Azithromycin Kills Invasive Aggregatibacter actinomycetemcomitans in Gingival Epithelial Cells

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Abstract

Aggregatibacter actinomycetemcomitans invades periodontal pocket epithelium and is therefore difficult to eliminate by periodontal scaling and root planing. It is susceptible to azithromycin, which is taken up by many types of mammalian cells. This led us to hypothesize that azithromycin accumulation by gingival epithelium could enhance the killing of intra-epithelial A. actinomycetemcomitans. [3H]-azithromycin transport by Smulow-Glickman gingival epithelial cells and SCC-25 oral epithelial cells was characterized. To test our hypothesis, cultured Smulow-Glickman cell monolayers were infected with A. actinomycetemcomitans (Y4 or SUNY 465 strains) for 2 hours, treated with gentamicin to eliminate extracellular bacteria, then incubated with azithromycin for 1 to 4 hours. Viable intracellular bacteria were released, plated and enumerated. Azithromycin transport by both cell lines exhibited Michaelis-Menten kinetics and was competitively inhibited by L-carnitine and several other organic cations. Cell incubation in medium containing 5 µg/ml azithromycin yielded steady-state intracellular concentrations of 144 µg/ml in SCC-25 cells and 118 µg/ml in Smulow-Glickman cells. Azithromycin induced dose- and time-dependent intra-epithelial killing of both A. actinomycetemcomitans strains. Treatment of infected Smulow-Glickman cells with 0.125µg/ml azithromycin killed approximately 29% of intra-epithelial CFU from both strains within 4 hours, while treatment with 8µg/ml azithromycin killed ≥ 82% of CFU from both strains (P < 0.05). Addition of carnitine inhibited killing of intracellular bacteria by azithromycin (P < 0.05). Thus, human gingival epithelial cells actively accumulate azithromycin through a transport system that facilitates killing of intra-epithelial A. actinomycetemcomitans and is shared with organic cations.
Introduction

Periodontitis is the result of infection by a specific group of subgingival bacteria. These pathogens induce host immunological and inflammatory responses in periodontal tissues, leading to destruction of connective tissues and alveolar bone (1). While nonsurgical periodontal treatment can usually eliminate most periodontal pathogens and arrest periodontal attachment loss, invasive pathogens like Aggregatibacter actinomycetemcomitans and Porphyromonas gingivalis are difficult to eliminate by conventional therapy (2, 3). If efforts to eliminate these bacteria are unsuccessful, they can multiply within gingival crevicular epithelial cells and re-colonize adjacent periodontal pockets.

A. actinomycetemcomitans is a Gram-negative facultative rod that possesses several virulence factors that can overwhelm the host defense. In addition to its ability to invade epithelial cells (4), A. actinomycetemcomitans resists phagocytic killing (5) and produces a leukotoxin that kills polymorphonuclear lymphocytes (PMNs) (6). Previous studies have shown that extra-crevicular reservoirs for A. actinomycetemcomitans exist and may contribute to recurrent or refractory diseases in some subjects (7, 8). Thus, it is rational to use a systemic antibiotic as an adjunct to nonsurgical periodontal treatment to facilitate elimination of pathogens from subgingival and extra-crevicular niches and enhance the response to therapy.

Azithromycin (AZM), a derivative of erythromycin, is effective against A. actinomycetemcomitans (9) and possesses a long half-life. In addition, AZM produces anti-inflammatory effects by inhibiting nuclear factor-kappa B in oral epithelium (10) and by reducing levels of pro-inflammatory cytokines in gingival crevicular fluid (11). Several clinical trials have demonstrated promising clinical and microbiological benefits of AZM in treating periodontal diseases (12-14). Unlike beta-lactam antibiotics, AZM is concentrated inside human
PMNs and fibroblasts (15, 16). Clarithromycin (CLR), a closely related macrolide, is also actively transported and accumulated by human oral epithelial cells, gingival fibroblasts and PMNs (17, 18). PMNs that have taken up CLR exhibit enhanced phagocytic killing of intracellular *A. actinomycetemcomitans* (18).

Although the mechanism by which AZM is taken up by epithelium is unclear, it is feasible that intracellular accumulation of AZM could be useful in eradicating invasive bacteria from gingival epithelial cells. AZM and other weak organic bases can potentially interact with organic cation transporters or organic anion transporting polypeptides, which have relatively broad substrate specificity. To the extent this occurs, substrates of these transport systems could competitively inhibit AZM transport. In the present study, we characterized AZM transport by two different cultured oral epithelial cell lines and utilized an *in vitro* model of epithelial invasion to examine the effect of intracellular AZM accumulation on elimination of two different strains of *A. actinomycetemcomitans* from cultured gingival epithelium.

