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Research Article

Cardiotoxic Potential of Hydroxychloroquine, Chloroquine and Azithromycin in Adult Human Primary Cardiomyocytes

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ABSTRACT

Substantial efforts have been recently committed to develop coronavirus disease-2019 (COVID-19) medications, and Hydroxychloroquine alone or in combination with Azithromycin has been promoted as a repurposed treatment. Although these drugs may increase cardiac toxicity risk, cardiomyocyte mechanisms underlying this risk remain poorly understood in humans. Therefore, we evaluated the proarrhythmia risk and inotropic effects of these drugs in the cardiomyocyte contractility-based model of the human heart. We found Hydroxychloroquine to have a low proarrhythmia risk, whereas Chloroquine and Azithromycin were associated with high risk. Hydroxychloroquine proarrhythmia risk changed to high with low level of K^+, whereas high level of Mg^{2+} protected against proarrhythmic effect of high Hydroxychloroquine concentrations. Moreover, therapeutic concentration of Hydroxychloroquine caused no enhancement of elevated temperature-induced proarrhythmia. Polytherapy of Hydroxychloroquine plus Azithromycin and sequential application of these drugs were also found to influence proarrhythmia risk categorization. Hydroxychloroquine proarrhythmia risk changed to high when combined with Azithromycin at therapeutic concentration. However, Hydroxychloroquine at therapeutic concentration impacted the cardiac safety profile of Azithromycin and its proarrhythmia risk only at concentrations above therapeutic level. We also report that Hydroxychloroquine and Chloroquine, but not Azithromycin, decreased contractility while exhibiting multi-ion channel block features, and Hydroxychloroquine's contractility effect was abolished by Azithromycin. Thus, this study has the potential to inform clinical studies evaluating repurposed therapies, including those in the COVID-19 context. Additionally, it demonstrates the translational value of the human cardiomyocyte contractility-based model as a key early discovery path to inform decisions on novel therapies for COVID-19, malaria, and inflammatory diseases.

Key words: hydroxychloroquine; adult human primary cardiomyocyte; proarrhythmia risk; contractility; translation; COVID-19.

An immunosuppressive drug and anti-parasitic, Hydroxychloroquine (Plaquenil), an analogue of Chloroquine, has long been used for the treatment of malaria, lupus and rheumatoid arthritis (Ben-Zvi *et al.*, 2012; Fava and Petri, 2019; Tripathy *et al.*, 2020). QT prolongation and Torsades de Pointes arrhythmia are uncommon adverse events with Hydroxychloroquine when used to treat malaria (Haeusler *et al.*, 2018) and inflammatory diseases (Ben-Zvi *et al.*, 2012), however

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there are published case reports of cardiotoxicity (Morgan *et al.*, 2013; O'Laughlin *et al.*, 2016; see also postings on the FDA Adverse Events Reporting System). Furthermore, the rapid advancement of the coronavirus disease-2019 (COVID-19) pandemic has created an urgent need for effective treatment and rapid repurposing of drugs previously assessed for acute respiratory syndrome coronavirus 2 (SARS-CoV-2) could accelerate the discovery of treatment options (Gordon *et al.*, 2020; Gysi *et al.*, 2020). Potential antiviral activity of Hydroxychloroquine alone or in combination with Azithromycin, an antibiotic with immunomodulating and antiviral properties, in SARS-CoV-2 has recently been predicted (Arshad *et al.*, 2020; Gautret *et al.*, 2020; Giudicessi *et al.*, 2020; Lagier *et al.*, 2020; Lauriola *et al.*, 2020; Risch, 2020; Yao *et al.*, 2020; Yu *et al.*, 2020). Consequently, Hydroxychloroquine has been promoted as a potential treatment of COVID-19. However, 4–61% of COVID-19 patients treated with Hydroxychloroquine and Chloroquine developed cardiotoxicity, primarily QT prolongation and Torsades de Pointes (Afsin *et al.*, 2020; Bernardini *et al.*, 2020; Borba *et al.*, 2020; Chorin *et al.*, 2020; Hooks *et al.*, 2020; Jankelson *et al.*, 2020; Lalabekyan *et al.*, 2020; Mercuro *et al.*, 2020; Saleh *et al.*, 2020; Szekely *et al.*, 2020).

The therapeutic environment of severe SARS-CoV-2 infection is unique: significant number of COVID-19 patients have high heart rates, elevated body temperature, low level of saturated oxygen over many days, and low level of serum K^+ featured prominently (Chen *et al.*, 2020). Furthermore, patients in intensive care units receive polytherapy, potentially including several QT-prolonging drugs (Crotti and Arbelo, 2020). To our knowledge, no comprehensive study has investigated the cellular cardiotoxic potential of Hydroxychloroquine, Chloroquine, and Azithromycin in cardiomyocytes of the adult human heart. The existing data on their cardiac risk potential are limited and come from very few nonhuman studies. The effects of Hydroxychloroquine and Chloroquine on hERG and hyperpolarizing-activated funny pacemaker If channels were investigated in heterologous systems (Capel *et al.*, 2015; Traebert *et al.*, 2004). Nonhuman cardiomyocytes from feline and guinea pig hearts were also used to examine the effects of Azithromycin on cardiac currents (Sánchez-Chapula *et al.*, 2001; Zhang *et al.*, 2017). Although drug development still depends on nonhuman systems for advancement of novel therapies, some of these systems have been associated with poor translation and predictivity (Gintant *et al.*, 2017; Holmes *et al.*, 2015; Sager *et al.*, 2014). It would therefore be valuable to get cellular insights into the human cardiac safety profiles of Hydroxychloroquine, Chloroquine, and Azithromycin, so potential clinical studies with these 3 drugs or novel innovative antiviral, antimalarial and antilupus medications are well supported.

Cardiotoxicity remains one of the leading causes of drug development discontinuation and withdrawal of approved drugs (Piccini *et al.*, 2009). Challenges in translating preclinical cardiac safety data to humans has accelerated the search for developing novel human-relevant platforms to bridge translational gaps (Abi-Gerges *et al.*, 2020a; Pang *et al.*, 2019). Predictive adult human cardiac tissue- and cardiomyocyte-based models from healthy organ donors are now available for preclinical discovery studies (Abi-Gerges *et al.*, 2020b; Britton *et al.*, 2017; Nguyen *et al.*, 2017; Otsomaa *et al.*, 2020; Page *et al.*, 2016; Qu *et al.*, 2018; Trovato *et al.*, 2020). The cardiomyocyte contractility-based model has been shown to simultaneously predict drug-induced inotropic and proarrhythmia risk (Nguyen *et al.*, 2017), and provide multiparametric mechanistic profiling of drugs (Abi-Gerges *et al.*, 2020b). We used this model to clarify the cellular proarrhythmia risk and potential inotropic activity of Hydroxychloroquine, Chloroquine, and Azithromycin. In particular, we set out in this investigation to: (1) determine the concentration dependence of effects; (2) establish human safety margins; (3) assess the influence of risk factors (low level of K^+ and elevated temperature) and the protective role of Mg^{2+}; (4) evaluate polytherapy (eg, combination of Hydroxychloroquine and Azithromycin); and (5) define the cardiac ion channel profiles of the 3 drugs.

MATERIALS AND METHODS

Donor Heart Procurement

All methods were carried out in accordance with relevant guidelines and regulations. All human hearts used for this study were nontransplantable and ethically obtained by legal consent (first person or next-of-kin) from cadaveric organ donors in the United States. Our recovery protocols and in vitro experimentation were preapproved by IRBs (Institutional Review Boards) at transplant centers within the U.S. OPTN (Organ Procurement Transplant Network). Furthermore, all transfers of the donor hearts are fully traceable and periodically reviewed by U.S. Federal authorities. Donor characteristics, heart number and donor identifier are shown in Supplementary Table 1 and exclusion criteria were previously described (Page *et al.*, 2016).

