Azithromycin/Chloroquine Combination Does Not Increase Cardiac Instability despite an Increase in Monophasic Action Potential Duration in the Anesthetized Guinea Pig

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Abstract. Prolongation of the electrocardiogram QT interval by some, but not all drugs, has been associated with increased incidence of sudden cardiac death. Current preclinical regulatory assays cannot discriminate the arrhythmia liability of these drugs. Consequently, many new medications that prolong the QT interval are not developed despite their potential therapeutic benefit. Alternans (action potential duration alternations) is a measure of cardiac instability in humans and animals associated with the onset of ventricular fibrillation. Due to potential arrhythmia risk from observed QT prolongation, alternans was assessed in the anesthetized guinea pig after azithromycin or chloroquine alone and after combination treatment at clinically relevant concentrations proposed for the management of malaria. Chloroquine alone, but not azithromycin, caused a profound increase in action potential duration but with only minimal effects on alternans (~10 ms). Azithromycin alone and in combination with chloroquine showed no increase in alternans beyond vehicle baseline responses indicating no additional arrhythmia liability.

INTRODUCTION

Since 1997, International Conference of Harmonization (ICH) regulatory guidelines have been established to assess properties of new drugs that cause QT prolongation because of the association with the increased occurrence of a rare but sometimes fatal ventricular arrhythmia, torsades de pointes. Many anti-bacterials such as erythromycin and moxifloxacin are known to prolong the QT interval but are associated with different likelihoods of torsades de pointes or cardiac risk. The current ICH regulatory required assays used in pharmaceutical development cannot accurately discriminate arrhythmia susceptibility below approximately 30-fold the intended therapeutic indices. Mixofloxacin and erythromycin, for example, are associated with a 1- to 8-fold therapeutic safety margin based on these assays and most likely would not be developed in today’s regulatory environment. There is no doubt that erythromycin and other antibacterial and antiparasitic agents have improved our lives by reducing the threat from infections, particularly malaria. Many of these highly valued agents may need to be replaced in the future with new generations of medications as organism resistance develops. Therefore, new methods to assess the arrhythmia potential are needed. To improve the confidence in their predictive value, these new methods can be benchmarked against drugs with clinical histories that are known/recognized by most physicians.

Azithromycin (Zithromax®) is being considered for use in combination with chloroquine in patients with chloroquine-resistant malaria because synergy has been reported in vitro. Preliminary studies indicated an increase in clinical success from approximately 30% with each drug used alone to 97% with combination therapy. Azithromycin and chloroquine have well-established safety profiles. However, each drug is known to delay cardiac repolarization through inhibition of rapidly activating delayed rectifier potassium current known as hERG. Which in the case of chloroquine, can result in QT prolongation.

Beat-to-beat alternations of the cardiac action potential duration (APD) known as alternans are thought to be a precursor/substrate to the development of ventricular arrhythmias. This occurs when a series of cardiac cycles oscillate between short-long duration and generates a reciprocating short-long sequence of APD as heart rate accelerates (see example in Figure 1). When the APD oscillations become large enough, they act as waves that break and may cause conduction block, reentry, and synchronous ventricular tachycardia that can transition to fibrillation. Our laboratory has developed a rapid pacing model (30 sequential cycles at increasing rates of stimulation after intermittent periods of rest) in the anesthetized guinea pig and shown it be predictive of the concentration-dependent onset of torsades de pointes type ventricular arrhythmias with a wide variety of both cardiovascular and non-cardiovascular commercial compounds including several anti-bacterials. To determine whether an arrhythmogenic effect could be detected with either azithromycin or chloroquine alone and in combination, studies were conducted to examine the generation of alternans in this model at concentrations within or exceeding the clinically relevant range for management of malaria.

