_{v}$/2.1, Cav1.2 and Nav1.5 which were induced overnight by exposure to 1 μg/mL doxycycline (in the presence of 3 μM verapamil for Cav1.2). Whole-cell recordings were performed at room temperature on a Qpatch® 48X planar patch-clamp workstation (Sophion, Denmark). The pulse-protocols used and cycle-times applied to elicit each current are schematized as insets in their respective figures. Leak currents were handled either on-line by P/ft subtraction or offline by subtracting currents remaining in the presence of a specific reference inhibitor added at the end of each recording session at a maximally active (i.e. “full-block”) concentration. Extracellular buffers for potassium channels contained (in mM) NaCl, 150; KCl, 4; CaCl$_{2}$, 2; MgCl$_{2}$, 1 and HEPES, 10. For the hERG channels, glucose (10) was added. The pH was adjusted to 7.4 with NaOH. The intracellular buffers were composed of (in mM): KF, 120; KCl, 20; EGTA, 10; MgCl$_{2}$, 1; HEPES, 10. For K$_{v}$/7.1 channels, 10 mM EDTA was added as a second chelator, EGTA was decreased to 5 mM and no MgCl$_{2}$ was added. The pH was adjusted to 7.2 with KOH. The extracellular buffer for Cav1.2 channels contained (in mM): NaCl, 145; KCl, 4; BaCl$_{2}$, 10 and HEPES, 10, and pH was adjusted to 7.4 with NaOH. The intracellular buffer contained: CsF, 27; CsCl, 112; EGTA, 8.2; NaCl, 2; HEPES, 10 and Mg-ATP, 4. The pH was adjusted to 7.2 with CsOH. For Nav1.5 channels, the extracellular buffer contained: NaCl, 137; KCl, 4; CaCl$_{2}$, 2; MgCl$_{2}$, 1 and HEPES, 10. The pH was adjusted to 7.4 with NaOH. The intracellular buffer contained: CsF, 150; EGTA/CsOH, 1/5; NaCl, 10; MgCl$_{2}$, 1; CaCl$_{2}$, 1; HEPES, 10. The pH was adjusted to 7.2 with CsOH. All channels were exposed to six concentrations of R(-)-HCQ or S(+)-HCQ applied cumulatively in an ascending order up to 90 μM. Specific inhibitors applied at full-block concentrations to establish assay sensitivity and isolate leak currents were E–4051 (10 μM) for the hERG channels, lidocaine (3 mM) for the Nav1.5 channels, CaCl$_{2}$ (0.2 mM) for Cav1.2, HMR-1556 (30 μM) for K$_{v}$/7.1, SKF-96365 (30 μM) for K$_{v}$/4.3 and BaCl$_{2}$ (3 mM) for the K$_{v}$/2.1 channels. Bepridil was used as a positive control and tested at concentrations up to 30 μM in parallel to the HCQ enantiomers for all channels which were insensitive to them. Treatments were done in buffer containing 0.3% or 1% DMSO, and 1% Pluronic F-68. All inhibitions were quantified as change in normalized peak current amplitude except for K$_{v}$/4.3 currents which were quantified as change in normalized integral charge transferred calculated as the area under the current trace versus time.

