Azithromycin Causes a Novel Proarrhythmic Syndrome

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Background—The widely used macrolide antibiotic azithromycin increases risk of cardiovascular and sudden cardiac death, although the underlying mechanisms are unclear. Case reports, including the one we document here, demonstrate that azithromycin can cause rapid, polymorphic ventricular tachycardia in the absence of QT prolongation, indicating a novel proarrhythmic syndrome. We investigated the electrophysiological effects of azithromycin in vivo and in vitro using mice, cardiomyocytes, and human ion channels heterologously expressed in human embryonic kidney (HEK 293) and Chinese hamster ovary (CHO) cells.

Methods and Results—In conscious telemetered mice, acute intraperitoneal and oral administration of azithromycin caused effects consistent with multi-ion channel block, with significant sinus slowing and increased PR, QRS, QT, and QTc intervals, as seen with azithromycin overdose. Similarly, in HL-1 cardiomyocytes, the drug slowed sinus automaticity, reduced phase 0 upstroke slope, and prolonged action potential duration. Acute exposure to azithromycin reduced peak SCN5A currents in HEK cells (IC50=110±3 μmol/L) and Na+ current in mouse ventricular myocytes. However, with chronic (24 hour) exposure, azithromycin caused a 2-fold increase in both peak and late SCN5A currents, with findings confirmed for INa in cardiomyocytes. Mild block occurred for IK+ currents representing IKr (CHO cells expressing hERG; IC50=219±21 μmol/L) and IKs (CHO cells expressing KCNQ1+KCNE1; IC50=184±12 μmol/L), whereas azithromycin suppressed L-type Ca++ currents (rabbit ventricular myocytes, IC50=66.5±4 μmol/L) and Kir2.1 (HEK cells expressing Kir2.1, IC50=44±3 μmol/L).

Conclusions—Chronic exposure to azithromycin increases cardiac Na+ current to promote intracellular Na+ loading, providing a potential mechanistic basis for the novel form of proarrhythmia seen with this macrolide antibiotic. (Circ Arrhythm Electrophysiol. 2017;10:e003560. DOI: 10.1161/CIRCEP.115.003560.)

Key Words: calcium channel ■ mice ■ pharmacology ■ potassium channels ■ sodium channels

Sudden cardiac death caused by noncardiac medications is a major public health issue, in particular given its preventable nature. The scope of this problem is illustrated by the fact that drug-related proarrhythmia has been the most common cause for removal of medications from the US market in recent decades. An improved understanding of the basic mechanisms causing drug-related sudden cardiac death would lead to safer pharmacotherapy.

See Editorial by Fishman

The macrolide antibiotics erythromycin and clarithromycin are associated with increased risk of serious ventricular tachyarrhythmias and sudden cardiac death, whereas azithromycin was long considered to have minimal adverse cardiac effects. However, because of occasional case reports of marked QT prolongation and serious ventricular arrhythmias, as well as concerning data from the US Food and Drug Administration Adverse Event Reporting System, we previously conducted a retrospective cohort study to examine azithromycin safety. We found that azithromycin users had an increased risk of cardiovascular mortality and sudden cardiac death, compared with users of amoxicillin.

Several mechanisms have been described whereby drugs can increase susceptibility to serious ventricular tachyarrhythmias and sudden cardiac death, analogous to inherited arrhythmia syndromes. First, a reduction in cardiac Na+ current (INa) can lead to slowed conduction, facilitating reentrant ventricular arrhythmias. This likely accounts for increased mortality in patients with coronary artery disease treated with Na+ channel blockers and in the Brugada syndrome. Secondly, drug-induced conduction delay can facilitate reentry by unmasking substrate. Azithromycin may cause slowed conduction by blocking L-type Ca++ channels (IC50=66.5±4 μmol/L) and Kir2.1 (IC50=44±3 μmol/L), which is consistent with its proarrhythmia. Third, some drugs can cause QT prolongation, which can predispose to torsade de pointes. Azithromycin also causes QT prolongation (IC50=219±21 μmol/L) and can cause a 2-fold increase in late Na+ current (IC50=110±3 μmol/L). Finally, some drugs can cause ventricular fibrillation via a direct effect on cardiac ion channels. Azithromycin causes both Na+ and K+ channel block, which could contribute to ventricular fibrillation. Further studies are needed to determine the relative contribution of these mechanisms to the proarrhythmia caused by azithromycin.
WHAT IS KNOWN

- The commonly used antibiotic azithromycin increases risk of cardiovascular and sudden cardiac death, but the underlying mechanisms are unclear.
- While torsades de pointes is rare, case reports indicate that azithromycin can also cause a novel proarrhythmic syndrome, characterized by polymorphic ventricular tachycardia in the setting of a normal QT interval.

WHAT THE STUDY ADDS

- Acutely, azithromycin causes diffuse depression of the cardiac conduction system and ion channel block, reminiscent of azithromycin overdose in humans.
- Chronically, the drug markedly potentiates cardiac sodium current to promote intracellular sodium loading, a condition associated with intracellular calcium overload and polymorphic ventricular tachycardia.
- These findings provide a unifying mechanistic basis for the novel form of proarrhythmia seen with azithromycin, which likely contributes to the increased risk of sudden cardiac death seen with this antibiotic.

proarrhythmic mechanism. Accordingly, we examined the electrophysiological effects of azithromycin in vivo and in vitro to investigate the molecular basis for this unusual form of drug-mediated proarrhythmia.

Methods

Reagents

Azithromycin was provided by Pfizer, Inc (Groton, CT) and dissolved in dimethyl sulfoxide to generate a 100 mmol/L stock solution (stored at \(-20^\circ\)C). The stock solution was serially diluted in bath solution to the final concentrations before each experiment. The drug was prepared for oral administration as described previously.7,22

Cell Preparations

The effects of azithromycin on the ionic currents under study were investigated using heterologously expressed human channels and cardiomyocytes. For cardiomyocyte studies, the species selected for experimentation was one that would optimize recording conditions for the specific current under study.