MATERIALS AND METHODS

Monophasic action potential duration (MAPD) alternans measurements in the anesthetized guinea pig. Surgical procedure. All studies were conducted in compliance with an Animal Care and Usage Protocol approved by the Institutional Animal Care and Use Committee (IACUC). Male Hartley guinea pigs weighing 400–700 grams were anesthetized with pentobarbital 40 mg/kg, intraperitoneally. Supplemental anesthetic was given if necessary and the guinea pigs were placed on a heating pad for the duration of the surgery and experiment. A tracheotomy was performed and the guinea pigs were ventilated with room air using a Harvard Apparatus Rodent Ventilator Model 683 (South Natick, MA). The right jugular vein and left carotid artery were cannulated with PE50 tubing for drug administration and blood pressure monitoring, respectively. A sternal (or medial) thoracotomy was performed allowing the heart to be suspended in a pericardial cradle. The heart was frequently moistened

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with lactated Ringer’s solution. Two Teflon-coated silver wires (0.013” diameter; A-M Systems, Inc., Carlsborg, WA) were sutured to the left ventricular apex for pacing with a Bloom Technologies DTU 215 Programmable Stimulator (Fischer Imaging Corporation, Denver, CO). Monophasic action potential (MAP) signals were recorded from the left ventricle with an EP Technologies EPT 200 Probe (San Jose, CA) that was held in position with a modified stereotaxic stand. Once the MAP signal was stable, the probe remained fixed in the same position throughout the entire study. Lead II ECG signals were recorded with 22-gauge needle electrodes placed subcutaneously around the thoracotomy. The MAP, pacing stimulus, ECG, and blood pressure waveforms were sampled at 1000 Hz and saved to disk using a PoNeMah data acquisition and analysis system (Transoma, St. Paul, MN).

Dosing protocol. Guinea pigs received one of the six infusion protocols outlined in Figure 2 and Table 1. These protocols were designed to achieve free plasma concentrations of azithromycin and chloroquine that were near or exceeded the clinical use concentrations of each drug without causing excessive hemodynamic effects that would compromise the study. Bolus infusions of drug or vehicle were administered over 15 minutes. The 4-step dose escalation was achieved by administering a loading dose of drug or vehicle over 5 minutes. All solutions were infused with a PHD 2000 Programmable Pump (Harvard Apparatus, South Natick, MA). Samples for assessment of azithromycin and chloroquine free plasma concentration were obtained from arterial blood at the end of each maintenance infusion phase during the prescribed intravenous infusion protocol.
Pacing protocol. Monophasic action potential (MAP) signals (> 15 mV) were filtered 0.05 Hz–1000 Hz and allowed to stabilize for at least 10 minutes before the pacing protocol. The minimum threshold current for ventricular capture was determined (0.15–0.4 mA). Pacing was performed at twice threshold current. A preconditioned pacing protocol, similar to a dynamic restitution pacing protocol, was applied as follows: a 50-beat preconditioning pulse train (S1/H1 220 ms or S1/H1 240 ms) was followed immediately by a 30-beat pulse train at S2/H1 200 ms. After a small pause to adjust the stimulator (∼5 sec), pacing was reinitiated at the 50-beat preconditioning S1 pulse train followed by a new 30-beat pulse train at S2/H1 190 ms. This protocol was repeated with a fixed S1 cycle length and from S2 = 200 ms to S2 = 140 ms by 10 ms decrements. Pacing was stopped if an arrhythmia (TdP) occurred, the heart became refractory, or the end of the protocol was reached. This pacing protocol was repeated twice during baseline to ensure stable control conditions, 10-minutes after the end of the bolus infusion, and during the last 5 minutes of each maintenance infusion during the 4-step dose escalation phase. The total duration of the experiment with pacing was approximately 120 minutes.

MAPD measurements. MAP duration (MAPD) measurements were automatically made by the PoNeMah system and defined as the time from the maximum rate of depolarization to 90, 50, and 30% repolarization. Signals recorded during each study were replayed on the PoNeMah system and the MAP signals were visually inspected to verify ventricular capture and to validate measurements during the pacing protocol. If necessary, manual measurements were made using a digital measure command on the PoNeMah. MAP signals that were accurately captured and measured during S2 pacing were used for alternans analysis. The first two pulsed beats at each cycle length were removed to account for possible incomplete capture. Beat-to-beat alternans were calculated as the difference between two consecutive MAPD measured at 50% repolarization (MAPD_{50}) measurements and averaged over the entire S2 pulse train to yield average MAPD alternans. Data were included for a given study only if the difference (< 5 ms) between the two baseline periods indicated a stable experimental preparation.