**Materials and Methods**

**Epithelial cell culture:** Smulow-Glickman (SG) gingival epithelial cells, originally derived from human attached gingiva (19), and SCC-25 epithelial cells (CRL-1628, ATCC, Manassas, VA), derived from oral epidermoid carcinoma (20), were used in this study. SG cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen Corp., Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (Atlanta Biologicals, Norcross, GA) while SCC-25 were grown in 50% DMEM/50% Ham’s F12 medium (Invitrogen Corp) containing 10% heat-inactivated fetal bovine serum and 0.4 μg/ml hydrocortisone. Both cell
lines were fed every 3 days and cultured to confluent monolayers at 37°C in the presence of 5% CO₂ in separate 24-well tissue culture plates.

**Bacterial culture:** Pure cultures of *A. actinomycetemcomitans* strains SUNY 465 (clinical isolate) and Y4 (ATCC 43718) were grown in brain heart infusion broth (Becton, Dickinson and Company, Sparks, MD) at 37°C in an environment containing 10% CO₂.

**Assay of AZM transport:** Confluent SG and SCC-25 cell monolayers were washed with Hank’s Balanced Salts Solution (HBSS, Invitrogen Corp.), harvested by brief treatment with 0.25% Trypsin-EDTA (Invitrogen Corp.), and suspended in HBSS at a density of 10⁶ cells/ml. AZM transport was assayed as previously described by measuring changes in cell-associated radioactivity over time (17, 21). Aliquots of cell suspension were incubated at 37°C with [³H]-AZM (American Radiolabeled Chemicals, St. Louis, MO) at concentrations of 10 µg/ml for time-course assays and 10-50 µg/ml in kinetic assays to determine the Michaelis constant (Kₘ) and maximal velocity of transport (Vₘₐₓ). After the indicated interval (1-20 min for uptake time-course and 3 min for kinetic assays), 0.5-mL aliquots of cell suspension were rapidly withdrawn, layered over 0.3 ml of a mixture of canola oil/dibutylphalate (3:10), and centrifuged for 30 sec at 15,000 x g in a microcentrifuge (22). After removal of the aqueous and oil layers, the cell pellets were recovered, lysed by agitation in 1 ml of sterile water and subjected to liquid scintillation counting.

The intracellular volume associated with identical cell suspensions was measured by incubation with [³H]-water (5 µCi/mL, NEN Life Science Products, Boston, MA) for 20 min at 37°C. Volume determinations were corrected for extracellular water trapped in the pellet, which was determined by incubation under the same conditions with [¹⁴C]-inulin (1µCi/mL, PerkinElmer, Waltham, MA) (23).
Several organic cations and organic acids were examined to assess their potential to inhibit AZM transport. All were purchased from Sigma Chemical Company (St. Louis, MO). Individual agents were added to 0.5-ml aliquots of cell suspension aliquots simultaneously with [3H]-AZM. Lineweaver-Burk analysis was used to determine the mechanisms of inhibition.

**Effect of AZM on killing of intra-epithelial *A. actinomycetemcomitans***: Epithelial invasion by *A. actinomycetemcomitans* was induced as described by Meyer et al (24). In this model, *A. actinomycetemcomitans* enters the epithelial cells in a host-derived membrane-bound vacuole and lysizes the vacuolar membrane soon after entry (25). Microtubules play a critical role in cell invasion by *A. actinomycetemcomitans*. Taxol, which stabilizes polymerized microtubules, enhances invasion and inhibits subsequent exit of *A. actinomycetemcomitans* from infected epithelial cells. Thus, the assay was conducted in DMEM containing 10 µM taxol (Sigma Chemical Company, St. Louis, MO) to help maintain the levels of intracellular *A. actinomycetemcomitans* over the course of the assay. Confluent SG cell monolayers in 24-well culture plates were washed and pretreated with assay medium for 30 min prior to the addition of bacteria. Bacterial cultures were harvested, washed, resuspended in assay medium and added to each culture plate well at a multiplicity of infection of 1000. After infection for 2 hours at 37° C, SG cell monolayers were washed five times with HBSS. Adherent extracellular bacteria were removed by treatment with 100 µg/ml gentamicin for 1 hour. After removal of gentamicin-containing medium, the infected monolayers were washed 5 times with HBSS. To confirm the absence of viable extracellular bacteria, an aliquot of the final wash was plated on BHI agar (Becton, Dickinson and Company, Sparks, MD). Infected SG cell monolayers were then cultured in the presence of AZM for periods of 1, 2 and 4 hours. As a positive control, infected monolayers were cultured under identical condition in the absence of AZM. At the indicated
intervals, monolayers were washed 4 times with HBSS and lysed in sterile water to release intracellular *A. actinomycetemcomitans*. Dilutions of the lysate were plated on BHI agar for enumeration of surviving CFUs. Data were expressed as a percentage of the colonies recovered from the positive controls. The effect of amoxicillin (AMX), which does not accumulate inside cells (26), was tested for comparison to AZM. Experiments were carefully monitored to rule out any cytotoxic effects of reagents on cultured epithelial cells or *A. actinomycetemcomitans*.