Human embryonic kidney (HEK 293) cells that stably expressed either human KCNH2 (hERG) or human SCN5A were kindly provided by Dr Craig January (University of Wisconsin) and Dr Alfred George (Northwestern University), respectively. A Chinese hamster ovary (CHO) cell line stably expressing KCNQ1 and KCNE1 to generate \( I_{\text{Ks}} \) currents was also provided by Dr George. The construct encoding the human Kir2.1 channel was kindly provided by Dr Antonin Lapoli, with transient transfection in HEK cells as reported previously.23,24 HEK293, CHO, and HL-1 cells were cultured as described.21–28

Isolation of rabbit ventricular myocytes was performed using the method of Bassani29 with minor modifications. Marine left ventricular myocytes were prepared from 10- to 12-week-old male mice as previously described.30 The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996).

Data Acquisition

Mouse ECGs

A DSI (Data Science International, St Paul, MN) telemetry system was used to monitor and collect ECG data from conscious, freely moving laboratory mice. C57BL/6 mice (age 10–12 weeks) were anesthetized using ketamine 100 µg/g and xylazine 10 µg/g injected IP to place a radio transmitter (EA-F20) in the abdominal cavity. The mouse ECG telemetry system contains 2 electrical ECG leads connected to a radio transmitter with subcutaneous electrodes in a lead I configuration. On activation of the transmitter by a magnet, the electrical signals were transmitted wirelessly to a nearby receiver (RPC-1) attached to an amplifier (MX2) and computer system for data acquisition (Ponemah v6.10; sampling frequency 2 KHz), storage, and analysis. Animals were allowed to recover for at least 5 days after surgery before experimentation. Each mouse served as its own control with resting ECG recorded for at least 15 to 30 minutes before any intervention. For IP administration, azithromycin 50 mg/kg was injected, followed by 100 mg/kg IP 1 hour later. ECG monitoring continued for at least 1 hour after the second injection. A separate group of mice were treated with oral azithromycin for 3 days, using a dose that was efficacious in treating infections.31 Baseline ECG was recorded, azithromycin 50 mg/kg was administered by oral gavage, and the ECG was recorded for 2 hours. This was repeated for 2 or 3 additional days.

Action Potentials

Spontaneous action potentials were recorded at 37°C from HL-1 cells as previously described (Data Supplement).32 Cells chosen for experimentation had a resting membrane potential of at least \(-55\) mV, overshoot exceeding 20 mV, regular rhythmicity, and a stable spontaneous cycle length for at least 5 minutes.
Ionic Currents
All currents were recorded at room temperature (22±1°C) in the whole-cell configuration with an Axopatch-200B amplifier (Molecular Devices, Sunnyvale, CA). Currents were low-pass filtered at 5 kHz and digitized with a DigiData 1320A. Capacitance and 80–95% series resistance were routinely compensated. Leak subtraction was completed using user-specified after-the-fact leakage correction of pCLAMP. Na+ current, rapidly activating (I_{Kr}) and slowly activating (I_{Ks}) delayed rectifier K+ currents, L- and T-type Ca2+ currents, and inward rectifier current (I_{K1}) were recorded as detailed in the Data Supplement. Pipettes were pulled (Sutter Instrument, Novato, CA) using Borosilicate glass having tip resistances of 0.5 to 1 MΩ.

Pulse Protocol and Solutions
These are described in the Data Supplement and illustrated in the representative figures.

Data Analysis
ECG Data
Mouse ECG waveforms over a 10-second period were averaged to obtain measurements of the RR, PR, QRS, QT, and QTc intervals and heart rate using LabChart (version 8.1.5; AD Instruments, Colorado Springs, CO) and are presented as mean±SEM. QTc was calculated using the normalized Bazett formula (QTc=QT/\[RR/100\]^{1/2}).

Action Potentials and Ionic Currents
Data were analyzed using Clampfit 10.4 software (Molecular Devices), Excel 2010 (Microsoft, Redmond, WA), and Origin 9.0 (OriginLab Corporation, Northampton, MA), with action potential measurements obtained as previously described (Data Supplement). Current amplitudes were normalized by cell capacitance and expressed as current density. All activation and inactivation curves were fitted with a single Boltzmann function as follows: y=A2+(A1−A2)/(1+exp((Vm−V_{1/2})/k)), where A1 is maximum current, A2 is the minimum current, V_{1/2} is the half-activation or half-inactivation potential (ie, half availability), Vm is test voltage, and k is slope factor. Fractional blockade was defined as follows: f=1−I_{drug}/I_{con}, where I_{con} and I_{drug} are current amplitudes in the absence and presence of azithromycin, respectively. Dose–response curves were fit by the equation: y=B_1+(B_2−B_1)/(1+10^{(logx_0−x)*p}), where B_1 is the bottom asymptote, B_2 is the top asymptote, logx_0 is IC_{50}, x is azithromycin concentration, and p is hill slope.

Whole-Exome Sequencing
After informed consent, DNA was extracted from peripheral blood leukocytes and protein-coding regions targeted using the Agilent SureSelect All Exon V3 capture kit. High-throughput DNA sequencing was then performed using the Illumina HiSeq 2500 instrument with the paired-end sequencing protocol generating just under 100 million short (75 mb) sequence read pairs. Raw sequence reads were aligned to the human reference (hg19) using the Burrows-Wheeler Aligner (0.6.9) an algorithm after hard trimming to 50 bp. Duplicates were flagged using the Picard suite of tools. Postprocessing of the aligned data was done using the (GATK v1.6–19) including local realignment and base quality recalibration. Variant detection was done using the UnifiedGenotyper from GATK and called collectively to a multisample variant call format file. Low-quality sites were flagged using the VariantFiltration Walker in agreement with the GATK BestPractices using filter thresholds Qual <50, QD <5, and AB >0.75.

mRNA Quantification
Real-time quantitative reverse transcription polymerase chain reaction was performed in HEK cells expressing SCN5A after 24-hour exposure to azithromycin or vehicle as described previously (Data Supplement).

Protein Quantification
Western analysis to quantify SCN5A protein in HEK cells was performed as described (Data Supplement). Both whole-cell lysate and biotinylated fraction were analyzed to quantify total cellular and plasma membrane–bound protein, respectively.