Cardiomyocyte Contractility Measurement

Upon arrival at our laboratory, hearts were reperfused with ice cold proprietary cardioplegic solution (AnaBios, California) and adult human primary ventricular myocytes were isolated enzymatically from the ventricles (Abi-Gerges *et al.*, 2020b; Nguyen *et al.*, 2017; Page *et al.*, 2016). Contractility transients were measured as previously described (Abi-Gerges *et al.*, 2020b; Harmer *et al.*, 2012; Nguyen *et al.*, 2017). Briefly, cardiomyocytes were placed in a perfusion chamber (FHC Inc., Bowdoin, Maine) mounted on the stage of an inverted Motic AE31E microscope (StellarScientific, Maryland) and continuously perfused from a gravity fed system at 2 ml/min with myocyte Tyrode solution (see composition below) heated to approximately 36°C using an inline heater (Cell MicroControls, Norfolk, Virginia). Two video-based cell geometry systems were used to measure sarcomere dynamics: IonOptix (www.ionoptix.com; Ren and Wold, 2001) and MyoBLAZER (www.anabios.com). To initiate a stimulated contraction, the myocytes were field stimulated at voltage 50% above threshold at a 1 Hz pacing frequency, with a biphasic pulse of 3 ms duration, using a pair of platinum wires placed on opposite sides of the chamber and connected to a MyoPacer EP stimulator (IonOptix). Images were acquired at rates of 148 Hz, using an Optronis CP70-16-M/C-148 camera, and 240 Hz, using an IonOptix MyoCam-S CCD camera. Digitized images were displayed within the IonWizard (v7.2.7.138; IonOptix) and MyoBLAZER (v2.4.4) acquisition software. Optical intensity data were collected from a user-defined rectangular region of interest placed over the myocyte image. The optical intensity data represent the bright and dark bands corresponding to the Z-bands of the cardiomyocyte. The IonWizard (v1.2.22) and MyoBLAZER software analyze the periodicity in the optical density along the myocyte detecting the Z-bands by means of a fast Fourier transform algorithm.

The stability of sarcomere shortening transients was assessed by continuous recording for 120 s in Tyrode's solution establishing the vehicle control (in 0.1% dimethyl sulfoxide,

DMSO). Subsequently, the test article concentration was applied for a minimum of 150-s period. Four to five ascending concentrations of the test article were used, providing cumulative concentration-effect (C-E) curves. Analysis was performed using the IonWizard software/Transient Analysis Tool and MyoBLAZER software. A series of polynomials were fitted to the 5 different phases of the monotonic transient (Nguyen *et al.*, 2017). For each test condition, contractility transients were analyzed for proarrhythmia (aftercontraction, premature contraction, contraction failure, pause-dependent arrhythmia) and inotropic effects (sarcomere shortening). An aftercontraction was visually identified as a change in slope of the contractility transient preceding the next stimulus-induced contraction (Figure 2 and Supplementary Figs. 1 and 3–7). Premature contraction was defined as a nonstimulated spontaneous contraction, and arrhythmia was defined as series of arrhythmic contractions (Figure 2 and Supplementary Figs. 4 and 5). Contraction failure was observed when the electrical stimulus failed to trigger a contraction transient (Supplementary Figs. 1 and 3). Pause-dependent arrhythmia was identified as an abnormally enhanced contraction following a pause-related contraction failure (Supplementary Figure 3). No incidence of proarrhythmia markers occurred at each pretreatment in all cardiomyocytes used in this investigation (representative examples are shown in Figures 2A, 2C, and 2E and Supplementary Figs. 1 and 3–7). Proarrhythmia events were recorded by examining individual transients during the 300-s application test article condition. Events were expressed as percentage incidence: number of transients with events/total number of transients and expressed as mean ± SEM. Sarcomere shortening was calculated from the average of the last 15 contractions and used to quantify test article-induced inotropic effects (Nguyen *et al.*, 2017) and treatment effects were expressed relative to the myocyte's specific baseline control period. Hill curves were fitted to sarcomere shortening C-E data using SigmaPlot v14.0, (Systat Software Inc., California, www.systatsoftware.com; Nguyen *et al.*, 2017) and used to determine IC_{50} (concentration inducing 50% decrease in sarcomere shortening).

The standard myocyte Tyrode solution contained (in mM): NaCl 145, KCl 4, $CaCl_2$ 1.8, $MgCl_2$ 1, glucose 11.1, and HEPES 10, pH 7.4 with NaOH. Reference drugs were obtained from Cayman Chemical Company (Chloroquine, an aminoquinoline) and Novartis International AG (Hydroxychloroquine, a Chloroquine derivative in which one of the N-ethyl group is hydroxylated at position 2, and Azithromycin), respectively. Drugs were initially formulated in DMSO as a 1000× stock solution. Stock solutions were diluted to the working concentrations in 0.1% DMSO on the day of the experiment.

Ion channel measurement

The automated whole-cell patch-clamp technique, using the QPatch HT system (www.sophion.com), was used to record hERG, Nav1.5, and Cav.12 currents (see Koci *et al.*, 2019).

hERG

CHO-K1 (Chinese Hamster Ovary) cells stably transfected with human hERG cDNA were used. The cells were harvested with Accutase and maintained in serum free medium (CHO- S-SFM II) at room temperature for 75 min before recording. Then, the cells were pipetted into each well of a 48-well plate in external solution (in mM): 137 NaCl, 4 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 10 HEPES, 10 Glucose, and pH 7.35 with NaOH. The internal solution contained (in mM): 70 KF, 60 KCl, 15 NaCl, 5 HEPES, 5 EGTA, 5 MgATP, and pH 7.3 with KOH. After whole-cell configuration was achieved, the cells were held at a resting membrane potential of −80 mV. A 500 ms pulse to −40 mV was then delivered to measure the leaking current, which was subtracted from the hERG tail current on-line. Next, the cells were depolarized to +40 mV for 500 ms and then to −80 mV over a 100 ms ramp to elicit the hERG tail current. This paradigm was delivered once every 8 s to monitor the current amplitude. The assay was conducted at room temperature. The external solution (control) was applied first and the cells were stabilized in this solution for 5 min. Each test compound was applied from low to high concentrations sequentially on the same cell. The cells were incubated with each test concentration for 5 min. The percent inhibition of the hERG channel was calculated by comparing the tail current amplitude before and after application of the test compound (ie, the current difference was normalized to vehicle control values). Hill curves were fitted to hERG C-E data using SigmaPlot v14.0 and used to determine IC_{50} (concentration inducing 50% decrease in the hERG current).

Nav1.5

HEK-293 cells stably transfected with human Nav1.5 cDNA were used. The cells were harvested with Accutase and maintained in serum free medium (CHO- S-SFM II) at room temperature before recording. Then, the cells were pipetted into each well of a 48-well plate in external solution (in mM): 137 NaCl, 4 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 10 HEPES, 10 Glucose, and pH 7.3 with NaOH. The internal solution contained (in mM): 100 CsF, 45 CsCl, 5 NaCl, 10 HEPES, 5 EGTA, and pH 7.3 with CsOH. Onset and steady-state block of peak Nav1.5 current was measured using a pulse pattern, repeated every 5 s, consisting of a hyperpolarizing pulse to −120 mV for a 200 ms duration, depolarization pulse to −15 mV for a 40 ms duration, followed by a step to +40 mV for 200 ms and finally a 100 ms ramp (1.2 V/s) to a holding potential of −80 mV. Peak current was measured during the step to −15 mV. Each concentration of test compound was applied for 5 min. The assay was performed at room temperature. Only current amplitudes more than 200 pA at the control stage were analyzed. The amplitude of the Nav1.5 current was calculated by measuring the difference between the peak inward current on stepping to −15 mV (ie, peak of the current) and the leak current. The Nav1.5 current was assessed in vehicle control conditions and then at the end of each 5-min compound application. Individual cell result was normalized to the vehicle control amplitude and mean ± SEM were calculated for each compound concentration. Hill curves were fitted to Nav1.5 C-E data using SigmaPlot v14.0 and used to determine IC_{50} (concentration inducing 50% decrease in the Nav1.5 current).