**Results**

**Epithelial AZM transport:** AZM accumulation by SCC-25 and SG cells saturated within 20 minutes (Figure 1), resulting in steady-state intracellular concentrations that were more than 20-fold higher than extracellular concentrations (Table 1). Transport activity exhibited Michaelis-Menten kinetics (Figure 1, inset). The observed $K_m$ values for AZM transport by SCC-25 and SG cells were similar, but SG cells transported AZM at approximately half the maximal velocity observed with SCC-25 cells (Table 1). At steady state, with an extracellular AZM concentration of 5µg/ml, the cellular/extracellular concentration ratio for SCC-25 cells was slightly higher than for SG cells. To examine the substrate specificity of the system that transports AZM, several organic cations and anions were tested as potential inhibitors. In kinetic studies with SCC-25 cells, the organic cations quinidine, pyrilamine, procainamide and L-carnitine acted as competitive inhibitors of AZM transport (Table 2 and Figure 1 inset). Probenecid, an organic acid, also produced competitive inhibition of AZM transport. At inhibitory concentrations, none of these agents altered the pH of the assay medium. The organic anions spironolactone, pravastatin, bromosulfopthalein, hydrocortisone, taurocholate, and estrone-3-sulfate produced little or no inhibition of AZM transport. Treatment with 10 µM taxol, used to enhance epithelial
infection by *A. actinomycetemcomitans*, also had no significant effect on AZM transport by SG cells.

**Killing of intra-epithelial *A. actinomycetemcomitans* by AZM:** Since *A. actinomycetemcomitans* exhibits strain-dependent differences in susceptibility to AZM, the effects of AZM on two different invasive strains were examined. Infected SG epithelial cells were treated with AZM concentrations similar to those found in blood (0.125 to 0.5 µg/ml) and gingival crevicular fluid (2 to 8 µg/ml) (27). AZM produced dose- and time-dependent intra-epithelial killing of both strains (Figure 2, P < 0.001, repeated measures ANOVA). Cells infected with SUNY 465 required four hours of treatment with 0.125 µg/ml AZM to produce a significant degree of bacterial killing (P < 0.01, Holm-Sidak test). Treatment with 0.5 µg/ml AZM produced significant killing after 2 hours, while treatment with ≥2 µg/ml produced significant killing after 1 hour (P < 0.01). Treatment with 8µg/ml AZM killed approximately 49% of control SUNY 465 CFU after 1 hour, 83% after 2 hours and 85% after 4 hours (P < 0.01, Holm-Sidak test). Under similar experimental conditions, treatment for 2 hours with 4µg/ml AMX killed only 14% of SUNY 465 (P > 0.05, data not shown).

Although AZM produced dose- and time-dependent bacterial killing in cells infected with the Y4 strain, concentrations of ≥0.5 µg/ml required a longer treatment time to produce the degree of inhibition observed with SUNY 465 (Figure 2, lower panel). Interestingly, treatment with 0.125µg/ml AZM produced significant killing after 1 hour (P < 0.01, Holm-Sidak test).

To determine whether inhibition of AZM transport impairs killing of intra-epithelial *A. actinomycetemcomitans*, 1 mM L-carnitine (alone or in combination with 2 µg/ml AZM) was added to the culture medium of invaded SG cells (Figure 3). Under these conditions, carnitine reduced the steady-state intracellular AZM concentration from 43.5µg/ml to approximately...
33.3µg/ml (data not shown). Treatment for 1 hour with carnitine had no significant effect on the survival of SUNY 465, while treatment with AZM killed 30% of the bacteria (P < 0.05, Holm-Sidak test). In the presence of a combination of carnitine and AZM, killing of SUNY 465 was reduced to approximately half of that produced by AZM alone (P < 0.05, Holm-Sidak test).