2.3. Current clamp on excised rabbit Purkinje fibers

Methods were reviewed and endorsed by Sanofi’s internal animal ethics committee and conducted in accordance with European Directives 86/609/EEC and 2010/63/EU under specific approval by the French Ministry of Research (APAFIS authorization N° 2018080110557740). As previously described (Le Marois et al., 2020), Purkinje fibers were dissected out from excised hearts of male New Zealand rabbits weighing 1.7–2.1 kg and fixed in a small ex vivo tissue bath continuously perfused with a buffer containing (in mM): NaCl 120; KCl 4; MgCl$_{2}$ 1; NaHPO$_{4}$ 1.8; NaHCO$_{3}$ 25; glucose 11; CaCl$_{2}$ 1.8 maintained at 36 ± 1 °C and delivered at a flow-rate of 10 mL/min. Oxygen supply and physiological pH 7.4 were obtained by bubbling 95% CO$_{2}$/5% O$_{2}$ in the buffer reservoirs. The tissue action potential (AP) firing was driven by electrical pulses delivered through stainless-steel electrodes. The membrane resting potential, AP amplitude, depolarization speed (V$_{max}$), and AP duration at 50% and 90% repolarization (APD$_{50}$ and APD$_{90}$, respectively) were recorded using 3 M KCl-filled fine glass micro-electrodes and standard signal amplification and computerized acquisition and monitoring devices. Concentrated (12 mM) stock solutions of S(-)HCQ or R(-)HCQ were prepared in 100% DMSO before being formulated in study buffer to the final concentrations tested. Each fiber (n = 6 per drug) was exposed to four increasing concentrations of R(-)HCQ or S(+)-HCQ according to the protocol depicted in Fig. 7. During the recordings, 1 mL samples of the formulated buffers were collected before (in the reservoirs at room temperature) and after the fibers (at the outlet of the tissue chamber 20 min following onset of each concentration perfusion) to determine their actual drug exposure.

2.4. Data analysis

Patch-clamp current-trace captures and current-size measurements were exported from Sophion’s proprietary Assay Software for Qpatch to a professional graphing package (Prism 8.3.0, GraphPad Software, San Diego, CA, USA) which served also for half-maximal inhibitory concentration (IC$_{50}$) estimations by fitting percent inhibition data (normalized to pre-drug levels) to a sigmoidal curve with minimum and maximum constrained to 0% and 100%, respectively. Calculated IC$_{50}$ values are reported along with their 95% confidence interval (95% CI). Statistical analyses of the Purkinje fiber data were performed by one-
way repeated factor analysis of variance (ANOVA) followed by Dunnett’s test versus baseline for each drug at each pacing rate using SAS 9.4 software (SAS Institute Inc., Cary, NC, USA). A rank-transformation was applied in case of normality hypothesis violation or lack of variance homogeneity. Due to a high number of missing values, the 30 µM data set for R(-)-HCQ was excluded from statistical analysis at all pacing rates and for all metrics except for the resting potential data. Likewise, the 10 µM data set for R(-)-HCQ at the lowest pacing rate was analyzed only for the resting potential due to missing data for the other parameters. Significance levels were set to 5%. Descriptive statistics and graphs were generated with Prism 8.3.0.

3. Results

3.1. Ion channel studies

Cloned human cardiac ion channels expressed in CHO or HEK cells were recorded on a QPatch® 48X automated patch-clamp workstation in population patch mode. Fig. 1A illustrates the effects of R(-)-HCQ and S(+)-HCQ on hERG current traces elicited by a classical two-step long-duration (5 s per step) voltage-protocol allowing ample time for channel blockade at room temperature and triggering large outward tail currents upon its +20 mV to −50 mV repolarizing step. Both R(-)-HCQ and S(+)-HCQ concentration-dependently inhibited these currents. The time-course of the effect of each of the six concentrations applied cumulatively indicated that the active concentrations could reach steady-state inhibition at the end of each exposure period (Fig. 1B). The final application of the selective and potent hERG blocker E-4031 (1 µM) eliminated all currents, thereby establishing the sensitivity of the assay. The half-maximal inhibitory concentrations (IC50) estimated from the sigmoidal curve fitting of the percent block data indicate a 3.7-fold greater potency of R(-)-HCQ over S(+)-HCQ (Table 1).

Fig. 2 summarizes the effects of the enantiomers on human NaV1.5 channels expressed in HEK cells. These were held at −80 mV outside of the depolarizing voltage-steps to populate both the closed and inactivated states of the channels as described (Donovan et al., 2011). The fast inactivating inward currents were generated by short depolarizing steps to −40 mV. Interestingly, neither R(-)-HCQ nor S(+)-HCQ affected NaV1.5 to a meaningful extent (i.e. below about 10% decrease which can be attributed to current rundown) up to the highest tested concentration of 90 µM. In contrast, the local anesthetic and Na+ channel blocker lidocaine at 3 mM systemically abolished all currents in each recording, confirming the sensitivity of the experimental conditions employed. Furthermore, we evaluated in parallel the multi-channel inhibitor bepridil, once used as an antianginal agent, and found an IC50 value of 2.5 µM along with a 95% confidence interval ranging from 2.3 µM to 2.7 µM, which is fully in accordance with published data (Crumb et al., 2016).