Cav1.2

Recombinant HEK-293 cells expressing the human Cav1.2 channel, the L-type voltage gated calcium channel, hCav1.2 α1C/β2a/α2δ1, were used. The cells were harvested with Accutase and maintained in serum free medium (CHO- S-SFM II) at room temperature between 15 and 60 min before recording. Then, the cells were pipetted into each well of a 48-well plate in external solution (in mM): 137 NaCl, 4 KCl, 1 $MgCl_2$, 5.0 $CaCl_2$, 10 HEPES, 10 Glucose, and pH 7.35 with NaOH. The internal solution contained (in mM): 67.5 CsF, 0.1 $CaCl_2$, 5 NaCl, 4 CsMSF, 2.25 $MgCl_2$, 2.5 Creatine, 2.5 Phosphocreatine, 2.5 Sodium Pyruvate, 2.5 Oxaloacetate, 17 HEPES, and 7.5 EGTA,

5 MgATP, pH 7.3 with CsOH

After whole-cell configuration was achieved, the human Cav1.2 currents were elicited from a holding potential of −90 mV. Currents were evoked following a 100 ms pulse to −60 mV followed by a 50 ms pulse to +10 mV before returning to the holding potential. This paradigm was delivered 3 times once every 20 s to monitor the current amplitude for each compound concentration tested. Cells were held at −90 mV with a 5 s pulse to −60 mV every 20 s for a total of 120 s between each set of 3 pulses. The assay was conducted at room temperature. The extracellular Solution (control) was applied first and the cells were stabilized in the solution for 3 min. Each test compound was applied from low to high concentrations sequentially on the same cell. The cells were incubated with each test concentration for 3 min. The maximum inward current elicited on stepping to +10 mV for 50 ms from −60 mV was measured. The percent inhibition of the Cav1.2 channel was calculated by comparing the Cav1.2 current amplitude before and after application of the test compound (ie, the current difference was normalized to vehicle control values). Hill curves were fitted to Cav1.2 C-E data using SigmaPlot v14.0 and used to determine IC_{50} (concentration inducing 50% decrease in the Cav1.2 current).

Stock solutions were prepared in DMSO at 300× the final assay concentrations and stored at −20°C until the day of assay. On the day of the assay, an aliquot of the stock solution was thawed and diluted into external solution to make final test concentrations. A final concentration of 0.33% DMSO is maintained for each concentration of the assay compounds and controls. E-4031, Tetracaine, and Nifedipine, which were tested at multiple concentrations, were used as positive controls for hERG, Nav1.5, and Cav1.2 channels, respectively.

RESULTS

Proarrhythmic Risk Assessment of Hydroxychloroquine and Chloroquine in Adult Human Primary Cardiomyocytes

With its ability to track parameters relevant to proarrhythmia risk, like the occurrence of aftercontraction, the adult human primary cardiomyocyte contractility-based model has been shown to differentiate torsadogenic from nontorsadogenic drugs (Abi-Gerges *et al.*, 2020b; Nguyen *et al.*, 2017). First, we used the model to address the cardiac safety of Hydroxychloroquine over a concentration range that covers multiples of the human therapeutic free plasma concentration (De Olano *et al.*, 2019; PharmaPendium, Elsevier) to provide sufficient scope for the cardiac safety margin of Hydroxychloroquine. The data show that Hydroxychloroquine exhibited a small increase in the incidence of proarrhythmia markers at the upper limit of the therapeutic free plasma concentration (0.3 μM; Figure 1A and Supplementary Figs. 1 and 2). However, higher incidence and additional

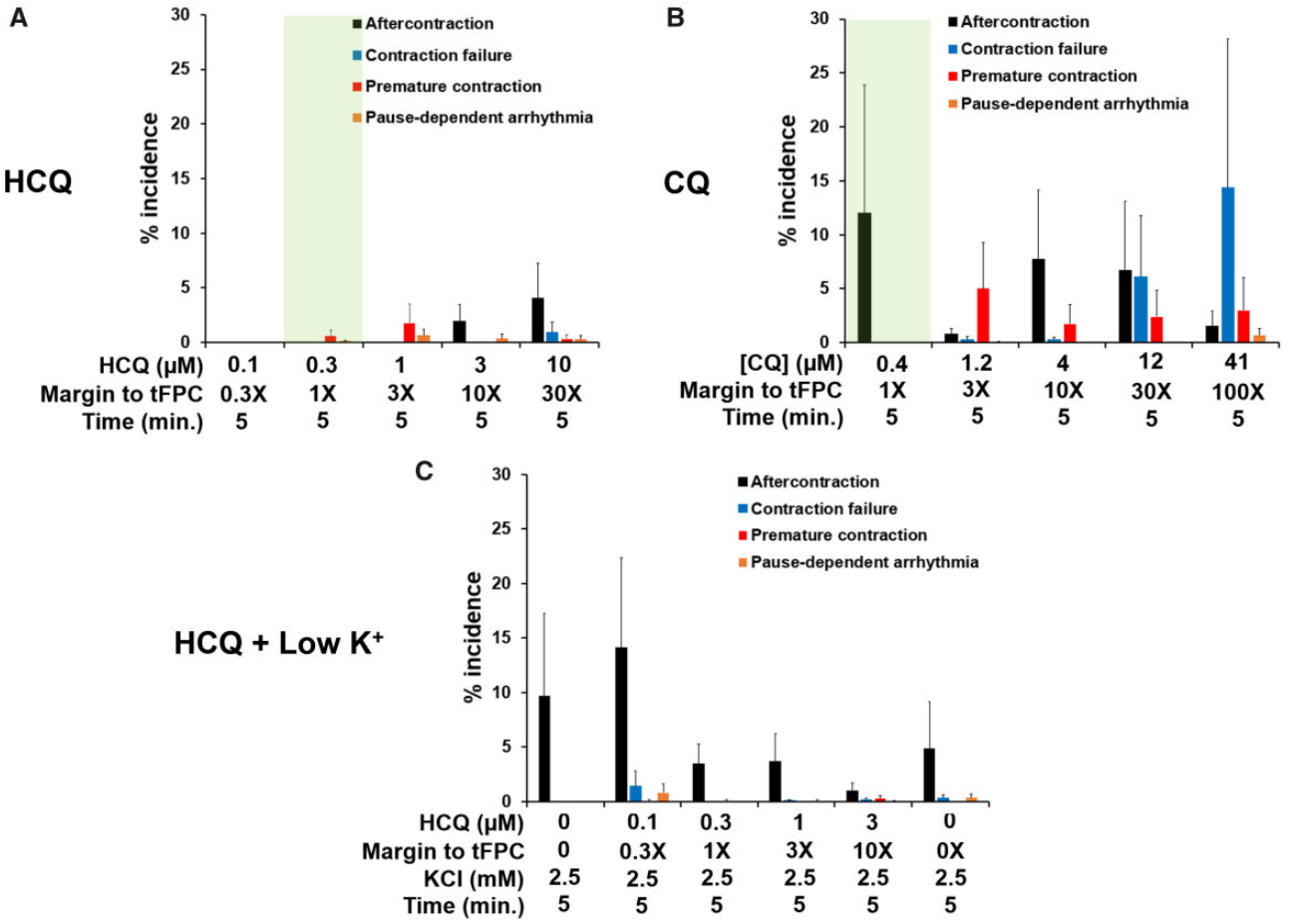

Figure 1. Human proarrhythmic potential of Hydroxychloroquine (HCQ) and Chloroquine (CQ). Mean % incidence in aftercontraction, contraction failure, premature contraction and pause-dependent arrhythmia when adult human primary cardiomyocytes were treated with HCQ ($n = 9$ cells, donor heart 200417HHA, A), CQ ($n = 8$ cells, donor heart 200321HHA, B) and HCQ in combination with low K^+ (2.5 mM KCl, $n = 12$ cells, donor heart 200425HHA, C) at a pacing frequency of 1 Hz. The light green zone represents therapeutic free plasma concentration. Abbreviation: min., minute.