**Discussion**

The results demonstrate that human oral epithelial cells lines possess an active transport system that concentrates AZM and facilitates killing of *A. actinomycetemcomitans* inside infected gingival epithelium. AZM transport by both epithelial cell lines exhibited Michaelis-Menten kinetics and yielded steady-state intracellular concentrations that were substantially higher than those in the extracellular medium. In addition to human phagocytes and fibroblasts, in which active cellular uptake of AZM has been reported (16, 28), previous studies provide evidence of AZM concentration inside epithelium (21, 29). The cellular/extracellular concentration ratios observed in the present study were approximately 3-fold higher than those reported for canine kidney, McCoy and Hep-2 epithelial cells with AZM. The differences might be associated with different origin of these epithelial cell lines and different levels of transporter gene expression. SCC-25 cells took up AZM at a 2-fold higher V_max than did SG cells and exhibited a significantly greater degree of intracellular AZM accumulation.

As a weak organic base, AZM is a candidate for interaction with transporters that carry organic cations. In this study, the organic cations quinidine, pyrilamine, procainamide and L-carnitine competitively inhibited epithelial AZM transport. Control experiments confirmed that none of these agents altered the pH of the assay medium. This suggests that AZM uptake by these oral cell lines is mediated by a transport system that accepts organic cations as substrates. Except for
probenecid, none of the organic anions we examined inhibited AZM transport. Probenecid reportedly interacts with organic cation transporters, possibly through binding to both the hydrophobic and the anionic binding sites of some organic cation transporters (30).

Antibiotics that can penetrate eukaryotic cell membranes and remain active in the intracellular environment are most suitable for treating infections by invasive bacteria (31). In the present study, AZM accumulation by SG cells was associated with time- and concentration-dependent killing of intracellular *A. actinomycetemcomitans*. While the more susceptible SUNY 465 strain was substantially inhibited after 2 hours of treatment with AZM, longer treatment times were required to produce comparable inhibition of Y4. Despite the limitation posed by a relatively short experimental treatment time with a bacteriostatic antibiotic, almost 90% of the SUNY 465 strain was killed inside SG cells incubated with AZM at concentrations comparable to those found in GCF (8 µg/ml). Inhibition of AZM transport by carnitine significantly impaired this killing. AMX, which does not concentrate inside cells, was significantly less effective at killing of intra-epithelial *A. actinomycetemcomitans* at concentrations found in GCF (4 µg/ml).

The connection between intracellular AZM accumulation and killing of invasive bacteria in this study is consistent with results obtained with *Listeria*- and *Staphylococcus*-infected macrophages (32). Inhibitors of the P-glycoprotein efflux pump increased intracellular accumulation of AZM and enhanced the killing of intracellular bacteria. Similarly, PMNs that take up and accumulate CLR exhibit enhanced phagocytic killing of the leukotoxin-producing Y4 strain of *A. actinomycetemcomitans* (18). Our findings can also be related to a previous study of antibiotic killing of *A. actinomycetemcomitans* NCTC 9710 within cultured KB epithelial cells (33). In that study, the effects of moxifloxacin and doxycycline, which are actively transported by human oral epithelial cells (23), were examined. Intracellular *A. actinomycetemcomitans* was
completely eliminated by treatment for 4 hours with 0.115 µg/ml moxifloxacin or 6.25 µg/ml
doxycycline. Thus, it appears that moxifloxacin, doxycycline and AZM can kill intra-epithelial
*A. actinomycetemcomitans* at concentrations near those attainable in GCF. One advantage of
AZM is that its therapeutic levels in GCF are sustained over an unusually long period (≥14 days
after the last oral dose) (34).

In summary, two cell lines derived from oral epithelium possess active transport systems for
AZM that exhibit Michaelis-Menten kinetics and accept other organic cations as substrates. The
resultant concentration of AZM inside cultured gingival epithelial cells is effective in killing
invasive infections by *A. actinomycetemcomitans*. To date, there have been no clinical studies
to examine the adjunctive effects of AZM in the treatment of localized aggressive periodontitis,
the variant of periodontitis that is most strongly associated with infection by *A. actinomycetemcomitans* (35). Our findings provide a rationale for conducting these studies.

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Kasten (East Tennessee State University) for providing Smulow-Glickman epithelial cells and
Dr. Joseph J. Zambon (SUNY at Buffalo) for providing *A. actinomycetemcomitans* SUNY 465.