Analytical procedure. Plasma samples were extracted by protein precipitation method and analyzed by high-performance liquid chromatography-mass spectroscopy LC/MS/MS (API-4000, MDS Sciex, Foster City, CA). Separation was achieved on a Phenomenex LUNA C18, 2.1 × 30 mm, 5-μm column at a flow rate of 0.4 mL/min. A 4-minute binary
Azithromycin and chloroquine are lipophilic drugs that distribute rapidly from the plasma compartment, have relatively long half-lives, and maintain long biologic activities. Consequently, comparisons to peak plasma concentrations may not always be appropriate because patients experience these levels for a short period of time relative to the duration of therapy. Therefore, the concentration of each drug at its first half-life in humans was also used in addition to the maximum concentration (Cmax) to adequately compare the drug concentrations in our studies to those that reflect the preponderance of therapeutic duration.

In humans, the mean Cmax total plasma concentrations of azithromycin are 805 ng/ml and 922 ng/ml when given alone as 1000 mg/day for 3 days and in combination with chloroquine (600 mg base for 2 days and 300 mg base on day 3), respectively. The half-life of azithromycin was determined to be 74 hours (100 ng/ml total). In that same study, subjects achieved mean Cmax total plasma chloroquine concentrations of approximately 200 ng/ml when given alone or in combination with azithromycin. The half-life of chloroquine was determined to be 206 hours and the total plasma concentration at that time was approximately 20 ng/ml. To compare the plasma concentrations in our guinea pigs to these clinical concentrations, the total plasma concentration was adjusted for protein binding with an experimentally determined unbound fraction of 0.79 for azithromycin and 0.44 for chloroquine in human plasma. Concentrations were targeted in these studies resulting in free Cmax concentrations of 636 and 90 ng/ml, respectively. Guinea pig free drug concentrations were obtained from total concentrations using the experimentally determined unbound fraction of 0.745 for azithromycin and 0.285 for chloroquine across the same concentration range in guinea pig plasma. Because these studies were based on preliminary intravenous experiments to determine the infusion concentrations required to obtain the targeted human clinical range for comparison in guinea pigs, no formal dose scaling was conducted.

In guinea pigs, azithromycin alone, given as a 4-step infusion at doses shown in Table 1 (0.17–10.67 mg active (adjusted for free base) compound mg/kg, intravenously) produced free drug concentrations of 32, 95, 343, and 1152 ng/ml, respectively (Table 2, top panel). The same infusion protocol for azithromycin after a 5 mg/kg bolus dose of chloroquine produced similar free plasma azithromycin concentrations of 33, 74, 310, and 1105 ng/ml. A similar step infusion protocol administering 0.22, 0.67, 2.0, and 6.0 mg chloroquine (Table 1, top panel) achieved free drug concentrations of 6, 13, 31, and 85 ng/ml alone and 6, 12, 34, and 121 ng/ml when given after a bolus 6.0 mg/kg dose of azithromycin.

Table 2 (bottom panel) also displays the relative multiples of each drug in comparison to clinically used concentrations. Azithromycin concentrations achieved in guinea pigs, when given alone, represent approximately 0.4- to 15-fold the therapeutic concentrations after one half-life (79 ng/ml unbound at 68±7.4 hours) and 0.05- to 1.8-fold the free Cmax concentrations of 636 ng/ml after a 1000 mg dose for 3 days in malaria treatment. The mean free drug concentrations achieved for chloroquine alone represents approximately 0.7- to 9-fold the therapeutic concentrations after one half-life (9 ng/ml unbound at 206 hours) and 0.07- to 0.94-fold the Cmax concentration (91 ng/ml) after 600 mg on days 1 and 2 and 300 mg on day 3 for management of malaria.