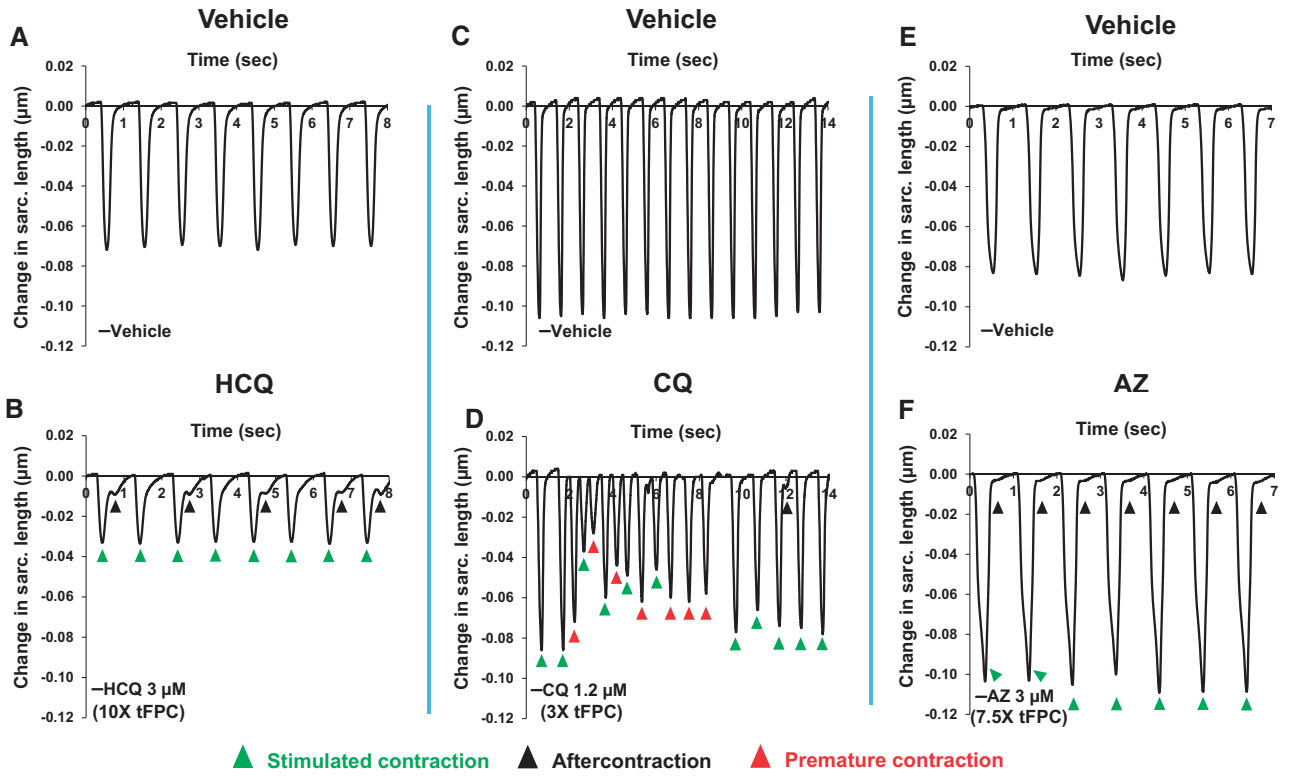

Figure 2. Typical human cardiomyocyte contractility transients after exposure to Hydroxychloroquine (HCQ), Chloroquine (CQ), and Azithromycin (AZ). A–F, transients recorded from 3 independent adult human cardiomyocytes in the presence of vehicle control and after exposure to HCQ (3 µM = 10× the tFPC), CQ (1.2 µM = 3× the tFPC), and AZ (10 µM = 7.5× the tFPC), respectively, at a pacing frequency of 1 Hz. Abbreviations: Sarc. length, Sarcomere length; tFPC, therapeutic free plasma concentration.

proarrhythmia markers were evident at multiples of the therapeutic free plasma concentration (Figs. 1A and 2A and 2B and Supplementary Figs. 1–3). Since Hydroxychloroquine is a derivative of Chloroquine, we next evaluated the cardiac safety of Chloroquine. When compared with Hydroxychloroquine, our data indicate that Chloroquine not only caused higher incidence of proarrhythmia markers at multiples of the therapeutic free plasma concentration (0.4 µM; Crumb *et al.*, 2016; PharmaPendium, Elsevier), but its increase of the incidence of proarrhythmia markers was already evident at the therapeutic free plasma concentration (Figs. 2B–D and Supplementary Figure 4). Taken together, our data suggest that Hydroxychloroquine exhibits a low proarrhythmia risk at the therapeutic exposure.

Impact of Risk Factors and Polytherapy on the Cardiac Safety of Hydroxychloroquine

Since it is well established that risk factors, including hypokalemia (plasma $K^+ < 3$ mM), can influence the occurrence of drug-induced proarrhythmia (Chen *et al.*, 2020; Osadchii, 2010, Osadchii and Olesen, 2009), and plasma electrolytes are known to affect cardiac function, we investigated the effects of low K^+ on Hydroxychloroquine-induced incidence of proarrhythmia markers. We first addressed the effects of 2.5 mM KCl on the incidence of proarrhythmia markers in the absence of Hydroxychloroquine. A 15-min application of 2.5 mM KCl exhibited an increase in proarrhythmia incidence (Supplementary Figs. 5A and 5B). Data summary for the effects of Hydroxychloroquine on markers of proarrhythmia in the continuous presence of 2.5 mM KCl is shown in Figure 1C and Supplementary Figure 2. The presence of 2.5 mM KCl impacted the cardiac safety profile of Hydroxychloroquine as Hydroxychloroquine-induced increase in proarrhythmia markers was observed at all concentrations tested, notably at clinically relevant concentrations (0.1 and 0.3 µM that correspond to 0.3- and 1-fold of the therapeutic free plasma concentration; Figure 1C and Supplementary Figure 2). As Mg^{2+} bolus is considered a preferred treatment option for patients with ventricular arrhythmia (Panchal *et al.*, 2018), we next investigated the effects of 3 mM $MgCl_2$ on Hydroxychloroquine-induced incidence of proarrhythmia markers. We first addressed the effects of 3 mM $MgCl_2$ on the incidence of proarrhythmia markers in the absence of Hydroxychloroquine. A 15-min application of 3 mM $MgCl_2$ caused no occurrence of proarrhythmia markers (Supplementary Figs. 5D and 5E). Data summary for the effects of Hydroxychloroquine on markers of proarrhythmia in the continuous presence of 3 mM $MgCl_2$ is shown in Figure 3A and Supplementary Figure 2. These data suggest that $MgCl_2$ attenuated the proarrhythmic effects of Hydroxychloroquine. This finding suggests that a Mg^{2+} bolus can augment the cardiac safety margin of Hydroxychloroquine and can be considered as a potential effective prophylactic way to treat proarrhythmia associated with high exposures to Hydroxychloroquine. Moreover, elevated body temperature is another factor that can affect the proarrhythmic potential of drugs. Our results show that a 2°C temperature elevation caused a significant increase in the incidence of aftercontraction, a proarrhythmia marker (Supplementary Figs. 7A–E). Data summary for the effects of Hydroxychloroquine on markers of proarrhythmia in the continuous presence of elevated temperature is shown in Figure 3B. Under the 2°C temperature increase condition; however, Hydroxychloroquine exposure at the therapeutic free plasma concentration did not further increase the incidence of