Azithromycin Plasma Concentrations
At 15 minutes after IP injection (when electrophysiological effects were maximal), animals were anesthetized with isofluorane, and blood was harvested by cardiac puncture. During oral administration, animals were euthanized at 90 minutes (the time to maximal plasma concentration) or at 24 hours (at trough or predose; n=4) after the last dose for measurement of azithromycin plasma concentration. Plasma was isolated for extraction and analysis of azithromycin concentration using liquid chromatography–tandem mass spectrometry as described.

Statistical Analysis
Data represented using a continuous variable were summarized using mean and SE for each group. For group comparisons of a repeated
measure, the nonparametric Wilcoxon signed-rank test was applied. For independent group comparisons, the nonparametric Wilcoxon rank-sum test was used. Exact $P$ values were calculated for these tests. For comparisons between multiple dose groups or time groups, $P$ values were adjusted using the Bonferroni correction method. For each parameter of mouse ECG data, a linear regression with robust SEs and consideration of mouse cluster analysis were also performed. The mean differences between dose groups and the confidence intervals were estimated. Two-sided nominal level of 0.05 was considered as statistically significant. Analyses were performed using R 3.2.4 (https://www.R-project.org/), Origin Pro 2016 (Origin Laboratory Corporation, Northampton MA 01060), and Microsoft Excel 2010.

**Results**

**Case Report**

The patient was a 24-year-old black female with no medical history who was treated for 5 days with azithromycin (Zithromycin Z-Pak) for an upper respiratory infection. She was taking no other medications or supplements. Within 24 hours after discontinuing azithromycin, she experienced recurrent episodes of syncope and presented to a local emergency department. Her ECG on admission was normal (Figure I in the Data Supplement). A rhythm strip obtained during a syncopal episode demonstrated rapid, polymorphic VT in the setting of a normal QT interval (Figure 1). She remained off azithromycin, and the VT resolved over 3 days. Cardiac magnetic resonance imaging was within normal limits. The patient received an implantable cardioverter-defibrillator before discharge. Whole-exome sequencing was performed, and there were no rare nonsynonymous variants identified in genes previously associated with inherited arrhythmia syndromes.

**Acute Electrophysiological Effects of Azithromycin in Conscious Mice**

As an initial step to investigate the electrophysiological effects of azithromycin in vivo, the drug was injected IP into conscious mice during continuous ECG monitoring. Within 15 minutes, azithromycin produced diffuse, dose-dependent depressant effects on cardiac automaticity and conduction (Figure 2; Table 1), with reversible slowing of heart rate (Figure II in the Data Supplement), and significant increases in the PR, QRS, QT, and QTc intervals (Table I in the Data Supplement). Similar findings were observed after oral administration, with sinus slowing and an increase in all ECG intervals.

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**Figure 2.** Effects of IP azithromycin (AZ) on the ECG of conscious adult mice. Mice received IP injection of AZ (50 mg/kg, followed in 60 min by 100 mg/kg). Representative ECG tracings (and interval measurements) are shown in the top, with parameter data for the group shown in the bottom ($n=7$). With both doses of AZ, heart rate declined, along with an increase in the PR, QRS, and QT/QTc intervals. $P<0.01$. 

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within 60 minutes of dosing (Figure III and Table II in the Data Supplement) and to a lesser extent 24 hours after dosing. These results are consistent with the effects of azithromycin overdose in humans, and they imply that acute administration of azithromycin blocks multiple depolarizing and repolarizing ionic currents in cardiomyocytes.

In separate groups of mice, plasma azithromycin concentrations were determined after IP injection (at 15 minutes) and oral dosing (at 90 minutes when plasma concentrations were maximal, and at 24 hours). Values were 0.09±0.03 and 0.26±0.4 μmol/L at 90 minutes and 24 hours post-dosing (n = 4 and 6, respectively). For oral administration, plasma concentrations were maximal,31 and at 24 hours). Values were 0.09±0.03 and 0.26±0.4 μmol/L at 90 minutes and 24 hours post-dosing (n = 4 and 6, respectively). For oral administration, plasma concentrations were maximal,31 and at 24 hours). Values were 0.09±0.03 and 0.26±0.4 μmol/L at 90 minutes and 24 hours post-dosing (n = 4 and 6, respectively). For comparison, peak plasma concentrations in humans are shown during chronic oral dosing with the 3 azithromycin regimens available commercially (Table III in the Data Supplement, from the product package insert). Thus, azithromycin concentrations resembling those seen clinically were achieved during these experiments. However, the depressant ECG effects seen in the mouse are not observed clinically with oral administration in humans, demonstrating substantial interspecies pharmacokinetic differences.

### Acute Effects of Azithromycin on Cardiomyocyte Action Potentials

To further confirm these effects for cardiomyocytes, we examined the effects of azithromycin on HL-1 cells, a cultured cardiac cell line that displays spontaneous automaticity. Acute exposure caused slowing of beat rate, a reduction in the phase 0 upstroke slope, and prolonged repolarization (Table 2; Figure IVA through IVC in the Data Supplement), consistent with the diffuse depressant effects observed in vivo.

### Reduction of SCN5A Currents With Acute Exposure

The QRS widening in vivo and reduced phase 0 upstroke in cardiomyocytes implied block of cardiac Na+ current by azithromycin. In HEK cells expressing human SCN5A channels, azithromycin caused rapid block of I Na with an IC 50 of ≈110 μmol/L, findings that were further confirmed in mouse ventricular myocytes (Figure 3A through 3D; Table 3). Suppression of Na+ current was accompanied by a depolarizing shift in the voltage dependence of channel activation and a hyperpolarizing shift in inactivation (Figure 3E and 3F; Table 3), changes that can account at least to some extent for the reduction in I Na observed.39

### Paradoxical Time-Dependent Increase in Peak I Na

Recent studies have highlighted the importance of time-dependent effects of electrophysiologically active agents on cardiac I Na.40-44 To determine whether such properties might also be present for azithromycin, we characterized Na+ current in cells chronically exposed to the drug (for 24 hours), compared with control (vehicle-exposed) cells. Interestingly, under these conditions, peak SCN5A current density was increased at all test potentials examined, with upregulation of ≈2-fold at −20 mV (Figure 4A and 4B; Table 4). As for acute exposure, the voltage dependence of channel gating was also modulated (Figure 4C and 4D; Table 4). However, in this case, these effects could not contribute to potentiation of cardiac I Na. These findings were replicated in HL-1 cardiomyocytes, with a substantial increase in