**Effects on BP, HR, and MAPD.** Mean arterial pressure was slightly reduced by 5–10 mm of Hg from baseline with the highest concentration achieved with azithromycin alone (1152 ng/ml) and during the combination (1105 ng/ml) with the chloroquine bolus (Table 3). Chloroquine alone showed spurious statistical increases in mean arterial pressure during periods 2 and 4 of less than 5 mm of Hg and were probably not of physiologic significance but most likely due to the low vehicle baseline effect (Table 4). Chloroquine did produce dose-dependent reductions in heart rate of 40 to 60 BPM both when given alone (31–85 ng/ml) and in combination (34–121 ng/ml) with the bolus administration of azithromycin (Table 4). Azithromycin alone showed only smaller reductions in
heart rate (30 BPM) at the highest concentration achieved of 1152 ng/ml (Table 3). The effect of chloroquine on heart rate also increased MAPD90 during sinus rhythm whereas the effect of azithromycin on heart rate did not. The chloroquine-induced prolongation of MAPD90 ranged from an increase of 20 ms at 13 ng/ml to 45 ms at 85 ng/ml. The prolongation in MAPD90 was also present with the combination of azithromycin but the magnitude was reduced approximately 8 ms. This occurred despite slightly higher levels of chloroquine, indicating the strong relationship between heart rate and sinus MAPD90.

To examine the impact on repolarization independent of heart rate, MAPD90 was examined at a pacing rate slightly above sinus rhythm (200 ms or BCL 200). Azithromycin alone showed no effect on MAPD90 up to 343 ng/ml but appeared to cause some slight shortening of approximately 5 ms at 1152 ng/ml (Figure 3). On the other hand, chloroquine alone caused a profound increase in MAPD90 with increasing plasma concentrations from 13 to 85 ng/ml (Figure 4). In cases when the two drugs were combined, the magnitude of the effect with chloroquine appeared primarily due to the concentration of chloroquine and was not increased by the presence of azithromycin (Figures 3 and 4).

**Effects on alternans during ventricular pacing.** No biologically significant (> 10 ms) effect on mean alternans was observed with the combination of azithromycin and chloroquine at any pacing basic cycle length (BCL) indicating no propensity toward arrhythmia liability (Figures 5B and 6B, respectively). In the azithromycin alone group, one animal had an atypical response of greater than 10 ms that resulted in large variation and statistical significance at the highest concentration and fastest pacing cycles of BCL 150 and 140 ms (Figure 7).

### Table 2

<table>
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<th>Treatment (bolus + step)</th>
<th>Free drug (ng/ml)</th>
<th>Ceff multiple (mg/ml)</th>
<th>Bolus (Period 1)</th>
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* Molecular weight: AZM = 749; CLQ = 320.
† Free fraction in guinea pig; AZM = 0.745; CLQ = 0.285.
‡ Ceff (free drug): AZM = 79 ng/ml; CLQ = 9 ng/ml.

### Table 3

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<tr>
<th>Treatment (bolus + step)</th>
<th>Baseline</th>
<th>Bolus (Period 1)</th>
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<th>Step 2 (Period 3)</th>
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</table>

* Denotes significant difference from pre-dose baseline compared with respective vehicle–vehicle difference from pre-dose baseline at each dose level within a given treatment.
† Denotes difference from chloroquine treatment alone, P < 0.05.

Refer to Table 1 for exact doses used for each step of the escalation protocol. Vehicle was administered in the same dose volume used for test compound. All values represent mean ± S.E.M.
5A). When this animal was removed from the data, the alternans effect was not statistically significant during this period. However, examination of the individual plasma concentration could not allow exclusion of this animal and it was included in the analyses as reported. Chloroquine alone did increase alternans at the highest concentration of 85 ng/ml in a rate-dependent manner (Figures 1 and 6A). The magnitude of this effect is considered to be the minimal physiologically relevant level required as a substrate for induction of reentry arrhythmia (i.e., > 10 ms). When chloroquine was examined after the bolus loading dose of azithromycin (Figure 6B), the magnitude of alternans was significantly reduced to a level not considered to be susceptible to arrhythmia and not different from vehicle baseline.