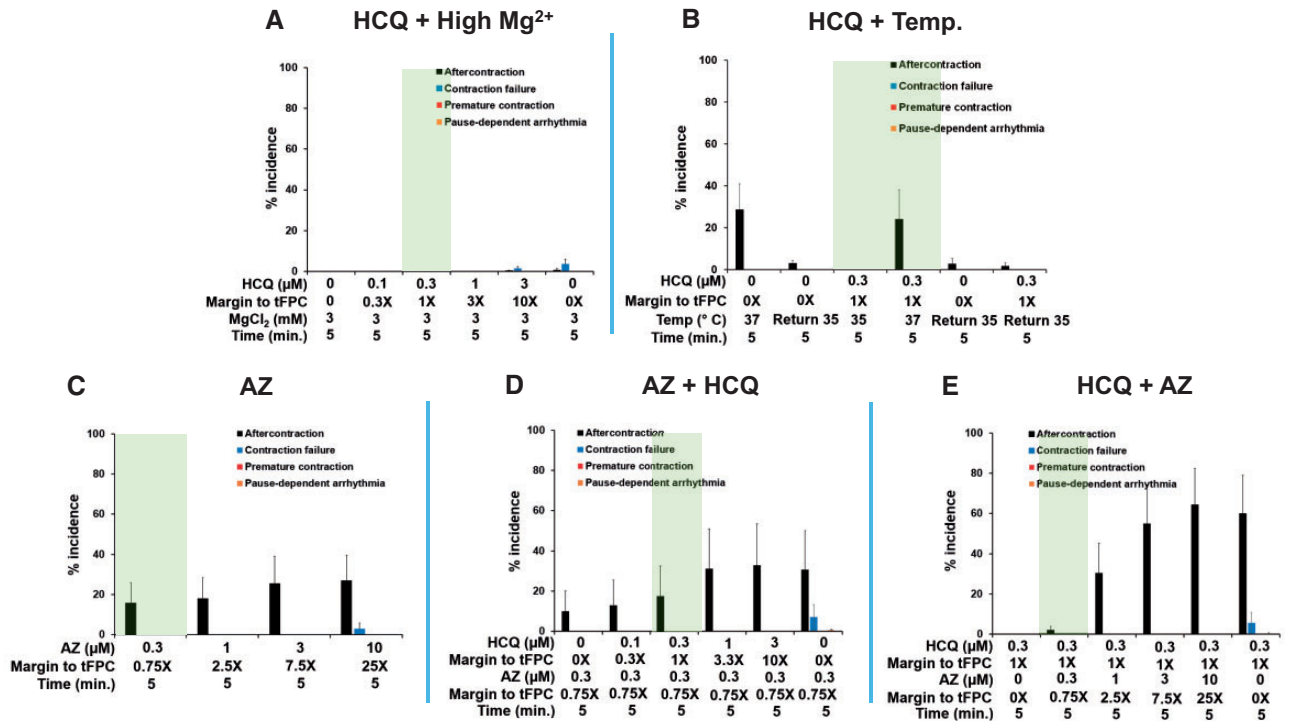

Figure 3. Effects of high Mg^{2+}, temperature and Azithromycin (AZ) on Hydroxychloroquine (HCQ) proarrhythmic potential. Mean % incidence in aftercontraction, contraction failure, premature contraction and pause-dependent arrhythmia when adult human primary cardiomyocytes were treated with HCQ in combination with high Mg^{2+} ($MgCl_2$ 3 mM, $n = 9$ cells, donor heart 200425HHA, A), and subjected to an increase in temperature after exposure to 0.3 μM HCQ ($n = 6$ cells, donor heart 200601HHB, B), AZ ($n = 9$ cells, donor heart 200518HHA, C), HCQ in combination with 0.3 μM AZ (0.75X the tFPC, $n = 6$ cells, donor heart 200518HHA, D) and AZ in combination with 0.3 μM HCQ (1× the tFPC, $n = 7$ cells, donor heart 200518HHA, E), at a pacing frequency of 1 Hz. The light green zone represents tFPC. Abbreviations: min., minute; Temp., temperature; tFPC, therapeutic free plasma concentration.

temperature-induced proarrhythmia (Figure 3B). Additionally, since polytherapy with QT-prolonging drugs can increase the risk of a drug to cause proarrhythmia (Sala *et al.*, 2005; Schrickel *et al.*, 2006) and Hydroxychloroquine has been combined with Azithromycin in COVID-19 patients (Chorin *et al.*, 2020; Lauriola *et al.*, 2020; Pani *et al.*, 2020), we investigated the effects of Azithromycin on Hydroxychloroquine-induced incidence of proarrhythmia markers. We first addressed the effects of Azithromycin, a macrolide antibiotic, on the incidence of proarrhythmia markers in the absence of Hydroxychloroquine. The data demonstrate that Azithromycin increased the incidence of proarrhythmia markers at all concentrations tested, including the therapeutic free plasma concentration (0.4 μM; Figs. 2E and 2F and 3C and Supplementary Figs. 2 and 7). Taken together, our data suggest that Azithromycin has a high proarrhythmia risk and this finding agrees with recent reports about the ability of Azithromycin to cause QT interval prolongation and proarrhythmia (Yang *et al.*, 2017; Zhang *et al.*, 2017). Data summary for the effects of Hydroxychloroquine on markers of proarrhythmia in the continuous presence of the therapeutic free plasma concentration (0.3 μM) of Azithromycin is shown in Figure 3D and Supplementary Figure 2. Data show that under this condition Hydroxychloroquine has a high proarrhythmia risk at all concentrations tested, including at clinically relevant concentrations. We also investigated the effects of Azithromycin on markers of proarrhythmia in the continuous presence of the therapeutic free plasma concentration (0.3 μM) of Hydroxychloroquine and found Azithromycin-induced increase in the incidence of proarrhythmia markers was only observed starting at 2.5-fold the therapeutic free plasma concentration (Figure 3E and Supplementary Figure 2). Hence, our findings with Hydroxychloroquine and Azithromycin suggest that polytherapy and the sequence of exposure may influence the proarrhythmia risk.

Inotropic Risk Assessment of Hydroxychloroquine, Chloroquine, and Azithromycin in Adult Human Primary Cardiomyocytes

Since the adult human primary cardiomyocyte model allows a simultaneous assessment of risks associated with proarrhythmia and inotropic activity, we assessed the effects of Hydroxychloroquine, Chloroquine, Azithromycin, and risk factors on cardiomyocyte contractility (sarcomere shortening). Data indicate that Hydroxychloroquine inhibited sarcomere shortening in a concentration-dependent manner (Figure 4A and Supplementary Figure 1). This concentration dependence of the negative inotropic effect of Hydroxychloroquine is also evaluated in the context of the therapeutic free plasma concentration (Figure 4B and Supplementary Figure 1). The same was true for Chloroquine (Figs. 4A and 4B and Supplementary Figure 4). Furthermore, we next evaluated the concentration-dependence of the negative inotropy of Hydroxychloroquine in the continuous presence of 2.5 and 3 mM of KCl and $MgCl_2$, respectively. Data show that while a 15-min application of 2.5 mM KCl had no effect on sarcomere shortening (Supplementary Figure 5C), a 15-min application of 3 mM $MgCl_2$ decreased sarcomere shortening (Supplementary Figure 5F). Additionally, we found that neither KCl nor $MgCl_2$ application affected the negative inotropic activity of Hydroxychloroquine and its IC50 values were found to be comparable (Figs. 4A and 4B). We also found that elevated temperature increased sarcomere shortening (Supplementary Figure 6F); however, no synergistic or antagonistic effect between elevated temperature and Hydroxychloroquine at the therapeutic free plasma concentration was observed on