We then went on to examine the effects of HCO’s enantiomers on Cav1.2 channels expressed in CHO cells along with their β2 and α2δ1 regulatory proteins. During the patch-clamp recordings, the cells were maintained at a depolarized holding of -50 mV to enrich the population of channels in the inactivated state as described (Kuryshiev et al., 2014). The slowly inactivating inward currents were obtained by stepping the membrane to 0 mV with 200 ms square pulses. To minimize current rundown over time, barium was used as a charge carrier. Fig. 3A depicts the current traces obtained in the presence of 90 µM R(-)-HCQ or 90 µM S(+)HCQ. Neither of the enantiomers was able to block Cav1.2 currents, while CdCl2 (0.2 mM) applied at the end of the recording sessions consistently inhibited all the currents (Fig. 3B). Moreover, the positive control bepridil tested in parallel with the enantiomers was active, exhibiting an IC50 value of 5.2 µM [95% CI: 4.4–6.2 µM] in agreement with published values (Crumb et al., 2016).

We then profiled R(-)-HCQ and S(+)HCQ on K+4.3 channels. CHO cells expressing these channels along with KChIP2.2 accessory subunits were held at −80 mV and stepped to +20 mV using 500 ms long square pulses. The latter generated large, rapidly inactivating outward currents as expected (Fig. 4A). Unlike bepridil, neither 90 µM R(-)-HCQ nor 90 µM S(+)HCQ were able to affect the current peak amplitude nor inactivation. Fig. 4B depicts the change of the total charge transferred (i.e. the integral of the area-under-the-current versus time curve) as a function of applications of each drug concentration and ultimately of 30 µM SKF-96365, a non-selective cationic channel blocker with micromolar potencies at several cardiac potassium channels including human K+4.3 (Liu et al., 2016). Bepridil, again tested in parallel, exhibited a concentration-dependent inhibitory effect on K+4.3 currents, yielding an IC50 value of 11 µM [95% CI: 9.7 µM–12 µM] (Fig. 4B).

To further explore the activities of R(-)-HCQ and S(+)HCQ on repolarizing currents of the cardiac AP, we patched CHO cells expressing K+7.1 subunits along with the ancillary protein minK which is needed to reproduce the native IKs current (Fig. 5A). The cells were stepped to +40 mV from a holding potential of −80 mV using prolonged 2 s square pulses. The latter triggered a slowly activating current that resisted the application of either 90 µM R(-)-HCQ or the same concentration of S(+)HCQ, while the positive control bepridil was active as expected, giving an IC50 value of 7.2 µM [95% CI: 6.6 µM–7.8 µM] (Fig. 5B).

| Channel | R(-)-HCQ | N | S(+)HCQ | N |
|---------|----------|---|---------|---|
| hERG    | 20 µM    | 19-22 | 74 µM    | 65-86 |
| NaV1.5  | >90 µM   | 6   | >90 µM   | 7   |
| Cav1.2/β2/α2δ1 | >90 µM | 6 | >90 µM | 5      |
| K+4.3   | >90 µM   | 7   | >90 µM   | 7   |
| K+7.1/minK | >90 µM | 4 | >90 µM | 5 |
| K+δ2.1  | 3.3 µM   | 2.9-3.7 | 10 | 6.5 µM [5.7-7.6] |

Subunit compositions of the human recombinant channels tested are detailed in the first column. IC50 values are shown along with their 95% confidence intervals.

![Fig. 1.](image1.png)

![Fig. 2.](image2.png)

![Fig. 3.](image3.png)

![Fig. 4.](image4.png)

![Fig. 5.](image5.png)
Finally, we examined the effects of the enantiomers on Kv2.1 expressed in CHO cells (Fig. 6). The cells were maintained at a depolarized holding of −20 mV, and the outward component of the current relevant to AP repolarization was elicited by a step-ramp protocol from +40 mV down to −120 mV at −0.46 V/s followed by a short plateau. As previously noted for hERG, both R(-)-HCQ and S(+)-HCQ inhibited the Kv2.1-mediated repolarizing currents (Fig. 6A), although with approximately one order of magnitude stronger potency (Fig. 6B), albeit with a similarly modest enantiomeric separation (Table 1).