### Table 1. Summary Data of Azithromycin Effects on the ECG of Conscious Mice

<table>
<thead>
<tr>
<th>Exposure</th>
<th>Heart Rate, beats per min</th>
<th>PR, ms</th>
<th>QRS, ms</th>
<th>QT, ms</th>
<th>QTc, ms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>715±7</td>
<td>32.4±0.7</td>
<td>9.5±0.1</td>
<td>25.4±1.2</td>
<td>27.7±1.2</td>
</tr>
<tr>
<td>AZ 50 mg/kg</td>
<td>637±8†</td>
<td>35.2±0.9 *</td>
<td>12.7±0.2 *</td>
<td>31.6±0.6</td>
<td>32.5±0.7 †</td>
</tr>
<tr>
<td>AZ 100 mg/kg</td>
<td>526±7†</td>
<td>37.9±0.4 †</td>
<td>14.4±0.3 †</td>
<td>33.9±0.2</td>
<td>31.7±0.4 *</td>
</tr>
</tbody>
</table>

n = 7 mice. Wilcoxon signed-rank test, normalized Bazett: QTc = QT/(RR/100) 1/2.

AZ indicates azithromycin.

*P < 0.05.
†P < 0.01.

### Table 2. Effects of AZ on Action Potentials

<table>
<thead>
<tr>
<th>Exposure</th>
<th>CL, ms</th>
<th>Peak, mV</th>
<th>MDP, mV</th>
<th>DDT, ms</th>
<th>Phase 0 Slope, mV/ms</th>
<th>APD 90, ms</th>
<th>APD 40, ms</th>
<th>Beat Rate, beats per min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Mean SE</td>
<td>308.3±2.9</td>
<td>107.7±1.5</td>
<td>−63.8±0.8</td>
<td>19.6±1.1</td>
<td>2.45±0.13</td>
<td>288.6±2.8</td>
<td>277.0±2.8</td>
</tr>
<tr>
<td>AZ 100 μmol/L</td>
<td>Mean SE</td>
<td>353.7†±3.7</td>
<td>89.2†±1.8</td>
<td>−59.1†±1.0</td>
<td>39.6*±2.4</td>
<td>1.18*±0.05</td>
<td>314.1†±4.1</td>
<td>299.9†±4.1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Exposure</th>
<th>CL, ms</th>
<th>Peak, mV</th>
<th>MDP, mV</th>
<th>DDT, ms</th>
<th>Phase 0 Slope, mV/ms</th>
<th>APD 90, ms</th>
<th>APD 40, ms</th>
<th>Beat Rate, beats per min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Mean SE</td>
<td>291.1±8.9</td>
<td>101.0±2.1</td>
<td>−63.2±0.5</td>
<td>18.8±0.8</td>
<td>2.5±0.1</td>
<td>272.3±8.3</td>
<td>256.4±8.1</td>
</tr>
<tr>
<td>AZ 100 μmol/L for 24 h</td>
<td>Mean SE</td>
<td>216.7†±13.9</td>
<td>99.5±1.2</td>
<td>−60.6±0.3</td>
<td>15.1*±1.2</td>
<td>4.0*±0.6</td>
<td>201.6†±12.9</td>
<td>185.5†±12.8</td>
</tr>
</tbody>
</table>

Wilcoxon signed-rank test. APD 90 indicates action potential duration at 90% of repolarization; APD 40, action potential duration at 100% of repolarization; AZ, azithromycin; CL, cycle length; DDT, diastolic depolarization time; and MDP, maximum diastolic potential.

*P < 0.05.
†P < 0.01.
Yang et al  Proarrhythmic Effects of Azithromycin

peak Na⁺ current and similar gating shifts on chronic azithromycin exposure (Table 4; Figure V in the Data Supplement). Consistent with this effect, the phase 0 upstroke slope was increased in HL-1 cardiomyocyte action potentials under similar conditions (Table 2; Figure IVD and IVE in the Data Supplement).

To explore the mechanism of azithromycin-mediated potentiation of Na⁺ current, experiments were performed using real-time quantitative reverse transcription polymerase chain reaction and Western analysis to determine whether the drug altered the production of SCN5A mRNA, total cellular channel protein, or protein at the cell surface (using biotinylation). However, azithromycin had no effect on any of these properties for SCN5A (Figure VI in the Data Supplement).

**Time-Dependent Increase in Late I_{Na}**

Rarely, azithromycin causes drug-induced long-QT syndrome. One potential mechanism is an increase in the Na⁺ window current, defined by overlap of the activation and inactivation curves, or a window of potentials over which a fraction of Na⁺ channels are activated but not inactivated. However,
Azithromycin caused either no change or a reduction, rather than an increase, in the window current with acute and chronic exposure (Figure VII in the Data Supplement).

Because an increase in late Na+ current can also prolong repolarization, we investigated the effects of azithromycin on this parameter using TTX subtraction in HEK cells expressing SCN5A (Figure 5A). Acute exposure to azithromycin had minimal effect on amplitude of late I_{Na} (Figure 5B; Table 3); however, chronic exposure for 24 hours caused doubling of the late Na+ current (Figure 5A and 5B; Table 4). Potentiation of late I_{Na} was greater than the increase in peak I_{Na}, illustrated by an increase in the ratio of these parameters (Figure VIII in the Data Supplement). The effects of chronic azithromycin exposure to increase both peak and late I_{Na} occurred at lower concentrations (IC_{50}=40.9 and 62.2 μmol/L, respectively; Figure 5C and 5D) than those causing acute I_{Na} block (IC_{50}=110–116 μmol/L; Figure 3) implying greater clinical relevance during therapeutic dosing.

### Minimal Effects on the Rapidly and Slowly Activating Delayed Rectifier K+ Currents

Most drugs prolong cardiac repolarization by blocking the rapidly-activating delayed rectifier K+ current, I_{Kr}. In HEK cells expressing hERG, K+ currents displayed only mild block by azithromycin, with an IC_{50} of 219±21 μmol/L (Figure 6; Table 5). Similarly, there was minimal effect of the drug on the slowly activating delayed rectifier, I_{Ks} (Figure 7; Table 5; IC_{50}=184±12 μmol/L). Given that these concentrations are 3- to 4-fold higher than that for potentiation of late I_{Na}, azithromycin-mediated QT prolongation most likely results from increased late Na+ current, rather than K+ channel block.