The effects on alternans relative to clinical use concentrations are highlighted at a single pacing rate of BCL 150. Azithromycin alone or in combination showed no effects over these concentrations (Figure 7). Chloroquine alone did cause a statistically significant increase at the upper end of its use concentration—an effect that was reduced with azithromycin bolus (Figure 8). There were no effects of either azithromycin or chloroquine vehicle–vehicle combinations on alternans at any cycle lengths tested.

### Table 4

<table>
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<tr>
<th>Treatment (bolus + step)</th>
<th>Baseline</th>
<th>Bolus (Period 1)</th>
<th>Step 1 (Period 2)</th>
<th>Step 2 (Period 3)</th>
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</tbody>
</table>

† Denotes difference from azithromycin treatment alone, *P < 0.05.

* Denotes significant difference from pre-dose baseline compared with respective vehicle–vehicle difference from pre-dose baseline at each dose level within a given treatment.

Refer to Table 1 for exact doses used for each step of the escalation protocol. Vehicle was administered in the same dose volume used for test compound. All values represent mean ± S.E.M.

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**Figure 3.** Effects of azithromycin, alone (○; dashed lines) and in combination with chloroquine (●; solid lines), on mean monophasic action potential duration at 90% repolarization (MAPD90) during ventricular pacing at BCL = 200 ms; data shown is difference from vehicle control. Vertical arrow indicates effect of vehicle (○) or chloroquine (●) bolus. Shaded box represents clinical efficacious concentration range of azithromycin between Cmax and the concentration at one half-life (t1/2). For chloroquine bolus plasma concentrations see Table 2. *Denotes significant difference *P < 0.05 from vehicle for each group; #Denotes significant difference between treatment groups.

**Figure 4.** Effects of chloroquine, alone (●; dashed lines) and in combination with azithromycin (■; solid lines), on mean monophasic action potential duration at 90% repolarization (MAPD90) during ventricular pacing at BCL = 200 ms; data shown is difference from vehicle control. Vertical arrow indicates effect of vehicle (■) or azithromycin (■) bolus. Shaded box represents clinical efficacious concentration range of chloroquine (CLQ) between Cmax and the concentration at one half-life (t1/2). For azithromycin bolus plasma concentrations see Table 2. *Denotes significant difference *P < 0.05 from vehicle for each group; #Denotes significant difference between treatment groups.
DISCUSSION

Azithromycin and chloroquine were studied in a guinea pig model of cardiac instability at clinically relevant therapeutic concentrations proposed for the management of malaria. The findings in this study are consistent with reported clinical incidences of QT prolongation with minimal propensity to produce ventricular arrhythmias for each drug. When azithromycin was added to chloroquine treatment, the magnitude of alternans observed was significantly reduced compared with chloroquine alone. To the extent that the magnitude of alternans predicts the likelihood of ventricular arrhythmia, these data suggest that the arrhythmia liability associated with the clinical occurrence of QT prolongation when two drugs are combined is not simply additive but may be mitigated by specific mechanisms related to the interaction between the drugs.

Chloroquine is associated with heart rate corrected QT(c) prolongation between 14 and 30 ms\(^{13,20}\) that exceeds the 10 ms upper limit threshold of concern specified in current regulatory ICH E14 guidance. Only three reports\(^{23–25}\) describe an event consistent with torsades de pointes ventricular arrhythmia with chloroquine administration. A confirmed diagnosis of torsades de pointes requires ECG confirmation, something unlikely to be available in resource-poor regions. However, given the billions of courses of therapy over the past 50 years, much of it in travelers and the military from developed countries, this remains a surprisingly low rate of ventricular arrhythmia. In fact, given the relatively profound QT prolongation associated with chloroquine, one would have expected a significantly greater number of reported episodes of ventricular arrhythmia if the mechanism whereby chloroquine prolongs the QT interval was associated with reentry currents. The relatively minor proarrhythmia potential predicted by a 10 ms alternans is consistent with the rare reports of...
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torsades de pointes despite pronounced QT prolongation in clinical trials and the profound MAPD90 increases observed in our study. These results are also consistent with the only other preclinical report in the literature examining the proar-