3.2. Purkinje fiber studies

Action potentials (AP) were recorded from rabbit Purkinje fibers with conventional KCl-buffer-filled fine glass microelectrodes manually inserted through the conductive tissue membrane. Fig. 7 schematizes the drug application sequence employed and pacing changes made to assess drug effects. Fig. 8 and Fig. 9 depict the effects of R(-)-HCQ and S(+)-HCQ, respectively, on five parameters analyzed at three different pacing rates, along with their statistical significance. Fig. 10 compares the percent change versus baseline observed for each of these metrics.
Overall, at the lowest tested concentration of 0.3 μM, neither R(-)HCQ nor S(+)-HCQ had a significant effect on any of the parameters whatever the beating rate imposed (compare first and second scatter dot plots in each graph labelled D through R in Figs. 8 and 9). In contrast, both enantiomers started to develop effects that occasionally reached statistical significance at 10-fold higher concentrations.

R(-)HCQ consistently decreased the resting potential of the conductive tissue membranes. This effect was concentration-dependent over the 3 μM–30 μM range, and of similar magnitude whatever the pacing rate driven by the electrical stimulations (Fig. 8, graphs D, E and F and Fig. 10, graphs A, B and C). Specifically, and for example, at the highest tested concentration of 30 μM, the median membrane potential was decreased by 23% from –91 mV to –70 mV at 180 beats/min (bpm), by 20% from –90 mV to –72 mV at 60 bpm and by 21% from –89 mV to –70 mV at 15 bpm. These effects were almost completely reversible after washout with vehicle buffer for most metrics (Fig. 6, compare baseline and washout scatter dot plots in graphs D, E and F). R(-)HCQ also produced a significant decrease in the AP amplitude and the depolarization velocity at 10 μM for the 180 bpm and 60 bpm pacing rates (Fig. 8, graphs G through L). However, the rather large membrane depolarization induced by R(-)HCQ resulted in the fibers escaping electrical pacing at the intermediate 60 bpm rate, and even more so at the very slow 15 bpm rate. Hence, AP amplitude, AP depolarization velocity, and APD<sub>90</sub> measurements could not be collected at 10 μM and 30 μM at
the slowest pacing rates, and at 10 μM at the intermediate pacing rate. A nearly complete data set could only be collected from fibers stimulated at the fastest rate (180 bpm) which overrode the autogenic activity, although several data-points were still missing at 30 μM. At this rapid 180 bpm pacing rate, R(-)HCQ significantly decreased the AP amplitude and its upstroke depolarization velocity at 10 μM. The latter was halved at the highest tested concentration (~51% at 30 μM, with n = 2 only, Fig. 10G). R(-)HCQ also elongated the AP duration by a small, but significant extent at near complete repolarization (Fig. 8, graphs P and Q), an effect culminating at 25% increase during exposure to 30 μM of the drug for the n = 2 readings that could be captured (Fig. 10, graph M). Similar effects could also be inferred at the intermediate (60 bpm) and
slow (15 bpm) pacing rates despite the high amount of lacking data, the effects on AP amplitude and depolarization velocity displaying some degree of use-dependency as suggested by the data available at 3 μM (Fig. 10, graphs D through O) while the AP elongating effects globally persisted upon washout. Of note, notwithstanding the few complete data sets, no torsadogenic early after depolarizations (EADs) events were observed in response to R(-)HCQ at any concentration and pacing rate.