### Table 3. Acute Effects of AZ on SCN5A Currents

<table>
<thead>
<tr>
<th></th>
<th>Peak I_{Na} (n=14–18)</th>
<th>Activation (n=14–32)</th>
<th>Inactivation (n=14–32)</th>
<th>Late I_{Na} (n=12–16)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pA/pF</td>
<td>V_{1/2} mV</td>
<td>k</td>
<td>V_{1/2} mV</td>
</tr>
<tr>
<td>CTL</td>
<td>−967±54</td>
<td>−44.6±1</td>
<td>5.0±0.5</td>
<td>−88.6±0.6</td>
</tr>
<tr>
<td>AZ 50 μmol/L</td>
<td>−769±49</td>
<td>−41.1±1</td>
<td>5.3±0.3</td>
<td>−94.2±0.6</td>
</tr>
<tr>
<td>AZ 100 μmol/L</td>
<td>−512±38†</td>
<td>−34.5±1†</td>
<td>5.6±0.3</td>
<td>−100.8±0.6†</td>
</tr>
</tbody>
</table>

Wilcoxon signed-rank test. AZ indicates azithromycin; CTL, control; and k, slope factor. †P<0.01.
Block of Cardiac Calcium Currents and $I_{K1}$

Because azithromycin slows sinus node automaticity and prolongs atrioventricular nodal conduction in vivo, we tested the hypothesis that the drug blocks cardiac $Ca^{++}$ currents. Using rabbit ventricular myocytes, nimodipine-sensitive L-type $Ca^{++}$ currents were suppressed by azithromycin ($IC_{50}=67±4\,\mu mol/L$; Figure 8A and 8B; Table 5), while mibefradil-sensitive T-type $Ca^{++}$ currents were minimally affected ($IC_{50}$ not determined; Figure 8C and 8D; Table 5).

Azithromycin increased the maximal diastolic potential in HL-1 cells during both acute and chronic exposure (Table 2), implying an effect on the inward rectifier current $I_{K1}$. Indeed, for HEK cells expressing Kir2.1 and for HL-1 cells, azithromycin suppressed Kir and $I_{K1}$ currents, respectively ($IC_{50}=43.8\,\mu mol/L$ for Kir2.1 currents; Table 5; Figure IX in the Data Supplement).

### Discussion

Azithromycin is a widely prescribed macrolide antibiotic that has been linked to an increased risk of sudden cardiac death and cardiovascular death. In contrast to other macrolides, torsades de pointes has been rarely reported for azithromycin. Thus, the cause of these adverse events has remained poorly defined, prompting the present investigation. As demonstrated by the case presented here, azithromycin can cause a unique, drug-mediated proarrhythmic syndrome characterized by...
by rapid, polymorphic VT in the absence of QT prolongation or demonstrable cardiac structural abnormalities. We found that chronic drug exposure markedly potentiates cardiac Na+ current, which would increase intracellular [Na+] and subsequently promote dysregulation of intracellular [Ca++]1, providing a unifying mechanism for this unusual syndrome that likely contributes to azithromycin-mediated sudden cardiac death.

It is well recognized that conditions associated with intracellular Na+ loading, most notably digoxin therapy and ischemia, alter intracellular Ca++ homeostasis via the Na+-Ca++ exchanger activity, with removal of Na+ in exchange for Ca++, leading to delayed afterdepolarizations and triggered arrhythmias.22 Abnormal intracellular Ca++ homeostasis due to sarcoplasmic reticulum Ca++ leak is the hallmark of catecholaminergic polymorphic VT, the clinical syndrome that most closely resembles this type of azithromycin proarrhythmia. Recent evidence further supporting the arrhythmogenic effects of cellular Na+ loading derives from a transgenic mouse model in which peak Na+ current was increased.42 Mutations in Scn1b, encoding the Na+ channel β1 subunit, have been linked to inherited arrhythmia syndromes, including sudden infant death syndrome and unexplained death in epilepsy.43,44 Mice with cardiac-specific deletion of Scn1b demonstrated enhanced peak INa, delayed afterdepolarizations, and polymorphic VT without APD prolongation, which was responsive to treatment with tetrodotoxin. Moreover, catecholaminergic polymorphic VT was recently linked to a gain-of-function mutation in Scn5a that increased Na+ influx by augmenting the Na+ channel window current.45 Along with other studies,46,47 these data provide compelling evidence that a generalized increase in cardiac Na+...
The current is arrhythmogenic by dysregulating intracellular Ca++, with clinical features exemplified by catecholaminergic polymorphic VT that mimic the unusual form of drug-mediated proarrhythmia described here.

Azithromycin can cause life-threatening arrhythmias by at least 2 other mechanisms, although these seem to be rare. First, cases of drug-induced long-QT syndrome have been reported, but as noted above, documented torsades is unusual. Based on our results, potentiation of late INa by azithromycin appears to be responsible for this adverse effect, occurring at much lower concentrations than those required for K+ channel block. Drug-induced long-QT syndrome is less common with azithromycin than with other drugs that also increase late INa, such as dofetilide. This is likely because many of the compounds that increase late Na+ current also block I Kr as well, and this is minimal with azithromycin. We speculate that concomitant block of cardiac Ca++ current by azithromycin might also provide an additional potential protective mechanism to prevent early afterdepolarizations and torsades.

Second, acute intravenous exposure (in one case causing overdose) can cause wide-spread depression of cardiac conduction, resulting in marked sinus bradycardia, slowing of AV nodal and infranodal conduction with heart block, and prolonged ventricular repolarization. For conscious mice and cardiomyocytes, we found that acute exposure to azithromycin had similar effects, with in vitro evidence of multi-ion channel block, providing a mechanistic basis for the electrophysiological effects of acute toxicity. Although these toxic effects have not been observed during oral dosing of azithromycin, we cannot rule out the participation of multi-ion channel block to drug-mediated sudden cardiac death. This is particularly true for acute Na+ channel block, as well as block of I Kr, which would elevate the myocyte resting potential leading to both enhanced automaticity and additional Na+ channel inactivation.