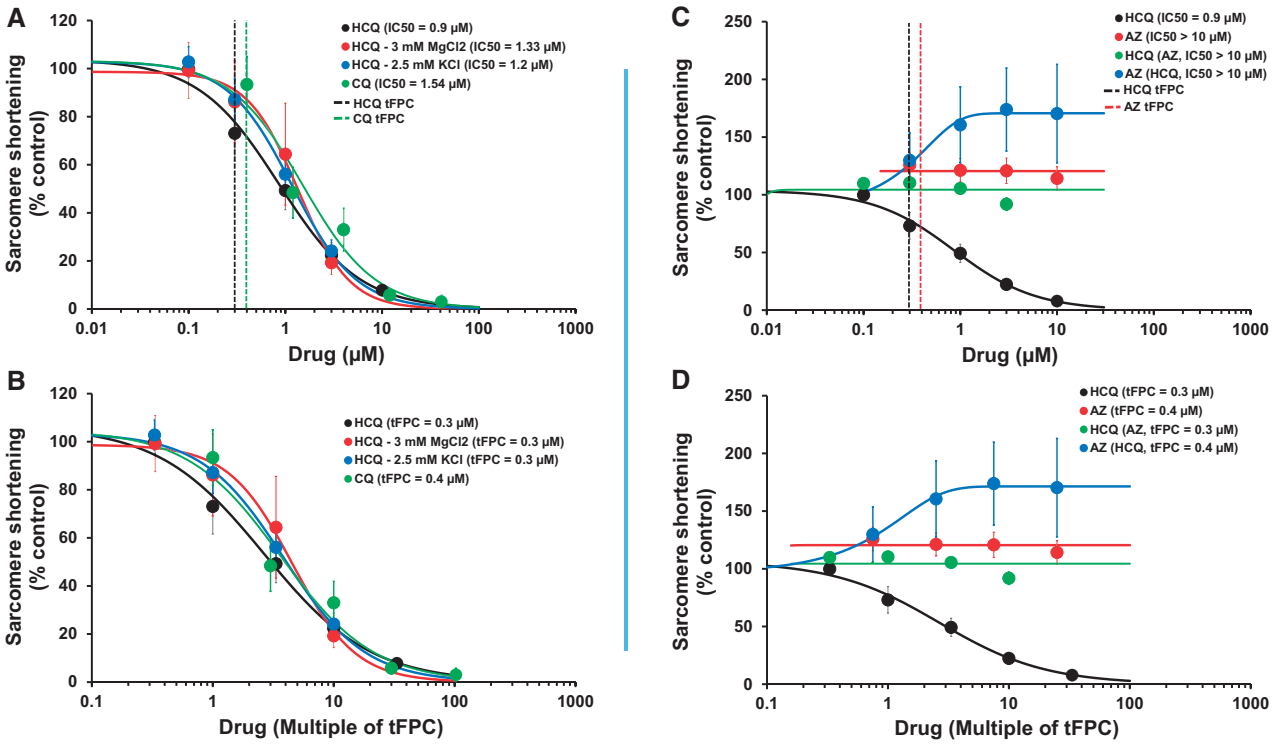

Figure 4. Human inotropic potential of Hydroxychloroquine (HCQ), Chloroquine (CQ), and Azithromycin (AZ). Cumulative concentration-effect curves for sarcomere shortening are shown as a function of concentrations tested (A and C) or multiple of tFPCs (B and D) at a pacing frequency of 1 Hz. Curves were fitted to sarcomere shortening data using SigmaPlot v14.0 and the concentrations inducing 50% decrease (IC50s) in sarcomere shortening were estimated. HCQ (AZ) and AZ (HCQ) represent HCQ curve in the presence of 0.3 μM AZ and AZ curve in the presence of 0.3 μM HCQ, respectively. The 0.01 μM represents the normalized vehicle data for drugs in (A) and (D). Abbreviation: tFPC, therapeutic free plasma concentration.

sarcomere shortening (Supplementary Figure 6G). We next evaluated the effects of Azithromycin and polytherapy on human cardiomyocyte contractility. Data demonstrate that Azithromycin had no effect on sarcomere shortening at all concentrations tested (Figs. 2E and 2F and 4C and 4D and Supplementary Figure 7). Remarkably, the negative inotropic activity of Hydroxychloroquine was significantly attenuated when the drug was applied in the continuous presence of Azithromycin at the therapeutic free plasma concentration (Figs. 4C and 4D). Another unanticipated finding was the concentration-dependent increase in sarcomere shortening caused by Azithromycin when it was applied in the continuous presence of Hydroxychloroquine at the therapeutic free plasma concentration (Figs. 4C and 4D). Hence, our findings with Hydroxychloroquine and Azithromycin suggest that polytherapy may influence the inotropic risk.

Effects of Hydroxychloroquine, Chloroquine, and Azithromycin on Cardiac Ion Channels

Given the critical contribution of hERG, Nav1.5, and Cav1.2 channels, the key cardiac ion channels for the CiPA initiative (Colatsky *et al.*, 2016; Yim, 2018), to normal and abnormal human ventricular repolarization and contractility, the cardiomyocyte effects of Hydroxychloroquine, Chloroquine, and Azithromycin may be facilitated by modulation of these 3 ion channels. Hence, we addressed the effects of Hydroxychloroquine, Chloroquine, and Azithromycin on hERG, Nav1.5, and Cav1.2 channels overexpressed in heterologous systems. Before constructing C-E curves for the effects of Hydroxychloroquine, Chloroquine, and Azithromycin on hERG, Nav1.5, and Cav1.2 currents, we first established time-control data using 5 additions of vehicle solution (0.33% DMSO) to mimic an experiment with an active drug. Neither the first to fifth vehicle addition significantly affected the amplitudes of hERG, Nav1.5, and Cav1.2 currents (Figure 5A). Thus, sequential DMSO additions do not significantly affect the function of hERG, Nav1.5, and Cav1.2 channels overexpressed in heterologous systems under the experimental conditions of this study, and illustrating that they can be used to generate meaningful C–E curves.

Figure 5B shows that Hydroxychloroquine and Chloroquine inhibited the hERG current with IC50 values of 9.7 and 7.77 μM, respectively, but Azithromycin had no effect. We used the hERG channel blocker, E-4031, as a typical positive control. E-4031's potency (IC50 = 0.036 μM) was left shifted compared with those with Hydroxychloroquine and Chloroquine. Next, the effects of the 3 drugs on Nav1.5 current are shown in Figure 5C. Although Chloroquine inhibited the Nav1.5 channel with an IC50 value of 8.48 μM, both Hydroxychloroquine and Azithromycin were found to have no significant effects. We used the Nav1.5 channel inhibitor, Tetracaine, as a typical positive control. Tetracaine's potency (IC50 = 1.23 μM) was left shifted compared with that with Chloroquine (Figure 5C). Next, we found that Chloroquine and Hydroxychloroquine, but not Azithromycin, inhibited the Cav1.2 channel with IC50 values of 3.05 and 7.64 μM, respectively (Figure 5D). We used the Cav1.2 channel inhibitor, Nifedipine, as a typical positive control. Nifedipine's potency (IC50 = 0.46 μM) was left shifted compared with those with Hydroxychloroquine and Chloroquine. Our ion channel data hence suggest that while Hydroxychloroquine and Chloroquine