rhythmia potential of chloroquine. The lack of proarrhyth-

mia potential with azithromycin alone or in combination with chloroquine, shown in this study by the absence of physiologi-

cally significant alternans, is consistent with results reported

by other investigators using different models of proarrrhyth-
mia assessment with azithromycin. Azithromycin is a weak inhibitor of hERG current (IC50 = 1,500 μM). Gintaní showed in canine Purkinje fibers that only small increases in MAPD of less than 10% could occur at 100-fold clinical levels compared with other antibiotics like moxifloxacin that caused 160% increases at 50-fold clinical levels. Recently azithromy-

cin was examined for effects on temporal variability of the left ventricular MAPD (similar to alternans but at low heart rates) in the anesthetized dog with chronic AV block. This animal model is highly susceptible to torsades de pointes ar-
rhythmia due to a lack of repolarization reserve developed during the remodeling process with chronic pacing. At clinically relevant intravenous doses of 2 and 8 mg/kg, azithromy-

cin did not induce any short-term variability in repolarization of the left ventricle, and the QT, QTc and left ventricular MAPD all showed non-significant trends toward reduction. However, in the isolated rabbit heart at concentrations exceeding clinical level by more than 300-fold (150–300 μM), whereas azithromycin did lead to increases in the QT interval and MAPD, it showed no signs of early afterdepolarizations (an arrhythmia triggering event) or torsades de pointes. In fact, when azithromycin (150 μM) was combined with concentra-
tions of erythromycin that cause torsades de pointes in this model, arrhythmia suppression occurred. These data to-

tgether indicate that azithromycin consistently shows a lack of arrhythmia potential when used alone and when combined with other agents that prolong QT and have proarrrhythmia potential.

The heart rate and blood pressure findings in this study appear to vary from clinical observations slightly. This is most likely due to the anesthetized state of the guinea pigs, which can affect baroreflexes and neural control of hemodynamics. Chloroquine administration was dose-limited by the reduc-
tion in heart rate whereas azithromycin was limited by a de-

cline in blood pressure. Clinically, chloroquine does cause hypotension at high doses with reflex tachycardia most com-

monly seen. Azithromycin, on the other hand, has not been reported to have adverse clinical hemodynamic effects. The difference in heart rate response, however, should not affect the results, given that the cardiac pacing used in our model would supersede this physiologic response.

The mechanisms for alternans leading to arrhythmia have been extensively studied and are attributed to increases in intracellular calcium mediated through changes in the dynam-

cics of any of a variety of calcium handling pathways. It has also been established that agents that increase intracellular calcium also increase cardiac contractility and are associated with increases in mortality. Azithromycin has been demon-

strated to produce physiologically significant suppression of contractility in smooth muscle tissue whereas other mac-
oplides have been shown to decrease cardiac contractility in isolated heart tissue and conscious dogs. Thus it is possible that azithromycin may mitigate arrhythmia liability through a reduction of intracellular calcium. More direct ex-

perimentation would be necessary to verify this speculation.

In summary, azithromycin alone or in combination with chloroquine showed no indication in this guinea pig model of cardiac instability that may lead to a ventricular arrhythmia liability at the adjusted free drug concentrations studied. These concentrations represented 1- to 2-fold the Cmax levels or 10- to 15-fold the upper free-drug concentrations antici-

![Figure 7. Effects of azithromycin, alone (●) or azithromycin + chloroquine (■) bolus.](image)

![Figure 8. Effects of chloroquine, alone (■) or azithromycin (■) bolus.](image)
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pated after dosing with chloroquine and azithromycin in a proposed clinical regimen for management of malaria. Dose-limiting reductions in heart rate or mean arterial pressure induced by chloroquine or azithromycin, respectively, prohibited higher concentrations being studied. These results are consistent with published data by other investigators using other models that assess proarrhythmia potential. Despite the ability of azithromycin to inhibit hERG current at high concentrations, it appears to lack proarrhythmia liability at or exceeding clinically relevant concentrations alone or when used in combination with chloroquine.

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