An important consideration is the range of azithromycin concentrations that we studied. Several oral preparations of azithromycin have been marketed, and peak plasma concentrations during oral dosing range from ≈0.4 to 1.1 μmol/L (Table III in the Data Supplement). However, plasma concentrations are misleading, as the drug accumulates within cells, achieving concentrations approaching 900 μmol/L in leukocytes and pulmonary tissue (Table III in the Data Supplement). A previous study by the pharmaceutical sponsor reported similar accumulation of the drug in cardiac cells for mice receiving oral azithromycin (200 mg·kg⁻¹·d⁻¹ for 10 days), with ≈30- to 220-fold increase in concentration compared with plasma. On the basis of these data and known azithromycin plasma concentrations in patients, we investigated azithromycin concentrations of 0.1 to 1000 μmol/L, to account for intracellular accumulation. Interestingly, amitriptyline also accumulates in...
cardiomyocytes. Intracellular concentrations were ≈5-fold higher than extracellular concentrations, explaining the drug’s higher potency in cardiomyocytes compared with RyR2 channels incorporated into lipid bilayers.

The mechanism for azithromycin-mediated potentiation of cardiac Na+ current remains unclear, as we found no evidence for increased production of SCN5A mRNA or total cellular protein, and there was no evidence for enhanced trafficking of channel protein to the cell surface. The alterations in channel gating, as well as the more selective increase in late INa, also argue against a nonspecific or generalized effect to increase Na+ channels at the membrane. We postulate that the increase in INa is mediated by effects that alter post-translational modification and protein–protein interactions, which could be complex.

In conclusion, chronic exposure to azithromycin markedly potentiates cardiac Na+ current to promote intracellular Na+ loading, providing a unifying mechanistic basis for the distinct type of proarrhythmia seen with this drug. These findings imply that preclinical screening of new compounds should include examination of their pharmacological effects on cardiac INa amplitude.

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Disclosures
None.

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12 Yang et al. Proarrhythmic Effects of Azithromycin


Azithromycin Causes a Novel Proarrhythmic Syndrome


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SUPPLEMENTAL MATERIAL

DETAILED METHODS

Data Acquisition

Mouse ECGs. Methods using a telemetry system to monitor and collect ECG data from conscious, freely moving laboratory mice have been described previously.¹

Action Potentials. Spontaneous action potentials were recorded at 37°C from HL-1 cells as previously described.² The pipettes were filled with an internal solution containing (in mM): 120 K-aspartate, 25 KCl, 1 MgCl₂, 5 HEPES, 10 EGTA, 2 Na₂phosphocreatine, 4 Na₂ATP, 2 NaGTP, pH 7.2 (adjusted with 1M KOH). The bath solution was Tyrode’s with: 145 NaCl, 4 KCl, 1 MgCl₂, 1.8 CaCl₂, 10 HEPES, pH 7.4 (adjusted with 1M NaOH).

Ionic currents. Na⁺ current, rapidly (Iₖᵣ) and slowly-activating (Iₖₛ) delayed rectifier K⁺ currents, L- and T-type Ca²⁺ currents, and inward rectifier current (Iₖᵢ) were recorded as described previously.³ ⁴.⁵.⁶.⁷

Recording Iₙa. To record Na⁺ currents, the holding potential was -120mV. The voltage dependence of channel activation was measured using 200ms test pulses from -90mV to +80mV in 10 mV increments. To assay the voltage-dependence of steady-state inactivation, the membrane potential was stepped from -160mV to 0mV in 10mV increments (200ms pulses) using a holding potential of -100mV, followed by 40ms test pulse to -20mV. Late Iₙa was averaged in a 3ms window (195-198ms after the pulse) before the capacity transient at the end of a 200ms depolarizing pulse at -20 mV and was quantified by subtraction of the current remaining after application of 30μM tetrodotoxin from the total current. For all preparations, the pipette solution contained the following: 10 NaF, 110 CsF, 20 CsCl, 10 EGTA, and 10 HEPES, pH 7.2
SCN5A currents in HEK 293 cells. The bath solution contained the following: 145 NaCl, 4 KCl, 1.8 CaCl₂, 1 MgCl₂, and HEPES, pH 7.4 (adjusted with 1M NaOH).

INa in mouse ventricular myocytes and HL-1 cells. The bath solution contained: 20 NaCl, 1 MgCl₂, 110 CsCl, 5TEA-Cl, 0.1 CaCl₂, 2µM nimodipine, 200µM NiCl, HEPES and 10 glucose, pH 7.4 (adjusted with 1M NaOH).

Recording INa and INa. To record K⁺ currents, the cycle time for pulse protocols was 15s. For INa, the holding potential was -90mV, and test potentials (2sec) were stepped from -80mV to +70mV, with tail currents recorded upon repolarization to -50mV for 2sec. For INa, the holding potential was -80mV, and test potentials (2sec) were stepped from -80mV to +90mV, with tail currents recorded upon repolarization to -50mV for 2sec. Patch electrodes were filled with: K-DL-aspartate 120; KCl 25; MgCl₂ 1; EGTA 10; Na₂ phosphocreatine 2; Na₂ATP 4; NaGTP 2 and HEPES 5, pH 7.2 (adjusted with 1M KOH). HEK 293 cells were superfused with Tyrode’s solution containing: 145 NaCl, 4KCl, 1 MgCl₂, 1.8 CaCl₂ and 10 HEPES, pH 7.4 (adjusted with 1M NaOH).