Compared to R(-)HCQ, its companion enantiomer S(+)HCQ had qualitatively similar effects on the AP metrics, although quantitatively it affected the AP duration more (notably at the very slow pacing rate) and the resting membrane potential less (see in particular Fig. 10, graphs B and O). The prolongation of the action potential at near complete...
repolarization was concentration-dependent, reverse use-dependent and only partially reversible upon washout (Fig. 9, graphs P through R, and Fig. 10, graphs M through O). As a quantitative illustration, the median APD$_{90}$ duration at the concentration of 10 μM was increased by 12% from 220 ms to 246 ms at 180 bpm, by 34% from 358 ms to 481 ms at 60 bpm, and by 56% from 531 ms to 827 ms at 15 bpm. Autogenic activity escaping electrical pacing was seen in two out of six fibers exposed to 30 μM at all rates. Of note, at the extreme slow pacing rate of 15 bpm, abnormal AP exhibiting EADs were seen in one of the six fibers exposed to 10 μM, and in one of the four fibers that could follow pacing in the presence of 30 μM S(+)HCQ, suggesting some degree of torsadogenic potential for this enantiomer beyond 10 μM (see AP waveform in Fig. 9C). Regarding the other AP metrics, S(+)HCQ also substantially decreased the speed of the action potential upstroke depolarization (Fig. 9, graphs J, K and L), an effect that was partially reversible and use-dependent (Fig. 10, graphs G, H and I). At the most rapid pacing rate (180 bpm), a depolarization of the resting potential was observed which culminated at 11% decrease from –91 mV to –81 mV in the presence of 30 μM S(+)HCQ (Fig. 9D), a significant and concentration-dependent change, although clearly less pronounced than the effect produced by R(-)HCQ on this same parameter (Fig. 10 A, B and C).

Table 2 compares concentration estimates made by standard LC-MS analytic methods on the R(-)HCQ and S(+)HCQ content in drug-formulated buffer samples collected in the reservoir and at the outlet points.
of the tissue chamber after 20 min perfusion of the Purkinje fibers. The data indicate that none of the tested compounds was lost by retention in the conductive tissue pieces or adherence to components of the experimental setup such as tissue chamber walls or the buffer circulation tubing and pumps.

4. Discussion

The present experiments indicate that, among the cardiac channels comprising the GIPA panel, the $R$-(-) and $S$- (+)-enantiomers of HCQ selectively inhibit the human $K_{v}2.1$- and hERG-potassium channels, leaving all four other major cardiac currents unaffected up to the highest tested concentrations. We also found that this inhibitory profile can translate into significant alterations of rabbit Purkinje fibers action potential (AP) parameters recorded in an ex vivo situation at concentrations above the therapeutically effective free circulating levels of HCQ.

The automated patch-clamp studies show that the $K_{v}2.1$ channels are the most sensitive to inhibition, with IC$_{50}$ values occurring within a low 1 μM–10 μM concentration range for both enantiomers, while the blockade of hERG channels by $R$-HCQ and $S$-HCQ developed within a tenfold higher concentration range. In contrast, neither $R$-HCQ nor $S$-HCQ affected Nav1.5, Cav1.2, $K_{v}4.3$ nor $K_{v}7.1$ channels by a meaningful amount up to 90 μM. This cardiac ion channel inhibitory profile agrees with the profile of chloroquine (CQ), the antiparasitic predecessor of HCQ. Indeed, using a manual patch-clamp approach (Crumb et al., 2016), observed less than 20% inhibition of human re-combinant Na$\textsubscript{v}1.5$, Ca$\textsubscript{v}1.2$, $K_{v}4.3$ and $K_{v}7.1$ channels exposed to 30 μM CQ, while hERG and $K_{v}2.1$ channels where inhibited with IC$_{50}$ values of 7 μM and 11 μM, respectively. Similarly, in thorough mechanistic study aimed at identifying the biophysical and molecular basis of $K_{v}2.1$ blockade by CQ, the IC$_{50}$ value on the outward component was found to be 9 μM (Rodriguez-Menchaca et al., 2008). Hence, hydroxylation of CQ’s side chain, which reduces its toxicity, does not affect its selectivity nor greatly change its potency at those of the CIPA cardiac ion channels that are sensitive to 4-aminoguanidines. More specifically, our data are also fully in line with those recently obtained in a new generation QPatch II platform, with IC$_{50}$ values for racemic HCQ on hERG and $K_{v}2.1$ reaching 17 μM and 30 μM, respectively (Okada et al., 2021).