Table 1. Kinetic Constants for AZM Transport by Smulow-Glickman and SCC-25 Cells\textsuperscript{a}

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>$K_m$ (µg/ml)</th>
<th>$V_{max}$ (ng/min/10\textsuperscript{6})</th>
<th>Cellular/Extracellular concentration ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>SG</td>
<td>198 ± 23.8</td>
<td>249 ± 13\textsuperscript{b}</td>
<td>23.7 ± 0.97\textsuperscript{b}</td>
</tr>
<tr>
<td>SCC-25</td>
<td>176 ± 10.5</td>
<td>486 ± 20.1\textsuperscript{b}</td>
<td>28.8 ± 1.53\textsuperscript{b}</td>
</tr>
</tbody>
</table>

\textsuperscript{a} $K_m$ and $V_{max}$ were determined by Lineweaver-Burk analysis of transport activity during the rapid initial phase of uptake (first 3 min). The cellular/extracellular concentration ratio was determined after incubation for 20 min in medium containing 5 µg/ml AZM. All data are expressed as the mean ± SEM of at least 3 experiments.

\textsuperscript{b} Values within columns are significantly different (P < 0.05, $t$ test).
### Table 2. Inhibition of SCC-25 Cell AZM Transport by Organic Cations and Probenecida

<table>
<thead>
<tr>
<th>Agent</th>
<th>$K_m$ (μg/ml)</th>
<th>$V_{max}$ (ng/min/10^6)</th>
<th>Mechanism of inhibition (Ki)</th>
<th>Chemical classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>176 ± 10.5</td>
<td>486 ± 20.1</td>
<td>Not applicable</td>
<td></td>
</tr>
<tr>
<td>Quinidine</td>
<td>382 ± 2.0</td>
<td>464 ± 8.5</td>
<td>Competitive (0.82 ± 0.09 mM)</td>
<td>Organic cation</td>
</tr>
<tr>
<td>Pyrilamine</td>
<td>266 ± 12.6</td>
<td>503 ± 6.6</td>
<td>Competitive (0.4 ± 0.07 mM)</td>
<td>Organic cation</td>
</tr>
<tr>
<td>Procainamide</td>
<td>269 ± 56.5</td>
<td>513 ± 112</td>
<td>Competitive (3.03 ± 0.46 mM)</td>
<td>Organic cation</td>
</tr>
<tr>
<td>L-Carnitine</td>
<td>314 ± 32</td>
<td>483 ± 46.5</td>
<td>Competitive (0.46 ± 0.05 mM)</td>
<td>Organic cation</td>
</tr>
<tr>
<td>Probenecid</td>
<td>236 ± 3.2</td>
<td>483 ± 30.9</td>
<td>Competitive (0.57 ± 0.08 mM)</td>
<td>Organic acid</td>
</tr>
</tbody>
</table>

* Derived from Lineweaver-Burk analysis of transport activity observed in the presence and absence of the indicated agents. All data are expressed as the mean ± SEM of at least 3 experiments.
Figure Legends

Figure 1. Time course of AZM accumulation by cultured SCC-25 and SG epithelial cells at 37°C. The assay was initiated by addition of [3H]-AZM to suspended cells. The data represent the mean of three experiments. Inset: Representative Lineweaver-Burk plot of the initial phase of AZM transport by SG cells in the presence and absence of 1 mm L-carnitine. The intercepts are consistent with competitive inhibition.

Figure 2. Effect of AZM on killing of intracellular *A. actinomycetemcomitans*. SG cell monolayers infected with either the SUNY 465 or the Y4 strain of *A. actinomycetemcomitans* were incubated in AZM-containing (treatment) or AZM-free medium (control). Intracellular *A. actinomycetemcomitans* was released by cell lysis, plated on BHI agar and enumerated. Data are presented as a percentage of the colonies recovered from the controls. The data represent the mean ± SEM of six to eight experiments. Treatments that failed to produce significant inhibition compared to control are denoted by * (P> 0.05, Holm-Sidak test).

Figure 3. Impairment of AZM killing of intracellular SUNY 465 with L-carnitine. Infected SG cell monolayers were incubated with carnitine alone, AZM alone, or a combination of AZM and carnitine for 1 hour. Cells cultured in the absence of treatment agents served as controls. Data are presented as percentage of the colonies recovered from the controls. The data represent the mean ± SEM of seven experiments. The results of all three treatments were significantly different from each other (P< 0.05, Holm-Sidak test).
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