Recording Ca⁺⁺ Currents. For recording ICa,L, a holding potential of -40mV was used, and the cycle time for pulse protocols was 5sec. Voltage was sequentially stepped for 200ms from -30mV to +80mV in 10mV increments. To record ICa,T, a holding potential of -90mV cells was used, with 200ms voltage steps from -80mV to +70mV (10mV increments). The pipette solution consisted of: 120 CsCl, 10EGTA-CsOH, 10 tetraethylammonium chloride (TEA-Cl), 1 Mg₂ATP, 5 phosphocreatine, 1 NaGTP, and 10 HEPES, pH 7.2 (adjusted with 1M CsOH). The bath solution contained: 140 TEA-Cl, 2 MgCl₂, 5CaCl₂, 10 HEPES, 10 glucose, pH 7.4 (adjusted with 1M CsOH).
**Recording I_{K1}.** For recording I_{K1}, a holding potential of -30mV was used, and the cycle time for pulse protocols was 5sec. Voltage was sequentially stepped for 150ms from -130mV to 0mV in 10mV increments. In HL-1 cells, I_{K1} was isolated by subtraction of the current remaining after application of 500 μM BaCl₂ from the total current. In HEK cells expressing Kir2.1, or HL-1 cells, the pipette solution consisted of: 140 KCl, 5 K₂ATP, 1 EGTA, and 10 HEPES, pH 7.2 (adjusted with 1M KOH). The bath solution was Tyrode’s with: 137 NaCl, 5.4 KCl, 0.5 MgCl₂, 0.3 CaCl₂, 0.16 NaH₂PO₄, 3 NaHCO₃, 5 HEPES, 5 glucose, pH 7.4 (adjusted with 1M NaOH).

**mRNA Quantification**

Total RNA was isolated from HEK cells expressing SCN5A after 24-hour exposure to azithromycin or vehicle using the RNAeasy Mini Kit with DNAse treatment (Qiagen). cDNA was synthesized from 2 μg of the RNA by use of the SuperScriptIII First Strand cDNA Synthesis System for RT-PCR with random hexamer primers (Life Technologies) and used as a template. To generate a standard curve for absolute quantification, SCN5A cDNA was PCR amplified and subcloned into the pGEM-T vector (Clontech, Mountain View, CA). cDNA and 5 different dilutions of the SCN5A vector were prepared with predesigned 6-carboxyfluorescein-labeled fluorogenic TaqMan probe and primers (Applied Biosystems) for SCN5A (Hs00165693.m1) or RPL19 (Hs02338565.qh) in triplicate in the same 94-well plate for real-time polymerase chain reaction amplification. Real-time quantitative RT-PCR was conducted with a 7900HT Real-Time Instrument (Applied Biosystems). Data were collected with instrument spectral compensation and analyzed by use of absolute quantification and a standard curve with SDS 2.4 software (Applied Biosystems). Each value was normalized to that for RPL19.
**Western Analysis**

Whole-cell lysates were prepared from HEK cells expressing SCN5A following 24-hour exposure to azithromycin or vehicle. Lysates were centrifuged at 10,000g for 5min, and protein content was analyzed with a bicinchoninic acid assay (Pierce Biochemicals, Rockford, IL). Proteins (20 μg) were separated by running the sample on a NuPage 4-12% Bis-Tris gel (Life Technologies, Carlsbad, CA). The protein was transferred to PVDF membranes (GE Healthcare Life Sciences, 0.45 um pore), blocked for 1hr with 10% nonfat dry milk in Tris-buffered saline with Tween 20 (TBST) buffer at room temperature and then incubated overnight with antibodies targeting SCN5A (anti-pan NaV rabbit polyclonal antibody, 1:250; Alomone Labs, Israel), GAPDH (mouse monoclonal antibody, 1:4000, Invitrogen, Rockford, IL), calnexin (rabbit polyclonal antibody, 1:1000, Stressgen Bioreagents, Belgium), or the Na^+-K^+ ATPase α-1 subunit (mouse monoclonal antibody [a6F-s], 1:1000, Developmental Studies Hybridoma Bank) in TBST with 5% nonfat dry milk at 4°C. Membranes were washed 4 times with TBST for 10min each and incubated with secondary HRP-conjugated antibodies sheep anti-mouse (1:5000, Amersham, Piscataway, NJ) and goat anti-rabbit (1:10000, Jackson Immunoresearch, West Grove, PA) in TBST buffer with 3% nonfat dry milk at room temperature for 1hr. The blots were then washed 4 times for 10min each in TBST, and antibody interactions were detected with the ECL substrate (Pierce, Rockford, IL).