While it is unwise to extend the differences in IC$_{50}$ values are significant, the reasons why the channel sensitivity is inverted in both studies is unclear.

Mechanistically, it is tempting to ascribe the non-use-dependen depolarizing effects of $R$-HCQ on the Purkinje fibers to its prominent potency at blocking non-voltage-gated $K_{v}2.1$ channels. These inwardly rectifying pores are chiefly involved in setting the diastolic resting membrane potential close to the potassium equilibrium potential between consecutive AP firings. $K_{v}2.1$ channels also contribute to the repolarization phase of the AP in tandem with hERG and $K_{v}7.1$ channels (Hibino et al., 2010), providing a way by which $R$-HCQ prolongs the AP$\textsubscript{D90}$. This prolongation of the AP duration could indeed be observed over the full $R$-HCQ concentration-range at the fastest pacing rate, and up to 10 μM and 3 μM at the intermediate and slow pacing rates, respectively. In the absence of direct inhibition of Na$\textsubscript{v}1.5$ channels, the slowing of the AP depolarization velocity and the decrease of the AP amplitude induced by both $R$-HCQ and $S$-HCQ can also be regarded as consequences of the $K_{v}2.1$ channel blockade, given that the rising membrane depolarization will engage fast inactivation of a substantially growing fraction of Na$\textsubscript{v}1.5$ channels, thereby removing them from the population available in the resting-state ready for activation. This implies that the effects of $S$-HCQ, which is slightly less potent at blocking $K_{v}2.1$ channels, on AP amplitude and depolarization velocity would be somewhat less pronounced. This is however difficult to establish given the incomplete sets of measurements we could acquire across pacing rates due to the autogenic activity induced by $R$-HCQ beyond 3 μM.

Overall, we found only a modest degree of enantiomer separation between $R$-HCQ and $S$-HCQ. Their effects on the cardinal AP metrics were largely similar (see Fig. 10) and in the patch-clamp experiments, their block-potency ratio against $K_{v}2.1$ was only two-fold, and hardly reached four-fold against hERG, although in both cases the levorotatory enantiomer was the most potent. The pharmacodynamics of HCQ’s enantiomers had previously been compared in other in vitro models without showing potency differences. For example, $R$-HCQ and $S$-HCQ were found equipotent at inhibiting human immune-deficiency virus serotype 1 replication in human T-cell lines and macrophage plaguevidoma (Chiang et al., 1996). Likewise, the proliferation of Plasmodium falciparum isolates grown in human erythrocytes was inhibited with identical IC$_{50}$ values by $R$-HCQ and $S$-HCQ (Warhurst et al., 2003). Our present in vitro data also do not suggest that racemic HCQ harbors a clear-cut estomer and distomer combination of drugs from the arrhythmogenic cardiac safety standpoint. Chirality affects somewhat more meaningfully the pharmacokinetics of HCQ. Indeed, besides the fact that its fate in the body is characterized by very large volumes of distributions and long half-life, the circulating levels at therapeutic doses display enantioselectivity. Specifically, in rheumatoid arthritis patients stably treated with racemic HCQ at the recommended dose, the $R$-/$S$ ratio in blood levels was found to be greater than one in all individuals despite a large two-to-three-fold variability between patients (Tett et al., 1994). Moreover, the pharmacokinetics of HCQ were confirmed to be stereoselective in rats (Wei et al., 1995) and rabbits (Ducharme et al., 1994) with no hints for in vivo racemization and a preferential accumulation of the $R$-HCQ enantiomer in ocular tissue (Wainer et al., 1994).