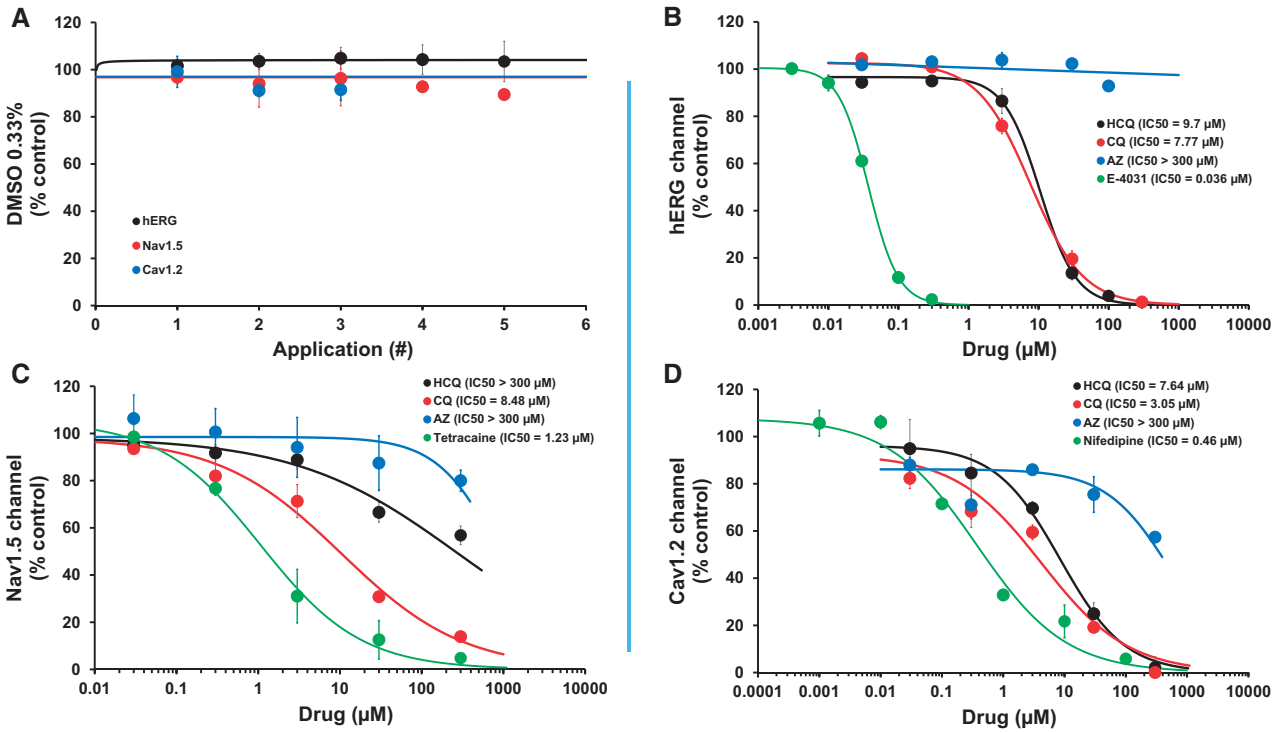

Figure 5. Effects of Hydroxychloroquine (HCQ), Chloroquine (CQ), and Azithromycin (AZ) on cardiac ion channels. A, Stability of current recordings over time in the presence of vehicle, 0.33% dimethyl sulfoxide. B–D, concentration-effect curves for HCQ, CQ, AZ and 3 positive controls (E-4031, Tetracaine and Nifedipine) on hERG, Nav1.5, and Cav1.2 currents, respectively. IC50, concentration inducing 50% decrease in the current ($n = 2$ replicates per concentration-effect curve).

inhibit multiple ion channels including the hERG channel, Chloroquine may inhibit more inward currents (Nav1.5 and Cav1.2) compared with Hydroxychloroquine (Cav1.2).

DISCUSSION

The main findings of the present investigation using adult human cardiomyocytes from healthy donor hearts can be outlined as follows: (1) Hydroxychloroquine was found to have a low proarrhythmia risk, whereas Chloroquine and Azithromycin were associated with high risk; (2) Hydroxychloroquine proarrhythmia risk changed to high at decreased level of K^+ or when combined with Azithromycin at therapeutic concentration; (3) High Mg^{2+} concentration protected against proarrhythmia caused by high concentrations to Hydroxychloroquine; (4) Hydroxychloroquine at therapeutic concentration caused no further increase in the incidence of temperature-induced proarrhythmia; (5) Hydroxychloroquine and Chloroquine, but not Azithromycin, displayed negative inotropic activity while exhibiting multi-ion channel block features; and (6) Hydroxychloroquine-induced decreases in contractility were abolished by Azithromycin.

Chloroquine, the most widely used antimalarial drug (Haeusler *et al.*, 2018), exhibited a high proarrhythmia risk in the human cardiomyocyte contractility-based model, and the risk was evident at clinically relevant concentrations. In agreement with our data, Chloroquine has been reported to have a relatively narrow therapeutic window, with multiple cases of overdoses leading to ventricular arrhythmias (Kamp *et al.*, 2020). Moreover, the ability of Chloroquine to induce proarrhythmia and cardiotoxicity has recently been reported in patients with COVID-19 disease (Jankelson *et al.*, 2020; Semedo *et al.*, 2020; Szekely *et al.*, 2020; van den Broek *et al.*, 2020). Like Chloroquine, Azithromycin is found to be associated with high proarrhythmia risk in the human cardiomyocyte contractility-based model. Our data with Azithromycin agree with recent clinical reports (Arellano-Rodrigo *et al.*, 2001; Huang *et al.*, 2007; Kezerashvili *et al.*, 2007; Kim *et al.*, 2005; Rao *et al.*, 2014; Yang *et al.*, 2017). In contrast to Chloroquine and Azithromycin, Hydroxychloroquine appears to be associated with low proarrhythmia risk in the human cardiomyocyte contractility-based model. This finding agrees with clinical reports of Hydroxychloroquine-induced QT prolongation with no incidence of proarrhythmia (Hooks *et al.*, 2020; Morgan *et al.*, 2013; O'Laughlin *et al.*, 2016; Saleh *et al.*, 2020; Sridhar *et al.*, 2020). Nevertheless, proarrhythmia has been reported in patients taking high doses of Hydroxychloroquine (Chen *et al.*, 2006; Ndukwu and Ghahramani, 2017). These reports correlate with the incidence of proarrhythmia caused by high concentrations of Hydroxychloroquine in the human cardiomyocyte contractility-based model. Additionally, our data show that Hydroxychloroquine, when combined with Azithromycin at therapeutic concentration, has a high proarrhythmia risk at all concentrations tested, including clinically relevant concentrations. Similarly, proarrhythmia has recently been reported in COVID-19 patients treated with Hydroxychloroquine plus Azithromycin therapy (Anupama *et al.*, 2020; Chorin *et al.*, 2020; Mercuro *et al.*, 2020). Likewise, polytherapy of Hydroxychloroquine and Moxifloxacin has been reported in patients with COVID-19 (Afsin *et al.*, 2020). Conversely, polytherapy of Azithromycin and Chloroquine has been shown not to be associated with arrhythmia liability in the anesthetized guinea pig model (Fossa *et al.*, 2007). Taken together, these findings highlight the ability of the human cardiomyocyte contractility-based model to identify true cardiotoxic effects and emphasize the challenges in cross-species translation for cardiac risk

assessment. Our data also indicate that Azithromycin, when combined with Hydroxychloroquine at therapeutic concentration, was associated with low proarrhythmia risk only at therapeutic exposure. Likely explanation why the proarrhythmia risk differs between sequences of combination of Hydroxychloroquine and Azithromycin at therapeutic exposures is proposed (see below), however the clinical significance of this finding remain to be explored.

Hypokalemia, a common risk factor in drug-induced proarrhythmia (Osadchii, 2010; Widimsky, 2008), has been reported in up to 60% of COVID-19 patients (Chen *et al.*, 2020). Our data show a synergistic proarrhythmia effect of Hydroxychloroquine and low level of K^+. At a K^+ concentration of 2.5 mM, we observed a 3-fold increase in Hydroxychloroquine proarrhythmia risk at therapeutic exposure. Moreover, elevated temperature has been observed in up to 77% of COVID-19 patients (Wang *et al.*, 2020). Our data indicate no interaction between the effects of temperature and Hydroxychloroquine at the therapeutic exposure. Our finding with elevated temperature-mimicking conditions is in agreement with other studies that identified elevated temperature as an independent risk for proarrhythmia in normal hearts (Pasquié *et al.*, 2004) and in patients with COVID-19 (Lagier *et al.*, 2020). Additionally, Mg^{2+} infusion is an established treatment for proarrhythmia, restoring normal repolarization and Ca^{2+} handling (Baker, 2017; Redwood *et al.*, 1996; Tzivoni *et al.*, 1984). In contrast to low level of K^+, we found that high level of Mg^{2+} reduced the incidence of proarrhythmia for Hydroxychloroquine. This finding reconfirms the effectiveness of Mg^{2+} administration for the prevention or treatment of proarrhythmia. Taken together, our data suggest that exposure of patients, specifically those with COVID-19, to Hydroxychloroquine should be avoided during hypokalemia, and that exposure to high Mg^{2+} may mitigate Hydroxychloroquine-induced proarrhythmia.