**Cell Surface Biotinylation**

HEK cells expressing SCN5A were washed with ice cold phosphate-buffered saline (PBS), and then treated with PBS containing 1.5 mg/ml sulfo-NHS-LC-biotin (Pierce) at 4 °C for
1 hr. Excess biotin was inactivated and removed by a 20 min incubation in 10 mM glycine/PBS at 4°C and three washes in PBS. The cells were lysed in lysis buffer containing (in mM) 10 Tris (pH 7.3), 150 NaCl, 1 EDTA, 1% Triton X-100, and 1% sodium deoxycholate and a mixture of mammalian protease inhibitors (Roche). Cell lysates were spun at 13,000 × g for 20 min, and the supernatants were incubated with streptavidin-coated beads (Pierce) for 1 hr at room temperature. Beads were washed four times with lysis buffer and resuspended in 1× SDS-PAGE loading buffer, boiled, and analyzed by Western blot analysis.
Figure 1: ECG of the patient on admission. Episodes of polymorphic ventricular tachycardia (Figure 1 in the main paper) were recorded the following day.
Figure 2: Reversible effects on IP azithromycin on sinus node automaticity/heart rate in a conscious telemetered mouse.
(Figure 3, continued on the next page)
**Figure 3: Effects of oral azithromycin (AZ) on the electrocardiogram of conscious adult mice.** Mice received azithromycin (50 mg/kg/day) by oral gavage for 3 days, with baseline recording followed by additional recording for 2h post dose. To obtain samples to measure peak plasma concentrations, 4 mice were dosed on day 4. Representative measurements of heart rate are shown in the upper panel, with parameter data for the group shown below (n=10). With each dose of AZ, heart rate declined, along with an increase in the PR, QRS, and QT/QTc intervals (see Supplemental Table 2; *P<0.05), with small but progressive effects on trough or pre-dose values. Each mouse is represented by one color in all panels.
Figure 4: Effects on HL-1 cell automaticity. Compared to control conditions (A), acute exposure of HL-1 cells to AZ (100μM; 25 min) significantly slowed spontaneous beat rate (B). (C) Summary data for the time course of automaticity slowing is shown. D and E. Similar data are shown before (D) and after (E) a 24hr exposure to azithromycin.
Figure 5: Increased peak Na$^+$ current in cardiomyocytes with chronic AZ exposure. A and B. In HL-1 cells, incubation with AZ (50µM) for 24 hr significantly increased I$_{\text{Na}}$ (from -81±19 pA/pF to -135±22 pA/pF at -20 mV; †P<0.01). C and D. In the same preparation, similar shifts were observed in the steady-state inactivation and activation curves, as those seen with acute exposure (for inactivation, -93.8±1.3 mV to -98.6±1.4 mV for control and AZ 50µM, respectively; for activation, -49.8±1 mV to -47.6±1 mV; *P<0.05 for both).
Figure 6: Lack of effect of chronic azithromycin exposure on mRNA, total cellular protein, and plasma membrane protein for SCN5A. Compared to control conditions, a 24hr exposure of HEK cells expressing SCN5A did not significantly change levels of mRNA (top panel), total cellular protein (middle panel), or SCN5A protein at the cell surface (lower panel, n=6).
Figure 7: Minimal effects of acute and a 24hr exposure to azithromycin on SCN5A window currents. A. Compared to control conditions, acute exposure of HEK cells expressing SCN5A to AZ (50µM; 25 min) had no effect on the area encompassed by overlap between activation and inactivation curves. B. With a 24hr exposure, azithromycin caused a small decrease in the SCN5A window current.
Figure 8: Effects of azithromycin following acute or 24hr exposure on the ratio of late $I_{\text{Na}}$/peak $I_{\text{Na}}$ for SCN5A currents. For acute exposure, peak $I_{\text{Na}}$ declined causing the ratio to increase, while for chronic exposure, the increase in late $I_{\text{Na}}$ exceeded that for peak Na$^+$ current. \[†P<0.01\]
Figure 9: Effects of azithromycin on Kir2.1 currents in HEK cells and I_{K1} in HL-1 cell cardiomyocytes. A, B, and C. Acute exposure to azithromycin suppressed both I_{K1} currents in HL-1 cells (A and B), as well as K^+ currents derived from expression of human Kir2.1 in HEK cells (C). The IC_{50} for suppression of Kir2.1 currents was 43.8±3.2μM (D). *P<0.05; †P<0.01
Table 1: Estimated mean ECG parameter differences between treatment and baseline and their confidence intervals using linear regressions

<table>
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<tr>
<th>Parameter</th>
<th>n</th>
<th>Estimated difference</th>
<th>Lower 95%</th>
<th>Upper 95%</th>
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<tr>
<td>Heart rate</td>
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<td>12.4</td>
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<td>&lt;0.001</td>
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<td>5.5</td>
<td>3.7</td>
<td>7.3</td>
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<td>QRS interval</td>
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<td>3.2</td>
<td>2.8</td>
<td>3.6</td>
<td>&lt;0.001</td>
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<td></td>
<td>4.9</td>
<td>4.3</td>
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<td>&lt;0.001</td>
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<td></td>
<td></td>
<td>30.2</td>
<td>27.7</td>
<td>32.7</td>
<td>&lt;0.001</td>
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<tr>
<td>QT interval</td>
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<td>6.2</td>
<td>4.6</td>
<td>7.8</td>
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<td></td>
<td></td>
<td>8.5</td>
<td>6.4</td>
<td>10.6</td>
<td>&lt;0.001</td>
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<tr>
<td>QTc interval</td>
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<td>15.1</td>
<td>8.9</td>
<td>21.2</td>
<td>&lt;0.001</td>
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<tr>
<td></td>
<td></td>
<td>12.8</td>
<td>5.7</td>
<td>19.9</td>
<td>0.001</td>
</tr>
</tbody>
</table>

The first line is for the comparison between 50mg/kg and baseline. The second line is for the comparison between 100mg/kg and baseline.
<table>
<thead>
<tr>
<th>Day</th>
<th>Treatment</th>
<th>Heart rate (bpm)</th>
<th>PR (ms)</th>
<th>QRS (ms)</th>
<th>QT (ms)</th>
<th>QTc (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>Control</td>
<td>720±16</td>
<td>32.7±0.7</td>
<td>9.4±0.1</td>
<td>25.9±0.3</td>
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<tr>
<td></td>
<td>n=10 AZ 50mg/kg</td>
<td>503±28*</td>
<td>37.0±0.8*</td>
<td>13.7±0.8*</td>
<td>31.9±0.4*</td>
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</tr>
<tr>
<td>Day 2</td>
<td>Control</td>
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<td>9.8±0.1</td>
<td>26.7±0.4</td>
<td>27.7±0.5</td>
</tr>
<tr>
<td></td>
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<td>524±19*</td>
<td>37.5±0.6*</td>
<td>15.0±0.4*</td>
<td>32.0±0.3*</td>
<td>32.2±0.5*</td>
</tr>
<tr>
<td>Day 3</td>
<td>Control</td>
<td>592±21</td>
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<td>10.2±0.3</td>
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<tr>
<td></td>
<td>n=10 AZ 50mg/kg</td>
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<td>37.3±0.5*</td>
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<td>34.6±0.4*</td>
<td>33.4±0.8*</td>
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<td>33.5±0.5</td>
<td>34.5±0.8</td>
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QTc = QT/(RR/100)^{1/2}, *adjusted P<0.05
Table 3: Serum and cellular concentrations of azithromycin in humans (Zmax package insert)

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<th>5-day</th>
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<td>Daily dose (mg/kg)</td>
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<td>7.14</td>
<td>3.6 (last 4 days)</td>
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<tr>
<td>$C_{\text{max}}$ (µg/ml) (µM)</td>
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<td>0.44</td>
<td>0.43</td>
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<tr>
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<td></td>
<td>178</td>
<td></td>
</tr>
<tr>
<td>Concentration in WBC/MN (µM)</td>
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<td></td>
<td>141</td>
<td></td>
</tr>
<tr>
<td>Concentration in lung/alveolar cell (µM)</td>
<td>669</td>
<td></td>
<td>815</td>
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</tr>
</tbody>
</table>

$C_{\text{max}}$ is maximal concentration; WBC/PMN is polymorphonuclear leukocytes; WBC/MN is monocyte white blood cells.
REFERENCES