The recent consideration of HCQ as a potential treatment for Covid-19 has generated concerns because of its 4-aminoguanidine structure common to CQ which is known to prolong the QT-segment of the ECG. In a meta-analysis of 28 clinical studies in which patients took HCQ with or without other QT-prolonging drugs, the occurrence of QT-prolongations associated with HCQ monotherapy was below 2%, although patients over 60 appeared significantly more sensitive (Oscaona et al., 2020). Our in vitro cardiodynamic data should be discussed with respect to the circulating drug levels at therapeutic doses. In a population pharmacokinetics model developed from rheumatoid arthritis patients receiving daily doses of 400 mg HCQ, average concentrations in the whole blood reached 2.8 μM (Carmichael et al., 2003). However, recent data comparing whole blood, serum and plasma levels demonstrated that the plasma concentrations of HCQ are two-fold lower than those in blood (Carlsson et al., 2020). Furthermore, in vitro data indicate that the enantiomer of HCQ bind plasma proteins, with the free fraction of $R$-HCQ being about twice as high as that of $S$-HCQ at 63% and 36%, respectively (McLachlan et al., 1993). Given that only the free fraction exercises pharmacodynamic effects such as those described here, a conservative estimate of the therapeutic free plasma concentrations of HCQ enantiomers for comparison with our electrophysiology findings are clearly below 1 μM. Specifically, such a sub-micromolar estimate is comforted by data calculated in a population PK model derived from lupus patients in which mean plasma HCQ ranges between 0.5 μM–0.9 μM for C$_{\text{max}}$ and 0.3 μM–0.4 μM for C$_{\text{rough}}$ (Morita et al., 2016). These conservative estimations may however gain relevance in specific circumstances where the cardiac AP repolarization reserve is altered, such

Table 2

| Drug   | Target (μM) | Actual (μM) | Loss |
|--------|-------------|-------------|------|
| $R$-HCQ | 0.3         | 0.29        | -3%  |
|         | 3           | 2.9         | -3%  |
|         | 10          | 9.8         | -2%  |
|         | 30          | 29.6        | -1%  |
| $S$-HCQ | 0.3         | 0.31        | +3%  |
|         | 3           | 3.1         | +3%  |
|         | 10          | 10.1        | +1%  |
|         | 30          | 30.1        | <1%  |
as for example in carriers of congenital long QT syndromes (the prevalence of which could be estimated to be as high as 1:2500 according to recent studies (Schwartz et al., 2009)), or when medications known to prolong the QT interval are administered concomitantly (Roden, 2004).

The present study has limitations, mostly inherent to the in vitro and ex vivo nature of our experimental approaches. First, the small sample size of the Purkinje fibers studies, that was driven by animal ethics considerations and the fastidiousness of manual Purkinje fiber recordings, may reduce the general relevance of our experimental observations. This applies notably to the effects of R(-)HCQ since several parameters could not be properly assessed over all pacing rates and concentrations because of the autogenic activity induced by this enantiomer. It would be relevant to compare the effects of both enantiomers in a more integrated model such as, for example, the rabbit whole-heart preparation perfused according to the Langendorff method, which might offer a better translation to the clinical situation given its higher degree of physiologic integrity (Ellermann et al., 2021). Second, there are uncertainties over the drug effective concentrations when attempting to translate to the clinical situation. Indeed, the defined buffers used in the in vitro patch-clamp and the ex vivo voltage-clamp experiments do not exhaustively mimic the plasma milieu, even if the enantiomers of HCQ bind plasmatic proteins relatively loosely (McLachlan et al., 1993) and are readily soluble, and not particularly suspected of stickiness due to lipophilicity, which can be a source of compound loss in automated patch-clamp experiments (Kramer et al., 2020). Third, the Purkinje fiber studies were performed in standard conditions. However, in real life, HCQ may need to be given to patients with associated pro-arrhythmic risk factors, such as for example hypokalemia, which is well known to promote cardiac arrhythmias (Weiss et al., 2017). It has been shown useful to evaluate the effect of R(-)HCQ and S(+)HCQ at concentrations that can reasonably be estimated for HCQ enantiomers at therapeutic doses, the present in vitro data agree with the low incidence of arrhythmogenic events such as TdP reported over the long-standing clinical practice of HCQ monotherapies in its approved indications. However, caution should be exercised when this drug is given in conjunction with other QT-prolonging drugs or in patients with additional risk factors such as electrolyte imbalance or congenital long QT syndromes.

Declaration of competing interest

All authors are current or former Sanofi employees and may hold shares and/or stock options in the company.

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