The negative inotropic effects of Hydroxychloroquine and Chloroquine are poorly understood, in contrast to their QT/proarrhythmia risk. Studies with intact mammalian hearts reported no effect or decrease in contraction (Blignaut *et al.*, 2019; Hughes, 2020; Mubagwa, 2020; Tona *et al.*, 1990), although the negative inotropy was reported at high exposures only (between 10 and 100 μM). Conversely, our data with Hydroxychloroquine and Chloroquine in the human cardiomyocyte contractility-based model indicate reproducible negative inotropic effects at concentrations 10- to 100-fold lower than in mammalian hearts. Our data are in agreement with negative inotropy being reported as the probable cause of sudden death following the rapid parenteral administration of chloroquine for the treatment of malaria in children (PK/PD assessment indicate high plasma concentrations; White *et al.*, 1988). Moreover, cardiac failure is a not-uncommon finding in COVID-19 patients treated with Hydroxychloroquine and Chloroquine (Chatre *et al.*, 2018). Azithromycin, in contrast to Hydroxychloroquine and Chloroquine, had no effect on contractility in the human cardiomyocyte contractility-based model. Azithromycin however has been reported to suppress ventricular contraction in halothane anesthetized dogs and Langendorff rat model (Galán *et al.*, 2017; Ohara *et al.*, 2015), although at much higher concentrations compared with the concentrations tested in our study. We furthermore report for the first time that Azithromycin at the therapeutic concentration attenuated Hydroxychloroquine-induced negative inotropy and it was also found to increase contractility in the presence of Hydroxychloroquine at the therapeutic concentration. These findings suggest that polytherapy influences drug-induced inotropy and may provide an explanation to why negative inotropy has yet to be reported in COVID-19 patients treated with Hydroxychloroquine plus Azithromycin.

Effects on ion channels can explain our findings with Hydroxychloroquine, Chloroquine, and Azithromycin. We found that Chloroquine inhibits hERG, Nav1.5, and Cav1.2 channels, whereas Hydroxychloroquine inhibits hERG and Cav1.2 channels. This indicates that both drugs can be considered as multi-ion channel blockers. Our ion channel data concur with other reports detailing the effects of these 2 drugs on multiple cardiac ion channels (Capel *et al.*, 2015; Kamp *et al.*, 2020; Mubagwa, 2020; Traebert *et al.*, 2004). Inhibition of hERG, Nav1.5, and Cav1.2 channels can explain effects of Chloroquine, although proarrhythmia and negative inotropy in the human cardiomyocyte contractility-based model were seen at Chloroquine concentrations approximately 30 times lower than ion channel potencies. Hydroxychloroquine negative inotropic effect was seen at concentrations approximately 8 times below the Cav1.2 potency; however, proarrhythmia effects were observed at concentrations that correlate with hERG potency, ie, approximately 30 times above the therapeutic exposure. Our data with Chloroquine and Hydroxychloroquine agree with the hypothesis that the activity of some drugs on certain ion channels in cell lines can be accomplished with high precision but often without adequate accuracy; ie, cell line IC50 values may not adequately represent the actual potency of drugs in primary cardiomyocytes (Abi-Gerges *et al.*, 2020a). Importantly, our data suggest that blockade of inward currents by multi-ion channel blockers may not always protect against proarrhythmia: blockade of the Cav1.2 channel may offer protection against proarrhythmia as it is the case with Hydroxychloroquine, while blockade of both Cav1.2 and Nav1.5 channels may not safeguard against proarrhythmia as it is the case with Chloroquine. In contrast to Hydroxychloroquine and Chloroquine, Azithromycin showed a weak affinity to hERG, Nav1.5, and Cav1.2 channels (this study; Yang *et al.*, 2017; Zhang *et al.*, 2017). Additionally, Azithromycin was found to increase peak and late Nav1.5 following 24-h exposure (Yang *et al.*, 2017). Increase in peak and late Nav1.5 can load cardiomyocytes with Na^+ and Ca^{2+} to produce Ca^{2+} overload. Since (1) Azithromycin-induced increase in the incidence of proarrhythmia events in primary human cardiomyocytes occurred at concentrations approximately 200–1000 times smaller than those tested in ion channel assays in transfected noncardiomyocytes and (2) cardiomyocytes in our study were exposed to each drug concentration for a 300-s period, it is plausible that Azithromycin-related proarrhythmia can be due to its strong affinity to ion channels in primary human cardiomyocytes (ie, left shift in the concentration-effect curves), including late Nav1.5 current without the need for chronic exposure. For the combination Azithromycin first (therapeutic concentration) followed by the application of Hydroxychloroquine, we suggest that double blockade of hERG (Azithromycin and Hydroxychloroquine) and enhancement of late Nav1.5 (Azithromycin) overwhelm Cav1.2 inhibition by Hydroxychloroquine and hence may provide an explanation for Hydroxychloroquine-induced proarrhythmia risk (at therapeutic exposure) with no effect on inotropy. For the combination Hydroxychloroquine first (therapeutic concentration) followed by application of Azithromycin, blockade of Cav1.2 (Hydroxychloroquine) may balance double hERG blockade (Hydroxychloroquine and Azithromycin) and enhancement of late Nav1.5 (Azithromycin) and hence the low proarrhythmia risk of Azithromycin at therapeutic exposure. With increasing concentrations of Azithromycin, Cav1.2 inhibition by

Hydroxychloroquine may not be able anymore to counteract hERG blockade and late Nav1.5 enhancement and this leads to proarrhythmia and positive inotropy. Future studies are needed to fully elucidate the relationship between proarrhythmia, inotropy, drug combination, and sequence of ion channel modulation.

There are, of course, caveats in the current work. Although 300-s exposure of each drug concentration in our study does not represent steady-state conditions in patients, the human cardiomyocyte contractility-based model with the 300-s exposure time has been successfully used to predict risk associated with proarrhythmia and inotropic activity of 33 reference drugs (Abi-Gerges *et al.*, 2020b; Nguyen *et al.*, 2017). Furthermore, the presentation of cardiotoxicity in patients, but not in cardiomyocytes, is subject to neurohormonal modulation including changes in contractility, heart rate and vascular impedance. Also, while single risk factor analysis provides mechanistic insight, several factors may be present simultaneously in patients and hence multifactorial risk assessment would provide additional insight.

In conclusion, our study is the first to profile the proarrhythmia risk and inotropic activity of Hydroxychloroquine, Chloroquine, Azithromycin, and the combination of Hydroxychloroquine plus Azithromycin in adult human primary cardiomyocytes. Data generated with these 3 drugs have the potential to inform studies evaluating repurposed therapies, including those in the COVID-19 context. Finally, this study demonstrates the mechanistic and translational value of the human cardiomyocyte contractility-based model as a key step in drug discovery to generate human cardiotoxicity data that can be used to make informed decisions on novel therapies for COVID-19, Malaria and inflammatory diseases.

SUPPLEMENTARY DATA

Supplementary data are available at *Toxicological Sciences* online.

AUTHOR CONTRIBUTIONS

All authors contributed to data interpretation and production of the article text and figures, and to critique.

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DECLARATION OF CONFLICTING INTERESTS

P.J., B.D., M.T., and L.U. are employees of Novartis and P.J., M.T., and L.U. own Novartis stocks or shares. N.A.G., P.E.M., and A.G. are employees of AnaBios Corporation and own AnaBios stocks or shares. Novartis Sandoz division is a non-exclusive manufacturer of Hydroxychloroquine, an off-patent medication.

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